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CHEMICAL AND GENETIC ANALYSIS OF LIPOPOLYSACCHARIDE SYNTHESIS IN ENTERIC BACTERIA

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 CHEMICAL AND GENETIC ANALYSIS OF LIPOPOLYSACCHARIDE SYNTHESIS IN ENTERIC BACTERIA, submitted by LAURENCE VINCENT COLLINS in partial fulfillment of the requirements for the degree of Master of Science.

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ABSTRACT

The lipopolysaccharide (LPS) surface component maintains the integrity of the outer membrane as a permeability barrier in addition to playing a major role in the conjugational, surface and phage adsorption, and motility functions of the cell envelope in enteric bacteria. In this study the structure of the LPS and the genetics of its biosynthesis were explored using LPS deficient mutants. Mutants of Escherichia coli K-12 which were isolated on the basis of resistance to phage U3 were found to be defective in the lipopolysaccharide (LPS) outer membrane component. These mutants, and those obtained from various sources were analysed: biologically, for sensitivity to a range of rough- and smooth-specific phages and to deoxycholate and chemically, the isolated LPS was analysed on SDS-PAGE by gas-chromatography. On the basis of these results and the postulated structure of the LPS of E. coli K-12 the mutants were assigned an LPS phenotype. Mutants with defects in the core LPS which gave rise to the chemotypes Re, Rd1, Rc, Rb3, and Rb2 were detected.

A plasmid pLC10-7, which carried a chromosomal segment of E. coli part of which consists of a number of the (rfa) genes for LPS biosynthesis, had previously been identified. In this study, a restriction enzyme cleavage map for this plasmid is presented. Complementation ability of this plasmid for mutants with defects in the rfaG,B,I and J genes of E. coli is demonstrated. The approximate location of

each of these genes on the plasmid was determined by Tn5 mutagenesis and by subcloning fragments of the DNA into pBR322. The tentative order of rfaG - rfaB - rfaI - rfaJ is postulated from polarity inactivation observations. Three separate operons are indicated. These operons include the rfaG and rfaB genes; the rfaI gene and the rfaJ gene. By using the plasmids carrying Tn5-inactivated genes it was possible to identify the genotypes of a number of LPS defective mutants.

Mutants which have severely shortened LPS are defective in a group of genes encoding enzymes involved in the biosynthesis or transfer of the heptose sugars. The chemistry of the heptose deficient LPS mutants is described. A restriction enzyme cleavage map of the plasmid pLC13-13 from the Clarke and Carbon library is presented in Chapter 3. The presence, on pLC13-13, of the rfaC and rfaF genes for heptosyltransferases and the rfaD gene for an enzyme required for epimerization of the heptosyl precursor was determined by virtue of the ability of this plasmid to correct the mutant phenotype of mutants defective in these genes to the wild-type phenotype. The location of the transcriptional units was determined by transposon Tn5 mutagenesis and a preliminary order of the genes and structure of the operon is proposed.

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To my parents and Ena for their support

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Abbreviations[1]

Kb	Kilobases
L	Litres
lam	Lambda phage
LPS	Lipopolysaccharide
min	Minutes
ml	Millilitres
moi	Multiplicity of infection
PBS	Phosphate buffered saline
pfu	Plaque forming units
SDS	Sodium dodecyl sulfate
rfa	rough A (Mutants which are LPS deficient)
Gal-	Galactose non-fermenting (unable to make UDP-galactose and therefore LPS deficient
galE	Galactose epimerase deficient and therefore LPS deficient
PAGE	Polyacrylamide gel electrophoresis

[1]Genotype acronyms are explained in Sanderson and Roth (1983) and in Bachmann (1983)

CHAPTER 1

CHEMICAL AND GENETIC DESCRIPTION OF A SET OF LIPOPOLYSACCHARIDE DEFICIENT MUTANTS IN *Escherichia coli* K-12

Introduction

Lipopolysaccharide (LPS) is a major macromolecular component of the outer membrane of Escherichia coli K-12 and other gram-negative species (Inouye, 1979). It is proposed that the polysaccharide structure consists in general of three major regions: O-somatic side chains, the core polysaccharide and the lipid A region (Luderitz et al, 1971).

The O-somatic side chains determine the O-serological specificity of the molecule and is characterised by a high diversity in the number and structure of repeating oligosaccharide units among different serotypes.

The core polysaccharide is more conservative in its structure and composition. Only six different core types have been identified in the Enterobacteriaceae (Jansson et al, 1981).Qualitative analysis of LPS revealed that all Salmonella spp. contained the same five basal sugars in the core region: KDO (3-deoxy-D-manno-octulosonic acid), L-glycero-D-mannoheptose, D-glucose, D-galactose, and N-acetyl-D-glucosamine (Luderitz et al, 1971: Hellerqvist and Lindberg,1971). In addition phosphate and ethanolamine are present. The sugars of the R-core are added sequentially, one at a time, from high energy UDP precursors in the cases of glucose, galactose and N-acetylglucosamine (Ginsburg, 1964). The structure of the core polysaccharide of Salmonella is well characterised and appears to be the

same in all strains studied (Hellerqvist and Lindberg, 1971; Luderitz, 1970; Osborn, 1979). The core structure of E. coli strains is not as well known. At least three different core types which differ from the Salmonella core, especially in the hexose region have been described (Orskov et al, 1977; Jansson et al, 1981). Schmidt et al (1969) found that the core polysaccharide of E. coli 08:K27- (R1 type core) has a galactose: glucose: heptose molar ratio of 2:3:3; while E. coli 08:K42- (R2) has a galactose: glucose: N-acetlyglucosamine: heptose molar ratio of 2:4:1:4. The core polysaccharide of E. coli 011:K58 (R3) differs from the R2 type core in that it has lower levels of galactose (Schmidt et al, 1970). The terminal N-acetylglucosamine of the Salmonella core is missing in E. coli core types R1, R3 and R4. The core LPS of E. coli B is an incomplete form of the E. coli K-12 LPS and may represent an rfa mutant of E. coli K-12 (Orskov et al, 1977; L.V.Collins, unpublished data). E. coli K-12 has an LPS structure similar to that of Salmonella but lacks the O polysaccharide and differs slightly in the composition of the core sugars (Fig 1-1). Lipid A is a fatty acyl and phosphorylated derivative of D-glucosamine disaccharide and which also may contain variable amounts of 4-amino-4-deoxy-L-arabinose (Osborn, 1979).

For structural and biological studies, mutant strains (R-mutants) have been of immense value. These mutants have a

genetic defect in the LPS biosynthetic genes and they contain incomplete LPS which lacks the O-side chains and may lack part of the core LPS. Loss of proximal sugars in the R-core results in inability to attach sugars distal to it.

Recognition of mutants in which particular steps in LPS synthesis are blocked has provided a series of simplified forms of LPS which have been used in investigating LPS structure and the process of LPS assembly as well as the recognition of the regions of the molecule essential for specific biological activities. The most complete set of LPS mutant chemotypes is available in S. typhimurium (Wilkinson and Stocker, 1968; Roantree et al, 1977; Luderitz et al, 1971; Makela and Stocker, 1984) and these mutants have been used to elucidate the genetic determinants of several genes involved in LPS biosynthesis (Kadam et al, 1985). Similar studies in E. coli K-12 have been hampered by the absence of a complete set of well characterised LPS mutants. Mutants of E. coli with defects in the lipopolysaccharide cell surface components have been previously used in studies determining: a) bacteriophage receptor sites (Monner et al, 1971, Rapin and Kalckar, 1971, Watson and Paigen, 1971, Tamaki et al, 1974, Hancock and Reeves, 1976, Sandulache et al, 1984), b) chemotypes with altered antibiotic sensitivities (Coleman and Leive, 1979, Eriksson-Greenberg, 1971, Monner et al, 1971, Tamaki et al, 1971, Hancock and Reeves, 1976), c) factors affecting mating pair formation (Reiner, 1974,

Havekes et al, 1977) and d) in many biochemical studies into the nature of cell surface LPS (for a review, see Handbook of Endotoxin, Vol 1, Ed.E.Th.Rietchel, Elsevier Pub., 1984). Detailed analysis of these mutants led to a tentative structure for the core polysaccharide of E. coli K-12 (Prehm et al, 1976). The reported structures, the glycosyltransferases and the genes involved in biosynthesis of the LPS of Salmonella typhimurium and Escherichia coli K-12 are indicated in Fig 1-1.

The synthesis of the core region of the LPS of E. coli K-12 is determined by the rfa cluster of genes at map position 81, close to pyrE and cysE , homologous to the location of the rfa genes of S. typhimurium and by genes such as the gal operon for galactose synthesis and degradation at map position 17 (Bachmann and Low, 1980). Characterisation of hybrid plasmids carrying some of the genes involved in LPS synthesis in E. coli K-12 is reported in Chapter 2 and Chapter 3.

In order to study the process of LPS synthesis in enteric bacteria we have chosen to work on the strain E. coli K-12. This strain has the following advantages: it is avirulent; it lacks capsular material, making it accessible to chemical and genetic manipulations; there is an immense accumulation of genetic and biochemical information on many topics on this strain. The LPS of E. coli K-12 contains a complete core but no side-chains, due to a mutation in the

complete core but no side-chains, due to a mutation in the rfb gene closely linked to the his locus (Jones and Stocker, 1972); thus the "wild-type" of E. coli K-12 with respect to LPS is not a smooth strain but a rough mutant. We have assembled core-defective LPS E. coli K-12 mutants representing different chemotypes and we have characterised these strains both chemically and genetically. In compiling this set of LPS chemotypes we have obtained strains from other investigators which have been partly characterised in previous studies and we have independently isolated mutants on the basis of resistance to bacteriophage U3.

Throughout this thesis the assignment of rfa mutations to specific gene loci is done on the following basis. A mutation in Salmonella typhimurium, previously defined to be in a specific locus, e.g., in the gene rfaG(S), is considered to be the reference mutant. (The symbol (S) following the locus designation indicates that the mutation is in S. typhimurium; the designation rfaG(K) indicates a mutation in the homologous gene of E. coli K-12). Plasmids carrying rfa genes which can complement only this mutation (or others in the same genes) are transferred into E. coli K-12 and tested for capacity to complement E. coli mutants; those mutations which can be complemented by the plasmid are also assigned to gene locus rfaG. Other aspects of the mutants, such as phage sensitivities, SDS-PAGE and gas chromatography analysis of the LPS are also considered in

assigning the gene locus of the strain.

Materials and Methods

Bacterial strains, plasmids and cultivation methods. The bacterial strains and plasmids used are listed in Table 1-1. All strains used were stored in 15% glycerol at -70C and were routinely single colony isolated on L-agar prior to use.

Chemicals. The chemicals used were from the following sources: acrylamide, N,N-methylene-bisacrylamide, sodium dodecyl sulfate (SDS), 3-N-morpholino propane sulfonic acid (MOPS), Trizma-base, Trizma-HCl, ethidium bromide, silver nitrate (Sigma); phenol (Mallinkrodt); Proteinase K (Bethesda Research Laboratories); sodium desoxycholate (Fisher Scientific Co.); antibiotic sensidisks (Becton Dickinson Co.). Other chemicals were of analytical grade, from various suppliers.

Media. Bacterial cells were routinely cultivated in L-broth (10g Bacto-tryptone, 1g glucose, 3.5 mls 1M NaOH, 1L water, pH 7.0). L-agar plates were made by the addition of Difco Bacto agar to a final concentration of 1.5% (w/v). Minimal medium contained 4.5 g/L KH_2PO_4, 10.5g/L K^2HPO^4, 1 g/L $(NH_4)_2SO_4$ and 0.1 g/L $MgSO_4$. Minimal glucose medium contained the above ingredients plus 20 g/L D-glucose. Solid medium was made by adding Difco Bacto agar at 1.5% (w/v).

Where necessary, media were supplemented at the following concentrations: carbon sources (usually glucose), 0.2%; amino acids,50ug/ml; tetracycline, 25 ug/ml; kanamycin sulfate, 50 ug/ml; sodium ampicillin 50 ug/ml.

Bacteriophages and Bacteriophage Sensitivity Tests. Bacteriophage sensitivity tests were carried out by the methods of Wilkinson et al (1972) using a battery of bacteriophages assembled by B.A.D Stocker and K.E.Sanderson. This set includes the following phages: FO, M13, f2, 6SR, Ffm, Br60, C21, Br2, Chi, U3, Lambda cI857, Lambda vir and Plvir. Phages were applied to a lawn of cells spread on L-plates, allowed to air dry and incubated at 37C for 4h. In the case of recombinants this test was performed on L-plates supplemented with the appropriate antibiotic.

Plasmid Isolation Methods. Plasmid DNA for use in transformations was isolated by modified versions of the large-scale and small-scale methods of Birnboim and Doly as described in Maniatis et al (1982).

Transformation and Conjugation Methods. Cells were made competent and transformed by the $CaCl_2$ method described in Maniatis et al (1982). Transfer by F-factor mediated conjugation of the mobilisable ColE1 derived plasmids was performed in a tri-parental cross. 0.1 ml of the plasmid

donor strain grown overnight in L-broth supplemented with the appropriate antibiotic was mixed in 4 ml of L-broth with 0.1 ml of the recipient strain and 0.1 ml of an F lac+ strain. After incubation overnight at 37C the cells were washed in sterile water before plating on selective media.

LPS Isolation and SDS-PAGE Methods. LPS for analysis on polyacrylamide gels was isolated by the proteinase K extraction method of Hitchcock and Brown (1983). Cells were grown overnight at 37C in 4 ml of L-broth, centrifuged and re-suspended in PBS (0.2M phosphate buffer, pH 7.2) to give an OD_{640} of 1.0. One and a half mls of this suspension were pelleted in a microfuge and the pellet was resuspended in 50ul of lysing buffer (2% SDS, 4% 2-mercaptoethanol, 1M Tris-HCl, pH 6.8 and 1% bromophenol blue). Cell lysates were heated for 10 min at 100C after which 25ug of proteinase K was added and incubated at 37C for 60 min. The isolated LPS was run on 16% polyacrylamide gels using the discontinuous system of Laemmli (1970). Electrophoresis was carried out in the Bio-Rad Protean I apparatus which had been modified to incorporate a water cooling coil. Gels were run at 35 mA constant current using a Tris-glycine buffer, pH 8.3, with 0.1% SDS. LPS preparations run on the SDS-PAGE gels were visualised by the silver staining method of Tsai and Frasch (1982). LPS samples for use in gas chromatographic analysis were isolated by the phenol-chloroform-petroleum ether (PCP)

method of Galanos et al, (1969).

Gas-Liquid Chromatography (GLC). The procedure used was that of Bryn and Jantzen et al (1982) as described in Kadam et al (1985).

Detergent Sensitivity Tests. L-plates containing 0.4% sodium desoxycholate were used to test for sensitivity to this detergent. Plates were streaked with the strain being tested and incubated overnight at 37C. Sensitivity to deoxycholate was assessed by failure to grow at 0.4% concentration.

RESULTS

Isolation and Assembly of Rough Mutants. Bacteriophages have long been used as probes of the bacterial surface structure (Wilkinson et al, 1972; Hancock and Reeves, 1976). Bacteriophages C21 and U3 both use LPS as a receptor for absorption (Rapin et al, 1971; Watson and Paigen,1971). Wild-type strains of E. coli K-12, which have the complete core but no side-chains, are sensitive to phage U3 and resistant to phage C21 (Beutin and Achtman, 1979). Spontaneous mutants of the E. coli K-12 wild-type strain Chi1715 were isolated by plating a saturated culture of cells on an L-agar plate which had been previously overlaid with approximately 10^8 pfu/ml of bacteriophage U3. The phage sensitivity patterns of the U3 resistant mutants are given in Table 1-2. The wild-type E. coli K-12 (Ra chemotype) strain Chi1715 was sensitive to phage U3 and all the rough mutants (except for the leaky rfaG mutant SAB2994, which is partially sensitive) were U3 resistant. The wild-type strain Chi1715 was resistant to phage C21 whereas the rough mutants were C21 sensitive, except in cases where the defect was determined to be in the inner core region; these strains (SAB2990 and SAB2992) showed resistance to phage C21.

The phage MuG(+) resistant mutants (MuR#35, MuR#38, D108-R#13 and S1652) obtained from D.Kamp all displayed resistance to U3 and sensitivity to C21, while the wild-type

strain from which these mutants were derived (C600) is sensitive to U3 and resistant to C21 (Table 1-2). The rough mutants D21e7 and D21f1, isolated by H.Boman on the basis of resistance to ampicillin are resistant to U3 and sensitive to C21, while strains D21f2 and D31m4 from the same source are resistant to both U3 and C21. The strain from which these mutants were originally derived (D21) is U3 - sensitive and C21 - resistant.

The strain CL29 has been postulated to have the deep rough LPS phenotype (Coleman and Leive, 1979). Strains BW228 and BW229 (obtained from B.Weiss via the CGSC) each carry an inserted Tn10 in the rfa gene cluster which results in the production of defective LPS. Both strains are U3 resistant and C21 sensitive.The strain JP5127, which has the galE mutation, (from R.Russell via CGSC) displays the rough phenotype of U3 resistance and C21 sensitivity.

The phage sensitivity results obtained for each of the groups of mutants obtained from sources outside this lab are consistent with the patterns of sensitivity seen for the spontaneous U3 resistant mutants. It is inferred that changes in LPS structure due to a mutational event are readily assayed by changes in the absorption capability of the LPS specific bacteriophages, U3 and C21.

Detergent Sensitivity Tests. Increased permeability to hydrophobic agents has been demonstrated in E. coli K-12

mutants lacking heptose (Coleman and Leive,1979) and in mutants having a less severe defect, lacking glucose or galactose (Tamaki and Matsuhashi,1974, Tamaki et al,1971). Deep rough mutants (Rd2 and Re chemotypes) display hypersensitivity to many hydrophobic agents including fatty acids (Sheu and Freese, 1973), anionic (Sanderson et al, 1974) and cationic (Vaara and Vaara, 1981) detergents. Resistance to sodium deoxycholate at 0.4% is evident in all strains tested (Table 1-2) except those which are postulated to have defects in the inner core region (strains SAB2990, SAB2992, D21f2 and D31m4) .

SDS-PAGE Analysis of Isolated LPS. LPS samples were prepared and analysed on SDS-PAGE by the procedures outlined in Materials and Methods. LPS chemotypes may be distinguished on SDS-PAGE on the basis of differences in sugar mobilities (Jann et al, 1975). Short chain polysaccharides have greater mobility in these gels, due to their smaller molecular weights, than long chain polysaccharides. By comparing the mobilities of unknown LPS preparations with those of known LPS samples from other species with similar LPS structures (eg. S. minnesota and S. typhimurium) one can partially deduce the chemotype of the LPS unknown.

