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MOLECULAR AND GENETIC ANALYSIS OF THE *ESCHERICHIA COLI* K-12 *rfaH* GENE

BY

SANDRA D. CARSON

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled:

MOLECULAR AND GENETIC ANALYSIS OF THE

ESCHERICHIA COLI K-12 rfaH GENE

submitted by

SANDRA CARSON

in partial fulfillment of the requirements for the degree of

Master of Science.

K.E. Sanderson

Supervisor, Dr. K.E. Sanderson, Dept. of Biological Sciences

Dr. M.M. Moloney, Dept. of Biological Sciences

M Kapoor

Dr. M. Kapoor, Dept. of Biological Sciences

Dr. P. Sokol - Dept. of Microbiology and Infectious Diseases

Date: May 7, 1990

ABSTRACT

rfaH mutants of *Escherichia coli* K-12 and *Salmonella typhimurium* LT2 have an incomplete lipopolysaccharide (LPS) core and are deficient in the F-mediated transfer functions (formation of sex pili, participation in mating aggregates, ability to act as donors in F-mediated conjugation, and sensitivity to the male-specific phages M13 and f2). The defects are due to the role of the *rfaH* gene product as a positive regulatory element acting on the genes of the *rfa* cluster (LPS) and F-factor *tra* operon (transfer functions). Previously made clones of the *E. coli* gene are capable of correcting these defects in *rfaH*$^-$ strains of *S. typhimurium*.

A putative *Salmonella rfaH* gene, carried on the plasmid pKZ25, was analyzed. The plasmid corrects defects in the *tra* and *rfa* functions in both species as shown by bacteriophage sensitivity patterns and analysis of LPS on silver-stained gels. The gene carried on this plasmid was located and analyzed by insertion mutagenesis using the Tn*1000* (gamma-delta) insertion. Several insertions, falling within a 1.2 kb fragment of pKZ25, caused partial or complete loss of *rfaH*-complementing activity in both species as shown by the above methods and by determination of donor capabilities in conjugation.

The 1.2 kb fragment was subcloned into pBluescript KS+ and pBluescript KS- to produce single-stranded DNA for sequencing. The subclones also complement the *rfaH*

defects in both species. DNA sequence was determined (by dideoxy sequencing) and a 483 base open reading frame (ORF) was found. The ORF codes for a 160 amino acid protein product of 17.9 kd. The size of the product is consistent with that seen by SDS-PAGE analysis of radiolabelled protein generated from the pBluescript T7 promoters.

Double stranded sequencing, using the primers made to the ends of the Tn1000 transposon, allowed for placement of the insertions within the ORF. An insertion in N-terminal third of the protein caused a complete loss of rfaH-complementation in both species while the losses seen with a group of insertions in the C-terminal portion were more variable, some showing loss of the rfa functions but not the tra or loss of complementation in Salmonella but not E. coli.

Towards the end of the study the sequence of the E. coli rfaH gene, carried on a second previously made plasmid, was determined by other investigators and the two sequences were found to be identical. To determine the source of the gene carried on pKZ25, fragments of its insert region were used to probe digests of the chromosomes of S. typhimurium and E. coli. The probes were found to hybridize with the E. coli genome and not with that of Salmonella, indicating that the gene studied is actually the E. coli rfaH gene.

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DEDICATION

To my parents, my brother and sister, Ron, and all my friends in Calgary.

And in memory of Alton Alexander and Shirlie Fage.

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ABBREVIATIONS

cm	Centimetre
dpm	Decays per minute
dATP	2′-deoxyadenosine-5′-triphosphate
dCTP	2′-deoxycytidine-5′-triphosphate
dGTP	2′-deoxyguanosine-5′-triphosphate
dITP	2′-deoxyinosine-5′-triphosphate
dNTP	Deoxynucleotide triphosphate
dTTP	2′-deoxythymidine-5′-triphosphate
fmol	Femptomoles
g	Gram, unless referring to centrifugation
g	Gravity when referring to centrifugation (ie 5000 x g)
gpN	N gene product
gpQ	Q gene product
kb	Kilobase
kv	Kilovolts
kDa	Kilodalton
l	Litre
mg	Miligrams
ml	Millilitres
mm	Milimetres
mA	Miliamperes
mM	Milimolar
ng	Nanograms
nm	Nanometres

pmol	Picomoles
rps	Revolutions per second
ug	Micrograms
ul	Microlitres
um	Micrometres
uv	Ultraviolet
uCi	Microcurie
uF	Microfarads
uM	Micromole
v	Volts
w/v	Weight/volume
BSA	Bovine Serum Albumin
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid (disodium salt)
GlcNAc	N-acetyl-D-glucosamine
GCL	Glycosyl carrier lipid
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
IPTG	Isopropyl-beta-D-thiogalactoside
KDO	2-keto-3-deoxy-D-manno-octulosonic acid
L	LB media with 1% glucose
M	Molar
MG	Minimal glucose media
MOI	Multiplicity of infection
MOPS	3-[N-morpholino]propanesulfonic acid

N	Normal
OD	Optical density
ORF	Open Reading Frame
PAGE	polyacrylamide gel electrophoresis
PEG	Polyethylene glycol ("Carbowax")
R	Rough
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulphate
SR	Semirough
U	Units (of enzyme)
W	Watts
X-gal	5-bromo-4-chloro-3-indolyl-beta-D-galactoside

INTRODUCTION

Lipopolysaccharide (LPS), or endotoxin, is a major component of the gram-negative bacterial outer membrane (Figure 1) and may comprise up to 5% of the total dry weight of a cell (Osborn et al, 1964). Historically, it has been of interest to researchers because of its pyrogenic properties. Structurally, the LPS of a smooth (wild-type) bacterial cell consists of a membrane-bound lipid A region attached to a hydrophilic O-somatic side chain (also known as the O-antigen) via an oligosaccharide core (Figure 2). Rough mutants lack the O-antigen and may also be missing portions of the core. The composition of the O-side chains varies from species to species and, to some extent, from strain to strain, however, the lipid A and core regions are less variable. The structures of the Salmonella typhimurium and Escherichia coli LPS are shown in Figure 2 (Hitchcock et al, 1986; Rick, 1987).

The core region is further divided into the inner and outer cores (Figure 2). The inner core is characterized by three molecules of 2-keto-3-deoxy-D-manno-octulosonic acid (KDO) and L-glycero-D-manno-heptose. While all portions distal to KDO are non-essential for growth, deep-rough mutants (those with loss of the inner core region) are more sensitive to adverse growth conditions and certain chemical agents. The inner core is structurally invariant among Salmonella and E. coli (Rick, 1987).

The LPS core portion is assembled by sequential addition of sugar molecules (Osborn et al, 1964) by catalyzed transfers of sugar residues from nucleotide sugars to the non-reducing end of the growing LPS chain. The glycosyltransferases are enzymes responsible for these transfers, and each is specific for one unique reaction (Rick, 1987).

Many of the glycosyltransferases are coded for by the genes of the rfa (rough A) cluster, between cysE and pyrE, at 79 minutes on the S. typhimurium linkage map (Kuo and Stocker, 1972; Sanderson and Saeed, 1972; Sanderson and Roth, 1988). Mutations causing loss of activity in one of these genes result in a rough LPS in which the lipopolysaccharide chain terminates at the point of the missing transferase.

Mutants in rfaH - located at 84 minutes and not part of the cluster - have heterogeneous rough LPS. They are also deficient in the F-mediated transfer functions as a result of premature transcription termination in the tra operon (Beutin et al, 1981; Sanderson and Stocker, 1981). Both defects are also seen in E. coli strains with mutations in the homologous gene, previously called sfrB (sex factor repression B) (Beutin and Achtman, 1979). Hereafter, I will be referring to both genes as rfaH.

Using a putative S. typhimurium rfaH clone prepared by Rehemtulla (1984), I attempted to study the S. typhimurium rfaH gene. This study has included insertion mutagenesis, protein analysis, and DNA sequencing. Towards the end of this work, the E. coli

rfaH gene, carried on a second plasmid (Rehemtulla, 1984) was sequenced (P.R. MacLachlan and P. Thirukkumaran, unpublished data) and the two were found to have identical DNA sequence. To confirm the origin of the clone which I had analyzed and sequenced, I used it to probe the chromosomes of *E. coli* K-12 and *S. typhimurium* LT2, and showed that the *rfaH* gene studied is actually that of *E. coli* K-12.

LITERATURE REVIEW

A. The structure of the lipopolysaccharide portion of the gram-negative bacterial outer membrane

LPS consists of a membrane-bound, hydrophobic portion, lipid A, which is attached to the hydrophilic O-side chains by means of a polysaccharide core region (Figure 2). Lipid A of E. coli, S. typhimurium, and other related gram-negative bacteria anchors the LPS molecule to the membrane and is the basis of the pyrogenic response raised by the endotoxin in susceptible organisms (Luderitz et al, 1982; Nikaido and Vaara, 1987; Rick, 1987). LPS is assembled in the inner membrane and subsequently transferred to the outer membrane (Osborn et al, 1972).

Although composition of the O-side chain varies widely between strains, the structure of the core is generally similar within a species (Osborn et al, 1964; Sutherland et al, 1965). E. coli cores are of a few different types (Figures 2,3) while a single core structure is seen in all S. typhimurium (Figure 2). Attached to lipid A, and forming a structural bridge to the remainder of the core, is the KDO region which appears necessary for the growth of the organism since mutants defective in its synthesis require exogenously

supplied KDO in order to survive and temperature sensitive mutants are lethal (Osborn, 1963; Brade and Reitschel, 1984; Goldman and Devine, 1987).

The KDO region, along with two or three attached molecules of L-glycero-D-manno-heptose (the heptosyl region), comprises the inner core. The first glucose residue (glucose I) of the outer core of S. typhimurium is attached to the second heptose (heptose II) and begins the outer core, or hexose portion. The outer core contains glucose, galactose, and N-acetyl-D-glucosamine (GlcNAc) with O-side chain attached to the sub-terminal glucose residue (glucose II) (Figure 2) (Jansson et al, 1981; Nikaido, 1970; Hellerqvist and Lindberg, 1971).

The O-somatic side chain extends from the outer membrane into the surrounding media and consists of multiple oligosaccharide units which may be composed of linear trisaccharides, pentasaccharides, or polymers of branched oligosaccharides. They normally contain neutral and amino sugars as well as other, more unusual sugars such as 3-deoxyhexoses and 3,6-dideoxyhexoses (Rick, 1987). Variations in the side chains are part of the basis for the Kauffman-White scheme of serogrouping Salmonella (Osborn et al, 1964; Hitchcock et al, 1986). The O-side chain is assembled separately from the lipid A-core structure and is later attached. Both structures are assembled in the cytoplasmic membrane and translocated to the outer membrane after

completion (Nikaido and Vaara, 1987; Osborn et al, 1969).

1. Lipid A

The structure of lipid A is shown in Figure 4. This is the most proximal portion of the LPS. Its structure has been described for several different bacteria and appears identical among enterics, consisting of a (beta)-1,6 linked diglucosamine backbone which is phosphorylated at positions 1 and 4′ and containing hydroxymyristoyl residues attached by amide bonds to carbons 2 and 2′ and by ester bonds to carbons 3 and 3′. In the complete structure, these are further acylated with saturated fatty acid residues. The polysaccharide portion of the core is linked, through KDO, to the 6′ carbon (Takayama et al, 1983).

