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The Biochemistry and Molecular Biology of Lipid Accumulation in Oleaginous Microorganisms

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I. Introduction

The concept of using microorganisms as sources of oils and fats has a long history. The commercial opportunities of such processes have been continuously examined for nearly a 100 years, though today such opportunities are confined to the production of the very highest valued

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oils—those containing nutritionally important polyunsaturated fatty acids. It has also long been known that some microorganisms have a greater propensity to accumulate substantial amounts of oil, sometimes up to and even in excess of 70% of their biomass weight, while other microorganisms remain stubbornly slim even when given the greatest encouragement to become obese. However, the reasons for microbial obesity, to adapt the common parlance for excessive lipid accumulation within an organism, have remained obscure until relatively recently. This review has therefore been undertaken to describe the work that has been carried out, mainly in the authors' laboratory, to elucidate this phenomenon: Why can some organisms accumulate lipids to a considerable extent and others cannot? There has to be a biochemical explanation for this difference. Knowing this reason should then lead to an understanding of the genetic constitution of the lipid-accumulating organisms and a determination of which genes are important for the process.

There is, though, a subsidiary problem that, perhaps, is even more intriguing than understanding the basic mechanism for lipid accumulation itself. Even among lipid-accumulating microorganisms there is a considerable difference between the extent to which lipid might accumulate to a maximal level. What determines these differences? Why should some microorganisms have an apparent limit to the accumulation of oil of, say, 30% of their biomass, whereas other organisms, even closely related ones, accumulate 50% oil and still others go even higher. Uncovering a biochemical explanation for these differences should then lead to elucidation of the genetical basis of microbial obesity.

We hope, therefore, in this review to present evidence that allows us to establish working hypotheses to explain the process of lipid accumulation, as well as the factors governing the extent to which this can take place, in both biochemical and molecular biological terms. We believe these explanations will also be applicable outside the microorganisms and may provide useful insights into the possible mechanisms of lipid accumulation in plant oilseeds and maybe even in animal cells as well. The questions that we addressed over many years of research are central to lipid accumulation processes in all cells even though the vast majority of our work has been confined to microbial systems. Ultimately, by being able to identify, first, the biochemical reasons for lipid accumulation and then the genes coding for the key enzymes, whatever they may turn out to be, we hope that it will become possible to modulate lipid accumulation processes. It should be possible to increase the amount of lipid that a cell might accumulate, but equally, it should also be possible to reverse this and curtail lipid accumulation. The first would be advantageous if the lipid itself were the product; the second would be

desirable if lipid represented a wasteful drain of carbon away from some other more valuable product as might happen during the production of secondary metabolites (see, for example, Jacklin *et al.,* 2000).

Because the study of lipid accumulation has a long historical record, we begin this review by a brief synopsis of the background to this subject. We then go on to describe, again fairly briefly, some of the more recent attempts to produce commercially useful microbial oils, now euphemistically known as *Single Cell Oils.* This then provides the background to the biochemical explanation of how it all happens. Finally, we indicate how the biochemistry of the process has helped show how the whole process of lipid biosynthesis is a closely integrated series of reactions.

II. The Development of Single Cell Oils

The study of microbial lipids has a long history going back to the mid-1870s (Ficinus, 1873; Nageli and Loew, 1878; see also Ratledge, 1984, 1992). Considerations for using microbial oils as sources of commodity oils and fats were made throughout most of the last century, with serious efforts being made in Germany during both world wars to develop processes that would provide useful amounts of oils and fats for a country denied access to major supplies of such commodities. Not surprisingly, major advances in identifying appropriate lipid-producing organisms took place in Germany from about 1920 to 1945 (Bernhauer, 1943; Bernhauer and Rauch, 1948; Hesse, 1949). Interest in other countries, including the United States and the United Kingdom, in the possible commercial aspects of developing microbial oils was though evident right up to the end of the 1950s (see Woodbine, 1959, for an authoritative review of the work that was done in the first half of the twentieth century). However, the considerable developments in agriculture that took place after 1945 meant that very cheap supplies not only of oils and fats could be assured but also for all other food sources as well. Consequently, it was realized that oils derived from microorganisms would never be able to compete in terms of price with the bulk commodity oils, such as soybean oil, sunflower oil, and more recently, rapeseed (or canola) oil. Interest in developing biotechnological processes for microbial oil production then virtually ceased, being considered a complete waste of time.

In the early 1960s, however, considerable interest was awakened by the prospects of producing protein by growing selected yeasts on cheap alkane feedstocks derived from petroleum refineries. The era of Single Cell Protein (SCP) arrived (see, for example, Rose, 1979). The concept

was taken up by many of the major oil companies as a simple means of producing a cheap animal feed, and its use for human consumption was not ruled out. SCP production was not only an innovative idea but it also led to major developments in bioprocess technology, with fermentation units up to 5 × 500 m^3 being developed, and for the conversion of natural gas (methane) or methanol derived from it into SCP, single fermenters of up to 1500 m^3 were eventually built.

It was then suggested (Ratledge, 1976, 1978) that, if SCP was an economic proposition with a value of no more than $300/tonne, then oils from microorganisms, which became known as Single Cell Oils (SCO) (Ratledge, 1976), could be an equally attractive commercial proposition. Unfortunately, historical events overtook both SCP and SCO processes, which led to major reconsiderations of their value. What had not been appreciated at the time of developing SCP processes was that agricultural developments would be enormous during the second half of the twentieth century. Greater yields of crops, with improved varieties of plants together with better agricultural technologies, drove down the price of major sources of animal feed materials, such as soybean meal, so that the prices in real terms hardly rose at all between 1960 and 1990. SCP processes became even more uneconomic when Organization of Petroleum Exporting Countries (OPEC) countries increased the world price of oil in the 1970s, thereby escalating the price of the very feedstock to be used in the these processes. Little has changed in the intervening years, and consequently there are no currently used SCP processes based on using alkanes as a feedstock.

With microbial oils there was, however, an alternative strategy. Although it was always evident that microbial oils could never compete commercially with the major commodity plant oils, there were commercially opportunities for the production of some of the higher valued oils. Although protein is always just "protein," with only minor variations in its nutritional qualities from different sources, oils are not just "oils." The price range of oils can vary enormously and the price of individual fatty acids that go to make up the oils can vary from as little as $0.30/kg to over $100/kg (Gunstone, 1997, 2001). If microorganisms could be identified that could produce some of the highest valued oils, then commercial development of them would still be a reality. And this is exactly what has happened to SCOs which, since the early 1980s, have moved increasingly toward the very highest valued materials, materials that are expensive simply because no abundant source of them currently exists.

Table I illustrates the wide range of fatty acids found in microorganisms, mostly eukaryotic species that produce triacylglycerol oils (see Fig. 1) and thus can be directly compared, in terms of their chemical

TABLE I

LIPID CONTENTS AND FATTY ACID PROFILES OF SOME OLEAGINOUS, HETEROPTROPHIC MICROORGANISMS USED, OR CONSIDERED FOR USE, AS SOURCES OF SCOs[a]

	Lipid (% w/w)	Major fatty acid residues (rel. % w/w)											
		14:0	16:0	16:1	18:0	18:1	18:2	18:3 (n-3)	18:3 (n-6)	20:4 (n-6)	20:5 (n-3)	22:6 (n-3)	Others
Yeasts													
Cryptococcus curvatus	58	—	32	—	15	44	8	—	—	—	—	—	
Lipomyces starkeyi	63	—	34	6	5	51	3	—	—	—	—	—	
Rhodosporidium toruloides	66	—	18	3	3	66	—	—	—	—	—	—	23:0 (3%) 24:0 (6%)
Rhodotorula glutinis	72	—	37	1	3	47	8	—	—	—	—	—	
Rhodotorula graminis	36	—	30	2	12	36	15	4	—	—	—	—	
Yarrowia lipolytica	36	—	11	6	1	28	51	1	—	—	—	—	
Molds													
Entomophthora coronata	43	31	9	—	2	14	2	—	4	4	—	—	20:1 (13%) 22:1 (8%)
Cunninghamella japonica	60	—	16	—	14	48	14	—	8	—	—	—	
Mortierella alpina	50	—	19	—	8	28	9	—	8	21	—	—	20:3 (7%)
Mucor circinelloides	25	—	22	1	5	38	10	—	15	—	—	—	
Pythium ultimum	48	7	15	—	2	20	16	1	—	11	14	—	20:1 (5%)
Algae(grown heterotrophically)													
Crypthecodinium cohnii	40	16	16	1	—	21	1	—	—	—	—	40	
Schizochytrium limacinum[b]	50	4	56	—	1	—	—	—	—	—	—	30	15:0 (2%) 22:5 (n-6) (6%)
Thraustochytrium aureum	15	3	8	—	—	16	—	—	—	3	—	52	

[a] Data mainly from Ratledge (1997, 2001).
[b] From Yokochi *et al.* (1998).

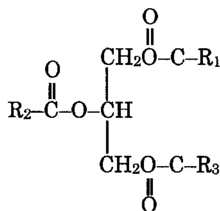

FIG. 1. Structure of a triacylglycerol and the nomenclature used to note the various fatty acids, where R_1CO—, R_2CO—, and R_3CO— are long acyl chains that may be either saturated, mono-unsaturated, diunsaturated, or polyunsaturated. *Nomenclature* of fatty acids is usually given in the from of *x*:*y*, where *x* denotes the number of C atoms and *y* the number of double bonds. As the double bonds in polyunsaturated fatty acids ($y = 3$ or more) are usually methylene interrupted (i.e., —CH:CH—CH_2—CH:CH—), it is only necessary to specify the position of the final bond in a chain. This is normally using the n-a (or ω-a) system where "a" denotes the number of C atoms from the methyl end of the chain to the position of the last double bond. Thus 18:3 (n-3) denotes a C_{18} chain with three double bonds at positions 9, 12, and 15 (counting the carboxyl group as no. 1) so that the final bond (between C15 and C16) is n-3 from the end. The alternative is to specify the position of each double bonds individually, i.e., 18:3(9, 12, 15). Unless stated otherwise, all double bonds may be arranged to be in the *cis* (or *Z*) configuration. (For further details and information, see Ratledge and Wilkinson, 1988.)

composition, to the oils and fats obtained from plant oilseeds. Another major consideration in evaluating the potential of a microorganism for oil production is the amount of oil it can produce. Obviously the more oil a microbial cell can accumulate, the more attractive it will be from a commercial viewpoint. Both the quality and quantity of the oil varied from organism to organism. Reviews on microbial lipids, and particularly on yeast lipids, divided species into high oil producers and low oil producers (Rattray *et al.*, 1975; Rattray, 1989; Ratledge and Evans, 1989). Some yeasts, such as *Saccharomyces cerevisiae* or *Candida utilis,* never accumulated much above 10% of their cell mass as lipid, but other yeasts, such as species of *Rhodotorula* and *Lipomyces,* could accumulate 70% and even more of their biomass as lipid. Moreover, the majority of this lipid was in the triacylglycerol form (Rattray, 1989), and therefore equivalent in chemical composition to the commercial oils and fats. Those microorganisms that could accumulate lipid to more than about 20% of their biomass (this was an arbitary, though useful, cutoff point dividing the accumulators from the nonaccumulators) were termed the *oleaginous* species (Thorpe and Ratledge, 1972), an epithet that seems to have stuck in just the same way that SCOs appears to have stayed in common use. [It should be pointed out, though, that the word *oleaginous* was first used in the 17th century (Oxford English Dictionary) and is not a neologism.]

III. Microorganisms as Sources of High-Valued Oils

A. Yeast Oils as Possible Cocoa Butter Equivalent Material

In the quest for microbial oils that could be produced economically, attention has increasingly focused on the highest valued materials. In the 1980s, selected yeasts were used to produce a cocoa butter equivalent (CBE) (see Table II)—that is, a triacylglycerol with equal amounts of stearate, oleate, and palmitate esterified to glycerol (see Smith, 2001). The research was undertaken principally in the Netherlands and New Zealand, but also in the United Kingdom and Canada (Moreton, 1988; Beaven *et al.,* 1992; Davies, 1992; Smit *et al.,* 1992), and used a variety of strategies to increase the amount of stearate in the yeast lipid as this fatty acid was normally less than 10% of the total fatty acids (see Table II). The most successful strategy used a mutant in which the Δ9 desaturase for the conversion of stearate to oleate (see also Fig. 2) was partially blocked so that stearate accumulated at the expense of oleate. The ensuing lipid then had the correct properties for its use as a CBE (Davies, 1992; Davies and Holdsworth, 1992). Unfortunately, during the time that it took to carry out this research the world price of cocoa butter, and to which of course the price of a CBE is related, fell from over $8000/tonne to less than $2500. The margin for profit from the yeast process then vanished.

TABLE II

Profile of Fatty Acid Composition of Yeast SCOs Used as Sources of CBE Fats and Compared to Cocoa Butter Itself[a]

	Relative fatty acyl composition (% w/w)				
	16:0	18:0	18:1	18:2	18:3
Yeast isolate K7-4[b]	20	24	40	7	2
Rhodosporidium toruloides[c]	28	7	40	18	5
Rhodosporidium toruloides[c,d]	20	47	22	5	2
Cryptococcus curvatus F33.10[e]	24	31	30	6	—
Cocoa butter	28	35	35	2	—

[a] (For further information, see Ratledge, 1994, 1997).
[b] From Davies and Holdsworth (1992).
[c] From Moreton (1988).
[d] With Δ9 and Δ12 cyclopropene $C_{18:1}$ fatty acids added each at 0.3 mg/liter to inhibit the Δ9 and Δ12 desaturases.
[e] From Verwoert *et al.* (1989): this is a hybrid yeast derived from an auxotrophic mutant with a diminished activity of the Δ9 desaturase (see Fig. 2).

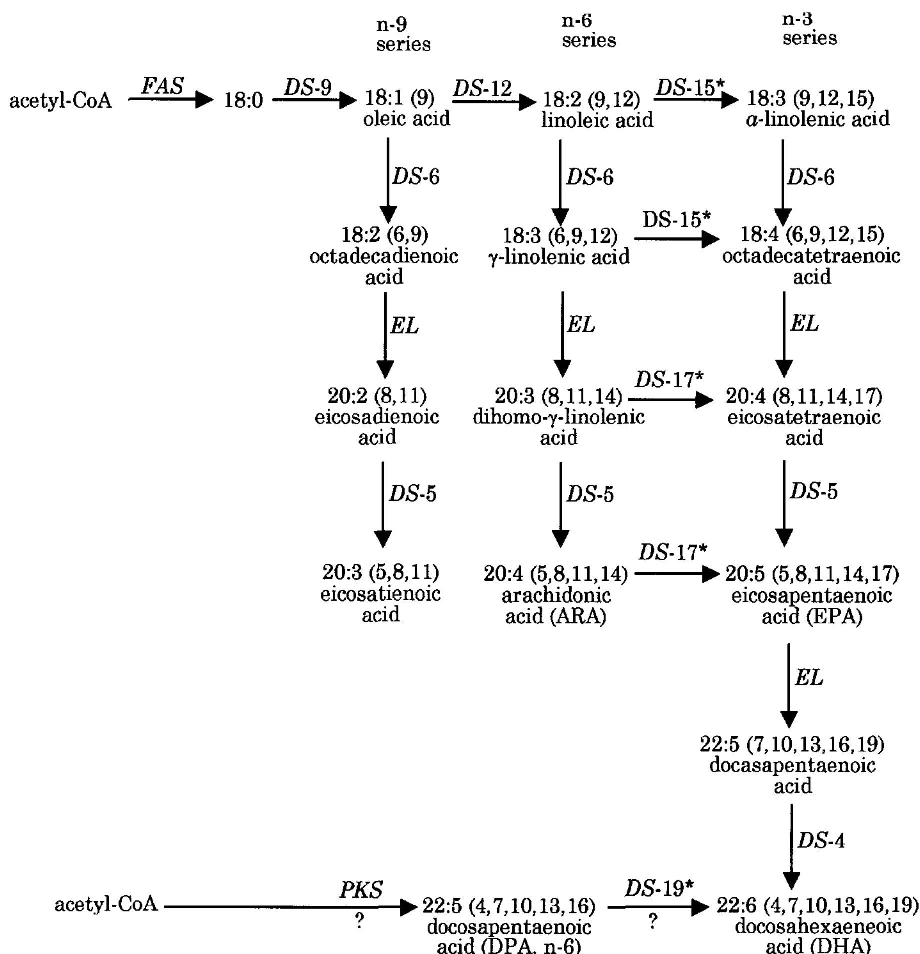

FIG. 2. Routes of polyunsaturated fatty acid biosynthesis in fungi, microalgae, and thraustochytrids (Updated from Ratledge, 2001). FAS: Fatty acid synthase; PKS: polyketide synthase. DS: A desaturase operating the carbon atom indicated (thus DS-15 is the Δ15 desaturase introducing a double bond between carbons 15 and 16 in the fatty acyl group). EL: An elongase; this is a four-component system involving a condensing enzyme, a reductase, followed by a dehydratase and a further reductase. Asterisks: These could all be regarded as n-3 desaturases—that is, introducing a double bond between the n-3 and n-2 carbon atoms. Question mark: Uncertain sequence in thraustocytrids leading to formation of DPA (n-6) and DHA. The synthesis of these fatty acids may not occur via the usual FAS route but by a complete separate PKS (see Metz *et al.*, 2001).

Nevertheless, the process know-how for the production of a CBE–SCO (i.e., a cocoa butter equivalent–single cell oil) as developed in New Zealand and the Netherlands (Davies, 1992; Smit *et al.,* 1992) is still valid today. The process is currently not considered to be economic even though the feedstock used for the growth of the yeast is whey, which is essentially zero cost. The fermentable substrate within the whey was lactose and the whey itself was derived from cheese creameries that, in New Zealand, posed environmental problems for its disposal. Even under these conditions, the operating costs of the fermentation plant coupled with the costs of oil extraction and refinement (which are still needed with microbial SCOs) were higher than could be borne by the final selling price that was dictated by the world price of cocoa butter itself (see Davies, 1992). Interestingly, it is predicted that by 2004, there is likely to be a shortfall in cocoa beans (and therefore of cocoa butter itself) of some 250,000 tonnes (Smith, 2001). The prospects therefore of the price of a CBE rising considerably in the next three years cannot be ruled out. Opportunities for a profitable CBE–SCO process may once more arise.

B. Possibilities for Producing Polyunsaturated Fatty Acids

Since the demise of the CBE–SCO process, interests in the commercial development of SCOs have concentrated almost exclusively on the newly developing market for polyunsaturated fatty acids. This area has been recently extensively reviewed (Certik and Shimizu, 1999; Ratledge, 2001), and readers who require details of the current approaches being taken to produce these materials are referred to one or both of these reviews for details that may not be provided here.

Polyunsaturated fatty acids (PUFAs), whose pathways of synthesis are given in Figure 2, are currently in increasing demand as dietary supplements, loosely termed *nutraceuticals,* for both adults as well as infants. Nutritional recommendations from a number of authoritative sources advise for the inclusion of PUFAs, especially the longer chained and more unsaturated fatty acids, in the diet for the prevention of coronary heart problems and also for the improvement of retinal and brain functions (Huang and Sinclair, 1998). At present, the nonmicrobial sources of these materials are from marine sources including many endangered species of fish. Thus, there is now a major activity in producing some of these PUFAs from alternate microbial sources.

1. γ-Linolenic Acid (18:3n-6)

The first PUFA–SCO that was produced commercially was γ-linolenic acid, 18:3n-6 (for the notation used to describe fatty acids, see Fig. 1).

γ-Linolenic acid (GLA) is found in a relatively small number of plant seed oils, principal among which is evening primrose oil, where it constitutes only about 8–10% of the total fatty acids. Evening primrose oil commands a price of about $15/kg. Borage oil, which contains 20–23% GLA, sells at about $35/kg (Clough, 2001). Both these oils sell as over-the-counter nutraceuticals in the United Kingdom and Europe for the relief or treatment of a number of minor complaints and problems, of which the relief of premenstrual tension is a major claim. It is also prescribed for the treatment of eczema, especially in children where it appears to be particularly effective (Huang and Ziboh, 2001). Development of a biotechnological route to produce an equivalent SCO rich in GLA has been described in detail elsewhere (Ratledge, 1992). This process, which was in commercial production in the United Kingdom from 1985 to 1990, and therefore was the world's first SCO to be offered for sale, used the filamentous fungus, *Mucor circinelloides.* The fatty acyl composition of the oil from this organism is given in Table I. The oil was given a clean bill of health and approved for human consumption as the fungus itself has long been associated with oriental food materials and therefore has a record of safe ingestion over several millennia.

Although the fungal oil contained about twice the concentration of GLA as did evening primrose oil, it experienced, perhaps not surprisingly, some marketing problems. Moreover, the price of evening primrose was deliberately decreased so as to become more competitive and, simultaneously, borage oil (also known as starflower oil) was developed as an improved plant source of GLA. "Oil of Javanicus," as the GLA–SCO was known, was thus forced out of the market by price reductions in the material it sought to replace and by the arrival of a cheaper product on the market. Should there come a demand for GLA at a high purity, then the fungal oil probably represents the best source of starting material as purification of GLA is easiest starting with an oil with a low content of other PUFAs that will then not interfere with the isolation of GLA. Both borage oil and evening primrose oil contain relatively high contents of linoleic acid (18:2)—40 and 70%, respectively—but this fatty acid is much less in the *Mucor* oil (see Table I). However, a commercial demand for a high-purity GLA material has not yet arisen.

2. Arachidonic Acid (20:4n-6)

After GLA, arachidonic acid (ARA) (20:4n-6) was the next PUFA–SCO to be developed and processes for its production continue today. ARA is incorporated into infant feed formula where it is considered a desirable component to be added along with docosahexaenoic acid (DHA)—see below. Traditional sources of ARA are egg yolks and animal livers. The former source yields various phospholipids rich in ARA that

are then used as such while the latter source, although containing ARA as a triacylglycerol, is not acceptable vegetarians. Microbial sources of ARA being actively developed have mainly used *Mortierella alpina* as the best producing organism. Currently, large scale processes exist in both Japan and Europe for its production. Although *Mortierella alpina* is related to *Mucor,* it has not been recorded as having a long term association with any oriential foodstuff as had *Mucor;* so a lot of work has been done to demonstrate its safety (summarized by Streekstra, 1997; see also Kyle, 1997a, 1997b). Approval for the use of this ARA–SCO has recently been given by the Food and Drug Authority (FDA) of the United States (Anonymous, 2001; see also FDA net link: www.cfsan.fda.gov/~rdb/opa-g041.html).

3. *Eicosapentaenoic Acid (20:5n-3) and Docosahexaenoic Acid (22:6n-3)*

Other PUFAs that are being produced by, or are capable of production by, microorganisms are eicosapentaenoic acid (EPA) (20:5n-3) and docosahexaenoic acid (DHA) (22:6n-3). These two PUFAs occur together in the oils of many fish, and recommendations for their use as dietary supplements for the prevention of cardiac problems in older people have long been advocated. Stocks of many fish species are now dwindling rapidly and the increasing presence of pollutants in the marine environment (many of which are concentrated in the livers of fish that are the major sources of these PUFAs) cause concern for the future and safe supplies of these fatty acids.

Additionally, and in favor of an SCO route to production, is the requirement for DHA to be produced in an oil without the presence of EPA, which is thought to compete against DHA for incorporation into key retinal and neural brain lipids (Gibson *et al.,* 1998). As it is very difficult to remove EPA from the mixture of DHA and EPA in fish oils, this has provided further impetus to develop microorganisms as sources of DHA as species are known that produce this PUFA without the presence of EPA (see Table II).

There are two principal organisms currently used commercially for production of an oil rich in DHA: *Crypthecodinium cohnii* and the thraustochytrid group of marine microorganisms. The former is a marine dinoflagellate that has been known for some time as a DHA producer (Harrington and Holz, 1968), but it is only within the last 10 years or so that it has been developed into a commercial process (Kyle, 1992). The organism is nonphotosynthetic and is therefore grown heterotrophically. The process is operated in the United States by Martek Biosciences Corp. and uses stirred tank fermenters up to 110 m^3 with glucose as the principal feedstock (Kyle, 1996, 2001). A refined triacylglycerol oil is

produced that contains 40% of the fatty acids as DHA. Its approval for incorporation into infant food formula has been given by the FDA (Anonymous, 2001) provided that it is given along with arachidonic acid (see above). The oil is also available in many countries as an over-the-counter nutritional supplement for adults (Becker and Kyle, 1998; Haumann, 1997, 1998).

The thraustochytrid group of marine organisms, originally classified as marine fungi, are now placed into a unique phyllum—*Heterokonta,* within the class of Labyrinthista (Dick, 2001). *Thraustochytrium* spp. and *Schizochytrium* spp. are the principal organisms that have been investigated for DHA production (Bajpai *et al.,* 1991a, 1991b; Barclay, 1991; Kendrick and Ratledge, 1992a; Barclay *et al.,* 1994; Nakahara *et al.,* 1996; Yaguchi *et al.,* 1997; Bowles *et al.,* 1999). In all cases, the oil not only contains DHA (see Table I) but also docosapentaenoic acid, DPA (22:5n-6). Although this particular PUFA is somewhat rare in oils from any source, and it is uncertain by which route it may be synthesized (see Fig. 2), it does not seem to be deletrious to the efficacy of DHA in its incorporation into key membrane lipids of the human body. While thraustochytrid oils are not yet incorporated into infant formula, the whole organism is currently used as a supplement for poultry feeding, which then produce eggs rich in DHA. The presence of DPA in the egg is very much lower than in the original oil (Abril and Barclay, 1998).

Both thrautochytrid and *Crypthecodium* biomass and oils can also be used in fish feeding, particularly to increase the rate of growth of young fish larvae and fry within hatcheries.

Opportunities to develop microorganisms for the production of oils rich in EPA have also been considered (Yongmanitchai and Ward, 1989; Vazhappilly and Chen, 1998; see also Ratledge, 2001) but the market for such oils is uncertain though various claims have recently been made for the efficacy of such materials in the treatment of certain mental disorders including schizophrenia (Fenton *et al.,* 2000; Peet *et al.,* 2000). At the moment, no process for the commercial production of such an oil is in operation, but this may have to be quickly rectified if current reports on the effectiveness of EPA to act against "wasting," which is symptomatic of cancer patients with a poor prognosis, prove to be substantiated in further clinical trials of this PUFA (Tisdale, 1999).

While emphasis for the production of desirable PUFAs has been placed on heterotrophic organisms (*Crypthecodium* and thrautochytrids are heterotrophs), considerable research is underway in many places throughout the world to develop processes to produce DHA and the other desirable fatty acids using photosynthetic algae. However, costs of both building and operating photobioreactors are prohibitively expensive (Borowitzka, 1999) and the prospects of using open lagoons for algae growth are unlikely to meet the stringent safety requirements for

subsequent use of the oils in baby foods or even for consumption by adults. Nevertheless, algae are considered by many proponents to be worthy of serious consideration as sources of PUFA-rich oils (Cohen, 1999). They also can be used for fish feeding and may represent the best sources of a range of nutrients for larvae and fish fry that might not be available from other sources.

IV. Biochemistry of Oleaginicity

For the details of fatty acid biosynthesis in cell systems, almost any standard college biochemistry textbook can be consulted by the erudite reader. In this review we are only concerned with using this information to help elucidate the key questions surrounding the causes of oleaginicity in microorganisms. We do not discuss in any detail the activity and organization of enzymes such as fatty acid synthetase or of acetyl-coenzyme A carboxylase except where these impinge upon other activities that we consider can explain the reasons of oleaginicity in microorganisms.

A. Patterns of Lipid Accumulation

Lipid accumulation in oleaginous microorganisms has long been known to be triggered by a nutrient imbalance in the culture medium. When cells run out of a key nutrient, usually nitrogen, excess carbon substrate continues to be assimilated by the cells and converted into storage fat. This is shown diagrammatically in Figure 3A. This pattern is observed in the lipid-accumulating yeasts and filamentous fungi, though it might not apply in photosynthetic algae, or the heterotrophic algae, *Crypthecodinium cohnii,* nor in thraustochytrids, that are of current interest for PUFA production (see above). In these organisms the growth rate is probably lower than the intrinsic rate of lipid biosynthesis. Cells assimilate carbon quicker than they can convert it into new cells so mechanism for storage the excess carbon is then found by converting it into lipid. A possible scenario for lipid accumulation in these organisms is shown in Figure 3B. This pattern of growth-associated lipid accumulation has also been found with a single strain of an oleaginous yeast, *Cryptococcus terricolus* (Boulton and Ratledge, 1984), but this seems to be an exception among yeasts.

With the "normal" oleaginous yeast or mold, the process of lipid accumulation can also be achieved in continuous culture (see Fig. 3C), where is it necessary to grow the cells at a sufficiently low dilution rate (= growth rate) to allow the cells to assimilate the glucose. The results from continuous cultivation studies clearly indicate that the rate of lipid synthesis is slower than the maximum growth rate.

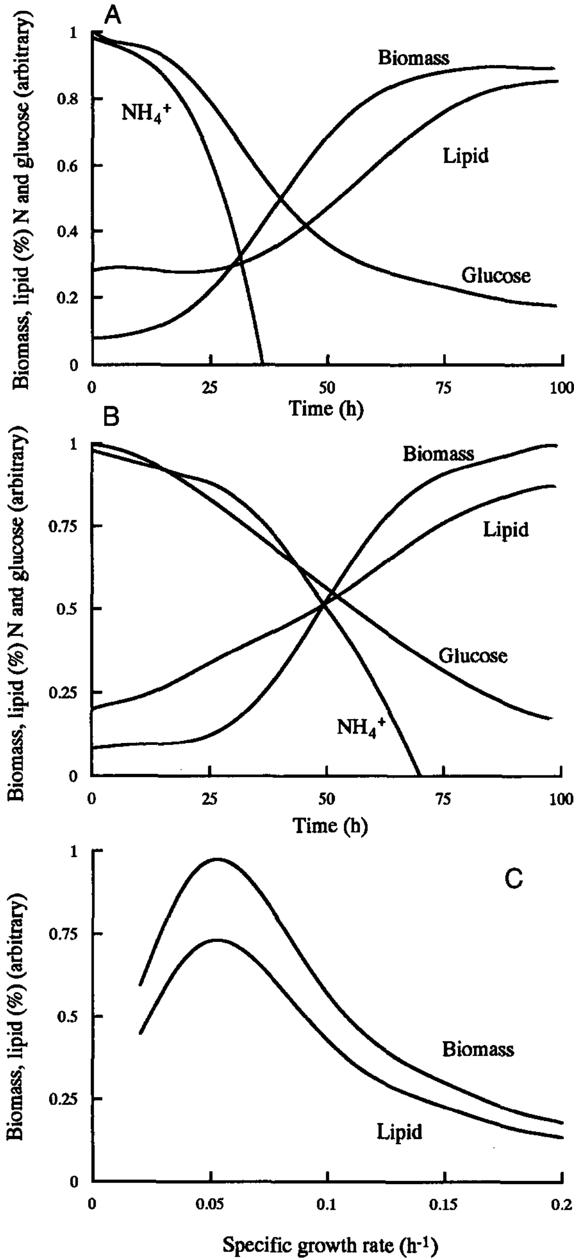

FIG. 3. Schematic representation of the course of lipid accumulation in oleaginous microorganisms. (A) Lipid accumulation in a batch culture system that is typical of oleaginous yeasts and filamentous fungi showing that lipid accumulation does not commence until nitrogen is exhausted from the medium. (B) Pattern of lipid accumulation in a

B. Possible Biochemical Reasons for Oleaginicity

With respect to lipid accumulation in yeasts and fungi, where a nutrient imbalance is need to engender the process, and where the pathway of fatty acid biosynthesis is the same in both oleaginous organism and nonoleaginous organisms (as indeed it is), the obvious question to ask is: What is the biochemical difference between these two groups of very distinct microorganisms?

Our laboratories starting studying this question over 20 years ago. We wanted to understand how two yeasts, placed in exactly the same growth medium, with the same nitrogen limitation after 24 h growth, would result in one accumulating in excess of 40% of its biomass as lipid, while the other would not. The yeasts used in this initial comparison were the nonoleaginous *C. utilis,* otherwise known as the food yeast, and an oleaginous yeast known as *Candida* sp. no. 107. We could have used *S. cerevisiae,* or indeed, any one of about 570 other species of yeast as controls. Only about 25–30 species are known to be capable of lipid accumulation—i.e., are "oleaginous" species (Ratledge and Evans, 1989; Rattray, 1989).

We considered four possible reasons why some yeasts might accumulate lipid (Botham and Ratledge, 1979):

- That upon nitrogen exhaustion from the medium, the nonoleaginous species would cease to assimilate glucose and thus no acetate units would be generated to act as the starting point for fatty acid biosynthesis.
- That acetyl-CoA carboxylase, the first committed reaction of fatty acid biosynthesis [considered by many at that time (Volpe and Vagelos, 1976), and even still today (Ivessa *et al.,* 1997; Davis *et al.,* 2000) to be the rate-limiting step of fatty acid biosynthesis] may be hyperactive in the oleaginous yeast. Alternatively, in the nonoleaginous yeast, this enzyme could be repressed or subject to feedback inhibition by a fatty acyl-CoA ester as the end product of fatty acid synthetase.
- That in the nonoleaginous yeast, there may be a futile cycle of lipid biosynthesis simultaneously accompanied by lipid oxidation so that there would be no net lipid accumulation.

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heterotrophically grown algae such as *Crypthecodium cohnii* or a thraustochytrid. Lipid accumulates during the growth phase and does not depend upon exhaustion of the nitrogen supply. (C) Pattern of lipid accumulation in continuous culture of a yeast or filamentous fungus growing in N-limited medium. Lipid accumulation requires a slow growth rate of the cells to allow the excess carbon to be assimilated faster than it can be converted into biomass so that the surplus carbon is channeled into lipid.

- Intermediary metabolism may be differently regulated in the two types of yeast so that in the oleaginous species there would be an increased flux of carbon into acetyl-CoA, or alternatively in the nonoleaginous species, this flux would be diminished by cellular regulatory processes.

We were able, by appropriate experimentation, to eliminate the first three possibilities: the two yeasts, the oleaginous and the nonoleaginous ones, were more or less the same with respect to glucose assimilation both before and after nitrogen exhaustion from the growth medium; both had equal activities of acetyl-CoA carboxylase, there was no discernable difference in their regulation, and there was no lipid turnover in either of them (Botham and Ratledge, 1979). This left the fourth possibility, which was not as clearly defined as the other options, and therefore a certain amount of guesswork had to take place as to what might be an appropriate experiment to carry out to determine if there were differences in cellular regulation between the two types of yeast.

C. The Role of AMP

Fortunately, at the time of the mid-1970s, the concept of the "energy charge," in which the prevailing cellular concentrations of ATP, ADP, and AMP were computed (Atkinson, 1977) to give a numerical value for the metabolic standing of the cell, was in current vogue. In order to calculate this energy charge, it was necessary to measure the intracellular concentration of the adenine nucleotides in the cell. This was done, and while the calculated energy charge values were different between the two yeasts, the most obvious difference between the yeasts was that in the oleaginous strain the content of AMP fell under N-limited growth conditions to less than 5% of its value under C-limited conditions. In the nonoleaginous yeast, *C. utilis*, the AMP concentration fell by very little (Botham and Ratledge, 1979).

In summary, we were able to see a massive change in the intracellular concentration of AMP during the lipid accumulation stage in the oleaginous yeast. These changes were subsequently confirmed by Boulton and Ratledge (1983b) using chemostat cultures of oleaginous yeasts undergoing a transition from C-limited growth (with N in excess) to N-limited growth (with C now in excess) (see Fig. 4). The AMP concentration in the cells fell abruptly as soon as the cells exhausted the nitrogen supply. This change, in fact, preceded the actual onset of lipid accumulation. N-limitation clearly started a cascade of biochemical events in the oleaginous yeast.

The sharp decrease in AMP concentration was not accompanied by an increase in either ADP or ATP (see Fig. 4B) and AMP deaminase

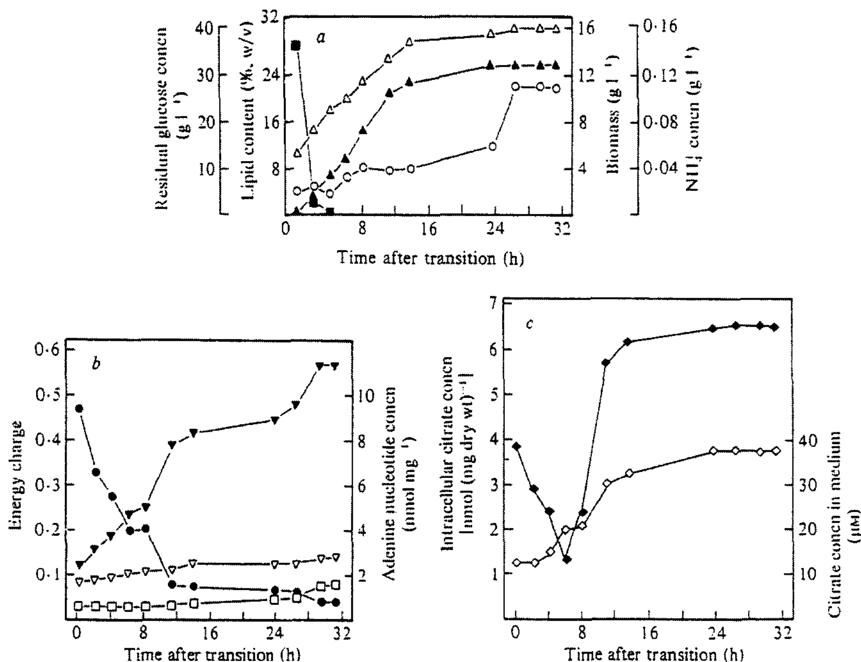


FIG. 4. Pattern of lipid accumulation in the oleaginous yeast, *Lipomyces starkeyi*, during transition from carbon-limited growth to nitrogen-limited growth. The yeast was in steady-state continuous culture growing at a constant rate of 0.06 h^{-1}; at zero time the medium was switched and effectively all residual NH_4^+ was consumed in about 3–4 h; the biomass began to increase immediately from time zero but lipid accumulation did not commence until after 8 h (A) during which time the AMP concentration had dropped by 80% (B) and citrate had begun to accumulate (C). (A) Biomass (△), lipid content of cells (○), concentration of NH_4^+ (■) and glucose (▲) in medium; (B) intracellular concentration of AMP (●); ADP (▽), ATP (□) and energy change (▼); c): intracellular (◆) and extracellular (◇) concentrations of citrate. (From Boulton and Ratledge, 1983a.)

was identified as the enzyme causing this change (Evans and Ratledge, 1985c):

$$AMP \longrightarrow IMP + NH_4 \quad (1)$$

where IMP = inosine monophosphate. *AMP deaminase*, which had been characterized in *S. cerevisiae* (Yoshino *et al.*, 1979; Yoshino and Murakami, 1985), showed a sharp increase in activity in the oleaginous yeast as soon as the cells ran out of available nitrogen in the medium. (How this increase in activity is brought about is still not understood. It could well involve posttranscriptional modification of the enzyme as a result of a change in the intracellular concentration of NH_4^+ or of some key amino acid at the onset of nitrogen exhaustion from the medium. The enzyme can be regarded as an NH_4^+-scavenging enzyme, and therefore its increased activity when cells enter N-limitation could

be viewed as a means of garnering further nitrogen for protein and nucleic acid biosynthesis.) A similar sharp increase in AMP deaminase activity has been noted in *Mucor circinelloides* at the point of nitrogen exhaustion from the medium and the onset of lipid accumulation (Wynn *et al.,* 2001). This may therefore be a common event in oleaginous microorganisms whose growth is limited by nitrogen availability in the medium (Solodovnikova *et al.,* 1998).

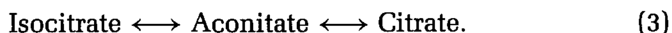
D. Events Leading to the Biosynthesis of Acetyl-CoA

The rapid drop in AMP concentration at the onset of nitrogen limitation profoundly affected the activity of *isocitrate dehydrogenase (ICDH) (NAD⁺-dependent)* within the oleaginous yeast (Botham and Ratledge, 1979). The reaction catalyzed by this enzyme,

$$\text{Isocitrate} + \text{NAD}^+ \longrightarrow \text{2-Oxoglutarate} + \text{NADH}. \quad (2)$$

is within the mitochondria, and in the oleaginous yeasts and molds, its activity is absolutely dependent upon AMP (Botham and Ratledge, 1979; Evans and Ratledge, 1985c; Wynn *et al.,* 2001). However in the non-oleaginous yeast, there was no discernable requirement of ICDH for AMP to be active (Botham and Ratledge, 1979). Related work with a citric acid-accumulating strain of *Candida lipolytica* (Mitsushima *et al.,* 1978) also found that nitrogen-limited growth of this yeast led to low AMP concentrations, a consequent shift-down in ICDH activity with the concomitant accumulation of citric acid in the cells and in the medium.

Nitrogen limitation would lead to an increase in AMP deaminase activity, which would then decrease the prevailing AMP concentration in the cells and the mitochondria (Mitsushima *et al.,* 1978; Bartels and Jensen, 1979), with the consequential drop in ICDH activity. The isocitrate, no longer effectively metabolised via the tricarboxylic acid cycle, would then equilibrate to citrate via the action of aconitase:



$$\text{Isocitrate} \longleftrightarrow \text{Aconitate} \longleftrightarrow \text{Citrate}. \quad (3)$$

Aconitase was found, as expected, to be equally active under both carbon-limited and nitrogen-limited growth conditions (Evans and Ratledge, 1983a; Evans *et al.,* 1983a, 1983b).

What we could not explain was the involvement of citrate in lipid biosynthesis. The obvious enzyme activity to investigate at this stage was the ATP:citrate lyase:

$$\text{Citrate} + \text{ATP} + \text{CoA} \longrightarrow \text{Acetyl-CoA} + \text{Oxaloacetate} + \text{ADP} + \text{P}_i. \quad (4)$$

A review on this enzyme (Srere, 1972) had stated that the enzyme had widespread distribution in most animal cell systems but "was absent

in yeast"! What was meant was that the enzyme was absent in *Saccharomyces cerevisiae*. However, ATP:citrate lyase was duly found in the oleaginous yeast but not in the nonoleaginous species, and was the first major biochemical difference to be identified between the two types of yeast. ATP:citrate lyase is discussed in further detail below as it has proved to be one of the key enzymes that must be present in a eukaryotic microbial cell for it to be able to accumulate substantial amounts of triacylglycerol lipids.

We could now provide a rational explanation as to how there could be metabolic channeling of carbon from glucose directly into fatty acid biosynthesis. Figure 5 shows how this information was used to describe the likely sequence of events in oleaginous yeasts; this metabolic channeling is, though, slightly different in oleaginous fungi (Wynn *et al.*, 2001).

As with yeasts, the process of lipid accumulation in the fungal species (*Mucor circinelloides* and *Mort. alpina*) begins when they run out of assimilatable N in the culture medium, which immediately causes a rapid increase in AMP deaminase activity. This, in turn, affects ICDH activity within the mitochondrion, causing a downturn in the TCA cycle

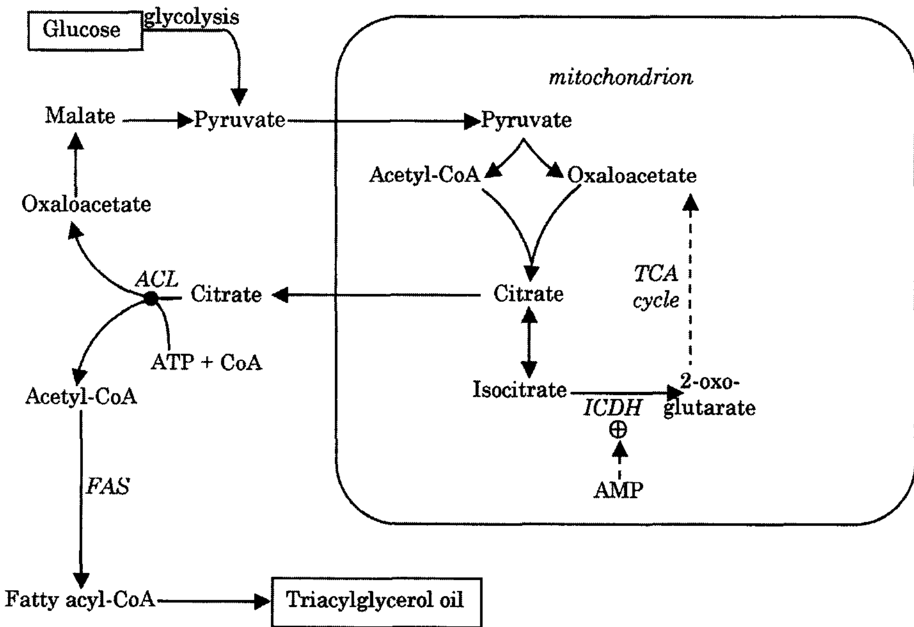


FIG. 5. Outline of the main sequence of events leading to lipid accumulation in oleaginous yeasts and molds. Lipid accumulation is triggered by a sequence of events described in the text. ICDH: isocitrate dehydrogenase (AMP dependent); TCA cycle: tricarboxylic acid cycle; ACL: ATP:citrate lyase; FAS: fatty acid synthase (see also Fig. 7).

activity, which can be detected by the falling output of CO_2 from the cultures. The time from N depletion to detecting a fall in levels of CO_2 was about 15–20 min. However, unlike the metabolic situation in yeasts, in the filamentous fungi the decline in AMP was matched by simultaneous downturns in concentrations of ADP and so that the overall energy charge with the cells remained largely unaltered during this transition into N-limited growth conditions. The key, though, to the initiation of lipid accumulation was considered to be the severe limitation to ICDH activity caused by the decrease in AMP concentration (Wynn *et al.*, 2001).

The following sections discuss the principal enzymes that are considered to be involved in the process of lipogenesis starting from glucose as the carbon source.

E. Glucose Uptake and Glycolysis

Very little is known about the regulation of glucose uptake in oleaginous microorganisms. It is likely to be a tightly regulated process. Work from Kubicek and his colleagues (Arisan-Atac *et al.*, 1996), examining the role of hexokinase in the regulation of glucose uptake into the citric acid-producing *Aspergillus niger*, showed that trehalose-6-phosphate was a major controlling metabolite for the activity of hexokinase. Genetic deletion of trehalose-6-phosphate synthetase led to a 20% increase in the productivity of citric acid accumulation by ensuring that hexokinase operated at its maximum possible activity. With respect to oleaginous yeasts, Botham and Ratledge, (1979) examined the rate of ^{14}C-labeled glucose into *Candida* sp. no. 107 and *Candida utilis*, and showed there was no evidence that glucose transport was a rate-limiting process for the growth of either yeast.

Of the various glycolytic enzymes, most attention has been paid to the possible regulation of *phosphofructokinase* (PFK) (see Fig. 6). Citrate acts as a strong inhibitor of this enzyme in most cells, but NH_4^+ can relieve this inhibition in both yeasts and fungi (Evans and Ratledge, 1984c; Wynn *et al.*, 2001). At physiological concentrations of fructose-6-phosphate and ATP (i.e., 1 mM in each case), the activity of PFK in *Rhodosporidium toruloides* was decreased to zero in presence of 5 mM citrate. However, in the presence of 10 mM NH_4^+ the K_i value for citrate was raised from 0.9 to 7.2 mM. What happens to the glycolytic flux once the nitrogen supply has been exhausted from the medium and the intracellular concentration of NH_4^+ is low? Glucose continues to be taken up by the cells and converted into lipid (see Fig. 3). In accordance with earlier work on this enzyme by Mavis and Stellwagen (1970), it was hypothesized that PFK could form a stable complex with ammonium

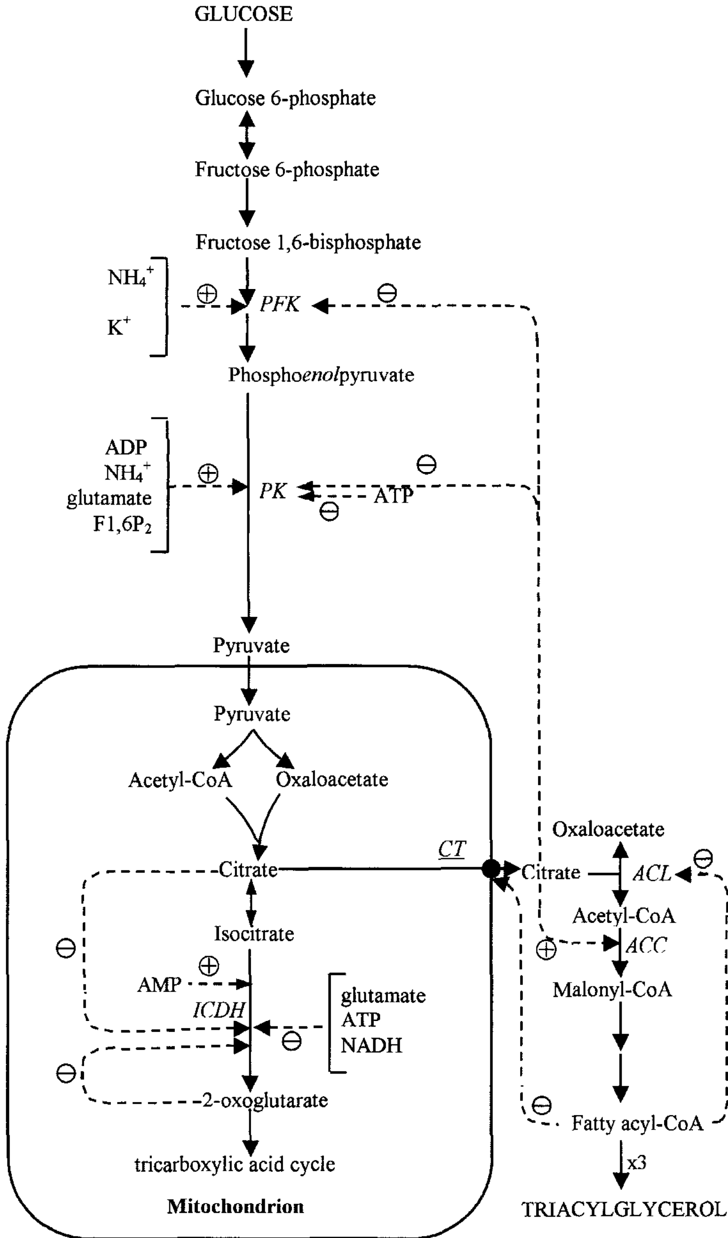


FIG. 6. Regulatory controls exerted by citrate and other metabolites on the flux of carbon to lipid. The key regulatory points appear to be at phosphofructokinase (PFK) pyruvate kinase (PK) and isocitrate dehydrogenase (ICDH). Acetyl-CoA carboxylase (ACC) may also be regulated by citrate. (Adapted from Evans and Ratledge, 1986.)

ions before the onset of nitrogen limitation. This complex was surmised not be susceptible to feedback inhibition by citrate (Evans and Ratledge, 1984c). However, the work of Evans and Ratledge (1984a, 1984b) was able to account for the increased concentration of lipid (up to 50% of the biomass) that accumulated in *Rh. toruloides* when it was grown with glutamate as a nitrogen source rather than ammonium salts (where the lipid content was less than 20% of the cell dry weight) by finding that the intracellular concentration of NH_4 was highest with the glutamate-grown cells and lowest with those grown on ammonium salts. Thus, in the glutamate-grown cells, the activity of PFK was not being controlled by feedback inhibition by citrate to the same extent that it was in the ammonium-grown cells.

Similar results have been recently reported for the feedback inhibition of PFK by citrate in the oleaginous mold, *Mucor circinelloides* (Wynn *et al.,* 2001), and these observations probably parallel those found in other fungi, including the citric acid producing *A. niger* (Roehr *et al.,* 1996). In both cases, it has to be argued that PFK is able to remain active in spite of the obvious increases in the concentration of intracellular citrate both in the oleaginous species and in the citrate producing one. It therefore seems reasonable to argue that PFK can indeed form a stable complex with NH_4, as was suggested above, which then resists the inhibitory effects of citrate.

Although PFK and pyruvate kinase (see below) are considered as the major regulatory enzymes for the control of the glycolytic flux, the over-expression of these enzymes by genetic manipulation (Ruijter *et al.,* 1997) has not led to any significant increase in the enhancement of the flux of carbon, at least in *A. niger,* suggesting that their regulation is more complex than previously thought.

F. The Role of Citrate

The key role of citrate was revealed through the work of Boulton and Ratledge (1983a) during an investigation into the changes in metabolite concentrations during the transition of oleaginous yeasts, *Candida* sp. no. 107 and *Lipomyces starkeyi,* from carbon limitation to nitrogen limitation (see Fig. 4). This work was done in continuous culture where the culture medium was changed abruptly from one that was low (limiting) in glucose, but with NH_4 in excess, to one that was high in glucose but with nitrogen now being the growth-limiting nutrient. One of the key events that occurred during the transition, and that preceded the onset of lipid accumulation, was in the large increase in intracellular citrate concentration (Fig. 4c). Biochemical events must have preceded

the accumulation of citrate itself; moreover, citrate was probably exerting metabolic control over the reactions described in Figure 6.

Besides regulating the activity of PFK (see above), citrate also regulates the activity of *pyruvate kinase* (Evans and Ratledge, 1985a). Like PFK, pyruvate kinase can be activated and inhibited by several metabolites (see Fig. 6). During lipogenesis it is crucial that the overall activity of the enzyme remains high so that a flux of carbon through pyruvate is maintained. Citrate also regulates the activity of several other key events (see Fig. 6 and also Evans and Ratledge, 1985d, where the role of citrate in the control of metabolism in the oleaginous yeast is discussed in detail). The intracellular concentration of citrate is a key determinant in both stimulating enzymes for fatty acid biosynthesis, e.g., acetyl-CoA carboxylase (see Fig. 6), and for down-regulating the activity of the tricarboxylic acid cycle, particularly at the level of isocitrate dehydrogenase (see above), as well as in regulating glycolysis (see Fig. 6).

Citrate must efflux from the mitochondrion, where it is formed, as a necessary prerequisite for its cleavage into acetyl-CoA by ATP:citrate lyase, which is a cytosolic enzyme. This efflux is a carefully controlled system. The *citrate translocase* (Evans *et al.*, 1983a, 1983b, 1983c) sometimes known as the *citrate/malate translocase,* is the translocating protein within the mitochondrial membrane, and involves the participation of malate moving into the mitochondrion as part of a concerted sequence of events. Malate is first synthesized in the cytosol by the action of malate dehydrogenase on oxaloacetate. The initial oxaloacetate to prime the cycle of events comes from the carboxylation of pyruvate. Once the citrate translocase has been primed with malate, then the effluxing citrate provides all the necessary oxaloacetate for the cycle to continue. Figure 7 shows how this process might operate in fungi (Wynn *et al.,* 2001).

In yeasts, the system may be slightly different as it is considered, though not universally accepted, that pyruvate carboxylase (PC) is within the mitochondrion (Evans and Ratledge, 1983a, 1983b) and a variation of Figure 7 therefore has been described (Evans and Ratledge, 1985c, 1985d) that takes account of this different location. The net reaction remains the same and the stoichiometry is unaltered.

PC has been described as a mitochondrial enzyme in certain oleaginous yeasts (Evans and Ratledge, 1983b), as well as be in animal cells (Bottger *et al.,* 1969; Taylor *et al.,* 1978). However, a cytosolic location for it has been reported in both oleaginous and nonoleaginous yeasts (van Urk *et al.,* 1989; Rohde *et al.,* 1991; Sokolov *et al.,* 1995). In filamentous fungi, in spite of an earlier report to the contrary (Purohit and Ratledge, 1988), the prevailing view is that in PC is a cytosolic enzyme

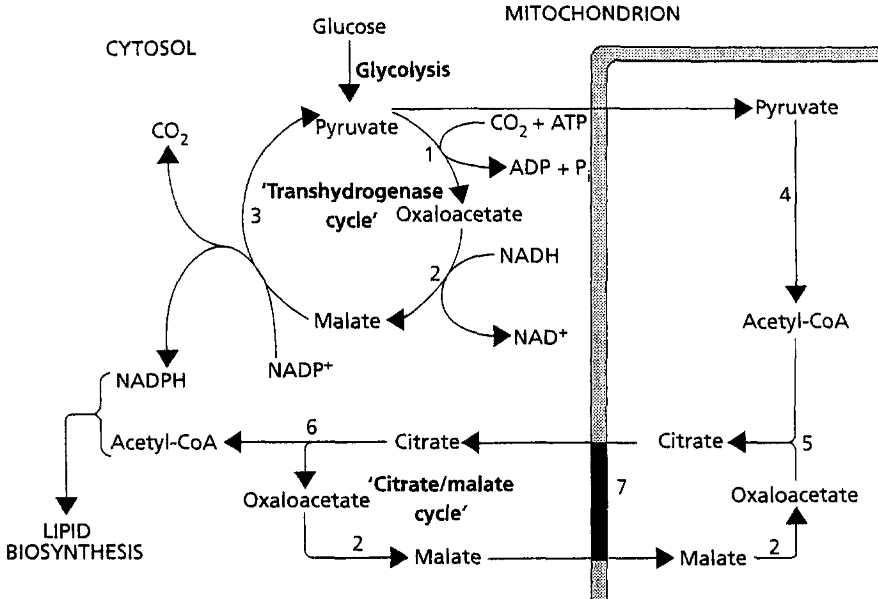


FIG. 7. Scheme showing how the proposed citrate/malate cycle and the cytosolic transhydrogenase cycle could provide sufficient precursors (acetyl-CoA and (NADPH) for lipogenesis in filamentous fungi. Enzymes: 1, pyruvate decarboxylase; 2, malate dehydrogenase; 3, malic enzyme; 4, pyruvate dehydrogenase; 5, citrate synthase; 6, ATP:citrate synthase; 7, citrate/malate translocase Net carbon balance: pyruvate $\longrightarrow$ acetyl-CoA + CO_2. Net reaction for NADPH production (the transhydrogenase cycle): NADH + $NADP^+$ + ATP $\longrightarrow$ NAD^+ + NADPH + ADP + P_i. The transhydrogenase cycle can operate independently of the citrate/malate cycle and provide all the NADPH required both for fatty acid synthesis and for fatty acid desaturation.

(Osmani and Scrutton, 1985; Jaklitsch *et al.*, 1991), and has recently been confirmed for two oleaginous molds, *Mucor circinelloides* and *Mortierella alpina* (Wynn *et al.*, 2001). The scheme shown in Figure 7 is proposed with of PC being considered a cytosolic enzyme.

On the basis of the combined details given in Figures 6 and 7, some stochiometry of the total carbon flux from glucose to triacylglycerol can also be suggested (see also Ratledge, 1997). One mole glucose, when metabolized exclusively via glycolyis, generates two moles pyruvate; thus, it can be calculated that approximately 15 mole glucose are needed to synthesize 1 mole triacylglycerol; i.e., 100 g glucose will provide maximally 32 g lipid, assuming that glucose is not used for the synthesis of any other product—which, of course, it is. Under the best growth condition (i.e., in a chemostat) the highest yields of lipid that have been obtained are 22 g/100 g glucose used (Ratledge, 1988; Ykema *et al.*, 1988; Davies and Holdsworth, 1992).