Figure 1-2 shows the silver stained SDS-PAGE gels of the LPS isolated by the Proteinase K method from the LPS mutants listed in Table 1-1. The direction of electrophoresis in these gels is from top to bottom. The standards run on these gels were LPS preparations of Salmonella typhimurium LPS chemotypes: rfaL (Fig 1-2A, lane d), rfaJ (Fig 1-2A, lane h) , rfaI (Fig 1-2A, lane m), rfaG (Fig 1-2B, lane i), rfaF (Fig 1-2B, lane n), and rfaE (Fig 1-2B, lane p) (For an explanation of genotype and chemotype designations, see Fig 1-1). The wild-type strains Chi1715, C600, and D21 (Fig 1-2A,lanes a,b,c) all have mobility similar to the rfaL(S) standard and are inferred to contain LPS of completed core without O antigenic side chains. Strains SAB2995 (Fig 1-2A, lane e) and SAB2997 (Fig 1-2A, lane f) have LPS migration which is slightly faster than

that of the wild-type strains, reflecting possibly a one sugar unit difference from the wild-type LPS. The LPS mobilities of strains SAB2998 (Fig 1-2A, lane i) and SAB3000 (Fig 1-2A, lane j) are expected to be similar to that of the rfaI(S) standard (Fig1-2A, lane m), however the SAB2998 and SAB3000 strains also appear to have some leakiness, indicating an incomplete block in LPS synthesis and producing significant amounts of both rough type LPS and wild-type LPS. The mutant D108-R#13 (Fig 1-2A, lane k) is a non-leaky version of the Rb3 chemotype; its LPS has mobility identical to that of the rfaI(S) standard in lane m (Fig 1-2A). The mutants inferred to be of Rc chemotype (galE or rfaB genotype): Mu-R#35 (rfaB(K)), S1652 (gal deletion (K)), SGSC163 (galE(S)), and D21e7 (rfaB(K)) (Fig 1-2A, lanes l,o,p and Fig 1-2B,lane b respectively) all have LPS which migrates the same distance. This confirms that the galE(S), the galE(K) and rfaB(K) mutants have similar chemotype. The LPS of strain S1652 (Fig 1-2A, lane o) shows some leakiness, due probably to the presence of trace amounts of galactose in the growth medium, allowing the strain to complete some LPS chains to the Ra chemotype. The isolated LPS of strain BW229 (Fig 1-2B, lane d) has mobility similar to that of D21e7 (Fig 1-2B, lane b), indicating a possible Rc chemotype for the Tn10-inserted strain. The LPS of the rfaG(S) standard is in lane i (Fig 1-2B). Strains SAB2994 (Fig 1-2B, lane e) and SAB2999 (Fig 1-2B, lane f) display a double LPS

band, a faint band which is similar to that of the rfaG chemotype in lane i, and a darker band which appears to be of the Ra chemotype. The doublet observed may reflect the leakiness of these mutants, resulting in the display of a large amount of wild-type LPS. Migration in the gel of the LPS of strain Mu-R#38 (Fig 1-2B, lane g) is slower than expected for its postulated chemotype of (rfaG(K)). Strain D21f1 (Fig 1-2B, lane h) has mobility slightly faster than that of the rfaG (Rd1) strain in lane i, however this may be an artefact of the amount loaded on the gel as prervious characterizations have classified this strain as an Rd1 chemotype (Prehm et al, 1976).

The heptoseless LPS standards rfaD and rfaE of S. typhimurium (Fig 1-2B, lanes o and p) have the fastest LPS mobilities of all the mutants. Strains displaying similar mobilities are SAB2990 (Fig 1-2B, lane j), SAB2992 (Fig 1-2B, lane k), D21f2 (Fig 1-2B, lane l) and D31m4 (Fig 1-2B, lane m). It is likely that all these strains have Re chemotype LPS. All of these strains appear to have LPS one sugar shorter than that of the rfaF chemotype (SL3789; Fig 1-2B, lane n). These strains are therefore inferred to have LPS of Re chemotype and the nature of the rfa defect may be rfaC, rfaD, or rfaE since each of these mutations produce heptoseless LPS. The rfaD defect is leaky by nature and some LPS chains are completed (to give a lightly stained band of Ra-like migration) even though the mutant is known to be

defective in the addition of the first heptose residue (Coleman and Leive, 1979). None of the four mutants in lanes j,k,l and m (Fig 1-2B) appear to have a significant amount of non-Re type LPS.

Gas Chromatography of LPS Preparations. Gas chromatographic analysis of LPS, purified by the PCP method from the mutants, was carried out using the procedures outlined in Materials and Methods. It should be stressed that the sugars listed in Table 1-3 are not the only sugars detected during gas-chromatographic analysis of LPS preparations. N-acetylglucosamine and KDO are also detected but their levels are relatively constant in these LPS mutants and are therefore not listed.

The chromatograph of Chi1715 (chemotype Ra from phage sensitivity tests and Ra-like LPS mobility in SDS-PAGE) gives a molar ratio for glucose, galactose, heptose of 3.12:0.61:2, close to the ratio of 3:1:2 predicted by other investigaters for wild-type LPS of E.coli K-12.

The LPS from SAB2992 lacks all the constituent sugars present in Chi1715 reflecting its deep rough nature (Table 1-3). This strain however shows a large peak which is possibly D,D-heptose (data not shown).This strain displays the deoxycholate supersensitivity characteristic of deep core mutants.

Sugar analysis of SAB2994 (which is sensitive to

rough-specific phages and has Rd1-like LPS mobility in SDS-PAGE) shows a reduced amount of galactose from the level in Chi1715. However, more glucose is observed than would be expected for an Rd1 chemotype (Table 1-3). This is probably due to the leakiness of this mutant. Strain Mu-R#38 has an LPS structure of the Rd1 chemotype and in gas chromatographic analysis of the sugars only shows the presence of two heptose residues (Sandulache et al, 1984).

Mutants of Rc chemotype have heptose and glucose but no galactose in their LPS (Fig 1-1). Strain Mu-R#35 and D21e7 have LPS of this type (Table 1-3). The amount of glucose is reduced by approximately one half to one third that of the wild-type. Strain S1652 carries a deletion of the gal operon and therefore cannot synthesise galactose-containing LPS. The amount of glucose in this strain is also reduced from that of the wild-type.

SAB3000 has rough phage type and Rb3-like LPS mobility in SDS-PAGE. In the gas chromatogram this strain shows almost equal amounts of glucose and galactose. The amount of glucose detected is approximately one third the amount seen for the wild-type. The amount of galactose is however unchanged (Table 1-3). Similarly sugar analysis of strain D108-R#13 has revealed the presence of two heptoses, one glucose and one galactose (Sandulache et al, 1984). These results are in good agreement with the postulated structure of Rb3 mutants (Fig 1-1).

The chromatographic results for SAB2995 shows normal amounts of galactose and heptose and two thirds of the wild-type level of glucose (Table 1-3). This shift in the ratios of glucose to galactose in favour of glucose implies that there is at least one glucose more in SAB2995 than in SAB3000. SAB2995 has the rough phage pattern and in SDS-PAGE resembles an Rb2 chemotype.

Complementation Tests. Introducing a plasmid-borne wild-type copy of the defective chromosomal locus allows the screening of the resultant diploid for restored wild-type phenotype. In this study monitoring of the complementation effect is facilitated by the specificity of the bacteriophages for either the rough or wild-type chemotype. SAB2995 (rfa-216) is resistant to phage U3 and sensitive to C21, but when transformed with plasmids pKZ48 or pKZ26, which have genes rfaG,B,I and J, it is restored to the wild-type phage sensitivity pattern (U3 sensitive and C21 resistant), while it is not restored to the wild-type phenotype by plasmid pKZ27 (rfaG,B,I); SDS-PAGE of the isolated LPS of this strain (Fig 1-3), with the above plasmids and without plasmid confirms the complementation seen in the phage sensitivity patterns these data indicate that the mutant gene is rfaJ(K), which can be complemented by either rfaJ(K) or rfaJ(S) (Table 1-4). The rfaJ(K) gene codes for the E. coli glucosyltransferase III (Fig 1-1).

The mutant gene (rfa-221) in SAB3000 is postulated to be rfaI(K) since this mutant is only complemented by plasmids carrying the rfaI(K) or rfaI(S) genes: pKZ39, pKZ26 and pKZ27 (Table 1-4). This complementation gives wild-type LPS (Ra chemotype) as assessed by phage sensitivity tests (shown in Table 1-4) and by SDS-PAGE of LPS extracted from the mutants with and without the plasmid (Fig 1-3). Phage sensitivity tests are a convenient method of determining LPS chemotype and gel analysis, where done, has confirmed the results obtained in the phage tests. The rfaI(K) locus encodes the glucosyltransferase II which catalyses the addition of the glucose II unit onto the core (Fig 1-1). When SAB2994 is transformed with each of the six plasmids, complementation of the defective (rfa-215) locus is observed only with plasmids which carry the rfaG gene (Table 1-4). Both pKZ14 (rfaG(K)) and pKZ15 (rfaG(S)) complement the defect of this mutant indicating that the glucosyltransferase I enzymes (Fig 1-1) are functionally similar in both species. The SAB2994 mutant is inferred to be defective in the addition of the glucose I unit to the heptose residues of the inner core.

Of the Mu-resistant strains (Mu-R#35, Mu-R#38, D108-R#13 and S1652), described by Sandulache et al (1984), only Mu-R#35, Mu-R#38 and D108-R#13 show positive complementation with pKZ48. This indicates that the genotype of these strains is within the group of genes: rfaG, rfaB,

rfaI and rfaJ. S1652 has a deletion of the galactose operon (Sandulache et al, 1984) and is therefore not complementable by these plasmids. Both D108R#13 and Mu-R#35 are complemented by pKZ48 and pKZ39, but not by pKZ14 . This implies that the defect in these two mutants may be in either the rfaB(K) or rfaI(K) activities - resulting in failure to add either the galactose I or the glucose II residues respectively, to the LPS core. The D108R#13 mutant however is not complemented by a plasmid derived from pLC10-7 containing a Tn5 insertion which inactivates the rfaI locus (described in Chapter 2). Strain Mu-R#35 fails to show complementation with either pKZ14 (Table 1-4) or a Tn5 insertion in pLC10-7 which has lost only rfaB activity (Chapter 2). It is therefore inferred that the defective locus in D108-R#13 is in the rfaI(K) and that the defect in Mu-R#35 represents a mutation at the rfaB(K) locus. When Mu-R#38 is transformed with pKZ39 (rfaB,I(K)) there is no change from the mutant phenotype, but pKZ14, pKZ26 or pKZ27 restore this mutant to wild-type phage sensitivity. This implicates the rfaG(K) gene as the defective gene in this mutant.

The mutants D21e7, D31m4, D21f1 and D21f2 all have defective LPS (Boman and Monner, 1975). When transformed with the plasmid pKZ48, only D21e7 and D21f1 show complementation to U3-sensitivity (Table 1-4). This is consistent with the published chemical data for these

mutants which determine D21e7 and D21f1 to have Rc and Rd1 chemotypes respectively (Boman and Monner, 1975). D21e7 has a single defective locus (rfa-1) which is complemented by pKZ39 but not complemented by a plasmid bearing an inactive rfaB gene (Chapter 2). D21e7 is therefore assigned the genotype rfaB1. D21f1, which is derived from D21e7 and has a two mutant alleles (rfa-1 rfa-21), is complemented by the genes on pKZ48, pKZ26 and pKZ27. The first defect has been determined to be rfaB (in D21e7). The second defect (rfa-21) is probably in the rfaG gene, since the chemotype is Rd1. Neither D31m4 (rfa-229 rfa-230) nor D21f2 (rfa-1 rfa-31) are complemented by any of the genes on pKZ48 (Table 1-4). This may indicate that the defects in these strains are of Rd2 or Re chemotype and therefore not complemented by genes on this plasmid. This postulate is supported by the chemical analysis of Boman and Monner (1975) which demonstrated a deep rough chemotype for both D21f2 and D31m4. Genetic analysis of plasmids bearing genes for the synthesis of the inner core region is discussed in Chapter 3.

DISCUSSION

The results of the chemical and genetic analyses for the mutants are discussed below on a chemotype - by - chemotype basis.

Re (rfaC,D,E). Mutants with defects in the heptose region of the core are of the Rd2 and Re chemotypes and show increased sensitivities to deoxycholate and to several antibiotics (Table 1-2). Isolated LPS preparations of strains SAB2990 (Fig 1-2B ,lane j), SAB2992 (Fig 1-2B,lane k), D21f2 (Fig 1-2B, lane m) and D31m4 (Fig 1-2B, lane n) have similar mobilities to that of Re type LPS of S. typhimurium (Fig 1-2B, lanes o and p). The gas chromatography of SAB2993 and SAB2992 confirms the absence of glucose, galactose, and heptose from these core-type, as expected for the Re chemotype (Table 1-3). The LPS of SAB2990 contains no glucose, galactose or L,D-heptose (Table 1-3). The chromatogram of this mutant does however contain an unknown peak which may indicate that the mutation in this strain is leaky.

D-glycero-D-mannoheptose is the precursor of L-glycero-D-mannoheptose, the form of heptose normally found in core LPS. This conversion depends on an epimerase encoded by the rfaD gene (Lehmann et al, 1973). If the epimerase is blocked (as in rfaD mutants) the precursor D,D-heptose accumulates and is partially incorporated into the LPS. This

form of heptose however is a poor acceptor for subsequent addition of the glucose I residue and therefore only a small fraction of the cores are completed. CL29 is an rfaD mutant of E. coli K-12 (Coleman and Leive, 1979). The LPS of strain CL29 is shown in Fig 1-2B, lane o and consists of an Re-like fraction and a leaky LPS fraction which has a longer LPS chain. This chemotype is consistent with a leaky defect in the heptose epimerase. The defective LPS of strains D21f2 and D31m4 is also of the Re chemotype. These strains display no leakiness either in SDS-PAGE or in chemical analysis (Table 1-3). These two mutants do not display complementation with pKZ48. Since these strains harbour double mutations, it is postulated that they may require the simultaneous presence, on plasmids, of genes for the inner and outer core of the LPS for complementation.

The plasmid pLC13-13 complements an rfaD mutant of E. coli K-12 (Coleman and Deshpande, 1985). This plasmid also complements rfaC, rfaD and rfaF mutations of S. typhimurium restoring the strain to the smooth phenotype, while the mutations of S. typhimurium affecting the outer core component of the LPS (rfaG, rfaB, rfaI, rfaJ, rfaK and rfaL) show no change in phenotype (L.V.Collins, unpublished data). Strain SAB2992 is complemented to wild-type by this plasmid - confirming the deep-rough genotype of this mutant (Table 1-4). Testing of the other deep rough mutants in E. coli to determine additional activities of this plasmid is described

in Chapter 3.

Rd1 (rfaG). Rd1 mutants are defective in the addition of the glucose I residue to the core (Fig 1-1). Strain Mu-R#38 is resistant to phages U3, C21 and MuG(+) and the LPS as determined by gas chromatography has no glucose or galactose but normal levels of heptose (Sandulache et al, 1984). This strain is complemented by pKZ14, pKZ48, pKZ26 and pKZ27 but not by the rfaB or rfaI genes on pKZ39, indicating that the defective rfa-225 locus is rfaG . The strains SAB2994, SAB2999 and D21f1 are sensitive to phage U3 and show a slight sensitivity to sodium deoxycholate (Table 1-2). The isolated LPS preparations of strains SAB2994 and SAB2999 appear leaky in SDS-PAGE (Fig 1-2B, lanes e and f) but this fails to confer wild-type phage-sensitivity characteristics on these strains. Gas chromatographic analysis shows the presence of glucose, due presumably to a partially active ("leaky") rfaG mutant resulting in some Ra type LPS. Very little galactose was detected, supporting the idea that the LPS of chemotype Rd2 strains contains only the heptose sugar. The LPS of SAB2994 is restored to wild-type by complementation with pKZ14; the only complementing activity known on this plasmid is rfaG.

GalE (galE). GalE mutants have a defect in the pathway for synthesis of the UDP-galactose required for assembly of the complete LPS core. In the presence of exogenous galactose, UDP-galactose is synthesised by the epimerisation

of UDP-glucose. A mutation in galE results in loss of the epimerase and results in an LPS core without galactose (Fukasawa and Nikaido, 1961). Sandulache et al (1984) observed that in minimal medium without any galactose, the galE strain synthesised an incomplete LPS core, whereas in broth medium it shows the complete wild-type core due probably to trace amounts of galactose in the broth providing for synthesis of UDP-galactose. In fact the GalE$^-$ phenotype may display a partial block, allowing the completion of a fraction of the LPS chains but prematurely terminating most of the cores at the glucose I residue by not adding the side chain galactose I residue. S1652 (galE) shows no complementation with pLC10-7 or derived plasmids since the galE gene does not map within the rfa cluster of genes.

Rc (rfaB). Mutants of the Rc chemotype are defective in the addition of the 1,6-galactose I residue. The precise chemotype of an rfaB mutant of E. coli has not previously been recognised, partly because of an inability to isolate a non-galE mutant which lacked the side-chain galactose, and partly because of the lack of a plasmid bearing the rfaB gene. Strain Mu-R#35 is resistant to U3 phage and sensitive to phage C21 (Table 1-2). Strain Mu-R#35 has a glucose to galactose to heptose ratio of 1:0:2.2 (Sandulache et al, 1984) and is thereby inferred to be of chemotype Rc. Absence of galactose from LPS can be due to a defect in either the

galE gene (as described above) or due to a defect in the gene encoding the galactosyltransferase (Osborn and Rothfield, 1971). Strain Mu-R#35 is Gal^+ in chemotype for it produces red colonies on MacConkey agar supplemented with 0.1% galactose (data not shown). The remaining possibility is that this mutant has some rfa defect(s). The rfa-224 mutation of Mu-R#35 is complemented by pKZ48 and pKZ39 indicating that the defective gene is either rfaB(K) or rfaI(K); since the mutation is also complemented by a plasmid having a Tn5 insert in pLC10-7 which inactivates the rfaI(K) locus but retains activity of the rfaB(K) locus (see Chapter 2). It is proposed that this mutant lacks the ability to attach the galactose I unit due to a defect in the E. coli galactosyltransferase I coding gene viz. rfaB(K).

Some mutants defective in the core LPS have decreased sensitivity to hydrophilic antibiotics (Boman and Monner, 1975). Mutant D21e7 is ampicillin resistant, partially sensitive to deoxycholate and phage C21, while able to ferment galactose (Gal^+), with LPS deficient in galactose (Mayer et al, 1976). It was postulated therefore to be defective in the incorporation of galactose (from UDPgal) into LPS and the defective locus in D21e7, designated lpsA, was linked with the xyl and mtl genes by transduction (Eriksson-Greenberg et al, 1971). Mayer et al (1976) suggested that D21e7 is an rfaP mutant since it resembled an

rfaP mutant of S. minnesota described in Muhlradt et al (1968), but direct proof is lacking. Mutant D21e7 is complemented by all plasmids carrying the rfaB gene and failed to show complementation with a plasmid (pKZ46; described in Chapter 2) derived from pLC10-7 which carried an inactivated rfaB gene; therefore its mutation is assigned as rfaB1. However, since it is not known if the rfaP locus is present on this plasmid, an rfaP assignment is possible.

Rb3 (rfaI). Chemotypes of the Rb3 class are defective in the addition of the glucose II residue to the LPS core. The defect in strain D108-R#13 renders this strain resistant to U3 and sensitive C21 phage (Table 1-2). On SDS-PAGE the isolated LPS has mobility similar to that of LPS of S.typhimurium Rb3 chemotype (Fig 1-2A). Gas chromatography of LPS of D108-R#13 (Table 1-3) indicates almost equal amounts of glucose and galactose. Genetic analysis of this strain reveals that the defect in this strain is alleviated when transformed with pKZ48 and pKZ39 (Table 1-4) both of which bear the rfaI gene (for glucosyltransferase II) of E. coli K-12 (Chapter 2) and also by the S. typhimurium plasmids, pKZ26 and pKZ27, both of which carry the rfaI(S) gene encoding the 1,3-galactosyltransferase II enzyme (Kadam et al, 1985).

