COLICINOGENIC PLASMIDS AND INHIBITOR SENSITIVITY IN *ESCHERICHIA COLI*

A THESIS SUBMITTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY AT THE UNIVERSITY OF LONDON

BY

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UNIVERSITY COLLEGE LONDON

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ABSTRACT

The effects of Col plasmids on sensitivities of *E.coli* K-12 derivatives, especially 1829 and P678-54, to hydrophobic, hydrophilic and aminoglycoside antibiotics and to other inhibitors such as copper ions and Tris-EDTA have been examined. It was found that the colicinogenic plasmid ColVIK-94 sensitised the strains to hydrophobic antibiotics as well as to some of the hydrophilic and aminoglycoside antibiotics whilst Col BK-98 sensitised the strains to rifampicin and some of the aminoglycosides and hydrophilic antibiotics but not to most of the hydrophobic agents.

The various effects of the above mentioned antibiotics and inhibitors were also tested on strains carrying mutant derivatives of the Col VIK-94. This was to investigate which plasmid components were responsible for sensitivities particularly to rifampicin and novobiocin. It was shown that both mutant plasmids sensitise 1829 to these two agents. It seems that both transfer and colicin components were important for increased rifampicin sensitivity whereas transfer components were needed for novobiocin sensitivity.

The effects of divalent cations, namely magnesium and calcium ions were investigated and it was found that they reversed the

inhibitory effects of the hydrophobic and aminoglycoside antibiotics on strain 1829 bearing the Col VIK-94 but there was no effect on those strains bearing Col BK-98. This probably indicates that the divalent cations in some way stabilise the outer membrane preventing the entry of some antibiotics into the cell.

A prior growth temperature of 25° C seemed to reduce the sensitisation effects to hydrophobic antibiotics of the ColV plasmids without affecting the sensitivity of the parent strain 1829 whereas the effects of the hydrophilic and aminoglycoside antibiotics were unaffected by a 25° C prior growth temperature. Synthesis of colicin and transfer components are reduced at 25° C, hence the results are in accordance with the conclusion concerning the components involved in sensitivity especially with regards to rifampicin and novobiocin.

The Col V and Col B plasmids sensitised the strains 1829 and P678-54 to copper sulphate and to the effects of the Tris-EDTA. The sensitivity to copper ions is due to the uptake of the ions by the Omp F porin. The plasmid may affect porin function or open up another entry pathway. The effects of the plasmids on the Tris-EDTA sensitivity was probably due to the weakening of the LPS-LPS bonds by this combination of agents.

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INTRODUCTION

Section 1

MOLECULAR BASIS OF OUTER MEMBRANE PERMEABILITY IN GRAM NEGATIVE BACTERIA

THE CELL ENVELOPE OF GRAM NEGATIVE BACTERIA

The cell envelope consists of two membranes separated by a layer of peptidoglycan and the periplasmic space. In order to study the two membranes, several techniques have been devised to separate them (Miura and Mizushima, 1968; Osborn *et al*, 1972a; Schnaitman, 1970).

When cells of *E.coli* are fixed, stained with suitable metal salts sectioned and examined under the electron microscope, the cytoplasmic membrane can be readily identified by its usual 'sandwich appearance' which comprises of two electron dense layers separated by a lighter space. Above the cytoplasmic membrane, the envelope appears as a structure containing three electron dense layers separated by clear layers (De Petris, 1967; Murray *et al*, 1965). The clear layer above the cytoplasmic membrane is the

periplasmic space. The electron dense layer above the periplasmic space is the peptidoglycan layer. The outer regions of the Gram-negative cells, which form the outer membrane have been difficult to characterize.

The outer membrane acts as a passive barrier to substances greater than a molecular weight of 600-700 daltons (Leive, 1974; Nakae and Nikaido; 1975). The outer membrane allows bacteria to survive the emulsifying action of bile salts, the degradative action of enzymes in the gut and aids resistance to the host defence mechanisms, for example, phagocytosis by white blood cells and complement mediated bactericidal action of the serum.

The outer membrane components are lipopolysaccharides (LPS), phospholipids, lipoproteins and other outer membrane proteins. Some of the outer membrane proteins are important in solute transport, others act as receptors for colicins and bacteriophages (reviewed by Reeves, 1979) while others are involved in conjugation (Skurray et al, 1974). The LPS is found exclusively in the outer membrane. 60% of the phospholipids of E.coli are found in the outer membrane (Lugtenberg and Peters, 1976).

Below the outer membrane is the peptidoglycan layer which is attached to the outer membrane by covalent links. The peptidoglycan layer is the rigid layer that confers shape and stability to the Gram-negative cell (Osborn, 1969; Osborn et al,

1974). The periplasmic space that lies between the peptidoglycan layer and the cytoplasmic membrane contains numerous and various soluble binding proteins involved in transport and chemotaxis (Osborn, 1969; Osborn et al, 1974; Adler, 1975). In a model proposed by Hobot et al (1984), it was suggested that the peptidoglycan and the periplasmic space form the periplasmic gel. The gel consists of an outer half in which the peptidoglycan chain is heavily cross-linked. This cross linking decreases towards the inner part of the gel. This gel is viscous and has large spaces which contain periplasm.

The inner or the cytoplasmic membrane is the primary permeability barrier. It contains enzymes for oxidative phosphorylation, biosynthesis of macromolecules and active transport (Schnaitman, 1970; Bell et al, 1971; White et al, 1971; Osborn et al, 1972a; Osborn et al. 1974).

All the components of the outer membrane are synthesised on the cytoplasmic membrane or in the cytoplasm. They are subsequently translocated across the cytoplasmic membrane and the peptidoglycan and assembled into the outer membrane structure (Osborn et al, 1972a; 1974). See figure 1 for the arrangement of the cell envelope.

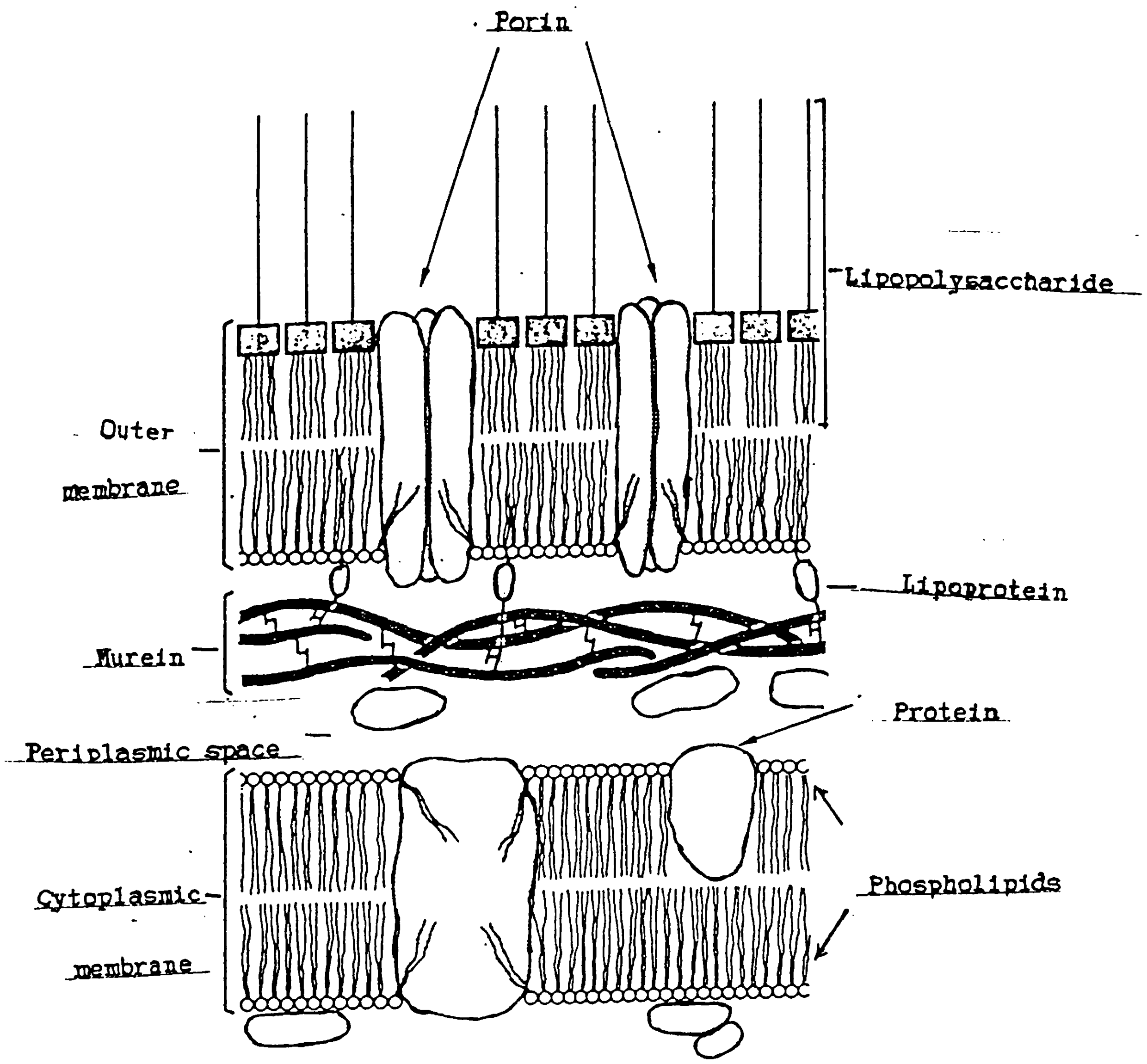


Fig.1 Model of the Gram-Negative cell envelope (Adapted from Schlegel,H.G. 1986.)

1.1.

LIPOPOLYSACCHARIDES

The structure of the LPS complex varies from strain to strain. The LPS molecule has three regions

(a) the Lipid A region

(b) the Core region

(c) the O-antigenic side chain

Figure 2 represents the LPS structure of S.typhimurium and corresponding chemotypes of mutants defective in various stages of core biosynthesis.

The LPS carries a net negative charge resulting in a strong negative surface charge on Gram-negative bacteria. The combination of this negative charge and divalent cross-bridging of LPS molecules provides the Gram-negative cells with many of their properties including resistance to hydrophobic antibiotics and detergents.

Structure of the Lipopolysaccharide (LPS)

1.1.1.

The O-antigen side chain

The O-antigen side chain is the serologically dominant part of the

←O-Antigen→ ←Core Region→

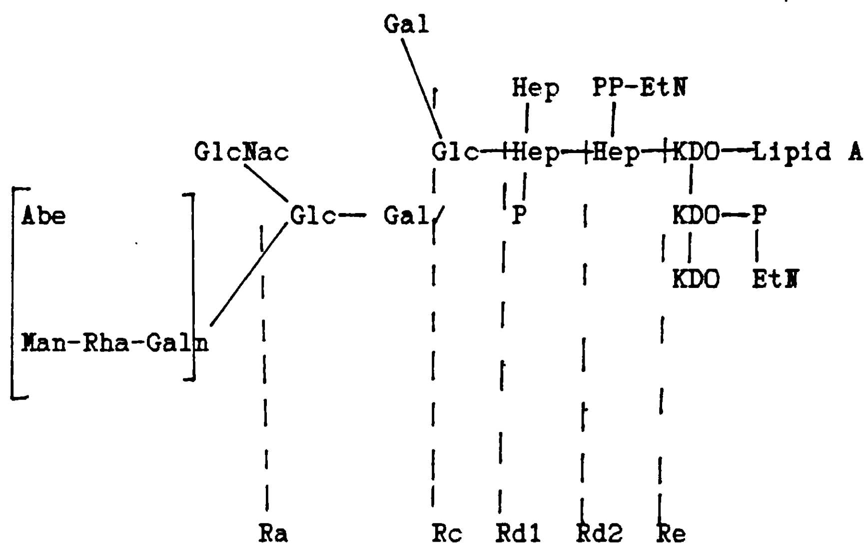


Fig.2 Structure of S.typhimurium lipopolysaccharide and some of its mutant chemotypes that are defective at various stages of core synthesis. These are indicated by the broken lines and associated letter designations. (From Rick, 1987).

LPS molecule. It consists of repeating oligosaccharide units, often containing rare sugars and it is in these sugars that the serological determinants reside. Individual repeat units may consist of linear trisaccharides or pentasaccharides or they may be polymers of branched oligosaccharides containing four to six sugars. The component monosaccharides include neutral sugars, amino sugars and in some cases rather unusual sugars such as 6-deoxyhexoses or 3, 6-dideoxyhexoses (Rick, 1987). In *S.typhimurium* the O-side chain consists of repeating units of the branched tetrasaccharide abequosyl

(Osborn *et al*, 1974).

The great degree of immunological diversity attributable to O antigens in many cases reflects pronounced structural differences. However, relatively minor differences in O-antigen structure can also be detected immunologically. In addition to the differences in O-antigen structure that are responsible for distinguishing between O serogroups, variations in the structure of the O side chains may also occur within a given O serogroup. Accordingly, the basic O-side chain repeat unit of a given strain may be structurally modified so that it is distinct from the parent strain.

In *S.typhimurium* the side chain is attached to the 4 position of the terminal glucose of the LPS core. The use of rough strains and

mutants defective in different stages of LPS biosynthesis has proved invaluable in the determination of the O antigen side chain structure. *E.coli* K-12 and its derivatives are rough organisms with no detectable O antigens (Orskov and Orskov, 1962). This is due to a defect in the *rfb* gene cluster that determines O-antigen specificity (Schmidt, 1973).

1.1.2.

The Core Region

The core is a complex oligosaccharide built from two unique sugars, L-glycero- D-mannoheptose and KDO (3-deoxy-D-manno-2-octulosonate). The core region of S.typhimurium has been found to consist in addition to KDO, of N-acetyl-glucosamine and D-glucose. The core also contains phosphate groups, O-pyrophosphorylethanolamine and O-phosphorylethanolamine. Recent studies of the KDO region has necessitated a reevaluation of the generally accepted structure of this region. It was originally proposed that the KDO residues of Salmonella and E.coli occur as a branched trisaccharide consisting of a single side chain of KDO and two residues in the main chain (Rick, 1987). Recent investigations of the KDO-Lipid A region of an Re mutant of E.coli by use of ^{13}C nuclear magnetic spectroscopy revealed the occurrence of only two KDO residues (Strain et al, 1983). Separate studies using chemical methods also revealed the occurrence of only two KDO residues in the LPS from Re mutants of Salmonella and E.coli (Brade et al, 1983 and Brade and Rietschel, 1984). Although the occurrence of a KDO disaccharide appears to be a structural characteristic of LPS from heptoseless mutants, a different structure has been found for the KDO from LPS with a complete core. Thus, evidence has been obtained which suggests the occurrence of a linear trisaccharide in the LPS of a Salmonella

Rb2 mutant (Brade *et al*, 1983 and Brade and Rietschel, 1984). The original and revised structures of the KDO region are given in diagramms A and B respectively.

A

αKDO- αKDO - Lipid A

|

α KDO

B

αKDO- Lipid A

|

αKDO

|

αKDO

1.1.3.

The Lipid A region

The Lipid A region consists of a β-1,6 linked glucosamine disaccharide molecule which is O-ac·,ylated by β-hydroxymyristic acid and saturated fatty acids predominantly C12 and C14 molecules with variable amounts of C16 molecules (see figure 3 for structure of the Lipid A). There are phosphate groups attached at the C-1 and C-4 positions of the disaccharide molecule of S.typhimurium. The glucosamine disaccharide is linked to the terminal KDO of the core region by a 6-hydroxyl ketosidic bond. The fatty acid chains of Lipid A along with those of the phospholipids align themselves to form the hydrophobic interior of the membrane. The outer membrane is as·ymetric with the LPS on the outer surface and phospholipids on the inner surface.

1.2.

Biosynthesis of Lipopolysaccharides

Advances in the biosynthesis of LPS have been made by studying mutants blocked at various points of the assembly sequence and by the use of specific inhibitors. Mutants were invaluable in the elucidation of the R-core and the O-antigenic side chains. The use of mutants was not possible in the elucidation of the Lipid A- KDO

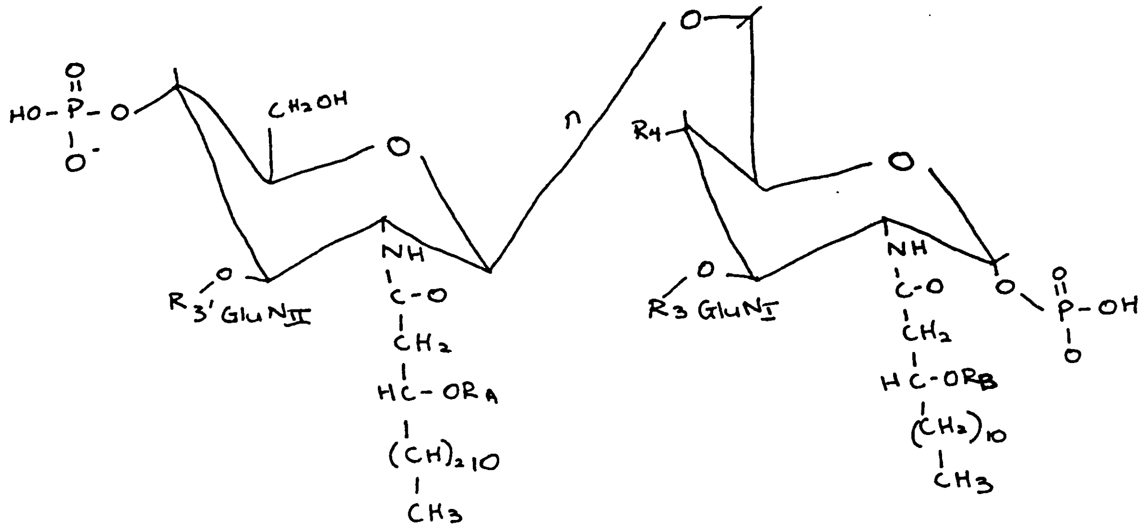


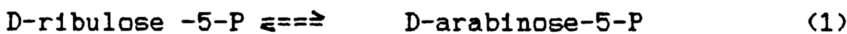
Fig.3. Structure of the glucosamine disaccharide backbone of the Lipid A region. R_A, R_B, R_3', R_3, R_4: fatty acid chains

region as this structure was essential for the structural and functional integrity of the cells. However, recently temperature sensitive mutants altered in Lipid A and the inner core have been obtained. The biosynthesis of the LPS is peculiar in that the core region is synthesised by the stepwise extension of the Lipid A while the O-antigen side chains are synthesised independently.

1.2.1.

Biosynthesis of Lipid A

Mutants of S.typhimurium defective in KDO synthesis have been used to provide an insight into the biosynthesis of Lipid A (Rick and Osborn, 1977; Lehmann et al., 1977). Its biosynthesis involves three steps


D-ribulose -5-P $\rightleftharpoons$ D-arabinose-5-P (1)

D-arabinose-5-P+P-enolpyruvate ----> KDO-8-P+Pi (2)

KDO-8-P ----> KDO+Pi (3)

The first reaction is catalysed by D-ribulose-5-P isomerase. The second reaction is catalysed by KDO-8-phosphate synthetase whilst the third reaction is catalysed by KDO-8-phosphatase (Lim and Cohen, 1966; Levine and Rackner, 1959; Ghalambor et al, 1966). The free KDO generated in (3) is converted to the nucleotide sugar CMP-KDO (Ghalambor and Heath, 1966) which serves as a donor of KDO in LPS biosynthesis. Recently, the existence of cytoplasmic KDO transferase in E.coli has been demonstrated (Brozek etal, 1989).

These transferases are capable of attaching 2 KDO residues to a Lipid A precursor. It has been shown that KDO is transferred to the N,O-β hydroxymyristoyl glucosamine disaccharide unit of Lipid A before the addition of the ester linked saturated fatty acids of a complete molecule (Munson et al, 1978; Rick et al , 1977; Walenga and Osborn, 1980).

Nishijima and Raetz (1979) have observed that certain phosphatidylglycerol deficient temperature sensitive mutants of E.coli accumulated two novel glycolipids, X and Y at 42°C. Comparison of X to the mature Lipid A provided the first indication that it might be a precursor of the reducing end of the molecule (Bulawa and Raetz, 1984; Ray et al, 1984). Ray et al (1984) searched for an enzyme in extracts of wild type E.coli catalysing the condensation of UDP 2,3 diacyl glucosamine with Lipid X and found that this reaction occurred efficiently in the cytoplasmic membrane fraction. The product is tetraacyldisaccharide -1-P. The disaccharide synthetase was found deficient in mutants harbouring lesions in the pgs B gene. Anderson et al (1985) have suggested the existence of a novel set of fatty acyltransferases in extracts of E.coli that attach two R-3-hydroxymyristoyl moieties to UDP-GLcNAc. Anderson and Raetz (1987) described an assay of the first enzyme in this pathway which catalyses the reaction : UDP-GLcNAc+R-3-hydroxymyristoyl-acyl carrier protein --> UDP-3-O-(R-3-hydroxymyristoyl)-GLcNAc+ acyl carrier protein. Anderson et al (1988) have also described

a novel enzyme capable of deacetylating UDP-3-O-(R-3-hydroxymyristoyl)-GLcNAc to form UDP-3-O(R-3-hydroxymyristoyl)glucosamine. A kinase has been demonstrated in extracts of wild-type E.coli (Ray and Raetz, 1985) that catalyses the phosphorylation of the 4-OH of the tetraacyldisaccharide -1-P. This enzyme functions best with ATP but also utilises GTP, CTP, UTP in the crude systems used. The final stages of Lipid A biosynthesis have not been studied extensively. It was first demonstrated by Munson et al (1978) that the presence of membrane bound enzymes were capable of adding at least two KDO units to the tetraacylsaccharide -1,4 biphosphate precursor. It was thought that two KDO transferases may be involved. Recently,Brozek et al, (1989) demonstrated the existence of cytoplasmic KDO transferases in E.coli capable of attaching 2 KDO residues from CMP-KDO to a Lipid A precursor. Figure 4 demonstrates the pathway for Lipid A biosynthesis.

1.2.2.

Biosynthesis of the core region

The pathway of the biosynthesis of the outer core region of the LPS of S.typhimurium is given in figure 5. It has been shown that the synthesis of the branched pentasaccharide of the outer core is performed by a series of glycosyl transferase reactions in which a single sugar residue is transferred from the appropriate

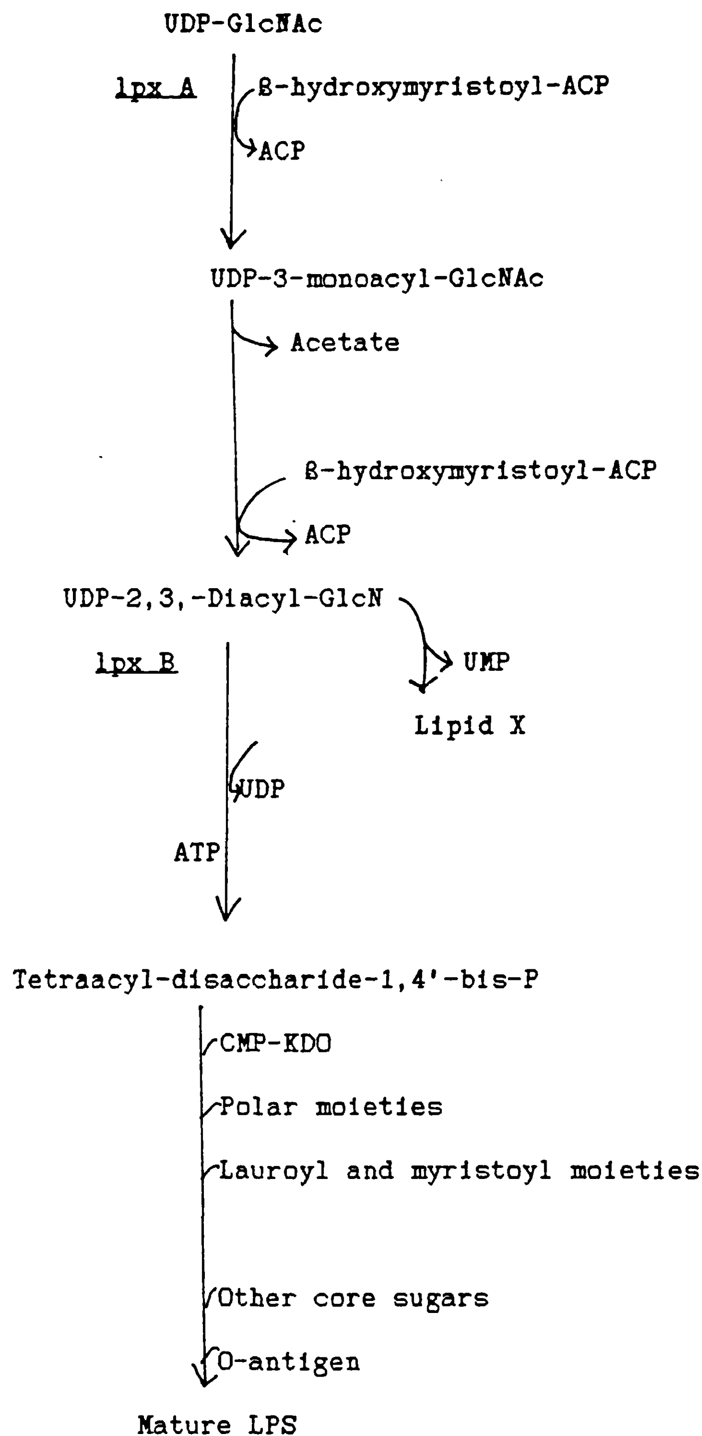


Fig. 4. Postulated pathway for the biosynthesis of Lipid A in E.coli. Synthesis is initiated by the acylation of the known metabolite, UDP-GlcNAc. (Modified from Anderson,M.S. and Raetz,C.R.H.,1987).

nucleotide sugar donor to the non-reducing terminus of the LPS acceptor (Osborn et al 1962; Osborn and D'Ari, 1964; Rothfield et al, 1964; Rosen et al, 1964). The enzymes responsible are primarily located in the cell envelope fractions.

Studies by Romeo et al (1970) and Hinkley et al (1972) have shown that the transferase enzymes are dependent on the phospholipids for activity. The active lipid species is phosphatidylethanolamine which interacts with LPS to form a mixed bilayer or monolayer essential for the binding of enzyme proteins to its LPS structure.

A tentative model for the biosynthesis of the core had been proposed by Rothfield and Romeo (1971). The model proposes that addition of sugars to the nascent core region occurs sequentially and is catalysed by a series of membrane associated glycosyltransferases. The LPS molecules are integrated into the inner leaflet of the cytoplasmic membrane such that the fatty acyl chains of lipid A are dissolved in the hydrocarbon interior. The polar groups of the lipid A disaccharide are aligned with the polar head groups of the phospholipids and the nascent polysaccharide chains oriented towards the cytoplasm or the interior. The fluid nature of the hydrocarbon interior allows mobility or lateral movement of LPS in the plane of the membrane. In contrast, the glycosyltransferases are envisioned as being considerably less mobile and they remain in a relatively fixed position. According to this model, lateral movement of LPS occurs

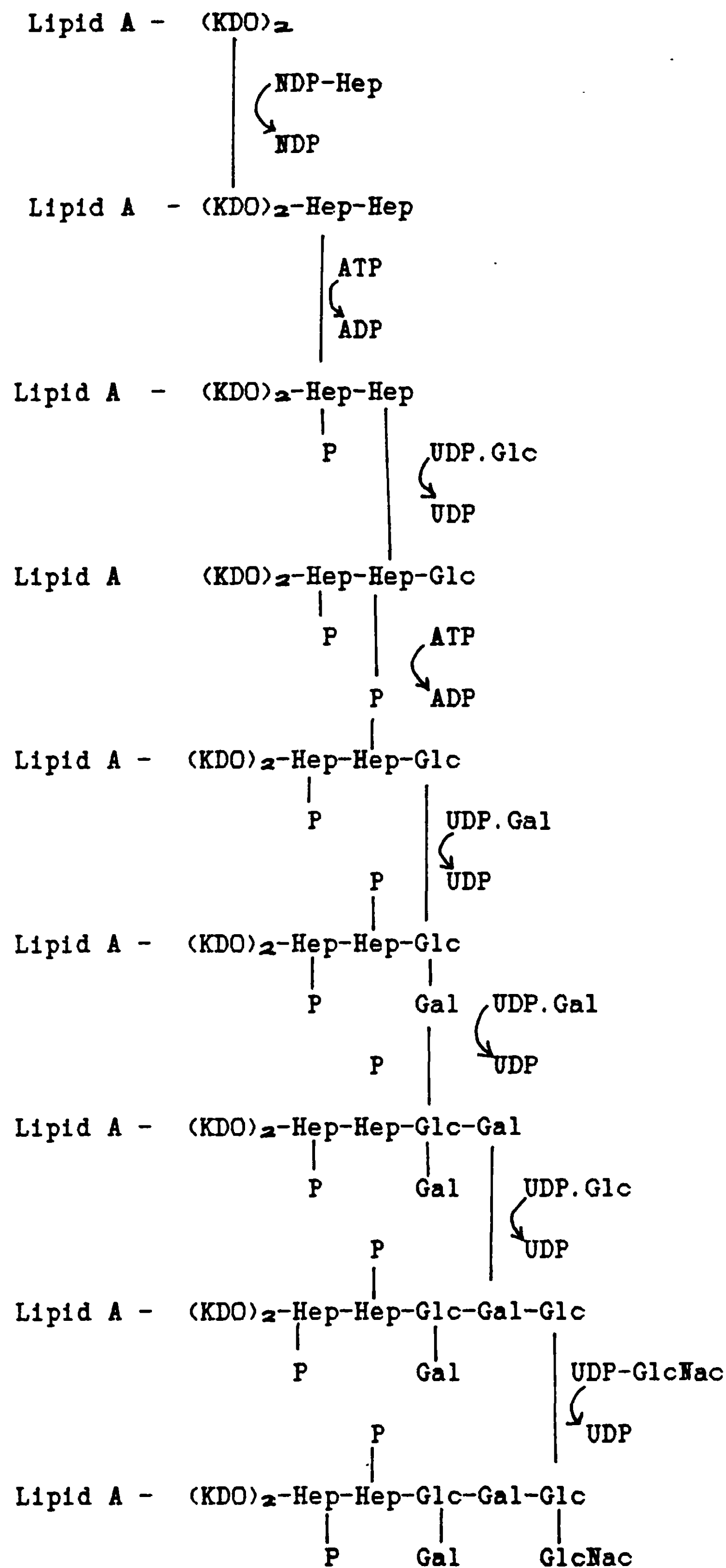


Fig 5: Biosynthesis of the core region of Lipopolysaccharide in <u>Salmonella</u> species. (From Bacterial Outer Membranes, Inouye).

until a specific complex is formed among the LPS, the phospholipid and the glycosyltransferase for which the acceptor is the substrate. After the transfer of the sugar the affinity of the enzyme for the LPS is decreased and the LPS becomes a substrate for the next glycosyltransferase. As the LPS molecule moves from one glycosyltransferase to the next, the core becomes elongated and the previous glycosyltransferase in the sequence is made available for the next molecule of acceptor.

Mutants of S.typhimurium defective in several core glycosyltransferases, as well as other enzymes involved in core synthesis are members of a group designated rough A or rfa genes have been mapped in a small region of the chromosome of S.typhimurium between cysE and pyrE; the remainder are located outside this region (Rick, 1987).

1.2.3.

Biosynthesis of the O-antigen side chain

This is mediated by a series of cytoplasmic membrane bound enzymes. The sugar units that make up the O-antigens are transferred from sugar nucleotide diphosphates to the C_{55} polyisoprenoid carrier. The first reaction is a transphosphorylation reaction where galactose-1-phosphate is transferred from UDP-galactose to produce carrier-lipid-P-P-galactose and UMP. The second stage is the transfer of rhamnose

from the sugar nucleotide TDP-rhamnose to the carrier-lipid-P-P-Gal to produce carrier-lipid-P-P-Gal-Rha. The third stage is the transfer of mannose from GDP-mannose to give carrier-lipid-P-P-Gal-Rha-Man. With S.typhimurium there is a transfer of abequose from CDP-abequose to give carrier-lipid-Gal-Rha-Man-Abe (Osborn and Weiner, 1968). The abequose residue is incorporated into the lipid linked oligosaccharide before polymerisation. The completed O-side chain attached to the lipid carrier appears to be freely mobile in the outer leaflet of the cytoplasmic membrane and will come into close association with the Lipid A core complex. The transfer of the O-side chain polysaccharide from the lipid carrier to the glucose 2 of the core is catalysed by LPS ligase. A long chain polymeric intermediate is synthesised while remaining linked at its reducing terminal to the lipid carrier. After each transfer the P-P-lipid carrier is liberated and the active monoester co-enzyme is regenerated by a pyrophosphatase. Figure 6 illustrates the pathway for O antigen biosynthesis.

Most of the genes required for the synthesis of the O side chain specific components are located in the his linked rfb cluster located at 44 to 45 min. on the Salmonella chromosomal map (Schmidt, 1973). This region includes information for nine of the enzymes involved in the synthesis of GDP-mannose, TDP-rhamnose and CDP-abequose as well as for the four glycosyltransferases required for the synthesis of a single O repeat unit. The rfc gene located near trp at 32 min. is believed to be the structural gene for the

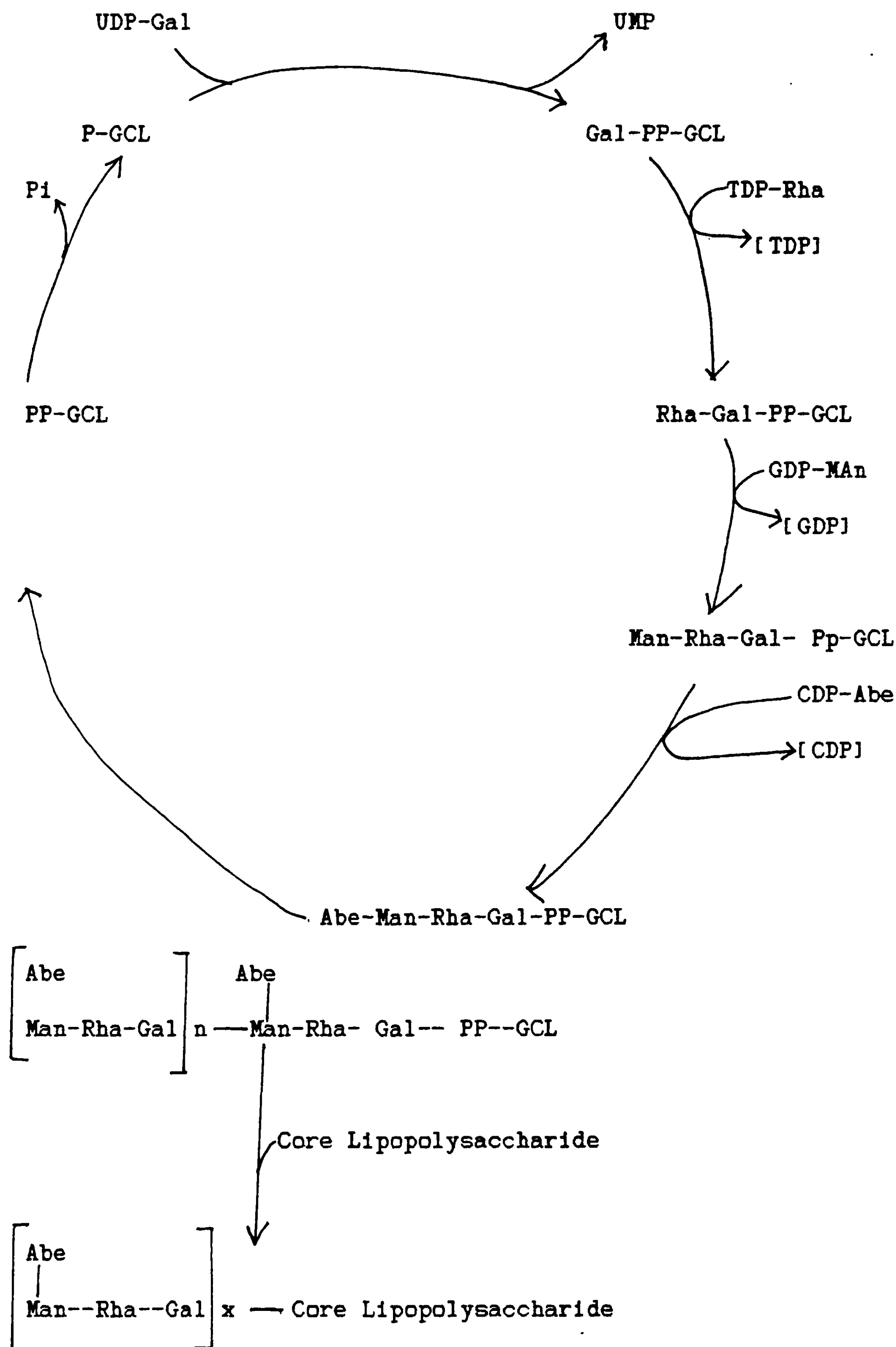


Fig. 6 Pathway of biosynthesis of O-antigen in S.typhimurium (From Osborn et al , 1974).

O-antigen polymerase (Rick, 1987). Recently, the entire rfb gene cluster from S.typhimurium LT2 was cloned (Brahmabatt et al, 1988).

1.3.

Translocation and assembly of Lipopolysaccharide

LPS molecules pulse labelled in vivo appeared initially in the cytoplasmic membrane but were rapidly transferred to the outer membrane during a subsequent chase (Osborn et al, 1972b). The enzymes of the O side chain biosynthesis were shown to be entirely in the cytoplasmic membrane and the enzymes that synthesise the core region were probably in the same location, though these tended to redistribute during fractionation (Osborn et al, 1972a). From these experiments Osborn et al (1972 a) concluded that the LPS was synthesised on the inner membrane and translocated to the outer membrane. The overall process of LPS translocation is unidirectional i.e. the LPS molecules that are integrated into the outer membrane are no longer accessible to the inner membrane enzymes (Osborn et al, 1972a,b). Muhlradt et al (1973) have shown that newly synthesised LPS is inserted at sites distributed all over the cell surface. They proposed that LPS is translocated at limited regions of contact between the two membranes, known as Bayer's junctions. Once they have emerged from these sites of adhesion, the LPS molecules are distributed over the entire surface of the cell with preferential localisation on the outside

face of the outer membrane (Kulpa and Leive, 1976; Bayer, 1975; Muhlradt et al, 1973; 1974; Funahara and Nikaido, 1980).

However, the detailed molecular mechanisms of translocation and integration of LPS are still poorly understood. As the carbohydrate portion must for biosynthesis reasons face inwards into the cytoplasm and then outwards after translocation, the translocation may require a transmembrane movement (flip-flop) as well as an intermediate transfer.

Neither completion of the core nor the O side chain is a necessary prerequisite for translocation and integration in the outer membrane since such translocation occurs in a variety of mutants including Re mutants. The KDO-deficient Lipid A precursors accumulated in the mutants are however only incorporated very slowly into the outer membrane (Osborn et al, 1980) providing strong evidence that the structural features of the LPS are indeed important for translocation (Osborn, 1979).

The LPS molecules inserted into the outer membrane are known to diffuse laterally within the membrane, from the insertion loci to cover the entire surface. This diffusion appears dependent on the structure of the LPS.

The LPS molecules interact covalently with major outer membrane proteins (Van Alpen et al, 1977; Schindler and Rosenbusch, 1978

and see section 2) . It is quite likely that these interactions account for the functional irreversibility of LPS translocation (Osborn _et al_, 1972b; Muhlradt _et al_, 1973).

Being complexed with outer membrane proteins is not however required for translocation since synthesis and integration of the LPS into the outer membrane occurs for several hours even in the absence of protein synthesis (Knox _et al_, 1967; Rothfield and Pearlman ~~and~~ Kothencz, 1969) and translocation rate is unaffected under these conditions (Osborn, 1979).

1.4.

The role of lipopolysaccharides

The LPS form a unique class of macromolecules representative of Gram-negative bacteria. Associated with protein, they are located in the outer leaflet of the outer membrane in the Gram-negative cell (Nikaido and Nakae, 1979). In this exposed position on the cell surface, LPS are involved in the interaction of the cell with the environment. Thus contact with the immune system leads to stimulation of specific antibodies directed predominantly against determinant structures of the LPS. Hence, LPS molecules represent the main surface antigens of Gram-negative bacteria.

A synonymously used term for LPS is endotoxin. The injection of

LPS containing bacteria or of purified LPS into experimental animals causes a wide range of endotoxic reactions. These include fever, changes in white cell counts, shock and death (Kadis et al, 1971; Kass and Wolff, 1973; Schlessinger, 1977).

LPS have the ability to activate and suppress lymphocytes (Koenig and Hoffman, 1979). They stimulate polyclonal B lymphocytes to differentiation, proliferation and secretion of immunoglobulins. LPS is also the most potent tumour necrosis factor (TNF) (Old, 1987). The endotoxic principle of LPS is the Lipid A region. Free Lipid A, obtained by mild acid hydrolysis and rendered water soluble by electrodialysis and subsequent neutralisation was shown to exhibit all the endotoxic reactions (Galanos et al, 1977a;b). Various Lipid A analogues have been chemically synthesised and one of them GLA-27 has been shown to play a part in the expression of mediator inducing and B-cell activation activity (Matsuura et al, 1986). It was shown that the separate expression of the various biological activities of Lipid A was through modification of its chemical structure.

The fundamental importance of the polysaccharides as recognition molecules rests in their property of conformational and structural diversity. LPS molecules are composed of a variety of sugar moieties that are linked in different ways. Inserted assymetrically into the outer membrane with the carbohydrate portions facing outwards, the LPS provide ideal recognition

structures for phages and antibodies. An important function of the cell surface carbohydrate is to increase the hydrophilicity of the cell surface. This is provided by the O antigen side chain. This increased hydrophilicity is crucial in enabling the cell to escape phagocytosis (Cunningham et al. 1975). The Salmonella species that contain O-antigen are also more resistant to complement than isogenic strains lacking O- antigen (Grossman et al, 1986). The protection afforded by O-antigen is due to steric hindrance of the C5,6-9 complement component affecting its access to the hydrophobic domains of the outer membrane.

The structure of the O-antigen side chain often shows tremendous diversity. Since attachment of specific antibodies to a bacterial surface enhances phagocytosis by promoting attachment to the Fc and C'3 receptors on the phagocyte surface (Silverstein et al, 1977) and by decreasing the hydrophilicity of the bacterial surface (Van Oss and Gilman, 1972), the appearance of a new surface structure to which the host has not been exposed will give a significant selective advantage to pathogenic bacteria.

LPS often acts as receptor sites for bacteriophages. Viruses requiring the short ("rough") LPS phenotype for adsorption include T3, T4, T7 and χ174 (Inouye, 1979). Bacteriophage T4 attaches to a glucosamine disaccharide linked to the heptose of the core region of the LPS. Removal of the terminal glucose leads to a 10%-50% reduction of receptor activity (Prehm et al, 1976). Considerably

longer LPS chains found in typical smooth strains do not permit rough specific phages to adsorb. The Salmonella phages such as ϵ15 and ϵ34 have a requirement for the smooth type of O-antigen. Without this activity, the phage would not be able to engage in close contact with the deeper region of the outer membrane and with membrane adhesion site. Phage ϵ15 adsorbs to the rhamnose-galactose linkage (Uetake and Hagewara, 1969) in the O antigen only when an α-linkage between repeat units is present. Phage ϵ34 adsorbs to the β-linkage between the Salmonella O antigen subunits only when the galactose is not substituted.

