STUDIES ON THE TolC PROTEIN OF *Escherichia coli* K-12 AND ITS EFFECT ON OmpF EXPRESSION

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ABSTRACT

TolC is a minor outer membrane protein of Escherichia coli K-12, the gene for which maps at 65 min on the chromosome. tolC mutants are tolerant to colicin E1 and hypersensitive to several dyes and detergents and to certain antibiotics. Membranes of tolC mutants lack three major outer membrane proteins; OmpF, NmpC and Lc. The tolC gene has been cloned and the nucleotide sequence determined. The TolC protein has also been partially characterised.

In this thesis, the work on the structural organisation of the TolC protein has been extended and biosynthesis of the TolC protein studied. In addition, the mechanism by which a mutation in the tolC locus exerts its affect on OmpF has been investigated.

Trypsin digestion of TolC in intact cells and in the membrane fraction gave different cleavage products, thus trypsin susceptible regions of the TolC protein appear to be different in these two situations. This suggests that the TolC protein is partly exposed to the outside of the cell surface and partly to the inside (periplasmic side) of the outer membrane and therefore traverses the outer membrane.

The TolC protein (Mr 52,000) is initially synthesised as a larger precursor (Mr 54,500). An additional polypeptide with an apparent molecular weight of 46,000 was also detected at early stages of synthesis. The presence of the rare codon AGA (at position 402) is the most likely reason for the temporary accumulation of the 46,000-Mr polypeptide since the rate of synthesis of the mature protein can be increased by providing extra $tRNA^{Arg}$ (AGA, AGG) in the cell.

The strength of the tolC effect on OmpF was studied in strains (tolC or ompR) carrying 1, or about 2, 7, or 51 copies of the ompF gene. When OmpF was synthesised from the single copy chromosomal gene, either

mutations (tolC or ompR101) reduced the amount of OmpF below the level which could be detected in whole cell envelopes. However, if the copy number of the ompF gene was increased, the effect of the ompR101 mutation remained essentially same, whereas the tolC mutation was increasingly unable to affect the level of OmpF. These results indicate that the TolC protein is not essential for OmpF synthesis, but is required for synthesis of OmpF at a normal rate.

Analysis of ompF-ompC chimeric genes suggests that the promoter function of the ompF gene is affected by the consequence of a tolC mutation. Direct measurment of the ompF transcript from tolC$^+$ and tolC strains showed that the amount of ompF mRNA in the latter was greatly reduced.

Other data presented in this thesis suggest that the primary affect of tolC is on the ompC and micF co-regulated genes and that their increased expression reduces ompF expression. The first evidence for this hypothesis came from the molecular characterisation of a suppressor mutation, Stc$^-$, which reverts the phenotype of tolC mutants from OmpF$^-$ to OmpF$^+$: the Stc$^-$ mutation was found to be a chromosomal deletion that removed the whole of the micF gene and part of the ompC gene.

The role of the ompC and micF genes in the tolC effect on OmpF was further studied in mutant strains carrying either chromosomal micF or ompC-micF deletions or by using an in vitro constructed micF$^-$ ompC$^+$ plasmid in the ompC-micF deletion background. The results have proved unambiguously that the tolC effect on OmpF is in large part mediated by the micF gene product.

STATEMENT

I state that this thesis contains no material which has been accepted for the award of any other degree or diploma in any university, and to the best of my knowledge and belief, this thesis contains no material previously published or written by any other person, except where due reference is made in the text of the thesis.

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CHAPTER-1

INTRODUCTION

Regulation and biosynthesis of the outer membrane proteins of Escherichia coli K-12 has been the subject of extensive investigation over the last several years. Some of these proteins, in association with peptidoglycan and lipopolysaccharide (LPS), contribute to the structural integrity of the membrane; others allow passive diffusion of nutrients, and provide phage and colicin receptor activities.

In this thesis biosynthesis of a minor outer membrane protein, TolC, and its role in the regulation of a major outer membrane protein, OmpF, has been studied. This chapter describes various properties of the outer membrane proteins of Escherichia coli K-12 and focuses on the regulation of two major outer membrane proteins, OmpF and OmpC.

1.1 The cell envelope

The cell envelope of Escherichia coli K-12 and other gram-negative enteric bacteria is composed of three morphogenically distinct layers (Murray et al., 1965): the cytoplasmic membrane layer, a rigid peptidoglycan layer and a second membrane structure, the outer membrane, at the outer surface of the cell. An aqueous zone between the inner and outer membranes is called the periplasmic space and contains various hydrolytic enzymes and binding proteins. These binding proteins are components of active transport systems and also play a role in chemotaxis (Rosen and Happel, 1973).

Sites with an apparent fusion of inner and outer membrane have been observed and are called "adhesion zones" or "Bayer patches" (Bayer,

1968a). Adhesion zones are present at 200 to 400 per cell and cover about 5% of the total cell surface (Bayer, 1979). These adhesion zones probably provide sites for nucleic acid injection by a number of phages (Bayer, 1968b; 1979) as well as sites for the export of newly synthesised components of the outer membrane such as lipopolysaccharides (LPS) (Muhlradt et al., 1973) and some outer membrane proteins (Smit and Nikaido, 1978), as well as sites for the production of sex pili (Bayer, 1979). More recently, these sites were also speculated to be involved in the uptake of macromolecules such as colicins (Konisky, 1982). Consistant data on the composition of these zones have not been obtained. De Leij et al. (1978; 1979) suggested that adhesion zones may exist temporarily and only during the synthesis of outer membrane proteins. Recently, Lugtenberg and van Alphen (1983) have proposed several models for the translocation of LPS and some outer membrane proteins which make use of the notion of transient adhesion sites.

The cytoplasmic membrane of gram-negative bacteria houses systems for active transport and biosynthesis of certain outer membrane components such as phospholipids, peptidoglycan and LPS (Costerton et al., 1974), besides providing a site for energy-requiring and energy-generating processes (Harold, 1977) many of which are involved in generating a proton gradient across this membrane. The bacterial chromosome and translating ribosomes have been shown to interact with this membrane (Worcel and Burgi, 1974).

Unlike the cytoplasmic membrane, the outer membrane contains a small variety of proteins present in rather large quantities. The outer membrane is very important in the physiology of gram-negative bacteria in making them resistant to several factors, especially in enteric gram-negative bacteria where it acts as a barrier against the

detergent action of bile salts, and enzymes in the digestive tract (Nikaido and Nakae, 1979). The outer membrane is responsible for establishing the high levels of resistance to several antibiotics such as novobiocin, rifamycins, lincomycin, clindamycin, and fusidic acid (Richmond and Curtis, 1974; Nikaido and Nakae, 1979). The most important component in the outer membrane in this regard is the LPS that exists exclusively in the outer membrane (see below). Another remarkable property of the outer membrane is its relatively non-specific permeability to small hydrophilic compounds of about 600 daltons (Nakae and Nikaido, 1975; Nakae, 1976). This permeability is facilitated by the "porins" (Nakae, 1976). The term "porin" was coined for outer membrane proteins which produce non-specific pores or channels that allow the passage of small hydrophilic solutes. Some outer membrane proteins also function as phage and colicin receptors, and some are involved in specific transport systems. These and other properties of the outer membrane proteins are discussed in a later section.

1.2 Components of the outer membrane: Phospholipids

Essentially all phospholipids of *E. coli* are located in the cell envelope (Cronan *et al.*, 1972). Phosphatidylethanolamine is the major species with phosphatidylglycerol and diphosphatidylglycerol present in relatively low amounts. Several reviews on various aspects of the phospholipids of gram-negative bacteria have been published (Cronan and Gelmann, 1975; Cronan, 1978; Op den, 1979). The phospholipid composition of the outer membrane is usually very simiar to that of the inner membrane with a slight but significant enrichment of phosphatidyl-ethanolamine in the outer membrane (Lugtenberg and Peters, 1976).

Transfer of phospholipids between the outer and inner membranes has been reported (Devor et al., 1976; Jones and Osborn, 1977). The phospholipid biosynthetic enzymes of E. coli are present exclusively in the inner membrane (Bell et al., 1971; White et al., 1971).

From an analytical study, Smit et al. (1975) concluded that the outer membrane contains hardly enough phospholipids to cover one monolayer. Inaccessibility of phospholipids by exogeneous agents such as cynogen bromide-activated dextran (Kamio and Nikaido, 1976), dansyl chloride (Schindler and Teuber, 1978) and phospholipases A_2 and C (van Alphen et al., 1977a) suggested that phospholipids are mainly or completely located in the inner leaflet of the outer membrane. In view of the evidence for a strong interaction between phosphatidylethanolamine and LPS, Fried and Rothfield (1978), suggested that phospholipids and LPS form a mixed bilayer. However, electron spin resonance studies (Nikaido et al., 1977b) have shown that LPS and phospholipids are completely segregated into the outer and inner leaflets in the outer membrane, thus making an asymmetric bilayer. In an excellent review by Nikaido and Vaara (1985) it was argued that such an asymmetric structure, where phospholipids are present in the inner leaflet of the outer membrane, protects enteric bacteria which live in an environment containing detergent cholates and ubiquitous phospholipases.

1.3 Components of the outer membrane: Lipopolysaccharide (LPS)

LPS is exclusively located in the outer leaflet of the bilayer (Muhlradt and Golecki, 1975; Funahara and Nikaido, 1980; Munford and Osborn, 1983). In this exposed position on the cell surface, LPS is involved in the interaction of the cell with the environment and hence,

LPS represents the main surface antigen of gram-negative bacteria. Of the various LPS types studied to date, those of Salmonella are probably the most thoroughly investigated (Nikaido, 1973; Galanos et al., 1977; Osborn, 1979; Luderitz et al., 1982; Jann and Jann, 1984; Makela and Stocker, 1984; Labischinski et al., 1985).

LPS molecules contain a lipid region, lipid A, and a long covalently linked heteropolysaccharide that can be subdivided into the core and the O-specific chain according to composition, structure and mode of biosynthesis. These three regions are not only distinct in their chemical structure, but also in their biological and functional properties.

LPS is synthesised on the inner face of the cytoplasmic membrane where it is inserted in the phospholipid bilayer (Rothfield and Romeo, 1971) and thought to pass through the adhesion zones initially described by Bayer (see above). A strong LPS-LPS interaction provides a very effective barrier against the penetration of hydrophobic molecules (Nikaido and Vaara, 1985). Deep rough mutants of Salmonella typhimurium do not make a complete core structure and also lack the O-specific chain; they are very sensitive to hydrophobic compounds (Sanderson et al., 1974; Nikaido, 1976; Roantree et al., 1977). However, certain mutants of S. typhimurium with increased sensitivity to hydrophopic agents do not show any obvious change in the LPS structure (Sukupolvi et al., 1984). The role of LPS in forming a barrier against the penetration of hydrophobic molecules has been discussed in great detail in a recent review by Nikaido and Vaara (1985).

LPS is strongly associated with proteins (Ames et al., 1974). A heptoseless strain of E. coli k-12 was reported to be almost totally devoid of OmpF protein (an outer membrane protein, see below) (Koplow and

Goldfine, 1974; Lugtenberg *et al.*, 1976). The *E. coli* "porins" (see below) OmpF and OmpC are known to have a strong affinity for LPS (Schindler and Rosenbusch, 1978; Overbeeke *et al.*, 1980). Another major outer membrane protein of *E. coli*, OmpA, also interacts with LPS and this interaction was reported to be essential for F plasmid-mediated conjugation (Skurray *et al.*, 1974; Manning and Achtman, 1979) and for the protein to function as a receptor for several phages (Skurray *et al.*, 1974, Datta *et al.*, 1977, van Alphen *et al.*, 1979).

1.4 Components of the outer membrane: Proteins

The outer membrane contains relatively few different species of protein. Proteins present in relatively large amounts are called "major" outer membrane proteins. These proteins and "minor" proteins constitute nearly half of the mass of the outer membrane (Osborn *et al.*, 1972). Wherever possible throughout this thesis the proteins described will be named according to the genetic symbol ascribed to their structural genes (Bachmann, 1983) as recommended by Reeves (1979).

1.4.1 OmpA protein

The OmpA protein consists of 325 amino acid residues (molecular weight of 35,159) and is synthesised as a high molecular weight precursor having a signal peptide of 21 amino acid residues attached at the amino-terminal end (Movva *et al.*, 1980). This protein exhibits anomalous "heat modifiable mobility" on SDS-gels: the protein is not denatured simply by addition of SDS and the unheated or non-denatured form of the protein runs faster (Mr 28,000) than the denatured or heat-modified form

(Mr 35,000) present after heating in the presence of SDS (Schnaitman, 1973; Nakamura and Mizushima, 1976). Susceptibility of the OmpA protein in the isolated cell envelopes to proteases (Henning et al., 1978; Schweizer et al., 1978) and its association with peptidoglycan as shown by cross linking experiments (Endermann et al., 1978) suggests that this protein is exposed to the inner surface of the membrane (periplasmic face). In addition the fact that it also functions as a receptor for bacteriophage K3 (Manning et al., 1976; van Alphen et al., 1977b) and Tull* (Datta et al., 1977) and can be labelled by a non-penetrating reagent in intact cells (Kamio and Nikaido, 1977), suggests that this protein is exposed at the external surface of the membrane and thus appears to span the thickness of the membrane.

The protein is rich in β-sheet structure (Nakamura and Mizushima, 1976) and can be cross-linked to another molecule of OmpA protein in the membrane (Palva, 1979) but is not present as a population of oligomers (Ueki et al., 1970). The OmpA protein contains a unique amino acid cluster (from position 176 to 187) which resembles the hinge region of immunoglobulins (Chen et al., 1980; Movva et al., 1980) and is exposed to the inner surface of the outer membrane. Recently, the study of several phage reisistant ompA mutants supported a model in which the OmpA protein repeatedly traverses the outer membrane in cross β-structure, exposing four areas to the outside and further suggests the importance of these regions in the binding of colicins, phages, and also in F plasmid-mediated conjugation (Morona and Henning, 1984; Morona et al., 1984; 1985).

Whether or not the OmpA protein produces a transmembrane pore is not yet clear (Nakae, 1976; Manning _et al_., 1977), further work has to be done to investigate this possibility. In association with the lipoprotein, OmpA was reported to be involved in outer membrane integrity and cell morphology (Sonntag _et al_., 1978).

1.4.2 Lipoprotein

Lipoprotein is a major outer membrane protein of _E. coli_ K-12. It was first discovered by Braun (1975) and is often referred to as Braun's lipoprotein. The lipoprotein is composed of 58 amino acid residues and has a molecular weight of 7,200. The amino-terminus is a cysteine residue to which glyceride and fatty acid are covalently attached. About one-third of the lipoprotein molecules are covalently bound to peptidoglycan and the other two-thirds are exclusively localised in the outer membrane as a free form (Inouye _et al_., 1972). The bound form of lipoprotein is involved in the interaction of the outer membrane with the peptidoglycan layer and this plays an important role in the cell surface structure (Suzuki _et al_., 1978). . Mutants lacking the bound form of lipoprotein also show increased sensitivity to EDTA, hydrophobic antibiotics and detergents and exhibit leakage of some periplasmic proteins (Suzuki _et al_. 1978). Lipoprotein deficient mutants have been isolated (Hirota _et al_., 1977; De Martini and Inouye, 1978), indicating that the lipoprotein is not essential for survival of the cell.

Fatty acid residues that are attached to the amino-terminal cysteine residue of lipoprotein are seemingly essential for the translocation of this molecule to the outer membrane (Mizushima, 1984). Mutants with a deletion through the structural gene for lipoprotein are

normal with regard to diffusion of small hydrophilic solutes (Nikaido et al., 1977a) and thus this protein probably does not form a transmembrane pore (Nakae, 1976).

1.4.3 Porin proteins: OmpF, OmpC and PhoE

The proteins encoded by the ompF, ompC and phoE genes in Escherichia coli K-12 are called "porins" because they produce relatively non-specific pores or channels that allow the passage of small hydrophilic molecules across the outer membrane (Nakae, 1976). OmpF and OmpC proteins are present in substantialy large quantities whereas, PhoE becomes a major outer membrane protein in phosphate-starved cultures (Argast and Boos, 1980; Overbeeke and Lugtenberg, 1980; Tommassen and Lugtenberg, 1980).

Over the last few years the genetics and other properties of these proteins have been extensively studied in several laboratories. The primary stucture of OmpF protein was studied by Chen et al. (1982). The ompF gene has been cloned (Mutoh et al., 1981; Tommassen et al., 1982b) and sequenced (Inokuchi et al., 1982). The ompC and phoE genes have also been cloned and sequenced (Mizuno et al., 1983: Overbeeke et al., 1983). The calculated molecular weights of OmpF, OmpC and PhoE proteins are 37,082, 37,306 and 36,782 respectively. The primary sequence of the porin genes suggests that they have evolved from a common ancestral gene (Mizuno et al., 1983). The porin proteins also share extensive structural homology. Porins exist as undenatured trimers when extracted with SDS (Palva and Randall, 1978; Nakae et al., 1979; Osborn and Wu, 1980). Circular dichromism, infrared spectroscopy and X-ray differaction analysis showed that they are rich in β-sheet structure and lack any

detectable α-helical segments (Rosenbusch, 1974; Nakamura and Mizushima, 1976; Garavito et al., 1983; Schindler and Rosenbusch, 1984; Kleffel et al., 1985; Paul and Rosenbuch, 1985). Infrared spectroscopic analysis has also shown that many of the β-sheet structures of porins are oriented so that the backbone is roughly perpendicular to the surface of the membrane (Garavito et al., 1982; Kleffel et al., 1985). Electron microscopic analysis of porin structure in the membrane revealed the presence of a trimer unit that contained a triplet of holes or channels (Dorset et al., 1983) on the outside, which fuse in the middle of the membrane and open on the other side of the membrane as a single central channel. Although the ultrastructure of the PhoE protein has not been studied, the regions of PhoE that are cell surface-exposed and determine the bacteriophage Tc45 receptor and channel specificities have recently been reported (Tommassen et al., 1985).

Although OmpF, OmpC and PhoE by and large act as general porin proteins, recent data suggest that they form more effecient channels for certain specific molecules. The PhoE porin forms an effecient channel for organic and inorganic phosphates and several other negatively charged ions (Korteland et al., 1982; Korteland et al., 1984). The OmpC channel was reported to be more specific for some peptides (Heller and Wilson, 1981). Recently, cation selectivity of OmpF pores has been reported (Kobayashi and Nakae, 1985). The size of the OmpF pore was reported to be larger than that of the OmpC pore (1.2 and 1.1 nm respectively, Nikaido and Rosenberg, 1983): the physiological and ecological importance of this difference was discussed by Nikaido and Vaara (1985).

At the outer surface porins provide receptor sites for bacteriophages: OmpF acts as a receptor for Tula and T2 (Datta et al., 1977;), OmpC acts as a receptor for Tulb, Mel and T4 (Datta et al., 1977;

Verhoef et al., 1977; Henning and Jann, 1979; Furukawa and Mizushima, 1982) and PhoE functions as a receptor for phage Tc45 (Chai and Foulds, 1978). At the inner surface porins interact with peptidoglycan and thus it appears that they protrude a little on either side of the membrane. Porin proteins (OmpF and OmpC) were initially called matrix proteins (Rosenbusch, 1974) and believed to be important in the maintenance of the cell surface structure, this notion was recently supported by Nogami and Mizushima (1983). Evidence that porins do not contribute to maintenance or determination of the cell shape however was presented by Schweizer et al. (1976). ompF mutants are tolerant to colicins A, K, L, N and S4 (Reeves, 1979) and ompF ompC double mutants are tolerant to colicins E2, E3 and X in addition to the above mentioned-colicins (Davies and Reeves, 1975; Pugsley and Schnaitman, 1978a). In the absence of OmpF and OmpC proteins, the PhoE protein can facilitiate the action of colicins E2 and E3 (Davies and Reeves, 1975; Pugsley and Schnaitman, 1978b).

Regulation of OmpF and OmpC proteins has been a subject of investigation for several years, and this aspect is discussed below in detail. Synthesis of PhoE is normally repressed and is derepressed when cells are grown under phosphate limitation conditions (Overbeeke and Lugtenberg, 1980; Tommassen and Lugtenberg, 1980). The PhoE protein is synthesised constitutively in strains carrying phoR, phoS, phoT and pst mutations (Tommassen and Lugtenberg, 1980).

1.4.4 Lc and NmpC proteins

The Lc protein (formerly called Protein 2) was first detected in cells lysogenic for phage PA-2 or its derivatives (Bassford et al., 1977; Pugsley and Schnaitman, 1978a). The nucleotide sequence of the lc gene

has not been determined yet, although its putative location in the phage DNA has recently been published (Highton et al., 1985). Synthesis of Lc protein in strains lysogenic for phage PA-2 results in a dramatic reduction in the amount of OmpF and OmpC proteins (Fralick and Diedrich, 1982). Suppression of OmpF and OmpC proteins in the PA-2 lysogens does not occur at the transcription level (Hall and Silhavy, 1979). However, this decrease in the amount of OmpF and OmpC proteins is in direct proportion to the increase in Lc protein (Fralick and Diedrich, 1982).

The NmpC protein (new membrane protein C) was first observed in extragenic pseudorevertants of E. coli double mutants lacking the OmpF and OmpC proteins (Pugsley and Schnaitman, 1978b). The NmpC protein is very similar to the Lc protein and because of this Lee et al. (1979) proposed that they arose from the same ancestral gene. The locus of a mutation resulting in the production of this protein was mapped at 12.5 min in the E. coli K-12 chromosome (Bachmann, 1983). This locus also houses a defective prophage (Anilionis et al., 1980; Kaiser, 1980) which contains an alternative set of lambda QSR genes called qsr'. The p4 region of this defective prophage (qsr') was reported to be responsible for the production of NmpC protein (Highton et al., 1985). From DNA heteroduplex analysis, Highton et al. (1985) have further shown the region of homology between the lc gene of phage PA-2 and defective prophage λqsr' and thus supported the previous proposal of Lee et al. (1979) of a common ancestral gene for these two proteins.

Lc and NmpC proteins are peptidoglycan associated and can function as pores. Ion selectivity by the NmpC pores was recently investigated by Benz et al. (1985). Both proteins can replace OmpF or OmpC functionally in colicin E2 and E3 action (Pugsley and Schnaitman, 1978a; 1978b).

1.4.5 LamB protein

When *E. coli* K-12 cells are grown in maltose, LamB becomes a major outer membrane protein. The *lamB* gene has been sequenced (Clement and Hofnung, 1981) and the deduced amino acid sequence suggests a molecular weight for the mature protein of 47,393. LamB, like other outer membrane proteins, is initially synthesised as a larger precusor with an amino-terminal extension of 25 amino acid residues as signal peptide. Like porins, this protein also forms a stable trimer (Palva and Westermann, 1979) which is rich in β-sheet structure (Garavito *et al.*, 1982; Neuhaus, 1982).

Several lines of evidence suggest that the LamB protein is partly exposed at both sides of the membrane. At the outer surface it acts as a specific receptor for bacteriophages lambda (λ) (Randall-Hazelbauer and Schwartz, 1973) and K10 (Rao, 1979) and binds to maltodextrin (Ferenchi *et al.*, 1980). At the inner face of the membrane it interacts with MalE (see below) and peptidoglycan (Gabay and Yasunaka, 1980). Experiments using monoclonal and polyclonal antibobies raised against the LamB protein (Schenkman *et al.*, 1983) and analysis of *lamB* missense mutants (Charbit *et al.*, 1984) have provided numerous data concerning the structural organisation of LamB in the membrane.

The LamB protein produces transmembrane diffusion channels (Boehler-Kohler *et al.*, 1979; Nakae, 1979; Luckey and Nikaido, 1980) which allow diffusion of maltose, maltotriose and larger oligosaccharides of the maltose series. LamB channels also allow diffusion of amino acids and unrelated sugars (Nakae, 1979; Luckey and Nikaido, 1980) and of metal cations (Boehler-Kohler *et al.*, 1979). As a result of these nonspecific properties of the LamB porin, it is often called "maltoporin".

Interaction of the LamB protein with the maltose binding protein (MalE) was shown by Wandersman et al., (1980); Heuzenroeder and Reeves, (1980); and Neuhaus et al. (1983). This interaction with MalE protein was suggested to confer specificity to the LamB pore in vivo (Heuzenroeder and Reeves, 1980) and facilitate diffusion of maltose and maltodextrins (Luckey and Nikaido, 1983; Neuhaus et al., 1983). Recently Brass et al. (1985) have claimed that the MalE protein does not interfere with, or stimulate the function of LamB protein as a general pore in vivo.

1.4.6 Tsx protein

The Tsx protein has an apparent molecular weight of 26,000, is not associated with peptidoglycan and functions as a receptor for bacteriophage T6 (Manning and Reeves, 1976; 1978). The Tsx protein is involved in the diffusion of nucleosides across the outer membmrane (Hantke, 1976; Krieger-Brauer and Braun, 1980). Heuzenroeder and Reeves (1981) have shown that the Tsx protein also allows the diffusion of serine, glycine and phenyalanine and, therefore, acts as a general pore. Part of the tsx gene has been cloned (Tommassen et al., 1982a).

1.4.7 BtuB protein

The btuB gene has recently been cloned (Heller et al., 1985) and the nucleotide sequence determined (Heller and Kadner, 1985). The molecular weight of the BtuB protein as determined from the DNA sequence is 66,400. This protein has been shown to facilitate the transport of vitamin B_{12} across the membrane (Dimasi et al., 1973; White et al., 1973) and also functions as a receptor for bacteriophage BF23 (Kadner and

coupling of transport processes (Bradbeer, 1979). A domain on the BtuB protein which was responsible for the interaction with the tonB gene product was reported by Bassford and Kadner (1977).

1.4.8 OmpT protein

The OmpT protein (formely called Protein a or 3b) has an apparent molecular weight of 42,000 and becomes a major outer membrane at 42°C (Lugtenberg et al., 1976; Manning and Reeves, 1977) and the ompT gene maps at 13 min on the E. coli chromosome. The ompT (omp Temperature-dependent) gene has been cloned (Gadya and Markovitz, 1978; Rupprecht et al., 1983). The OmpT protein has been shown to regulate the synthesis of capsular polysaccharide (Gadya et al., 1979) and also to be responsible for the proteolytic cleavage of the FepA protein (see below).

1.4.9 TolC protein

tolC mutants are among the first colicin tolerant mutants to be characterised (Clowes, 1965). tolC mutants are tolerant to colicin E1 but sensitive to other E group colicins such as E2 and E3 (Nagel de Zwaig and Luria, 1967; Davies and Reeves, 1975) and therefore represent a more specific class of tolerant mutants. Mutations in the tolC locus are pleiotropic and produced strains which are hypersensitive to detergents and dyes and to certain antibiotics (Clowes, 1965; Nagel de Zwaig and Luria, 1967; Davies and Reeves, 1975; Morona, 1982). A mutation in the tolC locus also results in a lowered growth rate but unlike some other colicin tolerant mutants (e.g. tolA and tolB, Nagel de Zwaig and Luria, 1967; Anderson et al., 1979; Bernstein et al., 1972), tolC mutants do not

and dyes and to certain antibiotics (Clowes, 1965; Nagel de Zwaig and Luria, 1967; Davies and Reeves, 1975; Morona, 1982). A mutation in the *tolC* locus also results in a lowered growth rate but unlike some other colicin tolerant mutants (*e.g.* *tolA* and *tolB*, Nagel de Zwaig and Luria, 1967; Anderson *et al.*, 1979; Bernstein *et al.*, 1972), *tolC* mutants do not show any leakage of periplasmic proteins (Nagel de Zwaig and Luria, 1967). Rolfe and Ondera (1971) showed that a *tolC* deletion mutant lacked a specific polypeptide band from gel profiles when compared with the wild type strain. Later it was shown that a mutation in the *tolC* locus affects synthesis of OmpF and also NmpC and Lc proteins (Morona and Reeves, 1982a; see below). A *tolC* mutant (P602) studied by Hancock *et al.* (1976) showed partial sensitivity to the LPS phage, C21, and were resistance to a group of bacteriophages, called Ktw, of which K2 is a member. All these properties of *tolC* mutants suggested that they have an altered membrane structure.

In spite of a good knowledge of the phenotypic properties of *tolC* mutants, very little had been known about the TolC protein itself. The *tolC* gene has recently been cloned (Morona and Reeves, 1981) and the nucleotide sequence determined (Hackett and Reeves, 1983). The mature TolC protein consists of 467 amino acid residues and has a molecular weight of 52,000. TolC is a minor outer membrane protein and is not associated with the peptidoglycan layer (Morona *et al.*, 1983).

1.4.10 Proteins related to iron transport

Several proteins in the region of "80,000-Mr" were observed in iron-starved cells (Braun *et al.*, 1976; Pugsley and Reeves, 1976; Ichihara and Mizushima, 1977). These proteins are receptors for several

ferric-iron siderophores (Greek: iron bearer) and some of them also function as the attachment site for several phages and colicins.

The FhuA (TonA) protein is a ferrichrome receptor of molecular weight 78,000 (Braun *et al.*, 1976) and is involved in ferric hydroxamate uptake (Hantke and Braun, 1975). The FhuA protein also facilitates the transport of ferrichrysin and ferricrocin (a siderophore closely related to ferrichrome) (Luckey *et al.*, 1975). A second locus, *fhuB*, which codes for a cytoplasmic membrane protein (Wookey *et al.*, 1981), interacts with FhuA protein for the transport of all hydroxamate-type siderophores (Hantke and Braun, 1978). The FhuA protein also performs a receptor function for phage T1, T5, ϕ80, and for colicin M.

The FepA protein facilitates the transport of enterobactin (ferri-enterochelin) (Pugsley and Reeves, 1977; Konisky, 1979) and acts as a receptor for colicin B and D (Hancock *et al.*, 1976; Pugsley and Reeves, 1977). The FepA protein (molecular weight 81,000) is very easily observed in outer membrane preparations from iron-depleted cultures. This protein is modified by the protease acitivity of the OmpT protein (Hollifield and Neilands, 1978; Fiss *et al.*, 1979) and the modified protein was unable to bind ferric enterobactin or colicin B.

The FecA protein has an apparent molecular weight of 80,500 and functions as a ferric-citrate receptor (Wagegg and Braun, 1981). Induction of this protein requires iron as well as citrate starvation.

Another protein, Cir, with an apparent molecular weight of 74,000 is strongly induced in iron straved cells. The Cir protein also functions as a colicin Ia and Ib receptor (Konisky *et al.*, 1973; Pugsley and Reeves, 1977). The *cir* gene has been mapped between *mgl* and *fpk* on the *E. coli* chromosome (Boos *et al.*, 1983). Recently, a gene, *cirR*, that

controls the transcriptional regulation of cir by iron has been reported (Worsham and Konisky, 1985).

A locus, fur, which maps close to nalG (16 min) was suggested to encode a protein (Fur) that represses the expression of all iron related genes in iron replete cells (Braun, 1985). Mutations that give Fur$^-$ phenotype have also been mapped at a slightly different position than that mentioned above (Bagg and Neilands, 1985).