V. The Key Enzymes of Lipid Accumulation

A. General Considerations

All enzymes within a cell could be regarded as essential for some function or other. But some are more essential than others. Considerable cellular organization must be in place for lipid biosynthesis and storage to occur. However, a simple examination of the various reactions leading from glucose, with its initial uptake into the cell via linkage to hexokinase thereby generating glucose 6-phosphate, right through to triacylglycerol biosynthesis does not immediately indicate which enzymes may be "more essential" than others. If we compare which enzyme activities are present in oleaginous microorganisms and which appear to be absent or different in the nonoleaginous organism, we would consider that two activities stand out as possible candidates for fulfilling important roles in microbial obesity. These are *ATP:citrate lyase* and *malic enzyme.* In addition, it is highly likely that *acetyl-CoA carboxylase* (ACC), the truly first committed enzyme of lipid biosynthesis, is also vitally important for lipid accumulation. But ACC, a ubiquitous enzyme, is found in all cells that generate their lipids from acetyl-CoA. Detailed studies have been carried out on the enzyme from *S. cerevisiae* (see, for example, Ivessa *et al.*, 1997), showing an integration of the enzyme with the endoplasmic reticulum and thereby suggesting a possible route for the product from ACC, malonyl-CoA, to be channeled directly into the fatty acid synthetase itself. If this is the case, then the commitment of acetyl-CoA for its subsequent conversion into fatty acids is absolute; the regulation of acetyl-CoA carboxylase therefore need not be "exceeding complicated" as has been suggested for the enzyme in animals (Allred and Reilly, 1997), as there is no flexibility in the use of the product if the conclusions of Ivessa *et al.* (1997) are correct. Overproduction of ACC activity in *Escherichia coli* led to an increase in the rate of fatty acid biosynthesis (Davis *et al.*, 2000), but even with a 100-fold increase in the content of intracellular malonyl-CoA, there was only a 6-fold increase in the rate of fatty acid biosynthesis. It was therefore concluded that the limitation (bottleneck) to lipid biosynthesis must be later in the pathway. No information was given, though, concerning whether the total lipid content of the genetically modified *E. coli* cells had been increased by increasing the activity of ACC—which is, of course, what this present review is seeking to clarify.

The gene coding for ACC in *Aspergillus nidulans* has been isolated, sequenced, and characterized (Morrice *et al.*, 1998). The enzyme itself is allosterically regulated with citrate being a positive effector. When the activity of the enzyme was inhibited *in vivo* by the fungicide, soraphen A, growth of the fungus was not restored by adding C_{16-18} fatty acids into

the medium, thereby suggesting that ACC maybe fulfilling an additional and essential function besides its involvement in fatty acid biosynthesis.

Other enzymes besides ACC have also been suggested as possible rate-limiting steps in fatty acid biosynthesis. (Indeed, it might be said that almost every single enzyme associated with lipid biosynthesis has been suggested by someone to be the rate-limiting step!) Thus, as an example, Heath and Rock (1996) have suggested that in *E. coli* it is the *condensation reaction* between acetyl-CoA and malonyl-CoA that controls the rate of fatty acid initiation, and therefore the total amount of fatty acid produced. However, up to now, no one has succeeded in substantially increasing the storage lipid content of a cell through genetic modification. This, though, remains a prime goal for many research teams dealing with both microbial and plant systems.

The following two sections give detailed information on two possible candidate enzymes that are, in the opinion of the authors of this review, critical for lipid accumulation to occur in oleaginous microorganisms.

B. ATP:Citrate Lyase

As indicated above, the first major biochemical difference delineated between an oleaginous and a nonoleaginous microorganism was the presence in the former of the citrate cleaving enzyme, ATP:citrate lyase. This enzyme had been known for some time to be of major importance in animal metabolism (Srere, 1972, 1975). There were two earlier reports (Attwood, 1973; Mahlen, 1973) that had shown the presence of this enzyme in, respectively, *Mortierella* spp. and in *Penicillium spiculisporum,* but without drawing the conclusion that the enzyme was essential for oleaginicity. While formation of citrate and its subsequent efflux from the mitochondria of the oleaginous yeast were clearly key events during lipogenesis (Evans *et al.,* 1983a, 1983b, 1983c), the detectable activity of ATP:citrate lyase (ACL) did not vary much between the balanced phase of growth and the lipid accumulation phase (Boulton and Ratledge, 1981a, 1981b, 1983b). Nevertheless, the ability of a yeast to accumulate lipid closely correlated with the possession of ACL (see Table III). In those yeasts without ACL, lipid contents of the cell were invariably low. However, some yeasts had ACL activity but did not accumulate lipid (Ratledge and Gilbert, 1985), thus indicating that other enzyme activities were needed to ensure lipid accumulation (see Table III). Interestingly, there was no correlation between the specific activity of ACL and the amount of lipid that a cell could accumulate (see Table III). In summary, while the possession of ACL activity will not automatically engender lipid accumulation in a microorganism, if the enzyme is absent the cells will be unable to accumulate lipid and

TABLE III

POSSIBLE CORRELATION OF ATP: CITRATE LYASE ACTIVITY WITH HIGH LIPID CONTENTS IN OLEAGINOUS YEASTS[a]

Yeasts	ACL activity (nmol min^{-1} mg $protein^{-1}$)	Lipid content (% dry wt)
Cryptococcus curvatus	7	34
Candida tropicalis	0	4
Candida utilis	0	4
Hansenula saturnus	11	25
Lipomyces lipofer NCYC 944	50	36
Lipomyces lipofer NCYC 692	0	2
Rhodosporidium toruloides CBS 6016	0	3
Rhodosporidium toruloides CBS 5490	42	26
Rhodosporidium toruloides ML 2590	45	4
Rhodosporidium toruloides ML 2921	52	5
Rhodotorula graminis	42	24
Saccharomyces cerevisiae	0	6

[a] From Boulton and Ratledge, 1981, and Ratledge and Gilbert, 1985. All filamentous fungi appear to possess ATP:citrate lyase activity irrespective of their lipid contents (Wynn *et al.*, 1998).

will therefore be nonoleaginous. Clearly, other enzyme activities must be in place to determine the extent to which lipids may accumulate in individual organisms.

In filamentous fungi, ACL appears to be universally distributed (Wynn *et al.*, 1998) irrespective of the lipid contents of these microorganisms. It is only in yeasts that ACL is of variable occurrence.

ACL, surprisingly, has even been found in the citric acid producing fungus, *A. niger* (Pfitzner *et al.*, 1987), but because ACL has a rather low affinity for citrate (2.5 m*M*) (in yeast the K_m value for citrate is 0.07 m*M*—Boulton and Ratledge, 1983a), it was considered that it did not significantly affect citrate accumulation but at the same time did function to supply acetyl-CoA units for lipid biosynthesis. ACL has been purified to homogeneity from the yeast, *Rhodotorula gracilis* (Shashi *et al.*, 1990). It consists of four identical subunits, each about 120 kDa in size, giving a total molecular weight of 520,000. Activity was stimulated by NH_4^+ and inhibited by long-chain acyl-CoA esters, as had been noted earlier by Boulton and Ratledge (1983a) for the enzyme from *Lipomyces starkeyi* where an M_r value of 510 kDa had been calculated for the molecular size of ACL. Evans and Ratledge (1985c) had calculated the molecular size of ACL from *Rh. toruloides* as being 480 kDa; this enzyme was stimulated by NH_4^+ ions at nonsaturating concentrations of citrate (0.1 m*M*) and was inhibited by fatty acyl-CoA esters. These features would then seem to be common among ACL from oleaginous yeasts. The inhibition by

fatty acyl-CoA esters was considered to be a rapid response mechanism to ensure, when the storage lipid of an oleaginous cell began to be broken down, as would occur under starvation conditions, that lipid biosynthesis would be instantly inhibited (Holdsworth *et al.*, 1988; Holdsworth and Ratledge, 1987; Naganuma *et al.*, 1987), thereby preventing both lipid biosynthesis and lipid degradation occurring simultaneously. [As well as inhibiting ACL activity, fatty acyl-CoA esters also inhibit citrate translocase—see Fig. 7 (Evans *et al.*, 1983c)—thereby reinforcing their stringent control over the initial steps leading to acetyl-CoA formation.]

In yeasts ACL consists of four homomeric subunits, each with 120 kDa polypeptides (Shashi *et al.*, 1990), and is similar to the structure of ACL in humans and rats (Elshourbagy *et al.*, 1990, 1992). The inference is, then, that the enzyme in yeasts will be coded for by a single gene, as has been shown to be the case for both human and rats (Elshourbagy *et al.*, 1990, 1992). This does not, though, seem to be the case for the enzyme from filamentous fungi. Both Adams *et al.* (1997, 2002) and Nowrousian *et al.* (2000), working, respectively, with *Aspergillus nidulans* and *Sordaria macrospora*, have found that ACL in these filamentous fungi consists of two different polypeptides. The gene (*acl1*) has been sequenced from *Sor. macrospora* and codes for a 73 kDa subunit of ACL. With *A. nidulans*, ACL has been isolated and purified to homogeneity, and shown to be a hexamer of 371 kDa comprised of three polypeptides, each 55 kDa, and three further ones of 70 kDa (Adams *et al.*, 2002). The gene that codes for the smaller subunit polypeptide remains to be identified and sequenced. In *Sor. macrospora*, ACL may be involved in fruiting body formation, possibly by providing additional fatty acyl-CoA esters which become the trigger for sexual reproduction (Nowrousian *et al.*, 2000). In *Asp. nidulans*, the role of ACL was considered to be for "normal" lipid accumulation (Adams *et al.*, 2002). In summary, in fungi each subunit of ACL is being coded for by separate genes instead of the single one found in yeasts and animals. (Plants are as yet unknown in this respect.)

The complexity of the structure of ACL from all cells suggests that it fulfills a key role in the generation of acetyl-CoA units. One might reasonably expect that its activity should be carefully controlled so that the changing needs of the cell for acetyl-CoA units can be met at all stages of cell growth and development. Indeed, initial work with ACL had suggested that it may be the key regulatory enzyme for lipid biosynthesis in yeast (Boulton and Ratledge, 1981b, 1983a; Evans and Ratledge, 1985c), and may even be the rate-limiting step of lipid biosynthesis in some cases (Boulton and Ratledge, 1981a).

Although no work has been done to increase the activity of ACL in microorganisms, either by gene cloning or by placing the existing gene/s

under different regulatory controllers, the cloning of the gene coding for ACL from rat into plastids of tobacco plant has been carried out (Rangasamy and Ratledge, 2000). Although a functional protein was produced in these genetically modified plants, with a 4-fold increase in ACL activity, there was only a 16% increase in the amount of lipid accumulated in tissue cultures of the modified plants. This would therefore suggest that ACL activity is not a major bottleneck for the production of lipid in this plant, although earlier work (Ratledge *et al.*, 1997) had suggested a positive correlation between ACL activity with lipid accumulation in the developing seeds of *Brassica napus*. However, until someone clones an additional gene for ACL into a microbial cell, we shall not know whether it is the rate-limiting step to fatty acid biosynthesis in microbes. But, as the following section indicates, we would not consider this to be likely.

C. Malic Enzyme

Malic enzyme catalyzes the reaction:

$$\text{L-Malate} + \text{NADP}^+ \longrightarrow \text{Pyruvate} + \text{CO}_2 + \text{NADPH}. \qquad (5)$$

It occurs in a range of fungi and yeasts, though is not ubiquitous. It also occurs in animals and its association with lipogenesis has been suggested for many years (see, for example, Wise and Ball, 1964). The considered view is that malic enzyme is just one of several activities (the others being glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, and NADP-dependent isocitrate dehydrogenase) that generate NADPH, which is used by fatty acid synthetase, or indeed, other enzymes requiring this cofactor. Malic enzyme would then contribute to a general cytosolic pool of NADPH, but would assume prime importance only if a substrate, such as pyruvate or even acetate, were being used instead of glucose (Flatt and Ball, 1964; Wise and Ball, 1964).

In fungi, malic enzyme appears to be of widespread distribution. It has been purified to homogeneity from *Mucor circinelloides* (Song *et al.*, 2001), where it occurs in a number of isoforms (see below). The native enzyme of the principal form has a molecular size of about 160 kDa (Savitha *et al.*, 1997), and is composed of two identical monomers (Song *et al.*, 2001). The function of malic enzyme, however, has never been too clear. Some workers have suggested that its main role is in the metabolism of pyruvate (Zink, 1972; Zink and Katz, 1973; McCullough and Roberts, 1974).

While malic enzyme activity has always been regarded as an essential component for the *transhydrogenase cycle* (see Fig. 7), its absolute requirement for fatty acid biosynthesis and for fatty acid desaturations has only been clear following the initial observations of Kendrick

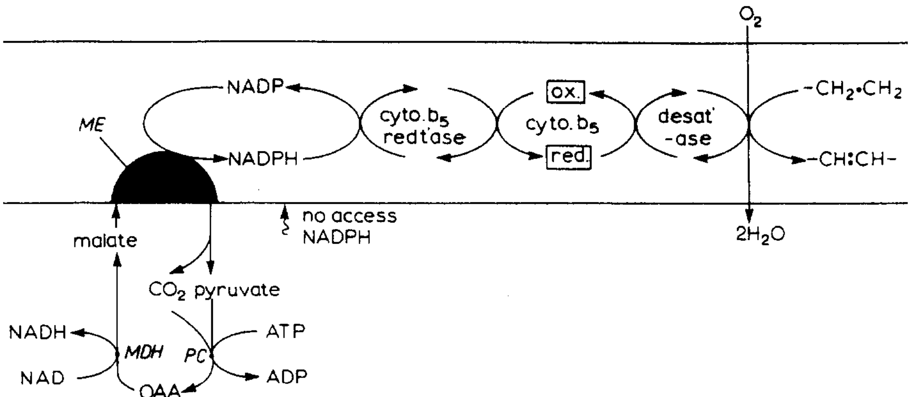


FIG. 8. Proposed structure of the election transfer chain involved in the microsomal membrane desaturation of fatty acids in *Mucor circinelloides*. Desaturation is driven by the provision of exogenous malic acid to the membranes. ME: malic enzyme (malic dehydrogenase decarboxylating-NADP linked); PC: pyruvate carboxylase (which is cytosolic enzyme in this fungus); and MDH: malate dehydrogenase (from Kendrick and Ratledge, 1992b).

and Ratledge (1992b) that the formation of polyunsaturated fatty acids using microsomal (endoplasmic reticulum) membranes from *Mucor circinelloides* could be achieved only when malate was included in the preparations and not NADPH. The added NADPH apparently was not able to access the desaturases within the membranes, but malate was. It appeared that there must be a second malic enzyme within the membrane beside the soluble one that occurred in the cytosol. This membrane-associated malic enzyme was indeed shown to be distinct from the cytosolic form (Kendrick and Ratledge, 1992b), and hypothesized to drive the desaturase reaction through the usual linkages of cytochrome b_5 and cytochrome b_5 reductase (see also Section V). This is shown schematically in Figure 8 (see also Kendrick and Ratledge, 1992c).

The suggestion that NADPH was not able to penetrate membrane preparations of *M. circinelloides,* and thus drive the desaturases directly, was surprising but should not have been in view of the pronounced lipophobic nature of this cofactor. On the other hand, a lipophilic enzyme, as this particular isoform of malic enzyme appeared to be, would certainly be able to penetrate, at least partially, into the membranes where fatty acyl desaturations were taking place. If the enzyme is indeed located as shown in Figure 8, then NADPH could easily be generated in close proximity to cytochrome b_5 reductase; malic enzyme could be even be physically associated with the reducatase, thereby ensuring direct channeling of NAPDH right through the

desaturases itself. Malic enzyme would function by the provision of malate on the "open" (cytoplasmic) side of the enzyme—a "transhydrogenase cycle" (see Fig. 7) would be in operation at this location.

While working on the membrane-bound malic enzyme, we attempted to block its activity by using selective inhibitors. Shimizu *et al.* (1989) had previously found that a nonoil component of sesame seed oil diminished the formation of arachidonic acid (20:4) in growing cultures of *Mortierella alpina.* This compound was identified as a bicyclic aromatic molecule, sesamin, which specifically inhibited the $\Delta 5$ desaturase of the fungus and caused the accumulation of the precursor of arachidonic acid, dihomo-γ-linolenic acid (20:3) (see Fig. 2). Kendrick and Ratledge (1996) and Wynn *et al.* (1997) then went on to test sesamol, which is 3,4-methylene-dioxyphenol, for its effect on fatty acid desaturases in several fungi. Wynn *et al.* (1997) showed that sesamol acted as a highly specific inhibitor of malic enzyme activity in *Mucor circinelloides.* When cultures were grown in its presence (at 3–5 m*M*), the content of lipid in the cells was decreased by almost 90%, from 24% of the cell biomass to 2% but—and this was of major significance—without causing any major effect on growth. Not only was the lipid content of the cells affected by sesamol, but the content of γ-linolenic acid (18:3n-6) dropped from 16% of the total fatty acids to 2%. Thus, as only malic enzyme activity appeared to have been affected by the inhibitor, it was concluded that sesamol was specifically inhibiting both the cytoplamic and membrane-bound malic enzymes, and that without malic enzyme the cell was unable to accumulate lipid or to carry out desaturations of it. The essentiality of malic enzyme for lipid biosynthesis appeared to be established.

Wynn and Ratledge (1997) went on to show that in a mutant of *Asp. nidulans* lacking malic enzyme activity, only half the lipid (12% of the cell dry weight) that had been produced by the competent strain under nitrogen-limited growth conditions was now produced. (Sesamol, for reasons of its impermeability into this fungus, had no effect on malic enzyme activity.) Interestingly, without the metabolic burden of having to synthesize the storage lipid in such large amounts, the mutant devoid of malic enzyme activity now grew slightly better than the parent cells. However, there was no diminution in the linoleic acid content of the oil from the mutant compared to the original culture having malic enzyme activity. Probably, therefore, a membrane-bound form of malic enzyme (see Fig. 8) is not universal among fungi though one has been detected in *Pythium ultimum* (Savitha *et al.,* 1997).

The hypothesis is advanced that only malic enzyme can provide the NADPH that is needed for fatty acid biosynthesis and, therefore, is vital to the process of lipid *accumulation.* If the actvity of the enzyme

is prevented, either by inhibition or by mutation, then lipid accumulaton ceases. Fatty acid biosynthesis per se is still functional and phospholipids can be produced. Thus, the cell can manage without malic enzyme—it is not absolutely vital—but the cell cannot produce storage triacylglycerols in any abundance. Without malic enzyme activity the flux of carbon, from glucose to lipid, is considerably diminished and only essential lipids are produced—presumably by using other sources of NADPH.

The activity of malic enzyme controls the extent of lipid accumulation. Thus any change in its activity, either upward or downward, would then change the extent of lipid accumulation in an oleaginous cell. And so it has proved to be.

Wynn *et al.* (1999) followed the course of lipid accumulation in two filamentous fungi showing different extents of lipid accumulation: *Mucor circinelloides* has a ceiling of approximately 25% lipid of its biomass when grown under nitrogen-limiting conditions whereas *Mortierella alpina* can accumulate about 50% but no more. When the activity of various enzymes were followed throughout growth (see Fig. 9), that of malic enzyme was the only one that paralleled the course of lipid accumulation. Other enzymes examined (but not shown in Fig. 9) included fatty acid synthetase, acetyl-CoA carboxylase, ATP:citrate lyase, and three other enzymes generating NADPH: glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, and the cytolsolic isocitrate dehydrogenase ($NADP^+$ dependent). All these other enzyme activities remained detectable throughout the growth of both fungi. Malic enzyme activity, however, ceased after 40 h growth in *M. circinelloides* and thereupon lipid accumulation also ceased; in *Mort. alpina* malic enzyme activity continued for up to 90 h but, when it could no longer be detected lipid accumulation stopped (see Fig. 9). Only malic enzyme activity correlated with the pattern of lipid accumulation.

A somewhat similar investigation in *Cunninghamella echinulata* (Certik *et al.*, 1999) concluded that the activities of ATP:citrate lyase and acetyl-CoA carboxylase paralleled lipid accumulation and could have caused the eventual cessation of lipid biosynthesis. However, malic enzyme activity also diminished along with these other two enzyme activities. The decrease in all three enzyme activities came *after* lipid accumulation had ceased and were not in parallel with the slowing down of lipid synthesis. This work was carried out in shake-flask cultures where O_2 transfer is always a problem, and this may then lead to changes in gene expression and possible down-regulation of proteins for reasons other than the limitation of lipid accumulation. In the work described above with *M. circinelloides* and *Mort. alpina,* the work had been done in aerated, stirred tank fermenters with appropriate O_2 and pH controls.

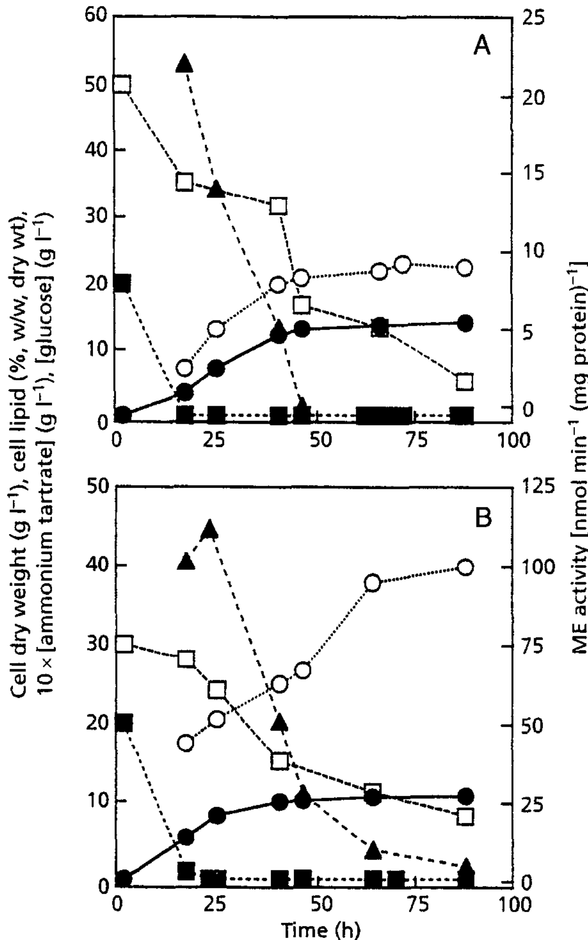


FIG. 9. The correlation of malic enzyme activity with the extent of lipid accumulation in *Mucor circinelloides* (A) and *Mortrerella alpina* (B). Each fungus was grown in a 4 liter stirred fermenter with a low amount of N in the medium but with glucose in excess throughout growth. Cell dry wt (●); cell lipid (○); ammonium tartrate (■), glucose (□) and malic enzyme activity (▲). (From Wynn *et al.*, 1999).

In the two filamentous fungi that we have studied, the cessation of malic enzyme activity is correlated with the onset of nitrogen limitation that is, of course, a necessary prerequisite for lipid accumulation to occur (see Fig. 3). If nitrogen (as ammonium tartrate) was added into the culture of *M. circinelloides* after the cessation of malic enzyme activity, then this resulted in the return of full activity within about 4 h (Wynn *et al.*, 1999). However, if an inhibitor of protein synthesis (cycloheximide) was added along with the extra nitrogen, the reinstatement of

malic enzyme activity was prevented. The conclusion was reached that malic enzyme activity was ceasing because of a down-regulation of the malic enzyme gene following nitrogen exhaustion from the medium.

When the malic enzyme gene has been identified, it should then be possible to place it under a constitutive promoter so that an active malic enzyme will continue to be synthesized after nitrogen depletion. Under such circumstances, it would then be expected that lipid accumulation would be substantially increased. Identification of the malic enzyme gene in *Mucor circinelloides* has been hampered by the identification of multiple forms of the enzyme, some of which are involved in anaerobic metabolism (Song *et al.*, 2001). The form of malic enzyme associated with lipid accumulation appears to occur in two isoforms: one form (Isoform III) is stable and occurs during the balanced growth phase of the organism. Upon the onset of nitrogen limitation, this isoform is converted (by some unknown posttranscriptional mechanism) into a second form (Isoform IV), which is unstable and then begins to lose activity so that eventually all activity is lost and lipid accumulation ceases. The stability of the malic enzyme, or the expression of the gene, determines the length of time over which lipid accumulation occurs and thus controls the ultimate content of lipid within the cell.

Based on knowledge of multiple forms of malic enzyme in *M. circinelloides*, Song *et al.* (2001) provided a biochemical explanation as to why acetate-grown cultures of this fungus accumulate about 35–40% more lipid than do glucose-grown cells (Du Preez *et al.*, 1997). In the acetate-grown cells, malic enzyme activity was consistently about 50% higher than in the glucose-grown cells throughout the lipid accumulation phase. The essential NADPH for fatty acid biosynthesis was thus provided at a higher rate and would explain why lipid accumulation continued for much longer when acetate was the growth substrate. The key isoform of malic enzyme (Isoform IV) apparently was more stable in acetate grown cells.

Interestingly, when sesamol, as the specific inhibitor of malic enzyme activity (see above), was added to cultures of *Fusarium moniliforme*, not only was there a substantial (35%) inhibition of lipid production but there was a simultaneous 20-fold increase in the formation of gibberellin, indicating that acetyl-CoA was being switched from fatty acid biosynthesis into the formation of the secondary metabolite (Jacklin *et al.*, 2000). Thus, it should be possible to control malic enzyme activity not only in a positive direction to increase lipid accumulation but also in a negative direction to divert essential acetyl-CoA units into preferred secondary metabolite products.

The correlation between malic enzyme and lipid accumulation therefore appears as *the* single event that controls the extent of lipid

accumulation in the oleaginous organisms studied. The absolute dependency of fatty acid synthetase for NADPH to be supplied directly from malic enzyme, means that there is no such thing as a "common pool of NADPH" at least as far as fatty acid synthesis is concerned (Wynn *et al.*, 1999). A coherent explanation can now be provided to explain not only the biochemistry of lipid accumulation in oleaginous organisms but also how the physical extent of lipid accumulation may be governed in different cells. This is discussed in Section IX.

VI. Desaturases

A. Background Information

Despite the key role fatty acid desaturases play in the biosynthesis of PUFA, and their regulatory importance in this process, relatively few biochemical studies of desaturases have appeared. The paucity of biochemical analysis of these systems reflects the difficulties these enzymes present for traditional purification/characterization (Lamascolo *et al.*, 1996; Michaelson *et al.*, 1998; Saito and Ochiai, 1999). Fatty acid desaturases are hydrophobic/membrane-bound proteins comprising at least three separate functions to yield the final desaturase activity (cytochrome b_5 reductase, cytochrome b_5 the terminal desaturase, and even perhaps malic enzyme—see Fig. 8). Furthermore, the substrates for these enzymes are either acyl-CoA or acyl residues attached to phospholipids. As the ^{14}C substrates required to assay these enzymes are either very expensive or simply not available commercially, many attempts to assay desaturases use labeled free fatty acids and rely on activating activities in the extracts to convert these into the required substrates for the desaturases.

The study of desaturase activities via the study of deficient mutants is likewise hampered by the fact that the deletion mutants (with the exception of mutants lacking Δ9 desaturase activity) often display no easily selectable phenotype (Das and Sen, 1983; Jareonkitmongkol *et al.*, 1992; Goodrich-Tanrikulu *et al.*, 1994). Often the mutants grow on defined medium as well, or nearly as well, as the wild type. Although the lack of cold tolerance has been used to select mutants deficient in the synthesis of PUFA in some systems (Jareonkitmongkol *et al.*, 1992), more often than not this approach is ineffective and many desaturase mutants have been identified by random screening of mutagenized populations for alterations in fatty acid profile (Jareonkitmongkol *et al.*, 1992). Using a range of techniques, various microorganisms, including cyanobacteria and fungi, deficient in Δ9, Δ12, Δ6, and Δ5 desaturases, have been isolated and studied (Jareonkitmongkol *et al.*, 1992; Certik and Shimizu, 1999).

The greatest success in studying fatty acid desaturases in microbial systems has come about using a molecular genetic approach. Indeed, the genes for all the desaturase activities involved with n-6 PUFA biosynthesis along with the elongases required for the synthesis of long-chain PUFA (LCPUFA), i.e., fatty acids with carbon chain lengths greater than 18, have been cloned. Although $\Delta 15$ (n-3) desaturases have been cloned from plant and animal systems (Kodama *et al.,* 1997; Meesapyodsuk *et al.,* 2000), and many fungi produce n-3 PUFA, the cloning of a microbial $\Delta 15$ desaturase has not been achieved to the authors' knowledge. The cloning of the desaturase genes has been greatly aided by the work of Stukey *et al.* (1990) and Shanklin *et al.* (1994), who identified key features that are common to all fatty acid desaturases. Desaturases from different species, and even different desaturases from a single species, often demonstrate a lack of overall similarity. However, certain key features, with structural and functional significance, are common to all, and are highly conserved between all desaturases. Most notable of these highly conserved areas is the histidine (HIS) boxes (Stukey *et al.,* 1990; Shanklin *et al.,* 1994). Three histidine-rich motifs, with a general structure HXXHH, are present in all desaturases characterized to date, although the exact sequence of the HIS boxes vary to some degree. The HIS boxes are separated by two long hydrophobic regions thought to be involved in anchoring the desaturases to the endoplasmic reticulum. It is envisaged that each hydrophobic region spans the endoplasmic reticulum (ER) membrane twice so that the three HIS boxes are positioned on the cytosolic face of the ER (Stukey *et al.,* 1990) where they are thought to play a crucial catalytic role in Fe^{2+} binding (Shanklin *et al.,* 1990).

The HIS boxes can be used to the design degenerate primers for PCR, to amplify a fragment of the desaturase gene to use as a probe for the cloning of intact desaturase genes from either cDNA or genomic DNA libraries. Likewise, searches of EST sequences for the presence of desaturase clones has often relied on the presence of sequences homologous to HIS box sequences for the identification of desaturase clones.

Despite the general lack of sequence homology between different desaturase genes, the structural similarity of the native proteins has been highlighted by the functional activity of heterologously expressed desaturases. The cross-species functionality of heterologously expressed desaturases applies not only to microbial desaturases but also to microbial desaturases expressed in plants and animals (Polashock *et al.,* 1992; Knutzon *et al.,* 1998; Kelder *et al.,* 2001). Indeed, heterologous expression of putative desaturase genes is one of the standard techniques employed to assign unambiguously a function to a cloned putative desaturase gene.

The heterologous functionality of microbial fatty acid desaturases provides the potential for the use of microbial systems as a reservoir of

genes for desaturases that could be transferred to plants to modify the fatty acid profile of seed oil crops to produce commercially attractive PUFAs that are not naturally synthesized by seed oil crops (Qiu *et al.*, 2001).

B. Δ9 Desaturases

The Δ9 desaturase catalyzes the insertion of the first double bond into saturated fatty acids between carbons 9 and 10 of the fatty acid chain. As such, it can be thought of as the first committed step in PUFA biosynthesis (see Fig. 2). The Δ9 desaturase can operate on either of the predominant saturated fatty acids in microbial cells, palmitate (16:0) and stearate (18:0), to produce palmitoleate (16:1n-7) or oleate (18:1n-9), respectively (Sakurdani *et al.*, 1999a). Although the preference for the Δ9 desaturase for these two potential substrates varies between systems, the Δ9 desaturase from microorganisms appears to utilize 18:0 preferentially (Sakuradani *et al.*, 1999b). The Δ9 desaturase is distinct from the other desaturases in a number of ways: it is the only desaturase that acts on saturated fatty acids and it is the only desaturase that has acyl-CoA rather than phospholipid-bound acyl groups as its substrate. In plant systems, the uniqueness of the Δ9 desaturase is further emphasized in that it is a soluble rather than a membrane-bound enzyme. In fungi, the Δ9 desaturase resembles the mammalian system with the Δ9 desaturase located within the ER. Nevertheless, the Δ9 desaturase from *S. cerevisiae* is functional when heterologously expressed in plant systems (Polashock *et al.*, 1992).

The Δ9 desaturase has been cloned from a number of yeasts and filamentous fungi, and several general features of these enzymes have been established. The gene has a ORF (open reading frame) of approximately 1500 base pairs (bp) and encodes a protein with between 440 and 500 amino acids with a molecular weight slightly greater than 50 kDa (Meesters and Eggink, 1996; Itoh *et al.*, 1998; Sakuradani *et al.*, 1999a; Lu *et al.*, 2000). The gene also encodes a cytochrome b_5 domain at the carboxy terminal of the native enzyme (Meesters *et al.*, 1997; Itoh *et al.*, 1998; Sakuradani *et al.*, 1999a; Wongwathanrat *et al.*, 1999; Lu *et al.*, 2000). This cytochrome b_5 is a prerequisite for activity, demonstrating that the Δ9 desaturase cannot accept electrons from an exogenous cytochrome b_5.

Of all the desaturases, the Δ9 appears to be the most conserved during evolution. The importance of this enzyme is confirmed by mutants lacking this activity, and are unable to grow in the absence of an exogenous supply of unsaturated fatty acids (Das and Sen, 1983; Goodrich-Tanrikulu *et al.*, 1994). Such unsaturated fatty acid auxotrophy is rare in mutants lacking other desaturases (Jareonkitmongkol *et al.*, 1992). The

Δ9 desaturase from *Cryptococcus curvatum* displays a high degree of identity, at the amino acid level, with both that of *S. cerevisiae* (72%) and rat (62%) (Meesters and Eggink, 1996).

Recently, multiple genes for the Δ9 desaturase in the oleaginous fungus, *Morti. alpina,* have been reported (Wongwathanrat *et al.,* 1999). One gene, Δ9-1, appears to be actively expressed in all strains of *Mort. alpina,* while expression and presence of the second gene varies between strains. Both genes encode a similar protein (86% identity) and contain a single intron whose position is conserved. Two clearly different genes encoding a functional Δ9 desaturase also have been cloned from *C. curvatum* (Meester and Eggink, 1996; Meesters *et al.,* 1997). A third Δ9 desaturase gene from *Mort. alpina* has been reported (MacKenzie *et al.,* 2002). Unlike the *ole1* and *ole2* genes (Wongwathanrat *et al.,* 1999), the third desaturase gene failed to complement the Δ9-desaturase (Δ9-3 gene) mutation in *S. cerevisiae.* The desaturase itself had a different substrate specificity than the other two Δ9-desaturases, and functioned only moderately on stearic acid (18:0) and not at all on palmitic acid (16:0). It was suggested that this desaturase may function with very long chain fatty acids (e.g., 26:0) though this remains to be substantiated.

The genetic regulation of the Δ9 desaturases still needs to be studied in detail, but expression of the gene(s) is repressed by exogenous fatty acids that have a Δ9 desaturation (Meesters and Eggink, 1996; Lu *et al.,* 2000). Furthermore, expression of the Δ9 desaturase in *Mucor rouxii* changes during cell growth and upon changes in cultivation temperature (Laoteng *et al.,* 1999), suggesting that the expression of this desaturase is involved in cold acclimation in microorganisms.

C. Δ12 Desaturases

The Δ12 desaturase catalyzes the conversion of oleic acid (18:1n-9), to linoleic acid (18:2n-6) by the insertion of a double bond between carbons 12 and 13 of the mono-unsaturated fatty acid chain. In the yeast, *L. starkeyi,* most of the Δ12 desaturase activity was in the cytosolic fraction after subcellular fractionation (Lomascolo *et al.,* 1996). Whether this is evidence of a truly cytosolic activity or an artifact caused by the disintegration of the endoplasmic reticulum during cell cleavage is uncertain, however. It is most likely that in this yeast, as in other systems, the Δ12 desaturase is a membrane-bound enzyme utilizing a phospholipid-bound 18:1 as its substrate.

The gene for the Δ12 desaturase has been cloned from number of microbial systems and, as with other desturases, the cloned gene is capable of expression and translation into a functional protein in other systems (Wada *et al.,* 1993; Huang *et al.,* 1999; Sakuradani *et al.,* 1999a). The gene

from the closely related oleaginous fungi, *Mort. alpina* and *M. rouxii*, have been cloned and found to possess ORFs encoding polypeptides of 399 and 396 amino acids, respectively (Huang *et al.*, 1999; Passorn *et al.*, 1999). Likewise, the ORFs of Δ12 desaturase genes from cyanobacteria appear to be relatively short, encoding proteins of approximately 350 amino acids (Wada *et al.*, 1993). As such, Δ12 desaturase genes encode smaller proteins than the Δ9 desaturases, since the Δ12 desaturase does not not possess a cytochrome b_5 domain (Sakuradani *et al.*, 1999b). Presumably, this desaturase (unlike desaturases with a cytochrome b_5 domain) can accept electrons from a discrete microsomal cytochrome b_5.

D. Δ6 Desaturases

The Δ6 desaturase converts linoleic acid (18:2n-6) to γ-linolenic acid as part of the n-6 fatty acid pathway and γ-linolenic acid (18:3n-3) to 18:4n-3 as part of the n-3 pathway (see Fig. 2). Although this enzyme is capable of taking part in two separate fatty acid desaturation/elongation pathways, in the vast majority of microbial systems only one of these pathways occur. The thraustochytrids appear to be an exception to this rule and are known to accumulate a cell lipid containing appreciable amounts of docosapentanoic acid (22:5n-6) and docosahexaenoic acid (22:6n-3) (Metz *et al.*, 2001). The genes encoding Δ6 deaturases from *Mort. alpina* and *M. rouxii* have been cloned, and their function confirmed by heterologous expression in yeast and *Aspergillus oryzae* (Huang *et al.*, 1999; Sakurdani *et al.*, 1999b, 1999c; Laoteng *et al.*, 2000) The *Mortierella* gene contains an open reading frame encoding a polypeptide of 457 amino acids, a similar size to the Δ9 desaturase, and possesses a cytochrome b_5 domain. However, in the Δ6 desaturases the cytochrome b_5 fusion protein is at the N-terminus of the expressed protein (Sakurdani *et al.*, 1999c; Laoteng *et al.*, 2000).

E. Δ5 Desaturases

Like the Δ6 desaturase, the Δ5 desaturase can potentially operate in either the n-3 or the n-6 fatty acid pathway, converting either dihomo-γ-linolenic acid (20:3n-6) to arachidonic acid (20:4n-6) or 20:4n-3 to eicosapentaenoic acid (20:5n-3—see Fig. 2). Furthermore, like the Δ6 desaturase it introduces a double bond into the fatty acid chain to the carboxyl-end relative to the initial Δ9 desauration site. As such, both the Δ6 and Δ5 desaturases belong to a subgroup of desaturases that have been designated the "front-end" desaturases. The front end desaturases have similar sequence homology and share distinct features that separate them from the desaturases that insert double bonds to the

methyl end of the fatty acid relative to the initial Δ9 desaturation. In particular, these desaturases possess an N-terminal cytochrome b_5 domain (Michaelson *et al.*, 1998; Saito and Ochiai, 1999; Saito *et al.*, 2000) and seem to differ slightly from other desaturases in that they have a H to Q substitution in the third HIS box to give a QXXHH consensus (Knutzon *et al.*, 1998; Michaelson *et al.*, 1998; Saito and Ochiai, 1999).

The gene from *Mort. alpina* coding for the Δ5 desaturase has been cloned. It encodes a sequence of 446 amino acids (Knutzon *et al.*, 1998). Duplicate genes for Δ5 desaturase occur in the slime mold *Dictylostelium*, one encoding a polypeptide of 464 amino acids and the other 467 amino acids (Saito and Ochiai, 1999; Saito *et al.*, 2000). In the slime mold, it appears that the Δ5 desaturase possesses an unusually long interval between the second and third HIS boxes (Saito and Ochiai, 1999).

F. A Δ4 DESATURASE?

The existence of a Δ4 desaturase that desaturates docosapentaenoic acid (22:5n-3) as the final step in docosahexaenoic acid (DHA; 22:6n-3) (see Fig. 2) has been debated. In mammals it has been suggested that this conversion is carried out via elongation of 22:5n-3 to 24:5n-3 which is then desaturated by a Δ6 desaturase to 24:6n-3 and this subsequently chain shortened to yield DHA (22:6n-3) (Voss *et al.*, 1991). Furthermore, in DHA-producing marine microorganisms (both bacterial and eukaryotic) the synthesis of DHA is thought to proceed along a "polyketide" pathway rather than via a fatty acid route (Metz *et al.*, 2001), obviating the need for a Δ4 desaturase.

A gene encoding a protein with a Δ4 desaturase activity (in yeast and plants) has been cloned from *Thraustochytrium* sp. (Qiu *et al.*, 2001). The existence of this Δ4 desaturase gene reignites the arguments as to the exact route for DHA biosynthesis, at least in eukaryotic marine microorganisms. That a Δ4 desaturase gene appears to be present and encodes a functional protein suggests a fatty acid route to DHA biosynthesis is possible, although the fact that EPA biosynthesis in the related *Schizochytrium* was uneffected by anaerobic growth is not compatible with a truly fatty acid route reliant on O_2-requiring desaturases (Metz *et al.*, 2001).

VII. Fatty Acid Elongases

The other conversion required for the synthesis of the long-chain PUFA (LCPUFA) that are of current biotechnological interest is the

elongation of the fatty acid chain by a two-carbon extension at the carboxyl end catalyzed by fatty acid elongases. Fatty acid elongases act primarily on 16:0 to form 18:0 and on either 18:3n-6 or 18:4n-3 during the production of ARA and DHA (or EPA) via the n-6 and n-3 routes, respectively (see Fig. 2).

Like the fatty acid desaturases, the elongases are membrane-associated proteins and rely for activity on the cooperation of several catalytic subunits. As a result, the fatty acid elongases are as recalcitrant to conventional biochemical study as the desaturases and remain relatively poorly characterized. Indeed the number, substrate specificity, and even the detailed mechanism of fatty acid elongation remain a matter of some debate.

The reaction sequence for the elongation of fatty acids is the same as that for their *de novo* synthesis (by the FAS complex), i.e., it involves a sequence of four linked reactions:

1. *Condensation* of an activated acyl chain with malonyl-CoA to give a ketoacyl product and evolving CO_2.
2. *Reduction* of the ketoacyl product to give a γ-hydroxyacyl species.
3. *Dehydration* to give the enoyl compound.
4. *A second reduction* to give the final two-carbon elongated fatty acyl chain.

The key differences from the FAS complex are the specificity of the initial condensing enzyme (which also appears to be the rate-limiting step for the entire elongation process) and the membrane-bound nature of the elongase complex.

Although the biosynthesis of long-chain PUFA is often thought of as a membrane-associated process (in contrast to the *de novo* synthesis of saturated fatty acids up to C_{18}, which occurs in the cytosol), all the enzymes, both desaturases and elongases, are membrane proteins and the intermediates appear to be phospholipid-bound acyl chains. However, this cannot be the case for the fatty acid elongation as the initial condensation reaction requires access to the carboxyl end of the acyl chain, access that would be impossible if the acyl chain was bound as part of a phospholipid group. Also, the condensation requires that the acyl chain be activated (probably in the form of a CoA thioester). The details as to how the acyl chain is removed from the phospholipid and is replaced thereon remains obscure.

Until the recent success in cloning genes for the fatty acid elongases from *Mort. alpina,* little was known about these enzymes from oleaginous microorganisms. However, recent evidence has suggested that during long-chain PUFA biosynthesis, the elongation process may be a

crucial rate-limiting step (Wynn and Ratledge, 2000), and would be the key enzyme activity to target in gene cloning if one wished to increase the productivity of, say, arachidonic acid (20:4n-6).

In the past two years, two genes encoding discrete elongases (probably just the "condensing enzyme") with different properties and substrate specifies have been identified in *Mort. alpina* (Parker-Barnes *et al.*, 2000). The first, which was cloned using a protocol based on conserved regions in plant and yeast elongaes genes (i.e. from systems that do not accumulate PUFA), identified a gene possessing 40% similarity at the amino acid level with the yeast elongase. This elongase exhibited a preference for saturated and monounsaturated fatty acids, and is probably involved in the conversion of 16:0 to 18:0.

Subsequently, a second gene encoding an elongase that specifically acted on C_{18} PUFA was isolated. This gene encoded a polypeptide of 318 amino acids that demonstrated heterologous activity using either 18:3n-6 or 18:4n-3 as a substrate, despite *Mortierella* not possessing an active n-3 pathway. The gene encoding the PUFA-specific elongase was clearly distinct from the saturated/mono-unsaturated fatty acid specific enzyme, exhibiting <25% identity with the yeast elongase (Parker-Barnes *et al.*, 2000).

It is therefore now apparent that elongations of C18 to C20 fatty acids and of C20 to C22 fatty acids (see Fig. 2) are each carried out by a specific elongase encoded for by a distinct gene. These conversions are therefore not a result of a single elongase with broad specificity capable of elongation of both 16:0 and 18:3n-6. It would also appear that only one enzyme function, that of the 'condensing enzyme' (see above), needs to be cloned; the other three enzyme activities of the two reductases and a dehydratase must be supplied by the existing activities of the FAS complex.

VIII. Lipid Bodies and Their Role in Lipid Accumulation

For a long time, the formation of lipid bodies in oleaginous microorganisms was thought to involve the simple coalescence of the nonsoluble neutral lipid. As such, lipid body formation was thought to be a predominantly physical process, and lipid bodies were thought to be physiologically inert structures. This idea has now been largely discredited, and it is accepted that, far from being a biochemically inert structure, lipid bodies play a key role in the regulation of storage lipid formation and reutilization (Murphy and Vance, 1998). The formation of lipid bodies is beyond the scope of the current review, but has been reviewed in detail elsewhere with particular emphasis on lipid bodies in plants (Murphy and Vance, 1998).

That lipid bodies were not purely cellular "puddles" of accumulated storage lipid was first suggested due to the observation that most oleaginous cells maintain many lipid bodies (all of a specific size) rather than a single large lipid vacuole (Kamisaka *et al.*, 1999). Indeed, in *Mortierella ramanniana* lipid bodies "mature" during the lipid accumulation phase, starting with a diameter of ≈ 1 μm increasing to a maximum of 2–3 μm (Kamisaka *et al.*, 1999). Further lipid accumulation is achieved by formation of more lipid bodies rather than by the size of the existing lipid bodies increasing (Kamasaka *et al.*, 1999).

In addition to specific physical properties, lipid bodies possess a distinct enrichment of enzymes associated with triacylglycerol (TAG) assembly in the membranes that are around the lipid bodies (Kamisaka and Nakahara, 1994; Pillai *et al.*, 1998). The final enzyme in TAG assembly, diacylglycerol acyltransferase (DAGAT), is especially enriched in the membranes most closely associated with lipid bodies (Kamisaka and Nakahara, 1994; Kamisaka *et al.*, 1997; Pillai *et al.*, 1998). The other TAG assembly enzymes are likewise enriched in the membranes associated with the lipid bodies though to a lesser extent. Indeed, a correlation between the order of the enzyme in TAG assembly and their enrichment in the lipid body fraction from oleaginous microorganisms has been suggested (Pillai *et al.*, 1998).

As well as enrichment in the TAG assembly enzymes, microbial lipid bodies possess a specific set of membrane proteins, having molecular sizes of 24, 29, and 54 kDa (Kamasaka and Nakahara, 1994). The role of these proteins is probably structural, maintaining the integrity of the lipid bodies and avoiding coalescence with neighboring lipid bodies. They may be analogous to the oleosins of desiccation-resistant plant seeds (Murphy and Vance, 1998).

The role of lipid bodies in the organisation of TAG biosynthesis is now becoming more evident. In *Mort. ramanniana,* phosphatidic acid appears to be incorporated preferentially into the lipid bodies for TAG biosynthesis while phosphatidylcholine is incorporated into the ER membranes (Kamisaka *et al.*, 1999). Therefore, TAG biosynthesis may occur predominantly via a "cytosolic" route rather than via phospholipid (Fig. 10). If this is the case, then a PUFA-rich TAG, as is commonly produced by oleaginous microorganisms, must occur via "acyl-shuttling" between diacyl glycerol and/or TAG and the phospholipid where PUFAs are formed (Kamisaka *et al.*, 1999).

IX. The Metabolon Concept for the Integration of Lipid Biosynthesis

One of the most intriguing aspects of the whole process of lipid accumulation (as with any other metabolic process) is the intracellular

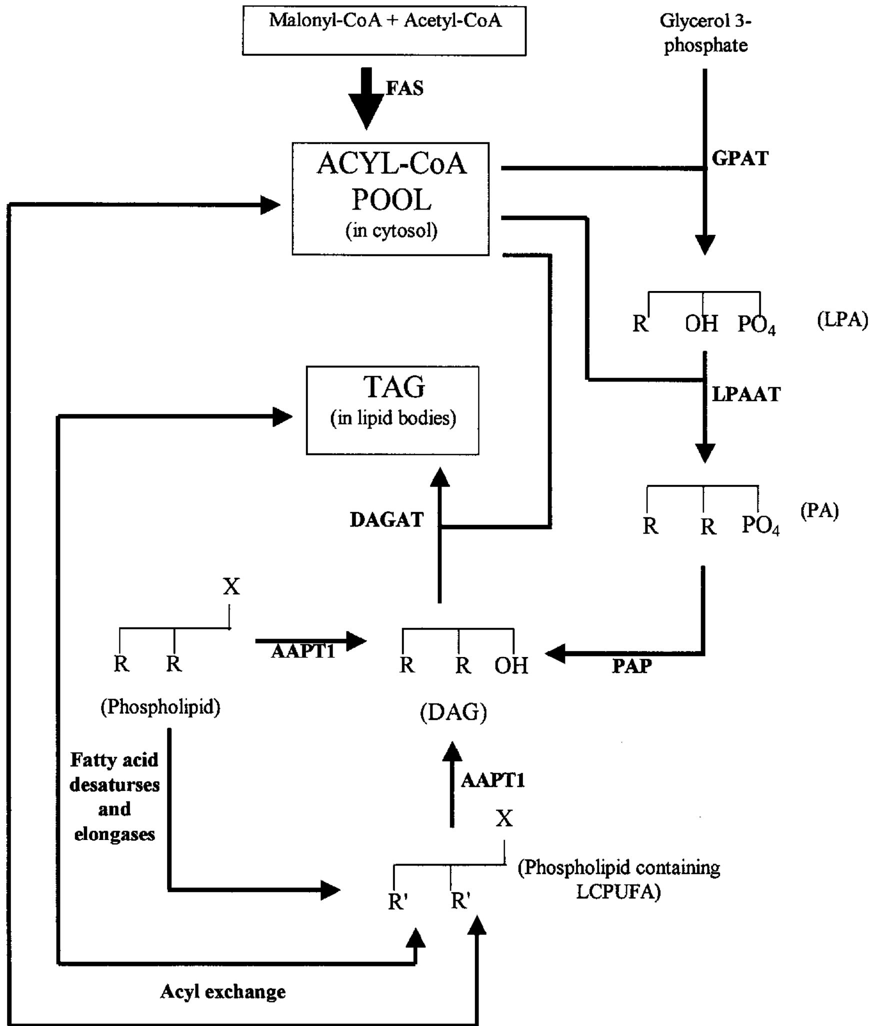


FIG. 10. The pathways for (PUFA)-rich triacylglycerol (TAG) biosynthesis. FAS: fatty acid synthase; GPAT: glycerol-3-phosphat acyltransferase; LPAAT: lysophosphatidic acid acyltransferase; PAP: phosphatidic acid phosphohydrolase; CDP-CPT: choline phosphotransferase; DAGAT: diacylglycerol acyltransferase; LPA: lysophosphatidic acid; PA: phosphatidic acid DAG: diacylglycerol; R: saturated fatty acid; R′: unsaturated fatty acid; X: phospholipid polar head group. Acyl exchange includes movement of acyl residues between phospholipid and TAG, by acyltransferases, and the activity of phospholipases to transfer unsaturated fatty acyl residues from phospholipid into the acyl-CoA pool.

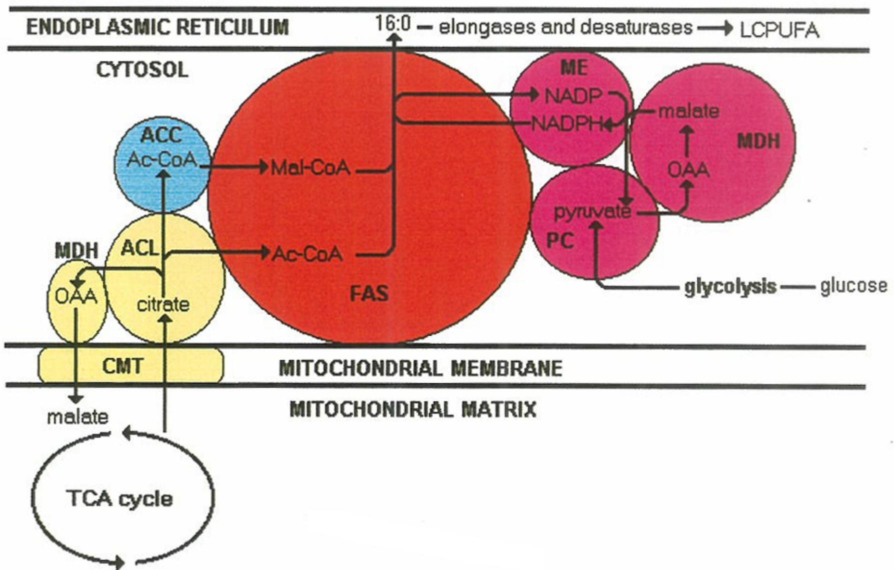


FIG. 11 A diagram of the hypothesized lipogenic metabolon with lines to demonstrate the routes of substrate channeling. OAA: oxaloacetate; Ac-CoA: acetyl-CoA; Mal-CoA: malonyl-CoA; FAS: fatty acid synthetase; ACL: ATP:citrate lyase; ACC: acetyl-CoA carboxylase; CMT: citrate/malate translocase; ME: malic enzyme; PC: pyruvate carboxylase; MDH: malate dehydrogenase. The "yellow" enzymes form the citrate/malate cycle while the "purple" enzymes represent the cytosolic transhydrogenase cycle.

organization that ensures the process continues in an efficient and closely regulated fashion. In this regard, we suggest that protein–protein interactions play a key role in the organization and regulation of lipid accumulation, and propose the existence of a "lipogenic metabolon" (Fig. 11, see color insert).

Protein interactions are clearly involved with the operation of the fatty acid desaturases (Fig. 8) and the clustering of triacylglycerol assembly enzymes in lipid body fractions as have been demonstrated by other authors (Kamisaka and Nakahara, 1994; Pillai *et al.,* 1998). Moreover, work in our laboratory has indicated that protein interactions may play a fundamental role in channeling substrates between lipogenic enzymes during *de novo* fatty acid synthesis.

It has long been apparent that malic enzyme plays a key role in the provision of NADPH for lipid synthesis (and desaturation) in fungal cells (Kendrick and Ratledge, 1992b; Wynn *et al.,* 1997, 1999; Wynn and Ratledge, 1997—see Section V.C). However, it is clear that malic enzyme is not the sole mechanism for NADPH generation in fungal cells (Wynn *et al.,* 1997; Wynn and Ratledge, 1997). Indeed, in the fungi studied the hexose monophosphate pathway appears the most active NADPH generating pathway (Wynn *et al.,* 1997; Wynn and Ratledge, 1997). As a consequence, it was hard to explain the specific role of malic enzyme in fatty acid synthesis unless a direct channeling of NADPH from malic enzyme to fatty acid synthase was assumed. Having become convinced of the interaction of malic enzyme and fatty acid synthase, it then seemed an obvious extension of our hypothesis to invoke other enzymes as potential members of the hypothesized lipogenic metabolon. This hypothesis, of course, is not completely novel. Srere, who originally developed the concept of the metabolon, had detected an apparent association between fatty acid synthase and another major lipogenic enzyme (ATP:citrate lyase) in animal cells (Finkelstein *et al.,* 1979; Linn and Srere, 1984) where this association was mediated via the endoplasmic reticulum.

Conclusive direct evidence of the hypothesized protein–protein interactions is still lacking. These interactions are likely to involve only weak noncovalent attractions between the various proteins, which would be readily disrupted by the physical stress and dilution effects that occur during cell breakage (Velot *et al.,* 1997). The metabolon concept is gaining ground in many areas of biochemistry. The energetic advantages of a high degree of intracellular organization (rather than separate enzymes floating around in a cytosolic substrate/product "soup") are obvious (Ovadi and Srere, 1996). Metabolon complexes are suggested to occur in the citric acid cycle (Verlot *et al.,* 1997), amino acid biosynthesis (Abadjieva *et al.,* 2001), pigment biosynthesis (Sugumaran *et al.,* 2000),

and β-oxidation (Eaton *et al.*, 2000). It is our firm assertion that fatty acid biosynthesis should (and will eventually) join this growing list of metabolons.

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Anonymous. (2001). *INFORM* **12,** 863–837; and see also Netlinks: Martek Biosciences DHA/ARA www.martekbio.com/dha.html and FDA response letter to Martek: www.cfsan.fda.gov/nrdb/opa-g041.html

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Bioethanol Technology: Developments and Perspectives

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I. Introduction

Accumulation of CO_2 in the atmosphere is long recognized as a major contributor to global warming and climate change (Revelle and Suess, 1957). Bioethanol used as a replacement for gasoline reduces vehicle CO_2 emissions by 90% (Tyson *et al.*, 1993). With respect to global warming, ethanol from biomass reduces net CO_2 emissions since fermentation CO_2, produced during ethanol production, is part of the global carbon cycle (Wyman, 1994). Ethanol has an important impact on automobile tailpipe emissions, producing a significant demand for use of ethanol as an oxygenate (Putsche and Sandor, 1996). With the phase out of the oxygenate methyl *tert*-butyl ether (MTBE), which reduces CO emissions by improving oxidative combustion, ethanol can replace MTBE as an oxygenate (Blackburn *et al.*, 1999; Unnasch *et al.*, 2001). A disadvantage of ethanol is that it has only 65–69% of the energy density of hydrocarbon fuels (Lynd, 1996).

Brazil produces 12.5 billion liters of ethanol from cane sugar, which is used as a 22% blend with gasoline or as neat ethanol fuel in their Otto cycle engine (Rosillo-Calle and Cortez, 1998). The United States produces 5 billion liters mainly from corn, used mainly as 10% in gasoline, but some as 85% ethanol that can be used in flexible fuel

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vehicles produced by Ford and Chrysler at no extra cost (Sheehan, 2001).

Because of the problems associated with conversion of lignocellulose to fermentable sugars, ethanol plants have relied on sugar- and starch-based substrates, and have been slow to take on the risks of lignocellulose-based fermentation (Claassen *et al.*, 1999). Nevertheless, several bioethanol production plants, having capacities in the range 1–20 million gal/year, are under construction or are being commissioned. These plants use microbial processes to produce ethanol from lignocellulose, sugar cane waste, and municipal solid waste. It has been estimated that the United States potentially could convert 2.45 billion metric tons of biomass to 270 billion gallons of ethanol each year, which is approximately twice the annual gasoline consumption in the United States (Gong *et al.*, 1999). Shell predicts that fuel from biomass will overtake oil by 2060 (Lynd *et al.*, 1999). The National Science and Technology Council predicts that 50% of organic chemicals will be produced from plant material by 2020 with biobased processes playing a central role (Lynd *et al.*, 1999).

The value of ethanol as an oxygenate and octane booster is $0.80–0.90 s/gal (Sheehan and Himmel, 1999). The U.S. highway bill includes an extension to the ethanol tax incentive program to 2007, which adds about $0.50/gal to the value of ethanol for the fuel market, allowing ethanol to sell for $1.20–1.40/gal (Sheehan and Himmel, 1999). Ultimately, technological developments must be such as to eliminate the need for the tax incentive.