Another important role of the LPS lies in the fact that the outer membrane is a passive barrier to various hydrophobic compounds (Nikaido and Nakae 1979). This role is further discussed in section 2.

1.5

Membrane Phospholipids

The cell envelope lipids of Gram-negative bacteria consist of Lipid A, phospholipids and lipoproteins. The phospholipids, all of which are membrane bound are the major fatty acid containing structures in Gram-negative bacteria. Their composition in the outer membrane and the cytoplasmic membrane are the same. 60% of the phospholipids are present in the outer membrane. Table 1 shows the various fatty acids in E.coli.

The membranes of E.coli K-12 cells normally contain three major phospholipids. They are phosphatidylethanolamine (about 70% of total extractable phospholipid), phosphatidylglycerol (about 25%) and cardiolipin (about5%) (Shibuya et al, 1985). All these molecules have similar fatty acid compositions consisting mainly of palmitic, myristic, palmitoleic and cis-vaccenic acids. Phosphatidylethanolamine is the major phospholipid. The relative amounts of the phosphatidylglycerol and cardiolipin depends on the growth phase of the cells, as do the relative amounts of the palmitoleic and cis-vaccenic acyl groups and their cyclopropane derivatives (Cronan, 1979). The phospholipids of S.typhimurium are essentially identical to those of E.coli (Ames, 1968).

The outer membrane has a higher proportion of phosphatidylethanol

-amine than the cytoplasmic membrane and is deficient in phosphatidylglycerol and cardiolipin relative to the cytoplasmic membrane (Osborn et al, 1972a; Lugtenberg and Peters, 1976; Jones and Osborn, 1977).

1.6.

Physical properties of membrane phospholipids

1.6.1.

Phase transitions

Phase transition is the temperature dependent change of the acyl moieties of membrane phospholipids from a solid state to a fluid state. Another term used is the order-disorder transition (Steim et al,,1969; Ladbrooke and Chapman, 1969; Engleman, 1970; Schechter and Letellier, 1974). At temperatures below phase transition the fatty acyl chains are in a tightly packed hexagonal form. When transition is complete, the fatty acyl chains are in a fluid state.

The temperature at the mid-point of the transition is the transition temperature, TE, while the temperature range between the initiation and completion of the phase transition is δt. These are both dependent on the fatty acyl content and polar head group composition of the phospholipids (Cronan and Gelman, 1975). This order-disorder transition can be investigated by several techniques such as X-ray diffraction, differential scanning

calorimetry, fluorescence probes and electron spin labelling techniques (Cronan and Gelman, 1975). Raman scattering has also been used to study phase transition in several unsaturated mixed-chain, and unsaturated symmetric phospholipids (Chen and Ho, 1985). Overath *et al*, (1975) revealed order-disorder transitions in both the outer and cytoplasmic membrane. The mid-transition temperatures and the range of transition are similar in both the outer and inner membranes. They showed that 60-80% of the hydrocarbon chains take part in transition in the cytoplasmic membrane whereas in the outer membrane only 25-40% become ordered.

Lipids bound to protein are called boundary lipids. Cronan (1979) argued that the boundary lipids make the phospholipids of the outer membrane more fluid than the cytoplasmic membrane; thus the fluidising effects of the high protein content of the outer membrane would prevent a sizeable fraction of the lipid from becoming ordered at low temperatures, so less phospholipid undergoes order-disorder transition. There are others who say that the phospholipids in the outer membrane are less fluid than those in the cytoplasmic membrane (Rottem and Leive, 1977; Cheng *et al*, 1974).

1.6.2.

Lateral diffusion

The movement of phospholipids in the plane of the membrane is

rapid. Sackmann et al. (1973) found a coefficient of lateral diffusion of 3.2×10^{-8} cm^2/sec in E.coli vesicles using a spin labelled fatty acid.

Dupont et al (1972), using an X-ray technique capable of kinetic resolution, reported that the time required for the phospholipid molecules of Kaback vesicles to complete order-disorder transition was about 10 minutes. Based on these data, the authors suggested that the diffusion of lipids in E.coli membranes is considerably slower than diffusion rate in pure lipid bilayers. However more recent data (Caron et al.1974; Mateu et al. 1978) suggests that a major portion of the time needed for completion of the disorder-order transition is consumed by nucleation rather than by diffusion and thus the conflict between the results of Dupont et al (1972) and the spin label results is probably only apparent. In conclusion, the diffusion rates of the phospholipids of both outer and inner membranes are very rapid and are typical of the fluidity in other biological and in artifical membranes.

Table 1

Structural type	General structural	Trivial name
Saturated	$CH_3-(CH_2)_x-COOH$	Lauric acid Myristic acid Palmitic acid Stearic acid
Unsaturated	$CH_3-(CH_2)_5\overset{H}{C}=\overset{H}{C}(CH_2)_x-COOH$	Palmitoleic acid cis-vaccenic acid
Cyclopropane	$CH_3-(CH_2)_5-\underset{H}{C}-\underset{H}{C}-(CH_2)_x-COOH$ (with CH_2 bridging the two C atoms)	None Lactobacillic acid
Hydroxy	$CH_3-(CH_2)_x-CHOH-CH_2-COOH$	β-hydroxymyristic acid

Structures and nomenclature of fatty acids of E.coli. All of the above fatty acids are components of the phospholipids of E.coli except β-hydroxymyristic acid which is only found in the Lipid A component of LPS (Cronan, 1979).

1.7

Membrane lipid biosynthesis

The phospholipid synthetic pathways can be considered the convergence of two pathways: fatty acid synthesis and a single step pathway for the synthesis of sn-glycerol-3-phosphate (G3P).

1.7.1.

Fatty acid biosynthesis

A soluble system of enzymes catalyses the synthesis of fatty acids in *E.coli* (Bloch and Vance, 1977; Volpe and Vagelos, 1976). A specific hydroxydecanoylacyl carrier protein dehydrase catalyses a key reaction at the point at which the biosynthesis of unsaturated fatty acids diverges from the saturated fatty acids (Raetz, 1978) (see figure 7). The dehydrase is defective in mutants designated *fab A* which consequently require unsaturated fatty acids for survival (Cronan *et al*, 1969; 1972). Other fatty acid auxotrophy in *E.coli* is due to mutations at the *fab B* and *fab F* loci; *fab F* strains have been shown to lack the β-ketoacyl acyl carrier protein (β-ketoacyl-ACP) synthetase II (Garwin and Cronan, 1978) and the *fab B* mutants lack β-ketoacyl-ACP-syhthetase I (Rosenfield *et al*, 1973). These enzymes catalyse the condensation reactions of the saturated fatty acid synthesis; β-ketoacyl-ACP synthetase I is needed for the synthesis of unsaturated fatty

acids from the cis-3-decanoyl precursor to the palmitoleic acid, whereas ß-ketoacyl synthetase 11 is required only for the elongation of the palmitoleate to the cis-vaccenic acid (Cronan, 1979).

1.7.2.

Phospholipid biosynthesis

The synthesis of phosphatidic acid is the first step in the formation of all the phospholipids of *E.coli*. G3P acyltransferase catalyses the first acylation of position 1 of G3P (Cronan and Rock, 1987). The second fatty acid is added by another enzyme(s), 1-acyl-G3P acyltransferase to form phosphatidic acid (Rock and Cronan, 1982). Phosphatidic acid composes only about 0.1% of the total phospholipid in *E.coli* and turns over rapidly and hence is an intermediate in phospholipid synthesis (Ganong *et al*, 1980). *In vitro*, the acylation of G3P to phosphatidic acid proceeds readily with either acyl-CoA or acyl-ACP donors (Cronan and Rock, 1987). This raises the question whether or not both these molecules are acyl donors *in vivo*. Recent work has shown that endogenously synthesised fatty acids are transferred to G3P as acyl ACPs since blocking phospholipid synthesis *in vivo* either through deprivation of a *plsB* or a *gpsA* strain for G3P results in accumulation of acyl-ACP molecules (Rock and Jackowski, 1982). However, the major pathway for incorporation of exogenously supplied fatty acids into phospholipids seems to be via acyl-CoA. Long-chain fatty acids are converted to CoA ester concomitant with transport and are incorporated into phospholipids even in

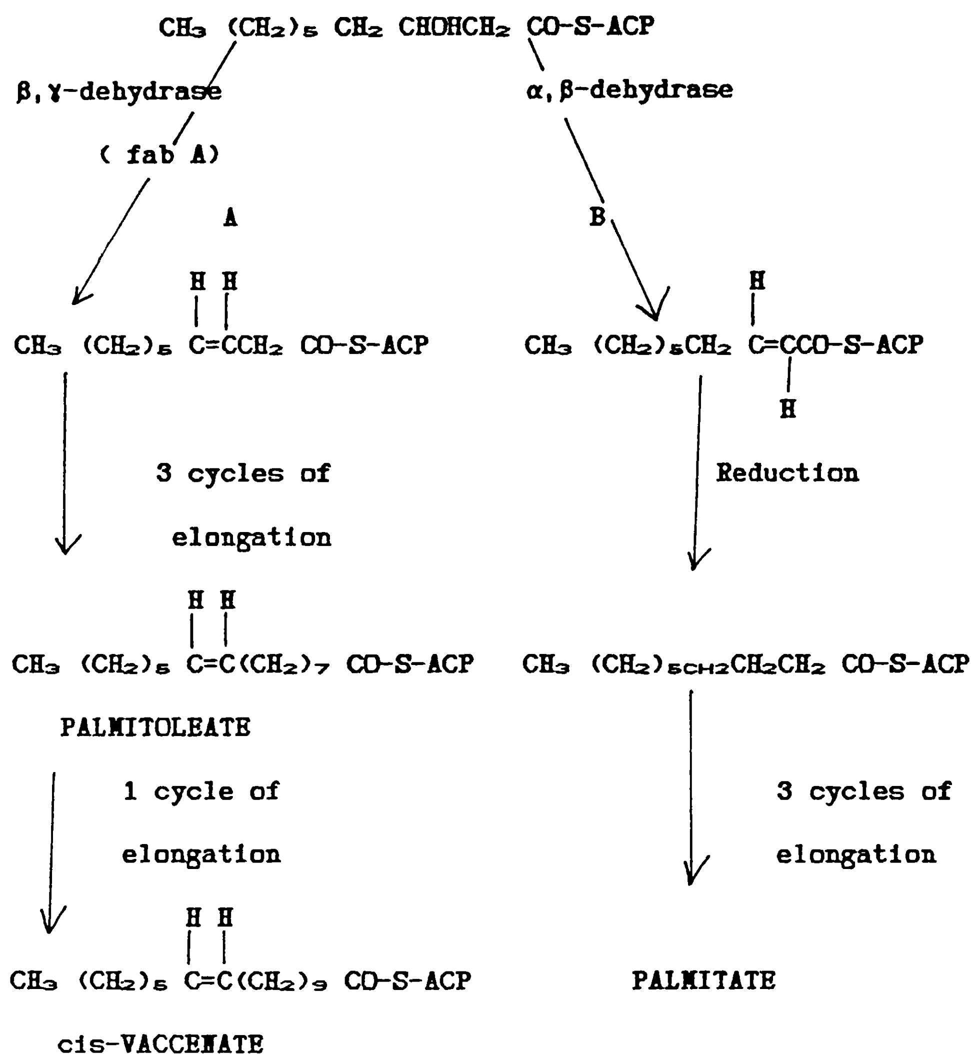


Fig.7. Fatty acid biosynthesis in E.coli. The dehydrase is situated at a metabolic branch point leading to the singly unsaturated palmitoleic and cis-vaccenic acids (Reaction A), or to fully saturated palmitic acid (Reaction B). (Modified from Raetz, 1978; Cronan and Vagelos, 1972).

cells depleted (>90%) of ACP (by use of an *acpS* mutant) (Cronan, 1984). Thus, the acyl donor probably depends on the source of the fatty acids. Next the phosphatidic acid is converted to a mixture of CDP-diglyceride and dCDP-diglyceride. This is catalysed by CDP-diglyceride synthetase (Ganong *et al*, 1980; Ganong and Raetz, 1983b). The CDP-diglyceride generated by this enzyme can react with serine to form phosphatidylserine or with G3P to form phosphatidylglycerolphosphate (PGP). The presence of ribo or deoxyribo forms of the liponucleotide could play a role in the determination of the relative rates of PS and PGP synthesis. The ratio of ribo to deoxyribo species is 3:1 (Ganong and Raetz, 1983b; Tuniatis and Cronan, 1973), a ratio similar to the relative rates of PS and PGP synthesis. However, *in vitro*, PS synthetase can favor either the ribo substrate or the deoxyribo substrate depending on the experimental conditions (Raetz,1978). Ganong and Raetz (1983b) report that a threefold change in the ratios of the ribo to deoxyribo species has no effect on the ratio of PE to PG synthesis.

The first step in the formation of phosphatidylethanolamine (PE) is the formation of PS by PS synthetase. The second step in the formation of PE is the decarboxylation of PS catalysed by PS decarboxylase (Cronan and Rock, 1987). The enzymes of PG synthesis, PGP synthetase and PGP phosphatase were readily demonstrated *in vitro* (Raetz, 1978). However, mutants deficient in the synthesis PGP and PG enzymes have only been recently

obtained (Icho and Raetz, 1983; Nishijima and Raetz, 1979). The dephosphorylation of PGP is catalysed by either of two enzymes, one of which seems to act as a phosphatidic acid phosphatase (Icho and Raetz, 1983). Figure 8 illustrates the synthesis of phospholipids.

Figure 8. Structures and biosynthesis of the phospholipids of *E. coli*

R1 and R2 denote the fatty acyl moieties of the phospholipids, R1 generally being palmitic acid and R2 being an unsaturated fatty acid or a cyclopropane fatty acid. The vertical line denotes the glycerol moiety. The biosynthesis is catalysed by the following enzymes: (1) sn-glycerol 3-phosphate and monoacylglycerol 3-phosphate acyltransferases (2) CTP-diglyceride synthetase (3) phosphatidylserine synthetase (4) phosphatidyl serine decarboxylase (5) phosphatidylglycerolphosphate synthetase (6) phosphatidylglycerol phosphate phosphatases (7) cardiolipin synthetase (catalyses the conversion of two phosphatidylglycerol molecules to cardiolipin plus glycerol). (Cronan, 1979).

GP-glycerophosphate

PA-phosphatidic acid

CDP-dGGP-CDP-diglyceride glycerolphosphate

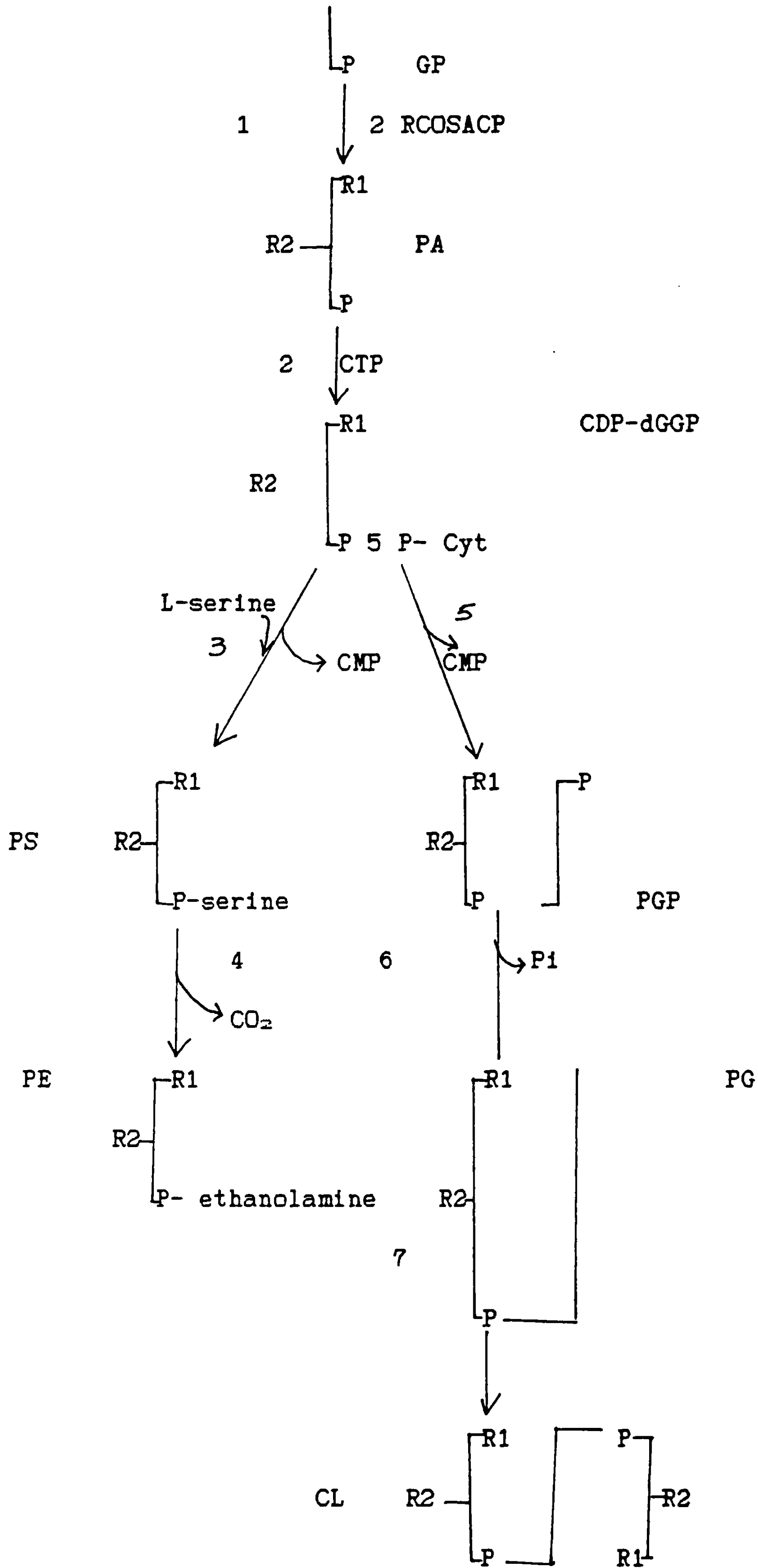
PGP- phosphatidylglycerophosphate

PG-phosphatidylglycerol

CL-cardiolipin

PE-phosphatidylethanolamine

PS-phosphatidylserine


GP
1
2 RCOSACP
R1
R2
PA
P
2
CTP
R1
CDP-dGGP
R2
P 5 P- Cyt
L-serine
3
CMP
5
CMP
R1
PS
R2
P-serine
R1
P
R2
P
PGP
4
6
Pi
CO_2
PE
R1
R2
P- ethanolamine
R1
PG
R2
7
P
R1
P
CL
R2
R2
P
R1

Regulation of phospholipid synthesis

The mechanisms which regulate the biosynthesis of membrane phospholipids of *E.coli* are not well understood. It seems that the rate of phospholipid synthesis is determined by the rate of fatty acid synthesis, since wild-type *E.coli* cells have small pools of free fatty acids or other precursors of phospholipid acyl groups. Jackowski and Rock (1983) have shown that after inhibition of phospholipid synthesis, acyl-compounds accumulate. These data were obtained by G3P starvation of a *plsB* or *gpsA* pantothenate auxotroph that had been prelabelled with a radioactive pantothenate precursor. Mindich (1972) first reported that fatty acid synthesis was completely inhibited when phospholipid synthesis was blocked in a *gpsA* strain. Starvation of glycerol auxotrophs, such as mutants defective in the *plsB* and *gpsA* genes, results in rapid inhibition of both fatty acid and phospholipid synthesis (Nunn *et al*, 1977). The inhibition of fatty acid synthesis occurs even though the level of ATP remains high and macromolecular synthesis continues for about 1 generation (Nunn *et al*, 1977; McIntyre *et al*, 1977).

Additional regulation of the overall pathway could occur at the level of formation of common intermediates, such as phosphatidic acid and CDP diglyceride. Since there is 20 times less CDP -

diglyceride than phosphatidic acid and both intermediates turn over rapidly, it is conceivable that CDP-diglyceride synthesis is at least partially limiting for the pathway (Raetz and Kennedy, 1973).

In wild-type strains of E.coli and S.typhimurium inhibition of protein synthesis by starvation for a required amino acid results in a strong inhibition of stable RNA synthesis. Inhibition of RNA synthesis is correlated with the presence of the novel nucleotide, guanosine 5'-diphosphate -3'-diphosphate (ppGpp), that accumulates after starvation of wild-type (rel) but not relA mutant strains. It has been reported that phospholipid synthesis decreased after starvation of rel and considerable evidence has accumulated that ppGpp is the effector of lipid synthesis in vivo (Rock and Cronan, 1982). When a stringent strain (rel) is starved for a required amino acid, there is usually a 2 to 4 fold inhibition of the rate of phospholipid synthesis (Golden and Powell, 1972; Sokowa et al, 1972). The inhibition of RNA synthesis that occurs during the stringent response is generally of a much greater magnitude than the effect on phospholipid biosynthesis (Sokowa et al, 1972). PpGpp accumulates during the stringent response (Cashel, 1975). Nunn and Cronan (1976) showed that the rate of lipid synthesis is proportional to the intracellular concentration of ppGpp. When very high concentrations of ppGpp are generated in vivo phospholipid synthesis is inhibited over 10-fold. The enzymes most likely to

be controlled are acetyl-CoA carboxylase and sn-glycerol-P-acyltransferase since these are the first commited steps at the levels of fatty acids and phospholipid synthesis.

Likely candidates for the site of chain length regulation were the 3-ketoacyl-ACP synthases I and II, which catalyse elongation reactions. Substrate specificity studies in vitro indicate that one reason why membrane phospholipids are devoid of chains of more than 18 carbons is in part the reduced activity of synthases I and II (Garwin et al, 1980; Greenspan et al, 1970). Moreover, synthase II mutants (fabF) are defective in the elongation of palmitoleate to cis-vaccenate (Gelmann and Cronan, 1972). Although these data indicated that the condensing enzymes play a significant role in determining chain length physiological experiments indicate that the level of G3P acyltransferase activity is also important. When phospholipid biosynthesis is slowed or arrested at the acyltransferase step [by using a G3P acyltransferase mutant (plsB) or a G3P synthase (gpsA) mutant], the fatty acids synthesised had abnormally long chains compared with the normal distribution of fatty acids synthesised in the presence of G3P acyltransferase activity (Cronan et al, 1975). These data indicate that competition between the rate of elongation and the rate of utilisation of the acyl-ACPs by the acyltransferase is a significant determinant of fatty acid chain length in E.coli.

As in the case of chain length, the temperature dependent control of fatty acid unsaturation (Bloch and Vance, 1977; De Siervo, 1969; Marr and Ingraham, 1962; Okuyama, 1969) can occur at the level of fatty acid synthesis and at the acylation of sn-glycerol-3-P (Cronan, 1974, ; Sinensky, 1971; Bloch and Vance, 1977; Volpe and Vagelos, 1976).

1.9

Translocation of phospholipids

The translocation of phospholipids would seem to require two different processes:

(1) lateral diffusion (2) transbilayer movement (flip-flop) of phospholipid molecules from the inner to the outer leaflets of membrane bilayers. Donohue-Rolfe and Schaecter (1980) using pulse-chase techniques showed that PE was translocated from the cytoplasmic membrane to the outer membrane with a half-life of 2.8 minutes at 37° C. PG and CL were translocated very rapidly, with half-lives shorter than 30 seconds. The translocation of PE proceeded by apparent first-order kinetics. The rate of PE translocation was not influenced by alterations in the cellular growth rate but cellular growth temperature had a pronounced effect on the rate of translocation; lower temperatures increased the half-life of PE translocation. These authors also showed that energy inhibitors which deplete the protonmotive force (CCCP

and 2,4-dinitrophenol) markedly inhibited translocation. In contrast, inhibitors of ATP, protein and lipid synthesis did not affect translocation. From this data, several possible mechanisms for PE translocation can be eliminated. Since the *E.coli* membrane has a constant viscosity (Sinensky, 1974), the rate of translocation of phospholipids by passive diffusion would vary with absolute temperature. The difference in translocation rate observed by Donohue-Rolfe and Shaecter (1980) was considerably greater than the ratio of absolute temperatures; thus PE translocation strictly by a diffusion mechanism is unlikely. The fact that chloramphenicol and tetracycline had no effect on translocation rules out the possibility that PE translocation is directly coupled with membrane protein synthesis.

PE translocation does not require the continued synthesis of phospholipid and ATP as driving forces. The addition of sodium arsenite depleted the ATP levels and inhibited phospholipid synthesis but had no effect on translocation of PE. The results of Donohue-Rolfe and Shaechter (1980) strongly suggest that translocation is driven by the cells protonmotive force, since uncouplers inhibited translocation.

The three major steps in phospholipid biosynthesis probably are: (1) the newly synthesised phospholipid molecule is inserted into the inner leaflet of the cytoplasmic membrane. (2) it moves into the outer leaflet of the cytoplasmic membrane (3)the phospholipid

molecule is transferred to the outer membrane. The final step could proceed via zones of adhesion between the cytoplasmic and outer membrane (Donohue-Rolfe and Shaechter, 1980).

It is also possible that PE translocation is inhibited at a step of transbilayer movement (flip-flop). In liposomes the rate of lipid flip-flop is extremely low, with a measured half-life of days (Kornberg and McConell, 1971) or many hours (Rothman and Dawidowicz, 1975).

In model systems the rate of flip-flop can be increased by (1) temperatures within the phase transition (de Kruijff and Van Zoelen, 1978) (2) as ymetry of the bilayers with respect to head group (de Kruijff and Baken, 1978) or fatty acid composition (de Kruijff and Wirtz, 1977) and (3) addition of a transmembrane protein to the vesicles (Van Zoelen et al.,1978; de Kruijff et al., 1978).

It thus appears that a combination of the influence of membrane proteins, phospholipid heterogeneity and fluidity above the phase transition could result in a rate of uncatalysed flip-flop sufficient for membrane biogenesis.

1.10

Peptidoglycan

The peptidoglycan of prokaryotic cells serves the following two main functions: (1) it preserves the integrity of the cell. Without the intact peptidoglycan the cytoplasmic membrane is subject to severe stress and ruptures as water enters the cell to equalise osmotic pressure. (2) it is intimately involved in the cell division process (Park, 1987). The formation of septa represents the specialised synthesis of entirely new murein in a precise location to form the poles of the daughter cells while retaining the integrity of the peptidoglycan. Without the peptidoglycan, the cell or sphaeroplast even when protected from osmotic shock has difficulty in dividing. An incidental role of the peptidoglycan is to determine the shape of the cell.

The chemical composition of peptidoglycan has been analysed by enzymatic degradation of isolated sacculi into low molecular weight sub-units. Until recently, descending paper chromatography or thin layer chromatography were employed (Primosigh et al, 1961). This way 11 different compounds were identified. Glauner and Schwarz (1983) revealed 80 different compounds using high performance liquid chromatography (HPLC). The structural data of Glauner et al (1988) agree best with a model of a thin multilayered peptidoglycan sacculus.

The chemical structure and composition of the peptidoglycan

The peptidoglycan consists of linear polysaccharide strands composed of alternating units of N-acetylglucosamine and N-acetylmuramic acid (see figure 9). Various chemical and enzymatic procedures have led to the belief that β-1.4 linkages between the amino sugars are uniform in bacteria and resemble those in chitin-like structures (Sharon *et al*, 1966; Chipman *et al*, 1968). The strands contain an average of 30 saccharides which is equivalent to a length of 30nm (Park, 1987). Each strand terminates in a 1,6-anhydromuramic acid residue which is non-reducing.

Attached to the carboxyl group of each muramic acid of *E.coli* by an amide linkage is a short peptide of L-alanine, D-glutamic acid, meso-diaminopimelic acid (DAP) and D-alanine. In the peptidoglycan of *E.coli* a small percentage of the peptides lack D-alanine and an even smaller percentage terminate with an additional D-alanine (Park, 1987). In some species the carboxyl groups of glutamic acid or DAP (or both) may be amidated, but in *E.coli* neither is amidated. A unique feature of the peptide backbone of all peptidoglycans exemplified in the mucopeptide of *E.coli*, is the alternating sequence of optical isomers D-L-D-L-D. Adjacent mucopeptide strands are cross linked to each other

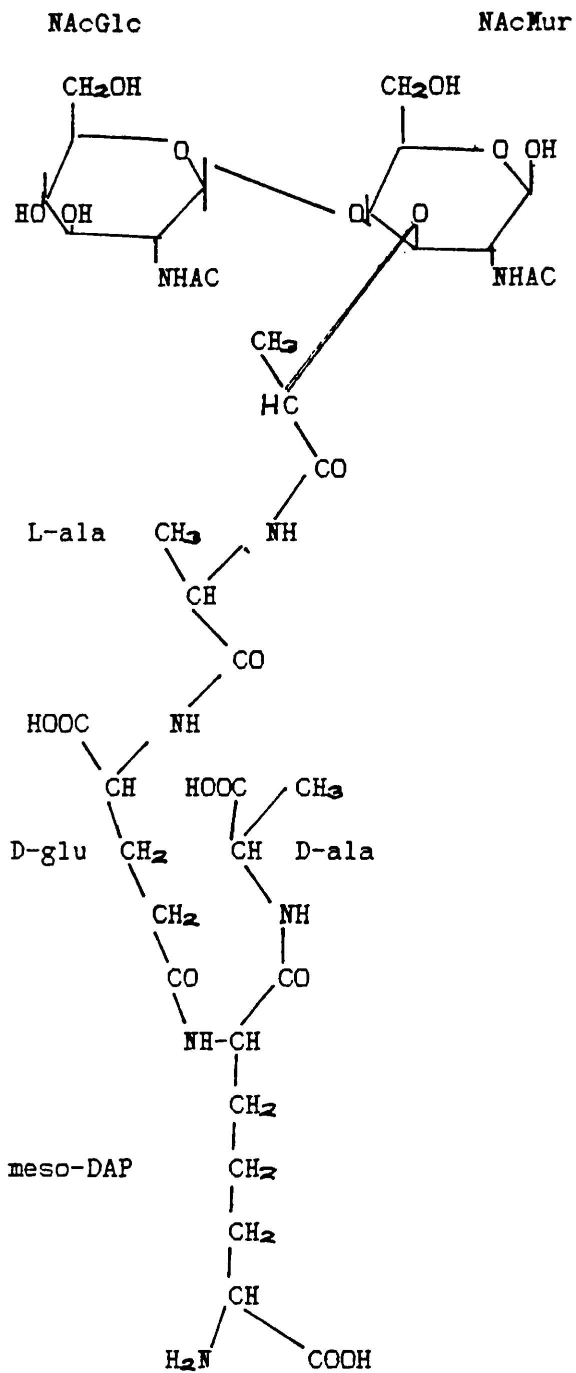


Fig. 9. Chemical structure of the principal muropeptide monomer present in the murein of E.coli and other Gram-negative bacteria. (From J. Park). L-ala=L-alanine; D-ala=D- alanine; Meso-DAP=meso-diaminopimelic acid; D-glu=D-glutamic acid.

through peptide side chains. Most of these cross-links are between the carboxyl group of D-alanine in position 4 of one peptide and the free amino group of DAP of a mucopeptide from the adjacent strand. It has recently been shown that about 20% of the cross-links do not involve D-alanine but rather link the DAP residues of neighbouring chains (Glauner and Schwarz, 1983).

In a model proposed by Barnickel et al (1983) the sugars do not lie in a flat plane. Instead they are tilted with respect to each other to accommodate the lactic acid moiety of muramic acid. As a consequence of this, mucopeptides are twisted in such a way that each strand is actually a spiral with each peptide chain being exposed 90° around the spiral relative to its neighbours. With this configuration only every fourth mucopeptide would be positioned to form a cross-link with a strand on its left. Similarly, a cross-link would occur within a strand on the right only with every fourth mucopeptide pointing to the right.

1.12

Biosynthesis of peptidoglycan

Synthesis of the peptidoglycan occurs in three stages: (1) Nucleotide activated precursors, in this case , UDP-N-acetylmuramyl-L-alanyl-D-isoglutamyl-(L)-meso-diaminopimelyl-D-alanyl- D-alanine (Park,1987) are synthesised. These precursors

are found in the cytoplasm and are synthesised by cytoplasmic enzymes. (2) the repeating unit of murein on the bactoprenyl phosphate is assembled. (3) the final stages are (a) the polymerisation of repeating units to form linear strands and (b) cross-linking of the strands to preexisting strands by transpeptidation. These enzymes are also associated with the cytoplasmic membrane but their active sites are located on the outer face of the cytoplasmic membrane.

1.12.1

Synthesis of the UDP-NAcMur pentapeptide

UDP-NacGlc is the branch point from which precursors unique to peptidoglycan synthesis are derived (see figure 9). UDP-NAcMur is formed by the condensation of phosphoenolpyruvate with UDP-NAcGlc, which is subsequently reduced to the 3-O-D-lactyl ether (Park, 1987). UDP-NAcMur pentapeptide is formed from UDP-NAcMur by sequential addition of the amino acids by specific enzymes. The enzymes are quite specific for the substrate and the amino acid to be added. In each case, the reaction is driven by ATP and requires either Mg or Mn. D-alanyl-D-alanine is synthesised separately and added to UDP-NAcMur tripeptide to form UDP-NAcMur pentapeptide. D-alanine is formed from L-alanine by a racemase. A specific D-alanine :D-alanine synthetase catalyses the formation of D-alanyl-D-alanine and a specific ligase ligates the dipeptide

to the precursor to give UDP-NAcMur pentapeptide.

1.12.2

The bactoprenyl cycle

The two reactions required for the formation of the repeating unit and also the reactions for the regeneration of the bactoprenyl phosphate carrier and polymerisation of the glycan are shown in figure 10.

Assembly takes place on a lipid carrier called bactoprenyl phosphate. It was shown to be a C_{55}-isoprenoid alcohol phosphate, undecaprenyl phosphate (Higashi et al, 1967; Wright et al, 1967). The first reaction of the cycle is the transfer of phospho-NAcMurpentapeptide to bactoprenyl phosphate with the release of UMP. This reaction is inhibited by vancomycin (Neuhaus et al, 1972; Perkins and Nieto, 1972) and tunicamycin (Takasuki et al, 1977). The second reaction which completes the formation of the muropeptides is the transfer of NAcGlc from UDP-NAcGlc to bactoprenyl -P- P- NAcMur pentapeptide to form the disaccharide pentapeptide repeating unit. The other product is UDP. The activated disaccharide pentapeptides are then utilised to form murein during the third stage of synthesis, and bactoprenyl pyrophosphate is released. Bactoprenyl phosphate is regenerated by a membrane bound pyrophosphate to complete the cycle.

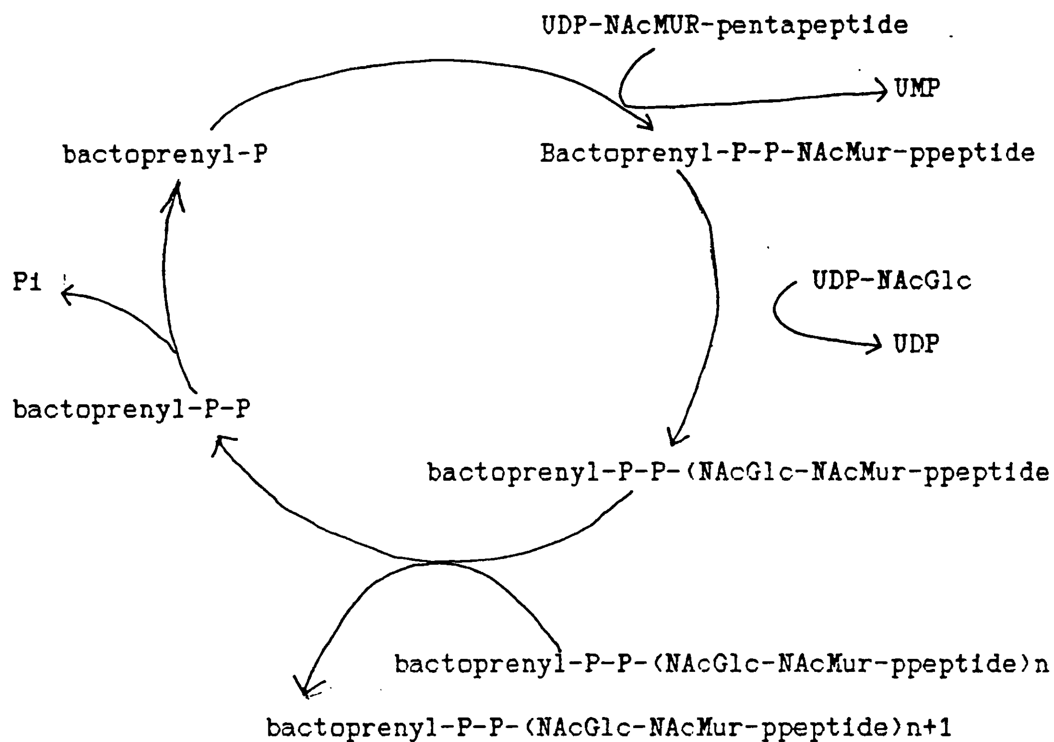


Fig.10. The bactoprenyl cycle: assembly of the mucopeptide repeating unit and polymerization of the glycan. ppeptide= pentapeptide. (From Park,J., 1987).

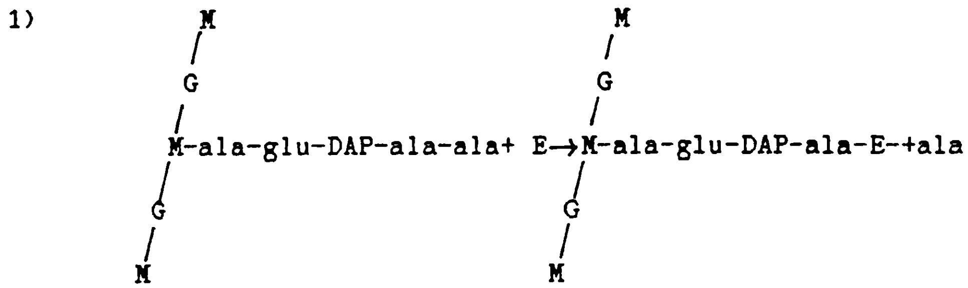
Bacitracin inhibits the pyrophosphatase by complexing with the substrate (Siewart and Strominger, 1967; Storm, 1974) and this prevents the regeneration of the bactoprenyl phosphate carrier.

1.12.3

Polymerisation and cross-linking of the repeating units

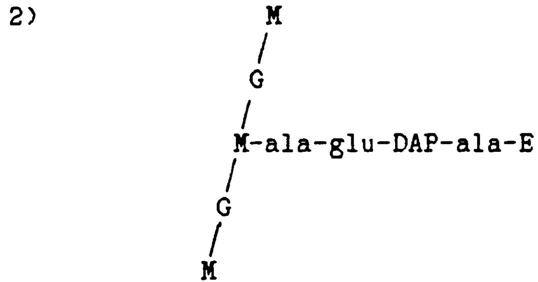
Study of the polymerisation and the cross-linking of the peptidoglycan by E.coli is complicated by the fact that two or more enzymes are present in the cell which are capable of carrying out these processes. In addition E.coli has multiple enzymes, each capable of both transglycosylation and transpeptidation (Matsuhashi et al, 1982). These are bifunctional or two-headed enzymes with two active sites. The transglycosylation reaction for lengthening the linear polysaccharide strand is believed to to proceed as illustrated in figure 9. A glycan chain still attached to bactoprenyl pyrophosphate is transferred to C4 of NAcGlc of a newly positioned bactoprenyl-P-P-(NAcGlc-Mur pentapeptide), leading to growth of the glycan at the reducing end (Ward and Perkins, 1973). Transpeptidation occurs as in figure 11. By virtue of being incorporated into a new, growing strand, mucopeptide that still carries a pentapeptide is positioned opposite a mucopeptide of an adjacent strand in such a way that transpeptidation can take place from the donor pentapeptide to the acceptor

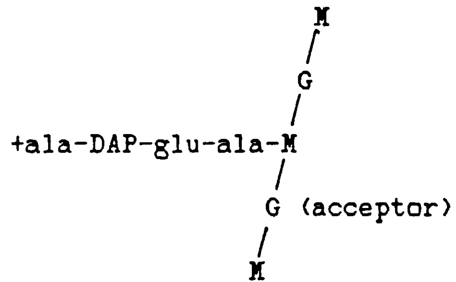
1)



(donor peptide)

2)





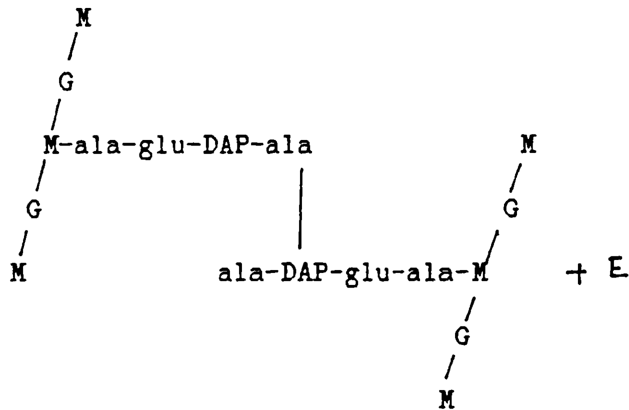


Fig.11. The transpeptidation reaction in peptidoglycan synthesis. G=N-Acetylglucosamine; M=N-acetylmuramic acid; E=transpeptidase. (From Park, J.; 1987)

mucopeptide. Transpeptidation involves cleavage of the D-alanyl-D-alanine bond of the pentapeptide with the concomitant formation of an acyl enzyme intermediate and the release of the terminal D-alanine. The activated intermediate reacts with the amino group of diaminopimelic acid of the available tetrapeptide on the neighbouring glycan strand to form a new peptide bond and to cross-link the two strands (Wise and Park, 1965; Tipper and Strominger, 1965; Strominger, *et al*, 1967). It may be noted that this reaction takes place in the periplasm. The energy of the D-ala-D-ala bond of the donor pentapeptide is used to drive this reaction (Park, 1987). Fractionation of the cross-linking enzyme complex of *E.coli* revealed that at least two distinct enzymatic activities are involved in the formation of the bridge structure (Strominger, 1970; Ghuysen, 1977; 1979): (1) the transpeptidase whose main function is to catalyse the formation of cross-linkages between the peptide units (2) the D-D carboxypeptidase that hydrolases the C-terminal D-alanyl-D-alanine peptide bond of the pentapeptide units.

