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KEVIN KAVANAGH

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Fungi

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Biology and Applications

Third Edition

Edited by

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Preface

Fungi make an enormous contribution to our life. The role of yeast in the production of alcohol and bread is well characterized. We consume fungi directly in the form of edible mushrooms and in "blue cheeses" which get their characteristic flavor and aroma from the presence of fungi. Fungi are also used for the production of antibiotics, such as penicillin, and enzymes for use in the food industry. Since the 1990s, fungi have been utilized for the production of recombinant proteins, some of which have great therapeutic potential. Although infrequently recognized as important decomposers of organic detritus, fungi play a significant role in degrading biological matter, such as fallen leaves. On a more negative note, some fungi (for example members of the genus *Candida* and *Aspergillus*) are capable of causing serious life-threatening infections in immunocompromised patients, and other fungi can be serious plant pathogens.

This is the third edition of *Fungi: Biology and Applications* which was first published in 2005. Since that date there have been enormous strides in our understanding of the biology of fungi, and their contribution to our life is becoming increasingly important. The aim of the current edition is to provide a detailed description of the biology, biotechnological applications, and medical significance of fungi. The book commences with an in-depth description of the physiology of fungi in which the structure, metabolism, and growth of fungi are described. This is followed by a chapter dedicated to the genetics of fungi in which the lifecycles of a number of representative fungi are described and the use of fungi for genetic analysis is outlined. The advent of genomics and proteomics has revolutionized our study of the cell. Chapters 3, 4, and 5 describe how genomics, transcriptomics, and proteomics, respectively, have increased our knowledge of fungi and made available new opportunities for exploiting fungi for the good of humanity. Chapter 6 describes the importance of fungi as food and highlights the different techniques for the commercial production of edible fungi. Chapters 7 and 8 describe how fungi can be utilized for producing commercially important antibiotics, enzymes, and a range of chemical

products such as citric acid. Chapter 9 focuses on the exploitation of fungi for the production of heterologous proteins and illustrates how yeast has been used for the production of hepatitis B antigens. Chapter 10 describes the main fungal pathogens of humans and Chapter 11 outlines the human immune response to fungi that restricts infection. Chapter 12 describes the main classes of antifungal drugs and their modes of action. Chapter 13 outlines the role of fungi in the environment where they play a significant role in recycling nutrients. Chapter 14 describes the main fungal pathogens of plants and assesses the impact of such pathogens on the global supply of food.

This book gives a comprehensive introduction to fungi in terms of their biology, genetics, medical significance, and biotechnological potential. Each chapter is written by internationally recognized experts, so the reader is given an up-to-date and detailed account of our knowledge of the biology and various applications of fungi.

Kevin Kavanagh

1

Introduction to Fungal Physiology

Graeme M. Walker and Nia A. White

1.1 Introduction

Fungal physiology refers to the nutrition, metabolism, growth, reproduction, and death of fungal cells. It also generally relates to interaction of fungi with their biotic and abiotic surroundings, including cellular responses to environmental stress. The physiology of fungal cells impacts significantly on the environment, industrial processes, and human health. In relation to ecological aspects, the biogeochemical cycling of carbon in nature would not be possible without the participation of fungi acting as primary decomposers of organic material. Furthermore, in agricultural operations fungi play important roles as mutualistic symbionts, pathogens, and saprophytes, where they mobilize nutrients and affect the physicochemical environment, or can be exploited as agents of biocontrol or as biofertilizers. Fungal metabolism is also responsible for the detoxification of organic pollutants and for bioremediating heavy metals and other recalcitrant chemicals in the environment (including wastewaters and groundwaters). The production of many economically important industrial commodities relies on the exploitation of yeast and fungal metabolism and these include such diverse products as whole foods, food additives, fermented beverages, antibiotics, probiotics, pigments, pharmaceuticals, biofuels, enzymes, vitamins, organic and fatty acids, and sterols. More negatively, fungi can cause considerable disease, spoilage, and decay of important artefacts, commodities, and materials, buildings, and of course food supplies.

In terms of human health, some yeasts and fungi represent major opportunistic life-threatening pathogens, while others are life-savers as they provide antimicrobial and chemotherapeutic agents. In modern biotechnology, several yeast

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species are being exploited as hosts for the expression of human therapeutic proteins following recombinant DNA and gene editing technologies (see Chapter 9). Recently, the application of gene editing using CRISPR/Cas is leading to a revolution in fungal genetic engineering (see Chapter 2). Furthermore, an international synthetic biology research consortium, called Sc-2.0, has embarked on the construction of a completely synthetic version of *Saccharomyces cerevisiae*. This would represent the world's first fully synthetic eukaryotic genome! In addition to the direct industrial exploitation of yeasts and fungi, it is important to note that these organisms, most notably the yeast *S. cerevisiae*, play increasingly significant roles as model eukaryotic cells in furthering our fundamental knowledge of biological and biomedical science. This is especially the case now that numerous fungal genomes have been completely sequenced and the information gleaned from fungal genomics and proteomics is providing valuable insight into human genetics and heritable disorders. However, knowledge of cell physiology is essential if the functions of many of the currently unknown fungal genes, including "synthetic" ones, are to be fully elucidated.

It is apparent, therefore, that fungi are important organisms for human society, health, and well-being, and that studies of fungal physiology are very pertinent to our understanding, control, and exploitation of this group of microorganisms. This chapter describes some basic aspects of fungal cell physiology, focusing primarily on nutrition, growth, and metabolism in unicellular yeasts and filamentous fungi.

1.2 Morphology of Yeasts and Fungi

There are a diversity of yeast and fungal cellular morphologies. Most higher fungi are filamentous, yeasts grow as unicells, and some primitive fungi such as the Chytridomycota grow as individual rounded cells or dichotomous branched chains of cells with root-like rhizoids for attachment to a nutrient resource. Here we consider the most common growth forms, the filamentous fungi and unicellular yeasts.

1.2.1 Filamentous Fungi

The gross morphologies of macrofungi and microfungi are varied and often apparent throughout the environment (Plate 1.1). For example, we can easily recognize a variety of mushrooms and toadstools, the sexual fruiting bodies of certain macrofungi (the higher fungi Ascomycota and Basidiomycota and related forms), during a walk through pasture or woodland. Microfungi (the molds) are also diverse and are often observed on decaying foods and detritus, whereas many, including the colored rusts, smuts, and mildews, are common plant pathogens. Closer inspection of these visible structures, however, reveals that all are

composed of aggregated long, branching threads termed hyphae (singular: hypha), organized to support spores for reproduction and dissemination. The hyphae of these aerial structures extend and branch within the supporting substratum as a network, termed a mycelium, from which the apically growing hyphae seek out, exploit, and translocate available nutrients. Apically growing hyphae usually have a relatively constant diameter ranging from 1 to 30 μm or more, depending on fungal species and growth conditions.

Filamentous fungi may be cultivated within the laboratory on a variety of different liquid or solid media. On agar, the radially expanding colonial growth form of the fungal mycelium is most evident, extending from an inoculum, on, within, and sometimes above the substrate, forming a near spherical three-dimensional (3-D) colony. This radiating, circular pattern is also visible during the growth of fairy ring fungi in grassland and as ringworm infections of the skin (Plate 1.1, parts a and b).

The hyphae of individual fungi may (theoretically) extend endlessly via apical growth, provided they are supported with appropriate nutrients and other environmental conditions. Eucarpic fungi are therefore spatially and temporally indeterminate organisms, and, unlike animal, plant, and other microbial individuals, have no predetermined maximum size or age. The mycelium is not, however, simply a homogeneously extending entity, but displays considerable developmental plasticity. Different interconnected regions of the fungal mycelium may grow, branch, anastomose (fuse), age, die, sporulate, and display varying physiological and biochemical activities at different times or even simultaneously, depending on local micro-environmental conditions. Thus, colonies growing on relatively homogeneous media may be pigmented, exhibit different morphological sectors, produce aerial structures, grow as fast-effuse or slow-dense forms, and even exhibit rhythmic growth.

As well as reproductive structures and substrate mycelium, certain higher fungi, most notably the basidiomycetes, when growing within an environment where nutrients are distributed heterogeneously, can differentiate into long string-like structures called rhizomorphs or cords. These linear organs have evolved to rapidly explore for, connect, and translocate water and nutrients between patches of resource (e.g. pieces of fallen timber on the forest floor or from tree root to tree root). Accordingly, many, particularly mature rhizomorphs, contain internal vessel hyphae which possess a wide diameter, forming a channel running along the organ. The peripheral hyphae are often closely packed and melanized for insulation (Plate 1.1, parts l and m).

Filamentous fungi and yeasts are simply different styles of fungal growth suitable for occupation of different habitats and produced by differing cell growth polarities. Many species termed dimorphic fungi can adopt either the hyphal or unicellular yeast forms according to environmental circumstances. For example, certain important human and animal pathogens exist as yeast forms mobilized in body fluids but are able to form hyphae or pseudohyphae for tissue invasion.

1.2.2 Yeasts

Yeasts are unicellular (mostly ascomycete, basidiomycete, or members of the deuteromycete group) fungi that divide asexually by budding or fission and whose individual cell size can vary widely from 2–3 μm to 20–50 μm in length and 1–10 μm in width. *Saccharomyces cerevisiae*, commonly referred to as brewer's or baker's yeast, is generally ellipsoid in shape with a large diameter of 5–10 μm and a small diameter of around 5 μm (Figure 1.1). There is great diversity in cell shapes and modes of cellular reproduction in the yeasts, as summarized in Table 1.1.

The morphology of agar-grown yeasts shows great diversity in terms of color, texture, and geometry (peripheries, contours) of giant colonies. Several yeasts are pigmented and the following colors may be visualized in surface-grown colonies: cream (e.g. *S. cerevisiae*); white (e.g. *Geotrichum candidum*); black (e.g. *Aureobasidium pullulans*); pink (e.g. *Phaffia rhodozyma*); red (e.g. *Rhodotorula rubra*); orange (e.g. *Rhodosporidium* spp.), and yellow (e.g. *Cryptococcus laurentii*). The pigments of some yeasts have biotechnological uses, including astaxanthin from *P. rhodozyma* in aquacultural feed supplements for farmed salmon (that are unable to synthesize these natural pink compounds).

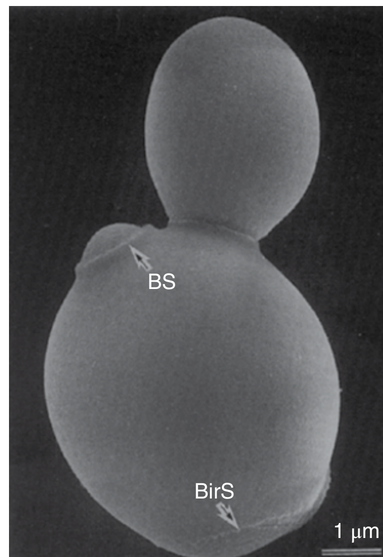


Figure 1.1 Scanning electron micrograph of a typical yeast cell (×10,000). BS, Bud scar; BirS, birth scar. (Reproduced with kind permission of Professor Masako Osumi, Japan Women's University, Tokyo.)

Table 1.1 Diversity of yeast cell shapes.

Cell shape	Description	Examples of yeast genera
Ellipsoid	Ovoid-shaped	*Saccharomyces*
Cylindrical	Elongated cells with hemispherical ends	*Schizosaccharomyces*
Apiculate	Lemon-shaped	*Hanseniaspora*, *Saccharomycodes*
Ogival	Elongated cell, rounded at one end and pointed at other	*Dekkera*, *Brettanomyces*
Flask-shaped	Cells divide by bud-fission	*Pityrosporum*
Miscellaneous shapes	Triangular	*Trigonopsis*
	Curved	*Cryptococcus* (e.g. *C. cereanus*)
	Spherical	*Debaryomyces*
	Stalked	*Sterigmatomyces*
Pseudohyphal	Chains of budding yeast cells which have elongated without detachment	*Candida* (e.g. *C. albicans*)
Hyphal	Branched or unbranched filamentous cells which form from germ tubes. Septa may be laid down by the continuously extending hyphal tip. Hyphae may give rise to blastospores	*Candida albicans*
Dimorphic	Yeasts that grow vegetatively in either yeast or filamentous (hyphal or pseudohyphal) form	*Candida albicans*, *Saccharomycopsis fibuligera*, *Kluyveromyces marxianus*, *Malassezia furfur*, *Yarrowia lipolytica*, *Histoplasma capsulatum*

1.3 Ultrastructure and Function of Fungal Cells

1.3.1 The Fungal Cell Surface

The cell envelope in yeasts and fungi is the peripheral structure that encases the cytoplasm and comprises the plasma membrane, the periplasm, the cell wall, and additional extracellular structural components (such as fimbriae and capsules). The cell wall represents a dynamically forming exoskeleton that protects the fungal protoplast from the external environment and defines directional growth, cellular strength, shape, and interactive properties (Figure 1.2).

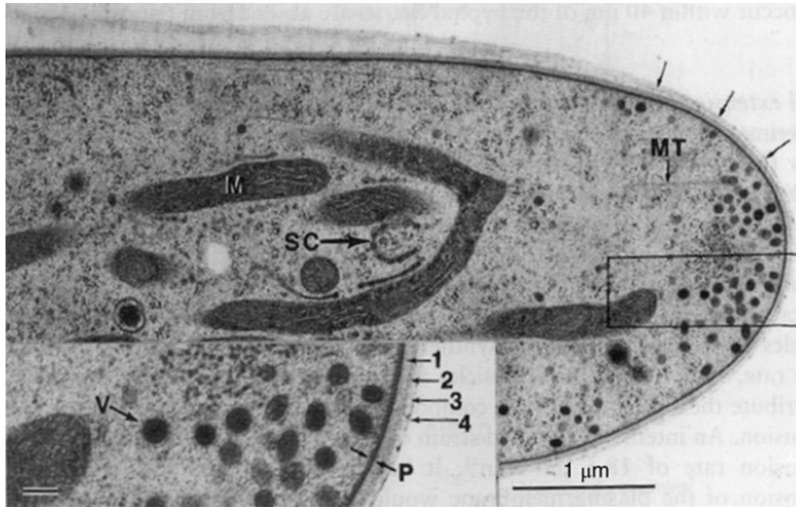


Figure 1.2 Transmission electron microscopy of ultrathin sections of a hyphal tip of *Fusarium* reveals intracellular fine structure. Layers of cell wall are shown in greater detail in lower image. M, Mitochondrion; V, vesicles; P, plasma membrane; MT, microtubules; SC, smooth Golgi cisternae; 1, 2, 3, 4, four layers of the cell wall. The Spitzenkörper appears as a region surrounded by vesicles containing many small particles (rectangle). (From Carlile *et al.* (2001).)

In filamentous fungi, cell wall formation and organization is intimately bound to the process of apical growth. Thus, for example in *Neurospora crassa*, the wall is thin (approximately 50 nm) at the apex but becomes thicker (approximately 125 nm) at 250 μm behind the tip. The plasma membrane component of the fungal cell envelope is a phospholipid bilayer interspersed with globular proteins that dictates entry of nutrients and exit of metabolites and represents a selective barrier for their translocation. Ergosterol is the major sterol found in the membranes of fungi, in contrast to the cholesterol found in the membranes of animals and phytosterols in plants. This distinction is exploited during the use of certain antifungal agents used to treat some fungal infections, and can be used as an assay tool to quantify fungal growth. The periplasm, or periplasmic space, is the region external to the plasma membrane and internal to the cell wall. In yeast cells, it comprises secreted proteins (mannoproteins) and enzymes (such as invertase and acid phosphatase) that are unable to traverse the cell wall. In filamentous fungi, the cell membrane and wall may be intimately bound as hyphae are often resistant to plasmolysis.

Fungal cell surface topological features can be visualized using scanning electron microscopy (SEM) and nanometre resolution achieved using atomic force microscopy (AFM). The latter is beneficial as it can be employed with unfixed, living cells and avoids potentially misleading artefacts that may arise when preparing cells for electron microscopy.

Table 1.2 Major polymers found in different taxonomic groups of fungi and fungus-like organisms, together with presence of perforate septa in these groups.

Taxonomic grouping	Fibrillar polymers	Matrix polymers	Perforate septa present or absent
Oomycetes (no longer considered to be true fungi)	β(1,3), β(1,6)-Glucan; cellulose	Glucan	Absent
Chytridomycetes	Chitin; glucan	Glucan	Absent
Zygomycetes	Chitin; chitosan	Polyglucuronic acid; glucuronomannoproteins	Absent
Basidiomycetes	Chitin; β(1,3)-β(1,6) glucans	α(1,3)-Glucan; xylomannoproteins	Present (mostly Dolipore)
Ascomycetes/ Deuteromycetes	Chitin; β(1,3)-β(1,6) glucans	α(1,3)-Glucan; galactomannoproteins	Present (mostly simple with large central pore)

Adapted from Deacon (2000); Carlile *et al.* (2001).

Ultrastructural analysis of fungal cell walls reveals a thick, complex fibrillar network. The cell walls of filamentous fungi are mainly composed of different polysaccharides according to taxonomic group. For example, they may contain chitin, glucans, mannoproteins, chitosan, polyglucuronic acid, or cellulose (absent from true fungi), together with smaller quantities of proteins and glycoproteins (Table 1.2). Generally, the semicrystalline microfibrillar components are organized in a network mainly in the central cell wall region and are embedded within an amorphous matrix. Bonding occurs between certain components behind the extending hyphal tip, thereby strengthening the entire wall structure. The processes of endocytosis and exocytosis occur around apical and subapical regions and serve to shape both hyphal growth and interactions with the environment (Figure 1.2). There is evidence to suggest that the cell wall is a dynamic structure where considerable quantitative and qualitative differences occur not only between different fungal species, but also between different morphological forms of the same species and even in response to environmental stress. For example, a class of hydrophobic proteins called hydrophobins are localized within the aerial growth or appressoria (terminal swellings involved in infection) of certain fungi, whereas pigmented melanins are often found within some fungal cell walls to insulate against biotic and abiotic stresses.

The hyphae of higher fungi extend via tip growth followed by cross-wall formation or septation, whereas the lower fungi remain aseptate (except when segregating spores or in damaged colony regions). Septa may offer some structural support to hyphae. Significantly, septa serve to compartmentalize hyphae

but are typically perforated, thereby permitting passage and communication of cytoplasm or even protoplasm between compartments. However, septal pores can become blocked by Woronin bodies or other materials. This aids morphological and biochemical differentiation and serves to seal-off stressed or damaged hyphae from undamaged colony regions. Again, different pore types are representative of different taxonomic groups and species (Table 1.2).

In yeasts, the cell wall provides stability and protection to the cells and its structure comprises polysaccharides (predominantly β-glucans for rigidity), proteins (mainly mannoproteins on the outermost layer for determining porosity), together with some lipid, chitin (e.g. in bud scar tissue), and inorganic phosphate material. Figure 1.3 shows the composition and structure of the *S. cerevisiae* cell wall. Hyphal cell walls generally contain fewer mannans than yeast cell forms, and such changes in composition are even observed during the transition from unicellular to mycelial growth of dimorphic fungi.

Chitin is also found in yeast cell walls and is a major constituent of bud scars (Figure 1.1). These are remnants of previous budding events found on the surface of mother cells following birth of daughter cells (buds). The chitin-rich bud scars of yeast cells can be stained with fluorescent dyes (e.g. calcoflour white) and this can provide useful information regarding cellular age, since the number of scars represents the number of completed cell division cycles. Outside the cell wall in fungi, several extramural layers may exist, including fimbriae and capsules. Fungal fimbriae are long, protein-containing protrusions appearing

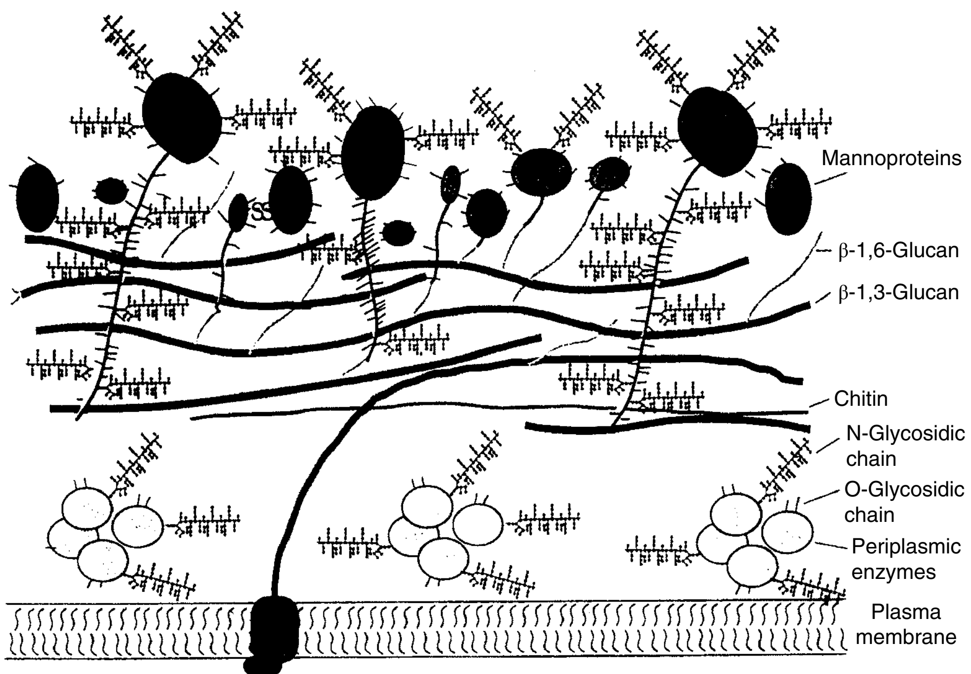


Figure 1.3 Cell envelope structure of the yeast *S. cerevisiae*. (From Walker (1998).)

from the cell wall of certain basidiomycetous and ascomycetous fungi that are involved in cell–cell conjugation. Capsules are extracellular polysaccharide-containing structures found in basidiomycetous fungi that are involved in stress protection. In *Cryptococcus neoformans* (the pathogenic yeast state of *Filobasidiella neoformans*) the capsule may determine virulence properties and evasion from macrophages. One extrahyphal substance, the polymer pullulan, is produced commercially from *Aureobasidium pullulans*, and is used in the production of oral hygiene products.

1.3.2 Subcellular Architecture and Organelle Function

Transmission electron microscopy of ultrathin sections of fungal cells reveals intracellular fine structure (Figures 1.2 and 1.4). Subcellular compartments (organelles) are bathed in an aqueous cytoplasm containing soluble proteins and other macromolecules together with low-molecular weight metabolites.

However, the hyphae of central (and therefore older) colony regions of filamentous fungi may become devoid of protoplasm and organelles, as protoplasmic components are driven forward or are recycled, to support the growth of actively growing hyphal tips. Cytoplasmic components additionally comprise microbodies, ribosomes, proteasomes, lipid particles, and a cytoskeletal network. The latter confers structural stability to the fungal cytoplasm and consists of microtubules and microfilaments. The following membrane-bound organelles may be found in a typical fungal cell: nucleus, endoplasmic reticulum (ER), mitochondria, Golgi apparatus, secretory vesicles, and vacuoles. Several of these

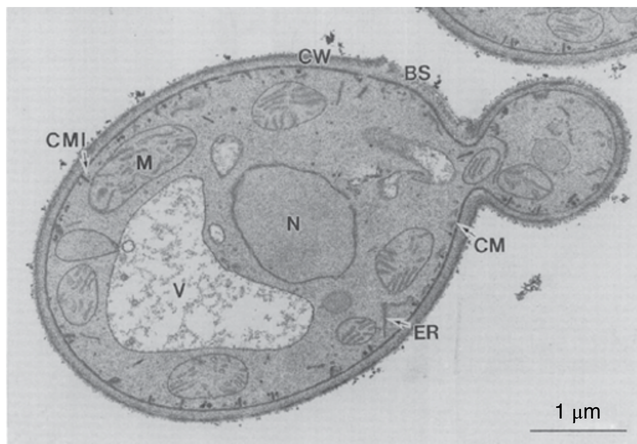


Figure 1.4 Electron micrograph of a typical yeast cell. CW, Cell wall; CM, cell membrane; CMI, cell membrane invagination; BS, bud scar; M, mitochondrion, N, nucleus; V, vacuole; ER, endoplasmic reticulum. (Reproduced with kind permission of Professor Masako Osumi, Japan Women's University, Tokyo.)

organelles form extended membranous systems. For example, the ER is contiguous with the nuclear membrane and secretion of fungal proteins involves intermembrane trafficking in which the ER, Golgi apparatus, plasma membrane, and vesicles all participate. The physiological function of the various fungal cell organelles is summarized in Table 1.3.

The nucleus is the structure that defines the eukaryotic nature of fungal cells. It is bound by a double membrane and encases the chromosomes in a nucleoplasm. Most yeasts and fungi are haploid (singular copies of each chromosome), although some (e.g. *S. cerevisiae*) may alternate between haploidy and diploidy.

Table 1.3 Functional components of an idealized fungal cell.

Organelle or cellular structure	Function
Cell envelope	Comprising: the plasma membrane which acts as a selectively permeable barrier for transport of hydrophilic molecules in and out of fungal cells; the periplasm containing proteins and enzymes unable to permeate the cell wall; the cell wall which provides protection and shape, and is involved in cell–cell interactions, signal reception, and specialized enzyme activities; fimbriae involved in sexual conjugation; capsules to protect cells from dehydration and immune cell attack
Nucleus	Relatively small. Containing chromosomes (DNA–protein complexes) that pass genetic information to daughter cells at cell division and the nucleolus which is the site of ribosomal RNA transcription and processing
Mitochondria	Site of respiratory metabolism under aerobic conditions, and, under anaerobic conditions, for fatty acid, sterol, and amino acid metabolism
Endoplasmic reticulum	Ribosomes on the rough ER are the sites of protein biosynthesis
Proteasome	Multi-subunit protease complexes involved in regulating protein turnover
Golgi apparatus and vesicles	Secretory system for import (endocytosis) and export (exocytosis) of proteins
Vacuole	Intracellular reservoir (amino acids, polyphosphate, metal ions); proteolysis; protein trafficking; control of cellular pH. In filamentous fungi, tubular vacuoles transport materials bidirectionally along hyphae.
Peroxisome	Oxidative utilization of specific carbon and nitrogen sources (contain catalase, oxidases). Glyoxysomes contain enzymes of the glyoxylate cycle

Many industrial strains of *S. cerevisiae* exhibit aneuploidy (odd numbers of chromosomes) or are polyploid (multiple chromosome copies). Chromosomes comprise DNA–protein structures that replicate and segregate to newly divided cells or hyphal compartments at mitosis. This, of course, ensures that genetic material is passed onto daughter cells or septated compartments at cell division. Yeasts usually contain a single nucleus per cell. However, the hyphal compartments of filamentous fungi may contain one or more nuclei. Monokaryotic basidiomycetes possess one nucleus per compartment, whereas dikaryons and heterokaryons possess two or more genetically distinct haploid nuclei. The maintenance of multiple nuclei within individual hyphal compartments allows fungi to take advantage of both haploid and diploid lifestyles. This is discussed further in Chapter 2.

In filamentous fungi, a phase-dark near-spherical region, which also stains with iron hemotoxylin, is evident by light microscopy at the apex during hyphal tip growth. The region is termed the Spitzenkörper, the apical vesicle cluster or centre, or apical body, and it consists of masses of small membrane-bound vesicles around a vesicle-free core with emergent microfilaments and microtubules (Figure 1.2). The Spitzenkörper contains differently sized vesicles derived from Golgi bodies, either large vesicles or microvesicles (chitosomes), with varying composition. It orientates to the side as the direction of tip growth changes, and disappears when growth ceases. This vesicle supply centre is involved in wall extension and hence tip growth, branching, clamp connection formation (in basidiomycetes), and germ tube formation.

1.4 Fungal Nutrition and Cellular Biosyntheses

1.4.1 Chemical Requirements for Growth

Yeasts and fungi have relatively simple nutritional needs and most species would be able to survive quite well in aerobic conditions if supplied with glucose, ammonium salts, inorganic ions, and a few growth factors. Exceptions to this would include, for example, obligate symbionts such as the vesicular-arbuscular mycorrhizal (VAM) fungi which require growth of a plant partner for cultivation. Macronutrients, supplied at millimolar concentrations, comprise sources of carbon, nitrogen, oxygen, sulfur, phosphorus, potassium, and magnesium; and micronutrients, supplied at micromolar concentrations, comprise trace elements like calcium, copper, iron, manganese, and zinc and would be required for fungal cell growth (Table 1.4). Some fungi are oligotrophic, apparently growing with very limited nutrient supply, surviving by scavenging minute quantities of volatile organic compounds from the atmosphere.

Being chemo-organotrophs, fungi need fixed forms of organic compounds for their carbon and energy supply. Sugars are widely utilized for fungal growth, and can range from simple hexoses like glucose to polysaccharides like starch and cellulose.

Table 1.4 Elemental requirements of fungal cells.

Element	Common sources	Cellular functions
Carbon	Sugars	Structural element of fungal cells in combination with hydrogen, oxygen, and nitrogen. Energy source
Hydrogen	Protons from acidic environments	Transmembrane proton motive force vital for fungal nutrition. Intracellular acidic pH (around 5–6) necessary for fungal metabolism
Oxygen	Air, O_2	Substrate for respiratory and other mixed-function oxidative enzymes. Essential for ergosterol and unsaturated fatty acid synthesis
Nitrogen	NH_4^+ salts, urea, amino acids	Structurally and functionally as organic amino nitrogen in proteins and enzymes
Phosphorus	Phosphates	Energy transduction, nucleic acid, and membrane structure
Potassium	K^+ salts	Ionic balance, enzyme activity
Magnesium	Mg^{2+} salts	Enzyme activity, cell and organelle structure
Sulfur	Sulfates, methionine	Sulfhydryl amino acids and vitamins
Calcium	Ca^{2+} salts	Possible second messenger in signal transduction
Copper	Cupric salts	Redox pigments
Iron	Ferric salts. Fe^{3+} is chelated by siderophores and released as Fe^{2+} within the cell	Heme-proteins, cytochromes
Manganese	Mn^{2+} salts	Enzyme activity
Zinc	Zn^{2+} salts	Enzyme activity
Nickel	Ni^{2+} salts	Urease activity
Molybdenum	Na_2MoO_4	Nitrate metabolism, vitamin B12

Some fungi can occasionally utilize aromatic hydrocarbons (e.g. lignin by the white-rot fungi). Table 1.5 outlines the variety of carbon sources that can be utilized by yeasts and filamentous fungi for growth.

Fungi are nondiazotrophic (cannot fix nitrogen) and need to be supplied with nitrogenous compounds, either in inorganic form such as ammonium salts, or in organic form such as amino acids. Ammonium sulfate is a commonly used nitrogen source in fungal growth media since it also provides a source of utilizable sulfur. Many fungi (but not the yeast *S. cerevisiae*) can also grow on nitrate,

Table 1.5 Diversity of carbon sources for yeast and filamentous fungal growth.

Carbon source	Typical examples	Comments
Hexose sugars	D-glucose, D-galactose,	Glucose metabolized by majority of yeasts and filamentous fungi
	D-fructose, D-mannose	If a yeast does not ferment glucose, it will not ferment other sugars. If a yeast ferments glucose, it will also ferment fructose and mannose, but not necessarily galactose
Pentose sugars	L-arabinose, D-xylose, D-xylulose, L-rhamnose	Some fungi respire pentoses better than glucose. *S. cerevisiae* can utilize xylulose but not xylose
Disaccharides	Maltose, sucrose, lactose, trehalose, melibiose, cellobiose, melezitose	If a yeast ferments maltose, it does not generally ferment lactose and vice versa. Melibiose utilization is used to distinguish ale and lager brewing yeasts. A large number of yeasts utilize disaccharides. Few filamentous fungi (e.g. *Rhizopus nigricans*) cannot utilize sucrose
Trisaccharides	Raffinose, maltotriose	Raffinose only partially used by *S. cerevisiae*, but completely used by other *Saccharomyces* spp. (*S. carlsbergensis*, *S. kluyveri*)
Oligosaccharides	Maltotetraose, maltodextrins	Metabolized by amylolytic yeasts, not by brewing strains
Polysaccharides	Starch, inulin, cellulose, hemicellulose, chitin, pectic substances	Polysaccharide-fermenting yeasts are rare. *Saccharomycopsis* spp. and *S. diastaticus* can utilize soluble starch; *Kluyveromyces* spp. possess inulinase. Many filamentous fungi can utilize these, depending on extracellular enzyme activity
Lower aliphatic alcohols	Methanol, ethanol	Respiratory substrates for many fungi. Several methylotrophic yeasts (e.g. *Pichia pastoris*, *Hansenula polymorpha*) have industrial potential
Sugar alcohols	Glycerol, glucitol	Can be respired by yeasts and a few fungi.
Organic acids	Acetate, citrate, lactate, malate, pyruvate, succinate	Many yeasts can respire organic acids, but few can ferment them
Fatty acids	Oleate, palmitate	Several species of oleaginous yeasts can assimilate fatty acids as carbon and energy sources

(*continued*)

Table 1.5 (*Continued*)

Carbon source	Typical examples	Comments
Hydrocarbons	n-Alkanes	Many yeast and a few filamentous species grow well on C_{12}-C_{18} n-alkanes
Aromatics	Phenol, cresol, quinol, resourcinol, catechol, benzoate	Few yeasts can utilize these compounds. Several n-alkane-utilizing yeasts use phenol as carbon source via the β-ketoadipate pathway
Miscellaneous	Adenine, uric acid, butylamine, pentylamine, putrescine	Some mycelial fungi and yeasts, e.g. *Arxula adeninivorans* and *A. terestre*, can utilize such compounds as sole source of carbon and nitrogen
	Lignin	Can be decayed only by white-rot fungi (basidiomycotina). Little net energy gained directly, but makes available other polysaccharides such as cellulose and hemi-cellulose
	"Hard" keratin	Keratinophilic fungi

Adapted from Walker (1998).

and if able to do so may also utilize nitrite. Nitrate reductase, followed by nitrite reductase, are the enzymes responsible for converting nitrate to ammonia. Most fungi can assimilate amino acids, amines, and amides as nitrogen sources. Most fungi (but not many yeasts) are also proteolytic and can hydrolyze proteins (via extracellularly secreted proteases) to liberate utilizable amino acids for growth. Urea utilization is common in fungi, and some basidiomycotenous yeasts are classed as urease-positive (able to utilize urea), while several ascomycotenous yeasts are urease-negative.

In terms of oxygen requirements, most fungi are aerobes and are often described as being microaerophilic (preferring an oxygen tension below that of normal atmospheric). Although yeasts like *S. cerevisiae* are sometimes referred to as facultative anerobes, they cannot actually grow in strictly anaerobic conditions unless supplied with certain fatty acids and sterols (which they cannot synthesize without molecular oxygen). In fact, there are thought to be very few yeast species that are obligately anaerobic. Unsaturated fatty acids (e.g. oleic acid) and sterols (e.g. ergosterol) are important constituents of the yeast cell membrane, and oxygen is required for their synthesis and to maintain membrane functional integrity and stress resistance. For aerobically respiring yeasts and fungi, oxygen is required as the terminal electron acceptor, where it is finally reduced to water in the electron transport chain. Different fungal species respond to oxygen availability in diverse ways and Table 1.6 categorizes fungi into different groups on this basis.

Table 1.6 Yeast and fungal metabolism based on responses to oxygen availability.

Mode of energy metabolism	Examples	Comments
Obligate fermentative	Yeasts: *Candida pintolopesii* (*Saccharomyces telluris*)	Naturally occurring respiratory-deficient yeasts. Only ferment, even in presence of oxygen
	Fungi: facultative and obligate anerobes	No oxygen requirement for these fungi. Two categories exist with respect to the effects of air: facultative anerobes (e.g. *Aqualinderella* and *Blastocladia*) and obligate anerobes (e.g. *Neocallimastix*)
Facultatively fermentative		
Crabtree-positive	*Saccharomyces cerevisiae*	Such yeasts predominantly ferment high sugar-containing media in the presence of oxygen
Crabtree-negative	*Candida utilis*	Such yeasts do not form ethanol under aerobic conditions and cannot grow anaerobically
Nonfermentative	Yeasts: *Rhodotorula rubra*	Such yeasts do not produce ethanol, in either the presence or absence of oxygen
	Fungi: *Phycomyces*	Oxygen is essential for such (obligately oxidative) fungi
Obligate aerobes	*Gaemannomyces graminis* (the take-all fungus)	Growth of these is markedly reduced if oxygen partial pressure falls below normal atmospheric

Adapted from Walker (1998), Deacon (2000), and Carlile *et al.* (2001).

Sulfur sources for fungal growth include sulfate, sulfite, thiosulfate, methionine and glutathione, with inorganic sulfate and the sulfur amino acid methionine being effectively utilized. Virtually all yeasts can synthesize sulfur amino acids from sulfate, the most oxidized form of inorganic sulfur.

Phosphorus is essential for biosynthesis of fungal nucleic acids, phospholipids, adenosine triphosphate (ATP), glycophosphates, and polyphosphates. Hence, the phosphate content of fungi is considerable (e.g. in yeast cells, this accounts for around 3–5% of dry weight; the major part of this is in the form of orthophosphate ($H_2PO_4^-$) which acts as a substrate and enzyme effector). The fungal

Table 1.7 Metals required for fungal growth and metabolic functions.

Metal ion	Concentration[1]	Main cellular functions supplied in growth medium
Macroelements		
K	2–4 mM	Osmoregulation, enzyme activity
Mg	2–4 mM	Enzyme activity, cell division
Microelements		
Mn	2–4 μM	Enzyme cofactor
Ca	<1 μM	Second messenger, yeast flocculation
Cu	1.5 μM	Redox pigments
Fe	1–3 μM	Heme-proteins, cytochromes
Zn	4–8 μM	Enzyme activity, protein structure
Ni	~10 μM	Urease activity
Mo	1.5 μM	Nitrate metabolism, vitamin B12
Co	0.1 μM	Cobalamin, coenzymes

[1] Concentration figures relate to yeast (*S. cerevisiae*) growth stimulation, and are dependent on the species/strain and conditions of growth, but they would be generally applicable for fungal growth. Adapted from Walker (2004).

vacuole can serve as a storage site for phosphate in the form of complexed inorganic polyphosphates (also referred to as volutin granules). Both nitrogen and phosphorus availability may be growth limiting in nature. Filamentous fungi have evolved a number of biochemical and morphological strategies allowing capture of often poorly available phosphorus within the natural environment. Plants exploit such efficiency during symbioses between their roots and certain mycorrhizal fungi. The major storage form of phosphorus in plants is phytic acid (myo-inositol hexa-dihydrogenphosphate) which is poorly utilized by monogastrics (e.g. humans, pigs, poultry), and fungal (and yeast) phytases have applications in reducing phytate content of foods and feeds (see Chapter 8).

Concerning requirements for minerals, potassium, magnesium, and several trace elements are necessary for fungal growth. K and Mg are macroelements required in millimolar concentrations primarily as enzyme cofactors, whereas other microelements (trace elements) are generally required in the micromolar range. These include Mn, Ca, Fe, Zn, Cu, Ni, Co, and Mo. Table 1.7 summarizes the main metals required for fungal growth. Toxic minerals (e.g. Ag, As, Ba, Cs, Cd, Hg, Li, Pb) adversely affect fungal growth generally at concentrations greater than 100 μM.

Fungal growth factors are organic compounds occasionally needed in very low concentrations for specific enzymatic or structural roles, but not as energy

sources. These include vitamins (e.g. thiamine, biotin), purines, pyrimidines, nucleosides, nucleotides, amino acids, fatty acids, and sterols. For fungi to have a growth factor requirement, this indicates that cells cannot synthesize the particular factor, resulting in the curtailment of growth without its provision in culture media. Some fungi (e.g. *Aspergillus niger*, *Penicillium chrysogenum*) have very simple nutritional needs and are able to synthesize their own growth factors from glucose.

1.4.2 Fungal Cultivation Media

Fungal nutritional requirements are important not only for successful cultivation in the laboratory but also for the optimization of industrial fermentation processes. In the laboratory, it is relatively easy to grow yeasts and fungi on complex culture media such as malt extract or potato-dextrose agar or broth, which are both carbon rich and in the acidic pH range. Mushrooms are cultivated on various solid-substrates depending on provincial availability. Therefore, *Agaricus bisporus* (common button mushroom) is grown in the United Kingdom, United States, and France on wheat-straw; the padi-straw mushroom (*Volvariella volvacea*) is grown in South-east Asia on damp rice-straw and in Hong Kong on cotton waste; and in Japan, the shiitake mushroom (*Lentinus edodes*) is cultivated on fresh oak logs (see Chapter 6). In industry, media for fungal fermentation purposes need to be optimized with regard to the specific application and production process. For some industrial processes, growth media may already be relatively complete in a nutritional sense, such as malt wort or molasses for brewing or baker's yeast production, respectively (Table 1.8). However, for other processes, supplementation of agriculturally derived substrates like corn steep liquor, molasses or malt broth with additional nutrients and growth factors may be necessary. For example, for penicillin production by *Penicillium* spp. the following may constitute a suitable fermentation medium – sucrose (3 g/L), corn steep liquor (100 g/L), KH_2PO_4 (1 g/L), $(NH_4)_2SO_4$ (12 g/L), $CaCl_2.2H_2O$ (0.06 g/L), phenoxyacetic acid (5.7 g/L) – whereas other industrial processes such as the growth of *Fusarium graminarium* for the production of Quorn™ mycoprotein require culture on a completely defined medium.

1.4.3 Nutrient Uptake and Assimilation

Fungal cells utilize a diverse range of nutrients and employ equally diverse nutrient acquisition strategies. Fungi are nonmotile, saprophytic (and sometimes parasitic), chemo-organotrophic organisms. They exhibit dynamic interactions with their nutritional environment that may be exemplified by certain morphological changes, depending on nutrient availability. For example, the filamentous mode of growth observed at the periphery of certain yeast colonies

Table 1.8 Principal ingredients of selected industrial media for yeasts and fungi.

Components	Molasses	Malt wort	Wine must	Cheese whey	Corn steep liquor
Carbon sources	Sucrose Fructose Glucose Raffinose	Maltose Sucrose Fructose Glucose Maltotriose	Glucose Fructose Sucrose (trace)	Lactose	Glucose, other sugars
Nitrogen sources	Nitrogen compounds as unassimilable proteins. Nitrogen sources need to be supplemented	Low molecular α-amino nitrogen compounds, ammonium ions, and a range of amino acids	Variable levels of ammonia nitrogen, which may be limiting. Range of amino acids	Unassimilable globulin and albumin proteins. Low levels of ammonium and urea nitrogen	Amino acids, protein
Minerals	Supply of P, K, and S available. High K^+ levels may be inhibitory	Supply of P, K, Mg, and S available	Supply of P, K, Mg, and S available. High levels of sulfite often present	Supply of P, K, Mg, and S	Supply of P, K, Mg, and S
Vitamins	Small, but generally adequate supplies. Biotin is deficient in beet molasses	Supply of vitamins is usually adequate. High adjunct sugar wort may be deficient in biotin	Vitamin supply generally sufficient	Wide range of vitamins present	Biotin, pyridoxine, thiamin
Trace elements	Range of trace metals present, although Mn^{2+} may be limiting	All supplied, although Zn^{2+} may be limiting	Sufficient quantities available	Fe, Zn, Mn, Ca, and Cu present	Range of trace elements present
Other components	Unfermentable sugars (2–4%), organic acids, waxes, pigments, silica, pesticide residues, caramelized compounds, betaine	Unfermentable maltodextrins, pyrazines, hop compounds	Unfermentable pentoses. Tartaric and malic acids. Decanoic and octanoic acids may be inhibitory. May be deficient in sterols and unsaturated fatty acids	Lipids, NaCl. Lactic and citric acids	High levels of lactic acid present. Fat and fibre also present

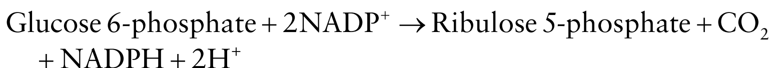
growing in agar is akin to a foraging for nutrients as observed in certain eucarpic fungi. Metabolic dynamism is also evident in yeasts which, although not avid secretors of hydrolytic enzymes like higher fungi, are nevertheless able to secrete some enzymes to degrade polymers such as starch (as in amylolytic yeasts like *Schwanniomyces* spp.).

Several cellular envelope barriers to nutrient uptake by fungal cells exist, namely the capsule, the cell wall, the periplasm and the cell membrane. Although not considered as freely porous structures, fungal cell walls are relatively porous to molecules up to an average molecular mass of around 300 Da, and will generally retain molecules greater than around 700 Da. Typically, fungi absorb only small soluble nutrients such as monosaccharides and amino acids.

The plasma membrane is the major selectively permeable barrier which dictates nutrient entry and metabolite exit from the fungal cell. Membrane transport mechanisms are important in fungal physiology since they govern the rates at which cells metabolize, grow, and divide. Fungi possess different modes of passive and active uptake at the plasma membrane: free diffusion, facilitated diffusion, diffusion channels, and active transport (Table 1.9). Active transport of nutrients such as sugars, amino acids, nitrate, ammonium, sulfate, and phosphate in filamentous fungi involves spatial separation of the ion pumps mostly behind the apex, whereas the symport proteins are active close to the tip. Thus, nutrient uptake occurs at the hyphal tip as it continuously drives into fresh resource, and the mitochondria localized behind the apex supply ATP to support the ion pump and generate proton motive force.

1.4.4 Overview of Fungal Biosynthetic Pathways

Anabolic pathways are energy-consuming, reductive processes which lead to the biosynthesis of new cellular material and are mediated by dehydrogenase enzymes which predominantly use reduced $NADP^+$ as the redox cofactor. NADPH is generated by the hexose monophosphate pathway (or Warburg–Dickens pathway) which accompanies glycolysis (see Section 1.5.1). In *S. cerevisiae*, up to 20% of total glucose may be degraded via the hexose monophosphate pathway. This pathway generates cytosolic NADPH (following the dehydrogenation of glucose 6-phosphate using glucose 6-phosphate dehydrogenase and $NADP^+$ as hydrogen acceptor) for biosynthetic reactions, leading to the production of fatty acids, amino acids, sugar alcohols, structural and storage polysaccharides, and secondary metabolites. Besides generating NADPH, the hexose monophosphate pathway also produces ribose sugars for the synthesis of nucleic acids, RNA, and DNA and for nucleotide coenzymes, NAD, NADP, FAD, and FMN. This is summarized as follows:

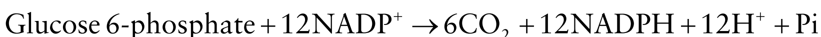


$$\text{Glucose 6-phosphate} + 2\text{NADP}^+ \rightarrow \text{Ribulose 5-phosphate} + \text{CO}_2 + \text{NADPH} + 2\text{H}^+$$

Table 1.9 Modes of nutrient transport in fungi.

Mode of nutrient transport	Description	Examples of nutrients transported
Free diffusion	Passive penetration of lipid-soluble solutes through plasma membrane following the law of mass action from a high extracellular concentration to a lower intracellular concentration	Organic acids, short-chain alkanes, and long-chain fatty acids by fungi and export of lipophilic metabolites (e.g. ethanol) and gaseous compounds)
Facilitated diffusion	Translocates solutes down a transmembrane concentration gradient in an enzyme (permease) mediated manner. As with passive diffusion, nutrient translocation continues until intracellular concentration equals that of the extracellular medium	In the yeast *S. cerevisiae*, glucose is transported in this manner
Diffusion channels	These operate as voltage-dependent "gates" to transiently move certain nutrient ions down concentration gradients. They are normally closed at the negative membrane potential of resting yeast cells but open when the membrane potential becomes positive	Ions such as potassium may be transported in this fashion
Active transport	The driving force is the membrane potential and transmembrane electrochemical proton gradient generated by plasma membrane H^+-ATPase. The latter extrudes protons using the free energy of ATP hydrolysis that enables nutrients to enter either with influxed protons, as in "symport" mechanisms, or against effluxed protons, as in "antiport" mechanisms	Many nutrients (sugars, amino acids, ions)

and complete oxidation of glucose 6-phosphate would result in:

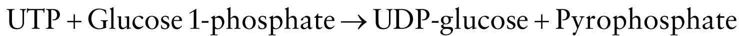


$$\text{Glucose 6-phosphate} + 12\text{NADP}^+ \rightarrow 6\text{CO}_2 + 12\text{NADPH} + 12\text{H}^+ + \text{Pi}$$

Fungal growth on noncarbohydrate substrates as sole carbon sources (e.g. ethanol, glycerol, succinate, and acetate) may lead to gluconeogenesis (conversion of pyruvate to glucose) and polysaccharide biosynthesis. Gluconeogenesis may be regarded as a reversal of glycolysis and requires ATP as energy and NADH as reducing power.

Concerning fungal amino acid biosynthesis, simple nitrogenous compounds such as ammonium may be assimilated into amino acid *families*, the carbon skeletons of which originate from common precursors of intermediary carbon metabolism.

The two main fungal storage carbohydrates are glycogen and trehalose. Glycogen is similar to starch with $\alpha(1 \rightarrow 4)$ glucan linear components and $\alpha(1 \rightarrow 6)$ branches. Trehalose (also known as mycose) is a disaccharide of glucose comprising an $\alpha,\alpha(1 \rightarrow 1)$ glucoside bond between two α-glucose units. Both trehalose and glycogen are synthesized following the formation of UDP-glucose, catalyzed by UDP-glucose pyrophosphorylase:


UTP + Glucose 1-phosphate → UDP-glucose + Pyrophosphate

Glycogen is synthesized by glycogen synthase. Glycogen may be metabolized by glycogen phosphorylase when nutrients become limited under starvation conditions and this contributes to the maintenance metabolism of cells by furnishing energy in the form of ATP. In yeast cells, glycogen breakdown is accompanied by membrane sterol biosynthesis (in the presence of some oxygen) and this is important for brewing yeast vitality and successful beer fermentations. The other major storage carbohydrate, trehalose, is synthesized from glucose 6-phosphate and UDP-glucose by trehalose 6-phosphate synthase and converted to trehalose by a phosphatase.

In addition to a storage role, trehalose is an important translocation material in filamentous forms and is also involved in stress protection in yeasts and fungi, accumulating when cells are subject to environmental insults such as heat shock or osmotic stress, or during plant host–fungal parasite interactions. Trehalose acts by protecting cell membranes against desiccation or thermal damage. Polyols, such as mannitol derived from fructose phosphate and glycerol from the glycolytic intermediate dihydroxyacetone phosphate, are also translocated by fungi. In particular, glycerol is produced as a “compatible solute” in response to osmotic stress to counteract the loss of intracellular water (see Section 1.6.1). Glycerol is also a yeast fermentation by-product and contributes to the viscosity or mouthfeel of alcoholic beverages such as beer and wine.

1.4.5 Fungal Cell Wall Growth

The structural polysaccharides in fungal cell walls include mannans, glucans, and chitin and are synthesized from sugar nucleotide substrates formed by pyrophosphorylase enzymes. For example:

Glucose 1-phosphate + UTP → UDP-glucose + PPi

Mannose 1-phosphate + GTP → GDP-mannose + PPi

Glucan synthesis involves plasma membrane-associated glucan synthetases for assembly of β-1,3 linkages and β-1,6 branches of cell wall glucan. Chitin (a polymer

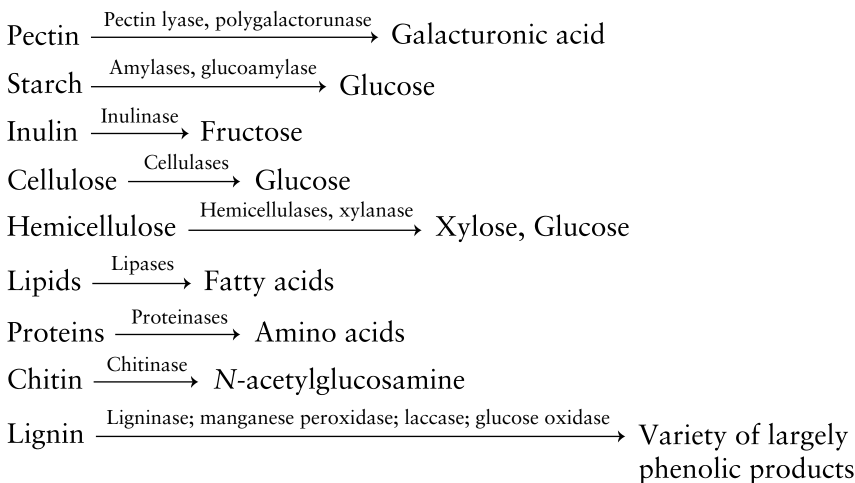
of N-acetylglucosamine) is an important fungal cell wall structural component and is involved in the yeast budding process and in dimorphic transitions from yeast to filamentous forms. Chitin synthetases catalyze the transfer of N-acetylglucosamine from UDP-N-acetylglucosamine to a growing chitin polymer within the fungal cell wall. The mannoproteins predominantly of unicellular forms are pre-assembled within the Golgi and are delivered to the cell wall via vesicles from the vesicle supply centre. Various vesicles containing cell wall-synthetic enzymes, wall-lytic enzymes, enzyme activators, and certain pre-formed wall components, are transported to the tip where they fuse with the plasma membrane and release their contents, which, together with substrates delivered from the cytosol, facilitate synthesis of the growing cell wall.

1.5 Fungal Metabolism

1.5.1 Carbon Catabolism

Being chemo-organotrophs, fungi derive their energy from the breakdown of organic compounds. Generally speaking, fungi, but few yeast species, extracellularly break down polymeric compounds by secreted enzymes prior to utilization of monomers as carbon and energy sources. Due to their relatively large size (20–60 KDa), enzymes assembled by the Golgi are transported in vesicles to be secreted from sites of cell growth, essentially from extending hyphal tips. Enzymes may either become linked to the cell wall as wall-bound enzymes, or may diffuse externally to decay substrates within the local environment.

Some examples follow of hydrolytic, oxidative, peroxidative, and free radical generating enzyme systems produced by fungi for the degradation of polymeric compounds:



Several lipolytic yeasts are known (e.g. *Candida rugosa*, *Yarrowia lipolytica*) which secrete lipases to degrade triacylgycerol substrates to fatty acids and glycerol.

In wood, the cellulose and hemicellulose components are embedded within a heteropolymeric 3-D lignin matrix, thus forming a complex lignocellulose material. Only certain filamentous basidiomycete or ascomycete fungi are able to degrade the recalcitrant lignin component, making available the cellulose or hemicellulose components. These are known as white-rot fungi due to resultant coloration of the delignified wood. Such fungi employ a cocktail of oxidative (including laccases) and peroxidative enzymes, together with hydrogen peroxide generating enzyme systems, to attack at least 15 different inter-unit bond types extant within the lignin polymer. The manganese and lignin peroxidase enzyme systems operate by releasing highly reactive but transient oxygen free radicals, which bombard and react with parts of the lignin molecule, generating a chain of chemical oxidations and producing a range of mainly phenolic end products. White-rot fungi have applications in, for example, upgrading lignocellulose waste for animal feed, paper production and bleaching, the bioremediation of contaminated land and water, and (potentially) for biofuel production (e.g. pre-treatment of lignocellulosic biomass for second-generation bioethanol). Brown-rot and soft-rot (in wet wood) fungi are only able to degrade the cellulose and hemicellulose components of wood. Cellulose decomposition involves the synergistic activity of endoglucanases (that hydrolyze the internal bonds of cellulose), exoglucanases (that cleave cellobiose units from the end of the cellulose chain), and glucosidases (that hydrolyze cellobiose to glucose). Initial attack of cellulose microfibrills within the cell wall may involve the generation of hydrogen peroxide. Commercially available cellulolytic enzymes are produced from filamentous fungal cultures, notably *Trichoderma reesei*.

Catabolic pathways are oxidative processes which remove electrons from intermediate carbon compounds and use these to generate energy in the form of ATP. The catabolic sequence of enzyme-catalyzed reactions that convert glucose to pyruvic acid is known as glycolysis, and this pathway provides fungal cells with energy, together with precursor molecules and reducing power (in the form of NADH) for biosynthetic pathways. Therefore, in serving both catabolic and anabolic functions, glycolysis is sometimes referred to as an amphibolic pathway. Glycolysis may be summarized as follows:

$$\text{Glucose} + 2\text{ADP} + 2\text{Pi} + 2\text{NAD}^+ \rightarrow 2\text{Pyruvate} + 2\text{ATP} + 2\text{NADH}^+ + 2\text{H}^+$$

During glycolysis, glucose is phosphorylated using ATP to produce fructose 1,6-biphosphate, which is then split by aldolase to form two triose phosphate compounds. Further phosphorylation occurs, forming two triose diphosphates from which four H atoms are accepted by two molecules of NAD^+. In the latter stages of glycolysis, four molecules of ATP are formed (by transfer of phosphate from the triose diphosphates to ADP) and this results in the formation of two molecules of pyruvic acid. ATP production (two molecules net) during glycolysis is referred to as substrate-level phosphorylation.

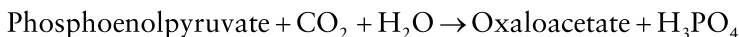
In yeast cells undergoing alcoholic fermentation of sugars under anaerobic conditions, NAD^+ is regenerated in terminal step reactions from pyruvate. In the first of these, pyruvate is decarboxylated (by pyruvate decarboxylase) before a final reduction, catalyzed by alcohol dehydrogenase (ADH) to ethanol. Such regeneration of NAD^+ prevents glycolysis from stalling and maintains the cell's oxidation–reduction balance and ATP production. Additional minor fermentation metabolites are produced by fermenting yeast cells, including glycerol, fusel alcohols (e.g. isoamyl alcohol), esters, (e.g. ethyl acetate), organic acids (e.g. citrate, succinate, acetate), and aldehydes (e.g. acetaldehyde). Such compounds are important in flavor development in alcoholic beverages such as beer, wine, and whisky.

Aerobic dissimilation of glucose by fungi leads to respiration, which is the major energy-yielding metabolic route and involves glycolysis, the citric acid cycle, the electron transport chain, and oxidative phosphorylation. Yeasts, in particular *S. cerevisiae*, are unique microorganisms in that they can switch from respiration to fermentation, and vice versa, depending on the prevailing growth conditions. In addition to glucose, many carbon substrates can be respired by fungi including: pentose sugars (e.g. xylose), sugar alcohols (e.g. glycerol), organic acids (e.g. acetic acid), aliphatic alcohols (e.g. methanol, ethanol), hydrocarbons (e.g. *n*-alkanes), and aromatic compounds (e.g. phenol). Fatty acids are made available for fungal catabolism following extracellular lipolysis of fats and are metabolized by β-oxidation in mitochondria.

During glucose respiration under aerobic conditions, pyruvate enters the mitochondria where it is oxidatively decarboxylated to acetyl CoA by pyruvate dehydrogenase, which acts as the link between glycolysis and the cyclic series of enzyme-catalyzed reactions known as the citric acid cycle (or Krebs cycle). This cycle represents the common pathway for the oxidation of sugars and other carbon sources in yeasts and filamentous fungi and results in the complete oxidation of one pyruvate molecule to: $2CO_2$, 3NADH, $1FADH_2$, $4H^+$, and 1GTP. Like glycolysis, the citric acid cycle is amphibolic since it performs both catabolic and anabolic functions, the latter providing intermediate precursors (e.g. oxaloacetate and α–ketoglutarate) for the biosynthesis of amino acids and nucleotides. The removal of intermediates necessitates their replenishment to ensure continued operation of the citric acid cycle. The glyoxylate cycle is an example of such an *anaplerotic* reaction and involves the actions of the enzymes pyruvate carboxylase:

$$\text{Pyruvate} + CO_2 + \text{ATP} + H_2O \rightarrow \text{Oxaloacetate} + \text{ADP} + \text{Pi}$$

and phosophoenolpyruvate carboxykinase:



$$\text{Phosphoenolpyruvate} + CO_2 + H_2O \rightarrow \text{Oxaloacetate} + H_3PO_4$$

During the citric acid cycle, dehydrogenase enzymes transfer hydrogen atoms to the redox carriers NAD^+ and FAD, which become reduced. On the inner membrane of mitochondria, these reduced coenzymes are then re-oxidized, and

Table 1.10 Respiratory chain characteristics of yeasts and fungi.

Type	Typical species	Sensitive to	Insensitive to
Normal respiration	All aerobic fungi	Cyanide and low azide[1]	SHAM[2]
Classic alternative	*Yarrowia lipolytica* (and in stationary phase cultures of several yeast species)	SHAM	Cyanide, high azide
New alternative	*Schizosaccharomyces pombe*, *Saccharomyces cerevisiae*, *Kluyveromyces lactis*, *Williopsis saturnus*	High azide	Cyanide, low azide, SHAM

[1]The azide-sensitive pathway lacks proton transport capability and accepts electrons from NADH but not from succinate.
[2]The SHAM (salycil hydroxamate)-sensitive pathway transports electrons to oxygen also without proton transport, and therefore does not phosphorylate ADP.
Adapted from Walker (1998).

oxygen is reduced to water via the electron transport chain. Energy released by electron transfer is used to synthesize ATP by a process called oxidative phosphorylation. The chemiosmotic theory describes proton pumping across the inner mitochondrial membrane to create a transmembrane proton gradient (ΔpH) and a membrane potential difference. Together, these comprise the proton motive force that is the driving force for ATP synthesis. Each pair of electrons in NADH yields about 2.5 ATP, while residual energy is largely dissipated as metabolic heat. Since mitochondria are impermeable to NADH, this reduced coenzyme generated in the cytoplasm during glycolysis is "shuttled" across the mitochondrial membrane using either the *glycerophosphate shuttle* (that uses NADH to reduce dihydroxyacetone phosphate to glycerol 3-phosphate) or the *malate shuttle* (that uses NADH to reduce oxaloacetate to malate). These processes enable molecules to be oxidized within mitochondria to yield reduced cofactors, which in turn are oxidized by the electron transport chain.

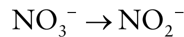
Fungi use molecular oxygen as a terminal electron acceptor in aerobic respiration in different ways (Table 1.10). Some yeasts, including *S. cerevisiae*, exhibit *alternative respiration* characterized by insensitivity to cyanide but sensitivity to azide.

1.5.2 Nitrogen Metabolism

Fungi assimilate simple nitrogenous sources for the biosynthesis of amino acids and proteins. For example, ammonium ions are readily utilized and can be directly assimilated into the amino acids glutamate and glutamine that serve as precursors for the biosynthesis of other amino acids. Proteins can also be utilized

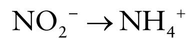
following release of extracellular protease enzymes. Glutamate is a key compound in both nitrogen and carbon metabolism, and glutamine synthetase is important as it catalyzes the first step in pathways leading to the synthesis of many important cellular macromolecules. Other important enzymes of fungal nitrogen metabolism include glutamate dehydrogenase and glutamate synthase (glutamine amide: 2-oxoglutarate-aminotransferase, or GOGAT), the latter requiring ATP. When glutamine synthetase is coupled with glutamate synthase this represents a highly efficient "nitrogen-scavenging" process for fungi to assimilate ammonia into amino acids and citric acid cycle intermediates. The particular route(s) of ammonium assimilation adopted by fungi depend on the concentration of available ammonium ions and the intracellular amino acid pools.

Some yeasts (but not *S. cerevisiae*) and fungi can use *nitrate* as a sole source of nitrogen through the activities of nitrate reductase:



$$NO_3^- \rightarrow NO_2^-$$

and nitrite reductase:



$$NO_2^- \rightarrow NH_4^+$$

The resulting ammonium ions can then be assimilated into glutamate and glutamine that represent end products of nitrate assimilation by yeasts.

Urea can also be utilized following its conversion to ammonium by urea aminohydrolase (urea carboxylase plus allophanate hydrolase):

$$NH_2CONH_2 + ATP + HCO_3^- \rightarrow NH_2CONHCOO^- \rightarrow 2NH_4^+ + 2HCO_3^-$$

Amino acids can either be assimilated into proteins or dissimilated by decarboxylation, deamination, transamination, and fermentation. Amino acid degradation by yeasts and fungi yields both ammonium and glutamate. During fermentation, yeasts may produce higher alcohols or *fusel oils* such as isobutanol and isopentanol following amino acid deamination and decarboxylation. These represent important yeast-derived flavor constituents in fermented beverages.

1.6 Fungal Growth and Reproduction

1.6.1 Physical Requirements for Growth

Most yeast and fungal species thrive in warm, sugary, acidic, and aerobic conditions. The temperature range for fungal growth is quite wide, but generally speaking most species grow very well around 25 °C. Low-temperature psychrophilic fungi and high-temperature thermophilic fungi do, however, exist in nature. Fungal growth at various temperatures depends not only on the genetic background of the species but also on other prevailing physical growth parameters and nutrient

availability. With regard to high temperature stress (or heat shock) on fungal cells, thermal damage can disrupt hydrogen bonding and hydrophobic interactions, leading to general denaturation of proteins and nucleic acids.

Fungi, of course, have no means of regulating their internal temperature, and the higher the temperature, the greater the cellular damage, with cell viability declining when temperature increases beyond growth optimal levels. Temperature optima vary greatly in fungi, with those termed "thermotolerant" growing well above 40 °C. Thermotolerance relates to the transient ability of cells subjected to high temperatures to survive subsequent lethal exposures to elevated temperatures, such that *intrinsic* thermotolerance is observed following a sudden heat shock (e.g. to 50 °C), whereas *induced* thermotolerance occurs when cells are pre-conditioned by exposure to a mild heat shock (e.g. 30 minutes at 37 °C) prior to a more severe heat shock. Heat-shock responses in fungi occur when cells are rapidly shifted to elevated temperatures, and if this is sublethal, induced synthesis of a specific set of proteins – the highly conserved "heat-shock proteins" (HSPs) – occurs. HSPs play numerous physiological roles, including thermoprotection.

High water activity, a_w, is required for growth of most fungi, with a minimum a_w of around 0.65. Water is absolutely essential for fungal metabolism, and any external conditions that result in reduced water availability to cells (i.e. "osmostress") will adversely affect cell physiology.

The term water potential refers to the potential energy of water and closely relates to the osmotic pressure of fungal growth media. Certain fungal species, for example the yeast *Zygosaccharomyces rouxii* and some *Aspergillus* species, are able to grow in low water potential conditions (i.e. high sugar or salt concentrations) and are referred to as osmotolerant or zerotolerant. By comparison, *S. cerevisiae* is generally regarded as a nonosmotolerant yeast. Mild water stress, or *hyperosmotic shock*, occurs in fungi when cells are placed in a medium with low water potential brought about by increasing the solute (e.g. salt, sugar) concentration. Conversely, cells experience a *hypo-osmotic shock* when introduced to a medium of higher osmotic potential (due to reducing the solute concentration).

Fungi are generally able to survive such short-term shocks by altering their internal osmotic potential (e.g. by reducing intracellular levels of K+ or glycerol). Glycerol is an example of a *compatible solute* that is synthesized in order to maintain low cytosolic water activity when the external solute concentration is high. Glycerol can effectively replace cellular water, restore cell volume, and enable fungal metabolism to continue. Trehalose, arabitol, and mannitol can similarly protect against osmotic stress. Evidence suggests that the accumulation of compatible solutes is attributed not only to their synthesis but also to control of membrane fluidity, thus preventing their leakage to the external environment.

As for pH, most fungi are acidophilic and grow well between pH 4 and 6, but many species are able to grow, albeit to a lesser extent, in more acidic or alkaline conditions (around pH 3 or 8, respectively). Fungal cultivation media acidified

with organic acids (e.g. acetic, lactic acids) are more inhibitory to growth compared with those acidified with mineral acids (e.g. hydrochloric, phosphoric acids) because organic acids can lower intracellular pH (following their translocation across fungal plasma membranes). Exposure to organic acids leads to cells exhausting their energy (ATP) when endeavouring to maintain pH homeostasis through the activities of proton-pumping ATPase in the plasma membrane. This forms the basis of action of weak acid preservatives in inhibiting the growth of food spoilage fungi. Many filamentous fungi can alter their local external pH by selective uptake and exchange of ions (NO_3^- or NH_4^+/H^+), or by excretion of organic acids such as oxalic acid.

Other physical parameters influencing fungal physiology include radiation (light or UV may elicit mycelial differentiation and sporulation in some fungi that produce airborne spores), aeration, pressure, centrifugal force, and mechanical shear stress.

1.6.2 Cellular Reproduction

Fungal growth involves transport and assimilation of nutrients, followed by their integration into cellular components, followed by biomass increase and eventual cell division (as in yeasts) or septation (as in higher fungi). The physiology of vegetative reproduction and its control in fungi has been most widely studied in two model eukaryotes, the budding yeast, *Saccharomyces cerevisiae*, and the fission yeast, *Schizosaccharomyces pombe*.

Budding is the most common mode of vegetative reproduction in yeasts and multilateral budding is typical in ascomycetous yeasts (Table 1.11). In *S. cerevisiae*, buds are initiated when mother cells attain a critical cell size and this coincides with the onset of DNA synthesis. The budding processes result from localized weakening of the cell wall and this, together with tension exerted by turgor pressure, allows extrusion of cytoplasm in an area bounded by a new cell wall. Cell wall polysaccharides are mainly synthesized by glucan and chitin synthetases. Chitin is a polymer of N-acetylglucosamine and this material forms a ring between the mother cell and the bud that will eventually form the characteristic *bud scar* after cell division. Under optimized growth conditions, budding yeasts, typified by *S. cerevisiae*, can complete their budding cell division cycle in around 2 hours.

Fission yeasts, typified by *Schizosaccharomyces* spp., divide exclusively by forming a cell septum, which constricts the cell into two equal-sized daughters. In *Schiz. pombe*, newly divided daughter cells grow in length until mitosis is initiated when cells reach a constant cell length (about 14 μm). The cell septum in *Schiz. pombe* forms by lateral growth of the inner cell wall (the primary septum) and proceeds inwardly, followed by deposition of secondary septa. Cellular fission, or transverse cleavage, is completed in a manner resembling the closure of an iris diaphragm.

Table 1.11 Modes of vegetative reproduction in yeasts.

Mode	Description	Representative yeast genera
Multilateral budding	Buds may arise at any point on mother cell surface, but never again at the same site. Branched chaining may occasionally follow multilateral budding when buds fail to separate	*Saccharomyces*, *Zygosaccharomyces*, *Torulaspora*, *Pichia*, *Pachysolen*, *Kluyveromyces*, *Williopsis*, *Debaryomyces*, *Yarrowia*, *Saccharomycopsis*, *Lipomyces*
Bipolar budding	Budding restricted to poles of elongated cells (apiculate or lemon-shaped) along their longitudinal axis	*Nadsonia*, *Saccharomycodes*, *Haneniaspora*, *Wickerhamia*, *Kloeckera*
Unipolar budding	Budding repeated at same site on mother cell surface	*Pityrosporum*, *Trigonopsis*
Monopolar budding	Buds originate at only one pole of the mother cell	*Malassezia*
Binary fission	A cell septum (cell plate or cross-wall) is laid down within cells after lengthwise growth and which cleaves cells into two	*Schizosaccharomyces*
Bud fission	Broad cross-wall at base of bud forms which separates bud from mother	Occasionally found in *Saccharomycodes*, *Nadsonia*, *Pityrosporum*
Budding from stalks	Buds formed on short denticles or long stalks	*Sterigmatomyces*
Ballistoconidiogenesis	Ballistoconidia are actively discharged from tapering outgrowths on the cell	*Bullera*, *Sporobolomyces*
Pseudomycelia	Cells fail to separate after budding or fission to produce a single filament. Pseudomycelial morphology is quite diverse and extent of differentiation variable depending on yeast species and growth conditions	Several yeast species may exhibit "dimorphism," e.g. *Candida albicans*, *Saccharomycopsis figuligera*. Even *S. cerevisiae* exhibits pseudohyphal growth depending on conditions

Adapted from Walker (1998).

In certain yeast species, the presence or absence of pseudohyphae and true hyphae can be used as taxonomic criteria (e.g. the ultrastructure of hyphal septa may discriminate between certain ascomycetous yeasts). Some yeasts grow with true hyphae initiated from *germ tubes* (e.g. *Candida albicans*), but

others (including *S. cerevisiae*) may grow in a pseudohyphal fashion when starved of nutrients or when subjected to environmental stress. Filamentous growth of yeasts by hyphal or pseudohyphal extension represents a different developmental pathway that is generally reversible. In other words, cells can revert to yeast unicellular growth in more conducive growth conditions, indicating that a filamentous mode of growth represents an adaptation by yeast to foraging when nutrients are scarce.

What constitutes a cell in filamentous fungi is ambiguous. The apical compartments of higher filamentous fungi are often multinucleate, and so the process of nuclear replication and segregation into a newly extended septated hyphal compartment is known as the duplication cycle. Thus, *Aspergillus nidulans* apical compartments contain approximately 50 nuclei per compartment produced during a 2-hour duplication cycle period. Continued septation results in the formation of subapical compartments containing fewer nuclei.

Hyphae also commonly branch, usually at some distance behind the leading growing hyphal tip and often just behind a septum in higher fungi. The processes that control branching are not fully elucidated, but branch initiation is associated with the appearance of a Spitzenkörper at the site of tip emergence and extension. Mathematical and computational models coupled with experimental data are being used to test our understanding of fungal growth not just at the hyphal tip but across multiple spatiotemporal scales and within communities. Branching allows filamentous fungi to fill space in an efficient and appropriate way, and according to local environmental circumstances. Therefore, fungi colonizing nutrient-rich substrata branch frequently, producing dense mycelia for resource exploitation, whereas hyphae colonizing nutrient-poor substrata branch less frequently, producing effuse mycelia appropriate for resource exploration.

Rates of branching and tip growth are related to the cytoplasmic volume. Thus, the hyphal growth unit is a measure of the average length of hypha required to support hyphal tip growth. It can be calculated from microscopic preparations growing on agar media as the ratio between the total length of mycelium and the total number of tips. The ratio becomes constant after the initial stages of growth, and is characteristic of each fungal species or strain.

1.6.3 Population Growth

When yeast or fungal cells are inoculated into a nutrient medium and incubated under optimal physical growth conditions, a typical batch growth curve will result comprising lag, exponential, and stationary phases. The *lag phase* represents a period of zero population growth and reflects the time required for inoculated cells to adapt to their new physical and chemical growth environment (by synthesizing ribosomes and enzymes). The *exponential phase* is a period of logarithmic cell (or mycelial biomass in the case of filamentous

growth) doublings and constant, maximum specific growth rate (μ_{max}, in dimensions of reciprocal time, per hour), the precise value of which depends on the prevailing growth conditions. If growth is optimal and cells double logarithmically, then

$$\frac{dx}{dt} = \mu^{x}_{max}$$

and when integrated this yields

$$\ln x - \ln x_0 = \mu^{t}_{max}$$

(where x_0 is the initial cell mass) or

$$x = x_0 \exp\left(\mu^{t}_{max}\right)$$

which is the fundamental equation for exponential batch growth. According to these kinetic expressions, a plot of ln *x* versus time is linear, with the slope being μ_{max}. Calculation of the doubling time (t_d) of a yeast or fungal culture can be acheved from knowledge of μ_{max} as follows:

$$t_d = \frac{\ln 2}{\mu_{max}} = \frac{0.693}{\mu_{max}}$$

During the exponential phase of balanced growth, cells are undergoing primary metabolism, explicitly those metabolic pathways that are essential for growth of the cell. Industrial fermentations requiring maximum cell biomass production or the extraction of primary metabolites or their products therefore aim to extend this phase of growth, often via fed-batch culture (incremental nutrient feeding) or continuous culture techniques (continuous nutrient input with concomitant withdrawal of the biomass suspension).

Following the exponential phase, cells enter a period of zero population growth rate, the stationary phase, in which the accumulated fungal or yeast biomass remains relatively constant and the specific growth rate returns to zero. After prolonged periods in stationary phase, individual cells may die and autolyze (see below). The stationary phase may be defined as cellular survival for prolonged periods (i.e. months) without added nutrients. In addition to nutrient deprivation, other physiological causes may promote entry of fungal cells into stationary phase, including toxic metabolites (e.g. ethanol in the case of yeasts), low pH, high CO_2, variable O_2, and high temperature. During the stationary phase of unbalanced growth, fungi may undergo secondary metabolism, specifically initiating metabolic pathways that are not essential for growth of cells but are involved in the survival of the organism. The industrial production of fungal secondary metabolic compounds such as penicillin and the ergot alkaloids therefore involves the controlled maintenance of cell populations within a stationary phase of growth. Recently, *S. cerevisiae* has been grown at near-zero growth rates in specialized cultivation systems called *retentostats*, in which cells can

retain high metabolic capacities and stress resistance. Retaining yeast cells under such maintenance-energy metabolic conditions may have relevance for industrial bioprocesses.

Filamentous fungi tend to grow as floating surface pellicles when cultivated in static liquid culture. In agitated liquid culture, fungi grow either as dispersed filamentous forms, or as pellets of aggregated mycelia, subject to species, inoculum size, agitation rate, and nutrient availability. Different growth forms will locally experience different micro-environmental conditions, which will affect fungal physiology and hence fermentation processes. In fungal biotechnology, cell morphology may directly influence fermentation progress. For example, the rheological properties of the growth medium, oxygen transfer, and nutrient uptake may adversely affect bioproduct formation. In the natural environment, fungal populations interact frequently to form often complex dynamic communities, which in turn shape ecosystem functioning. Understanding the population growth and functional (physiological) responses of fungi to their local environment is key to the development of predictive models and to our general understanding of the resilience and resistance of fungal communities to environmental perturbations such as climate change.

Yeast or fungal cell immobilization onto inert carriers has many advantages over free cell suspension culture in industrial processes. Cells may be successfully immobilized either by entrapment, aggregation, containment, attachment or deposition. Fungal biofilms represent a natural form of cell immobilization resulting from cellular attachment to solid support materials. Yeast biofilms have several practical applications in fermentation biotechnology and are also medically important with regard to colonization of human tissue. Regarding the former case, with dimorphic yeasts such as *Kluyveromyces marxianus*, filamentous cells with a large surface area may be better suited to immobilization compared with ellipsoidal unicellular yeast forms with a low surface area. In this latter case of pathogenic yeast biofilms, *C. albicans* has been shown to adhere to surgical devices such as heart pacemakers and catheters, human epithelial cells, and dental acrylic.

1.6.4 Fungal Cell Death

An understanding of the death of fungal cells is important from a fundamental viewpoint because fungi, especially yeasts, represent valuable model systems for the study of cellular aging and apoptosis (programed cell death). Recycling and redeployment of cellular material also helps drive the apical growth of filamentous fungi and the mycelium explores and extends through the environment. From a practical perspective, cell death in fungi is pertinent in relation to the following situations: industrial fermentation biotechnology (where high culture viabilities are desired), food preservation (regarding inhibition of spoilage fungal growth), food production (promotion of cellular autolysis for yeast extracts), and clinical mycology (where fungal death is the goal in treatment of human mycoses).

Numerous physical, chemical, and biological factors influence fungal cell death, which may be defined as complete and irreversible failure of cells to reproduce. Fungi will die if confronted with excessive heat, extreme cold, high-voltage electricity, ionizing radiation, high hydrostatic and osmotic pressures, and if exposed to chemical or biological fungicidal agents. When the cells' physiological protection responses are insufficient to counteract the cellular damage caused by physical stress, cells will die. In industrial situations, physical treatments can be used to eradicate contaminant fungi. For example, yeasts exposed to elevated temperatures may lead to their thermal death, and this is exploited in the pasteurization of foods and beverages to kill spoilage yeasts.

There are numerous chemical factors influencing survival of fungi. Several external chemical agents act as fungicides, including toxic organic compounds, oxygen free radicals, and heavy metals. Chemical preservatives are commonly employed as antifungal agents in foodstuffs, including weak acids such as sorbic, benzoic, and acetic acids. These agents, which are generally fungistatic rather than fungicidal, act by dissipating plasma membrane proton gradients and depressing cell pH when they dissociate into ions in the yeast cytoplasm. Similarly, sulfur dioxide which has long been used to eliminate undesirable yeasts (and bacteria) from wine, dissociates within the yeast cell to SO_3^{2-} and HSO_3^-, resulting in a decline in intracellular pH and this forms the basis of its antizymotic action. Fungicidal acids include medium-chain fatty acids (e.g. decanoic acid), which may cause rapid cell death of yeasts and fungi by disruption of cell membrane integrity. Endogenous chemical factors such as ethanol and other toxic metabolites (e.g. acetaldehyde) produced by fermentative activity, excessive intracellular acidity or alkalinity, or inability to protect against oxidative damage or sequester toxic metals, may also prove lethal to fungi. If fungal cells are unable to detoxify or counteract detrimental effects of chemicals, they may die.

Examples of lethal biotic interactions with fungi include direct ingestion (by insects, protozoa), engulfment and lysis (by mycoparasitizing fungi), direct predation (by haustoria-mediated processes), and intoxication (by killer toxin-producing yeasts). *Killer yeasts* secrete proteinaceous toxins that are lethal to other yeasts but to which the killers themselves are immune. Several yeast species have now been identified as possessing killer character, but the best known is the K1 system in *S. cerevisiae*. The K1 toxin from this species acts by binding to cell wall receptors in sensitive yeast cells, followed by plasma membrane channel formation. This latter event causes disruption of membrane permeability, which leads to the death of sensitive cells. Killer cells synthesize a membrane-bound immunity protein that prevents cellular suicide. In recent years, it has been established that some killer yeasts may also possess antimycotic activity against filamentous fungi. This has led to the potential use of killer yeasts and their toxins as novel antifungal biocontrol agents for combating important fungal pathogens in agriculture. For example, the killer yeast *Pichia anomala* (*Wickerhamomyces anomalus*) has been shown to inhibit the growth of grain-storage fungi (*Penicillium* spp.) and fungal spoilage of fruits (caused by *Botrytis cinerea*).

With regard to endogenous biotic factors influencing fungal cell survival, several physiological, morphological, genetic, and biochemical events may take place leading to "self-inflicted" death. For example, fungal autolysis may be described as cellular self-digestion and occurs when endogenous (vacuolar) hydrolytic enzymes, notably proteases and carbohydrases, cause dissolution of cytoplasmic proteins and cell wall polysaccharides, respectively. Autolytic enzymatic activity is encouraged during the production of yeast extracts in the food industry by using high temperatures (e.g. 45 °C), salt (to encourage plasmolysis) and solvents (to promote lipid dissolution). Exogenous hydrolytic enzymes such as papain can also be used to accelerate cell wall breakdown.

Genetic factors also influence fungal cell death. For example, cells may commit suicide following DNA damage, presumably to avoid the risk of producing genetically altered progeny. Cellular aging and apoptotic cell death has been widely studied in yeasts, especially *S. cerevisiae*, which is a valuable model organism for understanding molecular genetic basis of the aging process in eukaryotic cells. Beyond a certain finite limit (termed the Hayflick limit) of cell division cycles (generally around 20 in *S. cerevisiae*), this yeast can generate no further progeny and cells enter a senescent physiological state leading to death. Aged and senescent populations of this yeast can be isolated, together with mutants displaying age-related phenotypes. In *S. cerevisiae*, *UTH* (youth) genes have now been identified which appear to influence both stress resistance and longevity.

1.7 Conclusion

This chapter highlights the physiological diversity of yeasts and fungi in terms of morphology, growth, metabolism, and cell death. Understanding the ways in which fungi interact with their growth environment is crucial in medical mycology to control fungal pathogens, and also in industry to exploit yeasts and fungi for production of biotechnological commodities.

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2
Fungal Genetics

Malcolm Whiteway and Catherine Bachewich

2.1 Introduction

2.1.1 Fungi as Pioneer Organisms for Genetic Analysis

Genetic manipulation of organisms implies the ability to direct the formation of new combinations of traits within an individual. This process has historically been an important human endeavor, providing us with our breeds of domestic animals, economically important plants, and industrially important fungi. Genetic manipulation can be as simple as identifying and selecting, within a population, rare individuals that contain interesting traits, but is made more powerful by the ability to enhance the rate of individual variation, and more powerful still when traits identified in different individuals can be combined. Thus, mutagenesis and genetic recombination underpin the process of genetic manipulation. Such genetic manipulation can be used directly for practical ends (new varieties of tomatoes for example), or for more academic aims directed at an understanding of life.

Fungi were among the first organisms to be studied scientifically through genetics. Although peas and fruit flies provided the initial evidence for genes and for genetic linkage, some of the earliest fundamental insights into the genetic structure of organisms came from pioneering studies in fungal systems. One exceptional insight developed from analysis of fungal systems, in this case *Neurospora crassa*, was the recognition that individual enzymatic functions were encoded by the information from individual genes, a result rewarded by the 1958 Nobel Prize in Physiology or Medicine to G. Beadle, E. Tatum, and J. Lederberg. Other fundamental advances based on the genetic analysis of fungi

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included the dissection of the cell cycle of the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, and the analysis of *S. cerevisiae* telomeres, work that led to the 2001 Nobel Prize in Physiology or Medicine to L. Hartwell, P. Nurse, and T. Hunt, and the 2009 prize to J. Szostak, E. Blackburn, and C. Greider, respectively. These awards, more than 40 years after that to Beadle, Tatum, and Lederberg, show that fungal systems have maintained their utility in uncovering important biological truths.

More recently, large-scale, semi-industrialized international efforts have had an enormous impact on the field of fungal genetics. These efforts have been at the forefront of the development of the science of genomics. Many of the primary successes of genomics have come through studies on fungi, in particular the baker's yeast *S. cerevisiae*. Efforts directed at the analysis of the yeast genome have provided the first sequence of a eukaryotic chromosome, the first sequence of an entire eukaryotic genome, and the first development of a systematically created collection of null mutants of all the genes of an organism. Building on this pioneering work, the sequences of many fungal genomes are now fully completed or available as un-annotated draft sequences.

2.1.2 Significance/Advantages of Fungi as Model Organisms

Fungi have become among the pre-eminent models for the genetic investigation of basic cellular processes. There are many intrinsic characteristics of fungal systems that make them ideal model organisms for genetic studies. As unicellular organisms that can grow in simple defined media they are easy to culture. Because they often contain a stable, propagatable haploid phase they are easy to mutate, and in those organisms with a well-characterized sexual cycle the mutants can be readily combined. Finally, because they are eukaryotic cells they exhibit many of the properties and functions characteristic of human cells, and thus served as a better model for many cellular processes than the bacterial systems that had been investigated in depth previously.

The advent of the molecular biological revolution in the 1970s strengthened the importance of fungal systems as models for genetic studies of eukaryotic cell function. Because each individual fungal cell was autonomous, transformation with external DNA allowed the efficient genetic engineering of an entire organism. The rapid development of fungal transformation systems after the initial successes with *S. cerevisiae*, followed by the construction of efficient vectors for the transfer of genes and the cloning of the various components of chromosomes, allowed for effective manipulation of fungal cells. Ultimately, this technology permits the construction of strains of fungi genetically designed to differ from a standard strain by as little as a single selected nucleotide.

This chapter provides an overview of various aspects of fungi and their use as model organisms for genetic analysis. Representative species from the higher fungi, including ascomycetes and basidiomycetes, comprise the focus for

discussion, and comparisons between the yeasts (*S. cerevisiae*) and filamentous organisms (*Neurospora crassa*, *Aspergillus nidulans*, *Coprinus cinereus*) are emphasized to introduce the advantages of particular systems, as well as the diversity within the kingdom. Examples of dimorphic fungi, which live in both a yeast and filamentous form, are also discussed to highlight unique features and variations on themes within the fungi.

2.2 Fungal Lifecycles

2.2.1 Ascomycete Yeast (*Saccharomyces cerevisiae*)

Saccharomyces cerevisiae is an extremely well-studied organism, with a clearly defined and experimentally manipulable lifecycle. The lifecycle of yeast involves mitotically propagating haploid forms of two distinct mating types, and a diploid form that can either grow vegetatively or be induced into a meiotic developmental pathway through manipulation of the nutrient conditions of the growth media. The cellular pathways regulating processes such as mitotic proliferation, cell recognition and mating, meiosis, and sporulation have been extensively studied on a molecular level, and are generally well understood.

Mitotic growth of yeast cells involves budding (Figures 2.1 and 2.6c). During this process, growth of the cell is directed to a specific location on the surface of the mother cell, and a new cell is formed somewhat like blowing up a balloon through a hole in the mother cell. This involves highly polarized growth of the developing daughter cell, implicating both the actin- and microtubule-based cytoskeletal networks, and is tightly coordinated with the cell cycle. This coordination ensures that the daughter cell receives a complete copy of the genetic material. Both haploid and diploid cells divide by the budding process, although there are subtle differences in the choice of the sites of bud emergence between haploids and diploids. In addition, some diploid cells can modify the coordination of the cell cycle and polarized growth to switch to a pseudohyphal growth mode. In this growth pattern individual cells are more elongate, and the budding pattern leads to the formation of chains of cells rather than compact colonies characteristic of the true budding mode.

Genetic analysis is highly developed in *S. cerevisiae*. When vegetatively growing haploid cells of opposite mating types are brought into proximity, they communicate with each other by diffusible pheromones, synchronize their cell cycles, conjugate, and then fuse their nuclei to create non-mating, meiosis-proficient diploids. These diploids can be identified visually in their initial zygote form, and separated from the haploids by micromanipulation, or identified selectively because they contain a pattern of genetic traits not possessed by either haploid parent.

Under rich growth conditions, such diploid cells themselves propagate vegetatively, but under conditions of nitrogen and fermentable carbon limitation,

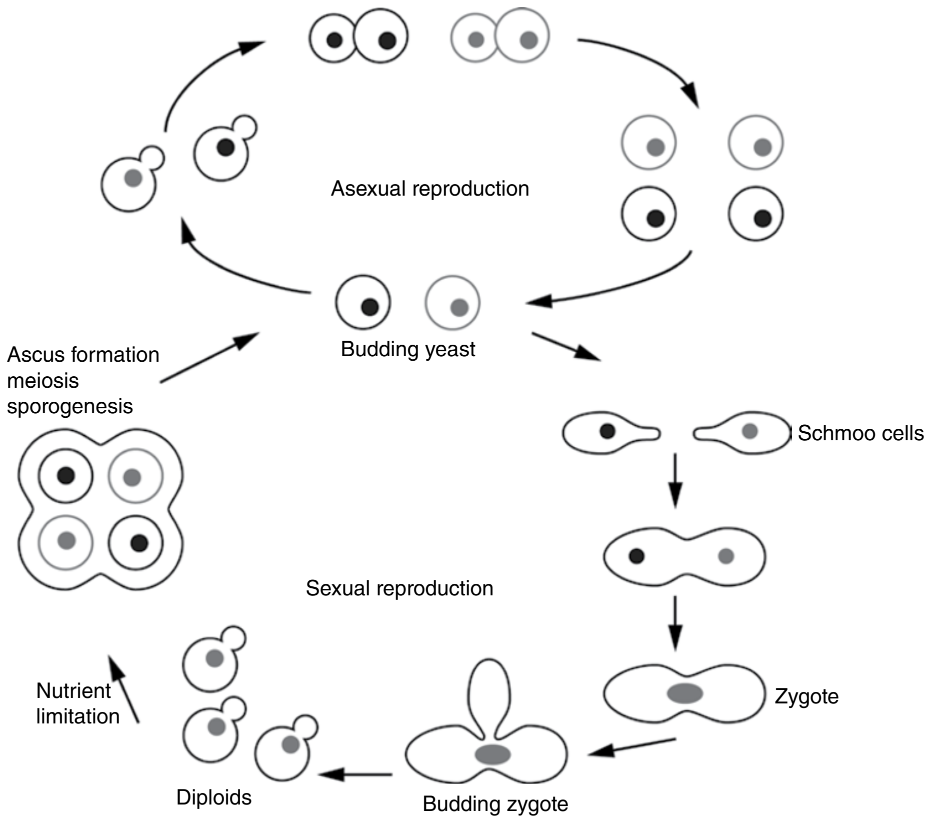

Figure 2.1 Lifecycle of *Saccharomyces cerevisiae.*

the diploid cells are induced to initiate meiosis and sporulation. The ability to propagate the diploid allows the amplification of the initial mating product, and provides an essentially unlimited source of potential meiotic events from a single mating.

2.2.2 Ascomycete Filamentous Fungi (*Neurospora crassa* and *Aspergillus nidulans*)

The filamentous fungi differ from the yeasts in that they grow vegetatively as hyphae, which are highly polarized filaments that extend indefinitely at their tips. The hypha initiates new tips in the form of branches from subapical regions, and together the growing mass constitutes the mycelium. Hyphae are predominantly multinucleated, with cross-walls called septa dividing the hypha into compartments. The compartments are connected through pores in the septa, and therefore display cytoplasmic continuity. The hypha functions primarily in acquisition of nutrients and exploration of the environment. Enzyme secretion at the tip assists both processes. In pathogenic fungi, the hyphal growth form can

also be important for virulence. In filamentous fungi, vegetative hyphal growth initiates from a spore. Spores are products of either sexual (ascospores, basidiospores) or asexual (conidia) reproduction. Conidia are typically produced from a differentiated structure called a conidiophore, whereas ascospores and basidiospores are produced within an ascus or basidium, respectively, contained within the fruiting body called an ascocarp or basidiocarp.

During asexual reproduction in the ascomycetes, such as *A. nidulans* (Figures 2.2 and 2.6a), a spore containing a single nucleus (monokaryotic) germinates into a multinucleate, homokaryotic hypha. The hypha grows and develops branches for a period of time, then initiates a specialized branch called

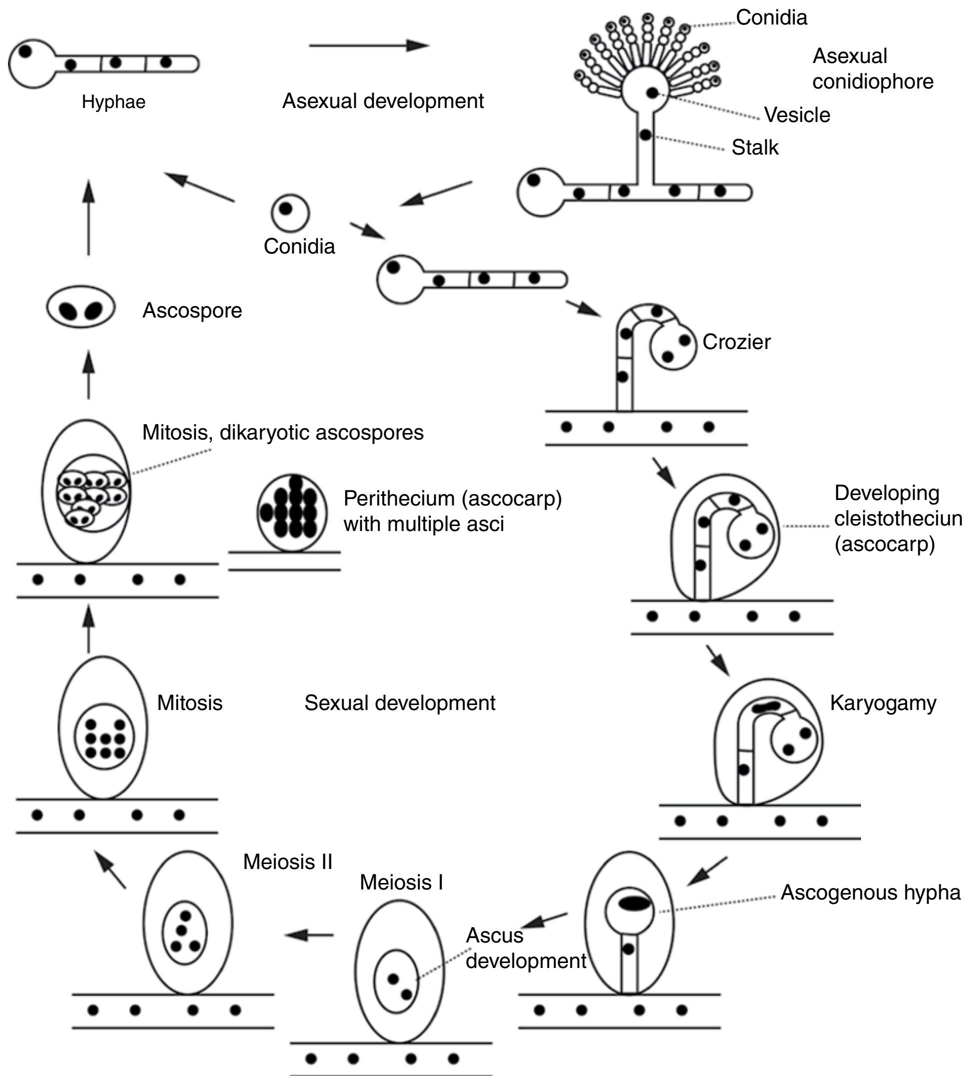

Figure 2.2 Lifecycle of *Aspergillus nidulans*.

the conidiophore. Development of the conidiophore involves numerous different cell types, and is investigated as a model developmental process. The nucleus divides mitotically within the conidiophore, allowing the ultimate production of asexual, haploid conidia. Upon release, conidia germinate into vegetatively growing hyphae, and the cycle continues. The factors that trigger initial conidiophore development in *Aspergillus* are not clear, but involve the supply of carbon and nitrogen. The process can normally only occur in cultures grown on solid media with an air interphase; conidiation does not occur in liquid.

Sexual reproduction in *A. nidulans* begins when vegetatively growing hyphae fuse to create a heterokaryon, or dikaryotic hypha (Figure 2.2). The dikaryotic hyphae differentiate into a developing fruiting body called a cleistothecia. The fruiting body is a complex structure composed of many cell types, including both sterile and fertile hyphae. The dikaryotic fertile hyphae within the cleistothecium develop into hooked structures called croziers, which then differentiate into developing asci. Karyogamy or nuclear fusion occurs within the crozier, creating a diploid. The diploid undergoes meiosis and the four meiotic products then undergo mitosis, creating eight haploid ascospores. The ascospores undergo another round of mitosis and are thus binucleate. Thousands of asci are contained within a cleistothecium and are fragile, hampering their individual isolation. Upon release, the ascospores germinate into hyphae as described.

Aspergillus is homothallic, or self-fertile, and sexual reproduction can be initiated within one colony containing genetically identical nuclei. In the absence of heterokaryon formation with another strain, the individual strain differentiates a cleistothecium as described, into which the hypha develops into a crozier and an ascogenous hypha. Unlike *S. cerevisiae*, *A. nidulans* does not undergo any mating-type switching. *Aspergillus nidulans* hyphae can also grow as heterokaryons and diploids as part of a parasexual cycle, which is discussed later in this chapter.

In *N. crassa* (Figure 2.3), asexual reproduction is triggered by circadian rhythms, or an internal clock mechanism, and produces both macro- and microconidia. Macroconidia are produced first from aerial hyphae, and are used for subculturing strains, while microconidia are produced later in the growth process and have poor viability. Macroconidia germinate into vegetatively growing hyphae, but also serve a function during sexual reproduction.

The sexual cycle is initiated in response to nitrogen starvation, or changes in temperature or light. *Neurospora crassa* is heterothallic, and therefore requires genetically different mating partners. Macroconidia or microconidia produced from hyphae serve as the "male" and produce a pheromone, which is a hydrophobic peptide. The opposite strain serving as the female develops a fruiting body intermediate called a protoperithecia. A polarized structure called a trichogyne grows from the protoperithecium of one mating-type female and fuses with the male conidia of the opposite mating type. The nucleus from the latter moves through the trichogyne into the ascogonium within the protoperithecium, which is then referred to as the perithecium. Nuclei from both mating partners divide

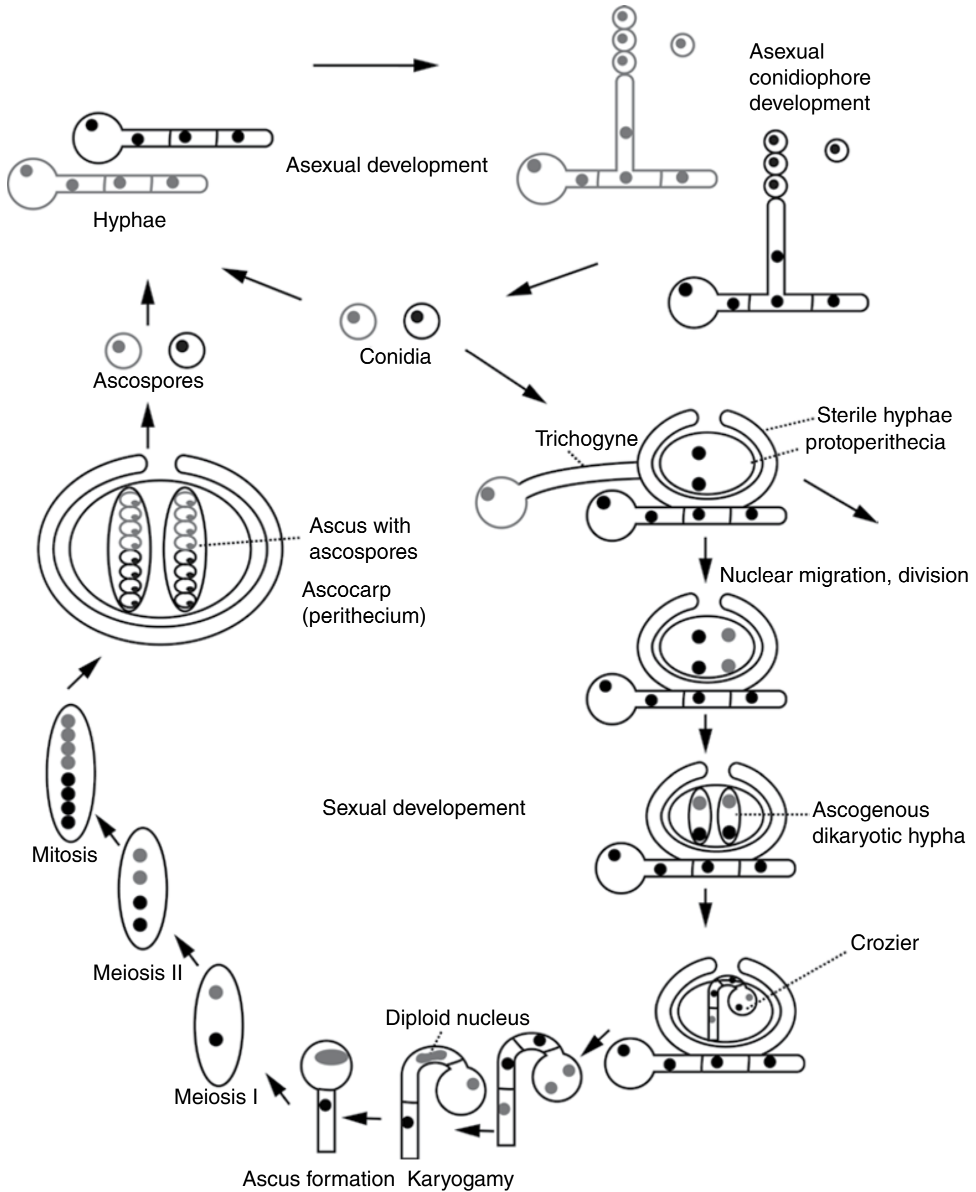

Figure 2.3 Lifecycle of *Neurospora crassa.*

within a developing dikaryotic ascogenous hyphal structure. The ascogenous hypha develops a crozier, where nuclear fusion or karyogamy takes place, followed quickly by meiosis within the developing ascus. Mitosis and subsequent ascosporogenesis results in eight spores within an ascus within the perithecium. Acsi are long and slender in *Neurospora*, allowing for individual dissection and separation of ordered ascospores.

2.2.3 Basidiomycete Filamentous Fungi (*Coprinus cinereus*)

The basidiomycete lifecycle is typically similar to the ascomycetes with a few exceptions. In *Coprinus cinereus* (Figure 2.4), a typical mushroom fungus, monokaryotic hyphae produce asexual spores called oidia, which germinate and form hyphae. To initiate sexual reproduction, monokaryotic hyphae fuse at their tips (anastomosis) to create a dikaryotic hypha. The dikaryotic hypha grows vegetatively, and is distinguished from hyphae of ascomycetes by the presence of hooked cells or clamp connections, which connect septated compartments of the hypha. Changes in temperature and light can trigger the hypha to undergo

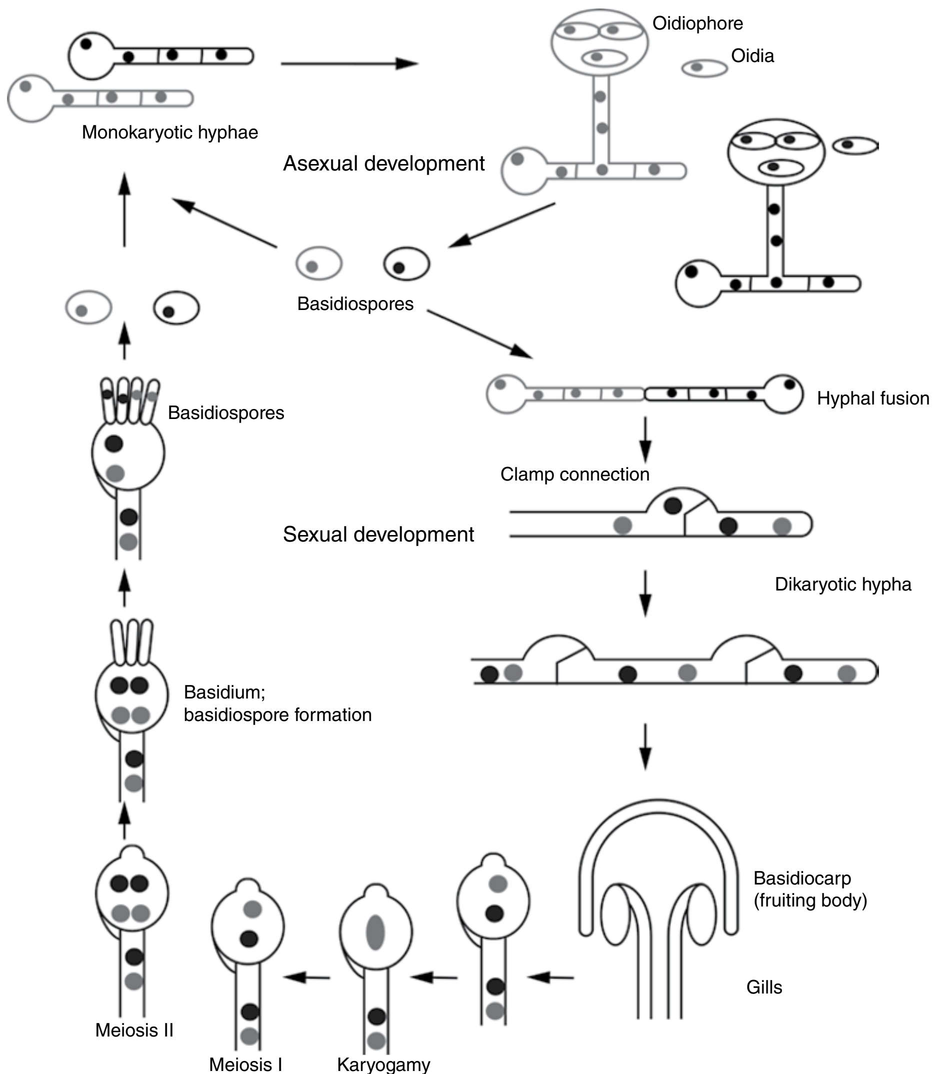

Figure 2.4 Lifecycle of *Coprinus cinerus*.

differentiation into the fruiting structure, or basidiocarp. Within the basidiocarp, basidium formation (the equivalent of an ascus) and karyogamy take place. Meiosis produces the basidiospores, which hang off the basidium contained within the gills of the mushroom cap, as opposed to being encased as in the asci of ascomycetes. Haploid basidiospores are then released, and germinate into monokaryotic hyphae and continue the cycle.

In dimorphic basidiomycetes that exist in both yeast and hyphal forms, such as the human pathogen *Cryptococcus neoformans* (Figure 2.5), vegetative growth occurs via budding yeast. Sexual reproduction involves the differentiation of yeast cells into hyphae upon exposure to pheromone, resulting in dikaryotic hyphae. Basidia differentiate from the ends of the hyphae, in which karyogamy followed by meiosis and mitosis occurs, producing haploid basidiospores. Upon release, the spores grow as budding yeast. The yeast cells can also undergo

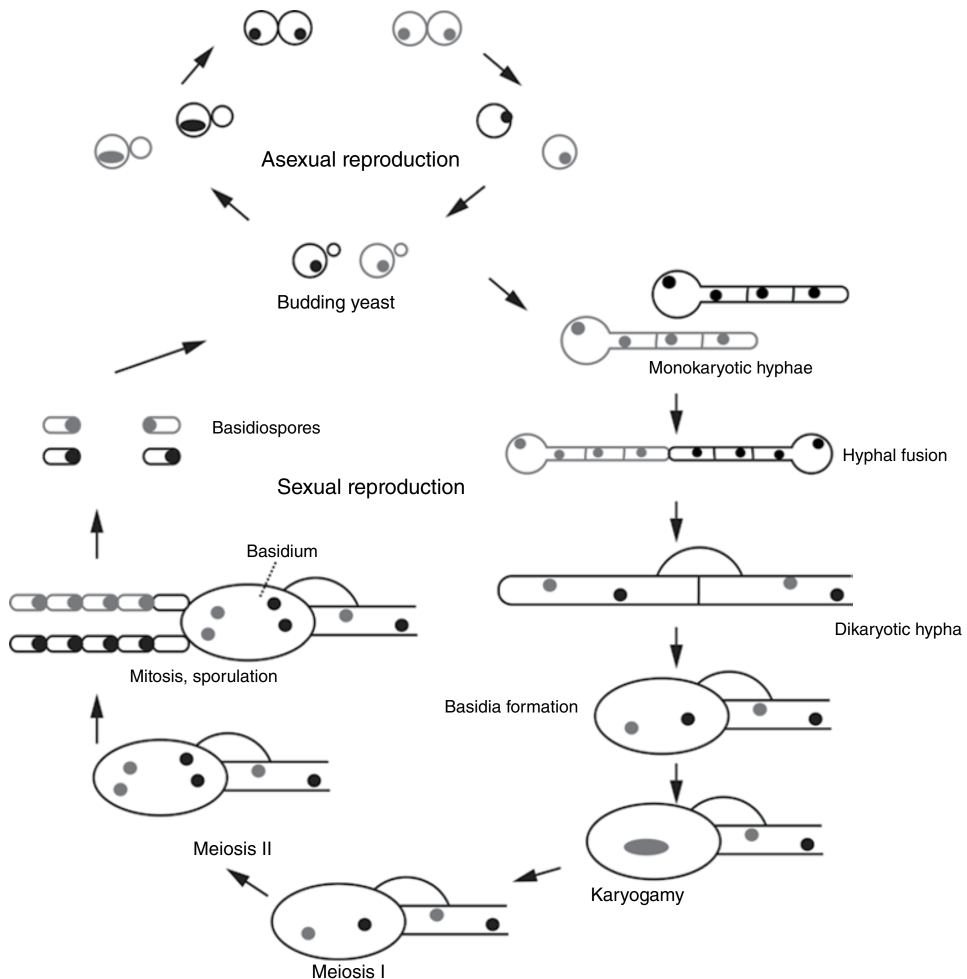

Figure 2.5 Lifecycle of *Cryptococcus neoformans*.

asexual sporulation in response to nitrogen limitation or desiccation. Under these conditions, yeast cells differentiate into hyphae, which differentiate monokaryotic basidia at their tips. Mitosis occurs within the basidia, producing haploid spores, which then germinate into yeast cells.

Filamentous fungi exhibit many variations in their lifecycles, and some do not exhibit a known sexual phase (the deuteromycetes). Others exploit parts of the lifecycle for pathogenesis, particularly in the pathogenic basidiomycetes. For example, the dimorphic corn smut fungus *Ustilago maydis* exists as a non-pathogenic yeast form, but upon mating yeast cells differentiate into dikaryotic hyphae, which are associated with virulence.

2.3 Sexual Analysis: Regulation of Mating

2.3.1 Ascomycete Yeast

The yeast *S. cerevisiae* has a simple mating system, with cells of two haploid mating types termed **a** and α. These cells can conjugate to form a diploid cell containing both **a** and α information. The **a**/α diploid is not capable of mating but can initiate meiosis to form four haploid products, two of which are mating type **a** and two of which are mating type α. Laboratory strains typically have stable mating types, and are termed heterothallic. In contrast, most wild strains are homothallic, and do not have stable mating types. Instead, during mitotic growth the cells are capable of switching their mating types, and thus a growing spore colony develops cells of both **a** and α. These cells mate with each other, resulting in a colony that grows up as an **a**/α diploid. The regulation of the stability of the mating type is controlled by a single locus, termed the homothallism locus (*HO*). The *HO* locus contains a functional endonuclease, while the stable mating types of the heterothallic strains result from a defective allele of this locus, designated *ho*.