1.4.11 Outer membrane enzymes

The outer membrane is much poorer than the cytoplasmic membrane in its enzymatic activities. The enzymes detected in the outer membrane are: phospholipase A1 (Scandella and Kornberg, 1971; Osborn et al., 1972); lysophospholipase; UDP-glucose hydrolase (Osborn and Munson, 1974); and certain proteases that cleave the precursor form of the secretory proteins into mature form (Wickner, 1980; Wolfe et al., 1982; Yamagata et al., 1983). Other proteases that cleave colicin Ia (Bowles and Konisky, 1981), modify the ferric enterobactin receptor (Fiss et al., 1979) and hydrolyze casein (Regnier and Thang, 1979) have also been reported in the outer membrane.

1.5 Regulation of OmpF and OmpC protein synthesis

Expression of the ompF and ompC genes is affected by several factors. Apart from mutations in the structural genes of these proteins, mutations in other loci and the osmolarity of the culture has a major effect on synthesis of the OmpF and OmpC proteins. Over the last decade regulation of ompF and ompC expression has been the subject of extensive

studies and since the results of this thesis are pertinant to this regulation, it is discussed in detail below.

1.5.1 Osmolarity of the culture media

The synthesis of OmpF and OmpC proteins are affected by the osmolarity of the culture media (Schnaitman, 1974; Lugtenberg et al., 1976; van Alphen and Lugtenberg, 1977; Kawaji et al., 1979). Although these proteins are chemically very similar (Nakamura and Mizushima, 1976; Minuno et al., 1983), the amount of OmpF and OmpC proteins vary differently with changing osmolarity of the media. Growth in high osmolarity media (or media containing high salt or sucrose levels), results in high levels of OmpC protein relative to OmpF. Conversely, low osmolarily media result in high levels of OmpF protein relative to OmpC. The total amount of these proteins remains approximately constant (van Alphen and Lugtenberg, 1977): that is, a decrease in the amount of one protein is compensated by a corresponding increase in the amount of the other protein. However, this medium-dependent variation in amounts of OmpF or OmpC protein does not occur in the absence of the other (OmpF or OmpC) protein, (Morona and Reeves, 1982a).

Ozawa and Mizushima (1983) have reported that osmolarity of the medium primarily regulates the expression of the ompF gene, which in turn regulates ompC gene expression, although this relationship was not as evident when a different background strain was used. However, later it was found that ompF mutants used by Ozawa and Mizushima (1983) also contained other mutations such as envZ and $ompC_p$ (a promoter mutation) which rendered mutants constitutive with respect to ompC expression and therefore their hypothesised mechanism for controlling reciprocal osmo-regulation has been questioned (Inokuchi et al., 1985).

By using ompF-lacZ and ompC-lacZ operon fusion strains (Hall and Silhavy, 1979; Hall and Silhavy, 1981a) and ompF-ompC chimeric genes (Matsuyama et al., 1984) it was established that the promoter region was primarily responsible for the osmoregulation switching of the expression of the two genes.

1.5.2 The ompB regulon

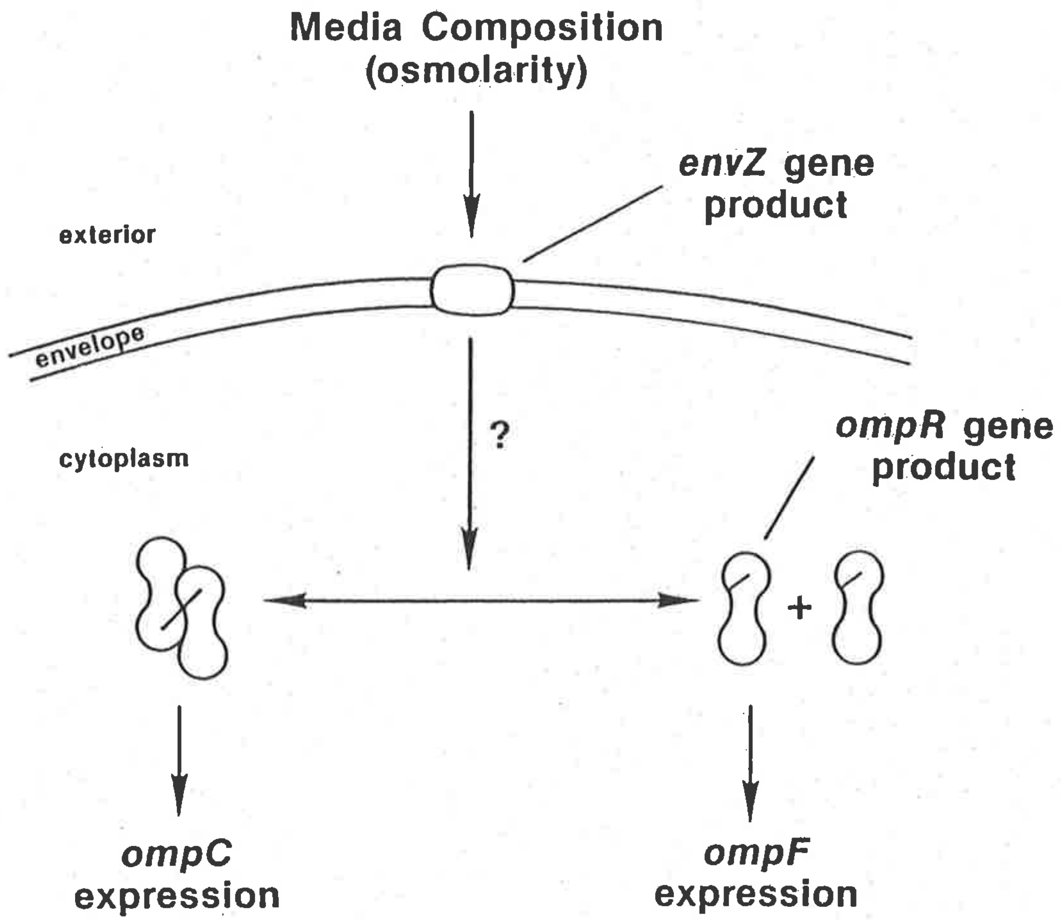
Genetic analysis of a class of mutants tolerant to several colicins (Davies and Reeves, 1975) led to the discovery of a regulatory locus (ompB) which affected synthesis of OmpF and OmpC proteins (Sarma and Reeves, 1977). Mutations in the ompB locus resulted in one of three phenotypes: $OmpF^-$, $OmpC^-$; $OmpF^-$, $OmpC^+$; or $OmpF^+$, $OmpC^-$ (Sarma and Reeves, 1977; Verhoef et al., 1979). The function of the ompB locus was studied by Hall and Silhavy (1979; 1981a) who showed that it acts as a positive regulatory element for the expression of the ompF and ompC genes. Further analysis of the ompB locus revealed the presence of at least two genes: ompR and envZ (Hall and Silhavy, 1981a).

The ompR gene product was postulated to be a soluble positive regulatory element which controls the expression of ompF and ompC genes at the transcription level, whereas the envZ gene product was postulated to be an essential envelope protein which is sensitive to the cell's external environment and influences OmpF and OmpC synthesis by controlling the multimerisation of the OmpR protein (Hall and Silhavy, 1981b). The model proposed by these investigators is illustrated in Fig. 1.1. According to this model, the envZ gene product in the cell envelope senses the external environment and then produces a cytoplasmic signal which regulates the expression of the ompF or ompC gene. In a high

Fig. 1.1

Osmoregulation of the ompF and ompC genes

A model proposed by Hall and Silhavy (1981b) for the role of the ompR and envZ gene products in osmoregulation of the ompF and ompC genes. See text for details.


Media Composition
(osmolarity)
envZ gene
product
exterior
envelope
?
cytoplasm
ompR gene
product
+
ompC
expression
ompF
expression

osmolarity medium, the EnvZ signal stimulates formation of multimeric OmpR protein which in turn stimulates ompC gene expression. In a low osmolarity medium, the monomeric OmpR protein predominates and stimulates ompF gene expression. However, no experimental evidence has yet been presented for the multimer or monomer forms of the OmpR protein and it is possible that it may be modified in a differnt way in response to the osmolarity of the medium.

The envZ and ompR genes have been cloned (Mizuno et al., 1982a) and the entire ompB operon sequenced (Mizuno et al., 1982b; Wurtzel et al., 1982; Comeau et al., 1985). The EnvZ protein consists of 449 amino acid residues and has a molecular weight of 50,339. Although its cellular location is not yet known, the amino acid composition suggested that EnvZ is an envelope protein (Mizuno et al., 1982b; Comeau et al., 1985). OmpR is a cytoplasmic protein, consisting of 239 amino acid residues and has a molecuar weight of 27,353 (Comeau et al., 1985). In vitro transcription studies have suggested that the ompR and envZ genes are cotranscribed as a single mRNA from a promoter located 5′ to the ompR gene (Wurtzel et al., 1982).

Several mutants having lesions in the region of the envZ gene have been isolated that were phenotypically OmpF^{-} OmpC^{+} and were repressed for the synthesis of several periplasmic (MalE and PhoA) and envelope proteins (LamB and iron-regulated proteins) (Wanner et al., 1979; Wandersman et al., 1980; Hall and Silhvay, 1981a; Lundrigan and Earhart, 1981). Mutations in these strains were originally known as tpo, perA and only later suspected to be at envZ. Recently Garrett et al. (1983) isolated amber mutations in the envZ gene that were phenotypically OmpF$^{-/+}$ OmpC^{+} and produced normal amounts of other proteins. The mutations in the envZ gene isolated previously showed some degree of

codominance with the wild type allele, suggesting that an altered EnvZ protein was synthesised as the result of a missense mutation in the envZ gene (Garrett et al., 1983). Based on these results, Garrett et al. (1983) suggested that loss of the envZ product decreases the expression of the ompF and ompC genes whereas an altered form of the EnvZ protein can interefere with the transcription of the structural genes of several export proteins. More recently, a deletion mutation in the envZ gene was isolated by Garrett et al., (1985) which behaves phenotypically as an envZ amber mutation. The EnvZ protein was also found to regulate the proca ine-mediated transcription control of several membrane proteins, including OmpF and OmpC (Garrett et al., 1983). A hypothesis proposed by Villarejo and Case (1984) suggested that expression of the target genes is regulated by a single environmentally responsive system that is sensitive to both medium osmolarity and procaine, and is dependent on the activity of the EnvZ protein. The role of the EnvZ protein in osmoregulation of OmpF and OmpC proteins was proposed by Hall and Silhavy (1981b, see above) but no direct evidence has yet been presented.

Strains carrying an amber mutation in the envZ gene still exhibited osmolarity dependent fluctuation in the amount of residual OmpC protein. Therefore, it has been suggested that EnvZ is not the only cellular component responsible for osmosensing and osmoregulation of the expression of OmpF and OmpC proteins (Villarejo and Case, 1984).

From the genetic analysis of a few ompR mutants, Hall and Silhavy (1981b) postulated two functional domains within the ompR gene. The amino-terminal domain was referred to as ompR1, and the carboxyl terminal domain as ompR2. The ompR1 domain was postulated to be a positive regulatory element required for the transcriptional expression of both ompF and ompC genes, since mutation in this region resulted in the OmpF$^-$,

OmpC$^-$ phenotype. Mutation in the ompR2 domain gave the OmpF$^+$ OmpC$^-$ phenotype. This domain was postulated to mediate in the multimerisation of OmpR. It was postulated that as a multimer, the ompR gene product turns on expression of the OmpC gene and a monomeric form of the ompR gene product turns on expression of the ompF gene (Hall and Silhlavy, 1981b; see above). Recently, Berman and Jackson (1984), have made several ompR-lacZ fusion strains that carry varying lengths of the ompR gene. One such fusion strain lacked 45 codons from the carboxyl terminal of the OmpR protein and conferred an OmpF$^+$, OmpC$^-$ phenotype, as did the ompR2 mutation above. These findings further confirmed the presence of the two functional domains in the ompR region, proposed originally by Hall and Silhavy (1981b). One mutation (ompR20) isolated by Nara et al. (1984) using nitrosoguanadin (NTG) reversed the normal osmoregulation of OmpF protein and the synthesis of OmpC protein was totally suppressed. The nature of the mutational changes is not known but it was predicted to alter the ompR2 domain.

1.5.3 OmpR interaction site of the ompF and ompC genes and their osmoregulation

Sato and Yura (1981) described a mutation (ompFp9) in the ompF gene presumed to be in the promoter region. Synthesis of OmpF protein in this mutant strain was constitutive and independent of the ompR positive control. A similar ompF promoter mutation (ompFp100) was isolated by Koga-Ban et al. (1983) that resulted in overproduction of the OmpF protein. However it was not reported whether OmpF protein was synthesised independently of ompR control. The nucleotide sequence of these two promoter mutants has not been reported.

Since the expression of both ompF and ompC is positively controlled by the ompR gene product, it was predicted that they may have a region of homologous DNA sequence where OmpR interacts. From the DNA sequence analysis of ompF and ompC, Mizuno et al. (1983) found three distinct regions of similarity upstream of the mRNA start site of both the genes. One such sequence ($CATC_AATAG$, ompF; $CATC_TATAG$, ompC) is present -87 to -78 in the ompF gene and -165 to -157 in the ompC gene and is 13 and 40 base pairs upstream of their putative -35 regions respectively. These very similar sequences of the two genes also form part of unique inverted repeats and are predicted to be involved in the recognition of the ompR gene product. The region that is responsible for the ompR-dependent functioning of the ompF promoter was located by Inokuchi et al. (1984). They generated a series of deletions both upstream and downstream of ompF promoter and connected them with the tet gene which lacked its promoter and hence expression of tet was dependent on the ompF promoter activity. In the $ompR^+$ background, all deletions which retained the ompF promoter region between positions -91 and +17, showed promoter activity. This essential segment includes the -35 region and the Pribnow box of the ompF gene.

An ompF promoter mutation was isolated in which the first base of the Pribnow box was changed from A to T. ompF expression from this mutant promoter was independent of OmpR and did not require the region upstream from the -35 region which is required for the OmpR-dependent functioning of the wild type ompF promoter (Dairi et al. (1985). The same authers also made a statistical survey of the known Pribnow box sequences of several genes which require an activator protein and concluded that bases other than T occupied the first position in the Pribnow box of such genes.

Although osmoregulation of ompF and ompC was reported to be under ompR positive control (Hall and Silhavy, 1981b; see above), ompR-independent osmoregulation of ompF has been reported recently (Inokuchi et al., 1985; Ramakrishnan et al., 1985). Expression of ompF under ompR-independent promoter(s) was osmoregulated, albeit only weakly, which suggested that a part of the ompF gene downstream of the Shine-Dalgarno sequence also plays some role in osmoregulation. However, since the upstream region of ompF which is required for the ompR-dependent expression of ompF (see above) was mainly responsible for osmoregulation of this gene, it has been suggested that the ompR-independent mechanism does not play a crucial role in osmoregulation of ompF (Inokuchi et al., 1985).

In spite of the accumulation of a large amount of data, a mechanism by which the ompR gene product regulates the transcriptional control of ompF and ompC is yet to be clearly elucidated.

1.5.4 The effect of the micF gene on ompC and ompF expression

Recently, a regulatory DNA sequence (micF) was located upstream from the ompC gene and is transcribed in the opposite direction to that of the ompC gene (Mizuno et al., 1984). The micF gene does not have a significant open reading frame but synthesises a small transcript of 174 base pairs. This micF RNA was found to have substantial sequence complementarity with the 5′ end of ompF mRNA. When the micF sequence was cloned into a multicopy vector plasmid and transformed into an $OmpF^+$ $OmpC^+$ E. coli K-12 strain, the resulting transformant showed an $OmpF^-$ $OmpC^+$ phenotype. A regulatory mechanism was proposed by Mizuno et al., (1984) in which mic RNA (mRNA interfering complementry RNA) forms a

stable duplex with the ompF mRNA, thereby inhibiting its translation. Forty-four base pairs of the 5′ untranslated region of the ompF mRNA, including the Shine-Dalgarno sequence, and twenty-eight base pairs of the coding part of the mRNA participate in duplex formation. The amount of ompF mRNA was also significantly reduced under these circumstances and it was suggested that this RNA-RNA interaction may cause premature termination of the ompF gene transcription and/or destabilisation of the ompF mRNA.

Expression of micF, like that of ompF and ompC, is controlled by the ompB locus and was proposed to be co-regulated with the ompC gene (Mizuno *et al.*, 1984). Schnaitman and McDonald (1984) also found a regulatory element upstream of ompC, presumably micF, with a product which inhibited OmpF synthesis and was co-regulated with ompC. More recently, Matsuyama and Mizushima (1985) constructed a strain in which the chromosomal micF gene was deleted. The expression of ompF in this mutant strain was reported to be osmoregulated normally. Based on this observation they concluded that the single copy of the micF gene on the chromosome does not play a critical role in ompF expression.

1.5.5 The tolC-mediated regulation of OmpF and OmpC proteins

TolC is a minor outer membrane protein of *E. coli* K-12 and its gene maps at 65 min on the chromosome. The tolC gene has been cloned (Morona and Reeves, 1981) and the nucleotide sequence determined (Hackett and Reeves, 1983). tolC mutants are tolerant to colicin E1 and hypersensitive to several dyes and detergents and to certain antibiotics (see above). Membranes of tolC mutants also lack detectable levels of the OmpF protein (Morona and Reeves, 1982a). Since only a small (2-3 fold)

reduction was detected in the β-galactosidase activity of a *tolC* derivative of an *ompF*-*lacZ* operon fusion strain, it was suggested that the *tolC*-mediated affect on OmpF synthesis occurs at the post-transcriptional level (Morona, 1982; Morona and Reeves, 1982a).

The OmpC protein is synthesised constitutively in a *tolC* mutant without an appreciable increase in the transcription of the *ompC* gene (Morona and Reeves, 1982a). The consititutive synthesis of the OmpC protein in *tolC* mutants was explained by an hypothesis which states that there are a limited number of sites in the membrane which may be occupied by proteins. This hypothesis predicts that a reduction in the amount of one protein will be compensated for by an increase in the amount of another protein (Halegoua and Inouye, 1979b; Scott and Harwood, 1980).

Another class of a mutation, *stc* (suppressor of *tolC*) has been identified (Morona, 1982; Morona and Reeves, 1982b) which reverts the phenotype of *tolC* mutants from OmpF$^-$ to OmpF$^+$. This mutation was mapped at 47.5 min on the chromosome, very close to the *ompC* gene (Morona and Reeves, 1982b). The *stc* mutation was isolated in a *tolC*, *ompC* background and the Stc$^-$ phenotype was observed only in the presence of a functional *ompR* gene product. Molecular characterisation of the *stc* mutation was not carried out but later it became apparant that phenotypically it resembled the phenotype which might be expected for mutations at *micF* (see above).

1.5.6 Other mutations that influence OmpF synthesis

Mutations in the *cpxA* and *cpxB* genes, which are involved in the synthesis of isoleucine and valine (McEwen and Silverman, 1980), also affect synthesis of OmpF and murein lipoprotein (McEwen *et al*., 1983). The mechanism by which *cpx* mutations reduce the *ompF* gene expression is not clear.

1.5.7 Thermal regulation of OmpF and OmpC proteins

The effect of temperature on *ompF* and *ompC* expression was first reported by Lugtenberg *et al*. (1976). The amount of OmpC protein in the cell envelope increased with increasing growth temperature with a concomitant decrease in the amount of OmpF protein. McIntosh *et al*. (1979) isolated mutants carrying DNA deletions from the 13 min region of the chromosome. These mutants also lacked the OmpT protein (Earhart *et al*., 1979; see above) and were found to be defective in thermoregulation of OmpF and OmpC synthesis (Lundrigan and Earhart, 1984). This thermoregulation defect of the deletion mutants was complemented by the structural gene for a new protein, EnvY, which was encoded by a vector plasmid. Lundrigan and Earhart (1984) concluded from this complementation, that the *envY* gene product was responsible for the thermoregulation of OmpF and OmpC proteins. The *envY* gene was mapped at 12.9 min on the chromosome (Bachmann, 1983) and resides next to the *ompT* gene. The molecular weight of EnvY protein was estimated to be about 25,000 in the minicell system but its cellular location could not be determined. The level at which the EnvY protein affects expression of OmpF and OmpC, and other thermoregulated proteins, is not known.

1.5.8 Other factors that influence synthesis of OmpF and OmpC

Several protease inhibitors such as tosyl-L-lysin chloromethyl ketone (TLCK), antipain, leupeptin and diisopropyl fluorophosphate inhibit synthesis of OmpF and OmC proteins (Ito, 1977; 1978). It was suggested that these protease inhibitors could inhibit the signal peptidase (see below) that processes the newly synthesised polypeptide

into the mature form or by their action at the level of transcription or translation. The lack of clear experimental evidence precludes an explanation for the mode of action of these protease inhibitors in the synthesis of OmpF and OmpC proteins.

Treatment of cells with local anaesthetics which react with the membrane such as toluene, polyethyl alcohol (PEA) and ethanol also influences synthesis of porins and other membrane proteins (Halegoua and Inouye, 1979a; Lory et al., 1983). It was suggested that these anaesthetics affect processing of the membrane proteins. Certain colicins and energy inhibitors are also known to influence the processing of membrane proteins (see below).

1.6 Export of the outer membrane proteins

Although this topic is related to this thesis, it is not the major theme of the thesis, and hence this aspect is dealt with only briefly.

The mechanisms involved in the export of outer membrane and periplasmic proteins have been described in several excellent reviews (Inouye and Halegoue, 1980; Michaelis and Beckwith, 1982; Silhavy et al., 1983; Randall and Hardy, 1984; Benson et al., 1985; Oliver, 1985; Pugsley and Schwartz, 1985).

The protein synthesising activity is located in the cytoplasm and from here proteins destined for export to the outer membrane or periplasmic space are exported or secreted through the cytoplasmic membrane. A number of ways in which this step might be accomplished have been proposed (see recent review by Oliver, 1985), of which three main models are described below.

1.6.1 The signal hypothesis

According to the signal hypothesis, proposed by Blobel et al. (1975), a protein destined to be secreted from the cell is synthesised initially as a larger precursor form containing an NH_2-terminal extension, the signal sequence of about 15-30 amino acid residues. The signal sequence serves as a signal to initiate protein secretion by facilitating the interaction between the ribosomes and the components of the membrane. Translation continues with the growing polypeptide chain being vectorially transferred through the membrane. The signal sequence is enzymatically cleaved off by a signal peptidase (see below) either prior to or immediately after translation is completed.

1.6.2 Membrane trigger hypothesis

This is an alternative hypothesis of protein secretion proposed by Wickner (1979; 1980). Accordi ng to this hypothesis the role of the signal sequence is to promote the folding of a newly made polypeptide in a water-soluble conformation. Binding of the precursor protein to the membrane triggers a conformational change, allowing the protein to insert into or through the lipid bilayer without the aid of any export machinary. Cleavage of the signal peptide would render this sequence of events irreversible.

1.6.3 The loop model

The loop model (Inouye and Halegoua, 1980) takes account of the presence in all signal sequences (see below) of a positively charged

amino-terminus followed by a stretch of 8-20 hydrophobic amino acid residues. According to this model, the initial interaction of the nascent signal peptide with the cytopasmic membrane is facilitated by an ionic interaction between the negatively-charged inner surface of this membrane and the positively-charged amino-terminus of the signal peptide. The hydrophobic region of the signal peptide then inserts into the hydrophobic lipid bilayer, forming a loop- or hairpin-like structure. As polypeptide chain elongation continues, an increasingly larger portion of the loop is located on the periplasmic side of the cytoplamic membrane. Cleavage at the processing site releases the translocated polypeptide chain, with the signal peptide remaining embedded in the cytoplasmic membrane.

1.6.4 The signal sequence

Almost all prokaryotic and many eukaryotic secretory proteins are made initially as a precursor containing an amino-terminal signal sequence of 15-30 amino acids. The signal sequence of many outer membrane and periplasmic proteins have been characterised over the years and was recently compiled by Watson (1984) and Oliver (1985). Although different prokaryotic signal sequences have the same distribution of charged and hydrophobic amino acids, they do not exhibit a high degree of DNA or amino acid sequence homology. Essential features of prokaryotic signal sequences are: (1) they all have a positively-charged and basic amino-terminal region of 2-8 amino acids; (2) a central long stretch (8-15) of hydrophobic or neutral amino acids; and (3) a region of cleavage site that usually contains alanine or glycine.

Although, all signal sequences have the same features, and are probably interchangeable, the information which determines the ultimate location of a protein is perhaps located in the structural sequence of the protein and not in the signal sequence (Tommassen et al., 1983; Yu et al., 1984b; Jackson et al., 1985).

1.6.5 Other components of the secretion machinary

The enzyme that cleaves the signal peptide, the signal peptidase, has been purified (Wolfe et al., 1982). The gene encoding the signal peptidase has been cloned and sequenced (Wolfe et al., 1983). A second peptidase that is specific for the processing of glyceride-modified lipoprotein precursor has also been purified and the gene encoding this enzyme cloned and sequenced (Yamagata et al., 1983; Yu et al., 1984a).

The genetic analysis of a number of export defective mutants have contributed to much of the current understanding of the secretory mechanism. A number of genetic loci have been defined which code for components of the cellular export machinary and are described in recent reviews by Benson et al. (1985) and Oliver (1985).

1.7 Aims of this thesis

A mutation in the *tolC* locus is pleiotropic, and *inter alia* *tolC* mutants are tolerant to colicin E1, extremely sensitive to detergents and dyes, and lack three major outer membrane proteins, OmpF, NmpC and Lc from their cell envelopes. These properties of *tolC* mutants indicate that the TolC protein either directly or indirectly plays an important role in the membrane structure. The *tolC* gene has now been cloned and sequenced. The TolC protein has also been partially characterised.

The aims of this thesis are to further characterise the TolC protein and to study the mechanism by which a *tolC* mutation exerts its affect on OmpF synthesis.

Chapter-2

MATERIALS AND METHODS

2.1 Growth media

Double strength Difco nutrient broth (Difco 0003, 16 g/L) with 5 g/L NaCl was the general growth medium which was referred to as ~nutrient broth" or ~high osmolarity medium". Low osmolarity nutrient broth contained 8 g/L of Difco nutrient broth. NA is nutrient agar (blood base agar, Difco, 0045) prepared without the addition of blood. Soft agar contained equal volumes of NA and nutrient broth.

For radio-active labelling experiments M9 minimal salt medium, (Miller, 1972) or phosphate limiting medium was used, the latter contained 20 mM KCl, 85 mM NaCl, 100 mM Tris, 20 mM NH_4Cl, 1 mM $MgCl_2$, 0.1 mM KH_2PO_4, 1 μg/ml thiamine, 1% casamino acids (dephosphorylated) and 5 mg/ml glucose. For dephosphorylation of the casamino acids solution, 2 ml of 1 M $MgCl_2$ and then 2 ml of concentrated NH_4OH was added to 20 ml of a 25% casamino acids (Difco) solution. The precipitate was filtered off by passing the solution through a Whatman (no. 1) filter paper and the precipitation procedure was repeated. Finally the pH of the solution was adjusted to 7.0 with concentrated HCl and the concentration of the casamino acids was adjusted to 17% by diluting with water.

Carbon sources for minimal media were glucose, 5 mg/ml or maltose, 10 mg/ml. Growth factors were added to a final concentration of 20 μg/ml, except vitamin B1 (thiamine) which was added to a final concentration of 1 μg/ml. Antibiotics were added to NA in the following concentrations: ampicillin, 25 μg/ml; chloramphenicol, 25 μg/ml; kanamycin, 50 μg/ml and tetracycline, 16 μg/ml. For <u>tolC</u> mutants, the

concentrations of chloramphenicol and tetracyline were 12 and 10 μg/ml respectively. Sodium deoxychlolate (DOC) was used at a final concentration of 0.05% (w/v). All cultures were incubated at 37°C unless specified.

2.2 Reagents

Reagents were obtained from the following sources: acrylamide and bisacrylamide, Eastman Kodak Co.; sodium dodecyl sulphate (SDS), prod. no. 30175 and 44244, BDH; urea, Merck; cesium chloride, KBI; agarose, Seakem Inc.; nitrocellulose, Schleicher and Schuell; restriction endonucleases, Bal31, DNaseI, DNA polymerase I, deoxy nucleotide triphosphates and di deoxy nucleotide triphosphates, Boehringer-Mannheim; large fragment of DNA polymerase I (Klenow), M13 primer and T4 ligase, BRESA; ampicillin, Beecham; chloramphenicol and pronase, Calbiochem; kanamycin sulphate and soyabean trypsin inhibitor, Sigma; tetracycline, Gist-Brocade; trypsin, Hopins and Williams.

[^{35}S]methionine (800 or 1000 Ci/mM) and Amplify (flour), Amersham; [α-^{32}P]dCTP (2000 Ci/mM), BRESA. All other chemicals were of analytical grade.

2.3 Bacterial strains, bacteriophages and plasmids

The bacterial strains, bacteriophages and plasmids used in this thesis are listed in Tables 2.1 and 2.2 respectively.

