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TEMPERATURE-SENSITIVE LIPOPOLYSACCHARIDE DEFICIENT MUTANTS OF *SALMONELLA TYPHIMURIUM* AND THEIR OUTER MEMBRANE PERMEABILITY

by

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "Temperature-Sensitive Lipopolysaccharide Deficient Mutants of Salmonella typhimurium and Their Outer Membrane Permeability" submitted by Dassanayake M. Sirisena in partial fulfillment of the requirements for the degree of Master of Science.

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ABSTRACT

Two mutants of Salmonella typhimurium LT2 (SA2903 and SA2904) which were temperature-sensitive (ts) for lipopolysaccharide (LPS) synthesis were isolated from a galactose epimerase-deficient (galE) strain based on their resistance to phage C21 and sensitivity to sodium deoxycholate (DOC) at 42 C. Phage sensitivity tests and gel electrophoretic data indicated that the mutants produce LPS of Rc chemotype at 30 C and LPS that is deficient in heptose at 42 C. The galE+ transductants of these mutants were also temperature-sensitive for the synthesis of LPS; they produced LPS with O-somatic side chains at 30 C and heptose-deficient LPS at 42 C. P22-mediated transductional analysis showed that the mutation responsible for their temperature sensitivity is located in the rfa cluster at 80 min on the linkage map where several rfa genes involved in the synthesis of the LPS core are mapped. The electrophoretic mobility of the LPS of these mutants, when grown at 42 C, corresponded with that of an rfaF mutant, making LPS of chemotype Rd2, while their LPS when grown at 30 C resembled the LPS of the galE parent. The galE+ transductants of these ts mutants produced LPS which showed O-somatic side chains on the polyacrylamide gel when they were grown at 30 C. The ColE1 plasmid pLC13-13 carrying the wild type allele of rfaC, D, and F genes of Escherichia coli K-12 complemented these ts mutants, restoring wild type LPS, while plasmids carrying rfaG, B, I,

J, K and L genes of E. coli K-12 and S. typhimurium LT2 failed to complement the ts mutants. Thus, it was inferred that the genètic defect which determines their temperature sensitivity is in the rfaF gene.

The sensitivity of these mutants to various hydrophobic compounds was tested using several different methods. Sensitivity disc tests indicated that they are more sensitive to hydrophobic agents when grown at 42 C than when grown at 30 C. Gentian violet uptake experiments, cell lysis tests with anionic detergents (DOC and sodium dodecyl sulfate (SDS)), and minimum inhibitory concentrations (MIC) of several hydrophobic compounds confirmed the increased sensitivity of these mutants to such compounds at high temperature. Thus, the defective LPS structure of these mutants at 42 C has a great effect on their outer membrane permeability. The presence of Mg^{2+} ions decreased their outer membrane permeability to DOC, gentian violet, and hydrophobic antibiotics, though Mg^{2+} ions could not reverse their permeability to SDS.

The permeability of these mutants to hydrophilic compounds was also tested with several beta-lactam antibiotics. The permeability to piperacillin, which is less hydrophilic compared to other tested beta-lactam antibiotics, increased when the mutants were grown at 42 C. In contrast, the highly hydrophilic beta-lactams had a low rate of diffusion when the cells were grown at 42 C.

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To

Indu, Hiruni, and my parents

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

Amp^R	ampicillin resistance
DOC	sodium deoxycholate
EDTA	ethylenediaminetetraacetic acid
g	grams
kb	kilobases
kd	kilodaltons
KDO	3-deoxy-D-manno-2-octulosonic acid
L	litres
L-amp	L-agar containing ampicillin
L-tet	L-agar containing tetracycline
Lgg	L-broth containing 1% glucose plus 1% galactose
LPS	lipopolysaccharide
M	molar
mM	millimolar (millimoles)
mg	milligrams
MG	minimal glucose
ml	milliliters
min	minutes
MOPS	Morpholinepropanesulfonic acid buffer
mw	molecular weight
NA	nutrient agar
NB	nutrient broth
nm	nanometers
OD	optical density
OM	outer membrane
PBS	phosphate buffered saline
PFU	plaque forming units
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
sec	seconds
Tc	tetracycline
Tc^R	tetracycline resistant
TES	Tris-HCl-EDTA-sodium chloride
Tris base	Tris (hydroxymethyl) aminomethane
Tris-HCl	Tris (hydroxymethyl) aminomethane hydrochloride
ts	temperature-sensitive
UDP	uridinediphosphate
$V_{(i)}$	rate of hydrolysis of beta-lactam antibiotics by intact cells
V(s)	rate of hydrolysis of beta-lactam antibiotics by sonicated cells
u	micron
ug	microgram
ul	microlitre

I. INTRODUCTION

Temperature-sensitive (ts) mutants isolated from several organisms have facilitated the analysis of many biological processes. Such mutants are usually unable to grow or carry out certain biological functions at an elevated (restrictive) temperature but are able to function normally at a lower (permissive) temperature. Therefore, ts mutants have been used as important tools in understanding genetics and other regulatory mechanisms of such temperature sensitive systems. Kadam et al. (1985) isolated and characterized two classes of temperature- sensitive mutants of Salmonella typhimurium for the production of lipopolysaccharide (LPS). These mutants, with defects in the outer core region of the LPS molecule, synthesized LPS deficient in O-side chains at the restrictive temperature. The temperature sensitivity of these mutants was due to defects in the rfaI and rfaJ genes (Figure 1).

The LPS is one of the major components in the outer membrane (OM) of gram negative bacteria, which functions as a barrier against certain antibiotics, detergents and other molecules. Many pieces of evidence have suggested that the structure of LPS has a significant effect on the penetration of hydrophobic molecules through the OM (Nikaido and Vaara, 1985; Hancock, 1984). The defects in the inner core region of the LPS core can breach this permeability barrier and

allow the diffusion of hydrophobic molecules into the cell (Roantree et al., 1977; Sanderson et al., 1974b). Since these mutants produced LPS with much deeper lesions at the restrictive temperature, they are useful to study the effects of changes in the inner core part of the LPS on OM permeability. The advantage of using ts mutants for such comparative studies is the possibility of testing the effect of changes of LPS structure without the influence of other factors, which may become a problem when two different strains are used.

A new class of mutants, temperature-sensitive for the LPS synthesis, was isolated in this study. These mutants produced semi rough LPS at the permissive temperature (30 C) and deep rough LPS at the restrictive temperature (42 C). Thus the mutation responsible for the temperature sensitivity of these strains hampered the synthesis of the inner core region of the LPS molecule. Transductional genetic analysis and complementation studies indicated that the ts alleles of these mutants map between cysE and pyrE on the linkage map (Sanderson and Saeed, 1972; Kuo and Stocker, 1792) in a region where rfaC, D,and F genes are located. The SDS-polyacrylamide gel electrophoresis of LPS from these mutants showed that they produce LPS of chemotype Rc at the permissive temperature and Rd2 LPS at the restrictive temperature.

Different approaches were taken to determine the

OM permeability of these mutants to several hydrophobic substances (detergents, antibiotics, and dyes). The severe defects of the LPS structure at high temperature increased the permeability of their OM towards these compounds. In order to examine the effect of changes within the inner core region of the LPS molecule on permeability to hydrophilic compounds, a crypticity assay was done using several beta-lactam antibiotics. Although not very pronounced a correlation between the lipophilic character of these beta-lactam antibiotics and their permeability through the OM of LPS mutants was observed.

Divalent cations, especially Mg^{2+} ions play an important role in organization of the OM. The addition of Mg^{2+} ions seems to stabilize the OM and mutants with defective LPS are substantially reduced in their sensitivity to hydrophobic compounds in the presence of Mg^{2+} ions (Stan-Lotter et al., 1979). Several other studies have shown the interaction between divalent cations and LPS (Hancock, 1984). The mechanism that has been proposed to explain the hydrophobic pathway greatly relies on cross bridging of LPS molecules by divalent cations (Nikaido and Vaara, 1985). However, the effects of divalent cations on permeability changes have not been studied in detail. Therefore, an attempt was made in this study to investigate the permeability changes caused by Mg^{2+} ions using these ts mutants.

II. LITERATURE REVIEW

A. Genetics of the synthesis of the LPS molecule with special reference to the region involved in the outer membrane permeability. The LPS component of the OM of S. typhimurium is composed of three regions,i.e. lipid A, an oligosaccharide core, and a chain of polymerized units carrying the O-antigenic determinants (Figure 2). These parts are made separately and then assembled into a complete molecule. The synthesis of the LPS molecule is determined by many genes (Sanderson and Roth, 1983; Makela and Stocker, 1984).A cluster of genes termed rfb at 42 units of the linkage map is responsible for the synthesis of several special sugar nucleotides and for assembly of the O-repeat unit (Beckman et al., 1964; Nikaido et al., 1964). Another group of genes known as rfa is involved in the synthesis of the LPS core. Now it is known that the cluster of rfa genes determines several glycosyltransferase enzymes used for synthesis of the core region as well as for some steps in the synthesis of sugars for the LPS and for the transfer of O-side chain to the core. Subbaiah and Stocker (1964) mapped the rfa cluster on the chromosome in the xyl region. The precise location of this gene cluster was determined by P22 mediated transduction (Sanderson and Saeed, 1972) and ES18 mediated transduction (Kuo and Stocker, 1972) to be between

pyrE and cysE at 79 map units. These studies and the conjugation studies involving insertion of the F factor into this gene cluster (Sanderson and Saeed, 1972) established the map order cysE-rfaF-(rfaJ, rfaI, rfaL)-rfaG-pyrE. The rfaG gene has been suggested to determine the enzyme glucosyltransferase I required to attach the first glucose unit of the LPS core (Wilkinson and Stocker, 1968). Transfer of the galactose unit to the α 1-6 position of the first glucose on the LPS is catalyzed by the rfaB gene product (Wollin et al., 1983). It has been proposed that the transfer of the α 1-3 linked galactose unit is determined by the rfaI gene (Makela and Stocker, 1981). Even though Beckmann et al. (1964) recognized that the rfaJ mutants were lacking the distal glucose unit of the core, the exact role of this gene product is not yet determined.

Cloning of rfaG,B,I, and J genes by Kadam et al., 1985) confirmed the glycosyltransferase activities of these genes and also established the gene order of the rfa region to be pyrE - rfaG -(rfaB - rfaI) rfaJ - rfaL - rfaF - cysE.

rfaH is another gene affecting the core region of LPS but this is not located between cysE and pyrE (Kuo and Stocker, 1972). It was mapped between metE and pepQ at 84 min on the linkage map (Stocker et al., 1980), thus not closely linked to the rfa cluster. This gene is homologous to the sfrB gene of E.coli K-12 (Sanderson and Stocker, 1981) which acts as a transcription antiterminator on the

tra operon (Beutin and Achtman, 1979; Beutin et al., 1981). Lindberg and Hellerqvist (1980) found that the rfaH mutants produce a heterogeneous mixture of LPS of chemotypes Rb2, Rb3, and Rc. This suggested that genes specifying glucosyltransferase I and II are repressed and that these are in limiting concentrations so that not all of the LPS is completed. The mechanism of regulation of other rfa genes by rfaH gene product is not yet known.

The inner core region of the LPS is the portion directly involved in the permeability of the OM to hydrophobic substances because mutants with LPS defects affecting the heptose region (called "deep rough" mutants) have increased sensitivity to these agents. Genetics of the inner core part is not well understood compared to that of the outer core. The Salmonella LPS core has two heptoses in the main chain and has a partial branch substitution with a third heptose unit (Hammerling et al., 1973). Four rfa genes, rfaC, D, E and F involved in the synthesis of the heptose region have been identified. The heptose units of the normal LPS core are L-glycero-D-mannoheptose. An intermediate compound, D-glycero-D-mannoheptose is converted to the L - D form by nucleotide diphosphate - D - glycero - D - mannoheptose epimerase of which synthesis is determined by the rfaD gene (Lehmann et al., 1973). This mutation was mapped by Kuo and Stocker (1972) in the cysE-pyrE segment close to cysE. Wilkinson et al., (1972) isolated rough

mutants producing LPS of type Rd2 with only one heptose unit. The mutations of these strains were found to be located in the rfa cluster closer to cysE than to pyrE (Kuo and Stocker, 1972), and this gene was designated rfaF. Presumably, rfaF is the structural gene for the heptosyltransferase which adds the heptose II unit. Other rough mutants producing the LPS of Rd2 chemotype also mapped in the same genetic location (Sanderson and Saeed,1972; Sanderson et al., 1974a).

The first heptoseless mutant (Re chemotype) of S. typhimurium was also isolated by Wilkinson et al. (1972). This mutation was mapped outside the rfa cluster near metC gene at 64 units on the linkage map (Kuo and Stocker, 1972; Sanderson et al., 1974a). The gene rfaE may be the structural gene for a heptosyltransferase or may be concerned with the synthesis of a heptosyl donor compound. In addition to rfaE mutants, another class of mutants producing heptoseless LPS resulted from a defect in the cysE-pyrE region, which was designated rfaC (Sanderson et al., 1974a). There is no evidence as to whether this gene specifies the transferase for addition of the heptose I unit or is involved in the biosynthesis of the heptosyl donor.

Several other genes like galE and galU are also involved in the synthesis of the LPS core. Strains with these mutations resemble rfa in LPS character but these loci are not closely linked to any known rfa locus. The galU gene

located at the map position 34 on the chromosome encodes the enzyme UDP-glucose pyrophosphorylase (Nakae and Nikaido, 1971a,b) which is essential to synthesize UDP-glucose. Therefore, like rfaG mutants, galU mutants produce LPS of chemotype Rd1. Mutation of the galE gene at 18 min leads to galactose deficient LPS of type Rc, because galE mutants are unable to synthesize UDP-galactose (Nikaido, 1962), due to the lack of the enzyme UDP-glucose-4-epimerase that determines the epimerization of UDP-glucose to UDP-galactose (Fukasawa and Nikaido, 1959).

Several phosphate groups are found in the heptose region and lipid A region (Droge et al., 1968; Muhlradt et al., 1977). A gene involved in the transfer of phosphate groups to the LPS, termed rfaP has been recognized in S.minnesota (Jousimies and Makela, 1974) and S. typhimurium (Vaara, unpublished data, cited in Makela and Stocker, 1984).

Genetics of the lipid A region linked to the LPS core through 3-deoxy-D-manno-2-octulosonic acid (KDO) is very poorly understood. Mutants with complete defects of this region are nonviable. The few mutants known to affect this region are conditional lethal mutants (temperature-sensitive), are unable to grow at high temperature due to non-synthesis of lipid A (Rick and Osborn, 1972; Rick and Osborn, 1977) but are able to grow and make lipid A at low temperature (Lehmann et al., 1977). It has been proposed

that the KDO defects of these mutants are due to mutations in the kdsA and kdsB genes.

B. Effect of LPS on OM permeability. The fact that wild type strains of Salmonella, E.coli and other related bacteria are naturally sensitive to a number of hydrophilic antibiotics such as ampicillin and neomycin, and that the sensitivity to these antibiotics is not affected much by alterations in LPS structure is well established (Nikaido, 1979). In contrast, these strains are much more resistant to hydrophobic antibiotics, dyes such as gentian violet and to such detergents as bile salts and sodium dodecyl sulfate (SDS). However, mutants of S. typhimurium and E.coli with defective LPS have been shown to be altered in their sensitivity to those antibiotics and detergents. The quantitative evaluation of antibiotic sensitivity of E.coli mutants with incomplete LPS indicated those mutants were highly sensitive to several hydrophobic antibiotics including novobiocin and actinomycin D (Tamaki et al., 1971 ; Roantree et al., 1977). Rough mutants of S. typhimurium having defects in the heptose region were shown to have greatly altered sensitivity to several antibiotics, sodium deoxycholate (DOC), SDS, and lysozyme (Sanderson et al., 1974b).

These studies led to the conclusion that the degree of permeability to hydrophobic substances is increased with the degree of lesions in the LPS. In addition to such genetic

changes leading to defective LPS the structure of the OM can be altered by removing up to 50% of LPS molecules by treating Salmonella or E.coli cells briefly with EDTA (Leive, 1965). The removal of LPS by this treatment increases the sensitivity of these strains toward several hydrophobic antibiotics; actinomycin D, novobiocin , rifampicin and detergents (Leive, 1965; Leive, 1974). These conclusions, however, were based only on the inhibitory effects measured by using whole cells. A more direct approach was taken by Gustafsson et al. (1973). They showed that the rates of uptake of crystal violet, a hydrophobic dye, by intact cells were dependent on the nature of LPS present. The rate of gentian violet uptake increased with the increasing deficiency of saccharide units of the LPS in the tested E.coli strains. Similar results have been obtained for dye uptake with different LPS mutants of S. typhimurium (Stan-Lotter et al., 1979). Even though the results with dye uptake experiments seemed to be complicated by the massive adsorption of the dye to unidentified cellular constituents, similar results were obtained with nafcillin, a hydrophobic solute showing negligible adsorption to cellular constituents (Nikaido, 1976).

Even certain beta-lactam antibiotics which are primarily considered to be hydrophilic have been found to diffuse through the OM of E.coli at varying rates depending

on the structure of LPS. When the lipophilic character of the beta-lactams increases their rate of diffusion tends to decrease (Zimmermann and Rosselet, 1977). In addition to these studies the permeability barrier of Pseudomonas aeruginosa to some beta-lactam antibiotics has also been correlated with changes of the LPS structure (Godfrey and Bryan, 1984).

However, if the LPS structure alone constitutes the major barrier against hydrophobic substances there must be a gradual decrease in permeability as the saccharide chain becomes shorter and shorter. A sudden and discontinuous rise in permeability from Rc to Rd mutants accompanying a loss of only one glucose residue suggested that the LPS is not the only component that makes the OM impermeable for lipophilic compounds. Several studies have shown that the OM proteins are also involved in the barrier function, to a greater extent.