The transpeptidation reaction was originally deduced from the observation that peptidoglycan formed by staphylococci in the presence of penicillin contained an excess of alanine (Wise and Park, 1965). On the basis of a different measure of cross-linking Tipper and Strominger (1965) came to the same conclusion, that penicillin must inhibit a transpeptidation reaction. They pointed out that penicillin may act as a structural analog of D-alanyl-D-

alanine so that the β-lactam antibiotic acylates the active site of the hypothetical transpeptidase and inactivates it. It is now apparent that any enzyme that metabolises a D-D peptide bond is likely to be sensitive to β-lactam antibiotics and may be inactivated by them. One evidence of inactivation is that penicillin acylates the enzyme stoichiometrically. Edwards and Park (1969) demonstrated that the rate of binding of a given β-lactam antibiotic correlates with its biological activity against staphylococci; this served to emphasize that binding was related to the mode of action of β-lactam antibiotics. This finding was fortuitous as it was found that staphylococci and other bacteria contain multiple penicillin binding proteins (Blumberg and Strominger, 1972). Spratt (1975) showed that E.coli has six penicillin binding proteins (PBP). Three of the high molecular weight PBPs, PBP-1C, PBP-7 and PBP-3 are bifunctional transglycosylase-transpeptidase enzymes. All three enzymes form cross-linked peptidoglycan in vitro from the lipid linked precursor: bactoprenyl-P-P-NAcGlc-NAcMur (pentapeptide) (Matsuhashi et al, 1982). With PBP-1B, about 23% cross-linkage occurred. With PBP-3, cross-linkage was only 6-10% and with PBP-1A, cross-linkage increased gradually to a level of 35%. The transpeptidase activity of PBP-3 appears to use either nascent or exogenously added UDP-N-acetylmuramyl tripeptide derived substrates as acceptors (Pisabarro et al, 1986). This peptidoglycan contained increased amounts of cross-linked peptidoglycan as well as a higher ratio of tripeptide containing

cross-linked subunits. In addition to these enzymes, an additional transglycosylase that synthesises cross-linked peptidoglycan and that does not bind penicillin has been purified (Hara et al, 1983).

PBP-2 has also been shown to have transpeptidase activity. The activity of this enzyme is apparently critical for the initiation of peptidoglycan synthesis (De la Rosa et al, 1985). Provided that PBP-2 remains active the other PBPs can be inhibited without any noticeable effect on the initial rate of incorporation of new precursors into macromolecular peptidoglycan. These precursors are, in addition inserted with a high degree of cross-linking. There also exists three low molecular weight DD-carboxypeptidases, PBP-4, PBP-5,PBP-6. These enzymes have been purified and PBP-5, the most abundant PBP of E.coli has been studied extensively (Waxman and Strominger, 1983). Strains with deletions in either PBP-5 or PBP-6 were found to grow normally (Spratt, 1983).

Genes of proteins for which there is a known role or activity related to peptidoglycan metabolism are listed in table 2. In addition to the genes listed in table 2 Dai and Ishiguro (1988) reported a new genetic locus involved in peptidoglycan synthesis which they designated murH.

Table 2 Genes of proteins involved in peptidoglycan metabolism

Gene symbol	Function
ftsI	PBP-3
murE	Meso-DAP adding enzyme
murF	D-alanyl-D-alanine adding enzyme
murG	Murein biosynthesis function unknown
murC	L-alanine adding enzyme
ddl	D-alanine:D-alanine ligase
envA	Required for cell separation
mrcB	PBP-1B
dacA	PBP-5
rodA	Required for rod shaped cell growth
lpp	Murein lipoprotein gene
amiA	N-acetylmuramyl-L-alanine amidase
mepA	Penicillin-insensitive endopeptidase gene
dacB	PBP-4
mrcA	PBP-1A
envC	Required for cell separation
mrbA	UDP-N-acetylglucosaminyl-3-enolpyruvate reductase
murA	UDP-GlcNAc:phosphoenolpyruvate transferase
mepB	Penicillin insensitive murein DD-endopeptidase gene
alr	Alanine racemase

This table includes only mapped genes for which a clear activity of function has been defined (Park, 1987).

1.13

Outer Membrane Proteins

Nearly half of the mass of the outer membrane is protein. Most outer membrane proteins are thought to be located exclusively in the outer membrane, although some are found in both the inner and outer membranes. The protein pattern of the outer membrane is dominated by a few protein species in large numbers. These are the major outer membrane proteins. For E.coli K-12 these include the porins and the OmpA proteins, protein a and lipoprotein.

1.13.1.

Lipoprotein

Lipoprotein is a small (7,200-dalton) protein that exists in a large number of copies per cell. The lipoprotein found in the outer membrane has been shown to exist in a free form (Inouye et al, 1972; Hirashima et al, 1973) and a bound form (Braun and Rehn, 1969) in the cell envelope. SDS gel electrophoresis of the E.coli membrane revealed that only the free form migrated on gels to give a peak at about 7,500 molecular weight. The bound form remains at the top of the gel because it is attached covalently to the peptidoglycan, which has a very high molecular weight. However, when the E.coli envelope is treated with lysozyme

followed by SDS gel electrophoresis the bound form can now migrate. The bound form has a higher molecular weight than the free form because it has a fragment of the peptidoglycan, cleaved by the lysozyme digestion attached to it.

The polypeptide chain of lipoprotein consists of 58 amino acid residues and exists in an α-helical form (Braun et al, 1976). Its amino terminal end consists of glycerylcysteine to which two fatty acid residues are linked by two ester linkages and one fatty acid residue linked by an amide linkage (Yamaguchi et al, 1988). The lipoprotein is produced from a secretory precursor, prolipoprotein containing a signal peptide of 20 amino acid residues (Inouye et al, 1977). This signal peptide is cleaved by a peptidase which requires the lipid modification at the amino terminal cysteine residue (Wu, 1987). Due to their hydrophobicity,the amino terminal ends of the lipoproteins are considered to be firmly associated with the membrane. It is, however, unlikely that the lipid modified amino terminal structure by itself directs the final location of the lipoprotein. Yamaguchi et al, (1988) demonstrated that a single amino acid residue of the lipoprotein molecule could determine the location of the molecule in the cell envelope.

The main function of the lipoprotein appears to be a structural one in that it stabilises the outer membrane-peptidoglycan complex by holding down the outer membrane to the surface of the

peptidoglycan (Nikaido and Vaara, 1985). A series of minor lipoproteins have been discovered (Ichihara _et al_, 1981).

1.13.2.

Porins

Porins are called peptidoglycan-associated proteins or matrix proteins. The proteins coded for by _ompC_, _ompF_ and _phoE_ are porins and produce relatively nonspecific pores or channels that allow the passage of small hydrophilic molecules across the outer membrane (Nikaido and Vaara, 1985). The porins are characterised by the fact that on isolation they show tight but noncovalent association with the peptidoglycan. They can be released from the peptidoglycan either by heating at 100° C for 5 minutes in SDS (Rosenbusch, 1974) or by extraction at 37° C with SDS containing 0.5 M NaCl (Nakamura and Mizushima, 1976; Hasegawa _et al_, 1976). The porins share various characteristics and were initially considered as one species. They were designated protein 1 by Schnaitman (1974) and protein A by Bragg and Hou (1972). Schmitges and Henning (1976) found that under certain electrophoretic conditions, protein 1 could be separated into two bands which they designated Ia (OmpF) and Ib (OmpC). They also showed that the OmpF and OmpC proteins differed in behaviour in isoelectric focussing. Comparison of the cyanogen-bromide fragments of these two proteins revealed that only one out of six fragments showed a

difference in electrophoretic mobility. Since this fragment did not correspond to the C-terminal or the N-terminal regions of the proteins (Garten *et al*, 1975), the possibility that they had a common precursor was excluded. In addition genetic analysis of OmpF and OmpC protein mutants identified three loci, designated *ompB*, *ompF* and *ompC*. The variability of phenotype in *ompB* mutants and the close structural and functional relationship between the OmpC and OmpF proteins, initially led to the idea that the proteins were encoded by one structural gene (*ompB*) whose product had undergone post-translational modification (Bassford *et al*, 1977; Henning *et al*, 1977). It is clear however, that although the overall amino acid sequences of the OmpF and OmpC proteins are similar , there are significant differences and their cyanogen bromide and proteolytic derivatives also differ significantly (Ichihara and Mizushima, 1978; Verhoef *et al*, 1979). Genetic evidence has confirmed that the proteins are products of two separate functional genes, *ompF* and *ompC* (Sato and Yura, 1979; Hall and Silhavy, 1979; 1981).

The similarity between these two proteins is extensive considering that they are the products of two separate genes; it has been shown that the anti-OmpC and anti-OmpF protein sera can react with both polypeptides (Overbeeke *et al*, 1980). In addition, the primary sequences as deduced from the DNA sequences

of respective genes show very strong homology (Inokuchi et al, 1982; Mizuno et al, 1983). These results suggest that they are derived from a common ancestral gene (Mizuno et al, 1983).

OmpF has a molecular weight of ca 37.2 K while OmpC has a molecular weight of ca 36 K. The structure of the pores of the bacterial outer membranes has been studied using electron microscopy and X-ray diffraction. Early electron microscopic studies have shown that the porins form a hexagonal lattice in the outer membrane (Steven et al, 1977). The ordered arrays suggest that the pore forming unit contains trimeric pores at the surface. More recent studies have shown that the pore is basically one channel with three openings facing the external surface (Engel et al, 1985). The three openings merge approximately in the centre of the membrane and the channel has only one outlet to the periplasmic side. The central constriction has a diameter of 1nm. (Engel et al, 1985). This is a rough approximation, better results are expected from X-ray diffraction of OmpF trimers but the analysis has not been completed (Benz, 1985). A striking feature of the OmpF is its extremely high content of β-structure.

In addition to the OmpF and OmpC porins which are present under normal growth conditions, another pore protein called PhoE protein is induced when cells are grown in phosphate limiting conditions. Phosphate limitation leads to the induction of outer membrane proteins that form anion selective channels (Poole and

Hancock, 1986; Van der Ley *et al*, 1987).

The porins function as passive diffusion pores which allow the rapid diffusion of hydrophilic molecules with small molecular weights. The permeability of the outer membrane for hydrophobic solutes has been studied in two ways. Firstly, radioactively labelled substrates were added to the external media and the uptake of the radioactivity into the cell was measured (Benz, *et al* 1988). Secondly, the β-lactamase activity was used to study the uptake of lactamase-sensitive β-lactam antibiotics through the outer membrane (Benz, 1988). The second method was used to study the function of OmpF, OmpC and PhoE in the *E.coli* outer membrane. The results indicated that PhoE had a preference for negatively charged solutes while the opposite was true for OmpC (Benz, 1988). Pleotropic transport mutants with decreased affinity for the uptake of substrates such as sugars, amino acids, uracil and inorganic anions were also found to be missing the porins (Bavoil, *et al*, 1977). Luktenhaus (1977) showed that the mutants from the *E.coli* B/r selected for resistance to copper lacked the OmpF and showed decreased ability to utilise a variety of low molecular weight metabolites such as amino acids and sugars when these are present in low concentrations. The OmpF is also involved in the uptake of low concentrations of adenosine monophosphate, guanosine monophosphate and bis-(para-nitrophenyl) phosphate and to a lesser extent cytidine monophosphate (Van Alpen, et al 1978).

The relative amounts of porins vary greatly depending on growth conditions; especially the composition of the growth medium (Schnaitman,1974; Lugtenberg et al, 1976; Bassford et al,1977; Hasegawa et al, 1976). Cells grown in nutrient broth (Bassford et al, 1977) or yeast broth (Lugtenberg et al, 1976) with no fermentable carbon source contained a decrease in amount of OmpC with concom itant increase in OmpF amounts. In contrast, the amount of OmpF decreases relative to OmpC in cells grown in complex media supplemented with carbon sources such as glucose and to a lesser extent glycerol (Bassford et al, 1977). OmpF is preferentially expressed if the cells grow in media of low osmolarity or high cAMP levels (Benz, 1988). OmpC is preferentially expressed in media of high osmolarity. It was shown that OmpF and OmpC directly affect the expression of one another which means that there is some feedback between both proteins (Schnaitman et al, 1984). It is unclear how the cells sense the osmolarity of their environment. In vitro experiments, have shown that the effective diameter of OmpF is a little larger than that of OmpC (Benz, et al, 1985).

Hall and Silhavy (1981) studied the ompB function using strains in which the lac gene was fused to the ompC and ompF gene. Their results indicated that both the absolute and relative levels of ompC amd ompF expression are positively regulated at the transcriptional level by the ompB locus, which defines at least two genes; ompR and ompZ. The protein products have been

identified (Taylor *et al*, 1981; Mizuno *et al*, 1982a, b). The *OmpR* gene product seems to be a positive transcriptional regulator required for expression of the OmpF and OmpC proteins. Mutations at the *envZ* are pleiotropic, affecting the production of OmpC protein, OmpF protein and some other exported proteins. The *envZ* gene product is necessary for optimal gene expression but not for expression of genes that encode other exported proteins (Garret *et al*, 1983). Hall and Silhavy (1981) have hypothesised that the role of the *envZ* gene product may be to sense conditions in the external environment of the cell and to direct the OmpR protein to the appropriate porin gene promoter. Ozawa and Mizushima (1983) have provided evidence suggesting that the OmpF protein is regulated by the OmpR protein and that the OmpF protein acts as a negative regulator of *ompC* expression. Ludrigan and Earhart (1984) have shown that fluctuations in porin ratios with growth temperature are controlled by a second locus, distinct from *ompB*

Recently, a mutant of *E.coli* K-12 which produced a new outer membrane protein OmpG was isolated, genetically and biochemically characterized (Misra and Benson, 1989). This mutation controls the synthesis of OmpG. Their data suggest that the protein was porin like.

Nikaido and Vaara (1985) speculated that the purpose of the environmental regulation of the porins is to let the bacteria sense whether they are inside or outside the hosts. Enteric

bacteria normally live in an environment where the osmotic pressure and temperature is quite high. Under these circumstances increased production of the OmpC porin at the expense of the OmpF porin would be beneficial to the bacteria, in that some of the inhibitory substances present in the gut would have difficulty penetrating the slightly marrower OmpC pore (Nikaido and Rosenburg, 1983). The OmpF porin would be advantageous when enteric bacteria have to survive in the external environment where the osmotic pressure and temperature are lower. The rate of assimilation of nutrients in such a dilute environment would be accelerated with increased production of the wider OmpF pore.

1.14

The OmpA Protein

The OmpA protein can be distinguished from other major outer membrane proteins on SDS-gels on the basis of several characteristics. Firstly, it cannot be isolated in association with the peptidoglycan from wild-type E.coli cells. Secondly, when cell envelope or outer membrane preparations are treated with trypsin the OmpA alone among the major outer membrane proteins is cleaved leaving a smaller fragment embedded in the membrane. The apparent molecular weight of the fragment has been

reported as 18,000 daltons (Overbeeke and Lugtenberg, 1980) or 24,000 daltons (Schweizer et al, 1978). The OmpA protein is similarly digested by pronase , leaving a fragment with a molecular weight of 19,000 daltons associated with the outer membrane (Bragg and Hou, 1972; Schweizer et al, 1978; Chen et al, 1980). Finally, on heating membranes below 70° C in SDS solution the apparent molecular weight of the OmpA protein on SDS-gels decreases from approximately 33,000 to 28,000 daltons (Bragg and Hou, 1972; Henning et al, 1973; Inouye and Yee,1973; Schnaitman, 1973a,b; Reithmeier and Bragg,1974). This has led to OmpA protein being termed a 'heat modifiable protein'. It should be noted that after sample preparation below 70° C, the OmpF and OmpC proteins remain at the top of the separating gel, as these are, oligomeric in their native state.

This protein is often linked to lipopolysaccharide and the lipoprotein and this contributes to membrane integrity. Mutants defective in OmpA function are pleiotropic; characteristic phenotypes include loss of viability in stationary phase, heat sensitivity in minimal media, inhibitor resistance and reduced transport rates for peptides and amino acids (Nikaido and Vaara, 1985; Manoil and Rosenbusch,1982; Reakes,et al 1988;Deeney et al 1986). A further function is the stabilisation of mating pairs during conjugation. The mechanism is not clearly understood. The protein also acts as a receptor for a group of T-even like bacteriophages including K3, Tu11 and Ox2 (Hancock and Reeves,

1975) and is involved in the uptake of colicin L (Chai and Foulds, 1974).

This protein has been purified (Hindenach and Henning, 1975) and the complete amino acid sequence determined (Chen et al, 1980). No marked internal homology or homology with ompF protein were revealed by computer analyses (Chen et al, 1980). However, examination of the predicted amino acid sequence of the OmpC protein in the region where it differs from that of the OmpF showed considerable homology with a region of the OmpA protein.

The -COOH terminal of 148 amino acids extends into the periplasmic space as does the $-NH_2$ terminus. The remainder of the molecule consists of eight amphipathic membrane spanning antiparallel β-sheets forming a β-parallel structure (Vogel and Jahnig, 1986).

A significant portion (6-24%) of the lysine residues of OmpA protein are present as allysine as a result of an enzymatic (Mirelman and Siegel, 1979) post-translational modification reaction (Diedrich and Schnaitman, 1978). It has been suggested that these residues could serve to cross-link the protein to other outer membrane components or to the peptidoglycan. It has been reported that some OmpA is covalently linked to the peptidoglycan in stationary phase cells (Diedrich and Schnaitman, 1978).

The *ompA* structural gene is located at 21.5 min. on the *E.coli* chromosome (Datta *et al*, 1976; Henning *et al.* 1976; Manning *et al* 1976; Henning *et al* 1978). All known mutations that result in the loss of the OmpA protein in *E.coli* as well as mutations that produce an altered OmpA, map at the *ompA* gene (Henning *et al*, 1976; Manning *et al*, 1976; Henning *et al*, 1978; Manoil and Rosenbusch, 1982). There is probably therefore no separate controlling region. Expression of the gene is therefore constitutive but some limit does appear to exist on the total amount of OmpA and other outer membrane proteins that can be incorporated into the outer membrane.

The production of the OmpA, OmpC, and OmpF proteins and of some other outer membrane poteins is somehow coordinated such that the combined copy number of these proteins remains relatively constant. For example, lack of the OmpA protein due to mutation is compensated for by increased amounts of the OmpC and OmpF proteins (Lugtenberg et al 1976) while mutants lacking the OmpC and OmpF proteins contain more OmpA protein (Schnaitman, 1974; Henning and Haller, 1975). These results have led to the proposal that production of these outer membrane proteins is under negative feedback control by these proteins at a post-transcriptional level (Datta et al 1976).

The *E.coli* K-12 *ompA* gene has been cloned and the DNA sequence determined (Henning *et al*, 1979; Beck and Bremer, 1980). This has

indicated the presence of an extensive secondary structure at the 5' end of the gene; the significance of this is unknown, as the gene shows no tendency to be regulated (Movva et al, 1980).

1.15.

VmpA Protein

The VmpA is a novel major outer membrane protein (Moores and Rowbury, 1982; de Pacheco et al, 1985; Rowbury et al, 1985). This is distinct from OmpA and is not heat modifiable . Like OmpA, VmpA is trypsin sensitive but it gives a larger major degradation product than OmpA (Moores and Rowbury, 1982). It has an apparent molecular weight of about 32,500 d and it is formed from a precursor of a 34,500 d apparent molecular weight (Rowbury et al, 1985). Certain OmpA properties are shared by VmpA; it is able to weakly replace the missing ompA function in conjugation stabilisation and in survival at 44°C (Moores, 1982; Deeney et al, 1986). Furthermore, it shows immunological cross-reactivity to OmpA protein. However, it does not act as a receptor for phages Tu1i or K3, or participate in uptake of colicin L (Moores and Rowbury, 1982). VmpA production does not seem related to derepressed transfer, colicin synthesis or colicin immunity.

This protein is present in strains carrying Col VIK-94 and Col VK-30 and some other Col V plasmids. VmpA production does not

correlate with colicin production or with the expression of derepressed transfer properties (Rowbury et al, 1985).

1.16.

Proteins involved in specific diffusion proceses

The outer membrane contains several inducible proteins involved in transmembrane diffusion of specific groups of solutes. One of the well known proteins is the LamB protein (maltoporin) of E.coli. It allows the passage of maltose and maltodextrins (Nikaido and Vaara, 1985). It was discovered at the cell surface as a receptor for several bacteriophages including bacteriophage lambda (Randall-Hazelbauer and Schwarz 1973). Like the porins OmpF, PhoE and OmpC, the LamB is an active trimer (142 kDa) whose secondary structure is of a β-pleated sheet (Neuhaus, 1982; Vogel and Jahnig, 1986).

Conflicting evidence has been reported concerning the physical basis of the selectivity of maltoporin; an interaction between the maltose binding protein, a soluble periplasmic protein and the size of the pore itself has been implied. It has been suggested that the maltose binding protein confers specificity on the maltoporin pore by physically impeding the passage of molecules other than maltose through the channel (Heuzenroeder

and Reeves, 1980). Brass *et al*, (1985) however, presented evidence *in vivo* that the maltose binding protein does not modulate activity of the maltoporin as a general pore. The permeability of maltose through maltoporin reincorporated in liposomes *in vitro* has been reported to be 225-fold higher than that through the general (OmpF) porin while glucose diffuses at the same rate through the two protein channels (Luckey & Nikaido, 1980a; Nikaido and Rosenburg, 1983). Also. maltose flux appears to be inhibited by maltodextrins (Luckey and Nikaido, 1980b). The existence of binding sites for maltodextrins has been suggested (Ferenci *et al*, 1980). The results of Dargent *et al*, (1987) confirm the results of Brass *et al*, 1985. Although it appears that maltose binding protein is not required to confer specificity of maltoporin we cannot rule out that interaction between it and maltoporin occurs *in vivo* and may play a role in a different step of the maltose uptake pathway.

The Tsx protein or T6 receptor is known to facilitate the diffusion of nucleosides and deooxynucleosides across the outer membrane. Recently, a pore forming activity of this protein has been discovered *in vitro* (Maier et al, 1988). It had been earlier hypothesised that the Tsx protein introduces a permeability pathway into the outer membrane by forming a pore (Hantke, 1976). This protein is also involved in the uptake of amino acids such as serine, glycine and phenylalanine (Heuzenroeder and Reeves, 1981).

A number of other outer membrane proteins are involved in highly specific transport processes. These proteins bind their transported substrates directly with high affinity, in contrast to the rather loose binding seen with LamB and Tsx proteins. An interesting find is that these systems require a functional _tonB_ gene product. Although its mode of action is unclear, it is generally assumed that it might serve to couple the energized cytoplasmic membrane to the proteins and thus regulate their functional state. Transport of iron (111) siderophore and vitamin B12 depend on the _tonB_ protein of the cytoplasmic membrane since these substances stay at their receptor proteins in _tonB_ mutants and unenergized _tonB_ cells (Fischer _et al._ 1989). Mutations in a single codon of the _tonB_ gene supressed the _btu_ 451 point mutation in the structural gene of the vitamin B12 receptor (Heller _et al._, 1988) and mutations in the _fhuA_ gene (Fischer _et al._, 1989), which encodes the receptor for ferrichrome, the antibiotic albomycin, colicin M and the bacteriophages T5, T1 and 80. Supression of mutations in receptor genes by _tonB_ mutations suggests a direct interaction between the TonB protein and the receptor proteins. The TonB protein seems to be involved in the transport across the outer membrane. There are a number of outer membrane proteins involved in the transport of chelates of ferric ion. The TonA (or FhuA) protein necessary for the uptake of ferrichrome, has a molecular weight of 78,000. Ferrichrome appears to have a high

affinity for this receptor on the basis of its interference with the adsorption of phages (Luckey *et al*, 1975; Wayne and Neilands, 1975). Mutations in gene *fhuB* were found to abolish utilisation of not only ferrichrome but also all other ferric hydroxymate siderophores; an intriguing observation is that inner membrane vesicles of *fhub* strains were not defective in ferric hydroxamate transport (Prody and Neilands, 1984). The FepA protein required for the transport of ferric enterochelin across the outer membrane is an 81,000 dalton protein and crude extract of FepA protein was shown to bind the ferric enterochelin (Hollifield and Neilands, 1978).

1.17.

Biosynthesis of the Outer Membrane proteins in Gram-Negative bacteria

It was thought that the examination of precursors or intermediate forms of these proteins would help elucidate mechanisms involved in biosynthesis of outer membrane proteins. Efforts to find these intermediates by short pulse experiments have failed (Lee and Inouye, 1974; de Leij *et al*, 1978). Therefore other approaches were taken to examine the intermediate steps in the biosynthesis of the outer membrane. Randall *et al* (1978) identified three proteins synthesised on membrane bound polysomes *in vitro*; two of these were soluble

periplasmic proteins and the other was an outer membrane protein (λ receptor protein). All these three proteins were larger than the mature protein which seemed to suggest that they might be precursor species. There were also investigations involving perturbants that inhibited different stages of outer membrane biosynthesis and assembly. One of the perturbants used was toluene that caused a breakdown of the cellular permeability with partial dissolution of the cytoplasmic membrane. Halegoua et al (1976) defined an ATP-dependent system, sensitive to tetracycline, chloramphenicol and puromycin which exclusively synthesised membrane proteins in the presence of toluene. When these proteins were treated with antilipoprotein serum two distinct peaks were found during SDS gel electrophoresis of the immunoprecipitate; one comigrated with the in vivo lipoprotein and the other appeared to be a new form of lipoprotein. The larger lipoprotein has the same carboxy terminal as the lipoprotein but the amino terminal amino acid was methionine (Halegoua et al 1977) instead of glycylcysteine of lipoprotein. It was found that the new lipoprotein contained 20 extra amino acids enriched in hydrophobic residues. It contained three glycine residues which lipoprotein lacks. This new lipoprotein was called prolipoprotein. It plays a role as a precursor in the biosynthesis and assembly of lipoprotein. Milder toluene treatment led to the accumulation of two new precursor proteins, giving rise to OmpF and OmpA proteins (Sekizawa et al 1977). Phenethyl alcohol (PEA) treatment resulted in the accumulation of the pro-OmpF protein. At higher concentrations of PEA, pro-

OmpA and pro-OmpF proteins were found. Removal of PEA led to the disappearance of the precursors and the OmpF and OmpA proteins were normally incorporated into the outer membrane, hence showing a direct precursor product relationship (Halegoua and Inouye, 1979a).

Furthermore failure to detect precursor proteins in undisturbed cells may be due to the methods used in the investigations. Crowlesmith _et al_ (1981) suggest that the cytoplasmic membrane may not be efficiently pelleted when cells are lysed by sonication, thus attempts to demonstrate the presence of membrane bound precursors would be unsuccessful. Furthermore, precursors are present in such low concentration that they may be detected in cytoplasmic membranes or whole cell lysates only when specific antisera are used. Crowlesmith _et al_ (1981) using antisera to the OmpF and OmpA proteins, detected precursors to these proteins _in vivo_ in pulse labelling experiments carried out at 25°C. Precursor molecules of both proteins were chased within 5 min. into mature proteins. When the pulse was at 37°C, no precursors were detected. The proproteins had higher molecular weights than the mature forms. The newly synthesised precursors comigrated in sucrose gradients with the cytoplasmic membrane, indicating that this may be their site of synthesis.

1.18.

Translocation and assembly of outer membrane proteins

One of the models proposed for protein translocation is the linear model or signal hypothesis proposed by Blobel and Dobberstein (1975) for the transfer of eukaryotic secretory proteins across the membrane. In this model, it is proposed that the hydrophobic nature of the peptide extension of the secretory protein precursors allows for initial interaction of the polysome with the membrane followed by interaction of the peptide in the membrane. The result is an activation of the receptor protein to mediate aggregation of proteins at the membrane bound polysome site to form a transmembrane hydrophilic channel. The peptide extension and the succeeding secreted protein are linearly translocated across the membrane through the channel during synthesis of the latter.

An alternative model, is the loop model proposed by Halegoua and Inouye (1979b). In this model a peptide extension for the translocation has been proposed. As the precursor protein is synthesised on the polysome the positively charged section 1 (see figure 12) allows the initial attachment of the peptide extension and consequently the polysome to the negatively charged inner surface of the cytoplasmic membrane through ionic interactions. The *E.coli* membrane surface is negatively charged

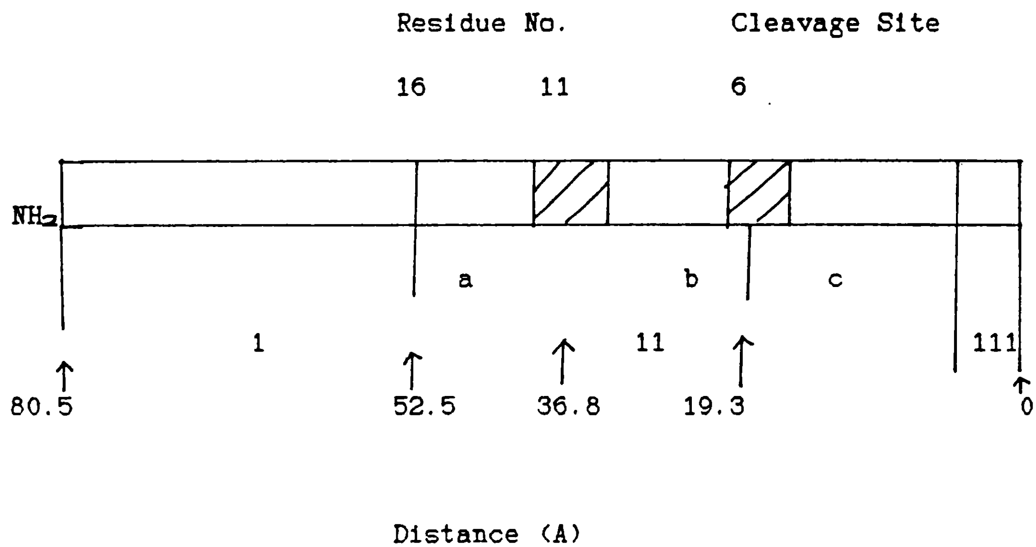


Fig.12. A generalised structure of the peptide extension. 1, basic region; 11, hydrophobic region; 111, recognition site for cleavage extension. Section 11 is divided into three subsections,a, b, c, by proline or glycine at positions 6 and 11. (From Halegoua and Inouye, 1979b).

at neutral pH as a result of the phosphatidyl glycerol. The next hydrophobic section 11 is then progressively inserted into the cytoplasmic membrane by hydrophobic interactions with the lipid layer. As the peptide further elongates, the loop formed by section 11 is further extended into the lipid bilayer. The processing or cleavage site is then exposed to the outside surface of the cytoplasmic membrane. The length of sections 11 and 111, about 53A, is long enough to extend across the lipid bilayer to expose section 111 to the outside surface of the membrane where processing of the cleavage site may take place. The role of the proline or glycine residues may be important in bending the peptide at that position to form the loop.

Although in vitro systems for protein translocation across the bacterial cytoplasmic membrane so far reported have facilitated the biochemical characterization of the translocation of proteins, the translocation activity was not necessarily reproducible enough to perform precise and quantitative studies. In the work by Yamane et al, (1988) an in vitro system exhibiting highly efficient protein translocation into inverted membrane vesicles of E.coli was established. In their study, the signal peptide cleavage site of the OmpF-lpp chimeric protein a model secretory protein, was changed from Ala-Ala to Phe-Pro through oligonucleotide directed site specific mutagenesis of the OmpF-lpp gene on a plasmid. The mutant was no longer processed by the signal peptidase. When proteinase K treatment

was adopted as a probe for protein translocation into inverted membrane vesicles the mutant protein exhibited rapid and almost complete translocation. This result seems to indicate that cleavage of the signal peptide is not required for translocation of the mature domain of the protein. This system also has its disadvantages as cleavage of the signal peptide cannot be observed during translocation. Failure of signal peptide processing may result in the protein molecule sticking to the translocation apparatus, which in turn affects the translocation of forthcoming protein molecules. Accumulation of the signal peptide precursor would perturb the membrane integration which would indirectly affect the translocation activity. It has been reported that accumulation of a precursor protein in the cytoplasmic membrane may lead to cell death (Hussain et al 1980; Date, 1983).

Emr et al. (1980) described mutants in which the unprocessed precursor form of the λ receptor of the outer membrane accumulated in the cytoplasm. In this mutation, there were changes in the peptide extension of the precursor proteins from hydrophobic to hydrophilic. The result supports the view that hydrophobicity is required in the extension peptide for the initiation of the export process.

A similar alteration in the prolipoprotein extension peptide sequence which affected the hydrophobicity of this section did

not have a dramatic effect on lipoprotein export (Lin et al, 1980). That processing is not necessarily coupled to the translocation process was demonstrated by Halegoua and Inouye (1979b) who showed that pro-OmpF could be translocated across the cytoplasmic membrane of PEA-treated cells in the absence of processing.

A signal peptide, a processing product of the precursor of the lipoprotein in the cytoplasmic membrane of E.coli has been purified through extractions with butanol and ethyl ether and chromatography with a Sephadex Lh-60 column Sep-Pak C18 (Suzuki et al, 1987). The signal peptide of lipoprotein is composed of 20 amino acid residues. The fact that the intact signal peptide is a major product of prolipoprotein processing indicates that the first step of the signal peptide catabolism is the endo-type cleavage of the signal peptide by signal peptidase. Ichihara et al, (1984) showed that protease 1V, an enzyme responsible for hydrolysis of the signal peptide in the cytoplasmic membrane, does not attack the signal peptide domain in the precursor protein. Taking these facts into consideration, it is plausible that proteases responsible for signal peptide hydrolysis do not function until signal peptides are released from the precursor proteins, although the full length of signal peptides is seemingly not required for the cleavage as demonstrated with leader peptidase (Darstein and Wickner, 1986).

One of the features of outer membrane translocation is the

presence of the 'trigger factor'. Crooke and Wickner (1987) purified pro-OmpA to determine if any other soluble factor catalysed the translocation of this protein. They found an activity in the cytosol (S100) fraction which is ne cessary for translocation. The trigger factor with an apparent molecular weight of 60,000 daltons appeared to act during the folding of the protein, yet its effects were not coupled to translocation. The trigger factor allows pro-OmpA to fold into an assembly competent form.

It has been suggested that the energy for protein translocation in prokaryotic cells comes from the protonmotive force (Bakker *et al*, 1984; Date *et al*, 1980) and membrane potential. Yamane *et al* (1988) studied the types of energy required for protein translocation across the membranes. They adopted an *in vitro* *E.coli* system consisting of inverted membrane vesicles and the OmpFlpp chimeric protein. Translocation of the protein was totally inhibited in the presence of carbonyl-cyanide m-chlorophenylhydrazone (CCCP) or valinomycin and nigericin and partially inhibited when either valinomycin or nigericin were added. Depletion of ATP with glucose and hexokinase resulted in complete inhibition of the translocation process and the inhibition was suppressed by the addition of ATP-generating systems such as phosphoenolpyruvate-pyruvate kinase or creatine phosphate -creatine-kinase. Their results indicated that both the protonmotive force and ATP are required for the

translocation process. The results also suggested that both the membrane potential and the chemical gradient of protons (δpH) of which the proton motive force is composed, participate in the translocation process. Geller et al (1986) also proposed that both ATP and the membrane potential are required for the optimal assembly of the OmpA into E.coli membrane vesicles. Enequist et al (1981) showed that the precursors for the outer membrane proteins, OmpF, OmpA amd LamB and for two periplasmic proteins, the maltose binding protein and arabinose binding proteins were not processed in the presence of compounds that dissipate the proton motive force, such as ethanol, dinitrophenol and CCCP. Thus it seems that an energized membrane is probably essential for export of most periplasmic and outer membrane proteins.

Porin, exists in a trimeric structure which is not dissociated during SDS-PAGE at 25°C. This unusual stability was utilised in the study of the conformational changes which accompany the targeting of the porin to the outer membrane in work by Reid et al, (1988). A delay of 16-44 seconds between completion of synthesis of a monomer and its assembly into a trimer was found from the ratio of monomers to trimers found in exponentially growing cells. They performed pulse chase experiments and showed that rapid processing of precursor OmpF molecules was followed by assembly into SDS-resistant oligomers with a half time of 20 seconds at 30°C. An intermediate in assembly was isolated by immunoprecipitation and SDS-PAGE below 10°C and was identified

as a metastable dimer.

The process of porin trimerization can be described as a pathway containing several sequential steps; (1) the removal of signal peptide (2) assembly of a partially stabilised dimer and (3) formation of the trimers (Reid et al, 1988). One of the rate limiting steps is presumably the reaction between independent subunits to form dimers because unassembled monomers were present in cells in steady state growth. Possibly the availabilty of another component such as LPS may affect the rate of porin insertion into the outer membrane.

BARRIER PROPERTIES OF THE OUTER MEMBRANE

Most biological membranes and phospholipid bilayers are known to allow the penetration of hydrophobic compounds (Stein, 1967; Bangham, 1972). The permeants cross the membrane by first dissolving in the hydrophobic interior, diffusing through the thickness of the hydrocarbon layer and finally partitioning into the aqueous phase on the other side of the membrane.

In contrast, the outer membrane of enteric bacteria such as E.coli and S.typhimurium confers on the bacteria a high resistance to many hydrophobic antibiotics such as novobiocin, erythromycin, rifampicin; detergents such as sodium dodecyl sulphate and hydrophobic dyes such as crystal violet (Leive, 1974; Nikaido and Nakae, 1979). In deep rough mutants of S.typhimurium, the permeability to the above compounds is substantially increased (Roantree et al, 1977; Nikaido and Nakae, 1979). A similarly marked increase in permeability to such hydrophobic compounds was observed in EDTA- treated cells of E.coli and S.typhimurium which also results in the release of half the cellular LPS (Leive, 1974; Nikaido and Nakae, 1979).

Permeation of hydrophobic molecules through the LPS would be difficult as the LPS molecules are highly charged in the Lipid

A- core region. Also the hydrocarbon chains of the LPS molecules are tightly packed and less mobile than those of the phospholipid molecules and hence would reduce further permeation of hydrophobic molecules. It has also been reported that isolated LPS, in particular the Lipid A component has a high state of order compared to phospholipid arrangements in cytoplasmic membranes (Labischinski et al, 1985). The assymetric arrangement of the outer membrane serves as an effective barrier to hydrophobic molecules.

Deep-rough mutants appear to be sensitive to the hydrophobic compounds. The molecular basis of this increased permeability is still poorly understood. It is unlikely that the carbohydrate chains of the LPS constitute a primary barrier to the hydrophobic compounds, as, in the Rc mutants up to 80-90% of the saccharide chains are lacking (Nikaido,1979a; Nikaido and Nakae,1979). Several pieces of evidence exist suggesting that the outer membranes of deep-rough mutants and EDTA-treated cells contain phospholipids in their outer leaflets, creating phospholipid bilayer regions in the outer membrane that allow diffusion of the hydrophobic compounds (Nikaido 1979a,b; Nikaido and Vaara, 1985).

The main evidence for this hypothesis was the finding that the outer membranes of the deep-rough mutants sensitive to hydrophobic compounds contained decreased amounts of outer

membrane proteins (Ames _et al_, 1974; Koplow and Goldfine, 1974; Smit _et al_, 1975) and significantly increased amounts of phospholipids at a level which was more than could be accommodated on one side of the membrane (Smit _et al_, 1975). These results were later confirmed by various investigators (Havekers _et al_, 1976; Lugtenberg _et al_ 1976; Van Alpen _et al_ 1976; Gmeiner and Schlecht, 1980).

Nakae and Nikaido (1979) proposed that in cells treated with EDTA, a space was created by the release of LPS which could be filled by the phospholipids either from the inner leaflet of the outer membrane or from the cytoplasmic membrane. Although there is no direct evidence for this, the observations that outer membranes free of peptidoglycan contain mixed phosphilipid-LPS bilayers (Muhlradt and Golecki, 1975) and that there appears to be a reversible flow of phospholipids between the cytoplasmic and outer membranes (Jones and Osborn, 1977) are consistent with this hypothesis.

This hypotheis may also explain other permeability changes, such as the increased sensitivity to hydrophobic compounds exhibited by mutants lacking both the OmpA protein and the porins (Schweizer _et al_, 1976) or with decreased levels of outer membrane proteins (Ames _et al_, 1974). These mutants have been reported to produce outer membranes with phospholipids in the outer leaflet (Kamio and Nikaido, 1976; Van Alpen _et al_, 1977b)

and apparently normal (Ames *et al*, 1974) levels of LPS respectively.

These findings are consistent with the occurence of phospholipid bilayer regions in the outer membrane of LPS mutants, There are some observations contrary to this. Deep rough mutants and cells treated with EDTA become sensitive to lysozyme (Repaske,1958; Sanderson *etal*, 1974; Tamaki and Matsuhashi, 1974). Deep-rough mutants also leak periplasmic enzymes into the medium (Chaterjee *et al*, 1976). It is unlikely that phospholipid bilayer regions would allow the enhanced passage of such macromolecules.

There is another explanation for the increased permeability of the deep-rough mutants and EDTA-treated cells. It is proposed that the strong LPS-LPS interaction, which is essential for the maintenance of the barrier property of the outer membrane becomes weakened or disrupted (Hancock, 1984; Nikaido and Vaara, 1985). This would allow penetration of the hydrophobic molecules regardless of the absence or presence of phospholipid bilayer. In favour of this hypothesis is the reversal of the phenotype of EDTA treated cells and deep-rough mutants on addition of magnesium which is requred for the stabilisation of the LPS in the outer membrane. As observed by Hancock in a recent review (1984) this phenomenon has been reported for some other hydrophobic permeable mutants and permeabilising agents (such as polymyxin); this author has proposed that all these permeability

alterations involve sites in which LPS molecules are stabilised by divalent cations. Furthermore, although the presence of phospholipid bilayer regions in the outer membrane of deep-rough mutants is well established (Smit et al,1975; Kamio and Nikaido,1976), these may in fact be rather limited, as a significant increase in LPS content has also been reported in such mutants (Hevekes et al,1976; Gmeiner and Schlecht,1979; 1980). This would leave only a small area, perhaps less than 10% of the outer membrane (Nikaido and Vaara, 1985) as a phospholipid bilayer.

The low hydrophobic permeabilty of the outer membrane of enteric bacteria such as E.coli and S.typhimurium can be explained very well by the as,ymetric nature of the outer membrane.

In addition to the deep rough mutants, other mutants which led to a hydrophobic sensitive phenotype have been described (Hancock, 1984; Nikaido and Vaara,1985). Benson and Decloux (1985) have recently described three mutants, two of which map in the ompF structural gene and the other in the ompC structural gene. The rate of migration of the respective proteins was altered on SDS gels. In addition to an increased uptake of hydrophilic molecules, these mutants exhibited an increased sensitivity to various hydrophobic antibiotics and detergents. They suggest that the alteration to the porins alters pore structure and function and hence disrupts the LPS-protein

interactions leading to a hydrophobic permeabilty.

2.1.

Interaction between LPS molecules

One of the most important features of LPS is that it appears to be stabilised in the outer leaflet of the outer membrane by noncovalent cross bridging between adjacent LPS molecules via divalent cations (Hancock 1984; Nikaido and Vaara,1985). The LPS molecule has a number of highly charged negative groups in the Lipid A-core region and the close association of the LPS molecule would lead to strong electrostatic repulsion between these molecules without neutralisation of and bridging between the anionic groups. Hence, the treatment of E.coli and S.typhimurium with EDTA results in the release of half of the LPS in the cell, probably due the weakening of the LPS-LPS interaction and disruption of the outer membrane permeabilty barrier function (Leive, 1965). Tris ions enhance the removal of LPS from the cell by EDTA by partially replacing other cations bound to LPS (Leive, 1974; Schindler and Osborn,1979).

There are many studies which show the importance of divalent cations in maintaining the structural organization of the outer membrane. Among the most convincing are those which show that the structure of the outer membrane can be destabilised and its permeability barrier function disrupted by removing or

displacing divalent cations with componds such as EDTA (Leive, 1974) and the antibiotic polymyxin B. Furthermore, exogenously added magnesium has been shown to stabilise the outer membrane and to reverse permeability effects of EDTA (Leive, 1974) and polymyxin (Nicas and Hancock, 1980). The sensitivity of deep rough mutants to hydrophobic inhibitors is similarly reduced by the addition of magnesium (Stan-Lotter et al 1979) as is the leakage of periplasmic enzymes from these mutants (Chaterjee et al, 1976) and from lipoprotein deficient mutants (Nikaido et al, 1977a).