Table 2.1

Bacterial strains and bacteriophages used in this thesis

Strain	Characteristics	Reference/Source
	Strains used in Chapter 3	
AB1133	F^- thr1 leu6 proA2 lacY1 supE44 galK2 his4 rpsL31 mtl1 argE3 thi1 xyl5 ara14 λ^-	A. L. Taylor
P602	AB1133 tolC203	Davies and Reeves, 1975
P2715	AB1133 (pPR42)	R. Morona
P2125	W1485F^- (see below) tonA208 pyrD34	P. Reeves
P2787	P2125 ompA (pPR42)	Morona *et al*., 1983
P2964	P602 (pPR258)	
P2970	P2964 (pGM4113)	
P3025	P2964 (pBR322)	
	Strains used in Chapter 4	
AB1133	see above	
P210	AB1133 ompF	Davies and Reeves, 1975
P530	AB1133 ompR101 (ompB101, ompR1)	Davies and Reeves, 1975
P602	see above	
P1533	AB1133 ompC	P. Reeves
W1485F^-	Prototroph	C. Schnaitman
P2731	W1485F^- tolC210::Tn10-48	Morona and Reeves, 1982a

Strain	Characteristics	Reference/Source
MC4100	F$^-$ araD139 Δ(arg-lac) U169 rspL150 relA1 flbB5301 deoC1 ptsF25	Casadaban, 1976
MH513	MC4100 araD$^+$ φ(ompF-lacZ$^+$) 16-13	Hall and Silhavy, 1981a
MH610	MC4100 araD$^+$ φ(ompF-lacZ$^+$) hyb. 16-10	Hall and Silhavy, 1981a
P3289	MH610 tolC	
P2983	AB1133 (pJP33)	
P2985	P210 (pJP33)	
P2986	P530 (pJP33)	
P2987	P602 (pJP33)	
P3030	AB1133 (pPR272)	
P3031	P210 (pPR272)	
P3032	P530 (pPR272)	
P3033	P602 (pPR272)	
P3053	AB1133 (pPR275)	
P3054	P210 (pPR275)	
P3055	P530 (pPR275)	
P3102	P602 (pPR275)	
P3224	P210 (pMAN007)	
P3225	P210 (pMAN009)	
P3226	P602 (pMAN007)	
P3227	P602 (pMAN009)	
P2770	P602 ompC	R. Morona

Strains	Characteristics	Reference/Source
P3228	P2770 (pMAN006)	
P3229	P2770 (pMAN007)	
P3230	P2770 (pMAN009)	
P3231	P2770 (pMAN010)	
P3183	P1533 ompF	
P3283	P3183 (pMAN006)	
P3284	P3183 (pMAN007)	
P3285	P3183 (pMAN009)	
P3286	P3183 (pMAN010)	
	Strains used in Chapter 5	
P2125	see above (chapter 3 list)	
P2716	P2125 ompC tolC210::Tn10-48	Morona and Reeves, 1982b
P2718	P2716 stc-2	Morona and Reeves, 1982b
CS1253	W1485F$^-$ ΔompC178 zei-198::Tn10	Scnaitman and McDonald, 1984
JM101	supE thi Δ(lac-pro) [F' traD36 proA$^+$ proB$^+$ lacI^q Z M15]	Messing and Vieira, 1982
W1485F$^-$	see above (chapter 4 list)	
P2731	see above (chapter 4 list)	
CS1253	see above (chapter 5 list)	

Strain	Characteristics	Reference/Source
P3398	CS1253 tolC	
P3183	see above (chapter 4 list)	
P3283	see above (chapter 4 list)	
P3427	P3183 (pPR426)	
P3418	CS1253 (pMAN006)	
P3419	P3398 (pMAN006)	
P3423	CS1253 (pPR426)	
P3424	P3398 (pPR426)	
SM3001	MC4100 Δ micF1	Matsuyama and Mizushima, 1985
P3493	SM3001 tolC	
MH760	MC4100 ompR472 (ompR2)	Hall and Silhavy, 1981a
P3394	MH760 tolC	
W4626 Phe⁻	purE pheA trp lac85 galK2 malA mtl xyl2 ara rpsL (λ⁻)	Nara *et al*., 1984
P3396	W4626 Phe⁻ tolC	
FN101	W4626 Phe⁻ ompR20 (res)	
P3393	FN101 tolC	
MC4100	see above (chapter 4 list)	
P3011	MC4100 tolC	
P3501	MC4100 (pmic B21)	
P3502	P3011 (pmic B21)	
P3503	MH760 (pmic B21)	
P3504	P3394 (pmic B21)	

Colicin producing strains

Strain	Colicin produced	Reference/Source
K53	E1	Davies and Reeves, 1975
CA42	E2	Davies and Reeves, 1975
CA38	E3	Davies and Reeves, 1975

Bacteriophages

Phage	Receptor	Reference/Source
K2	OmpF	Hancock and Reeves, 1975
Tula	OmpF	U. Henning
Tulb	OmpC	U. Henning

Table 2.2

Plasmids used in this thesis

Cloning vectors	Charateristics	Reference/Source
pACYC184	Cm^r, Tc^r	Chang and Cohen, 1978
pBR322	Ap^r, Tc^r	Boliver *et al.*, 1977
pUC18	Ap^r	Messing, 1983
pLG339	Km^r, Tc^r	Stoker *et al.*, 1982
pDF41	*trpE*$^+$	Kahn *et al.*, 1979
Other plasmids		
pPR42	Tc^r; vector, pBR322; cloned gene, *tolC*	Morona and Reeves, 1981
pPR178	Ap^r; vector, pUC9; cloned gene, *tolC*	P. Reeves
pPR258	Tc^r; vector, pACYC184; cloned gene, *tolC*	
pGM4113	Ap^r, Tc^r; vector, pBR322; cloned gene, T4 *tRNA*^{Arg}	Mazzara *et al.*, 1981
pJP33	Cm^r; vector, pACYC184; cloned gene, *ompF*	Tommassen *et al.*, 1982
pPR268	Ap^r; vector, pBR322; cloned gene, *ompF*	
pPR272	Km^r; vector, pLG339; cloned gene, *ompF*	

Other plasmids	Characteristics	Reference/Source
pPM431	Ap^r, Cm^r; carries Tn1725 (Cm^r gene)	P. Manning
pPR274	Cm^r; vector, pDF41; cloned gene, Cm^r gene from pPM431	
pPR275	Cm^r, vector, pPR274; cloned gene, ompF	
pLF11	Ap^r; vector, pBR322; cloned gene, 5'-terminal half of ompF	Inokuchi et al., 1982
pPR313	Ap^r; vector, pUC18; cloned gene, DNA from E. coli K-12 carrying the Stc^- mutation	
pMAN006	Ap^r; vector, pKEN403; cloned genes, ompC, micF (CpC)	Matsuyama et al., 1984
pPR426	Ap^r; vector, pKEN403; micF is deleted from pMAN006	
pMAN007	Ap^r; vector, pKEN403; cloned gene, ompC promoter controlled ompF gene (CpF)	Matsuyama et al., 1984
pMAN009	Ap^r; vector, pKEN403; cloned gene, ompF (FpF)	Matsuyama et al., 1984
pMAN010	Ap^r; vector, pKEN403; cloned gene, ompF promoter controlled ompC gene (FpC)	Matsuyama et al., 1984
pmicB21	Ap^r; vector, pKEN005; cloned gene, micF promoter controlled lacZ gene	Mizuno et al., 1984

2.4 Bacteriophage methods

2.4.1 Propagation of bacteriophages and plaque assay

Bacteriophages K2, Tula, Tulb and λ*vir* were propagated on large glass dishes (18 x 28 cm) using appropriate bacterial strains: 1 ml of an overnight bacterial culture was mixed with 5 x 10^4 plaque forming units (p.f.u.) of phage and after adding 24 ml of soft agar, the mixture was poured onto a NA dish and incubated for 6 to 8 hr at 37°C. Fifty ml of SM buffer (100 mM NaCl, 10mM $MgSO_4$, 50 mM Tris-HCl [pH 7.5], 0.001% gelatin) was added to the dish and left overnight at 4°C. The liquid was carefully collected, centrifuged in a bench centrifuge to remove agar fragments and incubated (shaking) with 2% chloroform for 30 min, centrifuged again as above and the supernatant stored at 4°C.

To perform the plaque assay, indicator bacteria (0.1 ml of a freshly grown culture) were incubated with 0.1 ml of bacteriophage (neat and serial 10^{-2} dilutions) at 37°C for 15 min, 3 ml of soft agar was added and the mixture poured onto a NA plate and incubated overnight.

2.4.2 Bacteriophage sensitivity test

A bacteriophage stock was streaked across the centre of a NA plate, then allowed to dry before cross streaking with bacterial strains from either liquid culture or single colonies.

2.4.3 Isolation of bacteriophage and colicin E1 resistant mutants

A high titre of bacteriophage stock was spread over half of a NA plate and allowed to dry. A fresh overnight bacterial culture was streaked once onto the dried phage area with the help of a sterile cotton

swab, and the plates were incubated for 24 hr. Bacteriophage resistant colonies were purified on NA plates and retested.

To isolate colicin resistant mutants, a fresh culture of colicin E1 producing strain (K53) was spread over half of a NA plate and incubated for 16 hr. Cells were killed by inverting the plate for 30 min over a tissue paper soaked with chloroform. The plate was overlayed with NA and fresh bacterial cultures were streaked across this plate and incubated for 24 hr. Colicin E1 resistant mutants were tested on NA plates containing DOC and against colicins E2 and E3 to detect tolC mutants. Colonies which were resistant to colicin E1 but sensitive to DOC and colicins E2 and E3 were purified on NA plates.

2.5 Protein methods

2.5.1 Small scale whole cell envelopes preparation

Whole cell envelopes from 10 ml bacterial cultures were isolated essentially by the lysozyme-sonication method described by Morona and Reeves (1982a) with the modification that after sonication of spheroplasts, unbroken cells were removed by low-speed centrifugation (5,000 rpm, 5 min) before the envelopes were pelleted by high-speed centrifugation (15,000 rpm, 1 hr).

2.5.2 Small and large scale preparations of outer membranes

Small scale preparation of outer membranes was performed according to the Triton X-100 extraction method of Manning et al. (1980). Whole cell envelopes isolated from 20 ml of fresh overnight cultures were

washed twice in 10 mM HEPES (pH 7.5) containing 2 mM $MgCl_2$, and resuspended in 10 mM HEPES (pH 7.5) containing 2% (v/v) Triton X-100 and incubated for 30 min at room temperature. The Triton X-100 insoluble outer membrane fraction was pelleted by centrifugation at 105,000 g for 30 min. Large scale isolation (from a 1L culture) of outer membranes was carried out essentially as described by Morona et al. (1983).

2.5.3 Trypsin treatment of whole cells and outer membranes

Bacterial cultures (50 ml) at log phase were pelleted by centrifugation (5,000 rpm, 10 min) in a SS34 rotor (Sorvall). The pellet was washed once in 10 ml of 10 mM Tris-HCl (pH 7.8) and resuspended in 10 ml of 10 mM Tris-HCl (pH 7.8) containg 0.1 mM EDTA. Samples (2 ml) were transferred to MacCarteny bottles and incubated with or without trypsin (250 μg/ml) for 4 hr at 37°C. The trypsin digestion was stopped by adding an excess of trypsin inhibitor. The cells were pelleted and washed in 30 mM Tris-HCl (pH 8.1) containing 100 μg/ml of the trypsin inhibitor and frozen. Preparation of outer membranes from the frozen pelletes was carried out as described above (see sec. 2.5.2).

Outer membranes, isolated from a 100 ml culture, were resuspended in 100 μl of 10 mM Tris-HCl (pH 7.8) and incubated with or without trypsin (200 μg/ml) in 1.5 ml Eppendorf microfuge tubes. Samples were taken at various time intervals and trypsin inhibitor (final conc. 200 μg/ml) was immediately added to each sample. Tubes were centrifuged at 15,000 rpm for 1 hr in a SS34 rotor and the resulting membrane pellet s were resuspended in sample buffer (62.5 mM Tris-HCl [pH 6.8], 2% SDS, 10% glycerol, 5% β-mercaptoethanol and 1 mg/ml bromo phenol blue).

2.5.4 Surface labelling of envelope proteins with ^{125}I

Surface exposed envelope proteins of whole cells were labelled with ^{125}I using lactoperoxidase according to the method of Manning et al. (1980)

2.5.5 Purification of the TolC and OmpF proteins

TolC protein was purified essentially as described by Morona et al. (1983) from a strain (P2787) carrying a high-copy number $tolC^+$ plasmid (pPR42). The OmpF protein was purified from an ompC mutant strain by the SDS-extraction method described by Rosenbusch (1974).

In both cases the contaminating proteins did not contribute more than 5-10% of the total protein as estimated by analysis of the purified proteins on SDS-polyacrylamide gels followed by Coomassie blue staining.

2.5.6 Pulse-chase experiments

Pulse-chase experiments were designed to label short-lived forms of TolC or OmpF and to chase these forms into the mature protein.

Strains used for pulse-chase experiments were all based on AB1133 and were grown in 10 ml of M9 minimal salt medium supplemented with L-arginine, L-leucine, L-proline, L-histidine, L-threonine, thiamine and glucose. Cells (at OD_{600}=0.5) were centrifuged and resuspended in one-tenth the volume of fresh M9 medium and preincubated at 25°C for 5 min prior to the addition of [^{35}S]methionine (100 μCi/ml). The radioactivity was chased with non-radioactive methionine (final conc. 20 mM) after the appropriate pulse period. Samples (100 μl) were taken at

various times and transferred into Eppendorf microfuge tubes containing crushed ice, chloramphenicol (250 μg/ml) and sodium azide (NaN_3, 0.2%), and quickly frozen at -70°C. Cells were pelleted at 4°C in an Eppendorf microfuge for 5 min and resuspended in 100 μl of sodium dodecyle sulphate (SDS) buffer (100 mM Tris-HCl [pH 7.4], 5m M EDTA, 0.5% SDS, 0.02% NaN_3), then heated for 3 min in boiling water for cell lysis prior to immunoprecipitation.

2.5.7 Labelling and Fractionation of [^{35}S]methionine labelled cells

A 10 ml culture was grown in M9 minimal medium to mid-log phase (OD_{600}=0.5), centrifuged and resuspended in 1 ml of fresh M9 medium containing 0.2 μg of L-methionine. Cells were then preincubated for 10 min prior to labelling with [^{35}S]methionine (100 μCi/ml) for 90 s. Radioactive isotope incorporation was stopped by adding non-radioactive methionine and NaN_3 to final concentrations of 20 mM and 0.02% respectively. A 0.1 ml sample was removed to prepare whole cell extract as above (sec. 2.5.6) and the remaining labelled cells were used to prepare cytoplasm, whole cell envelopes and outer and inner (cytoplasmic) membrane fractions. These fractions were prepared essentially by the method of Ito _et al_. (1977), which involved two sucrose step gradients. Each fraction thus obtained was used separately for immunoprecipitation.

2.5.8 Isolation of TolC and OmpF antisera and immunoprecipitation of [^{35}S]methionine labelled proteins.

To obtain antiserum to TolC or OmpF proteins, purified protein (0.75 mg) was suspended in Freund's complete adjuvant and injected into a

rabbit: booster shots (2 x 0.5 mg) followed at 14-day intervals and the rabbit was bled after five weeks.

SDS-solubilised [^{35}S]methionine labelled cells (approximately 2 x 10^8) were diluted 10-fold with Triton buffer (100 mM Tris-HCl [pH 7.4], 1 mM EDTA, 0.02% NaN_3, 1.0% Triton X-100). Antiserum (5-7.5 μl) was added and the mixture incubated at 37°C for 1 hr. Affinity-purified goat-anti-rabbit IgG (20 μl of 4 mg/ml, donated by Dr P. Ey) was added and after 3 hr of incubation at 37°C, the precipitate was collected by centrifugation (Eppendorf microfuge, 15 s). The precipitate was washed twice with Triton buffer and twice with Tris buffer (100 mM Tris-HCl [pH 7.4], 1 mM EDTA, 0.02% NaN_3), resuspended in sample buffer and heated for 3 min in boiling water.

2.5.9 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins were analysed on 11% linear SDS- polyacrylamide gels as previously described (Lugtenberg et al., 1975). Samples were heated in a boiling water bath for 3 min before loading. Gels were stained with Coomassie Brilliant Blue G250 (0.06%, w/v) in 3.5% perchloric acid and destained in 5% (v/v) acetic acid according to the method of Achtman et al. (1978). When required, quantities of major outer membrane proteins were estimated by scanning the stained gel on a Quick Scan densiometer (Helena labs, Beaumont, Texas) then measuring the area under a peak by tracing the scan onto butter paper, cutting out the peak area and weighing the piece of paper.

2.5.10 Fluorography and autoradiography

Gels containing [^{35}S]methionine labelled immunoprecipitates from pulse or pulse-chase experiments were fluorographed. Proteins were fixed in 7% acetic acid for 30 min and the gel soaked in "Amplify" fluor (Amersham) with agitation for 15 to 30 min, rinsed twice with water and dried in a Biolab gel drier. The gels were fluorographed at room temperature for 3 to 5 days using Fuji X-ray films. Stained gels with ^{125}I labelled samples were autoradiographed. Autoradiography was carried out at room temperature after drying the gel onto a Whatman (no. 1) paper.

2.6 DNA techniques

2.6.1 Small scale isolation of plasmid DNA

Small scale isolation of plasmid DNA was performed by the following two methods:

(a) When a large number of clones were to be screened, the method of Ish-Horowicz and Burke (1981) was used. Although the method was much quicker it was found that longer storage (more than a month) or longer incubation (more than 2 hr) with the restriction enzymes, resulted in partial or complete degradation of the plasmid DNA. The plasmid DNA isolated by this method was therefore extracted once with phenol or heated at 65°C for 10 min before digestion.

(b) A better quality plasmid DNA was isolated by the Triton X-100 cleared-lysate method as described by Kahn _et al_. (1979).

2.6.2 Cesium chloride (CsCl) purification of plasmid DNA

Large scale purification of plasmid DNA was carried out either by a two-step cesium chloride gradient method of Garger et al. (1983) or by isopycnic centrifugation of the nucleic acid fraction in CsCl as described by Davis et al. (1980).

2.6.3 Isolation of chromosomal DNA

A modified method of Nakamura et al. (1979) was used to isolate chromosomal DNA. A 20 ml overnight culture was centrifuged (bench centrifuge, full speed for 10 min), the pellet was resuspended in 10 ml of TES buffer (50 mM Tris-HCl [pH 8.0], 5 mM EDTA, 50 mM NaCl) and centrifuged as above. The pellet was resuspended in 2 ml of 25% sucrose, 50 mM Tris-HCl (pH 8.0) and after adding 1 ml of 10 mg/ml lysozyme in 0.25 M EDTA (pH 8.0), the mixture was incubated on ice for 20 min. Lysis was obtained after the addition of 0.75 ml of TE buffer (10 mM Tris-HCl [pH 8.1], 1 mM EDTA) and 0.25 ml of the lysis solution (5% sarkosyl, 50 mM Tris-HCl [pH 8.0], 62.5 mM EDTA). Pronase (10 mg) was added to this mixture and incubated at 56°C for 1 hr. The solution was extracted three times with phenol (saturated in TE buffer) and twice with diethyl ether at 4°C. The aqueous phase was decanted and 8 ml of cold 95% ethanol was added to the bottom phase. The precipitated DNA was washed twice in 70% ethanol, dried under vacuum and dissolved in 1 ml of TE buffer. Usually the dried precipitate was left overnight at 4°C in TE buffer. If the DNA was still not dissolved completely, it was heated at 56°C for 20 to 30 min.

2.6.4 Restriction enzyme digestions

DNA digestions were usually performed using SPK buffer (10x: 0.2 M Tris-HCl [pH 8.0], 1 mM EDTA, 50 mM $MgCl_2$, 0.5 M KCl, 50% glycerol, 5 mM dithioethritol) in a final volume of 20 μl. For some enzymes (*Acc*I and *Sma*I), the digestion buffer was made according to the manufacturer's specifications.

Usually 0.1 to 1 μg of the plasmid DNA was digested with 2 units of enzyme at 37°C in a 1.5 ml Eppendorf microfuge tube. For chromosomal DNA digestions, 5-20 units of enzymes were used. All digestions were terminated by heating the tubes at 65°C for 10 min. Before loading the samples on agarose gels, one-tenth volume of tracking dye (10x: 0.6% bromophenol blue, 15% ficoll, 100 μg/ml RNase) was added to the sample.

2.6.5 Agarose gel electrophoresis

Restriction enzyme digested or undigested DNA samples were analysed on 0.6%-1.5% horizontal agarose gels in 0.5x TBE buffer (10x: 0.89 M Tris, 0.89 M Boric acid, 0.025 M EDTA.) The gels were stained with ethidium bromide (0.5-1.0 μg/ml) and the bands were visualised and photographed under UV illumination (using Polaroid type 665 negative or 667 positive films). *Eco*RI digests of SPPI DNA and *Bgl*II digests of λcI857*sam*7 DNA were used as molecular size markers.

2.6.6 Purification of the restriction fragments from plasmid DNA

Plasmid DNA digested with the appropriate restriction enzyme was electrophoresed in a low gelling temperature (LGT) agarose gel containing

0.5 μg/ml of ethidium bromide. The bands were visualised under UV and segment(s) of the gel containing the DNA of interest was cut out with a sharp blade, mixed with 1-2 ml of TE buffer and heated at 65°C for 30 min (or until all the agarose pieces were completeley melted). This solution was then extracted twice with phenol (saturated in 50 mM Tris-HCl, [pH 7.5]) at room temperature, once with phenol/chloroform (50:50) and once with chloroform only. DNA was recovered by ethanol/sodium acetate precipitation: sodium acetate (pH 5.2) was added at a final concentration of 0.3 M and then mixed with 2.5 volumes of ice-cold 95% ethanol and left overnight at -20°C, or for at least 2 hr at -70°C, centrifuged at 4°C in an Eppendorf microfuge or SS34 rotor for 10 min, washed once in 70% ethanol, dried under vacuum and dissolved in TE buffer.

2.6.7 DNA ligation

Digested and heat-inactivated DNA samples were usually ligated in a 20 μl volume. The sticky-end ligations were carried out at 4°C for 1-2 hr (usually by this time 50% of the DNA molecules were circularised, as indicated by electrophoresis of a portion of the ligation mixture in agarose gels). This mixture was either used directly to transform competent cells or diluted 10-20 fold in water and ligation buffer (10x: 0.66 M Tris-HCl, [pH7.4], 10 mM EDTA, 0.1 M $MgCl_2$, 0.1 M dithiothreitol, 1 mM adenosine triphosphate) plus extra ligase (0.5 μl) and left for 12-16 hr at 4°C.

Blunt-end ligations were carried out in a volume of 10 μl (after adding 1 μl of 10x ligation buffer 200 mM Tris-HCl [pH 7.4], 100 mM $MgCl_2$, 100 mM dithiothreiotol and 6 mM adenosine triphosphate) at 4°C for 20-24 hr.

2.6.8 Dephosphorylation and *Bal*31 digestion of plasmid DNA

These techniques were performed essentially as described by Maniatis *et al*. (1982). After digesting DNA with *Bal*31 the mixture was desalted by passing through a sepharose CL-6B column.

2.6.9 Nick translation

Nick translation of plasmid DNA or DNA fragments extracted from agarose gels was performed essentially by the method of Rigby *et al*. (1977), but the DNA was first treated with DNaseI (10 μg/ml) for 10 min at 37°C. This treatment increased the radioactive incorporation into DNA up to five-fold.

2.6.10 DNA-DNA and colony hybridisations

These techniques were performed according to the methods of Southern (1975), and Grunstein and Hogness (1975) respectively, as described by Maniatis *et al*. (1982).

2.6.11 Transformation

Bacterial strains were made competent and transformed with plasmid DNA routinely by the method of Lederberg and Cohen (1974). When a *tolC* mutant was transformed, competent cells were prepared with 30 mM $CaCl_2$ and heat shocked at 25°C instead of 42°C.

2.6.12 M13 techniques and DNA sequencing

Subcloning of DNA fragments into M13mp8 and M13mp9 was performed as described by Messing and Vieira (1982). The resultant recombinant phages were used for DNA sequencing according to the method of Sanger et al. (1977).

2.7 RNA methods

2.7.1 Purification of RNA

RNA was purified from exponentially growing bacterial cultures by the method of Kalionis (Ph.D thesis, Department of Biochemistry, University of Adelaide, Adelaide). Briefly, 1 ml cultures were pelleted and washed twice in TE buffer. The pellet was resuspended in 200 µl of lysis buffer (10 mg/ml of lysozyme made up in TE buffer, directly before use), frozen (in dry ice/ethanol) and thawed by submerging the tube in water with occassional vortexing. To this mixture 150 µl of water and 50 µl of 5% SDS was added, vortexed and heated at 42°C until the cells were lysed (usually 2-3 min). Lysed cells were extracted twice with 400 µl of phenol (re-distilled phenol equilibriated in 20 mM sodium acetate [pH 5.2], 20 mM KCl and 10 mM $MgCl_2$). RNA was then precipitated from the aqueous phase with sodium acetate and ethanol (sodium acetate [pH 5.2] was added to 0.3 M, mixed well, 2.5 volumes of ice-cold 95% ethanol was added, and chilled at -70°C for 30 min.), pelleted (10 min, Eppendorf microfuge at 4°C), washed with 70% ethanol and stored in 0.1 mM EDTA (pH 7.5) at -20°C.

2.7.2. Northern transfer

Northern transfer of glyoxal-denatured RNA samples and hybridisation with [α-^{32}P]dCTP labelled DNA probe was performed essentially as described by Thomas (1980).

2.7.3 Isolation of specific mRNA species by hybridisation with DNA bound to nitrocellulose.

When a sensitive assay of hybridised mRNA was required, total cellular RNA was hybridised to plasmid DNA that had been denatured and immobilised on nitrocellulose filters. Specific mRNA that hybridised with DNA was then eluted out from the filters and analysed. This method is described below.

2.7.3.1 Binding of DNA to nitrocellulose

Approximately 10 μg of the denatured plasmid DNA was spotted and immobilised on nitrocellulose filters (small circular discs that fitted into a 2.2 ml screw top Eppendorf tubes) according to the method of Parnes *et al*. (1981).

2.7.3.2 *In vivo* labelling of RNA

Cells were grown in phosphate-limiting medium to late log-phase (OD_{600}=0.8). A portion of this culture (1 ml) was transferred to an Eppendorf tube (2.2 ml, screw top), incubated at 37°C with 100-150 μCi of [^{32}P]phosphoric acid for 5 min in a shaker, chilled (ethanol/dry ice) and pelleted (2 min in an Eppendorf microfuge). The supernatant was

carefully removed and RNA was extracted as described above (sec. 2.7.1). [^{32}P] labelled RNA was always stored at -20°C in 70% ethanol. When required, the labelled RNA from 70% ethanol was pelleted, dried under vacuum and resuspended in 0.1 mM EDTA.

2.7.3.3 Hybridisation and elution of RNA

Filters containing the immobilised DNA to be hybridised were soaked in 2 x SSC (17.53 g NaCl, 8.82 g tri-Sodium citrate) and then placed in a 2.2 ml screw top Eppendorf tube. To each tube was added 250 µl of hybridisation buffer (500 µl, deionised formamide; 250 µl, 4 M NaCl; 10 µl, 1 M Tris-HCl [pH 7.9]; 12.5 µl, E. coli t-RNA from a 10 mg/ml stock) and after 30 min of pre-hydridisation at 37°C, [^{32}P] labelled RNA ($\approx 5 \times 10^6 - 1 \times 10^7$ cpm) was added. Hybridisation was carried out at 37°C for 24 hr with gentle shaking, after which the hybridisation solution was carefully removed by aspiration. Filters were washed ten times with 1 ml of 2 x SSC containing 0.1% SDS at 65°C, four times in 2 x SSC and then transferred to fresh screw top Eppendorf tubes. To each tube was added 200 µl of water before being placed in a boiling water bath for 90 s. Tubes were spun for 10 min in an Eppendorf microfuge and the supernatant was extracted once with phenol, precipitated by sodium acetate/ethanol, washed in 70% ethanol, dried under vacuum and resuspended in 10 µl of 0.1 mM EDTA. The eluted mRNA was electrophoresed in a 5% acrylamide-8 M urea sequencing gel.

CHAPTER-3

SOME TOPOLOGICAL PROPERTIES AND BIOSYNTHESIS OF THE TolC PROTEIN

3.1 Introduction

tolC mutants of Escherichia coli K-12 are pleiotropic, being resistant to colicins El and A, extremely sensitive to several dyes and detergents, more sensitive to various antibiotics (Clowes, 1965; Nagel de Zwaig and Luria, 1967; Davies and Reeves, 1975; Hancock et al., 1976; Morona, 1982), and lack a detectable level of the OmpF protein (Morona and Reeves, 1982a). These phenotypic characteristics of tolC mutants suggest that the TolC protein plays an important role in the structural integrity of the outer membrane. TolC is a minor outer membrane protein but becomes a major outer membrane protein in strains carrying a high copy-number $tolC^{+}$ plasmid (Morona and Reeves, 1981; Morona et al., 1983). Previous studies (Morona et al., 1983) have shown that the undenatured TolC protein runs as high molecular weight aggregates which are not associated with the peptidoglycan layer. The TolC protein in isolated cell envelopes was also shown to be cleaved by trypsin, but no cleaved tryptic fragments were detected (Morona et al., 1983).

The work on the structural organisation of the TolC protein has been extended and biosynthesis of the protein studied: the results of these experiments are presented in this chapter.

3.2 Is the TolC protein exposed at the cell-surface?

To investigate the exposure of TolC at the cell-surface, bacterial cultures carrying a high copy-number <u>tolC</u>$^{+}$ plasmid, pPR42 (Morona and Reeves, 1981), were incubated with trypsin and the outer membrane fraction was analysed by SDS-PAGE and Coomassie blue staining. Fig. 3.1 shows that the TolC protein is cleaved in intact cells by externally added trypsin while OmpA, OmpF and OmpC remained unaffected: a large tryptic fragment of the TolC protein with a Mr of about 50,000 remained bound to the membrane. In a second experiment, bacterial cultures from TolC^{+} (P2715) and TolC^{-} (P602) strains were surface-labelled with ^{125}I and whole-cell extracts were analysed on a polyacrylamide gel which was stained with Coomassie blue, dried and autoradiographed. The results of this experiment are presented in Fig. 3.2 and show that TolC, along with the other major outer membrane proteins, can be surface-labelled with ^{125}I. These observations suggest that the TolC protein is exposed at the cell surface.

3.3 Trypsin susceptibility of the TolC protein in the outer membrane fraction

The experiment of Morona <u>et</u> <u>al</u>. (1983) was repeated but a different strain (P2787) was used which produces 3 to 4-fold more TolC protein (as demonstrated by SDS-PAGE; Morona, 1982). The outer membrane was isolated from this strain and from the parent strain (P2125) which carries only the chromosomal <u>tolC</u> gene and treated with trypsin for times indicated in Fig. 3.3. Trypsin digestions were stopped by the addition of trypsin inhibitor and the undigested membrane was re-isolated and

Fig. 3.1

Trypsin treatment of whole cells

Cells from strain P2715 were treated with or without trypsin and the outer membrane fraction from these cultures were analysed by SDS-PAGE. Lanes 2 and 1 contain samples from trypsin treated and untreated cultures repectively. Lane 3 contains samples from lanes 1 and 2. Arrows indicate the position of trypsin degraded (open arrow) and undegraded or native (closed arrow) TolC protein.