The reason that an endonuclease controls the stability of the mating type is the result of a sophisticated genetic exchange system involving expressed and unexpressed cassettes of information. The mating type of the cell is controlled by a single locus termed *MAT* close to the centromere of chromosome 3. When this locus contains the *MAT***a** allele the cells are of the **a** mating type; when the locus contains the *MAT*α allele the cells are mating type α. If there is no information at *MAT* the cells select **a** as the default mating type. However, the typical *S. cerevisiae* cell also contains an extra copy of both mating-type genes, typically **a** information near the right telomere of chromosome 3 at a locus called *HMR*, and α information at the left telomere of chromosome 3 at locus *HML*. The sequences at *HML* and *HMR*, although structurally identical to the sequences for **a** and α information at *MAT*, are not expressed due to the action of a series of proteins designated silent information regulators. In the presence of a functional *HO* endonuclease the information is exchanged

between the *HMR* or *HML* loci and the *MAT* locus as often as once per cell division. This exchange is recombinational; the *HO* endonucleases make a double strand cut at the *MAT* locus, and gene conversion then transfers the information from one of the silent loci to the *MAT* locus. The same machinery that keeps the information at *HML* and *HMR* unexpressed blocks the cutting of these DNA sequences by *HO*, so the informational exchange is typically unidirectional.

The *MAT* locus defines the mating type of the cell through direct transcriptional control. Each allele, *MAT***a** and *MAT*ααα, expresses transcription factors, and these transcription factors control the expression of blocks of genes defining the two cell types. *MAT*αα encodes two transcription factors, α1 and α2. The role of α1 is to stimulate the expression of α-specific genes such as *STE3*, encoding the receptor for the **a**-factor mating pheromone, and *Mf*α*1* and *Mf*α*2*, the genes specifying the α-factor mating pheromone. The α2 transcription factor is a repressor, serving to repress **a**-specific gene expression in the *MAT*α cells and, together with the **a**1 factor, to shut off haploid gene expression in *MATa/MAT*α diploid cells. Among the key genes shut off in the diploid cell is *RME1*, which encodes a repressor of meiosis; this regulatory circuit ensures that it is the **a**/α diploid cell that is uniquely capable of meiosis and sporulation.

2.3.2 Filamentous Ascomycetes

Neurospora crassa is heterothallic and requires two mating types, A and a, for sexual reproduction. The mating-type loci, mat A and mat a, control gene expression required for the mating process. The DNA sequences at the mating loci of opposite mating types are very different, and are therefore regarded as "idiomorphs" as opposed to alleles. The mat a idiomorph is 3.2 kb in length, and encodes one gene called mat a-1, while the mat A idiomorph is 5.3 kb long and encodes three genes, including mat A-1, A-2, and A-3. The mat a-1 and mat A-3 genes encode for HMG box-containing DNA-binding proteins, and are the major regulators of mating in both strains. Homologs of such factors are required for mating in other organisms, including some filamentous fungi and the fission yeast *Schiz. pombe*. The mat A-1 product is also a DNA-binding protein and similar to α1p from *S. cerevisiae*. mat A-3 encodes a protein with little homology in other organisms and of unknown function, but, together with mat A-2, is required for ascosporogenesis. mat A-2 and A-3 are expressed constitutively during both vegetative growth and sexual development. In contrast to the situation in *S. cerevisiae*, the downstream targets of the mating regulatory genes are unknown. They presumably control expression of the mating pheromone, but other processes must also be regulated, including nuclear migration, nuclear compatibility, and fruiting body development. Since filamentous fungi utilize multiple different cell types for sexual reproduction and contain complex fruiting body structures, including different thalli for male and female mating

partners, the regulation and function of the mating-type loci must be more complex than in yeast. In contrast to *S. cerevisiae*, mating-type switching does not occur in *N. crassa*.

The mating loci in *Neurospora* regulate additional processes, including vegetative, heterokaryon compatibility. Vegetative hyphae can fuse to form a heterokaryon, but only if they arise from opposite mating-type strains. If hyphal fusion occurs between incompatible cells, the fused hyphal compartment seals off from the rest of the hyphae through deposition of cross-walls, and the compartment undergoes a type of programed cell death characterized by DNA fragmentation, organelle and cytoplasmic breakdown, and vacuole production. Mutants of the *mat a* strain, which lost the incompatibility response, were affected in the *mat a-1* orf, and analysis of the *mat a-1* protein identified domains important for mating versus vegetative incompatibility.

Several filamentous fungi, including *A. nidulans*, are homothallic or self-fertile, where a colony derived from a single spore is able to undergo sexual reproduction. Although the genetic basis of homothallism is not fully understood, recent completion and analysis of the genome from *A. nidulans* has uncovered the presence of many conserved elements of mating from heterothallic species, suggesting that sexual reproduction may be regulated by similar genes in "selfing" fungi. For example, MAT-2 and MAT-1 genes encoding an HMG box-containing DNA-binding protein and an α1p domain homolog, respectively, have now been identified. In addition, genes encoding homologs to the hydrophilic pheromone alpha factor in *Saccharomyces*, the mating pheromone protease *KEX2*, and pheromone receptors *STE2* and *STE3* are present in the genome. However, no homolog to **a** factor mating pheromone was detected, despite the fact that its receptor was present. The MAT genes and factors associated with a pheromone response MAPK pathway were shown to be important for sexual development, suggesting that similar pathways underlie self and nonself mating.

2.3.3 Filamentous Ascomycete Dimorphic Fungi

Candida albicans (Figure 2.6b) is an important human pathogen and has been extensively studied for this reason. *Candida albicans* had been classified as an asexual deuteromycete, but recent genomic studies have provided convincing evidence for the potential for a sexual cycle. However, although a well-defined mating system has been identified that allows the conjugation of mating-type locus homozygous diploid cells, there is currently no evidence for a functional meiotic pathway that allows reductional division and a return to the diploid state from the tetraploid.

The detection of the potential mating ability of *C. albicans* arose through analysis of the genome sequence. A region of the genome was detected that encoded genes similar to those found at the mating-type locus of *S. cerevisiae*. Further analysis of this region uncovered a more complex locus than that found in

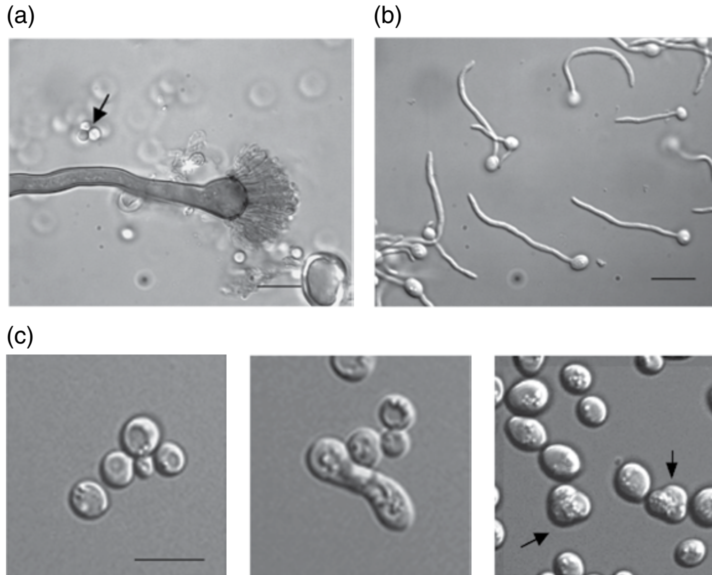

Figure 2.6 (a) Developing conidiophore composed of a vesicle giving rise to phialides and conidia in the filamentous fungus *A. nidulans*. Released conidia are indicated by an arrow. (b) Hyphae growing from yeast cells of the dimorphic fungus *C. albicans*. (c) Various stages in the lifecycle of the yeast *S. cerevisiae*. The first panel demonstrates vegetatively growing yeast, the second shows a zygote, and the third asci (arrows). Bars: 10 μm.

S. cerevisiae; in addition to the candidate cell-type regulating transcription factors, there were other genes whose function implied no obvious link to the mating process. Such complex loci are found in other fungi, such as *Cryptococcus neoformans*, and in algae such as *Chlamydomonas reinhardtii*; analysis across the spectrum of mating-proficient lower eukaryotes suggests that many organisms contain mating-type loci that mix genes with roles in controlling the mating type with genes involved in other cellular processes. In *C. albicans* these genes include oxysterol-binding proteins (*OBPa* and *OBP*α), poly-A polymerases (*PAPa* and *PAP*α), and phosphatidyl inositol kinases (*PIKa* and *PIK*α). The a and α versions of each protein are somewhat divergent, but the ability to homozygose each *MTL* allele shows that both versions of the proteins are capable of supporting cell viability.

In contrast to *S. cerevisiae*, where the *MATa* locus expresses one transcription factor and the *MAT*α locus encodes two factors, both the *MTLa* and *MTL*α loci of *C. albicans* express two transcription factors. Genetic analysis suggests that *MTLa2*, an HMG box-containing protein, is a positive effector of *MTLa* functions. This positive function (and gene) is missing from *S. cerevisiae* cells, where the *MATa* phenotype is expressed as the default state in the absence of any *MAT* information. This makes the *C. albicans MTL* loci structurally more complex than the *S. cerevisiae* loci, but the mating-type regulatory circuit is actually more direct. In *C. albicans* the *MTLa* locus expresses *MTLa2* that directs

a mating functions, and the *MTLα* locus expresses *MTLα1* controlling *α* mating functions. In the diploid state the other transcription factors, Mtla1p and Mtlα2p, combine to repress mating functions, as well as white-opaque switching. Thus, each locus has both positive and negative roles within the mating-type circuit.

The link between mating ability and the phenomenon of white-opaque switching represents another detail of the *C. albicans* mating process. White-opaque switching was identified as a high frequency event occurring in some *Candida* cells through which cells changed their morphology, some of their physiological characteristics, and their colony morphology. It is now appreciated that the ability to undergo this switching was occurring in cells that had homozygosed their mating type, thus relieving the repression of the switching process caused by the a1/α2 regulatory molecule. Efficient mating in *Candida* cells requires that the cells be in the opaque state, rather than the white state. Recent work has clarified this connection, as the major regulator of the opaque state, the transcription factor Wor1p, is repressed in the a1/α2 expressing cells, but has the potential to be transcribed in *MTL* homozygotes. Because WOR1 is autoregulated, it can set up a positive loop of high WOR1 expression generating the opaque state, which is epigenetically stable. However, fluctuations in Wor1p levels or function can break this loop, leading to a stable low-WOR1-expression state that generates the white form cell.

2.3.4 Filamentous Basidiomycetes

The basidiomycetes are novel in that they have multi-allelic mating-type genes. In *Coprinus cinereus*, for example, more than 12,000 mating types exist, demonstrating the diversity in mating systems and complex levels of control within the filamentous fungi. *Coprinus* has two unlinked mating-type loci, A and B, which are polymorphic and contain subloci called α and β. The mating system is therefore described as being tetrapolar. The α and β loci are redundant, but recombination occurs between them. Together these loci contribute hundreds of specificities to A and B loci, creating thousands of different mating types.

The A locus encodes genes for homeodomain proteins, and regulates nuclear pairing, clamp connection formation, and septation. At Aα, two classes of homeodomain proteins are produced. The HD1 and HD2 classes contain homologs to α2p and **a**1p, respectively, from *S. cerevisiae*. HD1 and HD2 proteins from different specificity loci dimerize and subsequently regulate the dikaryon. The B locus encodes six pheromone genes and three pheromone receptor genes. In contrast to ascomycetes where pheromone signaling stimulates the formation of mating-specific morphological shapes, pheromone signaling in *Coprinus* stimulates fusion of the monokaryotic hyphae to initiate the sexual cycle. The B locus also regulates nuclear migration and attachment between the clamp connection and the corresponding subapical hyphal compartment. As in the filamentous ascomycete *N. crassa*, the targets of the mating loci genes are currently not well characterized.

2.4 Unique Characteristics of Filamentous Fungi that Are Advantageous for Genetic Analysis

2.4.1 Parasexual Analysis

Parasexual genetics involves examination of recombination in the absence of sexual reproduction, and has been helpful in mapping genes to chromosomes. The unique feature of heterokaryosis, or maintaining two genetically distinct nuclei within one thallus in filamentous fungi, allows for this type of analysis. The parasexual cycle has been extensively utilized in *A. nidulans*, and involves heterokaryon formation, followed by karyogamy to produce a diploid that then undergoes spontaneous mitotic recombination. Thus, genetic recombination can occur within the vegetatively growing hypha. Diploids can be differentiated from heterokaryons in parasexual analysis in *Aspergillus* based on spore color. Heterokaryons formed from fusion of strains containing white or yellow spores will produce conidia of either color. Sections of the colony that undergo karyogamy and form a heterozygous diploid, however, can be recognized by the resulting spore color green, since the recessive mutations in spore color leading to white and yellow spores will complement each other. The diploid is then isolated and forced to undergo haploidization through treatment with drugs, such as benomyl, to induce chromosome loss, and the resulting haploid products are analyzed for evidence of mitotic crossing over. In *A. nidulans*, master strains containing unique markers per chromosome are used as a reference during "crossing" with the test strain.

Parasexual analysis has recently been utilized to great advantage in *C. albicans*, a diploid pathogen that does not have a known sexual phase involving meiosis, and therefore could not be analyzed through traditional genetic analysis involving crossing of strains and sexual reproduction. *Candida albicans* can mate and contains the necessary mating genes, as described earlier, but the resulting tetraploid products of mating break down to diploids through spontaneous chromosome loss, not meiosis. Therefore, *C. albicans* may naturally use a parasexual cycle to produce recombinant individuals. Parasexual analysis has been used for genetic linkage and construction of new strains in this organism, and holds promise for future mutagenic analysis.

2.4.2 Gene Silencing

A unique feature that has greatly facilitated genetic/molecular analysis in the filamentous fungus *N. crassa* is the process of repeat-induced point mutation (RIP). If more than one copy of a gene is introduced in tandem into the haploid strain prior to sexual reproduction, the tandem copies are inactivated through GC to AT mutations when passed through the sexual cycle. Therefore, gene inactivation can be achieved by simply introducing additional copies, and allowing

the strain to undergo sexual reproduction. *Neurospora crassa* was one of the first eukaryotic systems in which a form of RNA interference (RNAi) was investigated. The fungus demonstrates the process called quelling, where genes that are introduced at heterologous locations are silenced. This silencing involves degradation of mRNA through several factors including homologs of Argonaute and Dicer which are involved in RNAi in other systems. RIPing and quelling are therefore very useful for investigating gene function in *N. crassa*, and for understanding the related mechanisms of gene silencing in other eukaryotes, including plants and worms. Because of the utility of RNAi, recent efforts have been made to transfer the molecular machinery to organisms like *S. cerevisiae* and *C. albicans* that do not naturally possess the capacity.

2.5 Genetics as a Tool

2.5.1 Tetrad Analysis

2.5.1.1 Saccharomyces cerevisiae

The classic strategy for genetic analysis of *S. cerevisiae* meiosis involves tetrad analysis. Each meiotic event from a typical diploid cell generates four haploid spores. These spores are arranged in an ascus sac that is degraded by enzymatic treatment with an endoglucanase. This liberates the spores, which are then placed in separate locations on rich media plates by micromanipulation and allowed to divide to form a spore colony. The spore colonies are then analyzed to determine the distribution of the markers introduced into the initial cross. Recombination occurs when the diploid undergoes meiosis. During meiosis I, chromosomes from each parent pair up, then duplicate, creating two chromatids that remain attached. Chiasma formation between paired homologous chromosomes at this stage results in recombination of genetic material. Homologous chromosomes then separate to opposite poles of the meiotic spindle, and nuclear division results in two diploid nuclei. Independent assortment of chromosomes occurs at this stage. Meiosis II follows, which involves the splitting of the attached chromatids to opposite poles, and another round of nuclear division, resulting in four haploid nuclei. A critical advantage of the yeast meiotic process for genetic analysis is that all four products of the meioses are detected and available for analysis. This avoids questions of statistics in the analysis of segregation patterns, and allowed for the identification of "non-Mendelian" segregation patterns characterized by a 3:1 rather than 2:2 distribution of heterozygous markers. The recombinational replacement of information from one allele to the other that generates this pattern is termed gene conversion.

Because there is no pattern to the position of the spores within the ascal sac, *S. cerevisiae* does not provide ordered tetrads as are found in some of the filamentous fungi. However, the identification of markers tightly linked to centromeres

permits the indirect ordering of the spores relative to the actual meiotic event, because the centromere, and therefore all markers tightly linked to the centromere, segregate in the first meiotic division.

A modification of the classic tetrad analysis involves *selected tetrads*. This approach has been used during the analysis of meiotic recombination within a single locus. The frequency of intra-allelic recombinants is low, so selection is used to identify those infrequent meiotic events where a recombination has taken place. Typically, such intragenic recombination studies involve heteroalleles of an auxotrophic marker, so all the nonrecombinant products are auxotrophs. In contrast to standard tetrad analysis, the glucanase-treated ascal sacs are not spread on plates containing a rich growth medium, but rather are spread on plates that lack the nutrient required by the auxotrophic cells. The separated ascal groups are monitored microscopically to detect sets in which at least one member of the tetrad begins to germinate. These tetrads are then micromanipulated to permit subsequent analysis of the genetic structure of all four spores – this approach enriches for tetrads in which a recombination event is known to occur within the gene under study. This allows for a fine structure analysis of the recombination process, and the sophisticated ability to monitor all the consequences of the meiotic events has been critical to the development of models of the process of meiotic recombination.

In situations where the pattern of marker segregation in individual asci is not important, *random spore analysis* can be applied. In this approach, populations of asci are digested with glucanase *en mass*, and the mixture of haploid meiotic products and unsporulated diploid cells is spread on plates. These plates are designed to select against the initial diploid cells, typically by containing a recessive drug resistance marker that is only uncovered in the haploid cells. These haploid segregants can be rapidly screened to identify a cell containing a desired combination of markers, or the population can be scored to determine the overall patterns of segregation. An interesting recent development of the random spore strategy is found in the synthetic genetic array (SGA) approach pioneered by C. Boone and collaborators. In this approach, whole-genome-wide screens for synthetic lethal interactions are created by robotic replica plating, and sophisticated use of mating-type-specific gene expression is used to permit the growth of only a desired haploid cell type.

2.5.1.2 Neurospora crassa

Neurospora crassa is the pioneering organism for genetic analysis in microorganisms, pre-dating work with bacteria and with the yeast *S. cerevisiae*. It is an attractive model genetic system, since it is haploid and asci are large enough to remove ascospores, allowing the recovery of all products of meiosis and determining recombination of the parental genes within the progeny. *Neurospora* is particularly attractive for tetrad analysis since the ascospores are ordered within

the linear ascus, allowing genes to be mapped in relation to the centromere. In *N. crassa*, meiosis is followed by another mitotic division, producing eight haploid nuclei, or four pairs of sister nuclei. Sporogenesis then produces eight ascospores within the ascus (Figure 2.7). As in *Saccharomyces*, the individual spores are isolated and tested for traits such as nutrient requirements. Alternatively, spores from many asci are collected and analyzed as a random sample.

Due to independent assortment of chromosomes at meiosis I, if two strains that differ at loci A and B mate (A/b × a/B), and A and B are located very close together, the chances of recombination between these loci is small, and the resulting meiotic products will be 50% parental type (A/b and a/B). Such close genes are described as being linked. If A and B are far apart, crossing over or chiasma formation can occur during meiosis I, resulting in a proportion of recombinant progeny. The proportion increases the further apart the genes, to a maximum of 50% (25% Ab, 25% aB, 25% AB, 25%ab). In addition, if A and B were on different chromosomes, 50% recombinant progeny are expected. The tetrad of *Neurospora* is ordered, where the products of the first division are located within one half of the ascus. This feature contributes to the efficiency and convenience

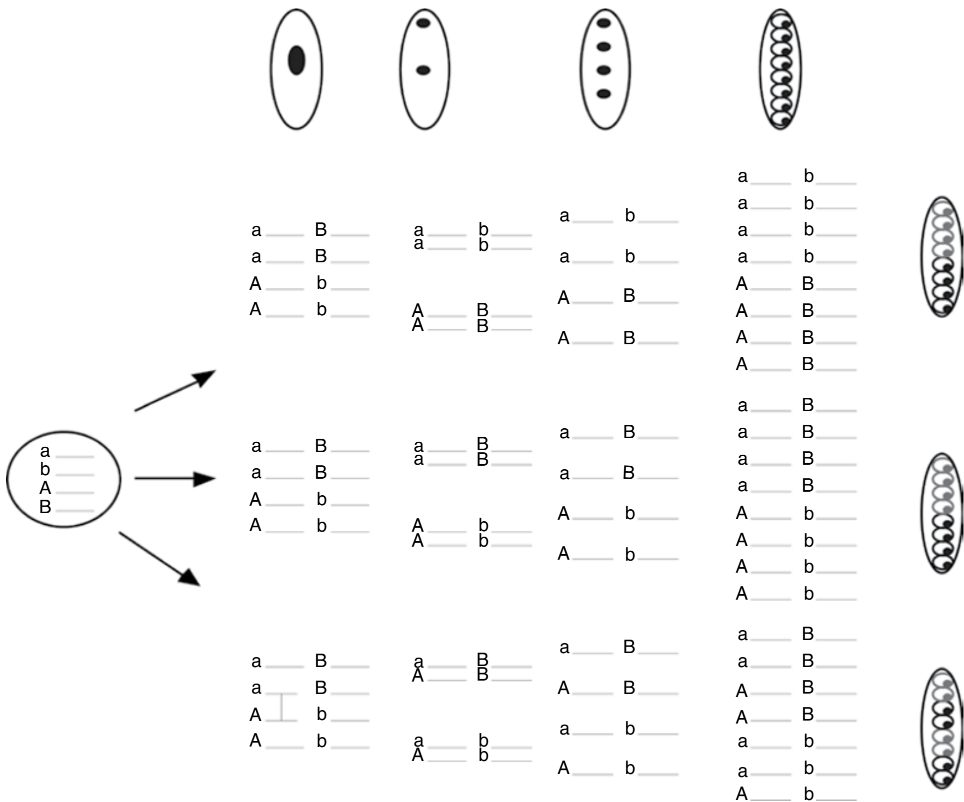

Figure 2.7 Chromosome assortment in *N. crassa*. (Adapted from Davis and De Serres (1970).)

of tetrad analysis in this organism. To set up strains for sexual reproduction and subsequent genetic analysis, the strain designated as the female is grown first, and conidia from the male are spread over the female culture. Perithecia develop within a few days, and acsi are mature and start to discharge ascospores by 10 days. Spores collect on the side of the incubation tube, and can be taken for random spore analysis. Alternatively, a perithecium is dissected out and crushed in a water droplet to release asci. An ascus is pressed with a needle to discharge a spore, break the ascus, and subsequently release the remaining spores. Spores are teased apart in order and separately analyzed.

Prior to sequencing of the genomes, tetrad analysis was used for linkage analysis and mapping of genes. Reference strains with known markers were crossed with a test strain containing the gene of interest to detect any linkage, and to map the gene relative to the known position of the marker gene in the reference strain.

2.5.1.3 Aspergillus nidulans

Analysis of ascospores is different in *A. nidulans*, since traditional tetrad analysis is not as feasible as in *N. crassa*. However, Mendelian segregation for all unlinked nuclear markers is demonstrated in random spore samples. Sexual reproduction for genetic analysis involves growing two strains of different auxotrophy and other genetically distinct markers, such as spore color, on media that selects against the individual strains and thus forces heterokaryon formation. The plate containing the resulting heterokaryons is sealed to prevent any aeration, and after 2–3 weeks, the fruiting bodies or cleistothecia form. Since the asci are extremely fragile, they are not dissected out from the cleistothecium, so traditional tetrad analysis is not usually performed. Instead, a cleistothecium is rolled onto a semi-solid water/agar surface to remove other cellular tissue, then crushed and spread across a plate to observe segregation of markers such as ascospore color. Some cleistothecia are derived from self-fertilization, which is evident upon analysis of ascospore colors of the products. A stock of the spores from the crushed cleistothecium is maintained and tested on different selective media to determine the genotype of the individual products and extent of recombination.

2.5.2 Molecular Methods for Genetic Screens

2.5.2.1 Transformation

Saccharomyces cerevisiae was the first fungus to be transformed with exogenous DNA, and the development and refinement of this technology has led to the ability to effectively manipulate the genome of this yeast. Initial proof of the transformation potential of *S. cerevisiae* involved the construction of a yeast

strain that contained two separate point mutants within a single locus, preventing any reversion of the gene to functionality, followed by introduction of a bacterial plasmid containing a cloned copy of the inactivated gene. A technique initially developed for protoplast (cells digested of their cell walls) fusion was modified to allow uptake of the DNA into the yeast cells, and proof that the exogenously added DNA was the source of the restored function of the missing gene was provided by the detection of the sequence of the bacterial plasmid in the clones that contained the restored function. There have been many modifications of the initial transformation protocol; currently, the use of electroporation or treatment of cells with lithium salts to trigger DNA uptake has essentially replaced the initial protocol of generating protoplasts which had to regenerate their walls in an osmotically stabilized medium.

Transformation of filamentous fungi is performed either in protoplasts or in asexual conidia. In the former, protoplasts are typically transformed using polyethylene glycol (PEG) and calcium to facilitate entry of DNA. Conidia in some fungi, including *A. fumigatus*, can be transformed without degrading the cell wall, through electroporation. Lithium acetate-based transformation has also been used. Transformation efficiencies and optimum methods vary between fungi.

2.5.2.2 Plasmids, Transforming DNA

Transformation requires that the DNA contains a selectable marker, which is normally a nutrition or drug-resistance gene. For example, if *pryG* encoding for orotidine-5′-phosphate decarboxylase, which is part of the uridine biosynthesis pathway in *A. nidulans*, is used as a marker on transforming DNA, the resulting positive transformants will grow on media lacking uridine and uracil, while untransformed cells will not. The use of such selectable markers is common to all fungal transformation systems.

The initial transformation of yeast cells was not highly efficient, and involved sequences that integrated into the genome. The great utility of episomal DNA sequences for the transformation of bacterial cells led to the search for equivalent tools for the manipulation of *S. cerevisiae*. These tools took two forms: episomes based on the backbone of the endogenous yeast 2α plasmid, and episomes that contained origins of replication from the chromosomes. The 2α plasmids contained sequences for efficient segregation, and thus were more stable than those based solely on the autonomous replication sequences (ARS) elements derived from chromosomes. However, a further introduction of centromeric sequences, which provide efficient segregation and maintain a low plasmid copy number, allows ARS-based plasmids to be very stable. Such plasmids can be maintained for many generations in the absence of selection, and now provide the workhorse tools for the molecular manipulation of yeast cells. The selection of the plasmids typically involves nutritional markers – the standard markers

(*URA3*, *HIS3*, *TRP1*, and *LEU2*) represent genes that were initially cloned by complementation of *Escherichia coli* mutations, and were available in cloned form prior to the development of yeast transformation. Dominant drug-resistant markers are also available for selection of plasmids; resistance to G418 has been a useful marker in *S. cerevisiae*. Essentially, all commonly used yeast transformation plasmids include selection markers and replication origins for propagation of the plasmid in *E. coli*, so the plasmids can be shuttled between the prokaryotic and eukaryotic hosts.

Most filamentous fungi lack the ability to maintain extrachromosomal plasmids, in contrast to the yeast *S. cerevisiae*, so transformed DNA typically integrates homologously or heterologously in the genome. The frequency of homologous recombination increases with increasing length of homologous DNA. Transforming DNA therefore typically involves a vector background containing an *E. coli* origin of replication and ampicillin resistance marker to allow replication and selection in bacteria for plasmid propagation, as well as a fungal-specific marker and the desired gene sequence.

Transformation is performed to accomplish things such as knocking out a gene, replacing a gene with a mutated version, or modifying gene expression. A gene knockout construct typically contains a marker gene surrounded by 5′ and 3′ flanking DNA of the gene of interest, so that the linear ends can recombine with the endogenous 3′ and 5′ flanking ends of the endogenous gene, allowing its replacement with the marker gene. Gene expression can be controlled by recombining a regulatable promotor in front of the endogenous ORF. In *S. cerevisiae*, the promotor for a galactose-regulated gene such as GAL1 is often used, where galactose or glucose in the medium regulates overexpression or repression, respectively. In *A. nidulans*, the *alcA* promotor is commonly used, and becomes overexpressed if the cells are grown on media containing ethanol as a carbon source, or repressed when cells are grown on media containing glucose.

2.5.2.3 Genetic Screens

In a post-genomic fungal world where genomes are sequenced and annotated, and genes no longer need to be mapped to chromosomes through traditional genetic techniques, genetic analysis is still a very powerful tool, particularly in construction of mutant screens. Genetic screens in filamentous fungi have uncovered an enormous amount of information about diverse cellular processes, and in many cases identified the first examples of conserved genes and their functions. Pioneering work in *N. crassa* by Beadle, Tatum, and Lederberg demonstrated that individual genes encoded for individual enzymes, bringing together genetic and biochemical analyses for the first time. Subsequent screens in this organism have provided novel information on gene conversion, recombination, circadian rhythms, gene silencing, and DNA methylation. Genetic screens in *A. nidulans*

and *S. cerevisiae* have uncovered and continue to uncover novel information in diverse areas including cell cycle regulation, cellular motors, and cytoskeletal dynamics, signaling, and development.

Classical screening involves mutagenizing cells, typically with UV light, radiation, or chemicals, then allowing growth of survivors. This approach was used by Beadle, Tatum, and Lederberg in 1941 to uncover the metabolic mutants in *N. crassa* (Figure 2.8). Single conidia colonies were exposed to X-irradiation on complex medium, and transferred to minimal medium. Growth on complex versus minimal medium was screened, and any colony that could not grow on minimal medium was considered to contain a nutritional mutation. The strain was maintained on complex media but subsequently tested for the restoration of growth on minimal media containing defined additives, such as tyrosine, leucine, or alanine. If growth was restored only when tyrosine was added, for example, the specific mutation could then be identified. From this approach, strains containing mutations in vitamin B6, vitamin B12, and para-aminobenzoic acid were uncovered. The mutant strains were crossed with wild-type parental strains to ensure that only a single gene was mutated.

Another classical mutagenesis screen identified many genes that control a circadian rhythm in *N. crassa*. Circadian rhythms are present in fungi to humans, and are biological processes that are sensitive to light and temperature, and therefore oscillate every 24 hours in the absence of environmental signals. The timing of conidia formation in *N. crassa* is regulated by such an internal clock, and genes involved in responding to the clock were uncovered by using mutagenesis and race tube assays. When inoculated at one end of a long glass horizontal tube, called a race tube, *Neurospora* hyphae grow to the other end, creating a periodic banding pattern along the tube from the colored conidia that form every 24 hours. Mutagenized conidia were placed at one end of the tube, and changes in banding pattern, reflecting changes in day length, relative to control strains were screened. The *frequency*, *period*, and *chrono* genes

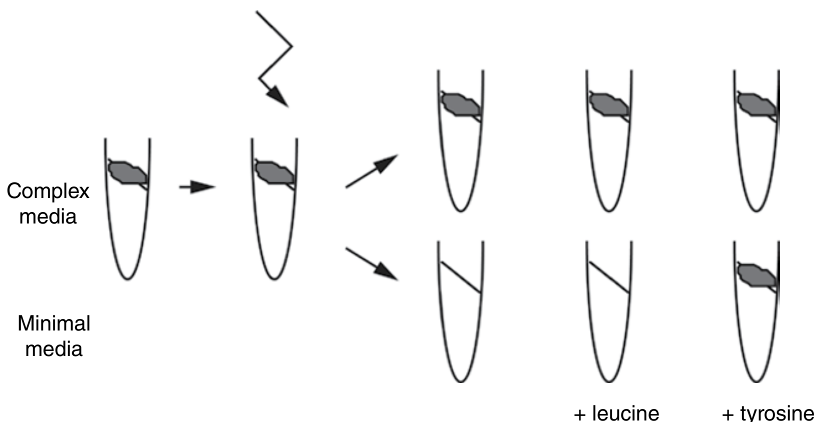

Figure 2.8 Genetic screen identifying metabolic mutants in *N. crassa*.

were uncovered, most of which have homologs involved in clock functions in *Drosophila melanogaster* and humans.

In *S. cerevisiae*, classical screens were applied to identify the cell division cycle (*cdc*) genes that uncovered many of the key controlling elements underlying what is now considered the universal eukaryotic cell cycle. Because defects in the cell cycle blocked cellular proliferation, it was necessary to identify conditional mutations, in this case temperature-sensitive mutants that arrested with a uniform terminal phenotype. In general, the uniform terminal phenotype arose because a particular cellular function necessary for completion of the cell cycle was missing at the restrictive temperature. Key proteins identified through this screening included Cdc28p, the cyclin-dependent protein kinase controlling both mitosis and the G1 to S transition, Cdc35p, which is adenylyl cyclase, Cdc12p, which is a septin, and Cdc9p, which is DNA ligase. Novel cellular processes were uncovered through the analyses of these mutations, and genes identified initially as *cdc* mutants form the underpinning of much of the current cell biology of *S. cerevisiae*.

Other strategies for gene identification in *S. cerevisiae* involved modifications of the classical screen approach that added enrichment protocols to improve the frequency of mutant identification. For example, screening for mutations that blocked the process of secretion was made more efficient by treating the mutagenized cell population to a density enrichment prior to looking for temperature-sensitive mutants.

The traditional screen for mutants has become more powerful and specific through introduction of various types of selection. A classic screen designed to identify mutants of the cell cycle in *A. nidulans* was designed by Ron Morris in 1975 (Figure 2.9a). Conidia were mutagenized with UV light, then allowed to grow at 32 °C. Cells were replica spotted onto media at 42 °C to screen for temperature sensitivity. Cells that could not grow at the restrictive temperature were analyzed for phenotype, and stained to visualize nuclei. Several classes of mutants were uncovered, and characterized as the nim (never-in-mitosis), bim (blocked-in-mitosis), and nud (nuclear distribution) mutants. These acronyms have subsequently been used for mutants in other systems, including *Schiz. pombe* and *S. cerevisiae*. The genes responsible for the mutant phenotypes were identified through complementation analysis. Several of the genes, including nima kinase which is essential for the G2/M transition, and bimc which is a kinesin motor, were the founding members of families of related proteins subsequently found in other organisms, from yeast to man. Another gene identified in the screen, nudf, has a homolog in humans that, when mutated, is the cause of the human neuronal disease lissencephaly.

In other landmark genetic screens performed in *A. nidulans*, resistance to the antimicrotubule drug benomyl was incorporated to select for mutations in tubulin. The subsequent *benA* mutants were confirmed to be β1- and β2-tubulin. Suppression analysis of the *benA* mutants was then used to identify mutations that affected proteins that interacted with β-tubulin. Based on work done in

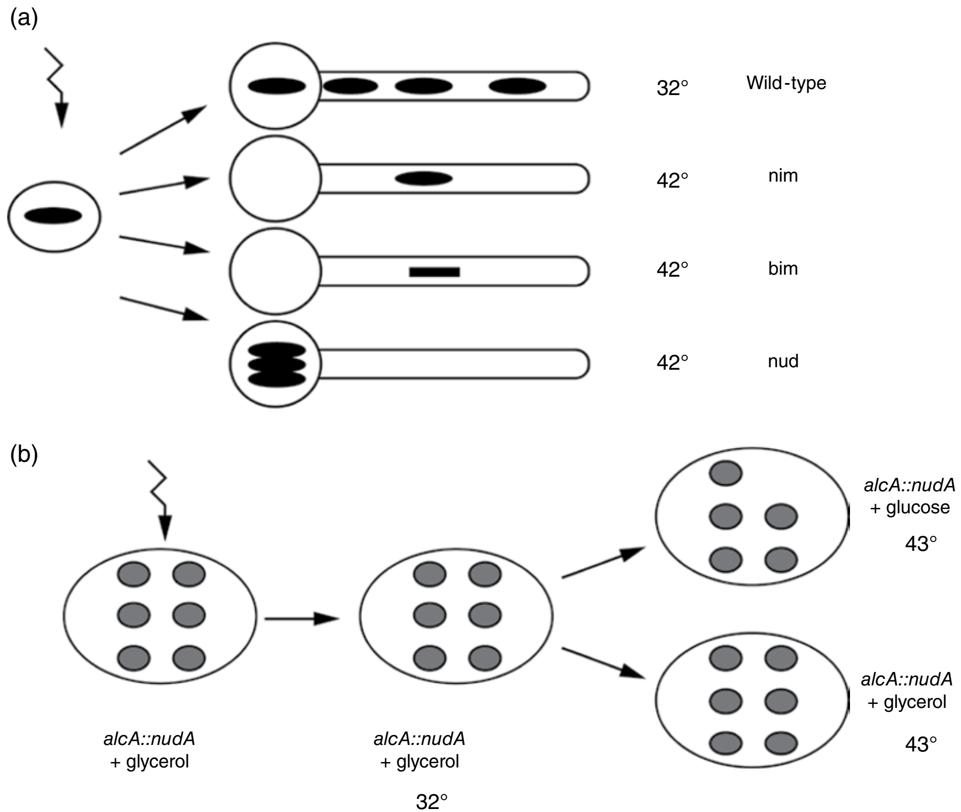

Figure 2.9 Selected genetic screens identifying cell cycle mutants in *A. nidulans*. (Adapted from Casselton and Zolan (2002).)

bacteriophage, if mutations of a protein prevented its interaction with another protein and thereby inactivated its function, a compensatory mutation in the second factor which restores the ability of the two proteins to interact would also restore function. The *benA* mutant was therefore subjected to another round of mutagenesis, and temperature-insensitive revertants were isolated. Through subsequent analysis, α-tubulin was identified, which forms a dimer with β-tubulin. Suppression screening of the *benA* mutant also identified the *mipA* mutants, which, upon mapping and cloning, provided the first example of γ-tubulin in any organism.

A classic selection approach was applied to identify mutants defective in the mating process in *S. cerevisiae*. This selection was based on the observation that a functioning mating response pathway resulted in cells arresting the cell cycle in the presence of the mating pheromone produced by cells of the opposite cell type. Thus, mutagenized *MATa* cells were treated with the mating pheromone α-factor, and the only cells that could grow to form colonies would be those that were insensitive to the pheromone. This selection allowed the identification of many of the key kinases and regulators of this pathway.

The mutagenesis screen has more recently been adapted to identify synthetic lethals, which can also uncover potential functionally interacting proteins. In one example, a strain of *A. nidulans* in which nudA (dynein) was placed under control of the *alcA* regulatable promotor was utilized (Figure 2.9b). On glucose, dynein expression was off, but on glycerol, dynein was expressed. After UV mutagenesis, the strain was grown on glycerol at 32 °C, then replica spotted onto glycerol or glucose at the restrictive temperature of 42 °C. The ability of temperature-sensitive mutations to grow on glycerol (+ dynein) but not on glucose (– dynein) was screened. The resulting strains were back-crossed to wild-type and *nudA* strains to isolate single and double mutants. The mutants, called *sld* for synthetic lethal with dynein, were cloned and determined to be homologs of Bub1p and Bub3p spindle checkpoint factors.

Insertional mutagenesis is highly advanced in *S. cerevisiae*. Initially, circular plasmids containing selectable markers were transformed into cells and were found to integrate at homologous sites quite efficiently. Markers inserted into repetitive sequences such as the ribosomal DNA allowed the analysis of both mitotic and meiotic recombination. The recognition that double-stranded breaks both dramatically enhanced the frequency of insertion and provided for efficient targeting to the homologous site in the genome greatly improved the technology of directed gene replacement. Several strategies are currently available for disrupting gene function by replacing part or all of a gene with foreign DNA, thus inactivating the function of the gene of interest. The use of such homology-driven gene inactivation has been extensively applied to investigations of individual genes. A modification of this approach has been used to provide efficient insertional mutations for random yeast sequences. In this approach, a library of random yeast DNA inserts in the 10-kb range is constructed in an *E. coli* plasmid vector. This library is transformed into *E. coli*, and the *E. coli* strain is then subjected to a transposon mutagenesis with a modified bacterial transposon containing at least a selectable marker for *S. cerevisiae*. Plasmids are selected which have picked up a transposon insertion, and many of these insertions have occurred in the yeast DNA portion of the plasmid. The yeast inserts are liberated from the vector by restriction digest, and the digestion products used to transform yeast cells. Selection for the yeast marker on the transposon allows detection of integration events, and these integration events represent transposon insertions into essentially random regions of the yeast genome – the overall distribution dependent on the randomness of the initial library of yeast sequences and the randomness of the transposon insertions into this library. Strategies that made use of the endogenous yeast transposable element to do the transposon hops directly in yeast have also been developed, but were limited by the nonrandom characteristics of the insertion locations.

Insertional mutagenesis has also been applied to genetic screens in filamentous fungi. In restriction enzyme-mediated insertions (REMI), a plasmid that has been linearized is transformed into protoplasts in the presence of the restriction enzyme that was used for cutting. The enzyme is thought to cleave the genomic

DNA at restriction sites that are compatible with the ends of the cut plasmid, allowing integration at multiple sites throughout the genome, and creation of multiple, potentially mutagenized transformants. To determine where the plasmid integrated, and the identity of the mutagenized gene, genomic DNA from the strain is isolated and cut with enzymes that would not cut within the plasmid itself. Fragments of DNA that contain the plasmid plus flanking genomic DNA are allowed to ligate, then transformed into *E. coli* to rescue the plasmid using a plasmid-specific drug resistance marker.

A more efficient system for random mutagenesis incorporates a tag on the mutagenic DNA, as is the case with the transposon mutagenesis library used in *Saccharomyces*. In the system termed TAGKO, a cosmid library containing the genome of *Magneporthe griseae* was mutagenized with a transposon. The individual mutated cosmids were sequenced off of the transposon and annotated. The cosmids with known mutated genes were then transformed into the fungus to determine the effect of replacing the endogenous gene with the mutated version. Transposon mutagenesis is also routinely used with other filamentous fungi, including *A. nidulans*.

2.5.2.4 CRISPR Gene Editing in Fungi

One of the most recent and powerful advances in molecular biology consists of the CRISPR/Cas system for genome editing. The system was originally identified in bacteria and serves as a memory and defense response against invading phage. One part of the system consists of direct repeats of DNA interrupted by short, unique nucleotide spacers. These "clustered regularly interspaced palindromic repeats" are referred to as CRISPR, and the unique spacer sequences are homologous to regions of viral or plasmid DNA. The transcripts from these regions are processed into small CRISPR RNAs (crRNA). The second part of the system consists of the Cas proteins (CRISPR-associated), which are encoded close to the CRISPR sequences and contain helicase and nuclease domains. The Cas proteins form a complex with the crRNA from the CRISPR spacer sequence, as well as a separate trans-activating RNA (tracrRNA). The Cas9 protein is then guided to homologous sequence in the cell from an invading virus via the crRNA, thus allowing targeting and cleavage of the foreign DNA via double-stranded (DS) breaks. Cas activity requires that additional conserved sequence of two to five nucleotides, called the protospacer-adjacent motif (PAM), is located within the target DNA sequence, 3′ to the crRNA binding region.

For use as a molecular tool, elements of the CRISPR/Cas system are introduced into eukaryotic cells. The system from *Streptococcus pyogenes* is most frequently used, and is comprised of a single Cas9 protein, the crRNA, and a tracrRNA. For ease, a two component system was developed consisting of expression of Cas9 and a single guide RNA (sgRNA) (Figure 2.10). The sgRNA contains the crRNA, which is made complementary to a target DNA of interest,

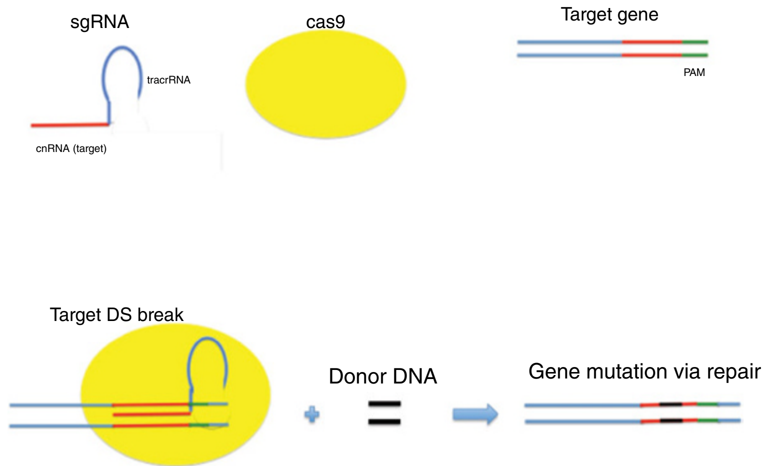

Figure 2.10 CRISPR/Cas9 for gene editing. (a) CRISPR components sgRNA and Cas9, and the target gene of interest containing a PAM sequence. (b) Cas9 interaction with the sgRNA and target locus, resulting in double-strand (DS) DNA breaks and introduction of mutation during repair.

fused to the tracrRNA. Cas9-induced DS breaks of the target DNA can result in insertions or deletions at the target locus, due to activity of the nonhomologous end-joining pathway (NHEJ). Alternatively, co-introduction of donor sequence homologous to the target can be incorporated to allow for replacement mutations (Figure 2.10). Cas9 activity has also been modified to allow for single strand breaks at the target locus, resulting in reduced deletions if a homologous repair sequence is introduced. Further, Cas9 nuclease activity can be eliminated, allowing for a diversity of modifications at the target locus, including introduction of effector domains and fluorescent localization, for example.

The CRISPR/Cas9 system has been used successfully in several filamentous fungi. For example, in *Aspergillus* species, autonomously replicating plasmids of different markers containing both codon-optimized *cas9* under control of the constitutive *tef1* promoter and an sgRNA nested within a larger transcript were constructed. The sgRNA was flanked by two ribozyme sequences for liberation, and driven by the *gpdA* promoter. Introduction of a single plasmid was successful in mutating various targets, including spore color genes as well as *pyrG*, through short and longer deletions. For example, plasmids with no sgRNA, or an sgRNA with a protospacer targeting an exon of the *yA* gene, were constructed via USER fusion cloning in *E. coli* and transformed into *A. nidulans*. Colonies expressing only *cas9* were green, whereas those also containing the sgRNA were yellow. Variations on the method were applied in *A. fumigatus*, where a construct containing *cas9* and sgRNA was integrated into the genome. Here, the *pksP* locus was successfully targeted in approximately 25–46% of transformants, in the form of single and large insertions and deletions Further, the CRISPR system was utilized to fuse calcineurin to GFP in a marker-free manner

in *A. fumigatus* as well as targeting two loci simultaneously Recently, CRISPR allowed for introduction of the gene cluster for trypacidin production in a non-producing strain of *A. fumigatus*. Despite the advantages of ease of manipulation, ability to simultaneously target several loci, and the powerful potential to be used as a screening tool, the CRISPR system in filamentous fungi requires additional validation to address issues including the extent of off-target and silent mutations, for example.

In the yeast *S. cerevisiae*, CRISPR technology was first applied in 2013, and has since been utilized successfully in a number of different applications. DiCarlo *et al*. (2013) constructed a strain carrying codon-optimized Cas9 gene under control of a constitutive promoter, which was subsequently transformed with sgRNA-containing cassettes and linear oligonucleotides as donor DNA for gene targeting. Importantly, off-target mutation was addressed by following a nontargeted locus and was found to be negligible. Subsequent studies have optimized the approach such that a single plasmid can be used to introduce Cas9 and gRNAs. In another study, a single plasmid coupled with multiple PCR-generated gRNA and donor DNA cassettes was achieved to allow for combinatorial gRNA delivery, underscoring the power and simplicity of the system. CRISPR has also been successfully utilized in other yeasts such as *Schiz. pombe* and *C. albicans*, for example.

2.6 Conclusion

Fungi have important interactions with humans. Many of these organisms are economically significant, such as the baker's or brewer's yeast *S. cerevisiae* generating our bread and alcohol, or medically important, such as the human pathogens *C. albicans* and *A. fumigatus*. They represent, as well, experimental systems that have been extensively exploited to investigate the molecular details of eukaryotic cell function. In particular, the fungi include some of the most highly developed eukaryotic genetic systems. From early work in *Neurospora* leading to recognition of the relationship of genes and enzymatic functions, to more recent work in the yeasts *S. cerevisiae* and *Schiz. pombe* that has uncovered the molecular basis of control of cell proliferation, we can see that the isolation and characterization of genetic variants of fungal cells has revolutionized our understanding of cellular function.

A key characteristic of genetic systems is that they can be exploited to create new combinations of variations. This ability is highly developed in fungal systems. The capacity to identify all the products of the meiotic event has allowed for such a sophisticated analysis of meiotic recombination that fungal systems have played leading roles in the development of molecular models of DNA recombination. Only through the analysis of complete individual meioses would we be able to detect the gene conversions and post-meiotic segregations that established the existence of heteroduplex DNA during the recombination process.

We have only touched the surface of the uses of fungi in uncovering details of the function of eukaryotic cells. Our current work focuses on a few fungi, but the ability to sequence whole genomes promises to allow many more organisms to be investigated through the tools of genetics and genomics. In the future, we should be able to study the characteristics of more and more of the fungal diversity, and investigate the current model organisms with even greater sophistication. Based on the success of the investigations of the prior decades, we can expect the fungi to continue to be in the forefront of research in providing new information on a diversity of cellular processes in the future.

Acknowledgment

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3 Fungal Genomics

David Fitzpatrick

3.1 Introduction

Genomics is defined as the study of an organism's complete genome sequence. The first complete (nonviral) genome to be sequenced was the bacterium *Haemophilus influenzae* in 1995. Today, more than 78,990 bacterial genomes have been sequenced or resequenced (source Genome Online Database; Table 3.1). Baker's yeast (*Saccharomyces cerevisiae*) was the first eukaryote to have its genome completely sequenced (released 1996). Since then, over 20,000 eukaryote genomes have been completed or resequenced, including our own (in 2001). Because of their relatively small genome size, roles as human/crop pathogens, and importance in the field of biotechnology, approximately three-quarters of all available eukaryote genome data belongs to the fungal kingdom. Some species, such as *S. cerevisiae*, have actually had over 400 strains sequenced at the time of writing. The majority of fungal species that have been sequenced belong to the Ascomycota phylum; furthermore, there is a significant bias towards species that are pathogens of humans. Reduced costs and continued improvements associated with new sequencing technologies (see Section 3.2) should mean that a wider range of evolutionarily, environmentally, and biotechnologically interesting organisms will become available in the coming years.

This abundance of genomic data has moved the fungal kingdom to the forefront of eukaryotic genomics. While some of the species sequenced are closely related, others have diverged one billion years ago. This enables us to use fungi to study evolutionary mechanisms associated with eukaryotic genome structure, organization, and content. Furthermore, doing a direct comparison between two

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Table 3.1 Useful online resources.

Database	URL address
SGD	www.yeastgenome.org
CGD	www.candidagenome.org
AspGD	www.aspgd.org
CADRE	www.cadre-genomes.org.uk
Aspergillus fumigatus database	www.aspergillusgenome.org
CandidaDB	www.candidagenome.org
NCBI	www.ncbi.nlm.nih.gov
Sanger Institute	www.sanger.ac.uk
EMBL	www.embl.de
DDBJ	www.ddbj.nig.ac.jp
Swiss-Prot	http://expasy.org/sprot/
Fungal Tree of Life	https://aftol.umn.edu/
CGOB	http://cgob.ucd.ie/
Genomes OnLine Database (GOLD)	https://gold.jgi.doe.gov/

or more closely related pathogenic and nonpathogenic species, a process called comparative genomics (see Section 3.4), permits us to locate metabolic pathways or genes associated with virulence.

3.1.1 The Fungal Kingdom

Fungi are eukaryotic organisms (contain a nucleus and membrane-bound organelles) and form one of the kingdoms of life. They lack chlorophyll and are saprobic (live on dead organic matter). Traditionally, fungi were thought to be closely related to plants; however, recent phylogenetic studies have shown that fungi are more closely related to animals than plants. The exact number of fungal species is unknown, but it is estimated to be 1.5 million.

Initial phylogenetic analyses of fungal species had revealed that there were four distinct phyla within the fungal kingdom: the Chytridiomycota, Zygomycota, Ascomycota, and Basidiomycota. Early diverging species were found in the Chytridiomycota and Zygomycota phyla. More recent phylogenetic analyses have suggested that neither the Chytridiomycota nor the Zygomycota phyla are monophyletic. Monophyletic species are descended from a common evolutionary ancestor

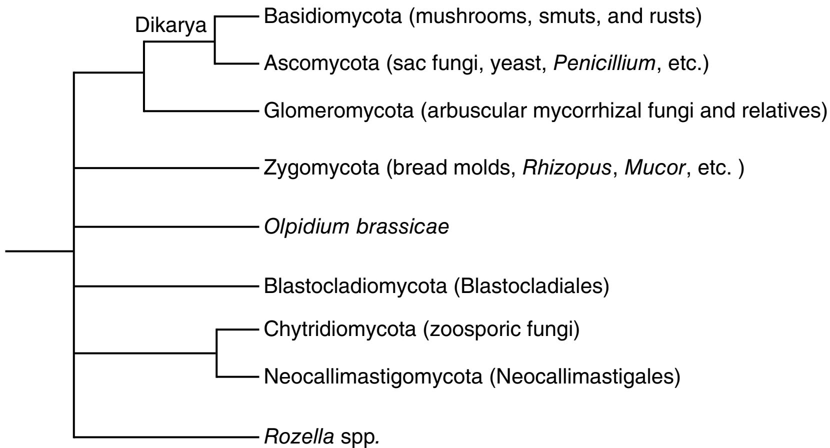

Figure 3.1 Schematic of the current consensus on fungal phylogeny. Fungal phylogenetics is currently in flux and the relationships between particular phyla are still unknown. (Phylogeny redrawn from the Tree of Life web project (Table 1).)

and are not shared with any other groups. Recent studies now suggest that there are actually six fungal phyla and four additional unplaced subphyla (Figure 3.1).

Fungal phylogenetics is far from static or resolved; in fact the placement of specific species within the fungal kingdom has been questioned. For example, gut inhabitants of arthropods (*Trichomycetes* species) that were thought to be members of the Zygomycota are actually protists. Furthermore, other species that were considered fungal as they display heterotrophic, mold-like growth morphology are in fact Stramenopiles (including algae, kelps, and diatoms). The genome sequences of sparsely sampled phyla should help resolve this ambiguity in future years. For clarity, only the four traditional fungal phyla are discussed here.

The Chytridiomycota (chytrids) are the only fungal phylum to produce zoospores and they require water for their dispersal. They are an ancient group of organisms and are thought to have changed little since fungi first diverged from the last common ancestor of all eukaryotes. Most chytrids live in soil or freshwater, although some are found in marine environments, where they have important roles in the decomposition of organic matter. The chytrid *Batrachochytrium dendrobatidis* has been shown to be responsible for a disease in amphibians called chytridiomycosis, which is responsible for declining frog populations in tropic regions.

The Zygomycota reproduce sexually and form thick-walled sexual spores called zygospores. Zygomycetes are morphologically diverse and account for 1% of all described fungal species. They are also the most ecologically diverse phyla of fungi, living as saprophytes on dung, fruit, and soil. They can also be found in the gut of arthropods and some are pathogens of plants, animals, and

other fungi. Some well-known members include *Mucor* and *Rhizopus* species, and these cause bread mold and fruit rots, respectively.

The Ascomycota is the largest fungal phylum, accounting for approximately 65% of all known fungal species. The distinguishing feature of this phylum is an ascus. The ascus is the sexual spore-bearing cell where meiosis, followed by one round of mitosis occurs to generate eight (or a multiple of eight) ascospores. Ascospores have thick walls and are therefore resistant to adverse conditions, but under favorable conditions will germinate to form a haploid fungus.

Three subphyla have been described in the Ascomycota: Saccharomycotina, Pezizomycotina, and Taphrinomycotina. The Saccharomycotina lack an ascoma, resulting in naked asci, and include important species such as *S. cerevisiae* (brewer's yeast) and *Candida albicans* (a human pathogen). Members of the Pezizomycotina include all filamentous fungi (molds) and include species such as *Aspergillus fumigatus* (a human pathogen) and *Penicillium chrysogenum* (produces penicillin antibiotic). The Taphrinomycotina phylum exhibits many diverse morphologies, including the fission yeast form of *Schizosaccharomyces pombe*.

The Basidiomycota phylum accounts for approximately 35% of the known fungal species. A number of basidiomycetes are instantly recognizable as they produce elaborate fruit bodies including puffballs and mushrooms. Well-known edible Basidiomycota mushrooms include *Agaricus bisporus* (common mushroom) and *Pleurotus ostreatus* (oyster mushroom) (see Chapter 6). The ability to degrade lignin (found in plant cell walls) by certain members (e.g. *Armillaria mellea*) of the Basidiomycota is significant as few microbes have this ability. Fungi that can degrade lignin are interesting in a biotechnological sense, as they have the potential to detoxify and delignify waste products (see Chapter 8).

3.2 Genome Sequencing

3.2.1 Sanger Sequencing

Fredrick Sanger won the Nobel Prize in 1958 for determining the amino acid sequence of the protein insulin. After this he turned his attentions to developing sequencing methods for RNA and DNA. In 1977, Sanger published a method for DNA sequencing commonly referred to as dideoxy sequencing (or chain termination) and won his second Nobel Prize for this work in 1980.

Sanger sequencing relies on DNA polymerase (a replication enzyme) to synthesize a new strand of DNA which in turn can reveal the sequence of the target DNA strand. DNA polymerase replicates a new DNA strand complementary to a piece of single-stranded DNA, by linking the 5′-hydroxyl end of a nucleotide to the 3′-OH group of the nucleotide at the end of a primer. A primer is a small piece of single-stranded DNA that can hybridize to one strand of the template DNA and be extended by successive additions of nucleotides. As well as a supply

of nucleotide triphosphates (dNTPs), the Sanger method requires 2′-3′-dideoxy-nucleotide triphosphates (ddNTPs) in small quantities relative to dNTPs. ddNTPs contain no reactive 3′-OH and therefore terminate DNA synthesis once they are incorporated into the primer extension.

A typical reaction mixture contains dATP, dTTP, dCTP, dGTP, and one ddNTP (ddGTP for example). Primer extension continues until an unmatched nucleotide is paired with a complementary ddNTP. Many fragments each ending with a ddNTP of varying lengths are produced from such a reaction (Figure 3.2). Radioactive sulfur or phosphorus isotopes are incorporated into the newly synthesized DNA template via labeled dNTPs, therefore making all fragments detectable by radiography. Fragments can then be separated based

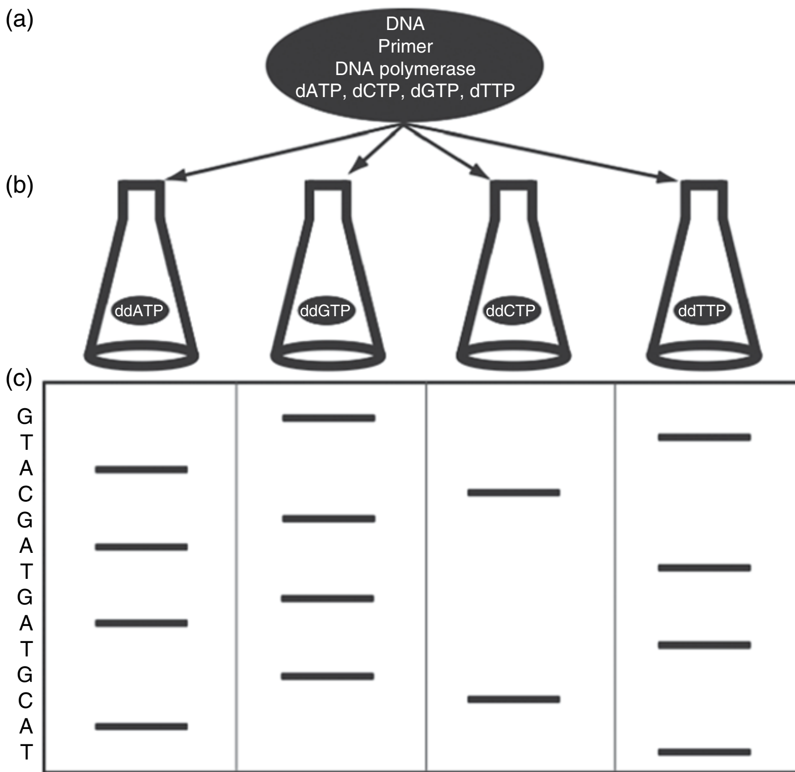

Figure 3.2 Schematic of the Sanger sequencing method. (a) Four separate DNA extension reactions are carried out. Materials required include single-stranded DNA, DNA polymerase, DNA primers, and all four dNTPs. One of the dNTPs is radioactively labeled to enable visualization in part c). (b) Each of the four reactions contains a different dideoxynucleoside triphosphate (ddNTP). Synthesis continues until a ddNTP is incorporated, terminating extension reaction. (c) Products are separated based on size on a polyacrylamide gel and the sequence can be determined.

on length with polyacrylamide gel electrophoresis and the sequence can be determined (Figure 3.2). To determine the relative position of all four nucleotides it is necessary to run four reactions (each with a different ddNTP) in parallel (Figure 3.2).

3.2.2 Next-Generation Sequencing

When Fred Sanger and co-workers first developed their enzyme-based chain termination method for DNA sequencing they could not have predicted the massive advances in sequencing technology that have taken place in recent years. Next-generation sequencing (NGS) refers to novel commercial technologies which make it possible to generate millions of sequence reads (hundreds of base pairs (bp) in length) in a single sequencing reaction. With respect to fungi, NGS has been used to resequence targeted strains such as *S. cerevisiae*, *Candida albicans*, and *Cryptococcus neoformans*, sequence *de novo* genomes, and analyse transcriptomes and characterize fungi in environmental samples.

In the following three sections, we examine some of the most popular NGS platforms. The Roche/454 GS FLX pyrosequencer and the Illumina genome analyser are considered second-generation sequencing platforms, while the more recent PacBio SMRT sequencing platform is considered as a third-generation sequencing platform.

3.2.2.1 Roche/454 GS FLX Pyrosequencer

The first commercial NGS was introduced in 2004 by 454 Life Sciences (now Roche Diagnostics). It utilizes pyrosequencing, a technique that ultimately emits light (using the firefly enzyme luciferase) after each incorporation of a nucleotide by DNA polymerase. With the latest instrument and sequencing kits and reagents, it is possible to generate more than one million reads (average length 400 bases) in a single 10-hour run.

The Roche/454 sequencer has three basic steps: single-stranded template DNA library preparation, emulsion-based clonal amplification of the library, and sequencing-by-synthesis. The DNA library preparation stage fragments sample DNA into small single-stranded DNA fragments (300–800 bp). Universal adapters specific for 3′ and 5′ ends are added to each fragment. Each universal adapter is 44 bases in length and consists of a 20-base PCR primer, a 20-base sequencing primer, and an initiating 4-base (TCGA) sequence.

For the clonal amplification stage the single-stranded DNA library is mixed with small DNA capture beads (~35 μm in size). The beads contain one of the adapter primers and ligate a single-stranded DNA library fragment. The ratio of capture beads to library DNA is chosen to ensure that each bead binds a single DNA fragment. The bead-bound library complexes are emulsified with amplification

reagents, resulting in microreactors containing just one bead with one unique sample-library fragment. In parallel, each library fragment is amplified using thermal cycling within its own microreactor. The end result is several million copies of unique library DNA per bead. At the end of this phase the emulsion is broken down. DNA-positive beads are enriched and deposited onto a PicoTater-Plate (PTP) (a solid surface containing wells (~44 μm)). The DNA-positive beads are overlaid with packing and enzyme (luciferase and sulfurylase) beads.

The final step is sequencing by synthesis. Nucleotides are flown across the PTP sequentially in a fixed order. Nucleotides that are complementary to the template strand are incorporated by the DNA polymerase, extending the DNA strand. If the template DNA contains three adjacent guanines (G), three cytosines (C) will be incorporated into the sequencing strand. As incorporation of nucleotides occurs at different rates, strands extend at different rates. Nucleotide incorporation generates free pyrophosphate, which is converted to ATP by the sulfurylase enzyme. ATP results in the oxidation of luciferin by the enzyme luciferase, and light whose intensity is proportional to the number of bases incorporated is emitted. Light photons are captured by a charge coupled device (CCD) camera and signal intensity per nucleotide is used to determine the sequence of template DNA.

3.2.2.2 Illumina Genome Analyzer

The Illumina genome analyzer (IGA) was released in 2006. Currently, read lengths (up to 150 bp) are shorter than those of 454 sequencing. The major advantage of the IGA over other sequencing platforms is the quantity of data produced at low cost. The sequencing process used by the IGA has three main steps: DNA library preparation, generation of clonal clusters, and sequencing by synthesis.

The DNA library is prepared by fragmenting sample DNA by sonication (ultrasound) or nebulization (vaporizing), and sequencing adapters are ligated to the fragments. Clonal clusters are generated by immobilizing sequencing templates on a flow cell. The flow cell is composed of silica and has eight lanes running lengthways. Separate DNA libraries can be loaded into different lanes, enabling eight individual sequencing runs per slide. Adapter ligated template is pumped into the flow cell and template DNA is captured by forward/reverse "lawn" primers that are covalently linked to the flow cell (Figure 3.3a). Free ends of DNA template attach to lawn primers forming U-shaped bridges (Figure 3.3b). Unlabeled nucleotides are added and solid-phase bridge amplification occurs, resulting in double-stranded clonal clusters. Reverse strands are removed from double-stranded DNA and sequencing primers are hybridized to free 3′ ends; this step ensures all clusters are sequenced in the same direction from the same end. The flow cell is then transferred to the IGA for sequencing.

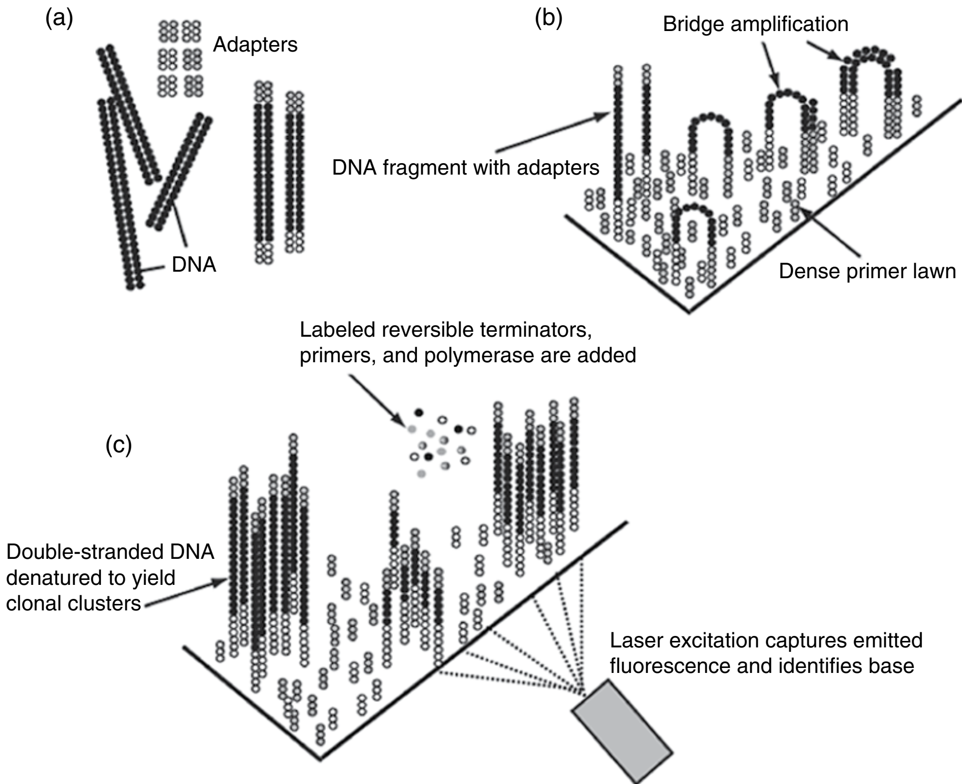

Figure 3.3 Schematic of the Illumina genome analyzer sequencing technology. (a) DNA is fragmented and adapters are ligated to both ends of the fragments. (b) Single-stranded fragments bind randomly to the surface of the flow cell (see main text). (c) Sequencing by synthesis (see main text).

IGA sequencing by synthesis involves the incorporation of fluorescent terminator deoxynucleoside triphosphate (dNTP) (Figure 3.3c). During each sequencing cycle, DNA polymerase incorporates a single dNTP to each of the growing nucleic acid chains (Figure 3.3c). After each cycle, the IGA images the relevant fluorescent dye identifying the base and then cleaves the terminator dye so addition of the next nucleotide can proceed. The sequence lengths of all clusters are identical as they are governed by the number of cycles (nucleotide incorporation, imaging, and cleavage) undertaken.

Recent developments in Illumina have led to the introduction of an ultra-high-throughput sequencer known as the HiSeq. The HiSeq produces over 50 gigabases a day and has a read length of 150-base paired-end reads. Illumina has also recently released the MiSeq sequencer. This platform is favored for focused genome projects such as bacterial genome sequencing or metagenomics and can produce over 1.5 gigabases of data in a 24-hour run with a read length of 150-base paired-end reads.

3.2.2.3 PacBio SMRT Sequencing

Pacific Biosciences (PacBio) has released a platform that is considered to be leading the way in third-generation sequencing platforms. Third-generation platforms aim to reduce the amount of DNA/RNA starting material and ultimately increase throughput. The PacBio RS was released in 2010 and allows for the detection of DNA synthesis by a single DNA polymerase known as single molecule real time (SMRT). It incorporates the use of a thick metal film containing microwave cavities. In this manner, SMRT sequencing differs from other NGS platforms as it involves real-time nucleotides and does not rely on reversible terminators that delay DNA synthesis. Furthermore, the PacBio platform does not require the amplification of template DNA before sequencing. Most importantly, the PacBio platform allows for the generation of reads up to thousands of kilobases in length, making the platform ideal for *de novo* genome assembly.

3.3 Bioinformatics Tools

3.3.1 Locating Homologs

Sequence similarity searches are an essential component of genomic studies. They allow researchers to identify homologs and conserved structural motifs, and help assign putative functions to unannotated genes in *de novo* genomes. Since 2002, there has been an exponential increase in the quantity of genetic data available in public databases such as NCBI (Table 3.1). To utilize this deluge of genetic data it is imperative we have efficient similarity search techniques.

3.3.1.1 Global and Local Alignments

The methods used to infer homology can be categorized into two main types. A global alignment attempts to align two sequences over their entire length. Global sequence alignment works best when the sequences being compared are approximately the same length and highly similar. A local alignment, on the other hand, attempts to align two sequences at regions where high similarity is observed, instead of trying to align the entire length of the two sequences being compared. Local alignments are usually more meaningful than their global counterparts, as they align conserved domains that may be important functionally even though the matched region is only a small proportion of the entire sequence length.

Needleman and Wunsch first implemented dynamic programing in 1970 to align sequences globally. Dynamic programing is a computational technique that determines the highest scoring alignment between two sequences. Smith and Waterman later adapted the Needleman and Wunsch method to align sequences locally. Both methods utilize a scoring matrix where rows and columns correspond to the bases/

amino acids being aligned. The first row and column of the matrix are filled with zeroes; the remaining cells are filled iteratively with values dependent on neighboring cell values. If a matrix cell corresponds to an identical base/residue, a match score is added to the score from the neighboring diagonal square. Alternatively, the maximum score is determined from cells above by adding a gap penalty. Gap penalties are generally negative numbers. Local alignments are produced by starting at the highest scoring cell in the matrix and following a trace path to a cell that scores a zero.

3.3.1.2 BLAST

The Basic Local Alignment Search Tool (BLAST) is the most commonly used method for locating homologs in a sequence database. BLAST is both sensitive and efficient at locating regions of sequence similarity between nucleotide or protein sequences.

The BLAST algorithm begins by "seeding" the search with a small subset of letters (query word) from the query sequence (Figure 3.4). The query word as well as related words (where conservative substitutions have been introduced) are located. All words are scored by a scoring matrix and this yields the "neighborhood" (Figure 3.4). BLAST uses a neighborhood threshold (T) to determine which words are closely related to the original query word. Increasing the value of T implies that only closely related sequences are considered, while decreasing it allows for distantly related sequences to be considered.

The original query word is aligned to a word above the neighborhood threshold (Figure 3.4). The BLAST algorithm then proceeds to extend the alignment in both directions, tracking the alignment score by addition of matches, mismatches, and gaps. The maximal length of the alignment is determined by the number of positions aligned versus the cumulative score of the alignment. The alignment extension continues until the number of mismatches starts to decrease the cumulative score of the alignment; if this decrease is large enough (above a predefined value X, Figure 3.4), the alignment procedure ceases and the resultant alignment is called the high scoring segment (HSP). A score threshold is defined by the algorithm, and if the HSP clears this score the alignment is reported in the BLAST result file.

Finally, the biological significance of an HSP is determined. BLAST uses the E-value to calculate the number of HSPs that would have a score greater than S by chance alone. Lower values of E imply greater biological significance; in essence, E can infer whether the HSP is a false positive.

3.3.1.3 FASTA

Like BLAST, FASTA is a program for rapid alignment of pairs of protein or DNA sequences and was the first widely used algorithm utilized for database similarity searching. FASTA begins by locating subsequences above a particular word length from the database sequence to subsequences of the query sequence. In FASTA

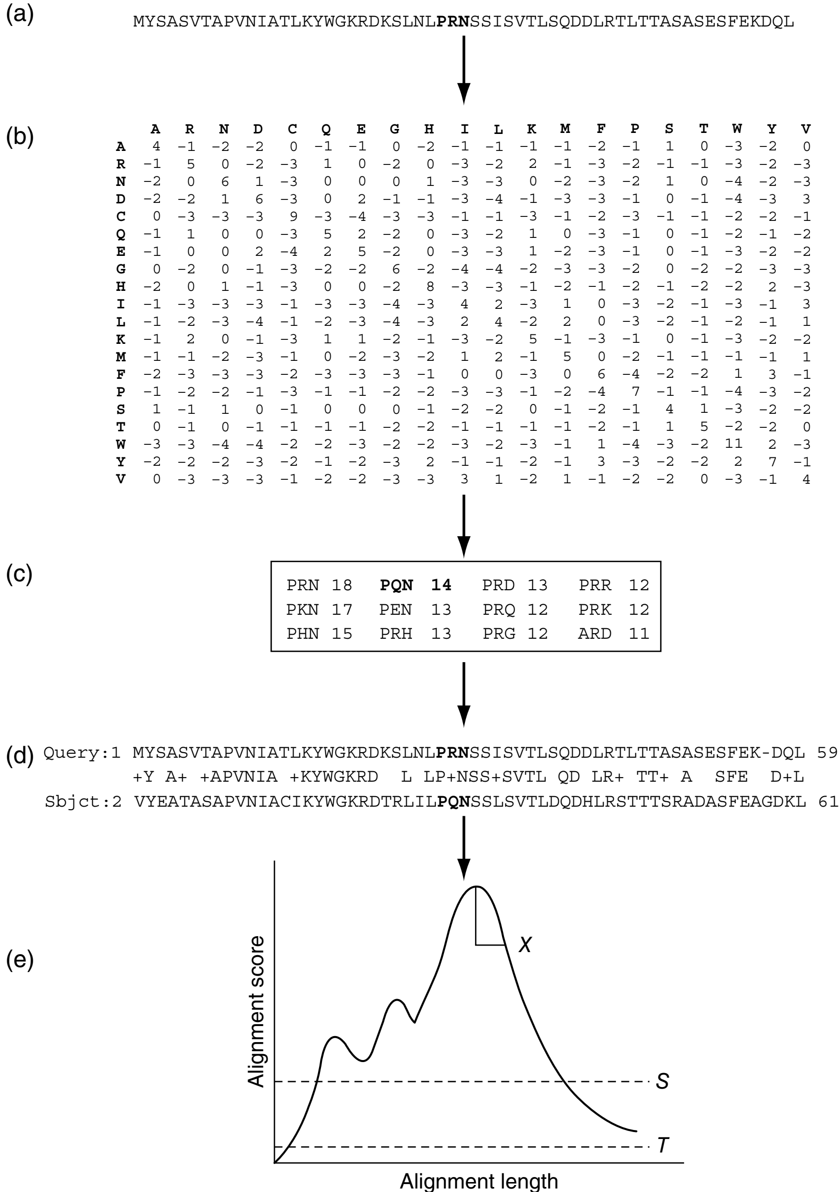

Figure 3.4 Steps taken by the BLAST algorithm when searching a database. (a) The query sequence is compared to a scoring matrix (b), and scores for query words of a given length (three in this case) are calculated (c); query words greater than a certain threshold (T) are used to search the database. (d) The algorithm attempts to extend the alignment either side of the query word that has a hit in the target database. (e) Extension continues until the alignment score falls off more than the allowable significance decay, X.

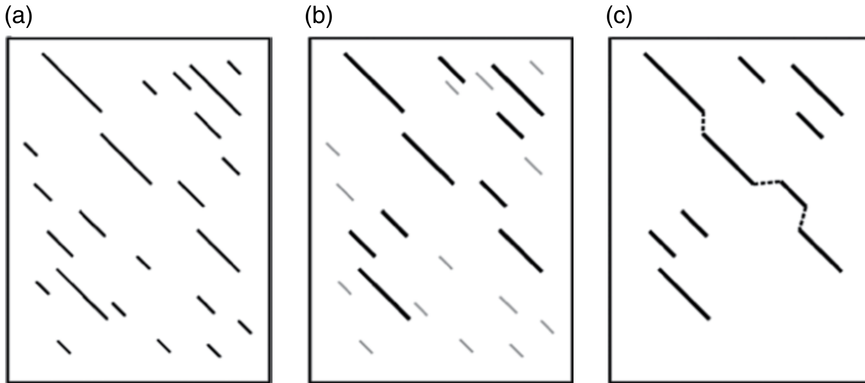

Figure 3.5 Steps taken by the FASTA algorithm when searching a database. (a) Words common to the query and target sequence are located. FASTA connects words close to one another and these are represented by diagonal lines. (b) The top 10 diagonals are selected for further analysis. (c) Diagonals are aligned optimally using a local alignment strategy.

the word length parameter is termed *ktup* and it is equivalent to W in BLAST searches. FASTA generates diagonal lines on a dotplot where residues match up (Figure 3.5). The FASTA algorithm next locates diagonal regions in the alignment matrix that contain as many *ktup* matches as possible with short distances separating them (Figure 3.5). The top 10 highest scoring diagonal regions are retained and correspond to high-scoring local alignments that do not contain gaps.

FASTA then determines which of the adjacent diagonals can be joined together, thereby increasing the overall length of the alignment. For each diagonal that is connected, a joining penalty is invoked and the overall score is determined by addition of the net scores of individual diagonals minus the joining penalties. The score of the enlarged diagonals is referred to as *initn*. All enlarged diagonals are ranked based on score and the highest scoring ones are aligned optimally using a local alignment strategy. Finally, FASTA assesses the significance of the alignment by randomly generating sequences of similar length and composition as the query sequences and calculates the probability that an alignment would be seen by random chance.

3.3.2 Multiple Sequence Alignment

Multiple sequence alignment (MSA) is a method that allows us to infer the interrelationships between DNA or protein families. While pairwise alignments are useful for locating homologs in databases and illustrating conservation between two sequences, they are not as informative as MSA. MSA has the ability to locate conserved residues/domains among thousands of sequences, which can provide insights into important evolutionary and physiochemical processes. MSA is the first step in phylogenetic analysis and is commonly used when designing primers for DNA amplification.

MSA is much more computationally intensive and difficult when compared to the pairwise strategy employed by BLAST and FASTA. One of the most commonly used MSA algorithms is CLUSTAL and it utilizes progressive alignment to efficiently align all sequences of interest. CLUSTAL follows three steps:

1 An initial assessment of how closely related different sequences are to one another by performing pairwise alignments.
2 A guide tree is generated based on the pairwise alignment scores.
3 Sequences are aligned progressively, guided by the phylogenetic tree. Closely related sequences are aligned first, and then additional sequences and groups are aligned.

CLUSTAL refines its progressive alignments by implementing a number of alignment penalties. For example, gap insertion and extension penalties exist to reflect that the chances of a gap within a hydrophilic region is more likely, as these are generally loops or random coil regions where gaps are more common. Similarly, residue-specific penalties are enforced so that domains that are rich in glycine are more likely to have an adjacent gap than positions that are rich in valine, for example.

3.3.3 Gene Ontology

When the first comparison between two complete eukaryotic genomes (yeast and nematode worm) was performed, researchers were surprised to discover that a high proportion of genes displayed orthology between these two distantly related organisms (which diverged ~ 1.6 billion years ago). Orthologs are genes that are derived from a common ancestor and commonly have the same function. Following from this, knowledge of the biological role of an ortholog in one species can be used to illuminate the putative function of the other ortholog. However, organizing biological data from multiple species databases is a major challenge and is made harder when different databases use different terminologies to describe the same process.

To overcome these difficulties, the Gene Ontology (GO) Consortium was set up in 2000 with the goal of producing a structured, precisely defined, common, controlled vocabulary for describing the roles of genes and gene products in any organism. Ontology terms provide a framework for storing and querying different databases using the same search terms. The GO Consortium provides detailed annotations for twelve important model organisms (*Arabidopsis thaliana*, *Caenorhabditis elegans*, *Danio rerio*, *Dictyostelium discoideum*, *Drosophila melanogaster*, *Escherichia coli*, *Gallus gallus*, *Mus musculus*, *Rattus norvegicus*, *Saccharomyces cerevisiae*, and

Schizosaccharomyces pombe). Collectively, those 12 species are referred to as the GO reference genomes. The GO consists of over 26,000 terms arranged in three branches:

1 *Cellular Component*: an individual component of a cell, but part of some larger object, such as an anatomical structure (nuclear membrane, for example).

2 *Biological Process*: describes broad biological goals, such as mitosis or purine metabolism.

3 *Molecular Function*: describes the roles carried out by individual gene products; examples include transcription factors and DNA binding.

The annotation of newly sequenced fungi can be greatly accelerated by comparisons to the GO reference genomes. *De novo* genes can be assigned putative functions based on sequence similarity to existing genes in one of the model organisms. The fact that two of the model organisms are fungi (*S. cerevisiae* and *Schiz. pombe*) makes the GO resource highly applicable to genome annotation in newly sequenced fungal genomes.

3.4 Comparative Genomics

3.4.1 Gene Families Associated with Disease

Comparative genomic analyses have shown that certain gene families are important for virulence in some fungal species. For example, a comparative analysis of 34 fungal genomes identified gene families that are specific to fungal plant pathogens (*Botryotinia cinerea*, *Ashbya gossypii*, *Magnaporthe grisea*, *Sclerotinia sclerotiorum*, *Stagonospora nodorum*, *Ustilago maydis*, and *Fusarium graminearum*). These families have expanded in terms of number (through duplication), during the evolution of phytopathogens. The same study also predicted the set of secreted proteins encoded by each phytopathogen and located gene families that were significantly enriched in the secretome (proteins secreted from a cell) of these species. Not surprisingly, many of the protein families identified are associated with pathogenic processes such as plant cell wall degradation and biosynthesis of toxins. Similarly, the complete genome sequence of the corn smut fungus, *U. maydis*, uncovered a large set of secreted proteins, many of which are arranged in clusters. These genes account for approximately 20% of all proteins secreted from *U. maydis*. Furthermore, the deletion of individual clusters seriously affects virulence, implicating the importance of these extracellular proteins.

In *Candida* species there are a number of gene families that are particularly enriched in highly pathogenic species (*C. albicans*, *C. parapsilosis*, and *C. tropicalis*) compared to nonpathogenic species. For example, comparative analysis has

shown that the Hyr/Iff proteins are present in all *Candida* species but are present in large numbers in the *Candida* pathogens (11, 17, and 18 copies, respectively). Members of this family are components of the cell wall, and based on sequence similarity are known to be evolving rapidly. They most likely play a role in host/fungal recognition, as rapid evolution of cell wall proteins is a common escape mechanism employed by microbial pathogens. Another family enriched in pathogenic *Candida* species is the agglutinin-like sequence (Als) family. ALS genes are well characterized in *C. albicans* and are important for adhesion to host cells, plastic surfaces, and biofilm formation. The ability to bind to plastic surfaces is a major problem in a hospital setting as it allows *Candida* species to enter the bloodstream via medical devices such as intravenous drips; similarly reduced susceptibility to antifungal drugs is observed when *Candida* species grow as biofilms.

3.4.2 Synteny

The term synteny was traditionally used by geneticists to indicate the presence of two or more loci on the same chromosome. In the post-genomic era the concept of synteny has been expanded to address the relative order of genes on chromosomes that share a common evolutionary history. Two regions are considered syntenic if multiple consecutive genes are found in a conserved order between the two genomes under consideration (Figure 3.6). Synteny between two species may break down due to genome rearrangements in one or both species since they last shared a common ancestor.

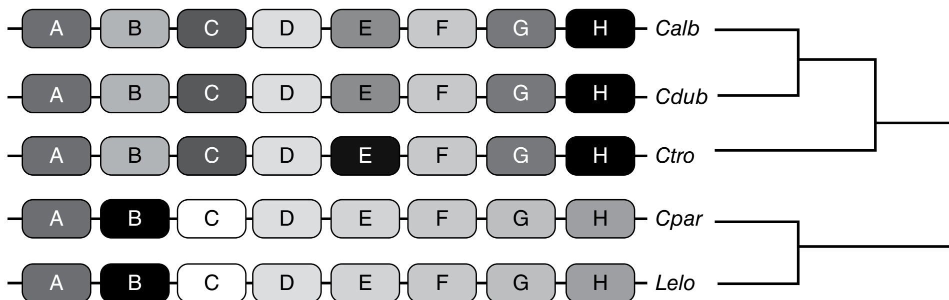

Figure 3.6 Synteny of eight orthologs in five *Candida* species. Genes A, D, and F are syntenic in all species; this is represented by conservation of color between them. Genes B, C, G, and H are syntenic in *C. albicans*, *C. dubliniensis*, and *C. tropicalis*. Orthologs of B, C, G, and H are present in *C. parapsilosis* and *L. elongisporus* and are syntenic with one another, although they are located in different genomic locations relative to the first three species. The degree of synteny between the five species closely matches the phylogeny of these species.

Comparative fungal genomic analyses have shown that syntenic structure is generally conserved between very closely related fungal species, but reduces as species become more distantly related. For example, the main subdivisions of Saccharomycotina yeasts share minimal synteny conservation between one another. However, large syntenic blocks are observed when members of the same subdivision are compared. For example, an analysis of nine *Candida* genomes (using the *Candida* genome order browser (CGOB); Table 3.1) showed they shared a high proportion of syntenic blocks. Conservation of gene order between closely related species correlates with phylogenetic analyses (Figure 3.6). Other studies have shown that homologous chromosomes between the S*accharomyces* "*sensu stricto*" species are almost collinear, differing only by a small number of translocations and large inversions (segment of DNA is reversed). A comparison of two basidiomycete genomes (*Coprinopsis cinerea* and *Laccaria bicolor*) showed they share extensive regions of synteny. The largest syntenic blocks occur in regions with low meiotic recombination rates and contain no transposable elements (cause translocations).

3.4.3 *In silico* Metabolic Analysis

The availability of fungal genomes allows us to compare and contrast the metabolic repertoire of different species *in silico*. Detailed information from a metabolic pathway in one organism allows us to locate similarities or differences in another. Comparative metabolic analysis provides us with insights into potential disease mechanisms between pathogenic and nonpathogenic fungal species. Similarly, they enable us to investigate the metabolic differences that allow one species to break down a particular substrate while another cannot.

Comparative studies of fungal species have shown that the genomic location of certain genes is not random. For example, an analysis of the *S. cerevisiae* genome revealed that there is a significant tendency for genes from the same metabolic pathway to cluster in its genome. Likewise, genes involved in secondary metabolism are often clustered in the genomes of filamentous fungi (such as *Aspergillus* species).

An analysis of synteny between nine *Candida* genomes showed that approximately 20% of metabolic pathways analyzed display some evidence of clustering (lie within 10 genes of one another). One of the clustered pathways is involved in the metabolism of N-acetylglucosamine (Nag) to fructose-6-phosphate. It had initially been proposed that the ability of pathogenic strains of *Candida* to utilize Nag as an alternative carbon sources is an important virulence factor. The three genes involved in the conversion of Nag to fructose-6-phosphate are hexokinase kinase (HXK1), Nag-6-phosphate deaminase (NAG1), and Nag-6-phosphate deacetylase (DAC1). These act sequentially on Nag and are present in *C. albicans* in a cluster termed the Nag regulon. Synteny analysis showed that the Nag regulon is conserved in nearly all *Candida* species. The conservation of the Nag

regulon in pathogens like *C. albicans*, *C. tropicalis*, and *C. parapsilosis* and nonpathogens such as *C. dubliniensis*, *Lodderomyces elongispors*, and *Debaryomyces hansenii* suggests that the ability to utilize Nag is not a virulence factor. *Saccharomyces cerevisiae* is missing the Nag regulon and cannot utilize Nag; however, it has been shown that expression of *C. albicans NAG* genes in *S. cerevisiae* enables it to utilize Nag.

3.4.4 Horizontal Gene Transfer

Horizontal gene transfer (HGT) is the exchange of genes between different strains or species. HGT introduces new genes into a recipient genome that either are homologous to existing genes, or belong to entirely new sequence families. Bacterial genomic sequencing has revealed that HGT is prominent in bacterial evolution and has been linked to the acquisition of drug resistance and the ability to catabolize certain amino acids that are important virulence factors. There are numerous methods to detect genes that have been transferred horizontally into a genome, including locating genes with an atypical base or codon usage pattern. Another approach is to perform a similarity search of candidate genes against a database and locate unexpected top database matches. These approaches have the advantage of speed and automation but do not have a high degree of accuracy. Some notable flaws with the similarity-based approach of detecting HGT were brought to attention when the initial publication of the human genome reported that there were 223 genes that have been transferred from bacterial pathogens to humans. These findings were based on top hits from a BLAST search, but subsequent phylogenetic analyses showed these genes were not recently transferred from bacterial species through HGT. Indeed, the most convincing method to detect HGT is by phylogenetic inference. Topological disagreement (incongruence) between trees inferred for one gene family and that inferred for another can often be parsimoniously explained only by invoking HGT.

The process of gene transfer has been assumed to be of limited significance to fungi. However, the availability of fungal genome data (Table 3.1) and subsequent comparative genomic analyses are showing the importance of HGT in the genome evolution of fungi. For example, *S. cerevisiae* has acquired 13 genes (from bacteria) via HGT since it diverged from its close relative *A. gossypii* (Figure 3.1). This number corresponds to a small minority of the *S. cerevisiae* genome (less than 1%). However, these 13 genes have contributed to important functional innovations, including the ability to synthesize biotin, grow under anaerobic conditions, and utilize sulfate from several organic sources. Other documented examples of HGT in fungi include the acquisition of bacterial metabolic genes by *C. parapsilosis* and the acquisition of a toxin gene (ToxA) by *Pyrenophora tritici-repentis* from *Stagonospora nodorum* resulting in *Pyrenophora* infestations of wheat.

Unlike prokaryotes, the mechanisms of gene transfer into fungi are poorly understood. To date, no DNA uptake mechanism has been identified. Interkingdom

conjugation between bacteria and yeast has been observed, however, and *S. cerevisiae* is transformant competent under certain conditions. However, HGT is probably facilitated by the fact that fungi are saprobes that live in close proximity with other organisms.

3.5 Genomics and the Fungal Tree of Life

3.5.1 Phylogenetics

The goal of phylogenetics is to arrange a set of populations, species, individuals, or genes into a logical arrangement that infers the evolutionary relationships among them. Evolutionary relationships infer the historical development of species and are usually presented as an evolutionary tree (Figure 3.1). Traditional methods of fungal systematics such as vegetative cell morphology, sexual states, physiological responses to fermentation, and growth tests can assign fungal species to particular genera and families. The fungal fossil record is poor, however, and fungi exhibit few morphological characters; therefore an alternative approach is desirable. Fungal sequence data (RNA, DNA, and protein) have been used successfully to infer evolutionary relationships among species. In many cases, aligned sequences (Section 3.3.2) are processed as a distance matrix. Species that are most closely related will have a small distance, while distantly related species will have a larger distance measure. Phylogenetic algorithms such as UPGMA (Unweighted Pair Group Method with Arithmetic Mean), minimum evolution, and neighbor joining are used to represent distance matrices as phylogenetic trees.

The choice of phylogenetic markers for inferring the fungal tree of life is a contentious issue. Ideally, a phylogenetic marker should be ubiquitous throughout the species under consideration, present in single copy, have slowly evolving sites, and be unlikely to undergo horizontal gene transfer. For this reason, a significant majority of accepted relationships between fungal organisms are determined using 18S ribosomal DNA. However, single-gene analyses are dependent on the phylogenetic markers having an evolutionary history that reflects that of the entire organism, an assumption that is frequently violated. Also, individual genes contain a limited number of sites and, in turn, limited resolution. An alternative approach to single gene phylogenies is multigene phylogenies. These attempt to combine all available phylogenetic markers. There are two commonly used methods to do this: concatenated multigene phylogeny reconstruction and supertree analysis.

3.5.1.1 Concatenated Multigene Phylogenies

Multigene concatenation essentially appends many aligned genes together to give a large super alignment. Combining the data increases their informativeness, helps resolve nodes and basal branching, and improves phylogenetic accuracy.

Numerous species phylogenies have been derived by concatenation of universally distributed genes. Recently, the Fungal Tree of Life consortium (Table 3.1) used six housekeeping genes (18S rRNA, 28S rRNA, 5.8S rRNA, elongation factor 1-alpha, and two RNA polymerase II subunits (RPB1 and RPB2)) from 199 fungal species to reconstruct the evolutionary history of the fungal kingdom. As well as showing the evolutionary history of all fungal phyla, this analysis showed that the loss of spore flagella from early diverging fungi (similar to extant chytrids) coincided with the development of novel spore dispersal mechanisms leading to the diversification of terrestrial fungi.

3.5.1.2 Supertrees

Supertree methods take all input trees and generate a single representative species phylogeny (Figure 3.7). Individual input trees are derived from single genes. Comparative fungal genomic analyses have shown that less than 1% of all fungal genes are universally distributed. This situation implies that when we reconstruct multigene phylogenies we are ignoring 99% of the genes found in fungi. Ideally we would use 100% of the gene data. Supertree methods enable us to do this.

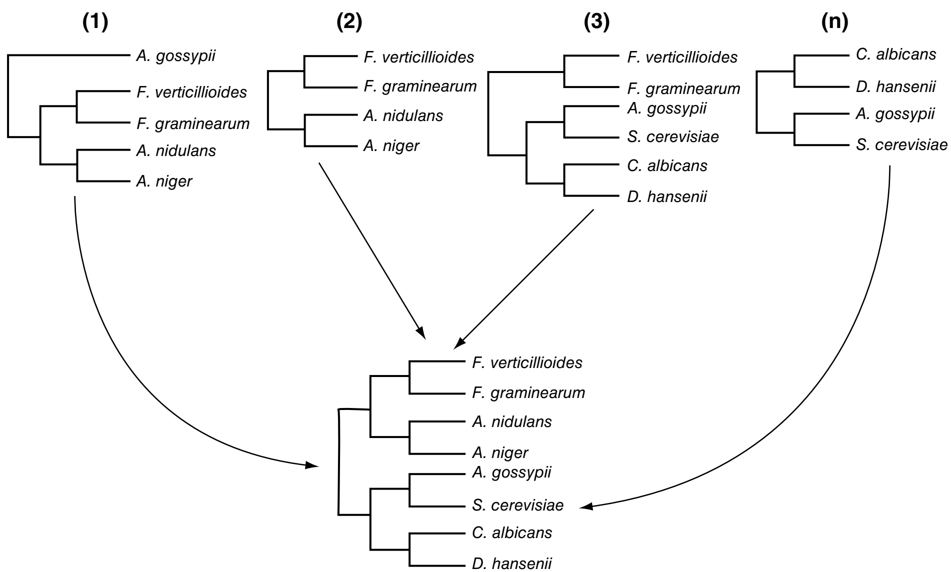

Figure 3.7 Representation of supertree reconstruction. Supertree methods take individual gene trees and express them as a single representative phylogeny. Thousands of trees (expressed as n) can be used as input for supertree techniques.

Supertree methods generate a phylogeny from a set of input trees that possess fully or partially overlapping sets of taxa (Figure 3.7). Therefore, supertree methods take as input a set of phylogenetic trees and return a phylogenetic tree that represents the input trees. This type of analysis yields a phylogeny that maximizes the number of genes used and therefore is truly representative of the entire genome. A supertree analysis of 103 complete fungal genomes identified 4,753 individual gene families. Individual phylogenies for each gene family were reconstructed and the complete set was summarized by supertree techniques. This analysis showed that within the Saccharomycotina, a monophyletic (single) clade containing *C. albicans* and close relatives is evident. Species within this clade translate the codon CTG as serine rather than leucine. A second monophyletic clade containing genomes that have undergone a whole-genome duplication (*S. cerevisiae* and close relatives) is also evident. Supertree techniques are becoming more popular in phylogenetic analysis and will be useful in reconstructing the Fungal Tree of Life as additional fungal genomes become available.

3.6 Online Fungal Genomic Resources

3.6.1 The Joint Genome Institute Fungi Portal

The continued use of fungi for the benefit of humankind requires an accurate understanding of how fungal species interact in different environments, both natural and synthetic. Metagenomics, the sequencing of all genetic material in a particular environmental sample gives us the ability to sample environments for complex fungal communities and will play an important part in harnessing fungi for energy, and industrial and potentially environmental management purposes. Our ability to accurately analyze metagenomic data relies on well-characterized and accurate reference genomes. To bridge the gap in our understanding of fungal diversity and help harness the power of metagenomics the Joint Genome Institute (JGI) of the Department of Energy and international collaborators have embarked on a project to sequence 1,000 fungal genomes from across the Fungal Tree of Life (Table 3.1). The consortium plans to fill in gaps in the Fungal Tree of Life by sequencing at least two reference genomes from the more than 500 recognized families of fungi.

Genome data are uploaded periodically to public web pages as they become available. As well as access to the raw sequence data and genome assemblies, researches can download predicted protein sequences. Furthermore, the JGI genome pipeline displays predictions for secondary metabolic clusters, orthologs in closely related species, and synteny between multiple species. Researchers can also undertake analyses by performing BLAST searches online or by conducting text searches for protein annotations of interest.

3.6.2 *Saccharomyces*, *Candida*, and *Aspergillus* Genome Databases

The *Saccharomyces* Genome Database (SGD) (Table 3.1) went online in 1997 and is a specialized database dedicated specifically to *S. cerevisiae*. It is housed at the Stanford Human Genome Centre and currently receives over 200,000 database hits a week. SGD provides users with access to the complete *S. cerevisiae* genome, its genes and their products, mutant phenotypes, and the literature supporting these data. It should be noted that SGD is not a primary sequence database (it contains information of the sequence alone), but instead collects DNA and protein sequence information from primary providers (such as GenBank, European Molecular Biology Laboratory (EMBL), DNA Data Bank of Japan (DDBJ), and SwissProt (Table 3.1)) and assembles all available information into datasets that are useful for molecular biologists. Therefore, SGD is considered a composite database as it amalgamates a variety of different primary database sources and cuts out the need to search multiple resources.

SGD is highly annotated, and supporting literature linked to each gene is curated by dedicated SGD curators. Weekly automated searches of PubMed locate literature associated with *S. cerevisiae* genes or products and these are refined by curators who assign a given publication with appropriate genes. SGD provides an excellent text-based search interface that allows users to search by gene name, gene information, protein information, author name, or full text. Among other things, SGD allows users to perform BLAST database searches, view yeast metabolic pathways, search yeast-specific literature, view gene expression data from multiple microarray studies, and view genes' relative positions on chromosomes.

SGD organizes gene information around locus pages. The gene name and associated systematic name are shown at the top of each locus page. Information about the feature of the gene is also given; genes can be "verified," meaning there is experimental evidence to show they are expressed, or "uncharacterized," implying a lack of experimental evidence. The "description" section details important information known about the gene and associated products. Each gene product is assigned gene ontology terms that describe its molecular functions, location within the cell, and putative biological processes in which it participates. A mutant phenotype section is also visible on the locus page. This section lists the type of mutation and any corresponding observable phenotype. Links to sequence information and literature describing the gene of interest are also available from the locus page.

The *Candida* Genome Database (CGD) went online in 2005 and is the central resource for researchers studying *Candida* pathogenesis and genetics. Before the launch of CGD, three independent web sites contained information about the *Candida* genome sequence and associated gene products. The Stanford Genome Technology Centre sequenced and distributed the genome; CandidaDB contained annotated genes for early assemblies of *C. albicans*, as did the *Candida* Working

Annotation group (see Table 3.1 for useful online resources). The information available in these three web sites was initially pooled together and has subsequently been expanded on. CGD is based on the SGD framework; therefore the software, user interfaces, and data structure in CGD are identical to that described for SGD above.

The *Aspergillus* Genome Database (AspGD; Table 3.1) went online in 2009 and is an online genomic resource for scientists studying the genetics and molecular biology of *Aspergillus* species. Currently there are a number of databases containing information for multiple *Aspergillus* genomes. For example, the Central *Aspergillus* Data Repository (CADRE) database (Table 3.1) contains clinical and patient-oriented information, the *Aspergillus* genome site at the Broad Institute (Table 3.1), the *Aspergillus fumigatus* database (Table 3.1), and other web sites that focus on sequencing projects of one or several *Aspergillus* species. AspGD aims to link the resources of these individual databases and complement them by implementing in-depth manual curation of the primary scientific literature associated with the data. As with CGD, AspGD is based on the SGD framework described above. AspGD is currently focussing on high-quality curation of *A. nidulans* – the best-characterized species of the *Aspergilli* – but will add information for other *Aspergillus* species (*A. fumigatus*, *A. flavus*, *A. oryzae*, *A. niger*, *A. clavatus*, *A. terreus*, and *Neosartotya fischeri*) in the near future.

3.7 Conclusion

The majority of fungi that have been sequenced to date are important biological pathogens (*Candida albicans*, *Aspergillus fumigatus*, and *Cryptococcus neoformans*, for example) or helpful species involved in brewing/fermentation (*Saccharomyces cerevisiae* and *Aspergillus niger*, for example). Because of this, there is an unintentional bias in terms of the phylogenetic distribution of species sequenced. Due to falling sequencing costs and their relatively small genome size, a deluge of fungal genomic data from all fungal phyla is expected in the years ahead. These data will allow us to address many new questions about fungal evolution and pathogenicity, and will undoubtedly help uncover novel proteins with medical and biotechnological potential.

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4

Fungal Genetics: A Post-Genomic Perspective

Brendan Curran and Virginia Bugeja

4.1 Introduction

Ushered in by the exponential accumulation of DNA sequences in databases throughout the world, the post-genome era is characterized by the application of computer technology to a deluge of information arising from the large-scale parallel analysis of biological molecules. With information accumulating from an ever-increasing network of resources, the challenge now is to reintegrate these molecular details to reveal the secrets of the dynamic processes they mediate within the cell – an experimental and theoretical approach referred to as systems biology.

4.2 The Yeast *Saccharomyces cerevisiae*: A Cornerstone of Post-Genomic Research

A forerunner of the much more ambitious project to sequence the human genome, the yeast *Saccharomyces cerevisiae* entered the history books in 1996 as the first eukaryotic organism to have its entire genome sequence deposited in a computer database. With other eukaryotic genomes entering the databases, and the development of computational tools for capturing, storing, displaying, distributing, and comparatively analyzing the rapidly accumulating information, biologists were able for the first time to analyze and compare entire eukaryotic genomes – the post-genomic era had begun. However, the DNA sequence of *S. cerevisiae* was just the starting point for large-scale molecular analysis of eukaryotic cells. Within a very few years, this extremely tractable model organism

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rapidly yielded a whole series of molecular secrets on a global scale: each of its genes was systematically deleted in search of phenotypes; technology to allow its global mRNA profiles to be identified was developed; all possible protein–protein interactions were examined; and cellular metabolites were exhaustively characterized. In short, this simple eukaryote became the key to post-genomic research.

4.3 Of *-omics* and Systems Biology

Whereas pre-genomic research was characterized by hypothesis-driven sequential experiments, post-genomic research is driven by the massively parallel analysis of biological information, followed by pattern recognition within datasets. Genomics, the accumulating and analysis of massive amounts of DNA sequence data, was possible because an appropriate technology platform was put in place. The components of this "platform" included automated preparation of DNA, automated sequencing of multiple DNA fragments, and the computational tools to store and then process the generated data. As the science of examining genomes is referred to as genomics, so too complementary global analyses of other biological molecules are given -omic suffixes: namely, transcriptomics, which is concerned with the accumulation of information on RNA sequences and their expressed levels; and proteomics, which deals with protein sequences, protein structures, protein levels, and protein interactions with both DNA and other proteins. Each one of these has required the development of automated and computationally intensive technology platforms to complement the high-throughput DNA sequence and analysis platform, which characterizes genomics. These include DNA array technology, which allows multiple DNA or RNA sequences to be simultaneously identified, and mass spectrometry (MS) for the identification of multiple protein samples. Once again, the yeast *S. cerevisiae* has led the way in the development of these platform technologies. *Saccharomyces cerevisiae* has also become a central player in the development of an entirely new approach to biological research – systems biology. This newly emerging field uses a cross-disciplinary approach involving biology, chemistry, physics, mathematics, computer science, and engineering to develop working models of how these molecules interact to generate biological phenomena.

4.4 Genomics

4.4.1 Analyzing Encoded Information

Genomics, the study of whole genomes, encompasses (1) searches for patterns of relatedness within and between genome sequences; and (2) attempts to ascribe specific biological functions to particular DNA sequences within those genomes. The successful completion of fungal genomes, in particular the annotated

genomes of the three model organisms *S. cerevisiae*, *Schizosaccharomyces pombe*, and *Neurospora crassa*, excited much interest within and beyond the fungal community.

Over 950 fungal genome sequencing projects can be accessed at http://www.ncbi.nlm.nih.gov/genome (Table 4.1), and fungal sequencing projects to date include model organisms, fungi with relevance to healthcare, and fungi of agricultural and commercial importance. Each genome project is powerful in its own right; however, comparative analysis of fungal genomes is set to revolutionize our understanding of this ancient and evolutionarily diverse group of organisms. Moreover, given the underlying unity of biological information, many of these findings will also be relevant to our understanding of plant and animal biology.

4.4.2 Pattern Recognition within and between Genome Sequences

Without ever revealing anything specific about biological function, computer analysis of a complete genome sequence provides lots of interesting and revealing facts about its topology and evolutionary history. For instance, computer programs use pattern recognition to identify tracts of DNA that start with a methionine codon and run for another 99 codons, without hitting a nonsense one (Figure 4.1). Such sequences, referred to as open reading frames (ORFs), are then annotated as highly likely to encode proteins. More advanced programs even allow for the presence of introns by being able to recognize intron–exon boundaries. Sequence recognition programs provide a detailed map of the genome and lots of useful information, including the number of genes, the size of the gene sequences, the presence or absence of introns, the spacing of genes within the chromosomes, and much more besides.

Such programs identified the precise position of approximately 6,000 genes in the *S. cerevisiae* genome – the best labors of yeast geneticists during the preceding 40 years had revealed the position of less than 1,000 genes! Sequence analysis also revealed that the yeast genome is extremely compact, with 70% consisting of protein-encoding sequences – protein-encoding sequences that are almost completely devoid of introns. In addition, the number and chromosomal locations of genes encoding noncoding RNA species such as ribosomal RNA, small nuclear RNAs, and transfer RNA (tRNA) were identified. A similar pattern-recognition analysis revealed that the *Schiz. pombe* genome carried 600 fewer protein-encoding genes (approximately 4,900 in all), whereas their multicellular filamentous cousin *N. crassa* requires twice that number – 10,000 genes. Unlike *S. cerevisiae*, the protein-encoding genes in both *Schiz. pombe* and *N. crassa* carry introns. The percentage of the genome consisting of protein-encoding sequences is also lower in both than in *S. cerevisiae*. Finally, the gene density of the latter two is significantly lower than that found in their cousin (Table 4.2).

Table 4.1 Details of the 19 completed fungal genomes.

Organism	Genome size (Mb)	Release date	Center/consortium and useful URL
Cryptococcus gattii WM276 [1]	18.3	01/12/2011	Canada's Michael Smith Genome Sciences Centre http://www.bcgsc.ca/project/cryptococcus/
Encephalitozoon intestinalis ATCC 50506 [2]	2.22	08/16/2010	University of British Columbia, Canada http://www.botany.ubc.ca/keeling/
Saccharomyces cerevisiae S288c	12.08	02/03/2010	*Saccharomyces* Genome Database www.yeastgenome.org
Aspergillus nidulans FGSC A4	29.8	09/24/2009	Eurofung http://mikrobiologie.eurofung.tu-berlin.de/
Lachancea thermotolerans CBS 6340	10.38	06/05/2009	Genoscope http://www.cea.fr/drf/ig/english/Pages/Genoscope.aspx
Zygosaccharomyces rouxii CBS 732	9.76	06/05/2009	Genoscope http://www.cea.fr/drf/ig/english/Pages/Genoscope.aspx
Pichia pastoris GS115	9.2	05/25/2009	Unit for Molecular Glycobiology, VIB/UGent, Belgium http://bioinformatics.psb.ugent.be/genomes/view/Pichia-pastoris
Candida dubliniensis CD36	14.61	02/16/2009	Wellcome Trust Sanger Institute www.sanger.ac.uk
Magnaporthe oryzae 70–15	3.99	01/30/2006	North Carolina State University (NCSU) http://www.cifr.ncsu.edu/
Aspergillus oryzae RIB40	37.08	12/20/2005	National Institute of Technology and Evaluation (NITE) http://www.bio.nite.go.jp/dogan/project/view/AO
Cryptococcus neoformans var. *neoformans* JEC21 [1]	19.05	01/07/2005	TIGR http://www.jcvi.org/cms/research/projects/tdb/overview/

Kluyveromyces lactis NRRL Y-1140	10.68	07/02/2004	Genoscope http://www.cea.fr/drf/ig/english/Pages/Genoscope.aspx
Yarrowia lipolytica CLIB122	20.50	07/02/2004	Genoscope http://www.cea.fr/drf/ig/english/Pages/Genoscope.aspx
Debaryomyces hansenii CBS767	12.22	07/02/2004	Genoscope http://www.cea.fr/drf/ig/english/Pages/Genoscope.aspx
Candida glabrata CBS 138 CBS138	12.28	07/02/2004	Genoscope http://www.cea.fr/drf/ig/english/Pages/Genoscope.aspx
Ashbya gossypii ATCC 10895	8.76	03/06/2004	Universität Basel, Zoologisches Institut Evolutionsbiologie http://evolution.unibas.ch/
Schizosaccharomyces pombe 972 h-	12.57	02/21/2002	*Schizosaccharomyces pombe* European Sequencing Consortium (EUPOM) http://www.sanger.ac.uk/Projects/S_pombe/EUseqgrp.shtml
Encephalitozoon cuniculi GB-M1 [2]	2.49	11/24/2001	Genoscope http://www.genoscope.cns.fr/spip/Encephalitozoon-cuniculi-whole.html
Saccharomyces cerevisiae S288c	12.08	10/25/1996	Wellcome Trust Sanger Institute http://www.sanger.ac.uk/research/projects/genomeinformatics/sgrp.html

Almost all of the completed fungal genomes are ascomycetes, two are basidiomycetes[1], and two are neither of these[2].

```
        GTC
       TGT
      ATG
    5 -ATGTCGAATTCGCCTATAG - 3
    3 -TACAGCTTAAGCGGATATC - 5
                       ATC
                      TAT
                     ATA
```

Figure 4.1 Finding an ORF. Finding the ATG methionine codon requires a computer search of six different reading frames. An ORF continues for a further 99 codons before hitting a nonsense codon.

Table 4.2 Genome topology of model fungi.