II. Feedstock Supply

The potential to use lignocellulose biomass for energy production derives from its position as the most abundant and renewable organic material in the biosphere, accounting for 50% of world biomass (Goldstein, 1981; Lutzen, 1983). Potential feedstocks include food crops, crop residues, and woody biomass. The chemical composition of various biomass feedstocks is presented in Table I. The low cost and chemical composition of crop residues make them attractive as feedstocks (Kaylen *et al.*, 2000). Oak Ridge National Laboratory's feedstock supply cost curves show costs ranging from $15–44/dry ton (dt) (Walsh, 1997) and a typical cost assumption is $25/dt (Wooley *et al.*, 1999). A scale that benefits from most of the economies of scale is 2000 dt/day feedstock with a feedstock collection radius of up to 40 miles. Current consumption of biomass in the corn-refining industry and the pulp and paper industry are 52 and 100 million tons/year, respectively (Lynd *et al.*, 1999). Annual available collectable waste cellulosic biomass at

TABLE I

PROXIMATE COMPOSITION OF BIOMASS FEEDSTOCK[a]

Components (% dry weight)	Agricultural residues	Forestry residues	Grasses	Grains
Hexosans	30–42	40–55	40–45	3–8
Pentosans	10–40	8–30	30–35	6–8
Lignin	10–30	15–30	5–12	<1%
Ash	2–20	0.2–1.0	4–6	1–2
Starch	<1%	<1%	<1%	10–70
Protein	<1%	<1%	4–13	10–60
Oil	<1%	<1%	<1%	10–25
Extraneous materials	7–10	0.5–8.0	<1%	<1%

<$45/ton is 140 million tons/year (Lynd, 1996). If available agricultural residues in the United States were converted to ethanol, production could expand to 38–53 billion liters per year, which would achieve a 10% blend of all gasoline used in the United States (Sheehan, 2001). By way of comparison, U.S. production of primary building blocks for organic chemicals (ethylene, propylene, benzene, toluene, xylene, butadiene) is 64 million tons/year 1997 (Lynd *et al.*, 1999).

Because type and availability varies with geographic region, climate, and environmental conditions, agriculture, and technology (Kuhad and Singh, 1993), generic processes for conversion to energy must be versatile and robust as well as cost effective. The more customized they have to be made to deal with variations in feedstock the less advanced each process is likely to be. For example, a major problem in lignocellulose pretreatment and hydrolysis is the variation in lignin and hemicellulose composition with plant species, cultivation method, and harvest time. Because of these problems, ethanol plants have relied on sugar- and starch-based substrates, and have been slow to take on the risks of lignocellulose-based fermentation (Claassen *et al.*, 1999).

III. Alternative Technologies for Biomass Conversion

Major options for production of energy and/or fuel from biomass are fermentation-based bioconversion processes and physicochemical technologies, and competition will occur between these approaches (Singh and Mishra, 1995). While the major target product in fermentation is ethanol, processes for methane production from waste are widely practiced. An acetone–butanol process was operated commercially in the

past and may be revived (Spivey, 1978; Lovitt *et al.*, 1988). Microbial production of hydrogen is at a less advanced state and commercial viability remains in question.

Major physicochemical processes are combustion, gasification, pyrolysis, and hydrothermal upgrading. Fermentation and physicochemical processes are differentiated with respect to manner of substrate utilization: thermal technologies can use total crops, producing heat and/or synthesis gas and/or pyrolysis oil, whereas fermentation processes produce specific energy/fuel products plus substantial valuable or waste by-products. Since the volume of by-products can be high (similar in magnitude to the volume of the main product), optimizing the beneficial use and economic value of the by-products is a significant challenge. Gasification-based power generation from lignin-rich residues is considered to be an attractive way to realize value from the residues of processes featuring enzyme hydrolysis (Lynd *et al.*, 1999).

IV. Biomass Conversion to Usable Fermentation Feedstock

There are five basic steps involved in bioethanol production: biomass production, conversion to usable fermentation feedstock, microbial fermentation of feedstock to ethanol, beneficial use of residual unfermented material, and environmental management of the process. These processes are currently implemented commercially using cane sugar and starch-based substrates. The intractable nature of cellulose, present in the native state as lignocellulose, is the key barrier to commercially viable use of cellulose as a fermentation feedstock, and development of effective methods for its conversion to fermentable feedstock is the top priority (Ghosh and Singh, 1993). Gasification, acid hydrolysis, and pretreatment/enzyme hydrolysis are options for overcoming recalcitrance. Various physicochemical pretreatment methods used in lignocellulose substrate preparation for bioethanol production are shown in Table II.

Acid hydrolysis of native cellulose with concentrated sulfuric acid, followed by dilution with water, results in an almost quantitative conversion of the substrate to glucose (Fan *et al.*, 1987). Commercial concentrated acid hydrolysis plants have operated since 1937 (Sheehan and Himmel, 1999). A key to process viability is the ability to recover and reuse the acid using membrane (Wenzl, 1970) or chromatographic techniques or the use of negative cost feedstocks by avoiding waste disposal costs (Sheehan and Himmel, 1999).

Dilute acid processes, first used in Germany in 1898 to hydrolyze cellulose to glucose, evolved into dilute acid hydrolysis percolation reactors that have been used in Germany, Japan, and Russia for 50 years, although they are not competitive in open markets (Hsu, 1996). Comparatively high glucose yields were achieved, but in dilute sugar

TABLE II

VARIOUS PRETREATMENT METHODS USED IN LIGNOCELLULOSE SUBSTRATE PREPARATION

Pretreatment type	Specific method
Mechanical	Weathering and milling—ball, fitz, hammer, roller
Irradiation	Gamma, electron beam, photooxidation
Thermal	Autohydrolysis, steam explosion, hydrothermolysis, boiling, pyrolysis, moist or dry heat expansion
Alkali	Sodium hydroxide, ammonium hydroxide
Acids	Sulfuric, hydrochloric, nitric, phosphoric, maleic
Oxidizing agents	Peracetic acid, sodium hypochlorite, sodium chlorite, hydrogen peroxide
Solvents	Ethanol, butanol, phenol, ethylamine, acetone, ethylene glycol
Gases	Ammonia, chlorine, nitrous oxide, ozone, sulfur dioxide
Biological	Ligninolytic fungi

streams (Sheehan and Himmel, 1999). Comparison of acid-based batch and flow-through processes illustrates that flow-through methods provide less sugar destruction, lower toxicity, and higher sugar yields, but result in greater sugar dilution (Jacobson and Wyman, 2000). A recent innovation is a countercurrent flow-through system, using low (0.07%) sulfuric acid concentrations. This method yields high hydrolysis levels while retaining high sugar concentrations, and produces 82% hydrolysis of cellulose and near total depolymerization of xylose while yielding a solution containing about 4% sugar (Lee *et al.*, 1999; Torget *et al.*, 2000). This process offers potential to eliminate the need for cellulase-mediated hydrolysis of cellulose. Detoxification of acid-hydrolyzed lignin and other extractables adds an additional cost to the total hydrolysis process (Lee *et al.*, 2000).

Mosier *et al.* (2001) recently characterized dicarboxylic acids for cellulose hydrolysis. Dilute maleic acid (50 m*M*) was shown to hydrolyze cellobiose and cellulose as effectively as dilute sulfuric acid. Higher glucose yields from cellulose hydrolysis also suggested that maleic acid does not denature glucose as easily as sulfuric acid. The hydrolyzate is also expected to be more easily fermented as a result of lower concentrations of fermentation-inhibiting degradation products. The results are significant since maleic acid is widely available in large quantities.

The cost of enzymatic cellulose hydrolysis using cellulases is high relative to other hydrolytic enzymes (Kuhad *et al.*, 1997). It relates largely to the microcrystalline nature of cellulose and the fact that biocatalysts are in general less effective on insoluble substrates (Himmel *et al.*, 1999; Esteghlalian *et al.*, 2000). One challenge is to reduce non-specific binding to lignin (Wooley *et al.*, 1999). The U.S. Department of Energy (DOE)

has attempted to accelerate progress in this area by awarding research contracts to Novozyme and Genencor to improve cellulases for biomass conversion to ethanol (Mielenz, 2001). Pioneer processes supported by the biofuels programs include a concentrated acid hydrolysis and two-stage dilute acid/enzyme processes (Wooley *et al.*, 1999; Kadam *et al.*, 2000; Mielenz, 2001).

Since the extent of native cellulose digestion by cellulolytic enzymes is very inefficient in most types of biomass, pretreatment is essential to make the cellulose accessible to the action of enzymes (McMillan, 1994; Kuhad *et al.*, 1997). Physical methods including irradiation, thermal treatment, and mechanical grinding are too energy intensive (Hsu, 1996). Biological ligninolytic processes are too slow and also degrade some of the utilizable sugars (Ghosh and Singh, 1993). Hemicellulose is hydrolyzed by steam heating, but acids are produced, reducing sugar yields to about 65% (Heitz *et al.*, 1991; Abatzoglou *et al.*, 1992; Ramos *et al.*, 1992). Although, pretreatment with dilute sulfuric acid appears to be the preferred option at present (Hinman *et al.*, 1992; Lynd *et al.*, 1999), use of sulfuric acid increases cost of materials of construction, requires costly neutralization chemicals, and increases chemical handling and disposal problems (Lynd *et al.*, 1996). In summary, general criteria for effective conversion of lignocellulose to fermentable sugars are as follows:

- Production of high yields of sugars with 90–100% in the hydrolysis reaction.
- Minimization of a corrosive processing environment to control construction costs.
- Minimization of a requirement for energy-intensive mechanical size reduction.
- Minimization of water addition, which contributes to the dilution of sugars and increases energy requirements.
- Minimization of the production of toxic products (for example, acetate and furfural). during hemicellulose hydrolysis by avoiding conditioning steps.
- Production of a cellulose substrate amenable to cellulase attack, if acid is used as a pretreatment for enzyme hydrolysis.

V. Toxicity of Acid-Treated Feedstock

Toxic compounds from acid-hydrolyzed lignocellulose biomass inhibit fermentation ethanol production (Palmqvist and Hahn-Hägerdal, 2000a, 2000b). The main inhibitors of ethanol fermentation from lignocellulose generated during steam pretreatment and hydrolysis are weak

acids, furan derivatives, and phenolic compounds (Palmqvist *et al.*, 1999a, 1999b). Phenolic compounds appear to be the major inhibitory problem. Major components are 4-hydroxybenzoic acid, vanillin, and catechol (Palmqvist *et al.*, 1999b). The lignin-derived compounds, syringaldehydes and vanillin, have have been shown to be particularly potent inhibitors of cell growth and ethanol production (Delgenes *et al.*, 1996). The woody hydrolyzate contains approximately 4-fold more syringyl-based monomers than does the herbaceous hydrolyzate, while guaiacyl- containing compounds are more evenly distributed (Fenske *et al.*, 1998). *Saccharomyces cerevisiae* has relatively high inhibitor tolerance, although different strains may vary in inhibitor tolerance (Linden *et al.*, 1992; Palmqvist *et al.*, 1998).

The toxicity of lignocellulose hydrolyzate can be removed by optimized "overliming" with $Ca(OH)_2$ (Martinez *et al.*, 2000). Toxicity of acid-hydrolyzed biomass is typically removed by addition of $Ca(OH)_2$ until the pH reaches 9–10, followed by pH neutralization (Hahn-Hägerdal, 1996). $Ca(OH)_2$ treatment of hydrolyzate to 9–10 and readjustment to 5.5 with H_2SO_4 precipitates the toxic compounds and produces better fermentability (van Zyl *et al.*, 1988). Sulphite and overliming treatment of hydrolyzate have been found best for fermentation by a recombinant *Escherichia. coli* strain (von Sivers *et al.*, 1994; Olsson *et al.*, 1995). Among the seven different detoxification methods tested on corn cob hemicellulose acid hydrolyzate, overliming produced the hydrolyzate that was most completely fermented by *Pichia stipitis* and *Candida shehatae* (Eken-Sarcoglu and Arslan, 2000). $NaHCO_3$ (0.5 mol/liter) treatment of inhibitors eliminated inhibition, while illustrating that the inhibitors were alkali unstable (Palmqvist and Hahn-Hägerdal, 2000a). Various detoxification methods are shown in Table III.

Enzymatic detoxification of wood hydrolyzate with peroxidase and laccase from *Trametes versicolor* increased maximum ethanol productivity by selective removal of phenolic monomers (Jönsson *et al.*, 1998). Larsson *et al.* (2001) developed a *S. cerevisiae* strain with enhanced resistance to phenolic inhibitors in lignocellulosic hydrolyzate by heterologous expression of laccase from *Trametes versicolor.* The laccase-producing transformant fermented dilute acid spruce hydrolyzate at a faster rate than the control transformant. *Trichoderma reesei* degraded inhibitors found in the acid hydrolyzate resulting in a 3–4 times increased maximum ethanol productivity and yield with evidence of removal of some inhibitors (Palmqvist *et al.*, 1997).

The need for detoxification decreased as inoculum cell density increased (Chung and Lee, 1984; Nishikawa *et al.*, 1988). Adding substrate in fed batch is another strategy that can eliminate the effect of inhibitors

TABLE III

LIGNOCELLULOSE HYDROLYZATE DETOXIFICATION PROCEDURES[a]

Detoxification method	Effect of treatment
Physicochemical	
Overliming	Reduction of acetic acid, furfurals, tannins, terpenes, phenolics and heavy metals
Neutralization with CaO, NaOH, KOH; activated carbon	Removal of acetic acid
Ether extraction	Removal of furfurals
Ethyl acetate extraction	Removal of lignin-degrading products
Vacuum evaporation	Removal of acetic acid
Steam stripping	Removal of furfurals, phenols, and acetic acid
Ion exchange chromatography	Removal of aromatic monomers and dimers, partial removal of acetic acid, furfurals, soluble lignin and metals
Enzymatic	
Laccase and peroxidase	Reduction of phenolics in hydrolyzate, rapid consumption of sugars, and increased ethanol productivity
Microbial	
Culture adaptation	Increased tolerance to acetic acid in *Pichia stipitis* with improved ethanol yield and productivity
Genetic	Development of laccase-expressing *Saccharomyces* with enhanced resistance to phenolic inhibitors

through their bioconversions (Taherzadeh *et al.*, 1999). Continuous systems with cell retention can maximize ethanol productivity and yield from lignocellulose hydrolyzate, but specific ethanol productivity can decrease with increasing cell mass concentration (Lee and Chang, 1987; Palmqvist *et al.*, 1998). Adaptation of *S. cerevisiae* (Chung and Lee, 1984), *Pichia stipitis* (Nigam, 2001), and *Candida guilliermondi* (Silva and Roberto, 2001) to lignocellulosic hydrolyzate has been reported to improve growth and fermentability.

VI. Cellulase Production

While commercial cellulases have been available for 50 years, they have only been used in specialty applications (Ward and Moo-Young, 1989; Bhat, 2000). Cellulose and lignocellulose in their native state are crystalline and water insoluble, and are recalcitrant to accelerated enzymatic attack (Kuhad *et al.*, 1997). A group or complex of different enzymes, which act synergistically in nature, mediates cellulose biodegradation, and one barrier to degradation is that most of the enzymes are

TABLE IV

SPECIFIC ACTIVITIES OF SOME COMMERCIAL CELLULASES[a]

Product name	Company	Microbial source	FPU activity (U/ml)	β-Glucosidase activity (U/ml)	Specific FPU activity (U/mg)	Specific β-glucosidase activity (U/mg)
Biocellulase TR1	Quest	*Trichoderma reesei*	68	200	0.24	0.72
Biocellulase A	Quest	*Aspergillus niger*	0.29	32	0.01	1.4
Celluclast 1.5L	Novo Nordisk	*T. reesei*	61	26	0.37	0.16
Cellulase TAP10[6]	Amano	*T. viride*	0.42	17	0.13	5.2
Cellulase AP30K	Amano	A. niger	0.17	60	0.03	10.0
Cellulase TRL	Solvay	*T. reesei*	95	170	0.57	1.0
Econase CE	Alko-EDC	*T. reesei*	40	46	0.42	0.48
Multifect CL	Genencor	*T. reesei*	64	30	0.42	0.20
Multifect GC	Genencor	*T. reesei*	65	59	0.43	0.39
Spezyme No. 1	Genencor	*T. reesei*	74	48	0.54	0.35
Spezyme No. 2	Genencor	*T. reesei*	72	53	0.57	0.42
Spezyme No. 3	Genencor	*T. reesei*	65	53	0.57	0.46
Ultra-Low Microbial	Iogen	*T. reesei*	88	176	0.48	0.96

subject to product inhibition (Singh and Hayashi, 1995). Although much has been learned concerning the structure, kinetics, catalytic action, and interactions of enzymes and their substrates, no single mechanism of total lignocellulose saccharification has been established (Mansfield *et al.*, 1999). The efficacy of cellulase to saccharify lignocellulose is linked to the structural characteristics of the substrate and the modifications that occur during hydrolysis. A comparison of specific activity values of some commercial cellulase preparations is shown in Table IV (Nieves *et al.*, 1998).

The high cost of cellulase is a major barrier to ethanol production from biomass mediated by enzymatic hydrolysis. While gasification, acid hydrolysis, and pretreatment/enzyme hydrolysis are options for overcoming recalcitrance, the potential for improvement in pretreatment/enzyme hydrolysis by an order of magnitude cost reduction has been predicted (Wyman *et al.*, 1993; Lynd *et al.*, 1996). However, it has been reasoned that only modest improvements are expected for acid hydrolysis and gasification. Some believe lignocellulose treatment with

cellulases has the lowest cost potential and is considered a first priority for bioethanol process design.

Commercially available cellulase preparations are currently employed in nonbiomass conversion applications such as food processing, detergents, and textiles. However, these markets command a much higher price for cellulase than can be afforded by any projected bioethanol process. The key challenge in lignocellulose biotechnology is to develop a robust enzyme production system that can produce adequate amounts of highly efficient cellulase to make biomass depolymerization more rapid and less expensive. Cellulase yield and productivity above 150 FPU/g cellulose and 55 FPU/liter/h, respectively, have been achieved using Solka floc as substrate (Himmel *et al.*, 1999). It has been estimated that a bioethanol process would require about 11 million FPU (19 kg, 42 lbs) of cellulase to process 1 ton of biomass (1000 lbs cellulose) to 84 gallons of ethanol at an enzyme loading of 25 FPU/g cellulose (Himmel *et al.*, 1997). It costs U.S.$ 3/gal of ethanol ($0.8/liter) for commercial enzyme formulations and $0.5/gal ($0.13/liter) for less processed formulations (Lynd *et al.*, 1999). To achieve total cost competitiveness, enzyme costs must be reduced to less than $0.07/gal of ethanol, requiring a 10-fold increase in specific activity or production efficiency (Himmel *et al.*, 1999).

Major improvements/breakthroughs in the efficiency of hydrolysis of cellulose by cellulases and in enzyme production yields are required to allow an enzymatic pretreatment step to make a major impact on production costs of bioethanol (a savings of $0.50/gal). The basis for arguments suggesting that such improvements are possible (Himmel *et al.*, 1999) relates to the shorter research history on cellulases (50 years) compared with physicochemical processes (200 years), and that biotechnology and the engineering of production strains and enzymes create the potential (Wooley *et al.*, 1999). Perhaps these cost reductions will occur, but there is a strong possibility they may not. Nevertheless, recent advances in protein engineering offer techniques targeted to increased thermal stability, improved cellulose binding domain, improved active site, reduced nonspecific binding, and improved microorganisms for bigher enzyme productivity (Arnold and Moore, 1997; Singh, 1999).

Prediction of a substantial specific activity improvement assumes that the action of cellulases on cellulose can be improved to more closely resemble the action of starch-degrading enzymes on soluble starch (Sheehan and Himmel, 1999), which is a very unlikely prospect. All high-volume commercial enzymes, including amylases, other carbohydrases, and proteases, manifest efficient biocatalytic activity on substrates solubilized in aqueous media, and the commercialization

processes for these enzymes occurred in a much shorter time scale. Physicochemical pretreatment appears to be an abiding prerequisite for making native cellulose accessible to cellulases, so achieving large increases in enzyme yield and improvements in enzyme-catalytic efficiency only reduces a part of the total substrate pretreatment cost. The product feedback inhibitory effects on cellulases may be overcome by engineering enzymes to be insensitive to products or by implementation of simultaneous saccharification and fermentation (SSF) processes whereby products are consumed by the fermenting strain thereby avoiding inhibition. Considering all of these barriers together, we are not optimistic that enzymatic pretreatment of cellulose represents a serious means to substantially reducing production costs of bioethanol, as some predict.

VII. The Fermentation

Improvements to the fermentation step involve efficient ethanol-producing strain development, a possible combination of saccharification and fermentation in one step, and fermentation optimization. A key requirement is that the fermentation organism must be capable of fermenting the xylose and other nonglucose sugars in hydrolyzates as well as glucose. Not all of these organisms ferment pentose sugars efficiently. *Saccharomyces cerevisiae* and *Zymomonas mobilis* are excellent ethanol producers from glucose and sucrose, but are incapable of fermenting pentose sugars (Mishra and Singh, 1993; Jeffries and Jin, 2000). *Pichia stipitis, Candida shehatae* and *Pachysolen tannophilus* are the most promising naturally occurring pentose-fermenting yeasts. Continuous ethanol production from a mixture of glucose and xylose using a coimmobilized *S. cerevisiae* and *C. shehatae* has also been attempted with little success (Lebeau *et al.*, 1998). Characteristics of some potential bioethanol-producing microorganisms are presented in Table V.

For developing suitable organisms, targets are broader substrate range and higher ethanol productivity, yield, ethanol tolerance, temperature tolerance, and increased tolerance to biocatalyst inhibitors in the lignocellulose hydrolyzate (Ingram *et al.*, 1998). Improvements in the fermentation step could be realized with ethanol-producing organisms capable of producing more than 5% ethanol at 50°C, improving cellulase activity in the SSF system, and creating organisms with capability of producing both ethanol and cellulase (Lynd *et al.*, 1996). Current cellulase/ethanol coproducers, such as certain species of *Fusarium* and *Clostridium* produce only low amounts of ethanol (Singh *et al.*, 1992; Lynd *et al.*, 1999).

TABLE V

CHARACTERISTICS OF POTENTIAL BIOETHANOL PRODUCING MICROORGANISMS

Organism	Fermentation characteristics	Optimum pH	Optimum temperature (°C)
Yeasts			
Saccharomyces cerevisiae	Ferments hexose sugars with high ethanol yield and productivity, high ethanol tolerance, unable to ferment pentose sugars, engineered to ferment xylose	3–7	30–35
Candida shehatae	Ferments both hexose and pentose sugars, moderate formation of xylitol, low ethanol tolerance	3.5–4.5	28–32
Pachysolen tannophilus	Efficiently ferments glucose and xylose, produces significant amounts of by-product xylitol, low ethanol tolerance	2.5–5	28–32
Pichia stipitis	Ferments all sugars found in wood or straw hydrolyzates, some strains ferment xylan, lack of anaerobic growth, genetically engineered to rapid anaerobic growth	4–5.5	28–32
Klyuveromyces marxianus	Ferments a wide range of sugars including xylose	3–7	30–35
Filamentous fungi			
Fusarium oxysporum	Ferments a wide range of carbon sources including xylose, some strains ferment xylan and cellulose	5–6	28–34
Neurospora crassa	Ferments cellulose and xylose to ethanol, low ethanol productivity and tolerance	5–6	28–37
Mucor sp.	Ferments hexose and pentose sugars	5–6	28–32
Bacteria			
Clostridium thermocellum	Ferments cellulose, glucose, and xylose, low ethanol production	4–8	65–70
Clostridium thermohydrosulfuricum	Ferments glucose, xylose, and arabinose	4.7–8	65–70
Thermoanaerobacter ethanolicus	Ferments a wide range of sugars including xylose and arabinose	4.4–9.5	65–70
Zymomonas mobilis	Rapid glucose and sucrose fermentation, genetically engineered to ferment xylose	4–6.5	30–37
Klebsiella oxytoca	Rapid xylose and cellobiose fermentation, genetically engineered to ferment cellulose to ethanol	6–8	30–37
Escherichia coli	Ferments xylose and glucose with organic acids, low ethanol tolerance, genetically engineered strains produce high ethanol yields from hydrolyzates	6–8	30–37

Biomass for fuels and chemicals requires cost-competitive technologies in metabolic engineering, chemical catalysis, and separations. Genomics and other continuing advances in biotechnology, in addition to the techniques of genetic engineering and mutation, provide tools for engineering of metabolic pathways. However, techniques for modification of cell properties are more complex and less well advanced. Hence, we believe it is important to exploit important unique native properties of cells—for example, alcohol tolerance in yeast. Chemical catalysis in biomass processing is less advanced and fundamentally different from petroleum-based catalysis, in that the latter generally uses a hydrophobic medium whereas the former uses an aqueous medium. In addition, chemical catalytic strategies must take account of the lower thermal stability of the products and intermediates as compared to petrochemicals (Lynd *et al.*, 1999). Separation processes must also address the unique properties of these molecules. Strains with saccharolytic/cellulolytic properties, which are thermophilic or possess broader substrate specificity, are required. The ideal microbial ethanol producer should be capable of fermenting all biomass sugars to ethanol, have good resistance to lignin monomers, acetate and other inhibitory by-products, and make the enzymes for substrate depolymerization.

Since no naturally occurring organism can satisfy all threse specifications, genetic engineering techniques are being utilized with the aim of constructing organisms with most desirable properties for bioprocesses (Ingram *et al.*, 1998; Aristidou and Pentillä, 2000). Ethanol genes have successfully been introduced into bacteria such as *Escherichia coli* and *Klebsiella oxytoca*. Efforts have been undertaken to incorporate pentose-metabolizing pathways into natural ethanol producers such as *S. cerevisiae* and *Z. mobilis*. However, the development/use of genetically engineered strains should take account of additional environmental or processing costs of using recombinant organisms.

Saccharomyces cerevisiae is the most effective and safest organism for fermenting sugars to ethanol. The advantages of *S. cerevisiae* as a host for ethanol production over other yeasts include tolerance to aerobic and anaerobic conditions, metabolic versatility, capacity to produce ethanol under anaerobic and aerobic (with excess carbon) conditions, and high ethanol tolerance (150 g/liter). Disadvantages include lack of cellulolytic activity to degrade lignocellulose, and lack of saccharolytic enzymes, resulting in a sugar utilization spectrum limited to hexoses such as glucose, fructose, maltose, and sucrose, and an inability to ferment pentose sugars.

Saccharomyces cerevisiae is incapable of using xylose because it lacks two key xylose-metabolizing enzymes, xylose reductase (*XYL1*) and xylitol dehydrogenase (*XYL2*). Several laboratories have engineered xylose

fermenting in *S. cerevisiae* through expressions of *XYL1* or both *XYL1* and *XYL2* (Tantirungkij *et al.*, 1993; Meinander *et al.*, 1996; Richard *et al.*, 1999; Ho *et al.*, 1999; Nissen *et al.*, 2000). Overexpression of *XYL3* (xylulokinase gene) along with *XYL1* and *XYL2* in *Saccharomyces* sp. strain 1400, a fusion product of *S. diastaticus* and *S. uvarum*, resulted in the production of 47 g/liter of ethanol with 84% theoretical yield from a 1 : 1, glucose: xylose mixture (Ho *et al.*, 1998).

The yeasts *Pichia stipidis, Candida shehatae,* and *Pachysolen tannonophilus* can efficiently ferment C_5 sugars, but ethanol production rates are five times lower than those observed with *S. cerevisiae* (Hahn-Hägerdal *et al.*, 1994; Singh and Mishra, 1995; Lee, 1997). These C_5-fermenting organisms require oxygen and are 3–4 times less alcohol tolerant than *S. cerevisiae* (Hinamn *et al.*, 1989; Picataggio and Zhang, 1996). Respiratory and fermentative pathways coexist to support growth and product formation in *P. stipitis.* The organism grows rapidly under aerobic conditions, and ferments xylose or glucose under oxygen-limited conditions, but stops growing after one generation under anaerobic conditions (Aristidou and Pentillä, 2000). *Pichia stipitis* cells express *ADH1* about 10 times higher under oxygen-limited conditions, than under fully aerobic conditions (Cho and Jeffries, 1999). Expression of *S. cerevisiae URA1* (encoding for dihydroorotate dehydrogenase) in *P. stipitis* enables rapid anaerobic growth in glucose-containing defined medium with 32 g/liter of ethanol from 78 g/L of glucose (Shi and Jeffries, 1998).

Most recent work in engineering of bacteria has focused on *K. oxytoca, E. coli,* and *Z. mobilis* (Bothast *et al.*, 1999; Ingram *et al.*, 1999; Joachimsthal and Rogers, 2000). *Klebsiella oxytoca* has the capability of metabolizing cellobiose and cellotriose, an interesting feature minimizing the need for addition of extracellular cellobiase (β-glucosidase) (Ingram *et al.*, 1998). Overexpression of *pdc* and *adh* of *Z. mobilis* in *K. oxytoca* resulted in a rapid and efficient ethanol production from both glucose and xylose in the recombinant strain (Ohta *et al.*, 1991a,b).

Escherichia coli is an attractive host for genetic manipulations aimed at making it useful in conversion of renewable resources. It can grow on a wide range of sugars including glucose, xylose, arabinose, mannose, and galactose. It can sustain high glycolytic fluxes, both aerobically and anaerobically, and has a reasonable ethanol tolerance up to 50 g/liter. When glycolytic fluxes in *E. coli* were redirected to ethanol by transforming with *Z. mobilis* genes *pdc* and *adhB* (preferred ethanologenic or PET operon), the recombinant organism sequentially utilized a mixture of sugars to produce near maximum theoretical yields of ethanol (Ohta *et al.*, 1991; Takahashi *et al.*, 1994; Ingram and Conway, 1998).

Replacement of the native fermentation pathway in *E. coli* B with a homo-ethanol pathway from *Z. mobilis* resulted in a 30–50% increase in growth rate and glycolytic flux during the anaerobic fermentation of xylose (Tao *et al.,* 2001) in the engineered strain KO11.

Zymomonas produces ethanol from glucose with only 1 mole of ATP, the lower energetic efficiency being an advantage, promoting a high ethanol flux and producing up to 120 g/liter of ethanol at yield of up to 97% of the theoretical value (Lynd *et al.,* 1996). When compared to yeast, *Z. mobilis* exhibits up to 5-fold higher volumetric productivity (Gunasekaran and Raj, 1999). Since *Zymomonas* has a higher temperature optimum, less cooling is required during the fermentation. The fermentation substrate specificity, being limited to glucose, fructose, and sucrose, is a drawback. Metabolic engineering of the pentose metabolic pathway from *E. coli* into *Z. mobilis* has been successfully attempted by different laboratories (Feldman *et al.,* 1992; Zhang *et al.,* 1995; Deanda *et al.,* 1996).

Thermophilic saccharolytic clostridia: *Clostridium thermohydrosulfuricum, C. thermosaccharolyticum,* and *C. thermocellum* produce almost 2 moles of ethanol from hexose, and can convert C_5 sugars and amino acids to ethanol, offering potential for simultaneous direct conversion of lignocellulose to ethanol (McMillan, 1997). The main disadvantage is the low ethanol tolerance of these strains with the maximum ethanol produced from *C. thermocellum* being only 30 g/liter.

VIII. Combined Saccharification and Ethanol Fermentation

Detailed analyses of bioprocesses for conversion of biomass to ethanol indicate a trend toward lower costs with greater consolidation of the hydrolysis and fermentation steps, namely in cost terms: CBP (consolidated bioprocessing) < SSF (simultaneous saccharification and fermentation) or SSCF (simultaneous saccharification and cofermentation) < SHF (separate hydrolysis and fermentation) (Wright, 1988; Lynd, 1996; Lynd *et al.,* 1999). All cases except CBP require a separate cellulase production process/facility. The research and development advances required to achieve more consolidated solutions, especially CBP, are greater. An integrated biomass processing and bioethanol production scheme is presented in Figure 1.

For CBP, cellulases must be produced at high rates anaerobically with simultaneous ethanol production, which is energetically challenging but apparently feasible (Weimer, 1996). Target hosts are *C. thermocellum* (native substrate strategy), *Lactobacillus, E. coli, K. oxytoca,* and *Saccharomyces* (recombinant substrate utilization strategy). With

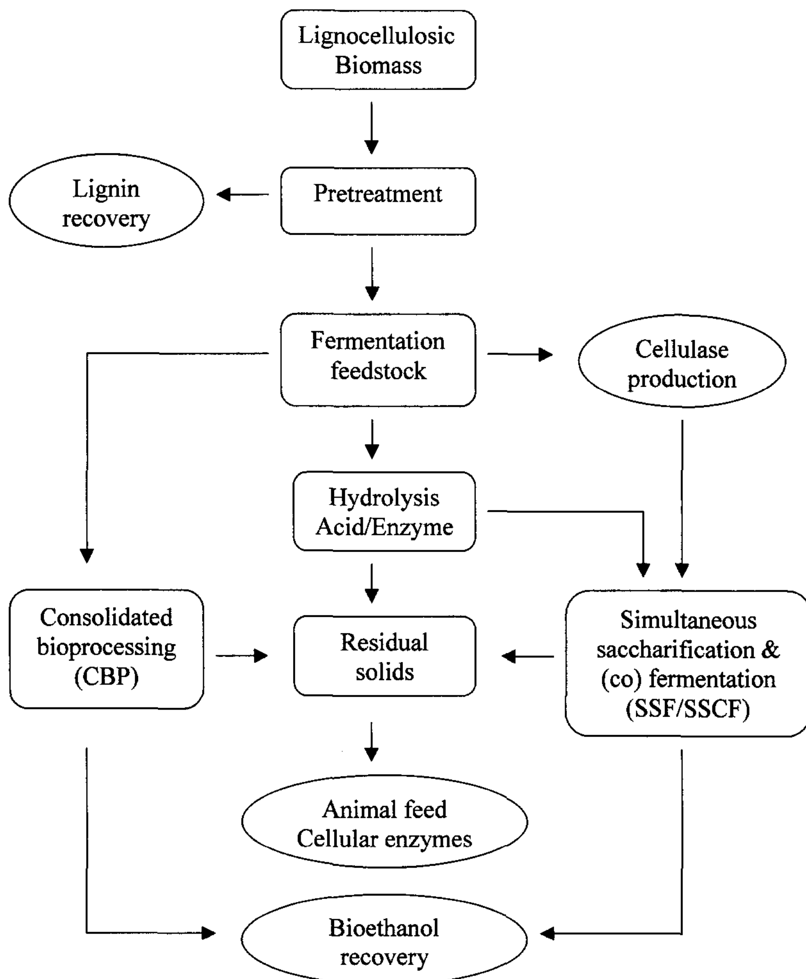

FIG. 1. Integrated biomass processing scheme.

yeast, expression and secretion of cellulases and utilization of cellulose has been reported (van Rensburg *et al.*, 1998; Peterson *et al.*, 1998). The overall metabolic energy burden of both cellulase production and ethanol from an anaerobe is being evaluated (van Walsum and Lynd, 1997; Desvaux *et al.*, 2001). Integrated bioprocessing may further contribute to improving yield and productivity where potentially inhibitory products are continuously removed from the fermentation broth as they are produced (Schügerl, 2000). Potential organisms and their ethanol yields from lignocellulosic hydrolyzate are presented in Table VI.

TABLE VI

ETHANOL YIELDS AND PRODUCTIVITIES OF VARIOUS MICROORGANISMS FROM THE FERMENTATION OF LIGNOCELLULOSIC HYDROLYZATE

Organism	Substrate/Pretreatment	Ethanol yield (g/g)	Ethanol productivity (g/L/h)	Reference
Yeasts				
Candida shehatae	Rice straw			Abbi *et al.* (1996)
	Acid hydrolysis	0.47	0.22	
	Autohydrolysis	0.50	0.24	
Candida shehatae	Whole barley enzymatic	0.48	1.16	Wayman and Parekh (1985)
Pachysolen tannophilus	Aspen Na_2SO_3	0.39	0.19	Deverell (1983)
Pichia stipitis	Aspen	0.45	0.85	Parekh *et al.* (1986)
	Steam/SO_2			
Pichia stipitis	Corn stover	0.41	0.79	Fenske *et al.* (1998)
	Dilute acid hydrolysis			
Saccharomyces cerevisiae	Softwood			Linden and Hahn-Hägerdal (1989)
	Na_2SO_3	0.34	0.26	
	Sulfite waste liquor	0.41	0.37	
Saccharomyces pombe	Sulfite waste liquor	0.30	0.21	Linden and Hahn-Hägerdal (1989)
Bacteria				
Clostridium thermocellum	SO_2-treated wood	0.08	0.01	Ng *et al.* (1981)
Clostridium saccharolyticum + *Zymomonas mobilis*	Aspen Acid hydrolysis	0.47	0.24	Murray and Asther (1984)
Clostridium thermocellum + *Clostridium thermohydrosulfuricum*	Steam-exploded wood	0.29	0.02	Ng *et al.* (1981)
Filamentous fungi				
Fusarium oxysporum	Wheat straw	0.40	0.20	Singh and Ghosh (1993)
	Acid hydrolysis			
Mucor sp.	Bagasse	0.33	0.16	Ueng and Gong (1982)
	Acid hydrolysis			

(*continues*)

TABLE VI (*Continued*)

Organism	Substrate/Pretreatment	Ethanol yield (g/g)	Ethanol productivity (*g/L/h*)	Reference
Recombinant organisms				
Escherichia coli K011	Corn fiber	0.41	1.16	Dien *et al.* (1997)
	Dilute acid hydrolysis			
Escherichia coli SL40	Corn fiber	0.42	1.12	Dien *et al.* (1997)
	Dilute acid hydrolysis			
Zymomonas CP4	Corn fiber	0.45	1.04	Bothast *et al.* (1999)
	Dilute acid hydrolysis			
Saccharomyces 1400	Corn fiber	0.50	1.60	Moniruzzaman *et al.* (1997)
	AFEX			

IX. Other Biological Processes for Energy Production

While ethanol is the dominant biofuel, processes for production of methane from municipal and agricultural wastes continue to be operated and developed. There is also renewed interest in an acetone–butanol process and in biological production of hydrogen.

A. Methane

Full-scale municipal landfill solid waste can produce 1000–2000 m^3 biogas/h with an energy content of 20–25 MJ/m^3 with methane and CO_2 contents of 55–70 and 30–45% by volume, respectively (Claassen *et al.*, 1999). In Denmark, annual production of biogas from available biomass resources is estimated at 25–30 PJ (700–800 million m^3 methane/year), with animal manure comprising 85% of the biomass (Tafdrup, 1994). H_2S is collected with the methane (200–4000 ppmv). Stabilized residues are used as soil conditioner/fertilizer. Biological production of methane is advantageous in that it involves sustainable use of organic waste, relatively cheap technology, and an additional energy source replacing electricity or fossil fuel. That an anaerobic process using methanogenic bacteria is susceptible to toxic compounds is a shortcoming, although it is less of a problem in anaerobic biofilms.

B. Acetone–Butanol

The acetone–butanol process, developed during World War I, was successfully operated for many years. Indeed, it was the second largest

fermentation process in first half of twentieth century, producing acetone for war-related activities and butanol for the lacquer industry. Currently, petroleum-based products have largely replaced these fermentation processes. Nevertheless, a production facility was operated until recently by National Chemical Products, South Africa, where petroleum was scarce due to the international embargo. The process is reported to be still operating in China (Durre, 1998). The main fermentation strains are *Clostridium acetobutylicum* and *C. beijerinckii*. The batch process, using starch or molasses as substrate, is followed by distillation. Barriers to its commercial viability include high substrate cost, low product concentration (20 g/liter) due to product toxicity, and high product recovery cost (distillation).

Interest in reviving the acetone–butanol process has gained momentum with increased knowledge of strain physiology and genetics, ability to use cheaper substrates (like whey and agricultural byproducts), and improvements in product recovery (Maddox *et al.*, 1993; Woods, 1995; Girbal and Soucaille, 1998).

Clostridia cannot degrade lignocellulose, so therefore physicochemical or enzymatic pretreatment is required. One approach involves simultaneous saccharification/fermentation systems by co-cultures of *C. cellulolyticum* or *C. thermocellum* and *C. acetobutylicum*, or use of cellulases plus *C. acetobutylicum*. A second approach involves using genetically engineering to create solvent-producing strains that simultaneously produce cellulases: by cloning the cellulases of *C. cellulolyticum* or *C. thermocellum* into *C. acetobutylicum* or *C. beijerinckii* (Minton *et al.*, 1993; Kim *et al.*, 1994). A third approach aims at increasing solvent production by other genetic manipulations and efforts to do this have shown some promise (Green and Bennet, 1998; Nair *et al.*, 1999; Parekh *et al.*, 1999).

C. HYDROGEN

The major advantage of hydrogen as a fossil fuel replacement is the lack of polluting emissions, since its use in fuel cells or combustion produces water. Microbial processes produce hydrogen from organic substrates through heterotrophic fermentation (Nandi and Segupta, 1998). A wide range of strains can be used including strict anaerobes (clostridia, ruminococci, and archaea), and facultative anaerobes including *E. coli* and *Enterobacter aerogenes* under anoxic conditions.

Light energy may be used to supplement chemical energy in processes known as photoheterotrophic fermentation. Microbes used in these processes include species of *Rhodopseudomonas* and *Rhodobacter*. Optimal hydrogen production may be obtained by growing the photosynthetic microbes in the dark followed by hydrogen formation

after exposure to light under anaerobic nongrowth conditions (Mao *et al.*, 1986; Sasikala *et al.*, 1993). Another approach is to produce CO from thermally gasified wood chips using a CO-linked hydrogenase in photosynthetic bacteria to convert CO_2 and H_2O into hydrogen (Uffen, 1976; Weaver *et al.*, 1998).

Biological production of hydrogen is at a very early stage of development. Hyperthermophiles offer perhaps the greatest potential since they produce biomass-degrading enzymes and the high temperatures can both protect against contamination and create a selective pressure for hydrogen production (Benemann, 1996).

X. Economics of Bioethanol Processes

The main factor impeding the biological productivity of commodity products is the high cost of current processing technology rather than the cost of raw materials. Challenges to development of a cost-competitive process may be grouped in terms of converting recalcitrant cellulose into reactive intermediates by overcoming the recalcitrance and converting reactive intermediates into useful products, using all the sugars and by-product value recovery of nonsugars (Lynd *et al.*, 1999; Kadam *et al.*, 2000).

A simultaneous saccharification and fermentation model has been developed by Gulf Oil–University of Arkansas. This model, which avoids the problem of cellulase product inhibition, is modified to exploit organisms capable of fermenting cellulose- and hemicellulose-derived sugars together. The process assumes a dilute acid (0.5%) pretreatment step, temperature of 190°C, 10 min residence time at a 22% solids concentration. Ethanol production is implemented in 18 × 950,000 gallon fermentors. Fermentation process design is based on bench scale results with *Z. mobilis* capable of fermenting glucose and xylose (Zhang *et al.*, 1995). Cellulase is used at a rate of 15 FPU/g cellulose. Cellulase production is implemented in eleven 264,000-gallon aerated 304-stainless stirred tank, nonsteam sterilizable fermenters giving an enzyme productivity of 75 FPU/liter/h. Total capital project investment was estimated to be $234 million (Wooley *et al.*, 1999).

The best industry near-term bioethanol production cost scenario, based on the current status of research and on a lignocellulose feedstock cost of $25/ton is $1.44/gal, higher than corn-derived ethanol at $1.20/gal. With improvements in the near term, it is argued that a 12% yield increase and some reduction in production cost could bring the lignocellulose derived fermentation ethanol price down to $1.16/gal. Best price predictions based on achievement of future research targets are Year 2005 with improved enzyme performance $0.94/gal; Year 2010 with development of improved ethanologens $0.82/gal; and Year 2015

with genetically engineered feedstock with higher carbohydrate content $0.76/gal.

There are some other challenges to the commercial viability of large-scale use of biomass in fermentation fuel ethanol production. Compared to petroleum, transport costs of biomass per energy unit are higher. Biomass supply is susceptible to weather fluctuations. There is a requirement for large volumes of water in both intensive production of biomass feedstock and in biomass environmental treatment. There are significant odor issues associated with distillation and drying in bioethanol operations. The greater molecular complexity of biobased materials requires that bioethanol processes are sufficiently robust and advanced to achieve high yields from variable raw materials. Volume of by-product from corn bioethanol is substantial, and the cost of its transport and distribution is high. The same drawback will apply to by-products from lignocellulose bioethanol production. Indeed, if raw materials used in lignocellulose feedstock are more variable than in corn ethanol, by-products will likewise be more variable, and their utilization in added value applications will require greater management of product specifications and standards. Finally, where feedstocks are from genetically engineered plants, there may be regulatory limitations on use of by-products as feed ingredients.

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Progress of *Aspergillus oryzae* Genomics

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I. Introduction

Very recently, the genome sequencing of *Aspergillus oryzae* has been initiated. The sequencing effort is being conducted through collaboration of several governmental institutes, universities, and companies in Japan. *Aspergillus oryzae,* which has a relatively large genome size for a microorganism, will require a relatively long time and great expense for the completion of the task.

Filamentous fungi have been widely used as hosts for the production of homologous and heterologous proteins (Christensen *et al.,* 1988; Barbesgaard *et al.,* 1992; Verdoes *et al.,* 1995; Gouka *et al.,* 1997) because of their great productive capacity with respect to the secretion of proteins (Finkelstein *et al.,* 1989). In addition, the bacterium, *Escherichia coli,* and yeasts including *Saccharomyces cerevisiae,* both of which have an advanced knowledge foundation established by extensive basic research, have been frequently used as hosts for heterologous protein production. However, proteins produced by *E. coli,* in which the proteins are produced in cytoplasm but are not secreted in general, often make insoluble "inclusion bodies," resulting in the loss of a majority of functional activity. Yeasts, on the other hand, can produce functional eukaryotic proteins, including those from plants and animals, through the secretory machinery compatible among eukaryotes. Yeasts can also glycosylate proteins at specific sites, which prokaryotic producers such as *E. coli* cannot do. However, the productivity of yeasts is

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generally insufficient for industrial production processes. Filamentous fungi, which like yeasts are eukaryotic microorganisms, have been used for the industrial production of many enzymes including *Mucor* renin (Gray *et al.,* 1986), phytase (van Gorcom *et al.,* 1991), lipase (Boel and Huge-Jensen, 1989; Huge-Jensen *et al.,* 1989), laccase (Saloheimo *et al.,* 1989), α-amylase (Cheng and Udaka, 1991), and alkaline protease. Filamentous fungi have a high potential for the secretory production of proteins. The productivity of enzymes is often several grams and reaches beyond 20 g in the case of glucoamylase from 1 liter culture medium.

Aspergillus oryzae, which is one of the most potent secretory producers of proteins among filamentous fungi, has been used for hundreds of years in Japanese traditional fermentation industries including oriental alcoholic beverages such as *sake* (rice wine) and *shochu* (spirits), *miso* (soybean paste), and *shoyu* (soy sauce). The long history of extensive use in the food industries placed *A. oryzae* on the list of Generally Recognized as Safe (GRAS) organisms by the Food and Drug Administration (FDA) in the United States (Tailor and Richardson, 1979). The safety of this organism is also supported by the World Health Organization (Food and Agriculture Organization/World Health Organization, 1987).

One remarkable feature of the traditional Japanese fermentation industries is the use of solid-state culture (*koji*), which allows *A. oryzae* to further enhance the productivity of proteins (Fig. 1). Steamed rice grains serve as the substrate for sake. Starch in the rice grain is degraded by amylases produced by *A. oryzae,* which is grown by sprinkling conidiophores on the surface of the steamed rice grains. Steamed ground soybeans together with ground wheat grains serve as the ingredient for soy sauce. Proteins and starch in the material are degraded into peptides or amino acids, mainly into maltose or glucose, respectively, by *A. oryzae* grown in a similar way as is done in the sake production. The solid-state culture used in the above fermentation often enhances enzyme production including amylases and proteases (Narahara *et al.,* 1982). It is said that the solid-state culture of filamentous fungi originated approximately 2000 years ago in China. The technology of the solid-state cultivation was imported to Japan at that period (*Yayoi* period) and was applied to food fermentation. Inocula from filamentous fungi for fermentation have been commercially available as koji seeds since A.D. 1400 (*Muromachi* period in Japan) (Murai, 1989). The inocula were produced by growing mycelia on the steamed rice grains mixed with wood ash which inhibited the contamination of other microorganisms and allowed efficient sporulation by supplementing minerals. *Aspergillus oryzae* can produce approximately 50 g of α-amylase from 1 kg of wheat bran, which is roughly equivalent to 1 l of the culture

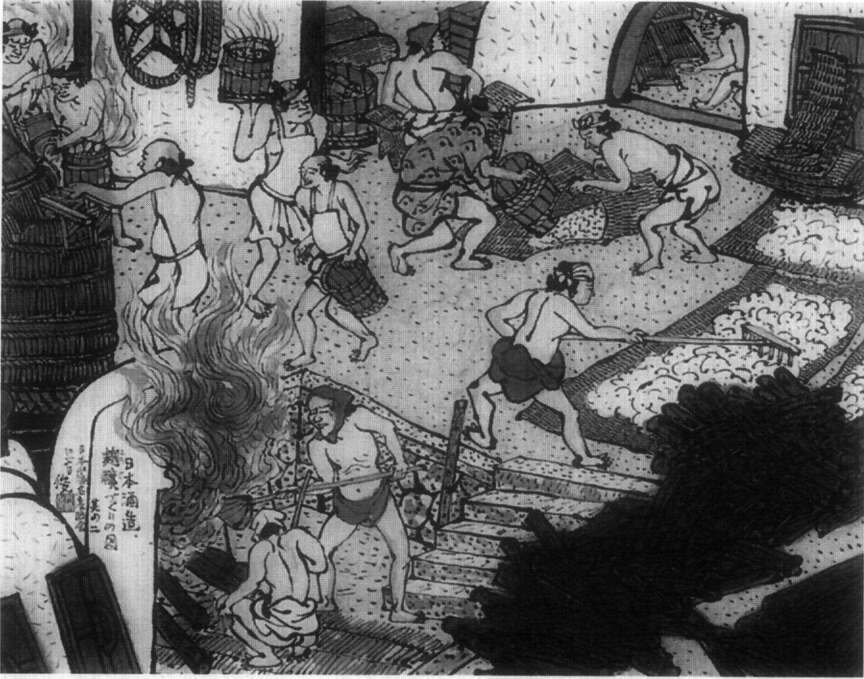

FIG. 1. Traditional fermentation process for sake brewing. Rice grains are steamed as shown in the bottom left section of the figure. Then koji is prepared by culturing *A. oryzae* mycelia on the steamed rice for approximately 48 h in the koji-making room, which is seen in the top right section of the figure. The *koji* is used as the ingredient for the successive fermentation stages mainly by yeast to produce alcohol. The traditional painting was kindly provided by Gekkeikan Sake (Kyoto, Japan).

medium. Studying the mechanism of enhancing enzyme yields in the solid-state culture is one of the most important issues not only for traditional fermentation industries but also for modern biotechnology.

In recent years, the technology for enzyme production by the traditional solid-state culture was applied to the industrial production of enzymes by microorganisms. For example, the Takadiastase production by the solid-state cultivation from wheat bran, first initiated by Dr. Jokichi Takamine in the 1890s and the first success in the industrial production of enzymes from microorganisms, has subsequently been extended to various other valuable enzymes including amylases and lipases. During the 20th century, development of liquid cultivation technology of filamentous fungi enabled the efficient production of enzymes in an industrial scale, resulting in a constant decrease in the use of solid-state culture production. However, the solid-state cultivation method, where productivity is often even higher than that of liquid cultivation, is still

preferable for the production of various enzymes. In spite of the high productivity, the solid-state culture method is more laborious and requires more skills than those required for liquid-culture production. A Japanese soy sauce company has developed a fully automated system for large scale solid-state cultivation. Improvement of the producing strains by biotechnology, together with improved mechanical engineering, is necessary further to improve the productivity.

The recent progress in genetic engineering has made it possible to apply molecular biology to industrial fungi. Transformation systems for the introduction of transgenes together with marker genes have been studied to harness *A. oryzae* for heterologous protein synthesis. Development of this transformation system of *A. oryzae* has made it possible to produce *Mucor* renin under the control of the α-amylase promoter, reaching a yield of approximately 3.3 g from 1 l of the medium (Christensen *et al.,* 1988). Although enzymes derived from other filamentous fungal species could be successfully produced in the heterologous hosts in industrially applicable amounts, some proteins derived from higher eukaryotes, such as human lactoferrin (Ward *et al.,* 1992), human lysozyme (Tsuchiya *et al.,* 1992), calf chymosin (Boel and Huge-Jensen, 1989; Tsuchiya *et al.,* 1993), or plant thaumatin (Hahm and Batt, 1990), were produced just from 50 μg to 25 mg of the 1 l culture. To improve the productivity of the proteins from higher eukaryotes, calf chymosin (Korman *et al.,* 1990; Dunn-Coleman *et al.,* 1991), calf phospholipase A2 (Roberts *et al.,* 1992), and human interleukin 6 (Contreras *et al.,* 1991) were fused at the carboxyl terminus of the *Aspergillus niger* glucoamylase or *Aspergillus awamori* α-amylase, which was used as a carrier. The productivity of the mammalian proteins was significantly improved by this modification. Furthermore, the fungal protein carriers could be processed off during secretion by inserting a –Lys–Arg– linker between the two proteins (Archer *et al.,* 1992) probably due to the yeast KEX2 proteinase-like processing activity existing in *A. oryzae.* This technique opens the possibility that higher eukaryotic proteins can be secreted in amounts similar to those of fungal proteins. Although technical barriers exist for the production of heterologous proteins, especially for human proteins of great medical value, filamentous fungi are robust hosts for protein production.

Aspergillus oryzae is used in the commercial production of industrially valuable enzymes, amylases, proteases, lipase, and so on, prompting extensive research on *A. oryzae,* its industrially important enzymes, and their corresponding genes. Since *A. oryzae* lacks a sexual generation in its life cycle and since it is difficult to obtain mutants due to the existence of multiple nuclei in conidia, traditional genetics is not

applicable to an extensive analysis of *A. oryzae* genes. The lack of basic knowledge about the *A. oryzae* genes and their organization has become a significant barrier to expanding the application of *A. oryzae* to modern biotechnology in spite of its prominent potential in industrial use.

II. Study of *A. oryzae* Genes

Gene cloning and sequencing generate fundamental information for genome sequencing. This section briefly summarizes the progress of sequencing *A. oryzae* genes in relation to the field most extensively studied for this organism.

A. Industrially Important Genes

Since *A. oryzae* has been exploited for the degradation of starch and proteins in the traditional Japanese fermentation industries, the hydrolytic enzymes have been studied most extensively. *Aspergillus oryzae* produces many enzymes that degrade various biomolecules of high molecular mass such as carbohydrate, polypeptide, and nucleic acids. α-Amylase randomly breaks down α-1,4-glycosyl bonds in starch yielding dextrin. Glucoamylase removes a glucose unit from the nonreducing terminus of dextrin chains, and then the product serves as a carbon source for alcohol production by yeast. The *A. oryzae* strains commonly used for research possess three α-amylase genes in the genome (Kitamoto *et al.*, 1994), probably because *A. oryzae* strains with strong glucose-producing activity have been screened down through the ages for efficient alcohol production in sake brewing. The *endo*-proteinases and *exo*-peptidases play the most important roles in yielding amino acids during soy sauce production from the proteins in soybeans and wheat grains. Since soy sauce productivity depends on how efficiently the proteins are utilized, strains possessing strong proteinase and peptidase activities have been screened and selected. In addition, many hydrolytic enzymes including nucleases, lipases, and amylases play important roles in the efficient production, and taste and flavor, of the products. Although yeasts and/or lactic acid bacteria that subsequently ferment after *A. oryzae* also significantly affect productivity and quality of the product, fermentation by *A. oryzae* is a key process in the entire process.

There are approximately 2000 sake brewers in Japan. The sake producers have their own tastes, which are clearly distinguishable from one another. Larger brewers may produce several lines of sake with different tastes or grades (more than 100 varieties in the case of a big company).

Interestingly, there are small variations in *A. oryzae* strains distributed for the brewing of sake, although soy sauce companies have their own strains selected by extensive breeding to increase protein-degrading activity. Furthermore, there are little choices for yeast and rice strains for the sake brewing. The superior taste of good-quality liquor is produced by the skillful control of the fermentation, mainly at the koji making stage, so the fermentation process still requires specialists with a wealth of experience. The decreasing number of specialists, and the danger of their aging without passing their knowledge onto the next generation, is a serious problem. Automated fermentation equipment with number of different sensors and controllers of various factors including temperature, pH, and glucose concentration have been developed to replace this specialized and laborious traditional work with a modern and standardized system.

Approximately 25% of the total genes in the genome of lower eukaryotes such as yeast are unexpressed or uninduced under ordinal culture condition in a laboratory (Naitou *et al.*, 1997). This means that one forth of the genes, an estimated 2000–3000 for *A. oryzae,* may remain unused under laboratory culture conditions. Therefore, these genes have little chance to be detected by the expressed sequence tag (EST) analysis, but could be identified by whole genome sequencing. It is expected that genes encoding valuable enzymes, which have not yet been known for the ordinary ingredients such as starch or protein, may be found in the genome because filamentous fungi have potential to degrade a variety of materials in general. These hitherto unidentified genes will be of great value to industry because *A. oryzae* is regarded as safe.

B. Transcription Regulation

It has recently become possible to prepare transformants with enhanced enzyme production capabilities by introducing multiple copies of a gene. Industrially valuable amylases produced by *A. oryzae,* including Taka-amylase, glucoamylase, and α-glucosidase, encoded by *amyB, glaA,* and *agdA,* respectively, have been cloned and produced at enhanced levels of expression in this way (Hata *et al.*, 1991; Tada *et al.*, 1991; Minetoki *et al.*, 1995). Minetoki *et al.* (1998) demonstrated that the introduction of multiple copies of Region III, the short regulatory element of the *amyB* promoter, into the *A. oryzae agdA* promoter dramatically enhanced the expression of the *agdA* gene (Minetoki *et al.*, 1998). On the other hand, the expression of both the *amyB* and *glaA* genes in the transformant was significantly repressed. These results suggest that the regulation of these amylase genes is governed by a common

transcription regulatory factor. The *amyR* gene encoding the transcription regulatory factor common to these amylase genes was obtained by screening the transformant in which the repression of the *amyB* and *glaA* expression by the introduction of the multiple short regulatory elements was restored (Gomi *et al.*, 2000). Further, these results indicate that an analysis of the relationship in transcriptional control among a number of genes is important for the artificial regulation of the genes of interest. Comprehensive analysis of transcriptional regulation will enable scientists to figure out the transcriptional network and to predict the response of *A. oryzae* to artificial modifications under industrial settings.

Many other species of *Aspergillus* produce extracellular proteases that are employed for protein degradation in the fermentation industries. Strains with increased protease activity have been screened to enhance productivity during the long history of traditional fermentation industries. On the other hand, the production of intrinsic proteases is undesirable for the use of *A. oryzae* as a host in the heterologous protein production because the proteases degrade the protein to be produced. Actually, the productivity of hen egg lysozyme secreted from the engineered *Aspergillus niger* decreased when cultivated in a rich medium (Archer *et al.*, 1990). The degradation of the enzyme occurred only when the enzyme was mixed with the supernatant of the medium after cultivation. Use of a medium that did not induce protease synthesis, and the addition of a protease inhibitor, increased the productivity of the enzyme. Therefore, efforts have been made to reduce protease activities by classical mutagenesis. The mutant *A. niger* strain that lacked the acid protease, aspergillopepsin, produced more chymosin than the wild-type strain. However, the remaining acid protease activity, insensitive to pepstatin, still degraded some proteins (Archer *et al.*, 1992). Marttern *et al.* prepared the mutant *A. niger* strain, which had very low activity of many proteases including aspergillopepsin and neutral proteases (Mattern *et al.*, 1992). The strain had increased productivity of lysozyme even after cultivation for 2–3 weeks (Jeenes *et al.*, 1993). However, the preparation of a multiple protease deficient mutant by random mutagenesis is likely to affect other phenotypes necessary for fermentation. *Aspergillus nidulans* was predicted to have approximately 80 protease genes in its genome (Gavrias *et al.*, 1999). The artificial control of a small number of transcription factors necessary for the protease expression seems a better way to reduce undesirable protease activity than the multiple disruption of undesirable protease structural genes. Understanding the transcriptional regulatory mechanisms in detail, including the factors regulating transcription, will facilitate the engineering of both desirable and undesirable enzymes for industries.