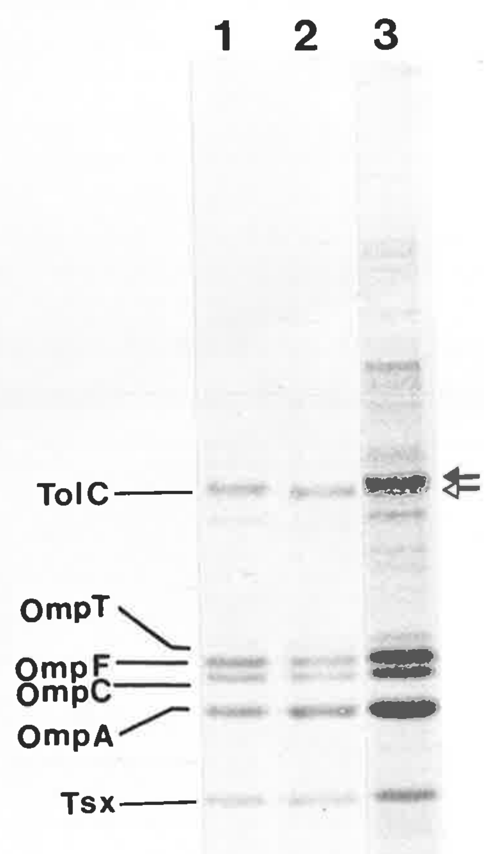
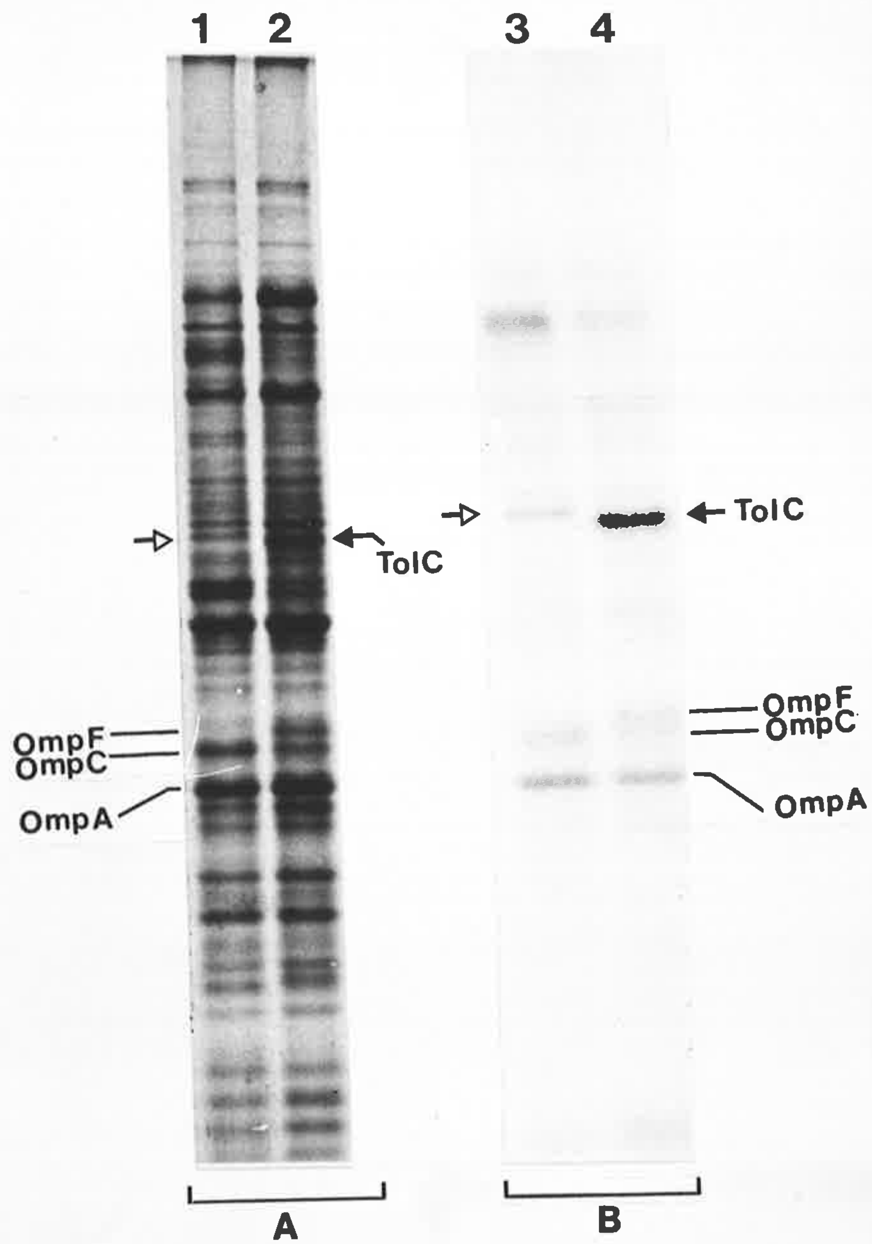

1
2
3
TolC
OmpT
OmpF
OmpC
OmpA
Tsx

Fig. 3.2

Labelling of proteins exposed to the cell surface

^{125}I-labelled cells from P602 (TolC^{-}; lanes 1 and 3) and P2715 (TolC^{+}; lanes 2 and 4) were solubilised in the sample buffer and analysed by SDS-PAGE. The gel was first stained with Coomassie blue (A) and then autoradiographed (B). Positions of TolC (closed arrows) and a protein observed only in P602 (open arrows) are shown.


1
2
3
4
TolC
TolC
OmpF
OmpC
OmpA
OmpF
OmpC
OmpA
A
B

analysed by SDS-PAGE and Coomassie blue staining (Fig. 3.3). Trypsin digestion resulted in almost complete disappearance of the TolC protein within 60 min with concomitant accumulation of a 27,000-Mr peptide in the digested membrane. The only other protein which was degraded, OmpA, was also completely digested with trypsin and its tryptic fragment of 20,000-Mr remained associated with the membrane (Fig. 3.3).

3.4 The signal sequence

Proteins that are exported by E. coli K-12 to the periplasmic space and outer membrane are synthesised in a precursor form containing an amino-terminal extension, the signal sequence, of about 20-25 amino acid residues (Blobel and Dobberstein, 1975; Inouye and Halegoua, 1980). The signal sequence is enzymatically removed from the protein by a "signal peptidase" (Silhavy et al., 1983) during, or shortly after the precursor protein inserts across the membrane.

The nucleotide sequence of the tolC gene (Hackett and Reeves, 1983; Hackett et al., 1983) showed that the amino terminus of the mature protein is preceded by a typical signal sequence of 22 amino acid residues as shown in Fig. 3.4.

3.5 Detection of TolC precursor

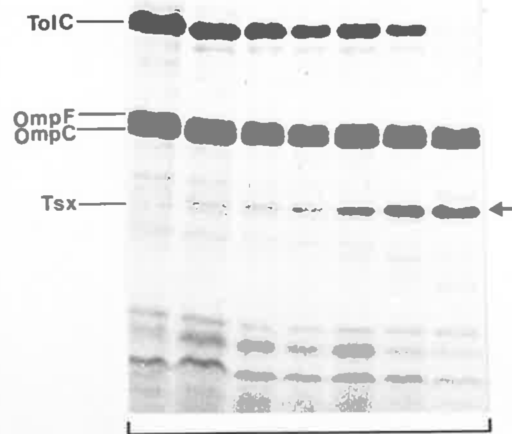
Pulse-chase experiments were designed to label short-lived forms of the TolC protein and chase these forms into mature protein. Cultures from P2715 (AB1133/pPR42, TolC$^+$) and P602 (AB1133, TolC$^-$) were pulse-labelled with [^{35}S]methionine and chased with non-radioacive methionine, samples were taken at various times and quickly frozen at

Fig. 3.3

Trypsin digestion of the outer membrane

The outer membrane fractions isolated from P2787 (P2125 ompA/pPR42) and P2125 were digested with trypsin and samples withdrawn at various times. Digestions were stopped by the addition of trypsin inhibitor and the undigested membrane was re-isolated and subjected to SDS-PAGE. Closed and open arrows indicate positions of tryptic fragments from the TolC and OmpA proteins respectively.

INCUBATION PERIOD (MIN.)



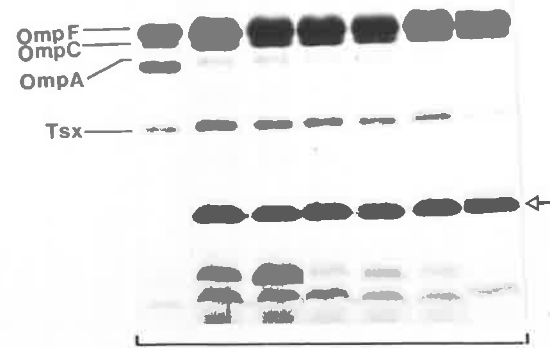


Fig. 3.4

The signal sequence of the TolC protein

A 22-amino acid signal sequence of the TolC protein. The sequence is taken from Hackett *et al*. (1983) and was deduced from the DNA sequence.

ATG AAG AAA TTG CTC CCC ATT CTT ATC GGC CTG AGC CTT TCT GGG TTC AGT TCG TTG AGC CAG GCC

Met Lys Lys Leu Leu Pro Ile Leu Ile Gly Leu Ser Leu Ser Gly Phe Ser Ser Leu Ser Gln Ala
-22 -1

-70°C. [^{35}S]methionine labelled cell lysates were immunoprecipitated with TolC antiserum and analyzed by SDS-PAGE. The gel was dried, autoradiographed and scanned to quantify the radioactivity that produced the bands. The molecular masses of the bands were determined by comparison with molecular mass standards run on an identical gel (or half of the same gel) stained with Coomassie blue. The result of this experiment is shown in Fig. 3.5. Although the detailed kinetics of processing was not studied since only a few chase samples were taken, the overall effect observed was the decrease of the higher-Mr band (54,500) from 76% of total label at 15 s chase to 1% at 300 s chase, while the lower-Mr band (52,000) rose from 24% of total label at 15 s to 99% of total label over the same period. As a control for the specificity of the TolC antiserum used, strain P602 was examined in a pulse-chase experiment as above. No immunoprecipitated labelled bands were detected on the gel (not shown). The tolC mutation therefore results in the disappearance of both the higher- and lower-Mr labelled bands of Fig. 3.5 in agreement with the proposal that the higher-Mr band is a precursor of the lower-Mr band.

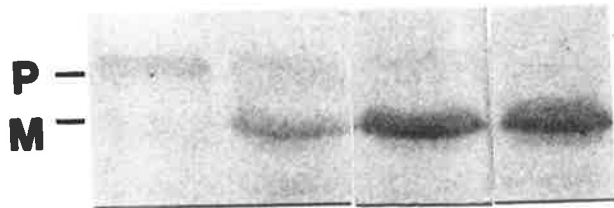
3.6 Pulse-chase experiment using shorter pulse time

The pulse-chase experiment carried out above showed that the TolC protein was first synthesised as a precursor with a signal sequence. The experiment was repeated using a shorter pulse time (10 s). Three major polypeptides with an apparent Mr of 46,000, 52,000 and 54,500 were immunoprecipitated using TolC antiserum (Fig. 3.6). None of these polypeptides were precipitated when a tolC^{-} strain (P602) was used in a control experiment (data not shown). A polypeptide with an apparent Mr of

Fig. 3.5

Pulse-chase experiment to show that the TolC protein is initially made in a high-Mr form

Strain P2715 (carrying a high copy number tolC$^+$ plasmid) was pulsed with [^{35}S]methionine for 15 s and chased, for varying times, with non-radioactive methionine. Solubilised cells were immunoprecipitated with antiserum to TolC protein and goat-anti-rabbit IgG. Immunoprecipitates were solubilised in the sample buffer, examined by SDS-PAGE and subjected to autoradiography (upper). The gel region containing polypeptides of Mr 50,000-56,000 was scanned (lower); P and M, proposed precursor and mature TolC protein, respectively. The open vertical arrows indicate the peak corresponding to precursor TolC protein; (1) 15 s chase; (2) 45 s; (3) 120 s; (4) 300 s.


P
M

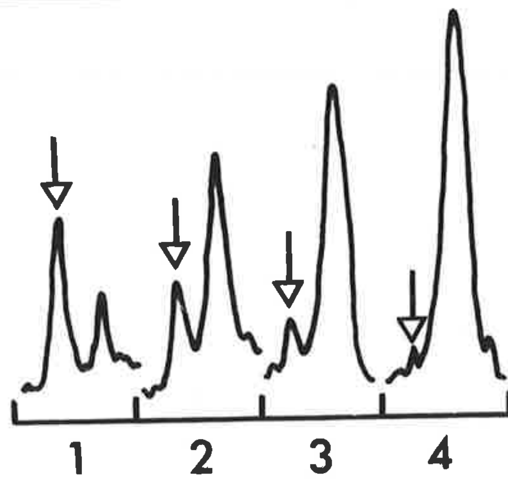
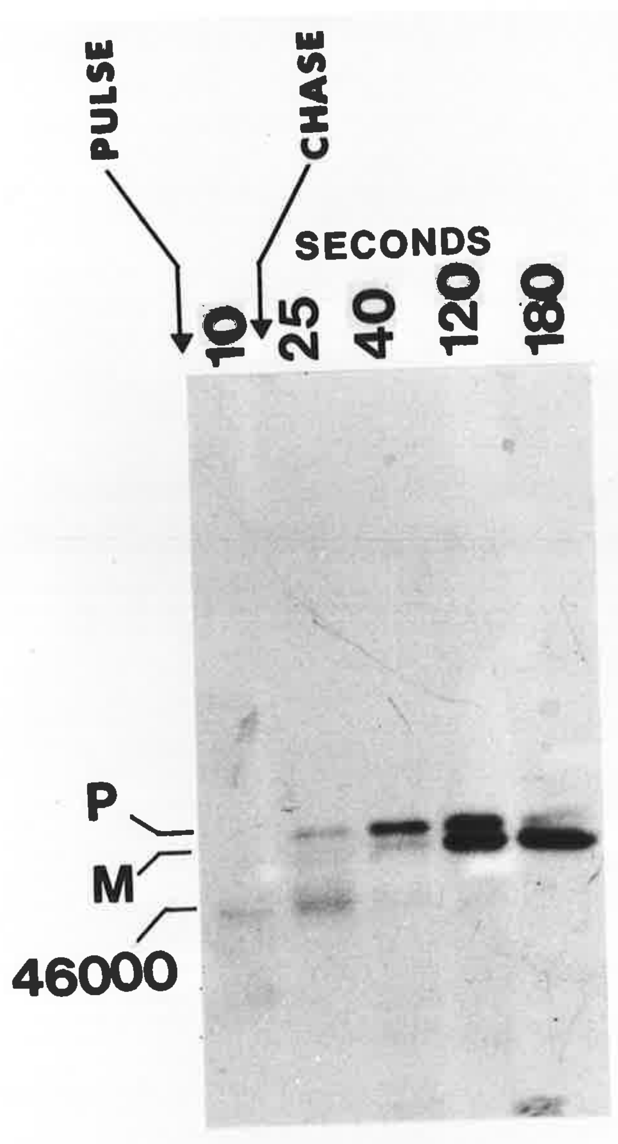

1
2
3
4
MIGRATION

Fig. 3.6

Immunoprecipitation of [^{35}S]methionine-labelled TolC and its precursor polypeptides

Strain P2715 was pulse-labelled (10 s) and chased as described in Materials and Methods (section 2.5.6). Samples, at indicated times, were immunoprecipitated and subjected to SDS-PAGE. The gel was dried and fluorographed. P, precursor; M, mature protein.


PULSE
CHASE
SECONDS
10
25
40
120
180
P
M
46000

46,000 was the first to appear at 10 s and was still the major band at 25 s but barely detectable by 40 s. A second polypeptide of 54,500-Mr (precursor) was immunoprecipitated at 25 s. This polypeptide was most abundant between 40 s and 120 s and disappeared during the chase while a concomitant increase in the amount of mature protein (Mr 52,000) was observed.

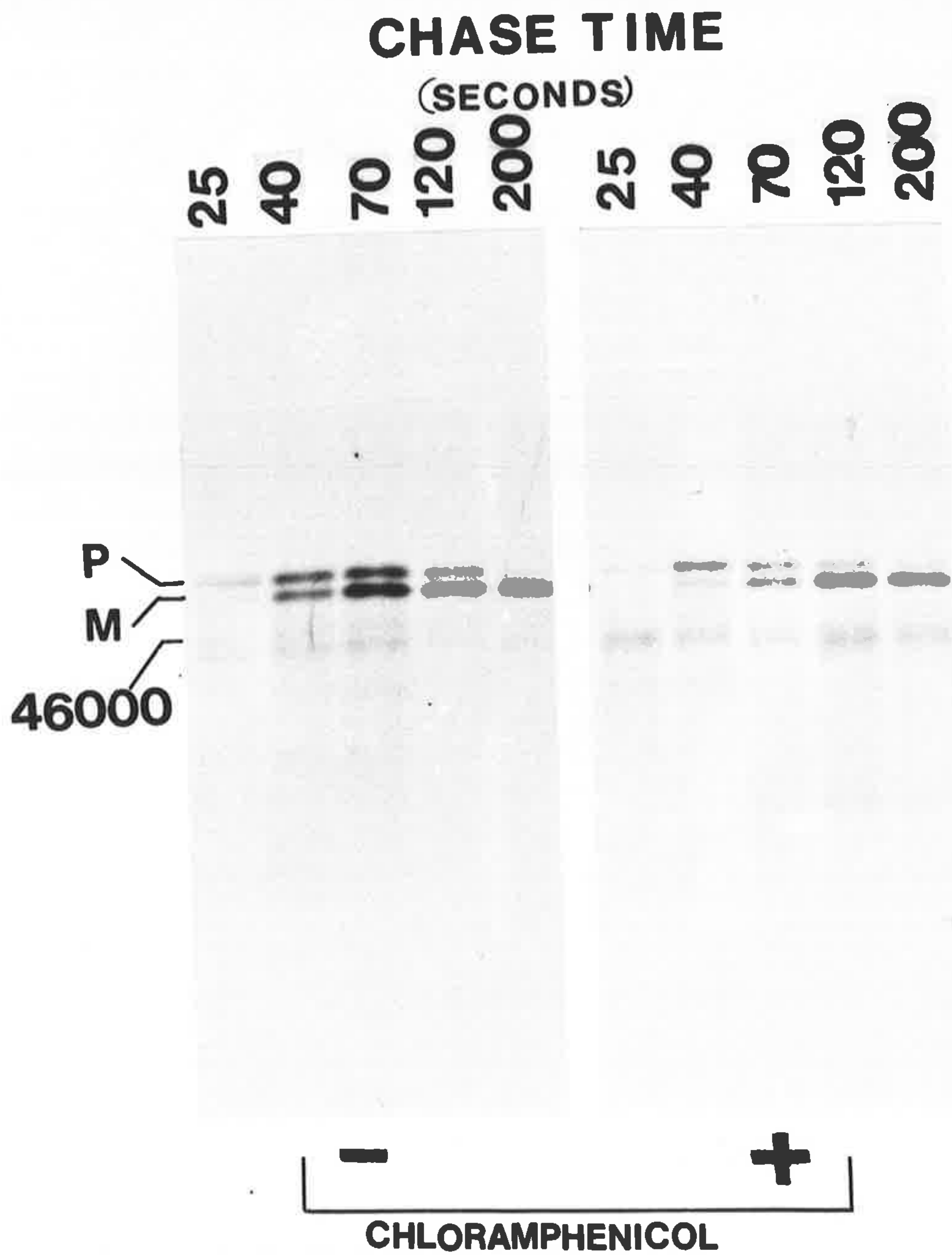
3.7 Is the 46,000-Mr polypeptide a biosynthetic intermediate of the mature TolC protein?

The kinetics of appearance of the 46,000-Mr polypeptide in the previous experiment suggested that it cannot be a degradation product. Rather, it could be an incomplete TolC peptide which requires further protein synthesis for completion. The following experiment was conducted to investigate this possibility. Cells were pulse-labelled for 10 s with [^{35}S]methionine and then chased with unlabelled methionine in the presence or absence of 250 μg/ml chloramphenicol (Fig. 3.7). In the absence of chloramphenicol, the 46,000-Mr polypeptide remained as a significant band up to 70 s. In the presence of chloramphenicol, the 46,000-Mr polypeptide was present in a much greater amount at 25 s and, instead of disappearing after 70 s, was still present in the final sample at 200 s. Furthermore, the 54,500-Mr completed precursor was fully chased into mature TolC protein, as it was in the absence of chloramphenicol. These results strongly support the hypothesis that the 46,000-Mr polypeptide is an incomplete TolC protein and not a degradation product.

Fig. 3.7

Fate of TolC biosynthetic intermediates in the presence or absence of chloramphenicol

Strain P2715 was pulse-labelled with [^{35}S]methionine for 10 s and chased with unlabelled methionine either in the absence or presence of chloramphenicol (final concentration, 250 μg/ml). Samples, taken at times indicated, were treated as for Fig. 3.6. P, precursor; M, mature protein.


CHASE TIME
(SECONDS)
25
40
70
120
200
25
40
70
120
200
P
M
46000
−
+
CHLORAMPHENICOL

3.8 Cause of the temporary accumulation of the 46,000-Mr polypeptide

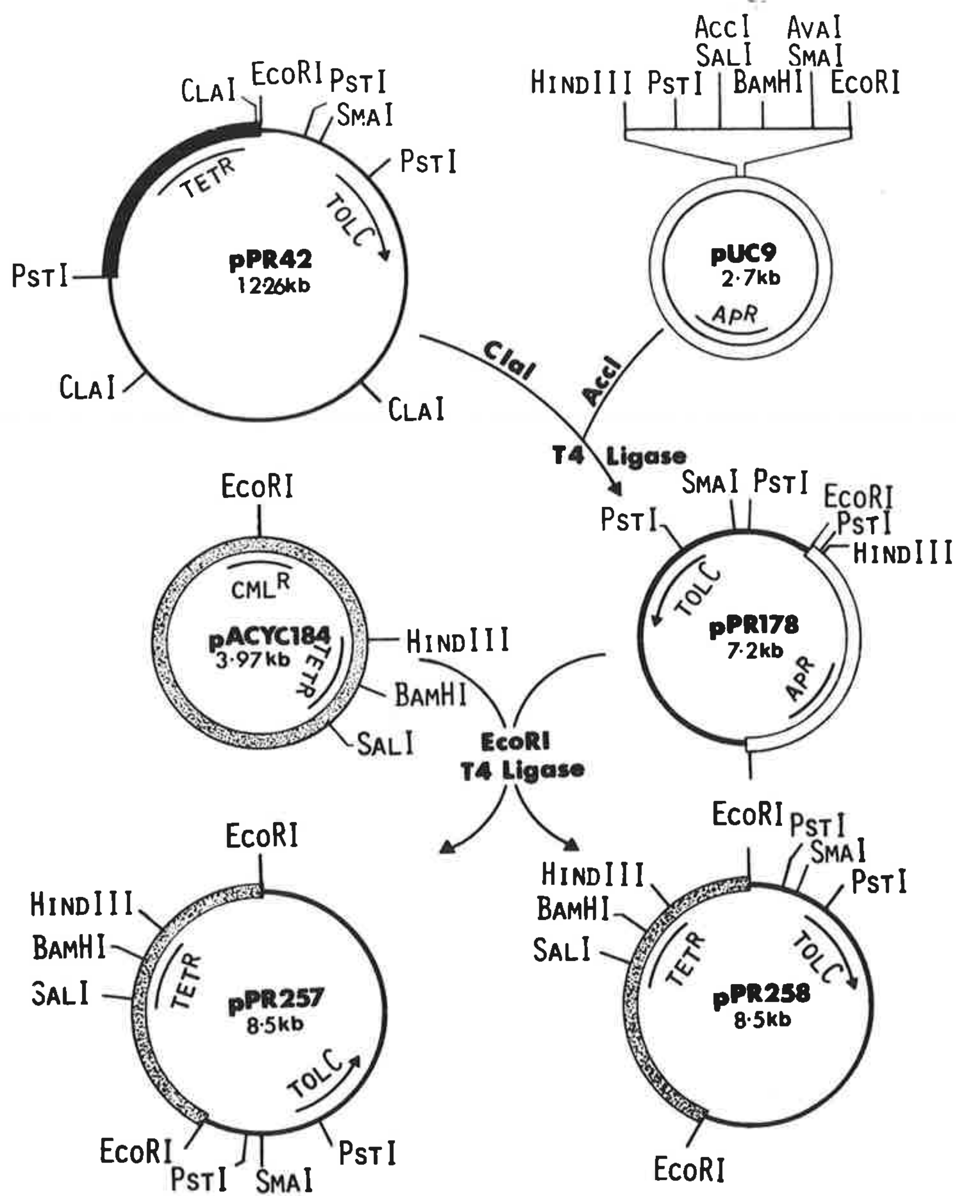
It has been suggested that secondary structures in mRNA may cause a temporary pause in the translation process. ompA mRNA is predicted to have about ten possible secondary structures (Movva *et al*., 1980). When the biosynthesis of the OmpA protein was studied (Crowlesmith and Gamon, 1982), several incomplete polypeptides were detected and this effect was attributed to temporary pausing in the translation process. A similar explanation was suggested for the existence of nascent intermediates in the synthesis of maltose-binding protein (Randall *et al*., 1980). The tolC DNA sequence predicts no possible secondary structure in mRNA (Hackett and Reeves, 1983) which could cause a pause in translation to give an incomplete peptide of 46,000-Mr. However, the presence of the rare codon AGA (arginine) at codon 402 of the tolC mRNA coding region may cause a translation delay owing to the limiting amount of the relevant $tRNA^{Arg}$ species in the cell (Ikemura, 1981). This would result in the temporary accumulation of an incomplete peptide with a relative molecular mass of 46,000. To test this hypothesis TolC biosynthesis was studied in a strain carrying a cloned T4 $tRNA^{Arg}$ gene. The $tRNA^{Arg}$ gene was originally cloned into pBR322 (Mazzara *et al*., 1981) and in order to overcome incompatibility the tolC gene was subcloned into pACYC184 to construct appropriate strains (Fig. 3.8).

The results of a pulse-chase experiment, using strains with or without $tRNA^{Arg}$ plasmid, are presented in Fig. 3.9. The presence of $tRNA^{Arg}$ plasmid had two effects. Firstly, the precursor (Mr 54,500) appeared earlier (10 s) and was subsequently processed quicker such that it was no longer detectable at 120 s. In the control strain, the

Fig. 3.8

Subcloning of the *tolC* gene into pACYC184

Plasmid pPR42 is pBR322 carrying a *Pst*I-*Eco*RI fragment from the *E. coli* K-12 chromosome which contains the *tolC* gene (Morona and Reeves, 1981). Plasmid pPR178 is pUC9 carrying a 4.5 kb *Cla*I fragment from pPR42. Other plasmids, pPR257 and pPR258 were made by subcloning the *tolC* gene from pPR178 into pACYC184 in both orientations. Equal amounts of the TolC protein were produced by strain P602 (*tolC*$^-$) carrying pPR257 or pPR258 (data not shown).

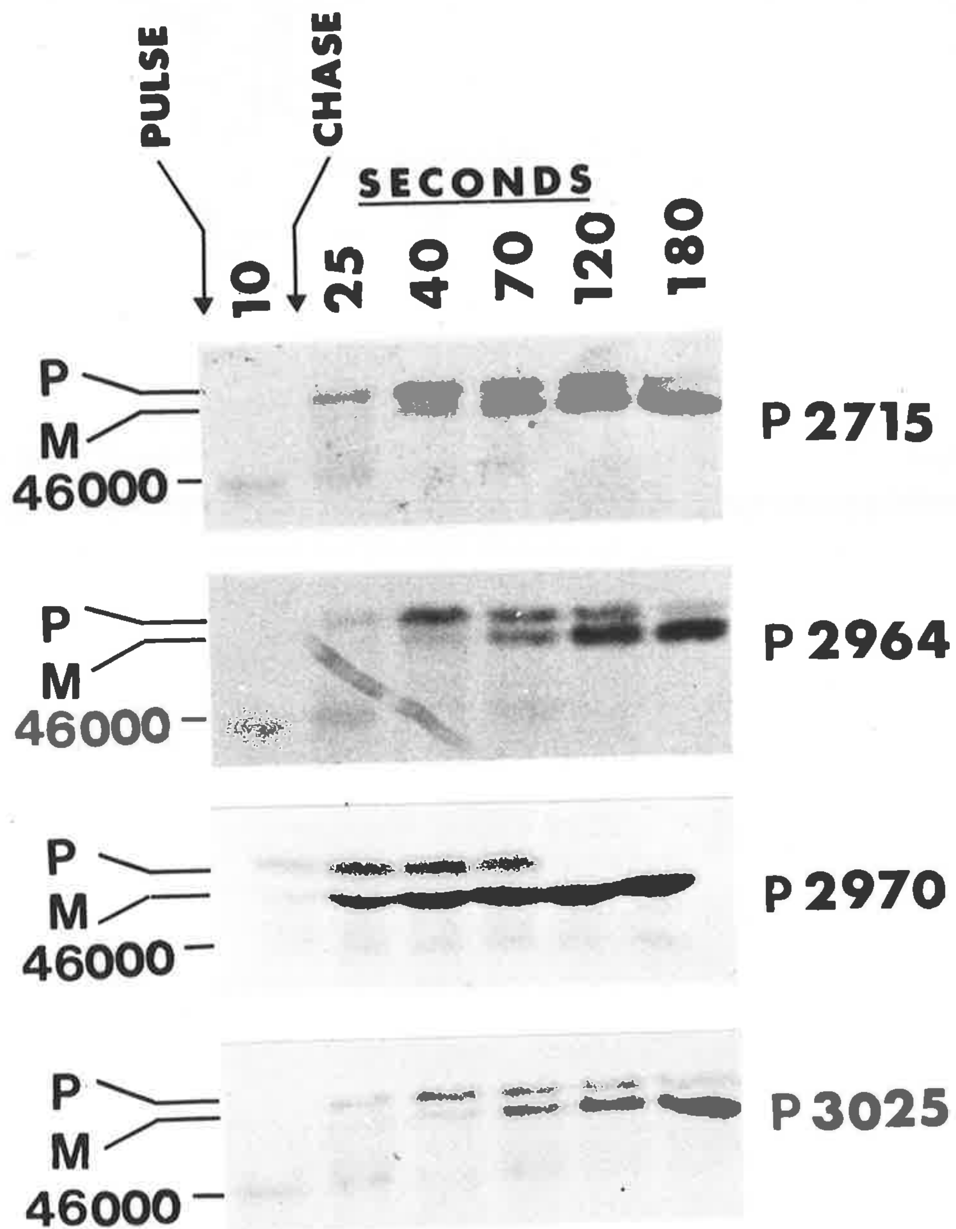


ClaI
EcoRI
PstI
SmaI
PstI
TetR
TolC
pPR42
12·26kb
PstI
ClaI
ClaI
AccI
SalI
AvaI
SmaI
HindIII
PstI
BamHI
EcoRI
pUC9
2·7kb
ApR
ClaI
AccI
T4 Ligase
EcoRI
CMLR
pACYC184
3·97kb
TetR
HindIII
BamHI
SalI
SmaI
PstI
EcoRI
PstI
PstI
HindIII
TolC
pPR178
7·2kb
ApR
EcoRI
T4 Ligase
EcoRI
HindIII
BamHI
SalI
TetR
pPR257
8·5kb
TolC
EcoRI
PstI
SmaI
PstI
EcoRI
PstI
SmaI
PstI
HindIII
BamHI
SalI
TetR
TolC
pPR258
8·5kb
EcoRI

Fig. 3.9

Effect of cloned T4 tRNA^{Arg} plasmid (pGM4113) on biosynthesis of the TolC protein

Strains indicated in this figure were treated as for Fig. 3.5. P2715 is AB1133/pPR42; P2964, P602/pPR258; P2970, P2964/pGM4113 (tRNA^{Arg}); P3025, P2964/pBR322. P, precursor; M, mature protein.


PULSE
CHASE
SECONDS
10
25
40
70
120
180
P
M
46000
P 2715
P
M
46000
P 2964
P
M
46000
P 2970
P
M
46000
P 3025

precursor appeared at 25 s, was still a major band at 120 s and was detectable at 180 s or later. Secondly, although the amount of the 46,000-Mr polypeptide detected at early sampling times was significantly reduced, it was still detected long after the chase commenced. In contrast, strains lacking the cloned tRNA^{Arg} gene, including a strain carrying pBR322 as a control, exhibited the 46,000-Mr polypeptide as a major component in the earlier stages of the pulse and chase, but then lost it completely.