Fungal species	Organismal complexity	Genome size (Mb)	Protein-encoding sequences	Gene density (excluding introns)
S. cerevisiae	Budding yeast	12.07	5,500	1 every 2.09 kb
Schiz. pombe	Fission yeast	13.8	4,900	1 every 2.53 kb
N. crassa	Filamentous fungus	41	12,000	1 every 3.1 kb

Having identified the majority of the ORFs in a genome, a computer-aided comparison that aligns each ORF against all other ORFs within the same genome can reveal much about the evolutionary history of a genome. One such study revealed that an ancient genome duplication had occurred during the evolutionary history of *S. cerevisiae*. ORF comparisons within the genome sequence identified 55 segments of chromosomes carrying three or more pairs of homologous genes – despite this being a haploid genome This supported a model in which two ancestral diploid yeast cells, each containing about 5,000 genes, fused to form a tetraploid. Most of the duplicate copies were then subsequently lost by deletion as this species evolved, leaving it in its haploid phase with approximately 5,500 genes. It is estimated that protein pairs derived from this duplication event make up 13% of all yeast proteins. The same study also revealed that transposable elements play an important role within the genome: there are 59 such elements, constituting 2.4% of the entire genome. On the other hand, intragenomic searches in the *Schiz. pombe* genome sequence failed to find evidence of large-scale genome duplications. However, they did find evidence suggesting that gene duplication played a key role in the evolution of this yeast. In fact, as many as 10% of the ORFs can be defined as having paralogous sequences within this genome (paralogous genes being homologous genes that arose by gene duplication within the same species). ORF comparisons also reveal that transposable

Table 4.3 Evolutionary history of model fungal genomes.

Fungal species	Evidence of former genome duplication	Evidence of gene duplication	Evidence of transposon activity
S. cerevisiae	Yes	Yes	Abundant
Schiz. pombe	No	Yes	Abundant
N. crassa	No	No	Very little

elements are important to this yeast; there are 11 intact transposable elements, which account for 0.35% of the genome (Table 4.3).

Within-genome comparison of ORFs in *N. crassa*, the multicellular distant cousin to both of these yeasts, fails to find evidence for large-scale genome duplication, gene duplication, or transposon activity. The level of redundancy among ORFs of *N. crassa* is very low; less than 4% can be defined as having a paralogous ORF within the genome. This analysis suggests that the genome did not undergo duplication, that gene duplication is rare, and that transposon activity is extremely limited (Table 4.3). The paucity of transposable elements and paralogs in the *Neurospora* genome can perhaps be explained by the process of repeat-induced point mutation (RIP), which involves the hypermutation of duplicated sequences of more than 1 kb in length during sexual development in this fungus. However, these results pose the interesting biological question: Can *Neurospora* currently utilize gene duplication as a means of gene diversification?

4.4.3 Assigning Biological Functions to Fungal Genome Sequences

Although analyses of individual genome sequence databases can be interesting and informative in their own right, biologists are more interested in using the sequence to understand the biology of the cell. Functional genomics, the name given to this process of assigning functions to ORF sequences, is multifaceted. Potential functions can be attributed to some ORFs by identifying similar sequences of known function in existing databases (*in silico* analysis). Alternatively, ORFs of unknown function can be disrupted in order to produce a phenotype (reverse genetics). Failing this, clues to the potential biological significance of unknown ORFs can be gleaned by large-scale comparisons of genomes from a variety of different organisms. This so-called *comparative genomics* can identify potential gene functions by virtue of the fact that certain sequences are present in some genomes but absent from others (identification by association).

4.4.4 Identification by *in silico* Analysis

Even before genome sequencing projects were initiated, research groups around the world had already set up databases containing large numbers of annotated DNA, RNA, and protein sequences. As the number of these grew, computational techniques were developed to make these resources readily available and searchable. These databases, which frequently annotate DNA sequences with encoded protein functions, provided invaluable information on the potential or actual function of proteins encoded by newly identified ORFs. Blast searches of these resources were used to annotate about 50% of the ORFs in the *S. cerevisiae* genome. This allowed biologists to discover the percentage of genes that this simple eukaryotic cell dedicated to various aspects of cellular biology: there are in excess of 600 metabolic proteins, in excess of 400 proteins involved in intracellular trafficking or protein targeting, and approximately 200 transcription factors. Specific genes whose existence in *S. cerevisiae* had hitherto been in doubt were also identified in this way: histone H1 was found on chromosome 16 (Figure 4.2), and a yeast γ-tubulin gene, which had previously eluded yeast geneticists despite intensive efforts, was identified on chromosome XII.

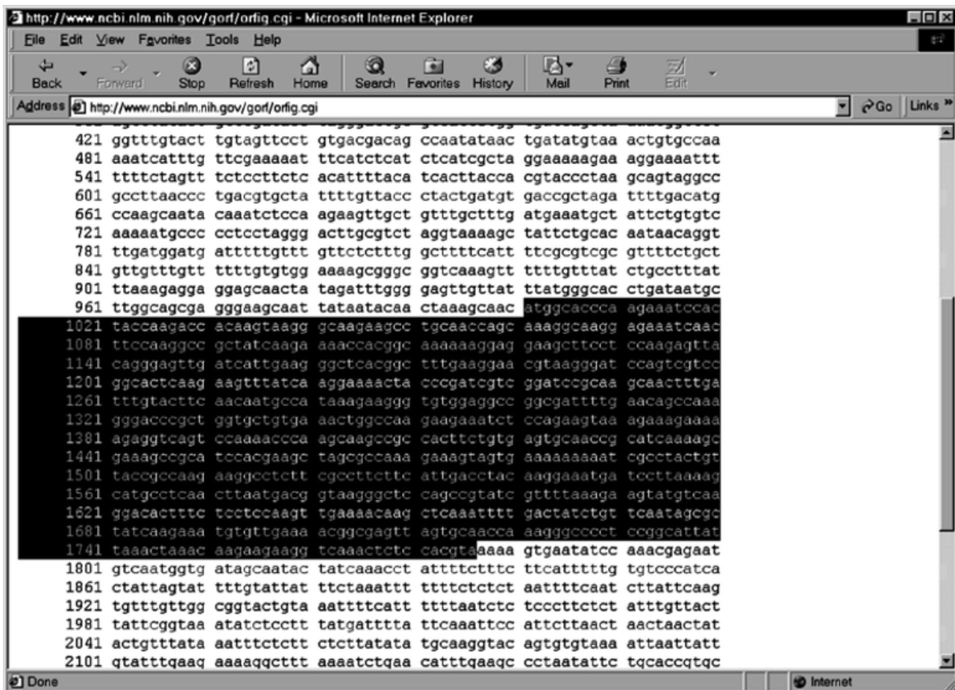

Figure 4.2 Sequence of the H1 gene in the *S. cerevisiae* database. Using an appropriate search, the bases encoding the H1 gene (blocked in above) were found as part of the sequence of chromosome 16.

4.4.5 Identification by Reverse Genetics

Although *in silico* analysis is useful, it can only annotate ORFs from new genome sequences using functions that had previously been identified elsewhere. Indeed, because it frequently identifies protein domains (rather than the entire protein sequence), the annotation is often no more than a general assignment (e.g. ORF "X" encodes a "protein kinase" or ORF "Y" encodes a "transcription factor"). A specific and unequivocal assignment is possible, however, if a phenotype can be identified when a particular ORF is deleted or disrupted.

It was for this reason that an international consortium of scientist undertook the daunting task of using *S. cerevisiae*'s ability to undergo homologous recombination to produce yeast strains in which specific ORFs had been deleted. It was hoped that such specific deletions would provide insight as to the biological function of the affected ORF. The EUROFAN project deleted and grossly characterized the mutant phenotypes from 758 ORFs of unknown function. A number of other European laboratories complemented this by undertaking a more focused range of deletion mutants. However, a transatlantic consortium undertook the most ambitious project: they used PCR to generate hybrid DNA molecules consisting of a selectable marker flanked by the 5′ and 3′ ends of the ORF of interest. They then transformed this into wild-type cells and selected for recombinants that had integrated the PCR fragment into the target ORF (Figure 4.3). By repeating this with each ORF in turn, they systematically deleted all 6,000 or so genes. A number of mutant yeast libraries have also been generated using transposons to randomly generate mutations by insertional mutagenesis. Many of these strains have subsequently been characterized for phenotypes. Of the approximately 3,000 ORFs of unknown function after the yeast sequence

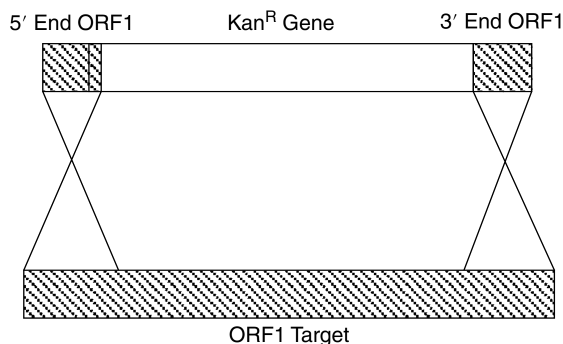

Figure 4.3 ORF knockout in the yeast *S. cerevisiae*. Appropriate PCR primers are used to generate hybrid DNA molecules consisting of the KanR selectable marker flanked by blocks of DNA consisting of the 5′ and 3′ ends of the ORF of interest. Homologous recombination in transformants knocks out the ORF of interest.

was released in 1996, such approaches have to date identified biochemical or physiological functions for more than 1,000 of them.

Gene knockout technologies using a variety of molecular approaches are also valuable in the study of other fungi. With appropriate selectable markers and transformation procedures available for many pathogenic fungi, including *Candida albicans*, *Candida dubliniensis*, *Cryptococcus neoformans*, *Aspergillus fumigatus*, *Histoplasma capsulatum*, and *Coccidioides immitis*, the application of gene knockout technology to investigate the basis of their pathogenicity is awaited with great interest. Such practical application of the basic approach taken in the investigation of *S. cerevisiae* will allow the development of antimycotic drugs in post-genomic research in these organisms. High-frequency homologous recombination is possible in a number of these, and complete or partial genome sequences for a number of pathogens are already available. However, the relationship between gene knockouts and pathogenicity is complicated by a number of factors, including the fact that high-frequency homologous recombination is not possible in all cases; there are different isolates; the same isolate under different nutrient conditions varies in its virulence; and, in the case of *Candida* (a diploid), knockouts require two independent deletion events. Nevertheless, the application of reverse genetics to some of these organisms is already underway.

However, as it is not feasible to analyze all fungi in this way, alternative strategies are being devised. One such strategy uses a process of "guilt by association" to link ORFs of unknown function (so-called orphans) with biological function.

4.4.6 Identification by Association

When the fission yeast *Schizosaccharomyces pombe* entered the history books as the second completed fungal genome, it became the sixth eukaryotic genome to enter the fully annotated genome databases. It also presented the first opportunity to undertake a comparative genomic analysis of fungal organisms viz. *S. cerevisiae* and *Schiz. pombe* – both to one another and to other genomes. Using the genome sequence of *Caenorhabditis elegans* as a simple multicellular eukaryote, this analysis revealed that 681 ORFs (14%) were uniquely present in *Schiz. pombe*, 769 (16%) were homologous to *S. cerevisiae* ORFs, and about two-thirds of the *Schiz. pombe* ORFs (3,281) had homologs in common with both *S. cerevisiae* and *C. elegans* (Figure 4.4).

With the availability of the *Neurospora* genome sequence it became possible to compare these single-celled yeasts with their multicellular cousins. Given that *Neurospora* produces at least 28 morphologically distinct cell types, and has a preponderance of 11,000 overwhelmingly nonparalogous genes, it is perhaps not surprising that a comparative genomic analysis reveals that a large proportion of *Neurospora* genes do not have homologs in the

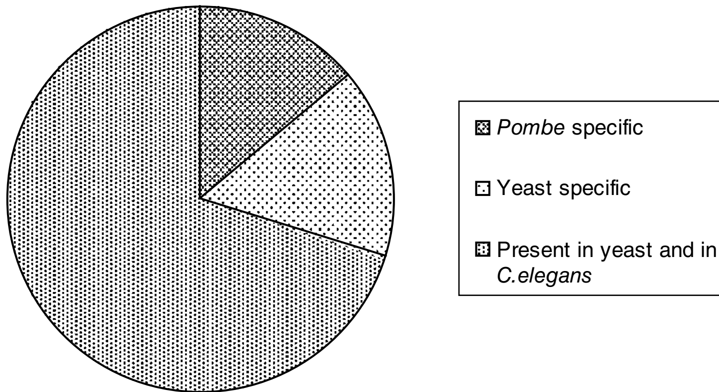

Figure 4.4 Using comparative genomics to assign biological significance to ORFs. BLAST searches of all the *Schiz. pombe* ORFs revealed that 681 ORFs (14%) were uniquely present in *Schiz. pombe*, 769 (16%) were homologous to *S. cerevisiae* ORFs, and approximately 3,300 *Schiz. pombe* ORFs (70%) had homologs in common with both *S. cerevisiae* and *C. elegans*.

yeasts *S. cerevisiae* and *Schiz. pombe*. Analysis of other genome databases reveals that in relation to yeasts, this particular filamentous fungus possesses an expanded group of sugar transporters, transcription factors, environmental sensing pathways, and a diversified metabolic machinery; *Neurospora* possess 38 proteins with a cytochrome P450 domain; *S. cerevisiae* and *Schiz. pombe* possess fewer than five such proteins.

As more fungal genomes become available it will become possible to identify subgroups of genes that are associated with specific types of fungal biology. Already, relatively crude comparisons between very distantly related fungi reveal much about the subset of fungal-specific genes, and genes that are yeast or multicellular specific. More accurate predictions become possible as more genomes and indeed more closely related genomes become available. However, the power of comparative fungal genomics does not stop with revealing the biology of this important and ubiquitous group of organisms. Over 200 predicted *Neurospora* proteins exhibit significant similarity to human gene products associated with disease states in humans. Many of these have counterparts in *S. cerevisiae* and *Schiz. pombe*, including 23 that are cancer-related proteins. Such findings lend support to the notion that fungi could be good model organisms for studying the associated human disease pathways – fungi have even been used to model neurodegeneration.

Annotated genomes reveal much about the topology, evolutionary history, and biological potential of cells, but not much about how this develops into an integrated biological entity. This requires a global view of how 2-D DNA information manifests in 4-D time and space. Experimental approaches to characterize global changes in cellular molecules, as they obey the central dogma DNA makes RNA makes protein, have therefore been devised.

4.5 Transcriptomics and Proteomics

4.5.1 Analyzing Encoded Information in Time and Space

In the pre-genome era, biologists used northern blot analysis of mRNA transcripts to explore the expression of the information stored in the DNA in the cell. Likewise, they relied on pattern changes in 1-D and 2-D PAGE to monitor changes in cellular protein profiles. Researchers were limited to analyzing, at best, time-lapsed gene expression from a very limited number of genes, and piecemeal revelations as to precise 3-D cellular context in which the encoded proteins carried out their functions. In the post-genomics world, biologists exploit the availability of genome sequences to examine simultaneous changes in gene expression profiles for every gene in the genome using array technology. Moreover, high-throughput protein analysis provides detailed information on cellular location, and protein–protein interactions, for thousands of characterized and uncharacterized proteins. The post-genomic era is distinguished therefore by massively parallel molecular analyses of information-rich molecules.

4.5.2 Transcriptomics

If genomics deals with the relationship between DNA sequence and its encoded function, transcriptomics looks at RNA sequences and their expressed levels. If the pre-genomic technique of northern blot analysis of mRNA acted as an index of expression for one or two genes, the post-genomic world demands a more global perspective on gene expression patterns. As was the case with fungal genome analysis, global fungal transcriptome analysis began with *S. cerevisiae*.

4.5.2.1 Dissecting the Diauxic Shift Using a Yeast Microarray

In a classic paper, DeRisi *et al.* (1997) PCR-amplified each of the 6,400 distinct ORFs described in the first edition of the yeast genome and printed these unique DNA molecules onto glass slides using a simple robotic printing device. The resulting DNA microarrays, in which each of the known DNA sequences was attached at a particular grid reference on an 18-mm^2 area of a glass slide, was then a multigene probe. This was used simultaneously to explore the expression profile of mRNA as yeast cells underwent a diauxic shift from fermentative growth to aerobic respiration. mRNA samples taken over a 21-hour period as the cellular metabolism changed from glucose fermentation to ethanol respiration were reverse-transcribed into cDNA molecules labeled with a red fluorescent dye. Green fluorescent cDNA was also prepared from the mRNA at the first time point to serve as reference. The expression level of each gene was measured by hybridizing fluorescently labeled cDNA to the probe, visualizing the fluorescence

pattern using a confocal microscope and using a computer to analyze the relative intensity of the spots. At the initial time point, the green and red signals were equal, and all spots appear yellow. At later time points, red color indicates that gene expression increased relative to the reference, while green color indicates that gene expression decreased relative to the reference (Plate 4.1).

The analysis revealed that a staggering 28% of all yeast genes underwent a significant alteration in gene expression level as a result of a diauxic shift: 710 genes were induced and 1,030 genes repressed by a factor of 2 or more. Moreover, cluster analysis identified groups of genes whose pattern of expression changed in association with one another. Such coordinated gene regulation points towards a common promoter element – a coordinated regulation that helps to identify possible cellular roles for ORFs that encode proteins of unknown function. The sequences upstream of the named genes in Plate 4.2 all contain stress response elements (STRE). When the promoter sequences of the 13 additional (not previously recognized as stress-inducible) genes that shared this expression profile were examined, nine were found to contain one or more recognizable STRE site. This suggests that many of these 13 additional genes, which were unidentified heretofore, have a role that is linked to stress response.

The same DNA microarrays were also used successfully to identify genes whose expression was affected by deletion of the transcriptional co-repressor *TUP1* or overexpression of the transcriptional activator *YAP1*, thereby demonstrating the feasibility and utility of this approach to the dissection and characterization of regulatory pathways and networks on a genome-wide basis.

4.5.2.2 The Vocabulary of Transcriptomics

Encouraged by the successful demonstration of this approach to global gene expression studies, other yeast studies rapidly followed. Then as genomes became available other fungi were explored in the same way. Fungal researchers found themselves embracing the new vocabulary of post-genomic analyses as they set about exploiting this approach in their organism of choice. Complementary DNA (cDNA) libraries, expressed sequence tags (ESTs), serial analysis of gene expression (SAGE), macro and microarrays, BLAST (Basic Local Alignment Sequence Tool) searches, and sequence alignments (Table 4.4) all entered the fungal literature within 5 years of DeRisi *et al*.'s paper. *Saccharomyces cerevisiae* has led the way in the microarray-based analysis of fungal transcriptomes because it was the first available fungal genome, and also because its genes lack introns, therefore allowing the ORFs to be PCR-amplified directly from genomic DNA. A number of other global approaches to mRNA analyses are also available including methods based on cDNA, SAGE, and EST analysis.

Next-generation sequencing (NGS) of DNA is poised to replace all of these techniques. NGS refers to a series of new technologies in which the DNA

Table 4.4 Post-genomic vocabulary.

Tool	Description
BLAST (Basic Local Alignment Sequence Tool)	An algorithm that searches a sequence database for sequences that are similar to the query sequence. There are several variations for searching nucleotide or protein databases using nucleotide or protein query sequences
cDNA (complementary DNA)	A DNA molecule synthesized by reverse transcriptase using an mRNA molecule as template. Hence, cDNA lacks the introns found in genomic DNA
NGS (next-generation sequencing)	Refers to a series of technologies in which the DNA sequence is recorded as DNA molecules are synthesized in real-time
ESTs (expressed sequence tags)	Short cDNA sequences that are derived from sequencing of all mRNAs present in a cell. ESTs represent the expression profile of the cell at the time point of RNA isolation
Microarray	An ordered grid of DNA sequences fixed at known positions on a solid substrate, e.g. glass slide
ORF (open reading frame)	The sequence of codons, in DNA or RNA, that extends from a translation start codon to a stop codon
SAGE (serial analysis of gene expression)	Extremely short ESTs that are linked together as DNA chimeras consisting of 15 bp sequences from 40 different mRNAs. The sequence analysis of thousands of these 40 × 15 bp chimeras permits a quantitative estimation of the mRNAs in the original sample
Sequence alignment	A linear comparison of nucleotide or protein (amino acid) sequences. Alignments are the basis of sequence analysis methods and are used to identify conserved regions (motifs)

sequence is recorded as the DNA molecules are synthesized in real-time. Sequence data are collected across millions of simultaneous reactions and powerful bioinformatics tools used to interpret them. This completely circumvents the traditional dideoxy-nucleotide (Sanger) sequencing which required labeled DNA fragments to be physically resolved by electrophoresis. The commercial companies involved in this include: 454 Genome Sequencer (Roche), Illumina (formerly Solexa) Genome Analyzer, the SOLiD system (Applied Biosystems/Life Technologies), and the HeliScope (Helicos BioSciences Corporation), and such technology will revolutionize SAGE and EST analysis. Moreover, because it can use mRNA as a primary template directly, NGS also has significant advantages over microarrays because the genome sequence is no longer needed to set up the DNA probe on the chip.

4.5.2.3 *From Clocks to Candidiasis: The "When" of Inherited Information*

DeRisi *et al.*'s seminal paper revealed the power of transcriptomics in dissecting out complex differential gene expression when cells are undergoing diauxic growth and when regulatory gene expression levels are altered. In short, it reveals the information stored in the genome as a dynamic process. This technology allows scientists simultaneously to analyze the expression levels of any number of individual genes as they change in time, thereby identifying coordinated gene expression, and most critically understanding what happens when environmental changes and gene perturbations impact on the flow of information through the cell.

Knowing the "when" of a gene's expression often provides a strong clue as to its biological role. SAGE and microarray technologies have already been used to identify: the set of fungal genes most highly expressed during cerebrospinal fluid infection by *Cryptococcus neoformans*; the pattern of differential gene expression that solved the problem of why glucose metabolism by *Trichoderma reesei* is so different to that of *S. cerevisiae*, and; identify 18 genes of unknown function implicated in the production of carcinogenic aflatoxins by *Aspergillus parasiticus*.

Conversely, the pattern of genes expressed in a cell "when" it has been subjected to various treatments can identify critical points of great biological significance. This approach has been used to identify potential antimycotic targets to combat the increasing threat posed by *Candida* infections. Microarray analysis has been used to: identify two transcription factors *EFG1* and *CPH1* induced when *C. albicans* is shifted to 37 °C in the presence of serum; establish that the final transcriptional profile found in some clinical isolates resistant to fluconazole could also be reproduced by allowing resistance to evolve in the laboratory; and demonstrate that the antimycotic itraconazole affected the expression of 296 ORFS in this fungus.

Transcriptomics has been used to accelerate research projects which could be solved without this technology, but in other cases it allows fungal biologists to explore biological phenomena that are quite simply too complicated to approach in any other way. Moreover, this applies to fungal biology at both basic and applied levels. In an example of basic research, *Schiz. pombe* workers used expression data for each *pombe* gene to reveal that many conserved genes are expressed at high levels, whereas a disproportionate percentage of the poorly expressed genes are organism specific. This analysis was only possible because the expression data were available and database searches were possible of all ORFs in S. *pombe*, *S. cerevisiae*, and the nematode worm *C. elegans*. In another example of basic research that was impossible by pre-genomic methods, microarrayed cDNA sequences of 1,000 different genes revealed that circadian clock control in *N. crassa* covers a range of cellular functions rather than preferentially belonging to specific pathways.

And then sometimes, unexpectedly, what was basic research to begin with turns out to have applied aspects: microarray analysis in *C. albicans* of the genes induced by α-factor in mating competent type **a** cells revealed 62 genes. Interesting in their own right as an insight into the cryptic mating pathway of this

fungus, the realization that seven of these genes encoded cell-surface or -secreted proteins that had previously been shown to be required for full virulence of *C. albicans* unexpectedly revealed a new potential target for the development of an antimycotic aimed at the factor(s) regulating the mating pathway.

An impressive example of purely applied research, on the other hand, is a microarray produced by the company Agilent Technologies. The first of its kind, this array carries 7,137 rice ESTs, and all 13,666 predicted ORFs from the major rice fungal pathogen *Magnaporthe grisea*. It therefore uniquely allows simultaneous analysis of gene expression profiles in both the host and pathogen. It has been responsible for identifying: a serine vacuolar protease involved in pathogenicity; the suite of genes induced by the Con7 transcription factor (which has a role in pathogenicity); and a comprehensive list of genes thought to be involved in the formation of the flattened hypha (appressorium) that this parasitic fungus uses in order to penetrate the host tissues.

4.5.3 Proteomics

Two-dimensional and 3-D analyses of biological information are extremely powerful (see Chapter 5). However, it is the 4-D manifestation of that information in time and space that biologists seek to define and understand. Small wonder that proteomics, which seeks to study biology in that extra dimension, faces challenges that are orders of magnitude beyond those posed by the global analysis of 2-D and 3-D information in DNA and mRNA, respectively. One has only to look at Table 4.5 to realize that, unlike genomics and transcriptomics, proteomics is in fact an entire suite of distinct but intricately interrelated

Table 4.5 Proteomics is much more challenging than transcriptomics or genomics.

DNA (information)	RNA (information in time)	Protein (information in time and space)
Nucleotide sequence	Nucleotide sequence	Amino acid sequence
	Level in cell	Level in cell
	Modifications (splicing)	Modifications (glycosylation, phosphate groups, etc.)
		3-D structure
		Function
		Location
		Interactions

technologies. Even though 2-D gel electrophoresis provided a platform for the global analysis of cellular proteins as early as 1978, it revealed nothing of the spatial arrangement of the proteins in the cell, little of post-translational modifications, only visualized moderately abundant proteins, and told the researcher nothing of the protein's structure and function. Developments such as microsequencing of the gel-separated proteins, and sensitive glyco-specific stains addressed some of these limitations in the pre-genomic era, but global analysis of proteins in the post-genomic era seeks to build on genomic and transcriptomic projects to provide databases of information that address all of the parameters listed in Table 4.5 and more.

4.5.3.1 Protein Sequence and Abundance

At its most basic level, proteomics seeks to identify at a given moment in time which ORFs in a genome are expressed as proteins in a cell, and what level of each one is present. Unlike RNA and DNA sequences that can be enzymatically amplified, large-scale parallel analysis of proteins must deal with unavoidable problems of limited sample material and abundance variation over six orders of magnitude. Nevertheless, a combination of tryptic digestion of protein mixtures, coupled with chromatographic separation of the resulting peptides allows increasingly sophisticated MS to identify the presence and abundance of ever smaller amounts of protein from increasingly complex protein mixtures. Peptide fingerprints (the spectrum of ionized molecules generated by the peptides in an MS system) can then be used to identify the same pattern already entered into the databases from MS analysis of known proteins (see Chapter 5). Alternatively, a further round of MS can be performed on each peptide to identify its constituent amino acids, and these sequences can be compared to entries in protein and DNA databases using the appropriate database searching tools. In a less high-throughput system, protein mixtures can first be separated by 2-D gel electrophoresis, and protein spots excised, digested with trypsin, and subjected to MS analysis in the same way.

This type of proteomic analysis, which essentially generates the same type of information on the protein level as transcript analysis does at the RNA level, has already been used to probe fungal biology; 2-D electrophoresis followed by MS analysis was used to assess the effect of concanamycin A, an antibiotic produced by *Streptomyces*, on protein levels in the filamentous fungus *Aspergillus nidulans*. Twenty spots were identified and five excised for tryptic digestion, followed by estimation of their complete amino acid sequence by MS. The functions of four of those proteins were identified using the protein sequence to search ORF databases; a fifth was identified as being homologous to a protein in *Aspergillus niger*, but of unknown function.

A recurring theme in protein sequence and abundance analyses is that proteomic findings differ with respect to transcriptomic findings. In some cases,

mRNAs are found but no corresponding protein, indicating that either the protein was in very low abundance, or unstable during extraction, or those transcripts were the subjects of post-transcriptional regulation. On other occasions, proteins are present but no corresponding RNA is found – indicating a less than representative RNA sample, or a protein with an unusually long half-life. However, despite these differences, large-scale parallel protein analyses are becoming a central theme in the fungal community. Indeed, progress has been so rapid that in the case of *S. cerevisiae*, global protein analysis projects have managed to address, with some success, the much more formidable question of how the proteins present in the cell interact with one another to form complexes, and how complexes are organized on a cellular scale. Moreover, given the power of comparative genomics, the findings in this model organism are already being extended (see Chapter 5).

4.5.3.2 From Locations to Interactomes

Once the presence and abundance of a protein has been determined for a given situation, the next parameter in the 4-D arrangement of biological information is the cellular location of the protein in question. Thereafter, a more complete description requires information on (1) the proteins that it interacts with, (2) the complexes it is involved with, and (3) how these complexes interact within the cell. The yeast *S. cerevisiae* is presently at the forefront of turning this dream into reality. This is because it is an extensively characterized model organism with a well-developed gene expression system, and its extremely accurate homologous recombination system allows novel gene constructs to be precisely targeted into the correct chromosomal locus, thereby ensuring that the gene is subjected to appropriate native gene regulation. Moreover, the majority of its ORFs are known and many have been characterized.

Using these cellular attributes, Won-Ki Huh *et al.* (2003) used PCR to amplify hybrid DNA molecules consisting of the coding sequence for green fluorescent protein (GFP) fused in-frame with the 3′ end of the coding sequence for each one of 4,100 yeast ORFs (Figure 4.5). They then used homologous recombination to target each hybrid molecule back into the correct chromosomal locus, thereby creating 4,100 yeast strains, each one carrying a different GFP-tagged gene sequence. Then, using fluorescence microscopy to find out where they were in the cell, they managed to classify these proteins into 22 distinct subcellular locations, and in doing so provided localization information for 70% of previously unlocalized proteins – a number that constitutes about 30% of the yeast proteome.

Hybrid DNA molecules encoding fusion proteins were also the basis of the first systematic search for interacting proteins in a yeast cell. Using a slight variation of the two-hybrid system, described in detail elsewhere in this book (see Chapter 9), they mated 192 cells expressing different "prey" proteins with each

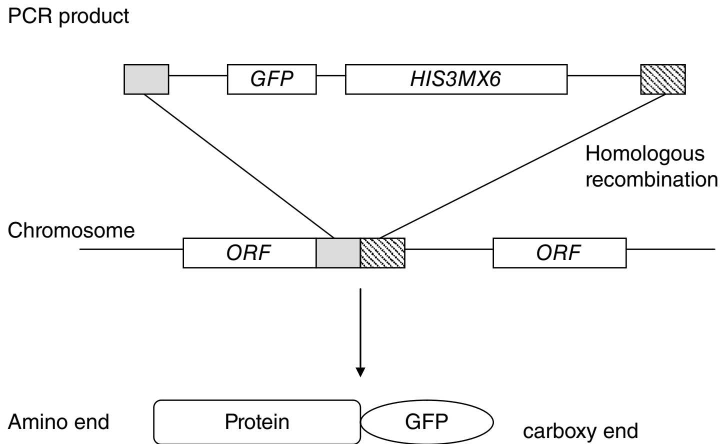

Figure 4.5 Creating a fluorescently labeled protein. PCR was used to create a hybrid DNA consisting of a selectable marker (*His 3*) and the coding sequence for GFP fused in-frame with the two adjacent sequences of DNA from the 3′ end of the coding sequence of the target ORF. Homologous recombination was then used to target each hybrid molecule back into the correct chromosomal locus, thereby creating a GFP tagged gene sequence.

of 6,000 strains expressing different "bait" proteins, and used reporter gene expression in the resulting diploid cells as an index of interaction. Although all two-hybrid studies identify false positive interactions and fail to identify weakly interacting protein ones, they do provide strong clues as to possible protein interactions and functions. In this study two proteins of unknown function were found to be intimately linked to arginine metabolism, a heretofore unknown cell cycle control circuit was discovered, and small networks of interactions traced a series of protein interactions joining a protein involved in the formation of double-stranded DNA breaks and one involved in the formation of the synaptonemal complex during meiosis.

In a subsequent paper using these and related data from other studies, Schwikowski and colleagues compiled a list of about 2,700 protein interactions in *S. cerevisiae* and found that 1,548 yeast proteins could be depicted in a single large network (Figure 4.6). Moreover, within the network it was found that proteins that could be allocated to specific cellular functions (e.g. DNA synthesis, amino acid metabolism, structural proteins) showed a high level of interactions and therefore clustered together into functional groups. This allowed them to ascribe potential functions to almost 40 yeast proteins previously of unknown function. Their global interactome revealed the great interconnectedness of the yeast proteome. It found that many members of protein clusters associated with central roles (e.g. cell cycle) had interactions with proteins from many other cluster classes (e.g. DNA synthesis, transcription, polarity), thereby connecting biological functions into larger cellular processes. On the other hand, not many

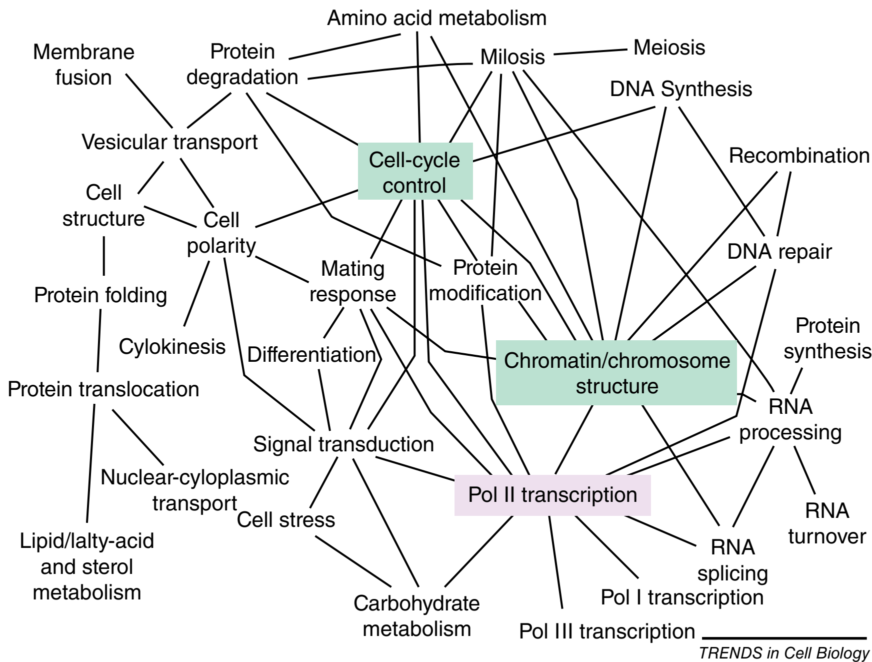

Figure 4.6 Meta-network consisting of 32 functions (and their 70 or so associated interlinking connections) in place of the 1,548 individual proteins and their 2,700 links which constitute the yeast interactome.

members of functional clusters for more peripheral cellular functions (e.g. RNA turnover) interacted with proteins from other clusters. This meta-analysis allowed a meta-network consisting of 32 functions (and their 70 or so associated interlinking connections) to be described in place of 1,548 individual proteins and their 2,700 links. The graphical representations of protein-interaction maps provide a rough outline of the complexity of protein associations.

This type of analysis only reveals protein–protein interactions arising under the artificial circumstances of being constitutively expressed at high levels in the same cellular compartment at the same time. However, a powerful complementary study provided a much more biologically relevant analysis of protein–protein interactions in this yeast: Gavin *et al.* (2002) exploited homologous recombination to integrate hundreds of ORFs fused to an affinity tag into their normal regulatory sequences. The transformed cells were then allowed to express the modified proteins, and tandem affinity purification (TAP) was used to isolate the tagged protein and any associated proteins with it. The co-eluting proteins were then identified using standard MS methods. This approach identified 1,440 distinct proteins within 232 multi-protein complexes in yeast. This study provided new information on 231 previously uncharacterized yeast proteins, and on a further 113 proteins to which the authors ascribe a previously unknown

cellular role. Other types of global analyses in yeast serve to complement and support these studies. These include:

- Protein microarrays, a technology analogous to DNA microarrays in which target proteins are immobilized on a solid support and probed with fluorescently labeled proteins. Proteins that bind to the target are thereby implicated as possibly interacting with them *in vivo*.
- Synthetic lethal screens, a genetic screening process which tests pairs of mutations together. This identifies mutations that alone are not lethal but together are incompatible with life and therefore provide strong evidence that the two gene products are functionally related.
- The identification of co-regulated mRNAs, which provides evidence that the encoded proteins are involved in related biological processes.

An overview of high-throughput systems designed to capture information on protein–protein interactions is provided in Figure 4.7.

Together, these global studies provide biological insights that are much deeper and dynamic than ever before. Moreover, comparisons of such data with similar information from other model organisms reveal that the basic constituents of cells, their interactions, and processes have been conserved across evolutionary time, and are therefore present in millions of organisms. By characterizing and annotating the constituent molecules and their interactions in one organism, it is often possible to extrapolate such information to other organisms. However, because the naming and description of genes and gene products varies widely in different organisms in this post-genomic era, it is even more vitally important for scientists to be able to communicate their findings to one another. That is why as far back as 1998, when there were still only three eukaryotic genomes available, genome researchers developed a shared species-independent controlled common vocabulary, and a carefully defined structural framework for organizing information, to allow communication across collaborating databases – the Gene Ontology (GO) project had been born. However, it is doubtful that even the original consortium foresaw just how important GO was to become. Today, it is a key element in post-genomic research in allowing scientists to produce standard annotations, improve computational queries, retrieve and analyze data from disparate sources, and, even more critically, computationally extract biological insights from enormous datasets.

4.5.3.3 Functional Annotations Are GO …

In the field of informatics, an ontology is essentially a graphical representation of knowledge (a knowledge map) consisting of nodes populated by terms from a strictly controlled vocabulary, joined by arrows which represent relationships

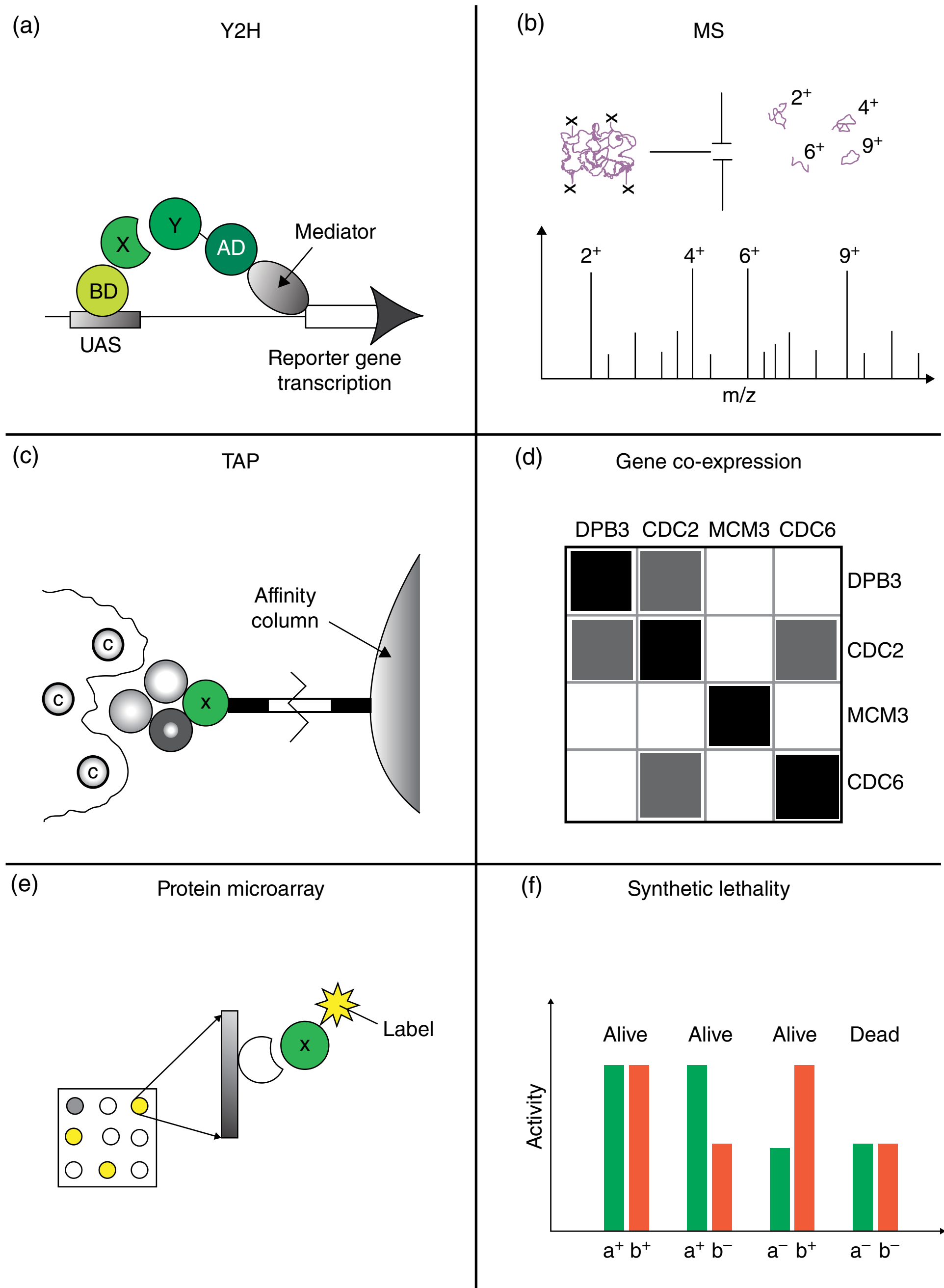

Figure 4.7 Overview of high-throughput systems designed to capture information on protein–protein interactions. (a) A yeast two hybrid (Y2H) detects interactions between proteins X and Y, where X is linked to a DNA binding domain which binds to upstream activating sequence (UAS) of a promoter. (b) MS identifies polypeptide sequence. (c) TAP purifies proteins associated with the target (x). (d) Gene co-expression analysis produces the correlation

between the terms based on strictly defined logical statements. The GO actually consists of three independently constructed ontologies: the Molecular Function is constructed with a vocabulary that defines the biochemical activity of a gene product, that is, what it does (e.g. transporter, ligand, enzyme); the Cellular Component is constructed with a vocabulary that defines where in a cell the gene product is active (e.g. mitochondrion, cytoplasm, mitotic spindle); the Biological Process is constructed with a vocabulary that defines cellular activities accomplished by multiple molecular events (e.g. growth, translation, signal transduction). A very low-resolution map of part of the Biological Process ontology is shown in Figure 4.8 (a higher resolution map would have much more detail and complexity). The nodes in this example are populated by terms from the controlled vocabulary of biological processes, joined by arrows representing the logical relationships statements "is_a" or "part_of".

It is important to realize that the GO project is *not* a nomenclature for genes or gene products, but a means of querying and retrieving genes and proteins based on their shared biology. The power of the GO project lies in the fact that its vocabulary describes biological phenomena – that is, attributes of biological objects (functions, processes, and locations), not the objects themselves. Nevertheless, access to the objects is made possible by associating a GO term from one of the three ontologies with a gene or gene product stored in keyword databases such as GenBank, the European Molecular Biology Laboratory (EMBL), or Munich Information Center for Protein Sequences (MIPS). The association must be further supported with a specific reference in the literature, plus an agreed evidence code, which is determined by the type of evidence presented in the reference (e.g. mutant phenotype, microarray expression) and the date that the annotation was assigned. The "chromatin binding" node, in a subset of the Molecular Function ontology, in Figure 4.9 is a case in point: it has been annotated with three columns of genes – six from *S. cerevisiae*, five from *Drosophila*, and five from *Mus*. Their assigned gene designations reveal how difficult it is to search for/compare gene functions

Figure 4.7 (Continued) matrix, where dark areas show high correlation between expression levels of corresponding genes. (e) Protein microarrays (protein chips) can detect interactions between actual proteins rather than genes: target proteins immobilized on solid support are probed with a fluorescently labeled protein. (f) Synthetic lethality method describes the genetic interaction when two individual, nonlethal mutations result in lethality when administered together ($a^- b^-$). (Source: Shoemaker BA, Panchenko AR (2007) Deciphering protein–protein interactions. Part I. Experimental techniques and databases. PLoS Comput Biol 3(3): 337–344. e42. doi:10.1371/journal.pcbi.0030042. This is an open-access article distributed under the terms of the Creative Commons Public Domain declaration which stipulates that, once placed in the public domain, this work may be freely reproduced, distributed, transmitted, modified, built upon, or otherwise used by anyone for any lawful purpose.)

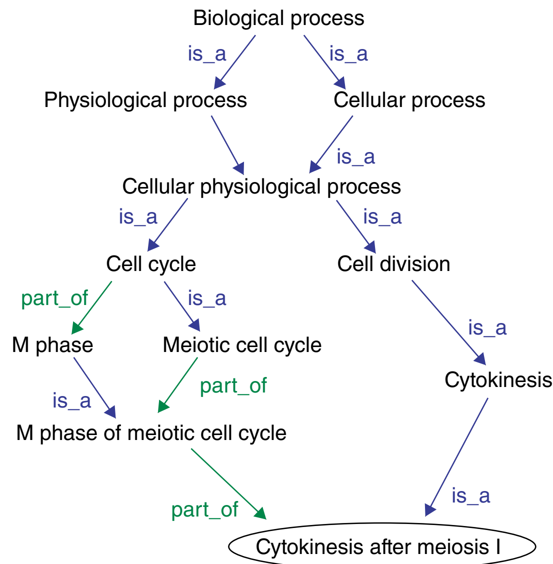

Figure 4.8 This low-resolution map of the Biological Process ontology is built by drawing up a carefully controlled vocabulary of terms about biological processes and then linking them with logical statements. This ontology is read from bottom to top. (See http://www.geneontology.org/GO.ontology.relations.shtml for a full description of logical nomenclature for all three ontologies. Source: http://www.yeastgenome.org/help/GO.html.)

between these model organisms; the GO term "chromatin binding," however, retrieves all related genes when applied across these three databases. The converse is also true: new genome sequences can be matched to GO terms by similarity with sequences in databases that have already been annotated. The GO project has allowed the computational transfer of biological annotations from the highly characterized and annotated yeast *S. cerevisiae* to a whole series of less well-characterized fungi, including *Ashbya gossypii* and the fungal pathogens *Pneumocystis carinii*, *Sclerotinia sclerotiorum*, and *C. albicans*. This affords biologists (fungal and others) the opportunity of understanding the biology of their favorite organism without necessarily developing technologies to undertake saturation analysis of its molecular interactions such as those developed to explore the biology of *S. cerevisiae*.

However, even in the case of this highly characterized organism, identifying the 4-D arrangement of all the intricate cellular protein interactions, complete with biochemical kinetics, is still a long way off. Nevertheless, it is now possible to develop theoretical models of the interactions between collections of elements responsible for carrying out well-defined biological tasks. Such models can provide a rational framework in which to design a focussed range of experiments – the data from which can in turn be used further to refine the model.

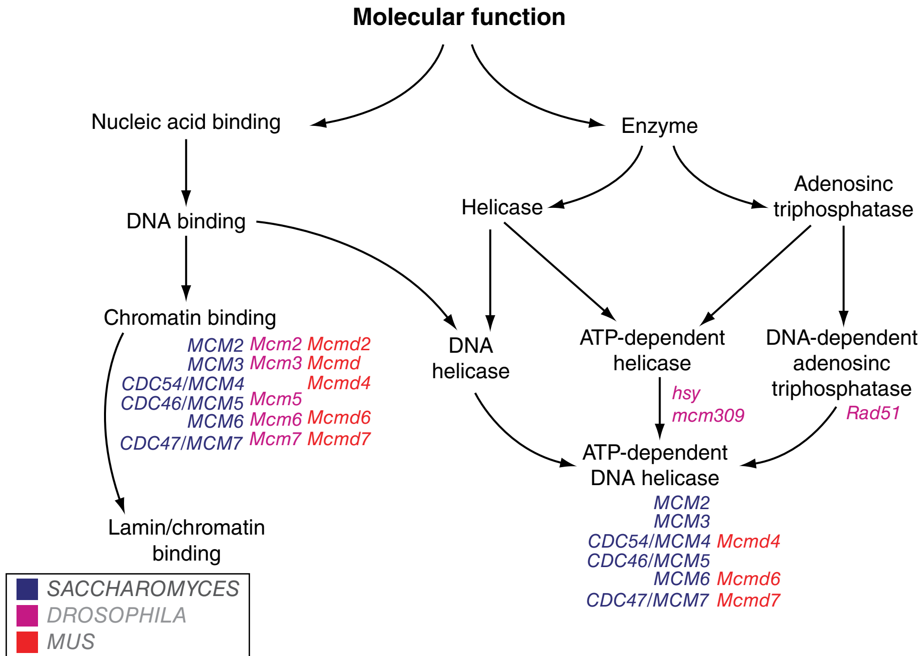

Figure 4.9 Using a subset of Molecular Function ontology to illustrate the difference between *annotation* and *ontology*. Biological terms populating nodes, and arrows between nodes, constitute the ontology. Gene designations for the three model organisms, linked to keyword databases such as GenBank, EMBL, and MIPS, are the annotations. In the case of the single ontological term "chromatin binding" there are 16 separate annotations arising from three different databases. (Source: Ashburner *et al. Nature Genetics* **25**, 25–29 (2000). Permission requests to: http://www.nature.com/reprints/permission-requests.html. Details: Michael Ashburner, *et al.* (2000) Gene Ontology: tool for the unification of biology. *Nature Genetics* **25**, 25–29 (2000) doi:10.1038/75556 Fig. 1 (b).)

Such attempts to reintegrate molecular details to reveal the secrets of the dynamic processes they mediate within the cell form an experimental and theoretical approach referred to as systems biology.

4.6 Systems Biology

4.6.1 Establishing Cause and Effect in Time and Space

The post-genomics era has been dominated to date by gigantic data-accumulating exercises – data that allow us to analyze encoded information in time and space. Now the challenge is to reintegrate these data to provide meaningful insight into biological phenomena.

4.6.2 Case Study: The Application of Systems Biology to Modeling Galactose Metabolism – A Basic Fungal Metabolic Pathway

The process of galactose utilization has been extensively studied for many decades at both the genetic and biochemical levels in the yeast *S. cerevisiae*. Induced by galactose in the absence of glucose, the nine genes, the gene products (one transporter protein, four enzymes, and four transcription factors), metabolic substrates, and key gene regulatory networks have all been worked out during 30 years of pre-genomic experimentation (Figure 4.10).

In step 1 of this systems approach all of this information was used to define an initial model of galactose utilization. In step 2 each pathway component was systematically perturbed, yielding 20 separate cellular conditions. These were wild-type cells and deletion strains for each of the nine genes grown in the presence and absence of galactose. The global cellular response to each perturbation was detected and quantified using array technology. Also, in the case of the wild-type cells, large-scale protein expression analysis was performed. In step 3 all of this new information was integrated with the initially defined model and also with the information currently available on the global network of protein–protein and protein–DNA interactions in yeast.

Amazingly this microarray analysis revealed that mRNA synthesis was significantly altered in the case of 997 yeast genes (i.e. approximately 20% of all ORFs) in one or more of these perturbations, and this despite the fact that only nine gene products are needed for galactose metabolism. It would therefore

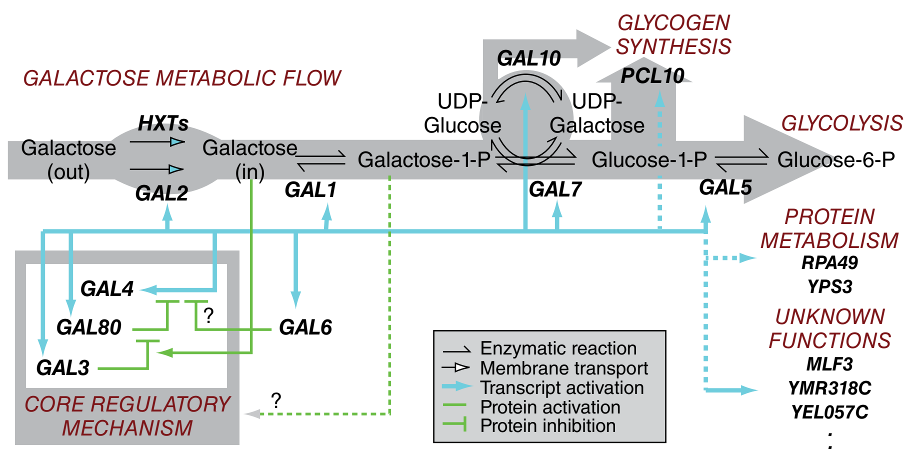

Figure 4.10 System analysis reveals new levels of regulation in a basic fungal metabolic pathway. Dotted interactions denote model refinements to galactose metabolism in yeast following a post-genomic global analysis of the yeast genome sequence, microarray-based mRNA analysis, databases of protein–protein interactions, and rapid MS analysis of proteins.

appear that the "great interconnectedness" of the yeast protein interaction network is extensively perturbed by a handful of perturbations to the galactose metabolic system.

In step 4 the researchers formulated new hypotheses to explain the observations not predicted by the model, and then designed and executed additional perturbation experiments to test the new "improved" model. For example, they detected an unexpected reduction in the expression levels in GAL enzyme genes (suggesting a regulatory effect) in cells carrying a deleted gal7 gene (which encodes the enzyme responsible for converting galactose -1-P to glucose -1-P). This suggests the presence of an additional heretofore unknown regulatory circuit which downregulates GAL gene expression when galactose -1-P accumulates in the cell. This new "improved" model was tested by additionally deleting gal 1 (which encodes the enzyme that converts galactose to galactose -1-P). As predicted, this essentially reversed the effect.

This seminal systems biology paper was only made possible because the yeast genome had been sequenced, microarray analysis of yeast mRNA had been developed, databases of protein–protein interactions were available, rapid MS analysis of protein presence and abundance was possible, and computing facilities to process all of the information were available. In short, systems biology is only possible in a post-genomic world.

4.7 Conclusion

From one gene, one enzyme in *Neurospora*, to cell cycle in yeast, fungal experiments have been at the forefront of altering the way biologists view their world. Yet the sum total of all the great discoveries in fungi, from Beadle and Tatum to Hartwell and Nurse, is set to pale into insignificance in the face of the potential offered by fungal systems biology in this, the post-genomic era. Led by *S. cerevisiae*, there is a paradigm shift towards massively parallel comparative biology in all types of organismal research. Even within the context of this book, such an approach offers rapid progress in every area of this field, including exploring native and transgenic fungal biology, identifying genomic differences for strain improvement, understanding the molecular consequences of fungal infection, and identifying targets for the development of new antimycotics. The opportunities offered by post-genomic research are quite simply limitless.

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5
Fungal Proteomics

Sean Doyle and Rebecca A. Owens

5.1 Introduction

The term "proteomics" refers to the large-scale study of the proteins present in an organism. Consequently, "fungal proteomics" can be defined as the study of the intracellular and extracellular protein complement of fungi. Historically, individual proteins were isolated by a combination of chromatographic techniques, enzymatically characterized, and subjected to N-terminal or partial amino acid sequence analysis for identification. However, the advent of genome sequencing and protein mass spectrometry (MS), allied to high-resolution separation techniques for proteins (e.g. molecular mass-based separation by electrophoresis) has meant that thousands of proteins can be simultaneously isolated, separated, and identified from an individual fungus. Although this large-scale approach has drawbacks, such as the generation of huge amounts of data, it represents the current situation with respect to the study of fungal proteomics.

The strategies for undertaking fungal proteomic investigations are continually evolving, but all share the following themes:

- Availability of a full or partial genome sequence (or cDNA) for the fungus of interest, or establishment of the extent of fungal genomic information on related fungi in publicly available databases (e.g. PubMed; http://www.ncbi.nlm.nih.gov/pubmed).
- Optimization of protocols for both fungal culture and intracellular and/or extracellular protein isolation.

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- Separation of proteins by techniques such as chromatography or electrophoresis (e.g. sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), Figure 5.1). This step is optional in the case of shotgun proteomics.
- Fragmentation of individual proteins into peptides by digestion with the proteolytic enzyme trypsin.
- Analysis of each peptide mixture by liquid chromatography–tandem mass spectrometry (LC-MS/MS). This technique separates the peptides from one another, determines the mass, and sequence, of each, and then compares these data to DNA or cDNA databases.

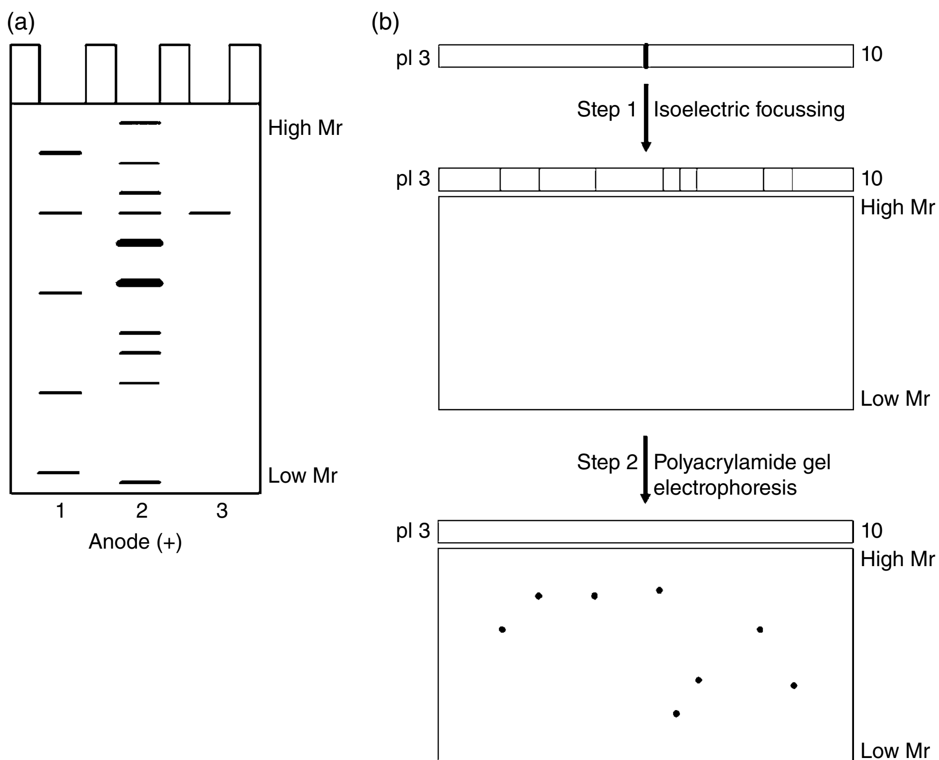

Figure 5.1 Schematic representation of SDS-PAGE and 2D-PAGE. (a) SDS-PAGE separation of proteins by molecular mass. Lane 1 illustrates proteins of known molecular mass ("marker proteins"), lane 2 contains a protein extract containing proteins of different molecular mass, and lane 3 contains a purified protein preparation (single band). (b) 2D-PAGE consists of two distinct steps: isoelectric focussing (IEF) followed by PAGE. In step 1, native proteins are separated according to charge on an IEF strip. This strip is then placed on top of a PAGE gel and step 2 involves the additional separation of proteins by molecular mass to yield protein "spots." These can be excised and digested with trypsin for mass spectrometric analysis.

- Subsequent identification of a peptide or set of peptides coded by a specific gene – following *in silico* translation – allows one to conclude that these peptides must have originated from the protein encoded by this gene.

This chapter focusses primarily on proteomics of filamentous fungi such as *Aspergillus* species, primarily because these organisms are reservoirs of valuable protein products and are effectively "black boxes" with respect to our knowledge of the detailed biochemical mechanisms used by these fungi to survive in the environment. Although the biotechnological potential of filamentous fungi in such areas as antibiotic and hydrolytic enzyme production has been exploited for a long time, research into fungal proteomics has lagged significantly behind that of bacteria and viruses for a number of reasons:

- No filamentous fungal genome sequences were available until the 2000s, which meant that protein MS was of limited use in facilitating protein identification.
- Filamentous fungal genes were known to contain multiple introns, unlike bacterial, yeast, and viral genes. This meant that interrogation of available genomic DNA databases (following *in silico* translation) with peptide mass data was often of limited value for protein identification, since inadequate bioinformatic tools were available for intron/exon splice site identification in genes.
- Intracellular protein isolation from fungi is particularly difficult due to the rigid nature of the fungal cell wall, allied to the presence of large amounts of interfering carbohydrate polymers.
- The level of proteins secreted by fungi is low in many wild-type organisms and is dependent on the culture medium and conditions used for growth.

Consequently, fungal proteomics is an emerging and highly important area, whereby modern proteomic techniques allow rapid identification of fungal proteins of biomedical or biotechnological importance. In addition, proteomics can be further defined in terms of either the study of protein modifications or protein–protein interactions. This chapter describes methods used for protein extraction and isolation, electrophoretic techniques used for protein purification prior to identification, protein MS, and selected examples of the uses of proteomics in the study of fungal virulence and commercial potential.

5.2 Protein Isolation and Purification

5.2.1 Cell Lysis Strategies

The extraction or isolation of proteins from fungi is a prerequisite for subsequent analysis. Unlike animal cells, which do not possess a cell wall, and where the cell membrane can be readily lysed using detergents, the rigid cell wall of fungi

represents a significant barrier to the efficient extraction of intracellular proteins. Consequently, a number of vigorous techniques have been devised to enable the release of proteins from fungal mycelia, including hyphal maceration in liquid N_2 using mortar and pestle, bead-beating, sonication, and rapid pressure changes (French Press technology). One or a combination of these physical techniques must be used to disrupt fungal mycelia, which, allied to strict temperature control (2–8 °C), use of protease inhibitors, pH control, and optimal mycelia:extraction buffer ratio, will yield efficient release of intracellular contents. Mycelial lysis is generally followed by high-speed centrifugation to remove insoluble material such as intact mycelia, cell wall fragments, or cell debris, to yield a clarified, protein-rich supernatant. This supernatant can then be further processed by ammonium sulfate fractionation, dialysis, or volume reduction by ultrafiltration, prior to protein purification by chromatographic techniques.

5.2.2 Chromatography

A detailed description of chromatographic techniques is beyond the scope of this chapter; however, ion-exchange chromatography (cation or anion exchange) facilitates protein separation by charge, gel filtration enables protein separation based on molecular mass, and affinity chromatography allows protein isolation by virtue of the specific affinity between the protein of interest and an immobilized ligand. Table 5.1 provides information of the relative merits and demerits of each chromatographic approach. Once the protein of interest has been

Table 5.1 Advantages and disadvantages of alternative chromatographic techniques for fungal protein isolation.

Chromatographic technique	Advantages	Disadvantages
Ion exchange	High resolution, high speed, high protein capacity resins, crude protein preparations suitable for use	Salt interference, total protein purity rarely achieved
Gel filtration	Separation based on molecular mass, high purity achievable, no salt interference	Low resolution, pre-fractionation required, time-consuming
Affinity	Excellent protein purity achievable, rapid, no pre-fractionation required	Requires affinity ligand, harsh protein release conditions
Immunoaffinity	Excellent protein purity achievable, rapid[1]	Requires protein-specific purified IgG, immunoaffinity-purified protein must be IgG-free

[1] Chromatography is rapid; however, antibody generation may be time-consuming.

isolated, it can then be characterized in terms of activity (if an enzyme), sequence, immunologically, or by protein–protein interaction. Chromatography is generally used for the preparative isolation of proteins for further use, but more recently these techniques have been coupled to MS-based proteomics for on-line or off-line separation of peptides prior to LC-MS/MS.

5.2.3 Protein Extraction Prior to 2-D Polyacrylamide Gel Electrophoresis (2D-PAGE)

Protein separation by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) is explained in detail in Section 5.3. This important separation technique exploits both protein charge and molecular mass to yield individually resolved proteins, in sufficient amount, which can be analyzed and identified by MS following enzymatic digestion by trypsin. This represents a significant advantage over chromatographic purification which can take days or weeks to complete and often yields only one or a few pure proteins. Fortunately, mycelial proteins can be extracted directly into reagents which are compatible with subsequent separation by 2D-PAGE. Here, mycelia are ground in liquid N_2 and sonicated in "solubilization buffers" which contain high concentrations of urea, thiourea, and detergents, along with ampholytes. Protein extracts prepared in this way can be applied directly to isoelectric focussing strips (pH range 4–7 or 3–10) to facilitate separation by isoelectric point prior to subsequent separation by molecular mass via protein electrophoresis (Figure 5.1).

5.2.4 Protein Extraction and Enzymatic Fragmentation without prior Purification

Protein fractionation in not always required prior to analysis, and the term "shotgun proteomics" has been introduced to describe the trypsin-mediated digestion of a complex mixture of proteins, followed by LC-MS/MS identification of most constituent proteins following comparison of individual peptide sequences to *in silico* databases. Such analyses require significant computing power and represent the limit of current proteomic approaches for global protein identification. In practice, this technique is carried out as follows: Total protein is first extracted from mycelia prior to in-solution digestion. Mycelia are ground in liquid N_2, suspended in lysis buffer with protease inhibitors, and sonicated to release intracellular protein. Following clarification by centrifugation, trichloroacetic acid (TCA)/acetone precipitation is carried out to concentrate extracted protein, and pellets are solubilized in solutions containing high concentrations of protein denaturing agents (e.g. 8 M urea). Protein samples are reduced with dithiothreitol (DTT), followed by alkylation using iodoacetamide (IAA) to prevent disulfide bridge re-formation. Samples are diluted using ammonium bicarbonate

to lower the concentration of the denaturing agent (e.g. to 1 M urea), followed by incubation with the protease trypsin. This enzymatically fragments all proteins present into constituent peptides by cleaving the peptide bond on the carboxyl side of lysine or arginine residues. This peptide mixture can then by subjected to LC-MS/MS analysis to facilitate global protein identification. Sample clean-up is often carried out using C18 resins (e.g. C18 ZipTips) to remove residual salts ahead of analysis.

5.2.5 Subcellular Fractionation

Separating mycelial lysates into distinct fractions can serve a dual purpose in proteomic analyses. This fractionation tool can allow targeted investigations of specific organelles (e.g. mitochondria) or subcellular domains (e.g. microsome) within the organism. In addition, this technique serves to reduce sample complexity, which in turn enables wider proteome coverage to be achieved. One method employed for subcellular fractionation is differential centrifugation, a tool that sequentially separates components in order of decreasing density. A sample protocol for recovery of the microsomal fraction from the filamentous fungus *Aspergillus fumigatus* is shown below. Total cell lysates are generated initially, followed by a medium-speed centrifugation step to pellet dense material (cell wall, nuclei, etc.), and finally high-speed centrifugation to pellet membrane-associated material (microsomal fraction). Using this protocol, 710 unique proteins were detected from microsomal fractions of *A. fumigatus* using the Orbitrap LTQ XL mass spectrometer, and this number has the potential to be increased using the higher resolution mass spectrometers such as the Thermo Q-Exactive instrument.

Materials:

- Lysis buffer: 200 mM tris-HCl, 20 mM EDTA, pH 8. Store at 4 °C. Bring to 1 mM PMSF immediately before use.
- Resuspension buffer: 6 M urea, 2 M thiourea, 0.1 M tris-HCl, pH 8.

Protocol:

1 Harvest mycelia through Miracloth, dry between tissue, and snap-freeze in liquid nitrogen. Lyophilize mycelia and transfer 100 mg into each of eight 2-mL microfuge tubes (i.e. process 800 mg per sample).

2 Add glass beads (400 mg) and one tungsten bead to each of the tubes and bead-beat at 30 Hz for 5 min.

3 Add cold lysis buffer (600 μL) to each tube and repeat the bead-beating step.

4 Add an additional 500 μL of cold lysis buffer to the samples and store on ice for 1 h.

5 Further lyse mycelia using sonication probe MS73 (3 × 10 sec, 25 % power, cycle 6), with samples cooled on ice between sonications.

6 Centrifuge samples at 10,000 g, 20 min, 4 °C.

7 Transfer supernatants to new microfuge tubes and repeat centrifugation to obtain clarified lysates.

8 Pool clarified lysates. Measure the protein concentration (whole cell extract).

9 Transfer clarified lysate to a 4.9-mL ultracentrifuge tube and adjust the volume using cold lysis buffer to fill the tube.

10 Perform ultracentrifugation at 150,000 g, 4 °C for 1 h.

11 Remove supernatant from the tube. Can retain as the "soluble" protein fraction for further analyses. Wash the pellet (microsomal fraction) by resuspending in ice-cold lysis buffer using a 27-G needle.

12 Transfer the resuspended sample to a fresh 4.9-mL ultracentrifuge tube, adjust the volume with lysis buffer to fill the tube, and repeat centrifugation at 150,000 g, 4 °C, 1 h. Repeat wash step as necessary.

13 Resuspend microsomal pellets in urea/thiourea resuspension buffer (approx. 200–300 μL) using a 27-G needle.

14 Measure protein concentration and proceed with in-solution digestion for gel-free proteomic analyses. Note: MS-compatible detergents (e.g. ProteaseMAX™ Surfactant) can be included in the digestion procedure to aid solubility of microsomal proteins.

5.2.6 Protein Recovery from Culture Supernatants

In addition to the production and presence of mycelial (intracellular) proteins, fungi secrete a wide range of enzymes into the extracellular environment; indeed the pattern of secreted enzymes is often dependent on the available carbon source. Fungal culture can take place on either solid matrices (solid-state fermentation (SSF)) or in liquid culture, sometimes referred to as submerged fermentation. In both scenarios, the concentration of secreted enzymes is generally low (nanograms to micrograms per milliliter) and so a concentration step is often required prior to subsequent analysis and characterization. Initial enzyme recovery from SSF is generally via resuspension in aqueous buffered solutions (occasionally containing low concentrations of detergents). Once resuspended SSF material or submerged culture supernatants are available, enzyme concentration

is effected by (1) ammonium sulfate precipitation, (2) protein ultrafiltration, (3) lyophilization, or (4) TCA precipitation. Once one or more of these concentration steps has been performed, and the volume has been reduced by up to a factor of 50–100, analytical tests such as protein estimation or protein electrophoresis are performed to estimate and visualize all proteins present. It should be noted that many fungi, especially basidiomycete species, produce large amounts of extracellular carbohydrate polymers which can interfere with the isolation of secreted enzymes. Consequently, a high-speed centrifugation step is often used to remove this material prior to protein concentration when investigating the extracellular proteome (secretome) of many fungal species.

5.3 Electrophoretic Techniques

5.3.1 Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis

SDS-PAGE is one of the most useful and straight-forward techniques for protein fractionation or separation. SDS is a powerful detergent which contributes an overall negative charge to the proteins and effectively denatures proteins following co-incubation at 95 °C for 3–5 minutes. Moreover, complete protein denaturation is enabled by inclusion of reducing agents during this denaturation step which serve to cleave all intra- and inter-molecular disulfide bridges. SDS-PAGE is based on the principle that if all protein molecules present in a porous polyacrylamide matrix are fully denatured and reduced, and possess equivalent (negative) charge, then they will migrate towards the anode (+) when subjected to an electric field. Proteins of low molecular mass will migrate furthest, while those with the highest molecular mass will migrate least. In this way, proteins are separated from one another by size, to yield a "ladder" of individual proteins in the polyacrylamide gel (Figure 5.1). Following electrophoresis it is necessary to fix and stain the proteins in the gel using specific staining reagents such as Coomassie Brilliant Blue (CBB), silver staining, or Amido Black. Staining is essential to allow protein visualization in the gel and, fortunately, the most commonly used stain, CBB, does not interfere with subsequent trypsin digestion of excised protein bands and MS identification of fractionated proteins. Silver stains can interfere with trypsinization and subsequent LC-MS identification, but occasionally good results can be achieved.

The immunological identification of proteins separated by SDS-PAGE, using polyclonal or monoclonal antibodies, is known as western blotting. Here, an SDS-PAGE gel containing separated but unstained proteins is sandwiched against either nitrocellulose or polyvinylidene fluoride (PVDF) membrane and subjected to electrotransfer to move the proteins from the gel onto the membrane. Once all proteins are replica-transferred to the membrane, a "blocking" step is carried out to minimize nonspecific antibody binding to the membrane, followed by visualization

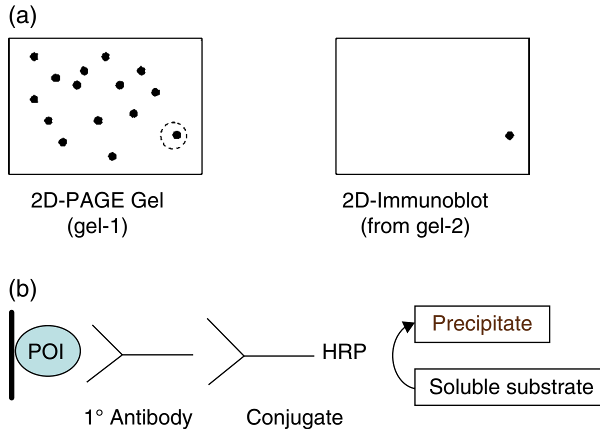

Figure 5.2 Immunoproteomics. (a) Duplicate 2D-PAGE analysis is performed and gel-1 is stained with Coomassie brilliant blue dye, while gel-2 is subjected to electrotransfer onto a suitable membrane. This membrane is subsequently probed with antisera using an immunoblot procedure as shown in (b) to detect immunoreactive proteins (2D-immunoblot). The corresponding protein (circled) in gel-1 can then be excised and identified by protein MS.

of immunoreactive proteins by a combination of antibody, antibody–enzyme conjugate, and substrate addition (Figure 5.2). Immunoproteomics is a term used to describe the combined used of immunological and proteomic techniques for protein identification. It will be appreciated that duplicate SDS-PAGE analysis, whereby one gel is CBB-stained (gel 1) and the other subjected to western (immuno) blot analysis (gel-2), will allow MS identification of a protein from gel-1 and co-analysis of the immunoreactivity of the same protein in gel-2. This approach is widely used to identify immunoreactive fungal antigens associated with disease-states in humans, such as allergy.

Although SDS-PAGE is an extremely powerful and widely used technique in fungal proteomics, it does not possess sufficient resolving power to completely separate every protein in a complex mixture prior to further MS analysis. In addition, because MS detection is such a high-sensitivity technique, it can detect protein presence even if a protein band is not visualized by CBB staining. Consequently, 2D-PAGE is the method of choice for gel-based fungal protein separation prior to analytical MS.

5.3.2 2D-PAGE

2D-PAGE is actually two techniques, isoelectric focussing (IEF) and SDS-PAGE, combined into a single process. It is also referred to as 2-DE (two-dimensional electrophoresis). Here, either total mycelial or secretome protein extracts are obtained from fungi under native conditions and without significant salt

contamination. Following addition of IEF sample buffer, protein extracts (250–400 μg) are applied to isoelectric focussing strips, followed by high-voltage conditions (up to 8,000 V) for 24 hours. Under these conditions, the charged proteins migrate in the electric field until they reach their isoelectric point (p*I*), at which point they cease movement as a result of loss of charge – that is, the proteins become focussed. Once the IEF stage is completed, the entire strip is removed and placed adjacent to an SDS-PAGE gel, electrophoresis is commenced, and the proteins leave the IEF strip, enter the SDS-PAGE gel, and migrate according to molecular size. After electrophoresis, the gel is stained using either CBB or the more sensitive colloidal Coomassie dye (detection limit: 0.1 μg protein/spot), to detect protein spots (Figure 5.3); fluorescent stains including SYPRO® Ruby or Deep Purple™ dyes also allow protein visualization, but these require fluorescent scanners for detection.

Once protein visualization is complete, individual protein spots can be either manually or automatically excised from 2D-PAGE gels for trypsin digestion and MS analysis. It should be noted that proteins present in mycelial or secretome extracts can also be labeled with fluorescent dyes (termed Cy2, Cy3, and Cy5) prior to IEF and SDS-PAGE separation, and that this approach not only removes

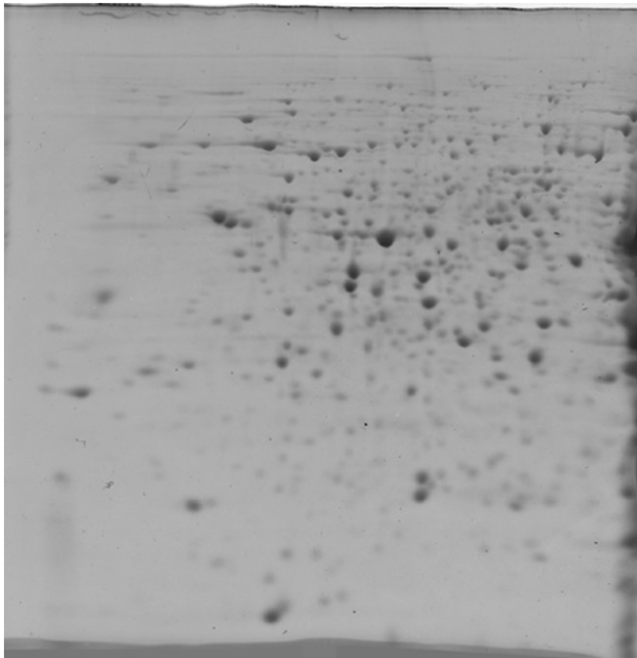

Figure 5.3 2D-PAGE of a mycelial extract from an ascomycete stained with Coomassie Brilliant Blue protein stain. IEF was carried out over a pH range 4–7 and 10% PAGE was employed for molecular mass separation. Each "spot" corresponds to an individual protein monomer from the organism. In general, different protein spot intensity corresponds to different relative amounts.

the requirement for post-2D-PAGE gel staining, but also facilitates the quantitative assessment of differential protein expression under different experimental conditions on a single PAGE gel. This process is known as 2D-DIGE (two-dimensional difference in gel electrophoresis) and requires an extensive instrumentation environment, including fluorescent scanners and automatic protein "spot-picking" devices. In fungal proteomics, 2D-DIGE has been primarily employed for the quantitative assessment of differential protein expression when the organism has been exposed to either environmental stress (e.g. oxidative stress) to identify protective proteins, or different carbon sources to identify hydrolytic enzyme expression.

Like SDS-PAGE, 2D-PAGE gels can be electrotransferred and subjected to immunological interrogation using antisera. Thus, immunoproteomic approaches are possible for proteins fractionated by both SDS-PAGE and 2D-PAGE, although the resolution and sensitivity of 2D-PAGE far exceeds that of SDS-PAGE.

5.4 Protein Mass Spectrometry

5.4.1 Genome Databases

Before detailing protein MS techniques and, in particular, the further consideration of fungal proteomics, it is necessary to be aware of the availability of computer databases which store large amounts of DNA and cDNA sequences from fungi, and indeed other organisms. These databases contain huge amounts of publicly available nucleic acid sequence data, including that from approximately 700 sequenced fungal genomes, which has been deposited by researchers from all over the world. The internet addresses of, and portals to, many of these sites are given in Table 5.2. The pace at which fungal genomic and cDNA data are deposited at these locations is rapidly accelerating due to the advent of high-throughput next-generation DNA sequencing (see Chapter 3) and the increased interest in fungi as a source of enzymes for carbohydrate degradation with a view to biofuel production. Most importantly, these databases represent the source of raw data, often referred to as *in silico* information, which is interrogated by, or compared against, fungal peptide information (e.g. peptide masses or sequences) to yield definitive fungal protein identification. Thus, at the heart of fungal proteomics and protein identification lies the ability to compare the mass or sequence of tryptic peptides isolated from a particular protein against the theoretical tryptic peptides present in a predicted protein sequence encoded by a gene in the aforementioned *in silico* databases (Figure 5.4).

The data present in these databases can either be downloaded to local servers or accessed or interrogated over the internet. In addition, it is important to note that although extensive DNA sequence information is available, there can often be minimal information with respect to gene structure (intron/exon presence or splicing), whether or not a particular gene is expressed, and what, if any,

Table 5.2 Genomic, proteomic, and general fungal websites.

Database	URL address
Aspergillus genomes (CADRE)	www.cadre-genomes.org.uk
Aspergillus genomes (AspGD)	www.aspergillusgenome.org
Multifungal genome database	http://mips.gsf.de/genre/proj/fungi/fungal_overview.html
Aspergillus nidulans	www.broadinstitute.org/fungal-genome-initiative/aspergillus-genome-projects
Aspergillus niger	http://genome.jgi-psf.org/Aspni5/Aspni5.home.html
US Government Genome Sequencing Initiative	www.nhlbi.nih.gov/research/resources/nhlbi-precision-medicine-initiative/topmed/wgs
Fungal genomes	http://fungalgenomes.org/blog/
FungalGenetics Stock Centre	www.fgsc.net
UniProt	www.uniprot.org
ExPASy	http://expasy.org/
Mascot	www.matrixscience.com
MycoCosm at JGI	http://genome.jgi.doe.gov/programs/fungi/index.jsf

post-translational protein modifications occur to the encoded proteins. A specific issue with respect to fungal genomic data is that many predicted genes are only classified as encoding "hypothetical" or "predicted" proteins, because either the proteins have never been identified or, even if they have previously been detected, no function has been assigned to them. This "functional proteomics" challenge represents one of the major issues in fungal biology today.

5.4.2 Protein Digestion

Prior to MS analysis of either a gel-purified protein or protein mixture, it must be digested into constituent peptides using trypsin. Trypsin is the enzyme of choice for protein fragmentation as it cleaves specifically at the C-terminal side of either K or R to yield a population of peptides, each terminating in a positively charged amino acid residue. The presence of this positive charge is key to peptide flight during MALDI-ToF MS (see Section 5.4.4). Moreover, many of the peptides generated fall within the mass range 500–2,500 Da, which is compatible with the optimal detection range of most MS instrumentation. High-grade trypsin is almost always used, which means that no contaminating protease activities will be present and trypsin is chemically treated to prevent auto-digestion and inhibit endogenous chymotryptic activity. Any of these interferences could reduce the efficiency and sensitivity of subsequent peptide detection by MS.

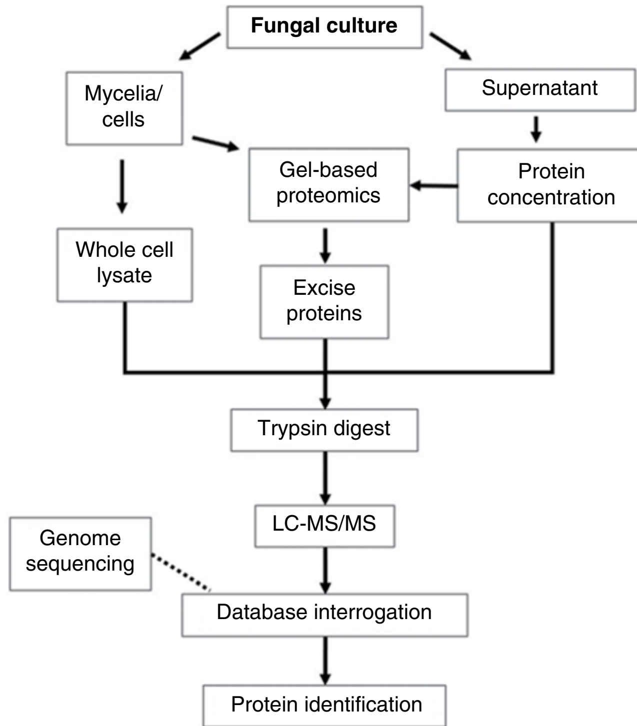

Figure 5.4 Schematic diagram of the overall process involved in fungal proteomics. Fungal cultures are initially separated into mycelia and culture supernatants. Protein fractionation can then be carried out using gel-based electrophoresis (SDS-PAGE or 2D-PAGE), or chromatography (not shown). Alternatively, whole cell lysates can be trypsin-digested without prior fractionation. Ultimately, peptide mixtures derived from either individual proteins (following purification) or protein mixtures (shotgun proteomics) are analyzed by LC-MS/MS to generate mass or sequence information. These data are then used to interrogate publicly available or local genomic/cDNA databases to facilitate protein identification.

Target protein amounts can range from nanograms/micrograms to milligram amounts and the substrate protein:trypsin is generally 20:1. It should be noted that in theory, digestion of a protein should result in release and detection of all tryptic peptides. However, in practice this rarely if ever occurs. From a chemical perspective this inefficient detection of all constituent tryptic peptides occurs for the following reasons: (1) inhibition of peptide bond cleavage, (2) loss of large hydrophobic peptides by adherence to plastic or insolubility, (3) release of multiple short peptides of low molecular mass (<300 Da), (4) nonspecific binding of peptides to filters used for sample preparation prior to MS analysis, and (5) modified K or R residues. From an instrumentation viewpoint, peptides may not efficiently ionize during MALDI MS analysis or can be irreversibly bound to LC columns during LC-MS, thereby resulting in absence of detection.

5.4.3 Mass Spectrometry

It should be clear at this point that when reference is made to protein MS, that one is generally inferring identification of proteins by comparison of peptide data to genomic or cDNA databases. The fidelity of protein identification is therefore significantly dependent on the accuracy of peptide mass, and sequence, determination by MS. Modern mass spectrometers can accurately determine the masses of either peptides or peptide fragments, for subsequent interrogation against the corresponding theoretical tryptic peptide masses or sequences present in predicted protein sequences encoded by genes or cDNA in *in silico* databases. Peptides must generally be ionized to facilitate detection in a mass spectrometer and this is achieved by either laser or electrospray techniques.

5.4.4 MALDI-ToF Mass Spectrometry

MALDI-ToF MS stands for matrix-assisted laser desorption ionization–time-of-flight mass spectrometry. In this type of protein MS, a peptide digest, derived from a purified protein, is mixed with an energy-absorbing matrix material (e.g. α-cyano-4-hydroxycinnamic acid (HCCA)) and spotted onto a metal target plate. After drying, this sample is placed into the vacuum chamber of a MALDI-ToF MS, where it is subjected to ionization by laser light (337 nm), in a vacuum and at high voltage. The HCCA facilitates laser energy transfer to constituent peptides, which vaporize and, because they are positively charged, electrostatically "fly" in the time-of-flight detector. Peptide ions are thereby separated according to mass:charge, or *m/z* ratio, to yield a peptide mass fingerprint or MS spectrum. It is worthwhile noting that not all peptides present in the protein will be detected, and that MALDI MS is suitable for analyzing other biomolecules (e.g. nucleic acids and carbohydrates) and intact proteins also. However, alternative matrix materials, such as 2,5-dihydroxy benzoic acid or sinapinic acid, and target deposition strategies need to be employed to enable such analyses.

While MALDI MS is a rapid and automated technique, whereby multiple specimens can be analyzed sequentially and individual specimen analysis can take as little as 5 minutes, it tends to be less sensitive than other types of protein MS and requires relatively pure protein samples to identify proteins with high confidence.

5.4.5 Electrospray Mass Spectrometry

Unlike MALDI MS, electrospray or nanoelectrospray ionization (ESI or nanoESI) techniques generate gaseous ionized molecular species (e.g. peptides) directly from a liquid phase. This type of MS for analysis of peptide mass and sequence has grown in popularity since 2007 due to the ability to link the initial liquid

chromatographic (LC) separation of peptides via ESI to high mass accuracy analyzers. In practice, peptides from digestion of either a pure protein or a complex mixture are initially fractionated by reverse phase–high performance liquid chromatography (RP-HPLC) (Figure 5.5). As peptides elute from the LC column, they are ionized by nanoESI or ESI, and subject to highly accurate mass determination, prior to interrogation of *in silico* databases to enable protein identification. This so-called LC-MS approach is superior to MALDI MS insofar as highly confident protein identification is possible using significantly smaller amounts of starting material. Moreover, since peptide purification occurs during the LC step, sample purity is less of an issue than when using MALDI ionization. Optimal LC run times range from 30 minutes for simple mixtures to 2–3 hours duration for complex samples, and so throughput is less than with MALDI MS when considering pure protein samples.

Peptides detected by both MALDI MS and LC-MS can also be subjected to repeat ionization to yield smaller peptide fragments. This is sometimes referred to as MS^n, MS/MS, or tandem MS. The observed peptide fragmentation pattern can be used to determine the amino acid sequence of the parent ion. These data can be compared to a protein database to determine if a peptide matching the predicted amino acid sequence would be generated in a theoretical digest. Multiple peptides matching the same protein provide additional support for an identification. This type of analysis is not carried out manually but conducted using commercially available software which comes as standard with most commercial instruments.

5.4.6 Shotgun Proteomics

So far in this chapter, emphasis has been on the requirement to trypsin-digest a purified or semipurified protein prior to MS analysis. However, due to improvements in LC fractionation for the separation of peptides and, more importantly, in the algorithms used to assign detected peptides to a corresponding protein in a database, it is now possible to simultaneously identify many of the proteins present in a complex mixture by protein MS. This approach is known as "shotgun proteomics" and bypasses the need to purify the protein of interest prior to trypsinization and MS analysis. Figure 5.4 outlines how shotgun proteomics analyses can be carried out. The resultant mixture, after trypsin digestion, contains peptides derived from all proteins present in the initial sample, whereby thousands of proteins may yield many tens of thousands of discrete peptides. Following fractionation and detection of this mixture by LC-MS/MS, a list of proteins is obtained, which have been identified by comparison against the appropriate *in silico* database. Some proteins may be identified even from as little as a single peptide, while in most cases detection of two peptides per protein, or sequence coverage of 5–10%, is necessary for confident identification of constituent proteins. Extended gradients (2–3 hours) are required for shotgun

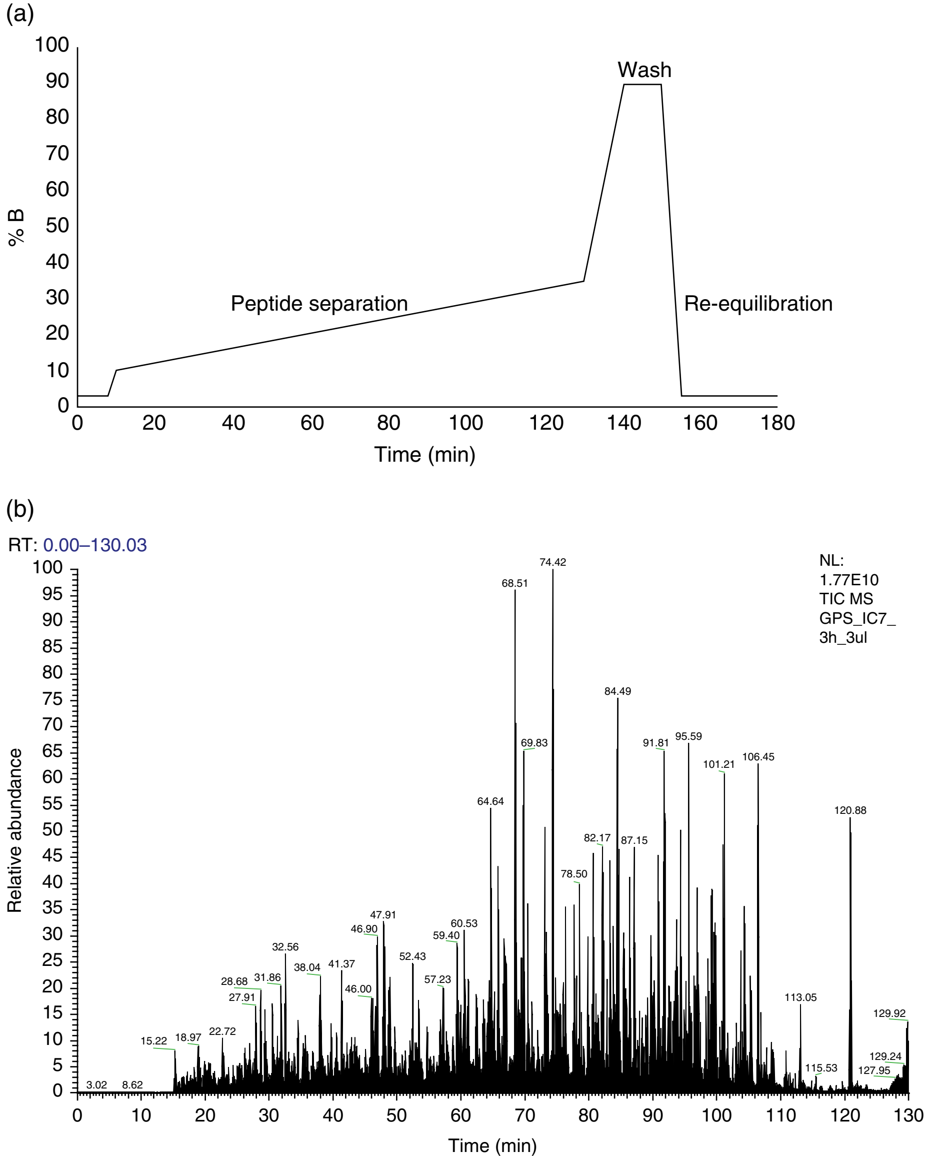

Figure 5.5 Gradient profile for reverse phase LC-MS and total ion chromatogram, respectively. (a) Typical gradient profile used in qualitative or quantitative MS-based proteomics. A polar solvent (Solvent A: 0.1 % (v/v) formic acid in water) is used initially for peptide loading to the column. Peptides bound to the column are eluted by increasing concentration of the nonpolar solvent (Solvent B: 0.1 % (v/v) formic acid in acetonitrile) across a 2-hour gradient. Most peptides typically elute by 30–40% B, and this is followed by a nonpolar wash step to remove any tightly bound molecules, before re-equilibration into a low % B for loading of the next sample. (b) MS-based proteomics: this total ion chromatogram (TIC: ion intensity versus time (min)) shows separation of peptides

proteomics, to facilitate resolution of complex mixtures (Figure 5.5). This extended analysis time is offset by the large amounts of data obtained from a single experiment, especially when high-resolution MS instruments are used.

5.4.7 Quantitative Proteomics

Protein identification using the proteomics strategies described so far is primarily a qualitative event. In others words, we can detect the presence of a protein, but generally have no quantitative estimate as to how much is actually present, or if the amount of a particular protein changes under different conditions. Quantitative proteomics is in part enabled by comparative 2D-PAGE or 2D-DIGE analysis of two experimental conditions, followed by image analysis to detect differential protein abundance; however, gel-free strategies have become more prominent in recent years. Protein quantification can be achieved by computation of ion signal intensities of differentially labeled peptide pairs in comparative mass spectra. The importance of this technique in fungal proteomics cannot be overestimated, as it provides a means (1) to investigate the effect of culture conditions on the extent of protein expression, (2) to study altered global or specific protein expression following gene deletion, and (3) of altered virulence factor expression during infection.

5.4.8 Label-Free Proteomics

With the development of mass spectrometers capable of high mass accuracy and high resolution, MS-based proteomics has taken over the field in both qualitative and quantitative comparative proteomics. Label-free methods represent the most straight-forward of these techniques, in that protein samples do not have to undergo chemical or metabolic labeling prior to analysis. Whole-protein digests from replicate samples are analyzed consecutively on extended LC gradients (Figure 5.5). Relative abundance of a peptide is determined by correlating the peak area or spectral counts of the precursor peptide ion within a retention time window (peak matching) (Figure 5.6). MS/MS analyses subsequently reveal the identity

Figure 5.5 (Continued) by nanoLC, with peptide detection and identification by MS/MS (via electrospray ionization), following trypsin digestion of a total fungal protein lysate (Figure 5.4). Sample amount used for analysis is generally 0.7 μg peptide and an LC gradient time of 120 min is necessary to enable optimal peptide fractionation. Instrument software (primarily) and operator expertise then matches peptide mass/sequence data to translated DNA/cDNA sequences in *in silico* databases to enable multiple protein identification. Thousands of individual proteins in a single sample can be confidently identified using this approach.

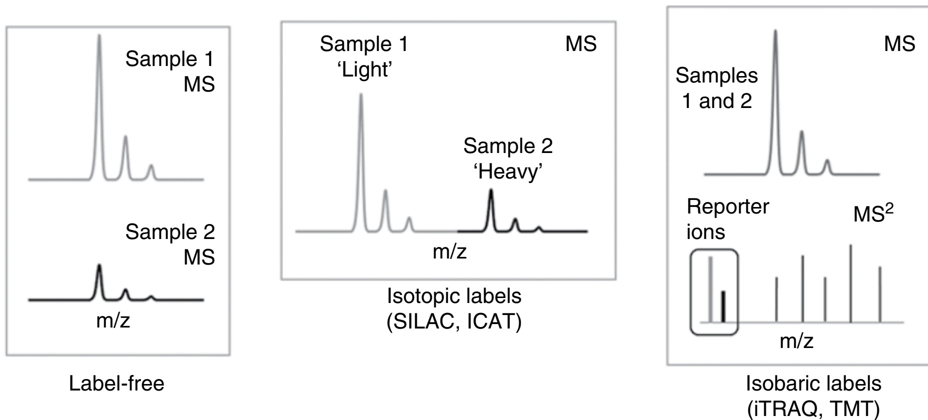

Figure 5.6 Quantitative MS-based proteomics methods. In label-free workflows, proteins from distinct samples are extracted, processed, and analyzed separately. MS spectra from each sample are compared within specific windows of time, and relative peptide abundance is calculated based on the "intensities" of precursor ions (area under the curve or spectral counts). Isotopic or isobaric labeling allows samples possessing different labels to be combined once the label/tag has been introduced (metabolically or chemically). In the case of isotopic labels, each type of label will add a distinct mass upon incorporation into the peptide. "Heavy" peptides can be differentiated from their "light" counterparts based on a defined increase in the m/z value. Relative abundance of a peptide originating from comparator samples is calculated by comparing the MS spectra for each isotope. In contrast, each isobaric label adds the same mass to the labeled peptides, and so no distinction can be made from MS spectra following multiplexing. Fragmentation of the precursor peptide ions prior to MS^2 leads to release of reporter ions of a specific m/z. By comparing the intensities of reporter ions, the relative abundance of the peptide can be determined for each sample.

of the peptide based on fragmentation data and correlation to a supplied protein database. Peptides and their corresponding abundance data are collated to determine the relative protein abundance across comparator samples. Due to the absence of internal standards and lack of multiplexing in label-free proteomic analyses, care must be taken to limit variation at all stages of the workflow, from sample preparation to analysis. Moreover, comparator groups should not exhibit extreme differences in their protein profiles, as label-free quantitation operates on the premise that samples have sufficient correlation to enable peak matching and normalization.

5.4.9 Label-Based Proteomics

Various methodologies can be used to carry out label-based proteomics, with the main strategies involving the use of isotopic or isobaric tags introduced

Table 5.3 Features of various MS-based methods of quantitative proteomic analysis.

Quantitative method	Multiplexing	Number of comparator samples	Labeling of proteins or peptides	Source of peptide abundance data	Challenges
Label-free	No	***	n/a	MS	Variation between LC-MS/MS analyses
Metabolic labeling	*	*	Proteins	MS	Limited multiplexing
Isotopic tags	***	***	Both	MS	Crowding of MS with multiple isotopes of same peptide
Isobaric tags	***	***	Peptides	MS/MS	Appropriate instrument required to measure low mass reporter ions in MS/MS

*Low capacity, ***high capacity.

by chemical labeling, or metabolic labeling. All of these methods allow multiplexing of samples, albeit at different stages in the proteomic workflow. This reduces technical variation introduced during sample preparation and/or LC-MS/MS analyses. Chemical labeling of proteins or peptides is generally performed using either cysteine-specific (isotope-coded affinity tag (ICAT)) or amine-reactive, commercially available reagents (isobaric tag for relative and absolute quantitation (iTRAQ), or tandem mass tag (TMT)). Peptide abundances are quantified through comparison of heavy and light isotopes of the precursor ion using the MS spectrum (isotopic tags), or through the intensities of released reporter ions in the MS/MS spectrum (isobaric tags). Metabolic labeling produces isotopic peptides through the introduction of either heavy or light isotopes of lysine or arginine to the culture medium (stable isotope labeling with amino acids in cell culture (SILAC)). Quantitation of relative peptide abundance is similar to that described for chemical isotopic tags. While metabolic labeling does allow multiple samples to be combined, processed, and analyzed at once, a higher multiplexing capacity is associated with chemical labeling kits. An overview of the quantitative aspects of these various methods is shown in Figure 5.6, with a summary of their respective attributes outlined in Table 5.3.

5.5 Fungal Proteomics

5.5.1 *Trichoderma* Proteomics

Trichoderma harzianum is a mycoparasitic fungus and can protect plants against the deleterious effects of plant pathogenic fungi like *Botrytis cinerea* and *Rhizoctonia solani*. This means that *T. harzianum* can be considered as a biocontrol agent. Moreover, since *Trichoderma* spp., in general, secrete valuable carbohydrate-degrading enzymes, they have attracted much interest from the fungal proteomic community.

Prior to 2004, little data were available on *T. harzianum* proteomics. Thus, initial proteomic investigation of *T. harzianum* involved 2D-PAGE fractionation and MALDI and LC-MS/MS identification of 25 intracellular proteins, including heat shock proteins and glycolytic enzymes. These findings were followed by those that examined the types of proteins produced by *Trichoderma atroviride* (formerly *T. harzianum* P1) in response to exposure to cell wall extracts of the plant pathogenic fungus *R. solani*. Here, increased expression of eight *T. atroviride* enzymes was detected by protein MS techniques and the identified proteins included *N*-acetyl-β-D-glucosaminidase, endochitinase, vacuolar protease A, superoxide dismutase, trypsin-like protease, a serine protease, and a hypothetical protein. It will be immediately apparent that the majority of proteins identified represent hydrolytic or protective enzymes which were most likely produced to degrade *R. solani* biomolecules, including cell wall material and constituent proteins. Thus, it is clear that cell wall extracts from one fungus can generate a biological response in another fungal species and that proteomics can reveal these types of interactions. Interestingly, many of the *T. atroviride* enzymes were present in multiple "spots" on 2D-PAGE gels, which is a characteristic of this technique and arises because of the high resolution associated with IEF which enables separation of different charged forms of the same protein. It will be noted that a single "hypothetical protein" was also detected, which means a protein with no known function or homology to a previously identified protein. Detection of this type of protein during fungal proteomic studies is common, and represents a significant functional genomics/proteomics challenge for the future.

Proteomics can also provide an insight into the complex pattern of protein expression in mixed cultures involving *T. atroviride*, *B. cinerea*, and plant leaves. These type of mixed-proteomic studies use the high specificity of detection of protein MS (mainly LC-MS/MS) to identify the types of proteins produced by each of three species under conditions that mimic infection and biocontrol conditions. For instance, expression of cyclophilin A, a protein involved in protein folding, and superoxide dismutase, a free radical scavenger, was upregulated in *B. cinerea* upon co-incubation with *T. atroviride* and bean plant leaves.

Subproteomic investigations of *Trichoderma* spp. have also been enabled by protein MS. In particular, the mitochondrial proteome of *T. harzianum* and that of the 26S proteasome of the industrially important species *T. reesei* have been

dissected by combined electrophoretic and MS analyses. The relevance of these studies lies in the fact that organelle purification preceded fractionation and identification, and thus served as an enrichment step to improve the detection of low abundance and highly localized proteins within the cell. These studies identified many unique proteins, many of which appear to serve regulatory, as opposed to catalytic functions in *Trichoderma* spp. Importantly, since *T. reesei* is used for the commercial production of enzymes, it is imperative that the protein degradation system (which includes the 26S proteasome) in this microorganism is intensively and comprehensively studied. This will ultimately ensure that any barriers to protein stability and/or release can be circumvented, and protein production optimized to improve yields of secreted enzymes.

5.5.2 *Aspergillus* Proteomics

Aspergillus fumigatus is an opportunistic human pathogen, and causes significant mortality in immunocompromised patients. *Aspergillus niger* and *A. oryzae*, respectively, produce enzymes and metabolites that have applications in the food industry. Many genome sequences from *Aspergillus* spp. are now available (55 genomes from fifty species; genome size range: 24.2–42.8 Mb) and it is clear that many genes encode proteins of unknown function, thus highlighting the requirement for both functional proteomic and genomic studies of these species to illuminate the biological roles of these proteins. The difficulty associated with investigating *Aspergillus* spp. proteomics is exemplified by the fact that only about ten reports of 2D-PAGE and protein MS in these species were published between 2002 and 2007. The increased interest in these important species for both biomedical (need for improved diagnostics and new drug targets) and industrial reasons (valuable source of enzymes) has intensified the research, and consequently the number of publications now emerging.

Proteomic and immunoproteomic studies of *A. fumigatus* have investigated the intracellular proteome, the types of proteins secreted by the organism under different culture conditions, conidial- and biofilm-associated proteins, and immunoreactive or antigenic proteins present in the organism. Moreover, method development for optimal protein extraction from, and investigation of the intracellular proteome of *A. fumigatus* have received much attention. Combined, the work of many groups has resulted in the identification of thousands of intracellular proteins in *A. fumigatus*, many of which are of unknown function. Predominant among the proteins that have been identified are heat shock proteins, glycolytic enzymes, mitochondrial enzymes, and catalases. Pre-enrichment of proteins present in *A. fumigatus* cell lysates, by affinity chromatography, has revealed the unexpected identification of glutathione *s*-transferase activity in a putative translation elongation factor protein; Gel permeation chromatographic enrichment, prior to 2D-PAGE and MALDI-ToF MS, led to the detection of low-abundance siderophore synthetases, produced in response to iron-depleted conditions.

In an effort to identify mechanisms by which *A. fumigatus* may become tolerant or resistant to antifungal drugs such as voriconazole, caspofungin, and amphotericin B, a number of research groups have investigated the fungal proteomic response subsequent to drug exposure. The rationale here is that alteration (elevation or decrease) of intracellular or extracellular protein expression will improve our understanding of fungal drug resistance, and, more importantly, enable researchers to develop strategies to combat resistance, mediated by these proteins, and thereby enhance the potency of the aforementioned antifungal drugs. In amphotericin B exposure studies, the differential expression (at least a twofold difference in expression) of 85 proteins (76 upregulated and 9 downregulated) was detected, compared to normal growth conditions. These included cell stress proteins, transport proteins, and enzymes involved in ergosterol biosynthesis (which is targeted by amphotericin B). Moreover, the unexpected alteration in the levels of enzymes involved in protein secretion was evident and the significance of this finding remains unclear.

Although possible, ethanol (i.e. biofuel) production from lignocellulose biomass via enzymatic hydrolysis and fermentation is quite constrained due to the relatively high cost, and associated minimal efficiency, of the enzymes required to break down cellulose to fermentable sugars. Proteomic studies on *Aspergillus* spp. have focussed on identification of carbohydrate-degrading enzymes for use in substrate generation with potential for biofuel production, and one approach has led to the identification of two thermostable β-glucosidases, secreted by *A. fumigatus*, which could degrade cellulose in a superior fashion to pre-existing enzymes from other *Aspergillus* spp. In general, fungi represent a rich source of commercially relevant enzymes for use in a range of biotechnological processes – as described in detail in Chapter 8.

2D-PAGE and MALDI-ToF MS enabled identification of 57 proteins in *A. fumigatus* conidia which were overrepresented compared to those present in fungal mycelia. In particular, enzymes associated with anaerobic fermentation (e.g. alcohol dehydrogenase and pyruvate decarboxylase) were evident, suggesting a nonaerobic-type metabolism in resting conidia. Also, stress-resistance enzymes such as those responsible for pigment biosynthesis, inactivation of reactive oxygen species (catalase A, thioredoxin reductase, and peroxiredoxins), and conidial surface formation were found and it is thought that these may contribute to the protection of *A. fumigatus* conidia against the innate or adaptive animal immune system. Conidial germination in *A. nidulans* has also been studied using proteomics, and expression of 241 proteins was altered ($P < 0.05$) at the early phase of germination; 40 of 57 which were identified by MALDI-ToF MS were associated with detoxification of reactive oxygen species (as also noted for *A. fumigatus* above), energy metabolism, protein synthesis, and protein folding process, respectively. Simultaneous analysis of gene expression using molecular techniques such as northern blot and reverse transcriptase (RT)-PCR analyses confirmed the altered protein levels found in *A. nidulans* conidia. However, it

should be noted that coincidence of gene and protein expression is not seen on all occasions during combined fungal genomic and proteomic analyses.

As noted earlier, fungal genes contain multiple introns which can make the absolute delineation of the start and stop codon of a gene difficult to define, even using the most sophisticated bioinformatics tools. Moreover, although highly reliable software tools are used to predict intron/exon splice sites (>95% confidence), they may not always correctly predict (*in silico*) the corresponding cDNA sequence of a fungal gene. In this regard, it will be recalled that a protein sequence corresponds to the cDNA sequence; therefore, the protein sequence will contain tryptic peptides which derive from both the corresponding internal regions of an exon and from exon/exon splice regions (Figure 5.7). Thus, proteomics has a significant role in confirming the accuracy of genes identified by high-throughput bioinformatics tools used for gene identification (sometimes called "gene or gene model calling") because tryptic peptide detection can only result from transcription and translation of actual genes. Moreover, tryptic peptide sequences are often identified that do not correspond to the internal region of an exon and therefore must result from an exon:exon fusion, thereby confirming the existence of an intron/exon splice site, and the accuracy of the splice site prediction software (Figure 5.7). These important concepts have been clearly elucidated using LC-MS/MS analysis of tryptic peptides derived from *A. niger*

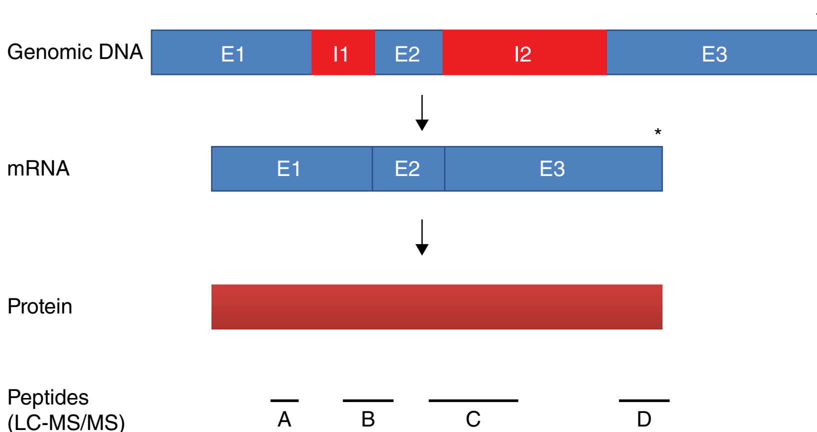

Figure 5.7 The role of protein MS in confirming *in silico* splice site identification in fungal genes. Following fungal genome sequencing, *in silico* analysis had predicted the presence of two introns (I1 and I2), and a stop codon in exon 3 (E3), in the gene of interest. LC-MS/MS analysis of tryptic peptides from the protein revealed the presence of four peptides (A–D). Peptide A was originally encoded by exon 1. Peptides B and C could only have been detected if I1 and I2, respectively, had been present in DNA and subsequently excised during mRNA synthesis. Peptide D did not contain a C-terminal K or R residue and so its detection by LC-MS/MS strongly indicates correct stop codon (*) annotation in genomic DNA.

proteins separated only by SDS-PAGE. In this study, tryptic peptides were mapped against two *A. niger* genome sequences, one of which (ATCC1015) was sequenced by the Joint Genome Institute (Table 5.2) and the other (CBS 513.88) by the Dutch biotechnology company DSM. Data from 19,628 mass spectra yielded 405 peptide sequences which were mapped to 214 different *A. niger* genomic locations, of which (6%) were not found to be the best predicted gene model. Consequently, the peptide data were used to modify, or correct, the *in silico* genome annotation process and, in addition, confirm the prediction of 54 intron/exon splice sites. This experimental approach shows how proteomic data can be used to validate fungal genome annotation, intron/exon splice site identification, and mRNA translation.

The secretome of *A. niger*, following culture in different carbon sources, has also been explored using protein fractionation by SDS-PAGE, subsequent LC-MS/MS shotgun proteomics, and interrogation of fungal genomic databases. This work resulted in the identification of 200 secreted proteins which were encoded by genes containing *in silico* predicted signal peptides. Secretome composition was also observed to respond to changes in culture conditions, whereby protease secretion was upregulated under carbon starvation conditions and pectinolytic enzyme secretion increased when galacturonic acid was used as the carbon source. Interestingly, one protein that was quite similar to Asp-hemolysin from *A. fumigatus*, and lacked a signal peptide, was detected in the secretome.