C. Effect of proteins on OM permeability. In order to act as an efficient barrier there must be a strong interaction between LPS and OM proteins. The first evidence for such an interaction came from the findings of Ames et al. (1974) and Koplow and Goldfine (1974) that levels of major OM proteins were drastically decreased in S. typhimurium and E.coli mutants synthesizing very defective LPS. These mutants were extremely sensitive to various hydrophobic agents. Later

Smit et al. (1975) confirmed this and they reported that in deep rough mutants (Rd or Re chemotype) the amount of OM proteins per unit surface area decreased to about 60% of the value of the wild type. However, in Ra, Rb, or Rc mutants (Figure 1) very little change was seen in the proteins levels and the decrease occurred suddenly when the non-reducing terminal glucose residue of the Rc LPS was lost by mutation. This indicated the involvement of OM proteins as well as LPS in the barrier function of the OM. Furthermore, mutants with a decreased amount of OM proteins but with apparently normal LPS were shown to be sensitive to hydrophobic inhibitors (Ames et al., 1974). Although the identity of major OM proteins of S. typhimurium has not been known, the decrease is most pronounced in two porin species, OmpF and OmpD, in such mutants (Nikaido and Vaara, 1985).

D. The mechanism of diffusion of hydrophobic compounds through the OM. It is known that the rate of diffusion of hydrophobic molecules across a membrane in a range of systems is faster with molecules of small size, with those of higher hydrophobicity and at higher temperature. These molecules first dissolve in the hydrophobic interior of the membrane, diffuse through the thickness of the hydrocarbon layer and then cross the membrane by partitioning into the aqueous phase on the other side of the membrane (Stein, 1967). The diffusion of hydrophobic molecules through the OM

of deep rough mutants of S. typhimurium seems to have properties similar to those noted above except that there is no clear-cut size limit (Nikaido, 1976). In the wild type strains of gram negative bacteria, the outer leaflet of the OM is almost occupied by LPS and proteins (Figure 2). The LPS molecules are distributed only on the outer leaflet. (Muhlradt and Golecki, 1975). Since the inner core region of the LPS molecule is highly charged the outer surface of the gram negative bacteria is usually electrically charged. The water filled porin channels of the OM formed by specific OM protiens allow the passage of only small hydrophilic molecules through the OM (Nakae, 1976). The amount of phospholipid content in the outer leaflet is very low compared to the inner leaflet. Hydrophobic molecules cannot pass through the aqueous pores or penetrate the charged LPS layer. Therefore, these molecules have difficulty in going through the OM of wild type strains of gram negative bacteria.

Nikaido (1976) proposed that in LPS defective mutants there is a different mechanism to allow the passage of hydrophobic substances through their OM. The opening of this hydrophobic pathway is mainly attributed to the fact that deep rough mutants showing increased permeability (Rd1, Rd2 and Re chemotypes) have a significantly increased phospholipid content. In wild type S. typhimurium the phospholipid content per unit surface area was quite low and

even in Rc mutants missing most of the polysaccharide chain in LPS the phospholipid content remained constant (Smit et al., 1975). The increased phospholipid content of deep rough mutants was more than that could be accommodated by one side of the membrane. Furthermore, the OM of these mutants contained considerably reduced levels of proteins, but the number of LPS molecules per unit area of surface remained constant (Smit et al., 1975). The incorporation of proteins into the OM of such mutants is impaired presumably because many of the major OM proteins interact with LPS (Yu and Mizushima, 1977; Schweizer et al., 1978) and the interaction is rendered difficult because of the extremely defective structure of the mutant LPS. Nikaido (1976) proposed that the decrease in proteins is compensated by the increased incorporation of phospholipids, which then produce phospholipid bilayer regions in the OM. Hydrophobic molecules can easily diffuse through these phospholipid bilayer patches, though they cannot penetrate the areas in which LPS comprises the outer leaflet.

This model was favored over the hypothesis that the hydrophobic permeability of the OM is primarily due to the deletion of the polysaccharide chain of LPS, which acts as the major barrier. such a hypothesis predicts a gradual increase in permeability as the polysaccharide chain becomes shorter and shorter. A sudden and discontinuous increase in permeability from Rc to Rd1 mutants accompanying a loss of

only one glucose residue cannot be easily explained by this hypothesis. The finding of Ames et al. (1974) that mutants with a decreased amount of OM proteins but with apparently normal LPS were sensitive to hydrophobic inhibitors supports the proposal that saccharide layers of LPS are not the primary factor that makes the OM impermeable for hydrophobic compounds. Despite such observations, many pieces of evidence have confirmed that structure of LPS has a quantitative influence on permeability.

In contrast, Gmeiner and Schleicht (1979) have observed an increase in LPS content concomitant with the degree of LPS sugar deficiency in different mutants. In the deep rough mutants of Rd1 and Re types almost a four fold increase in LPS was observed along with a less dramatic increase of phospholipid . This suggests that the asymmetric nature of the OM is maintained even in deep rough mutants. At the same time the major OM proteins with molecular weight of 30,000-40,000 daltons were greatly reduced. Their results strongly suggest that the protein deficiency in the OM of deep rough mutants is compensated for not only by phospholipids but mainly by multiple amounts of LPS. Thus it has proven difficult to fully explain OM permeability by any single model of the architecture of the OM of gram negative bacteria.

E. The effect of divalent cations on OM permeability. It has been shown that cations (Mg^{2+}, Ca^{2+}, Na^{+}) have a profound effect on the permeability properties of the gram negative OM. Leive(1965) demonstrated that the treatment of gram negative bacteria with the chelating agent ethylene diamine tetra-acetate (EDTA) could release about 50% of LPS due to the removal of divalent cations. Further, EDTA treatment makes the cells sensitive to a number of hydrophobic compounds including actinomycin D, novobiocin, etc. (Leive, 1974). Thus, the removal of divalent cations destabilizes the OM facilitating the passage of hydrophobic molecules into the cells. On the other hand, addition of Mg^{2+} ions seems to reverse the permeability changes stabilizing the OM. The leakage of periplasmic proteins to the surrounding medium is also caused by defective OM. Na^{+} and Mg^{2+} can prevent the leakage of periplasmic enzymes in rough mutants of S. typhimurium (Chatterjee et al., 1976).

Effect of cations in restoration of resistance to the hydrophobic antibiotics such as novobiocin, and to the dye gentian violet has been studied in more detail using LPS mutants of S. typhimurium and E.coli (Stan-Lotter et al., 1979). Their investigations revealed that Ca^{2+} ions had about the same effect as Mg^{2+} ions in reducing the uptake of gentian violet in rough mutants whereas Na^{+} ions had to be present in much higher concentrations. Further the require-

ment for growth in the presence of Mg^{2+} to reduce the permeability strongly suggested that new cell wall components are produced in the presence of Mg^{2+} ions or that there is a reorganization of the cell envelope, rather than merely binding of Mg^{2+} to the existing envelope to develop a barrier to penetration. However, the missing OM proteins of rough mutants are not restored in the presence of cations (Stan-Lotter et al., 1979). These studies led to the hypothesis that the divalent cations form non-covalent cross bridging of adjacent LPS molecules. Since LPS carries a net negative charge contributing to the strong negative surface charge of gram negative cells (Shirbert and Lakshmi, 1973) it is reasonable to expect such cross bridging of LPS. Divalent cations have a high affinity for LPS (Leive, 1975) and the analysis of cation interaction with LPS from E. coli using a cationic electron spin resonance probe clearly demonstrated that there are two binding sites for Mg^{2+} and Ca^{2+} ions. Although other cations also have the ability to interact with LPS Mg^{2+} and Ca^{2+} were the most effective (Coughlin et al., 1981; Schindler and Osborn, 1979).

Several charged groups are found in the inner core and lipid A regions of the LPS, phosphate groups being predominant (Droge et al., 1968; Muhlradt et al., 1968). Some of these phosphate groups are linked to the heptose (Lehmann et al,1971), some to KDO units (Gmeiner et al., 1971). Some Rough mutants of Salmonella have fewer phosphate

groups (Droge et al., 1968) bound to their LPS; such mutants of S. minnesota lacking the phosphate substituents are not able to add galactose or subsequent sugars to the LPS, thus they produce LPS of type RcP$^-$. As a consequence of this defect they also lack sugar units distal to glucose 1 (Hammerling et al., 1973). These mutants were also found not to contain the third heptose unit.

Two other substituents found in the inner core region of the LPS are ethanolamine (Droge et al., 1970; Lehmann et al., 1971) and 4-aminoarabinose (Volk et al., 1970). P^{31} nuclear magnetic resonance studies showed that many of the phosphate groups found on the LPS are linked to ethanolamine or 4-aminoarabinose and the substitution of these phosphate groups may vary considerably (Muhlradt et al., 1977) (Figure 2). Due to the presence of phosphate groups and free carboxyl groups of KDO the LPS molecule has a net negative charge. Positive charge of the free amino groups of ethanolamine and aminoarabinose reduce this negative charge. It has been suggested that these ionic groups may participate in ion exchange and in the control of the access of ions to the cell membrane (Heptinstall et al., 1970). These charged groups may affect the permeability of the OM because they may alter the binding of a cation like Mg^{2+} which has a profound effect in organizing the OM.

III. MATERIALS AND METHODS

A. Bacterial strains and media. The bacterial strains used are described in Table 1. All the strains were stored in L-broth with 15% glycerol at -76 C. They were used routinely after single colony isolations. L-broth (10 g Bacto-tryptone, 5 g Bacto yeast extract, 10 g NaCl, 3.5 ml 1 M NaOH, 1 litre water) containing 0.1% glucose (w/v) was used for the cultivation of all strains. Lgg-broth, in addition to the ingredients in L-broth contained 1% glucose (w/v) plus 1% galactose (w/v). Solid medium also contained 1.5% Difco Bacto agar. Tetracycline (25 ug/ml), ampicillin (50 ug/ml), kanamycin (50 ug/ml) and sodium deoxycholate (DOC) (0.4% w/v) were used with L-agar and minimal agar when required. Minimal medium contained per liter: 1g $(NH_4)_2SO_4$, 3 g KH_2PO_4, 7 g K_2HPO_4, 0.1 g $MgSO_4.7H_2O$, plus carbon source added to a final concentration of 0.2% (glucose or galactose). Solid medium was made by adding Difco Bacto agar at 1.5% . Minimal medium was supplemented with 1% L- amino acids when needed. Difco MacConkey agar, Difco Bacto nutrient broth and Difco Bacto nutrient agar were used as other growth media.

B. Chemicals and antibiotics. Antibiotics were from the following sources. Ampicillin, novobiocin, erythromycin, vancomycin, kanamycin sulfate and bacitracin from Sigma;

piperacillin (Cyanamid Canada Inc), carbenicillin (Ayerst Laboratories); ticarcillin (Beecham Inc.); nafcillin (Wyeth Ltd.) and antibiotic discs (Becton Dickinson, Canada). Acrylamide, N,N'-Methylene- bis-acrylamide, N,N,N',N'-tetramethylenediamine and sodium dodecyl sulfate (SDS) were from Bio-Rad laboratories . DOC, gentian violet and soluble starch were from Fisher Chemicals. All other chemicals were of analytical grade from various suppliers.

C. Bacteriophage sensitivity tests. The phage sensitivity tests were done according to the method of Wilkinson et al. (1972), using a set of phages assembled by B.A.D. Stocker and K.E. Sanderson. Cells grown to saturation in L-broth overnight at 37 C were flooded on plates of L-agar, the surface of the plate was allowed to dry at room temperature, phage suspensions at about 10^8 plaque forming units (pfu) per ml were applied using a multiprong replicator, and the plates were incubated at 30 or 42 C.

D. Sensitivity disc tests. Sensitivity to different detergents, antibiotics and gentian violet was tested employing the following method. DOC, SDS, Triton X-100, nafcillin and gentian violet discs were prepared by dispensing a known amount of each substance onto each disc. Bacterial strains were grown in L-broth or nutrient broth at 30 or 42 C to give an OD_{640} of 0.2-0.3. 0.7 ml of this culture was plated

on L-agar or nutrient agar, the discs were placed on the dry bacterial lawn, and incubated at 30 or 42 C for 24 hrs. The size of the zone of inhibition around each disc was measured.

The effect of Mg^{2+} ions was tested using the same method, but the cells were grown in L- broth plus 20 mM $MgCl_2$ or nutrient broth plus 20 mM $MgCl_2$ and plated on L-agar or nutrient agar to which $MgCl_2$ was added to the same concentration.

E. Gentian violet uptake experiment. The method of Gustafson et al. (1973) was slightly modified. The strains were grown into the logarithmic growth phase (50 klett units, Klett Summerson colorimeter, yellow filter) in L-broth at 30 or 42 C. The OD_{640} of all the cell suspensions was adjusted to 0.45 (LKB Ultrospec-4050 spectrophotometer), 0.5 ml of cell suspension was mixed in an Eppendorf tube with 0.5 ml of distilled water containing gentian violet (20 ug/ml), and incubated in a 37 C water bath for 15 min. The tubes were then chilled in ice and centrifuged (microcentrifuge, at 4 C). The absorbance of the supernatant at 590 nm was measured, and the dye uptake was calculated based on the estimated amount of dye left in the supernatant.

F. Determination of the rate of cell lysis by detergents. The rate of cell lysis by different detergents (DOC, SDS,

and Triton X-100) was determined by a spectrophotometric method. Cells were grown in L-broth to 0.4-0.5 OD_{640}, centrifuged , washed with 0.07 M potassium phosphate buffer, pH 7.2, and resuspended in the same buffer. The OD_{640} of the cell suspension was adjusted to 0.5. 1.9 ml of this suspension was incubated in a cuvette at 37 C for 30 min for equilibration, then 0.1 ml of 10% DOC solution was added to yield a final concentration of 0.5%, and the decrease in absorbance was continually monitored in an LKB Ultrospec-4050 spectrophotometer. The lysis test with Triton X-100 was essentially the same as the DOC test, but the cells were washed and resuspended in 0.9% NaCl solution rather than phosphate buffer when the test was done with SDS.

G. Determination of minimum inhibitory concentrations (MIC). Serial dilutions of each of the agents to be tested was made in L-broth. The final volume in each test tube was 1 ml. Cells were grown to exponential phase at 30 or 42 C in L-broth and diluted to 0.1 OD_{640}. 0.1 ml of this diluted culture of each strain was added to each tube of the serial dilution (approximately 10^7 cells) and incubated overnight at 30 or 42 C. The greatest dilution of the antibiotic or detergent which inhibited the growth was considered to be the MIC. When it was difficult to determine the right dilution the growth was compared by measuring the absorbance

at 640 nm.

H. Determination of partition coefficient. Partition of each compound into an organic phase (1-octanol) and an aqueous phase (0.05M sodium phosphate buffer, pH 7) was determined to calculate the partition coefficients. Since 1-octanol (Aldrich, gold label) showed a slight decrease in absorbance when mixed with pure phosphate buffer, 1-octanol was shaken with phosphate buffer and separated by centrifugation. This sodium phosphate buffer saturated with 1-octanol was used to prepare the aqueous solutions. Each compound was dissolved in phosphate buffer to a known concentration, shaken with an equal volume of 1-octanol, centrifuged at room temperature, and finally the concentration of each chemical in both aqueous and organic phases was determined spectrophotometrically (LKB Ultrospec-4050 spectrophotometer). The wave length of an absorbance peak of each compound was used: 590 nm for gentian violet (Gustafson et al., 1973), 227 nm for nafcillin (Nikaido,1976), and 210 nm for ampicillin, ticarcillin and carbenicillin. The absorbance peak of these beta-lactam antibiotics was determined in a Perkin-Elmer 552A UV/VIS spectrophotometer.

I. Isolation of LPS and gel electrophoresis. The cells grown at 30 or 42 C were resuspended in PBS (0.2 M phosphate buffer, pH 7.2, 0.9% NaCl) to give an OD_{640} of 1.0 and LPS

was extracted by the method of Hitchcock and Brown (1983), as follows. Aliquots of the cell suspensions (1.5 ml) were centrifuged for 2 min in a micro centrifuge, the pellets were solubilized in 50 ul of lysing buffer (2% SDS, 4% beta-mercaptoethanol, 1 M Tris HCl, pH 6.8 and 0.1% bromophenol blue), the resulting lysates were heated in a boiling water bath for 10 min, then 25 ug of proteinase K was added in 10 l lysing buffer and this mixture was incubated at 60 C for 2hrs. Then the samples were again heated in a boiling water bath for 5 min and frozen.

Electrophoresis was done using Mini Protean II dual slab cell (Bio-Rad). LPS samples were run on 12% SDS-polyacrylamide gels at a constant voltage (90V) in Tris-glycine buffer, pH 8.3, with 0.1% SDS. The LPS was visualized by silver staining using the Bio-Rad silver stain protocol, as follows. Gels were first fixed with 40% methanol (v/v) in 10% acetic acid (v/v) for 30 min and then fixed with 10% ethanol (v/v) in 5% acetic acid (v/v) for 30 min. LPS was oxidized for 5 min using 1.05 g periodic acid in 150 ml of second fixative. Gels were then washed three times with deionized water. Silver reagent (28 ml 0.1 M NaOH, 1 ml concentrated NH_4OH, 5 ml 20% $AgNO_3$ (w/v), 115 ml distilled water) was used to stain the gel for 15 min. After washing the gels with 400 ml distilled water (1 min) they were developed using the developer (50 mg citric acid, 0.5 ml 35% formaldehyde in 1 liter distilled water), and treated with

stop solution (5% acetic acid, v/v) for 5 min. The gels were photographed using Polaroid # 55 or # 57 film.

J. Genetic methods. (a). Propagation of bacteriophages. The strains in which phages were propagated were grown overnight in L-broth and diluted 1:50 into L-broth. Phage P22 HT105/int was added to a multiplicity of infection (MOI) of 1 and grown at 37 C overnight. The phage lysate was cleared by centrifugation (12,000g, 20 min, room temperature), mixed with chloroform and stored at 4 C. The phage lysate was titered by the "drop on lawn" method. Other phages for testing sensitivity to lysis were propagated similarly.

(b). Transduction. 1 ml of cells of each temperature sensitive rough mutant grown in Lgg-broth at 30 C was mixed with 10 ul of the phage suspension (at a titer of 10^8 pfu/ml) and incubated at room temperature for 20 min for preadsorption, plated on selective medium and incubated at 37 C. These strains were grown in Lgg at 30 C to make them smooth because only smooth strains are sensitive to the transducing phage P22 HT105/int; the Lgg medium causes the galE mutants to be phenotypically converted to smooth, and 30 C is the permissive temperature for LPS synthesis.

(c). Conjugation. Conjugation was carried out either by mixing broth cultures or by plate mating. In the former

method 1 ml of overnight cultures of donor and recipient strains were mixed and incubated at 37 C overnight, plated on the selective medium and incubated at appropriate temperature. Plate mating was done by mixing 10 ul of the donor and recipient cultures grown overnight at 37 C, on a plate of selective medium. If the donor strain is F^- the conjugation was done through another F^+ strain (three way cross). An equal volume of broth culture of the F^+ strain i.e. 0.5 ml in broth mating or 10 ul in plate mating, was mixed with donor-recipient conjugation mixture.

(d). Transformation. This was according to the method of MacLachlan and Sanderson (1985). Bacterial cultures grown at 37 C in L-broth were used to inoculate 100 ml of L-broth in which cells for transformation were grown. This was incubated in a shaking incubator at 37 C until OD_{640} of the culture reached 0.2-0.3, then the flask was chilled on ice for 15 min. A 1.5 ml sample was taken into an Eppendorf tube and centrifuged at 4 C, the cell pellet was washed with 1 ml of 0.1 M $MgCl_2$ and resuspended in 1 ml of 0.1 M $CaCl_2$ in 50 mM 3-N-morpholinepropanesulfonic acid buffer, pH 6.5, (MOPS), incubated on ice for 40 min, pelleted and resuspended in 0.3 ml $CaCl_2$. The cells were transformed with the plasmid DNA sample in TES buffer (50 mM Tris-hydrochloride, 10 mM EDTA, 50 mM NaCl, pH 8). 75 ng of DNA was added to the Ca^{2+} treated cells, the cell-DNA mixture was

then incubated on ice for 30 min, heat shocked for 2 min in a 42 C water bath, chilled on ice for 5 min, mixed with 1 ml chilled L-broth and incubated in a 37 C water bath for 10 min. L-amp (50 ug/ml) plates were used to grow the transformed cells incubating at 37 C, because all the plasmids used for transformation had been derived from pBR322 which carries resistance to ampicillin. All glassware and solutions were chilled to 4 C, and all operations before the heat-shock step were performed in a 4 C cold room.

K. Determination of the OM permeability barrier by beta-lactamase assay. Permeability of the OM to several beta-lactam antibiotics was determined as described by Zimmermann and Rosselet (1977) with slight modifications. In order to make the bacterial strains synthesize beta-lactamase, the plasmid R68.45 (incompatibility group P1) which carries resistance to ampicillin, kanamycin, and tetracycline was introduced into each strain by conjugation. The Pseudomonas aeruginosa strain PAO25 carrying R68.45 was used as the donor (Godfrey et al., 1984). The recombinants were selected for ampicillin (100 ug/ml) and kanamycin (100 ug/ml) resistance.

(a). Preparation of cells for enzyme assay. R^+ strains carrying plasmid R68.45 were grown in L-broth at 30 or 42 C

to an optical density of 0.5 at 640 nm. 1 ml samples were taken into Eppendorf tubes (3 samples from each strain), centrifuged for 3 min and washed three times in phosphate buffer (0.1 M, pH 7.2). One tube was left for 10 min, centrifuged, and the supernatant was chilled on ice; this is called the 10 min supernatant. 0.5 ml from each of the other washed cell suspensions was taken into another Eppendorf tube and the cells were ruptured by sonication using two 30 s bursts at 350 watt (Braun-Sonic 1510 sonicator). The tubes were kept on ice during and after sonication. The rest of the cell suspension was used to assay the enzyme activity of the intact cells.

(b). Enzyme assay. beta-lactamase activity of intact cells, sonicated cells and the 10 min supernatant was determined by the microiodometric method of Ross and O'Callaghan (1975). Iodine reagent (0.08 M I_2 in 3.2 M KI) was made by dissolving 2 g of I_2 and 53.29 g of KI in 100 ml distilled water. 0.2% (wt/v) hydrolyzed starch was prepared by dissolving 0.2 g soluble starch in 100 ml distilled water. 0.15 ml of iodine reagent was added to 100 ml of starch solution to prepare the starch-iodine solution. The substrate, a beta-lactam antibiotic at 0.2 mM in 0.1 M phosphate buffer, pH 7.2 was prepared immediately before the assay and kept on ice. The reaction mixture contained 1 ml of starch-iodine solution, 0.9 ml of phosphate buffer (0.1

M, pH 7.2), 0.1 ml of intact cell suspension (or sonicated sample or 10 min supernatant) and 1 ml of the substrate solution. Following addition of the substrate into the cuvette, the reaction was monitored in a recording spectrophotometer (LKB Ultrospec-4050 spectrophotometer). Absorbance at 620 nm decreases as the starch-iodine complex is reduced when beta-lactamase hydrolyzes the beta-lactam antibiotic. The very slow rate of the decrease of OD620 with boiled cells was also determined for each beta-lactam antibiotic and this was used to correct the rate of the reaction. The dry weight of the cells was determined using duplicate 25 ml sample from a culture grown to 0.5 OD_{640}. The samples were centrifuged, washed in distilled water, resuspended in 5 ml distilled water, filtered using a pre-weighed Millipore filter (0.45 u), then the filter was dried and weighed.

IV RESULTS

A. Mutant isolation and phage sensitivity tests. The mutants used for this study were isolated from S. typhimurium LT2 LB5010, a strain which has a mutation in the galE gene and is thus unable to ferment galactose since it is defective in the synthesis of the enzyme UDP-glucose-4-epimerase (Nikaido, 1962). Therefore, this strain produces incomplete LPS of chemotype Rc and is sensitive to the phage C21 as well as other rough specific phages like Ffm and Br60 (Wilkinson et al., 1972). Since mutants of S. typhimurium having LPS with only the first glucose unit (chemotype Rc) or the second heptose unit (chemotype Rd1) are sensitive to the phage C21, while mutants with more defective LPS are resistant, deep rough mutants from a galE line can be isolated by selecting for C21 resistance (Sanderson et al., 1974). This strategy was used to isolate the mutants used in this study, with the additional feature that temperature sensitive mutants were selected, which were expected to be smooth at the permissive temperature (30 C) but rough at the restrictive temperature (42 C). The parent strain LB5010 was grown in L-broth overnight at 37 C, plated on L-agar, flooded with a suspension of phage C21 and incubated at 42 C. C21 resistant colonies which grew on these plates were then replica plated onto L-agar plus 0.4% DOC (w/v), and incubated at 42 C; all the colonies were DOC-sensitive. This

sensitivity to DOC further confirmed their deep rough phenotype since mutants with defects in the heptose region of the LPS are unable to grow in the presence of surface active agents such as DOC (Wilkinson et al., 1972). When these colonies were replica plated on L-agar plus DOC and incubated at low temperature (30 C) a few of the colonies were able to grow, and were isolated and named SA2903 and SA2904. When these mutant strains were grown on L-agar at low temperature their phage sensitivity pattern was the same as that of the parent strain, LB5010 (Table 2) and they were resistant to DOC (Table 3), but at 42 C they showed the deep rough phenotype (sensitive to Ffm, Br60, and resistant to C21). Therefore, these mutants, SA2903 and SA2904, appeared to be temperature sensitive for LPS synthesis; semi-rough LPS at permissive temperature and deep rough LPS at restrictive temperature. When galE mutants are grown in Lgg-broth which contains 1% glucose plus 1% galactose in addition to other constituents found in L-broth, they can utilize this galactose to synthesize a complete LPS core; hence they give the smooth phenotype. The two temperature sensitive (ts) mutants, SA2903 and SA2904, when grown in Lgg at low temperature, became sensitive to smooth specific phages including P22 and FO, and resistant to DOC. However,these two strains grown in the same medium but at high temperature were sensitive to phages Ffm, Br60, and resistant to C21; and highly sensitive to DOC. The galE

parent (LB5010) showed smooth phage pattern and resistance to DOC at both temperatures. Thus, even in the presence of galactose these two mutants show their temperature sensitivity. This further indicates that only the galE mutation is affecting the synthesis of LPS at low temperature whereas another genetic block affecting the inner core region of the LPS molecule prevents the synthesis of complete LPS core at the restrictive temperature.

B. Determination of the genetic locus of the ts mutation by transduction. The mutant alleles of these two ts mutants SA2903 and SA2904 which are responsible for the synthesis of defective LPS at high temperature are designated rfa-3077 and rfa-3078 respectively. In order to determine the appropriate location of these mutant alleles on the linkage map the ts mutants were transduced with the phage P22 HT105/int propagated in an rfa+ strain (SA2703) carrying transposon Tn10 known to be inserted between cysE and pyrE close to the rfa cluster (C. Clark and K.E. Sanderson, unpublished data). The transductants were selected on L-agar plus tetracycline (Tc) medium to select recombinants carrying transposon Tn10. Strain SA2903 gave 130 Tc resistant transductants of which 38 (29%) were donor type for the rfa gene,i.e., rfa+, resistant to DOC and sensitive to phage C21 at 42 C, while the remaining 71% had the phenotype of the rfa-3077 allele (Figure 3). SA2904 gave 120

Tc resistant transductants and 47 (39%) were donor type (*rfa*$^+$) and 61% were recipient type. This proved that the *ts* alleles in the recipients are replaced by the transducing fragments so that such recombinants are no longer temperature sensitive. Since sensitivity to DOC is closely associated with some *rfa* mutants and the Tn10 in the donor strain is inserted close to the *rfa* cluster it could be inferred that the transducing fragments are carrying at least part of the *rfa* cluster so that the recipient *ts* mutants give recombinants which resemble the parental genotype. Thus, the donor *rfa*$^+$ allele is jontly transduced to the Tn10 transposon insertion by 29% in SA2903 and 39% in SA2904. Since the transducing fragment of P22 is about 50 kb in size and the chromosome of *S. typhimurium* is about 5000 kb this transduction analysis localized the *ts* mutation in a region only 1% of the entire chromosome. Since the frequency of transducing this region of the *rfa* cluster for SA2903 and SA2904 was 29% and 39% respectively, the *rfa-3078* allele in SA2904 is closer to the *pyrE* locus than *rfa-3077* allele in SA2903 (Figure 3).

In order to examine whether the *galE* mutation has any effect on the temperature sensitivity, and to eliminate the influence of this mutation on the LPS, the *galE*+ allele was transduced into SA2903 and SA2904 using phage P22 HT/105*int* propagated in the *galE+* strain, SA2703. These transductants selected on minimal medium with galactose as

carbon source, were designated SA3046 and SA3047. They were smooth at low temperature showing sensitivity to phages P22 and FO, but were rough at 42 C where only the rough specific phages (Ffm, Br60) but not the C21 were able to lyse them (Table 2) and they were very sensitive to DOC. Thus, the galE+ derivatives were also temperature sensitive indicating that galE mutation is not involved in the temperature sensitivity.

C. Determination of the LPS chemotype. As it was evident from the phage sensitivity tests the LPS extracted from these two mutants, SA2903 and SA2904, after growth in the presence of galactose produced smooth LPS at low temperature, showing the characteristic "ladders" on the sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) indicating many O-somatic side chains (Figure 4, lanes 1,3), but at 42 C their LPS had no side chains (Figure 4, lanes 2,4). Thus the SDS-PAGE data confirmed that these mutants are temperature sensitive even when grown in the galactose containing medium. The gel electrophoretic analysis (SDS-PAGE) of the LPS extracted from these mutants grown in L-broth also clearly indicated their temperature sensitivity for LPS synthesis. At permissive temperatures SA2903 (Figure 5, lane 1) and SA2904 (Figure 5, lane 3) produced LPS with mobility resembling that of parent LB5010 (Figure 5, lane 5) which is known to be of chemotype Rc. LPS from these two mutants

grown at 42 C (Figure 5, lanes 2,4) ran faster than Rc LPS did and produced a discrete band which resembled Rd2 LPS extracted from standard strain SL3789 (rfaF511) (Figure 5,lane 8). The proposal that the ts mutants produce LPS of chemotype Rd2 was confirmed on Figure 6, when the LPS of the four mutants SA2903, SA2904, SA3047, and SA3046 in lanes 6,7,9, and 10 had mobility identical to LPS from SL3789 (rfaF511) (lane 5) but different from other mutants such as SA1377 (lane 3), SL3769 (lane 4), and the parent strain LB5010 (lane 8, Rc chemotype). As expected, LPS from galE+ derivatives of the ts mutants SA3046 and SA3047 also showed side chains on the gels confirming that they synthesized complete LPS at permissive temperature (Figure 7,lanes 8,6). However, LPS from SA3047 (Figure 7, lane 6) has a high proportion of rough or semi-rough LPS molecules. This indicates a greater heterogeneity of the individual LPS molecules produced by this strain.

D. Determination of the rfa gene mutated in ts strains. In order to confirm that the ts mutations are in the rfaF gene, the ability of plasmids carrying different rfa genes to complement the ts mutants was tested. Plasmids carrying rfa genes of S. typhimurium and of E.coli K-12 (Figure 8) were transferred into these strains. Three different plasmids carrying rfa genes of S. typhimurium, pKZ15 (rfaG), pKZ26

(rfaG,B,I,J) (Kadam et al., 1985) and pKZ33 (rfaK,L) (P.R. MacLachlan and K.E. Sanderson, unpublished data) were transformed into the ts mutants SA3046 and SA3047, and selected for ampicillin resistance which is determined by a gene on the pBR322 vector. Complementation by pLC10-7 (ColE1 - rfaG, B, I, J) (Creeger and Rothfield, 1979) and pLC13-13 (ColE1- rfaC, D, F) (Coleman and Deshpande, 1985), two plasmids from Clarke and Carbon library with E. coli K-12 DNA inserted into a ColE1 vector (Clarke and Carbon, 1976) was also tested. Recombinants from these crosses were selected directly based on the nutritional requirements of the donor and recipient strains (Table 1). In addition to these plasmids three Tn5 insertions at different locations in pLC10-7, i.e., pKZ45 ($rfaG^+$, B^+, I^-, J^+), pKZ47 ($rfaG^-$, B^-, I^+, J^+),and pKZ48 ($rfaG^+$, B^+, I^+, J^+) (L.V. Collins and K.E. Sanderson, unpublished data) were tested for their complementing activity. These ColE1 plasmids were transferred by conjugation; they were mobilized by the F factor through the addition of another S. typhimurium strain (SA2197) which is F^+, because the donor strains lack the F factor. The recombinants were selected for kanamycin resistance determined by the transposon Tn5 inserted into each plasmid.

Complementing activity of the introduced genes was determined by testing the phage sensitivity pattern and DOC sensitivity of the recombinants. Only one plasmid, pLC13-13

carrying rfaC, D, and F genes of E. coli K-12 was able to complement the ts mutations of SA3046 and SA3047 (Table 4). These recombinants bearing pLC13-13 (SA3241 and SA3242) were sensitive to the smooth specific phages and resistant to DOC at high temperature. LPS extracted from these strains (grown at 42 C) clearly showed the presence of O-side chains when run on SDS-PAGE (Figure 7, lanes 9,10). This confirmed that the ts mutants are complemented by pLC13-13 allowing the synthesis of complete LPS. However, it was not possible to determine the affected genes of the ts mutants exactly because rfaC, D, and F genes are not yet well characterized or cloned separately.

E. Sensitivity disc tests. The sensitivity to several hydrophobic and hydrophilic antibiotics, detergents and gentian violet was initially determined using a disc containing the agent on a lawn of bacterial cells on a plate of L-agar. The test was performed on L-agar as well as on nutrient agar (NA); it was also tested with the addition of Mg^{2+}, but this will be discussed below. The two ts mutants SA2903 and SA2904 and their galE+ derivatives SA3046 and SA3047 showed an increased sensitivity to highly hydrophobic antibiotics, i.e., novobiocin, nafcillin, and oleandomycin and to the hydrophobic dye gentian violet when compared to the rfa^+ control strains LB5010 and SL3770 (Table 3). The size of the zone of inhibition around the

discs containing these compounds was usually two or three times greater at 42 C than at 30 C, and was substantially greater in the deep rough mutants than in the control strains. Since at high temperature these mutants produce deep rough LPS (Table 2, Figure 6) the increased sensitivity to hydrophobic antibiotics and gentian violet can be expected, for sensitivity to these agents has been reported earlier for rough mutants of S. typhimurium (Nikaido, 1976; Stan-Lotter et al., 1979; Ames et al., 1974). DOC and SDS also had the same effect, with only ts mutants showing zones of inhibition, and with much larger zones at the restrictive temperature (42 C), except for the deep rough mutant SA1377 (chemotype Re LPS) which is sensitive at both temperatures. The sensitivity of ts mutants towards bacitracin and vancomycin, which are less hydrophobic (Nikaido, 1976; Coleman and Leive, 1979) is not very much pronounced in L-agar, except for unusually high sensitivity of SA3046 at 42 C. However, when these two antibiotics were tested in nutrient agar they had a greater effect on ts mutants at high temperature (Table 3). Although ts mutants and deep rough strain SA1377 showed similar pattern of sensitivity to erythromycin, the smooth strain SL3770 also became sensitive to this agent. Despite its high hydrophobicity erythromycin seems to somehow overcome the OM barrier of these smooth cells. This strain showed a slight sensitivity to novobiocin too when the cells were grown in NA. All strains

irrespective of the LPS chemotype were equally sensitive to ampicillin (10 ug) and ticarcillin (75 ug) discs at both temperatures (data not shown).

In nutrient agar medium a similar pattern of sensitivity was observed. Even at low temperature the sensitivity levels of almost all strains were slightly higher compared to the values with L-agar medium. Bacitracin, vancomycin, and the neutral detergent Triton X-100 (data not shown) which did not have a clear effect in L-agar behaved like other hydrophobic compounds when the test was done in nutrient agar medium. SA1377 did not grow in nutrient agar at 42 C; this effect has been observed earlier by Chatterjee et al. (1976). Sensitivity to the polycationic antibiotic polymyxin B (300 units) and the cationic detergent cetyl pyridinium chloride (400 ug) was also tested in the same manner; both compounds had a very slight effect (zones of inhibition of 1-2 mm) on all strains regardless of the growth temperature (data not shown).

F. Spectrophotometric determination of cell lysis by detergents. Effect of detergents on ts mutants was studied more precisely by following the rate of cell lysis using a spectrophotometer. In this experiment the cells were produced at 30 and 42 C, then resuspended in a non-growth medium, and the lysis test was done at 37 C. Thus, the effect of the agent on the cells is independent of growth

during lysis experiment. This is a problem in other tests where the cells are tested for sensitivity at 30 or 42 C. Even smooth strains may be more sensitive due to growth at 42 C. But in this test rates of cell lysis clearly indicated the difference between sensitive and non-sensitive cells. Further, changes of permeability solely dependent on the temperature may affect the results of other sensitivity tests since they were performed at 30 and 42 C. This problem was avoided by doing the lysis test at 37 C. Each test was repeated three times and the results were very similar. Figure 9 and 10 show detailed data from such tests with a limited number of strains and agents. Data from one test was used to produce each curve shown in these figures.

In the lysis tests done with DOC (Figure 9) and SDS (Figure 10) ts strain SA3047 grown at 42 C and the Re mutant SA1377 showed a dramatic drop in optical density indicating a very rapid lysis. These data and other data derived from similar curves are shown in Tables 5, 6, and 7 as the percentage decrease in OD_{640} during the first 5 min (for DOC and Triton X-100) or during the first 2 min (for SDS) of the detergent treatment. The smooth strain, SL3770, was not lysed at all by DOC (Figure 9), but there was 2-3 % lysis in the presence of SDS (Figure 10). SA3047 grown at 30 C in L-broth gave 15.9% reduction in OD_{640} in the presence of DOC, but after growth at 42 C gave 50.6% reduction; similarly in SDS following growth at 30 C this strain gave

3.2% reduction in OD_{640}, but after growth at 42 C the reduction was 70.1%. The Other galE+ ts strain SA3046 also gave similar results although percentage reductions in optical density in the DOC test were less (4.4% at 30 C and 37.2% at 42 C) compared to the values obtained with SA3047. These data indicate the very low level of sensitivity of cells with smooth LPS to these two detergents, while the cells which have deep rough LPS are highly susceptible to them. SA2903 and SA2904 also had low rates of lysis by DOC at 30C, i.e., percentage reduction of OD_{640} was 6.5 and 10.6 respectively, but when grown at 42 C their reduction in optical density was about 35% (Table 5). The lysis by SDS was similar (Table 6) for the smooth strains SL3770 and SA3240, the rfa ts mutants SA3046 and SA3047 at 30 C and the Rc parent LB5010 all gave low rates of cell lysis (less than 7%), while SA3046 and SA3047 grown at 42 C gave 70-80% lysis, and SA1377 gave about 80% lysis when grown at both temperatures. Surprisingly, SA2903 and SA2904 gave a high rate of lysis when grown at either temperature. Thus the results of the cell lysis tests performed with DOC and SDS confirmed the increased sensitivity of these ts mutants to these anionic detergents at high temperature, when their LPS structure becomes more defective. The effect of the neutral detergent Triton X-100 (Table 7) was not very pronounced even on deep rough mutant SA1377 or on ts mutants grown at 42 C, but the percentage decrease in OD_{640} given by each of

these ts strains when grown at 42 C was higher than those given by the cells grown at 30 C (Table 7).

G. Gentian violet uptake experiment. Gentian violet uptake was determined by the method previously described by Gustafson et al. (1973). As observed by these workers, about 20% of the gentian violet added to the medium binds to the surface of the cells regardless of the nature of their OM. Thus uptake values of 20% or less than 20% obtained in this experiment represent this passive uptake. Only values greater than this limit show the active uptake of dye through the OM. Two smooth strains, SL3770 and SA3240 and galE parent LB5010, grown in L-broth, showed only this initial passive uptake at both temperatures, with uptake frequently less than 20% (Table 8). The comparison of uptake values in ts mutants indicate that their dye uptake is greatly dependent on the growth temperature, and therefore on the LPS chemotype, for SA3046 and SA3047 showed uptake of about 20% at 30C, but at 42 C about 40% of the gentian violet was taken up. The permeability of the other two ts mutants, SA2903 and SA2904 to gentian violet also increased tremendously so that the amounts of dye taken up at 42 C were double the amounts absorbed at 30 C (Table 8). Strains which were not temperature sensitive for LPS synthesis such as the smooth strain SA3240, the Rc chemotype LB5010, and the Re chemotype mutant SA1377 gave same gentian violet

uptakes at both 30 C and 42 C. The data obtained from this experiment compared very well with results of the gentian violet disc test (table 3).

H. Determination of the minimum inhibitory concentrations (MIC). The visible growth of cells in L-broth containing serial dilutions of the compound was compared to determine the MIC of several hydrophobic compounds. MICs of all tested compounds (DOC, gentian violet, novobiocin, nafcillin, vancomycin, and bacitracin were lower for ts mutants grown at the restrictive temperature compared to the MIC for wild type strain SL3770 (Table 9). DOC, gentian violet, novobiocin, and nafcillin which are highly hydrophobic (Nikaido, 1976; Coleman and Leive, 1979) showed a sharp difference in MICs with ts mutants when they were grown at permissive and restrictive temperatures. At 42 C the MICs of DOC and novobiocin for these four strains were half or less than half the values at 30 C. The MIC of gentian violet for the ts mutant SA3046 was 6.25 ug/ml when the cells were grown at 30 C, but when grown at 42 C the MIC decreased to <1.56 ug/ml (Table 9). This sharp difference in MIC of this highly hydrophobic dye, which depends on growth temperature was seen in other ts mutants too. With the smooth strain SL3770 the same MICs were obtained for each compound except nafcillin at low and high temperatures; the high MIC of nafcillin for 42 C grown cells may be due to the temperature

dependent permeability of this antibiotic (Nikaido, 1976). In contrast, MICs of all compounds with Re strain (SA1377) decreased drastically at high temperature, although it did not show such temperature sensitivity in cell lysis tests or gentian violet uptake experiment. A possible reason for this is the very slow growth rate of this strain at 42 C. The different growth rate of this strain at 30 C and 42 C does not allow a true comparison of its susceptibility to these agents in a this type of experiment which is dependent of the growth of cells. The increased sensitivity of this strain to novobiocin, DOC, and SDS was also observed in the sensitivity disc tests done on L-agar medium.

I. Effect of Mg^{2+} ions on permeability to detergents and antibiotics. The effect of Mg^{2+} ions on permeability of ts mutants was studied by performing the sensitivity disc test, gentian violet experiment, and cell lysis tests in the presence of Mg^{2+} ions. It has been shown that the divalent cations exert a great effect on OM permeability of gram negative bacteria (Leive, 1974) and Mg^{2+} ions seem to be effective in the stabilization of the OM against the passage of hydrophobic substances (Stan-Lotter et al., 1979; Hancock, 1984). There was a substantial decrease in sensitivity to DOC, SDS, gentian violet and other hydrophobic compounds in the disc tests done on L-agar and nutrient agar containing 20 mM $MgCl_2$. This protection was evident in all

The presence of Mg^{2+} caused a dramatic decrease in gentian violet uptake in smooth as well as rough strains (Table 8). Despite this protective effect the ts mutants had higher uptake values at restrictive temperature compared to their uptake values at permissive temperature. Cell lysis by DOC showed a general decrease when Mg^{2+} was present in the growth medium (Figure 9), especially in the Re strain SA1377 (Table 5). In contrast, growth in $MgCl_2$ could not protect against lysis by SDS in any case, and certain strains (SA2903, LB5010, and SA3047) became more sensitive to SDS when they were grown with $MgCl_2$. However, in the disc test Mg^{2+} ions had the opposite effect on these strains, for the sensitivity to SDS discs of all tested strains decreased when Mg^{2+} ions were incorporated into L-agar or nutrient agar (Table 3). Growth with Mg^{2+} ions did not have a clear effect on the action of Triton X-100, for some strains, such as SA2903 and LB5010 became more susceptible to this detergent whereas SA3046 and SA3047 were less sensitive to lysis following growth in Mg^{2+} containing medium (Table 7). Therefore, growth in Mg^{2+} ions reduces sensitivity to lysis by DOC, but it does not protect against SDS. The galE strain, LB5010, was very sensitive to SDS when Mg^{2+} is present, this might be due to some other change in the OM caused by the high Mg^{2+} concentration used in this experiment.

J. Determination of the OM permeability by"crypticity" assay. "Crypticity" assay in which hydrolysis of beta-lactam antibiotics by periplasmic beta-lactamase is assessed, is considered to be a relatively unambiguous technique of measuring hydrophobic permeability (Zimmermann and Rosselet, 1977). This method was used to examine whether the ts mutants with truncated LPS alter their OM permeability towards beta-lactam antibiotics and thereby correlate the degree of permeability to their LPS structure. The incompatibility group P1 plasmid R68.45 which carries resistance to ampicillin and several other antibiotics was introduced into the ts mutants and other test strains to make them synthesize periplasmic beta-lactamase. The ratio between the rate of hydrolysis of beta-lactams by intact cells and sonicated cells ($V_{intact}/V_{sonicated}$) was compared since it is a measure of OM permeability. Four beta-lactam antibiotics were used and the $V_{(i)}/V(s)$ ratio for each strain grown at 30 and 42 C are given in Table 10. Each $V_{(i)}$ or $V_{(s)}$ value given in Table 10 is an average of two independent tests.

The $V_{(i)}/V(s)$ values for the hydrolysis of piperacillin suggests that the ts mutants have an increased permeability to that antibiotic at 42 C than at 30 C. For example, for 30 C grown cells of SA2903/R^+ cells $V_{(i)}/(Vs)$ was 0.007, but for 42 C grown cells this increased to 0.07. Similarly the $V_{(i)}/V(s)$ for SA2904/R^+ increased from 0.02

to 0.073 when the growth temperature was changed from 30 C to 42 C. SA2903/R^+ and SA2904/R^+ showed higher ratios also with carbenicillin at 42 C. Although $V_{(i)}/V(s)$ of SA2903/R^+ with ticarcillin at 42 C is almost doubled compared to that of 30 C the values for SA2904/R^+ are opposite. For ampicillin also the relationship between $V_{(i)}/V(s)$ and growth temperature is opposite, because all strains except LB5010/R^+ when grown at 42 C had lower ratios. However, the rate of hydrolysis of ampicillin by the sonicated cell suspension of 30 C grown wild type strain SL3770R^+ was surprisingly low (0.04 umol/min/mg of dry weight of cells) compared to the rates with sonicated cells of other strains grown at the same temperature (Table 10). This cannot be due to the low level of synthesis of the enzyme, because $V_{(s)}$ value of the same strain grown at 30 C with piperacillin was much higher (0.564 umol/min/mg dry weight of cells) than that with ampicillin. Since ampicillin is a better substrate for this enzyme than piperacillin a higher rate of hydrolysis is expected with ampicillin. Due to the very low $V_{(s)}$ this strain had a very high crypticity value (0.842) for ampicillin at 30 C indicating a higher permeability. This strain is also showing a fairly high permeability to carbenicillin at 42 C. These differences observed in ts mutants and other strains cannot be solely attributed to the LPS structure of their OM.

In order to understand these differences in

permeability towards each of these beta-lactams the relative hydrophobicity of each of these compounds was determined by estimating their partition coefficients in an octanol/phosphate buffer system. Piperacillin, which showed an increased permeability in ts mutants at 42 C had the highest partition coefficient (0.037) with respect to other beta-lactams (Table 11) Ampicillin had the lowest partition coefficient (0.003); therefore highly hydrophilic. Thus, although the hydrophobicity of piperacillin and carbenicillin is very low compared to gentian violet or nafcillin the increased permeability of ts mutants to these two antibiotics at 42 C might be associated with changes in the LPS chemotype.

Most of the beta-lactam antibiotics do not show significantly greater penetration into ts rfa mutants at high temperature than at low temperature. However, this may be because these antibiotics are hydrophilic. Therefore, a beta-lactam antibiotic which is more hydrophobic was tested. The ts mutants grown at restrictive temperature had increased sensitivity to the beta-lactam antibiotic nafcillin in the sensitivity disc test (Table 3) and minimum inhibitory concentration test (Table 9). The partition coefficient of nafcillin in the octanol/phosphate buffer system, 0.407, was greater than those of other beta-lactam antibiotics (Table 11), indicating nafcillin is very hydrophobic. This compound seemed to be suitable to deter-

mine the hydrophobic permeability of the ts mutants by the method used to estimate the gentian violet uptake as well as by beta-lactamase (nafcillinase) assay.

The uptake experiment was performed exactly the same way as the gentian violet uptake test, except that the cells were washed and resuspended in the phosphate buffer (0.1 M, pH 7.2). The amount of nafcillin left in the supernatant was determined by the absorbance at 227 nm, the absorbance peak of nafcillin. However, the absorbance of the supernatant was higher than that of the original nafcillin solution. This could be due to the diffusion of some cellular material into the external medium, which is able to give high absorption at 227 nm. This experiment was not successful due to this complication.

An attempt was made to determine the rates of hydrolysis of nafcillin using the ts mutants carrying R factor R68.45. But these strains were unable to hydrolyze nafcillin at a rate greater than the background rate detectably in the microiodometric method, though the strains carrying the R factor is resistant to nafcillin on plates. Therefore, another plasmid pMG202 (obtained from George Jacoby, Massachusetts General Hospital) carrying genes for resistance to several beta-lactam antibiotics and expected to produce a beta-lactamase efficient in degradation of nafcillin was conjugated into the ts mutants, and the hydrolysis of nafcillin was tested using these new R^+

strains. The highest rate given in this experiment was 0.006 umol/min/mg dry weight of cells for the hydrolysis of nafcillin by the 42 C grown sonicated cells of the ts mutant SA2904. This is a very low rate compared to the rates obtained for other beta-lactam antibiotics with sonicated cells. This level of activity was too low for experiments done using the Zimmermann and Rosselet technique. Therefore the amount of cells used in the assay was increased by 30 times. Under these conditions the sonicated cell suspension reduced (decolorized) the starch-iodine complex, the indicator for the enzyme reaction, even without the addition of the substrate, nafcillin. Some cellular component of the sonicated cell suspension seems to act as the substrate for beta-lactamase in vitro, or such a component itself reduces the starch-iodine complex. Therefore nafcillin was not usable as a substrate in the Zimmermann and Rosselet method to determine the hydrophobic permeability of ts mutants.

V. DISCUSSION

A. Isolation and characterization of temperature sensitive (ts) mutants. Mutants with defects in the heptose region of the LPS core have been isolated from galE strains by selecting C21 resistant colonies (Wilkinson et al., 1972; Sanderson et al., 1974). The mutants used in this study, SA2903 and SA2904, were also isolated by the same method from the galE strain LB5010 by selecting for resistance to phage C21 at 42 C. The galE parent and these two mutants when grown in L-broth at 30 C were sensitive to rough specific phages including C21 (Table 2) and they were resistant to sodium deoxycholate (DOC). Following growth at high temperature (42 C) these mutants showed the deep-rough phenotype for they became sensitive to DOC and sensitive to rough specific phages, but resistant to C21. Thus they were identified as temperature-sensitive (ts) rough mutants.

Following growth in L-broth containing glucose plus galactose (Lgg) at low temperature the parent strain LB5010 and the mutants SA2903 and SA2904 had the smooth phenotype (as determined by phage sensitivity), and when grown at 42 C the phenotype of the mutants changed to deep rough. Therefore, regardless of the growth medium the mutants show their temperature sensitivity. Since the adsorption of rough specific and smooth specific phages is mainly determined by

the nature of the LPS on the cells, the changes in phage sensitivity of these mutants are inferred to be due to changes of the LPS structure which is dependent on growth temperature. Thus 30 C appeared to be a permissive temperature and 42 C a restrictive temperature for the synthesis of LPS in these mutants.

The ability of these rough mutants to produce a smooth LPS at permissive temperature in Lgg medium allowed absorption of transducing phage P22 and the isolation of galE+ derivatives by P22 mediated transduction. The galE+ strains, SA3046 and SA3047, also showed the same response to the temperature shift. This is a clear evidence that the galE mutation in these strains does not affect their temperature sensitivity. Therefore, the mutation responsible for temperature sensitivity affects the synthesis of the inner core region of the LPS molecule.

B. the genetic location of the ts mutation. Several genes are involved in the synthesis of the inner core part of the LPS molecule. The rfaG is the structural gene for glucosyltransferase I which catalyzes the addition of the first glucose unit in the core (Kadam et al., 1985) (Figure 1). The transfer of the second heptose unit requires the activity of the rfaF gene which is presumed to be the structural gene for the enzyme heptosyltransferase II (Sanderson et al., 1974a). The linkage between KDO and the

first heptose unit is determined by the rfaC, D, and E genes. The rfaG,F,C, and D genes have been mapped in the rfa cluster between cysE and pyrE loci whereas rfaE is located outside the rfa cluster (Kuo and Stocker, 1972; Sanderson and Saeed, 1972; Sanderson et al., 1974a). The transduction of the ts mutants using phage P22 propagated in an rfa$^+$ donor carrying transposon Tn10 close to the rfa gene cluster (C. Clark and K.E. Sanderson, unpublished data) gave recombinants which were rfa$^+$; they had the galE phenotype at both temperatures. Since these transductants were selected for Tc resistance they all carried transposon Tn10 from the donor, but only 29% of the transductants obtained from SA2903 and 39% from SA2904 lost the temperature sensitivity suggesting that the rfa$^+$ donor allele is jointly transduced with Tn10. The transduced rfa$^+$ allele could replace the mutant allele in ts strains; therefore, they are phenotypically similar at both temperatures. Thus, P22 mediated transduction localized the ts mutation in the rfa gene cluster.

C. LPS chemotype of ts mutants. When strains SA2903 and SA2904 are grown at 30 C their LPS has electrophoretic mobility corresponding to that of a strain of Rc chemotype, but when grown at 42 C they produced LPS corresponding to mutants of Rd2 chemotype (Figure 5 and 6). This confirmed that they are temperature-sensitive for LPS synthesis. The

SDS-PAGE of LPS from the galE+ derivatives of ts mutants, SA3046 and SA3047, clearly indicated the effect of temperature on the synthesis of LPS, for the LPS of 30 C grown SA3046 and SA3047 had O-somatic side chains, but LPS of 42 C grown cells resembled the Rd2 chemotype (Figure 7). Since all four strains, SA2903, SA2904, SA3046 and SA3047, produce LPS with electrophoretic mobility which corresponds to that of Rd2 mutant SL3789, which has a mutation in the rfaF gene and makes LPS of Rd2 chemotype, the temperature sensitivity of these strains is inferred due to a mutation in the rfaF gene.

The ability of the plasmid pLC13-13 that carries the wild type alleles of rfaC, D, and F genes of E. coli K-12 to complement the ts mutations in these strains further confirmed that their genetic defects are located in a region which determines the synthesis of the inner core part of the LPS molecule. However, at this time it is not possible to determine which of these three genes are mutated, since the complementing activity of these three genes cannot yet be tested individually.

D. OM permeability of ts mutants. Several studies on OM permeability of S. typhimurium have demonstrated that the structure of LPS has a great effect on resistance to antibiotics and detergents (Nikaido, 1976; Roantree et al., 1977; Stan-Lotter et al., 1979). These investigations

established the fact that the permeability to hydrophobic compounds is mainly due to the defects of the LPS structure. Stan-Lotter et al. (1979) determined the permeability barrier of S. typhimurium mutants by estimating their efficiency of taking up the hydrophobic dye gentian violet, a method considered to be more accurate to determine the hydrophobic permeability. However, in many studies the inhibitory effect on growing cells is taken as a measure of permeability. In this study, different approaches were taken to compare the OM permeability of ts mutants at permissive and restrictive temperatures. A clear correlation between LPS structure and the permeability to hydrophobic compounds was observed. The preliminary observations made in the sensitivity disc tests are in agreement with the results of the cell lysis and gentian violet uptake experiments. However, the results of the sensitivity disc tests seemed to be dependent on the growth medium. This has been reported earlier by Sonntag et al. (1978). The increased sensitivity to less hydrophobic antibiotics like bacitracin and vancomycin in the nutrient agar medium may be due to high rate of diffusion of these agents in this medium. The growth of the cells during the test and the varying rates of diffusion of the testing agent into the medium as well as into the cells could affect the results of the disc test. Since cell lysis tests were done in a non-growth medium at one temperature (37 C) it was possible to minimize such

problems.

The sensitivity disc tests indicated a high susceptibility of ts mutants grown at restrictive temperature to hydrophobic agents such as novobiocin, nafcillin, DOC, and gentian violet (Table 3) and these results agree very well with Roantree et al. (1977) and Stan-Lotter et al. (1979). The low minimum inhibitory concentrations (MIC) of the hydrophobic compounds for ts mutants grown at 42 C, compared to those for cells grown at 30 C provide strong evidence for increased permeability of these mutants at restrictive temperature (Table 9). The comparison of the gentian violet uptake is a direct way of measuring the hydrophobic permeability of these mutants. As suggested by the results of the disc test and MIC test the increased uptake of gentian violet at 42 C correlates very well with their deep rough LPS chemotype at high temperature (Table 8). These mutants grown at 30 C and the parent strain LB5010 showed about 20% uptake of the dye as expected in galE mutants. A greater portion of the uptake values of smooth strains must be representing surface bound dye. The MICs of gentian violet (Table 9) support this explanation because the MIC for smooth strain SL3770 at 42 C is about 50 times greater than those for ts mutants.

The most convincing and unambiguous data to support the varying level of OM permeability of ts mutants that depends on the growth temperature came from spectrophotometrically

determined rates of cell lysis by detergents. As described in the results section the level of sensitivity to DOC is directly related to the LPS chemotype. The smooth strain SL3770 is completely resistant to DOC while ts mutants grown at restrictive temperature and the deep rough strain SA1377 are extremely sensitive (Figure 9 , Table 5). The highest rate was obtained with SA1377 and this indicates the difference of the level of permeability to DOC between the chemotypes Rd2 (ts mutants grown at 42 C) and Re. The average increase of the rate of cell lysis when going from smooth to galE phenotype is less than 10% (Table 5). This supports the fact that Rc mutants are not hyperpermeable despite the absence of 80 to 90% of the saccharide chain found in the wild type LPS (Nikaido and Vaara, 1985). The rates from the SDS test for smooth strain and galE strain LB5010 are also very low (Table 6). In contrast, cells of SA2903 and SA2904 produced at low temperature, therefore phenotypically galE, are lysed very rapidly. Furthermore, the rates for 42 C grown cells of these two strains are greater than that of SA1377 in which LPS lesion is much deeper. This may be due to some other changes associated with the galE phenotype of these two mutants. Therefore, it is not possible to determine the level of hydrophobic permeability only by the LPS chemotype.

The resistance of these mutants to the cationic detergent cetyl pyridinium chloride and to the polycationic

antibiotic polymyxin B, probably indicates that the ionic charge of these molecules determines their passage through the OM. This discrimination cannot be over the size of the molecule because DOC (m.w.414) or novobiocin (m.w.613) which are readily taken into these cells are larger than cetyl pyridinium chloride (m.w.358). Since these molecules are fairly hydrophobic the LPS component of the OM must have a significant effect on their diffusion through the OM barrier. The self promoted pathway proposed by Hancock (1984) as an alternative means of uptake of antibiotics like polymyxin also greatly relies on their interaction with LPS. Therefore, it can be argued that the LPS of these mutants does not allow such interactions with cationic molecules, probably because the ionic environment of their OM mainly determined by LPS does not favor the binding of cationic substances. Coleman and Leive (1979) isolated a novobiocin supersensitive mutant in E. coli which is more sensitive to cationic agents and the sole detectable biochemical alteration in that mutant was the greater than 90% reduction in the phosphate content of the lipid A region of the LPS. Thus, the resistance to cationic agents in this S. typhimurium strains might be due to a change of phosphate content associated with the LPS. If this is true the resistance to cationic agents can be caused by increased amount of phosphate groups. Positively charged ethanolamine and 4-amino arabinose constituents are always attached to

the phosphate groups found in the inner core region of the LPS molecule (Muhlradt et al., 1977) (Figure 1). The increased amounts of phosphate groups might result in increase of such positively charged groups and subsequently hindering the interaction of LPS with cationic compounds. If these positively charged groups are not responsible for this permeability change another possibility is when phosphate content is higher the high negative charge on the surface of the OM can bind the cationic agents in the aqueous phase and thereby decrease their lipid/water partition coefficient which results in a decreased permeability.

E. Effect of Mg^{2+} ions on OM permeability. Although Leive (1974) demonstrated that the removal of divalent cations could increase the OM permeability, the findings of Chatterjee et al. (1976) and Stan-Lotter et al. (1979) revealed that the role of cations, especially Mg^{2+}, is to stabilize the OM by interacting with OM components and thereby develop a barrier to penetration. The OM permeability of the ts mutants showed a substantial decrease in their sensitivity to hydrophilic compounds in the presence of Mg^{2+} ions (Table 3). The restoration of the barrier layer by Mg^{2+} ions was quite clear in their dramatic drop of gentian violet uptake (Table 8). However, Mg^{2+} ions could not protect either the cells of mutant strains or of control strains against the lytic action of SDS. Thus, despite the

reorganization of the OM by Mg^{2+} ions, SDS molecules somehow get into the OM. SDS has the least molecular weight (288) among the various compounds tested and structurally it is not very complex compared to other compounds. These properties would probably allow these molecules to pass through the reorganized barrier layer. As evident from the data of the lysis test with DOC (Table 5) the changes of the OM caused by Mg^{2+} ions seem to be incomplete, because even in the presence of Mg^{2+} OM of the LPS mutants does not become completely impermeable like in wild type strains.

F. The permeability of the ts mutants towards beta-lactam antibiotics. The penetration of hydrophilic molecules through the OM is not much affected by LPS structure (Nikaido, 1979). However Godfrey and Bryan (1984) and Godfrey et al. (1984) demonstrated that the increased barrier function in certain P. aeruginosa strains to beta-lactam antibiotics is correlated with changes within their LPS. The data of the crypticity assay for the hydrolysis of piperacillin shows a similar correlation in this S. typhimurium ts mutants. The increased permeability of ts mutants to piperacillin at restrictive temperature, compared to other tested beta-lactam antibiotics might be associated with defects of the LPS structure at this temperature. Since relative to other beta-lactams the octanol/water partition coefficient of piperacillin is fairly high (Table 11) it is

reasonable to predict such a correlation. As observed by Zimmermann and Rosselet (1977) when $V_{(i)}/V_{(s)}$ ratios at 30 C or 42 C are considered in these mutant strains also the rate of diffusion of the beta-lactams tends to decrease with the increasing lipophilic character (octanol/water partition coefficient) (Table 10 and 11). Further, increased permeability of highly hydrophilic ampicillin at low temperature indicates that diffusion of such compounds is independent of the LPS structure. The diffusion of beta-lactam antibiotics through the OM of S. typhimurium occurs through the OM proteins which form porin channels (Kobayashi et al., 1982). Two OM proteins, 36k and 34k, have been identified as the ones that form porins through which ampicillin is taken into the cells (Van Alphen et al., 1978). Therefore the low levels of diffusion of ampicillin at high temperature is possibly due to the reduction of such proteins, because defects of the LPS are often accompanied by the reduction of OM proteins (Ames et al., 1974; Koplow and Goldfine, 1974). Alternatively, even if the OM proteins are present in the normal levels in these mutants at restrictive temperature, the impaired LPS structure might not allow the formation of stable porin channels (Schlindler and Rosenbusch, 1978; 1981).

G. Future experiments. In order to confirm the exact LPS chemotype of these ts mutants at restrictive temperature the

amount of heptose present in them must be determined at high temperature. The gas-liquid chromatography of LPS would be the best approach for this. The mutation involved in the temperature sensitivity has to be further confirmed when the rfaC, D, and F genes are cloned so that the complementing activity of these genes can be tested individually. Such plasmids are not constructed at this time.

The different levels of permeability of these mutants to anionic and cationic substances must be due to the changes in the charged groups attached to the inner core region of the LPS molecules. A quantitative analysis of these charged groups, especially phosphate groups, would help understand those discrepancies. Such analysis will also help to explain the role played by cations in restoration of the OM permeability barrier against some compounds, but not for compounds like SDS. Since changes in the LPS structure, hence changes in the OM permeability are closely associated with the levels of OM proteins, it is important to isolate and study the OM proteins of these mutants at permissive and restrictive temperatures, to understand the phenomenon of temperature sensitivity properly.

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Table 1. Bacterial strains used in this study.

Strain	Genotype	Source/Reference
S. typhimurium		
SL3770	*pyrE*$^+$ *rfa*$^+$	Roantree et al., 1977
LB5010	*trpC2 metA22 H1-b h2-e,n,x nml*$^-$ (Fel-2)$^-$ *flaA66 rpsL120 xyl-404 metE551 hsdL6 hsdSA29 hsdSB121 ilv-452 leu-3121 galE856*	Bullas and Ryu, 1983
SA2903	Same as LB5010, but also *rfa-3077*	This study[a]
SA2904	Same as LB5010, but also *rfa-3078*	This study[a]
SA3046	Same as SA2903, but *galE*$^+$	This study[b]
SA3047	Same as SA2904, but *galE*$^+$	This study[b]
SA3240	Same as LB5010, but *galE*$^+$ (P22)$^+$	This study[b]
SA1377	*rfaC630* (P22)$^+$/F251 *lac*$^+$	Sanderson et al., 1974
SA2703	*zhi-1403*::Tn10	K.E. Sanderson
SL3749	*pyrE*$^+$ *rfaL446*	B.A.D. Stocker
SL3769	*pyrE*$^+$ *rfaG471*	B.A.D. Stocker
SL3789	*pyrE*$^+$ *rfaF511*	B.A.D. Stocker

....continued

Table 1 (continued)

Strain	Genotype	Source/Reference
SL3600	*rfaD657 metA22 trpE2 H1-b H2-e,n,x flaA66 rpsL120 xyl-404 metE551* ('Cured of Fels 2')	B.A.D. Stocker
SA2197	*purC7*/F42 *finP301 lac*$^+$	Sanderson et al., 1983
SL3770/R$^+$	Same as SL3770, but with R68.45	This study[c]
SA1377/R$^+$	Same as SA1377, but with R68.45	This study[c]
LB5010/R$^+$	Same as LB5010, but with R68.45	This study[c]
SA2903/R$^+$	Same as SA2903, but with R68.45	This study[c]
SA2904/R$^+$	Same as SA2904, but with R68.45	This study[c]
SA3046/R$^+$	Same as SA3046, but with R68.45	This study[c]
SA3047/R$^+$	Same as SA3047, but with R68.45	This study[c]
SL3749/R$^+$	Same as SL3749, but with R68.45	This study[c]
SA3241	Same as SA3046, but with pLC13-13	This study[d]
SA3242	Same as SA3047, but with pLC13-13	This study[d]

....continued

Table 1 (continued)

Strain	Genotype	Source/Reference
E. coli		
SGSC360	F^+ *trpE5 thr leu recA*/pLC10-7	L. Rothfield
SAB3105	Same as SGSC360 but with pLC13-13	Coleman and Deshpande, 1985
SAB3068	F^- *hsdS* ($\bar{}_B$ m^-_B) *recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1 supE44*/pKZ45 (pLC10-7::Tn5 *rfaG*$^-$ *B*$^-$ *I*$^+$ *J*$^+$)	L.V. Collins and K.E. Sanderson
SAB3070	Same as SAB3068, but with pKZ47 (pLC10-7::Tn5 *rfaG*$^-$ *B*$^-$ *I*$^+$ *J*$^+$)	L.V. Collins and K.E. Sanderson
SAB3072	Same as SAB3068, but with pKZ48 (pLC10-7::Tn5 *rfaG*$^+$ *B*$^+$ *I*$^+$ *J*$^+$)	L.V. Collins and K.E. Sanderson
C600	*leu thi thr*/pMG202 [Ap(OXA-7) Cm Gm Km Su Tn Hg Inc C]	G. Jacoby
Pseudomonas aeruginosa		
PA025	*arg10 leu10*/R68.45	Godfrey et al., 1984

....continued

Table 1 (continued)

[a] SA2093 and SA2904 were selected as temperature-sensitive rough mutants from LB5010. The method of selection is described in the text.

[b] SA3046, SA3047 and SA3240 are *galE*$^+$ transductants selected after treatment with bacteriophage P22, as described in Materials and Methods.

[c] The R$^+$ strains were obtained by mating PA025 with each of the *S. typhimurium* strains and selecting for ampicillin resistance.

[d] The strains carrying the plasmid pLC13-13 were obtained by means of a conjugation cross using SAB3105 as a source of the F-factor and of the plasmid, and conjugating into SA3046 and SA3047.

Table 2. Sensitivity of *S. typhimurium* strains to bacteriophages.[a]

Strain	Partial Genotype[b]	LPS Chemotype	Bacteriophages P22 30°	P22 42°	F0 30°	F0 42°	Ffm 30°	Ffm 42°	6SR 30°	6SR 42°	Br60 30°	Br60 42°	C21 30°	C21 42°	Phage Sensitivity Pattern 30°	Phage Sensitivity Pattern 42°
SL3770	*rfa*$^+$ *galE*$^+$	S	+[c]	+	+	+	-	-	-	-	-	-	-	-	smooth	smooth
SA3240	*rfa*$^+$ *galE*$^+$	S	-	-	+	+	-	-	-	-	-	-	-	-	smooth	smooth
SA3046	*rfa3077 galE*$^+$	S/Rd2(ts)[d]	+	-	+	-	-	+	±	-	-	+	-	-	smooth	deep rough
SA3047	*rfa3078 galE*$^+$	S/Rd2(ts)	+	-	+	-	-	+	±	-	-	+	-	-	smooth	deep rough
LB5010	*rfa*$^+$ *galE856*	Rc	-	-	-	-	+	+	-	-	+	±	+	+	rough	rough
SA2903	*rfa3077 galE856*	Rc/Rd2(ts)	-	-	-	-	+	+	+	+	+	+	+	-	rough	deep rough
SA2904	*rfa3078 galE856*	Rc/Rd2(ts)	-	-	-	-	+	+	+	+	+	+	+	-	rough	deep rough
SA1377	*rfaC630*	Re	-	-	-	-	+	+	-	-	+	+	-	-	deep rough	deep rough

[a] L-agar plates were flooded with cells grown overnight at 37°C in L-broth, phage suspensions (10^8 PFU/ml) were spotted on the dried bacterial lawn and incubated at 30°C or 42°C.

[b] The complete genotype is in Table 1.

[c] +, lysis; -, no lysis; ±, intermediate

[d] Temperature sensitive, therefore different LPS chemotypes at low and high temperatures.

Table 3. Sensitivity to various antibiotics and detergents in disc tests.[a]

Compound	Conc. µg	Plating Medium	SL3770		SA3046		SA3047		LB5010		SA2903		SA2904		SA1377	
			30°	42°	30°	42°	30°	42°	30°	42°	30°	42°	30°	42°	30°	42°
Novobiocin	30	L	0	0	2	9	(3)[c]	6	(1.5)	2	(3)	8	(2)	6	(2)	4
		L+Mg^{2+}	0	0	(1.5)	8.5	0	5	0	2	0.5	6	(2.5)	7.5	0	1
		NA	1	1	1	16	(4)	8.5	(3)	(2)	(5)	11	(4)	9	5	NG[d]
		NA+Mg^{2+}	0	1	(2)	6	1.5	6.5	1	3	3	7.5	2	7	4	NG
Oleandomycin	15	L	0	0	1.5	3.5	2	3	(1)	0	0.5	4	(0.5)	3	1	3.5
		L+Mg^{2+}	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		NA	0	0	4	8	2	5	0	0	2.5	5.5	3	4	5	NG
		NA+Mg^{2+}	0	0	0	1	0	0	0	0	0	0	0	0	0	NG
Bacitracin	10	L	0	0	0	7	0	1	0	0	0	1	0	1	0	(2.5)
		L+Mg^{2+}	0	0	0	6	0	0	0	0	0	0	0	0.5	0	0
		NA	0	0	5	11	2	4.5	0	1	2.5	5	3	6	5	NG
		NA+Mg^{2+}	0	0	0	5	0	1	0	0	0	1.5	0	1	0	NG
Vancomycin	30	L	0	0	0	1	0	0	0	0	0	1	0	1	0	0
		L+Mg^{2+}	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		NA	0	0	3.5	8	1	3.5	0.5	1	1	4	2	3	5.5	NG
		NA+Mg^{2+}	0	0	0.5	2.5	0	1	0	0	0	1	0	1.5	0	NG

....continued

Table 3 (continued)

Compound	Conc. µg	Plating Medium	SL3770		SA3046		SA3047		LB5010		SA2903		SA2904		SA1377	
			30°	42°	30°	42°	30°	42°	30°	42°	30°	42°	30°	42°	30°	42°
Nafcil-line[e]	100	L	0	0	2	6	1	5.5	0	1	3	6	3	6	5	6
		L+Mg^{2+}	0	0	0	5	0.5	3	0	0.5	1	4.5	1.5	5	2	6
		NA	0	0	1.5	9	1.5	7	0	2	2	7	2	6	3.5	NG
		NA+Mg^{2+}	0	1	2.5	7	0.5	5	0	2	2	6	1.5	6	3	NG
Erythro-mycin	15	L	2	0	4	5	4	5	2	1	3	6	4	5	5	5
		L+Mg^{2+}	0	0	0	(7)	1	(6)	0	0	0	1(5)	0	1.5	0	2
		NA	1	2	8	ND	7	7	2	1.5	ND	7.5	1	7	8	NG
		NA+Mg^{2+}	0	0	6	3	1	1.5	0	0	0	2	1	3	1	NG
DOC[e]	400	L	0	0	0	2.5	0	1(3)	0	0	0	2	0	3	1.5	3.5
		L+Mg^{2+}	0	0	0	0	0	(9)	0	0	0	2(7)	0	2(7)	1	3
		NA	0	0	0	5	0	3	0	0	0	2.5	0	2	1.5	NG
		NA+Mg^{2+}	0	0	0	1	0	0	0	0	0	0	0	0	0.5	NG

....continued

Table 3 (continued)

Compound	Conc. µg	Plating Medium	SL3770		SA3046		SA3047		LB5010		SA2903		SA2904		SA1377	
			30°	42°	30°	42°	30°	42°	30°	42°	30°	42°	30°	42°	30°	42°
SDS[e]	400	L	0	0	0	4	0	4.5	0	0	1	5	1	5	4.5	8
		L+Mg^{2+}	0	0	0	2	0	3	0	0	0	2.5	0	3	2.5	5
		NA	0	0	0	4.5	0	4.5	0	0	0	5	0	4.5	1.5	NG
		NA+Mg^{2+}	0	0	0	3	0	3	0	0	1	3	1	2.5	2.5	NG
Gentian violet[e]	100	L	0	0	1	3	1.5	3	1	0.5	2.5	4.5	2	4	5	5
		L+Mg^{2+}	(1.5)	0	0	0.5	0	1	0.5	0	1	1	2.5	1	4	4
		NA	2.5	0	2	6	2	4	1.5	0	2.5	4	3	4	5	NG
		NA+Mg^{2+}	0	0	0	2.5	0	2	0	0	0	2	0	2	4	NG

[a] The sensitivity is shown as the radius in millimeters of the zone of inhibition around the antibiotic disc minus the radius of the disc.

[b] Cells were grown in each medium (broth) at 30° or 42°C, plated on the same medium and incubated at 30° or 42°C for 24 hrs.

[c] Numbers within parentheses indicate that the zone of inhibition is not very clear.

[d] NG, No growth.

[e] The discs of these compounds were prepared by dispensing the given amount of each compound onto the filter paper discs. Other discs of antibiotics were from Becton Dickinson Canada.

Table 4. Results of the complementation tests performed by introducing different plasmids carrying *rfa* genes of *S. typhimurium* and *E. coli* K-12.[a]

Plasmid		Complementation			
Name	Complementing activity carried by the plasmid	Recipient strain			
		SA2903	SA2904	SA3046	SA3047
A. pBR322 vector, *S. typhimurium* inserts					
pKZ15	*rfaG*	-[b]	-	-	-
pKZ26	*rfaG,B,I,J*	-	-	-	-
pKZ33	*rfaK,L*	-	-	-	-
B. ColE1 vector, *E. coli* K-12 inserts					
pLC13-13	*rfaC,D,F*	NR[c]	NR	+	+
pLC10-7	*rfaG,B,I,J*	-	NR	-	-
pKZ45[d]	*rfaG,B*	-	NR	-	-
pKZ47[d]	*rfaI,J*	-	NR	-	-
pKZ48[d]	*rfaG,B,I,J*	-	NR	-	-

[a] *rfa* genes of *S. typhimurium* and *E. coli* K-12 are cloned into pBR322 vector and ColE1 vector respectively. The plasmids are shown in Fig. 8.

[b] -, no complementation; +, complementation. Complementation is observed through a change in phage sensitivity of the mutant from the rough pattern to smooth pattern in the recombinants carrying the plasmid.

[c] NR, no recombinants were obtained.

[d] Transposon Tn5 insertions at different sites into the plasmid pLC10-7 gave rise to plasmids with differing ability to complement *rfa* mutants; this is postulated to be due to polar mutations caused by the Tn5 insertion (L.V. Collins, unpublished data).

Table 5. Cell lysis by sodium deoxycholate (DOC).[a]

Strain	Partial Genotype[b]	LPS Chemotype	% Decrease in OD_{640} in First 5 Min			
			L-broth		L + $MgCl_2$	
			30°[c]	42°	30°	42°
SL3770	*rfa*$^+$ *galE*$^+$	S	0.0	2.6	2.9	6.1
SA3240	*rfa*$^+$ *galE*$^+$	S	20.5	18.0	4.0	3.4
SA3046	*rfa3077 galE*$^+$	S/Rd2(ts)[d]	4.4	37.2	5.2	33.5
SA3047	*rfa3078 galE*$^+$	S/Rd2(ts)	15.9	50.6	12.8	20.5
LB5010	*rfa*$^+$ *galE856*	Rc	8.6	18.9	18.7	12.6
SA2903	*rfa3077 galE856*	Rc/Rd2(ts)	6.5	36.9	8.9	39.5
SA2904	*rfa3078 galE856*	Rc/Rd2(ts)	10.6	33.0	8.4	30.7
SA1377	*rfaC630*	Re	57.4	61.4	19.8	34.6

[a] Cell lysis was studied by following OD_{640} after adding DOC to a cell suspension (in 0.07 M phosphate buffer, pH 7.2) to give a final concentration of 0.5%.

[b] Complete genotype is in Table 1.

[c] Cells were grown at 30° or 42°C in L-broth or L-broth plus 20 mM $MgCl_2$, and the lysis test was done during incubation at 37°C.

[d] Temperature sensitive, therefore different LPS chemotypes at low and high temperatures.

Table 6. Cell lysis by sodium dodecyl sulfate (SDS).[a]

Strain	Partial Genotype[b]	LPS Chemotype	% Decrease in OD_{640} in First 2 Min			
			L-broth		L + $MgCl_2$	
			30°[c]	42°	30°	42°
SL3770	*rfa*$^+$ *galE*$^+$	S	2.1	3.6	8.6	5.2
SA3240	*rfa*$^+$ *galE*$^+$	S	4.4	6.0	9.1	16.1
SA3046	*rfa3077 galE*$^+$	S/Rd2(ts)[d]	4.5	81.9	20.4	73.2
SA3047	*rfa3078 galE*$^+$	S/Rd2(ts)	3.2	70.1	8.6	89.1
LB5010	*rfa*$^+$ *galE856*	Rc	5.1	7.0	52.8	89.2
SA2903	*rfa3077 galE856*	Rc/Rd2(ts)	68.0	89.9	73.6	90.1
SA2904	*rfa3078 galE856*	Rc/Rd2(ts)	51.6	91.4	69.0	87.5
SA1377	*rfaC630*	Re	82.7	79.1	84.3	89.7

[a] The rate of cell lysis was studied by following OD_{640}. SDS was added to a cell suspension (in 0.9% NaCl) to give a final concentration of 0.5%.

[b] The complete genotype of each strain is in Table 1.

[c] Cells were grown at 30° or 42°C (in L-broth or L + $MgCl_2$) and the lysis test was done at 37°C.

[d] Temperature sensitive; therefore different LPS chemotypes at low and high temperatures.

Table 7. Cell lysis by Triton X-100.[a]

Strain	Partial Genotype[b]	LPS Chemotype	% Decrease in OD_{640} in First 5 Min			
			L-broth		L + $MgCl_2$	
			30°[c]	42°	30°	42°
SL3770	*rfa*$^+$ *galE*$^+$	S	4.9	3.5	5.5	1.7
SA3240	*rfa*$^+$ *galE*$^+$	S	4.0	7.0	2.9	ND[e]
SA3046	*rfa3077 galE*$^+$	S/Rd2(ts)[d]	5.5	53.7	1.2	21.6
SA3047	*rfa3078 galE*$^+$	S/Rd2(ts)	5.2	22.7	1.1	8.1
LB5010	*rfa*$^+$ *galE856*	Rc	9.2	4.7	9.5	22.9
SA2903	*rfa3077 galE856*	Rc/Rd2(ts)	6.8	12.7	18.5	28.8
SA2904	*rfa3078 galE856*	Rc/Rd2(ts)	6.4	24.6	7.9	23.1
SA1377	*rfaC630*	Rc	19.3	9.8	17.6	31.8

[a] Cell lysis was studied by following OD_{640} after adding Triton X-100 to a cell suspension (in 0.07 M phosphate buffer) to a final concentration of 0.5%.

[b] Complete genotype of each strain is in Table 1.

[c] Cells were grown at 30° or 42°C in L-broth or L-broth plus 20 mM $MgCl_2$ and the lysis was tested incubating at 37°.

[d] Temperature sensitive; therefore different LPS chemotypes at low and high temperatures.

[e] ND, Not Determined.

Table 8. Uptake of gentian violet.

Strain	Partial Genotype[b]	LPS Chemotype	% Uptake[a] L-broth 30°	L-broth 42°	L + $MgCl_2$ 30°	L + $MgCl_2$ 42°
SL3770	*rfa*$^+$ *galE*$^+$	S	11.6	19.2	0.58	1.2
SA3240	*rfa*$^+$ *galE*$^+$	S	5.2	5.2	4.8	1.1
SA3046	*rfa3077 galE*$^+$	S/Rd2(ts)[d]	23.7	39.1	1.9	10.6
SA3047	*rfa3078 galE*$^+$	S/Rd2(ts)	20.1	42.7	3.4	7.9
LB5010	*rfa*$^+$ *galE856*	Rc	20.8	20.8	1.0	2.3
SA2903	*rfa3077 galE856*	Rc/Rd2(ts)	30.7	57.6	4.9	20.0
SA2904	*rfa3078 galE856*	Rc/Rd2(ts)	26.0	53.8	4.0	9.8
SA1377	*rfaC630*	Re	49.8	39.7	22.6	9.0

[a] The numbers indicate the percentage of gentian violet taken up by cells when the dye was added to a final concentration of 10 μg/ml. Each value is an average of three independent tests.

[b] The complete genotype of each strain is in Table 1.

[c] Cells were grown at 30° or 42°C and the dye uptake experiment was carried out at 37°C.

[d] Temperature sensitive, therefore different LPS chemotypes at low and high temperatures.

Table 9. Minimum inhibitory concentrations (MIC).[a,b]

Strain	Partial Genotype[c]	LPS Chemotype	DOC		Gentian Violet		Novobiocin		Nafcillin		Vancomycin		Bacitracin	
			30°	42°	30°	42°	30°	42°	30°	42°	30°	42°	30°	42°
SL3770	*rfa*$^+$ *galE*$^+$	S	>1000	>1000	50	50	100	100	500	>500	>400	>400	>1000	>1000
SA3046	*rfa3077* *galE*$^+$	S/Rd2(ts)[d]	>1000	400	6.25	<1.56	50	<6.25	200	150	200	100	700	500
SA3047	*rfa3078* *galE*$^+$	S/Rd2(ts)	>1000	500	6.25	3.125	50	25	250	50	200	100	>700	ND[e]
SA2903	*rfa3077* *galE856*	Rc/Rd2(ts)	>1000	500	3.125	<1.56	12.5	6.25	250	100	200	100	>700	500
SA2904	*rfa3078* *galE856*	Rc/Rd2(ts)	>1000	500	6.25	1.56	25	12.5	150	100	250	200	>700	>500
SA1377	*rfaC630*	Re	<1000	<125	<1.0	<0.125	12.5	<1.25	150	12.5	200	<25	500	<125

[a] Minimum inhibitory concentrations expressed as μg per ml.

[b] A set of serial dilutions (in duplicate) for each of the compounds was made using L-broth and 0.1 ml cell suspension of each strain grown to 0.1 OD_{640} was added to each tube. One set of tubes was incubated at 30°C and the other at 42°C. The greatest dilution which inhibited the growth was considered as minimum inhibitory concentration.

[c] Complete genotype is in Table 1.

[d] Temperature sensitive, therefore different LPS chemotypes at low and high temperatures.

[e] ND, Not determined.

Table 10. Rates of hydrolysis of β-lactam antibiotics by intact cells (V_{intact}) and sonicated cells ($V_{sonicated}$)[a] expressed as $V_{(intact)}/V_{(sonicated)}$.

		$V_{(intact)}/V_{(sonicated)}$							
		Piperacillin		Ticarcillin		Carbenicillin		Ampicillin	
Strain	LPS Chemotype	30°[b]	42°	30°	42°	30°	42°	30°	42°
SL3770/R+	S	0.0067 / 0.564	0.0027 / 0.732	0.0013 / 0.0236	0.0034 / 0.106	0.0021 / 0.0324	0.0169 / 0.1064	0.0337 / 0.04	0.1591 / 1.1721
		0.0118	0.0036	0.055	0.032	0.0648	0.1588	0.842	0.1357
SA3046/R+	S/Rd2(ts)[c]	0.01 / 0.571	0.036 / 1.114	ND[d]	ND	ND	ND	ND	ND
		0.018	0.032						
SA3047/R+	S/Rd2(ts)	0.0 / 0.905	0.074 / 1.458	ND	ND	ND	ND	ND	ND
		0.0	0.05						
LB5010/R+	Rc	0.019 / 0.768	0.015 / 0.928	0.0005 / 0.1064	0.0025 / 0.0432	0.0021 / 0.1101	0.0012 / 0.0493	0.0246 / 1.335	0.0108 / 0.5641
		0.0247	0.016	0.005	0.057	0.019	0.0243	0.018	0.019

....continued

Table 10 (continued)

		$V_{(intact)}/V_{(sonicated)}$							
		Piperacillin		Ticarcillin		Carbenicillin		Ampicillin	
Strain	LPS Chemotype	30°[b]	42°	30°	42°	30°	42°	30°	42°
SA2903/R+	Rc/Rd2(ts)	0.006/0.836	0.07/0.999	0.002/0.0675	0.002/0.0351	0.003/0.0787	0.0034/0.0435	0.2084/0.9721	0.0496/0.5465
		0.007	0.07	0.0296	0.057	0.038	0.078	0.214	0.0907
SA2904/R+	Rc/Rd2(ts)	0.022/1.073	0.132/1.80	0.0034/0.108	0.00067/0.0358	0.0099/0.1189	0.0039/0.0405	0.2259/1.3775	0.0317/0.5357
		0.02	0.073	0.031	0.019	0.0832	0.096	0.164	0.059
SL3749/R+	Ra	0.0067/0.499	0.0013/0.753	ND	ND	ND	ND	ND	ND
		0.0134	0.0017						
SA1377/R+	Re	0.027/0.946	0.036/1.144	0.0093/0.126	0.0024/0.0916	0.0135/0.1452	0.0021/0.1108	0.1755/1.516	0.0223/1.3647
		0.028	0.031	0.0738	0.026	0.0929	0.0189	0.1157	0.0163

....continued

Table 10 (continued)

[a] Rate of hydrolysis of β-lactam antibiotics was determined by following the rate of decolorization of starch-iodine complex, using a recording spectrophotometer, at 520 nm. $V_{(intact)}$ was determined using intact cells suspended in the phophate buffer (0.1 M, pH 7) and $V_{(sonicated)}$ after sonicating the cells suspended in the same medium. One ml of each β-lactam antibiotic dissolved in phosphate buffer to give a final concentration of 0.2 mM was used in the reaction mixture. The slope of the line was used to calculate the rate. Rates are expressed as micromoles per minute per milligram of dry weight of cells.

[b] Cells were grown at 30^{o} or 42^{o}C and enzyme assay was done at room temperature.

[c] Temperature sensitive; therefore different LPS chemotypes at low and high temperatures.

[d] ND, Not determined.

Table 11. Partition coefficients of gentian violet and β-lactam antibiotics.[a]

Compound	Partition coefficient[b]
Gentian violet	13.28
Nafcillin	0.407
Piperacillin	0.037
Carbenicillin	0.028
Ampicillin	0.003
Ticarcillin	0.006

[a] Partition was determined in the 1-octanol/0.05 M sodium phosphate buffer (pH 7.0) system at room temperature. The concentration of each compound in aqueous and organic phase was determined spectrophotometrically at the wavelength of an absorption peak of each compound.

[b] Average value of two separate tests.

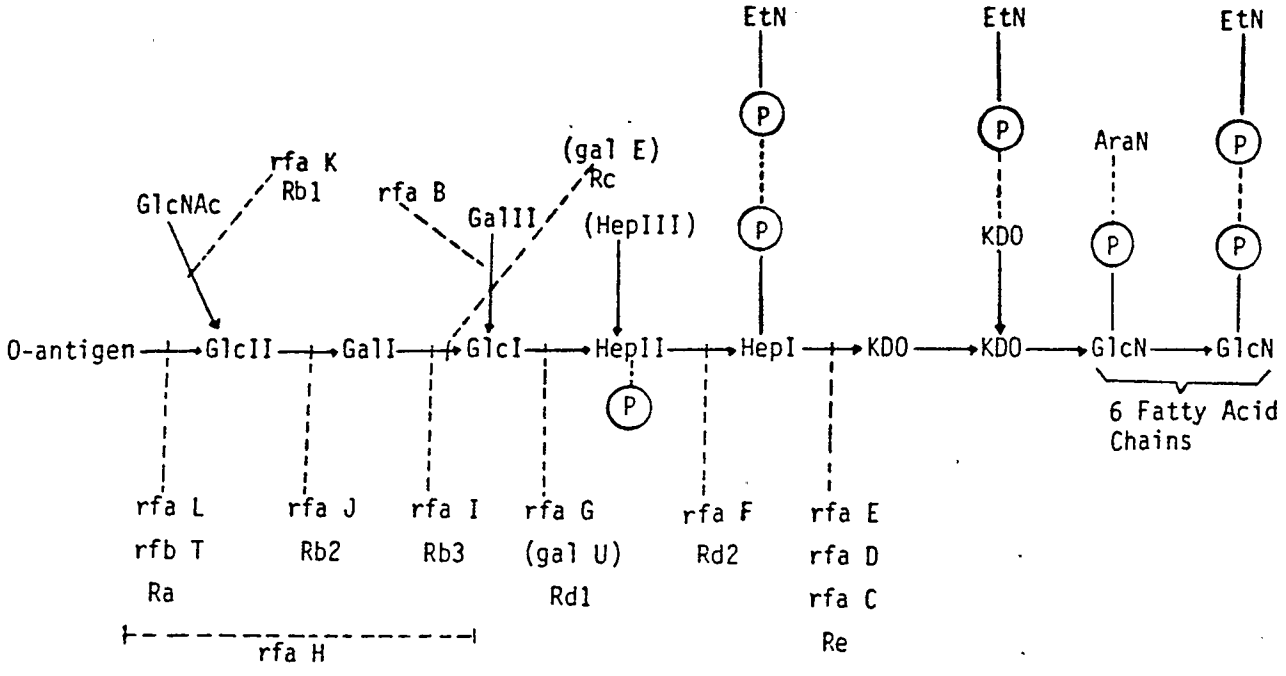


Fig.1. Structure of LPS of S.typhimurium and known genes required for its synthesis (From Kadam et al.1985). The structure of inner core and lipid A regions is as proposed by Muhlradt et al.(1977).

Abbreviations: AraN,4-amino-4-deoxy-l-arabinose;EtN,ethanolamine; Gal,galactose;Glc,glucose; GlcN, glucosamine; GlcNAc, N-acetyl glucosamine; Hep, heptose; KDO, Ketodeoxyoctonic acid; P,phosphate.

Substituents that may vary are indicated in broken bonds.

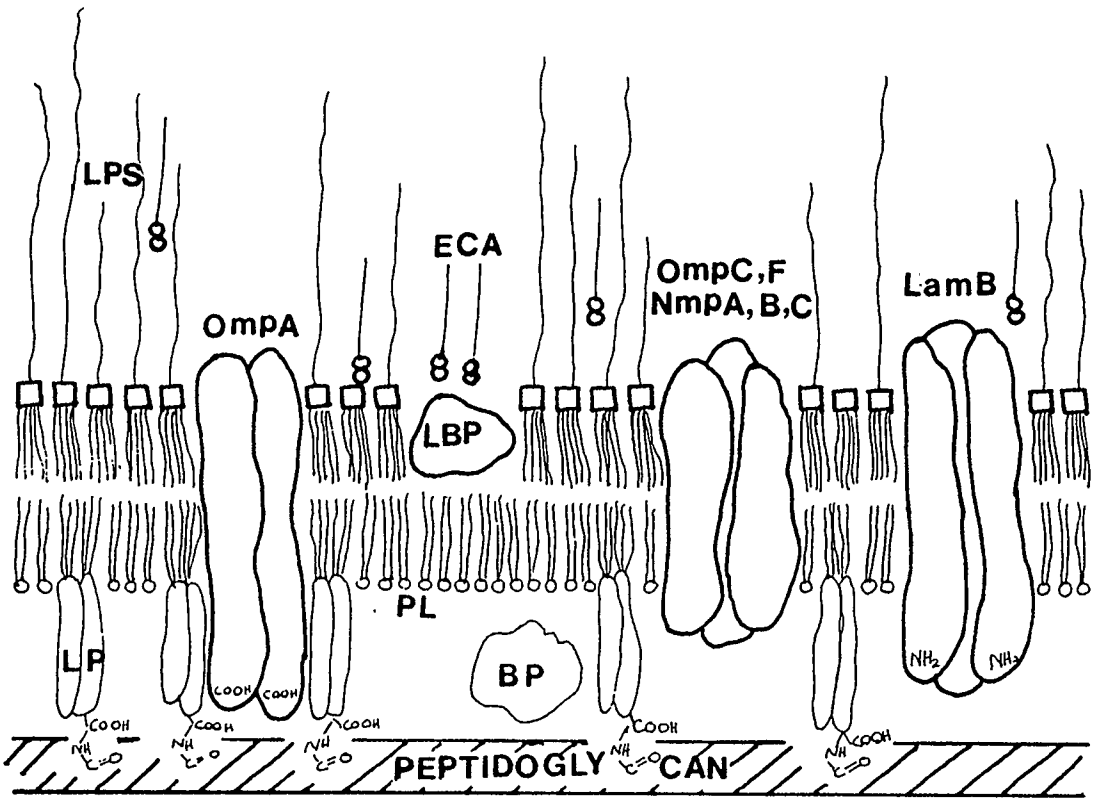


Figure 2. A schematic representation of the outer membrane of Gram negative bacteria.
(Adapted from Osborn and Wu, 1980)

LPS	lipopolysaccharide
PL	phospholipids
ECA	enterobacterial common antigen
LBP	LPS binding protein
BP	periplasmic binding protein
OmpA	outer membrane protein A
OmpC,F,LamB,NmpA,B,C	outer membrane proteins
LP	lipoprotein

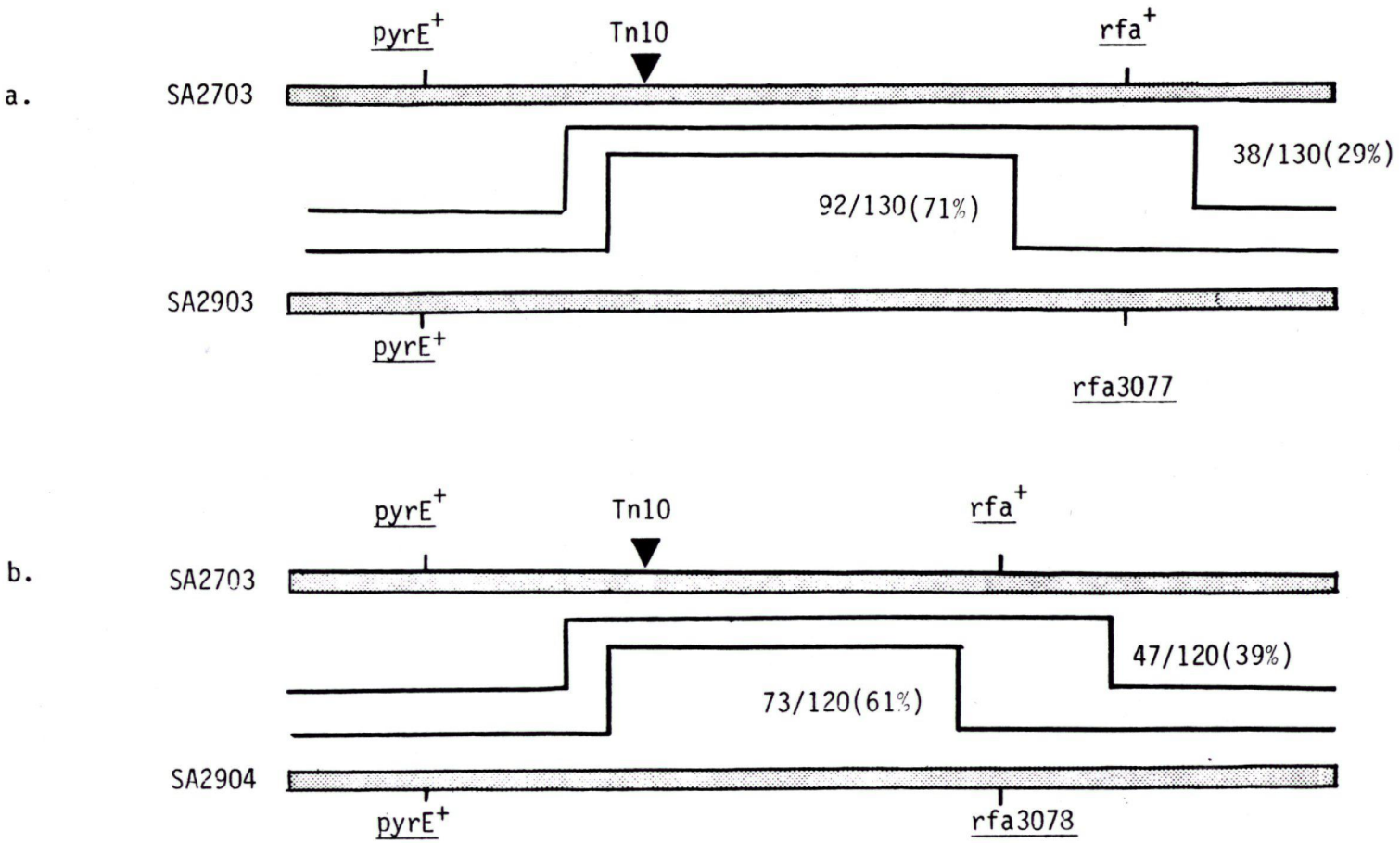


Fig. 3 . Cross over diagrams for the transductions of the regions of the rfa cluster linked to transposon Tn10 from SA2703 into SA2903 (a), and SA2904 (b).

Figure 4. SDS-PAGE of LPS from *ts* mutants and their *galE* parent LB5010 grown in L-broth containing 1% glucose plus 1% galactose (Lgg). Partial genotype and LPS chemotype are shown for each strain; growth temperature is given in parentheses. Lanes 7-9 are LPS from mutants of *S. typhimurium* LT2 which have the mutations in the *rfa* genes.

Lane	Strain	Partial Genotype	LPS Chemotype of Reference Strains	LPS Chemotype Inferred from Mobility on SDS-PAGE
1	SA2903 (30°C)	*rfa3077 galE856*		S
2	SA2903 (42°C)	*rfa3077 galE856*		Rd2
3	SA2904 (30°C)	*rfa3078 galE856*		S
4	SA2904 (42°C)	*rfa3078 galE856*		Rd2
5	LB5010 (30°C)	*rfa*$^+$ *galE856*		S
6	LB5010 (42°C)	*rfa*$^+$ *galE856*		S
7	SL3789 (37°C)	*rfaF511*	Rd2	
8	SA1377 (37°C)	*rfaC630*	Re	
9	SL3770 (37°C)	*rfa*$^+$	S	

Figure 5. SDS-PAGE of LPS from SA2903 and SA2904 grown at 30°C and 42°C in L-broth. Lanes 6-11 are LPS from reference strains of *S. typhimurium* LT2. Partial genotype and LPS chemotype are shown for each strain; growth temperature is in parentheses.

Lane	Strain	Partial Genotype	LPS Chemotype of Reference Strain	LPS Chemotype Inferred from Mobility on SDS-PAGE
1	SA2903 (30°C)	*rfa3077 galE856*		Rc
2	SA2903 (42°C)	*rfa3077 galE856*		Rd2
3	SA2904 (30°C)	*rfa3078 galE856*		Rc
4	SA2904 (42°C)	*rfa3078 galE856*		Rd2
5	LB5010 (42°C)	*rfa*$^+$ *galE856*		Rc
6	SL3749 (37°C)	*rfaL446*	Ra	
7	SL3769 (37°C)	*rfaG471*	Rd1	
8	SL3789 (37°C)	*rfaF511*	Rd2	
9	SA1377 (37°C)	*rfaC630*	Re	
10	SL3600 (37°C)	*rfaD657*	Re	
11	SL3770 (37°C)	*rfa*$^+$	S	

Fig 4

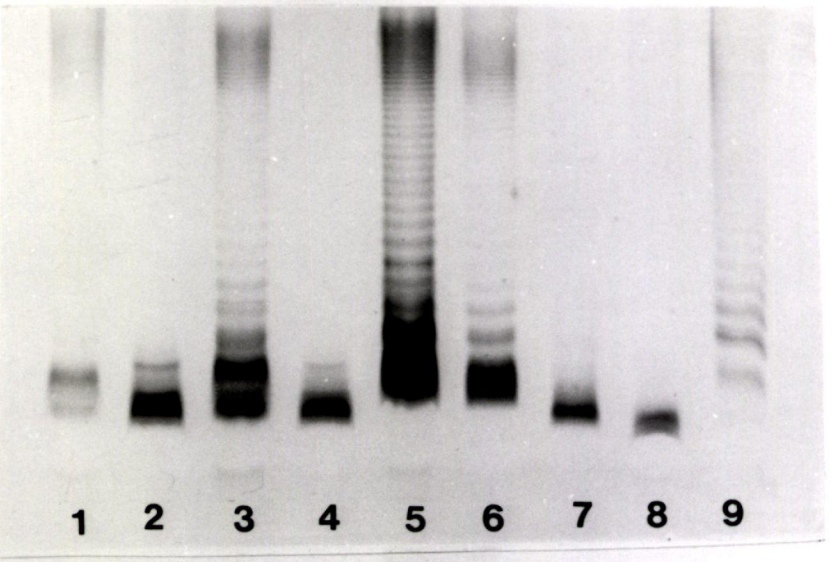


Fig 5

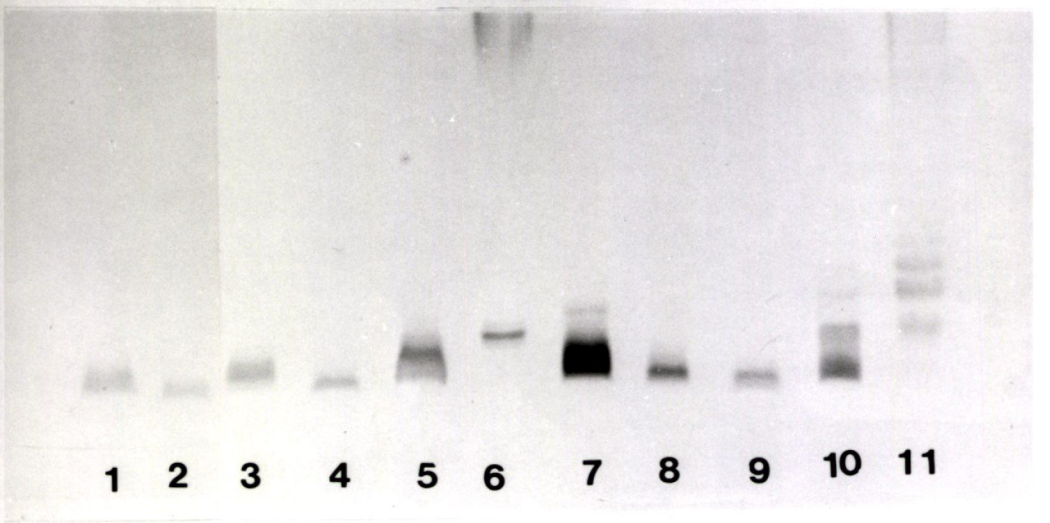


Figure 6. SDS-PAGE from *ts* mutants grown at restrictive temperature (42°C). Lanes 1-5 are LPS from reference strains of *S. typhimurium* which have the mutations in *rfa* genes. Partial genotype and LPS chemotype are shown.

Lane	Strain	Partial Genotype	LPS Chemotype of Reference Strain	LPS Chemotype Inferred from Mobility on the SDS-PAGE
1	SL3770	*rfa*$^+$	S	
2	SL3600	*rfaD657*	Re	
3	SA1377	*rfaC630*	Re	
4	SL3769	*rfaG471*	Rd1	
5	SL3789	*rfaF511*	Rd2	
6	SA2904	*rfa3078 galE856*		Rd2
7	SA2903	*rfa3077 galE856*		Rd2
8	LB5010	*rfa*$^+$ *galE856*		Re
9	SA3047	*rfa3078 galE*$^+$		Rd2
10	SA3046	*rfa3077 galE*$^+$		Rd2

Figure 7. SDS-PAGE of LPS from *galE*$^+$ transductants of *ts* mutants and of parent strain LB5010. Lanes 3 and 4 are LPS from mutants of *S. typhimurium* which have the mutation in the *rfa* genes. Lanes 9 and 10 are *ts* mutants carrying pLC13-13, grown at 42°C.

Lane	Strain	Partial Genotype	LPS Chemotype of Reference Strain	LPS Chemotype Inferred from Mobility on SDS-PAGE
1	SA3240 (42°C)	*rfa*$^+$ *galE*$^+$		S
2	SA3240 (30°C)	*rfa*$^+$ *galE*$^+$		S
3	SL3770 (37°C)	*rfa*$^+$	S	
4	SL3789 (37°C)	*rfaF511*	Rd2	
5	SA3047 (42°C)	*rfa3078 galE*$^+$		Rd2
6	SA3047 (30°C)	*rfa3078 galE*$^+$		S
7	SA3046 (42°C)	*rfa3077 galE*$^+$		Rd2
8	SA3046 (30°C)	*rfa3077 galE*$^+$		S
9	SA3241 (42°C)	*rfa3077 galE*$^+$/ pLC13-13		S
10	SA3242 (42°C)	*rfa3077 galE*$^+$/ pLC13-13		s

Fig 6

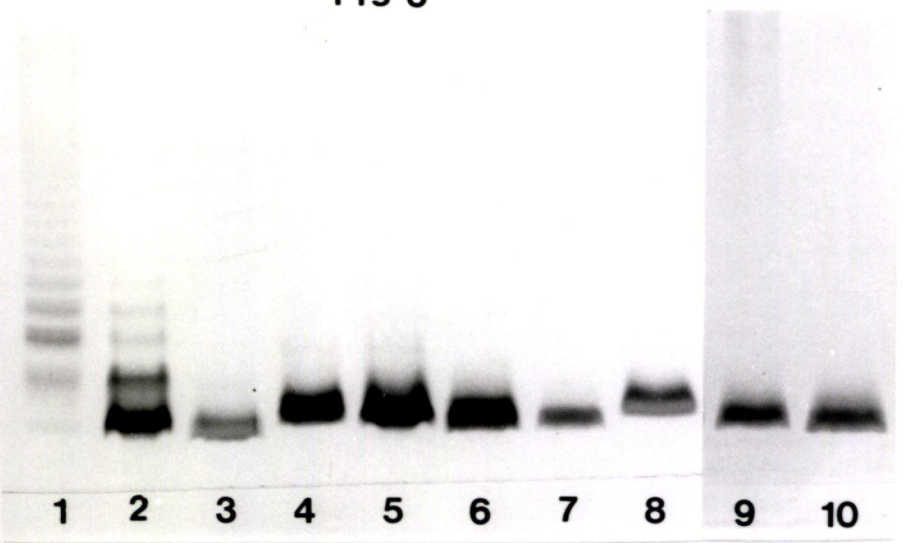
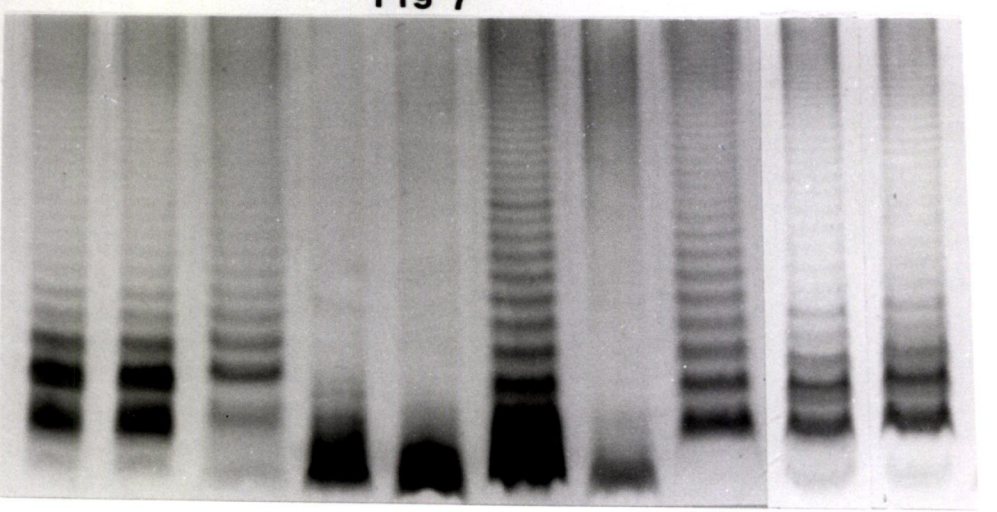


Fig 7



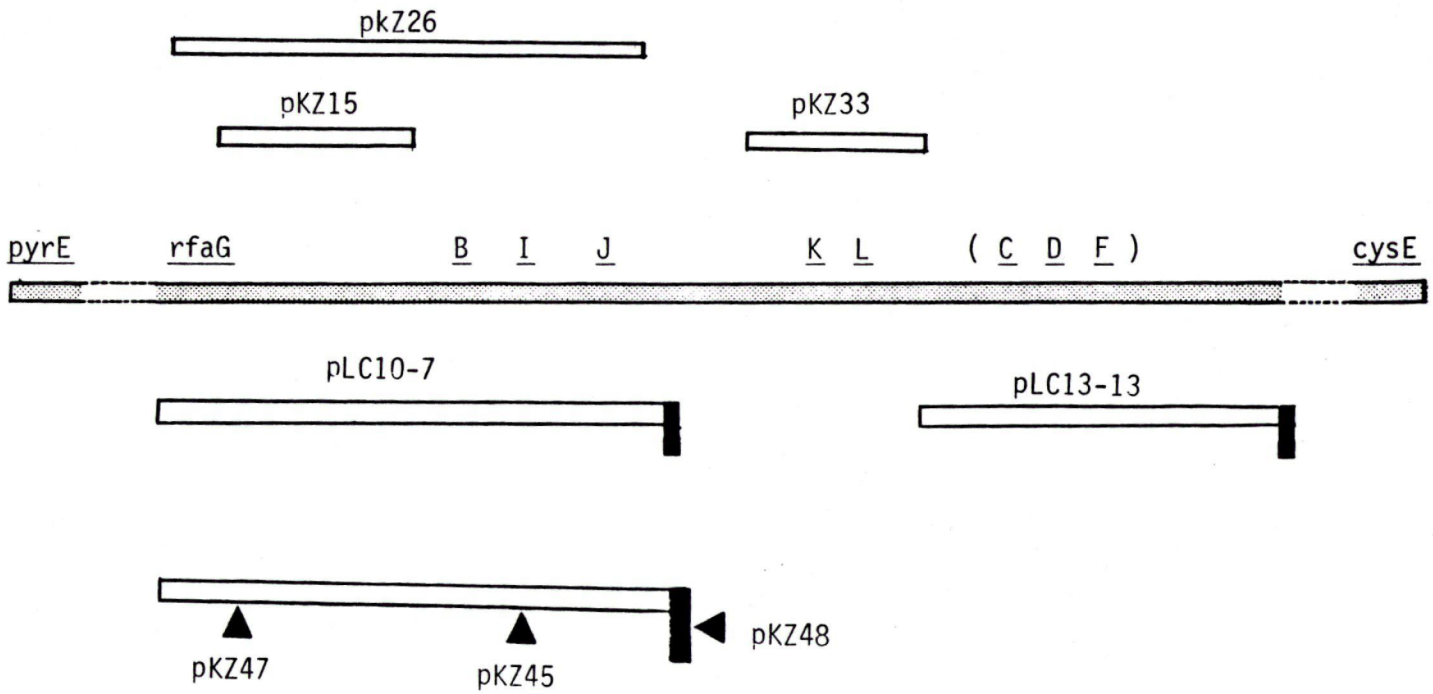


Fig. 8. Structure of the rfa cluster of S. typhimurium and different plasmids carrying rfa genes of Salmonella and E. coli. The stippled bar represents the chromosomal fragment carrying the rfa cluster. The open bars at the top show the chromosomal fragments from S. typhimurium cloned into pBR322 (Kadam et al. 1985). The open bars at the bottom represents ColE1 plasmids carrying E. coli K-12 genes; pLC10-7 (Creeger and Rothfield, 1979); pLC13-13 (Coleman and Deshpande, 1985). pKZ47, pKZ45 and pKZ48 are three Tn5 insertions at different locations in pLC10-7 (L.V. Collins and K.E. Sanderson, unpublished data). The solid vertical bar shows a part of the ColE1 vector, with the Tn5 insert into the vector in pKZ48.

Figure 9. Rates of cell lysis by sodium deoxycholate (DOC) determined by the spectrophotometric method. Cells grown at 30°C or 42°C in L-broth with or without 20 mM $MgCl_2$ were resuspended in potassium phosphate buffer (0.07 M, pH 7.2), DOC was added to a final concentration of 0.5% and then OD_{640} was monitored during incubation at 37°C. Growth temperature and medium are given at the end of the curve for each strain.

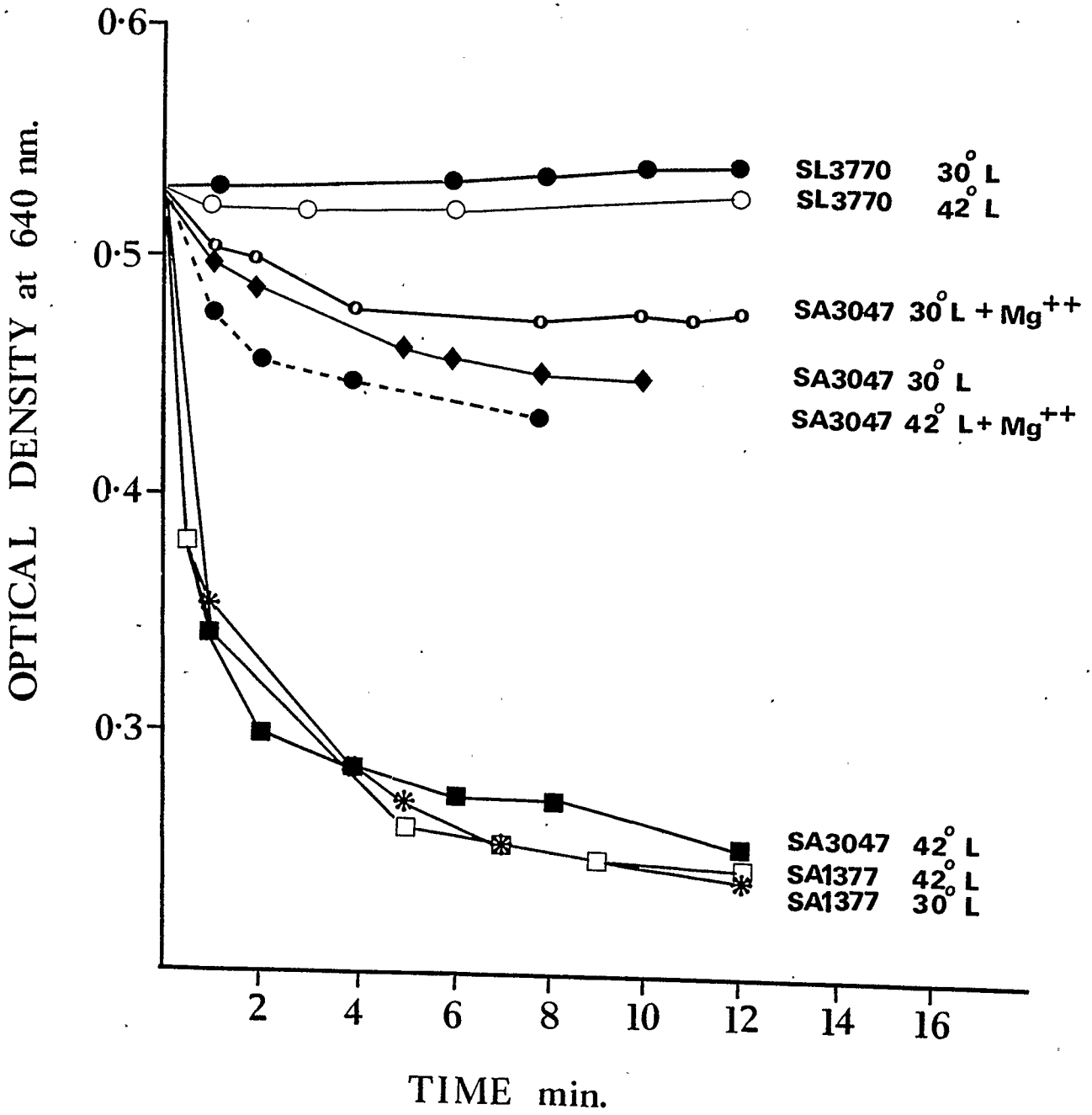
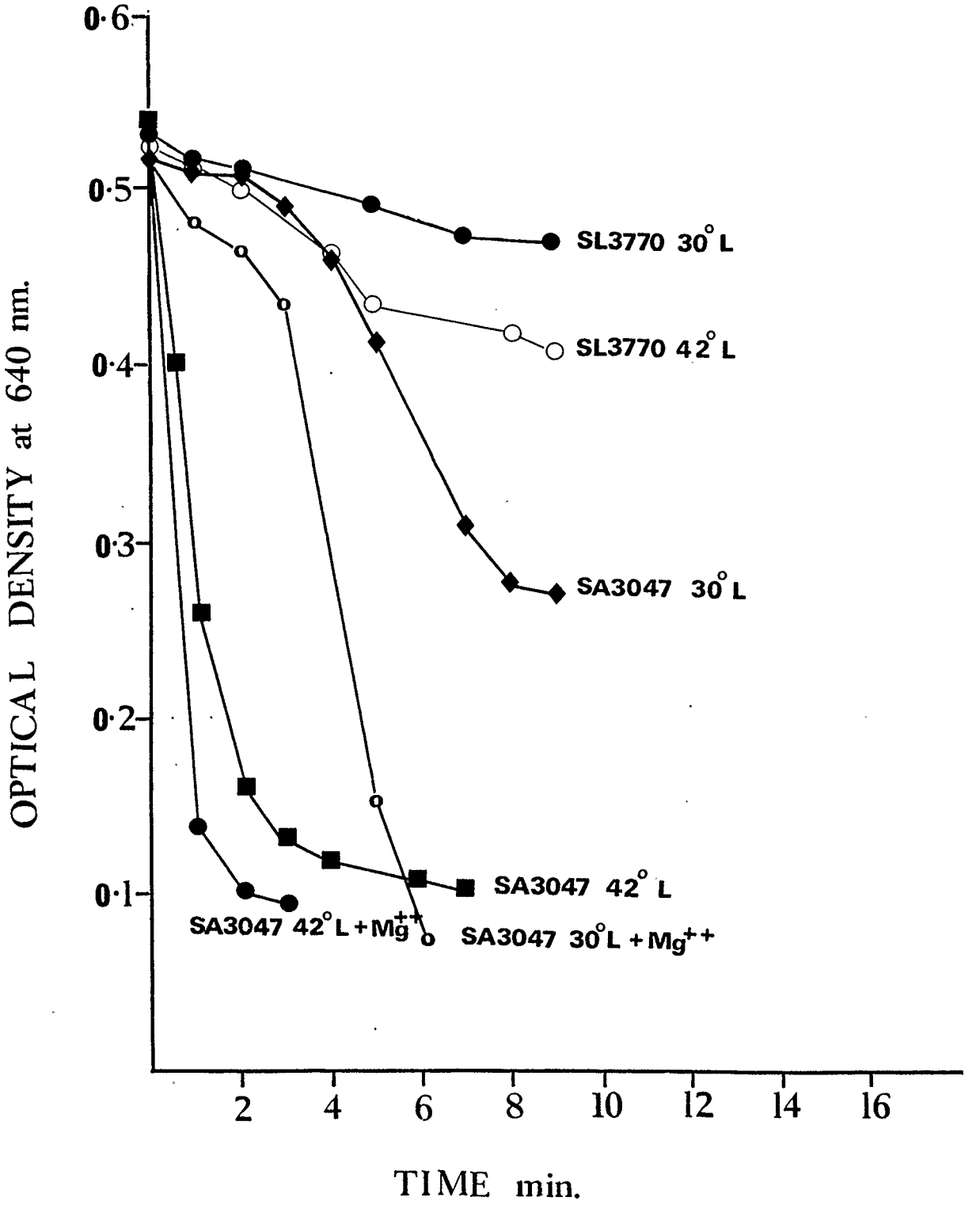

OPTICAL DENSITY at 640 nm.
0·6
0·5
0·4
0·3
2
4
6
8
10
12
14
16
TIME min.
SL3770 30° L
SL3770 42° L
SA3047 30° L + Mg++
SA3047 30° L
SA3047 42° L + Mg++
SA3047 42° L
SA1377 42° L
SA1377 30° L

Figure 10. Rates of cell lysis by sodium dodecyl sulfate (SDS) determined by the spectrophotometric method. Cells grown at 30°C or 42°C in L-broth with or without 20 mM $MgCl_2$ were resuspended in 0.9% NaCl, SDS was added to a final concentration of 0.5% and then OD_{640} was monitored during incubation at 37°C. Growth temperature and medium are given at the end of the curve for each strain.


0·6
0·5
0·4
0·3
0·2
0·1
OPTICAL DENSITY at 640 nm.
SL3770 30° L
SL3770 42° L
SA3047 30° L
SA3047 42° L
SA3047 42° L + Mg++
SA3047 30° L + Mg++
2
4
6
8
10
12
14
16
TIME min.