3.9 Location of the 46,000-Mr polypeptide, precursor and mature TolC proteins.

[^{35}S]methionine-labelled cells were fractionated as described in Materials and Methods. The TolC protein and related poplypeptides in each fraction were immunoprecipitated and analysed by SDS-PAGE (Fig. 3.10). The 46,000-Mr polypeptide was present in the whole cell extract (track 6), cell sonicate (track 7), and whole cell envelopes (track 8) fractions. Furthermore, it was present in the inner membrane (track 9) but not in the outer membrane (track 10) fraction. These results show that the 46,000-Mr polypeptide is associated with the inner membrane. As expected, the precursor protein (Mr 54,500) was present mainly in the inner membrane (track 9). The mature TolC protein precipitated mainly from the outer membrane fraction (track 10).

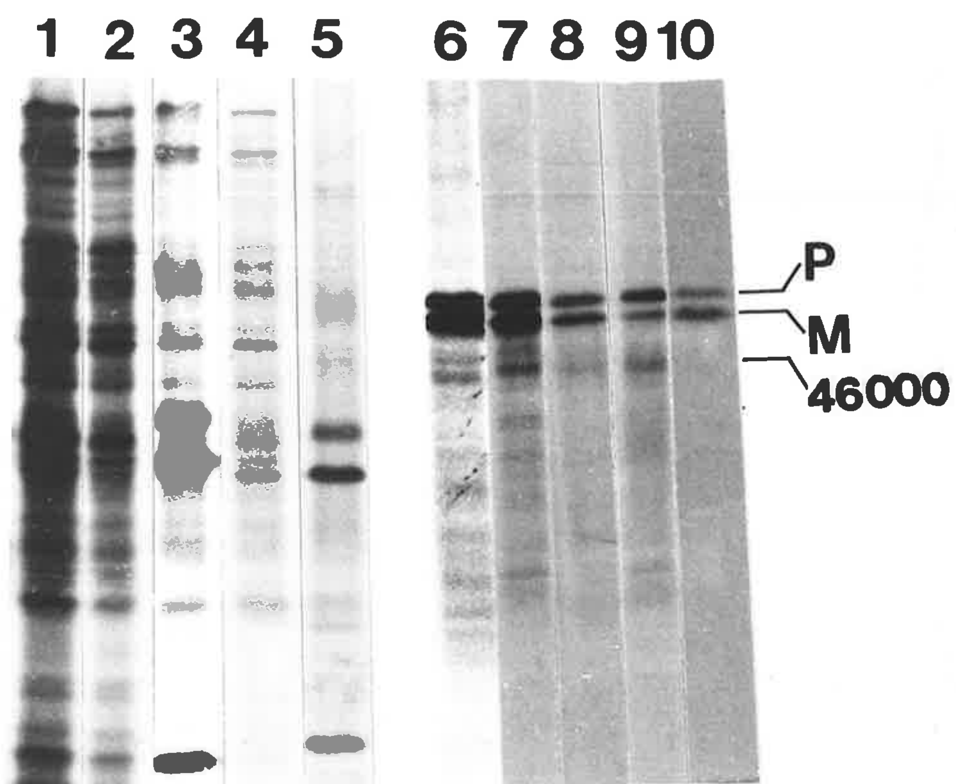
3.10 Summary and discussion

The TolC protein of *E. coli* K-12 can be cleaved in intact cells by externally added trypsin whereas other outer membrane proteins such as

Fig. 3.10

Fractionation of [^{35}S]methionine-labelled cells.

Cells were labelled with 100 μCi [^{35}S]methionine for 90 s. Radioactive isotope incorporation was stopped by adding non-radioactive methionine and sodium azide to final concentations of 20 mM and 0.02% respectively and freezing the labelled cells at -70°C. Labelled cells were fractionated into whole cell extract (track 1), cell sonicate (track 2), whole cell envelopes (track 3), and inner membrane (track 4) and outer membrane (track 5) by two sucrose step gradients. These fractions were then immunoprecipitated separately with TolC antiserum (tracks 6-10 respectively). and examined by SDS-PAGE. The gel was dried and fluorographed. P, precursor; M, mature protein.


1
2
3
4
5
6
7
8
9
10
P
M
46000

OmpA, OmpF and OmpC remain unaffected. When whole cells were incubated with ^{125}I, several major outer membrane proteins were radioactively labelled in addition to TolC. These experiments suggested that the TolC protein is exposed at the cell surface. The TolC protein was also cleaved by trypsin in the purified outer membrane fraction and its tryptic fragment of 27,000 Mr remained associated with the membrane.

Since cleavage of the TolC protein by trypsin in intact cells and in the membrane fraction gave two different size products, it appears that trypsin susceptible regions of the TolC protein in the two situations are different: one such region is exposed to the cell surface and the other towards the periplasmic face of the membrane. These results suggest that the TolC protein traverses the outer membrane.

The results presented in sections 3.4 to 3.9 of this chapter suggested that synthesis of the mature TolC protein takes place in at least three steps. A polypeptide with an apparent Mr of 46,000 appears within 10 s and is converted into the conventional precursor (Mr 54,500) with a half-life of about 30 s. The precursor is first detected 25 s after labelling and is itself converted into mature protein with a half-life of about 60 s. The mature TolC protein (Mr 52,000) is first detected 40 s after the beginning of labelling and the whole of the [^{35}S]methionine pulse is completely chased into this form by about 180 s.

The presence of the rare codon AGA (codon 402 of the coding region of <u>tolC</u> mRNA) is the most likely reason for the temporary accumulation of the 46,000-Mr polypeptide since the rate of synthesis of mature protein can be increased by providing extra $tRNA^{Arg}$ (AGA, AGG) in the cell. The significance of rare condons in regulatory proteins and minor proteins is discussed in chapter 7.

CHAPTER-4

EFFECT OF A *tolC* MUTATION ON *ompF* EXPRESSION

4.1 Introduction

A mutation in the *tolC* locus of *E. coli* K-12 affects expression of a major outer membrane protein, OmpF (Morona and Reeves, 1982a): *tolC* mutants lacked detectable levels of OmpF from their outer membranes and it was not present in any cellular fractions. Experiments using an *ompF*-*lacZ* operon fusion strain indicated that the *tolC*-mediated effect on the expression of *ompF* is at the post transcriptional level (Morona and Reeves, 1982a).

In this chapter the strength of the *tolC* effect on OmpF and the manner in which a mutation in the *tolC* gene affects *ompF* expression are studied and compared with the effect of the previously characterised *ompB* regulation.

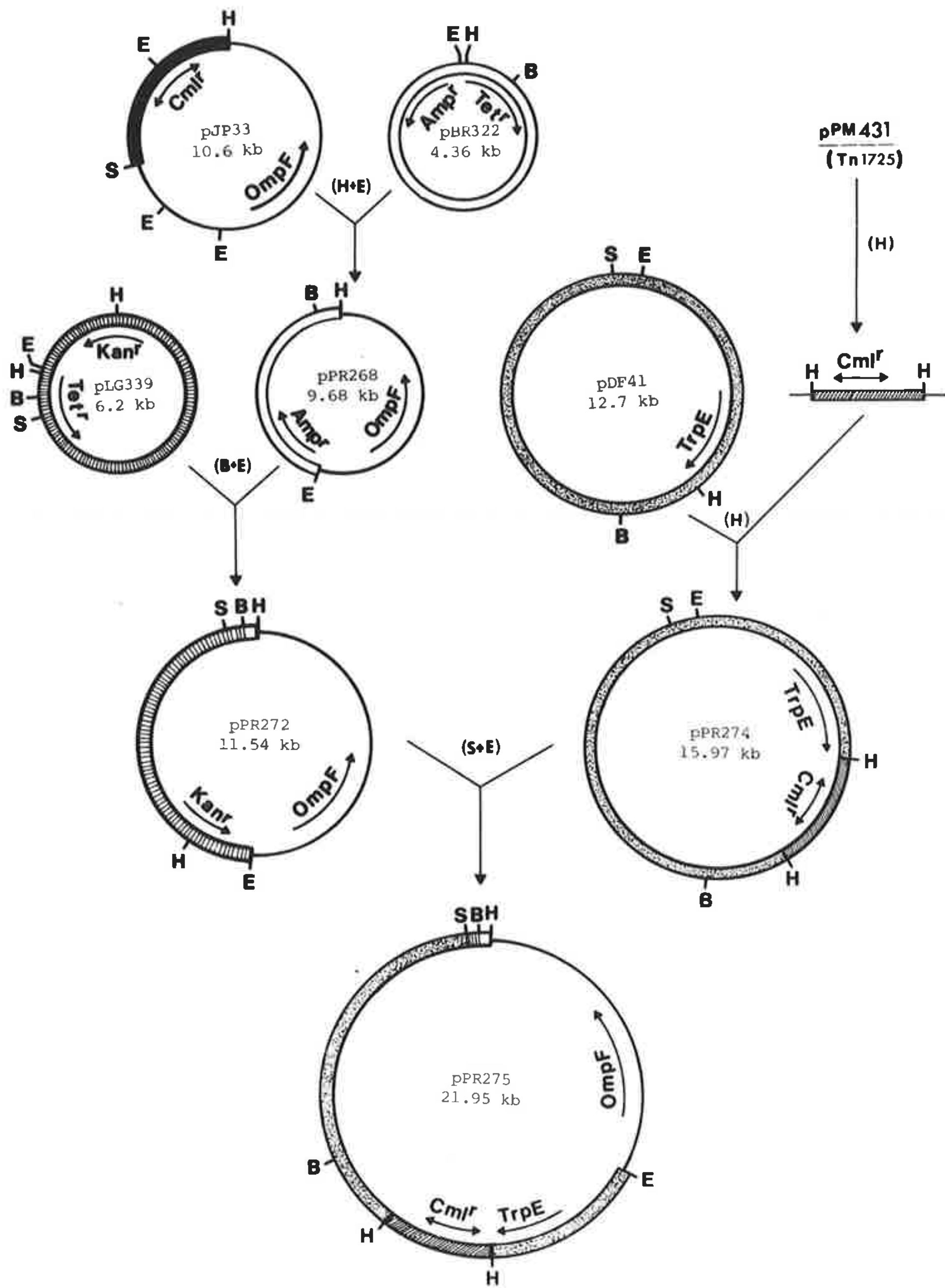
4.2 Subcloning of the *ompF* gene into different copy number vector plasmids

The *ompF* gene from *E. coli* K-12 has been cloned into the high copy number plasmid pACYC184 (Tommassen *et al.*, 1982b) and was subcloned into plasmid vectors of various copy number as shown in Fig. 4.1.

Fig. 4.1

Subcloning of the ompF gene into different copy number vector plasmids

The ompF gene from E. coli K-12 was cloned into a multi-copy plasmid, pACYC184, by Tommassen et al. (1982b). The EcoRI-HindIII fragment from pJP33 (ompF/pACYC184) that carries the ompF gene, was inserted between the EcoRI and HindIII sites of pBR322, resulting in pPR268. The EcoRI-BamHI fragment from pPR268 was inserted between the EcoRI and BamHI sites of a 6-copy number plasmid, pLG339, resulting in pPR272. To obtain a single-copy number ompF plasmid, the chloramphenicol resistance gene of Tn1725 obtained from pPM431 was inserted into the HindIII site of pDF41, resulting in pPR274 and then the EcoRI-SalI fragment from pPR272 was inserted between the EcoRI and SalI sites of pPR274, resulting in pPR275. Abbreviations: B, BamHI; E, EcoRI; H, HindIII; S, SalI



pJP33
10.6 kb
CmlR
OmpF
pBR322
4.36 kb
AmpR
TetR
(H+E)
pPM431
(Tn1725)
(H)
pLG339
6.2 kb
KanR
TetR
pPR268
9.68 kb
AmpR
OmpF
pDF41
12.7 kb
TrpE
CmlR
(B+E)
(H)
pPR272
11.54 kb
KanR
OmpF
pPR274
15.97 kb
TrpE
CmlR
(S+E)
pPR275
21.95 kb
OmpF
CmlR
TrpE

4.3 Synthesis of OmpF protein in strains carrying ompF$^+$ plasmids of varying copy number

The effect of tolC and ompR101 mutations on ompF expression was studied in mutant (tolC or ompR) strains carrying ompF$^+$ plasmids derived from miniF, pSC101 or pACYC184 replicons, which have approximate copy numbers of 1, 6 or 50 respectively. Whole cell envelopes of these strains were prepared and analysed by SDS-PAGE (Fig. 4.2). When OmpF was synthesised from the single copy chromosomal gene, either mutation (tolC or ompR101) reduced the amount of OmpF protein below the level which could be detected in whole cell envelopes. However, when the copy number of the ompF gene was increased, the effect of the ompR101 mutation remained essentially the same, whereas the tolC mutation was increasingly unable to affect the level of OmpF. Thus for 2, 7 and 51 copies of ompF, the tolC mutation produced a 20-fold, 4 to 5-fold and negligible reduction respectively in the OmpF level.

4.4 Transcription and transcription-translation studies of the ompF gene in ompF-lacZ fusion strains

Morona and Reeves (1982a) have previously reported that ompF transcription, as determined by β-galactosidase activity of an ompF-lacZ operon fusion strain, was reduced only 2 to 3-fold by a tolC mutation compared to the more than 35-fold reduction observed in an ompR101 mutant. In this study a protein fusion strain (MH610) was included in which the lacZ gene was placed under ompF transcription-translation controls and synthesised a hybrid OmpF-LacZ protein containing about the first 35 amino acid residues from the NH_2-terminus of the OmpF protein

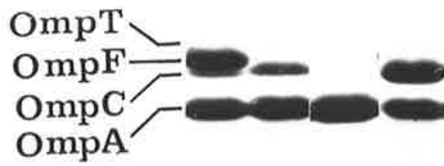
Fig. 4.2

Effect of the tolC mutation on OmpF synthesised by varying copy number plasmids

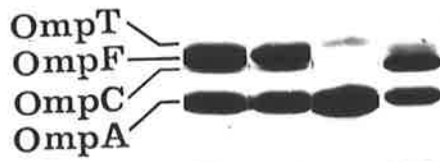
Whole cell envelopes from the parent (AB1133) an from ompF (P210), tolC (602) and ompR101 (P530) strains harbouring varying copy number $ompF^+$ plasmids were analysed by SDS-PAGE. Only the relevant part of the gels is shown.

AB1133 P210 P530 P602

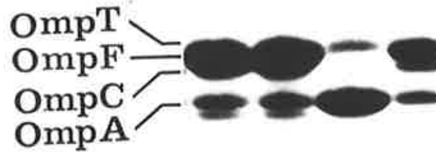
ompF copy number



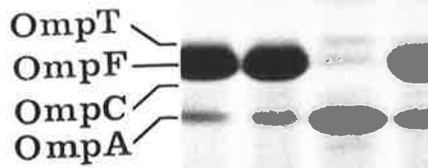
One



Two



Seven



≈ Fifty

and most of the LacZ protein (Hall and Silhavy, 1981a).

When a tolC mutation was introduced into the protein fusion strain, β-galactosidase activity was reduced by 10-fold. This decrease was also reflected by the amount of hybrid OmpF-LacZ protein observed when whole cell extracts from parental and tolC derivative strains were compared by SDS-PAGE (Fig. 4.3). The tolC mutation had the same effect in the operon fusion strain as observed before (see above, Morona and Reeves, 1982a). As previously observed in the operon fusion strain (Morona and Reeves, 1982a), the ompR101 mutation reduced β-galactosidase activity substantially: a similar reduction (by over 50-fold, data not shown) was observed in the protein fusion strain.

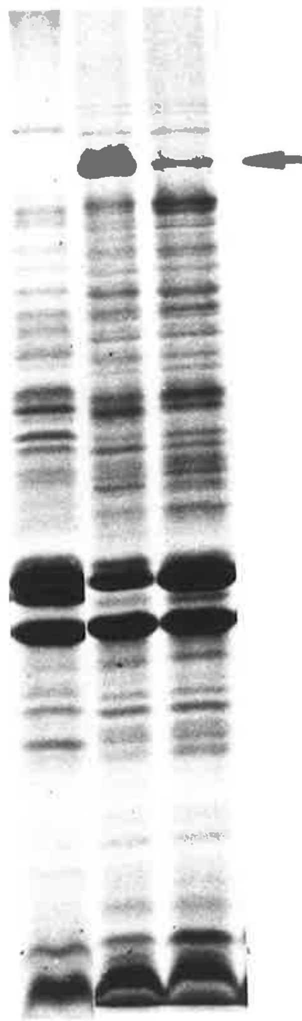
The greater effect of a tolC mutation on β-galactosidase activity in the ompF-lacZ protein fusion strain than in the ompF-lacZ operon fusion strain suggested that in addition to the ompF promoter itself, part of the sequence downstream of the promoter or even part of the ompF gene itself may be required for tolC-mediated regulation of ompF expression. The tolC effect in the protein fusion strain, however, was still not as dramatic as in the $ompF^+$ strain (compare AB1133 and P602, Fig. 4.2), where OmpF protein could not be detected in any cellular fractions of P602 under similar growth conditions (Morona and Reeves, 1982a).

It appeared possible from the above data that a functional ompF gene is required for the full tolC effect on ompF expression. If the OmpF protein itself was involved in the tolC effect, one might expect its effect to occur in cis. To test this possibility, a single copy $ompF^+$ plasmid (pPR275) was transferred into ompF-lacZ fusion strains and into their tolC and ompR101 derivatives, and the amount of OmpF protein in whole cell envelopes and β-galactosidase activity were measured. The

Fig. 4.3

Effect of the tolC mutation on the OmpF-LacZ fusion protein

Whole cell extracts from MC4100 (lane 1), MH610 (lane 2) and P3289 (MH610 *tolC*, lane 3) were analysed by SDS-PAGE. The arrow indicates the position of the OmpF-LacZ fusion protein.


1 2 3

addition of the plasmid had no effect on the level of β-galactosidase, but unexpectedly, the amount of OmpF protein synthesised by the single copy *ompF*$^+$ plasmid was reduced only 2 to 3-fold in *tolC* mutants of partial diploid strains carrying either fusions (data not shown) compared to a 20-fold reduction observed in the *tolC* mutant (P602) of a non-fusion *ompF*$^+$ strain (AB1133) carrying pPR275 (Fig. 4.2). Thus in the *ompF-lacZ* fusion/*ompF*$^+$ diploid strains, both products (β-galactosidase and OmpF protein) were reduced only 2 to 3-fold by a *tolC* mutation. It thus appears that the presence of the fusion interferes with the *tolC* effect on OmpF. The OmpF protein was not detected in the *ompR101* derivatives of either fusion strain carrying the plasmid (data not shown).

It is interesting to note that *ompF-lacZ* fusion strains carrying a single copy *ompF*$^+$ plasmid (pPR275) were resistant to the OmpF specific phage K2, even though the normal amount of OmpF protein was observed in their cell envelopes. The effect, similar to that observed by Emr *et al.* (1980) for LamB protein and lambda phage, is presumably due to the fusion product interacting adversely with the normal protein and/or the phage. Strains carrying a multi-copy *ompF*$^+$ plasmid were sensitive to phage K2.

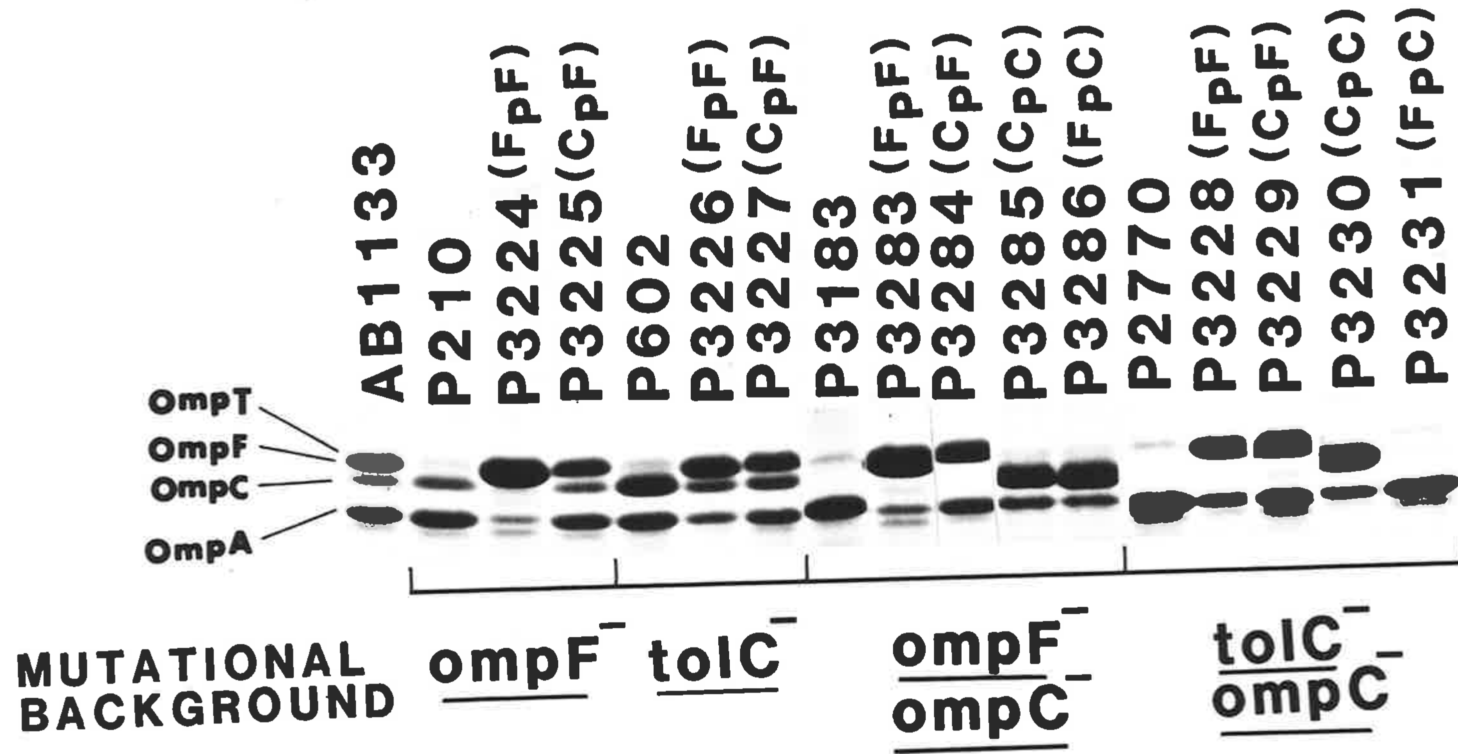
4.5 Use of *ompF-ompC* chimeric genes to determine the region of the *ompF* gene affected by the *tolC* mutation

To determine whether a *tolC* mutation produces its effect via the promoter or the coding region of the *ompF* gene, chimeric plasmids in which the *ompF* structural gene was placed under *ompC* promoter control or vice versa (Matsuyama *et al.*, 1984) were used. Strains carrying *tolC* or *ompF* mutations were transformed with plasmids pMAN007 (*ompF* promoter and *ompF* structural gene: FpF) or pMAN009 (*ompC* promoter and *ompF* structural

Fig. 4.4

Determination of the region of the ompF gene affected by the tolC mutation

Bacterial strains of different mutational background, as indicated in the figure, were transformed with pMAN007 (FpF), pMAN009 (CpF), pMAN006 (CpC) and pMAN010 (FpC) and whole cell envelopes prepared from these strains were analysed by SDS-PAGE. Only the relevant part of the gel is shown. See text for plasmid details.



AB1133
P210
P3224 (FpF)
P3225 (CpF)
P602
P3226 (FpF)
P3227 (CpF)
P3183
P3283 (FpF)
P3284 (CpF)
P3285 (CpC)
P3286 (FpC)
P2770
P3228 (FpF)
P3229 (CpF)
P3230 (CpC)
P3231 (FpC)
OmpT
OmpF
OmpC
OmpA
MUTATIONAL BACKGROUND
ompF⁻
tolC⁻
ompF⁻ ompC⁻
tolC⁻ ompC⁻

gene: CpF). In addition, ompF, ompC or tolC, ompC double mutants were transformed with plasmids pMAN007, pMAN009, pMAN006 (ompC promoter and ompC structural gene: CpC) or pMAN010 (ompF promoter and ompC structural gene: FpC). Whole cell envelopes of these strains were prepared and analysed by SDS-PAGE (Fig. 4.4). Only when a gene was under ompF promoter control, was its product reduced by a tolC mutation, with the greater effect being on OmpC under ompF promoter control. These results suggested that the effect of the tolC mutation is exerted at a point upstream of the chimera junction (at amino acid 11) of the mature OmpF protein. It should be noted that the presence or absence of the OmpC protein (compare P602 and P2770, Fig. 4.4) does not alter the tolC effect on OmpF.

4.6 Direct measurement of the ompF transcript

The data presented above suggested that it is the promoter function of the ompF gene which is affected by a mutation in the tolC locus. Transcription of the ompF gene was studied by directly analysing ompF mRNA from tolC, ompR101 and ompF mutants. RNA was purified from two different parent strains and their mutant derivatives. These RNA preparations were electrophoresed in an agarose gel and subjected to Northern transfer to nitrocellulose filters, hybridised with a ^{32}P labelled DNA fragment of the ompF gene and autoradiographed. The protein profile of whole cell envelopes of these strains and the autoradiograph are shown in Figs. 4.5 and 4.6 respectively. The ompF transcript was present in the parent strains (AB1133 and W1485F^-) but was not detected in tolC, ompF or ompR101 mutants (Fig. 4.6). When a more sensitive RNA-DNA hybridisation method (see sec. 2.7) was employed, the tolC mutant was shown to have 50-fold less ompF transcript than was present in the parent strain and none was detected in the ompR101 mutant (Fig. 4.7).

Fig. 4.5

Effect of tolC on OmpF

Whole cell envelopes prepared from parental (AB1133 and W1485F$^-$), ompF (P210), tolC (P602 and P2731) and ompR101 (p530) strains were analysed by SDS-PAGE.

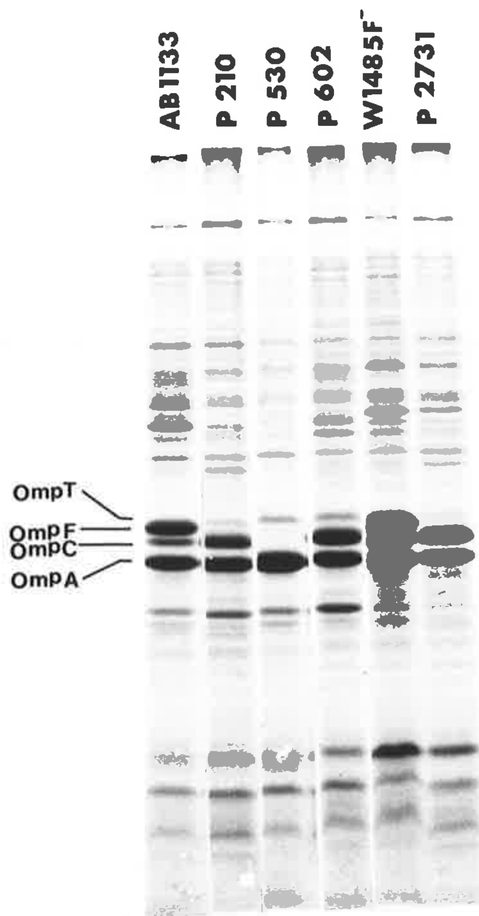

AB1133
P 210
P 530
P 602
W1485F⁻
P 2731
OmpT
OmpF
OmpC
OmpA

Fig. 4.6

Analysis of ompF mRNA

Cellular RNA isolated from the strains, indicated in the figure, were electrophoresed on an agarose gel and subjected to Northern transfer. The filter was hybridised with a ^{32}P-labelled ompF probe and autoradiographed. Arrows indicate the position of ompF mRNA.

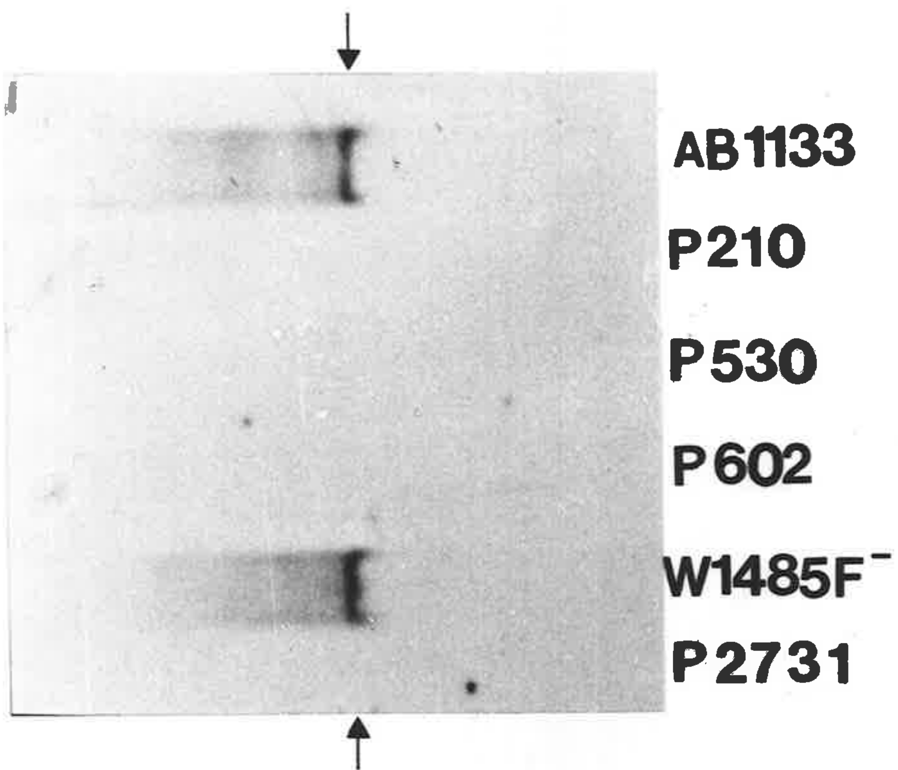
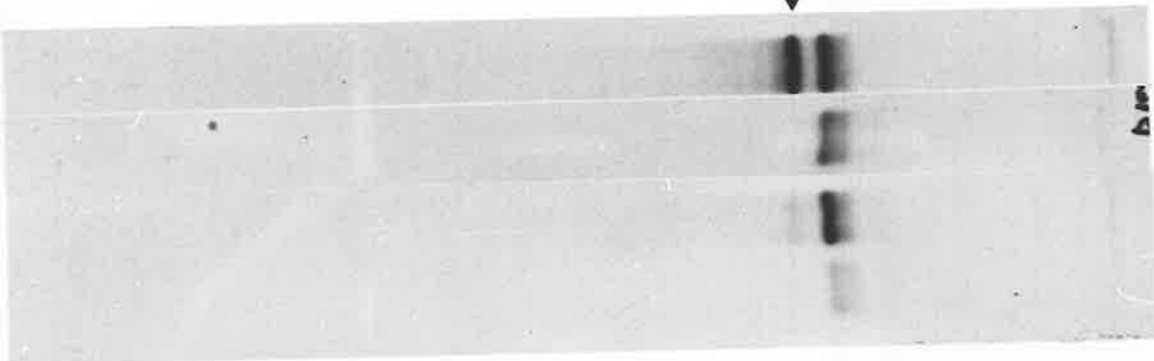

AB1133
P210
P530
P602
W1485F⁻
P2731

Fig. 4.7

A sensitive assay for ompF mRNA

RNA isolated from [^{32}P]phosphoric acid labelled cultures of the parent (AB1133), tolC (P602) and ompR101 (P530) strains were hybridised with ompF DNA bound to nitrocellulose filters. ^{32}P-labelled RNA, which hybridised with ompF DNA, was eluted from filters, electrophoresed on a 5% acrylamide-8M urea gel and autoradiographed. As a control, the radioactive RNA isolated from AB1133 was hybridised to a filter with no DNA. The arrow indicates the position of ompF mRNA. A band running above ompF mRNA is the contaminating chromosomal DNA extracted with the crude labelled RNA preparations.