Rb2 (rfaJ). Mutants of this class in E. coli K-12 are resistant to U3 and partially sensitive to C21. Isolated LPS of strains SAB2995 (Fig 1-2A, lane e) and SAB2997 (Fig 1-2A,

lane f) has slightly faster mobility than that of the wild-type E. coli K-12 (Fig 1-2A, lane a). In the gas chromatograph the LPS of SAB2995 gives a glucose: galactose: heptose ratio consistent with two heptose, one galactose, and two glucose residues (Table 1-3). Therefore the LPS of this mutant is terminated at the glucose II residue and is inferred to be of the Rb2 chemotype. When transformed with plasmids carrying the rfaJ gene (pKZ26 and pKZ48), strain SAB2995 was restored to wild-type phage sensitivity (Table 1-4). Transformation however, with the pKZ27 and pKZ39 plasmids, neither of which carry the rfaJ gene, resulted in failure to correct the mutant phenotype. The genotype of this strain is inferred to be rfaJ216. This gene codes for the E. coli glucosyltransferase III which adds the terminal glucose to the LPS core chain (Fig 1-1).

Rb1 (rfaK). Mutants lacking the terminal N-acetylglucosamine were not detected in this study. It has been reported that the C6 position of the glucose III residue in E. coli K-12 is only partially substituted by the N-acetylglucosamine (Prehm et al, 1976). Sandulache et al (1984) postulated thst the acceptor for phage MuG(+) was in the terminal GlcNAc - Glucose III region of the LPS of E. coli. Mutants of the Rb1 chemotype might retain part of the receptor for phage MuG(+) - this would explain the failure to the detect this type of mutant in the selection for

Mu-resistant mutants.

Ra (*Rfa*$^+$). The postulated structure of the LPS of wild-type *E. coli* K-12 strains comprises the sugar sequence in Fig 1-1. The completed core or Ra chemotype displays sensitivity to bacteriophage U3 and resistance to C21 (Table 1-2). The isolated LPS of strains Chi1715 (Fig 1-2A, lane a), C600 (Fig 1-2A, lane b) and D21 (Fig 1-2A, lane c) have similar mobility in SDS-PAGE gels to that of the Ra chemotype of an *rfaL* mutant of *S. typhimurium* (Fig 1-2A, lane d). These three strains are inferred to have wild-type LPS and to have the *rfa*$^+$ genotype. Gas chromatography of the purified LPS of Chi1715 reveals a ratio of sugars (Table 1-3) consistent with the three glucose to one galactose to two heptose ratio expected for LPS of wild-type *E. coli* K-12.

Table 1-1. Bacterial strains and plasmids used in this study

Strain	Genotype	Source	Reference
E. coli K-12			
BW228	*zia-204*::Tn10 *gltC10 pyrE metB thi lac rpsL*	B. Weiss (via CGSC)	
BW229	*rfa-209*::Tn10 *gltC10 pyrE metB thi lac rpsL*[a]	B. Weiss (via CGSC)	
BW322	*rfa-210*::Tn10 *thi-1 relA1 spoT1 pyrE* Hfr[b]	B. Weiss (via CGSC)	
C600	*thr-1 leu-6 thi-1 supE44 lacY tonA21*	D. Kamp	
CL29	*tfrB rfaD thi*	L. Leive	Coleman and Leive, 1979
D21	*pro trp his lac rpsL ampA tsx*	H.G. Boman	Boman and Monner, 1975
D21e7	Same as D21 except *rfa-1*[c]	H.G. Boman	
D21f1	Same as D21 except *rfa-1 rfa-21*[d]	H.G. Boman	
D21f2	Same as D21 except *rfa-1 rfa-31*[e]	H.G. Boman	
D31m4	Same as D21 except *rfa-229 rfa-230*[f]	H.G. Boman	
D108-R #13	Same as C600 but *rfa-222*[g]	D. Kamp	Sandulache, Prehm and Kamp, 1984
HB101	F$^-$ *hsdS (r-B,m-B) recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1 supE44*	J. Duerkson	
Mu-R#101,St1-R	Same as C600 except *rfa-223*[g]	D. Kamp	

....continued

Table 1-1 (continued)

Strain	Genotype	Source	Reference
Mu-R #35	Same as C600 except *rfa-224*[g]	D. Kamp	Sandulache et al., 1984
Mu-R #38	Same as C600 except *rfa-225*[g]	D. Kamp	Sandulache et al., 1984
S1652	Δ(*gal*)	D. Kamp	Sandulache et al., 1984
Chi1715	F⁻ del41(*pro-lac*) *glnV42*(*supE42*) *his53 xyl14 cycA1 cycB2*	R. Curtiss	
JP5127	*trp-356 xyl-5 mtl-1 mal-358 rpsL704 tfr-3 tsx-358 supE44 fuc-3 ilvC7 argE3 thi-1 his-4 galE45*	R. Russell (via CGSC)	
SAB2990	As for Chi1715, except *rfa-211*[h]	This study	
SAB2992	As for Chi1715, except *rfa-213*[h]	This study	
SAB2994	As for Chi1715, except *rfaG215*[h]	This study	
SAB2995	As for Chi1715, except *rfa-216*[h]	This study	
SAB2997	As for Chi1715, except *rfaJ218*[h]	This study	
SAB2998	As for Chi1715, except *rfaI219*[h]	This study	
SAB2999	As for Chi1715, except *rfaG220*[h]	This study	
SAB3000	As for Chi1715, except *rfaI221*[h]	This study	
SAB3002	Same as HB101 with pKZ14	This study	
SAB3051	Same as HB101 with pKZ39	This study	
SAB3072	Same as HB101 with pKZ48		
SAB3073	*trpE5 thr leu recA*/pLC10-7	L. Rothfield	Creeger and Rothfield, 1979

....continued

Table 1-1 (continued)

Strain	Genotype	Source	Reference
S. typhimurium			
SA2738	Same as SL1655 (*rfaG3073*) with pKZ15	This lab	Kadam et al., 1985
SA2857	Same as SL3769 (*rfaG471*) with pKZ26	This lab	Kadam et al., 1985
SA2858	Same as SL3769 (*rfaG471*) with pKZ27	This lab	Kadam et al., 1985
SGSC163	*galE503*	G. Ames	
SL1655	*metA22 metE551 trpC2 H1-B H2-e,n,x flaA66 rpsL120 xyl-404 ilv-452 hsdLT6 hsdSA29 rfaG3037*	B. Stocker	
SL1102	*metA22 metE551 trpE2 H1-B H2-e,n,x flaA66 rpsL120 xyl-404 rfaE543*	B. Stocker	
SL3748	*rfaI432*	B. Stocker	Roantree et al., 1977
SL3749	*pyrE*$^{+}$ *rfaL446*	B. Stocker	Roantree et al., 1977
SL3750	*rfaJ417*	B. Stocker	Roantree et al., 1977
SL3769	*rfaG471*	B. Stocker	Roantree et al., 1977
SL3789	*pyrE*$^{+}$ *rfaF511*	B. Stocker	Roantree et al., 1977
SL4807	*rfaB707* (ColE1-30) *leu-1051 malB479 hisC527* (amber) *cysI1173*	B. Stocker	Roantree et al., 1977

....continued

Table 1-1 (continued)

Strain	Genotype	Source	Reference
Plasmids			
pKZ14	(pBR322-*rfaG(K)*)	This lab	Chapter 2, this study
pKZ15	(pBR322-*rfaG(S)*)	This lab	Kadam et al., 1985
pKZ26	(pBR322-*rfaGBIJ(S)*)	This lab	Kadam et al., 1985
pKZ27	(pBR322-*rfaGBI(S)*)	This lab	Kadam et al., 1985
pKZ39	(pBR322-*rfaI(K)*)	This lab	Chapter 2, this study
pKZ48	pLC10-7::Tn5(ColE1-*rfaGBIJ(K)*)	This lab	Chapter 2, this study
pLC10-7	(ColE1-*rfaGBIJ(K)*)	This lab	Creeger and Rothfield, 1979
pLC13-13	(ColE1-*rfaCDF(K)*)	This lab	Chapter 2, this study

....continued

Table 1-1 (continued)

[a] BW229 has a Tn10 insert, originally called *zib-205*, now known to be in the *rfa* gene (see Results).

[b] BW322 has a Tn10 insert, originally called *zia-207*, now known to be in the *rfa* gene (see Results).

[c] The mutation in D21e7 was originally called *lpsA1*; this was changed to *rfa-1*.

[d] D21f1 carries *rfa-1*; a second mutation was selected, originally called unknown 2 (Boman and Monner, 1975); this was changed to *rfa-21*.

[e] D21f2 carries *rfa-1*; a second mutation was selected, originally called unknown 4 (Boman and Monner, 1975); this was changed to *rfa-31*.

[f] D31m4 is derived from parental strain D3; two mutations affecting LPS were isolated, originally called unknown 1 and unknown 2 (Boman and Monner, 1975). These are now called *rfa-229* and *rfa-230*.

[g] These mutants were obtained from D. Kamp. They were assigned allele numbers.

[h] These are spontaneous mutants of strain Chi1715, selected for resistance to bacteriophage U3.

[i] To facilitate selection of the pLC10-7 plasmid a Tn5 (Km-res) transposon was inserted into the ColE1 sequences of this plasmid; pKZ48 has the same *rfa* gene activities as pLC10-7.

Table 1-2. Characterisation of *E. coli* K-12 mutants affecting LPS structure

Strain	Genotype[a]	Phage Sensitivities[b]			Deoxycholate Sensitivity[c]	Inferred Chemotype[d]
		U3	C21	MuG(+)		
Chi1715	*rfa*$^+$	S	R	S	R	Ra(K)
SAB2995	*rfaJ216*	R	S		R	Rb2(K)
SAB2997	*rfaJ218*	R	S		R	Rb2(K)
SAB2998	*rfaI219*	R	S		R	Rb3(K)
SAB3000	*rfaI221*	R	S		R	Rb3(K)
SA2907	*galE45*	R	S		R	GalE(K)
SAB2994	*rfaG215*	R	S		R	Rd1(K)
SAB2999	*rfaG220*	R	S		P	Rd1(K)
SAB2990	*rfa-211*	R	R		S	Re(K)
SAB2992	*rfa-213*	R	R		S	Re(K)
CL29 (SA2895)	*rfaD*	R	R		S	Re(K)
C600	*rfa*$^+$	S	R	S	R	Ra(K)
Mu-R #35	*rfaB224*	R	S	R	R	Rc(K)

....continued

Table 1-2 (continued)

Strain	Genotype	Phage Sensitivities			Deoxycholate Sensitivity	Inferred Chemotype
		U3	C21	MuG(+)		
Mu-R #38	*rfa-228*	R	R	R	R	Rd1(K)
D108-R #13	*rfa-222*	R	R	R	R	Rb3(K)
St1-R,Mu-R101	*rfa-223*	R	S	R	R	
S1652	*gal* deletion	R	S	R	R	GalE(K)
D21	*rfa*$^{+}$	S	R		R	Ra(K)
D21e7 (SA1731)	*rfaB1*	R	S		P	Rc(K)
D31m4 (SA1733)	*rfa-229 rfa-230*	R	R		S	Re(K)
D21f1 (SA1732)	*rfaB1 rfa-21*	R	S		P	Rd1(K)
D21f2 (SA1734)	*rfaB1 rfa-31*	R	R		S	Re(K)
BW229 (SAB2805)	*rfa-209*::Tn10	R	S		R	
BW322 (SAB2806)	*rfa-210*::Tn10	R	S		R	

....continued

Table 1-2 (continued)

[a] Partial genotypes are shown; full genotypes are given in Table 1-1.

[b] Phage sensitivities for U3 and C21, "S" = lysis; "R" = no visible lysis on lawn of cells. Other phages tested, F0, M13, B42 and Chi: all resistant; 6SR, Ffm and B460: all sensitive.

[c] Growth on L-agar + deoxycholate 0.4%. R = Resistant (normal growth); S = Sensitive (no growth); P = Poor growth.

[d] Chemotype is inferred from the data in this table, from the mobilities of the LPS in SDS-PAGE (Fig. 1-2), and from gas chromatography data in Table 1-3.

Table 1-3. Sugar analysis of LPS mutants by gas-liquid chromatography[a]

Strain	Molar Ratios: Experimental[b] Glucose / Glucose	Galactose / Galactose	L,D-Heptose	Inferred Chemotype of Strain[c]	Molar Ratios: Theoretical[d] Glucose	Galactose	L,D-Heptose
Chi1715[e]	3.12	0.61	2	Ra	3	1	2
SAB2992[e]	0	0	0[h]	Re	0	0	0
SAB2994[e]	0.83	0.36	2	Rd1 (leaky)	0	0	2
SAB2995[e]	1.32	0.62	2	Rb2	2	1	2
SAB3000[e]	0.81	0.63	2	Rb3	1	1	2
SAB2990[e]	0	0	0	Re	0	0	0
C600[f]	2.7	1	3.2	Ra			
Mu-R #35	1	0	2.2	Rc			

....continued

Table 1-3 (continued)

Strain	Molar Ratios: Experimental Glucose	Galactose	L,D-Heptose	Inferred Chemotype of Strain	Molar Ratios: Theoretical Glucose	Galactose	L,D-Heptose
Mu-R #38[f]	0	0	2.1	Rd1			
D108-R #13[f]	1.4	1	2.7	Rb3			
S1652[f]	2	0	3.2	Rc			
D21[g]	2	1.25	1.95	Ra			
D21e7[g]	0.93	0	2	Rc			
D21f1[g]	0	0	1.95	Rd1			

....continued

Table 1-3 (continued)

[a] Sugar analysis by gas-liquid chromatography of Chi1715 and derived mutants as described in Materials and Methods. For methods of analysis of other mutants see References below.

[b] For Chi1715, SAB2992, SAB2994, SAB2995 and SAB3000 the molar ratio of constituents is based on two heptoses. The area percent of glucose and galactose was obtained from the integrated printout from the chromatograph of an unknown LPS, divided by the area percentage for the L_1D_1 heptose to normalise the values (consistent with 2 moles heptose) and then multiplied by the appropriate molar response factor. (The molar response factor for glucose and galactose was determined from authentic standards by determining the integrated areas for known amounts of each sugar.)

[c] Chemotype is inferred from the data in this table, from the mobilities in SDS-PAGE (Fig. 1-2) and from the data in Table 1-2.

[d] Theoretical molar ratios of glucose, galactose and heptose in LPS of *E. coli* K-12, based on LPS structure proposed by Koplow and Goldfine (1974), and on the inferred chemotype.

[e] Chi1715 (*rfa*$^+$) has wild-type *E. coli* K-12 LPS. SAB2990 (*rfa-211*), SAB2992 (*rfa-213*), SAB2994 (*rfaG215*), SAB2995 (*rfaJ216*) and SAB3000 (*rfaI221*) are all derived from Chi1715 by selection for phage U3 resistance.

[f] C600 (*rfa*$^+$) has wild-type LPS. Mutants derived from this strain with defective LPS, Mu-R #35 (*rfaB224*), Mu-R #38 (*rfaG225*) and D108-R #13 (*rfa-222*) were derived by selection for resistance to phage MuG(+). Strain S1652 has a deletion in the *gal* region. The molar ratios of sugars and LPS structure of these strains have been calculated from the results given in Sandulache et al. (1984).

[g] D21 (*rfa*$^+$) is the parental LPS wild-type strain of D21e7 (*rfaB1*) and D21f1 (*rfaB1, rfa-21*). Chromatographic analysis of sugar composition of these strains is described in Prehm et al. (1976). The values for the molar ratio of LPS sugars have been calculated from the results given in Prehm et al. ((1976).

[h] For SAB2992 an unknown peak (possibly D,D-heptose) was detected. No L,D-heptose was observed.

Table 1-4. Complementation patterns of *E. coli* K-12 mutants

Strain	Complementation Analysis[a]							Inferred Genotype[b]
	pKZ48 (pLC10-7/Tn5)	pKZ14	pKZ39	pLC13-13	pKZ15	pKZ26	pKZ27	
	rfaGBIJ(K)	*rfaG(K)*	*rfaBI(K)*	*rfaCDF(K)*	*rfaG(S)*	*rfaGBIJ(S)*	*rfaGBI(S)*	
SAB2995	+	-	-		-	+	-	*rfaJ216*
SAB3000	+	-	+		-	+	+	*rfaI221*
SAB2994	+	+	-		+	+	+	*rfaG215*
SAB2990				+				*rfaC211*
SAB2992				+				*rfaC213*
CL29				+				*rfaD*
Mu-R #35	+	-	+			+	+	*rfaB224*
Mu-R #38	+	+	-			+		*rfaG225*
D108-R #13	+	-	+			+	+	*rfaI222*
S1652	-							*Δgal*
D21e7	+	-	+					*rfaB1*
D31m4	-							*rfa-229 rfa-230*
D21f1	+	-	-			+	+	*rfaB1 rfa-21*
D21f2	-							*rfaB1 rfa-31*
BW229	+							*rfa-209*::Tn10
BW322	+							*rfa-210*::Tn10

....continued

Table 1-4

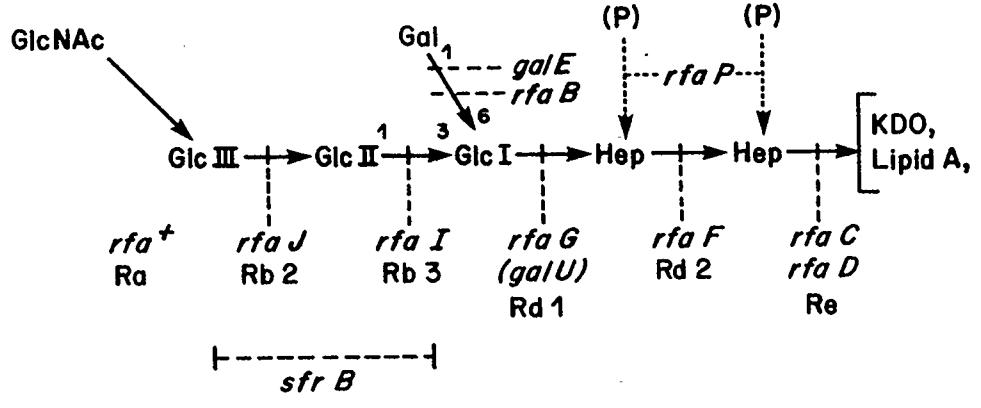
[a] Complementation tested by conjugation or transformation of plasmids into mutants and testing of recombinants for changes in the mutant phage phenotype (U3-resistant and C-21 sensitive) to the wild-type phage phenotype (U3-sensitive and C-21 resistant). Positive complementation of mutant gene(s) is denoted by "+".

[b] Inferred genotype based on ability of plasmid genes to complement mutations in *E. coli*.

Figure 1-1

The structures of the LPS of E. coli K-12 and S. typhimurium LT2 indicating the genes required for their synthesis. Genes in brackets have functions other than LPS synthesis. Dashed lines indicate positions at which rfa or galE mutations shorten the LPS structure. Ra through Re refer to the chemotypes of the LPS produced. Symbols: GlcNAc, N-acetyl-D-glucosamine; Glc, glucose; Gal, galactose; Hep, L-glycero-D-mannoheptose; KDO, 2-keto-3- deoxyoctulosonic acid.