Aspergillus niger is used by the biotechnology industry because of its extensive protein secretion ability, via a process involving intracellular secretory organelles, which greatly facilitates downstream processing and enzyme recovery. Indeed, both transcriptomic and proteomic investigations of the factors affecting protein secretion by this filamentous fungus have been undertaken; however, only discussion of proteomic approaches will be detailed here. Following D-xylose induction of cellulase and hemicellulase secretion, the protein composition of secretory organelles in *A. niger*, compared to sorbitol presence only (i.e. no induction), was investigated. Microsomal membrane fractions, enriched in ER and Golgi component, were isolated by subcellular fractionation, followed by shotgun proteomics involving LC-MS/MS. This led to the identification of over 1,000 proteins which were predicted to partake in protein secretion. Some of the most abundant types of proteins identified included protein disulfide isomerases, mannosyltransferases, chaperones, and GTPases. Interestingly, recruitment of the 20S proteosomal complex to the microsomal fraction was noted only under conditions of D-xylose induction of protein secretion, which suggests involvement of this complex under conditions of induced protein secretion. Since no altered gene expression of 20S proteosomal components was observed, quantitative PCR or microarray analysis could not have been used to reveal this interaction. Thus, this work not only demonstrates a role for the 20S complex in protein secretion, but also clearly shows the essential, and complementary, role of proteomics for elucidating protein–protein interactions, *in vivo*.

Many hypothetical proteins, more correctly called proteins of unknown function, were also detected by LC-MS/MS. Detection of such unknown function proteins both confirms expression of cognate genes and leads to hypotheses that they may play a role in protein secretion. This type of supposition can be further investigated by gene knockout technologies whereby genes of interest can be specifically deleted and mutant phenotypes, in this case with respect to protein secretion, assessed by comparison to wild-type *A. niger*. Any alteration in mutant ability to secrete proteins would contribute to assignment of gene function. Specifically, the quantitative proteomic investigation of *A. niger* wild-type and mutant protein secretion has also been achieved using iTRAQ allied to LC-MS/MS. Although full details of the mutant *A. niger* strain were unavailable, there was a statistically significant increase ($P < 0.001$) in secretion of a range of glycosidases, cellulases, hemicellulases, and a pectinase compared to wild-type cultures. This approach clearly highlights the potential of gene knockout strategies to positively influence global protein secretion, allied to highly sensitive quantitative proteomic analysis, to maximize evaluation of the effect of gene loss on protein secretion.

5.6 Label-Free Quantitative Proteomic Applications

In a very short timeframe, label-free quantitative (LFQ) proteomics has revolutionized the field of fungal proteomics, especially with respect to fungal secretome analysis for both protein identification and determination of relative abundance between comparative scenarios: (1) *Botrytis cinerea*: comparative analysis of different wild-type fungal strains; and (2) *Fusarium graminearum*: assessment of the impact of deficient mycotoxin biosynthetic capacity.

Botrytis cinerea is a plant pathogen, infects over 200 species, and causes major economic losses worldwide. While genome sequencing has contributed to our understanding of this pathogen, more recently, proteomic investigations have begun to illuminate the virulence and pathogenicity arsenal of *B. cinerea*. Comparative proteomics of six wild-type *B. cinerea* strains with different host ranges was analyzed by, among other techniques, LFQ proteomic approaches. Differences in both mycelial and secretome protein profiles between strains were observed whereby 47 and 51 variable proteins, respectively, were identified. Interestingly, secretome-located endopolygalacturonase, aspartic protease, and a cerato-platanin protein exhibited differential abundance between strains. These enzymes are known to be involved in host-tissue invasion and pathogenicity, and so can be classified as virulence factors. It is possible that interference with their activity could attenuate fungal virulence and overcome the pathogenic effects of *B. cinerea*.

Fusarium graminearum is also a plant pathogen, produces the mycotoxin trichothecene deoxynivalenol (DON), and infects many major cereal crops (wheat, barley, and maize). There is limited understanding of the nature of virulence

factors associated with *F. graminearum*. As a consequence, proteomics is at the forefront of investigations into organismal virulence mechanisms, especially as DON contamination of foodstuffs is toxic to both humans and livestock. The secretomes of *F. graminearum* wild-type and two DON-deficient, nonpathogenic mutants were compared using a gel-free LC-MS approach to identify proteins, with relative quantification by spectral counting. Interestingly, proteins exhibiting decreased abundance in DON-deficient mutants included cell wall-degrading enzymes, metabolic enzymes, pathogenesis-related proteins, and unknown function proteins. Thus, these are now identified as potential candidate virulence factors whose presence is associated with DON production.

5.7 Specialized Proteomics Applications in Fungal Research

Three specific proteomics applications, (1) detection of post-translational modifications (PTM), (2) whole protein or "top-down" proteomics, and (3) intact conidial proteomics, merit some additional commentary.

PTM of fungal proteins include, but are not limited to glycosylation, phosphorylation, acetylation, phosphopantetheinylation, ubiquitination, and methylation. While DNA sequence analysis may predict PTM sites or motifs within proteins, direct biochemical analysis of the protein is necessary to confirm that the modification actually occurs *in vivo*. Glycosylation aids protein stability or helps target localization, while phosphorylation is generally a regulatory switch to activate protein–protein interaction and ubiquitination directs proteins for degradation within the cell. Methodologies for detection of these PTM are well described in the scientific literature.

However, protein acetylation and phosphopantetheinylation have emerged as significant PTM of fungal proteins, as the former appears to regulate chromatin activation and hence expression of fungal gene clusters, while the latter is essential for natural product or secondary metabolite biosynthesis (e.g. antibiotics, siderophores, or toxins) via nonribosomal peptide synthesis. Modern mass spectrometers can readily detect acetyl mass (42 Da) within peptides and thereby confirm protein acetylation. Nonribosomal peptide synthetases must be post-translationally activated by a 4′-phosphopantetheinyl transferase (4′-PPTase) activity, by attachment of 4′-phosphopantetheine (from coenzyme A) to specific S residues within the synthetase. Nonribosomal peptide synthetase-derived peptides, containing 4′-phosphopantetheine (358.09 Da), can also be detected by MS, and this approach has been successfully used in *A. fumigatus* proteomics to confirm both synthetase activation and 4′-PPTase activity.

Protein PTM can also be detected using whole-protein MS. This technique is known as Fourier transform ion cyclotron resonance (FT-ICR) MS and is the optimal approach for the analysis of intact proteins because it can reveal subtle differences in mass, associated with PTM, without prior trypsin fragmentation.

FT-ICR MS has been used in fungal proteomics to determine the extent of histone acetylation and deacetylation, and the nature of the amino acids bound to the 4′-phosphopantetheine arms of nonribosomal peptide synthetases for subsequent nonribosomal peptide formation.

Finally, MALDI-ToF MS strategies have been developed using intact conidial proteomic analysis for strain typing, based on analysis of species- or strain-specific mass spectrum patterns observed. Here, fungal conidia are mixed with MALDI-compatible matrices and spotted onto target plates for ionization. The technique, also called MALDI-ToF intact cell MS (MALDI-ToF ICMS), uses matrix material such as 2′,5′-dihydroxybenzoic acid and sinapinic acid to enable specimen ionization. The spectra obtained appear to be specific for particular fungal species (e.g. *Trichoderma*, *Fusarium*, *Penicillium*, or *Aspergillus* spp.) and may also facilitate strain identification (e.g. *Fusarium* spp.). Although many species-specific ions, in the m/z range 1,500–15,000, can be detected by MALDI-ToF ICMS, the nature of the actual compounds is often unknown.

5.8 Conclusion

Fungal proteomics has come of age. Advances in proteomic technologies for individual and large-scale protein identification, allied to the plethora of fungal genome sequences becoming available, have created ideal conditions for the exploration of fungal proteomes. It is clear that fungal proteomes are highly dynamic and demonstrate exquisite responsiveness to the environment in which the organism finds itself. This phenomenon will accelerate our understanding of both fungal biology and disease-causing mechanisms, and yield identification of many new enzymes for biotechnological exploitation.

Acknowledgments

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6
Fungi as Food

Johan Baars

6.1 Introduction

Mushrooms have been used by people since Neolithic times for food, medicinal purposes, as hallucinogenic agents in rituals, or as a means to start a fire (tinder mushroom). For example, the Iceman (popularly named Ötzi), who lived between 3350 and 3100 BC, was found in 1991 in an alpine glacier at the Hauslabjoch and carried three fungal objects. The mushrooms he carried were two differently shaped fruit body pieces of the polypore fungus *Piptoporus betulinus*, each mounted separately on a leather thong, and, found in his girdle bag, a relatively large quantity of tinder material prepared from the "true tinder bracket" *Fomes fomentarius*. The purpose of the other two mushroom objects is much less clear. Researchers suggest that it may have been important to the Iceman for medical–spiritual reasons. The best known use of mushrooms in the western world is as a food material. Explicit mention of fungi as food can be found with ancient Roman and Greek writers. In the East Asian world, mushrooms are known both as food and for their medicinal purposes in traditional Chinese medicine.

Currently, there are at least 1,100 species of mushrooms eaten in more than 80 countries. Most of these are collected in nature and there are nearly a hundred species of fungi for which some kind of cultivation system is known. All of these cultivated species are saprophytes. The first written record on the cultivation of mushrooms is from China. Wang Zeng (AD 1313) in *The Agriculture Book* described the culture of shiitake (*Lentinula edodes*). The cultivation of the button mushroom (*Agaricus bisporus*) was described for the first time in France by Tournefort in 1707. However, for most mushroom species that are used as food, there is no cultivation method even today. Most of the highly appreciated

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mushrooms are mycorrhizal species which live in a symbiotic relation with trees. Especially for such mycorrhizal species, no cultivation methods are available. All cultivated species of mushrooms are either litter degraders (like *A. bisporus*) or degraders of woody materials (like *L. edodes*) and oyster mushroom (*Pleurotus ostreatus*)).

Commercial cultivation of mushrooms is a relatively recent activity and has developed since the 1960s (Figure 6.1). In 2013 the main mushroom producing countries were China, Italy, the United States, the Netherlands, Poland, Spain, and France. Among these countries, China is by far the biggest producer, providing about 70% of the world production (Table 6.1).

The main mushroom species cultivated in China are oyster mushroom (*Pleurotus* species), shiitake (Xianggu by the Chinese name; *Lentinula edodes*), enokitake (*Flammulina edodes*), straw mushroom (*Volvariella volvacea*), wood ear mushroom (*Auricularia* species), and button mushroom (*A. bisporus*). Italy produces 792,000 tonnes of mushrooms and the main crops are button mushrooms (*A. bisporus*) and oyster mushrooms (*P. ostreatus*). Next to this, porcini mushrooms (*Boletus edulis* group) and truffles (*Tuber* species) are collected in nature. In the United States, the main areas that produce mushrooms are Pennsylvania and California and the main species grown is the button mushroom (*A. bisporus*). Next to this, small volumes of shiitake (*L. edodes*) and oyster mushroom (*Pleurotus* species) and even smaller volumes of maitake (*Grifola frondosa*, also called “hen of the woods”), beech mushrooms (*Hypsizygus marmoreus*), enokitake (*Flammulina velutipes*), and pom pom blanc (*Hericium erinaceus*, also called “lion’s mane mushroom”) are produced. The mushroom industries in the Netherlands, Poland, France, Spain, Iran, Canada, and the United Kingdom produce mainly button mushroom (*A. bisporus*), with smaller amounts of oyster mushroom (*Pleurotus* species) and shiitake (*L. edodes*).

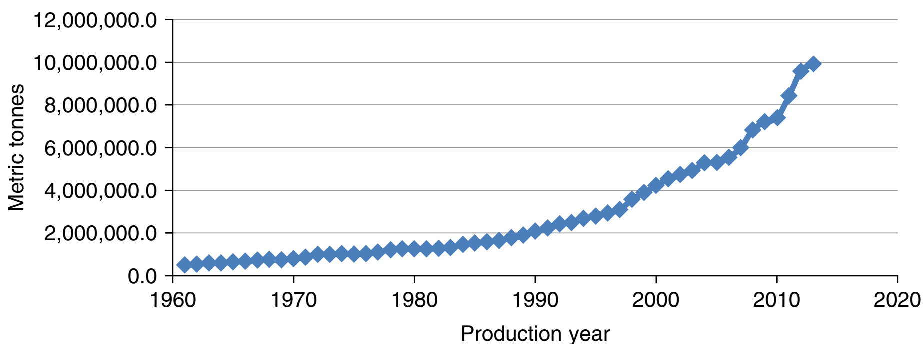

Figure 6.1 Development of world mushroom production. Source: FAOStat.

Table 6.1 Amounts of mushrooms and truffles* produced by the top 10 producers in the world.

Country	Mushroom production in 2013 (tonnes)
China	7,076,842
Italy	792,000
United States	406,198
Netherlands	323,000
Poland	220,000
Spain	149,700
France	104,621
Iran (Islamic Republic of)	87,675
Canada	81,788
United Kingdom	79,500
World (total)	9,935,706

*Truffles are only a very minor portion of the amounts of mushrooms listed in this table.
Source: FAOStat.

6.2 The Main Cultivated Mushroom Species

When considering mushroom production on a global scale, about 85% is believed to be covered by five main species or genera: *A. bisporus* (estimated at 30% of the world's mushroom production), the genus *Pleurotus* (five to six cultivated species at about 27%), *L. edodes* (about 17%), *Auricularia* (about 6%), and *Flammulina* (5%). However, due to incomplete statistics this ranking is not very reliable.

6.2.1 Button Mushroom/Champignon de Paris (*Agaricus bisporus*)

Agaricus bisporus is grown in two varieties, producing either white (Plate 6.1) or brown mushrooms. The mushrooms are marketed under a variety of names. White mushrooms that have not yet opened to spread spores are sold as "closed cups;" when they have matured they are known as flats. The brown varieties of *A. bisporus* are sold by the names of cremini or crimini mushroom. Alternatively

they are called chestnut mushroom. If matured with the cap fully opened they are called portobello. Portobello mushrooms are quite large, with a cap diameter up to 15 cm.

As a crop, *A. bisporus* has a quite narrow genetic base. Virtually all present-day white hybrids are closely related to the first hybrid strains Horst®U1 and Horst®U3. Brown strains used to be genetically less similar. However, currently the market is dominated by a few new strains released to the market in the early 2000s.

Agaricus bisporus is grown in a process involving six sequential steps, involving phase I composting and phase II composting, spawning, and spawn-run, casing, pinning, and cropping. Substrate production starts with the preparation of a mixture of raw materials. In Europe, mainly wheat straw or horse-bedded wheat straw, chicken manure, gypsum, and water are used to prepare mushroom compost. In the United States, also hay, corncobs, cottonseed hulls, or cocoa bean hulls are added to the raw materials.

Conventional phase I composting begins by mixing and wetting the ingredients and stacking them in a large pile for several days to soften. Once the pile is wetted and formed, aerobic fermentation (composting) starts. Heat, ammonia, and carbon dioxide (CO_2) are released as by-products from microbial metabolism. As temperatures increase above 70 °C, microorganisms cease growing and a chemical reaction begins which is believed to make the cellulose and hemicellulose components in the straw more accessible for microbial degradation later in the process. As temperatures rise to 80 °C during phase I composting, any food pathogens that might have been present in the manure will be killed. In Europe, phase I composting is performed in bunkers or tunnels in batches of up to 250 tonnes (Plate 6.2).

Once Phase I is complete, the substrate is filled into a system for phase II composting. Some growers perform phase II composting on the shelves of a growing room, others perform it in a tunnel (Plate 6.2). In northern Europe, phase II composting is performed in bulk in tunnel systems on aerated floors. The first objective of phase II is to pasteurize the composted substrate and kill insects and nematodes that can adversely affect the mushroom crop. These organisms may have survived the high temperatures during phase I composting by hiding in the outer layers of the compost heap which do not become as hot as the inside. In phase II composting, the compost is uniformly heated to 56 °C and kept at this temperature for about 8 hours. The second goal of phase II is to complete the composting process by allowing the development of a thermophilic microflora (thermophilic fungi (especially *Scytalidium thermophilum*), bacteria, and actinomycetes ("firefang")). The thermophilic microflora consumes all freely available carbohydrates from the compost and incorporates gaseous and soluble ammonia into its biomass. At the end of phase II, volatile ammonia should be below 0.05%, as ammonia is toxic to the mushroom mycelium. This usually takes about 5 days in a tunnel system. If phase II composting is performed in a mushroom bed, it may take anywhere from 7 to 18 days, depending on how the air and compost temperatures are managed to control microbial activity.

At the end of phase II composting, the compost has become "selective" for the growth of *A. bisporus*. The next step in the cultivation process is spawning, that is, inoculation of the compost with *A. bisporus* mycelium (spawn). Spawn is produced by specialized companies which inoculate pure cultures of mushroom strains onto sterilized grains. *Agaricus bisporus* is most often inoculated on sterilized rye grains. The grains allow easy seeding of the mycelium into the compost. After cooling the phase II compost to 25 °C, spawn grains are mixed evenly through the compost. After this, the mycelium is allowed to colonize the compost at 25 °C (spawn-run). Relative humidity of the air should be high to minimize drying of the substrate surface. Spawn-run takes 14–17 days in a tunnel system. If spawn-run is performed in mushroom beds, it usually requires 14–21 days. At the end of spawn-run, supplements can be added. Many of these supplements consist of a high-protein oil material, such as soybean meal, cornmeal, or feather meal, which has been treated to delay the availability of the nutrients for the mushroom. Supplements have to be treated to make them hard to degrade. If not treated, bacteria and competitor molds in the compost can use the nutrients in the supplements to outcompete the *A. bisporus* mycelium. Addition of supplements to the compost at the end of spawn-run increases the yield of mushrooms.

After addition of the supplements to the compost, a casing layer of about 5 cm is loaded on top of the compost (Plate 6.3). In Europe and the United States, the casing layer consists of a mixture of sphagnum peat and limestone. Limestone serves to neutralize the low pH of the peat (pH 3.5–4.5) to a value of about 7.5. *Agaricus bisporus*-producing countries that lack a source of peat (Australia, New Zealand) import casing soil from Europe, mainly the Netherlands.

Alternatives for a peat-based casing soil are pasteurized clay loam field soil; reclaimed, weathered, spent compost; and coir fibers. The function of a casing layer is to trigger the mycelium to switch from vegetative growth to the development of mushrooms. The casing also functions as a moisture supply for the mushrooms and their rhizomorphs (thicker threads of mushroom mycelium). Colonization of the casing soil can take 14 days at 25 °C and relative humidity of the air of 93–95%. To shorten the time needed to colonize the casing soil, small amounts of compost are mixed through the casing.

During the colonization of the casing soil, a bacterial population (especially bacteria from the genus *Pseudomonas*) develops near the fungal strands, living on the sugars, amino acids, and volatile compounds that are released by the fungus. These bacteria are important for the fructification of *A. bisporus*. In order to allow *A. bisporus* to produce mushrooms, the growing rooms need to be vented. The mushroom growers gradually lower the air temperature to about 18 °C and the carbon dioxide content in the air to about 1,000–1,200 ppm. To achieve a lower carbon dioxide content in the air, they need to let in fresh air from outside the growing room. The relative humidity is lowered to about 90%. On the rhizomorphs that have developed in the casing soil, small primordia start to develop. From the onset of venting till the harvest of closed mushrooms takes

about 10 days. Especially in the last days before harvest, the mushrooms double in weight every 24 hours.

Mushrooms are harvested over a 2- to 4-day period, and after harvest, new mushrooms start to develop and these will be ready for picking about 7–10 days later. Each crop of mushrooms is called a flush. The first two flushes account for the majority of the total yield. In later flushes, much less mushrooms can be harvested. In most countries, the mushrooms are harvested by hand and are picked at a time before the cap becomes soft. When the cap becomes soft, it is an indication that the mushroom is starting to lose its quality and starts to open up and release spores. In the Netherlands, about 60% of the crop is harvested mechanically. Mushrooms that are harvested mechanically are produced for the canning industry and frozen mushrooms.

6.2.2 Oyster Mushroom (*Pleurotus* Species)

Oyster mushrooms (*Pleurotus* species) are produced throughout the entire world. A number of different species including *P. ostreatus* (Plate 6.4, left), *P. sajor-caju*, *P. cystidiosus*, *P. cornucopiae*, *P. pulmonarius*, *P. tuber-regium*, *P. citrinopileatus* (Plate 6.4, middle), and *Pleurotus flabellatus* (Plate 6.4, right) are all called oyster mushroom. The two main cultivated species are *P. ostreatus* and *P. pulmonarius*. The popularity of oyster mushrooms is partly due to the vast number of agricultural waste streams that can be used as a substrate for their cultivation. They have been reported to grow on most hardwoods, wood by-products such as sawdust, paper, pulp sludge, all the cereal straws, corn and corn cobs, coffee residues such as coffee grounds, hulls, stalks, and leaves, banana fronds, waste cotton, Azolla ferns, bean straw and pods, on *Brassica* crop residues like rape and mustard, on cactus, agave, and yucca, on cardamom pulp, coconut husks, groundnut shells, and water hyacinth, cassava stipes, leaves, and peels, potato foliage, quinoa plants, sesame stems, sunflower peels, and many more.

There are a number of different cultivation systems in use. Large companies, mainly in China, Japan, and Korea, use a bottle system. In this system, bottles of 750–1,250 mL are filled (often mechanically) with a sawdust-based substrate mixed with water and a nitrogen-rich supplement such as rice bran. After being capped, the substrate is sterilized. Either spawn grains or a liquid form of spawn is added to the substrate once it has cooled down to room temperature. Subsequently, spawn-run is performed, which usually takes about 14 days at 25 °C. Once the substrate is fully colonized, the top layer of the substrate is scratched. Initiation of fruit body production depends on carbon dioxide, light, and temperature. Venting involves lowering the temperature of the air in the growing room to 15–18 °C, lowering the amount of carbon dioxide in the air to values below 800 ppm, and providing light to the mycelium. The growth of the fruiting body requires light of 50–500 lux intensity. The color of the caps is closely

related to the intensity of light, and if it is low, then the color will be pale. Also temperature has an effect; with lower temperatures darker mushroom caps develop. Primordia of mushrooms usually appear in a few days. About 8–10 days after venting the mushrooms can be picked.

In Europe, bag systems predominate and most growers use wheat straw as a substrate for oyster mushroom cultivation. The wheat straw is chopped into short pieces (3–4 cm in length) and wetted. After this, the straw is fermented for a few days in order to become thoroughly wetted. During this fermentation the temperature in the straw can rise up to 60 °C. After 3–4 days of fermentation the straw substrate is pasteurized for 8 hours at 70 °C. Once the substrate has cooled down to room temperature, the substrate is inoculated with spawn (either rye grain or millet) and packed into plastic bags with a microperforation (for gas exchange). After a 14-day spawn-run at a substrate temperature of 25–28 °C, slits are made in the plastic. After one day of recovery growth, the bags of substrate are vented as described above.

Oyster mushrooms are known to produce large amounts of basidiospores (between 200 and 660 million spores per gram tissue per 24 hours). These basidiospores are highly allergenic to people and can cause extrinsic allergic alveolitis when inhaled on a regular basis. Assuming that approximately 3 kg of fruit bodies are produced per meter squared of growing surface and the ventilation in a growing room is $10\,m^3/m^2$ of cultivation area, spore concentration in the air might increase to 10^{10} spores/m^3. The minimum amount of basidiospores leading to extrinsic allergic alveolitis is believed to be 10^8 spores/m^3. Because of this occupational hazard associated with the cultivation of oyster mushroom, most growers in the northwest of Europe use a sporeless strain of *P. ostreatus* that has been on the market since 2004.

6.2.3 Shiitake (*Lentinula edodes*)

Shiitake (or Xianggu in Chinese) is produced in large amounts in China, Japan, and Korea. In other parts of the world, this mushroom is also produced commercially, but at much lower volumes. There are mainly two cultivation methods: log cultivation (Plate 6.5) and bag cultivation (Plate 6.6).

Log cultivation involves inoculating tree trunks with the fungus. This traditional method of cultivation has been described in China by Wang Zeng as early as AD 1313 in *The Agriculture Book*. In China, Korea, and Japan, the growers mainly choose oak (*Quercus*) trees for cultivation of *L. edodes*, but this mushroom can be grown on various other hardwood and softwood tree species around the world. The preference for oak originates from the strong type of bark on this tree which allows it to keep its shape for many years while being consumed by the mushroom. As a result, the growers are able to harvest for as long as 4–5 years from this type of log. The logs need to be 5–15 cm in diameter, about 100 cm in length, and dried slightly. After selection, the logs are inoculated

with the mycelium of *L. edodes*. In contrast to *A. bisporus*, there is a wide genetic variation among *L. edodes* strains. Traditionally *L. edodes* strains are classified according to their fruiting temperatures into four groups: fruiting at low (below 5 °C), low to medium (5–10 °C), medium (10–15 °C), and high temperatures (able to fruit at 20–25 °C).

To be able to inoculate, holes of about 2 cm depth are drilled in the log. Mostly the inoculation holes are spaced at 15- to 20-cm intervals along the length of the log, with rows 3–4 cm apart. Either sawdust or wooden plugs colonized with the fungus are inserted into the holes, after which they are plugged (e.g. with Styrofoam plugs or sealing with warm wax). Inoculated logs are arranged in places with suitable humidity, good drainage, and indirect sunlight (e.g. underneath trees or a shading net). Optimal temperature for mycelial growth is 22–26 °C. Direct sunlight must be avoided because this can raise the log temperature too high, causing heat damage to the mycelium. Inoculated logs are stacked and restacked during spawn-run.

The period needed for full colonization of logs by the mycelium depends on temperature, humidity, type of strain, type of spawn, and the properties of the log, and can last between half a year and a year and a half. Once fully colonized, the logs are transferred to another location (a so-called raising yard) and placed in an almost upright position at a small distance from each other. This is usually done at the end of winter in order to allow fruit body production in spring (and again in autumn). In order to initiate mushroom production, the logs are subjected to different forms of shock. Taking the logs apart from each other from the stack creates a temperature shock. Some growers beat the logs, as this is also an effective method to induce fruiting. Other growers soak the logs in cold water at 15–20 °C. Generally, soaking combined with beating induces much more fruit bodies. The mushrooms appear in flushes, but the mycelium needs to rest for at least a month between flushes. A log of 10 litres volume could produce about 2.5 kg of mushrooms in the course of a number of years.

Since 1987, cultivation of *L. edodes* in bags filled with substrate was developed in China. Sawdust is the most popular basal ingredient for shiitake bag cultivation, which is mixed with supplements such as wheat bran, rice bran, millet, rye, and/or maize and minor quantities of calcium carbonate, gypsum, and sometimes table sugar. After mixing the ingredients, water is added to bring the moisture content to about 60%. The substrate is then filled into plastic bags. Some growers use plastic bags with microporous breathing filters, partly filled with the substrate and leaving an air chamber above the substrate (Plate 6.6). Other growers fill the bags completely and close the bag with a ring-neck and a plug. No air space is left in such bags. Growers with advanced equipment sterilize their bags at 121 °C for 2 hours. Growers with less advanced equipment use oil drums to boil water and lead the steam into a steam chamber filled with bags of substrate for prolonged periods of time. After sterilization the substrate is cooled to ambient temperatures and inoculated. In bags with an air chamber,

spawn is mixed with the substrate in the closed bag. In bags without an air chamber, spawn is added in a cavity left in the substrate.

Colonization of the substrate (spawn-run) takes place at 25 °C and lasts for 1–4 months. During the spawn-run period of *L. edodes*, the substrate first becomes white as it is covered by a thick white mycelial coat and subsequently turns brown. Although the mycelium can grow in darkness, light exposure in the first 3 weeks of spawn-run is critical for the browning of the mycelial blocks. Eventually the mycelium forms a dark brown and dry outer surface. At this point, the mycelium is fully matured and ready for induction of fructification. Fruiting can be induced by lowering the temperature, increasing the relative humidity, soaking the substrate block in water (overnight), supplying fresh air through ventilation (removing carbon dioxide), and increasing the light intensity. Once the mushroom primordia are formed, they are allowed to develop into mature mushrooms. Mushrooms are harvested when the caps have not yet fully expanded. After harvest, the mycelium is allowed to rest for 7–10 days. After this, the blocks are soaked overnight to induce a second harvest. Depending on the size of the substrate block, five to six flushes of mushrooms can be harvested. Bag cultivation makes it possible to produce shiitake all year (as opposed to log cultivation).

6.3 The Main Species of Mushroom Collected in Nature

In 2004, the FAO published an overview of the use and importance to people of wild edible mushrooms and stated that wild edible mushrooms are collected and traded in at least 80 countries. A small group of species are of economic importance in terms of exports. Commercial harvesting is an important business in countries such as Zimbabwe, Turkey, Poland, the United States, the Democratic People's Republic of Korea, and Bhutan. The export trade is driven by a strong and expanding demand from Europe and Japan.

Among the 2,500 species of edible fungi recorded, more than 400 are mycorrhizal species (as mentioned earlier, a mycorrhiza is a symbiotic association between a fungus and the roots of a plant). The most valuable species of wild edible mushrooms are ectomycorrhizal, such as truffles (*Tuber* species), porcini mushrooms (*Boletus edulis*), and chanterelles (*Cantharellus cibarius*).

Unfortunately, harvests of many edible mushrooms collected in nature have declined over the past century possibly due to changes in their natural environment caused by various factors such as climate change, deforestation, changed forest management practices, air pollution, soil acidification, and fertilization. For example, *Tuber melanosporum* (Périgord black truffle) harvests have fallen from around 2,000 tonnes in the 1900s to less than 100 tonnes annually. Similarly, current matsutake production in Japan is just 5% of 1940s harvests. This decline has increased the interest in the development of methods for cultivating

mycorrhizal mushrooms. However, cultivation of edible mycorrhizal mushrooms is difficult because of the extremely slow *in vitro* growth rate of the mycelium on nutrient media which causes problems in producing "spawn." A second problem is the absence of fruiting body formation without mycorrhiza formation with a host plant. It is also difficult to sustain a mycorrhizal association with a host plant. Once planted in nature in a "mushroom orchard," the symbiosis between the mycorrhizal fungus and the plant may be lost. And last but not least, there is a lack of information on fruiting mechanisms. As a result, production of mushrooms is a chance process. Currently, only six species of symbiotic edible fungi can actually be successfully produced in the presence of a plant: *Tuber melanosporum*, *Tuber aestivum*, *Tuber borchii*, *Terfezia claveryi*, *Lactarius deliciosus*, and *Lyophyllum shimeji*.

6.3.1 Chanterelle (*Cantharellus cibarius*)

The golden chanterelle and allied species are highly appreciated edible mushrooms. It is a mycorrhizal species which is often associated with trees older than 25 years. It has been attempted to form mycorrhizas with pine seedlings to develop a cultivation system for this mushroom species. However, so far attempts at this have not been successful. Chanterelles (girolles in French) are still collected in nature from June till October in both pine forests and deciduous forests. The mushroom can be found in Asia, North America, Europe, and Australia.

6.3.2 Morels (*Morchella* species)

Morel mushrooms (*Morchella* spp.) include a range of species growing in temperate zones throughout the world. Black (*M. angusticeps*, *M. costata*, and *M. conica*) and yellow (*M. esculenta* and *M. deliciosa*) morels are the most popular edible species. However, morels exhibit considerable morphological diversity and there is much disagreement on the identification of morel species. Morels have long been considered saprobes, although recent evidence indicates that some might be facultatively mycorrhizal.

In the wild, morel fruit bodies are generally found growing in forests for a few weeks in early spring. Morels are known to produce fruit bodies under two distinct ecological environments: undisturbed and disturbed. Stable undisturbed ecosystems produce a limited number of fruit bodies each spring, with production continuing over many years. Disturbed habitats (after wildfire, deforestation, volcanic eruptions, etc.) produce numerous fruiting bodies in the spring following the disturbance, but production rapidly declines over following years. A study on morel productivity in forests located in northeastern Oregon showed yields ranging from 0.5 to 9.1 kg/ha. Productivity was highest in wildfire-burned forests, followed by insect-damaged forests, and lowest in healthy forests.

Claims that morels can be cultivated commercially have been made for more than a century. A method for cultivating morels (*M. esculenta*) was published in 1982 and patented in the years after. Many people have tried, without success, to repeat the cultivation process described in 1982. The patent has been copied, but ascocarp production has been unsuccessful.

In 2016, Chinese researchers reported the commercial cultivation of *M. importuna* and *M. sextelata* in Sichuan province. Their cultivation system produces mushrooms in 80–100 days. Average yields of *M. importuna* and *M. sextelata* were reported to be 1,200 and 1,500 kg/acre, respectively. The maximum yields of *M. importuna* reached 3,048 kg and 3,120 kg/acre for *M. sextelata*.

6.3.3 Truffles (*Tuber* species)

The name "truffle" is used for hypogeous fungi belonging to different genera: *Tuber*, *Terfezia*, *Tirmania*, etc. However, the popular names "truffe" in French, "tartufo" in Italian, or "trafa" in Spanish are only used for fruit bodies of *Tuber*. The first efforts to grow truffles started about 200 years ago when Pierre Mauléon and later Joseph Talon in France independently found that after transplanting seedlings from under a Périgord black truffle-producing tree into a new field, they also produced truffles. This method remained in use in Europe until the early 1970s when a new method of producing truffle mycorrhized plants was developed using spores as inoculum. From that moment on there was a shift from collecting truffles from nature to "growing" truffles in "truffle plantations." At this moment, only *T. melanosporum*, *T. uncinatum*, and *T borchii* are considered as really cultivated. In the year 2000, it was reported that 350,000–400,000 mycorrhized trees were sold annually in France. The other species are still provided from picking in forests where they naturally occur.

Because of the lack of official data, it is difficult to provide accurate figures on European production. There are large variations from one year to the next, mainly depending on weather factors. Annual harvests of *T. melanosporum* range from 40 to 150 tonnes/year at market prices ranging from 300 to 450 €/kg. For *T. magnatum* and *T. uncinatum* the annual harvest ranges from 10 to 30 tonnes at a mean market price, respectively, of over 1,000 €/kg and between 100 and 180 €/kg. For *T. aestivum* the annual harvest ranges from 50 to 100 tonnes at a market price ranging from 50 to 100 €/kg. Next to this, considerable amounts of *T. brumale*, *T. borchii*, and *T. mesentericum* are sold on the markets. Since 1993, truffles have been imported into Europe from China, at 10–20 tonnes/year and sold between 50 and 120 €/kg. Next to this, *T. melanosporum* is emerging as a product in New Zealand. Recently, truffle cultivation (*T. aestivum*) has been introduced in "Upper Galilee" in calcareous soil in a mountainous region.

Tuber melanosporum is the well-known "black truffle" or "Périgord truffle," naturally fruiting in the south of France, northeast Spain, and centre of Italy. The

mushroom is found buried in basic soils (pH over 7.8) in a hot and dry climate, associated with oaks or hazelnut trees. The season for collecting starts in December and ends in March. *Tuber uncinatum* is found more often in the northeast of France (Burgundy truffle) and in the centre of Europe. This species is collected in October and November from oak (or hazel or hornbeam) forests. *Tuber aestivum* (the summer truffle) is a related species which is common throughout Europe and collected from March to October. *Tuber magnatum* is the white Italian truffle and is collected in summer and autumn under oaks, willow, or poplar. The species is only known in Italy. If the cultivation of *T. magnatum* is not really successful, another white truffle is grown in artificial plantations, *T. borchii*, which has a lower value on the market. Competition between cultivated *Tuber* species and truffles of less or no commercial interest is a major problem during the first few years after planting. *Tuber aestivum* and *T. brumale* often replace *T. melanosporum* in French, Italian, and Spanish truffle orchards.

Specialists generally recognize the morphological features of truffles. However, sometimes, identification is unreliable and this is a problem for edible *Tuber* species, especially for the expensive species *T. magnatum* (the "white truffle") and *T. melanosporum*, (the "black truffle"). *Tuber magnatum* has morphological features quite similar to the much less expensive *T. borchii* complex species, grouping "whitish" truffles (*T. borchii*, *T. puberulum*, *T. dryophilum*, and *T. maculatum*). Similarly, *T. melanosporum* shares morphological features with *T. brumale* and *T. indicum*. Therefore molecular tools have been developed for identifying truffles.

6.3.4 Boletes (*Boletus* Species)

Boletes are mostly offered in a dried form to consumers. Boletes are known as porcini mushrooms, cepes, steinpilze, penny bun mushrooms, zhutui mo (pig leg mushroom, north China), and dajiao gu (fat feet mushroom, south China). It is estimated that between 20,000 and 100,000 tonnes are eaten annually worldwide. More than 3,000 tonnes are estimated to be sold in Europe each year. These mushrooms actually form a complex of species, called the *Boletus edulis* species complex. Within this species complex, species delineation is very difficult because it is based exclusively on a few, highly variable morphological features. Using molecular analysis of the internal transcribed spacer of the nuclear rDNA region, it has been possible to discriminate *B. edulis*, *B. aestivalis*, *B. pinophilus*, and *B. aereus*. Also *B. reticulatus* is grouped within this species complex which is collectively known as *B. edulis sensu lato*.

Boletus edulis is found predominantly in the northern hemisphere in an area ranging from the north of Scandinavia to the south of Italy and Morocco, and throughout Asia and North America including Mexico, and in a wide range of habitats. *Boletus edulis sensu lato* form mycorrhizae with a wide range of trees

in a wide range of habitats. Although it does not occur naturally in the southern hemisphere, it has been accidentally introduced to New Zealand and South Africa.

Boletus edulis is collected from forests throughout the world. Yields vary by year, depending on climatic conditions. For a Spanish region, it has been reported that some families collected more than 1,000 kg/year. The mean annual yield per harvester was 500 kg (as much as 40 kg/person/day), with a resulting income of €6,000 annually. Many attempts to cultivate edible boletes have been made. However, a protocol for their commercial production has not yet evolved and the results have been very poor until now.

6.4 Nutritional Value of Mushrooms

The nutritional value of various kinds of mushrooms (along with other foods) is shown in Table 6.2. The nutritional value of mushrooms is closely linked to their moisture content. *Agaricus bisporus* mushrooms have been reported to have moisture contents ranging from 86% to 95% of their fresh weight. This relatively wide range of moisture contents may be attributed to differences in cultivation technique (applying much water or less water), the developmental stage at which the mushroom was analyzed, or differences between mushroom strains. In scientific literature, oyster mushroom (*P. ostreatus*) has been reported to have moisture contents ranging from 85% to 95%. Moisture contents of shiitake mushrooms range from 89% to 95%. As seen in Table 6.2, mushrooms are comparable to vegetables with respect to their moisture content.

Energy content is closely linked to the dry matter content; lower moisture contents (dried corn, meat) are related to higher energy contents. The energy content of mushrooms is comparable to that of vegetables like carrots, cabbage, spinach, and tomatoes.

Crude protein contents of mushrooms vary widely. For *A. bisporus*, protein contents have been reported ranging from 1.6 to 4.5 g per 100 g fresh weight, and for *P. ostreatus*, 0.9 to 4.9 g per 100 g fresh weight. The values for protein content of shiitake mushrooms range from 0.86 to 3.73 g per 100 g fresh weight. Based on dry weight, protein content ranges between 35 and 40%. As a result of this, some researchers consider mushrooms a good source of protein. Next to protein content, also protein digestibility and protein quality are of importance to the nutritional value of mushrooms. Protein quality is linked to the presence of essential amino acids. Essential amino acids are indispensable amino acids that humans cannot synthesize themselves, but have to be taken up from food sources. The essential amino acids are phenylalanine, valine, threonine, tryptophan, methionine, leucine, isoleucine, lysine, and histidine. Levels of essential amino acids in cultivated mushrooms are about 30% of the total amino acid content. In *A. bisporus* mushrooms the levels of threonine, valine, and phenylalanine are comparable to those in meat, while the levels of isoleucine, leucine,

Table 6.2 Proximate composition per 100 g of fresh weight of raw fresh mushrooms compared to other foods.

	Water	Energy	Protein	Total lipid (fat)	Ash	Carbohydrate, by difference	Fiber, total dietary	Sugars, total
	g	kcal	g	g	g	g	g	g
Mushrooms								
White mushrooms	92.45	22	3.09	0.34	0.85	3.26	1	1.98
Oyster mushrooms	89.18	33	3.31	0.41	1.01	6.09	2.3	1.11
Shiitake	89.74	34	2.24	0.49	0.73	6.79	2.5	2.38
Enokitake	88.34	37	2.66	0.29	0.91	7.81	2.7	0.22
Maitake	90.37	31	1.94	0.19	0.53	6.97	2.7	2.07
Morels	89.61	31	3.12	0.57	1.58	5.1	2.8	0.6
Other foods								
Corn grain, yellow (dried)	10.37	365	9.42	4.74	1.2	74.26	7.3	0.64
Hamburger, raw	67.13	198	19.42	12.73	1.71	0	0	0
Soybeans, green, raw	67.5	147	12.95	6.8	1.7	11.05	4.2	No data
Chicken, broilers or fryers, meat only, raw	75.46	119	21.39	3.08	0.96	0	0	0
Potatoes, flesh and skin, raw	79.25	77	2.05	0.09	1.11	17.49	2.1	0.82
Carrots, raw	88.29	41	0.93	0.24	0.97	9.58	2.8	4.74
Cabbage, raw	92.18	25	1.28	0.1	0.64	5.8	2.5	3.2
Spinach, raw	91.4	23	2.86	0.39	1.72	3.63	2.2	0.42
Tomatoes, red, raw	94.52	18	0.88	0.2	0.5	3.89	1.2	2.63
Lettuce, green leaf, raw	94.98	15	1.36	0.15	0.62	2.87	1.3	0.78
Cucumber, with peel, raw	95.23	15	0.65	0.11	0.38	3.63	0.5	1.67

Data from the USDA National Nutrient Database.

lysine, and histidine are somewhat lower. Levels of methionine and cysteine in mushroom proteins are considerably lower than those in meat proteins.

Mushrooms are low in lipids. For *A. bisporus*, *P. ostreatus*, and *L. edodes* various sources report lipid contents ranging below 0.5 g per 100 g fresh weight. The majority of the lipids are fatty acids, for the largest part the polyunsaturated fatty acid linoleic acid. The main saturated fatty acid is palmitic acid.

For *A. bisporus*, carbohydrate contents have been reported ranging from 4.2 to 5.3 g per 100 g fresh weight. The carbohydrate fraction in *A. bisporus* comprises glucans, glycogen, chitin, mono- and disaccharides, and sugar alcohols. Glycogen is a polysaccharide which resembles starch. Different values for the glycogen level are reported and these differences are partly explained by the different extraction techniques used and partly by the developmental stage of the mushrooms that were analyzed. Glycogen contents range from 2 to 4% of dry weight in buds to 5 to 8% of dry weight in fully developed mushrooms (flats). Next to glycogen, the main carbohydrates are mannitol, trehalose, fructose, glucose, and sucrose. Especially mannitol is present in high amounts, up to 10% of the dry matter.

Carbohydrate contents of oyster mushrooms (*P. ostreatus*) range from 5 to 6.7 g per 100 g fresh weight. Compared to *A. bisporus*, the mushrooms of *P. ostreatus* contain far less mannitol and much more trehalose (ranging from 4 to 9.6% of the dry matter) and glycogen (about 11% of the dry matter). Carbohydrate content of shiitake ranges between 3.4 and 11.3 g per 100 g fresh weight. Mannitol is a main carbohydrate in shiitake at levels of 8.4 to 13.4% of the dry matter. Levels of trehalose (2.9% of dry matter) and glucose (1.4–2.8% of dry matter) are much lower. Soluble carbohydrates are metabolized during the post-harvest period and therefore the levels decline with time after harvesting.

Dietary fiber in mushrooms mainly consists of its cell wall components chitin, β-glucans, and heteropolysaccharides (pectins, hemicelluloses, polyuronides, etc.). Cell walls of *A. bisporus* mushrooms contain, on a fresh weight basis, 43% chitin, 14% alkali-soluble glucans, 27% β-glucans, 16% proteins, and 1.5% lipids. As can be seen in Table 6.2, the fiber content of oyster mushroom, shiitake, and the other mushroom species is higher than that of *A. bisporus*. Mushrooms can be regarded as a rich source of dietary fiber.

6.4.1 Mushrooms as a Source of Vitamins

Mushrooms are rich in vitamins from the vitamin B complex (Table 6.3). For an adult, a portion of 100 g of fresh mushrooms contains about 10% of the recommended dietary allowance (RDA) of thiamine, about 30% of the RDA of riboflavin, about 25% of the RDA of niacin and pantothenic acid, 6% of the RDA of pyridoxine and folate, and 2% of the RDA of cobalamin. With respect to vitamins from the vitamin B complex, mushrooms are about as good a source as meat.

Table 6.3 Contents of vitamins in 100 g of raw fresh mushrooms.

		White mushrooms	Oyster mushrooms	Shiitake	Enokitake	Maitake	Morels
Vitamin A (RAE)	μg	0	2	No data	0	0	0
Thiamin (vitamin B1)	mg	0.081	0.125	0.015	0.225	0	0.069
Riboflavin (vitamin B2)	mg	0.402	0.349	0.217	0.2	0.146	0.205
Niacin (vitamin B3)	mg	3.607	4.956	3.877	7.032	0.242	2.252
Pantothenic acid (vitamin B5)	mg	1.497	1.294	1.5	1.35	6.585	0.44
Pyridoxin (vitamin B6)	mg	0.104	0.11	0.293	0.1	0.056	0.136
Folate, total (vitamin B9)	μg	17	38	13	48	0.056	9
Cobalamine (vitamin B12)	μg	0.04	0	No data	0	0	No data
Vitamin C (total ascorbic acid)	mg	2.1	0	No data	0	0	No data
Vitamin D2 (ergocalciferol)	μg	0.2	0.7	0.4	0.1	28.1	5.1
Vitamin D3 (cholecalciferol)	μg	0	0	0	0	0	0
Vitamin E (alpha-tocopherol)	mg	0.01	0	No data	0.01	0.01	No data

Data from the USDA National Nutrient Database.

However, the high contents of vitamins from the vitamin B complex can only be found in fresh mushrooms, canned mushroom contain much lower quantities.

Mushrooms contain low amounts of vitamin D2 which is an essential nutrient for humans. There are two forms of vitamin D: vitamin D3 (cholecalciferol) and vitamin D2 (ergocalciferol). Normally, vitamin D3 is produced in our skin as a result of the UV-B component (~295–315 nm) in sunlight. However, several environmental factors, such as latitude and prevailing weather conditions, determine

whether sunshine of sufficient strength is available to stimulate the production of vitamin D3 in the skin. Many European residents are at risk of not maintaining a healthy vitamin D status all year round. There are only a few dietary sources of vitamin D, all of animal origin (fish like herring, salmon, mackerel, sardines, tuna, eel, cod-liver oil, egg yolks, and liver). It was found that edible mushrooms collected in nature, like chanterelles and porcini mushrooms, contain considerable amounts of vitamin D2 (up to 58 μg per 100 g fresh weight). Cultivated mushrooms contain much lower levels of vitamin D. All mushroom species contain large amounts of ergosterol (tens to hundreds of milligrams per 100 g dry matter). When exposed to UV, ergosterol is converted to provitamin D2, tachysterol, and lumisterol. Provitamin D2 spontaneously converts into vitamin D2. Several research groups have demonstrated that it is possible to increase the content of vitamin D2 in mushrooms by illumination with UV light. In June 2008, Dole Food Company in the United States was the first to market *A. bisporus* mushrooms with an enhanced vitamin D content (more than 100% of the RDA per serving). Currently, also in Europe, vitamin D mushrooms are marketed by Monaghan Mushrooms. In most dietary sources of vitamin D, the vitamin content is associated with a high energy content. Mushrooms with an enhanced vitamin D content provide an opportunity to add dietary vitamin D to a meal without adding much calories.

6.4.2 Mushrooms as a Source of Minerals

The main minerals present in mushrooms are listed in Table 6.4. Mushrooms contain considerable amounts of potassium, phosphorus, copper, and iron, but relatively low amounts of calcium and sodium. In comparison to meat and most vegetables, mushrooms contain a lot of copper. The copper content of *A. bisporus* compares well to that of pulses. The copper content of oyster mushroom and shiitake is a bit lower. The RDA for copper for adults is 0.9 mg. A portion of 100 g of *A. bisporus* mushrooms is able to provide a reasonable amount of the RDA for copper.

The amounts of minerals in commercially cultivated mushrooms are relatively constant. Mushrooms collected in nature, however, show much more variation. Especially minerals like lead, mercury, cesium, selenium, cadmium, and arsenic can be present at high levels in mushrooms collected from nature. Research has shown that mushrooms are able to actively accumulate heavy metals. Oyster mushrooms (*P. ostreatus*) grown on a substrate containing mercury were shown to contain 140 times more mercury in their mushrooms compared to the levels in the substrate. This illustrates the necessity for commercial companies preparing substrates for mushroom cultivation to monitor the levels of heavy metals. Surveys of the heavy metal content of commercially cultivated mushrooms of *A. bisporus*, *P. ostreatus*, and *L. edodes* have shown that their levels of cadmium, lead, mercury, and arsenic are low and comply with EU standards.

Table 6.4 Contents of minerals in 100 g of raw fresh mushrooms and other raw foods.

	Calcium, Ca	Iron, Fe	Magnesium, Mg	Phosphorus, P	Potassium, K	Sodium, Na	Zinc, Zn	Copper, Cu	Manganese, Mn	Selenium, Se
	mg	mg	mg	mg	mg	mg	mg	mg	mg	μg
White mushrooms	3	0.5	9	86	318	5	0.52	0.318	0.047	9.3
Oyster mushrooms	3	1.33	18	120	420	18	0.77	0.244	0.113	2.6
Shiitake	2	0.41	20	112	304	9	1.03	0.142	0.23	5.7
Enokitake	0	1.15	16	105	359	3	0.65	0.107	0.075	2.2
Maitake	1	0.3	10	74	204	1	0.75	0.252	0.059	2.2
Morels	43	12.18	19	194	411	21	2.03	0.625	0.587	2.2
Hamburger	12	1.99	19	175	289	68	4.55	0.063	0.01	14.2
Soybeans	197	3.55	65	194	620	15	0.99	0.128	0.547	1.5
Chicken	12	0.89	25	173	229	77	1.54	0.053	0.019	15.7
Potatoes	12	0.81	23	57	425	6	0.3	0.11	0.153	0.4
Carrots	33	0.3	12	35	320	69	0.24	0.045	0.143	0.1
Cabbage	40	0.47	12	26	170	18	0.18	0.019	0.16	0.3
Spinach	99	2.71	79	49	558	79	0.53	0.130	0.897	1.0
Tomatoes, red	10	0.27	11	24	237	5	0.17	0.059	0.114	0.0
Lettuce	36	0.86	13	29	194	28	0.18	0.029	0.25	0.6
Cucumber with peel	16	0.28	13	24	147	2	0.2	0.041	0.079	0.3

Data from the USDA National Nutrient Database.

Compared to vegetables, mushrooms contain a fair amount of selenium. Selenium content of mushrooms is lower than that in meat. Selenium is an important trace element in the diet, but poisonous at high concentrations. It is part of seleno-proteins like glutathione peroxidase and acts as an antioxidant. Next to this it is involved in the production of active thyroid hormone. Many studies suggest that the body needs selenium for the immune system to work properly. Furthermore, it has a positive influence on male fertility.

Compared to the cultivated mushrooms, mushrooms collected in nature contain higher amounts of selenium. In porcini mushrooms, selenium contents ranging from 5.9 to 37 mg/kg dry matter were found. It is possible to increase the selenium content of *A. bisporus* mushrooms by adding selenium to the water used for spraying the crop. The mushrooms that are produced in this way can satisfy 100% of the RDA for selenium with a single serving of mushrooms. Currently, there are no selenium-enriched mushrooms on the market.

6.5 Potential Medicinal Properties of Mushrooms

There is an increased interest in obtaining medicinal compounds from mushrooms and several molecules have been identified that possibly exert health effects. Most research has been performed on mushroom polysaccharides, more notably on protein–polysaccharide complexes and the β-glucans. Cereals, like oats and barley, are an important dietary source of β-glucans. Mushrooms also contain β-glucans, but they differ structurally from the β-glucans in cereals. Fungal β-glucans contain a backbone of β-(1 → 3) linked glucose residues, which branches with β-(1 → 6) linked glucose residues. The frequency of branching differs between fungal species. For instance, in *G. frondosa* there are two β-(1 → 6) branched glucose residues for every five β-(1 → 3) glucose residues. In *L. edodes* there is one β-(1 → 6) branched glucose residue for every three β-(1 → 3) glucose residues.

The fungal β-glucans are able to modulate the activity of the immune system and exert effects on both the innate and adaptive immune systems. All multicellular organisms possess receptors called "pattern recognition receptors" (PRRS), to detect innately nonself structures (including pathogen-associated molecular patterns, or PAMPs). Thus, fungal β-glucans probably act as PAMPs and are recognized by appropriate cell-surface receptors, initiating immune responses. There is a lot of scientific interest in the use of fungal β-glucans to influence the mode of action of the immune system. One of the applications is the potential use of fungal β-glucans in cancer therapies. The fungal β-glucans lentinan (from *L. edodes*), schizophyllan (from *Schizophyllum commune*), and maitake D-fraction (from *G. frondosa*) are currently used as side treatments in cancer therapies.

Fungal β-glucans also exert an influence on various risk factors collectively associated with "metabolic syndrome," such as high blood pressure, diabetes

type II, and high cholesterol linked with obesity. The pathogenesis of metabolic syndrome is not known yet. Some scientists have hypothesized that the various symptoms of metabolic syndrome are linked together as a result of a systemic inflammation reaction of the innate immune system. In animal models, fungal β-glucans have been shown to lower blood cholesterol concentrations. Fungal β-glucans have also been shown in animal models to reduce blood glucose concentrations after eating.

Nevertheless, most research on the health-promoting effects of mushrooms or mushroom components has been performed using tissue cultures and animal studies. Such studies do not necessarily reflect the effects in humans. When eating mushrooms, the gastro-intestinal system digests and the relevant molecules that are responsible for effects in tissue cultures may be destroyed, or may never reach the relevant cell types in the human body. There have been only limited human trials on the medicinal effects of mushroom consumption. Those that have been carried out to date have primarily been smaller observational studies, or studies without appropriate placebo or other matched controls. Larger, double-blind, placebo-controlled human studies are required before clear effects on human health can be unequivocally demonstrated. In general, the data from scientific tests on medicinal effects of mushroom consumption suggest that the mushrooms and mushroom extracts tested are safe and generally well tolerated. In rare cases, consumption of mushrooms can trigger allergic reactions, as reported for shiitake (*L. edodes*), enokitake (*Flammulina velutipes*), and porcini mushrooms (*B. edulis*).

6.6 Conclusion

There are estimated to be at least 1,100 species of edible mushrooms, possibly even over 2,500, and most of these are collected in nature. Only a very limited number of species are cultivated on an industrial scale: button mushroom (*Agaricus bisporus*), oyster mushroom (mainly *Pleurotus ostreatus* and *Pleurotus pulmonarius*), shiitake mushroom (*Lentinula edodes*), wood ear (*Auricularia auricula-judae* and related species), and enokitake (winter mushroom, *Flammulina velutipes*). All these species are saprophytes. The species for which large quantities are collected in nature are morels (*Morchella* species), truffles (*Tuber* species), porcini mushrooms (*Boletus edulis* species complex), and chanterelles (*Cantharellus cibarius* and related species). Most of these species are mycorrhizal species.

Mushrooms are nutritionally low in energy content, but rich in dietary fibre and vitamins from the vitamin B complex. Mushrooms that are collected outdoors are rich in vitamin D, and mushrooms that are grown commercially can become rich in vitamin D by mild treatment with UV light. Mushrooms that are collected in nature may contain elevated levels of heavy metals, depending on the

location where they were picked. Mushrooms contain substances that potentially have a medicinal effect. Whether consumption of mushrooms actually has a positive effect on human health is a current topic of research.

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Useful Websites

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7

Pharmaceutical and Chemical Commodities from Fungi

Karina A. Horgan and Richard A. Murphy

7.1 Introduction

The economic significance of fungal biotechnology cannot be overstated; indeed, as this chapter will outline, fungi have been exploited to yield a range of valuable products, some of which have proved invaluable to mankind. Since the time of the pharaohs, fungi have been utilized for simple food processing; however, the last century has seen the development of fungal biotechnology for the subsequent production of valuable commodities such as antibiotics, enzymes, vitamins, pharmaceutical compounds, fungicides, plant growth regulators, hormones, and proteins. As we move forward into the twenty-first century, this list will surely expand further, and it is beyond the scope of this chapter to fully appreciate the enormous benefits and economic impact of fungi in the area of biotechnology. Instead, we will concentrate on a number of the more economically significant production processes which have been developed through the utilization of fungi. The diverse natures of some of the economically important products produced by fungi are listed in Table 7.1.

7.2 Fungal Metabolism

A common link between all fungi is their heterotrophic nature; they cannot manufacture their own food and depend on the organic material in other organisms for their survival. In a broad sense, however, it is possible to ascribe fungi

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Table 7.1 Fungal products of economic importance.

Class of product	Typical examples	Industrial/commercial applications	Common production organisms
Enzymes	Amylase	Starch processing Fermentation application	*Aspergillus niger* *Rhizopus oryzae*
	Cellulase	Animal feed industry Brewing	*Trichoderma longibrachiatum*
	Protease	Meat/leather industry Cheese manufacture	*Aspergillus oryzae* *Rhizopus oligosporus*
Organic acid	Citric acid	Soft drinks industry	*Aspergillus niger*
	Itaconic acid	Chemical industry	*Candida/Rhodoturula*
	Malic acid	Beverage/food industry	*Candida*
	Fumaric acid	Food industry	*Candida*
Vitamins	Riboflavin	Health industry	*Candida*
	Pyridoxine	Health industry	*Pichia*
	D-erythro-ascorbic acid	Health industry	*Candida*
Antibiotics	Penicillin	Human/animal health	*Penicillium chrysogenum*
	Cephalosporin	Human/animal health	*Cephalosporium acremonium*
Fatty acids	Stearic	Food industry	*Cryptococcus*
	Dicarboxylic	Chemical industry	*Candida*
Alcohol	Industrial alcohol	Fuel industry	*Saccharomyces*
	Beverage alcohol	Beverage industry	*Saccharomyces*
Pharmaceuticals	Lovastatin	Human health	*Monascus ruber*
	Cyclosporin	Human health	*Tolypocladium inflatum*
Amino acids	Lysine	Health industry	*Saccharomyces*
	Tryptophan	Health industry	*Hansenula*
	Phenylalanine	Health industry	*Rhodoturula*
Recombinant proteins	Insulin	Treatment of diabetes	*Saccharomyces cerevisiae*
	Phytase	Phosphate liberation	*Aspergillus niger*
	Hepatitis B surface antigen	Vaccine preparation	*Saccharomyces cerevisiae*

into two main groups depending on how they obtain and assimilate nutrients. One group, the parasitic and mutualistic symbionts, obtain their nutrients in an effective manner from living organisms. The second group, saprotrophs, have the ability to convert organic matter from dead organisms into the essential nutrients required to support their growth. It is this second group that we are

particularly interested in, as this collective of organisms give rise to the production of the main bulk of the commodities commonly associated with fungi. However, regardless of this division, within the fungal lifecycle one can clearly delineate the production of certain products or metabolites into two phases, namely primary and secondary metabolism.

Primary metabolites are those that are essential for growth to occur and include proteins, carbohydrates, nucleic acids, and lipids. Indeed, the precursors of these primary products must be synthesized if they cannot be obtained from the growth medium. These primary metabolites have essential and obvious roles to play in the growth of the fungus. Typically, primary metabolites are associated with the rapid initial growth phase of the organism and maximal production occurs near the end of this phase. Once the fungus enters the stationary phase of growth, however, primary metabolites may be further metabolized. Examples of primary metabolites produced in abundance include enzymes, fats, alcohol, and organic acids. Economically speaking, primary metabolites are easily exploited, as the biochemical pathways involved in their production are widespread throughout the fungi, with common metabolites occurring in a wide range of fungal organisms. This allows for the rapid screening of classes of fungi for such products and the easy development of production processes for their utilization. Primary metabolic processes have also been extensively usurped through the use of recombinant DNA technologies to the extent that heterologous proteins can be routinely produced by the host fungus as part of its primary metabolic phase.

In contrast to the primary metabolites, secondary metabolites are not essential for vegetative growth and indeed may have little or no primary function within the organism. Secondary metabolites are produced when the organism enters the stationary phase, once the initial phase of rapid growth has declined. The metabolites produced in this phase are often associated with differentiation and sporulation and can have profound biological activities, which in some instances have been exploited economically. A number of distinct differences are apparent between primary and secondary metabolites. First, they have been shown to possess an enormous variety of biosynthetic origins and structures that are not in general found among the primary metabolites. Second, their occurrence tends to be restricted to a small number of organisms and indeed can vary between isolated strains of the same species. Finally, their production is characterized by the generation of groups of closely related compounds which may have very different biological properties.

Important examples of secondary metabolites include medically important compounds such as antibiotics, statins, cyclosporins, and ergot alkaloids. Agriculturally important secondary metabolites include strobilirubin, an antifungal compound, and plant hormones such as gibberellic acid (see Table 7.2).

Fungal biotechnology has developed to allow the utilization of the metabolic processes inherent to the organisms in a commercially viable manner. In this

Table 7.2 Examples of primary and secondary metabolites.

	Examples	Production organisms
Primary metabolites		
	Enzymes	*Aspergillus*
	Industrial alcohol	*Saccharomyces cerevisiae*
	Organic acids	*Aspergillus/Candida*
	Fats	*Candida*
	Polymers	*Yarrowia*
Secondary metabolites		
	Antibiotics: Penicillin	*Penicillium*
	Fusidic acid	*Fusidium coccineum*
	Cholesterol-lowering agents: Lovastatin	*Monascus ruber*
	Mevastatin	*Penicillium citrinum*
	Immunosuppressing drugs: Cyclosporin A	*Tolypocladium inflatum*
	Plant hormones: Gibberellic acid	*Gibberella fujikuroi*

chapter we detail a number of the more important commercial commodities produced by fungi and outline the production processes for them.

7.3 Antibiotic Production

The most well-known and possibly best-studied secondary metabolites are a class of compounds known as antibiotics. These low-molecular-mass compounds are so called because at low concentrations they inhibit the growth of other microorganisms. While many thousands of antibiotics have been discovered, their use has been limited to perhaps 60 at most due to the toxic properties they exhibit towards humans. Clinically speaking, the majority of antibiotics are produced by actinomycetes, a bacterial order, and will not be dealt with here. While several fungal genera produce antibiotics, only two do so to a commercially viable extent and these include *Aspergillus* and *Penicillium*. The β-lactams, of which penicillin is the most infamous not least because of its fortuitous discovery by Fleming in 1928, comprise a very large group of antibiotics and include both the

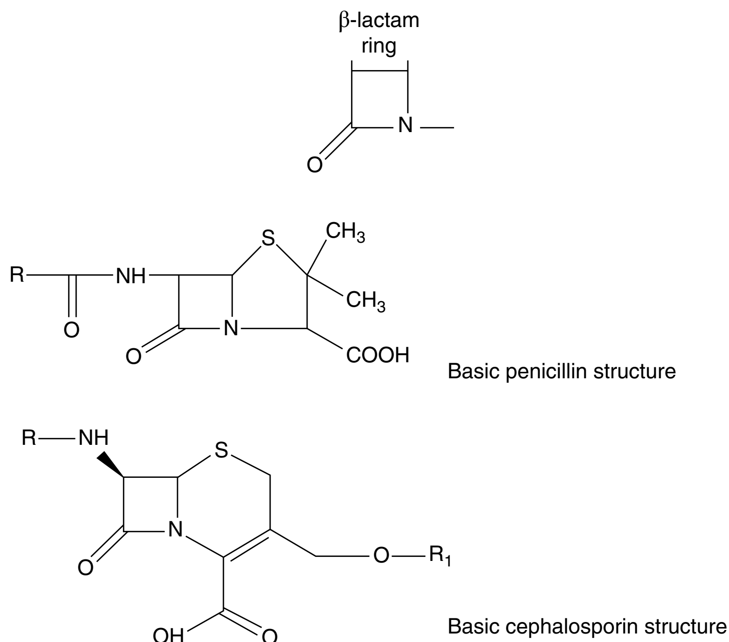

Figure 7.1 Structures of the core β-lactam ring and β-lactam antibiotics.

cephalosporins and penicillins. The global systemic antibiotics market was valued at \$39.6 billion in 2013 and is expected to reach \$41.2 billion by 2018, which underlies the importance both medically and economically of these metabolites.

At their core they all possess a β-lactam (four-atom cyclic amide) ring on which side chain substitutions and differences give rise to a series of antibiotics, each with differing antibacterial activity. In addition to the so-called classical β-lactams, semisynthetic varieties can be manufactured by the removal of the naturally occurring side chains and the subsequent chemical derivatization of the core β-lactam ring (Figure 7.1).

Gram-positive bacteria have on the outside aspect of the cell wall a layer that is composed of characteristic groupings of proteins and carbohydrates that comprise the antigenic determinants responsible for generating an immune response. Inside this outermost layer there is a polymeric structural layer known as peptidoglycan which is composed of repeating units of N-acetylglucosamine (NAG) and N-acetylmuramic acid (NAM). Associated with this cell wall structure are a number of proteins known as penicillin binding proteins (PBP), some of whose functions are as yet unclear. During cell wall biosynthesis, a crosslinking process occurs whereby peptidoglycan strands become linked, leading to the structural stability of the wall. It is this crosslinking that is extremely sensitive to β-lactam antibiotics. For instance, various penicillins bind to the PBPs through their different side chains, leading to a variety of effects. Reaction with PBP-1

(a transpeptidase) produces cell lysis, while binding to PBP-2 (also a transpeptidase) leads to the generation of oval cells which are unable to replicate. Cephalosporins act in a very similar fashion to the penicillins and are also able to react with the PBPs by forming covalent bonds, thus leading to cellular lysis.

Gram-negative cells have a more complex cell wall structure and usually contain an outer membrane and a complex periplasm consisting of lipopolysaccharides. While the gram-negative cell wall also contains a peptidoglycan layer, it is not as extensive as that of gram-positive bacteria, but is sensitive to β-lactam antibiotics due to the presence of PBPs.

The word penicillin can be regarded as a generic term used to describe a large group of natural and semisynthetic antibiotics that differ only by the structure of the side chains on the core aminopenicillanic acid ring. As a rule, the basic penicillin molecule consists of a β-lactam ring, a five-membered thiazolidine ring, and a side chain. β-Lactams with nonpolar side chains such as phenylacetate and phenoxyacetate are hydrophobic in nature and include penicillin G (benzylpenicillin) and penicillin V (methylpenicillin). The nonpolar penicillins are synthesized only by filamentous fungi.

Penicillins with polar side chains, such as D-α-aminoadipate, include penicillin N and possess hydrophilic characteristics. They are more widely synthesized by a range of microorganisms including fungi, actinomycetes, and unicellular bacteria. The production of semisynthetic penicillins is quite easy and involves the removal of the side chain from naturally occurring penicillin and its subsequent replacement with a different side chain to yield a novel β-lactam derivative. Examples of semisynthetic varieties include methicillin and ampicillin (see Figure 7.2).

One serious limitation to the use of penicillins relates to the highly reactive nature of the β-lactam ring which can result in their being susceptible to a variety of degradation processes. Factors that can affect their stability include their reactivity with hydroxide ions which can result in the formation of inactive penicilloic acid, and their acid-sensitive nature which can lead to their degradation at low pH. Acid sensitivity can be overcome clinically by use of the compounds in a buffering solution. A more serious limitation to their use, however, is their susceptibility to a group of enzymes known as penicillinases which are produced by bacteria and can result in the generation of antibiotic resistance. The most common of these enzymes is β-lactamase which cleaves the β-lactam ring and thus inactivates the antibiotic. A variety of acylases have also been identified whose mode of action is to cleave the acylamino side chain of the antibiotic, thus rendering them inactive. To combat these enzymes a number of compounds such as clavulinic acid or the sublactams have been developed which when given in combination with the susceptible antibiotic result in the permanent inactivation of the antibiotic-degrading enzymes. Of more importance, however, has been the development of the semisynthetic penicillins, many of which are resistant to β-lactamase and other penicillinases. For instance, methicillin is completely resistant to these enzymes, though it does have the

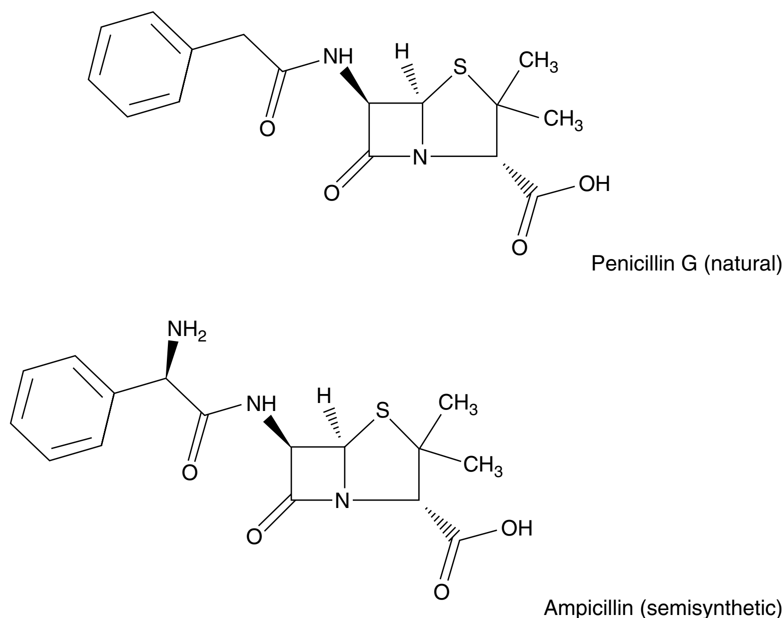

Figure 7.2 Natural and semisynthetic penicillins.

disadvantage that it is less effective. Almost all β-lactamase-resistant penicillins are less potent than the parent molecules.

Cephalosporins are very closely related to the penicillins and indeed were initially discovered soon after; however, unlike the penicillins, their use was limited for a long period until a clinically useful agent was found. Cephalosporin C is regarded as the prototypical cephalosporin, and following its structural elucidation it was found to be a β-lactam with a six-membered dihydrothiazine ring instead of the five-membered thiazolidine ring characteristic of the penicillins. Chemical removal of the side chain of cephalosporin C results in the generation of 7-aminocephalosporonic acid (7-ACA), which can be used as a synthetic starting point for most of the cephalosporins available today. Indeed, it is more economically feasible to produce 7-ACA from penicillin G by a series of synthesis reactions rather than to incur the prohibitive costs of fermentation to produce the antibiotic.

One notable feature of the structure of the cephalosporins is their reduced chemical reactivity relative to the penicillins. However, some β-lactamases are more efficient at cleaving cephalosporins than penicillins and this has led to the development of second, third, and fourth generation cephalosporins. These compounds all differ in their antimicrobial properties, susceptibility to microbial resistance, absorption, metabolism, and side effects. Examples of first generation cephalosporins include cephalothin and cefazolin, second generation cephalosporins include cefamandole and cefachlor, third generation cephalosporins include cefotaxime and cefixime, and fourth generation examples include cefapime (Figure 7.3).

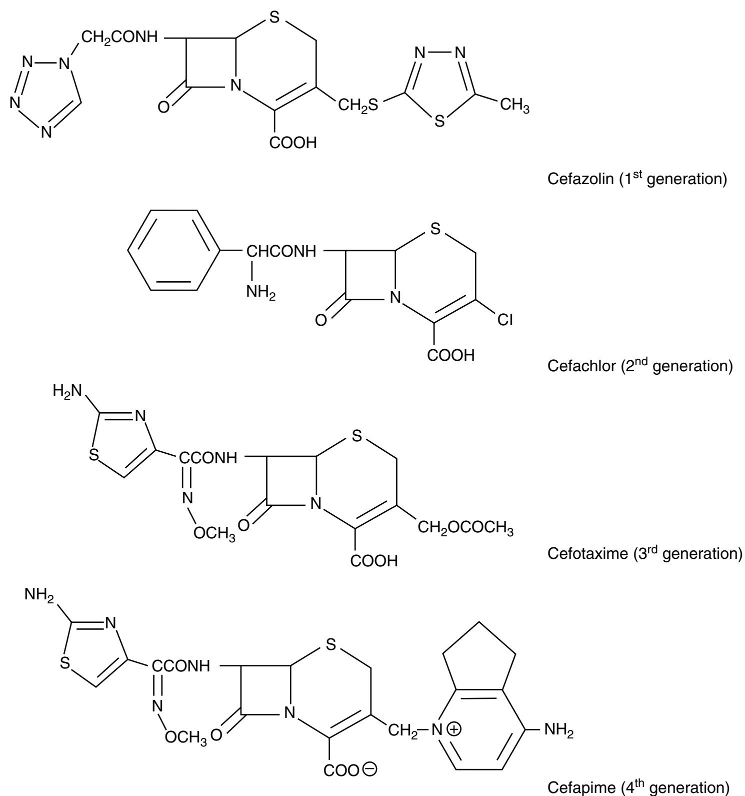

Figure 7.3 Cephalosporin structures.

7.3.1 Antibiotic Production Cycles

At a cellular level, the production pathways for the cephalosporins and penicillins share some similarities, and indeed the first two steps are common to both classes of antibiotic. Initially, a tripeptide known as ACV is formed from the amino acids L-cysteine, L-α-aminoadipic acid, and L-valine. This key intermediate is then converted to isopenicillin N (IPN) by the enzyme IPN synthase. It is this intermediate that gives rise to both the penicillins and the cephalosporins. In the case of penicillin formation, IPN is hydrolyzed to 6-amino penicillanic acid (6-APA), which can be used subsequently to give rise to specific penicillins. Alternatively, in the formation of the cephalosporins, IPN is epimerized to penicillin N, which is further reacted enzymatically to yield deoxycephalosporin C. This last molecule can then undergo further modification to give rise to cephalosporin C and cephamycin C.

As discussed earlier, the generation of semisynthetic varieties of penicillins and cephalosporins is a simple process. By reacting the core penicillin compound 6-APA with a variety of organic acids, numerous penicillins can be duly formed. Indeed, the production of 6-APA is now carried out through the removal of the

side chain from penicillin G which is then reacted to yield a range of antibiotics. Similarly, the removal of the side chain from cephalosporin C to yield 7-amino cephalosporonic acid (7-ACA) can lead to the generation of numerous cephalosporins through the reaction of this compound with a variety of acids.

7.3.2 Industrial Production of Antibiotics

Penicillin production industrially is a relatively inefficient process, where it is estimated that only 10% of the carbon source utilized in the fermentation ends up as antibiotic. Production of β-lactam antibiotics occurs best under conditions of carbon, nitrogen, and phosphorus limitation and at low growth rates. Each manufacturer uses a different production process, the details of which are closely guarded. Overall, though, the basics of the production process are similar in nature. Production starts with the inoculation of a primary culture from a preserved culture stock. Typically, the culture stock can be in the form of lyophilized spores or spores preserved in liquid nitrogen. Numerous other preservation methods exist and will not be outlined here.

Primary culturing can utilize either agar slants or liquid culture, with agar slants being the most common. The primary culture is then used to inoculate a secondary culture, which in the case of antibiotic production is aimed at the generation of spores. Secondary culturing can take place in agar-coated bottles or on particulate material, both of which result in the generation of a large quantity of spores.

A spore suspension prepared from the secondary culture is subsequently used to inoculate liquid media as part of an inoculum build-up process. It should be pointed out that stringent aseptic techniques are used throughout the process to prevent the contamination of the antibiotic-producing culture with a more robust microorganism. Industrial strains of antibiotic-producing fungi are less robust than naturally occurring fungi due to the aggressive mutation and selection pressures placed on them when they were originally isolated.

Depending on the size of the process, the scale-up procedure can have as many as three or four stages. Typically, the initial seed culture produced from the secondary spore suspension is less than 10 liters. Following a defined period of growth, this can be used to inoculate a culture of less than 20,000 liters, which in the final stages of the production cycle will serve to start a culture of up to 300,000 liters. One important point is the nature of the product that the production cycle is centered on. Antibiotics are secondary metabolites and in order to obtain the maximum productivity from the final stage culture, it is necessary to ensure that growth of the organism is limited and the organism enters its secondary metabolism phase. This is usually achieved by designing the growth medium to ensure that a key nutrient becomes limiting at the right time to effect the change in metabolism necessary for antibiotic production. In the case of penicillin production this is usually achieved by limiting the supply of glucose.

At the end of the fermentation it is necessary to separate the antibiotic material from the fungal mycelia, medium constituents, and any other metabolites produced during the process. This is known as downstream processing and the types of steps involved will depend on the antibiotic in production and also on the production process. Typically, it will involve some form of centrifugation or filtration to remove the fungal biomass, and additional steps such as solvent extraction, ultrafiltration, chromatography, and drying to produce a relatively pure antibiotic which can then be used for the manufacture of pharmaceutical preparations. It is estimated that over 10,000 tonnes of penicillin G alone are produced by fermentation each year.

7.3.3 Additional Fungal Antibiotics

Fungi also produce a number of other antibiotics, which are structurally unrelated to the β-lactams. Griseofulvin, a natural organic compound containing chlorine, is produced by *Penicillium griseofulvin*. This compound is interesting as it inhibits the growth of fungi by preventing the assembly of fungal microtubules and thus mitosis. Another unrelated antibiotic is the steroidal compound fusidic acid which is produced by *Fusidium coccineum*. This antibiotic is active against gram-positive bacteria and has clinical use against β-lactam-resistant strains of bacteria.

7.4 Pharmacologically Active Compounds

In addition to antibiotics, fungi, as illustrated in Table 7.2, produce a range of other secondary metabolites. Some of these compounds are very significant in terms of their medical importance, including cyclosporin A and a group of compounds with cholesterol-lowering properties known as statins. Other compounds, which will be discussed, include the alkaloids and the gibberellins.

7.4.1 Cyclosporin A

Immunosuppressive drugs have transformed modern transplant surgery by vastly reducing the incidence of organ rejection. The discovery and exploitation of the powerful immunosuppressant cyclosporin A has relied almost completely on fungal biotechnology. Indeed, attempts at chemically synthesizing the drug have served to illustrate the complexity of fungal secondary metabolism. Cyclosporin A is produced by the fungus *Tolypocladium inflatum* and was initially isolated from a Norwegian soil sample. The compound inhibits the production of interleukin-2 by T-lymphocytes and in so doing inhibits any potential immune response stimulated by antigens produced against transplanted organs. Cyclosporin

A has also found use in the treatment of medical conditions such as psoriasis and eczema, due to the role of interleukin-2 in mediating inflammatory responses.

The structure of cyclosporin A has shown it to be a heavily methylated cyclic peptide. In a similar fashion to other secondary metabolites, a range of over 25 cyclosporin analogs are produced by *T. inflatum* and while 17 have antifungal activity only two are immunosuppressants. Following a series of strain improvements using mutagenesis and culture optimizations, gram-quantity yields per liter have been achieved under optimized fermentation conditions. Despite the best efforts at chemical synthesis of the drug, production of cyclosporin A is still only economically feasible by natural means.

7.4.2 Statins

The so-called statins are a group of compounds that act as potent competitive inhibitors of 3-HMG-CoA-reductase, a key enzyme in the biosynthesis of cholesterol. These organic acids interact with the enzyme through their acidic side groups and in doing so effect a reduction in plasma cholesterol levels. The most important statins commercially are the mevinic acids, with the most notable being lovastatin from *Monascus ruber* and mevastatin from *Penicillium citrinum*. Both mevastatin and lovastatin can be converted into the compounds ML-236A and monacolin J, respectively, by chemical means or by microbial transformation. Each of these compounds differs in its affinity for 3-HMG-CoA-reductase and thus in its effectiveness. Research and development since the 1990s has shown that a number of fungi produce a range of similar compounds with cholesterol-lowering effects. One interesting aspect of their commercial production lies in the ability to produce these compounds by liquid fermentation (e.g. lovastatin from *Aspergillus terreus*) or by using solid-state fermentation, the so-called Koji process (e.g. lovastatin from *M. ruber*).

7.4.3 Alkaloids

Members of the genus *Claviceps*, a parasitic fungus which grows on a wide variety of grains, synthesize numerous secondary metabolites known as alkaloids. These compounds are produced in the sclerotia of the fungus, the resting structure with which the fungus ensures its survival over winter. Many of these alkaloids are pharmaceutically important and can act in a variety of ways, but they particularly affect the central nervous system, causing hallucinations or convulsions. The common core of these compounds is the tetracyclic alkaloid ring structure (ergoline nucleus) in which the nitrogen atom at position 6 is usually methylated. This core structure is derived from tryptophan and mevalonic acid and can be modified with varying degrees of complexity to give rise to a multitude of alkaloids, each differing in their potency and toxicity. These derivatives

can then be used in the chemical synthesis of additional pharmacologically active compounds. For instance, lysergic acid diethylamide (LSD) is synthetically derived from lysergic acid, an alkaloid produced by *Claviceps paspali*. Medically speaking, many of these alkaloids are important due to their negative effects; indeed, alkaloid-contaminated bread caused numerous outbreaks of ergotism until the eighteenth century. There is also evidence to suggest that the Salem witch trials were brought about following outbreaks of ergotism.

Medically useful alkaloids have been isolated and the most useful of these are the alkaloids ergometrine and its methylated derivative methylergometrine. Both of these compounds stimulate contractions of uterine smooth muscle and can also be used as vasoconstrictors to control excessive bleeding after childbirth.

7.4.4 Gibberellins

The gibberellins are a group of diterpenoid compounds containing 19 or 20 carbon atoms and are capable of promoting numerous developmental processes in plants. Examples of effects that may be mediated by these compounds include the induction of bolting, production of hydrolytic enzymes, and stimulation of both cell elongation and cell division. These secondary metabolites are biosynthesized from mevalonic acid by *Gibberella fujikuroi*, though they have also been isolated from *Sphaceloma manihoticola*, *Neurospora crassa*, *Rhizobium phaseoli*, and *Azospirillium lipoferrum*. They have numerous agricultural applications, including their use in malting, fruit ripening, and improving fruit set and size. On an industrial scale, the most important production organism is *G. fujikuroi*, from which mutated strains secrete gibberellins at gram per liter quantities.

7.4.5 Endophytic Compounds

The development of drug resistance in infectious microorganisms (e.g. species of *Staphylococcus*, *Mycobacterium*, and *Streptococcus*) to existing antibiotic compounds has led to a requirement for new chemotherapeutic agents that are highly effective, possess low toxicity, and have a minor environmental impact. In the continual search by both pharmaceutical and agricultural industries for new products, natural selection has been found to be superior to synthetic chemistry for discovering novel substances that have the potential to be developed into new industrial products. Since natural products are adapted to a specific function in nature, the search for novel secondary metabolites should concentrate on organisms that inhabit novel ecosystems.

In the past few decades, plant scientists have begun to realize that plants may serve as a reservoir of untold numbers of organisms known as endophytes. Endophytic fungi, a polyphyletic group of highly diverse, primarily ascomycetous

fungi that are defined functionally by their occurrence within tissues of plants without causing any immediate negative effects are found in liverworts, hornworts, mosses, lycophytes, equisetopsids, ferns, and seed plants from the arctic tundra to the tropics. Once inside their host plant, endophytes usually assume a dormant state either for the whole lifetime of the infected plant tissue or for an extended period of time, that is, until environmental conditions are favorable for the fungus or until the ontogenetic state of the host changes to the advantage of the fungus which may then turn pathogenic. Colonization of host plants by endophytic fungi is believed to contribute to the host plant an adaptation to biotic and abiotic stress factors. It is of special interest that in many cases host plant tolerance to biotic stress has been correlated with fungal natural products.

Although work on the utilization of this vast resource of poorly understood microorganisms has been initiated, the enormous potential for the discovery of a wide range of extremely beneficial products holds exciting promise. This is witnessed by the discovery of a wide range of products and microorganisms (Table 7.3). There are other characteristics of endophytic fungi that also render them desirable for manipulation in an industrial screening program. Most screening has focused on soil-dwelling fungi; little attention has been directed toward endophytes. As a consequence, they have not been subjected to intensive screening programs, which suggests the majority remain largely undiscovered.

An array of natural products has been characterized from endophytes, which include anticancerous, antifungal, antibacterial, antiviral, and anti-insecticidal products, and immunosuppressants. The discovery of the paclitaxel (Taxol®)-producing endophytic fungus *Taxomyces andreanae* from the western yew plant *Taxus brevifolia* has led to a more comprehensive examination of other *Taxus* species and plants for the presence of paclitaxel-producing endophytes. Paclitaxel, a multibillion dollar anticancer compound, has activity against a broad band of tumor types, including breast, ovarian, lung, head, and neck cancer. Paclitaxel, a tetracyclic diterpene lactam (shown in Figure 7.4), was first isolated from the bark, roots, and branches of *T. brevifolia* in the late 1960s. The main natural source of paclitaxel is found in the bark, where it exists in low concentrations of 0.01–0.05%; however, *Taxus* species are endangered and grow very slowly, and traditional methods of extracting paclitaxel from the bark are inefficient and environmentally costly. Microbial fermentation has demonstrated that the isolation and identification of paclitaxel-producing endophytic fungi is a new and feasible approach to the production of Taxol. The development and utilization of these fungi have brought about significant progress worldwide. For industrial production purposes, optimization of fermentation conditions is necessary to increase the yields of paclitaxel-producing fungi. In addition to reducing costs and increasing yields, fungal fermentation as a way of producing Taxol is beneficial as it protects natural *Taxus* tree resources. Taxol produced by fungi using large-scale industrial fermentation has attractive development prospects, and it will have enormous market and social benefits.

Table 7.3 Isolated endophytic fungi and their bioactive products.

Endophyte	Host plant	Metabolite	Bioactivity
Colleotrichum gloeosporiodes	*Artemisia mongolica*	Colletotric acid	Antimicrobial
Muscodor roseus	*Erythophelum chlorosachys*	Volatile antibiotic	Antimicrobial
Muscodor albus	*Cinnamomum zeylanicum*	1-Butanol, 3-methylacetate	Antimicrobial
Phoma spp.	*Taxus wallachina*	2-Hydroxy-6-methylbenzoic acid	Antibacterial
Fusarium spp.	*Selaginella pallescens*	CR377	Antifungal
Cryptosporiopsis quercina	*Tripterigeum wilfordii*	Cryptocandin	Antifungal
Nodulisporium spp.	*Bontia daphnoides L.*	Nodulisporic acids	Anti-insecticidal
Semiatoantlerium tepuiense	*Maguireothamnus speiosus*	Taxol®	Anticancer
Taxomyces andreanae	*Taxus brevifolia Nutt.*	Taxol®	Anticancer
Tubercularia spp.	*Taxus mairie*	Taxol®	Anticancer
Pestalotiopsis microspora	*Taxus wallachina*	Taxol®	Anticancer
Sporomia minima	*Taxus wallachina*	Paclitaxel	Anticancer
Rhinocladiella spp.	*Tripterygium wilfordii*	22-Oxa-[12]-cytochalasin	Antitumor
Pestalotiopsis guepinii	*Wollemia nobilis*	Taxol®	Antitumor
Fusarium subglutinans	*Tripterygium wilfordii*	Subglutinols A and B	Immunosuppressive

Adapted from Tejesvi, M.V. et al. (2007).

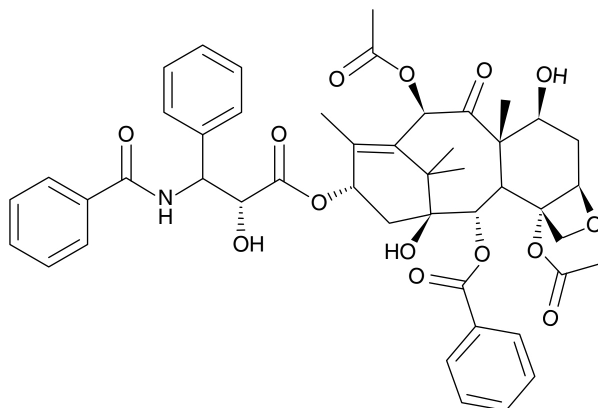

Figure 7.4 Chemical structure of paclitaxel.

Natural products from endophytic microbes have been observed to inhibit or kill a wide variety of harmful disease-causing agents, including, but not limited to, phytopathogens, as well as bacteria, fungi, viruses, and protozoans that affect humans and animals. *Cryptosporiopsis quercina*, a fungus commonly associated with hardwood species in Europe, has been isolated as an endophyte from *Tripterigeum wilfordii*, a medicinal plant native to Eurasia. On Petri plates, *C. quercina* demonstrates excellent antifungal activity against some important human fungal pathogens, for example *Candida albicans* and *Trichophyton* species. Since infections caused by fungi are a growing health problem, especially among AIDS patients and those who are otherwise immunocompromised, new antimycotics are needed to combat this problem. A unique peptide antimycotic, termed cryptocandin, has been isolated and characterized from *C. quercina*. This compound contains a number of hydroxylated amino acids and a novel amino acid (3-hydroxy-4-hydroxy methylproline). The bioactive compound is related to known antimycotics such as the echinocandins and the pneumocandins. Cryptocandin is also active against a number of plant pathogenic fungi, including *Sclerotinia sclerotiorum* and *Botrytis cinerea*, and is currently being tested and developed by several companies for use against a number of fungi causing diseases of skin and nails.

Endophytes produce substances that can influence the immune system of animals. Subglutinols A and B are immunosuppressive compounds produced by *Fusarium subglutinans*, an endophyte of *T. wilfordii*. The compounds both have IC50 values of 0.1 μM in the mixed lymphocyte reaction assay. In the same assay, cyclosporin is roughly as potent as the subglutinols. These compounds are being examined more thoroughly as immunosuppressive agents. Their role in the endophyte and its relationship to the plant are unknown.

In the continuous search for novel drug sources, endophytic fungi have proven to be a largely untapped reservoir of bioactive products, with great chemical diversity. These compounds have been optimized by evolutionary, ecological,

and environmental factors. The development of drugs from endophytes with high potency will offer much needed new remedies for acute and chronic human diseases. As so many bioactive compounds have been isolated from endophytes which only occupy a small portion of total endophyte species, it is obvious that there is a great opportunity to find reliable and novel bioactive natural products in endophytes, which may be used as clinically effective compounds in the future.

7.5 Chemical Commodities

Several industrially important chemicals are produced via biological processes using molds and yeasts. In terms of world production volume, the most important of these are citric acid. From 1978 to 1984, the average rate of increase of total consumption of citric acid in western countries was about 3.5–6% per year.

7.5.1 Citric Acid

Citric acid is the principal organic acid found in citrus fruit. To meet with increasing demands, it is produced from carbohydrate feedstock by fermentation with the fungus *Aspergillus niger* and the yeast *Candida*. The initial commercial production of citric acid was achieved using *A. niger* in a surface fermentation process. The development of the process of submerged fermentation in the 1950s was a major turning point in citric acid production. Citric acid's main use is as an acidulant in soft drinks and confectionery. A more recent application of citric acid is as a metal complexing agent, to reduce oxidative metal deterioration, and for metal cleaning. With the increasing requirement for citric acid, its production by fermentation is increasing continually, with about 500,000 tonnes of it being produced annually.

A number of fungi and yeasts have been used over the years for the production of citric acid, but *A. niger* remains the preferred fermentation organism for commercial production. The main advantages of using this organism are its ease of handling, its ability to ferment a wide variety of cheap raw materials, and high yields.

A variety of raw materials such as molasses, starchy materials, and hydrocarbons have been employed as substrate for the production of citric acid. Sucrose, cane molasses, or purified glucose syrup from maize are sometimes used, according to availability and price. Molasses has been acclaimed as a low-cost raw material and it contains 40–55% of sugars in the form of sucrose, glucose, and fructose. There are considerable variations in the culture conditions reported in the literature for citric acid production by *A. niger*. To ensure high productivity it is essential that the media contain major nutrients such as carbon, nitrogen, and phosphorus, and also trace elements. The fermentation process is also influenced by aeration temperature and pH.

The use of different carbon sources has been shown to have a marked effect on yields of citric acid by *A. niger*. *Aspergillus niger* can rapidly take up simple sugars such as glucose and fructose. Sucrose is usually the sugar of choice; at industrial scale the fungus possesses an extracellular mycelial bound invertase, which under the acidic conditions of citric acid fermentation hydrolyzes sucrose to its monomers. A sugar concentration of 14–22% is considered the optimal level for maximum production yields. Lower sugar concentrations lead to lower yields of citric acid as well as the accumulation of oxalic acid.

The nitrogen sources for citric acid production by *A. niger* are generally ammonium sulfate, ammonium nitrate, sodium nitrate, potassium nitrate, and urea. The presence of phosphorus in the fermentation medium has a profound effect on the production of citric acid. Too high a level of phosphorus promotes more growth and less acid production. Potassium dihydrogen phosphate (0.1%) has been reported to be the most suitable phosphorus source. Maintenance of a low pH is essential for production; generally, a pH below 2 is required for optimal fermentation. Citric acid fermentation is an aerobic process and increased aeration rates have resulted in enhanced yields and reduced fermentation times. Trace elements are also a major factor in the yields obtained in citric acid fermentation. When trace elements are growth limiting, citric acid accumulates in larger quantities.

7.5.1.1 Production of Citric Acid by Filamentous Fungi

A number of different fermentation processes exist for the production of citric acid, which are outlined below.

7.5.1.1.1 Surface Fermentation

Surface culturing was the first process employed for the large-scale production of microbial citric acid. Despite the fact that more sophisticated fermentation methods (submerged process) have been developed, surface culturing techniques are still employed, as they are simple to operate and install. Another advantage of this culturing method is that energy costs for surface fermentation are lower than those of submerged fermentation. The mycelium is grown as a surface mat in shallow 50- to 100-liter stainless-steel or aluminum trays. The trays are stacked in stable racks in an almost aseptic fermentation chamber.

The carbohydrate source (usually molasses) for the fermentation medium is diluted to 15% sugars, the pH is adjusted to 5–7, and any required pre-treatment is carried out. After the addition of the nutrients the medium is sterilized, cooled, and pumped into the trays. Inoculation is performed by introducing spores, either by generating a spore suspension or by blowing spores over the surface of the trays along with air. Spores subsequently germinate and form a mycelial mat. The temperature is maintained at 28–30 °C and the relative humidity between 40 and 60%.

During fermentation considerable heat is generated, necessitating high aeration rates. Air provides oxygen to the organism and also controls the fermentation temperature and the relative humidity. As the fermentation progresses the pH decreases to below 2.0. If the fermentation pH rises to 3.0, oxalic acid and gluconic acid may be formed in considerable amounts. Fermentation progresses for 8–12 days, after which time the fermented liquid is poured out of the pans and separated from the mycelium for further processing. Fermentation yields are in the range of 70–75%.

7.5.1.1.2 Submerged Fermentation

Submerged fermentation is now more popular for the commercial production of citric acid. It requires less space, is less labor intensive, and higher production rates are obtained. With submerged fermentation a stirred tank reactor or a tower fermenter may be used (Figure 7.5).

In view of the low pH level that develops during fermentation and the fact that citric acid is corrosive, the use of acid-resistant bioreactors is desirable. An important consideration with bioreactors designed for citric acid production is the provision of an aeration system, which can maintain a high dissolved oxygen level. With both types of bioreactors, sterile air is sparged from the base, although additional inputs are often used in tower fermenters.

The medium preparation in submerged fermentations involves appropriate dilution of the carbon source, pre-treatment addition of the appropriate nutrients, and sterilization in line or in the bioreactor. Inoculation is performed by the addition of either a suspension of spores or pre-cultivated mycelia. When spores

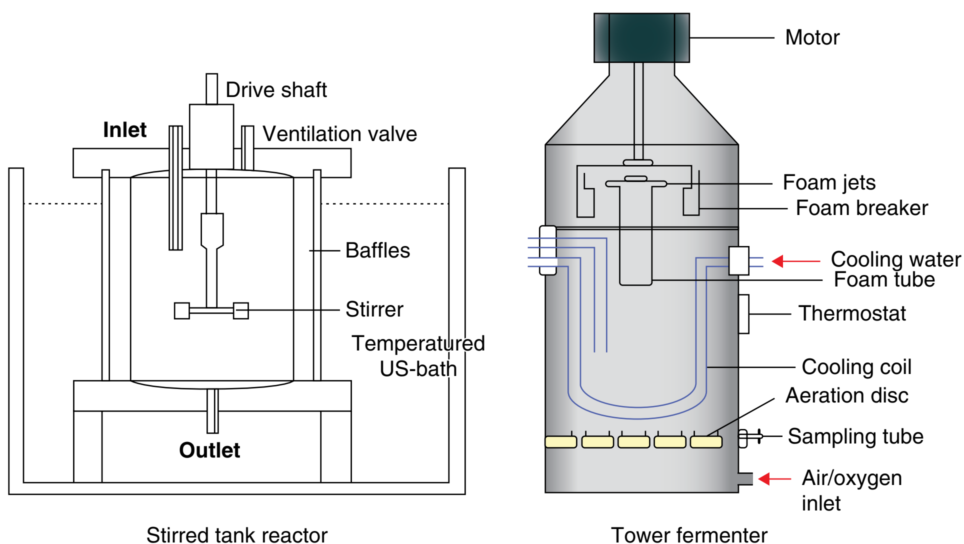

Figure 7.5 Types of reactor for citric acid production.

are used they need to be dispersed in the medium, and therefore addition of a surfactant is usually necessary. With pre-cultivated mycelia the inoculum size is usually about 10% of the fresh medium. Air is sparged through the medium at a rate of 0.5–1.5 VVM throughout the fermentation. Under optimal conditions fermentation is completed in 5–10 days. Submerged fermentation can be performed by continuous and fed batch modes, but generally it is carried out by the batch feed mode.

7.5.1.1.3 Solid-State Fermentation

The Koji process or solid-state fermentation, developed in Japan, is the simplest process for production of citric acid. This process is the solid-state equivalent of surface fermentation. The raw materials used are sweet potato fibrous residues, rice or wheat bran, and fruit wastes. The carbohydrate source is moistened with water to about 70% moisture. The moist carbohydrate is then steamed for sterilization, placed in trays, and inoculated using conidia of *A. niger*. The pH at the start of fermentation is 5.5. The starch is hydrolyzed by amylase produced by the fungus and subsequently converted to citric acid. The fermentation is complete in 4–5 days. The main problem with this process is the presence of trace elements, which cannot be removed by standard methods.

7.5.1.2 Production of Citric Acid by Yeast

Yeasts are also employed in the commercial production of citric acid from various carbon sources. Yeast strains that are used in the production of citric acid include *Saccharomyces lipolytica*, *Candida tropicalis*, *C. olephila*, *C. guillermondii*, *C. citroformans*, and *Hansenula anomala*.

There are a number of advantages when using yeast in comparison with filamentous fungi. Yeasts can tolerate high initial sugar concentration; they are insensitive to trace metals and can thus ferment crude carbon sources without any treatment; they have a great potential for being used in continuous culture; and they have a high fermentation rate. For commercial production of citric acid by yeast, tower fermenters with efficient cooling systems are employed. To inoculate the fermentation an inoculum is prepared in a smaller fermenter and is subsequently transferred into the production fermenter. The temperature of the fermentation is maintained between 25 and 37 °C, depending on the type of strain employed. The pH is generally > 5.5, but can fall during fermentation. A continuous process for citric acid production using *Candida* cultured on cane molasses has been developed. Processes employing normal paraffin (n-paraffin) as a carbon source, with strains of *Candida* as the fermenting organism, have been fully developed, but have become uneconomic with the rise in prices of petroleum products and have never been run on a large scale.

7.5.1.3 Citric Acid Metabolic Pathways

The exact mechanism for citric acid production is not clearly understood but involves an incomplete version of the tricarboxylic acid cycle. Possibly during the initial metabolism of glucose there is an increase in cellular oxaloacetate levels, which decrease the catabolism of citrate by α-ketoglutarate dehydrogenase and simultaneously increase the rate of citrate synthetase. The condensing enzyme citrate synthetase brings about the biosynthesis of citric acid by the condensation of acetyl-CoA and oxaloacetic acid. This condensation of C2 and C4 compounds is the major route of citrate synthesis (Figure 7.6). Citrate synthetase has been shown to have allosteric regulation. Oxaloacetic acid for citric acid formation is achieved by way of the citric acid cycle and by anaplerotic reaction for the high yield of citric acid. Once the concentration of citric acid in the cells is high enough the acid has to be excreted.

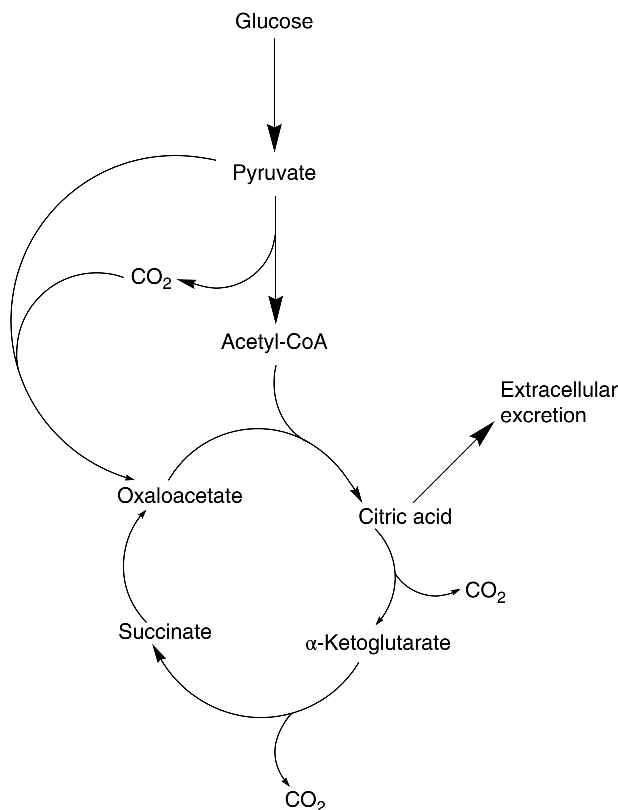

Figure 7.6 Citric acid biosynthetic pathway.

7.5.1.4 Citric Acid Recovery

Following the 5–10 days fermentation the microbial cells are separated from the fermented liquor by centrifugation or filtration. Yeast-based fermentation liquors kept neutral with $CaCO_3$ or lime need to be acidified at this point with mineral acid before this step. Citric acid is then precipitated from the filtrate or supernatant as insoluble calcium citrate tetrahydrate by the addition of lime. The filtered washed calcium salt is treated with sulfuric acid in an acidulator. A precipitate of calcium sulfate is formed and filtered off. The remaining citric acid solution is treated with active carbon, passed through cation exchangers, and concentrated by evaporation before it crystallizes. Alternatively, citric acid may be extracted from the filtered broth using either tributyl phosphate or long chain secondary or tertiary amines. The acid is extracted into solvent at low temperatures and re-extracted into water at a higher temperature. The purified solution is concentrated and crystallized.

7.5.2 Itaconic Acid

Itaconic acid is used to alter the dyeing characteristics of vinyl polymers and also in the manufacture of polymers used in emulsion paints. Itaconic acid accumulation was originally observed in *Aspergillus itaconicus* and *Aspergillus terreus*, and mutants of this strain are now more widely used. The main carbon sources used in the commercial production of itaconic acid include glucose, together with inorganic salts, purified molasses, or media containing a portion of beet molasses. Calcium and zinc are also essential in the growth medium.

7.5.2.1 The Fermentation Process

Once the medium is prepared and sterilized, inoculation is performed by the addition of either a suspension of spores or pre-cultivated mycelia. Though surface fermentation in trays has been used, the submerged method is more widely preferred. Aerated and agitated stainless-steel tanks are employed and provision for cooling is necessary.

Fermentation temperatures for itaconic acid production are quite high at approximately 40 °C. The pH of the media must be reduced to 2 to initiate production; once the accumulation of itaconic acid is well under way higher yields are obtained if the medium is partially neutralized. The fermentation is highly aerobic and continuous aeration is required to decrease production losses. Following 72 hours of fermentation, yields of 60% can be obtained based on the carbohydrate source supplied. Carbohydrate is metabolized, in *Aspergillus itaconicus* cells, by glycolysis to pyruvate, which is further converted through the citric acid cycle to aconitic acid. Aconitic acid is then converted to itaconic

acid by the enzyme aconitic acid decarboxylase, an enzyme that has been reported to be extremely oxygen dependent.

7.5.2.2 Itaconic Acid Recovery

The mycelium is separated from the fermentation medium by filtration and the resultant liquor clarified. The itaconic acid is then recovered by evaporation and crystallization, ion exchange, or solvent extraction.

7.5.3 Vitamins

Vitamins, essential nutrients required in small quantities, have a documented and accepted value to the health of humans and animals. Indeed, vitamins and related biofactors belong to the few chemicals that have a direct appeal to people. There is a large need for extra vitamins, other than those derived from plant and animal food sources, due to unbalanced food habits or processing, food shortage, or disease. Added vitamins are prepared either chemically or biotechnologically via fermentation or bioconversion processes (Table 7.4). Several vitamins are at the moment only produced via organic chemical synthesis. However, for many of these compounds, microbiological processes for their production are rapidly emerging and some are already taking over. Compounds such as riboflavin (B2), ergosterol (provitamin D2), cyanocobalamin (B12), orotic acid (B13), vitamin F group, and vitamin C are now produced exclusively via fermentation.

7.5.3.1 Vitamin B2 (Riboflavin)

Riboflavin is commonly used in animal feed and human nutrition. It is produced by both synthetic and fermentation processes, with the latter recently increasing in application. The first fermentations were initially carried out in 1965, but were shut down after 3 years as they proved more expensive than chemical processes. With improvement in producer organisms the microbial process was revived in 1975 and is now increasingly used for riboflavin production. Although bacteria (*Clostridium* spp.) and yeasts (*Candida* spp.) are also good producers, currently two closely related ascomycete fungi, *Ashbya gossypii* and *Eremothecium ashbyi*, are considered the best riboflavin producers. *Ashbya gossypii* is the preferred strain for production as *E. ashbyi* is genetically unstable.

Soya bean oil and soya bean meal are the substrates most commonly used in *A. gossypii* fermentations. Riboflavin production occurs during the late phase of growth when all the glucose in the medium is exhausted. While glucose remains in the medium, during the early phase of growth it is converted to lipid droplets, which are used later in riboflavin production. Supplementation of the culture medium with glycine or ribitol stimulates riboflavin formation. Both compounds

Table 7.4 Industrial production of vitamins.

Vitamin	Organic extraction	Chemical synthesis	Microbial synthesis			
			Bacterial	Fungal	Algal	World Production (tons per year)
Vitamin B1	+					2,000
Vitamin B2	+		+	+		2,000
Niacin (B3, PP)	+		+			8,500
Pantothenic acid (B5)	+					4,000
Vitamin B6	+					1,600
Biotin (B8)	+		+			3
Folic acid (B9)	+					300
Vitamin B12			+			10
Vitamin B13			+			100
Vitamin C	+		+			70,000
Vitamin A	+					2,500
Provitamin D2				+		
Provitamin D3	+	+				25
Vitamin E	+	+		+	+	6,800
Vitamin F		+		+	+	1,000
Vitamin K2	+					2

are precursors in the riboflavin synthetic pathway and their effects suggest a limitation of central metabolites.

An alternative biotechnological process for the commercial production of riboflavin is through the fermentation of yeast, that is, *Candida famata*; mutants of this strain also exist which can produce up to 200 g of riboflavin per liter following 8 days of fermentation.

7.5.3.2 Vitamin D

Vitamins D2 and D3 are used as antirachitic treatments and large amounts of these vitamins are also used for fortification of food and feed. Vitamin D2 (ergocalciferol) is obtained by the UV irradiation of yeast ergosterol (provitamin D2)

(Figure 7.7). Efficient fermentation processes for ergosterol accumulation have been established. *Saccharomyces cerevisiae* is known to accumulate high levels of sterols. Of about 20 sterols encountered in *S. cerevisiae*, ergosterol, ergosta-5,7,22,24(28)-tetraen-3β-ol, zymosterol, and lanosterol are considered to be the major sterols, of which ergosterol makes up over 90%.

Yeast cells consume carbohydrate as energy and carbon sources by aerobic and anaerobic metabolism. The concentration of carbohydrate and the supply of oxygen determine which metabolic pathway yeast cells utilize. To overcome the repression caused by insufficient nutrients or an oversupply of carbohydrate, fed batch methods have been used in the process of ergosterol fermentation. In the yeast culture process, glucose is preferred and when glucose concentration reaches a low level the cell growth is confined. Then during a short period of adaptation, cells continue to grow by consuming the ethanol, produced in the first phase, as the carbon source. The whole process appears to be a two-phase process, with the ergosterol content increasing when the specific growth rate is decreased.

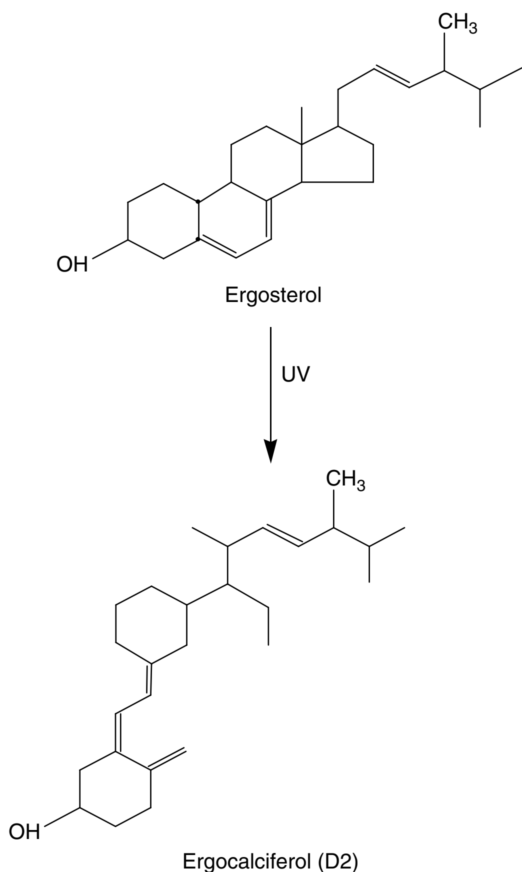

Figure 7.7 Vitamin D biosynthesis.

7.5.4 Fungal Pigments

Fungi have the potential for use in production processes that are themselves less polluting than traditional chemical processes. For instance, many fungi produce pigments that have application in both the textile and food industries, as evidenced by the way in which mildew growth can lead to permanent staining of textiles and plastics. They could therefore be used for the direct production of textile dyes or dye intermediates, replacing chemically synthesized forms which have inherent environmental effects during their production and waste disposal. The approval of fungal carotenoids as food colorants by the European Union has served to strengthen the prospects and global market for use of noncarotenoid fungal polyketide pigments. Fungal production of colorants not only has environmental and safety benefits but also confers on the manufacturer the benefit of making the production process independent of the seasonal supply of raw materials and minimizes batch-to-batch variations. Fungal pigments are known to exhibit unique structural and chemical diversities and have an extraordinary range of colors.

Carotenoids such as β-carotene are produced by a wide range of *Mucorales* fungi and are suitable for addition to a variety of foods. The yeast *Phaffia rhodozyma* has become the most important microbial source for the production of the carotenoid pigment astaxanthin and is responsible for the orange-pink color of salmonid flesh and the reddish color of boiled crustacean shells. Feeding farmed salmonids with a diet containing this yeast induces pigmentation of the white muscle and imparts the red color normally associated with wild fish. Economically its importance is very high

Polyketide pigments are produced in abundance by filamentous fungi, and include quinones such as anthraquinones and naphthaquinones, dihydroxy naphthalene melanin, and flavin compounds such as riboflavin. Yellow-colored anthraquinone pigments are produced by many fungi including *Eurotium* spp., *Fusarium* spp., *Curvularia lunata*, and *Drechslera* spp. Anthraquinone is an important member of the quinone family and is a building block of many dyes, examples of which include catenarin, chrysophanol, cynodontin, helminthosporin, tritisporin, and erythroglaucin. *Emericella* species have been shown to produce alternative yellow-colored pigments such as the epurpurins, falconensins, and falconensones.

Monascus species produce orange, water-insoluble pigments such as monascorubrin and rubropunctatin. These well-characterized compounds can be converted to high purity red, water-soluble pigments by reaction with amino acids, yielding monascorubramine and rubropunctamine. These pigments are suitable as colorants for a broad variety of foodstuffs and often serve as suitable replacements for the food dyes FD&C Red No. 2 and Red No. 4. Interestingly, pigment derivatives with improved functional properties in the color range of orange-red to violet-red can be produced by *Monascus* fermentations through the inclusion of different amino acids in the growth media. One concern with the

use of *Monascus* for the purpose of dye production, however, is that the fermented rice substrate used in the process has been found on occasion to contain the mycotoxin citrinin. For the present, this limits the use of *Monascus* as a producer of natural food colorants. Clearly there is significant scope for the identification and development of food colorants and dyes from fungi.