III. *A. oryzae* Genomics

A. Overview of Genomics of Filamentous Fungi

The far-reaching goal of sequencing the entire human genome was initiated in 1985. In addition, microorganisms that had already been extensively studied such as *E. coli* and *Saccharomyces cerevisiae* were selected as models for the pioneering work. The genome sequencing was accelerated by rapid advances in sequencing technology in the 1990s. As a result, the entire genome sequence of the yeast, *S. cerevisiae* (Mewes *et al.,* 1997), was published in 1996; *E. coli* (Blattner *et al.,* 1997) and *B. subtilis* (Kunst *et al.,* 1997) became public in the following year. Genome sequencing of higher eukaryotes with a genome size much larger than microorganisms, *Caenorhabditis elegans* (The C.elegans Sequencing Consortium, 1998), *Drosophila melanogaster* (Adams *et al.,* 2000), and *Arabidopsis thaliana* (The Arabidopsis Genome Initiative, 2000), also became public in 1998, 2000, and 2000, respectively. Excitingly, the draft sequence of the human genome became public at the beginning of 2001, the first year of the twentieth century. Simultaneously, development of the whole genome shotgun strategy enabled the quick completion of microorganisms with small genomes [i.e., approximately 1–12 Megabase (Mb)]. The hierarchical approach analyzes the sequence of a DNA fragment typically 15–300 kilobase (kb) in length cloned on a lambda, a cosmid, or bacterial artificial chromosome (BAC) vectors, one by one. Before the sequencing, the positions of the cloned fragments on the chromosomes are analyzed, and the clones with minimum overlaps are selected serving as a template for the sequence analysis. Although the sequencing performance has been dramatically improved, mainly owing to the development of a capillary array sequencer, preparation of the clones having minimum overlaps still takes a long time and requires laborious work. The whole genome shotgun sequencing approach omits this time-consuming tiling step, enabling the completion of genome sequencing in a short period. Since Archaea have small genome sizes (0.5–2 Mb) and have GC contents suitable for the widely used Sanger sequencing protocol in general, the genomes of more than 5 species of archaea have been completely determined by the whole genome shotgun approach to date (Bult *et al.,* 1996; Klenk *et al.,* 1997; Smith *et al.,* 1997; Kawarabayasi *et al.,* 1999; Ng *et al.,* 2000). Development of the high-performance algorithm of multiple alignment (Ewing *et al.,* 1998; Ewing and Green, 1998; Gordon *et al.,* 1998; Gordon *et al.,* 2001) has made the whole genome shotgun sequencing applicable to organisms with larger genome size. Genomics venture companies have revealed that the whole genome shotgun is highly effective in the analysis of higher eukaryotes including human and plant for industrial

use—that is, fishing out industrially important genes and then applying for a patent on them. Approximately 90% of the entire nucleotide sequence of a fungi genome of 30 Mb in size could be completed within one month by a sequencing facility with 30 capillary array sequencers, appropriate peripherals, and staff.

The genome size of most filamentous fungi is estimated to be 30–40 Mb, and expected to encode 9,000 to 13,000 genes. Since most of the filamentous fungal genomes consist of several chromosomes ranging in size from 2 to 10 Mb, the entire genome may be sequenced similarly to those of archaea and bacteria without significant improvement of the equipment, protocol, and computers when the chromosomes are analyzed one by one. The number of sequence reads 5–10 times larger than those in the case of archaea and bacteria genomes are required in total for the completion of the filamentous fungal genome. The progress of fungal genomics has been reviewed in detail by Bennett and Arnold (Bennett and Arnold, 2001). Important background and recent progress of the genomics of filamentous fungi is briefly surveyed below in relation to *A. oryzae* genomics.

1. Aspergillus nidulans

The genome sequencing of the genetic model, *A. nidulans,* had been extensively discussed since 1996 and was initiated by the collaboration of researchers in the United States, Europe, and Asian countries including Korea and Japan. A cosmid, SW06E08 on chromosome VIII, was sequenced by Prade *et al.* at Oklahoma State University (Stillwater, OK) as the first step (Kupfer *et al.,* 1997), and the neighboring cosmid, SW06H01 (GenBak ID: AF188714), was sequenced by Akeno *et al.* at the National Brewing Institute (Higashi-Hiroshima, Japan) and Kunihiro *et al.* at the Advanced Institute of Industrial Science and Technology (AIST) (Tsukuba, Japan). N. Keller at the University of Texas A&M University [College Station, Texas, moved to Wisconsin (Madison, WI)] sequenced the chromosome IV of *A. nidulans.* The sequences are downloadable at Asergillus-Genomics.org (http://aspergillus-genomics.org/groups/bioinfo/PipeOnline_db/ancivsearch.fls/acivdownload.html). A large number of ESTs has also been collected by D. Kupfer at the University of Oklahoma (Norman, OK) in collaboration with R. Prade and R. Aramayo at Texas A&M University. They have prepared a unigene database that is available from their web site with the annotation searched by BLASTX of GenBank (http://www.genome.ou.edu/fungal.html). Cereon Genomics announced the completion of a proprietary *A. nidulans* genome sequencing project in August 1998 by a whole genome shotgun approach. They have collected the sequence of approximately 3× depth of coverage of the *A. nidulans* genome (Gavrias

et al., 1999). Recently, the sequence became publicly available, but only for academic use at public organizations, from the Cereon web site (http://microbial.cereon.com/).

2. Aspergillus fumigatus

Recently, Elitra Pharmaceuticals at San Diego, California announced the completion of genome sequencing of *Aspergillus fumigatus,* a major human fungal pathogen, by accumulating approximately 10× depth of coverage in collaboration with Celera Genomics (Rockville, MD) in May 2001 (http://www.elitra.com/). Excluding the private sectors, *A. fumigatus* genome sequencing work is also underway at the Pasteur Institute, the Sanger Centre, and The Institute for Genomic Research (TIGR) (Rockville, MD) coordinated by D. Denning at The University of Manchester (Manchester, UK). They have already collected 4× sequence coverage of *A. fumigatus,* the clinical isolate Af293, and these data are searchable at http://www.tigr.org/cgi-bin/BlastSearch/blast.cgi?organism = a_fumigatus.

3. Aspergillus niger

The Gene Alliance (Geleen, The Netherlands and Hilden, Germany), a division of DSM (Amsterdam, The Netherlands) has completed an *A. niger* genome sequencing project during 2001 using a BAC by BAC sequencing approach. The Gene Alliance consists of five German and Dutch companies, QIAGEN GmbH (Hilden), GATC Biotech AG (Konstanz), AGOWA GmbH (Berlin), Biomax Informatics GmbH (Munich), and MediGenomix GmbH (Munich). Genencor has announced gaining access to the *A. niger* genome sequence data of Integrated Genomics (http://www.genencor.com/webpage_templates/sec.php3?page_name=pr_1001434970).

4. Aspergillus flavus

Aspergillus flavus EST data are accessible through http://www.genome.ou.edu/fungal.html at the University of Oklahoma. A physical map has been partly constructed by Kelkar and Arnold at the University of Georgia (Bennett and Arnold, 2001).

5. Neurospora crassa

The genome sequencing of *N. crassa* has been conducted at six German universities coordinated by U. Schulte at Heinrich-Heine University (Düsseldorf, Germany) and at the Whitehead Institute (Cambridge, MA) by sequencing cosmid/BAC clones and by the whole genome shotgun approach. A complementary DNA (cDNA) sequencing project was also initiated at the University of New Mexico and Oklahoma State University. The draft of the entire genome was released

in February 2001. The improved assembly after the addition of end sequences from cosmid and BAC libraries has been released in September 2001 (http://www. genome.wi.mit.edu/annotation/fungi/ neurospora/background.html).

In general, genome sequences accomplished at a private company are proprietary. The sequence may be available only for public organizations only under special agreements that a part of the results and intellectual properties obtained from the sequence belong to the company.

B. Current Progress of *A. oryzae* Genomics

Large-scale EST sequencing efforts were initiated by the collaboration of M. Machida at the National Institute of Advanced Industrial Science and Technology (AIST) (Tsukuba, Japan), O. Akita at the National Research Institute of Brewing (NRIB) (Higashi-Hiroshima, Japan), Y. Kashiwagi at the National Food Research Institute (NFRI) (Tsukuba, Japan), T. Kobayashi at the Nagoya University (Nagoya, Japan), N. Kitamoto at the Food Research Institute of Aichi Prefectural Government (Nagoya, Japan), K. Kitamoto and H. Horiuchi at The University of Tokyo (Tokyo, Japan), M. Takeuchi at Tokyo University of Agricultural Technology (Tokyo, Japan), and K. Gomi and K. Abe at Tohoku University (Sendai, Japan). The project was partly supported by private companies, Amano Enzyme (Nagoya, Japan), Ozeki (Nishinomiya, Japan), Gekkeikan Sake (Kyoto, Japan), Higashimaru (Tatsuno, Japan), Higeta (Chyoshi, Japan), Kikkoman (Noda, Japan), Yamasa (Chyoshi, Japan), Miso-Kyokai (the society of soybean paste producing companies), and Tanekoji-Kumiai (the association of koji seed companies that produce *A. oryzae* conidiophores). Most of these companies have relations to traditional Japanese fermentation industries.

For the sequencing of ESTs, the *A. oryzae* strain RIB40 (ATCC-42149) was selected. In general, soy sauce companies have their own strains, selected after extensive breeding, most of which are patented. *Aspergillus oryzae* RIB40 is a wild-type strain, similar to those used for sake brewing, but still having the ability to produce proteinases, an important characteristic for soy sauce fermentation. Messenger RNA (mRNA) was prepared from *A. oryzae* mycelia grown in several different culture conditions including in complete medium, at high temperature and without any carbon source (see Table I). It was expected that the chance of finding new genes from a limited number of ESTs will be increased using these different culture conditions. In addition, two libraries were prepared from the mycelia grown in solid-state culture, using rice bran or wheat bran. More amylases and proteases are produced in solid-state culture than in liquid culture; thus, the information from the libraries

TABLE I

A. oryzae ESTS ANALYZED TO DATE

Culture condition	Analyzed ESTs
Liquid complete medium (+glucose)	2,693
Liquid complete medium (+glucose, 37°C)	2,072
Liquid synthetic medium (−glucose)	1,953
Liquid complete medium (+maltose)	932
Liquid complete medium (pH 10)	751
Solid-state cultivation (wheat bran)	6,309
Solid-state cultivation (Shoyu koji 25°C)[a]	1,049
Liquid complete medium (germination)	1,049
Total	16,808

[a] Solid-state cultivation is the same as that using wheat bran except that a mashed and steamed complex of soybean and wheat was used as the medium instead of mashed and steamed wheat.

is expected to be important for improvement of the productivity of *A. oryzae*.

cDNAs synthesized from the mRNA using oligodeoxythymidine (oligo(dT)) as a primer were unidirectionally inserted onto plasmid vectors such as pBluscript and were sequenced specifically from their 5′-termini. Although protein-coding regions can be more effectively sequenced by sequencing from a 5′-terminus than from a 3′-terminus, the truncation of cDNAs often caused by the nuclease digestion of mRNA during preparation and by incomplete synthesis by reverse transcriptase may result in incomplete clustering based on the sequence similarity. This causes an overestimation of the number of genes discovered by the EST sequencing. To decrease the overestimation, the average insert size of the cDNA library was maintained for as long as possible, typically exceeding 1.5 kb. Furthermore, the long-read protocol of LI-COR (Lincoln, NE), the average sequenced length of which was greater than 800 bases (b), was used for approximately one third of the total ESTs. Distribution of sequence lengths of ESTs and that of contigs after assembling are shown in Figure 2. The number of clones with a relatively short size of the inserted cDNA increased in the final phase of the project because of the use of a culture condition from which mRNA preparation was much more difficult than from the liquid culture. The total number of ESTs sequenced to date is 16,808 and the total length analyzed reached 9.83 Mb with the average length of a single-pass sequence being 585 b (unpublished data). After clustering, the total number of the nonredundant sequences was approximately 6,000 (Fig. 3) with the total length of the contigs (nonredundant sequence) being 4.5 Mb. The length of the nonredundant sequence was equivalent

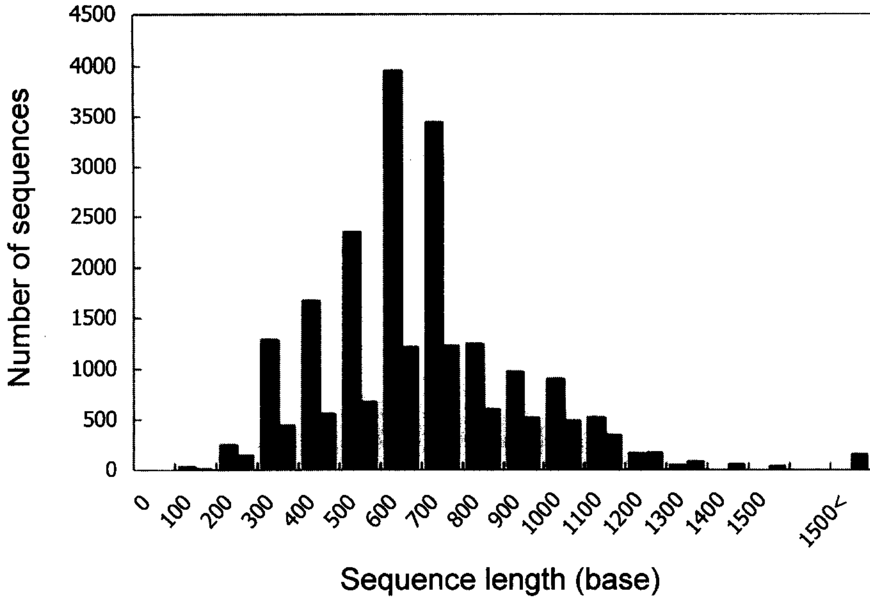

FIG. 2. Distribution of sequence lengths of ESTs and contigs. Hatched and solid bars indicate the distribution of the sequence length of ESTs and contigs after clustering and successive multiple alignment. Average lengths of the ESTs and contigs are 585 and 671 b, respectively. Approximately 140 contigs greater than 1.5 kb in length appeared after clustering.

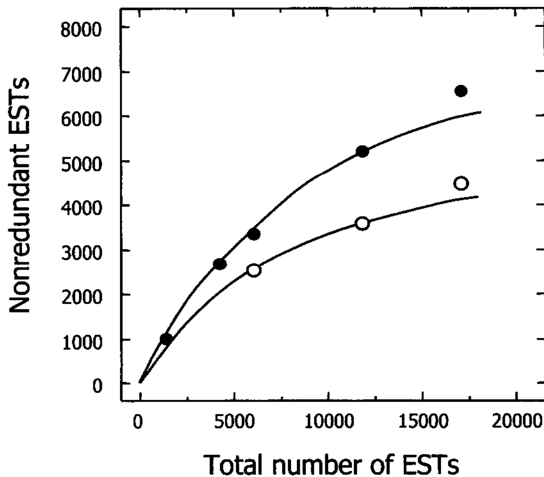

FIG. 3. Growth of nonredundant ESTs vs total number of analyzed ESTs. The *A. oryzae* ESTs were clustered according to the overlap of their nucleotide sequences. Closed and open circles designate the numbers of resulting contigs and singletons, respectively.

to 13–15% of the *A. oryzae* genome (estimated size = 30–35 Mb). Thus, the nucleotide sequence of approximately 40% of the protein coding region has been sequenced, assuming that the average length of the protein coding region is 1.5 kb as in *S. cerevisiae*. The total number of genes already sequenced has not been precisely determined due to possible incomplete clustering as mentioned above. Considering the number of contigs after clustering, and the ratio of already sequenced protein coding regions, approximately 50% of the total genes are estimated to have been sequenced. The polypeptides encoded by 47% of the contigs had significant homology to those found in the public database by a BLASTX search. The contigs of the *A. oryzae* ESTs are made available from the Database of Genomes and Transcriptional Regulations for Filamentous Fungi on the web site of the Research Information Database (RIO-DB) at the Advanced Institute of Industrial Science and Technology (AIST) (http://www.aist.go.jp/RIODB/ffdb/index.html).

The contigs were sorted according to the number of ESTs involved in the contig and were plotted vs the redundancy of the ESTs (Fig. 4). The number of highly expressed ESTs (i.e., highly expressed genes) is less than 500, which is roughly 5% of the total number of estimated

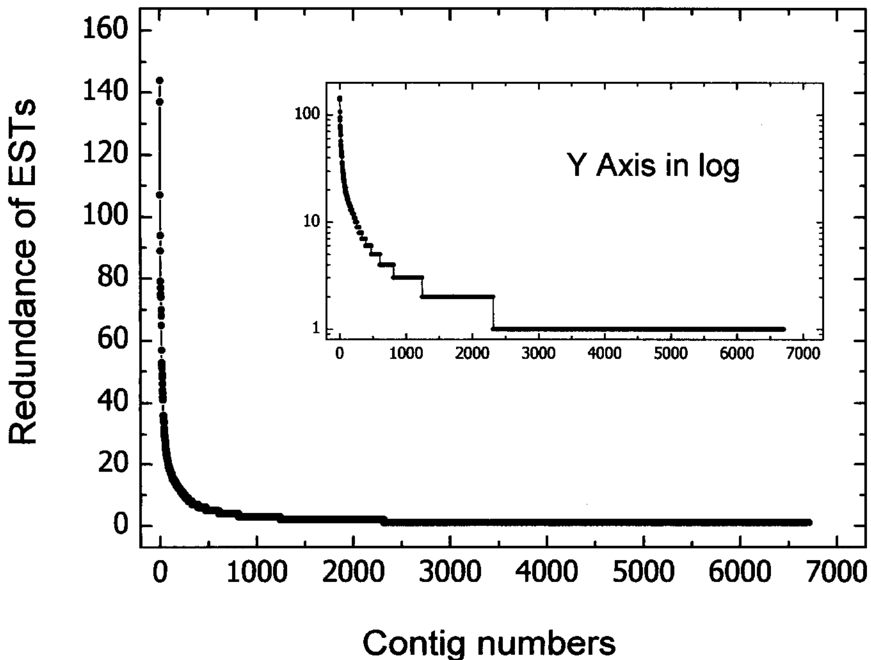

FIG. 4. Redundance of ESTs in each contig. Contigs were sorted according to the number of ESTs included in each contig. The redundance of ESTs is plotted vs contigs. The small graph was redrawn using a logarithmic scale for the *Y* axis instead of a linear scale.

A. oryzae genes. The same result was obtained from the ESTs from any particular condition, but also from all the ESTs sequenced using the 8 different libraries. The number of the highly expressed genes, which were found to express higher than 10% of the gene with the highest expression, was only 167, which was 2.5% of the total number of contigs. The number of singletons still occupied 66% of the total contigs, even after the collection of 16,808 ESTs from various culture conditions.

The frequently observed ESTs are thought to be highly expressed in the particular condition under which the mycelium was cultivated. Because strong and/or inducible promoters are useful for the expression of genes artificially introduced into the cell, high-expression ESTs can be used for identifying new strong promoters. Based on the observation that nearly half of the top 20 ESTs with the highest expression were glycolytic genes and other so-called house keeping genes, all the glycolytic genes were cloned from *A. oryzae* and the entire protein coding regions were sequenced (Nakajima *et al.*, 2000b). Among these 12 glycolytic genes cloned from *A. oryzae,* six genes were found among approximately 1,400 ESTs that had been sequenced until that time. Amino acid identities between *A. oryzae* glycolytic genes and those from other fungi were 41–72, 58–78, 81–92, and 86–93%, for *S. cerevisiae, N. crassa, A. nidulans,* and *A. niger,* respectively (Nakajima *et al.*, 2000b). Before the analysis of ESTs, the cloning of genes from *A. oryzae* was most frequently achieved by using degenerate primers and PCR. Primers were designed by taking advantage of the amino acid and/or nucleotide sequences conserved among the corresponding genes from other organisms (Nakajima *et al.*, 2000a; Sano *et al.*, 2000).

For comprehensive analysis of the nucleotide sequence of *A. oryzae* genes and their organization on the chromosomes, we started to promote a *A. oryzae* genome sequencing project in 1998. At the same time, we started the large-scale EST sequencing project. The *A. oryzae* genome consists of 8 chromosomes ranging from 2.8 to 7 Mb in length, and is estimated to have a total genome size of 35 Mb (Kitamoto *et al.*, 1994) as shown in Figure 5. The shortest band at 2.8 Mb has approximately 2 times stronger intensity than other bands, indicating the band derives from two chromosomes, VII and VIII. The second shortest band (VI) is obviously weaker than the others and is smeared. The band was found to hybridize with the DNA fragment having a ribosomal DNA (rDNA) sequence from *A. oryzae* (Chang *et al.*, 1991). These results strongly suggest that chromosome VI possesses rDNA, which repeats in variable numbers as is observed in chromosome XII of *S. cerevisiae* (Rustchenko *et al.*, 1993) and in the other organisms (Ritossa, 1968; Russel and Rodland, 1986).

We have constructed an *A. oryzae* genomic DNA library containing *Hin*dIII or *Eco*RI complete digests specific to chromosome V.

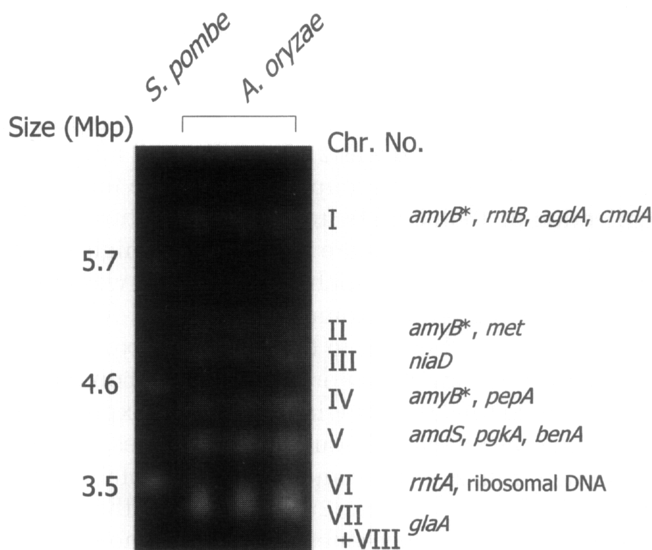

FIG. 5. Pulse field gel electrophoresis of the *A. oryzae* chromosomes. Protoplasts and agarose plugs containing *A. oryzae* chromosomes were prepared according to the method as described by Kitamoto *et al.* (1994). The *A. oryzae* chromosomes were electrophoresed on a 0.8% agarose gel in 0.5× Tris-acetate/EDTA buffer by CHEFF Mapper (Bio-Rad, Hercules, CA) at 1.5 V/cm with 50 and 45 min intervals of angle switch (60°/−60°) for an initial 36 h and the successive 300 h, respectively. The temperature was kept at 12°C. After electrophoresis for 14 days, the migration positions of the chromosomes were visualized by ethidium bromide staining under UV irradiation. The chromosomes from *Schizosaccharomyces pombe* were used as the size standard. The chromosome numbers and the genes already identified on each chromosome are indicated on the right side of the gel image. There are three α-amylase genes with more than 98% sequence similarity to each other in the *A. oryzae* genome. The *amyB**, which was first identified as the gene encoding Taka-amylase, represents one of the three α-amylase genes, *amyA*, *amyB*, and *amyC*. The other genes encode proteins as follows: *agdA*, α-glucosidase; *amdS*, acetamidase; *benA*, β-tubulin; *cmdA*, calmodulin; *glaA*, glucoamylase; *met*, methionine synthesis; *niaD*, nitrate reductase; *pepA*, acid protease; *pgkA*, phosphoglycerate kinase; *rntA*, ribonuclease T1; *rntB*, ribonuclease T2. The genes were assigned on each chromosome by Kitamoto *et al.* (1994).

Approximately 500 clones were selected and sequenced from either or both ends of the inserts, yielding 525 single-pass sequences of chromosome V (unpublished data). The total analyzed sequence was 423 kb with an average length of 807 b. Approximately 35% of the sequences had sequence similarity with nucleotide sequences in the public databases. No sequences were markedly difficult to sequence such as a long-repeated sequences or sequences with an extremely high or low GC content. The overall GC content was 45.6%. These results indicate that the *A. oryzae* genome is an appropriate target for a large-scale sequencing project.

Very recently, a whole genome sequencing project for *A. oryzae* was launched at the National Institute of Technology and Evaluation (NITE) (Tokyo, Japan) by the cooperation of The Consortium for *A. oryzae* Genomics consisting of the National Institute of the Advanced Institute of Industrial Science and Technology (AIST), the National Research Institute of Brewing (NRIB), the National Food Research Institute (NFRI), The University of Tokyo, Tokyo University of Agricultural Technology, Tohoku University, Nagoya University, Axiohelix (Tokyo, Japan), Amano Enzyme, Gekkeikan Sake, Higeta, Intec Web and Genome Informatics (Tokyo, Japan), Kikkoman, Kyowa-Hakko Kogyo (Tokyo, Japan), Ozeki, and the Brewing Society of Japan (Tokyo, Japan). The DNA libraries are being prepared by AIST, NRIB, and NFRI, and the large-scale sequencing is being done by NITE. The members of the consortium will focus on the analysis of gene function and the utilization of data derived from the *A. oryzae* genome.

Because there are no genetic maps of the *A. oryzae,* the sequencing will be done mainly by a whole genome shotgun sequencing approach in combination with some chromosome-specific shotgun sequencing. Because the *A. oryzae* chromosomes have sizes similar to those of bacterial genomes, they may be analyzed in the same way as bacterial genomes. A rough draft of the *A. oryzae* genome was completed in January 2002 by accumulating sequences of approximately 6× depth of coverage. Tentatively, the total genome size of *A. oryzae* was estimated to be 37 Mb. The completely fixed sequences will be published for each chromosome without significant delay.

C. Related Technologies and Applications

1. *Gene Manipulation and Disruption*

Gene manipulation in *A. oryzae* can be achieved by incubating *A. oryzae* protoplasts with DNA in the presence of polyethylene glycol (Gomi *et al.,* 1987; Iimura *et al.,* 1987). The introduced DNA is integrated into chromosomes through homologous/nonhomologous recombination or double crossover displacement. There are nearly 10 host-marker gene systems available, among which *niaD* (Unkles *et al.,* 1989), *argB* (Berka *et al.,* 1990), *pyrG* (Ruiter-Jacobs *et al.,* 1989; Wu and Linz, 1993) and *amdS* (Gomi *et al.,* 1992) based systems are preferred. Recently, a pyrithiamine resistant gene (*ptrA*) was successfully used as a dominant selectable marker for the transformation of wild-type *A. oryzae* strains (Kubodera *et al.,* 2000). The typical transformation efficiency of *A. oryzae* is 10–100 transformants per microgram of plasmid DNA. The low transformation frequency is apparently due to the integration of DNA into an indispensable gene on the chromosome and/or

the removal of the marker DNA during integration. The autonomously replicating fragment, *AMA1*, from *A. nidulans* functions in *A. oryzae*, and is able to maintain the external DNA without integration into the chromosome and to allow a 30-fold increase in the transformation efficiency (Gems *et al.*, 1991).

Gene disruption is one of the most important approaches for analyzing the function of genes. Unfortunately, disruption of *A. oryzae* genes is very difficult and it takes a long time to obtain disruptants because of the low efficiency at both transformation and homologous recombination. Furthermore, complete disruption is not guaranteed due to the existence of multiple nuclei in *A. oryzae* cells and conidiophores (Ushijima and Nakadai, 1987; Ushijima *et al.*, 1990). Even after purification by several single-spore isolations making the nuclei homogeneous, the transformed cell may still possess a nucleus in which the target gene remains undisrupted. To date, there are only a limited number of reports of successful disruption of *A. oryzae* structural genes (Gomi *et al.*, 1991; Doumas *et al.*, 1998). Recently, however, a novel method for the efficient disruption of *A. oryzae* genes has been developed by Hatamoto *et al.* (1999a). The vector consists of a positive selection marker, a negative selection maker and a marker for indicating the copy number of the integrated gene, which are *pyrG*, *oliC31*, and the polyphenol oxidase (laccase) gene (Hatamoto *et al.*, 1999b), respectively. The two markers, *pyrG* and the laccase gene, were put between the two DNA fragments derived from upstream and downstream of the target gene, yielding a cassette to be integrated in the genome. The negative selection marker, *oliC31*, is located outside the integration cassette and is removed during homologous recombination. The transformants that possess the integration cassette are positively screened in the presence of 5-fluoroorotic acid using the *pyrG*-disrupted *A. oryzae* strain as a host. The transformants with the cassette by homologous integration are selected on the medium containing triethyltin as Ward *et al.* reported (1986). The transformants with the darkest brown color grown on the plates containing gallic acid were selected. When the brown color reaches a plateau after several rounds of color selection, the transformants are thought to have the cassette in all the nuclei in their cells (Hatamoto *et al.*, 1999a).

An alternative approach to inactivate a particular gene of interest is to use an anti sense technique. For example, the carboxy peptidase (CPase O) structural gene inserted between its promoter and terminator in the reverse orientation was introduced into *A. oryzae*. The resultant transformant showed approximately 30% activity of the wild-type and a higher level of lysozyme expression than that obtained with the wild type (Zheng *et al.*, 1998). The anti sense control is also applicable to conditional inactivation of indispensable genes; however, expression of the target gene cannot be completely suppressed.

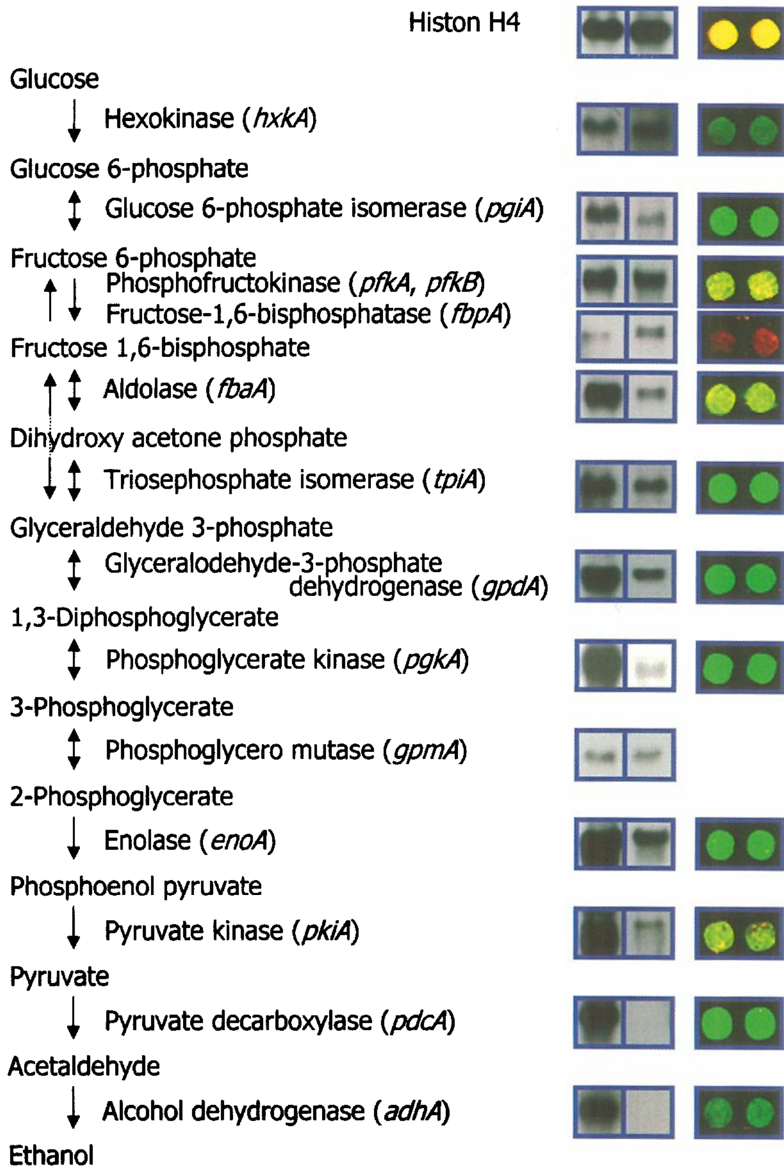

FIG. 6 Expression profiling of *A. oryzae* glycolytic genes by Northern hybridization and a cDNA microarray. *Aspergillus oryzae* cDNAs were amplified by PCR using the *A. oryzae* EST clones as templates and were spotted onto a glass slide. The mRNAs from mycelia grown in the modified CD medium (Czapek-Dox medium with 1% polypepton) (Hata *et al.*, 1992) containing 3% glucose and CD medium without any carbon source were labeled by Cy3 and Cy5 fluorescent dyes, respectively, combined, and then were hybridized to the cDNAs immobilized on the slide. The results from duplicate spots of the DNA microarray for each glycolytic gene (H. Maeda, Y. Maruyama, K., Abe, K. Gomi, F. Hasegawa, M. Sano, M. Machida, T. Akao, O. Akita, T. Nakajima, and Y. Iguchi, unpublished data) were compared with those from Northern hybridization (Nakajima *et al.*, 2000b).

2. Gene Expression

Comprehensive analysis of gene expression using a DNA microarray is one of the most powerful and up-to-date methods for both investigating gene function and for discovering useful genes. The oligonucleotide microarray fabricated using semiconductor-based technologies (Fodor *et al.*, 1993) has been applied to sequencing by hybridization (SBH) (Pease *et al.*, 1994) and to the analysis of mutations including single nucleotide polymorphisms (Chee *et al.*, 1996; Hacia *et al.*, 1996; Kozal *et al.*, 1996). Gene expression profiling is often performed by using the microarray prepared by spotting and immobilizing complementary DNAs onto a glass slide (Schena *et al.*, 1996; DeRisi *et al.*, 1996; Schena *et al.*, 1998; Heller *et al.*, 1997; DeRisi *et al.*, 1997; Chu *et al.*, 1998). The differential expression of mRNAs extracted from cells grown under different culture conditions, from different tissues, or from tissues of patients including those in a healthy condition have been analyzed by cDNA microarrays using the two-color fluorescence hybridization technique (Schena *et al.*, 1995). An *A. oryzae* cDNA microarray should prove extremely useful not only for basic research but also for monitoring and controlling the fermentation process by the analysis of the mRNA expression profile. A first-generation *A. oryzae* microarray was prepared by Maeda *et al.* at Tohoku University (Sendai, Japan) consisting of approximately 2,000 cDNAs amplified from EST clones (unpublished data). Figure 6 (see color insert) shows the comparison of expression of some of the glycolytic genes between the cDNA microarray and Northern hybridization. Most of the genes except *fbpA*, which encodes fructosebisphosphatase, were confirmed to be induced by glucose by the microarray and by Northern hybridization.

Analyses of the regulatory elements and the transcription factor of a particular gene are important for utilizing its function by predicting expression in the production condition. However, the difficulty in the *in vivo* analysis, including the reporter gene assays, significantly delays the analysis of the promoters of *A. oryzae* genes. One way to circumvent the problem is to use *A. nidulans* for *in vivo* analysis. Alternatively, *in vitro* analysis using an electrophoretic mobility shift assay (EMSA) generates useful information about transcription regulatory elements (*cis* elements). Scanning of the element(s) based on the sequence-specific binding of cellular factors(s) by EMSA with highly sensitive fluorescence detection has remarkable potential for the rapid determination of these elements (Sano *et al.*, 2001). Identification of cellular factor(s) associated with the elements can also be accelerated by the use of an *in vitro* techniques such as phage display. High-throughput identification of *A. oryzae* factors from a cDNA phage display library is underway. The combination of the DNA microarray and analyses based

on DNA–protein interaction may be a useful way to generate information about transcription of industrially important organisms for which *in vivo* analysis techniques have not yet been well established.

Hydrolytic enzyme productivity by *A. oryzae* is enhanced in solid-state cultivation, which is called koji in Japan. However, the solid-state production process generally is much more labor intensive than the liquid cultivation process, and is harder to automate. Nevertheless, Kikkoman has successfully developed a large-scale and continuous automated fermentation system for solid-state cultivation. However, for further improvement of productivity of the solid-state cultivation process and also for the enhancement of productivity in liquid cultivation, the molecular mechanism leading to high productivity in solid-state cultivation must be deciphered. Therefore, genes enhanced specifically in the solid-state cultivation were cloned by preparing a cDNA library specific to the solid-state cultivation using the DNA subtraction technique. A number of genes, including those encoding the solid-state-specific enzymes already found, were detected in the library (Akao *et al.*, 2000). Ishida *et al.* found that *glaB*, one of the genes encoding the solid-state culture-specific enzymes was strongly induced when grown on a nitrocellulose membrane on a Czapek-Dox medium plate with low water activity in the presence of 50% maltose. These findings indicate that the solid-state specific induction is mainly due to low water activity and physical barriers (Ishida *et al.*, 1998). Solid-state cultivation seems to induce the potential for secretion of extracellular proteins. Suites of different genes were induced in solid-state cultivation, suggesting that the enhanced secretion in the solid-state cultivation may be accomplished by a global change of cell metabolism. The comprehensive expression analysis by the DNA microarray may provide remarkably important information to reveal the mechanism of enhanced productivity in solid-state cultivation.

3. *Proteomics*

Proteome analysis using two-dimensional electrophoresis is underway by Takeuchi *et al.* at Tokyo University of Agricultural Technology and Yamagata *et al.* at Tohoku University. The analysis will be accelerated by the use of mass spectrometry when the *A. oryzae* genome becomes available. The proteomics should be extremely useful for the control of a fermentation process because they provide information and technologies directly applicable to monitoring the fermentation process.

IV. Conclusions

Fermentation results from a variety of biological functions of microorganisms, including metabolism, biosynthesis, and reactions

by the secreted enzymes—some of which may be undesirable to accomplish high fermentation efficiency and to obtain high quality of the product. Huge efforts have been made already to improve the fermentation efficiency, by both the breeding of *A. oryzae* and modifying the fermentation conditions. Comprehensive analyses of genes, proteins, gene expression, and protein interactions, such as have been extensively done with the model organisms, will establish an indispensable knowledge for research on *A. oryzae,* which still has a significant disadvantage in classical genetics and molecular biology when compared to the model organisms. The comprehensive genomic analyses will enable us to reconstruct biological systems including transcription networks and metabolic pathways *in silico. Aspergillus oryzae* genomics, in combination with experimental data accumulated by research and development in the fermentation industries, will enable us to simulate and predict the responses of *A. oryzae* cultures in particular environmental conditions. Considering that *A. oryzae* is proven to be safe for human consumption, efficient in decomposing a variety of organic materials, and tolerant against extracellular conditions, the *A. oryzae* genomics project will lead to a new generation of fermentation processes, which can be used to increase energy efficiency in industrial processes and to protect environmental conditions.

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Transmission Genetics of *Microbotryum violaceum* (*Ustilago violacea*): A Case History

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I. Introduction

Three saprophytic ascomycetous fungi became "model species" for geneticists interested in exploiting their unique constellation of technical advantages for transmission, and later, molecular genetic research: *Neurospora crassa* (bread mold), *Aspergillus nidulans,* and *Saccharomyces cerevisiae* (yeast). Their advantages as genetic models include the following: (1) heterothallism with two mating-type alleles for *N. crassa* and *S. cerevisiae* and homothallism for *A. nidulans,* selfing and also relative heterothallism when auxotrophic mutants are used; (2) haploid, uninucleate vegetative cells to induce mutations; (3) readily separated ordered or random haploid products of meiosis; (4) relatively simple complex and minimal media; (5) completion of life cycle on laboratory medium; and (6) vegetative units not requiring a rest period or special treatment or conditions for germination. In time, geneticists involved these species in their investigations, and created a very large database and literature for their transmission genetics, which was later exploited by molecular geneticists. *Neurospora crassa* ($n = 7$) and *A. nidulans* ($n = 8$) have low chromosome numbers, and *S. cerevisiae*

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($n = 16$), a large chromosome number. Recently, the genomes of all three species have been sequenced (Bennett and Arnold, 2001).

Once the technology of molecular genetics was available, plant pathologists added molecular geneticists to their research groups in the expectation that a molecular approach would yield the desired information on the components of pathogenicity.

The seminal papers by Day and Jones (1968, 1969) on the transmission genetics of *Microbotryum violaceum,* formerly named *Ustilago violacea,* using both the sexual and parasexual cycles, indicated that this species might become a model species for basic investigations on the transmission and molecular genetics of a fungal phytopathogen (Perkins, 1991). Few phytopathogens have the same constellation of technical advantages as *M. violaceum* (Sidhu, 1988; Bennett and Lasure, 1985, 1991; Bos, 1996). Day and Jones (1969) identified 12 linkage groups and Baird and Garber (1981) added two more linkage groups, indicating a haploid chromosome number of at least 14 chromosomes and perhaps $n = 20–21$. In view of this large haploid chromosome number, Garber *et al.* (1982, 1983) and Saltiel and Garber (1984a) devised efficient meiotic and mitotic genetic mapping strategies to detect linked (syntenic) and independently segregating genes by exploiting some of the unique array of characteristics of this species. An efficient method to obtain auxotrophic mutants was devised to increase the number of detectable genes. Moreover, it was possible to determine the centromere-linkage values (CLVs) for selected mutant genes. Once syntenic genes were identified, it was possible to identify "new" linkage groups, thereby estimating the haploid chromosome number. Random sporidial analysis could be used to construct linkage maps. Consequently, *M. violaceum* seemed worthy of an effort to create a large database for transmission genetics as efforts were made to initiate programs involving molecular technology. The molecular genetics of *Ustilago maydis* investigated by the Leong group at the University of Wisconsin (Leong *et al.,* 1991) offers a model for comparable investigations in *M. violaceum.*

II. Taxonomy

As originally constructed the genus *Ustilago* included species pathogenic for both monocot (particularly the cereal crop host-species) and dicot host-species. For the dicots, diseased (= smutted) anthers contained uninucleate diploid teliospores instead of pollen grains. Deml and Oberwinkler (1982) and Vanky (1998) recognized significant differences, including the production of different siderophores, between the monocot and dicot pathogens, by retaining *Ustilago* as the genus

for the moncot pathogens and *Microbotryum* as the genus for the dicot pathogens. Consequently, *Ustilago violacea* (Pers.) Roussel is now termed *Microbotryum violaceum* ([Pers.:Pers.] Deml & Oberw.). Because most of the published literature dates prior to the nomenclatural change and uses *Ustilago violacea,* this change may confuse those new to the field.

III. Life Cycle

In nature, the teliospores are disseminated by insect vectors. The teliospores are harvested from smutted anthers and germinate on the surface of leaves or flowers, water, or laboratory media. Cummins and Day (1977) present a detailed description and diagram of the life cycle. The germinating teliospore produces a short promycelium into which the nucleus migrates and undergoes meiosis. One nucleus returns to the teliospore and the other three nuclei are separated by septa. *Microbotryum violaceum* is a bipolar species with mating types termed a-1 and a-2, determined by two alleles. Thus, after meiosis, the two a-1 and two a-2 basidiospores form a linear tetrad on a short promycelium and can be separated by micromanipulation for tetrad analysis. Garber *et al.* (1981) presented a method for tetrad analysis without micromanipulation. Each basidiospore buds to produce a sporidium. The multiplication of sporidia by budding yields a yeast-like teliospore colony with four sporidial clones, each with the same genotype as the progenitor basidiospore. These teliospore colonies have been used for genetic analysis.

Haploid sporidia with different mating type alleles conjugate on water agar. More than 70% of the sporidia conjugate after 12 h at 15°C. Conjugation pegs are produced from a circle close to the ends of the tapered sporidia and involve sporidia in contact or up to 30 μm apart. The dissolution of the sporidial walls and membranes to open the conjugation bridge for the migration of the a-2 nucleus into the a-1 sporidium to produce a dikaryon has not yet been investigated at the biochemical level.

The dikaryon produces a dikaryotic hypha that penetrates the intercellular spaces to reach and enter the immature anthers where the teliospores are produced (Day and Cummins, 1982; Kokontis and Ruddat, 1980). The development of a thick gelatinous material, and later, the deposition of thick, purple, reticulated wall, were responsible for the inability to identify stages from dikaryotic hyphae to the dry teliospores by light or electron microscopy. Nevertheless, genetic observations have been used to provide a plausible description of cellular events.

Assuming that the fragmentation of the dikaryotic hyphae yields individual rather than connected cells and that the fragment cells produce

"preteliospores" committed to morphogenic changes leading to the diploid teliospores, the following sequence of cellular events in the immature anthers can be deduced: (1) hyphae enter the anther sac; (2) hyphae fragment, yielding single fragment cells with two nuclei of different genotypes; (3) fusion of the haploid nuclei signals the presence of the preteliospore; and (4) diploid preteliospores develop into mature teliospores. These events occur as overlapping waves (Garber *et al.*, 1984). The black smutted anthers indicate mature teliospores which readily germinate on laboratory medium.

The Chicago collection: Day and Jones (1968) isolated strains 1.A1 a-1 pink thiamin-less (*thi*) and 2A.2 a-2 pink *thi*, each originating from one sporidium in one teliospore colony. The teliospore was obtained from a smutted anther of *Silene alba*. These strains were used by Day and Jones (1969) and by the Garber group (Garber and Day, 1985) for their genetic analysis of *M. violaceum* and have been termed "the laboratory strains." The strains have been stable for more than 30 years, retaining their mating type alleles, thiamin requirement, and with rare exceptions, their original pink colonies. The Chicago collection was assembled by Garber *et al.* (1978) from teliospores of herbarium specimens, and later, of field collections. One herbarium specimen with viable teliospores was dated to 1893. It should be possible to exploit other smutted herbarium specimens of dicot species to assemble collections for each species. Such a collection could yield information on polymorphisms for natural populations of each dicot pathogen.

IV. Cytology

An accurate count of the haploid chromosome number provides the expected number of linkage groups. Chromosome morphology can assign specific groups to specific chromosomes, and for favorable species, genes to a specific chromosome arm. The chromosomes of most fungal species, however, are notoriously small and difficult to count. Poon and Day (1974) could not get a precise count of the haploid chromosomes of *M. violaceum*, using phase contrast and fluorescence microscopy.

Pulse field gel electrophoresis (PFGE) provides an alternate method for separating fungal chromosomes (= bands in a gel). Perlin (1996) surveyed 18 strains of *M. violaceum* by PFGE and reported extensive chromosome polymorphism. The number of bands (= chromosomes) ranged from 6+ to 17–19. Based on genetic data, Day and Jones (1969) and Baird and Garber (1981) reported 14 linkage groups for the laboratory strains, indicating at least $n = 14$ and an estimated $n = 20$–21. Crosses between strains with the least and the most bands have not yet been made to determine teliospore viability and the number of bands in the viable meiotic products.

The origin of extensive chromosome polymorphism in fungal species is a problem yet to be resolved. A plausible explanation assumes that ectopic recombination, i.e., crossing over for homologous nucleotide sequences in different chromosomes, may be responsible. For *M. violaceum*, the *Faust* transposable element (see later discussion) presumably occurs in different chromosomes and could provide the proposed homologous nucleotide sequences (Garber and Ruddat, 1995). Families of transposable elements are found in the genomes of a number of fungal species (Daboussi, 1997).

V. Mutations in *M. violaceum*

Large numbers of mutations with different phenotypes are absolutely necessary to perform an effective transmission genetic study of a species. The common mutagenic agents, e.g., UV irradiation and nitrosoguanidine, used for the model saprophytic species are readily applied to the uninucleate haploid sporidia of *M. violaceum* (Tanabe and Garber, 1980; Bolker, 2001). The minimal medium of salts, glucose, and thiamin allows for the isolation of auxotrophic mutations with loci in different chromosomes as well as in the same chromosome (Day and Jones, 1968). In the following sections, different types of mutation will be presented separately and evaluated in terms of their worth in the different mapping strategies.

A. Colony Color

The sporidial colony colors are pink (+), orange (*o*), pumpkin (*p*), yellow (*y*), white (*w*), and magenta (*m*). The color mutations have been used for tetrad and fine structure analysis, and to determine CLVs. The pink color reflects a tenfold increase in cytochrome *c* in the mitochondria compared with the level in the white sporidia (Will *et al.*, 1982); the orange, pumpkin, and yellow phenotypes result from the accumulation of different carotenes (Will *et al.*, 1984). The biochemical basis for the magenta phenotype is not known.

B. Colony Morphology

The sporidial colonies are yeast-like with a smooth shiny surface and regular periphery, usually with solitary sporidia. Colony morphology (*morph*) mutants may have a "ropy" surface, an irregular periphery and pseudophyphal sporidia (Garber *et al.*, 1981; Zielinski and Garber, 1982), a dull surface and a regular periphery, a dull surface with numerous "bumps", a dull surface with shallow dimples, a dull fuzzy surface, small colonies with large bumps, or a dull speckled surface (Saltiel and Garber, 1984a). The morphology or size of the sporidia may be altered.

Chains of two to seven sporidia are usually present. Finally, the conjugation frequencies for solitary *morph* and wild-type sporidia range from 1 to 7%.

The *morph* sporidia may be overgrown by wild-type sporidia during the formation of the teliospore colony. Consequently, in order to detect the phenotypes of segregants from crosses involving *morph* mutants, sporidia must be harvested from "very small" to "small" teliospore colonies, and sporidial suspensions spread on the surface of the complex medium. Crosses involving *morph* mutants resulted in obviously diseased plants that did not produce flowers in the first flowering (Baker, 1947). However, cutting back the plants above the crown sometimes gave smutted flowers in the new shoots.

Temperature-sensitive morphology (*t-morph*) mutants are incubated at the permissive temperature and then scored at the restrictive temperature, thereby avoiding the need to harvest sporidia from "very small" teliospore colonies. The *morph* mutants have been used for tetrad analysis and single and double selection.

C. Auxotrophy

Nutritionally deficient mutations provide numerous mutant alleles at loci throughout the genome. Descriptions of different mutagenic agents, methods to detect auxotrophs, to identify their nutritional requirements and strategies to increase the frequencies of mutants are available from numerous articles on the transmission genetic analysis of the model saprophytic species. Stevens and Garber (1987) developed a limited enrichment method to get amino acid, vitamin, or growth factor mutants of *M. violaceum*. Using UV mutagenesis to avoid producing multiple mutations, different complex supplements, such as malt agar, V-8 juice, and pureed green beans, are added to the minimal medium and the mutagenized sporidia (ca. 5–10% survival) are incubated for 7–10 days at room temperature or 24°C. Sporidia from the very small to obviously small colonies are transferred to the standard complex and minimal media, and incubated for 7–10 days. Only transfers that either do not multiply or multiply only slightly on the minimal medium are potential deficiency mutants. Approximately half of the candidate mutants are indeed nutritionally deficient, although not all of these mutants necessarily have defined requirements. Each complex supplement generally has its characteristic spectrum of requirements that may be changed by reducing or doubling the initial concentration of the supplement.

Baird and Garber (1979, 1981) used the phenol-red urea medium to detect a bright red zone around colonies on the bright yellow medium, indicating urease activity (Christensen, 1946). Colonies from mutagenized

sporidia had three phenotypes: bright red, very small and pale red, or no detectable zone. Sporidial extracts from the latter two colony types were assayed for urease activity. Sporidia from many colonies with pale red zones had obviously reduced urease activity. Sporidia from the colonies with apparently no red or a pink zone either had wild-type activity or no activity (Baird and Garber, 1981). The former mutants were presumably the result of missense mutations. The latter mutants were products either of a presumptive permease because they had wild-type activity or of a nonsense mutation. It may be possible to adapt this method for detecting certain types of nutritional mutants when a chemical indicator shows activity, reduced activity or no activity by a color change around the colonies from mutagenized cells.

Auxotrophic mutations have been used to get diploid sporidia, for single and double selection and tetrad analysis. Screening colonies after sporidial mutagenesis often yields colony morphology mutants.

D. Fungicide Resistance

Mutants resistant to fungicides are easily isolated. After determining the lethal concentration, the fungicide is added to complex medium. Mutagenized sporidia are incubated on the fungicide and control media to detect colonies (Garber *et al.*, 1982).

Garber *et al.* (1982) devised a teliospore test method to identify dominant (*Dfr*), semidominant (*SDfr*), or recessive (*rfr*) fungicide-resistant mutations. Aliquot samples of teliospores heterozygous for a fungicide-resistant mutation are added to fungicide and control complex media and incubated. Comparable numbers of colonies indicate dominance; obviously reduced numbers indicate semidominance; and no colonies on the fungicide medium, recessiveness. Dominant and semidominant mutants can be used to get diploid sporidia and for single or double selection.

E. Selected Mutations

Additional mutant phenotypes for the different genetic mapping strategies include temperature sensitivity (*ts*) and ultraviolet light-sensitivity (*uvs*). Day and Day (1970) used *uvs* mutants for fine structure analysis by intact tetrad analysis.

VI. Genetic Mapping Strategies

Efficient mapping strategies are necessary for the genetic analysis of a species such as *M. violaceum* with a relatively large number of haploid

chromosomes. Once a collection of different types of mutants is assembled, the next tasks identify syntenic genes and then construct linkage maps. Assigning genes to different linkage groups can lead to an estimate of the haploid number of chromosomes. Finally, *M. violaceum* has a readily manipulated sexual cycle and a complete parasexual cycle (diploidization, haploidization and UV-induced somatic crossing over). Consequently, numerous technical advantages are available for the genetic analysis of this species, and presumably, other species of *Microbotryum* (Garber and Ruddat, 1992).

A. Meiotic Genetic Mapping Strategies

1. *Making Crosses*

The preferred host species for making crosses is *Silene latifolia* (= *S. alba*). Seeds readily germinate. Mature plants require relatively little space in the greenhouse and conditions for their flowering are easily controlled (Garber *et al.*, 1987). Numerous, large unopened buds with ten smutted anthers instead of bright yellow anthers are readily recognized. Such buds are continuously produced until the plants no longer flower. Flowers of both male and female plants produce smutted anthers. Relatively large number of plants, usually 4–5/inoculum, can be processed during a single crop. Smutted anthers can be stored in silica gel should additional teliospores be need for further analysis.

Large drops of a very turbid suspension of a-1 and a-2 sporidia in distilled water are added to the base of petioles in seedlings at the rosette stage. A flame-sterile needle is used to stab the petiole tissue through the drops, which are then sucked into the petiole base. This method yields at least one smutted plant/4–5 inoculated plants, regardless of the source of seeds. Plants are initially grown under natural day length during November–December in Chicago and for at least 10 days after inoculation. Plants are then placed under 16 h photoperiods, supplemented with fluorescent light to induce flowering so that the dikaryotic hyphae can grow in the host tissue and enter the immature buds. An inoculation may fail when the main stem bolts so that the hyphae have not invaded the immature buds. In such plants, cutting the main stem almost to the base can induce shoots to develop and to bear smutted flowers.

2. *Intact Tetrad Analysis*

Intact tetrad analysis refers to the colony of four sporidial clones formed by the four basidiospores on the short promycelium. Each clone has the same genotype as the progenitor basidiospore. The genetic

analysis of the (1) colony color mutants, (2) the UV-induced white (*uvs*) mutants, and (3) urease-negative (*ure*-1) provided examples of intact tetrad analysis. In each case, the teliospore colony was examined for a new phenotype. Crosses involving the different color mutants indicated that the loci were very closely linked (<1 c*M*) in the following linkage map: orange (*o*)—pumpkin (*p*)—yellow (*y*)—centromere-white (*w*A-*w*B)—magenta (*m*) The white "locus" is complex with two cistrons (loci?). The assignment to different chromosome arms was accomplished by UV-induced somatic crossing over (Garber *et al.*, 1975, 1980; Garber and Ruddat, 1996).

Diploid sporidia heterozygous for different color mutations gave pink colonies, indicating that pink (+) was the dominant allele for each mutation. Crosses between color mutants usually produced bisectored colonies with the colors of the parental strains. Occasional colonies had a pink sector, indicating crossing over between the mutant loci and the color loci (Cattrall *et al.*, 1978); other occasional colonies were uniformly pink. The uniformly pink colonies had $n + 1$ (disomic) sporidia, indicating that these *w* mutations were in contiguous cistrons (*wA, wB*), i.e., complementation (Garber, 1980). Using such readily related phenotypes, it should be possible to adapt this method to get recombinants for allelic mutations and data for meiotic nondisjunction for different chromosomes.

Teliospores from crosses between *ure*-1 mutants were incubated on phenol red-urea medium, and after incubation, occasional wild-type colonies represented products of intragenic recombination (Baird and Garber, 1981). The numbers of these colonies divided by the number of plated teliospores provided the data to construct a fine structure map for this locus.

3. *Random Sporidial Analysis*

Sporidia from "very small" teliospore colonies are pooled by flooding the surface of the agar and an appropriate number of sporidia is spread on complex medium for auxotrophic mutants (Day and Jones, 1969) and their genotypes are determined. The data are then used to construct linkage maps. Day and Jones (1969) present linkage maps for three chromosomes (Table I).

4. *Tetrad Analysis*

The basidiospores of the linear tetrad can be removed by micromanipulation. Garber *et al.* (1981) devised another less tedious method by crossing yellow (*y*) and white (*w*) mutant strains with a morphological (*morph*) marker in one strain. Each very small teliospore colony provides a sporidial suspension for spreading on complex medium. The

TABLE I

LINKAGE GROUPS IN *MICROBOTRYUM VIOLACEUM*[a]

Linkage group	Mutation	CLV[b]	Linkage group	Mutation	CLV
1	*arg*-1	7.2	5	*met-3*	2.4
	arg-2	2.4	6	MT[c]	0
	orn-1	4.5	7	*o*	<0.5
	orn-2	19.4		*p*	<0.5
2	*his*-1	21.7		*y*	<0.5
	met-2	8.7		*w*A	<0.5
	ade-2	—		*w*B	<0.5
3	*lys*-2	15.2		*m*	<0.5
	glu-1	2.5	8	*his*-3	—
4	*pdx*-1	5.2	9	*lys*-3	—
	lys-1	5.3		*met*-1	10.5
	arg-3	7.7	10	*his*-2	5.0
	Bot-2	10.0	11	*his*-4	—
	Bot-3	5.1	12	*arg*-5	2.0(?)
	ph	4.3	13	*ure*-1	—
	t-morph	8.9	14	*ure*-2	—
	lys-3	9.6			
	arg-4	7.0(?)			
	cf	31.3			
	mph	31.0			
	ino-1[d]	—			

[a]Revised Table I, Day and Garber (1988). Mutations with the same phenotype and comparable CLVs were arbitrarily assigned to the same locus. Crosses are needed to demonstrate allelism, different loci in the same or different chromosomes.

[b]Refer to Table II for phenotype.

[c]MT: mating-type locus.

[d]*ino:* Inositol.

segregants usually express one of the three possible phenotypic combinations, indicating ditype (N), nonditype (ND), and tetratype (T) tetrads. *Morph* genes with CLVs distal from the centromere yield greater numbers of T tetrads. Sporidia from one colony with each phenotype from T tetrads are used for the genetic analysis of other marker genes in the parental strains. The genotypes of the four products of tetrad analysis indicated centromere linkage for the mating type locus (Saltiel and Garber, 1984b), thereby confirming an earlier report by Castle and Day (1981), using a different approach involving polyploid teliospores.