Polymyxin B which is bactericidal to most Gram-positive bacteria is a large amphipathic molecule and so cannot penetrate the narrow pores of enteric bacteria. It is thought to gain access to the cytoplasmic membrane by displacing divalent cations from LPS thereby destroying LPS crossbridging and permeabilising the outer membrane. The antibiotic binds to the outer membrane (Teuber and Bader, 1976), most probably to the divalent cation binding sites on the LPS (Schindler and Osborn, 1979; Nicas and Hancock, 1980) causes extensive alterations to the outer membrane structure (Schindler and Teuber, 1975, 1978; Lounatmaa et al, 1976) and disrupts the outer membrane permeability barrier to agents such as detergents (Vaara and Vaara, 1981), hydrophobic antibiotics (Rosenthal and Storm, 1977) and lysozyme (Teuber, 1970).

2.2

Interaction between proteins and lipopolysaccharides

For the outer membrane to act as an efficient barrier, the interstices between the protein molecules and the LPS molecules should remain tightly sealed. It would be expected that the outer membrane and proteins interact strongly. The first indication of such an interaction between protein and LPS came from the findings that levels of major outer membrane proteins were drastically reduced in S.typhimurium and E.coli mutants synthesising very defective LPS which became extremely sensitive to various hydrophobic agents as a consequence of this structural reorganization of the outer membrane (Ames et al, 1974; Koplow and Goldfine, 1974). The existence of LPS-protein interactions was then clearly established by experiments which showed that LPS was required for reconstitution of the phage receptor activities of the OmpA, OmpF and OmpC proteins (Datta et al, 1977; Van Alpen et al, 1977a; 1979a).

Additional evidence for an interaction between the OmpF and OmpC proteins and LPS comes from observations that purified porin preparations contain significant amounts of LPS (Schindler and Rosenbusch, 1978; Overbeeke et al, 1980). Furthermore, heating of a porin-LPS complex at 100°C in SDS was found to dissociate the LPS (Yamada and Mizushima, 1980) suggesting that a native protein

conformation is necessary to absorb LPS. The presence of LPS also stimulates the binding of these proteins to the peptidoglycan *in vitro* (Hasegawa *et al*, 1976; Yu and Mizushima, 1982).

The role of LPS in the formation and activity of the OmpC and OmpF protein channels is unclear still. Schindler and Rosenbusch (1981) showed that LPS was required for the formation of stable, open channels *in vitro*. Full channel activity can be reconstituted with the porins or the LamB protein and phospholipids alone (Nikaido, 1983; Nikaido and Rosenburg, 1983).

Rocque *et al* (1987) isolated LPS bound to isolated porin and this was detected on SDS-PAGE by using a carbohydrate specific silver stain and on Western blots by using anti-lipid A monoclonal antibodies. Porin was isolated from Ra and Re chemotypes. Isolated porin was separated from loosely associated LPS by SDS-PAGE. It had been reported that when LPS is removed from the complex, porin maintains its structure and integrity but Rocque *et al* (1987) were unable to completely remove the LPS without denaturing the protein. Crude porin readily separated from loosely bound LPS by electrophoresisbut the trimer band detected in SDS-PAGE was shown to contain LPS.

The specific interaction between an outer membrane protein and LPS has been shown for the OmpA protein. Several lines of evidence have suggested that the OmpA protein is the specific receptor

for phage Tull *in vivo* (Datta *et al*, 1977). However, the isolated OmpA protein, dissolved in Triton X-100 bicarbonate buffer, did not activate this phage (Datta *et al*, 1977; Schweizer *et al*, 1978). The inactivation occurred when LPS or Lipid A of *E.coli* was added to the OmpA protein and was maximal when the LPS/OmpA mixture was precipitated by magnesium or phospholipids.

OmpA protein appears to be needed in F-plasmid mediated conjugation to stabilise the mating aggregates (Manning and Achtman, 1979; Skurray *et al*, 1974). However, purified OmpA inhibits conjugation only when it is added with LPS (Achtman *et al*, 1978; Schweizer and Henning, 1977; Van alpen *et al*, 1977). LPS also protected isolated OmpA protein from proteolytic cleavage. This variety of experimental evidence strongly suggests the LPS-OmpA protein association demonstrated *in vitro* reflects a similar interaction *in vivo* which may be required for stabilisation of the OmpA protein in the outer membrane.

In most of the above cases Lipid A, but not the saccharide chain, substituted effectively for intact LPS in reconstitution of biological function indicating that the relevant interactions may be largely hydrophobic. The importance of the Lipid A region in the LPS-OmpA protein interaction has also been demonstrated by the finding that partially deacylated LPS will not bind the OmpA protein (Beher *et al*, 1980a). In addition, the isolated core portion of LPS

when added separately to a Lipid A -OmpA protein mixture was found to inhibit the effect of Lipid A in reconstititution of phage receptor activity (Schweizer et al, 1978).

However, the OmpA may also interact with the core portion of the LPS. Pupurs et al, (1983) have recently described a group of OmpA mutants that were resistant to phage K3 only when the background LPS was defective in some core sugar residues. The LPS mutants were K3 sensitive and produced near normal amounts of OmpA as did the double mutants. It has also been reported that the adsorption of phage K3 to vesicles containing OmpA protein and Lipid A was strongly reduced compared to the rate when complete LPS was used (Lugtenberg and Van Alpen, 1983).

Rick et al (1983) have reported that when a temperature sensitive mutant of S.typhimurium that cannot synthesise a complete Lipid A under non-permissive conditions (42°C) was shifted to 42°C the rate of OmpA protein synthesised was markedly increased. The results suggest that the inability to synthesise a complete Lipid A affects the regulation of the OmpA synthesis.

PLASMIDS

Plasmids are molecules that can be stably inherited without being linked to the chromosome. Plasmids are important in both medicine and agriculture as they confer antibiotic resistance in both man and animals (Hardy, 1984). They also code for toxins and other proteins which increase the virulence of pathogens. Certain plasmid genes are beneficial; some code for antibiotics while others code for a wide range of metabolic activities and enable bacteria to degrade compounds which would accumulate as pollutants if not degraded (Hardy, 1984)

Bacteria often contain two or more plasmids that can co-exist and are therefore said to be compatible. Incompatible plasmids are closely related to each other and cannot co-exist; after a few generations one or the other plasmid is lost. Plasmids are classified into incompatibility groups on this basis.

Plasmids may be either conjugative or non-conjugative. Conjugative plasmids transfer copies of themselves from one bacterium to another and many of them code for surface protein structures called sex pili. Large, stringent, plasmids are conjugative whereas relaxed ones are not although they can be transferred using the transfer apparatus of the conjugative ones.

Plasmids consist of double stranded covalently closed circular DNA (CCC). Most of the CCC plasmids isolated from bacteria are twisted to form supercoiled molecules that have superhelical twists. The CCC molecules range in size from 1md to more than 100md. For stable maintenance a plasmid must initiate and regulate its own replication and segregation. The replication of most large plasmids is stringently controlled with few copies per cell. Smaller plasmids are under relaxed control with many copies per cell.

3.1

Isolation of Plasmid DNA

Marmur et al (1961) who worked with S.marcesens utilised the differences in density between the plasmid, F lac, DNA and chromosomal DNA as a basis of plasmid DNA isolation. This method was disadvantageous as the putative plasmid had to be transferred to a host with DNA of different GC and also there was breakage of DNA molecules.

Hickson et al (1967) formed bacterial sphaeroplasts and then lysed them with an ionic detergent e.g. sodium dodecyl sulphate. Protein was removed by extraction with buffered phenol. Phenol was removed by dialys-is and the DNA was centrifuged in a caesium chloride gradient.

There are many procedures that involve a short, clearing spin after cell lysis to pellet cell debris and chromosomal DNA. The cleared lysate is then purified of protein and centrifuged in a caesium-chloride gradient. (Katz et al, 1973).

A method involving ethidium bromide was used (Hickson et al, 1967).This chemical binds to DNA by intercalating between the base pairs, a process causing the helix to unwind. CCC plasmid DNA has no free ends and thus can only unwind to a limited extent. This limits the amount of dye that the plasmid DNA can bind. No such constraint applies to chromosome DNA. As the binding of the ethidium bromide reduces the density of the molecule the plasmid DNA acquires a higher buoyant density than chromosomal DNA. Hence the two DNA types band at different points in a caesium chloride density gradient during ultracentrifugation.

Alternative methods for removal of chromosomal DNA include heat (Kado and Liu, 1981) or alkali denaturation (Cohen and Miller, 1969) which are dependent on the resistance of CCC DNA to denaturing by these agents. Some authors have combined alkali denaturation with the clearing centrifugation (Hansen and Olsen, 1978; Birnboim and Doly, 1979). Protein can be removed at this stage, either enzymatically with pronase, or chemically with chloroform and phenol, and RNA can be removed with RNAase prior to concentration of the remaining DNA. This is commonly done by precipitation with ethanol (Currier and Nester, 1976) or with

polyethylene glycol(Humphreys et al ,1975).

Although plasmid DNA can readily be separated on CsCl gradients, more usually, these days, if it is merely required to demonstrate the presence of the plasmid, then separation by agarose gel electrophoresis is used.

3.2

Plasmid Replication

For its stable maintenance in bacterial cells, a plasmid must replicate at least once in each cell cycle and ensure segregation at cell division, so that at least one copy is passed to each daughter cell. Any replicating plasmid must have an origin of replication (Rowbury, 1977).

Plasmid replication can proceed either unidirectionally or bidirectionally. For larger plasmids e.g. R6K, replication is bidirectional; there appears to be a fixed terminus which is located 20% of the genome size from this origin. In the majority of molecules replication proceeded unidirectionally from the origin until 20% of the molecule was reached and then from the origin to the terminus in the other direction to complete the replication of the R6K molecule (Lovett et al, 1975). In this case , replication is as ymetrically bidirectional.

Plasmid replication can be divided into three stages. In those plasmids in which it has been studied, initiation proceeds from a single origin, followed by elongation which may be unidirectional or bidirectional and finally termination occurs preceeding segregation.

Plasmids can be placed in two classes depending on control of their replication, The replication of one class is strictly controlled in relation to chromosome replication such that there are one or two plasmid copies per chromosome (stringent replication). The plasmids showing relaxed control are however present at 10-50 copies per chromosome.

3.3

Incompatibility

Incompatibility is defined operationally as the inability of two (or more) closely related plasmids to co-exist stably in the same cell-line (Timmis, 1979). The phenomenon has provided a basis for plasmid classification and in Gram-negative bacteria more than 20 incompatibility (Inc) groups have been defined in this way (Datta, 1975). When two plasmids belonging to the same Inc group are present in the same cell only one will be stably inherited. Members of the same Inc group are usually closely related and at least partially homologous; they usually encode related pili and conjugation systems.

3.4

Conjugation

Many plasmids are conjugative. They have a cluster of genes that enables them to transfer copies of themselves from one bacterium to another. Plasmids can also be transferred in ways that do not depend on plasmid encoded products, that is by bacteriophage mediated transduction or transformation.

Conjugative plasmids have been isolated from a diverse range of Gram-negative bacteria. Most of these plasmids are known to encode pili (sex or conjugative pili) which are required to initiate cell to cell contact which precedes DNA transfer. This probably involves recognition of a suitable receptor on the recipient cell surface. The aggregate pairs thus formed are then stabilised by an unknown process, which may involve the OmpA protein (Manning and Achtman, 1979). Although it is clear that binding of the pilus tip to the recipient cell is essential in conjugation (Ou and Anderson, 1972a, b; Ou, 1973) the subsequent function of the sex pili is still unclear. One suggestion is that the pilus acts as a conduction tube or bridge through which DNA passes fron the donor to the recipient cell (Brinton, 1971). Another is that pilus retraction brings the mating cells together in intimate contact and that close contact is essential for DNA transfer (Curtiss, 1969; Marvin and Hohn,

1969). The retracted pili might serve as a channel to allow DNA transfer; alternatively this could involve a direct interaction between donor and recipient cell envelopes at the point of contact (Willets and Wilkins, 1984).

Genetic analysis has identified a region of the plasmid (tra region) which is required for conjugation and comprises at least 20 genes, almost all of which lie in a single operon. These genes are involved in the synthesis and assembly of pili, the stabilisation of mating aggregates, some aspects of DNA transfer itself and in the control of transfer functions. Most of the Tra proteins are located in the cell envelope (Manning and Achtman, 1979; Willets and Skurray, 1980; Willets and Wilkins 1984). The organisation of the tra operon of the F plasmid is given in figure 13.

The DNA transfer itself in conjugation has only been studied in detail for a few plasmids in E.coli primarily for F and F-like plasmids and to a lesser extent some I-like plasmids (Willets and Wilkins, 1984). This process involves nicking and initiation at the origin of transfer site, separation of the two plasmid DNA strands single strand transfer, complementary strand synthesis in both donor and recipient and recircularization of the plasmid. In addition to plasmid specified products, host cell proteins are also required.

The F-like and I-like plasmids of *E.coli* specify pili and transfer systems which resemble those encoded by F and ColI respectively. The transfer properties of most of these plasmids are naturally repressed. The F plasmid and several F-like ColV plasmids are naturally derepressed. Many F-like plasmids not only repress their own transfer properties but inhibit those specified by F (or other derepressed F-like plamids) when present in the same host.

3.5. Properties specified by plasmids

Some of the properties specified by plasmids are resistance to antibiotics, bacteriocin production, and degradation of organic compounds.

3.5.1. Resistance to antibiotics

Classically there are four mechanisms of antibiotic resistance: (a) Inactivation of the antibiotic (b) modification of the target site of the antibiotic (c) alteration to cell permeability (d) bypass of the inhibited step, for example by enzyme substitution (Foster, 1983).

3.5.2.

Bacteriocin Production

Many plasmids specify the production of extracellular toxins termed bacteriocins. Such plasmids are called bacteriocinogenic factors and are given names indicating their origins; e.g. Col factors from *E.coli*

Col factors specify colicin production (Hardy 1975). Some are large, conjugative and present as only a few copies per cell; others may be small and non-conjugative. Colicinogenic strains are usually immune to the specific colicin they produce because they produce a protein immunity factor which binds the colicin.

The colicins produced are generally much larger than antibiotics such as streptomycin and chloramphenicol and the cell walls are of course, impermeable to proteins (Hardy, 1984). The question as to how these large proteins kill bacteria is particularly interesting. Some of the colicins kill cells by affecting intracellular target sites like the ribosomes or DNA whilst others increase the permeability of the cytoplasmic membrane (Hardy, 1984). In almost all cases so far studied, colicin action is initiated by adsorption of colicin molecules to specific outer membrane receptor proteins. In many instances colicin resistance is due to alteration or loss of these receptors. However, the adsorption of a particular

colicin is not sufficient to ensure strain sensitivity. The so-called colicin tolerant strains retain an active receptor but are blocked in some step subsequent to receptor binding. Many of these mutants show an alteration in cell envelope structure or function.

Receptors are frequently common to several colicins and serve as attachment sites for phages. In addition many colicin receptors are involved in the uptake of various metabolites such as vitamin B_{12}, ferri-enterochelin, or nucleosides.

3.5.3

Other plasmid specified properties

The degradation of many organic compounds such as salicylate, camphor, octane and benzoate by pseudomonads has been shown to be plasmid specified (Wheelis,1975) as is resistance to many metal ions. Enterotoxin and haemolysin production and formation of adherence factors in enterobacterial pathogens are plasmid specified; Col V plasmid is associated with increased pathogenicity of E.coli (Williams Smith and Huggins, 1976).

3.6.

Properties of ColV IK-94 plasmid

3.6.1.

Preliminary remarks

Historically Col V plasmids have been found associated with extraintestinal E.coli infections. It was not until 1974 that the possible significance of this association was recognized, following a study by Williams Smith of transferable virulence properties in E.coli (Williams-Smith 1974). Seven Col V plasmids when transferred to a non-pathogenic recipient ,each gave transconjugants of increased lethality for chickens and mice. This was not found with the other Col plasmids examined. In a survey in the same study the high frequency of Col V+ strains among bacter aemic isolates from various sources was revealed. Subsequent studies of human clinical isolates tended to confirm this (Minshew et al, 1978a;b; Davies et al, 1981; Milch, et al, 1984).

There are significant differences in the behaviour of otherwise isogenic Col V+ and ColV- strains during the course of experimental infections. in animals. Following injection into chickens,mice or colostrum-deprived calves, ColV+ organisms were found in greater abundance than the corresponding Col V- in various tissues,

especially the blood and peritoneal fluid, but including lymph nodes, liver, spleen, lung, muscle, kidney, brain, cerebrospinal fluid, (Smith, 1974 and Smith and Huggins, 1976). This effect was not due to killing of sensitive organisms *in vivo* by colicin V, showing that the Col V plasmid somehow contributes to invasivity. Similarly, the presence of either ColV,IK-94 or ColV- H247 allowed K12 strains to grow in diffusion chambers implanted into the peritoneal cavity of rabbits, which isogenic Col- strains were unable to do (Finn *etal*, 1982).

Col V,IK-94 was first isolated by Fredericq in a strain (*E.coli* K-94) isolated from a patient suffering from a *S.paratyphi* B infection (Fredericq and Joiris, 1950). It is a large conjugative plasmid F-like with a molecular weight of approximately $95x10^6$. It is present in the cell as one or two copies per chromosome. It codes for both colicin V and colicin Ia and their immunity. Col VIK-94 can be termed a virulence plasmid in that it confers enhanced pathogenicity on some *E.coli* strains that harbour it.

Col VIK-94 resembles the F plasmid in both transfer properties and incompatibilty. There is a virtual homology between the tra regions of the F plasmid and the ColVIK-94 (Sharp *et al* 1973) although they are not identical as they have different surface exclusion systems (Willets and Maule, 1973). The transfer properties are derepresed and susceptible to repression by fin+ plasmids (Hardy, 1975).

3.6.2

Adhesive properties conferred by Col VIK-94

A widely recognized and well characterized virulence property of most pathogenic bacteria is their ability to adhere to the host cell (Smith,1984; Smith, 1977). This property usually correlates with the presence of pili or fimbriae (Ottow, 1975). An increased ability of K-12 strains to adhere to mouse intestinal epithelium has been correlated with the presence of Col V-711 and was accompanied by the presence of pili of longer, thicker dimensions than common pili (Clancy and Savage, 1981). Adhesion to cultured mammalian cells in greater numbers compared to Col- cells has also been observed for organisms bearing Col VIK-94 (Tewari, 1986). A possible related ColV, IK-94 specified phenomenon is autoagglutination apparent as the formation of clumps in static culture (Tewari et al, 1985; Rowbury et al, 1985). This is probably mediated by the sex pili since it correlated to the derepressed transfer properties. It is associated with a general increase in hydrophobicity of the cell surface. One possible effect of autoagglutionation if active in serum is an inhibition of phagocytosis, by sheer size of clumps and by reduction in motility of clumped organisms.

3.6.3.

Serum resistance

The bactericidal effect of serum is due to the complement system (Taylor, 1983). The two major effects of complement are a deposition of C3b on the activation surface leading to susceptibilty to phagocytosis and in the case of the lipid bilayer membranes, the formation of a membrane attack complex (MAC) composed of C5b, C6, C7, C8, and C9 in the membrane. In the case of Gram-negative bacteria this would lead to a disruption of the outer membrane (Wright and Levine, 1981). There may also be damage to the cytoplasmic membrane.

Plasmid encoded serum resistance of Col V strains was first observed by Smith (1974) who measured survival of *E.coli* strains in chicken serum. The difference between otherwise isogenic Col- and Col+ strains was lost in heated serum indicating that complement activity was responsible. This serum resistance phenotype has been confirmed in studies of Col-/ColV+ pairs of wild type and K-12 strains (Quakenbusch and Falkow, 1979; Binns *et al*, 1979; Binns *et al*, 1982; Aguero and Cabello, 1983). However, in surveys of clinically isolated strains, serum resistance does not ne cessarily correlate with carriage of Col V plasmids (Hughes *et al* 1982) indicating the multifactorial nature of *E.coli* virulence.

The serum resistance determinant of Col VIK-94 is iss; another outer membrane serum resistance factor found associated with Col V and other F-like plasmids is Tra T, a transfer component involved in surface exclusion.

3.6.4.

Iron chelation

A major factor inhibiting the growth of micro-organisms in body fluids is the low availability of iron. This is due to the iron being complexed with carrier proteins such as transferrin or lactoferrin. Enterobacteria specify one or more of the three high affinity iron chelation mechanisms, all of which serve to extract iron from the environment; namely the enterobactin (enterochelin) system; the ferrichrome system; the citrate system (Rosenberg and Young, 1974).

A recently recognized bacterial virulence property is the possession of additional, sometimes plasmid specified iron chelation mechanisms as observed in E.coli (Williams, 1979; Stuart et al, 1980; Williams and Warner, 1980). Such a system is specified by Col VK-30 and various Col V plasmids but not Col VIK-94. The importance of this novel mechanism is shown by the ability of ColV-H247 organisms to outgrow isogenic Col- in experimental infections of mice in the absence but not in the presence of 20mM ferric

ammonium citrate, and by their ability to grow in mimimal medium in the presence of transferrin (Williams, 1979).

The siderophore involved is aerobactin (Braun, 1981; Warner et al, 1981) a hydroxamate consisting of two N-acetylated N-hydroxylated lysine derivatives linked to the α and γ carboxyl groups of a single residue of citric acid (Konopka et al, 1982). The advantages of such a system over the more usual enterobactin system may be due to the lower aqueous solubility, higher antigenicity, lower stability and greater pH dependence of iron complexes of enterobactin compared with those of aerobactin and to enterobactin being more expensive in terms of energy.

3.7

Colicin V

Colicin V was the first colicin to be described. It was described following the observation that E.coli V, a strain isolated from an infected rabbit and so named for its virulence inhibited growth of other E.coli strains (Gratia, 1932). It is the smallest colicin known; about 4 k while others are 27-80 k (Hardy, 1975). The small size leads to greater diffusibility and hence the zones in the agar overlays are large (Davies et al, 1981) though size depends on the host. Due to the colicin's small size it may be classified as a microcin, a class of antibiotic polypeptides which are produced by

some *E.coli* strains (Baquero and Morena, 1984; Yang and Konisky, 1984).

The mechanism by which colicin V kills sensitive cells involves disruption of the energy transducing cytoplasmic membrane, leading to dissipation of proton motive force and leakiness of the membrane (Yang and Konisky,1984). Successful interaction between colicin V and sensitive cells is dependent on several membrane components. These are *tonB*, *exbB*, *cir*, *cvt*, *crt* (Davies and Reeves, 1975b) and *feuA* (Hancock and Braun, 1976). Colicins V and I seem to share a common receptor; there is much controversy over which of the above performs this role, *cir* and *feuA* being the contenders. Membranes isolated from mutants lacking either of these proteins failed to absorb colicin V. Both these proteins probably have a role in ferric-enterochelin uptake. Colicin V resistant strains are unable to transport enterochelin (Hantke and Braun, 1975).

Following the cloning of immunity and structural genes for colicin V into well-characterized amplifiable cloning vectors (Frick *et al*, 1981), and development of methods to stabilise colicin activity, Konisky and co-workers have initiated studies on the mode of action of colicin V (Yang and Konisky,1984). These investigators have found that colicin V treated *E.coli* was inhibited in active transport of proline and was unable to generate a membrane potential. Colicin V also prevented membrane potential formation by isolated cytoplasmic membrane vesicles. These results strongly

suggest that the primary target of colicin V is the cytoplasmic membrane.

3.8.

Col BK-98

The Col BK-98 plasmid has a molecular weight of 70×10^{6} . It may exist as one or two copies a cell. It belongs to the F111 incompatibility group and it has repressed transfer properties. It codes for colicins B and M. At present very little is known about this plasmid.

3.9.

Colicin B

Very litle is known about the mode of action of colicin B. Crude preparations of colicin B inhibited oxygen uptake, oxidative phosphorylation, uptake of o-nitrophenyl-β-galactosides and synthesis of RNA, protein and DNA (Arima et al, 1968) suggesting general impairments of energy metabolism. Pressler et al, (1986) managed to isolate a pure sample of colicin B. They started from a strain that contained the colicin activity and immunity genes

cloned on a multicopy plasmid. This enabled them to provide evidence that the bactericidal action of colicin B is based on the inhibition of the membrane potential by channel formation in the cytoplasmic membrane. Colicin B was shown to form small, ion permeable channels. They showed that active colicin B consisted of a single polypeptide of Mwt about 60 000. Pressler et al (1986) showed that colicin B inhibited the membrane potential dependent transport of proline and enhanced the uptake of α-methylglucoside via the phosphoenolpyruvate dependent phosphotransferase system.

Cells of E.coli containing the cbi locus are immune to colicin B. The nucleotide sequence of the cbi region was determined. It contains an open reading frame for a polypeptide of 175 amino acids. The immunity protein was identified by the overproduction of cbi (Schramm et al, 1988). The protein contains four large hydrophobic regions. The colicin B immunity protein was found mainly in the cytoplasmic fraction (Schramm et al, 1988).

SECTION 4

AIMS OF THIS STUDY

The aims of this study are:

(a) to investigate the effects of colicinogenic plasmids on sensitivity of *E.coli* to hydrophilic, hydrophobic and aminoglycoside antibiotics.

(b) to study whether growth inhibition by these antibiotics can be reversed by divalent cations such as magnesium and calcium.

(c) to study how prior growth with phosphate influences on growth inhibition.

(d) to study the effects of temperature on growth inhibition.

(e) to investigate as far as possible any changes in the outer membrane components between the Col+ and the Col- strains.

(f) to study the effects of storage temperature on the production of colicin zones by the Col B plasmid.

Section 5

MATERIALS AND METHODS

5.1

Bacterial strains and plasmids used

The identities and properties of the bacterial strains and plasmids used in this study are given in tables 3 and 4. An agarose gel showing Col VIK-94 and Col BK-98 is given in figure 13a.

5.2.

Maintenance of stocks

For long term storage, the bacteria were stored in nutrient broth containing 15% glycerol at -70°C. For shorter term storage, cultures, nutrient agar plates or slopes stored at 4°C were used. The latter were regularly sub-cultured at monthly intervals from glycerol stocks.

5.3

Media

Oxoid nutrient broth No.2 was used as a rich medium at the recommended concentration of 25g/litre. The minimal medium was

Table 3

Bacterial strains used in this study

Strain	Genotype	Characteristics	Reference or Source
1829	trp		Finnegan and Willets (1971)
P678-54	thr, leu, thi lacY, S^r ara, xyl	Minicell producer	Adler et al (1967)
D21	pro, his, trp	Parent strain	Boman
D21f1	pro, his, trp	Mutant of D21e7 glucose deficient LPS.	Boman
D21e7	pro, his, trp	Mutant of D21; Galactose deficient LPS	Boman

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S: streptomycin

trp: tryptophan; thr: threonine; thi: thiamine; ara: arabinose; xyl: xylose; pro: proline; his: histidine.

Table 4

Plasmids used in this study

Plasmid	Incompatibility Group	Genetic Markers	Transfer Properties	Reference or Source
Col VIK-94	Inc F1	Colicins V&Ia	Derepressed	Hardy
Col VK-30	IncF 1	Colicin V	Derepressed	Williams
Col BK-98	IncF111	Colicins B & M	Repressed	Puig& Nagel de Zwaig (1964)
Col VM50(1)	Inc F1	Colicins V & Ia	Absent	This lab.
Col VM40(5)	Inc F1	None	Derepressed	This lab.
R124	Inc F1V	Tc	Repressed	Hedges & Datta(1972)

Tc= tetracycline resistance

Col VIK-94
Col BK-98

Fig. 13a

Agarose gel of the Col VIK-94 and Col BK-98 plasmids.

Lanes 2) the marker strain λ

4) 1829

5) Col VIK-94

6) Col BK-98

Table 5

Antibiotics used in this study

Antibiotic	Mechanism of action
Rifampicin	Rifampicin is a 3-(4-methylpiperazinyl-iminomethyl) derivative of rifamycin SV. It binds to the ß-subunit of RNA polymerase. Inhibits initiation of RNA synthesis.
Novobiocin	Active on subunit B of DNA gyrase. The swivelase component of DNA gyrase is not affected by this antibiotic.
Erythromycin	Binds to the 50S ribosomal subunit. Inhibits protein synthesis. Interferes with positioning of peptidyl-tRNA and protein translocation.
Rifamycin	Inhibitor of RNA polymerase (see above)

Vancomycin	Acts on the acyl-D-ala-D-ala termini of pentapeptide of N-acetyl-muramyl-pyrophophoryl C_{55} -isoprenoid, hence interfering with peptidoglycan synthesis
Oxacillin	
Ampicillin	Act on transpeptidation reaction involved in the cross-linking process of peptidoglycan synthesis.
Carbenicilin	
Cephalothin	Its main action is to inhibit the cross-linking process of peptidoglycan synthesis.
Minocycline	Acts on protein synthesis. It is a tetracycline derivative.
Tetracycline	Prevents enzymatic and non-enzymatic binding of aminoacyl tRNA on the A site of the ribosome. Chelates divalent cations. Inhibits polypeptide chain termination.
Spectinomycin	Inhibits the translation process

	of protein synthesis. Binds reversibly to the 30S subunit of the ribosome.
Streptomycin	Binds to the 30S ribosomal protein S12, thus preventing chain initiation and elongation. Streptomycin binding causes release of peptidyl-tRNA and concomittant polysome breakdown.
Kanamycin	Inhibitor of protein synthesis. Produces mis-reading of mRNA.
Gentamicin	Inhibitor of protein synthesis
Polymyxin B	Produces disorganization of the cytoplasmic membrane. Binds in place of divalent cations to the negatively charged amphipathic molecules causing disruption of the membrane lipid packing.

adapted from the recipe of Davis and Mingioli (1950). It contained 30mM dipotassium hydrogen phosphate, 22mM potassium dihydrogen phosphate, 0.4mM magnesium sulphate, 7mM ammonium sulphate and 1mM sodium citrate supplemented with 0.2% glucose as a carbon source. The appropriate amino-acids were added at 20µg/ml. Where appropriate, antibiotics, detergents and other inhibitors were added to the above media as shown in the relevant results section.

5.4.

Growth conditions

Cultures were initially grown overnight with shaking at 37° C. In most experiments exponential cultures were used. To prepare these, overnight cultures were diluted into fresh medium and shaken for 2-3 hours at 37°C.

5.5.

Measurement of optical density

The optical density of cultures was measured with a Hilger photoelectric colorimeter. Filter used for broth cultures was 550µm filter.

5.6.

Antibiotic sensitivity tests

There were two methods used in this study: (a) Multo disc - 0.1 ml. of an overnight nutrient broth culture was added to 4ml. soft agar and used as an overlay on a nutrient agar plate. Once the soft agar was set, an Oxoid multodisc was placed on the nutrient agar surface. Zones of inhibition were measured after an overnight incubation at 37°C to give the relative sensitivities to antibiotics. (b) growth curve method- overnight cultures were diluted into flasks containing nutrient broth and grown to exponential phase at 37°C. These exponential cultures were then diluted down into flasks containing 27ml.nutrient broth. Appropriate antibiotics or other inhibitors were added at suitable concentrations and the flasks were shaken at 37°C for 3 hours. Optical density readings were taken every 30 minutes. Comparisons were made with flasks that contained no antibiotic. Where appropriate, 0.05M magnesium sulphate or 65mM phosphate were added to the flasks. In some experiments the strains were grown overnight at 25°C and then grown to exponential phase at 25°C to investigate the effect of temperature on bacterial growth in the presence of antibiotics. Where the standard errors have been given in section 6 the experiments have been performed seven times; otherwise the results given are from a single experiment performed three times with consistent results.

5.7.

Colicin sensitivity tests

Colicin-producing strains were streaked onto nutrient agar plates and incubated at 37°C, then surface killed by exposure to chloroform vapour for ten minutes. After allowing the chloroform vapour to evaporate, the plates were overlaid with 4ml. soft agar containing 0.1ml. indicator cells. The plates were left to incubate overnight at 37°C. The zones of inhibition produced indicated sensitivity to colicin. In this way both colicin production and immunity to colicin were determined. Unless otherwise stated, strain 1829 was used as indicator.

5.8.

Effect of temperature on colicin production of Col BK-98

Strain 1829 Col BK-98 was streaked onto nutrient agar plates and left to incubate overnight at 37°C. The next day the plates were left to incubate at temperatures of 10°C, 15°C, 20°C, 25°C and 30°C for an overnight and a seven day incubation period after which they were tested for colicin production. The sizes of their zones of inhibition were measured. The controls used consisted of 1829 Col BK-98 being tested for colicin production after an overnight and a one week incubation at 37°C. The zones of

inhibition were measured and compared with those produced when 1829 Col BK-98 was incubated at the other temperatures.

5.9.

Effect of storage at 4°C for one week on the colicin production production of 1829 Col BK-98

Strain 1829 Col BK-98 was streaked onto nutrient agar plates and left to incubate overnight at 37°C. The next day the plates were left at 4°C for one week. At days 0, 1, 3, 5, and 7 the plates were tested for colicin production and their zones of inhibition measured and compared with the zones of inhibition of the control cultures. The controls consisted of 1829 ColBK-98 incubated at 37°C and tested for colicin production at days, 1, 3, 5 and 7.

5.10.

Effect of sodium deoxycholate on the colicin production of 1829 ColBK-98 after storage for one week at 4°C

Strain 1829 ColBK-98 was streaked onto nutrient agar plates containing 0.12% sodium deoxycholate and grown overnight at 37°C. The plates were then stored at 4°C for one week. At days 0,1, 3, 5 and 7 the plates were tested for colicin production and their zones of inhibition were measured and compared with

control cultures. The control cultures were 1829 Col BK-98 grown on nutrient agar containing 0.12% sodium deoxycholate incubated at 37°C and tested for colicin production at days 0, 1, 3, 5, and 7 and cultures grown on nutrient agar overnight at 37°C and then stored at 4°C.

5.11.

Plasmid transfer

Plasmid transfer was carried out in two ways;

(a) Broth mating- this was for easily transferable plasmids. Exponential cultures of overnight grown donor and recipient strains were mixed in the ratio 1:2 (2.5ml. donor, 5ml. recipient and 7.5ml. nutrient broth). The mixtures were incubated statically at 37°C for approximately 4 hours, after which time dilutions were made in 0.7% saline , plated out on selective media and incubated at 37°C.

(b) Filter mating-this was used for plasmids that were poorly transferable. Donor and recipient strains were grown overnight at 37°C, diluted down the next day and grown to exponential phase with shaking for two to three hours at 37°C. 1 ml. aliquots of donor and recipient strains were filtered through a 'Multipore' membrane filter disc. The filter discs were placed on nutrient agar plates and left to incubate for approximately 5 hours at 37°C. The cells were then dislodged from the filter paper by

vortexing in 0.7% saline solution. Dilutions of the mixture were made and plated out on selective media as 0.1 ml. aliquots. Controls of donor and recipient cultures were treated in the same way and plated out separately on selective media to check on growth.

5.12.

Estimation of the % viability on copper sulphate

Strains of bacteria were inoculated into 10ml. nutrient broth and grown overnight at 37°C. Dilutions of these were then made and plated out on nutrient agar containing copper sulphate (from 0-800µg/ml) and incubated overnight at 37°C. The colonies were counted the next day and the % colony formation was subsequently calculated. All experiments were repeated 4 times with consistent results. The results tabulated were from a single experiment.

5.13.

Isolation of the cell envelope

10 ml. nutrient broth was inoculated and left to shake overnight at 37°C. Cells were harvested the next day by centrifugation at 9000r.p.m. for 15 minutes. The pellets were then washed with 5 ml. 0.9% saline solution and resuspended in 1.4 ml. 50mM Tris-HCL and frozen at -70°C for 15 minutes. The suspensions were then

left to thaw at room temperature and then the cooled cell suspensions were sonicated (6 × 15 sec) at maximum current of 1.5 A. The unbroken cells and large debris were pelleted by spinning for 2 minutes at 12000 r.p.m. in an MSE 'Microcentaur' microcentrifuge. The supernatants were removed and respun at the 12000 r.p.m. for 15 minutes. The resulting pellet, was resuspended in 20µl of 2mM Tris.

5.14.

Separation of the cytoplasmic and outer membrane

Cytoplasmic and outer membranes were separated using a modification of the method used by Schnaitman (1974). 0.5 ml. of the above suspension was diluted by 3ml. of 10mM Tris-HCL, pH 8.0 containing 5mM magnesium chloride. TritonX-100 was added to a final concentration of 2% and the mixture was shaken for 15 minutes at 23°C. The Triton insoluble fractions which contained the outer membrane were collected by centrifugation at 50,000 r.p.m. for 1 hour at 4°C. The process was repeated again. The pellet was resuspended in 2mM Tris-HCL, pH7.8 and stored frozen. The pooled supernatants which contained the cytoplasmic membrane were added to 2 volumes 95% ethanol and allowed to stand overnight at -20°C to precipitate the proteins. The cytoplasmic membranes were collected by centrifuging at 38,000 r.p.m. for 1 hour at 4°C.

5.15.

Analysis of proteins by the SDS-Polyacrylamide Gel Electrophoresis System

Gels were prepared according to the method used by Laemmli (1970) with some modifications. 10% separating gels were made by mixing 14ml. stock acrylamide solution containing 30% acrylamide and 0.8% bis-acrylamide with 20ml. 0.75M Tris-HCl, pH 8.8; 3.6ml. distilled water; 0.4ml. 10% SDS; 2ml. 10mg/ml ammonium persulphate and 60μl TEMED. The stacking gel contained 3ml. stock acrylamide; 10ml. 0.25M Tris of pH 6.8; 0.2ml. 10% SDS; 5.8 ml. distilled water and 1ml. 10mg/ml ammonium persulphate and 60μl TEMED. Gels were run on a vertical apparatus using an electrode buffer that consisted of 25mM Tris, 200mM glycine and 0.1% SDS at 20mA constant current at room temperature.

Samples were first denatured by mixing 10μl sample with 40μl sample buffer and incubated at 100°C for 5 minutes. The sample buffer consisted of 60mM Tris-HCl of pH 6.8; 2% SDS; 10% glycerol; 0.001% bromophenol blue and 5% 2-mercaptoethanol.

The gels were stained overnight with Coomassie Blue (10% glacial

acetic acid; 0.1% Coomassie Brillant Blue R250 [Sigma] and 50% methanol. The gels were destained using several changes of 40% methanol and 10% glacial acetic acid.

5.16.

Isolation of plasmid DNA

Phenol chloroform: 5g phenol was dissolved in 5ml. chloroform.
Lysis solution: 3% SDS in 50mM tris-HCl; pH to 12.6 with NaOH.
TE buffer: 10mM Tris-HCl, 1mM EDTA; pH 8.0.

The miniprep method used was a modification of the method used by Kado and Liu (1981). The bacterial cells were grown overnight with good aeration at 37°C in L-broth. 1.5 ml. of the cultures was centrifuged in sterile microcentrifuge tubes for 5 minutes at maximum speed in a microcentrifuge. The pellets were washed once and resuspended in 350µl of distilled water. 700µl of lysis solution were added and mixed gently. The cells lysed and the suspension became viscous. The cell suspensions were then incubated at 55°C for 1 hour. 350µl of phenol-chloroform were added and shaken well to obtain a uniform suspension which was then centrifuged at maximum speed for 30-60 minutes. The upper aqueous layer was carefully removed and transferred to a fresh tube. 1.25 volumes of isopropanol were added and the tubes held at -20°C for 30 minutes. The microcentrifuge tubes were then

centrifuged at maximum speed for 15 minutes, and the tube then drained, re-filled with 70% ethanol and drained. The tubes were then inverted over some paper tissues and left to dry for several minutes. The pellets were then resuspended in 100µl of TE buffer.

5.17.

Agarose gel electrophoresis

Minigels, 5×7 cm. were run in a BRL horizontal minigel kit. Gels consisted of 0.7% agarose (Sigma) in a Tris borate electrode buffer (90mM Tris, 90mM boric acid, 2mM EDTA). The sample buffer used contained 4M urea, 50% sucrose, 50mm EDTA, pH 7.0, 0.1% bromophenol blue and was added to samples in a 1:3 ratio. Gels were run at 1-4V/cm until the tracking dye was within 1cm. of the end of the gel, then stained for 10 minutes with 1µg/ml ethidium bromide. Viewing was with a medium wave length ultra violet transilluminator and photography was with a Polaroid 600 SE camera through a Wratten no.9 filter.

RESULTS

SECTION 6

EFFECTS OF PLASMIDS ON INHIBITOR SENSITIVITY

A wide range of plasmids are known to enhance the virulence of pathogenic strains of E.coli and even to confer pathogenicity on strains which are non-pathogenic without them. The Col V plasmid seems to enhance virulence (Williams Smith, 1974; Williams Smith and Huggins, 1976). There is no similar evidence for Col B but like Col V+ strains, Col B+ strains have been isolated from clinical specimens including those from serious diseases like septicaemia and meningitis (Mercer et al, 1984). Accordingly, Col B elements like Col V may be virulence plasmids. It is very important to understand how these plasmids affect the survival of the cell both inside and outside the animal host.

In this study, the effects of Col B and Col V plasmids on inhibitor resistance and sensitivity have been studied in the hope that the presence of the plasmid may make it easier to eradicate the pathogen.

Initially the sensitivity of the Col V+ strains carrying the well- studied plasmids Col VIK-94 and Col VK-30 to hydrophobic

agents such as detergents, antiseptics, and hydrophobic antibiotics was tested. Tests were made in liquid medium at 37 °C because the results are often more reproducible than on solid media and they more closely resemble growth *in vivo*.

Normally *E.coli*, because of its outer membrane is resistant to hydrophobic antibiotics that are normally feasible for clinical use; so therefore *E.coli* infections have to be treated with hydrophilic agents such as β-lactams, tetracyclines or 4-quinolines; occasionally aminoglycosides are administered but their toxicity limits their usefulness.

Prior to this work, it had been noticed that ColV+ strains were more sensitive to detergents and the hydrophobic antibiotic erythromycin (de Pacheco *et al*, 1985) than Col- ones. It seemed possible therefore that ColV+ strains might be more sensitive than Col V-ones to hydrophobic agents and this was tested in the following experimental work.

6.1 EFFECTS OF COL V PLASMIDS ON INHIBITOR SENSITIVITY

6.1.1

Sensitivity of Col VIK-94+ strains to hydrophobic agents

Initially the work was done using strain 1829. Later we studied

Table 6

Sensitivity of 1829 Col VIK-94 to hydrophobic agents

Plasmid in strain 1829	% Growth inhibition			
	SDS %		Rifampicin (μg/ml)	
	3	5	7.5	15
None	35(±1.5)	51(±1.5)	26(±2.4)	45(±2.5)
Col VIK-94	95(±0.5)	98(±0.5)	58(±1.9)	79(±0.6)
	Novobiocin (μg/ml)		Oxacillin (μg/ml)	
	20	50	50	200
None	19(±1.7)	34(±1.7)	10(±1.2)	26(±1.5)
Col VIK-94	50(±1.2)	100(±0.5)	20(±1.0)	51(±1.2)
	Erythromycin (μg/ml)			
	5	10		
None	20(±2.1)	30(±2.1)		
Col VIK-94	50(±1.9)	54(±1.8)		

Col VIK-94 in P678-54. The hydrophobic agents studied in most detail were rifampicin, novobiocin, oxacillin, erythromycin and SDS. With each of these, Col VIK-94 produced a marked increase in sensitivity. Figures 14-15 show the results of individual experiments with rifampicin and novobiocin. Table 6 gives the means and standard errors for these agents tested in seven separate experiments.