7.6 Yeast Extracts

Maintenance of an adequate food supply has challenged humans during much of our early existence. Since ancient times, both western and oriental cultures have used microorganisms to transform or produce food. Fungi have a crucial role to play in the processing of many foods, improving the texture, digestibility, nutritional value, flavor, or appearance of the raw material used. The first industrial production of microorganisms for nutritional purposes took place in Germany, when *Torula* yeast was produced and incorporated into soup and sausages. Yeast cells may be solubilized either partially or completely by autolysis and several other techniques. On further processing, the slurries can be converted into a variety of preparations and products, which are useful in the laboratory and as ingredients in food, feeds, and fermentation media. Among the principal products are concentrates of yeast invertase and β-galactosidase, soluble yeast components in liquid, paste, powder, or granular form, and isolated fractions of yeast cell constituents, such as protein and cell walls (glucan, manno-oligosaccharide, emulsifiers, and mannoprotein), liberated by cells fractured mechanically. The major commercial products are clear water-soluble extracts, known generally as yeast extract, autolyzed yeast extract, and yeast hydrolysate.

During the early part of the last century, studies on spent yeast from breweries led to the development of yeast extracts for the food industry. At present, this technology has been extended to several other types of yeast, thus providing a much wider range of yeast extract to the food industry. As natural flavorings approved by the FDA, yeast extracts are used as condiments in the preparation of meat products, sauces, soups, gravies, cheese spreads, bakery products, seasonings, vegetable products, and seafoods. A reliable economical source of peptides, amino acids, trace minerals, and vitamins of the B complex group, yeast extracts are nutritional additives in health food formulations, baby foods, and feed supplements, and for enrichment of growth and production media for microorganisms and other biological culture systems.

7.6.1 Yeast Extract Production

There are three distinct manufacturing practices for yeast extract production: autolysis, plasmolysis, and hydrolysis. Autolysis is a process by which the cell components within the cells are solubilized by activation of enzymes, which are

inherently present in the cell. This is achieved by carefully controlling temperature, pH, and time, with the careful addition of enzymes or reagents to stimulate degradation and release of the cell contents into the medium. The amount of free amino acid present in the extract can serve as a rough guide to indicate the degree of hydrolysis. Free amino acids are known to directly or indirectly exert major influences on food flavor.

A yeast extract manufacturing process (Figure 7.8) which has gained more acceptance in Europe than the United States is plasmolysis. In this process yeast cells are treated with salt and begin to lose water, and the cytoplasm separates from the cell wall. When this happens cells die and the degradative process begins. The advantages of this process are the fact that no specialized equipment

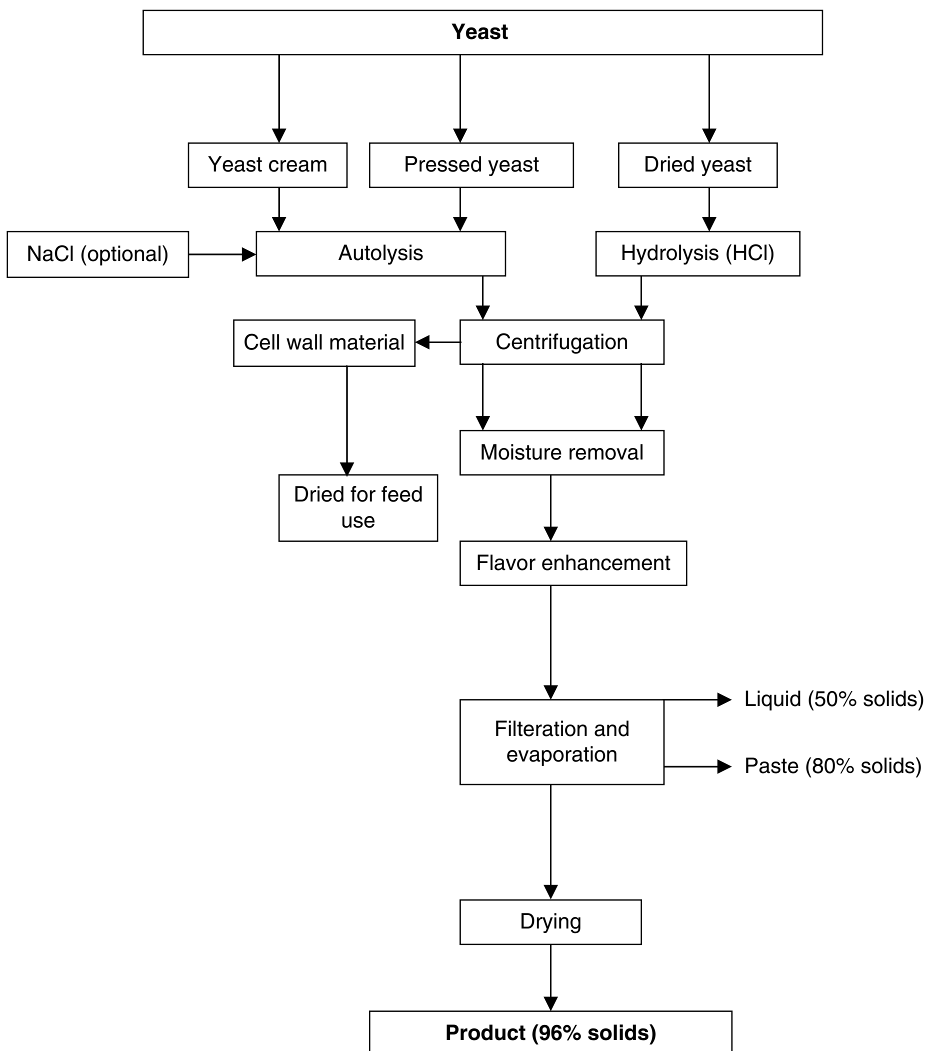

Figure 7.8 Yeast extract manufacturing process.

is required and salt is relatively cheap to purchase and readily available. One drawback is the high salt content of the extract. The hydrolytic process utilizes the action of hydrochloric acid on yeast at specific temperatures and pressure. Hydrolysis is carried out until the required concentration of free nitrogen is achieved; this usually takes 6–12 hours, and shorter more efficient hydrolysis can be achieved at higher temperature and pressure. The hydrolysate is neutralized with sodium hydroxide; the extract is then filtered and concentrated.

7.7 Enriched Yeast

With increasing demand for organic and nongenetically modified additives in human and animal feeds, interest in the production of "organic vitamins and minerals" has increased in recent years. One of the major concerns with the production of these organic products is economic viability. Thus, to overcome this concern, the incorporation into or enrichment of yeast (*S. cerevisiae*) with minerals has developed as a common industrial process. One of the most commonly available of these yeast-type products is selenium-enriched yeast, such as Sel-Plex®.

7.7.1 Selenium Yeast Production

Since selenium and sulfur exist in the same group within the periodic table and have many similar chemical characteristics, microbes such as the yeast *S. cerevisiae* have been shown to be unable to distinguish between either atom. Indeed, *S. cerevisiae* has the ability to metabolize selenium and incorporate it into molecules where sulfur would normally exist as the native atom. When propagated in a nutrient-enriched medium containing reduced levels of sulfur but enriched with selenium as inorganic selenite or selenate salts, *S. cerevisiae* can utilize selenium as it does sulfur, resulting in the biosynthesis of various organic selenium compounds. The majority of the selenium in selenium yeast exists as analogs of the organo-sulfur compounds such as selenomethionine, selenocysteine, and selenocystine. Small amounts are also thought to exist as selenohomocystenine, selenocystathione, methylselenocysteine, S-adenosylselenomethionine, selenotrisulfide, selenoglutathione, and various selenothiols (Figure 7.9). The biosynthesis of such organo-selenium compounds is thought to be achieved through the biochemical pathways of organo-sulfur biosynthesis, which have been well characterized. These seleno-amino acids can then be utilized by the protein synthesis machinery of the yeast cell and incorporated into protein molecules.

Crude protein, shown to account for approximately one-half of the dry weight of yeast, was characterized and was shown to be comprised of approximately 80% amino acids, 12% nucleic acids, and 8% ammonia. Manipulation of these

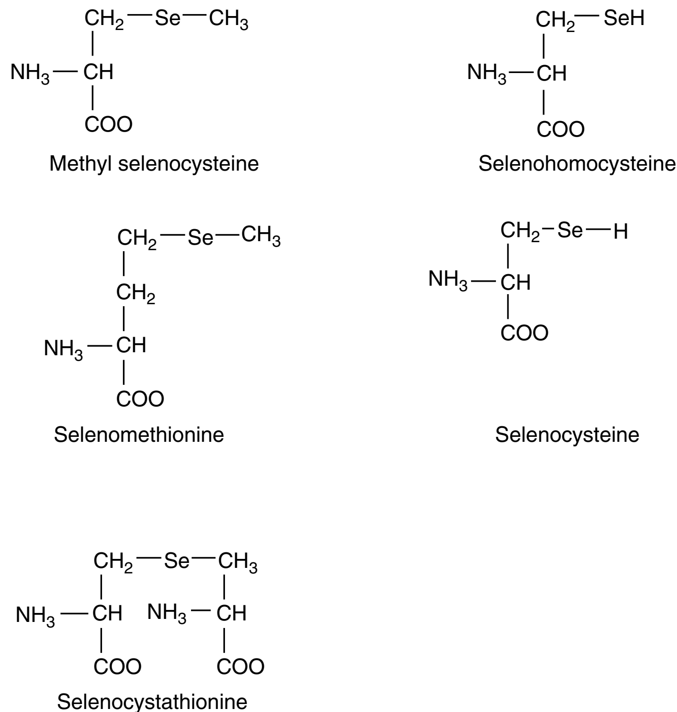

Figure 7.9 Selenium compounds in selenium yeast.

data shows the total sulfur-containing amino acid content of *S. cerevisiae* to be 1.99% (w/w), with 1.21% as methionine and 0.78% as cysteine. This represents 2,600 and 2,080 ppm of organically bound sulfur in these forms, respectively. Studies have shown that up to 50% of the methionine moieties in proteins can be replaced with selenomethionine while retaining biological activity. These findings would indicate that well in excess of 2,000 ppm of yeast sulfur could be replaced by selenium with possibly no adverse effects to protein synthesis or growth characteristics of the yeast cells. Therefore, if propagation conditions are carefully controlled, a growth pattern can be induced which allows the incorporation of selenium in the yeast to levels in excess of 2,000 ppm – over 50,000 times the normal level of 0.04 ppm (Figure 7.10).

Selenium yeast has many advantages over traditional inorganic selenium. These include a lessening of environmental concerns pertaining to the toxicity of selenium, as selenium yeast displays reduced toxicity over inorganic selenium forms. Another advantage associated with increased bioavailability is a reduction in the level of unabsorbed selenium excreted by the fecal route. This would prevent toxic selenium build-up by concentration in the feces and is of significance where intensive farming techniques are employed. Economically, too, benefits are to be seen with increased bioavailability, and lower quantities of selenium would be required to supplement selenium-deficient diets, resulting in less expensive feeds.

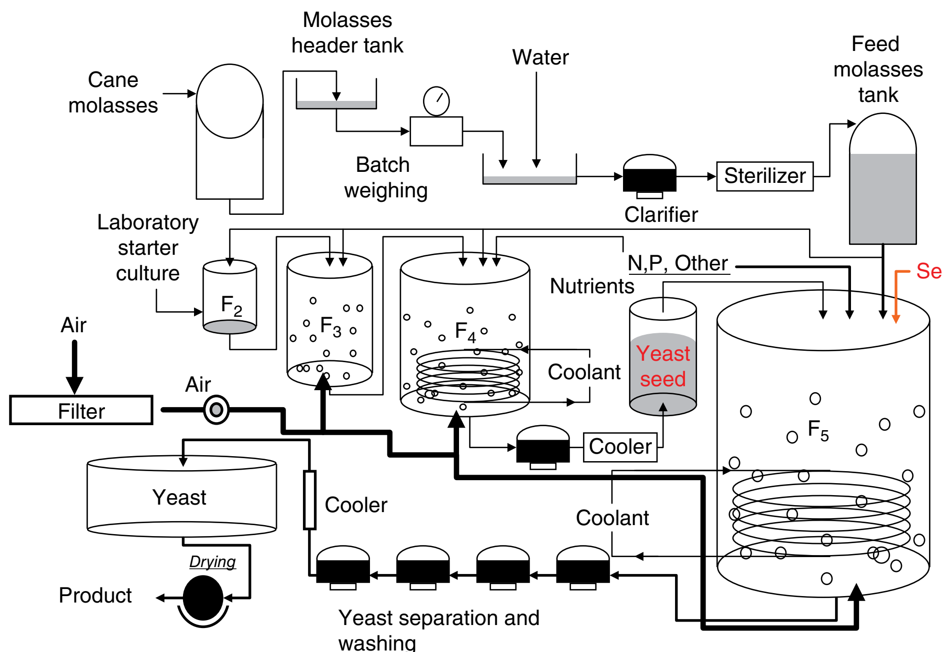

Figure 7.10 Selenium yeast production.

7.8 Conclusion

Within the fungal lifecycle one can clearly delineate the production of certain products or metabolites into two phases, namely primary and secondary metabolism. Fungal biotechnology has developed, to allow the utilization of these metabolic processes in a commercially viable manner. The economic significance of this cannot be understated when one considers the use of fungi in the production of valuable commodities such as antibiotics, enzymes, vitamins, pharmaceutical compounds, fungicides, plant growth regulators, hormones, and proteins.

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8
Biotechnological Use of Fungal Enzymes

Shauna M. McKelvey and Richard A. Murphy

8.1 Introduction

The roots of modern enzymology may be traced back to the nineteenth century when it was shown that an alcohol precipitate of malt extract contained a thermolabile substance, which converted starch into fermentable sugars. The enzyme responsible was termed diastase because of its ability to yield soluble dextrins from insoluble starch granules. By the mid-nineteenth century the existence of several additional enzymes including polyphenol oxidase, peroxidase, and invertase was recognized. An amylolytic preparation, termed Taka-Diastase was the first enzyme preparation to be patented for industrial use. It was produced by the filamentous fungus *Aspergillus oryzae* when grown on rice. The patent was lodged in 1884 by Dr Jokichi Takamine, a Japanese immigrant to the United States. Since then, the technology to identify, extract, and produce enzymes on a commercial scale has progressed dramatically and they are now used in many industrial processes.

Of all the commercially utilized enzymes, approximately half are of fungal origin. Numerous factors favor microbially sourced enzymes over those derived from plants or animals, including the wide variety of catalytic activities, higher yields, ease of genetic manipulation, and rapid production in inexpensive media. Other appealing factors include the fact that they are biodegradable and are active under mild conditions with respect to temperature and pH. Enzymes have long played a central role in mankind's attempts to utilize biological systems for a variety of purposes. Examples of harnessing their power include cheese making, brewing, baking, the production of antibiotics, and in the manufacture of

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commodities such as leather, indigo, and linen. They also find applications in areas such as detergent and paper production, the textile industry, and in the food and drinks industry in products ranging from fruit juice, coffee, and tea, to wine.

8.2 Enzymes in Industry

The first serious attempt at the beginning of the twentieth century to use enzymes for industrial purposes met with limited success. Disappointing initial results were due largely to a lack of understanding of enzyme activity. Only since the 1960s have enzymes been characterized and their kinetics understood. This increased knowledge has in turn facilitated the application of enzymes in a variety of industrial processes.

The majority of enzymes currently used in industry may be described as hydrolytic depolymerases (pectinases, lipases, carbohydrases, etc.), and the single most significant industrial application of enzymes is the inclusion of proteases and amylases in detergent preparations. Carbohydrases (in particular amylases and pectinases) account for a significant portion of the remaining market. The glucose isomerase enzyme, which is utilized industrially in the production of high-fructose syrup from glucose syrups, is one of the few nondepolymerases to command significant industrial volume.

Rapid developments have occurred in the enzyme supply market over the past few years due to the evolution of the biotechnology industry. Volume growth for this thriving sector is currently between 4 and 5% of the annual growth rate and annual sales figures in excess of $4.2 billion were reported in 2014. Current advancements in protein engineering and recombinant gene technology have revolutionized enzyme production and commercialization by extending the list of enzymes now available (Table 8.1).

Three main segments dominate the industrial enzyme market: technical enzymes, food enzymes, and animal feed enzymes. Technical enzymes including protease, amylase, and cellulase account for the highest percentage of sales. These enzymes are extensively used in the detergent, starch, textile, leather, paper and pulp, and personal care industries. The second largest segment in the market is for food enzymes. Included in this group are enzymes employed in the dairy, brewing, wine and juice, fats, oils, and baking industries, such as lipase and pectinase. The final area is that of feed enzymes comprising of enzymes such as phytase, xylanase, and β-glucanase.

8.3 Current Enzyme Applications

Given the wide and varied applications for industrial enzymes, discussion will be limited to the most important in financial terms.

Table 8.1 Sources and applications of industrial enzymes.

Enzyme	Organism	Main application areas
α-Amylase	*Aspergillus niger*, *A. oryzae*	Starch processing and food industry
Chymosin	*A. niger*	Food industry
Cellulase	*Trichoderma viride*, *T. reesei*	Textiles, pulp and paper industry
Cellobiohydrolase	*T. viride*, *T. reesei*	Textiles, pulp and paper industry
Glucoamylase	*A. phoenicis*, *Rhizopus delemar*	Starch processing industry
Glucose oxidase	*A. niger*, *A. oryzae*	Textiles, biosensor
Laccase	*Trametes versicolor*	Textiles, pulp and paper industry
Lipases	*A. niger*, *A. oryzae*	Food and detergent industries
Pectin lyase	*T. reesei*	Food industry
Proteases	*A. niger*, *A. oryzae*, *R. delemar*	Food and detergent industries
Phytase	*A. niger*, *A. oryzae*	Food industry
Rennin	*Mucor miehei*	Food industry
Xylanases	*T. reesei*, *T. konignii*, *A. niger*	Textiles, pulp and paper industry, bakery industries
Amyloglucosidase	*A. niger*	Starch syrups, dextrose, food industry
Invertase	Yeast spp.	Confectionery industry

Adapted from Meyer (2008).

8.3.1 Detergent Industry

The use of enzymes originates back to 1913 when pancreatic enzymes were used in a pre-soaking product. Shortly after this, the first enzymatic detergent, Brunus, was marketed. Since then the application of enzymes in detergents has become widespread, with different enzymatic formulations continuously emerging, such as mannanase, which aids in the removal of various food stains containing guar gum. Detergent additives still represent the largest application of industrial enzymes, and protease is the most dominant enzyme, accounting for 30% of the market. Cellulases have also been employed in household washing powders; however, they are considered a more expensive enzyme source. They function by allowing the removal of small, fuzzy fibrils from fabric surfaces such as cotton and improve the appearance and color brightness.

The increased usage of these enzymes as detergent additives is mainly due to their cleaning capabilities in environmentally acceptable, nonphosphate detergents. Important factors to be considered when including enzymes in detergents include:

- They should have a high activity and stability over a wide pH and temperature range.
- They should be effective at low levels (0.4–0.8%).
- They should be compatible with various detergent components.
- They must have a long shelf life.

8.3.2 Bioethanol and Biodiesel

Due to recent increased environmental concerns, rising oil prices, and fast-diminishing resources of fossil fuels, there has been a surge of interest in the production of biofuels such as bioethanol and biodiesel. Ethanol is the most widely used biofuel today and intense efforts are currently being undertaken to develop a technically viable process for conversion of cellulose and lignocellulose into bioethanol. As an incentive to reduce the price of cellulase enzymes used in processing cellulosic materials, the US Department of Energy awarded a research grant of $32,000,000 to Genencor and Novozyme in an attempt to make bioethanol production more economically feasible.

Biodiesel is an ester derived from oils such as soybean, rapeseed, vegetable, and animal fats. Vegetable-derived biodiesel is much cleaner than that of petroleum as it does not produce sulfur dioxide and the soot particulate is minimized by one-third. Through trans-esterification with lipase enzymes, organically derived oils are combined with ethanol or methanol to form fatty esters and these esters can then be blended with conventional diesel fuel or used as 100% biodiesel.

8.3.3 Tanning Industry

Traditionally, lime and sodium sulfide mixtures have been used in the leather industry for the dehairing of skins and hides. This method causes pollution and is unpleasant. However, through the advent of biotechnology, safer, more environmentally friendly means of treating animal skins have been developed. A cocktail of proteases and lipases extracted from fungal strains such as *Aspergillus* has now become common practice. The enzyme mixture causes the swelling of hair roots, which allows the hair to be removed easily. Elastin and keratin are then degraded and hair residues removed in a process called bating. The end product is of a higher quality when compared to leather manufactured using traditional methods.

8.3.4 Effluent and Waste Treatment

With respect to the waste treatment industry, enzymes are now playing a significant role. For instance, lipases are used in activated sludge and other aerobic waste processes as they aid in the breaking down of solids and prevention of fat blockages. Proteases are employed in the processing of waste feathers from poultry slaughterhouses. Approximately 5% of the body weight of poultry is feathers, which can be considered a high protein source of feed, provided their keratin structure is completely degraded. Total solubilization of feathers can be achieved through enzymatic hydrolysis along with sodium hydroxide addition and mechanical disintegration.

Vast amounts of wastewater are produced each year in the textile industry, with China producing in the region of 0.65 billion tonnes per annum. Disposal of this effluent causes environmental pollution, so it is with great interest that a solution is found. Currently, textile effluent is mainly treated using biological methods or a combination of biological and chemical methods. However, enhancements of biological treatment techniques, such as bio-augmentation, immobilized microorganisms, and microorganism activity enhancements are currently being studied with a view to improving the efficiency of this system.

8.3.5 Food Processing

The food industry is one of the most significant utilizers of enzymatic activities, ranging from catalases and lipases in cheese making and cheese ripening, pectinases in wine and fruit juice clarification, and α-amylases in dextran liquefaction, to proteases in meat tenderization. The main reasons for the increased interest in the use of enzymes in food-related processes include the following:

- They are very specific and controllable chemical catalysts and as such the production of undesirable by-products may be avoided.
- Catalysis takes place under mild conditions of temperature, pH, and pressure compared with chemical methods of conversion.
- Enzymes are biodegradable and chemical toxicity problems are greatly reduced.
- Their immobilization on solid supports can provide technological advantages in processing and also avoids the presence of the enzyme in the final product, thus reducing the possibility of allergenicity.

Lipases find application in the production of leaner fish, refining rice flavor, and modifying soybean milk. Other applications include the flavor enhancement of cheeses and the production of cheese products such as soft cheeses. Proteases

play a vital role in meat tenderization, as they possess the ability to hydrolyze connective tissue proteins as well as muscle fiber proteins. Enzymes are applied directly to the meat or injected directly into the bloodstream. Bread making is a technique that has been around for thousands of years. Currently, amylase, protease, and cellulase are being used as dough improvers. Their application results in improved texture, volume, flavor, and freshness of the bread, as well as improved machinability of the dough. In addition, current efforts are being made to further understand bread staling and the mechanisms behind the enzymatic prevention of staling when using α-amylases and xylanases.

8.3.6 Fruit Juice Maceration

A combination of pectinases, cellulases, and hemicellulases (collectively called macerating enzymes) is used in the extraction and clarification of fruit and vegetable juices. In addition, α-amylases, amyloglucosidase, and laccase have been used to prevent haze formation in starch-containing fruits such as apples. Treatment of fruit pulps with pectinase also produces an increase in fruit juice volume from bananas, grapes, and apples. The demand for enzymes such as these is bound to increase as the range of applicable fruits and vegetables rises.

A process called vacuum infusion has been developed using pectinase to ease the peeling of citrus fruits. This process was developed from the observation that the infusion of certain impure naringinase preparations into grapefruit peel in an attempt to reduce bitterness also softened the albedo (the white portion of citrus peel). This application may also apply to the pickling process where excessive softening may occur during fermentation and storage. Thus, enzyme infusion to alter the sensory attributes of fruits, vegetables, and other foods has enormous potential in food biotechnology.

8.3.7 Animal Feed

The animal feed industry is an extremely important part of the world's agro-industrial activities, with an annual production of more than 950 million tonnes of feed, worth in excess of US $50 billion. It is an industry that has gone through many changes in the past few years, as consumers and the industry itself have looked more closely than ever before into how compound animal feeds are produced, how the animals are reared, and how the systems of animal husbandry in use today affect the environment. The incorporation of enzymes including β-glucanase, cellulase, amylase, and protease into feedstuffs has been demonstrated to contribute to enhanced production performance. Perhaps the best example is the addition of β-glucanase and xylanase to barley-based diets in the poultry industry. Noted positive effects include improvements in digestion and absorption of feed components, as well as weight gain by broiler chicks and egg-laying hens.

Other positive effects of these enzymes are evident in the digestion of nonstarch polysaccharides (NSP) by barley-fed piglets. Table 8.2 lists enzyme activities that may contribute to enhanced animal performance when incorporated into animal feedstuffs.

Supplementation of the diet with selected enzyme activities may also promote a decrease in the overall pollutive effect of animal excreta. This is particularly true in the case of dietary phosphorus, a large proportion of which remains unassimilated by monogastrics. The inclusion of suitable, microbially derived phytases in the diet can improve the efficiency of nutrient utilization and reduce waste. Current research has identified new fungal enzymes with 4- to 50-fold higher specific activities than previously reported.

Phytic acid is an organic compound of phosphate and is the main storage form of phosphorus in plants (60–65% of phosphorus present in cereal grains). Phytate-bound phosphorus is poorly hydrolyzed, as broilers have a low capacity for its hydrolysis. Phytic acid also hinders the assimilation of other nutritionally important proteins and metals such as calcium, zinc, and magnesium, as it binds tightly to these. The majority of this form of phosphorus is therefore excreted in the manure, causing environmental problems in areas of intensive livestock production. Inclusion of phytate-degrading enzymes such as phytase has yielded many dramatic beneficial results on an environmental basis, and the addition of such enzymes to animal diets is well documented. Use of phytase has a threefold beneficial effect: the antinutritional properties of phytic acid are destroyed, a lesser requirement for feed supplementation with inorganic phosphorus results, and

Table 8.2 Enzyme activities and associated supplementational effects on animal performance.

Enzyme(s)	Substrate	Beneficial effects
β-Glucanases	Mixed linked glucans	Reduction in viscosity of digesta. Reduction in incidence of pasted vents. Improvements in litter characteristics. Reduction in dirty egg problems
Xylanases	Pentosans	Reduction in viscosity of digesta
Cellulases	Cellulose	Promotion of a more comprehensive digestion of vegetative matter
Phytases	Phytic acid	Removal of antinutritional effects of phytic acid
Proteases Amylases	Proteins Starch	Supplementation of endogenous proteolytic and amylolytic capacity to benefit young or sick animals whose digestive function may not be operating maximally

Adapted from Walsh (2003).

reduced phosphate levels are present in the feces, which leads to less loading of the environment with phosphorus. Currently, phytase utilization is restricted to locations where there is a considerable pollution load, but if increases in efficiency can be achieved then the application of this enzyme will become more widespread.

8.4 Enzymes and Sustainability

Many industries produce waste that can be hazardous for nature. This is not the case with the use of enzymes as they are fully biodegradable. For instance, when fungal enzymes have acted on their respective substrates, they break down into amino acids that are naturally recycled in the environment. Enzymes enable the production of high quality products, increasing overall yields and avoiding unwanted by-products. The use of fungal enzymes has greatly enabled various industries to increase production efficiency. Additionally, their use provides for the development of more environmentally friendly products and processes through the use of less energy, water, and raw materials. Processes that utilize fungal enzymes will also result in the generation of less waste. Estimates by the World Wildlife Fund (WWF) have indicated that enzymes enable efficiency improvements that potentially could reduce carbon dioxide emissions by up to 139 MtCO2e in the food industry and up to 65 MtCO2e in traditional industries (detergents, textiles, pulp and paper) by 2030. This is equivalent to taking almost nearly 40 million cars off our roads or using 430 million barrels of oil less than at present.

8.5 Future Direction of Industrial Enzymes

There is little doubt that in the near future the use of industrial enzymes will expand dramatically in areas such as biopulping, food processing, carbohydrate conversions, chemical conversions, food and animal feed additives, cleaning, and detoxification of environmental toxins. Prior to the advent of the tools of molecular genetics, the use of enzymes as industrial catalysts was limited to perhaps 20 enzymes that could be produced inexpensively in large amounts. The use of genetic engineering to clone the genes of nonabundant enzymes for subsequent overexpression in heterologous hosts promises to greatly expand the opportunities for using enzymes in industrial applications.

8.6 Applications of Specific Fungal Enzymes

In a commercial sense, the main enzymes include protease, cellulase, xylanase, lipase, amylase, and phytase, and these can be produced by many different genera of microorganism including fungal strains of *Aspergillus*, *Rhizopus*, and *Penicilium*. The following section deals specifically with these enzymes.

8.6.1 Proteases

Proteases have been widely described as a diverse class of enzymes that are known to hydrolyze the peptide bond (CO-NH) in a protein molecule. They vary with respect to their pH optima, and in general alkaline proteases are produced by bacteria and acid proteases by fungi. Proteolytic enzymes occur naturally in all living organisms and represent 2% of all the proteins present. They have long been applied in numerous industries and the vast diversity of proteases has attracted worldwide attention in attempts to exploit their physiological and biotechnological applications.

Proteases are physiologically necessary for living organisms and are found in a wide diversity of sources such as plants, animals, and microorganisms. However, microbes possess certain qualities that see them as a preferred choice, including their broad biochemical diversity and susceptibility to genetic manipulation. Microbial proteases have therefore been extensively studied and their molecular properties are understood in detail.

Filamentous fungal strains such as *Aspergillus* have been widely used for industrial production of protease. Compared to bacteria, fungi produce a wider variety of protease with broad pH activity ranges.

8.6.1.1 Sources

Proteases are subdivided into two major groups, exopeptidases and endopeptidases, depending on their site of action. Exopeptidases, also known as peptidases, are classified according to whether they split off single amino acids from the N-terminus or C-terminus of peptide chains and are specific for dipeptide substrates. Additional exo-acting peptidases cleave dipeptide units from the N-terminus or the C-terminus of proteins. Endopeptidases, also known as proteinases, cleave peptide bonds internally within a polypeptide. Based on the functional group present at the active site, endoproteinases are further classified into four prominent groups, namely, serine, cysteine, aspartic, and metalloproteases (Table 8.3).

Aminopeptidases are exo-acting peptidases that catalyze the cleavage of amino acids from the N-terminus of protein or peptide substrates. They are widely distributed throughout the animal and plant kingdoms and are found in a variety of microbial species including bacteria and fungi.

As the name implies, carboxypeptidases cleave amino acids from the C-terminus of the polypeptide chain and liberate a single amino acid or a dipeptide. They can be divided into three groups based on the nature of the amino acid residue at the active site of the enzyme: serine carboxypeptidases, metallocarboxypeptidases, and cysteine carboxypeptidases.

Serine proteases are characterized by the presence of a serine group in their active site and as endopeptidases with a strong proteolytic activity coupled with low specificity. The basic mechanism of action of serine proteases involves transfer

Table 8.3 Classification of proteases.

Protease	Mode of action[1]	EC no.
Exopeptidases		
Aminopeptidases	•↓-o-o-o-o—	3.4.11
Dipeptidyl peptidase	•-•↓-o-o-o—	3.4.14
Tripeptidyl peptidase	•-•-•↓-o-o—	3.4.14
Carboxpeptidase	—o-o-o-o-o↓-•	3.4.16–3.4.18
Serine-type protease		3.4.16
Metalloprotease		3.4.17
Cysteine-type protease		3.4.18
Peptidyl dipeptidases	—o-o-o-o↓-•-•	3.4.15
Dipeptidases	•↓-•	3.4.13
Omega peptidases	*-•↓-o-o—	3.4.19
	—o-o-o↓-•-*	3.4.19
Endopeptidases	—o-o-o↓-o-o-o—	3.4.21–3.4.34
Serine protease		3.4.21
Cysteine protease		3.4.22
Aspartic protease		3.4.23
Metalloprotease		3.4.24
Endopeptidases of unknown catalytic mechanism		3.4.99

[1]Open circles represent amino acid residues in the polypeptide chain, solid circles indicate terminal amino acids, and asterisks signify blocked termini. Arrows show sites of action of enzyme. Adapted from Rao *et al.* (1998).

of the acyl portion of a substrate to a functional group of the enzyme. They are widespread among bacteria, viruses, and eukaryotes, suggesting that they are vital to all organisms. Serine proteases have also been found in the exopeptidase, oligopeptidase, and omega peptidase groups. Based on their structural similarities, they have been further divided into 20–30 families.

Proteases with low pH optima are abundant in filamentous fungi and a large proportion of these have been shown to have properties consistent with aspartic proteinases. These molecules contain an aspartic residue at the active site and are unaffected by chelating agents, thiol-group reagents, or serine protease inhibitors. They share similarities with the animal digestive enzymes pepsin and rennin and are therefore employed as replacements for animal proteases and in cheese manufacture.

Cysteine proteases occur in both prokaryotes and eukaryotes and about 20 families have been recognized; however, reports of their occurrence in fungi are very limited. The activity of all cysteine proteases depends on a catalytic dyad consisting of cysteine and histidine, and the order of Cys and His residues differs among the families. Cysteine proteases have a highly similar mechanism of action to that of serine proteases.

Metalloproteases are the most diverse of the catalytic types of protease and about 25 families have been recognized. The metalloproteases typically contain an essential metal atom and show optimal activity at neutral pH. Ca^{2+} is essential for stability and the molecules are therefore inactivated and destabilized by chelating agents.

8.6.1.2 Application

The use of proteases in the food industry dates back to antiquity; for instance, in ancient Greece they utilized enzymes from microorganisms for various purposes such as baking, alcohol production, and meat tenderization. The importance of proteases in industry has been widely investigated and applications have been found in sectors such as detergent, feed, pharmaceutical, animal feed, diagnostics, and fine chemical industries. Of these industries, the detergent and feed sectors are the highest exploiters of proteolytic activity.

There is a wide application for proteolytic enzymes in the detergent industry due to a number of attractive characteristics, including stability over a broad temperature range and optimal activity in the alkaline pH range. Proteases added to laundry detergents enable the release of proteinaceous stains such as keratin, blood, milk, and gravy. In response to the current energy crisis and awareness for energy conservation, researchers are continually screening for new proteases. Attempts have been made to produce protease enzyme, using various substrates such as shrimp and crab shell powder, soybean meal, and fish waste.

With respect to the dairy industry, the main application for proteases is in the manufacture of cheese, as they have high milk clotting properties. Calf rennet, extracted from the fourth stomach of calves, has traditionally been used in the manufacturing of cheese; however, increased demand resulted in a shortage of rennet and the search began for alternative sources. Chymosin has successfully replaced calf rennet and is now produced from fungal strains including *Mucor miehei* and *Mucor pusillus*. In addition, fungal proteases from *Penicillium roqueforti* and *Penicillium caseicolum* play fundamental roles in the cheese ripening process. *Aspergillus oryzae* proteinases are widely employed in the baking industry to help control break texture and gain dough uniformity.

The wide diversity and specificity of proteases are used to great advantage in developing effective therapeutic agents. Collagenases are increasingly used for therapeutic applications in the preparation of slow-release dosage forms. Proteolytic enzymes from *A. oryzae* have been used as digestive aids to correct lytic enzyme

deficiency. Overall, the wide diversity of proteases allows for further exploitation of these microbial powerhouses.

8.6.2 Cellulase

Cellulose is the principal component of plant cell walls and is the most abundant, renewable polymer on earth. Its structure consists of a linear polymer of 1,4-β-linked glucose residues. Individual cellulose molecules are linked together by hydrogen bonds to produce larger, crystalline structures. Due to the complexity of this structure, crystalline cellulose is not amenable to attack by single enzymes. As a consequence, cellulolytic microorganisms have been utilized to secrete mixtures of cellulolytic activities which degrade cellulose.

It is widely documented that complete degradation of cellulose requires the coordinated action of three main enzymes including endoglucanases, exoglucanases, and β-glucosidases. While cellulase is an endoglucanase that hydrolyzes cellulose randomly, producing numerous oligosaccharides, cellobiose, and glucose, exoglucanases hydrolyze β-1,4-D-glucosidic linkages in cellulose-releasing cellobiose from the nonreducing end. On the other hand, β-glucosidases hydrolyze cellobiose to glucose. As it is a major waste by-product both in nature and from man's activities, cellulose holds tremendous potential as a renewable energy source (Table 8.4).

Table 8.4 Components of aerobic fungal cellulases and their mode of action on the cellulose chain.

Enzyme type	EC no.	Synonym	Mode of action
Endo-(1-4)-β-D-glucanase	3.2.1.4	Endoglucanase or endocellulase	—G—G—G—G— ↑ ↑ Cleaves linkages randomly
Exo-(1-4)- β-D-glucanase	3.2.1.91	Cellobiohydrolase or exocellulase	G—G—G—G—G— ↑ Releases cellobiose either from reducing or nonreducing end
Exo-(1-4)-β-D-glucanase	3.2.1.74	Exoglucanase or glucohydrolyase	G—G—G—G—G— ↑ Releases glucose from nonreducing end
β-Glucosidase	3.2.1.21	Cellobiase	G—G, G—G—G—G ↑ ↑ Releases glucose from cellobiose and short chain xylo-oligosaccharides

Adapted from Bhat and Bhat (1997).

8.6.2.1 Sources

Cellulolytic enzymes are produced by a wide variety of bacteria and fungi, aerobes and anerobes, mesophiles and thermophiles. The most detailed studies have been on the cellulolytic enzyme systems of the aerobic fungi *Trichoderma reesei*, *Trichoderma viride*, *Penicillium pinophilium*, *Sporotrichum pulverulentum*, and *Fusarium solani*.

8.6.2.2 Application

Active research on cellulases and related polysaccharides began in the early 1950s owing to their enormous potential to convert lignocellulose to glucose and soluble sugars.

Biotechnology of cellulases and hemicellulases began in the early 1980s, first in animal feed, followed by food applications. Subsequently, these enzymes were used in the textile, laundry, and pulp and paper industries.

Cellulose is one of the most abundant polymers on earth, with exceedingly good potential as a renewable energy source. During World War II the US army was alarmed at the rate of deterioration of cellulosic materials including clothing, tents, and sand bags in the South Pacific. Several organizations within the Army set up laboratories to find an immediate solution to this problem. As a result, a parent strain QM6A was isolated and identified as *T. viride* and later recognized as *T. reesei*. The immediate benefit of the Army's program led to further research on selection and characterization of hypercellulolytic *T. reesei* strains. These projects not only improved the production of cellulase by *T. reesei* but also aroused worldwide activity.

Fungal enzyme systems involved in cellulose degradation have been extensively studied due to their potential value in biotechnology. Applications are found in food sectors such as juicing, in which cellulases, along with numerous other enzymes, are used in juice extraction and clarification. In the farming industry, cellulolytic activities are used as supplements for livestock, which in turn increases yield and performance. In the textile industry, cellulases have achieved success because of their ability to modify cellulosic fibers in a controlled and desired manner. Cellulases have only been applied to the textile industry since 2007, but are now the third largest group of enzymes used. Combinations of cellulase and hemicellulase have been used in the pulp and paper industry in areas such as biomechanical pulping and biobleaching.

One of the main applications for cellulolytic enzymes is in the conversion of biomass to ethanol for fuel production. Unlike fossil fuels, ethanol is a renewable energy source produced through fermentation of sugars. Ethanol is widely used in the United States as a partial gas replacement, with approximately 1 billion gallons of fuel ethanol manufactured from corn starch using a traditional yeast-based process. This process, using a high value grain, is made possible by the production of animal feed and other co-products. Fuel ethanol that is produced

from corn has been used in gasohol and oxygenated fuels since the 1980s; however, the cost of ethanol as an energy source still remains relatively high compared to fossil fuels. As this industry is quite mature there is limited opportunity for major process improvements. A potential source for low-cost ethanol production is to utilize lignocellulosic materials such as crop residues (straws, hulls, stems, stalks), grasses, sawdust, wood chips, and solid animal waste. Development and implementation of such technologies would provide employment, reduce oil imports, improve air quality, and provide a natural solution to the disposal of solid wastes. An improvement in the organisms and processes for the bioconversion of lignocellulose to ethanol offers the potential to increase efficiency and reduce the production cost of fuel ethanol to that of petroleum.

Cellulase costs are a critical factor with respect to improving the economics of ethanol production. One approach to increase the effectiveness of cellulase utilization is to develop recombinant microorganisms, which provide some of the enzymes necessary for cellulose solubilization, minimizing the accumulation of soluble inhibitory products and reducing the requirement for fungal cellulase.

8.6.3 Xylanase

Xylan is a major structural polysaccharide in plant cells and one of the most abundant organic substances in nature, with a high potential for degradation to useful end products. It is a heteroglycan composed of a linear chain of xylopyranose residues bound by β(1 → 4) linkages, with a variety of substituents linked to the main chain by glycosidic or ester linkages. Due to the structural heterogeneity of xylan, complete degradation requires the synergistic action of different xylanolytic enzymes such as endoxylanase, β-xylosidase, α-glucuronidase, α-arabinofuranosidase, and esterase. Endo-1-4-β-xylanase is the most important as it initiates the degradation of xylan into xylo-oligosaccharides and xylose. Xylan is found in large quantities in hardwoods from angiosperms (15–30% of the cell wall content) and softwoods from gymnosperms (7–10%), as well as in annual plants (<30%), and is typically located in the secondary cell wall of plants.

8.6.3.1 Sources

Xylanases are widespread among an array of organisms including bacteria, algae, fungi, protozoa, gastropods, and anthropods. Some of the most important fungal sources include strains of *Aspergillus* and *Trichoderma*. The habitats of these microorganisms are diverse and typically include environments where plant materials accumulate and deteriorate, as well as in the stomach of ruminants.

Although numerous organisms produce this class of enzyme, filamentous fungi are the preferred choice for commercial production due to their high

specificity, mild reaction conditions, and high level of enzyme production compared to other sources. Fungal xylanases are generally active at mesophilic temperature (40–60 °C) with a slightly acidic pH; however, xylanases have also been reported to be active in extreme environments. The majority of these have been found to be members of families 10 and 11. Psychrophilic fungi such as *Penicillium* spp., *Alternaria alternate*, and *Phome* spp. have been isolated from the Antarctic environment. Common features of these xylanases are a low temperature optimum, high catalytic activity at low and moderate temperatures, and poor stability.

8.6.3.2 Application

Xylanases are produced by a large number of different fungal strains; however, commercial production is more or less restricted to *Trichoderma* spp. and *Aspergillus* spp. Advancements such as increased activity, thermostability, and stability under acidic and alkaline conditions may enhance the repertoire of fungal xylanases utilized in industry. The potential biotechnological applications of xylan and xylanases have been of major interest to researchers. The major end products of xylan, furfural, derived from agricultural residues, and xylitol, obtained from wood residues, are of major importance.

Inclusion of xylanolytic activities in industry has been widely documented and they have found application in the food and beverage industries, for example, to improve the properties of dough and the quality of baked products, as well as in the clarification of juice and wines. In addition, they are used in the poultry industry to increase feed efficiency and improve the nutritional value of silage and greenfeed. Other less well-documented applications include in brewing to increase wort filterability in addition to reducing haze in the final product, and in coffee extraction in the preparation of soluble coffee.

The widest application for xylanase is in the paper and pulp industry where environmental regulations have put a restriction on the usage of chlorine in the bleaching process. The by-products formed during chemical processing are toxic, mutagenic, and bioaccumulating, and cause numerous harmful disturbances in biological systems. Biobleaching has now replaced traditional methods and involves using microorganisms and enzymes to bleach pulp. Microbial xylanase cleaves the xylan backbone and enhances the accessibility of lignin in wood fibers to bleaching chemicals, such as chlorine dioxide (ClO_2), and can reduce the amount of bleaching chemicals required to produce pulps of desired brightness value. Besides bleaching, the use of xylanase helps increase pulp fibrillation, reduces the beating times, hence reducing energy consumption, and increases the freeness in recycled fibres.

The number of xylanase enzymes in production is on the increase; for instance, the United States Patent and Trademark Office has listed 468 patents since 2001. Stringent environmental regulations and awareness to reduce the

emission of greenhouse gases have added an incentive for future research developments in the study of xylanases.

8.6.4 Amylase

Amylases, including α-amylase, β-amylase, and glucoamylase, are perhaps the most important enzymes in present-day biotechnology due to their wide-ranging application in numerous industrial processes, including the food, fermentation, textiles, and paper industries. Such is their success that microbial amylases have successfully replaced the chemical hydrolysis of starch in starch-processing industries. The starch biopolymer consists of α-D-glucose joined together in two polymeric units, amylose and amylopectin.

Amylose can be considered as a linear molecule with glucose units linked through α-l,4 bonds with a double helical crystalline structure containing six D-glucose molecules per turn. In contrast, amylopectin is a highly branched structure with an average branch chain of 20–25 glucose units in length and with 4–6% α-1,6 bonds at branch points.

Amylopectin may have a molecular weight in excess of 10^8, making it one of the largest molecules in nature. Starch granules are round or irregular in shape and in the raw or unprocessed state are between 1 and 100 μm long, being held together with internal hydrogen bonds.

Amylolytic enzymes occur widely and are produced by many species of fungi. While many different microorganisms produce amylases, obtaining a strain capable of producing commercially acceptable yields remains challenging. Commercial production of amylolytic enzymes is carried out by two main methods, submerged fermentation and solid-state fermentation. In recent times, solid-state fermentation has gained in popularity for the production of starch-saccharifying enzymes.

Thermostability of amylase enzymes has become a feature of most of the enzymes with industrial application. As a consequence, thermophilic microorganisms are of special interest for the production of thermostable amylases.

8.6.4.1 α-Amylase

α-Amylases (also known as endo-1,4-α-D-glucan glucohydrolyase, 1,4-α-D-glucan glucanohydrolase, and glycogenase) are extracellular enzymes which randomly cleave 1,4-α-D-glucosidic linkages that occur between adjacent glucose units in linear amylase chains. These endo-acting enzymes hydrolyze amylase chains in the interior of the molecule and are classified according to their action and end products. Those that produce free sugars are termed "saccharogenic" and those that liquefy starch without producing free sugars are known as "starch-liquefying." By hydrolyzing randomly along the starch chain, α-amylase breaks down

long-chain carbohydrates such as starch, yielding maltotriose and maltose from amylose, or maltose, glucose and limit-dextrin from amylopectin. Due to its ability to cleave starch indiscriminately, α-amylase tends to be faster acting than β-amylase.

α-Amylase is produced commercially from bacterial and fungal sources including strains of *Aspergillus* and *Bacillus*. In general, bacterial α-amylase is preferred over fungal amylase due to its characteristic thermostability. Typically, *Bacillus amyloliquefaciens* and *Bacillus licheniformis* are employed for commercial applications. The use of thermostable α-amylase can minimize contamination risk in addition to reducing reaction time and also minimize the polymerization of D-glucose to iso-maltose. Filamentous fungi have been assessed for their ability to produce α-amylase. The thermophilic fungus *Thermomyces lanuginosis* has been shown to produce high levels of α-amylase with interesting thermostability properties. A strain of *Aspergillus kawachii* has been used to produce high levels of acid-stable α-amylase using solid-state fermentation. *Pycnoporus sanguineus* has been identified as a source of α-amylase and cultivation in solid state resulted in higher enzyme production than in submerged fermentation. Finally, a strain of *Rhizopus* has been found that produces a thermostable α-amylase under solid-state conditions.

8.6.4.2 β-Amylase

β-Amylases (also known as 1,4-α-D-glucan maltohydrolase, glycogenase, and saccharogen amylase) are typically of plant origin, but a few microbial strains which produce this enzyme have also been identified. Being an exo-acting enzyme, it cleaves nonreducing chain ends of amylose, amylopectin, and glycogen molecules through hydrolysis of alternate glycosidic linkages, yielding maltose. As this enzyme is unable to cleave α-1,6-glycosidic linkages in amylopectin, it results in incomplete degradation of the molecule, producing only 50–60% maltose and a β-limit dextrin. Unlike other amylase enzymes, only a small number of β-amylases of microbial origin are produced commercially. The enzyme has primarily been characterized from bacterial strains, although fungal strains such as *Rhizopus* have been reported to synthesize β-amylase.

8.6.4.3 Glucoamylase

Glucoamylase (also known as glucan 1,4-α-glucosidase, amyloglucosidase, exo-1,4-α-glucosidase, lysosomal α-glucosidase, and 1,4-α-D-glucan glucohydrolase) hydrolyzes single glucose monomers from nonreducing ends of amylose and amylopectin. Unlike α-amylase, however, most glucoamylases are also able to cleave the 1,6-α-linkages at the branching points of amylopectin, albeit at a reduced rate. Glucose, maltose, and β-limit dextrins are the end products of

glucoamylase hydrolysis. The majority of glucoamylases are multidomain enzymes consisting of a catalytic domain connected to a starch-binding domain. Glucoamylase enzymes have been isolated from numerous microbial sources. Filamentous fungi, however, constitute the major source of glucoamylase which is widely used in the manufacture of glucose and fructose syrups.

Commercially speaking, glucoamylase is typically produced from solid-state cultures of *A. niger* using numerous agro-industrial waste residues such as wheat bran, rice bran, rice husk, gram, wheat, and corn flour, tea waste, and copra waste. In addition, strains of *Aspergillus awamori* have been frequently utilized for glucoamylase production, as have numerous *Rhizopus* isolates. Interestingly, *Rhizopus* strains can be classified into four groups based on their soluble-starch-digestive glucoamylase (SSGA) and raw-starch-digestive glucoamylase (RSGA) activities. Glucoamylase is also produced by yeast cultures such as *Saccharomyces cerevisiae*, *Saccharomycopsis fibuligera*, *Candida fennica*, *Candida famata*, and *Endomycopsis fibuligera*.

8.6.4.4 Application of Amylolytic Enzymes

Starch-containing agricultural biomass can be used as a potential substrate for the production of gaseous or liquid fuels, feed proteins, and chemicals through microbial conversion. These substrates include corn (maize), wheat, oats, rice, potato, and cassava, which on a dry basis can contain around 60–75% (wt/wt) of starch.

Starch liquefaction is achieved by dispersion of insoluble starch granules in aqueous solution, followed by a partial hydrolysis using thermostable amylases. Industrially, the starch suspension for liquefaction is generally in excess of 35% (w/v) and the viscosity is extremely high following gelatinization. Thermostable α-amylase is used to effect a reduction in viscosity and the partial hydrolysis of starch. Before the introduction of thermostable amylases, starch liquefaction was achieved by acid hydrolysis (hydrochloric or oxalic acids, pH 2, and 140–150 °C for 5 minutes). The introduction of thermostable amylases has brought about the use of milder, more environmentally friendly processing conditions. The formation of by-products is reduced and refining and recovery costs are lowered

Starch represents a high-yielding ethanol resource and production has been reported from numerous materials such as corn, wheat, potatoes, cassava, and corn stover. Ethanol production is reliant on the use of fungal saccharifying enzymes, as the carbohydrates in the raw materials are not directly fermentable by most yeast. Starch is first hydrated and gelatinized by milling and cooking, and then broken down by amylolytic enzymes which pre-treat the starch and hydrolyze it into simple sugars. These sugars can then be converted by yeast into ethanol for use in many different applications.

Starch conversion into high-fructose corn sweeteners or syrups (HFCS) is of major industrial importance. Due to their high sweetening property they can be

used to replace sucrose syrups in foods and beverages. HFCS is produced by first milling corn to produce corn starch. This is then treated with α-amylase to produce shorter oligosaccharide sugars. Glucoamylase from strains of *Aspergillus* breaks the sugar chains down even further to yield glucose. This is subsequently converted by xylose isomerase into a fructose-rich syrup containing approximately 42% fructose. A final purification step yields a high fructose corn syrup with a fructose content of 90%.

Amylase enzymes also find use in bread making. The main component of wheat flour is starch, from which amylases can produce smaller weight dextrins for the yeast to utilize, imparting flavor and causing the bread to rise. Amylase enzymes are added into the bread improver, thus making the overall process faster and more practical for commercial use.

8.6.5 Lipases

Lipases (triacylglycerol acylhydrolases) are a class of hydrolytic enzyme which are widely distributed among microorganisms, plants, and animals. Their principal function is to hydrolyze triglycerides into diglycerides, monoglycerides, fatty acids, and glycerol, but they can also catalyze esterification, interesterification, and transesterification reactions in nonaqueous media. Approximately 35 different lipases are commercially available and one of the main advantages of lipases is the fact that they can be used not only in water, but also in water–organic solvent mixtures, or even in pure anhydrous organic solvents. This versatility enables lipases to have potential applications in the food, detergent, pharmaceutical, leather, textile, cosmetic, and paper industries.

8.6.5.1 Sources

Lipases from a large number of fungal sources have been characterized and shown to have a wide range of properties, depending on their source, with respect to positional specificity, fatty acid specificity, thermostability, and activity optima. Strains of fungi producing lipase of commercial interest include *Rhizopus* spp., *Rhizomucor miehei*, *Geotrichum candidum*, *Pichia burtonii*, and *Candida cylidracae. Rhizomucor miehei* lipase is probably the most used lipase obtained from fungi, even being used as a model for the determination of the structure of some other lipases.

8.6.5.2 Application

Microbial lipases are an important group of biotechnologically valuable enzymes and it is anticipated that the market for lipase will continue to grow, mainly in

detergent and cosmetics markets. At present, lipases are considered to be one of the largest groups of enzymes, based on their commercial use in a billion-dollar business that comprises a wide variety of different applications. Lipases are mainly used as biological catalysts to manufacture food ingredients or in the making of fine chemicals. Novel biotechnological applications for lipases have been demonstrated, such as in the synthesis of biopolymers and biodiesel, the production of enantiopure pharmaceuticals, agrochemicals, and flavor compounds. The potential also exists for the manufacture of industrially important chemicals which are typically manufactured from fats and oils by chemical processes and that could be generated by lipases with greater rapidity and better specificity under mild conditions.

Their value as biocatalysts lies in their ability to act under mild conditions, their high stability in organic solvents, broad substrate specificity, and a high degree of regio- and/or stereoselectivity in catalysis. These enzymes are active under ambient conditions and this reduces the impact of reaction conditions on sensitive reactants and products.

Lipases have found extensive application in the dairy industry, where they are used for the hydrolysis of milk fat. Current uses include enhancement of flavor in cheese, acceleration of cheese ripening, and lipolysis of butterfat and cream. The use of lipase in milk fat hydrolysis endows many dairy products, such as soft cheese, with specific flavor characteristics. For example, the addition of lipases that primarily release short-chain (mainly C4 and C6) fatty acids promotes a sharp, tangy flavor, while the release of medium-chain (C12, C14) fatty acids imparts a soapy taste to the product.

The promotion of free fatty acid release by lipolytic enzymes enables their participation in simple chemical reactions and can initiate, for instance, the synthesis of flavor ingredients such as acetoacetate, beta-keto acids, methyl ketones, flavor esters, and lactones. A whole range of fungal lipase preparations have been developed for the cheese manufacturing industry, including enzymes from *M. miehei*, *A. niger*, and *A. oryzae*.

In the food industry, some fats have greater value because of their structure. However, lipase-catalyzed transesterification of cheaper oils can enable cheaper fats to be used, a good example of which is the production of cocoa butter from palm mid-fraction. Lipase-catalyzed transesterification in organic solvents is an emerging industrial application and has been used for the production of cocoa butter equivalent, human milk fat substitute, and pharmaceutically important polyunsaturated fatty acids, and in the production of biodiesel from vegetable oils.

Mucor miehei and *Candida antarctica* lipase have been successfully employed in the esterification of free fatty acids in the absence of organic solvent and in the transesterification of fatty acid methyl esters in hexane with isopropylidene glycerols. Interesterification using an immobilized *M. miehei* lipase has been utilized for the production of vegetable oils such as corn oil, sunflower oil, peanut oil, olive oil, and soybean oil containing omega-3 polyunsaturated fatty acids.

The use of lipases to carry out industrial hydrolysis of tallow has a number of advantages, and significantly the heat requirement is reduced, thereby decreasing the consumption of fossil fuels and obviously the environmental impact. Additionally, there is less degradation of unsaturated fatty acids due to the lowered reaction temperature and, as a consequence, pure, natural fatty acids can be obtained from highly unsaturated oils. With regard to their nutritional value, undegraded polyunsaturated fatty acids may be important to preserve in the production of food additives such as mono- and diglycerides.

Lipases play a number of roles in the textile industry, where they have been used for the removal of size lubricants and as commercial preparations for the desizing of cotton fabrics. The most commercially significant application of lipases is in their addition to household and industrial detergents. Nowadays, detergents typically contain one or more enzymes, such as protease, amylase, cellulose, and lipase. These can reduce the environmental impact of detergent products, since they not only save energy by enabling a lower wash temperature to be used but also allow the content of other chemicals in detergents to be reduced. Additionally, lipase and other enzymatic components are biodegradable, leave no harmful residues, have no negative impact on sewage treatment processes, and do not present a risk to aquatic life.

The first commercial lipase, Lipolase, originated from the fungus *Thermomyces lanuginosus*. Due to the low yield of enzyme, the gene encoding the protein was cloned and subsequently expressed in *A. oryzae*. This enzyme has been extensively used in the detergent and textile industries. One of the most commercially successful lipase preparations is a recombinant *Humicol*a enzyme which has been heterologously expressed in *A. oryzae* and again has application in the detergent sector.

8.6.6 Phytase

Phytase is an enzyme that has found widespread use as a feed additive in the animal industry due to its absence in the gastrointestinal tracts of monogastric species such as pigs and poultry. The enzyme hydrolyzes an antinutritional factor known as phytate or phytic acid to liberate inositol and inorganic phosphorus.

As an essential element, phosphorus is necessary for the growth and development of all organisms, playing key roles in skeletal structure and in vital metabolic pathways too numerous to mention. The negative effects of phosphorus-deficient diets on livestock performance are multifold and are well documented, including reduced appetite, bone malformation, and lowered fertility. To counteract this, an external source of phosphorus must be supplied in sufficient quantity to meet the daily requirements of the animal. This can result in the environment becoming overloaded with phosphorus in areas of intensive livestock production. The development of enzyme technology based on supplementing

diets with sources of microbial phytase has proven to be a practical and effective method of improving phytate digestibility in monogastric animal diets.

The principal storage form of phosphorus in feedstuffs of plant origin is the hexaphosphate ester of myoinositol, more commonly known as phytic acid, and it accounts for up to 80% of the phosphorus in grains and seeds. Phytic acid and its salts and esters are considered to be antinutritional factors due to their ability to bind essential minerals such as calcium, zinc, magnesium, and iron. They may also react with proteins, thereby decreasing the bioavailability of protein and other nutritionally important factors. Considerable interest has been focussed on the supplementation of animal feeds with exogenous (mainly microbial) sources of phytases, with the specific aim of liberating phytate-bound phosphorus in the gastrointestinal tract.

8.6.6.1 Sources

A broad range of microorganisms produce phosphohydrolases or phytases capable of catalyzing the hydrolysis of phytate. These include yeast such as *S. cerevisiae* and fungi such as *Aspergillus ficuum* which produce a highly thermostable, highly active phytase enzyme. Phytases have also been found in bacteria; however, they tend to display pH optima that are in the neutral to alkaline range and are therefore relatively inactive at monogastric stomach pH values.

Over 200 fungal isolates belonging to the genera *Aspergillus*, *Mucor*, *Penicillium*, and *Rhizopus* have been tested for phytase production. An additional survey of 84 fungi from 25 species for phytase production indicated that the incidence of phytase production is highest in *Aspergillus*. Of all the organisms surveyed, *A. niger* NRRL 3135 produces the most active extracellular phytase. This fungus produces two different enzymes, one with pH optima at 5.5 and 2.5, and one with a pH optimum of 2.0, which are designated PhyA and PhyB, respectively. In light of these considerations, the favored microbial source of exogenous phytase is filamentous fungi such as *A. niger*.

8.6.6.2 Application

The addition of microbial phytase to the feedstuffs of monogastric animals was described in the early 1980s. Published research shows that enzymatic treatment of feed using microbial phytase sources increases the bioavailability of essential minerals and proteins and provides levels of growth performance as good as or better than those with phosphate supplementation.

Obviously there is significant potential for the use of fungal phytase in view of the pollution caused by inorganic phosphorus supplementation and the legislative regulations to reduce this by at least 50% in the EU alone. In Europe, livestock production, and in particular pig production, has received much of the blame for

phosphorus pollution and has been targeted in an attempt to relieve the phosphorus burden of the land. Legislation against phosphorus pollution has been adopted in many countries; farmers must operate within a legal limit per unit of land. For instance, in the Vendée region of France, the amount of phosphorus may not exceed 44 kg/ha (100 kg P_2O_5/ha).

Current global estimates are that animal feed producers with combined annual production of 550 million metric tonnes of animal feed presently use (or will soon use) phytase as a supplementary enzyme in diet formulations. The commercial potential for supplementation with phytase preparations is therefore quite obvious. However, a major drawback to the widespread use of phytases in animal feed is the constraint of thermal stability (65–95 °C) required for these enzymes to withstand inactivation during the feed-pelleting or expansion processes. As such, a commercially successful phytase must be able to withstand brief heating at elevated temperatures prior to being administered to monogastric target species. In addition, for industrial applications in animal feed, a phytase of interest must be optimally active in the pH range prevalent in the digestive tract.

8.7 Enzyme Production Strategies

Fungal enzyme production is mainly through the use of submerged fermentation strategies, although a second method known as solid-state fermentation or the Koji process is used extensively in Asia. We will concentrate on submerged fermentation strategies; a typical flow diagram of the process is depicted in Figure 8.1.

Broadly speaking, the fermentation stages involved in the production of fungal enzymes are relatively similar. Large batch fermentations using inexpensive culture media are scaled up in a similar manner to antibiotic production. The downstream processing steps though can vary widely and this is dependent upon the ultimate process in which the enzyme preparation will be used. Similarly, those that are produced intracellularly, such as lactase, will require additional processing steps to those that are produced on an extracellular basis. Generally, though, most industrial enzymes are produced extracellularly in large batch fermentations and require little in the way of downstream processing. In fact, fewer processing steps are preferable as this will lead to a reduction in enzyme losses. Only enzymes and material likely to interfere with the catalytic process for which the enzyme is required will be removed. Unnecessary purification is to be avoided, as each step is costly in terms of equipment, manpower, and enzyme loss. As a consequence, some industrial enzyme preparations are a simple mix of concentrated fermentation broth and additives to stabilize the preparation. If required, though, a powder preparation can be prepared from a fermentation broth. Treatments such as salt or ethanol precipitation will result in the generation of a protein suspension from the spent culture media, which can then be filtered and dried to recover an extremely concentrated enzyme preparation.

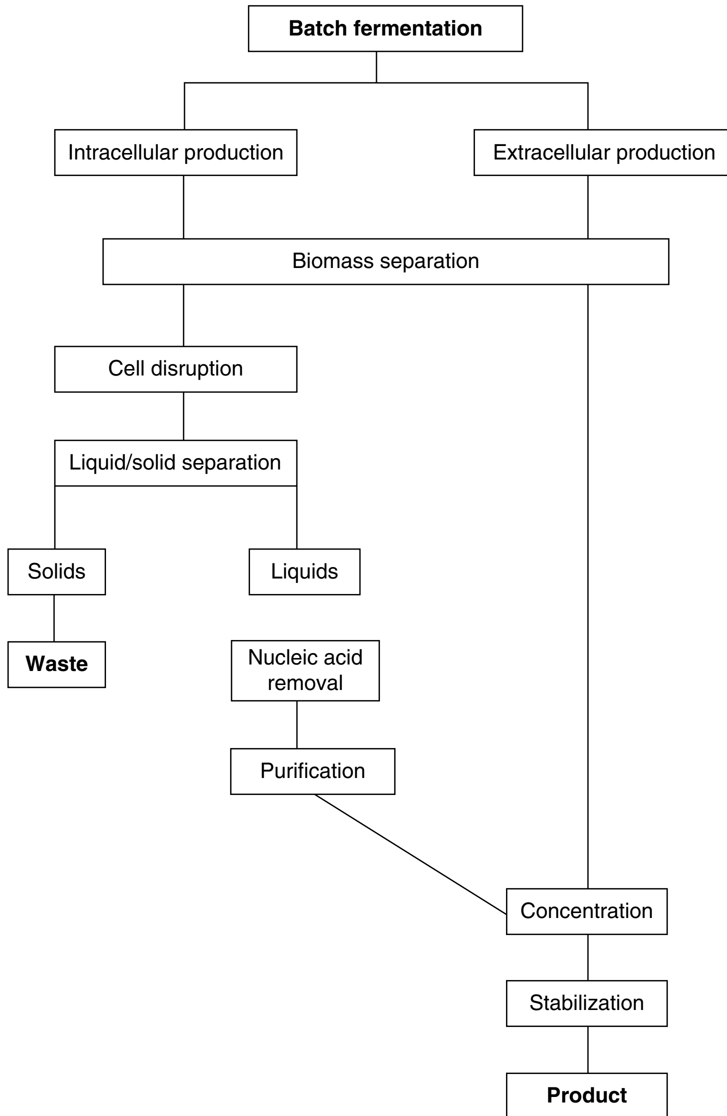

Figure 8.1 Flow diagram of fungal enzyme production.

8.8 Conclusion

Enzymes have long played a central role in mankind's attempts to utilize biological systems for a variety of purposes. Examples of harnessing their power include cheese making, brewing, baking, the production of antibiotics, and in the manufacture of commodities such as leather, indigo, and linen. They also find applications in areas such as detergent and paper production, the textile industry, and in the food and drinks industry in products ranging from fruit juice, coffee and tea, to wine.

Further Reading

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9

Biotechnological Exploitation of Heterologous Protein Production in Fungi

Brendan Curran and Virginia Bugeja

9.1 Introduction

Heterologous, or recombinant, proteins are produced using recombinant DNA technology to express a gene product in an organism in which it would not normally be made. Unlike the synthesis of recombinant DNA, a relatively simple procedure involving the cutting and joining together of DNA sequences from different organisms, recombinant protein production is fraught with difficulty because of the need to transcribe and then translate heterologous DNA into a correctly folded protein with the appropriate biological activity.

Initially developed in *Escherichia coli*, heterologous protein expression technology is now possible in scores of prokaryotic and eukaryotic host systems, including gram-positive and -negative bacteria, yeasts, filamentous fungi, insect cells, plants, mammalian cells, and transgenic animals.

Heterologous gene expression systems are available for an extremely wide range of fungi, including yeasts such as *Saccharomyces cerevisiae* and *Pichia pastoris*, and many of their filamentous cousins including *Neurospora*, *Aspergillus*, and *Penicillium* species. Some heterologous proteins have been produced for use in basic research, others for commercial exploitation. Here we restrict ourselves to a very specific brief: the production of biotechnologically relevant heterologous proteins in fungi.

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9.2 Heterologous Protein Expression in Fungi

Regardless of their intended use, heterologous protein production requires the following steps: the insertion of the desired heterologous DNA coding sequence into appropriate regulatory sequences in specialized expression vectors, and a transformation procedure for the introduction of the construct into the desired host species. This has then to be followed by the transcription and translation of this sequence into biologically active protein molecules – a process that can require post-translational modifications such as glycosylation.

9.2.1 Heterologous DNA

The DNA sequence due to be expressed into heterologous protein can be genomic in origin, thereby possessing introns and/or regulatory sequences from the original organism from which it has been cloned. Alternatively it can be a cDNA sequence derived by reverse-transcription of the heterologous mRNA, in which case it will lack introns and regulatory sequences. Filamentous fungi and many yeast species are capable of excising introns accurately from the mRNA transcripts of heterologous genes, and indeed some filamentous species can recognize heterologous regulatory signals from human DNA. However, efficient expression almost always requires the heterologous mRNA to be driven from the promoter of a strongly expressed host cell gene. Therefore, although heterologous genomic DNA has been successfully expressed in a small number of fungi, commercially important heterologous protein expression is normally initiated in these organisms by inserting the appropriate cDNA sequence into an expression vector that already encodes appropriate promoter and terminator sequences.

9.2.2 Vectors

In addition to the backbone of bacterial plasmid DNA, which is common to expression vectors in all systems and facilitates DNA manipulation/large-scale plasmid purification in *E. coli*, fungal expression vectors carry:

- A selectable marker for the intended fungal host.
- A strong promoter to drive the production of the heterologous mRNA.
- Appropriate DNA sequences to ensure efficient termination of transcription and polyadenylation of the mRNA.
- Appropriate sequences to ensure the correct initiation and termination of translation.

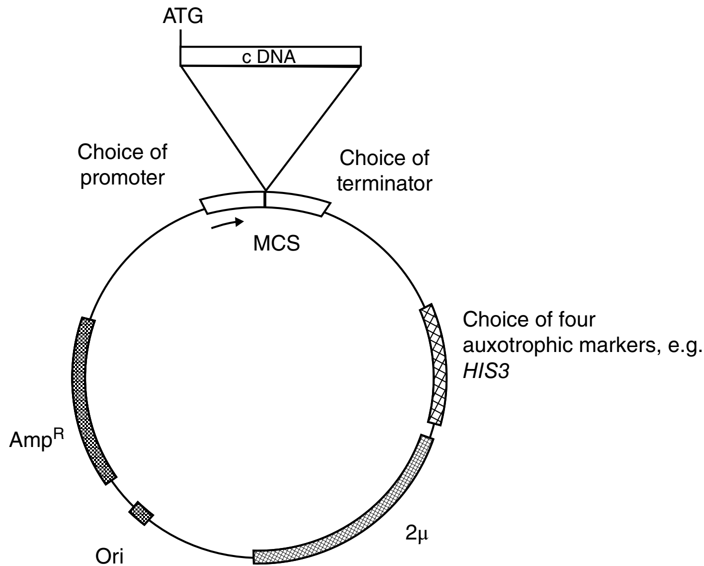

Figure 9.1 A generalized expression vector for use in *S. cerevisiae.*

The most commonly used shuttle vectors for biotechnological applications in the yeast *S. cerevisiae* are autonomously replicating because they carry appropriate sequences (Figure 9.1) from yeast 2 μ DNA – a native yeast plasmid. On the other hand, many of the vectors designed for use in the methylotrophic yeast *Pichia pastoris* (see below), and in filamentous fungi such as *Penicillium* and *Aspergillus* species, are integrative vectors (Figure 9.2a) that require the heterologous DNA to be incorporated into the host cell chromosomal DNA (Figure 9.2b).

9.2.3 Transformation and Selection

Although DNA manipulation can be achieved easily in *E. coli*, for protein expression to occur the heterologous DNA construct has to be transferred into the fungal host. The constructs are therefore transformed into the fungal host and colonies of transformants identified using appropriate selective agar plates. Although there are a number of dominant selectable markers conferring antibiotic resistance on the transformed cells (hygromycin B resistance is a particularly versatile one), many yeast and filamentous systems exploit auxotrophic marker complementation for selection. In these cases, the plasmids carry the appropriate wild-type information to complement auxotrophic alleles (*Leu2*$^-$, *His3*$^-$, *Trp1*$^-$, etc.) in the host cells. The type of transformation processes used include: enzymatically removing the cell walls and exposing the resulting protoplasts to the

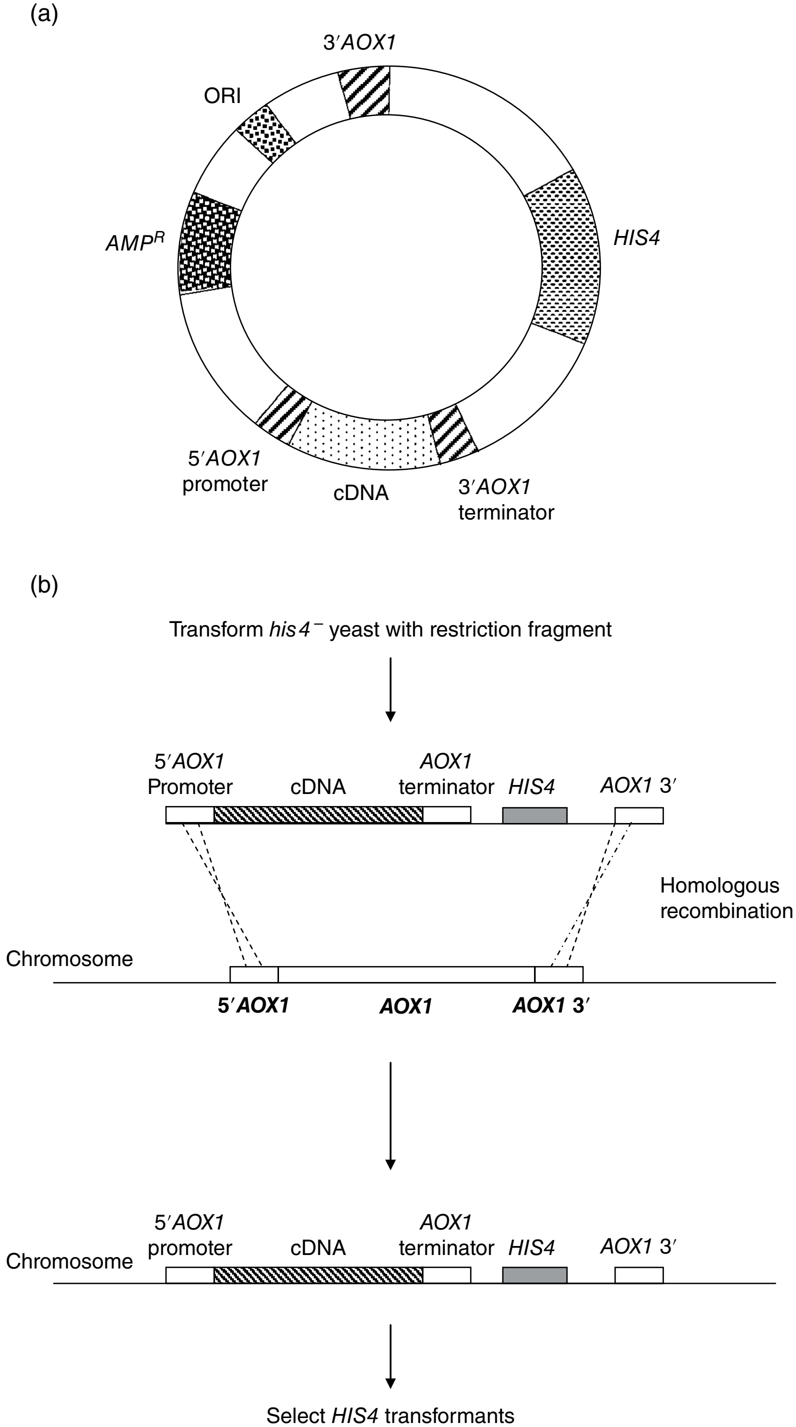

Figure 9.2 Structure (a) and integration (b) of a *P. pastoris* expression vector.

DNA in the presence of calcium ions and polyethylene glycol; electroporation of yeast cells and fungal protoplasts; and transformation of yeast cells by treating them with alkali cations (usually lithium) in a procedure analogous to *E. coli* transformation.

9.2.4 Host Systems

The type of protein product required determines the fungal host that is used. There are two basic types of biotechnologically relevant proteins: enzymes, used in a wide variety of industrial applications, which have a low unit value and are required in bulk, and proteins of therapeutic value, which have a high unit value and are required in more limited amounts.

Filamentous fungi (especially *Aspergillus* species) are the host systems of choice for the high-level production of a wide variety of enzymes for industrial applications. The enzymes produced in this way include amylases (used to convert corn starch for ethanol fermentation), proteases and lipases in detergents, cellulases used in paper production, and many others. This invariably involves the expression of heterologous enzymes from other fungal species, and these products can be recovered at levels up to grams per liter (Table 9.1). However, for a variety of reasons, including protease contamination and inefficient secretion of these products, filamentous fungi are relatively inefficient as hosts for the expression of heterologous proteins of animal/human origin.

Therefore yeasts, and in particular *S. cerevisiae*, are preferred as hosts for the production of therapeutically important proteins (Table 9.2). Here we focus on the biotechnological development of the two most important members of this

Table 9.1 Representative selection of heterologous proteins produced in filamentous fungi.

Host	Product	Origin	Industrial use
A. niger	Glucoamylase	*Aspergillus*	Clarifying fruit juices
A. oryzae	Lipase	*Candida*, *Fusarium*, *Rhizomucor*, *Thermomyces*	Food, textiles, detergent, leather, pulp and paper
A. oryzae, *T. reesei*, *T. longibrachiatum*	Cellulase	*Trichoderma*	Textiles, fruit processing
A. niger, *A. oryzae*	Protease	*Aspergillus*, *Rhizomucor*, or calf stomach	Food, leather
A. niger	Glucose oxidase	*Aspergillus*	Stabilizing food and beverages

Table 9.2 Representative selection of heterologous proteins produced in yeast.

Host	Product	Origin	Therapeutic use
S. cerevisiae	Human papillomavirus vaccine	Human papillomavirus	Vaccine to protect against papillomavirus infection
S. cerevisiae	Glucagon	Human cDNA	Hormone involved in blood glucose level regulation
S. cerevisiae	Hepatitis B vaccine	Hepatitis virus	Vaccine to protect against hepatitis B infection
P. pastoris	Hepatitis B vaccine	Hepatitis virus	Vaccine to protect against hepatitis B infection
P. pastoris	Ecallantide	Human cDNA	A 60 amino-acid protein inhibitor of plasma kallikrein

group: *S. cerevisiae*, the oldest and most studied star, and *Pichia pastoris*, "the new kid on the block" but increasingly the host of choice. These yeast hosts are much more tractable in terms of genetic manipulation and product recovery than their filamentous cousins, and therefore most of the high-value heterologous protein production occurs in these fungi.

9.3 Use of *Saccharomyces cerevisiae* for Heterologous Protein Production

Saccharomyces cerevisiae was the first eukaryotic cell engineered to express heterologous proteins because it shared with *E.coli* many of the characteristics that make the latter such a useful host for recombinant DNA technology. *Saccharomyces cerevisiae* grows rapidly by cell division, has its own autonomously replicating plasmid, can be transformed as intact cells, and forms discrete colonies on simple defined media. In addition, *S. cerevisiae* can carry out post-translational modifications of expressed proteins – essential features of many heterologous proteins that *E. coli* is unable to provide. Furthermore, it secretes a small number of proteins into the growth medium, which can be exploited to simplify the purification of heterologous proteins. Finally, it has a long safe history of use in commercial fermentation processes, and unlike *E. coli*, *S. cerevisiae* does not produce pyrogens or endotoxins. These parameters taken together make it particularly suitable for approval by regulatory bodies charged with the responsibility of ensuring the safe production of medically important heterologous proteins.

Despite the versatility of yeast expression systems, the production of high levels of biologically active heterologous proteins is still largely a matter of trial and error. The recovery of satisfactory levels of authentic heterologous protein depends on a number of factors, including the type of expression vector used, the site of protein expression, and the type of protein being expressed.

9.3.1 Expression Vectors

A wide range of yeast cloning vectors is available for use in *S. cerevisiae*, but here we restrict ourselves to the self-replicating variety most commonly used for biotechnological applications (Figure 9.1). These YEp (yeast episomal plasmid) vectors are based on the ARS (autonomously replicating sequence) from the endogenous yeast 2 μ (so called because of its unique length) plasmid. They are present at 20–200 copies per cell and under selective conditions are found in 60–95% of the cell population. Integrative vectors similar to those used in *Pichia* (Figures 9.2a and 9.2b) can also be used in *S. cerevisiae*, and indeed one variant of this uses homologous recombination to target multiple copies of the gene construct into middle-repetitive δ DNA sequences generated by the activity of the Ty transposable element. However, in the majority of cases, plasmid-borne expression vectors are used commercially.

9.3.2 Regulating the Level of Heterologous mRNA in Host Cells

The overall level of heterologous mRNA in the cell is determined by the copy number of the expression vector, the strength of the promoter used to drive transcription, and the stability of the specific mRNA sequence.

Expression vectors based on YEp technology have a high copy number but require selective conditions to ensure their stable inheritance. High-level mRNA production is also dependent upon the type of promoter chosen to drive expression (Table 9.3). The most frequently encountered are based on promoters from the highly expressed genes that encode glycolytic enzymes. These include phosphoglycerate kinase (*PGK*), alcohol dehydrogenase 1 (*ADH1*), and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), all of which facilitate high-level constitutive mRNA production. Constitutive expression can be disadvantageous when the foreign protein has a toxic effect on the cells. This can be circumvented by using a regulatable promoter to induce heterologous gene expression after cells have grown to maximum biomass. There are a number of regulatable promoters available. One of the most useful ones is based on the promoter of the galactokinase gene (*GAL1*), which undergoes a 1,000-fold induction when glucose is replaced by galactose in the medium.

Table 9.3 Promoters used to direct heterologous gene expression in *S. cerevisiae.*

Promoter	Strength[1]	Regulation	Example of heterologous gene expressed using promoter
PGK (3-phosphoglycerate kinase)	++++	Constitutive	Human β-interferon
ADH1 (alcohol dehydrogenase 1)	+++	Constitutive	Human β-interferon
GAPDH (glceraldehyde-3-phosphate)	++++	Constitutive	Human epidermal dehydrogenase growth factor
GAL1 (galactokinase)	+++	1,000 × induction	Calf chymosin by galactose
PHO5 (alkaline phosphatase)	++	500 × repression	Hepatitis B surface antigen by phosphate
CUP1 (copperthionein)	+	20 × induction by copper	Mouse IG kappa chain

[1] Relative levels of mRNA expression when promoter is active.

Regardless of the choice of promoter, it is important that transcription of the heterologous mRNA is terminated properly; otherwise, abnormally long mRNA molecules, which are often unstable, can be generated by read-through along the plasmid DNA. It is for this reason that expression vectors frequently contain the 3′ terminator region from a yeast gene (e.g. *CYC1*, *PGK*, or *ADH1*) to ensure efficient mRNA termination (Figure 9.1).

9.3.3 Ensuring High-Level Protein Production

The level of heterologous protein produced by the host depends upon the efficiency with which the mRNA is translated, and the stability of the protein after it has been produced.

It is vitally important to address control of protein translation and subsequent translocation when choosing the expression vector. The sophisticated translation initiation mechanism found in mammalian cells is absent from yeast. Therefore, in order to ensure efficient initiation of translation, it is important to genetically engineer mammalian cDNAs to remove regions of dyad symmetry and noncoding AUG triplets in the leader sequence of heterologous mRNAs upstream of the AUG encoding the first methionine in the protein.

Once expressed, some proteins form insoluble complexes in *S. cerevisiae*, but many others do not. Other proteins can be produced as denatured, intracellular complexes, which can be disaggregated, and renatured after harvesting. The first

Table 9.4 Signal sequences used to direct secretion of heterologous proteins from *S. cerevisiae.*

Signal sequence	Cellular location of gene product	Example of secreted heterologous protein
Invertase	Periplasm	α-1-Antitrypsin
Acid phosphatase	Periplasm	β-Interferon
α-Factor mating pheromone	Culture medium	Epidermal growth factor
Killer toxin	Culture medium	Cellulase

recombinant DNA product to reach the market was a hepatitis B vaccine produced in this way. Some proteins are rapidly turned over by ubiquitin degradative pathway in the cell, while others are degraded by vacuolar proteases. This can be especially true during cell breakage and subsequent purification. The powerful tools provided by a detailed knowledge of yeast genetics and biochemistry can be used to minimize this problem in *S. cerevisiae*.

The use of protease-deficient host strains can improve both the yield and the quality of heterologous proteins. One mutant (*PEP4-3*) is widely used because it is responsible for the activation of inactive vacuolar zymogen proteases; in its absence a wide range of proteinase activities is therefore prevented. Even more impressively, our detailed knowledge of the yeast secretory pathway can be exploited to genetically engineer the heterologous protein so that it is smuggled out of the cell before it can be degraded by either vacuolar proteases or the ubiquitin degradative pathway. Indeed, secretion not only minimizes the exposure of heterologous proteins to protease activity but also, because *S. cerevisiae* only secretes a handful of proteins, facilitates the recovery and purification of heterologous proteins. Two of these (invertase and acid phosphatase) are targeted to the periplasmic space, which lies between the cell membrane and cell wall; the other two (α factor and killer toxin) are secreted out beyond the wall into the culture medium (Table 9.4). Entry into the secretory pathway is determined by the presence of short hydrophobic "signal" sequences on the N-terminal end of secreted proteins. The DNA sequence for these signal peptides can be genetically engineered onto the DNA sequence for the heterologous protein of choice – thereby ensuring that it is targeted for export after being synthesized. The "signal" sequences from all four of S. *cerevisiae*'s secretion proteins have been used in this way with varying degrees of success. A secretion vector that encodes the α-factor signal peptide is shown in Figure 9.3. A number of medically important proteins, including insulin, interferon, and interleukin-2, have been successfully secreted using this type of signal peptide.

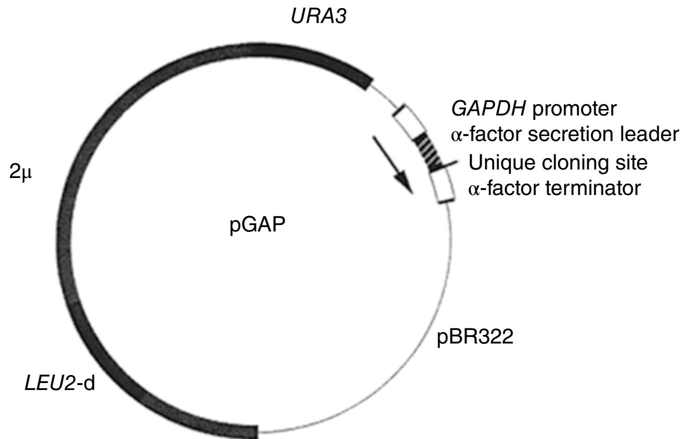

Figure 9.3 A secretion vector for use in *S. cerevisiae.*

9.3.4 Ensuring Authentic Protein Structure and Function

The objective of heterologous gene expression for commercial purposes is not just the high-level transcription and translation of the appropriate DNA. Proteins are produced because of their structure, and therefore what is required are appropriately folded, biologically active, authentic protein molecules. Many proteins of therapeutic importance undergo sophisticated post-translational modifications in mammalian cells. These vary from the removal and/or addition of small chemical moieties, such as the removal of the N-terminal methionine, or the addition of acetyl group to the N-terminal amino acid (acetylation), through the addition of large lipid molecules to generate lipoproteins, to the complex addition of countless sugar moieties to proteins as they are synthesized and passed through the cell's endoplasmic reticulum (ER) and Golgi apparatus to produce glycosylated proteins. Simple prokaryotic expression systems like *E. coli* are unable to carry out many of these processes and it was for that reason that eukaryotic expression systems were developed. Although not ideal in all respects, *S. cerevisiae* offers solutions to at least some of these problems. When *E. coli* failed to produce properly acetylated human superoxide dismutase, *S. cerevisiae* obliged by intracellularly expressing a soluble active protein identical to that found in human tissue – complete with acetylated N-terminal alanine.

Moreover, the yeast secretion system affords options with respect to ensuring that proteins are folded correctly, N-terminal methionines are removed, and sugar residues are added to glycoproteins. A direct comparison between the intracellular production and extracellular secretion of prochymosin and human serum albumin resulted in the recovery of small quantities of mostly insoluble, inactive protein when they were produced intracellularly, but the recovery of soluble, correctly folded, fully active protein when they were secreted.

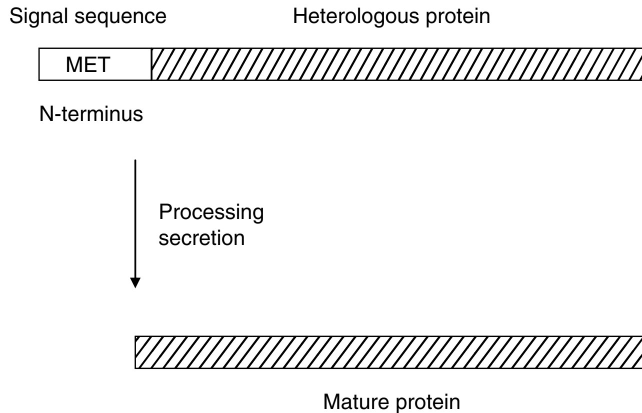

Figure 9.4 Cleavage of a secretory signal sequence from a heterologous protein.

Secretion can also be used to produce proteins that have an amino acid other than methionine at their N-terminus. If a secretory signal is spliced onto the heterologous gene at the appropriate amino acid (normally the penultimate one), then the N-terminal methionine, which is obligatory for translation initiation, will be on the secretory signal. Proteolytic cleavage of this signal from the heterologous protein in the ER will generate an authentic N-terminal amino acid (Figure 9.4). Glycosylation in yeast is of both the N-linked (via an asparagine amide) and O-linked (via a serine or threonine hydroxyl) types, occurring at the sequences Asn-X-Ser/Thr and Thr/Ser respectively. However, it is important to note that the number and type of outer core carbohydrates attached to glycosylated proteins in yeast are different to those found on mammalian proteins. Therefore if the protein is being produced for therapeutic purposes, they may cause unacceptable immunogenicity problems. One approach to overcoming this problem is to prevent glycosylation of the protein by using site-directed mutagenesis of the DNA in *E. coli* to alter one amino acid, thereby removing the glycosylation recognition site before expressing the protein in *S. cerevisiae*. This strategy was successfully used to produce urokinase-type plasminogen activator.

9.3.5 Limitations

Despite its ability to express a wide variety of proteins, *S. cerevisiae* has limitations. Its very primitive glycosylation system frequently hyperglycosylates heterologous proteins. Also the production of alcohol during glucose metabolism limits the generation of biomass and therefore heterologous protein product. However, another yeast species, *Pichia pastoris*, which can grow to much higher cell densities and does not hyperglycosylate its proteins, has been developed as an alternative host system for the production of heterologous proteins. It has yet to

Table 9.5 Heterologous proteins of therapeutic use produced in yeasts.

Period	Fungal hosts	Number of products	Types of products
1985–1990	*S. cerevisiae*	1 (1986)	First recombinant hepatitis B vaccine
1991–1995	*S. cerevisiae*	1	Growth factor for stimulating bone marrow
1996–2000	*S. cerevisiae*	10	Five vaccines, an anticoagulant, a tissue growth factor, three hormones
	P. pastoris	1	Hepatitis B vaccine
2001–2005	*S. cerevisiae*	8	Five vaccines, two hormones, one therapeutic enzyme
	P. pastoris	2	Insulin and interferon
2006–2010	*S. cerevisiae*	4	Three hormones, one vaccine
	P. pastoris	1	Ecallantide, a protein inhibitor of plasma kallikrein

outscore *S. cerevisiae* in terms of the number of therapeutic proteins produced to date (Table 9.5), but new technological developments in glycobiology, which are explained below, mean that it is set to eclipse its cousin in the very near future.

9.4 Use of *Pichia pastoris* for Heterologous Protein Production

Pichia pastoris is one of the methylotrophs, a small number of yeast species belonging to the genera *Candida*, *Torulopsis*, *Pichia*, and *Hansenula* which share a specific biochemical pathway that allows them to utilize methanol as a sole carbon source. The promoters of the genes that encode the enzymes for this pathway are extremely strong and exquisitely sensitive to the presence or absence of methanol, making them ideal for the regulation of heterologous gene expression. Offering the ease of genetic manipulation associated with *S. cerevisiae*, these species have a number of advantages over their ethanol-producing cousin:

- They grow to much higher cell densities in fermenters due to the absence of toxic levels of ethanol.
- They use integrative vectors, which removes the need for selective media to be used in fermenters while at the same time offering greater mitotic stability of recombinant strains.
- They have a more authentic type of glycosylation pattern for heterologous products.

Of these species, *P. pastoris* is by far the most popular choice when it comes to producing high-value heterologous proteins of therapeutic value. With a popular commercial kit (Invitrogen, San Diego) widely available, *P. pastoris* has been used to express over 100 heterologous proteins for both research and commercial purposes. Selection of transformants for heterologous gene expression commonly relies on complementation of an auxotrophic *his4* marker in the host cells, although a number of dominant selectable markers are also currently available. Unlike *S. cerevisiae*, integrative vectors (as opposed to autonomously replicating plasmids) are normally used in this yeast species. The gene of interest is spliced in between the promoter and terminator sequences of the *AOX1* gene in an *E. coli* vector, which also carries the *His4*+ gene and further downstream of this the 3′ end of the *AOX1* gene (Figure 9.2). A linear fragment bounded by AOX1 sequences is then transformed into a *His4*$^-$ host. This DNA construct can then undergo homologous recombination targeting the gene of interest into the chromosomal locus of the *AOX1* gene (Figure 9.2b). Such cells can grow either on methanol using an alternative alcohol oxidase locus, in which case the heterologous protein is continuously expressed, or on glucose, in which case the heterologous gene is repressed until induced by methanol. The tight level of regulation allows for extremely precise control of the expression of the heterologous gene. Integrative vectors are also available that target the constructs to the *His4* locus. Quite apart from the fact that this easily regulated promoter has practical advantages over the more cumbersome galactose-inducible ones used to regulate heterologous expression in *S. cerevisiae*, *P. pastoris* is regarded as a more efficient and more faithful glycosylator of secreted proteins. The most widely used secretion signal sequences include the *S. cerevisiae* α-factor pre-pro sequence and the signal sequence from *Pichia*'s own acid phosphatase gene.

A number of proteins of therapeutic importance (Table 9.5) have been successfully made in *Pichia*. However, this modest number is set to increase sharply because *Pichia* has been subjected to extensive genetic manipulation to engineer strains of yeast that can produce human glycoproteins which are identical in every detail to those found in the human body. This genetic manipulation entailed knocking out four genes (to prevent yeast-specific glycosylation) and introducing 14 additional glycosylation genes to generate strains of *P. pastoris* capable of producing uniformly glycosylated, sialic acid-capped proteins. Many human proteins of therapeutic value are glycoproteins that must possess the correct sugar structures in order to have authentic biological activity and to prevent unwanted immunological complications. Heretofore, yeast cells have not been able to reproduce the required pattern of sugar moieties on heterologous proteins, but now *P. pastoris* can do so, and indeed their ability to produce essentially uniform *N*-glycosylation is superior to that even of mammalian cells, which are the usual hosts of choice when glycosylation is a key issue of concern in heterologous protein production.

9.5 Case Study: Hepatitis B Vaccine – A Billion-Dollar Heterologous Protein from Yeast

The production of recombinant hepatitis B subunit vaccines in yeast is one of the major success stories of molecular biotechnology. Produced as a heterologous protein originally in the yeast *S. cerevisiae* and more recently in both *P. pastoris* and *Hansenula polymorph*, the phenomenal success of the hepatitis B vaccine makes it an ideal candidate with which to illustrate the research and development of a biotechnologically important heterologous protein.

9.5.1 Hepatitis: A Killer Disease and Huge Market Opportunity

Hepatitis B (HB), a double-shelled virus in the class Hepadnaviridae (Figure 9.5), is responsible for the death of more than 250,000 people per annum. The liver infections caused by this organism can manifest in one of two different modes: either acute or chronic viral hepatitis. In acute hepatitis, the virus is completely cleared from the body when the symptoms disappear. In chronic hepatitis, the virus persists in the liver after infection, and the patient becomes a carrier of the disease. The chronic mode of the infection is regularly associated with progressive cirrhosis and primary hepatocellular carcinoma. Worldwide, a staggering 200 million people carry the disease.

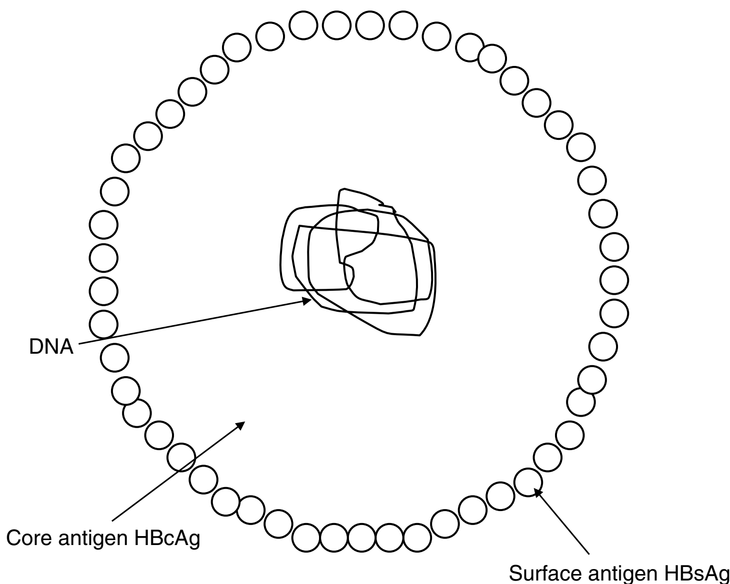

Figure 9.5 Hepatitis B virus.

In the majority of cases, antiviral interferon therapy is either unavailable or, in the cases where symptoms have already developed, ineffective. Therefore vaccination offers the only useful medical intervention with respect to this disease. In brutal biotechnological terms, the production of an effective HB vaccine constitutes a huge market opportunity. That is why so many companies develop and sell these products.

9.5.2 A Vaccine from Infected Carriers of the Disease

The first commercially available HB vaccine was prepared from protein particles of viral origin isolated from the plasma of chronic carriers of the disease. These extremely immunogenic 22-nm subviral particles, noninfective byproducts of viral replication, consist of multiple molecules of a glycoprotein, called the HB surface antigen (HBsAg), embedded in a phospholipid membrane of cellular origin. These noninfective particles appear as if they are complete 42-nm viral particles to the immune system. They therefore elicit a strong immune response against the coat of the virus and confer resistance to subsequent viral infection.

First licensed in 1981 and used for over 10 years in the United States, this vaccine was safe, effective, and well tolerated. It was less than ideal, however, because it depended upon a continuous supply of plasma, and required extensive processing and safety testing. Although expensive to produce, the vaccine would have been more widely acceptable except for unbased fears that, despite elaborate safety precautions to prevent contamination, it had the potential to contain infective HB viruses, or other bloodborne diseases (such as HIV), originating from the donor plasma.

Biotechnologists therefore turned to recombinant DNA technology to circumvent the problems associated with the human-derived product. However, given that genetic manipulation was in its infancy in the early 1980s and that at that time heroic efforts were needed to express even a simple human protein in *E. coli*, the expression of an effective heterologous HB vaccine was a tall order indeed. Such a challenging objective required genetic engineers to separate the DNA sequence encoding the HBsAg protein from the rest of the viral genome and then to arrange to have the information transcribed and translated into an immunogenic protein in a suitable host cell.

9.5.3 Genetically Engineering a Recombinant Vaccine

Using the previously sequenced 3,200-base HB genome, the short DNA sequence encoding the 226 amino acids that comprise the major surface protein was isolated, spliced in frame with a strong promoter in an *E. coli* expression vector, and transformed into the appropriate cells. Although the viral

protein was expressed, it was not glycosylated. Furthermore, the host cells failed to produce the 22-nm phospholipid-protein particles. The unassembled human HBsAg protein was known to be 1,000 times less immunogenic than the 22-nm plasma-derived particles, and scientists were not surprised therefore when the *E. coli* recombinant protein failed to elicit an appropriate immune response in animals.

However, undaunted by this failure, scientists then attempted to exploit recombinant DNA technology that had just been developed to facilitate basic gene cloning in the simple eukaryotic organism *S. cerevisiae*. It was hoped that this yeast, a eukaryote capable of glycosylating and secreting proteins, would be able to produce immunogenic particles of glycosylated proteins where *E. coli*, a prokaryote, had failed. In a proof of principle experiment, the DNA encoding the HBsAg was spliced downstream of the yeast alcohol dehydrogenase (*ADH1*) promoter in an *E. coli*-based shuttle vector carrying a 2 μ replication origin and the *TRP1* gene (Figure 9.6a). After transformation and selection in a *trp1* host, not only were the resulting transformants found to express substantial levels of HBsAg protein, but also the proteins aggregated into phospholipid particles similar to those found in the plasma from human carriers of the disease. Unlike the majority of HBsAg proteins synthesized in humans, however, the yeast-expressed protein lacked glycosylation, and rather than being secreted, it accumulated inside the cells. Despite the lack of appropriate glycosylation, these particles elicited the appropriate immunological response when tested in animals, indicating that glycosylation was not needed for assembly of the particles or for immunogenicity.

9.5.4 From Proof of Principle to Industrial Scale-up

Having used a basic expression vector to demonstrate that yeast could produce immunogenic 22-nm phospholipid-protein particles, thereby circumventing the problems associated with the bloodborne source of the vaccine, an improved expression vector (Figure 9.6b) was developed as a prelude to industrial scale-up of vaccine production. A comparative analysis of the industrial vector and the vector used in the proof of principle experiments illustrates many of the molecular subtleties associated with ensuring high-level heterologous protein production in yeast.

As can be seen in Figure 9.6, both plasmids are shuttle vectors carrying segments of plasmid DNA from *E. coli* and both are based on the yeast 2 μ plasmid. However, whereas the proof of principle vector has a *TRP1* marker, the industrial one has a *LEU-2d* gene. This gene has a truncated promoter and, as it is ineffectively transcribed, the cell requires a higher copy number of the plasmid encoding it in order to be able to grow in the absence of leucine. On average each cell has 150 –300 copies of a *LEU-2d*-carrying plasmid per cell, as against approximately 30 copies of the *TRP1*-carrying plasmid. The industrial vector

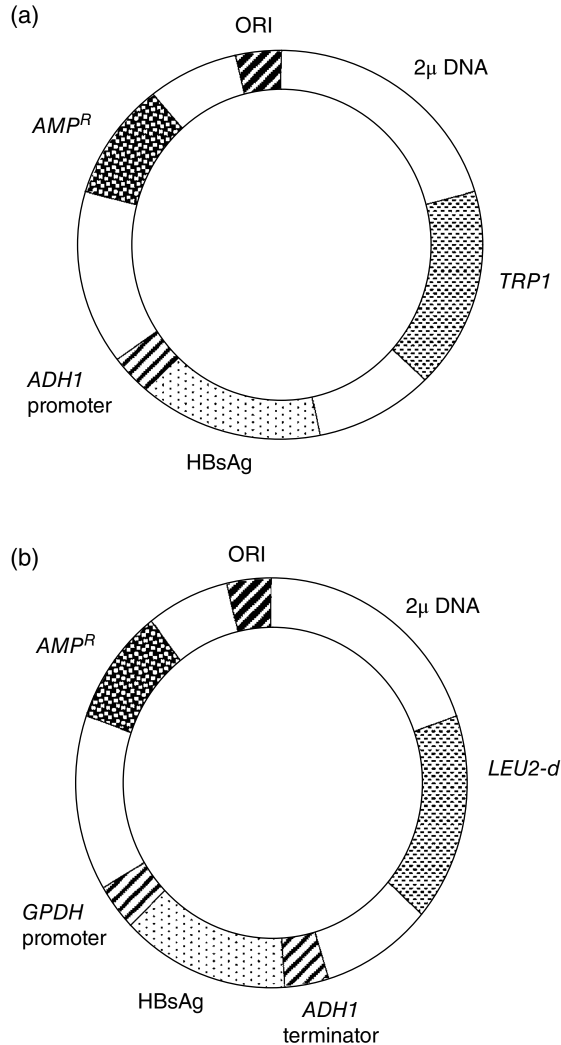

Figure 9.6 Proof of principle (a) and scale-up (b) plasmids.

also has a terminator sequence to ensure efficient termination, whereas the proof of principle vector lacks such a sequence. Finally, the reasonably strong *ADH1* promoter of the proof of principle vector has been replaced in the industrial one by an extensively characterized, highly efficient *GPDH* promoter.

In short, in addition to the basic expression modules found in the proof of principle vector, the industrial one has DNA sequences to ensure a higher plasmid copy number, more efficient transcription initiation, and more efficient termination of transcription. All of these lead to high-level mRNA production, thereby ensuring that the level of protein expressed is significantly higher in the industrial strain than in the proof of principle host.

9.5.5 Much More than a Development in Expression Technology

The development of high-level HB vaccine production in *S. cerevisiae* illustrated some of the subtleties of gene regulation and heterologous protein expression – aspects of which can still confound biotechnologists today. It also marked the first commercial exploitation of gene expression in a eukaryotic host, and a new era in vaccine development. However, above and beyond expression technology, the greatest impact of this billion-dollar protein has been the protection it affords millions of individuals against a debilitating, often deadly, disease.

9.6 Further Biotechnological Applications of Expression Technology

Quite apart from producing proteins of commercial value, protein expression can be manipulated in *S. cerevisiae* to provide *in vivo* tools with which to probe molecular interactions. These sophisticated heterologous protein expression systems re-engineer promoter elements, transcription factors, and signal cascade proteins to transduce heterologous molecular interactions into easily scoreable phenotypes. Such assays enable biotechnologists to screen for molecules that interfere with/enhance these interactions – so-called lead molecules in drug development. Reporter genes and growth on selective media have been used to examine the molecular biology of expressed heterologous steroid and peptide receptor proteins, respectively, whereas the elegant two-hybrid technique, which exploits the modular nature of transcription factors, provides a window into intracellular interactions between proteins.

9.6.1 Expression and Analysis of Heterologous Receptor Proteins

Oestrogen is an important human hormone that has been linked to breast cancer. As with many steroid hormones, oestrogen affects gene expression by binding to a cytoplasmically sequestered receptor protein, and this complex then enters the cell's nucleus, where it binds to promoters containing a specific receptor recognition sequence referred to as the oestrogen receptor element (ERE). Normal yeast cells do not contain either the receptor or ERE sequences. However, yeast cells have been re-engineered so that a β-galactosidase gene fused to a disabled *CYC1* promoter carrying the ERE resides at the *URA3* chromosomal locus (Figure 9.7a). The same cells also carry a plasmid constitutively expressing the receptor protein intracellularly (Figure 9.7b). When these cells are now treated with oestrogen, the hormone binds to the receptor and enters the nucleus where it then binds to the ERE cloned into the promoter in front of the β-galactosidase gene, thereby inducing expression. This enzyme can be easily assayed. The level of enzyme

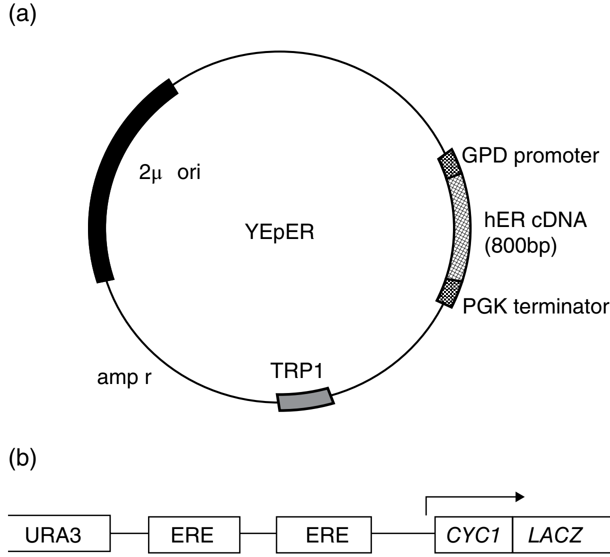

Figure 9.7 Analysis of oestrogen receptor proteins.

detected in the cells is then an index of oestrogen-induced receptor activity, allowing antagonists to be tested on the cells to identify which ones are best at inhibiting the hormone's activity. This reporter system was sufficiently sensitive to analyze the effect of site-specific mutations on hormone binding efficiency and to measure the effectiveness of agonists and antagonists on hormone action.

Unlike steroid hormone receptors, which when activated by hormone bind directly to promoter elements in the DNA, peptide hormones bind to receptor proteins that are embedded in the cell membrane. These molecular interactions are then communicated to the nucleus by kinase cascades (enzymes that add phosphate groups to proteins), ultimately resulting in the phosphorylation of a transcription factor which induces gene expression via specific promoter elements. This type of signal transduction is controlled by so-called G-proteins (a heterotrimeric protein complex activated when a constitutively bound GDP molecule is replaced by a GTP molecule) that reside in the membrane next to the receptors. These protein complexes consist of three subunits: α, which is the subunit in contact with the receptor protein; and β and γ, which initiate the kinase cascade. When the receptor is activated by binding of the appropriate peptide, the α subunit, which has a GDP molecule bound to it, undergoes a conformational change during which the bound GDP molecule is replaced by a GTP molecule with concomitant dissociation of the β and γ subunits. The latter translocate to yet another membrane protein that then initiates the kinase cascade – ending in the phosphorylation of a specific transcription factor and activation of gene expression. The precise structure of the receptors and G proteins, and the TF/promoter elements that they influence, varies from one organism to the next. Nevertheless, this overall cellular strategy for the transduction of a membrane signal into altered gene expression is highly conserved. It is

for this reason that the extremely well-characterized mating-signal-transduction pathway of *S. cerevisiae* can be re-engineered to analyze the interactions of human peptides and their target membrane receptors (Figure 9.8).

Haploid cells secrete small peptides, which bind to receptors in the membranes of cells of the opposite mating type in order to synchronize their cell cycles for mating. The binding of α-factor from α cells to the receptor protein of a normal haploid cell of "a" mating type causes the activation of a G-protein in close contact with the receptor. The resulting kinase cascade causes a number of

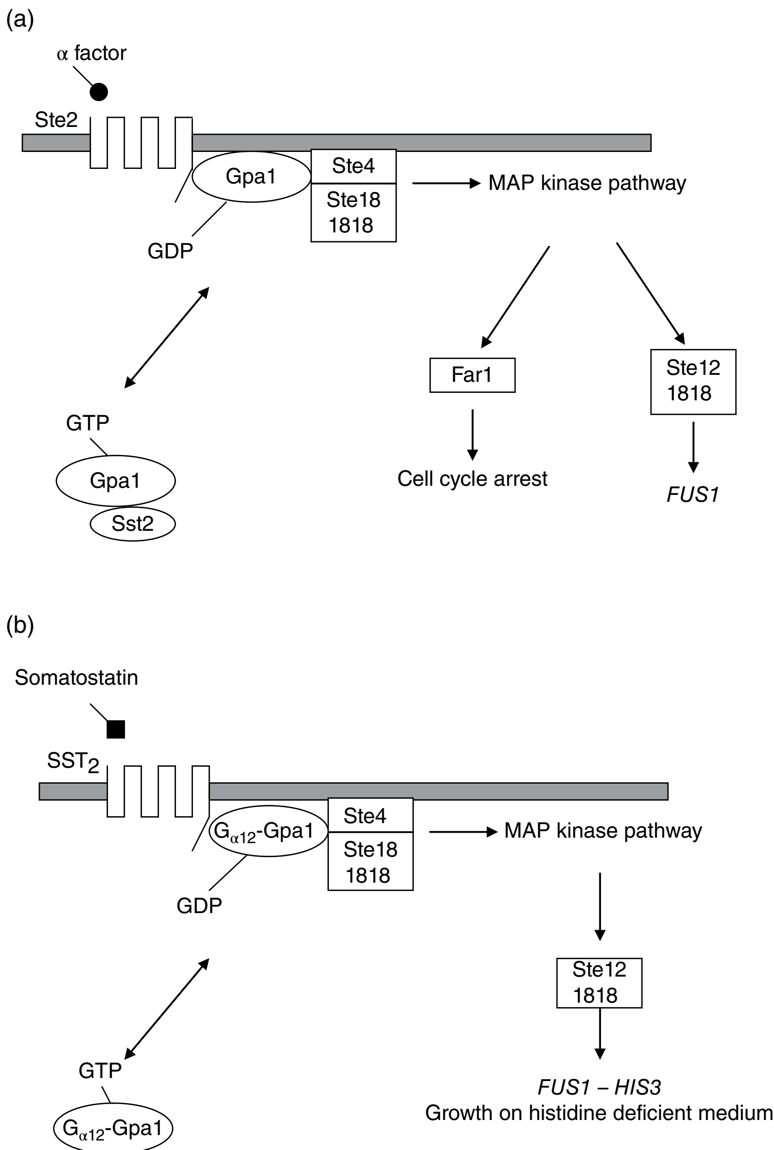

Figure 9.8 Heterologous receptor analysis using a re-engineered yeast pathway.

alterations in the cell's gene expression pattern. These include the activation of genes to arrest the cell in G1 of the cell cycle, and the expression of gene products ready for cell and then nuclear fusion as the synchronized cells mate together. With a view to using yeast cells in a high-throughput screening format, yeast cells have been manipulated to provide a "readout" of cell growth in a selective medium. The success of this extremely elegant approach to developing tools for screening drugs hinges on the fact that the mating pathway of yeast cells of "a" mating type could be re-engineered such that they:

- Expressed a human receptor protein instead of the α-factor receptor.
- Expressed an α subunit of the G-protein re-engineered so it could interact with the human receptor protein while retaining the segment that interacted with the yeast β and γ subunits.
- Carried an *HIS3* wild-type gene driven by a promoter sensitive to activation by the TF normally activated by the mating pathway kinase cascade.
- Carried a deleted *FAR1* gene in order to prevent cell-cycle arrest when the kinase cascade is activated.