5. Half-Tetrad Analysis

Half-tetrad analysis refers to the loss of two sporidial clones in the teliospore colony. The key to half-tetrad analysis is a phenotypically

obvious, centromere-linked mutation. Cattrall *et al.* (1978) detected no crossing over between the *y* locus and the centromere, and greatly reduced crossing over for the other color mutations and the centromere. These observations are responsible for half-tetrad analysis to determine CLVs for such selected mutations as auxotrophy (*aux*), temperature sensitivity, and dominant/semidominant fungicide resistance mutations. For example, after crossing a-1 *y* and a-2 *w* strains with either strain carrying an auxotrophic mutation, the teliospores are incubated on the standard minimal medium (Garber *et al.*, 1982). Two of the four basidiospores with the *aux* mutation in each tetrad do not multiply and produce colonies with two sporidial clones. Uniformly colored colonies with one or the other parental color indicate a parental (PD) or nonparental (NPD) ditype. A PD/NPD ratio of one indicates independent segregation for the color loci and the selected mutation. A ratio greater than one indicates linkage. The bisectored colonies with parental colored sectors result from a crossing over between the selected mutation locus and the centromere. For independently segregating genes, the bisectored colonies reflect T tetrads. The frequency of T tetrads is used to calculate the CLV for the selected mutation: $T \times 100/2N$. The CLVs do not exceed 33.3 c*M*, regardless of the genetic distance that a locus occupies (Fincham *et al.*, 1979). Two of forty-two tested mutations show linkage with the color mutations, but their CLVs are 24 and 27 c*M* and require confirmation (Garber *et al.*, 1987).

A *morph* mutation could be used to determine CLVs provided that (1) the mutation is centromere-linked or proximal to the centromere and (2) the *morph* sporidia in the teliospores are not overgrown by the *morph*$^+$ sporidia.

The CLVs for 59 loci in *M. violaceum* show a marked pericentric clustering (Table II). Approximately 40% of the loci are within 0–5 c*M* of their centromere and approximately 60% are within 0–10 c*M*. The remaining loci are evenly distributed along the chromosome arms in the following intervals: 5, 11–15 c*M*; 7, 16–20 c*M*; 5, 21–25 c*M* and 5, 25–33 c*M*. The pericentric clustering of loci in *M. violaceum* has no counterpart in the saprophytic Ascomycetes with the most detailed linkage maps.

6. *Double Selection Analysis*

In double selection analysis, the sporidial clones in the intact teliospore colony are subjected to selection so that only the selected clones give rise to a colony. Two types of double selection have been used for different purposes (Garber *et al.*, 1982, 1983). As an example of the first type, dominant/semidominant fungicide-resistant (*Dfr*/*SDfr*) and an auxotrophic (*aux*) mutations are present in one parental strain.

TABLE II

MUTANT GENES OF *MICROBOTRYUM VIOLACEUM* AND THEIR CLVs (IN cM)[a]

Gene	Symbol	CLV[b]	Gene	Symbol	CLV
Nutritional requirement			Colony color		
Alanine	*ala*	5.0	Orange	*o*	<0.5
Arginine	*arg*-1	7.2(9)	Pumpkin	*p*	<0.5
	arg-2	2.4(9)	Yellow	*y*	<0.5
	arg-3	7.7(2)	WhiteA	*w*A	<0.5
Aspartic acid	*asp*	26.5	WhiteB	*w*B	<0.5
Citrulline	*cit*	26.7	Magenta	*m*	<0.5
Glutamic acid	*glu*-1	2.5	Fungicide resistance[e]		
	glu-2	13.7	Botran	Bot-1	0.5
Glutamine	*gln*-1	4.6		Bot-2	10.0
	gln-2	12.4(4)		Bot-3	5.1
Glycine	*gly*	16.3	Chloroneb	chl-1	1.1(4)
Histidine	*his*-1	21.7(2)		CHL-2	11.5(3)
	his-2	3.5		CHL-3	9.5(2)
	his-3	0.9(2)	Thiabendazole	Tbz-1	1.6(4)
	his-4	—	Carboxin	CAR	27.2(3)
Isoleucine	*ile*	3.9	Colony morphology[f]		
Leucine	*leu*-1	7.7	Pseudohyphal	*ph*	4.3
	leu-2	31.3	Cauliflower	*cf*	31.3
Lysine	*lys*-1	5.3(4)	Dull	*dl*	15.8
	lys-2	15.3	Chain	*ch*	16.3
Methionine	*met*-1	2.4(2)	T-morph	*ts*-m	8.9
	met-2	10.5(2)	Morph	*m*-1	15.0
Ornithine	*orn*-1	4.5		*m*-2	25.2(4)
	orn-2	19.4(2)	Temperature sensitive		
Proline	*pro*-1	9.0	Temp-sens	*ts*-1	3.5(5)
	pro-2	23.9(2)		*ts*-2	8.3(2)
Tryptophan	*trp*	9.9		*ts*-3	25.7(3)
Tyrosine	*tyr*-1	4.9		*ts*-4	32.3
	tyr-2	19.2			
p-Aminobenzoic acid	*pab*-1	1.0			
	pab-2	7.5			
	pab-3	21.3			
Pyridoxin	*pdx*	5.2			
Casamino acids[c]					
cas	*cas*-1	1.8			
	cas-2	5.9(3)			
	cas-3	18.7(2)			
Complex supplement[d]					
Unknown	*unk*-1	2.8(2)			
	unk-2	10.1(4)			
	unk-3	28.8			

[a] Garber *et al.* (1975); Cattrall *et al.* (1978); Garber *et al.* (1980); Garber (1980); Baird and Garber (1981); Garber *et al.* (1981); Garber (1982); Garber *et al.* (1982); Zielinski and Garber (1982); Garber *et al.* (1983); Saltiel and Garber (1984a, 1984b); Garber and Day (1985); Stevens and Garber (1987); Garber *et al.* (1987).

[b] Number in parentheses for CLVs is a mean value for independent mutations with the same phenotype.

[c] Minimal medium supplemented with casamino acids.

[d] Minimal medium with V-8 juice, yeast extract, nutrient broth, or pureed beans.

[e] All capital letters: dominant; first capital letter; semidominant; all letters lowercase = recessive.

[f] Crosses of certain combinations of colony morphology mutants with comparable CLVs to test for allelism resulted in nonflowering, obviously diseased plants.

Aliquot samples of heterozygous teliospores (*Dfr aux*/+ +) are added to fungicide-containing minimal medium and to complex medium. After incubation yields colonies under the restrictive condition, the numbers of colonies on the two plates are compared. When the two selected mutations are linked, the number of colonies is greatly reduced on the restrictive medium.

The second type of double selection tests for allelism and intragenic crossing over for mutations with the same phenotype, such as arginine-deficient mutants, which may be responsible for different blocks in the biosynthetic pathway, and fungicide-resistant mutant, which may occur at different loci. Such mutant strains are crossed and the teliospores are incubated under restrictive and permissive conditions. For auxotrophic mutations, aliquot samples of teliospores are incubated on minimal and complex media, and the numbers of colonies on each medium are compared. Relatively few colonies or no colonies on minimal medium indicate allelism and the relatively few colonies for alleles, intragenic recombination. Numerous colonies on the minimal medium indicate independent segregation, although distant linkage cannot be ignored.

Mutations with comparable CLVs are particularly useful for double selection. The analysis of large numbers of teliospores is necessary to construct a fine structure map for pseudoalleles and to identify independently segregating or loosely linked genes.

B. Mitotic Genetic Mapping Strategies

The mitotic genetic mapping strategies use the different components of the parasexual cycle, which is readily manipulated in *M. violaceum*. This species has a complete parasexual cycle. Pontecorvo *et al.* (1953) discovered and exploited a complete parasexual cycle (diploidization, haploidization, and somatic crossing over) for the genetic analysis of *Aspergillus nidulans* by mitotic genetic *mapping*. Pontecorvo and Kafer (1958) used somatic crossing over to assign loci to a specific chromosome arms and to sequence the mutations with reference to the centromere and to each other in *Aspergillus nidulans*. Day and Jones (1968, 1969, 1971) demonstrated a complete and readily manipulated parasexual cycle in *M. violaceum*. Garber and Ruddat (1992) reported such a cycle in *Ustilago scabiosae*. Garber and Day (1985) and Day (1998) reviewed the parasexual cycle in *M. violaceum*, noting that few fungal species have a complete parasexual cycle and even fewer have a readily manipulated cycle. Mitotic mapping strategies yield considerable information for the genetic analysis of a species with a readily manipulated, complete parasexual cycle without resorting to plant inoculation for phytopathogens that must complete their life cycle *in planta*, and in

some cases, may be more efficient than meiotic mapping strategies for species with a relatively large, haploid chromosome number.

Mitotic mapping strategies for *M. violaceum* have been used for the following purposes: (1) complementation tests, (2) identifying independently segregating and syntenic genes, (3) assigning syntenic genes to a specific chromosome arm, and (4) sequencing mutations in the same arm relative to the centromere and to each other.

1. Diploidization

Complementing colony color mutations, usually yellow (*y*) and white (*w*), and auxotrophic (*aux*) mutations, preferably diauxotrophic mutations, facilitate the isolation of colonies with diploid sporidia from conjugating haploid sporidia (Garber *et al.*, 1975). When plated on media containing inhibitory concentrations of fungicides, complementing, dominant fungicide-resistant mutants with complementing colony color mutations yield pink colonies with diploid sporidia. Diploid sporidia are readily distinguished from haploid sporidia by their obviously larger size and dumbbell shape.

Conjugants and solitary sporidia from the water agar medium are suspended in water and an aliquot containing a known number (ca. 3×10^5) of conjugants) is spread on the surface of minimal medium supplemented with thiamin. After two weeks of incubation, the pink colonies with diploid sporidia are likely candidates; the frequency of such colonies is 3.4×10^{-4} (Day and Jones, 1968). The pink colonies with sporidia heterozygous for the different recessive color mutations indicate complementation (Garber *et al.*, 1975, 1983). Kokontis and Garber (1983) used the recessive pseudohyphal (*ph*) mutation in heterozygous diploid sporidia to demonstrate the UV induction of somatic crossing over at the cellular level.

2. Haploidization

Haploidization provides segregant sporidia so that it is possible to identify independently segregating or linked genes. Diploid sporidia are stable and rarely yield haploid sporidia unless a haploidizing agent is used. When diploid sporidia (*y*+/+ *w*) are incubated on complex medium containing fluorophenylalanine, numerous papillae with haploid or aneuploid ($n + 1$) sporidia eventually appear on each plate. The yellow papillae are readily identified, and provide the sporidia for transfer to complex medium and for genotyping. Approximately 70% of the sporidia are haploid. The sporidia from the remaining papillae are aneuploid ($n + 1$) with slowly multiplying sporidia that produce small colonies (Day and Jones, 1969, 1971). The aneuploid sporidia form colonies with the dominant phenotype for the pertinent mutation

and eventually yield haploid sporidia with either the dominant or recessive allele. Haploid sporidia result from the random loss of one of the pair of chromosomes during sporidial multiplication.

Data from haploidization indicate independent segregation or synteny for pairs of mutations. Sporidia with neither two dominant nor two recessive alleles indicate synteny. Random sporidial analysis yields linkage maps for syntenic genes. Segregants with both mutant genes in coupling can be saved for sequencing with respect to the centromere and to each other by somatic crossing over, particularly for closely linked genes (Garber *et al.,* 1975, 1980). Day and Jones (1969, 1971) and Baird and Garber (1981) used data from haploidization to identify 14 linkages and speculated that additional linkage groups may be expected.

3. *Somatic Crossing Over*

Sexual crosses involving linked genes in repulsion usually yield haploid segregants with the genes in coupling. Such segregants are candidates for parasexual crosses with a strain carrying the dominant alleles to assign them to the same or different chromosome arms. When such genes are in the same arm, the segregants from somatic crossing over indicate their sequence relative to the centromere. The UV irradiation of diploid sporidia sufficiently increases the frequency of somatic crossing over to warrant using somatic crossing over as a tool for genetic mapping analysis. While somatic crossing yields a linear sequence for genes in the same chromosome, the data do not provide linkage values.

VII. The *Faust* Transposable Element

The pink 1.A1 a-1 *thi* and 2.A2 a-2 *thi* laboratory strains have been stable during numerous cycles of storage in silica gel, revival, mass transfers, and storage for over 30 years, except for occasional spontaneous color mutants. Mutant laboratory strains with other phenotypes have also been stable under the same regime. Crosses involving certain combinations of Chicago strains, the orange (*o*) and magenta (*m*) mutants, however, gave bisectored teliospore colonies in which a specific parental color was replaced with a specific nonparental color (Garber and Owens, 1980; Garber and Ruddat, 1995, 1996). The frequencies of such unexpected colonies ranged from 70 to 100%, depending on the specific combination of strains. Garber and Ruddat (1995) proposed two types of *Faust* transposable elements to account for the extensive colony color and the bisectored colonies in the Chicago strains: cryptic and transactive.

Seven unexpected observations were noted during the transmission genetic analysis of *M. violaceum* (Table III). For example, two

TABLE III

UNEXPECTED OBSERVATIONS ENCOUNTERED DURING THE GENETIC INVESTIGATION OF *MICROBOTRYUM VIOLACEUM*

Observation	Reference
Extensive colony color polymorphism and bisectored teliospores colonies in Chicago collection of strains	Garber *et al.* (1978)
Failure to detect recombinants from crosses of white (*w*) Chicago strains	Garber and Owens (1980)
Failure to detect recombinants from crosses of UV-induced *w* mutants in laboratory strains and *w* Chicago strains	Garber (1982)
Extensive pericentric clustering of genic mutations	Garber and Day (1985); Garber *et al.* (1987)
Replacement of a specific parental color by a specific nonparental color in teliospore colonies from crosses involving Chicago strains	Garber and Ruddat (1996) [Garber and Owens (1980)]
Relatively high frequency of color mutations after UV mutagenesis of magenta (*m*) sporidia	Garber and Ruddat (1996)
Mutable thiamin (*thi*) and pyridoxin (*pdx*) loci (*thi* ↔ *pdx*) in Chicago strains C429 and C449 during mitosis	Garber and Ruddat (1998)

observations involved crosses between *w* Chicago strains, and between UV-induced *w* mutants from the laboratory strains and the *w* Chicago strains, and no recombinants were recovered. Crosses between UV-induced *w* mutants from the laboratory strains, on the other hand, gave recombinants whose frequencies were used to construct a fine structure map for this "locus" (Garber, 1980). Complementation tests, however, indicated that the white locus was complex and included two contiguous cistrons (loci?), termed *w*A and *w*B. Crosses between *w* Chicago strains, on the other hand, gave no indication of a complex locus by complementation test.

All of the observations in Table III could be explained by proposing a transposable element, termed *Faust,* which presumably occurs in different chromosomes. The first author, in honor of the Faust legend, named the elements.

Mutable loci furnish acceptable indicators for transposable elements, provided that other possible sources can be eliminated, e.g., disomy (y +/+ w), ring chromosomes with a dominant allele in a disomic cell with heterozygous genes or in diploid or trisomic ($2n + 1$) cells with a recessive allele or mutator genes(s). None of these alternative possibilities accounted for *all* of the observations (Table III).

The closely linked, centromere-linked color mutations provided marker sites to detect the transposition of the *Faust* elements in the

same chromosome. Transposition was transcentric and the preferred sites of insertion were more likely to be proximal than distal from the centromere.

Crosses involving stable combinations of Chicago strains and the 1.A1 and 2.A2 laboratory strains are assumed to have cryptic elements in each strain. The strains in combinations producing teliospore colonies with a nonparental color sector presumably involve one strain with a cryptic element and the other strain with a transactive element or both strains with transactive elements. The UV irradiation of magenta (*m*) sporidia gave color mutants with frequencies significantly greater than expected from spontaneous or induced mutation. The UV irradiation may be a "stress" factor to inducing transposition. The UV-induced orange (*o*) and spontaneous magenta (*m*) mutations in the laboratory strains are likely to be insertional mutations. The appearance of "spontaneous" color mutations from laboratory strains subjected to silica gel storage may also reflect a "stress" factor inducing transposition. Polymorphism for the haplo-lethality mutations may also result from insertional mutations (Garber *et al.*, 1978). Finally, it is probable that at least some of the UV-induced centromere-linked auxotrophic and other mutations may also be insertional.

Garber and Ruddat (1998) presented genetic evidence indicating that transposable elements function during the mitotic multiplication of sporidia and during meiosis in different chromosomes. In all of these cases, the elements appear to be pericentric and to have the same characteristics as the *Faust* elements in the chromosome with the color mutations. This speculation does not preclude the possibility that other types of elements may also be present in strains of the Chicago collection.

All of the apparently disparate genetic observations in Table III may be explained by implicating the proposed *Faust* elements assumed to occur pericentrically throughout the genome of *M. violaceum*.

Once the proposed *Faust* element is characterized at the nucleotide level, it should be possible to compare this unusual element with other elements found in fungi (Daboussi, 1997). The nucleotide sequence(s) should yield a probe for hybridization with the chromosomes separated by pulse field gel electrophoresis (Perlin, 1996) to determine the distribution of such elements in the genome. A probe could also be used to hybridize chromosomes of the *Ustilago* species carrying the haplo-lethal mutation (*U. bullata*, Fischer, 1940; *U. kolleri*, Grasso, 1955; *U. nigra*, Darlington and Kiesling, 1975). Finally, the probe could be used to compare the postulated cryptic and transactive elements. The molecular genetic analysis of *M. violaceum* has been initiated by Perlin *et al.* (1997) and Henry *et al.* (1999).

VIII. Haplo-Lethality

Haplo-lethality refers to the spontaneous lysis (apoptosis?) of haploid sporidia in two clones with the same mating-type allele. After several mitotic divisions of the sporidia in each clone in the teliospore colony, spontaneous lysis occurs. Two sporidial clones from separated basidiospores of a linear tetrad also express haplo-lethality (Fischer and Holton, 1957), indicating that the phenotype is monogenically (*hl*) determined. Consequently, sampling sporidia from relatively large teliospore colonies yields sporidia with only the a-1 or a-2 mating-type allele (Garber *et al.*, 1978; Antonovics *et al.*, 1998; Hood and Antonovics, 1998).

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Molecular Biology of the *Koji* Molds

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I. Introduction

Fungi are a diverse group of organisms that are ubiquitous in natural environments and traditionally used in different industrial processes including food and fermentation industries. *Koji* molds (*Aspergillus oryzae, Aspergillus sojae, Aspergillus kawachii, Aspergillus shirousamii,* and *Aspergillus awamori*) have been widely used as the starter in preparation of koji for over a thousand years in Japanese traditional fermentation industry involving the production of *sake* (rice wine), *shoyu* (soy sauce), *miso* (soy bean paste), and *shochu* (distilled spirits). Recently the koji mold, *A. oryzae,* has been recognized as Japan's national microorganism in line with *sakura* (cherry blossom) as the national flower. The koji, in addition to being a saccharifying and diastatic agent, also contributes to the color, flavor, and aroma of the fermented foods that are important for their overall attributes. The quality of the koji greatly depends on the properties of the fungal strains used, and these

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TABLE I

JAPANESE FERMENTED BEVERAGES AND FOODS WITH KOJI MOLDS

Fermented beverages and foods	Koji molds
Sake	*Aspergillus oryzae*
Shochu (distilled spirits, *Awamori*)	*A. awamori*
Shochu (distilled spirits, other than Awamori)	*A. kawachii, A. shirousamii*
Shoyu (soy sauce)	*A. oryzae, A. sojae*
Miso (soy bean paste)	*A. oryzae*
Mirin (seasoning made of rice)	*A. oryzae*
Katsuobushi (dried bonito fish)	*A. glaucus*

strains have been provided by *tane koji* manufacturers for a long time (at least about 600 years).

Although koji molds are very important microorganisms, research on their molecular biology began only 15 years ago and progressed dramatically for the last 5 years. In this review I describe the molecular biology of koji molds, focusing specifically on one of the most important koji mold, *A. oryzae*.

II. Koji Making: Characteristics of Solid-State Cultures of *Aspergillus*

Each and every fermentative product made by utilizing koji, has its own distinctive process and material for koji making. Table I summarizes typical Japanese fermented beverages and foods with the respective *Aspergillus* species used in their manufacture. Figure 1 shows brown

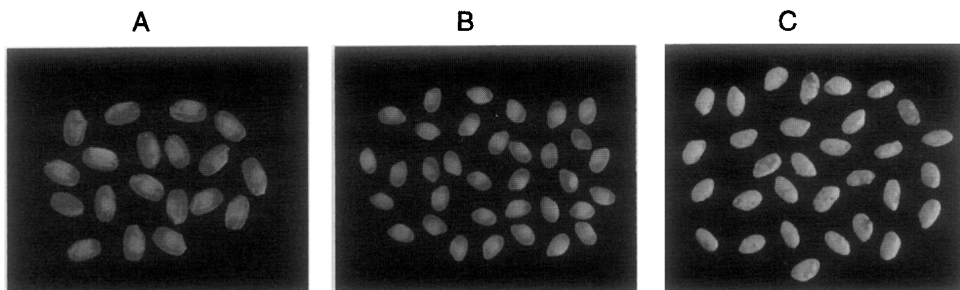

FIG. 1. (A) Brown rice (*Gohyakumangoku*); (B) polished rice (60% of polishing rate); (C) koji. *Gohyakumangoku* is one of the typical rice varieties for sake brewing, which contains white part in the center of the grain.

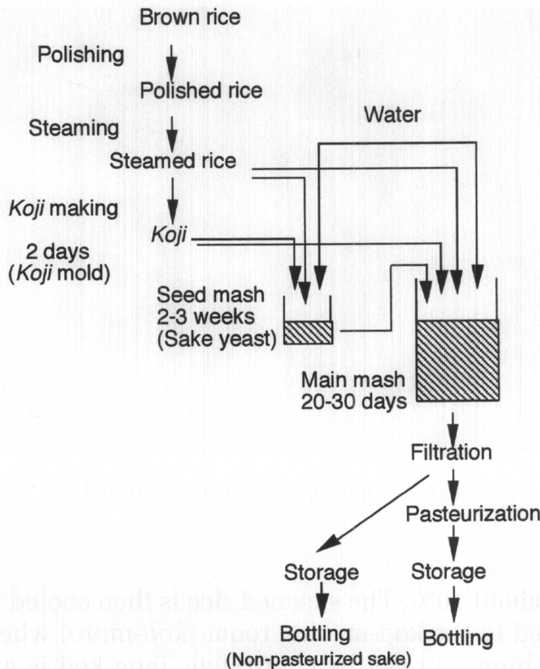

FIG. 2. An overview of the sake production process.

rice (A), polished rice (B), and koji (C) used for sake brewing (Fig. 2). A brief outline of koji preparation for sake and tane koji (conidia of *Aspergillus,* Fig. 3) manufacture is given below.

A. KOJI MAKING FOR SAKE BREWING

In general, steamed rice with a polishing rate of 40–70% is used in sake brewing. For koji preparation, 20% of the total rice is utilized and then the remaining is used for sake fermentation.

1. *First Day*

After washing, the polished rice is soaked in water for several hours to about 28–30% moisture content. The soaked rice is then drained and transferred into a tank, called *koshiki,* that has a hole at the bottom in order to let out steam. The koshiki is placed over a large kettle containing boiling water and the rice is steamed for about an hour under atmospheric pressure. Recently, new machinery for continuous steaming of rice has been adoped in large-scale sake industries. The steaming procedure increases the moisture content of the starch granules within

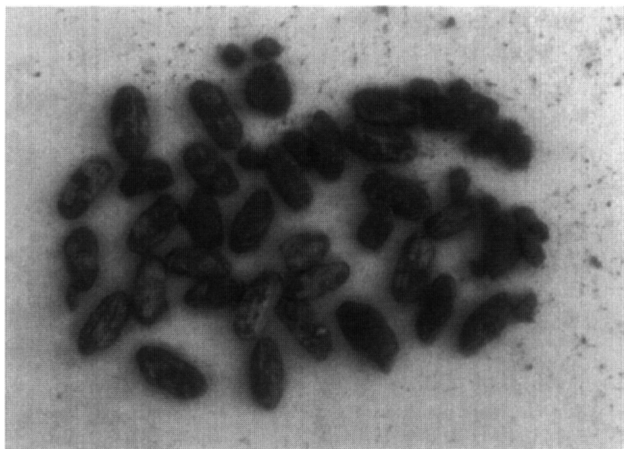

FIG. 3. Tane koji (conidia of *A. oryzae*).

the kernel to about 10%. The steamed rice is then cooled to about 36°C and transferred to the koji-making room (*koji-muro*) wherein the temperature and humidity can be controlled. Tane koji is available from about 10 tane koji manufacturers in Japan, who provide the product to various sake, shoyu, and miso companies. This tane koji is scattered over the surface of the rice, with an approximate moisture content of 35% at an inoculation rate of 1 g of tane koji preparation per kilogram of raw rice, at about 30°C, and then mixed thoroughly and covered with a cloth. After 10–12 h, the rice is remixed and simultaneously heaped on the table.

2. *Second Day*

About 20 h after inoculation, the growth of mold mycelia on the grains gives rise to small white spots, visible to the naked eye; simultaneously, the temperature of the heaped grains begins to rise. At this stage the developing koji is transferred into many shallow wooden trays that are stacked on shelves and covered with a cloth. As the koji mold grows rapidly and vigorously, the temperature of the koji rice increases. This preparation is stirred twice in every 4 h so as to control the temperature below 38–42°C. At this stage an unpleasant odor known as *ohaguro-shu* can be sensed. In order to obtain maximal yield of amylase it is essential to maintain the temperature at about 42°C for several hours.

3. Third Day

Forty to forty-five hours after inoculation, the koji rice in the trays is shifted from the warm koji-muro so that the low temperature (outside temperature; about 0–7°C) restricts the growth of the mold. The resulting koji is white and has a pleasant odor (*kuri-ka*) resembling roasted chestnuts. Ohaguro-shu and kuri-ka are characteristic odors during koji making process, and are empirically considered to be important for proper control of the process.

Although recently most sake factories have adopted the use of a koji-making machine, the koji for high-quality sake named *ginjo-shu* is still prepared by the traditional method described above.

Finished koji (Fig. 1C) is white in color, since the growth phase is halted prior to sporulation of the mold. The mycelial growth is observed not only on the surface of the grain, but also in the center of the kernel. Growth of the mold within the kernel is considered desirable for the slow and progressive release of α-amylase and glucoamylase in the parallel fermentation of sake.

In sake brewing, the following mycological characteristics have been found empirically to be of major importance in koji mold, *A. oryzae:*

1. rapid growth on and into the kernel of the steamed rice;
2. production of abundant amylases (α-amylase and glucoamylase), a little acid carboxypeptidase, and low tyrosinase;
3. production of good fragrance and accumulation of flavoring compounds;
4. low production of colored substances such as deferriferrichrome (a kind of siderophore), flavines, etc.

B. Tane Koji Making

In all sake breweries, tane koji (Fig. 3) is purchased from the tane koji manufacturers, some of which had been established in the *Muromachi* era (for 600 years). It is really surprising that almost pure conidia had been prepared without use of equipment such as an autoclave or a clean hood and even before the development of modern microbiology by L. Pasteur and R. Koch. As can be seen by the tane koji production process is described below, the differences between koji making for sake, etc., and tane koji preparation are that brown rice is not polished as much as raw material, that culture time is much longer, and that wood ash is added in order to change to pH alkaline condition in tane koji manufacture.

1. First Day

Polished rice (more than 95% of polishing rate) is washed and soaked in water for a few hours. The rice is then drained and allowed to stand for 2 h. Washed rice is put into a rotary-type cooker and steamed for about an hour under pressure. The steamed rice is cooled in a cooker and then the tane koji (0.03% of raw material) with ash (1% of raw material) is inoculated and the cooker is rotated to ensure uniform distribution of the conidia. The inoculated rice is incubated at 30°C in the cooker for a night.

2. Second Day

The rice is sifted into the incubation room, and mixed thoroughly and transferred to koji trays. About 1 kg of the rice is placed in each tray and spread evenly by hand. Room temperature and humidity are controlled at about 30°C and 95%, respectively.

3. Third Day

The rice is mixed several times in order to maintain its temperature and to promote conidial formation.

4. Fourth to Fifth Day

The positions of the trays are rotated in order to keep their temperature constant.

5. Sixth Day

No work is done.

6. Seventh Day

The finished koji is then moved to a drying room, and dried by filtered air blown at 40°C at 20 ~ 25% humidity for 17 ~ 18 h. Drying the tane koji, and reducing the water content to less than 7%, can make the storage time longer.

III. Basic Systems of Molecular Biology in Koji Molds

A. Host Vector Systems

The first successful transformation of the koji mold, *A. oryzae,* was reported in 1987 by Gomi *et al.* (1987) and Mattern *et al.* (1987) using *argB* and *pyrG* genes as selectable markers from *A. nidulans.* To date, several host vector systems have been developed as shown in Table II. A host strain, NS4 (Yamada *et al.,* 1997), with double auxotrophic markers, *niaD* and *sC,* is useful for analyzing cloned genes. Koji molds are

TABLE II

SELECTABLE MARKER GENES FOR TRANSFORMATION OF THE KOJI MOLD, *A. oryzae*

Enzyme name (gene name)	References
Ornithine carbamoyltransferase (*argB*)	Gomi *et al.* (1987); Nagashima *et al.* (1998)
ATP sulphurylase (*sC*)	Yamada *et al.* (1997)
Acetamidase (*amdS*)	Gomi *et al.* (1991, 1992)
Pyrithiamine resistance gene (*ptrA*)	Kubodera *et al.* (2000)
Orotidine-5′-phosphate decarboxylase (*pyrG*)	de Ruiter-Jacobs (1989); Mattern *et al.* (1987); Kitamoto *et al.* (1995)
Nitrate reductase (*niaD*)	Unkles *et al.* (1992)
5-Aminolevulinate synthase (*hemA*)	Elrod *et al.* (2000)

generally tolerant to antibiotics such as hygromycin, phleomycin, and aureobasidin A that are used for selection in some filamentous fungi. Recently the dominant selectable marker *ptrA* (Kubodera *et al.,* 2000) for koji molds was reported, and is available from Takara Shuzo Co, Japan. Thus, using NS4- and *ptrA*-based plasmids, three kinds of genes can be introduced into *A. oryzae.*

B. TRANSFORMATION

Transformation of koji molds is carried out by the normal protoplast formation method described for other filamentous fungi such as *A. nidulans* and *A. niger* (Punt and van den Hondel, 1992). In general, transformation frequency is relatively low (about a few to ten transformants per microgram DNA). Recently, an electroporation method using restriction enzyme mediated integration (REMI) in *A. oryzae* (Yaver *et al.,* 2000) and *Agrobacterium*-mediated transformation in *A. awamori* (Gouka *et al.,* 1999) were reported. These methods may become common for koji molds in the near future. A typical transformation procedure by protoplast formation for the koji mold, *A. oryzae,* is shown in Figure 4.

C. PROMOTERS USED FOR EXPRESSION OF HETEROLOGOUS AND HOMOLOGOUS GENES IN KOJI MOLD

To date, about 100 genes have been isolated and analyzed from *A. oryzae.* In addition, almost the same number of genes are estimated to be isolated by use of the *A. oryzae* expressed sequence tag (EST) project database. Among these isolated genes, strong promoters have been used for expression of selected genes in *A. oryzae.*

Usually strong promoters such as α-amylase (*amyB*) and glucoamylase (*glaA*) are used (Tsuchiya *et al.,* 1992, 1993; Lee *et al.,* 1996a; Hirozumi

1. Conidiospores are inoculated in DPY media [2% dextrin, 1% polypeptone, 0.5% yeast extract, 0.5% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, pH5.5]. The cultures are incubated for 18-20 hours at 30℃.
2. Mycelia are collected by filtration with sterile Myracloth (Calbiochem, La Jolla, CA) and rinsed with sterile ddH_2O.
3. Protoplasts are prepared by incubation of the mycelia with Solution I [1% Yatalase (TaKaRa, Shuzo, Kyoto, Japan), 0.6M $(NH_4)_2SO_4$, 50 mM Maleate buffer (pH 5.5)] at 30℃ by mild agitation for 3 hours. Protoplast formation is checked by microscopic observation.
4. Protoplasts are separated from mycelia by filtration through sterile Myracloth. The protoplast suspension is diluted (1:1) in Solution Ⅱ [1.2M sorbitol, 50 mM $CaCl_2$, 35 mM NaCl, 10 mM Tris-HCl (pH 7.5)].
5. Protoplasts are collected by centrifugation (2000 rpm, 8 min, 4℃) and washed twice with Solution Ⅱ. Finally, protoplasts are resuspended (2.5×10^8 /ml) in Solution Ⅱ.
6. The protoplast suspension (200 μl) is mixed with transforming DNA (5-10 μg/10 μl) and incubated on ice for 30 min.
7. In three serial steps 250, 250 and 850 μl of Solution Ⅲ [60% PEG4000, 50 mM $CaCl_2$, 10 mM Tris-HCl (pH 7.5)] are carefully mixed with DNA-protoplast mixture and kept at room temperature for 20 min.
8. PEG-treated protoplast suspensions are diluted with addition of 5-10 ml Solution Ⅱ and protoplasts are collected by centrifugation (2000 rpm, 8 min, 4℃) and resuspended in 500 μl of Solution Ⅱ.
9. Aliquots of this suspension are added in 5 ml Top agar (selective media including 1.2M sorbitol and 0.8% agar) and plated onto selective agar media containing an osmotical stabilizer (1.2 M sorbitol).

After 5-7 days cultivation at 30℃, transformants can be seen. These are inoculated onto new selective media. This inoculation is repeated more two times to generate homokaryotic transformants.

FIG. 4. Transformation procedure of koji mold, *A. oryzae.*

et al., 1999; Sakuradani *et al.,* 1999; Maruyama *et al.,* 2000a). Recently, Minetoki *et al.* (1998) found the conserved sequence, Region III in the promoter regions of the amylase-encoding genes *amyB, glaA,* and *agdA* (α-glucosidase) of *A. oryzae.* Introduction of multiple copies of the Region III fragment into the *agdA* promoter resulted in a significant

increase in promoter activity at the transcriptional level. The expression plasmids (pNAN8142, and pNGA142) with this improved promoter were used for the production of several useful proteins including 1,2-α-mannosidase (Ichishima *et al.*, 1999).

The tyrosinase-encoding gene, *melO* is highly expressed in later phases of submerged culture of *A. oryzae*. Glucoamylase encoded by the *A. oryzae glaB* gene, which is not expressed in the submerged culture, was used as a model target protein. The *melO* promoter fused with the coding region of *glaB* was introduced into *A. oryzae*. The maximum yield by the transformant was 0.8 g/liter broth, and the *glaB* product amounted to 99% of the total extracellular protein. This experiment provided a high-level and high-purity protein overproduction system in *A. oryzae*. (Ishida *et al.*, 2001). For constitutive gene expression in *A. oryzae, tef1* (Kitamoto *et al.*, 1998) and *pgkA* (Ogawa *et al.*, 1994) promoters were also used.

D. Gene Disruption and Antisense Inhibition

In order to elucidate the function of cloned genes, gene disruption experiments are frequently carried out. Since the koji mold *A. oryzae* has multinucleate conidia, it is much more difficult to obtain disruptants than it is for *A. nidulans*, which has a single nucleus in a conidium. Nevertheless, successful disruption of many genes including *amdS, dppIV, arpA, brlA, dhcA, vmaA,* and *vmaC* from *A. oryzae* was reported (Gomi *et al.*, 1991; Doumas *et al.*, 1998; Hirozumi *et al.*, 1999; Yamada *et al.*, 1999; Maruyama *et al.*, 2000; Kuroki *et al.*, 2001a, 2001b).

Further, using an antisense control strategy, an *A. oryzae* mutant that produced low levels of carboxypeptidases (CPases) was isolated (Zheng *et al.*, 1998). The mutant expressing the antisense RNA of the structural gene of CPase O showed about 30% of the CPase activity in the parent strain. Antisense RNA expression of *melB* gene encoding tyrosinase (Obata *et al.*, 2000) reduced melanization of the resulting koji as described below.

IV. Cloning and Analysis of Genes Essential for the Fermentative Process in *A. oryzae*

As mentioned above, the most important role of koji molds during fermentation is hydrolysis of high polymer compounds such as starch, protein, and cellulose. Therefore, initial targets of gene analyses in koji molds were the structural genes for hydrolytic enzymes, especially amylases including α-amylase and glucoamylase from strains of *A. oryzae*, essential for sake brewing. Recently, the target has shifted to genes related to regulation and secretion of these hydrolytic enzymes.

Moreover, in addition to these fermentation-related genes, many important genes for elucidating cell biological processes have also been cloned and analyzed.

A. Hydrolytic Enzymes

α-Amylase is also known as Taka-amylase A, named after its discoverer J. Takamine, and its structure has been analyzed in great detail, which contributed to advances in protein chemistry. Genes encoding the α-amylase were independently isolated from four laboratories in 1989 (Gines *et al.*, 1989; Tada *et al.*, 1989; Tsukagoshi *et al.*, 1989; Wirsel *et al.*, 1989). Unexpectedly, *A. oryzae* has three genes encoding α-amylase with the same amino acid sequence. Since α-amylase is one of the most important enzymes for sake brewing, *A. oryzae* has evolved to have three copies of genes due to the selection of strains with high amyloytic activity at the tane koji preparation companies for more than a several hundred years. These three copies (*amyA, amyB, amyC*) are located on different chromosomes and separated from each other by physical mapping using pulse field gel electrophoresis (Kitamoto *et al.*, 1994). Interestingly, other koji molds (*A. shirousamii* and *A. awamori*) have the same α-amylase gene with high identity of more than 98% at the nucleotide level. Since most genes cloned and analyzed from *shochu koji* mold (*A. awamori* and *A. shirousamii*) have 60–80% of identity with sake koji mold (*A. oryzae*), it has been suggested that horizontal transmission from *A. oryzae* to *A. awamori* and *A. shirousamii* occurred during the past few hundred years or less. A transposase encoding gene has been found near an amylase gene in *A. oryzae* (Gomi, 2001).

As shown in Table III, four amylase genes including α-amylase, two types of glucoamylase, and α-glucosidase have been isolated from *A. oryzae*. Glucoamylase activity is a very important factor for the production of a high-quality sake named *ginjou-shu*. Therefore, after Hata *et al.* (1991b) cloned the glucoamylase gene (*glaA*), transformants with high copies of *glaA* gene were constructed and examined for their glucoamylase activity. The transformants showed about 20–30-fold higher activity of glucoamylase in submerged culture, but almost no increase in the solid-state culture associated with koji making. Next, Hata *et al.* (1998) isolated another glucoamylase gene (*glaB*) from *A. oryzae*, and showed that *glaB* is expressed specifically under solid-state culture conditions (Ishida *et al.*, 1998, 2000). Since GlaA contains the glucoamylase catalytic domain along with a starch-binding domain, which is comparable to GlaA from *A. awamori*, it can hydrolyze raw starch. On the contrary, GlaB, without a starch-binding domain, hydrolyzes only steamed

TABLE III

AMYLASE GENES CLONED FROM KOJI MOLDS

Enzyme name (gene name)	References
A. oryzae	
α-Amylase (*amyA, myB, amyC*)	Tada *et al.* (1989); Tsukagoshi *et al.* (1989); Gines *et al.* (1989); Wirsel *et al.* (1989)
Glucoamylase (*glaA*)	Hata *et al.* (1991a, 1991b)
Glucoamylase (*glaB*)	Hata *et al.* (1998)
α-Glucosidase (*agdA*)	Minetoki *et al.* (1995)
A. kawachii	
Acid-stable α-amylase (*asaA*)	Kaneko *et al.* (1996)
A. shirousamii	
α-Amylase (*amyA*)	Shibuya *et al.* (1992)
α-Amylase (*glaA*)	Shibuya *et al.* (1990)

starch-like materials during sake brewing. An *A. oryzae* transformant with multiple copies of *glaB* revealed higher glucoamylase activity in solid-state culture like koji making. Thus, *A. oryzae* has two types of glucoamylases, *glaA* is prominently expressed in submerged culture, while *glaB* is expressed in solid-state conditions. It is expected that *A. oryzae* most likely contains similar genes specifically expressed under solid-state culture conditions. In fact, several genes encoding tyrosinase, acid protease, hydrophobin, etc., were identified as solid-state culture specific genes by the *A. oryzae* EST project (Obata *et al.*, 2000; Akao *et al.*, 2001; Hata, 2001).

In comparison to the two amylases, α-amylase and glucoamylase, it was long thought that α-glucosidase did not contribute as much to sake brewing. However, after the α-glucosidase encoding gene (*agdA*) was isolated and analyzed (Minetoki *et al.*, 1995), it was found that it plays an important role during the formation of ethyl-α-D-glucoside and α-D-glucosylglycerol compounds, which contribute to the taste and flavor of sake (Hayakawa *et al.*, 2000, Takenaka *et al.*, 2000). Kaneko *et al.* (1996) isolated another gene (*asaA*) encoding α-amylase from the koji mold, *A. kawachii,* utilized for shochu making, which contained the catalytic domain and the starch-binding domain akin to that of GlaA. This acid stable α-amylase has raw starch-digesting activity. *Aspergillus oryzae* and *A. shirousamii* may also have homologs for *asaA* and *glaB* genes.

Proteinases are important for shoyu and miso making. Proteinase genes cloned from *A. oryzae* are summarized in Table IV. Several studies on these neutral and alkaline proteinases have been reported (Ikegaya *et al.*, 1992; Tatsumi *et al.*, 1994; Fushimi *et al.*, 1999; Ichishima, 2000).

TABLE IV

PROTEINASE AND GLUTAMINASE GENES CLONED FROM *A. oryzae*

Enzyme name (gene name)	References
Alkaline protease (*alpA*)	Tatsumi *et al.* (1989); Murakami *et al.* (1991); Cheevadhanarak *et al.* (1991)
Neutral proteaseII (*mep20*)	Tatsumi *et al.* (1991)
Acid protease (*pepA*)	Gomi *et al.* (1993)
Aspergillopepsin O	Berka *et al.* (1993)
Serine carboxypeptidase	Blinkovsky *et al.* (1999)
Prolyl dipeptidyl peptidase gene (*dppIV*)	Doumas *et al.* (1998)
Aspartic proteinase	Takeuchi *et al.* (1995)
Carboxypeptidases	Zheng *et al.* (1998)
Calpain-like protease (*palB*)	Futai *et al.* (1999)
Glutaminase (*gtaA*)	Koibuchi *et al.* (2000); Thammarongtham *et al.* (2001)

Plant cell wall degrading enzymes such as cellulase, xylanase etc. play vital role during the filtration stage of shoyu making. Genes encoding these enzymes from *A. oryzae* are shown in Table V. During miso making, lipases of koji contribute to flavoring of the final products and such genes encoding the lipases from *A. oryzae* are shown in Table VI.

B. GENES REGULATING THE QUALITY OF FERMENTED PRODUCTS

Breeding of koji molds for the purpose of making more preferable fermented products such as sake, shoyu, and miso has a long history of

TABLE V

PLANT CELL WALL DEGRADING GENES CLONED FROM *A. oryzae*

Enzyme name (gene name)	References
endo-1,4-β-glucanase (*celA*)	Kitamoto *et al.* (1996)
endo-1,4-β-glucanase (*celB*)	Kitamoto *et al.* (1996)
Major xylanase (*xynG2*)	Kimura *et al.* (2000)
Xylanase (*XynF1*)	Kitamoto *et al.* (1998a, 1999b)
Xylanase gone (*xynG1*)	Kimura *et al.* (1998)
β-Xylosidase (*xylA*)	Kitamoto *et al.* (1999a)
Polygalacturonase (*pgaA*)	Kitamoto *et al.* (1993)
Pectinmethyl esterase (*pmeA*)	Kitamoto *et al.* (1999c)
Pectin lyase gene (*pel1*)	Kitamoto *et al.* (2001a)
Pectin lyase gene (*pel2*)	Kitamoto *et al.* (2001b)
Polygalacturonase (*pgaB*)	Kitamoto *et al.* (1998b)
Cellulase (*celD*)	Matsui *et al.* (2001)

TABLE VI

LIPASE GENES CLONED FROM *A. oryzae*

Enzyme name (gene name)	References
Lipolytic enzyme (*cutL*)	Ohnishi *et al.* (1995)
Monodiacy llipase (*mdlB*)	Tsuchiya *et al.* (1996)
Triacylglycerol lipase (*tglA*)	Toida *et al.* (2000)
Phospholipase A1	Watanabe *et al.* (1999)

over a 100 years. Although strains derived from mutation, cell fusion, and screening were all adopted (Hara *et al.*, 1992), little is known about the molecular mechanism of these improved varieties. Recently, however, several genes that are closely involved in the quality of fermented products, especially sake, have been isolated and illustrated.

1. *Off-Flavor Formation in Sake*

An unfavorable and characteristic odor, called *mureka,* is often formed in nonpasteurized sake during storage and commercial distribution at room temperature. The main component involved in such off-flavoring has been identified as iso-valeraldehyde, which is formed by the activity of a novel enzyme known as isoamyl alcohol oxidase (Yamashita *et al.*, 1999). Recently, this isoamyl alcohol oxidase encoding gene was isolated from *A. oryzae* and designated as *mreA*. The derived protein was comprised of 567 amino acids with a presumed signal peptide consisting of 24 amino acids at the N-terminus. Moreover, nine potential N-glycosylation sites were also present (Yamashita *et al.,* 2000). It was confirmed that *mreA* gene was not only involved in mureka formation but also in the formation of a key desirable smell named *kurika.*

2. *Browning of* Sake-Kasu *(Sake Cake)*

Following the completion of the main fermentation of sake *moromi* mash, filtration on a filter press is carried out. The resultant filtrate is sake, and the residue is called sake-kasu (sake cake), which is used for making a kind of soup and also as a raw material for Japanese pickles. When koji mold with high tyrosinase activity is used, the resultant sake-kasu turns black after storage for a few days, which is unpreferable and called *kuro-kasu.* The tyrosinase catalyzes both mono-oxygenation of tyrosine to DOPA (3,4-dihydroxyphenylalanine) and oxidation of DOPA for first step of melanin biosynthesis. The tyrosinase-encoding gene, *melO,* has been isolated from *A. oryzae* (Fujita *et al.,* 1995). Another tyrosinase-encoding gene expressed in solid-state culture has also been

isolated and named *melB* (Obata *et al.*, 2000). Northern analysis revealed that the *melB* gene is specifically expressed in solid-state culture. The *melB* gene carries six exons interrupted by five introns and has an open reading frame encoding 567 amino acids. When tyrosinase activity was reduced by the antisense or excess immature RNA of *melB* in solid-state culture, melanization of the resulting koji was successfully repressed, indicating that the *melB* gene encodes the tyrosinase related to *kuro kasu*.

3. *Deterioration of Sake by Ferrichrysin Formation*

Water with very low iron content should be used for sake brewing. When water containing more than 0.02 ppb of iron ions is used, the color of sake turns brown-red and quality of taste deteriorates. Both the color and the off-flavor are due to deferriferrichrysin, a compound that is produced by *A. oryzae,* which binds iron ions to form red-colored ferrichrysin. Recently, ferrichrysin, a kind of siderophore, regulating the genetic element *sreAo* was isolated from *A. oryzae* (Watanabe *et al.*, 2001). Gene disruptants (Δ*sreAo*) showed constitutive expression of deferriferrichrysin even in iron-rich media. It is expected that in the future the details of ferrichrysin formation at a molecular level will be elucidated.

4. *Glutamic Acid as a Key Amino Acid in Shoyu*

Glutaminase, which converts glutamine to glutamic acid, is one of the most important enzymes in flavor enhancement. While glutamine has no taste, glutamic acid has a good taste called *umami*. When the glutaminase gene was isolated from *A. oryzae* and bred into an *A. oryzae* transformant, a marked increase in glutaminase activity was observed (Koibuchi *et al.*, 2000).

5. *No Production of Aflatoxin in Koji Molds*

Although the mycotoxin-producing species, *A. flavus* and *A. parasiticus,* are closely related to koji molds, the koji mold, *A. oryzae,* has been certified safe on epidemiological aspects and has a GRAS status (Generally Regarded As Safe). For instance, it is well known that *A. oryzae* has been used in Japanese food fermentations for a long time, and that the Japanese people, who have consumed the fermented foods, such as miso, shoyu, and sake, have a very long life compared to other populations in the world. Nevertheless, the possibility that the koji molds may produce mycotoxins has been of considerable concern. Recently, the sterigmatocystin gene cluster was identified in *A. nidulans* and its regulation system was also elucidated (Brown *et al.*, 196). Several reports show that the structural genes for the aflatoxin–sterigmatocystin

gene pathway exist in strains of *A. oryzae,* although most of them have deletions in the gene cluster, suggesting that koji molds do not have the ability to produce these mycotoxins. In one intensive study Kusumoto *et al.* (2000) examined the structure of the aflatoxin gene cluster in *A. oryzae.* Thirty-nine strains belonging to this species were examined for the presence of *pksA, fas1A, aflR,* and *vbs,* and the results compared with those for *ver-1* obtained previously. These five genes are involved in aflatoxin biosynthesis in *A. parasiticus.* The strains examined were categorized into three groups; group 1, having the five homologs; 2, having *ver-1* and *vbs;* and 3, having *vbs* homologs. Long-polymerase chain reaction (PCR) analysis of the regions between the five homologs in *A. oryzae* Institute for Fermentation, Osaka (IFO) 4135, coupled with Southern hybridization analysis, showed that those homologs are clustered in a similar arrangement as that found in *A. parasiticus.* Directed deletions of the cluster occurred in strains of the koji mold, *A. oryzae,* apparently as a result of long time selection by tane koji manufacturers. To date all reports (Kusumoto *et al.,* 1998a, 1998b; Motomura *et al.,* 1999; Watson *et al.,* 1999; Geiser *et al.,* 2000) on aflatoxin pathway genes from koji molds suggest that *A. oryzae* is safe. The genome sequence project of *A. oryzae* will confirm that this organism is truly unable to produce mycotoxins.

C. OTHERS

Genes encoding nucleases (Table VII and transcription factors (Table VIII) have also been cloned and analyzed from *A. oryzae.* The catabolite repressor (*creA*), a positive regulator of amylase (*amyR*), and nitrogen metabolism (*areA*), involved in amylase and protease production, could be important for koji molds. DNA binding protein (*blrA*) involved in conidial formation is one of the key factor in tane koji manufacturing, as suggested by Yamada *et al.* (1999), who showed that overexpression of *blrA* promotes conidial formation even in submerged culture. In tane koji preparation, koji molds are cultured at alkaline pH

TABLE VII

NUCLEASE GENES CLONED FROM *A. oryzae*

Enzyme name (gene name)	References
Ribonuclease T2 (*rntB*)	Ozeki *et al.* (1991)
Ribonuclease T1 (*rntA*)	Fujii *et al.* (1995)
Nuclease S1 (*nucS*)	Lee *et al.* (1995)
Nuclease O (*nucO*)	Sano *et al.* (1996)

TABLE VIII

TRANSCRIPTION FACTOR GENES CLONED FROM *A. oryzae*

Enzyme name (gene name)	References
Positive regulator of *amdS* (*amdR*)	Wang *et al.* (1992)
Catabolite repressor (*creA*)	Lee *et al.* (1996b)
DNA binding protein (*facB*)	Todd *et al.* (1997)
Positive regulator of amylase (*amyR*)	Petersen *et al.* (1999); Gomi *et al.* (2000)
Nitrogen metabolism (*areA*)	Christensen *et al.* (1998)
DNA binding protein (*blrA*)	Yamada *et al.* (1999)
CCAAT-binding complex (*hapC*)	Tanaka *et al.* (2001)
pH Regulating protein (*pacC*)	Sano *et al.* (2000)

condition (around pH 10), whereas koji making process for sake brewing is carried out at acidic or neutral pH, suggesting that the transcription factor for pH regulation (PacC) is important. The *palB(ory)* gene, encoding calpain-like protease (Futai *et al.*, 1999), may be involved in processing of PacC in *A. oryzae*. Juvvadi *et al.* (2001) isolated the *cnaA* gene encoding the catalytic subunit of calcineurin from *A. oryzae*, and antisense expression of *cnaA* caused reduced growth of *A. oryzae* under various stress conditions including the presence of 1*M* NaCl, alkaline pH 10, and high temperatures (37 and 42°C). Recently, Kuroki *et al.* (2001a, 2001b) also found some genes encoding the vacuolar membrane ATPase expressed under alkaline pH-mediated growth of *A. oryzae*, and cloned *vmaA*, the gene encoding a 69-kDa catalytic subunit, and *vmaC*, the gene encoding a 16-kDa proteolipid subunit of the vacuolar H^+-ATPase. Disruption of these genes caused very poor growth at alkaline pH condition, indicating that the vacuolar H^+-ATPases may play an important role in the tane koji manufacturing process.

V. Visualization of Organelles in *A. oryzae*

A. OBSERVATION OF NUCLEI IN *A. oryzae* CELLS

It is difficult to isolate recessive mutants from *A. oryzae* due to the presence of multiple nuclei not only in hyphae but also in conidia. Figure 5 shows the number of nuclei in a population of conidia of different *A. oryzae* strains used in sake brewing. This multinucleate characteristic contributes to the maintenance of genetic stability of koji molds. However, it makes genetic manipulation involving the isolation of auxotrophic mutants and gene disruption extremely difficult. Maruyama *et al.* (2001) succeeded in visualizing nuclei in *A. oryzae* using EGFP (Enhanced Green Fluorescent Protein) fused with histone H2B. Since

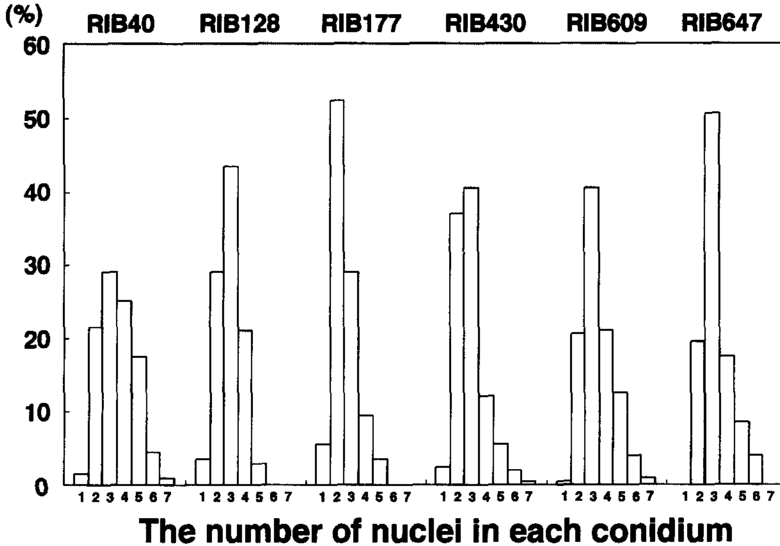

FIG. 5. The number of nuclei in each conidium of *A. oryzae* strain. Percentages of the number of nuclei in each conidium are presented. Two hundred conidia of the wild-type strain, RIB40, and five *A. oryzae* strains used in sake brewing were collected and scored with 4′,6-diamino-2-phenylindole dihydrochloride (DAPI) staining.

the EGFP system enables the observation of living cells of koji mold, it should be possible to isolate mutants with uninucleate conidia by FACS (fluorescence activated cell sorter) (Ishi *et al.*, 2001).

B. OBSERVATION OF VACUOLES, ER, AND SECRETED PROTEIN IN *A. oryzae*

The EGFP system was also utilized for visualization of organelles such as vacuoles and endplasmic reticulum (ER) in *A. oryzae*, using fusion with *A. nidulans* carboxypeptidase Y (CPY) (Ohsumi *et al.*, 2001) and *A. oryzae* BiP (Kasuya *et al.*, 1999), respectively (Kitamoto, 2000). Using EGFP fusion protein with RNase T1 (Fujii *et al.*, 1995), analysis of the protein secretion pathway in *A. oryzae* was facilitated (Masai *et al.*, 2001).

VI. The *A. oryzae* EST and Genome Projects

An *A. oryzae* EST project was successfully completed in 2000 by a Japanese consortium including several universities, national research institutes, and companies. The EST database was constructed using several cDNA libraries made under varied growth conditions such as rich, poor, liquid, solid, acidic, and alkaline media, and germlings from

conidia. About 6,000 clustering groups were identified from a total of 17,000 clones sequenced. Since *A. oryzae* is estimated to have about 9,000–10,000 genes, about 60% of the genes are now available. In addition to this EST project, an *A. oryzae* genome sequence project has begun at Biotechnology Center, National Institute of Technology and Evaluation (NITE) Japan in 2001 (see article by Machida, Chapter 3, this volume).

VII. Conclusion and Future Prospects

The koji molds are the most popular and important microorganisms for Japanese cuisine, and excellent strains of koji molds have been selected for centuries. By virtue of their industrial applicability and useful metabolite production, it can be certain that there are no other filamentous fungi as useful as the koji molds throughout the world. In particular, *A. oryzae* has the ability to produce copious amounts of useful enzymes, and has a history of industrial use for both enzymes and Asian alcoholic beverage production. The gene manipulation techniques drived from yeast paved ideas for the application of similar techniques to economically unimportant fungi such as *N. crassa* and *A. nidulans*. I anticipate that similar gene manipulation techniques on economically important koji molds have a highly promising future. Although at this juncture the understanding of these organisms at a molecular level is in its infancy, in the near future, annotated sequences of *A. oryzae* genomes will become available and the cell biology of this organism will be established in detail. Further, these molds, along with *A. nidulans* and *A. niger*, will be commercially exploited for the production of vital mammalian proteins. *Aspergillus oryzae* is poised to become one of the most established and refined models among the filamentous fungi, proving worthy of its epithet of "Japan's national microorganism."

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Noninvasive Methods for the Investigation of Organisms at Low Oxygen Levels

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I. Introduction

Living organisms may be regarded as machines that control and harness the chemistry of dioxygen. It is therefore not surprising that an almost infinite diversity of measured responses have evolved to the almost ubiquitously present, energetically useful, but potentially catastrophic oxidant. Even those that do not use it cannot afford to be indifferent to it. O_2 produces major effects at almost infinitesimally low concentrations geared either to its disposal or utilization. It is not widely recognized that the air we breathe (20.9% O_2; 21.2 kPa O_2; 158 mm Hg; equivalent to 278 μM O_2 at 37°C) represents a huge excess of O_2, even for the most highly aerobic life processes. When studied *in vitro*, O_2-reactive systems have highly avid binding characteristics and become saturated at much lower partial pressures than ambient. Figure 1 shows the O_2 dependencies for human gas transport systems. Although the alveolar air in equilibrium with arterial blood contains the equivalent of 100 μM O_2, discharge from blood oxyhemoglobin, depending upon CO_2 tension, occurs within a range of 80–20 mm Hg ≡ 60–30 μM O_2. The oxymyoglobin store of red muscle begins to unload at below 10 μM O_2. The working

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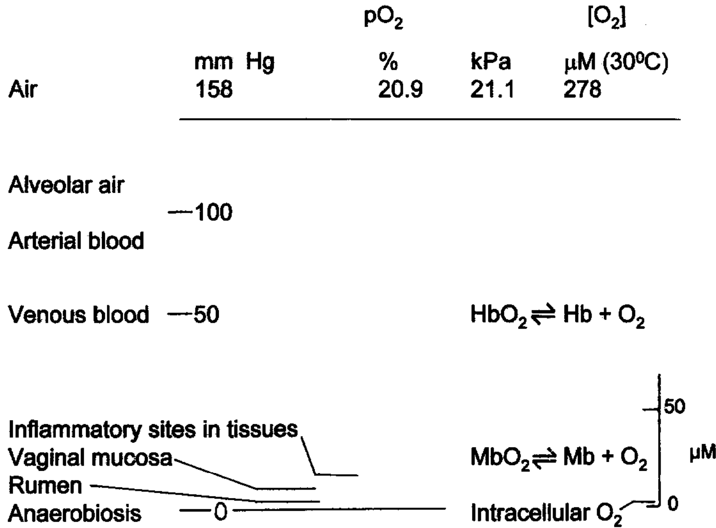


FIG. 1. Oxygen concentration decreases during transport from lungs to mitochondria in the human body.

pO_2 there, is about 3 mm Hg (Wittenberg and Wittenberg, 1989). Steep gradients of O_2 diminish this working range over which intracellular O_2 is poised, down through an estimated two orders of magnitude, so that intramitochondrial O_2 may be as little as 100 n*M*. The intracellular environment is thus highly microaerobic, to such an extent that these values have not yet been measured directly, but rather are derived from estimates obtained by *in situ* observation of the redox states of respiratory chain components using noninvasive fluorimetric (for NADH) and spectrophotometric (for myoglobin and cytochromes) readout (Chance *et al.*, 1989).