E. coli K-12



S. typhimurium

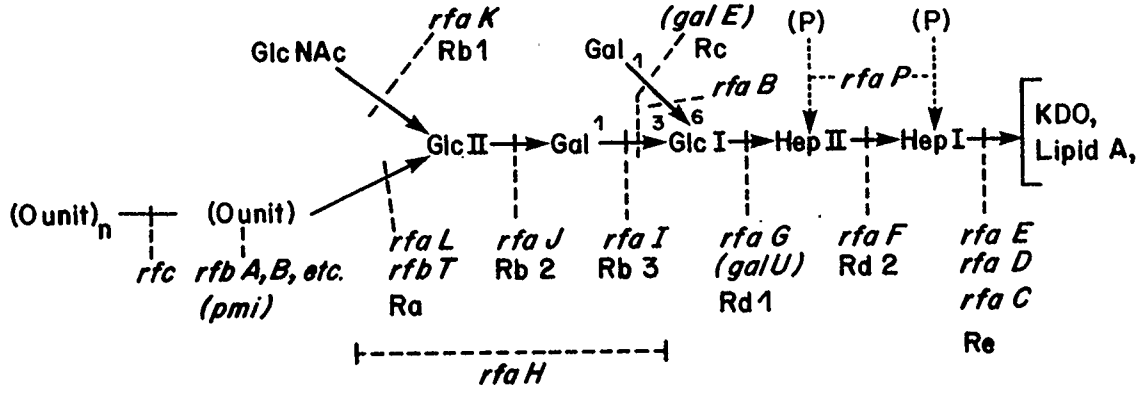
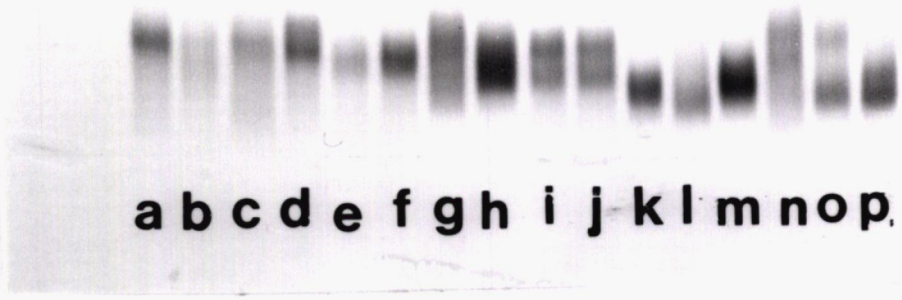


Figure 1-2

Migration patterns of the LPS of mutants of E. coli K-12 and S. typhimurium in a 16% polyacrylamide gel. Whole cell proteinase K - digested lysates were prepared and the LPS was stained by the method of Tsai and Frasch (1982). LPS from the following strains were run in the letter-indicated lanes: Fig. 1-2A: a, Chi1715 (rfa$^+$); b, C600 (rfa$^+$); c, D21 (rfa$^+$); d, SL3749 (rfaL(S)); e, SAB2995 (rfaJ216(K)); f, SAB2997 (rfaJ218(K)); g, BW322 (rfa210::Tn10); h, SL3750 (rfaJ417(S)); i, SAB2998 (rfaI219(K)); j, SAB3000 (rfaI221 (K)); k, D108-R#13 (rfaI222(K)); l, Mu-R#35 (rfaB224(K)); m, SL3748, (rfaI432(S)); n, JP5127 (galE45(K)); o, S1652 (del-gal(K)); p, SGSC163 (galE503(S)). Fig 1-2B: a, St1-RMu-R101 (rfa-223(K)); b, D21e7 (rfaB1(K)); c, SGSC163 (galE503(S)); d, BW229 (rfa209::Tn10(K)); e, SAB2994 (rfaG215(K)); f, SAB2999 (rfaG220(K)); g, Mu-R#38 (rfa-225(K)); h, D21f1 (rfaB1,rfa21(K)); i, SL3769 (rfaG471(S)); j, SAB2990 (rfaC211(K)); k, SAB2992 (rfaC213(K)); l, D21f2 (rfaB1,rfa-31(K)); m, D31m4 (rfa-229,rfa230(K)); n, SL3789 (rfa F511(S)); o, CL29 (rfaD(K)); p, SL1102 (rfaE543(S)).

1-2A



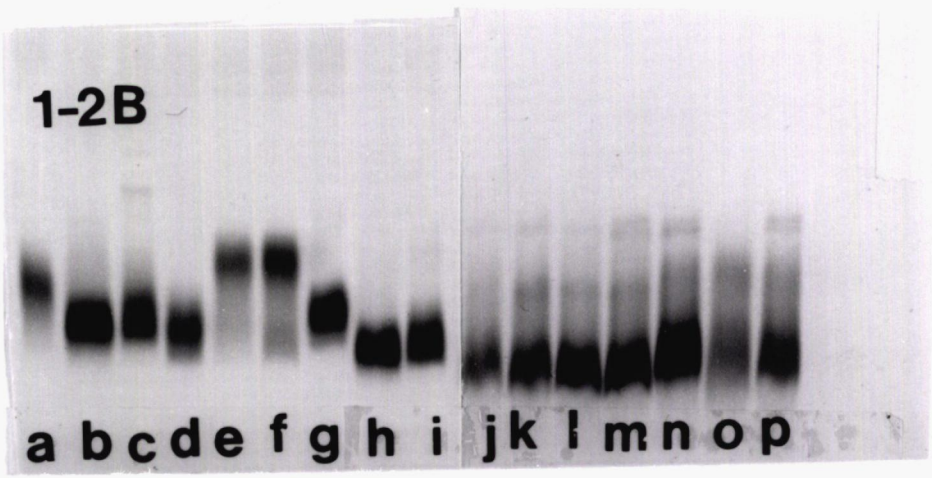
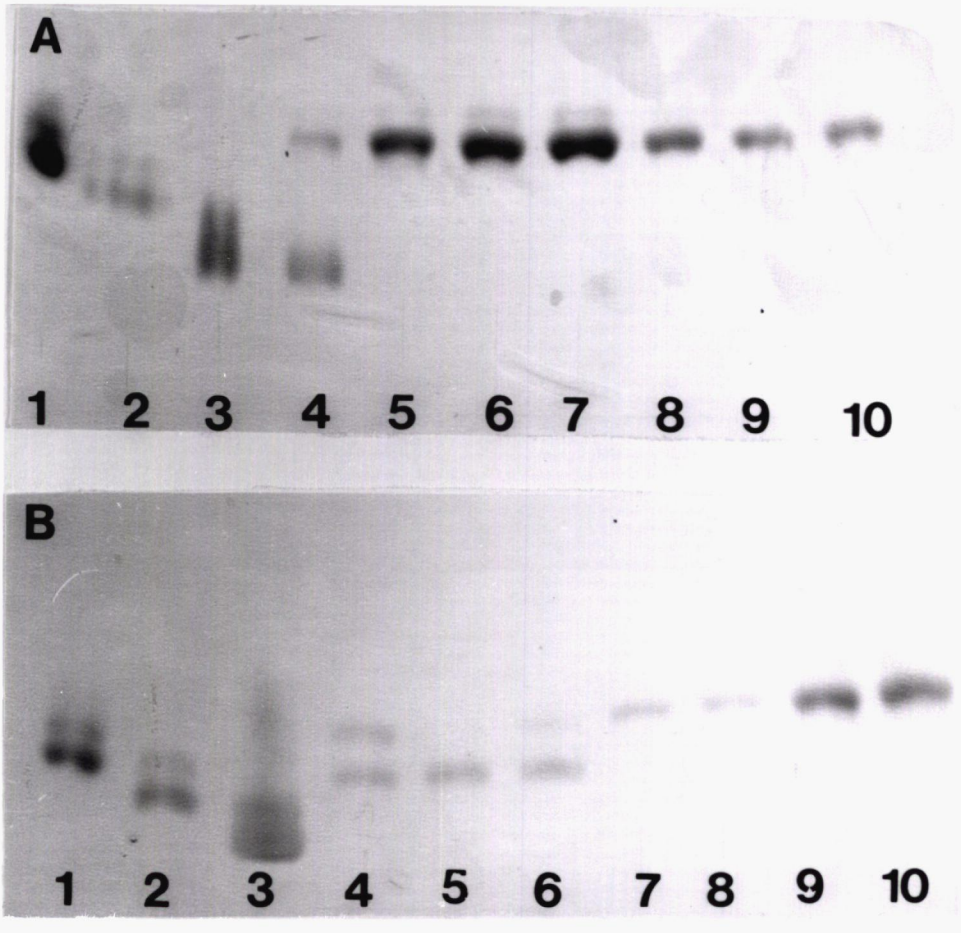


Figure 1-3

Mobility of isolated LPS of mutants of E. coli K-12 carrying plasmids with rfa genes of E. coli or S. typhimurium. The plasmids are pKZ14 (rfaG(K)); pKZ15 (rfaG(S)); pKZ26 (rfaGBIJ(S)); pKZ27 (rfaGBI(S)); pLC10-7 (rfaGBIJ(K)). Whole cell proteinase K digests were prepared and run on 16% polyacrylamide gels and LPS was visualised by the silver staining method of Tsai and Frasch (1982). The first three lanes and the last lane of each gel contain standard LPS preparations for comparison. The LPS of the following strains was loaded in the indicated lane. Fig 1-3A: 1, SL3750 (rfaJ417(S)); 2, SL3748 (rfaI432(S)); 3, SL3769 (rfaG471(S)); 4, SAB2994 (rfaG215(K)), 5, SAB2994/pKZ14; 6, SAB2994/pKZ15; 7, SAB2994/pKZ26; 8, SAB2994/pKZ27; 9, SAB2994/pLC10-7; 10, Chi1715, (rfa^+(K)). Fig 1-3B: 1, SL3750 (rfaJ417(S)); 2, SL3748 (rfaI432(S)); 3, SL3769 (rfaG471(S)); 4, SAB3000 (rfaG215(K)), 5, SAB3000/pKZ14; 6, SAB3000/pKZ15; 7, SAB3000/pKZ26; 8, SAB3000/pKZ27; 9, SAB3000/pLC10-7; 10, Chi1715, (rfa^+(K)).


A
1 2 3 4 5 6 7 8 9 10
B
1 2 3 4 5 6 7 8 9 10

Chapter 2

PHYSICAL AND FUNCTIONAL ANALYSIS OF HYBRID PLASMIDS WHICH ENCODE THE *Escherichia coli* K-12 *rfaG*,*rfaB*,*rfaI* (*rfaM*) AND *rfaJ* GENES FOR GLYCOSYLTRANSFERASE ENZYMES FOR LIPOPOLYSACCHARIDE BIOSYNTHESIS, WHICH HAVE ANALOGOUS ACTIVITIES IN *Salmonella typhimurium* LT2.

INTRODUCTION

The cell envelope of gram negative bacteria is crucial to the organism as a barrier to noxious substances and as a permeable membrane system through which nutrients may enter the cell. Many components (proteins, phospholipids, polysaccharides) interact to maintain the integrity of the envelope. Lipopolysaccharide (LPS), also termed endotoxin, is an essential component of the outer leaflet of the cell membrane. In cells not possessing an extracellular capsule, the LPS matrix is the outermost constituent of the outer membrane and is thus the primary obstacle encountered by environmental agents. LPS has therefore been extensively studied for its role in; a) bacteriophage attachment, b) antibiotic resistance, c) resistance to detergents, d) the conjugational mechanism and in e) pathogen-host interactions.

The polysaccharide structure in LPS consists of three major regions: the Lipid A, the rough (R) core and the O-somatic (antigenic) side chains (Luderitz et al, 1971) (Fig 1-1). This report will emphasise research on the core region. For S. typhimurium only one core type (has been observed, whereas in E. coli five different core structures have been detected, R1, R2, R3, R4 and K-12 (Orskov et al, 1977). All core types in E. coli and S. typhimurium share a common basal structure consisting of the heptose units and

an adjoining glucose residue. A large number of O serotypes of Salmonella have been detected which produce O-specific polysaccharide. Some strains of E. coli (eg E. coli O111) also attach the O polysaccharide. The reported structures for the LPS of E. coli K-12 and S. typhimurium LT2 are shown in Fig 1-1. Two major differences exist in the LPS structures of these two species; E. coli K-12 lacks the O-specific side chains and it has a glucose II residue in the core region in place of the galactose II residue of S. typhimurium. Since the two species are closely related the differences observed in surface structures are of interest from a chemical and genetic standpoint. Genetic studies of this structural heterogeneity should reveal whether the glycosyltransferase enzymes involved in LPS assembly are functionally homologous. If the gene products of the LPS of the two species are similar, it should be possible to create oligosaccharide hybrids by recombining the genetic regions specifying these proteins. Before we can address these questions we need to elucidate the chemical nature of the LPS and the genes which specify LPS synthesis from both species. (The assembly of a set of LPS mutants and description of their structures is described in Chapter 1).

Synthesis of the core region of the LPS is catalysed by a series of cytoplasmic membrane bound glycosyltransferases. These glycosyltransferases are named and described in Table 2-1. The glycosyltransferase nomenclature was proposed by

Creeger and Rothfield (1979) to distinguish similar enzymatic activities in the two species and to indicate the acceptor molecule (in brackets for each enzyme). In addition, we propose the use of the bracketed initials K and S, ie. (K) and (S), to indicate enzymic and genetic activities associated with E. coli K-12 and S. typhimurium, respectively.For example the rfaI activity in S. typhimurium is designated as rfaI(S) in order to distinguish this activity (galactosyltransferase II) from that of the rfaI activity of E. coli (glucosyltransferase II), which is designated rfaI(K).

A series of genes encoding glycosyltransferases involved in LPS biosynthesis in S.typhimurium has been described (Makela and Stocker, 1984). The rfaK,J,I,G, and B genes are organised into the main rfa cluster. The cloning of a number of these genes has recently been reported (Kadam et al, 1985).

A ColE1 hybrid plasmid isolated from a Clarke and Carbon genomic library (Clarke and Carbon, 1976) which contained E. coli K-12 genes which complemented rfaG and rfaI mutants has been reported (Creeger et al, 1979; Creeger and Rothfield, 1979). The genomic library of E. coli K-12 hybrid plasmids was constructed by annealing a population of poly(dA)-tailed fragments (produced by shearing E. coli genomic DNA) to poly(dT)-tailed DNA of the plasmid ColE1. Average size of insert is ca. 12Kb, while the ColE1 vector

is 7Kb (Oka and Takamani, 1976). The hybrid plasmids are efficiently mobilised by F mediated conjugation and can be selected for a gene on the vector which determines colicin E1 resistance (Clarke and Carbon, 1976). Creeger and Rothfield (1979) demonstrated that a plasmid pLC10-7, from that library, had complementing activities for the 1,3-glucosyltransferase I (rfaG(S)) and the 1,3-galactosyltransferase I (rfaI(S)) activities of S. typhimurium, since they converted the bacteriophage sensitivities of the S. typhimurium strains from those which are characteristic of rough strains to those characteristic of smooth strains, and they showed that glycosyltransferase activity could be detected in crude extracts of the complemented cells. In addition they postulated the activity of the E. coli 1,3-glucosyltransferase I (rfaI(K)) in S.typhimurium for this plasmid. The activity of the rfaI gene is particularly intriguing since the acceptor residues in E. coli and S. typhimurium are different and the glycosyltransferase enzymes are also presumably different but the genes involved in coding for these enzymes apparently cross-complement. Creeger and Rothfield (1979) also identified the plasmid pLC17-24 which converted an rfaG(S) mutant from the rough to the smooth state. This plasmid was also found to contain the pyrE$^+$ gene for it complemented a pyrE mutant (P.R.MacLachlan, unpublished data). I have attempted to elucidate the genetic

determination of LPS structure in E. coli K-12 by characterising the functional genetic units of the plasmid pLC10-7, in both E. coli K-12 and S. typhimurium LT2 LPS mutants affected in genes for LPS synthesis. I demonstrate the complementation of four non-identical rfa genes of E. coli K-12 and of four homologous rfa mutations of S. typhimurium. The existence of the rfaG(K), rfaI(S) and rfaI(K) activities suggested by Creeger and Rothfield (1979) is confirmed. In addition the fourth and fifth glycosyltransferase activities are described (the rfaB(S) and rfaB(K) genes for the 1,6-galactosyltransferase activity of S. typhimurium and E. coli K-12, respectively) as well as the sixth and seventh activities to be assigned to this plasmid (that of the rfaJ(S) and rfaJ(K) genes for the 1-3,galactosyltransferase II of S.typhimurium and for the 1-3,glucosyltransferase III of E. coli K-12, respectively). An endonuclease restriction map of pLC10-7 is presented. Subcloning of fragments of pLC10-7 into pBR322 results in two new plasmids: pKZ14, with rfaG(K)+ and rfaG(S)+ activities, and pKZ39, with rfaB(K)$^+$, rfaI(K)$^+$, rfaB(S)$^+$ and rfaI(S)$^+$ activities.

Transposon mutagenesis of the plasmid genes with Tn5 has confirmed the approximate locations of some of the gene activities and has provided a tentative order of the transcriptional units on pLC10-7.

MATERIALS AND METHODS

Bacterial Strains, Plasmids and Cultivation Methods. The bacterial strains and plasmids used are listed in Table 2-1. All strains used were stored in 15% glycerol at -70C and routinely single colony isolated on L-agar (10 g of tryptone [Difco], 10 g of NaCl, 5 g of yeast extract [Difco], 3.5 ml of 1M NaOH, 1 liter of water, 1.5% (w/v) bacto-agar [Difco]) prior to use. Lambda broth, used in the propagation of lambda phage and in transduction experiments using this phage, consisted of: 10 g tryptone, 5 g yeast extract, 5 g NaCl in 1 liter distilled water. The pH was adjusted to 7.0 with 1.7 ml of 1M NaOH prior to autoclaving. L-soft agar was constituted as for L-agar except that the final concentration of Bacto-agar was reduced to 0.75%, while L-broth contained no agar. Minimal medium contained 4.5 g potassium phosphate (monobasic), 10.5 g potassium phosphate (dibasic), 1 g ammonium sulphate, 0.1 g magnesium sulphate, 1 l water. Carbon sources were added to 0.1% w/v as required. Minimal medium with glucose is designated MG. Solid medium was prepared by the addition of Difco Bacto agar at 1.5%.

When necessary, media were supplemented at the following concentrations: amino acids 200 ug/ml, kanamycin sulfate and sodium ampicillin 50 ug/ml, sodium desoxycholate 0.4% (w/v).

Chemicals. Chemicals used in this study were obtained from the following suppliers: ampicillin, agarose, SDS, MOPS, Trizma base, ethidium bromide (Sigma Chemical Co.), ammonium sulphate, magnesium sulphate, calcium chloride, potassium phosphate (Fisher), phenol (Mallinkrodt), restriction endonucleases (used as recommended by the suppliers; Pharmacia, Bethesda Research Labs. and Boehringer Mannheim). DNA molecular weight marker III (Boehringer Mannheim), sodium desoxycholate (Fisher), ligase (Bethesda Research Labs.).

Production of Colicin. Colicin E1 was prepared from strain CL136 (E. coli K-12; (colE1-K30)+ producer) by the method of Foulds and Barrett (1973). Cells of strain CL136 were grown in L-broth to late log phase and mitomycin C added to a final concentration of 2 ug/ml. The culture was reincubated for eight hours. The cells were pelleted by centrifugation and the cell bound colicin was extracted by the addition of 0.1M NaCl in 0.02M potassium phosphate buffer. Solid ammonium sulphate was added to 75% saturation and the precipitate collected by centrifugation. The pellet was resuspended in 10mM potassium phosphate buffer, pH7.0, 1mM sodium azide and 1mM EDTA and stored frozen at -70 C. The activity of the protein was assayed by adding drops at different dilutions to a lawn of cells of HB101, a colicin sensitive indicator strain, on an L-agar plate.