The results in this and subsequent experiments are given as % growth inhibition compared to the control at 150 min. after adding the inhibitor.

The results in table 6 showed that Col VIK-94 in strain 1829 produced increased sensitivity to a range of detergents and hydrophobic antibiotics.

Other hydrophobic agents such as rifamycin, vancomycin, deoxycholate and Triton X-100 were also tested on strain 1829 and for each of those used in the experiments on table 7, Col VIK-94 again conferred increased sensitivity.

In order to show that the sensitivity to the hydrophobic compounds was due to the plasmid and not to transfer of the plasmid into an altered mutant form of 1829, a 1829 strain cured of its Col VIK-94 plasmid was used and rifampicin and novobiocin were tested on this strain. The results obtained are given in

Table 7

Sensitivity of 1829 Col VIK-94 to other hydrophobic compounds

Plasmid in	% Growth Inhibition							
strain 1829	Rifamycin		Vancomycin		Sodium Deoxycholate		Triton X-100	
	(μg/ml)		(μg/ml)		(%)		(%)	
	50	100	100	200	3	5	3	5
None	2	5	15	35	23	47	4	19
Col VIK-94	19	40	21	57	75	90	16	43

Table 8

Sensitivity of the cured 1829 Col VIK-94 to hydrophobic antibiotics

Plasmid in	% Growth Inhibition			
strain 1829	Rifampicin		Novobiocin	
	(μg/ml)		(μg/ml)	
	7.5	15	20	50
None	23	40	18	30
Col VIK-94	50	70	55	100
Cured strain	25	37	16	34

table 8. The results showed that the cured strain was as resistant as 1829 to the rifampicin and novobiocin.

It is clear from the results in tables 6 and 7 that the Col VIK-94 plasmid markedly increased the sensitivity of strain 1829 to hydrophobic antibiotics; it is possible that some hydrophobic antibacterials not normally used clinically against Gram-negative bacteria could be used against strains which have become virulent by the presence of the Col V plasmids. To ascertain whether this is feasible another strain carrying Col VIK-94 has been tested (see Table 9).

This strain used was P678-54. The Col VIK-94 plasmid was transferred into this strain by the filter mating method. The hydrophobic compounds used were novobiocin, rifampicin, SDS, oxacillin and erythromycin at concentrations the same as those used for 1829.

Col VIK-94 in P678-54 seems to increase sensitivity to rifampicin but not novobiocin (see Table 9). Subsequently other hydrophobic agents were tried on P678-54 Col VIK-94 and the results are tabulated in table 10. Col VIK-94 increased the sensitivity of P678-54 to each of the three new agents.

Table 9

Sensitivity of P678-54 Col VIK-94 to hydrophobic agents rifampicin and novobiocin

Plasmid in	% Growth Inhibition			
strain P678-54	Novobiocin (µg/ml)		Rifampicin (µg/ml)	
	20	50	7.5	15
None	6(±1.4)	16(±1.7)	15(±2.1)	23(±2.1)
Col VIK-94	7(±1.2)	18(±1.5)	43(±1.5)	75(±1.2)

Table 10

Sensitivity of P678-54 Col VIK-94 to other hydrophobic compounds

Plasmid in	% Growth Inhibition					
strain P678-54	SDS (%)		Oxacillin (µg/ml)		Erythromycin (µg/ml)	
	3	5	50	200	5	10
None	9	12	6	28	17	40
Col VIK-94	64	79	20	47	39	61

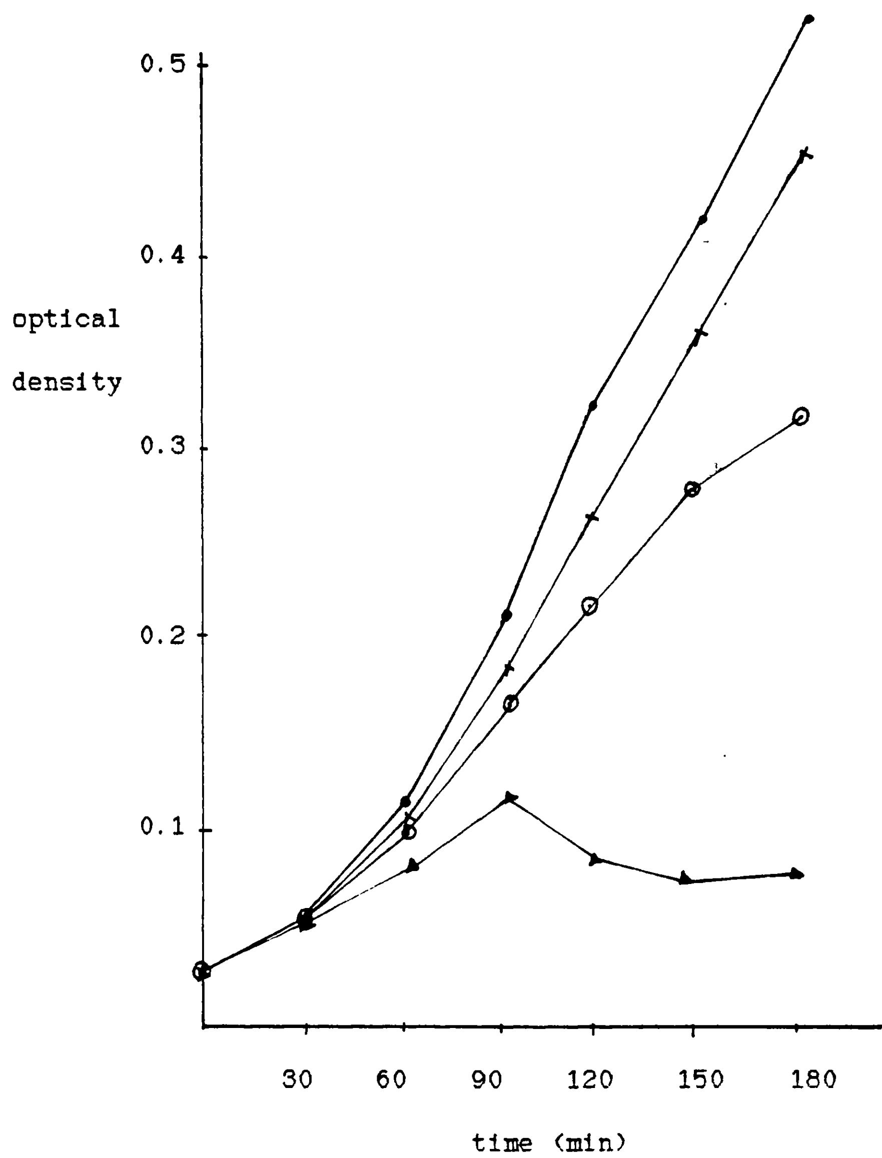


Fig.14. Effect of rifampicin on 1829 and 1829 Col VIK-94.

Standard growth curves performed in absence and presence of 15μg/ml of rifampicin.

•1829; - rifampicin

⊙1829;+ 15μg/ml rifampicin

× 1829 Col VIK-94;- rifampicin

▲1829 Col VIK-94 ;+15μg/ml rifampicin

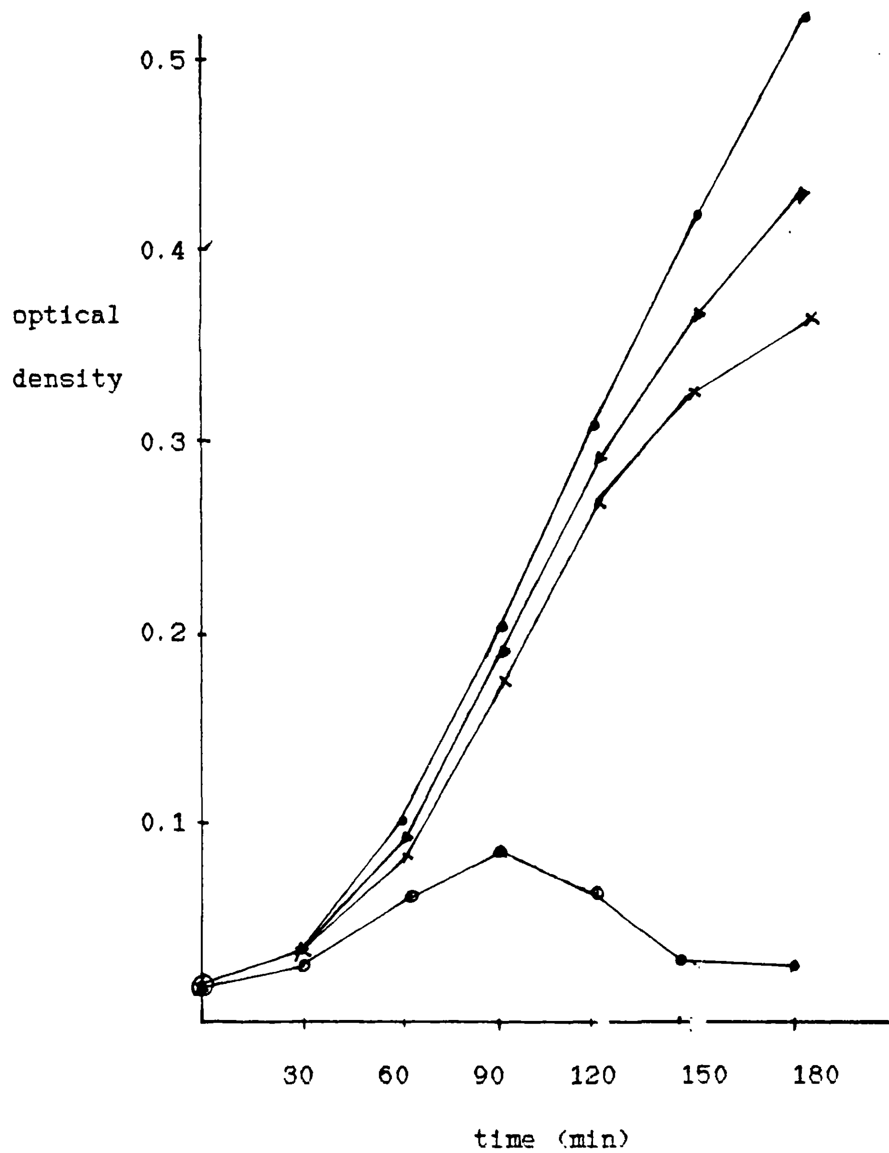


Fig.15. Effect of novobiocin on 1829 and 1829 Col VIK-94.
Standard growth curves performed in absence and presence of 50μg/ml of novobiocin.

•1829; - novobiocin

×1829;+ 50μg/ml novobiocin

▲ 1829 Col VIK-94;- novobiocin

○ 1829 Col VIK-94 ;+50μg/ml novobiocin

6.1.2

Sensitivity of Col VIK-94 bearing bacteria to other inhibitory agents

The finding that Col VIK-94 confers sensitivity to hydrophobic agents suggests a possible alteration in the LPS probably due to the presence of the plasmid.

If the LPS structural arrangement or amounts were altered without the porins being affected then Col VIK-94 bearing strains might be more sensitive than Col - ones to agents that enter *E.coli* via the LPS but unaltered in sensitivity to hydrophilic agents and metal ions that use the OmpF and Omp C pores.

6.1.2a

Sensitivity of Col VIK-94 bearing strains to hydrophilic compounds

If the presence of ColVIK-94 alters the LPS of strain 1829 on introduction but has no effect on the porins, then it may not sensitise the strain to the hydrophilic agents. Table 11 shows the results for ampicillin and carbenicillin. Both ampicillin and carbenicillin inhibited the 1829 Col VIK-94 substantially more than the Col- strain. Other hydrophilic compounds were subsequently tested. These were cephalothin, minocycline and tetracycline. Their results are given in table 12. Figure 16 shows the results of an individual experiment with ampicillin.

These results show that only cephalothin seemed to have a marked effect on the Col V bearing strain. Minocycline had a slightly greater effect on the Col V+ strain at 0.5 μg/ml but there was no increase in sensitivity with regards to tetracycline.

Two hydrophilic agents were tested on strain P678-54; these results are given in table 13. Col VIK-94 did not seem to alter the sensitivity to carbenicillin and ampicillin appreciably.

Table 11

Sensitivity of 1829 Col VIK-94 to ampicillin and carbenicillin

Plasmid in strain 1829	% Growth Inhibition			
	Ampicillin (μg/ml)		Carbenicillin (μg/ml)	
	1.0	2.0	2.5	5.0
None	20(±1.5)	25(±1.7)	25(±1.4)	40(±1.6)
Col VIK-94	42(±1.6)	67(±1.6)	43(±1.5)	84(±0.5)

Table 12

Sensitivity of 1829 Col VIK-94 to other hydrophilic antibiotics

Plasmid in strain 1829	% Growth Inhibition					
	Cephalothin (μg/ml)		Minocycline (μg/ml)		Tetracycline (μg/ml)	
	2.5	5.0	0.5	1.0	0.5	1.0
None	19	98	35	63	60	75
Col VIK-94	52	100	50	66	69	79

Table 13

Sensitivity of P678-54 Col VIK-94 to carbenicillin and ampicillin

Plasmid in strain P678-54	% Growth Inhibition			
	Carbenicillin (μg/ml)		Ampicillin (μg/ml)	
	2.5	5.0	1.0	2.0
None	12(±0.5)	24(±0.4)	8(±0.6)	7(±0.6)
Col VIK-94	20(±0.5)	40(±0.5)	13(±0.6)	20(±0.6)

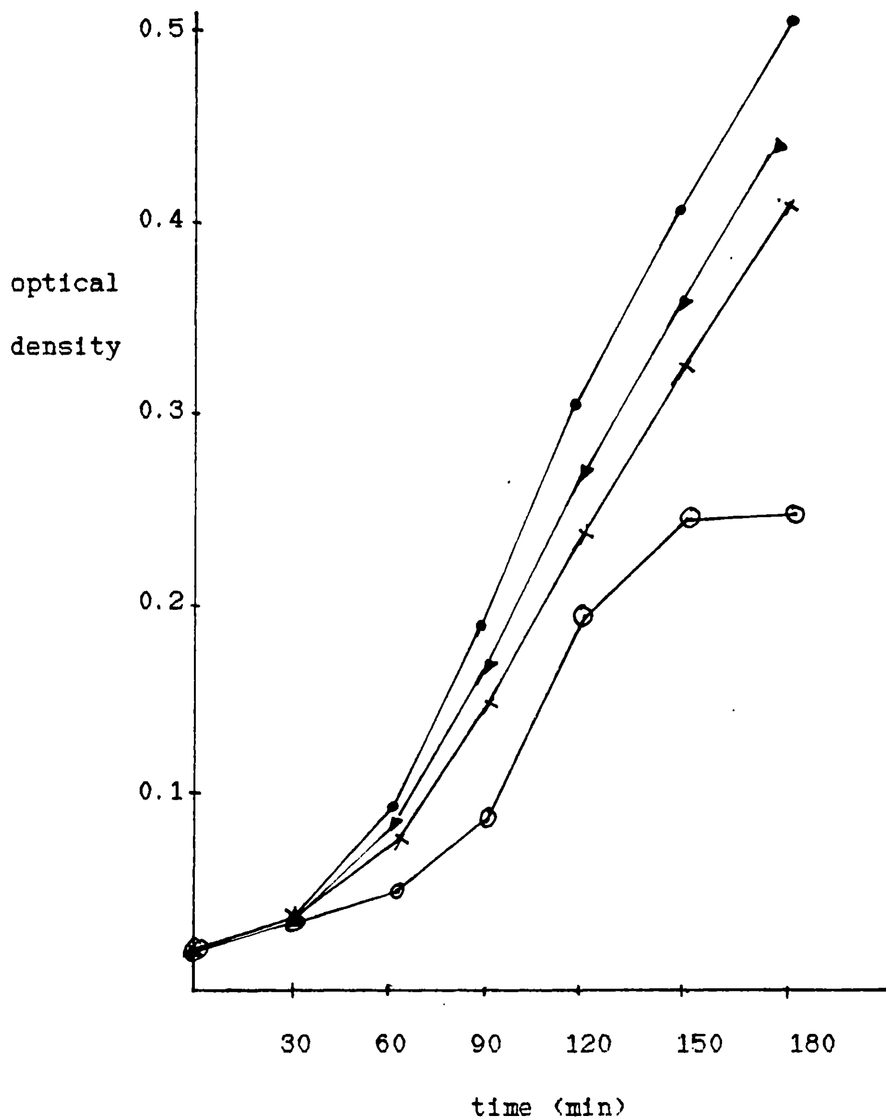


Fig.16. Effect of ampicillin on 1829 and 1829 Col VIK-94.

Standard growth curves performed in absence and presence of 1μg/ml of ampicillin.

• 1829; - ampicillin

× 1829; + 1μg/ml ampicillin

▲ 1829 Col VIK-94; - ampicillin

⊙ 1829 Col VIK-94 ; +1μg/ml ampicillin

6.1.2b.

<u>Sensitivity to aminoglycosides and other cationic compounds</u>

The aminoglycoside compounds tested here may be able to penetrate the outer membrane by passing through the LPS layer using the self-promoted pathway (Hancock, 1984). Any changes in the LPS structure or arrangement due to Col V encoded outer membrane components might therefore sensitise the cell.

The effects were studied in most detail for both spectinomycin and kanamycin (see Table 14). Figure 17 is the result of a single experiment with spectinomycin. The Col VIK-94 plasmid increased the sensitivity of 1829 to both compounds but especially to spectinomycin. Other cationic compounds were also tested and the results are recorded in table 15.

Col VIK-94 increased the sensitivity to streptomycin, gentamicin and 0.5 μg/ml polymyxin. Various experiments were also carried out with P678-54 Col VIK-94. The results are given in table 16. These results show that Col VIK-94 did not seem to alter sensitivity of P678-54 to aminoglycoside antibiotics appreciably.

Table 14

Sensitivity of 1829 Col VIK-94 to the aminoglycoside antibiotics

Plasmid in strain 1829	% Growth inhibition			
	Spectinomycin (µg/ml)		Kanamycin (µg/ml)	
	5	10	1.25	2.0
None	20(±1.7)	49(±1.7)	40(±1.5)	68(±1.5)
Col VIK-94	44(±1.5)	85(±0.7)	66(±1.2)	75(±1.4)

Table 15

Sensitivity of 1829 Col VIK-94 to other cationic antibiotics

Plasmid in strain 1829	% Growth Inhibition					
	Streptomycin (µg/ml)		Gentamicin (µg/ml)		Polymyxin (µg/ml)	
	0.25	0.5	0.1	0.2	0.5	1.0
None	10	17	23	41	70	98
Col VIK-94	33	61	70	82	98	98

Table 16

Sensitivity of P678-54 Col VIK-94 to aminoglycoside antibiotics

Plasmid in strain P678-54	% Growth Inhibition			
	Spectinomycin (µg/ml)		Streptomycin (µg/ml)	
	5	10	0.25	0.5
None	35(±2.0)	59(±1.7)	8(±1.7)	15(±1.3)
Col VIK-94	45(±2.2)	67(±1.8)	10(±0.9)	20(±1.7)

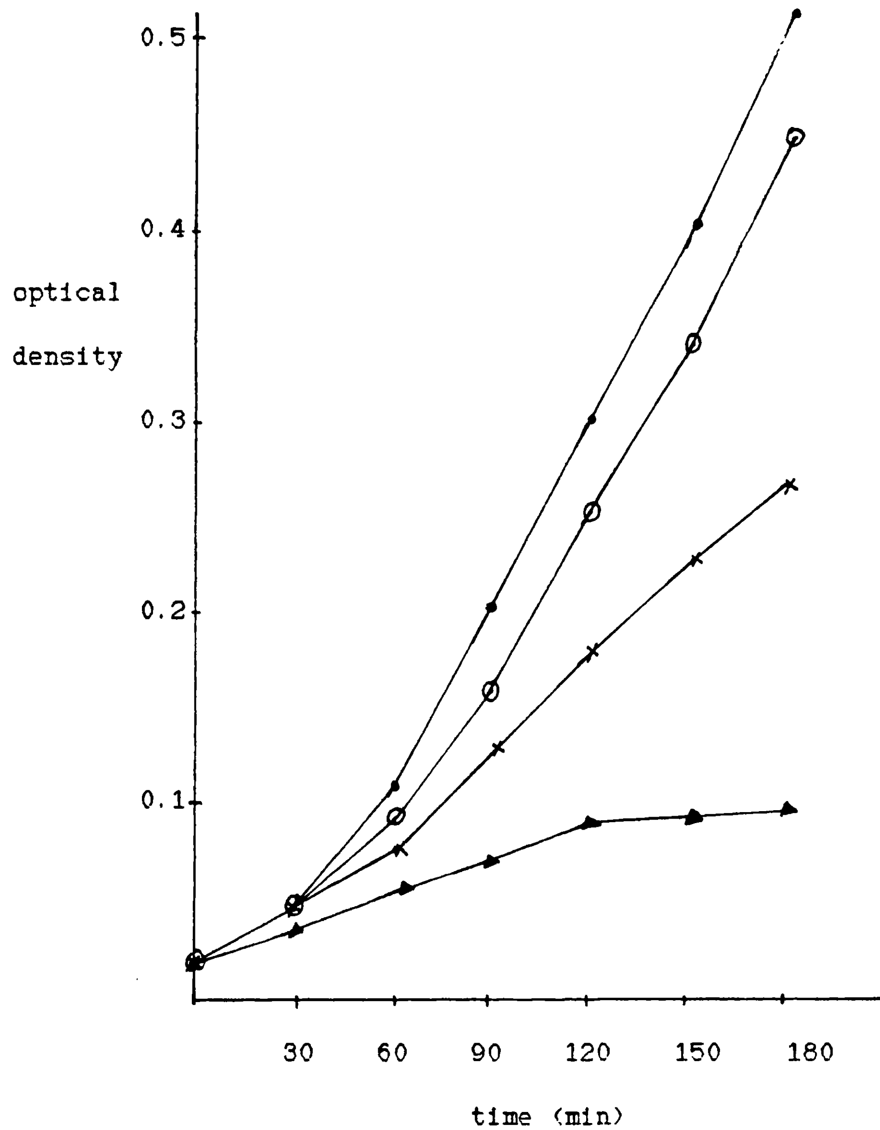


Fig.17. Effect of spectinomycin on 1829 and 1829 Col VIK-94.

Standard growth curves performed in absence and presence of 10μg/ml of spectinomycin.

• 1829; - spectinomycin

× 1829;+ 10μg/ml spectinomycin

⊙ 1829 Col VIK-94;- spectinomycin

▲ 1829 Col VIK-94 ;+10μg/ml spectinomycin

6.1.2c.

Sensitivity of Col VIK-94 bearing strains to copper ions

Apart from looking at the effect of antibiotics and detergents on Col V containing bacteria the effects of copper sulphate were investigated. The % viability of the Col V bearing strains and the Col V- strains on copper sulphate nutrient agar were calculated and tabulated as shown in table 17. Again the two parental strains used were 1829 and P678-54.

The results in table 17 were performed four times with consistent results. However, the results given in this table are from a single experiment. Col VIK-94 sensitised strain 1829 and P678-54 to copper ions. Copper sulpate enters the cell via the OmpF or OmpC porins so that increased OmpF or OmpC induced by ColV could explain these results. From figure 18 it seems that there maybe more OmpF/OmpC in the Col V+ strain than in 1829 (Fig18, tracks 7&9) but Figure 19 shows the cell envelope protein profiles of P678-54, P678-54 Col BK-98 and P678-54 Col VIK-94; there does not seem to be much difference between the OmpF/OmpC levels of P678-54 and P678-54 Col VIK-94.

The results in this section show that Col VIK-94 sensitises 1829 to hydrophobic compounds, aminoglycosides and some of the hydrophilic antibiotics. Col VIK-94 did not sensitise strain

P678-54 to carbenicillin or ampicillin appreciably. It also did not affect the sensitivity of P678-54 to the aminoglycosides. To ascertain whether a specific Col V component was responsible for the sensitivity, various growth experiments were carried out with strains carrying mutant Col V plasmids as seen in the following sections.

Table 17

Sensitivity of Col V bearing strains to copper sulphate

Bacterial strains	Concentration of copper sulphate (μg/ml)	% Colony formation
1829	0	100
	200	93
	400	80
	600	80
1829 Col VIK-94	0	100
	200	62
	400	48
	600	40
P678-54	0	100
	200	100
	400	90
	600	90
P678-54 Col VIK-94	0	100
	200	80
	400	71
	600	66

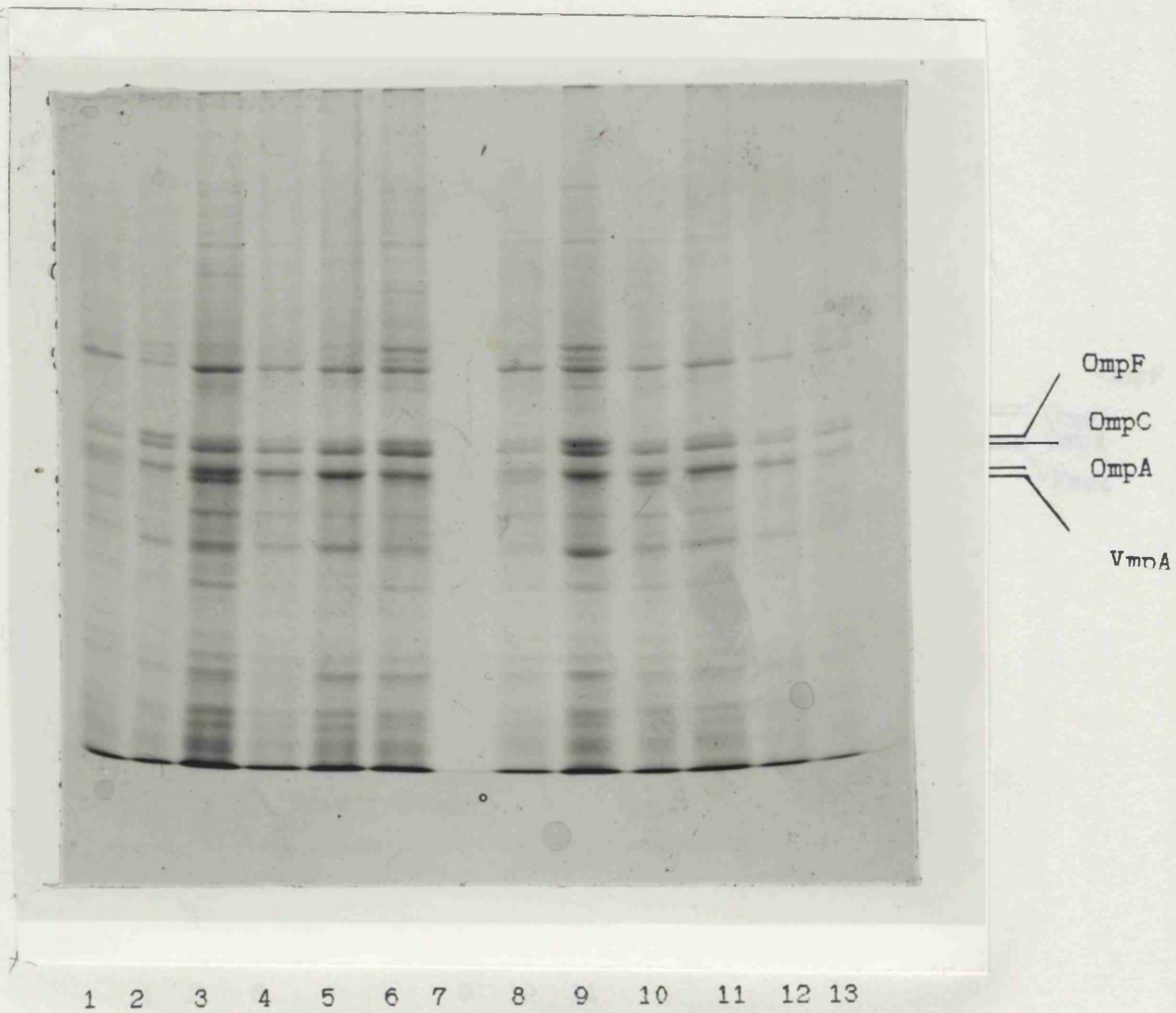
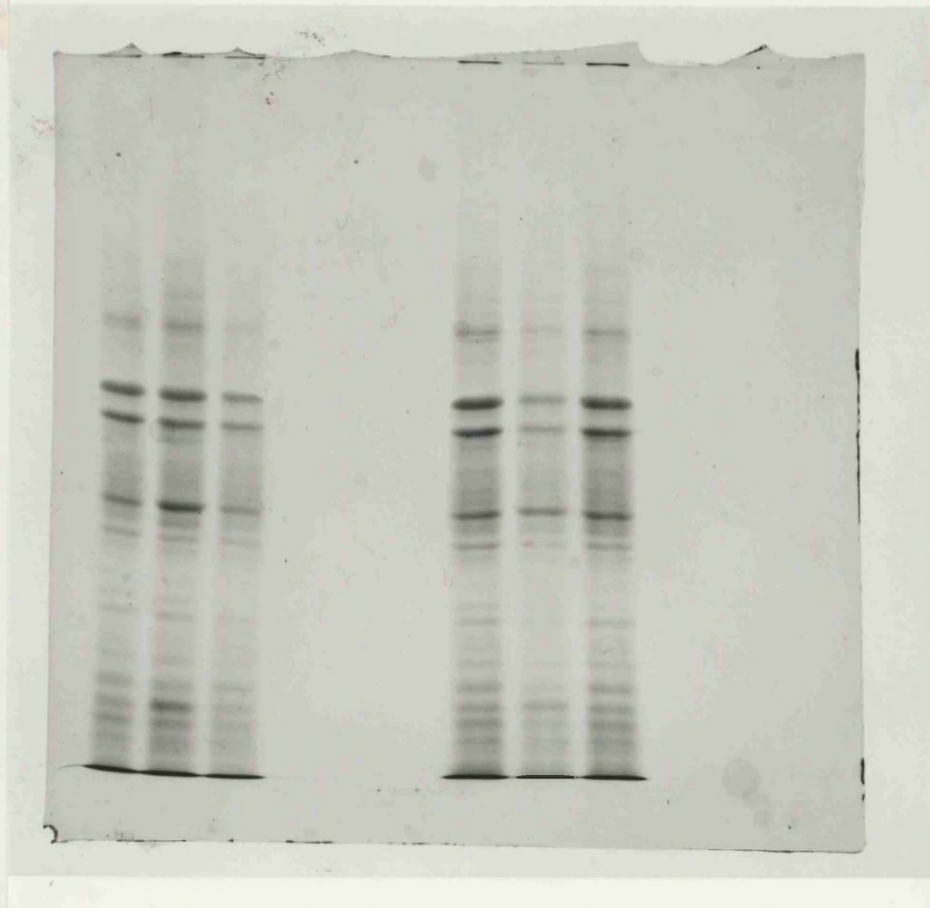


Fig.18.

Cell envelope protein profiles of 1829, its ColV and Col B bearing strains. 1&8. 1829 2&9. 1829 ColBK-98 3&10. 1829 ColVIK-94, 4&11. 1829 Col VM 40(5) 5&12. 1829 Col VM50 (1) 6&13. 1829 ColVK-30.



OmpF
OmpC
OmpA
VmpA

1 2 3 9 10 11

Fig.19.

Cell envelope protein profiles of P678-54 and its ColV and ColB derivatives. 1&9 . P678-54 2&10. P678-54 ColBK-98 3&11. P678-54 ColVIK-94.

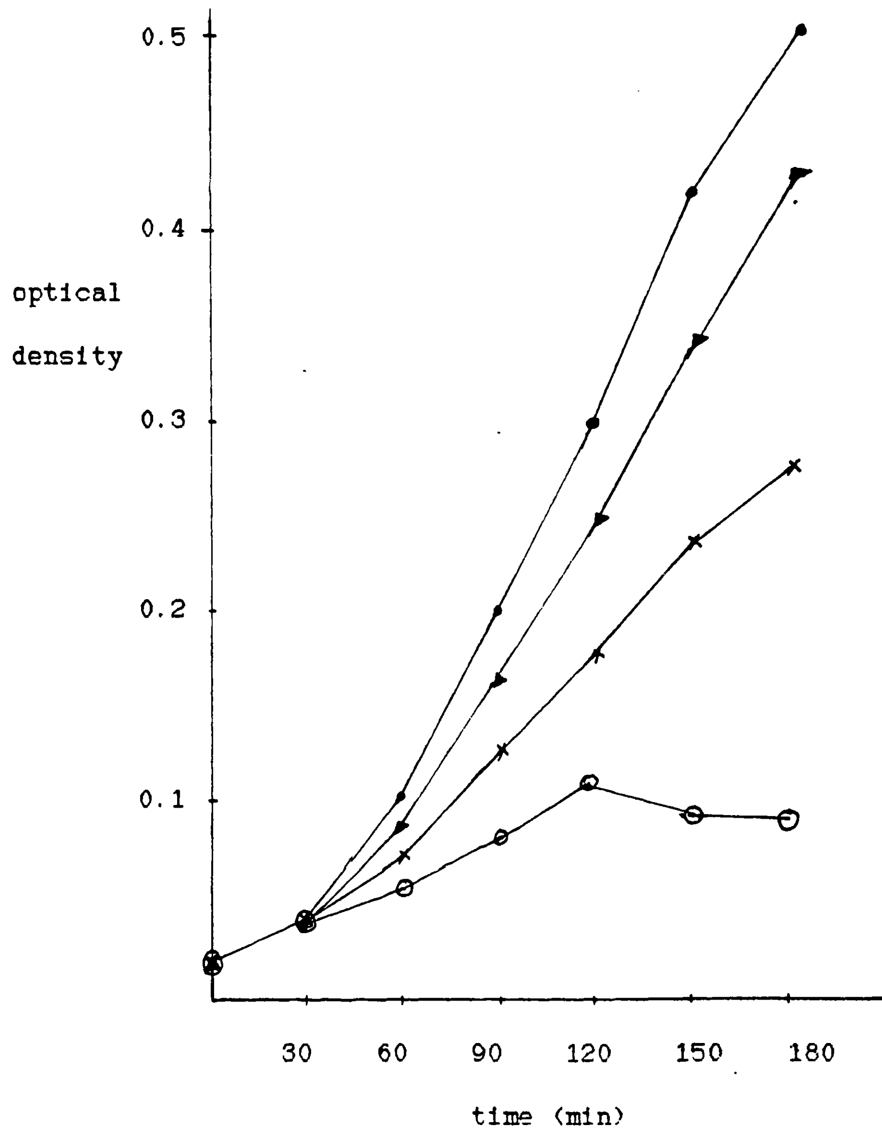


Fig.20. Effect of rifampicin on 1829 and 1829 Col VM50(1).

Standard growth curves performed in absence and presence of 15µg/ml of rifampicin.

•1829; - rifampicin

× 1829; + 15µg/ml rifampicin

▲1829 Col VM50(1);- rifampicin

⊙1829 Col VM50(1) ;+15µg/ml rifampicin

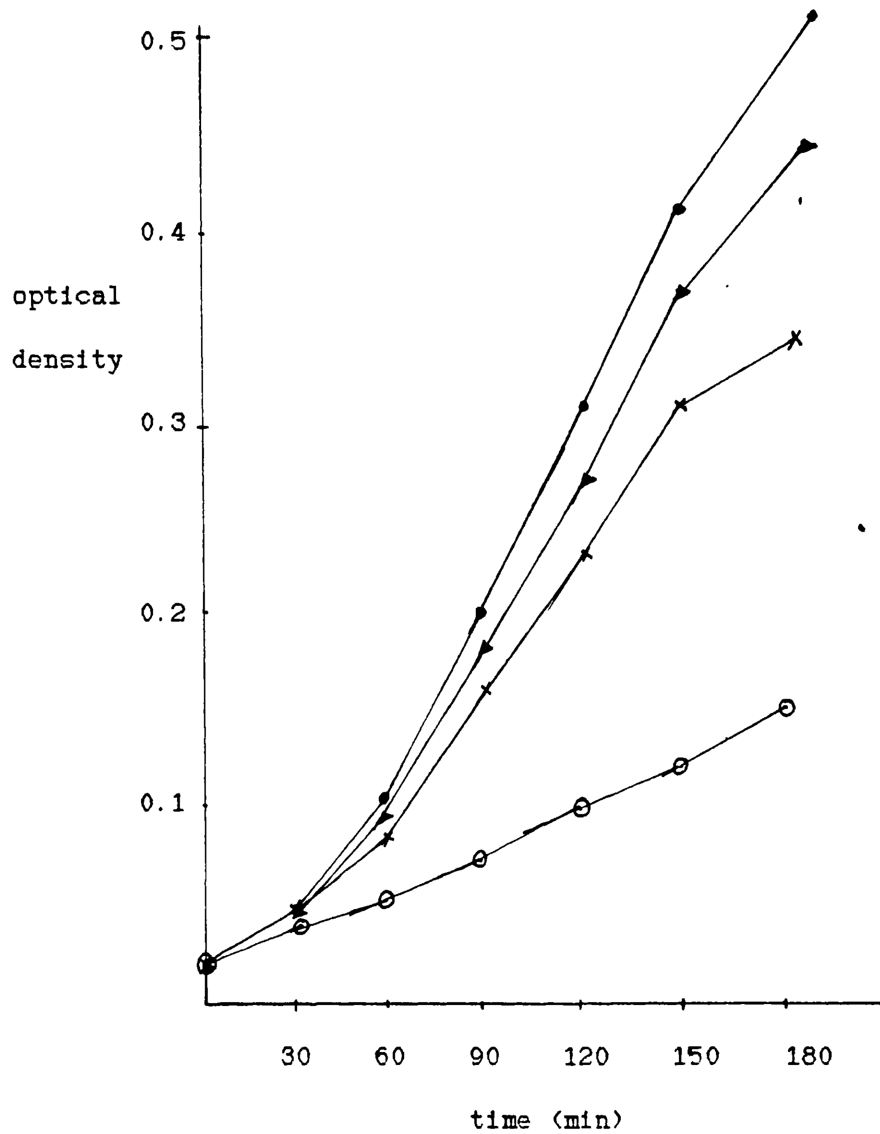


Fig.21. Effect of novobiocin on 1829 and 1829 Col VM50(1).

Standard growth curves performed in absence and presence of 50μg/ml of novobiocin.

•1829; - novobiocin

×1829; + 50μg/ml novobiocin

▲ 1829 Col VM50(1);- novobiocin

ʘ 1829 Col VM50(1) ;+50μg/ml novobiocin

6.2.

EFFECTS OF INHIBITORS ON COL VIK-94 MUTANT STRAINS

6.2.1

Sensitivity of strains carrying Col VM50(1) to hydrophobic, hydrophilic, aminoglycoside antibiotics and copper ions

Col VM50(1) is a mutant derived from Col VIK-94. This mutant plasmid confers production of colicins V and Ia; however its transfer properties are absent. In order to find out whether the colicin or transfer components are involved in sensitivity of Col V+ strains to hydrophobic compounds, we tested a range of hydrophobic compounds, antibiotics and detergents on Col VM50(1)+ strains.

The results in table 18 showed that Col VM50(1) sensitised the strain 1829 to all hydrophobic compounds tested except oxacillin. The effect of the mutant plasmid seemed to be somewhat less than that of Col VIK-94 (cf table 6). Figures 20 and 21 are representative of a single experiment performed using novobiocin and rifampicin.

The results in table 19 show that Col VM50(1) sensitised strain 1829 to ampicillin and carbenicillin. The effect on carbenicillin sensitivity was somewhat less than that of Col VIK-94 (cf Table

11). Figure 22 is representative of a single experiment with ampicillin.

The results on table 20 show that Col VM50(1) sensitises strain 1829 to spectinomycin and not kanamycin. The Col VIK-94 strain (table 14) was more sensitive to spectinomycin and kanamycin than the strain bearing Col VM50(1). Figures 23 is representative of an individual experiment with spectinomycin.

There did not seem to be any difference between the sensitivities of 1829 and 1829 Col VM50(1)to copper sulphate (Table 21). This maybe associated with slightly less porin in 1829 Col VM50(1) than in 1829 Col VIK-94 (See figure 18).

Also Col VM50(1) seemed to have a lesser effect on sensitivities of strain 1829 to SDS, oxacillin, novobiocin, carbenicillin, kanamycin and spectinomycin than Col VIK-94. However, the effects of the mutant plasmid on rifampicin and ampicillin and sensitivities were similar to the effects of Col VIK-94. As previously described, Col VM50(1) is a mutant derivative of Col VIK-94.

Table 18

Sensitivity of 1829 Col VM50(1) to hydrophobic antibiotics

Plasmid in strain 1829	% Growth Inhibition					
	Rifampicin (μg/ml)		Novobiocin (μg/ml)		Oxacillin (μg/ml)	
	7.5	15	20	50	50	200
None	26(±2.4)	45(±2.5)	19(±1.7)	34(±1.7)	10(±1.2)	26(±1.5)
Col VM50(1)	46(±2.0)	76(±1.5)	43(±1.5)	70(±1.5)	8(±1.0)	38(±1.4)

	SDS (%)	
	3	5
None	35(±1.5)	51(±1.5)
Col VM50(1)	64(±1.6)	76(±0.9)

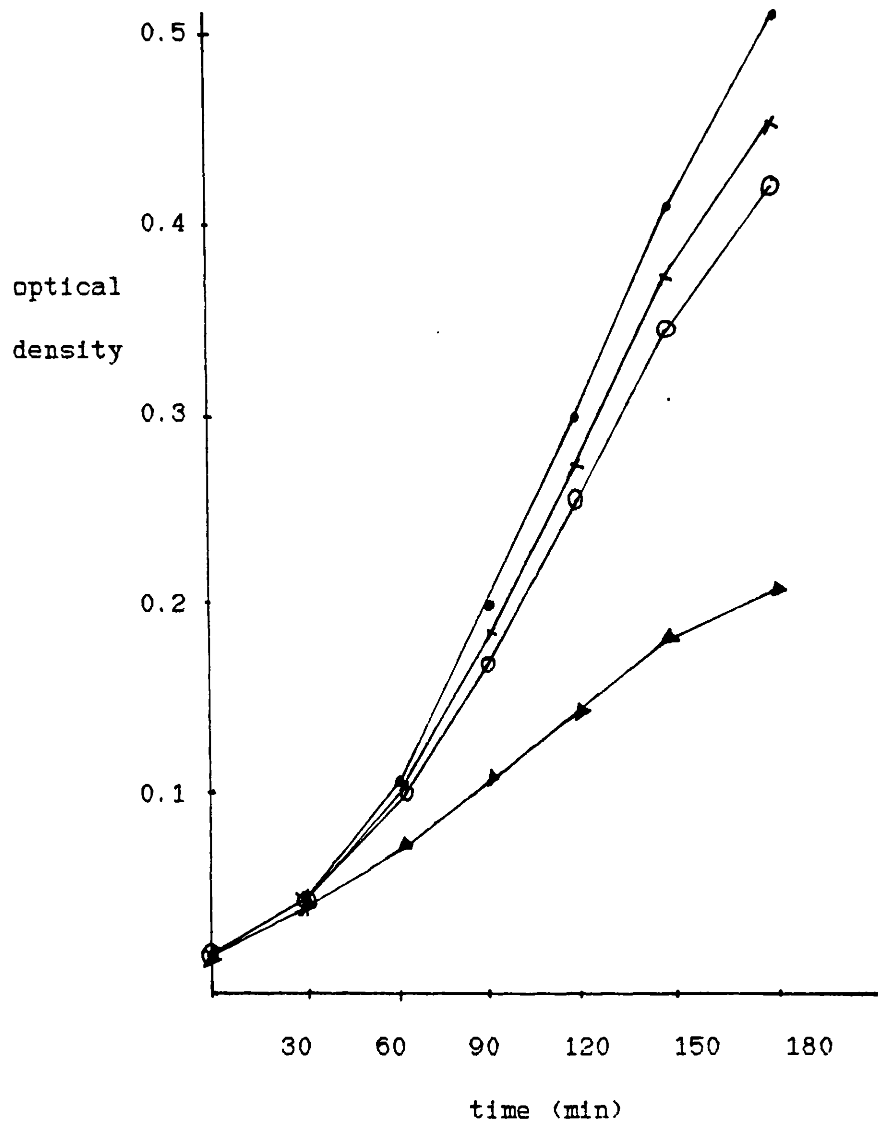


Fig.22. Effect of ampicillin on 1829 and 1829 Col VM50(1).