Awareness of this hierarchy of oxygenation states in the circulatory system as well as in mammalian organs, tissues, and cells can provide fundamental insights (Vanderkooi *et al.*, 1991; Silver and Erecinska, 1998). It also provides an indirect appreciation of the likely lifestyles of invasive microorganisms and conditions necessary for their pathogenicity. It has been suggested that sometimes the "nonculturability" of some putative pathogens may be traced to the inadvertant experimental provision of toxic levels of O_2. Microaerophily is a comparatively neglected, yet potentially pivotal, aspect of microbiology (Hungate, 1966; Bryant, 1991; Williams and Coleman 1992; Fenchel and Finlay, 1995; Lloyd and Biagini, 1998).

In this article, I outline the ways currently available for studies of the O_2 relationships of microbes, using noninvasive methods of observation.

II. Noninvasive Continuous Readout of Cellular Functions

The requirement for monitoring functions *in situ* in living organisms continues to attract new technology; the increasing sensitivity and specificity thereby afforded improve our capabilities for control of biotechnological processes as well as our understanding of fundamental issues. For studies of microrganisms, as for other biomedical applications, nonintrusive as well as noninvasive observation is essential to minimize perturbation of the object of attention. The list of physical techniques that can be applied to intact organisms grows as sensitivities are refined: optical methods include ultraviolet (UV), visible and infrared (IR) spectroscopies, as well as fluorimetry (Tamura *et al.,* 1989). Ion-specific electrodes, especially those for Ca^{2+}, K^{+}, pH, and redox potential, can be used in combination with other techniques (Pressman, 1968). Nuclear magnetic resonance (NMR) spectrometry, especially of ^{13}C and ^{31}P nuclei, provide well-established ways of tracing metabolic transformations *in vivo.* Microcalorimetric monitoring provides an extremely sensitive measure of metabolic activity (Tensink *et al.,* 1996). Less exploited has been electron spin resonance spectroscopy (Lloyd and Pedersen, 1985). As well as providing kinetic information, recent innovations enable high resolution imaging in three dimensions.

Combinations of these physical methods with continuous monitoring of controlled environmental conditions provides powerful new approaches to functional analysis of microbial metabolic performance. It also makes possible the elucidation of interactions between species in mixed cultures, in biofilms, and in some natural ecosystems. The scale of investigation can be chosen so as to be appropriate at the single cell level or in highly concentrated populations in large-scale fermenters.

Continuous readout methods provide data that are much more amenable to analysis than are series of discrete time measurements. As well as providing high-quality information for kinetic analyses, especially valuable for the interpretation of periodic phenomena, automated data acquisition with frequent sampling of continuous output enables reliable assessments of long-term (days, weeks, months, or years) behavior. Where multiple-parameter (variable) monitoring is feasible, new insights into microbial processes *in vivo* and activities become available.

This review summarizes application of noninvasive physical methods as a means of determination of activities of organisms at low oxygen levels.

III. Techniques for Low O_2 Studies

A. Oxygen Measurements

Of the available techniques (Table I) for monitoring O_2, criteria for choice of a method depend primarily on whether gas or liquid phase levels are to be determined, the total time scale of the experiment, the rapidity of changes to be observed, and the sensitivity of detection required. The traditional manometric measurements (Umbreit *et al.*, 1964) or their more recent adaptations (e.g., the Gilson respirometer) are still

TABLE I

Methods for Oxygen Determination

	Continuous liquid phase measurement	Lower limit of sensitivity	Response time
Gasometric			
Warburg manometry			3 min
Cortesian diver			5 min
Chemical			
Hemoglobin	√	10 n*M*	10 s
Physical			
Mass spectrometry (membrane inlet)	√	0.1 μM	10 s
Phosphorescence quenching	√	0.1 μM	
Fluorescence quenching	√	1.0 μM	20 s
Paramagnetic analysis			
Gas-liquid chromatography			
Electron spin resonance			
Electrochemical			
Winzler			
Lehninger			
Longmuir/Hagihara (rotating gold)		10 n*M*	
Clark (membrane)	√	0.1 μM	30 s
Chance			
Joliot			
Hersch (Cd Galvanic cell)			
ZnO_2			
Zirconia			
Biological			
Photobacterium	√	0.1 n*M*	1 s

useful where many parallel measurements of O_2 consumption are made over short periods (hours) from a gas phase. Oxygen electrode methods (Degn *et al.*, 1980; Gnaiger and Forstner, 1983) use either polarographic sensors, in which the polarized electrodes produce a current in an external circuit, or the galvanic principle, whereby a current is generated directly. These two devices, represented respectively by the Clark and Mackereth electrodes, are invaluable for measurements of O_2 in the liquid phase in fermenters, especially in their steam-sterilizable modifications. Reliable over long periods up to many months and through many cycles of sterilization, these large-scale devices continue to outperform other newer O_2 monitoring techniques [e.g., fluorescence-quenched fluorophore films interfaced to fiber optic light leads, or phosphorescence quenching of soluble phosphors (Lo *et al.*, 1996)].

An especially useful electrode that has provided the O_2-measuring device central to the applications described here is that manufactured by Radiometer (Copenhagen). The advantages of this O_2 electrode include:

1. reasonably rapid response ($t_{90\%} < 30$ s, depending on membrane material and thickness);
2. high sensitivity ($\sim 0.1\ \mu M$, again depending on the membrane employed);
3. stability (adequate for experiments over many hours);
4. low consumption of O_2 (due to very small area Pt cathode).

It can be sterilized by using 70% ethanol; like all Ag-Pt based Clark electrodes, it can be affected by some other gases (CO_2, H_2S, Cl_2, Br_2, I_2) and high-intensity illumination.

Where other gases (or some membrane-permeable low molecular weight volatile organic compounds) are to be measured alongside O_2, membrane inlet mass spectrometry (MIMS) provides an ideal monitoring technique. The specifications provided by a small quadrupole mass spectrometer (we have found ideal the ones that scan to mass 200 from Hiden Analytical), fitted with a MIMS probe devised by Bohátka *et al.* (1983) (Table II), are adequate for many biomedical or environmental applications. The theory and practice of this device have been reviewed (Lloyd *et al.*, 1983b, 1985a, 1992b, 1996; Boddy and Lloyd, 1989). O_2 measurements using this mass spectrometric method and the electrode method are similar in sensitivities, as it is the unstirred (Nernstian) layer on the membrane that limits sensitivities and response times for liquid phase measurements for both techniques. However, the facility for parallel measurements of other gases of biological interest (CO_2, H_2, CH_4, N_2, N_2O, etc.) as well as many low molecular weight volatiles (CH_3OH, C_2H_5OH, CH_3SH, etc.) (Harland *et al.*, 1987), immediately suggest endless applications in microbiology (see Section IV below). As

TABLE II

SPECIFICATIONS FOR A MEMBRANE-INLET MASS SPECTROMETER

Specification	Details
1. Multispecies monitoring (single probe)	H_2, CH_4, O_2, CO_2, N_2, NO_x, H_2S, CO, C_2H_2, H_2O, C_2H_4, CH_3SH, CH_3OH CH_3CH_2OH, CH_3COOH, and other low mol. wt. volatiles
2. Gas- or liquid-phase (single probe)	
3. Multiprobe inlet feasible	(Up to 64 have been employed)
4. Steam sterilizable	(Many tens of cycles using silicone rubber membrane)
5. Small sensing zone (>4 μM)	(Makes probe stirring rate insensitive)
6. Highly specific	(Resolution on mass scale better than 1 amu) (Must be aware of overlap of cracking patterns)
7. Highly sensitive	(e.g., 0.1 μM O_2)
8. Wide dynamic range	(From ppm → 10% gives linear output)
9. Continuous readout	(Effectively: scan rate 10^3 amu/s)
10. Noninvasive	(Gas consumption can be made negligible)
11. Long-term stability	(<1% drift per week; filament life >1 year)
12. Fast response	(1 s for inlet response; probe response depends on membrane material and thickness, and temperature. In liquid phase, may be stirring rate dependent)
13. Compact and portable	3 × 20 kg, limited by vacuum system
14. Reliability in adverse conditions	Good; output affected by temperature fluctuations
15. Ease of interfacing	RS 232 Port
16. Cost	£25 k in 2001

for membrane-based electrodes, a permeable membrane serves as the interface between the sensor and the biological system.

For monitoring very low O_2 levels, the bioluminescence of luminous bacteria provides an extremely sensitive method (Lloyd *et al.*, 1981) provided that conditions are optimal (Waters and Lloyd, 1985). Thus, the photoemission of these marine species is O_2 limited at concentrations less than 10 μM, and is proportional to concentrations over several orders of magnitude below this (Lloyd, 1990). The lower limit of sensitivity differs between species, depending on the affinity of the bacterial luciferase for O_2. Thus monitoring of levels of around 1 nM O_2 are feasible using those bacteria with the most avidly O_2-binding luciferase systems (Lloyd *et al.*, 1985b). Spectrophotometric monitoring of the deoxygenation of oxyhaemoglobin provides another extremely sensitive method for very low O_2 concentrations (Barzu, 1984).

Detection of photoemission traditionally employed a photomultiplier, but the availability of highly sensitive charge-coupled devices (CCD) provides an alternative with the added advantage of provision of spatial information. Imaging of oxygen (and nitric oxide) by electron

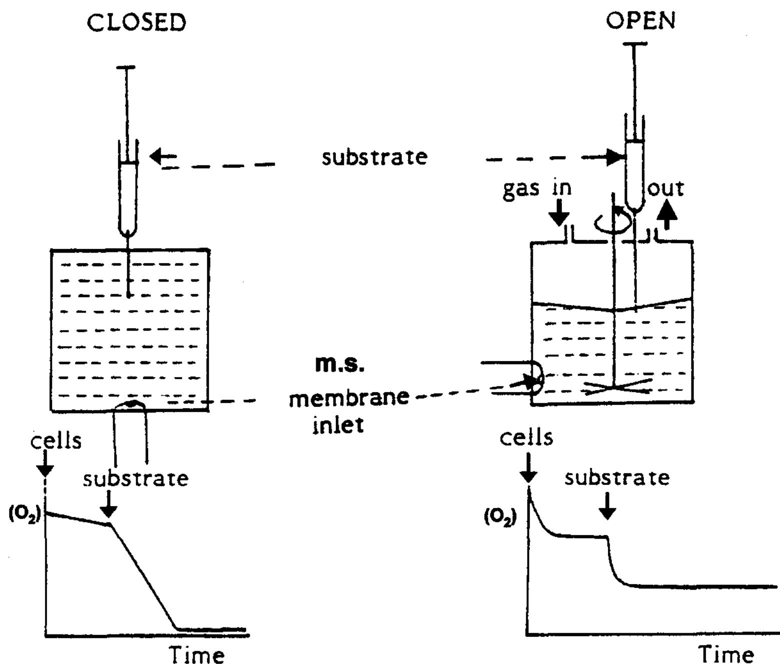


FIG. 2. Closed and open gas-flow systems. In the former, O_2 concentration decreases during its consumption, whereas in the latter it can be maintained at a constant steady state.

spin resonance in tissues and organs is now possible (James *et al.*, 1999; Jackson *et al.*, 2001), but resolution is not adequate for cellular studies.

B. Open vs Closed Systems

Maintenance of low O_2 environments for organisms during experimental procedures over extended time periods necessitate systems open for gas flow (Degn *et al.*, 1980) (Fig. 2). Both the measuring method and the biomass consume O_2, and thus in a closed system O_2 levels fall continuously. O_2 control for extended periods and measurement in a steady state requires an open system. A second advantage is that the presence of adventitious small air leaks become less important in an open system under a small positive pressure provided by the mobile gas flow. Stainless steel reaction vessels designed for different applications are fitted with quartz glass observation windows.

Flow lines are also of stainless steel (e.g., 3.2 mm o.d., 1.0 mm i.d.), as are all fittings. Black butyl rubber is less gas permeable than red, and it is always worth a reminder that even a short run of silicon rubber tubing

acts almost as a window for air gases. Traces of O_2, always present in cylinders of N_2 or Ar, must be removed by passage over heated Cu, through Fieser's solution (20 g KOH, 2 g anthaquinone β-sulfonate, 100 ml water, 15 g Na hydrosulfite absorbs 788 ml O_2 until it changes from clear to dull red or brown), through a thick suspension of baker's yeast, or by using a commercially available O_2-scavenging column, (e.g., those supplied by Alltech).

Measurement of dissolved O_2 in a suspension of organisms stirred at constant rate under a gas phase, kept at fixed O_2 tension in a thermostatically enclosed reaction vessel, enables long-term experiments to be carried out. It is better not to bubble, but rather to stir (using a cross-shaped impellor) to produce a stable liquid vortex for gas–liquid exchange. The level of dissolved O_2 represents the balance between supply and demand, so that by choosing appropriate gas phase and cell concentration, organisms can be maintained for extended periods at any required O_2 concentration. The accuracy of control attainable in such a system is extremely good, even for O_2 concentrations $<1\ \mu M$. These systems can be constructed for liquid volumes at any scale, (e.g., 5 ml to 5 liter). The practical limit for using small liquid volumes is reached when evaporative losses become considerable. Hence a requirement for humidification of the mobile gas phase becomes critical. A hanging-drop version of the system (Fig. 3) has been used using a confocal scanning laser microscope or CCD camera. A working volume of 5 ml with a stirring rate of 200 $\text{rev} \cdot \text{min}^{-1}$ gives a response time ($t_{1/2}$, the half time for equilibration in the liquid phase after a step change in gas phase O_2 in the absence of organisms), of about 1.5 min. Calculation of the respiration rate of organisms (V_r) in this system requires determination of the gas exchange coefficent (k):

$$k = \frac{\ln 2}{t_{1/2}} = \frac{0.693}{t_{1/2}},$$

$$V_r = k(T_G - T_L),$$

where T_G, T_L are respectively the O_2 tensions in gas and liquid phases.

For these calculations it is convenient to measure time in min and gas tensions as μM (for T_L) and as the equivalent of μM (for T_G), where 100% (i.e., gas saturation) is the gas solubility at the temperature of the experiment. This value is obtained from the published literature (e.g. Wilhelm *et al.*, 1977).

The dissolved O_2 is measured by an electrode, photobacterium, or MIMS probe fitted through a port below the level of the stirred vortex, or by including photobacterium in the reaction mix and measuring light emission through the window: fluorometric spectrophotometric,

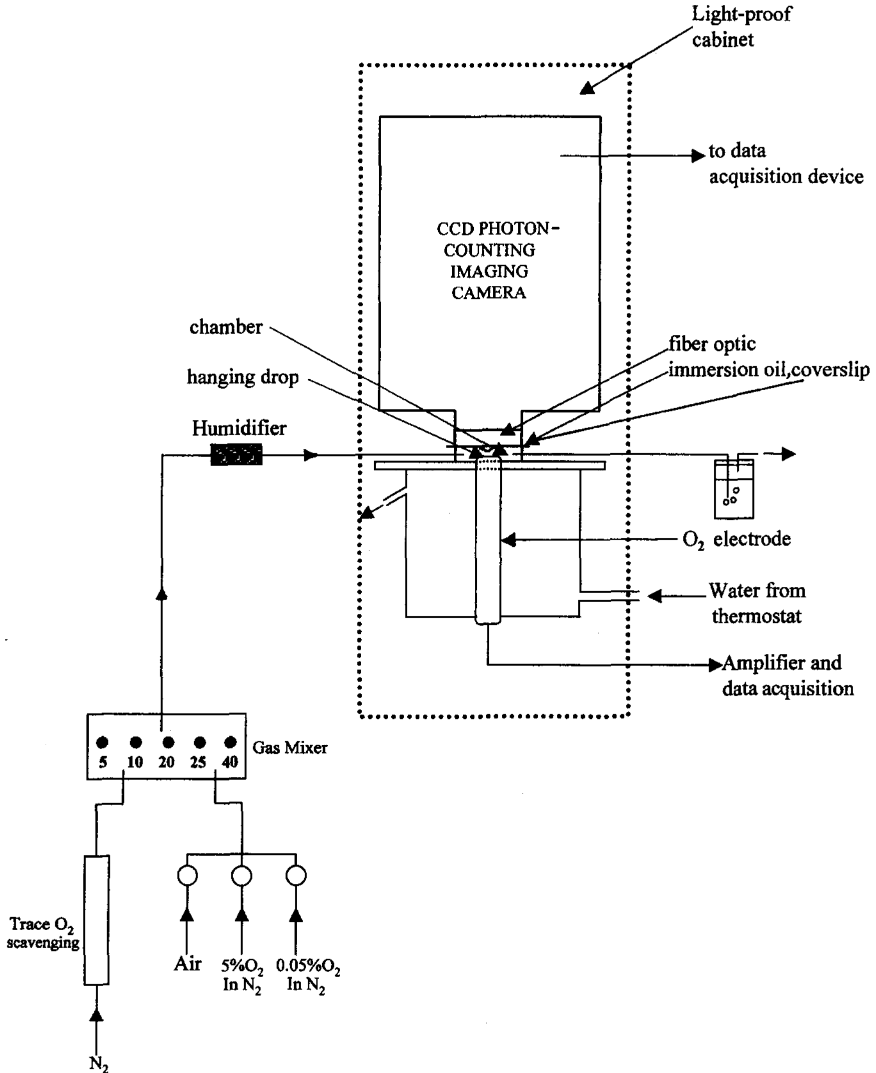
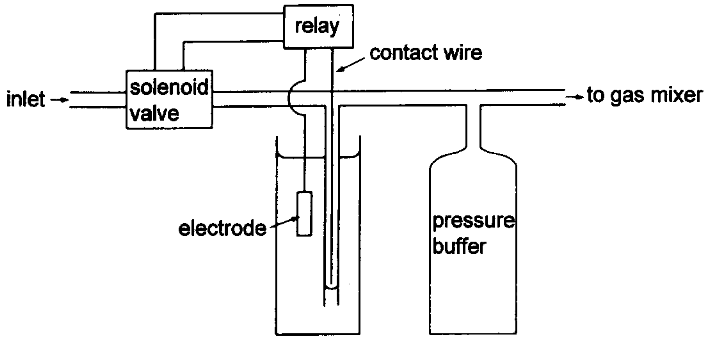


FIG. 3. Hanging-drop chamber open for gas flow. In the configuration illustrated, pO_2 in the gas phase is monitored continuously and simultaneously with images of the organisms under study. These may be labeled with specifically targeted luciferases. Inversion of the system enables dissolved O_2 monitoring in the extracellular buffer. Imaging involves use of a CCD photon-counting imaging camera [e.g., one that stores 385 × 288 pixels at video rates (50 Hz) using microchannel plates, with a background count of about 10 cps over the whole pixel array (D. Lloyd and A. K. Campbell, unpublished data). Alternatively, a confocal laser scanning microscope is used to resolve intracellular events after fluorescent labeling (Lloyd *et al.*, 1999).

A



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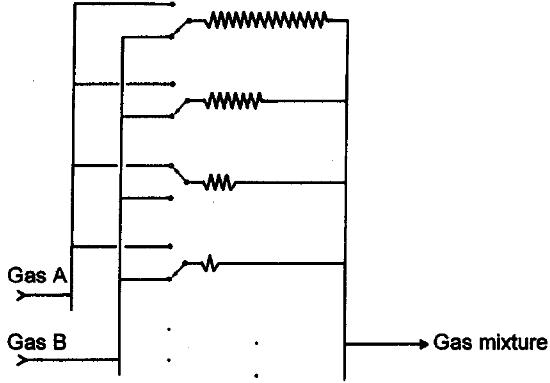

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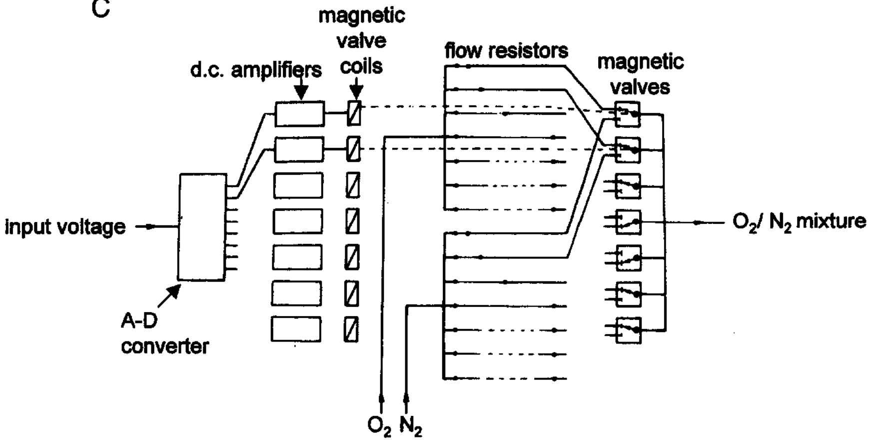


or photon counting observations are made through a second window, using appropriate optical filters. Alternatively, an electron paramagnetic resonance (EPR) or NMR tube serves as the reaction vessel (Lloyd and Pedersen, 1985; Lloyd *et al.*, 1993).

A closed O_2 electrode system, specially constructed to avoid the O_2-leakage problems inherent in many commercially available systems and capable of measuring O_2 to 0.1 μM, has recently been developed (Haller *et al.*, 1994) and used extensively for studies of mitochondria at low O_2 levels and for the inhibitory effects of NO (Boveris *et al.*, 2000; Gnaiger *et al.*, 2000). This system uses viton O-ring seals, a glass rather than perspex vessel and titanium stoppers. Polyetheretherketone-coated stirrers are much preferable to Teflon. A Teflon stirrer bar acts as an O_2 buffer and O_2 leaks back at up to 30 pmol $\cdot$ $s^{-1} \cdot cm^{-3}$ when its concentration is lowered quickly.

C. Gas Mixing

A simple, reliable, and versatile device for mixing gases is that described by Lundsgaard and Degn (1973). It uses a combination of stainless steel capillary tubes acting as flow resistors to apportion the blending of a pair of commercially available gases from cylinders. A series of two-way switches can be used to change the composition of the gas stream; five switches enables generation of a mixture that can be altered from A to B in 5% steps (Fig. 4). The two gases must be provided at equal pressures to their inlets; the most convenient method for this involves the use of water columns to provide pressure heads. The columns are adjusted in a ratio inversely proportional to the respective dynamic viscosities of the two gases (Table III). For most purposes this easily constructed device is adequate. However, a further refinement that uses seven valves and automated switching has been described for the generation of gas gradients (Degn and Lundsgaard, 1980). Another automatic mixer that works on a similar principle, but involves time-shared

FIG. 4. Mixing devices for gases. (Modified after Lundsgaard and Degn, 1973; Degn and Lundsgaard, 1980.) (a) Pressure regulator for input to gas mixer. A T-piece with Pt-tipped contact wire immersed in saline solution (0.9% NaCl) serves as a pressure switch: a solid state relay activates a solenoid valve when contact is made. (b) Digital mixer based on Poiseilles' Law. Parallel tubes with flow conductivities $2^n, \ldots, 4, 2, 1$ are connected to either of the two gases to be mixed by two-way valves. Activating the valves in the sequence of binary counting (0000, 0001, 0010, 0011, etc.) gives the sum of conductivities of one gas to increase and that of the other to decrease with unit step, provided the input pressures are inversely proportional to the dynamic viscosities of the two gases. (c) Automated version of (b) giving a constant gas flow with varying proportion of two gases depending on electrical input voltage.

TABLE III

DYNAMIC VISCOSITIES OF GASES

Gas	Viscosity at 20°C (Micropoise)
Air	183
Argon	222
Carbon dioxide	148
Carbon monoxide	175
Helium	194
Hydrogen	87
Methane	109
Nitrogen	175
Oxygen	202

energization of two valves, is invaluable for the fast changes required in computer-controlled gas composition supply to fermenters that operate under automated control. Mixing of three gases can be achieved by using two manual mixing boxes in series. This configuration is useful for generation of a gas mixture of fixed relative humidity that can be altered instantly from one value to another by switching gas streams.

For most purposes, adequate flow rates of gases (e.g., up to 100 ml min^{-1}) are obtained from the mixing devices described here by using short columns of water (e.g., <1 m) as pressure heads. Where larger gas throughput is required (e.g., for fermentation vessels of 1 liter liquid volume or more) longer water columns (2–3 m) are necessary.

IV. Examples of Applications

A. O_2 KINETICS

Measurement of respiratory rates as a function of O_2 concentration gives an indication of the importance of the functional activity of each terminal oxidase. The use of specific inhibitors (e.g., cyanide, CO, azide for cytochrome aa_3; salicylhydroxamic acid for the alternative oxidase) facilitates the discrimination between oxidases (Lloyd *et al.*, 1979a, 1980). Where more than two oxidases are expressed (e.g., in *Escherichia coli*), the use of mutant organisms with deleted haemproteins has been invaluable. Apparent K_mO_2 values give some indication of the relative importance of each oxidase in terms of the affinities of O_2 binding and hence physiological role over specific ranges of O_2 concentration: cultured mammalian cells can be studied as well as microorganisms (Edwards *et al.*, 1983). Table IV shows the K_mO_2 values for some microbial eukaryotic organisms. H_2O_2 production can

TABLE IV

APPARENT OXYGEN AFFINITIES AND INHIBITION THRESHOLDS OF "ANAEROBIC" PROTOXZOA FROM VARIOUS SOURCES

Organism	Habitat	*In situ* O_2 (μM)	Apparent K_m O_2 (μM)	Inhibition threshold O_2 (μM)	Reference
Hexamita sp.	Free living limnic	0–30	13.97	100	Fenchel *et al.* (1995); Biagini *et al.* (1997)
Metopus contortus	Marine sediment	<2.6	~2.6	<10	Fenchel and Finlay (1990)
Plagiopyla frontata			~2.6	<10	
Giardia intestinalis	Human jejunum	0–60 μM	0.5–6.40	80	Atkinson (1980); Paget *et al.* (1989a); Ellis *et al.* (1994); Paget *et al.* (1989a)
Giardia muris	Mouse small intestine	ND	2.0	15	
Entamoeba histolytica			5.5	16	T. A. Paget (unpublished results)
Trichomonas vaginalis	Human genito-urinary (g-u) tract	13–56	3.2	19	Wagner and Levin (1978) Yarlett *et al.* (1986)
Tritrichomonas foetus	Bovine g-u tract	ND	1.6	8	Lloyd *et al.* (1982b, 1987c)
Dasytricha ruminantium	Ovine rumen	<0.25–3.2	0.33	ND	Hillman *et al.* (1985a);
Eudiplodinium maggi			5.2	5	Scott *et al.* (1983a)
Isotricha spp.			2.33	ND	Hillman *et al.* (1985b);
Polyplastron multivesiculatum			1.7	10	Ellis *et al.* (1989)
Neocalimastix patriciarum			4.0		Yarlett *et al.* (1987)

be monitored spectrophotometrically alongside O_2 consumption (Paget *et al.*, 1987) as can redox states of respiratory chain components (Lloyd *et al.*, 1983a; Lloyd 1985; Hill and Lloyd, 1985; Edwards and Lloyd, 1986).

Escherichia coli cytochromes have extremely high affinities for O_2 binding; thus very low K_mO_2 values have been measured using the spectrophotometric method that follows the course of the deoxygenation of oxyhemoglobin or oxyleghemoglobin (D'Mello *et al.*, 1995, 1996) *Bacillus cereus* too can show extremely high O_2 affinity (Contreras *et al.*, 1999). Cytochrome *bd* has a K_m in the range 3–8 n*M*: in membrane preparations, cytochrome *bo'* gives values between 16 and 85 n*M*. Values of K_mO_2 for intact organisms are 0.5–1.8 μM. The N_2-fixing root nodule endosymbiont *Bradyrhizobium japonicum* has a K_mO_2 of 7 n*M* to cope with the 10–20 n*M* ambient O_2 (Preisig *et al.*, 1996). The proposal that the flavohemoglobin of *E. coli* (Hmp) is an oxygen sensor conflicts with recent measurements of its O_2 affinity; this is the case even in the presence of nitric oxide (Mills *et al.*, 2001). These values correspond to 15 and 80 μM [with and without added flavin adenine dinucleotide (FAD), respectively].

Photochemical action spectra for the relief of inhibition of respiration by CO-liganded hemoprotein oxidases provides a direct confirmation of functional activity (Lloyd and Scott, 1983). This approach reveals those CO-reacting components not contributing to respiration (e.g., the *b*-type cytochrome mistakenly referred to as cytochrome *o*) present ubiquitously in bacteria (Poole *et al.*, 1983), lower eukaryotes (Lloyd *et al.*, 1982a; Unitt *et al.*, 1983; Scott and Lloyd, 1983; Lloyd and Scott, 1986), and nematodes (Paget *et al.*, 1988a, 1988b, 1989b). The oxidases of leucocytes have also been studied using these methods (Edwards and Lloyd, 1986, 1987, 1988). In methylotrophic bacteria (Joergensen and Degn, 1983), cytochrome aa_3 is the sole terminal oxidase in those assessed by the determination of photochemical action spectra (Joergensen and Lloyd, unpublished data), despite the presence of huge amounts of CO-binding *c*-type cytochrome.

The method employing a system open for gas flow also reveals sensitivity to respiratory inhibition by O_2 (substrate inhibition) in microaerophilic organisms (e.g., in the parasitic protozoa *Giardia intestinalis* (Paget *et al.*, 1989) and *Trichomonas vaginalis* (Yarlett *et al.*, 1986). In such cases, as well as giving a value for the apparent K_m of the oxygen-consuming system, a threshold for respiratory inhibition can be determined (Table IV).

O_2 scavenging as a means of diminishing intracellular O_2 (and hence O_2 toxicity) often yields photoemissive species assigned to singlet $O_2{}^1$. Chemiluminescence measurements provide a measure of these.

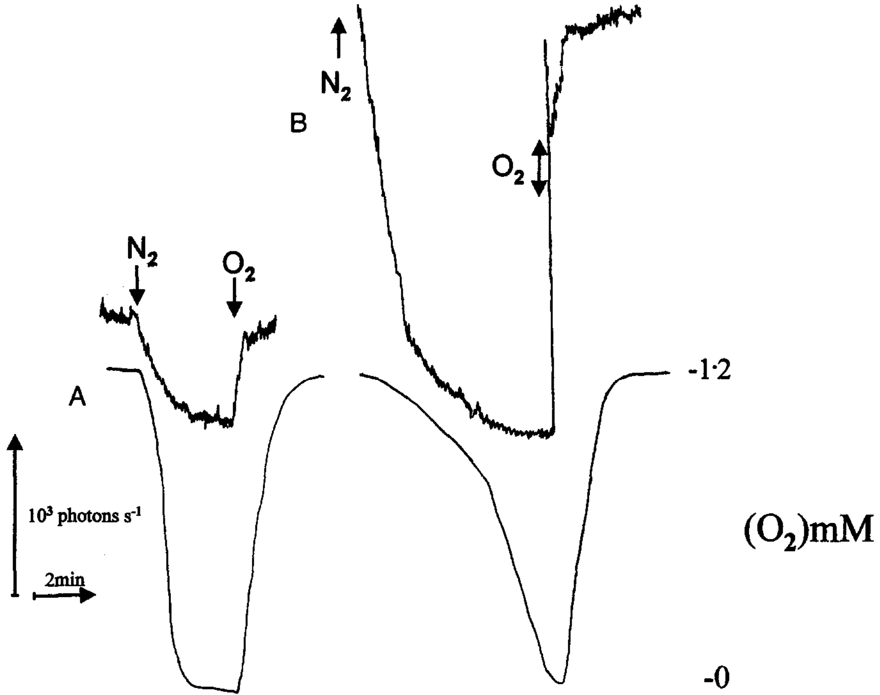


FIG. 5. Weak chemiluminescence emission from a washed intact cell suspension of *Acanthamoeba castellanii* during cycles of oxygenation and deoxygenation. The mobile gas phase was switched from O_2 to N_2 as indicated. In (b) the experiment was repeated in the presence of the mitochondrial electron transport inhibitor dibromothymoquinone (2, 5-dibromo-6-isopropyl-3-methyl-*p*-benzoquinone) (40 μM), an analogue of ubiquinone. Cells were in 50 mM $MgCl_2$ at a concentration of 4×10^7 organisms per ml; temperature 30°C. (Unpublished data of D. Lloyd, A. Boveris, and B. Chance.)

Figure 5 shows the weak photoemission from a washed suspension of the soil amoeba *Acanthamoeba castellanii,* as a function of O_2 concentration (Lloyd *et al.,* 1979b). The red-sensitive photomultiplier was kept at 77 K and as close as possible to the stirred oxygenated organisms. At hypoxic (hyperbaric O_2), a strong signal was recorded and decreasing O_2 gradually diminished this to a background level under N_2. Similar indications of the presence of singlet $O_2{}^1$ have been in microaerophilic protozoa, e.g., *T. vaginalis* (Lindmark and Lloyd, unpublished results), *G. intestinalis* (Paget, unpublished results), and *Hexamita inflata* (Biagini *et al.,* 2001), as well as in *Saccharomyces cerevisiae* (Quickenden and Tilbury, 1991), and in plant and animal tissues (Cutrin *et al.,* 2000). Use of a specially designed photomultiplier (Hamamatsu Photonics K. K.) now makes this assay for an important

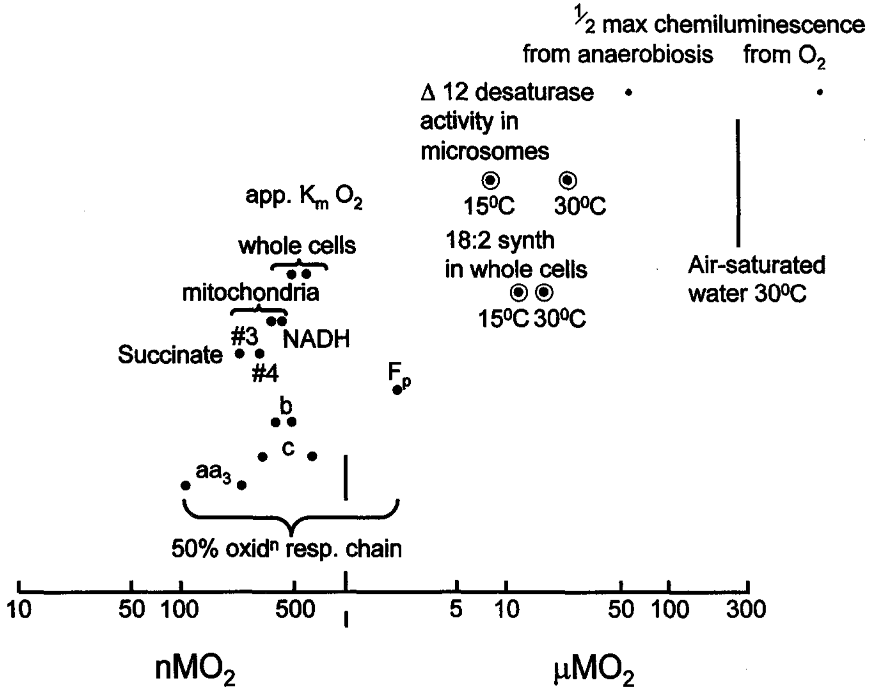


FIG. 6. Oxygen dependencies of various cellular functions in the soil amoeba *A. castellanii* (trophozoites). Note that mitochondria operate at submicromolar oxygen, whereas microsomal fatty acid oxidation and that of whole cells, especially at the higher temperature is half maximal at extracellular $O_2 > 20\ \mu M$. Reactive species of O_2, indicated by $O_2{}^1$ formation (weak chemiluminescence) is half-maximal at 50 μM O_2 or higher (Lloyd *et al.*, 1983a).

component of oxidative toxicity highly sensitive and specific. Detection of other reactive O_2 species has been reviewed by Chance *et al.* (1979).

A summary of work on *A. castellanii* with regard to its O_2 relationships is shown in Figure 6. Note the extremely low intracellular O_2 concentrations as indicated by the redox titrations of spectrophotometrically monitored electron chain components. Higher levels of O_2 in the culture are necessary to produce microsomal desaturases rather than to affect mitochondrial redox states (Thomas *et al.*, 1997).

The oxygen dependence of microbial growth has been extensively studied for almost 150 years and has generated a vast literature. Unfortunately most of these investigations have been qualitative rather than quantitative. Oxygen tensions resulting in half-maximum growth rates

of *Pseudomonas putida* were 7–8 μM only for nonaromatic compounds like succinate or glucose (Arras *et al.*, 1998). These observations suggest that the oxygenases required for the initial steps in aromatic degradation have a lower affinity for O_2 than the bacterial oxidases in this system.

It has been widely asserted that bacterial denitrification is an anaerobic process as O_2, the most thermodynamically advantageous terminal oxidant, will always take precedence over NO_3^- (Lloyd, 1993). However, the common occurrence of aerobic denitrification carried out by many different bacteria indicates that this is not invariably so (Lloyd *et al.*, 1987b; Davies *et al.*, 1989; Lloyd *et al.*, 1990; Thomas *et al.*, 1994; Thomas and Lloyd, 1995). MIMS monitoring of N_2O and N_2 alongside O_2 and CO_2 provides a powerful technique for studies in this area.

B. O_2 Dependence of Alcoholic Fermentation (Pasteur Effect)

The open system provides a unique method for the direct measurement of the Pasteur effect. Thus, simultaneous measurement of CO_2 output and O_2 consumption by MIMS reveals, in a most striking way, the increasing aerobic inhibition of fermentation even at low O_2 tensions (1–5 μM). This method has so far only been applied to yeasts (Lloyd *et al.*, 1983a, 1983b, 1983c, 1983d; Lloyd and James 1987; Gaunt *et al.*, 1988; Carlsen *et al.*, 1991; Lloyd *et al.*, 1992a, 1992b), although it is known to be a widespread phenomenon in mammalian tissues and elsewhere.

C. O_2 and H_2 Production

MIMS provides a highly convenient method for measurement of hydrogen evolution. The group of Berlier *et al.* (1985) have used this technique at very low O_2 to study the mechanisms of hydrogenase in relation to the nitrogenase of *Azotobacter chroococcum, Azospirillum brasiliense,* and *Klebsiella pneumoniae.* This research group have also studied hydrogen recycling by *Rhodopseudomonas capsulata* and *Paracoccus denitrificans. Desulphovibrio gigas* hydrogenase requires very strictly anaerobic conditions (Berlier *et al.*, 1982).

Hydrogen evolution has been studied in crude rumen fluid (Scott *et al.*, 1983a) microaerophilic protozoa, and fungi from the rumen (Yarlett *et al.*, 1982, 1987; Ellis *et al.*, 1989; Hillman *et al.*, 1985a; Rees *et al.*, 1998) (see Fig. 7). In anaerobic digestion systems, H_2 accumulation (Scott *et al.*, 1983b; Whitmore *et al.*, 1987a) is an early diagnostic of system failure; control of feed rate of continuous processes by mass spectrometrically monitored H_2 is feasible, at least at laboratory scale (Whitmore *et al.*, 1987b).

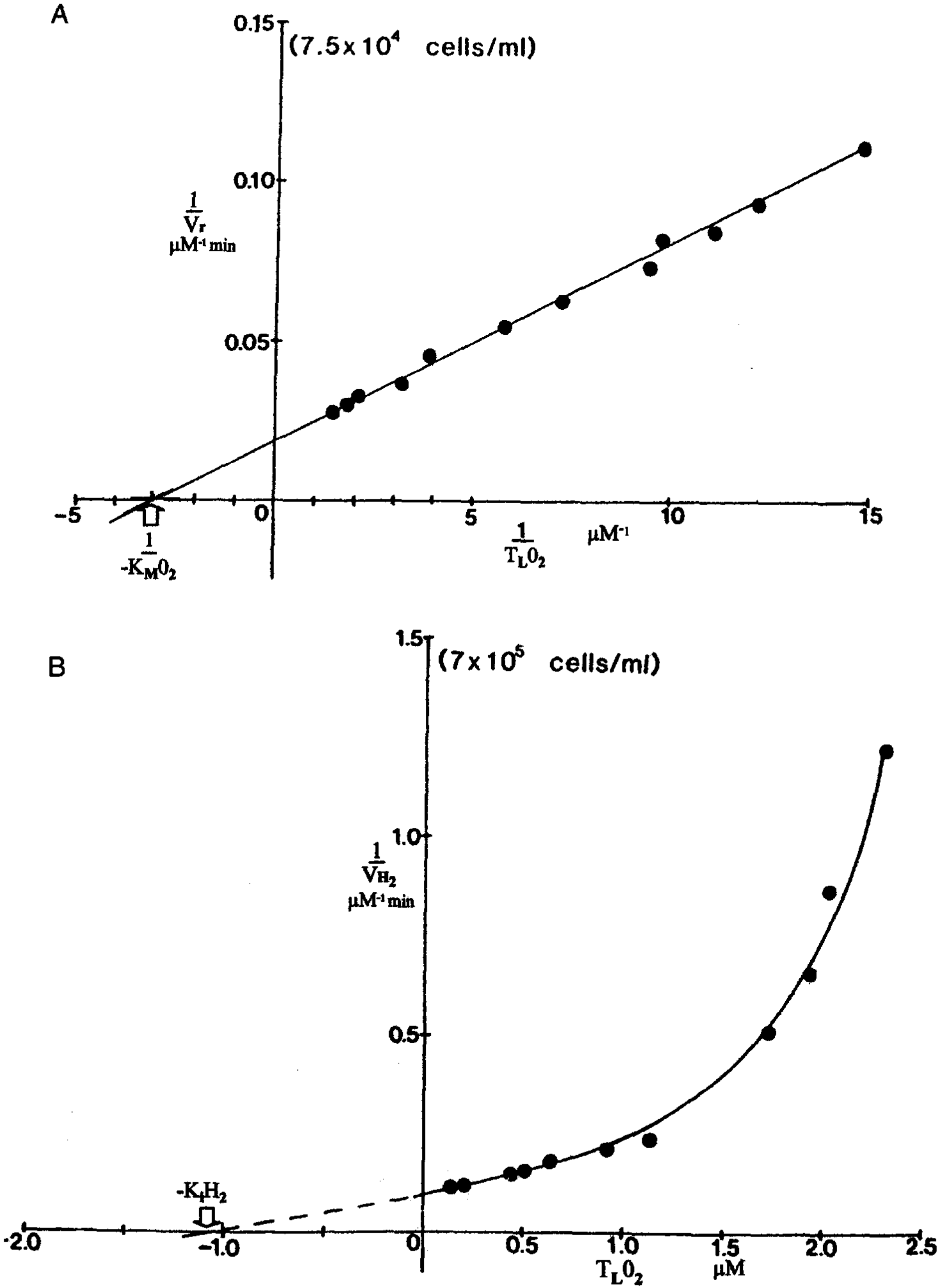


FIG. 7. Inhibition of H_2 production by O_2 in the rumen protozoan, *Dasytricha ruminantium* (Hillman *et al.*, 1985a).

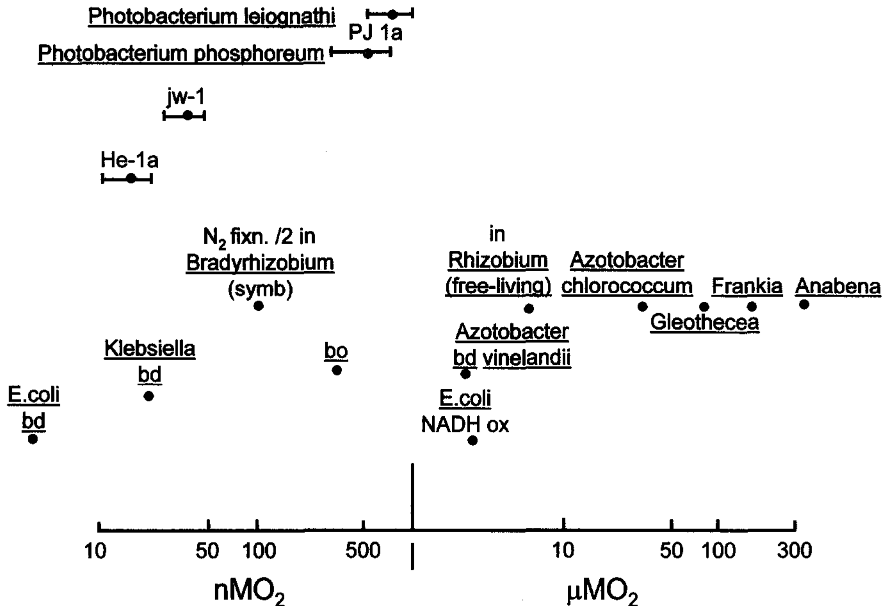


FIG. 8. O_2 sensitivities of some N_2-fixation systems, various light-emitting bacteria, and K_mO_2 values for some bacterial cytochromes.

D. THE INHIBITORY EFFECTS OF O_2 ON N_2 FIXATION

Figure 8 shows the O_2 sensitivities of various N_2-fixation systems in bacteria. For comparison, the extreme efficiency of oxygen binding to *E. coli* cytochrome *bd* is shown as well as the ranges of O_2 measurement using various species of luminous bacteria. Dinitrogen uptake can be measured directly using MIMS (Jensen and Cox, 1983).

V. Future Prospects

A. MICROBIAL PHYSIOLOGY AND BIOTECHNOLOGY

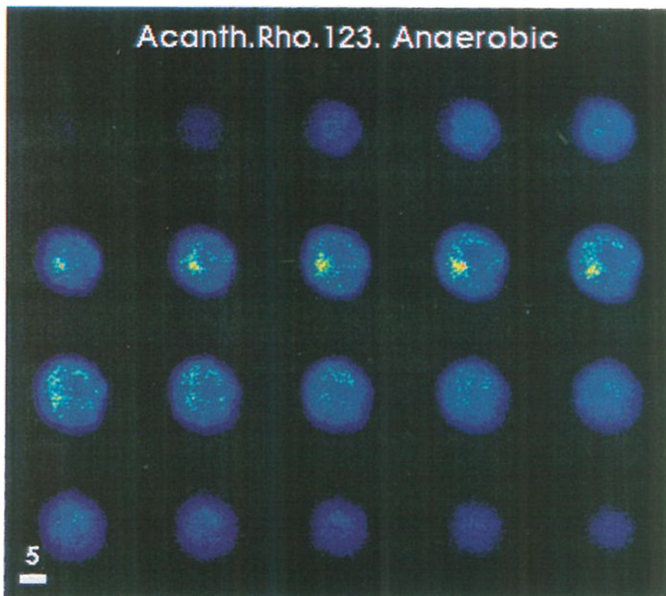
Studies already undertaken confirm the usefulness of combining noninvasive physical techniques with oxygen measurement. Many of these on-line methods are the same as those increasingly used to maintain laboratory cultures of microorganisms under controlled conditions. Thus, direct interfacing to "biological reactors" can give continuous information on metabolic states. This technology can thereby provide opportunities for immediate corrective action and for optimization of metabolite production. Although rather few variables have been exploited until

now, examples where monitoring and modulation of O_2 play a key part in process control of biotechnology and bioremediation are legion (White *et al.*, 1998; Gadd, 2000).

Direct fluorometric observation of NADH as an indicator of intracellular redox state is another prime example that has proved useful (Harrison and Chance, 1970; Murray *et al.*, 1998). On-line permittivity monitoring for biomass (Yardley *et al.*, 2000), especially useful for filamentous organisms, and already widely employed for yeast fermentations in the brewing industry, will undoubtedly become more widely adopted. Use of tryptophan fluorescence monitoring, as a measure of total protein in growing biofilms (Wimpenny *et al.*, 1999), alongside direct-on-line IR measurement of polymer accumulation (Palmer and White, 1997; Hansen *et al.*, 2000) is another exciting development. The increasing sensitivity of NMR, ESR, and mass spectrometric techniques will no doubt provide new noninvasive methods for the continuous interrogation of live organisms and developments in the medical applications of imaging technology, and already provide ground-breaking new ways of looking at and measuring the activities of microorganisms. The revolution in optical microscopy provided by confocal scanning laser technology, diversification of fluorophore applicability, and most recently the promise of two-photon excitation, are all of revelatory dimensions. Improvements in the spatial resolution of NMR and ESR imaging will, perhaps, afford further opportunities.

B. Microbial Ecosystems Research

The ecological and environmental applications of the techniques outlined here are slow to be adopted. The suggestion is that the only place to study microbial ecology is in the natural environment (Holmes and Lloyd, 1996), but still is mostly pursued in the laboratory (Edwards, 1999). The extraordinary power of noninvasive methods has become evident in some recent experiments with cores of natural sediments (Lloyd *et al.*, 1987a; Thomas and Lloyd, 1995; Cartaxana and Lloyd, 1999; Cowie and Lloyd, 1999), peat (Benstead and Lloyd, 1994; Thomas *et al.*, 1995, 1996, 1998; Lloyd *et al.*, 1998a, 1998b; Upton *et al.*, 2000; Beckmann and Lloyd, 2001), and soil (Sheppard and Lloyd, 2001); these are but preliminary first steps toward real *in situ* environmental monitoring. The three approaches involve electrode-based methods (Revsbech and Jørgensen, 1986), biosensors (Lorenzen *et al.*, 1998; Liesack *et al.*, 2000) or MIMS (Hillman *et al.*, 1985b; Lloyd *et al.*, 1987a; Boddy and Lloyd, 1989); for robustness, MIMS is superior. Improvements in spatial resolution by miniaturization of the MIMS probe (Lloyd *et al.*, 1996) widens the interest of monitoring gases in structured ecosystems,



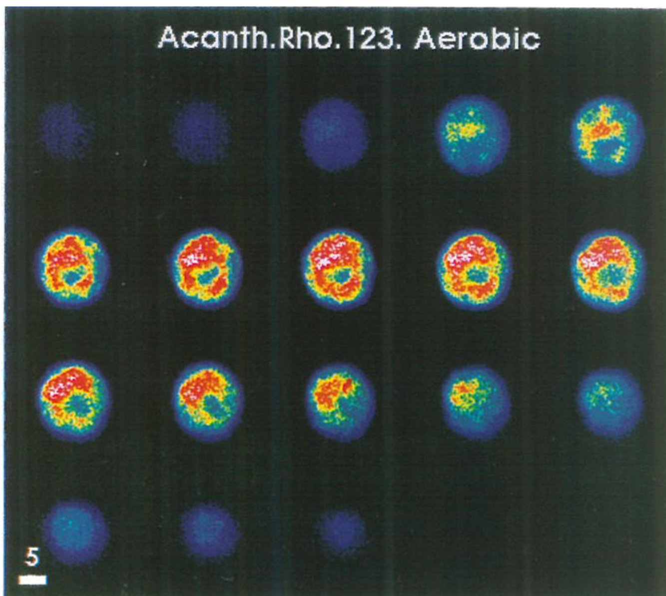


FIG. 9 O_2 dependence of mitochondrial membrane potential in *A. castellanii*. Confocal scanning laser microscopy; a series of optical sections of a single cell after 5 min incubation with 1 μg/ml rhodamine 123 in a hanging-drop preparation. (a) Under N_2 gas and (b) 30 s after switching gas phase to air. (Unpublished images markers 5μm, obtained in collaboration with Dr. A. J. Hayes.)

although rivaling the resolution, already attainable using microelectrodes, will be a challenge.

Exciting new O_2-monitoring measurements for the determination of dissolved gas gradients utilize fluorescence quenching or lifetime methods with specially developed fluorophores interfaced to fiber optic detection (Pringault *et al.*, 1998; Glud *et al.*, 1999). Two-dimensional methods for O_2 will follow. Confocal laser scanning microscopy gives three-dimensional (3D) spatial information on intracellular processes in a single cell (Fig. 9, see color insert) as well as on complex structured ecosystems at a bacterial-sized resolution (Lloyd *et al.*, 1999). In photocounting mode imaging systems can now detect photoemission from single bacterial cells (Phiefer *et al.*, 1999). The use of green fluorescent protein fluorescence as a molecular reporter for gene transcription is a method becoming widely used for all sorts of applications; it is still possible at low O_2 (0.1 ppm $\equiv$ 0.1 n*M*) but does not prove possible at extremely low O_2 (0.025 ppm) levels attainable in the presence of cysteine as reducing agents (Hansen *et al.*, 2001) on account of the O_2 requirement of the light-emitting reaction.

Biogeochemical processes occurring in subsurface or deep sediments are often microaerobic. Recent examples where low O_2 dependence of key processes have been investigated include the metal-metabolizing bacteria *Geobacter* and *Magnetospirillum* species (Bazylinski *et al.*, 2000).

Gradients of O_2 in natural ecosystems are clearly pivotal in determining the distribution of microorganisms (see Amaral and Knowles, 1995) and their cytodifferentiation (Bonner *et al.*, 1998).

C. Clinical Microbiology

Techniques described here have not yet been applied to many organisms of interest and importance in clinical and food microbiology. Many of the most important of these are microaerophiles, e.g., *Salmonella typhimurium* (Sevcik *et al.*, 2001), *Pseudomonas aeruginosa* (Xiong *et al.*, 2000), *Campylobacter jejuni* (van Vliet *et al.*, 2001), *Helicobacter pylori* (Ge *et al.*, 2000), *Treponema pallidum*, and *Plasmodium falciparum*. Here, O_2 relationships of even the most common pathogens in the context of their natural environment are not completely resolved. For instance, the virulence of *Staphylococcus aureus* depends on O_2 (Yarwood *et al.*, 2001). It seems likely that the modular design and variable expression of electron transport chains (and especially of terminal oxidases) is a reflection of the adaptable life styles of these bacteria, (Poole and Cook, 2000; Richardson, 2000). The intracellular pathogens almost certainly experience low O_2 environments within their hosts, as

the range of intracellular O_2 concentration is necessarily constrained to minimize the possibility of free radical damage by reactive O_2 species (Lloyd and Biagini, 1998). It seems likely that this is also so for those living interstitially between the cells of many different mammalian tissues and organs.

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The Development of the Penicillin Production Process in Delft, The Netherlands, During World War II Under Nazi Occupation

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I. Introduction

The aim of this paper is to highlight the development of the penicillin production process at the Nederlandsche Gist- en Spiritusfabriek (NG&SF) in Delft, The Netherlands (Netherlands Yeast and Spirit Factory, subsequently Gist-brocades, presently part of DSM) in the period 1943 and onward. Whereas the Anglo-American war project (Herion, 2000; Strohl *et al.*, 2001) involved many research institutes and pharmaceutical companies, the Dutch project was carried out by only a few research personnel under the severely restricted conditions of Nazi-occupied Holland in the last years of World War II. While much has been written on the wartime experiences of The Netherlands as it endured almost exactly five years of occupation, little has been recorded of the successful research and production of penicillin at NG&SF during the war years. The Delft team remained unlauded. Their place in the history of penicillin is not generally known. However, this paper[2] sets out not "just to tell the story" but to bring the wartime experience of those at NG&SF into perspective and highlight the achievements of those who laid the foundation for DSM's present leading position in the world of bulk penicillin.

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[2]Most of the research on this subject is contained in Burns (2000).

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II. The Pre-Penicillin Years

In 1869, Jacques C. van Marken, a young chemical engineer, the first graduate from the newly established Delft Polytechnic School (now Delft Technical University), founded a factory in Delft called the Nederlandsche Gist- en Spritusfabriek to produce yeast for baking bread using a new production process that had been developed in Vienna by a local brewer named Mautner. Mautner's method was based on the use of a special type of top-fermenting yeast, which could simply be skimmed from the top of the fermentor (Elema, 1970). Up until then it was common practice in The Netherlands to use brewer's yeast as a by-product of the grain-distilling industry, although the baking quality of such products was highly variable and bakers frequently complained. In 1882, van Marken appointed his nephew, a young Delft technologist, F. G. Waller, as head of factory production. In 1885, van Marken and Waller appointed Dr. Marinus W. Beijerinck, then teaching at the Advanced School of Agriculture in Wageningen, head of the bacteriological laboratory. Beyerinck thus became the first industrial researcher in the NG&SF factory. Beijerinck obviously experienced great freedom in his research because in the ten years he stayed at NG&SF more than 30 scientific papers on different subjects were published. In 1895, Beyerinck was appointed full Professor in Microbiology at Delft Technical University (TU) and laid the foundation of what later became known as the Delft School of Microbiology (Bennett and Phaff, 1993; Bennett, 1996). In 1896, H. P. Barendrecht, who had graduated from the University of Amsterdam with the famous scientists van 't Hoff (the first Nobel Laureate in Chemistry in 1901) and van der Waals (Nobel Laureate in Physics in 1910), was appointed at NG&SF as Beyerinck's successor, and, being more a physical-chemist, also became head of the chemical laboratory. Here he developed, for example, a simple hydrogen electrode to measure pH, which remained in use in the factory until after World War II, when reliable glass electrodes became available. Meanwhile, the yeast produced in the factory was of such high quality that it deserved its brand name "Koningsgist" (Royal Yeast). After the death of van Marken in 1906, Waller became President and started to expand the company. New production facilities were established outside The Netherlands, first in Bruges, Belgium, later in Mathoshinos, Portugal, and in Monheim and Werne, Germany. Yeast was supplied to the bakers, while the fermentation liquor was distilled to obtain alcohol. The remaining product was supplied to the animal feed industry as fodder.

During World War I, there was a shortage of raw materials and NG&SF adopted a fed-batch process developed in Denmark, with the advantage that almost no alcohol was formed but all carbon sources were

utilized to make yeast. In addition, they shifted from grain mash as the principal carbon source to beet and sugar cane molasses. Alcohol production, distilling, and the production of Jenever (a Dutch brand of gin) thus became a separate activity from normal baker's yeast production, and NG&SF had several production plants in the Netherlands and Belgium devoted to this activity. These processes had been worked out at pilot plant scale by a self-made microbiologist and biochemist, L. J. van der Lek, who had become head of the "Investigation Department," which also included the pilot plant. On the factory side, the implementation for industrial scale was carried out by F. G. Waller's son, F. G. Waller, Jr., who had joined the company in May 1923 after finishing his academic training with Beyerinck's successor at Delft University, Prof. Albert Jan Kluyver. Kluyver's more biochemically oriented research also yielded a new production process for NG&SF using an *Enterobacter cloacae* strain to produce 2,3-butylene glycol from sugar and conversion into acetyl-methylcarbinol and diacetyl (Kluyver and Scheffer, 1929a, 1929b). In the late 1920s, production processes were also developed for butanol and acetone, using microbial strains isolated by Beyerinck and extensively studied both in Kluyver's group and at NG&SF (Donker, 1926; Scheffer, 1928; Van der Lek, 1930). Both the glycol and the butanol fermentation processes at production scale proved to be very sensitive to phage infections, whereas the butanol fermentation proved to be highly sensitive to infection by other microorganisms. This required the development of new fermentation technologies—the need for pure culture fermentations on an industrial scale—with intense support from microbiologists who had studied the processes in Kluyver's laboratory.