Bacteriophage Propagation. Bacteriophages were grown as follows: an overnight broth culture of phage-sensitive cells was diluted 1:50 with L-broth and phage was added at a multiplicity of infection of 0.1. This mixture was incubated at 37C for twelve hours with gentle shaking. The phage lysates were cleared by centrifugation (12,000 g; 20 min; 4C) and sterilised by the addition of chloroform. Phage lysates were titered by the "drop on lawn" method of Gemski and Stocker (1967). Phages U3 and Ffm were stored frozen at -70C in 15% glycerol, all others were stored at 4C.

Bacteriophage Sensitivity Tests. Bacteriophage sensitivity tests were carried out by the methods of Wilkinson et al (1972) using a battery of phages assembled by B.A.D Stocker and K.E. Sanderson. Two batteries of phages were used. In testing the S. typhimurium strains the smooth-specific phages P22.c2, P22h.c2, 9NA and FO and the rough-specific phages Br60, P221.c2, 6SR, Ffm and C21 were used. E. coli K-12 strains were phage typed with the wild-type specific phage U3 and the rough-specific phage C21. The phage sensitivity patterns were determined by flooding L-agar plates with 1 ml of an overnight broth culture and applying a drop of phage containing approximately 10^8 pfu/ml onto the dried surface. Plates were read after incubation at 37C for four hours.

Mating Methods. Conjugation by F factor mediated transfer of plasmid DNA was done by adding 0.1 ml each of stationary phase donor and recipient cells on an L-agar plate, mixing the cultures and incubating them overnight. Cells were streaked from the mating mixture onto media selective for the transconjugants. Plasmids with a ColE1-vector, such as pLC17-24 and pLC10-7 and derivatives, are mobilizable by F while plasmids derived from pBR322 are not. Derivatives of pBR322 must therefore be transferred by transformation.

Plasmid Isolation, Transformation and Cloning Methods. Large-scale and small-scale plasmid preparations were made by a modification of the method of Birnboim and Doly, as described by Maniatis et al (1982). Ligation of DNA fragments and vector DNA and transformation of competent cells with plasmid DNA was achieved by the methods of Maniatis et al (1982). Restriction endonuclease digests and gel electrophoresis of DNA fragments were done according to the methods of Maniatis et al (1982). DNA fragments were run on 0.7% agarose gels and stained with ethidium bromide for visualisation with a UV-light source. Sizes were calculated from a standard curve using the formula: (Molecular weight) log2/3 vs. mobility (Bearden, 1979), and using lambda-HindIII fragment mobilities as standard sizes. DNA fragments used for cloning were electroeluted from the gels

and prepared for ligation using the methods of Maniatis et al (1982).

Tn5-Mutagenesis Methods .a) Propagation Method. Strain LE392 (E. coli K-12 strain which carries the amber suppressor mutation supE44) was used for propagation of the lambda-delivery phage: (lambda::Tn5:b221 Pam cI857 rex::Tn5 ;Received from D.Weiner, U. of Alberta). The amber suppressor mutation in the LE392 allows expression of the lytic functions of a phage which carries an amber mutation (Pam). LE392 was grown in lambda broth supplemented with 0.1% maltose and 10mM magnesium sulphate at 37C overnight with shaking. 0.1 ml of phage (diluted to give an moi between 1 and 5) was added to 0.1ml of the bacterial cells and adsorbed at room temperature for 30 min. The mixture was added to 3 ml of molten L-soft agar and overlaid on L-plates preheated to 40C. The plates were incubated at 30C until plaques appeared (4-5 hours) at which time 5 ml of L-broth was added and the soft agar removed to a centrifuge tube. After centrifugation at 10,000g for 10min the supernatant was removed and stored in a sterile glass vial with a small amount of chloroform added. The phage lysate was titered by dropping increasing dilutions of phage onto a lawn of LE392 cells on an L-agar plate. Plaque forming units could be counted after incubation at 37C for 4-5 hours.

b) Lambda Tn5 Transduction Method. The plasmid pLC10-7 was

transformed into SAB2906 which was used as the host in the transposon mutagenesis of this plasmid. This strain was grown in lambda broth supplemented with 0.1% maltose and 10mM MgSO4 and tested for sensitivity to lambda-cI857, lambda-vir and lambda-Tn5. SAB2906/pLC10-7 displayed sensitivity to lambda-vir but was not lysed by lambda-Tn5; this indicates that it does not have an amber suppressor mutation, thus blocking the lytic cycle in lambda::Tn5 which has an amber mutation. This strain was subsequentally grown to mid-log phase (1.5-2 hours with shaking) in lambda broth + 0.1% maltose + 10mM magnesium sulphate at 30C, centrifuged to pellet the cells and resuspended in 0.1 ml of 10mM magnesium sulphate. The lambda-Tn5 phage was diluted to give an moi of 1. 0.1 ml of phage and 0.1 ml of cells were added and adsorbed at room temp for 30 min. The mixture was then added to 10 ml of L-broth and grown for 2 hours at 30C. The cells were then pelleted and plated on L-agar containing kanamycin sulfate. Kanamycin-resistant colonies appeared after overnight incubation at 37C. Plasmid DNA was isolated as described and the pooled DNA was transformed into strain HB101 with selection for kanamycin resistance.

c) Mapping of the Site of the Tn5 Insert. A restriction map of transposon Tn5 has been published (Jorgensen et al, 1979). Based on those restriction sites present in the transposon and those in plasmid pLC10-7 (Fig 2-2) the plasmids bearing the inserts were cleaved with the enzymes

BglII, HindIII, SalI and PvuII in individual digests and in double digests following the methods in Maniatis et al (1982) and the manufacturers' recommendations. DNA fragments were visualised and the sizes calculated using the methods described previously.

LPS Methods. Cells grown overnight in 4 ml of L-broth were resuspended in PBS (0.2M Phosphate buffer, pH7.2, 0.9% NaCl) to a final O.D. at 640nm of 1.0. LPS was then isolated by the method of Hitchcock and Brown (1983). One and one half mls of the cell suspension was pelleted in a microfuge and solubilised in 50 ul of lysing buffer (2% SDS, 4% mercaptoethanol, 1M Tris-HCl, pH6.8, 0.1% bromophenol blue). This mixture was heated at 100C for 10 min, then 25ul of proteinase K was added in 10 ul lysing buffer and incubated at 60C for 120 min. Before loading on a 15% SDS-polyacrylamide gel, the samples were again heated at 100C for 5 min. The SDS-PAGE gels were run according to the discontinous buffer system of Laemmli (1970). The LPS was visualised by silver staining using the methods of Hitchcock and Brown (1983). Gels were fixed overnight in 25% (v/v) isopropanol in 7% (v/v) acetic acid. LPS was oxidised for 5 min using 1.05 g periodic acid in 150 ml distilled water plus 4 ml 25% (v/v) isopropanol in 7% (v/v) acetic acid. Each gel was washed six times for 30 min with 200 ml distilled water. Silver stain (28 ml 0.1M NaOH, 1 ml

concentrated ammonium hydroxide, 5 ml 20% silver nitrate, 115 ml distilled water) was added for 10 min. The gels were washed four times with 200 ml distilled water and developed until bands appeared with 50 mg citric acid monohydrate, 0.5 ml 35% formaldehyde in 1 l distilled water. The process of development was stopped by washing the gels in 200 ml distilled water plus 10 ml 7% (v/v) acetic acid.

RESULTS

The activities assigned to the pLC10-7 plasmid by Creeger and Rothfield (1979) included the rfaG(K) and rfaG(S). In addition Creeger and Rothfield (1979) postulated the activity of the rfaI(S) and rfaI(K) genes for this plasmid. We confirm these three activities by complementation analysis of this plasmid in LPS mutants of E. coli and S.typhimurium. Strain SGSC360 which carries pLC10-7 was conjugated with each of the E. coli mutants (SAB2994 (rfaG215), SAB2995 (rfaJ216) and SAB3000 (rfaI221)) using ED1714 as the source of the mobilizing F factor in a triparental cross. Transconjugants were selected on MG supplemented with histidine, proline and colicin E1. When tested with the E. coli battery of phages all three mutants bearing the plasmid showed the Ra (wild-type) phage sensitivities. It is inferred that the pLC10-7 plasmid has the activities: rfaG(K), rfaI(K), and rfaJ(K) (Table 2-3).

Complementation analysis of S.typhimurium mutants was hampered by the fact that Salmonella LT2 strains are colicin resistant. In order to overcome this problem a transposon Tn5 carrying the kanamycin resistance determinant was inserted in this plasmid (see Functional Analysis, below).

Determination of a Restriction Map of pLC10-7. Using a series of single and double restriction enzyme digests of

(rfaI432) but produces a change to wild-type sensitivities in strains SL3750 (rfaJ417), SL3769 (rfaG471) and SL4807 (rfaB707). It is inferred that the rfaI gene alone is inactivated. The plasmid pKZ46 returns SL3769 (rfaG471), SL3750 (rfaJ417) and SL3748 (rfaI432) to smooth phage type but strain SL4807 (rfaB707) into which the plasmid was conjugated had rough phenotype (Table 2-4). Thus the inserted Tn5 in pKZ46 is inferred to inactivate the rfaB gene only. The Tn5 insert in pKZ47 results in loss of complementing activity when the plasmid is transformed into SL3769 (rfaG471) and SL4807 (rfaB707) but has retained complementing activity in strains SL3748 (rfaI432) and SL3750 (rfaJ417), indicating inactivation by the inserted Tn5 of both the rfaG and rfaB genes. The insertion in pKZ53 allows complementation of SL3748 (rfaI432), SL3769 (rfaG471) and SL4807 (rfaB707) but has no effect on the mutant phenotype of SL3750 (rfaJ417), indicating an inactivated rfaJ gene. In summary, pLC10-7 with Tn5 inserts at different locations have the following complementation activities: pKZ45(pLC10-7::Tn5) is rfaG+B+I-J+; pKZ46(pLC10-7::Tn5) is rfaG+B-I+J+ ; pKZ47(pLC10-7::Tn5) is rfaG-B-I+J+ ;pKZ48(pLC10-7::Tn5) is rfaG+B+I+J+ and pKZ53(pLC10-7::Tn5) is rfaG+B+I+J-. The postulated gene activities of each of the Tn5 inserts is shown in Table 2-4.

The positions of the individual inserts based on a series of single and double restriction enzyme digests is

summarized in Fig 2-2. Based on the insert location and the gene(s)-inactivation data the inferred position of the gene loci are also indicated (Fig 2-2). Since the transposon can have polar effects on neighbouring genes it is not possible to indicate what portion (ie promoter or structural genes) of the operon contains the insert.

Construction of pKZ14 and pKZ39. The 4.8Kb (BglII-C) and the 5.3Kb (BglII-B) fragments of pLC10-7 (Fig 2-2) were electroeluted from agarose gels and ligated into the BamHI site of pBR322 to give recombinant plasmids pKZ14 and pKZ39 respectively. Both plasmids were checked for insert size by cutting with restriction enzyme HindIII. The 5.3Kb BglII-B insert in pKZ39 and the 4.8Kb BglII-C insert in pKZ14, which are contiguous BglII fragments in pLC10-7 (Fig 2-2) are inserted in pBR322 in orientations opposite to each other.

Introduction of plasmid pKZ14 into the E. coli strains SAB2994 (rfaG215), MuR#35 (rfaB224), SAB2995 (rfaJ216) and SAB3000 (rfaI221) resulted in restoration of normal phage sensitivities in SAB2994 (rfaG215) only. The S.typhimurium mutants, SL3748 (rfaI432), SL3750 (rfaJ417), SL3769 (rfaG471) and SL4807 (rfaB707), when transformed with plasmid DNA of pKZ14, displayed complementation for SL3769 (rfaG471) alone. This plasmid is therefore proposed to carry the rfaG gene only.

Transformation of the same E. coli strains with plasmid

DNA of pKZ39 restored wild type phage sensitivity in SAB3000 (rfaI221) and in MuR#35 (rfaB224) only (Table 2-3). Transformation of the S.typhimurium mutants with pKZ39 gave the corrected phage pattern for SL3748 (rfaI432) and for SL4807 (rfaB707). In all cases the uncomplemented recombinants showed the unchanged mutant phenotype (Table 2-3).

DISCUSSION

The rfa cluster of genes which determines genes for LPS synthesis is mapped between the cysE and pyrE on the S. typhimurium linkage map, and the rfaG,B,I,J,K,L,F,C,D genes have been located in this cluster (Makela and Stocker, 1984; Sanderson and Roth, 1983). An allele for the rfaD gene (earlier called the nbsB gene) was located in a homologous genetic location by P1-mediated joint transduction studies between cysE and pyrE (Coleman and Leive, 1979; Coleman, 1983). The seventh edition of the linkage map of E. coli K-12 (Bachmann, 1983) shows two other genes in the rfa region, rfaC and rfaP (based on the work of Beher and Schnaitman (1981). Coleman and Deshpande (1985) have recently mapped a mutant described as rfa-2 (with heptose-deficient LPS) in the same region by transduction.

The published data show that genes for inner core synthesis (rfaC,D,P) are at homologous locations in E. coli K-12 and S. typhimurium LT2. Mutations affecting outer core synthesis have been reported (Sandulache et al, 1984; Hancock and Reeves, 1976; Boman and Monner, 1975) but the exact degree of correspondence between the E. coli and S. typhimurium genes had not been shown. There was, however, evidence that E.coli K-12 has genes which are functionally homologous to S. typhimurium genes. Creeger and Rothfield (1979) showed that the plasmid pLC10-7 from the Clarke and

Carbon library would complement rfaG mutants of S.typhimurium, and that this plasmid also amplified enzymatic activities postulated to be associated with the rfaI gene and Creeger and Rothfield (unpublished data) showed that pLC10-7 will complement rfaI and rfaJ mutants of S. typhimurium.

This thesis shows that the rfa cluster of genes of E. coli is homologous to that of S. typhimurium and includes most (though possibly not all) of the genes present in S. typhimurium and also present in E. coli K-12, and that the order of the genes is equivalent.

Creeger and Rothfield (1979) identified the rfaG(S) gene on the chromosomal segments present in the Clark and Carbon plasmids pLC10-7 and pLC17-24. However they did not determine the map location of the rfa genes on the chromosome of E. coli, or the sequence of the genes on the E. coli map. The plasmid pLC17-24 also contains the pyrE marker (P.R.MacLachlan, unpublished data), suggesting linkage of the rfaG and pyrE genes. We have confirmed the presence of the rfaG(K) gene on pLC10-7, as predicted by Creeger and Rothfield (1979), and in addition we have indicated the presence, on the chromosomal fragment of pLC10-7, of the rfaB(K), rfaI(K) and rfaJ(K) genes. We infer therefore the linkage of each of these genes to the pyrE locus (Fig 2-1). The location of the inserts in pKZ14 and pKZ39 are indicated in relation to pLC10-7, from which they

were derived. The rfaD(K) gene has been shown by transduction to be closely linked to the cysE locus (Coleman and Deshpande, 1985). The rfaD(K) and rfaF(K), along with the rfaC(S), rfaD(S) and rfaF(S) defective loci are complemented by the plasmid pLC13-13 (L.V.Collins, unpublished data), indicating that these loci are closely linked both to one another and to cysE.(The pLC13-13 plasmid is discussed in detail in Chapter 3). The above data suggests the order of rfa loci for E. coli K-12 as cysE - rfa(C,D,F) - rfaJ - rfaI - rfaB - rfaG - pyrE. This order resembles that obtained for S.typhimurium: cysE - rfaF - rfa(K,L) - (rfaJ,I,B) - rfaG - pyrE (Kuo and Stocker, 1972; Sanderson and Saeed, 1972a; Sanderson and Saeed, 1972b; Kadam et al, 1985) The proposed order of the rfa genes of E. coli K-12 between pyrE and cysE and the postulated sites of activity of the encoded enzymes in the process of LPS core assembly are outlined in Fig 2-1.

The restriction map of pLC10-7 including the subcloned fragments comprising pKZ14 and pKZ39 is included in Fig 2-2. The pLC10-7 plasmid in the linear conformation is approximately 22Kb in size; this indicates that the insert of E. coli DNA is of approx. 15Kb. pLC10-7 was used as a source of DNA to subclone E. coli K-12 genes which complement defective loci in both E. coli K-12 and S. typhimurium. Complementation was determined by the fact that recombinants carrying complementing plasmids had the

wild-type phage sensitivity and had LPS mobilities in SDS-PAGE identical to that of LPS isolated from wild-type strains (Fig 1-3).

All the complementing activities attributed to the insert of E. coli DNA in pLC10-7 are present in pKZ48 which has the Tn5 insert in the ColE1 sequences (Fig 2-2). This insert in the pLC10-7 plasmid was constructed to facilitate selection of recombinants in the S.typhimurium strains, which are colicin resistant. The 4.3Kb BglII-C fragment of pLC10-7 (Fig 2-2) which was used to construct pKZ14 contained the rfaG(K) and the rfaG(S) activities (Fig 2-1; Table 2-3). The 5.3Kb BglII-B fragment (Fig 2-2), which comprises the E. coli DNA in pKZ39, contained the rfaI(K), rfaB(K), the rfaB(S) and the rfaI(S) activities (Fig 2-1; Table 2-3).

Further evidence for the presence of rfa genes on these fragments is provided by the Tn5 inserts. In Fig 2-2 the restriction enzyme sites indicated above the line are those which were used in mapping the position of the Tn5 inserts The insert in pKZ45 is within the 5.3Kb BglII-B fragment (Fig 2-2) and inactivates the rfaI(K) and rfaI(S) genes without affecting the rfaG(K), rfaG(S), rfaB (K), rfaB(S), rfaJ(K) or rfaJ(K) activities (Table 2-4). Plasmid pKZ46 has an insertion which completely inactivates the rfaB locus locus but has no effect on the rfaG or rfaJ, and rfaI complementing activities (Table 2-4). The location of the

Tn5 element in pKZ46 is within the same 5.3Kb BglII-B fragment as pKZ45, but on a different HindIII fragment as indicated in Fig 2-2. The insert in pKZ47 maps within the 4.3Kb BglII-C fragment (Fig 2-2) and has retained ability to complement rfaI and rfaJ mutants of both E. coli and S.typhimurium, but has lost ability to complement rfaG and rfaB mutants (Table 2-4). The Tn5 in plasmid pKZ53 maps within the 9.4Kb BglII-A fragment (Fig 2-2) and affects only the rfaJ(K) and rfaJ(S) activities (Table 2-4).

The rfaG gene has been postulated to be the structural gene for S.typhimurium glucosyltransferase I (Makela and Stocker, 1981) and has been shown to map between the cysE and pyrE genes (Kuo and Stocker, 1972). In E. coli K-12 the analogous enzymatic activity is that of the E. coli glucosyltransferase I (Fig 1-1). Two ColE1 plasmids carrying E. coli genes capable of complementing rfaG mutants of S.typhimurium, pLC10-7 and pLC17-24, have been identified (Fig 2-2). The location of both the rfaG(S)+ and pyrE+ activities on pLC17-24 (P.R.MacLachlan, unpublished data) confirms that the rfa cluster of genes is close to the pyrE locus (Creeger and Rothfield, 1979) (Fig 2-1). We have confirmed, in this study, both the E. coli and S. typhimurium rfaG complementing activities for pLC10-7, since this plasmid is capable of restoring wild-type phage sensitivities to these mutants (Table 2-3). It seems likely

that the rfaG gene is functionally homologous in both species. This is confirmed by the fact that the subcloned 4.3Kb fragment of pLC10-7 in pKZ14 has rfaG(K) and rfaG(S) activities i.e. the rfaG gene of E. coli (on pKZ14) complements the rfaG mutant of S.typhimurium and the rfaG mutant of E. coli K-12 (Table 2-3). Extracts from the S.typhimurium mutant SL3769 (rfaG471) have been shown to lack ability to transfer glucose from UDP-glucose to LPS core stubs, but extracts from strains carrying the plasmid pKZ15 (pBR322-rfaG+(S)) had this activity amplified ca. five-fold over the level found in wild-type strains (Kadam et al, 1985). Similarly, pLC10-7 (Col E1-rfaG+(K)) restored and amplified glucosyltransferase I activity in an S.typhimurium rfaG strain (Creeger and Rothfield, 1979).