The addition of the fatty acids is not necessary either for production of the polysaccharide core region, or transfer of LPS to the outer membrane (Walenga and Osborn, 1980a, b). That microheterogeneity exists in lipid A isolated from mature LPS, based on the degree of acylation, was supported by the observation that LPS isolated from a single strain of S. abortusequi showed three varieties of molecule; a long-chain form containing 20 or more O-antigen units, a short-chain species with 1-6 units, and an R form with no attached side chains. The three lipid A species contained identical amounts of phosphate but

their fatty acid contents differed with the R form identical to that previously described for rough Salmonella while the long and short-chain forms showed a 40% reduction in fatty acids (Jiao et al, 1989).

2. The inner core

KDO is an eight carbon sugar found as a disaccharide in Salmonella and E. coli Re mutants and as a linear trisaccharide in smooth strains and rough mutants with a more complete core (Figure 5) (Ghalambor et al, 1976). The addition of KDO to the lipid A precursor appears to be essential for the translocation of the LPS molecule to the outer membrane (Osborn et al, 1980). Since translocation of LPS in heptoseless mutants is as efficient as that of more complete molecules, this addition is also apparently sufficient for the transfer. It was originally proposed that the KDO residues were linked to position 3′ of lipid A but ^{13}C and ^{31}P nuclear magnetic resonance have shown the attachment to be at carbon 6′ (Figure 4) (Strain et al, 1983a, b).

KDO forms a linear trimer in which one residue joins lipid A to heptose I while the other two form a side chain (Figure 5) (Brade et al, 1983a, b). The linkage between the KDO residues is 2,4 and the residues are in the alpha configuration (Munson et al, 1978; Brade and Rietschel, 1984). KDO II is non-stoichiometrically substituted at position 7 with

phosphorylethanolamine. In heptoseless (Re) mutants, however, only two KDO residues are seen (Prehm et al, 1975; Strain et al, 1983b).

The inner core also contains three residues of L-glycero-D-manno-heptose (Figure 2). The synthesis of this region involves several steps beginning with the conversion of sedoheptulose-7-phosphate to D-glycero-D-manno-heptose-7-phosphate and ending with the conversion of ADP-D-glycero-D-manno-heptose to ADP-L-glycero-D-manno-heptose by an epimerase. This last form is the donor of heptose residues to the core (Eidels and Osborn, 1974).

The heptose region is completed after the outer core has been begun. After the first glucose residue has been attached to heptose II, the third, or branch, heptose (heptose III) is added and phosphate is incorporated into the region (Muhlradt, 1969; Hammerling et al, 1973).

3. The outer core

The core region in S. typhimurium and E. coli is assembled by a series of sequential transfers of sugar residues from nucleotide-sugar donors to the non-reducing end of the growing LPS chain (Edstrom and Heath, 1964; Osborn et al, 1964; Osborn and Rothfield, 1971). Its structure (Figure 2) has been determined using rough (R) mutants, which have defects in different steps in synthesis of the core region producing incom-

plete cores which end at the point of the defect. R mutants show varying sensitivities to certain antibiotics, dyes, and bile salts and R strains may be differentiated by means of serological specificity (haemagglutination or haemagglutination inhibition) (Beckmann et al, 1964), chemotype (Luderitz et al, 1965), or phage sensitivity. Mutants of S. typhimurium are classified into chemotypes Ra-Re depending on the types and quantities of sugar residues present (Figure 2) (Lindberg and Hellerqvist, 1971; Luderitz et al, 1971). E. coli cores are classified as R1-R4, K-12, and B (Figures 2,3) (Jansson et al, 1981).

All of the core sugars appear to be alpha-linked (Jansson et al, 1981). Heterogeneity may be seen in the core region relating to substitution with GlcNAc, galactose II, heptose III, and phosphorylethanolamine and pyrophosphorylethanolamine groups (Hellerqvist and Lindberg, 1971; Hussey and Baddily, 1985). The presence or absence of the GlcNAc residue is apparently responsible for the difference between RI and RII serotypes in S. typhimurium as LPS from RI strains lacks this residue (Sutherland et al, 1965).

4. The O-antigen

E. coli K-12 lacks side chains. The construction of the O-side chain in S. typhimurium is carried out in the cytoplasmic membrane by a series of membrane-bound enzymes (Zeleznick et al, 1965; Osborn et al, 1972).

It begins with the reversible attachment of galactose-1-phosphate to a glycosyl carrier lipid (GCL), a C_{55} polyisoprenoid alcohol which is identical to carriers used in the assembly of capsular polysaccharides and other bacterial polymers (Osborn and Tze-Yuen, 1968; Osborn et al, 1972; Luderitz et al, 1982; Rick, 1987). This is followed by the sequential additions of mannosyl and rhamnosyl residues from GDP-mannose and TDP-rhamnose, respectively, to form a linear trisaccharide (Weiner et al, 1965; Zeleznick et al, 1965). A branch chain abequose residue is then transferred from CDP-abequose to give the tetrasaccharide structure shown in Figure 2 (Weiner et al, 1966). After completion of the tetrasaccharide, repeating units are polymerized by attaching the reducing galactose of one unit to the non-reducing mannose of the next to give a polymer attached to the GCL. The number of oligosaccharide units is not constant and while most S. typhimurium LPS molecules contain no side chains at all, the majority of those with side chains have between nineteen and thirty-four repeating units (Goldman and Lieve, 1980; Munford et al, 1980; Palva and Makela, 1980; Ishiguro et al, 1986). Polymerase deficient (rfc) mutants of S. typhimurium do not polymerize the repeating units, therefore, their LPS has a 20-30% reduction in O-side chain sugar content and each individual molecule contains only one repeating unit. These strains are called SR (for semi-rough) and are intermediate in their characteristics between smooth and rough (Makela et al, 1984).

The various antigenic determinants are then added to form haptens, precursors of the O-side chain showing a high turnover rate in smooth strains (Osborn et al, 1972; Luderitz et al, 1982). Haptens represent the last stage of O-side chain synthesis prior to attachment to the core (Kent and Osborn, 1968a, b). They contain the basic repeating units as well as any modifications or antigenic factors (Makela, 1966; Takeshita and Makela, 1971), including O-acetylations and the addition of glucosyl side branches, but are not substituted on the reducing galactose molecule (Stocker and Makela, 1971). The polymerized side chain of the hapten is transferred to the completed core by the O antigen:lipopolysaccharide ligase, giving a 1,4 linkage between the unsubstituted reducing galactose and the subterminal core glucose (Figure 2) (Stocker and Makela, 1971). This transfer is followed by translocation of the LPS molecule to the outer membrane (Osborn et al, 1972).

B. The genetics of core assembly

1. The glycosyltransferases

The transfer of sugar residues in the assembly of the LPS core is carried out by a group of membrane-associated enzymes known as glycosyltransferases (Rothfield et al, 1964; Osborn et al, 1969). Unlike the membrane-bound enzymes involved in the synthesis of the

O-antigen, the core transferases are easily solubilized. Failure in any of the steps of core synthesis in S. typhimurium results in rough LPS with core defects occurring at different points (Nikaido, 1962; Schmidt et al, 1970). Several of these enzymes are coded for by the genes of the rfa (rough A) cluster which maps between cysE and pyrE at 79 minutes on the S. typhimurium linkage map (Kuo and Stocker, 1972; Sanderson and Saeed, 1972) and at 81 minutes in E. coli K-12 (Schmidt, 1973; Bachmann, 1987). Genes for other enzymes involved in core synthesis, especially those for "housekeeping" functions, map outside of this region. The first characterized S. typhimurium rough mutants were in one of these "housekeeping" genes, galE (18 minutes), coding for the epimerase which transforms UDP-glucose to UDP-galactose (Fukasawa and Nikaido, 1961). Thus galE mutants, in addition to producing LPS lacking galactose (type Rc) (Nikaido, 1962), are unable to ferment galactose (Makela and Stocker, 1984).

Functions of several of the genes of the cluster have been determined. The chemotypes expected from loss of various rfa genes are summarized in Figure 2 and the currently known order of genes in the clusters of E. coli K-12 and S. typhimurium are shown in Figure 6. rfaC is the structural gene for heptosyltransferase I (Sirisena, 1990) and mutants in this gene do not add the first heptose residue to KDO (Sanderson et al, 1974). rfaF appears to be the structural gene for heptosyltransferase II (Kuo and Stocker, 1972; Jousimies and Makela, 1974). Other genes affecting the

formation of the heptose region are rfaE, which is not included in the cluster, and rfaD. While the actual role of rfaE is not known, mutants in this gene are unable to incorporate any heptose into the core. rfaD mutants in S. typhimurium (Lehmann et al, 1973; Lehmann, 1977) and E. coli (Coleman and Leive, 1979; Coleman, 1983) are deficient in the epimerase catalyzing the switch from D-glycero-D-manno-heptose to the L-glycero form. Half of their LPS molecules terminate at KDO and the remainder contain D-glycero-D-manno-heptose which is incorporated by heptosyltransferase I at a reduced rate (Lehmann et al, 1973; Lehmann, 1977; Makela and Stocker, 1981; Coleman, 1983).

The incorporation of the first glucose of the outer core in both S. typhimurium and E. coli K-12 is catalyzed by the product of rfaG, the structural gene for glucosyltransferase I (Creeger and Rothfield, 1979; Makela and Stocker, 1981; Kadam et al, 1985). This is followed, in S. typhimurium, by the addition of galactose I, the main chain galactose residue which is catalyzed by the product of gene rfaI. LPS of rfaI mutants is of the chemotype Rb_3 and this gene was formerly called rfa(R-res-2) based on the phage sensitivities of its mutants (Wilkinson et al, 1972). In E. coli K-12, the incorporation of glucose I is followed by the addition of glucose II (Figure 2) by glucosyltransferase II, coded for by rfaM (Creeger and Rothfield, 1979).

Mutants in gene rfaP have been reported in E. coli (Beher and Schnaitman, 1981) and S. minnesota (Jousimies and Makela, 1974) and rfaP activity has been demonstrated in S. typhimurium (Muhlradt et al, 1968). These mutants fail to phosphorylate the inner core region and are unable to incorporate sugar residues distal to glucose I. Their chemotype is therefore RcP^-. Extracts of $rfaP^-$ membrane fractions can catalyze the transfers of galactose I and galactose II to a galactose-deficient phosphorylated core (Muhlradt, 1969; Jousimies and Makela, 1974; Beher and Schnaitman, 1981; Rick, 1987).

rfaB in S. typhimurium is the structural gene for galactosyltransferase II (Wollin et al, 1983). Mutants in this gene are unable to add the branch chain galactose in core formation and produce heterogeneous LPS - some complete chains which lack galactose II, some of chemotype Rc (Figure 2) (Wollin et al, 1983). Glucosyltransferase II, which adds the second glucose in the main chain, is presumed to be the product of rfaJ (formerly rfa(R-res-1)) (Makela and Stocker, 1984). The homologous gene in E. coli, glucosyltransferase III, which adds the third main-chain glucose residue (Figure 2) is predicted to be the product of rfaN (Figure 6) (Austin et al, 1990).