These cells no longer respond to the addition of α-factor because they lack the α-factor receptor protein. However, when the appropriate human peptide is added to the cells, it binds to the heterologous human receptor protein in the membrane (Figure 9.8). The conformational change this causes is detected by the "humanized" α subunit of the G-protein. When GTP replaces GDP in this subunit the β and γ subunits dissociate, thereby initiating the kinase signal cascade to the nucleus. The activated transcription factor binds to the promoters of the genes normally induced by α-factor and to the promoter driving the *HIS3* gene product, thus conferring an HIS+ phenotype on the cell. *Far1* activation would normally ensure that the cells arrest in G1 of the cell cycle, but as it has been deleted in this strain the cells progress through G1, allowing them to divide in His-selective medium.

In short, this re-engineered yeast cell transduces the heterologous human receptor–agonist interaction into a scoreable HIS+ phenotype. As G-protein-coupled receptors represent the targets for the majority of presently prescribed pharmaceutical drugs, this system has exciting potential for the development of high-throughput screening technology.

9.6.2 Hybrid Analysis: An Expression System that Turns Protein–Protein Interactions into a Scoreable Phenotype

This extremely clever expression system hinges on the fact that transcription factors consist of two separate domains: the DNA binding and activation domains (DBD and AD, respectively). When expressed separately from different expression

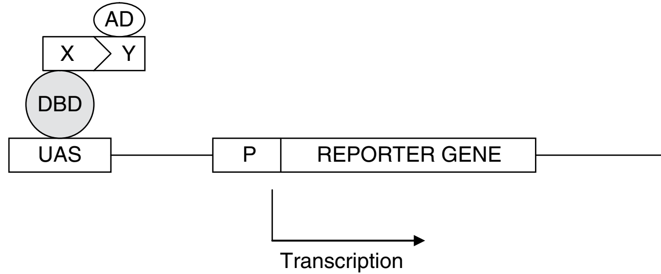

Figure 9.9 The two-hybrid system – only when "X" interacts with "Y" can the reporter gene get activated.

vectors the two domains cannot interact and therefore a functional TF cannot be constructed. However, if the domains are expressed as fusion proteins with two proteins (X and Y) and the two proteins interact with one another in the cell, then the two TF domains are brought into contact with one another and a functional TF is regenerated, which can then drive gene expression (Figure 9.9).

Yeast vectors are available in which the DNA encoding the DBD of the yeast *GAL4* transcription factor and the DNA sequences encoding the AD of the same protein are on separate 2 μ-based expression plasmids carrying different selectable markers. The genes for the proteins under analysis for potential interaction with one another are inserted as gene fusions with the sequence for the DBD and AD respectively in these vectors. The plasmids are transformed into separate haploid cells carrying complementary genetic markers, defective in *HIS4*, and one of which has integrated into its chromosomal DNA a gene construct consisting of a GAL4-inducible promoter fused to the coding sequence for HIS4+. The two strains are then mated. If the two proteins interact then this will bring the DBD and AD together in the cell, thereby reassembling the dissociated TF. Such cells will express the His4 gene product and be able to grow on selective His medium. In cases where the expressed fusion proteins do not interact, the two TF domains remain apart and although the DBD can bind to the His4 promoter it cannot activate transcription because it lacks the AD (Figure 9.9). Such a diploid strain will be unable to express *HIS4* and will therefore not grow on the selective medium. Not only has this so-called yeast two-hybrid system been successfully applied to study a broad spectrum of protein–protein interactions from many different species, but also it can be used to detect small molecules that interfere with the targeted protein–protein interaction, thereby providing yet another tool for drug discovery.

9.7 Conclusion

The production of heterologous proteins in fungi includes the production by filamentous fungi of heterologous proteins (mainly of fungal) origin for industrial applications, the production of high-value therapeutic proteins mainly by yeast

species, and the use of yeast cells to probe the protein–protein interactions of heterologous proteins from a wide range of organisms. Although heterologous proteins production was initially an empirical science, with hosts being tested to identify which one(s) provided the most authentic and highest yielding protein production, the next-generation technologies promise to be much more considered in their approach. The most impressive example of this to date is the successful re-engineering of the yeast *Pichia pastoris* to produce human proteins with glycosylation patterns that are more reproducible than those produced by mammalian host cells. However, this is just the beginning: global transcriptomic analyses of fungal cells are identifying the rate-limiting factors in the protein folding and secretion pathways, which are two rate-limiting aspects of heterologous expression. Guided by this much more rational approach, fungi in general, and yeast in particular, key players in this technology, are set to assume an even more central role in the production of heterologous proteins in the years to come.

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10
Fungal Infections of Humans

Derek J. Sullivan, Gary P. Moran, and David C. Coleman

10.1 Introduction

The ubiquity of fungi in the environment has already been alluded to in earlier chapters of this book. Fungal species have evolved and adapted to live in a wide variety of environments and ecological niches and consequently constitute a very diverse group of organisms. There are fewer than 100,000 species of fungi that have been identified to date; however, this is likely to be just the tip of the iceberg and it has been conservatively estimated that there are probably at least 1.5 million fungal species inhabiting our planet. Given this vast number of species and their prevalence in the environment it is hardly surprising that humans unwittingly come into contact with many different types of microscopic fungi every day and that humans offer a potential source of nutrients for some of these fungal species.

For the most part, transient exposure to fungi or fungal colonization occurs without the knowledge of the affected individual. This is primarily due to the inherent low virulence of most fungi, especially when confronted with the full arsenal of the human immune system. However, some species of fungi are long-established members of the human microflora (i.e. commensals in the oral cavity and the gastrointestinal tract) and under certain conditions some fungi can cause disease and even death. As many as 200 fungal species have been associated with human infections (known as mycoses); however, only a handful of these species are responsible for the majority of infections. Although, less well known than bacterial or viral pathogens, over a billion people are affected by fungal disease and approximately 1.5 million deaths are attributable to fungi annually. To put this in context, a similar number of people die from tuberculosis each year.

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Some of the most common microbial infections in humans are caused by microscopic fungi (e.g. thrush, dandruff, and "athlete's foot"). These are superficial and relatively innocuous; however, fungi can also cause far more devastating diseases, such as invasive aspergillosis and systemic candidiasis, both of which have very high associated mortality rates. The incidence of these latter infections has been increasing in recent decades and this has fueled a heightened interest in mycoses and the fungal species responsible for them among the clinical and scientific communities.

The types of infections caused by fungi can be classified in a number of ways. One division is based on whether the infection occurs in an otherwise healthy host (i.e. primary mycoses) or whether the host has an underlying medical condition causing impaired immune function (i.e. opportunistic mycoses). These groups of diseases can be further subdivided depending on whether the infection is confined to the outer layers of the epithelia (i.e. superficial mycoses) or whether the infecting organisms penetrate through this barrier into the bloodstream and disseminate throughout the body (i.e. systemic or disseminated mycoses).

One of the most exciting developments in the field of microbiology in recent years, made possible by the introduction of next-generation sequencing, has been the ability to study the entire microbial populations present in specific parts of the body (i.e. the microbiome). These studies, which have largely been directed towards the bacterial component of the microbiome, have identified that fungi in general only represent approximately 0.1% of the total human microbiota. However, despite being present in comparatively small numbers, these studies have confirmed the diversity of fungi and have also shown that a significant proportion of the mycobiome (all of the fungal species) present in niches such as the oral cavity are noncultivatable and taxonomically unclassified. Studies are currently underway to investigate how the mycobiome evolves and varies in health and disease and to identify how fungi interact with other members of the microbiome.

10.2 Superficial Mycoses

The human body is covered by skin, hair, and nails which, given their location in the body, are continuously exposed to the environment and, consequently, a wide variety of environmental microbes. Consequently, at any one time, approximately 25% of the human race has a fungal infection. For the most part, the keratinized epithelia which comprise the outer layers of the skin constitute an effective barrier that excludes microorganisms from gaining entry to deeper tissues and causing more dangerous diseases. In addition, the skin produces secretions, including sweat, sebum, transferrin, and antimicrobial peptides known as defensins that have the ability to kill many bacterial and fungal species. The skin is also equipped with intra-epithelial T and B cells as well as a range of phagocytes.

Table 10.1 Examples of superficial mycoses.

Site of infection	Disease	Examples of causative species
Skin	Pityriasis versicolor	*Malassezia furfur*
	Dandruff	*Malassezia globosa*
	Tinea nigra	*Hortaea werneckii*
	Ringworm (e.g. tinea capitis)	*Trichosporon/Microsporon*
	Athlete's foot (e.g. tinea pedis)	*Trichosporon/Microsporon*
Hair	White piedra	*Trichosporon beigleii*
Nail	Tinea unguium	*Trichophyton rubrum*
Subcutaneous	Chromoblastomycosis	*Fonsecaea*
	Sporotrichosis	*Sporothrix schenkii*
	Mycetoma	*Pseudallescheria boydii*

However, a small number of fungal species have evolved strategies for overcoming these defensive mechanisms and can actively colonize the skin surface, becoming established as members of the normal skin microbial flora.

From time to time (mainly for reasons that are still unclear) these fungi can cause disease (Table 10.1). Two examples of such infections are pityriasis versicolor and tinea nigra. The former is caused by a yeast-like organism known as *Malassezia furfur*. This species thrives on the fatty acids found in sebum secreted by the skin and affects pigment-producing cells, resulting in a pink rash on pale skin and hypopigmentation in darker skin. Interestingly, this species and the related species *Malassezia globosa* have also been associated with dandruff, a common ailment characterized by increased shedding of skin cells from the scalp. Tinea nigra, a rare dermatomycosis characterized by a rash caused by the mold species *Hortaea werneckii*, results from the production of melanin by the fungus that causes the formation of brown macular patches on the palms and soles of the feet. As well as infecting the skin, fungi can infect hair and nails. For example, fungi belonging to the genus *Trichosporon* cause a disease in hair known as white piedra (from the Spanish for stone), while *Trichophyton rubrum* causes the nail infection onychomycosis, also known as tinea unguium.

Due to the confinement of these infections to the extreme outer layers of the body there generally is no cellular immune response to the pathogens responsible for the disease. However, if the infecting fungi penetrate deep enough into the tissues to elicit an immune response the infections are referred to as cutaneous mycoses. The most important examples of these infections are known collectively as tinea (more commonly known as ringworm). These infections can occur in various locations in the body, ranging from the feet (i.e. tinea pedis, better known as athlete's foot) to the head (i.e. tinea capitis) and are caused by keratin-degrading

fungi collectively known as the dermatophytes, a group of organisms that includes species such as *Trichophyton* and *Microsporum*. These infections are usually self-limiting and can be treated relatively easily using topical antifungal drugs, such as members of the azole family and terbinafine, although in severe cases oral drugs may be administered.

Rarely, fungi manage to penetrate deeper through the epidermis and cause infection in the underlying subcutaneous tissues (sometimes penetrating as deep as underlying muscle and bone). These infections are usually the result of the fungus gaining access to these tissues following trauma (e.g. wounds, splinters, and bites). Examples of these infections are chromoblastomycosis, sporotrichosis, and mycetoma.

10.3 Opportunistic Mycoses

As mentioned previously, the innate and adaptive human immune systems are remarkably adept at protecting the human body from infection by fungi. Consequently, in normal healthy individuals systemic fungal infections are relatively uncommon. However, some fungi can capitalize on defects in the host (see Table 10.2 for specific risk factors) and overgrow and cause infection. These infections (Table 10.3) are known collectively as opportunistic mycoses, due to

Table 10.2 Examples of risk factors for opportunistic fungal infection.

Risk factors
HIV infection and AIDS
Solid-organ transplantation
Anticancer chemotherapy
Granulocytopenia
Premature birth
Old age
Use of corticosteroids
Use of broad-spectrum antibiotics
Central vascular catheters
Gastrointestinal surgery
Colonization with fungus (e.g. *Candida* spp.)

Table 10.3 Examples of opportunistic mycoses.

Causative species	Disease
Candida	Oropharyngeal candidiasis/denture stomatitis (OPC) Vulvovaginal candidiasis (VVC) Chronic mucocutaneous candidiasis (CMC) Invasive candidiasis (IC)
Aspergillus	Invasive aspergillosis (IA) Aspergilloma Allergic bronchopulmonary aspergillosis (ABPA)
Cryptococcus neoformans	Cryptococcal meningitis
Pneumocystis jiroveci	Pneumocystis pneumonia

the fact that the fungi that cause them are opportunists that exploit the imbalance between the host and the pathogen that occurs, for instance, when patients' defense systems are not functioning adequately. The two most important opportunistic fungal pathogens are yeast species belonging to the genus *Candida* (which cause candidiasis) and molds belonging to the genus *Aspergillus* (which cause aspergillosis).

10.3.1 Candidiasis

The genus *Candida* comprises approximately 200 yeast species, most of which have no known teleomorphic (i.e. sexual) reproductive phase. They are ubiquitous in the environment (often associated with plants and animals), but little more than a dozen have been associated with human commensalism or disease (Table 10.4).

Most of these *Candida* species are carried innocuously by a large proportion of humans, particularly on the epithelial surfaces of the mouth, gastrointestinal tract, vaginal tract, and skin. They typically grow as ovoid blastospores; however, under specific conditions, most can produce filamentous cells known as pseudohyphae, while *Candida albicans* and *Candida dubliniensis* can produce true mycelium and refractile spore-like structures known as chlamydospores (Figure 10.1).

Although these species are usually harmless in healthy individuals, when the host's immune defenses are compromised in any way they have the potential to overgrow and cause infection, which can, depending on the circumstances, be severe. Predisposing factors (Table 10.2) to candidiasis include immunosuppression

Table 10.4 *Candida* species most commonly associated with human disease.

Species	Frequency
Candida albicans	Common
Candida glabrata	Common
Candida parapsilosis	Common
Candida tropicalis	Common
Candida dubliniensis	Infrequent
Candida krusei	Infrequent
Candida guilliermondii	Infrequent
Candida lusitaniae	Infrequent
Candida kefyr	Rare
Candida norvegensis	Rare
Candida famata	Rare
Candida inconspicua	Rare
Candida metapsilosis	Rare
Candida orthopsilosis	Rare

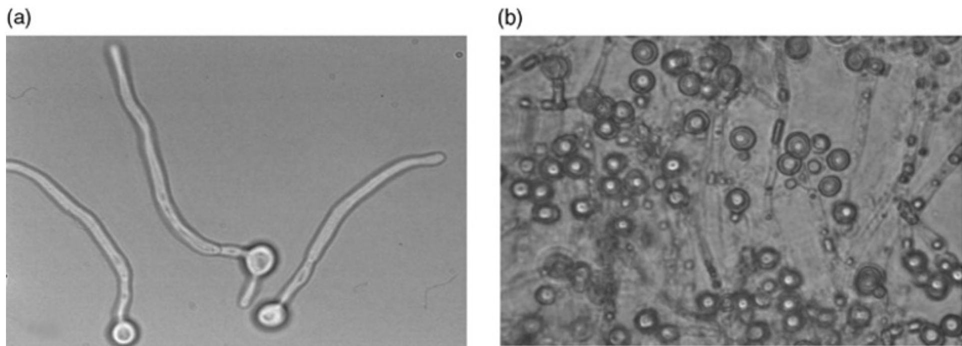

Figure 10.1 *Candida albicans* yeast cells (blastospores) producing (a) hyphae and (b) chlamydospores.

(e.g. due to HIV infection, anticancer therapy, and treatment with immunosuppressive drugs used in organ transplantation), catheterization (which allows the direct inoculation of the yeast cells into tissue and blood vessels), premature birth (immature immune system), extreme old age (defective immune system),

use of broad-spectrum antibiotics (disruption of the normal bacterial microflora), use of corticosteroids (disruption of local immune response), gastrointestinal surgery (direct inoculation of yeast cells into the bloodstream), and prior colonization with *Candida* species (most cases of candidiasis are acquired endogenously from the patient's normal microbial flora). Once *Candida* cells have overcome the (usually impaired) immune response they can cause a wide range of infections. These range from superficial infections of the skin and the mucous membranes of the oral cavity and the vagina to cases when the cells penetrate through the epithelia and are disseminated throughout the body by the blood to infect a wide variety of organs, including the kidney, liver, and brain.

One of the most common fungal infections is vulvovaginal candidiasis (VVC), an infection of the vulva and vaginal area, also known as vaginal thrush. This infection can occur in apparently otherwise healthy women, approximately 75% of whom will become infected at least once during their reproductive years. The symptoms of the disease include discomfort, itching, erythema, and the production of a white discharge. While VVC is usually easily treated using topical antifungal agents, a small proportion of women suffer from recurrent infections which are particularly recalcitrant to conventional therapies. The predisposing factors for VVC are not clear; however, an association with pregnancy, diabetes, antibiotic use, or HIV infection has been suggested.

Candida species are also associated with infections of the mucosal tissues of the mouth and oropharynx. These infections are known as oropharyngeal candidiasis (OPC) and can occur following antibacterial therapy or in individuals whose immune systems have been compromised, such as those infected with HIV, neonates with immature immune systems, patients receiving steroid therapy for asthma, and patients receiving head and neck radiotherapy for the treatment of cancer. In addition, patients wearing dentures who practice poor oral hygiene can present with overgrowth of *Candida* species and inflammation of oral tissues in contact with the denture (i.e. denture stomatitis).

There are several forms of OPC (Plate 10.1), the most common of which is known as pseudomembranous candidiasis (more commonly known as oral thrush). This is characterized by the presence of creamy-white patches (comprised of buccal cells, host protein exudate, and candidal yeasts and hyphae) overlying red patches primarily found on the palate and dorsum of the tongue. Other forms of OPC include erythematous candidiasis and angular cheilitis. OPC causes a high degree of discomfort, particularly when eating and swallowing.

It was originally treated using topical agents such as nystatin lozenges; however, severe cases are now treated with systemic oral azole drugs, especially fluconazole. Recurrent infections (due to acquired or intrinsic resistance to antifungals) can also occur in specific patient populations, and in these cases amphotericin B is often the most effective treatment option. OPC is most commonly observed in individuals infected with HIV. The majority of AIDS patients suffer from oral candidiasis at some stage during their disease progression, and

in the early stages of the AIDS epidemic OPC was often used as one of the diagnostic markers for HIV infection. On occasion the infection can extend into the esophagus, resulting in esophageal candidiasis. Fortunately, the introduction of highly active antiretroviral therapy (HAART) for the treatment of AIDS in the developed world has led to a significant reduction in the incidence of OPC in HIV-infected individuals.

Candida species can also cause infections of the nail bed and skin (e.g. nappy rash and intertrigo). In addition to these infections, *Candida* species can cause a more severe skin disease that is known as chronic mucocutaneous candidiasis (CMC). This infection can occur in HIV-infected patients, individuals with certain types of cancer (e.g. thymoma), and patients with endocrine and immune dysfunction (e.g. autoimmune polyendocrinopathy candidiasis ectodermal dystrophy (APECED)).

The infections described so far are superficial and confined to the outer layers of the skin and mucosal surfaces. However, under specific circumstances *Candida* cells can sometimes penetrate through these barriers, eventually reaching the bloodstream, thus causing transient candidemia and ultimately resulting in disseminated infections in a wide range of organs (e.g. kidney, spleen, liver) and systems (e.g. urinary tract). Invasive candidiasis (IC) usually occurs in patients with severe neutropenia (i.e. patients with severely depleted neutrophil levels often due to treatment for cancer and hematological malignancy), patients who have undergone major abdominal surgery, patients receiving prolonged broad-spectrum antibacterial therapy, and patients with catheters (Table 10.2). Consequently, these infections are usually only encountered in hospitalized individuals who are already very ill. How the yeast cells gain access to the bloodstream is not entirely clear. However, antineoplastic chemotherapy can damage the epithelia of the gastrointestinal tract sufficiently to allow the yeasts to translocate across the gut wall into the local blood vessels. Similarly, intestinal surgery and intravenous catheters can allow the yeasts direct access to the bloodstream.

Under most circumstances the human immune system can eradicate low numbers of invading candidal cells (transient candidemia); however, in patients with reduced numbers of neutrophils, the yeasts are allowed to overgrow and spread throughout the body. The symptoms associated with invasive candidiasis are very difficult to discriminate from those of systemic infections caused by other pathogens (e.g. bacteria). One of the earliest indicators of systemic fungal infection is persistent fever that does not respond to broad-spectrum antibacterial therapy; however, in some cases skin lesions can also appear. If left untreated candidemia can result in candidal cells being distributed to organs such as the kidney, liver, and brain, ultimately leading to death. Because of the underlying illness of most invasive candidiasis patients, it can be difficult to attribute patient death to *Candida* infection. However, attributable mortality rates have been estimated to range between 14.5 and 49%, far higher than for other common causes of systemic infection (e.g. MRSA). In addition to the personal costs of candidal infection, in the United States alone in 2002 the attributable financial costs

associated with candidemia were calculated to be as high as $1.7 billion per year. Invasive candidiasis is usually treated with azole and polyene drugs (especially expensive lipid formulations of amphotericin B). In addition, novel azoles (e.g. voriconazole) and the new class of drugs known as the echinocandins offer the chance of improved survival rates in infected patients.

10.3.1.1 Diagnosis and Epidemiology of Candida *Infections*

In order to effectively treat candidal infections it is imperative that the yeast is detected and identified as rapidly as possible. In the case of superficial infections, such as VVC and OPC, this is relatively straightforward. Swabs can be taken from the affected area and inoculated onto routine mycological agar plates. The chromogenic medium CHROMagar Candida™ has been shown to be particularly useful in enumerating and identifying the yeast species present, with different species of *Candida* yielding colonies with characteristic colors (Plate 10.2).

However, the diagnosis of candidemia is more problematic. As mentioned earlier, the symptoms of candidemia are nonspecific, and can easily be confused with other bloodstream infections. When candidemia is suspected, a blood sample should be taken and inoculated into culture medium and analyzed using manual or automated culture methods. However, in some cases it can take up to 48 hours for growth to be detected, and it has also been reported that in many cases of candidemia blood culture fails to detect evidence of *Candida*, mainly due to the relatively low levels of *Candida* cells usually present in the blood of patients with candidemia. This can result in a delay in the provision of appropriate therapy and increased mortality. Other tests to detect *Candida* species in blood include the detection of anti-*Candida* antibodies and *Candida* antigens (e.g. galactomannan and β-D-glucan) in blood samples.

In order to improve the speed, sensitivity, and specificity of fungal infection diagnosis, molecular methods based on the polymerase chain reaction (PCR) are currently being assessed. This test relies on the detection and amplification of very small amounts of candidal DNA in blood samples using the thermostable enzyme *Taq* polymerase. PCR is exquisitely sensitive (it can detect as little as ten *Candida* cells per milliliter of blood) and results can be obtained within a few hours. However, the exquisite sensitivity of PCR can lead to problems, with false positive results occurring due to problems associated with contamination and the detection of nonviable *Candida* cells. The combination of PCR and antigen detection methods has great potential to revolutionize the early diagnosis of these infections and to reduce the associated levels of morbidity and mortality.

The epidemiology of candidiasis is constantly changing and mirrors developments in other aspects of human disease. The incidence of superficial forms of the disease, particularly OPC in the developed world, increased dramatically during the 1980s and early 1990s. This increase was due primarily to the

HIV pandemic, which began in the 1980s and which resulted in large numbers of individuals at risk of infection. However, the introduction in the late 1990s of combination therapies, including novel anti-HIV agents such as protease inhibitors, which lead to a reconstitution of the T helper cell count and inhibit candidal proteinases, has resulted in a marked decline in the prevalence of OPC in developed countries. Despite this, candidal infections can still occur in HIV-infected patients in whom HAART has failed, or in cases where the patient does not follow the full course of therapy (i.e. noncompliance). Unfortunately, due to their high cost, these therapies are rarely provided in developing countries around the world, where HIV infection continues to be a major health problem.

While the incidence of invasive candidiasis was observed to increase during the 1980s, recent studies suggest that it has now leveled off and *Candida* species are now recognized as the fourth most common cause of nosocomially acquired (hospital-based) bloodstream infections (the leading causes are gram-positive bacteria such as *Staphylococcus aureus* and *Staphylococcus epidermidis*). The most likely reasons for the persistence in the prevalence of candidal systemic infection are the development of more aggressive anticancer therapies and more powerful immunosuppressive drugs associated with organ transplantation. These have resulted in a greater number of individuals surviving life-threatening diseases; however, these patients are usually extremely ill and are immunocompromised for increasingly long periods of time. Consequently, these patients provide an ever-growing population of at-risk individuals in our hospitals who are very susceptible to fungal infection. In order to protect patients from these infections and to decrease the incidence of systemic candidal disease many hospitals treat their "at-risk" patients prophylactically with antifungal drugs, although this runs the risk of causing the development of resistance in colonizing strains or in the selection of species such as *C. glabrata* and *C. krusei*, both of which have reduced susceptibility to azole drugs.

The *Candida* species most frequently associated with human infection is *C. albicans*. This species is widely regarded as being the most important human yeast pathogen, and when people refer to *Candida* it is often assumed that the species in question is *C. albicans*. However, in many epidemiological studies it is clear that *Candida* species other than *C. albicans* can cause both superficial and systemic disease (Table 10.4).

The exact proportion of species responsible for cases of candidiasis can vary from country to country and even between different hospitals in the same geographical region. In the majority of studies *C. albicans* is the most commonly identified species in clinical samples. However, species such as *C. glabrata* and *C. parapsilosis* are often also identified as contributors to human disease, while in some studies in Asia the most commonly isolated *Candida* species in blood samples was found to be *C. tropicalis*. The reasons for the apparent increased prevalence of these species are not clear. However, since *C. glabrata* can rapidly develop resistance to antifungal drugs it has been suggested that prior treatment with these drugs can select for infections caused by this species. In the case of

C. parapsilosis this species is often found in biofilms growing on plastic surfaces and has been primarily associated with infections resulting from procedures requiring the use of intravenous lines and catheters.

The fact that some species are inherently resistant (e.g. *C. krusei*) or can develop resistance (e.g. *C. glabrata*) to many antifungal drugs has significant implications for the choice of antifungal therapy. Therefore, in order to prescribe the optimum therapy it is important to be able to identify the species responsible for a particular infection as rapidly as possible. Unfortunately, it is quite difficult to readily discriminate between different *Candida* species. Their cell and colony morphologies are for the most part very similar, with the yeast cells of most species being similar in size and shape, while colonies cultured on routine diagnostic agar (such as Sabouraud's dextrose agar) are often very similar in size, shape, texture, and color. However, as mentioned earlier, the chromogenic agar medium CHROMagar Candida has been shown to be a useful aid in species identification, with the most clinically important *Candida* species being distinguishable on the basis of colony color.

In diagnostic medical mycology laboratories there are two "gold standard" tests that have been used for decades for the identification of *C. albicans*. These rely on the fact that *C. albicans* produces germ tubes (the first stage of hyphal development as the hyphae emerge from the cell) when incubated in serum, and chlamydospores (thick-walled refractile spores of unknown function) when cultured on particular nutrient-depleted media. Until recently, *C. albicans* was the only species known to produce germ tubes and chlamydospores; however, in 1995, a novel, closely related species, *C. dubliniensis*, was identified in the oral cavities of HIV-infected individuals, which can also exhibit these two morphological characteristics. Other commercially available tests used routinely to identify specific *Candida* species include the analysis of carbohydrate assimilation profiles and serological tests. Molecular tests, in particular those based on PCR and microarray technology, have also been developed to allow the rapid identification of specific species and offer great potential for future rapid and accurate diagnostic tests.

While it is certainly very important to be able to discriminate between *Candida* species, for epidemiological studies it is often also very useful to be able to distinguish between strains within a species. This is particularly important when (1) tracking the source of an infection in an outbreak, (2) determining if a recurrent infection is due to re-infection with the original or a new strain, or (3) determining whether more than one strain is present in a clinical sample, all of which can have a significant effect on the course of therapy for an infection.

A wide variety of methods of differentiating between strains have been developed. The earliest methods were based on the comparison of phenotypic characteristics (e.g. sugar assimilation profiles, colony morphology, antifungal drug resistance profiles); however, these tests have poor discriminatory power and have largely been superseded by molecular methods, which are far more discriminatory. Molecular strain typing methods are mainly based on the comparisons

of the genetic content of individual strains. Until recently, the commonly used method for strain discrimination was DNA fingerprinting using species-specific probes homologous to regions of DNA repeated in the candidal genome. This generates a barcode-like pattern which is strain-specific and, with the aid of computer software, can be compared quantitatively to the patterns obtained for other strains. DNA fingerprint analysis allows strains to be tracked epidemiologically and has revealed that the *C. albicans* species is comprised of more than 17 clades. Discriminatory DNA fingerprinting probes have been used in the epidemiological analysis of *C. albicans*, *C. glabrata*, *C. parapsilosis*, and *C. dubliniensis*, among others. The karyotype (chromosome content) and the electrophoretic mobility of certain enzymes can also be used as a means of strain comparison.

Due to problems concerning the subjectivity of interpreting and comparing fingerprint and karyotype patterns, these methods have largely been superseded by multilocus sequence typing (MLST) analysis. This technique relies on the comparison of sequences of approximately 500-bp segments of seven housekeeping genes, and the data, which can be generated rapidly, are reproducible and not subjective. Because the sequence data can be stored in publicly accessible databases, many hundreds of strains can be compared. MLST has been shown to be as discriminatory as DNA fingerprinting and has identified the same clade structure in *C. albicans* and *C. dubliniensis*. As the cost of whole genome sequencing decreases, the prospect of strain typing by sequencing whole genomes is now within reach.

These methods have been applied widely to investigate the origin of *Candida* infections. In the case of the majority of superficial and systemic infections it appears that the infecting strains are mainly acquired endogenously from the patients' own colonizing flora, confirming the observation that prior colonization with *Candida* is a risk factor for candidiasis. However, there are numerous cases in the literature describing the identification of strains present in a patient, on environmental surfaces, and on the hands of healthcare workers, suggesting that in some cases of disease the source of infection may be acquired from an exogenous source.

10.3.1.2 Candidal Virulence Factors

The capacity of a pathogenic microorganism to cause disease is usually the result of a complex interaction between the microbe and its host. Since *Candida* species mainly cause disease in immunocompromised individuals, it would be easy to conclude that the most important factors contributing to the establishment of candidal infections are purely related to the host. While host factors undoubtedly play a significant role in the development of candidiasis, traits associated with the yeast are also very important. The capacity of a microorganism to cause disease in a host is known as its virulence. Clearly, certain microbes are more predisposed to cause disease than others, and this is usually due to the possession

Table 10.5 Virulence factors of *C. albicans.*

Adhesins (e.g. Hwp1, Als proteins)
Yeast ↔ Hyphal dimorphism
Phenotypic switching
Extracellular hydrolases (e.g. Sap proteinases and lipases)

of specific attributes related to the ability to grow *in vivo* and to cause damage to host tissue. These attributes are often referred to collectively as virulence (or virulence-associated) factors. In the case of some bacterial species, it is very easy to identify virulence factors (e.g. the toxins produced by *Vibrio cholerae* and *Clostridium botulinum*); however, in the case of *Candida* species it is quite difficult to establish which of its phenotypic characteristics contribute to pathogenesis. Since *C. albicans* remains the most important yeast pathogen, its virulence mechanisms have been studied far more intensively than any other fungal pathogens of humans. From these studies a number of traits expressed by *C. albicans* have been identified as being putative virulence factors (Table 10.5).

One of the most important requirements for any microbe, whether commensal or pathogenic, is the ability to adhere to host tissue. This requires that the microbial cell be able to recognize and to stick to host ligands, such as extracellular matrix proteins and cell membrane components. This allows the organism to establish a foothold on the host surface, which prevents it from being dislodged by blood or host secretions, such as saliva or sweat. So far, a number of such proteins have been identified in *C. albicans*. Adhesins have been identified that bind to a range of host proteins, such as fibronectin and components of complement, as well as to host cell surface carbohydrate moieties of membrane glycolipids and glycoproteins. In addition, a large family of related proteins, known as the Als (agglutinin-like sequence) family have been shown to be involved in yeast–host cell interactions. One of these proteins, Als3, which is only expressed by *C. albicans* hyphae, has been proposed to act as an invasin and has been shown to sequester iron, suggesting that this protein is an important multifunctional virulence factor. The surfaces of hyphal *C. albicans* cells have also been shown to express a protein called hyphal wall protein (Hwp1) which appears to act as a substrate for host enzymes (transglutaminases) that can covalently link the hyphal Hwp1 proteins directly to proteins on the surfaces of the epithelial cells.

One of the most important phenotypic characteristics of *C. albicans* (and *C. dubliniensis*) is its ability to grow as ovoid blastospores and elongated hyphal filaments; the transition between yeast and hyphal forms is known as dimorphism. Both yeast and hyphal cells have been observed in tissue samples and it is possible that each form may contribute to different stages or different types of candidal infection as mutants locked in either the yeast or filamentous forms

have a reduced capacity to cause disease. A range of environmental conditions have been shown to induce hypha formation (e.g. incubation in serum, growth temperature greater than 35 °C, nutrient starvation, and pH > 6.5); however, the precise nature of the genetic switch(es) involved are complex and as yet not fully understood. Interestingly, several virulence factors have been associated with specific morphological forms (e.g. the adhesin Hwp1, the candidalysin Ece1, and several aspartyl proteinases (see below)). In reality, *C. albicans* is in fact polymorphic, as, in addition to yeast and hyphae, it can grow as pseudohyphae and chlamydospores; however, the role of the latter morphological forms in pathogenesis is unclear.

As in the case of most other pathogens, *C. albicans* has been shown to produce a wide range of extracellular enzymes that can digest host proteins. In particular, *C. albicans* possesses a family of ten closely related secreted aspartyl proteinases (Saps) that have the ability to hydrolyze a wide range of host proteins, ranging from matrix components to immune system proteins such as antibodies. In addition, *C. albicans* encodes a family of lipases and phospholipases that have also been implicated in virulence. Interestingly, genes encoding individual proteinases and lipases are quite stringently regulated, only being expressed in specific morphological or switch phenotypes.

Clearly, candidal pathogenesis is a complicated and multifactorial process, with a complex array of different virulence factors interacting in specific microenvironments to contribute to the survival and proliferation of the organism (see Plate 10.3 for a summary of events). However, it is becoming clear that it is naïve to think of *Candida* cells acting in isolation. In nature it is now believed that the majority of microbes exist in complex communities of microorganisms attached to surfaces and covered in extracellular matrix. These communities are known as biofilms, and it is now believed that the ability to form biofilm is an integral component of the candidal offensive armory. Candidal biofilms are of particular interest because it has been demonstrated that cells in biofilm are more resistant to the activity of antifungal drugs. In addition to *C. albicans*, *C. parapsilosis* has been demonstrated to form biofilms, particularly on the surfaces of plastic catheters, possibly explaining the high level of association between this species and catheter-related infections.

The recent completion of the genome sequences for a range of pathogenic and nonpathogenic yeast species offers great potential to further our understanding of these species and how they cause disease. Genome sequence analysis has allowed the discovery of a parasexual mating cycle in *C. albicans* that is related to the ability of this species to undergo phenotypic switching between white and opaque cells. The availability of these genomic sequences also allows comparisons to be made between species and has confirmed that virulence is associated with expansion of specific gene families, such as the ALS and SAP families. Post-genomic studies investigating global transcriptional and proteomic responses in a range of infection models are also currently being used to identify the functions of previously uncharacterized genes and novel patterns of gene expression in disease.

10.3.2 Aspergillosis

In addition to pathogenic yeast species, some filamentous fungi and molds, under specific circumstances, have the ability to cause serious infections in humans. The most important of these opportunist molds belong to the genus *Aspergillus*. Approximately 20 *Aspergillus* species have been associated with human infections; however, the majority of cases of aspergillosis are caused by only a handful of species, especially *A. fumigatus*, *A. niger*, *A. terreus*, and *A. flavus*, with *A. fumigatus* being the most commonly identified cause of infection. *Aspergillus fumigatus* is a saprophytic fungus that is ubiquitous in the environment, and is particularly associated with soil and decaying vegetable matter. Although it has been thought that this fungus is asexual, in 2009 it was shown to have the capacity to reproduce sexually in the laboratory. In the wild, *A. fumigatus* grows as a mass of branching hyphae; however, it also produces vast numbers of asexual spores, known as conidia, in structures known as conidiophores (Figure 10.2).

Conidia are released into the environment (especially in construction and demolition sites) and can be carried great distances by air currents, thus providing the means to contaminate food and water sources. *Aspergillus fumigatus* conidia are dispersed very easily and the concentration of conidia in air can range from 1 to 100/m^3. Therefore they are routinely inhaled by humans, and their size (approx. 2–3 μm) allows them to penetrate deep into the lower respiratory tract. In normal healthy individuals the conidia are easily detected and destroyed by alveolar macrophages present as part of the innate pulmonary immune system; however, in certain subsets of the population the spores can settle, germinate, and invade, ultimately leading to a wide range of diseases.

The most important form of aspergillosis is known as invasive aspergillosis (IA). Patients at risk of acquiring this infection are generally profoundly neutropenic for prolonged periods of time. Patients most at risk of acquiring IA include those receiving immunosuppressive treatment for organ transplantations (especially bone marrow transplants) and those receiving cytotoxic antineoplastic therapy. Reduced neutrophil counts and impaired alveolar macrophage function prevent

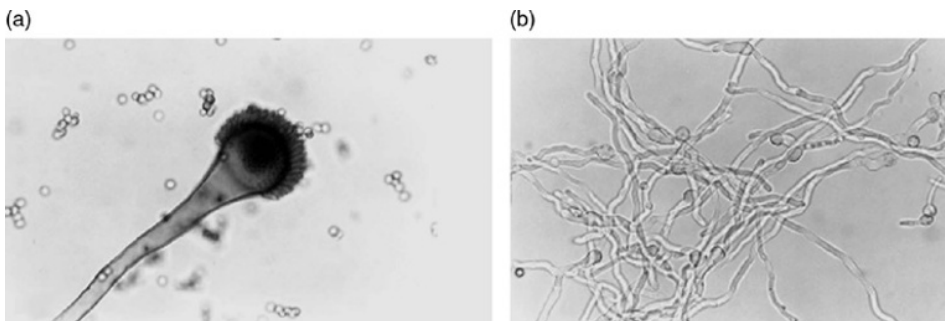

Figure 10.2 *Aspergillus fumigatus* (a) conidiophore and (b) germinating conidia.

the host from destroying *Aspergillus* conidia that are inhaled into the alveoli, allowing the conidia to germinate and the developing germlings to proliferate. If not detected and treated in time the hyphae produce hydrolytic enzymes and cause tissue damage, eventually breaching the alveolar wall, before ultimately reaching and penetrating into the circulatory system. Fragments of hyphae can then be spread to a wide range of organs throughout the body, resulting in disseminated aspergillosis.

Clearly, the human body represents a very different environment for aspergilli in comparison with their normal habitat in compost. However, these species have the ability to adapt to growth *in vivo* when the immune response is compromised (e.g. in cases of neutropenia). Identification of virulence factors or virulence-associated traits in opportunistic pathogens, such as *A. fumigatus*, is notoriously difficult. However, virulence-associated traits identified in these fungi include thermal tolerance, production of proteinases, production of gliotoxin, and rapid response to environmental stress. Completion of its genome sequence in 2005 has facilitated the investigation of the molecular basis of *A. fumigatus* pathogenesis. Global transcriptional profiling studies using these arrays have revealed that alterations in temperature are an important trigger for virulence, and that genes involved in iron and nitrogen assimilation as well as alkaline stress response play an important role in adaptation to growth *in vivo*.

The symptoms of IA are nonspecific, but usually include fever and sometimes chest discomfort and cough (sometimes with blood). The mortality rate of IA is very high (50–100%), especially once the fungus has spread to other organs, particularly the central nervous system. Clearly, early diagnosis of IA is essential to allow optimum treatment, but this is very difficult to achieve as specific symptoms occur relatively late during infection and the available diagnostic methods are not sufficiently effective. The gold standards of diagnosis include histopathological analysis and culture of biopsy and bronchoalveolar lavage fluid taken from the infected area of the lung. However, improved radiographic imaging methods (e.g. CT scans) and serological tests to detect fungal galactomannan have been demonstrated to facilitate accurate and early diagnosis. The potential usefulness of PCR as a diagnostic tool is still under intensive investigation. Despite all of the tests available definitive diagnosis of IA is still often only obtained at *post mortem*.

Because of the problems associated with diagnosis, cases of suspected aspergillosis are often treated empirically (e.g. in patients with persistent fever that is unresponsive to antibacterial therapy) with antifungal agents. Liposomal amphotericin B is one of the most commonly used drugs to treat aspergillosis; however, other antifungal drugs, such as itraconazole, voriconazole, and caspofungin, have been shown to be effective. Since acquired resistance to these drugs is relatively rare in *A. fumigatus*, attempts can be made to minimize the risk of IA by using prophylactic doses of drug during periods of profound neutropenia. Other strategies to reduce infection risk in susceptible patients include pre-emptive

antifungal therapy and the use of high-efficiency air filters to remove conidia from the air in the patients' rooms.

Aspergillus species can cause a range of other diseases, often in nonimmunocompromised individuals. One example is aspergilloma, an infection that develops due to *Aspergillus* species colonizing areas in the lung that have been damaged (e.g. by tuberculosis and sarcoidosis scars and cavities). The fungus proliferates and forms a large ball-like hyphal mass (the aspergilloma). The infection is often asymptomatic; however, fever and coughing up blood (hemoptysis) can also develop. It is usually treatable using conventional antifungal drugs, but in extreme cases the fungal ball has to be surgically excised. Another disease caused by *Aspergillus* species is allergic bronchopulmonary aspergillosis (ABPA). This is an allergy to toxic products produced by *Aspergillus* cells that chronically colonize the upper respiratory tract, especially in asthmatic and cystic fibrosis patients.

There are of course many other filamentous fungi in the environment that can cause infection in severely immunocompromised individuals. These include the dematiaceous fungi (so called because of their dark color) (e.g. *Alternaria* and *Pseudallescheria* spp.), *Fusarium* spp., and the zygomycetes, such as *Mucor* and *Rhizopus* spp. While infections caused by these species are still relatively rare, their incidence is increasing due to the growing numbers of patients with severely compromised immune systems for longer periods of time. It is particularly noteworthy that it is often difficult to identify many of these molds in clinical samples, and in any case many of them are not susceptible to most of the commonly used antifungal drugs. Consequently, these mycoses represent an increasing challenge for clinicians.

10.3.3 Cryptococcosis

Cryptococcus neoformans is the most pathogenic *Cryptococcus* species, although the closely related species *Cryptococcus gatii* is also an opportunistic human pathogen. Cryptococci are sexually reproducing basidiomycetous yeast-like fungi that produce a characteristic carbohydrate capsule (an important virulence factor) which is required for pathogenesis. The natural environmental reservoir of *C. neoformans* is believed to be the soil, particularly in areas with high levels of pigeon guano. Infectious propagules of the fungus are thought to be carried by air currents and are regularly inhaled by humans.

In the majority of cases, the fungi are cleared by the immune system, or a transient nonsymptomatic infection can sometimes occur. However, in susceptible individuals, especially HIV-infected and AIDS patients, the yeast cells can disseminate from the lungs into the bloodstream. Yeasts ultimately reach the central nervous system and the meninges, resulting in cryptococcal meningitis, a very serious infection with high mortality rates. The most common treatment is amphotericin B, often combined with 5-fluorocytosine.

While the levels of cryptococcosis were very high during the 1980s and 1990s, the introduction of HAART for the treatment of AIDS has resulted in a marked decrease in the incidence of the disease in developed countries; however, the global incidence of cryptococcal meningitis is estimated to be approximately 1 million, with more than 600,000 of these occurring in Sub-Saharan Africa.

10.3.4 Pneumocystis Pneumonia

Pneumonia caused by the ascomycetous fungal species *Pneumocystis jiroveci* (previously known as *Pneumocystis carinii* or *P. carinii* f. sp. *hominis*) is one of the most commonly encountered opportunistic infections associated with AIDS, although a wide range of other immunocompromised individuals can also acquire the disease, which is commonly referred to as PCP. PCP has also been associated with outbreaks of infection in malnourished children in crowded institutions. *Pneumocystis jiroveci* was originally considered to be a protozoan; however, in-depth phenotypic and genotypic analyses have confirmed it is more closely related to fungi. It is believed that this species is prevalent subclinically in the lungs of a large proportion of the human population (particularly young children) and is either latent or continuously encountered by individuals through person-to-person exposure. In either case, when the human T-cell count becomes depleted (e.g. by HIV infection or immunosuppressive therapy) the infection becomes activated, resulting in pneumonia-like symptoms, which if untreated can lead to death due to hypoxia.

PCP is difficult to diagnose because *P. jiroveci* cannot be cultured *in vitro*, and it is difficult to treat because *P. jiroveci* cell membranes do not contain ergosterol (the target of common antifungal drugs such as the azoles and amphotericin B). Consequently, the disease is treated using trimethoprim-sulfamethoxazole, which can have serious side-effects. Fortunately, due to the recent success of anti-HIV HAART the incidence of PCP is decreasing, although it still remains as the most common AIDS-defining opportunistic infection in developed countries.

10.4 Endemic Systemic Mycoses

Not all systemic fungal infections require an immunocompromised host. There are several examples of fungi that are primary pathogens (i.e. naturally virulent for humans) and which can cause symptoms of disease in individuals who are apparently otherwise healthy, as well as in patients who are immunocompromised (Table 10.6). These infections are usually most often found in very specific geographic locations and hence are often referred to as endemic mycoses.

Table 10.6 Examples of endemic systemic mycoses.

Causative species	Disease
Histoplasma capsulatum	Histoplasmosis
Blastomyces dermatitidis	Blastomycosis
Coccidioides immitis	Coccidioidomycosis
Paracoccidioides brasiliensis	Paracoccidioidomycosis

10.4.1 Histoplasmosis

Histoplasmosis is caused by the dimorphic fungus *Histoplasma capsulatum*. This sexually reproducing fungal species is naturally found in the soil (often associated with avian and bat guano) and is endemic in tropical areas of the world and in the Ohio and Mississippi river basins in the United States. In the soil and at 25 °C in the laboratory the fungus exists in a hyphal/mold form; however, in the human host and at 37 °C *in vitro* it exists as small yeast-like spherules (conidia). It is interesting to note that in *C. albicans* both the yeast and hyphal forms are thought to be important in virulence, while in *H. capsulatum* (and other systemic mycoses) only the conidial form is associated with disease. Inhalation of the infectious propagules by normal healthy individuals usually results in a self-limiting subclinical respiratory infection, although in a small number of cases the patient may complain of mild flu-like symptoms. In cases where the patient happens to be immunocompromised (e.g. infected with HIV) histoplasmosis can be life-threatening. An important virulence factor of *H. capsulatum* is the ability of this species to survive and proliferate within macrophages following phagocytosis. In certain circumstances, the *H. capsulatum* cells can remain latent and become reactivated in tissue years following the original exposure to the fungus, appearing once the immune system begins to deteriorate.

10.4.2 Blastomycosis, Coccidioidomycosis, and Paracoccidioidomycosis

The causative agent of blastomycosis, *Blastomyces dermatitidis*, is similar to *H. capsulatum* in that it is also a sexually reproducing dimorphic fungus that can cause primary human infections. As with histoplasmosis, blastomycosis is acquired by inhaling conidia from contaminated damp soil and is endemic in large areas of North America. In the majority of cases, the infection causes little or no symptoms; however, progressive pulmonary and/or systemic infection can rarely occur.

Coccidioides immitis and *Coccidoides posadasii* are the causative agents of coccidioidomycosis (also known as Valley fever) which grow in the hyphal form in their natural habitat (alkaline soils in dry and arid regions of the southwest United States and northern Mexico). These fungi produce spore-like structures known as arthroconidia, which when inhaled into the lungs of normal healthy individuals develop into multinucleate spherical structures, called spherules, which are filled with endospores. This results in respiratory infection, which is normally self-limiting, although infected individuals sometimes complain of cough and fever. However, in approximately 5% of cases the disease can disseminate and progress to become a more serious disease. Another disease caused by a dimorphic fungus is paracoccidioidomycosis, which is caused by the dimorphic pathogen *Paracoccidioides brasiliensis*. This infection is endemic in Central and South America and, as occurs with the other systemic mycoses, results from the inhalation of spores originating from the soil. The primary disease is usually subclinical; however, for reasons as yet unknown, symptomatic infections are primarily diagnosed in males.

10.5 Mycotoxicoses

Like all organisms, fungi produce by-products of metabolism as they grow. The majority of these low molecular weight secondary metabolites are harmless to humans, but some can deleteriously affect human health and these are referred to as mycotoxins. It should also be remembered that some fungal metabolites are beneficial to humans (e.g. antibiotics produced by fungi). While there are approximately 300–400 recognized mycotoxins, only 10 or so are commonly observed in disease, which are collectively known as mycotoxicoses. The best known mycotoxins are the aflatoxin family and the ergot alkaloids. There are four major aflatoxins (B_1, B_2, G_1, and G_2); however, B_1 is the most toxic (Figure 10.3).

In biochemical terms the aflatoxins are difuranocoumarin derivatives and are produced by a range of *Aspergillus* species, particularly *A. flavus*. These fungi frequently contaminate and produce toxins while growing on crops such as corn and peanuts that are subsequently consumed by animals and humans. Consumption of meat and milk from cows that have been exposed to the mycotoxins can

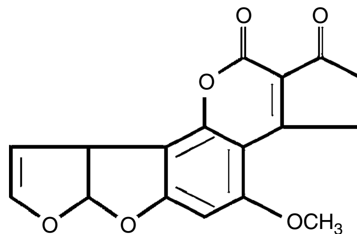

Figure 10.3 Chemical structure of aflatoxin B_1.

also result in exposure of humans to the toxins. While acute aflatoxicosis is a relatively rare phenomenon, ingested aflatoxins are notorious for their carcinogenicity as a result of chronic exposure. The mutagenic nature of aflatoxins is believed to be due to the DNA-damaging properties of aflatoxin metabolic derivatives. The most common form of disease associated with dietary exposure to aflatoxins is liver cancer; however, it may also be implicated in other forms of cancer. Because *Aspergillus* species are ubiquitous in the environment it is impossible to prevent foodstuff contamination with fungi. However, this contamination can be minimized by the use of stringent production, storage, and monitoring procedures.

Ergotism (also known as St Anthony's fire) is a disease associated with the ingestion of cereals contaminated with the fungus *Claviceps purpurea*. This fungus, which infects the flowers of grasses and cereals, produces a range of alkaloids, of which ergotamine is the best known. Ergotamine is related to the hallucinogen LSD, and ingestion of cereals and cereal-derived products such as rye bread that are contaminated with the fungus can result in serious symptoms of disease, such as convulsions and gangrene.

It has been suggested that mycotoxins might also exert a damaging effect through inhalation, rather than by ingestion. Most homes, office buildings, and factories provide many niches suitable for the growth of a myriad of filamentous fungi, including *Aspergillus*, *Claviceps*, and *Stachybotrys* spp., and it has been suggested that mycotoxins produced by these species might contribute to the phenomenon known as "sick building syndrome." This syndrome has been associated with an ill-defined group of nonspecific symptoms (usually including fatigue, minor respiratory problems, and headache) that are only experienced within a particular building. The etiology of sick building syndrome is not known; however, poor air quality and ventilation, cleaning chemicals, and microbial contamination have all been suggested as contributory causes. The role of fungi in the syndrome is a source of considerable conjecture.

Spores and volatile by-products from a wide range of filamentous fungal species can also act as allergens. Since most fungi come into contact with humans by inhalation into the respiratory tract, the symptoms of allergy usually occur in the sinuses and lungs. Symptoms are similar to other allergies such as hay fever and asthma and are usually caused by IgE-mediated type I hypersensitivity reactions.

10.6 Conclusion

Considering the vast number of fungi that most of us come into contact with on a daily basis, it is surprising that fungal diseases are less prevalent than perhaps might be expected. The fact that fungal infections are relatively rare is testament to the amazing efficiency of the human immune system, and from the descriptions of the mycoses described above it is clear that when the immune system

fails, the fallout can be catastrophic for the patient. As great improvements continue to be made in medical science, especially in organ transplantation and cancer treatment, there will be an ever-increasing number of severely ill patients with profound immunosuppression for longer time periods in our hospitals. Therefore the number of individuals at risk of acquiring life-threatening fungal infections is growing all the time. Mycologists are becoming increasingly able to prevent, diagnose, and treat these diseases, thus improving the prognosis for a wide range of patients.

In recent years the prevalence of many fungal infections has changed dramatically for a variety of reasons. For instance, in the early 1980s it would have been impossible to forecast the huge increase (and subsequent decrease during the late 1990s) in the incidence of oral candidiasis associated with AIDS. Therefore it is essential that clinicians, epidemiologists, and mycologists remain vigilant and ready for future changes in the epidemiology of fungal diseases. Future medical advances, viral epidemics, and bioterrorism could all alter the spectrum of fungal pathogens plaguing mankind in the future. The armamentarium of antifungal agents available to clinicians continues to grow (e.g. the echinocandins, voriconazole, isavuconazole); however, the greatest challenge facing mycologists is the development of improved, rapid diagnostic techniques to allow antifungal drugs to be administered in sufficient time to maximize the chances of survival of infected patients. It is hoped that new developments in the fields of nanotechnology and microfluidics will lead to the development of automated, miniaturized, minimally invasive, and accurate tests which will revolutionize the point-of-care diagnosis of fungal infections and ultimately reduce the high morbidity and mortality rates associated with these diseases.

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Useful Websites

Aspergillus and Aspergillosis Website: www.aspergillus.man.ac.uk
Doctor Fungus: http://mycosesstudygroup.org/
Fungal Infection Trust: www.fungalinfectiontrust.org
Mycology Online: www.mycology.adelaide.edu.au

11

Immunity to Human Fungal Infections

Mawieh Hamad, Mohammad G. Mohammad, and
Khaled H. Abu-Elteen

11.1 Introduction

During their evolutionary histories, mammals have evolved elaborate defense mechanisms to defend against microbial infections. This is well demonstrated by the observation that susceptibility to fungal infections is far greater in hosts with defective, deficient, or compromised immunity compared with healthy hosts. Increased susceptibility to opportunistic fungal infections in an ever-increasing number of immunocompromised hosts is known to be a significant contributing factor to the rising incidence of human mycoses. Added to this is the ability of fungi to employ sophisticated mechanisms to evade host immunity and develop resistant strains to antifungal chemotherapy. Adaptive T cell-mediated immunity (CMI) plays a major role in protection against most fungal infections. However, mounting evidence suggests that innate immunity and adaptive responses other than those mediated by T cells play a more significant role in protection against fungal infections than previously thought. The current working paradigm in fungal immunity is that adaptive and innate components of the immune system collaboratively generate a broad range of immune responses to limit the spread of and clear fungal pathogens.

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11.2 Compromised Immunity Increases Host Susceptibility to Fungal Infections

The majority of opportunistic fungal infections occur in patients with compromised immunity (Table 11.1). This is based on numerous clinical and experimental findings documented in hosts (humans and animal models) with naturally occurring or acquired immune defects. Cancer patients on chemo- or radiotherapy, patients with acquired immune deficiency syndrome (AIDS), diabetics, and organ transplant recipients on immunosuppressive therapy are highly susceptible to opportunistic fungal infections.

11.2.1 Naturally Occurring or Acquired Immunodeficiencies

Mammalian hosts with deficient immunity resulting from severe combined immunodeficiency (SCID), selective T cell deficiency (e.g. nude mutation (*nu/nu*) in mice) or granulocyte deficiency show increased susceptibility to opportunistic fungal infections. Chronic granulomatous disease (CGD), which results from nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase or myeloperoxidase deficiency, is associated with increased susceptibility to aspergillosis and pulmonary candidiasis. High susceptibility to aspergillosis in CGD patients has been attributed to restricted activation of Th17 cells and unrestrained inflammation. Increased susceptibility to recurrent vulvovaginal

Table 11.1 Commonly encountered opportunistic fungal infections in patients with impaired immunity.

Immune impairment	Opportunistic fungal infections
Defective CMI	Cryptococcosis, disseminated candidiasis, mucosal candidiasis
Drug-induced immunosuppression[1]	Aspergillosis, cryptococcosis, *Fusarium* spp. infections, *Zygomycete* spp. infections
Neutropenia	Aspergillosis, *Fusarium* spp. infections, disseminated candidiasis, *Zygomycete* spp. infections, trichosporonosis
Skin tissue injuries or disrupted mucosal surfaces	*Fusarium* spp. infections, trichosporonosis
Diabetic ketoacidosis	*Zygomycete* spp. infections

[1] Corticosteroids (dexamethasone), cyclosporins and tacrolimus (calcineurin inhibitors), and sirolimus (mTOR pathway inhibitors).

candidiasis (RVVC) has recently been linked to an early stop-codon mutation (Tyr238X) in the β-glucan receptor dectin-1.

11.2.2 Genetically Manipulated Animal Models

Besides naturally occurring or acquired immunodeficiencies, genetically manipulated animal models have been immeasurably valuable in deciphering the role of different immune components in protection against mycotic infections (Table 11.2).

For instance, epsilon 26 transgenic mice (Tgε26) that lack natural killer (NK) and T cells are highly susceptible to alimentary tract colonization by wild-type and mutant *Candida albicans* strains. Germ-free BALB/c mice deficient for the IL-8 receptor homolog (IL-8Rh$^{-/-}$) are more susceptible to gastric and acute systemic candidiasis compared with wild-type counterparts. Increased susceptibility in this model has been attributed to slow trafficking of polymorphonuclear (PMN) cells into infected tissues and reduced representation of PMN cells among peritoneal exudate cells (PECs). The killing activity of PECs from IL-8Rh$^{-/-}$ mice against *C. albicans* was shown to be lower than that mediated by PECs isolated from IL-8Rh$^{+/+}$ mice. Monocyte chemokine receptor (CD192) deficiency in mice was shown to precipitate defective inflammatory cell recruitment, increased IL-4 production, and progressive histoplasmosis (*Histoplasma capsulatum*). Mice deficient for group V secretory phospholipase A2 (PLA2), which hydrolyzes cell membrane phospholipids and releases fatty acids and lysophospholipids, show increased *C. albicans* fungal burden in kidneys, liver, and spleen, along with increased mortality. IL-4 and IL-13 double knockout (IL-4$^{-/-}$/IL-13$^{-/-}$) mice challenged with *Cryptococcus neoformans* H99 strain develop Th1/Th17 but not Th2-type immunity, which prevents pulmonary eosinophilia and airway goblet cell metaplasia, elevates serum IgE, and activates macrophages. Toll-like receptor (TLR) 2$^{-/-}$ mice intratracheally infected with *Paracoccidioides brasiliensis* develop mild pulmonary infection and decreased nitric oxide (NO) synthesis associated with reduced CD4$^+$ and CD8$^+$ cell numbers and impaired expansion of CD4$^+$CD25$^+$FoxP3$^+$ Tregs. Dectin-1-knockout mice show higher susceptibility to pneumocystis infections than wild-type mice.

11.3 Shaping of the Antifungal Immune Response

11.3.1 Host-Contributed Factors

The genetic background of the host plays a significant role in shaping the immune response against fungal infections. For example, C57BL/6 and BALB/c mouse strains are equally susceptible to gastric candidiasis. However, the infection upregulates the expression of β-defensins (mBD1, mBD3, mBD4), chemokines (MIP-2, KC), and cytokines (IL-12, TNF-α) in a more pronounced manner in

Table 11.2 Commonly used transgenic and knockout mouse models in antifungal immunity research.

Affected cell subsets	Mouse model	Associating phenotypic profile	Susceptible to[1]
Mostly Th2 cell subset	CD192$^{-/-}$	CCR2 deficiency, diminished Th1 immunity, hyper Th2 cell activity	Histoplasmosis
	CCR2$^{-/-}$	Defective DC recruitment, active nonprotective Th2 immunity	*C. neoformans*
	CD40L$^{-/-}$	CD154-deficient T cells, disrupted Th2 immunity (decreased numbers of CD4^{+}, CD19^{+}, and CD68^{+} cells)	PCP (*Pneumocystis murina*)
Mostly Th1 cell subset	MyD88$^{-/-}$	MyD88 deficiency, defective production of Th1 cytokines IL-1β, IL-6, IFN-γ	*A. fumigatus*
	IFN-α/βR$^{-/-}$	IFN-α/βR deficiency, defective functioning of IFN, disrupted Th1 immunity	Cryptococcosis
	IFN-γ$^{-/-}$ (GKO nude mice)	IFN- γ deficiency, defective Th1 immunity	PCP
	IL-23p19$^{-/-}$/IL-17RA$^{-/-}$	IL-23 deficiency, disruption of Th17 cell differentiation and activity	OPC, skin candidiasis
	IL-12p40$^{-/-}$	IL-12 deficiency, defective Th1 immunity, reduced production of IFN-γ and other proinflammatory cytokines	*P. brasiliensis*
	CD28$^{-/-}$	Defective co-stimulation of T cells, disrupted T cell signaling and function	*P. brasiliensis*
	Tg-ε26	Marked deficiency in NK and T cells	Alimentary tract candidiasis (*C. albicans*)

Phagocytes	TLR2$^{-/-}$	TLR2 deficiency, disrupted recognition of pathogens	*P. brasiliensis*
	TLR4$^{-/-}$	TLR4 deficiency, disrupted recognition of pathogens	*Sporothrix schenckii*
	Clec4n$^{-/-}$	Dectin-2 deficiency, disrupted recognition of pathogens	Candidiasis
	G-CSF$^{-/-}$/IL-6$^{-/-}$	G-CSF/IL-6 double deficiency, defective granulopoiesis, neutropenia, defective neutrophil function	Systemic candidiasis (*C. albicans*)
	IL-8Rh$^{-/-}$	IL-8 receptor deficiency, defective recruitment of PMNs	Gastric and acute systemic candidiasis
	Gp91phox$^{-/-}$/iNOS$^{-/-}$	iNO-synthase deficiency, defective production of reactive oxygen and nitrogen intermediates	Mucosal and systemic candidiasis, *P. brasiliensis*
	CnB$^{-/-}$	Calcineurin B deficiency, neutrophil function impairment	Candidiasis
Complement	Factor B$^{-/-}$	Complement factor B deficiency, defective activation of alternative complement pathway	Cryptococcosis (*C. gattii*)
	Bf/C2$^{-/-}$	Complement factor B and C2 deficiency, defective activation of complement pathways	Systemic candidiasis (C. *albicans*)

[1] Listed fungal pathogen(s) to which the animal model is susceptible is only tentative; susceptibility to other fungal pathogens is possible.

C57BL/6 mice compared with BALB/c mice. Age of the host is also a factor to consider regarding the robustness of antifungal immune responses. Intravenous infection with *C. albicans* hyphae associates with low survival rates, high fungal burden, low IFN-γ-producing $CD4^+$ T cell numbers, and reduced ability of macrophages and splenocytes to produce TNF-α and IFN-γ in aged but not young C57BL/6 mice.

11.3.2 Pathogen-Contributed Factors

Fungal pathogens also participate in shaping the antifungal immune response, since immune response profiles associated with different mycotic infections show considerable temporal and spatial variations. Immunity against aspergillosis and pneumocystis is characterized by rapid pathogen recognition and deployment of effective innate responses followed by a robust but delayed adaptive immune response. The immune response profile against *C. albicans* infections varies depending on whether the infection is mucosal or systemic. Resistance to mucosal candidiasis associates with Th1 responses, while that to systemic forms associates with differential involvement of Th1 and Th2 responses. Although humoral (antibody-specific) and innate responses are involved in protection against invasive and disseminated candidiasis, mucocutaneous candidiasis is mainly countered by CMI. The role of CMI in dermatophytosis is to destroy the pathogen and render the host immune against subsequent infections; hence resolution of such infections is associated with delayed-type hypersensitivity (DTH). In fact, disparities in the immune response against *Cryptococcus* species infection allow for species differentiation.

These observations suggest that protective immunity against fungal infections proceeds in spatial and temporal sequences that are influenced by the genus, species, and strain of the pathogen, its morphotype (mold, yeast, pseudohyphae, hyphae, mycelium, etc.), and the anatomic site of the infection (localized, mucosal, systemic). This is probably so because such factors could influence the antigenic repertoire and pathogenic determinants expressed during a specific fungal infection. Morphotype switching, a phenomenon common to several fungal species, may also alter fungal immunity through its ability to reshape the profile of morphotype-specific virulence factors and pathogenic determinants that are recognized by immune receptors. Fungi that undergo phenotype switching include dimorphic fungi (*H. capsulatum*, *P. brasiliensis*, *Coccidioides immitis*, and *Blastomyces dermatitidis*) that transform saprobic filamentous molds into unicellular yeasts, filamentous *Aspergillus* species that transform into multicellular mycelia following inhalation of unicellular conidia, and the yeast *C. albicans* which produces blastospores or hyphae. While *C. neoformans* infections generally elicit robust Th1-mediated responses, *C. neoformans* H99 strain infections induce nonprotective Th2-type responses that associate with significant lung fungal burden.

Type switching-related changes in *A. fumigatus* cell wall structure or synthesis influence its ability to activate, suppress, or subvert host immunity. For example, exposure to *A. fumigatus*-derived polysaccharides induces IL-10 production, while exposure to *A. fumigatus*-derived secreted or membrane proteins and glycolipids activates Th2, Th1/Treg, and Th17 cells. Recognition of different fungal morphotypes results in the activation of distinct intracellular pathways in different dendritic cell (DC) subsets. While inflammatory DCs initiate a myeloid differentiation primary response gene (MyD88)-dependent Th17/Th2 immune response against *C. albicans* yeast infections, *C. albicans* hyphae infections induce tolerogenic DC TLR/interleukin 1 receptor (IL-1R) domain-containing adaptor-inducing interferon-beta (IFN-β) (TRIF)-dependent Th1/Treg responses. The ability of different DC subsets to vary their response to different fungal antigens has been attributed to the involvement of the signal transducer and activator of transcription 3 (STAT3) that affect the activation of nuclear factor (NF)-κβ and indoleamine 2,3-dioxygenase (IDO) (see Section 11.6).

11.4 Paradigm Shifts in Antifungal Immunity

11.4.1 Adaptive Cell-Mediated Immunity

The working paradigm in fungal immunity over many decades has been "*T cells versus the rest.*" In other words, systemic and local adaptive CMI has long been regarded as the main player in the defense against medically important fungi. Historical emphasis on antifungal adaptive CMI is not without merit. T cells play a major role against systemic candidiasis, VC, chronic mucocutaneous candidiasis (CMC), aspergillosis, and cryptococcosis. Depletion of $CD4^+$ T cells increases susceptibility to *Pneumocystis pneumonia* and other mycotic infections as well. One of the important determinants of resistance to fungal infections is the differentiation of Th1-type responses. Induction of Th1-mediated immunity by IL-12, IFN-γ, and IL-6 confers significant protection against different forms of mycoses. According to the old paradigm, however, the role of humoral immune (antibody (Ab)) responses has been considered as minor. Furthermore, the standing of innate immunity has not fared any better as it has traditionally been viewed as a negligible and passive aspect of antifungal immunity. Fortunately, this old and exhausted paradigm has gone through a significant shift in recent years.

11.4.2 Shifting of the Paradigm

Deeper understanding of the workings of antifungal immunity derives from work that has uncovered the existence and role of pathogen-associated molecular patterns (PAMPs), pattern recognition receptors (PRRs), antimicrobial peptides, the role of

DCs in the immune response, and the signal transduction pathways involved in adaptive and innate immune responses. The development and utilization of experimental animal models in elucidating the exact role of various immune components in antifungal immunity has been instrumental in this effort. Now we know that various components of innate and adaptive immunity differentially participate in recognizing, handling, and clearing fungal pathogens.

11.4.3 The Role of Antibodies Revisited

Defects in Ab production or function rarely associate with increased susceptibility to fungal infections. However, multiple supportive roles of Abs during mycoses have been identified and characterized over the years. Furthermore, work with monoclonal antibodies (mAbs) as preventive or therapeutic agents against fungal infections has shed more light on the importance of Abs in antifungal immunity.

11.4.4 Innate Immunity Revisited

The observation that each fungal pathogen expresses multiple antigens that can be recognized by various PRRs expressed on different phagocytes highlights the importance of PRRs and innate immune cells (antigen-presenting phagocytes in particular) and suggests that subsequent interactions between the pathogen and adaptive immunity are pre-conditioned by PRR/PAMP interactions. Neutrophils and other phagocytes mount effector responses against a wide range of local and disseminated forms of mycoses. In fact, high rates of opportunistic fungal infections often associate with immunosuppression- or chemotherapy-induced neutropenia. Expression of innate immunity genes involved in the recruitment and activation of neutrophils and monocytes (MNs) significantly upregulates during the early phase of fungal infections. Although epithelial cells are not immune cells *per se*, vaginal epithelium secretes inflammatory chemokines (e.g. IL-8) following exposure to *C. albicans*. Likewise, inflammatory cytokines are produced by bronchial and corneal epithelial cells following exposure to *A. fumigatus*.

Circulating monocytes are also important in antifungal immunity, as evidenced by the observation that the expression of several genes involved in cell function and survival (TNF-α, IL-1, IL-6, IL-8) and cell viability (BCL2-related protein, metallothioneins, SOCS3) upregulate within a few hours following incubation of human MN cells infected with *C. albicans*. In contrast, the expression of genes that negatively impact MN cell function or viability (IL-15, IL-13Ra1, CD14) downregulate within a few hours of incubation. The functional role of NK cells in fungal infections has yet to be clearly demonstrated; however, a possible role in protection against *C. albicans*, *A. fumigatus*, *C. neoformans*, *Pneumocytis murina*, and *P. brasiliensis* infections has been suggested. Other innate immunity

components including antimicrobial peptides, mannose binding lectin (MBL), collectins, and complement components differentially participate in protection against fungal infections.

11.5 Anatomy of the Antifungal Immune Response

Intact epithelial and endothelial surfaces, microbial antagonism, physiologic and biochemical barriers, and antimicrobial peptides (AMPs) function to ward off fungal infections (Figure 11.1). Upon pathogen entry through breached skin, mucosal lining injuries, or inhalation, innate immune cells sense the pathogen and mount an effector response to kill it or prevent it from pathologically colonizing invaded tissues or disseminating to other tissues. As noted previously, professional phagocytes, namely PMN cells (neutrophils), mononuclear (MN) cells (monocytes and macrophages), and DCs, all participate in this process. These cells recognize common fungal PAMPs, especially cell wall-derived sugars, glycoproteins, and glycolipids, via different sets of PRRs. PRRs involved in fungal PAMP recognition include TLR/IL-1R receptors (TLRs 2, 4, and 9 in particular) and C-type lectin receptors (CLRs; dectin-1, dectin-2, DC-specific intercellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN or CD209), galectin, and mannose receptor (MR)). Complement receptors (CR3; also known as CD11b/CD18), Ab Fc receptors (FcγR and the newly described FcR for IgG3), and MBL also play a role in fungal recognition. Lastly, professional phagocytes (mainly DCs and to a lesser extent macrophages), functioning as APCs, engage T cells (CD4+ helper T cells in particular), enabling a full-blown immune response to ensue.

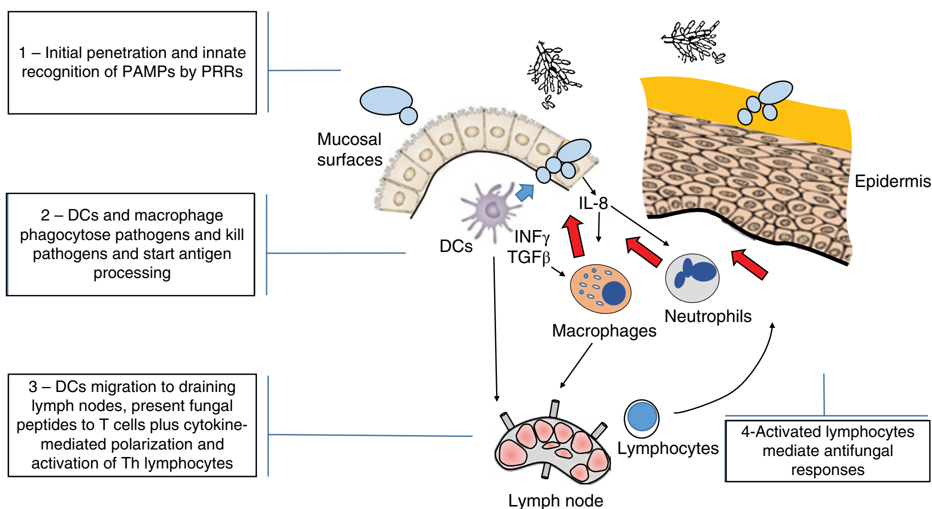

Figure 11.1 General overview of cell-mediated antifungal immunity.

11.5.1 Pattern Recognition Receptors

11.5.1.1 Toll-Like Receptors

PRRs recognize fungal cell wall-derived glucans, mannose, mannans, and chitins, among other fungal sugars, proteins, glycoproteins, glycolipids, and specific fungal DNA sequences. To recognize fungal pathogens and initiate intracellular signals within phagocytes, TLR2 forms a homodimer, or a heterodimer with TLR1 or TLR6, or it combines with other PRRs. TLR2$^{-/-}$ mice show increased susceptibility to *P. brasiliensis* infection associated with a skewed Th17-type response characterized by increased production of KC, a CXC chemokine involved in neutrophil chemotaxis, TGF-β, IL-6, IL-23, and IL-17. Co-culturing of *A. fumigatus* with immortalized corneal epithelial cells (THCE) which recognize *A. fumigatus* zymosans via TLR2 and lipopolysaccharides via TLR4 initiates intracellular signals that induce IL-1β and IL-6 production. Recognition of *C. albicans* or *A. fumigatus* antigens by TLR2 and TLR4 activates members of the G protein-coupled protease-activated family of receptors and triggers downstream signals that differentially influence the immune response. Recognition of *C. neoformans*-derived DNA by TLR9 mediates MyD88-dependent intracellular signals important to anti-*C. neoformans* host immunity.

11.5.1.2 C-Type Lectin Receptors

While TLRs remain the main inducers of inflammation, CLRs and other non-toll-like PRRs are also involved in antifungal immunity. CLRs (dectin 1, dectin 2, mincle, etc.) modulate the inflammatory response by enhancing (or inhibiting) cytokine synthesis. Dectin-1, a CLR that is predominantly expressed on DCs and macrophages to recognize β-glucan, plays an important role in protection against *C. albicans*. Dectin-1-β-glucan interaction triggers signals that upregulate cytokine production among other cellular responses. Mincle-deficient mice show reduced TNF-α production and high susceptibility to systemic candidiasis. An early stop-codon mutation (Tyr238X) in dectin-1 has been shown to result in poor expression of the receptor, thus compromising its ability to bind β-glucan in patients with RVVC. Mutated cells show defective production of IL-17 and IL-6 following stimulation with β-glucan or *C. albicans in vitro*. Although dectin-1-knockouts are more susceptible to pneumocystis infections than wild-type mice, both mouse models are equally susceptible to candidiasis. This suggests that dectin-1 is perhaps involved in the recognition and handling of some but not all fungal infections. This is further supported by the observation that disruption of dectin-1 does not impair the production of protective cytokines during cryptococcosis.

The CLR receptor galectin-3 is also involved in host immunity against a wide range of microbial infections. For example, galectin-3-deficient (gal-$3^{-/-}$) C57BL/6 mice show disrupted Th1/Th2 balance and increased susceptibility to the pathogen. Gal-$3^{-/-}$ mice also exhibit a Th polarization pattern that favors Th2 responses characterized by high IL-4 and GATA-3 expression and low NO production in infected organs.

11.5.1.3 Soluble PRRs

Besides membrane-anchored PRRs, soluble PRRs play a role in fungal antigen recognition. For example, administration of pentraxin3 (PTX3), a multimeric pattern-recognition protein, following challenge with *A. fumigatus* has been shown to restore antifungal resistance and limit inflammation in mice with CGD. The therapeutic potential of PTX3 against *A. fumigatus* could be related to its ability to downregulate IL-23 production in DCs and epithelial cells.

11.5.2 Phagocytes, Phagocytosis, and Fungal Killing

Phagocytosis of fungal pathogens is mainly mediated by neutrophils, macrophages, and DCs. Tissue-resident phagocytes (mainly macrophages) can carry out antigen processing and function as APCs. They also modulate antifungal immunity by producing cocktails of cytokines in response to fungal infections. Macrophages arising from circulating monocytes migrate to tissues and differentiate into distinct functional phenotypes in a cytokine-dependent manner. Pro-inflammatory cytokines (IFN-γ) induce differentiation of the classical M1 type and anti-inflammatory cytokines (TGF-β) induce that of M2 type. In general terms, differentiation of an M1 type correlates with protection (e.g. pulmonary cryptococcosis), while that of M2 correlates with increased susceptibility. Phagocytes kill fungi by both oxidative and nonoxidative mechanisms.

11.5.2.1 The Oxidative Burst

The oxidative burst, which assists in the killing of internalized fungal pathogens, utilizes the NADPH oxidase- and the inducible nitric oxide (iNO) synthase-dependent pathways. The lysosomal hemoprotein myeloperoxidase, which is abundant in azurophilic granules of neutrophils and MN cells, is another potent oxygen-dependent mediator of fungal killing. NADPH oxidase deficiency, which disrupts the formation of reactive oxygen species and other free radicals, increases host susceptibility to fungal infections (e.g. aspergillosis) and is associated with CGD. Impaired phagocyte function due to deficient production or function of

oxidases (Phox) or NO synthase 2 (NOS2) or due to deficient production of reactive oxygen or nitrogen intermediates increases host susceptibility to *C. albicans* infections.

11.5.2.2 Nonoxidative Killing of Fungi

Nonoxidative killing of fungal pathogens is mediated by antimicrobial peptides (AMPs) (collectins, defensins, neutrophil cationic peptides, etc.) that target and disrupt fungal cell membranes. Besides their ability to kill phagocytosed pathogens, neutrophils degranulate and release antimicrobial proteins and produce neutrophil extracellular traps (NETs). NETs antifungal activity has been demonstrated against *C. albicans*, *C. neoformans*, and *A. fumigatus*. The NETs structure contains a significant number (around 24) of associated proteins, which tend to localize to the cytoplasm in an unstimulated state. A critical step in the induction of the NETs antifungal activity is dependent on the ability of pathogens like *C. albicans* to induce the release of the antimicrobial heterodimer calprotectin. Therefore, calprotectin-deficient animals fail to clear *C. albicans* infections. Additionally, depletion of calprotectin in experimental animals results in increased susceptibility to fungal infection. PMNs including neutrophils can also exact lethal hits against several fungal pathogens by releasing elastases and cathepsin-G enzymes. Restricting fungal growth and minimizing pathogen infectivity by phagocytes can also be achieved by immune mediators that deprive fungi of iron, inhibit dimorphism, and/or block phenotype switching.

11.5.3 Iron Sequestration as an Innate Antifungal Immune Response

The significant redox potential of iron along with its ability to readily alternate between the ferrous (Fe^{2+}) and ferric (Fe^{3+}) states make it an optimal cofactor in a multitude of proteins involved in cellular energetics, DNA replication, gene expression, and cell proliferation, among other aspects of cell metabolism. As such, iron is essential for the survival and growth of almost all kinds of cells and organisms. Iron availability enhances the growth and virulence of microbial pathogens including fungi. At the same time, it is essential for the activation and proliferation of host immune cells. Immune cells sequester iron by upregulating the expression of pro-inflammatory cytokines (IL-1 and IL-6) and PRRS (TLR4) that enhance hepcidin synthesis – a peptide hormone that acts as a master regulator of iron homeostasis. Hepcidin then binds ferroportin, the main membrane protein that channels iron out of the cells, triggering its internalization and degradation. This blocks intracellular iron efflux and reduces systemic iron availability. In this context, it is interesting to note that increased levels of estrogen (17-β estradiol; E2) which associate with increased systemic iron availability through the ability of E2 to block hepcidin synthesis also associates with

increased susceptibility to fungal infections, in VC and RVVC in particular. To counter early inflammation-dependent iron sequestration responses by the host, pathogens secrete several iron chelating agents including ferric reductase, siderophores, and hemophores (desferricoprogen, desferrichrome, desferrirubin, desferrichrysin). For instance, *H. capsulatum* enhances its pathogenic potential over a broad pH range by sequestering iron through the secretion of glutathione-dependent ferric reductase.

In both iron deficiency and iron overload, especially in immunocompromised hosts, the fight seems to favor the pathogen. For example, susceptibility of liver transplant patients to invasive aspergillosis, cryptococcosis, and zygomycosis has been shown to correlate with iron overload. In healthy hosts with homeostatic iron status, however, microbial infections trigger a race for iron between the pathogen and the host that is ultimately won by the host, but only after the pathogen exhausts all means of securing iron.

The struggle for iron *in vivo* is under evaluation as a potential therapeutic target especially in immunocompromised hosts who are highly susceptible to opportunistic fungal infections. Enhanced protection against mucormycosis in immunocompromised hosts has been achieved with the use of the nonsiderophoric iron chelator deferasirox in place of the siderophoric iron chelator deferoxamine. Combination therapy involving liposomal amphotericin B in conjunction with deferasirox in mice with invasive pulmonary aspergillosis (IPA) can improve treatment outcome by increasing survival rates and lowering tissue fungal burden.

11.5.4 The Role of Autophagy in Antifungal Immunity

Autophagy is a conserved mechanism in eukaryotic cells that clears nonfunctional, old, or damaged organelles so as to maintain intracellular structural and functional integrity and enhance cell survival. Autophagy influences adaptive immunity by modulating fungal antigen presentation via DCs and macrophages. Engagement of different PRRs with their respective fungal PAMPs has been shown to directly induce autophagy in phagocytes. Phagocytic autophagy can also be indirectly induced through the activity of pro-inflammatory cytokines (IL1, IL6, INF-γ, and TNF-α). Recent studies have suggested that autophagy and autophagy-related proteins play a role in protection against fungal infections. Zymosans derived from *A. fumigatus* or *Saccharomyces cerevisiae* spores induce the LC3-associated phagocytosis (LAP) autophagy protein. However, the extent to which autophagy contributes to antifungal immunity is still not known.

11.5.5 Antimicrobial Peptides

The human innate immune response produces several potent AMPs as a means of protection against a wide variety of microbial infections. Hundreds of AMPs have been identified and characterized in humans and other mammals including

α- and β-defensins, human neutrophil peptides (HNP), gallinacin, and cathelicidin cationic peptides (e.g. hCAP-18 and LL-37). Tissues and surfaces in direct contact with the outside (skin, ears, eyes, mouth, lungs, intestines, and urinary tract) express extensive but different profiles of AMPs. For example, the skin of newborn babies usually contains detectable amounts of cathelicidin LL-37 and that of adults contains detectable amounts of β-defensin (hBD-2). Both infection and inflammation (IL-6 and TNF-β) induce the expression of multiple AMPs, especially cationic peptides (cathelicidins) as means of modulating host immunity by upregulating the expression of pro-inflammatory and/or anti-inflammatory cytokines.

AMPs differ in many aspects including residue sequence and length of the peptide; however, they are generally moderately hydrophobic with a net positive charge. As such, AMPs tend to adopt an amphipathic arrangement where the hydrophobic and positively charged faces assume opposite orientations. Experimental work has indicated that amino acid substitutions that increase the hydrophobicity of cationic AMPs tend to enhance their antifungal activity. The hydrophobic nature of AMPs helps them to primarily target the plasma membrane of fungal cells. Hence, the antifungal (cytocidal) activity of most hydrophobic AMPs involves the disruption of plasma membrane integrity and cytoplasmic leakage. Other AMPs inhibit fungal cell growth by adversely interacting with cytoplasmic or nuclear targets and disrupting cell signaling cascades.

11.6 The Role of DCs in Antifungal Immunity

DCs arise from a common macrophage-dendritic cell progenitor (MDP) in the bone marrow. The MDP gives rise to monocytes and bone marrow-restricted common DC progenitors (CDCPs). CDCPs differentiate into conventional or mature DCs, plasmacytoid DCs (pDCs), and migratory or immature DCs (iDCs) that continuously survey mucosal surfaces and solid organs and respond to pathogenic insult. DCs phagocytose pathogens, process them, and load pathogenic peptides onto the MHC (mainly MHC-II) for presentation to naïve lymphocytes at the site of insult or in draining lymph nodes. They migrate to draining lymph nodes, where they activate effector lymphocytes and contribute to controlling inappropriate immune responses. Animals lacking pDCs are susceptible to *P. brasiliensis* infections and invasive aspergillosis; in fact, pDCs tend to accumulate in lung tissues during pulmonary fungal infections. Following activation via TLR9, pDCs secrete significant amounts of IL-6 and IL-23 as a means of polarizing Th17 cells. Recognition of the recombinant *A. fumigatus*-derived Aspf1 antigen by iDCs upregulates the expression of pro-inflammatory cytokines and augments the activation of NF-κβ. Activated iDCs which reside in the skin, lungs, and gut migrate to lymph nodes where they interact with T cell zones. Following *C. albicans* infection in mice and humans, heavy presence of dermal $CD11b^+$ and epidermal $MHC\text{-}II^+CD103^+$ DCs (Langerhans cells) has been demonstrated in

the skin. These cells produce IL-23 which is required for the recruitment of TCR-$\gamma\delta^+$ T cells to the dermis and epidermis. The role of resident or conventional DCs ($CD8^+$ or $CD4^+CD8^-$ subsets) in antifungal immunity remains largely unknown, although recent work has shown that they help initiate protective immunity against *B. dermatitidis*.

11.6.1 Unique Functional and Phenotypic Features of DCs

A better understanding of how DCs recognize fungal pathogens and initiate and orchestrate innate and adaptive responses has re-focused the attention on innate responses as a significant participant in antifungal immunity. It is well accepted now that DCs play a central role in initiating and orchestrating antifungal innate and acquired immune responses. Their involvement in antifungal immunity and their potential use as vaccines or "active" vaccine delivery systems stems from the fact that DCs possess unique pathogen recognition, processing, and handling potential, as discussed below.

11.6.1.1 PRRs on DCs

DCs express a diverse set of PRRs capable of recognizing and binding to a wide range of fungal antigens. The expression of such a diverse repertoire of PRRs endows DCs with a pathogen-handling and -internalization plasticity unmatched by other phagocyte or APC populations. DCs express TLRs, CLRs, and other membrane-anchored and -soluble PRRs (CR3, MBLs, etc.), all of which can mediate distinct downstream signals to induce the synthesis of cytokines, AMPs, and other signaling and effector responses. The presence of Toll/interleukin-1 receptor (TIR) domains in the cytosolic region of all TLR/IL-1R receptors on DCs allows for the activation of the transcription of nuclear factor β (NFβ) and the stress-activated protein kinase family. These pathways are important for the activation of multiple inflammatory and adaptive immune responses.

11.6.1.2 DCs as Phagocytes

As professional phagocytes, DCs internalize and process diverse sets of fungal peptides. Both human and mouse DCs can internalize *A. fumigatus*, *C. albicans*, *C. neoformans*, *H. capsulatum*, *Malassezia furfur*, and *S. cerevisiae* using different receptors and modes of phagocytosis. Yeasts and conidia are predominantly internalized by coiling phagocytosis, while hyphae internalization occurs by zipper-type phagocytosis. DCs can also recognize and internalize unopsonized yeasts and conidia via SIGN, dectin-1, CR3, and other cell wall sugar receptors. Therefore, the expression of a diverse set of PRRs coupled with the capacity to

cross-present internalized and exogenous fungal antigens equip DCs with the unique ability to decode and translate fungus-associated information into varied immune responses.

11.6.1.3 PRR–PAMP Interaction and signal Transduction in DCs

Signals generated following the engagement of PRRs on DCs with PAMPs on fungal pathogens recruit several adapter proteins, especially the ones that associate with the MyD88 pathway. CLRs and other non-toll-like PRRs also generate distinct intracellular signals to activate and regulate antifungal immunity. For instance, dectin-1-transduced signals involve downstream signaling proteins including Src/Syk kinases, CARD9, and Raf-1. Signals mediated by TLR/IL-1R in an MyD88-dependent manner and those mediated by CLR in Src/Syk/CARD9-dependent manner mobilize multiple downstream signaling molecules and kinases that mostly converge at the NF-κβ step. Activation of NF-κβ triggers the production of cytokines and the expression of ligands on DCs that govern their ability to interact with T cells. Production of cytokines (IL-12, IL-4, and IL-10) and expression of MHC-II and ligands for T cell co-stimulatory molecules such as CD80/CD86 (B7.1/B7.2) enables DCs to engage adaptive immunity in the fight against fungal infections. CD80/CD86 on DCs engages T cells via the co-stimulatory molecules CD28 or the inhibitory molecule CTLA-4 (CD152). Activated DCs upregulate the expression of MHC-II as a means of restricting their APC activity to $CD4^+$ T cells, which position them (DCs that is) to actively participate in regulating antifungal immunity. Cytokines produced by DCs (IL-10) activate $CD4^+CD25^+$ T regulatory (Treg) cells, which downregulate the production of IFN-γ and dampen antifungal CMI against chronic candidiasis and endemic mycoses.

11.7 Adaptive Immunity to Fungal Infections

Adaptive immunity to fungi is comprised of humoral and cell-mediated responses. Adaptive humoral immunity involves the production of antibodies from B cells, which subsequently act to enhance innate immunity and inflammation by facilitating fungal antigen recognition, complement activation and fixation, and pathogen opsonization and neutralization (Table 11.3). Furthermore, B cells, like other APCs, recognize and bind fungal pathogens via cell-surface immunoglobulins (Igs), phagocytose the pathogen, and process and present it to Th cells. Th cells then polarize to Th1, Th2, and others so as to differentially modulate downstream innate and adaptive responses. T cells participating in antifungal immunity are cytotoxic T (Tc) cells, Th cells, and regulatory T (Treg) cells. Although evidence in support of the engagement of the latter two subsets in orchestrating and regulating antifungal immunity in overwhelming, evidence demonstrating the involvement of the former is sporadic.

Table 11.3 Tentative summary of the role of various immune cells and mediators in antifungal immunity.

Immune cells or mediators			Major function	Antifungal protective role[1]
Adaptive Immunity	T lymphocytes	TCR-αβ⁺ CD4⁺ T cells	From which arises Th cells (Th1, Th2, Th17, and Treg cells), cytokine secretion, regulation of antifungal immunity	*B. dermatitidis*, *P. pneumonia*, *C. albicans*, *S. schenckii*, *A. fumigatus*, *P. brasiliensis*
		TCR-αβ⁺ CD8⁺ T cells	Cytotoxic effector function (infected cells), cytokine secretion	*C. albicans*, *P. brasiliensis*, *H. capsulatum*, *C. neoformans*
		TCR-γδ⁺ (CD8αβ ⁺ or CD8αα⁺ T cells)	Cytotoxic effector functions at mucosal surfaces, cytokine secretion	*C. albicans*, *P. brasiliensis*, *H. capsulatum*, *C. neoformans*
	B cells	Abs	Opsonization, neutralization, complement fixation, Ab-dependent cytotoxic activity (ADCC)	*C. albicans*, *C. neoformans*, *P. carinii*, *F. pedrosoi*
Innate Immunity	Professional phagocytes	DCs	Recognize, process, and present fungal antigens to Th cells, orchestrate innate and adaptive immunity	Majority of human fungal infections
		Neutrophils	Phagocytosis and killing of fungal pathogens by oxidative and nonoxidative mechanisms, NETs, degrading enzymes, etc.	*C. albicans*, *A. fumigatus*
		Macrophages	Fungal pathogen recognition and phagocytosis, fungal antigen presentation, oxidative and nonoxidative killing of pathogens, cytokine secretion	Majority of human fungal infections
	Soluble mediators	Complement cascade	Opsonization, effector cell recruitment, direct killing of fungi	*C. albicans*, *P. carinii*, *P. brasiliensis*, *H. capsulatum*
		AMPs (defensins, collectins, cathelicidins, hepcidin, HNPs, etc.)	Cytostatic and cytocidal activities against pathogen, iron sequestration, recruitment and regulation of innate responses	Majority of human fungal infections

[1] Listed fungal pathogen(s) against which a specific immune component shows significant activity is tentative; other fungal pathogens could also be susceptible.

11.7.1 Humoral Immunity

Antifungal immunity often associates with the generation of protective antibodies. For example, systemic candidiasis (*C. albicans*), cryptococcosis (*C. neoformans*), *Fonsecaea pedrosoi* (chromoblastomycosis), and other fungal infections associate with detectable levels of opsonizing and complement-fixing Abs. Protective Abs against mycotic infections such as disseminated candidiasis target several pathogen-related antigens including membrane and cytoplasmic proteins (e.g. heat shock protein 90) along with multiple cell wall sugar moieties. *Cryptococcus neoformans* infections elicit protective Abs that interfere with capsular polysaccharide release from fungal cells and prevent biofilm formation. Expression of IgG3 receptor (Ig-γ3R) on phagocytes enhances opsonization-dependent protective Ab-mediated responses against *C. neoformans*. Sensitization of mice against IFN-γ-inducing *C. neoformans* strain H99γ results in the production of significant amounts of *C. neoformans*-specific Abs upon re-exposure. Anti-*Pneumocystis* Ab therapy confers significant preventive and therapeutic potential in patients with *P. carinii* pneumonia (Pcp). Use of hyperimmune sera from mice immunized with *P. carinii* or mAbs specific for *P. carinii* surface antigens resolves *P. carinii* infection in SCID mice. Use of complement-fixing 4 F11(G1) mAb also protects against *P. carinii* infections in mice.

11.7.2 T Cell-Mediated Immunity

Mice lacking $TCR\alpha\beta^+$ T cells are highly susceptible to various forms of mycoses. Patients infected with *P. brasiliensis* show increased numbers of peripheral $CD4^+$ and $CD8^+$ memory T cells. Stimulation of peripheral blood monocytes (PBMC) with heat-inactivated *C. albicans* yeast or hyphae enhances the ability of $CD4^+CCR6^+$ T cells to produce IL-22, IL-17, and other Th1, Th2, or Th17 cytokines (Figure 11.2). Immune patients with coccidioidomycosis show polyfunctional $CD4^+$ cells that produce IL-2, IFN-γ, and TNF-α at much higher levels compared with nonimmune patients. Cell cultures isolated from healthy individuals receiving cellular extracts of *A. fumigatus*, *A. fumigatus*-derived dipeptidase, or catalase antigens tend to overproduce lymphocyte-derived IFN-γ. IL-17 receptor (IL-17R) null mice show impaired clearance of *C. albicans* and decreased survival rates during systemic candidiasis. In fact, genetic polymorphism in human genes regulating Th17 differentiation (IL-17, IL-17A, STAT1, or STAT3) correlates with variable susceptibility to mucocutaneous candidiasis. $TCR\gamma\delta^+$ T lymphocytes also play a role in immunity against localized fungal infections. The $TCR\gamma\delta^+$ vaginal T lymphocyte (VTLs) population, which is part of the $CD3^+TCR^+CD90^+$ T cell subset, exhibits unique phenotypic, functional, and distribution features that enable VTLs to tailor-make localized immune responses against microbial and yeast infections.

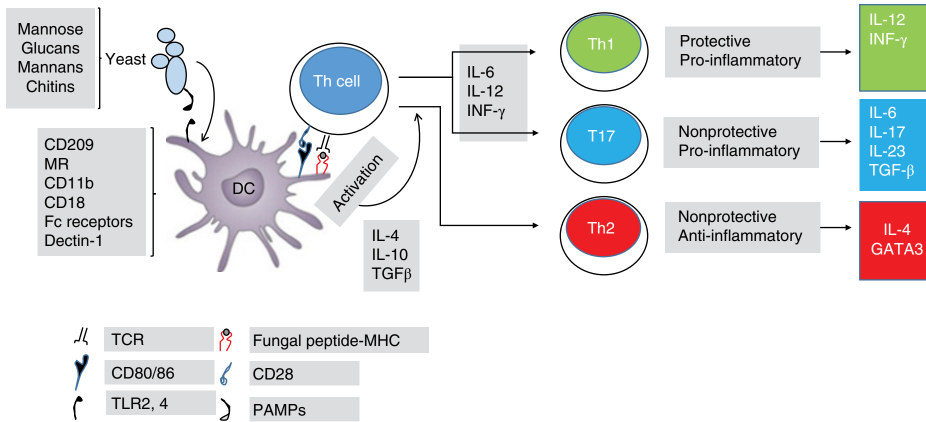

Figure 11.2 Differentiation and production of pro- and anti-inflammatory cytokines.

11.7.2.1 The Role of T Helper Cells in Antifungal Immunity

Differentiation of naïve Th cells into Th1, Th2, or T17 cells is largely determined by the kinetics of peptide-MHC-II/TCR interaction and the cocktail of cytokines in the milieu (Figure 11.3). Fungal morphotype and associated antigens that engage and activate DCs could also influence the differentiation of naïve Th cells. For example, the intracellular pathways that mediate the sensing of conidia by lung DCs *in vitro* induce Th1 protective responses, while those mediating the sensing of hyphae induce Th17 inflammatory responses. Differentiation into Th1, Th2, or T17 results in varied consequences depending on the pathogen and site of infection. IL-12-driven differentiation of Th1 results in the production of pro-inflammatory cytokines (IFN-γ, IL-6, IL-12, and TNF-β) and is protective against most fungal infections, mucosal ones in particular. IFN-γ enhances Ab production and function; it also recruits and activates innate effector components. In contrast, IL-4-driven differentiation of Th2 permits fungal persistence and allergy. Elevated levels of Th2 cytokines (IL-4, IL-5, IL-10, IL-13) positively correlate with disease severity and poor prognosis and associate with reduced IFN-γ synthesis, suppressed DTH responses, increased synthesis of nonprotective Abs (IgE, IgG4, and IgA), and eosinophilia.

IL-23-driven differentiation of Th17 cells is thought to be distinct from the Th1 differentiation pathway; although IL-23, like IL-12, induces the production of pro-inflammatory cytokines including IFN-γ, unlike IL-12, IL-23 induces the production of IL-17. Th17 cells also produce IL-17F, IL-21, and IL-22. The immune consequences of Th17 cytokines vary depending on multiple factors. For example, IL-17 along with IL-17F, IL-22, and other Th-17 cytokines induce protective responses against viral, bacterial, and fungal infections at various

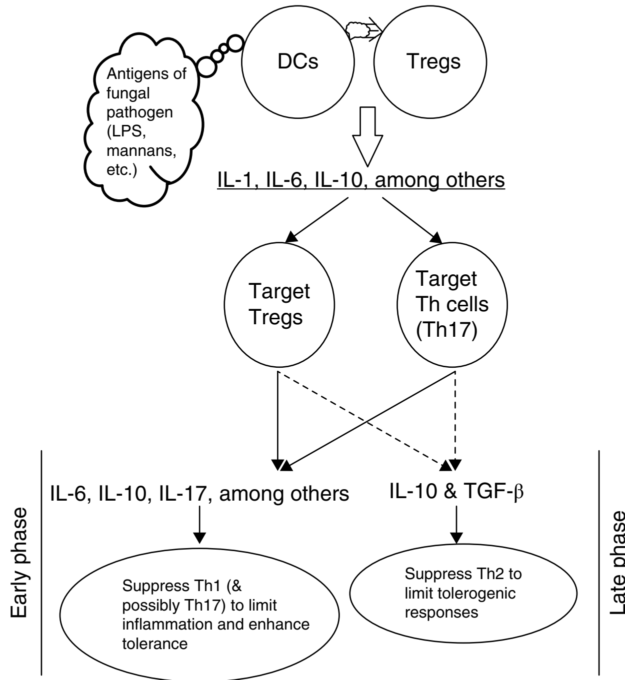

Figure 11.3 Balancing inflammation vs. tolerance: following interaction with fungal antigens, DCs and Tregs produce cytokines like IL-1 and IL-10 to influence the activity of downstream T cells that regulate the pro-inflammatory activity of Th1 (and perhaps Th17) cells and those that regulate the activity of Th2 cells. Subsequently, Th1 inflammatory response is subdued during the early phase of the T cell response while that of Th2 is subdued during the late phase of the Th2 response.

mucosal surfaces. $CD3^+$ T cell-derived IL-17 plays an essential role in the induction of inflammation and clearance of *H. capsulatum* in mice. Defective Th17 function associates with increased susceptibility to recurrent pneumonia and mucocutaneous candidiasis in patients with primary immunodeficiency diseases. IL-17 may also function as a negative regulator of Th1-mediated protective responses, thus impairing immune resistance. For instance, Th17 responses associating with *C. albicans* and *A. fumigatus* infections can subvert neutrophil inflammatory responses, promote fungal virulence, and exacerbate inflammation and infection.

11.7.2.2 Tolerance Versus Inflammation

Successful fungal commensalism and latency dictates that antifungal immunity needs to be weak enough not to completely clear the pathogen, yet strong enough to keep it under control. This delicate balance between inflammation and tolerance

is achieved through extensive cooperation between various effectors and regulators deriving from innate and adaptive components. Th1 responses recruit and activate effector immune mediators to clear the pathogen while causing significant inflammation. This minimizes tissue colonization and prevents pathogen persistence. Th17 responses subdue Th1 protective immunity but permit further inflammation and tissue injury. Th2 responses, however, impair Th1 immunity by enhancing anti-inflammatory responses, thus providing for significant tissue colonization and persistence. Under normal conditions, optimal antifungal immunity is achieved by balancing pro-inflammatory and anti-inflammatory responses.

However, the "Th1/Th17/Th2" paradigm may not be sufficient to fully appreciate how such an exquisite and subtle balance between inflammation and tolerance is attained. In this respect, Tregs may also play a role in balancing protective (Th1) and nonprotective (Th17) pro-inflammatory responses and nonprotective anti-inflammatory (Th2) responses. Naturally occurring thymus-derived Tregs ($CD4^+CD25^+FoxP3^+$) and adaptively differentiated Tregs ($CD4^+CD25^+FoxP3^-$) produce cytokines (IL-10, tumor growth factor-beta (TGF-β), and IL-4) that participate in establishing the balance between Th1, Th17, and Th2 responses. For example, IL-10 produced by DCs and Tregs in response to cell wall mannans and other polysaccharides downregulates IFN-γ production, suppresses CMI, and subdues Th1 responses against chronic candidiasis, severe forms of endemic mycoses, and aspergillosis in neutropenic patients. Indoleamine 2,3-dioxygenase 1 (IDO1), which catabolizes tryptophan to N-formylkynurenine, depletes tryptophan in target cells, thus halting their growth and activity. During the early phase of mycoses, IL-10 and CTLA-4, by acting on IDO, suppress neutrophil activity and limit inflammation and tissue damage. During the late phase of the infection, IL-10 and TGF-β inhibit Th2 responses and hence limit pathogen persistence.

11.8 Immunity to Dermatophytes

Dermatophytes are fungal molds that infect the skin and skin-derived keratinized structures such as hair and nails. Several aspects of dermatophytes influence the way in which host immunity protects against dermatophytosis.

11.8.1 Dermatophytes – Atypical Pathogens

Unlike typical opportunistic fungal infections, the incidence of dermatophytosis is as frequent in healthy immunocompetent hosts as it is in immunocompromised patients (atopic individuals, AIDS patients, etc.). Infection-causing dermatophytes are not commensals in the infected host, but rather exogenous pathogens. Some dermatophytic infections present with inflammation (erythema, skin infiltration,

pustule formation), but others (tinea pedis (*Trichophyton rubrum*), tinea capitis (*T. tonsurans*), and tinea imbricata or Tokelau (*T. concentricum*)) associate with little or no inflammation, suggestive perhaps of variable levels of neutrophil infiltration at the site of infection. Dermatophytes tend to infect the outer layers of skin and hair, and can survive under harsh conditions. Once attached to skin epidermis, however, they secrete tissue-degrading enzymes (e.g. proteases, metalloproteases) to breach body surfaces, and infect and colonize internal organs. Common dermatophytic infections including tinea pedis, tinea capitis, tinea corporis, and tinea cruris are superficial; cases of disseminated dermatophytosis (those that involve internal organs) are rare and often associate with defective immunity.

11.8.2 Cell-Mediated Innate Immunity to Dermatophytes

Interaction with unsaturated transferrin, activation of epidermal peptides, and the inhibitory effects of undecylenic acid and other fatty acids in sebum are innate responses that participate in the defense against dermatophytosis. Epidermal keratinocytes function as physical barriers and secrete immune mediators such as pro-inflammatory cytokines, chemokines (IL-1, IL-6, IL-8, TNF-α, etc.), and AMPs. Macrophages release TNF-α and IL-10 in response to dermatophytes (*T. rubrum*). During acute dermatophytosis, leukocyte effector infiltrates are detectable in infected areas. Epidermal cell infiltrates ($CD8\alpha\alpha^+$ and $CD8\alpha\beta^+$ T cells, $HLA\text{-}DR^+$ cells, and Langerhans cells) are detectable as well. Neutrophils are recruited to the site of infection by basal cell-derived chemotactic leukotriene derivatives and dermatophyte-derived cell wall antigens that activate the alternative complement pathway. Neutrophils and other PMN cells infiltrate the site of infection to kill invading pathogens. As histidine and other inhibitors of free radicals (superoxide) inhibit dermatophyte killing, it is likely that neutrophils kill dermatophytes by an oxidative burst-dependent mechanism. However, immunity against dermatophytosis not associating with inflammation is likely to be independent of neutrophil activity, given the lack of cellular infiltration common to such infections.

11.8.3 Adaptive Immunity to Dermatophytes

Mammals mount secondary immune responses to several previously encountered dermatophytes. This suggests that adaptive immunity could be involved in protection against dermatophytosis. Several dermatophyte-derived antigens including cell wall-derived glycopeptides, HSP-60, metalloproteinases, and subtilases induce adaptive immunity. Epidermal DCs that recognize and engage dermatophyte-derived antigens via the SIGN innate receptor have been shown to help orchestrate T cell-mediated immunity against keratinophilic dermatophytes.

Such DCs present antigens to local T cells or to T cells residing in peripheral lymph nodes. Dermatophyte-derived antigens like *Trichophyton* antigens that contain TQ-1 reactive epitopes may downregulate the expression of ligands like CD50 and CD80 on DCs and increase host susceptibility to infection. Oligosaccharides derived from dermatophyte cell wall glycoproteins can also disrupt T and B lymphocyte activation.

11.9 Evasion of Host Immunity by Fungal Pathogens

Fungal pathogens employ a number of strategies to evade innate and/or adaptive immune responses. Differential expression or masking of fungal PAMPs to prevent recognition by PRRs is a common strategy. For instance, masking of β-1,3-glucan beneath a layer of mannose helps hyphal forms of *C. albicans* to evade PRR recognition. Coupling this with reduced dectin-1 affinity for the sugar on *C. albicans* hyphae ensures effective evasion by the pathogen. Immune evasion through masking has also been observed in *H. capsulatum* and *C. neoformans*. *Cryptococcus neoformans* evades innate immune recognition by masking its cell wall PAMPs under a thick capsular layer of polysaccharides. Reduced expression of PAMPs has been documented in *P. brasiliensis*. During its endospore differentiation stage, *Coccidioides posadasii* secretes metalloproteinases (Mep1) that digest immune-dominant antigens including the spherule outer wall glycoprotein (SOWgp). This enables the pathogen to evade phagocytosis and killing at such a vulnerable stage of its development. Other mechanisms involving the modulation of inflammatory signals, shedding of decoy components, persistence in intracellular environments, and complement evasion have been documented. For example, *C. neoformans* undergoes phenotypic switching to a mucoid colony form that synthesizes a thick capsular polysaccharide (GXM) which helps the pathogen to reduce phagocytic efficacy of alveolar macrophages.

11.10 Conclusion

In recent decades, antifungal immunity has witnessed breathtaking advances. Interest in antifungal immunity continues to grow, given the numerous breakthroughs it has contributed to our basic understanding of how immunity in general works. Research on antifungal preventive and therapeutic modalities has contributed in a tangible way to progress in the general field of immunotherapy. However, our understanding of antifungal immunity is still incomplete and/or lacking in several specific areas. For example, the role of antibodies and that of Tc remains poorly understood despite decades of intensive work in these areas. The differential contribution of local and systemic immunity, though reasonably worked out in some instances, is still ambiguous for many of the less medically important fungal infections. Despite significant progress in elucidating the

biology of Th cell polarization and the contribution of various Th subsets to fungal immunity, the regulatory mechanisms responsible for balancing inflammation and tolerance are not fully understood. Current and future work in these as well as other areas of antifungal immunity should help antifungal immunotherapy to bear fruit.

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12

Antifungal Agents for Use in Human Therapy

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12.1 Introduction

The treatment of human fungal infections is a real challenge mainly because of drug resistance, morphological changes of fungi, and homology of fungal proteins to host proteins. Historically, treatment of fungal infections is started with the use of chemicals such as potassium iodide in 1903, followed by the use of Whitefield's ointment in 1907, and undecylenic acid in the 1940s. Other antifungal chemicals used in the treatment of fungal infections include benzoic acid, zinc, and selenium. Medicinal plant extracts, including garlic, cinnamon, *Origanum*, tea, orange, and olives, were used traditionally for the same purpose. In fact, some of these agents are still in use as folk medicines.

The development of systemic antifungal chemotherapy since the 1960s has been a significant contribution to the treatment of the majority of human fungal infections. Antifungal chemotherapy relies on the ability of drugs to inhibit the synthesis or disrupt the integrity of the cell wall, plasma membrane, cellular metabolism, and/or mitotic activity (Table 12.1). Antifungal agents used to treat human mycosis are either topical (treat local infections) or systemic (treat systemic and disseminated infections) and they can be naturally derived or chemically synthesized antibiotics. Antifungals differ in structure, molecular target, solubility, mode of action, pharmacokinetic profile, spectrum of activity, therapeutic effect (fungistatic or fungicidal), degree of toxicity, and ability to induce resistance. Based on structural differences, antifungals can be grouped into four major classes, namely polyenes, azoles, allylamines, and echinocandins. Other compounds including flucytosine, nikkomycin, sordarins, and griseofulvin do not belong to any of these classes and are arbitrarily grouped as miscellaneous antifungal compounds.

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Table 12.1 Nature, target, mode of action, and fungal resistance mechanisms of the major antifungal drugs used in human therapy.