AB1133
P530
P602
CONTROL

4.7 Processing of the OmpF protein

The results presented in Figs. 4.6 and 4.7 showed that a mutation in the tolC locus affects transcription of the ompF gene. Since TolC itself is a minor outer membrane protein, it is not clear how it could affect transcription of the ompF gene directly. The TolC protein may affect ompF expression indirectly by interacting with OmpF protein during processing or insertion of OmpF protein into the membrane. This hypothesis would predict that the OmpF protein may not process or assemble properly in the absence of TolC protein and that the unprocessed OmpF protein then inhibits transcription of its own gene by a feed back mechanism. To investigate this possibility the processing of OmpF protein was studied in a tolC mutant strain and its parent. [^{35}S]methionine pulse-labelled cells were chased with cold methionine and samples were withdrawn at various times, lysed and immunoprecipitated with purified OmpF antiserum. Immunoprecipitates were analysed by SDS-PAGE and autoradiographed. In the parent strain, OmpF precursor was processed properly into the mature protein (Fig. 4.8, lanes 1-3) whereas no precursor or mature OmpF protein was detected in the tolC mutant (Fig. 4.8, lanes 5-7). This suggested that there was no accumulation of the OmpF precursor and therefore the inhibition of ompF gene expression did not occur during the processing or assembly of the OmpF protein but at some stage during or immediately after transcription.

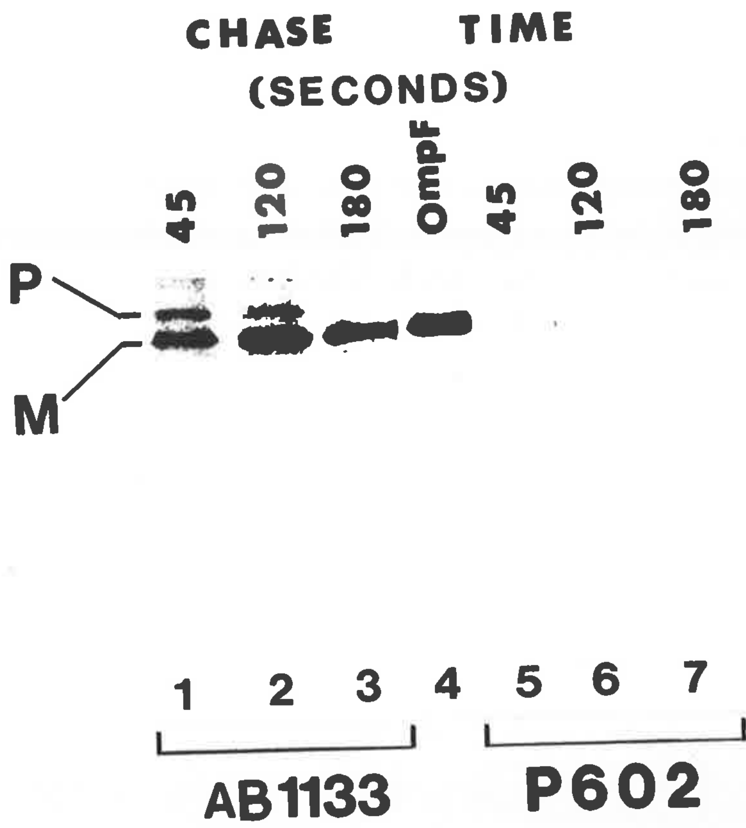
4.8 Summary and discussion

In this chapter the effect of a tolC mutation on strains which carry different copy numbers of the ompF gene was studied. A tolC

Fig. 4.8

Detection of the OmpF precursor from *tolC*$^{+}$ (AB1133) and *tolC* (P602) strains

[^{35}S]methionine labelled (15 s) cultures were chased with non-radioactive methionine. After solubilisation in SDS buffer, samples were immunoprecipitated with OmpF antiserum, analysed by SDS-PAGE and autoradiographed. P, precursor and M, mature OmpF protein.


CHASE TIME
(SECONDS)
45
120
180
OmpF
45
120
180
P
M
1
2
3
4
5
6
7
AB1133
P602

mutation in a normal E. coli K-12 strain reduces the amount of OmpF protein to a level undetectable on stained polyacrylamide gels, whereas it has no effect on the OmpF level if there are 51 copies of the ompF gene present: there was an intermediate reduction if 7 or 2 copies were present. It is clear that TolC protein is not essential for OmpF synthesis: this conclusion was also drawn by Morona and Reeves (1982a) who showed that growth in a low salt medium results in detectable levels of OmpF in tolC mutants.

The tolC mutation affects the promoter function of the ompF gene as shown by the use of plasmids carrying hybrid ompF-ompC genes. A mutation in the tolC locus also drastically reduces the amount of ompF transcript in an ompF$^+$ strain and this reduction was not due to the improper processing of the OmpF precursor in tolC mutants.

The tolC effect on OmpF is comparable to the effect exerted by ompR (Hall and Silhavy, 1981a; Matsuyama et al., 1984) or by micF (Mizuno et al., 1984), both of which act on the amino terminal end of the ompF gene. Therefore, it is possible that the tolC effect on OmpF may be exerted via ompR or via micF. However, regulation of OmpF by the tolC locus appeared to be independent of that exerted by the ompR locus because it has a similar effect on two other outer membrane proteins (NmpC and Lc; Morona and Reeves, 1982a) which are not under ompR positive control (Pugsley and Schnaitman, 1978b; Hall and Silhavy, 1981a). The other possibility, that the tolC effect on OmpF is exerted via micF, is supported by the observation that tolC mutants have higher levels of OmpC present than in ompF mutants (for example compare P602 and P210 in Fig. 4.1). As the expression of ompC and micF is thought to be co-regulated (Mizuno et al. 1984; Schnaitman and McDonald, 1984), increased expression of ompC may reflect increased expression of micF in tolC mutants. Since

the presence or absence of the OmpC protein itself does interfere with the _tolC_ effect on OmpF, it seems possible that _micF_ is in large part responsible for the reduction of OmpF synthesis in _tolC_ mutants. This possibility is investigated in the following chapters.

CHAPTER-5

MOLECULAR CHARACTERISATION OF THE Stc$^-$ MUTATION OF *Escherichia coli* K-12

5.1 Introduction

The results presented in the previous chapter suggested that a mutation in the *tolC* locus affects promoter function of the *ompF* gene. It was postulated that the *micF* gene product may be responsible for this effect of *tolC* on *ompF* expression.

Morona and Reeves (1982b) identified a suppressor mutation, *stc*, which reverted the phenotype of *tolC* mutants from OmpF$^-$ to OmpF$^+$. The *stc* (suppressor of *tolC*) mutation was mapped very close to the *ompC* and *micF* genes. For the reason given in sec. 5.5 of this chapter, the *stc* mutation is referred to here as by its phenotypic designation, Stc$^-$. In this chapter the original Stc$^-$ mutation has been further characterised in order to investigate the role of the *ompC* and *micF* genes in the *tolC* effect on OmpF.

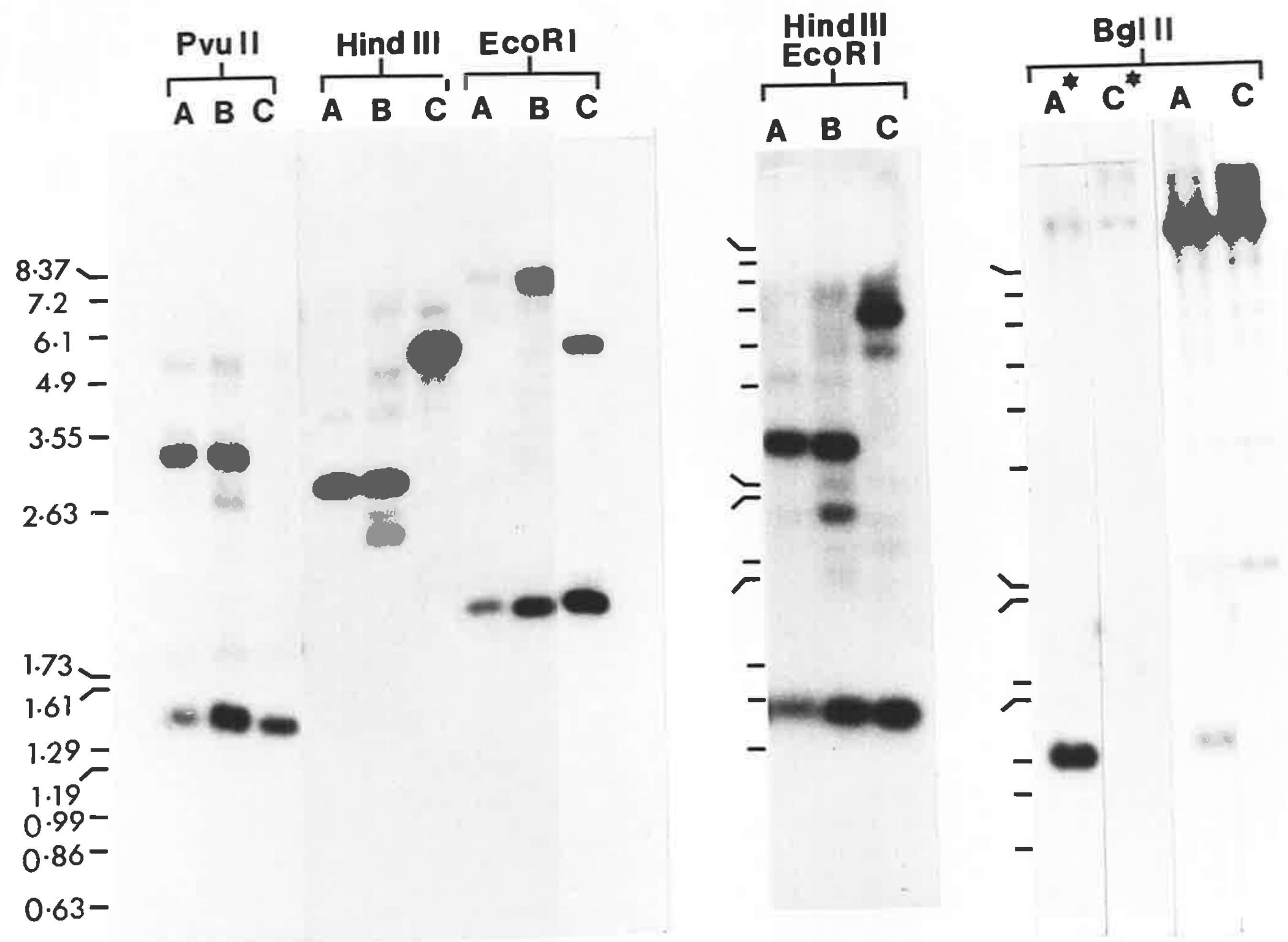
5.2 Restriction analysis of chromosomal DNA from Stc$^+$ and Stc$^-$ strains

Chromosomal DNA from strains P2125 (Stc$^+$, *ompC*$^+$, *tolC*$^+$), P2716 (Stc$^+$, *ompC*$^-$, *tolC*$^-$) and P2718 (Stc$^-$, *ompC*$^-$, *tolC*$^-$) was digested with various restriction enzymes and subjected to Southern transfer. Nitrocellulose filters were hybridised with ^{32}P-labelled *ompC*-*micF* probe. Results of this experiment are shown in Fig. 5.1 and summarised in Table 5.1. The relevant sites, which are predicted from the DNA sequence of

Fig. 5.1

Restriction mapping of chromosomal DNA from Stc^{+} and Stc^{-} strains

Autoradiogram of a Southern blot of chromosomal DNA from strains P2125(A), P2716(B), and P2718(C) digested with the indicated restriction enzymes and hybridised with the ompC-micF probe (or micF probe*; see Fig. 5.2). Phage SPP1 DNA, digested with EcoRI was used as a M_r standard. Fragment sizes (in kb) of the standard are shown at the left; the same standards were used for the HindIII+EcoRI and BglII digests with their positions indicated. Note that some bands resulted from partial DNA digests.



Pvu II
A B C
Hind III
A B C
EcoR I
A B C
Hind III
EcoR I
A B C
Bgl II
A* C* A C
8·37
7·2
6·1
4·9
3·55
2·63
1·73
1·61
1·29
1·19
0·99
0·86
0·63

TABLE 5.1

Size (in kb) of the DNA fragments which took up the ompC-micF probe or micF probe (*) after cutting with the indicated enzymes[a]

Strains	Restriction enzymes				
	PvuII	HindIII	EcoRI	EcoRI + HindIII	BglII
P2125 (Stc^+, $ompC^+$, $tolC^+$)	1.4;3.0	2.7	2.0;8.5	0.8;1.9	*1.0;12.0
P2716 (Stc^+, $ompC^-$, $tolC^-$)	1.4;3.0	2.7	2.0;8.5	0.8;1.9	n.t.[b]
P2718 (Stc^-, $ompC^-$, $tolC^-$)	1.4	5.4	2.0;5.7	0,8;4.6	12.0

[a] Only the BglII digests were hybridised with the micF probe (*) in addition to the ompC-micF probe (Fig.5.1). Only the 1.0 kb fragment from P2125 hybridised with the micF probe. [b] n.t., not tested.

this region from a Stc$^+$ strain (Mizuno et al., 1983), are shown in Fig. 5.2. The probe hybridised with fragments of the predicted lengths when DNA from Stc$^+$ strains was used (Fig. 5.1, Table 5.1), but with DNA from a Stc$^-$ strain (P2718) only some of the predicted fragments hybridised and some additional bands were seen. It was clear that there had been a rearrangement, possibly a deletion, in the micF region. We therefore used a micF-specific probe to hybridise BglII-digested chromosomal DNA fragments from Stc$^+$ and Stc$^-$ strains. This probe lit up the 1.0-kb fragment (which includes the micF gene) from the Stc$^+$ strain but did not light up any fragment in the Stc$^-$ strain (Fig. 5.1, Table 5.1). When similar digests were hybridised with the ompC-micF probe, an additional 12.0-kb DNA fragment, present in both strains, took up the probe (Fig. 5.1, Table 5.1). These results confirmed the presence of a deletion in the Stc$^-$ strain upstream from the BglII site of the ompC gene; the deleted DNA includes the micF gene and must extend either about 2.8 kb or greater than 7.5 kb to account for the 5.7-kb EcoRI fragment lit up by the ompC-micF probe. The location of the deletion is shown in Fig. 5.2.

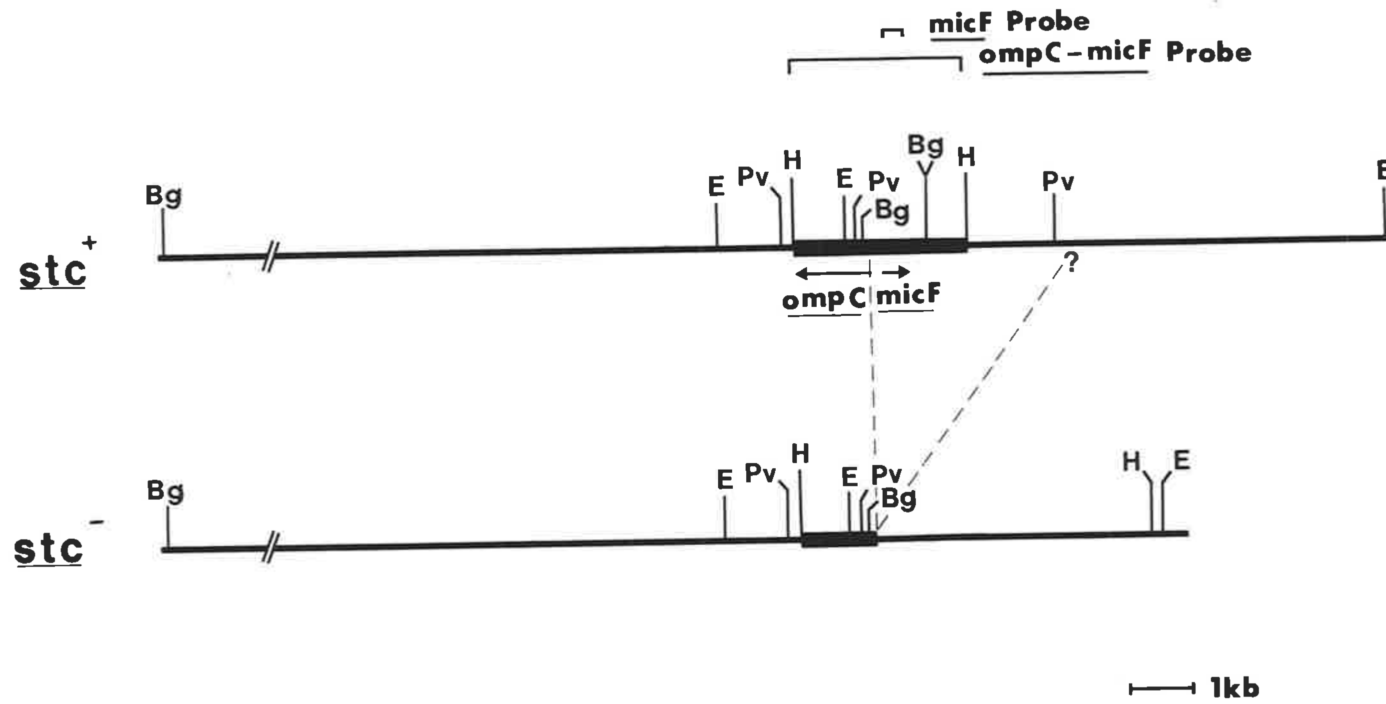
5.3 Cloning of the Stc$^-$ mutation

As indicated in Fig. 5.2, a 5.4-kb HindIII fragment from the Stc$^-$ strain encompasses the possible deletion. Chromosomal DNA from the Stc$^-$ strain was therefore digested with HindIII and ligated to pUC18 DNA which had been digested with HindIII and treated with alkaline phosphatase. The ligated DNA mixture was then used to transform an ompC deletion strain (CS1253) and colonies selected on Ap plates were transferred to nitrocellulose filters and hybridised with a 2.7-kb ompC-micF probe. Plasmid DNA was prepared from colonies giving a positive signal and

Fig. 5.2

A comparative restriction map of the chromosomal DNA of Stc^{+} and Stc^{-} strains around the *ompC*-*micF* genes.

The dotted lines shows probable extent of the deletion in the Stc^{-} strain. Note that this experiment would not give the location of *Hin*dIII sites outside of the probe in the Stc^{+} strain. Bg, *Bgl*II; E, *Eco*RI; Pv, *Pvu*II; H, *Hin*dIII.


micF Probe
ompC-micF Probe
Bg
E
Pv
H
E
Pv
Bg
Bg
H
Pv
E
stc+
ompC
micF
?
Bg
E
Pv
H
E
Pv
Bg
H
E
stc-
1kb

subjected to restriction analysis. One such plasmid, pPR313, contained the desired 5.4-kb DNA fragment. A partial restriction map of this plasmid is shown in Fig. 5.3.

5.4 Nucleotide sequence of the EcoRI-PstI DNA fragment which includes the Stc$^-$ mutation

A 650-bp EcoRI-PstI fragment from pPR313 was subcloned into M13mp8 and M13mp9 (Messing, 1983) and sequenced in both orientations. This sequence was then compared with the parental DNA sequence from the Stc$^+$ strain (Fig. 5.4). As predicted from the restriction analysis of the Stc$^-$ strain, a deletion started upstream from the BglII site of the ompC gene. The deletion start was located 49 bp upstream of the ompC gene start AUG codon. The deletion thus removes the -35 region of the ompC promoter, and the whole of the micF gene and promoter.

5.5 Summary and discussion

The suppressor mutation, Stc$^-$, which allows OmpF protein to be produced at detectable levels in a tolC ompC mutant background, has been characterised. Restriction mapping of chromosomal DNA from Stc$^+$ and Stc$^-$ strains was performed to investigate the nature of the mutation: it was shown to be a deletion upstream of the ompC gene. DNA from the region of the deletion was cloned into pUC18, and a 650-bp PstI-EcoRI fragment was sequenced. The deletion started 49 bp upstream of the AUG start codon of the ompC gene, thus removing part of the ompC gene and the entire micF gene.

Fig. 5.3

Partial restriction map of plasmid pPR313.

This plasmid carries the 5.4-kb "Stc" fragment (shown by thick line) from P2718 (Stc^-). A 650-bp EcoRI-PstI fragment (↔) was subcloned into M13 vectors for sequencing. The numbers are in kb.

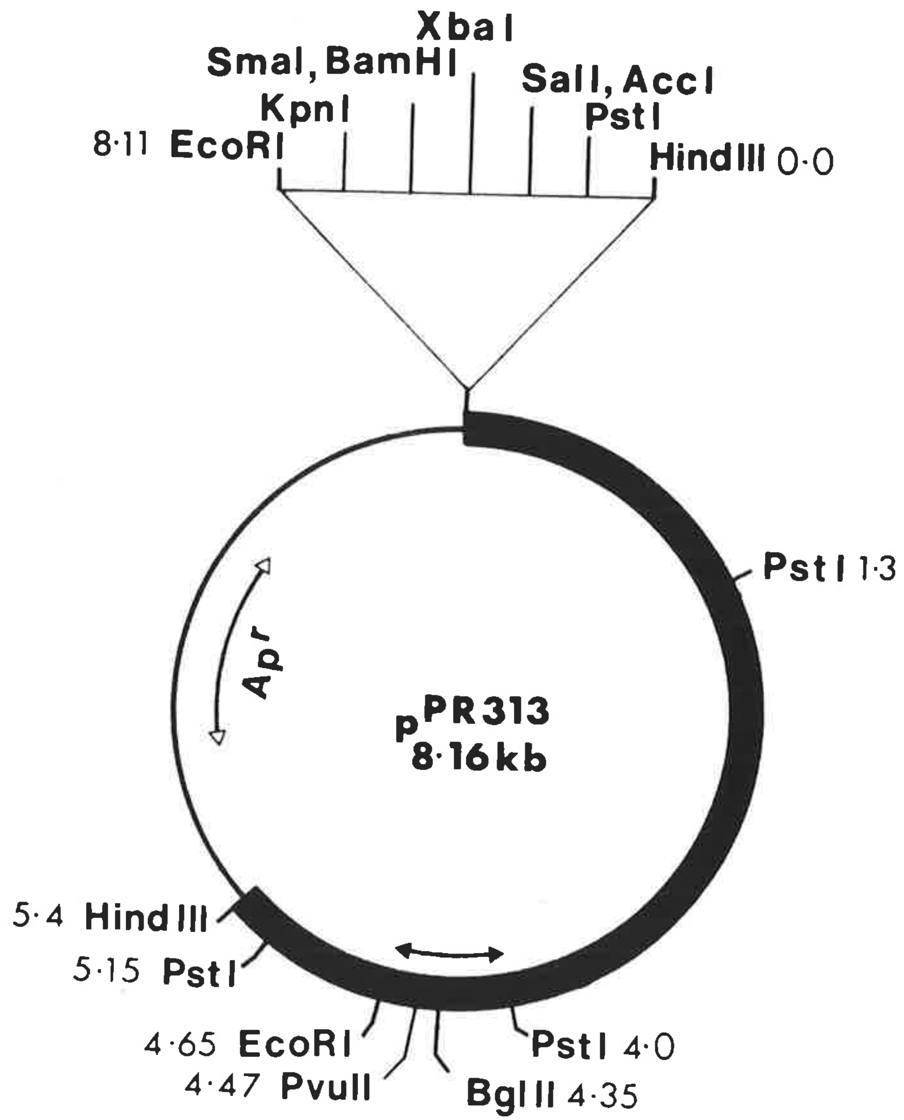
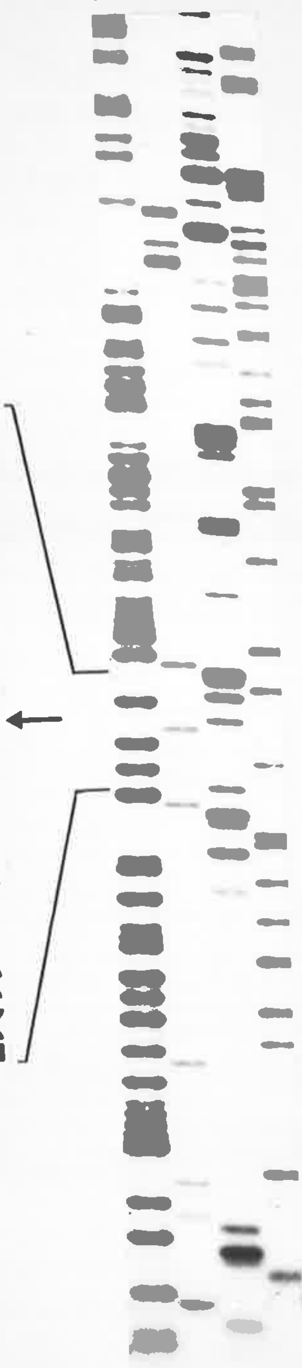

Xba I
Smal, BamHI
Sal I, Acc I
Kpn I
Pst I
8·11 EcoRI
HindIII 0·0
Pst I 1·3
Ap r
pPR313
8·16 kb
5·4 Hind III
5·15 Pst I
4·65 EcoRI
4·47 PvuII
Pst I 4·0
Bgl II 4·35

Fig. 5.4

Nucleotide sequence of a 650-bp EcoRI-PstI DNA fragment from pPR313.

Only the relevant portion of the sequence is shown. Arrow indicates start of the chromosomal deletion in the Stc^- strain. Nucleotide sequence of this region from the Stc^+ strain was taken from Mizuno *et al*. (1983).



A C G T
stc+
GGTGACGTATGACTA
stc-
GGTGACGCCACTACG

Since the Stc^- mutation was isolated in a tolC ompC mutant background, the data obtained suggest that the $OmpF^+$ phenotype of Stc^- mutants is not due to the lack of ompC but due to the deletion of micF: for this reason the term "Stc" has been retained for the phenotypic description only, as the Stc^- phenotype is probably due to the loss of the gene now known as micF. However, as the Stc^- deletion extends at least 2.2 kb beyond micF and may clearly encompass other gene(s), analysis of shorter deletions will be necessary to establish unequivocally if the effect on ompF expression is solely due to the deletion of micF. Mutants that are lacking only micF have been used in the following chapter.

If the Stc^- phenotype (which reverses the tolC effect on OmpF) is due to deletion of micF, it is reasonable to ask if the reduction in ompF expression in a tolC mutant is due to increased micF expression. This possibility is investigated in the following chapter.

CHAPTER-6

ROLE OF micF IN THE tolC-MEDIATED REGULATION OF OmpF

6.1 Introduction

The results of the previous chapters suggest that the tolC effect on OmpF is in large part mediated by the micF gene product. However, as the Stc$^-$ deletion described in chapter-5 extends beyond micF and clearly encompasses other gene(s), shorter deletions were required to determine unambiguously the role of micF in tolC-mediated regulation of OmpF.

In this chapter the tolC effect is studied in a micF-ompC deletion mutant strain carrying either a plasmid that contains both micF and ompC genes or a plasmid carrying only the ompC gene. After the completion of this work, construction of a chromosomal micF deletion mutant strain (SM3001) was reported (Matsuyama and Mizushima, 1985) and this strain (kindly donated by Dr. S. Mizushima) is included in this work to study the tolC effect on OmpF.

In addition, the interaction of tolC and ompR mutations is studied in order to further understand the regulation of ompF and ompC-micF expression.

6.2 Effect of the tolC mutation on OmpF synthesis in a micF-ompC deletion mutant

Schnaitman and McDonald (1984) constructed an ompC deletion mutant (CS1253) in which the micF gene was also completely deleted. A tolC mutation, which normally reduces the OmpF level in an ompF$^+$ ompC$^+$

micF^{+} strain to a level undetectable in stained gels, was unable to exert such a dramatic effect on OmpF in this micF-ompC deletion mutant: the tolC mutation resulted in only a 2 to 3-fold reduction in the level of OmpF when strains were grown in high osmolarity medium and no reduction in strains grown in low osmolarity medium (Fig. 6.1). This experiment indicated that the tolC effect on OmpF is largely mediated via the micF and/or ompC genes.

6.3 Effect of tolC on OmpF in micF deletion mutants

In chapter-4 of this thesis it is shown that the presence or absence of the OmpC protein itself does not interfere with the tolC effect on OmpF. This, together with the conclusion from the above experiment, strongly suggests that the micF gene alone is involved in the suppression of ompF expression observed in tolC mutants. To test this hypothesis, a micF^{-} ompC^{+} plasmid, pPR426 (Fig. 6.2), was constructed. The deletion of the micF gene from pMAN006 (micF^{+} ompC^{+}) was confirmed by nucleotide sequencing (Fig. 6.2): the deletion ended 61 bp upstream of the putative -35 region of the ompC gene and thus removed the entire micF gene from pMAN006. An equal amount of the OmpC protein was produced by strain P3183 (ompF ompC double mutant) carrying pMAN006 (micF^{+} ompC^{+}) or pPR426 (micF^{-} ompC^{+}) (Fig. 6.3). Both of these plasmids were transformed into a micF-ompC deletion strain (CS1253) and its tolC derivative (P3398) to give strains effectively micF^{+} ompC^{+} (P3418 and P3419) or micF^{-} ompC^{+} (P3423 and P3424).

A comparison of the outer membrane protein profiles of strains P3418 and P3419 (Fig. 6.4, lanes 1 and 2), grown in low osmolarity medium, shows that the tolC mutation in P3419 has the same major effect

Fig. 6.1

Effect of tolC on OmpF in micF$^+$ ompC$^+$ and micF$^-$ ompC$^-$ strains

Whole cell envelopes from strains W1485F$^-$ (wild type; lanes 1 and 5), P2731 (W1485F$^-$ tolC; lanes 2 and 6), CS1253 (W1485F$^-$ ompC micF; lanes 3 and 7) and P3398 (CS1253 tolC; lanes 4 and 8) were analysed by SDS-PAGE. Strains were grown either in a low osmolarity medium (lanes 1 to 4) or in a high osmolarity medium (lanes 5 to 8).