Standard growth curves performed in absence and presence of 1μg/ml of ampicillin.

• 1829; - ampicillin

⊘ 1829; + 1μg/ml ampicillin

× 1829 Col VM50(1);- ampicillin

▲ 1829 Col VM50(1) ;+1μg/ml ampicillin

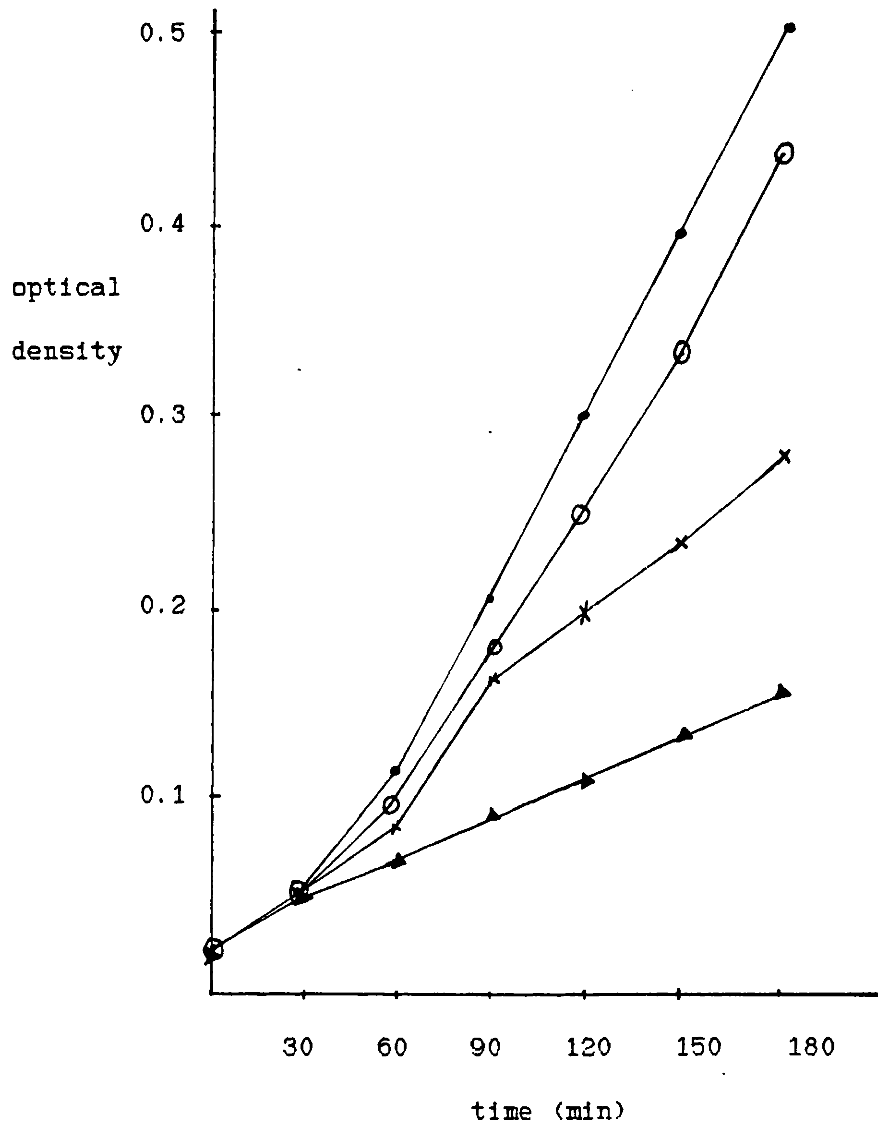


Fig.23. Effect of spectinomycin on 1829 and 1829 Col VM50(1).

Standard growth curves performed in absence and presence of 10µg/ml of spectinomycin.

• 1829; - spectinomycin

× 1829;+ 10µg/ml spectinomycin

⊙ 1829 Col VM50(1);- spectinomycin

▲ 1829 Col VM50(1) ;+10µg/ml spectinomycin

Table 19

Sensitivity of 1829 Col VM50(1) to hydrophilic agents

Plasmid in strain 1829	% Growth Inhibition			
	Ampicillin (μg/ml)		Carbenicillin (μg/ml)	
	1.0	2.0	2.5	5.0
None	20(±1.5)	25(±1.7)	25(±1.4)	40(±1.6)
Col VM50(1)	35(±2.3)	65(±2.1)	29(±2.2)	58(±2.1)

Table 20

Sensitivity of 1829 Col VM50(1) to aminoglycoside antibiotics

Plasmid in strain 1829	% Growth inhibition			
	Spectinomycin (μg/ml)		Kanamycin (μg/ml)	
	5	10	1.25	2.0
None	20(±1.7)	45(±1.7)	40(±1.5)	68(±1.5)
Col VM50(1)	40(±2.1)	65(±1.7)	48(±1.5)	74(±1.0)

Table 21

Sensitivity of 1829 Col VM50(1) to copper sulphate

Bacterial Strains	Concentrations of copper sulphate used (µg/ml)	% Colony Formation
1829	0	100
	200	93
	400	80
	600	80
1829 Col VM50(1)	0	100
	200	93
	400	83
	600	80

6.2.2.

Sensitivity of 1829 Col VM40(5) to hydrophobic, hydrophilic and aminoglycoside antibiotics

Col VM40(5) is a mutant derived from Col VIK-94 like Col VM50(1). It does not confer production of either colicin V or Ia (or immunity components) but still confers derepressed transfer properties. Various hydrophobic compounds, hydrophilic and aminoglycoside antibiotics were tested out on strain 1829 carrying Col VM40(5) and compared with the effects of Col VIK-94 and Col VM50(1).

Col VM40(5) sensitised strain 1829 to rifampicin and novobiocin (Table 22). Col VM40(5) sensitised the strain to almost the same extent as Col VIK-94 for novobiocin but to a lesser extent with rifampicin (cf Table 6). The plasmid also sensitised the strain to SDS but not appreciably to oxacillin. The Col VM40(5) seems to sensitise the strain 1829 to novobiocin (50μg/ml) to a greater extent than does Col VM50(1) (cf table 18). Figures 24 and 25 are representative of a single experiment with rifampicin and novobiocin respectively.

The results in table 23 showed that Col VM40(5) did not seem to sensitise the strain to either hydrophilic agent but it did seem to make strain 1829 slightly more resistant to carbenicillin.

This may be due to LPS- porin interactions. Figure 26 is representative of single experiment with ampicillin.

From the results in table 24 it seems that Col VM40(5) sensitised the strain 1829 to spectinomycin at 10µg/ml but there did not seem to be much of a difference in response to kanamycin. Figure 27 is representative of a single experiment with spectinomycin.

It seems as if Col VM40(5) does in fact sensitise 1829 to copper sulphate (Table 25) unlike Col VM50(1) (cf Table 21). Also the Col VM40(5) plasmid does not seem to sensitise 1829 to hydrophilic compounds as did Col VM50(1).

For the two hydrophobic antibiotics studied in most detail, rifampicin sensitivity is altered by loss of either colicin or transfer components but mostly by loss of colicin components (Table 22) whereas sensitivity to novobiocin seemed to depend much more on transfer components (Table 19).

In contrast, sensitivity to the hydrophilic agents used appeared to be dependent on the presence of colicin components.

Table 22

Sensitivity of 1829 Col VM40(5) to hydrophobic antibiotics

Plasmid in strain 1829	% Growth inhibition			
	Rifampicin (μg/ml)		Novobiocin (μg/ml)	
	7.5	15	20	50
None	26(±2.4)	45(±2.5)	19(±1.7)	34(±1.7)
Col VM40(5)	35(±2.5)	61(±2.1)	26(±1.4)	100(±0.5)
	SDS (%)		Oxacillin (μg/ml)	
	3	5	50	200
None	35(±1.5)	51(±1.5)	10(±1.2)	26(±1.5)
Col VM40(5)	52(±1.2)	78(±0.9)	22(±1.1)	36(±1.7)

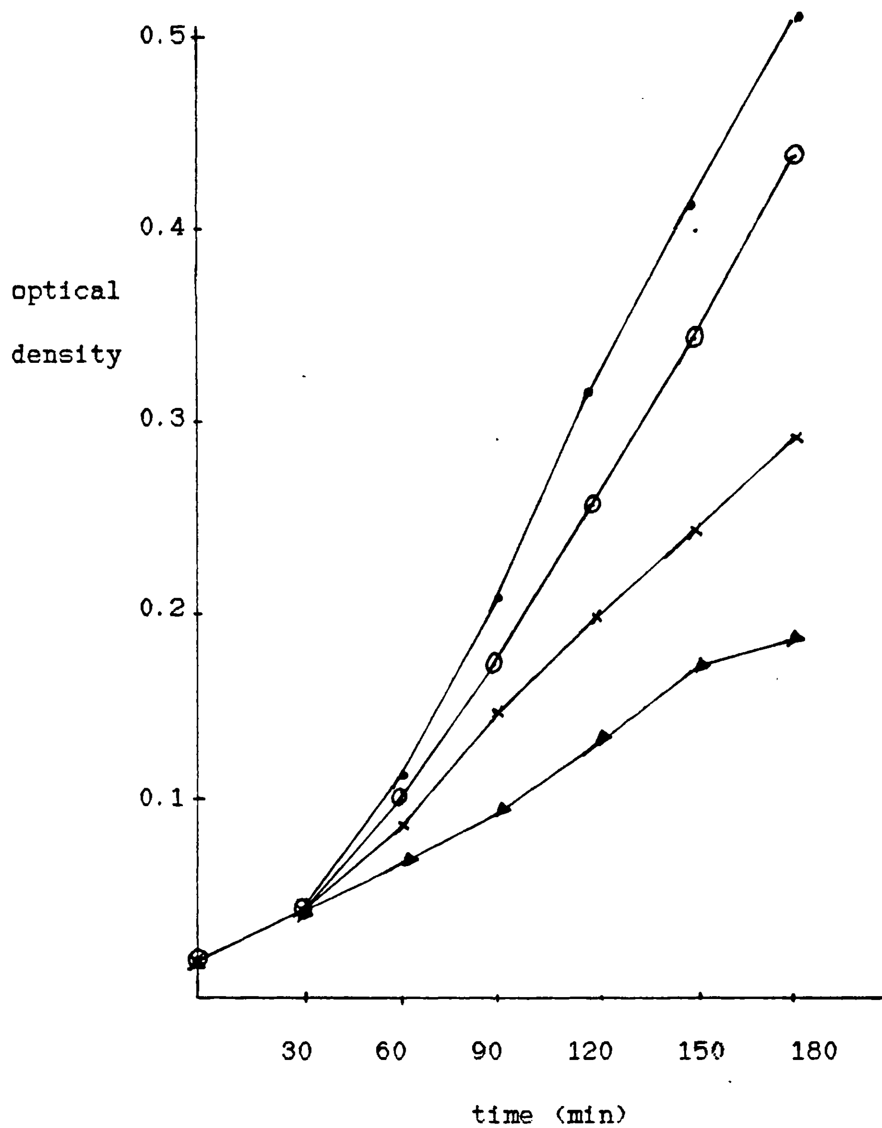


Fig.24. Effect of rifampicin on 1829 and 1829 Col VM40(5).

Standard growth curves performed in absence and presence of 15μg/ml of rifampicin.

• 1829; - rifampicin

× 1829; + 15μg/ml rifampicin

⊙ 1829 Col VM40(5); - rifampicin

▲ 1829 Col VM40(5) ; +15μg/ml rifampicin

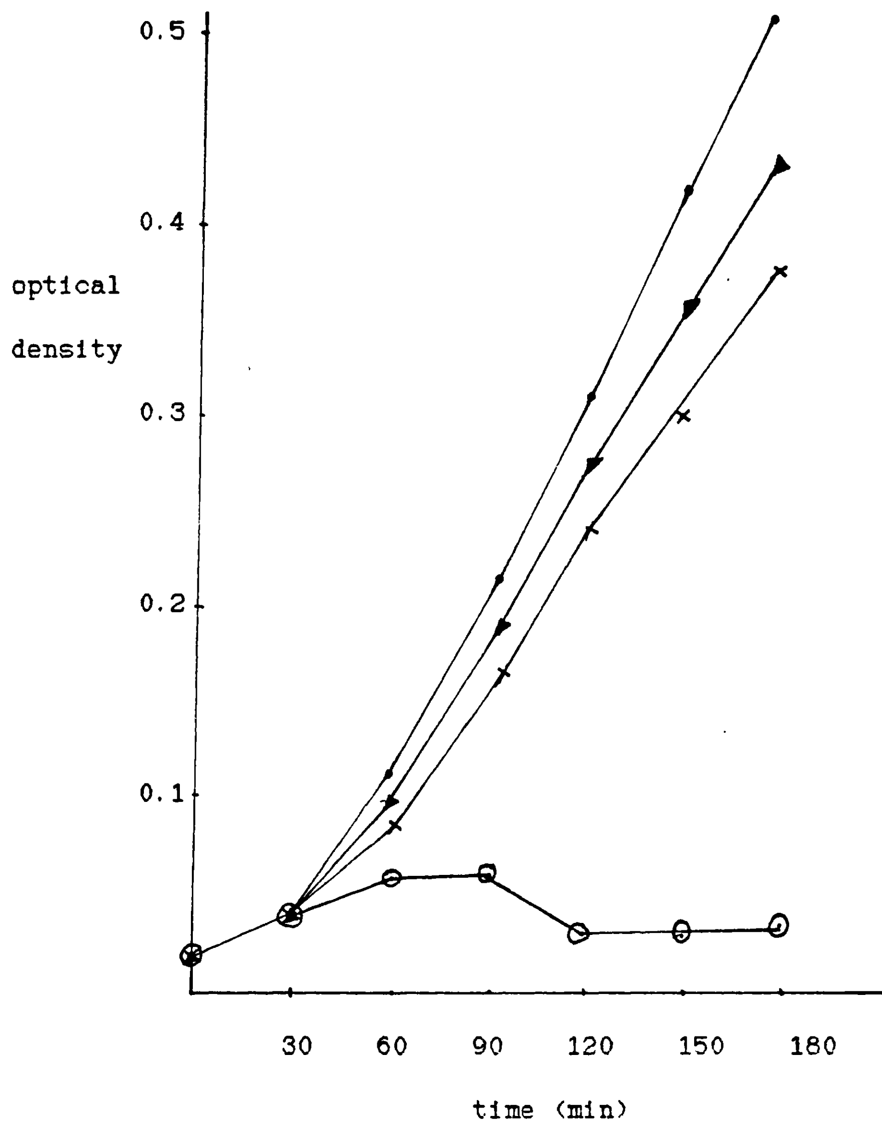


Fig.25. Effect of novobiocin on 1829 and 1829 Col VM40(5).

Standard growth curves performed in absence and presence of 50μg/ml of novobiocin.

• 1829; - novobiocin

× 1829; + 50μg/ml novobiocin

▲ 1829 Col VM40(5); - novobiocin

⊙ 1829 Col VM40(5) ; +50μg/ml novobiocin

Table 23

Sensitivity of 1829 Col VM40(5) to hydrophilic antibiotics

Plasmid in strain 1829	% Growth Inhibition			
	Ampicillin (μg/ml)		Carbenicillin (μg/ml)	
	1.0	2.0	2.5	5.0
None	20(±1.5)	25(±1.7)	25(±1.4)	40(±1.6)
Col VM40(5)	13(±1.3)	19(±1.4)	13(±1.4)	28(±1.8)

Table 24

Sensitivity of 1829 Col VM40(5) to the aminoglycoside antibiotics

Plasmid in strain 1829	% Growth Inhibition			
	Spectinomycin (μg/ml)		Kanamycin (μg/ml)	
	5	10	1.25	2.0
None	20(±1.7)	49(±1.7)	46(±1.5)	68(±1.5)
Col VM40(5)	19(1.5)	73(±1.6)	40(±1.0)	75(±0.9)

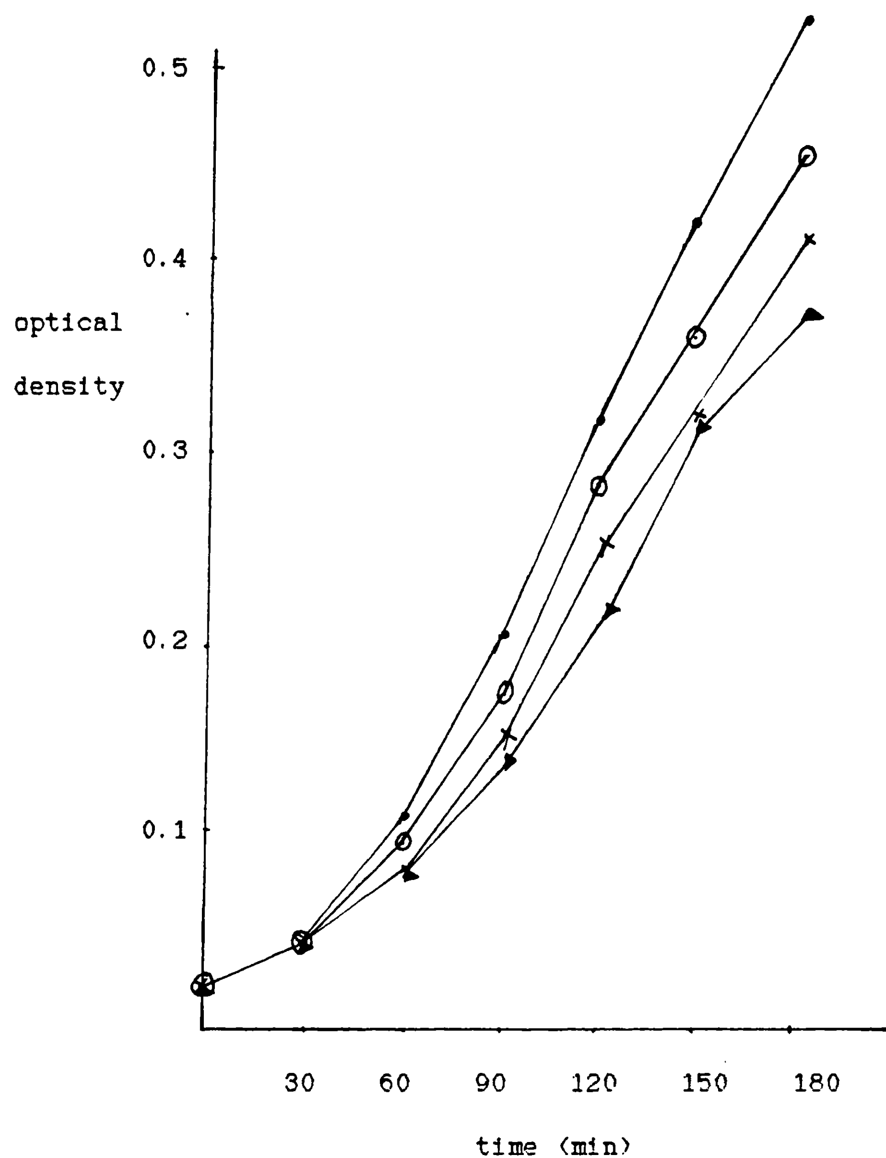


Fig.26. Effect of ampicillin on 1829 and 1829 Col VM40(5).

Standard growth curves performed in absence and presence of 1μg/ml of ampicillin.

• 1829; - ampicillin

× 1829;+ 1μg/ml ampicillin

⊙ 1829 Col VM40(5);- ampicillin

▲ 1829 Col VM40(5) ;+1μg/ml ampicillin

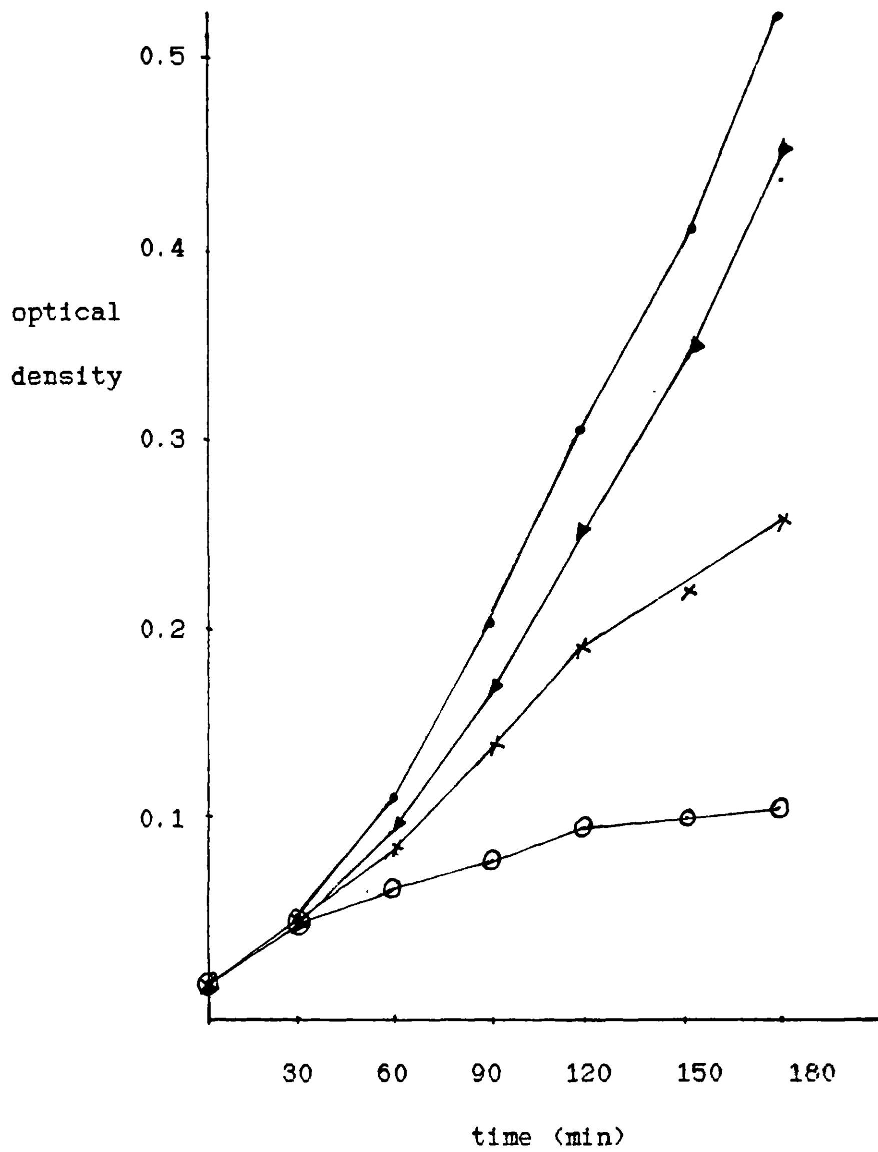


Fig.27. Effect of spectinomycin on 1829 and 1829 Col VM40(5).

Standard growth curves performed in absence and presence of 10µg/ml of spectinomycin.

1829; - spectinomycin

1829;+ 10µg/ml spectinomycin

1829 Col VM40(5);- spectinomycin

1829 Col VM40(5) ;+10µg/ml spectinomycin

Table 25

Sensitivity of 1829 Col VM40(5) to copper sulphate

Bacterial Strains	Concentrations of copper sulphate used (µg/ml)	% Colony formation
1829	0	100
	200	93
	400	80
	600	80
1829 Col VM40(5)	0	100
	200	55
	400	44
	600	33

6.3

Effect of another Col V plasmid on inhibitor sensitivity

We also looked at the effects of another Col V plasmid, Col VK-30. Col VK-30 only produces colicin V and has derepressed transfer properties. Again, the effects of some hydrophobic and hydrophilic agents and aminoglycosides were investigated on Col VK-30+ and Col- derivatives of strain 1829.

It seems that Col VK-30 plasmid has less of an effect on 1829 as regards sensitivity to rifampicin and SDS than did Col VIK-94 (see Table 26 and cf Table 6) possibly because colicin Ia and its immunity component are not formed or possibly due to some other component formed by K-94+ but not K-30. With novobiocin the response was the same for the two Col V+ strains at least for 50 µg/ml. Fig.28 and 29 are representative of a single experiment with rifampicin and novobiocin.

The Col VK-30 plasmid did not seem to have any significant effect on the strain 1829 with regards to sensitivity to the hydrophilic compounds of ampicillin and carbenicillin (Table27; fig.30) suggesting that colicin Ia (+ immunity component) may affect sensitivity to hydrophilic agents; or to have any appreciable effect on the response of strain 1829 to kanamycin; however, with spectinomycin there seems to be some effect at 10µg/ml(see Table 28 and fig.31).

The effects of copper sulphate were also investigated. It was found that there was no appreciable difference in the sensitivities of the two strains to copper sulphate (Table 29).

From figure 18 it seemed that there was no appreciable reduction in the amounts of OmpF protein in 1829 Col VK-30 compared to 1829 Col VIK-94 but there was less VmpA protein.

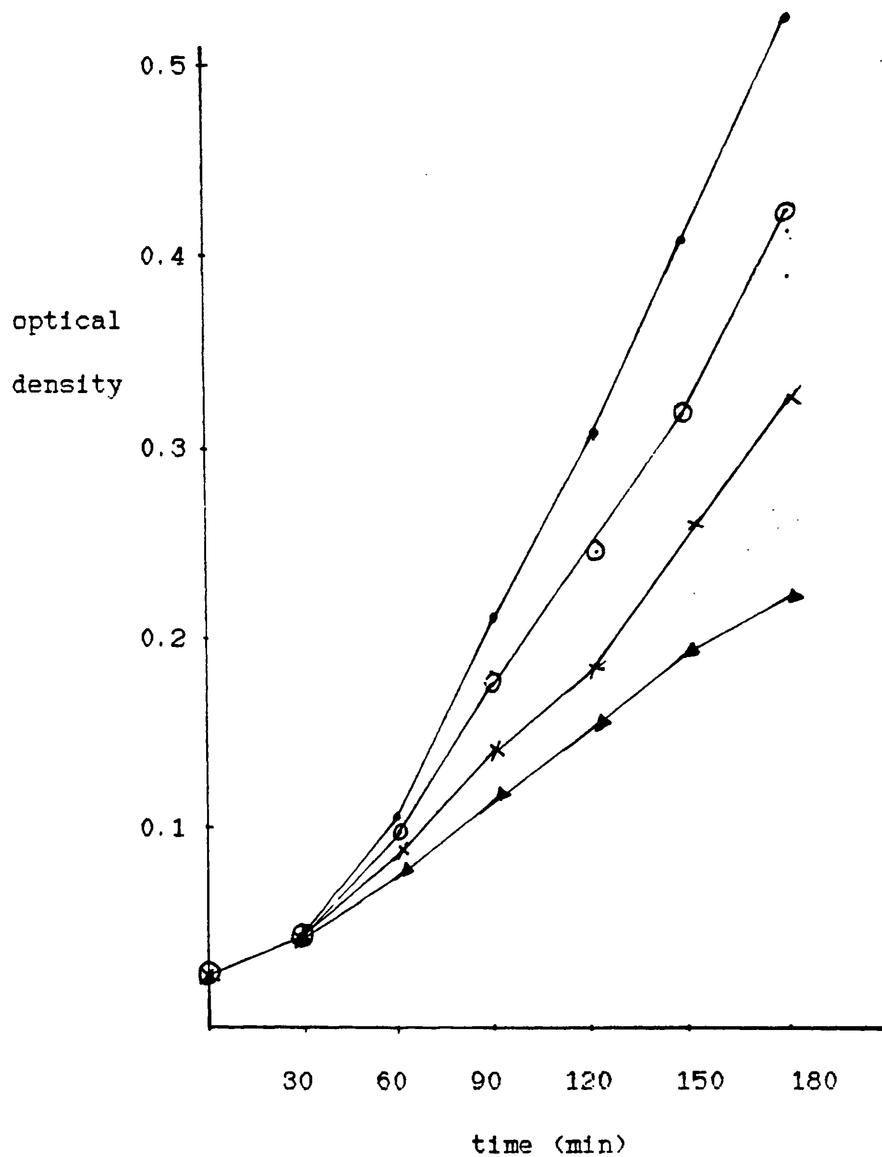


Fig.28. Effect of rifampicin on 1829 and 1829 Col VK-30.

Standard growth curves performed in absence and presence of 15μg/ml of rifampicin.

• 1829; - rifampicin

× 1829;+ 15μg/ml rifampicin

⊙ 1829 Col VK-30;- rifampicin

▲ 1829 Col VK-30 ;+15μg/ml rifampicin

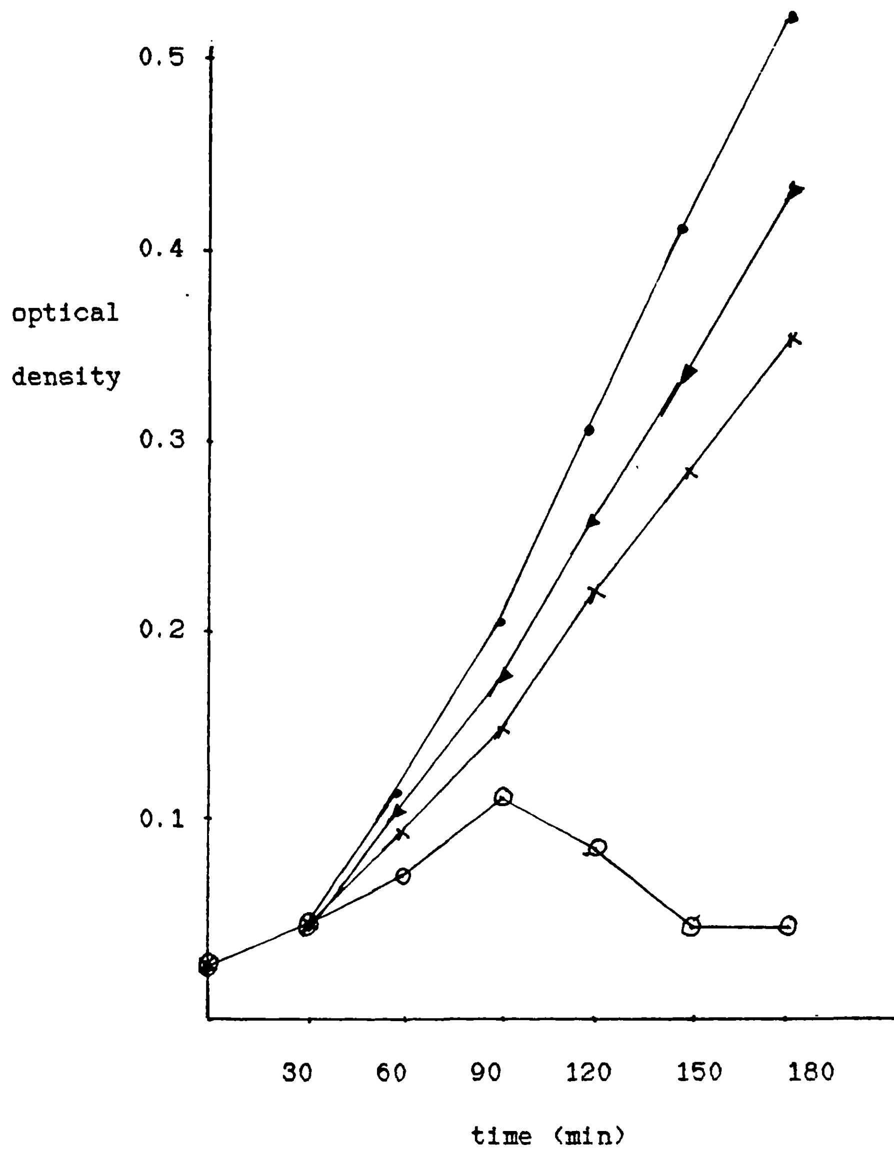


Fig.29. Effect of novobiocin on 1829 and 1829 Col VK-30.

Standard growth curves performed in absence and presence of 50µg/ml of novobiocin.

- • 1829; - novobiocin
- × 1829;+ 50µg/ml novobiocin
- ▲ 1829 Col VK-30;- novobiocin
- ⊙ 1829 Col VK-30 ;+50µg/ml novobiocin

Table 26

Sensitivity of Col VK-30 bearing organisms to the hydrophobic antibiotics

Plasmid in strain 1829	% Growth Inhibition			
	Rifampicin (μg/ml)		Novobiocin (μg/ml)	
	7.5	15	20	50
None	26(±2.4)	43(±2.5)	20(±1.7)	34(±1.7)
Col VK-30	38(±2.0)	58(±2.1)	22(±1.5)	100(±0.5)

	% Growth Inhibition	
	SDS (%)	
	3	5
None	24(±1.2)	46(±1.3)
Col VK-30	53(±1.4)	74(±1.3)

Table 27

Sensitivity of 1829 Col VK-30 to the hydrophilic antibiotics

Plasmid in strain 1829	% Growth Inhibition			
	Carbenicillin (µg/ml)		Ampicillin (µg/ml)	
	2.5	5.0	1.0	2.0
None	26(±1.4)	48(±1.5)	18(±1.5)	29(±1.5)
Col VK-30	38(±1.2)	57(±1.3)	19(±1.6)	38(±1.7)

Table 28

Sensitivity of strain 1829 Col VK-30 to the aminoglycoside antibiotics

Plasmid in strain 1829	% Growth Inhibition			
	Spectinomycin (µg/ml)		Kanamycin (µg/ml)	
	5	10	1.25	2.0
None	24(±1.3)	40(±1.2)	45(±1.4)	67(±1.5)
Col VK-30	39(±1.4)	67(±1.5)	60(±1.4)	78(±1.5)

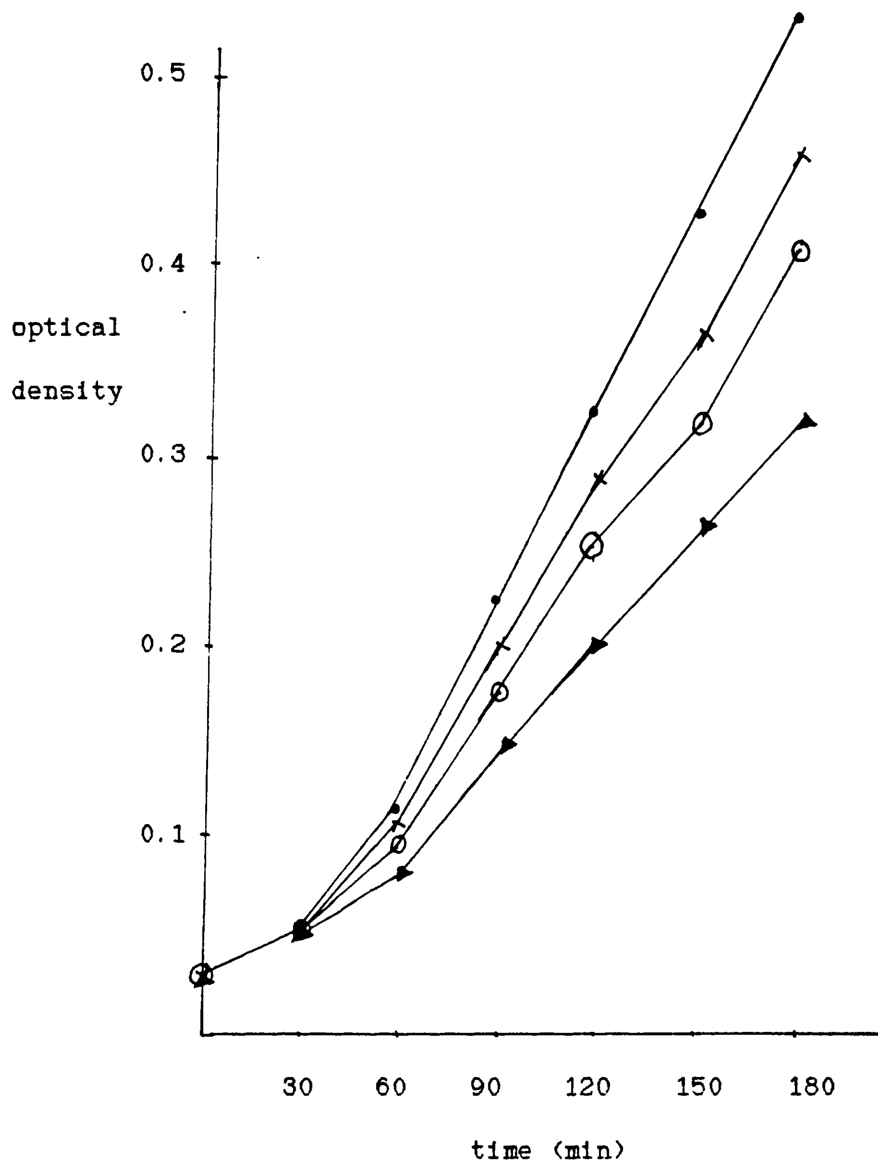


Fig.30. Effect of ampicillin on 1829 and 1829 Col VK-30.

Standard growth curves performed in absence and presence of 1μg/ml of ampicillin.

• 1829; - ampicillin

⊙ 1829; + 1μg/ml ampicillin

× 1829 Col VK-30; - ampicillin

▲ 1829 Col VK-30 ; +1μg/ml ampicillin

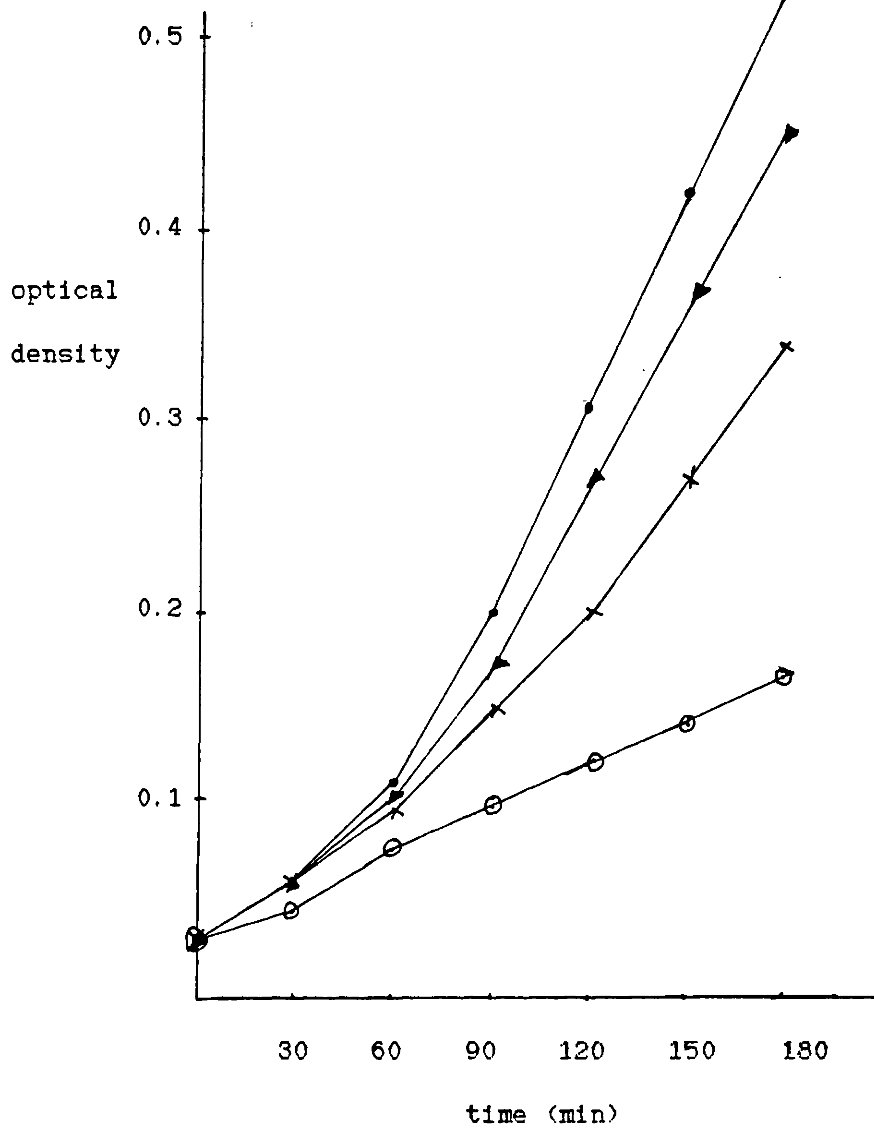


Fig.31. Effect of spectinomycin on 1829 and 1829 Col VK-30. Standard growth curves performed in absence and presence of 10μg/ml of spectinomycin.

• 1829; - spectinomycin

× 1829; + 10μg/ml spectinomycin

▲ 1829 Col VK-30; - spectinomycin

⊙ 1829 Col VK-30 ; +10μg/ml spectinomycin

Table 29

Sensitivity of 1829 Col VK-30 to copper sulphate

Plasmid in strain 1829	Concentration of copper sulphate used (μg/ml)	% Colony formation
None	0	100
	200	90
	400	83
	600	80
Col VK-30	0	100
	200	82
	400	80
	600	75

6.4.

F like plasmids and antibiotic sensitivity

The effects of rifampicin and novobiocin were tested out on strain 1829 carrying other F-like plasmids, such as R-124, R1 and R124 plus Col VIK-94. The results are given in table 30.

Of the F-like plasmids tested, R124 had a fairly marked effect on rifampicin and novobiocin sensitivity at the higher concentration of 15 and 50 μg/ml respectively. The effects of the plasmid R1 on the sensitivity of 1829 to both rifampicin and novobiocin were only slight.

R124, a fi+ plasmid, abolishes transfer component synthesis by Col V; this greatly reduced the effect of Col V on novobiocin sensitivity in accord with earlier results.

Table 30

Effects of other F-like plasmids on sensitivity to hydrophobic compounds

Plasmid in strain 1829	% Growth Inhibition			
	Rifampicin (μg/ml)		Novobiocin (μg/ml)	
	7.5	15	20	50
None	22(±1.5)	44(±1.6)	17(±1.2)	35(±1.6)
R1	36(±2.0)	59(±2.1)	10(±2.1)	57(±2.0)
R124	33(±1.5)	62(±1.9)	20(±1.0)	70(±0.9)
R124 Col VIK-94	44(±2.0)	78(±1.6)	24(±1.6)	65(±1.9)
Col VIK-94	55(±1.5)	80(±1.6)	55(±1.5)	100(±0.6)

6.5.