Antifungal agent	Currently available	Mode of action and cellular target	Susceptible fungi	Mechanism of resistance	Resistant fungi
Active against plasma membrane integrity or synthesis					
Polyenes	Amphotericin B deoxycholate and lipid formulations (amphotericin B lipid complex, amphotericin B colloid dispersion, liposomal amphotericin B)	Auto-oxidation of ergosterol and formation of free radicals which compromise plasma membrane integrity and increase permeability	Different species of *Candida*, *Aspergillus*, *Histoplasma*, *Coccidioides*, *Sporothrix*, *Cryptococcus*	Absence of ergosterol (loss of function mutation in ERG3 or ERG6), decrease of ergosterol content in cells	*Aspergillus fennellial*, *S. cerevisiae*
Azoles	Fluconazole, itraconazole, voriconazole, posaconazole, isavuconazole, ketoconazole, ravuconazole	Inhibition of cytochrome p450 function: 14α-lanosterol demethylase (ERG11) sterol Δ^{22} desaturase (ERG5)	Different species of *Candida*, *Aspergillus*, *Coccidioides*, *Cryptococcus*	Efflux mediated by multidrug transporters Decrease of affinity in Erg11p by mutations Upregulation of ERG11 Alterations in ergosterol biosynthetic pathway	*C. glabrata*, *C. krusi* for fluconazole, *A. fumigatus*
Allylamines	Terbinafine, naftifine	Inhibition of squalene epoxidase (ERG1)	Different species of *Aspergillus*, *Fusarium*, *Penicillium*, *Trichoderma*, *Acremonium*, *Arthrographis*	Unknown	*S. cerevisiae*
Octenidine and pirtenidine	Octenidine, pirtenidine	Affect ergosterol biosynthesis by inhibition of 14α-demethylase	*C. albicans*, *S. cerevisiae*	Unknown	–[1]

Sphingofungin	Sphingofungin	Interrupts sphingolipid synthesis by inhibiting serine palmitoyltransferase activity	Different fungal species	Unknown	–
Morpholines	Pradimicin A, pradimicin B, benanomycin A	Inhibition of sterol Δ^{14} reductase (ERG24) and Δ^{78} isomerase (ERG2) Calcium-dependent complexing with saccharides of mannoprotein and disruption of membrane, causing leakage of intracellular potassium	Different species of *Candida*, *Aspergillus*, *C. neoformans*	Unknown	–
Active against cellular anabolism					
5-Fluorocytosine	5-Fluorocytosine	Inhibition of nucleic acids and protein synthesis	Different species of *Candida*, *Aspergillus*, *Cryptococcus*	Defect in cytosine permease Deficiency or lack of enzymes implicated in metabolism of 5-FC Deregulation of pyrimidine biosynthetic pathway	1–2% of *C. neoformans* clinical isolates are resistant

(*continued*)

Table 12.1 (*Continued*)

Antifungal agent	Currently available	Mode of action and cellular target	Susceptible fungi	Mechanism of resistance	Resistant fungi
Sordarin (sordaricin methyl ester)	Sordarin, isosordarin	Disruption of displacement of tRNA from A site to P site and movement of ribosomes along mRNA thread by blocking activity of elongation factor EF2 and large ribosomal subunit stalk rpPO	*C. albicans*, *S. cerevisiae*	Unknown	–
Active against cell wall components					
Echinocandins	Caspofungin, anidulafungin, micafungin	Inhibition of β-1,3 glucan synthase (FKS1 and 2)	Different species of *Candida*, *Aspergillus*	Alteration of affinity of echinocandins for β(1,3)-glucan synthase	*C. neoformans*, *Trichophyton*, *Fusarium* genera
Nikkomycin, aureobasidin, polyoxins	Nikkomycin, aureobasidin, polyoxins	Inhibition of chitin synthesis and assembly	*Candida* spp., *C. neoformans*	–	–

[1] Dashes indicate no resistance fungi known and no resistance mechanism known.

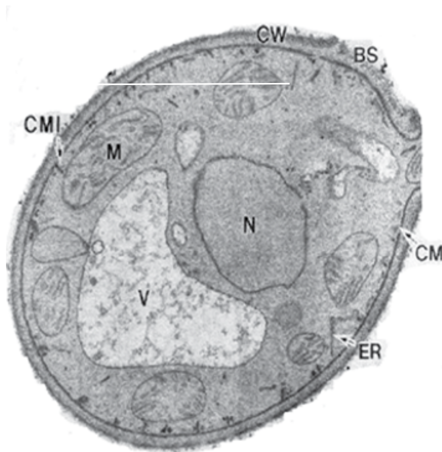


Figure 12.1 Sites of action and mechanisms of systemic antifungal agents. CW, Cell wall; BS, bud scar; CM, cell membrane; ER, enodplasmic reticulum; CMI, cell membrane invagination; M, mitochondrion; N, nucleus; V, vacuole.

A more inclusive classification of antifungals is based on their respective molecular targets, including agents that target the synthesis or structural integrity of plasma membrane (polyenes, azoles, and allylamines, among others), cell wall (echinocandins, aminocandin, and nikkomycin), synthesis of nucleic acids and/or proteins (sordarin, its derivatives, and 5-fluorocytosine (5-FC)), and novel antifungals (mitosis inhibitors) (Figure 12.1).

12.2 Drugs Targeting the Plasma Membrane

Agents that disrupt plasma membrane integrity and/or synthesis make up the majority of Food and Drug Administration (FDA)-approved drugs that are commonly prescribed to treat pre-systemic and systemic fungal infections. This group includes polyenes, azoles, allylamines, thiocarbamates, octenidine, pirtenidine, and morpholines.

12.2.1 Polyenes

Nystatin (Fungicidin; $C_{47}H_{75}NO_{17}$) and amphotericin B (AMB or Fungizone; $C_{47}H_{73}NO_{17}$) were the first polyenes used commercially as systemic antifungals. AMB was first isolated from *Streptomyces nodosus* in 1957 and is considered as lead for the production of more than 200 polyene antifungals. Furthermore, a promising polyene is SPK-843 (N-dimethylaminoacetyl-partricin A 2-dimethylaminoethylamide diascorbate) which is superior to AMB because it is a water-soluble polyene with a heptane structure, with significant antifungal activity

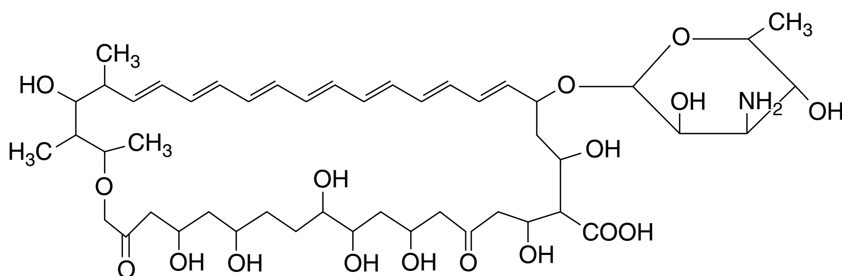
against *Aspergillus* spp. and other filamentous fungi. In fact, compared with AMB, SPK-843 has superior *in vitro* inhibitory activity against *Candida* spp., *Cryptococcus neoformans*, and *Aspergillus* spp. However, stability, solubility, toxicity, and absorption concerns of polyene compounds limit their clinical approval and use.

12.2.1.1 General Properties

Polyenes are macrolides made up of 12–37 carbons with an internal lactone ring and 6–14 hydroxyl groups arranged alternately (Figure 12.2). AMB is an amphoteric compound which contains free carboxylic and amine groups, making it capable of forming channels across the cell membrane. Some polyenes have sugar moiety, including mycosamine ($C_6H_{13}O_4N$) connected to the amine group through glycosidic linkage such as candicidin, trichomycin, pimaricin, candidin, and nystatin. Polyenes are sparingly soluble in water and nonpolar organic solvents, but easily soluble in polar solvents such as dimethyl sulfoxide and dimethyl formamide.

Despite the low water solubility, high toxicity, and ineffectiveness of AMB in immunocompromised hosts, it remains the drug of choice to treat systemic mycoses. To improve its therapeutic potential, lipid formulations such as AMB lipid complex (ABLC), AMB colloidal dispersion (ABCD), and liposomal AMB (L-AMB)

Amphotericin B



Nystatin

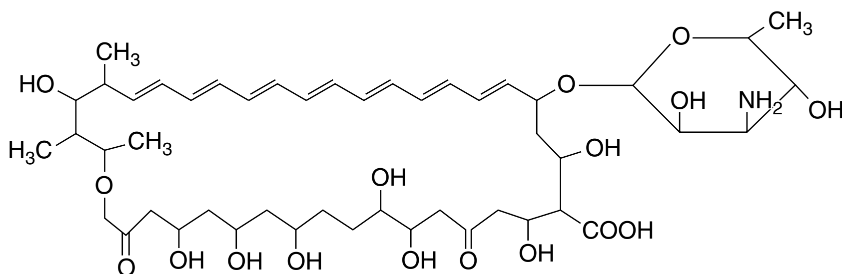


Figure 12.2 Chemical structure of polyene antifungal agents.

have been developed and approved for clinical use. The lipid-formulated AMB offers a delivery system with sustained release of the drug at higher doses (up to 10-fold), especially in primary reticuloendothelial organs and with lower toxicity. All three formulations are indicated for patients with systemic mycoses, including those who are intolerant to conventional AMB or have pre-existing renal dysfunction. Deoxycholate-AMB (D-AMB) and its lipid formulations (ABLC, L-AMB, and ABCD) are currently licensed for the treatment of invasive aspergillosis (IA). The utility of lipid AMB formulations in treating invasive mold infections (aspergillosis and zygomycosis) in immunocompromised hosts is still under study.

12.2.1.2 Mechanism of Action

AMB acts by binding the major sterol component, ergosterol, of the fungal cell membrane. This binding generates annulus pores with a diameter of 0.4–1.0 nm. Each pore consists of eight AMB molecules linked to sterols by hydrophobic bond and with hydroxyl groups facing inward. Perforation of fungal cell membrane leads to leakage of cytoplasmic components, inhibition of aerobic and anaerobic respiration, and induction of cell lysis and death. Similarly, nystatin in sufficient amounts can lead to pore formation in the fungal membrane and hence potassium (K^+) ion leakage and cell death.

Notwithstanding that pore formation is an essential mechanism of polyene antifungal activity, it is a part of a complex, multifaceted process that produces the full extent of polyene activity against target cells. For instance, polyenes induce oxidative damage to fungal plasmalemma, an effect that contributes to their fungicidal activity. AMB also induces oxidation-dependent stimulation of macrophages, an effect that permits better engagement of antifungal innate immunity. The ability of polyenes to induce lethal effects on fungal but not host cells is attributed to their selective binding affinity to ergosterol, but not to the mammalian sterol, cholesterol; hence this minimizes the adverse effects of polyenes on mammalian hosts. Fungal resistance to polyenes can be attributed to the ability of some fungi to strategically incorporate sterols into the growth media which is competitively bound to polyenes, protecting the fungal cells from the inhibitory effects of polyenes. On the other hand, polyene toxicity is also dependent on the fatty acyl composition of membrane phospholipids; changes in the ratio of various phospholipids may affect the internal viscosity and molecular motion of lipids within the membrane.

12.2.1.3 Spectrum of Activity

Polyenes are known for their significant broad antifungal activities, including most *Candida* spp., *C. neoformans*, dimorphic fungi (*Sporothrix schenkii*), and some dematiaceous fungi. Synergistic effects of polyenes have been reported; for example, AMB in combination with 5-FC showed significant activity in mice infected with

C. neoformans, *C. albicans*, *Aspergillus* spp., and *Histoplasma capsulatum*. Combination of AMB and tetracycline or rifampicin showed a synergistic effect in mice infected with *Coccidioides immitis*. Moreover, nystatin is currently used in the treatment of cutaneous, vaginal, and esophageal candidiasis, as well as cryptococcosis. Nystatin is also used as a prophylactic agent in immunocompromised patients at risk of developing fungal infections, such as acquired immune deficiency syndrome (AIDS) patients with low cluster of differentiation ($CD4^+$) counts.

12.2.1.4 Pharmacokinetics

Following intravenous (IV) administration, AMB dissociates from its deoxycholate form, and more than 90% of the free AMB binds a variety of host serum proteins, which are then redistributed to body tissues. An IV infusion of 0.6 mg/kg in adults yields a peak serum concentration (PSC) of 1–3 mg/L, which rapidly falls to a prolonged plateau phase of 0.2–0.5 mg/L. AMB is found primarily in the liver and spleen and to a lesser extent in the kidneys and lungs. Tissue reservoirs of AMB elute the drug back to blood as plasma levels fall. The concentration of AMB in peritoneal, pleural, and synovial fluids is less than half that in serum.

AMB follows a biphasic pattern of elimination from serum: an initial half-life (t½) of 24–48 hours, followed by a prolonged elimination t½ of up to 15 days owing to slow release from tissues. Detectable levels of AMB can stay in bile for up to 12 days and in urine for up to 35 days following administration. AMB can remain in the liver and kidneys for as long as 12 months following cessation of therapy. At therapeutic doses, 2–5% of AMB is excreted in urine and bile. Elimination rates of AMB are unchanged in anephric patients and those on hemodialysis. Drug concentration in hyperlipidemic patients on hemodialysis may drop due to AMB–lipoprotein complex binding to dialysis membranes. In children, the volume of distribution of AMB is smaller (greater than 4 L/kg) than in adults and rate of clearance is larger (less than 0.026 L/h/kg); PSC is approximately half that reached in adults receiving an equivalent dose.

On the other hand, the pharmacokinetic profile of L-AMB is significantly different from that of the conventional micellar form; it is selectively taken up by the reticuloendothelial system and concentrated in the liver, spleen, and lungs. AMB lipid formulations have significantly lower nephrotoxicity than D-AMB even at higher doses. ABLC and ABCD are approved for clinical use at 5 mg/kg/day and 3–4 mg/kg/day doses, respectively; L-AMB is approved as salvage therapy to IA at a dose of 3–5 mg/kg/day.

12.2.1.5 Administration and Dosage

D-AMB is available as a lyophilized powder containing 50 mg AMB and 41 mg deoxycholate and as an infusion solution of 5% dextrose in water. An infusion of 1 mg is often used to test AMB immediate reactions (fever, hypotension, chills,

and dyspnea), especially in patients with cardiac or pulmonary complications. Depending on severity of illness, two to three escalating doses can be given every 8 hours or once daily until a full therapeutic dose is reached. Doses can then be given once a day or once every other day. For most indications, the therapeutic dose is 0.4–0.6 mg/kg daily or 1 mg/kg every other day. Tolerance is assessed by starting the treatment with a lower dose (0.05–0.1 mg) that is then gradually increased as tolerated. The addition of 10–15 mg hydrocortisone hemisuccinate to the infusion can decrease some of the acute reactions. Injectable formulations of nystatin have been discontinued due to high toxicity; however, the drug is safe to use in oral (0.1–1.0 million units/dose) and topical forms because its absorption through gut and skin mucocutaneous membranes is minimal.

12.2.1.6 Adverse Effects

Nephrotoxicity is the most significant adverse effect of AMB treatment. Manifestations of nephrotoxicity may include decreased glomerular filtration rate, decreased renal blood flow, casts in urine, hypokalemia, hypomagnesemia, renal tubular acidosis, and nephrocalcinosis. The drug can cause changes in tubular cell permeability to ions; tubuloglomerular feedback (increased delivery and reabsorption of chloride ions in the distal tubule) may reduce glomerular filtration rates. The effect of tubuloglomerular feedback is amplified by sodium deprivation and is suppressed by sodium administration. This may explain renal arteriolar spasm and calcium deposition during periods of ischemia and direct tubular or renal cellular toxicity following intake of AMB. Sodium administration is effective in attenuating AMB nephrotoxicity and glomerular filtration rates. Cancer patients concurrently receiving AMB and high sodium content antibiotics (e.g. carbenacilin) experience reduced nephrotoxicity compared to those receiving AMB alone or in combination with low sodium content antibiotics. However, sodium administration requires close monitoring to avoid hypernatremia, hyperchloremia, metabolic acidosis, and pulmonary edema. Renal toxicity caused by aminoglycosides and cyclosporin is often worsened by AMB therapy.

Other adverse effects can include AMB-induced azotemia. Prostaglandin and tumor necrosis factor (TNF-α) may play a role in renal azotemia. Azotemia is reversible following cessation of therapy; however, return to pre-treatment levels usually takes several weeks to a few months. Anemia (normocytic normochromic) commonly develops due to suppressed erythropoietin synthesis and is exacerbated by reduced renal function and red blood cell (RBC) production. Anemia is reversible within several months after treatment cessation. Renal toxicity caused by aminoglycosides and cyclosporin is often worsened by AMB therapy.

AMB-induced hypokalemia may result from increased renal tubular cell membrane permeability to potassium or from enhanced excretion via activation of sodium/potassium (Na^+/K^+) exchange. Hypokalemia due to AMB requires parenteral administration of 5–15 mmol/L supplemental potassium/hour. AMB therapy may associate with magnesium loss, mainly with cancer patients who

show divalent cation loss during antineoplastic (cisplatin) therapy. Furthermore, AMB therapy may be accompanied by fever, chills, and rigors that are mediated by TNF and interleukin-l (IL-1) production. Infusion-related adverse effects of fever, chills, and rigor are less frequent with L-AMB compared with D-AMB. Mild increase in serum bilirubin and alkaline phosphatase levels has been observed with AMB lipid formulations. Clinical trials with SPK-843 indicate that it results in lower renal toxicity than AMB or liposomal AMB.

Acute reactions to AMB can include nausea, vomiting, anorexia, headaches, myalgia, and arthralgia, which are infusion-related reactions that associate with the initiation of AMB therapy. Acute adverse effects due to AMB can be minimized with corticosteroids, paracetamol (acetaminophen), aspirin, or pethidine (meperidine). Aspirin should be avoided in AMB infusion-associated thrombocytopenia. Slow infusion of the drug, rotation of infusion site, small dose of heparin in the infusion, hot packs, inline filters, avoidance of AMB concentrations greater than 0.1 g/L, or infusion of AMB through a central venous line can minimize thrombocytopenia.

12.2.1.7 Resistance to Polyenes

Resistance to polyenes remains insignificant despite decades of use. Decreased binding to mutant strains of *C. albicans* occurs due to one of several mechanisms, including (1) decreased total ergosterol content without concomitant changes in sterol composition, (2) presence of low affinity polyene-binding sterols (3-hydoxysterol or 3-oxosterol) instead of ergosterol, or (3) reorientation of existing ergosterols in such a way that masks their binding with polyene. The majority of polyene-resistant *Candida* are *C. glabrata*, *C. guilliermondii*, *C. lusitaniae*, and *C. tropicalis*, which all have low membrane ergosterol content. Similarly, *A. fennellial* mutants are resistance to polyenes since they have sterols other than ergosterol. Nystatin-resistant strains of *Saccharomyces cerevisiae* have 5, 6-dihydroergosterol instead of ergosterol as the main sterol of cell membrane.

12.2.2 Azoles

Inhibition of fungal growth by azoles was first described in the 1940s and the fungicidal properties of N-substituted imidazoles were described in the 1960s. Since then, several azoles such as itraconazole, fluconazole, voriconazole, lanconazole, ravuconazole, posaconazole, isavuconazole, and efinaconazole (also known as KP-103) have been developed to treat various forms of mycosis. More than 40 β-substituted 1-phenethylimidazoles are reported for their potent antifungal activities. Imidazoles in clinical use include clotrimazole, miconazole, econazole, ketoconazole, and sertaconazole. Luliconazole is a novel imidazole that is currently being tested for potential clinical use in the United States.

12.2.2.1 General Properties

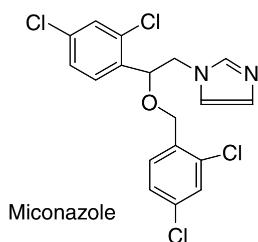
Imidazoles are five-membered ring compounds with two nitrogen atoms ($C_3H_4N_2$) and complex side chain attached to one of the nitrogen atoms. Triazoles have a similar structure but contain three nitrogen atoms ($C_2H_3N_3$). Moreover, the ring structure in both imidazoles and triazoles contains a short aliphatic chain in which the second carbon is linked to a halogenated phenyl group (Figure 12.3). The molecular weight of azoles is around 279–700 kilodalton (kDa). The unsubstituted imidazole ring and the N-C covalent linkage between the imidazole and the rest of the molecule are essential for antifungal activity. Imidazoles are white crystalline or microcrystalline powders and soluble in most organic solvents.

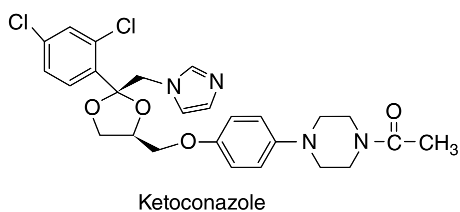
12.2.2.2 Mechanism of Action

Antifungal activity of azoles is dependent upon their ability to inhibit cytochrome P450 (CYP)-driven ergosterol synthesis. CYP is an integral component of the smooth endoplasmic reticulum and the inner mitochondrial membrane of eukaryotic cells. Its iron protoporphyrin moiety plays a key role in metabolic and detoxification reactions. CYPs are required for ergosterol biosynthesis by catalyses of the oxidative removal of the 14α-methyl group in lanosterol and/or eburicol. Azoles target CYP-Erg-11-P and lead to inhibition of 14α-demethylase and hence depletion of ergosterol and accumulation of sterol precursors (Figure 12.4). Depletion and replacement of ergosterol with unusual sterols alters the membrane permeability and fluidity. Furthermore, the drug binds to iron protoporphyrin via a nitrogen atom in the imidazole or triazole ring. The antidermatophyte abafungin (Abasol; $C_{21}H_{22}N_4OS$) can also disrupt ergosterol synthesis in a way similar to azoles. Fluconazole and itraconazole can affect the reduction of obtusifolione to obtusifoliol, resulting in accumulation of methylated sterol precursors. Luliconazole is both fungicidal by inhibiting ergosterol biosynthesis and fungistatic by inhibiting extracellular protease secretion. Mammalian cholesterol synthesis is also blocked by azoles at the 14α-demethylation step (Figure 12.5). However, the dose required to reach the inhibition in mammalian cells is much higher than that required in fungi; the inhibitory concentrations (IC_{50}) of voriconazole against CYP-dependent 14α-demethylase in rat liver cells is 7.4 μM compared with 0.03 μM in fungi.

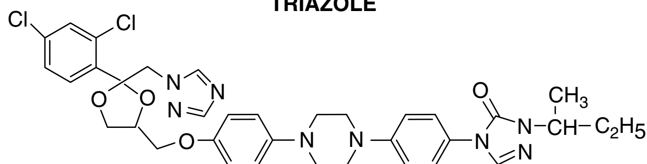
Miconazole and ketoconazole inhibit the membrane ATPase in *C. albicans* and other yeasts, causing a rapid collapse of the electrochemical gradient and drop of intracellular ATP level. At growth ICs, miconazole and ketoconazole can block the activity of *C. albicans* membrane glucan synthase, chitin synthase, adenylcyclase, and 5-nucleotidase. Incubation of *C. albicans* and other yeasts at fungistatic concentrations with clotrimazole, miconazole, econazole, voriconazole, posaconazole, or ketoconazole results in extensive changes in plasma and

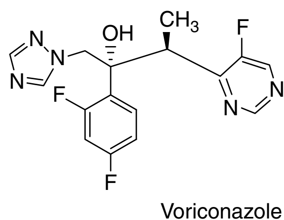
IMIDAZOLE

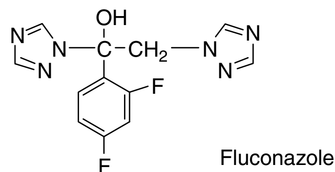


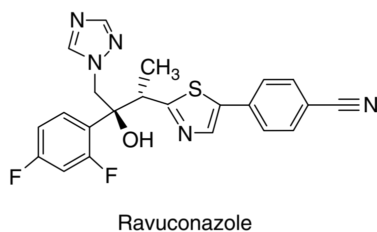


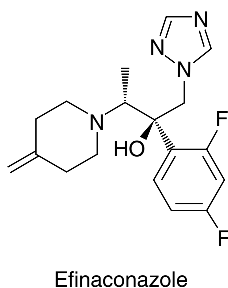
TRIAZOLE

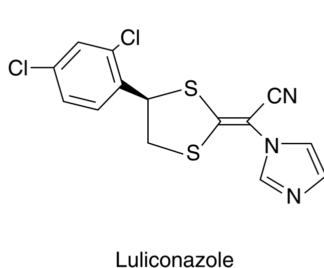












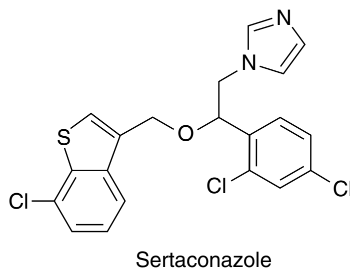


Figure 12.3 Chemical structure of imidazole and triazole antifungal agents.

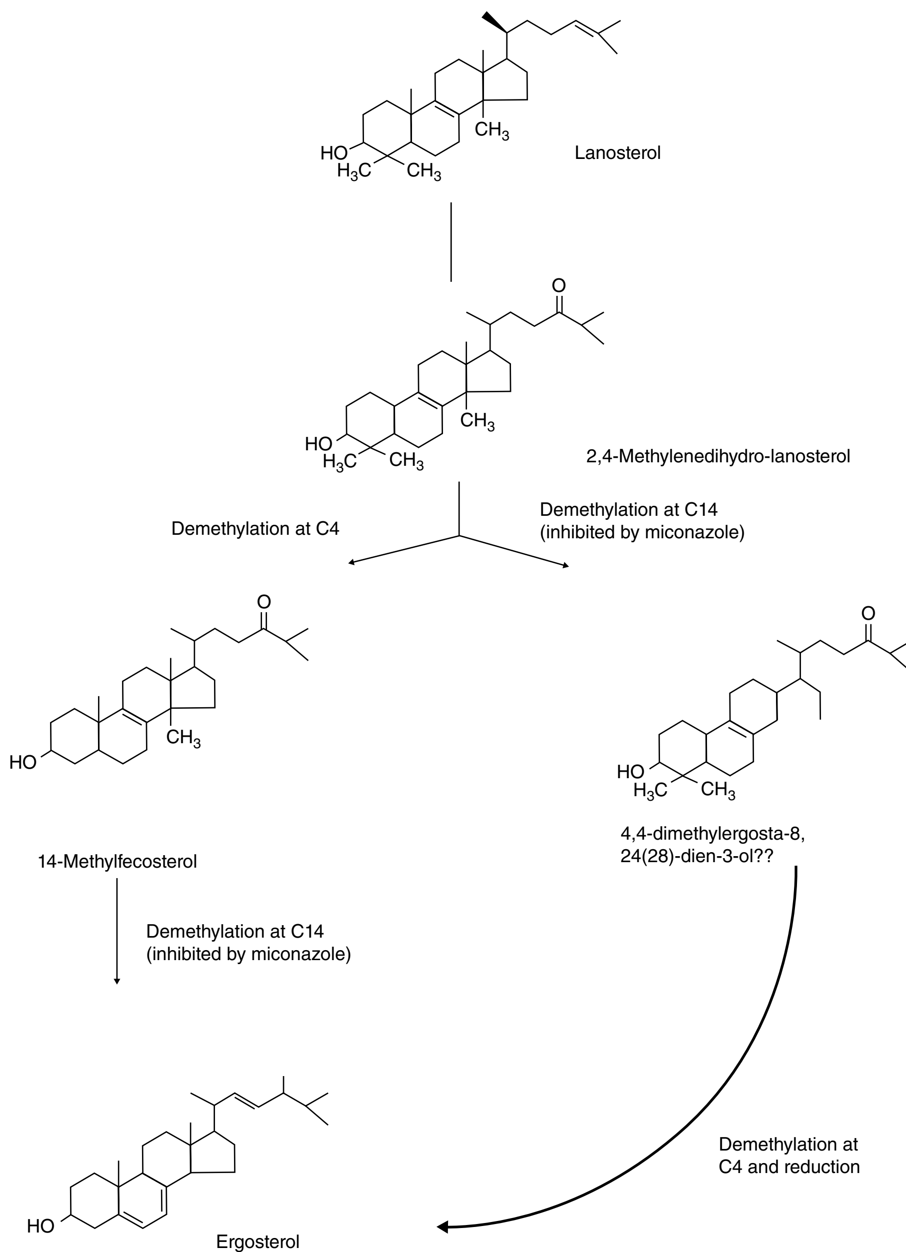


Figure 12.4 Inhibition of ergosterol synthesis by 14-α demethylase by miconazole.

nuclear membranes. Furthermore, azoles inhibit cytochrome C oxidative and peroxidative enzymes, alter cell membrane fatty acids (leakage of proteins and amino acids), inhibit catalases, and decrease fungal adhesion to the host cell. Ketoconazole tends to disrupt the transformation of *C. albicans* from budding

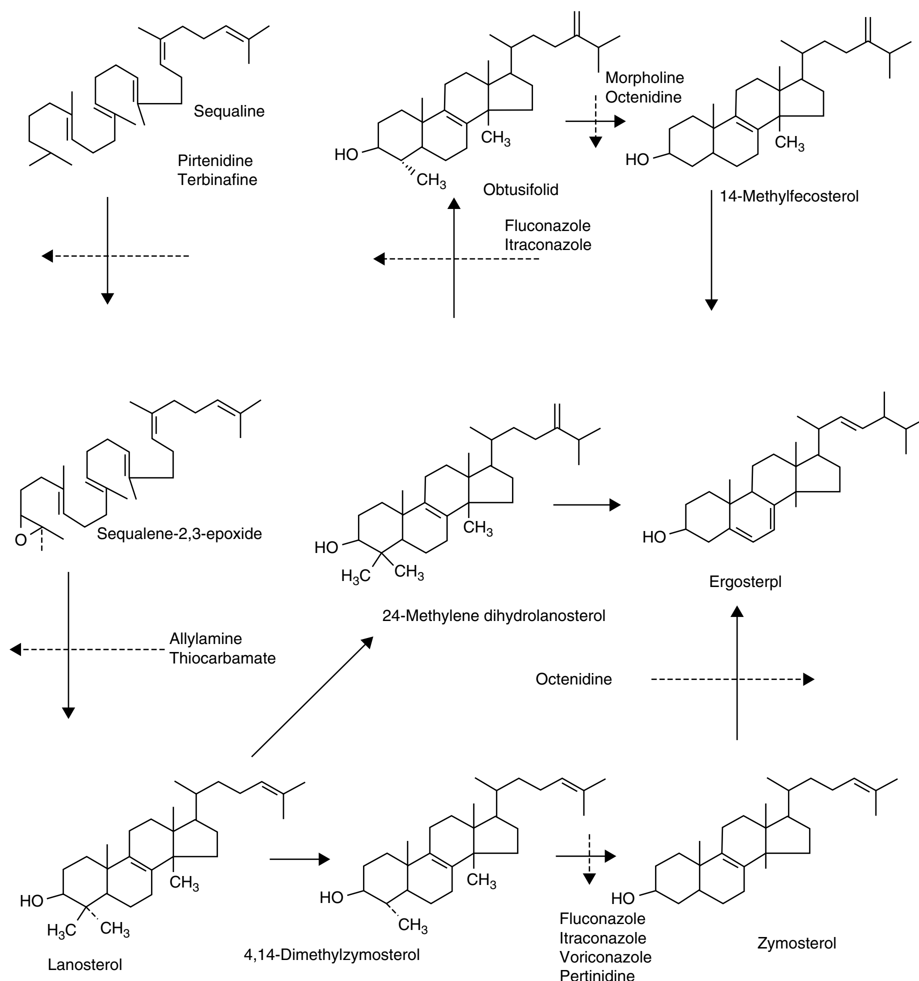


Figure 12.5 Ergosterol biosynthetic pathways and inhibition sites of clinically important antifungal agents.

to pseudomycelial form. At fungicidal concentrations, azole treatment induces the disintegration of nuclear membranes and mitochondrial internal structures.

12.2.2.3 Spectrum of Activity

Azoles exhibit significant activity against candidiasis, cryptococcosis, coccidioidomycosis, blastomycosis, and histoplasmosis. Partial therapeutic response is achievable with itraconazole in experimental animal models of aspergillosis and sporotrichosis. Voriconazole and fluconazole synergistically interact with neutrophils and monocytes to enhance the killing effect on *A. fumigatus* and *C. albicans*. Voriconazole, itraconazole, and posaconazole are currently approved for

the treatment and prevention of IA. Azoles are also active against fusariosis, *Scedosporium prolificans* infections, and clinical isolates of filamentous species. The new triazole, posaconazole, and isavuconazole are active against several species of *Candida*, *Cryptococcus*, *Aspergillus*, *Absidia*, *Rhizopus*, *Rhizomucor*, and dimorphic fungi. Their activity against *Candida* spp. is superior when compared to itraconazole or voriconazole. Moreover, benzimidazole derivatives (e.g. chlormidazole), efinaconazole, luliconazole, and sertaconazole are active against dermatophytes including *Trichophyton*, *Microsporum*, and *Epidermatophyton* spp., and hence are used in the treatment of tinea unguium, tinea corporis, tinea pedis, and tinea capitis. Efinaconazole is also active against nail and skin infections caused by *Candida* and *Malassezia* spp.

12.2.2.4 Pharmacokinetics

Available azoles differ substantially in their pharmacokinetic properties. For example, clotrimazole is unstable to sustain adequate blood concentrations. Miconazole should be administered IV to establish adequate blood concentrations; this leads to local and systemic toxicity and restricted ability to reach the cerebrospinal fluid (CSF), urine, and joints. Ketoconazole produces appropriate serum concentrations following oral administration, but the levels of absorption are dependent on gastric acidity, and hence anti-acids or H_2-blockers greatly reduce its absorption. Administration of 200 mg ketoconazole in combination with 400 mg cimetidine in an acid solution increases posaconazole (PSC) concentration from baseline levels (1.3 μg/mL) to 5.6 μg/mL. Administration of 200 mg ketoconazole showed 3.9 μg/mL PSC in young adults compared to 2.6 μg/mL in older patients. Patients with bone marrow transplantation receiving prophylactic ketoconazole therapy showed serum concentration drop to 0.5 μg/mL after 3 weeks of daily intake. Absorption of ketoconazole in patients on peritoneal dialysis is greatly reduced and its distribution into peritoneal fluid is negligible. About 85% of ketoconazole binds plasma proteins, while only a small percentage binds to hemoglobulin. Greater than 70% of the normal dose is excreted within 4 days, with about 80% excreted in feces and the rest in urine.

Fluconazole is available in oral and IV dosage forms and an appropriate milligrams per kilogram dose adjustment is required depending on the patient's clinical status. The drug is rapidly and completely absorbed; serum concentrations using the oral route are comparable to those of the IV route. Steady-state serum concentration is attained within 5–10 days, but an initial loading dose is recommended (twice the daily dose). Fluconazole is evenly distributed in body tissues, crosses the blood–brain barrier, and penetrates into the vitreous and aqueous humors of the eye. It is minimally metabolized in the liver and excreted mainly unchanged in the urine. Terminal elimination t½ is about 60 hours if glomerular filtration rates are 20–70 mL/min. Three hours of hemodialysis generally reduces drug serum concentration by 50%, but peritoneal dialysis clears the drug.

Itraconazole is highly lipophilic and almost insoluble in water or diluted acids; it is only ionized at low pH (gastric pH). High concentration is reached in polar organic solvents, cyclodextrins, and acidified polyethylene glycol. Average bioavailability after a single oral dose is about 55% depending on whether the drug is administrated in capsule or in solution form. Its absorption is dependent on stomach acidity and is enhanced by the presence of food in the stomach but reduced in the presence of anti-acids. Serum elimination t½ of itraconazole is dose-dependent within 15–25 hours after the first dose, and increases to 34–42 hours once a steady state is reached (approximately 2 weeks). Acute leukemia and AIDS patients show reduced drug absorption. Itraconazole concentration in plasma is low, two to three times greater in tissues, and reaches up to 20 times in adipose tissue compared to plasma levels. Drug concentration in eye fluid, CSF, and saliva is negligible. The drug persists in tissues for long periods, is highly metabolized in the liver, and is about 55% excreted in feces and the rest in urine. The pharmacokinetic profile of itraconazole is not affected by renal impairment and the drug is not removed by hemodialysis; however, its metabolism is reduced in patients with hepatic impairment.

Both voriconazole and posaconazole are relatively poorly aqueous soluble; hence they are formulated with sulfobutyl-ether-β-cyclodextrin (SBECD). The FDA approved delayed-release tablets and IV formulations of posaconazole in November 2013 and March 2014, respectively. The three formulations of posaconazole (oral suspension, delayed-release tablets, and IV) are widely distributed, highly protein bound, and have a relatively long t½, between 20 and 60 hours.

12.2.2.5 Adverse Effects

Azoles' toxicity includes gastrointestinal, hepatic, metabolic, hematological, and endocrinological complications (Table 12.2). Carcinogenic side-effects are also reported due to drug–drug interactions. At conventional doses, azoles are well tolerated even if administered for long periods; however, nausea and vomiting may occur in up to 10% of patients receiving ketoconazole at conventional doses and up to 50% of patients receiving higher doses (>1,600 mg/dL). Other adverse effects include headaches, fever, fatigue, abdominal pain, diarrhea, nonfatal urticaria, exfoliative dermatitis, and anaphylaxis. Hypersensitivity reactions like pruritus, rash, and eosinophilia rarely occur.

Transient nonfatal elevations of hepatic transaminase and alkaline phosphate enzymes during the first 2 weeks occasionally occur in about 10% of patients receiving ketoconazole. Transient abnormalities of liver function also occur in about 3% of patients receiving fluconazole. Endocrinological toxicity is associated with ketoconazole in a dose- and time-dependent manner. Serum testosterone levels are reduced and may cause decreased libido and potency in male subjects. Decreased serum cortisol in response to adrenocorticotropic hormone

Table 12.2 Tolerability and drug interactions of membrane-targeting antifungals.

Drug	Adverse effects	Drug–drug interactions
AMB deoxycholate	Nephrotoxicity, fever, chills, phlebitis, hypokalemia, anemia, GI disturbance	Azotemia with aminoglycosides, cyclosporine, pulmonary toxicity with granulocyte transfusion
Lipid AMB formulations	Reduced azotemia	Not defined
5-FC	Bone marrow suppression, hepatotoxicity, GI disturbance	Minimal
Imidazoles		
Miconazole	Headache, pruritus, thrombophlebitis, hepatotoxicity, autoinduction of hepatic degrading enzymes	Not defined
Ketoconazole	GI disturbance, hepatotoxicity	Drugs that induce hepatic microsomal enzymes, e.g. rifampicin, cyclosporine, antacids, H_2-receptor blockers
Itraconazole	GI disturbance, rare hepatotoxicity	Rifampicin, phenytoin, cyclosporine
Fluconazole	GI disturbance, rare hepatotoxicity, rare Stevens–Johnson syndrome	Phenytoin, warfarin, cyclosporine
Saperconazole	GI disturbance, rare hepatotoxicity	Rifampicin, phenytoin, cyclosporine

(ACTH) stimulation occurs in patients receiving ketoconazole at doses greater than 400 mg/day. At doses greater than 600 mg/day, itraconazole can cause endocrinological toxicity due to accumulation of steroid precursors with aldosterone-like effects. At doses greater than 1,200 mg/day, ketoconazole reduces the mean serum low-density lipoprotein (LDL)-cholesterol levels by about 25% with no effect on triglycerides; lanosterol concentration increases by about 45%. Miconazole causes normocytic or microcytic anemia in up to 45% of patients and thrombocytosis in 30% of patients; however, thrombocytopenia due to fluconazole therapy is rare. A daily dose of 100 mg ketoconazole increases serum concentration of sulfonylurea; hence close monitoring of serum concentration is advised. Adverse reactions following voriconazole intake include transient visual disturbance, hepatoxicity, skin rash, and visual hallucination.

Table 12.3 Biochemical basis of azole resistance.

Mechanism	Cause	Comments
Alteration in drug target (14α-demethylase)	Mutations that alter drug binding but not binding of endogenous substrate	Target is active (i.e. can catalyze demethylation) but has reduced affinity towards azoles
Alteration in sterol biosynthesis	Lesions in $\Delta^{5(6)}$-desaturase	Results in accumulation of 14α-methyl fecosterol instead of ergosterol
Reduction in intercellular concentration of target enzyme by active efflux	Change in membrane lipid and sterols, overexpression of specific drug efflux pumps (CDR1, CDR2, PDR5, BEN^r)	Poor penetration across fungal membrane, enhances drug efflux, and reduces azole accumulation
Overexpression of antifungal drug target	Increased copy number of target enzyme	Results in increased ergosterol synthesis, contributes to cross-resistance between fluconazole and itraconazole
Sequesters drug within extra- or intracellular compartments	Formation of biofilms	Results in dense network matrix containing different elements including several cell wall polymers

Modified from Ghannoum, M.A. and Rice, L.B., Clinical Microbiol. Rev., 12(4), 501–517, 1999, © 1999 American Society for Microbiology.

12.2.2.6 Resistance to Azoles

Candida albicans resistance to azoles appears only with prolonged exposure and may involve reduced permeability to the drug, along with possible alterations to cell membrane but not to cytoplasmic enzymes (Table 12.3). Pathogenic strains resistant to both polyenes and azoles have higher lipid content and lower polar/neutral membrane lipid ratios compared to doubly sensitive or only azole-resistant strains. Alterations in membrane sterols resulting in reduced binding to polyenes and reduced permeability to azoles may provide a common basis for double resistance.

12.2.3 Allylamines and Thiocarbamates

Drugs available for clinical use in this synthetic class include the allylamines naftifine ($C_{21}H_{21}N$) and terbinafine ($C_{21}H_{25}N$) and the thiocarbamate tolnaftate ($C_{19}H_{17}NOS$). Naftifine is applied topically, while terbinafine is taken orally. In general, allylamines and thiocarbamates have a naphthalene ring substituted at one position with an aliphatic chain (Figure 12.6). Naphthalene noncompetitively

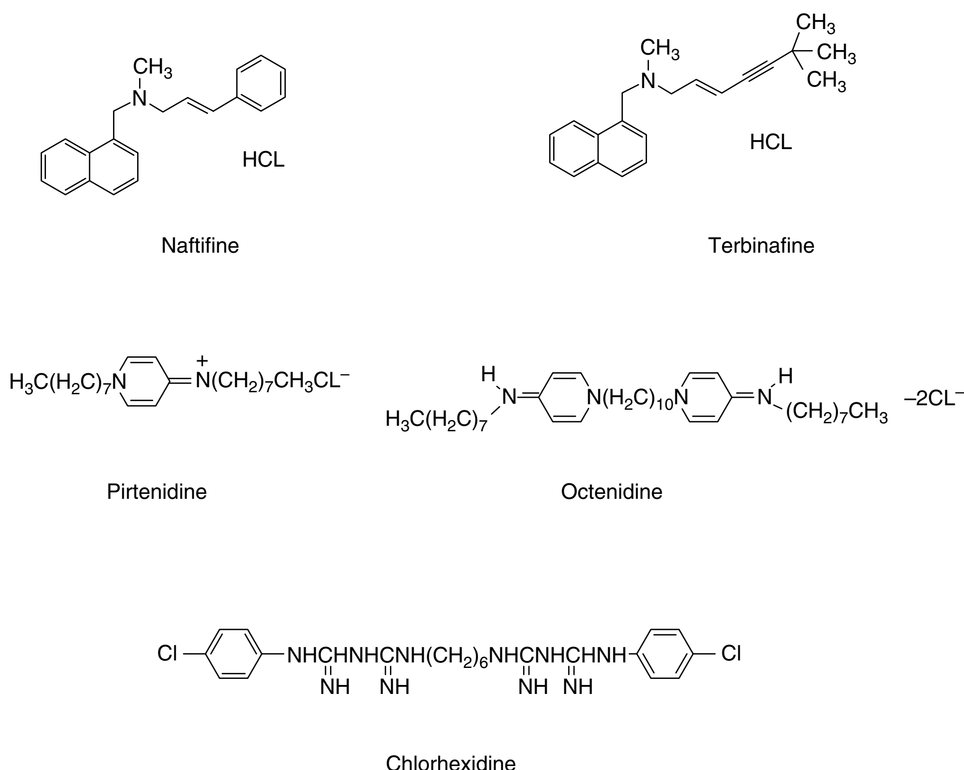


Figure 12.6 Chemical structure of allylamines (naftifine and terbinafine), pirtenidine, octenidine, and chlorhexidine.

inhibits fungal squalene epoxidase (mono-oxygenase), an enzyme that uses nicotinamide adenine dinucleotide phosphate oxidase (NADPH) to oxidize squalene to squalene epoxide as a preliminary step in membrane ergosterol biosynthesis. Similarly, butenafine hydrochloride ($C_{23}H_{27}N$) is a synthetic benzylamine derivative structurally related to synthetic allylamines and can disrupt ergosterol synthesis by inhibiting squalene epoxidase.

Accumulation of squalene at high levels increases fungal membrane permeability and disrupts cellular organization, leading to cell death. Terbinafine inhibits the growth of dermatophytes *in vitro* at concentrations less than 0.01 μg/mL. Naftifine hydrochloride cream (1%) is active against tinea cruris and tinea corporis, while 1% butenafine cream is active against *Microsporm canis*, *M. gypseum*, *Trichophyton mentagrophytes*, *T. rubrum*, and *Epidermophyton floccosum*. Butenafine has superior fungicidal activity against many fungi including *C. albicans*, *C. neoformans*, and *Aspergillus* spp. when compared to that of terbinafine, naftifine, clotrimazole, or tolnaftate. The rise of allylamine-resistant strains of *S. cerevisiae* and *Ustilago maydis* (a plant pathogen) has been documented; however, resistance to allylamines and/or thiocarbamates among human fungal pathogens is rare.

12.2.4 Octenidine and Pirtenidine

Octenidine ($C_{36}H_{64}Cl_2N_4$) and pirtenidine ($C_{21}H_{38}N_2$) are structurally similar to chlorhexidine, and were first developed as mouthwashes (Figure 12.6). They are both alkylpyridinylidine–octanamine derivatives and can cause extensive leakage of cytoplasmic contents from *C. albicans* and *S. cerevisiae*. Such changes lead to gross morphological and ultrastructural changes in treated cells. Treatment of *C. albicans* with sub-ICs of octenidine or pirtenidine alters membrane lipid and sterol contents, with significant increase in squalene and 4,14-dimethylzymosterol in pirtenidine-treated cells and an increase in zymosterol and obtusifoliol content in octenidine-treated cells.

12.2.5 Morpholines and Other Agents

Morpholines and phenylmorpholine are synthetic derivatives, with amorolfine ($C_{21}H_{35}N$) as the sole representative in clinical use. The drug was discovered in the 1980s, and shows a broad range of MICs for *Candida* isolates *in vitro*. The drug disrupts the ergosterol biosynthetic pathway by inhibiting Erg24P (Δ14-reductase) and Erg2P (Δ8-Δ7 isomerase enzyme) reactions. Amorolfine derivatives are used as a topical treatment for superficial mycoses. Drug resistance is rare. Pradimicin A (BMY-28567) and pradimicin B (BMY-28864) were identified in the late 1980s as metabolic products of *Actinomadura hibisca*. Along with the recently described pradimicin B analog (BMS-181184), these agents represent a new class of antifungal compounds with broad-spectrum activity. BMS-181184, a water-soluble derivative of pradimicin, has an MIC between 1 and 8 μg/mL against most clinical isolates of *C. albicans*, *C. glabrata*, *C. tropicalis*, *C. krusei*, *C. lusitaniae*, *C. neoformans*, and *A. fumigatus*. The definitive mode of action of pradimicin remains unclear but appears to involve initial calcium-dependent complexing of the free carboxyl group with the saccharide moiety of cell surface mannoproteins. This could presumably perturb the cell membrane and cause intracellular K^+ leakage and cell death.

Quinoline nitroxoline ($C_9H_6N_2O_3$) (Figure 12.7), a urinary antiseptic, has significant activity against pathogenic *Candida* spp., with MIC ranging between 0.25 and 2 μg/mL depending on the pathogenic strain. A39806 is a non-azole compound (Figure 12.7) with significant activity against several *Candida* spp., *Cortinarius albidus*, and *A. niger*. Both compounds increase the 4,14-dimethyl sterol concentration and concurrently decrease desmethyl sterol concentration, indicative of lanosterol 14α-demethylase inhibition.

Ciclopirox olamine, ciclopirox, and rilopirox (Figure 12.7) are hydroxypyridones with *in vitro* activity against several medically important dermatophytes, yeasts, and molds. Hydroxypyridones do not affect fungal sterol synthesis but alter membrane permeability at high concentrations and hence inhibit cellular

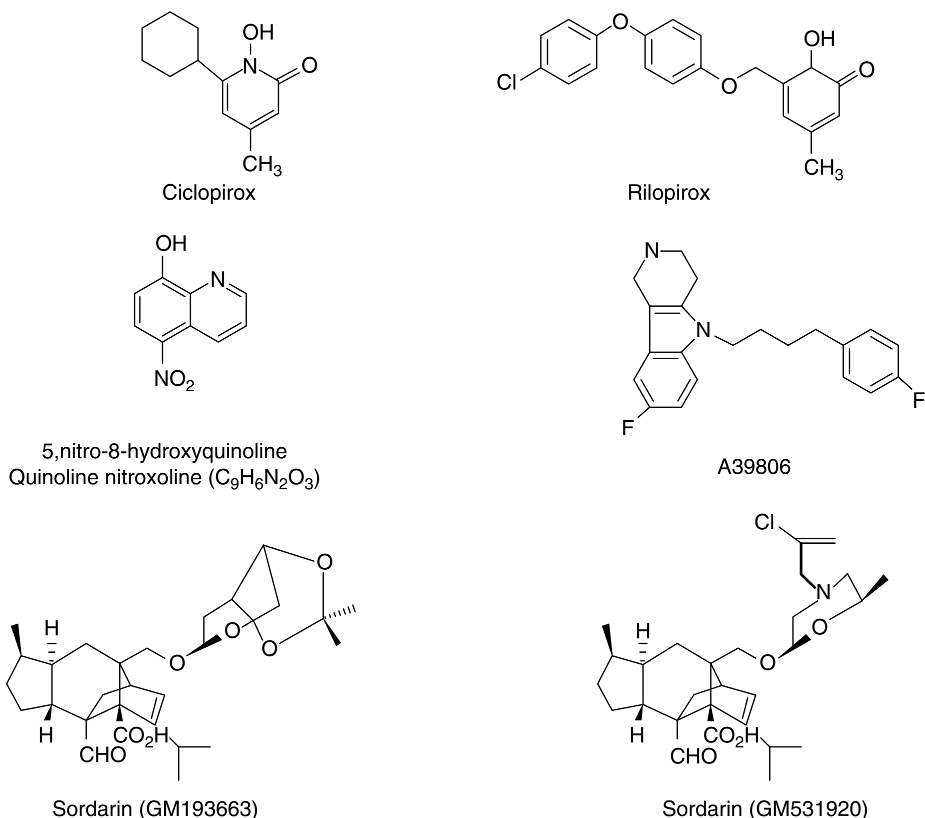


Figure 12.7 Chemical structure of two sordarin derivatives (GM193663 and GM531920) in addition to some promising antifungal compounds under development.

uptake of essential compounds. Cilofungin ($C_{49}H_{71}N_7O_{17}$), the first clinically applied echinocandin drug, is an *Aspergillus*-derived antifungal that disrupts fungal cell membrane (and possibly cell wall) synthesis by specifically inhibiting the conversion of lanosterol to ergosterol.

12.3 Drugs Targeting the Cell Wall

In fungi, the cell wall serves as the interface between the cell and its environment; it protects against osmotic pressure and controls the passage of molecules in and out of the cells. Disruption of cell wall integrity and/or synthesis is detrimental to cell viability and survival. Compounds that affect fungal cell wall glucans include candins (echinocandin and aminocandin), and those that target cell wall chitins include nikkomycin and aureobasidins.

12.3.1 Echinocandins

Echinocandins or pneumocandins are semisynthetic lipopeptides consisting of a cyclic hexapeptide core and a fatty acid side chain responsible for antifungal activity. Caspofungin, anidulafungin, and micafungin are FDA-approved echinocandins administered in the treatment of candidiasis and some other forms of mycosis (Figure 12.8). They are specific noncompetitive inhibitors of β-(1,3)-glucan synthase, a 210-kDa integral membrane heterodimeric protein, hence the name penicillin antifungals. Although Fks1p is the component to which echinocandins bind, it may not necessarily be the catalytic subunit that mediates the noncompetitive inhibition of glucan synthesis. Glucan synthesis inhibitors have secondary effects, including the reduction of ergosterol and lanosterol content and increase of cell wall chitin content; this leads to cytological and ultrastructural changes of the target fungi to pseudohyphae, with thickened cell wall and buds that fail to separate from the mother cells. The cells also become osmotically sensitive, with significant lysis of the growing tips of budding cells.

Echinocandins have low oral bioavailability; therefore, they are only available as IV infusions. Nonetheless, owing to their broad spectrum of activity, minimal toxicity, and negligible resistance, they are becoming the drugs of choice to treat certain forms of mycosis alone or in combination with other antifungal agents. They are also becoming a first-line treatment for *Candida* infections before species identification and for anti-*Candida* prophylaxis in hematopoietic stem cell transplantation (HSCT) recipients.

In addition to echinocandins, genetic analysis of resistant *S. cerevisiae* mutants to the pyridobenzimidazole derivative D75-4590 suggests that this agent can specifically inhibit β-1,6 glucan synthesis (KRE6p). Interestingly, KRE6p gene homolog presents in *A. fumigatus*, and hence *A. fumigatus* is susceptible to the activity of D75-4590. D75-4590 can also inhibit *C. albicans* hyphal elongation in a dose-dependent manner. Aminocandin is structurally similar to echinocandins, but its t½ has the advantage of being three to four times that of echinocandins. The drug has demonstrated significant efficacy against *Candida* and *Aspergillus* spp. in animal models. Overall, it has a similar *in vitro* spectrum of activity to that of echinocandins.

12.3.1.1 Caspofungin

Caspofungin (Cancidas; $C_{57}H_{88}N_{10}O_{15}$) was approved for clinical use in 2001 to treat fungal infections in febrile neutropenic adults, patients with refractory IA, and patients with IA intolerant to AMB and/or itraconazole therapy. It is also used for the treatment of candidemia and *Candida*-related infections (intra-abdominal abscesses, pertussis, pleural cavity infections, and esophagitis). The duration of treatment ranges between 1 day and 4 months, with an average greater than 1 month. In general terms, duration of treatment is determined by disease severity, response to treatment, and immune status of the patient.

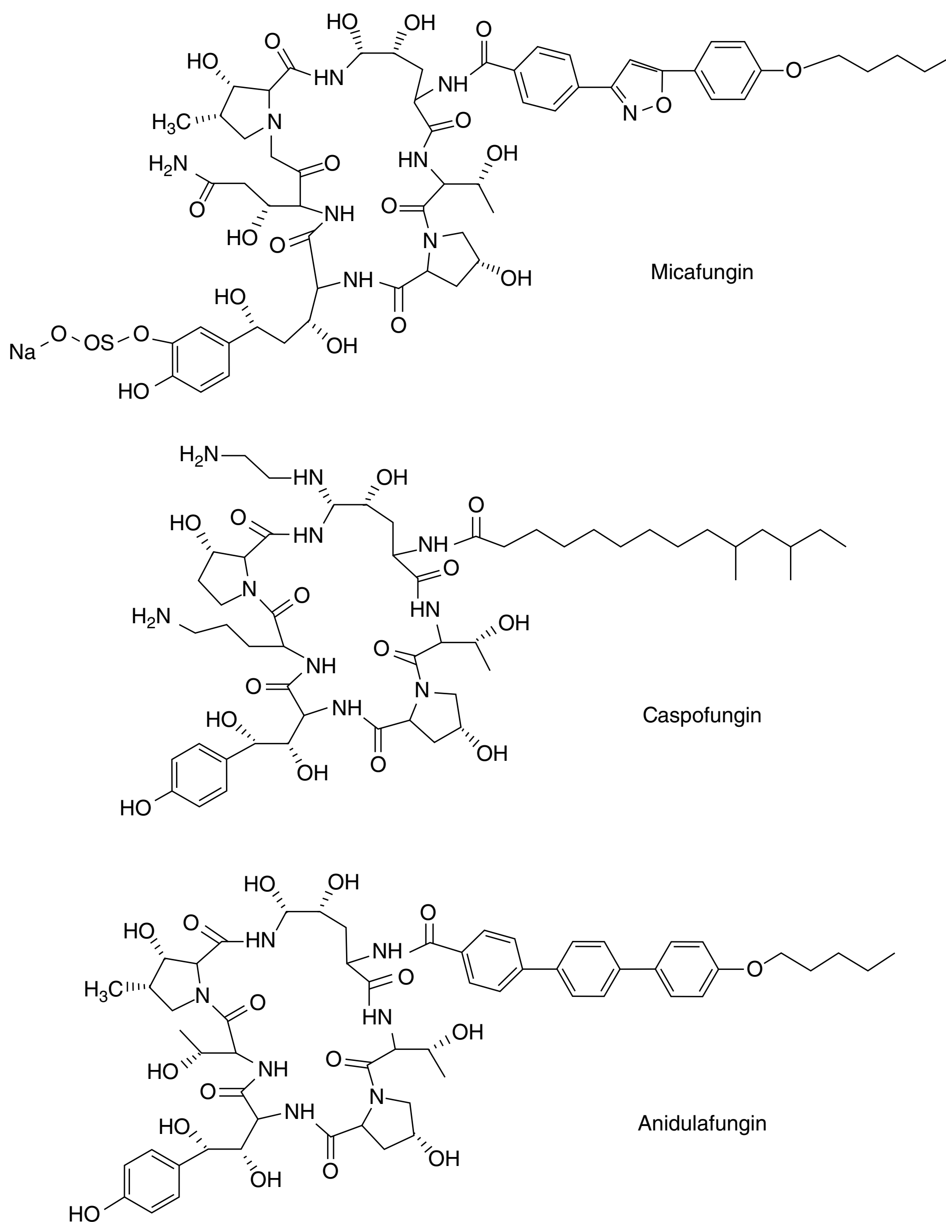


Figure 12.8 Chemical structure of three echinocandins.

Caspofungin shows minimal side-effects compared to AMB. Rare cases of symptomatic liver damage, peripheral edema, swelling, and hypercalcemia have been reported. Hypersensitivity and histamine release-related reactions such as rashes, facial swelling, pruritus, and sensation of warmth have been reported and should be closely monitored. Embryo-toxic effects have been reported following treatment of pregnant animals with caspofungin; therefore, the drug should be given to pregnant women only if benefits to the pregnant

mother outweigh potential risks to the unborn baby. Adverse interactions with cyclosporine and tacrolimus are also possible and require attention. Use of caspofungin in combination with cyclosporine may increase liver aminotransferase enzyme production to abnormal levels, and result in gastrointestinal upset and headaches. Dosage adjustment in patients with moderately impaired liver function is recommended. Although resistance in *C. albicans* has been recorded, it remains rare.

12.3.1.2 Anidulafungin

Formerly known as LY303366, anidulafungin (Eraxis; $C_{58}H_{73}N_7O_{17}$) is an echinocandin that received FDA approval as an antifungal in 2006. The drug has significant activity against invasive and esophageal candidiasis and other infections caused by *Candida* and *Aspergillus* spp. Although anidulafungin is active against experimental pulmonary aspergillosis, little is known regarding its efficacy in treating IA in humans. The drug significantly differs from other antifungals as it undergoes chemical degradation to an inactive form at body pH and temperature. Because it does not rely on enzymatic degradation and hepatic or renal excretion, it is safe to use in patients with hepatic or renal impairments. Levels of distribution are in the range of 30–50 liters and t½ is about 27 hours. Approximately 30% of the drug is excreted in feces and less than 1% in urine.

12.3.1.3 Micafungin

Micafungin (Mycamine; $C_{56}H_{71}N_9O_{23}S$) is a natural antifungal produced by the fungus *Coleophoma empetri*, and approved for clinical use by the FDA and European Medicines Evaluation Agency (EMEA) in 2005 and 2008, respectively. It is used for the treatment of candidemia and acute disseminated candidiasis, as well as *Candida*-related pertussis, abscesses, and esophagitis. Recently, micafungin has received approval for use as a prophylaxis in HSCT recipients at risk of developing candidiasis. Micafungin and anidulafungin exhibit significant activity against *Aspergillus* spp. The mechanism of fungicidal activity by micafungin is concentration-dependent inhibition of 1,3-β-D-glucan synthase, which significantly reduces 1,3-β-D-glucan formation and leads to osmotic instability and cell lysis. The drug is administered IV and dosage per day varies depending on condition; 100 mg for candidemia, 150 mg for esophageal candidiasis, 110 mg for IA, and 50 mg for prophylaxis in HSCT recipients. The drug is metabolized in the liver by Cyt P450 as a substrate for the enzyme CYP3A4. Hypersensitivity reactions may associate with micafungin therapy.

12.3.2 Nikkomycin and Chitin Synthesis

Polyoxin ($C_{17}H_{25}N_5O_{13}$) and nikkomycin ($C_{20}H_{25}N_5O_{10}$) are *Streptomycete*-derived nucleoside antibiotics capable of inhibiting chitin synthesis. They specifically and competitively inhibit chitin synthase by acting as decoys in place of the substrate uridine diphosphate-N-acetylglucosamine. *In vitro* testing of nikkomycins X and Z against various fungi shows moderate susceptibility of *C. albicans* and *C. neoformans*. The activity of nikkomycin Z against *C. albicans* and *C. neoformans* improves significantly when used in combination with fluconazole or itraconazole. Nikkomycin shows fungicidal activity against the dimorphic fungi *C. immitis* and *B. dermatitidis*. Aureobasidins alter chitin assembly and sphingolipid synthesis; the drug is mainly active against *Candida* spp. and *C. neoformans* and has some potency against *H. capsulatum* and *B. dermatitidis*.

12.4 Drugs Targeting Nucleic Acid and Protein Synthesis

Antifungals that target protein and nucleic acid synthesis can include sordarin, azosordarin, and 5-FC. 5-FC inhibits pyrimidine metabolism by interfering with RNA and protein synthesis and is considered to be the sole antimitotic antifungal in clinical use. The drug is active against *Candida*, *Aspergillus*, and *Cryptococcus* spp., as well as dematiaceous fungi that cause chromomycosis (*Phialophora* and *Cladosporium*).

12.4.1 Sordarin

Sordarin and other derivatives including azosordarin and icofungin are a class of antifungals that inhibit protein synthesis. Sordarin was isolated in 1969 from the fungus *Sordaria araneosa*. It inhibits protein synthesis by disrupting the polypeptide elongation factor 2 (EF2) and the displacement of tRNA, and hence blocks the movement of ribosomes along the mRNA. In the case of candidiasis, azosordarin disrupts polypeptide chain elongation more effectively than sordarin. *Candida albicans* EF2 shows 85% amino acid sequence similarity to the human EF2, making these compounds toxic to host cells; however, they have high specificity for fungal targets that minimize their toxicity. Icofungin (PLD-118 or BAY-10-8888) is a synthetic derivative of the naturally occurring β-amino acid cispentacin and it targets isoleucyl-tRNA synthase, which inhibits protein synthesis and cell growth. Icofungin has poor *in vitro* activity against *C. albicans*, *C. glabrata*, and *C. tropicalis*, but shows significant activity against disseminated and invasive candidiasis in neutropenic animals.

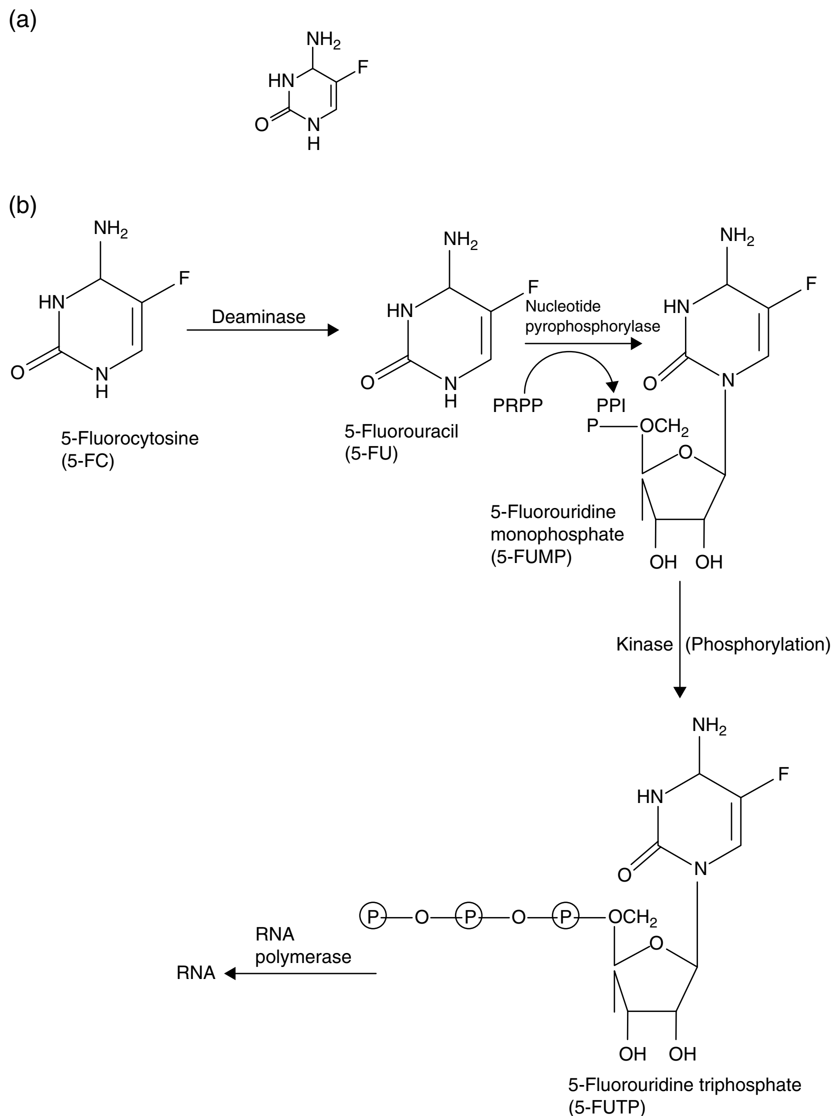


Figure 12.9 (a) Chemical structure of 5-fluorocytosine and (b) the metabolic action of 5-FC.

12.4.2 5-Fluorocytosine

The oral antifungal 5-FC (Ancobon; $C_4H_4FN_3O$) is a synthetic fluorinated pyrimidine analog. Although it lacks cytostatic and antineoplastic activities, for which it was first identified in the 1950s, 5-FC has noticeable antifungal activity. 5-FC is a 4-amino-5-fluoro-2-pyrimidine with a molecular weight of 129 kDa (Figure 12.9), comes as an odorless white crystalline, is relatively stable at normal temperatures, and is soluble in water up to 1.2%. The drug tends to crystallize if

kept at low temperatures and partially deaminates to 5-fluorouracil (5-FU) when stored at high temperatures or once taken up by cells. It is commonly used as an adjunct to polyene therapy as AMB potentiates the uptake of 5-FC by increasing membrane permeability.

12.4.2.1 Mechanism of Action

5-FC disrupts nucleic acid and protein synthesis and alters the amino acid pool of susceptible cells (Figure 12.9). It first enters the cells by the help of cytosine permease, which is usually responsible for the uptake of cytosine, adenine, guanine, and hypoxanthine. It is then converted by cytosine deaminase to 5-fluorouracil (5-FU), and by uridine monophosphate pyrophosphorylase to 5-fluorouridylic acid (5-FUMP), which is then phosphorylated and incorporated into RNA instead of uracil. The drug incorporates in large quantities into the 80S ribosomal subunits. Extensive replacement of uracil by 5-FC in fungal RNA leads to alterations in the amino acid pool and inhibition of protein synthesis. 5-FU can also be converted to 5-fluorodeoxyuridine monophosphate, which functions as a potent inhibitor of thymidylate synthase, one of the enzymes involved in DNA synthesis. Inhibition of DNA synthesis in *C. albicans* can take place before incorporation of 5-FU into RNA and hence inhibition of protein synthesis. Resistant strains of *C. neoformans* incorporate 5-FC into RNA at lower levels compared to that in sensitive strains. *Candida albicans* cells growing at subinhibitory 5-FC concentrations show increased cell diameter due to excessive carbohydrate and protein synthesis. Additional morphological changes include enlarged and translucent nucleus with filamentous components, thinner cell wall, and increased budding both in *C. albicans* and *C. neoformans*. Such changes are attributable to unbalanced growth activities where DNA synthesis is halted and residual metabolism is retained.

12.4.2.2 5 Spectrum of Activity

5-FC displays significant activity against different species of *Candida*, *Torulopsis*, *Cryptococcus*, and *Geotrichum*. It also exhibits moderate activity against *Aspergillus* spp. and chromomycosis-causing dematiaceous fungi. However, *Coccidioides*, *Histoplasma*, dermatophytes, and other medically important fungi do not respond well to 5-FC treatment. 5-FC exerts both fungicidal and fungistatic activities against *C. albicans*, *C. glabrata*, *C. parapsilosis*, *C. tropicalis*, and *C. neoformans*. Fungicidal effects usually occur at relatively high concentrations and prolonged exposure. Combining 5-FC with various AMB formulations is useful in clearing the CSF in non-HIV patients with cryptococcal meningitis, candidiasis, *Candida* endophthalmitis, renal and hepatosplenic candidiasis, *Candida* thrombophlebitis of the great veins, aspergillosis, and CNS phaeohyphomycosis.

It is also effective against large cryptococcal intracerebral masses, minimizing the need for surgical intervention. 5-FC activity against *C. neoformans* and *C. albicans* is enhanced when used in combination with fluconazole.

12.4.2.3 Pharmacokinetics and Dosage

5-FC is available in 250- and 500-mg capsules. Regarding its absorption, 5-FC is completely absorbed from the intestine following oral intake, with distribution approximately equal to the total body water. Administration of 150 mg/kg/day in adults with normal renal function produces a PSC of 50–80 mg/L within 1–2 hours. CSF concentration can reach greater than 70% that of serum concentration, making the drug suitable for treatment of CNS mycoses. Initial dosage in patients with normal renal function is 37.5 mg/kg every 6 hours (150 mg/kg/day). Regarding the drug elimination, its t½ in adults with normal renal function is 3–6 hours, and patients on hemodialysis can be given 37.5 mg/kg as a dosage adjustment. The drug accumulates in patients with azotemia, which could be toxic unless dosage is reduced to keep blood levels within the acceptable range (50–100 μg/mL). About 80% of 5-FC is excreted unchanged in the urine, while negligible quantities bind to serum proteins.

12.4.2.4 Adverse Effects

5-FC toxicity can involve gastrointestinal tract upsets including diarrhea, nausea, and vomiting. Colonic bacteria such as *Escherichia coli* rich with cytosine deaminase can deaminate 5-FC and can then get reabsorbed. This may explain why 5-FC enteritis is largely confined to the colon. Variations in colonic microflora among patients may explain why some patients tolerate 5-FC blood levels of more than 150 μg/mL for up to 6 weeks, while others show signs of toxicity much earlier. 5-FC does not deaminate in human cells, but its metabolites can be found in urine. However, serious side-effects can include hepatitis, bone marrow suppression, and rarely death. Hepatitis can occur at concentrations more than 50 μg/mL which can be resolved in days to weeks. Bone marrow suppression includes neutropenia, thrombocytopenia, and pancytopenia. Death occurs from sepsis or intracerebral hemorrhage.

12.4.2.5 Resistance to 5-FC

5-FC is often administered in low doses in combination with other drugs to limit drug resistance and toxicity. Many fungi are either inherently resistant to 5-FC or develop resistance following exposure. Drug resistance is mainly developed due to loss of cytosine permease or cytosine deaminase, deficiency in uridine

monophosphate pyrophosphorylase, or increased *de novo* synthesis of pyrimidine via increased orotidylic acid pyrophosphorylase and orotidylic decarboxylase. Furthermore, loss of feedback regulation of aspartic transcarbamylase by ATP leads to an increase in *de novo* synthesis of pyrimidine, which also facilitates the development of resistance to 5-FC. The frequency of *C. albicans* resistance to the drug at concentrations of more than 25 μg/mL is generally greater among strains of serotype B than serotype A. Serotype B, though, comprises a small minority of clinical isolates, and is usually responsible for the majority of primary resistance cases.

12.5 Novel Therapies

Despite the great therapeutic benefits of antifungals, the incidence of opportunistic and invasive fungal infections continues to rise. Infections caused by *A. fumigatus*, *C. albicans*, and *C. neoformans* remain very common and those caused by other *Candida* and *Aspergillus* spp., opportunistic yeast-like fungi (*Trichosporon* spp., *Rhodotorula* spp., *Geotrichum capitatum*), zygomycetes, hyaline molds (*Fusarium*, *Acremonium*, *Scedosporium*, *Paecilomyces*, *Trichoderma* spp.), and dematiaceous fungi are also on the rise. The ever-increasing number of hosts with compromised immunity due to underlying disease states (cancer, AIDS, and diabetes) and those on immunosuppressive therapy is partly responsible for this trend. Furthermore, toxicity, resistance, narrow spectrum of activity, and inability to fully clear sites of infection continue to limit the clinical applicability and therapeutic efficacy of available antifungals.

To overcome the limitations of currently available antifungals there is a real need for research focussing on developing antifungals with enhanced potency, broad spectrum of activity, minimal toxicity, flexible mode of administration, and favorable pharmacokinetics (bioavailability and effective tissue penetration). So far as can be foretold, new generations of triazoles, lipid formulations of AMB, and echinocandins are meeting most of these requirements. However, resistance and toxic side-effects following the use of these new drugs has been reported.

New insights into the molecular basis of mycotic infections and deeper understanding of fungal immunity, along with major advances in immunotherapy are furnishing the essential tools to search for safer and more effective molecular and immune-based antifungal therapeutic approaches. Since the 1990s, hundreds of experimental antimicrobial peptides, vaccines, monoclonal antibodies (mAbs), adoptive cell transfer procedures, and cytokine cocktails have been developed and tested as antifungal therapies (Table 12.4). However, more work needs to be done to translate benchtop findings into bedside therapies. In this regard, work on antifungal immunotherapy could benefit from the numerous success stories of cancer immunotherapy, where more than 20 monoclonal antibodies and several cytokine therapies have been granted approval for clinical use recently.

Table 12.4 Tentative list of promising alternative approaches to prevent and/or treat fungal infections.

	Specific examples	Antifungal activity	Mechanism(s) of action
Antimicrobial peptides	Pentraxin 3 (PTX3)	Pulmonary aspergillosis	Modulate cytokine production and complement responses
	Cathelicidin and other cationic peptides	*A. fumigatus* infections in animal models	Produce IL-6 and TNF-α, minimize inflammation
	Kininogen-derived peptides GKH17 and HKH17	Candidiasis	Fungicidal activity
	Synthetic lactoferrin-derived peptides Lfpep and kaliocin-1	Fluconazole- and AMB-resistant strains of *C. albicans*	Fungicidal activity (membrane permeabilization)
	Synthetic analogs of cationic polypeptide CAP37	Fluconazole-sensitive and -resistant isolates of many *Candida* spp.	Fungicidal activity
	Cystatin	Azole-resistant *C. albicans* isolates overproducing multidrug efflux transporters Cdr1p and Cdr2p	Fungicidal activity
	Histatins 1, 3, and 5	*C. albicans*, *C. glabrata*, *C. krusei*, *S. cerevisiae*, *C. neoformans*, and *A. fumigatus*	Fungistatic and fungicidal activity (target mitochondria and initiate loss of transmembrane potential)
	Trappin-2 (endogenous serine protease inhibitor)	*A. fumigatus* and *C. albicans*	Fungistatic and fungicidal activities
Monoclonal antibodies	Anti-CD40	Systemic *C. neoformans* infections	Produce IFN-γ and TNF-α, expression of MHC-II
	Anti-β-glucan in *C. albicans*	Candidiasis	Inhibits synthesis of β-glucan
	G5 (anti-*C. albicans* IgA)	Candidiasis	Fungicidal activity
	G15 (IgM-recognizing epitopes on capsular polysaccharide GXM of *C. neoformans*)	Virulent *C. neoformans* in pre-sensitized mice	Fungistatic and fungicidal activities
	A9 (IgG1 with high affinity for surface peptides)	Invasive aspergillosis in mice	Inhibit hyphal development
	4F11 (anti-*P. carinii* IgM)	Protect SCID mice against development of pneumocystis pneumonia	Fungistatic/fungicidal activities

Table 12.4 (*Continued*)

	Specific examples	Antifungal activity	Mechanism(s) of action
	Anti-gp70	*P. brasiliensis*	Abolish lung granulomas
	Anti-KT mAb KT4	KT-sensitive *C. albicans* strains	Anti-idiotype activity
	nmAb-KT (IgG1κ specific to peptide sequence 41GST-DGK46 of HM-1 KT in *Williopsis saturnus*)	Aspergillosis in neutropenic T cell-depleted bone marrow-transplanted mice	Neutralizing antibody (reduces HM-1 killing and glucan synthase activity)
Vaccines	P13 decapeptide mimotope of cryptococcal capsular polysaccharides	*C. neoformans*	Upregulates expression of IFN-γ
	HSP90-DNA vaccine	Systemic candidiasis in mice	Produces protective IgGs
	A. fumigatus-derived rAspf3	Invasive aspergillosis	T cell-dependent immunity
	N-terminus of *Candida* adhesin Als3p	Lethal candidemia and *S. aureus* infections	T cell-dependent immunity
Cytokines	IFN-γ, IL-12, anti-IL-4, G-CSF, or GM-CSF	*C. neoformans*, *C immitis*, *P. carinii*, and others	Modulation of Th1-dependent immunity
	GM-CSF and G-CSF plus conventional antifungals	Polyene- and azole-sensitive and -resistant strains of *C. albicans* and *A. fumigatus*	Modulate Th1-dependent immunity, synergism with chemotherapy
	Human lymphocytes cultured with IL-2	*C. neoformans in vitro*	Conjugation and direct inhibition of fungal growth
Cell transfer therapy	Human lymphocytes cultured with encapsulated *C. neoformans*	*C. neoformans*	Fungistatic (involves NK, CD4⁺, CD8⁺, and CD16/56⁺ cell activity)
	Blood cells stimulated by Aspf16-pulsed DCs	*Aspergillus* spp. *in vitro*	Cytotoxic activity and IFN-γ production
	Granulocyte transfusion	In neutropenic patients	Innate immunity-dependent clearance of infecting agent
	G-CSF-activated granulocyte transfusion	Clears *Zygomycete* infections	
	CD4⁺ or CD8⁺ T cells from immunized animals	Immunizing pathogen in naïve mice	Modulate Th1- or Th2-dependent responses

(*continued*)

Table 12.4 (*Continued*)

	Specific examples	Antifungal activity	Mechanism(s) of action
Oligonucleotides	2′-O-methyl backbone (19-mer2′-OMe) hairpin	*C. albicans*	Antisense oligonucleotide
	^{L}(TACCTTTC) and $T^{L}CT^{L}AC^{L}GA^{L}CG^{L}GC^{L}C$	*C. albicans*	Oligonucleotide-directed misfolding of RNA
	30 bp oligonucleotide targeting calcineurin A (CNA1)	*C. neoformans*	Antisense repression
	Olidos targeting PI3K/Akt/mTOR inflammatory pathway in lung DCs	Aspergillosis-related inflammation	Antisense small interfering RNAs (siRNA)
Drugs and inhibitors in use for other indications	Deferasirox (iron chelator)	Mucormycosis in neutropenic mice	Fungistatic, especially if combined with LAMB
	Indinavir (antiretroviral protease inhibitor)	*C. neoformans* infections	Expands splenic CD8α^{+} DCs, expression of co-stimulatory molecules and proinflammatory cytokines
	Daucosterol (beta-sitosterol glycoside)	Disseminated candidiasis	Induction of Th2-dependent.protective immunity
	Amiodarone (sodium channel blocker)	*C. albicans*, *C. neoformans*, and *S. cerevisiae* infections	Depletion of cellular Ca^{2+} stores, especially if used with miconazole or fluconazole
	CPT (camptothecin, $C_{20}H_{16}N_2O_4$)	*C. neoformans*	Topoisomerase I inhibitor
	Eflornithine ($C_6H_{12}F_2N_2O_2$)	*C. neoformans*	Inhibitor of ornithine decarboxylase
	FK506 and CsA (calcineurin inhibitors)	*C. albicans*, *C. neoformans*, *A. fumigatus*, cryptococcal meningitis	Suppress T cell-mediated immunity
Probiotic therapy	*Lactobacillus rhamnosus* GR-1 and *L. reuteri* RC-14	Mucosal and vaginal candidiasis in humans and animals	Restore and/or improve microbial balance Modulate immunity to better respond to infection
	L. casei GG	Vaginal candidiasis during human pregnancy	
	L. acidophilus (NCFM and LA-1)	Candidiasis in immunodeficient bg/bg-nu/nu mice	
	Bifidobacteria animalis	Mucosal candidiasis	

12.6 Conclusion

The eukaryotic nature of fungal pathogens and the complex fungal pathogen–host interactions make the development of safe and effective antifungals very challenging. The rising number of hosts susceptible to invasive and opportunistic mycosis and the ever evolving repertoire of human fungal pathogens add to the complexity of this issue. Additionally, the surprising lack of preventive and/or therapeutic vaccine against human fungal pathogens despite massive efforts since the 1960s makes the case for novel antifungals extremely urgent. Therefore, research geared towards the development and evaluation of preventive and therapeutic agents that engage the development of immunotherapy is as important as the development of conventional antifungals.

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13
Fungi in the Environment

Richard O'Hanlon

13.1 Introduction

Fungi are ubiquitous in the natural environment. They have been found deep in the oceans, in the earth's upper atmosphere, and in all of the other habitats in between. Experts have estimated that there are more than 1.5 million species of fungi, of which only 7% (ca. 100,000) have been discovered. It is thought that the fungal biomass in soils exceeds the biomass of all other soil organisms combined, except plant roots. A fungus (*Armillaria solidipes*) is the largest living organism ever recorded (spreading for over 965 ha in an Oregonian forest), recently reclaiming the title from the massive genetically identical clonal tree (a poplar species) commonly known as "Pando."

Yeasts, molds, mildews, mushrooms, puffballs, and truffles are some of the common names given to different groups of fungi. While many fungi are used as food (see Chapter 6) or in biotech applications (e.g. in brewing) (see Chapters 7, 8, and 9), the majority of fungi are found in the wider environment and are yet to be harnessed for human enterprise. These fungi have important functions in the environment including:

- in symbiotic mutualisms
- in nutrient cycling, retention, and formation of soil structure
- as food in food webs
- in the creation of microhabitats and aiding in successional processes in habitats.

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It takes a number of different fungi with different ecological lifestyles to maintain ecosystem health and provide the functions listed above. These fungi can be highly specialized in both their ecosystem function and their ecological requirements. For example, the dung fungus *Panaeolus papilionaceus* is usually found on animal dung, where it is important in recycling the nutrients in the dung back into the environment. This chapter aims to highlight the diversity of fungi found in the environment and describe some of their ecosystem functions.

In order to examine the fungi that provide these ecosystem functions, it is helpful to divide the fungi into three groups:

- Symbiotic fungi form a close relationship with another nonfungal organism, in which both partners gain.
- Saprobic fungi break down debris (e.g. leaf litter and deadwood) and utilize the nutrients released.
- Parasitic fungi infect and take nutrition from another living organism.

While many of the examples in this chapter come from forest ecosystems (the author's primary area of interest), the information and concepts are mostly applicable to other habitats too (e.g. grasslands). Furthermore, while the majority of known fungal species are microfungi, examples of macrofungi predominate in this chapter. This focus is partly one of preference of the author, but also because macrofungi have inherent characteristics that make them especially important for some ecosystem services, such as in food webs.

13.2 Macrofungi, Mushrooms, and Sporocarps

The main component in terms of biomass in most fungi is the microscopic filaments (hyphae). While mushrooms are often the most noticeable part of the fungus, they only make up a small portion of the biomass of the fungal species. The majority of the fungus is growing in the substrate (e.g. soil, deadwood) in the form of a web of hyphae known as the fungal mycelium. In some species this mycelium can grow over many hundreds of meters in search for nutrients. When the mycelium encounters a food source, it releases a suite of powerful enzymes to break down the substrate and extract nutrients for the fungus.

Fungal colony establishment starts as a result of a spore landing in a suitable habitat, from which hyphae emerge in search of nutrients. Each fungal species has ecological requirements necessary for its establishment and survival. Fungal ecologists have conceptualized the effect of these ecological requirements as a series of environmental filters. Before establishing in an environment, a fungal species must first pass through a number of these filters. These filters can be

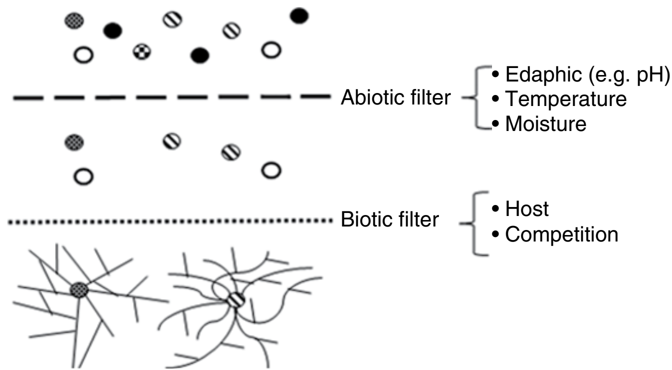

Figure 13.1 Schematic of abiotic and biotic filters that affect the establishment of fungi in a habitat.

broadly grouped into biotic and abiotic filters (Figure 13.1). Abiotic filters include edaphic conditions (e.g. soil pH); for example, acidophilic (acid-loving) fungi are less likely to establish in alkaline soils. Biotic filters include whether a suitable host is present or not; for example, if the tree species sycamore (*Acer pseudoplanatus*) is present, then the tar spot fungal parasite (*Rhytisma acerinum*) can become established in the leaves of the sycamore.

In some groups – known as macrofungi – sporocarps are produced from the mycelium as a result of sexual reproduction between two compatible individual mycelia. Sporocarps can be formed above ground (epigeous) or below ground (hypogeous), depending on the fungal species. There are estimated to be more than 9,000 species of macrofungi worldwide, with over 1,200 recorded in Ireland alone. Macrofungi take a variety of forms, including mushrooms, truffles, puffballs, and bracket fungi (Plate 13.1a–k). A rough definition of macrofungi is that they produce sporocarps that are larger than 5 mm; the largest recorded (*Fomitiporia ellipsoidea*) weighed over 400 kg and was discovered in a forest in China. The function of these sporocarps is to disperse the fungal spores, and macrofungal sporocarps are mainly formed by members of the phyla Ascomycota and Basidiomycota. Fungi, like plants, are immobile, and so need to spread their spores over large distances to find suitable nutrient sources. Spores can be dispersed in the wind over many kilometers; however, in general, it has been shown that the majority of spores of most macrofungi, particularly in the basidiomycetes, fall within 5 m of the sporocarp. Often the shape and characteristics of the sporocarp reflect the ecological traits of the fungus. Bird's nest fungi, for example, have sticky spore masses that are launched into the air when rain or wind causes disturbance to the sporocarp. Fungi have evolved to form sporocarps suited to spore dispersal in many different environments. Examples of these specific evolutionary adaptations are dealt with in the sections below.

13.3 Symbiotic Fungi

The term symbiosis is used to describe a situation where two organisms form an intimate relationship in which both partners gain. There are many types of symbioses involving fungi in the natural world, of which the lichen and mycorrhizal (Plates 13.2 and 13.3, respectively) symbioses are the most well-known types. Across all fungi, the symbiotic lifestyle evolved from either a saprobic or in some cases a pathogenic lifestyle. This is indicated by the fact that many symbiotic fungi possess genes commonly found in saprobic fungi, an indicator of carry-over from a previous lifestyle.

Fungal genera that contain individual species of symbiotic and saprobic lifestyles (e.g. the mycorrhizal genera *Amanita*, and *Laccaria*) offer fascinating opportunities to study the evolution of the mycorrhizal habit. The mycorrhizal fungus *Amanita thiersii* was thought to be an example of a species that may have reverted back to a saprobic lifestyle, having originally been saprobic but evolving with the rest of the genus *Amanita* to be mycorrhizal. However, new synthesis of existing research has indicated that this species may in fact be an ancestral saprobic sister group to the mycorrhizal members of the genus *Amanita* (i.e. it never evolved the mycorrhizal lifestyle). The same picture has emerged from a 2016 global study of the genus *Laccaria*. Many symbiotic fungi have evolved to become obligate symbionts – that is, being unable to complete their lifecycle in the natural environment in the absence of their nonfungal partner. This points to the large benefits that some fungal species gain from choosing a symbiotic lifestyle.

Another fact that indicates the benefits to be gained by the fungus from a symbiotic lifestyle is that in both mycorrhizal and lichen-forming fungi, the symbiotic lifestyle is polyphyletic. This means that a number of distantly related groups of fungi evolved over a period of time to be symbiotic, independent of each other (Figure 13.2). In one group of mycorrhizal symbiotic fungi (the ectomycorrhizas; see below), the symbiotic lifestyle is said to have evolved independently in over 70 different and distantly related phylogenetic lineages. From an environmental point of view, the lichen and mycorrhizal symbioses are two of the most important symbioses in terms of their biomass and their effect on ecosystem functioning in terrestrial ecosystems, and they are discussed further here.

A lichen is a symbiosis between a fungus (the mycobiont) and an alga/cyanobacteria (the photobiont). The mycobiont provides water and nutrients, as well as forming a protective micro-environment in which the photobiont can generate food via photosynthesis. In return, the photobiont provides some of its photosynthetically generated food to the mycobiont. In lichens, the photobiont gains nutrients and environmental stability and the mycobiont gains a reliable food source. The lichen lifestyle is polyphylectic across fungi, with the majority (>90%) of mycobionts coming from the ascomycetes. Some mycobionts can associate with a range of different photobionts depending on the ecological situation, and in some cases two or more photobionts can be contained in the

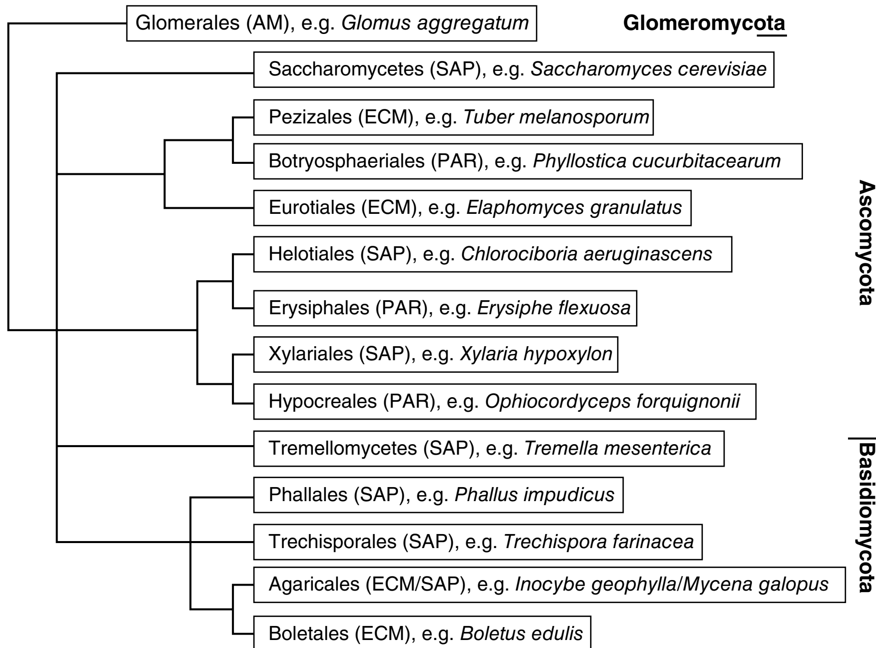

Figure 13.2 Illustrative molecular phylogeny of several macrofungal taxonomic orders from fungal phyla Glomeromycota, Ascomycota, and Basidiomycota, showing the polyphyletic nature of mode of nutrition arbuscular mycorrhizal (AM), (saprobic (SAP), ectomycorrhizal (ECM), parasitic (PAR)) in fungal orders.

same lichen structure (i.e. thallus). In general, lichens are divided into three groups based on their gross morphology: crustose, foliose, and fruticose. Crustose lichens form a crust-like growth, foliose form a flattened leafy thallus, while fruticose lichens form shrub-like growths. Lichens are negatively affected by high concentrations of sulfur dioxide in the atmosphere. As sulfur dioxide is a common by-product of atmospheric pollution, lichens can be used as reliable indicators of good ecosystem health.

Mycorrhizas are another important form of symbiosis involving a fungus. The term mycorrhizal comes from the Greek words *myco* (fungus) and *rhiza* (root). As the name suggests, mycorrhiza describes a symbiotic relationship between a fungus and a vascular plant. The fungus attaches to the plant roots and gains photosynthetically generated food from the plant host (sometimes as much as 20% of the plant's total). In return for this, the fungus provides the plant host with increased access to nutrients from the soil, and increased defenses against root-infecting pathogens. Mycorrhizal symbioses are thought to have been vital in the colonization of land by the first land plants. Mycorrhizas are still very important to the survival of many plants, with over 80% of plants known to be involved in mycorrhizal symbioses. The two most frequent forms of mycorrhizal symbioses are the arbuscular mycorrhizas (AM) and the ectomycorrhizas (ECM).

In AM fungi, the fungus grows within and between the plant root cells. In ECM, the fungus exists as an outer sheath (mantle) surrounding the plant root, with limited growth within the plant roots. The mycelium of both AM and ECM fungi extends into the rhizosphere (i.e. root zone) and scavenges for nutrients, which it then provides to its plant host (Plate 13.3).

For forest trees in the boreal and temperate zone where soil fertility is relatively low, ECM symbiosis is vital for establishment and survival. Studies have shown that ECM fungi can extract nutrients directly from decomposing plant litter and wood, from insects such as springtails, nematodes, and even from stone particles in the soil. There are over 7,700 known ECM fungal species, and these can account for around 40% of the species richness of sporocarp-producing species found in temperate and boreal forests. The ECM genera *Cortinarius* (webcaps), *Russula* (brittle gills), and *Lactarius* (milkcaps) are all very species rich in temperate forests. In addition to surveying for sporocarps of ECM fungi, researchers often examine root samples to measure the ECM species richness and community composition in the ecosystem. Below-ground surveys of ECM diversity on plant roots in forests often find that more than 90% of the live fine roots of trees are colonized by ECM fungi. New root growth is actively colonized by ECM species present in the rhizosphere. These ECM species compete for root space, the result of these competitive interactions often depending on which species "gets there first." This priority effect has been shown through experiments on two *Rhizopogon* species. Glasshouse trials using the truffle species *Rhizopogon occidentalis* and *Rhizopogon salebrosus* found that *R. salebrosus* was competitively inferior to *R. occidentalis*, only being able to colonize the roots where *R. occidentalis* was not already present.

Glasshouse trials and field studies have shown that a single fungal species can form ECM associations with a number of individual trees at the same time, and the resulting network is known as the common mycorrhizal network (CMN). Food and nutrients (e.g. nitrogen, phosphorus) can be shared between networked trees via the CMN. A number of experiments using pulses of labeled CO_2 have shown that this transfer can go in both directions between two plants. A source-sink hypothesis has been used to explain this bidirectional movement of nutrients between plant hosts and ECM fungi (Figure 13.3). Larger plants build up nutrients (e.g. nitrogen) in their tissues every year. ECM fungi actively search for and absorb nitrogen from the soil, and so have higher nitrogen than the plants. In the example shown (Figure 13.3), the young seedlings have the lowest amount of nitrogen in their tissues, and so nitrogen flows preferentially from the high nitrogen ECM fungus to the low nitrogen seedlings. From an ecological perspective, this nutrient sharing can promote stability in plant ecosystems by ensuring there are healthy young seedlings ready to replace mature plants when they die. On the other hand, recent results from AM fungal experiments have indicated that AM fungi preferentially supply nutrients to the larger plants in their network, thus exacerbating the competition for nutrients between host plants in AM networks.

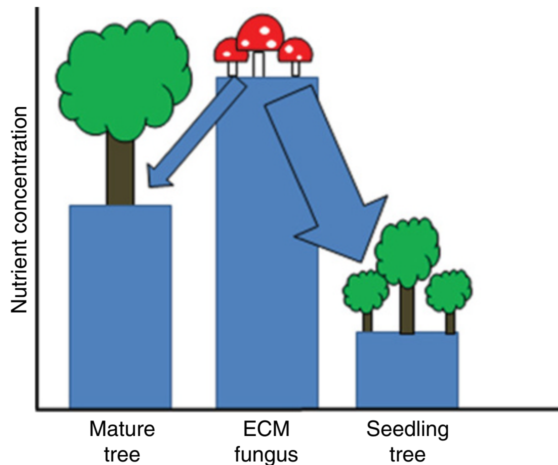

Figure 13.3 Illustrative representation of one of the hypotheses to explain the source-sink mechanism in distributing nitrogen between trees of different size in a common mycorrhizal network. Arrow indicates direction of resource transfer; width of arrow indicates magnitude of transfer.

The study of CMN transfers of nutrients is an area that has benefited greatly from modern techniques in recent years, allowing these experiments to be carried out in the field under real conditions. This is an area where some researchers have called into question how reliably results from artificial environments (e.g. glasshouse microcosms) can be extrapolated to the situation in the field. Another very important consideration of these experiments is the experimental set-up used. The topic of experimental design and replication is discussed in more detail below. A fascinating area of current research is in the study of signal transfer via the CMN. Separate groups of researchers have shown that plants under attack by herbivores can send warning signals to other plants through the CMN. At the ecosystem level this could allow neighboring plants to prime their defenses when a harmful organism is in their vicinity. Other than aiding plant communities in defending against harmful organisms, the CMN also encourages successional processes in forest ecosystems. Succession in ecosystems describes the process by which fast-growing ruderal species are replaced by more competitive species.

13.4 Saprobic Fungi

Saprobic fungi are very important in the recycling of nutrients in terrestrial and aquatic environments. The fungal mycelium releases extracellular enzymes that break down the remains of plants and animals, and in turn release these nutrients back into the soil for reuse by other organisms when the fungal biomass decomposes. With up to 70% of the above-ground biomass of forests in the form of perennial plants, it is important that the nutrients stored in this biomass are

recycled into the forest soil. Saprobic fungi can harness a wide range of enzymes and extracellular metabolites to break down complex organic matter. Because of this they are often studied for their biotechnology applications, with some species being able to decompose petroleum and plastic-based refuse. With hundreds of thousands of saprobic fungal species in existence, the potential to harness their specialized metabolic arsenal is a major driving factor in fungal biotechnological research.

In natural ecosystems there is often a noticeable chronosequence of fungal species involved when plant material is broken down. From the moment a piece of plant material (e.g. a leaf) is cut off from the main plant, it is colonized by saprobic fungi. In some cases, the fungi that begin the process of decay are already present before the leaf is shed by the plant. These fungi are known as endophytes – that is, an organism (usually fungal or bacterial) that lives within a plant for some of its lifecycle without causing apparent disease. Some fungal endophytes even provide protection to the host plant from other pathogenic organisms. However, in other cases endophytes can also be pathogenic to the plants under certain conditions, or at certain stages in the endophytes' lifecycle. Shortly after coming into contact with the ground the leaf will be colonized by terrestrial fungal species. Many of these colonizing fungi are transient, and are outcompeted once their preferred nutrient source is used up within the leaf.

As fungal species differ in their ability to break down certain polysaccharides, several species of fungi can co-exist in this decaying material. The general trend is that the first fungi to colonize a piece of detached plant material have a high level of host specificity. This has been reflected in an analysis of fungal records from Denmark. It was found that deadwood in early stages of decay had a rather specific community, linked to the identity of the plant host. This may be because the fungus has become specialized to that host in order to be able to avoid the still-active host defenses. Another was that the pH of the material had a strong effect on the fungal community found. As the plant material decays further, generalist fungal saprobes become more frequent, while the specialist species are outcompeted. The sulfur tuft fungus (*Hypholoma fasciculare*) is one such generalist saprobic species that is commonly recorded on deadwood in temperate coniferous forests. During the colonization of a piece of plant debris (e.g. a tree branch), the competition between fungal species for space and nutrients is intense. The saprobic fungus *Oudemansiella mucida* produces a number of powerful antifungal compounds including strobilurins. These strobilurins have since been synthesized and are a commonly used class of fungicides in plant protection products. Other saprobic fungi are active in the decomposition of macrofungal sporocarps. Members of the genera *Penicillium*, *Trichoderma*, and *Spinellus* are common species found on decaying sporocarps.

In aquatic environments, fungi such as those from the aquatic hyphomycetes are very important in nutrient cycling. Often described as "Ingoldian fungi," after the mycologist who devoted his career to the group (C.T. Ingold), the spores of these microfungi attach to the surface of leaves that fall into the water. Some

groups of these fungi have evolved to have grappling hook-like spores, increasing their ability to attach to leaves and detritus. The spores then germinate and penetrate within the leaf. Leaves colonized by these fungi have been found to be more palatable to invertebrate detritivores, thus aiding in the recycling of nutrients from plant material in these aquatic ecosystems.

13.5 Parasitic Fungi

Parasitic fungi infect and absorb nutrition from another living organism. This term is often interchangeable with pathogenic; however, pathogenic is a term used more frequently in agriculture and medicine where the emphasis is on the outcome of the interaction (i.e. disease) between the pathogen and host. Use of the term parasitic is more frequent where the interaction itself is of primary interest (i.e. one organism living off a host). Fungi such as the honey fungus (*Armillaria mellea*) and many bracket fungi (e.g. *Ganoderma applanatum*) are said to be fungal parasites on trees. On the other hand, *Fusarium graminearum* (cause of *Fusarium* head blight of wheat) and *Botrytis fabae* (cause of chocolate spot on beans) are said to be pathogens of plants.

Fungi parasitic and/or pathogenic on plants are dealt with in Chapter 14. However, there are some examples of fungi that can switch from mutualistic to parasitic depending on the circumstances. In the discipline of plant pathology, the pathogenic behavior of an organism is often examined in regards to the disease triangle. This states that the interaction between the host, pathogen, and the environment controls the manifestation of disease. A susceptible host, an especially virulent pathogen, or a conducive environment can all lead to disease development. Ash dieback disease (causal agent *Hymenoscyphus fraxineus*) is a result of a very susceptible host (*Fraxinus excelsior*) meeting a virulent pathogen in a favorable environment. In its native range of northeastern Asia, *H. fraxineus* is not thought to be a strong pathogen to the native ash (*Fraxinus manshurdica*) trees, and is more commonly seen as an economically unimportant endophyte. The ash dieback pathogen may be an example of when an endophyte becomes a pathogen when it encounters a particularly susceptible host.

While fungal parasites are often seen as negative in agriculture and forestry, in natural ecosystems these parasitic fungi play an important role in regulating the ecosystem. Under natural conditions, parasitic fungi often infect and kill old trees, which opens up the canopy of the forest for younger trees to thrive and replace the fallen tree. In other circumstances, parasitic fungi kill groups of trees, which opens up large areas within the forest which can be colonized by a different tree species, thus increasing the tree species diversity of the forest at the landscape scale. Parasitic fungi also create habitats for many bird and small mammal species through the process of heart-rot in large trees, with an estimated 40% of the bird species in some North American forests nesting in tree cavities. Furthermore, fungal parasites encourage diverse plant communities to

form in natural ecosystems. In monocultures, a parasite can quickly spread from plant to plant in cases where the dominant plant is susceptible. In mixed plant communities, the presence of nonsusceptible hosts adds a buffering effect to the ecosystem, limiting the damage the parasite causes to the plant community by limiting its spread capacity.

Fungi can also be parasitic and/or pathogenic on other fungi. The mycoparasite *Chalciporus piperatus* is thought to be parasitic on its host the macrofungus *Amanita muscaria*, with mycologists commonly noting the tendency of these species to fruit side by side. *Asterophora parasitica* is a mycoparasite on *Russula* sporocarps, an example of a parasite that is very host restricted. The "lobster fungus" is a sought-after mushroom for eating; however, this is not a single fungus but the result of one fungus parasitizing another. The lobster fungus is produced as a result of the mycoparasite *Hypomyces lactifluorum* infecting either a *Russula* or *Lactarius* sporocarp and causing abnormal sporocarp formation. The macrofungal species *Tolypocladium longisegmentum* (syn. *Elaphocordyceps longisegmentis*) is mycoparasitic on sporocarps of the hypogeous genus *Elaphomyces* (Plate 13.1f). In horticultural production of button mushrooms (*Agaricus bisporus*), the fungal pathogen *Trichoderma aggressivum* (cause of green mold) can result in total crop losses in severe infestations.

13.6 Fungi in Food Webs

Apart from cases where fungal mycelium is formed into a foodstuff (e.g. the mycelium of *Trichoderma venenatum* is processed to become the food product "Quorn"), in most cases it is the sporocarp of the fungus that is eaten by humans and animals. Sporocarps of macrofungi are collected or grown for eating by humans in over 80 countries. Some species of fungi can be farmed under controlled conditions, leading to a reliable source of mushrooms year round (Chapter 6). Farmed mushrooms are produced by inoculating a sterilized nutrient source (e.g. compost and peat) with the spores of the mushroom, and the inoculated medium is then stored under closely controlled (e.g. humid and cool) conditions. The sterilization process along with the closely controlled environment is vital in preventing contamination and crop losses due to fungal parasites such as *Trichoderma aggressivum*. The most commonly farmed fungus in Europe is the button mushroom (*Agaricus bisporus*); less common varieties including the shitake (*Lentinula edodes*) and oyster mushroom (*Pleurotus ostreatus*).

Due to their ecologies, some edible fungal species cannot be "farmed" and are only available when collected from the wild. Some of the most expensive edible macrofungi are ECM species, such as the matsutake (*Tricholoma matsutake*) and several hypogeous truffle fungi (e.g. *Tuber melanosporum*) (Table 13.1). These edible ECM species are inherently difficult to farm because they are obligate ECM symbionts; that is, they require a suitable tree host to be grown in close proximity. While there has been success in "growing" truffle orchards in

Table 13.1 Notable macrofungal species frequently grown or collected worldwide.

Species	Ecology	Commercially grown	Regions where eaten
Agaricus bisporus (button mushroom)	Saprobic	Yes	Worldwide
Cantharellus cibarius (golden chanterelle)	Ectomycorrhizal	No	Temperate
Lentinula edodes (shitake)	Saprobic	Yes	Worldwide
Pleurotus ostreatus (oyster mushroom)	Saprobic	Yes	Worldwide
Boletus edulis (cep)	Ectomycorrhizal	No	Worldwide
Ophiocordyceps	Parasitic (on insects)	No	Asia (medicinal uses)
Tuber melanosporum (black truffle)	Ectomycorrhizal	No	Worldwide
Tricholoma matsutake (matsutake)	Ectomycorrhizal	No	North America, Japan
Tuber aestivum (summer truffle)	Ectomycorrhizal	No	Mediterranean, worldwide
Ganoderma applanatum (artist's bracket)	Parasitic	Yes	Asia (medicinal uses)

some countries by planting trees that already have the truffle fungus on the roots, a common problem is that the truffle fungus is replaced over time by inedible indigenous ECM species once the trees are planted in the soil. Indeed, studies of tree seedlings grown in plant nurseries and planted in the wider environment have shown that the ECM community can change entirely a few years after out-planting.

There are many organisms other than humans that rely on macrofungi as a food source. Soil micro-arthropods (e.g. springtails) "graze" on fungal mycelium in the soil, and show a distinct preference for fungal mycelia over other food sources. Macrofungal sporocarps are quickly colonized by many insects, much to the dismay of hungry mushroom enthusiasts. Some fungi have evolved physical and chemical protective mechanisms to deter these predators. Physical deterrents include scaly stems to slow crawling insects or slimy caps to prevent predators from eating the fungus. Chemical deterrents include the production of unpleasant or even toxic chemicals to ward off predators. The "deathcap," "destroying

angel," and "fool's webcap" are all examples of macrofungi that produce toxic chemicals and are responsible for many of the human deaths worldwide due to mushroom poisoning.

Other fungi have evolved to encourage predation by insects and animals, in order to use the predator as a vector to disseminate their spores. Two examples of this are the hypogeous fungi (e.g. truffles; Plate 13.1d) and the stinkhorn fungi (Plate 13.1i). Truffle fungi produce aromas and flavors that are very attractive to certain predators. The use of pigs or trained dogs to find these truffles is practiced in many countries, as the truffles are often very valuable and highly sought after for culinary purposes. In the Pacific Northwest of North America, truffles form a large part of the diet of some mammals, including squirrels and chipmunks. These animals seek out, store, and eat truffles. Truffle spores can survive passage through the gut of the animal, and so are disseminated in their droppings. Truffles that are stored, but subsequently forgotten about, can mature and also release their spores. These spores percolate into the soil and can go on to form ECM symbiosis with a host.

While truffle fungi produce pleasant aromas to attract predators, other fungi (e.g. stinkhorn fungi) produce unpleasant aromas to attract insects. The stinkhorn fungus produces a slimy substance that smells like rancid meat, and this substance also contains thousands of spores. Insects are attracted to the sporocarp by the scent, and leave with spores attached to them. These spores later fall off and can germinate to form a new fungal colony, which may result in a new stinkhorn sporocarp. Many thousands of insects can visit a mature stinkhorn during its fruiting period, indicating that this evolved dispersal strategy is highly successful.

13.7 Fungi and Nutrient Cycling

Fungi affect nutrient cycling in terrestrial ecosystems by breaking down organic matter stored in plant and animal cells, and by making nutrients available to other organisms that are otherwise inaccessible. Fungi play an important role in the cycling of many nutrients including carbon, nitrogen, phosphorus, sulfur, and trace nutrients in terrestrial ecosystems. The carbon in plant debris and animal remains is stored as complex polysaccharides such as cellulose and lignin. These substances are highly recalcitrant, and are unavailable to most organisms in the ecosystem unless they are first broken down into simpler forms by microorganisms. Many fungi can produce a wide range of extracellular enzymes to break down these compounds. Once these complex sugars are broken down into simpler forms, the fungus can absorb these and use the energy to produce fungal biomass. Fungal cells in turn die, or are eaten, and their organic matter is once again cycled back into the environment. Some macrofungi, such as the brown rot fungus (*Phanerochaete chrysosporium*), have been studied intensively to map their enzyme repertoire. The potential to harness the enzymatic powers of fungal

species opens up many biotechnological prospects, for example in the areas of biofuel generation or environmental remediation.

Nitrogen (N) and phosphorus (P) are two of the most limiting nutrients on plant growth in natural ecosystems. Fungi and bacteria are two of the main groups of organisms involved in N and P cycling in the rhizosphere. The part that ECM fungi play in N cycling in terrestrial ecosystems has already been discussed. Phosphorus exists in a variety of different organic and inorganic forms in the terrestrial environment. The inorganic form orthophosphate ($H_2PO_4^-$) is the only form taken up in significant amounts by plants and fungi. Fungal-absorbed P is transferred to the plant host in exchange for plant-derived carbon.

Fungi are also thought to have an influence on environmental sulfur (S) cycles. In terrestrial ecosystems S is mainly present in two organic forms, sulfate-esters and sulfonates. Sulfate-esters are only accessible to bacteria and fungi, while bacteria alone are able to access sulfonates. Although hypothesized as playing a part in the terrestrial S cycle, direct transfer of S from fungi to plants has not yet been shown to occur. Indirect transfer occurs when soil fauna (e.g. micro-arthropods, nematodes) graze on fungal and bacterial communities, thus releasing the sulfate (SO_4^{2-}) into the soil solution for uptake by plants. Fungi also bio-accumulate several trace nutrients from the environment. Several studies have found that after radioactive fallouts, fungi can accumulate more radiocesium isotopes than are present in the rest of the environment. This is why it is often advised that mushrooms in the vicinity of previous nuclear fallouts are not eaten.

While fungi can have strong effects on nutrient cycling in environments, they themselves are also strongly affected by the nutrient status of the environment. As discussed earlier, many fungi have a range of conditions at which they can establish and persist. Atmospheric nitrogen deposition in northern Europe increased during the twentieth century due to increasing industrialization and intensive agricultural production. These two developments led to increasing amounts of nitrogen oxide and ammonia released into the atmosphere. The atmospheric deposition of these molecules contributed to increased levels of acid rain across Europe. These nitrogen compounds and the resultant acid rain caused changes in community composition of ECM fungi in some European forests. At the taxonomic level, it is well known that some ECM species are nitrophobic, while others show nitrophilic tendencies. The fetid species from the genus *Russula*, so called because they often have nauseating odors, are a group with strong nitrophilic tendencies. The frequent nursery stage ECM species *Amphinema byssoides* is nitrophobic, and has been shown to decrease in frequency in forest plots that have received nitrogen fertilization. Many of the emblematic grassland waxcap fungi (genus *Hygrocybe*) are strongly nitrophobic, and have become less frequent in recent years due to increased fertilizer use in intensively managed grasslands and atmospheric nitrogen deposition.

13.8 Quantifying Fungi in the Environment

What is known about fungal biology and ecology could not have been revealed if not for scientists conducting carefully planned reproducible studies to answer specific questions. Fungal ecology investigations often seek to describe the fungal community by recording the numbers/frequency of fungal individuals (e.g. individuals of the same species, individuals of different species) in a set area (e.g. a forest). In order to study fungi in an environment, samples of the fungal community must be collected and the fungal taxa (e.g. species, individuals) need to be classified. As is evident from the above sections, fungi are very heterogeneously distributed in the environment. This means that samples of the fungal community need to be replicated across space and time in order to allow generalizations about the fungal community of a site to be made. An investigator cannot learn much about the fungal community in a forest from examining the fungal species present in a single soil sample from that forest. Likewise, collecting sporocarps on one occasion will only record what macrofungal species were fruiting at that time in the study area. Replication is also vital in the more advanced techniques, such as in next-generation sequencing (NGS) studies. Often it can help to consult a statistician about an experimental plan and the number of samples needed to generate enough statistical power to find a significant result.