The addition of the branch GlcNAc, completing the core region, is carried out by the product of rfaK. rfaK mutants are generally part-rough including LPS of chemotype Rb_1 (Figure 2) and a few molecules with a full O region but lacking the GlcNAc side chain of the

core (Osborn and D'Ari, 1964). The product of rfaL is not involved in the transfer of sugar residues but is responsible for the attachment of the O-side chain to the completed core through the action of the O antigen:lipopolysaccharide ligase. Mutants in this gene have LPS of chemotype Ra and accumulate O haptens (Makela and Stocker, 1981; Makela and Stocker, 1984).

Two of the glycosyltransferases have been recovered and purified from the membrane fraction of sonicated cells. A galactosyltransferase was purified approximately 6000-fold by Endo and Rothfield (1969) and glucosyltransferase I about 1000-fold by Muller et al (1972). In the production of the normal LPS, glucosyltransferase I transfers a glucose residue in alpha configuration from UDP-glucose to the third carbon of heptose II. The resulting chain acts as the substrate for the galactosyltransferase which adds a galactose residue in alpha configuration from UDP-galactose to the previously transferred glucose (Endo and Rothfield, 1969). The identity of the galactosyltransferase purified, and hence the position of the attached galactose, are uncertain (Wollin et al, 1983). These two enzymes are highly substrate specific. Glucosyltransferase I will only accept as a substrate LPS containing a complete inner core (although this region need not be phosphorylated since rfaP mutants do show attachment of glucose I) and will only catalyze the transfer of glucose (Muller et al, 1972). In vitro,

the galactosyltransferase will only use the product of the glucosyltransferase reaction as a substrate and will transfer only galactose to this substrate.

Both of these purified enzymes require divalent cations, preferably Mg^{++}, and the presence of phospholipids in the form of phosphatidylethanolamine (Endo and Rothfield, 1969; Muller et al, 1972). These same requirements have been seen for the activities of other glycosyltransferases studied from membrane extracts (Osborn et al, 1962; Romeo et al, 1970). If purified LPS is used as a substrate, transfer is not catalyzed unless phosphatidylethanolamine is first incorporated (Rothfield and Horecker, 1964; Rothfield and Takeshita, 1965). In the membrane it is believed that LPS and phosphatidylethanolamine molecules are interdigitated to produce the optimum conformation for glycosyltransferase activity (Rothfield and Takeshita, 1966; Rothfield et al, 1966).

Closer study of other glycosyltransferases has been hindered by difficulties in producing sufficient quantities of these proteins to allow for their isolation and purification. Overproduction of these products is extremely difficult, possibly due to rate limiting effects of the rfaH gene product (below).

2. rfaH

Gene rfaH is located outside of the rfa cluster, between metE and pepQ, at 84 minutes on the S. typhimurium linkage map (Wilkinson et al, 1972; Stocker et al, 1980). rfaH mutants are sensitive to the rough-specific phage C21 and resistant to Felix O. Their phage sensitivities are characteristic of galE mutants, yet they are capable of utilizing galactose as a carbon source (Osborn, 1968). rfaH mutants also show increased sensitivity to a variety of antibiotics including bacitracin, polymyxin, novobiocin, nafcillin, and oxacillin (Stocker et al, 1980). Since these mutants are deficient in galactosyltransferase I, the rfaH gene was originally believed to represent the structural gene for this protein (Osborn, 1968; Creeger et al, 1979).

Clarke and Carbon (1976) created a bank of plasmids with E. coli K-12 DNA inserted in ColE1. Creeger et al (1979) reported that the Clarke and Carbon plasmid pLC14-28 carries a region of the E. coli chromosome which can restore galactosyltransferase I activity in rfaH$^-$ S. typhimurium based on incorporation of ^{14}C-labelled galactose. They also reported the same transferase activity in E. coli (Creeger et al, 1979). This was unexpected since the E. coli core region does not contain the alpha 1,3-linked galactose found in S. typhimurium. Their findings are explained, however, when one realizes that they were probably monitoring galactosyltransferase II in S. typhimurium (the incor-

poration of the branch alpha 1,6-linked galactose) (Wollin et al, 1983), which is seen in both organisms (Figure 2).

The sfrB gene has a map position in E. coli similar to that of rfaH in S. typhimurium (Beutin and Achtman, 1979; Beutin et al, 1981). It affects the F-mediated transfer functions coded for by the tra operon through a positive regulatory mechanism (Beutin et al, 1981). Mutants in this gene have few or no sex pili and thus are resistant to the male-specific phages M13 and f2. They are also deficient in surface exclusion (Beutin and Achtman, 1979). The functioning of the tra operon (Figure 7) is under positive control of the product of the traJ gene, therefore, mutations decreasing F-factor function may exert their effect either on traJ or on the operon itself. sfrA (sex factor repression A), located at 100 minutes on the E. coli map, represses traJ while examination of mRNA transcripts indicates that the product of sfrB prevents premature rho-dependent termination of transcription in the tra operon (Beutin et al, 1984). Based on beta-galactosidase activity in tra-lacZ fusions it was determined that, in sfrB mutants of E. coli, 85% of transcripts terminate at each of two sites, one between traC and traG and the other between traG and traD (Figure 7) (Gaffney et al, 1983). The presence of the sfrB gene product also decreases termination in the rplKAJL-rpoBC cluster of genes for ribosomal proteins and RNA polymerase (Ralling and Linn, 1987).

The similarities in phage sensitivities between mutants in rfaH and sfrB, along with the close linkage of both genes to metE (Beutin and Achtman, 1979; Stocker et al, 1980) and the ability of pLC14-28 to complement rfaH defects (Creeger et al, 1979) indicate that the genes are homologous (Sanderson and Stocker, 1981). The first evidence that rfaH might not represent the structural gene for a glycosyltransferase enzyme came with the observation that the LPS of rfaH mutants is not homogeneous. Rather, it shows a mixed core containing LPS molecules of the chemotypes Rc, Rb_3, Rb_2, Rb_1 and Ra (Figure 2) (Lindberg and Hellerqvist, 1980). This heterogeneity is also seen in SDS-PAGE gels of rfaH mutants of E. coli K-12 LPS (Rehemtulla et al, 1986).

E. coli strains carrying the rfaH mutation also have defective LPS (Beutin et al, 1984). E. coli sfrB mutant strains have reductions in the activities of two glycosyltransferases - the 1,6-galactosyltransferase previously misidentified and reported (Creeger et al, 1979) as well as 1,3-glucosyltransferase (Creeger et al, 1984). rfaH amber mutations in S. typhimurium have a 97% reduction in galactosyltransferase I activity (Creeger et al, 1984). The hypothesis that rfaH(sfrB) is not a structural gene is supported by the observation that, in both of these cases, antibody precipitation showed that the loss of activity was a result of a reduction in the amounts of transferase present and not in the size of individual molecules (Creeger et al,

1984). Although the effect of rfaH mutations on the tra operon is rho-dependent, the effect on LPS may be rho-independent.

Defects in F-mediated transfer functions are also seen in S. typhimurium rfaH mutants. These include reductions in; the transfer of Flac, the numbers of male-specific phage f2 infective centers formed, lysis by bacteriophages M13 and f2, the presence of visible F pili, and the formation of mating aggregates. These losses are not merely side effects of the incomplete LPS phenotype since other mutants affecting core synthesis do not show similar reductions in F-mediated functions. Core mutants including rfaC, rfaF, rfaG - which affect the inner core - rfaJ, and rfaL - affecting the outer core have wild-type donor capacities (Sanderson and Stocker, 1981).

A plasmid (called pKZ17) carrying the E. coli gene, which corrects rfaH defects in E. coli as well as S. typhimurium, was made from pLC14-28 (Rehemtulla, 1984; Rehemtulla et al, 1986). Minicell analysis of this new plasmid shows the synthesis of an 18 kDa protein which is not seen in the pBR322 vector. The identity of the protein was confirmed since minicells with plasmids containing rfaH-inactivating Tn5 insertions show its loss (Rehemtulla et al, 1986). The size is inconsistent with that of galactosyltransferase I which is expected to be approximately 39 kDa (Endo and Rothfield, 1969). The role of this product as a regulatory element is indicated by its size, its ability to affect more than one cellular function, and

the fact that rfaH(sfrB) mutants, when complemented with pKZ17, do not show galactosyltransferase amplified above normal cellular levels (Beutin et al, 1984; Rehemtulla et al, 1986).

C. Antitermination

Transcription of DNA to produce messenger RNA occurs in three stages. In the initiation stage, RNA polymerase binds to the DNA at a recognition sequence, the promoter. An area of local unwinding forms allowing the polymerase to associate with the template DNA strand. The first few nucleotides of the RNA message are incorporated and the "transcription bubble", consisting of the polymerase, partially unwound DNA, and the growing RNA chain is formed. This bubble is stabilized, thus reducing its tendency to abort, after reaching its mature size with the addition of eight or nine RNA nucleotides. It then enters the elongation phase in which the bubble moves down the template strand as the RNA chain grows. This phase ends when the polymerase encounters a transcription termination site where the messenger RNA is released and the RNA polymerase dissociates from the DNA template (Yager and Von Hippel, 1987). The most common method of transcriptional control involves regulation at initiation, by prevention of RNA polymerase binding; however, some systems are regulated by control at termination. Attenuation involves the premature

termination of transcription before mRNA for the complete gene or operon has been made. In operons, its effect is polar, meaning that termination in an early gene prevents transcription of downstream genes. Attenuation may be countered by antitermination which avoids the premature termination.

Antitermination has been extensively studied in the lytic cascade of bacteriophage lambda (Figure 8) which is normally transcribed as two large operons. The cascade begins with the initiation of transcription at the p_L and p_R promoters leading to transcription of the immediate early genes N and cro, respectively, after which it is terminated. The N gene product, a small, highly basic protein, allows transcription of the delayed early genes in the course of the cascade and is currently the best studied antiterminator. After sufficient gpN has accumulated, transcription is able to continue into the genes to the left of N and right of cro to produce the proteins needed for phage replication. Transcription mediated by gpN does not extend into the late gene region coding for the head, tail, and lysozyme needed for the lytic life cycle (Luzzati, 1970; Lozeron, 1976; Ptashne, 1986) and the prevention of termination acts at both rho-dependent and rho-independent sites (Adhya and Gottesman, 1987). Transcription of the genes for assembly of the head and tail region, as well as the R gene for lysozyme, is not required if the phage is entering a lysogenic life cycle. If a lytic life cycle is to be completed however, these gene products are needed and are trans-

cribed under the control of a second antiterminator, the product of gene Q, one of the delayed early genes (Figure 8) (Ptashne, 1986). There is an antitermination resistant termination site at the end of the p_L transcript, which prevents anti-sense mRNA from being produced against the late genes, and another which terminates the p_R transcript (Honigman, 1981) (Figure 8).

The protein products of N and Q do not influence the initiation of transcription but interact with the elongating RNA polymerase to prevent its premature release (Greenblatt, 1984). For gpN, this interaction occurs at the nut (N utilization) sites - found between p_L and the beginning of N and to the right of cro (Salstrom and Szybalski, 1978; Barik et al, 1987). Chromatographic studies show that gpN becomes a stable component of the elongating transcription apparatus at this point. The nature of the promoter region is not important and the presence of a nut site appears to be sufficient for N action (deCrohmbrugge et al, 1979). Several host factors, the Nus proteins, are also required for gpN action (Friedman et al, 1981). NusE, the ribosomal S10 protein, is also required (Freidman et al, 1981). The mutation identified as NusC falls within the E. coli rpoB gene and actually affects the structure of RNA polymerase, possibly rendering it incapable of interactions with gpN (unpublished data cited by Friedman et al, 1981).