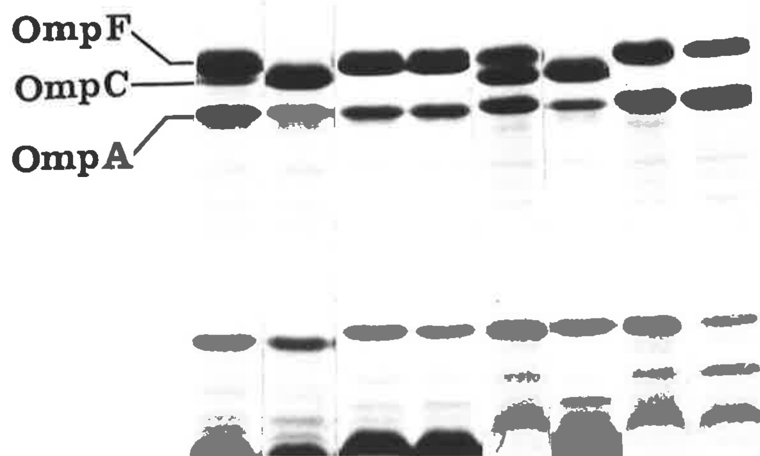
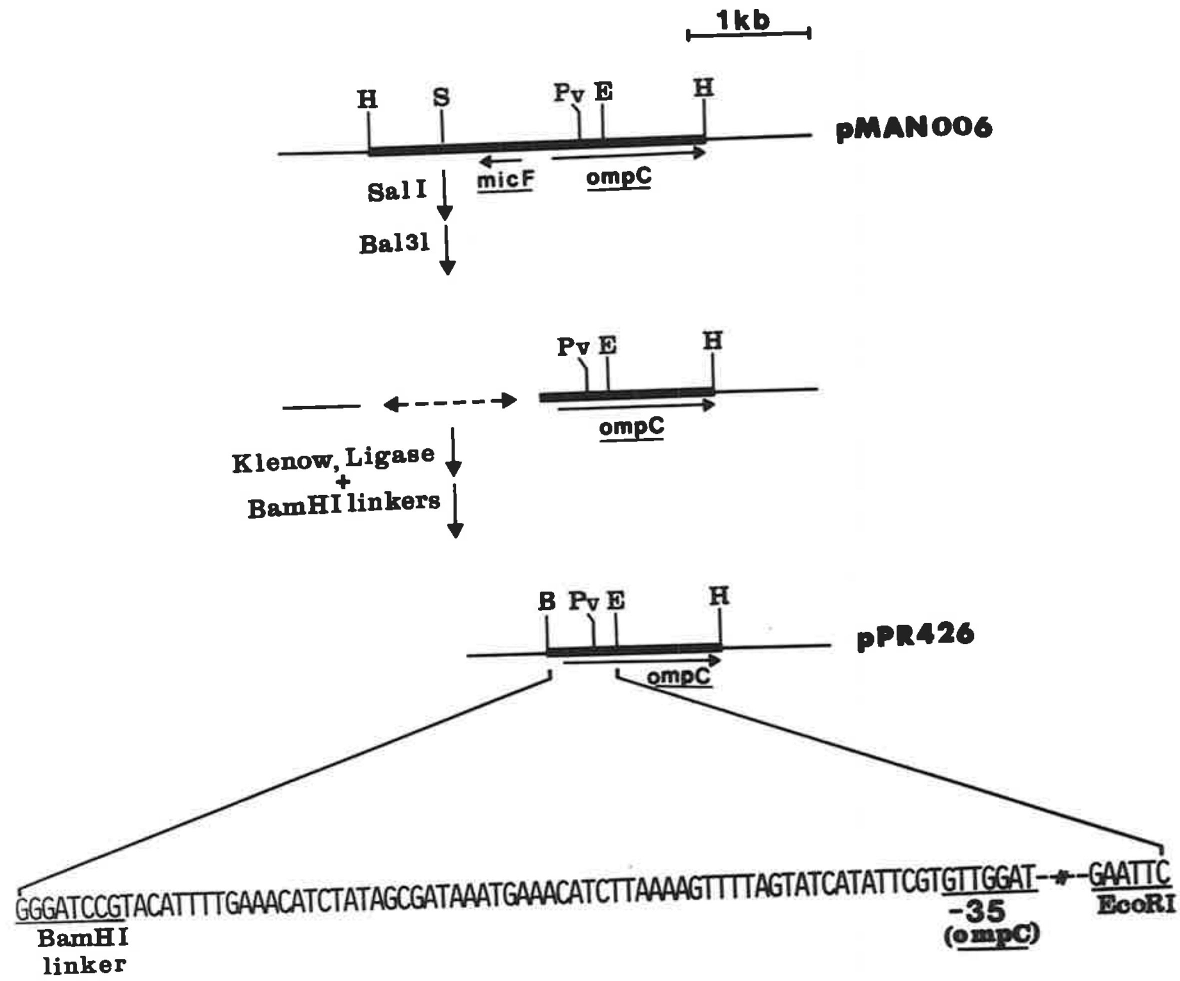

1 2 3 4 5 6 7 8
OmpF
OmpC
OmpA

Fig. 6.2

Construction of the micF delete plasmid, pPR426, and the nucleotide sequence downstream of the newly created BamHI site in pPR426.

Plasmid pMAN006 contains both micF and ompC genes, and pPR426 ($micF^-$ $ompC^+$) was derived from this plasmid by Bal31 deletion as shown in the figure. Briefly, pMAN006 was cut at an unique SalI site (located approximately 700 bp from the start of the micF gene), and then digested with Bal31: samples were taken at various times and the reaction was stopped by the addition of 5mM EGTA. The DNA was incubated with Klenow fragment in the presence of all four deoxyribonucleotides (dCTP, dATP, dGTP and dTTP) and ligated in the presence of phosphorylated BamHI linker (8-mer, pdCGGATCCG). To determine the deletion end point, a 571-bp BamHI-EcoRI fragment from pPR426 was cloned into M13mp9 (Messing, 1983) and sequenced. Abbreviations: B, BamHI; E, EcoRI; H, HindIII; Pv, PvuII; S, SalI.



1kb
H
S
Pv
E
H
pMAN006
micF
ompC
Sal I
Bal31
Pv
E
H
ompC
Klenow, Ligase
+
BamHI linkers
B
Pv
E
H
pPR426
ompC
GGATCCGTACATTTTGAAACATCTATAGCGATAAATGAAACATCTTAAAAGTTTTAGTATCATATTCGTGTTGGAT--//--GAATTC
BamHI
linker
-35
(ompC)
EcoRI

Fig. 6.3

Expression of OmpC by pMAN006 (micF$^+$ ompC$^+$) and pPR426 (micF$^-$ ompC$^+$)

Whole cell envelopes from strain P3183 (ompF ompC; lane 1) carrying either pMAN006 (lane 2) or pPR426 (lane 3) were analysed by SDS-PAGE. Cultures were grown in a high osmolarity medium.

1 2 3

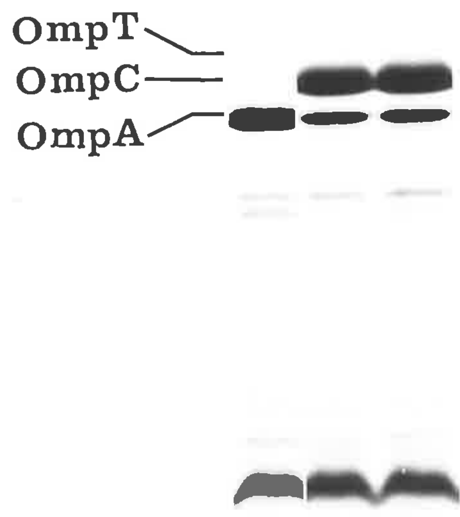


Fig. 6.4

Effect of tolC on OmpF, in the presence of pMAN006 ($micF^+$ $ompC^+$) or pPR426 ($micF^-$ $ompC^+$) in a $micF^-$ $ompC^-$ (CS1253) background

Whole cell envelopes from P3418 (CS1253/pMAN006; lanes 1 and 5), P3419 (CS1253 tolC/pMAN006; lanes 2 and 6), P3423 (CS1253/pPR426; lanes 3 and 7) and P3424 (CS1253 tolC/pPR426; lanes 4 and 8) were analysed by SDS-PAGE. Strains were grown either in a low osmolarity medium (lanes 1 to 4) or in a high osmolarity medium (lanes 5 to 8). Only the relevant part of the gel is shown.

1 2 3 4 5 6 7 8

OmpF

OmpC

OmpA

on the OmpF level in the presence of pMAN006 as it has in the wild type background (Figs. 6.1 and 6.5A, lanes 1 and 2). However, comparison of the outer membrane protein profiles of strains P3423 and P3424, which carry the micF delete plasmid, pPR426, shows that in this background the tolC mutation has a negligible effect on OmpF synthesis (Fig. 6.4, lanes 3 and 4). Furthermore, the tolC mutation has only a 2-fold effect on the OmpF level in the presence of a chromosomal micF deletion (compare SM3001 and P3493; Fig. 6.5A, lanes 3 and 4). Thus the micF gene is required for the tolC effect to be exerted on ompF expression.

In a wild type strain, such as MC4100 or W1485F^{-}, growth in a high osmolarity medium (high salt) causes a 2 to 3-fold increase in the OmpC level and a 2 to 3-fold reduction in the OmpF level (Fig. 6.1, lanes 1 and 5; Figs. 6.5A and 6.5B, lane 1). A tolC mutation in these strains causes a substantial reduction in the level of OmpF (Fig. 6.1, lanes 5 and 6; Fig. 6.5B, lanes 1 and 2). Strain P3418, which differs from a wild type strain in having approximately six copies of micF and ompC, shows a greater reduction in OmpF level when grown in a high osmolarity medium (Fig. 6.4, lanes 1 and 5), than does a wild type strain (Fig. 6.1, lanes 1 and 5; Figs. 6.5A and 6.5B, lane 1). Under these conditions, a tolC mutation further reduces the residual level of OmpF to a barely detectable level in P3419 (Fig. 6.4, lanes 5 and 6).

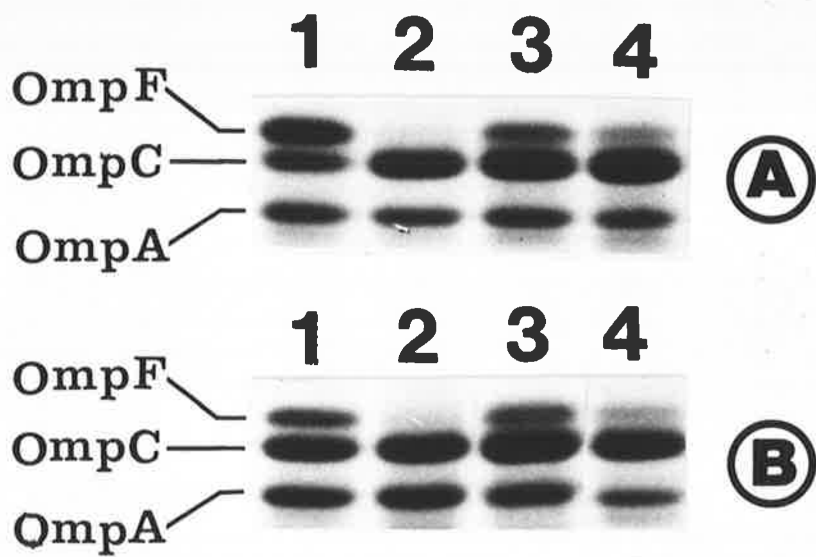
In case of strain P3423, which carries pPR426 and hence carries about six copies of ompC but none of micF, growth in a high osmolarity medium only reduces the level of OmpF by 2 to 3-fold (Fig. 6.4, lanes 3 and 7) and a tolC mutation further reduces the level of OmpF by 4 to 5-fold (Fig. 6.4, lanes 7 and 8).

By comparing the effect of osmolarity on the wild type strain and on those strains carrying either pMAN006 or pPR426, it can seen that the

Fig. 6.5

Effect of tolC on OmpF in the presence or absence of the chromosomal micF gene

Whole cell envelopes from MC4100 (wild type; lane 1), P3011 (MC4100 tolC; lane 2), SM3001 (MC4100 Δ micF; lane 3) and P3493 (SM3001 tolC; lane 4) were analysed by SDS-PAGE. Strains were grown either in a low osmolarity medium (A) or in a high osmolarity medium (B).



1 2 3 4
OmpF
OmpC
OmpA
A
1 2 3 4
OmpF
OmpC
OmpA
B

reduction in OmpF levels is much greater if the micF gene is present in the plasmid. The effect of osmolarity on OmpF and OmpC levels in strain SM3001 (which carries a chromosomal micF deletion) is less marked than in a wild type strain (MC4100). It should also be noted that the micF deletion itself reduces the OmpF level and increases the OmpC level (Matsuyama and Mizushima, 1985; Figs. 6.5A and 6.5B, lane 3). A tolC mutation in SM3001 causes only a 2 to 3-fold reduction in the level of OmpF when the cultures were grown in a high osmolarity medium whereas a tolC mutation in a micF$^+$ strain (MC4100) resulted almost in the total loss of OmpF under similar growth conditions.

Thus whereas in a low osmolarity medium, the effect of tolC on the OmpF level in a micF deletion background was undetectable, in a high osmolarity medium the effect was always detectable but was much less than that observed in a corresponding micF$^+$ strain.

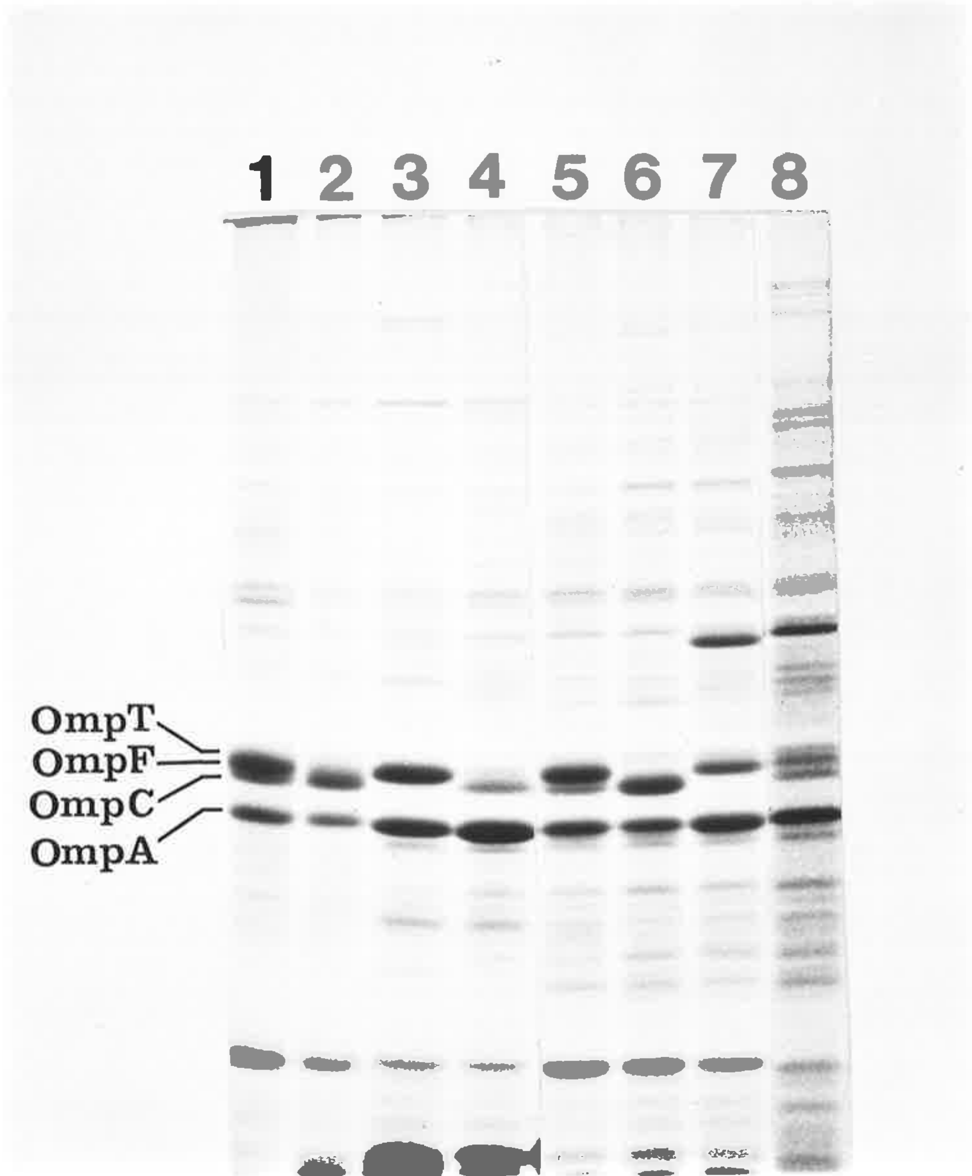
6.4 Interaction of tolC and ompR mutations and expression of the ompC and micF genes

Mutations in the ompR gene affect synthesis of one or both of the OmpF and OmpC proteins. The original ompR101 mutation (Sarma and Reeves, 1977) results in the absence of both OmpF and OmpC proteins; ompR472 and ompR20 mutations result in a greatly reduced level of OmpC, with OmpF synthesised at a high level (regardless of osmolarity) in the strain carrying the ompR472 mutation (Hall and Silhavy, 1981b) and at a reduced level with reverse osmolarity effect in the strain carrying the ompR20 mutation (Nara et at., 1984). By comparison, a mutation in the tolC locus results in a greatly reduced level of OmpF and constitutive synthesis of OmpC (Morona and Reeves, 1982a; see also chapter-4).

Fig. 6.6

Effect of tolC on OmpF and OmpC in ompR and ompR$^+$ strains

Whole cell envelopes from MC4100 (wild type; lane 1), P3011 (MC4100 tolC; lane 2), MH760 (MC4100 ompR472; lane 3), P3394 (MH760 tolC; lane 4), W4629F$^-$ (ompR$^+$; lane 5), P3396 (W4626F$^-$ tolC; lane 6), FN101 (W4626F$^-$ ompR20; lane 7) and P3393 (FN101 tolC; lane 8) were analysed by SDS-PAGE.


1 2 3 4 5 6 7 8
OmpT
OmpF
OmpC
OmpA

In this experiment two mutations, tolC and ompR, were combined to determine their interaction. The addition of a tolC mutation to two different ompR mutants (MH760, ompR472; FN101, ompR20) leads to a substantial increase in the level of OmpC and a decrease in the OmpF level (Fig. 6.6).

Expression of micF, which is co-regulated with ompC (Mizuno et al., 1984; Schnaitman and McDonald, 1984), is also under ompR positive control (Mizuno et al., 1984). It was therefore of interest to see whether a mutation in the tolC locus also increases micF expression. To investigate this, a micF-lacZ fusion plasmid, pmic-B21 (Mizuno et al., 1984), was obtained and transformed it into wild type, tolC, ompR472 and tolC ompR472 strains, and micF transcription was estimated by measuring β-galactosidase activity (Table 6.1). The tolC mutant had 8-fold more β-galactosidase activity present than the wild type strain: the tolC ompR double mutant had a level very similar to that of the tolC mutant while the ompR mutant, as expected, had a very low level, such that there was 60-fold more β-galactosidase activity present in the tolC ompR double mutant than in an ompR mutant. The 10-fold reduction in micF expression in an ompR mutant confirms the results obtained by Mizuno et al. (1984). These observations clearly demonstrate that a mutation in the tolC locus enhances the expression of both the ompC and micF genes.

6.5 Summary and discussion

The results obtained in this chapter show that the tolC effect on OmpF was almost negligible in an ompC-micF deletion background when cultures were grown either in a high osmolarity medium (nutrient broth plus NaCl) or in a low osmolarity medium (nutrient broth without NaCl).

TABLE 6.1

β-galactosidase activities of strains carrying

micF-lacZ fusion plasmid (pmicB21)

Bacterial strains	β-galactosidase units
MC4100	0
P3501	169
P3502	1378
P3503	18
P3504	1121

Similarly, in a _micF_ deletion background, a _tolC_ mutation has virtually no effect on the level of OmpF when cultures are grown in a low osmolarity medium. The effect in a high osmolarity medium is difficult to interpret since _tolC_ mutants are sensitive to high salt levels (unpublished observations), as they are to many other environmental factors, but certainly the effect of _tolC_ is much less in a _micF_ mutant than in a _micF_$^{+}$ strain. These observations clearly suggest that the _tolC_ effect on OmpF is largely mediated by _micF_. The role of _tolC_ in osmoregulation of _ompF_ and _ompC_ expression is discussed in the following chapter.

In addition, it has been found that a mutation in the _tolC_ locus enhances the expression of the _ompC_ and _micF_ genes in an _ompR_$^{+}$ strain and more interestingly in an _ompR_ mutant strain where their expression had been suppressed. This indicates that the increased expression of the _ompC_ and _micF_ genes in a _tolC_ mutant results in the reduced expression of _ompF_. However, in previous chapters it has been shown that the presence or absence of the OmpC protein itself does not interfere with the _tolC_ effect on OmpF. Thus it can safely be concluded that the effect of _tolC_ on OmpF is mediated by activating the _micF_ gene, which has an RNA product known to inhibit expression of _ompF_ (Mizuno _et al_., 1984).

CHAPTER-7

FINAL DISCUSSION AND CONCLUSIONS

7.1 Introduction

In this thesis some aspects of the structural organisation and biosynthesis of the TolC protein have been studied. In addition, the mechanism by which a mutation in the tolC locus exerts its affect on OmpF has been investigated. In this chapter the results obtained on these topics are analysed.

7.2 Structural organisation of the TolC protein

Much of the knowledge about the structural organisation of many outer membrane proteins such as LamB, lipoprotein and OmpA, has come from the use of biochemical and genetical techniques. One technique which has yielded meaningful data is the use of proteolytic enzymes. In this thesis use was made of a protease, trypsin, to study some topological properties of the TolC protein.

Trypsin digestion of the TolC protein in intact cells and in a purified outer membrane fraction gave two different sets of cleavage products and thus the trypsin susceptible regions of the TolC protein appear to be different in the two situations: one such region is present close to one end of the polypeptide chain and is accessible to trypsin digestion in intact cells whereas the other region is present near to the middle of the polypeptide chain and accessible to trypsin digestion only in the isolated outer membrane fraction and not in intact cells.

The amino acid sequence of the mature TolC protein (Hackett and Reeves, 1983) reveals only two relatively highly charged (hydrophilic) segments. One such segment of 18 amino acid residues is present near the amino terminus, (^{18}Arg-Lys-Ser-Ala-Ala-Asp-Arg-Asp-Ala-Ala-Phe-Glu-Lys-Ile-Asn-Glu-Ala-Arg^{35}), and contains 3 arginine and 2 lysine residues and may represent the trypsin-sensitive segment of the TolC protein (trypsin cleaves peptide bonds on the carboxyl side of arginine and lysine only) exposed on the outer surface. The other relatively charged stretch of amino acid residues,(^{214}Lys-Glu-Ala-Glu-Lys-Arg-Asp-Leu-Ser-Leu-Leu-Gln-Ala-Arg-Leu-Ser-Gln-Asp-Leu-Ala-Arg-Glu-Lys-Phe-Ala-Arg-Arg-Arg^{241}), is also rich in arginine and lysine and is therefore a likely candidate for the trypsin sensitive segment on the inner face of the outer membrane. A trypsin cleavage in this segment will result in peptide fragments of approximately 23,500 to 26,500 Mr. This expected size is compatible with that of the TolC fragment detected in trypsin-treated purified membranes.

These results suggest that the TolC protein is partly exposed on the outside of the cell surface and partly on the inside (periplasmsic side) of the outer membrane and therefore traverses the outer membrane.

On the cell surface, TolC, like some other outer membrane proteins, may provide phage receptor activity. However, attempts to isolate TolC specific phage were unsuccessful. The cell surface exposed region(s) of TolC may be involved in colicins action. Other phenotypic properties of *tolC* mutants such as slow growth rate, hypersensitivity to detergents and dyes suggest that on the inside, the TolC protein is closely associated with other components of the membrane, and thus plays an important role in the membrane structure (see also sec. 7.3.2).

7.3 Biosynthesis of the TolC protein

The data presented in this thesis suggested that the TolC protein, like other outer membmrane proteins, is initially synthesized as a higher molecular weight precursor which is located in the cytoplasmic membrane. At early stages of synthesis, a polypeptide of apparent Mr of 46,000 was detected which during the chase converted into the conventional precursor. The experiments demonstrated that this polypeptide is not a degradation product but is an incomplete biosynthetic intermediate of the TolC protein which requires further protein synthesis for completion.

Several incomplete nascent polypeptides were detected in case of OmpA (Crowlesmith and Gamon, 1982) and MalE (Randall *et al*., 1980). It was suggested that several stable secondary structures in *ompA* mRNA and some unusual structures in *malE* mRNA were causing blockage in peptide elongation and accumulation of biosynthetic intermediates. *tolC* mRNA has no stable secondary or unusual structures in its main body (Hackett and Reeves, 1983). However, the presence of the rare co don AGA (co don 402 of the coding region) is the most likely reason for the temporary accumultation of the 46,000-Mr polypeptide.

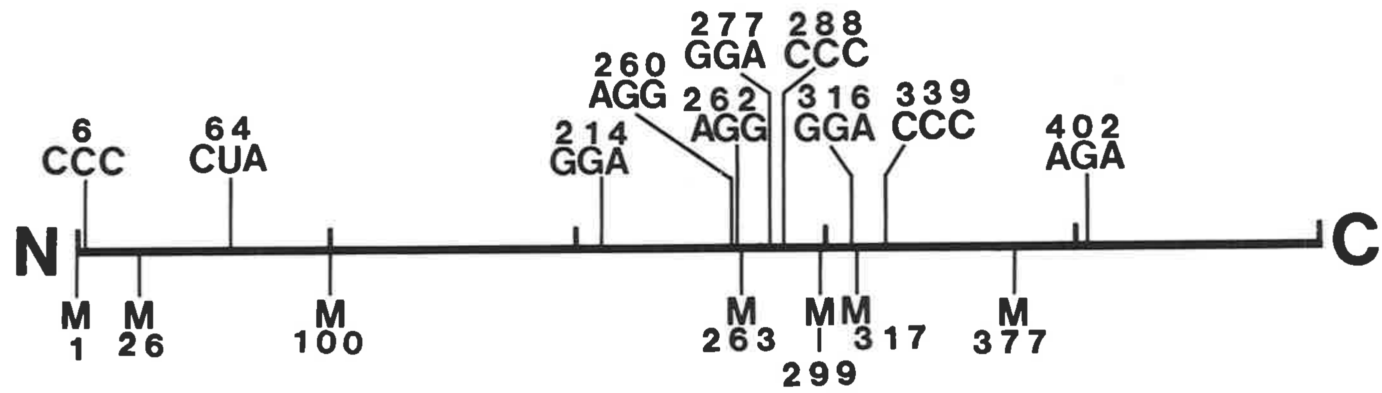
The significance of rare codons in regulatory and minor proteins has been studied before (Farabaugh, 1978; Singleton *et al*., 1980; Grantham *et al*., 1981; Grosjean and Fiers, 1982; Stoner and Schleif, 1982; Konisberg and Godson, 1983), and recently it has been shown (Robinson *et al*., 1984) that the insertion in tandem of four of the extremely rare codon AGG (Arg) into the *E. coli cat* gene significantly reduces the level of expression. For some *E. coli* proteins the synonymous codons are used in a non-random manner and the codons preferred are those

recognised by the most abundant tRNA species in the cell; the concentration of each tRNA and the frequency of usage of the synonymous codons have been listed by Ikamura (1981a; 1981b). Codons AGA, AGG and CGG for arginine, CCC for proline, GGA for glycine, and CUA for leucine were considered to be rare codons and are also not present in the mRNA of major outer membrane proteins: lipoprotein (Nakamura et al., 1979), OmpA (Movva et al., 1980), OmpF (Inokuchi et al., 1982), OmpC (Mizuno et al., 1983), or LamB (Clement and Hofnung, 1981). However, these codons and rare codons for other amino acids are present in relatively high proportions in minor proteins and regulatory proteins (Table 7.1), which are maintained in the cell in low concentrations. The rare codons in such protein may cause the translating ribosome to pause as it proceeds along the mRNA, owing to the limiting amount of corresponding tRNA, thereby reducing expression of the gene. Absence of these rare codons from the mRNA of the major proteins would facilitate a high level of expression.

In this thesis evidence for such a mechanism has been presented. TolC is a minor outer membrane protein and has ten amino acid residues encoded by the rare codons listed above. TolC is synthesised in large amounts in strains carrying the tolC gene in multicopy plasmids and a 46,000 Mr polypeptide was consistently observed at the earlier stages of biosynthesis in such circumstances. This would be expected if translating ribosomes pause at codon 402 (AGA). The results presented in this thesis have confirmed that the 46,000-Mr plypeptide is a nascent biosynthetic intermediate of the mature TolC protein and that the rate of synthesis of mature protein can be increased by providing extra $tRNA^{Arg}$ (AGA, AGG). Under this hypothesis one would expect the translating ribosome to pause also at the other rare codons shown in Fig. 7.1. However, in the experimental conditions of this work other small peptides were not

Fig. 7.1

Position of rare codons and methionine (M) residues in the *tolC* gene



277
GGA
288
CCC
260
AGG
262
AGG
316
GGA
339
CCC
6
CCC
64
CUA
214
GGA
402
AGA
N
C
M
1
M
26
M
100
M
263
M
299
M
317
M
377

Table-7.1

The usage of rare codons in *E. coli* genes

The usage of 6 rare codons was examined. Figures are given for a total of 22 highly expressed genes. A total of 16 weakly expressed genes were surveyed as were the genes for 5 regulatory proteins. omp, major outer membrane proteins: genes included are *ompF*, *ompC* *ompA*, *lamB* and *lpp*.

Rare codons	Genes			Major *omp*	*tolC*
	highly expressed	weakly expressed	regu- latory		
AGA (Arg)	3	9	3	0	1
AGG (Arg)	1	4	6	0	2
CGG (Arg)	0	8	14	0	0
CCC (Pro)	2	8	13	0	3
GGA (Gly)	5	7	13	0	3
CUA (Leu)	3	5	7	0	1
Total	14	41	56	0	10
Average frequency per gene	0.636	2.56	11.2	0	10

consistently detected. It is possible that the other tRNA species involved are not in such limiting levels as that for $tRNA^{Arg}$ (AGA, AGG). However, one might at least expect the rare arginine codons at residues 260 and 262 to lead to accumulation of a small peptide of 28,000 Mr, as the same tRNA species is involved for codons AGA and AGG (Celis and Maas, 1971). It should be noted (Fig. 7.1) that four of the seven methionine residues are encoded between codons 262 (AGG) and 402 (AGA). Therefore, one would expect the translating ribosomes to synthesise 46,000 Mr polypeptides, which are labelled to more than twice the specific activity of 28,000 Mr ploypeptides. Furthermore, ribosomes which have passed codon 100 at the commencement of the pulse will not incorporate [^{35}S]methionine into the 28,000 Mr polypeptide. Together, these observations predict that during a short pulse significantly less radioactive label will be incorporated into the 28,000 Mr polypeptide than into the 46,000 Mr polypeptide resulting in difficulty in detecting the smaller polypeptide. The fortunate circumstance of four methionine codons preceding AGA codon at position 402 may account for the relative ease of detecting it. It is also possible that other small polypeptides do not fold in a way which can be recognised by TolC antiserum raised against the mature TolC protein. Some small polypeptides besides the 46,000 Mr polypeptide were observed but only when immunoprecipitates were not washed thoroughly with buffer before loading on the gel (data not shown). This may reflect the weak antigenic reactivity of these additional small polypeptides with TolC antiserum; this possibility was not further investigated.