In the period 1928–1933, in view of the imminent retirement of both Barendrecht and Van der Lek, NG&SF Research and Development was strengthened with three young microbiologists/biochemists recruited directly from Kluyver's Delft School, A. P. Struyk, A. A. Stheeman, and B. Elema. In addition, a physical chemist, L. M. Rientsma, was appointed. In 1938 NG&SF research and development (R&D) was enhanced by the appointment of Dr. Jacomina Lodder, an expert from the "Yeasts" department of the "Centraal Bureau voor Schimmelcultures" located in Kluyver's laboratory in Delft (CBS, the Dutch National Culture Collection in Baarn, now Utrecht). She later became very well known for her handbook on the taxonomy of yeasts (Lodder, 1970). Ultimately, NG&SF developed a new yeast product, active dry yeast, marketed under the brand name "Engedura." In 1926, Dr. W. H. van Leeuwen succeeded F. G. Waller, Sr., as President-Director, while F. G. Waller, Jr., was placed in charge of the Delft factories. Technologically, therefore, the NG&SF focused on yeast fermentation with the exception of a limited knowledge

of bacterial fermentations. This was more or less the industrial position for NG&SF when The Netherlands, with a long history of neutrality, entered World War II.

III. The World War II Period

Following the trauma of occupation in 1940, the NG&SF, being a vital economic element to the Dutch baking industry, was allowed to continue production under the surveillance of a German army overseer. Also, as the fermentation process requires highly specialized skills, most NG&SF personnel were protected through their "essential" worker status. While this may also have protected the NG&SF work force from being taken as forced labor to work in Germany, it is also known that some of those hiding from the Nazi regime found safety undercover within the NG&SF production site. At the same time, the fermentation process meant working around the clock, even during the curfew, and this too offered possibilities for clandestine activities to be carried out unknown to the German occupier.

On the other hand, yeast production was strongly reduced due to the loss of export possibilities. In addition, the production of distilled alcohol at NG&SF was curtailed as it became deemed a luxury good. Such alcohol and Jenever production as there was largely went to the Wehrmacht. By the end of the war, alcohol was being requisitioned for use as rocket fuel by the German military forces. Consequently, the NG&SF had to embark on other activities to fill its fermentors, and during the war years there followed a period of rapid product diversification for the company.

At the request of the Dutch administration, and coordinated by the Dutch Organisation for Nutrition and Food Research (TNO), NG&SF started production of vitamin C, in collaboration with Dutch Shell and Chemische Fabriek Naarden (now Quest ICI). In this process, NG&SF converted the sorbitol, supplied by Shell, to sorbose by the action of *Acetobacter suboxydans*. Naarden then oxidized the sorbose to ketogulonic acid and NG&SF hydrolyzed the (diacetone) ketogulonic acid to ascorbic acid. The combined efforts of the three companies led to many tons of vitamin C being supplied to the acting Dutch governmental authority.

Other NG&SF products developed in this wartime period were yeast extracts (Gistex) and acid-hydrolyzed vegetable proteins (HVPs) as an alternative to meat in soups and sauces. Vitamin shortages provided the incentive to explore yeast-derived vitamins such as vitamin B1, while ergosterol extracted from yeast served as raw material for the production of vitamin D_2. Some of the fermentors were used to ferment cabbage to

sauerkraut for its high levels of vitamin B_{12}. Butanol production was increased and chemical derivatives such as butylacetate, dibutylphthalate, and other butanol esters for use as solvents and plasticizers in the paint and lacquer industry were also developed.

IV. The Development of the Penicillin Production Process

How, therefore, did the NG&SF researchers turn their attention to a fungal product like penicillin during the war? To begin with, news of the success of the British and American penicillin reached NG&SF clandestinely. The first reports came from "*De Vliegende Hollander*" (The Flying Dutchman), leaflets dropped by British bombers, and through the underground Radio Orange based in London. According to F. G. Waller, Jr. (Elema, 1970), it was while listening to an illegal British radio transmission in the summer of 1943 that he first heard of the new "wonder drug," penicillin, which was so quickly restoring the health of war-wounded soldiers. It is, however, also possible that his principal scientific advisor Kluyver played a role in the use Waller and his R&D staff made of this information. From sources in the CBS archive we know that Kluyver had been the go-between for two requests to CBS for Fleming's *Penicillium notatum* penicillin strain in 1943 and that Kluyver had weekly meetings with NG&SF R&D (Kamp *et al.*, 1959). Nonetheless, under the instruction of F. G. Waller, Jr. (Fig. 1a, see color insert), a small group of researchers lead by Dr. A. P. Struyk (Fig. 1b), head of NG&SF's microbiology laboratory, started work. Other members were Dr. A. A. Stheeman (Fig. 1c), Dr. J. Rombouts, and their technicians Messr. Knotnerus, Langendijk, and Mathu, and Miss A. Addeson.

Although as a result of wartime conditions current foreign technical literature was unobtainable in The Netherlands, prewar publications had remained within easy access. From these, Struyk obtained a copy of Fleming's original publication (1929) and also a copy of the inconclusive results of a biochemical investigation conducted by Clutterbuck *et al.* (1932). Following their clandestine information, however, what had become clear to the NG&SF researchers was the fact that from these basic works others had gone on, not only to prove the feasibility of isolating Fleming's "Penicillin" but also to manufacture it. However, in addition to having little information, the team had not much space to house such new research. At the time there was only one small laboratory building at the NG&SF, originating from the days of Beijerinck (Fig. 2).

Ultimately, the article that was to prove invaluable to the Delft team was a publication by Kiese (1943). How the researchers at NG&SF had managed to gain access to this current German academic journal remains unclear. Although Kiese did not give any further detailed information

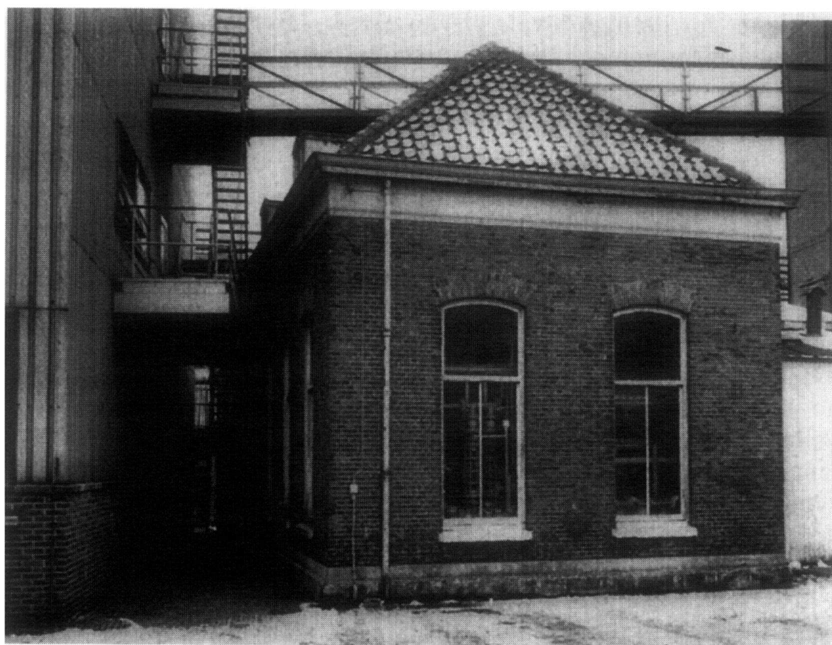


FIG. 2. The NG&SF research building, where the penicillin research by Struyk, Stheeman, and other members of the Bacinol team was carried out up to 1945. The photo was taken around 1970 and published by Elema (1970).

on how penicillin could be produced, his article was quite clear about the methodology that had to be followed.

Another influence on the research and development of penicillin at the NG&SF came by sheer chance in a meeting by one of their scientific advisors, Dr. A. Querido[3] (Fig. 1e, see color insert). As a young postgraduate in medicine, Querido had interrupted his studies at the Pasteur Institute in Paris (Fig. 1f) to return home to Amsterdam in 1939 when the Polish crisis brought with it the threat of war. At that time, F. G. Waller, Jr., on the advice of the NG&SF advisor and associate of the Pasteur Foundation, Prof. A. J. Kluyver (Fig. 1d), had offered Dr. Querido a permanent position. However, Querido's main interest was not in industrial production. His preference was to stay within his academic field. Rather than lose Querido's experience, Waller had offered him a part-time position as scientific advisor, which Querido had accepted. However, shortly thereafter his Jewish ancestry had brought with it internment for Querido, his wife, and his young son in the transit camp

[3]Marlene Burns interviewed Prof. Querido in December 1999; A. Querido died at the age of 88 on January 30, 2001.

Westerbork near the Dutch–German border. But as an employee of the NG&SF Querido had gained "essential worker" status for Querido from the German authorities. As a result, he was allowed to attend meetings at the NG&SF one day each month. This was a double-edged sword for Querido; he had a day of freedom during which he no restrictions, but his family were held hostage until his return.

It was on one of these day visits when chance played its part. While changing trains in Amsterdam Central Station to go to Delft, Querido met a former colleague, by then the Professor of Paediatrics at Amsterdam. Also of Jewish origin and under German supervision, Querido's colleague was like himself still free to move around. He was also allowed to receive foreign visitors. Bursting with excitement, he told Querido he had just had a visit from a colleague from neutral Portugal who had brought with him the whole issue of a 1944 Swiss medical journal. The journal had only recently been printed, and whereas previously information on penicillin had only been available in broad terms, this journal gave precise information. The article by A. Wettstein (1944), a researcher of CIBA in Basel, simply entitled "Penicillin," clearly showed the results the Allies had achieved. It was agreed that Querido could borrow the publication for copying. The journal would then be returned via an undercover route. This copying was done at NG&SF probably by hand. Although German photocopying facilities were available, they could not be used in this case for obvious reasons. With this last building block in place, the Delft team had something against which to compare their research.

In his first report on the research undertaken between March and June 1944, Struyk (1944a) clearly sets out his method of investigation, the scientific literature consulted, his work scheme, and his subsequent conclusion. Using his experience in yeast fermentation, Struyk describes his task as an investigation into the possibility of growing a mold culture on the NG&SF fermentation mash, *Liquitex,* a mixture of bran and malt, which would produce an antibacterial substance. As his scientific sources Struyk lists Fleming (1929), Clutterbuck, Lovell, and Raistrick (1932), and Kiese (1943). However, he also refers to Waksman (1940), Vonkennel *et al.* (1943), and Penau *et al.* (1943). These citations clearly show that the team had access to recent scientific literature, and that they were aware of the fact that many fungal cultures could produce antibacterial activities and not only penicillin.

The fungal cultures used were ordered from the CBS in Baarn, The Netherlands. In a copy of the 1943 CBS catalogue present in the NG&SF archives, the strains requested are marked by pencil (Fig. 3, see color insert). In total, 18 *Penicillium* and 3 *Aspergillus* strains were received, and their receipt was acknowledged in a letter, dated January 25, 1944,

TABLE I

OVERVIEW OF THE STRAINS SCREENED[a]

Code	Strain	Origin
P1	*Penicillium corylophilum* Thom[b]	Thom (CBS)[c]
P2	*Penicillium notatum* Westling	Thom
P3	*Penicillium cyano-fulvum* Biourge	Biourge
P4	*Penicillium chrysogenum* Thom	L.M.[d]
P5	*Penicillium meleagrinum* Biourge	CBS
P6	*Penicillium baculatum* Westling	Thom
P7	*Penicillium corylophilum* Thom[b]	Thom (France)[c]
P8	*Penicillium chloro-phaeum* Biourge	Biourge
P9	*Penicillium brunneo-rubrum* Dierckx	Biourge
P10	*Penicillium citreo-roseum* Dierckx	Biourge
P11	*Penicillium griseo-roseum* Dierckx	Biourge
P12	*Penicillium expansum* (Link) Thom	CBS, van Luijk, Nieth.
A13	*Aspergillus clavatus* Desm. (Abott)[c]	CBS, Wolf
A14	*Aspergillus giganteus* Wehmer	Thom
P15	*Aspergillus flavus* Link	Nattrass, Thom, Walker
P16	*Penicillium commune* Thom	Thom (CBS)[c]
P17	*Penicillium corymbiferum* Westling	CBS
P18	*Penicillium citrinum* Thom	Thom
P19	*Penicillium cyclopium* Westling	Thom
P20	*Penicillium baculatum* Westling	April CBS[c]
P21	*Penicillium notatum* Westling	CBS[c]
	Cacao fungus 1	NG&SF isolate
	Cacao fungus 2	NG&SF isolate

[a] Taken from Struyk (1994a).
[b] Struyck mentions in his report that this probably should be *P. coryphilum* Dierckx.
[c] This additional information is from Struyck; it is not mentioned in the CBS catalogue.
[d] Laboratory of Microbiology Delft (Kluyver's lab).

from Rombouts to Prof. Johanna Westerdijk, the Director of CBS. The Delft team also included 2 *Penicillium* strains isolated in-house from molded cacao powder, simply called cacao fungus 1 and 2. (See Table I) The correspondence retrieved in the archive of the CBS shows that Prof. Westerdijk offered the strains free of charge. Although a generous offer, it was declined by the NG&SF management as they felt an obligation to support CBS financially in those hard times (letter from Rombouts to Westerdijk, dated March 15, 1944).

Struyk and his technician L. P. Lagendijk used the microorganism *Micrococcus aureus* (Rosenbach) Migula, an old name for *Staphylococcus aureus*, obtained from Professor Kluyver's collection as test organism to screen for the formation of antibiotics. A peptone agar plate was coated with a thick suspension of *Micrococcus aureus* to which spores were added, and the plate was incubated at 26°C for several days.

This method was unsatisfactory, time-consuming, and nondiscriminating, since both the spores and the bacteria continued to grow. Struyk then changed his methodology with the use of an "agar block" test that was quicker and more discriminating. Here, the spores were first germinated on the agar plate and after different time periods a small section of the agar layer with the mould on top was inverted onto another plate into which the bacterial culture had been inoculated. After incubation, Struyk looked to see if "clear" areas had formed around the mold, indicative of a substance diffusing out of the "agar block" with an inhibiting effect on the growth of the bacteria. Fleming had called such an inhibiting substance "Penicillin."

From the results, Struyk concluded that the *Penicillium* strains P6, P7, P9, P11, P13, the *Aspergillus* strain A14, and, his own, cacao 1, were active in the production of an antibacterial substance. However, Struyk also concluded that the substance produced by P7 resembled one of Penau's antibacterial agents rather than Fleming's "Penicillin." Identifying P7 as "*Penicillium corylophilum* (source France)," Struyk noted that Penau's substance, "Notatine," contrasted with penicillin in its instability when heated, and in its more enzyme-like properties. Ultimately, the mold culture with the highest yielding antibacterial substance overall and the one chosen for further study had been sixth on the Baarn list, P6: *Penicillium baculatum* Westling.

According to Struyk's following reports (1944b, 1944c), when flasks with NG&SF's *Liquitex* bran and malt medium were inoculated with a suspension of *Penicillium baculatum* conidia grown on malt agar for approximately five days at 26°C (shaken once a day), the results from *P. baculatum* appeared to be identical to those reported by Fleming for *Penicillium notatum*. In addition, the substance produced by P6 was soluble in acetone and alcohol, which facilitated extraction from the broth, and when mixed with water, its properties were also resistant to boiling. Unsure, however, whether P6 produced the same active compound as the wonder drug penicillin, Struyk named the antibiotic substance "Bacinol" after its producing organism *P. baculatum*. At the same time this meant that the German occupiers was kept unaware of their research into the wonder drug. Ultimately, Struyk's results were reported only to F. G. Waller, Jr., and discussed with his two colleagues in the microbiology laboratory, A. A. Stheeman and J. Rombouts.

However, further research was needed before enough Bacinol was produced to allow recovery and isolation of the product. Dr. Rombouts showed that the strain also could be grown on the surface of a liquid medium in Roux flasks. Stheeman and his colleagues Knotnerus and Mathu investigated a method of extraction of the harvested culture fluid. Following Kiese's paper, they applied the methodology of Clutterbuck

et al. (1932). Using this method, however, at a pH of the broth of 4.83, almost all Bacinol remained in the broth. Therefore, they returned to Kiese's article (1943) and found Abraham's Oxford team methodology of extraction with ether after acidification to pH 2.02. The ether extract was washed with a phosphate buffer of pH 6.48 and the resulting yellowish-colored Bacinol was predominantly found in the water layer. In June 1944, the team at NG&SF produced its first small amount of a gold-brown substance of which 50% was Bacinol, the rest impurities.

The next step for the Delft team was to test for toxicity. Dr. Rombouts and his assistant, Ans Addeson, as Wettstein had reported,[4] chose to test in *S. aureus* infected rabbits and mice. Rombouts had experience in this field. In a letter to Prof. Westerdijk, discussing the NG&SF request for the *Penicillium* and *Aspergillus* strains, dated March 15, 1944, Rombouts mentions that he was glad to return to research on microbes after a period of four years of animal testing for safety studies. Fortunately, Rombouts did not choose guinea pigs, since, although unknown in Delft, Oxford experiments had found guinea pig testing ill-fated, failure being caused by guinea pig allergenicity to penicillin. The NG&SF trials, however, proved Bacinol's effectiveness and its safety. The animals recovered. Once more Struyk could report that the substance Bacinol was active against the same bacteria as those named by Fleming in 1929. However, for Stheeman the harvested substance was more a vindication of the Delft team's research, as he was heard to remark, "See, I always knew that those little creatures would murder each other."

That being said, for the Delft team, the success of their efforts was not yet certain and further research required the production of larger amounts of Bacinol. In order to enhance growth, Struyk tried various types of flat glass and enamel containers. In the end he chose milk bottles (Fig. 4). Bearing in mind the wartime shortages and the lack of laboratory equipment, milk bottles were still accessible, and they were relatively easy to clean and sterilize. They are also known, as Struyk would have known, as the "natural" fermentor.

Having found the optimum conditions for the growth of Bacinol, Struyk decided that he could supervise his experiment better if his milk bottle containers were placed in one room. Accordingly, the Head of the Fermentation Plant, Klokgieters, received the order to empty a room in the fermentation pilot plant for "hundreds" of milk bottles. Lying on their side, the milk bottles were partly filled with different liquid nutrients, and assistants from Rombouts' laboratory regularly came to inoculate spores taken from *P. baculatum* into the milk bottles.

[4]Wettstein (1944) reviews in his paper the experiments on infected mice of Chain *et al.* (*Lancet*, 1940), and those of Robson and Scott (*Nature*, 1942; *Lancet*, 1943), on rabbits.

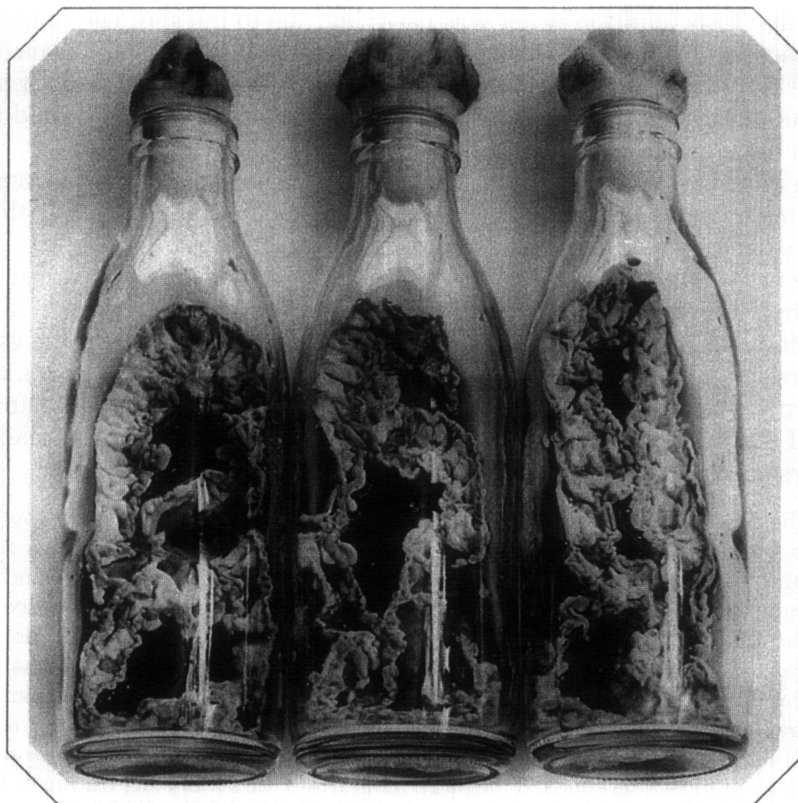


FIG. 4. *Penicillium baculatum* growing in surface cultures in milk bottles. Photo reproduced from the Gist-brocades brochure "35 jaar Penicilline" (Gist-brocades, 1978).

The bottles were then closed with sterilized cotton wool and the production of Bacinol awaited (Fig. 4). In order to follow the day-by-day formation of Bacinol, a quantitative biological test was developed in which the concentration of the solution was expressed in "Delftsche Eenheden" (D.E.) ("Delft Units"). The "Delft Unit" was defined (Struyk, 1944d) as the amount of bacteriostatic substance, which just completely suppresses the growth of the test organism *Micrococcus aureus* strain 6 (obtained from Dr. L. E. Den Dooren de Jong from Kluyver's lab) in 1 ml of peptone water at 37°C. The assay was as follows: The test solution was diluted 1:20 with peptone water. Of this diluted solution, 0.1, 0.2, 0.3, 0.4, 0.5, and 1.0 ml samples were made up to 7.4 ml with peptone water. To this, 0.1 ml of a 1:10 diluted 24-h-grown test organism was added, and the total mixture of 7.5 ml was kept at 37°C for 48 h before it was evaluated. A tube in which the growth of the bacteria was just completely suppressed will therefore contain 7.5 D.E. It is stated that in

the first tests bouillon water was used, but due to the shortage of meat extract the team switched to peptone, from Rhone Poulenc (France), later from Organon (Netherlands). Four strains from Kluyver's lab were tested for their suitability as indicator organisms. Strain 6 appeared to give the most reproducible results.

While hindsight reflects the similarity of this method of measurement to the Oxford Unit method, it has to be recalled at this point that the existence of Oxford Units were as yet unknown to the Delft team (Elema, 1970). Up to that time, information gleaned from the research of the Delft team remained entirely their own.

Moreover, that this penicillin research was kept secret during the war years acts as a marker to the determination of the Delft team. As Mr. de Horn, an employee of NG&SF since 1933 and a member of the laboratory staff that implemented the production process in the factory, explains (personal communication).[5]

> The NG&SF was a family concern. They knew their business and how to keep trade secrets. During the war we were working with Shell on a joint vitamin project. Apart from that, we kept up our research in the food industry and in yeast and solvents, but because of wartime shortages we had to develop pieces of equipment from what we could get. It was not unusual to see things like milk bottles on their side growing "stuff." That was fermentation and we knew more about the fermentation industry than the Germans. We did have a German supervisor whose job it was to keep us under surveillance, but he liked Jenever gin, so we made sure he got a lot. He slept most afternoons. If we had to come onto the factory site during the curfew, we all lived close enough to walk or cycle, and we used the "back" gate on the west side away from the main, east entrance. Fermentation plants have to be kept going 24 h of the day. Waller, Jr., known to us as "Mr. F. G.," often joined the night shift. He was the driving strength behind us. Every day we had meetings. We always had a list of suggestions, a list of things to do. What we decided one day, we tried out the next. Producing Bacinol was not a duty, it was a pleasure and also exciting. We wanted to keep it secret. We wanted to succeed.

From July 1944 until March 1945 research with Bacinol continued on a regular basis. As R&D reports show, Stheeman and Knotnerus (1945) and Stheeman (1945a) reported on the buffer for the ether extraction of Bacinol from the broth culture; on the trials to improve growth cultures for *P. baculatum* in the well-known laboratory "Jena" and "Roux" bottles; on the further purification of their antibacterial substance using the bioassay with *S. aureus* to follow the purification; and finally, on the search for an improved production medium for Bacinol, using sugars, beet pulps, and grain mixes.

[5]Marlene Burns interviewed Mr. De Horn in October 1999.

Change came at the end of the notorious "Hunger Winter" of 1944/1945. In April 1945, the west of The Netherlands, as yet isolated from the rest of the country, was close to liberation. Agreement between the German occupiers and the Allies was reached that Allied bombers be allowed to drop food parcels over four airfields: Ypenburg, Duindigt, Valkenburg, and Waalhaven. The German administration agreed to stand aside. Distribution would be undertaken by the Dutch themselves. In that food drop American penicillin made up part of the included medicines. Dr. E. Verschuyl (Fig. 1g), a local Delft GP, managed to obtain some of the American compound. However, Dr. Verschuyl was also a surgeon at the Delft Bethel Hospital and was the NG&SF company doctor. As such, he knew of NG&SF penicillin research, and consequently, he brought a sample of the American substance to the NG&SF.[6]

Analyses of the U.S. sample enabled Stheeman to equate the ratio of Units used for the Delft measurements with those of Oxford as 10:1 respectively. However, his analysis also enabled him to report (Stheeman, 1945b) to Deputy Director F. G. Waller that the American penicillin, made by Chas Pfizer & Co. and supplied by Upjohn of Kalamazoo, Michigan, had been found to possess the same properties as Bacinol. The only difference was that the American product was white, while the NG&SF product still retained a yellow color[7] (Dr. J. de Flines, personal communication). At the end of the war, therefore, the NG&SF Delft team was in possession of an antibacterial substance that not only matched the published attributes of Fleming's penicillin but also mirrored the actual penicillin that had been mass produced in the United States.

Critically, however, the NG&SF had researched and developed their own penicillin using their own mold culture, *P. baculatum*. In the United States and Britain, research and development had continued with strains of *Penicillium notatum* and *P. chrysogenum*. Further, the NG&SF had developed their own production techniques. There would be no patent clash.

In later NG&SF R&D reports, the possibility of using submerged cultures are also discussed. This in itself is not surprising since Kluyver's Delft TU group had pioneered submerged culturing of another fungus, *Aspergillus niger*, for citric acid production in the early 1930s (Kluyver and Perquin, 1932). During the war years Kluyver, as the principal scientific advisor at NG&SF, met with NG&SF research staff weekly. Therefore, in addition to the milk bottle production, it was decided to use a

[6]Interview with Verschuyl in a documentary entitled "50 Years of Penicillin" broadcasted on Dutch television in 1991.

[7]Later it was found that this colored compound was due to an alkaloid called chrysogine.

carefully hidden supply of stainless steel to design a dedicated fermentor to produce penicillin in a submerged culture. The first attempt, however, was disastrous. Based on their knowledge of yeast fermentation, the Delft team had designed a 1 m^3 air-lift fermentor without an impeller. Productivity of penicillin was almost negligible. Despite this initial failure F. G. Waller, Jr., immediately ordered the construction of two new 15 m^3 stainless steel fermentors, this time with turbine impellers and a working volume of 10 m^3 (A. Langejan, personal communication). Ultimately, an 80 h fermentation process from one fermentor yielded 15 g of 50% pure penicillin.

In November 1945, Dr. Verschuyl treated the first 2 patients successfully in Delft's Bethel Hospital with the new NG&SF product (Fig. 5, see color insert). Both were critically ill with staphylococcal infections.

V. The Postwar Period

In January 1946, half a year after the end of World War II, NG&SF introduced their first penicillin produced on a technical scale onto the Dutch market (Fig. 6, see color insert). For large-scale production (Fig. 7)

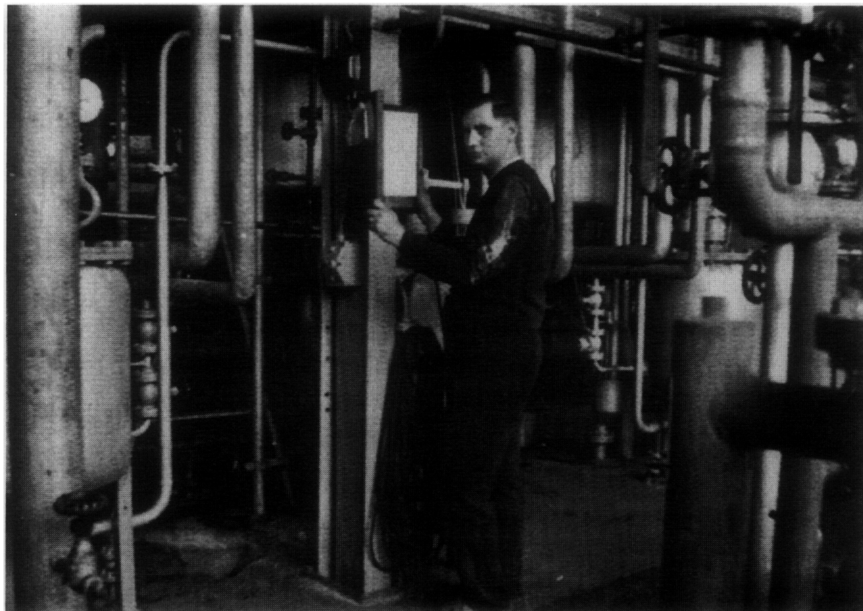


FIG. 7. From the onset of large-scale penicillin production by NG&SF. Mr. C. A. van den Berg working in the area of the 6 m^3 seed fermentors in the production plant. Photo taken at around 1955 and reproduced from the Gist-brocades brochure "35 jaar Penicillin."

THE BACINOL RESEARCH TEAM

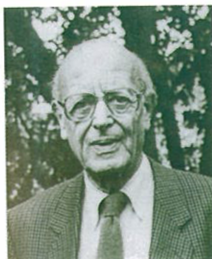
a

b

c

THE ADVISORS

d



e

f

g

FIG. 1

FIG. 1a-g The Bacinol research team and the Advisors.

The team: (a) F. G. Waller, Jr. He joined NG&SF in 1923 and retired as CEO of the company in 1965. The photo is reproduced from the *Fabrieksbode* (NG&SF's magazine for the employees) of 1965. (b) Dr. Piet (Albertus Petrus) Struyk. He joined the company in 1928 and retired in 1967. Piet Struyk died in 1978. This photo was published in the *Fabrieksbode* in 1953 to commemorate his 25 years of service. (c) Dr. A. A. Stheeman. He joined NG&SF in 1930, retired in 1958, and died in 1977. The photo was published by Gist-brocades in 1978 in a brochure entitled "35 jaar Penicilline" (35 Years of Penicillin) to commemorate 35 years of penicillin production by the company. Of the other member of the team, Dr. J. E. Rombouts, no photo is available. He joined the company in 1940 and left in 1948 to migrate to the Carribean.

The Advisors: (d) Dr. Albert Jan Kluyver, Professor of Microbiology at the Delft Technical University (then it still was called Delft Polytechnical School). He was NG&SF's scientific advisor until his death in 1956. The photo was published in the Fabrieksbode. (e) Dr. Andries Querido, Professor of Medicine at the University of Leiden and Rotterdam. The photo (Klaas Koppe, Amsterdam) is reproduced with permission from the autobiography of Querido: *Andries Querido. De binnenkant van de geneeskunde: een autobiografie* (Andries Querido. The inside of Medicine: a autobiography), Meulenhoff Publishers, Amsterdam (1990). (f) Dr. Andries Querido as a young post-doc at the Pasteur Institute in Paris (1938). Photo reproduced from Querido's autobiography, with permission. (g) Dr. Evert Verschuyl, NG&SF's company doctor and surgeon at the Delft Bethel Hospital. This picture was taken at the occasion of the making of a TV documentary "De revolutie van het geneesmiddel. 50 jaar Penicilline" (the Revolution of the Cure. 50 Years of Penicillin), directed by Willy Lindwer and made by AVA Productions, Amstelveen, The Netherlands, which was broadcasted by the Dutch Television channel NCRV in 1991.

M24C14-1943

BIBLIOTHEEK D.B.M.

NEDERLANDSCHE AKADEMIE VAN WETENSCHAPPEN

CENTRAALBUREAU VOOR SCHIMMELCULTURES
BAARN
(NEDERLAND)

LIJST VAN SCHIMMELCULTURES
1943

Penicillium baculatum Westl.	Thom
„ *Bialowiezense* Zaleski	Zaleski
„ *biforme* Thom	Thom
„ *Biourgei* Arnaudi (name antedated)	Inst. Sierot. Milan.
„ *Biourgei* Dierckx	Biourge, C. B. S.
„ *Brefeldianum* Dodge,	
cf. Carpenteles Brefeldianum (Dodge) Shear	
„ *brevicaule* Sacc., cf. Scopulariopsis	
(Syn.: Acaulium anomalum Olsen-Sopp)	
„ *brevi-compactum* Dierckx	Biourge
„ *brunneo-rubrum* Dierckx	Biourge
„ *brunneo-violaceum* Biourge	Biourge
„ *brunneo-viride* v. Szilvinyi	Janke
„ *camemberti* Thom	Thom
„ „ var. *Rogeri* Thom	Thom
(Syn.: Penicillium candidum Roger et Mazé)	
„ *candido-fulvum* Dierckx	Thom
„ *canescens* Olsen-Sopp	C. B. S., Thom
„ *capreolinum* Biourge	Biourge
„ *carmino-violaceum* Dierckx	C. B. S., N. C. T. C.
„ *carneo-lutescens* G. Smith	G. Smith
„ *caseicolum* Bain.,	
cf. Penicillium camemberti Thom var. Rogeri Thom	
„ *Charlesii* G. Smith	Thom
„ *chermesinum* Biourge	Biourge
„ *chloro-leucon* Biourge	C. B. S.
„ *chloro-phaeum* Biourge	Biourge
„ *chrysitis* Biourge	Biourge
„ *chrysogenum* Thom	L. M.
„ *Chrzaszczi* Zaleski	Zaleski
(Syn.: Penicillium citreo-sulfuratum Biourge)	
„ *cinerascens* Biourge	Biourge
„ *citreo-nigrum* Dierckx	Biourge
„ *citreo-roseum* Dierckx	Biourge
„ *citreo-sulfuratum* Biourge	Biourge
„ *citreo-viride* Biourge	C. B. S.
„ *citrinum* Thom	Thom
„ *claviforme* Bain.	C. M. Doyer
(Syn.: Coremium silvaticum Wehmer)	
„ *clavigerum* Dem.	C. B. S.
„ *commune* Thom	Thom
„ *corylophilum* Dierckx	Thom
„ *corymbiferum* Westl.	C. B. S.
„ *crateriforme* Gilman et Abbott	Abbott, Dattilo-Rubbo
„ *crustosum* Thom	Thom
„ *cyaneo-fulvum* Biourge	Biourge
„ *cyclopium* Westl.	Thom
„ *Daleae* Zaleski	Zaleski
„ *decumbens* Thom	C. B. S.
„ *Dierckxii* Biourge	Biourge

FIG. 3 A photo of the front cover and page 87 of the CBS 1943 catalog of fungal cultures from the NG&SF Library. With colored pencils several *Penicillium* strains are marked which were used in Struyk's screening in 1944. Photo taken by Piet Geijssendorpher (2001).

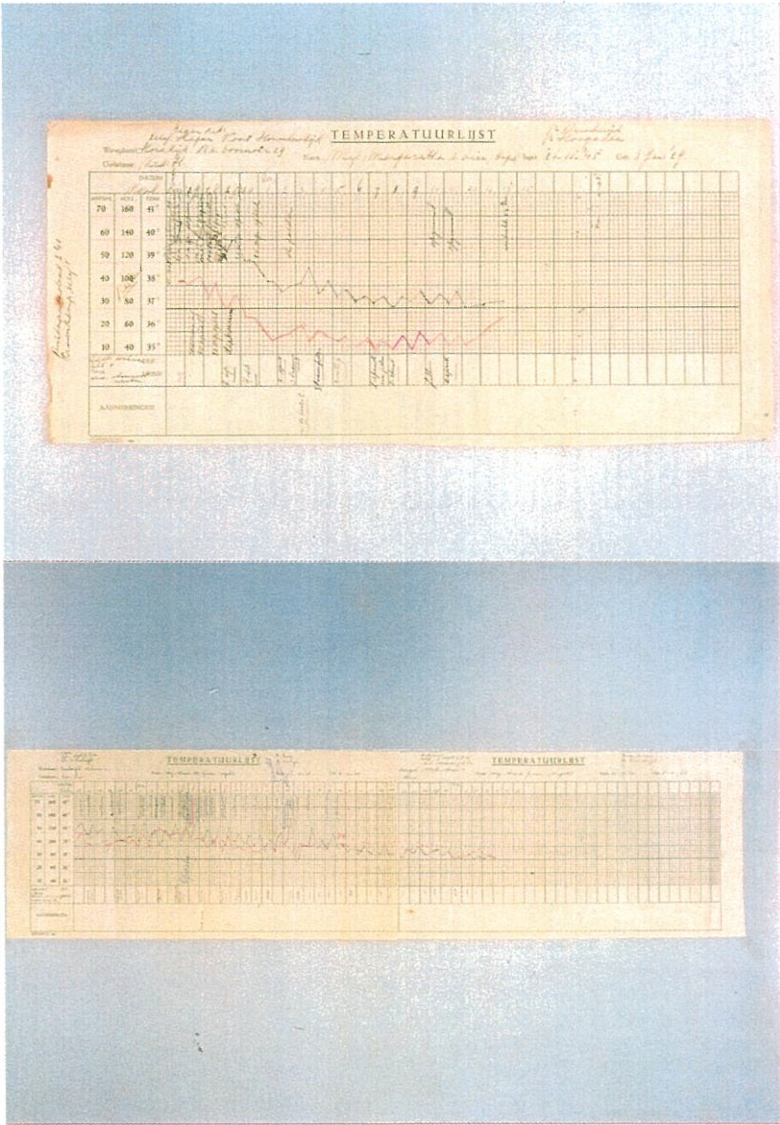


FIG. 5 Patient temperature charts of the first two patients treated by Dr. Verschuyl in the Bethel Hospital in Delft successfully with the NG&SF penicillin product. Photo Freek Zieck, published in the last issue of *Fabrieksbode* (2001).

a

b

FIG. 6 (a) This picture shows two samples of the first penicillin products made by NG&SF in 1945 and taken by Dr. Spiers to the United States in October 1945 to convince U.S. companies of the quality of the NG&SF Penicillin. In addition, a sample of a commercial product of 100,000 Units of Penicillin G calcium salt from 1949 and a sample of distilled water to prepare an aqueous solution of the product is shown in the picture (Photo: Freek Zieck, 2001). (b) A selection of labels from the early days of penicillin production by NG&SF (Photo: Freek Zieck).

in 1947, 60 m^3 fermentors were taken from the butanol fermentation plant and were adapted for the penicillin production process (Dr. J. de Flines, personal communication). By 1948 the NG&SF production of penicillin met the total demand of the Dutch population (Hoogerheide, 1980). In later years, NG&SF, by then Gist-brocades, grew to attain a dominant position in the world market of bulk penicillin (Hersbach *et al.,* 1984). In 1998 Gist-brocades was merged with DSM. Its activities in bulk β-lactam antibiotics are now concentrated in the DSM Anti-Infectives business group.

Acknowledgments

The authors would like to thank Jos van Leeuwen of the Central Archive, Piet Geijsendorpher of the R&D Archive and Wil van den Ende of the R&D Library of DSM in Delft (formerly Gist-brocades). We also would like to thank Karin van den Tweel and Rob Samson of the Centraal Bureau voor Schimmelcultures (CBS) for their help in retrieving documentation. Furthermore, we are indebted to Hans van Suijdam, formerly Director of DSM Gist in Delft, for granting Marlene Burns the permission to use all sources in the archives of the former Gist-brocades for the M.A. (History) research.

Finally, our thanks go to all retired NG&SF personnel for their willingness to share personal recollections of the early days of the NG&SF penicillin production at the NG&SF.

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Genomics for Applied Microbiology

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I. Introduction
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I. Introduction

The field of cell biology was transformed in 1995 when the complete 1.8 Mb genome of *Haemophilus influenzae* was sequenced by The Institute for Genomic Research (TIGR) in Rockville, Maryland (Fleischmann *et al.,* 1995). For the first time, all of the genes and predicted proteins necessary to operate a living cell were revealed. Since 1995, an additional 63 microbial genomes have been completely sequenced (http://www.tigr.org/tdb/mdb/). Of these, 35 are human, animal, or plant pathogens, and 28 are nonpathogenic bacterial and archaeal species. Of greater significance, more than 180 microbial genome sequencing projects are currently in progress worldwide.

Initial choices for whole genome sequencing projects were clearly geared toward organisms of medical importance such as *H. influenzae* (Fleischmann *et al.,* 1995) and *Mycoplasma genitalium* (Fraser *et al.,* 1995), the characterization of the major pathogens allowing for an

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increased understanding of the biology of these species; identification of new antimicrobial targets, and ultimately, the identification of new vaccine candidates (Hoffmann *et al.*, 1998). An initial focus on pathogenic species, however, later changed to include the sequencing of microbes of agricultural, environmental, evolutionary, and biotechnological importance (for review, see Nelson *et al.*, 2000).

The completed genome projects have produced the sequence of over 110 DNA molecules (inclusive of chromosomes and plasmids) and greater than 159,000 annotated genes (see http://www.tigr.org/tigr-scripts/CMR2/CMRHomePage.spl). Since the biochemical and metabolic diversity within the microbes vastly exceed that of plants and animals, the genes represented within the microbial universe constitute a wealth of resources for various applications that employ microbial species or their enzymes.

Beyond generating sequencing data, many new developments are enabling our understanding of the basic biology and genetics of many of these species. Improvements in abilities to engineer microorganisms to synthesize desired molecules or to have desired biodegradative pathways, as well as in our abilities to increase and optimize enzyme activity, are positive developments. New expression vectors and methods to monitor survival of engineered organisms in the environment are also being developed. The complete genome sequences of two well-characterized organisms *Bacillus subtilis* (Kunst *et al.*, 1997) and *Escherichia coli* (Blattner *et al.*, 1997), will also contribute significantly to the development of genetic tools and new approaches for assigning functional roles to the many unknown open reading frames (ORFs). These new developments will accelerate the rate at which potential applications of completed microbial genome sequences can be put into practical use.

II. Applied Microbiology Overview

Applied microbiology involves the use of microorganisms to produce commercial products, or to carry out useful chemical transformations. Biology and genetics provides the raw material that facilitates the development of useful applications. Technology and engineering accomplish the application of that knowledge to practical problems. Yet it is clear that science and technology are interdependent and often inextricably linked. Major examples of the applications of the science of microbiology include the production of cells themselves as biomass for food, or yeast for brewing and baking. The bioconversion of substrates to useful products such as that of cellulose into fuels, and the production of primary and secondary metabolites such as amino acids, alcohol, antibiotics, and enzymes, are also major examples. While some applied

microbiology applications date from antiquity, the biological sciences and engineering have brought these applications to their present state of productivity.

One component of research in applied microbiology that has relevance for genomic considerations is strain improvement and/or the identification of new strains related to useful distinctive properties. The traditional approach to strain improvement is to treat a strain with a mutagen and to select from the resultant population of mutant organisms those possessing favorable traits. A more directed approach employs recombinant DNA technology to add, eliminate, or modify the capabilities of the strain under study. Having completed genomes also allows for the identification of novel pathways that may not have been identified by traditional biochemical methods. Genomics can bring immense value to applied microbiology by revealing the global gene content of organisms and the ways in which the genes are used.

III. Microbial Genomics

A. Genomics Overview

What is generally understood by the term genomics is the study of biology from the perspective of the entire genetic content of organisms. Genomics derives from genetics, the science of heredity. The triumph of biology in the past century has been the elucidation of the mechanism of heredity, the encoding of the heredity instructions in DNA sequences. Thus for bacteria and other organisms, genomics begins with the DNA sequencing and the detailed analysis of the sequence of the chromosomes and plasmid molecules in the organism. The DNA sequencing establishes the unambiguous order of the A's, T's, G's, and C's in the DNA molecules, and their physical structure (absolute size and linear vs circular structure). During the sequencing process the DNA is translated into digital form and stored in a computer for analysis. Whole genome analysis includes annotation—the identification of all DNA encoded (ORFs) or candidate genes in the DNA sequence and the tentative assignment of gene names and associated functions to these ORFs. This process is accomplished by using ORF-finding software (e.g., GLIMMER, Salzberg *et al.*, 1998a, 1999), and by using sequence comparisons of DNA and amino acid sequences encoded in the ORFs to that of all known genes (e.g., BLAST, Altschul *et al.*, 1990). Whole genome annotation also includes the identification of rRNA (ribosomal RNA) and tRNA (transfer RNA) sequences as well as *IS* (insertion sequence) elements. Statistical analysis of nucleotide frequency across the genome is conducted to locate such features as the origin of replication (Salzberg *et al.*, 1998b), regions of atypical composition, and

regions of the genome that may have resulted from lateral gene transfer from unrelated species (Nelson *et al.*, 1999). Reconstruction of biochemical pathways and transporter profiles allow for an overview of the metabolic capacity of the cell, and often reveal new aspects of the basic biochemistry of the species of interest (Figs. 1 and 2, see color inserts).

Genomics is observational as opposed to hypothesis driven; the annotated genome sequence is only the beginning point for comparative and functional genomics-based studies. In contrast to comparative studies that rely heavily on bioinformatic tools to interpret the genomic data, functional genomic studies aim to establish a function for each gene in the genome as well as to develop an understanding of the regulatory circuits that control the metabolic and other activities of the organism. The tools of functional genomics include expression profiling, identification and analysis of protein–protein interactions, deletion phenotype analysis and proteomics. All of these tools allow for the exploration of how the organism deploys its genetic information, thus providing function related information.

Genomic studies exhibit characteristic attributes. They are high-throughput, technology-intensive, and specifically informatics intensive. These characteristic attributes derive from the large number of data elements that make up the genetic content of even the simplest bacterium. It is only through the application of high-throughput technology and informatics that genomic analysis of organisms containing millions of DNA bases and thousands of genes can be economically feasible. Beyond the economic issue, only the application of sophisticated informatics tools allows for the analysis of a genome sequence. The same consideration is true for the simultaneous mRNA (messenger RNA) expression analysis of the thousands of genes in a genome via a glass slide microarray.

B. The Genome Sequencing Process

As illustrated in Figure 3 (see color insert), the process of sequencing a microbial genome is accomplished in four phases. The first phase is library construction, during which genomic DNA from the organism of choice is fragmented into pieces of two to ten thousand base pairs (bp) in length. These DNA fragments are ligated to a plasmid vector to create DNA constructs (plasmids) that can be inserted into the laboratory bacterium *E. coli*, one plasmid per cell, for propagation and amplification. The aggregate of clones resulting from this activity is termed a shotgun library.

The second phase of the process is termed random sequencing. In this phase the *E. coli* cells containing the plasmid built during the library

construction phase are grown and plasmid DNA is purified from the cells. DNA sequence is obtained from the two ends of the insert fragment in the purified plasmid by performing a sequencing reaction for each end and analyzing the reaction products on a sequencing machine. As seen in Figure 3, approximately 15,000 sequences are obtained for each million base pairs of DNA comprising the genome from the organism that is being sequenced. This results in an average of 8-fold redundancy of the genome. This level of redundancy and the subsequent editing of any remaining ambiguities accounts for the high accuracy of sequence that is obtained.

In the third phase, called the closure or finishing phase, all of the sequences obtained are sorted into their order in the genome by an assembly program that puts sequences together based on overlaps and known distances between the sequences from both ends of the plasmid insert. Since there is 8-fold redundancy in the sequences, the vast majority of the genome is correctly put together by the assembly software. As a consequence of the random nature of the ends of the insert fragments that are sequenced, and the fact that many regions of a genome are often underrepresented in the shotgun library or unclonable in *E. coli,* there will often remain gaps in the sequence at the end of the assembly process. These gaps are termed "sequencing" or "physical" gaps depending on whether there is linking clone information across the gap. The gaps are closed by a combination of methods that include the sequencing of spanning clones across the gaps, multiplex and combinatorial polymerase chain reaction (PCR), and the sequencing of newly generated PCR products from across these gaps. An array of informatics tools is often employed to assist in the closure process.

C. Genome Annotation

Once the sequence of the entire genome is obtained, the final phase of the project—genome annotation—is initiated. This portion of the project is essentially an analysis of the raw sequence of A's, T's, G's, and C's, with an overall goal to identify all the putative genes, pseudogenes, transposons, repetitive elements, etc. and any other features of the genome, and to organize them into structural and functional groups. Unlike the initial phases of a genome project that are highly dependent on laboratory techniques, the annotation phase is highly informatics intensive.

1. Gene Finding

The first task in annotation of a genome sequence is to find all the potential genes encoded by the sequence. Computational programs such as Glimmer or Genmark (Hagiwara *et al.,* 1995) are used to predict

ORFs. The Glimmer algorithm uses interpolated Markov models for gene identification (Salzberg *et al.*, 1998a; Delcher *et al.*, 1999a). In tests on a set of 10 microbial genomes, Glimmer was shown to correctly identify 98–99% of all genes (Delcher *et al.*, 1999b). A eukaryotic version of Glimmer, called Glimmer M, has been developed as a gene-finding tool for finding *Plasmodium* genes in genomic sequence, and is now also trained for fungal and protozoan genomes (Salzberg *et al.*, 1999).

2. *Gene Assignments*

Assigning gene names and functions to the ORFs identified by gene finding software requires a variety of robust tools for sequence similarity searches and gene function assignment. A correct annotation process also necessitates human intervention to ensure that assignments are correctly made. A variety of processes are carried out by different centers when annotating genome sequences, and here we describe the process as it is conducted at TIGR.

Initial searches of the predicted coding regions are performed with BLASTP (Fleischmann *et al.*, 1995). The protein–protein matches are aligned with a modified Smith–Waterman algorithm that maximally extends regions of similarity across protein coding frameshifts in the DNA sequence (Waterman, 1988). Gene identification is facilitated by searching against a database of nonredundant proteins (nraa) developed at TIGR and curated from the public archives GenBank (http://www.ncbi.nlm.nih.gov/), Genpept (http://helix.nih.gov/apps/bioinfo/), PIR (http://pir.georgetown.edu/pirwww/dbinfo/pirpsd.html), and SwissProt (http://www.expasy.ch/sprot/sprot-top.html). Searches matching entries in nraa have the corresponding role, gene common name, percent identity and similarity of match, the pairwise sequence alignment, and taxonomy associated with the match assigned to the predicted coding region and stored in the database. Regions of the genome without predicted coding regions and Glimmer predictions with no database match are reevaluated using blastx as the initial search; new genes are then extrapolated from regions of alignment. Finally, each putatively identified gene is assigned to one of 102 role categories adapted from a system of gene role categories created for *E. coli* (Riley, 1993). The Gene Ontology Consortium has initiated a project to develop "a structured, precisely defined, common controlled vocabulary for describing the roles of genes and gene products in any organism" (Ashburner *et al.*, 2000). At TIGR and other places performing annotation on microbial genome sequences, the Riley *E. coli* based gene role categories are being converted to the Gene Ontology (GO) system.

These methodologies for gene identification depend primarily on pairwise alignments. In order to enhance the ability to make potential

gene identifications, approaches and tools based on multiple sequence alignment and family building are employed (Eddy *et al.,* 1998; Sonnhammer *et al.,* 1998). Paralogous gene families are created from multiple sequence alignments made with the target genome's predicted amino acid sequences and built with the MKDOM software (Gouzy *et al.,* 1997). The multiple-sequence alignments group similar proteins into families for verification of annotation and identification of family members not recognized by simple pairwise alignment. The ORFs are also aligned with a variety of tools against a growing database of Hidden Markov Models built on protein family/superfamily multiple sequence alignments (pfam v3.1, Bateman *et al.,* 2000; TIGRfam, Haft *et al.,* 2001).

In addition to ORF analysis and gene discovery by similarity searches, a number of other features of the genome are analyzed and documented. TopPred is used to identify potential membrane-spanning domains in proteins (Claros and von Heijne, 1994). Signal peptides and the probable position of a cleavage site in secreted proteins are detected with SignalP (Nielsen *et al.,* 1997). Genes coding for untranslated RNAs can be identified by database searches at the nucleotide level, and searches for tRNAs can be performed using tRNAScan-SE (Lowe and Eddy, 1997). Repetitive sequences in the genome can be identified using an algorithm based on suffix trees (Delcher *et al.,* 1999b), which can very rapidly identify all repeats in large genome sequences. An initial set of repeats can be further grouped into classes based on size and complexity.

In order to analyze particular aspects of the large quantities of data that are being generated, data analysis sites that allow the user to query for information related to their organism of choice have become essential (Table I). One excellent database that has recently been constructed is the Comprehensive Microbial Resource (CMR) (http://www.tigr.org/tigr-scripts/CMR2/CMRHomePage.spl), which was introduced primarily to reduce annotation inconsistency across completed genomes (Peterson *et al.,* 2001). With the CMR, the user can access data from all completed genomes with annotation available from both the sequencing center that sequenced the genome and automated annotation from TIGR. Complex queries based on role assignments, database matches, protein families, membrane topology, and other features are feasible. In addition to the CMR, other databases such as the The University of Minnesota Biocatalysis/Biodegradation Database (Ellis *et al.,* 2000; http://www.labmed.umn.edu/umbbd/) which contains more than 100 pathways for microbial catabolic metabolism of primarily xenobiotic compounds, are extremely useful for generating biochemical information on an organism. This database is particularly relevant, as it has been developed specifically to capture information relevant to environmental applications. The database currently contains more than

TABLE I

SOME OF THE CURRENTLY AVAILABLE DATABASES FOR GENOMIC DATA ANALYSES

The Comprehensive Microbial Resource	http://www.tigr.org/tigr-scripts/CMR2/CMRHomePage.spl
University of Minnesota Biocatalysis/Biodegradation Database	http://www.labmed.umn.edu/umbbd/
TIGRFams	http://www.tigr.org/TIGRFAMs/
TIGR Software Tools	http://www.tigr.org/softlab/
National Center for Biotechnology Information	http://www.ncbi.nlm.nih.gov/
DNA Structural Analysis of Sequenced Prokaryotic Genomes	http://www.cbs.dtu.dk/services/GenomeAtlas/index.html
KEGG: Kyoto Encyclopedia of Genes and Genomes	http://www.genome.ad.jp/kegg/
ARCHAIC: ARCHAebacterial Information Collection	http://www.aist.go.jp/RIODB/archaic/
The DEAMBULUM	http://www.infobiogen.fr/services/deambulum/english/menu.html

110 pathways, 643 compounds, 462 enzymes, and 303 microorganism entries as well as information on 50 organic functional groups. Graphical presentations of pathways and reaction mechanisms are also provided. Of the 110 pathways in the database, 53 are from organisms for which there is complete or partial genome sequences available. Some useful databases are provided in Table 1.

3. *Reconstruction of Physiology and Transport Profiles from Complete Genomes*

The availability of the complete microbial genome sequence allows for a reconstruction of the physiological and transporter profiles of the cell. The recently completed *Agrobacterium tumefaciens* genome, for example, has shown that the bacterium is able to utilize a range of substrates including 17 amino acids, and sugars such as glucose, fructose, sucrose, ribose, xylose, xylulose, and lactose (Wood *et al.*, 2001). Physiology and transporter profiles have been reconstructed for other organisms including *Thermotoga maritima* (Nelson *et al.*, 1999), *Streptococcus pneumoniae* (Tettelin *et al.*, 2001), and *Chlorobium tepidum* (Eisen *et al.*, in press). These reconstructions allow the reader to have easy access to the physiological basis of the organism in question. They rely heavily on having an accurate curation of the genome annotation, at least in the major role categories presented such as energy metabolism and transport. Automated annotation without human intervention may miss genes, make miss calls, and is not able to recognize operonic structures. An example of metabolic reconstructions based on accurate annotation

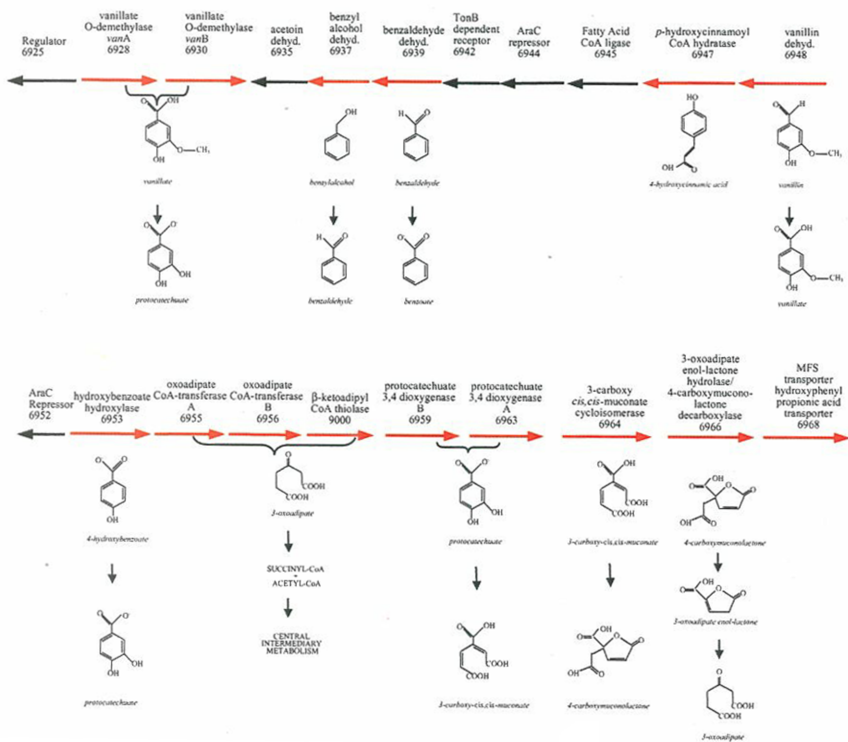

FIG. 1 Previously uncharacterized pathway for metabolism of aromatics, identified from genome of *Caulobacter crescentus* (Nierman *et al.*, 2001).

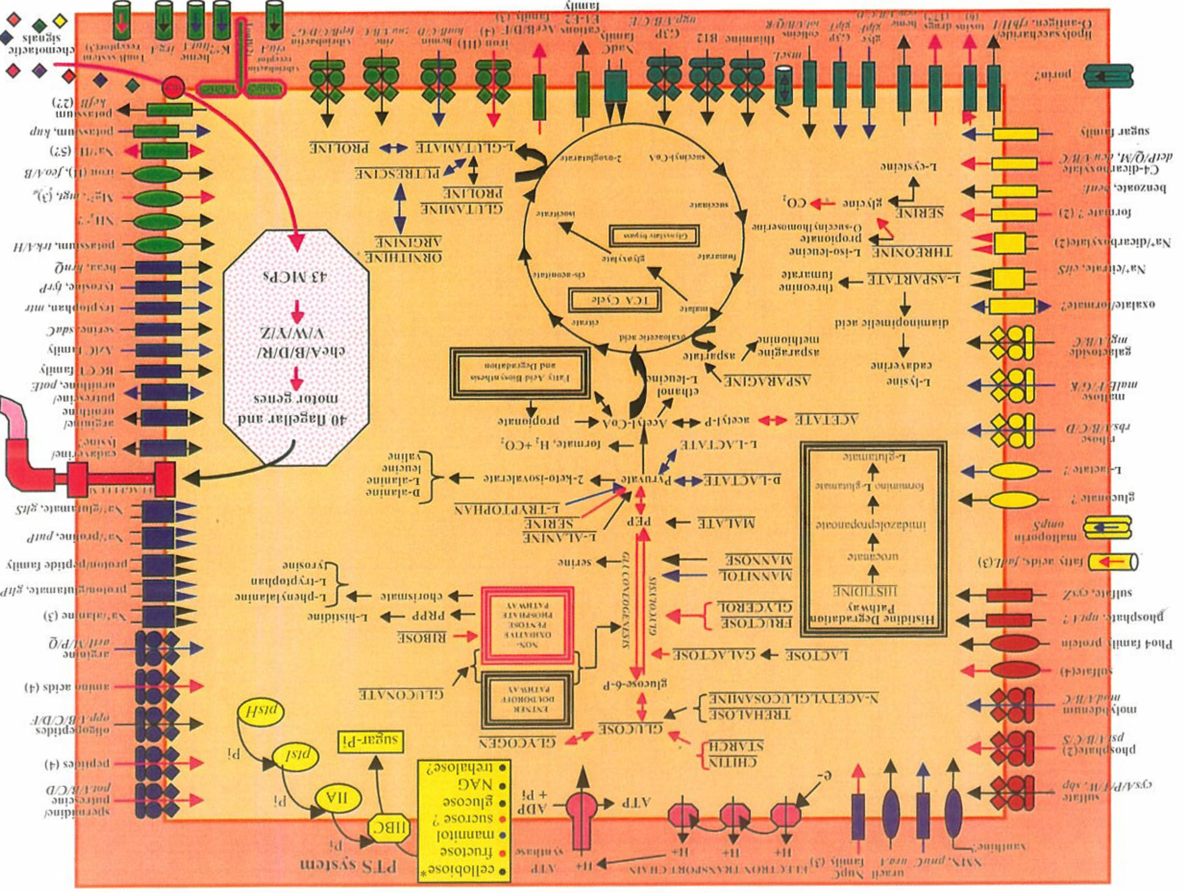

FIG. 2 Reconstruction of physiology and metabolism in *V. cholerae* (reprinted with permission from Nature, Heidelberg *et al.*, 2000).

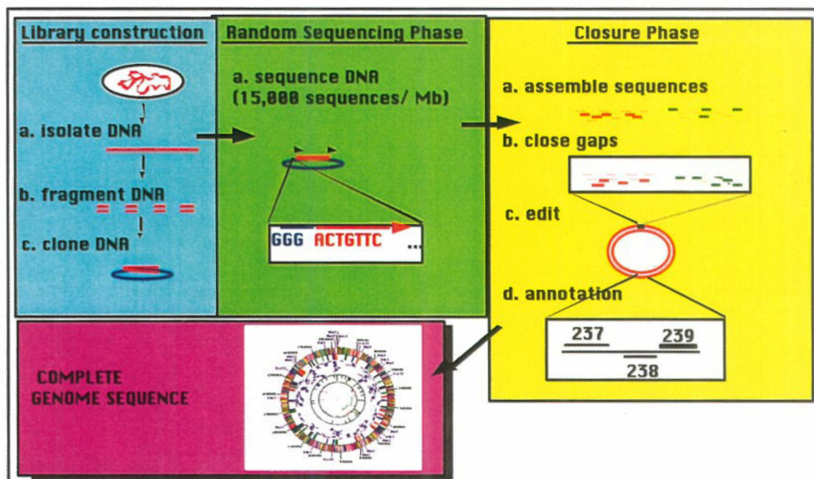

FIG. 3 An overview of sequencing and analysis of a microbial genome.

of the recently completed *Vibrio cholerae* genome (Heidelberg *et al.*, 2000) is presented in Figure 2.

IV. Functional Genomics

The main premise for sequencing the entire genome of any organism is the gain in terms of potential application that will be realized by having the complete sequence. Whole genome data significantly increases our abilities to address biological questions. However, many potential applications cannot be realized unless methods are available to validate and assign function to the many unknown ORFs that remain at the end of a genome project, as well as methods to interpret the functions of the many predicted genes in these genomes. Below is a synopsis of three well-established technologies for biological interpretation of the genomic sequence data.

A. Microarray Expression Analysis

While the genome sequence of an organism provides an immense amount of biological information, it represents only the beginning for a more complete understanding of all cellular functions. Of particular note is the observation that on average, 30% or more of the genes in sequenced organisms encode proteins with no known function (Nelson *et al.*, 2000). Examination of the patterns of gene expression on a whole genome scale under a variety of conditions provides a powerful resource for assessing function for these genes. Through microarray analysis of patterns of gene expression in the cell cycle control pathway of the bacterium *Caulobacter crescentus,* several general conclusions could be drawn relative to assessing genome function (Nierman *et al.*, 2001; Laub *et al.*, 2000). (1) Genes involved in a given cell function are transcriptionally activated at the time of execution of that function; (2) genes encoding proteins that function in complexes are coexpressed; and (3) temporal cascades of gene expression control multiprotein structure such as flagellar biogenesis. Thus, by analyzing when genes are expressed and what clusters of genes exhibit specific patterns of expression, hypotheses can be developed and tested about the roles of the individual cluster members and of all of the genes in a cluster. Transcriptional profiling techniques can be used to develop a more complete understanding of gene function, regulation, and interactions.

A variety of techniques, including serial analysis of gene expression (SAGE) (Velculescu *et al.*, 1995), differential display (Liang and Pardee, 1992), oligonucleotide arrays (Lockhart *et al.*, 1996) and ORF-specific PCR product and cDNA (DNA complementary to mRNA) microarrays (Schena *et al.*, 1995) have been developed that allow mRNA expression

to be assessed on a global scale for hundreds or thousands of genes in a single experiment. The most common use of these techniques is to determine patterns of differential gene expression or to compare differences in mRNA expression levels between identical cells subjected to different stimuli or between different cellular phenotypes or developmental stages.

Microarray expression analysis has features that make it a widely used method for profiling mRNA expression (Schena *et al.*, 1995). DNA segments representing the set of genes to be assayed are amplified by PCR and mechanically spotted at high density on glass microscope slides using relatively simple *x–y–z* stage robotics systems to create a microarray containing thousands of elements. Microarrays containing the entire set of genes from a microbial genome or tens of thousands of genes from a eukaryote can be easily constructed. The microarrays are queried in a cohybridization assay using two or more fluorescently labeled probes prepared from mRNA from the cellular conditions of interest (Shalon *et al.*, 1996; Hedge *et al.*, 2000). The kinetics of hybridization allows the determination of relative expression levels based on the ratio with which each probe hybridizes to the individual array element. Hybridization is assayed using a confocal laser scanner to measure fluorescence intensities, which allows the simultaneous determination of the relative expression levels of all the genes represented in the array. Whole genome microarray expression studies have been initiated for several pathogenic and environmental microorganisms, and extensive informatics infrastructure have been developed and implemented to support these studies.

B. Heterologous Gene Expression

The completed genome sequence, with all predicted protein coding sequences and annotation, presents a convenient resource for cloning any gene from that genome. The gene of interest can be PCR amplified from genomic DNA, cloned into an expression vector, and electroporated into a heterologous host such as *E. coli* to produce analytical or production quantities of an enzyme or protein encoded by the gene. This becomes a particularly useful path for producing and characterizing proteins from organisms such as the extremophiles that can present severe difficulties in laboratory growth in terms of sufficient cell mass for enzyme or protein purification. For single proteins that require accessory proteins or for multisubunit enzymes, the coding sequences can be introduced to the heterologous host using constructed operons requiring coexpression of the coding sequences. Of late, the heterologous expression approach has frequently been used for the functional characterization of enzymes from sequenced organisms.

C. Proteomics

For completing the analysis of genome function, nucleic acids-based methods such as annotation of genome sequence and DNA-based microarray expression technology are often inadequate. The gene products, proteins, and stable RNAs, execute genome function in cells. Analysis of the complete protein complement of cells is essential to functional analysis of the genome, and includes individual protein identification, localization, and quantification. Included in this analysis is characterizing protein modifications, interactions, and activities in order to understand their functions, and functional and regulatory interactions. This is a much more difficult task than comparative genomic analysis. Proteins are modified posttranslationally in several ways, and single genes can encode multiple different proteins. In response to internal or external signals, proteins can change their intracellular location, undergo modifications and/or cleavages, or have their stability or functional properties altered. Protein levels frequently do not directly correlate with mRNA levels (Gygi *et al.,* 1999). Since there is no strict linear relationship between genes or even mRNA levels and the protein complement of a cell, proteome analysis is a necessary component for the functional characterization of a genome.

Proteomics is the large-scale study of the proteins of an organism (for review, see Pandey and Mann, 2000). The field really began in the 1970s with the cataloging of protein spots revealed by the then new technique of two-dimensional gel electrophoresis (O'Farrell, 1975). In the first dimension, proteins are sorted by isoelectric point, and in the second, they are sorted by mass. This technology has resulted in the construction of databases of the resultant spots to serve as resources of expressed proteins. The technological limitation of this approach has been the lack of a sensitive and analytical method for characterization of all the proteins in the spots. However, in the 1990s, mass spectrometry emerged as a powerful analytical method. Coupled with the availability of complete genome sequence, mass spectrometry procedures now allow for the identification and quantification of proteins in a complex mixture of proteins isolated from a cell.

The technology for high-throughput proteomics analysis is presently undergoing rapid development. First the protein sample is obtained fractionated in some manner, and treated to reduce the complexity of the protein mixture. The complexity reduction step is required because the dynamic range of protein abundance is very high and because two-dimensional gels can resolve on the order of only up to 1,000 spots. Once separated, proteins are recovered and digested with a sequence-specific protease such as trypsin. A peptide mass fingerprint is obtained using

relatively simple mass spectrometric methods (Henzel *et al.,* 1993). These mass spectra can be compared automatically to a database of peptide fragments generated using the protein sequence of each protein coded in the organism's genome. Thus, a peptide fragment mass fingerprint can be used to identify the protein or proteins contained in a spot on the two-dimensional gel (Jensen *et al.,* 1997; Berndt *et al.,* 1999). Adaptations of this methodology allow for the quantification of the levels of the individual proteins in the cell.

V. Application Potential of Completed Microbial Genomes

For the nonpathogenic microorganism, the choice for sequencing is usually dictated by some anticipated environmental or biotechnological gain that cannot be realized without having a significant portion of the genome sequence in hand. Below in Tables II and III is a list of the ongoing and completed genome sequencing projects for organisms of biotechnological and environmental significance, as well as a synopsis of some of their main potential applications.

A. Bioremediation and Environmental Engineering

Bioremediation is an effective, environmentally benign method for the treatment of waste- and radiation-exposed sites, contaminated soils, and groundwaters, and for the disposal of sewage and solvents. It has many significant advantages over conventional techniques for pollution control such as incineration and chemical treatment. In addition, bioremediation also can be used to create a renewable source of energy through conversion of plant biomass. The genome sequences of *Dehalococcoides ethenogenes, Deinococcus radiodurans, Pseudomonas putida,* and *T. maritima* hold promise for significant advances in the areas of bioremediation.

Dehalococcoides ethenogenes isolated in 1999 (Maymo-Gatell *et al.,* 1999), efficiently degrades tetrachloroethene, one of the most commonly found compounds in contaminated ground water. It can persist for an extended time in these subsurface environments. This compound has been widely used in dry-cleaning, and as a degreasing solvent, and is considered to pose a public health risk. Tetrachloroethene resists aerobic breakdown, and *D. ethenogenes* strain 195 is the only bacterium isolated to date that can completely dechlorinate tetrachloroethene. Currently, growth of this organism on tetrachloroethene requires extracts from mixed microbial cultures. It is anticipated that the genome sequence and subsequent functional analyses (based primarily on microarray studies) will provide significant information on the basic physiology of this

TABLE II

COMPLETELY SEQUENCED GENOMES FOR ORGANISMS OF ENVIRONMENTAL AND BIOTECHNOLOGICAL SIGNIFICANCE[a]

Organism	Size (Mb)	Completed	Interest/Application
Aeropyrum pernix	1.67	1999	Aerobic hyperthermophile, biotechnology applications
Agrobacterium tumefaciens	5.67	2001	Plant pathogen, Plant genetic engineering
Aquifex aeolicus	1.50	1997	Hyperthermophile, biotechnology applications, evolutionary implications, chemolithoautotroph
Archaeoglobus fulgidus	2.18	1998	Hyperthermophile, sulfate reducer, oil well problem, biotechnology applications
Bacillus halodurans	4.25	1999	Alkaliphilic, deep sea adaptations, industrial applications
Bacillus subtilis	4.20	1997	Industrial applications
Caulobacter crescentus	4.01	2001	Cell cycle regulation
Clostridium acetobutylicum	4.10	2001	Solvent production, cellulose degradation
Deinococcus radiodurans	3.28	1999	Radiation resistance, environmental clean up
Escherichia coli	4.60	1997	Biotechnology applications
Escherichia coli	5.50	2000	Biotechnology applications
Escherichia coli	5.60	2001	Biotechnology applications
Halobacterium sp.	2.57	2000	Halophile, biotechnology applications
Methanobacterium thermoautotrophicum	1.75	1997	Hyperthermophile, methanogen, waste digestion, biogas production, biotechnology applications
Methanosarcinia mazei	2.8	1999	Hyperthermophile, methylotroph, sewage digestion, biogas production, biotechnology applications
Lactococcus lactis	2.36	2001	Dairy industry applications
Methanococcus jannaschii	1.66	1996	Methane production, hyperthermophilic enzymes
Mesorhizobium loti	7.59	2000	Plant symbiont
Pyrococcus abyssi	1.80	1999	Hyperthermophile, biotechnology applications
Pyrococcus horikoshii	1.80	1998	Hyperthermophile, biotechnology applications
Saccharomyces cerevisiae	12.07	1996	Food industry, biotechnology applications
Sinorrhizobium meliloti	6.68	2001	Nitrogen fixation
Sulfolobus solfataricus	2.99	2001	Thermophile, sulfur oxidizer, biotechnology applications
Synechocystis sp.	3.57	1996	Photosynthetic reactions
Thermoplasma acidophilum	1.56	2000	Thermoacidophilic archaeon
Thermoplasma volcanium	1.58	2000	Thermoacidophilic archaeon
Thermotoga maritima	1.86	1999	Hyperthermophile, evolutionary implications, bioremediation, biotechnology applications
Thermus thermophilus	1.82	1999	Thermophile, biotechnology applications

[a] For a complete list of references, see www.tigr.org/tdb/mdb/.

TABLE III

ONGOING MICROBIAL GENOME PROJECTS OF ORGANISMS OF SIGNIFICANCE TO APPLIED MICROBIOLOGY[a]

Organism	Size (Mb)	Interest/application
Aspergillus niger	30	Removal of toxic compounds
Bacillus stearothermophilus	?	Thermophilic enzymes
Burkholderia fungorum	8.1	Polychlorinated biphenyl biodegradation
Carboxydothermus hydrogenoformans	2.1	Carbon monoxide utilization
Chlorobium tepidum	2.1	Model for evolution of photosynthesis
Chloroflexus aurantiacus	3.0	Photoautotroph
Chromobacterium violaceum	4.2	Antibiotic, polyhydroxyesters and polysaccharide biosynthesis
Corynebacterium glutamicum	3.3	Amino acid biosynthesis
Dehalococcoides ethenogenes	1.5	Solvent detoxification
Desulfitobacterium hafniense	4.6	Anaerobic chlorophenol reduction
Desulfobacterium autotrophicum	4.4	Sulfate reduction
Desulfotalea psychrophila	4.0	Psychrophilic enzymes, sulfate reducer
Desulfovibrio vulgaris	3.2	Sulfate reduction
Ferroplasma acidarmanus	2.0	Iron-oxidizing archaeon
Fibrobacter succinogenes	3.6	Cellulose degradation
Geobacter sulfurreducens	2.5	Ferric-iron reduction
Halobacterium salinarium	4.0	Photochemical reactions, salt tolerant enzymes
Lactobacillus acidophilus	1.9	Probiotic bacteria
Magnetospirillum magnetotacticum	4.5	Model for biomineralization, magnetite producer
Methanococcus maripaludis	?	Methanogen, nitrogen fixer
Methanogenium frigidum	?	Psychrophilic enzymes, methanogen
Methanosarcina barkeri	2.8	Methanogen, anaerobic conversion of cellulose in co-culture
Methanosarcina mazei	2.8	Methanogen
Methylobacterium extorquens	?	Methylotroph
Methylococcus capsulatus	4.6	Methane oxidizer
Myxococcus xanthus	9.5	Model for fruiting body development
Nitrosomonas europaea	2.2	Bioconversion of ammonia and halogenated compounds
Nostoc punctiforme	10	Photosynthetic nitrogen fixer
Pseudomonas fluorescens	5.5	Secondary metabolites, antibiotics, bioremediation
Pseudomonas putida	6.1	Secondary metabolites, bioremediation
Pyrobaculum aerophilum	2.2	Hyperthermophilic, organotrophic, nitrate reducing archaeon
Pyrococcus abyssi	1.8	Hyperthermophilic, organotrophic, archaeon

(continues)

TABLE III—*Continued*

Organism	Size (Mb)	Interest/application
Pyrococcus furiosus	2.1	Hyperthermophilic, organotrophic, archaeon
Ralstonia metallidurans	5.0	Heavy metal resistance, selenite reduction
Rhodobacter capsulatus	3.7	Phototrophic, bioremediation
Rhodopseudomonas palustris	5.5	Polyhydroxybutyrate production, plant polymer remediation
Ruminococcus albus	4.0	Fiber digester, anaerobe
Schizosaccharomyces pombe	14	Single celled yeast
Shewanella oneidensis (putrefaciens)	4.5	Dissimilatory metal reduction
Sphingomonas aromaticivorans	3.8	Aromatic degradation
Streptococcus thermophilus	1.8	Economically important thermophilic lactic acid bacteria
Streptomyces coelicolor	8.0	Antibiotic production
Thermobifida fusca	3.6	Thermophilic degrader of plant cell walls
Thermus thermophilus	1.8	Thermophilic enzymes
Thiobacillus ferrooxidans	2.9	Ferrous ion precipitation, wastewater detoxification

[a] For a complete list of sequencing centers, see http://www.tigr.org/tdb/mdb/mdbinprogress.html.

bacterium and reveal requirements to stimulate growth under varying conditions.

Deinococcus radiodurans withstands high levels of radiation, efficiently repairing breakage of its own DNA without loss of viability (White *et al.*, 1999). This organism shows remarkable resistance to damage caused by extreme levels of ionizing radiation, dessication, ultraviolet (UV) radiation, oxidizing agents, and electrophilic mutagens. It can grow continuously in the presence of chronic radiation (6,000 rads/h) as well as survive acute exposures to γ radiation exceeding 1,500 kilorads without undergoing induced mutations. The resistances stem from extremely efficient DNA repair mechansisms, but a detailed understanding of these processes has not yet evolved. The sequencing of *D. radiodurans* has facilitated the continued development of this organism as an agent for use in bioremediation of radioactive waste (Makarova *et al.*, 2001), and with a very efficient genetic manipulation system, the organism is poised for rapid development to become an effective bioremediation agent. Although the genome sequence did not give significant insights into the DNA repair mechanisms employed by this bacterium, the complete genome does allow for an increased understanding of its global physiology under varying growth conditions such as may be found in radioactive environments. This is significant if advances are to be made

in the development of this organism for bioremediation. Venkateswaran and co-workers (1999) have described nutritional compounds capable of restoring growth of *D. radiodurans* in nutritionally limiting radioactive environments. Additionally, the gene for toluene dioxygenase has been inserted into the chromosome of *D. radiodurans* (Lange *et al.*, 1998), and the resultant recombinant bacterium is capable of degrading toluene and other organic compounds in high radiation environments. This organism has significant potential for use in the bioremediation of waste sites simultaneously contaminated by toxic chemicals and exposed to radiation. It is anticipated that new functional assignments for unknown genes will be gleaned from the complete genome sequence.

Pseudomonas putida is a ubiquitous, metabolically and physiologically diverse bacterium mineralizing many organic wastes and pollutants including ring-based organic compounds. The genome sequence has recently been completed, and functional studies employing microarray technology have been initiated. With more than 200 strains of *P. putida* already described, all with diverse abilities to degrade compounds in the environment, pathways for the catabolism of new compounds will likely be identified through a combination of genome annotation, and functional characterization of all the proteins in the bacterium.

Thermotoga maritima has numerous pathways for the degradation of simple and complex plant polymers including xylan and cellulose (Nelson *et al.*, 1999). Both polymers accumulate in the environment and have significant potential as renewable energy sources. Current microarray projects for *T. maritima* MSB8 have a primary emphasis on the proteins involved in carbohydrate and plant polymer metabolism. Again, assignment of function to the unknown ORFs in this genome will increase our knowledge of the basic physiology of this bacterium, and the potential it holds for the conversion of plant polymers. It is anticipated that mutants can be engineered that are specific for desired environmental settings.

B. Enzymes from Extremophiles

Proteins from organisms that live under extreme conditions may be chemically and physically more stable than those from mesophiles. Their stability often increases the efficiency of biotechnological reactions. These enzymes, therefore, tend to be more suitable for industrial processes than those derived from mesophilic organisms. The most widely used thermophilic enzymes in industry are DNA polymerases (e.g., *Taq* polymerase, derived from the thermophilic bacterium *Thermus aquaticus*). Other potential enzymatic applications from

environmental thermophiles include proteases in the laundry industry, amylases in food processing, and cellulases for paper softening.

While the limits of life in extreme environments are not established, it is clear that microbes can flourish at temperatures of 113°C, at pH conditions approaching zero, and at salinities approaching saturation (Delong, 2000). Many of the completed genome sequences are from microorganisms that occupy extreme ecological niches of high temperature, low pH, or high salinity. At least 10 completed genomes are from such extremophiles and another 10 will be completed shortly. In addition to the thermophilic bacteria *T. maritima* and *Aquifex aeolicus*, the thermophilic Archaea have the potential to provide thermostable products with industrial applications. Other Archaea such as the *Halobacterium* species have high salt level requirements for survival. These organisms can provide the biotechnology industry with a source of enzymes useful for extreme conditions, or processes that require organic solvents. In addition, enzymes from the *Halobacteria* (Robb *et al.*, 1995) and *P. putida* (Garcia *et al.*, 1999) have potential for the production of biodegradable plastics.

It should be pointed out, however, that although proteins from extremophilic bacteria and Archaea have generated considerable interest since their discovery in the 1970s (Brock, 1978), most have not found wide acceptability for industrial applications due to their low activity at more moderate temperatures. Some also exhibit short half-lives at high temperatures. Although it has been established that activity and thermal stability are not linked (Van den burg *et al.*, 1998; Zhao and Arnold, 1999), there is concern that the structural constraints of thermal stability may restrict the activity at lower temperatures. The availability of complete annotated genome sequences for 10 thermophilic microbes has afforded, however, the opportunity for greater exploration of the properties of enzymes from these organisms.

Several of these thermophilic enzymes exhibit high activity and stability at moderate temperatures as well (Kengen *et al.*, 2001; Helianti *et al.*, 2001; Ishikawa *et al.*, 2001; Ando *et al.*, 1999). For example, the DNA polymerase IV from *Sulfolobus solfataricus* works well at 37°C as well as under PCR-like conditions (Zhou *et al.*, 2001). For others, this is not true. Glutamate dehydrogenase from *Aeropyrum pernix* K1 exhibits only 15% activity at 45°C (Helianti *et al.*, 2001) and the phosphofructokinase from the same organism is a mere 11% activity at 65°C (Rominus *et al.*, 2001). Pyruvate carboxylase from *Methanococcus jannaschii* has optimal activity at 80°C but is also unstable at this temperature (Mukhopadhyay *et al.*, 2000). In summary, each thermostable enzymes must be carefully evaluated for the targeted industrial application (see Table IV).

TABLE IV

EXAMPLES OF RECENTLY CHARACTERIZED ENZYMES FROM VARIOUS SPECIES[a]

Organism	Enzyme	References
A. pernix K1	Glutamate dehydrogenase	Helianti *et al.* (2001)
A. pernix K1	Phosphofructokinase	Ronimus *et al.* (2001)
A. pernix K1	Isocitrate dehydrogenase	Steen *et al.* (2001)
A. pernix K1	Pernilase (heat stable protease)	Croocker *et al.* (1999)
A. fulgidus	Recombinase Rad H51	McIlwraith *et al.* (2001)
A. fulgidus	Catalase-peroxidase	Kengen *et al.* (2001)
A. fulgidus	8-oxoguanine glycosylase	Chung *et al.* (2001)
M. jannaschii	Pyruvate carboxylase	Mukhopadhyay *et al.* (2000)
M. jannaschii	Phosphofuctokinase	Verhees *et al.* (2001)
M. jannaschii	Acid phosphatase	Graham *et al.* (2001)
P. furiosus	Isocitrate dehydrogenase	Steen *et al.* (2001)
P. furiosus	Citrate synthase	Arnott *et al.* (2000)
P. horikoshii	Mannosyl-3-phosphoglycerate synthase	Empadinhas *et al.* (2001)
P. horikoshii	Mannosyl-3-phosphoglycerate phosphatase	Empadinhas *et al.* (2001)
P. horikoshii	Carboxypeptidase/aminoacylase	Ishikawa *et al.* (2001)
P. horikoshii	Aminopeptidase	Ando *et al.* (1999)
S. solfataricus	DNA polymerase IV	Boudsocq *et al.* (2001)
S. solfataricus	DNA polymerase UmuC/DinB	Zhou *et al.* (2001)
S. solfataricus	α-Xylosidase	Trincone *et al.* (2001); Moracci *et al.* (2001)
S. solfataricus	Amidase	d-Abusco *et al.* (2001)
S. solfataricus	Glycosynthase	Moracci *et al.* (2001)
S. solfataricus	Alcohol dehydrogenase	Raia *et al.* (2001)
S. solfataricus	Glyceraldehyde-3-phosphate dehydrogenase	Littlechild and Isupov (2001)
S. solfataricus	Nicotinamide-mononucloetide adenyltransferase	Raffaelli *et al.* (2001)
T. acidophilum	Proteasome (N-terminal hydrolase–protease)	Beadell *et al.* (2001)
T. acidophilum	Orotidine-5′-monophosphate	Yaoi *et al.* (2000)
T. acidophilum	Citrate synthase	Arnott *et al.* (2000)
T. maritima	β-Mannanase	Parker *et al.* (2001)
T. maritima	β-Manosidase	Parker *et al.* (2001)
T. maritima	Topoisomerase I	Viard *et al.* (2001)
T. maritima	Isocitrate dehydrogenase	Steen *et al.* (2001)
T. maritima	Endonuclease V	Huang *et al.* (2001)
T. maritima	DNA gyrase	Guipaud and Forterre (2001)
T. maritima	Chorismate synthase	Fitzpatrick *et al.* (2001)

[a] Full genus names are provided in Tables II and III.

C. Microbial Genomics and Food Biotechnology

Microorganisms play important roles in producing food ingredients, as processing aids, or in producing fermented foods. In addition, organisms responsible for food spoilage and food poisoning are important components of the processes for controlling food quality and safety. Some organisms retain important activities that contribute to human health even after they are consumed. Thus, the genomics of microorganisms used in food production, microbial food pathogens, and spoilage organisms, and the genomics of humans themselves, are all relevant to the production of healthy and safe foods. It is not surprising that the progress in genomics has extended into food biotechnology with the sequencing of the genomes of many organisms important to the food industry (Table V; for review, see de Vos, 2001).

1. *Saccharomyces cerevisae*

Saccharomyces cerevisae (yeast) is important both as a model organism as well as for the significant role it plays in the food and beverage industry. In 1996 an international group of 600 scientists reported the completed sequence of its 12 Mb genome (Goffeau *et al.,* 1996). Despite the popularity and importance of *S. cerevisae,* the genome sequence revealed that only about one third of the 6,000 genes predicted by the sequence had been functionally characterized. Five years later, about 3,800 genes were characterized by genetic or biochemical means, and an additional 600 genes have homologues in other organisms that provide some indication of function (Kumar and Snyder, 2001; http://genome-www.stanford.edu/Saccharomyces), leaving about 1,900 genes that encode proteins of unknown function. Within the yeast scientific community, coordinated genome scale technologies have been employed to elucidate genome function as well as to establish information on regulatory networks within the organism. The *S. cerevisae* system is the best example of the application of post-sequencing genomic technologies, and illustrates the range and power of the technologies that can be applied to the functional characterization of the genome of any organism.

a. Genome-Wide Gene Deletions. An international consortium has undertaken the systematic deletion of each predicted coding region in the *S. cerevisae* genome followed by exhaustive phenotype analysis of each deletion strain (Winzeler *et al.,* 1999). The high rate of homologous recombination in yeast facilitates the precise deletion of the coding region of each gene from start codon to stop codon (Baudin *et al.,* 1993). Deletion cassettes are constructed that contain 45 bp of sequence identical

TABLE V

ONGOING AND COMPLETED GENOME SEQUENCING PROJECTS FOR FOOD-RELATED MICROBES

Species	Application	References
Fungi		
Saccharomyces cerevisiae	Beverage and bread production	Goffeau *et al.*, 1996; http://genome-www.stanford.edu/Saccharomyces
Kluyveromyces lactis	Food enzymes and dairy fermentations	Bolotin-Fukuhara *et al.*, 2000
Aspergillus niger	Food enzymes and plant fermentations	http://www.gene-alliance.com
Aspergillus oraziae	Soy fermentations	http://www.aist.go.jp/RIODB/ffdb/EST-DB.html
Aspergillus flavus	Toxigenic plant pathogen	http://www.tigr.org
Neurospora crassa OR74A	Model fungus, rots	http://www-genome.wi.mit.edu/
Magnaporthe grisea	Rice blast disease agent	http://www-genome.wi.mit.edu/
Bacteria		
Bacillus subtilis	Food enzymes and soy fermentations	Kunst *et al.*, 1997
Lactococcus lactis	Cheese and other dairy fermentations	Bolotin *et al.*, 1993; 2001
Lactobacillus plantarum	Intestinal isolate, various fermentations	http://www.wcfs.nl
Bifidobacterium longum	Intestinal isolate and probiotic activity	de Vos, 2001
Bifidobacterium breve	Intestinal isolate	de Vos, 2001
Lactobacillus acidophilus	Intestinal isolate and probiotic activity	de Vos, 2001
Streptococcus thermophilus	Yogurt and cheese fermentations	http://www.biol.ucl.ac.be
Lactobacillus bulgaricus	Yogurt fermentations	http://www.genoscope.fr
Bacterial pathogens		
Escherichia coli O157:H7	Food poisoning	Perna *et al.*, 2001; Hayashi *et al.*, 2001
Campylobacter jejuni	Food poisoning	Parkhill *et al.*, 2000a, http://www.tigr.org
Pseudomonas syringae pv. Tomato	Plant pathogen	http://www.tigr.org
Staphylococcus aureus	Food poisoning	Kuroda *et al.*, 2001; http://www.tigr.org
Listeria monocytogenes	Listeriosis and meningitis	http://www.tigr.org
Bacillus cereus	Food spoilage and poisoning	http//www.integratedgenomics.com

to the region upstream and downstream of the targeted gene and an antibiotic resistance cassette. Introduction into yeast and selection for the antibiotic resistance yields colonies deleted for the targeted sequence. In this way, a set of deletion strains has been constructed and made available to yeast investigators through distribution resources such as the American Type Culture Collection (ATCC).

The deletion cassettes include an additional element that has proved to be very valuable in functional analysis, a sequence tag upstream and downstream of the antibiotic resistance gene (Shoemaker *et al.*, 1996). These tags are unique for each gene deletion. They serve as markers or barcodes for each deletion strain. The upstream and downstream tags are flanked by common 18 bp PCR priming sites, with distinct sequences for the upstream and downstream tags, allowing amplification of the upstream tags or the downstream tags from a mixed population. The presence of the tags can be scored by hybridization of the PCR products to a glass slide microarray of complementary oligonucleotides, providing a method by which the surviving individual deletion strains can be analyzed in parallel (Shoemaker *et al.*, 1996).

The deletion strains have been extremely useful in characterizing deletion phenotypes (Winzeler *et al.*, 1999). In addition, the haploid deletion strains and some heterozygous diploid deletion strains have been used to study drug responses and drug targets (Giaever *et al.*, 1999; Chan *et al.*, 2000). Heterozygous deletion strains and the deletion barcode tags were used with the oligonucleotides array to identify strains with reduced fitness when competitively grown with a sublethal concentration of a given drug; strains showing reduced fitness were heterozygous at putative drug target loci. This methodology was validated in identifying a known tunicamycin target (Giaever *et al.*, 1999). Ooi and co-workers (2001) used the barcode tags to screen for components of the nonhomologous end-joining pathway in *S. cerevisiae.*

b. Expression Profiling. Microarray expression profiling has been used extensively to contribute to the functional analysis of the *S. cerevisiae* genome. For example, DNA glass slide microarrays can identify genes whose expression at the mRNA level are induced or repressed in response to an experimental treatment, or for genes differentially expressed during the sporulation process (Chu *et al.*, 1998), during the cell cycle (Cho *et al.*, 1998; Spellman *et al.*, 1998), and during the transition from anaerobic fermentation to aerobic respiration (Derisi *et al.*, 1997). In *S. cerevisae,* more than 400 genes were induced or repressed in response to a DNA alkylating agent (Jelinsky and Samson, 1999). These and other studies identified genes that function in common regulatory pathways or are modulated by specific regulatory factors (Holstege *et al.*, 1998; Madhani *et al.*, 1999).

A major expectation of expression profiling is that such data will be valuable for identifying the function of genes otherwise lacking any experimental sequence similarity based indication of function. The underlying assumption is that a function can be assigned if a strain deleted for that gene gives an expression profile identical to that generated by deleting a gene of know function. The two genes would likely be components of the same process or pathway. To identify gene function by this approach, expression data must be sorted and analyzed. By using clustering algorithms (for review, see Quackenbush, 2001), genes are grouped together that show similar patterns of expression and thus similar functions. The other component required to apply this kind of analysis genome-wide is to build a library of expression profiles that are readily searchable and comparable. The set of deletion strains provides a ready set of loss-of-function mutants for such a project. Hughes and co-workers (2000) have initiated a pilot project to develop a set of 300 expression profiles from yeast deletion mutants or from cultures treated with different chemical compounds. The expression is compared to wild-type or mock-treated cultures. Despite the lack of validation of the basic assumption that a mutation of any or many of the functions in a pathway will give a uniquely characteristic expression profile, this high-throughput compendium-based approach is promising particularly for identifying signaling pathway components and drug targets (Hughes *et al.*, 2000; Roberts *et al.*, 2000; Jelinsky *et al.*, 2000).

c. Two-Hybrid Studies. Yeast two-hybrid analysis is an established technology for the analysis of proteins that interact physically as protein pairs (Fields and Song, 1989). The method is based on the transcriptional activation of a reporter gene by the Gal4 transcription factor. In a typical assay, each of two potentially interacting proteins is fused to one of the two functionally distinct protein domains of the Gal4 protein, the DNA binding domain, or the activation domain. The protein fused to the DNA binding domain is termed the bait protein and that fused to the activation domain is termed the prey protein. When the two fusion proteins bind in *S. cerevisiae* containing a Gal4 reporter gene construct via the interaction between the bait and prey proteins, the Gal4 hybrid protein is functionally reconstituted and reporter gene expression is induced.

This assay has recently been implemented on a genome-wide scale (Fromont-Racine *et al.*, 1997; Tucker *et al.*, 2001). Two pilot-scale studies have been reported. Ito and co-workers (2000) cloned 5,700 annotated yeast ORFs as both bait and prey Gal4 fusions into strains of opposite mating type that were then organized into pools for mating. The resulting diploid strains were screened for Gal4-induced responses from three reporter genes. Analysis from the positive clones identified

175 independent interacting pairs from 430 matings. A variation to this approach was described by Uetz and Hughes (2000). A selected set of bait fusions was individually mated in a microtiter array to 6,000 strains containing prey fusions. Proteins scoring for Gal4 induction were identified by their position in the mating array. In a second screen, 6,000 strains containing bait fusions were each mated to a pool of 6,000 strains carrying prey fusions. The prey protein was identified by sequencing from the positive colonies. These screens identified 957 candidate interactions involving 1,004 proteins. Ito and co-workers (2001) reported the completion of their project cited above, identifying 4,549 two-hybrid interactions among 3,278 proteins. These studies show the promise of developing a protein–protein interaction map in *S. cerevisiae,* and the potential for applying this technology to other organisms.

d. Other Genome-Scale Technologies. Because the *S. cerevisiae* genome sequence has been available since 1996 and because of the importance of this organism as a model system, a range of additional genome-wide technologies are under development for yeast. The ability to spot DNA on glass slides as hybridization targets has allowed the exploration of DNA binding domains such as transcription factor binding sites in genomic DNA (Ren *et al.,* 2000; Iyer *et al.,* 2001). By crosslinking proteins to DNA, shearing the DNA, immunoprecipitating targeted proteins and bound DNA, preparing a labeled probe from precipitated DNA, and hybridizing to DNA spots on a glass slide, DNA binding sites for the immunoprecipitated protein can be identified. This approach has been used to identify binding sites for over 200 cell cycle transcriptional activators (Iyer *et al.,* 2001), 10 galactose-induced target genes, and 29 pheromone-induced target genes (Ren *et al.,* 2000).

Functional analyses of the proteins encoded in the yeast genome have been undertaken. Such studies are facilitated by the availability of purified proteins that correspond to the entire protein complement of the organism. Overexpression constructs of the *S. cerevisiae* ORFs have been prepared with a carboxyterminal V5 epitope and polyhistidine tags (Heyman *et al.,* 1999) or glutathione-S-transferase (GST) to allow rapid purification. Martzen and co-workers (1999) purified GST-tagged proteins from pools of strains that expressed GST-tagged constructs and screened the pools for new biochemical activities. Genome-wide sets of purified proteins allow the development of protein microarrays that can be processed for functional assays, e.g., secreted and membrane associated gene products (Maximilian *et al.,* 2000). Printing and other formats that preserve the functional activity of the proteins are under development (Arenkov *et al.,* 2000; MacBeath and Schreiber, 2000; Zhu *et al.,* 2000). An integrated analysis of microarray expression profiling

and quantitative proteomics was used to study pertubation in the yeast galactose utilization pathway (Ideker *et al.,* 2001). This approach identified 997 mRNAs responding to 20 systematic perturbations. The study reported that 15 of the 289 detected proteins were posttranscriptionally regulated, an observation that could only be made by protein analysis.

As a last topic for the genome-wide study of function of the *S. cerevisiae* genome, a study by Raamsdonk and co-workers (2001) employs a process that they term *metabolomics.* For many of the systematically constructed deletion mutants, the deletion strain shows no overt phenotype in altered growth rate. By measuring the intracellular concentration of metabolites, phenotypes for deleted proteins active in metabolic regulation can be revealed. Metabolite quantitation can reveal the site of action of a deleted gene in a metabolic network and can reveal function when strains deleted for an unknown gene are compared to those deleted for known genes. The authors demonstrate the approach using deletion strains for several genes for glycolytic enzymes, and measuring the levels of glycolytic metabolites using electrospray mass spectrometry, nuclear magnetic resonance spectroscopy, and Fourier-transform infrared spectroscopy. This approach may prove to be extremely useful for engineering strains to maximize the production of metabolites in industrial applications.

2. Bacteria Used in Food Production

Bacteria used in food production are all Gram-positive. Genome sequencing and analysis projects have been undertaken for many of these organisms (Table V). In addition, genetic tools are being developed that will allow the efficient use of the genome data in strain development. An expression system has been developed for production of proteins in low-GC Gram-positive bacteria (de Vos *et al.,* 1997). Expression systems have also been developed for lactic acid bacteria (de Vos, 1999) and genetic systems for thermophilic lactic acid bacteria (Delcour *et al.,* 2000). The *B. subtilis* genome was the first of these food-related bacteria to be sequenced, and the *Lactococcus lactis* genome sequence was recently reported (Table II). The *L. lactis* genome is half the size of the *B. subtilis* genome but the genes involved in basic information processes are similar. The *B. subtilis* genome analysis revealed more σ factors and two-component regulatory proteins than *L. lactis*—52 vs 11 in *L. lactis*—probably reflecting the differences in life styles of the two organisms. *L. lactis* resides in a stable nutritional environment such as milk, while *B. subtilis* is a metabolically versatile soil bacterium.

Lactic acid bacteria play a significant role in the production of fermented foods that are estimated to comprise a fourth of the human diet. For example, many strains of *Lactobacillus* are used in traditional

mozzarella cheese production. Demonstrated benefits from the consumption of products containing *L. acidophilus* (see section below on Probiotics) include the control of intestinal infections, improved lactose digestion, controlled serum cholesterol levels, and anticarcinogenic activity (Hooper and Gordon, 2001). The plasmids from *L. lactis* may provide a framework for the design of novel plasmids to be incorporated into nutrition improvement programs for the dairy industry.

The functional genomic analysis of food-relevant bacteria is not nearly so developed as for *S. cerevisiae,* and *B. subtilis* is the bacterium for which functional analysis is the most advanced. For *B. subtilis,* two studies using transcript profiling have been reported. One is a transcript analysis through the time course of sporulation (Fawcett *et al.,* 2000) and the other a response to catabolite repression (Moreno *et al.,* 2001). The latter revealed a major regulatory role for CcpA, a major control protein present in lactic acid and other low-GC Gram-positive bacteria (Van den Bogaard *et al.,* 2000). A coupled proteomics and transcript study has explored bottlenecks in the production of heterologous proteins in *B. subtilis* (Jurgen *et al.,* 2001). In *L. lactis,* transcript and protein studies have been undertaken to analyze metabolic flux and stress response (Wouters *et al.,* 2000; Even *et al.,* 2001). A database of *B. subtilis* transcription factors and promoters is currently available (Ishii *et al.,* 2001; http://elmo.ims.u-tokyo.ac.jp/dbtbs/).

3. Probiotics

Foods containing living microorganisms can add to the functional components of the food. These organisms are termed *probiotics.* The probiotic products traditionally incorporate intestinal species of *Lactobacillus* because of their long history of safe use in the dairy industry and their natural presence in the human intestinal tract. *Bifidobacterium* spp. have more recently been added to foods for probiotic purposes, probably encouraged by the discovery of their consistent presence as part of the normal microbiota of the human intestine. Probiotics are presumed to have a beneficial influence on the intestinal ecosystem, which in turn may provide protection against gastrointestinal infections and inflammatory bowel diseases. The desirable effects on human health include antagonistic activity against pathogens, anti-allergic effects, and other effects on the immune system.

Bacteria that are found in the human intestinal tract have potential for use as probiotics (Vaughan *et al.,* 1999; Verrips, 2001), and the genomes of these species may assist in an understanding of why and how certain intestinal and lactic acid bacteria have beneficial properties such as the stimulation of the immune system (Scheinbach, 1998; Isolauri *et al.,* 2000), the conversion of carcinogenic compounds into harmless

products, and the production of beneficial peptides (Hate *et al.*, 1996). In the future, screening of metabolites from microorganisms will identify a range of components in food that will be of benefit to humans.

4. *Genomics of Foodborne pathogens*

Complete genome sequences have been determined for several foodborne pathogens (Table V). These sequences will provide tools for developing diagnostic reagents, and for drug and vaccine development. The availability of these genome sequences will also be useful for developing food surveillance protocols, and for optimization of food processing and storage conditions to minimize the potential of food contamination with these organisms.

D. ENVIRONMENTALLY PROBLEMATIC ORGANISMS

Some sequenced nonpathogenic species are associated with problems in the environment. Their genome sequences will lead to greater understanding of ways that can be used to control these environmental pathogens. One prominent example is *Archaeoglobus fulgidus* (Klenk *et al.*, 1997), a sulfate reducer that has been associated with the contamination of oil wells. The organism also serves as a model for understanding corrosion and the souring of hydrocarbon reserves. Inhibition of certain essential biochemical pathways can disrupt its normal metabolism, and therefore limit its detrimental effects in the environment. The genome sequence revealed numerous pathways that could serve as candidates for initial inhibitory studies (Klenk *et al.*, 1997).

E. NITROGEN METABOLISM IN WASTEWATER TREATMENTS

Microbes play a crucial role in the nitrogen cycle of the earth. Microbial nitrogen fixation converts atmospheric nitrogen gas to ammonia for biological assimilation. Microbial denitrification, the process of converting nitrate to nitrous oxide or nitrogen gas, and oxidation of ammonia are the major pathyways that convert nitrogen compounds back to atmospheric nitrogen. Denitrification allows microbes to use alternative electron acceptors to gain energy under conditions of oxygen limitation (Zumft, 1997). These microbial activities are carried out by a broad assortment of microorganisms that range from archaea to proteobacteria, to Gram-positive bacteria, and to fungi. Considerable progress had been made in the understanding of the physiology of microbial metabolism of inorganic nitrogen compounds (Ye and Thomas, 2001). A partial listing of sequenced microorganisms containing nitrogen cycle pathways is provided in Table VI.

TABLE VI

MICROORGANISMS CONTAINING NITROGEN CYCLE PATHWAYS FOR WHICH A GENOME SEQUENCING PROJECT IS COMPLETED OR UNDERWAY

Species	Pathways or enzymes	References
Methylomonas sp. 16a	Ammonia oxidation	Ye and Thomas (2001)
Nitrosomonas europaea	Ammonia oxidation Dissmilatory nitrite and nitric oxide reductases	http://spider.jgi.psf.org/ JGI_microbiol/html
Neisseria meningitidis	Dissmilatory nitrite and nitric oxide reductases	Tettelin *et al.* (2000); Parkhill *et al.* (2000b)
Synechocystis sp. PCC6803	Cytochrome *b* nitric oxide reductase	Kaneko *et al.* (1996)
Bacillus subtilis 168	Dissimilatory nitrate reduction to ammonia	Kunst *et al.* (1997)
Pseudomonas aeruginosa PAO1	Denitrification	Stover *et al.* (2000)
Azoarcus tolulyticus Tol-4	Denitrification	Ye and Thomas (2001)
Paracoccus denitrificans ATCC 19367	Denitrification	Ye and Thomas (2001)
P. denitrificans strain SANVA100	Denitrification	Ye and Thomas (2001)
Rhodobacter sphaeroides	Denitrification, nitrogen fixation	http://spider.jgi.psf.org/ JGI_microbiol/html
Methanococcus maripaludis	Methogen, nitrogen fixation	http://www.tigr.org/tdb/mdb/ mdbinprogress.html
Nostoc punctiformes	Photosynthetic nitrogen fixer	http://www.tigr.org/tdb/mdb/ mdbinprogress.html
Pyrobaculum aerophilum	Hyperthermophilic, organotrophic, nitrate reducing archaeon	http://www.tigr.org/tdb/mdb/ mdbinprogress.html
Sinorrhizobium meliloti	Nitrogen fixation	http://www.tigr.org/tdb/mdb/

A microarray expression analysis study of *B. subtilis* was conducted to investigate the changes in mRNA levels from 4,020 genes when *B. subtilis* was grown under anaerobic conditions with nitrate or nitrite as the electron transport receptor (Ye *et al.,* 2000). Of the several hundred genes that were induced or repressed, the most highly induced genes under these conditions were *narGHIJ* and *narK,* both of which are involved in dissimilatory nitrate reduction. Other genes were involved in carbon metabolism, electron transport, iron uptake, antibiotic production, and stress response, and a significant number of genes were of unknown functions. Dissimilatory nitrate reduction is regulated by the two-component regulatory proteins ResDE. The *B. subtilis* strains

mutant at *resDE* cannot grow on nitrate or nitrite under anaerobic conditions. Additional expression profiling was performed in a *resDE* mutant strain during aerobic exponential growth and under oxygen-limiting conditions. These data provide a great deal of suggestive information on genes related to anaerobic growth using nitrate and nitrite as electron acceptors and on the regulation of the genes involved in these processes.

The need to reduce the eutrophication of estuaries has resulted in an effort to reduce the nutrient content of the discharge from wastewater treatment facilities (Bricker *et al.,* 1999). Nitrogen-containing compounds, especially ammonia, nitrate, and nitrite, are major contributors to the eutrophication process. Industrial and municipal wastewater treatment facilities meet the discharge limits for these compounds by converting organic and inorganic nitrogen to atmospheric nitrogen gas using microbes. Commercial applications have used the ammonia oxidation and dissimilatory denitrification physiologies for existing processes. New technologies are under development to improve wastewater processing. These approaches include aerobic nitrification (Gradly and Lim, 1980; Caulet *et al.,* 1998), ammonia oxidation coupled to nitrate reduction (Strous *et al.,* 1999; Jetten *et al.,* 1998), and the use of denitrifying bioreactors (Dos Santos *et al.,* 1998; Verstaete and Phillips, 1998; Beun *et al.,* 2000). These approaches coupled with the detailed view of the nitrogen metabolic pathways revealed by genomics should provide for the rapid advancement of these technologies.

F. Ecotoxicity Assessment

DNA hybridization arrays can serve as high-throughput tools to assess chemical contaminants in soil (Fredrickson *et al.,* 2001). By spotting sequences of microbial genes that are induced by specific soil contaminants by resident soil microorganisms, the expression profiles of the aggregate soil microorganisms can be used to detect and classify the contamination. While the analysis hardware and software is directly transferable from the more traditional glass slide microarray applications, questions remain about the environmental relevance of microbiological mRNA level responses. The increasing quantities of genomic information available on environmental organisms makes the development of this technology more feasible.

G. Industrial Microbial Production and Biotechnology

Aspergillus niger is a fungal microbe of great industrial importance. This mold is used extensively in the production of citric acid and in the

production of several enzymes such as amylases, pectinases, and proteases (Godfrey and West, 1996). The 30 Mb genome of this organism has been sequenced by an industrial genome sequencing contract from the DSM N.V. (Amsterdam: DSM.ASX), to the German genomics consortium Gene Alliance (http://www.gene-alliance.com). Another proprietary sequence is available from Integrated Genomics, Chicago, IL. For the production of enzymes and other biological products, *E. coli* and *S. cerevisiae* are the two most commonly used organisms for which there is a complete genome sequence. While these organisms could benefit by strain improvement projects that would leverage the genomic information available for these organisms to improve industrial productivity, the existing fermentation systems have already reached a level of maturity that would discourage extensive genomic-based strain modification.

On the other hand, the sequence of *Caulobacter crescentus* was conducted largely to support the use of this organism as a model system for cell cycle control. Indeed, a report on the genes regulated in a cell cycle dependent manner by glass slide microarray expression profiling was reported in 2000 (Laub *et al.,* 2000). This study used the prepublication genome sequence to bring genomic data to this endeavor. Even before the publication of the sequence, *C. crescentus* was being developed both as a bioremediation candidate organism and as a heterologous protein expression system. Since the sequence was published in early 2001 (Nierman *et al.,* 2001), the pace of work on this application has increased dramatically.

The paracrystalline surface (S)-layer of *C. crescentus* completely envelops the cell by forming a geometric arrangement of a single protein monomer, the RsaA protein, on the outer membrane (Smit *et al.,* 1992). This protein has been exploited for surface display of peptides and proteins, and for heterologous protein production. About 10–12% of the cell's protein synthetic capacity is devoted to S-layer production, covering the cell with about 40,000 RsaA monomers on the cell surface. Bingle and co-workers (1997a, 1997b) first demonstrated the use of this system to display large quantities of epitopes on the cell surface and to serve as a fusion system for the synthesis and secretion of foreign proteins into the culture medium. The alternative of surface display or secretion is selected by the point of insertion of the heterologous sequence into the *rsa*A gene. The secretion mechanism requires a proteolytic C-terminal cleavage of the RsaA protein freeing the heterologous protein as a fusion product.

This system has recently been used to produce a vaccine against infectious hematopoietic virus for Atlantic salmon (Simon *et al.,* 2001), and to produce a vaccine candidate protein for *Pseudomonas aeruginosa* (Umelo-Njaka *et al.,* 2001). The availability of the genome sequence has

been coupled to a transposon mutagenesis strategy to identify genes and proteins involved in the attachment of the S-layer via an O antigen polymer attached to the LPS (lipopolysaccharide; Awram and Smit, 2001). This so-called S-LPS is required for attachment of the S-layer to the outer membrane. Understanding the molecular details of this interaction and other details of the biology through the use the genome data may further aid in the development of *C. crescentus* as a heterologous expression system for biotechnology applications.

H. Metabolic Engineering—Strain Improvement

An exclusive reliance on reductionist biological studies has now shifted to studies of entire biological systems. The tools for these studies, genome sequencing and analysis, mRNA expression profiling, proteomics, metabolite flux analysis, high-throughput protein–protein interaction studies, deletion analysis, and molecular genetic manipulation systems, etc., are in various stages of development and implementation. This refocusing of basic biological studies is both inevitable and appropriate.

Metabolic engineering uses genetic tools to improve industrial organisms (Bailey, 1991; Stephanopoulos *et al.*, 1998). Genetic manipulations targeting strain improvement have been conscious, of necessity, of biological complexity. Yet, here also, the success of the process has been due to simplifying assumptions. The basic assumptions are that the function of a single gene product is known, that it does what it does and has no other effect on the biochemical and regulatory networks that operate within a cell. A derivative assumption is that metabolic pathways operate such that a single biochemical conversion catalyzed by a single enzyme is the rate-limiting step for the pathway. New very successful tools for metabolic engineering include rational design, and evolutionary or combinatorial approaches for engineering pathways in microbes (Rohlin *et al.*, 2001). The rational design strategy is the one that will benefit most by advances in genomic technology and information. Rational design developments in the metabolic engineering of carotenoid and polyketide biosysthesis is reviewed to illustrate these approaches.

Industrial biosysthesis of carotenoids are of interest due to the increasing potential of these compounds as antioxidants and food additives. Gene clusters for carotenoid biosynthesis have been identified in various microorganisms (Sandmann *et al.*, 1999; Barkovich and Liao, 2001), most notably in the completed genome sequence of *Streptomyces avermitilis* (Omura *et al.*, 2001). Carotenoids are synthesized from a C20 compound, geranylgeranyl diphosphate (GGPP). The identification of

an isoprenoid precursor synthesis pathway in *E. coli* and the engineering of carotenoid synthesis in the yeast *Candida utilis* (Shimada *et al.*, 1998) has led to the introduction of heterologus carotenoid gene clusters in *E. coli* to serve as a rational design for a whole carotenoid biosynthesis network in *E. coli*. After the introduction of the heterologous carotenoid gene cluster, successive rate-limiting steps in the biosynthetic pathway were identified and eliminated by the overexpression of additional heterologous enzymes in the synthetic pathway (Albrecht *et al.*, 1999; Wang *et al.*, 1999; Matthews and Wurtzel, 2000; Wang *et al.*, 2000; Albrecht *et al.*, 2001; Kim and Keasling, 2001) all leading to increased yields of carotenoids. Farmer and Liao (2000) expanded the approach beyond rate-limiting step management by installing a novel control loop in the metabolic pathway that balances cell growth and protein expression. The new circuit not only improved the yield of the carotenoid lycopene but also overcame growth inhibition under protein overproduction. The same group overexpressed the enzyme phosphoenolpyruvate synthase as a way of shunting excess pyruvate to glyceraldehyde-3-phosphate, thus balancing these two precursors of the engineered carotenoid pathway, and significantly increasing carotenoid production (Farmer and Liao, 2001). A limitation of rational design is the lack of knowledge of genes in the pathway and of the range of enzymes available from heterologous sources. Additional steps in the *E. coli* pathway leading to the synthesis of GPP have been identified (Rohdich *et al.*, 2000; Campos *et al.*, 2001), as have been some of the steps leading to the biosynthesis of taxol that remains an attractive target for microbial biosysthesis development (Walker *et al.*, 2000; Walker and Croteau, 2000; Walker and Croteau 2001). Genes encoding enzymes involved in the synthesis of novel carotenoids were also identified in *Corynebacterium glutamicum* (Krubasik *et al.*, 2001a, 2001b).

A second microbial biosynthetic process of great interest for industrial microbiology is the synthesis of polyketide compounds as candidate pharmaceuticals. The polyketide synthesizing enzymes, polyketide synthases (PKS), are encoded in modular clusters of genes whose linear organization of functional subunits catalyze specific steps in polyketide chain elongation. A single organism can contain multiple PKS clusters encoding enzymes that produce different polyketide compounds. The primary goal of molecular engineering is to employ combinatorial techniques to diversify polyketide-synthesizing enzymes to produce a large variety of bioketides for screening for biological activity. Once a desired polyketide is identified, production yield becomes an important consideration. Several recent reports have used natural or engineered constructs to synthesize numerous polyketide variant compounds (Gokhale *et al.*, 1999; McDaniel *et al.*, 1999; Olano, *et al.*, 1999; Shen *et al.*, 1999;

Xue *et al.,* 1999; Burson and Khosla, 2000; Xue and Sherman 2000; Xue and Sherman, 2001). Engineering polyketides pathways to achieve high yields can be accomplished by strain development and by increasing the availability of precursor compounds. As an example of strain development, Tang and co-workers (2000) produced epothilone, a candidate taxol successor that is not a polyketide but a sterol, in *Streptomyces coelicolor,* a strain that grows 10-fold faster than the natural producer, *Sorangium cellulosum.* Cropp and co-workers (2000) supplied a gene cluster for the synthesis of cyclohexanecarboxylic acid (CHC) to *S. avermitilis,* which is used to produce the polyketide, doramectin. *S. avermitilis* requires CHC as a precursor to this compound that is normally supplied in the producing medium. The engineered strain does not require exogeneous CHC.

The successes described for the metabolic engineering of microbes have been accomplished in spite of our inability to model the complexity of biological systems. Genome sequencing and analysis has revealed that a substantial fraction of the genes even in well-studied organisms code for proteins whose function is unknown. About half of these genes have homologs in distantly related microbes. Such evolutionary persistence indicates that those genes are likely to be performing essential functions. Related to this is the observation that many single gene mutations do not affect phenotype or their influence on phenotype is not obvious. The phenotype screens undertaken in conjunction with the systematic deletion of each annotated ORF in the *S. cerevisiae* genome have provided many examples of genes without obvious phenotypes (Winzeler *et al.,* 1999). Microarray expression profiling has also revealed an unanticipated level of complexity. In *C. crescentus,* the listing of cell cycle regulated genes was expanded from a set of about 70 identified through classical studies to over 550, by monitoring whole genome expression mRNA expression levels at 15-min time points through the 150-min cell cycle in synchronized cells (Laub *et al.,* 2000; Nierman *et al.,* 2001). A loss of function mutation in a control gene, *ctr*A, resulted in the altered expression of 84 genes while a gain of function mutation in the same gene altered the expression levels of 125 genes. Similar levels of complexity have been revealed by expression profiling and proteomics in *B. subtilis.* In the sporulation process, over 580 genes were found to be at least 3-fold dependent on SpoOA and/or σ F regulatory proteins (Fawcett *et al.,* 2000). Several hundred genes were induced or repressed in cells grown anaerobically relative to those grown aerobically (Ye *et al.,* 2000). The repertoire of known glucose repressible genes in *B. subtilis* was expanded from 22 to 66 in a combined microarray expression profiling–proteomics study (Yoshida *et al.,* 2001).

So what can genomic information and tools bring to microbial strain improvement for industrial processes? The overproduction of proteins, and of primary or secondary metabolites, is a complex process. For the core activities of cells, there appears to be a redundancy and robustness toward preserving the metabolic homeostasis that is observed when genes are knocked out or heterologous pathways are introduced, and no significant effect on phenotype is observed. The successful development of improved strains requires knowledge of physiology, pathway regulation and control, and efficient screening procedures. Genome sequence and analysis of an organism reveals the entire gene complement. This information and the subsequent functional analysis will ultimately establish metabolic pathyways and all of the component enzymes of relevance to any metabolic engineering project. Microarray expression profiling, proteomics analysis, and metabolome flux analysis will further the functional analysis of genes and will additionally reveal information on the pathway control components and mechanisms. As strain engineering progresses, these tools will help evaluate the consequence of implementing a genetic alteration beyond simple quantitation of an outcomes such as product yield. Genomic and proteomic tools have potential to greatly increase the productivity of industrial strains with greatly reduced development time lines.

VI. Conclusions and Perspectives

The triumph of modern molecular biology has been the accumulation of information derived from an intensive analysis of the individual components of complex biological systems. Biological complexity necessitated this simplifying approach. In this reductionist paradigm there has been no role for systematic quantitative methods for building an understanding of the complexities of biological system properties. We have accumulated an impressive inventory of molecular mechanisms, components, knowledge of metabolic pathways and regulatory networks from reductionist biological research, without making parallel advances toward a conceptual or theoretical framework of living systems. While this approach was essential historically to avoid being buried by the complexity of biology, the severe limitation of the approach was revealed in the observation that greater than 30% of the genes, even in extensively studied organisms, code for proteins of unknown function. With the new technologies that are available and model systems such as yeast where community efforts to completely assess this genome have been successful, it is anticipated that tremendous gains soon will be made in the field of applied microbial genomics.

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