13.8.1 Sampling Methods for Fungal Studies

A number of sampling methods have been used in the past to get a representative sample of the fungal community in a terrestrial site (Table 13.2). The samples would then be examined using one or more of the methods of analysis listed in Table 13.3. Each of the methods listed (Table 13.2) has its pros and cons.

In the past, studies of fungi in ecosystems often focussed on monitoring of sporocarps as an indicator of fungal diversity. This method was relatively cheap, and provided samples (i.e. sporocarps) that could be stored long term (i.e. by drying) and re-examined by other mycologists. The dried fungal material stored in herbaria around the world is a priceless source of information for mycologists, and its value has been rediscovered by scientists as they seek to apply modern molecular biology techniques to reveal the true depth of fungal biodiversity. However, the use of fungal sporocarps alone as a measure of the fungal community present had obvious disadvantages. Many fungal species do not produce noticeable sporocarps, while those that do may only do so irregularly. This means that depending on the time of the year when the survey is carried out, the resulting macrofungal community recorded can vary widely. It is common in studies of macrofungal diversity to see the species richness increasing with every year of survey, as was found in a macrofungal survey of Irish forests (Figure 13.4). A study of macrofungal diversity in a forest in Switzerland is still recording new

Table 13.2 Sampling methods for the collection of fungi in the environment.

Method	Brief description
Sporocarp collection	Sporocarps are collected and recorded
Root sampling	Root samples are taken and root-associated fungi are identified and recorded
In-vitro isolation	Soil or debris samples are taken from the site and fungi are isolated onto agar media using serial dilutions and/or selective media/isolation conditions
Sample incubation	Soil or debris is incubated at different temperatures in the laboratory in order to encourage fungi to grow from the sample
Spore trapping	Mechanical or passive traps are established on a site to capture the fungal spore community present in the air
Baiting	Plants or plant material (e.g. wood) are positioned throughout the site. These samples are collected after a predetermined time to identify the fungi colonizing them

Table 13.3 Identification methods for quantifying fungi in an environmental sample.

Method	Brief description
Morphological identification	Species are identified by comparing their morphological characters to published descriptions of species
Sanger sequencing	A single taxon is identified by sequencing a region of its genome (often the ITS region) and comparing to sequences of identified taxa stored in a repository
Phospholipid-derived fatty acids (PLFA)	Content of certain indicator PLFA for fungi in samples is measured and used as a proxy for abundance of fungal groups
Next-generation sequencing (NGS)	Numerous taxa are identified and their frequency measured in samples by barcoding based on amplification of a specific marker gene region
Matrix-assisted laser desorption/ionization–time-of-flight (MALDI-ToF)	Uses mass spectrometry to compare microorganism profiles to known species stored in a reference database
Carbon source metabolism microplates	A standard range of carbon-containing compounds are provided to a pure culture of fungus in a microplate. Based on the carbon sources that are used by the fungus, the species can be identified by comparison to a reference database
Stable isotope analysis	Isotopic fractions of carbon and nitrogen in the fungus are examined and compared to isotopic fractions in plants, soil, and wood in the habitat. This technique is useful for understanding how the fungus gains its carbon and nitrogen, for example in differentiating between mycorrhizal or saprotrophic lifestyles in fungi

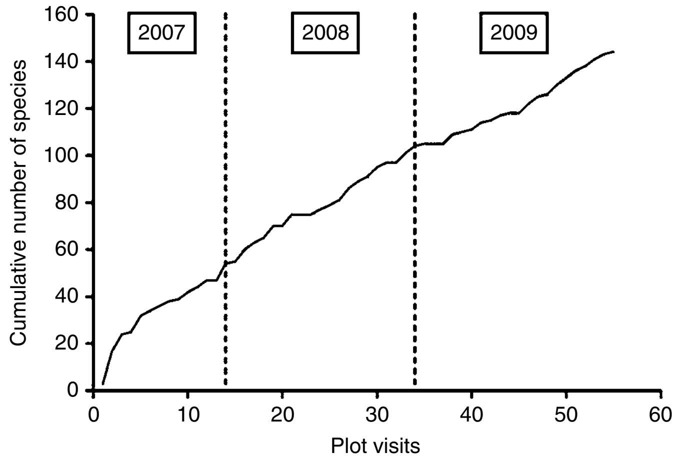

Figure 13.4 Species accumulation curve for macrofungi collected in nine spruce forests in Ireland over 3 years.

species after over 30 years of regular sampling. Whether these species were always present yet rarely fruit, or whether they represent immigration of species is not yet known. Despite these limitations to sporocarp surveys, most mycologists still regard them as being an important method of recording fungal diversity, especially when combined with other survey techniques.

Given that a large proportion of the fungus is in the form of mycelium in the substrate, most studies now sample the soil or plant debris when examining the fungal community. For ECM fungi, root samples of the hosts within the site are often taken, and these have shown that the correspondence between the fungal communities of a location based on above-ground (sporocarp) versus below-ground (root) surveys is often low. A 2012 study in Irish oak, pine, and spruce forests found only 10% similarity between the species recorded as sporocarps and as ECM roots. This may be because not all ECM species form noticeable sporocarps, and because short of examining every root of the tree, it is practically impossible to fully record all of the ECM species on the roots of even a single tree. A mature European aspen (*Populus tremula*) tree in Estonia was found to have over 100 different species of ECM fungi present on its roots.

In-vitro isolations from sample dilutions are a useful way of sampling the fungal community in soil or water samples. Samples are diluted prior to plating onto agar media, because in most cases the samples will be dominated by one or a few species that will quickly overgrow most agar plates. By increasingly diluting the samples an investigator can isolate rare species and reduce the effect of high competition between co-occurring species. Varying the incubation temperature, the media used, or the sample pre-treatments can have a further effect on

the fungi isolated from samples. With this method it is very important to closely monitor the plates throughout the study. The different fungal individuals should be aseptically transferred to new plates as soon as they produce hyphae to avoid contamination and overgrowth by the fast-growing fungi in the sample. By varying the growing conditions (e.g. incubation temperature or the agar media used) it is possible to encourage the growth of some species while discouraging the growth of others. The use of varying incubation temperatures (15 °C rather than the standard 20 °C temperature normally used) was key in discovering that the fungus-like oomycete *Phytophthora austrocedri* was the causal agent of a recent disease of cedar trees in forests in Argentina.

In examining the fungal community present within a plant sample, *in-vitro* isolation from plant material is a vital technique to identify the fungi present. In general, the plant material is plated onto a nonselective nutrient-poor media agar plate (e.g. water agar) after first being surface sterilized with a dilute bleach solution. The plates are incubated in sterile conditions and the fungi that grow from the plant material are examined and aseptically removed and cultured on nutrient-rich agar plates in order to identify them using the identification techniques below (Table 13.3). Studies of this kind are often used in pathological examinations or in surveys of the fungal endophytes in a plant.

Spore trapping is a useful method for surveying what fungal species are actively spreading in a site by aerially disseminated spores. There are two types of spore traps commonly used. The passive type is simply a container that collects spores in air, rainwater, or soil splash. These environmental samples typically need to be pre-treated (e.g. filtered) to remove debris, before being examined using one of the identification methods listed below. The second type of spore trap is an actively sampling spore trap. These traps have a motor that draws in samples of the environment (e.g. air, water) and collects the spores. The Burkhart spore trap is one such trap, using either solar or battery power to draw in air from the atmosphere. This air is directed towards a strip of clear plastic onto which petroleum jelly and an antifungal chemical have been placed. The petroleum jelly holds the spores to the strip of plastic, while the antifungal stops the spores from germinating and makes it easier to study them microscopically. The strip moves according to time passed, and can run for 7 days. This allows the researcher to calculate the time at which the spores were deposited on the strip. Assigning a time to when the spores were collected is important in linking fungal sporulation to recent weather events, or in linking sporulation to time of day.

13.8.2 Identification Methods Used in Fungal Studies

The most common method of identifying a fungus is by studying its characteristics and comparing it to descriptions of published species. Taxonomy is the science

of the principles and practice of classifying organisms into groups of closely related individuals. Taxonomy includes:

- Classification – arranging organisms into hierarchically arranged groups (known as taxa – singular taxon).
- Nomenclature – the rules of naming species.
- Systematics – the study of the diversity and differentiation of organisms and the relatedness between them.

In fungal taxonomy, species are named and described based on a combination of morphological, ecological, and biological characteristics. Microscopic characteristics such as spore shape, size, and attachment are a frequently used characteristic to classify fungi. At a high phylogenetic level, for example, ascomycetes produce several (often eight) spores in asci (i.e. a sac-like enclosure), while basidiomycetes produce spores (often four) on a basidium (a club-like stalk). A fungal species is named and described based on a comprehensive description of its morphology and biology, leading to a species hypothesis being proposed. The hypothesis states that the investigating scientist believes the description provided is an accurate and useful way of grouping together individual samples (e.g. of mushrooms) based on their (1) high similarity to each other and (2) high dissimilarity to other published species.

More recently, the rules (the International Code of Nomenclature for algae, fungi, and plants) governing the classification of fungi have been expanded to include genetic characteristics of the taxon. This code was originally designed for the identification of animals and plants, and so its applicability to fungi and other microorganisms that show high levels of phenotypic plasticity is not always straight-forward. For example, the rust fungi (order Pucciniales) have been particularly troublesome to classify because they often produce several different spore types depending on the stage of their lifecycle – a factor that sometimes led to different life-stages of the same rust fungus being given different names. Similarly, the ash dieback pathogen produces different spore types depending on whether it is in its asexual or sexual state. This led to the organism first being named *Chalara fraxinea* based on the characteristics of its asexual state, until further study identified the sexual state of the taxon as being a member of the genus *Hymenoscyphus*, and it was subsequently named *Hymenoscyphus fraxineus*.

These and many other historical and biological issues in fungal taxonomy have led to the present state where the taxonomy of fungi at present is rather disorganized, with many thousands of the described species probably being synonyms of other described species. It will take a lot of time for these taxonomic issues to be resolved in mycology, and it is expected that molecular biology methods will assist in resolving the classification of many groups of fungi. For further reading on taxonomy, classification, and nomenclature in fungi see the Further Reading section.

The application of DNA identification methods for fungal species has revolutionized the science of fungal ecology. These methods changed how scientists studied fungi in the environment, by allowing researchers to rapidly and accurately compare fungal taxa across the globe. This led to the finding that many ECM species have smaller than expected geographic ranges, and that ECM taxa that were once thought to represent a single species constituted different species (i.e. a species aggregate). Molecular analysis is often a good starting point to identify fungal groups in need of taxonomic revision, which when studied closely can also be objectively separated according to their morphological or ecological characteristics. In many modern studies of fungal ecology, NGS technologies are being utilized to reveal the massive diversity of fungi in environmental samples. In NGS studies of fungi in terrestrial ecosystems it is common to find hundreds of operational taxonomic units (OTU) of fungi present in a relatively small sample area (e.g. in the phyllosphere of an oak tree, or the rhizosphere of a beech tree). While they may be roughly equal to species in some NGS studies, these OTUs are not always equivalent to species.

The scientific discourse is ongoing between mycologists over the amalgamation of traditional systematics based on morphological/ecological characteristics with more modern systematic approaches based on molecular characteristics. Molecular phylogenetic classification involves barcoding taxa based on a selected gene region. If the barcode gene region differs significantly between two individuals, then the two individuals are taken to represent different taxa. There is much philosophical debate as to how much molecular difference (percentage) should constitute a fungal species; that is, what is the threshold at which a difference in the barcodes of the two organisms becomes significant? Furthermore, which gene region should be adopted as the barcode region? While researchers that study other groups (e.g. animals and plants) have chosen the COX1 (cytochrome oxidase I) region of the genome, mycologists have adopted the ITS (internal transcribed spacer) region as the barcode region for fungi. Some taxa show clear phenotypic differences despite the fact that they are highly similar according to the ITS gene region. In the genus *Phytophthora*, the *Phytophthora* taxon oaksoil and the species *Phytophthora boodjera* are 99% similar according to the ITS region, but the former is sexually sterile while the latter is homothallic (potentially inbreeding) with regards to its mating system. Given this high level of similarity in the barcode region, the two taxa would not be classed as significantly different based on a molecular classification. However, based on their morphological and ecological differences the taxa could be considered significantly different enough to warrant naming as two separate species. Whether or not these two taxa are the same species or different is a matter for the mycology community to agree upon.

For identifying ECM species present on roots, there is a large body of scientific literature on the morphological description of the ECM mantle on the host roots. Known as morphotyping, this relies on comparison of the gross morphology and the pattern of the mantle layers with that of published species descriptions. This

process was rather time consuming and often led to an underestimation of the true ECM species richness in a sample. Thankfully, it has now largely been replaced by molecular characterization methods, with morphotyping sometimes used as a preliminary sorting technique prior to molecular analysis. While several other identification/quantification methods are listed (Table 13.3), a detailed explanation of how they work would be too onerous and is not provided here. Descriptions of these methods and their application to fungal ecology can be found in the sources listed in Further Reading. One point to remember is that no matter what the method used, it is still vital that researchers take a representative sample of the roots/soil from a location. This point is as valid for relatively "low-tech" identification using a microscope as it is for "high-tech" identification using NGS. In particular with studies of ECM fungi, it is important to take a representative sample, as the community is known to vary both vertically and horizontally in the rhizosphere, as well as showing high temporal variation.

Most studies of fungal diversity in the environment acknowledge that they probably have not recorded all of the fungi present. In these cases, a statistical technique known as species richness estimation can be used. These methods extrapolate from actual data to estimate the likely species richness of a location, within certain bounds of confidence. Some of the estimation methods most commonly used for fungi include Chao1, ACE, and ICE estimates. The older estimators such as Chao1 use the ratio of singletons (species only found in one sample) to doubletons (species found in two samples) to calculate the species estimate. The more recent estimators, the coverage estimators ACE and ICE, use the ratio of rare to frequent species to estimate the richness. The definition of what is "rare" and at which threshold to set the rarity value has been discussed (see Further Reading) and cannot be dealt with in any great detail here. What can be said is that rarity depends on a number of considerations, including the make-up of the community being examined and the value (e.g. monetary, inherent, ecological) of the species being assessed. Another problem that often faces fungal ecologists is that there are unequal numbers of samples taken from different habitats or units. Thus, comparing the species richness of one unit to another would not be logical because more effort has gone into examining one of the units. In these cases, another type of statistical extrapolation, known as rarefaction, is often used.

13.9 Conclusion

Fungi are widespread in the natural and man-made environment. Fungi provide direct benefits to humans as a food source and in biotechnological applications. Indirectly, fungi support the normal functioning of many environments, on which humanity relies. To control and harness fungi has been an aim of human enterprise for more than 2,000 years. It is only recently that we are becoming aware of the important functions that fungi perform in the environment. Despite

the fact that these fungi underpin many important environmental functions, many fungi are under threat globally due to increasing globalization, industrialization, and land-use change. While some studies indicate that there may be a high degree of redundancy in some fungal communities when it comes to the provision of ecosystem services, the dearth of knowledge on fungal diversity in many habitats worldwide has led to many scientists urging caution in our treatment of fungal conservation issues. Once a fungal species is removed from the environment (i.e. extirpated) it may be very difficult to restore the fungus or replace the ecosystem services it provided. In general, there is an inverse proportional relationship between the size of microorganisms and their effect on ecosystem functioning globally. Mycology and fungal ecology are undergoing a revolution with the addition of powerful molecular biology methods to the mycologist's toolkit. Hopefully this will help reverse the trend noted in recent years where mycologists have become so scarce that they were at a level that qualified them as being considered members of an endangered species.

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14
Fungal Pathogens of Plants

Fiona Doohan and Binbin Zhou

14.1 Introduction

A wide range of fungi cause diseases of plants (see Chapter 1 for a description of fungal taxonomy, morphology, and reproduction). To be classified as a fungal plant pathogen or phytopathogen (*phyto* = plant), a fungus should, if possible, satisfy Koch's postulates or rules. Koch determined that an organism was the cause of an infectious disease if it can: (1) be isolated from a diseased host, (2) be cultured in the laboratory, (3) cause the same disease upon re-introduction into another host plant, and (4) be re-isolated from that host. However, some fungal pathogens cannot be cultured, or in some cases it is not easy to carry out Koch's tests.

Since the early nineteenth century, thousands of fungi have been recognized as parasites of plants. *Parasitism* occurs where one species lives off another, as distinct from *symbiosis* where different species live in harmony with each other and the relationship is mutually beneficial, or *saprophytism* where organisms grow on dead organic matter.

Plant-pathogenic fungi are classified as:

- *Biotrophs*: only grow and multiply when in contact with their host plants (and therefore cannot be cultured on nutrient media); for example, the fungi that cause rusts, powdery mildews, and downy mildews.
- *Non-obligate pathogens*: grow and multiply on dead organic matter (and can therefore be cultured on nutrient media) as well as on living host tissue. These can be further distinguished as facultative saprophytes or facultative pathogens.

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Facultative saprophytes complete most of their lifecycle as parasites, but under certain conditions they grow on dead organic matter. Conversely, facultative parasites complete most of their lifecycle on dead organic matter, but under certain conditions they attack and parasitize living plants.

14.2 Disease Symptoms

Plant disease symptoms caused by fungal parasitism include:

- Spots – localized lesions on host leaves.
- Wilts – fungal colonization of root or stem vascular tissue and subsequent inhibition of translocation, leading to wilting.
- Blight – browning of leaves, branches, twigs, or floral organs.
- Rots – disintegration of roots, stems, fruits, tubers, fleshy leaves, corms, or bulbs.
- Cankers – localized, often sunken, wounds on woody stems.
- Dieback – necrosis of twigs initiated at the tip and advancing to the twig base.
- Abnormal growth – enlarged gall-like or wart-like swelling of plant stems, roots, tubers, leaves, or twigs, root and shoot proliferation, etc.
- Damping off – rapid death and collapse of young seedlings.
- Decline – loss of plant vigour, retarded development.
- Anthracnose – necrotic (often sunken) ulcer-like blemishes on stem, fruit, leaf, or flower.
- Scab – localized lesions of scabby appearance on host fruit, leaves, tubers.
- Rusts – many small, often rust-colored lesions on leaves or stems.
- Mildews – chlorotic or necrotic leaves, stems, and fruits covered with mycelium and fruiting bodies of the fungus, often giving a white “woolly” appearance.

Many diseases are associated with more than one of these symptoms. Table 14.1 lists some of the economically significant fungal pathogens, associated symptoms, and host plants.

14.3 Factors Influencing Disease Development

The relationship between a phytopathogen and plant and the development of disease is influenced by the nature of the pathogen and host, and the prevailing environmental conditions; all three interact and form integral parts of the disease

Table 14.1 Examples of economically significant fungal diseases, causal organisms, hosts, and associated symptoms*.

Diseases	Fungus	Hosts	Symptoms
Vascular wilts			
Fusarium wilt	*Fusarium oxysporum*	Most vegetable and flower crops, cotton, tobacco, banana, plantain, coffee, turf-grass, ginger, soybean	Wilting, vein clearing in younger leaflets, epinasty, stunting and yellowing of older leaves
Verticillium wilt	*Verticillium dahliae*, *Verticillium albo-atrum*	Many vegetables, flowers, field crops, fruit trees, roses, forest trees	Similar to *Fusarium* wilt
Dutch elm disease	*Ophiostoma ulmi*, *Ophiostoma novo-ulmi*	Elm	Wilting, yellowing/browning of leaves, brown/green streaks in infected sapwood underlying bark of infected branches
Oak wilt	*Ceratocystis fagacearum*	Oaks	Downward wilting and browning of leaves, defoliation, brown discoloration of sapwood underlying bark of infected branches
Blights			
Late blight of potato	*Phytophthora infestans*	Potato, tomato	Water-soaked lesions turning to dead brown blighted areas on lower leaves, white woolly growth on underside of infected leaves, tubers have dark surface blotches, internal watery dark rotted tissue
Downy mildews	Several genera, e.g. *Unicinula*	Wide range of crops, ornamentals and shrubs	Powdery growth covering shoots, leaf surfaces, and sometimes flowers
Alternaria blight (early blight)	*Alternaria solani*	Potato, tomato	Dark lesions on stems and leaves, potato tuber lesions and internal dry rotting, tomato blossom blight, fruit rot, stem lesions

(*continued*)

Table 14.1 (*Continued*)

Diseases	Fungus	Hosts	Symptoms
Helminthosporium leaf blight	*Cochliobolus sativus*	Cereals, grasses	Dark brown spotting of leaves (also causes root rot, seedling blight, and head blight)
Botrytis blossom blight (and rots)	*Botrytis* spp. (e.g. *Botrytis cinerea*)	Ornamentals, fruit trees	Water-soaked and rotting blossoms, gray/brown powdery lesions on fruit, leaves, stems, and bulbs, rotting of fruit
Fusarium head blight	*Fusarium* spp.	Cereals	Premature bleaching of cereal spikelets, shrivelled pale/pink grain
Rots			
Phytophthora root rots	*Phytophthora* spp.	Fruit, forest, ornamental trees and shrubs, annual vegetables, ornamentals	Rotting of roots, plant stunting and wilting, death in severe cases
Damping off and associated rots	Several genera, e.g. *Pythium* spp.	Many hosts; broad-leaved weeds and grasses are very susceptible	Seedling death, rotting of seed, roots, and fruit in contact with soil
Soft rot	*Rhizopus* spp.	Fruit, vegetables	Softening and rotting of soft fleshy organs and fruit
Brown rot of stone fruits	*Monilia* spp.	Stone fruits	Brown rotting of stone fruits
Anthracnose	Several genera, e.g. *Colletotrichum* spp.	Fruits, fruit, some forest trees, beans, cotton, ornamentals, rye, etc.	Dark sunken lesions on stems or fruits, may cause rot of fruit
Leaf and stem spots			
Septoria leaf spot	*Zymoseptoria tritici*	Cereals (primarily wheat)	Gray to brown water-soaked leaf lesions that, when mature, bear black visible pycnidia

Table 14.1 (*Continued*)

Diseases	Fungus	Hosts	Symptoms
Scabs			
Apple scab	*Venturia inaequalis*	Apples	Dark lesions on fruit, leaves, and sometimes stems and bud scales
Rusts			
Black stem rust of cereals	*Puccinia graminis*	Cereals	Diamond-shaped raised orange/red powdery lesions on leaves, stems, and cereal heads, that when mature bear black teliospores
Coffee rust	*Hemileia vastatrix*	Coffee	Yellow/orange oval lesions on leaves with powdery orange/yellow lesions on leaf undersurface, infected leaves eventually drop off
Smuts			
Covered smut of oats	*Ustilago hordei*	Oats	Grain appears black due to its replacement with black fungal spore masses
Covered smut (bunt) of wheat	*Tilletia* spp.	Wheat	As above
Powdery mildews			
Powdery mildew of cereals	*Blumeria graminis*	Cereals	Chlorotic or necrotic leaves, stems, and heads covered with mycelium and spores of fungus, often giving a white "woolly" appearance
Cankers, galls, and malformations			
Club-root of brassicas	*Plasmodiophora brassicae*	Brassicas	Wilting of leaves, swelling and distortion of roots, stunted growth; when severe, roots rot and plant dies

* Many fungi cause more than one of the above diseases (e.g. *Botrytis* spp. can cause blights and rots).

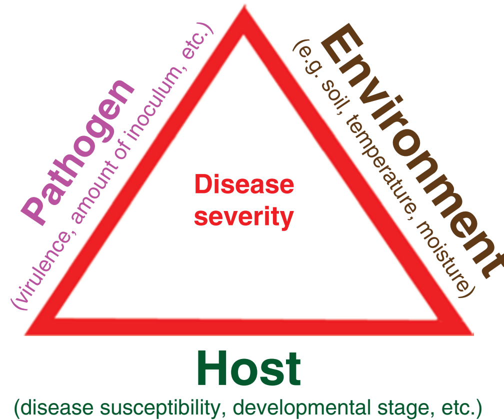

Figure 14.1 The disease triangle.

triangle (Figure 14.1). Each component represents one side of the triangle. For example, if environmental conditions are unfavorable for disease development, for instance wrong temperature, wind, or moisture conditions, then the environment side of the triangle would be shorter and therefore the overall disease level would be low or nonexistent.

14.3.1 The Pathogen

In order for disease to occur, a pathogen must be virulent towards, and compatible with, its host. The aggressiveness of a pathogen will influence disease development. Some pathogens have a broad host range, while others attack relatively few plant species. Some fungal species comprise *formae specialis* (f. sp.), each of which parasitize and cause disease of one or a small number of host plants. For example, there are many different f. sp. of *Fusarium oxysporum*, which cause wilt disease of many host species. Also, races may exist within a fungal species; races differ in their pathogenicity towards different genotypes (i.e. genetically distinct variants) of the same host species.

Infective propagules (e.g. fungal spores) must be present for disease to occur, and, to some extent, the amount of inoculum influences disease development. The length of time that a pathogen is in contact with a host is also critical for disease development. Also, the pathogen must be able to compete with other organisms present on or in the plant.

14.3.2 The Host

Disease development requires that the host plant be at a stage of development susceptible to infection. For example, damping-off disease only affects seedlings.

Infection and colonization will not occur unless the host is susceptible or tolerant to disease. Plant species and even genetic variants of the same species differ in their susceptibility to disease. For example, wheat cultivars or genotypes differ in their susceptibility to *Fusarium* head blight (FHB) disease caused by *Fusarium* species (Plate 14.1); this is a serious disease of cereals worldwide). Also, resistance to one pathogen does not mean immunity; that is, plants may be susceptible to other fungal diseases (e.g. the FHB-resistant wheat cultivar Sumai 3 is susceptible to the potentially serious stem rust disease caused by *Puccinia graminis*). Plants may be disease tolerant; that is, even though infected, they survive and grow and symptoms are not visible or at a nondestructive level.

14.3.3 Environmental Conditions

There is a great degree of uncertainty regarding (1) what plants will be grown and what diseases will prevail under future climatic conditions, and (2) how climate change will influence disease development. Temperature, wind, moisture, sunlight, nutrition, and soil quality are environmental factors that have a major impact on disease development. Fungal pathogens differ in the optimal environmental conditions required for inoculum production, dispersal, and disease development. Often, disease development by fungal pathogens requires a minimum exposure time to a specific temperature and moisture combination. For example, low relative humidity can reduce the development of powdery mildew disease of tomato caused by *Erysiphe lycopersicon.* Host susceptibility to disease may be influenced by future environmental conditions; for example, acclimation to elevated carbon dioxide was shown to reduce the resistance of wheat to pathogens.

14.4 The Disease Cycle

The disease cycle describes the events that occur from the initiation of disease to the dispersal of the pathogen to a new host plant. This is distinct from the lifecycle of either the pathogen or plant; lifecycle describes the stage or successive stages in the growth and development of an organism that occur between the appearance and reappearance of the same state (e.g. spore or seed) of the organism. Pathogenesis describes events that occur in the disease cycle from infection to final host reaction.

Figure 14.2 depicts a generalized disease cycle. Inoculum is produced and disseminated, and on reaching its target host plant tissue (inoculation) it penetrates the host. The type and mode of production of inoculum (e.g. sexual and asexual spores, resting spores, mycelium) and the method of dissemination (e.g. wind, water, insect) depend on the specific pathogen. For many important plant pathogens, a sexual stage has not been identified.

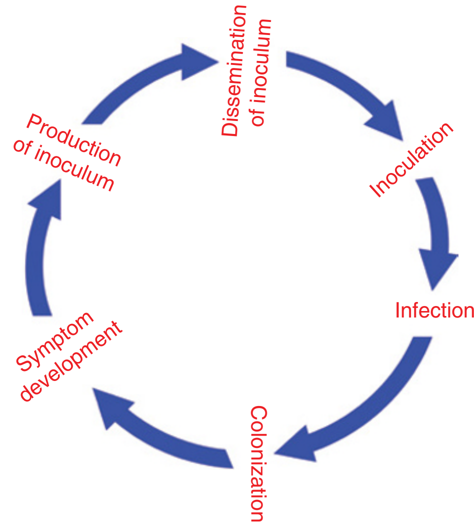

Figure 14.2 Generalized fungal disease cycle.

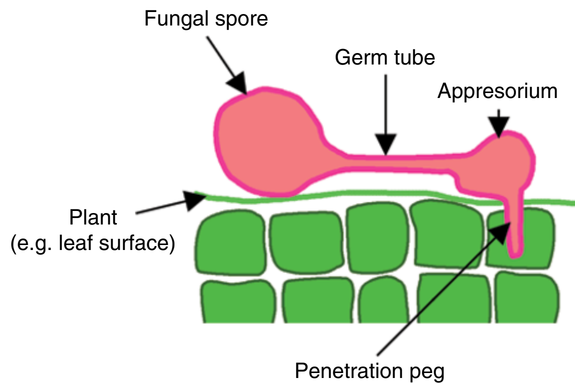

Figure 14.3 Fungal penetration of a host plant by means of a specialized appresorium and penetration peg.

Penetration is through wounds or natural plant pores (e.g. stomata), and some fungi produce specialized penetration structures called appressoria (see Section 14.6.1 and Figure 14.3). Having penetrated its host, the fungus then grows within plant tissue (infection). Incubation period defines the period between inoculation and infection. As it grows within the host, it utilizes the plant's cellular resources as a nutrient source and the damage inflicted on the plant manifests as disease symptoms. Latent period defines the period between infection and symptom development. The pathogen forms survival structures such as spores that are then disseminated. During the plant pathogenesis, some diseases only involve one such cycle (primary cycle), while others have the potential to do more damage as they involve a secondary or multiple cycles of disease.

14.5 Genetics of the Plant–Fungal Pathogen Interaction

Fungal genes encode proteins that make the fungus specific and virulent to specific hosts, and, in turn, host plants have genes that make the plant susceptible or resistant to a specific fungus. The gene-for-gene hypothesis explains the interaction between cognate sets of pathogen and host genes. The host has dominant genes for resistance (*R*) and recessive genes for susceptibility (*r*). The pathogen has recessive genes for virulence (*a*) and dominant genes for avirulence or inability to infect (*A*). The interaction between specific sets of pathogen avirulence/virulence and host resistance/susceptibility genes determines whether disease develops. Table 14.2 explains the possible interactions between such plants and pathogens. Of the possible combinations of genes, only *AR* interactions are resistant, and in all other cases, disease will occur. *R* genes encode receptors that interact with *A* genes. Many avirulence genes (*A*) act as virulence genes (*a*) in the absence of the corresponding host resistance gene (*R*).

14.6 Mechanisms of Fungal Plant Parasitism

Fungi parasitize plants using physical, chemical, and biological means. In doing so they adversely affect the photosynthesis, translocation of water and nutrients, respiration, transcription, and translation in host tissue.

14.6.1 Mechanical Means of Parasitism

Mechanical means of plant parasitism include the adherence to host tissue and forceful penetration; chemical and biological molecules often facilitate such steps. Biotrophic plant pathogens have evolved specialized structures called

Table 14.2 Gene-for-gene interaction between fungal pathogens and plants*.

Host →	Resistant (*R*)	Susceptible (*r*)
Pathogen ↓ Avirulent (*A*)	*AR* (incompatible)	*Ar* (compatible)
Virulent (*a*)	*aR* (compatible)	*Ar* (compatible)

* Incompatible interactions result in no disease, i.e. plant is resistant to that pathogen, while compatible interactions result in disease, i.e. plant is susceptible (Flor, 1956). Only *AR* interactions are resistant: in all other cases, disease will occur.

appressoria (singular = appressorium) – a highly organized enlarged end of a hypha (Figure 14.3). Once the hypha senses an appropriate site, it enlarges and adheres itself to the leaf surface. This adherence is necessary to support the amount of mechanical force used to penetrate the plant via a hypha called a penetration peg.

14.6.2 Pathogen Metabolite-Mediated Parasitism

14.6.2.1 Enzymes

Fungi produce a range of enzymes that facilitate host plant infection and colonization by degrading the cellular and intercellular constituents of plants (certain fungal pathogens also produce nonenzymatic proteins that inhibit the activity of plant enzymes involved in the host defense response). The cuticle forms a continuous layer over aerial plant parts and is an important barrier against pathogens and other stresses. Cutin is the major structural component of the cuticle barrier and some fungi secrete cutinases that hydrolyze ester linkages between cutin molecules. In doing so, they release monomers as well as oligomers, thus breaking the integrity of the cuticle barrier and facilitating plant parasitism. Indeed, their capacity to degrade plant cell walls makes them attractive organisms to exploit as agents to degrade waste plant residue.

The plant cell wall presents a complex and important physical barrier against invading fungi. Pectinaceous substrates form part of the cell wall and are usually a major constituent of the middle lamella adhering adjacent plant cells. They are polysaccharides consisting mostly of galacturonan molecules interspersed with rhamnose molecules and side chains of galacturonan and other sugars. There are many pectin-degrading enzymes that attack different parts of the polysaccharide, including pectin esterases, polygalacturonases, and pectate lyases, resulting in a general disintegration of host tissue.

Cellulose, the most abundant natural polymer and a component of plant cell walls, consists of repeating units of glucose molecules and these chains cross-link to form fibrils embedded in a matrix of other polymers such as pectin and lignin. Fungi often produce cellulose-degrading enzymes, or cellulases, in the latter stages of plant parasitism. Different cellulases attack the cross-linkages, and the polymeric, or degraded oligomeric or dimeric cellulose chains in a series of steps, resulting in the degradation of cellulose into small oligomers or the monomer glucose. Hemicellulose represents a complex mix of polysaccharides that forms part of the plant cell wall matrix and is also present in the middle lamella. The primary polysaccharide component is xyloglucan, and others include glucomannan, galactomannans, and arabinogalactans. Fungi produce an array of hemicellulose-degrading enzymes necessary for complete hydrolysis of the substrate. Thus, cellulases and hemicellulases reduce the host cell wall integrity.

Lignin is the second most abundant natural polymer on earth, being ubiquitous in monocots and dicots and a major component of woody plants. The basic lignin polymer consists of chains of substituted phenylpropanoid molecules, and this polymer is substituted with a variety of side chains. Certain fungi, mostly basidiomycetes, can degrade lignin. The white-rot fungi produce ligninase enzymes that degrade most of the lignin in the world.

Various other fungal enzymes facilitate plant pathogenesis. These include protein-degrading proteases, chitinases, and lipid and starch-degrading enzymes. Proteases can interfere with membrane integrity by degrading the protein component of the plasma membrane. Chitinases (that degrade the chitin component of fungal cell walls) are recognized as important pathogenicity determinants in some plant diseases. Some enzymes degrade plant compounds involved in the host defense response (e.g. β-glucosidases cleave and detoxify glucoside residues).

14.6.2.2 Polysaccharides, Toxins, and Growth Regulators

Certain fungi also produce polysaccharides that facilitate host colonization or deactivation of the host defense response. Some fungi produce toxic compounds that seriously damage or kill the host cells. Toxins vary in composition, from low molecular weight metabolites to proteins. Examples of fungal toxins, their producers, and the associated disease and hosts are listed in Table 14.3. Fungal toxins may be host-specific or non-host-specific. Host-specific toxins are usually required for pathogenicity, but are only toxic to the host plants of a pathogen, and show no or low toxicity to other plants. In contrast, non-host-specific toxins affect a wide range of host plants, and may act as virulence factors, increasing the extent of the disease, but are not essential for disease development. In addition to being phytotoxic, some non-host-specific toxins are also classified as mycotoxins, that is, they are harmful to human and animal health (e.g. trichothecenes, fumonisins, and fusaric acid). Fungal toxins may cause visible symptoms such as wilting, chlorosis, and stunting (Plate 14.2), but may operate at the biochemical level and not cause visible effects.

Increased levels of growth regulators or hormones are often associated with diseases of plants; whether these increases originate from the fungus or the plant is often unclear. Growth regulators affect plant growth in many ways. Increased cytokinin production is associated with tumor formation (e.g. clubroot of brassicas caused by *Plasmodiophora brassicae* (Plate 14.3) and peach leaf curl caused by *Taphrina deformans*). The pathogen *Gibberella fujikuroi* secretes gibberellins, causing "foolish seedling" disease of rice characterized by overgrown, weak spindly plants. Ethylene is a volatile hormone and increased levels are sometimes associated with fungal diseases, but its origin is often unclear, that is, fungus or plant.

Table 14.3 Examples of fungal toxins, their specificity, producer fungi, and associated diseases.

Toxin	Producer fungi	Associated diseases
Host-specific		
Victorin	*Cochliobolus victoriae*	Victoria blight of oats
HS toxin	*Bipolaris sacchari*	Eyespot disease of sugar cane
HC toxin	*Cochliobolus carbonum* race 1	Northern leaf spot and ear rot of maize
T-toxin	*Cochliobolus heterostrophus* race T	Southern corn (maize) leaf blight
AK-toxin, AF-toxin, ACT-toxin, AM-toxin, AAL-toxin, ACR(L) toxin	*Formae speciales* of *Alternaria alternate*	Diseases of Japanese pear, strawberry, tangerine, apple, tomato, rough lemon, respectively
PM-toxin	*Mycosphaerella zeae-maydis*	Yellow leaf blight of maize
Peritoxin (PC-toxin)	*Periconia circinata*	Sorghum root rot
Ptr ToxA, Ptr ToxB	*Pyrenophora tritici-repentis*	Tan spot of wheat
Non-host-specific		
Tentoxin	*Alternaria* spp.	Various
Solanopyrones	*Alternaria solani* Didymella rabiei (anamorph: *Ascochyta rabiei*)	Early blight of potato Blight of chickpea
Trichothecenes	*Fusarium* spp.	Head blight, seedling blight, and root rot of wheat
Fumonisins	*Gibberella fujikuroi* (anamorph: *Fusarium moniliforme*) *Anternaria alternata*	Ear rot of maize
Enniatins	*Fusarium* spp., e.g. *F. avenaceum*	Dry rot of potato
Fusicoccin	*Fusicoccum amygdali*	Fusicoccum canker of peaches, nectarine, and almonds
Sirodesmin PL, depsipeptide HST, phomalide	*Leptosphaeria maculans*	Blackleg of crucifers
Oxalic acid	*Sclerotium*, *Sclerotinia*	
Fomannoxin	*Heterobasidion annosum*	Root and butt rot of conifers
Cerato-ulmin	*Ophiostoma novo-ulmi*	Dutch elm disease
Cercosporin	*Cercospora* spp.	Various

14.7 Mechanisms of Host Defense

Different host species and genotypes of a host plant species vary in their susceptibility to fungal diseases, and non-hosts are disease resistant. This variation in disease resistance is due to structural and/or metabolic differences among plants. Table 14.4 highlights some of the pre-formed and induced structural and metabolic defense strategies used by different plants or plant cultivars to combat fungal diseases. The host defense response may be localized or systemic.

Plant disease resistance (*R*) genes encode receptors that interact with fungal avirulence (*A*) genes. The protein products of *R* genes both perceive and activate signals, that is, they recognize the pathogen signal (*A* gene) and activate a plant defense response. Many *R* genes encode structurally related proteins with motifs that target their intracellular or extracellular localization (leucine-rich repeat (LRR) motifs and nucleotide binding (NB) motifs). Having perceived the pathogen, *R* gene products in disease-resistant hosts induce an active host defense response. Fungi produce pathogen-associated molecular patterns (PAMPs) that are recognized by pattern recognition receptors (PRRs) to initiate PAMPs-triggered immunity. Many fungal virulence (a) effectors can interfere with PAMPs-triggered immunity, resulting in effector-triggered susceptibility. Some avirulence (A) effectors are recognized by NB-LRR-resistant (R) proteins to initiate effector-triggered immunity. This gives rise to the so-called zig-zag model of plant defense, which is summarized in Plate 14.4. Although it has limitations, it is a useful expository model of the plant immune system.

Plant defenses vary in their time of activation in response to fungal attack. Rapid defense responses include the oxidative burst, callose synthesis and deposition, generation of nitric oxide (NO), and cross-linking of cell wall proteins. Nitric oxide and oxidative burst-generated O_2^- and H_2O_2 possess antimicrobial activity. Callose, a β-1,3-linked glucan that is deposited as papillae (localized wall appositions), together with the cross-linked cell wall proteins, increases the cellular resistance to fungal penetration.

Slower responses include the production of phytoalexins, pathogenesis-related proteins (PRPs), and hydroxyproline-rich glycoproteins (HGRPs), and the induction of lignification, suberization, the hypersensitive response (HR), and induced or systemic acquired resistance (ISR or SAR). Phytoalexins are low molecular weight compounds that, nonspecifically, inhibit a wide range of organisms. HGRPs are a constitutive part of cells, but can also be induced by wounding and infection. Although it is known that they play a role in cross-linking of cell wall proteins, much remains to be discovered as to their role in plant defense. Lignin and suberin are both constitutive components of healthy plant cell walls; enhanced synthesis and deposition of these molecules in response to infection is thought to increase the resistance of cell walls to penetration and degradation. HR involves a genetically programed suicide of the cells surrounding the infecting fungus and both O_2^- and H_2O_2 may participate in this response. The HR is particularly debilitating to biotrophic and hemibiotrophic pathogens that require living plant tissue to survive, and its role in response to necrotrophic pathogen invasion is not so

Table 14.4 Pre-formed and induced defense strategies used by plants to combat fungal diseases.

Type	Time of formation	Examples
Structural	Pre-formed	Thick wax or cuticle layer covering the epidermis.
		Thickness and/or tough cell walls (e.g. highly lignified walls impede pathogen advancement).
		Size, shape, and location of stomata
	Induced	Cork cells impeding pathogen advancement.
		Isolation of infected area by formation of abscission layers.
		Tylose formation in xylem vessels (blocking pathogen advancement through the vascular system).
		Gums deposited in intra- and intercellular spaces forming an impenetrable barrier to the pathogen.
		Morphological cell wall alterations (e.g. lignification).
		Hypersensitive response (destroying cells in contact with the pathogen and thus starving the fungus of nutrients).
		Fungitoxic exudates
Metabolic	Pre-formed	Lack of elicitor production.
		Lack of host receptor production.
		Low content of essential pathogen nutrients.
		Antifungal phenolics, tannins, saponins, or antifungal enzymes (e.g. glucanases and chitinases).
		Plant defense proteins
	Induced	Formation of callus and cork cells around infection.
		Production of phenolics and phytoalexins (that may be hypersensitive reaction-associated metabolites).
		Transformation of nontoxic to toxic compounds, e.g. phenolics, cyanides.
		Production of fungal-degrading enzymes.
		Production of compounds that inhibit pathogen enzymes.
		Detoxification of pathogen toxins.
		Complexing of plant substrates to resist fungal enzyme attack.
		Metabolites involved in localized and systemic induced resistance

clear. Also, HR is not an obligatory component of disease resistance and is absent, or occurs as a late event, in some resistant responses of plants to pathogens.

SAR describes the resistance that develops in plants at a distance from the initial infection point, that is, a process that is induced by infection and immunizes the plant against future pathogen attack. A salicyclic acid-dependent process mediates the SAR response, and mitogen-activated protein kinase (MAPK) signal transduction cascades have been implicated as negative regulators of salicyclic acid accumulation and inducers of resistance. PRPs are acidic, protease-resistant proteins, some of which possess antifungal activity (e.g. glucanases and chitinases that degrade the chitin component of fungal cell walls). Some increase the permeability of fungal cell membranes (thionins), or affect membrane transport (defensins) or inactivate fungal ribosomes (ribosome-inactivating proteins). SAR results in the formation of plant PRPs. In response to infection, PRPs are known to accumulate in resistant plants in tissue distant from the infection point and their accumulation is correlated with the development of SAR. ISR differs from SAR, at least in some systems, in that it is mediated by a jasmonic acid/ethylene-dependent process and is not accompanied by the accumulation of PR proteins.

14.8 Disease Control

14.8.1 Cultural Practices that Aid Disease Control

Removal of crop debris, stubble, or alternative host plants such as weeds helps control fungal diseases, as many causal organisms survive and overwinter on these materials. The most famous example of a fungus with an alternative host was that of *Puccinia graminis* f. sp. *tritici*, the cause of black stem rust disease of wheat. This fungus completes its lifecycle on barberry bush, and for this reason, barberry bushes are eradicated in the United States. Plant rotation in cropping systems will help prevent inoculum build-up on condition that all hosts are not susceptible or do act as alternative hosts for prevalent diseases. Good sanitary practices will help prevent dissemination of inoculum by mechanical means such as contaminated equipment. The date and method of sowing and the quality of the seedbed can influence disease development. As a general rule (but dependent on the pathogen, host, and environmental conditions), earlier-sown seed tends to suffer more disease problems than does late. Planting seed too deep will increase the time to emergence and increase the susceptibility to root rot and damping-off diseases.

14.8.2 Fungicidal Control of Plant Pathogens

For a more complete overview of modern fungicides, please refer to Oliver and Hewitt (2014). There is a huge array of fungicides, many variants of the same active ingredient(s), on the market for controlling fungal diseases. Depending on

the disease and the host, fungicides are applied as a fumigant, spray, dust, paint, soil treatment, etc. Fungicides are classified in several different ways, depending on how they penetrate plant tissue, what effect they have, and their mode of action. Fungicides may act as:

- *Protectants*: which do not penetrate the plant, but affect pathogen viability and germination on the surface of the host plant. Such fungicides must adhere to the plant surface and resist weathering and are most effective when applied as a preventative measure, that is, before plant inoculation with the fungus.
- *Systemics*: which can act on the plant surface and be translocated throughout the plant vascular system to kill the fungus. Systemic fungicides may also exhibit translaminar movement within the leaves.
- *Eradicants*: which are applied post-infection and act on contact by killing the organism or by preventing its further growth and reproduction.

Strobilurins represent a class of broad-spectrum fungicides that exhibit translaminar properties. Some are systemic and some are mesostemic; that is, they act on the plant surface, penetrate plant tissue, show translaminar movement, are absorbed by the waxy layer, and exhibit vapor movement and redistribution within the crop canopy.

Fungicides are further classified into numerous groups based on their target sites. For example, systemic sterol biosynthesis-inhibiting (SBI) fungicides interfere with ergosterol production in fungi, and hence with the integrity of the cell membrane. Strobilurins disrupt the production of ATP. Some chemicals induce systemic disease resistance in the plant (defense activators).

Many fungal pathogens have evolved resistance to fungicides by changing the target site of the fungicide. For example, many target pathogens have evolved resistance to the broad-spectrum methyl benzimidazole carbamate (MBC) fungicides. These fungicides inhibit β-tubulin assembly (and hence mitosis), and a point mutation in the β-tubulin gene of some fungi renders them unaffected by such fungicides. Strains of *Zymoseptoria tritici* (formerly known as *Mycosphaerella graminicola*) that cause *Septoria tritici* blotch (STB) disease of wheat have developed tolerance to SBI fungicides and resistance to strobilurin fungicides. These are the major classes of fungicide used to control this pathogen and because STB is one of the most economically important diseases of wheat, this poses a significant threat for the future security of wheat grain supply.

14.8.3 Host Resistance to Disease

If screening studies identify genotypes of a host that are disease resistant, the resistance can be introgressed into host cultivars that possess other desirable

characteristics such as high yield. If the resistance is already in a cultivar of the required plant species, or in closely related species, this may be achieved by conventional plant breeding techniques. Also, identified gene(s) associated with resistance can be incorporated into the required plant via genetic engineering. Ongoing research attempts to identify such resistance genes using a variety of molecular biology approaches. Candidate genes for enhancing disease resistance include those that code for proteins that either are antifungal or regulate pathways involved in the host defense response (see Section 14.7). Some plant genes render them more susceptible to fungal diseases and new molecular technologies that enable the plant to turn off or silence genes involved in host susceptibility or pathogen virulence (RNA silencing and CRISPR/Cas9 technology (see Section 2.5.2.4)) will increase the number of potential target genes for genetic engineering. As an alternative to GM technology, a screen of plant populations in which genes are randomly mutated via non-GM means (e.g. chemical treatment) offers the potential to identify lines with enhanced resistance due to the disruption of a disease susceptibility gene. This approach is being used to identify wheat germplasm with enhanced resistance to many diseases, and this material can then be used by breeders to introgress the genes into their elite germplasm via traditional crossing techniques.

The decline in the effectiveness of genes to confer resistance over time to one or more races of a pathogen in the field led to the quest for durable (long-lasting) non-race-specific resistance. Vertical resistance describes resistance to specific races of a fungal pathogen (i.e. race specific) and is usually conferred by one (monogenic) or a few genes. Horizontal resistance describes intermediate resistance to all races of the pathogen (i.e. non-race-specific) and is usually conferred by many genes (polygenic). This is achieved by pyramiding *R* genes into a single or multiple isogenic plant lines (genotypes that are genetically identical except for a single character, e.g. the *R* genes), or by growing mixes of genetically distinct genotypes that each contains one or more different *R* gene.

14.8.4 Biological Control of Fungal Pathogens

Biological control uses organisms (i.e. biocontrol agents (BCAs)) to directly or indirectly control fungal diseases of plants. Most BCAs used to control fungal diseases of plants are environmentally acceptable and non-plant-pathogenic bacteria or fungi. Biological control is not a new phenomenon as it occurs naturally. For example, the natural microbial population of certain soils suppresses the development of wilt diseases caused by vascular wilt fungi (e.g. *F. oxysporum*). Also, many organic matter soil amendments (e.g. compost) provide nutrients for, and therefore increase, the resident microbial population, some of which may be disease suppressive. Biological control can therefore be attempted either by the introduction of foreign organisms or, less commonly,

through the manipulation of the natural microbial population to enhance the activity of resident disease-suppressive organisms.

There are three means by which microbes can inhibit the development of a fungal disease (Figure 14.4):

- *Direct:* the BCA colonizes directly, suppressing the pathogen by one or more of the following means: hyphal interference, secretion of antifungal compounds (e.g. antibiotics), or enzymes. For example, the fungus *Trichoderma harzianum* is used for the biocontrol of various fungi, which it achieves by means of hyphal interference and the secretion of hydrolytic enzymes that attack the causal fungi.
- *Indirect:* the BCA physically excludes the pathogen from contact with its host or out-competes the pathogen for nutrients in a niche environment; for example, in the rhizosphere, hypovirulent (nonvirulent) strains of *F. oxysporum* can outcompete some virulent strains associated with certain vascular wilt diseases.
- *Induction of host defense*: the BCA induces the host defense mechanisms; for example, *Pseudomonas* spp. induce SAR to vascular wilt fungi in various hosts.

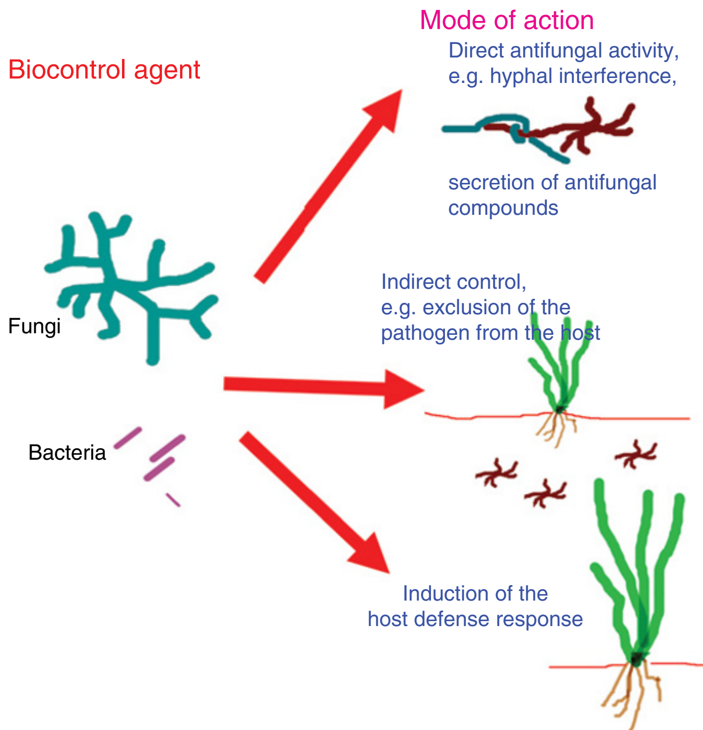

Figure 14.4 Means by which biological control agents can inhibit development of a fungal disease.

An effective BCA must be produced, formulated, and delivered in such a way that it can reach its target ecological niche in a viable and active form. Although many potential BCAs are effective under experimental conditions, many prove erratically effective under field conditions. Many potential BCAs are isolated from a habitat similar to that in which they will be applied (Plate 14.5).

One of the most promising developments in recent years is the use of endophytic fungi as BCAs. Endophytes are organisms that colonize the internal tissues of plants with no associated disease symptoms and they have demonstrated benefits for cereals and other crops. It is theorized that wild relatives of crops harbor many beneficial endophytes which their cultivated counterparts do not carry. A fungal endophyte strain isolated from a wild barley relative completely suppressed the development of seed-borne barley pathogens. Ongoing research is investigating the endophyte populations of crop wild relatives and their potential to improve the disease resistance of their cultivated relatives.

14.9 Disease Detection and Diagnosis

Host disease symptoms may enable the assessor to make a preliminary disease diagnosis and give the first clue as to the causal organism. Isolation and purification of the pathogen on culture media and subsequent macroscopic and microscopic analysis of fungal structures (e.g. hyphae, spores, resting structures) may confirm its identity. For new pathogens, their ability to fulfil Koch's postulates should be determined.

These traditional diagnostic techniques, although invaluable, often require a considerable amount of time and plant pathology expertise. Also, the disease will have advanced to the stage where symptoms are expressed before detection and diagnosis can occur, and at this stage, control measures adopted may be reduced in their efficacy. These disadvantages have led to the development of more rapid diagnostic techniques for many diseases, which can often detect as little as a few propagules of the pathogen in asymptomatic plant material. Such techniques include the commonly used pathogen-specific polymerase chain reaction (PCR) tests that detect DNA sequences specific to the pathogen. Future developments are likely to include biosensors and microchips for disease diagnosis. Biosensors convert a biological reaction (for example, between a pathogen cell wall component and an enzyme) into a detectable, quantifiable signal. Microchips contain nucleic acid, protein, or antibody probes for a range of pathogens on a single micro slide. A reaction between a cellular extract from a plant suspected to be diseased and an entity on the probe indicates a positive reaction. A positive reaction can be detectable via several methods (most often via fluorescence chemistry).

A recent development is the use of phenomics to diagnose disease based on changes in plant phenotype. Plant phenomics approaches aim to measure traits such as growth and development using a suite of noninvasive sensing technologies

and have the potential to nondestructively identify the diseased plants within a large field area. Given the fact that disease occurrence is often patchy within a field, this technology has great potential to help farmers apply fungicides in a more precise way ("precision agriculture") and thus reduce the costs and environmental concerns associated with chemical usage. It can also be used by crop breeders and agrochemical companies to expedite the screening of new crop varieties and fungicides.

14.10 Vascular Wilt Diseases

Wilt diseases arise from a water deficiency in plant foliage. This wilting results because fungus infection and colonization of the host results in a blocking of the xylem vessels and consequent inhibition of water translocation. The fungus may directly or indirectly mediate the blocking of the xylem vessels:

- *Direct*: the fungus colonizes the xylem vessels and physically blocks water movement within the vessel.
- *Indirect*: the host responds to fungal infection by secreting substances that block its xylem vessels to limit the spread of the pathogen.

Fusarium and *Verticillium* species cause vascular wilt diseases of a range of host plants; other fungal wilts of economic significance include Dutch elm disease and oak wilt (Table 14.1).

14.10.1 *Fusarium* Wilts

Vascular wilt caused by soil-borne *Fusarium* species, especially *F. oxysporum*, is common in many parts of the world. It produces asexual macroconidia (characteristic of *Fusarium* species) (Plate 14.6a) and resting spores called chlamydospores (hyphal swellings) and smaller asexual microconidia characteristic of select *Fusarium* species. Mycelia are yellow/red pigmented when grown in culture on potato dextrose agar (Plate 14.6b). The sexual state of this fungus, if it exists, has not yet been found.

Fusarium oxysporum attacks and causes serious economic losses of most vegetable and flower crops, cotton, tobacco, banana, plantain, coffee, turfgrass, ginger, and soybean, in warm and temperate climates. More than 120 f. sp. have been identified (e.g. wilt disease of tomato is caused by *F. oxysporum* f. sp. *lycopersici*, while wilt disease of banana is caused by *F. oxysporum* f. sp. *cubense*). Some f. sp. are primarily associated with root rots, foot rots, or bulb rots of plants, rather than with vascular wilt disease. Within many f. sp., *F. oxysporum* isolates are further classified into races based on their virulence against different

host genotypes. Nonpathogenic populations of *F. oxysporum* also exist in soils, some of which have provided biological control of wilts and other fungal diseases of plants.

Plate 14.6c depicts the typical disease symptoms associated with vascular wilt disease of tomato plants caused by *F. oxysporum* f. sp. *lycopersici*. In addition to wilting, other symptoms associated with this disease include vein clearing in younger leaflets in the early stages of disease, epinasty, stunting, and yellowing of older leaves.

Figure 14.5 depicts the typical wilt disease cycle for *F. oxysporum*. Conidia of the fungus germinate in response to root exudates, producing penetration hyphae that attach to the root surface and penetrate it directly. Mycelium then advances intercellularly through the root cortex and enters the xylem vessels through the pits. Within the xylem vessels, the fungus produces microconidia that are carried upwards in the sap stream. Upon germination, microconidia penetrate the upper wall of the vessels, producing more microconidia in the next vessel. The plant is subjected to water stress and subsequently wilts due to both the accumulation of fungal mycelium and/or toxin production, and the host defense responses induced because of pathogen attack (e.g. production of gels, gums, tyloses, and physical crushing of the vessels due to induced proliferation of adjacent parenchyma cells). Once the plant is killed, the fungus spreads to invade the parenchymatous tissue and sporulates profusely on the plant surface. These conidia can be returned to

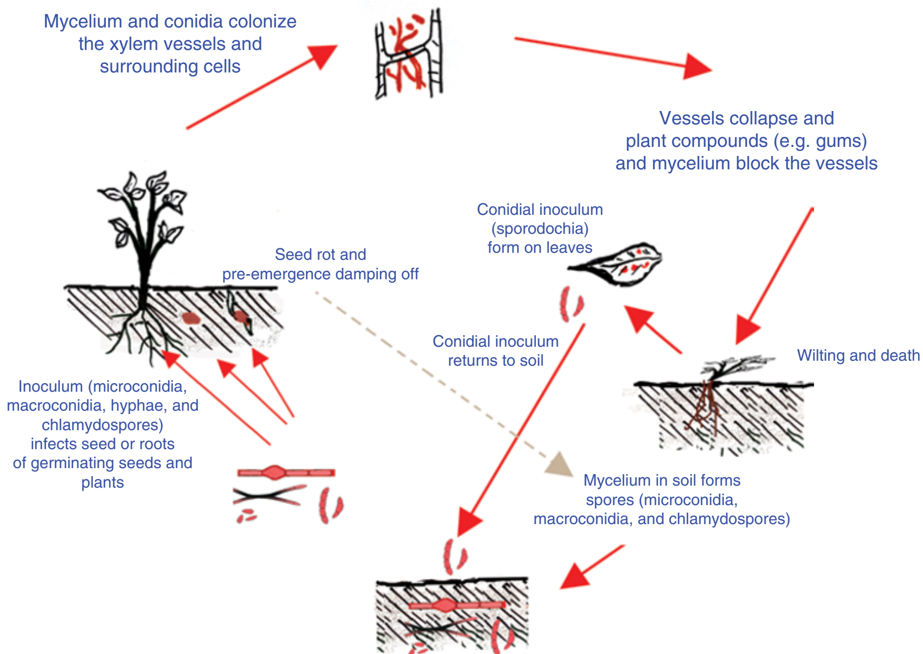

Figure 14.5 *Fusarium* wilt disease cycle.

the soil where they can survive for extended periods. Conidia are disseminated to new plants or areas by wind, water, etc.

Plant infection and colonization by *F. oxysporum* is facilitated by the pathogen's perception of signals present in root exudates, hyphal adherence to the root surface, secretion of a battery of cell wall-degrading enzymes, and the pathogen's possession of multiple mechanisms of overcoming host defense mechanisms. Root exudates elicit a fungal response and evidence suggests that cellular signal transduction cascades mediate this response. Host molecules that activate such cascades and the mechanisms by which the fungus perceives such signals – that is, the signal receptors – are not yet known. *Fusarium oxysporum* secretes an array of cell wall-degrading enzymes that may contribute to infection and colonization (e.g. polygalacturonases, pectate lyases, xylanases, and proteases). Pectate lyases are thought to play an important role in the virulence of *Fusarium* wilt fungi. Plants have evolved a number of physical, chemical, and biological defense strategies against *Fusarium* wilt disease, including the deposition of callose adjacent to infected cells, the "blocking off" of colonized vessels by gels, gums, and tyloses, the synthesis of fungitoxic compounds, and antifungal enzymes.

Control is difficult due to the soilborne nature of the pathogen and its ability to persist for extended periods in the absence of a host. Disease control is usually achieved via an integrated pest management system – that is, the combined use of one or more of the following: cultural practices, resistant host genotypes, fungicides, and biological control agents. Cultural practices that help control this disease include using rotating land with nonhosts such as cereals (avoiding solanaceous crops such as potato and tomato), removal of crop debris, nonexcessive irrigation, and the maintenance of vigorous plants via fertilizer application. Using *Fusarium* wilt-resistant host cultivars is an option: many host cultivars differ in their susceptibility to this disease. Resistance is thought to be mediated by a gene-for-gene relationship, and Takken and Rep (2010) reviewed the arms race that is ongoing between *F. oxysporum* and tomato. Wilt resistance genes can be introgressed into cultivars with other desirable characteristics (e.g. carrying resistance genes for other races, good yield).

Traditionally, chemical control was often attempted by soil fumigation with methyl bromide to kill the soilborne conidial inoculum. Over recent decades, environmentally friendly alternatives have been revisited, such as soil steam sterilization of a limited amount of substrates (e.g. for glasshouse use) and reduction of seedborne inoculum using hot water treatment. Fungicide seed treatment can protect the plant during the early stages of establishment. Certain systemic fungicides can protect the plant at later developmental stages, but their use is not encouraged for food plants. Biological control of *Fusarium* wilt has received a lot of attention in recent years as an attractive means of disease control.

14.10.2 Other Wilts of Economic Significance

Other wilts of economic significance include *Verticillium* wilt, Dutch elm disease, and oak wilt. *Verticillium* wilt is commonly caused by the soilborne *V. albo-atrum* and *V. dahliae*. These lack known sexual reproductive structures (deuteromycetes) and asexual conidia are produced. *Verticillium albo-atrum* grows best at 20–25 °C, while *V. dahliae* grows better at 25–28 °C. *Verticillium* wilt affects a wide array of vegetables, flowers, field crops, fruit trees, roses, and forest trees. Both *V. albo-atrum* and *V. dahliae* exist as various strains or races whose host range, virulence, and other characteristics vary considerably.

The symptoms and disease cycle of *Verticillium* wilt are like those of *Fusarium* wilt. Symptoms, often not obvious until either dry weather or later in the growing season, include wilting, stunting, vascular discoloration, leaf epinasty, and chlorosis and necrosis of leaves. Compared to *Fusarium* wilt, *Verticillium* wilt symptoms develop more slowly, at lower temperatures, often only develop on one side of a plant, and are usually confined to lower plant parts. Both *Verticillium* species can be spread in contaminated seed, propagative plant parts, by wind, water, and soil, and can overwinter and persist as mycelium in perennial hosts, plant debris, and vegetative propagative parts. As with *Fusarium* wilt, control is usually based on an integrated pest management strategy similar to that described above for *Fusarium* wilt. Resistant or partially resistant cultivars of some susceptible plant species are available and durable resistance has been incorporated into many commercial tomato cultivars.

Dutch elm disease has devastated elm populations worldwide, and devastation continues particularly in the United States. Two species of the ascomycetous heterothallic *Ophiostoma* genus are responsible for the disease, O. *ulmi* and the more aggressive *O. novo-ulmi* (the causal organism was traditionally called *Ceratocystis ulmi*). Disease symptoms include wilting, yellowing/browning of leaves of individual branches or of the entire tree (Plate 14.7), and the formation of brown or green streaks in the infected sapwood underlying the bark. The disease is most destructive when trees are infected early in the growing season.

The fungus overwinters as a saprophyte in dying or dead elm trees or logs as mycelium or conidia-bearing coremia. The fungus is spread by emerging adult elm bark beetles that become coated with sticky conidia. These beetles wound and colonize other elms, to which they transfer their conidial loads (the fungus can also spread through grafted root systems). The conidia germinate and rapidly parasitize and colonize the wood until the fungus reaches the large xylem vessels of the spring wood, where it may produce more conidia, which are carried up in the sap stream and initiate new infections. Control of Dutch elm disease spread may be facilitated by proper sanitation, removal of localized branch infections by pruning, avoidance of contact between healthy and infected roots, and immediate removal of infected elms. Root injection with systemic fungicides may give protective or short-term curative control, but this may be expensive.

Elimination of the disease vector, the elm bark beetles, can be attempted (using pesticides, pathogenic nematodes, or trapping using pheromones), but, in the past, this has not proved to be very effective in controlling Dutch elm disease. Control via innate host resistance is unlikely, as most elm species are susceptible to this disease.

The ascomycetous fungus *Ceratocystis fagacearum* causes oak wilt disease. Infected trees rapidly wilt from the top of the canopy downward and trees usually die within 2 months. Leaf symptoms include wilting, clearly delineated browning, and defoliation. These symptoms are sometimes accompanied by a brown discoloration of the sapwood underlying the bark of symptomatic branches. Red oaks are very susceptible to the disease; members of the white oak family are generally more resistant. The fungus overwinters as mycelium in still-living, infested trees and as fungus pads on dead trees. Insects such as sap- and bark-feeding beetles can spread the fungus. Control of oak wilt disease spread may be facilitated by means similar to those described above for Dutch elm disease.

14.11 Blights

Fungal blight diseases of plants are characterized by a rapid browning and death of the plant leaves, branches, twigs, and floral organs. Table 14.1 lists some of the economically important blights caused by plant-pathogenic fungi (many of these fungi cause additional disease symptoms, such as rots, on these and other hosts). The most famous example is late blight of potatoes.

14.11.1 Late Blight of Potatoes

Phytophthora comprises a genus of fungi that cause a range of disease symptoms on different host plants. Of the different species, *P. infestans* is best known as the causal agent of late blight of potatoes and tomatoes. This is a ubiquitous disease in most potato-growing regions of the world and is very destructive in the potato-growing regions of Europe and the United States. This disease precipitated the Irish potato famine in the nineteenth century.

Phytophthora infestans is a heterothallic oomycete that produces motile zoospores (that have two flagella) in lemon-shaped sporangia that form at the top of sporangiophore branches (Plate 14.8a). At higher temperatures (>15 °C), sporangia can germinate directly by producing a germ tube, while at lower temperatures (>12–15 °C) sporangia germinate almost entirely by means of zoospores. Sexual reproduction requires the mating of an A1 and A2 type of the fungus, whose hypha on contact differentiates to form an antheridium and oogonium. The antheridium fertilizes the oogonium (a process called karyogamy) which develops into a thick-walled oospore that germinates by means of a germ tube (Plate 14.8b). This germ tube then forms a sporangium or,

occasionally, directly forms a mycelium. Several races of *P. infestans* exist that vary in their pathogenicity towards different host cultivars.

This disease affects stem, leaf, and tuber tissue. Leaf symptoms include water-soaked spots on lower leafs that, under moist conditions, subsequently enlarge to form brown areas (Plate 14.8c). On the underside of these infected leaves a white woolly fungal growth appears and soon all the leaflets on the leaf die. Under prolonged periods of wet or damp weather, all aerial plant parts blight and rot, giving a foul odor. Affected tubers have brown/black blotches on their surface, and internally they exhibit water-soaked dark-brown rotted tissue (Plate 14.8d). Such tissue may be colonized by secondary soft-rotting invaders (bacteria and fungi), resulting in a foul odor.

The pathogen overwinters as mycelium in infected potato tubers. Overwintering mycelium infects and spreads though the cortical region of the stems of plants arising from infected tuber seed, and later colonizes the pith cells of the stem. Once the mycelium reaches the aerial plant parts it produces sporangiophores that protrude through the leaf and stem stomata and project into the air. Sporangia are produced on the sporangiophores and, when mature, they are released into the air or are dispersed by rain. These sporangia land on potato leaves or stems where they form appressoria, and under moist conditions they germinate and the arising penetration peg penetrates the plant and causes new infections. The penetrating hypha grows intercellularly, and the resulting mycelium penetrates and forms intracellular haustoria (i.e. hyphae) that enter and draw nutrients from the host cells. These colonized cells die and the disease spreads to fresh tissue.

Many of these asexual generations and new infections can occur in a short period. These infections result in the blighting and premature death of foliage and a reduction in tuber yield. Tuber infection begins when, under wet conditions, sporangia are washed downwards from infected foliage into the soil, releasing zoospores that reach the tubers near the surface of the soil. Zoospores germinate and the germ tube penetrates the lenticels or wounds of tubers. The fungus colonizes the tuber cells by growing intercellularly and producing haustoria within the cells. Infected and colonized tuber tissue decays and rots, either in the field or in storage. Also, seemingly healthy tubers inoculated with sporangia from soil can become infected and rot during storage.

Relatively warm days and early evenings promote infection and sporulation; lowering of temperature during the night induces dew formation and zoospore production; gradually increasing temperature during the day promotes zoospore encystment and penetration. Infection and colonization of potato by *P. infestans* is facilitated by adhesion of the pathogen to the host surface, penetration and infection via physical force, degradative enzymes and other virulence molecules, and suppression of host defense responses. Zoospores of other *Phytophthora* species exhibit electrotactic swimming towards weak electric fields generated by plant roots, chemotaxis (i.e. attraction towards plant-derived compounds), and chemotrophic and contact-induced responses.

Control of late blight of potato has received much attention. It is generally attempted by an integrated approach involving cultural, chemical, and disease forecasting control strategies. Proper sanitation, including the use of disease-free seed and removal of plant debris and volunteer potato plants, helps control late blight disease. Regular fungicide applications are often used as a preventative measure under late blight-favorable conditions, that is, wet or humid weather and cool nights. Disease forecasting systems are widely used to indicate environmental conditions conducive to blight. Advanced systems consider the resistance of the potato cultivar to late blight, the effectiveness of the fungicide, and, in some cases, the local disease risk. Most commercial cultivars are susceptible to the disease. However, some do offer partial resistance, and research is ongoing to develop new cultivars with enhanced disease resistance via both traditional breeding and transgenic approaches. *R* genes (for single-gene resistance and genes for quantitative resistance) are known to be present in both wild and cultivated potato germplasm. The potential of controlling late blight using biological control methods is being extensively investigated. Isolates of several fungal species (e.g. *Aspergillus*, *Fusarium*, *Mucor*, *Penicillium*, *Rhizoctonia*, and *Trichoderma*) and bacterial species (e.g. *Bacillus subtilis*) have shown promise for disease control and some have been commercialized.

14.12 Rots and Damping-Off Diseases

Many fungi cause rots of a wide variety of plants. These fungi vary greatly in terms of their taxonomic classification, their host and tissue specifics, and mechanisms of parasitism. Different fungi cause soft rots of fruits, tubers, corms, bulbs, root rots, stem rots, and so on, and Table 14.1 lists some of the economically important rots of plants caused by fungal diseases. Rots can occur in the field, or in stored fruits, tubers, and bulbs. In addition to causing a rotting of roots and stems of young plants, some fungi cause rapid death and collapse of young seedlings – that is, damping-off disease.

14.12.1 *Pythium* Damping-Off Disease

Species of the genus *Pythium* include *P. irregulare*, *P. aphanidermatum*, *P. debarynum*, and *P. ultimum*. *Pythium ultimum* can cause root rot, seedling damping off, and seed rot of many plants, and soft rot of fleshy fruits that are in contact with the soil. Broadleaf and grass plant species are particularly susceptible to the disease. *Pythium ultimum* prevails in cooler to cold soils, while *P. irregulare* and *P. aphanidermatum* are adapted to higher soil temperatures. Like *Phytophthora*, *Pythium* species are soilborne oomycetous fungi that produce asexual motile sporangia-derived zoospores (Plate 14.9a) and sexual

oospores (Plate 14.9b), although some pathogenic isolates have no known sexual reproduction stage, and most species are homothallic.

The symptoms caused by damping-off fungi such as *Pythium* species depend on the plant age and developmental stage. Seeds sown into infested soil can become infected and disintegrate. Post-germination seedling infections manifest as either a poor rate of emergence above the soil line (i.e. pre-emergence damping off) or the collapse of emerged seedlings (i.e. post-emergence damping off) (Plate 14.9c). *Pythium* species can attack and cause lesions of the stems or root rot of older plants that, if severe, can cause plant stunting, leaf yellowing, wilting, and death. *Pythium* soft rots of fleshy vegetable organs manifest as woolly fungus growth on the surface (often referred to as "cottony leak"), accompanied by internal soft rotting of the organ. These symptoms are also commonly caused by species of the genera *Rhizoctonia*, *Fusarium*, and *Phytophthora*, other fungi, and some bacteria.

Sporangia and thick-walled oospores constitute important soilborne survival structures of *Pythium* species. Seed, root, and stem infection (at the soil line) occurs when, under moist conditions, soilborne mycelium or released zoospore germ tubes come into contact with, and penetrate the tissue (either directly or through cracks, wounds, or natural openings). Exudates from seeds and roots can induce fungal spore germination, hyphal growth, and penetration. The fungus then uses physical force and an enzymatic degradation of middle lamellae, cell walls, and protoplasts to colonize the host, leading to pre-emergence death or to post-emergence damping off of the seedlings. Once the tissue is colonized by mycelium, sporangia and oospores are produced inside or outside the host tissue and these return to the soil with decaying plant material, providing a new source of inoculum for future infections. The disease is spread through soil water and through the movement of infected plant debris.

Good resistance to *Pythium* damping off and associated diseases is virtually unknown among cultivars of many hosts. Cultural practices, including good sanitary practice, shallow seed planting, provision of adequate plant nutrition, and maintaining well-drained plots or beds, help prevent or reduce the risk of *Pythium* diseases. Because many plants are susceptible to these diseases, crop rotation may not prevent disease and inoculum build-up in soil. Pre-plant soil fumigation was commonly used in contained conditions (e.g. glasshouses) to kill soilborne inoculum. Soil sterilization, and soil solarization in warmer countries, may reduce inoculum build-up. Systemic fungicides can be applied as a preventative seed dressing or as a water-soluble preventative or curative soil drench. However, as with many diseases, when applied post-infection, the fungicide efficacy in controlling disease will be reduced. The success of biological control agents in controlling *Pythium* diseases has been very variable, especially under field conditions. Bacteria that have showed potential to control *Pythium* diseases include species of *Pseudomonas*, *Burkholderia*, *Streptomyces*, and *Bacillus*; fungal antagonists include species of *Trichoderma*, *Gliocladium*, and nonpathogenic *Pythium* and *F. oxysporum* isolates.

14.13 Leaf and Stem Spots, Anthracnose, and Scabs

Leaf and stem spots are characterized by the occurrence of localized lesions on either the host leaves or stems. Several fungi cause such foliar and stem diseases of cereals that manifest as localized spots (Table 14.1). *Septoria tritici* blotch (Plate 14.10) (and glume blotch) are considered among the most serious diseases of cereals, especially in maritime climates.

Anthracnoses are diseases in which spots or sunken lesions with a slightly raised rim occur on the stems, foliage, or fruits of plants; other symptoms of this disease may include dieback of twigs or branches and infected fruit may drop and rot (Table 14.1). Anthracnose diseases of curcurbits and common beans caused by *Colletotrichum* species cause significant yield losses worldwide; the latter is particularly serious in Africa, Central and South America, and Asia (Plate 14.11).

Scabs are characterized by localized lesions of scabby appearance on host fruit, leaves, and/or tubers. Fungal scab is the most important disease of apples, reducing quality and size of infected fruit (the causal organism is *Venturia inaequalis*).

14.13.1 Spot and Blotch Diseases Caused by *Mycosphaerella* Species

Mycosphaerella comprises an ascomycetous genus of fungi with anamorphs of various types, for example *Cercospora*, *Septoria*, *Ascochyta*, *Ramularia*, and *Didymella*. Associated disease names often incorporate the anamorph state. Between them, members of this genus, or their anamorph state, cause serious leaf spots of cereals, banana, pea, and other vegetable crops. Economically important diseases caused by fungi from this genus include leaf spot diseases of banana caused by *Mycosphaerella* species, *Ramularia* blotch of barley caused by *Ramularia collo-cygni* (telomorph not known), and *Septoria* blotch of wheat caused by *Mycosphaerella graminicola* (anamorph: *Septoria tritici*). The latter has been recently renamed as *Zymoseptoria tritici.*

Septoria tritici blotch of wheat is among the most serious diseases of cereals, particularly in maritime climates. This fungus produces sexual ascospores and asexual conidia called pycnidiospores (produced in a spore case called a pycnidia). Disease symptoms include elongate oval lesions on leaves, running parallel to the leaf. These become water-soaked and brown, and a chlorotic halo may develop around the lesion. Black pycnidia develop on mature lesions and cirri may form on the lesions if the weather is dry for prolonged periods. The fungus overwinters on seed, stubble, debris, and overwintering cereal crops. In the spring, it reproduces sexually to form ascospores that are wind-dispersed to wheat leaves. However, asexual pycnidiospores that are water-splashed to host leaves provide the major source of inoculum for disease development. Spore germ tubes directly penetrate the plant or enter via stomata, where they colonize

host tissue and produce pycnidia. Moisture in the form of rain, dew, or irrigation stimulates pycnidiospore release from conidia that are water-splashed to leaves to cause new infections.

Control of this disease is attempted by good sanitary practices such as removal of crop debris, crop rotation, using resistant wheat cultivars, and chemical control. As stated earlier (see Section 14.8.2), strains of the causal fungus have developed resistance to the fungicides that were used to control the disease and strains are emerging that have enhanced tolerance to the main fungicide group currently used to control the disease.

14.14 Rusts, Smuts, and Powdery Mildew Diseases

Rusts, smuts, and powdery mildew diseases are among the most common and devastating fungal diseases of plants. Control is achieved by good clean cultural practices, using resistant hosts, and fungicide application.

Rust diseases have caused devastating losses on coffee and grain crops, but also of pine, apple, other field crops, and ornamentals. There are thousands of rust fungi that attack different plants. They are basidiomycetes and most are obligate parasites. The most destructive include stem rusts of wheat and other cereals caused by *Puccinia graminis* (Plate 14.12), coffee rust caused by *Hemileia vastatrix*, and *Puccinia* rusts of field crops, vegetables, and ornamentals. Rust diseases appear as yellow to brown, white or black rusty spots; the rusty appearance results from epidermal rupture by the emerging spore masses that often have a powdery appearance. Wheat stem rust caused by *P. graminis* threatens food security at present. International research efforts are focussed on developing new wheat varieties that resist the new aggressive rust strains that have evolved in the last few decades.

Smut diseases, also caused by basidiomycetes, were a serious problem prior to the development of contemporary fungicides. These fungi are not obligate parasites; that is, they can be cultured on media, but in nature they exist almost exclusively as parasites. Most smut fungi attack and devastate the ovaries of cereal grains and grasses, turning seed to black powdery masses (e.g. covered smut of oats caused by *Ustilago hordei* and covered smut or "bunt" of wheat caused by *Tilletia* species; Plate 14.13), but some attack leaves, stems, or seeds. Smut diseases either destroy the affected tissue or replace it with black spores.

Powdery mildew diseases are among the most conspicuous plant diseases affecting all kinds of plants. They are caused by obligate parasites that are members of the fungal family Erysiphaceae. Plate 14.14 depicts the symptoms of powdery mildew disease of wheat caused by *Blumeria* (formerly known as *Erysiphe*) *graminis*. Grasses and cereals are among the plants severely affected by this disease, as are ornamentals such as roses, field crops, and trees. Symptoms include chlorotic or necrotic leaves, and stems and fruits covered with mycelium and fruiting bodies of the fungus, often giving a white "woolly" appearance.

14.15 Global Repercussions of Fungal Diseases of Plants

The economic implications of plant disease are a reduction in crop or harvest value due to decreased quality and/or quantity of produce. Plant disease epidemics can directly or indirectly impact on the health of humans and animals. Direct impacts include mycotoxicosis associated with the consumption of mycotoxin-contaminated foods. Mycotoxins have also been investigated as potential biological warfare agents; but, conversely, some mycotoxin derivatives have provided medicinal compounds beneficial to human health. Historic examples that clearly illustrate the far-reaching and indirect repercussions of fungal diseases of plants include ergot disease of rye and other cereals and the Irish potato famine. A more recent example is sudden oak death caused by *Phythophthora ramorum*.

Ergot is a disease of cereal heads caused by *Claviceps* species, including *C. purpurea* and *C. fusiformis*. In infected grain, *C. purpurea* produces ergotamine ergocristine alkaloid mycotoxins and *C. fusiformis* produces clavine alkaloid mycotoxins; these are responsible for gangrenous and convulsive ergotism diseases, respectively, of humans and animals. Gangrenous ergotism is characterized by a loss of extremities, and convulsive ergotism normally manifests as a nervous dysfunction of the victim. In the Middle Ages these compounds caused many disease epidemics and outbreaks of ergotism have been documented throughout history. They were responsible for many thousands of deaths and social upheaval in Europe. In France in AD 800–900, the consumption of bread contaminated with toxins (and the ergot alkaloid derivative lysergic acid diethylamide (LSD) produced during the baking of contaminated wheat) caused what was termed ignis sacer (sacred fire) or St Anthony's fire disease of humans. This ergotism epidemic was so-called because it was believed that a pilgrimage to St Anthony's shrine would alleviate the intense burning sensation associated with ergotism disease. It is theorized that women accused of witchcraft and sentenced to death in European trials and in the Salem trials of 1692 were suffering from ergotism. An outbreak of gangrenous ergotism occurred in Ethiopia in 1977–1978; convulsive ergotism occurred in areas of India in 1975. However, contemporary grain processing and cooking processes employed in the western world eliminate or detoxify most ergot alkaloids present in grain. Contrastingly, ergot alkaloids have yielded beneficial medicinal compounds; the commonly used derivative ergotamine is often prescribed for patients suffering from vascular headaches.

The Irish potato famine (1845–1850) was the consequence of *P. infestans* late blight of potato. Although this epidemic of late blight was not unique to Ireland, the consequences for Ireland were more severe because, being nutritious, and easy to grow and to store, the potato then constituted the bulk of the diet of the impoverished Irish. This dependence on potato, particularly on the blight-susceptible cultivar lumper, together with the blight-favorable weather conditions in Ireland,

rendered the population at the mercy of potato blight disease that arrived from North America in 1845. More than one million people died because of the famine and many tried to escape from hunger by emigrating to the United States, Canada, and Britain (reducing Ireland's population from eight to five million in a matter of years). The famine highlighted the need for cultural change in Ireland and heralded the end of the traditional practice of dividing family estates into small plots among descendants. It also heralded the beginning of political upheaval in the then British-ruled Ireland.

The advent and development of fungicides over the last century means that, today, the repercussions of fungal diseases are not as widespread as those of the Irish potato famine or ergotism in the Middle Ages. However, such compounds do not easily control some pathogens and others evolve resistance to fungicides. Also, new pathogens and associated disease outbreaks are continuously emerging. Sudden oak death caused by *Phytophthora ramorum* is an example of such a disease. This has emerged recently as a devastating disease of oak and other forest trees in cooler wet climatic regions and has reached epidemic proportions along the Californian coast. It has spread to Europe, and, more recently, this pathogen has been identified as the causal agent of disease of other broadleaved trees and larch. In 2010 it devastated larch plantations in Britain. In more recent years, a dieback disease of ash caused by the fungus *Hymenoscyphus fraxineus* has spread rapidly across Europe and this disease can be fatal, particularly for younger trees.

Plant pathogens and their toxic metabolites have been investigated as terrorism and military weapons and thus have raised public and security concerns. However, the usefulness of such organisms as biological weapons is open to debate. Some suggest that yellow rain caused thousands of deaths in South-east Asia (1974–1981) and that T-2 toxin, which belongs to the trichothecene group of mycotoxins produced by some *Fusarium* species, was the agent of disease, but this is highly controversial. Strains of the non-trichothecene-producing *Fusarium* species *F. oxysporum* and the fungus *Pleospora papaveracea* that are natural pathogens of poppies were investigated for their potential control of narcotic-yielding poppy fields to aid in the "war on drugs." However, the consequences of the deliberate release of such organisms or derivative compounds is highly controversial and a subject of world debate. In the future, genetic engineering provides the potential for improved virulence or altering the metabolite profile of a fungus, thus increasing its usefulness as a biological weapon.

14.16 Conclusion

This chapter is too concise to cover all fungal plant diseases of economic significance, and some important diseases (e.g. those that cause cankers and galls) did not even feature. From this insight into plant pathology, it is obvious that fungal diseases of plants have, and will in the future, put tremendous stress on world

food production. From the wilts to the gall diseases, these fungi have evolved, and are continually evolving diverse mechanisms for infecting and colonizing the world plant population. Plant pathology researchers have made a tremendous contribution to the science of understanding these diseases.

The "global economy" and changing cultural and breeding practices (e.g. organic farming and genetic engineering) are now placing further challenges on plant pathologists to determine how these virulent pathogens are moving around the world (i.e. gene flow), if host or non-host disease resistance exists, and how it can be introgressed into agronomically desirable crops, and if new environmentally friendly and durable chemical and biological disease control methods can be developed. Traditional pathology, molecular biology techniques, and more recent phenomics technologies are being adopted to answer these questions to safeguard the world plant population. However, pressures on food and feed supply are ever increasing due to both a growing population and climate change. There is an urgent need to test models to get a clearer picture as to how climate change will impact upon agronomic practices, pathogen dynamics and population biology, and disease epidemiology. If we are to feed the growing world population under changing climatic conditions, and in a sustainable manner, we need to improve our understanding of fungal–plant interactions so that we can try to outwit the fungi in what is a never-ending arms race.

Acknowledgments

Thanks to Seamus Kennedy for help with graphical illustrations and to Brian Fagan and Gerard Leonard for supplying diseased material.

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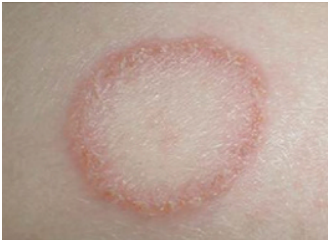
(a)

(b)



(c)

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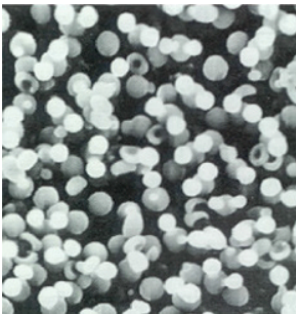

(e)

(f)

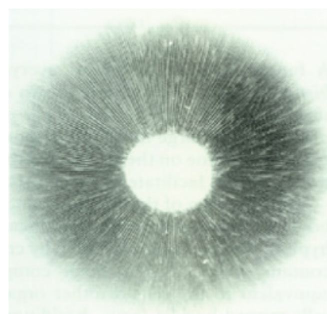

Plate 1.1 Radiating circular pattern of (a) *Chlorophyllum molybdites* fruit bodies forming a fairy ring in grassland, and (b) *Tinea corporis* infection of the skin; (c) fruiting bodies of *Agaricus bitorquis*; (d) scanning electron micrograph of *Agaricus bisporus* gill surface (P.T. Atkey); (e) light micrograph of basidium bearing two spores (T.J. Elliott); (f) spore print from underside of fruit body (M.P. Callen); (g) germ tubes emerging from spores (T.J. Elliott; published by John Wiley & Sons Ltd); (h) branching hyphae growing on agar plate (T.J. Elliott) (all from Carlile, Watkinson, & Gooday (2001) Academic Press). Dry-rot fungus *Serpula lacrymans* (i) colony decaying timbers in a wall void and forming red-brown fruiting structure; (j) close-up of underside of a basidiocarp (from Ingold & Hudson printed by Chapman & Hall); (k) exploratory fan-shaped mycelium with connected rhizomorphs; (l) fast-growing and (m) slow-growing colonies on agar media.

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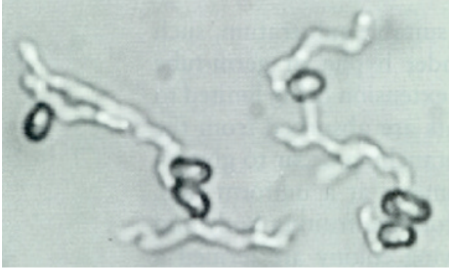
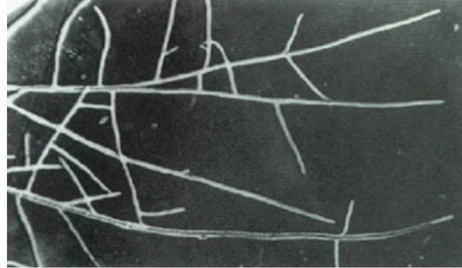
(g)



(h)



(i)

(j)

(k)

(l)

(m)

Plate 1.1 (*Continued*)

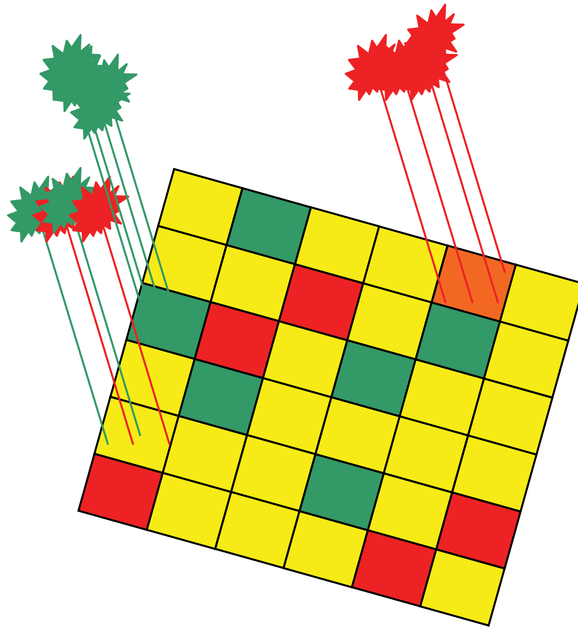


Plate 4.1 Two-color microarray technology of 6,400 distinct ORFs, arrayed on slide (only 36 are shown here). Two separate cDNA samples, one from yeast growing in glucose at beginning of fermentation labeled with green fluorescent dye, the other from later in the fermentation labeled with red dye. Red color indicates gene expression increased relative to the reference, green indicates gene expression decreased relative to reference, and yellow indicates no change in expression level. Three ORFs are shown in detail here.

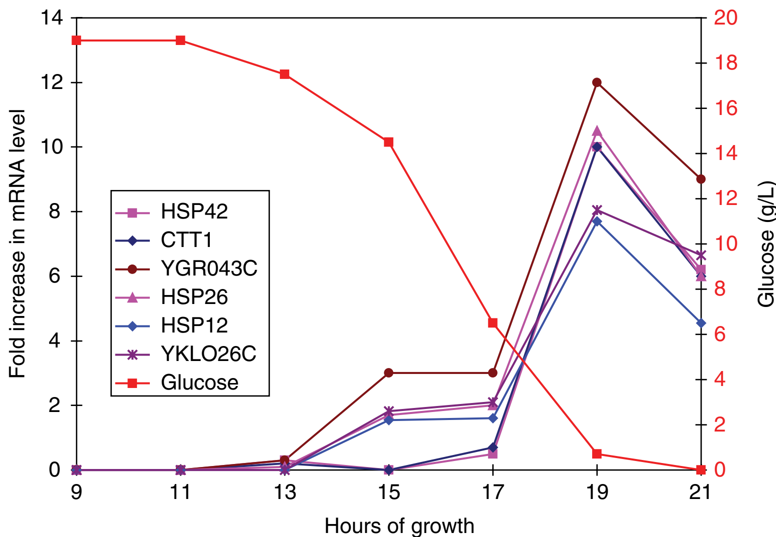


Plate 4.2 Pattern interpretation of microarray (after de Risi (1997)). This shows how expression levels of genes known to be stress-induced via STREs in their promoters were identical to one another. (Promoter sequences of other genes with the same profile but not previously identified as stress-inducible were than examined – many contain one or more recognizable STRE sites.)

Plate 6.1 Button mushroom (*Agaricus bisporus*).

Plate 6.2 Interior of a composting tunnel. Compost is filled in a layer about 2.5 m deep on top of a mat. Air is blown in underneath the compost to allow an aerobic composting process.

Plate 6.3 Cross-section of a mushroom bed. On top of the compost (brown layer), a black layer of casing soil can be seen, on top of which mushrooms develop.

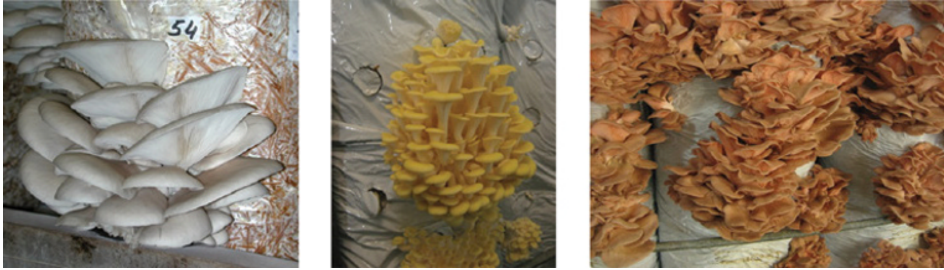


Plate 6.4 Three species of oyster mushroom. From left to right: *Pleurotus ostreatus*, *Pleurotus citrinopileatus*, and *Pleurotus flabellatus*.

Plate 6.5 Example of log cultivation of shiitake mushroom (*Lentinula edodes*).

Plate 6.6 Example of bag cultivation of shiitake mushroom (*Lentinula edodes*).

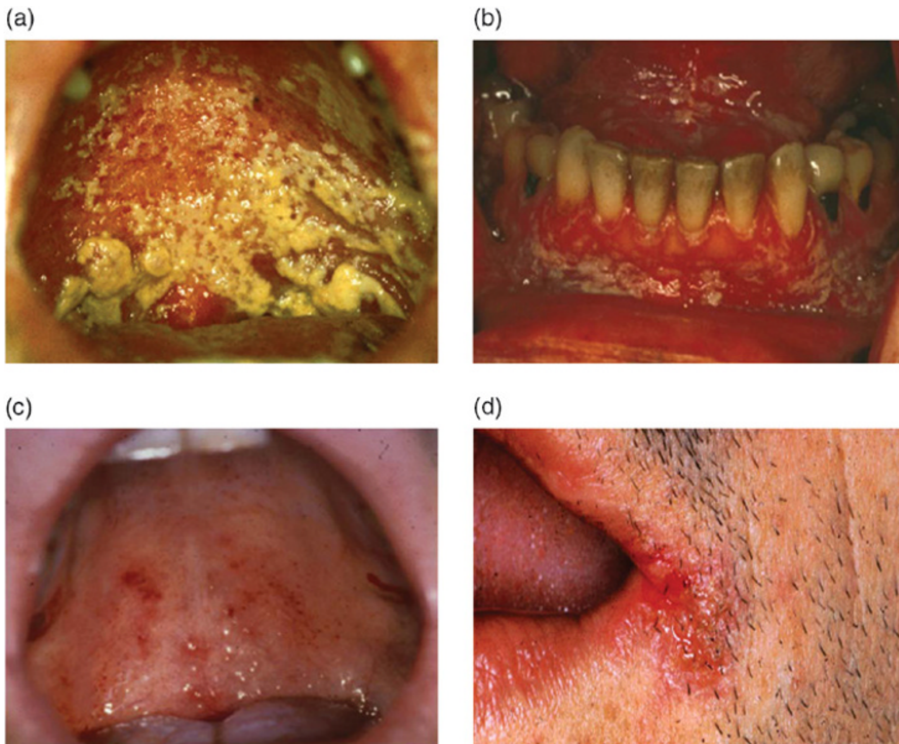


Plate 10.1 Different forms of oral candidiasis: pseudomembranous candidiasis of the palate (a) and oral mucosa (b), erythematous candidiasis of the palate (c), and angular cheilitis (d).

Plate 10.2 Colonies of *Candida* spp. grown from a clinical sample taken from the oral cavity of a patient with oral candidiasis and grown on CHROMagar Candida™ plate. Most *Candida* spp. can be identified on the basis of color of colonies produced (e.g. *C. albicans* colonies are green/blue, *C. glabrata* pink, and *C. parapsilosis* pale cream).

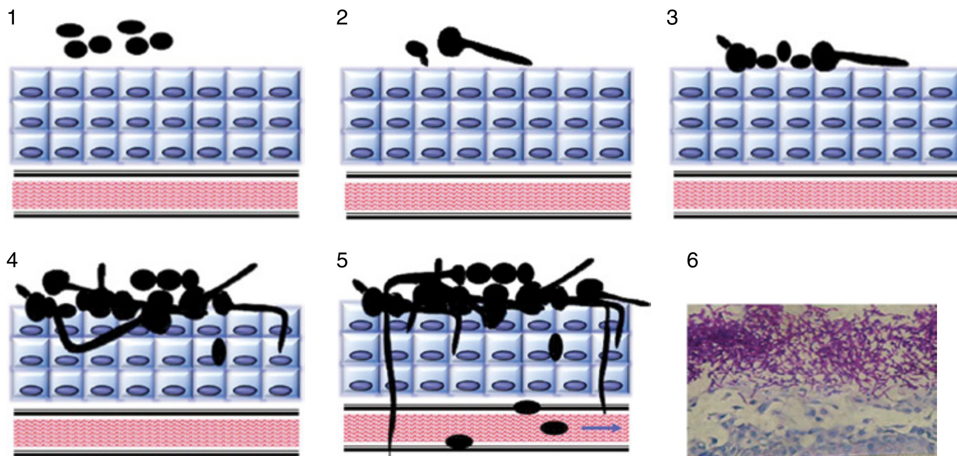


Plate 10.3 Schematic diagrams indicating stages of candidal infection. (1) Candida cells (black) come into contact with host epithelium (blue). (2) Candida cells, particularly germ tubes and hyphae, adhere to epithelial cell surface via specific interactions between candidal adhesins (e.g. Hwp1) and host ligands. (3) Once they have bound to the tissue, *Candida* cells begin to proliferate and produce hyphae. (4) Growth continues by budding and production of extensive levels of hyphae (growth is often associated with formation of biofilm). (5) Fungal hyphae and yeast cells eventually penetrate through the epithelial layer to tissues below, ultimately reaching the bloodstream (red), via which they disseminate throughout the body to cause disseminated candidiasis. (6) Photograph of reconstituted human epithelial tissue infected with *C. albicans*.

(a)

13.1a *Laccaria amethystina* – an ectomycorrhizal basidiomycete.

(b)

13.1b *Lycoperdon perlatum* – a saprobic basidiomycete with the puffball-type sporocarp.

(c)

13.1c *Piptoporus betulinus* – a parasitic basidiomycete with a shelf-type sporocarp.

(d)

13.1d An unidentified *Rhizopogon* sp. – an ectomycorrhizal basidiomycete with a hypogeous truffle-type sporocarp.

(e)

13.1e An unidentified *Clavulinopsis* sp. – a saprobic basidiomycete with a coral-type sporocarp.

(f)

13.1f *Tolypocladium longisegmentum* – a parasitic ascomycete with a club-type sporocarp that only grows on sporocarps of the hypogeous fungal genus *Elaphomyces*.

Plate 13.1 Some fungal species that represent the more common fungal sporocarp types. The types include mushroom (*Laccaria amethystina*), puffball (*Lycoperdon pyriforme*), bracket (*Piptoporus betulinus*), truffle (*Rhizopogon* sp.), coral (*Clavulinopsis* sp.), club (*Tolypocladium longisegmentum*), jelly (*Tremella mesenterica*), bird's-nest (*Cyathus* sp.), stinkhorn (*Phallus impudicus*), glebal (*Daldinia concentrica*), and resuprinate crust (*Pullchericium cearullium*).

(g)

13.1g *Tremella mesenterica* – a saprobic basidiomycete with a jelly-type sporocarp.

(h)

13.1h An unidentified *Cyathus* sp. – a saprobic basidiomycete with a bird's-nest-type sporocarp.

(i)

13.1i *Phallus impudicus* – a saprobic basidiomycete with a stinkhorn-type sporocarp.

(j)

13.1j *Daldinia concentrica* – a parasitic ascomycete composed of a glebal mass of perithecia on a large stromata.

(k)

13.1k *Pullchericium cearullium* – a saprobic basidiomycete with a resupri-nate crust-like fruiting mass.

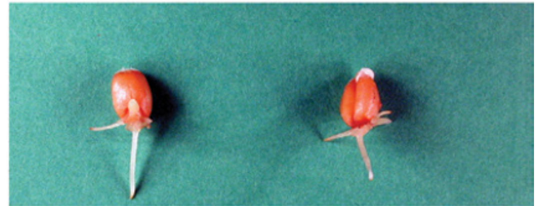
Plate 13.1 (*Continued*)

Plate 13.2 A lichen growing on a tree branch.

Plate 13.3 Ectomycorrhizal root tips and mycelium on roots of a potted spruce plant.

Plate 14.1 Variation in head blight susceptibility of two barley cultivars to *Fusarium* head blight disease. Both heads were inoculated with *Fusarium culmorum*, but only the cultivar on the left shows disease symptoms (premature bleaching of head spikelets).

(a)



(b)

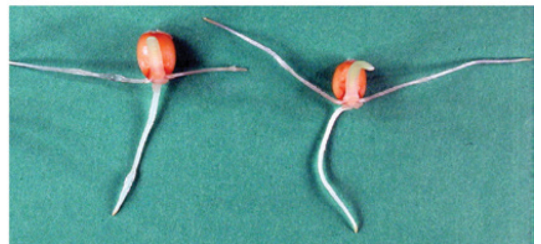


Plate 14.2 Effect of deoxynivalenol, a non-host-specific fungal trichothecene toxin, on germination of wheat seedlings. Seeds germinated on toxin solution (a) show reduced root and coleoptile growth compared to those germinated on water (b).

Plate 14.3 Club-root of brassicas caused by *Plasmodiophora brassicae*. Roots swell and develop tumors due to increased hormone (e.g. cytokinin) production.

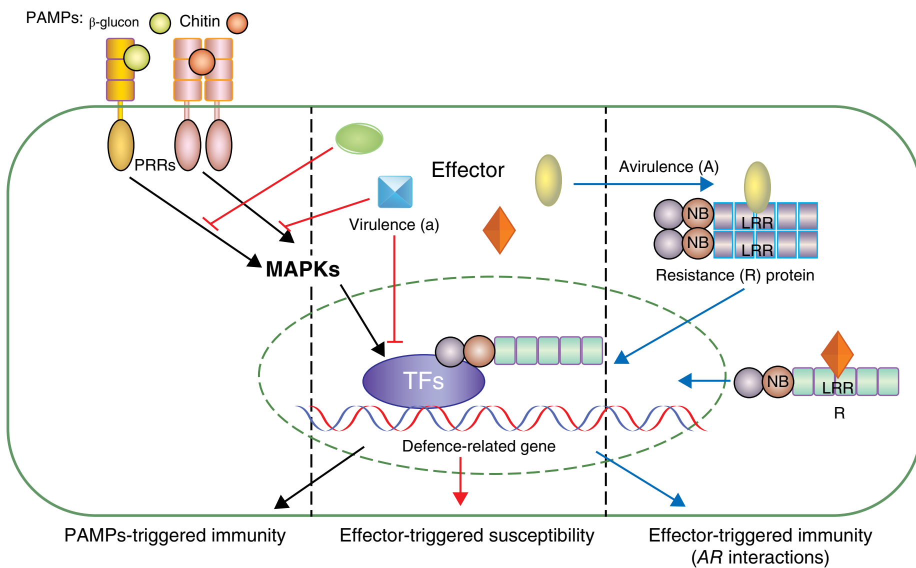


Plate 14.4 Means by which biological control agents can inhibit development of a fungal disease.

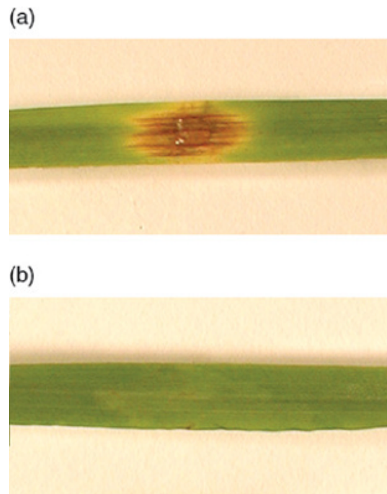


Plate 14.5 Biological control of net blotch disease of barley (caused by *Perenophora teres*) by a bacteria (*Bacillus* spp.) originating from a cereal field. Blotch disease symptom severity caused by this pathogen on inoculated leaves in the absence (a) and presence (b) of the biocontrol agent.

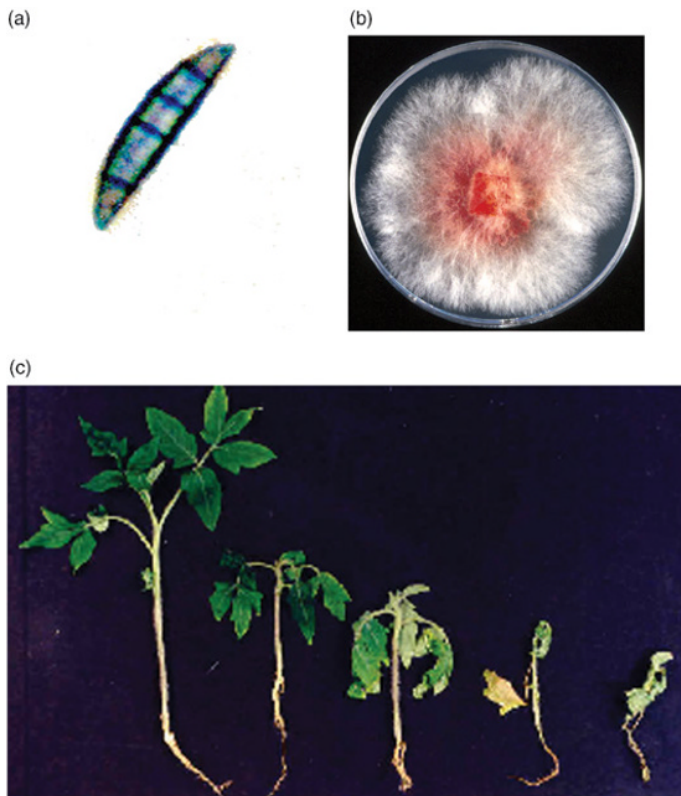


Plate 14.6 *Fusarium* wilt. Microscopic analysis of typical asexual macroconidia (a) and mycelial growth on potato dextrose agar (b) of *Fusarium* sp. (*F. oxysporum* also produces microconidia and chlamydospores). (c) *Fusarium oxysporum* f. sp. *lycopersici* wilt of tomato: symptoms range from healthy (left) to severe wilting and stunting (right). (Courtesy of Dr Antonio Di Pietro, Cordoba, Spain: Di Petro *et al.*, 2003.)

Plate 14.7 Yellowing/browning of leaves of an elm branch due to Dutch elm disease. The entire tree subsequently wilted and died.

Plate 14.8 *Phytophthora infestans* late blight of potatoes. Microscopic analysis of asexual sporangia containing zoospores (a) and sexual oospore (b). Disease symptoms: water-soaked dark lesions on leaves (c) and diseased tubers exhibit brown/black blotches on their surface, and internally they exhibit water-soaked dark brown rotted tissue (d).

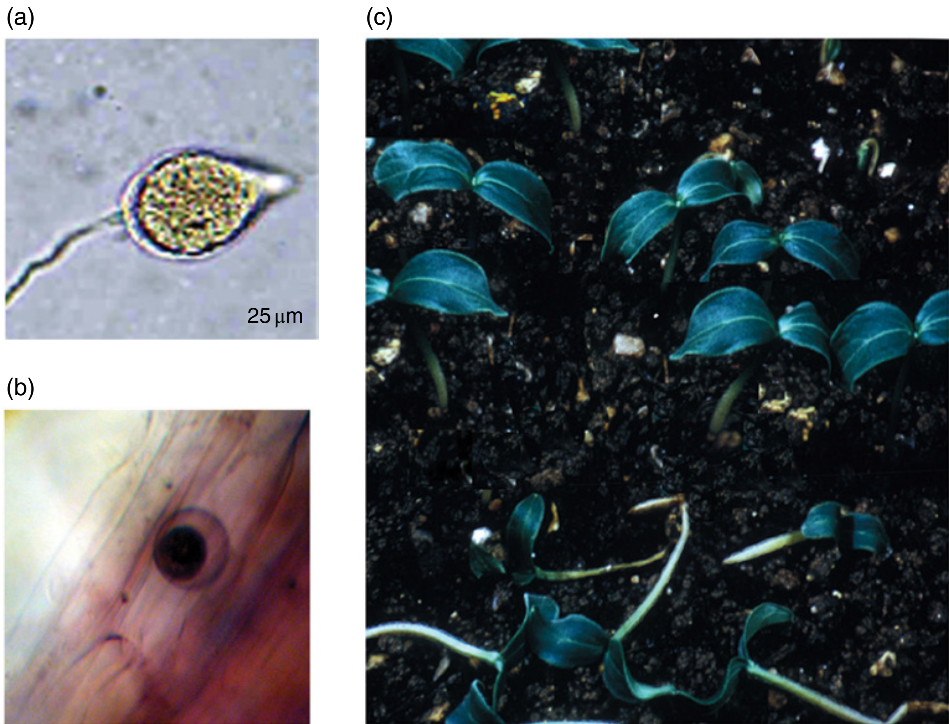


Plate 14.9 *Pythium* damping-off disease. (a) Asexual sporangia containing zoospores; (b) sexually produced oospore formed on the surface of a tomato seedling; (c) seedlings of cress that are healthy (above) and others exhibiting damping-off symptoms (below).

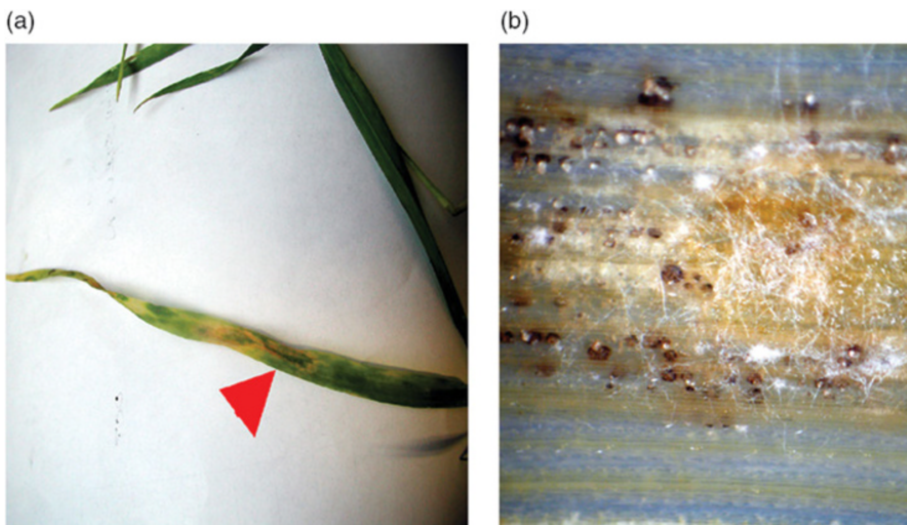


Plate 14.10 *Septoria* leaf blotch of wheat caused by *Mycosphaerella graminicola*. (a) Oval disease lesions (surrounded by chlorotic halo) on an infected leaf, running parallel to leaf blade; (b) black pycnidia develop on mature lesions and cirri may form on lesions if weather is dry for prolonged periods.

Plate 14.11 Sunken lesions on broad bean pod due to anthracnose disease caused by *Colletotrichum lindemuthianum*.

Plate 14.12 Black stem rust of mature wheat stems caused by *Puccinia graminis* f. sp. *tritici*. The three stems possess black pustules of teliospores; these pustules arise from red urediospore pustules that erupt through the stem epidermis earlier in the growing season.

Plate 14.13 Smut diseases of cereals. Covered smut of oats caused by *Ustilago hordei* (a) and covered smut or "bunt" of wheat caused by *Tilletia* sp. (b). Grains are replaced with black spore masses and hence destroyed.

Plate 14.14 Powdery mildew disease of wheat caused by *Blumeria* (= *Erysiphe*) *graminis*.

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