The rfaB gene was initially identified in S.typhimurium on the basis of resistance to Felix O phage (Hudson et al, 1978). Wollin et al (1983) demonstrated that the defective enzyme in rfaB(S) mutants was UDP-D-galactosyltransferase (S. typhimurium galactosyltransferase I: formerly called galactosyltransferase II, but changed to correspond to E. coli K-12 and to conform with Creeger and Rothfield (1979)). The rfaB(S) gene is situated between the cysE and pyrE (Hudson et al, 1978). Plasmids bearing the rfaB(S) gene have been isolated (Kadam et al ,1985). Creeger et al (1984) observed an increase in galactosyltransferase levels in strains carrying pLC10-7 and postulated that this was due to

the presence of the rfaB gene on this plasmid. We have used the Tn5-derivative of pLC10-7, pKZ48, to test the hypothesis that pLC10-7 has an rfaB activity. Plasmid pKZ48 restored the phage phenotype of SL4807 (rfaB707) to that of wild-type (Table 2-4) and is thus postulated to carry the structural gene for the S. typhimurium galactosyltransferase II. Identification of the rfaB(K) activity on the pKZ39 plasmid was carried out by transformation of an rfaB mutant of E. coli (SAB3063); plasmid pKZ39 has both the rfaI and rfaB activities. Only the rfaB(K) and rfaB(S) activities are fully inactivated in the plasmid pKZ46 while in pKZ45 only the rfaI(K) and rfaI(S) activities have been totally eliminated by the insertion (Table 2-4). Therefore these plasmids may be employed to distinguish between rfaB and rfaI mutants. The activities of rfaB in both species are possibly similar since both enzymes recognise the same glucosyl acceptor. It seems likely that the 1,6-galactose is added in S.typhimurium before the 1,3-galactose (Creeger and Rothfield, 1979) however the absence of the side-chain galactose I residue does not seem to inhibit the addition of the remainder of the core or the O antigen. One major difference exists between rfaB mutants of S.typhimurium and E. coli K-12; whereas in S.typhimurium rfaB mutants retain their smooth character, in E. coli there does not appear to be any of the wild-type LPS. This may be due to the fact that there is a stringent specificity requirement for the

gene product of rfaI(K) which prevents addition of the glucose II residue in the absence of the galactose I side-chain.

S.typhimurium rfaI (rfaI(S)) mutants produce LPS of chemotype Rb3 and are defective in the galactosyltransferase I activity (Kadam et al, 1985) (Fig 1-1, Chapter 1). The rfaI(S) locus has been localized on plasmids (Kadam et al, 1985). Previously Creeger and Rothfield (1979) demonstrated rfaI(K) activity for pLC10-7 in S.typhimurium (which they termed rfaM activity). By transforming an rfaI(K) mutant (SAB3000 rfaI221) it is demonstrated here that pLC10-7 carries complementing activity which restores wild-type phage sensitivity (Table 2-3) and wild-type LPS mobility (Fig 1-3, Chapter 1). This indicates that the gene responsible for the E. coli 1-3,glucosyltransferase is present. The rfaI gene(s) present on pKZ39 plasmid have two different activities: complementing mutants defective in 1-3,galactosyltransferase in S.typhimurium and mutants lacking the 1-3,glucosyltransferase activity in E. coli (ie. the rfaI(S) and rfaI(K) activities respectively) (Table 2-3). The appearance of both these activities on a plasmid carrying E. coli genes suggests that pLC10-7 carries both the rfaI(K) and rfaI(S) alleles. It is surprising to find cross-complementation for two genes which have very different activities for their gene products.

Three models are put forward here in an attempt to explain the unexpected cross-complementation of rfaI(K) and rfaI(S) genes. If the specificity of the enzyme resides in a primary structure determined by the rfaI gene, then the rfaI(K) gene encodes a glucosyltransferase and rfaI(S) encodes a galactosyltransferase (Model A, Fig 2-3). Explanation of the cross-complementation in this case would require a highly relaxed specificity of each glycosyltransferase for the sugar acceptor on the LPS. When transferred on a plasmid into an rfaI(S) mutant, the rfaI(K) encoded glucosyltransferase II for example would add a glucose residue to the glucose I on the S. typhimurium core where normally a galactose unit was added by the rfaI(S) encoded galactosyltransferase II. This complete absence of acceptor specificity would produce LPS structures of extreme heterogeneity. This heterogeneity is not observed however in chemical analyses.

The second model is depicted in Fig 2-3B. This model proposes that there are two allelic forms of the rfaI gene in each species which have diverged during evolution. The rfaI(K) and rfaI(S) alleles in this case would produce intracellular levels of both the glucosyltransferase II and the galactosyltransferase II. The activity of the two enzymes has been demonstrated in vivo in E. coli (Creeger and Rothfield, 1979). This led to the postulate that the absence of the structure associated with the

galactosyltransferasese II activity of S.typhimurium in the LPS of E. coli was reflected in a greater efficacy on the part of the E. coli glucosyltransferase II to bind the acceptor than that of the (rfaI(S) encoded) galactosyltransferase II (Creeger et al, 1979).

The third model (Fig 2-3C) put forward here proposes the existence of a proenzyme, the product of a gene equivalent to both the rfaI(K) and rfaI(S) genes. The proenzyme takes the form of a glycosyltransferase which is modified by a species-specific cofactor. This cofactor is encoded by a gene distal to the rfaI gene. Two allelic forms of the cofactor must exist: in E. coli the $rfaI(K)_C$ converts the glycosyltransferase to the glucosyltransferase II; in S. typhimurium the $rfaI(S)_C$ converts the inactive glycosyltransferase to the active galactosyltransferase II. This model is effective in explaining the cross-complementation observations; however nothing is known about the nature of the putative cofactors. The "cofactor" may in fact represent a conformational change in the glycosyltransferase to recognise a different UDP-sugar donor.

There is some precedent for the type of cofactor process described in the third model. Hill and coworkers (1975) discovered a system of regulation in the mammalian lactose synthesis system. The enzyme lactose synthetase catalyses the conversion of UDP-galactose and D-glucose to

lactose. The lactose synthetase is composed of two components; the Protein A component is only weakly active with D-glucose and usually catalyses the reaction involving conversion of UDP-galactose and N-acetyl-D-glucosamine to acetyllactosamine; Protein B has no catalytic activity but when complexed with Protein A, it induces Protein A to utilise D-glucose as substrate rather than N-acetyl-D-glucosamine. The end product is therefore lactose instead of acetyllactosamine.

Differentiation between the models outlined above may be possible when the identity of the sugar incorporated into the LPS when an rfa gene on a plasmid is introduced into a cell which carries a mutation in the analogous gene for that species and complements the LPS defect, is known. Analysis of the LPS from strains with a mutation in rfaI(S) carrying a plasmid which has the rfaI(K) gene, for example, with NMR techniques to identify the units of the polysaccharide would identify which glycosyltransferase was active. It is possible to identify indirectly, by crude extraction of total cell glycosyltransferases, which glycosyltransferases are present; however incorporation experiments using enzymes in cell extracts do not differentiate LPS-specific glycosyltransferases. Purification of the glycosyltransferase protein and dissociation of subunits might provide evidence for a conformational change in one sub-unit leading to altered enzyme specificity.

Mutants of the rfaJ class in S. typhimurium are defective in the addition of the glucose II residue to the core and rfaJ is assumed to be the structural gene for glucosyltransferase II (Makela and Stocker,1984). The analogous activity for rfaJ in E. coli K-12 is for the glucosyltransferase III enzyme (Fig 1-1, Chapter 1). Restoration to wild-type of rfaJ mutants of both S.typhimurium and E. coli K-12 by pLC10-7 (Table 2-3) indicates the presence of both the rfaJ(K) and rfaJ(S) activities on this plasmid. A plasmid, pKZ26, bearing the rfaJ(S) gene of S.typhimurium (Kadam et al, 1985) shows similar complementation of both species (Table 1-4, Chapter 1). There is evidence therefore that the rfaJ gene of both species is either identical or that the genes though not identical have functionally homologous gene products. The acceptor residues in the LPS for the rfaJ(K) and rfaJ(S) are glucose II and galactose II, respectively. It is not clear how the gene product of the rfaJ gene(s) on pLC10-7 recognises a different acceptor residues on the LPS structures of S.typhimurium and E. coli.

The transposon gene inactivation data are summarised in Table 2-4. The Tn5 inactivation experiments indicate that an insertion in the ColE1 sequences has no effect on the gene activities associated with the original pLC10-7 plasmid. The insert in pKZ47 has lost the rfaG(K) and rfaG(S) but has had

no effect on the other activities . The pKZ53 plasmid has an insert which has lost activity of the rfaJ(K) and rfaJ(S) but which has normal activity of the other loci. Inserts in the rfaI locus do not block the rfaB activity and conversely inserts which inactivate rfaB have no polar effect on the rfaI gene. Inserts in rfaG have polar activity on the rfaB locus but not on any of the other genes; an insert in either rfaI or rfaB have no effect on each other or on any of the other gene activities. When taken together the data suggests that the four gene activities may be grouped into at least three transcriptional units. The order of genes appears to be rfaG - rfaB - rfaI - rfaJ. There is preliminary evidence for three operons with rfaG and rfaB in the first operon, rfaI in the second operon and rfaJ in a possible third operon (this proposed order of transcriptional activity closely resembles the order of LPS core assembly); we have however no evidence as yet on the directionality of the genes.

Table 2-1. Nomenclature for some of the glycosyltransferase enzymes for the synthesis of the LPS core region in *E. coli* and *S. typhimurium* LT2.[a]

Genotype[b]	Name of Glycosyltransferase[c]	Abbreviated Name[d]	LPS Chemotype[e,f]
E. coli K-12			
rfaG(K)[f]	UDP-glucose:(heptosyl)lipopolysaccharide 1,3-glucosyltransferase	*E. coli* glucosyltransferase I	Rd1(K)
rfaB(K)	UDP-galactose:(glucosyl)lipopolysaccharide 1,6-galactosyltransferase	*E. coli* 1,6-galactosyltransase I	rfaB(K)
rfaI(K)	UDP-glucose:(glucosyl)lipopolysaccharide 1,3-glucosyltransferase	*E. coli* glucosyltransferase II	Rb3(K)
rfaJ(K)	UDP-glucose:(glucosyl)lipopolysaccharide 1,2-glucosyltransferase	*E. coli* glucosyltransferase III	Rb2(K)
S. typhimurium			
rfaG(S)	UDP-glucose:(heptosyl)lipopolysaccharide α1,3-glucosyltransferase	*S. tm.* glucosyltransferase I	Rd1(S)
rfaB(S)	UDP-galactose:(glucosyl)lipopolysaccharide α1,6-galactosyltransferase	*S. tm.* α1,6-galactosyl transferase	rfaB(S)
rfaI(S)	UDP-galactose:(glucosyl)lipopolysaccharide α1,3-glucosyltransferase	*S. tm.* α1,3-galactosyl transferase	Rb3(S)
rfaJ(S)	UDP-glucose:(galactosyl)lipopolysaccharide 1,2-glucosyltransferase	*S. tm.* glucosyltransferase II	Rb2(S)

....continued

Table 2-1 (continued)

[a] The site of action of individual enzymes is indicated in Fig. 1.

[b] Name of gene postulated as structural gene for the glycosyltransferase. The stock numbers of representative strains with mutations in these genes is given in Table 2-2.

[c] Adapted from Creeger and Rothfield (1979). These names were constructed in accordance with the naming system proposed by the International Union of Biochemistry.

[d] Name used throughout text.

[e] Chemotype of attenuated LPS chain produced by a strain carrying the *rfa* mutation.

[f] The letter (K) or (S) indicates that the gene or the chemotype represents *E. coli* K-12 or *S. typhimurium* LT2, respectively.

Table 2-2. Strains used

Strain	Genotype	Source
S. typhimurium LT2		
SL3770	*pyrE*$^{+}$ *rfa*$^{+}$	B. Stocker
SL3748	*rfaI432*	B. Stocker
SL3750	*rfaJ417*	B. Stocker
SL3769	*rfaG471*	B. Stocker
SL4807	*leu-1051 malB479 hisC527 cysI1173 rfaB707* (ColE1-30)$^{+}$	B. Stocker
SA2698	$\Delta his(DCBHAFIE)712_{u}$ *rfaG*/pSLT^{-}/pBR322	This lab
E. coli K-12		
ED1714	*lac(del)X74 trp* sup^{+} lam^{+}/F42 *finP301 lac*$^{+}$	N.S. Willetts
LE392	F^{-} *hsdR514*(r-K, m-K) *supE44 supF58 lacY1 galK2 galT22 metB1 trpR55* lam^{-}	D. Weiner
Mu-R #35	*thr-1 leu-6 thi-1 supE44 lacY tonA21 rfaB224*	D. Kamp
Chi1715	*pro his lac xyl rpsL tsx cycB cycA*	R. Curtiss
SAB2906	*galE15 relA1 rpsL150* lam^{-}	S. Brenner
SAB2995	Isolated from Chi1715, but also *rfaJ216*	This lab

....continued

Table 2-2 (continued)

Strain	Genotype	Source
SAB2994	Isolated from Chi1715, but also *rfaG215*	This lab
SAB3000	Isolated from Chi1715, but also *rfaI221*	This lab
SGSCC360	F^+ *trpE5 thr leu recA* F^+/pCL10-7	L. Rothfield
HB101	F^- *hsdS,(r-B,m-B) recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1 supE44*	J. Duerksen
SAB3002	Same as HB101 with pKZ14 (pBR322-*rfaG*$^+$)	This study
SAB3051	Same as HB101 with pKZ39 (pBR322-*rfaI*$^+$)	This study
SAB3236	Same as HB101 with pKZ45 (pLC10-7::Tn5 (*rfaG*$^+$*B*$^+$*I*$^-$*J*$^+$)),F*lac*$^+$	This study
SAB3237	Same as HB101 with pKZ46 (pLC10-7::Tn5 (*rfaG*$^+$*B*$^-$*I*$^+$*J*$^+$)),F*lac*$^+$	This study
SAB3238	Same as HB101 with pKZ47 (pLC10-7::Tn5 (*rfaG*$^-$*B*$^-$*I*$^+$*J*$^+$)),F*lac*$^+$	This study
SAB3239	Same as HB101 with pKZ48 (pLC10-7::Tn5 (*rfaG*$^+$*B*$^+$*I*$^+$*J*$^+$)),F*lac*$^+$	This study
SAB3080	Same as HB101 with pKZ53 (pLC10-7::Tn5 (*rfaG*$^+$*B*$^+$*I*$^+$*J*$^-$))	This study

Table 2-3. Complementation tests with plasmids pLC10-7, pKZ14 and pKZ39 when tested in *rfa* mutants of *S. typhimurium* LT2 and *E. coli* K-12.

Strain	Partial Genotype[a]	Complementation By:[b] pLC10-7	pKZ14	pKZ39
S. typhimurium LTS				
SL3769	rfaG471	+	+	-
SL4807	rfaB707	N/D	-	+
SL3748	rfaI432	+	-	+
SL3750	rfaJ417	+	-	-
E. coli K-12				
SAB2994	rfaG215	+	+	-
Mu-R #35	rfaB224	+	-	+
SAB3000	rfaI221	+	-	+
SAB2995	rfaJ216	+	-	-
Inferred Genotype of Plasmid:		ColE1-*rfaGBIJ(K)*	pBR322-*rfaG(K)*	pBR322-*rfaBI(K)*

....continued

Table 2-3 (continued)

[a] Partial genotype only. For full genotype, see Table 2-2.

[b] Complementation assessed after transformation or conjugation with individual plasmids by procedures described in Materials and Methods. "+" indicates restoration of mutant phage phenotype to wild-type (U3-sensitivity and C21 resistance for *E. coli*; Ffm-resistance and FO-sensitivity for *S. typhimurium*). "-" indicates no change from *E.coli* mutant phage phenotype (U3-resistance and C21-sensitivity) or from *S. typhimurium* mutant phage phenotype (Ffm-sensitivity and FO-resistance). In all cases at least two recombinant colonies were tested for phage sensitivities and in representative cases the LPS structure inferred from phage sensitivities was confirmed by SDS-PAGE of isolated LPS.

[c] Inferred genotype of plasmid based on complementation patterns of *rfa* mutants with plasmids carrying different *rfa* genes or groups of *rfa* genes.

Table 2-4. Complementation tests with plasmids derived by insertion of transposon Tn5 into pLC10-7 (ColE1-*rfaGBIJ(K)*) when tested in *rfa* mutants of *S. typhimurium* LT2 and *E. coli* K-12

Strain	Partial Genotype[a]	Complementation By:[b]				
		pKZ48	pKZ47	pKZ46	pKZ45	pKZ53
S. typhimurium LT2						
SL3769	*rfaG471*	+	-	+	+	+
SL4807	*rfaB707*	+	-	-	+	+
SL3748	*rfaI432*	+	+	+	-	+
SL3750	*rfaJ417*	+	+	+	+	-
E. coli K-12						
SAB2994	*rfaG215*	+	-	+	+	+
Mu-R #35	*rfaB224*	+	-	-	+	+
SAB3000	*rfaI221*	+	+	+	-	+
SAB2995	*rfaJ216*	+	+	+	+	-
Inferred Genotype of Plasmid:		ColE1::Tn5 *rfaGBIJ (K)*	ColE1::Tn5 *rfaIJ (K)*	ColE1::Tn5 *rfaGIJ (K)*	ColE1::Tn5 *rfaGBJ (K)*	ColE1::Tn5 *rfaGBI (K)*

....continued

Table 2-4 (continued)

a
Partial genotype only. Full genotype is listed in Table 2-2.

b
Complementation of LPS mutants assessed after conjugation with listed plasmids and selection for Kanamycin resistance. "+" indicates restoration of *E.coli* K-12 wild-type phage sensitivities (i.e. U3-sensitivity and C21-resistance) or *S. typhimurium* wild-type phage sensitivities (FO-sensitivity and Ffm-resistance). "-" indicates no change from mutant phage phenotype (U3-resistance and C21-sensitivity for *E. coli* and FO-resistance, Ffm-sensitivity for *S. typhimurium* strains).

c
Assignment of *rfa* genes to individual plasmids based on ability to complement mutants carrying the defective gene.

Figure 2-1

The Structure of the LPS of *E. coli* K-12 and chromosomal region encoding enzymes involved in biosynthesis of the core lipopolysaccharide. The *rfaG,B,I,J,F,C* and *D* genes are postulated to encode glycosyltransferases and their proposed positions in the *cysE* - *pyrE* region of the chromosome of *E. coli* K-12 at 81 min. (Bachmann, 1983) are indicated. The order of the *rfaC,D,F* region has not yet been fully clarified (Chapter 3).