Effect of divalent cations on the antibacterial sensitivity of strains carrying Col VIK-94 and mutant Col VIK-94 and other Col V plasmids

Magnesium ions were initially added in order to investigate whether there would be a reversal in sensitivity to rifampicin and novobiocin. Rifampicin sensitivity appears to depend mainly on the presence of colicin whilst sensitivity to novobiocin depends mainly on transfer components. Magnesium is supposed to have an inhibitory effect on colicin synthesis/secretion (Davies et al, 1986) possibly by enhancing the LPS-LPS bonding.

The addition of 50mmol/l of magnesium salts to nutrient broth in the presence of antibiotics has been studied on plasmid containing bacteria. The results in table 31 suggest that there was no effect on the sensitivity of strain 1829 but rifampicin inhibition was partially reversed by the magnesium salts in the plasmid bearing strain. The effects on novobiocin sensitivity with magnesium salts were slight (see table 31).

Table 32 suggests that magnesium had no effect on the sensitivities to carbenicillin and minocycline. Table 33 shows that the magnesium salts had an effect on sensitivity of the Col V+ strains to gentamicin and polymyxin. In the case of

polymyxin, (and slightly with gentamicin) strain 1829 seemed to be affected by the presence of the magnesium salts too.

The effects of magnesium salts on the sensitivity of Col VM40(5) and Col VM50(1) strains to rifampicin and novobiocin were also investigated (Tables 34 and 35). Magnesium salts did not reverse the effects of inhibition in 1829 Col VM40(5) possibly since colicin is not present (magnesium may act by inhibiting colicin synthesis). It did, however partially reverse the effects in 1829 Col VM50(1) possibly because this strain produces colicin, and therefore magnesium can affect it. The effect of magnesium on novobiocin sensitivity was somewhat more than expected since transfer components confer most sensitivity to this agent (although they are absent from the VM50(1)+ strain). Col VM50(1) and Col VM40(5) both had less effect on the sensitivity of 1829 to these two antibiotics than did Col VIK-94 (cf tables 6, 18 and 22) presumably because both transfer components and colicin components play a role in sensitisation of the Col VIK-94+ strain to these agents.

Magnesium salts also affected the inhibition by rifampicin and novobiocin, of the Col VK-30+ strain (Table 36); again the effect on novobiocin sensitivity was surprisingly large. Polymyxin and gentamicin inhibition of this strain (Table 37) was also reversed.

The other cation used in this study was 10mM calcium. The effects of calcium ions on the sensitivity of Col VIK-94+, Col VM40(5)+, Col VM50(1)+ and Col VK-30+ strains to rifampicin and novobiocin was studied. The results indicated that calcium had a similar reversal effect to that of magnesium ions (Tables 38, 39, 40 & 41). Calcium like magnesium acts as a divalent cross-bridge between adjacent LPS molecules.

One of the effects of these divalent ions is that they act as a divalent cationic bridge between LPS molecules and one of the effects of polymyxin and the aminoglycoside is to actually displace the divalent ions thus making the cells more permeable to the antibiotics. Addition of magnesium salts to the medium would enable the magnesium ions to reform a divalent cationic bridge.

Another property which magnesium salts have is that they reduce colicin synthesis by Col V strains but have little or no effect on transfer properties (Davies, et al, 1986). This probably explains why there seems to be an effect of magnesium ions on sensitivities of the Col VM50(1) strain but no effect on Col VM40(5) with novobiocin or rifampicin.

Table 31

Effects of magnesium ions on the sensitivity to hydrophobic antibiotics of Col VIK-94 bearing strains

Plasmid in strain 1829	% Growth Inhibition			
	Rifampicin (μg/ml)		Novobiocin (μg/ml)	
	7.5	15	20	50
None	20(±2.0)	47(±2.0)	15(±1.2)	42(±1.5)
None+Mg++	16(±1.7)	40(±1.9)	10(±1.4)	39(±1.4)
Col VIK-94	55(±1.6)	79(±0.9)	36(±1.7)	88(±1.5)
Col VIK-94+Mg++	12(±1.7)	34(±1.8)	36(±1.5)	71(±1.6)

Table 32

The effects of magnesium salts on the sensitivity to hydrophilic antibiotics of Col VIK-94 bearing strains

Plasmid in strain 1829	% Growth Inhibition			
	Minocycline (μg/ml)		Carbenicillin (μg/ml)	
	0.5	1	2.5	5.0
None	36	64	25	40
None+Mg++	33	57	20	35
ColVIK-94	43	53	38	65
ColVIK-94+Mg++	45	49	35	60

Table 33

The effects of magnesium salts on aminoglycoside and polymyxin sensitivity in a Col VIK-94+ strain

Plasmid in	% Growth Inhibition			
strain 1829	Polymyxin (μg/ml)		Gentamicin (μg/ml)	
	0.5	1.0	0.1	0.25
None	70	98	23	41
None+Mg++	10	15	16	17
Col VIK-94	98	98	70	82
Col VIK-94 +Mg++	7	10	16	25

Table 34

The effects of magnesium salts on the sensitivity of a Col VM40(5)+ strain to hydrophobic antibiotics

Plasmid in	% Growth Inhibition			
strain 1829	Rifampicin (μg/ml)		Novobiocin (μg/ml)	
	7.5	15	20	50
None	18(±2.1)	45(±2.0)	20(±1.5)	49(±1.8)
None+Mg++	20(±2.0)	40(±1.9)	16(±1.4)	46(±1.5)
ColVM40(5)	35(±2.2)	61(±1.8)	30(±1.9)	95(±1.2)
ColVM40(5)+Mg++	49(±2.1)	73(±1.5)	29(±1.8)	88(±1.2)

Table 35

The effects of magnesium salts on the sensitivity of a Col VM50(1)+ strain to hydrophobic antibiotics

Plasmid in strain 1829	% Growth Inhibition			
	Rifampicin (µg/ml)		Novobiocin (µg/ml)	
	7.5	15	20	50
None	20(±2.1)	49(±2.1)	15(±1.7)	40(±1.5)
None+Mg++	21(±1.8)	40(±2.0)	15(±1.5)	36(±1.4)
ColVM50(1)	32(±2.1)	78(±1.6)	43(±1.2)	76(±1.4)
ColVM50(1)+Mg++	27(±2.2)	47(±2.1)	24(±1.4)	54(±1.5)

Table 36

The effects of magnesium salts on the sensitivities of a Col VK-30+ strain to hydrophobic antibiotics

Plasmid in strain 1829	% Growth Inhibition			
	Rifampicin (µg/ml)		Novobiocin (µg/ml)	
	7.5	15	20	50
None	15(±2.2)	43(±2.0)	18(±1.5)	40(±1.4)
None+Mg++	14(±2.2)	40(±2.1)	20((±1.2)	34(±1.5)
Col VK-30	28(±1.9)	73(±1.6)	29(±2.0)	97(±1.6)
Col VK-30+Mg++	21(±2.1)	47(±2.1)	10(±1.5)	25(±1.4)

Table 37

The effects of magnesium salts on the sensitivities of 1829 Col VK-30 to polymyxin and an aminoglycoside antibiotic

Plasmid in strain 1829	% Growth inhibition			
	Polymyxin (μg/ml)		Gentamicin (μg/ml)	
	0.5	1.0	0.1	0.25
None	70	98	25	45
None+Mg++	4	17	14	19
ColVK-30	80	98	83	92
ColVK-30+Mg++	7	20	15	24

Table 38

The effects of calcium salts on the sensitivity of Col VIK94+ strains to hydrophobic antibiotics

Plasmid in strain 1829	% Growth Inhibition			
	Rifampicin (μg/ml)		Novobiocin (μg/ml)	
	7.5	15	20	50
None	16	50	20	34
None+Ca++	14	45	18	35
Col VIK-94	70	86	33	96
Col VIK-94+Ca++	30	33	28	55

Table 39

The effects of calcium salts on sensitivity of a Col VM40(5)+ strain to hydrophobic antibiotics

Plasmid in strain 1829	% Growth Inhibition			
	Rifampicin (μg/ml)		Novobiocin (μg/ml)	
	7.5	15	20	50
None	20	47	20	34
None+Ca++	22	45	18	32
ColVM40(5)	52	74	58	79
Col VM40(5)+Ca++	50	76	54	74

Table 40

The effects of calcium salts on sensitivity of a Col VM50(1)+ strain to hydrophobic antibiotics

Plasmid in strain 1829	% Growth Inhibition			
	Rifampicin (μg/ml)		Novobiocin (μg/ml)	
	7.5	15	20	50
None	20	44	22	32
None+Ca++	22	45	17	34
Col VM50(1)	45	70	43	76
ColVM50(1)+Ca++	44	52	23	55

Table 41

The effects of calcium salts on 1829 Col VK-30 in the presence of hydrophobic antibiotics

Plasmid in strain 1829	% Growth Inhibition			
	Rifampicin (μg/ml)		Novobiocin (μg/ml)	
	7.5	15	20	50
None	15	39	12	43
None+Ca++	18	34	12	40
ColVK-30	28	70	27	94
ColVK-30+Ca++	24	45	22	65

6.6.

Effects of Phosphate on antibiotic sensitivity

The nutrient broth used in these experiments contained low but detectable amounts of magnesium and calcium ions. The broths were therefore treated with phosphate to precipitate the divalent ions present. Accordingly, organisms grown in phosphate containing medium should be magnesium and calcium deficient.

It seemed that the phosphate ions did slightly affect the sensitivity of 1829 to novobiocin and rifampicin (Table 42). In the experiment in table 42 it was difficult to see whether there was increased sensitivity of Col VIK-94+ to rifampicin with 7.5 µg/ml and 15 µg/ml so I used lower concentrations of rifampicin of 2.5 and 5µg/ml when testing out 1829 Col VIK-94 (Table 43). Phosphate also affected the sensitivity of the other plasmid bearing strains to rifampicin (Tables 44, 45, 46). Even the Col VM 40(5)+ strain was affected; it may be that whereas increasing the level of divalent cations only reduces colicin secretion, reducing them may affect secretion of both colicin and transfer components. Alternatively the effect on this strain may be independent of the plasmid since 1829 is also sensitised by phosphate.

Table 42

The effects of phosphate on 1829 Col VIK-94 in the presence of 7.5 and 15 µg/ml rifampicin

Plasmid in strain 1829	% Growth Inhibition Rifampicin (µg/ml)	
	7.5	15
None	18	46
None+PO_4^{2-}	28	57
Col VIK-94	73	82
Col VIK-94+PO_4^{2-}	100	100

Table 43

The effects of phosphate on 1829 Col VIK-94 in the presence of hydrophobic antibiotics

Plasmid in strain 1829	% Growth Inhibition			
	Rifampicin (μg/ml)		Novobiocin (μg/ml)	
	2.5	5	20	50
None	15(±2.1)	19(±2.3)	19(±1.7)	42(±1.4)
None+PO_4^{2-}	10(±1.6)	20(±2.3)	39(±1.7)	68(±1.5)
ColVIK-94	12(±1.5)	24(±1.8)	53(±1.5)	96(±0.9)
ColVIK-94+PO_4^{2-}	38(±1.5)	94(±0.6)	92(±1.2)	100(±0.8)

Table 44

The effects of phosphate on 1829 Col VM40(5) in the presence of hydrophobic antibiotics

Plasmid in strain 1829	% Growth Inhibition			
	Rifampicin (μg/ml)		Novobiocin (μg/ml)	
	7.5	15	20	50
None	24(±2.2)	45(±2.3)	16(±1.7)	33(±1.4)
None+PO_4^{2-}	38(±2.3)	57(±2.1)	41(±1.9)	84(±1.3)
ColVM40(5)	37(±2.3)	73(±1.8)	27(±1.7)	96(±1.2)
ColVM40(5)+PO_4^{2-}	64(±2.2)	89(±1.3)	67(±1.5)	100(±0.8.)

Table 45

The effects of phosphate on 1829 Col VM50(1) in the presence of hydrophobic antibiotics

Plasmid in strain 1829	% Growth Inhibition			
	Rifampicin (μg/ml)		Novobiocin (μg/ml)	
	7.5	15	20	50
None	27(±2.2)	50(±2.1)	17(±1.8)	39(±1.7)
None+PO_4^{2-}	38(±2.0)	62(±2.0)	22(±1.5)	57(±1.7)
Col VM50(1)	41(±1.8)	92(±1.0)	52(±1.7)	79(±1.5)
Col VM50(1)+PO_4^{2-}	90(±1.2)	98(±0.9)	87(±1.2)	98(±0.8)

Table 46

The effects of phosphate on 1829 Col VK-30 in the presence of hydrophobic antibiotics

Plasmid in strain 1829	% Growth Inhibition			
	Rifampicin (μg/ml)		Novobiocin (μg/ml)	
	7.5	15	20	50
None	24(±2.2)	47(±2.3)	16(±1.7)	39(±1.6)
None+PO_4^{2-}	38(±1.9)	56(±2.3)	24(±1.4)	57(±1.6)
Col VK-30	28(±2.0)	73(±1.9)	33(±1.4)	96(±0.9)
Col VK-30+PO_4^{2-}	49(±1.2)	90(±0.9)	55(±1.7)	98(±0.5)

6.7

Temperature effects on antibiotic sensitivity

All the previous experiments were performed with organisms grown at 37° C. It is known that growth temperatures can affect the cell envelope properties. Colicin and transfer components are greatly reduced at 25°C (Tewari *et al*, 1985). Hence sensitivities to the various antibiotics have also been tested on 25° C grown cells. The experiments from tables 47-52 were performed with cells grown at the prior growth temperature of 25 °C but tested for antibiotic sensitivity at 37°C. Prior growth temperature seemed to reduce the sensitising effects of the Col V plasmids to rifampicin and novobiocin without affecting the sensitivity of 1829. Col VIK-94 and Col VM50(1) bearing strains were most affected i.e. effects on colicin components are likely to be the most significant effect of low temperatures. Also in these two strains, rifampicin sensitivity was reduced most by growth at 25°C, in accord with Col V-encoded sensitivity being mainly dependent on colicin components. The prior growth temperature of 25° C seemed to virtually reverse the effects of Col VIK-94 and its mutants and also the effects of Col VK-30.

The effects of the hydrophilic antibiotics and aminoglycosides were unaffected by the prior growth temperature of 25 °C in 1829 Col VIK-94. In view of this I did not proceed to test the

effects of a 25 °C prior growth temperature on the mutant Col V plasmid bearing strains in the presence of the hydrophilic and aminoglycoside antibiotics.

Table 47

Effect of a 25° C prior growth temperature on sensitivity of the Col VIK-94+ strain to hydrophobic antibiotics

Plasmid in strain 1829	% Growth Inhibition			
	Rifampicin (μg/ml)		Novobiocin (μg/ml)	
	7.5	15	20	50
None(37°C)	20(±2.0)	47(±1.9)	19(±1.6)	36(±1.6)
None(25°C)	19(±2.2)	40(±2.3)	16(±1.7)	40(±1.7)
Col VIK-94(37°C)	86(±1.5)	90(±0.7)	35(±1.7)	96(0.9)
ColVIK-94 (25°C)	34(±2.0)	59(±2.1)	33(±1.9)	73(±1.5)

Table 48

Effect of a 25°C prior growth temperature on sensitivity of Col VIK-94+ strain to hydrophilic antibiotics

Plasmid in strain 1829	% Growth inhibition			
	Ampicillin (μg/ml)		Minocycline (μg/ml)	
	1.0	2.0	0.5	1.0
None(37°C)	12	23	33	60
None(25°C)	14	25	30	54
ColVIK-94(37°C)	49	75	47	65
ColVIK-94(25°C)	45	67	50	56

Table 49

Effect of a 25° C prior growth temperature on sensitivity of Col VIK-94+ strains to aminoglycoside and polymyxin

Plasmid in strain 1829	% Growth inhibition			
	Streptomycin (μg/ml)		Polymyxin (μg/ml)	
	0.25	0.5	0.5	1.0
None(37°C)	13	25	56	95
None(25°C)	14	24	55	95
ColVIK-94(37°C)	40	73	95	99
ColVIK-94(25°C)	35	65	94	97

Table 50

Effect of a prior growth temperature of 25° C on the sensitivity of a 1829 bearing Col V mutant to the hydrophobic antibiotics

Plasmid in strain 1829	% Growth Inhibition			
	Rifampicin (μg/ml)		Novobiocin (μg/ml)	
	7.5	15	20	50
None (37°C)	22(±1.9)	46(±2.0)	19(±1.8)	34(±1.8)
None(25° C)	24(±2.1)	44(±1.9)	22(±1.9)	33(±1.8)
ColVM40(5)(37°C)	35(±2.3)	76(±2.2)	33(±1.4)	100(±0.9)
ColVM40(5)(25°C)	47(±1.9)	65(±1.9)	46(±1.8)	74(±2.0)

Table 51

Effect of a 25° C prior growth temperature on the sensitivity of a ColVM50(1)+ strain to hydrophobic antibiotics

Plasmid in strain 1829	% Growth Inhibition			
	Rifampicin (µg/ml)		Novobiocin (µg/ml)	
	7.5	15	20	50
None(37°C)	22(±2.0)	45(±2.0)	18(±1.5)	33(±1.8)
None(25°C)	25(±2.2)	44(±2.2)	22(±1.7)	34(±1.8)
ColVM50(1)(37°C)	34(±2.0)	78(±1.5)	52(±1.7)	72(±1.2)
ColVM50(1)(25° C)	26(±2.0)	49(±2.2)	39(±1.8)	60(±2.0)

Table 52

Effect of a 25° C prior growth temperature on the sensitivity of a Col VK-30+ strain to hydrophobic antibiotics

Plasmid in strain 1829	% Growth Inhibition			
	Rifampicin (µg/ml)		Novobiocin (µg/ml)	
	7.5	15	20	50
None(37°C)	22(±2.1)	44(±2.3)	18(±1.5)	33(±1.5)
None(25°C)	25(±2.1)	46(±2.2)	22(±1.5)	34(±1.7)
Col VK-30(37°C)	28(±2.1)	73(±1.6)	29(±1.8)	96(±1.8)
Col VK-30(25°C)	26(±2.2)	48(±2.2)	26(±1.8)	49(±1.7)

6.8

Effects of hydrophobic, hydrophilic and aminoglycoside antibiotics on lps mutant strains carrying Col V plasmids

The effects of hydrophobic, hydrophilic and aminoglycoside antibiotics were studied on two lps mutants of D21. They were D21e7 which had a galactose-deficient LPS and D21f1 which had a glucose-deficient LPS.

Most of the following experiments were repeated three times with consistent results and the results given in the tables are those of a single experiment. To these mutant strains were transferred Col VIK-94. The parent strain was D21.

The effect of Col V may be to alter LPS; accordingly, its ability to sensitise strains to antibiotics was tested in lps mutants. Both mutants were more sensitive than D21 itself to the hydrophobic antibiotics tested and D21e7 was more sensitive than D21 to 5.0 μg/ml spectinomycin; the two lps lesions did not however, sensitise to the hydrophilic agents tested (Tables 54 and 57). From table 53 it seemed that Col VIK-94 increased sensitivity of D21e7 to rifampicin and novobiocin. It may have also increased sensitivity to spectinomycin (Table 55) but not to the hydrophilic antibiotics (Table 54) and kanamycin (Table 55). Col VIK-94 also increased sensitivity to rifampicin and novobiocin in strain D21f1 (Table 56). Col VIK-94 also seemed to increase sensitivity to the hydrophilic and aminoglycoside antibiotics.

Table 53

Effects of hydrophobic antibiotics on D21e7 and D21e7 Col VIK-94 strains

Bacterial strain	% Growth Inhibition			
	Rifampicin (μg/ml)		Novobiocin (μg/ml)	
	5.0	7.5	20	50
D21	25	34	17	24
D21e7	48	80	64	84
D21e7 ColVIK-94	80	95	85	99

Table 54

Effects of hydrophilic antibiotics on D21e7 and D21e7 Col VIK-94 strains

Bacterial strain	% Growth Inhibition			
	Carbenicillin (µg/ml)		Ampicillin (µg/ml)	
	2.5	5.0	1.0	2.0
D21	15	17	7	10
D21e7	10	15	10	12
D21e7ColVIK-94	14	17	14	16

Table 55

Effects of aminoglycoside antibiotics on D21e7 and D21e7 Col VIK-94 strains

Bacterial Strain	% Growth Inhibition			
	Spectinomycin (µg/ml)		Kanamycin (µg/ml)	
	5	10	0.25	0.5
D21	61	74	4	9
D21e7	64	95	10	20
D21e7VIK-94	81	95	15	25

Table 56

The effects of hydrophobic antibiotics on D21f1 and D21f1 Col VIK-94

Bacterial Strain	% Growth Inhibition			
	Rifampicin (μg/ml)		Novobiocin (μg/ml)	
	5	7.5	20	50
D21	26	37	10	24
D21f1	60	85	70	85
D21f1 Col VIK-94	90	98	94	100

Table 57

The effects of hydrophilic antibiotics on D21f1 and D21f1 Col VIK-94

Bacterial STrain	% Growth Inhibition Carbenicillin (μg/ml)		Ampicillin (μg/ml)	
	2.5	5	1	2
D21	4	12	23	27
D21f1	5	11	17	15
D21f1VIK-94	43	51	50	70

Table 58

The effects of aminoglycosides on D21f1 and D21f1 Col VIK-94

Bacterial Strain	% Growth Inhibition Kanamycin (μg/ml)		Spectinomycin (μg/ml)	
	0.25	0.5	5	10
D21	27	46	37	50
D21f1	26	39	26	60
D21f1VIK-94	35	73	37	75

THE EFFECTS OF COL B PLASMIDS ON INHIBITOR SENSITIVITY

The effects of Col BK-98 on antibiotic sensitivity

Col B plasmids have been isolated from patients with serious disease (Mercer *et al*, 1984); hence the effects of Col BK-98 on strains 1829 and P678-54 were investigated. Col BK-98 has repressed transfer properties and produces colicins B and M.

The effects of hydrophobic, hydrophilic and aminoglycoside antibiotics were investigated and the results are shown in the following tables.

Tables 59 and 60 show the effects of hydrophobic compounds on Col B+ and B- strains. It seems that Col BK-98 only sensitises strain 1829 to rifampicin (fig.32). In order to find what the effects of the plasmid would be on sensitivity to hydrophobic antibiotics in another strain, P678-54 was examined. The results obtained from these experiments are shown in table 61. They show that even in P678-54 the plasmid sensitised the strain to rifampicin but not to novobiocin.

Tables 62 and 63 show the effects of Col BK-98 on 1829 in the presence of hydrophilic antibiotics. Figure 34 is the result of a

single experiment with ampicillin. It seems that the plasmid does sensitise the strain to the higher concentrations of ampicillin and carbenicillin but not to tetracycline and minocycline appreciably. Table 64 shows the effects of the plasmid in P678-54 on sensitivity to the hydrophilic compounds. There does not seem to be any major difference between the Col B+ and Col B- strains in sensitivity to hydrophilics and this is because there do[illegible] not seem to be any significant differences in the outer membrane proteins (Figure 19).

Tables 65 and 66 show the sensitivities of the Col B+ and the ColB- strains to the aminoglycoside and polymyxin antibiotics. Figure 34 shows the result of a single experiment with spectinomycin. The Col BK-98 plasmid seemed to sensitise the strain 1829 to spectinomycin, gentamicin, kanamycin and polymyxin. In contrast Col BK-98 does not appear to appreciably sensitise strain P678-54 to the two tested aminoglycosides (Table 67). Experiments were also carried out using copper sulphate (see Table 68). The results show that Col BK-98 slightly sensitised 1829 and P678-54 to copper sulphate.

Table 59

Effects of the ColBK-98 plasmid on sensitivity to the hydrophobic agents

Plasmid in strain 1829	% Growth Inhibition					
	Rifampicin (µg/ml)		Novobiocin (µg/ml)		Erythromycin (µg/ml)	
	7.5	15	20	50	10	20
None	20(±1.9)	48(±2.1)	11(±1.2)	30(±1.5)	20(±1.8)	35(±1.4)
Col BK-98	68(±1.8)	86(±0.9)	18(±1.1)	35(±1.5)	21(±1.1)	38(±1.5)

	SDS (%)		Oxacillin (µg/ml)	
	3	5	50	200
None	42(±1.8)	60(±1.4)	19(±1.0)	33(±1.5)
Col BK-98	54(±1.9)	68(±1.7)	15(±1.6)	30(±1.7)

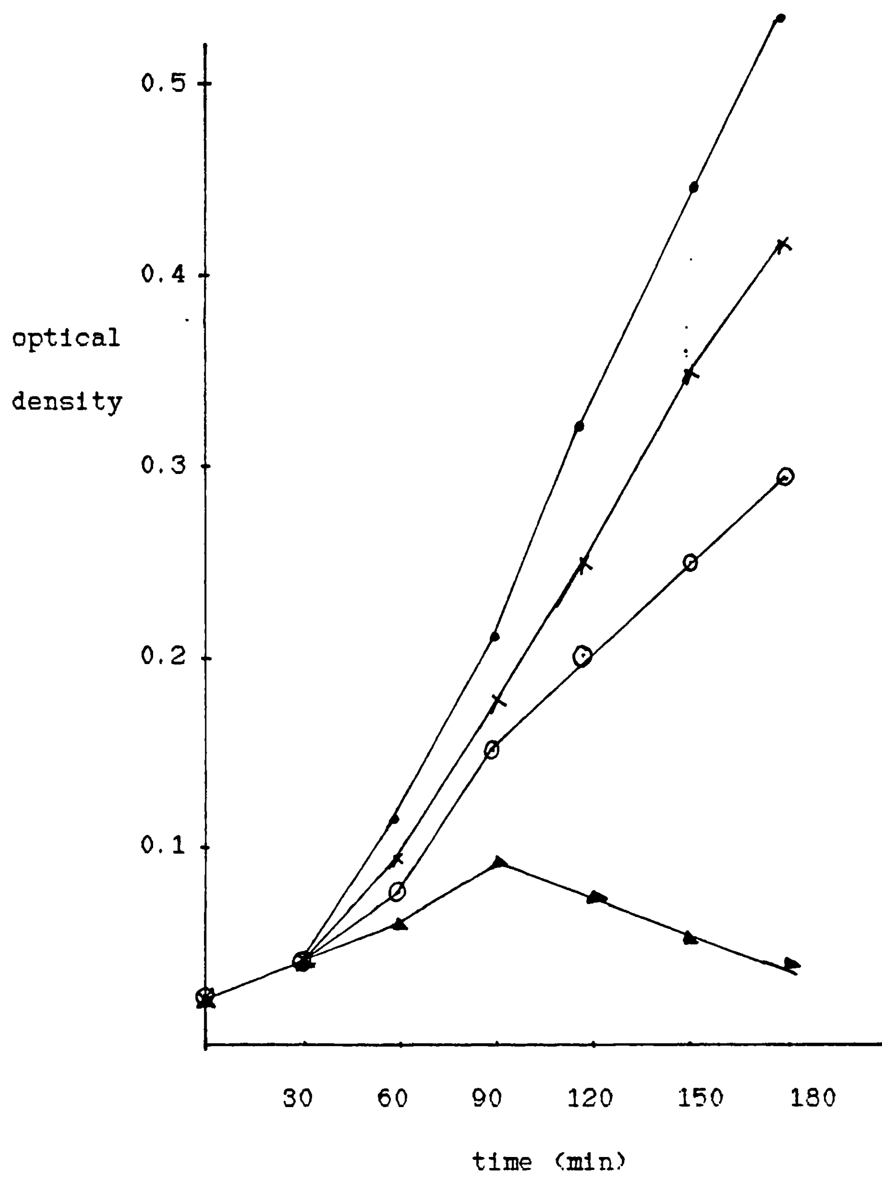


Fig.32. Effect of rifampicin on 1829 and 1829 Col BK-98.

Standard growth curves performed in absence and presence of 15μg/ml of rifampicin.

• 1829; - rifampicin

⊙ 1829;+ 15μg/ml rifampicin

× 1829 Col BK-98;- rifampicin

▲ 1829 Col BK-98 ;+15μg/ml rifampcin

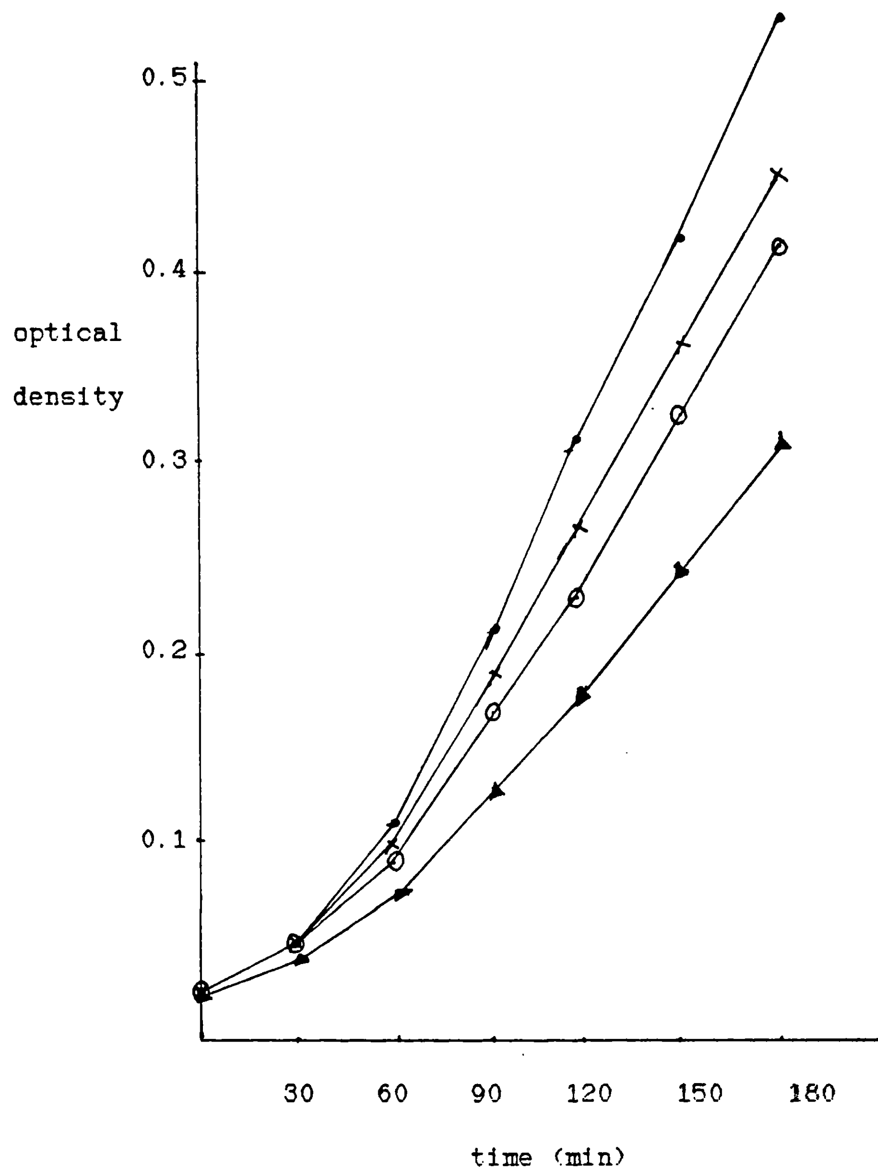


Fig.33. Effect of novobiocin on 1829 and 1829 Col BK-98.

Standard growth curves performed in absence and presence of 50µg/ml of novobiocin.

1829; - novobiocin

1829; + 50µg/ml novobiocin

1829 Col BK-98; - novobiocin

1829 Col BK-98 ; +50µg/ml novobiocin

Table 60

Effects of other hydrophobic inhibitors on the Col BK-98 plasmid bearing E.coli

Plasmid in strain 1829	% Growth Inhibition Rifamycin (μg/ml)		Deoxycholate (%)		Triton X 100 (%)		Vancomycin (μg/ml)	
	50	100	3	5	3	5	100	200
None	0	8	23	42	4	19	12	24
Col BK-98	8	12	29	54	8	20	8	20

Table 61

Effects of hydrophobic antibiotics on P678-54 Col BK-98 strain

Plasmid in strain P678-54	% Growth Inhibition			
	Novobiocin (µg/ml)		Rifampicin (µg/ml)	
	20	50	7.5	15
None	0(±0.6)	9(±0.9)	18(±1.2)	29(±1.1)
Col BK-98	8(±0.6)	12(±1.1)	47(±1.5)	79(±1.5)

Table 62

Effects of hydrophilic antibiotics on 1829 ColBK-98 strain

Plasmid in strain 1829	% Growth Inhibition			
	Ampicillin (µg/ml)		Carbenicillin (µg/ml)	
	1.0	2.0	2.5	5.0
None	13(±1.4)	21(1.3)	34(±1.5)	49(±1.5)
Col BK-98	18(±1.4)	49(±1.9)	41(±1.2)	84(±0.8)

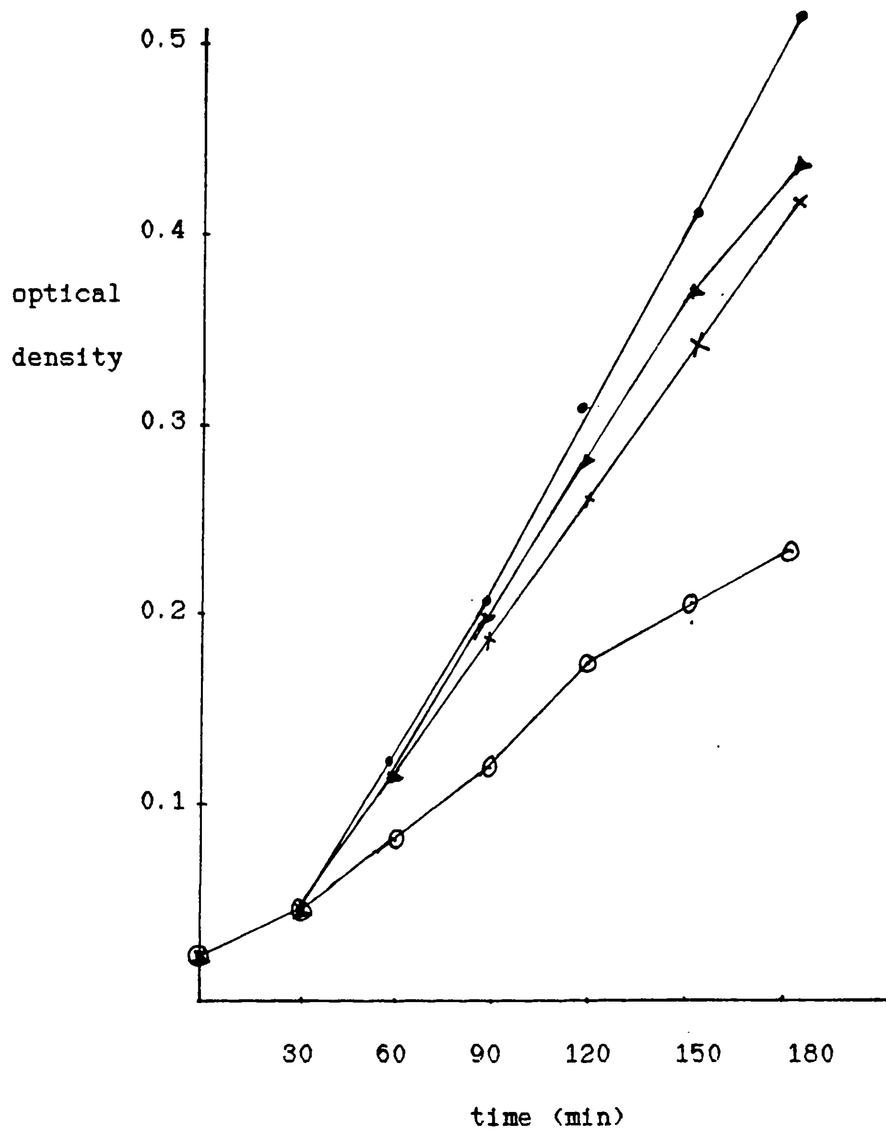


Fig.34. Effect of ampicillin on 1829 and 1829 Col BK-98.

Standard growth curves performed in absence and presence of 1μg/ml of ampicillin.

• 1829; - ampicillin

× 1829;+ 1μg/ml ampicillin

▲ 1829 Col BK-98;- ampicillin

⊘ 1829 Col BK-98 ;+1μg/ml ampicillin

Table 63

The effects of other hydrophilic antibiotics on 1829 ColBK-98

Plasmid in strain 1829	% Growth Inhibition			
	Tetracycline (μg/ml)		Minocycline (μg/ml)	
	0.5	1.0	0.5	1.0
None	60	79	35	63
Col BK-98	67	75	51	70

Table 64

The effects of hydrophilic antibiotics on P678-54 ColBK-98

Plasmid in strain P678-54	% Growth Inhibition			
	Ampicillin (μg/ml)		Carbenicillin (μg/ml)	
	1.0	2.0	2.5	5.0
None	15(±1.7)	23(±1.4)	16(±1.5)	25(±1.4)
Col BK-98	25(±1.5)	30(±1.4)	22(±2.0)	40(±1.3)

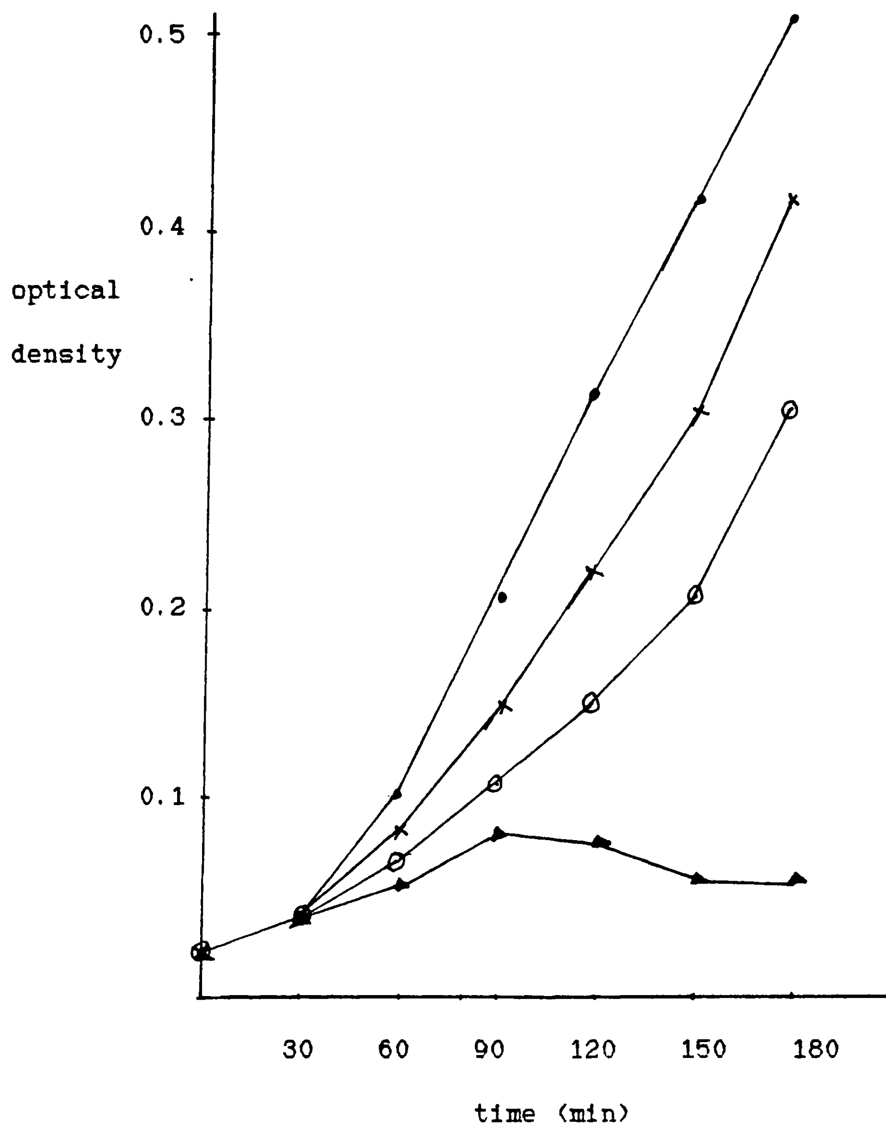


Fig.35. Effect of spectinomycin on 1829 and 1829 Col BK-98.
Standard growth curves performed in absence and presence of 10μg/ml of spectinomycin.

• 1829; - spectinomycin

⊙ 1829; + 10μg/ml spectinomycin

× 1829 Col BK-98; - spectinomycin

▲ 1829 Col BK-98 ; +10μg/ml spectinomycin

Table 65

The effects of Col BK-98 on sensitivity to the aminoglycoside antibiotics

Plasmid in strain 1829	% Growth Inhibition			
	Spectinomycin (µg/ml)		Gentamicin (µg/ml)	
	2.5	5.0	0.1	0.25
None	23(±1.5)	49(±1.5)	24(±1.4)	45(±1.2)
Col BK-98	27(±1.2)	95(±0.8)	81(±0.9)	94(±0.7)

Table 66

The effects of another aminoglycoside antibiotic and polymyxin on a Col BK-98 + strain

Plasmid in strain 1829	% Growth Inhibition			
	Kanamycin (µg/ml)		Polymyxin (µg/ml)	
	1.25	2.0	0.5	1.0
None	47	70	58	97
Col BK-98	78	92	92	100

Table 67

The effects of aminoglycosides on P678-54 Col BK-98

Plasmid in strain P678-54	% Growth Inhibition			
	Spectinomycin (μg/ml)		Kanamycin (μg/ml)	
	5.0	10.0	1.25	2.0
None	35(±1.6)	60(±1.5)	10(±1.5)	20(±1.3)
Col BK-98	48(±1.8)	73(±1.7)	14(±1.2)	25(±1.6)

Table 68

Effects of copper sulphate on Col BK-98 bearing strains

Bacterial Strain	Concentration of copper sulphate used	% Viability
1829	0	100
	200	93
	400	80
	600	80
1829 Col BK-98	0	100
	200	90
	400	75
	600	68
P678-54	0	100
	200	100
	400	90
	600	90
P678-54 Col BK-98	0	100
	200	75
	400	65
	600	65

EFFECTS OF MAGNESIUM AND CALCIUM SALTS ON SENSITIVITY OF COL BK-98+ TO INHIBITORY AGENTS

The addition of magnesium and calcium had no effect on the sensitivity of strain 1829 Col BK-98 to rifampicin (Table 69 & 72). This is in accord with B and/or M colicin components being involved in sensitivity because their synthesis is essentially unaffected by magnesium. Magnesium salts as previously mentioned reduced colicin synthesis in ColV strains but had no effect on colicin synthesis in Col BK-98 strains.

The addition of magnesium salts had no effect on the sensitivity of 1829 Col BK-98 to hydrophilic compounds (Table 71) but did have an effect on the sensitivity of 1829 Col BK-98 to gentamicin and polymyxin (Table 70). Magnesium as previously mentioned acts as a divalent cationic bridge between adjacent LPS molecules. As gentamicin and polymyxin may penetrate by displacing the divalent cations between the adjacent LPS molecules, addition of magnesium may compete with gentamicin and polymyxin and hence make the cells more resistant to the antibiotics. In agreement with this, magnesium affected the sensitivity of the plasmid - free strain also.

Table 69

The effects of magnesium salts on the sensitivities of a Col BK-98+ strain to hydrophobic antibiotics

Plasmid in strain 1829	% Growth Inhibition			
	Rifampicin (μg/ml)		Novobiocin (μg/ml)	
	7.5	15	20	50
None	26(±2.1)	55(±2.2)	19(±1.5)	36(±1.4)
None+Mg++	23(±2.2)	50(±2.1)	14(±1.6)	34(±1.7)
Col BK-98	74(±2.0)	92(±2.2)	25(±1.8)	30(±1.7)
ColBK-98+Mg++	75(±1.9)	84(±1.5)	28(±1.9)	35(±1.7)

Table 70

The effects of magnesium salts on the sensitivities of a Col BK-98+ strain to an aminoglycoside and polymyxin

Plasmid in strain 1829	% Growth Inhibition			
	Polymyxin (μg/ml)		Gentamicin (μg/ml)	
	0.5	1.0	0.25	0.5
None	68	75	28	42
None+Mg++	14	16	12	15
ColBK-98	92	100	81	94
ColBK-98+Mg++	10	18	15	18

Table 71

The effects of magnesium salts on the sensitivities of a Col BK-98+ strain to hydrophilic antibiotics

Plasmid in strain 1829	% Growth Inhibition			
	Minocycline (µg/ml)		Ampicillin (µg/ml)	
	0.5	1.0	1.0	2.0
None	30	55	13	18
None+Mg++	28	57	13	20
Col BK-98	49	75	28	37
Col BK-98+Mg++	45	70	25	35

Table 72

The effects of calcium on the sensitivity of a Col BK-98+ strain to the presence of rifampicin

Plasmid in strain 1829	% Growth inhibition	
	Rifampicin (µg/ml)	
	7.5	15
None	19	47
None+Ca++	22	44
Col BK-98	82	91
Col BK-98+Ca++	80	92

7.2

EFFECT OF PHOSPHATE ON THE SENSITIVITY OF COL BK 98+ STRAIN TO RIFAMPICIN

The addition of phosphate increased the sensitivity of 1829 Col BK-98 to rifampicin (Table 70).The concentrations of rifampicin used in these experiments were 2.5μg/ml and 5.0 μg/ml. Like the experiments with the ColV strains the nutrient broth used contained low amounts of magnesium and calcium ions. The phosphate that was added to the medium precipitated the divalent ions and hence the organisms grew in an environment deficient in calcium and magnesium ions.

This deficiency in the divalent cations may have acted by reducing the amount of divalent cationic bridging between the LPS molecules, thus allowing the easier permeation of the hydrophobic antibiotics but since the Col- strain was not affected, sensitivity may result from increased synthesis of some Col B component. Although adding magnesium does not appreciably reduce colicin B/M synthesis, reducing magnesium may increase it.

Table 73

The effects of phosphate on a Col BK-98+ strain in the presence of rifampicin

Plasmid in strain 1829	% Growth Inhibition Rifampicin (µg/ml)	
	2.5	5.0
None	0(±0.6)	8(±1.5)
None+PO_4^{2-}	0(±0.6)	14(±1.5)
Col BK-98	9(±1.2)	22(±1.7)
Col BK-98+PO_4^{2-}	30(±1.9)	60(±1.8)

EFFECTS OF GROWTH AT 25°C ON SENSITIVITY OF 1829 COL BK-98 TO RIFAMPICIN AND NOVOBIOCIN

Prior growth at 25°C had no effect on reducing the sensitivity to rifampicin. It was previously mentioned that the synthesis of colicin and transfer components is greatly reduced at 25 °C in Col V+ strains. Hence prior growth at 25°C greatly reduced the sensitising effects of the ColV plasmids. As regards 1829 Col BK-98, colicins B and M components are probably involved in the sensitivity to rifampicin. Presumably therefore the lower temperature of 25°C does not affect the synthesis of colicin and transfer components.

Table 74

The effects of a 25° C growth temperature on the sensitivity of a Col BK-98+ strain to hydrophobic antibiotics

Plasmid in	% Growth Inhibition			
strain 1829	Rifampicin (μg/ml)		Novobiocin (μg/ml)	
	7.5	15	20	50
None(37°C)	23(±2.2)	45(±2.3)	18(±1.6)	33(±1.7)
None (25°C)	25(±2.2)	46(±2.1)	22(±1.7)	34(±1.5)
Col BK-98(37°C)	68(±2.0)	90(±0.9)	10(±1.6)	33(±1.4)
ColBK-98(25°C)	67(±1.6)	85(±1.9)	8(±1.8)	36(±1.6)

7.4

EFFECTS OF COL BK-98 ON SENSITIVITY OF LPS MUTANTS TO HYDROPHOBIC, HYDROPHILIC AND AMINOGLYCOSIDE ANTIBIOTICS

As previously mentioned, D21e7 has a galactose deficient LPS and D21f1 has a glucose deficient LPS. The effects of hydrophobic, hydrophilic and aminoglycoside antibiotics have already been studied using Col VIK-94 in *lps* mutants. Again, most of the experiments were repeated three times and the results in Tables 75-80 were obtained from a single experiment.

Col BK-98 sensitised D21e7 to rifampicin at 5.0 μg/ml but not appreciably to novobiocin (Table 75). Col BK-98 had no effect on the sensitivity of D21e7 to hydrophilic antibiotics and aminoglycosides (Table 75 and 76).

Col BK-98 sensitised the strain D21f1 to rifampicin and novobiocin (Table 78). It also sensitised D21f1 to kanamycin (Table 80) but had no effect on the sensitivities to hydrophilic antibiotics (Table 79) and spectinomycin (Table 80).

Table 75

Effects of hydrophobic antibiotics on D21e7 and D21e7 Col BK-98 strains

Bacterial strains	% Growth Inhibition			
	Rifampicin (µg/ml)		Novobiocin (µg/ml)	
	5.0	7.5	20	50
D21	27	40	17	29
D21e7	60	90	66	82
D21e7ColBK-98	82	95	75	91

Table 76

The effects of hydrophilic antibiotics on D21e7 and D21e7 Col BK-98

Bacterial Strain	% Growth Inhibition			
	Carbenicillin (µg/ml)		Ampicillin (µg/ml)	
	2.5	5.0	1.0	2.0
D21	14	18	8	10
D21e7	15	27	10	14
D21e7 ColBK-98	15	30	12	18

Table 77

The effects of aminoglycosides on D21e7 and D21e7 Col BK-98

Bacterial	% Growth Inhibition			
Strain	Spectinomycin (µg/ml)		Streptomycin (µg/ml)	
	5.0	10	0.25	0.5
D21	60	72	4	9
D21e7	64	94	6	18
D21e7 ColBK-98	60	84	17	22

Table 78

The effects of hydrophobic antibiotics on D21f1 and D21f1 ColBK-98

Bacterial Strain	% Growth Inhibition			
	Rifampicin (μg/ml)		Novobiocin (μg/ml)	
	5	7.5	20	50
D21	24	38	10	24
D21f1	43	70	60	90
D21f1Col BK-98	92	100	92	100

Table 79

The effects of hydrophilic antibiotics on D21f1 and D21f1 Col BK-98

Bacterial Strain	% Growth Inhibition			
	Carbenicillin (μg/ml)		Ampicillin (μg/ml)	
	2.5	5	1	2
D21	8	12	24	27
D21f1	5	11	17	15
D21f1 Col BK-98	7	10	12	17

Table 80

The effects of aminoglycosides on D21f1 and D21f1 ColBK-98

Bacterial strains	% Growth Inhibition			
	Kanamycin (µg/ml)		Spectinomycin (µg/ml)	
	0.25	0.5	5.0	10
D21	27	46	35	50
D21f1	26	39	26	60
D21f1 Col BK-98	65	60	30	66

Section 8

COL PLASMIDS AND LPS ALTERATIONS

Effects of Tris-EDTA on bacterial strains carrying the ColV and ColB plasmids.

Further methods were used to investigate whether LPS alterations occurred in the presence of Col VIK-94 and Col BK-98. Tris and EDTA are important divalent cation chelators. They chelate magnesium ions and weaken bonds between the LPS molecules and hence destabilise the outer membrane. Both the ColV+ and the ColB+ were more sensitive than the Col- to inhibition by Tris-EDTA (Table 81); this may indicate that the binding between the LPS molecules in the Col V+ strain and the ColB+ strain may be weaker than in 1829, hence more easily disruptible and hence a greater percentage of growth inhibition.

There was not much difference observed between the p- and Col V+, ColB+ strains in growth inhibition by Tris-EDTA in the presence of benzylpenicillin; this antibiotic sometimes sensitises cells to the effects of Tris-EDTA (Table 82).

Table 81

Effect of Tris-EDTA on ColV and ColB bearing strains

Plasmid in strain 1829	% Growth Inhibition by Tris-EDTA	
	14mMTris,25mM EDTA	14mM Tris, 13mMEDTA
None	50	55
ColVIK-94	80	70
ColBK-98	71	55

Table 82

Effect of Tris-EDTA on ColV and ColB bearing strains in the presence of benzyl-penicillin

Plasmid in strain 1829	% Growth inhibition				
	Tris-EDTA	Benzylpenicillin (Tris-EDTA) (µg/ml)		Benzylpenicillin (µg/ml)	
		0.5	1.0	0.5	1.0
None	41	41	45	6	8
Col VIK-94	70	75	77	10	20
Col BK-98	53	58	60	8	12

The concentrations of Tris and EDTA used are 14mM Tris and 7mm EDTA.

THE EFFECTS OF TEMPERATURE AND STORAGE ON COLICIN B PRODUCTION

It was noticed that plates with Col B+ colonies gave larger inhibitory zones if incubated for a few days prior to addition of the sensitive strain. This was investigated further.

We looked at the effects of storage and temperature on the zone size. We wanted to see whether zone size changed when the B+ strain was stored at various temperatures and on agar plates containing sodium deoxycholate.

9.1. The effect of storage at 4° C on colicin B production

Storage of Col B+ before overlaying seemed to increase the zones produced (Table 83). In both cases (i.e. storage at 4°C or 37°C) the zone sizes seemed to have increased. This was probably due to continued colicin synthesis or to better release with extended storage.

9.2

<u>The effect of storage on medium containing sodium deoxycholate for one week at 4° C</u>

Zone sizes are smaller on nutrient agar + sodium deoxycholate (cf Tables 83 and 84). This is because sodium deoxycholate stops colicin M production by 1829 Col BK-98. Thus zone sizes on nutrient agar + sodium deoxycholate represent colicin B. Strikingly, zone sizes increased (on nutrient + sodium deoxycholate) with prior storage at 4°C but not at 37°C. Thus presumably the effect of storage at 37°C on 1829 col BK-98 plated on nutrient agar was due to increased colicin M production. Presumably at 4°c both colicin M and B increase on storage.

9.3

<u>The effect of temperature on colicin production</u>

In this experiment the zone sizes were measured after storage at different temperatures. Higher temperatures seemed to slightly increase the zones of inhibition (Table 85) although this was not so at 37°C (Table 83).

Table 83

The effect of storage for a week at 4° C on colicin B production

Day	Zone size (cm) for 1829 Col Bk-98 after storage on nutrient agar at:	
	37° C	4 °C
0	1.4	
1	1.5	1.5
2	1.5	1.4
3	1.7	1.5
4	1.9	1.8
5	2.7	2.9
6	3.7	3.9
7	4.2	4.5

The above experiment was repeated four times with consistent results.

Table 84

The effect of sodium deoxycholate on 1829 ColBK-98 incubated for one week at 4 C.

Day	Zone size (cm) after storage on nutrient agar + sodium deoxycholate			
	1829 ColBK-98 at		Pugsley B at	
	37C	4C	37C	4C
0	0.5		0.5	
1	0.5	0.7	0.5	0.7
3	0.6	1.2	0.5	1.0
5	0.6	2.0	0.6	1.4
7	0.6	2.4	0.6	1.9

The above experiment was repeated four times with consistent results.

Table 85

The effect of temperature on the colicin production of 1829 ColBK-98

Day	Zone size (cm) on nutrient agar after storage at (°C)				
	4	15	20	25	30
0	0.6	0.6	0.7	0.7	0.8
7	2.1	2.4	2.8	3.0	3.5

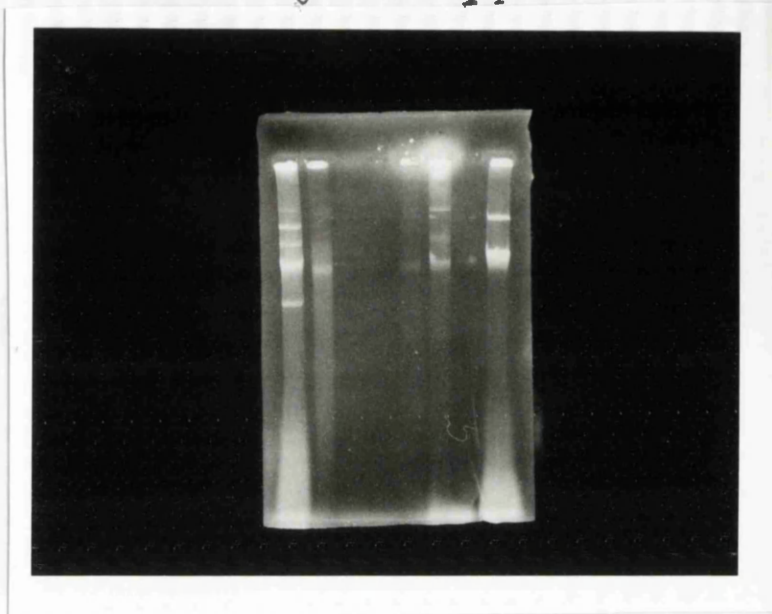
The above experiments have been repeated four times with consistent results.

Identification of novel colicin B-producing clinical isolates

A search was made in this laboratory for novel ColB plasmids of clinical origin. Col plasmids have been implicated in some aspects of *E.coli* virulence.

A collection of *E.coli* isolates from urinary tract infections was kindly made available by Dr. R.N.Gruneberg of the Dept.of Medical Microbiology at University College Hospital, London. These strains had been collected in 1982 at the hospital and in the general catchment area. One hundred and seventy four strains were screened but it was only strain 4469 that produced colicin B.They were screened by first doing a general colicin test using as indicator 1829 to identify firstly the colicin producers. Then strains 1829 ColBK-98 and the Pugsley Col B+ strain were used as indicators. The plasmid content of strain 4469 was examined by agarose gel electrophoresis. It contained a large plasmid of similar mobility to the Col BK-98 plasmid and a smaller plasmid. A typical gel is shown in figure 36.

8 2 1



Large Plasmid
Col BK-98
Chromosomal DNA

Fig.36.

Plasmid content of the colicin producing strain.

Lanes from right

1) 1829 Col BK-98

2) 1829

3)the colicin producing strain 4469

4)Pugsley B+ strain

8) Marker strain 39R861

DISCUSSION

Plasmids are of considerable medical importance because, in addition to often conferring antibiotic resistance, they are also frequently linked to virulence. Thus, isolates of *E.coli* from patients with serious disease are often p+ and Col V and Col B plasmids are particularly prevalent in such isolates (Mercer, *et al*, 1984).

Accordingly, it is important to know whether such p+ (including Col+) strains show increased inhibitor sensitivities which might aid their control. These might be linked to the outer membrane changes which plasmids frequently cause. Studies of such plasmid induced changes may also throw some light on how bacteria survive in the environment.

11.1. Effect of Col VIK-94 on the permeability of E.coli K-12 to hydrophobic antibiotics

It has been found that the outer membranes of *E.coli* and *S.typhimurium* confer a high resistance to hydrophobic antibiotics such as rifampicin, novobiocin, lincomycins, clindamycin, fusidic acid, SDS and hydrophobic dyes like crystal violet (Leive, 1974; Nikaido and Nakae, 1979; Nikaido and Vaara, 1985).

The hydrophilicity of the O-antigen region is unlikely to be the cause of this increased resistance since E.coli K-12 which lacks the O-antigen region shows resistance. It has also been suggested that the combined effects of divalent cation bridging of LPS molecules and high negative charge may be responsible for the absence of the hydrophobic pathway in most Gram-negative bacteria (Hancock, 1984). Certainly, the lipid regions of the LPS are tightly packed and would greatly reduce penetration of the hydrophobic agents.

Deep-rough mutants of E.coli and S.typhimurium demonstrate enhanced uptake of hydrophobic antibiotics, detergents and the hydrophobic dye ,gentian violet (Newton,1954; Lotter et al, 1979) and enhanced susceptibility to EDTA (Singh and Reitheimer,1975), Tris-lysozyme (Benz-and Hancock, 1981) and phospholipases (Gmeiner and Schlecht, 1979; Kamio and Nikaido, 1976).The outer membrane can also be permeabilised by chemical means e.g. with Tris-EDTA (Leive, 1974). One suggestion is that these genetic or chemical methods reduce the packing of LPS and permit penetration. The other hypothesis is that these changes may involve phospholipid bilayer regions in the outer membrane, created by the insertion of phospholipids in the outer leaflet (Nikaido and Vaara, 1985).

Other agents e.g. cationic ones, which permeabilise the outer membrane, may do so by displacing divalent cations and hence

weakening the LPS-LPS bonds leading to discontinuities in the outer membrane (Hancock, 1984).

Accordingly, other genetic or chemical treatments which permeabilise the outer membrane may act similarly to lps lesions and chemical treatments such as the above.

Initially used in this study were rifampicin and novobiocin to investigate the sensitivity of Col VIK-94 bearing strains. It is unlikely that this plasmid alters sensitivity of the target sites to these agents (Alfa, et al, 1987) and hence, it may affect the penetration of e.g. hydrophobic compounds into Col V+ strains. Col VIK-94 produced a marked sensitivity to rifampicin and novobiocin in 1829 (Table 6). It was proposed that either the colicin or transfer components or both were involved in sensitivity to these compounds.

Other hydrophobic compounds such as erythromycin, SDS, rifamycin, vancomycin, and Triton-X-100 were also tested. They all showed an increased effect on the Col VIK-94 bearing 1829 strain (Tables 6 and 7). The Col VIK-94 conferred sensitivity on strains 1829 and P678-54 to most of the hydrophobic compounds.

It may be that the enhanced ability of these hydrophobic compounds to act better results from the plasmid in some way altering the LPS structurally or in the weakening of the bonds between the LPS molecules.

We also used mutant derivatives of Col VIK-94 which were Col VM40(5), and Col VM 50(1). Col VM 40(5) does not encode synthesis of colicins V or Ia or their immunity components. Col VM50(1) does encode synthesis of colicins V, Ia and their immumity components but not of the transfer components.

Initially the effects of these mutant plasmids were investigated on the bacterial response to rifampicin and novobiocin and then we investigated the response to the other hydrophobic antibiotics and detergents. Both Col VM40(5) and Col VM50(1) sensitised strain 1829 to rifampicin but not to the same extent as Col VIK-94 (Table 6, cf Tables 18 and 22). This suggested that both the colicin and transfer components are needed for sensitivity to rifampicin unless the lesions which alter colicin synthesis in Col VM40(5) and transfer components in Col VM50(1) also affect other regions of the Col VIK-94 genome. We shall assume here that the mutants are specifically affected in the properties mentioned above. If anything, colicin components were most critical as factors in rifampicin sensitivity as indicated by studies with the mutant plasmids. Transfer components were needed for novobiocin sensitivity conferred by Col VIK -94 because mutational loss of transfer properties (in Col VM50(1) increased novobiocin resistance (Table 18) and repression of transfer properties had a similar effect (Table 30). Colicin components were less important because 1829 Col VM40(5) was nearly as sensitive as 1829 Col VIK-94 to novobiocin. There may be some

other Col V component involved in sensitivity to novobiocin as loss of transfer components as with Col VM50(1) did not restore full resistance.

The absence/presence of colicin or transfer components could lead to an alteration in the outer membrane properties and /or structure if these components have to pass through the LPS for secretion (e.g. colicin/ immunity components) or if membrane associated molecules alter LPS bonding or interactions. Such alterations could in turn affect permeation of the hydrophobic antibiotics through the outer membrane.

The sensitivity to oxacillin and SDS conferred by both mutant plasmids is lower than that observed for Col VIK-94 which may indicate that the transfer and colicin components are almost equally responsible for the increased sensitivity to these agents (Table 6 cf Tables 18 and 22).

11.2

Effects of Col VIK-94 on the permeation of hydrophilic compounds into E.coli K-12.

Hydrophilic compounds can pass through the outer membrane via the water filled channels formed from proteins called "porins". The hydrophilic compounds used in this study were ampicillin, carbenicillin, cephalothin, minocycline and tetracycline. Col

VIK-94 seems to sensitise the strain 1829 to ampicillin, carbenicillin and cephalothin (Tables 11 and 12). However, in strain P678-54 little effect of the plasmid was seen with ampicillin and carbenicillin (Table 13). The effects of Col VIK-94 might be directly on porin levels. Strain 1829 Col VIK-94 may have slightly more OmpF and OmpC than strain 1829 and this could cause increased sensitivity to the hydrophilic agents; in contrast, Col VIK-94 does not seem to affect the porin levels of P678-54 (Figs. 18 and 19). Also the VmpA protein might have a role in the penetration of hydrophilic compounds. If it did, then 1829 Col VIK-94 would be likely to be more sensitive than P678-54 Col VIK-94 as the VmpA level is greater in 1829 Col VIK-94 than in P678-54 Col VIK-94. VmpA is absent from the p- strains. Alternatively components produced by the plasmid might affect the proportion of open pores; if so, then there is much less effect on strain P678-54.

The rate of uptake by specific porins of different hydrophilic compounds is determined by the molecular properties of the porin channel, channel size and the ionic selectivity relative to the size and charge of the compound (Benz et al, 1982; Nikaido and Nakae, 1979; Nikaido and Rosenberg,1983) as well as the total number of available channels/cell (Nicas and Hancock, 1983).

Col VM50(1) sensitised 1829 to both ampicillin and carbenicillin but the effect with carbenicillin was less than that observed

with the Col VIK-94 plasmid. Accordingly, transfer components have some effect on sensitivity but not much. Col VM 40(5) did not seem to sensitise the 1829 to either agent. Strain 1829 Col VM40(5) produces little OmpF and there may be a small proportion of open channels available for the entry of hydrophilic antibiotics. This mutant plasmid fails to encode secretion of colicins or immunity components and it may be that it is the presence of these components which causes changed outer membrane interactions which produce more porin to be inserted and / or more open channels although magnesium failed to reverse the Col V effect on sensitivity to hydrophilic compounds which is contrary to expectations since magnesium reduces colicin V synthesis/ secretion (Davies et al, 1986).

At this stage it is difficult to say exactly what contributes to the sensitivity / resistance to the hydrophilic antibiotics. According to figure 18 the Col VM 40(5) strain does not seem to produce much OmpF protein. However, the strain is sensitive to copper sulphate. It could be that copper sulphate enters the cell by two routes, both by the porins and possibly via the LPS. This latter route could be enhanced in 1829 Col VIK-94 and in 1829 Col VM40(5) but not in 1829 Col VM50(1).

11.3.

Effects of Col VIK-94 on the permeation of aminoglycosides into E.coli K-12.

Aminoglycosides and polymyxins cross the outer membrane of Pseudomonas aeruginosa via the self-promoted transfer pathway (Hancock, 1984). It involves the displacement of divalent cations from the LPS by these polycations which results in the destruction of the LPS-LPS cross bridging and hence the destabilisation of the outer membrane (Hancock and Nicas, 1984; Hancock et al, 1981). From the results of the experiments it seems that Col VIK-94 sensitises 1829 to all these compounds. It is possible that in 1829, the porins are used for entry but possibly the self- promoted pathway is used for the entry of cationic compounds in E.coli 1829 Col VIK-94. The Col VIK-94 probably brings about changes in the LPS structure such as weakening of the bonds that exist between the LPS molecules hence making the p+ derivative of 1829 more permeable than 1829 itself to polycations and aminoglycosides. P678-54 Col VIK-94 was sensitive to spectinomycin but not streptomycin. Perhaps spectinomycin can use both porins and the LPS pathway for entry in P678-54 ColV+ but streptomycin cannot enter via the LPS in this strain but only enters the cell via the porins; studies with the hydrophilic compounds suggest that Col VIK-94 does not alter uptake via the porins in P678-54.

In this case the mutant plasmids both had a distinct effect on the sensitivity to spectinomycin; presumably therefore it is both colicin and transfer components which contribute to its sensitivity by possibly weakening the LPS-LPS bonds.

11.4.

Effects of copper on Col VIK-94+ bearing strains

Col VIK-94 sensitised 1829 and P678-54 to copper ions (Table 14). Copper sulphate can enter the cell via the Omp C and Omp F porins. It seems that there might be more Omp C and Omp F (Fig.18,tracks 7 and 9) induced by Col VIK-94 in 1829; hence this could explain the greater sensitivity of the Col VIK-94+ bearing strains but since porins levels do not appear to change in P678-54 Col VIK-94, there may be a different explanation; possibly Col VIK-94 enhances copper penetration via LPS in both strains.

Col VM 40(5) sensitised 1829 to copper ions whereas Col VM50(1) did not; possibly the Col V- encoded LPS penetration by copper is greatly reduced in ColVM 50(1) but not in Col VM40(5). This would suggest that it is dependent on the presence of transfer components.

11.5.

Effects of another Col V plasmid on inhibitor sensitivity

We looked at the effects of Col VK-30 on sensitivities of 1829 to hydrophobic, hydrophilic and aminoglycoside antibiotics. It seems to have less effect on 1829 than did Col VIK-94 as regards sensitivity to rifampicin (Table 26 cf Table 6). Hence, it could be that complete sensitivity could be due to both colicin V and Ia components. Col VIK-94+ but not Col VK-30 confers ability to produce Ia components as well as colicin components. Since, Col VK-30 lacks ability to form colicin Ia it may not sensitise the cell as much as Col VIK-94. With novobiocin, the sensitivity of 1829 Col VK-30 was the same as for 1829 Col VIK-94 as expected, since transfer components (which are present in derepressed levels in 1829 Col VK-30) appear most important for novobiocin sensitivity (see section 11.1).

The Col VK-30 did not seem to sensitise 1829 to the hydrophilic antibiotics. To ascertain whether this was due to the absence of any of the outer membrane proteins, the outer membrane porins were analysed on an SDS-PAGE gel. The outer membrane protein profile is given in figure 18. 1829 Col VK-30 has all the outer membrane porins and even the VmpA protein. Accordingly, the effects with the hydrophilic compounds are hard to explain but

colicin Ia components may be required for the full plasmid effects e.g. on porin activity.

Col VK-30 sensitised 1829 to spectinomycin at a concentration of 10μg/ml and kanamycin at 1.25μg/ml. Col VK-30 sensitised strain 1829 to copper sulphate but not to the same extent as Col VIK-94. Both effects may be due to enhancement of LPS penetration conferred by Col VK-30.

11.6.

Effects of divalent cations on inhibitor sensitivity in Col V+ strains

(a)

Rifampicin sensitivity in ColV+ strains

The addition of magnesium and calcium salts had a marked reducing effect on the rifampicin sensitivity conferred on 1829 by Col VIK-94 (Tables 31 and 38). Studies with strains carrying the mutant Col V plasmids had already suggested that for rifampicin sensitivity both the colicin and transfer components seemed to be important (Tables 18 and 22) but that colicin components had the most effect; presumably the presence of one or more colicin components in the envelope aids rifampicin entry. The above studies with divalent cations on 1829 Col VIK-94 were in accord with this. Thus magnesium salts (which appear to reduce colicin synthesis and secretion, Davies et al, 1986) reduced rifampicin

sensitivity. The divalent cations could act directly on the passage of rifampicin through the LPS, reducing the process, but since such cations have very little effect on the rifampicin sensitivity of strain 1829 itself,this is likely to be a minor effect; presumably it is mainly the lowered level of colicin components in the membranes in the presence of magnesium that reduces rifampicin sensitivity of the Col VIK-94 derivative. The colicin components might normally destabilise the LPS layer by themselves interacting with LPS molecules and reducing LPS-LPS interactions. Alternatively, colicin components might interfere with the association of LPS with other outer membrane proteins. In both cases, if magnesium greatly reduced colicin component secretion, increased rifampicin sensitivity would be abolished.

The studies of magnesium effects on rifampicin sensitivity of the strains with mutant Col Vs confirmed the above; sensitivity was unaffected by magnesium in the Col VM40(5) strain (which secretes no colicin components) whereas sensitivity of the Col VM50(1) strain was greatly reduced by magnesium.

(b)

Novobiocin sensitivity in Col V+ strains

Magnesium salts had only a slight reversing effect on novobiocin sensitivity with strains carrying Col VIK-94 and its mutants. This is in accordance with the fact that colicin

components have less effect on novobiocin sensitivity while transfer properties are not appreciably affected by growth with magnesium ions. The effects of magnesium on novobiocin sensitivity that did occur are presumably due to its effect on colicin components as the Col VM 40(5) strain was almost entirely unaffected. The results with the Col VK-30+ strain are surprising as novobiocin inhibition was markedly reversed by magnesium. One possibility (which should be tested) is that magnesium ions inhibit expression of the transfer properties of Col VK-30 but not Col VIK-94. Alternatively, the other plasmid component which appears to be involved in novobiocin sensitivity, (as complete loss of transfer components in Col VM50(1) strain did not restore full resistance) may play a bigger role in the sensitivity conferred by Col VK-30 and its synthesis may be affected by magnesium (e.g. as is synthesis of VmpA protein).

(c)

Hydrophilic and Cationic agent sensitivity of Col V+ strains

Magnesium had no effect on the sensitivity of any of the strains to the hydrophilic antibiotics (Table 32). Presumably, this is because entry of hydrophilic agents is via the porins. Presumably

ColVIK-94 enhances porin levels or porin efficiency by mechanisms which are not affected by magnesium ions. There is no sign of a new Col V encoded porin (and VmpA level is reduced by magnesium, Davies et al , 1986).

Magnesium ions did have an effect on the strain sensitivities to the aminoglycosides (Tables 33 and 37). This was probably due partly to the magnesium ions enhancing bridge formation between the LPS molecules making it more difficult for the aminoglycosides entering the cell. This is probably the major reason why magnesium reversed the effects of cationic agents since 1829 was affected as much as the ColV+ derivatives.

Where tested, calcium ions had essentially the same effects on the reversal of inhibition as did magnesium ions. The effect of calcium ions on colicin synthesis is unknown. Calcium ions like magnesium ions could act as a divalent cationic bridge between the LPS molecules and contribute to tighter packing of the LPS molecules, or could (like Mg++) affect secretion of Col V encoded components.

Effect of phosphate on sensitivity of Col V+ strains to the hydrophobic antibiotics

One way to starve organisms of magnesium , calcium and other divalent cations is to grow the cells in medium with added phosphate. Any magnesium or calcium ions are precipitated out as phosphates. Such organisms starved of these divalent cations proved to be more sensitive to the hydrophobic antibiotics rifampicin and novobiocin. Strain 1829 itself was slightly sensitised to rifampicin and markedly to novobiocin by phosphate. It seems likely that the phosphate effect on novobiocin sensitivity is unrelated to the presence or absence of Col V; the p- strain is affected at least as much as the p+ strain by the phosphate. Presumably the treatment either leads to envelope changes or to changes in the novobiocin target site (which are independent of Col V). Phosphate starvation also increases sensitivity of Col VM 50(1), Col VM40(5) and Col VK-30 to novobiocin (Table 44,45,46).

Phosphate also increased the sensitivity of these strains to rifampicin; this effect is enhanced by Col VIK-94 but the increase in sensitivity with Col VM40(5) and Col VK-30 is less than that observed for Col VIK-94 in the presence of 7.5μg/ml rifampicin (Tables 42,44, and 46).

Effect of temperature on antibiotic sensitivity of Col V+ strains

Col V+ organisms grown at a prior growth temperature of 25°C seemed to be less sensitive to the hydrophobic compounds rifampicin and novobiocin than those grown at 37°C. Col VIK-94, Col VM50(1) and Col VK-30 seemed to have less effect on sensitivity to rifampicin after a prior growth temperature of 25°C. As already stated rifampicin sensitivity appears to be dependent on both colicin and transfer components and since lower temperatures reduce the amounts of colicin and transfer components, the results with these strains confirm the earlier conclusions on sensitivity to rifampicin. With 1829 Col VM 40(5) there was no effect of low temperature on rifampicin sensitivity. This is probably because Col VM40(5) is deleted for the colicin components and it is these which play the main role with transfer components playing a subsidiary role.

With novobiocin, the effects of low temperature on sensitization by Col VIK-94, Col VK-30, Col VM 50(1) and Col VM40(5) are all similar. In each case, the 25°C culture was more resistant than that grown at 37°C. Novobiocin sensitivity is dependent more on the transfer components but another ColV component is also involved and this presumably is affected by low temperature in strain 1829 Col VM50(1).

Low temperature growth had no effect on the sensitivity of 1829 Col VIK-94 to hydrophilic and cationic compounds. It is therefore likely that the Col V components which enhance sensitivity (Tables 11, 14 and 15) are ones whose synthesis is not greatly reduced at 25°C.

11.9.

Effects of antibiotics on Col V+ derivatives of *lps* mutants

Both the strains D21e7 and D21f1 seem to be more sensitive to the hydrophobic compounds than D21 (Tables 53 and 56). The LPS of D21e7 lacks the phosphate groups on the heptose residues although it contains the glucose residue linked to the heptose while the LPS of D21f1 lacks all the glucose, galactose and phosphate molecules (figure 37) (Nikaido and Vaara, 1985). The presence of Col VIK-94 seemed to cause an increase in the sensitivity in both mutants to these hydrophobic compounds. This plasmid presumably acted by causing further alteration in the outer membrane, probably via its colicin and transfer components, which may have further weakened the LPS-LPS bonds in the already abnormal outer membranes of these *lps* mutants.

The plasmid-less deep rough mutants D21f1 and D21e7 did not show an increased sensitivity to the hydrophilic compounds compared to D21. However, when the plasmid Col VIK-94 was present there was

a distinct increase in sensitivity of D21f1 to the hydrophilic compounds. Col VIK-94 was unable to achieve a similar effect in D21e7. Col VIK-94 was also able to sensitise D21f1 to kanamycin. Presumably these effects of Col VIK-94 on sensitivity to hydrophilic compounds and kanamycin reflect increased porin level or activity.

Work in our laboratory has shown that in D21e7 there was virtually no Omp F whilst in D21f1 there was very little Omp F and OmpC porin. With D21f1 Col VIK-94 there was more OmpF and OmpC than in D21f1 or in D21. This probably explained the increased sensitivity to the hydrophilic antibiotics and kananycin.

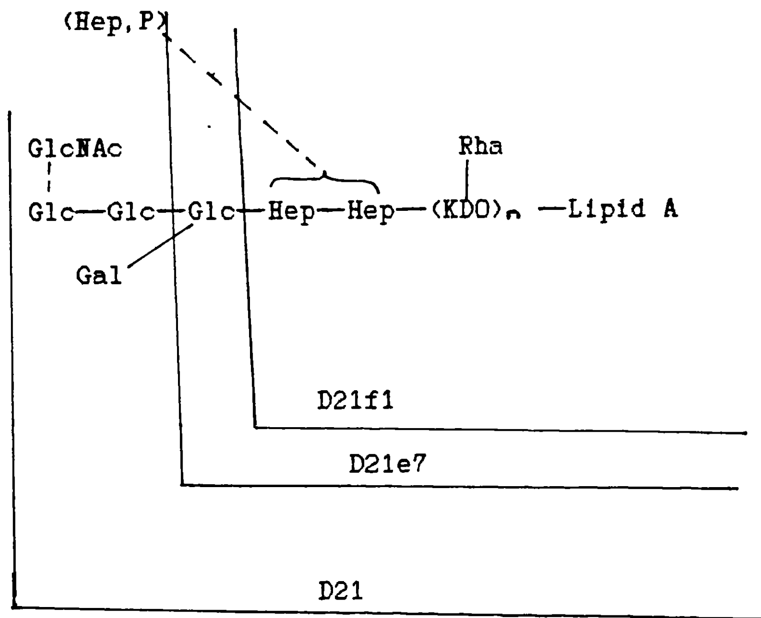


Fig. 37. Structure of LPS of E.coli K-12 (Nikaido and Vaara, 1985).GlcNAc,N-acetylglucosamine; Glc,D-glucose; Gal,D-galactose; Hep,l-glycero-manno-heptose; EtN, ethanolamine; P,phosphate. The broken lines show incomplete substitution.

11.10.

Effect of Col BK-98 on sensitivity to hydrophobic, hydrophilic and aminoglycosides antibiotics

Col BK-98 only sensitised 1829 and P678-54 to rifampicin of the hydrophobic agents. It confers repressed transfer properties and accordingly as with Col VM 50(1), sensitivity to antibacterials may depend on the presence of colicin components. However, there is no effect with the other hydrophobic antibiotics other than rifampicin. It may be that the change produced by Col BK-98 in the envelope allows only agents of a specific size and /or charge and/or hydrophobicity to penetrate, with only rifampicin fulfilling the requirements.

Col BK-98 also seemed to sensitise 1829 to the hydrophilic antibiotics ampicillin, carbenicillin and minocycline (0.5μg/ml). However, as with Col VIK-94 it does not seem to sensitise P678-54 to ampicillin and carbenicillin. This could be because the plasmid associated LPS changes in the outer membrane affect the porins e.g. the amount of available open porins and that this effect is specific to 1829. Col BK-98 did sensitise both strains to copper sulphate that normally enters via the porins This could mean that apart from entering the cell via the Omp F porin, copper sulphate could enter the cell via the LPS at least in the Col B+ strains.

Col BK-98 also sensitised strain 1829 to the aminoglycosides. However, it only sensitised P678-54 to spectinomycin and not kanamycin. Col VIK-94 did not sensitise P678-54 to streptomycin. This result is expected if these antibiotics (kanamycin and streptomycin) enter the cell via the porin pores (Nakae and Nakae, 1982) since plasmid effects on these maybe specific to 1829.

11.11.

Effect of divalent cations and phosphate on inhibitor sensitvity in Col B+ strains

Magnesium salts had no effect on the sensitivity of Col BK-98 strains to rifampicin (Table 69). This is in accordance with B and M colicin components being essentially unaffected by magnesium.

With the Col BK-98+ strains the effects of phosphate on sensitivity were less than that observed with Col VIK-94+ strains (Tables 70 cf Table 42).

11.12

Effect of a prior growth temperature of 25°C on inhibitor sensitivity in Col B+ bearing strains.

The sensitising effect (with respect to rifampicin) produced by Col BK-98 was as great for organisms grown at 25°C as for those grown at 37 °C. Presumably the prior growth temperature of 25°C had no effect on the formation of the colicin components by Col BK-98; it is probably these which cause increased permeation of rifampicin. This should be tested.

11.13.

Effects of antibiotics on Col B+ derivatives of lps mutants

Col BK-98 sensitised D21e7 and D21f1 to rifampicin (Tables 75 and 78). However, there was no appreciable increase in sensitivity observed with novobiocin in the case of D21e7. Col BK-98 sensitised strain D21f1 to novobiocin (Table 75). This is the only strain where Col BK-98 increases novobiocin sensitivity. The change produced by Col B, presumably to the LPS, only enhances rifampicin sensitivity in most strains. Possibly the LPS lesion in D21f1 allows more marked permeation to be conferred by the plasmid. Possibly the proteins inserted in the outer membrane by Col BK-98 further destabilise the already weakened LPS-LPS interactions in D21f1.

Col BK-98 like Col VIK-94 sensitised D21f1 to kanamycin and the hydrophilic compounds. These plasmids may have had an effect on the LPS-protein interaction thus making it easier for the ampicillin, carbenicillin, and the kanamycin to enter the cell. The changed protein - LPS interaction may have made available more open channels for the permeation of the above compound. With D21e7 these plasmids are presumably unable to bring about an altered protein-LPS interaction. Col BK-98 was unable to sensitise D21e7 to the hydrophilic compounds but oddly enough it sensitised the cell to kanamycin. Presumably in this case the kanamycin may enter through another route. Probably in this mutant the LPS-LPS interaction has been weakened by the Col BK-98 such that kanamycin finds its entry into the cell easier.

11.14.

Effect of Tris-EDTA on Col V and Col B strains

Leive (1965) showed that EDTA in the presence of Tris buffer released about one-half of LPS but litle else from E.coli and made the cells more sensitive to several hydrophobic compounds. One theory (Nakae and Nikaido, 1979) is that the region normally occupied by LPS then becomes filled by phopholipids. The presence of the phospholipids in the leaflet would create regions like those in the outer membrane of the deep-rough mutants thus making cells more sensitive to hydrophobic antibiotics.

An alternative explanation is that the LPS-LPS interaction is weakened by the removal of the divalent cations and that the altered LPS monolayer then allows the penetration of hydrophobic molecules. The removal of divalent cations increases the electrostatic repulsion between neighbouring molecules or between LPS and acidic proteins. Thus the packing of the LPS molecules becomes less tight, facilitating penetration. Tris, a bulky primary amine further contributes to destabilisation by partially replacing other cations bound to LPS (Nikaido and Vaara, 1985). High concentrations of Tris alone are sufficient to release significant amounts of LPS from smooth S.typhimurium (Nikaido and Vaara, 1985). Tris also sensitises smooth bacteria to complement (Reynolds and Pruul, 1971). Tris also renders the outer membrane

of an Rc mutant of S. typhimurium susceptible to labelling with dansyl chloride (Schindler and Teuber, 1978)

The results obtained here suggest that the presence of Col V and Col B may have themselves weakened LPS-LPS interactions thus making the p+ cells more susceptible to the Tris-EDTA. There seemed to be slightly more sensitisation by the ColV plasmid than by the Col B plasmid. This may be because the LPS-LPS bonds that exist in the Col B+ are slightly stronger than those which exist in the Col V+ possibly because of the absence of transfer components from the outer membranes of Col B+ bacteria (Col BK-98 confers repressed transfer properties).

11.15.

Effect of storage, sodium deoxycholate and temperature on colicin production

Storage at 4°C and at 37°C for one week before overlaying seemed to increase the production of colicin by Col BK-98+ strains. Col BK-98 encodes both colicins B and M. The effect may have been due to better colicin synthesis or better release of colicin after storage for one week. We also looked at the effects of sodium deoxycholate on zone sizes produced by 1829 Col BK-98 at 4°C and 37°C.

Sodium deoxycholate has the effect of inhibiting colicin M synthesis but has no effect on colicin B. As expected the zone sizes produced are smaller than when in the absence of sodium deoxycholate. Also, at 37°C there was no increase in zone size on storage in the presence of sodium deoxycholate. It may be that the increased colicin produced during storage at 37°C is only colicin M.

Higher temperatures during storage also allowed larger zones than lower temperatures . Perhaps growth at higher temperatures causes a greater disruption in the outer membranes thus allowing increased colicin release or possibly colicin continues to be

synthesised during storage and synthesis is better at 37°C.

11.16.

The New Col+ strain

As Col B plasmids have been implicated in clinical infections (Mercer *et al*, 1984) a search was made in this laboratory for the presence of ColB amongst clinical *E.coli* strains obtained from urinary tract infections. Only one strain of the 174 strains tested seemed to have a Col B plasmid. It was a little difficult to isolate the ColB+ strains as a lot of the cultures were contaminated with Proteus.

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