One of the Nus proteins, NusA, is bound by a column of purified gpN and appears to be involved in the initial recognition of the polymerase by gpN. NusA is also required for E. coli growth (Schauer et al, 1987). In the absence of gpN, it enhances pausing and termination in several bacterial operons (Greenblatt et al, 1981) and is required for transcription of the rif operon coding for four ribosomal proteins and the genes for the beta and beta' RNA polymerase subunits as well as being involved in trp termination (Nakamura and Mizasawa, 1985; Grunberg-Manago, 1987). In the absence of gpN, NusA enhances Rho-dependent pausing at t_{R2} in lambda (Greenblatt et al, 1981, Greenblatt, 1984). NusA binds to the core of RNA polymerase and may act as a bridge between the polymerase and gpN (Horwitz et al, 1987).

The nut site has three components; BoxA, BoxB, and BoxC (Olson et al, 1982; Schauer et al, 1987). BoxA appears to be required for recognition by the nusA gene product. This recognition is essential since a modification in the phage lambda BoxA site, which allows S. typhimurium NusA to recognize it, will allow lambda to grow in S. typhimurium (Freidman and Olson, 1983). BoxAs from phages lambda, 21, and P22 have also been determined and the sequences CGC and TT appear to be common characteristics of nut sites (Olson et al, 1982). BoxB is a palindromic region which is capable of forming a short stem-loop structure in which the loop typically contains the sequence GPPPA (in which P represents a purine). It is found downstream of BoxA

in bacteriophage lambda but may be upstream in other systems. Since a single base change in BoxB prevents gpN binding (Doelling and Franklin, 1989), this may be the recognition sequence for gpN (Schauer et al, 1987).

The second lambda antiterminator, Q, was originally believed to code for a transcriptase (Dove, 1966) but was found to be non-catalytic and, therefore, more likely a regulatory gene (Dambly et al, 1968). The activity of gpQ requires the DNA region surrounding the lambda late promoter (Yang et al, 1987). A qut (Q utilization) site, containing BoxA and BoxB sites resembling those of the nut, in the first twenty nucleotides of the coding region following this promoter suggests that gpQ is also an antiterminator. The qut site contains no BoxC and purified lambda gpQ requires only NusA for function (Yang and Roberts, 1989). gpQ binds to RNA polymerase at the qut site, allowing readthrough of the t_R, terminator found 194 nucleotides downstream from the late gene promoter (Figure 8) (Yang et al, 1987). gpQ speeds the RNA polymerase along the template, neutralizing the pausing leading to termination which is otherwise caused by NusA (Yang and Roberts, 1989). gpQ from the related phage 82, does not actually require the presence of NusA although it increases its efficiency (Yang and Roberts, 1989).

Bacterial operons so far implicated in antitermination include those for ribosomal proteins (Almond et al, 1989), rRNA operons (Askoy et al, 1984), and those involved in macromolecular synthesis - such as the rfa cluster. NusA and the N gene product can also enhance

expression in the E. coli rpsU-dnaG-rpoD operon (Lupski et al, 1983; Peacock et al, 1985). Transcription of rpoD (sigma subunit gene) is increased in the presence of N but eliminated in the absence of NusA. These effects involve control of transcription termination at the Rho-independent termination signal found thirty-six bases 5′ of the dnaG initiation codon. 5′ of this termination site are a pair of tandem promoters and a nut-like region (Smiley et al, 1982). In the absence of the antiterminator, termination occurs at the t_1 terminator between rpsU and dnaG. A part of rpsU resembling the lambda nut site (called dnaG nut) is involved in reduction of termination at t_1 (Smiley et al, 1982; Lupski et al, 1983). The dnaG nut includes a conserved core region (BoxA) followed by a short stem loop (BoxB) and a repeated GT region comprising a possible BoxC 3′ of BoxA. The presence of the dnaG nut site, the effect of gpN on dnaG and rpoD transcription, and gpN's utilization of E. coli host factors suggest the existence of antitermination systems in E. coli (Lupski et al, 1983).

Thirty-one of the fifty-two genes coding for individual protein components of E. coli ribosomes are found in a single cluster, at 73 minutes, containing four operons. Antitermination is suggested in the eleven ribosomal proteins of the S10 operon, since transposons inserted in upstream genes are not completely polar (Jinks-Robertson and Nomura, 1987). There is a transcription termination sequence upstream of the first gene in the operon which is preceded by a

region resembling the BoxA, BoxB, and BoxC of the nut site. Readthrough of the terminator occurs if transcription begins from the correct promoter. Ralling and Linn (1987) measured transcription of genes for other ribosomal proteins and RNA polymerase found in the rplKAJL-rpoBC chromosomal segment. Termination of transcription was increased by the presence of Rho protein and decreased by the products of both the nusA and rfaH genes, giving further evidence for antitermination in E. coli and for a possible antiterminator role for rfaH (Ralling and Linn, 1987).

Potential NusA utilization sites are found in the leader regions of all seven rrn operons (unpublished data cited in Holben and Morgan, 1984). rrnG, rrnB, and rrnC are known to be protected from Rho-mediated termination by an antitermination mechanism although the antiterminator has not been determined (Brosius et al, 1981; Kingston and Chamberlin, 1981; Askoy et al, 1984; Holben and Morgan, 1984; Li et al, 1984). In the presence of the nusA gene product alone, these transcripts are terminated prematurely. There is a nut-like sequence seen in the 67 base pair fragment immediately following the p_2 promoter which is required for this effect in rrnG (Li et al, 1984). There is also a potential BoxA site (TGCTCTTTAA) seen 154 bases downstream from the start of the rrnB promoter and 100 bases upstream from the NusA-dependent terminator (Olson et al, 1982; Li et al, 1984). Although it has been suggested that NusA may itself be an antiterminator in at least one system (Peacock et al, 1985), its

tendency to promote both pausing and termination in other systems (Greenblatt et al, 1981; Nakamura and Mizisawa, 1985; Grunberg-Manago, 1987) makes this unlikely. Possibly NusA may form a bridge between RNA polymerase and an as yet unidentified E. coli antiterminator.

Another bacterial operon recently found to be regulated by antitermination is the bglG operon coding for genes which catabolize beta-glucosides. This is normally a cryptic operon but is activated by insertions into the bglR locus. The first gene in the operon is bglG and the product of this gene acts as an antiterminator to allow transcription of the remainder of the operon. Without this product, there is Rho-dependent termination stopping more than ninety percent of transcripts in the leader region before the translational start. This product has no effect on transcription if the bglG leader region is not present giving further evidence that the bglG product is an antiterminator (Mahadevan and Wright, 1987).

MATERIALS AND METHODS

A. strains used

The E. coli and S. typhimurium strains used in this study are listed in Table 1. All strains were maintained frozen in 25% glycerol at -70°C and were single colony isolated on plates containing supplemented minimal media plus glucose or L agar (below) with appropriate selections prior to use.

B. media

L broth (10 g/l tryptone (Difco), 5 g/l yeast extract (Difco), 10 g/l NaCl, 0.1% glucose, adjusted to pH 7 with NaOH) and L agar (as above but also containing 1.5% w/v Bacto-agar) with appropriate antibiotic selection were routinely used to grow strains. The selections used included: ampicillin, 100 ug/ml, streptomycin 100 ug/ml; tetracycline, 12.5 or 25 ug/ml; and kanamycin, 40 ug/ml. The ability to ferment lactose was tested on Difco MacConkey agar (17 g/l Bacto-peptone, 3 g/l proteose-peptone (Difco), 1.5 g/l Bacto bile salts No. 3, 0.03 g/l Bacto neutral red, 0.001 g/l crystal violet, 13.5 g/l Bacto agar) containing 0.1% lactose. 2xYT (16 g/l Bacto-tryptone, 10 g/l Bacto-

yeast extract, 5 g/l NaCl) broth was used to grow cells for rescue of single-strand DNA, conjugation efficiency assays, and phage propagation.

A modified form of Davis and Mingioli (1950) minimal agar was used (4.5 g/l KH_2PO_4, 10.5 g/l K_2HPO_4, 0.1 g/l $MgSO_4.7H_2O$, 1 g/l $(NH_4)_2SO_4$; 1.5% Bacto-agar). Glucose, lactose, or sorbitol were used as carbon sources at 10 g/l and the medium was supplemented with amino acids at concentrations of 50 ug/ml as required.

Incubations were carried out at 37°C unless otherwise stated.

C. chemicals

The enzymes used in this study were obtained from Bethesda Research Labs (BRL), Boeringher-Mannheim, New England Bio-Labs (NEB), Pharmacia, and Promega. Radioisotopes were purchased from Amersham and New England Nuclear (Dupont). Electrophoresis quality chemicals (urea, SDS, acrylamide, and bis-acrylamide) were BRL's "ultra-pure" reagents. Sequenase and Sequenase version II were purchased from United States Biochemicals (USB). The TaqTrack sequencing system was obtained from Promega. All other chemicals are of laboratory grade.

D. plasmids used

1. pKZ25

This 6.5 kb plasmid was reportedly created by shotgun cloning of 2-4 kb Sau3A fragments from the rfaH-complementing F'-ilv-met plasmid pDU450 into the BamHI site of the vector pBR322 (Figure 9) (Rehemtulla, 1984). It contains a 2.2 kb insertion and shows ampicillin resistance as well as the ability to complement rfaH defects in both E. coli and S. typhimurium. pKZ25 does not show the tetracycline resistance of the pBR322 vector due to the location of the insertion within the tetracycline resistance gene.

2. pBluescript KS+ and pBluescript KS-

These modified pUC-based cloning vectors were obtained from Stratagene Cloning Systems (San Diego, Cal.). They have a multiple cloning site flanked by the lambda T7 and T3 promoters which are inserted into the coding region of the lacZ gene. The interruption of the lacZ gene by an insert into the multiple cloning site allows for identification of subclones by a blue/white color selection in the presence of IPTG and X-gal. A 454 nucleotide fragment of the intergenic region of M13 has also been inserted between the AatII and NarI sites of the parent plasmid, allowing for isolation of single-stranded DNA. The KS+ and KS-

forms differ only in the orientation of this fragment. The vectors are ampicillin resistant and, in the presence of the M13 helper phage R408, produce a single-stranded infective form which is preferentially packaged by the helper.

E. plasmid isolation

1. Portnoy method (Portnoy et al, 1981)

For the large-scale method, 1 l of L broth, supplemented with ampicillin, was inoculated with a strain carrying the plasmid to be isolated. After overnight incubation with agitation, the cells were pelleted by centrifugation (4000 x g, 5 minutes), washed with 1 volume TE(50/10) (50 mM tris, 10 mM EDTA, pH 8.0), and resuspended in 12 ml TE(50/10) divided between eight Oakridge tubes. The cells were lysed with the addition of 15 ml freshly prepared lysis buffer (4% SDS in TE(50/10), pH 12.2) per tube followed by incubation at 37^{o}C for 15 minutes. The solution was then neutralized by the addition of 750 ul of 2.0 M tris HCl (pH 7.0) and 4 ml 5 M NaCl followed by incubation at 0^{o}C for 4 hours or 4^{o}C for 16 hours.