A second effect of the cloned $tRNA^{Arg}$ was also observed in that a portion of the 46,000 Mr polypeptide persisted much longer than in the absence of the cloned $tRNA^{Arg}$ gene. It appears that the additional tRNA may have one of two effects when a ribosome reaches condon 402: it may

either enable rapid addition of arginine and avoidance of stalling at this site, or it may in some way terminate chain elongation. The later aspect was not investigated but assumed it to be due either to the T4 tRNA being not fully functional or not fully charged. The cloned tRNA may for example not be properly processed to the mature form or may be at a high concentration and which saturates the charging process.

7.4 The tolC effect on OmpF

Mutations in the tolC locus affect the normal synthesis of OmpF, a major outer membrane protein of Escherichia coli K-12. Experiments examining the effect of a tolC mutation on OmpF expression in strains carrying different copy numbers of the ompF gene (chapter-4) suggested that unlike ompR, the tolC locus is not essential for OmpF production. The same conclusion was drawn by Morona and Reeves (1982a) who showed that growth in a low salt medium results in detectable levels of OmpF in tolC mutants.

Analysis of ompF-ompC chimeric genes showed (chapter-4) that a tolC mutation exerts its effect either at the promoter, or at the amino terminal end of the ompF gene. This is comparable to the effect exerted via ompR which acts on the promoter, or via micF which acts on a region overlapping the promoter and amino terminal of the ompF gene. Indeed, as found for the ompR mutation (ompR101) or in the presence of the micF gene at high levels, a tolC mutation drastically reduces the amount of ompF transcript in $ompF^{+}$ strains. The earlier observation of Morona and Reeves (1982a) that the tolC mutation has a relatively slight effect on transcription of an ompF-lacZ operon fusion strain, is now shown to be due to a reduction in the fusion strain of the tolC effect itself: in a

heterzygote, a tolC mutation has a relatively little effect on expression of either the fusion or the wild type ompF allele. The tolC effect is greater in the protein fusion strain but still much less than in a non-fusion strain where OmpF could not be detected in a tolC mutant under similar growth conditions.

The tolC effect on OmpF is possibily independent of ompR regulation as tolC affects NmpC and LC proteins (Morona and Reeves, 1982a), which do not require OmpR as a positive control element (Pugsley and Schnaitman, 1978b).

The tolC effect may be to activate micF expression. This possibility was supported by the observation that in addition to reducing the amount of OmpF, tolC mutation increases the level of OmpC present in the membrane. This increase is much greater than that observed in an ompF mutant, and hence is not a simple compensation for the lack of OmpF protein. Rather, this increase is presumably an effect of the tolC mutation at the ompC locus itself. A locus, micF, maps very close to ompC and is probably co-regulated with ompC expression (Mizuno et al., 1984; Schnaitman and McDonald, 1984). High levels of micF expression have been shown to exert a strong negative effect on OmpF expression (Mizuno et al., 1984). Therefore, it is possible that tolC mutations exert their effect on OmpF expression by co-ordinately stimulating the expression of the ompC and micF genes, rather than directly acting on the ompF gene itself. It should be noted that the presence or absence of the OmpC protein itself does not interfere with the the tolC effect on OmpF. It thus appears possible that micF is in large part responsible for the suppression of OmpF in tolC mutants.

The above possibility was supported by analysis of the Stc$^-$ mutation (Morona and Reeves, 1982b) which reverted the phenotype of tolC

mutants from OmpF$^-$ to OmpF$^+$. The nature of this mutation was shown (chapter-5) to be a deletion, removing part of the ompC promoter and the entire micF gene. Since this suppressor mutation was isolated in an ompC tolC background, it is reasonable to assume that the OmpF$^+$ phenotype of the Stc$^-$ mutation is not due to the lack of ompC but due to the lack of the micF gene.

The role of micF in tolC-mediated regulation of OmpF was clearly shown (chapter-6) by using strains in which micF alone is deleted, being ompC functional either in the chromosome or in a plasmid. In a micF deletion background, a tolC mutation had virtually no effect on the level of OmpF when cultures were grown in a low osmolarity medium: the effect in a high osmolarity medium is difficult to interpret since tolC mutants are sensitive to high salt levels (data not shown), as they are to many other enviromental factors, but the effect of tolC was clearly much less in a micF mutant than in a corresponding micF$^+$ strain.

The notion that the effect of tolC on OmpF is mediated by activation of the micF gene was further supported by the finding that a tolC mutation substantially increased the transcription of micF in a micF-lacZ fusion strain.

7.4.1 Pleiotropic effects of the tolC mutation

The tolC mutation is pleiotropic and has three major effects: cells become (1) tolerant to colicin E1, (2) extremely sensitive to detergents and dyes and (3) lack detectable levels of OmpF in the membrane. These properties of tolC mutants suggest that they have a membrane defect.

If the tolC effect on OmpF is due essentially to increased micF expression, then it resembles the effect of increased osmolarity of the medium on OmpF, as growth in a high osmolarity medium favours OmpC (and presumably micF) expression and reduces OmpF expression. Therefore, it appears that the tolC effect on OmpF could be due to a membrane defect which leads to the modification of the cell's osmosensing system such that the OmpF to OmpC ratio is pushed even further in favour of OmpC/micF than is the case for normal strains grown in a high osmolarity medium. It thus seems possible that the tolC effect on OmpF is brought about by this regulatory system which affects the ompC and micF genes and indirectly affects the ompF gene.

If the effect of tolC is due to modification of the osmosensing system of the cell, then it could well be mediated by OmpR, which is thought to be involved in osmoregulation of ompF and ompC, besides being a positive regulatory element of these genes. The data presented in this thesis suggest that the major part of the tolC effect on OmpF is indirect and due to increased micF expression which is also under OmpR positive control. The effect of the tolC mutation then can be considered as primarily an effect on the membrane function which leads to the OmpR-mediated system being pushed in favour of ompC and micF, with the apparent effect being the concomitant loss of OmpF from the outer membrane. It thus appears that the three pleiotropic effects of the tolC mutation are due to only two main effects i.e. (1) tolerance to colicin E1 and (2) a membrane defect. The latter property, which leads to the sensitivity to detergents and dyes, may also affect OmpF by affecting the osmosensing system of the cell.

7.4.2 Speculations on the membrane defect of tolC mutants

It is unlikely that the absence of the TolC protein itself, which is a minor outer membrane protein, would be responsible for the membrane defect unless it is interacting with other membrane components such as LPS, phospholipids or other envelope proteins. Increased sensitivity of tolC mutants towards hydrophobic agents, a property similar to that observed in "deep rough" LPS mutants of Salmonella typhimurium (Kamio and Nikaido, 1976), suggests that they may have a defect in their LPS structure but chemical composition of LPS from $TolC^+$ and $TolC^-$ E. coli strains showed no differnce (Drs. J. Redmond and P. Reeves, unpublished data). This, however, does not rule out the possbility that tolC mutants have differences in LPS structure which were not detected and which lead to the exposure of phospholipids on the cell surface and thus contribute to the increased sensitivity towards hydrophobic compounds. Several minor envelope proteins were also reported to be affected in tolC mutants but their roles remained unidentified (Morona, 1982). At this stage the nature of the membrane defect in tolC mutants is not known and further investigation on this will help to understand the effect of the tolC mutation on OmpF and its other phenotypic properties.

7.4.3 Role of micF in osmoregulation of ompF

Although osmoregulation of ompF and ompC is reported to be mediated mainly by the ompR and envZ genes (Hall and Silhavy, 1981b; Inokuchi et al., 1985), a third gene, micF, also regulates ompF expression directly, at least when present in a high copy number plasmid (Mizuno et al., 1984). However, Matsuyama and Mizushima (1985) reported

that the micF gene, when present as a sigle chromosomal copy or when is present in a low copy number plasmid (six copies), does not play any significant role in osmoregulation of ompF. The experiments reported in this thesis have shown that micF, when present in a six-copy-number plasmid (pMAN006), can be important for osmoregulation of ompF expression: the amount of OmpF was substantially reduced in the presence of pMAN006 ($ompC^+$ $micF^+$) but not in the presence of pPR426 ($ompC^+$ $micF^-$) when cells were grown in a high salt medium. This discrepency could be due to the following two reasons: firstly, strains that were used in the two studies had a different genetic background and secondly, different growth media were used. However, it should be noted that the high osmolarity medium used by Matsuyama and Mizushima for their micF experiments contained less than half the amount of sucrose (8%) than that used in their normal high osmolarity medium which contained 20% sucrose. Although, there is still conflicting data on the role of micF in osmoregulation, results presented in this thesis suggest that micF is involved as a major factor in the osmoregulation of ompF, and by tolC mutation.

7.4.4 Final remarks

Several genetic loci such as ompR, envZ, micF, and tolC affect expression of ompF and ompC. Some of these loci interact with each other in complicated ways which makes it difficult to determine the primary effect of each of these regulatory elements. Too little is known as yet of the molecular mechanisms involved in osmoregulation, or of the role of ompR and envZ (Inokuchi et al., 1985; Ramakrishnan et al., 1985), to speculate on the primary effect of tolC at the molecular level.

The role of these regulatory components can perhaps be more precisely determined by setting up an *in vitro* system where only one factor can be varied at a time without affecting others.

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Molecular characterisation of the Stc$^-$ mutation of *Escherichia coli* K-12

(Gene expression; restriction mapping; cloning; sequencing)

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SUMMARY

The previously described Stc$^-$ (suppressor of TolC) mutation modifies the phenotype of *tolC* mutants from OmpF$^-$ to OmpF$^+$. Restriction mapping of chromosomal DNA from Stc$^+$ and Stc$^-$ strains was performed to investigate the nature of the mutation which was shown to be a deletion, upstream of the *ompC* gene. DNA from the region of the deletion was cloned into pUC18 and a 650-bp *Pst*I-*Eco*RI fragment was sequenced. The deletion started 49 bp upstream of the AUG start codon of the *ompC* gene, thus removing part of the *ompC* promoter and the whole of the *micF* gene. We suggest that the deletion of *micF* gives rise to the Stc$^-$ phenotype since the effect of *micF* expression is assumed to reduce *ompF* expression, and the Stc$^-$ phenotype involves increase in *ompF* expression.

INTRODUCTION

OmpF and OmpC are both major outer membrane proteins, and are the two porins present constitutively to allow diffusion of nutrients through the outer membrane. These two proteins are regulated to vary the relative amounts under different growth conditions, with osmotic pressure of the medium having a substantial influence (Van Alphen and Lugtenberg, 1977). This regulation is thought to be mediated in large part by two genes, *ompR* and *envZ* (Hall and Silhavy, 1981a,b) which map at the *ompB* locus described earlier (Sarma and Reeves, 1977). The nature of this regulation is not yet understood, but see Nikaido and Vaara (1985) for a recent review.

TolC is a minor outer membrane protein of *Escherichia coli* K-12. The *tolC* gene has been sequenced and the TolC protein characterised in our laboratory (Hackett and Reeves, 1983; Morona et al., 1983). *tolC* mutants are pleiotropic being resistant to colicin E1, extremely sensitive to detergent and various dyes and other agents, and have greatly reduced levels of OmpF protein: under certain conditions *tolC* mutants lack detectable levels of OmpF protein (Morona and Reeves, 1982a).

A further gene, *stc* (supressor of TolC) was de-

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Abbreviations: Ap, ampicillin; bp, base pair(s); Δ, deletion; EtdBr, ethidium bromide; kb, kilobase pairs; ^{R}, resistant; Stc, suppressor of TolC phenotype; [ ], indicates plasmid-carrier state.

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TABLE I

Bacterial strains and plasmids used

	Characteristics	Source/Reference
Strains		
P2125	W1485 F^-, *tonA*208, *pyrD*34	P. Reeves
P2716	P2125, *ompC*, *tolC*210 : : Tn*10*-48	Morona and Reeves (1982b)
P2718	P2716, *stc*-2	Morona and Reeves (1982b)
CS1253	W1485 F^-, *gyrA*$^+$, *ompC*178-*zei*-198::Tn*10*[a]	Schnaitman and McDonald (1984)
JM101	*supE*, *thi*, *Δ*(*lac-pro*), [F′ *traD*36, *proA*, *proB*, *lacIZΔ*M15]	Messing and Vieira (1982)
Plasmids		
pMAN006	*ompC*$^+$, *micF*$^+$, Ap^R	Matsuyama et al. (1984)
pCX28	*micF*$^+$, Ap^R	Mizuno et al. (1984)
pUC18	Ap^R	Messing (1983)

[a] *zei* symbol (min 48 on *E. coli* map) is according to Chumley et al. (1979; p. 644).

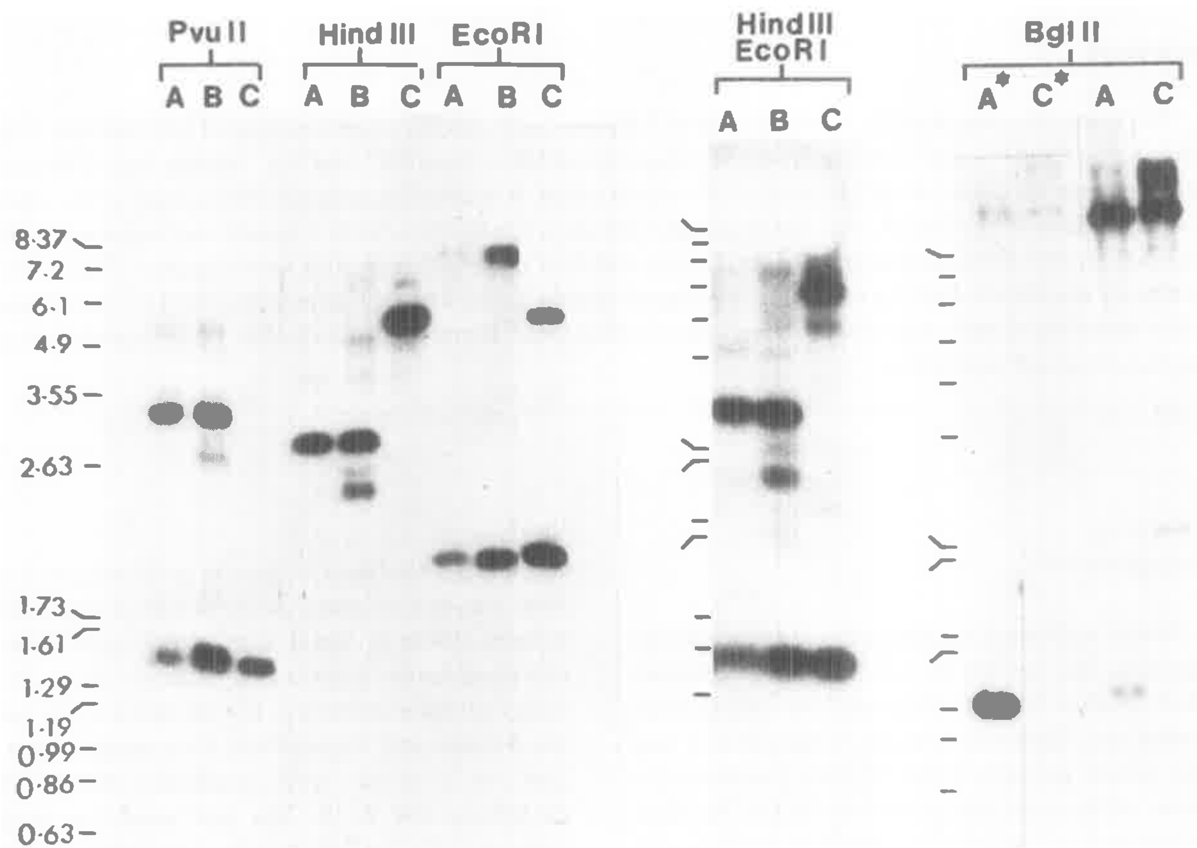

Fig. 1. Autoradiogram of a Southern blot of chromosomal DNA from strain P2125 (A), P2716 (B), and P2718 (C) digested with indicated restriction enzymes and hybridized with the *ompC-micF* probe (or *micF* probe*; see Fig. 2). Phage SPP1 DNA, digested with *Eco*RI was used as M_r standard. Fragment sizes (in kb) of the standard are shown at the left; the same standards were used for the *Hin*dIII + *Eco*RI and *Bgl*II digests and their positions indicated. Note that some bands resulted from partial DNA digests. Chromosomal DNA was purified essentially as described by Nakamura et al. (1979). Plasmid DNA was isolated by the two-step CsCl-EtdBr gradient method of Garger et al. (1983). DNA-DNA hybridizations were performed as described by Southern (1975). A 2.7-kb (*Hin*dIII fragment) *ompC-micF* probe was isolated from plasmid pMAN006 (Matsuyama et al., 1984) and a 300-bp (*Xba*I fragment) *micF* probe was isolated from plasmid pCX28 (Mizuno et al., 1984). Nick translation of plasmid DNA or DNA fragments extracted from agarose gels was performed by the method of Rigby et al. (1977).

scribed by Morona and Reeves (1982b), which modifies the effect of *tolC* mutation on *ompF* expression. However, we now use Stc only as a phenotype description for reasons given under EXPERIMENTAL AND DISCUSSION, section **d**. *tolC*, *ompC* mutants lack OmpC and under some conditions lack OmpF due to the *tolC* mutation. Stc$^-$, *tolC*, *ompC* mutants produced significant amounts of OmpF protein under the same growth conditions. The Stc$^-$ mutation was isolated in a porin producing revertant of an *ompC*, *tolC* strain and mapped very close to the *ompC* gene. A regulatory gene *micF*, has since been located in the same region and has been sequenced (Mizuno et al., 1984): *micF* RNA has a long sequence that is complementary to the 5′ end of *ompF* mRNA and is postulated to interfere with its translation by forming a stable RNA-RNA hybrid (Mizuno et al., 1984) although the significance of this effect in normal regulatory processes has been questioned (Matsuyama and Mizushima, 1985). In this communication we have characterised the nature of the Stc$^-$ mutation in relation to the *micF* gene in order to investigate its role in the TolC phenotype.

EXPERIMENTAL AND DISCUSSION

(a) Restriction analysis of the chromosomal DNA from Stc$^+$ and Stc$^-$ strains

Chromosomal DNA from strains P2125 (Stc$^+$, *ompC*$^+$, *tolC*$^+$), P2716 (Stc$^+$, *ompC*$^-$, *tolC*$^-$) and P2718 (Stc$^-$, *ompC*$^-$, *tolC*$^-$) (Table I) was digested with various restriction enzymes and subjected to Southern transfer. Nitrocellulose filters were hybridized with ^{32}P-labelled *ompC-micF* probe. Results of this experiment are shown in Fig. 1 and summarised in Table II. The relevant sites predicted from the sequence of the wild-type (Stc$^+$) DNA (Mizuno et al., 1983) are shown in Fig. 2. The probe hybridized with fragments of the predicted lengths when DNA from Stc$^+$ strains was used (Fig. 1, Table II), but with DNA from a Stc$^-$ strain (P2718) only some of the predicted fragments hybridized and some additional bands were seen. It was clear that there had been a rearrangement, possibly a deletion, in the *micF* region. We therefore used a *micF*-specific probe to hybridize *Bgl*II-digested chromosomal DNA fragments from Stc$^+$ and Stc$^-$ strains. This probe lit up the 1.0-kb fragment (which includes the *micF* gene) from the Stc$^+$ strain but did not light up any fragment in the Stc$^-$ strain (Fig. 1, Table II). When similar digests were hybridized with the *ompC-micF* probe, an additional 12.0-kb DNA fragment, present in both strains took up the probe (Fig. 1, Table II). These results confirmed the presence of a deletion in the Stc$^-$ strain upstream from the *Bgl*II site of the *ompC* gene; the deleted DNA includes the *micF* gene and must extend either about 2.8 kb or greater than 7.5 kb to account for the 5.7-kb *Eco*RI fragment lit up by the *ompC-micF* probe. The location of the deletion is shown in Fig. 2.

TABLE II

Size (in kb) of the DNA fragments that took up the *ompC-micF* probe or *micF* probe (*) after cutting with the indicated enzymes[a]

Strains	Restriction enzymes				
	*Pvu*II	*Hin*dIII	*Eco*RI	*Eco*RI + *Hin*dIII	*Bgl*II
P2125 (Stc$^+$, *ompC*$^+$, *tolC*$^+$)	1.4; 3.0	2.7	2.0; 8.5	0.8; 1.9	*1.0; 12.0
P2716 (Stc$^+$, *ompC*$^-$, *tolC*$^-$)	1.4; 3.0	2.7	2.0; 8.5	0.8; 1.9	n.t.[b]
P2718 (Stc$^-$, *ompC*$^-$, *tolC*$^-$)	1.4	5.4	2.0; 5.7	0.8; 4.6	12.0

[a] Only the *Bgl*II digests were hybridized with the *micF* probe (*) in addition to the *ompC-micF* probe (Fig. 1). Only the 1.0-kb fragment from P2125 hybridized with the *micF* probe.

[b] n.t., not tested.

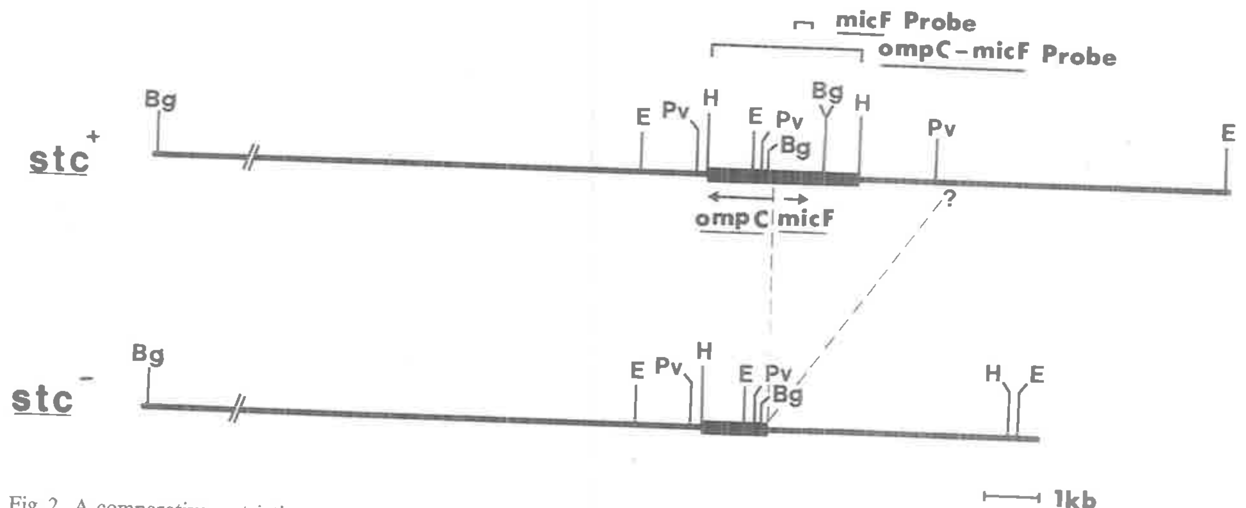

Fig. 2. A comparative restriction map of the chromosomal DNA of Stc$^+$ and Stc$^-$ strains around the *ompC-micF* genes. The dotted lines show the probable extent of the deletion in the Stc$^-$ strain. Note that this experiment would not give the location of *Hin*dIII sites outside of the probe in the Stc$^+$ strain. Bg, *Bgl*II; E, *Eco*RI; Pv, *Pvu*II; H, *Hin*dIII.

(b) Cloning of the Stc$^-$ mutation

As indicated in Fig. 2, a 5.4-kb *Hin*dIII fragment from the Stc$^-$ strain encompasses the possible deletion. Chromosomal DNA from the Stc$^-$ strain was therefore digested with *Hin*dIII and ligated to pUC18 DNA which had been digested with *Hin*dIII and treated with alkaline phosphatase. The ligated DNA mixture was then used to transform an *ompC* deletion strain (CS1253) and colonies selected on Ap plates were transferred to nitrocellulose filters and hybridized with a 2.7-kb *ompC-micF* probe. Plasmid DNA was prepared from colonies giving a positive signal and subjected to restriction analysis. One such plasmid, pPR313, contained the desired 5.4-kb DNA fragment, and a partial restriction map of this plasmid is shown in Fig. 3.

(c) Nucleotide sequence of the *Eco*RI-*Pst*I DNA fragment which includes the Stc$^-$ mutation

A 650-bp *Eco*RI-*Pst*I fragment from pPR313 was subcloned into M13mp8 and M13mp9 (Messing, 1983) and sequenced in both orientations. This sequence was then compared with the parental DNA sequence from the Stc$^+$ strain (Fig. 4). As predicted from the restriction analysis of the Stc$^-$ strain, a deletion started upstream from the *Bgl*II site of the *ompC* gene. The deletion start was located 49 bp upstream of the *ompC* gene start AUG codon. The deletion thus removes the −35 region of the *ompC* promoter, and the whole of the *micF* gene.

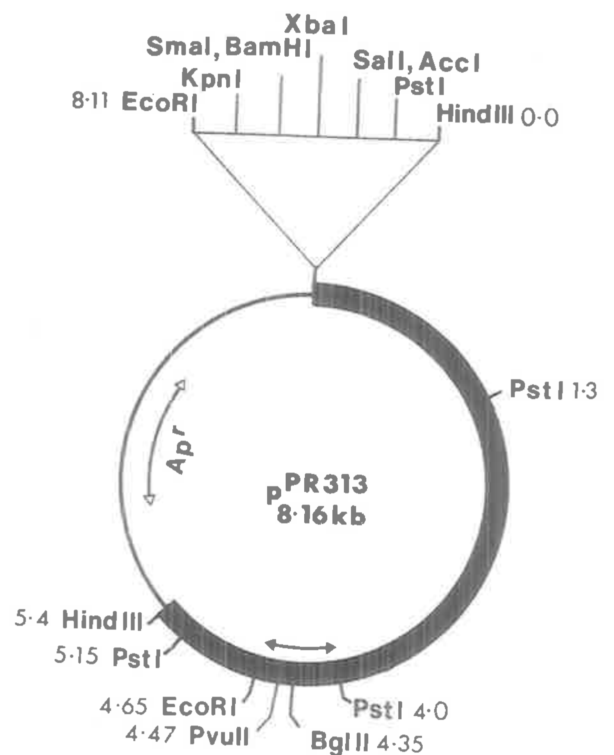

Fig. 3. A partial restriction map of plasmid pPR313, which carries the 5.4-kb "*Stc*" fragment (thick line) from P2718. A 650-bp *Eco*RI-*Pst*I fragment (⟷) was subcloned into M13 vectors for sequencing. The numbers are in kb.

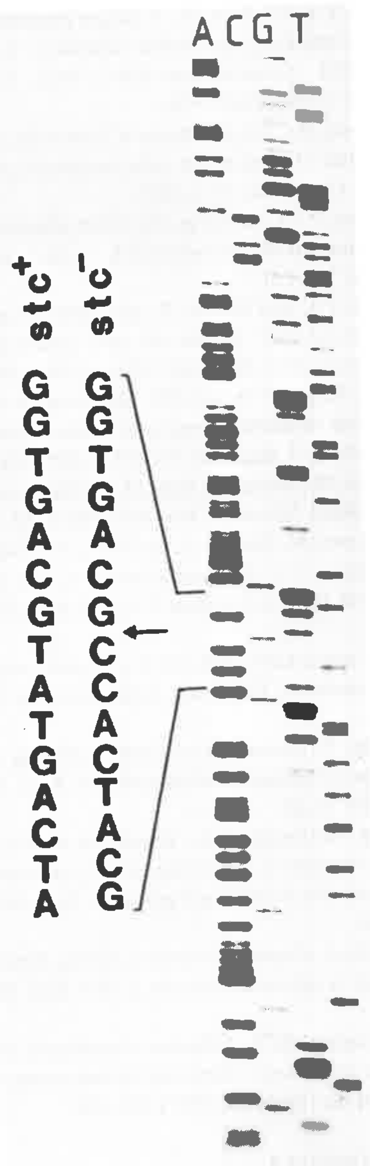

Fig. 4. Nucleotide sequence of a 650-bp *Eco*RI-*Pst*I DNA fragment from pPR313. Only the relevant portion of the sequence is shown. Arrow indicates start of the chromosomal deletion in the Stc⁻ strain. Nucleotide sequence of this region from the Stc⁺ strain was taken from Mizuno et al. (1983). Sequencing was performed essentially according to the method of Sanger et al. (1977).

(d) The nature of the Stc⁻ phenotype

In this paper we show that the Stc⁻ mutation studied previously (Morona and Reeves, 1982b) is a deletion which extends from within the *ompC* promoter region upstream of *ompC* for at least 2.8 kb, thus deleting the *micF* gene. Although the role of *micF* in *ompF* expression is not yet fully resolved, it seems that under some condition at least, *micF* RNA can inhibit *ompF* expression and we suggest that the effect of the Stc⁻ mutation on *ompF* expression in P2718 is due to deletion of the *micF* gene: for this reason we now use the "Stc" designation for the phenotype only and the effect of *tolC* mutation may also be in part at least mediated via the *micF* and *ompC* co-regulated genes.

However, as the Stc⁻ deletion extends at least 2.2 kb beyond *micF* and may clearly encompass other gene(s), shorter deletions will be necessary to establish unequivocally if the effect on *ompF* expression is solely due to the deletion of *micF*.

(e) Speculation on the nature of the *tolC* effect on *ompF* expression

If the Stc⁻ phenotype is due to *micF* deletion, it is reasonable to ask if the reduction in *ompF* expression in a *tolC* mutant is due to increased *micF* expression. This possibility is supported by the observation (R.M. and P.R., manuscript in preparation) that *tolC* mutants have increased *ompC* expression: if *ompC* and *micF* are co-regulated, as suggested by both Mizuno et al. (1984), and Schnaitman and McDonald (1984), such that there is indeed a similar increase in *micF* expression, then this could account for the decrease in OmpF protein in *tolC* mutants as high level *micF* expression certainly inhibits expression of *ompF* (Mizuno et al., 1984).

The sequence data presented in this paper show that the Stc⁻ mutation deleted a part of the *ompC* promoter, and we have not as yet been able to study the effect of deletion of *micF* in the presence of a normal *ompC* gene. We are currently investigating the cause of the higher expression of the *ompC* gene in *tolC* mutants to further elucidate the regulation of *ompC* and *ompF* gene expression and are reinvestigating the recombinants previously thought to be Stc⁻, *ompC*⁺ recombinants (Morona and Reeves, 1982b).

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APPENDIX

Material from this thesis has been published or submitted for publication in the following journals:

1. Hackett J., R. Misra and P. Reeves. 1983. The TolC protein of *Escherichia coli* K-12 is synthesised in a presursor form. *FEBS Lett*. 156 : 307-310.

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