Plasmids carrying portions of the *cysE* - *pyrE* region are depicted at the bottom of the figure by bars which cover the genes which are carried on the plasmid. The plasmids are not shown to scale of the amount of *E. coli* K-12 DNA carried. They are further described in the text of Chapters 2 and 3.

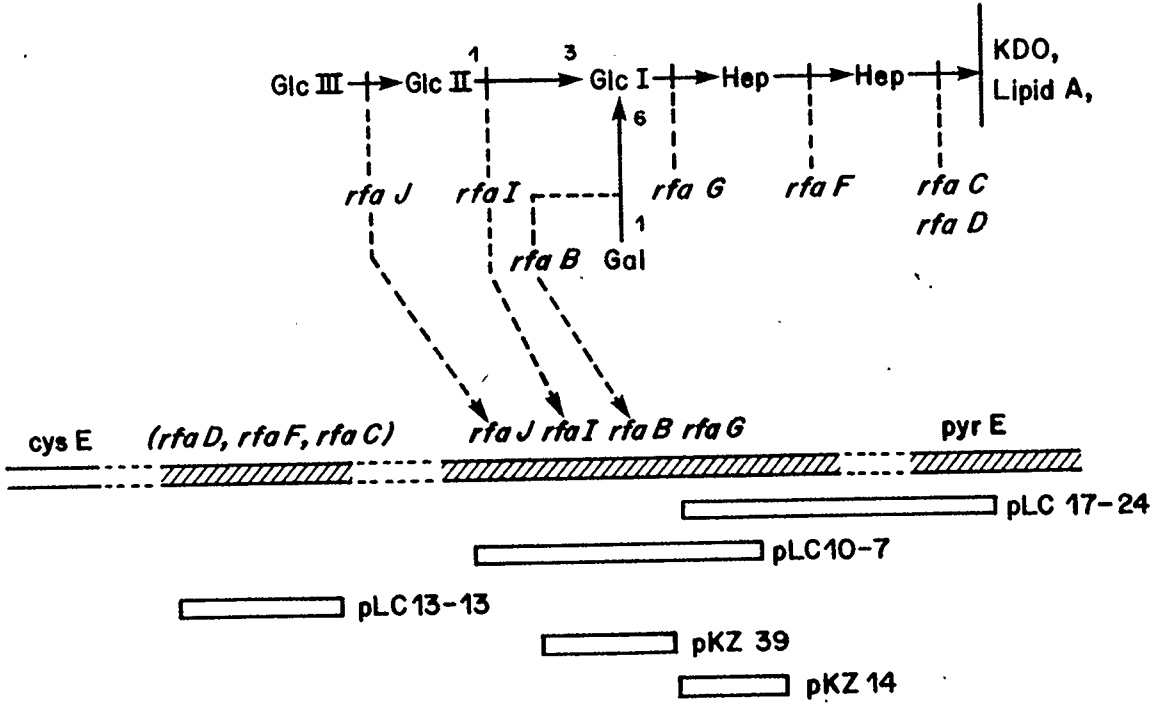

Glc III
Glc II
Glc I
Hep
Hep
KDO,
Lipid A,
1
3
6
1
rfa J
rfa I
rfa G
rfa F
rfa C
rfa D
rfa B
Gal
cys E
(rfa D, rfa F, rfa C)
rfa J rfa I rfa B rfa G
pyr E
pLC 17-24
pLC10-7
pLC13-13
pKZ 39
pKZ 14

Figure 2-2

Restriction Map of pLC10-7 (ColE1-rfaG,B,I,J(K)). The restriction enzymes represented on the top of the figure are: B, BglII; H, HindIII; P, PvuII. The symbol (▽) indicates sites of insertion of the transposon Tn5 in the plasmids pKZ45, pKZ46, pKZ47, pKZ48 and pKZ53. The locations of the individual gene activities shown below the map are based on the inactivation of complementation ability which results from each insert. The BglII fragments (A-D) are indicated at the top and correspond to the order of sizes of the BglII fragments in pLC10-7. The BglII-C fragment and the BglII-B fragment have been subcloned to pBR322 to produce pKZ14 and pKZ39 respectively (see Fig 2-1). The ColE1 sequences are indicated by hatched lines. Plasmid pKZ48 results from an insertion of Tn5 into the portion of pLC10-7 which controls colicin immunity, thus strains carrying this plasmid are colicin sensitive. The map distances in kilobases (Kb) are indicated on scale at the bottom.

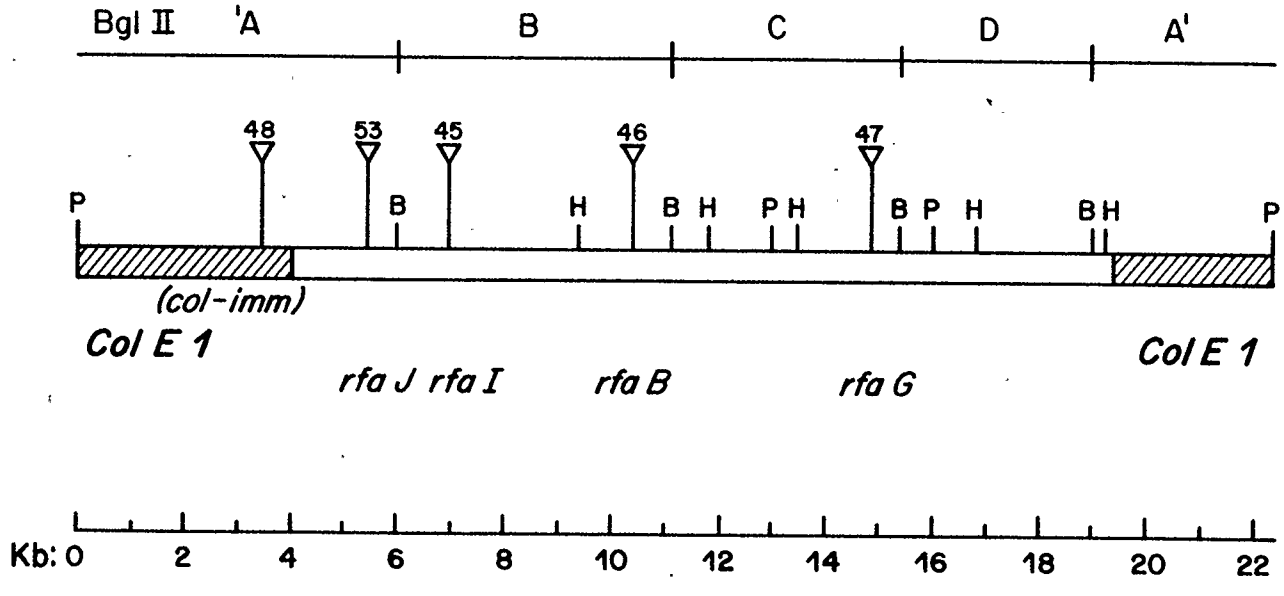
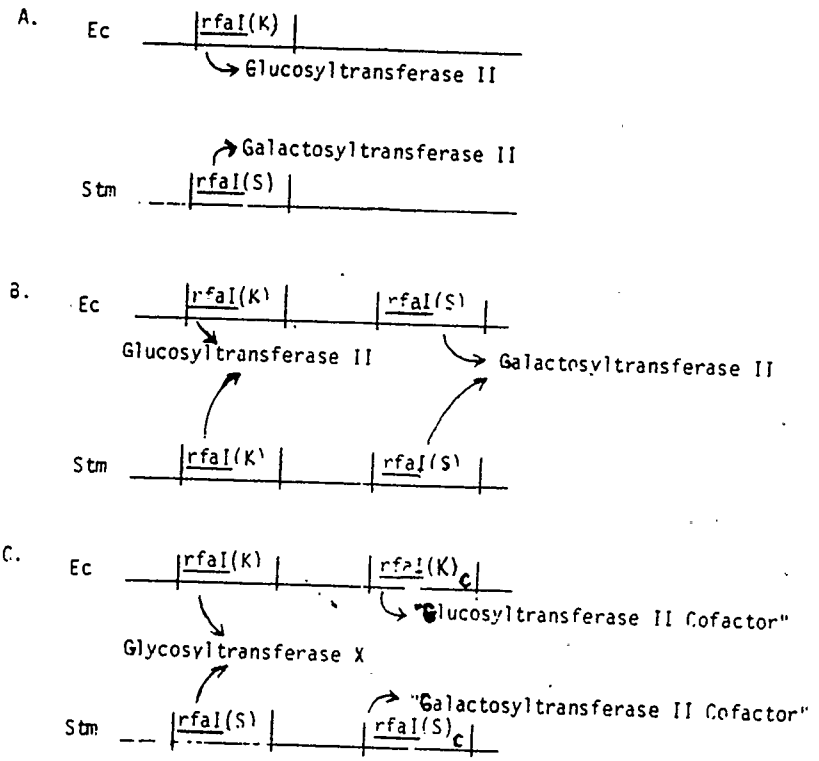

Bgl II
'A
B
C
D
A'
48
53
45
46
47
P
B
H
B H
P H
B P H
B H
P
(col-imm)
Col E 1
Col E 1
rfa J rfa I
rfa B
rfa G
Kb: 0 2 4 6 8 10 12 14 16 18 20 22

Figure 2-3

Proposed models for the rfaI gene activities in E. coli K-12 and S. typhimurium LT2. Horizontal lines indicate chromosomal regions of E. coli K-12 (Ec) or S. typhimurium (Stm) with the rfaI genes indicated between vertical lines. Arrows indicate the presumptive end product of each gene. The models A, B, and C are evaluated in the Discussion section, Chapter 2.


A.
Ec
rfaI(K)
Glucosyltransferase II
Galactosyltransferase II
Stm
rfaI(S)
B.
Ec
rfaI(K)
rfaI(S)
Glucosyltransferase II
Galactosyltransferase II
Stm
rfaI(K)
rfaI(S)
C.
Ec
rfaI(K)
rfaI(K)c
"Glucosyltransferase II Cofactor"
Glycosyltransferase X
"Galactosyltransferase II Cofactor"
Stm
rfaI(S)
rfaI(S)c

Chapter 3

PHYSICAL AND FUNCTIONAL ANALYSIS OF pLC13-13, A HYBRID PLASMID ENCODING THE rfaC, rfaD, AND rfaF GENES OF Escherichia coli K-12, INVOLVED IN BIOSYNTHESIS OF THE DEEP CORE REGION OF THE LIPOPOLYSACCHARIDE OF ENTERIC BACTERIA.

Introduction

It has become increasingly evident that the cell envelope of gram negative bacteria is essential to the physiology of the organism in providing a protective barrier. Many antibiotics which are lethal to gram positive bacteria are ineffective against gram-negatives except at high concentrations (Tamaki et al, 1971). The barrier function is especially important to the gut colonizing bacteria which must resist the action of bile salts and digestive enzymes (Nikaido and Nakae, 1979). In addition the outer membrane presents a strong permeability barrier to many antimicrobial macromolecules, especially antibiotics.

Strains harbouring defects in the deep core region of the LPS, the portion containing heptose (the mutants are often called "deep rough" mutants), are seriously impaired, having lost much of their outer membrane and periplasmic protein content in addition to having increased susceptibility to a variety of hydrophobic compounds and to the complement component of serum. For these reasons these mutants are valuable in studying outer membrane structure and function and membrane permeability phenomena. It has been reported that mutations affecting the biosynthesis of the LPS increase the permeability of the cell to a variety of compounds :antibiotics (Tamaki et al, 1971), deoxycholate (Sanderson et al, 1974), dyes (Leive, 1974), polycyclic mutagens (Ames et al, 1973), and predisposes the cell to

leakage of periplasmic enzymes (Lopes et al, 1972; Chatterjee et al, 1976; Lindsay et al, 1972). Increased permeability to hydrophobic agents has been demonstrated for a group of mutants of E. coli lacking heptose and other sugars from the LPS (Tamaki and Matsuhashi, 1974). Deep rough strains have been used in screening of mutagenic and carcinogenic compounds (Ames et al, 1973) and in the detection of trace amounts of antibiotics in fermentations (Brown, 1981). Since LPS is a major component of the outer membrane of gram negative bacteria, strains with defective LPS are expected to have a debilitated membrane function. For example, flagella are not produced in deep rough mutants while they are still present in galE mutants (Ames et al, 1974). The importance of the phosphate diester bridges in maintaining a cell surface resistant to antibiotic penetration in E. coli was demonstrated by the isolation of novobiocin-supersensitive mutants which had defective LPS lacking phosphate (Coleman and Leive, 1979). These mutants were also shown to have altered bacteriophage sensitivities (Tamaki et al, 1971). In E. coli at least, the presence of the phosphate diester bridges in the LPS seems more important than the sugar content in determining resistance to antibiotics such as novobiocin, coumeromycin, spiramycin, and actinomycin D (Tamaki et al, 1971). Isolation of a series of mutants in S. minnesota with increasingly defective LPS however indicated that the sensitivity of

these mutants to erythromycin, rifamycin, actinomycin D, and bacitracin increased with loss of sugar units (Schlecht and Westphal, 1970). Hydrophobic antibiotics such as novobiocin, fusidic acid and clindamycin also have increased toxicity in inner core LPS mutants (Roantree et al, 1977; Sanderson et al,1974).

In contrast to the increased sensitivity to hydrophobic antibiotics, deep rough mutants do not show hypersensitivity to hydrophilic agents. In some rough mutants a decrease in sensitivity is found to hydrophilic antibiotics such as ampicillin (Boman and Monner,1975; Roantree et al, 1977; Sanderson et al, 1974).

In order to provide an efficient barrier it is postulated that specific interactions between outer membrane proteins and LPS are required for outer membrane integrity and that the charged phosphate groups on the heptose residues play a major role in maintaining this integrity (Nikaido and Vaara, 1985). Evidence for an association between outer membrane proteins and LPS is provided by the observation that inner core mutants have lost certain outer membrane proteins (Ames et al, 1974; Koplow and Goldfine, 1974). With one heptoseless (Re) mutant of E. coli the protein to LPS ratio was found to be decreased threefold (Koplow and Goldfine, 1974). The OmpA protein has been shown to bind LPS in vitro (Beher et al, 1980).

Several genetic abnormalities have been characterised

that affect the barrier function of the outer membrane. Mutants with defects in the ompF or ompC genes are deficient in outer membrane porins (the channels which allow passive diffusion of small hydrophilic molecules across the outer membrane) and therefore have altered membrane function (Nikaido and Vaara, 1985). Mutants which lack the OmpA protein (required for F-plasmid-mediated conjugation) produce a defective outer membrane and are defective in conjugational capabilities (Manning and Achtman, 1979). Mutations in the acrA locus of E. coli display increased sensitivity to crystal violet, acriflavine and SDS (Nakamura, 1968). envA mutants of E. coli make this organism more sensitive to hydrophobic agents. Although the LPS structure is unaltered, the LPS content is reduced by 25% (Grundstrom, 1980).

Deep rough mutants can be easily isolated in strains with galE (galactose epimerase) mutations which are unable to synthesize galactose and are sensitive to C21; using selection for reistance to that phage, these mutants become sensitive to bile salts (Wilkinson et al, 1972). Deep rough mutants of S. typhimurium with defects in the heptose region have been characterised for phage sensitivity and by genetic techniques (Sanderson et al, 1974). Examination of the LPS of mutants which were sensitive to bile salts revealed that some of them contained only one heptose (Rd2), and others were completely lacking in heptose (Re) (Wilkinson et al,

1972). In summary, four genes of S. typhimurium involved in synthesis or transfer of the heptose units were determined: mutants defective in the heptosyltransferase II activity, producing LPS of Rd2 chemotype, were postulated to be due to a defect at the rfaF(S) locus. The genetic loci of mutants having the Rd2 chemotype have been mapped, presumably at rfaF, between cysE and pyrE, but closer to cysE, and are postulated to control the heptosyltransferase II enzyme (Kuo and Stocker, 1972; Sanderson and Saeed, 1972; Sanderson et al, 1974). Three different mutations, rfaC(S), rfaD(S) and rfaE(S) produced heptoseless (Re) LPS (Sanderson et al, 1974). The rfaE locus maps near metC, outside the cysE-pyrE sector (Kuo and Stocker, 1972; Sanderson et al, 1974). The rfaC locus maps between cysE and pyrE (Sanderson et al, 1974); the rfaD locus is in the same region (Lehmann et al, 1973). Mutants in rfaD produce a part-rough LPS and these strains are bile salt sensitive. An rfaD mutant of S. typhimurium produced LPS containing a small amount of the normal L-glycero-D-manno-heptose and also some of the precursor D-glycero-D-manno-heptose. It has been suggested that the final step in the biosynthesis of the core heptose is the conversion of the ADP-D-glycero-D-manno-heptose to the corresponding L-D compound (Eidels and Osborn, 1971). It was postulated that the epimerase required for this reaction was the product of the rfaD gene (Lehmann et al, 1973; Coleman and Leive, 1979; Coleman, 1983). The locus of the

defective epimerase gene, rfaD, has been mapped close to cysE in both S. typhimurium (Kuo and Stocker, 1972) and in E. coli K-12 (Coleman and Leive, 1979).

The genetic analysis of heptose-deficient mutants of E. coli K-12 is much less advanced than in S. typhimurium. Hancock and Reeves (1976) identified bacteriophage resistant, heptose-deficient mutants of E. coli K-12. Mutants which displayed super-sensitivity to novobiocin and which had simultaneously become resistant to phage T4 were isolated, some of which were heptose deficient (Tamaki et al, 1971). Prehm et al (1976) identified strains with reduced amounts of heptose in their LPS from among a group of ampicillin resistant mutants. Recently, Coleman and Deshpande (1984) have identified an LPS mutant which is heptoseless and for which the nature of the enzymatic defect is unknown. Mutants similar to the rfaD mutants of S. typhimurium have been isolated and the rfaD locus in E. coli was mapped close to the cysE locus (Coleman, 1983). Two plasmids were identified from a set of hybrid plasmids containing E. coli DNA, constructed by Clarke and Carbon (1976). These plasmids, pLC13-13 and pLC32-45, both have complementing activity for an rfaD mutation in E. coli K-12, but were not tested for complementation for other genes involved in LPS synthesis (Coleman, 1983). In this study I tested the pLC13-13 plasmid for ability to complement deep rough mutants of E. coli and S. typhimurium and thus

identified additional gene activities to that of the rfaD(K) reported earlier by Coleman (1983). In addition, a restriction map of the pLC13-13 plasmid has been determined. Transposon Tn5 insertions have been constructed, mapped and studied for complementation activity to determine the location of the rfaC,D and F genes on the plasmid and the polarity patterns.

Materials and Methods.

Bacterial Strains, Plasmids and Cultivation Methods. The bacterial strains and plasmids used in this study are listed in Table 3-1. All strains were stored and grown as described in Materials and Methods, Chapter 1. The media used are as described in Materials and Methods, Chapter 1 and Chapter 2.

Chemicals. The chemicals used in this study and the preparation of colicin are listed in the relevant sections of Materials and Methods, Chapter 1.

Bacteriophage and Deoxycholate Sensitivity Tests. Bacteriophages U3, C21, FO, P22, and Ffm were propagated and used to test the phenotype of mutant and recombinant strains in the manner described in Materials and Methods, Chapter 1. Strains were tested for sensitivity to deoxycholate by streaking for growth on L-agar plates containing 0.4% sodium deoxycholate.

Mating Methods. F-mediated conjugation, using strain ED1714, as a source of F factor, was performed in transfers involving the ColE1 derived plasmid pLC13-13 and its derivatives. The mating mixture was inoculated on an L-agar plate and consisted of 0.1 ml each of the donor, recipient and F^+ strains. Transconjugants were subsequentally selected for colicin resistance in crosses with pLC13-13 and for

kanamycin resistance in the cases involving the transfer of plasmids carrying Tn5 inserts. In both cases the auxotrophic requirements of the donor strain and F^+ strain were counter-selected.

Plasmid Isolation and Restriction Enzyme Mapping. Plasmid DNA preparations were made using the modified method of Birnboim and Doly, as described in Maniatis et al, (1982). Restriction enzyme digests of plasmid DNA, electrophoresis of fragments on 0.7% agarose gels and visualization using ethidium bromide were performed according to the methods of Maniatis et al, (1982).

Tn5 Mutagenesis Methods. Plasmids carrying inserted DNA of the transposon Tn5 were isolated using the methods outlined in Materials and Methods, Chapter 2. Plasmids containing Tn5 inserts were transferred by F-mediated conjugation into strain SL3600 (rfaD(S)) and tested for complementation. Selected insertions were subsequently transferred into other mutants for assessment of active genes. Plasmid DNA was isolated and restriction fragment size analysed as described above. Inserts in pLC13-13 were located based on increased fragment sizes of pLC13-13::Tn5 derivatives and on the sizes and location of appropriate restriction fragments in Tn5 as reported by Jorgensen et al, (1979).

Results

The rfaD(K) activity has previously been assigned to the plasmid pLC13-13 and the rfaD(K) gene has been mapped on the chromosome of E. coli K-12 as being close to cysE (Coleman, 1983; Coleman and Deshpande, 1984). We confirm that this plasmid has complementing capability in an rfaD(K) strain, CL29, the original strain used by Coleman (1983). Using F-mediated conjugation with the F factor supplied by strain ED1714 in the tri-parental cross the plasmid pLC13-13 was transferred from the strain JA200/pLC13-13 into recipient strains carrying mutations in the genes rfaD(S), rfaC(S), rfaF(S) or rfaC(K) mutants and complementation was tested. The mating protocol and the subsequent analysis of complementation by phage sensitivity testing is described in Materials and Methods. Selection of S. typhiumurium transconjugants was made for resistance to phage Ffm - all strains of E. coli K-12 and rough mutants of S. typhimurium are sensitive to this phage thus only S. typhimurium strains with a corrected LPS defect by virtue of the plasmid can grow under this selection. Confirmation of successful complementation in the Ffm resistant colonies was made by detection of sensitivity to phages FO (which requires complete core LPS) and P22 (which requires O side chains on the LPS for adsorption). Crosses involving the E. coli strains, CL29 and SAB2990 (rfaC(K)) with JA200/pLC13-13 were

selected on MG agar, supplemented with the appropriate amino acids and overlaid with colicin. Colicin resistant colonies were screened for a change in the sensitivities of the transconjugants to wild-type (U3 sensitivity and C21 resistance). Strains SA1377 (rfaC(S)), SL3789 (rfaF(S)), SL3600 (rfaD(S)), SAB2990 (rfaC(K)), and CL29 (rfaD(K)) all show the corrected phenotype when crossed with the pLC13-13 containing strain (Table 3-2). The results indicate that the pLC13-13 plasmid, which is known to carry the rfaD(K) gene (Coleman, 1983), also carries the rfaC(K), and in addition is able to complement rfaD(S), rfaC(S) and rfaF(S) mutants. A number of other strains available in this lab with mutations in each of the rfaG,B,I,J,K,L and E genes of S. typhimurium were also tested in the way described above. However no change in the phenotype of any of these strains mated with JA200/pLC13-13 was observed (data not shown).

In cases where selection of wild-type revertants is favoured (i.e. phage resistance selection for complemented recombinants) it is desirable to use selection for a plasmid inserted antibiotic resistance determinant, thus obviating the need for direct selection of positive complementing types only. An easy way in which to construct a derivative plasmid of pLC13-13 with an inserted antibiotic resistance marker is to use the transposon Tn5 mutagenesis method described in Materials and Methods. Using this method, pKZ57, a colicin sensitive derivative of pLC13-13, was

obtained which has presumably identical genetic activities to those of pLC10-7, except that the colicin immunity gene is disrupted in pKZ57. The location of the Tn5 insert in pKZ57 was determined by carrying out a series of single and double restriction digestions (described in Tn5 mutagenesis section of Maaterials and Methods) of the plasmid DNA (Fig 3-1).

Selection of this plasmid in mutant strains into which it is conjugated, is facilitated by the presence of the kanamycin resistance determinant.

Restriction Map of pLC13-13. Plasmid DNA of pLC13-13 was isolated from JA200/PLC13-13 and cut with a series of restriction endonucleases. The fragment sizes were calculated by comparison with the sizes of HindIII-generated fragments of lambda phage. A restriction map indicating the restriction sites and sizes for the plasmid was generated (Fig 3-1).

Analysis of the Functional Units on pLC13-13 by Transposon Tn5 Insertions. In the same manner as described above for the construction of pKZ57, two independent insertions of Tn5 were isolated which affected the genes on pLC13-13. Plasmids pKZ55 and pKZ56 were isolated from a pool of random Tn5 inserted pLC13-13 plasmids which had been conjugated into strain SL3600 (rfaD(S)) with selection for kanamycin. The

transconjugants were screened for complementation by the phage sensitivity test. One each of a smooth (complemented) strain (SA3244) and a rough (non-complemented) strain (SA3245) were chosen for further analysis and assigned the plasmid designations pKZ55 and pKZ56 respectively. The SA3244 (pLC13-13::Tn5; rfaD+) and SA3245 (pLC13-13::Tn5; rfaD-) strains were conjugated with each of the strains: SAB2990 (rfaC(K)), CL29 (rfaD(K)), SA1377 (rfaC(S)) and SL3789 (rfaF(S)). Both plasmids displayed non-complementation with SAB2990 (rfaC(K)), SA1377 (rfaC(S)) and SL3789 (rfaF(S)). pKZ55 complemented the defect in strain CL29 (rfaD(K)) but plasmid pKZ56 did not have this capability (Table 3-2).

Mapping of Tn5 Inserts. The Tn5 inserts in pLC13-13 represented by pKZ55 and pKZ56 were mapped in the same way as described above for the mapping of the insert in pKZ57. The location of the inserts is indicated in Fig 3-1.

Discussion

The biochemical characterization of the deep rough mutants of *E. coli* K-12 used in this study are described in Chapter 1. These mutants and the deep rough strains of *S. typhimurium* listed in Table 3-1 have in common the deoxycholate sensitive phenotype which is indicative of a defect in the core heptose region. All of these strains have the phage sensitivity phenotype of C21 resistance and U3 resistance (for *E. coli*) or C21 resistance and FO resistance (for *S. typhimurium*).

Mutants classified as *rfaD* have previously been demonstrated as defective in the epimerase activity responsible for conversion of the D-glycero-D-manno-heptose to the equivalent L-D- form incorporated normally in the LPS (Coleman, 1983). The presence of a gene capable of complementing this mutation was identified on a plasmid from the Clarke and Carbon library of hybrid plasmids (Coleman, 1983). In this study it has been demonstrated that the additional activities of the *rfaD*(S), *rfaC*(K), *rfaC*(S) and *rfaF*(S) are present on this plasmid. Mutants, characterised as defective in these loci, have shown the corrected wild-type phage phenotype when mated with a strain carrying the pLC13-13 plasmid (Table 3-2).

Heptoseless mutants having the Re chemotype (Fig 1-1, Chapter 1) have previously been isolated in *S. typhimurium* and found to be sensitive to bile salts. The *rfaC*(S) locus

has been mapped between cysE and pyrE (Sanderson et al, 1974). It is not known if the rfaC gene encodes the heptosyltransferase I activity or an enzyme involved in the earlier steps of heptose biosynthesis (Makela and Stocker, 1984). The fact that deep rough mutants of E. coli K-12 and S. typhimurium are phenotypically similar (Chapter 1) and the fact that plasmid pLC13-13, carrying E. coli genetic information, corrects the LPS defect in both SAB2990 (rfaC(K)) and in SA1377 (rfaC(S)) indicates that the activity of this gene is similar in both species.

A second mutation in S. typhimurium, termed rfaE, which produces Re type LPS has been mapped outside the rfa cluster of genes, near metC (Kuo and Stocker, 1972; Sanderson et al, 1974). Gene rfaE may be the structural gene for a heptosyltransferase or may be concerned with the synthesis of a heptosyl donor compound (Makela and Stocker, 1984). The plasmid pLC13-13 has no complementing ability for S. typhimurium strain SL1102 (rfaE(S)) (data not shown).

LPS mutants of the Rd2 chemotype produce the heptose I unit but not the heptose II unit (Wilkinson et al, 1972). The defective locus in this type of mutant in S. typhimurium is postulated to be rfaF and has been mapped by ES18 transduction to a position between cysE and pyrE, but closer to cysE (Kuo and Stocker, 1972). The nature of the gene product of this gene is unproven but assumed to be the heptosyltransferase II. The rfaF(S) mutant (SL3789) is

complemented to wild-type by pLC13-13 (Table 3-2). The rfaF(S) gene is therefore postulated to be present on this plasmid.

The presence of the rfaC,D, and F genes on plasmid pLC13-13 supports the existing data which positions these genes close to one another within the cysE - pyrE region. Identification and location of these gene activities makes possible the sub-cloning of individal genes from pLC13-13. Plasmids carrying individual activities of these genes would be useful in analysing the structure and activities of the gene-products.

Transposon mutagenesis using Tn5 is an effective method of obtaining polar, non-leaky mutants. Insertions of the transposon element into plasmid DNA sequences occurs randomly and produces new plasmids which have the kanamycin resistance determinant and which have lost, by virtue of the Tn5 inserting into structural or regulatory genes, some genetic activity. Assignment of approximate gene location and operon structure is possible if the genetic inactivation is measurable in terms of a phenotypic change. In this study inactivation of genes encoding a cell surface structure are assayable due to the unique chemical and phage sensitivity profile of deep rough LPS mutants. Loss of genetic activity on the plasmid is reflected in inability of the plasmid to restore resistance to deoxycholate or sensitivity to wild-type specific phages to a deep rough mutant. The loss

of activities of the rfaC and rfaF genes in pKZ55 indicates a possible common promoter region but does not indicate the order of these two loci. The insertion in pKZ56, which inactivates the rfaC, rfaD, and rfaF encoded regions indicates a possible single operon structure for the pLC13-13 plasmid. The results from the Tn5 experiments point towards a tentative gene order on the plasmid of rfaD - (rfaF,C), with the promoter for all three genes proximal to the rfaD encoding region. Characterization of a further group of Tn5 inserts in pLC13-13 is underway and results from this should further elucidate the location and order of the genes. Comparison of this order with that of analogous genes in other enteric species is not yet possible due to the lack of information on the deep rough genes of these species.

Several other mutants have been reported whose LPS phenotype is similar to that of the deep rough mutants, but the genotype of which is unknown. A mutant defective in the synthesis of sedoheptulose-7-P, a precursor of L-glycero-D-manno-heptose, has been identified (Eidels and Osborn, 1971). It is possible that the gene responsible for the transketolase activity which is missing in this mutant is encoded on plasmid pLC13-13. A mutant defective in phosphorylation of the core and which as a consequence appears to lack ability to synthesise the core LPS beyond the first glucose residue, has been reported for S.minnesota

(Muhlradt, 1968) and has been mapped close to xyl locus and to the rfa genes (Jousimies and Makela, 1974). Strains of E. coli K-12 which lacked phosphate substitution in the heptose region and whose LPS cores were shortened as a result, were identified by Blache et al, (1981). There is some reason therefore, to believe that some of these mutations might be complemented by a gene on the chromosomal insert of E. coli DNA in either pLC10-7 or pLC13-13.

A mutation designated as rfa-2 which gives rise to a heptoseless LPS structure has recently been reported (Coleman and Deshpande, 1985). The defective genetic locus of this mutant is located close to the rfaD locus but complementing activity for the rfa-2 mutation has not been found on pLC13-13 (Coleman and Deshpande, 1985). It is not known if the rfa-2 defect involves a blocked step in aldoheptose biosynthesis or represents a defective enzyme involved in the transfer of the heptose to the core or involved in the translocation of the LPS from its site of assembly in the periplasm to the outer leaflet. Cloning of the rfa-2 gene should be possible using the rfaD gene from pLC13-13 to probe chromosomal DNA fragments.

Mutant strains which have outer membranes deficient in certain proteins are unable to incorporate the LPS into the outer membrane. Mutants of this type might have the properties of deep rough mutants even though their LPS is normal. Removal of LPS by treatment with EDTA made these

cells sensitive to a range of hydrophobic antibiotics, which included actinomycin D and novobiocin (Leive, 1974).It has been postulated that the space left by the released LPS is filled with phospholipids and that this results in the formation of a phospholipid bilayer resembling that found in deep rough mutants (Nikaido and Nakae, 1979). However, the data of Gmeiner and Schlecht (1979) suggests that the the asymmetric nature of the outer membrane (LPS in the outer monolayer, phospholipid in the inner) is maintained in deep rough mutants.

Table 3-1. Strains used

Strain	Genotype	Source
E. coli K-12		
CL29	F^- *tfr-8 rfaD thi*	L. Leive
ED1714	*lac(del)X74 trp* sup^+ lam^+/F42 *finP301* lac^+	N.S. Willetts
JA200/pLC13-13	F^+ *trpE5 thr leu recA*/pLC13-13 (ColE1-*rfaD(K)*)	J. Carbon
HB101	F^- *hsdS (r-B,m-B) recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1 supE44*	J. Duerksen
SAB2906	*galE15 relA1 rpsL150* lam^-	S. Brenner
SAB2990	*pro his lac xyl rpsL tsx cycB cycA*	This lab
SAB3247	Same as SAB2990 with pKZ55 (pLC13-13::Tn5, $rfaC^-D^+F^-$)	This study
SAB3248	Same as SAB2990 with pKZ56 (pLC13-13::Tn5, $rfaC^-D^-F^-$)	This study
SAB3249	Same as HB101 with pKZ57 (pLC13-13::Tn5, colicin-sens)	This study
S. typhimurium		
SL1102	*rfaE543 metA22 trpE2 H1-b H2-e,n,x flaA66 rpsL120 metE551*	B.A.D. Stocker

....continued

Table 3-1 (continued)

Strain	Genotype	Source
SA1377	*rfaC630* (P22)$^{+}$	
SL3600	*rfaD657 metA22 trpE2 H1-b H2-e,n,x flaA66 rpsL120 xyl-404 metE551* (Cured of Fels2)	B.A.D. Stocker
SL3789	*pyrE*$^{+}$ *rfaF511*	B.A.D. Stocker
SAB3244	Same as SL3600 with pKZ55 (pLC13-13::Tn5, *rfaC*$^{-}$*D*$^{+}$*F*$^{-}$)	This study
SAB3245	Same as SL3600 with pKZ56 (pLC13-13::Tn5, *rfaC*$^{-}$*D*$^{-}$*F*$^{-}$)	This study

Table 3-2. Complementation testing of pLC13-13 and its derivatives in *S. typhimurium* and *E. coli* K-12

Recipient Strain		Plasmid			
Strain	Genotype[a]	pLC13-13(ColE1-) *rfaC*$^{+}$*D*$^{+}$*F*$^{+}$ [b]	pKZ55(ColE1-) *rfaD*$^{+}$(*C*$^{-}$,*F*$^{-}$::Tn5)	pKZ56(ColE1-) *rfa*(*D*$^{-}$,*C*$^{-}$,*F*$^{-}$::Tn5)	pKZ57(ColE1::Tn5)
SA1377	*rfaC(S)*	+	-	-	
SL3600	*rfaD(S)*	+	+	-	
SL1102	*rfaE(S)*	-	-	-	
SL3789	*rfaF(S)*	+	-	-	
SAB2990	*rfaC(K)*	+			
CL29	*rfaD(K)*	+			

....continued

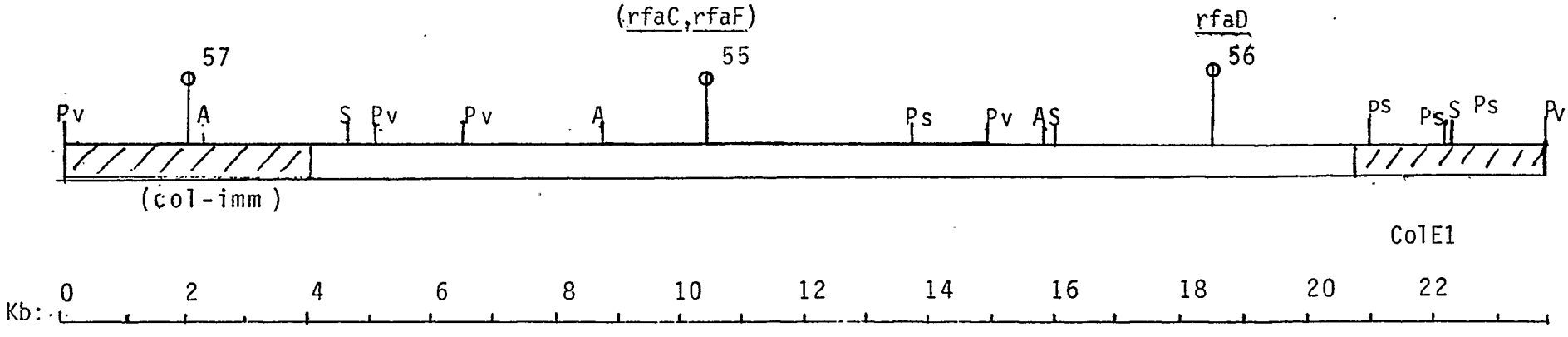
Table 3-2 (continued)

[a] Partial genotype only; fill genotype is given in Table 3-1.

[b] Gene activities assigned to plasmids based on ability to complement mutants of *E. coli* K-12 and *S. typhimurium*. "+" indicates U3-sensitivty, deoxycholate-resistant for *E. coli* K-12 and FO-sensitive, deoxycholate-resistant for *S. typhimurium*. "-" indicates no change in mutant phenotype when conjugated with plasmid (U3-resistant or FO-resistant and deoxycholate-sensitive).

Figure 3-1

Restriction map of plasmid pLC13-13 (ColE1-*rfaC,D,F*(K)). The restriction enzymes represented are: A, *AvaI*; Pv, *PvuII*; S, *SmaI*; Ps, *PstI*. The symbol (Φ) indicates the transposon Tn5 insertion represented in plasmids pKZ55, pKZ56 and pKZ57. The hatched segment indicates the ColE1 vector sequences and includes the genes for colicin immunity (col-imm). Plasmid pKZ57 has a Tn5 insertion in the col-imm region, thus strains carrying this plasmid are colicin sensitive. The location of the genes shown at the top of the figure is inferred from the ability of the individual Tn5 insertions to abolish activity of some or all of the genes on pLC13-13. pKZ55 is *rfaD*$^{+}$*C*$^{-}$*F*$^{-}$ and pKZ56 is *rfaD*$^{-}$*C*$^{-}$*F*$^{-}$. The map distances in kilobases (Kb) are indicated on the scale at the bottom.


(rfaC,rfaF)
rfaD
57
55
56
Pv
A
S
Pv
Pv
A
Ps
Pv
A
S
Ps
Ps
S
Ps
Pv
(col-imm)
ColE1
Kb:
0
2
4
6
8
10
12
14
16
18
20
22

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