The lysate was cleared by centrifugation at 36,600 x g for 30 minutes and treated with 4 ug/ml RNAse for 30 minutes at 37^{o}C. The supernatant was then extracted repeatedly with buffer-saturated phenol:chloroform to give a clear interface, then once with 24:1 chloro-

form:isoamyl alcohol (hereafter referred to as "chloroform") to remove excess phenol. The DNA was precipitated with 1 volume isopropanol then dried and resuspended in TE(10/1) (10 mM tris, 1 mM EDTA, pH 8.0) and purified by PEG precipitation or CsCl dye-buoyant density centrifugation followed by dialysis (see below).

2. Small scale DNA preparation by alkaline lysis (Maniatis et al, 1982)

Cells were pelleted from 3 ml of a 4 ml broth culture, which had been grown from four hours to overnight, and resuspended in 100 ul freshly prepared lysozyme solution (10 mM EDTA, 25 mM tris HCl (pH 8.0), 50 mM glucose, 4 mg/ml lysozyme). The suspension was incubated at room temperature for 5 minutes and 200 ul of a 0.2 M NaOH, 2% SDS solution was added. The mixture was incubated on ice for 5 minutes. 150 ul 5 M potassium acetate was added, the suspension was vortexed briefly, and ice incubation was continued for 5 minutes.

The lysate was cleared by centrifugation (10 minutes in a microfuge) and the DNA was precipitated from the supernatant with 2 volumes of 95% ethanol. The DNA was washed with 1 ml 70% ethanol, dried, and resuspended in 50 ul TE(10/1). DNA isolated by this method was normally used directly but could be stored at -20^{o}C.

3. Qiagen preparation (Diagen, 1988)

This method is similar to the small-scale alkaline lysis method (above) but the use of the Qiagen columns prohibits ionic detergents such as SDS. Cells were pelleted from 5 ml overnight culture and suspended in 400 ul freshly prepared 50 mM tris HCl (pH 7.4) with 2 mg/ml lysozyme. The suspensions were incubated on ice for 20 minutes with the addition of 100 ul 0.5 M EDTA after the first 10 minutes. This was followed by addition of 20 ul 2% Triton X-100.

The lysate was cleared by centrifugation (30 minutes in a microfuge) and 450 ul of the supernatant was removed to a new tube. 90 ul 5 M NaCl and 25 ul 1 M MOPS buffer (pH 7.0) were added and the DNA was purified and concentrated using Qiagen columns (below).

F. plasmid purification

1. CsCl dye-buoyant density centrifugation (Radloff et al, 1967)

The DNA was suspended in a small (8 ml/tube) volume of TE(10/1). 1 g/ml CsCl and 100 ul of a 10 mg/ml ethidium bromide solution were added. The use of less ethidium bromide than recommended by Radloff allows for the bands to be visualized without the use of ultraviolet light (P.R. MacLachlan, personal communication).

This sample was then transferred to a quick-seal ultracentrifuge tube and balanced. Samples were layered with light paraffin oil, sealed, and rebalanced.

After centrifugation at 20°C in a Beckman 70.1 Ti rotor (36,000 rps, 40 hours) three bands were visible representing chromosomal DNA, plasmid, and RNA. The plasmid band was recovered from the tube using a syringe and the ethidium bromide removed by repeated extraction with CsCl/water-saturated isobutanol. The CsCl was then removed by dialysis against three changes of TE(10/1) buffer. The DNA was aliquoted and stored at -20°C.

2. PEG precipitation of plasmid DNA (Lis, 1980; Sadhu and Gedamu, 1988)

The DNA was precipitated with ethanol and suspended in 2 ml sterile distilled water or TE(10/1). 800 ul PEG solution (30% PEG6000 in 1.8 M NaCl) was added and incubation was carried out for 4 hours at 0°C or 16 hours at 4°C. The DNA was then pelleted by centrifugation (30 minutes in a microfuge) and resuspended in 400 ul TE(10/1). The solution was extracted repeatedly with buffer-saturated phenol:chloroform to give a clear interface, and once with chloroform to remove remaining phenol.

The remaining PEG was removed by precipitation with 0.5 volumes 7.5 M ammonium acetate prior to precipitation of the DNA. It is important that all the

PEG be removed since it will interfere with most enzyme activity. The remaining liquid was transferred to a new tube and plasmid DNA was precipitated with an additional 2 volumes of 95% ethanol. The pellet was washed twice with 70% ethanol, dried, and resuspended in 200 ul TE(10/1).

3. Qiagen column purification (Diagen, 1988)

These columns are available from Qiagen, Inc., Studio City, CA. The column was first equilibrated with 2 ml buffer A (400 mM NaCl, 50 mM MOPS, 15% ethanol, pH 7.0) and the sample adsorbed by repeatedly pipetting through the column. Proteins, chromosomal fragments, and RNA were removed by washing with 2 ml buffer C (1 M NaCl, 50 mM MOPS, 15% ethanol, pH 7.0), and the DNA was eluted into a 600 ul volume of buffer F (1.5 M NaCl, 50 mM MOPS, 15% ethanol, pH 7.5). DNA was then precipitated with 0.8 volumes isopropanol, washed twice with 1 ml 70% ethanol, dried, and resuspended in TE(10/1). The suspensions were stored under 2 volumes 95% ethanol at -20^{o}C and reprecipitated prior to use. This method gives high recovery of very clean DNA but requires that no ionic detergents (such as SDS) be used in the preparation of the lysate.

G. Digestions with restriction endonucleases

Digestions for mapping were carried out in 20 ul volumes using the conditions recommended by the manufacturers. Digestions for fragment isolations (for subcloning) used larger volumes (100-150 ul). Incubations lasted 1-4 hours. In the case of double digestions requiring different buffers, the DNA was cut first with the enzyme requiring a lower salt concentration, precipitated with 95% ethanol, washed with 70% ethanol, and dried. The DNA was then resuspended in the appropriate volume of salts and buffers for the second enzyme and reincubated with this enzyme.

H. Agarose Gel Electrophoresis

DNA from restriction digestions was mixed with 1/6 volume of tracking dye (bromophenol blue, 0.25%; xylene cyanol, 0.25%; Ficoll 400, 15%) (Maniatis et al, 1982) and loaded onto horizontal gels containing 0.7% agarose in TAE buffer (40 mM tris-acetate, 1 mM EDTA with 0.5% ethidium bromide) for electrophoresis. Electrophoresis was performed with constant voltage of 4-10 volts/cm for 1-4 hours in chambers purchased from Pharmacia or Bio-Rad.

Bands were visualized using a uv-transilluminator and photographed using black-and-white Polaroid Type 57 Land film and a red filter. DNA Molecular Weight Marker II (lambda/HindIII) was used for size and concen-

tration comparison. The marker consists of seven bands of the following sizes (in kb): 23.1, 9.4, 6.6, 4.4, 2.3, 1.0, and 0.56. For size comparison, 200 ng of marker in a volume of 10 ul was loaded. Differing amounts of marker, varying between 200 and 25 ng, were loaded for concentration determination.

I. Southern transfer of genomic DNA (Reed and Mann, 1985)

Restriction digestions containing 30 ug chromosomal DNA were loaded onto a 150 ml 0.7% agarose gel and electrophoresed at 50 v for 7.5-8 hours. S. typhimurium LT2 (SL3770) and E. coli K-12 (NK5992) chromosomal DNA were prepared by D. Sirisena and P. Thirukkumaran, respectively. After electrophoresis, the gel was photographed and treated twice with 0.25 M HCl for 15 minutes to depurinate the DNA. The DNA was denatured by soaking the gel twice, for 15 minutes, with 0.5 N NaOH, 1.5 M NaCl, then neutralized with two changes of 0.5 M Tris-HCl (pH 7.5), 1.5 M NaCl (15 minutes each).

For transfer, two pieces of Whatman 3MM paper were placed on a stand in a pyrex baking dish so that their edges hung into the dish, forming wicks. The paper was wetted with 0.4 N NaOH and the gel was placed, upside down, on the stand. A piece of Zeta-probe nylon membrane, cut to the size of the gel and prewetted in distilled water, was placed on top of the gel and covered with two or three pieces of Whatman paper. A stack of

paper towels, cut slightly smaller than the membrane, were placed on top of the paper and a weight was added to provide pressure. Transfer was carried out in 0.4 N NaOH for a minimum of 8 hours.

The membrane was then placed, DNA side up, on a pad of Whatman paper saturated with 0.5 N NaOH, 1.5 M NaCl for 5 minutes, the neutralized on a second pad saturated with 0.5 M Tris-HCl (pH 7.5), 1.5 M NaCl for 5 minutes. The membranes were rinsed briefly in 2 x SSC buffer (1 x SSC contains 0.15 M NaCl, 0.015 M trisodium citrate) to remove any agarose. The DNA was fixed to the membrane by baking 2 hours at 80^{o}C in a vacuum oven.

J. Preparation of probes

Probes for genomic hybridizations were prepared using the BRL random primers DNA labelling system. 25 ng of the fragment to be labelled was denatured by boiling 5 minutes. dATP, dTTP, dGTP, random primer buffer, and 50 uCi ^{32}P-dCTP were added and the mixture was brought to 49 ul with sterile distilled water. 3 U Klenow (DNA polymerase I, large fragment) were added and the reaction was incubated at room temperature for 2 hours. 5 ul STOP buffer were added and the incorporation was measured as follows. 2 ul reaction mix were diluted in 498 ul distilled water. 5 ul of this dilution was spotted on each of two Whatman GF/C filters. One filter was washed three times with 50 ml 15% tri-

chloroacetic acid with 1.5% sodium pyrophosphate and once with 50 ml 95% ethanol to remove unincorporated nucleotides. The second filter was untreated. Incorporated and unincorporated counts were determined from these filters in a tricarb series 4000 scintillation counter.

Unincorporated nucleotides were removed by repeated precipitation with 0.5 volumes 7.5 M ammonium acetate and 2 volumes 95% ethanol. The probe was then dried and resuspended in TE(10/1).

K. Probing of genomic digests

Membranes with transferred genomic DNA were prehybridized in a sealed bag at 65°C for 4-8 hours in: 5 x SSC, 50 mM sodium phosphate (pH 7.0), 5 x Denhardt's (100 x Denhardt's contains 2 g Ficoll, 2 g polyvinyl pyrrolidone, 2 g BSA, adjusted to 100 ml with 3 x SSC), 1% SDS, 5 mM EDTA, 1 mg/ml denatured salmon sperm DNA. After prehybridization, the solution was removed and replaced with 3-5 x 10^6 dpm/ml probe in 1 ml prehybridization solution. Prior to addition, the hybridization mix was boiled for 5 minutes.

Hybridization was carried out at 68°C for 12-24 hours. The filter was then removed from the bag and washed once in 2 x SSC for 15 minutes, twice in 2 x SSC, 0.1% SDS for 30 minutes, then twice in 0.1 x SSC, 0.1% SDS for 30 minutes. The washes were carried out

at 68-72°C. The filters were blotted, wrapped in Saran wrap, and exposed to Kodak X-Omat AR film 12-48 hours at -70°C, with intensifying screens.

L. Tn1000 insertion mutagenesis

The Tn1000 (gamma-delta) insertion sequence is carried on an F-factor and inserts itself throughout the genome of the host cell, including the chromosome and other plasmids, showing a bias for A-T rich regions (Guyer, 1978; Guyer, 1983; Liu et al, 1987). Thus, the plasmid may be used for insertional inactivation.

Since the sequences at the ends of the insertion are known, they may be used as primers for bidirectional sequencing (Liu et al, 1987). Four sets of Tn1000 insertions were created by two different methods (broth matings and plate matings), using two donors (SAB3490 and SAB3491) - both of which are recA$^-$, F$^+$ E. coli carrying pKZ25.

1. Broth matings (Wang et al, 1987)

The insertions were with SAB3490 or SAB3491 as a donor and SAB2562 as the recipient. Beginning with overnight broth cultures of each, the donor and recipient were diluted (1:40 and 1:10, respectively) with L broth and incubated with shaking for 2 hours. 4 ml of the donor were then mixed with 10 ml recipient and 26

ml warm L broth. Conjugation was carried out, without shaking, in a 37°C water bath for one hour followed by plating in 2.5 ml saline soft agar (0.8% NaCl, 0.7% Bacto-agar).

Dilutions of donor, recipient, and mating mix were plated on various media with selection for resistance to both ampicillin (indicating transfer of the pKZ25 ampicillin resistance gene) and streptomycin (from the chromosomal resistance carried by the recipient).

2. Plate matings

100 ul of overnight cultures of each of the donor (SAB3490 or SAB3491) and recipient (SAB2562) were plated on L agar without antibiotic selection and allowed to grow 4 hours. The confluent growth was then streaked to L agar containing ampicillin and streptomycin for isolation of single colonies resistant to both antibiotics.

M. Phage sensitivity tests (Wilkinson et al, 1972; Hancock and Reeves, 1975)

LPS and tra functions were screened using a battery of phages. The phages were prepared as follows: bacteriophage was added to a 1:50 dilution of an overnight broth culture of sensitive cells (MOI = 2-5 for temperate phages, 0.1 for lytic phages). After growth

at 37°C, the lysates were cleared by centrifugation (12,000 g, 20 min, 4°C), filter sterilized (0.45 um Millipore filter), and stored at 4°C (over chloroform for all except M13). Bacteriophage U3 was stored in 15% glycerol at -70°C. Phage titre was determined using the drop-on-lawn method (Gemski and Stocker, 1967).

For phage pattern tests 1 ml of an overnight broth culture of the strain to be tested was used to flood a plate of L agar with the appropriate antibiotic selection. The excess liquid was removed and the plate was allowed to dry. Phage lysates were held in vials arranged in a grid pattern which was duplicated on the lawn using a multi-pronged phage pattern replicator to deposit 10 ul drops. The replicator was first rinsed in 95% ethanol and flamed, then placed on the grid so that one prong was dipped in each vial. The replicator was then placed briefly on the dried bacterial lawn so that drops of the phage solutions were applied in a consistent pattern. After drying the spots, the plate was incubated for 4-16 hours and the resulting sensitivity patterns were recorded.

N. Testing of F-transfer efficiency

F-transfer efficiency was determined for strains carrying pKZ25::Tn1000 plasmids. The conjugations were carried out as previously described (section I).

Mixes using SL3770 as a recipient were plated on minimal agar with lactose to select for lactose utilization (from F'42lac) and the ability to grow in the absence of amino acid supplements. For the E. coli recipient, SAB3457, transformants were plated on MacKonkey agar plus lactose and tetracycline to determine transfer of the F'42lac to the tetracycline-resistant strain.

O. Isolation of LPS

Following the method of Hitchcock and Brown (1983) 10 ml 2xYT broth was inoculated with 500 ul of an overnight culture and grown, with agitation, for 4 hours (to an OD at 650 nm of 0.2). Cells were pelleted from 7.5 ml of this solution and washed in 1.5 ml phosphate buffer (50 mM sodium phosphate, pH 7.5). Cells were pelleted from this suspension and resuspended in 50 ul lysing buffer (1 M tris, pH 6.8, 10% glycerol, 2% SDS, 4% 2-mercaptoethanol, 0.01% bromophenol blue). The suspension was boiled for 10 minutes and 25 ug of proteinase K was added in 10 ul lysing buffer. Incubation was carried out at 60^{o}C for 1-2 hours and the solution was boiled again for 5 minutes to remove the enzyme. The solutions were loaded on 20 cm long, 1.0 mm diameter SDS-polyacrylamide gels (below).

P. fragment isolations for subcloning

Fragments were isolated using one of three methods; GENECLEAN purification, electroelution, or "Freeze-Squeeze" purification. In each case, the fragment to be isolated was visualized on a 0.7% agarose gel using uv light (either a long-wave light source or a short-wave transilluminator with the majority of the gel shielded to prevent the formation of thymine dimers). The fragment was then excised from the gel by slicing with a razor blade and the DNA was purified as follows:

1. GENECLEAN purification of fragments

Purifications used the instructions and materials of Bio101, Inc., La Jolla, CA. Gel slices were weighed and dissolved at 55°C in 2.5 volumes NaI solution. 5 ul GLASSMILK suspension was added to each tube and allowed to bind to DNA for 5 minutes on ice. The GLASSMILK with bound DNA was then pelleted and washed three times with 250 ul NEW wash (NaCl/ethanol/water) to remove contaminants. The pellet was then resuspended in 20 ul TE(10/1) and the DNA eluted at 55°C for 5 minutes. The excess GLASSMILK was pelleted out and the DNA-containing supernatant removed to a new tube. The elution step was normally carried out twice for a final volume of 40 ul.

2. Electroelution of fragments

Electroelution used an International Biotechnologies unidirectional electroeluter (analytical). The agarose band containing the desired fragment was excised as described above and placed in one of the six sample wells on the device. The chamber was filled with 0.5 x TBE buffer (50 mM tris, 1.25 mM EDTA, 5 mM boric acid, pH 8.8) so that the liquid level was even with the top of the gel slice. 75 ul of salt cushion (1 mg/ml Bromophenol blue in 7.5 M ammonium acetate) was loaded and 125 volts applied for 30-45 minutes to drive the DNA into the salt cushion.

Approximately 500 ul liquid was removed from the salt cushion reservoirs and the DNA was precipitated from this using 0.04 volumes 2.5 ug/ml calf thymus tRNA and 2 volumes 95% ethanol. The DNA was washed in 70% ethanol and resuspended in TE(10/1).

3. Freeze-squeeze purification

The excised gel slice was wrapped in parafilm and frozen for a minimum of 20 minutes at -20°C (no maximum). The DNA was removed from the slice by crushing in a garlic press and collecting the resulting liquid. 100 ul TE(10/1) was then added to the pressed agarose and the slice was squeezed again. All liquid was collected in a 1.5 ml microfuge tube, extracted three times with buffer-saturated isobutanol, then precipi-

tated with 2 volumes 100% ethanol. The DNA was washed in 70% ethanol, dried, and resuspended in 0.5 M NaCl in TE(10/1).

The DNA was further purified and concentrated using Elutip-d columns (Mandel Scientific Co., Rockwood, Ont.). The column was prepared with a high-salt prewash (1 M NaCl in TE(10/1)) then primed with a low-salt solution (0.2 M NaCl in TE(10/1)). The DNA solution was then forced through a prefilter and the column in order to allow it to adsorb to the matrix. Impurities were removed by washing with the low salt solution, the DNA was eluted into a microfuge tube using the high salt solution, precipitated with 2 volumes 100% ethanol, washed with 70% ethanol, dried, and resuspended in TE(10/1).

Q. Subcloning

Fragments to be subcloned were prepared as described (above). Ligation reactions were carried out using 20 fmol vector with vector:insert ratios of 1:3, 1:1, and 3:1 in a 20 ul volume of ligation buffer (50 mM tris, pH 7.6, 5% PEG8000, 10 mM $MgCl_2$, 1 mM ATP, 1 mM DTT) (King and Blakesly, 1986). 2 U T4 DNA ligase were added and ligations were carried out at room temperature for 4 hours. Reactions were stopped by the addition of 0.5 ul 5 M EDTA and were stored at $4^{o}C$.

1 ul of each ligation mix was used to transform XL1Blue by electroporation (below). Transformants were screened for ampicillin resistance and lack of blue color when grown on media containing IPTG and X-gal, then for the desired plasmids.

R. Production of competent cells / transformation

1. $CaCl_2$ Heat-shock method (Lederberg and Cohen, 1974; MacLachlan and Sanderson, 1985)

In the $CaCl_2$ shock transformation method, 100 ml L broth was inoculated with 1 ml of an overnight culture of the strain to be transformed. This was grown with shaking approximately two hours (OD at 640 nm of 0.2) and chilled on ice for 20 minutes to stop growth. Cells were then pelleted from 40 ml and washed with 40 ml of 0.1 M $MgCl_2$, repelleted, and resuspended in 20 ml of 100 mM $CaCl_2$ in 50 mM MOPS buffer. The suspension was incubated on ice for 25 minutes and the cells were pelleted and resuspended in 1 ml of the $CaCl_2$ solution. These cells were kept on ice until used (same day).

For transformation, 100 ul of the competent cells were placed in a tube with a small volume of DNA (less than 1 ug in 20 ul). This mixture was subjected to heat shock at 42°C for 2 minutes, and cooled on ice for 5 minutes. 1 ml chilled L broth was then added to the

mixture and incubation was carried out, without shaking, at 37°C (32°C for recipients carrying the cI857 repressor (Section U)) for 1-2 hours prior to washing in minimal salts solution and plating on selective media.

2. Electroporation (Dower et al, 1988)

Cells exposed to high voltages become capable of taking up free DNA from their environment. The efficiency of this procedure is affected by the concentration of cells, phase of cell growth, concentration of DNA, pulse strength, and ionic strength of the suspension (McIntyre and Harlander, 1989). In this case, the charge was produced using a Bio-Rad Gene Pulser set at 25 uF and 2.5 kv. The output was directed through a Pulse Controller Unit (Bio-Rad) and the electroporations were carried out in Bio-Rad cm cuvettes with 0.2 cm between electrodes.

1 ml of an overnight culture of the cells to be transformed was used to inoculate 500 ml 2xYT or L broth and the culture was incubated to an OD at 640 nm of 0.75. Cells were then chilled on ice for 10 minutes to stop growth and pelleted.

The conductivity of the cells was reduced by washing in one volume 1 M HEPES (pH 7.0), followed by 0.5 volumes 1 M HEPES, 0.1 volumes 10% glycerol, and final

resuspension in 3 ml 10% glycerol. Electrocompetent cells were kept on ice and used the same day or aliquoted and frozen at -70°C.

For each transformation, 40 ul competent cells and a small amount of DNA (i.e., 1 ul of ligation mixes) were mixed in a cold electroporation cuvette. After the pulse, 1 ml SOC broth (2% Bacto tryptone, 0.5% Bacto Yeast Extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) was added and the cells were incubated at 37°C, without shaking, for 2 hours prior to plating.

S. Single-strand DNA rescue

25 ml 2xYT broth was inoculated with 500 ul of an overnight culture of XL1Blue or JM109 carrying the desired M13-based plasmid. Incubation was carried out with vigorous shaking to an OD of 0.3 at 600 nm and the helper phage R408 was added at an MOI of 20:1. Incubation was continued for a minimum of 8 hours.

The cells were pelleted (12,000 x g, 15 minutes) and the supernatant was removed to a fresh tube. 1/10 volume of 3 M NaCl, 25% PEG was added and the mix was allowed to sit at room temperature for 15 minutes.

The phage particles were then pelleted (12,000 x g, 20 minutes) and the supernatant was thoroughly drained. The pellet was resuspended in 750 ul

TE(10/1), and extracted repeatedly with phenol:chloroform to give a clear interface, then extracted once with chloroform.

Excess PEG was removed as described previously (section F-2). The DNA was resuspended in 20 ul TE(10/1) and concentration was determined by absorbance at 260 nm using 5 ul of the suspension diluted 1/200 in TE(10/1) (1.0 OD_{260} indicates 50 ug DNA/ml (Maniatis et al, 1982)).

T. DNA sequencing

Sequencing was done by the Sanger method (Sanger et al, 1977; Sanger et al, 1980). All double-stranded and most single-stranded DNA sequencing was done using Sequenase or Sequenase version II. This modified form of T7 DNA polymerase is highly processive and has no 3′ to 5′ exonuclease activity. Sequenase is also capable of incorporating nucleotide analogs (dITP) which reduce compressions in sequence by decreasing the potential for secondary structure (Tabor and Richardson, 1987).

In some cases, Taq polymerase was used for single-stranded sequencing. This enzyme is capable of functioning at 70°C - normally high enough to melt secondary structures.

1. Double-stranded sequencing

4-5 ug of the desired plasmid, prepared by Qiagen or PEG precipitation, was denatured at room temperature with 0.1 M NaOH, 0.1 M EDTA for five minutes, then precipitated with 0.4 volumes 5 M ammonium acetate and 4 volumes 100% ethanol. The DNA was washed with 70% ethanol and resuspended in sterile water.

Annealing was carried out for 30 minutes at the Td-5 appropriate for the primer (based on AT/GC content). Primer was added to a ratio of 10:1 (primer:template). One fifth volume of 5 x Sequenase buffer (200 mM tris, pH 7.5, 50 mM $MgCl_2$, 250 mM NaCl) was added and the volume increased with sterile water to 10 ul.

Labelling and sequencing were carried out as for single-stranded DNA (below). (Korneluk et al, 1985; Toneguzzo et al, 1988).

2. Single-stranded sequencing

For each labelling reaction, 0.5 pmol single-stranded DNA was annealed in a 1:1 ratio with the desired primer. One fifth volume of 5 x Sequenase buffer was added to the reaction and annealings were carried out for 30 minutes at the appropriate temperature. Ordinarily, dGTP and dITP reactions were car-

ried out simultaneously, so the typical annealing reaction held 1 pmol in a volume of 20 ul (10 ul for each of the two labelling reactions).

For the Sequenase labelling reactions, 1 ul 0.1 M DTT and 2 ul of the dGTP or dITP labelling mix (1.5 uM dGTP or 3 uM dITP and 1.5 uM dCTP, 1.5 uM dTTP) were added to 10 ul annealed template:primer mix. 0.5 uCi ^{35}S(dATP) in 0.5 ul was added as label and the incorporation was begun with the addition of 3.25 U Sequenase in 2 ul. Labelling was continued at room temperature for 5 minutes or 2 minutes in the case of dITP reactions since the secondary structure is destabilized during synthesis and this may lead to unwanted degradation (USB product information).

3.5 ul of the labelling reaction was then added to each of four prewarmed tubes containing 2.5 ul of the appropriate termination mix (ddGTP, ddATP, ddCTP, or ddTTP). Incubation was continued at 37^{o}C for 5 minutes (again, 1 minute in the case of dITP reactions) and 4 ul STOP solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FF) was added. Reaction mixes were stored at -20^{o}C and heated to 90^{o}C just prior to loading on sequencing gels (Toneguzzo et al, 1988).

3. Sequencing gels

DNA sequence was resolved using thin acrylamide gels (Sanger and Coulson, 1978). The gels were typically 6 or 8% acrylamide using a 38:2 ratio of acrylamide:bis-acrylamide. Gels contained approximately 50% (w/v) urea for denaturation of the DNA. The gels were wedge-shaped ranging in width from 0.4 mm to 1 mm and were run on a Bio-Rad S2 sequencing apparatus at a constant power of 50 W for 3-11 hours in 1 x TBE buffer (100 mM tris pH 8.0, 90 mM boric acid, 2 mM EDTA).

Gels were fixed in 5% acetic acid, 5% methanol for a minimum of 1 hour to remove urea (interferes with beta emissions) and dried 90-120 minutes with heating to 80^{o}C for the first 60 minutes. Autoradiography was performed at room temperature using Kodak XAR-5 X-ray film.

U. Expression of rfaH from the T7 promoter

The plasmid pGP1-2 carries the bacteriophage T7 RNA polymerase gene under control of the lambda p_L promoter. This is made heat-inducible by the use of the mutant cI857 repressor (Tabor and Richardson, 1985). When induced, the polymerase may comprise 20% of the soluble protein of the cell. This system, coupled with inhibition of the normal cell polymerases, permits exclusive expression of genes under control of the T7 promoter (Figure 10).

The strain DLT111, which carries plasmid pGP1-2, was transformed with plasmids pKZ87 and pKZ91, which carry the gene of interest under the control of the T7 promoter, and grown overnight at 32°C. 2 ml of this culture was used to inoculate 20 ml of fresh LB broth and the culture was grown at 32°C for approximately 2 hours (OD_{550} of 0.6). The cells were pelleted from 2 ml of this culture, washed twice with 1 volume 10 mM phosphate buffer (pH 7.4), and resuspended in 0.75 volume M9 media (22 mM Na_2HPO_4, 22 mM KH_2PO_4, 8.6 mM NaCl, 18.7 mM NH_4Cl, 2 mM $MgSO_4$, and 0.1 mM $CaCl_2$ (Maniatis et al, 1982)) containing 1% glucose and 1% methionine assay powdered media (Difco) (Latour and Weiner, in press). The cells were then incubated at 32°C for 90 minutes to starve them for methionine.

Expression from the T7 RNA polymerase promoter was begun by placing the cultures at 45°C for 15 minutes. 40 ul of a freshly prepared 10 mg/ml solution of methanolic rifampicin was added to suppress transcription from promoters for bacterial RNA synthesis, and incubation continued at 45°C for 20 minutes. The cultures were cooled to 32°C for 20 minutes and labelled with 5 uCi [^{35}S]methionine (15 minutes, 32°C). The cells were then pelleted, washed twice with 1 volume phosphate buffer, and resuspended in 60 ul cracking buffer (60 mM tris, 10% glycerol, 1% SDS, 0.01% bromophenol blue, pH 6.8). Samples were boiled 3 minutes prior to loading.

The resulting mixes were run on an 18 cm long, 1.5 mm thick 12% acrylamide gel to resolve proteins (below).

V. SDS Polyacrylamide Gel Electrophoresis (Ames, 1974; Tsai and Frasch, 1982; Hitchcock and Brown, 1983)

1. Silver Staining for LPS (Tsai and Frasch, 1982)

SDS-PAGE was performed as described by Hitchcock and Brown (1983) using 12% or 15% separating and 6% stacking gels except that the gels contained 0.1% SDS. 1 mm thick gels were run at 24 mA/gel for 5 hours and fixed at least one hour in 40% methanol, 10% acetic acid.

The gels were washed twice with 10% ethanol, 5% acetic acid, then oxidized with 30 mM periodic acid in 10% ethanol, 5% acetic acid. The gels were washed repeatedly in distilled water, stained (in 0.02 N NaOH, 0.2% ammonium hydroxide, 0.7% silver nitrate), washed again in water, and developed (50 mg citric acid (monohydrate), 500 ul 37% w/v formaldehyde in 1 l distilled water). After developing, gels were placed in 5% acetic acid STOP solution, and stored in distilled water.

2. Coomassie blue staining and autoradiography for proteins

SDS-PAGE was performed using 12% acrylamide gels. 1.5 mm gels for proteins were run at 35 mA/gel for 3 hours then stained 2-3 hours with Coomassie blue (2% R250 Coomassie blue in 25% isopropanol, 7% acetic acid). Gels were destained with 5% acetic acid, using Kimwipes to absorb dye from the liquor, and treated with AMPLIFY (Amersham) prior to drying and autoradiography. Gels were dried 2.5-3 hours with heating to 80^{o}C for the first 90 minutes. Autoradiography was carried out at -70^{o}C using Kodak XAR-5 X-ray film.

W. Preparation of primers

Appropriate primer sites were determined by examination of single-stranded DNA secondary structure using the program PCFOLD (Michael Zucher, National Research Council of Canada). 17-base primer sites for which the Td-5 was sufficient to melt nearby secondary structure were noted. Primers were produced by the Regional DNA Synthesis Laboratory in Calgary. Stocks of primers were maintained at 0.5 pmol/ul in TE(10/1). The Td-5's for primers used are listed in Table 2.

X. Analysis of DNA sequence

Sequence analysis was carried out using a variety of computer programs. Principal among these were the ESEE sequence editor (E. Cabot, Burnaby, B.C.) for comparison of sequences from gels, and DM5 (Genetics Software Center, Dept. of Molecular and Cellular Biology, University of Arizona) for determination of open reading frames, protein size, and amino acid composition of the gene product. Homology with other known proteins was also checked using the MicroGenie data base (Queen and Korn, 1984).

RESULTS

A. Identification of the pKZ25 insert as E. coli sequence

Two 0.5 kb fragments of the 2.2 kb insert region of the rfaH-complementing plasmid pKZ25, one from either side of the HindIII site (Figure 11) were used to probe genomic digests of E. coli K-12 and S. typhimurium LT2. Under stringent hybridization conditions (see Materials and Methods), both hybridized with the E. coli chromosome (Figure 12). Similar digests of the S. typhimurium showed no hybridization (data not shown). These data indicate that the rfaH coding region and flanking sequence of pKZ25 originate from E. coli and not S. typhimurium, as was previously believed.

Figures 12a and 12b represent probings of the same membrane (containing E. coli K-12 chromosomal digests) with two different probes from pKZ25 (PvuII-SacI and PstI-NarI, respectively). Lane 1 on Figure 12a is a positive control for hybridization with the PvuII-SacI fragment of pKZ25 (0.5 kb), the probe used in this Figure. There is a great deal of DNA in this lane (25 ng) leading to a very bright spot on the autoradiogram. The hybridization in this area of the membrane was so intense that it could not be completely removed

resulting in a spot in the same position on Figure 12b (lane 1). Lane 1 of Figure 12b contains uncut pKZ25 as a hybridization control on this gel.

On both autoradiograms (Figure 12a and b), the probes hybridize to a NarI fragment of greater than 23 kb (lane 2 on both autoradiograms). This fragment contains the hybridizing PvuII fragment since PvuII and PvuII-NarI both show bands of 4.9 kb (lanes 3 and 4 on both autoradiograms). With the pKZ25 PvuII-SacI probe, BamHI shows a single hybridizing fragment of 9.6 kb and HindIII a slightly larger 10.7 kb fragment (Figure 12a, lanes 6 and 7). The apparent bands found around 23 kb in both of these lanes are artifacts representing poorly digested chromosomal DNA. The PvuII-HindIII double digestion (Figure 12a, lane 5) does not show a hybridized band with the PvuII-SacI probe, possibly as a result of insufficient distribution of the probe. The second probe, containing an internal NarI-PstI fragment which includes 350 nucleotides of the rfaH coding region (Figures 11,14) shows a small hybridizing HindIII chromosomal fragment (1.6 kb) which is contained within the hybridizing PvuII fragment (Figure 12b, lanes 5 and 6). The second probe also hybridizes to a BamHI fragment of more than 23 kb (Figure 12b, lane 7).

The E. coli sfrB gene maps at 86 minutes (Beutin and Achtman, 1979; Bachmann, 1987). A comparison of hybridizing fragment sizes with the map of the E. coli chromosome (Kohara et al, 1987) (Figure 13) shows a 9.4 kb BamHI fragment within a 10.7 kb HindIII fragment at this position. There is a 1.7 kb overlap between the

HindIII fragment and a larger PvuII fragment. Adjacent to the 9.4 kb BamHI fragment is a fragment of 26.7 kb which corresponds to the large BamHI fragment seen with the second probe. The small HindIII fragment also maps in this area as 1.3 kb and is contained within the same PvuII fragment. The PstI site seen in both pKZ25 and pKZ17 is found within the 1.3 kb HindIII fragment (Figure 13). The map does not include data for NarI. The disparity in size between the map PvuII fragment (7 kb) and the hybridizing band (4.9 kb), may be an artifact of electrophoresis or the result of a second PvuII site which is not seen in the E. coli W3110 strain used by Kohara et al (1987). The adjacent BamHI sites, which hybridize to different probes, indicate that the BamHI in pKZ17, but not in pKZ25, is also found in the chromosome.

B. Characterization of pKZ25

Two rfaH$^-$ E. coli K-12 strains, SAB2562 and SA2885, were previously transformed with pKZ25 to create SAB2845 and SA2888, respectively (Rehemtulla, 1984). An rfaH$^-$, F$^+$ S. typhimurium LT2 strain, SA2887 was transformed with the plasmid to give SA3444 (Table 1). rfaH-complementing activity was confirmed using phage sensitivity, and the restriction map was determined (below).

1. Complementation of *rfaH* by phage sensitivities

Wild type strains of *E. coli* K-12 are sensitive to the *E. coli* LPS core-specific phage U3 and resistant to the rough-specific phage C21 (Table 3, line 1). *rfaH*$^-$ strains show opposite sensitivities to these two phages. The phage patterns in Table 3 confirm the presence of *rfaH*-complementing activity of pKZ25 in *E. coli* resulting in a pattern of phage sensitivity similar to that of *rfaH*$^+$ K-12 (lines 3,5).

In the absence of other mutations, *rfaH*$^+$ *S. typhimurium* are sensitive to the smooth-specific phages: P22, P22h, KB1, 9NA, and FO. The non-lysogenizing variants P22.*c1*, P22h.*c1*, and KB1*int*, were used (Boro and Brenchley, 1971; McIntire, 1974). Felix O (FO) lyses smooth *S. typhimurium* strains as well as rough strains with core regions terminating in GlcNAc (chemotype Ra) (Wilkinson et al, 1972; Lindberg, 1973). *rfaH*$^-$ strains are generally resistant to these phages, although some susceptability to FO is seen, and sensitivity to the rough-specific phages: 6SR, Ffm, Br60 and C21 but Br60 is also female-specific (K.E. Sanderson, personal communication). An *rfaH*$^-$ *S. typhimurium* strain carrying pKZ25 has phage sensitivity resembling the smooth strain (Table 3, line 9).

In both species, the presence of F-pili is indicated by sensitivity to the male-specific phages M13 and f2. In the *rfaH*$^-$ cells, which lack *tra* function, the numbers of pili are greatly reduced, so F$^+$ *rfaH*$^-$

strains are resistant to these phages (Table 3, lines 4,8). The plasmid converts these strains to sensitivity to both these phages (Table 3, lines 5,9). These data indicate that the gene carried by pKZ25 complements both the LPS$^-$ and Tra$^-$ properties of the rfaH$^-$ mutants.

2. Restriction mapping of pKZ25

Using the large-scale Portnoy method, pKZ25 was isolated from SAB2845 for restriction mapping. 34 digestions were carried out with enzymes reported to have no more than one site in the pBR322 vector (Maniatis et al, 1982; BRL and Pharmacia catalogues) (Table 4). The reaction mixtures were analyzed on 0.7% agarose gels and fragments resulting from the digestions were counted. Fragment sizes were determined by comparing their mobility on agarose gels with that of known standards and insert sites were mapped relative to known vector sites. Nine of the 34 enzymes showed higher numbers of fragments from digestion of pKZ25 than are predicted from the vector sites. These extra fragments are the result of sites within the 2.2 kb insert region.

One enzyme, BamHI, showed fewer restriction sites in pKZ25 than in pBR322 (Table 4, line 5) due to the loss of the vector site during the creation of pKZ25 (Figure 9). Those enzymes which were shown to cut only

in the insert region of the plasmid were mapped relative to the vector HindIII and PvuII sites. The resulting restriction map of pKZ25 is shown in Figure 11.

C. Tn1000 insertion mutagenesis

1. Determination of complementation by phage sensitivities

Tn1000 insertions were generated as described (Materials and Methods, section L). Sixty-eight of the resulting colonies (ampicillin and streptomycin resistant) were picked to broth and rfaH-complementing activity was determined based on phage sensitivities. The low numbers of donor cells and the short conjugation period made it likely that each individual colony was the result of an independent conjugational event. The recipient strain used in the conjugation contained the mutation sfrB14 (Table 1) and thus had an rfaH$^-$ phenotype which was complemented by the rfaH gene on pKZ25. If a Tn1000 had been inserted into this gene, this complementation would be lost resulting in the resistance to U3 and sensitivity to C21 seen in the uncomplemented recipient.

Based on phage sensitivity tests, 63 of the isolated colonies, 22 of which are shown in Table 5 (lines 3-11,19-31) did not lose rfaH-complementing activity (i.e., were resistant to C21 and sensitive to U3). Seven of the colonies no longer showed full complement-

ation. Since the transfer of pKZ25 carrying the Tn1000 insertion requires that the donor cell F-factor also be transferred, Br60 resistance is expected in all strains since this phage is "female specific". Sensitivity to M13 and f2 is expected in strains with rfaH-complementing activity from restoration of the tra functions. Loss of complementation, as a result of insertion of the transposon into the plasmid-borne rfaH gene results in a loss of this sensitivity. The plasmids were separated into three groups based on their rfaH-complementing ability and the locations of the Tn1000 insertions (Figure 15). Those insertions designated with "rfaH" (e.g., rfaH3118::Tn1000) cause loss of rfaH complementation and, as expected, all of these insertions map within the insert of chromosomal DNA in pKZ25. Those designated "zhj" do not inactivate the pKZ25 rfaH gene but do map within the insert region of the plasmid. The remainder, designated "pKZ25", do not inactivate rfaH and map within the pBR322 vector (Figure 15).

Eight of the recombinant plasmids, three showing rfaH complementation and five which were non-complementing, were then transformed into SA2887, an F^+, rfaH$^-$ S. typhimurium strain (Table 1, line 30). Analysis of phage sensitivities in the resulting strains (Table 6) showed varying degrees of loss of rfaH-complementing activity in the non-complementing plasmids which were similar but not identical to complementation in E. coli. One insertion, called rfaH3118::Tn1000 causes a complete lose of complementation in both strains (Table 5, line 18; Table 6, line 6).

rfaH3122::Tn1000 shows complete complementation in E. coli (Table 5, line 14) but only complements the Tra defect in S. typhimurium (i.e. it is LPS$^-$) (Table 6, line 10). rfaH3123::Tn1000 and rfaH3119::Tn1000 complement the tra but not the LPS defects in both strains (Table 5, lines 13,17; Table 6, lines 7,9). rfaH3121::Tn1000 shows no complementation in S. typhimurium (Table 6, line 4) but the E. coli strain carrying this plasmid is U3 sesitive (Table 5, line 15) indicating a complete LPS core.

2. Mapping of insertions

Plasmids from thirty-nine of the colonies recovered from conjugation were mapped using SalI and EcoRI, each of which cuts pKZ25 once and has known sites in Tn1000. Generally SalI, which divides the transposon in half (Figure 16), was used to determine the location of the insert and EcoRI, which cuts asymmetrically, to determine orientation. No obvious preference for one orientation over another was seen with eighteen of the insertions in the gamma-delta orientation and eleven in delta-gamma (Figure 15). The insertions were located throughout the plasmid with the exception of areas around 4 kb, 5 kb and between 5.5-6.5 kb (Figure 15).

One of the resulting plasmids, pKZ25-5::Tn1000 was shown to carry two insertions (labelled pKZ25-5::Tn1000a and b on Figure 15), both in the pBR322 vec-

tor. Since the transposon is replicated many times and inserts itself randomly, there is no reason that this should not occur.

3. Determination of conjugation efficiency

Restoration of the tra-mediated transfer functions was examined using rfaH$^-$ F$^+$ strains carrying pKZ25::Tn1000 plasmids as donors in conjugation. Conjugation efficiencies were determined for eight of the Tn1000 insertions, three complementing (zhj::Tn1000) and five non-complementing (rfaH::Tn1000) (Table 6), using strains created by transformation of the recombinant plasmids into SA2887. The results of these conjugations are shown in Table 7. The only strains giving the frequency of conjugation resembling the strain SA3797 (rfaH3074 with no pKZ25 plasmid) (i.e. showing no complementation for the tra functions) were those carrying rfaH3118::Tn1000, rfaH3119::Tn1000, and rfaH3121::Tn1000 (lines 5,7,8). Two of the strains in Table 7, rfaH3123::Tn1000 and rfaH3122::Tn1000, did show complementation of the tra functions, but were LPS$^-$ from phage sensitivities and appearance on silver-stained gels.

4. Determination of LPS chemotype

Using four complementing and six non-complementing (from phage sensitivities shown in Table 5, lines 3-5, 7, 12-14, and 16-18) pKZ25::Tn1000 recombinant plasmids carried in rfaH⁻ strains of E. coli and S. typhimurium (SAB2885 and SA3797, respectively), LPS was extracted and mobilities determined on silver-stained SDS-PAGE gels. The resulting gels are shown in Figures 17 (E. coli) and 18 (S. typhimurium). On these gels, smooth Salmonella LPS (consisting of a complete core region with attached side chains) is separated into as many as forty components, resulting in a ladder-like appearance which normally fills the entire length of the lane (Figure 18a, lane 10; Figure 18b, lane 2). Each band in the ladder represents a side chain with one more repeating unit than the one below it. Rough LPS may show a few bands or migrate as a single spot, representing the core region (Figure 18a, lanes 1, 7-9; Figure 18b, lanes 1, 3, 4, 9). E. coli K-12, although rough, has a heterogeneous core (Figure 17a, lane 1; