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MICROBIAL PHYSIOLOGY

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Physiological Responses of *Bacteroides* and *Clostridium* Strains to Environmental Stress Factors

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I. Introduction

The two genera *Bacteroides* (non-spore-forming Gram-negative bacilli) and *Clostridium* (endospore-forming Gram-positive bacilli) comprise important groups of obligate anaerobic bacteria. Since bacteria from both genera are

causative agents of disease in humans and animals (Dowell and Lombard, 1981; Hill, 1981), they are among the most intensively studied of the obligate anaerobes.

Within the obligate anaerobic bacteria, members of the genus *Bacteroides* constitute the single most important group of human pathogens and usually occur as mixed infections (Bartlett, 1983; Salyers, 1984). The unique patterns of resistance to antibiotics displayed by these organisms make them difficult to treat (Bawdon *et al.*, 1982). Non-pathogenic strains of *Bacteroides* are the common inhabitants of the alimentary tract of warm-blooded animals and account for a major portion of the bacteria in certain regions of the gut (Macy, 1981). Their importance is emphasized since they inhabit parts of the gastro-intestinal tract where there is little or no host digestive capability (e.g. mouth, caecum, colon, rumen) (Gorbach and Levitan, 1970; Hungate, 1966, 1977).

Since the early days of bacteriology, the clostridia have been known to cause diseases in man and domestic animals. The ability of many species to produce potent toxins has given these organisms special significance in the field of public health (Crowther and Baird-Parker, 1983). The clostridia are widely distributed in soils and aquatic sediments (Slepecky and Leadbetter, 1984). They have been reported as inhabitants of the alimentary tracts of both ruminants and non-ruminants, although their role and importance in these environments still remains uncertain. The clostridia, which have diverse fermentative abilities (Nakhmanovich and Shcheblykina, 1959; Barker, 1961; Stadtman, 1973) and produce a variety of organic acids and solvents (Spivey, 1978; Zeikus, 1980; Zeikus, 1980, 1983; Rogers, 1985), have great potential in the fields of biotechnology and industrial microbiology.

In spite of the importance of obligate anaerobes in general, and *Bacteroides* and *Clostridium* strains in particular, studies on these bacteria have tended to lag behind those on aerobes. This disparity is most apparent in the fields of molecular biology and genetics. The major reason for the neglect of the anaerobic bacteria has been the technical difficulties of working with these organisms. However, modern technical developments have overcome many of the difficulties associated with the study of anaerobes. Furthermore, molecular biologists are beginning to appreciate the importance of the genetic diversity of bacteria, and as a result there is a rapidly growing interest in anaerobic bacteria.

Cultures in a state of exponential growth, produced under near optimum conditions, are the stock in trade of the experimental microbiologist (Mandelstam, 1971). They are easily produced and the results obtained with them are fairly reproducible from day to day. It is easy to forget that, in the natural state, periods of exponential growth must be rare and short. Most of the time, bacteria are subjected to environmental stresses and are in a state of partial or total starvation where little or no growth can occur.

A field of research that has resulted in major advances in the molecular biology of aerobes involves their response to environmental stress. Studies on nutrient depletion in bacteria have resulted in an understanding of the stringent response and of the way in which metabolism of the cell is regulated and co-ordinated by enzyme induction and repression and feedback inhibition. Similar studies on the effect of physical stress factors such as radiation and heat have facilitated an understanding of how organisms are able to withstand and repair damage resulting from such environmental stresses. For example, studies on stress induced by UV irradiation and UV-induced phage reactivation have played an important role in the characterization of DNA repair, recombination and mutagenic systems in *Escherichia coli*, *Salmonella typhimurium* and other Gram-negative aerobes (Bernstein, 1981; Walker, 1984). These pioneering studies with UV-irradiation have formed the basis of recent comparative molecular biology studies involving responses to heat, radiation, oxygen, oxidizing agents, methylating agents and ethanol (Lee *et al.*, 1983; Varshansky, 1983; Walker, 1984).

In the endospore-forming Gram-positive bacilli the organization and regulation of metabolism during vegetative growth is similar to non-spore-forming bacteria, but these organisms are able to exhibit the additional response of producing a highly resistant and dormant spore in response to environmental stress. This serves as a means of preserving viability through long periods when the environmental conditions are inimical to growth. Extensive studies of spore formation and germination in *Bacillus subtilis* and a number of other species of *Bacillus* have enhanced our understanding of the regulation of differentiation in prokaryotes (Halvorsen, 1962; Schaeffer *et al.*, 1965; Vinter, 1969; Dawes and Mandelstam, 1970; Young and Mandelstam, 1979).

Molecular biology studies in prokaryotes have been facilitated by the extensive work on the physiological responses of the aerobic bacteria, in particular *E. coli* and *B. subtilis*, to stress factors. Before similar comparative molecular biology studies can be undertaken in anaerobes it is necessary that the basic physiological responses of anaerobes to environmental stress are characterized. In view of the importance of the genera *Bacteroides* and *Clostridium*, and the obvious analogy between *Bacteroides* and the enterobacteria (*E. coli* in particular) and between *Clostridium* and *Bacillus*, we have confined our review to these two anaerobic genera. It is often tempting to regard the responses of *E. coli* and *Bacillus* spp. as typical for non-spore-forming Gram-negative and spore-forming Gram-positive bacteria, respectively. In this review we have emphasized the diversity of bacterial responses to selected stress factors and hope that it will provide a basis for future comparative molecular biology studies.

With the exception of the response of anaerobes to oxygen toxicity,

information regarding the other types of environmentally imposed stress is limited. The environmental stresses that have been chosen for review are those that affect macromolecular synthesis and induce specific gene products in Gram-negative non-spore-forming bacteria, and factors that regulate cellular differentiation and related stationary-phase events in Gram-positive spore formers.

II. Physiological responses of *Bacteroides fragilis* to Stress Factors

The stress factors that have been chosen for emphasis in this section of the review involve agents that, among other activities, are known to damage DNA and result in the induction of specific gene products. Studies on the responses of enterobacteria to DNA-damaging agents have contributed greatly to an understanding of recombination, DNA repair and mutagenesis in Gram-negative aerobes. Although data are still limited at present, evidence suggests that there may be novel and interesting features regarding chromosomal recombination, DNA repair systems and mutagenesis in *Bacteroides*.

Although non-plasmid transfer of antibiotic resistance (conjugative transposition) has been described in *Bacteroides fragilis* (Mays *et al.*, 1981, 1982; Tally *et al.*, 1981; Smith *et al.*, 1982), homologous chromosomal recombination has not been reported for *Bacteroides*. Recombination between a phage genome and the *Bacteroides* chromosome also has not been demonstrated, and despite extensive searches by a member of our laboratory and by Booth *et al.* (1979), lysogeny and transduction have not been shown. Pseudolysogeny appears to be common in *Bacteroides* spp. (Keller and Traub, 1974; Burt and Woods, 1977), but true lysogeny seems to be rare or absent. Silver *et al.* (1975) reported phage-like particles which were observed in thin sections of *B. fragilis* cells associated with larger spherical bodies 300 nm in diameter. Phage heads were always present in association with these bodies, and the intact phages appeared to belong to Bradley's group B. These structures were presumed to be temperate phages although no sensitive strain was found and it was not possible to induce the phage. Lysogenic phages were reported by Prévot *et al.* (1970) and Nacesu *et al.* (1972), but pseudolysogeny was not ruled out in these earlier reports and more recent attempts to isolate prophages have been unsuccessful.

Studies on the mutagenesis of *B. fragilis* and other obligate anaerobes indicate that they are difficult to mutate (Van Tassell and Wilkins, 1978; Droffner and Yamamoto, 1983). Droffner and Yamamoto (1983) investigated the effect of stringent anaerobic conditions on mutagenesis in *S. typhimurium*. Error-prone repair was not expressed in *S. typhimurium* grown under strictly anaerobic conditions. This was due to the lack of expression of *recA* and

recBC functions. They proposed the hypothesis that the anaerobic environment might establish a physiological condition causing the lack of expression or malfunction of the cellular activities necessary for error-prone repair activity in facultative anaerobes.

In an excellent and comprehensive review on the responses of *E. coli* to DNA damage, Walker (1984) identified three regulatory networks that can be induced by exposure to DNA-damaging agents. Ultraviolet radiation and nalidixic acid induce the SOS response which involves the induction of at least 15 different genes and is regulated by the *recA* and *lexA* genes. The heat-shock response is induced by heat and ethanol and at least 13 genes regulated by the *htpR* gene are induced. Methylating agents induce an adaptive response involving at least two genes regulated by the *ada* gene. Studies by Demple and Halbrook (1983) suggest that there may be a fourth regulatory system induced by oxidizing agents and concerned with the repair of oxidative DNA damage. In view of the toxicity of oxygen to anaerobes, the responses of *Bacteriodes* spp. to oxygen and its radicals will be considered first.

A. OXYGEN AND HYDROGEN PEROXIDE

Oxygen is toxic to all living organisms (Haugaard, 1968; Gottlieb, 1971) but bacteria differ in their sensitivity to oxygen and a continuous spectrum of oxygen tolerance, from the most strict anaerobe to the least sensitive hyperaerobe, can be discerned (Morris, 1976). The topic of oxygen toxicity has been reviewed by Fridovich (1975). Morris (1975, 1976), Rolfe *et al.* (1978), Hassan and Fridovich (1979), Privalle and Gregory (1979), Gregory and Dapper (1980), Fee (1982) and Singh and Singh (1982).

Oxygen most commonly exists in the inactivated ground state which is called triplet oxygen ($^3O_2$). Singlet oxygen, which is a higher energy form of oxygen, is very reactive and is one of the forms of oxygen that is toxic to living organisms. The reduction of oxygen to water requires the addition of four electrons. The reduction of $^3O_2$ by a single electron generates the superoxide radical (O_2^-). This radical is highly reactive and can damage DNA by causing single-strand breaks (Moody and Hassan, 1982) either directly or by the secondary production of other radicals (peroxyanion) that attack DNA. Superoxide radicals also cause oxidative destruction of lipids and other biochemical components. The superoxide radical can disproportionate spontaneously to form H_2O_2 and singlet oxygen, and can interact with iron chelates and H_2O_2 in a catalytic cycle to generate the hydroxyl radical (OH^0) which is the most reactive of the various oxygen radicals. Hydroxyl radicals are capable of indiscriminately reacting with and damaging nucleic acids (Cadet and Teoule, 1978; Ito, 1978; Lynch and Fridovich, 1978; Totter, 1981).

They abstract hydrogen atoms from saturated carbon atoms of both purines and pyrimidines at a constant rate close to the limit set by diffusion.

Although anaerobes differ markedly in their sensitivity to oxygen (Fredette *et al.*, 1967; Loesche, 1969; Tally *et al.*, 1975; Walden and Hentges, 1975; Hoshino *et al.*, 1978; Rolfe *et al.*, 1977, 1978), the presence of molecular oxygen inhibits growth of all obligate anaerobes (Loesche, 1969; O'Brien and Morris, 1971; Walden and Hentges, 1975; Hoshino *et al.*, 1978; Rolfe *et al.*, 1978; Wimpenny and Samah, 1978; Samah and Wimpenny, 1982) and prolonged exposure to oxygen invariably leads to cell death. Obligate anaerobes were divided into two groups by Loesche (1969): strict anaerobes, which are unable to grow on plates when there is more than 0.5% oxygen in the atmosphere, and moderate anaerobes, which can grow on plates in the presence of 2–5% oxygen. Certain factors may, however, affect the classification of anaerobes into the different groups: several strict anaerobes may become more aerotolerant after two or three subcultures (Willis, 1969), and cells in the exponential growth phase in batch culture are usually more oxygen-sensitive than cells in the stationary phase (Morris, 1976; Jones *et al.*, 1980a). Loesche (1969) reported that *B. fragilis* can withstand exposure to air for periods of at least 60 minutes without loss of viability, and Onderdonk *et al.* (1976) reported that the effect of oxygen on *B. fragilis* cells growing in chemostat culture is only bacteriostatic in nature. Jones and Woods (1981) isolated a *B. fragilis* Bf-2 strain which was maintained in various aerobic holding solutions for 1 to 6 hours without loss in viability.

The survival of anaerobes in air may be assisted by oxygen free radical scavenging enzymes. McCord and Fridovich (1969) were the first to report the existence in bacteria of superoxide dismutase (SOD) enzymes. Superoxide dismutase very efficiently catalyses the safe dismutation of superoxide radicals to yield hydrogen peroxide and triplet oxygen (Morris, 1976). This enzyme also can effectively quench singlet oxygen (Fridovich, 1974), and in *E. coli* cells a definite correlation seems to exist between oxygen sensitivity and a lack of SOD production in mutants of this organism (Hassan and Fridovich, 1979).

In 1971, McCord *et al.* proposed the superoxide dismutase theory of obligate anaerobiosis in which they stated that the lack of this important enzyme accounted for the aero-intolerance of anaerobes. The production of this enzyme is, however, not restricted to aerobic and aerotolerant bacteria and a few years after McCord *et al.* (1971) proposed their theory, SOD was discovered in several aero-intolerant and strictly anaerobic bacteria. These bacteria often contain at least two electrophoretically distinct SOD enzymes (Hewitt and Morris, 1975; Ashley and Shoesmith, 1977; Gregory *et al.*, 1978). The aero-intolerant bacteria in which the presence of SOD has been demonstrated include *Chlorobium*, *Desulfovibrio*, *Clostridium* (Morris, 1976) and several *Bacteroides* species (Carlsson *et al.*, 1977; Gregory *et al.*, 1977a;

Tally *et al.*, 1977; Rolfe *et al.*, 1978). In *Bacteroides* spp. (Carlsson *et al.*, 1977; Tally *et al.*, 1977), *Clostridium* spp. (Ashley and Shoesmith, 1977) and *Selenomonas ruminantium* (Wimpenny and Samah, 1978; Samah and Wimpenny, 1982) the levels of SOD present in the cells correlate well with the oxygen tolerance of these organisms. The SOD level in certain *Bacteroides* species is similar to, or even greater than, that reported for *E. coli* (Gregory *et al.*, 1978).

The presence of SOD in strict anaerobes has been explained in different ways. The most plausible one is that the presence of SOD enables them to survive transient exposures to oxygen, albeit with growth inhibition (Tally *et al.*, 1977; Halliwell, 1982). Lumsden and Hall (1975) considered that, in anaerobes, SOD provides protection against superoxide radicals formed by cosmic radiation, while Tally *et al.* (1977) proposed that SOD is used as a virulence factor by pathogenic anaerobes.

There are four different types of SOD enzymes known. Copper- and zinc-containing SOD enzymes (Cu/Zn SOD) are characteristic of the cytosol of eukaryotic cells, although similar enzymes have been found in the prokaryotes *Photobacterium leioyhathi* and *Micrococcus denitrificans* (Bannister and Bannister, 1981). Manganese SOD enzymes (MnSOD) are common to both prokaryotes (Vance *et al.*, 1972; Weisiger and Fridovich, 1973) and eukaryotes (Bannister and Bannister, 1981), but SOD enzymes containing iron (FeSOD) have so far only been isolated from prokaryotes (Bannister and Bannister, 1981). In *E. coli*, MnSOD is found in the cell matrix (Keele *et al.*, 1970; Gregory *et al.*, 1973) and presumably protects the organism against endogenously produced superoxide radicals (Rolfe *et al.*, 1978), while FeSOD is found in the periplasmic space (Gregory *et al.*, 1973; Yost and Fridovich, 1973) and may protect against exogenously generated radicals. This latter type of protection may be important for anaerobes that reduce little or no oxygen internally, and will also be advantageous in the establishment of infections in well oxygenated tissues (Rolfe *et al.*, 1978).

The level of SOD in *E. coli* can be induced by oxygen to 25 times the anaerobic level (Gregory and Fridovich, 1973a), while the maximum level of SOD in aerobic *Streptococcus faecalis* is 16-fold higher than that present in the cells during anaerobic growth (Gregory and Fridovich, 1973a). An increase in intracellular SOD correlates well with a gain in resistance to hyperbaric oxygen in *E. coli*, *S. faecalis* and *Saccharomyces cerevisiae* (Fridovich, 1972, 1974, 1975; Gregory and Fridovich, 1973a,b, 1974; Hassan and Fridovich, 1977). *Bacteroides* species contain a low level of SOD in cells grown under anaerobic conditions: when these cells are exposed to 2% oxygen, growth is inhibited and the SOD content increases to five times the anaerobic level (Gregory *et al.*, 1977b).

One of the by-products of oxygen reduction by NADH oxidase is the

superoxide radical (Wimpenny and Samah, 1978). In the anaerobe *Selenomonas ruminantium* a SOD enzyme is induced at the same time as the NADH oxidase and complements the NADH oxidase action in protecting these organisms against oxygen toxicity (Wimpenny and Samah, 1978; Samah and Wimpenny, 1982). Wimpenny and Samah (1978) reported that the inducible SOD responded to higher oxygen concentrations than the NADH oxidase and that SOD levels were highest in cells whose growth was completely inhibited by oxygen. In 1982, however, they modified this statement and reported that the activity of SOD declines at high oxygen concentrations. The induction of NADH oxidase reaches a maximum at a higher aeration level than the aeration level necessary to evoke maximum SOD production. Both NADH oxidase and SOD activities decrease at oxygen concentrations high enough to inhibit growth in *Selenomonas ruminantium* (Samah and Wimpenny, 1982).

Hydrogen peroxide is one of the most stable oxygen radicals and is produced in most aerobically grown cells (Lemberg and Legge, 1949) through the two-electron reduction of oxygen which is generally mediated by reduced flavoproteins (Malström, 1982). Furthermore, H_2O_2 is almost ubiquitous in autoclaved culture media exposed to air. The heating of glucose and phosphate together in culture media produces intermediates that generate H_2O_2 on aeration of the media (Carlsson *et al.*, 1978). The oxidation of the thiol group of the cysteine present in anaerobic media also produces H_2O_2 when these media are exposed to air (Carlsson *et al.*, 1969).

An increased number of single-strand DNA breaks and enhanced lethality were observed in *E. coli* (Pollard and Weller, 1967; Ananthaswamy and Eisenstarck, 1977; Hartman and Eisenstarck, 1978; Carlsson and Carpenter, 1980), *S. typhimurium* (Yoakum and Eisenstarck, 1972; Carlsson and Carpenter, 1980), and T7 phage (Ananthaswamy and Eisenstarck, 1976), after treatment with H_2O_2. Hydrogen peroxide affects isolated DNA by altering it such that all four bases are liberated (Uchida *et al.*, 1965; Yamafugi and Uchida, 1966; Freese *et al.*, 1967; Rhaese and Freese, 1968; Massie *et al.*, 1972). All studies on isolated DNA, however, were carried out using relatively high concentrations of H_2O_2 (0.05 to 0.1 M) and long periods of incubation in the presence of ferric chloride. In heat-treated bacteria, H_2O_2 appears to interfere with the ability of the cells to recover after stress (Martin *et al.*, 1976; Brewer *et al.*, 1977; Flowers *et al.*, 1977; Rayman *et al.*, 1978). It also displays a synergistic effect with near-UV irradiation and Hartman and Eisenstarck (1978) proposed that H_2O_2 decreases *recA*$^+$-dependent repair of UV-induced DNA damage. The latter synergism was thought to be specific for near-UV irradiation (Hartman and Eisenstarck, 1978), but Bayliss and Waites (1979) found that the simultaneous treatment of dormant spores of *Bacillus subtilis* with far-UV radiation and H_2O_2 resulted in a 2000-fold greater kill than that

produced by irradiation alone or followed by a treatment with H_2O_2. This synergism was not due to the hydroxyl radicals formed during the decomposition of H_2O_2 by UV irradiation, since hydroxyl radical quenchers failed to protect the spores.

It was thought that the possession of the H_2O_2 scavenging enzymes, catalase and/or peroxidase, is essential for survival of living organisms in air (Saunders, 1973; Stadtman, 1980) and that the lack of these enzymes in anaerobes accounts for their oxygen sensitivity. Holdeman and Moore (1972) showed, however, that a large number of obligate anaerobes produce these enzymes and Hansen and Stewart (1978) recently recommended a catalase test as a rapid screening procedure to identify members of the "*B. fragilis*" group of anaerobic Gram-negative bacilli.

Bacteroides distasonis is the anaerobe that produces the most catalase. The catalase concentrations reported for this organism are comparable with those observed in aerobic *E. coli* (Gregory and Fridovich, 1973b) and aerotolerant *Streptococcus faecalis* organisms (Gregory and Fridovich, 1973a). The *B. distasonis* catalase enzyme has a molecular weight of 250,000 and its uninduced production reaches peak values in the late logarithmic growth phase (Gregory *et al.*, 1977b).

Gregory and Fridovich (1973b) claimed that catalase is not induced by oxygen in *E. coli* cells, but Hassan and Fridovich (1977) reported a 2.4-fold increase in catalase and a 12.7-fold increase in peroxidase levels on aeration of *E. coli* K12 cells. The induction of catalase by oxygen in anaerobes is markedly influenced by the growth medium.

Catalase is a non-dialysable, cyanide- and azide-sensitive heat-labile protein (Gregory *et al.*, 1977b) which, with a few exceptions (Johnston and Delwiche, 1965), contains heme as a biologically active-centre component (Bomberg and Luse, 1963; Schonbaum and Chance, 1976). *Bacteroides fragilis* cannot synthesize its own heme and must therefore transport into the cell preformed hemin for synthesis of heme proteins like catalases and cytochromes (Sperry *et al.*, 1977). Several other *Bacteroides* species also require preformed hemin for catalase production, and catalase levels of *B. distasonis* were found to vary with the amount of hemin supplied in the medium (Gregory *et al.*, 1977b). Ten times as much hemin is required for catalase production than for optimum growth (Wilkins *et al.*, 1978). Since it is heme availability, and not total heme concentration, that is the important factor in determining catalase production, higher concentrations of catalase can be induced in some media if hemin is added after autoclaving. The effect of hemin on catalase production is very specific and cannot be duplicated by ferrous sulphate or ferrous ammonium citrate (Gregory *et al.*, 1977b). Vitamin K apparently acts synergistically with hemin in elevating the catalase-specific activity (Gregory *et al.*, 1977b). The requirement for exogenously

supplied hemin was not recognized until recently, and is one of the reasons why catalase production in anaerobes remained undetected for such a long time.

Gregory *et al.* (1977a) showed that catalase production and induction in a number of *Bacteroides* species is suppressed by the presence of glucose and other carbohydrates in the medium. When cells, grown in the absence of carbohydrates, are transferred to media containing glucose, the residual catalase activity already in the cells is not destroyed, but is diluted out as the cells multiply without producing more enzyme. In the absence of carbohydrates, *B. fragilis* produces relatively large amounts of catalase (25–50 units (mg protein)$^{-1}$; Gregory *et al.*, 1977a). Anaerobes are routinely cultured in media that contain carbohydrates as energy sources and this may be another reason why catalase production was not previously detected in these organisms.

Several investigators have stated that catalase induction does not render cells more tolerant towards oxygen (Gregory and Fridovich, 1973a,b; Fridovich, 1974; Rolfe *et al.*, 1978) or less sensitive to H_2O_2 (Adler, 1963; Ananthaswamy and Eisenstark, 1977). Although catalase significantly lowers the concentration of H_2O_2 present in cells during aerobic metabolism (Schonbaum and Chance, 1976), its activity *in vivo* is affected by several environmental conditions. The endogenous catalase in thermally stressed *Staphylcoccus aureus* cells is inactivated by the combined action of heat and NaCl in selective *Staphylococcus* media; exogenously added catalase was found to cause as much as a 15,000-fold increase in the enumeration of these thermally stressed *Staphylococcus aureus* cells (Martin *et al.*, 1976). Catalase also is inactivated both by high concentrations of H_2O_2 and by cysteine, the latter being a standard component of anaerobic media (Boeri and Bonnichsen, 1952; Alexander, 1957). Peroxidase enzymes, which are effective scavengers of H_2O_2 and are capable of using cysteine as a substrate in the reaction (Olsen and Davis, 1976), are therefore much better suited to protect anaerobes against the bacteriocidal effect of aerated cysteine-containing media (Carlsson *et al.*, 1969).

B. EFFECT OF OXYGEN AND HYDROGEN PEROXIDE ON MACROMOLECULAR SYNTHESIS

Although numerous reports have shown that oxygen inhibits the growth of obligate anaerobes, this does not necessarily imply that macromolecular synthesis is immediately blocked. There are examples of stationary phase Gram-negative aerobic bacteria which continue to synthesize proteins and nucleic acids for long periods (weeks) after growth has ceased (Robb *et al.*, 1980). Furthermore, aeration of these cells affects the rate of synthesis of nucleic acids and proteins but does not totally inhibit their production.

The effect of oxygen on macromolecular synthesis has been investigated in three *Bacteroides* species, and the results of these studies indicate that *Bacteroides* strains react differently to oxygen stress. Glass *et al.* (1979) investigated the effect of oxygen on nucleic acid and protein synthesis in *Bacteroides thetaiotaomicron*. The incorporation of [^{3}H]thymidine and [^{3}H]guanosine into nucleic acids, and of L-[^{35}S]methionine into proteins, was blocked immediately on exposure of growing cultures to air. Synthesis of guanosine tetraphosphate (ppGpp) and guanosine pentaphosphate (pppGpp) occurred concomitantly with the cessation of DNA, RNA and protein synthesis. By analogy with *E. coli*, these experiments on the production of ppGpp and pppGpp during oxygen stress, and the abrupt cessation of macromolecular synthesis, suggest that *B. thetaiotaomicron* exhibits a classical stringent response. Although the authors suggest that mutants unable to synthesize ppGpp and pppGpp should be isolated in order to confirm their suggestion, no mutants have yet been reported. Nevertheless, the importance of these and similar mutants for molecular biology studies on anaerobes must be stressed, and it is to be hoped that they will be isolated and investigated.

Nierlich (1978) found that bacteriostatic hyperoxia induced the stringent response in bacteria. The induction of SOD and catalase by oxygen has been seen by many investigators as a form of adaptation of living organisms to hyperoxia (Fridovich, 1981). Stringency is a pleiotropic response under the control of the *rel* genes (Gallant, 1979) and subjects the whole of the cellular metabolism to major changes. An inability to exhibit a stringent response, and thus to adapt to hyperoxia, leads to cell death in hyperoxygenated *relA*$^-$ *E. coli* cells (Fee, 1981). Chloramphenicol, tetracycline and rifampicin can interfere with the onset of stringency (Sokawa and Sokawa, 1978; Glass *et al.*, 1979) and Fee (1981) suggested that the induction of MnSOD is part of this response.

Stevenson (1979) investigated amino-acid uptake systems in *Bacteriodes ruminicola* and reported that the exposure of *B. ruminicola* to air inhibited uptake of amino acids.

In contrast to the previous reports, Schumann *et al.* (1983) reported that DNA, RNA and protein synthesis in a *B. fragilis* strain were decreased by oxygen but not blocked immediately. The reduction in the rates of synthesis of DNA, RNA and protein was proportional to the amount of aeration.

The reason for the difference between *B. fragilis* Bf-2 and the other two *Bacteroides* species is not known, but it suggests that there are substantial differences between species of *Bacteriodes* and their responses to oxygen. *Bacteriodes fragilis* Bf-2 was isolated in 1977 and has been regularly exposed to air over the last 7 years, which may account for its observed response to oxygen.

Schumann *et al.* (1984) investigated the effect of H_2O_2 on DNA synthesis in

B. fragilis Bf-2 cells. Under anaerobic conditions, DNA synthesis was unaffected by H_2O_2 concentrations below 0.0025% but was decreased by 0.01% H_2O_2 and completely inhibited by 0.1% H_2O_2. There was a synergistic effect between oxygen and H_2O_2, and concentrations of H_2O_2 (0.001%) that had no effect on DNA synthesis under anaerobic conditions inhibited DNA synthesis immediately in the presence of oxygen. Hydrogen peroxide (0.001%) did not markedly affect the exponential growth rate of *B. fragilis* Bf-2 cells, but 0.1% H_2O_2 lowered the growth rate initially, and growth was totally inhibited after 90 minutes.

Studies on H_2O_2 sensitivity in *E. coli* indicate that *recA* mutants were approximately 10 times more sensitive to peroxide than *recA*$^+$ strains (Ananthaswamy and Esisenstarck, 1977; Carlsson and Carpenter, 1980). *Escherichia coli* mutants lacking exonuclease III (*xthA*) are exceptionally sensitive to H_2O_2 and are killed by this agent at 20 times the rate of wild-type bacteria (Demple *et al.*, 1983). Both the *recA* and *xthA* gene products seem to be more important than catalase and superoxide dismutase in protecting *E. coli* against H_2O_2 toxicity.

C. ULTRAVIOLET RADIATION

The major lesions induced by far-UV irradiation in *E. coli* cells are the formation of intra-strand pyrimidine dimers (Beukers and Berends, 1960; Ben-hur and Ben-Ishai, 1968). The repair of these lesions occurs by photoreactivation, excision repair or postreplication repair.

Photoreactivation brings about the direct reversal of pyrimidine dimer damage and is an accurate repair process. A single cellular enzyme specifically binds to cyclobutyl pyrimidine dimers to generate a new enzyme–DNA chromophore (Harm, 1975; Rupert, 1975). This complex can form in the absence of light, but activation of the complex to catalyse the cleavage of the joined bases, and to monomerize the pyrimidines, requires the absorption of visible light of wavelength 320 to 410 nm (Rupert, 1975). This mechanism does not break any phosphodiester bonds and is error free. Although photoreactivation has been reported for many aerobic bacteria, not all bacterial species are able to carry out this process. The only anaerobe studied to date is *B. fragilis* which seemingly does not possess a photoreactivation system (Jones *et al.*, 1980a). In view of the habitats of obligate anaerobes it is, perhaps, to be expected that they will not have photoreactivation systems.

Excision repair in *E. coli* is also an accurate repair process. An incision is made at or near the DNA dimer on the damaged strand, which is excised, and the missing DNA is then resynthesized by using the opposite strand as a template (Hanawalt *et al.*, 1979; Grossman, 1981). Excision repair is initiated directly by the action of a damage specific endonuclease (UvrABC endonuc-

lease; Sancar and Rupp, 1983) or by the sequential action of a damage-specific glycosylase and an apurinic/apyrimidinic endonuclease (Grossman, 1981; Lindahl, 1982).

Postreplication repair involves a recombination interchange of strands and it repairs gaps left in daughter strands opposite dimers when replication proceeds past a dimer lesion in the parental strand (Rupp and Howard-Flanders, 1968; Hanawalt *et al.*, 1979; Hanawalt, 1982). Postreplication repair can occur in an error-free way (Walker, 1984).

Error-prone repair in *E. coli* gives rise to mutations after UV irradiation and requires the induction of a special inducible system (Radman, 1975; Witkin, 1976). This inducible system is known as the SOS system and is under the co-ordinate control of the *recA* and *lexA* genes which affect the expression of the *din* (damage-inducible) genes (Kenyon and Walker, 1980). The physiology of SOS responses were reviewed by Witkin (1976), and recently Walker (1984) reviewed the genetics and regulatory aspects of the SOS system.

The recent advances made with *E. coli* in understanding its response to UV radiation damage at the molecular level have been facilitated by extensive physiological studies which were carried out over the last 30 years. These studies were reviewed by Swenson (1976). An unfortunate consequence of the detailed physiological studies carried out on *E. coli* is that the physiological responses of this organism to UV irradiation have come to be regarded as typical for bacteria in general. An object of the present review is to demonstrate that this is not correct and to emphasize the importance of physiological studies on *Bacteroides* which provide a starting point for future work on the molecular biology of UV repair, recombination and mutagenesis in this important anaerobe.

Inactivation of aerobic bacteria by far-UV radiation has been shown to be independent of the presence of oxygen (Zetterberg, 1964; Webb and Lorenz, 1970; Webb, 1977). A novel situation exists in *B. fragilis* where it was shown by Jones *et al.* (1980a) that these cells were more sensitive to far-UV radiation under aerobic conditions than under anaerobic conditions. The cells were more sensitive to UV damage when in the exponential growth phase than in the stationary phase. The dose-enhancement factor (Jagger, 1967) under aerobic conditions varied between 1.75 and 1.90 for stationary-phase cells and between 1.15 and 1.45 for exponential-phase cells. This effect of oxygen is not specific for *B. fragilis* cells damaged by UV irradiation, as oxygen had the same effect on *B. fragilis* cells treated with *N*-methyl-*N'*-nitrosoguanidine, ethylmethane sulphonate, acriflavine or mitomycin C (Slade *et al.*, 1984).

The initial report on survival of UV-irradiated *B. fragilis* (Jones *et al.*, 1980a) did not establish whether the oxygen effect was due to an increased sensitivity to far-UV radiation under aerobic conditions or an increased sensitivity to oxygen after far-UV irradiation. Since cellular metabolism and

photochemical oxidation in the environment generate a variety of toxic derivatives of oxygen, and as *B. fragilis* is an obligate anaerobe, it is important to distinguish between these two possibilities. Slade *et al.* (1981) investigated the effect of H_2O_2 and quenchers of oxygen radicals on the survival of *B. fragilis* cells after far-UV irradiation. Quenchers of toxic oxygen derivatives did not increase the resistance of *B. fragilis* cells to far-UV irradiation under aerobic conditions. Furthermore, H_2O_2 did not render anaerobically irradiated cells more sensitive to far-UV irradiation. It is concluded that *B. fragilis* cells are more sensitive to UV irradiation in the presence of oxygen rather than to oxygen after irradiation.

The number of pyrimidine dimers present in the DNA of the irradiated *B. fragilis* cells was assayed to determine whether a difference in the intrinsic sensitivity of DNA contributed to the different levels of survival after irradiation under anaerobic and aerobic conditions (Jones *et al.*, 1980a). The percentages of radioactivity in the dimer regions from the acid-insoluble fractions, when the cells were irradiated anaerobically and aerobically, were 1.35 and 1.45%, respectively. It does not appear that differences in the number of pyrimidine dimers formed are responsible for the differences in survival obtained under anaerobic and aerobic conditions.

Caffeine has been reported to inhibit excision repair processes in *E. coli* (Rupert and Harm, 1966; Setlow, 1967; Swenson, 1976). When *B. fragilis* cells were plated on sub-inhibitory concentrations of caffeine, after irradiation under both anaerobic and aerobic conditions, a marked decrease in the number of surviving colony-forming units occurred (Jones *et al.*, 1980a). Although it is not known whether the mechanisms of action of caffeine in *B. fragilis* are similar to those reported for *E. coli*, these results suggest that different repair processes may function in *B. fragilis*.

In *B. fragilis*, colony formation was not inhibited immediately by UV irradiation and there was an inverse relationship between the dose of UV radiation and inhibition of colony formation (Schumann *et al.*, 1984). An initial transient increase in colony formation after UV irradiation has also been reported in *E. coli* under conditions where DNA synthesis was inhibited in virtually all the cells (Okagaki, 1960; Smith, 1969). Smith (1969) observed that with both UV-sensitive and UV-resistant *E. coli* mutants there was an inverse relationship between the dose of UV radiation and inhibition of colony formation.

When UV-irradiated cells of certain *E. coli* strains are kept in buffer for a number of hours before plating on nutrient medium, they show a much greater survival than if plated immediately. This phenomenon, known as liquid-holding recovery (LHR), was discovered by Roberts and Aldous (1949) using *E. coli* B. Wild-type *E. coli* K-12 cells do not show LHR but it does occur in *recA* strains of *E. coli* K-12 (Ganesan and Smith, 1968a,b). LHR only

occurs in excision repair-competent cells and *uvrA*, *uvrB* or *uvrC* mutations block this recovery (Ganesan and Smith, 1969). Acriflavine and caffeine, which inhibit excision repair, also prevent LHR (Swenson, 1976). Tang and Patrick (1977) have shown that excision repair alone is sufficient for LHR in *recA* strains and that it is *polA* dependent. Tang *et al.* (1979) have suggested that LHR depends upon a delicate balance between DNA turnover and repair of UV-induced damage.

Jones and Woods (1981) investigated the effect of LHR in UV-irradiated *B. fragilis* cells and showed that LHR occurred under aerobic conditions but was inhibited by anaerobic conditions. Dimer excision was demonstrated during aerobic LHR and was inhibited by caffeine and acriflavine. Under aerobic conditions LHR was not affected by H_2O_2 or quenchers of toxic oxygen derivatives (Slade *et al.*, 1981). In addition to LHR, minimal medium recovery (Ganesan and Smith, 1968 a,b) also occurs in *B. fragilis*. Although there are a number of similarities between LHR in *E. coli* and *B. fragilis*, there are significant differences; in *B. fragilis* LHR only occurs under aerobic conditions and is completely inhibited under anaerobic conditions. A second difference is that LHR in *E. coli* is completely inhibited by the addition of low concentrations of yeast extract to the holding buffer (Ganesan and Smith, 1968a,b; Jones and Woods, 1981). The mode of action of yeast extract is not known, but it appears to bring about increased DNA degradation. The addition of yeast extract, even at concentrations that were 10-fold higher than the concentration that inhibited LHR in *E. coli*, only caused a slight decrease in the LHR of *B. fragilis* cells.

Since *B. fragilis* cells are more sensitive to UV irradiation under aerobic conditions (Jones *et al.*, 1980a), LHR occurs when repair processes are operating least efficiently. A similar situation exists in *E. coli* where LHR is only observed in cells that have a decreased repair capacity (*E. coli* B or *E. coli* K-12 *recA* mutants; Smith, 1978).

In *B. fragilis* cells, filament production was dependent on the fluence and less than half the cells produced filaments. Filament formation in *E. coli* B and *E. coli* K-12 *lon*$^-$ mutants is different in that filament formation is an inducible process and the majority of cells form filaments after exposure to low fluences of UV radiation (Witkin, 1967). *Bacteroides fragilis* filaments also differed from *E. coli* filaments in that they always contained a certain number of septa. Filamentous growth in *E. coli* is lethal unless cell division is resumed before a critical length is reached, and it is the cause of the increased UV sensitivity exhibited by *E. coli* B. and *E. coli* K-12 *lon*$^-$ mutants. The increase in sensitivity is represented by the absence of a shoulder region in UV survival curves. The differences in filament formation between *E. coli* strains and *B. fragilis*, and the shape of the survival curve obtained with *B. fragilis* cells, suggest that the differences in the survival of irradiated *B. fragilis* cells under

different conditions are not directly related to filament formation as in *E. coli* B and *E. coli lon*$^-$ mutants.

Although inactivation of aerobic bacteria by far-UV (254 nm) radiation is not affected by oxygen, inactivation by near-UV (310–400 nm) radiation is strongly enhanced by oxygen (Webb, 1977). Since *B. fragilis* is more sensitive to far-UV radiation in the presence of oxygen (Jones *et al.*, 1980a), Slade *et al.* (1982) investigated the role of oxygen in near-UV survival of *B. fragilis*. The anaerobe *B. fragilis* is similar to *E. coli* (Peters and Jagger, 1981) in that both bacteria possess an inducible system which repairs a major fraction of lethal damage produced by near-UV radiation. There are two important differences between the induction of a near-UV repair system in *B. fragilis* as compared with *E. coli*. The first difference is that, with *E. coli*, a "V"-shaped survival curve was only obtained with an *E. coli* AB 1157 *recA* mutant but not with wild-type *E. coli* AB 1157. A "V"-shaped survival curve was obtained with wild-type *B. fragilis* cells in air. LHR only occurs in wild-type *B. fragilis* cells exposed to air (Jones and Woods, 1981), whereas in *E. coli* LHR is only observed in cells that have a decreased repair capacity (*E. coli* B or *E. coli* K-12 *recA* mutants; Smith, 1978). It is suggested that there may be an analogy between wild-type *B. fragilis* cells exposed to air and *E. coli recA* mutants. In *B. fragilis* oxygen produces phenocopies that resemble *E. coli recA* mutants. *Bacteroides fragilis* cells exposed to oxygen have a decreased repair capacity since they are more sensitive to far-UV irradiation under aerobic conditions (Jones *et al.*, 1980a).

The second difference is the threshold fluence for induction in *B. fragilis* as compared with *E. coli*. In *B. fragilis* the system is induced by 1.5 kJ m^{-2} and in *E. coli* by 70–100 kJ m^{-2}. Peters and Jagger (1981) concluded that the near-UV inducible repair system in *E. coli* may be an adaptation to environments exposed to near-UV solar radiation. At sea level under conditions of maximal solar UV-A (320–400 nm) a fluence of 200 kJ m^{-2} is delivered in about 1 hour of exposure (Parrish *et al.*, 1978). As an obligate anaerobe, *B. fragilis* would not normally be exposed to UV-A but it nevertheless has an inducible capacity to become resistant to the toxic effects of near-UV radiation plus oxygen.

D. ULTRAVIOLET IRRADIATION AND MACROMOLECULAR SYNTHESIS

The synthesis of DNA in *E. coli*, and in a number of other aerobic bacteria, is inhibited by far-UV radiation (254 nm) (Kelner, 1953; Swenson and Setlow, 1966; Swenson, 1976; Trgovcevic *et al.*, 1980). Inhibition of DNA synthesis occurs almost immediately and after a dose-dependent lag it recovers and proceeds at the same rate as in unirradiated cells. Photoreactivation largely relieves this inhibition, indicating that the inhibition is caused primarily by pyrimidine dimers (Hall and Mount, 1981). The inhibition of DNA synthesis

in *E. coli* cells is dependent on a functional *recA* gene product (Trgovcevic *et al.*, 1980).

In contrast to *E. coli* cells, irradiation of *B. fragilis* cells with far-UV radiation resulted in the immediate, rapid and extensive degradation of DNA which continued for 40 to 60 minutes after irradiation (Schumann *et al.*, 1984). During the degradation phase, DNA synthesis was decreased but was never totally inhibited. Although the characteristics of DNA synthesis in *B. fragilis* cells after UV irradiation differ from those in wild-type *E. coli* cells, they resemble those in *E. coli recA* mutant cells where extensive degradation occurs following UV irradiation (Clark, 1973). In *E. coli recA* mutants this DNA degradation results from the action of an uncontrolled recBC deoxyribonuclease (DNAase) (Williams *et al.*, 1981). RecA protein and recBC DNAase are not the only two enzymes involved in DNA degradation in *E. coli*. Excessive degradation following UV- or X-irradiation has been reported in *ras*, *polA* and *uvrD* mutants (Ogawa *et al.*, 1968; Boyle and Setlow, 1970; Youngs and Bernstein, 1973). In these strains, 75–90% of the degradation is accounted for by recBC DNAase, with the remainder being attributed to other nucleases (Youngs and Bernstein, 1973). Degradation of DNA in *E. coli recA* mutants is greatly decreased by caffeine (Shimada and Takagi, 1967), which also inhibited DNA degradation in *B. fragilis*.

The inhibition by chloramphenicol of the extensive DNA degradation that occurs after UV irradiation in *B. fragilis* suggests that protein synthesis is required for degradation (Schumann *et al.*, 1984). In *E. coli*, excision occurs in the presence of chloramphenicol (Swenson and Setlow, 1966). Chloramphenicol decreased DNA synthesis in irradiated *B. fragilis* cells but did not inhibit it completely. Although chloramphenicol prevents the resumption of DNA synthesis in irradiated *E. coli* (Swenson, 1976), Swenson and Setlow (1966) reported that, in a radiation-resistant *E. coli* B strain, DNA synthesis resumed at the same time in irradiated cells with or without chloramphenicol. In *Haemophilus influenzae*, exposure to X-rays induced degradation of DNA, but the DNA break down was not inhibited by chloramphenicol (Stuy, 1961). Synthesis of DNA in irradiated *B. fragilis* cells treated immediately with chloramphenicol is analogous to stable DNA replication in *E. coli* in that it is an abnormal UV-induced DNA synthesis, which can occur in the absence of protein synthesis (Kogoma and Lark, 1970, 1975; Lark and Lark, 1978). However, *B. fragilis* differs from *E. coli* in that, in *E. coli*, protein synthesis is necessary during a 40-minute period after irradiation for the initiation of stable DNA synthesis (Kogoma *et al.*, 1979).

In *B. fragilis* cells, RNA and protein syntheses are less sensitive to UV irradiation than is DNA synthesis (Schumann *et al.*, 1984). The syntheses of RNA and protein are decreased by UV irradiation, and the degree of inhibition is proportional to the dose of UV radiation. Similar results have

been obtained with *E. coli* (Hanawalt and Setlow, 1960; Swenson and Setlow, 1966).

In order to determine why *B. fragilis* is more sensitive to far-UV irradiation under aerobic conditions than under anaerobic conditions (Jones *et al.*, 1980a), Schumann *et al.* (1983) investigated the effects of oxygen and UV irradiation on DNA, RNA and protein synthesis. The syntheses of RNA and protein, after UV irradiation, were relatively unaffected by oxygen. However, the pattern of DNA synthesis in cells irradiated in the presence of oxygen differed from that in cells irradiated anaerobically. The extensive degradation of DNA that occurred under anaerobic conditions, immediately after UV irradiation, was not observed in aerobically irradiated cells. It is suggested that the inhibition of DNA degradation and DNA synthesis by oxygen and UV irradiation could be a reason for the increased sensitivity of *B. fragilis* cells to UV irradiation in the presence of oxygen.

E. INDUCTION OF PHAGE REACTIVATION SYSTEMS BY STRESS FACTORS

Studies on the survival of phages treated with DNA-damaging agents have contributed greatly to our understanding of DNA repair systems and the genetic mechanisms involved in repair. DNA repair in phages of aerobic bacteria has been reviewed by Devoret *et al.* (1975), Witkin (1976) and more recently and comprehensively by Bernstein (1981). A number of different modes of reactivation are known to occur in the host cell. These systems are responsible for increasing the survival of irradiated phage (Swenson, 1976; Bernstein, 1981). Phages of the T series code for functions required for excision, recombination and postreplication repair (Setlow and Carrier, 1968; Symonds, 1975). However, reactivation also requires host cell functions. There is no evidence that phage lambda codes for any functions that promote repair, and repair processes can be considered to be a reflection of bacterial, rather than phage, repair systems (Bernstein, 1981). Phage reactivation processes occurring in host cells involve prereplication repair, recombination repair and reactivation processes that are induced in the host cell by stress factors. It is the induced phage reactivation processes that are the concern of this review.

Phage reactivation induced by UV radiation (Weigle reactivation) has been extensively studied in phage lambda and involves the increased survival of damaged phage when the host cell has been exposed to a low dose of UV radiation before infection (Weigle, 1953; Bernstein, 1981; Walker, 1984). Reactivation by UV irradiation is one of the manifestations of the SOS response, and is regulated by the *recA* and *lexA* genes. Although uninduced phage reactivation processes are not mutagenic, UV-induced reactivation is mutagenic for phage lambda (Weigle mutagenesis). The introduction of UV-

damaged lambda DNA into an unirradiated host cell has very little effect on the phage mutation frequency, and infection with the damaged DNA does not cause a sufficient degree of induction of the SOS system for the SOS processing response to be expressed (Walker, 1984).

Phage reactivation studies of anaerobes have only been carried out with *B. fragilis* Bf-2. Slade *et al.* (1983a) showed UV-induced reactivation of irradiated phage b-1 in exponentially growing *B. fragilis* cells irradiated anaerobically and infected under anaerobic conditions. The highest level of UV reactivation occurred at a fluence of 120 J m^{-2}, and a tenfold increase in phage survival was obtained. Chloramphenicol inhibited UV-induced reactivation. In contrast to UV reactivation of phage lambda, the induction of mutations in phage b-1 by UV irradiation was not observed.

Two novel induced phage-reactivation systems have been reported in *B. fragilis*. Slade *et al.* (1983a) described an oxygen-induced reactivation system that was inhibited by chloramphenicol. The oxgyen-induced reactivation system required a 20-minute exposure to air and had a biological half-life of approximately 15 minutes. Although synthesis of phage DNA was not investigated, DNA synthesis was not inhibited in the host cells held under aerobic conditions during phage reactivation. This suggests that the increased survival of phage b-1 under aerobic conditions is not a result of the inhibition of DNA synthesis but is due to the induction by oxygen of a phage reactivation system.

Slade *et al.* (1983b) also reported a H_2O_2-induced phage reactivation system in *B. fragilis* Bf-2. The H_2O_2-induced phage reactivation system was affected by chloramphenicol but differed from both the UV- and oxygen-induced phage reactivation systems in certain respects. *Bacteroides fragilis* therefore has three inducible phage reactivation systems. However, it has not been established whether these DNA repair systems are distinct, or whether they share common pathways.

An important feature of the three inducible systems is that they do not result in mutagenesis and appear to be error-free repair systems. This contrasts with the inducible UV reactivation system in *E. coli*, which involves the SOS system, which is mutagenic, and affects the phage mutation frequency.

The oxygen- and H_2O_2-induced phage reactivation systems in *B. fragilis* are not specific for DNA damaged with far-UV radiation, since oxygen- and H_2O_2-induced reactivation of phage treated with methyl methane sulphonate, H_2O_2 and X-rays was observed (Slade *et al.*, 1984).

F. INDUCTION OF PROTEINS BY AGENTS THAT DAMAGE DEOXYRIBONUCLEIC ACID

Exposure of *E. coli* to agents that damage DNA or interfere with its

replication results in the increased expression of genes that are part of the SOS regulatory network (Walker, 1984). The many and varied induced SOS responses are regulated by the *recA* and *lexA* genes, and the basic regulatory mechanism mediated by the *recA* and *lexA* gene products is now understood at the molecular level. The recA protein, which has a molecular weight of 37,800 (Horii *et al.*, 1980; Sancar *et al.*, 1980), is one of the prominent proteins induced in *E. coli* by far-UV radiation. Schumann *et al.* (1982) investigated the induction of proteins by far-UV radiation in *B. fragilis* under anaerobic conditions.

Exposure of *B. fragilis* cells to far-UV radiation resulted in the induction of one protein (molecular weight 95,000) and the increased synthesis of two proteins (molecular weights of 90,000 and 70,000) that were synthesized in small amounts in unirradiated cells. The induction of a 37,000 to 40,000 molecular-weight protein was not observed in irradiated *B. fragilis* cells. Caffeine, which affected the survival of irradiated *B. fragilis* cells and decreased host cell-mediated UV reactivation, specifically inhibited the induction of the 95,000, 90,000 and 70,000 molecular-weight proteins. Sodium arsenite did not affect the induction of these three inducible proteins or the survival of irradiated *B. fragilis* cells. The effects of caffeine and sodium arsenite on irradiated *B. fragilis* cells differ from those in *E. coli*. In *E. coli*, excision repair and host-cell reactivation are inhibited by caffeine, whereas *recA*-dependent repair is thought to be inhibited by sodium arsenite (Rossman *et al.*, 1975; Swenson, 1976; Fong and Bockrath, 1979; Rothman, 1980). These differences suggest that the far-UV-induced repair systems in *B. fragilis* differ from those in *E. coli*, which tends to be accepted as typical for bacteria in general.

The induction of proteins by the DNA-damaging agents, oxygen and H_2O_2, which also induced phage reactivation (Slade *et al.*, 1983a,b), were investigated by Schumann *et al.* (1984). Oxygen induced six proteins with apparent molecular weights of approximately 106,000, 90,000, 70,000, a doublet at 37,000, and 34,000. Hydrogen peroxide induced four proteins with apparent molecular weights of approximately 106,000, 95,000, 90,000 and 70,000.

Although similarity in molecular weights does not prove identity, the induction of two proteins with apparent molecular weights of approximately 90,000 and 70,000 by UV, oxygen and H_2O_2, all of which induce phage reactivation systems in *B. fragilis*, is striking. This suggests that in *B. fragilis* two proteins (molecular weights 90,000 and 70,000) may be involved in phage reactivation processes induced by UV, oxygen and H_2O_2. This suggestion is supported by the inhibition by caffeine of the synthesis of the 90,000 and 70,000 molecular-weight proteins after UV irradiation (Schumann *et al.*, 1982) under anaerobic conditions and after exposure to oxygen. Caffeine completely inhibited UV- and oxygen-induced phage reactivation (Slade *et*

al., 1983a,b). At present the mode of action of caffeine in *B. fragilis* cells is not known, but these results suggest that it may be different from that in *E. coli* where it does not seem to affect the synthesis of specific proteins but inhibits excision repair processes (Fong and Bockrath, 1979; Rothman, 1980).

Although oxygen induces a phage reactivation system in *B. fragilis* (Slade *et al.*, 1983a), the survival of *B. fragilis* cells after far-UV irradiation is interesting because this organism is more sensitive to UV radiation in the presence of oxygen (Jones *et al.*, 1980a). In other bacteria, it has been shown that inactivation by far-UV radiation is independent of the presence of oxygen. Hydrogen peroxide does not decrease the survival of UV-irradiated *B. fragilis* cells (Slade *et al.*, 1983b), and it is interesting that a protein with an apparent molecular weight of approximately 95,000 is induced by both UV irradiation and H_2O_2, but is not induced by oxygen. Furthermore, the induction of this protein by UV irradiation and H_2O_2, under anaerobic conditions, is inhibited when the cells are treated in the presence of oxygen. Since UV radiation only induces three proteins, two of which are also induced by oxygen, it is suggested that the inhibition of the induction of the 95,000 molecular-weight protein by oxygen may be involved in the decreased survival of *B. fragilis* cells irradiated under aerobic conditions.

High concentrations of SOD are induced in various *Bacteroides* species by exposure to oxygen (Tally *et al.*, 1977; Gregory *et al.*, 1977b, 1978). The molecular weight of inducible SOD in *Bacteroides distasonis* is 40,000 and in *E. coli* the inducible dimeric MnSOD is composed of subunits with molecular weights of 40,000 (Fee, 1981). Oxygen induced the synthesis of a doublet with an apparent approximate molecular weight of 37,000 in *B. fragilis* Bf-2. The synthesis of the 37,000 molecular-weight doublet was a specific effect of oxygen since under anacrobic conditions H_2O_2 and UV irradiation did not induce this protein, and it is suggested that either of these two proteins may be an inducible SOD.

G. HEAT-SHOCK STRESS

When prokaryotic and eukaryotic cells are exposed to high temperature, the rate of synthesis of a specific set of proteins increases (Schlesinger *et al.*, 1982; Daniels *et al.*, 1984). This heat-shock response may be part of a general cellular adaptation to stress since other stimuli, including anoxia, ethanol and other chemical agents, induce the synthesis of heat-shock proteins in various organisms (Ashburner and Bonner, 1979; Plesset *et al.*, 1982). In *E. coli* the rate of synthesis of at least 17 polypeptides is enhanced after a temperature shift (Neidhart *et al.*, 1983; Phillips *et al.*, 1984). The induction is under the control of the *htpR* gene (Neidhardt and Van Bogelen, 1981). A link between the heat-shock (*htpR* controlled) regulatory network and the DNA-damage

inducible responses (SOS responses controlled by *recA* and *lexA*) was suggested by Walker (1984) because SOS-inducing agents (UV irradiation and nalidixic acid) induced heat-shock proteins (Krueger and Walker, 1984). *Escherichia coli* not only responds to UV irradiation by turning on the SOS system, but also by inducing a second independent regulatory system.

The heat-shock response has not been studied previously in anaerobes, but the response of *B. fragilis* to various DNA-damaging stress factors has been reported and discussed in this review (Jones *et al.*, 1980a; Schumann *et al.*, 1982, 1984; Slade *et al.*, 1983a,b). Since UV radiation, oxygen and H_2O_2 all induced proteins and phage reactivation systems in *B. fragilis*, the possibility of a link between the heat-shock phenomenon and the responses to UV, oxygen and H_2O_2 was recently investigated by Goodman *et al.* (1985). The induction of heat-shock proteins, and their ability to induce phage reactivation, were studied.

In common with other organisms, *B. fragilis* exhibited a heat-shock response. The wild-type cells responded quickly to stress and synthesized heat-shock proteins immediately on exposure to heat. Seven heat-shock proteins, with apparent molecular weights of 125,000, 80,000, 74,000, 65,000, 56,000, 52,000 and 20,000, were observed. Densitometric comparisons of [^{35}S]methionine-labelled protein bands after sodium dodecyl sulphate–polyacrylamide gel electrophoresis, of extracts of heat-shocked cells with those that had not been heat shocked indicated that, except for the seven heat-shock proteins, the other proteins showed relatively minor variations after exposure to heat.

The apparent molecular weights of the heat-shock proteins in *B. fragilis* differed from those observed in *E. coli* (Neidhardt *et al.*, 1982) and the archaebacteria (*Halobacterium* strains) (Daniels *et al.*, 1984). In *E. coli*, the heat-shock proteins are induced by ethanol (Schlesinger *et al.*, 1982), but no proteins were induced by similar concentrations of ethanol in *B. fragilis*. Heat and ethanol stress did not induce phage reactivation.

Both chemical and environmental stresses have been shown to induce the heat-shock proteins in a number of organisms (Ashburner and Bonner, 1979). From these studies it has become apparent that heat shock is representative of a general stress response (Daniels *et al.*, 1984). Because O_2 and H_2O_2 are likely to be natural stresses for anaerobic bacteria, it is not unreasonable to expect that similar proteins would be induced by heat, O_2 and H_2O_2. However, this does not seem to be the case in *B. fragilis* where proteins with apparently different molecular weights are induced by heat in comparison with exposure to O_2 or H_2O_2 (Schumann *et al.*, 1984). Although similarity in molecular weight does not prove identity, the common induction of three proteins, with apparent molecular weights of approximately 106,000, 90,000 and 70,000, by exposure to either O_2 or H_2O_2 (out of a total of six and four induced proteins, respectively), suggests the existence of a common response and control for O_2

and H_2O_2 stress. Although, as an obligate anaerobe, *B. fragilis* would not normally be exposed to UV irradiation, nevertheless when so exposed, it produces two proteins with molecular weights of approximately 90,000 and 70,000 (Schuman *et al.*, 1982). Proteins with similar molecular weights are induced by O_2 and H_2O_2, but not by heat. Ultraviolet irradiation, O_2 and H_2O_2 induce phage reactivation systems but heat stress does not. Since UV irradiation, O_2 and H_2O_2 cause DNA damage and induce DNA repair mechanisms, it is suggested that the physiological responses to these stress factors have aspects in common with, but differ from, the heat-shock response. A further difference, suggesting different control systems, involved the rate of protein induction. The cells responded quickly to heat stress and induced high levels of the heat-shock proteins immediately (i.e. within 1 minute) on exposure to heat. The UV, O_2 and H_2O_2 response took longer and the proteins were induced over a 15-minute period (Schumann *et al.*, 1982, 1984). It is concluded that the heat-shock response and responses to UV irradiation, O_2 and H_2O_2 represent two independent groups of stress responses in *B. fragilis*. In this respect, *B. fragilis* differs from *E. coli* where there is a link between the heat-shock regulatory network and the DNA-damage inducible responses (Krueger and Walker, 1984; Walker, 1984).

Goodman *et al.* (1985) isolated two *B. fragilis* heat-resistant mutants after ethyl methane sulphonate mutagenesis and selection involving cycles of heat stress and growth. The synthesis of the seven heat-shock proteins was investigated in the *B. fragilis* HS5 and HS9 mutant strains. The 125,000 and 52,000 molecular-weight proteins, which were induced in the wild-type strain, were not induced in the mutant strains. The 80,000 and 74,000, 65,000 and 56,000 molecular-weight proteins were induced both in the wild-type strain and in the mutant strains. In the wild-type strain and the HS5 strain, the 20,000 molecular-weight protein was induced but it was not induced in the HS9 mutant. In both mutant strains the basal concentration of the 56,000 molecular-weight protein, prior to heat induction, was very high in comparison with the concentration in the wild type.

The *B. fragilis* heat-resistant mutants are interesting in that both mutants, which were isolated from different mutation experiments, had lost the ability to synthesize the same two proteins after heat treatment. These two proteins are synthesized by the wild type after exposure to heat. This suggests that the proteins with apparent molecular weights of 125,000 and 52,000 are not involved in heat resistance in *B. fragilis*. Both mutants also showed a high constitutive level of a protein with an apparent molecular weight of approximately 56,000 which is an inducible heat-shock protein in the wild-type strain. The high concentrations of this protein in the mutant cells prior to heat treatment may contribute to the heat resistance of the mutants.

H. ERROR-FREE REPAIR IN *Bacteroides fragilis*

Some major conclusions can now be drawn on the responses of *B. fragilis* to DNA-damaging agents. Although there are DNA repair systems in *B. fragilis* which are induced by DNA-damaging agents, the nature of the repair pathways seem to be accurate and largely error-free. Furthermore, the *B. fragilis* strains studied to date are similar in many respects to *E. coli recA* mutants and some results suggest that *B. fragilis* may not have a typical *E. coli recA*-type mediated response. The observations which may indicate the absence of a *recA*-type controlled SOS processing and mutagenesis system in *B. fragilis* are: (i) this low level of *B. fragilis* cells and phage mutagenesis after treatment with DNA-damaging agents; (ii) a protein with a molecular weight of 37,000 to 40,000 is not induced by UV radiation in *B. fragilis*; (iii) the response of *B. fragilis* cells to LHR and near-UV radiation resembles that of *E. coli recA* mutants, and (iv) extensive DNA degradation after UV radiation occurs in *B. fragilis* and *E. coli recA* mutant cells. If the absence of an error-prone system is confirmed in *B. fragilis*, it would be of interest to determine whether this is a general phenomenon among other *Bacteroides* species and other anaerobic bacteria. It is interesting that the response of *E. coli* to UV irradiation and nalidixic acid is even more complex than previously thought. Krueger and Walker (1984) discovered that these agents induce the expression of several genes that are not regulated by the *lexA* and *recA* gene products but rather by a different regulatory system.

Studies regarding the presence or absence of error-prone repair, which implicates the *lexA*, *recA* system, have been carried out by assessing the mutability of bacteria by both direct and indirect mutagens (Campbell and Yasbin, 1984). Direct mutagens cause mutations by mispairing mechanisms either involving template or nucleotide precursors (Miller, 1983). Alternatively, indirect mutagens (UV) act by inducing a postreplication repair system that is error-prone (Miller, 1983). In *E. coli*, error-prone repair is dependent on *recA*, *lexA* and *umuC* gene products (Walker, 1984). As defined by UV immutability, *Neisseria gonorrhoeae* (Campbell and Yasbin, 1984), *Micrococcus radiodurans* (Sweet and Mosely, 1974), *Streptococcus pneumoniae* (Gase *et al.*, 1980), *Streptococcus mutans* (Sicard, 1983), *Streptococcus sanguis* (Sicard, 1983) and *H. influenzae* (Kimball *et al.*, 1977; Setlow and Notani, 1981) all lack error-prone repair. A further common characteristic of all these bacteria is that they are naturally competent organisms for DNA uptake and transformation. Campbell and Yasbin (1984) suggested the intriguing possibility that competency is incompatible with error-prone repair and that high levels of competency and error-prone repair might result in an unfavourable genetic load. An exception to the correlation between mutability and competence is the demonstration of chromosomal mutagenesis by UV

irradiation and methyl methane sulphonate in *B. subtilis* (Rudner, 1981; Campbell and Yasbin, 1984). However, *B. subtilis* does differ from the other competent strains in the level of competency. In *B. subtilis*, only 10 to 20% of the cell population becomes competent (Dubnau, 1981), whereas in the other strains the competent population can approach 100% (Campbell and Yasbin, 1984). Although the hypothesis is attractive that the majority of competent bacteria evolved in such a way as to remove error-prone repair from the phenomena associated with the SOS response, Campbell and Yasbin (1984) point out that it should be approached with caution. Both *S. typhimurium* and *Proteus mirabilis*, two non-competent organisms, appear to be deficient in some aspects of error-prone repair (Walker, 1978; Hofemeister *et al.*, 1979). *Bacteroides fragilis* is another bacterium that also appears to differ since it is a non-competent strain and appears to lack an error-prone system.

I. REPAIR-DEFICIENT MUTANTS

Although it has been stressed that the isolation of mutants of *B. fragilis* is difficult, nevertheless it is important that mutants are obtained and characterized. The use of mutants has been instrumental in accelerating the progress of research on DNA repair systems in *E. coli*. After an extensive search, Abratt *et al.* (1985) have recently been successful in the isolation of a *B. fragilis* UV-sensitive mutant.

Since a correlation between UV sensitivity and mitomycin C sensitivity has been reported in *E. coli* (Boyce and Howard-Flanders, 1964), *M. radiodurans* (Moseley, 1967), *Bacillus subtilis* (Okubo and Romig, 1966) and *S. typhimurium* (Ames *et al.*, 1973), an indirect selection procedure utilizing mitomycin C was investigated. Mitomycin C has been reported to be chemically activated by reduction with hydrogen in the presence of a palladium catalyst, but that it is highly unstable in this form and rapidly loses its cross-linking ability (Szybalski and Iyer, 1976). Its suitability as a selective agent under anaerobic conditions was established, and two different DNA repair-deficient mutants of *B. fragilis* were isolated after mutagenesis with ethyl methane sulphonate. The MTC25 mutant is markedly sensitive to mitomycin C but is unaffected as regards its sensitivity to UV irradiation. The UVS9 mutant is sensitive to UV radiation, but is only moderately sensitive to mitomycin C. A similar correlation between the repair of mitomycin C and UV-induced lesions has been observed with *M. radiodurans* (Moseley and Copeland, 1978). In *M. radiodurans* it has been shown that two separate DNA excision repair pathways exist, one requiring a functional *mtcA* gene and the other functional *uvsC*, *uvsD* and *uvsE* genes, and that mutational blocks in both must exist for an excisionless phenotype to be expressed (Evans and Moseley, 1983; Moseley and Evans, 1983).

The *B. fragilis* UVS9 mutant showed a fluence reduction factor (Harm, 1980) of 0.6 at 1% survival, as compared with the wild type. The main difference between the UV survival curves of the wild-type and UVS9 strains was in the shoulder region where repair of the DNA damage occurs at low doses of UV radiation.

With *E. coli*, liquid-holding recovery is observed in cells having functional *uvr* and *polA* gene products, but only if they have some other decreased repair capacity as is found in *E. coli* K12 or *E. coli* B *recA* mutants (Ganesan and Smith, 1968a,b, 1969). With *B. fragilis*, aerobic liquid-holding recovery occurs in the wild type as well as in both mutant strains. This indicates functional excision repair but decreased repair capacity of another, as yet unidentified, repair system when the cells are exposed to aerobic conditions. Under anaerobic conditions, where the wild type is functioning at its optimum level, a decrease in cell viability is observed. This negative liquid-holding phenomenon is usually found in cells that are repair proficient and where holding is detrimental to recovery (Harm, 1980). The MTC25 mutant also showed a negative liquid-holding effect that correlates with the wild-type level of UV resistance found in this strain. The UV-sensitive UVS9 strain, however, showed a low level of recovery on holding under anaerobic conditions, which correlates with its UV-sensitive phenotype.

In *E. coli*, host-cell reactivation of UV-irradiated phage is mediated by cellular excision repair enzymes and recombination repair plays a minor role (Howard-Flanders *et al.*, 1966). There is therefore a correlation between uvr^- and hcr^- phenotypes in *E. coli* strains. In *B. fragilis* there does not seem to be a similar correlation as the MTC25 mutant showed decreased levels of host-cell phage reactivation, but had wild-type levels of excision repair. This is in agreement with previous observations that treatments such as UV radiation, H_2O_2 and O_2, which induce phage reactivation in *B. fragilis*, have little or a negative effect on the hosts ability to repair its own damaged DNA (Jones *et al.*, 1980a; Slade *et al.*, 1983a,b).

Hydrogen peroxide-induced phage reactivation revealed a further difference between the two *B. fragilis* mutant strains. Phage reactivation in the MTC25 mutant was induced to the same level as in the wild type by H_2O_2, but the UVS9 mutant showed a reduced level of H_2O_2-induced reactivation. The mutants showed the same level of UV-induced phage reactivation as the wild-type strain.

It is hoped that the isolation and physiological characterization of the mitomycin C- and UV-sensitive mutants, as well as other repair-deficient mutants, will result in a greater understanding of repair and recombination processes in *B. fragilis*.

III. Responses of *Clostridium* Species to Stress Factors

When environmental conditions become inimical to growth, the Gram-positive spore-forming bacteria manifest the additional property of being able to produce dormant endospores. These highly resistant structures serve as a means to preserve viability under adverse environmental conditions. Spore formation and germination has been extensively studied and is best understood in aerobic species such as *B. subtilis* (Murrell, 1967; Warren, 1968; Hanson *et al.*, 1970; Piggot and Coote, 1976; Young and Mandelstam, 1979; Piggot *et al.*, 1981; Szulmajster, 1982). The convenience of working with aerobic species (e.g. the availability of suitable culture media to study sporulation events and the early development of effective genetic exchange systems), coupled with the isolation of sporulation mutants, facilitated rapid advances in the understanding of sporulation in the aerobic bacilli. The known number of genetic loci specifically concerned with sporulation and germination in *B. subtilis* is approximately 50 (Young and Mandelstam, 1979; Piggot *et al.*, 1981). Recently a number of sporulation genes have been cloned and sequenced (Hirochika *et al.*, 1981; Bonamy and Szulmajster, 1982a; Jenkinson and Mandelstam, 1983; Fort and Piggot, 1984; Savva and Mandelstam, 1984).

The technical difficulties of working with the anaerobic spore formers has meant that, in comparison with *B. subtilis* and a number of other aerobic species, relatively little is known about the regulation of sporulation in the clostridia. However, due to their importance in the fields of medicine and public health, a great deal of research has been carried out on the resistance and inactivation of clostridial spores. The extensive literature dealing with the effects of a variety of physical and chemical factors that cause injury or destruction of bacterial spores has been reviewed by a number of authors (Schmidt, 1955; Roberts and Hitchins, 1969; Adams, 1978; Busta *et al.*, 1981; Foegeding and Busta, 1981; Russell, 1982; Hurst, 1984). Environmental factors resulting in injury or destruction of clostridial spores will not be discussed further in this review.

With the exception of the effect of O_2 on the growth of vegetative clostridial cells, which has been reviewed by Morris and O'Brien (1971) and Morris (1975), very little work has been reported on the effects of other physical and chemical factors which result in injury to vegetative clostridial cells (Hurst, 1984). Results suggestive of possible heat injury and recovery of vegetative cells of *Clostridium botulinum* type E were reported by Pierson *et al.* (1974). Late-exponential-phase cells were heated at 40.5°C in peptone yeast extract broth. The number of vegetative cells and spores did not change for 3 hours at this temperature when enumerated on peptone yeast extract agar, but the counts decreased in the same medium containing 0.07% bile salts. *Clostridium*

perfringens cells became sensitive to neomycin after rapid cooling (Tracy and Duncan, 1974), but incubation for only 60 minutes under optimal conditions enabled these cells to regain their neomycin resistance.

In most reviews dealing with the regulation of sporulation in bacteria it has been generally assumed that the factors involved in the onset and regulation of sporulation in the bacilli and the clostridia are similar. Although great advances have been made in the understanding of the regulation of sporulation in the aerobic species, there is still a paucity of information relating to the anaerobic species, and the way in which sporulation is regulated remains poorly understood.

A. COMPLEX AND DEFINED SPORULATION MEDIA FOR THE CLOSTRIDIA

In addition to the technical problems associated with working with obligate anaerobes, an important reason for the paucity of information on sporulation in the clostridia are the complex nutrient requirements displayed by most of the medically important clostridia, and the consequent lack of suitable culture media in which to study the sporulation process. In contrast, the ability of *B. subtilis* and other aerobic species to sporulate in defined media has facilitated the sophisticated physiological and genetic studies performed with this organism.

In general, clostridia will sporulate in common anaerobic media containing protein hydrolysates and yeast extract (Gibbs and Hirsch, 1956; Perkins, 1965; Roberts, 1967). However, sporulation frequencies vary significantly (0 to >80%) from one strain to another, and sporulation is often highly asynchronous. Nevertheless, studies on sporulation in complex media have been undertaken with a number of *Clostridium* species. Complex media devised for *C. perfringens* sporulation have been used for enterotoxin formation (Ellner, 1956; Tsai *et al.*, 1974; Gyobu and Kodama, 1976; Skjelkvåle *et al.*, 1979), but the most widely used complex sporulation medium for producing enterotoxin was developed by Duncan and Strong (1968) and modified by Labbe and Rey (1979). Recently, Craven and Blankenship (1982) improved this medium by replacing protease peptone with peptone and starch with raffinose. An alternative medium for *C. perfringens* sporulation (which supported sporulation of 138 out of 141 strains) was reported by Tórtora (1984). This medium gave a greater percentage of sporulation than five other media described previously. A variety of complex sporulation media have been described for putrefactive and proteolytic clostridia (Gibbs and Hirsch, 1956; Uehara *et al.*, 1965; Collee *et al.*, 1971; King and Gould, 1971; Bagadi, 1977), saccharolytic clostridia (Halvorson, 1957; Hitzman *et al.*, 1957) and for the thermophilic strains *Clostridum thermosaccharolyticum* (Pheil and Ordal,

1967; Hsu and Ordal, 1969b) and *Clostridium thermohydrosulfuricum* (Hollaus and Klaushoffer, 1973).

Defined media have been specifically developed to determine the nutritional and physiological requirements of a few sporulating clostridia. The synthetic medium of Ting and Fung (1972) supported the growth of nine out of the eleven strains of *C. perfringens* which were tested. However, only two strains sporulated and the level of spore formation was low (approximately 8%). The D-medium (Sacks and Thompson, 1978) was an improvement of the Ting and Fung medium and contained a more slowly metabolizable energy source (dextrin) which partially replaced glucose. The carbohydrate concentration and the buffering capacity were adjusted to maintain a pH value that allowed sporulation. Calcium, which is generally regarded as essential for the production of viable spores, was incorporated together with zinc and copper ions. Theophylline, a methylxanthine, stimulated sporulation and prevented the formation of a precipitate which was undesirable during the recovery of purified spores. The D-medium supported growth and sporulation of seven strains of *C. perfringens* including one that had failed to sporulate in the Ting and Fung medium. The yields of heat-resistant spores compared favourably with those obtained in a complex medium (Duncan and Strong, 1968).

Since dextrin is not a well-defined compound, Sacks (1983) surveyed the effect of carbohydrates on the growth and sporulation of *C. perfringens* in D-medium and showed that six strains could be made to sporulate at high levels with the use of well-defined sugars such as glucose, maltose, melibiose, or raffinose.

Attempts to obtain sporulation in chemically defined media in other species of *Clostridium* with complex nutritional requirements have met with varying success. Chemically defined media which supported growth, but not sporulation, of *Clostridium tertium*, *Clostridium septicum* (Hasan and Hall, 1976) and *Clostridium thermocellum* (Fleming and Quinn, 1971) have been reported. Frank and Lum (1969) defined the nutritional requirements of a putrefactive anaerobe growing in a complete synthetic medium. Some compounds essential for spore development were not necessary for vegetative growth. Extensive autolysis, which occurred during the stationary phase, was a major disadvantage of the sporulation medium. Approximately 10% of the vegetative cells resisted lysis and sporulated. Cultures of *Clostridium bifermentans* followed a similar fate when grown in a synthetic medium (Holland and Cox, 1975). The development and use of defined media for the growth and sporulation of *C. botulinum* was reported by Perkins and Tsuji (1962) for type A strains, and by Ward and Carroll (1966) and Strasdine and Melville (1968) for type E strains.

Owing to the complex nutritional requirements of many of the clostridia the synthetic media described thus far all contain most of the commonly occurring

amino acids and an unwieldy supply of vitamins and minerals. Less complicated minimal media have been developed for the saccharolytic clostridia where the amino acids may be replaced by a single inorganic nitrogen source.

A number of minimal media have been described for the growth of *Clostridium pasteurianum* (Carnahan and Castle, 1958; Daesch and Mortenson, 1972; Sargeant *et al.*, 1968). These media were designed for the production of large amounts of vegetative cells and do not appear to support sporulation. However, cultures of *C. pasteurianum* were found to sporulate rapidly in a simple defined medium containing salts, glucose, cysteine and ammonium ions (Mackey and Morris, 1971). The sporulation developmental stages occurred near-synchronously rendering the system amenable to correlative morphological and biochemical studies.

A glucose/mineral salts/biotin medium incorporating ammonium sulphate as the sole source of nitrogen has been recommended for the growth and sporulation of *Clostridium butyricum* and *Clostridium beijerinckii* (Holdeman *et al.*, 1977).

Minimal media for the study of solvent production in *Clostridium acetobutylicum* and related solvent producing species have been reported (Monot *et al.*, 1982; Nishio *et al.*, 1983; Gottschal and Morris, 1981a,b; Bahl *et al.*, 1982). A chemically defined minimal medium which allowed both the production of significant concentrations of solvents and high levels of sporulation in an industrial strain of *C. acetobutylicum* (strain P262), was described by Long *et al.* (1983). The developmental sequence was sufficiently synchronous in this medium to enable the system to be used for correlative physiological and biochemical studies. This study also showed that there was considerable variation among *C. acetobutylicum* strains in their ability to grow, sporulate and produce solvents in various media. These variations emphasize the importance of strain differences and indicate that it may be necessary to determine the optimum conditions for sporulation for the various strains of solvent-producing clostridia.

B. MORPHOLGICAL EVENTS DURING SPORULATION IN THE CLOSTRIDIA

The morphological changes associated with spore formation have been described for a number of different species of clostridia (Hashimoto and Naylor, 1958; Robinow, 1960; Takagi *et al.*, 1960a,b; Fitz-James, 1962; Propst and Mose, 1966; Bayen *et al.*, 1967; Hoeniger *et al.*, 1968; Santo *et al.*, 1969; Mackey and Morris, 1971; Rousseau *et al.*, 1971; Stevenson and Vaughn, 1972; Johnstone and Holland, 1977; Long *et al.*, 1983). Spore development in the genus *Clostridium* is essentially the same as that described for the genus *Bacillus* (Murrell, 1967; Kay and Warren, 1968; Fitz-James and Young, 1969;

Walker, 1970). The sequence of morphological changes that result in the formation of the mature endospore can be divided into seven easily distinguishable stages in both groups (Murrell, 1967). However, there are some differences in spore formation between the two genera as well as differences between individual species and strains of clostridia. For example, forespore engulfment appears to be similar in both *Bacillus* and *Clostridium* species but after engulfment the process of cortex formation followed by coat formation is reversed in many *Clostridium* species (Robinow, 1960; Pope and Rode, 1969; Mackey and Morris, 1971; Samsonoff *et al.*, 1970; Stevenson and Vaughn, 1972; Johnstone and Holland, 1977; Long *et al.*, 1983). Many clostridia contain varied and elaborate spore appendages that arise from the outer spore coat (Krasil'nikov *et al.*, 1964; Hodgkiss and Ordal, 1966; Hodgkiss *et al.*, 1967; Pope *et al.*, 1967; Yolton *et al.*, 1968). Similar appendages are rare or absent from members of the genus *Bacillus*.

Spore formation in the genus *Clostridium* is often accompanied by characteristic changes in cell shape. Unlike sporulating cells of *Bacillus*, *Clostridium* cells often become swollen during sporulation resulting in the production of characteristic clostridial, plectridial, clavate or navicular forms (Gottschalk *et al.*, 1981). These changes in cell shape are associated with the accumulation of vegetative growth and the onset of sporulation, and has been reported to occur in a wide variety of species of *Clostridium* including *C. acetobutylicum* (Jones *et al.*, 1982), *C. beijerinckii* (Bergère *et al.*, 1975), *C. bifermentans* (Johnstone and Holland, 1977), *C. botulinum* (Takagi *et al.*, 1960a; Strasdine, 1968, 1972; Emeruwa and Hawirko, 1973), *C. butyricum* (Rousseau *et al.*, 1971), *C. histolyticum* (Dworcznski and Meisel-Mikolajczyk, 1964), *C. pasteurianum* (Mackey and Morris, 1971), *Clostridium pectinovorium* (Fitz-James, 1962), *C. perfringens* (Svec and McCoy, 1944; Darby *et al.*, 1970; Baine and Cherniak, 1971), *C. oedomatiens* (Ivanov et al., 1956) and *C. thermosaccharolyticum* (Pheil and Ordal, 1967). In the majority of clostridia where the nature of storage granules has been investigated, the reserve material has been shown to be polyglucans which have been given the generic name of granulose (Darvill *et al.*, 1977). In strains that accumulate large amounts of storage material, granulose accumulation may be associated with the swollen phase-bright clostridial stage (Mackey and Morris, 1971; Jones *et al.*, 1982; Long *et al.*, 1984a). The presence of poly-β-hydroxybutyrate has been reported to occur as a storage product in *C. botulinum* type E (Emeruwa and Hawirko, 1973). However, this is in conflict with an earlier report (Strasdine, 1968, 1972), which noted that the storage material was a polyglucan. In most species, storage granules appear at the time when forespore septation occurs. The storage granules are usually excluded from the developing forespore but occasionally granules may be enclosed in the early forespore compartment (Mackey and Morris, 1971; Long, 1984). The

appearance of the granules possibly indicates some phase-specific change in the mother-cell cytoplasm during the shift from vegetative growth to sporulation (Johnstone and Holland, 1977). In addition to the accumulation of intracellular polysaccharides, some species of *Clostridium* produce extracellular slime or capsules during the shift from vegetative to sporulating cells (Fitz-James, 1962; Jones *et al.*, 1982).

As well as changes in cell shape during sporulation, cells of some species of *Clostridium* continue to elongate after normal cell division has halted (Fitz-James, 1962; Pheil and Ordal, 1967; Hsu and Ordal, 1969a). Increases in length of sporulating cells of up to threefold the length of the vegetative cells have been observed. During sporulation the turbidity of the culture may continue to increase for some hours after cell division has stopped. This may be due to the cell elongation, increase in cell size or accumulation of storage products (Hsu and Ordal, 1969a; Mackey and Morris, 1971; Jones *et al.*, 1982).

In the clostridia the formation of the forespore septum is frequently associated with the presence of mesosomes. Normally only a single forespore septum is produced at one pole of the cell. The presence of mutant strains exhibiting abnormal membrane configurations have been reported in *Bacillus* species and several classes of mutants showing atypical septation and bipolar sporulation sites have been characterized (Young and Mandelstam, 1979). Cells with multiple sporulation sites do not contain genomic material within the forespore septa and are unable to develop further. In the clostridia there have been a number of reports of cells showing aberrant bipolar septation. Takagi *et al.* (1960b) described the presence of multiple sporulation loci in several species of *Clostridium*. Cells containing random multiple septa and bipolar forespores containing nuclear material were observed by Santo *et al.* (1969). A small proportion of these cells produced cells with bipolar mature spores. Cells containing refractile bipolar endospores also have been observed in *Clostridium oceanium* (Smith, 1970) and *C. acetobutylicum* (Long, 1984). Mature bipolar spores were also reported to occur in about 10% of the cells of a strain of *C. botulinum* type E (Mann, 1966). The symmetry and synchrony in spore development at the two ends of the cells exhibiting bipolar sporulation indicates that these events are under a common control (Long, 1984).

C. PHYSIOLOGICAL EVENTS DURING SPORULATION IN THE CLOSTRIDIA

The morphological developments that occur during sporulation are directly linked to corresponding physiological events within the cell. In *B. subtilis*, and in other species of *Bacillus* that have been extensively studied, it has been possible to divide these events into those that are essential for sporulation and

those that are dispensable, as well as distinguishing between sporulation-specific and non-specific events (Young and Mandelstam, 1979).

After the induction of sporulation, many species of *Bacillus* produce a number of distinct enzymes, some of which may be excreted from the cell. The appearance of specific enzymes during sporulation has provided useful markers for establishing the sequence of events during sporulation. Although the production of a number of specific enzymes has been linked to the induction of sporulation in some species of *Clostridium* (Simmons and Costilow, 1962; Green and Sadoff, 1965; Lee and Ordal, 1967; Hsu and Ordal, 1970; Duncan, 1973; Emeruwa *et al.*, 1974; Mackey and Morris 1974b; Emeruwa, 1979; Clark and Morris, 1980), the establishment of a comprehensive series of marker events has not been achieved for any species of *Clostridium* (Mackey and Morris 1974b; Emeruwa, 1979).

In addition to the production of exoenzymes, some species of *Bacillus* also synthesize a variety of substances with antibiotic activity. The majority of these enzymically synthesized molecules are linear or cyclic peptides (Hurst, 1969; Schaeffer, 1969). The synthesis of similar antibiotic products during sporulation appears to be uncommon amongst the clostridia.

Many species of *Clostridium* are characterized by their ability to produce potent exotoxins and endotoxins. The toxins produced by several *Clostridium* species first appear when the active growth phase has been completed (Schaeffer, 1969). However, a direct link between toxin production and sporulation has only been found in a few species. In other instances the release of toxins at the end of the growth phase has been linked to cell lysis (Boroff, 1955; Bonventre and Kempe, 1960a) and may involve the activation of toxin precursors by proteases (Bonventre and Kempe, 1960b; Das Gupta and Sugiyama, 1972; Löffler and Labbe, 1983). In *C. botulinum* and *C. novyi*, the production of certain toxins is dependent on the presence of prophages in the producer strain (Inoue and Iida, 1970; Eklund *et al.*, 1971, 1974; Eklund and Poysky, 1974; Takumi *et al* ., 1980).

In *C. histolyticum*, a direct correlation between the production of a toxin and sporulation was established by Sebald and Schaeffer (1965). Sporulation mutants blocked at stage II of sporulation, or later, all produced toxin in normal amounts, whereas mutants blocked earlier produced little or no toxin. These findings were confirmed by Nishida and Imaizumi (1966) who found that toxin production in both freshly isolated strains, as well as stock strains of *C. histolyticum*, were closely associated with the potential for sporulation.

A variety of toxins are synthesized by different types of *C. perfringens*. At least 12 toxins have been identified (Andrews and Bulla, 1981) but only the enterotoxin responsible for food poisoning in *C. perfringens* is directly related to sporulation (Sebald and Cassier, 1969; Duncan *et al.*, 1972). The quantity of enterotoxin produced by various strains of *C. perfringens* was related to the

degree of sporulation (Uemura *et al.*, 1973; Tsai *et al.*, 1974; Craven and Blankenship, 1982). In mutants blocked at stage 0, enterotoxin production was absent whereas mutants blocked at stage III or later produced enterotoxin (Duncan *et al.*, 1972). Enterotoxin synthesis did not begin until stage III and continued to accumulate intracellularly until lysis of the sporangium released both the mature spore and enterotoxin (Duncan, 1973; Labbe and Duncan, 1977b). The enterotoxin of *C. perfringens* is a sporulation-specific gene product consisting of a protein with an approximate molecular weight of $3.5 \cdot 10^4$ (McDonel, 1980). An enterotoxin-like protein occurs in spores of *C. perfringens* (Frieben and Duncan, 1975) and Duncan *et al.* (1973) reported parasporal crystalline inclusions in sporulating cells, which were assumed to be aggregates of enterotoxin protein. Frieben and Duncan (1975) identified three enterotoxin-like proteins in the coat of *C. perfringens* with apparent molecular weights of 14,500, 23,000 and 36,500. All three proteins had biological and serological activities similar to those of *C. perfringens* enterotoxin. McDonel and Smith (1981) reported an *in vitro* system which was able to synthesize enterotoxin and identified three proteins with molecular weights of approximately 17,000, 35,000 and 52,000. All were precipitated by anti-enterotoxin serum and they hypothesized that the 35,000 protein was a precursor of a smaller spore-coat component.

Although a discrepancy exists in the molecular weights reported by the two groups, it appears that all of the products are sporulation-specific coat components which probably contribute to the structural integrity of the spore. Excess enterotoxin may accumulate in the cell cytoplasm of food-poisoning strains as the result of defective regulation of spore coat synthesis, and may be absent from strains not causing food poisoning because of more efficient regulation (Frieben and Duncan, 1973).

In *C. botulinum* a correlation between sporulation and the production of the type C_2 neurotoxin has been reported (Boroff and Das Gupta, 1971). However, because a number of other types of neurotoxin are known to be synthesized by asporogenous strains of *C. botulinum*, the significance remains unclear.

Whether toxins are important compounds in cellular differentiation is not known, but some toxin accumulation may represent an overproduction of a protein required for sporulation, a waste product of the sporulation process, or may provide a reserve storage product of a selective advantage similar to antibiotic production.

The extensive literature relating to the metabolism of intracellular reserves and changes in energy-generating metabolic pathways during sporulation in various species of *Bacillus* has been included in a number of reviews (Murrell, 1967; Vinter, 1969; Young and Mandelstam, 1979; Freese and Heinze, 1984).

Much less information is available about changes in biochemical pathways during sporulation in the clostridia.

Although the presence of cytoplasmic granules consisting of intracellular reserve material has been reported in many species or strains of *Clostridium* (see Section III.B), the role of these intracellular reserves has only been investigated in a few species. Bergère *et al.* (1975) reported that the production and utilization of a polyglucan reserve material was closely related to sporulation in *C. butyricum*. Polyglucan accumulation, which began just before the end of exponential growth, reached a maximum at about stage III of sporulation, just before the first refractile spores appeared and then decreased by about 50% during the later stages V and VI of sporulation.

In *C. pasteurianum*, intracellular polyglucans accumulate in cells in the latter part of the exponential phase of growth and constituted up to 60% of the dry weight of the cell at the time of the onset of sporulation (Robson *et al.*, 1972, 1974; Laishley *et al.*, 1973, 1974). The appearance of storage granules within the cytoplasm correlated well with the derepression of synthesis of ADP-glucose pyrophosphorylase (Otto *et al.*, 1972) which is the initial enzyme in the biosynthetic pathway. Robson *et al.* (1974) also reported that polyglucan deposition correlated with a threefold increase in the activity of this enzyme. These investigators also isolated mutants unable to synthesize polyglucan which lacked either ADP-glucose pyrophosphorylase or granulose synthase, the two constitutively synthesized enzymes required for biosynthesis. The ability of granulose-negative mutant strains to sporulate was inhibited in glucose-rich media. However, they were able to sporulate normally when the rate of supply of glucose was decreased to a level that was sufficient to sustain sporulation in the wild-type organism without concurrent production of polyglucan. The rate of granulose synthesis in *C. pasteurianum* is probably regulated by the adenylate energy charge and the availability of glucose 1-phosphate (Robson *et al.*, 1974). The polyglucan was degraded when cells were transferred to a medium lacking alternative sources of carbon and energy (Dawes and Senior, 1973; Robson and Morris, 1974). Mobilization of polyglucan in *C. pasteurianum* was favoured under conditions of low adenylate charge and did not occur in the presence of exogenous glucose (Robson and Morris, 1974), and the addition of a variety of nucleotide sugars also was found to inhibit mobilization. The isolation of mutants that were able to accumulate polyglucan in the normal manner, but were unable to utilize the storage product, was reported by Mackey and Morris (1974a). Robson and Morris (1974) suggested that, in addition to functioning as a carbon and energy source, polyglucan synthesis itself may serve as an overflow mechanism that functions to moderate the increase in the intracellular concentrations of potentially inhibitory metabolites, including suppressors of sporula-

tion which might occur during the early stationary phase in glucose-rich cultures.

The role of polyglucan as an endogenous substrate for the supply of carbon and energy has also been reported to occur in some strains of *C. botulinum* type E (Strasdine, 1968, 1972). Strasdine (1972) reported that the intracellular polyglucan appeared in the cytoplasm during the latter part of the growth phase and reached a maximum just before the onset of sporulation and was then lost during subsequent spore formation in glucose/trypticase medium. When non-proliferating cells, grown in a glucose-rich medium, were transferred to a medium lacking an exogenous supply of glucose, the polyglucan reserve was rapidly utilized concurrently with the formation of mature spores. The addition of exogenous glucose delayed the loss of the polyglucan and stimulated the production of CO_2, and either inhibited or greatly decreased the formation of mature spores.

The relationship between the accumulation of intracellular polyglucan granules, solvent production and sporulation also has been investigated in a solvent-producing industrial strain of *C. acetobutylicum* (Jones *et al.*, 1982; Long *et al.*, 1983, 1984b). The conventional batch fermentation for the production of acetone and butanol by *C. acetobutylicum* occurs in two distinct phases. During the initial phase, rapid vegetative growth is accompanied by the fermentation of substrate to acetate and butyrate. Following a pH breakpoint, the fermentation shifts to the solvent-producing phase during which growth ceases (Davies and Stephenson, 1941; Spivey, 1978). In *C. acetobutylicum* P262 the accumulation of intracellular polyglucan granules was observed to be associated with the shift from the acid-producing metabolism to the solvent-producing metabolism at the end of the exponential growth phase (Jones *et al.*, 1982). The accumulation of intracellular granules was linked to the production of a swollen phase-bright clostridial stage, to capsule production and to the cessation of cell division, and these events appeared to be a prerequisite for the onset of sporulation. Sporulation mutants blocked both before and after forespore septum formation were still able to accumulate storage material, produce a clostridial stage and form solvents (Jones *et al.*, 1982; Long *et al.*, 1984a). However, clostridial stage mutants, which did not form the typical clostridial stage, did not undergo the shift to the solventogenic phase and were unable to accumulate polyglucans or extracellular polysaccharide capsules and were unable to sporulate. Granulose mutants that were defective in the ability to synthesize polyglucans were still able to undergo the shift to the solventogenic phase and were able to produce mature spores in a glucose-rich medium. The observation that the clostridial mutants did not exhibit any of the physiological and morphological changes that normally occur at the end of exponential growth indicates that these events may share common regulatory features.

A similar shift in metabolism associated with the inhibition of cell division and the onset of sporulation has been observed with *C. thermosaccharolyticum* (Hsu and Ordal, 1970). Under conditions of restricted growth, *C. thermosaccharolyticum* was found to go through an intermediate elongated non-dividing morphological form that produced mainly ethanol instead of the acetate, butyrate and lactate produced by the vegetative forms (Hsu and Ordal, 1970; Hoffman *et al.*, 1978). Wild-type strains of *C. thermosaccharolyticum* initially form elongated cells when they are grown in continuous culture, and produce mainly ethanol (Landuyt *et al.*, 1983). These cells can, however, either revert back to dividing cells, which shift back to an acid-producing metabolism, or they can go on to sporulate. Landuyt and Hsu (1984) isolated a mutant strain that remained as an elongated cell and continued to produce ethanol. The shift to the production of solvents may provide a relief-valve for the need to lower pools of excess NADH and NADPH which may accumulate after the cessation of cell division, and may be associated with decreased hydrogenase activity (Kim *et al.*, 1984; Datta and Zeikus, 1985).

Although the shift in metabolic pathways associated with the cessation of cell division and the onset of sporulation has been most extensively studied in solvent-producing strains of clostridia, with potential applications in biotechnology, metabolic shifts have been reported in some other species of the clostridia. This suggests that shifts in biochemical pathways involved in energy generation and the dissipation of excess reducing power may be relatively widespread amongst the clostridia. Many species of saccharolytic butyrate-producing clostridia are known to produce varying amounts and ratios of solvents during the later stages of batch culture (Gottschalk, 1979).

Clostridium botulinum type E also shows a switch in a metabolic pathway associated with the transition to sporulation. Using a sporulation mutant blocked at the early forespore stage, Emeruwa *et al.* (1974) demonstrated that both sporulating and non-sporulating cells were able to accumulate intracellular storage granules and acetate, which reached maximum levels during the early stationary phase of growth. Butyrate synthesis, however, was blocked in the asporogenic mutant.

Day and Costilow (1964a,b) also reported a switch in metabolic activity during sporulation in *C. botulinum*. An increase in the catabolic rate occurred at the onset of sporulation and both acetic and valeric acids were produced at increasing rates until over 90% of the cells contained forespores. In addition, a small amount of propionate also was produced at this stage. During maturation and the production of refractile spores, the rate of valeric acid production slowed and there was a significant decrease in the acetic acid concentration.

The use of metabolic inhibitors has proved useful in investigating the relationship between metabolic changes and sporulation in *Bacillus*, and

Campbell and Ordal (1968) reported the use of such inhibitors in a preliminary study of *C. thermosaccharolyticum*. Fluoroacetic acid was the only inhibitor of those tested that did not substantially inhibit vegetative growth of this organism. Their results showed that fluoroacetic acid partially inhibited glucose catabolism of vegetative cells and completely inhibited the formation of spores.

Inhibitors were also utilized by Emeruwa *et al.* (1974) to investigate the role of specific fermentation pathways in relation to sporulation in *C. botulinum*. The metabolic analogues β-phenethyl alcohol, fluoroacetic acid and 2-picolinic acid inhibited anaerobic sporogenesis, butyrate biosynthesis and acetate accumulation. Although the site of inhibitory action was not determined, the results suggest a direct relationship between the butyric type of fermentation and sporulation.

In many species of *Bacillus* the onset of sporulation is associated with the induction of both intracellular and extracellular proteases. Intracellular proteases are presumed to be involved in the extensive turnover of protein that occurs throughout sporulation in many species of *Bacillus*. Very little is known about the turnover of protein during sporulation in clostridial species. However, the butyric clostridia which have been investigated exhibited virtually no extracellular protease activity during sporulation (Mackey and Morris, 1974b; Long, 1984). Intracellular proteolytic activity has, however, been reported in cell-free extracts of two strains of *C. perfringens* (Löffler and Labbe, 1983). Most of the protease activity was related to sporulation in these strains. Two types of protease were detected, an alkaline serine protease and a neutral metallic protease. Kinetic studies, and studies using a stage 0 sporulation mutant, indicated that only the alkaline serine protease was sporulation specific. These authors suggest that proteases may play a role in the processing of the enterotoxin precursor. Very little is known about other changes in protein and nucleic acid synthesis during sporulation in the clostridia.

It has been reported that the onset of sporulation in many species of *Clostridium* is accompanied by the autolysis of a substantial proportion of the vegetative cells (Wooley and Collier, 1966; Frank and Lum, 1969; Hasan and Hall, 1976; Holland and Cox, 1975). Cell components released during autolysis may provide an important source of nutrients for sporulating cells. Autolysis at the end of the vegetative growth phase also may be an important mechanism for toxin release in some species of *Clostridium* (Boroff, 1955; Bonventre and Kempe, 1960a). Labbe and Tang (1983) reported that a germination initiation protein produced by *C. perfringens* appeared to be an autolysin which was overproduced by vegetative cells. The initiation protein appeared to be a vegetative cell product because it was still produced by a sporulation mutant blocked at stage 0.

D. THE TRIGGERING OF SPORULATION

In *Bacillus* spp. it has been firmly established that spore formation is triggered when cells are starved of nutrients. Sporulation in *Bacillus* spp. is normally studied in cells which, after initially multiplying rapidly in a liquid medium, are suddenly exposed to a nutritionally poor medium where they differentiate to form endospores. As most clostridia grow and sporulate only under anaerobic conditions in complex media, where synchrony is often poor, it has been much more difficult to investigate the requirements for sporulation. As a consequence, detailed investigations have only been attempted with a few species and the factors involved in the triggering of sporulation have been poorly defined. It has been generally assumed that spore formation in the clostridia is also triggered by starvation (Young and Mandelstam, 1979; Freese and Heinze, 1984), but there is little evidence to support this assumption. Unlike *Bacillus* species, *Clostridium* spp. appears to require an exogenous carbon and energy source throughout the sporulation cycle (Murrell, 1967; Hickey and Johnson, 1981). Within the clostridia, vegetative growth does not necessarily continue until the nutrients are exhausted. A number of saccharolytic clostridia have been shown to sporulate in the presence of high concentrations of glucose, and Bergère and Hermier (1965a) demonstrated that the sporulation-inducing factor in *C. butyricum* was not the depletion of a nutrient.

Cultures of *C. pasteurianum* also were observed to sporulate efficiently in the presence of high concentrations of glucose, and an exogenous source of carbon and energy also was needed for sporulation (Bowen and Smith, 1955; Mackey and Morris, 1971). In *C. acetobutylicum,* glucose or ammonium limitation did not result in the induction of either solvent production or sporulation, and both glucose and ammonia were required for their initiation and development (Long *et al.*, 1983, 1984b). At intermediate concentrations of glucose or ammonia, the yield of endospores was proportional to the substrate concentration.

In studies carried out on a thermophillic saccharolytic clostridium, Pheil and Ordal (1967) and Hsu and Ordal (1969a,b) also showed that sporulation was not a response to starvation, and a carbon and energy source was required for endospore formation in this species.

The requirement for an exogenous energy source also has been demonstrated amongst the proteolytic species of *Clostridium.* Frank and Lum (1969) showed that with the putrefactive anaerobe 3679, methionine, glycine and glucose were essential, and histidine, proline and sodium acetate were stimulatory for spore production in a chemically defined medium. An exogenous source of energy also was required for spore maturation in a variety of strains of *C. botulinum* (Perkins and Tsuji, 1962; Day and Costilow, 1964b). With *C. perfringens* the presence of an energy source during

sporulation again is obligatory (Labbe and Duncan, 1975; Hickey and Johnson, 1981).

It is not apparent whether the accumulation of intacellular reserve material, which occurs during the early stationary growth phase in many strains of *Clostridium*, can wholly replace the requirement for an exogenous carbon source during sporulation, but there is evidence that it may at least do so partially in some strains (Emeruwa and Hawirko, 1973).

In *Bacillus* strains, sporulation can be completed endotrophically when cells are transferred to distilled water or other non-nutrient media. This is in marked contrast to the anaerobic spore formers where endotrophic sporulation has not been demonstrated (Day and Costilow, 1961, 1964b; Murrell, 1967; Hsu and Ordal, 1969a; Labbe and Duncan, 1975).

A clear distinction can be made between those species of *Clostridium* in which catabolic inhibition of sporulation by glucose occurs and those where such catabolic inhibition is absent. Catabolic repression of sporulation by glucose is absent from a number of species of saccharolytic butyric acid-producing clostridia such as *C. butryricum* (Bergère and Hermier, 1965a), *C. pasteurianum* (Mackey and Morris, 1971) and *C. acetobutylicum* (Long *et al.*, 1984b). When *C. butyricum* was grown in medium containing excess glucose, only 15% of the substrate was used during the exponential growth phase and 55% was used after forespore formation (Bergère and Hermier, 1965a). A similar pattern of glucose utilization was observed with *C. acetobutylicum* grown in a minimal medium containing 50 g of glucose l^{-1}, where only 25–40% of the glucose was consumed in the exponential growth phase and the remainder was utilized during the solventogenic phase associated with endospore formation (Long *et al.*, 1984b). An enhancement of glucose consumption occurred during the stationary growth phase and during endospore formation in this organism.

The catabolic repression of sporulation by glucose was observed in a saccharolytic thermophile which produced acetate and ethanol as end products (Hsu and Ordal, 1969a). When *C. thermosaccharolyticum* was grown in the presence of excess glucose, endospore formation was repressed (Hsu and Ordal, 1969b). However, glucose could serve as an exogenous energy and carbon source for sporulation provided a limited amount was fed continuously (Hsu and Ordal, 1969a). In addition to glucose, high concentrations of fructose, maltose, mannose, sucrose and D-pentoses also induced catabolic repression of sporulation (Hsu and Ordal, 1969b). Sporulation was stimulated, however, when cells were grown in media containing L-arabinose, L-xylose, galactose, methylglucoside, β-methylglycoside, cellobiose, salicin and starch. The L-pentose sugars, and the carbohydrates containing glucosidic linkages which stimulated sporulation, lowered the growth rate of *C. thermosaccharolyticum* and resulted in specific growth rates of less than 0.465

h^{-1}. The carbohydrates that repressed sporulation supported unrestricted growth. Sporulation only commenced when the supply of exogenous energy and carbon was lowered but not absent. The limitation in supply resulting in a lowered growth rate could be achieved either mechanically, by the slow feeding of glucose, or by using substrates that were less readily utilized. Glucose consumption also was enhanced during sporulation in *C. thermosaccharolyticum*, and consumption rates that were three to four times higher in sporulating cells were observed (Hsu and Ordal, 1970).

In *C. perfringens*, sporulation is also decreased or inhibited by the mechanism of catabolite repression (Labbe and Duncan, 1975; Labbe and Rey, 1979; Hickey and Johnson, 1981). Glucose concentrations of 1% (w/v) resulted in repression of sporulation (Walker, 1975; Hickey and Johnson, 1981). In both defined and complex media a poorly utilized carbon source such as starch, dextrin or raffinose appeared to be critical for good sporulation (Labbe and Duncan, 1975; Labbe *et al.*, 1976; Sacks and Thompson, 1977; Labbe and Rey, 1979; Labbe, 1981; Craven and Blankenship, 1982; Sacks, 1983). The carbohydrate that resulted in the greatest stimulation of sporulation varied from strain to strain and according to the type of culture media used.

Media containing both glucose and starch resulted in significantly improved spore yields compared with media containing starch alone (Hickey and Johnson, 1981). Glucose-induced catabolite repression could be circumvented if the initial glucose concentration was kept low (below about 20 mM). A combination of low levels of glucose and the presence of starch resulted in biphasic growth which produced high levels of sporulation (Labbe and Duncan, 1975; Hickey and Johnson, 1981). The biphasic growth response was dependent on the starch concentration, and the omission of either starch, glucose or maltose resulted in an approximately 100-fold decrease in viable spores.

Clostridium botulinum also has been shown to utilize a variety of carbohydrates including glucose, maltose and sucrose, and neither stationary-phase metabolism nor endospore production were detected when carbohydrates were omitted from the growth medium (Emeruwa *et al.*, 1974). Strasdine and Melville (1968) also reported a carbohydrate requirement for sporulation in this species and demonstrated that spore development was markedly influenced by the source of available carbohydrates, which ranged from complete sporulation in cultures grown on glucose and maltose to complete inhibition with galactose. Emeruwa *et al.* (1974) suggested that sporulation was suppressed until the monosaccharide was depleted. An increase in the rate of catabolism after the onset of sporulation again was observed with *C. botulinum* (Day and Costilow, 1964a).

Glucose also was reported to be essential for spore production in the

putrefactive anaerobe 3679, when grown in a chemically defined medium (Frank and Lum, 1969). However, if glucose was added in amounts that exceeded the optimal concentration, sporulation was drastically decreased. In other proteolytic species such as *C. tetani* and *C. histolyticum*, sporulation again has been reported to be inhibited by the presence of sugars (Leifson, 1931; Nishida and Imaizumi, 1966).

In addition to the requirements for an energy and carbon source during spore formation, the presence of a nitrogen source may be necessary in many species of clostridia. Many clostridial species are only able to utilize an organic nitrogen source for growth and sporulation, but amongst the saccharolytic butyric acid-producing clostridia a number of species are capable of using inorganic nitrogen as their sole source of nitrogen (Perkins, 1965; Mackey and Morris, 1971; Long *et al.*, 1983, 1984b). In *C. acetobutylicum* P262, the cessation of growth brought about by nitrogen limitation inhibited both the shift to solvent production and sporulation (Long *et al.*, 1984b). Perkins (1965) reported that the butyric acid-producing clostridia seemed to sporulate best when grown on quite low concentrations of an organic nitrogen source. In those species of *Clostridium* that require organic nitrogen for growth, the role of a nitrogen source during spore formation has been more difficult to determine. However, in a number of species, the composition and concentration of the nitrogen source has been observed to be important in determining the frequency of sporulation (Perkins, 1965; Roberts, 1967).

In studies on the putrefactive anaerobe 3679 grown in minimal medium, Frank and Lum (1969) reported that methionine and glycine were essential for sporulation and that histidine and proline stimulated sporulation. The nature of the nitrogen source was also important in determining the frequency of sporulation in various strains of *C. perfringens* (Duncan and Strong, 1968; Adams, 1973; Sacks and Thompson, 1978; Levinson and Feeherry, 1978) and in *C. thermosaccharolyticum* (Pheil and Ordal, 1967). Two sporogenic peptides that were components of tripticase were reported to be required specifically for sporulation in *C. roseum* (Wooley and Collier, 1966). In *C. botulinum*, arginine was reported to be essential for sporulation (Perkins and Tsuji, 1962) and the onset of sporulation coincided with the cessation of rapid arginine consumption. Arginine was metabolized to citrulline and then to ornithine. Perkins and Tsuji (1962) suggested that the role of arginine was as an energy source which generated ATP during the breakdown of citrulline to ornithine. Day and Costilow (1964a,b) investigated the ability of *C. botulinum* cells to utilize L-alanine and L-proline during sporulation after the cells, initially grown in a complex medium, were transferred to a replacement medium. These cells were only able to utilize mixtures of L-alanine and L-proline, L-isoleucine and L-proline or L-alanine and L-arginine. L-Ornithine and L-citrulline were unable to function as substitutes, and single amino acids

did not support sporulation in the replacement medium. The amino acids were fermented most actively after the onset of sporulation and these authors considered that the major function of the amino acids was to provide energy during spore maturation by means of the Stickland reaction. However, Emeruwa *et al.* (1974) suggested that, in *C. botulinum* type E, amino-acid metabolism did not contribute significantly to the energy-generating pathways.

In the clostridial species that require organic nitrogen both for growth and for sporulation, the role of the nitrogenous compounds remains largely undetermined. Although there is substantial evidence to suggest that in some species the major role of these compounds is in energy generation during sporulation, it is not clear whether there is an additional requirement for specific nitrogenous compounds for endospore development. It is also not known whether there is a general requirement for a source of nitrogen during sporulation in some or all species of *Clostridium*.

In addition to the effects exerted by the nutrient composition and concentration, end products and other metabolites that are generated during vegetative growth play a key role in the regulation of sporulation in the clostridia. In aerobic species such as *Bacillus*, a large proportion (about one-third) of the available substrate is converted into biomass during aerobic growth. Under conditions where the substrate is oxidized efficiently, little or no build-up of toxic end products occurs. Inhibition of growth due to the accumulation of toxic metabolites prior to the onset of sporulation is therefore unlikely. However, in the clostridia, the fermentation pathways utilized during growth under anaerobic conditions result in the bulk of the substrate being catabolized to end products, whereas only a small proportion is converted into biomass. The highly reduced nature of the organic acids and alcohols produced during anaerobic fermentation make them harmful to biological systems. As a consequence, anaerobic bacteria tend to be strongly inhibited when the end products of metabolism accumulate to relatively low concentrations (Leung and Wang, 1979, Herrero, 1983).

The acidic nature of many of the end products produced during fermentation by clostridia results in a lowering of the pH value during growth. The pH value of the culture at the end of vegetative growth has been shown to play a major role in the outcome of sporulation in many species of *Clostridium*. A number of species of saccharolytic butyric acid-producing clostridia sporulate well in the presence of high concentrations of glucose, but only if the medium is well buffered with calcium carbonate or similar buffering agents (Collier, 1957; Bowen and Smith, 1955; Bergère and Hermier, 1964; Mackey and Morris, 1971; Jones *et al.*, 1982; Long *et al.*, 1983). The extent of the drop in the pH value of the culture depends on the buffering capacity of the medium and the initial carbohydrate concentration. If the former is insufficient or the latter is too high, the pH value drops too low (below pH 5) and the culture fails

to exhibit an increase in pH value at the end of the exponential growth phase and does not sporulate successfully (Collier, 1957; Bergère and Hermier, 1964; Mackey and Morris, 1971; Jones *et al.*, 1982; Long *et al.*, 1984b). Among the saccharolytic clostridia, the optimum pH value for sporulation is usually within the range 5.8–5.0 and if the pH value falls much below pH 5.0, sporulation and other metabolic activities are inhibited. A similar critical pH range at the end of the exponential growth phase, also has been reported for *C. thermosaccharolyticum* cultures (Hsu and Ordal, 1969a).

In contrast, proteolytic species and species that exhibit both proteolytic and saccharolytic metabolism are known to sporulate best at near neutral pH values (Perkins, 1965; Roberts, 1967), and pH 7.0 was found to be optimum for sporulation in *C. perfringens* type A (Labbe and Duncan, 1974). Sporulation was decreased at pH 6.0 or 8.0 and completely inhibited at pH 5.5. However, Labbe and Duncan (1974) reported that the type A strain sporulated after a long lag period following exposure for a short period to pH values as low as pH 2.0. A neutral or slightly acidic pH value provided the optimum conditions for sporulation of *C. botulinum*, *C. tetani* and *C. bifermentans* (Leifson, 1931; Holland and Cox, 1975). In species that exhibit both proteolytic and saccharolytic metabolism, an increase in the culture pH value at the onset of sporulation can be due to either a decrease in free acid or the production of ammonia or basic ammonium compounds. The fermentation of excess amounts of carbohydrates in these species may also result in the pH value of the culture decreasing to a level that inhibits sporulation completely (Perkins, 1965; Roberts, 1967; Holland and Cox, 1975; Sacks and Thompson, 1978). Under such conditions, adequate buffering by calcium carbonate, or by other buffers, has resulted in improved spore yields (Holland and Cox, 1975; Sacks and Thompson, 1978).

Where the inhibition of sporulation occurs in the presence of high concentrations of glucose, it is important to distinguish between those effects that may be due to catabolic repression and those effects that result from inhibition due to the low pH value of the culture medium.

In addition to influencing the pH values of the culture at the end of the exponential growth phase, a specific role of end-product accumulation in the induction of sporulation was demonstrated by Strasdine and Melville (1968). The fermentation of various carbohydrates with concurrent acid production was characteristic of those cultures of *C. botulinum* giving good endospore formation. Bacterial growth in the absence of acid production yielded cells with a reduced ability to form spores. A substantial amount of evidence has been provided to support the conclusion that acid end-product production is directly involved in the initiation of the solventogenic phase in *C. acetobutylicum* (Häggström and Molin, 1980; Gottschal and Morris, 1981a, 1982; Bahl *et al.*, 1982; Martin *et al.*, 1973; Yu and Saddler, 1983; Long *et al.*, 1984b).

Emeruwa *et al.* (1974) also proposed that acetate accumulation at the end of the exponential growth phase may induce enzymes associated with acetate metabolism and with the butyric acid type of fermentation associated with the transition from vegetative growth to sporulation in *C. botulinum* type E. They suggested that acetate accumulation is involved in the derepression of synthesis of the specific enzymes of these pathways. Acetate, and other acid products, may also accumulate during vegetative growth of *C. botulinum* as a result of the deamination of amino acids (Day and Costilow, 1964a). The accumulated acetate was shown to be neutralized during spore formation, but since clostridia are not able to oxidize such compounds, some role other than that of energy generation was indicated (Day and Costilow, 1964b).

In cultures of *C. acetobutylicum* P262 cell division was inhibited when 4–5 g of acid end products were present (Long *et al.*, 1984b). At this stage none of the nutrients were growth limiting and the enhanced glucose consumption that was observed following the cessation of growth was indicative of metabolic uncoupling by acid end products. The inhibition of cell division by acetate and butyrate was associated with the initiation of solvent production and differentiation. The involvement of acetate and butyrate in triggering solvent production and differentiation is indicated by the results of studies on the resuspension of vegetative cells in the acids, and by the addition of the acids to non-differentiated cultures. At low glucose or ammonium concentrations, growth was inhibited by nutrient limitation rather than by the accumulation of acid end products, and a shift to the solventogenic phase and cell differentiation did not occur. Gottschal and Morris (1981a) and Long *et al.* (1984b) used a non-metabolizable acid to replace acetate and butyrate and showed that the attainment of a low pH value alone is not sufficient to initiate the shift to the second phase of the fermentation.

Although a threshold concentration of acid end products and a limited pH range are essential, they are not sufficient on their own to ensure the development of the solventogenic phase and induce differentiation. These developments also require the presence of glucose and ammonium at the end of the acidogenic phase.

In addition to the accumulation of fermentation end products, an unknown product was excreted into the medium by exponentially growing *C. butyricum* cells (Bergère and Hermier, 1965b). This substance depressed growth and induced sporulation. Little information is available on the effect of the accumulation of end products resulting from the fermentation of proteinaceous substrates by proteolytic clostridia. Murrell (1967) pointed out that the critical limiting factors that may determine sporulation are difficult to detect in the complex media that are often required for the cultivation and sporulation of clostridia.

Although a number of studies have been carried out on the toxicity of

oxygen to *Clostridium* (Morris and O'Brien, 1971; Morris, 1975, 1976), very little work has been done on the effects of oxygen in relation to sporulation in the clostridia. Jones *et al.* (1980b) reported the formation of simple fruiting body-like structures in *C. acetobutylicum* which contained a high proportion of sporulating cells and free spores enclosed in a tough pliable sheath. This structure seemed to be intermediate between the ordinary bacterial colony and the elaborate multicellular fruiting bodies formed by myxobacteria, and could be regarded as an example of primitive multicellular differentiation. The production under aerobic conditions of an extracellular proteinaceous substance, which rapidly hardened on exposure to air to form a sheath, suggests a protective function for this material.

Recently, Long (1984) investigated the induction of sporulation in *C. acetobutylicum* in liquid media after exposure to air. No sporulation occurred in a glucose/mineral salts/biotin medium under anaerobic conditions but, on exposure of vegetative cells to air, 30% sporulation was observed. In a complex medium sporulation was increased two- to three-fold by aeration. The induction of sporulation in air did not appear to be dependent on the Eh value of the culture, or to be a consequence of either H_2O_2 or the generation of other toxic oxygen derivatives by *C. acetobutylicum* P262. Hydrogen peroxide did not enhance sporulation under anaerobic conditions, and neither catalase nor SOD inhibited sporulation in aerobic cultures.

There is little information on the effect of temperature on sporulation in *Clostridium*. In general, temperatures that produce optimum growth conditions appear to be optimum for spore production (Perkins, 1965; Roberts, 1967). Roberts (1967) stated that, in general, the incubation temperature for spore production was unimportant, but some species of *Clostridium* did sporulate better at 30°C than at 37°C. With *C. perfringens*, sporulation was inhibited by temperatures that were only slightly higher than the optimum temperature for growth (Labbe and Duncan, 1974; Labbe and Tang, 1983). A similar inhibitory effect of high temperature on sporulation of *C. acetobutylicum* was observed by Long (1984). The propagation of cells at temperatures that do not significantly affect growth, but which inhibit sporulation, have provided a useful system for the study of sporulation physiology (Labbe and Tang, 1983).

E. THE REGULATION OF SPORULATION

In cultures of *Bacillus* strains the probability that a cell will be induced to sporulate rather than to continue vegetative growth is determined by two factors (Young and Mandelstam, 1979). Firstly, the onset of sporulation is determined by the nutritional state of the culture, which directly influences the growth rate and the concentration of one or more intracellular biochemical

effectors. Secondly, the onset of sporulation is also dependent on the individual cells being in the right physiological condition to respond to the biochemical effector concentration (determined by the nutritional state of the culture). The capacity of the cell to respond to the biochemical effector is determined by the state of chromosome replication in the individual cells.

It is apparent that the factors responsible for the initiation of sporulation in *Clostridium* differ substantially from those in *Bacillus* where sporulation is triggered by nutrient limitation. Schaeffer *et al.* (1965) showed that when *B. subtilis* was grown in media containing readily utilizable carbon and nitrogen sources, the probability of endospore formation was very low, but it increased with poorer nitrogen sources. Using chemostat cultures, Dawes and Mandelstam (1970) subjected *B. subtilis* to nutritional limitation at a variety of growth rates in a defined medium. In these experiments some degree of sporulation was found at all growth rates, but sporulation was highest at the lowest growth rates. They concluded that the relationship between the incidence of sporulation and growth rate was a continuous function with no evidence of a threshold value. Furthermore, at any specified growth rate a definite fraction of the cells was induced to sporulate. These results were consistent with the assumption that sporulation is a repressible function.

In cultures of *Clostridium* spp. the probability that cells will sporulate also appears to be directly related to the growth rate, and extensive sporulation only occurs in cultures with decreased growth rates. However, the clostridia appear to differ from *Bacillus* strains in that an exogenous supply of energy, a carbon source and possibly a nitrogen source are required throughout the sporulation process (possibly due to less efficient anaerobic metabolism). Thus, in *Clostridium*, nutrient starvation resulting in a decreased growth rate does not generally lead to the production of spores except, possibly, in the case of cells possessing endogenous reserve material. Instead, sporulation appears to be favoured by conditions that lead to a decrease in growth rate in the presence of substantial energy and carbon-source reserves. In a number of species, the utilization of a poorly metabolized carbon source results in decreased growth rates in the presence of excess nutrients. In species where catabolic repression appears to be absent, the fermentation of a readily utilizable substrate results in the accumulation of inhibitory end products that cause a lowering of the growth rate in the presence of excess nutrients. In many cases the decline in growth rate leading to the onset of sporulation is accompanied by an enhancement in catabolic activity and substrate consumption. This is often accompanied by a shift in the pathways involved in energy generation, resulting in the production of other types of end products.

Mutations and antimetabolites that enable cells to sporulate under normal or adverse conditions have proved to be very useful in analysing the mechanisms controlling differentiation (Freese and Heinze, 1984). Although

sporulation in *Bacillus* normally requires starvation, the induction of sporulation events has been achieved in growth media containing excess ammonium ions, glucose and phosphate by mutation and by the addition of antimetabolites. The mutations and antimetabolites all directly or indirectly affected the synthesis of guanine nucleotides and resulted in a decrease in the levels of GTP and GDP. Sporulation can be induced or inhibited in *Bacillus* spp. by inhibitors that cause a decrease or increase, respectively, in GTP (Ochi *et al.*, 1982; Ochi and Freese, 1983).

Amino-acid limitation, which initiates sporulation, also evokes the stringent response. This response includes the accumulation of guanosine tetraphosphate (ppGpp) and guanine nucleotides, GTP and GDP (Lopez *et al.*, 1981). Sporulation is absent from relaxed (*relA*) mutants that lack the stringent response (Freese, 1981). Ochi *et al.* (1981) demonstrated that this was a consequence of an insufficient decrease in the level of GTP which did not suffice to initiate sporulation. Stringent and relaxed strains can be induced to sporulate by inhibitors of GMP (and thus GTP) synthesis, which indicates that the initiation of sporulation resulted from the decrease of GTP (or GDP) and not from the accumulation of ppGpp and pppGpp (Lopez *et al.*, 1981).

The role of GTP in the regulation of sporulation and enterotoxin production has been studied indirectly in *C. perfringens* by examining the effect of purine analogues and other compounds that partially inhibit the synthesis of purine nucleotides (Sacks and Thompson, 1975, 1977, 1978; Labbe and Nolan, 1981; Craven and Blankenship, 1982; Sacks, 1982, 1983). Methylxanthines (eg. caffeine and theobromine) and papaverine are phosphodiesterase inhibitors (Tsuboi and Yanigishima, 1973) and as such could affect the concentration of certain nucleotides. These compounds were effective in increasing sporulation and enterotoxin production in various *C. perfringens* strains, but no individual compound was effective in all strains.

Although Sacks and Thompson (1975) suggested that the iron-binding function of these compounds might account for their ability to enhance sporulation, methylxanthines are also known to inhibit the pathway of purine synthesis in some organisms. It is probable therefore that these compounds initiate sporulation in *C. perfringens* by inhibiting the synthesis of purine nucleotides. Decoynine, which is a known inhibitor of purine synthesis, also enhanced sporulation in *C. perfringens* (Sacks, 1980) and *C. acetobutylicum* (Long, 1984). Guanosine and other ribosides are known to repress sporulation in *C. perfringens* (Sacks and Thompson, 1975; Labbe and Nolan, 1981; Sacks, 1983).

It has been demonstrated with *Bacillus* spp. that, even when sporulation has been induced by exposure of cells to a suitable environment, there is, in addition, a requirement for DNA synthesis (Young and Mandelstam, 1979). The initiation of sporulation only seems to occur when a critical region of the

chromosome, situated at about 15 to 20 minutes from the chromosome origin, undergoes replication.

With *B. subtilis*, inhibition of DNA synthesis blocks sporulation and this has been used to distinguish between sporulation-specific events that are dependent on chromosomal replication and events that are not sporulation specific, but may be essential for the sporulation process (Mandelstam and Higgs, 1974; Dunn *et al.*, 1978; Young and Mandelstam, 1979). Similar experiments with *C. acetobutylicum* indicated that the inhibition of DNA synthesis also blocked sporulation in this species. The first sporulation-specific event that could be distinguished was the production of the forespore septum (Long *et al.*, 1984a). Clostridial stage, granulose, capsule and solvent formation, which were associated with the solventogenic phase, were not sporulation-specific events as they were induced in the absence of chromosomal replication. However, the shift to the solventogenic phase appeared to be essential for the production of mature endospores which were never observed in cultures that had not undergone the transition to the clostridial stage.

The isolation and characterization of mutants in which sporulation is blocked at distinct stages, and which differentiate abnormally, have formed the basis of studies on the genetic regulation of sporulation in *Bacillus* spp. The genetic analysis of differentiation in *Bacillus* species has been focused primarily on *B. subtilis* as many of the loci involved in sporulation have been identified by genetic mapping. Transformation, generalized transduction and marker frequency analysis have lead to the identification of some 35 sporulation loci in this strain. The application of recombinant DNA technology has resulted in the cloning and sequencing of a number of these genes, and to a study of their expression during sporulation (Hiroshika *et al.*, 1981; Bonamy and Szulmajster, 1982a; Jenkinson and Mandelstam, 1983; Fort and Piggot, 1984; Savva and Mandelstam, 1984). Although numerous sporulation mutants have been isolated in many other species of *Bacillus*, less effort has been devoted to the analysis of genes associated with sporulation in these strains.

In contrast to the bacilli, there has been almost no investigation of genetic regulation in the clostridia, due largely to the lack of genetic transfer systems in these organisms. Transduction systems have not been reported for any of the clostridia, and only recently has transformation (Reid *et al.*, 1983; Heffner *et al.*, 1984; Lin and Blaschek, 1984) and protoplast fusion (Jones *et al.*, 1985) been reported in a few species. To date the isolation and characterization of sporulation mutants in the clostridia have been used almost exclusively to study the relationship between sporulation and toxin production (Sebald and Schaeffer, 1965; Sebald and Cassier, 1969; Duncan *et al.*, 1972; Sacks and Thomas, 1979; Löffler and Labbe, 1983), and the relationship between sporulation and the shift in metabolism that occurs at the beginning of the

stationary phase in cultures of some species (Emeruwa and Hawirko, 1972; Hawirko *et al.*, 1973; Emeruwa *et al.*, 1974; Jones *et al.*, 1982; Long *et al.*, 1984a; Landuyt and Hsu, 1984).

Sporulation can be viewed as a dependent and essentially linear sequence of gene expression. During the initial stages of sporulation approximately 20 individual sporulation-specific genetic loci are activated and appear to be expressed in a number of separate transcriptional steps (Piggot and Coote, 1976; Young and Mandelstam, 1979; Piggot *et al.*, 1981). It has been proposed that some or all of these transcriptional changes are mediated by alterations in the template specificity of the RNA polymerase which enables it to recognise new classes of promoters or allows read through of termination sites. The role of sporulation-specific sigma factors in altering RNA polymerase during sporulation has been reviewed by Losick and Pero (1976), and by Losick (1981).

RNA polymerase structural mutants were isolated as mutants that were resistant to RNA polymerase inhibitors such as rifampicin, streptolydigin and streptovaricin. Many of these mutants also exhibit sporulation defects (Piggot and Coote, 1976; Bonamy and Szulmajster, 1982b; Sumida-Yasumoto and Doi, 1977; Leighton, 1977). In *C. acetobutylicum* the isolation of mutants resistant to rifampicin, streptolydigin and streptovaricin also gave a high proportion of isolates that exhibited sporulation defects (Jones *et al.*, 1982; Long *et al.*, 1984a). However, it has not been established whether alterations in the RNA polymerase result in a general imbalance in transcription or in specific changes affecting the transcription of sporulation genes.

There have been numerous reports suggesting that changes in translation and in the protein-synthetic machinery also occur during sporulation in *Bacillus* spp. Translational controls have been suggested to operate via stable mRNA, alteration in ribosomes or alteration in rRNA or tRNA synthetases. However, there is still no compelling evidence to show that any of these changes are essential for sporulation. Almost nothing is known about the role of translational changes in *Clostridium*, but evidence has been put forward for the involvement of stable mRNA in enterotoxin synthesis and spore formation in *C. perfringens* (Labbe and Duncan, 1977a).

IV Summary

Studies on the responses of anaerobes to environmental stress are limited, and within the *Bacteroides* group they are virtually restricted to one strain of *B. fragilis* (Bf-2). This strain has been shown to exhibit a variety of physiological responses and to induce new proteins in response to various stress factors and DNA-damaging agents. However, available evidence suggests that *B. fragilis*

lacks a *recA* controlled error-prone SOS processing system which is characteristic of *E. coli*. The DNA repair pathways in *B. fragilis* are largely error-free. In *E. coli* the regulatory networks controlling the SOS response and the heat-shock response are interconnected. Ultraviolet irradiation and nalidixic acid, which induce the SOS response, also induce the heat-shock response in *E. coli* (Walker, 1984). In *B. fragilis* there is no connection between the regulatory networks controlling the heat-shock response and the responses to UV irradiation, oxygen and H_2O_2 (the latter being interconnected since they involve the induction of apparently similar proteins). The absence of any overlap between heat-shock response and the responses to oxygen and H_2O_2 is particularly interesting since heat shock and oxidative stress have been shown to be linked in aerobic bacteria. It is concluded that, in *B. fragilis*, the response to DNA-damaging stress factors and other environmental stress factors, such as heat shock, involve separate regulatory networks.

The study of sporulation in *Clostridium* has lagged behind that in *Bacillus* due to (1) the technical difficulties in working with anaerobes, (2) the requirements for complex media for growth and sporulation in many species, (3) the difficulty in obtaining sporulation and the lack of synchrony during sporulation in many strains, and (4) the lack of suitable genetic transfer systems in these organisms. However, defined media giving synchronous sporulation are now available for a number of species.

Morphological changes during sporulation are essentially similar to those observed in *Bacillus*, but some differences are apparent. The production of mature spores at both poles of the cell has been reported in various clostridia. During spore maturation, coat production begins earlier in clostridia than in *Bacillus*, and in many species there is a reversal in the order so that coat development occurs before cortex development. The production of an exosporium is common, and considerable variation in structure and form exists. The production of elaborate spore appendages is also widespread. The accumulation of intracellular storage granules, which in most cases consist of a polyglucan, occurs at the end of the growth phase in many clostridia and may serve as an energy and carbon reserve during sporulation.

Attempts to establish biochemical marker events during sporulation have met with limited success. Toxin production is common amongst the clostridia, and in some cases a direct link between toxin production and sporulation exists. In a number of species a shift in cell metabolism occurs concurrently with the onset of sporulation which may involve a shift from acid production to the production of alcohol or other solvents. Enhanced substrate utilization associated with the onset of sporulation has also been reported. From the limited data available it appears that rapid protein turnover during sporulation is limited or absent, but extensive autolysis associated with the onset of sporulation has been reported in a number of species.

There is little evidence implicating nutrient starvation in the triggering of sporulation in *Clostridium*, and many species have been shown to require a source of energy (carbon substrate) and possibly nitrogen throughout the sporulation process. Endotrophic sporulation has not been reported to occur in the clostridia. Catabolic repression occurs in species utilizing mixed carbohydrates and the presence of a poorly utilized carbohydrate appears to play a critical role in the triggering of sporulation in these species. In strains where catabolic repression is absent, the accumulation of acid end products or other inhibitory metabolites appear to play a major role in the onset of sporulation. In all cases the resulting decrease in growth rate appears to be important in the triggering of sporulation. The accumulation of acid end products during fermentation also results in a lowering of the pH value of the culture, and in poorly buffered media the resulting low pH value completely inhibits sporulation.

The biochemical events involved in the regulation of sporulation in *Clostridium* have been little studied and are poorly understood. Studies employing inhibitors suggest that the internal effectors involved in the induction of sporulation may be similar to those that occur in *Bacillus*. Chromosome replication may also be a general prerequisite for the induction of sporulation in *Clostridium* strains. Little is known about the genetic regulation of sporulation in *Clostridium*. The study and use of sporulation mutants has been almost entirely restricted to investigating the relationships between sporulation and toxin production, and between sporulation and solvent formation. The isolation of drug-resistant mutants with altered RNA polymerase, which exhibit sporulation defects, suggest that altered RNA polymerase specificity may be involved in the regulation of sporulation in *Clostridium*.

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Note Added in Proof

Although the available evidence suggested that *Bacillus fragilis* lacked a *recA* controlled error-prone SOS processing system which is characteristic of *Escherichia coli*, our laboratory has recently cloned a *recA*-like gene from *B. fragilis*. The *B. fragilis* gene renders an *E. coli recA* mutant more resistant to ultraviolet irradiation and mitomycin C, and also enables phage Lambda to form an inducible prophage relationship in the *E. coli recA* mutant. The cloned *B. fragilis* gene produces two proteins with apparent molecular masses of 37,000 and 39,000 Da. Both proteins cross-react with antiserum prepared against purified *Escherichia coli recA* protein. The reason for the apparent absence of *recA* controlled error-prone SOS processes in *B. fragilis* is not known.

The Fimbrial Adhesins of *Escherichia Coli*

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I. Introduction

Escherichia coli is known as a normal inhabitant of the intestinal tract of man and several animals but also as an aetiological agent of a wide variety of both intestinal and extra-intestinal pathological conditions. Commensal *E. coli* strains are residents of the large intestine, which contains, at any one time, many different *E. coli* strains usually with one dominant serotype. In principle, each animal species is characterized by its own serotypes, though some serotypes do occur in more than one species.

Pathogenic *E. coli* strains can be distinguished from the commensal strains by their ability to colonize habitats where commensal strains usually do not survive. Like commensal strains, pathogenic strains tend to be host-specific. Despite the existence of a large number of different serotypes, only a very limited number of serotypes is associated with a particularly pathological condition in man or domestic animals (Sojka, 1973; Evans and Evans, 1983).

The most common manifestations of *E. coli* infections are diarrhoeal conditions associated with enteropathogenic strains and urinary tract infections caused by uropathogenic strains. The enteropathogenic *E. coli* strains are classified into enterotoxigenic, enterotoxemic, and entero-invasive or "shigella-like" strains (Moon, 1974). The enterotoxigenic strains are characterized by the production of heat-stable (ST) and/or heat-labile (LT) enterotoxins. In man, these strains are recognized as aetiological agents of "infantile diarrhoea" and of "traveller's diarrhoea", a diarrhoeal disorder in adults. In pigs, enterotoxigenic strains are associated with neonatal diarrhoea and with colibacillosis that occurs in 3-week-old pigs, or immediately post-weaning. In calves, the enterotoxigenic strains are frequently found in neonatal diarrhoea commonly known as "white scour". The enterotoxigenic strains colonize the small intestine where they elaborate enterotoxins that cause a massive secretion of fluid from the tissues into the intestinal lumen leading to watery diarrhoea and dehydration.

Enterotoxemic *E. coli* strains, usually indicated as enteropathogenic strains, also colonize the small intestine. They generally produce toxins which do not respond in the usual tests for enterotoxins. In man, classical enteropathogenic strains that cause a "Salmonella-like" disease may be classified as enterotoxemic. In pigs, the oedema disease is a prototype of an enterotoxemic infection. This disease is characterized by acute neurological disturbances and the responsible toxin has been referred to as *E. coli* neurotoxin, or as oedema disease principle (EDP).

Entero-invasive *E. coli* strains colonize and proliferate primarily in the colon and invade the epithelial cells causing a "dysentery-like" syndrome. These strains do not produce enterotoxins. Their invasiveness is the main virulence factor.

In extra-intestinal disease, *E. coli* is known as an aetiological agent in urinary tract infections in man (cystitis, acute pyelonephritis), neonatal meningitis, septicaemic colibacillosis in calves and other syndromes of Gram-negative bacteraemia (e.g. nosocomial infections, peritonitis, appendicitis).

An important and often crucial event in the colonization of host epithelia by commensal or pathogenic *E. coli* strains is the adherence of the bacteria to the mucosal surfaces or to the brush-borders of epithelial cells lining the intestinal or urinary tract. Adherence of enterotoxigenic or uropathogenic *E. coli* strains seems to be a relatively simple process in which fimbrial adhesins located at the surface of the bacteria interact with specific receptor substances on the epithelial cells. In general, each adhesin is more or less characteristic for a particular type of *E. coli* disease in man, or in a limited number of animal species (Gaastra and de Graaf, 1982). In addition to these so-called host-specific adhesins, most pathogenic and commensal strains carry a common type of adhesin (type 1 fimbriae). It should be emphasized that many *E. coli* strains possess the ability to produce more than one type of adhesin.

Although attachment has been recognized as a conclusive initial event in the colonization of different epithelia by a wide variety of *E. coli* strains, only a limited number of strains have been studied in sufficient depth with respect to the mechanisms of adherence at the molecular level.

The aim of this review is to evaluate current knowledge of *E. coli* adhesins and their receptors. The main emphasis will be on the genetic and physiological aspects of adhesin production, the primary structure of adhesin subunits, their adhesive properties, and the characterization of adhesin receptors.

II. Terminology and Classification

Adhesins are macromolecular structures present on the bacterial cell and mediate the attachment of these cells to a surface. The terms adhesion, adherence or attachment are used to indicate the interaction between bacteria and receptor structures present in the mucosa or in the glycocalyx of epithelial cells. Most adhesins studied are polymeric structures and can be described as non-flagellar filamentous appendages assembled from protein subunits. On the basis of their morphology, these filaments have been named fimbriae (Latin for threads or fibres) by Duguid *et al.* (1955) or pili (Latin for hairs) by Brinton (1959). Both terms appeared to be linguistically correct (Duguid and Anderson, 1967) and can be used as synonyms. We have chosen to use the terms fimbriae, fimbrial and fimbriated because of priority in usage. The term fimbrial adhesin is used to denote both morphology and function.

The ability to produce fimbriae is distributed among a wide variety of

mainly Gram-negative bacteria. Variation in morphology and adhesive properties has stimulated attempts at their classification, but these classification schemes are rather incomplete and provisional. Ottow (1975) divided fimbriae into six different groups on the basis of their morphology, arrangement and adhesive properties. Earlier classifications, mainly based on data obtained with *Enterobacteriacae*, were presented by Brinton (1965) and by Duguid *et al.* (1966).

The majority of the commensal and pathogenic *E. coli* strains produce type 1 fimbriae, classified after Duguid *et al.* (1966) and also designated as *E. coli* "common" fimbriae. They consist of aggregates of a single repeating protein subunit with a molecular weight of 17,000 (Brinton, 1965; Salit and Gotschlich, 1977a). Type 1 fimbriae confer on the bacterial cells the ability to attach to a wide variety of animal, plant, fungal and yeast cells, indicating a role for these structures as a general colonization factor.

In addition to type 1 fimbriae, most pathogenic *E. coli* strains possess the ability to produce a variety of other fimbriae with a definite role in colonization of certain epithelial surfaces. These adhesins were not included in previous classification schemes (Brinton, 1965; Duguid *et al.*, 1966; Ottow, 1975).

The interaction of fimbriae with "specific" receptor substances argues in favour of a classification based on the chemical nature of receptor structures, for instance adhesins recognizing mannose residues (M fimbriae) or galactose residues (G fimbriae) or those recognizing sialylated carbohydrates (S fimbriae). At present, however, such a classification would be very incomplete since only a very few receptor structures have been analysed. Moreover, such a classification may be confusing since, for instance, fimbriae associated with certain uropathogenic strains are already designated according to the blood-group antigens they recognize (i.e. P fimbriae recognizing P blood-group antigens, M fimbriae recognizing blood-group M antigens).

A quite different classification of *E. coli* adhesins is based on their antigenic properties. Strains of *E. coli* are classified primarily by serological identification of their three major types of surface antigens: O (lipopolysaccharide), K (capsular polysaccharide) and H (flagella) (Orskov *et al.*, 1977). Orskov and Orskov (1983) have proposed to use the fimbrial adhesins as a fourth group of surface antigens. They used crossed immunoelectrophoresis for the serological analysis of fimbriae and proposed to use the prefix F to designate fimbrial antigens. Type 1 fimbriae from different strains are closely related and all share a common antigenic determinant designated as F1. Some strains, however, possess additional group-specific antigens (Duguid *et al.*, 1979). The host-specific fimbrial antigens of pathogenic *E. coli* strains are antigenically diverse and usually show a high degree of species specificity. Orskov and Orskov (1983) proposed the following designations: F2 = CFA I,

F3=CFA II, F4=K88, F5=K99, F6=987P and F7–F12=various serotypes of P fimbriae present on uropathogenic strains. Some properties of the host-specific adhesins are summarized in Table 1.

The K88 adhesin of porcine enterotoxigenic strains was analysed by Stirm *et al.* (1967a,b) and characterized as a thin flexible fimbriae composed of protein subunits with a molecular weight of 26,500. The diameter of K88 fimbriae has been estimated to be 2.1 nm (Wadström *et al.*, 1979). Three serologically different K88 variants have been detected: K88ab, K88ac and K88ad (Orskov *et al.*, 1964; Guinée and Jansen, 1979) with an apparent molecular weight of 23,500 to 26,000 depending on the K88 variant (Mooi and de Graaf, 1979).

Another fimbrial adhesin occurring on porcine enterotoxigenic strains is the 987P adhesin characterized by Isaacson and Richter (1981). Morphologically the 987P fimbriae cannot be distinguished from type 1 fimbriae, having a diameter of 7 nm and an apparent axial hole. The molecular weight of the 987P fimbrial subunit is approximately 20,000. A slightly different molecular

TABLE 1. Characteristics of some fimbrial adhesins of *Escherichia coli*

Adhesin	Subunit molecular weight	Diameter fimbriae (nm)	Host
A. Enterotoxigenic strains			
type 1	17,000	7	
K88 (ab, ac, ad)	23,000–26,000	2.1	pig
987 P	20,000	7	pig
K99	18,500	5	calf, lamb, pig
F41	29,500	3.2	calf, lamb, pig
CFA I	15,000	7	human
CFA II			
CS1	16,300	7	human
CS2	15,300	7	human
CS3	14,800	2	human
CFA III	16,000	7	human
B. Uropathogenic strains			
type 1	17,000	5–7	human
F7 (1, 2)	22,000	5–7	human
F9	19,500	5–7	human
F12	18,200	5–7	human
KS 71A	21,300	5–7	human
KS 71C	16,600	5–7	human
ER 2B2	16,200	5–7	human

weight of 18,900 was reported by Fusco *et al.* (1978). Strains producing both K88 fimbriae, as well as 987P fimbriae, have been detected (Schneider and To, 1982).

The K99 adhesin can be detected on many enterotoxigenic strains isolated from calves, lambs or pigs. The K99 fimbriae have a diameter of about 5 nm (Wadström *et al.*, 1979: de Graaf *et al.*, 1980b) and consist of protein subunits with an apparent molecular weight of about 18,500 (de Graaf *et al.*, 1980b; Isaacson *et al.*, 1981). The K99 adhesin is the only adhesin described to date with a strong positive charge at neutral pH values (Isaacson, 1977; de Graaf *et al.*, 1980b).

Many strains carrying K99 fimbriae, in particular strains of serogroup O101 and O9, produce a second adhesin F41 (Morris *et al.*, 1982b). Purified F41 fimbriae have a diameter of about 3.2 nm and are composed of protein subunits with a molecular weight of 29,500 (de Graaf and Roorda, 1982). A different apparent molecular weight of 34,000 was reported by Chanter (1982).

Human enterotoxigenic *E. coli* strains produce a larger variety of adhesins than strains isolated from various animal species. The CFA I adhesin originally described by Evans *et al.* (1975) consists of fimbriae with a diameter of 7.0 nm (Freer *et al.*, 1978; D.G. Evans *et al.*, 1979). CFA I isolated by Evans and coworkers was described as being composed of protein subunits with a molecular weight of 23,500. However, CFA I purified by Klemm (1979) appeared to be composed of subunits with an apparent molecular weight of 14,500. Recently, the molecular weight of CFA I subunits was calculated to be 15,058 by amino-acid sequence analysis (Klemm, 1982).

Other human enterotoxigenic strains belonging to serogroup O6 or O8 possess the CFA II adhesin (Evans and Evans, 1978). On testing a variety of strains of serotype O6:K15:H16 or O6:K15 for the presence of CFA II, Smyth (1982) identified three immunologically distinct antigens designated CS1, CS2 and CS3. The CS1 antigen was found on strains of biotype A (rhamnose-negative), CS2 on strains of biotype B, C and F (rhamnose-positive), and CS3 was detected on most strains possessing CS1 or CS2 and thus was independent of the biotype. The CS3 antigen was also detected on enterotoxigenic strains of serotype O8:K40:H9 which lack the CS1 and CS2 antigens. Some strains of serogroup O6 producing only CS2 were identified (Smyth, 1982). Subtypes of CFA II also have been detected on *E. coli* serotype O78 (Svennerholm and Ahrén, 1982). The molecular weights of the protein subunits of CS1, CS2 and CS3 are 16,300, 15,300 and 14,800, respectively (Smyth, 1982). CS1 and CS2 fimbriae are indistinguishable from type 1 fimbriae, whereas CS3 fimbriae are very thin structures with a diameter of 2 nm (Mullany *et al.*, 1983; Levine *et al.*, 1984; Smyth, 1984). The relationship between the originally described CFA II antigen and the CS antigens is still

uncertain, but most probably CFA II is identical to CS1. According to Smyth (1984) the fimbriae described by Evans and Evans (1978) are CS1, whereas the antiserum they isolated is directed against CS3.

Another adhesin, designated CFA III, was discovered by Darfeuille *et al.* (1983). CFA III fimbriae are 7 nm in diameter and composed of subunits with a molecular weight of 16,000.

Some fimbrial adhesins of uropathogenic *E. coli* strains have been isolated and characterized. The P fimbriae of *E. coli* strain 3048 (serotype O4:K3) and strain 3669 (serotype O2:K5:H1) are composed of protein subunits with a molecular weight of 19,500 (Svanborg Edén *et al.*, 1983). In addition to these P fimbriae, which recognize globoseries glycolipids, strain 3048 produces type 1 fimbriae (Korhonen *et al.*, 1980b, 1981). Purified P fimbriae have a diameter of 5–7 nm. The P fimbriae of strain 3669 have been classified as the F9 antigen (Orskov and Orskov, 1983). Another type of P fimbriae, designated F12 antigen, was isolated and purified from strain C1979 (serotype O16:K1:H6). These fimbriae are composed of 18,200 Da subunits (Klemm *et al.*, 1983). The fimbrial antigen F7, isolated from strain C1212 (serotype O6:K2:H1), is composed of 22,000 Da subunits (Klemm *et al.*, 1982). In addition to antigen F7, this strain produces type 1-like fimbriae called 1C (Orskov *et al.*, 1982).

Generally, uropathogenic strains produce two or three different types of fimbriae in addition to type 1 fimbriae (Jann *et al.*, 1981a; Orskov and Orskov, 1983; Rhen *et al.*, 1983b,c). The fimbriae of two strains (KS71 and ER2) have been further characterized (Table 1). All these fimbriae are 5–7 nm in diameter and closely related in amino-acid composition (Rhen *et al.*, 1983b).

Recently, Korhonen and coworkers (1984) characterized another type of fimbriae isolated from strains associated with neonatal sepsis or meningitis, and recognizing sialylgalactosides as receptor. These so-called S fimbriae are morphologically identical to the type 1 and P fimbriae and are composed of subunits with an apparent molecular weight of 17,000.

III. Adhesive Properties

A. HAEMAGGLUTINATION

Haemagglutination was the first observed manifestation of the adhesive properties of bacteria and most strains of *E. coli* possess this activity. Duguid *et al.* (1955) showed that haemagglutinating activity was related to the presence of fimbriae. *Escherichia coli* strains carrying type 1 fimbriae were able to agglutinate a large variety of erythrocytes of different animal species, although variations were observed in the strength of haemagglutinating activity (Duguid *et al.*, 1955; Collier *et al.*, 1955; Collier and Jacoeb, 1955).

Remarkably, ox erythrocytes were not agglutinated. Growth conditions have a marked effect on haemagglutinating activity of type 1-fimbriated bacteria (see Section VIII). Some strains were shown to be variable in their haemagglutinating activity and readily underwent genetic variation between agglutinating and non-agglutinating forms.

With most *E. coli* strains, the haemagglutinating activity can be inhibited with D-mannose (Collier and de Miranda,1955; Duguid and Gillies, 1957), indicating the presence of type 1 fimbriae (Brinton, 1965). However, pathogenic strains of *E. coli*, carrying host-specific adhesins, were able to agglutinate erythrocytes in a way that was not sensitive to mannose. This type of haemagglutination, also called "mannose-resistant" haemagglutination, is developed when the strains are grown at 37°C but not at 18°C. Haemagglutination tests for mannose-insensitive adhesins should be done at 3–5°C. On raising the temperature of the agglutination mixture, the bacteria are generally eluted from the agglutinated erythrocytes. Therefore, mannose-insensitive haemagglutinins are also designated as eluting haemagglutinins. A large number of *E. coli* strains of various O serogroups and origin were tested by Duguid *et al.* (1979) for the presence of mannose-sensitive as well as mannose-insensitive haemagglutinating activity with erythrocytes obtained from various animal species. The majority of the strains showed mannose-sensitive haemagglutination associated with the presence of type 1 fimbriae. About half of these strains also exhibited mannose-insensitive haemagglutination when grown and tested under the appropriate conditions. A limited number of strains carried mannose-insensitive haemagglutinins only. Obviously, many strains are able to produce more than one type of haemagglutinin. Different types of haemagglutinins require different cultural conditions for their development and different test conditions and types of erythrocytes for their demonstration. It should be noted that, although the mannose-insensitive haemagglutinins are generally identified as fimbriae, some *E. coli* strains appear to produce non-fimbrial haemagglutinins (Ip *et al.*, 1981).

Since *E. coli* strains carrying one particular type of mannose-insensitive haemagglutinin (adhesin) appear to bind to a limited number of erythrocyte species, it has been proposed to use the haemagglutination for typing of *E. coli* strains from human sources (D.J. Evans *et al.*, 1979, 1980). It should be noted, however, that although a particular type of adhesin is able to agglutinate only certain types of erythrocytes, the haemagglutination pattern as such cannot always be used to identify any particular adhesin (Salit *et al.*, 1983). Similar patterns of haemagglutination exhibited by human strains carrying CFA I, for instance, were also observed with strains from extra-intestinal sources (Cravioto *et al.*, 1979b, 1982; Minshew *et al.*, 1978). Moreover, one particular haemagglutinating activity (as, for instance, the agglutination of bovine

erythrocytes by strains carrying CFA II) appeared to vary with the erythrocyte sample collected from different animals, which indicates that the CFA II-induced reaction may be limited to only certain specific blood-group antigens of bovine erythrocytes (Faris *et al.*, 1982).

Instead of using erythrocytes, the adhesive properties of strains carrying type 1 fimbriae also can be demonstrated by the agglutination of yeast cells containing mannans at their surface (Duguid and Gillies, 1957; Ofek *et al.*, 1977). Yeast agglutination has been used for the screening of bacterial isolates for mannose-sensitive adhesins (Korhonen, 1979; Mirelman *et al.*, 1980).

Although agglutination of erythrocytes or yeast cells is generally accepted as an indication for the presence of cell-surface adhesins, the question remains as to whether surface components present on erythrocytes or yeast cells, and which are recognized by the bacterial adhesin, are always identical with the "natural" receptor for *E. coli* strains on the host epithelial cells. Jann *et al.* (1981b) have shown that there is no correlation between the ability of a particular *E. coli* strain to agglutinate erythrocytes or yeast cells and the capacity of this strain to adhere to different types of mammalian cells. Obviously, the adherence of bacteria to eukaryotic cells is a rather complex phenomenon which not only depends on the type of adhesin(s) expressed by a particular strain under a defined growth condition, but also on a variety of different receptor structures present on the eukaryotic cells.

B. ADHESION TO EPITHELIAL CELLS

On infection, *E. coli* cells become attached to the glycolipids or glycoproteins present on the epithelial cell surfaces (the glycocalyx), or to the mucus layer composed of other glycoproteins secreted by specialized cells (goblet cells). This mucus gel may form an extra barrier which impedes free access of bacteria to the epithelial cells. Strains carrying only type 1 fimbriae are believed to adhere to the mucus gel. Enterotoxigenic or uropathogenic strains are supposed to penetrate the mucus either passively or actively and become attached to the brush-borders of the epithelial cells. The strong interaction between bacteria and the brush-border will then prevent the bacteria being washed away. It should be noted that glycoproteins present in the mucus gel, or in saliva, may share receptor specificities with the glycolipid and glycoproteins present in the brush-border membranes.

1. Enterotoxigenic Strains of Domestic Animals

Strains of *E. coli* possessing K88 fimbriae colonize the pig small intestine via adherence to the epithelial cells (Arbuckle, 1970; Bertschinger *et al.*, 1972; Hohmann and Wilson, 1975). Bacteria were shown to be present contiguous

to the brush-borders along the villi, but not in the crypts. K88-negative strains did not attach to the epithelia of the small intestine but were distributed in the lumen (Jones and Rutter, 1972; Wilson and Hohmann, 1974; Hohmann and Wilson, 1975).

Sellwood *et al.* (1975) developed an *in vitro* assay in which direct interaction between K88-positive bacteria and brush-borders isolated from pig intestinal epithelial cells was measured using phase-contrast microscopy. It appears that brush-borders isolated from some pigs lack receptor activity and that these animals are resistant to infection by K88-positive enterotoxigenic *E. coli* strains (Rutter *et al.*, 1975). The receptor activity was inherited in a simple Mendelian way involving one locus and two alleles (Gibbons *et al.*, 1977), and the allele coding for the receptor was dominant over the non-adhesive allele. Bijlsma *et al.* (1981) used the brush-border adhesion test to investigate whether adhesion-positive pigs possess different receptors for the three serological K88 variants ab, ac and ad. They were able to show that, in addition to the adhesion-negative phenotype, to which none of the K88 variants adhere, the brush-borders of adhesion-positive pigs could be subdivided into four groups. Phenotype A is recognized by all three K88 variants, phenotype B adheres K88ab- and K88ac-positive strains, phenotype C adheres K88ab- and K88ad-positive strains, and phenotype D adheres only cells carrying K88ad. Preincubation of type A brush-borders with purified K88ad did not prevent adhesion of K88ab- or K88ac-positive bacteria, but in most cases strains K88ab and K88ac blocked subsequent adhesion of K88ad-positive bacteria (Bijlsma *et al.*, 1982). The existence of different variants of "adhesive" pig phenotypes is a further confirmation of results obtained by Wilson and Hohmann (1974) who showed that adhesiveness is associated with the variable antigenic determinants K88b and K88c and that their adhesive ability can only be neutralized by the homologous antisera. Parry and Porter (1978) confirmed this conclusion, in general. The results could be explained by assuming the existence of separate receptors specific for each of the K88 variants. This assumption does not explain why blocking with a particular K88 variant also inhibits the adhesion of other variants. Therefore, it was assumed that only one K88 receptor exists which, depending on its modification(s), has a different affinity for the various K88 variants.

Another group of porcine enterotoxigenic *E. coli* strains, which also adhere to the pig intestinal epithelium *in vivo* and to isolated epithelial cells *in vitro* (Hohmann and Wilson, 1975; Nagy *et al.*, 1976; 1977; Moon *et al.*, 1977a), was shown to produce another fimbrial adhesin called 987P. The 987P-mediated adhesion *in vitro* is not restricted to porcine intestinal epithelial cells alone. Strains carrying this adhesin also adhered to epithelial cells or brush-borders isolated from adult, but not from infant, rabbits, although they did not colonize or cause diarrhoea in rabbits (Dean and Isaacson, 1982).

Addition of purified 987P fimbriae to adult rabbit brush-borders, or antiserum prepared against purified 987P fimbriae, prevented the adhesion of 987P-positive bacteria (Isaacson *et al.*, 1978).

Bovine enterotoxigenic strains carrying K99 fimbriae attached to the intestinal epithelium of calves, lambs and pigs (Moon *et al.*, 1977b; Bellamy and Acres, 1979; Hadad and Gyles, 1982; Chan *et al.*, 1982). Tests to measure adhesion to intestinal villi *in vitro* have been developed (Girardeau, 1980; Dean and Isaacson, 1982). It appears that *in vitro* attachment of K99-producing enterotoxigenic strains occurs with villi preparations from the jejunum of calves, lambs, pigs, rabbits, rats and mice, indicating that K99-positive cells are also able to adhere to the intestinal epithelial cells of various animals that are not colonized *in vivo*. Infant mice, however, appear to be infected by K99-positive strains and thus present a very suitable model to study the pathogenicity of K99-positive enterotoxigenic strains *in vivo* (Duchet-Suchaux, 1980). Epithelial cells isolated from older pigs, calves and mice were resistant to adhesion by K99-positive strains (Runnels *et al.*, 1980). Remarkably, also K88-positive strains adhered in high numbers to calf intestinal epithelial cells, even though such strains did not colonize the calf intestine *in vivo*. However, the observed adhesion of K88-positive bacteria occurred predominantly on basolateral membranes of the calf epithelial cells rather than on their brush-borders.

Another fimbrial adhesin associated with enterotoxigenic *E. coli* strains isolated from domestic animals is the F41 adhesin. Adhesin F41 occurs either in combination with K99 fimbriae, or alone. Strains carrying both F41 and K99 have been isolated from calves, lambs and piglets (Morris *et al.*, 1982b). Strains carrying only adhesin F41 have been isolated from piglets (Morris *et al.*, 1983a; Moon *et al.*, 1980), but are also able to colonize the villi of the small intestine of lambs (Morris *et al.*, 1983b) and cause diarrhoea. Recently, To (1984) described virulence studies with a number of strains producing F41 and type 1 fimbriae but lacking the other mannose-insensitive adhesins K88, K99 and 987P. The F41-producing strains were found to be virulent in newborn pigs and calves and induced diarrhoea. The production of F41 fimbriae in the intestine can be demonstrated by immunofluorescence staining of ileal villi from infected animals. The type 1 fimbriae produced by these strains were not involved in the adhesion (Awad-Masalmeh *et al.*, 1982).

2. Human Enterotoxigenic Strains

Several methods have been developed to investigate the adhesive properties of human enterotoxigenic strains *in vitro*. Buccal cells, as well as isolated foetal

enterocytes, have been used in these studies (McNeish *et al.*, 1975; Ofek *et al.*, 1977). In general, adhesion to foetal enterocytes correlated with adhesion to buccal epithelial cells of patients from whom the strains were isolated (Candy *et al.*, 1978). Comparison of adherence to buccal epithelial cells with adherence to foetal enterocytes indicated that type 1 fimbriae may play an important role in adhesion to buccal cells but a very limited role in adhesion to foetal enterocytes (McNeish *et al.*, 1975; Candy *et al.*, 1981). Also, Thorne *et al.* (1979) have shown that human enterotoxigenic strains adhere to buccal epithelial cells, but they found no correlation between this binding and mannose-insensitive haemagglutination of erythrocytes. In agreement with this observation, Jann *et al.* (1981b) found no correlation between the haemagglutinating ability and the adherence to monolayers of cells derived from the human intestine. These results indicate that different adhesins function in mannose-insensitive haemagglutination and adherence to enterocytes versus adherence to buccal epithelial cells.

Recently, a more appropriate adhesion test for the natural target cells of human enterotoxigenic strains was developed by Deneke *et al.* (1983) using human intestinal epithelial cells isolated from patients with an ileostomy. Enterotoxigenic strains isolated from humans and possessing fimbrial adhesins (Deneke *et al.*, 1979) were found to adhere to a greater extent than control strains of different origin and carrying various other adhesins. Binding activity was demonstrated in the presence of D-mannose and appeared to be much stronger than that observed with buccal epithelial cells (Thorne *et al.*, 1979). The adhesive properties were not attributed to a particular type of adhesin. Remarkably, strains carrying K88 fimbriae bound equally well to the epithelial cells as did strains of human origin. Also Lafeuille *et al.* (1981) used human intestinal epithelial cells to study adherence of *E. coli* isolates obtained from children with or without diarrhoeal disease. Most strains isolated from the children with diarrhoea adhered to the brush-borders of the epithelial cells, some of which possessed either CFA I or CFA II fimbriae.

Using human intestinal brush-borders, Cheney and Boedeker (1983) observed that the adherence of the human enterotoxigenic strains H10407, which produces both type 1 and CFA 1 fimbriae, was dependent on the expression of CFA I. When grown under conditions that promote expression of only type 1 fimbriae, the strain did not adhere. Recently, Knutton *et al.* (1984a) described an assay system for human enterotoxigenic strains with isolated human enterocytes prepared from duodenal biopsies. The fimbrial adhesins CFA I and CFA II, but not type 1 fimbriae, promote the attachment of these strains to the enterocyte brush-borders. On the other hand, the type 1 fimbriae produced by many of these strains promote basolateral attachment to the isolated enterocytes. The basolateral surface, however, is normally not exposed to bacteria *in vivo*. Electron microscopy showed the formation of

many interactions between the tips of the fimbriae and the enterocyte brush-border (Knutton *et al.*, 1984b). As with K99 and 987P, also the human enterotoxigenic strains carrying CFA I or CFA II were shown to adhere to the intestinal villous surfaces of infant rabbits (Evans *et al.*, 1978; Evans and Evans, 1978), preferentially in the upper small intestine.

3. Enteropathogenic Strains

Some enteropathogenic *E. coli* strains produce diarrhoea without detectable production of the classical *E. coli* enterotoxins, LT and ST, and without the ability to invade the mucosa. The pathogenic mechanisms of such strains are rather obscure, but these strains do colonize the intestinal epithelium, as described by Cantey and Blake (1977) for an enteropathogenic strain associated with diarrhoea in rabbits. This strain, designated RDEC-1, is fimbriated and was shown to adhere only to epithelial cells that had lost their brush-border (Takeuchi *et al.*, 1978). The strain colonized the ileum, caecum and colon of the infected rabbits (Cantey and Hosterman, 1979). The adherence was not inhibited by D-mannose or other monosaccharides (Cheney *et al.*, 1979). *In vitro* adherence and *in vivo* colonization and infectivity were shown to be specific for rabbits, indicating the presence of specific receptors on the intestinal mucosa of these animals (Cheney *et al.*, 1980).

Enteropathogenic strains of human origin were investigated for adhesive properties using HEp-2 (human epithelial) cells (Cravioto *et al.*, 1979a). It appeared that the majority of the traditional infantile enteropathogenic strains possess a mannose-insensitive adhesin distinct from CFA I or CFA II. Possibly this adhesin is non-fimbrial (Scotland *et al.*, 1983), although it was not excluded that the adhesin detected is identical to either CS1 or CS3 (Smyth, 1982).

4. Entero-invasive Strains

In a survey for fimbriae and flagella on human pathogenic *E. coli* strains that cause diarrhoea by a variety of mechanisms, O'Hanley and Cantey (1978) showed that entero-invasive strains do not produce such surface structures. Nevertheless, adherence of invasive strains to the mucosal surfaces is likely to precede invasion, and cell-surface structures involved in the initial stages of tissue invasion should be considered as virulence factors. Strains of *E. coli* isolated from patients with dysentery-like diarrhoeal disease have been shown to adhere, in the presence of D-mannose, to both human erythrocytes and cultured HEp-2 cells (Nandadasa *et al.*, 1981). This haemagglutination

reaction is specific for human erythrocytes (Williams *et al.*, 1984). The haemagglutinins of two of these strains have been described as non-fimbrial in nature and probably they recognize different receptor structures. Mutants deficient in haemagglutinating activity do not produce the cell-surface adhesin and show no adherence to cultured human epithelial cells (Williams *et al.*, 1984). In an ultrastructural study of the two strains they investigated, Knutton *et al.* (1984c) found that cultures of these strains contained bacteria with type 1 fimbriae, bacteria with thin flexible fimbriae, and cells surrounded by a glycocalyx. The majority of the bacteria, however, appeared to be devoid of surface appendages, and only a minority of the cells reacted with antiserum prepared against purified haemagglutinins. Since a positive reaction could not be attributed to a particular surface structure, the authors suggest that the mannose-insensitive haemagglutinins are non-fimbrial structures. Each of the structures observed (type 1 fimbriae, fimbriae, glycocalyx and non-fimbrial haemagglutinin) may function to promote attachment to intestinal epithelial cells and erythrocytes.

Bacteraemic strains of *E. coli*, which invade the host tissue causing septicaemia, often harbour a so-called Vir plasmid, encoding a very potent toxin and a mannose-insensitive adhesin, which enable these cells to adhere to various types of epithelial tissues (Morris *et al.*, 1982a; Lopez-Alvarez and Gyles, 1980). The fimbriae encoded by this plasmid are composed of protein subunits with a molecular weight of 20,000 (F.K. de Graaf, unpublished results).

5. *Uropathogenic Strains*

Strains of *E. coli* causing urinary tract infections often originate from the faeces. The bacteria colonize the outer genital area followed by an ascending infection of the urinary tract. Urinary-tract infections vary from a relatively mild or even unnoticed asymptomatic bacteriuria to symptomatic infections, as in acute cystitis or pyelonephritis, dependent on the virulence factors of the invading strain and the susceptibility of the host. Only certain O:K:H serotypes are frequently encountered in cystitis or pyelonephritis, indicating that such strains possess a special pathogenicity for the human urinary tract.

Most of the *E. coli* strains isolated from patients with urinary-tract infection show adhesive properties, and the capacity to attach to the uroepithelial cells is considered an important factor determining which faecal strains of *E. coli* colonize the urinary tract. Adhesive capacity measured *in vitro* with isolated epithelial cells appeared to be related to the severity of the infection *in vivo* (Svanborg Edén *et al.*, 1976, 1979a). Adhesiveness is high in symptomatic, and low in asymptomatic, bacteriuria (Svanborg Edén *et al.*, 1978a,b). Generally, strains from urinary-tract infections have stronger

adhesive properties than faecal isolates (Varian and Cooke, 1980). Furthermore, adhesion *in vitro* varies between individuals and epithelial cell types, with the viability of the epithelial cells, the growth medium, growth phase, ratio of bacteria to epithelial cells used and the incubation conditions of the adhesion test (Svanborg Edén *et al.*, 1977; Fowler and Staney, 1977, 1978; Schaeffer *et al.*, 1979). Although a relatively simple test can be used to study adhesiveness *in vitro*, it should be kept in mind that it is uncertain to what extent epithelial cells isolated from the urine are representative of the intact epithelial surface of the urinary tract. Generally it may be questioned whether optimal conditions for adhesion *in vitro* apply for the situation *in vivo* in the infected host.

Some host predisposition appears to play a role in the colonization by uropathogenic strains. These strains, for instance, adhere more readily to the uroepithelial cells of women or girls with recurrent urinary-tract infections than to cells isolated from control individuals with no history of bacteriuria (Fowler and Staney, 1977; Källenius and Winberg, 1978; Svanborg Edén and Jodal, 1979). Most of the recurrent infections were caused by reintroduction of bacteria from the faeces.

Adhesive capacity in the urinary tract is related to the presence of fimbriae (Svanborg Edén and Hansson, 1978; Källenius and Möllby, 1979). Adhesion to periurethral cells is not affected by D-mannose and is correlated with the ability for mannose-insensitive agglutination of human erythrocytes. However, it should be noted that, besides fimbriae, non-fimbrial adhesins also may exist on uropathogenic strains.

Hagberg *et al.* (1981) investigated a large collection of uropathogenic *E. coli* strains for their capacity to adhere to human uroepithelial cells and to agglutinate human erythrocytes and yeast cells. They found three main combinations of adhesive properties: (i) strains showing mannose-sensitive as well as mannose-insensitive agglutination, which attach in high numbers to uroepithelial cells, (ii) strains possessing only mannose-sensitive agglutination, which attach in low numbers, and (iii) strains without agglutinating capacity, which do not bind to uroepithelial cells. Strains producing mannose-insensitive adhesins attach in high numbers and are found in high proportion among isolates from acute pyelonephritis. These observations suggest that type 1 fimbriae may be less important in the establishment of symptomatic urinary tract infections although these fimbriae contribute to the adhesive properties of a particular strain. The association between mannose-insensitive adhesins and acute pyelonephritis was further established by Källenius and his coworkers (1981a). They showed that, in cases of acute pyelonephritis, strains carrying these adhesins also predominate in the faecal flora. The persistence of strains carrying the mannose-insensitive adhesins in the intestine is considered to be a continuous threat for further attacks of

pyelonephritis. Orskov *et al.* (1980a) proposed that type 1 fimbriae constitute a general adhesive property that promotes the adherence of *E. coli* strains to mucus layers. Uropathogenic *E. coli* with type 1 fimbriae may be trapped in the urinary slime. Tamm-Horsfall glycoprotein (or urinary slime) is rich in mannose residues and hence this glycoprotein is supposed to act as receptor for type 1 fimbriae in the urinary tract (Orskov *et al.*, 1980b). These authors suggested that production of mucus is an important part of the non-immunological antibacterial host defense mechanism. Svanborg Edén *et al.* (1982) found that *E. coli* strains possessing type 1 fimbriae adhered to purified Tamm-Horsfall glycoprotein. In contrast to type 1 fimbriae, the mannose-insensitive adhesins present on uropathogenic strains promoted the attachment of these strains to the uroepithelial cells (Svanborg Edén *et al.*, 1982). Further evidence for the role of mannose-insensitive adhesins in the attachment to the human uroepithelial cells was given by Korhonen *et al.* (1981) who found that antibodies against these adhesins inhibited the binding of these strains to the uroepithelium. Furthermore, purified mannose-insensitive fimbrial adhesins bound specifically to human uroepithelial cells (Korhonen *et al.*, 1980a). In conclusion, (uro)pathogenic *E. coli* strains produce a combination of different fimbrial adhesins which, together with the distribution of various receptors on the target cells, determines the specificity of bacterial adherence to a particular tissue. Binding occurs only when the appropriate adhesin and receptor are present.

Mice are suitable as an animal model to study the role of different fimbriae in urinary-tract infections. Van de Bosch *et al.* (1979) showed that the mouse model can be used to determine differences in the virulence of haematogenous nephropathogenic *E. coli* strains. More recently, Hagberg *et al.* (1983a) used an experimental mouse model to study the role of various adhesins in the pathogenicity of infections induced by *E. coli* strains associated with urinary-tract infections in humans. Mice were selected because the globoseries glycolipids, which serve as receptor for the mannose-insensitive adhesins of these strains, are the predominant non-acid glycolipids in the mouse kidney (Adams and Gray, 1968). Strains associated with urinary-tract infections in human are recovered in higher numbers and persist longer in mouse kidneys and bladders than commensal *E. coli* strains isolated from human faeces.

Hagberg *et al.* (1983b) used pairs of genetically defined strains to study the role of both type 1 and of mannose-insensitive P fimbriae in the experimental infection of mice. The homogeneic strains consisted of three mutants derived from one human pyelonephritic strain. One mutant produces only P fimbriae, a second only type 1 fimbriae and a third mutant produces both types of fimbriae. The other pair of strains consisted of two transformants of a faecal *E. coli* strain, one transformant harbouring a recombinant plasmid coding for P fimbriae, the other one a plasmid coding for type 1 fimbriae. It appeared

that production of P fimbriae enhanced bacterial recovery from both kidney and bladder. Simultaneous production of type 1 and P fimbriae improved the bacterial recovery from the bladder but not from the kidneys. Apparently, bacterial survival in the mouse bladder is enhanced by both types of adhesins, while P fimbriae only were sufficient for persistence in the kidneys. The strains producing only type 1 fimbriae were recovered in higher numbers from the bladder than the strains producing only P fimbriae. It was concluded that the adhesive properties of a particular strain, represented by the type(s) of fimbriae produced, at least in part determined the localization and retention of bacteria in the mouse urinary tract. Mutants derived from the pyelonephritic strain were recovered in higher numbers than the transformants obtained from the commensal *E. coli* strain, indicating a difference in colonization capability. This difference may be due to a requirement for additional virulence factors. Hagberg *et al.* (1983b) also reported an apparent selection in adhesin expression *in vivo*. Expression of type 1 fimbriae by a mutant strain producing both types of fimbriae decreased in the kidneys but not in the bladders of infected mice. It has been suggested that type 1 fimbriae play a part in the initiation of infection rather than in its persistence. This implies that faecal *E. coli* strains present in the faeces possess type 1 fimbriae to allow the initial colonization of the uroepithelia. In agreement with this hypothesis, Iwahi *et al.* (1983) have found that mutants obtained from a highly virulent *E. coli* strain that had lost the ability to produce type 1 fimbriae also lost their capacity to adhere to mouse bladder epithelial cells. The authors concluded that type 1 fimbriae probably facilitate urinary tract infection in mice via adherence to bladder epithelial cells.

In conclusion, binding of bacteria to the globoseries glycolipids, mediated by P fimbriae, appears to be preferentially involved in the adhesion of *E. coli* strain causing pyelonephritis (Källenius *et al.*, 1981a; Leffler and Svanborg Edén, 1981; Hagberg *et al.*, 1981, 1983b). However, most uropathogenic strains, including strains associated with acute pyelonephritis, also produced type 1 fimbriae. A competitive binding to uromucoid, mediated by type 1 fimbriae, or to globoseries glycolipids, mediated by P fimbriae, may determine the site of infection.

IV. Adhesin-Receptors

A. RECEPTORS FOR TYPE 1 FIMBRIAE

The first indications regarding the nature of mammalian cell-surface components that recognize the fimbrial adhesins of *E. coli* came from studies on the inhibitory activity of various substances on the haemagglutinating and

adhesive capacity of these bacteria. Collier and de Miranda (1955) investigated the effect of various carbohydrates on the haemagglutinating activity of *E. coli*. They observed a strong inhibitory effect of D-mannose on the agglutination of chicken erythrocytes. A large variety of other sugars had little or almost no effect on the haemagglutinating activity. In a subsequent paper, Collier and Tiggelman-van Krugten (1957) described a comparable strong inhibition of agglutination of both chicken and guinea-pig erythrocytes by D-mannose and methyl-α-D-mannoside. Duguid *et al.* (1955) found the haemagglutinating activity to be associated with the presence of type 1 fimbriae and confirmed the inhibitory effect of D-mannose and methyl-α-D-mannoside on haemagglutination induced by type 1 fimbriae. The inhibitory effect of both carbohydrates was not restricted to the bacteria–erythrocyte interaction. Pellicle formation, induced by subculturing type 1-fimbriated bacteria in aerobic static broth, also was inhibited by D-mannose or methyl-α-D-mannoside (Old *et al.*, 1968) which indicates that the binding of these sugars to type 1 fimbriae alters the properties of the fimbriae and prevents aggregation. In a more detailed study of the inhibitory activity of D-mannose and its derivatives, Old (1972) showed that, besides D-mannose, 1,5-anhydromannitol, D-mannoheptulose and α-D-mannose 1-phosphate also exhibited a strong inhibition of haemagglutination at concentrations between 0.001 and 0.02% (w/v). D-Fructose and yeast mannan had a moderate inhibitory effect at 0.05–0.2% (w/v). All mannose derivatives that are active inhibitors are substituted at C-1. Derivatives modified at any of the hydroxyl groups at C-2, C-3, C-4 or C-6 have no inhibitory activity. Furthermore, the pyranose ring form of D-mannose, and the α-configuration of the C-1 position, appear to be necessary since open-chain derivatives like D-mannitol, or carbohydrates with a β-configuration (e.g. methyl-β-D-mannoside), were not inhibitory.

All previous studies were done with fimbriated whole bacteria. Rivier and Darekar (1975) and Salit and Gotschlich (1977a) tested purified type 1 fimbriae and demonstrated that the fimbriae themselves can agglutinate guinea-pig erythrocytes. As with whole cells, the haemagglutinating activity of purified type 1 fimbriae was inhibited by D-mannose, methyl-α-D-mannoside and yeast mannan. Treatment of erythrocytes with trypsin enhanced their agglutinability, in particular with human erythrocytes. Also, neuraminidase treatment enhanced type 1 fimbriae-induced haemagglutination, whereas treatment of erythrocytes with mannosidase slightly diminished their agglutinability. Antibodies against type 1 fimbriae prevented their haemagglutination (Salit and Gotschlich, 1977a).

The nature of the type 1 fimbriae receptors not only has been studied using erythrocytes but also using epithelial cells. Attachment of type 1-fimbriated bacteria to epithelial cells was demonstrated *in vitro*. Ofek *et al.* (1977) described the binding of *E. coli* to isolated human buccal epithelial cells. Both

D-mannose and methyl-α-D-mannoside inhibited the binding, and rapidly displaced bacteria from epithelial cells to which they were bound (Ofek and Beachey, 1978) indicating that inhibition is reversible. Saturation of binding sites on the bacterial adhesin by the inhibitor prevented attachment of the bacteria to receptors containing mannose or mannose-like residues. Treatment of epithelial cells with periodate completely destroyed their affinity for fimbriated bacteria. Furthermore, concanavalin A, a lectin that is known to bind to mannose residues, also inhibits the binding of *E. coli* to epithelial cells (Ofek *et al.*, 1977) as well as their haemagglutinating activity (Salit and Gotschlich, 1977a). Apparently, attachment to both erythrocytes and epithelial cells is mediated by the same "mannose-specific" adhesin identified as type 1 fimbriae. Using yeast-cell agglutination, Firon *et al.* (1982) tested the inhibitory effect of a variety of linear and branched oligosaccharides as well as glycosides containing D-mannose residues. It appeared that both phenyl-α-D-mannoside and *p*-nitrophenyl-α-D-mannoside are 20–30 times more potent inhibitors than methyl-α-D-mannoside. Likewise, the trisaccharide Man-α-(1→3)-Man-β-(1→4)-GlcNAc and α glycosides of branched oligosaccharides containing five or six 1→3, 1→6 or 1→2-linked mannose residues are very strong inhibitors. Linear disaccharides, tetrasaccharides and pentasaccharides are poor inhibitors. The inhibitory activity determined with whole bacteria correlated with that observed with purified fimbriae. In general, the "mannose-specific" adhesins preferentially bind to relatively short oligosaccharides as are present in *N*-glycosyl-linked glycoconjugates. For *E. coli* type 1 fimbriae, the results indicate an elongated binding site recognizing a trisaccharide and probably containing a hydrophobic region (Firon *et al.*, 1983). The binding site might have the form of a pocket present on the fimbrial surface.

Since mannose-containing glycoconjugates are found on most mammalian cell surfaces, the "mannose-specificity" of type 1 fimbriae may provide bacteria carrying this adhesin with the ability to bind to a variety of eukaryotic cells (Salit and Gotschlich, 1977b). On the other hand, the interaction between type 1 fimbriae and mannose-containing receptors does not explain the selective colonization of only particular epithelia by *E. coli*. Apparently, the physiological conditions in the environment which strongly affect the production of type 1 fimbriae, as well as the mammalian cell properties and the presence of other adhesins, are of paramount importance in determining selective adherence.

Although type 1 fimbriae isolated from different genera belonging to the *Enterobacteriaceae* can be classified as a family of mannose-sensitive (or "mannose-specific") fimbrial adhesins this does not necessarily mean that the different mannose-containing glycoconjugates present on the epithelial cell surfaces are equally well recognized by the type 1 fimbriae present on bacteria

of various genera and species. Recent data published by Firon *et al.* (1984) show that type 1 fimbriae of different genera exhibit differences in their ability to bind to a variety of mannose-containing oligosaccharides, although all strains within a given genus possess the same oligosaccharide specificity. This observation is an indication of the existence of different classes of type 1 fimbriae among the *Enterobacteriaceae*.

B. RECEPTORS FOR K88, K99 AND 987P FIMBRIAE

As with type 1 fimbriae, the first indications regarding the chemical nature of receptors involved in the binding of enterotoxigenic strains mediated by mannose-insensitive adhesins, came from studies on the inhibitory effect of various carbohydrates or glycoconjugates on the haemagglutinating activity of these strains.

Strains of *E. coli* carrying the K88 adhesin cause mannose-insensitive haemagglutination of guinea-pig erythrocytes at 4°C (Stirm *et al.*, 1967a; Jones and Rutter, 1974). The haemagglutination reaction was shown to be associated with the K88 fimbriae. Gibbons *et al.* (1975) used the agglutination of guinea-pig erythrocytes by K88-positive strains as a model for testing the inhibitory effect of various substances on haemagglutinating activity. They observed that mucus glycoproteins isolated from the pig intestine, but not the structurally different serum glycoproteins or glycosaminoglycans, inhibit the haemagglutination reaction. By means of chemical modification of isolated glycoproteins, indications were obtained that sugar residues, in particular unsubstituted β-D-galactosyl residues, are an essential part of the K88 receptor structure. Monosaccharides known as constituents of the oligosaccharide chain of these glycoproteins have no effect, indicating that more than a single sugar residue is necessary for recognition by K88 fimbriae. Kearns and Gibbons (1979) were able to isolate a fraction containing polar glycolipids from brush-borders isolated from adhesion-positive and adhesion-negative pigs. Analysis of this glycolipid fraction showed a major glycolipid component with slightly different mobility, dependent on whether the glycolipids were isolated from adhesion-positive or adhesion-negative brush-borders. Interaction of K88 fimbriae with their natural target cells was studied by Sellwood (1980) using ^{125}I-labelled K88 fimbriae and brush-borders isolated from pig intestinal epithelial cells. A high level of non-specific binding was observed which could be abolished after treatment of the brush-borders with 1% formaldehyde. Treatment with 2.5% glutaraldehyde, however, completely inhibited the attachment of K88-positive cells or isolated labelled K88 fimbriae. Variations in temperature had little effect on the rate of binding, and periodate oxidation destroyed the receptor activity of the brush-borders indicating that either glycoproteins or glycolipids are part of the receptor

structure. Inhibition experiments with mono- and oligosaccharides confirmed the observations of Gibbons *et al.* (1975) that galactosyl residues may be important in binding activity. By using another *in vitro* binding assay, with ^{125}I-labelled K88 fimbriae and isolated brush-borders, based on differential centrifugation, Anderson *et al.* (1980) concluded that glycoproteins with terminal *N*-acetylhexosamine residues play a role in the attachment of K88 fimbriae to brush-borders isolated from hog intestinal epithelial cells.

Nilsson and Svensson (1983) isolated glycolipids from the pig small intestine and separated the carbohydrate moiety from the various glycolipids by trifluoroacetolysis. They observed that isolated oligosaccharides consisting of about 20 sugar residues completely inhibit the haemagglutination of guinea-pig erythrocytes by purified K88 adhesin. One of these large purified oligosaccharides contains fucose, galactose, glucose and *N*-acetylglucosamine in the relative molar proportions 0.22:1.00:0.40:0.81 and shows complete inhibition of haemagglutination at a concentration of 5–10 μg ml^{-1}. Furthermore, other oligosaccharides consisting of galactose residues also inhibit haemagglutination.

Staley and Wilson (1983) used affinity chromatography of detergent-solubilized brush-borders on agarose coupled with K88 fimbriae to identify the K88 receptor. Brush-border components with a wide range of molecular weights are bound in a saturable process that can be inhibited with anti-K88 antibodies and stimulated by Ca^{2+} ions. Also, monosaccharides were tested for inhibitory activity and galactose, galactosamine, glucose and *N*-acetylglucosamine showed a partial inhibition of the binding reaction. Analysis of bound brush-border components by sodium dodecyl sulphate–polyacrylamide gel electrophoresis revealed the presence of two glycoproteins with molecular weights of 23,000 and 32–35,000, respectively. On recovery of these components from the affinity gel, they retained their affinity for K88 fimbriae. The authors suggested that the K88 receptor consists of glycoprotein which in its native state exists in multimeric forms. Differences in these multimeric forms may result in different affinities for the three K88 variants, as observed by Bijlsma *et al.* (1982).

Material that caused a visible aggregation of *E. coli* strains carrying the 987P adhesin was released from the brush-borders of rabbit intestinal epithelial cells, when stored at 4°C (Dean and Isaacson, 1982). Periodate oxidation or prolonged incubation with pronase destroys the receptor activity, indicating the possibility of there being a glycoprotein receptor. Recently, Dean and Isaacson (1984) purified the receptor material from the solubilized fraction and identified a low molecular-weight glycoprotein consisting of 81% carbohydrate and 19% amino-acid residues. Galactose, fucose and mannose were found in a proportion of 6:4:1. No sialic acid could be detected. The ability of the purified receptor material to aggregate *E. coli*

strains carrying 987P fimbriae was inhibited by relatively high concentrations of amino sugars, their *N*-acetylated derivatives, or compounds with free amino groups like lysine or ethanolamine. Also lectins, in particular those specific for galactose or fucose residues, were inhibitory when preincubated with purified receptor material.

Enterotoxigenic strains carrying the K99 adhesin caused haemagglutination of sheep and horse erythrocytes (Tixier and Gouet, 1975; Burrows *et al.*, 1976). Faris *et al.* (1980) used sheep erythrocytes to study the inhibitory effect of various gangliosides on K99-induced haemagglutination and observed that the monosialylganglioside GM2 abolished the haemagglutination reaction at relatively high concentration. The gangliosides GM1 and GM3 and the cerebrosides type I and II had no effect. Lindahl and Wadström (1983) tested various glycoconjugates and found that bovine submaxillary mucin strongly inhibited K99-induced haemagglutination. They suggested that the K99 fimbriae probably recognize the terminal *N*-acetylgalactosamine and sialic acid residues present in complex glycoconjugates.

Recently, Smit *et al.* (1984) have isolated, purified and characterized the K99 receptor present on horse erythrocytes. This receptor was identified as being the glycolipid Neu5Gc-α-(2→3)-Gal-β-(1→4)-Glu-β-(1→1)-ceramide (Table 2) and identical with the so-called haematoside known as the major glycolipid component of horse erythrocytes. Purified receptor material

TABLE 2. Human P blood-group system

Blood-group phenotype	P-antigens present on erythrocytes	Frequency
P_1	P, P_1, P^k	75%
P_2	P, P^k	25%
P_1^k	P_1, P^k	very rare
P_2^k	P^k	very rare
p	none	–

Structure of P antigens

P^k = α-D-Gal*p*-(1→4)-β-D-Gal*p*-(1→4)-β-D-Glc*p*-Cer (globotriaosyl ceramide)

P = β-D-GalNAc*p*-(1→3)-α-D-Gal*p*-(1→4)-β-D-Gal*p*-(1→4)-β-D-Glc*p*-Cer (globoside)

P_1 = α-D-Gal*p*-(1→4)-β-D-Gal*p*-(1→4)-β-D-GlcNAc*p*-(1→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*-Cer (globopentaosyl ceramide)

Structure of the K99 receptor

Neu5Gc-α-(2→3)-Gal*p*-β-(1→4)-Glc*p*-β-(1→1)-ceramide

inhibited both haemagglutination by K99-positive bacteria, as well as by purified K99 fimbriae, and prevented the attachment of K99 carrying cells to the intestinal epithelial cells. Coating of guinea-pig erythrocytes, normally not agglutinated by K99 fimbriae, with the isolated glycolipid receptor conferred on these erythrocytes the ability to bind both K99-positive cells and K99 fimbriae.

Almost nothing is known yet about the structure of the receptors for the CFA fimbriae in the human intestine. Using human erythrocytes, Faris *et al.* (1980) observed that CFA I-induced haemagglutination, like K99-induced haemagglutination, was inhibited by monosialylganglioside GM2.

In summary, it seems that relatively simple glycosphingolipids appear to function as receptor for certain mannose-insensitive adhesins of enterotoxigenic *E. coli* strains, in particular K99 and CFA I. The presence of such glycolipids in the intestinal mucosa of man has been described (Falk *et al.*, 1979). Glycolipids with one sugar residue (galactosylceramide and sulphatides) and with more than four sugar residues (blood-group glycolipids) are almost exclusively located on the intestinal epithelial cells, whereas di-, tri- and tetraglycosylceramides are predominantly located in the underlying tissue.

C. RECEPTORS FOR MANNOSE-INSENSITIVE ADHESINS OF UROPATHOGENIC STRAINS

The specificity of the interaction of *E. coli* strains carrying mannose-insensitive adhesins to the human uroepithelium suggests the existence of specific receptors on the epithelial cells. A glycolipid fraction extracted from these cells was found to inhibit adherence of *E. coli* cells (Svanborg Edén and Leffler, 1980). This fraction contained a special class of glycosphingolipids known as the globoseries glycolipids which were later shown to act as receptor for the mannose-insensitive adhesins (Leffler and Svanborg Edén, 1980). The most effective glycolipid extracted from the uroepithelial cells is globotetraosylceramide (globoside) but globotriaosylceramide also exhibited receptor activity (Table 2). The activity of both glycolipids in preventing adhesion of *E. coli* to uroepithelial cells was confirmed with model glycolipids of the same structure but isolated from different sources.

Globoseries glycolipids are present on human erythrocytes of the normal P_1 and P_2 phenotype. Individuals with erythrocytes of the very rare blood-group p phenotype, however, lack these glycolipids because of the absence of galactosyltransferase that adds the terminal galactosyl residue to lactosylceramide (Marcus *et al.*, 1976; Koscielak *et al.*, 1976). Subsequently, Leffler and Svanborg Edén (1980) were able to demonstrate that the total non-acid glycolipid fraction from the normal P_1 or P_2 erythrocytes completely inhibits

the attachment of uropathogenic strains to uroepithelial cells, whereas no such inhibition was found when an identically prepared glycolipid fraction from p erythrocytes was tested. The mannose-insensitive adhesins recognizing the glycolipids of the P blood group are designated P fimbriae. Guinea-pig erythrocytes, which normally do not interact with *E. coli* strains carrying only P fimbriae, can be coated with isolated globoside. This coating induces specific agglutination of these erythrocytes by strains possessing P fimbriae. Also, Källenius *et al.* (1980a,b) described the ability of pyelonephritic *E. coli* strains to agglutinate human erythrocytes from individuals with commonly occurring blood groups. Erythrocytes of the rare p phenotype lacking the P, P_1 or P^k antigen were not agglutinated, which confirmed the affinity of P fimbriae for P blood-group antigens. Since $P_1{}^k$ erythrocytes lacking the common P antigen (Table 2) were agglutinated, Källenius and his coworkers suggested that the P antigen is not the receptor. The P_2 erythrocytes, lacking the common P_1 antigen, were also agglutinated, indicating that P_1 also does not serve as receptor. The authors concluded that the P^k antigen (globotriaosylceramide) is the most likely candidate as receptor for P fimbriae. Further evidence for this conclusion came from the observation that reduced oligosaccharide obtained from globotetraosylceramide did not inhibit agglutination, whereas the reduced oligosaccharide obtained from globotriaosylceramide is a highly active inhibitor of haemagglutination.

Leffler and Svanborg Edén (1981) investigated a large collection of strains isolated from patients with urinary tract infections and found that the majority of the strains that bind to uroepithelial cells also bind to globotetraosylceramide. The recognition of globoside was especially frequent among strains causing pyelonephritis. Preincubation of these strains with globoside prevented their attachment to the human uroepithelial cells. They suggested that strains carrying P fimbriae recognize a common feature of the oligosaccharide chains present in the globoseries glycolipids, i.e. the α-Gal-(1→4)-β-Gal- part. Källenius *et al.* (1980b, 1981b) were able to demonstrate that the synthetic disaccharide α-D-Gal*p*-(1→4)-β-D-Gal*p*-O-NO_2 blocks the binding of pyelonephritic strains to human uroepithelial cells. Coating of these cells with this disaccharide increased the binding of bacteria.

Since globoseries glycolipids are present on many types of human cells, including uroepithelial cells, the binding of strains causing pyelonephritis to human uroepithelial cells isolated from individuals with and without P blood-group antigens was investigated. Källenius *et al.* (1981a) observed that the uroepithelial cells from individuals lacking P blood-goup antigens adhere pyelonephritic strains to a significantly lesser extent than cells from individuals of the P_1 or P_2 phenotype. Interestingly, individuals of the p phenotype, which lack globoseries glycolipids in their erythrocytes and

epithelial cells, are not sensitive to urinary tract infections by strains carrying P fimbriae.

Purified P fimbriae agglutinate neurominidase-treated human P_1 and P_2^k erythrocytes but not p erythrocytes (Korhonen *et al.*, 1982). Coating of the last type of erythrocytes with globoside renders these erythrocytes agglutinable by P fimbriae. The haemagglutinating reaction could be inhibited by the synthetic disaccharide. Obviously, purified P fimbriae recognize the same carbohydrate sequence as shown for P-fimbriated whole cells. In conclusion, the α-D-Gal*p*-(1→4)-β-D-Gal*p* sequence appears to be the minimal *P* fimbriae receptor structure (Källenius *et al.*, 1980b; Leffler *et al.*, 1983). This sequence is part of both globotriaosyl-(P^k) and globotetraosyl-(P) ceramide. Both glycolipids are recognized as receptors by the same uropathogenic strains. On the basis of their ability to recognize a common receptor structure, the P fimbriae of uropathogenic strains may be considered as a family of related fimbriae with affinity for galactose residues. Instead of using the prefix "P" we prefer to designate this family according to their receptor specificity as "G" fimbriae exhibiting "galactose-specificity" in contrast to "mannose-specificity". Svanborg Edén *et al.* (1984) use the term globotetraosylceramide-sensitive adhesins. Not all uropathogenic strains, however, appear to recognize globoseries glycolipid. Mett *et al.* (1983) described a mannose-insensitive adhesin, isolated from a patient with urinary tract infection and expressing fimbriae, that recognized galactose-containing glycolipids and digalactosyl diglyceride but no globoseries glycolipids.

Some pyelonephritic strains recognize a different structure on human erythrocytes, indicated as X (or unknown) specificity (Vaisanen *et al.*, 1981). P and X specificity may occur on the same strains but some strains show X specificity only. X-specific recognition is not inhibited by the α-D-Gal*p*-(1→4)-β-D-Gal*p* disaccharide. P specificity occurs significantly more frequently among strains isolated from younger patients, or among individuals with relatively few episodes of urinary tract infection, whereas X specificity occurs almost equally among patients irrespective of age or number of urinary-tract infection. Strains with X specificity are also fimbriated (Vaisanen *et al.*, 1981). One of the X-specific strains isolated from a case of acute pyelonephritis was further investigated and showed blood group M specificity (Vaisanen *et al.*, 1982). A strong agglutination was obtained with both MM and MN erythrocytes and a very weak or no agglutination of NN erythrocytes. This weak interaction with NN erythrocytes could be inhibited with methyl-α-D-mannoside, indicating that the presence of type 1 fimbriae are responsible for this interaction. Agglutination of MM and MN erythrocytes is not inhibited by this carbohydrate. The observations suggest that this particular strain probably recognizes the M blood-group antigen known as type M glycophorin A. Differences between glycophorin A from M and N erythrocytes are

in the *N*-terminal amino-acid sequence of this protein (Blumenfeld and Adamany, 1978) consequently the M specificity appears to depend on recognition of the *N*-terminal amino-acid sequence in type M glycophorin A. This sequence is Ser-Ser-Thr-Thr-Gly in type M, and Leu-Ser-Thr-Thr-Glu in type N glycophorin A. Glycophorin A isolated from MM erythrocytes inhibited the agglution of MN erythrocytes, whereas glycophorin A isolated from NN erythrocytes did not (Vaisanen *et al.*, 1982).

Other strains with X-specific adherence, and isolated from various sources (urine, faeces, cases of meningitis and others), show another P blood group-independent binding specificity (Parkkinen *et al.*, 1983). These strains completely lose their haemagglutinating activity when p erythrocytes are treated with neuraminidase. Different neuraminic acid residues containing glycoproteins and their desialylated derivatives were subsequently tested for haemagglutination inhibition. Orosomucoid was found to be the most effective glycoprotein. Asialoglycoproteins showed no effect. By testing various purified oligosaccharides only $\alpha(2\rightarrow3)$-linked isomers of sialyllactose and sialayl-*N*-acetyllactosamine, present in orosomucoid, showed complete inhibition of haemagglutination, indicating that glycoproteins or glycolipids containing these structures function as receptor for these strains.

Treatment of erythrocytes with enzymes that modify their surface structure seems to be a useful tool in the identification of receptors for bacterial adhesins. Vaisanen-Rhen *et al.* (1983) used endo-β-galactosidase-treated human erythrocytes to screen *E. coli* strains from patients with urinary tract infections, as well as healthy persons for yet unidentified adhesins. One of the strains carrying M-specific fimbriae showed a strong haemagglutination with the enzyme-treated erythrocytes in which *N*-acetyl-D-glucosamine residues had become exposed. Addition of *N*-acetyl-D-glucosamine, but no other monosaccharides, inhibited this agglutination. Orosomucoid, and its desialylated derivative had no effect on haemagglutination, but became inhibitory after treatment with β-galactosidase. Apparently some *E. coli* strains possess adhesins that recognize terminal *N*-acetyl-D-glucosamine residues. The nature of this adhesin is unknown. Fimbriae isolated from this strain exhibited M specificity but lacked affinity for *N*-acetyl-D-glucosamine, indicating that the adhesin specific for *N*-acetyl-D-glucosamine is non-fimbrial.

D. RECEPTORS FOR OTHER STRAINS OF *Escherichia coli*

Recently, Korhonen *et al.* (1984) isolated the adhesin of a X-specific strain obtained from a patient with neonatal meningitis. The adhesin consisted of a fimbria that was morphologically identical to type 1 and P fimbriae. The fimbrial subunits have an apparent molecular weight of 17,000. Bacteria, as

well as purified fimbriae, caused mannose-insensitive haemagglutination of human erythrocytes. Treatment of these erythrocytes with neuraminidase abolished this haemagglutination. Also, desialylation of orosomucoid abolished the inhibition of haemagglutination caused by this glycoprotein. Antibodies against purified fimbriae reacted strongly with the homologous antigen and with other strains isolated from cases of neonatal meningitis, but not with type 1 or P fimbriae. On the basis of their ability to recognize sialylgalactosides, Korhonen *et al.* (1984) proposed to call these fimbriae S fimbriae. Since K99 fimbriae recognize the same type of oligosaccharide, they also should be classified as S fimbriae. Within the heterogeneous group of X fimbriae the S fimbriae appear to form an homologous group of fimbriae.

E. INTERACTION WITH PHAGOCYTIC CELLS

Binding of *E. coli* strains carrying type 1 fimbriae to mannose-containing glycoconjugates on the host mucosal surfaces on the one hand promotes bacterial colonization, but on the other hand the interaction of these strains with other cells, e.g. phagocytic cells, may promote the elimination of the bacteria. Phagocytic cells are supposed to bind bacteria and become activated, in the absence of immunoglobulins, provided that these bacteria possess surface components that are recognized by the phagocytic cell (van Oss, 1978). A dual role for type 1 fimbriae in the host–parasite relationship was first described by Bar-Shavit *et al.* (1977) who showed that phagocytes possess surface receptors containing mannose residues that interact with type 1 fimbriated bacteria in the absence of serum. The binding was inhibited by D-mannose, methyl-α-D-mannoside and yeast mannan. Attachment of opsonized bacteria was not inhibited by these sugars, indicating that normal recognition of opsonized bacteria does not involve mannose residues. Silverblatt *et al.* (1979) found that the susceptibility of *E. coli* strains to phagocytosis by human polymorphonuclear leucocytes (PMNL), in the absence of opsonins, correlated with the degree of fimbriation. Removal of the fimbriae abolished phagocytosis.

Binding of fimbriated strains to phagocytic cells activates the release of lysosomal enzymes, as well as the oxidative metabolism of phagocytes, as detected by enhanced chemoluminescence (Mangan and Snyder, 1979a,b). These processes are inhibited if binding of bacteria is prevented by the addition of methyl-α-D-mannoside. Ingestion of *E. coli* by PMNL, and the inhibition of this process by methyl-α-D-mannoside was demonstrated by Rottini *et al.* (1979). Surprisingly, purified type 1 fimbriae agglutinated leucocytes but did not prevent the attachment of the fimbriated bacteria to the phagocytic cells, nor the activation of phagocytosis. These data very well may mean that binding of type 1 fimbriated bacteria to phagocytic cells is much

more efficient than binding of isolated fimbriae. However, it is also possible that binding of fimbriated bacteria is mediated by another mannose-sensitive adhesin. Blumenstock and Jann (1982) observed that some strains capable of producing mannose-sensitive fimbriae, but grown under conditions in which fimbriae were not expressed, adhered to phagocytes. They suggested that the mannose-sensitive adhesion of *E. coli* to phagocytic cells does not necessarily involve type 1 fimbriae but may be also mediated by other mannose-sensitive cell-surface adhesins. Nevertheless, a striking correlation exists between the mannose binding activity of bacteria and their degree of association with phagocytic cells (Bar-Shavit *et al.*, 1980).

Stimulation of phagocytic cells by strains with mannose-sensitive adhesins was enhanced when these bacteria were opsonized by antifimbrial antibodies (Perry *et al.*, 1983). Treatment with antifimbrial antibodies induced formation of bundles of fimbriae on the bacterial surface which are supposed to be more effective in stimulating the phagocytic cells by aggregating the mannose-containing receptors on these cells. The increase in stimulation can be decreased with methyl-α-D-mannoside. Cross-linking of the fimbriae by glutaraldehyde induced a comparable stimulation of the phagocytic cells.

The results obtained with mannose-sensitive adhesins, and their role in the interaction between *E. coli* and phagocytic cells, has stimulated investigations into a possible role of other adhesins in this process. Feilberg Jorgensen and Heron (1980) found that CFA I and K99-positive bacteria, in contrast to K88-positive cells or bacteria without fimbriae, interacted with a higher percentage of human lymphocytes than did CFA I-negative or K99-negative cells. However, a possible role of type 1 fimbriae was not excluded. Avril *et al.* (1981) reported that CFA-positive cells adhere to a very limited portion of monocytes, when these cells are properly washed to prevent non-specific adherence. Using radioactively labelled bacteria and chemiluminescence as a measure of bacteria–leucocyte interaction, Blumenstock and Jann (1982) showed that *E. coli* carrying mannose-insensitive adhesins were unable to attach to rat peritoneal macrophages and human polymorphonuclear granulocytes in the absence of opsonizing antibodies, and did not induce a chemiluminescence response. This observation indicates that phagocytic cells generally do not possess or express receptors for mannose-insensitive fimbriae and consequently bacteria carrying mannose-sensitive adhesins are more readily eliminated than bacteria possessing mannose-insensitive adhesins. Björkstén and Wadström (1982) confirmed these conclusions, in general. Strains carrying CFA fimbriae, in particular CFA I, induced less chemiluminescence in PMNL than their non-fimbriated variants.

Recently, Svanborg Edén *et al.* (1984) described the influence of P and type 1 fimbriae on the interaction of *E. coli* with human phagocytes. Two sets of homogenic strains were constructed that expressed either, both or none of

the fimbriae. It appeared that the strains required type 1 fimbriae for binding to, and activation of, PMNL. Removal of type 1 fimbriae from strains carrying both types of adhesins abolished both processes. Poor binding and activation were found with strains carrying only P fimbriae, but coating of PMNLs with globoseries glycolipid induced binding and phagocyte activation.

In summary, it may be concluded that, in contrast to mannose-insensitive adhesins, the mannose-sensitive adhesins have a dual effect as virulence factor by enhancing colonization and phagocytosis.

F. PHYSICOCHEMICAL ASPECTS OF ADHESION

The surface charges of both prokaryotic and eukaryotic (epithelial) cells are, in sum, negative. Yet adhesion occurs, i.e. there are attractive forces that act to overcome the repulsive forces between the like-charged surfaces of the cells. To understand the adhesion behaviour of bacteria to epithelial cells it is necessary to consider the forces operating between both cells, at larger and at shorter distance, as well as the role of cell-surface appendages like capsules, fimbriae and flagellae.

Interactions between like-charged particles at a long distance can be considered in terms of colloidal theory, as described generally by Deryagin and Landau (1941) and by Verweij and Overbeek (1948) (the DLVO-theory). In this theory, the total interaction energy between two particles (V_T) is described as the summation of the electrostatic energies of repulsion (V_R) and the energies of attraction (V_A), which are largely due to Van der Waals forces and partly to electrostatic attraction. Much attention has been focused on deducing equations for V_R and V_A (Hogg *et al.*, 1966; Wiese and Healy, 1970; Weiss and Harlos, 1972; Visser, 1976; Nir, 1977). A qualitative diagram for V_T is presented in Fig. 1. Two regions of attraction can be discerned. A shallow minimum at a distance of approximately 5–8 nm, called the secondary minimum, and at much shorter range (< 2 nm), another steep minimum indicated as the primary minimum. In between these two minima, a maximum is observed. The height of this maximum is related to the radius of the particle. The smaller the particle (bacteria) the lower the height of this maximum (Weiss and Harlos, 1977). In the case of a bacterium approaching an epithelial cell, this maximum is probably too high to be overcome and the bacterium will stay in the secondary minimum in which the attractive forces are rather weak and not likely to account for the strong interactions as probably required for an irreversible adhesion. Filamentous surface appendages like fimbriae, however, have a rather small diameter and they can easily overcome the small energy maximum. Furthermore, they are long enough to attach the bacterium

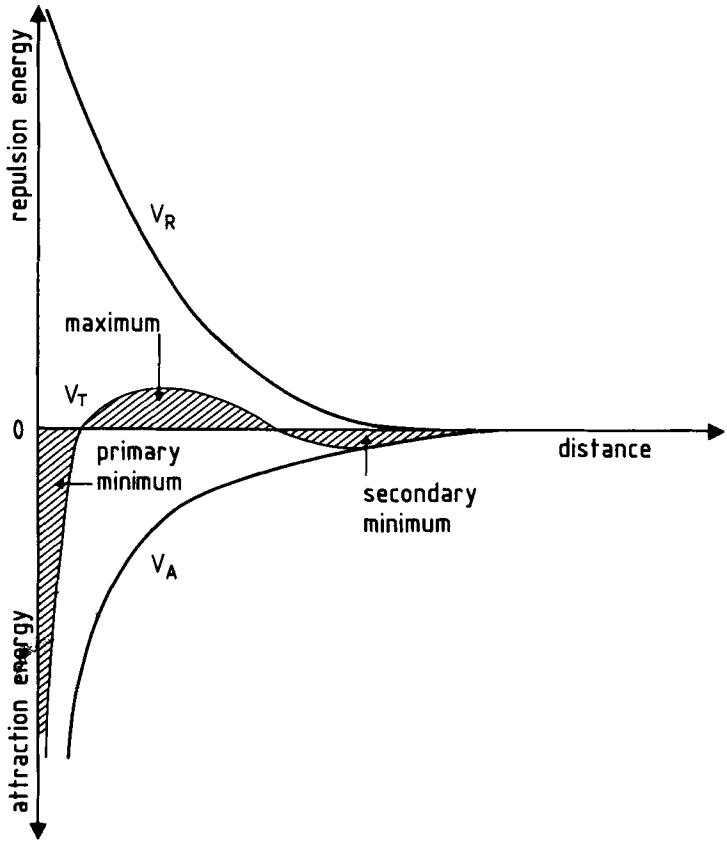


FIG. 1. Schematic presentation of the total interaction energy (V_T) between two particles (i.e. bacteria and epithelial cells), as the summation of the electrostatic energy of repulsion (V_R) and the energy of attraction (V_A).

to the epithelial cell surface whereas the bacterium itself stays in the secondary minimum.

Interactions between the two particles at a short distance may be described by using surface thermodynamics (Absolom *et al.*, 1983). First, non-specific short-range interactions are considered as in systems where the effect of electric charges and specific biochemical interactions may be neglected. In this case, bacterial adhesion will be favoured if the process causes the free energy of adhesion (F^{adh}) to decrease. The change in this function is, per unit area, given by the formula:

$$\Delta F^{adh} = \gamma BS - \gamma BL - \gamma SL$$

where γ is the interfacial tension and B, S and L refer to bacterium, substratum, and the surrounding liquid medium, respectively. With the aid of Young's equation and a formula for γSL (Neumann *et al.*, 1974) this equation can be solved. It follows that when the surface tension of the bacterium is larger than that of the medium, adhesion is greater to substrata with high surface tension. When the surface tension of the bacterium is lower than that of the medium, adhesion will occur to substrata with lower surface tension. In the case of bacterial adhesion to epithelial cells, one should consider the surface tension of the extracellular materials (e.g. polysaccharides) that encapsule the bacterium and not the surface tension of the bacterium itself. The bacterium, staying at a distance predicted by the secondary minimum (DLVO theory) will attach very strongly to the epithelial cell by the glue of capsular polysaccharides.

Second, one should consider specific short-range interactions involving molecular interactions, which require the interplay of electrostatic bonds, hydrogen bonds, van der Waals bonds, hydrophobic interactions, and perhaps chemical bonds.

Finally, it should be stressed that, in case of bacterial adhesion to epithelial cells, hydrodynamic forces also should be considered, applied by the flow of secretions such as saliva, intestinal juice or urine. For a given velocity of flow of solvent, the hydrodynamic force acting on attached particles has been shown to be roughly proportional to the third power of the dimension of this particle (Dahneke, 1975). This means that the hydrodynamic forces easily shear off bacteria from surfaces at low velocities of flow when these bacteria are attached only by forces predicted by the DLVO theory for the secondary minimum.

V. Structure of Fimbriae

A. CHEMICAL CHARACTERIZATION OF FIMBRIAE

Chemical analysis of fimbriae has shown that they are composed of a single major polypeptide species, with carbohydrates or phosphate occasionally present as minor components (Table 3). However, only in a few cases has it been plausibly argued that the carbohydrate or phosphate detected was not derived from contaminating cell-wall material. McMichael and Ou (1979) have shown that type 1 fimbriae contain approximately one residue of reducing sugar per subunit. At least some of the sugar is thought to be linked to a serine, threonine or tyrosine residue. Glucosamine (2 to 3 residues per subunit) has been detected in fimbriae from an *E. coli* strain (strain 7714) isolated from blood (Salit *et al.*, 1983). An unidentified amino-sugar has been

TABLE 3. Chemical analysis of fimbriae

Strain[a]	Designation fimbria	Sugars bound by fimbriae[b]	References	Amino-acid composition[c]											
				Lys	His	Arg	Asp	Asn	Thr	Ser	Glu	Gln	Pro	Gly	Ala
Escherichia coli K12	type 1	D-Mannose	1, 2, 3	3	2	3	8	11	19	10	3	10	2	16	31
UTI (346)	type 1	D-Mannose	4	5	2	2	20		18	9	13		2	15	25
UTI O4:K6 (J96)	type 1	D-Mannose	5	4	2	2	18		20	9	16		2	21	35
UTI O6:K2:H1 (C1214)	type 1A	D-Mannose	6	8	1	3	22		16	12	14		4	17	19
UTI O4:K2:H1 (C1212)	type 1C	?	6	7	2	3	22		18	12	14		4	17	18
UTI O4:K12 (KS71)	KS71C	?	7	7	2	2	20		20	12	13		4	18	20
UTI O4:K12 (ER2)	ER2B2	?	7	7	2	2	20		19	12	13		4	18	18
UTI O6:K2:H1 (C1214)	type 1B	?	6	8	1	3	23		19	13	15		4	18	20
UTI O4:K12 (3048)	3048	Gal-Gal	8	6	2	4	22		22	12	14		4	18	32
UTI O2:K5:H1 (3669)	F9	Gal-Gal	8	6	2	3	19		26	12	15		3	17	25
UTI O6:K2:H1 (C1212)	$F7_2$	Gal-Gal	9	10	2	2	13	9	17	12	3	9	8	21	16
UTI O4:K12 (KS71)	KS71A	Gal-Gal	7	11	2	2	23		15	20	27		5	28	20
UTI O4:K6 (J96)	Pap	Gal-Gal	10	12	2	0	11	10	13	12	6	7	5	22	17
Escherichia coli (7714)	7714	Gal-Gal	11	12	2	3	18		13	14	16		7	19	20
UTI O16:K1:H6 (C1979)	F12	Gal-Gal	12	12	2	0	24		16	12	14		7	18	19
UTI O4:K3 (3048)	3048	Gal-Gal	8	6	2	4	22		22	12	14		4	18	32
UTI (SS142)	SS142	?	13	9	4	4	25		23	15	15		0	23	24
UTI (O18ac)	F8	Gal-Gal	14	14	3	0	22		16	16	15		7	23	25
UTI (O1:H$^-$) (GV-12)	V-A adh[f]	?	15	6	4	13	17		13	8	9		0	28	6
ETEC 078:H11 (H-10407)	CFA I	?	16, 17	8	1	1	5	7	15	17	5	6	7	10	19
ETEC 0101 (B41)	K99	Neu-lac	18, 19	8	3	4	6	17	21	16	3	3	4	18	17
ETEC 0101 (B41)	F41	?	20	22	6	4	27		18	33	24		11	39	16
ETEC 09:K103:H$^-$ (987)	987P	?	21	10	0	2	34		28	23	16		8	26	26
ETEC O8:K87:H19 (G7)	K88ab(p17.6)	?	22	5	2	11	13	3	12	10	2	9	6	17	21
ETEC O8:K87:H19 (G7)	K88ab(p26)	B-D-Gal-X	23, 24	11	1	8	15	16	28	18	9	6	5	36	29
Escherichia coli O?:H4 (444-3)	Adh444-3[f]	?	25	3	4	0	17		10	25	20		2	24	10
Escherichia coli O21:H$^-$ (469-3)	Adh469-3[f]	?	25	4	3	2	15		18	21	17		4	20	12

Strain[a]	Designation fimbria	Sugars bound by fimbriae[b]	References	Amino acid composition[c]										
				Cys	Val	Met	Ile	Leu	Tyr	Phe	Trp	Total	Mod[d]	pI[e]
Escherichia coli K12	type 1	D-Mannose	1, 2, 3	2	15	0	4	10	2	7	0	158	RS	3.9
UTI (346)	type 1	D-Mannose	4	2	14	0	5	10	3	5	0	151	ND	ND
UTI O4:K6 (J96)	type 1	D-Mannose	5	2	14	0	5	14	2	8	0	174	ND	ND
UTI O6:K2:H1 (C1214)	type 1A	D-Mannose	6	2	13	1	7	10	4	6	ND	159	ND	ND
UTI O4:K2:H1 (C1212)	type 1C	?	6	2	18	0	6	8	3	7	ND	161	ND	ND
UTI O4:K12 (KS71)	KS71C	?	7	2	21	0	5	8	3	7	ND	164	ND	ND
UTI O4:K12 (ER2)	ER2B2	?	7	2	19	0	5	8	3	7	ND	159	ND	ND
UTI O6:K2:H1 (C1214)	type 1B	?	6	2	14	1	7	11	4	5	ND	168	ND	ND
UTI O4:K12 (3048)	3048	Gal-Gal	8	2	16	1	6	12	0	8	0	181	ND	ND
UTI O2:K5:H1 (3669)	F9	Gal-Gal	8	2	17	1	5	10	3	7	0	173	ND	ND
UTI O6:K2:H1 (C1212)	$F7_2$	Gal-Gal	9	2	15	1	8	7	2	10	0	167	ND	ND
UTI O4:K12 (KS71)	KS71A	Gal-Gal	7	2	14	2	10	10	4	11	ND	206	ND	ND
UTI O4:K6 (J96)	Pap	Gal-Gal	10	2	17	1	7	9	2	8	0	163	ND	ND
Escherichia coli (7714)	7714	Gal-Gal	11	2	13	2	10	11	3	7	ND	171	GA	ND
UTI O16:K1:H6 (C1979)	F12	Gal-Gal	12	2	13	2	10	12	3	9	0	175	ND	ND
UTI O4:K3 (3048)	3048	Gal-Gal	8	0	16	0	6	12	0	8	ND	178	ND	4.9
UTI (SS142)	SS142	?	13	3	9	0	5	14	3	3	ND	179	ND	ND
UTI (O18ac)	F8	Gal-Gal	14	2	17	0	12	10	3	8	ND	193	P	5.1–5.6
UTI (O1:H^-) (GV-12)	V-A adh[f]	?	15	0	7	1	4	8	3	4	ND	131	ND	ND
ETEC O78:H11 (H-10407)	CFA I	?	16, 17	0	19	3	5	12	4	2	1	147	ND	4.8
ETEC 0101 (B41)	K99	Neu-lac	18, 19	2	6	3	8	7	5	7	1	159	ND	9.5
ETEC 0101 (B41)	F41	?	20	ND	17	3	10	14	13	9	ND	266	HL	4.6
ETEC O9:K103:H^- (987)	987P	?	21	2	15	1	12	17	5	3	1	229	AS	3.7
ETEC O8:K87:H19 (G7)	K88ab(p17.6)	?	22	2	22	0	2	7	6	10	0	160	ND	ND
ETEC O8:K87:H19 (G7)	K88ab(p26)	B-D-Gal-X	23, 24	0	21	3	13	19	10	12	4	264	ND	4.2
Escherichia coli O?:H4 (444-3)	Adh444-3[f]	?	25	ND	6	2	4	7	4	5	ND	143	ND	4.7
Escherichia coli O21:H^-(469-3)	Adh469-3[f]	?	25	ND	7	2	5	8	2	5	ND	135	ND	5.0

[a] Strain numbers are given in parenthesis.

[b] For more details concerning the sugars recognized by fimbriae see Section IV.

[c] If no values are given for Asn or Gln, the values in the Asp and Glu columns refer to the amount of Asx or Glx detected, respectively. ND, not determined.

[d] Post-translational modification; RS, reducing sugar; GA, glucosamine; P, phosphate; AS, amino sugar; HL, hydroxylysine; ND, not determined. [e] Isoelectric point.

[f] Non-filamentous adhesions.

[g] References: 1, Brinton, 1965; 2, McMichael and Ou, 1979; 3, Klemm, 1984; 4, Eshdat *et al.*, 1981; 5, O'Hanley *et al.*, 1983; 6, Klemm *et al.*, 1982; 7, Rhen *et al.*, 1983d; 8, Svanborg-Eden *et al.*, 1983; 9, Van Die *et al.*, 1984; 10, Baga *et al.*, 1984; 11, Salit *et al.*, 1983; 12, Klemm *et al.*, 1983; 13, Mett *et al.*, 1983; 14, Wevers *et al.*, 1980; 15, Sheladia *et al.*, 1982; D.G. Evans *et al.*, 1979; 17, Klemm, 1982; 18, De Graaf *et al.*, 1980b; 19, Roosendaal *et al.*, 1984; 20, De Graaf and Roorda, 1982; 21, Isaacson and Richter, 1981; 22, Mooi *et al.*, 1984; 23, Mooi and De Graaf, 1979; 24, Gaastra *et al.*, 1981; 25, Williams *et al.*, 1984.

detected in preparations of 987P fimbriae (Isaacson and Richter, 1981), and fimbriae isolated from an uropathogenic strain (strain O18ac) have been shown to contain phosphate (Wevers *et al.*, 1980). Another example of post-translational modification is found in F41 fimbriae which appear to contain hydroxylysines (De Graaf and Roorda, 1982). The function of the various post-translational modifications might be to enhance the stability or solubility of these proteins, or to protect them against proteolytic enzymes ubiquitous in the intestinal tract (Wold, 1981). For the K88ab fimbriae it has been suggested that post-translational modification might be necessary for receptor recognition (Mooi *et al.*, 1983).

Most fimbrial subunits are composed of 140–200 amino-acid residues (Table 3). Exceptions are the subunits of the K88ab and F41 fimbriae, which are composed of 264 and 266 residues, respectively. Fimbria are generally acidic proteins with pI 4–5 (Table 3). A notable exception is formed by the K99 fimbria which is a very basic protein with pI 9.7. The observation that most fimbriae have a net negative charge at pH 7 is unexpected, because this might hamper the approach of the fimbriae to the negatively charged surface of eukaryotic cells. Possibly, the charge density of the fimbriae is very low. It is also possible that cations shield the negative charge, or are involved in the formation of ionic bridges between fimbriae and the eukaryotic cell surface. Indeed, divalent metal ions have been implicated in K88 fimbriae-mediated adhesion (Sugarman *et al.*, 1982).

Most fimbrial subunits contain two cysteine residues. For type 1, 987P and K99 fimbrial subunits it has been found that addition or removal of reducing agents affects their apparent molecular weight as determined by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (McMichael and Ou, 1979; Isaacson *et al.*, 1981; Isaacson and Richter, 1981). This suggests that the two cysteine residues are involved in the formation of an intramolecular disulphide bridge. The PapA subunit also contains a disulphide bridge, because reducing conditions are required to separate fragments obtained after cleavage of the subunit with CNBr (O'Hanley *et al.*, 1983 cited in Baga *et al.*, 1984). The existence of an intrachain disulphide bond has also been shown in P fimbriae derived from the uropathogenic strains 3048 and 3669 (Svanborg Edén *et al.*, 1983). Schoolnik *et al.* (1983) have shown that gonococcal fimbrial subunits contain an intrachain disulphide bridge, which confers the conformational requirements for antigenicity.

Type 1 fimbriae derived from different *E. coli* strains show small differences in amino-acid composition, indicating small differences in primary structure. Type 1 fimbriae and fimbriae associated with urinary tract infections (UTI fimbriae) appear to be very similar in amino-acid composition suggesting a close evolutionary relationship between these proteins. In contrast, fimbriae

associated with diarrhoeal disease are more heterogeneous with respect to their amino-acid composition.

B. PRIMARY STRUCTURE OF FIMBRIAE

Fimbriae of *E. coli* form a group of functionally and structurally closely related proteins that might have evolved from common ancestral genes. Therefore, it is of interest to compare the primary structure of different fimbrial subunits. From this comparison one may infer evolutionary relationships. Furthermore, it allows one to discern and distinguish between conserved and variable sequences, to correlate these with similarities and differences in function and ultrastructure, and ultimately, to link certain sequences to particular functions. Also, identification of conserved sequences may facilitate the development of synthetic vaccines directed against a number of different fimbriae.

The primary structure of a number of fimbrial subunits has been partially or completely resolved. In particular, a relatively large number of type 1 and P fimbriae have been characterized with respect to their *N*-terminal sequences (Table 4). Comparison of the *N*-terminal sequences of type 1 fimbrial subunits derived from different *E. coli* strains, reveals that these sequences are virtually identical. Sequence differences between these subunits do exist in other regions, because small but significant variations in amino-acid compositions are found between these polypeptides (Table 3). Type 1 fimbrial subunits derived from *Salmonella typhimurium* LT2 and *Klebsiella pneumoniae* share a large degree of homology at their *N*-termini with *E. coli* type 1 fimbrial subunits (Table 4). The *N*-termini of P fimbrial subunits also share a large degree of homology. Furthermore, comparison of type 1 and P fimbriae shows homology at their *N*-termini, suggesting a close evolutionary relationship between these two groups of proteins. The *N*-termini of the 3048 and 3669 P fimbrial subunits are somewhat more homologous with type 1 subunits than with the other P fimbrial subunits, and might represent transitional forms between the two.

When comparing the *N*-terminal sequences of type 1 and P fimbrial subunits, a pattern can be seen which consists of highly conserved hydrophobic amino-acid residues, alternated by less conserved and generally hydrophilic residues. Hydrophilic residues are generally exposed at the surface of proteins, and therefore accessible to antibodies. It might be expected that a selective pressure exists to vary these exposed residues as much as is allowed within the functional and structural constraints imposed upon them. In contrast, hydrophobic amino-acid residues are generally not exposed at the surface of proteins, and therefore do not interact with the host immune system. Furthermore, hydrophobic amino-acid residues play an important role in maintaining the structure of proteins (Thomas and Schechter, 1980)

TABLE 4. *N*-Terminal amino-acid sequences of fimbrial subunits[a]

Strain[b]	Designation fimbriae	Sugars bound by fimbriae[c]	References[d]	Amino acids											
Escherichia coli K12	Type 1	D-Mannose	1				A	A	T	T	V	N	G	—
Salmonella typhimurium LT2	Type 1	D-Mannose	2	A	D	P	T	*	V	S	.	S	.	—
Klebsiella pneumoniae	Type 1	D-Mannose	3				N	T	.	.	.	.	.	—
UTI O6:K2:H1 (C1214)	Type 1A	D-Mannose	4				.	.	.	.	.	.	.	—
UTI 2239	2239A	?	5				.	.	.	.	.	.	.	—
UTI O4:K6 (J96)	Type 1	D-Mannose	6		A	A	T	T	V	.	.	.	.	—
UTI O4:K12 (KS71)	KS71C	?	7					V	.	.	.	.	.	—
UTI O4:K12 (ER2)	ER2B2	?	7					V	.	.	.	.	.	—
UTI O6:K2:H1 (C1212)	Type 1C	?	4					V	.	.	.	.	.	—
UTI 2239	2239B	?	5					V	.	.	.	.	.	—
Escherichia coli 9353	9353A	D-Mannose	5	D	D	T	T	*	.	.	.	.	.	—
UTI O4:K6 (J96)	Pap	Gal-Gal	9				.	P	.	I	P	Q	.	Q
UTI O4:K12 (3048)	3048	Gal-Gal	8					V	P	.	I	.	.	—
UTI 02:K5:H1 (3669)	F9	Gal-Gal	8		E	T	T	*	.	.	.	.	.	—
UTI O6:K2:H1 (C1212)	$F7_1$	Gal-Gal	4				.	.	.	*	*	*	.	*
UTI O4:K12 (KS71)	KS71A	Gal-Gal	7				.	.	.	*	*	*	.	*
UTI O6:K2:H1 (C1212)	$F7_2$	Gal-Gal	10				.	*	.	*	*	*	.	*
Escherichia coli 7714	7714	Gal-Gal	5				.	*	.	*	*	*	.	*
UTI O16:K1:H6 (C1979)	F12	Gal-Gal	11				.	*	.	*	*	E	.	*
ETEC 0101 (B41)	F41	?	12										A	D
ETEC O8:K87:H19 (G7)	K88ab(p26)	β-D-Gal-X	13											W
ETEC O101(B41)	K99	Neu-lac	14,15										N	T
ETEC O8:K87:H19 (G7)	K88ab(p17.6)	?	16									A	V	*
ETEC O78:H11 (H-10407)	CFA I	?	17										V	E

[a] The amino-acid sequences are given in the single letter code. Dots and asterisks indicate amino-acid residues that are identical to residues in the *E. coli* K12 type 1 and the Pap A fimbrial subunit sequence, respectively. Dashes indicate gaps introduced to increase the number of matches.

[b] Strain numbers are given in parenthesis.

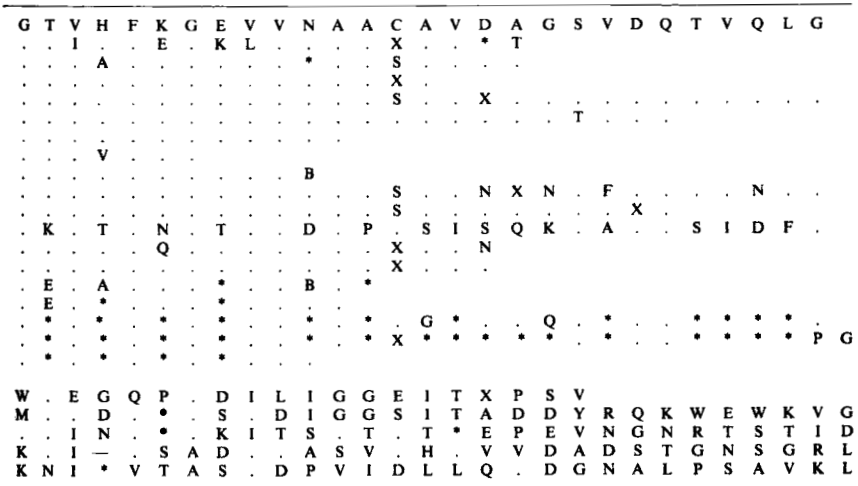
[c] For more details concerning the sugars recognized by fimbriae see Section IV.

[d] References: 1, Klemm, 1984; 2, Waalen *et al.*, 1983; 3, Fader *et al.*, 1982; 4, Klemm *et al.*, 1982; 5, Salit *et al.*, 1983; 6, O'Hanley *et al.*, 1983; 7, Rhen *et al.*, 1983d; 8, Svanborg-Eden *et al.*, 1983; 9, Baga *et al.*, 1984; 10, Van Die *et al.*, 1984; 11, Klemm *et al.*, 1983; 12, De Graaf and Roorda, 1982; 13, Gaastra *et al.*, 1981; 14, De Graaf *et al.*, 1980b; 15, Roosendaal *et al.*, 1984; 16, Mooi *et al.*, 1984; 17, Klemm, 1982.

and are therefore generally highly conserved. It has been suggested (Klemm *et al.*, 1983) that at the *N*-termini of type 1 and P fimbrial subunits, the hydrophobic and hydrophilic residues respectively form the outward and inward facing side of a β-sheet. In fact, secondary structure predictions reveal a high probability for a β-sheet at the *N*-termini of these polypeptides (F.R. Mooi, unpublished observations). Thus, the conservation of hydrophobic, and the variation of hydrophilic, residues might allow the conservation of a common structure still exhibiting a large degree of antigenic variation. In the light of the aforementioned, it seems likely that the *N*-termini of type 1 and P fimbrial subunits are in part exposed at the surface of the fimbriae.

Compared with type 1 and P fimbriae, the fimbriae associated with diarrhoeal disease form a much more diverse group with respect to *N*-terminal sequences (Fig. 2). Nevertheless, some homology is evident at the *N*-termini of these proteins. Some of the highly conserved residues found in type 1 and P fimbriae, also appear to be conserved in fimbriae associated with diarrhoeal disease.

The primary structure of only a few fimbrial subunits has been determined



completely. The K88ab fimbrial subunit was the first fimbrial subunit of which the amino-acid sequence was resolved. Compared with most *E. coli* fimbrial subunits, it has a high molecular weight (Table 1). Nevertheless, the K88ab fimbrial subunit shows homology at its *N*- and *C*-terminus (Fig. 2) with other fimbrial subunits, suggesting an evolutionary relationship. Possibly, the K88ab fimbrial subunit has evolved from a putative ancestral fimbrial subunit gene by gene fusion. At least three serological variants of the K88 fimbria have been described, which have been designated K88ab, K88ac and K88ad (Orskov *et al.*, 1964; Guinée and Jansen, 1979). Apparently, the K88 fimbriae contain regions that are conserved and form the a determinants, and regions that are variable and form the b, c and d determinants. Chemical analysis of the K88 variants revealed only small differences in amino-acid composition (Mooi and De Graaf, 1979). The primary structure of the K88ab fimbrial subunit was determined by DNA (Gaastra *et al.*, 1981), and protein sequencing (Klemm, 1981). Later the primary structure of the K88ad and K88ac fimbrial subunits were resolved (Gaastra *et al.*, 1983; Josephsen *et al.*, 1984). Comparison of the three primary structures (Fig. 2) reveals highly conserved and highly variable regions. For the three variants, the first 37 and last 28 amino-acid residues are completely identical. A large stretch of amino-acid residues showing complete identity also is found between residues 106 and 133. Two antigenic determinants have been predicted within these conserved sequences (Fig. 2; Klemm and Mikkelsen, 1982), possibly representing the a determinants. If we confine ourselves to areas containing differences between all three variants, two highly variable regions can be

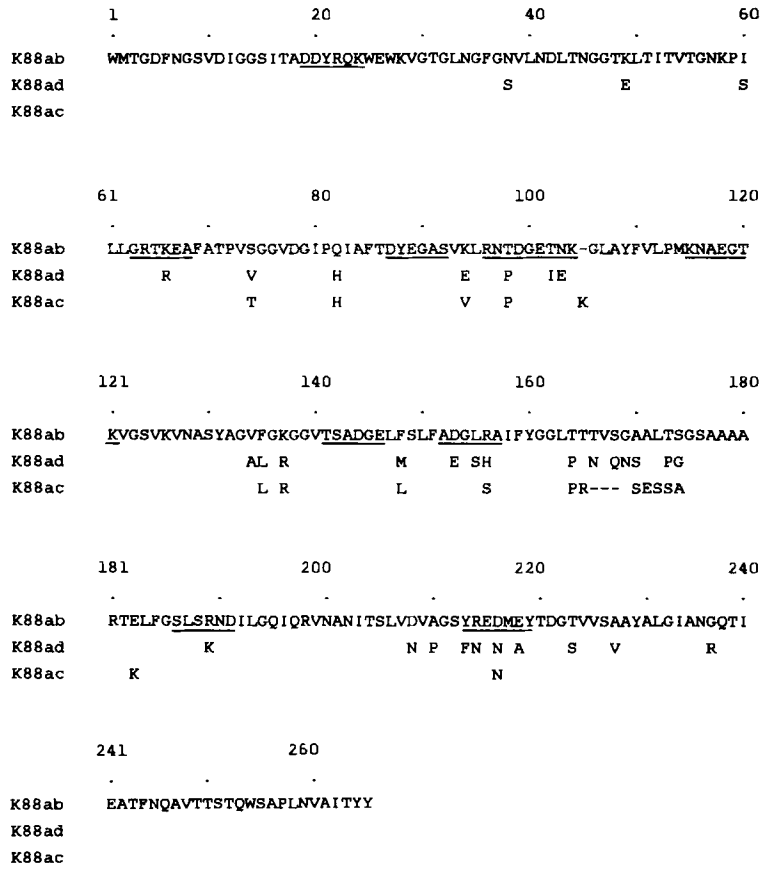


FIG. 2. Comparison of the primary structures of the K88ab, K88ad and K88ac fimbrial subunits. The amino-acid sequences are given in the single-letter code. For the K88ad and K88ac subunits, only amino-acid residues that differ from K88ab are indicated. Dashes indicate gaps introduced to increase the number of matches. The horizontal bars indicate predicted antigenic determinants. See the text for references.

discerned, located between residues 94 and 105, and 148 and 174, respectively. In both regions antigenic determinants have been predicted. Since all three variants show mutual differences here, these regions probably contribute to the b, c and d antigenic determinants. We assume that the conserved sequences of the K88 fimbrial subunits are involved in assembly and maintenance of the fimbrial structure, and in receptor recognition. The variable regions of the subunits might have evolved by genetic drift, or to evade the immune response of the host.

Apart from the K88 fimbrial subunits, the primary structure of the CFA I

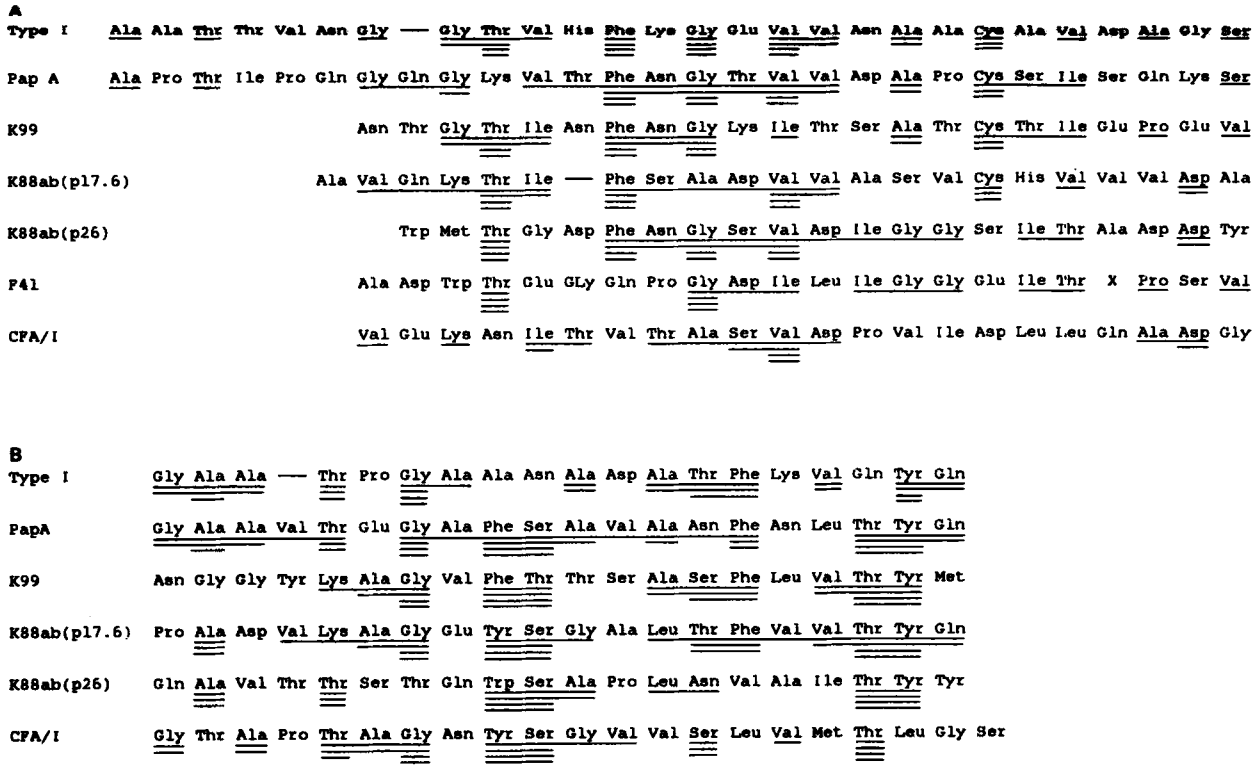


FIG. 3. Comparison of *N*- (A) and *C*- (B) terminal amino-acid sequences of fimbrial subunits. Identical or functionally identical amino-acid residues have been underlined. The number of times a residue is underlined, indicates how often it occurs at that particular position. The amino acids (Ser, Thr) and (Phe, Trp, Tyr) were assumed to be functionally identical. The amino-acid sequences were obtained from the following references: K88ab (p26), Gaastra *et al.* (1981); CFA I, Klemm (1981); F41, De Graaf and Roorda (1982); K99, Roosendaal *et al.* (1984); PapA, Baga *et al.* (1984); K88ab (p17.6), Mooi *et al.* (1984); type 1, Klemm (1984).

(Klemm, 1982), PapA (Baga *et al.*, 1984), K99 (Roosendaal *et al.*, 1984), type 1 (Klemm, 1984) and $F7_2$ (Van Die and Bergmans, 1984) fimbrial subunit have been resolved. Furthermore, the primary structure of a fimbria-like subunit (p17.6) encoded by the gene cluster for K88ab has also been determined (Mooi *et al.*, 1984). Comparison of the primary structures of these polypeptides reveals that they are similar in a number of aspects. With the exception of the CFA I subunit, these polypeptides are composed of approximately the same number of amino-acid residues, and contain two cysteine residues located at approximately the same position. Furthermore, all these polypeptides, as well as the K88ab fimbrial subunit, show homology at their *N*- and *C*-termini (Fig. 3). Homology between the PapA and $F7_2$ polypeptides is not confined to their *N*- and *C*-termini, but is also found in more central parts of the molecules (Fig. 4). Furthermore, the PapA and $F7_2$ fimbrial subunits share homology with central parts of the type 1 fimbrial subunit polypeptide chain (Fig. 4), again suggesting a close evolutionary relationship between type 1 and P fimbriae. Homology between the central parts of these three proteins and the K99 fimbrial subunit is much less pronounced, suggesting a more distant evolutionary relationship (Fig. 4). Except for their *C*-termini, the CFA I and the K88ab fimbrial-like subunit (p17.6) show very little homology with the above-mentioned fimbrial subunits. Furthermore, the CFA I subunit is approximately 15 amino-acid residues shorter, and contains no cysteine residues. This might indicate that it has evolved from the putative ancestral gene by one or more deletion events, possibly involving its *N*-terminus.

C. ULTRASTRUCTURE OF FIMBRIAE

In general, the fimbriae of *E. coli* can be separated into two morphological classes or types. One morphological type is represented by type 1, 987P, CFA I, CS1, CS2 and P fimbriae. These fimbriae are rigid structures with a diameter of approximately 7 nm and with an apparent axial hole. The second morphological type is represented by K88, K99, F41 and CS3 fimbriae. These fimbriae are flexible and very thin with a diameter of 2–5 nm. Based on their thin and flexible nature these fimbriae are also designated as fibrillae or as "fibrillar" fimbriae (Jones, 1977). A fimbriated bacterium may carry several hundreds of fimbriae peritrichously arranged on its surface. The length of these fimbriae varies between 0.5 and 2 μm or even longer.

The structure of the rigid type 1 fimbriae has been studied extensively by Brinton (1965) using electron microscopy, crystallography and X-ray diffraction. Type 1 fimbriae tend to form aggregates of parallel bundles and angled layer crystals in the presence of Mg^{2+} ions. The most simple model for explaining the observed crystalline forms is to suppose a rigid right-handed

FIG. 4. Comparison of the primary structure of the type 1, papA, $F7_2$ and K99 fimbrial subunits. The amino-acid sequences are given in the single-letter code. Identical or functionally identical amino-acid residues have been underlined once or twice if they occur in three or four fimbrial subunits, respectively. (R, K) (S, T) and (F, W, and Y) were assumed to be functionally identical. Asterisks indicate $F7_2$ amino-acid residues that are identical to PapA amino-acid residues. Dashes indicate gaps introduced to increase the number of matches. See text for references.

helical structure. X-ray diffraction patterns are in agreement with such a model. A helical repeat of 2.32 nm (23.2 Å) was calculated which fits with $3\frac{1}{8}$ subunits per turn. The diameter of the axial hole is about 2 nm.

Also, fimbriae belonging to the second morphological type (i.e. "fibrillae") sometimes appear to form aggregates. However, as far as they have a helical structure (as for instance with the K99 fimbriae), this helix has an open structure without an axial hole (De Graaf *et al.*, 1980b). Whether the differences in morphology reflect an adaptation to different functions remains to be determined.

It has been suggested by Watts *et al.* (1983), that a likely way for fimbriae to be assembled is to use the same packing arrangement repeatedly. Thus one would expect all subunits to occupy an equivalent environment and be dissociated into monomers under denaturating conditions. However, *Pseudomonas* sp. fimbriae dissociate into dimers in the presence of octyl glycoside (Watts *et al.*, 1983). Eshdat *et al.* (1981) have shown that, after denaturation in the presence of guanidinium hydrochloride, type 1 fimbrial subunits reassociate into dimers when the guanidine hydrochloride is removed by dialysis. Thus, it is possible that the basic building block of these fimbriae is a dimer, and not a monomer. Watts *et al.* (1983) have presented possible packing arrangements of dimers in fimbrial structures.

An interesting question is whether type 1 fimbriae adhere only with their tip or alternatively with binding sites along the fimbrial structure. Brinton (1965), using polystyrene latex spheres, showed that this artifical substrate was agglutinated by end-wise attached fimbriae which indicated a binding site at the tip of type 1 fimbriae. However, latex beads do not contain specific receptors for these fimbriae, and the binding could simply be mediated by the hydrophobic subunit–subunit binding site exposed at the tip. The effect of D-mannose on the binding of type 1 fimbriae to latex beads was not investigated, but it is unlikely that such an interaction displays mannose sensitivity. Salit and Gotschlich (1977b) observed that purified type 1 fimbriae stick to the cell membrane of monkey kidney (Vero) cells over a considerable length along their structure, indicating a lateral- rather than terminal-wise attachment. Ultrasonication of fimbriae resulted in shortening and a strong decrease in haemagglutinating activity (Salit and Gotschlich, 1977a; Sweeney and Freer, 1979). The ability of shortened fimbriae to bind to erythrocytes, however, appeared to be much less affected (Sweeney and Freer, 1979), indicating that type 1 fimbriae have lateral binding sites along their structure but require a critical length to induce haemagglutination.

Not all adhesins may be classified as fimbriae. Eshdat *et al.* (1981) described an *E. coli* strain, isolated from a case of urinary tract infection, producing type 1 fimbriae as judged by different criteria. The mannose-sensitive yeast-agglutinating activity, however, was found not to be associated with the type 1

fimbriae only, but also with defimbriated cells and isolated outer-membrane vesicles. This observation may indicate that fimbrial subunit monomers are inserted into the outer membrane without polymerization into fimbriae or, alternatively, that another mannose-sensitive adhesin is present in these membranes.

In another study, Eshdat *et al.* (1978) isolated a "mannose-specific" adhesin from an *E. coli* strain (isolated from a urinary tract infection) with other characteristics as type 1 fimbriae. The isolated adhesin was identified as a high molecular-weight protein aggregate present at the cellular surface and consisting of protein subunits with a molecular weight of 36,500. The purified adhesin agglutinated yeast cells and mouse spleen lymphocytes. This activity was inhibited by D-mannose, methyl-α-D-mannoside, mannan and *p*-nitrophenyl-α-D-mannoside, the latter being the most potent inhibitor. The adhesin was identified as a flagellar-like structure (Eshdat *et al.*, 1980). Other examples of non-fimbrial adhesins (Table 3) have been reported (Williams *et al.*, 1984; Sheladia *et al.*, 1982; Labigne-Roussel *et al.*, 1984).

D. STRUCTURE–FUNCTION RELATIONSHIPS

Comparison of the primary structure of a large number of fimbrial subunits shows that the conservation of amino-acid residues is most apparent at their *N*- and *C*-termini. The conservation of homology in limited areas of the fimbrial subunit polypeptide chains could be explained if these areas define domains involved in functions shared by all these subunits. Such functions could involve transport across the outer membrane, anchorage, and subunit–subunit binding. Conservation of particular amino-acid residues within a large group of fimbriae is most pronounced at the *C*-terminus. For several reasons it seems possible that the *C*-termini of fimbrial subunits are involved in subunit–subunit binding. With the exception of the *C*-terminus of type 1 fimbriae, the *C*-termini are hydrophobic and therefore likely to be buried inside the fimbrial structure. Furthermore, at three positions aromatic amino-acid residues appear to be conserved, which have been implicated in the maintenance of quarternary structure of fimbriae. Watts *et al.* (1983) have shown that two tyrosine residues, and at least one tryptophan residue, become exposed when fimbriae of *Pseudomonas* sp. are dissociated in dimers, suggesting that these amino-acid residues are located at the dimer–dimer interface. In addition, McMichael and Ou (1979) have shown that disruption of type 1 fimbriae of *E. coli* K12 by acid treatment results in unmasking of tyrosine residues, also implicating an aromatic amino acid in subunit–subunit binding. In this context it is interesting to note that the type 1 fimbrial subunit derived from *E. coli* K12, contains only two tyrosine residues, both located at the *C*-terminus of the molecule (Klemm, 1984).

Homology at the *N*-termini is not so widespread as homology at the *C*-termini, and this might indicate a somewhat more specialized function for the *N*-terminus compared to the *C*-terminus. Homology at the *N*-termini is most evident within a group of morphologically similar fimbriae, possibly suggesting that this region may correspond to a section of the polypeptide chain determining a specific conformation (flexible/rigid) of the fimbria.

The more central parts of the fimbrial subunit polypeptide chains show a higher degree of variability than the termini. These parts might have become adapted to more specialized functions such as receptor recognition, or may simply have diverged as a response to immunological pressure because less stringent structural or functional constraints are imposed on them.

In the context of structure–function relationships, some remarks should be made concerning the (location of the) receptor-binding domain. Until recently it was generally assumed that fimbriae were composed of one subunit species which contained the receptor-binding domain. This assumption was based on a strong correlation between the presence and absence of fimbriae and adhesive capacity, and on biochemical data which showed that purified fimbria preparations revealed only one polypeptide species and retained the same building properties as the cells from which they were derived. The genetic analyses of the gene clusters coding for Pap (Norgren *et al.*, 1984) and X (J. Hacker, personal communication) fimbriae have shown that the correlation between fimbria and adhesion may be more complex than hitherto anticipated. These analyses have shown that at least some fimbriae of uropathogenic strains may attach to their receptors by means of an adhesin that is distinct from the fimbrial subunit that constitutes the major component of the fimbria. The gene cluster coding for Pap fimbriae appeared to contain three genes (*papE*, *papF* and *papG*) essential for P-specific binding but dispensable for fimbrial assembly (Lindberg *et al.*, 1984). The DNA sequence of these genes has revealed that the structure of the *papE* and *papF* gene products resembles the structure of the fimbrial subunit (encoded by *papA*) constituting the major component of the Pap fimbriae. Furthermore, biochemical and serological data indicate that the PapE and PapF proteins are minor components of the Pap fimbriae (Lund *et al.*, 1984). The K88ab fimbriae also might be composed of more than one fimbrial subunit species since, in addition to the K88ab fimbrial subunit, the gene cluster for K88ab codes for a fimbria-like subunit (p17.6) (Mooi *et al.*, 1984). As yet it is not clear whether this fimbria-like subunit is part of the K88ab fimbriae, nor which subunit contains the receptor-binding domain. Thus it is possible that some fimbrial subunits do not contain a receptor-binding domain and only have a structural function; i.e. to expose the adhesin molecule at some distance from the bacterial cell surface, presumably to facilitate interaction with the host receptor. For several reasons the incorporation of minor fimbrial subunits,

containing the receptor-binding site, in the fimbrial structure might confer a selective advantage. First, antibodies produced by the host will be primarily directed against the presumably immunodominant, major, structural, component of the fimbriae, and be less likely to block adhesion. Second, it allows a larger degree of antigenic diversity of the structural component of fimbriae.

Although some fimbriae might contain a minor subunit containing the receptor-binding site, this is clearly not always the case. For example, there is evidence that mannose-specific binding of type 1 fimbriae is mediated by the type 1 fimbrial subunits. Eshdat *et al.* (1981) have shown that when type 1 fimbriae are dissociated into dimers, 25% of these dimers bind to a mannan–Sepharose column and can be eluted with methyl-α-D-mannoside. It is not clear why only 25% of the dimers bind to the mannan column. Possibly only a portion of the subunits may have an active binding site due to heterogeneity of individuals subunits in the fimbriae, or to denaturation caused by the treatment used to dissociate the fimbrial subunits. It has also been shown that the receptor-binding domain of gonococcal fimbriae is part of the fimbrial subunit constituting the gonococcal fimbriae (Schoolnik *et al.*, 1983).

VI. Genetics

A. TYPE 1 FIMBRIAE

Swaney *et al.* (1977) have performed a genetic complementation analysis of non-fimbriated mutants of a type 1 fimbriated strain of *E. coli* K12 (strain AW405). The pattern of complementation defined three structural genes (*pilA*, *pilB*, *pilC*) (pil stands for pilus) involved in fimbriae formation. It seems likely that one of these genes codes for the fimbrial subunit, whereas the other two might be involved in regulation, transport or assembly. All three genes are located at a position corresponding to 98 minutes on the *E. coli* K12 linkage map (Bachmann, 1983).

The genes coding for type 1 fimbria formation have been cloned using an *E. coli* clinical isolate J96 (Hull *et al.*, 1981). Orndorff and Falkow (1984a) have examined this clone in minicells, and found it to encode five polypeptides associated with type 1 fimbria production (Fig. 5). Three of these polypeptides (p17, p86 and p30) are involved in assembly of the fimbriae; assembly of the fimbrial subunit (p17) is not seen in mutants lacking either p86 or p30, but synthesis and proteolytic processing of p17 are not affected. A fourth polypeptide (p23) appears to be involved in the regulation of fimbria expression, because mutants lacking this polypeptide exhibit a 40-fold increase in the amount of fimbriae per cell. The gene coding for p23 was termed *hyp* for hyperfimbriation (Orndorff and Falkow, 1984b). A function

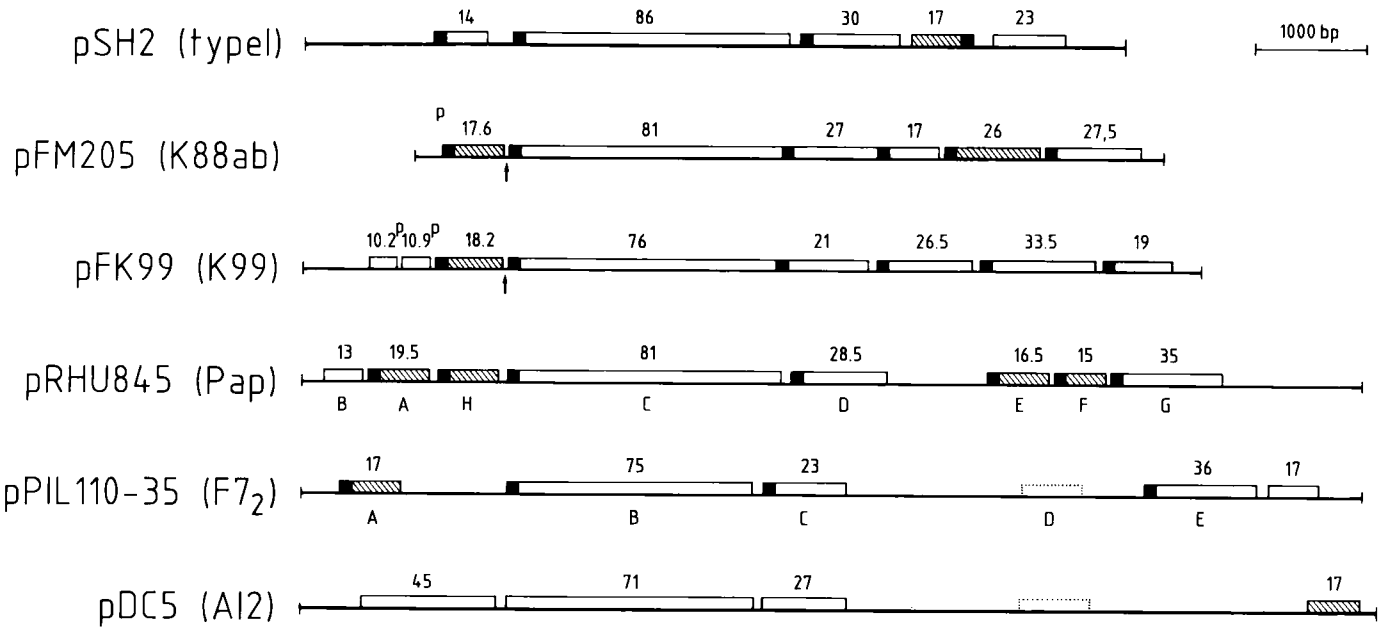


FIG. 5. Genetic organization of DNA regions involved in fimbria formation and adhesion. The locations of the various structural genes have been indicated by boxes. The black ends of the boxes indicate the parts of genes coding for the signal peptide. Dotted boxes indicate structural genes that encode products that have not yet been identified. Fimbrial subunit genes have been shaded. The numbers above the boxes refer to the molecular weights ($\times 10^{-3}$) of the corresponding polypeptides. The vertical arrows indicate regions with dyad symmetry; P, promoter sequence; bp, base pair. See text for references.

for the fifth polypeptide (p14) was not found. However, this polypeptide could be immunoprecipitated together with type 1 fimbriae, suggesting that it is associated with type 1 fimbriae, or immunologically cross-reactive with the type 1 fimbrial subunits. Except for polypeptide p23, all these polypeptides are synthesized as precursors containing a signal peptide, indicating that they are located in the cell envelope. The genes encoding the polypeptides are probably located in two or more transcriptional units.

It is not yet clear what the relation is between the *pil* genes defined by Swaney *et al.* (1977) and the genes identified by Orndorff and Falkow (1984a). It seems likely that gene clusters encoding type 1 fimbriae in different *E. coli* strains are closely related. Orndorff and Falkow (1984a) have found that *E. coli* K12 and *E. coli* Bam strains contain DNA with homology to their type 1 fimbria gene cluster. Furthermore, the type 1 fimbria gene cluster of *E. coli* K12 is organized similarly to the cluster derived from the clinical isolated J96 (P. Klemm and H. Bergmans, personal communication).

B. FIMBRIAE ASSOCIATED WITH DIARRHOEAL DISEASE

1. CFA I Fimbriae

The CFA I fimbria is encoded by a group of closely related plasmids (size approximately 89 kilobase pairs (kbp)) that also carry the genes for heat-stable enterotoxin, ST (Willshaw *et al.*, 1982). The genes coding for CFA I have been mapped by transposon mutagenesis, and it appeared that two regions on the plasmid, separated by 37 kbp, are required for CFA I production (Smith *et al.*, 1982). Both regions were isolated by molecular cloning (Willshaw *et al.*, 1983), and it was shown that the ST genes are closely linked to one of these regions (designated region 1). Region 1 is contained within a DNA fragment of 6 kbp. The second region (region 2) is much smaller and contained within a 2 kbp fragment. Cell-free extracts of bacteria harbouring only region 1 contained protein that reacted with antibodies directed against CFA I fimbriae, suggesting that the CFA I fimbrial subunit gene is located within this region. The separate location of region 2 might indicate that it is not only involved in the production of CFA I, but also is required for other adhesins encoded by CFA I plasmids.

2. CFA II fimbriae

The genes responsible for production of the three CFA II components CS1, CS2 and CS3 reside within the same plasmid (size approximately 89 kbp), which generally also codes for heat-labile (LT) and heat-stable (ST) enterotoxin (Penaranda *et al.*, 1980; Mullany et al., 1983; Smith *et al.*, 1983).

Although CFA II plasmids code for all three components, a strain containing a CFA II plasmid may produce CS1 and CS3, CS2 and CS3, or CS2 or CS3 only. The decisive factor determining whether CS1 or CS2 is produced, is the genetic character of the host, which is recognized by its serotype and biotype. Only enterotoxigenic strains of serotype O6:H16 have been shown to produce CS1 or CS2. Furthermore, CS1 is produced only by O6:H16 strains of biotype A (rhamnose negative), whereas CS2 is produced only by O6:H16 strains of biotype B, C and F (rhamnose positive) (Cravioto *et al.*, 1982; Smyth, 1982). CS3 is produced independent of these biotypes and serotypes (Cravioto *et al.*, 1982). When CFA II plasmids are transferred to *E. coli* K12, only CS3 is produced (Mullany *et al.*, 1983). It is not clear what mechanism underlies the selective phenotypic expression of the CFA II components.

3. K99 Fimbriae

K99 fimbriae are encoded by conjugative plasmids of approximately 75 kbp in size (Smith and Linggood, 1972; Orskov *et al.*, 1975). The genes coding for K99 fimbria production have been isolated by molecular cloning (Van Embden *et al.*, 1980). The cloned DNA contained within plasmid pFK99 has been shown to contain eight structural K99 encoding genes, seven of which at least have been implicated in the biosynthesis of the K99 fimbria (Fig. 5; De Graaf *et al.*, 1984). Originally, an additional structure gene, coding for a 21,500-Da polypeptide, was assigned to the DNA region located between the genes for p76 and p21 (Fig. 5; De Graaf *et al.*, 1984). However, DNA sequence data indicate that the gene for p76 is directly followed by the gene for p21 (B. Roosendaal and F.K. De Graaf, unpublished work). The polypeptide p21.5 is produced in very low amounts in minicells, and probably translated from within the *C*-terminal part of the gene for p76.

DNA sequence data have revealed two possible promoters, which are located proximal to the genes for p10.9 and p18.2 (the K99 fimbrial subunit; Roosendaal *et al.*, 1984; B. Roosendaal and F.K. De Graaf, unpublished work). Especially the promoter proximal to the gene for p10.9 complies well with the consensus sequence of strong *E. coli* promoters, and the K99 genes are probably mainly transcribed from this promoter in pFK99. Thus it seems likely that the K99 genes are transcribed from their natural promoter(s) in the constructed recombinant plasmid. This is also suggested by the observation that expression of the genes for K99 is independent of the orientation of the cloned DNA within the cloning vehicle used (Van Embden *et al.*, 1980).

K99 fimbria production by wild-type strains is repressed at temperatures below 30°C, and by L-alanine (De Graaf *et al.*, 1980a,c). The effect of temperature and L-alanine on K99 fimbria production is still observed with strains containing the recombinant plasmid pFK99 (Van Embden *et al.*,

1980), indicating that the genes involved in these regulatory phenomena are contained within this plasmid.

The K99 operon contains a small intracistronic region, showing dyad symmetry, between the fimbrial subunit gene and the gene for p76 (Roosendaal *et al.*, 1984). The stem and loop structure that can be formed by the K99 transcript of this region (Fig. 6) might function as a *rho*-dependent terminator, which introduces a natural polarity in the K99 operon. The polypeptides encoded by the DNA located downstream from the putative terminator are

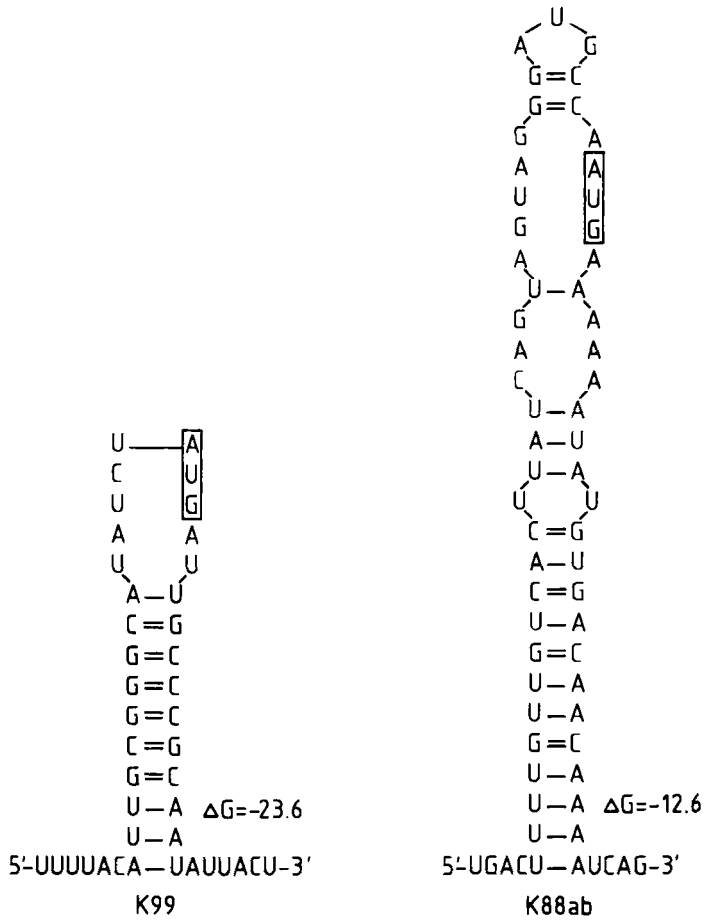


FIG. 6. Possible secondary structures formed by K99 and K88ab mRNA. The DNA regions coding for these structures are indicated by vertical arrows in Fig. 5. The putative initiation codons for the genes for p81 and p76 have been blocked. ΔG was calculated according to Tinoco *et al.* (1973).

probably required in lower amounts than the K99 fimbrial subunit, the gene of which is located upstream from this terminator. Another possible function for this region is suggested by the finding that the putative initiation codon for the gene encoding p76 is located within the stem and loop structure (Fig. 6). Therefore, this region also might be involved in temperature-dependent regulation, because a low temperature will stabilize the stem formed by the transcript and thus prevent initiation of translation of the gene for p76.

4. K88 Fimbriae

K88 fimbriae are encoded by plasmids (approximately 75 kbp) which frequently also code for the ability to utilize raffinose (Raf) (Orskov and Orskov, 1966; Shipley *et al.*, 1978). The genes coding for K88 and Raf are not closely linked, but are separated by 30 kbp (Mooi *et al.*, 1979). The association of K88 and Raf encoding genes might reflect the abundance of raffinose or related sugars in the pig intestine. It is also possible that this association endows porcine enterotoxigenic strains with a selective advantage, because Raf encoding genes also code for enzymes involved in the degradation of sugars that compete with the intestinal receptor for the adhesin binding site.

At least three different K88 variants, K88ab, K88ac and K88ad, have been described (Orskov *et al.*, 1964; Guinée and Jansen, 1979), which can be distinguished serologically. The K88ab and K88ac genetic determinants have been cloned (Mooi *et al.*, 1979; Shipley *et al.*, 1981) and studied extensively. Six structural genes have been located on the cloned K88ab DNA contained within the recombinant plasmid pFM205, at least five of which are located within a single transcriptional unit (Fig. 5) (i.e. the genes for p17.6, p81, p27, p17, and the K88ab fimbrial subunit p26; Mooi *et al.*, 1981, 1982, 1983; Mooi, 1982). In contrast, Kehoe *et al.* (1981) have proposed that the gene cluster coding for K88ac is organized in two operons. One operon is believed to contain the structural gene for K88ac fimbrial subunit (designated *adhD*). A second operon is thought to contain three structural genes; *adhA*, *adhB* and *adhC*. These genes code for polypeptides homologous to the K88ab polypeptides p81, p27 and p17, respectively. Kehoe *et al.* (1981) have suggested that *adhC* codes for a positive regulator (p17) necessary for transcription of *adhD*. However, this hypothesis was later shown to be incorrect, because p17 is located in the periplasmic space (Van Doorn *et al.*, 1982; Dougan *et al.*, 1983). It seems likely that the only significant differences between the gene clusters coding for K88ab and K88ac reside within the fimbrial subunit genes. In addition to the K88ab fimbrial subunit gene, the operon coding for K88ab has been shown to contain a gene for a fimbria-like subunit (p17.6; Fig. 5) (Mooi *et al.*, 1982, 1984). The existence of this fimbria-like subunit was inferred from DNA sequence data, and the analysis of K88ab mutants. It is produced in too

low amounts to be detected and might be a minor component of the K88ab fimbria (see Section VII).

Although the K88ab recombinant plasmid pFM205 contains all the structural genes necessary for production of functional fimbriae, this plasmid does not contain all the genes for K88ab. Expression of the genes for K88ab in plasmid pFM205 is mainly dependent on the pBR322 promoter P1 (Stueber and Bujard, 1981), indicating that the natural K88ab promoter is not contained within this plasmid (Mooi, 1982). When P1 was deleted, a 10–20-fold drop in K88ab production was observed. A weak promoter proximal to the gene for p17.6 (Fig. 5) might be responsible for the low level of K88ab production still observed in the absence of P1 (Mooi *et al.*, 1984). It is interesting that, although the genes for K88ab in plasmid pFM205 are mainly transcribed from P1, strains containing this plasmid still show a temperature-dependent production of K88ab fimbriae (Mooi *et al.*, 1979). This suggests that temperature does not (only) affect initiation of transcription of genes for K88ab. The K88ab operon contains a region with dyad symmetry located at the same relative position as in the K99 operon (Fig. 5). However, the stem and loop structure that can be formed by the K88ab transcript of this region is less stable than that of K99 transcript (Fig. 6). In the K88ab operon, the most highly expressed gene (the K88ab fimbrial subunit gene) is located downstream from the region of dyad symmetry. Thus, since there is no evidence for the presence of a promoter between the region of dyad symmetry and the gene for the fimbrial subunit (Mooi, 1982), it is unlikely that the stem and loop structure functions as a terminator. However, since the initiation codon of the gene for p81 is located within the stem and loop structure (Fig. 6), it might be involved in temperature-dependent regulation, as was suggested for the K99 operon.

C. FIMBRIAE ASSOCIATED WITH URINARY TRACT INFECTIONS

In contrast to most (but not all) fimbriae associated with diarrhoeal disease, the genes encoding fimbriae associated with urinary tract infections are located on the bacterial chromosome (Hull *et al.*, 1981; Berger *et al.*, 1982; Clegg, 1982; Rhen *et al.*, 1983a; Van Die *et al.*, 1983; Low *et al.*, 1984). Analysis of these fimbriae is complicated by the fact that uropathogenic strains may produce up to four different fimbriae, some of which are serologically related and recognize the same receptor (Rhen *et al.*, 1983c; Orskov and Orskov, 1983). The diversity of uropathogenic strains with respect to fimbria production may be illustrated by two well characterized strains of serotypes O6:K2:H1 (strain C1212; Orskov *et al.*, 1982), and O4:K12 (strain KS71; Rhen *et al.*, 1983a,d). Apart from type 1 fimbriae (designated type 1A and KS71D, respectively), these strains produce two

different P fimbriae (designated $F7_1$, $F7_2$ and KS71A, KS71B, respectively), and a fimbria that lacks demonstrable haemagglutinating activity (type 1C and KS71C, respectively) (Rhen *et al.*, 1983b; Klemm *et al.*, 1982). The four P fimbriae are clearly related, since they have very similar amino-acid compositions, and nearly identical *N*-terminal amino-acid sequences (Table 3 and Fig. 4). The same applies for the pairs type 1A and KS71D, or type 1C and KS71C fimbriae (Table 3 and Fig. 4). The gene clusters coding for the $F7_2$, KS71A and KS71B fimbriae have been cloned and appear to have very similar restriction maps (Van Die *et al.*, 1984; Rhen *et al.*,1983c; Rhen, 1985), suggesting a close evolutionary relationship between them.

There is a high incidence of alpha-haemolysin production among uropathogenic strains, and it has been confirmed that haemolysins play an important role in the pathogenesis of extra-intestinal *E. coli* infections (Welch *et al.*, 1981). Therefore, it is interesting that, in a number of uropathogenic strains, the gene clusters coding for haemolysin and UTI fimbriae are closely linked on the bacterial chromosome (Hacker *et al.*, 1983; Low *et al.*, 1984). In five O6 strains (designated 536, A55, 6754a, 3443 and C1212) the distance between the two clusters appears to be a 4–5 kbp. In one O4 strain (designated J96), this distance was found to be much larger and amounted to 14–15 kbp. It has been suggested that the linkage of haemolysin and UTI fimbriae gene clusters confers a selective advantage, because both factors act in a synergistic or complementary way (Low *et al.*, 1984). Linkage of these gene clusters may have occurred by transposition events. Indeed, a number of investigators have found evidence for the presence of homologous DNA segments flanking the haemolysin and UTI fimbriae gene clusters (Hacker *et al.*, 1983; Low *et al.*, 1984; Rhen, 1985), suggesting that they are contained in a transposon-like structure.

1. Pap Fimbriae

Hull *et al.* (1981) have isolated a chromosomal fragment from an *E. coli* O4:K6 strain (designated J96) coding for the Pap fimbria (Pap–pyelonephritis associated pilus). The 8.5 kbp region required for Pap fimbria formation and haemagglutination was shown to contain eight structural genes involved in fimbria formation and haemagglutination (*papA–H*, Fig. 5; Norgren *et al.*, 1984; Lindberg *et al.*, 1984). The genes have the same transcriptional orientation, and are probably transcribed from a promoter located proximal to *papB*. The region located upstream from the structural gene for the Pap fimbrial subunit (*papA*) contains regulatory functions involved in temperature-dependent production of Pap fimbria. This regulation probably occurs at the level of transcription (Göransson and Uhlin, 1984). As in the gene clusters for K99 and K88ab, a region of dyad symmetry is observed just downstream

from *papA* (S. Normark, personal communication). The Pap DNA contains several genes (*papE*, *F* and *H*) which code for fimbria-like subunits (Lund *et al.*, 1984).

2. $F7_2$ Fimbriae

Van Die *et al.* (1984) have cloned the genetic determinant for the $F7_2$ fimbria. Six structural genes were located on the cloned DNA, five of which are necessary for fimbria production and haemagglutination (i.e. genes denoted by A–E in Fig. 5). Gene D is probably expressed at a very low level, because no product from this gene was detected in minicells. The structural gene for the $F7_2$ fimbria is located at the same relative position as the structural gene for the Pap fimbria. Van Die *et al.* (1984) have concluded that the genes encoding $F7_2$ genes are organized in at least four different transcriptional units. However, this conclusion may be premature, because it was based on the lack of observed polarity of some Tn*5* insertions, and it has been observed often that Tn*5* insertions in multicopy plasmids show incomplete or even no polarity (De Bruijn and Lupski, 1984).

3. *IA2 Fimbriae*

Clegg (1982) has isolated a chromosomal DNA region coding for the synthesis of a fimbria involved in mannose-insensitive haemagglutination using the uropathogenic strain IA2 (serotype O6). This fimbria has not been named, therefore we will refer to it as the IA2 fimbria. The smallest recombinant plasmid still expressing the IA2 fimbria and haemagglutinating activity, contains a chromosomal DNA fragment of approximately 6 kbp, and was found to encode at least four polypeptides (Fig. 5; Clegg and Pierce, 1983). Genetic evidence suggests the presence of at least one additional structural gene on the cloned DNA, which might be involved in regulating the expression of the genes encoding p71 and p45. The production of this gene is not detected in minicells and probably is produced in low amounts. It might be similar to gene D of the $F7_2$ gene cluster, which is also expressed at a very low level and located at an equivalent position. The IA2 fimbria genes are suggested to be organized in more than one operon. Furthermore, the genes encoding p76, p45 and p27 are transcribed in the same direction. The gene for the IA2 fimbrial subunit is thought to be located at a relative position totally different from the genes for the Pap and $F7_2$ fimbrial subunits. The large open spaces in the genetic maps of the IA2 and $F7_2$ encoding gene clusters suggest the presence of unidentified structural genes involved in fimbria formation or adhesion.

D. PHASE VARIATION

Phase variation may be defined as the random on and off switching of the expression of a particular phenotype in a single bacterial cell. Phase variation might have evolved to enable bacteria to adapt to rapidly changing conditions that do not coincide with predictable or reproducible changes in the chemical or physical characteristics of their environment. In *Enterobacteriaceae*, several genetic loci involved in phase variation have been described: *gin*, involved in host–range variation in phage Mu (Plastkerk *et al.*, 1983); *pin*, involved in inversion in the *E. coli* chromosomal element e14 (Van de Putte *et al.*, 1984); *hin*, involved in flagellar variation in *Salmonella typhimurium* (Silverman *et al.*, 1979); *flu*, involved in variation of surface properties (including fimbriation) of *E. coli* (Diderichson, 1980); *sis1*, involved in suppression of polar effects due to insertion of IS1 (Rak, 1983). For Gin, Hin and Pin phenotypes the mechanism of phase variation has been resolved, and shown to be the result of DNA inversions. These inversion systems are closely related in both recombination sites and the amino-acid sequence of the recombinases, and have been shown to complement each other (Kutsukake and Ino, 1980; Kahmann and Kamp, 1981; Van de Putte *et al.*, 1984).

The production of fimbriae by *E. coli* can also be subject to phase variation. It should be noted that this phenomenon has only been described for chromosome-encoded fimbriae. For example, uropathogenic strains may produce several different fimbriae, and Rhen *et al.* (1983b) have shown that a rapid phase variation may take place between alternate fimbriae on a particular strain. Also, the production of 987P and type 1 fimbriae has been shown to be subject to phase variation (Nagy *et al.*, 1977; Brinton, 1959). Eisenstein (1981) has isolated a *lac* operon fusion within a locus on the *E. coli* chromosome presumed to be involved in type 1 fimbria phase variation. Two findings suggest that the locus (designated *fimD*) affected by the operon fusion is indeed involved in expression of type 1 fimbria genes. First, *fimD* is located at approximately the same position on the chromosome as *pilA*, *pilB* and *pilC* which have been implicated in type 1 fimbria production (Freitag and Eisenstein, 1983; Swaney *et al.*, 1977). Second, strains containing the *fimD–lac* fusion have lost their ability to produce type 1 fimbriae (Eisenstein, 1981). Expression of the *fimD–lac* fusion was found to oscillate between lac^+ and lac^- phenotypes, demonstrating that *fimD*-dependent phase variation is under transcriptional control. The transition rates of lac^+ to lac^- is $1.05 \cdot 10^{-3}$ per bacterium per generation, and for lac^- to lac^+ $3.12 \cdot 10^{-3}$ per bacterium per generation. As mentioned above, *flu* is also involved in the control of fimbriation. The *flu* and *fimD* loci are not identical, since they map at different positions (44 and 98 minutes, respectively) on the *E. coli* chromosome. However, *sis1*, another locus implicated in phase variation, has been mapped close to *fimD* (i.e. at approximately 95 minutes). At present, the relation

between *fimD*, *flu* and *sis1* is not clear. According to Van de Putte *et al.* (1984), the proteins involved in phase variation of type 1 fimbriae cannot catalyse G-inversion, and are therefore probably not closely related to the Gin, Pin and Hin phenotypic inversion systems.

Orndorff and Falkow (1984b) have identified a gene (termed *hyp*) involved in regulation of type 1 fimbria production. It is not clear how the hyp protein and phase variation are related. Strains containing the type 1 fimbria recombinant plasmids constructed by Orndorff and Falkow (1984b) do not show phase variation; however, this may be due to the high copy number of the vector containing the type 1 fimbria DNA.

VII. Biogenesis of Fimbriae

A. INTRODUCTION

Fimbriae are supramolecular structures composed of low molecular-weight subunits, and this section will deal with the problem of how these low molecular-weight subunits are transported across the inner and outer membrane, and assembled into a filamentous structure at the cell surface. Generally five to eight polypeptides have been implicated in the phenotypic expression of *E. coli* fimbriae (Kehoe *et al.*, 1981; Mooi *et al.*, 1981, 1982; Norgren *et al.*, 1984; Van Die *et al.*, 1984; De Graaf *et al.*, 1984; Orndorff and Falkow, 1984a). Therefore, fimbriae are simpler subjects to study biogenesis than, for example, F pili or flagellae, which require a much larger amount of gene products for their biogenesis (Manning and Achtman, 1979; Iino, 1977; Silverman and Simon, 1977). Most information available on biogenesis of fimbriae has been gained with the K88ab fimbria, and we will concentrate here on this fimbria. However, it seems likely that other fimbriae are produced in a similar way, because fimbrial genetic determinants have probably evolved by divergent evolution from common ancestral genes, and it seems likely that this is reflected in similar routes for their biogenesis.

B. FUNCTIONS OF K88AB POLYPEPTIDES

Insight into the functions of the K88ab polypeptides was obtained by the analyses of derivatives of pFM205 (Fig. 5) which contain small deletions in the various genes encoding K88ab (Mooi *et al.*, 1982, 1983). It appeared that at least five of the six K88ab polypeptides encoded by pFM205 are involved in the biogenesis of the K88ab fimbria (Table 5). It is not clear whether the sixth polypeptide (p27.5) also is involved in this process. A deletion in the gene for p27.5 results in a decrease in K88ab fimbriae production, but does not affect

TABLE 5. Properties of K88ab polypeptides and the effects of mutations in their genes

Polypeptide	Subcellular location	Effect of a mutation in the gene for this polypeptide on: Haemagglutination (HA)	Biogenesis K88ab fimbriae	Putative function polypeptide
p17.6	Extracellular?	No HA	1. No fimbriae produced 2. Intracellular accumulation of p26	Minor component of the K88ab fimbria
p81	Outer membrane	No HA	1. No fimbriae produced 2. Accumulation of assembly precursors (p26–p27–p17) in periplasmic space	Transport of p26 (and p17.6?) across the outer membrane and anchorage of fimbria
p27	Periplasmic space	No HA	1. No fimbriae produced 2. Degradation of p26 and p27.5	Transport p26 across outer membrane
p17	Periplasmic space	No HA	1. Transport of p26 across outer membrane, and assembly of fimbriae reduced 2. Accumulation of assembly precursors (p26–p27) in periplasmic space	Modification of p26
p26	Extracellular	No HA	No fimbriae produced	Major component of the K88ab fimbria
p27.5	Periplasmic space	Reduced HA	Decrease in p26 synthesis, and fimbriae production	?

their function since p27.5 mutants still bind to pig intestinal epithelial cells and agglutinate erythrocytes (Table 5). The low amount of K88ab fimbriae produced by the mutant lacking p27.5 cannot be enhanced by complementation in *trans*, suggesting that the effect of the mutation is not (only) due to the absence of p27.5, but (also) to the deletion of a *cis*-acting locus. One observation suggests that the K88ab fimbrial subunit and p27.5 are somehow connected; a deletion in the gene for p27 affects the stability of both polypeptides (Table 5). More mutants lacking p27.5 will have to be analysed to clarify the role of this polypeptide.

All six polypeptides encoded by the cloned K88ab DNA are synthesized as precursors containing a signal peptide indicating that they are located in the periplasmic space or outer membrane (Mooi *et al.*, 1981, 1982, 1984). This has been confirmed by determining the subcellular localization of these polypeptides (Table 5; Van Doorn *et al.*, 1982). It appeared that p27, p17 and p27.5 are located in the periplasmic space, whereas p81 is located in the outer membrane. Of course, p26, the major component of the K88ab fimbria, is normally located on the outside of the cell attached to the outer membrane. The subcellular location of p17.6 could not be determined, because it is produced in a too low amount to be detected. The fact that p17.6 resembles a fimbrial subunit suggests that it is located on the cell surface.

Since p26 is synthesized as a precursor containing a signal peptide and accumulates transiently in the periplasmic space (see below), it is probably transported across the inner membrane via the same route as *E. coli* periplasmic proteins (Silhavy *et al.*, 1983). In the periplasmic space, p26 associates with p27 before it is transported across the outer membrane. Normally, the p26–p27 complexes are too transient to allow analysis; however, mutants lacking p17 or p81 accumulate these assembly precurors in the periplasmic space (Table 5), from where they can be isolated and analysed. Electrophoretic analysis suggests that the assembly precursors are composed of p26–p27 dimers that have a low affinity for each other and continuously associate and dissociate to form oligomers of different size. This process might facilitate subsequent assembly of p26. In mutants lacking p27, p26 is synthesized but subsequently rapidly degraded (Table 5). Apparently the association of p26 with p27 affects the conformation of the fimbrial subunit, which becomes more resistant to proteolysis. The effect of p27 on the conformation of p26 suggests several possible functions for p27, which might not be mutually exclusive. First, as is shown clearly with phage tail proteins (King, 1980) and flagellin subunits (Iino, 1977), structural proteins are often synthesized in a form that does not spontaneously assemble so as to prevent the formation of a structure at the wrong site. The function of p27 could be to induce or stabilize such a form of p26, to prevent the fimbrial subunits from premature polymerization in the periplasmic space. Indeed, fimbrial subunits

present in the periplasmic space have a different, more thermolabile, conformation than fimbrial subunits assembled into fimbriae (F.R. Mooi, personal communication), confirming that they occur in different conformations in the course of fimbrial assembly. Second, association with p27 might be required to fold the p26 polypeptide chain into a particular conformation necessary for transport across the outer membrane. For example, p26 might be transported as an extended or relatively unstructured polypeptide chain through channels formed by p81 molecules in the outer membrane (see below, p. 123). Finally, association of p26 with p27 might be required to deliver the energy necessary for transport or assembly of p26. For example, if p27 catalyses the folding of the p26 polypeptide chain into a strained and energetically unfavourable conformation, some of the energy built into the protein during polymerization of amino acids might be retained. The conformational energy thus built into the p26–p27 complexes might be used to drive transport or assembly of p26.

Considering its instability in the absence of p27, it seems likely that p26 associates with p27 within a very short span of time after its synthesis is completed. Possibly, association takes place as the fimbrial subunit polypeptide chain emerges from the inner membrane. Polypeptide p27 is synthesized in large amounts, and a fast association between p26 and p27 is probably facilitated by a high concentration of p27 in the periplasmic space. Furthermore, p26 has a low isoelectric point (pI 4.2), whereas p27 has a high isoelectric point (pI $\geqslant$9.3; Mooi *et al.*, 1983). Therefore, these proteins are expected to have opposed net charges at physiological pH values, also facilitating their association in the periplasmic space.

Mutants lacking p17 are still able to transport p26 to the cell surface and to assemble them into fimbriae, albeit very inefficiently (Table 5). However, these mutants do not agglutinate erythrocytes nor do they bind to intestinal epithelial cells. This observation might be attributed to the small amounts of fimbriae produced by these cells. Alternatively, the fimbriae produced might not be functional. If the latter is true, this suggests that p17 (which is located in the periplasmic space) is involved in modification of p26 during its passage through the periplasmic space.

In mutants lacking p17, most p26 molecules are found in the periplasmic space associated with p27. Apparently, p17 is necessary for efficient transport of p26 across the outer membrane. Possibly only modified p26 molecules are transported efficiently across the outer membrane. Another possibility is suggested by the observation that, in mutants lacking p81, p17 is found associated with the p26–p27 complexes in low amounts (Table 5); this association might promote transport of p26 across the outer membrane by enhancing the affinity of these complexes for export sites. Both possibilities

prevent unmodified p26 molecules from being assembled into fimbriae in significant amounts.

The location of p81 in the outer membrane, and the accumulation of p26 in the periplasmic space of mutants lacking p81, suggests that p81 is involved in the transport of fimbrial subunits across the outer membrane. It is conceivable that p81 is a transmembrane protein that forms channels specific for fimbrial subunits. Association of complexes composed of p26, p27 and p17 with p81 in the periplasmic space might induce conformational changes in these proteins resulting in modification of p26, opening of the channel, transport of p26 across the outer membrane, and release of p17 and p27. Released p17 and p27 molecules are probably re-used for further cycles of assembly. At the cell surface p26 probably folds into a conformation with a high affinity for other fimbrial subunits, enabling it to assemble into the fimbrial structure. At the base of the fimbria, some p26 molecules presumably remain associated with p81, anchoring the fimbria to the cell.

It is difficult to obtain insight into the function of p17.6 because it is produced in too low amounts to be detected. Since the primary structure of p17.6 suggests that it is a fimbrial subunit, it is probably located at the cell surface. Furthermore, mutants lacking p17.6 do not produce K88ab fimbriae and accumulate p26 inside the cell (Mooi *et al.*, 1984). These observations suggest that p17.6 might be a minor component of the K88ab fimbria.

A model for the biogenesis of the K88ab fimbria is shown in Fig. 7.

C. FUNCTIONS OF K99 POLYPEPTIDES

With the exception of p10.2, all K99 polypeptides encoded by pFK99 (Fig. 5) have been implicated in the biogenesis of the K99 fimbria (De Graaf *et al.*, 1984; B. Roosendaal and F.K. De Graaf, unpublished work). It is not clear whether p10.2 is also involved, because mutants lacking this polypeptide have not yet been isolated. The polypeptides p10.2 and p10.9 are not detected in minicells suggesting that they are produced in very low amounts. Their existence was inferred from DNA sequence data. The predicted amino-acid sequence of p10.9 shows that is a highly charged polypeptide which does not contain an *N*-terminal signal peptide. Thus, p10.9 is probably located in the cytoplasmic space. It might be involved in regulation at the level of transcription or translation, since non-polar insertions in its gene result in a decrease in K99 fimbria production.

Several observations suggest that p76 and p21 have similar functions as the K88ab polypeptides p81 and p27, respectively (Table 5). First, the genes for p76 and p21 have the same relative position as the genes for p81 and p27, respectively (Fig. 5). Second, the two large and the two small polypeptides show homology (F.R. Mooi and B. Roosendaal, personal communication).

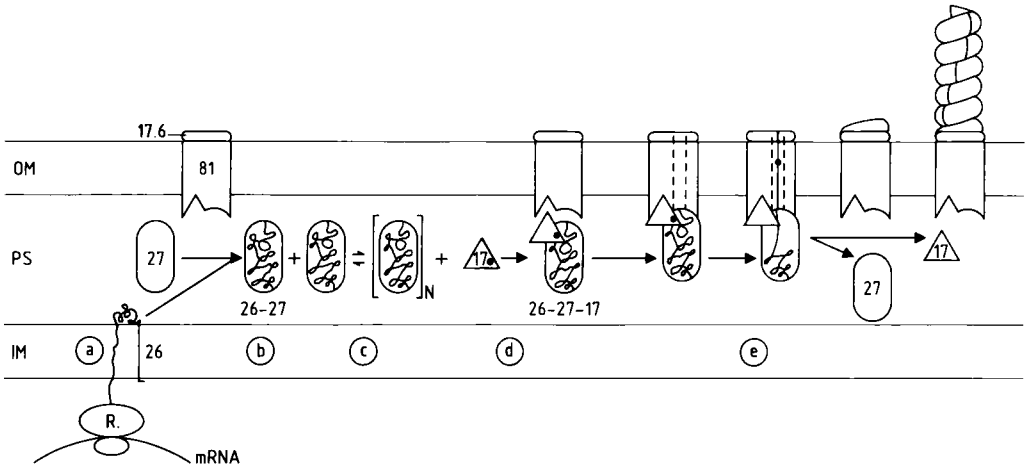


FIG. 7. Model for the biogenesis of the K88ab fimbria. (a) p26 (the K88ab fimbrial subunit) is transported across the inner membrane following the normal secretory pathway of periplasmic proteins. (b) In the periplasmic space p26 is associated with p27. This association induces a conformation of p26 that is required for transport across the outer membrane. (c) The p26–p27 dimers have a low affinity for each other and form oligomers of different size. This process might facilitate the subsequent assembly of p26. (d) p17 binds to the oligomers and enhances their affinity for p81 (for simplicity only p26–p27 dimers are depicted). (e) Binding of the p26–p27–p17 complexes to p81 induces conformational changes in these proteins which result in modification of p26 by p17, transport of p26 across the outer membrane through channels formed by p81, and dissociation of p27 and p17. The polypeptides p27 and p17 are re-used for further cycles of assembly. At the cell surface p26 obtains a conformation with high affinity for other p26 molecules, allowing it to assemble into the fimbrial structure. The fimbrial-like subunit (p17.6) has been assumed to be part of the basal structure of the fimbria. The numbers refer to the molecular weights ($\times 10^{-3}$) of the polypeptides. The thick black line indicates the signal peptide of p26. The thick black spot indicates the hypothetical group used to modify p26. IM, inner membrane; PS, periplasmic space; OM, outer membrane; R, ribosome.

Finally, a small non-polar deletion in the gene for p21 results in degradation of the K99 fimbrial subunit (De Graaf *et al.*, 1984). Thus this mutant resembles a K88ab mutant lacking p27 (Table 5).

Polypeptide p19 might have a similar function to the K88ab polypeptide p17 (Table 5). The two polypeptides have similar molecular weights, and are both produced in low amounts. Furthermore, p19 can be co-precipitated with the K99 fimbrial subunit from cell-free extracts using antibodies directed against the K99 fimbrial subunit (De Graaf *et al.*, 1984). Since p19 is not found associated with purified K99 fimbriae, this observation suggests that p19 associates with the K99 fimbrial subunit prior to its assembly. A similar observation has been made for p17 and the K88ab fimbrial subunit. Finally, a K99 mutant lacking p19 resembles a K88ab mutant lacking p17 in that it shows a lower number of fimbrial subunits on the cell surface, and does not adhere to epithelial cells (De Graaf *et al.*, 1984).

No clues regarding the functions of p26.5 and p33.5 have yet been obtained. Double mutants lacking p33.5 and p19 have the same phenotype as mutants lacking p19 only (see above). As with p19, p26.5 probably associates with the fimbrial subunit prior to its assembly, because it co-precipitates with the fimbrial subunits from cell-free extracts, but is not found in purified K99 fimbria preparations.

In mutants lacking one or more of the polypeptides p76, p21 or p26.5, fimbrial subunits are synthesized and subsequently degraded. Thus, like the K88ab fimbrial subunit, the K99 fimbrial subunit may require other polypeptides to fold into a stable (proteolysis resistant) conformation subsequent to its synthesis.

D. FUNCTION OF PAP POLYPEPTIDES

The genetic organization and function of genes involved in the biogenesis of the Pap fimbria and P adhesion has been studied by the group of Normark (Normark *et al.*, 1983; Lindberg *et al.*, 1984; Lund *et al.*, 1984; Norgren *et al.*, 1984). In addition to the gene coding for the major subunit of the Pap fimbria (*papA*), the gene cluster encoding Pap contains three other genes coding for fimbrial subunits, or closely related polypeptides (*papH*, *papE* and *papF*; Fig. 5). Biochemical and serological data indicate that the *papE* and *papF* gene products are minor components of the Pap fimbria (Lund *et al.*, 1984).

Molecular genetic analysis of the Pap gene cluster has revealed that it is possible to separate genetically fimbria formation and P-specific haemagglutination. Mutations in *papA* abolish fimbria formation but do not affect P-specific haemagglutination by whole cells. Conversely, mutations in *papF* or *papG* abolish P-specific haemagglutination, but do not affect fimbria formation. Furthermore, fimbriae isolated from *papF* or *papG* mutants have a

very low or no P-specific haemagglutinating ability. These results indicate that the *papF* and *papG* products are directly or indirectly involved in binding to the P receptor. It has been suggested that the *papF* product contains the P receptor-binding domain (S. Normark, personal communication). The *papG* product might be involved in transport or modification of the P adhesin.

A mutation in *papE* does not affect the P-specific haemagglutinating activity of whole cells. However, fimbriae isolated from these mutants show a reduced or complete absence of P-specific haemagglutinating activity. Together with the finding that the *papE* gene product is a minor component of the Pap fimbria, this suggests that the *papE* product is necessary to link the adhesin to the fimbria.

Although *papA* mutants are devoid of fimbriae, they still show P-specific haemagglutination. Apparently, the P adhesin is not only found associated with the Pap fimbria, but may also be located directly on the cell surface, where it could form a non-filamentous structure possibly similar to surface layers (see Sleytr and Messner, 1983 for a review of surface layers). Evidence exists that surface layers may be involved in colonization (Evenberg *et al.*, 1982). The *papA* or *papE* gene products are probably involved in the biogenesis of the non-filamentous form of the P adhesin, because *papA*, *papE* double mutants do not agglutinate erythrocytes. Since mutations in *papA* or *papE* alone do not affect haemagglutination by whole cells, it has been suggested that the *papA* and *papE* gene products have partially overlapping functions. Both may be able to function as carriers for the adhesin.

Two polypeptides (the *papC* and *papD* gene products) are required for both fimbria formation and P-specific haemagglutination. On the basis of their molecular weight and the relative positions of their genes, it seems likely that these polypeptides have similar functions to the K88ab polypeptides p81 and p27 (Fig. 5 and Table 5) and are involved in transport and anchorage of fimbrial and adhesin subunits. *papB* has a similar location as the K99 gene for p10.2, and might also be involved in regulating the expression of Pap genes (Norgren *et al.*, 1984).

E. FUNCTIONS OF $F7_2$ POLYPEPTIDES

Van Die *et al.* (1984) have studied the organization and function of genes coding for another P fimbria, designated $F7_2$. Five structural genes have been implicated in the phenotypic expression of the $F7_2$ fimbria and P-specific haemagglutination (gene A–E, Fig. 5). Transposon insertions in each of these five genes abolishes both fimbria formation and P-specific haemagglutination. Thus, in this respect the gene cluster encoding $F7_2$ clearly differs from that for Pap, since Norgren *et al.* (1984) have isolated transposon mutants that produce fimbriae, but do not show P-specific haemagglutination. The fact that

fimbria formation and P-specific haemagglutination cannot be separated genetically in the $F7_2$ gene cluster does not necessarily mean that both functions are encoded by the same gene. For example, in both the Pap and the $F7_2$ gene clusters, phenotypic expression of the P adhesin may require the presence of a carrier molecule. The difference between the gene clusters encoding Pap and $F7_2$ could be caused by the presence of only one gene coding for such a carrier polypeptide in the $F7_2$ cluster (gene A), while the Pap cluster contains two such genes (i.e. *papA* and *papE*). Of course, this does not explain why no $F7_2$ mutants have been isolated that produce fimbriae, but do not show P-specific haemagglutination.

VIII. Influence of Growth Conditions on Adhesin Production

Growth conditions affect the synthesis of fimbrial adhesins. For instance, a common feature of probably all mannose-insensitive fimbrial adhesins is the strong repression of their synthesis at 18–20°C (Orskov *et al.*, 1961; Jones and Rutter, 1974; Burrows *et al.*, 1976; Evans *et al.*, 1978; Guinée and Jansen, 1979; Wevers *et al.*, 1980; Awad-Masalmeh *et al.*, 1982; Björkstén and Wadström, 1982; De Graaf and Roorda, 1982; Morris *et al.*, 1982b; Smyth, 1982; Thomas *et al.*, 1982; Darfeuille *et al.*, 1983; Honda *et al.*, 1984; Williams *et al.*, 1984). Above 20°C the production of these fimbriae gradually increases, reaching its optimum at 37°C (Gaastra and De Graaf, 1982). In contrast to many mannose-insensitive fimbriae, type 1 fimbriae are expressed at low temperatures (Duguid and Gillies, 1957; Duguid and Old, 1980; Dodd and Eisenstein, 1984). It is not yet clear whether repression of fimbriae production at low temperatures is related to a slower growth rate or due to a more direct effect of the temperature on transcription–translation. For Pap fimbriae it has been shown that temperature affects transcription of the *pap* genes (Göransson and Uhlin, 1984).

Another important regulatory phenomenon affecting the expression of fimbriae, known as phase variation, has been discovered by Brinton (1959) in a study of type 1 fimbriae production. Brinton (1965) has defined phase variation as mutational in nature being an all-or-nothing, sudden, random, spontaneous and reversible event resulting in a change of fimbriated to non-fimbriated cells or vice versa. The rate of phase change is different in both directions, varies from strain to strain and is affected by environmental conditions (Duguid and Wilkinson, 1962; Brinton 1965). The non-fimbriated phase predominates under the usual conditions of laboratory growth. When grown on solid medium, strains able to produce type 1 fimbriae develop two different morphological types of colonies. Fimbriated cells form smaller, more opaque colonies with sharper edges than colonies of non-fimbriated cells.

These differences in colonial morphology are very useful in selecting for fimbriated cells. Growth in unshaken liquid minimal media under conditions of limiting oxygen and high cell concentration promotes the growth of fimbriated cells. When grown under unshaken aerobic conditions, fimbriated cells are able to form a pellicle at the culture surface probably as a result of the hydrophobic properties of type 1 fimbriae (auto-agglutination). Once a pellicle has been formed, a subsequent renewed post-logarithmic growth phase occurs and the fimbriated cells outgrow the non-fimbriated cells which cease growing because of a depletion of dissolved oxygen and fermentable substrates (Old *et al.*, 1968; Old and Duguid, 1970). The renewed growth is probably due to a free access to atmospheric oxygen and depends on early pellicle formation. Addition of D-mannose or methyl-α-D-mannoside prevents or delays pellicle formation suggesting that mannose-containing receptors are involved in pellicle formation. Binding of mannose may change the hydrophobicity of type 1 fimbriae. Unshaken anaerobic cultures, shaken aerobic cultures or growth on aerobic solid media do not promote the selective outgrowth of fimbriated cells. Brinton (1977) also observed a more rapid relative growth rate of fimbriated-phase cells versus non-fimbriated cells at low oxygen tension, but considered pellicle formation as a secondary effect of growth rate differences. As well as for type 1 fimbriae, phase variation has been observed for 987P and some P fimbriae (Nagy *et al.*, 1977; Rhen *et al.*, 1983b).

Most studies on the effects of environmental conditions on the production of mannose-insensitive fimbrial adhesins concern the synthesis of K99 fimbriae. Production of K99 fimbriae among wild-type enterotoxigenic strains shows a considerable variation. Strains of serogroup O101 generally produce more K99 fimbriae than strains belonging to serogroup O8, O9 or O20. K99 production appears to be independent of the presence of particular K polysaccharide antigens (De Graaf *et al.*, 1980c; Girardeau *et al.*, 1982b). The frequently encountered difficulties in the detection of K99-positive bacteria in the faeces of infected calves has stimulated the search for media for optimal development of K99 fimbriae. Guinée *et al.* (1976) discovered that the K99 adhesin is readily detectable when the cells are grown on a buffered semi-synthetic medium at pH 7.5 (Minca medium). Minca medium, as well as a minimal medium with glucose, appear to be the most suitable ones in which to obtain a high K99 production (De Graaf *et al.*, 1980c). In rich medium the production of K99 is strongly decreased.

During investigations into a possible inhibitory effect of certain nutrients on K99 production, it was observed that L-alanine caused a specific inhibition (De Graaf *et al.*, 1980a; Girardeau *et al.*, 1982a). A concentration of 1 mM L-alanine is sufficient for an almost complete repression of K99 synthesis in minimal media without casamino acids, 8 mM is required to suppress K99

production in Minca medium (containing 0.1% casamino acids), and cells grown in minimal media containing a higher concentration of casamino acids are less sensitive to the L-alanine-mediated repression. Recently, Isaacson (1983) found that the latter effect was caused by the presence of relatively higher concentrations of L-threonine and L-isoleucine, which partially reverse the inhibitory effect of L-alanine.

Variable results have been published regarding the possible effect of glucose and glucose-induced catabolite repression on the synthesis of K99 fimbriae. When grown in Trypticase soy broth, low concentrations of glucose stimulate K99 production with an optimum at 0.15% glucose, but higher concentrations suppress K99 production (Isaacson, 1980). This suppression is not due to the accumulation of acid fermentation products. Glucose-mediated repression can be overcome by addition of extracellular cyclic AMP (adenosine 3′,5′-monophosphate). Although these experiments might indicate that the synthesis of K99 fimbriae is subject to catabolite repression and requires an appropriate concentration of intracellular cyclic AMP, it should be mentioned that rich media (usually containing sugars) are not suitable for studying the effects of glucose and cyclic AMP. In a more recent study, Isaacson (1983) repeated the experiments on the possible effect of glucose in minimal media, and observed that addition of a variable concentration of glucose to this medium does not affect the production of K99 fimbriae. Both the synthesis of K99 subunits and the assembly of subunits into fimbriae appears to be independent of variation in the glucose concentration. However, when studied in an adenylate cyclase (*cya*) mutant, incapable of synthesizing cyclic AMP, it appeared that subunit synthesis was repressed although normal amounts of fimbriae were produced. Isaacson presumed that subunit synthesis, but not assembly, is subject to catabolite repression. The ability of the *cya* mutant to produce normal amounts of K99 fimbriae was explained by assuming that this mutant, despite the repression of subunit synthesis, is still able to produce enough subunits for efficient assembly into fimbriae. According to Isaacson, the wild-type strain preserves a large pool of (membrane-associated) subunits not assembled into fimbriae. The existence of such a subunit pool appeared likely since addition of chloramphenicol to a K99-producing culture arrested subunit synthesis but the production of K99 fimbriae still continued. It should be noted that DNA sequence analysis of the K99 gene cluser did not show the presence of a binding site for the cyclic AMP binding protein (B. Roosendaal and F.K. De Graaf, unpublished results). Also, Contrepois *et al.* (1983) found no catabolite repression of K99 biosynthesis. On the contrary, they observed that high concentrations of intracellular cyclic AMP correlated with a low production of K99. Therefore, the absence of a K99 subunit pool in the *cya* mutant might be due to some secondary effect of the *cya* (or other mutations) in this particular strain.

In another study of the effect of glucose on K99 production Girardeau *et al.* (1982b) described the existence of two groups of K99-positive strains. In one group, K99 production appeared to be constitutive and unaffected by the presence or absence of glucose. Strains belonging to the second group only produced K99 in the presence of glucose and were designated glucose-dependent. Unfortunately, the relatively insensitive slide agglutination test was used to distinguish glucose-constitutive from glucose-dependent strains. Using the more sensitive ELISA, F.K. De Graaf and coworkers (unpublished results) were able to demonstrate that the glucose dependent strains represent a group of wild-type strains that produce small, but nevertheless detectable, amounts of K99 fimbriae when grown without glucose. Addition of glucose enhanced K99 production to a level detectable by slide agglutination. The glucose-constitutive strains produced comparable amounts of K99 fimbriae in the absence of glucose as glucose-dependent strains did in the presence of glucose. Both types of strains showed a pronounced increase in K99 production in media with 0.05–0.2% (w/v) glucose. Many K99-positive strains also produced F41 fimbriae. The synthesis of this adhesin was also inhibited by L-alanine, but no effect of glucose has been observed (De Graaf and Roorda, 1982; Ollier and Girardeau, 1983).

It seems reasonable to assume that the variable effects of glucose-containing media on K99 production by different strains might, at least in part, be explained by an effect of growth rate on the synthesis of K99 fimbriae. Isaacson (1980) reported that K99 fimbriae were only produced during the logarithmic phase of growth in aerated cultures. During the stationary phase the amount of fimbriae declined. Non-aerated cultures produced little or no K99 fimbriae. This relation between growth phase and K99 production appears to be restricted to the assembly of K99 subunits into fimbriae because, contrary to the production of fimbriae, the K99 subunits are synthesized throughout the entire life cycle (Isaacson, 1983).

In a recent study on K99 and F41 production with cells growing in the chemostat and in the recycling fermenter, van Verseveld *et al.* (1985) were able to demonstrate that the expression of both adhesins is highly dependent on the specific growth rate. Production of both adhesins started at a specific growth rate (μ) of about 0.2 h^{-1}, and subsequently gradually increased, reaching its maximum at the maximal growth rate. Production during anaerobic growth was comparable or somewhat higher than during aerobic growth, provided that the pH value of the cultures was regulated at pH 7. Expression of both fimbriae was strongly dependent on the pH value of the cultures. Below pH 7, production rapidly declined. At higher pH values the decrease in production was less drastic. This observation may explain a previous observation that addition of sodium acetate suppressed K99 production (Francis *et al.*, 1983), as well as the very low K99 production observed in batch cultures without pH

regulation (De Graaf *et al.*, 1980c). The use of the recycling fermenter has established the occurrence of three growth domains in *E. coli* (van Verseveld *et al.*, 1984; Chesbro *et al.*, 1979). Domain 1 occurs at specific growth-rate values higher than 0.05 h^{-1}, domains 2 and 3 occur at rates between 0.05 and 0.01 h^{-1} and lower than 0.01 h^{-1}, respectively. Production of both K99 and F41 fimbriae was shown to be a typical domain 1 phenomenon. This domain gives the highest biomass yield and is characterized by a low intracellular concentration of the regulatory nucleotide guanosine 5′-diphosphate 3′-diphosphate (ppGpp). This regulatory activity of ppGpp is known to influence the synthesis of many proteins (Gallant, 1979; Riedel, 1983). O'Farrell (1978) analysed the amount of each of about 300 different cellular proteins and observed that the synthesis of 25% of these proteins is stimulated by the stringent control mechanism, whereas the synthesis of another 25% of the cellular proteins is inhibited by this response. Apparently, the synthesis of K99 fimbriae is subjected to stringent control.

Since neither van Verseveld *et al.* (1985) nor Contrepois *et al.* (1983) have distinguished between subunit synthesis and assembly of fimbriae it is possible that the correlation between expression of K99 fimbriae and relatively high growth rates is restricted to subunit assembly. Synthesis of K99 subunits may be unaffected by growth rates resulting in the accumulation of a pool of subunits at slow growth rates.

The significance of these findings for the situation *in vivo* may be explained as follows. Ingested bacteria are likely to be in the stationary phase (stringent control) and possess relatively few K99 fimbriae because ppGpp suppresses their assembly. However, these cells may have preserved a pool of fimbrial subunits. Once entering the intestine of the newborn animal, the ingested bacteria meet favourable growth conditions which permit the cells to proliferate at high growth rates characteristic for domain 1. The existing subunit pool is immediately assembled into fimbriae and further synthesis of fimbriae is initiated which in turn will promote attachment to the intestinal epithelium and stimulate further colonization. In this manner, the possession of a K99 plasmid enables enterotoxigenic strains to colonize a habitat where *E. coli* normally does not survive. This hypothesis may, in part, explain why only newborn calves are susceptible to infection with K99-positive enterotoxigenic strains, since the favourable growth conditions necessary to permit a high growth rate are caused by the relatively high gastric pH values, sluggish motility, high oxidation–reduction potential, lack of microbial antagonism and unrestricted feeding of newborn calves (Moon, 1974).

Also, the expression of type 1 fimbriae has been studied in relation to possible effects of glucose or cyclic AMP. Glucose, and other fermentable sugars, suppress the synthesis of type 1 fimbriae in *Salmonella typhimurium* (Saier *et al.*, 1978) as well as in *E. coli* (Eisenstein and Dodd, 1982). The effect

was not associated with the low pH value developed in unshaken broth cultures with glucose since, also in buffered media at pH 6.8 to 7.0, addition of increasing amounts of glucose induced a parallel decrease in fimbriation. Further analysis showed that addition of glucose induced a decrease in the number of fimbriated cells but does not affect the degree of fimbriation per cell, indicating that transcription of the fimbrial operon is most likely insensitive to catabolite repression (Eisenstein and Dodd, 1982). The independency of type 1 fimbriae production on cyclic AMP was further demonstrated with an adenylate cyclase (*cya*) mutant which is still able to produce type 1 fimbriae. Also in this mutant, glucose represses the number of fimbriated cells even in the presence of extracellular cyclic AMP. In shaken cultures where fimbriated cells have no advantage of selective outgrowth, glucose does not affect the number of fimbriated cells. Furthermore, addition of glucose to static broth cultures increased the final amount of growth by non-fimbriated cells to a greater extent than for fimbriated cells, showing that this disadvantage of non-fimbriated cells relative to fimbriated cells does not occur in the presence of glucose (Eisenstein and Dodd, 1982). Apparently glucose has no effect on the synthesis of type 1 fimbriae but prevents selective outgrowth of fimbriated cells in unshaken cultures.

We like to hypothesize that, in shaken aerobic cultures, fimbriated and non-fimbriated cells grow at comparable rates resulting in an overall relatively low production of type 1 fimbriae in the stationary phase. Also, in static cultures without glucose, both fimbriated and non-fimbriated cells start growing at a comparable rate, but non-fimbriated cells cease growing after a relatively short period of time due to depletion of nutrients that provide energy for anaerobic growth. However, cells that have produced type 1 fimbriae in the pre-logarithmic growth phase are able to form a pellicle which gives them access to free oxygen and permits subsequent outgrowth for a relatively long period of time. When, however, glucose is added to unshaken cultures, both fimbriated and non-fimbriated cells have the possibility for exponential growth during a much longer period of time, thereby consuming the available nutrients at comparable rates. Consequently, fimbriated cells have a very limited period of selective outgrowth under these conditions.

In a recent study of the kinetics of type 1 fimbriae production, Dodd and Eisenstein (1984) found that the assembly of type 1 subunits into fimbriae takes almost three minutes in log-phase cultures at 37°C. In the absence of protein synthesis, the free subunits continue to assemble until the pool is depleted. The subunit pool, however, is small in comparison with the total fimbrial protein already assembled into fimbriae and not sufficient to regenerate new fimbriae after removal of pre-existent ones by blending. Synthesis and assembly of subunits is slowed down at lower temperatures, or

as the cultures enter the stationary phase. Under the latter conditions, the rate of subunit synthesis slows sooner than that of total cellular proteins.

It should be mentioned again that also pathogenic *E. coli* strains generally have the capacity to produce type 1 fimbriae. The significance of this capacity in the establishment of disease is less clear. Although type 1 fimbriae confer adhesive properties on the bacteria, it remains to be investigated whether these fimbriae are actually produced at the time and site of infection. Ofek *et al.* (1982) reported that only a very small number of uropathogenic strains able to produce type 1 fimbriae actually carry this adhesin when isolated from the urine of patients. They suggest that growth in the bladder may suppress the production of type 1 fimbriae.

Sublethal concentrations of aminoglycoside antibiotics differentially interfere with the production of fimbriae. *Escherichia coli* grown in sublethal concentrations of streptomycin lose their ability to aggregate mannan-containing yeast cells and to adhere to human buccal epithelial cells (Eisenstein *et al.*, 1979). The effect of streptomycin is dose related between 0.5 and 10 μg streptomycin ml^{-1}. Once the fimbriae are produced, the mannose-binding activity is not modified by subsequent exposure to high concentrations of streptomycin. The results indicate that streptomycin affects the synthesis of type 1 fimbriae already at concentrations with little or no effect on biomass synthesis. Furthermore, the effect of streptomycin is most pronounced when added during the early logarithmic growth phase and has no effect after the exponential phase of growth. The streptomycin effect does not occur in streptomycin-resistant mutants. The authors suggest that the streptomycin effect is probably a result of the production of aberrant fimbrial proteins caused by misreading.

Other antibiotics that interfere with protein synthesis, in particular chloramphenicol, have very little affect on the expression of mannose-binding activity, measured by yeast cell aggregation, and degree of fimbriation, even at 50% of their minimum inhibitory concentration. Nevertheless, chloramphenicol also decreases adherence to human epithelial cells (Eisenstein *et al.*, 1980). Moreover, Brinton (1965) observed that the regeneration of type 1 fimbriae on defimbriated cells is inhibited by streptomycin but not by chloramphenicol.

Comparable effects of subinhibitory concentrations of antibiotics on the adhesiveness of pathogenic *E. coli* strains have been described (Vosbeek *et al.*, 1982; Shibl and Gemmell, 1983).

IX. Concluding Remarks

Pathogenic *E. coli* strains associated with intestinal or urinary tract infections are able to produce one or more different types of fimbrial adhesins that

enable the bacteria to colonize the host epithelia. The majority of these fimbriae are composed of a single repeating protein subunit but some fimbriae may contain other (minor) polypeptides as well.

Fimbriae encoded by the bacterial chromosome have a similar morphology (a rod-like rigid structure with a diameter of about 7 nm) and their production is generally subject to phase variation. The majority of the plasmid-encoded fimbriae are very thin and flexible structures whose production has not been shown to be affected by phase variation.

All fimbriae appear to recognize and bind to relatively simple glycolipids or to glycoproteins present on the host epithelial cell surface or on certain types of erythrocytes. The fimbriae may be classified according to their affinity for a particular oligosaccharide structure present on these glycoconjugates.

The production of fimbriae is affected by environmental conditions. In particular, growth rate appears to affect the amount of fimbriae produced.

The analysis of a number of fimbrial gene clusters has revealed that they all carry two consecutive genes, the first coding for a 70–86 kD polypeptide, and the second for a 21–28.5 kD polypeptide. DNA sequence data and mutational analysis strongly suggest that these two genes have similar functions in different gene clusters, and are involved in the transport of subunits across the outer membrane, and assembly and anchorage of fimbriae. Other similarities are also observed between the fimbrial gene clusters analysed to date. The two genes mentioned above are generally preceded by a DNA region showing dyad symmetry and, subsequently, a fimbrial subunit gene. Furthermore, fimbrial subunits derived from different *E. coli* strains appear to share homology at their *C*-termini and, to a lesser extent, at their *N*-termini. The homology observed at the *N*- and *C*-termini of fimbrial subunits suggests that these parts of the polypeptide chain are involved in functions shared by different fimbrial subunits. There is some evidence that the *C*-termini are involved in subunit–subunit interactions. All these findings suggest that fimbrial gene clusters of *E. coli* are evolutionarily closely related.

Studies of several fimbrial gene clusters have indicated that the receptor binding domain may be located within a polypeptide distinct from the subunit constituting the major component of the fimbria. Furthermore, there is evidence that these adhesin subunits resemble fimbrial subunits in primary structure, and are therefore probably derived from them. Another interesting observation is that two or more fimbrial subunit genes may be found clustered around a single set of genes coding for export and anchorage proteins. These findings might suggest that these gene clusters have acquired several fimbrial genes in the course of evolution which have become specialized in particular functions (i.e. carrier or adhesion functions). The separation of carrier and adhesion functions in different subunits might confer a selective advantage, because it allows a greater flexibility in evading the host immune response.

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The Molecular Biology of Amino-Acid Transport in Bacteria

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I. Introduction

Transport systems control the flow of molecules between a cell and its environment thus mediating intercellular communication. Early studies on biological transport processes were either biochemical or genetic in nature; however, more recently, researchers have been utilizing recombinant DNA techniques to determine the molecular mechanisms of biological transport systems. This review will focus on transport systems in prokaryotes in which recent significant advances in our understanding of transport have been made. The first section will discuss some general aspects of biological transport as well as the physiological role of amino-acid transport in prokaryotes. Additional sections will review in detail the work done on five different amino-acid transport systems operating in prokaryotes.

A. GENERAL ASPECTS OF BIOLOGICAL TRANSPORT

Transport systems in prokaryotes serve to move many different types of solute into or out of the cell in order to maintain the optimum physiological cellular concentration for each nutrient. The types of solute transported include ions, sugars and amino acids. Transport processes fall into three major classifications: passive diffusion, facilitated diffusion and active transport. The distinction between passive or facilitated diffusion and active transport is made on the basis of the energy requirements of the system. Passive and facilitated diffusion processes do not require energy input, whereas active transport requires an energy source. In bacteria, a common source of energy for active transport processes is the flow of protons across the membrane (often referred to as the proton-motive force). Under physiological conditions, the proton gradient is generated by the flow of electrons from the respiratory chain. Another source of energy for active transport is derived from the coupling of ATP hydrolysis. Sodium ion gradients across the membrane are a third means for providing energy for active transport processes, although this is a somewhat uncommon mechanism in prokaryotes. In this review, only active systems that transport amino acids will be considered.

Some active amino-acid transport systems consist solely of one or more membrane components, whereas other transport systems have membrane components as well as at least one binding protein which resides in the

periplasmic space of the bacterial cell (Oxender, 1972, 1974, 1975; Slayman, 1973; Boos, 1974; Halpern, 1974; Simoni and Postma, 1975; Wilson, 1978; Iaccarino *et al.*, 1980; Anraku, 1980; Furlong and Schellenberg, 1980; Landick *et al.*, 1985). Two examples of membrane-bound transport systems discussed in this review are those for proline (Sections V and VII.C) and tyrosine (Section VI). Other systems examined in this review have both membrane-bound and periplasmic components. Table 1 lists the transport systems to be discussed along with some of their more general features.

B. PHYSIOLOGICAL ASPECTS OF AMINO-ACID TRANSPORT

The physiology of amino-acid transport in prokaryotes can be quite complex. For example, transport of branched-chain amino acids in *Escherichia coli* is carried out by multiple systems. One of the systems, LIV-II, has a low affinity for substrate which sets the basal level of branched-chain amino-acid transport for the cell. This system is not highly regulated. Another system, LIV-I, has a high affinity for substrate and is regulated by the intracellular concentration of leucine. This system is activated in times of stress (i.e. during leucine starvation) and functions primarily as a scavenger of the branched-chain amino acids.

When bacterial cells are grown in nutrient media, LIV-I transport activity is repressed. Moreover, biosynthesis of the branched-chain amino acids is also shut down. In these cells, the low-affinity LIV-II transport system provides sufficient amounts of these amino acids. When the cells are shifted to media with lower concentrations of leucine, the high-affinity LIV-I transport system becomes functional and serves to scavenge amino acids that have diffused into the periplasmic space from the extracellular environment. As the leucine concentration in the media decreases further, the biosynthetic operon for the branched-chain amino acids is now derepressed while the LIV-I transport activity remains high. At this stage, though, the LIV-I system is primarily recycling branched-chain amino acids which have leaked into the periplasm from the cell's cytoplasm.

This physiological concept is consistent with observations of genetic mutants. For example, when cells that are constitutively derepressed for LIV-I transport are grown in nutrient media, the branched-chain amino-acid biosynthesis is non-functional as expected, yet the LIV-I transport system is fully activated because it is no longer regulated. When these cells are subsequently shifted to minimal media, they experience a lag time for growth. The LIV-I system remains active but the isoleucine–leucine–valine (*ilv*) biosynthetic operon cannot initiate expression. If isoleucine or valine is added to the medium, the *ilv* biosynthetic operon begins to function (Quay *et al.*,

TABLE 1. Characteristics of amino-acid transport systems in prokaryotes

System	Substrates	Organism	Comments	Genes/locations
Glutamine-specific	Glutamine	*Escherichia coli*	One periplasmic-binding protein	*glnP*, min 17.7
PP-I	Proline	*Salmonella typhimurium*	High-affinity, membrane-bound	*putP*, membrane component/min22 *putA*, repressor/min22
	Proline	*Escherichia coli*	High-affinity, membrane-bound proton-motive energy	*putP*, membrane component/min22 *putA*, repressor/min22
PP-II	Proline	*Salmonella typhimurium*	Low-affinity, membrane-bound	*proP*, membrane component/min92
	Proline	*Escherichia coli*	Low-affinity, membrane-bound proton-motive energy	*proT*, membrane component/min83
PP-III	Proline	*Salmonella typhimurium*	Only functions in high osmotic strength extracellular media	*proU*, min59
Histidine-specific	Histidine	*Salmonella typhimurium*	High-affinity, periplasmic and membrane-bound components, ATP energy	*hisJ*, periplasmic b.p./min48.5 *hisP*, membrane component/min48.5 *hisM*, membrane component/min48.5 *hisQ*, membrane component/min48.5
LAO	Lysine, arginine, ornithine	*Salmonella typhimurium*	One periplasmic protein binds three substrates and utilizes same membrane components as is-specific system, ATP energy	*argT*, periplasmic b.p./min48.5 *hisP*, membrane component/min48.5 *hisM*, membrane component/min48.5 *hisQ*, membrane component/min48.5
	Lysine, arginine ornithine	*Escherichia coli*	One periplasmic-binding protein	*argP*, periplasmic b.p./min63
Arginine, ornithine	Arginine, ornithine	*Escherichia coli*	Low-affinity, one periplasmic-binding protein	*abpR*, repressor/min61–63 *abpS*, periplasmic b.p./min61–63

TABLE 1 (continued)

System	Substrates	Organism	Comments	Genes/locations
Aromatic amino acid	Tyrosine, phenylalanine, tryptophan	*Escherichia coli*	—	*aroP*, min2 *tyrR*, repressor/min29
Tyrosine-specific	Tyrosine	*Escherichia coli*	One membrane component, proton-motive energy	*tyrP*, membrane component/min42 *tyrR*, repressor/min29
Phenylalanine-specific	Phenylalanine	*Escherichia coli*	—	*pheP*, min13
LIV-I	Leucine, valine, isoleucine, threonine, alanine	*Pseudomonas aeruginosa*	Low-affinity, periplasmic binding protein (LIVAT-BP)	*braC*, periplasmic b.p./min60+ *braB*, membrane component/min60+ *braA*, repressor/min60+
	Leucine, isoleucine, valine, alanine, threonine	*Escherichia coli*	Low-affinity, periplasmic-binding protein and three membrane components, proton-motive energy	*livJ*, LIV-BP/min76 *livH*, membrane component/min76 *livM*, membrane component/min76 *livG*, membrane component/min76 *livR*, repressor/min20
LS	L-,D-Leucine	*Escherichia coli*	Low-affinity, periplasmic-binding protein and same membrane components as LIV-I, proton-motive energy	*livK*, LS-BP/min76 *livH*, membrane component/min76 *livM*, membrane component/min76 *livG*/membrane component/min 76 *lstR*, repressor/min20
LIV-II	Leucine, isoleucine, valine	*Escherichia coli*	High-affinity, membrane component(s)	*livP9*, min74–78 *liv-12*, min74–78
	Leucine, isoleucine, valine	*Pseudomonas aeruginosa*	High-affinity, membrane component(s)	—

1977). It has been suggested that this effect could be due to feedback inhibition by leucine on the first enzyme of the biosynthetic *ilv* pathway.

Moreover, a double transport mutant (LIV-I-/LIV-II-), has no transport activity so that the biosynthetic pathway is active and leucine is able to leak out of the cells. Because other mutants that are leucine auxotrophs can grow in the vicinity of these cells by feeding on the leucine that leaks out, the LIV-I-/LIV-II- mutants are considered leucine secretors.

Leucine seems to have a special physiological role in the bacterial cell. One of the amino acids to which bacteria exhibit sensitivity is leucine (Quay *et al.*, 1977) and leucine is the only amino acid that regulates the rates of branched-chain amino-acid transport. Moreover, it has been reported that the intracellular concentration of leucine also regulates expression of other bacterial operons.

II. Branched-Chain Amino-Acid Transport Systems in *Escherichia coli*

A. INTRODUCTION

There are three major systems operating in *E. coli* for the transport of the branched-chain amino acids. The LIV-I system transports L-leucine, L-isoleucine and L-valine with K_d values of approximately 1 to 0·1 pM (Piperno and Oxender, 1968; Rahmanian *et al.*, 1973; Wood, 1975; Anderson and Oxender, 1978). In addition, this system is responsible for transport of threonine and alanine, but with somewhat lower affinities (Rahmanian *et al.*, 1973). The LIV-II system also transports the three branched-chain amino acids leucine, isoleucine and valine; this system demonstrates a much lower affinity for the substrates than does the LIV-I system (Rahmanian *et al.*, 1973; Anderson and Oxender, 1978). The third transport system to be considered is the LS or leucine-specific transport system. The LS system transports L-leucine with a K_d value of approximately 1 pM; D-leucine is also an acceptable substrate but with a lower affinity (Piperno and Oxender, 1968; Rahmanian *et al.*, 1973; Wood, 1975; Anderson and Oxender, 1978). These three transport systems have been mapped with genetic mutants and it was found that the LIV-I and LS systems were genetically inseparable and were located at the minute 74–76 region on the *E. coli* chromosome (Anderson and Oxender, 1978). The LIV-II system mapped close to the other two transport systems in the minute 74–78 region of the *E. coli* chromosome but the LIV-II system was genetically distinct from the LIV-I and LS systems (Anderson and Oxender, 1978).

The LIV-II system has been studied and several of its features have been characterized. The LIV-II system remained active in cells that had undergone

osmotic shock; thus no periplasmic components are required for this transport system. Two independent mutations, *liv-12* and *livP9*, were isolated which abolished the LIV-II transport activity (Anderson and Oxender, 1978). Both mutations mapped to the same region on the *E. coli* chromosome and they were very closely linked. In strains carrying each of the mutations, both the LIV-I and the LS transport systems were functional indicating that these defects occurred in a component specific to the LIV-II system. Regulatory mutants that affect the LIV-I and LS transport systems (Anderson *et al.*, 1976) do not have an effect on the LIV-II system. In summary, the data indicate that the LIV-II transport system is a distinct transport system which sets the basal level of branched-chain amino-acid transport activity in the cell.

Before the advent of molecular biology, the LIV-I and LS transport systems had been studied quite extensively using kinetic, biochemical and genetic techniques. It was found that, on osmotic shock, both the LIV-I and the LS transport activities were lost indicating that each contained a periplasmic component (Penrose *et al.*, 1968; Furlong and Weiner, 1970). The periplasmic protein for the LIV-I system was shown to bind leucine, isoleucine and valine as well as alanine and threonine with less affinity (Penrose *et al.*, 1968), whereas the periplasmic protein for the LS system specifically bound isomers of leucine (Furlong and Weiner, 1970). Genetic experiments had been done in which the Mu phage had been randomly inserted into the *E. coli* chromosome, and LIV-I and/or LS transport-deficient mutants had been selected. One of these mutants, AE4107, retained the periplasmic-binding components of the two transport systems. Hence, it was concluded that the LIV-I and LS systems shared components in common and that these components were probably located in the inner membrane of the bacterial cell (Anderson and Oxender, 1977; Oxender and Quay, 1976b). The same genetic experiments also resulted in the isolation of mutants that had lost one or other high-affinity transport activity as well as one or other periplasmic-binding protein (Anderson and Oxender, 1977). Thus, correlations were observed between the *livJ*- mutation and the periplasmic-binding protein that bound all of the branched-chain amino acids (LIV-binding protein) and between the *livK*- mutation and the periplasmic-binding protein which was specific for leucine (LS-binding protein).

B. GENE ORGANIZATION

The region of the *E. coli* chromosome encoding the branched-chain amino-acid transport systems was cloned first into a phage lambda vector and subsequently into the multicopy plasmic pACYC184 (Oxender *et al.*, 1980a). The gene order and organization for the LIV-I and LS transport systems were determined (Oxender *et al.*, 1980a; Landick and Oxender, 1982; Nazos *et al.*,

1984) and is shown in Fig. 1. The *livJ* gene encodes the periplasmic LIV-binding protein and the *livK* gene encodes the periplasmic LS-binding protein. The genes *livH*, *livM* and *livG* produce the membrane-associated components which are shared by both the LIV-I and the LS transport systems. The *livH* and the *livG* genes had been identified previously via genetic mutants (Anderson and Oxender, 1977; Oxender *et al.*, 1980a), but the *livM* gene was not predicted from earlier studies. The presence of the *livM* gene was determined by a combination of subcloning and complementation studies utilizing the cloned transport operons and the previously isolated genetic mutants for various transport components (Nazos, 1984; Nazos *et al.*, 1984). The protein products for *livH*, *livM* and *livG* genes have been identified by using a combination of a minicell expression system (Clark-Curtiss and Curtiss, 1983) and sodium dodecyl sulphate (SDS)–polyacrylamide-gel electrophoresis (Nazos, 1984; Naxos *et al.*, 1984, 1985a,b) and their molecular weights have been estimated to be approximately 30,000, 28,000 and 22,000, respectively. The *livL* gene was first identified as an open reading frame after DNA sequencing (Landick, 1983). In subsequent experiments to determine whether this open reading frame was expressed, gene fusions were constructed and a fused product was identified (Landick, 1983). The function of this gene is unknown and its relation to the branched-chain amino-acid transport systems is presumed because of its position. Sequencing of DNA has been carried out for most of the cloned transport operons, including the entire *livJ*, *livL*, *livK* and *livH* genes (Landick, 1983; Landick and Oxender, 1985; Nazos *et al.*, 1985a).

C. SECRETION OF THE LEUCINE-BINDING PROTEINS

The subject of the secretion of the periplasmic binding proteins has been studied at some length by Oxender and his associates (Daniels *et al.*, 1980, 1981; Oxender *et al.*, 1980b, 1984; Landick and Oxender, 1982; Copeland *et*

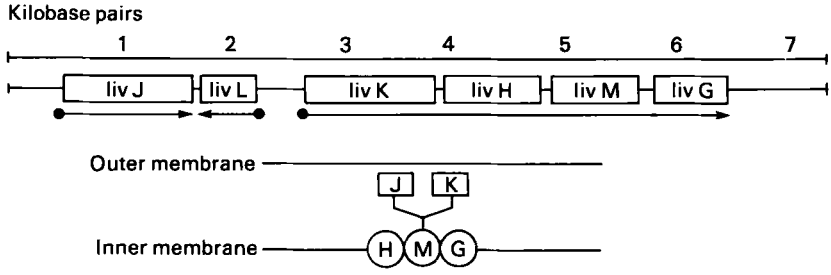

FIG. 1. Organization of the high-affinity branched-chain amino-acid transport genes in *Escherichia coli*. Arrows represent the direction of transcription for the operons.

al., 1984; Landick *et al.*, 1984; Su *et al.*, 1985). Subclones were constructed which contained either the *livJ* or *livK* genes (Daniels *et al.*, 1980; Oxender *et al.*, 1980b). These plasmids were then subjected to a transcription/translation expression system *in vitro* and the results showed that each binding protein was synthesized and detected in this sytem. Moreover, the proteins had molecular weights that were approximately 2,500 greater than their previously identified mature forms (Oxender *et al.*, 1980b). After incubating the proteins in the presence of membrane fractions, posttranslational processing was observed which yielded the mature forms of the two binding proteins (as determined by their molecular weights) (Oxender *et al.*, 1980b; Daniels *et al.*, 1980). The processing activity in the membrane fractions could be removed by centrifugation which indicated that the processing activity was membrane-associated; this association has been suggested by several groups (Inouye and Beckwith, 1977; Chang *et al.*, 1978, 1980; Randall *et al.*, 1978; Mandell and Wickner, 1979). A combination of DNA sequence analysis and of amino-acid sequence determination has shown that the signal sequence of each binding protein is 23 amino acids in length, although the sequences of the two signal regions are only 60% homologous (Oxender *et al.*, 1980b; Landick, 1983). This low degree of homology is in contrast to the 80% homology observed throughout the mature sequences of the two binding proteins (Landick, 1983). Conformational differences have been demonstrated to exist between the precursor and mature forms of the two binding proteins with proteolytic cleavage experiments (Oxender *et al.*, 1980b). These data have been interpreted to indicate that the signal sequence may possibly play a role in determining the conformation of the binding protein precursor that is recognized by the membrane.

There is a difference in the rate of processing for the two binding proteins, with that for LIV-binding protein being faster than that for LS-binding protein (Daniels *et al.*, 1980). Moreover, deletion mutants of the gene for LS-binding protein have been constructed with altered or missing amino-acid sequences in the *C*-terminal portion of the protein (Landick *et al.*, 1984). The deletion products were processed and secreted normally but they were rapidly degraded in the periplasm. Two major conclusions from this study were that the *C*-terminal sequence of the LS-binding protein is not required for secretion of the protein into the periplasm and that it is probably the altered conformation of the deleted product that renders it susceptible to proteases after secretion.

Furthermore, it has been observed that, when a proton ionophore (such as CCCP (carbonylcyanide-*m*-chlorophenyl hydrazone)) is present at concentrations that dissipate the membrane potential, it can prevent the processing of periplasmic proteins and the precursor forms of the proteins accumulate in the inner membrane. This result suggests that the binding-protein precursors are

initially folded into a conformation that exposes hydrophobic regions that permit them to associate with the membrane, and that the transmembrane potential plays a role in the processing of the precursors (Daniels *et al.*, 1981; Copeland *et al.*, 1984; Oxender *et al.*, 1984).

After consideration of all the data, a model (Oxender *et al.*, 1984) has been proposed for secretion of the binding proteins into the periplasmic space (see Fig. 2). In the model, step 1 represents the initial association of the binding protein with the inner membrane. Step 2 indicates formation of a helical hairpin structure between the leader peptide and the *N*-terminal portion of the mature protein. This helical hairpin assumes a transmembrane orientation in the presence of a proton-motive force. Step 3 indicates cleavage of the leader sequence by the leader peptidase. Finally, steps 4 and 5 correspond to a refolding of the mature protein and its translocation into the periplasmic space.

D. REGULATION OF LIV-I AND LS TRANSPORT

The potential regulatory effect of each of the branched-chain amino acids has been examined, and it was found that the rate of transport responds only to changes in the concentrations of leucine (Quay and Oxender, 1976). In the presence of high concentrations of leucine, the transport activity is decreased from the wild-type level (Penrose *et al.*, 1968; Rahmanian *et al.*, 1973; Oxender and Quay, 1976a; Quay and Oxender, 1976); this repression of transport is due to a decrease in the rate of synthesis of the periplasmic binding proteins (Quay and Oxender, 1976). It is currently thought that two types of regulation are involved in decreasing the rate of synthesis of the binding proteins in response to high concentrations of leucine: the major form of regulation is probably caused by a *trans*-acting repressor similar in action to other prokaryotic repressors (Anderson *et al.*, 1976), whereas the residual leucine regulation is credited to *rho*-dependent transcriptional attenuation (Oxender and Quay, 1976b).

Two strains of *E. coli* harbouring spontaneous regulatory mutations were isolated and partially characterized (Rahmanian *et al.*, 1973). One of the strains, EO311, carried the mutation known as *livR*− which affected both the LIV-I and the LS systems of transport. The other strain, EO318, carried the mutation called *lstR*− which was only known to affect the LS transport system. Both of these mutations result in derepression of transport activity in the presence of high concentrations of leucine (conditions under which wild-type bacterial cells show repressed rates of transport). These mutations were crossed into isogenic strains of *E. coli* for more rigorous analysis (Anderson *et al.*, 1976). They were mapped and found to reside at minute 20 on the *E. coli* chromosome. Moreover, the two mutations were inseparable by genetic

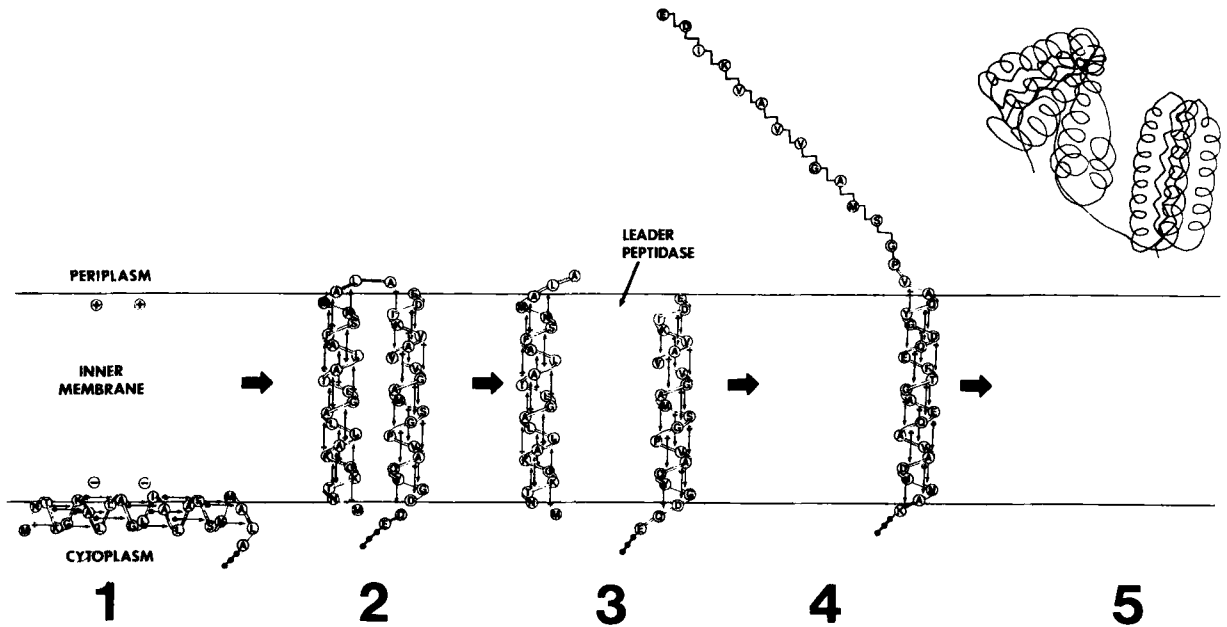

FIG. 2. Model for export of the periplasmic leucine-binding protein by bacteria. The amino acids are represented by single-letter codes in the circles. The small arrows indicate the directions (positive to negative) of helix dipoles. The steps are described in the text.

techniques. Because the regulatory loci are unlinked to the transport regulon, these loci must exert their effects in a *trans*-acting mechanism. When F prime f106, carrying the wild-type region including the *livR/lstR* loci, was crossed into a strain bearing either the *livR-* mutation or the *lstR-* mutation, wild-type leucine regulation was restored. This result indicates that the mutations reside in a gene or genes encoding a negative controlling element for branched-chain amino-acid transport. All of these data taken together suggest that *livR/lstR* encodes a diffusible repressor.

Currently, the *livR/lstR* region has been cloned and a plasmid that complements both mutations has been identified (T.K. Antonucci and D.L. Oxender, unpublished observations). Experiments are under way to identify the gene or genes involved in the repressor regulation as well as their products.

The *rho* factor attenuates transcription of many operons by a mechanism that is not yet fully understood. Probably the *rho* factor first recognizes a particular site along the non-translated region of the RNA which is at least 85 nucleotides in length and which contains few ($< 14\%$) guanosine residues (Ceruzzi *et al.*, 1985). Apparently, the *rho* factor then becomes involved in dislodging the RNA polymerase from the DNA strand and consequently results in release of the RNA transcript (Andrews and Richardson, 1985). The process of *rho* factor-mediated transcription attenuation is coupled to the hydrolysis of ATP.

The first studies that implicated the *rho* factor in the regulation of branched-chain amino-acid transport were carried out in a strain of *E. coli* that was lacking the *rho* factor (Oxender and Quay, 1976a). In this strain, it was shown that the branched-chain amino-acid transport capacity increased approximately fourfold. Subsequent work on the kinetics of leucine transport showed that the LIV-I system (which utilizes LIV-binding protein) increased its activity by a factor of 8.5, whereas the LS system (which employs LS-binding protein) experienced a fourfold increase in activity (Quay and Oxender, 1977). In addition, it was shown that only systems that had binding proteins were affected by the *rho* factor. Three membrane-bound transporst systems were tested (those for proline, tryptophan and LIV-II) and were not found to be derepressed in the strain carrying the *rho*— mutation (Quay and Oxender, 1977).

It has also been shown that the concentrations of charged leucyl-tRNA play a role in regulation of both the LIV-I and LS transport systems (Oxender and Quay, 1976b; Oxender *et al.*, 1977, 1980c; Quay and Oxender, 1980a,b). When transport rates were measured in a strain of *E. coli* containing a temperature-sensitive leucyl-tRNA synthetase, it was observed that the transport activity increased at the non-permissive temperature (Oxender and Quay, 1976b). To determine whether the observed effects were due to the internal cellular concentrations of activated leucyl-tRNA or to the leucyl-tRNA synthetase

itself, another genetic mutant, *hisT*, was utilized (Quay *et al.*, 1978). The *hisT* locus in *Salmonella typhimurium* codes for an enzyme that converted uridine into pseudouridine in the tRNA species for leucine, histidine and isoleucine. Regulation of the histidine, isoleucine–valine and leucine biosynthetic operons in the presence of this mutation was altered so that these operons were no longer sensitive to a limitation for their cognate amino acids (Cortese *et al.*, 1975). The concentration of leucine in the growth medium did not affect the rate of transport in the *hisT* strain thus indicating that repression of transport activity required fully maturated tRNA which was amino-acylated with leucine.

The "stringent phenomenon" in bacteria is a mechanism that regulates synthesis of rRNA and tRNA co-ordinately with general protein synthesis (Cashel and Gallant, 1968). Wild-type bacteria accumulated guanosine tetraphosphate (ppGpp) during amino-acid limitation (Block and Haseltine, 1974). The product of the *relA* gene, as well as deacylated tRNA, is required for synthesis of ppGpp *in vitro* (Haseltine and Block, 1973). It has been demonstrated that ppGpp is a positive regulator in the *in vitro* or *in vivo* expression of several biosynthetic operons, including those for histidine (Stephans *et al.*, 1975), tryptophan (Morse and Morse, 1976), methionine (Somerville and Ahmed, 1977) and isoleucine–valine (Smolin and Umbarger, 1975). In addition, in *relA* strains starved for leucine, maturation of $tRNA^{leu}$ was incomplete (Kitchingman *et al.*, 1976; Kitchingman and Fournier, 1977). Leucine starvation led to accumulation of the major leucyl-tRNA species, $tRNA^{leu}$, in an undermodified state with uridine in place of dihydrouridine in position 16 and pseudouridine in positions 39 and 66. Similarly, *hisT* mutants, which failed to increase the rate of leucine transport after leucine deprivation, contain $tRNA^{leu}$ with uridine in place of pseudouridine in positions 39 and 41 (Allandeen *et al.*, 1972). Furthermore, the *relA* locus was required for derepression of the synthesis of branched-chain amino-acid transport proteins (Kitchingman and Fournier, 1977). The authors concluded that, since mutations affecting $tRNA^{leu}$ maturation such as *hisT* or *relA* prevent transport derepression, it appeared that mature, fully modified $tRNA^{leu}$ was a regulator of transport derepression.

Two types of double regulatory mutants were constructed and their rates of leucine transport tested. One strain carried the *hisT*− and the *rho*− mutations (Quay *et al.*, 1978). The level of derepression for the double mutant strain was similar to those found for each of the single mutants. Hence, this finding, that *rho* did not elevate expression in a *hisT* mutant, suggested that *rho* interacted with $tRNA^{leu}$. The other type of double mutant that was examined had both *rho*− and *livR*− (Landick *et al.*, 1980). When the level of derepression of transport activity was measured in this double mutant strain, it was observed that it was significantly higher than that found for a strain

carrying either of the single mutations. Thus, the effects of mutations in the *rho* and *livR* genes were additive and therefore appeared to be involved in independent regulatory mechanisms. Mutations in the *rho* gene affect both the LIV-I and LS transport systems by increasing expression of *livJ* and *livK* which are the genes encoding the binding proteins for LIV and LS, respectively.

Since both the biosynthetic enzymes and the transport activity were repressed by growth on medium containing leucine, it was first important to examine if these two distinct processes were regulated by the same mechanism. Reciprocal experiments were done in which transport regulation was measured in strains with operator–promoter mutations in the *leu* operon or with mutations causing alterations in the regulation of the *ilv* biosynthetic operon (Oxender and Quay, 1976b; Quay *et al.*, 1978). In all such strains studied, transport was found to be regulated in a normal fashion. These experiments showed that regulation of transport was separate from regulation of the biosynthetic operon.

A model illustrating interaction of all of the known regulatory factors (Landick *et al.*, 1980) has been presented for the branched-chain amino-acid transport regulon (see Fig 3). In addition to this illustration, a mechanism for *rho*-dependent transcriptional attenuation has been suggested for the *livJ* gene (Landick, 1984). This mechanism attempts to integrate all of the known genetic mutations as well as to utilize potential functional regions in the DNA sequence of the promoter of the *livJ* operon. In this proposed mechanism, the

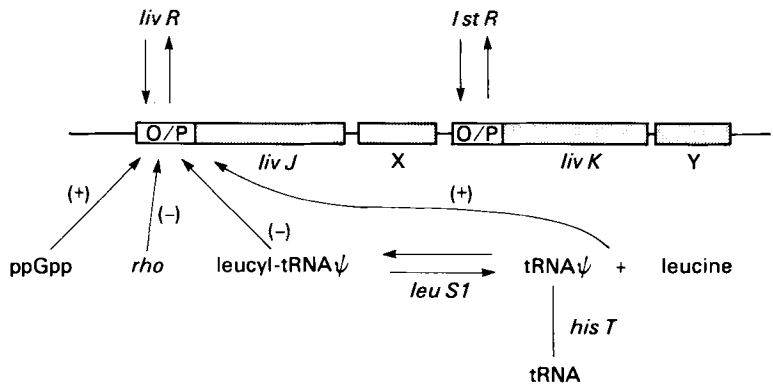

FIG. 3. Model of regulation of leucine transport in *Escherichia coli*. This model illustrates the effects of the regulatory mutants on the expression of the two binding-protein genes, *livJ* and *livK*.

livJ promoter encodes a leader polypeptide (see Fig. 4) with tandem leucine codons that might allow translational coupling of the level of charged leucyl-tRNA with transcription termination. In addition to the potential leader polypeptide sequence, the promoter of the *livJ* operon contains a probable binding site for the *rho* factor (noted in Fig. 4 as R; Ceruzzi *et al.*, 1985). If a cell is starved of leucine and contains low concentrations of charged leucyl-tRNA, ribosomes translating the small peptide in the LIV-binding protein transcript leader may become stalled on the tandem leucine codons and thus block *rho*-factor binding. When adequate leucine is available, ribosomes would rapidly translate the leader peptide, release at the stop codon, and thereby not hinder *rho*-factor binding (see Fig. 5). The net result would be that leucine starvation would block *rho*-dependent attenuation while leucine excess would allow *rho*-dependent attenuation.

We are currently attempting to alter the two adjacent leucine codons in the putative leader polypeptide by oligonucleotide-directed mutagenesis (T.K. Antonucci, R.C. Landick and D.L. Oxender, unpublished observations). This alteration should provide a direct test of the model proposed for *rho*-dependent transcription attenuation. If the model is correct, the alterations should abolish the residual leucine regulation.

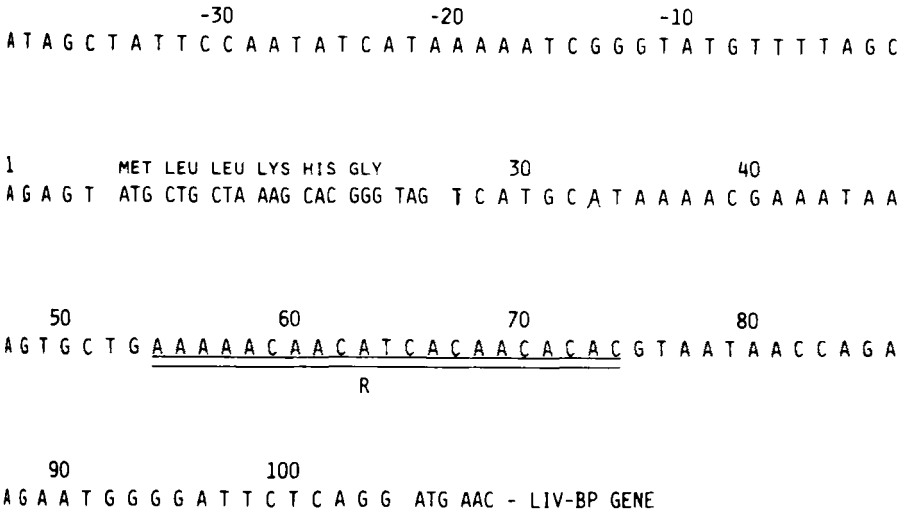

FIG. 4. The nucleotide sequence of the promoter and leader regions of the *livJ* gene. The underlined sequence labelled R is the potential *rho*-binding site. The amino-acid sequence of the putative leader polypeptide is shown above the DNA strand. Abbreviation: LIV-BP, LIV-binding protein.

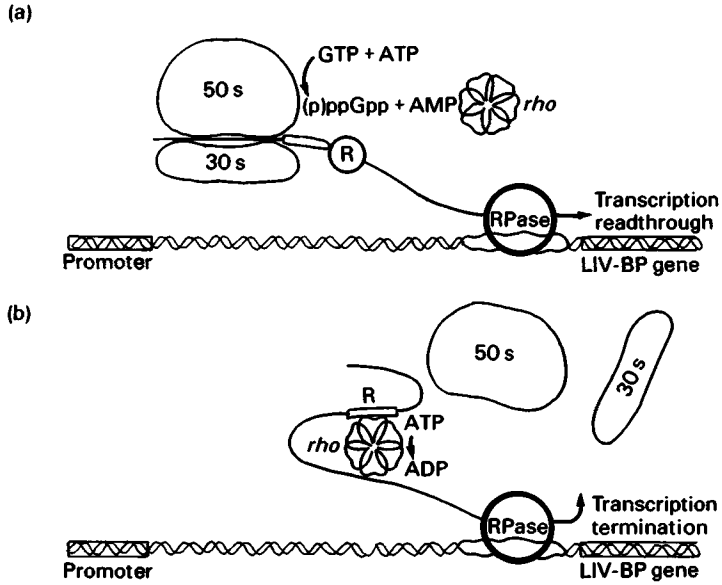

FIG. 5. Model for the attenuation of *livJ* transcription by the *rho* factor in *Escherichia coli*. (a) shows that, under conditions of leucine deprivation, the *livJ* gene is expressed because the stalled ribosome interferes with *rho* binding. (b) shows that, in the presence of excess leucine, the ribosome completes translation of the leader peptide and dissociates thus allowing *rho* to bind to the mRNA transcript. Once *rho* binds, transcription is uncoupled from translation and the *livJ* gene is not expressed. A more detailed description of this model is given in the text.

III. Branched-Chain Amino-Acid Transport Systems of *Pseudomonas aeruginosa*

A. INTRODUCTION

There exist at least two distinct systems in *Pseudomonas aeruginosa* for transporting branched-chain amino acids (Hoshino, 1979). One of the systems, LIV-I, displays a low affinity for the substrate leucine (K_m 0.34 μM) and is sodium independent. The LIV-I system transports leucine, isoleucine and valine and, to a lesser extent, also threonine and alanine. On osmotic shock, bacterial cells experience a decrease in activity of LIV-I transport suggesting that the periplasmic space contains a component important for the transport process. The second system for transporting branched-chain amino acids is called LIV-II and it has affinity only for leucine, isoleucine and valine. The apparent K_m value of this high-affinity system is 7.6 μM for leucine. Transport activity of LIV-II seems to require the presence of sodium ions

because it is only in their presence that the high-affinity system for leucine transport is observed. The LIV-II system was the only one that was funtional in membrane vesicles (Hoshino and Kageyama, 1979) and the K_m value of LIV-II in vesicles agreed with the sodium-dependent value obtained with whole cells. The LIV-II system was unaffected by osmotic shock indicating that it does not require periplasmic components.

Much work has focused on the purification and characterization of the periplasmic component(s) of the LIV-I system in *Ps. aeruginosa* (Hoshino, 1979; Hoshino and Kageyama, 1980). The specific activity of the purified periplasmic component was comparable to that of the LIV-I system. The protein was purified from shock fluid and was found to be a monomer with pI 4.1 and a molecular weight of approximately 40,000. The protein did not contain sugar, and analysis of the amino-acid composition of the protein showed that the major residues were alanine, glycine, aspartic acid and glutamic acid. In addition, one residue each of cysteine and of tryptophan were identified. The protein bound leucine, isoleucine and valine with dissociation constants of 0.4, 0.3 and 0.5 μM, respectively. Alanine and threonine were also bound but with significantly lower affinities. Because of the specificity of binding, this protein was called the LIVAT-binding protein.

B. TRANSPORT MUTANTS

Genetic studies were also carried out on the LIV transport systems in *Ps. aeruginosa* (Hoshino and Kageyama, 1982; Hoshino and Nishio, 1982; Hoshino *et al.*, 1983). Two types of mutant were described for LIV-I, and two types of mutant were also isolated for LIV-II (Hoshino and Kageyama, 1980, 1982). Two of the LIV-I mutants were found to be deficient in producing the LIVAT-binding protein as shown by shock-fluid purification and subsequent SDS–polyacrylamide-gel electrophoresis. These mutants exhibited a decrease in LIV-I activity. Revertants were obtained that showed wild-type rates of LIV-I transport and, after purification from shock fluids, also yielded relatively normal amounts of the LIVAT-binding protein (Hoshino and Kageyama, 1980). The other class of LIV-I mutant identified produced normal amounts of the LIVAT-binding protein as demonstrated by SDS–polyacrylamide-gel electrophoresis of shock fluids; moreover, the LIVAT-binding protein seemed to be funtional when binding assays were carried out. Thus, it was concluded by the authors that this type of mutant carries a defect in another gene required for LIV-I transport activity (Hoshino and Kageyama, 1980).

The two classes of mutant for LIV-II had the following phenotypes (Hoshino and Kageyama, 1980, 1982). One strain showed a decrease in rates of LIV-II transport while maintaining a normal rate of LIV-I transport. Thus,

this strain was probably carrying a defect in a gene required for LIV-II. The second type of mutant showed an enhancement of LIV-II activity. Membrane-vesicle preparations for each of these two types of mutation were tested for their LIV-II transport. It should be recalled that membrane-vesicle preparations exhibit only LIV-II activity and not LIV-I activity. It was shown that vesicle preparations from the LIV-II-defective mutant had no measurable rate of leucine transport. In addition, vesicle preparations from the LIV-II-enhanced mutant also had higher rates of LIV-II transport than that observed in wild-type cells. Hence, the experiments carried out on membrane-vesicle preparations confirm the results obtained from the mutants with regard to LIV-II activity.

From the experiments done on the mutants described thus far, the authors concluded that the LIV-I and LIV-II transport systems operate independently of each other. They pointed out, however, that these results do not preclude the possibility that the two transport systems share some components in common (Hoshino and Kageyama, 1982).

Another type of mutant was isolated (Hoshino and Nishio, 1982) which produced a non-functional LIVAT-binding protein. The shock fluid from this mutant had normal amounts of the LIVAT-binding protein as determined by both SDS–polyacrylamide-gel electrophoresis and immunological cross-reactivity to wild-type LIVAT-binding protein. Moreover, a denaturing gel demonstrated that the mutant LIVAT-binding protein was of approximately the same molecular weight as the wild-type protein. However, almost no binding activity was detectable for the branched-chain amino acids in the shock fluids from the mutant strain. The mutation was most probably a defect in the structural gene for the LIVAT-binding protein (*braC*). This mutation was called *braC310* and was mapped in a region of the *Ps. aeruginosa* chromosome later than minute 60 (Hoshino and Nishio, 1982; Hoshino *et al.*, 1983).

Recently, the nature of the *braC310* LIVAT-binding protein has been examined with the following results (T. Hoshino, personal communication). The LIVAT-binding proteins from wild-type and mutant shock fluids were purified to homogeneity and several characteristics of the two were compared. The proteins showed the same properties for ultraviolet and circular dichroism spectra, immunological cross-reactivity, electrophoretic mobilities on both denaturing and non-denaturing gels, and two-dimensional tryptic peptide maps. In addition, alanine was identified as the *N*-terminal amino acid for both proteins. However, the K_m values for the two proteins were quite different, namely 0.2–0.3 μM for the wild type and 20–30 μM for the mutant. Moreover, the proteins showed differential heat-stabilities. The denaturation temperatures for the wild-type protein was 58°C whereas, for the mutant protein, it was 53°C. Although the two proteins showed similar amino-acid

compositions, they displayed a difference in cysteine contents. The wild-type protein had two cysteine residues with no free -SH group but the mutant protein had three cysteine residues and one free -SH group. The author thereby suggested that *braC310* is a missense mutation in the *braC* gene which resulted in a cysteine residue substituted for another, to date unknown, amino acid.

Yet another set of mutations, *bra311–314*, were found which cause a decrease in LIV-I activity and map near to the *braC310* mutation. However, in these mutants, there were normal amounts of the functional LIVAT-binding protein; consequently, it seemed that this set of mutations affected a locus required for LIV-I transport but not the structural gene for the LIVAT-binding protein. The region containing these mutations was named *braB* (Hoshino *et al.*, 1983).

The last type of mutation discussed was one that affected both the LIV-I and LIV-II transport sytems. Branched-chain amino-acid transport was significantly decreased in the presence of this mutation, but examination of shock fluids showed that the mutation altered the amount of the LIVAT-binding protein by lowering the expression of the *braC* structural gene at either the transcriptional or translational levels. The other proteins present in shock fluids were not affected by this mutation indicating that it specifically acted on the gene for LIVAT-binding protein. In addition, this mutation mapped in a region unlinked to the other previously described mutations. Hence, this mutation was considered to have occurred in a regulatory gene for the LIV transport operons and was designated *braA* (Hoshino *et al.*, 1983).

C. RECONSTITUTION OF TRANSPORT COMPONENTS

In a recent study, the LIV-II transport system was solubilized and reconstituted into liposomes (Uratani, 1985). It was observed than an H^+-concentration gradient in the presence of Na^+ did not drive leucine transport. This result suggested that the transport component may not couple with H^+ ions.

IV. Histidine Transport in *Salmonella typhimurium*

A. INTRODUCTION

The histidine-transport system in *S. typhimurium* is one of the better characterized binding protein-dependent transport systems in prokaryotes. Until 1980 when the histidine-transport genes of *S. typhimurium* were cloned, much information had already been determined on the components and

mechanism of histidine transport. Although histidine is transported by several different systems, each with its own patterns of specificity and affinity, the high-affinity osmotic shock-sensitive transport system had been analysed most rigorously (Ames, 1964, 1972; Ames and Lever, 1972; Ames *et al.*, 1977; Ames and Nikaido, 1978). Three required components for the high-affinity system had been identified. The *hisP* gene product, the P protein, had been established as a membrane protein; the *hisQ* gene product did not yet have a known cellular location; and the *hisJ* gene product or J protein was a periplasmic component which bound histidine and which had been purified (Lever, 1972). These three genes had been located on the chromosomal linkage map of *S. typhimurium* to minute 48.5 (Ames *et al.*, 1977).

The fundamental steps in the mechanism of histidine transport, while not fully established, were rather well-understood. It had been shown that the J protein contained two binding sites essential for transport activity (Ames and Kutsu, 1974), one for histidine as well as one that interacted with the P protein (Ames and Spudich, 1976). The P protein was found to be involved in transporting several molecules in addition to histidine, such as histidinol and arginine (when required as a nitrogen source; Kutsu and Ames, 1973). Interestingly, arginine transport utilizing the P protein also required the lysine–arginine–ornithine binding protein (LAO-binding protein, discussed in Section VII. A of this review; Kutsu *et al.*, 1979). The LAO-binding protein is the product of the *argT* gene which was closely linked genetically to the *hisJ* gene encoding the histidine-binding protein. Moreover, it had been shown that the histidine-binding protein and the LAO-binding protein cross-react immunologically and that the cellular concentrations of both of these proteins are regulated in response to availability of nitrogenous nutrients. Thus it was hypothesized that interaction of the LAO-binding protein with the membrane-bound P protein occurs via the same mechanism as interaction of the histidine-binding protein with the P protein (Kutsu *et al.*, 1979).

B. GENE ORGANIZATION AND CHARACTERIZATION

The histidine-transport operon was cloned into a lambda phage vector and identified by genetic complementation studies, by physiological transport activity measurements, and by demonstrating that the lambda-cloned DNA encoded the J and P protein products (Ardeshir and Ames, 1980). The 12.4 kb lambda clone was shown to contain all of the known histidine-transport genes as well as the *argT* gene encoding the LAO-binding protein and the *ubiX* locus. The positions of the histidine-transport genes were initially determined by detailed restriction mapping, by deletion mapping and by transposon insertion mutagenesis (Ardeshir *et al.*, 1981). Deletion mapping was carried out using *S. typhimurium* strains containing genetically defined deletions.

These strains are characterized in two ways. First, the transport component rendered non-functional or missing entirely because of the deletion is identified. Then the location and extent of the deletion along the DNA are determined by restriction-enzyme mapping. The consequence is a correlation of a particular region of the DNA sequence with a transport component. The final method already referred to, namely transposon-insertion mutagenesis, is a very similar analysis to deletion mapping, except that strains which contain multiple insertions of a transposable element in various positions are characterized; in this way, the non-functional transport component resulting from the insertion event is identified. Subsequently, the position of the transposable element is determined and, hence, the position of the now non-functional transport component's gene.

The entire DNA sequence of the histidine-transport operon and of the *argT* gene was determined as well as the exact number and location of the genes involved (Higgins and Ames, 1981; Higgins *et al.*, 1982). Consequently, a model was constructed for histidine transport, and Fig. 6 presents the gene organization and model for the histidine transport system. The DNA sequences of the histidine- and LAO-binding proteins are about 70% homologous indicating that these two genes are products of an ancestral gene duplication (Higgins and Ames, 1981). This high degree of homology may initially be considered surprising in light of the fact that the two proteins have different substrate specificities, but it must be remembered that both proteins have been shown to interact with the same membrane component, namely the P protein. The *hisQ* gene encodes a basic protein with a molecular weight of approximately 21,000 which has been located in the inner membrane of the bacterial cell (Higgins *et al.*, 1982). Furthermore, the DNA sequence of the transport genes revealed a previously undiscovered gene involved in histidine transport, namely *hisM* (Higgins *et al.*, 1982). The *hisM* gene is located in an open reading frame distal to the *hisP* gene and proximal to the *hisQ* gene. Mutants isolated previously which map to the *hisM* open reading frame have

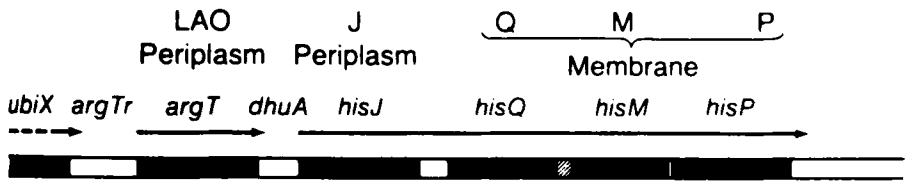

FIG. 6. Organization of the histidine-transport genes. Arrows represent the direction of transcription of the operons. The regions designated *argTr* and *dhuA* are regulatory regions for the *argT* and for the histidine-transport operons, respectively.

the same phenotype as mutants in either *hisP* or *hisQ*, and the *hisM* mutants produce wild-type concentrations of the normal P protein. Thus, the *hisM* gene is not regulatory in function, but probably produces a protein that interacts with the P and/or Q proteins and may, itself, reside in the membrane. The length and codon sequence of the *hisM* gene suggest that the M protein is a basic component with a molecular weight of about 26,000.

All of the preceding information is brought together in a model for histidine transport in *S. typhimurium* (Fig. 7). In the periplasm, the J protein binds histidine and carries it to the other proteins in the membrane. The act of binding histidine has been shown to induce a conformational change in the J protein (Robertson *et al.*, 1977) thus allowing it to interact with the P protein (Ames and Spudich, 1976). The P protein resides on the periplasmic side of the membrane (Ames and Nikaido, 1978), presumably in a complex with the Q and M proteins (Higgins *et al.*, 1982). Interaction of the J and P protein results in passage of histidine through the membrane and into the cytoplasm of the cell. Currently, there are two proposed mechanisms for transport of histidine through the membrane. One possibility is that histidine may move through the membrane in a pore that has been formed by the P, Q and M proteins (see Fig. 7a). In this mechanism, the membrane components would be induced to form the pore because of docking of the histidine–J protein complex. Alternatively, each of the transport components may have a binding site for histidine and, by this mechanism, histidine would be passed from one component to the next until it was through the membrane (see Fig. 7b).

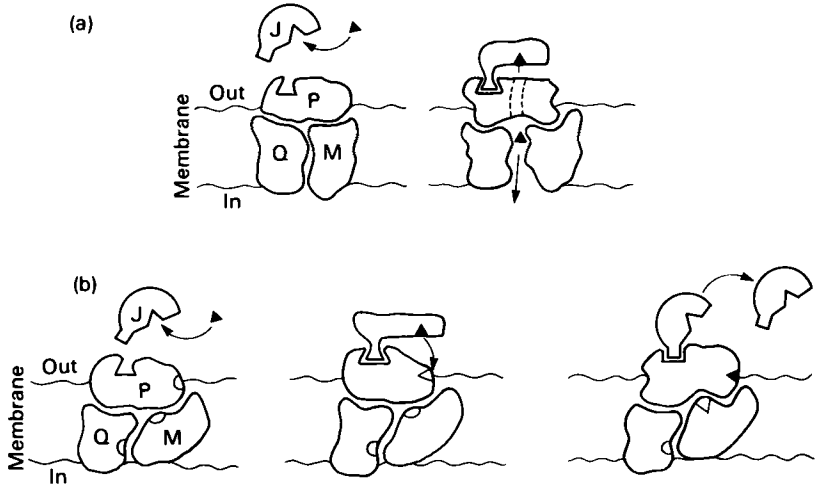

FIG. 7. Two possible models for histidine transport (a and b). The two models are described in the text.

C. REGULATION

Four major aspects of regulation of expression of the histidine-transport genes have been examined to date. First, DNA sequencing has revealed no structures (series of stem-loops or small open reading frames signalling leader polypeptides) in the promoters of either the histidine or LAO binding-protein genes (Higgins and Ames, 1981, 1982). This result indicates that the transcriptional attenuation type of regulation, which exists for many amino-acid biosynthetic operons (Keller and Calvo, 1979; Oxender *et al.*, 1979; Johnston *et al.*, 1980), does not appear to have a role for the histidine or LAO transport operons. Second, all of the promoter mutations that resulted in increased expression of the histidine operon were located to a single base in the Pribnow box (Lee and Ames, 1984), thus suggesting that no unusual regulatory regions exist in the promoter sequences.

Third, it had previously been observed that the level of expression of the genes for the membrane components was substantially (30 times) lower than the level of expression of the J (histidine binding) protein (Higgins *et al.*, 1982). It had been postulated that the presence of a potential stem-loop structure located 3′ to the *hisJ* gene and 5′ to the *hisP* gene could be a signal for transcriptional attenuation of the distal genes encoding the membrane components (Higgins and Ames, 1982; Stern *et al.*, 1984a). Subsequently, it was shown that the presence of this stem-loop does not correlate with a significant transcriptional attenuation of those genes. However, it was found also that the 3′ to 5′ degradation of the mRNA is halted at the stem-loop structure, presumably because the nuclease enzyme cannot read through the stem-loop obstruction. In the absence of the stem-loop, the degradative process procedes through the total length of the mRNA resulting in a twofold decrease in expression of the J protein (Stern *et al.*, 1984a). So part of the explanation of the attenuation of expression of the membrane components has been elucidated.

Fourth, the sequences involved in nitrogen regulation of the operons for histidine transport and LAO-binding protein were examined (Higgins and Ames, 1982; Stern *et al.*, 1984b). With fusions created by recombinant DNA techniques between the two promoters of interest and the β-galactosidase structural gene, it was found that the observed nitrogen regulation occurred at the level of gene transcription. Moreover, a consensus sequence of mirror symmetry in each of the promoters was identified which may serve as the site of nitrogen regulation for these two operons. This same consensus sequence has been observed in the promoters of two other operons also regulated by nitrogenous nutrients (Drummond *et al.*, 1983), thus lending support to this hypothesis.

D. ENERGETICS

The final aspect of histidine transport to be discussed is the energy which must drive the system. One of the latest publications from the Ames' laboratory probes the source of energy for this transport process (Hobson *et al.*, 1984). Briefly, the paper describes studies carried out with the membrane-associated proteins in isolated membranes. It was determined that both the P and M proteins react with the reagent 8-azido-ATP, used in photo-affinity labelling. The amount of labelling is decreased by the presence of ATP and is slightly decreased by the presence of either GTP, CTP, UTP or ADP. The authors thus proposed that the M and/or P proteins have a site that can bind to an adenine nucleotide. Consequently, these two proteins may be involved in the energy-coupling step of histidine transport with ATP hydrolysis.

V. Proline Transport in *Escherichia coli*

A. HIGH-AFFINITY PROLINE TRANSPORT (PP-I)

A single, membrane-associated component appears to be responsible for the high-affinity proline transport in *E. coli* (Wood and Zadworny, 1979). Some genetic evidence suggests that a second proline transport system, homologous to the low-affinity proline transport system (PP-II) of *S. typhimurium*, may exist in *E. coli*. An *E. coli* episome, which contains a segment of the chromosome corresponding to the PP-II region of *S. typhimurium*, complements mutations in the PP-II transport system of *S. typhimurium*, (Menzel, 1980). In addition, double mutants (*putP*−, *putA*−) are defective for the PP-I transport system and for proline dehydrogenase activity, but these mutants still transport proline via PP-II (Stalmach *et al.*, 1983). It is generally thought that proline transport derives its energy from a proton-motive force (Harold, 1977; Ramos and Kaback, 1977). Moreover, proline transport as well as proline degradation are inducible and repressible by either L-proline or glycyl-L-proline (Brenchley *et al.*, 1973; Ratzkin and Roth, 1978a; Ratzkin *et al.*, 1978).

Genetic selections were carried out to identify mutations in the *putP* locus which encodes the membrane-associated protein of the high-affinity proline transport system (PP-I) (Wood *et al.*, 1979). The Clarke–Carbon gene bank (Clarke and Carbon, 1976) was screened by complementation analysis and several clones were isolated which complemented the *putP*− mutation (Wood *et al.*, 1979; Wood and Zadworny, 1980). A strain of *E. coli* which produces minicells during normal cellular replication was used to examine the products of the isolated plasmids (Wood and Zadworny, 1980; Wood, 1982). The PutP protein was identified in the cytoplasmic membrane of the minicells and its

molecular weight was estimated to be approximately 25,000 by SDS–polyacrylamide-gel electrophoresis.

B. LOW-AFFINITY PROLINE TRANSPORT (PP-II)

The low-affinity proline-transport system (PP-II) has been studied using similar strategies to those discussed for PP-I. Mutations in the PP-II system, consisting of the *proT* locus at minute 82, have been selected for and characterized (Motojima *et al.*, 1978, 1979). The observed phenotype of the *proT*− mutant is a defect in the activity of the proline transporter (Motojima *et al.*, 1978). Two recombinant plasmids were isolated from the Clarke–Carbon gene bank (Clarke and Carbon, 1976) which complement the *proT*− mutation. Restriction-enzyme maps of these two plasmids were made and it was found that the insert segments are identical. A minicell expression system was used to determine whether the plasmids produced a unique protein product. The results indicated that the plasmids do indeed produce a specific product of molecular weight 24,000 and that, in addition, the product resides in the cytoplasmic membrane. In addition, using a newly established reliable method for measuring binding activity of the proline carrier in the membrane, it was estimated that cells carrying the recombinant *proT*+ plasmids produced six times more proline transport carrier than the wild-type cells (Motojima *et al.*, 1979).

The final locus affecting proline transport in *E. coli* to be considered is the *putA* gene which maps at minute 22 on the chromosome and which is adjacent to the *putP* gene (Wood and Zadworny, 1980). The *putA* locus encodes the L-proline and pyrroline carboxylic acid dehydrogenases (Ratzkin and Roth, 1978b; Anderson *et al.*, 1980). In addition, the *putA* gene product controls its own expression as well as that of the *putP* gene by acting as a repressor. Two types of *putA*− mutants have been observed: one type lacked proline dehydrogenase activity and was constitutive for proline transport, as expected for a traditional *trans*-acting repressor mutation; the second type displayed normal proline dehydrogenase activity and was constitutive for both proline dehydrogenase and proline transport, once again a phenotype predicted for a repressor mutation (Menzel, 1980). The first kind of mutation was caused by an insertion of the transposable element Tn*5* into the *putA* gene, whereas the second kind of mutant was classified as artificial because it was due to strains carrying multicopy hybrid plasmids containing the *put* genes (Wood and Zadworny, 1980).

Once again, the Clarke–Carbon bank (Clarke and Carbon, 1976) was screened for cells harbouring recombinant plasmids which would complement the *putA*− mutations (Wood and Zadworny, 1980). Such plasmids were isolated and found to contain the *putPA* region of the *E. coli* chromosome.

These plasmids were used in minicell experiments in order to identify the *putA* gene product(s). The only product detectable for *putA* had a molecular weight of approximately 130,000. It should be noted here that the *putA* gene products from both *E. coli* and *S. typhimurium* had been purified to homogeneity, and their molecular weights were determined to be 124,000 and 132,000, respectively (Menzel, 1980; Scarpulla and Soffer, 1978).

Wood and her colleagues also examined chemotaxis of *E. coli* to proline (Clancy *et al.*, 1981). Proline chemotaxis showed inducibility somewhat similarly to that of proline transport, but more similar to that of proline dehydrogenase activity. The chemotactic response was abolished in strains carrying mutations in either the *putA* or *putP* loci. Likewise, in the absence of a proline concentration gradient, no chemotactic response to proline was detectable. The authors concluded from these and other data that the effect of proline oxidation on the energy metabolism of the bacterial cell results in a chemotactic response to proline. Moreover, the chemotactic response of the cell to proline probably requires proline-transport activity so that proline may be taken up and oxidized by the cell.

Another aspect of proline transport examined by Wood and her colleagues involves proline excretion from strains genetically designed to overproduce proline (Rancourt *et al.*, 1984). Proline excretion, which has been looked upon as a model chemical production system, has been shown not to be a response to uncontrolled biosynthesis of proline. Rather, proline excretion occurred only if either catabolism and/or transport are defective. Thus, the authors believed that this study showed that metabolite (in this case proline) excretion may be specifically induced if active transport of the metabolite is eradicated.

C. ENERGETICS OF PROLINE TRANSPORT

Some of the more recent work from Anraku's laboratory on proline transport involves the energetics that drive the PP-I system (Mogi and Anraku, 1984a,b,c). In order to investigate the kinetics of energization of proline transport, membrane vesicles were prepared from a proline carrier-overproducing strain (Mogi and Anraku, 1984a). In the presence of an imposed membrane potential, the Michaelis constant for transport (K_t) was decreased and the V_{max} value was concomitantly increased. The stoicheiometry of proline transport in the presence of an imposed membrane potential was two protons for one of substrate (at pH 8.0). Hence the following symmetrical model for proline transport was proposed (Mogi and Anraku, 1984a). In the model, $2H^+$/proline symport occurs via formation of a carrier/H^+/H^+/substrate intermediate. Thus the effect of the membrane potential on the K_t value can be explained as formation of the transport intermediate. Moreover, the effect of the membrane potential on the V_{max} value is due to an increase in

movement of loaded carriers between the two sides of the membrane. In another study (Mogi and Anraku, 1984b), it was shown that alkaline cations, such as Na^+ and Li^+, can stimulate proline transport. However, such cations in high concentrations can inhibit proline transport (Mogi and Anraku, 1984c). The authors conclude that high concentrations of Na^+, Li^+ or H^+ can regulate transport by inhibiting the transition from the carrier intermediate, thus controlling the amount of active carrier which is responsible for the $2H^+$/proline symport in the presence of a membrane potential.

VI. Aromatic Amino-Acid Transport in *Escherichia coli*

A. INTRODUCTION

Although the region responsible for tyrosine-specific transport has only recently been cloned (Kasian and Pittard, 1984; Wookey *et al.*, 1984), a significant body of work had been accomplished on genetic aspects of transport of tyrosine and other aromatic amino acids (Whipp and Pittard, 1977; Whipp *et al.*, 1980). Several systems have been identified for transporting aromatic amino acids, including a common system which transports all three of them as well as three other systems which are each specific for one aromatic amino acid (Brown, 1970). The amino acid tryptophan also has another specific transport system which differs from that already referred to in that it is inducible (Boezi and DeMoss, 1961; Burrows and DeMoss, 1963).

B. REGULATION

Regulation of some of these systems has been studied in some detail in biochemical and genetic experiments. It has been shown that growing bacteria in the presence of phenylalanine or tyrosine decreased the rate of the common transport system (Kuhn and Somerville, 1974; Harrison *et al.*, 1975). Moreover, cells grown in the presence of tyrosine experienced a retardation of the tyrosine-specific transport system (Kuhn and Somerville, 1974).

The *tyrR* gene product was known to be involved in the regulation of biosynthesis of several enzymes which play a role on the aromatic amino-acid biosynthetic pathways (Wallace and Pittard, 1969; Brown and Somerville, 1971; Im *et al.*, 1971; Camakaris and Pittard, 1973; Ely and Pittard, 1975). Therefore, the potential role of *tyrR* in aromatic amino-acid transport was investigated (Whipp *et al.*, 1976; Whipp and Pittard, 1977). A strain carrying a mutation in the *tyrR* locus was tested for its ability to transport aromatic amino acids by the common system as well as by the substrate-specific

systems. It was found that the common transport system and the tyrosine-specific transport system were significantly derepressed in their activities when tested in the mutant strain. Thus it appeared that the *tyrR* locus was a negative regulator of these two transport systems. The phenylalanine-specific system was not affected by *tyrR*. It was suggested that tyrosine (not the charged tyrosine tRNA) acted as a corepressor (Bachmann, 1983).

Genetic mutants have been isolated for the common aromatic amino-acid transport system and for the specific (non-inducible) systems (Hiraga *et al.*, 1968; Brown, 1970; Oxender, 1975; Whipp *et al.*, 1976). The common transport system is inactivated by several mutations, all of which reside in the *aroP* gene located at minute 2 on the *E. coli* chromosome (Brown, 1970; Whipp *et al.*, 1980). Mutations in genes for the phenylalanine-specific transport system were located in the *pheP* gene which was found to reside at minute 13 (Whipp and Pittard, 1977; Bachmann, 1983). Mutations that affect tyrosine-specific transport have been placed in the *tyrP* gene which was mapped at minute 42 (Whipp and Pittard, 1977; Kasian and Pittard, 1984).

C. GENE ORGANIZATION AND CHARACTERIZATION

The *tyrP* gene has been cloned on a 2.8 kb insert (Wookey *et al.*, 1984) and identified by genetic selection for *tyr*+. The 2.8 kb insert was mapped using restriction-enzyme digestions in various combinations. The *tyrP* gene was determined to be approximately 1.1 kb in length by transposon-insertion mutagenesis using the transposable element Tn*1000*. Seven different plasmids carrying *tyrP* were transformed in a maxicell strain of *E. coli*. Six of these plasmids contained Tn1000 inserted into the *tyrP* gene thus inactivating it, while the seventh plasmid contained the intact *tyrP* gene. Maxicells were prepared, and their newly expressed proteins were labelled with [^{35}S]methionine and separated by SDS–polyacrylamide-gel electrophoresis. The TyrP product was identified and its molecular weight was estimated to be approximately 24,500. The cellular location of the TyrP protein was determined by sedimentation analysis and was found to be associated with the membrane. It was observed that cells containing multiple copies of the *tyrP* gene exhibited accelerated tyrosine-specific transport, but the system is still regulated by both tyrosine and phenylalanine in the presence of a functional *tyrR* gene product.

In another recent series of studies from Pittard's laboratory, a plasmid was constructed carrying a *tyrP–lac* operon fusion (Kasian and Pittard, 1984). This plasmid was used to isolate *tyrP* promoter mutants exhibiting altered expression from the *tyrP* promoter. In particular, in these mutants, tyrosine repression from the *tyrP* promoter was less effective or else completely eradicated. In addition, in some of the mutants, the presence of phenylalanine

was still able to increase the level of expression from the *tyrP* promoter (as in the wild-type condition). Consequently, the authors suggest from these results that there are two distinct sites in the *tyrP* promoter region which are involved in tyrosine-specific transport regulation. One site would be associated with the repressing effect of the TyrR protein in response to tyrosine, while the other site would be involved in the induction effect of the TyrR protein in response to phenylalanine. Furthermore, it had been observed previously (Whipp and Pittard, 1977) and confirmed using the *tyrP–lac* operon fusion plasmid (Kasian and Pittard, 1984) that, with a wild-type *tyrP* promoter, repression by tyrosine is dominant over induction by phenylalanine. Thus, a model was proposed (Kasian and Pittard, 1984) in which the tyrosine–TyrR complex binds to a particular site in the *tyrP* promoter resulting in attenuation of *tyrP* transcription. Moreover, when the tyrosine–TyrP complex is bound, it may cause the other binding site (for the phenylalanine–TyrR complex) to be inaccessible to its complex, consequently exerting dominance over the phenylalanine–TyrR complex.

D. ENERGETICS

Some preliminary experiments have been undertaken to determine the energy source for tyrosine-specific transport (Wookey *et al.*, 1984; Wookey and Pittard, 1984). The authors assert that this transport system is driven by a proton-motive force primarily because the system is sensitive to the energy uncoupler CCCP as accumulated radioactive tyrosine is rapidly effluxed on the addition of CCCP. However, unlike other transport systems which are energized by a proton-motive force, energization of the tyrosine-specific transport system is not directly mediated by thiol groups. Furthermore, it was shown that the V_{max} values and levels of steady-state accumulation of tyrosine are dependent on the copy number of the *tyrP* gene and, by extrapolation, on the amount of TyrP protein produced. This contrasts with the chemi-osmotic hypothesis which states that the steady-state concentration of substrate should be controlled by the magnitude of the proton-motive force (Mitchell, 1979). Thus, the authors conclude that, although a proton-motive force may drive tyrosine-specific transport, this transport system is not in thermodynamic equilibrium with it. Work is currently underway better to understand the energetics of tyrosine-specific transport.

VII. Other Amino-Acid Transport Systems

The five transport systems already described in this review have been studied extensively, and they exemplify the current level of understanding of amino-

acid transport in prokaryotes. However, there are several other transport systems which have been examined and which will be discussed more briefly.

A. ARGININE AND ORNITHINE TRANSPORT IN *Escherichia coli*

Transport of ornithine and arginine by *E. coli* is an active process and is carried out by two independent transport systems (Celis *et al.*, 1973). The high-affinity system transports lysine, arginine and ornithine (LAO system) and is repressible only by lysine (Rosen, 1971; Celis *et al.*, 1973). The low-affinity system transports arginine and ornithine and is repressible by both amino acids (Celis *et al.*, 1973; Celis, 1981). There are three periplasmic binding proteins associated with these two systems. The binding proteins are distinguished by their substrates: one binds lysine, arginine and ornithine (LAO-binding protein; Rosen, 1971), another binds arginine and ornithine (Celis, 1981), and the third binds only arginine (Rosen, 1971). Regulatory mutants were constructed which demonstrated that regulation of the low-affinity arginine/ornithine transport activity is independent from regulation of arginine biosynthesis (Celis, 1977). Moreover, two mutations affecting synthesis and structure of the arginine/ornithine periplasmic binding protein have been mapped in the region around *argA–serA* at minutes 61–63 on the *E. coli* chromosome (Celis, 1982). Lastly, in a study involving phosphorylation of the arginine/ornithine-binding protein, it was concluded that ATP is a direct energy donor for the low-affinity arginine/ornithine-transport system (Celis, 1984).

B. GLUTAMINE TRANSPORT IN *Escherichia coli*

The glutamine-transport system of *E. coli* is another example of a high-affinity transport system (Weiner and Heppel, 1971; Berger and Heppel, 1973, 1974). The system contains a periplasmic binding protein which is glutamine-specific and which has been very well characterized physically (Weiner and Heppel, 1971; Weiner *et al.*, 1971; Kreishman *et al.*, 1973; Marty *et al.*, 1979). A mutant defective in the binding protein and a regulatory mutant were constructed, and both loci were shown to map to minute 17.7 on the *E. coli* chromosome (Masters and Hong, 1981a). Reconstitution of the glutamine-transport system in sphaeroplasts of *E. coli* showed conclusively that the periplasmic glutamine-binding protein is an essential component of this transport system (Masters and Hong, 1981b).

C. PROLINE TRANSPORT IN *Salmonella typhimurium*

Proline transport has been studied rather thorougly in *S. typhimurium*. One of

the genes responsible for high-affinity proline transport (PP-I) in *S. typhimurium*, *putP*, has been mapped at minute 22 and is adjacent to the gene encoding proline dehydrogenase, *putA* (Ratzkin and Roth, 1978a,b; Menzel and Roth, 1980). Moreover, it appears that the *putA* gene product acts as the repressor for itself as well as for the *putP* gene (Menzel, 1980). The low-affinity proline-transport system (PP-II) in *S. typhimurium* is encoded by the *proP* gene which maps at minute 92 (Anderson *et al.*, 1980; Menzel and Roth, 1980). A third proline-transport system (PP-III) which functions when cells are grown in media containing elevated concentrations of solute has been mapped at the *proU* locus at minute 59 (Csonka, 1982). Regulation of PP-III was examined in mutants and was found to influence transcription from *proU* (Dunlap and Csonka, 1985a,b). Many of the genetic and biochemical characteristics of the proline-transport system of *S. typhimurium* are analogous to those described for *E. coli* (Wood and Zadworny, 1980; Wood, 1981). However, there is evidence that shows that PP-I transport is coupled to Na^+ ions in both *S. typhimurium* and in *E. coli* (Stewart and Booth, 1983; Cairney *et al.*, 1984). These reports are in conflict with those by Anraku and his colleagues (Mogi and Anraku, 1984a,b,c) referred to in Section V of this review.

D. ALANINE TRANSPORT IN THERMOPHILIC BACTERIA

Transport of alanine in the thermophilic bacterial strain PS3 is carried out by a single membrane-associated component (Hirata *et al.*, 1984). In earlier studies, the alanine carrier had been partially purified from membranes (Hirata *et al.*, 1976, 1977). When this preparation was reconstituted into proteoliposomes, active alanine transport was observed to be driven by both a proton gradient and a sodium-ion gradient (Hirata, 1979). Using a more sophisticated purification protocol, the alanine carrier was isolated and was found to be a single polypeptide of molecular weight 42,500. This polypeptide, when reconstituted into proteoliposomes, effected alanine transport in response to a proton gradient as well as to a sodium-ion gradient (Hirata *et al.*, 1984).

VII. Concluding Remarks

We have presented in this review current information available on the molecular biology of bacterial amino-acid transport. Some elegant experiments have been carried out with the result that we now have a better understanding of the mechanisms and the regulation of amino-acid transport. Future studies in this field should lead to a thorough understanding of the interrelation of the factors involved in amino-acid transport in bacteria.

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Regulation of Carbon Metabolism in *Saccharomyces cerevisiae* and Related Yeasts

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I. Introduction

Metabolism of growing yeasts depends on the yeast strain, the carbon source and the physicochemical factors in the environment. In a defined cultivation system, different types of yeasts exhibit a distinct growth pattern (Fiechter *et al.*, 1981). All yeast species studied so far are able to use glucose as the sole source of carbon and energy (Barnett, 1976). Therefore utilization of glucose by different yeasts has been studied most extensively.

This review is dedicated to Professor Armin Fiechter on the occasion of his 60th birthday.

On the basis of the predominant carbon-containing end products of glucose catabolism, fermentation (ethanol and carbon dioxide) and respiration (carbon dioxide) have been distinguished. The main factors affecting the nature of glucose metabolism are glucose itself and oxygen. Yeasts may be grouped according to their glucose metabolism under carbon and oxygen limitations as follows: (1) purely respirative yeasts (e.g. *Trichosporon cutaneum*; Käppeli and Fiechter, 1982) not showing ethanol formation even under oxygen limitation; (2) oxygen-sensitive yeasts (e.g. *Candida tropicalis*; Fiechter *et al.*, 1981) which produce ethanol under oxygen limitation; and (3) glucose-sensitive yeasts exhibiting aerobic ethanol production in the presence of excess glucose (e.g. *Saccharomyces cerevisiae*; Fiechter *et al.*, 1981). Without any oxygen, only little more than half of the yeast species studied were able to grow on glucose (229 out of 434; Barnett, 1976).

Saccharomyces cerevisiae is possibly the most widely investigated yeast. This is a consequence not only of its importance in industry but also of its distinct glucose metabolism. It has attracted the curiosity of many scientists who investigated this yeast as a model eukaryote. From these investigations hypothetical explanations of aerobic ethanol formation and its regulation emerged.

It is the aim of this article to discuss the knowledge that resulted from early investigations on the regulation of glucose metabolism in *Sacch. cerevisiae* and related yeasts in view of more recent data. I hope that this venture will contribute to a better understanding of the subject and stimulate further research which will proceed from new and more accurately defined positions.

II. Physiology of Growth

A. AEROBIC BATCH CULTURE

In 1954, one of the first qualitative studies of aerobic growth of baker's yeast was undertaken by Lemoigne and his coworkers who showed a diauxic growth on glucose. Later on, this growth behaviour in an aerobic batch culture was confirmed by Beck and von Meyenburg (1968) with a well-documented investigation. As schematically represented in Fig. 1, it is characterized by two sequential biomass increases. In a first phase, biomass is formed and ethanol is accumulated at the expense of glucose. In the second growth phase, which begins after glucose is completely exhausted, ethanol serves as substrate for further growth. This growth pattern is characteristic for yeasts which are the subject of this article. Formation of ethanol in aerobic batch cultures is one of the main characteristics of these yeasts.

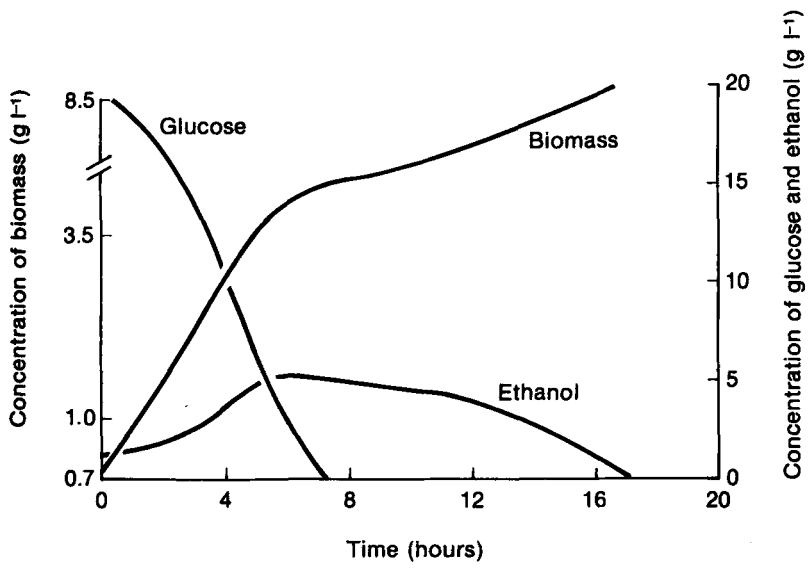

FIG. 1. Time-course of aerobic growth of *Saccharomyces cerevisiae* and related yeasts in batch culture. Growth is characterized by two subsequent growth phases (diauxie). In the first phase, glucose is consumed and biomass and ethanol formed. The latter is subsequently used in the second growth phase.

B. AEROBIC CONTINUOUS CULTURE

When biomass formation by *Sacch. cerevisiae* and related yeasts (Beck and von Meyenburg, 1968; Petrik *et al.*, 1983) is recorded as a function of dilution rate in a continuous culture, two metabolically different regions can be distinguished (Fig. 2). In the first range of low dilution rates, breakdown of glucose occurs oxidatively. No other carbon-containing products other than biomass and carbon dioxide are formed in appreciable amounts. Accordingly, the yield of biomass is high (approximately 0.5 g dry cell weight (g of glucose^{-1})).

When the dilution rate is successively increased, a particular yeast-specific value is reached above which ethanol is produced by the cells. Owing to formation of the energy-rich product, ethanol, the yield of biomass drops to approximately 0.15 g dry cell weight (g of glucose)$^{-1}$. This yield is comparable to that found in the first phase of a batch culture. Hence, growth of these yeasts at high dilution rates resembles that of the first phase in the batch culture.

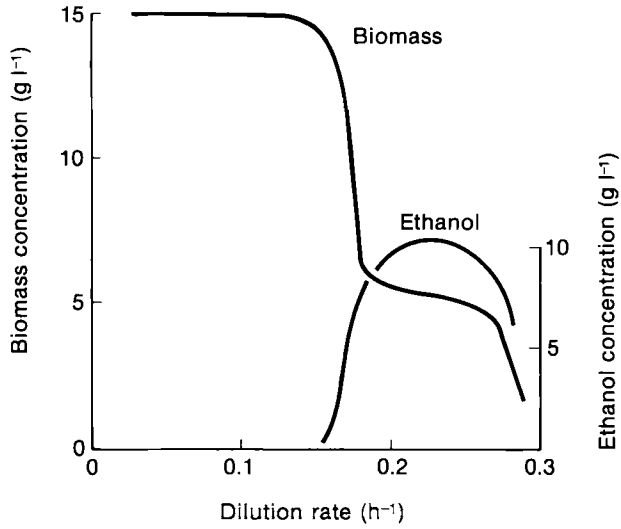

FIG. 2. Effect of dilution rate on aerobic growth of *Saccharomyces cerevisiae* and related yeasts in continuous culture (glucose concentration in feed, 30 g l^{-1}). Growth was controlled by two ranges of dilution rates with distinct glucose metabolism. At low dilution rates, high steady-state biomass yields and lack of ethanol formation were observed. At a species-specific dilution rate ethanol formation began as indicated by accumulation of ethanol in the culture liquid and decreased biomass concentrations. This shape of the biomass–dilution rate curve is characteristic of *Saccharomyces*-type yeasts, with the dilution rate at which aerobic ethanol formation was first obtained being the only parameter to change depending on the yeast species used.

C. ANAEROBIC GROWTH

Yeasts of the *Sacch. cerevisiae* type belong to that group of yeasts which are able to grow anaerobically. Since molecular oxygen is required for synthesis of ergosterol and unsaturated fatty acids (Hunter and Rose, 1971), these compounds need to be added as medium supplements in order to promote anaerobic growth (Fiechter *et al.*, 1981).

Under anaerobic growth conditions, energy originates exclusively from glycolysis since respiration is impossible in the absence of oxygen. Pyruvate is decarboxylated to acetaldehyde which serves as acceptor for reducing equivalents with concomitant reduction to ethanol. A linear relationship between growth rate and ethanol production rate is therefore observed in anaerobic continuous cultures (Fig. 3). Alternatively, glyceraldehyde may function as an electron acceptor yielding glycerol as an end product of energy metabolism. In fact, glycerol is a by-product which is formed in appreciable amounts in anaerobic yeast cultures (Fiechter *et al.*, 1981).

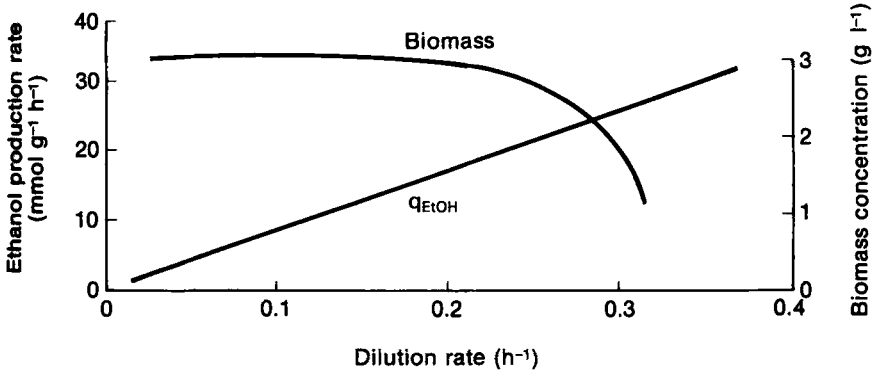

FIG. 3. Effect of dilution rate on anaerobic growth of *Saccharomyces cerevisiae* and related yeasts in continuous culture (glucose concentration in feed, 30 g l^{-1}). Energy generation is purely glycolytic which is expressed as a linear increase in ethanol production rate (q_{EtOH}) with increasing dilution rate. The parameter that is specific for the yeast strain used is the maximum obtainable anaerobic dilution rate.

Biomass yield in anaerobic cultures growing on glucose is considerably lower than in aerobic ones. It amounts to approximately 0.1 g dry cell weight (g of glucose)$^{-1}$, which is lower than in a culture that exhibits aerobic ethanol formation. Likewise, maximum specific growth rates observed in anaerobic cultures are lower than in aerobic ones. As an example, the corresponding values for *Sacch. cerevisiae* H1022 (ATCC 32167) are given in Table 1.

III. Molecular Basis of Growth

The different pathways for glucose breakdown exhibited by *Sacch. cerevisiae* and related yeasts as outlined in the previous section of this article have raised

TABLE 1. Maximum specific growth rates and biomass yields recorded for *Saccharomyces cerevisiae* H1022 grown under different conditions. The values were measured in chemostat cultures. From Fiechter *et al.* (1981) and Rieger *et al.* (1983)

	Respirative growth	Anaerobc growth	Aerobic growth with ethanol formation
μ_{max} (h^{-1})	0.3	0.35	0.45
Y (−)	0.5	0.1	0.17

the question of the molecular basis for the observed flexibility. Problems of adaptation to changes in environmental conditions have been studied for many years. For example, readjustment of carbohydrate metabolism with the transition from aerobic to anaerobic conditions (and vice versa), the so-called "Pasteur effect", has repeatedly attracted the interest of biochemists. A certain similarity in the metabolism of aerobically ethanol-forming yeasts and that of tumour cells as described by Crabtree (1929), further stimulated yeast research. It was assumed that basic mechanisms of adaptation function in micro-organisms just as in higher living forms. However, they may be investigated in micro-organisms without the imposition of hormonal and nervous mechanisms (Krebs, 1956).

A. INITIAL OBSERVATIONS: PASTEUR, CRABTREE AND GLUCOSE EFFECTS

If an organism is able to metabolize sugars under aerobic as well as anaerobic conditions, anaerobically by using an organic electron acceptor (fermentation) and aerobically by both a fermentation and a respiratory process, the fermentation process is in general less active under aerobic than under anaerobic conditions. The decreasing efficiency of fermentation in the presence of air is called the "Pasteur effect" (Warburg, 1926; Burk, 1939). It is the oxygen that, via respiration, is responsible for inhibition of fermentation as initially described by Pasteur (1861).

The Pasteur effect is an expression of the close interrelation of the two dissimilation processes mentioned. Both follow the reactions of glycolysis, pyruvate being the stage where the pathways part. In fermentation, pyruvate is decarboxylated and the acetaldehyde formed is used as an organic electron acceptor and reduced to ethanol. During respiration, pyruvate is oxidatively decarboxylated and coupled to coenzyme A, thus entering the tricarboxylic acid cycle and undergoing endoxidation.

The Pasteur reaction, acting as a brake on dissimilation of sugars, limits aerobic ethanol formation but in most cases does not eliminate it completely as will be shown in detail in the next section. Although, in general, the Pasteur effect is more pronounced with increasing respiratory activity, there is no quantitative relation between the two. Nor is there a completely satisfactory hypothesis to explain how the Pasteur reaction is regulated (Johnson, 1941; Lynen, 1941; Lynen and Koenigsberger, 1951; Lynen *et al.*, 1959; Sols *et al.*, 1971; Holzer, 1959). The reaction catalysed by phosphofructokinase has been identified as the main regulatory step (Sols *et al.*, 1971). It is noteworthy that the regulation is based exclusively on allosteric feedback mechanisms, with citrate and ATP acting as inhibitors and ammonium ions, ADP and AMP being activators of the reaction. With this regulation concept, a reasonable explanation of the Pasteur effect was possible.

In 1929, Crabtree published his results on carbohydrate metabolism of tumour cells. In his article, he made two important statements. First, that respiration is ineffective in checking glycolysis and second that glycolytic activity of tumour cells exerts an inhibitory effect on their respiration. These conclusions were derived from measurements of the glycolytic and respiration rates of tumour cells.

In 1966, De Deken carried out analogous experiments with growing *Sacch. cerevisiae*, as did Crabtree (1929) with tumour cells. In essence, De Deken (1966) found that the Crabtree effect is also active in yeasts. Glucose and fructose degradation were found to proceed mainly via aerobic fermentation. Only when galactose or mannose was used as a carbon and energy source did fermentation and respiration occur simultaneously. It was concluded that the Crabtree effect in yeasts actually consists of a repression of an energy source (respiration) by another energy source (fermentation).

At this point, it should be added that the observations related to the Crabtree effect contradict those on the Pasteur effect. When the transition from anaerobiosis to aerobiosis was considered, an inhibition of glycolysis by respiration was postulated whereas the Crabtree effect describes an inhibition of respiration by excess glycolytic rates.

In parallel with the measurements of overall metabolic rates in yeasts, enzymic studies were carried out. In the presence of glucose, remarkably lower activities of enzymes of the tricarboxylic acid cycle (Polakis *et al.*, 1965), the glyoxylate shunt (Barnett and Kornberg, 1960) and the respiratory chain (Polakis *et al.*, 1965) were observed as compared to cells growing on ethanol. Additional enzyme systems were found to be less active when glucose served as the carbon source for the cells (gluconeogenic enzymes (Gancedo *et al.*, 1965; Witt *et al.*, 1966; Gancedo and Schwerzmann, 1976), α-glucosidases (Wijk *et al.*, 1969) and invertase (β-fructofuranosidase; Gascon *et al.*, 1968)). By analogy with a comparable phenomenon in bacteria, the term catabolite repression, introduced by Magasanik (1961), was used for the effect of glucose in yeasts, although the mechanism is generally assumed to be different from the cyclic AMP (adenosine 3′,5′-monophosphate)-mediated process in *Escherichia coli* (de Crombrugghe and Pastan, 1978).

As far as the metabolite-triggering repression is concerned, no glycolytic intermediate could be identified serving this function. In 1967, Holzer stated that only glucose itself, glucose 6-phosphate, ATP, ADP and orthophosphate come under consideration as possible triggers of repression. Since the presence of glucose was essential, the phenomenon was called the "glucose effect". Although Holzer (1967) pointed out that the glucose effect may consist of a variety of different repression effects, repression of respiratory enzymes by glucose has come to characterize the Crabtree effect in yeasts. The mechanism of regulation generally accepted as the basis of the Crabtree effect is depicted

in Fig. 4. The terms glucose effect and Crabtree effect are often used as synonyms.

B. NEW ASPECTS OF REGULATION GAINED BY MEANS OF IMPROVED CULTIVATION TECHNIQUES

If the regulation mechanism outlined above is correct, it should be comprehensive and explain all types of metabolism observable when yeast cells are cultivated with glucose as the carbon source. The first metabolic state contradictory to this claim was observed at low dilution rates in a continuous culture. At low substrate-feed rates, glucose is degraded oxidatively as depicted in Fig. 2. It follows that the presence of glucose *per se* is not the primary cause of aerobic ethanol formation and enzyme repression supposed to be the underlying regulatory mechanism. Beck and von Meyenburg (1968) concluded that not the presence of glucose but the rate of glucose consumption has a regulatory effect on enzyme activities.

Furthermore, a detailed quantitative analysis of the overall metabolism of growing yeast cultures indicated that respiration is not repressed completely by glucose. A significant oxygen uptake by cells is measured both in the first phase of batch culture as well as in the corresponding metabolic state at high dilution rates in continuous culture (Fig. 5).

It follows that there is a branched glucose breakdown when cells exhibit aerobic ethanol formation. Depending on the terminal electron acceptors, part of the glucose is catabolized respiratively (electron acceptor oxygen) and part fermentatively (electron acceptor acetaldehyde). Consequently, the type of metabolism corresponding to aerobic ethanol formation is respiro-fermentative. Taking into account the growth patterns of *Sacch. cerevisiae* and related yeasts in aerobic and anaerobic batch and continuous cultures, the types of glucose catabolism are summarized in Table 2.

C. PHYSIOLOGICAL CHARACTERISTICS OF CELLS EXHIBITING DIFFERENT TYPES OF GLUCOSE METABOLISM

The different types of metabolism in yeasts utilizing glucose as the carbon source suggest that there are significant differences in the physiology of the cells. All studies originating from batch cultures compared respiro-fermentatively growing cells, growing for example on ethanol. From a comparison of these two physiological states, most data and the concept of the glucose effect were derived (Goerts, 1967; Polakis and Bartley, 1965; Polakis *et al.*, 1965; Witt *et al.*, 1966). Inhibition of formation of respiratory, tricarboxylic acid cycle and glyoxylate bypass enzymes, already referred to, was observed when glucose was the carbon and energy source. Carbohydrate substrates which

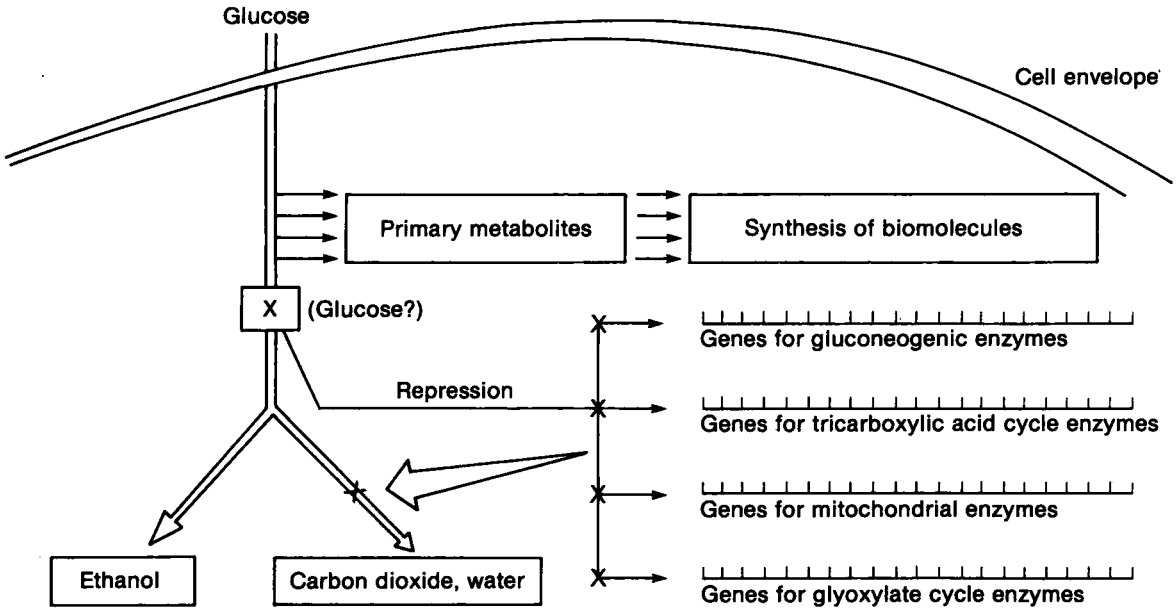

FIG. 4. Diagram showing glucose repression as a regulatory mechanism to explain aerobic ethanol formation by yeasts. In the early stages, a catabolite (or eventually glucose itself) is formed which triggers repression of several enzymes, mainly of the oxidative branch of catabolism. As a consequence, respiration is cut off and ethanol formation serves as the alternative pathway for energy generation and re-oxidation of reducing equivalents.

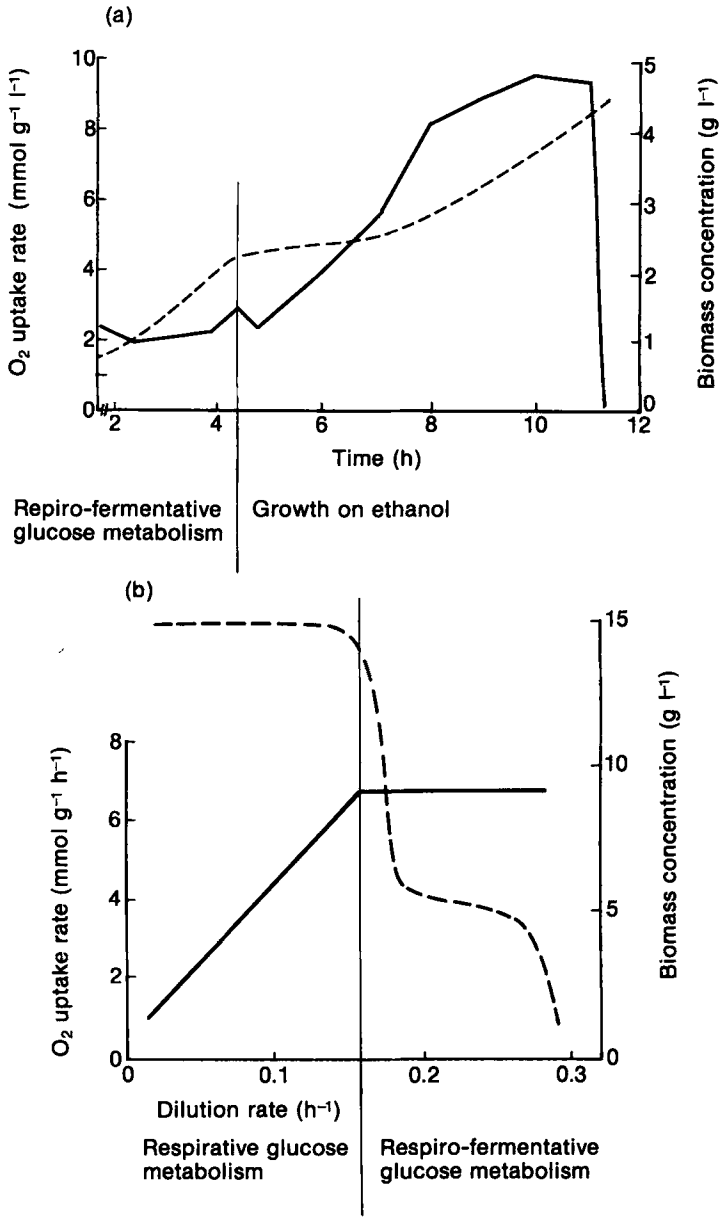

FIG. 5. Oxygen uptake by *Saccharomyces cerevisiae* exhibiting aerobic ethanol formation. (a) Time-course of oxygen uptake in batch culture. During growth on glucose, oxygen uptake rates (——) were substantial. (b) Oxygen uptake rates as function of dilution rate in continuous culture. In the range of dilution rates, where ethanol was formed by cells, the oxygen uptake remained at the maximum rate reached for oxidative glucose metabolism. The parameter which changes with different yeast species is the maximum oxygen uptake rate. – – – –, biomass formation under the corresponding conditions.

TABLE 2. Types of glucose utilization by *Saccharomyces cerevisiae* and related yeasts in aerobic and anaerobic batch and continuous cultures

Conditions		Type of glucose catabolism
Batch culture		
Aerobic	growth phase 1	Respiro-fermentative[a]
	growth phase 2	Assimilation of ethanol
Anaerobic		Fermentative
Continuous culture		
Aerobic	low dilution rates	Respirative[b]
	high dilution rates	Respiro-fermentative
Anaerobic		Fermentative

[a] Alternatively the term 'oxidoreductive' was used (Käppeli *et al.*, 1985b). Respiro-fermentative is, however, the more accurate term.

[b] Alternatively, the term oxidative may be used.

supported growth at lower specific growth rates, e.g. maltose or galactose, caused a less marked inhibition (Goerts, 1967; Polakis and Bartley, 1965; Polakis *et al.*, 1965). Derepression of the same enzymes was observed when the culture was growing at the expense of the ethanol accumulated during the first growth phase.

Using continuous cultures (chemostats), different growth rates with populations of *Sacch. cerevisiae* were maintained and respirative glucose metabolism was observed (Beck and von Meyenburg, 1968). In a batch culture, it is not observed and therefore could not be analysed. Actually, in batch culture, it represents a transitionary state only at the end of the first growth phase near the point of glucose exhaustion.

Enzyme activities on respirative pathways were significantly lower in cells exhibiting respiro-fermentative glucose metabolism as compared with cells growing respiratively. In cells with oxidative glucose breakdown, activities were comparable to those of cells growing on ethanol.

Käppeli *et al.* (1985b) further characterized the physiology of respiratively growing *Sacch. cerevisiae*. Since these cells were able to utilize ethanol as a cosubstrate with glucose, the activities of key gluconeogenic enzymes were recorded. It was found that they exhibited significant activities. With regard to the activities of these enzymes, as well as that of malate dehydrogenase and mitochondrial cytochrome content, the physiology of cells respiratively degrading glucose was distinguishable from that of cells showing respiro-fermentative glucose metabolism and that of ethanol-grown cells. The activities of the measured enzymes and the cytochrome contents showed

intermediate levels with those of ethanol-grown cells forming the upper, and respiro-fermentative cells forming the lower limits (Table 3).

Enzyme activities and cytochrome contents were relatively closely dependent on growth rate. As an example, steady-state cytochrome contents of *Sacch. uvarum* growing at different growth rates are given in Table 4. From these and other data (Petrik *et al.*, 1983), it becomes clear that enzyme expression not only depends on the type of cellular metabolism but also on growth rate. Furthermore, seemingly unnecessary enzyme activities, as represented here by gluconeogenic enzymes, are expressed under appropriate growth conditions.

TABLE 3. Comparison of mitochondrial cytochrome contents, and activities of fructose 1,6,-bisphosphatase (FBPase), phospho*enol*pyruvate carboxykinase (PEPCK) and malate dehydrogenase (MDH) of *Saccharomyces cerevisiae* utilizing glucose respiratively ($D = 0.10\ h^{-1}$), respiro-fermentatively ($D\ 0.35\ h^{-1}$) or growing at the expense of ethanol ($D = 0.10\ h^{-1}$). From Käppeli *et al.* (1985a)

	Cytochrome content (pmol (mg dry wt)$^{-1}$)			Enzyme activities (nmol substrate consumed (mg protein)$^{-1}$ min^{-1})		
Substrates	Cyt *a*	Cyt *b*	Cyt *c*	MDH	PEPCK	FBPase
Glucose ($D = 0.1\ h^{-1}$)	45	41	119	2700	19	7
Glucose ($D = 0.35\ h^{-1}$)	24	45	97	740	6	4
Ethanol ($D = 0.1\ h^{-1}$)	46	56	143	16600	672	10

TABLE 4. Dependence of cytochrome contents on growth rate during respirative and respiro-fermentative glucose metabolism of *Saccharomyces uvarum*. From Käppeli *et al.* (1985a)

	Cytochrome content (pmol(mg dry wt.)$^{-1}$)		
Glucose metabolism	Cyt *a*	Cyt *b*	Cyt *c*
Respirative:			
$D = 0.05\ h^{-1}$	30	60	150
$D = 0.15\ h^{-1}$	50	100	310
Respiro-fermentative			
$D = 0.17\ h^{-1}$	20	65	170
$d = 0.25\ h^{-1}$	10	50	120

Another unique property of respiratively growing *Sacch. cerevisiae* and related yeasts is spontaneous synchronization of growth (Käppeli *et al.*, 1985b). Whereas von Meyenburg (1969) needed to initiate synchronous growth by small shifts in dilution rate, no such measures were needed by Käppeli and his colleagues. Synchronization of the culture occurred without any obvious triggering when a continuous culture was started after a batch culture.

Metabolism during the cell cycle exhibits some important features. First ethanol formation always takes place when cells enter a reproduction phase. It coincides with mobilization of the reserve carbohydrates, trehalose and glycogen (Küenzi and Fiechter, 1969). This mobilization represents the action of an internal glucose pulse which leads to short-term ethanol formation. In asynchronously growing cultures, ethanol is not detected during oxidative glucose metabolism. Ethanol does not accumulate because of the different phases of the division cycle the cells are in. Second, gluconeogenic enzyme activities oscillate during the cell cycle. When the reproduction phase begins, activities of these enzymes increase (Käppeli *et al.*, 1985b). It seems that the preceding ethanol formation derepresses synthesis of gluconeogenic enzymes. Third, cytochrome contents did not oscillate. This indicates that different regulatory mechanisms occur for gluconeogenic enzymes and cytochromes.

As far as the basis for self-synchronization is concerned, no mechanism has been established so far. It could be that short-term ethanol formation represents the signal for synchronization. This notion is supported by the fact that spontaneous synchronization is mainly observed with yeasts that exhibit a *Sacch. cerevisiae* type of glucose metabolism. Furthermore, synchronization is related to cells that utilize glucose respiratively, i.e. under conditions where ethanol formation is limited to a short period of time and represents a continuously returning signal. The involvement of an extracellular component may also be deduced from the observation that oscillation is best achieved in high-quality equipment that produces a well-mixed and homogeneous culture (Meyer and Beyeler, 1984).

This brief summary of available knowledge on the physiology of *Sacch. cerevisiae* and related yeasts indicates the flexibility of these yeasts in response to changing growth conditions. Further analysis will provide more insight into the regulation of enzyme expression in yeasts, especially when the advantages of synchronous cultures are fully exploited.

D. A LIMITED RESPIRATORY CAPACITY AS THE BASIS FOR RESPIRO-FERMENTATIVE GLUCOSE METABOLISM

Whereas the transition from respiro-fermentative to purely fermentative glucose metabolism is governed by the availability of oxygen, occurrence of

respirative glucose breakdown is less obvious. Since it can be represented in feed-controlled cultivation systems only (fed batch or continuous culture) it was observed only recently, i.e. long after the repression/derepression mechanism as an explanation of aerobic ethanol formation emerged. As a consequence, physiological states were obtained in continuous culture which could not be explained satisfactorily as already discussed.

For the first time, Beck and von Meyenburg (1968) established a dynamic view of regulation in that they indicated that glucose repression depended on metabolic rates and not solely on the presence or absence of glucose. But, they also concluded that repression of respiratory pathways led to respiro-fermentative glucose breakdown. Glucose repression was a regulatory concept that was mainly deduced from enzymic studies based on batch cultures and steady-state analyses in continuous cultures.

Barford and Hall (1979) quantified the control of glycolysis and respiration in a strain of *Sacch. cerevisiae* in continuous culture. They did not find repression of respiration when the cells exhibited respiro-fermentative glucose metabolism. A maximum value for the respiration rate was reached independent of the nature of substrate (glucose or galactose). To achieve maximum respiratory capacity, a considerable adaptation was necessary particularly during batch growth.

Rieger *et al.* (1983) hypothesized that the occurrence of respiro-fermentative glucose metabolism is a consequence of a limited respiratory capacity in cells. This was concluded from continuous-culture experiments with glucose alone and glucose together with ethanol as substrates. Since, under all conditions, maximum oxygen uptake rate was reached and the ethanol co-consumption rate was governed by the limiting respiration rate, it was concluded that a step in the oxidative branch of catabolism is responsible for incomplete oxidation of glucose at high growth rates or in the presence of excess glucose.

The hypothesis put forward by Rieger *et al.* (1983) was substantiated by Petrik *et al.* (1983). By applying pulse and shift techniques in continuous yeast cultures, it was demonstrated that the primary regulatory process leading to ethanol formation is not a repression of oxidative pathways. Pulsing excess glucose into continuous cultures of yeast oxidatively degrading glucose induced respiro-fermentative glucose metabolism with ethanol formation before a decrease in respiration rate or mitochondrial cytochrome content (Fig. 6). As pyruvate represents the metabolite where fermentative and respirative degradation pathways of glucose separate, it was concluded that ethanol formation is primarily due to an overflow reaction on the pyruvate level when oxidative pathways are saturated. By such a mechanism the formation of ethanol and acetate can be explained. It is also consistent with kinetic data available for the two enzymes standing at the beginning of the two

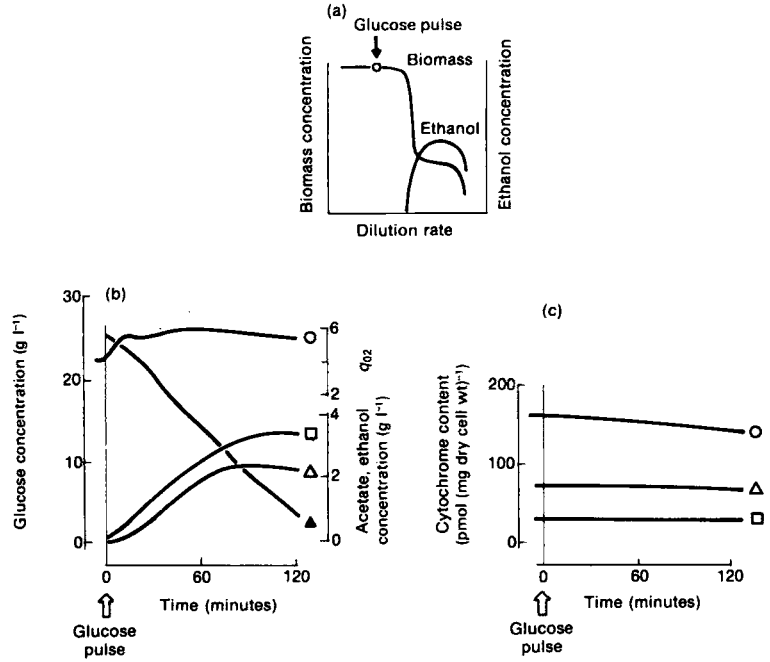

FIG. 6. The effect of glucose pulse to a respiring *Saccharomyces uvarum* culture. For orientation see (a). (b) Response of overall culture parameters to the glucose pulse. Glucose (▲) is taken up immediately before any decrease of oxygen uptake rate (○, q_{O_2} in mmol $g^{-1}h^{-1}$) is manifest. Ethanol (□) and in the early stages of the experiment acetate (△) accumulate. (c) Response of mitochondrial cytochrome content to a glucose pulse. There is little short-term change in the contents of cytochrome *a* (□), cytochrome *b* (△) and cytochrome *c* (○). From Petrik *et al.* (1983).

metabolic routes, pyruvate dehydrogenase for pyruvate oxidation and pyruvate decarboxylase leading to ethanol production. The K_m value for pyruvate dehydrogenase is approximately ten times lower than that for pyruvate decarboxylase (Holzer and Goedde, 1957) indicating that the oxidative pathway is the preferential one.

Long-term adaptation of cells to respiro-fermentative glucose metabolism was investigated by a shift in dilution rate from the range of respirative to respiro-fermentative growth (Fig. 7). An adaptation of the cellular physiology to respiro-fermentative glucose metabolism takes place within 24 to 48 hours as manifested by a decrease of mitochondrial cytochrome content.

These results were interpreted to mean that the occurrence of respiro-fermentative glucose metabolism in yeasts and the subsequent physiological adaptation are due to two independent mechanisms. There is a short-term

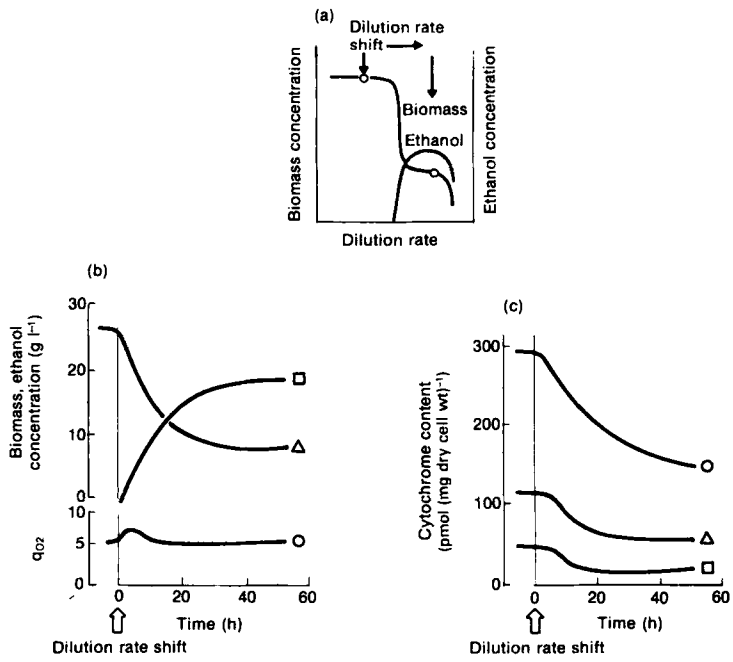

FIG. 7. Effect of a dilution rate shift in a continuous culture of *Saccharomyces uvarum* from the range of respirative to that of respiro-fermentative glucose metabolism. For orientation, see (a). (b) Response of the overall culture parameters biomass (△) and ethanol (□) concentrations and the oxygen uptake rate (○, q_{O2}, in mmol g^{-1} h^{-1}). (c) Response of mitochondrial cytochrome content to the dilution rate shift. Cytochrome *a* (□), cytochrome *b* (△) and cytochrome *c* (○) decreases indicate an adaptation of the cells to respiro-fermentative glucose metabolism. From Petrik *et al.* (1983).

regulation yielding ethanol, which is primarily based on a limited oxidative capacity of the cells, leading to an overflow reaction on the level of pyruvate as the glycolytic flux exceeds the maximum possible respiratory flux. Long-term adaptation of cellular functions (including enzyme activities of respiratory pathways and cytochrome contents) occurs when the cells are kept under conditions of respiro-fermentative glucose metabolism. It has to be assumed that this type of catabolism affects the activities of the susceptible pathways by mechanisms presently unknown.

A new regulatory concept derived from the data reviewed is depicted in Fig. 8. Comparing it to the regulation mechanism based on glucose repression (Fig. 4), it becomes obvious that first, catabolite repression of the respiratory pathways as the basis for aerobic ethanol formation is abandoned and replaced by the overflow reaction resulting from a limited respiratory capacity

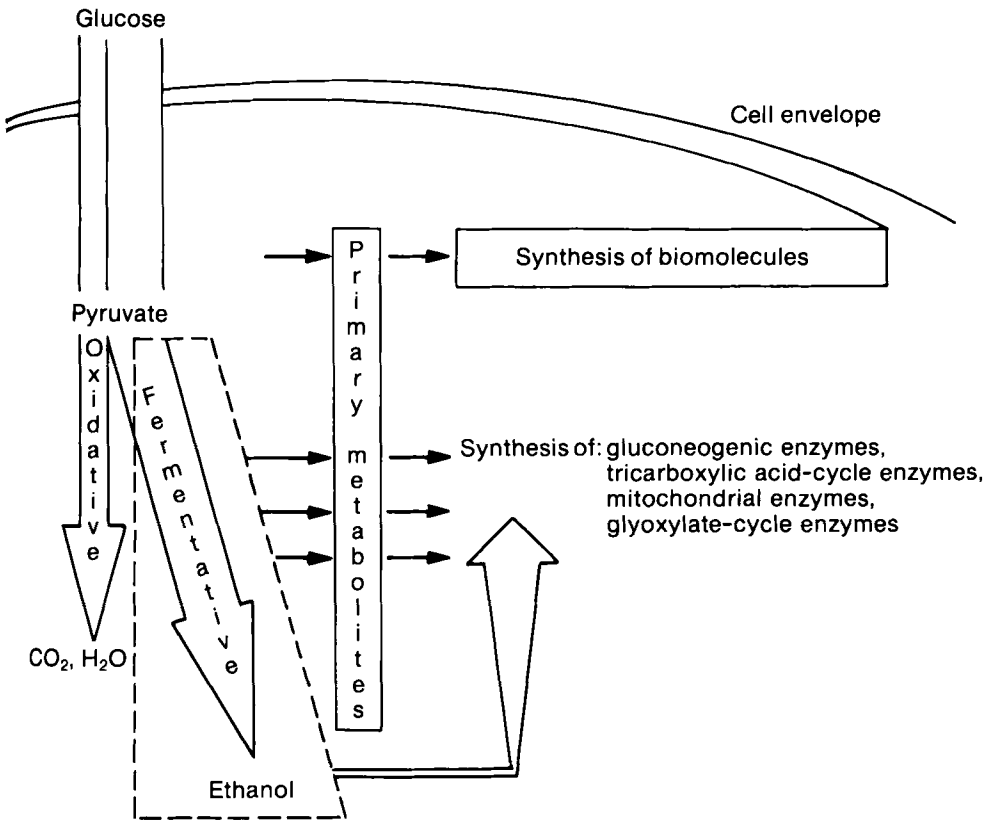

FIG. 8. Diagram showing regulation of glucose metabolism in *Saccharomyces cerevisiae* and related yeasts based on the concept of a limited respiratory capacity. A species-specific glucose flux is degraded oxidatively. When the respiratory capacity is saturated, ethanol formation begins. Respiro-fermentative metabolism affects synthesis of enzymes by unknown mechanisms. Activities characteristic of this type of metabolism result.

of the cells and second, that long-term adaptation of cellular physiology to respiro-fermentative glucose metabolism is based on effects of the fermentative branch of catabolism. Glucose or catabolite repression may account for regulation of certain enzymes but it is hardly the only regulatory mechanism involved. Therefore, an investigation of the influence of aspects that hitherto have received little attention needs to be promoted. There is for example ethanol, the end product of the fermentative branch of catabolism. It is well known that ethanol has an inhibitory effect on growth or fermentation rate itself (Aiba *et al.*, 1968). It has to be assumed that ethanol also affects other

cellular functions including mitochondrial ones. Other possible effectors whose cellular concentration or rate of production change significantly with onset of respiro-fermentative metabolism are NADH (Fig. 9) and carbon dioxide (Käppeli *et al.*, 1985b). Changes in NADH concentration may shift the intracellular redox balance and therefore contribute to cellular regulation. Effects of carbon dioxide on yeast metabolism, particularly growth processes, have been described (Jones and Greenfield, 1982). Regulatory influences on yeast function by all of these metabolites may well account for the observed long-term adaptation of yeasts to respiro-fermentative glucose metabolism. A comprehensive quantification of the effect of any of the above-mentioned metabolites is, however, not available at present. Nevertheless, a global decrease in the activities of respiratory, gluconeogenic and other enzymes in the presence of excess glucose or high metabolic turnover rates is to be attributed to its appropriate causes.

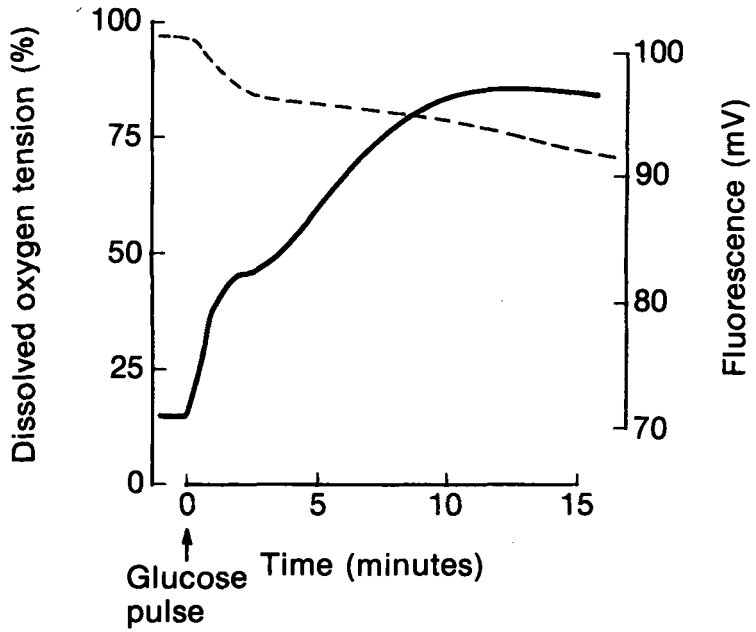

FIG. 9. Response of NADH pool concentration to a glucose pulse as recorded by a NADH-specific fluorescence probe (Beyeler *et al.*, 1981). Glucose was pulsed to respiratively growing cells (as in Fig. 6) and the signal recorded directly from the bioreactor. —, fluorescence; – –, dissolved oxygen tension.

E. CRABTREE AND PASTEUR EFFECTS IN VIEW OF THE LIMITED RESPIRATORY CAPACITY REGULATION CONCEPT

If limited respiratory capacity exerts the main regulatory function determining the type of glucose metabolism of cells, the phenomena referred to as Crabtree and Pasteur effects should be explainable adequately by this concept.

The Crabtree effect was defined as an inhibition of respiration by glycolytic activity of cells (Crabtree, 1929; De Deken, 1966). Inhibition of respiratory pathways is observed as outlined in the preceding section (p. 197). Steady-state oxygen uptake rates of cells growing respiro-fermentatively in continuous culture remained, however, at their maximum values observed at the onset of this type of glucose metabolism (Barford and Hall, 1979; Rieger *et al.*, 1983; Petrik *et al.*, 1983). Whether or not maximum respiratory activity is maintained under respiro-fermentative growth conditions may depend on the adaptation procedure (Barford and Hall, 1979), glucose concentration and consequently metabolite concentrations and the specific rates of product formation. Although it has to be accepted that respiro-fermentative glucose metabolism needs further research for a full explanation of this particular physiological state, the assumption that glycolytic activity as such affects respiration is rather unlikely.

Energy generation by respiration represents a significant part of the overall energy requirement for respiro-fermentative growth. By comparing yield coefficients and maximum growth rates of respiro-fermentatively growing cells with cells growing fermentatively (anaerobic growth or petite mutants) this becomes obvious (see Table 1). For petite mutants (respiration-defective mutants), the yield was similar to that of anaerobically growing cells (Schatzmann, 1975), whereas the maximum specific growth rate was even lower ($0.15\ h^{-1}$) for the petite mutant.

It follows that respiration is never completely absent during aerobic glucose metabolism of wild-type *Sacch. cerevisiae* and related yeasts. As already pointed out, the traditional conclusions of Crabtree (1929) do not adequately describe respiro-fermentative glucose metabolism. It is therefore suggested that the terminology introduced here be adopted (see Table 2) for describing the corresponding types of glucose metabolism.

That the fermentative branch of metabolism unmistakably has an influence on cellular physiology can also be derived from data of Rieger (1983). In a chemostat culture, respiro-fermentative growth can be initiated before maximum oxidative capacity is reached by applying dilution-rate shifts (Fig. 10). It was found that cells continue to metabolize glucose respiro-fermentatively when this type of metabolism is triggered at dilution rates close to the maximum respirative one. Although the cells should theoretically exhibit respirative growth, glucose is used respiro-fermentatively even after an adaptation of 24 hours. It seems that the physiology of the cells is irreversibly

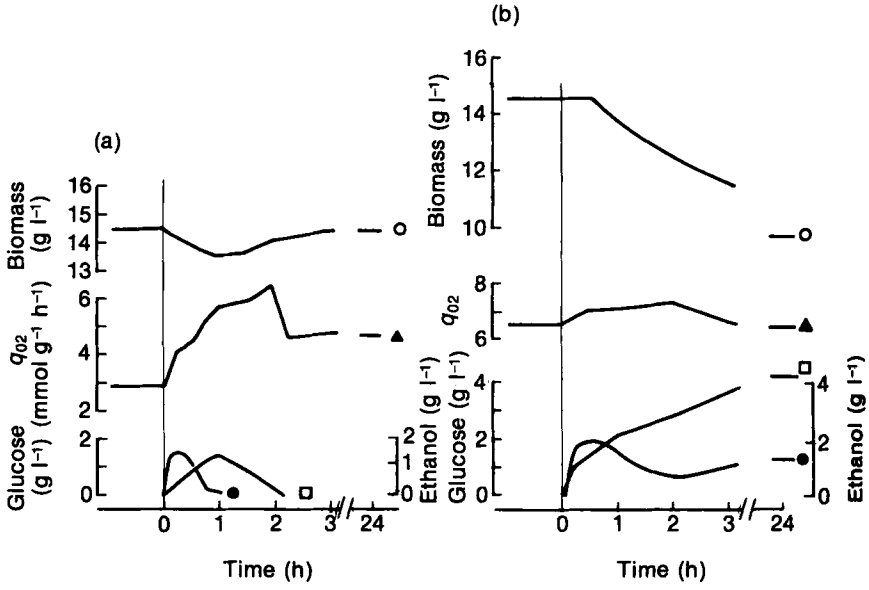

FIG. 10. Dilution rate shifts in a continuous culture of *Saccharomyces cerevisiae* initiated at two different starting dilution rates: (a) 0.10 h^{-1}, (b) 0.22 h^{-1}. The dilution-rate increment was 0.05 h^{-1}. In both experiments, ethanol formation was triggered. At the lower dilution rate, respirative glucose metabolism, was soon restored. At the higher dilution rate, the cells did not return to respirative glucose breakdown although the theoretical maximum capacity was not reached. ○ indicates biomass, ▲ specific oxygen uptake rate, ● residual glucose concentration, and □ ethanol concentration.

changed by the fermentative branch of metabolism. Respirative breakdown of glucose can only be recovered when the dilution rate is lowered to approximately below 0.20 h^{-1} for the yeast used in this study.

In the established concept of a limited respiratory capacity, saturation of respiration is the primary cause for onset of respiro-fermentative glucose metabolism. Since oxygen represents the final electron acceptor of respiration, its action can be regulated by varying the degrees of oxygen availability. Such experiments are of importance in evaluating the validity of the concept and an assessment of the general influence of oxygen on cellular physiology. Early studies by Pasteur (1861) lead to formulation of the Pasteur effect which describes an inhibition of ethanol formation (fermentation) by oxygen.

When cells of *Sacch. uvarum* oxidatively metabolizing glucose were submitted to oxygen limitation by lowering the oxygen content of the inlet air from 21 to 2% (v/v), a change to respiro-fermentative glucose metabolism was observed (Fig. 11). Immediate formation of ethanol and, in the early stages, of

acetate occurred. As a consequence, a decrease in biomass concentration became apparent. The specific oxygen-uptake rate was diminished due to limited availability of oxygen. Generally, the response of the overall culture parameters very closely resembled that seen after the dilution-rate shift (see Fig. 5). The same was true for the responses in mitochondrial cytochrome contents (see Figs 11 and 5, respectively). Minor differences noticed in the dynamics may originate from the different dilution rates employed in the

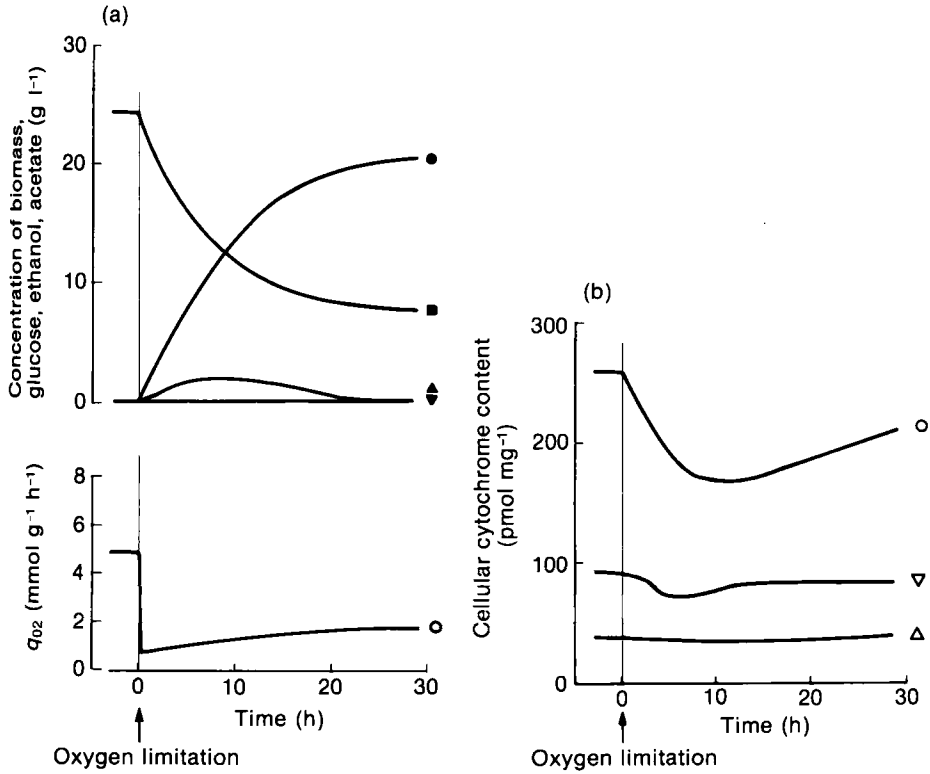

FIG. 11. Effect of introducing oxygen limitation in a respiratively growing culture of *Saccharomyces uvarum*. (a) Response of the overall culture parameters. Respiro-fermentative glucose metabolism was triggered as indicated by the beginning of ethanol (●) formation and, in the early stages of the experiment, acetate (▲) formation. Biomass concentration (■) decreased accordingly. The specific oxygen uptake rate of the cells (○) decreased as a consequence of the decreased availability of oxygen. (b) Response of mitochondrial cytochrome content. Cytochrome *a* (△) and cytochrome *b* (▽) only slightly decreased, whereas cytochrome *c* content (○) showed a significant decrease. The transient response is comparable to that of the dilution-rate shift illustrated in Fig. 7. From Käppeli *et al.* (1985a).

experiments. These results clearly indicate that respiro-fermentative glucose metabolism can be initiated by artificially lowering the respiratory capacity of the cells. It follows that the so-called "glucose effect" (i.e. the initial explanation for the occurrence of respiro-fermentative glucose metabolism by glucose repression) and the Pasteur effect are closely related in that the latter is a premature triggering of respiro-fermentative glucose metabolism by oxygen limitation. Therefore, the Pasteur effect represents a special case in that the cell's inherently limited respiratory capacity can be decreased and eventually extinguished completely under anaerobic conditions.

The decrease of cytochrome contents after introduction of oxygen limitation shows that there is the same cellular adaptation as that observed after exceeding the maximum respiratory capacity (see Fig. 5). This represents further evidence that it is respiro-fermentative glucose metabolism as such which affects cellular regulation and not glycolytic activity as assumed in the earlier explanation for the Crabtree effect. This is further substantiated by Käppeli *et al.* (1985a) who showed that other medium limitations (iron and nitrogen limitations) also lead to respiro-fermentative glucose metabolism with the observed effects on cytochrome content also occurring. It is concluded that any imbalance between glucose flux and the biosynthetic potential of the cells causes respiro-fermentative glucose breakdown with the predictable physiological changes.

Using the concept of limited respiration as the basis for regulation of glucose metabolism, a comprehensive interpretation of the phenomena occurring during growth of yeasts is possible. The apparent contradiction of the observations referred to as Pasteur and Crabtree effects is explainable and can be considered properly. It is advisable, however, not to use these terms any longer since they are connected with regulatory concepts that do not correspond to the actual situation. The same is true for the term "glucose effect". As already pointed out, there may be enzyme regulation by glucose or catabolite repression but usually the term is used in a general sense and then becomes inaccurate and eventually misleading.

The importance of the contribution of respirative energy formation during aerobic growth has recently been questioned. Lagunas (1981) concluded that *Sacch. cerevisiae* does not obtain much energy from respiration during its aerobic growth and that it does not have a noticeable Pasteur effect. An analysis of metabolic rates (Table 5) and the distribution between respirative and fermentative fluxes shows the following. (1) In terms of fluxes, the respirative part is low. It amounts to less than 20% of the maximum glucose uptake rate in the two yeasts shown. (2) In terms of energy generation, the respirative part is substantial even if the theoretical values given in Table 5 need to be corrected downwards. The significance of respiration during aerobic growth in yeast can also be derived from a comparison of aerobic and

TABLE 5. Maximum respirative and respiro-fermentative glucose uptake rates of *Saccharomyces cerevisiae* and *Saccharomyces uvarum* and theoretical rates of energy formation from respiration and fermentation. From Petrik *et al.* (1983) and Rieger *et al.* (1983)

Yeast	*Saccharomyces uvarum*	*Saccharomyces cerevisiae*
Maximum glucose uptake rates (mmol $g^{-1}h^{-1}$):		
respirative	2.0	3.4
respiro-fermentative	10.5	20.0
Maximum theoretical rate of energy generation (mmol ATP $g^{-1}h^{-1}$):		
respirative[a]	30	48
fermentative[b]	17	33

[a] Calculated from a maximum oxidative CO_2 production rates of 5 and 8 mmol $g^{-1}h^{-1}$ respectively and a respirative ATP formation of 36 mol (mol glucose)$^{-1}$.
[b] Calculated from fermentative glucose fluxes of 8.5 and 16.6 mmol $g^{-1}h^{-1}$ respectively and a fermentative ATP formation of 2 mol (mol glucose)$^{-1}$.

anaerobic growth in continuous culture (Fig. 12). When all of the necessary reservations originating from such a simplification are taken into account, it cannot be denied that respiration must play an important role in aerobic growth of yeasts. This may not become obvious when batch growth is analysed. As already indicated, respirative flux is low as compared with the maximum respiro-fermentative glucose uptake rate with the latter being close to the maximum anaerobic one (Schatzmann, 1975; Ohno *et al.*, 1981). Therefore, difficulties in assessing the contribution of respiration may arise.

IV. Regulation of Glycolysis

Having discussed the concept of saturation of respiration as the basic principle for the occurrence of respiro-fermentative glucose metabolism in *Sacch. cerevisiae* and related yeasts, it is now important to discuss regulation of glycolysis. As was shown, repression of respiratory pathways cannot account for the occurrence of ethanol formation. Simultaneous kinetic analysis of ethanol production, rate of oxygen uptake and mitochondrial cytochrome content revealed that ethanol release is based on an extremely fast reaction. There have been many attempts to explain glucose repression in yeasts. All of these reports initially used the term "glucose repression" in its general sense, which refers to the repression of enzymes of the tricarboxylic

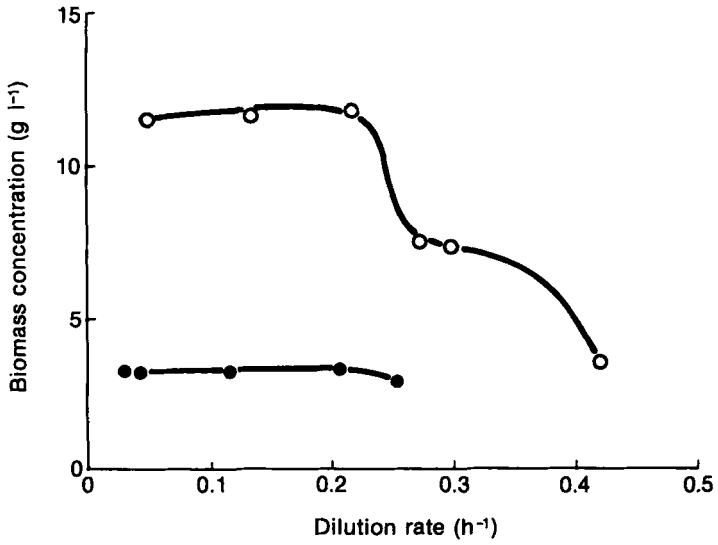

FIG. 12. Growth of *Saccharomyces cerevisiae* in aerobic (○) and anaerobic (●) continuous culture. In both experiments the glucose concentration in the inflowing medium was 30 g l^{-1}. The effect of respirative glucose metabolism on the potential of the cells for biomass formation is clearly manifest. From Ohno *et al.* (1981).

acid (TCA) cycle, glyoxylate cycle, respiratory enzymes, gluconeogenic enzymes and enzymes necessary for utilization of sugars such as maltose, sucrose and galactose. Usually, glucose repression is then assessed by measuring particular enzyme activities, mostly maltase, invertase or galactokinase. On this basis, mutants have been isolated in which synthesis of two or more unrelated enzymes was resistant to carbon catabolite repression. The first such mutant was isolated by Montenecourt *et al.* (1973) which was found to be "hexose resistant" for both invertase and α-glucosidase synthesis. Several other investigators have independently isolated mutants which also turned out to be insensitive to glucose repression for synthesis of maltase and invertase (Lemont, 1977; Wickner, 1974; Rothstein and Sherman, 1980). Recently, several classes of mutations have been described which result in derepression of not only cytoplasmic enzymes, such as invertase and maltase, but also mitochondrial enzymes like cytochrome *c* oxidase. In the main, three mutant classes, corresponding to three unlinked genes, were identified. One class of mutants, *hex 1*, had decreased hexokinase activity (Zimmermann and Scheel, 1977; Entian *et al.*, 1977) and was shown to be altered in the structural gene for hexokinase isoenzyme PII (Entian, 1980; Entian and Mecke, 1982). A second class of mutants, *hex 2*, had elevated hexokinase PII synthesis when grown on glucose (Entian, 1981). It has been postulated that hexokinase PII,

the *HXK-2* gene product, has not only a catalytic site but also a regulatory site which is involved in controlling the repression of susceptible genes (Entian *et al.*, 1984). Ciriacy (1978) has isolated mutants that were specific in their ability to alleviate catabolite repression of mitochondrial enzymes. These mutations were dominant, of nuclear origin, and almost completely inhibited aerobic ethanol formation from glucose. Boeker-Schmitt *et al.* (1982) have also reported isolation of phenotypically similar dominant nuclear mutants in which growth on, and fermentation of, glucose were normal.

From these data on catabolite (glucose) repression in *Sacch. cerevisiae*, a rather complicated picture emerges. A total of three genes have now been identified which release certain enzymes (invertase and maltase) from glucose repression. There is no question that pleiotropic phenotypes exist for different catabolite repression-insensitive mutants (Bailey and Woodword, 1984). A trivial explanation could be that the rate of glucose uptake is decreased sufficiently to prevent intracellular accumulation of repressive amounts of glucose or, perhaps more accurately, the onset of respiro-fermentative glucose metabolism. In most instances, growth rates of mutants on glucose were lowered, a fact hardly taken into account. A comprehensive analysis of growth of mutant strains should be carried out in order to understand the influence of mutations on glucose metabolism. If this is done, generalizations about data from specific enzymes should be avoided. In any case, glucose repression is not the mechanism that leads to respiro-fermentative glucose utilization. It may merely be involved in long-term adaptation of certain enzymes leading to the observed physiological states under respiro-fermentative glucose metabolism.

As far as the fast response of the metabolism to a glucose pulse is concerned (see Fig. 6), no regulation of the level of enzyme synthesis can be imagined. A study of regulation of glycolysis needs to be related to mechanisms coupling glycolytic reactions to other cellular processes to which glycolysis donates phosphoryl groups, reducing equivalents and carbon fragments for further oxidation, reduction and biosynthesis. Any change in the latter processes will lead to corresponding and well-balanced changes in the activity of glycolysis.

Since the occurrence of respiro-fermentative glucose metabolism was related to inherent saturation of respiration in cells, a regulatory mechanism following the lines of the explanation of the Pasteur effect seems reasonable. Allosteric feedback regulation of glycolysis established by Sols *et al.* (1971) can, therefore, still serve as a basic pattern. Since its formulation, further observations on regulatory interactions have evolved (for a review see Hess *et al.*, 1983), the most important being the discovery of fructose 2,6-bisphosphate as an important effector (Hers and Van Schaftingen, 1982). This compound is also present in yeasts (Lederer *et al.*, 1981) and acts at the level of phosphofructokinase by a mechanism described by François *et al.* (1984).

Figure 13 summarizes the mechanisms by which glycolysis is regulated in *Sacch. cerevisiae*. As far as the fast regulation leading to onset of respiro-fermentative glucose metabolism is concerned, the following controls occur. Glucose enters the cells by a mechanism that has been described as facilitated diffusion (Heredia *et al.*, 1968) but could also be more complex (Thevelein, 1984). It then becomes available for glycolysis. Under feed-controlled conditions, glycolytic rate and respiration rate are balanced. Excess glucose and high feed rates will stimulate formation of fructose 2,6-bisphosphate which is a positive effector of phosphofructokinase 1 (François *et al.*, 1984). Glycolytic activity increases and leads to an imbalance in energy generation due to the limited respiratory capacity which causes a decrease in the cellular content of energy-rich phosphate compounds (ATP) and a decrease in TCA-cycle intermediates (citrate). A further stimulation of glycolysis results which activates the overflow reaction at the level of pyruvate. Ethanol formation is initiated. Further regulation of glycolytic flux may be exerted by alternative pathways for glucose degradation as proposed by Breitenbach-Schmitt *et al.* (1984). Whereas existence of one such pathway seems to be proven, its role in regulation of glucose metabolism is not yet elucidated.

V. Concluding remarks

Glucose breakdown in *Sacch. cerevisiae* and related yeasts proceeds via different pathways. Principally respirative, fermentative (anaerobic) and respiro-fermentative glucose metabolism have to be considered, but growth on ethanol is also to be considered due to the diauxic growth pattern observed in batch cultures. Ethanol accumulated during the first growth phase with respiro-fermentative glucose catabolism is used for subsequent growth. The type of glucose metabolism depends on cultivation conditions.

Respirative glucose catabolism is possible in feed-controlled systems only (fed batch or continuous culture). The shift to respiro-fermentative glucose breakdown is governed by the respirative capacity of cells and therefore occurs above a certain glucose-feed rate. Respiro-fermentative glucose metabolism is primarily the consequence of an overflow reaction at the level of pyruvate when respiration is saturated. Following onset of respiro-fermentative glucose metabolism, an adaptation of cellular physiology takes place. Purely fermentative glucose metabolism is limited to anaerobic growth conditions.

Metabolic regulation exhibited by *Sacch. cerevisiae* and related yeasts represents an impressive example of the necessity of proper methodical investigations for the elucidation of regulatory mechanisms. It is important to notice that metabolic turnover rates are decisive for the type of metabolism exhibited by cells and not the presence of glucose as such. This is the most

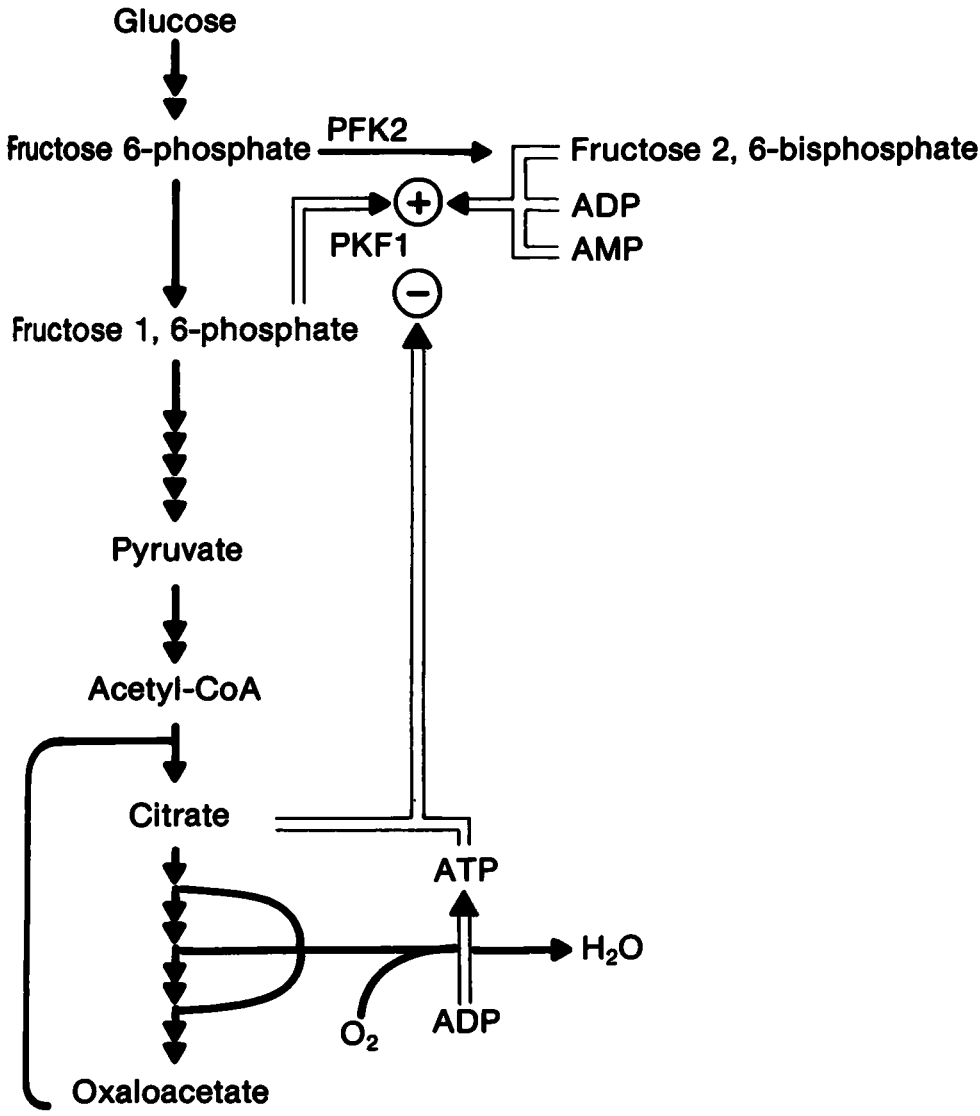

FIG. 13. Proposed control of glycolysis in *Saccharomyces cerevisiae* by feedback regulation. This schematically represented regulation is based on the limited respiratory capacity concept as the cause for aerobic ethanol formation in *Saccharomyces cerevisiae* and related yeasts. − indicates inhibition and + activation. Adapted from Hess *et al.* (1983).

important result gained by dynamic analyses of glucose metabolism under different growth conditions. This should be considered performing any metabolic studies. It is hoped that the terminology used here will be accepted and replace the hitherto employed less accurate one.

The examples of *Sacch. cerevisiae* and related yeasts also demonstrate that environmental conditions influence cellular physiology. This applies to the most common factors, namely growth rate, nutrient limitation and oxygen availability. These yeasts and possibly most micro-organisms possess a remarkable potential to adapt to changes in their environment. It indicates that gene expression is influenced by environmental conditions, and that this forms the basis for biotechnological applications of micro-organisms. The study of such interactions presents very much of a challenge and appears to be very exciting and rewarding.

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The Antibacterial Effects of Low Concentrations of Antibiotics

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I. Introduction

It has been known for nearly 50 years that growth of bacteria in the presence of low concentrations of antibiotic can affect bacterial metabolism (e.g. Tunnicliff, 1939; Gardner, 1940). However, until the 1970s, little interest was devoted to evaluating the therapeutic significance of *in vitro* observations (Atkinson and Amaral, 1982). Thus, it was generally assumed that antibiotics eradicated infections because the drug concentrations achieved at the site of infection *in vivo* always exceeded the minimum inhibitory concentration (MIC), or minimum bactericidal concentration (MBC) values determined by *in vitro* assays. However, there is now a growing body of evidence which strongly suggests that antibiotic concentrations below the conventionally determined MIC or MBC values can be effective in the treatment of infections (O'Grady, 1982). Consequently there has been renewed interest in the effects of exposing bacteria to low concentrations of antibiotics, which in turn has improved our understanding of disease processes, the therapeutic efficacy of antibiotics and the feasibility of decreasing the duration of therapy.

Several reviews concerning the antimicrobial effects of low concentrations of antibiotics have already been published (Washington, 1979; Zak and Kradolfer, 1979; Lorian, 1980; Ahlstedt, 1981; Atkinson and Amaral, 1982). Nevertheless, even since the most recent review, many relevant research papers have been published. For this reason we believe that another review of the subject is warranted. Furthermore, although the concept of sublethal concentrations of antibiotics seems straightforward, the effects on bacteria are varied and for the most part uncharacterized at the molecular level. In this

review we shall, wherever possible, point to the likely molecular basis for a particular response.

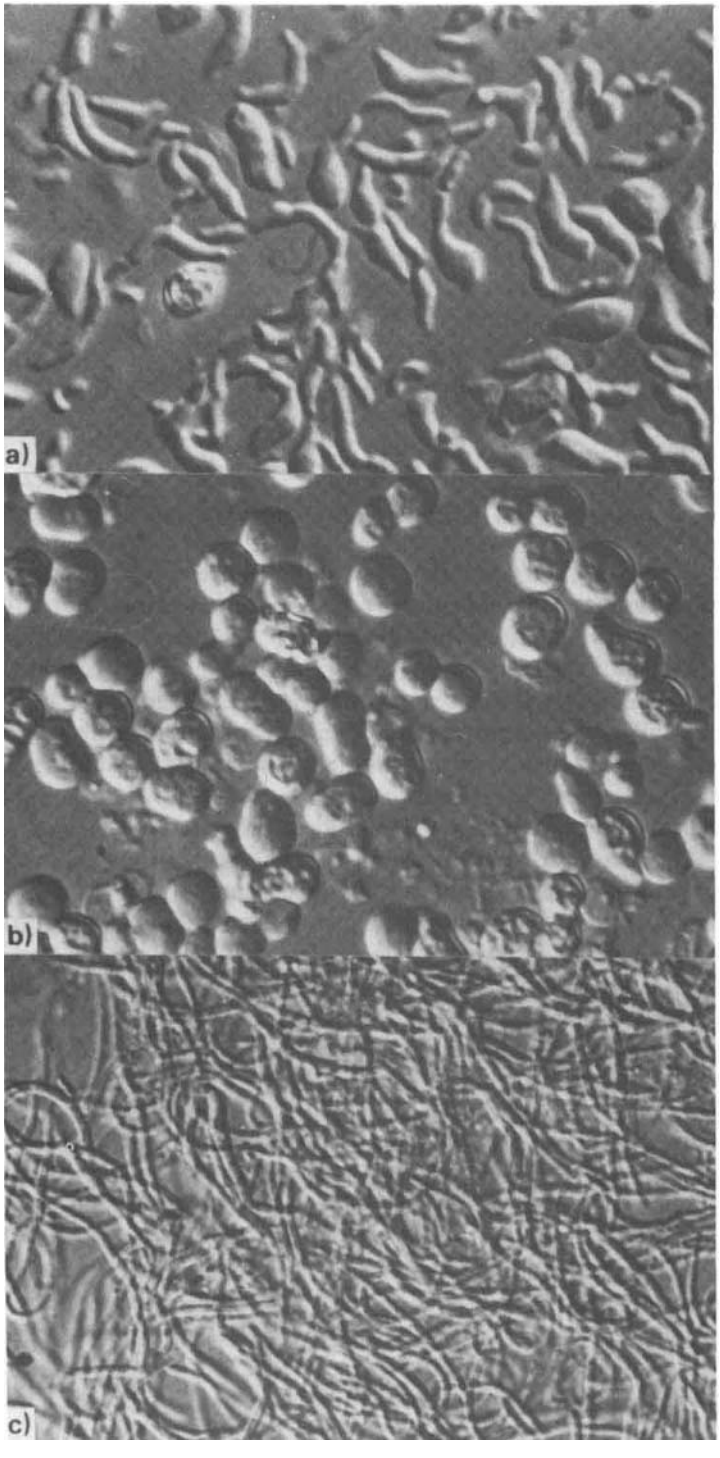
Apart from their antimicrobial activity, low concentrations of antibiotics may exhibit immunological side effects in the host by either directly inhibiting or stimulating immune-response mechanisms. We will not specifically consider the effect of antibiotics on host immune responses, but readers interested in this topic can consult a number of recent papers which deal with the subject (Gnarpe and Belsheim, 1981; Ahlstedt, 1981; Gillissen, 1982; Bassi and Bolzoni, 1982; Chaperon, 1982; Glette *et al.*, 1982; Mandell, 1982; Kowolik *et al.*, 1982; Adam, 1982; Limbert *et al.*, 1982; Pickering *et al.*, 1982; Seger *et al.*, 1982; Midtvedt *et al.*, 1982; Raff *et al.*, 1982).

II. Morphological Alterations

The earliest observations on the effects of exposing bacteria to low concentrations of antibiotics were concerned with antibiotic-induced morphological alterations (Washington, 1979; Lorian, 1980, 1985; Atkinson and Amaral, 1982). This is not surprising because these are the most obvious changes to occur (Lorian, 1985). Extensive studies have now been made and it is apparent that almost all antibiotics produce morphological alterations, although the β-lactam antibiotics produce them at concentrations significantly below those that prevent bacterial multiplication (Lorian, 1985). Since the subject of morphological alterations has already been extensively reviewed (Washington, 1979; Lorian, 1980, 1985; Atkinson and Amaral, 1982) we will restrict ourselves to a few remarks concerning β-lactam antibiotics.

Exposure of Gram-negative bacilli to low concentrations of β-lactam antibiotics can result in production of filaments, oval cells or irregularly shaped cells (see reviews already cited; Fig. 1). However, formation of filaments appears to be the most common effect produced by β-lactam antibiotics at subminimum inhibitory concentrations (Ellis *et al.*, 1976; Guss and Bawdon, 1984). These morphological alterations tend to be characteristic for specific β-lactam antibiotics and are related to the affinity of the antibiotics for particular penicillin-binding proteins (Atkinson and Amaral, 1982; Lorian, 1985). Indeed, the discovery of penicillin-binding proteins (PBPs) and characterization of their roles in the maintenance of morphological integrity in Gram-negative bacteria has, to a major extent, provided a biochemical basis for the alterations induced by low concentrations of β-lactam antibiotics in Gram-negative bacilli.

Exposure of Gram-positive cocci to low concentrations of β-lactam antibiotics usually results in formation of abnormally large cells with thickened cross walls, but morphologically normal outer walls (Fig. 2).


a)
b)
c)

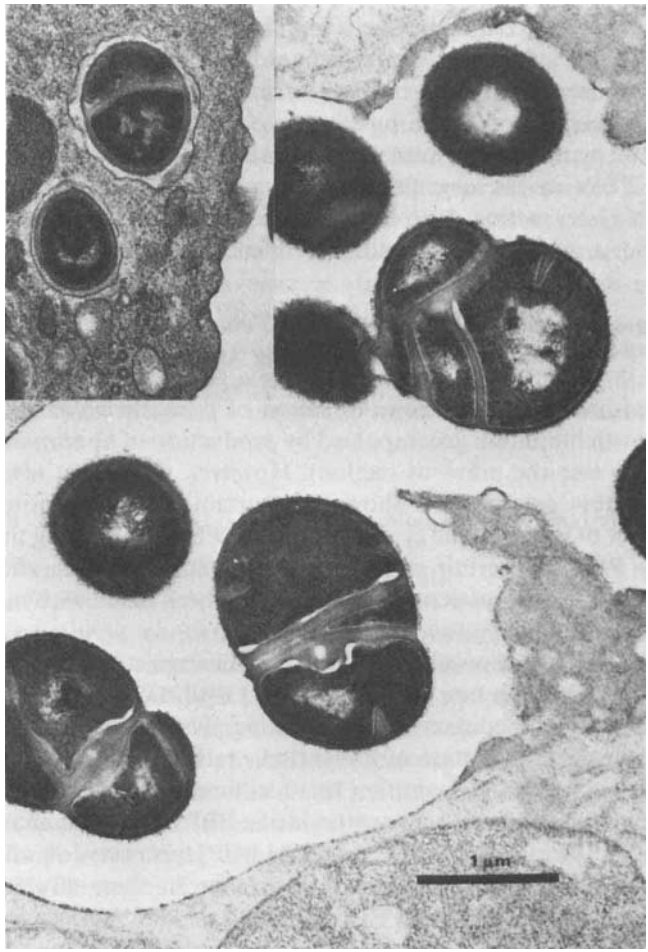


FIG. 2. Large cells of staphylococci resulting from exposure to cloxacillin. The specimen was isolated from the spleen of a mouse treated with cloxacillin, but the effect of the β-lactam on staphylococcal morphology is typical of that which occurs when Gram-positive cocci are grown in the presence of low concentrations of antibiotic (Washington, 1979; Atkinson and Amaral, 1982). Inset shows control staphylococci. From Lorian (1985).

FIG. 1. Typical morphological changes that can result from exposure of Gram-negative bacilli to low concentrations of β-lactam antibiotics. The panels show the effect on *Proteus mirabilis* of (a) 6-aminopenicillanic acid, (b) mecillinam and (c) ampicillin. From Lorian (1985).

Detailed examination of the large cells frequently shows them to be clusters of cocci that have failed to separate (Washington, 1979; Atkinson and Amaral, 1982). The observation that most β-lactam antibiotics produce similar morphological effects on Gram-positive cocci probably reflects the fact that there is little variation between the affinities of β-lactam antibiotics for individual PBPs in the organisms concerned (Lorian, 1985). As will be described in a later section, morphological changes of the type described often render bacteria more susceptible to host defences.

III. Changes in Penicillin-Binding Proteins Following Growth of Bacteria in the Presence of β-Lactam Antibiotics

Exposure of bacteria to low concentrations of β-lactam antibiotics usually leads to growth inhibition accompanied by production of abnormal morphological forms (see the previous section). However, this is not always so as reports are now emerging to show that certain β-lactam antibiotics are capable either of inducing the synthesis of novel PBPs, or altering the balance of existing PBPs to permit normal growth in the presence of β-lactam antibiotics. These observations have so far only been made with methicillin-resistant strains of *Staphylococcus aureus*.

Rossi *et al.* (1985) showed that one methicillin-resistant strain of *Staph. aureus* (R2) produced a new PBP (designated PBP2a) when methicillin was added to the culture medium. Data concerning production of the novel PBP were consistent with induction of its synthesis rather than with activation of pre-existing molecules. In another methicillin-resistant strain (R1), the antibiotic did not induce *de novo* synthesis of a PBP but caused an increase in the amount of PBP3. Both R2 PBP2a and R1 PBP3 have very low affinities for methicillin suggesting that methicillin resistance in these strains may be related to acquisition of these PBPs. Ubukata *et al.* (1985) have reported findings which, in essence, are identical to those observed for the strain described as R2 (Rossi *et al.*, 1985). However, Ubukata *et al.* (1985) demonstrated that other β-lactam antibiotics are capable of inducing the new staphylococcal PBP. The molecular basis of the β-lactam-induced changes in PBP content are currently unclear as there is little information on the mechanisms by which syntheses of PBPs are regulated.

IV. Effects on Bacterial Adhesion

A. INTRODUCTION

Bacteria possess many adhesive surface structures (Gaastra and De Graaf, 1982; Jones and Isaacson, 1983; Vosbeck and Mett, 1983; De Graaf and Mooi,

this volume). Adhesion of pathogenic Gram-negative bacteria is usually mediated by specific bacterial surface appendages (pili or fimbriae) that recognize specific receptors located on the epithelial cell surface. In other instances, Gram-negative bacteria lacking fimbriae can nevertheless adhere specifically to human or animal tissues. In these circumstances, other surface macromolecules (e.g. lipopolysaccharides and outer membrane proteins) are implicated in adhesion (Jones and Isaacson, 1983; Vosbeck and Mett, 1983). Adhesion of Gram-positive bacteria is also usually mediated by surface macromolecules rather than discrete surface organelles such as fimbriae (Jones and Isaacson, 1983; Vosbeck and Mett, 1983).

Many studies (to be discussed more fully later in this section) indicate that cultivation of bacteria in the presence of low concentrations of antibiotics can interfere with production or retention of bacterial adhesins without affecting bacterial viability.

B. TYPE 1 FIMBRIAL ADHESION IN GRAM-NEGATIVE BACTERIA

Type I fimbriae are found on the surface of many Gram-negative species and, at least in *Escherichia coli*, are encoded by chromosomal genes (Hull *et al.*, 1981). The fimbriae, consisting of protein subunits, interact with mannose-containing receptors on the surface of eukaryotic cells (Jones and Isaacson, 1983). Serologically, all type I fimbriae tested so far appear to be similar, although quantitative differences exist in their cross-reactivity with a particular antiserum, especially between fimbriae isolated from organisms in different genera (Korhonen *et al* ., 1981; Klemm *et al.*, 1982). Despite their undoubted involvement in promoting adhesion to eukaryotic cells, the role of type I fimbriae as virulence determinants has been questioned (Vosbeck and Mett, 1983). This arises from the observation that phagocytic cells contain a polymer containing mannose residues, and that infecting organisms bearing type I fimbriae should therefore be highly susceptible to phagocytosis. The apparent dilemma has been resolved by studies on the expression of type I fimbriae which indicate that the level of piliation varies and is regulated by a specific control system (Ofek *et al.*, 1981; Orndorff and Falkow, 1984). It has been proposed that, during the early stages of infection, bacteria may benefit from their ability to bind to mannose residues on epithelial cells, whereas at later stages loss of this adhesiveness (exerted by the control system) is also beneficial since the bacteria no longer adhere to mannose residues on phagocytes (Ofek *et al.*, 1981).

Several studies (primarily in *E. coli*) have been conducted to examine the influence of low concentrations of antibiotic on adhesion mediated by type I fimbriae (Table 1). Exposure of *E. coli* during growth to sub-lethal concentrations of penicillin and several translational inhibitors markedly suppressed

TABLE 1. Inhibition of bacterial adhesion by low concentrations of antibiotics

Adhesive bacteria	Receptor-containing material	Inhibitory antibiotics	References
(a) Gram-negative bacteria			
Escherichia coli: type I fimbriae	Human leucocytes, buccal epithelial cells. Guinea-pig erythrocytes. Yeast cells.	Benzylpenicillin, chloramphenicol, gentamicin, neomycin, spectinomycin, streptomycin, tetracycline	Ofek *et al.* (1979); Beachey *et al.* (1981, 1982); Eisenstein *et al.* (1982).
Uropathogenic *Escherichia coli*	Human buccal epithelial cells, intestinal cells, erythrocytes, uroepithelial cells.	Ampicillin, chloramphenicol, clindamycin, streptomycin, sulphadiazine, sulphamethoxazole, sulphathiazole, tetracycline, trimethoprim	Sanberg *et al.* (1979); Vosbeck *et al.* (1979, 1982); Vosbeck (1982); Stenqvist *et al.* (1982); Vaisanen *et al.* (1982); Bassaris *et al.* (1984a,b).
Uropathogenic *Pseudomonas aeruginosa*, *Proteus mirabilis*	Human buccal epithelial cells	Cefotaxime	Bassaris *et al.* (1984b).
Uropathogenic *Klebsiella pneumoniae*	Human buccal epithelial cells	Clindamycin	Lianou *et al.* (1985).
Enterotoxigenic *Escherichia coli*: K88 fimbriae	Pig erythrocytes	Colistin	Sogaard *et al.* (1983).
Enterotoxigenic *Escherichia coli*: K99 fimbriae	Pig and sheep erythrocytes	Chloramphenicol, colistin, gentamicin, tetracycline	Sogaard *et al.* (1983); Ferreiros and Criado (1983).
Enterotoxigenic *Escherichia coli*: CFA I, II, III*	Human and bovine erythrocytes, human intestinal epithelial cells	Benzylpenicillin, doxycycline, minocycline, olandeomycin	Forestier *et al.* (1984).
Neisseria meningitidis	Human erythrocytes, buccal epithelial cells.	Ampicillin, benzylpenicillin, colistin, erythromycin, lincomycin, nystatin, rifampin, tetracycline, tobramycin	Kristiansen *et al.* (1983); Salit (1983); Stephens *et al.* (1984).

TABLE 1 (continued)

Neisseria gonorrhoeae	Human buccal epithelial cells	Benzylpenicillin, tetracycline	Stephens *et al.* (1984).
Vibrio proteolytica	Polystyrene	Ampicillin, oxacillin, streptomycin	Paul (1984).

* The entity originally referred to as CFA II is now known to consist of three distinct fimbrial antigens (see text for further details).

(b) Gram-positive bacteria

Staphylococcus aureus	Fibronectin	Chloramphenicol, erythromycin, lincomycin	Proctor *et al.* (1983).
Streptococcus pyogenes	Human pharyngeal cells	Benzylpenicillin, tetracycline, rifampicin	Tylewska *et al.* (1981).
Streptococcus sanguis	Constituents of thrombotic endocarditis	Benzylpenicillin, chloramphenicol, tetracycline, vancomycin	Bernard *et al.* (1981); Scheld *et al.* (1981); Lowy *et al.* (1983).

adhering ability (Beachey *et al.*, 1981; Eisenstein *et al.*, 1982). In general, the depressed adherence correlated with diminished levels of fimbriae (Table 2) and the antibiotics were unable to suppress adhesiveness of *E. coli* once the organisms acquired this property (Ofek *et al.*, 1979). Furthermore, exposure of non-dividing bacteria to the antibiotics had no influence on their subsequent ability to adhere in the test systems used (Beachey *et al.*, 1982). The effect of low concentrations of penicillin on mannose-specific binding of *E. coli* correlated with conversion of rod-shaped organisms into filaments devoid of type I fimbriae, as revealed by electron microscopy. The filamentous forms also lacked the ability to agglutinate yeast cells or to adhere to epithelial cells (Ofek *et al.*, 1979). The relationship between filament formation and lack of fimbriae production was also demonstrated by the use of a thermosensitive division mutant of *E. coli* that undergoes filamentation at restrictive temperatures (e.g. 39°C). This mutant (probably containing a thermosensitive PBP3; see Spratt (1983) for details of similar mutants), when grown at restrictive temperatures, formed filaments and lacked fimbriae but, when shifted to permissive temperatures, it formed normal rods, produced surface fimbriae and regained its adhesive properties (Ofek *et al.*, 1979; Beachey *et al.*, 1981).

In most situations, streptomycin also suppressed expression of type I fimbriae (see Table 1). However, one notable exception was reported (Beachey *et al.*, 1981; Eisenstein *et al.*, 1982) where exposure of a streptomycin-resistant mutant of *E. coli* to low concentrations of streptomycin (0.03 of its MIC value) led to decreased adhesiveness, but without concomitant

TABLE 2. Antibiotic-mediated decrease in epithelial cell adherence, yeast cell agglutination and fimbriation after growth of *Escherichia coli* in the presence of low concentrations of antibiotics. From Beachey *et al.* (1981) and Eisenstein *et al.* (1982).

Antibiotic	Adhering ability (% of control)		Degree of fimbriation (% of control)
	Epithelial cells	Yeast cells	
Chloramphenicol	62	100	100
Gentamicin	49	20	65
Neomycin	NR	20	50
Spectinomycin	53	62	97
Streptomycin	17	2	31
Tetracycline	40	77	88

Values refer to mannose-sensitive adhesins (i.e. principally type I fimbriae) and are compiled from data presented by Beachey *et al.* (1981) and Eisenstein *et al.* (1982). Bacteria were grown for 48 hours in the presence of antibiotics at half their MIC values. The cultures were harvested and then assayed for the properties indicated, which were compared to those of drug-free controls.

NR, not reported.

depression of fimbrial synthesis. Further studies revealed that organisms grown in the presence of streptomycin did indeed form surface fimbriae, but the length of the organelles was greater than those produced by organisms grown in the absence of the antibiotic. When isolated and purified, these modified type I fimbriae also failed to agglutinate guinea-pig erythrocytes under conditions where normal isolated fimbriae promoted agglutination. The authors (Beachey *et al.*, 1981) suggested that the streptomycin-resistant mutant studied may have acquired a second mutation, possibly analogous to *ram* (Gorini, 1974), which results in streptomycin-promoted misreading of mRNA during translation of type I fimbrial messenger at the ribosome.

C. UROPATHOGENIC GRAM-NEGATIVE BACTERIA

The importance of bacterial adhesion in the pathogenesis of human urinary-tract infections caused by certain Gram-negative bacteria is well established (Bassaris *et al.*, 1984b; Svanborg Eden *et al.*, 1976, 1978; Vosbeck and Mett, 1983; Kallenius *et al.*, 1981). In some cases, specific proteinaceous fimbrial adhesins have been unequivocally associated with the ability of bacteria to

cause infections. For example, P fimbriae contribute to virulence of pyelonephritogenic *E. coli* (Kallenius *et al.*, 1981; Vaisanen *et al.*, 1981). Their receptor on both human erythrocytes and uroepithelial cells is the α-D-Gal (1→4)-β-D-Gal disaccharide moiety of the P-blood group-specific glycosphingolipids (Kallenius *et al.*, 1981; Korhonen *et al.*, 1982; Vosbeck and Mett, 1983). The genes encoding P fimbriae production reside on both plasmids and transposons (Hales and Amyes, 1983). The observation that fimbrial genes in uropathogenic strains are located in transposons is also consistent with recent analysis of DNA encoding P fimbriae, where the fimbrial structural genes were found to be flanked by inversely orientated DNA segments of about 1.2 kilobases (kb) (Rhen, 1985).

Several studies have been performed which describe the effects of antibiotics on adhesion of uropathogenic bacteria. Low concentrations of trimethoprim and various sulphonamides have been reported by several authors to decrease adhesion of uropathogenic *E. coli* (see references in Table 1). The molecular basis of this response has not been studied in detail, but Vaisanen *et al.* (1982) examined some aspects of the mechanism of inhibition. Decreased adhesion corresponded to a decrease in the number of P fimbriae per cell, suggesting that the antimicrobial agents interfered with formation of fimbriae. However, the authors postulated that the antibiotics do not directly affect fimbrial synthesis, but rather exert their effects as a result of changes in the cell envelope that interfere with secretion or assembly of the fimbriae, or both. This hypothesis is based primarily on the observation that both inner and outer membranes in *E. coli* exposed to sublethal concentrations of trimethoprim showed disorganization when examined by freeze-fracture electron microscopy (Vaisanen *et al.*, 1982). Streptomycin, tetracycline, chloramphenicol and clindamycin also decrease adhesion of many uropathogenic *E. coli* strains (see Table 1). However, since these are translational inhibitors (Gale *et al.*, 1982; Lianou *et al.*, 1985), they probably affect adhesion by suppressing synthesis of the fimbrial adhesins themselves, or the products of accessory genes required for fimbrial export into the outer membrane (see Section IV.J. for further comments).

Sanberg *et al.* (1979) and Stenqvist *et al.* (1982) reported that low concentrations of ampicillin decreased the attachment of uropathogenic *E. coli* to human uroepithelial cells. As already noted, synthesis or insertion of type I fimbriae into the cell envelope of *E. coli* is inhibited by β-lactam antibiotics, and a similar explanation may account for the mediation by fimbriae of adhesion of uropathogens. Sanberg *et al.* (1979) suggest that elongated bacterial forms (i.e. induced by β-lactam antibiotics) may fit less well into the region of the uroepithelial cell surface that contains fimbrial receptors. In contrast to the studies just mentioned, Vosbeck *et al.* (1979) and Bassaris *et al.* (1984b) reported that adhesion of other uropathogenic strains

of *E. coli* was not influenced by growth in the presence of ampicillin, nor indeed by several other β-lactam antibiotics. Despite the apparent discrepancy concerning the effects of β-lactam antibiotics on adhesion of uropathogenic strains of *E. coli*, it should be mentioned that the conditions used by the various authors were not identical and that, although *in vitro* adhesion systems usually give comparable results, there is no absolute correlation between different assay systems (Vosbeck, 1982; Vosbeck *et al.*, 1982). Furthermore, since several distinct types of fimbriae are associated with adhesion of uropathogenic strains of *E. coli* (Jones and Isaacson, 1983; Vosbeck and Mett, 1983), it is possible that the differences observed are due to variations in the composition of the fimbriae on the strains examined.

The effects of antibiotics on adhesion of uropathogens other than *E. coli* have also been examined (Table 1). When *Pseudomonas aeruginosa* and *Proteus mirabilis* were cultured in the presence of the β-lactam antibiotic cefotaxime (at 0.25 MIC values) decreased adhesion resulted, but similar treatment of *Klebsiella pneumoniae* had no effect on adherence of this organism (Bassaris *et al.*, 1984b). In contrast, addition of low concentrations of clindamycin to the growth medium enhanced adhesion of *Ps. aeruginosa* and *P. mirabilis*, but suppressed *K. pneumoniae* adhesion (Lianou *et al.*, 1985). These observations, together with those already referred to for β-lactam antibiotics and *E. coli*, show that the effects of antibiotics on adhesion of Gram-negative bacteria can be species and even strain dependent.

D. ENTEROTOXIGENIC STRAINS OF *Escherichia coli*

As already stated, the first step in the establishment of many gastrointestinal infections caused by bacteria is attachment of the organisms to mucosal or epithelial cells in order to localize bacterial multiplication at a favourable site. The importance of such localization has been clearly established for enterotoxigenic strains of *E. coli*, where the organisms have to attach to the mucosal surface of the intestine to establish an infection (Gaastra and deGraaf, 1982). These organisms invariably produce fimbriae that are responsible for attachment and, with several organisms, the genes encoding fimbriae, e.g. K88,K99 and CFA/I, are located on plasmids (Gaastra and DeGraaf, 1982; Jones and Isaacson, 1983; De Graaf and Mooi, this volume).

Despite their importance as pathogens few, if any, studies had been performed prior to 1983 to determine whether low concentrations of antibiotics inhibit adhesion of enterotoxigenic *E. coli*. Since 1983, several studies have been conducted in this area and a variety of antibiotics including β-lactam and translational inhibitors have been shown to inhibit adhesion following growth of bacteria in the presence of low concentrations of the drugs (see Table 1). However, the effects of colistin, apparently not previously

studied in other adhesion systems, are particularly interesting. Sogaard *et al.* (1983) provide data to suggest that colistin may be a more potent inhibitor of K88- and K99-mediated adhesion than translational inhibitors. Since colistin is a cationic surface-active compound, it may prove possible effectively to inhibit adhesion of a variety of bacteria by other surface-active compounds that have, or may have, clinical applications, e.g. chlorhexidine (Hugo and Russell, 1982) and the polymyxin analogue, polymyxin B nonapeptide (PMBN; Vaara and Vaara, 1983). Indeed, research into these aspects is currently being conducted in the authors' laboratories.

In their studies on CFA-mediated adhesion, Forestier *et al.* (1984) claim that several antibiotics, including translational inhibitors, decrease adhesion of *E. coli*, but nevertheless fail to prevent formation of fimbrial adhesins on the bacterial cell surface. The authors suggest that, although the CFAs are still synthesized, their structures may be modified to render them non-functional as adhesins. This hypothesis will be considered further in Section IV.J. Nevertheless, it should be mentioned at this point that the entity originally referred to as CFA/II was later found to consist of three distinct antigens, namely CS1, CS2 and CS3 (Smyth, 1982; Cravioto *et al.*, 1983). Antigens CS1 and CS2 are on very similar rigid fimbriae, 6–7 nm long and, although morphologically indistinguishable, are immunologically distinct (Smyth, 1984). Antigen CS3 is distinguishable from CS1 and CS2 since CS3 fimbriae are only 2 nm long (Levine *et al.*, 1984). It is therefore clear that the studies of Forestier *et al.* (1984) on CFA/II did not involve expression of a single fimbrial adhesin as assumed by these authors, but were concerned with expression of several adhesins. Undoubtedly this situation complicates interpretation of some of the data they obtained.

E. *Neisseria meningitidis* AND *Neisseria gonorrhoeae*

Attachment of *Neisseria gonorrhoeae* and *Neisseria meningitidis* to human mucosal surfaces is probably the first step in the pathogenesis of gonococcal and menigococcal infections (McGee *et al.*, 1977; Stephens and McGee, 1981). Attachment of gonococci appears to be mediated by specific adhesins because fimbriae have been identified on the surface of *N. gonorrhoeae* strains that facilitate attachment of the organisms to human fallopian tube mucosa (McGee *et al.*, 1977). Furthermore, fimbriate organisms of colonial types 1 and 2 produce disease in human volunteers, whereas non-fimbriated gonococci are avirulent (Kellogg *et al.*, 1968). Fimbriae also appear to have an important role in adhesion of *N. meningitidis*. Primary isolates possess fimbriae, fimbriate organisms attach to human cells in greater numbers than non-fimbriated strains, and fimbriated meningococci attach in greater numbers to nasopharyngeal and oropharyngeal cells, the presumed sites of

meningococcal invasion (Craven and Frasch, 1978; Devoe and Gilchrist, 1975; Salit and Morton, 1981; Stephens *et al.*, 1983; Stephens and McGee, 1981).

The effect of exposure to low concentrations of antibiotics on gonococcal adhesion has been studied by Stephens *et al.* (1984). Tetracycline and penicillin both lowered the number of fimbriae on each gonococcus, which was also accompanied by decreased adhesion to human epithelial cells. Tetracycline appeared to decrease synthesis of fimbrial subunits, as would be expected for a translational inhibitor, but penicillin acted in a different manner probably resulting either in defective assembly of fimbriae or their release from the bacterial cell surface.

The effect of antibiotics on fimbriation and adhesion of meningococci has been more extensively studied than in gonococci. Kristiansen *et al.* (1983) showed that lincomycin decreased fimbriation and adhesion of *N. meningitidis*, but growth in the presence of other antibiotics, e.g. trimethoprim, colistin and vancomycin did not affect adhesion. In contrast, Salit (1983) found that low concentrations of several antibiotics, including vancomycin and colistin, decreased meningococcal fimbriation and adhesiveness. This situation again suggests that the effects of certain antibiotics on adhesion may be strain-specific. Salit (1983) did not conduct detailed experiments to determine the basis of decreased adhesion but, in more recent work, Stephens *et al.* (1984) have shown that low concentrations of antibiotics seem either to inhibit synthesis of meningococcal fimbriae or lead to their loss from the outer membrane of the organism.

F. *Vibrio proteolytica*

Although *Vibrio cholerae* is an important human pathogen that probably adheres to host tissue by surface-located flagellae, there are apparently no studies on the effects of antibiotics on adhesion of this organism (Vosbeck and Mett, 1983). Recently, however, work has been performed on the effects of antimicrobial agents on adhesion of another *Vibrio* species, namely *V. proteolytica* an estuarine organism (Paul, 1984). Growth of this organism in the presence of ampicillin, oxacillin or streptomycin at concentrations corresponding to 0.25 of the MIC value led to inhibition of adhesion to a polystyrene surface. The mechanism(s) by which these antibiotics cause decreased adhesion is unknown since the bacterial components promoting adhesion are uncharacterized (Paul, 1984).

G. *Staphylococcus aureus*

Investigations of the effects of low concentrations of antibiotics on the

adhesion of *Staphylococcus aureus* have been concerned with staphylococcal–fibronectin associations (Proctor *et al.*, 1983). Fibronectin is a large glycoprotein (about 440,000 Da) found in the extracellular matrix of loose connective tissue and in plasma. Although the major biological function of fibronectin is probably to mediate adhesion of eukaryotic cells to the extracellular matrix, it may also serve as a matrix for bacterial colonization because several pathogenic Gram-positive bacteria (e.g. staphylococci and streptococci) bind fibronectin (Kuusela, 1978; Switalski *et al.*, 1982, 1983; Myhre and Kuusela, 1983). Growth of *Staph. aureus* in the presence of low concentrations of lincomycin, erythromycin and chloramphenicol decreased fibronectin binding to the staphylococci (Proctor *et al.*, 1983).

H. *Streptococcus pyogenes*

The pathogenicity of *Streptococcus pyogenes* is associated with its ability to adhere to and invade tissues. The cell envelope of this organism is complex, and there are several components that might be candidates for adhesins. Various surface components have been examined to identify the adhesin which is now believed to comprise lipoteichoic acid (LTA) stabilized by interaction with the type-specific surface-located M protein antigen (Ofek *et al.*, 1975; Beachey, 1981; Beachey and Ofek, 1976). Apparently the streptococci bind to uncharacterized receptors in eukaryotic cells by means of secreted LTA molecules linked to surface-bound LTA molecules which themselves are stabilized or bound to a network of M protein.

Several studies show that exposure of *Strep. pyogenes* to low concentrations of antibiotics alters their adhesiveness. Alkan and Beachey (1978) found that exposure of non-dividing *Strep. pyogenes* to low concentrations of penicillin led to loss of ability to adhere to epithelial cells, and that the effect was accompanied by loss of bacterial LTA. Penicillin did not, however, have any effect on streptococcal adhesion when growing cultures were exposed to the drug (Alkan and Beachey, 1978). Tylewska *et al.* (1981) examined the effects of several antibiotics on adhesion of *Strep. pyogenes* to human pharyngeal cells. Growth in the presence of benzylpenicillin, tetracycline or rifampicin decreased bacterial adhesiveness and this was ascribed to changes in surface hydrophobicity. This again may be associated with changes in the quantity of surface-located LTA.

I. *Streptococcus sanguis*

Streptococcus sanguis is important in the pathogenesis of endocarditis. Three major stages in the pathogenesis of this disease have been identified: (a) bacteraemia, (b) adherence of micro-organisms to the cardiac valve and (c)

vegetative growth after valve colonization (Drake *et al.* 1985). A model system simulating the adhesion step (i.e. the second stage) in the progression of endocarditis has been developed (Scheld *et al.*, 1981). Using this model, Scheld *et al.* (1981) demonstrated that *Strep. sanguis* adhesion was decreased after treatment of the bacteria for 4 hours with low concentrations of vancomycin, penicillin, tetracycline, chloramphenicol or streptomycin. Scheld *et al.* (1979) have also assessed the effect of vancomycin on the development of endocarditis due to *Strep. sanguis* after catheter-induced aortic valve trauma in rabbits. Vancomycin decreased adhesion of the organism, resulting in its inability to produce endocarditis *in vivo*. Similar observations were made by Bernard *et al.* (1981). Lowy *et al.* (1983) have shown that decreased adhesion of *Strep. sanguis* mediated by penicillin is accompanied by release of LTA from the cell wall. This mechanism may also be responsible for the decreased adherence demonstrated by vancomycin (Scheld *et al.*, 1981; Bernard *et al.*, 1981) which, like penicillin, interferes with cell-wall synthesis.

J. MECHANISMS BY WHICH ANTIBIOTICS INFLUENCE BACTERIAL ADHESION

The examples discussed in the previous sections illustrate that sublethal concentrations of antibiotics may exert their anti-adhesive effects in at least four different ways. The antibiotic may: (a) alter the overall shape of the bacterium and in so doing modify the ability of the organism to approach receptors located on the surface of eukaryotic cells, (b) promote release of an adhesin from the bacterial cell surface, (c) induce formation of functionally deficient adhesins, or (d) suppress synthesis or secretion of surface-located adhesins.

Alteration of overall cell shape by antibiotics is not usually suggested as a reason for decreased bacterial adhesion, but it may nevertheless be a significant cause, especially for β-lactam antibiotics many of which are widely recognized to cause cell-shape alterations. Antibiotic-promoted release of adhesins has been quite well documented with penicillin-promoted loss of LTA from streptococci and the same antibiotic has also been suggested to cause loss of adhesins from the gonococcal cell surface. Formation of functionally deficient adhesins has been clearly demonstrated in the case of streptomycin and type I fimbrial adhesion in *E. coli* and similar mechanisms have been invoked to explain the effects of other translational inhibitors (e.g. see the studies of Forestier *et al.* (1984) which were discussed earlier). Apart from streptomycin, which is known to induce RNA misreading and hence provides the opportunity for insertion of abnormal amino acids into a protein, the molecular basis of aberrant adhesin synthesis in the presence of other translational inhibitors is obscure. Nevertheless, it is clear that exposure of bacteria to low concentrations of certain translational inhibitors can cause

changes in the properties of surface-located polypeptides; for example, tetracycline causes alterations in the electrophoretic mobility of certain gonococcal and meningococcal outer-membrane proteins (Stephens *et al.*, 1984). Possibly these changes reflect antibiotic-mediated inhibition of post-translational polypeptide modification processes, such as protein phosphorylation or glycosylation. Thus, alterations to adhesin structure may be secondary events that follow inhibition of synthesis of unrelated proteins (enzymes).

The mechanisms operating to prevent synthesis or secretion of adhesins following exposure to antibiotics will probably prove to be the most complex to unravel in molecular terms. With respect to translational inhibitors and proteinaceous adhesins the simplest explanation would be provided by direct inhibition of synthesis of the adhesin or its component polypeptides. Although this may indeed be responsible for some of the observed effects, it has become increasingly clear that so-called "helper" proteins, located at different positions in the cell envelope, play essential roles in secretion of many fimbrial adhesins in bacteria (Oudega *et al.*, 1984; Dodd *et al.*, 1984). The role of "helper" proteins has been most closely examined in *E. coli* (Oudega *et al.*, 1984; Michaelis and Beckwith, 1982; Randall and Hardy, 1984; Pugsley and Schwartz, 1985). In this organism, many proteins (including fimbrial adhesin subunits) that are exported from the cytoplasm are initially synthesized as precursor forms with *N*–terminal extensions consisting of an extra 20–30 amino-acid residues. These precursor proteins are processed by one of two signal peptidases during or immediately after translocation of the nascent polypeptides through the cytoplasmic membrane. In *E. coli*, the signal peptidase for prolipoprotein (lipoprotein signal peptidase, LSP) is distinct from the enzyme that appears to process all other proteins containing leader sequences (leader peptidase, LP). Therefore, in *E. coli*, decreased secretion of fimbrial adhesins caused by exposure to translational inhibitors could to some extent reflect decreased synthesis of the enzyme LP. Many other chromosomal products of *E. coli* are also involved in protein export (Michaelis and Beckwith, 1982; Pugsley and Schwartz, 1985; see also Table 3). The function of these products is not yet totally clear, but they probably aid protein secretion through the envelope. In mutants that are defective for these products, precursor forms of exported proteins frequently accumulate in mutant cells. In the context of adhesion, it is already known that secretion of type 1 fimbrial adhesin subunits depends on at least one of these additional chromosomal products, namely the *secA* gene product (Dodd *et al.*, 1984). Therefore, in general, decreased secretion of fimbrial adhesins caused by exposure to translational inhibitors could to some extent reflect decreased synthesis of these "helper" products.

In addition to "helper" products which are encoded by genes dispersed

around the chromosome (see Table 3), it is clear that the structural genes for some fimbrial adhesins may be directly flanked by genes that encode further "helper" proteins specific to export or assembly of that particular adhesin (Oudega *et al.*, 1984). Examples of this situation are provided by the K88ab and K99 fimbrial determinants, but most is known about the K88ab system (Oudega *et al.*, 1984). In addition to encoding the K88ab fimbrial adhesin, the K88ab determinant contains structural genes for another four polypeptides of molecular weights 81,000, 27,500, 27,000 and 17,000 (Gaastra and deGraaf, 1982; Oudega *et al.*, 1984). The last three of these polypeptides are located in the periplasmic space (p27.5, p27 and p17) and the first in the outer membrane (p81). Information about the function of these additional K88ab polypeptides was provided by examination of mutants containing deletions in the respective structural genes (Mooi *et al.*, 1983). These studies resulted in a model to explain biosynthesis and secretion of K88ab fimbriae (Fig. 3) from which it can be seen that polypeptides p81, p27 and p17 each act as "helper" proteins during secretion or assembly of the K88ab fimbriae. A specific function for p27.5 has not been proposed (Oudega *et al.*, 1984). Mutants defective in p27.5 synthesize normal K88ab fimbriae, albeit in a smaller quantity (Mooi *et al.*, 1983). The K99 determinant encodes five polypeptides

TABLE 3. Some chromosomal genes that affect protein export in *Escherichia coli*. From Michaelis and Beckwith (1982) and Pugsley and Schwartz (1985).

Gene	Map position (min)	Product[a]	Function
Lep	54	LP	Processes most signal peptides
LspA	0.5	LSP	Processes Braun lipoprotein signal peptide
SecA	2.5	92,000 protein	Not specifically known
SecB	80.5	Not characterized	Not specifically known
SecY	72	49,000 protein	Signal peptide recognition and export
CpxA	87.8	Not characterized	Not specifically known
CpxB	41	Not characterized	Not specifically known

[a] LP, leader peptidase; LSP, lipoprotein signal peptidase.

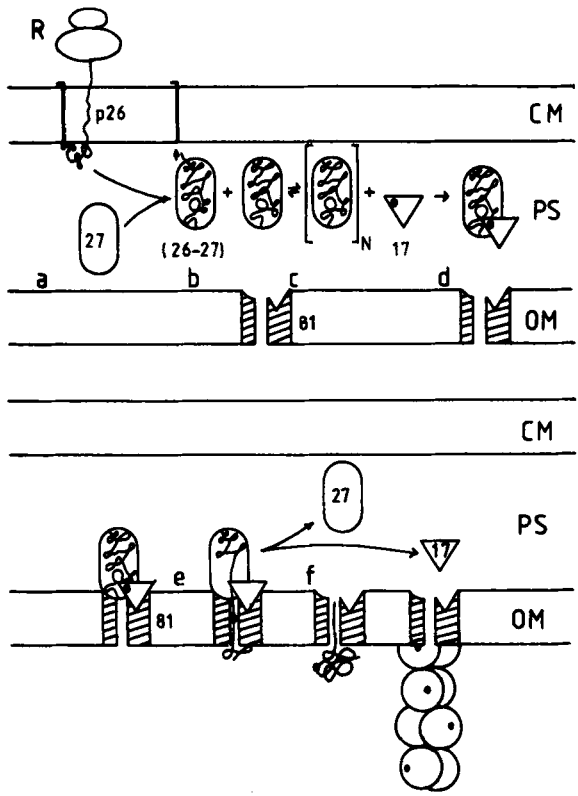


FIG. 3. Model for the biosynthesis of K88ab fimbriae. (a) The fimbrial subunit precursor (molecular weight 26,000; 26K) is translocated across the cytoplasmic membrane cotranslationally. (b) The 27,000-molecular weight (27K) polypeptide associates with the fimbrial subunit in the periplasmic space and stabilizes a conformation of the subunit that is required to translocate it across the outer membrane. (c) The 26K–27K dimers associate and dissociate to form oligomers of different size. (d) The 17,000-molecular weight (17K) polypeptide binds to the oligomers and enhances their affinity for the 81,000-molecular weight (81K) polypeptide (for simplicity only a 26K–27K dimer has been depicted). (e) Once the 17K–26K–27K complexes are bound to the 81K polypeptide, the fimbrial subunit is modified by the 17K polypeptide and translocated through channels formed by one or more 81K polypeptides in an extended conformation. The 17K and 27K polypeptides dissociate from the membrane and are re-used for further cycles of assembly. (f) The 81K polypeptide catalyses folding of the fimbrial subunits into a confirmation that polymerizes spontaneously with other subunits and also serves to anchor the growing fimbriae to the cell. The signal peptide of the fimbrial subunit precursor is indicated by the thick black line. The small vertical arrow indicates cleavage of the signal peptide. The numbers refer to the molecular masses in (kilodaltons) of the polypeptides. The thick black spot is the hypothetical group used to modify the fimbrial subunit. CM indicates cytoplasmic membrane, PS the periplasmic space, OM the outer membrane and R a ribosome. From Mooi *et al.* (1983).

of molecular weights 76,000, 21,000, 26,500, 33,500 and 19,000 which are additional to the K99 fimbrial protein subunit of molecular weight 18,200 (Oudega *et al.*, 1984). The exact functions of p76, p21, p26.5, p33.5 and p19 are not known, but they are very likely to be similar to the "helper" proteins described for K88ab fimbrial protein synthesis and secretion (Oudega *et al.*, 1984). It can therefore be seen that inhibition of K88ab and K99 mediated adhesion, by translational inhibitors (Ferreiros and Criado, 1983; I. Chopra and K. Hacker, unpublished work) could result from decreased synthesis of one or more of the following: (a) the fimbrial adhesin itself, (b) leader peptidase, (c) "helper" products such as those described in Table 3, and (d) specific "helper" products encoded by genes flanking the structural genes for K88ab and K99 fimbrial adhesins. The extent to which inhibition of each class of protein contributes to decreased adhesion has yet to be determined.

Although discussion has been focused on K88ab- and K99-mediated adhesion of *E. coli*, it seems likely that "helper" products analogous to those already described will be responsible for secretion and assembly of other fimbrial adhesins both in *E. coli* and other bacteria. Therefore decreased adhesion mediated by translational inhibitors in these other systems may also result from decreased synthesis of "helper" products.

Another two important effects of translational inhibitors on bacterial adhesion are worthy of consideration, namely why are some antibiotics more active than others in inhibiting adhesion mediated by a particular adhesin, and secondly how can certain antibiotics mediate anti-adhesive effects at concentrations that often do not appear grossly to affect other cellular metabolic processes? The answers to each of these questions are to some extent interconnected and probably arise from the ability of some antibiotics to become compartmentalized within the bacterial cell envelope (particularly the cytoplasmic membrane) resulting in localized regions where the antibiotic concentrations are greater than those in the cytoplasm. If some of these membrane regions are also domains that contain polysomes engaged in synthesis of secreted proteins, then a general explanation is provided for the ability of some antibiotics to inhibit preferentially synthesis of secreted proteins (e.g. fimbrial adhesins). Data supporting this general hypothesis have indeed been reported (Hirashima *et al.*, 1973; Randall and Hardy, 1977; Piovant *et al.*, 1978; Yasumura *et al.*, 1981). The plausibility of the hypothesis is strengthened by two further observations. The first of these is that hydrophobic translational inhibitors, which would be expected to partition into the cytoplasmic membrane, preferentially inhibit synthesis of envelope and secreted proteins in *E. coli* (Piovant *et al.*, 1978); the second is that compartmentalization of antibiotics and/or protein synthetic sites cannot be maintained following cell disruption (Randall and Hardy, 1977). In addition to compartmentalization of antibiotics within the cytoplasmic membrane, the

molecular weight of a protein is another factor that may dictate its susceptibility to inhibition because synthesis of low molecular-weight envelope proteins seems to be, in general, more resistant to antibiotics than that of high molecular-weight proteins (Yasumura *et al.*, 1981). The molecular basis of this particular phenomenon is not understood.

Mention should also be made of the possible mechanism by which low concentrations of penicillin prevent production of type I fimbriae in *E. coli* (Ofek *et al.*, 1979). As already noted in Section IV. B, this phenomenon appears to be related to filament formation induced by the antibiotic. Since filament formation in turn reflects inhibition of septum formation, it seems that synthesis or insertion of type I fimbriae into the envelope during normal growth might be regulated to occur at the time of septum formation. Although formal proof of this hypothesis is lacking, it is interesting to note that type I fimbriation in *E. coli* is subject to transcriptional regulation (Orndorff and Falkow, 1984).

Finally, it should be noted that exposure of bacteria to low concentrations of certain antibiotics can enhance adhesiveness. Antibiotics that can produce this response are clindamycin, penicillin and other β-lactam antibiotics, trimethoprim, erythromycin, vancomycin and naladixic acid (Shibl, 1985). In contrast to the wealth of information on antibiotics that inhibit adhesion, very little is known about the basis of antibiotic-stimulated adhesion. Therefore we do not intend to consider it further in this review, but readers wishing to follow up the subject should consult Shibl (1985) to gain access to the original papers describing the work. We stress that virtually nothing is known about the molecular basis of the enhanced adhesive effects, and this area would seem to be very worthy of study.

V. Effects on the Synthesis or Excretion of Extracellular Products

A. INTRODUCTION

This section will deal with the effects of antibiotics on synthesis or excretion of bacterial products that are freely liberated into the environment by physiologically functional secretion systems, i.e. it will not consider products that are released as a consequence of gross cell lysis associated with bacterial death. The products to be considered are principally proteins. Gram-positive bacteria secrete a large variety of soluble extracellular proteins but, in Gram-negative bacteria, fewer proteins are exported across both the cytoplasmic membrane and the outer membrane because the latter forms an extra barrier for protein secretion (Oudega *et al.*, 1984). Detailed consideration of protein export by bacteria cannot be attempted in this article, but readers requiring

fundamental knowledge in this area are recommended to consult recent reviews (Oudega *et al.*, 1984; Pugsley and Schwartz, 1985). It should also be noted that excretion of bacterial extracellular products shares features that are common to secretion of fimbrial adhesins. Aspects of secretion of fimbrial adhesins were considered in the previous section.

B. *Staphylococcus aureus*

Staphylococcus aureus produces a variety of extracellular proteins many of which appear to be involved in pathogenesis of staphylococcal disease, i.e. they are probably toxins (Stephen and Pietrowski, 1981). In addition to toxins, certain strains of *Staph. aureus* also produce extracellular β-lactamases which confer resistance to penicillins (Arvidson, 1983). Various experiments have been conducted to examine the effects of antibiotics on production of these extracellular staphylococcal products.

In an early study, Hinton and Orr (1960) examined the effect of antibiotics on α-haemolysin production. They observed that haemolysin production was susceptible to inhibition by streptomycin and bacitracin to a greater extent than could be merely attributed to lower growth rates. Other studies on α-haemolysin production were subsequently performed. Kobayashi *et al.* (1966) showed that low concentrations of lincomycin, chloramphenicol and erythromycin decreased production of the protein at concentrations well below those required to inhibit bacterial growth. In contrast, low concentrations of methicillin, bacitracin and vancomycin were found to increases α-haemolysin production (Kobayashi *et al.*, 1966). Note that the observations made by Hinton and Orr (1960) and Kobayashi *et al.* (1966) with respect to bacitracin are clearly discrepant. Shibl (1982), using a mouse subcutaneous abscess model to examine the effects of low concentrations of antibiotics on production of staphylococcal extracellular proteins, found that lincomycin and clindamycin were particularly effective inhibitors of α-haemolysin production. Subsequent *in vitro* studies demonstrated that the effect of lincomycin on α-haemolysin production is probably attributable to direct inhibition of synthesis of the protein rather than inhibition of secretion (Shibl, 1984). Low concentrations of cerulenin also suppress α-haemolysin production (Altenbern, 1977). This antibiotic is a specific inhibitor of β-oxo-acyl thioester synthetase and therefore interferes with both *de novo* fatty acid synthesis and with fatty-acid chain elongation from palmityl-CoA (D'Agnolo *et al.*, 1973). The possible mechanism by which cerulenin interfers with α-haemolysin production will be considered in Section V.L. The effect of antibiotics on β and δ haemolysin production has also been examined. Vymola and Lochman (1974) found that penicillin, chloramphenicol, tetracycline, erythromcyin and carbenicillin all depressed production of the haemolysins.

Mates (1973, 1974, 1975) examined the effects of low concentrations of tetracyclines and lincomycin on lipase production by *Staph. aureus*. Most of the tetracyclines were able to inhibit lipase production without affecting bacterial growth, but the most active inhibitors of lipase production were demethylchlortetracycline and minocycline (Mates, 1974). Mates (1975) also found that low concentrations of lincomycin inhibited lipase production without affecting bacterial growth, a finding subsequently confirmed by Gemmell and Shibl (1976) and Shibl (1984).

The effects of antibiotics on production of other potential staphylococcal toxins has also been examined; they include coagulase, agressin, exfoliative toxin and enterotoxins. Low concentrations of clindamycin interfered with production of the first three proteins (Gemmell and Shibl, 1976; Shibl, 1981, 1982, 1984). Low concentrations of lincomycin decreased production of deoxyribonuclease (DNAase) and coagulase (Gemmell and Shibl, 1976; Shibl, 1982, 1984), whereas low concentrations of oxytetracycline suppressed coagulase production (Peazi *et al.*, 1966). Production of staphylococcal enterotoxins B and C was completely inhibited by low concentrations of cerulenin which also suppressed coagulase production (Altenbern, 1977).

Resistance of *Staph. aureus* to penicillins is usually caused by production of β-lactamases (Barber, 1962; Dyke and Richmond, 1967). Despite the medical importance of these enzymes, which are frequently extracellular, few studies have been conducted to examine the effects of antibiotics on their production. We are only aware of two relevant studies. Michel *et al.* (1980) studied the effects of low concentrations of erythromycin, clindamycin and pristinamycin on penicillinase production by several strains of *Staph. aureus*. The effect of the antibiotics on enzyme production was strain dependent; in some strains production was enhanced by a particular antibiotic, whereas in others it was suppressed by the same drug. Chopra and Anderson (1985) examined the effects of minocycline on β-lactamase production in a single strain of *Staph. aureus*. Some inhibition of β-lactamase synthesis was detectable in organisms exposed to 0.15 of the single-cell MIC value and complete inhibition of synthesis was observed at half of the MIC value. In contrast, total cell protein synthesis was less susceptible to inhibition by minocycline.

C. STREPTOCOCCI

Most of the important human pathogens are β-haemolytic and can be classified into Lancfield groups by the nature of the polysaccharide present on the surface of the organism. *Beta*-haemolytic streptococci produce a range of extracellular toxins and enzymes which include streptolysin-O, streptolysin-S, erythrogenic toxins, DNAase, nicotinamide adenine dinucleotidase

(NADase), fibrinolysin, lipoproteinase and hyaluronidase. The extent to which these products may be involved in pathogenicity is unclear (Stephen and Pietrowski, 1981). Nevertheless, various studies have been conducted to examine the effects of antibiotics on production of streptococcal extracellular products. Gemmell and Abdel-Amir (1979) found that low concentrations of lincomycin and clindamycin inhibited production of streptolysin-S but only partially inhibited growth of *Strep. pyogenes*. Further experiments showed that the antibiotics probably had a direct effect on the synthesis of the streptolysin. Low concentrations of chloramphenicol or erythromycin did not affect streptolysin-S production (Gemmell and Abdel-Amir, 1979; Ginsburg *et al.*, 1982), but streptolysin production was enhanced by low concentrations of chloramphenicol (Ginsburg *et al.*, 1982). Production of streptococcal DNAase was strongly inhibited by chloramphenicol (0.25 of the MIC value), but not by erythromycin (Ginsburg *et al.*, 1982).

D. *Bacillus* SPECIES

Bacillus species secrete a variety of soluble extracellular proteins. Although the effects of antibiotics on exoprotein production by *Bacillus* spp. have not been extensively studied, cerulenin-mediated inhibition of protein export has been noted in several *Bacillus* species. Low concentrations of the antibiotic affect production of the following proteins: penicillinase in *B. licheniformis* and *B. cereus*, alkaline phosphatase in *B. licheniformis* and *B. subtilis*, protease in *B. cereus* and *B. amyloliquefaciens*, haemolysin in *B. cereus* and levansucrase in *B. subtilis* (Fishman *et al.*, 1978, 1980; Paton *et al.*, 1980; Caulfield *et al.*, 1979). In general, the concentrations of cerulenin affecting production of these exoproteins had little effect on cell growth or total protein synthesis.

E. *Clostridium difficile*

Clostridium difficile is a Gram-positive anaerobic bacterium whose extracellular cytotoxin has been implicated as one of the major causes of pseudomembranous colitis (Gemmell, 1982). George *et al.* (1980) reported that low concentrations of both clindamycin and lincomycin enhanced toxin production, and that the results were not simply due to enhancement of bacterial growth. Gemmell (1982) observed that clindamycin had a similar effect in some strains, but not in others. In fact, in some strains clindamycin at concentrations corresponding to only 12.5% of the MIC value suppressed cytotoxin production. These results clearly imply that the effects of clindamycin on cytotoxin release by *Cl. difficile* appear to be strain dependent. Gemmell (1982) also found that vancomycin inhibited cytotoxin production,

and 50% inhibition could be achieved with drug concentrations corresponding to 6.25% of the MIC value.

F. *Propionibacterium acnes*

It is generally agreed that skin-borne micro-organisms, in particular *Propionibacterium acnes* a Gram-positive organism, play an important role in generation of acne vulgaris (Holland *et al.*, 1981). Development of acne lesions depends, at least partially, on production of an extracellular lipase by *P. acnes* (Holland *et al.*, 1981). This enzyme releases from sebum triglycerides fatty acids that are irritating and comedogenic (Kligman and Katz, 1968). Weaber *et al.* (1971) reported that low concentrations of tetracycline caused a delay in lipase synthesis by *P. acnes*. Subsequently, Webster *et al.* (1981) confirmed these findings and also showed that erythromycin behaved similarly. The effects of tetracycline on lipase production may explain the clinical success of the antibiotic for treatment of acne vulgaris (Chopra *et al.*, 1981).

G. *Escherichia coli*

Up to this point, the micro-organisms discussed in relation to exoproduct formation have been Gram-positive bacteria. As already noted, Gram-negative bacteria are not usually recognized for their ability to liberate products into the environment, but a number of species, including *E. coli*, are able to secrete macromolecules.

Heat-labile enterotoxin (HLET) of *E. coli* is a true extracellular protein (Pugsley and Schwartz, 1985). It comprises two subunits, each of which initially contain hydrophobic leader sequences. Both subunits are exported to the periplasmic space, but the precise mechanism of assembly of HLET and its release into the medium are not well understood (Pugsley and Schwartz, 1985). It is possible that the HLET subunits are assembled in the outer membrane and then released in vesicles that are blebbed off from the cell surface (Gankema *et al.*, 1980), but not all the available data are consistent with this model (Pugsley and Schwartz, 1985). Production of HLET by *E. coli* is increased when bacteria are grown in the presence of low concentrations of lincomycin or tetracycline (Levner *et al.*, 1977; Yoh *et al.*, 1983). The molecular basis of these effects is not understood but, at least in the case of lincomycin, it is not due to an increase in the copy number of the plasmid encoding HLET (Levner *et al.*, 1977).

H. *Vibrio cholerae*

The cholera toxin (CT) of *Vibrio cholerae* is similar in many respects to the

HLET of *E. coli* described in the previous section (Pugsley and Schwartz, 1985). Production of CT, like HLET, is also increased by growing *V. cholerae* in the presence of low concentrations of lincomycin (Levner *et al.*, 1980). Production of enterotoxin by *V. cholerae* is also stimulated by low concentrations of lincomycin (Yamamoto *et al.*, 1981).

I. *Pseudomonas aeruginosa*

Pseudomonas aeruginosa secretes a number of extracellular enzymes that contribute to its pathogenicity, including at least two proteases, namely elastase and alkaline protease (Warren *et al.*, 1985). Protease production is inhibited by low concentrations of tetracycline (Shibl and Al-Sowaygh, 1980), gentamicin and tobramycin (Warren *et al.*, 1985). Whether or not these antibiotics affect production of both proteases has not been directly examined, but it seems that the mechanism by which gentamicin and tobramycin affect protease production may differ from the tetracycline-mediated inhibition (see Section V.L.)

J. *Klebsiella pneumoniae*

Apparently the only studies conducted to examine the effects of low concentrations of antibiotics on extracellular product formation in *Kl. pneumoniae* concern siderophore production. To obtain essential iron from the environment, bacteria synthesize and secrete iron-chelating compounds known as siderophores (Lankford, 1973). The chelated iron is delivered to the cell by means of envelope receptors which bind the siderophores. Kadurugamuwa *et al.* (1985) showed that low concentrations of a variety of β-lactam antibiotics inhibited production of the siderophore enterochelin, but did not affect bacterial growth.

K. MECHANISMS BY WHICH ANTIBIOTICS INFLUENCE EXTRACELLULAR PRODUCT FORMATION

Essentially three classes of antibiotic have been examined for their effects on bacterial extracellular product formation. These are (a) translational inhibitors, (b) cell-wall synthesis inhibitors, principally β-lactam antibiotics, vancomycin and bacitracin, and (c) the inhibitor of fatty-acyl residue synthesis, cerulenin.

The ability of low concentrations of translational inhibitors to inhibit exoprotein production almost certainly results from mechanisms similar to those already discussed for inhibition of proteinaceous fimbrial adhesins. Therefore, as described for adhesins, inhibition of exoprotein production could result either from direct effects on synthesis of the specific protein concerned, or indirectly by inhibition of "helper" products required for

protein secretion through the bacterial cell envelope. As for adhesins, a number of "helper" products have already been identified for exoprotein secretion in a variety of bacteria, ranging from leader peptidases to more poorly characterized products (Pugsley and Schwartz, 1985). As already noted in the discussion on adhesion, hydrophobic translational inhibitors tend to cause greater inhibition of envelope and secreted-protein synthesis than hydrophilic drugs. These conclusions are further supported by some studies on exoprotein production. For instance, as already noted, Chopra and Anderson (1985) reported that relatively low concentrations of minocycline (a hydrophobic member of the tetracycline group) caused inhibition of β-lactamase production in *Staph. aureus*, but had little effect on bulk protein synthesis. These results are consistent with the hypothesis that β-lactamase is synthesized on membrane-bound polysomes which are more accessible than cycloplasmic ribosomes to hydrophobic antibiotics such as minocycline. A similar explanation probably applies to the findings of Mates (1974) who observed that low concentrations of minocycline inhibited production of staphylococcal extracellular lipase without affecting bacterial growth.

Although inhibition of exoprotein production by translational inhibitors probably results usually from inhibition of polypeptide translation, it has been suggested that low concentrations of such antibiotics can act in a different manner. For example, Warren *et al.* (1985), in their studies on protease production by *Ps. aeruginosa*, suggest that gentamicin and tobramycin may affect synthesis of the enzymes by lowering the concentrations of cyclic nucleotides rather than selectively inhibiting protein synthesis. Although *Ps. aeruginosa* protease production is not apparently controlled by catabolite repression (see Warren *et al.*, 1985), their suggestion is to some extent supported by earlier studies on *E. coli* where low concentrations of streptomycin were reported to cause selective inhibition of β-galactosidase production (a cytoplasmic enzyme) by lowering cyclic AMP concentrations (Pinkett and Brownstein, 1974; Artman *et al.*, 1972). It is known that expression of the gene for β-galactosidase is dependent on the concentrations of 3′,5′-AMP in the cell.

In the discussion of translational inhibitors, mention should also be made of several reports where low concentrations of antibiotics have been found to stimulate exoprotein production. For example, lincomycin stimulated production of cytotoxin by *Cl. difficile*, *E. coli* HLET and *V. cholerae* CT, tetracycline stimulated production of *E. coli* HLET, and chloramphenicol stimulated production of streptolysin-S in streptococci. Nothing is known about the molecular events leading to enhanced toxin production, and this would seem to be an important area for future research.

Little is known about the mechanisms by which low concentrations of cell-wall synthesis inhibitors decrease production of extracellular products, but it has been suggested that changes in the topography of bacteria induced by

these drugs might impair secretion of exoproteins (Gemmell, 1982). Cerulenin, which inhibits fatty-acyl residue synthesis, affects production of a number of bacterial exoproteins. At least one of the proteins affected, the penicillinase of *B. licheniformis*, is known to be post-translationally modified by addition of fatty acyl and glyceride groups to the *N*-terminal cysteine of the mature polypeptide (Pugsley and Schwartz, 1985). The role of the lipid groups is unclear, but they may be required to orientate correctly the enzyme in the membrane for cleavage at one or more sites prior to release into the medium (Pugsley and Schwartz, 1985). Inhibition of fatty-acyl residue synthesis by cerulenin presumably results in inhibition of attachment of lipid to penicillinase and therefore a decrease in liberation of the enzyme into the environment. A similar explanation may apply to the other exoproteins whose production is affected by cerulenin, but it is not known in these instances whether the proteins concerned are synthesized as lipoproteins.

VI. Effects on Lipid Synthesis

The lipid composition of bacteria grown in the presence of low concentrations of antibiotics has been examined by several groups (e.g. Berminghan *et al.*, 1970; Starka and Moranova, 1970; Veerkamp, 1976; Brisette *et al.*, 1982; El-Khani and Stretton, 1983). With β-lactam antibiotics, changes in both the rate of lipid synthesis and composition of lipids have been reported (Starka and Moronova, 1970; Brisette *et al.*, 1982; El-Khani and Stretton, 1983). In their studies on *Salmonella typhimurium*, El-Khani and Stretton (1983) found that growth of the organism in the presence of benzylpenicillin, ampicillin or mecillinam did not affect the total lipid content. However, the proportions of individual lipids were affected in the bacteria exposed to β-lactam antibiotics; phosphatidylserine, diphosphatidylglycerol (cardiolipin) and palmitoleyl residue proportions were higher, but the proportions of cyclopropane C_{17} residues were decreased. Since similar effects were observed with tetracycline (El-Khani and Stretton, 1983), it seems that the reported alterations in lipid composition observed in bacteria exposed to low concentrations of antibiotics could be due to secondary non-specific events, caused by a general decrease in rates of bacterial multiplication.

VII. Effects on Host Defences

A. INTRODUCTION

As already noted in Section II, antibiotics at concentrations equal to or at a

fraction of their MIC values produce morphological and ultrastructural changes in bacterial cells that are likely to render them more susceptible to host defences (Lorian, 1985). Host defences (specific and non-specific) include serum components (e.g. antibody and complement) and cellular responses (particularly phagocytic cells). Each will be considered separately.

B. EFFECTS ON ANTIGENICITY AND AGGLUTINABILITY OF BACTERIA

Morphological changes resulting from exposure to subminimal concentrations of antibiotics are often associated with changes in the biochemical composition of the cell envelope. Since the specific antigenic constituents of Gram-negative bacilli are located in lipopolysaccharides at the surface of the bacteria, it is possible that the immunogenic responses of the cells could be affected by subinhibitory concentrations of antibiotics.

Lorian *et al.* (1976) treated normal and abnormal *Salmonella* sp., prepared by growing in the presence of gentamicin, tetracycline, chloramphenicol or ampicillin, with complimentary antiserum to the same organism. The abnormal cells produce a higher titre (1/2500) than normal cells (1/320). Observed under a phase-contrast microscope, agglutination of abnormal cells was floccular due to a loose clumping of the filamentous organisms, compared with a typical granular O-type reaction observed with the normal cells. It was concluded that the higher titre was a consequence of changes in the physical distribution of the antigens on the surface of the antibiotic-grown cells, and not to a change in the nature of the antigens. This conclusion was confirmed by Lorian and his group (Atkinson and Amaral, 1982) who raised antisera against both normal and mecillinam-grown *Sal. typhimurium*. Fluorescent labelling of both antisera failed to show any antigenic differences between normal and abnormal cells. Also, extracts of cell walls of both types of bacteria failed to show differences in precipitin reaction on Ouchterlony plates.

Viano *et al.* (1979) also showed that, while the antigenic determinants in *Sal. wien* were qualitatively the same, their antigenicity in rabbits differed. The abnormal ovoid-filamentous bacteria produced a greater immunogenic response than that found with normal cells; this was also attributed to the physical distribution, or greater availability, of the antigens on the surface of abnormal cells.

C. SERUM EFFECTS *in vitro*

A number of components of normal serum have a bactericidal action on bacteria. In the presence of opsonin (often naturally occurring antibodies), complement is one of the most efficient bactericidal factors. But can this effect be increased in cells treated with subminimum inhibitory concentrations of

antibiotics? Normal human serum has been shown to have little effect on antibiotic-treated Gram-positive bacteria (Ahlstedt, 1981). However, since complement is known to kill Gram-negative bacilli by first attacking the outer membrane, changes in this structure resulting from exposure to low concentrations of antibiotics could affect the susceptibility of cells to normal serum.

Various workers have reached different conclusions. A synergistic killing effect on *E. coli* by subminimum inhibitory concentrations of a number of antibiotics and the complement factor of normal human serum has been observed. They include polymixin B (Rowley and Wardlaw, 1958), nafcillin (Warren and Gray, 1967), ampicillin, trimethoprim, streptomycin, tetracycline (Dutcher *et al.*, 1978) and rifampin (Alexander *et al.*, 1980). In contrast, Forsgren and Gnarpe (1973a,b) reported that tetracycline inhibited the killing effect by normal human serum, and neither chloramphenicol (Traub and Sherris, 1970; Dutcher *et al.*, 1978) nor nalidixic acid (Dutcher *et al.*, 1978) produced a synergistic effect against *E. coli.*

Working with serum-resistant *E. coli*, Taylor *et al.* (1981) reported that growing organisms in medium containing subminimum inhibitory concentrations of mecillinam rendered them susceptible to the complement-mediated bactericidal action of normal human serum. This was not observed with other β-lactam antibiotics and was thought to be related to the unique binding of mecillinam to penicillin-binding protein 2 (Spratt, 1983). Similar treatment of serum-sensitive *E. coli* did not alter their response to serum.

Hart *et al.* (1982) also separated the application of the two agents in time by first incubating *E. coli* K12, for various periods, in the presence of bacteriostatic concentrations of antibiotic and subsequently incubating a sample of the mixture, diluted beyond an effective antibiotic concentration, with normal human serum or heat-inactivated human serum. Survival was determined at the beginning and end of incubation. Tetracycline and chloramphenicol potentiated the killing of *E. coli* by normal human serum, whereas nalidixic acid and rifampin inhibited the killing effect. Killing of cells did not occur with heat-inactivated human serum (i.e. complement-deficient serum), either on normal or antibiotic-treated bacteria, indicating that the killing effect was dependent on complement in the presence of "natural" antibody in the serum. It was concluded that changes in susceptibility to complement were due to surface changes, probably at different locations, on the bacterial surface.

Lorian and Atkinson (1979), in a study of five genera of Enterobacteriaceae, namely. *E. coli*, both serum-sensitive and resistant, *P. mirabilis*, *Serratia marcescens*, *Sal. typhimurium* and *K. pneumoniae*, each grown in the presence of subinhibitory concentrations of ampicillin and mecillinam, found that the killing effects of normal human serum or whole blood were less than their effects on bacteria grown in the absence of the antibiotics. These contradictory

findings are thought to be due, in part, to the different criteria used to evaluate the effects at subminimum inhibitory concentrations of different antibiotics. Furthermore, the different methods used to obtain the antibiotic-treated organisms may result in different degrees of osmotic stress being imposed on the fragile cells which may influence their susceptibility to host defences.

D. EFFECTS ON PHAGOCYTOSIS

Polymorphonuclear leucocytes, by virtue of their ability to ingest and kill micro-organisms, serve as one of the more important host defences against infection. Foci of infection, such as abscesses, are often surrounded by these leucocytes (Joiner *et al.*, 1981) which may also limit penetration of bacteria by antibiotics. This may result in the causal organisms being exposed to low concentrations of drug which in turn may influence the phagocytosis of bacteria. It is important, therefore, to consider whether subminimum inhibitory concentrations can influence the phagocytic process.

The degree of phagocytosis and subsequent intracellular killing may be affected by the size, shape and nature of the infecting bacteria, features that can change as a result of growth in the presence of subminimum inhibitory concentrations of different antibiotics. Drugs that affect cell wall synthesis may or may not potentiate phagocytosis depending on whether filament formation is induced by low concentrations of antibiotic. Phagocytes have been shown to ingest filamentous bacteria (Lorian and Atkinson, 1984; Fig. 4). This does not imply, however, that the filamentous forms are more susceptible than individual bacilli to opsonization and phagocytosis (Milatovic, 1984).

Friedman and Warren (1974, 1976) showed that penicillin-resistant staphylococci, after incubation *in vitro* with low concentrations of nafcillin or cyclacillin were more susceptible to phagocytosis by mouse macrophages. Similar enhancement of phagocytic function was reported by Root *et al.* (1981). Exponentially growing *Staph. aureus*, pretreated with subminimum inhibitory concentrations of penicillin and vancomycin, were more suceptible to phagocytosis by human polymorphonuclear leucocytes but gentamicin showed no enhancement. Lorian (1985) reported that large staphylococci resulting from the action of β-lactam antibiotics were phagocytosed as readily as staphylococci grown in the absence of antibiotic. Similarly, phagocytes could engulf filamentous forms of Gram-negative bacilli. However, the mass of the individual bacteria following exposure to subminimum inhibitory concentrations of antibiotics exceeded that of normal cells resulting in a more efficient phagocytic response (Lorian and Atkinson, 1984).

Many organisms resist the host defences, and phagocytosis in particular, by synthesizing surface structures. The M-protein on the surface of strains of

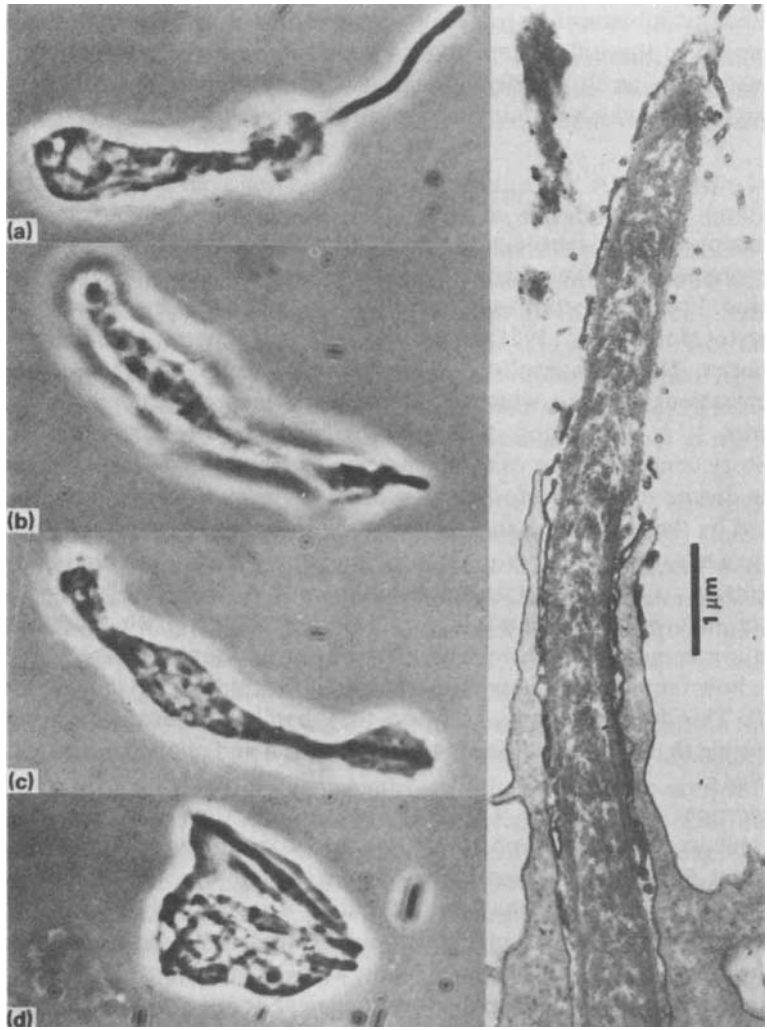


FIG. 4. Phagocytosis by a polymorphonuclear leucocyte of a cell of *E. coli* grown in the presence of a subminimal inhibitory concentration of ampicillin. (a–c) shows the initial stages, (d) two hours after phagocytosis the filament has been degraded inside the leucocyte and (e) shows a detailed picture of a leucocyte engulfing a filament. From Lorian (1985). Similar phenomena have been observed with other Gram-negative bacterial species and with other β-lactam antibiotics (Lorian and Atkinson, 1984).

Strep. pyogenes is typical. By growing an M-protein-positive streptococcus in the presence of subminimum inhibitory concentrations of clindamycin, Gemmell *et al.* (1981) demonstrated that the hair-like structures of M-protein were not produced and the resultant streptococci were more readily phagocytosed and killed by polymorphonuclear leucocytes than normal ones. Similar findings were subsequently reported for the action of penicillin on Group-B streptococci (Horne and Tomasz, 1981). Milatovic (1982) demonstrated that clindamycin and doxycycline enhanced phagocytic uptake of *Staph. aureus* grown in the presence of these drugs.

It is clear that not all workers in this field have obtained the same effects or reached the same conclusions. Differences may be due to the experimental conditions under which the assays were undertaken, such as the bacterial species used, the nature of exposure of the cells to the antibiotic, and the source of polymorphonuclear leucocytes. Caution is therefore necessary in drawing conclusions relative to clinical significance. Morphologically abnormal bacteria have been seen in clinical material following antibiotic therapy (Lorian and Atkinson, 1975), but it is not clear whether the abnormal cells are more or less susceptible to phagocytosis (Gemmel, 1984).

VIII. Bacterial Recovery After Exposure to Low Concentrations of Antibiotics

Much of the work described so far has involved experiments in which organisms have been cultivated in the laboratory with continuous exposure to low concentrations of antibiotics. However, antibiotic concentrations within the body frequently diminish exponentially during therapy, and thus do not remain constant as in the usual *in vitro* test systems (Bergan and Carlsen, 1985). Initially, antibiotic concentrations at the site of infection may exceed the *in vitro* MIC value for a particular organism, falling after some time to values corresponding to the MIC value, and then finally to subminimum inhibitory concentrations (Bergan and Carlsen, 1985). The overall effects on bacteria during a period of exposure to continuously decreasing subminimum inhibitory concentrations of antibiotic, and the possible subsequent recovery of the bacteria have been studied by several workers (e.g. see Bergan and Carlsen, 1985; Haag, 1985; Vogelman and Craig, 1985 for recent reports).

One approach to studies of this kind involves *in vitro* experiments where dilution or dialysis of antibiotic is employed to provide conditions approximating to a physiological decrease in the concentration of the antimicrobial compound. Bergan and Carlsen (1985) studied the response of several bacterial species to a variety of bactericidal and bacteriostatic agents added at various multiples of the MIC values and eliminated at different rates. The

main findings were: (a) for most drugs a drop in bacterial numbers persisted while the drug concentrations remained above the MIC value; (b) bacteria did not resume multiplication until antibiotic concentrations fell to at least the IC_{50} value of the antibiotic; (c) variable periods of time elapsed before bacterial multiplication resumed at the same rate as in control antibiotic-free cultures. Apparently this pattern of events occurred during exposure both to bactericidal and bacteriostatic agents.

Detailed examination of the physiology of bacteria during exposure to decreasing concentrations of antibiotics has not yet been attempted. However, interpretation of data that might be obtained on the physiological status of the organisms during periods of decreasing antibiotic concentrations are likely to be complicated by a number of factors. For instance, it will probably be difficult to distinguish the effects that result from exposure to subminimum inhibitory concentrations of antibiotic from the post-antibiotic effects that follow limited exposure to higher concentrations of an antimicrobial agent (Vogelman and Craig, 1985). Therefore, the principal value of this type of research will probably be the provision of data relevant to the clinical situation, e.g. whether intermittent antibiotic dosage schedules might be appropriate (Bergan and Carlsen, 1985; Haag, 1985).

IX. Effects of Low-dosage Antibiotic Administration on the Selection of Resistant Bacteria

A. INTRODUCTION

One of the adverse effects of the *in vivo* use of antibiotics has been the selection of resistant strains. Beside being used therapeutically and prophylactically, antibiotics have also been used at subtherapeutic concentrations since the 1950s for growth promotion in meat animals (Linton, 1981). Not all antibiotics are good growth promoters, but those that are effective include the tetracyclines, penicillin, bacitracin, tylosin, oleandomycin, virginiamycin and streptomycin (Kiser, 1984). It was assumed that these antibiotics inhibited certain gut bacteria which had a deleterious effect on growth of the animal (i.e. food conversion) and health, enabling the animal to grow at its full potential. The expectation that this beneficial effect might decline with time due to the emergence of resistant strains has not been realized in spite of many years of use.

But can these subtherapeutic doses, administered continuously in feed over many weeks, select for antibiotic resistance? This question has occupied the thoughts of the various licensing authorities in different countries for many years and provoked the setting up of the Swann Committee in the UK who

reported their findings in 1969 (Report, 1969). Even now, an answer to the problem and its significance for human health remain elusive. In 1980, the Food and Drugs Administration commented, "After reviewing the evidence, the committee concluded that the postulated hazards to human health from the subtherapeutic use of antimicrobials in animal feeds were neither proven nor disproven ..." and concluded, "... it is not possible to conduct a feasible, comprehensive epidemiological study of the effects on human health arising from the subtherapeutic use of antimicrobials in animal feed." (Report, 1980).

There are many reasons for this. One problem is due to the fact that antibiotics have been used for nearly 40 years for therapy and prophylaxis in man and animals simultaneous with their use in animals for growth promotion. Where antibiotic resistance has arisen, it has not been possible to separate the effects of these different usages. Another problem is the difficulty of obtaining animals free from antibiotic-resistant bacteria in which the effects of subtherapeutic concentrations may be determined. The problem is very real since, as Langlois *et al.* (1983) have shown, even after discontinuing the use of all antibiotics in a herd of swine for over 10 years, 40% of the gut *E. coli* were still resistant to tetracycline.

B. EFFECTS OF SUBINHIBITORY CONCENTRATIONS ON BACTERIA *in vitro*

To determine whether subminimum inhibitory concentrations of antibiotics could select for drug-resistant mutants, a number of workers have grown pure cultures of bacteria repeatedly in media containing low concentrations of antibiotics with various modes of action. Grassi (1978, 1979) cultured *Staph. aureus*, *E. coli* and *Ps. aeruginosa* separately in the presence of 25% of the minimum inhibitory concentrations of penicillin, ampicillin, cloxacillin, carbenicillin, chloramphenical, gentamycin and fosfomycin, respectively. The organisms were subcultured for up to 30 times in the presence of each antibiotic. With few exceptions, the minimum inhibitory concentrations for each organism were not altered and mutation to resistance was not observed.

Korting *et al.* (1984) repeatedly subcultured *Neisseria gonorrhoeae* in the presence of subminimum inhibitory concentrations of cefotiam, ceftizoxime, rifampicin and penicillin G. Each time the most resistant colonies were propagated up to 25 times. While resistance to rifampicin increased readily, the same was not true of the cephalosporins and penicillin G.

Tomich *et al.* (1980) reported that low concentrations of erythromycin had a marked influence on resistance in *Streptococcus faecalis*. They investigated the erythromycin transposon Tn*917*. *Streptococcus faecalis* was cultured in the presence of 0.5 μg erythromycin ml^{-1} for 1, 2, 3 and 4 hours, after which

the concentration was increased to 500 μg ml^{-1}. The period of induction using 0.5 μg ml^{-1} led to an efficient increase in resistance to the high dose. It was also shown that concentrations of erythromycin to 1% of the MIC value increased the transposition frequency of the resistance gene.

In 1978, Petit *et al.* speculated that there may be resistance factors whose conjugative abilities are inducible by the antibiotic to which they mediate resistance. Such a mechanism would result in efficient spread of the plasmid and thus a rapid adaptation of a bacterial population to changes in environmental conditions. Subsequently, Privitera *et al.* (1979, 1981) found such a system in *Bacteroides fragilis*, where transfer of tetracyline-resistance plasmids depends on induction by subinhibitory concentrations of tetracycline. Increased conjugal activity in the presence of tetracycline is not, however, directly due to expression of resistance as there is independent control of both resistance and its transferability (Privitera *et al.*, 1981). A distinct but related phenomenon may apply to transposition of the tetracycline-resistance genes contained in transposon Tn*10*. Schmidt *et al.* (1981) detected a tetracycline-inducible RNA species encoded by the inverted repeat of Tn*10*, which they suggest may be involved in the transposition process itself. Thus, low concentrations of tetracycline may increase the transposition frequency of Tn*10*.

Lebek and Egger (1983) conducted experiments with a controlled laboratory system in a chemostat. They incubated three strains of *E. coli* in mixed culture including a sensitive, plasmid-free strain, a sensitive plasmid-carrying one and a resistant R-factor-carrying strain, in the presence of different concentrations of tetracycline. They observed that at 10% of the MIC value (0.25 μg ml^{-1}) preferential growth of the tetracycline-resistant strain occurred.

C. EFFECTS OF SUBINHIBITORY CONCENTRATIONS ON BACTERIA *in vivo*

The same three strains of *E. coli* used by Lebek and Egger (1983) were tested by D.E. Corpet (personal communication) in gnotobiotic mice. The mice were given the three strains simultaneously *per os* and their excretion was monitored. In the absence of antibiotic, the tetracycline-resistant strain became dominant and the streptomycin-resistant strain was eliminated. Mice were infected with the rifampicin-resistant strains and given tetracycline continuously in their drinking water at concentrations of 1, 4 and 16 μg ml^{-1}; they were then challenged with the tetracycline-resistant strain. This resulted in the creation of a strain, by genetic transfer, which was resistant to rifampicin and tetracycline. This strain became dominant and replaced the rifampicin-resistant one. The rapid spread of the R-plasmid has not been demonstrated *in vitro* which suggested that interactions *in vitro* between *E. coli*

strains cannot be directly extrapolated to situations *in vivo*. Further work indicated that this R-plasmid transfer also occurred in the gnotobiotic mice in the absence of tetracycline selection.

Goldberg *et al.* (1971) investigated the influence of oxytetracycline administered to test persons over a period of 6 months. They showed that a dose of 10 μg a day was sufficient to cause the selection of even highly resistant coliform bacteria in some of the test persons. Other workers have shown a dose-related selective response. For instance, Hirsh *et al.* (1974) demonstrated that 1000 mg of oxytetracycline a day in four equal doses for 9 days favoured selection of tetracycline-resistant *E. coli* in humans, whereas 50 mg a day did not. Rollins *et al.* (1974) also demonstrated that 2 p.p.m. tetracycline in the diets of dogs, guinea pigs and rats failed to select antibiotic-resistant lactose-fermenting microflora.

The different conclusions of these studies must reflect the differences in composition of microflora present in the animals studied (whether conventionally raised or axenic) as well as the concentrations of antibiotics achieved in the gut of the animals by the various doses used and the method of administration. It must be appreciated that the conclusions reached for studies *in vitro*, using pure cultures of bacteria, cannot be extrapolated to studies *in vivo* where the dynamics of growth and the complexity of bacterial competition and interaction are so very different.

X. Effects on the Expression of Antibiotic Resistance in Bacteria

It is well established that expression of many antibiotic resistance mechanisms, particularly those encoded by plasmids, are inducible (i.e. the level of resistance increases in response to the presence of subinhibitory concentrations of the drug). Although such responses do indeed comprise another aspect of the effects of low concentrations of antibiotics on bacteria, we will not consider them in detail here. Several reviews are available which deal with induction of antibiotic resistance (i.e. regulation of expression) and interested readers are referred to these (Davies and Smith, 1978; Foster, 1983; Chopra, 1985).

XI. Effects of Low-dosage Antibiotic Administration on the Outcome of Bacterial Infection

A. INTRODUCTION

There are many *in vivo* sites (e.g. sputum, fibrin clots, abscesses and

endocarditis vegetations on heart valves) where the antibiotic concentrations achieved during therapy never attain, or rapidly fall below, the *in vitro* MIC value (Lorian *et al.*, 1984). O'Connel and Plaut (1969) showed that, in artificially produced fibrin colts *in vitro*, the peak concentrations of penicillin were significantly lower than concentrations maintained in plasma. Nevertheless, antibiotics frequently produce a clinical response in patients where these situations obtain. Such responses raise questions concerning the correlation between *in vitro* MIC values and the sensitivity of the infecting pathogen *in vivo*. Although the concept of an MIC value for a particular antibiotic and organism seems to be relatively straightforward, the conventional *in vitro* methods for determining the MIC value may significantly overestimate the concentration of the agent required to inhibit growth of the organism on first exposure *in vivo* (O'Grady, 1982). Apart from this, the *in vitro* and *in vivo* systems differ fundamentally (Zak *et al.*, 1985). *In vitro* MIC determinations are made under simple, predominantly static, conditions with relatively heavy inocula. In contrast, the *in vivo* system is dynamic because continuous variations are arising from host–parasite–antibiotic interactions. The infection process develops through a series of stages with different virulence factors assuming importance at each stage (Drake *et al.*, 1985). Subminimum inhibitory concentrations of antibiotics, which are known to have a profound effect on the morphology and function of the invading pathogen, may affect the sensitivity of the organism to the various host factors, such as phagocytosis, or render the bacteria incapable of adhesion, an essential step in the full expression of virulence by many pathogens. The combined effects of subminimum inhibitory concentrations of an antibiotic and the host defences may explain the success of many therapeutic procedures even where MIC values are not attained.

B. EFFECTS OF SUBMINIMUM INHIBITORY CONCENTRATIONS OF ANTIBIOTIC ON BACTERIAL MORPHOLOGY OBSERVED *in vivo*

Some of the effects of subminimum inhibitory concentrations of antibiotics observed under laboratory conditions, such as changes in bacterial morphology, have also been shown to occur *in vivo*. These particular changes may render the bacteria more susceptible to host defences (Washington, 1979) and result in a clinical response.

Elongated and filamentous forms of *Ps. mirabilis*, *E. coli* and *Salmonella typhimurium* were observed in the peritoneal exudate of rabbits in which a steady state of β-lactam antibiotics was maintained over the range 50 to 12.5% of the MIC value for each organism (Zak and Kradolfer, 1979). Enlarged forms of these bacteria were also observed with gentamicin. Ryan and Monsey (1981) studied the effects of subminimum inhibitory concentrations

of seven cephalosporins on mice infected with *E. coli*. Different morphological changes in the bacteria were observed in the peritoneal exudate with each cephalosporin dependent on the affinity of the cephalosporin for the penicillin-binding proteins (PBP 1a, 1b, 2 and 3). However, with each agent, subminimum inhibitory concentrations rapidly decreased the number of viable organisms in the exudate by more than 90%.

Lorian *et al.* (1982a,b) observed large cells of staphylococci in the peritoneal fluid of infected mice treated with subminimum inhibitory concentrations of oxacillin. Similar large forms were also observed in the sputa of patients treated with β-lactam antibiotics. Both normal-sized and large staphylococci have also been demonstrated in endocarditis heart valve vegetations in rabbits treated with low-dosage cloxacillin (Lorian *et al.*, 1984). Sections of the vegetations revealed large cells in areas nearest to the blood supply from which low concentrations of antibiotic had reached the fibrin mass by diffusion. However, normal-sized bacteria were embedded in the fibrin mass furthest from the blood supply to which little, if any, antibiotic had diffused. The ultrastructure of the large staphylococci was comparable with that shown in staphylococci grown in the presence of subminimum inhibitory concentrations of cloxacillin *in vitro*.

C. EFFECTS OF SUBMINIMUM INHIBITORY CONCENTRATIONS OF ANTIBIOTICS ON EXPERIMENTAL INFECTIONS

Since 1977 many reports on the successful treatment of bacterial infections in experimental animal models have been published. Using a mouse model, Comber *et al.* (1977) showed that 50% of the mice were protected from infection with *E. coli* when 25% of the MIC value of amoxicillin was maintained in the blood, although a similar concentration of ampicillin, in blood and peritoneal fluid, failed to protect mice (Zak and Kradolfer, 1979). The difference was attributed to the greater potency of amoxicillin in mice (Rollinson *et al.*, 1977). Zak and Kradolfer (1979) also showed that ampicillin and gentamicin maintained in the rabbit peritoneal fluid at 33% of the MIC value for 6 hours, gave 50% survival against artificial *E. coli* infections and prolonged the survival time of those that died. Similar therapeutic success was obtained with subminimum inhibitory concentrations of ampicillin, cephaloridine or gentamicin against *Ps. mirabilis* infections in rabbits. Subminimum inhibitory concentrations of ampicillin, however, were not effective against artificial *S. typhimurium* infections in rabbits.

Lam *et al.* (1984) sought to determine whether the observation *in vitro*, that a subminimum inhibitory concentration of penicillin renders staphylococci more sensitive to host defences, also occurred *in vivo* and thereby contributed to the therapeutic efficacy of the drug. Diffusion chambers were implanted

subcutaneously in rabbits and intraperitoneally in mice. Staphylococci and polymorphonuclear leucocytes could be localized in these chambers which were impermeable to cellular host factors but allowed diffusion of soluble factors. They found that staphylococci, sensitized *in vitro* by pretreatment with subminimum inhibitory concentrations of penicillin, retained their hypersensitivity to phagocytosis *in vivo* during their implantation in the chambers. Similarly, normal staphylococci and polymorphonuclear leucocytes in implanted chambers, when exposed to subminimum inhibitory concentrations administered to the animal, became hypersensitive to the polymorphonuclear leucocytes. However, because the staphylococcal growth rate was considerably decreased *in vivo*, the degree of penicillin-induced sensitivity to polymorphonuclear leucocyte-killing was less than that obtained *in vitro*. A decrease in the virulence of penicillin-treated staphylococci in mice was also observed.

D. CLINICAL RESPONSE TO SUBMINIMUM INHIBITORY CONCENTRATIONS OF ANTIBIOTICS

There is a growing body of clinical evidence that many patients are given doses of antibiotics in excess of the amounts necessary to achieve a cure (O'Grady, 1982). For instance, Reymann *et al.* (1979) reported that a group of patients with sepsis due to Gram-negative bacilli, when treated with gentamicin at serum concentrations corresponding to 12.5% of the MIC value or lower, showed the same rate of mortality compared with a group given higher doses. A number of clinical trials have shown that, in the treatment of urinary-tract infections, regimens either of much shorter duration (even to the point of requiring only a single dose; Anderson, 1980) are as efficacious as conventional treatments. Ben-Redjeb *et al.* (1984) reported on two similar groups of patients with symptomatic urinary-tract infections caused by *E. coli* . One group was treated with 10 mg of ampicillin and 2 litres of fluid daily for 3 days. The ampicillin concentration in the urine reached a peak at 80% of the MIC value of the infecting organism and declined to a trough at 12% of the MIC value. Sixteen of the twenty patients were cured as determined by laboratory testing. The other group received only 2 litres of fluid for 4 days and these continued to have high *E. coli* numbers and leucocyte counts.

Rosenstock *et al.* (1985) reported a 75% cure with a single dose of 2 g of tetracycline in a group of women with signs and symptoms of lower urinary-tract infections. Multi doses of 500 mg of tetracycline, four times a day for 10 days achieved a 94% cure, whereas another group given a single dose (3 g) of amoxicillin realized a 54% cure. These observations indicate that low concentrations, often below the MIC values, achieved at the target site *in vivo*, frequently effect a clinical cure in serious infections.

XII. Conclusions

The studies described in this review have revealed an almost bewildering array of responses that can occur in bacteria following their exposure to low concentrations of antibiotics. Furthermore, in the majority of cases, the underlying molecular events associated with these changes are unknown. Clearly, one of the goals for those engaged in future research in this area is a more comprehensive understanding of the fundamental alterations in bacterial metabolism and structure following exposure to low concentrations of antibiotics. Nevertheless, despite gaps in our understanding of the molecular basis of the changes, data are accumulating which show that low-level, or single-dose, antibiotic administration can be used for the successful therapy of certain bacterial infections. Indeed, a decrease in antibiotic dosage, achieved for a number of important diseases, probably constitutes the most important applied aspect of research into the effects of low concentrations of antibiotics on bacteria (O'Grady, 1985). Further steps towards decreasing the concentrations of antibiotics administered to man and animals offer the prospect of minimizing the adverse side effects that can be experienced during the therapeutic administration of these agents (Midtvedt, 1985a,b).

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Note Added in Proof

Since the main part of this article was prepared, additional relevant publications have come to our notice. One paper (Seidl *et al.*, 1985) deals with the effects of low concentrations of penicillin G on peptidoglycan metabolism in *Streptococcus pyogenes*. Several papers deal with antibiotics and adhesion. Deneke *et al.* (1985) examined the effects of very low concentrations of tetracycline on attachment of K88 enterotoxigenic *Escherichia coli* to pig intestinal cells, whereas Hales and Amyes (1985) studied the effects of a wider range of antibiotics on the *in vitro* adhesion of Gram-negative uropathogens. Work recently completed in Bristol demonstrates concomitant suppression of synthesis and secretion of K88ac fimbriae in *E. coli* exposed to low concentrations of certain translational inhibitors (Chopra and Hacker, 1986). Effects of other translational inhibitors on K88 antigen synthesis have also been reported by Yoshida *et al.* (1986). With respect to adhesion of Gram-positive bacteria, Nealon *et al.* (1986) provide further evidence that low concentrations of penicillin release lipoteichoic acid (LTA) from the *Strep. pyogenes* cell surface. In the context of extracellular product formation, Young and Broadbent (1986) demonstrated that lincomycin had a differential effect on exoprotein production by *Vibrio cholerae*. The production of cholera toxin and deoxyribonuclease (DNAase) was stimulated by low concentrations of the drug, whereas production of other extracellular proteins was unaffected

Recent studies indicate that before the influence of antibiotics on the gut flora can be evaluated, it is necessary to differentiate strains. For instance, difficulties in monitoring antibiotic resistance in enterococci of pigs and chickens fed diets containing various feed additives (antibiotics and other

growth promoters) were attributed to the heterogeneous composition of the gut streptococci (Linton *et al.*, 1985). Further work (Kaukas *et al.*, 1986) demonstrated an age-related rotation of different *Enterococcus* spp. in young chickens not receiving antibiotics, each species exhibiting differences in their natural insusceptibility to a range of antibiotics. This need to speciate or biotype gut streptococci in pigs and chickens in order to interpret the effects of dietary chlortetracycline has been confirmed by Molitoris *et al.* (1986a,b). Similarly the ecology of antibiotic-resistant *E. coli* in calves requires detailed indentification of strains by O-serogrouping, biotyping and resistogram determinations (Hinton *et al.*, 1985a,b). The *E. coli* flora was shown to be in continuous flux, with new strains (both sensitive and resistant) displacing those previously dominant.

Most reports on the selection of antibiotic-resistant gut organisms have been concerned with the effects following the use of feeds incorporating antibiotics. Other routes of administration may also be of importance. Recently, Hinton and Linton (1986) demonstrated that sufficient trimethoprim was excreted in sow's milk to select resistant *E. coli* in suckling piglets.

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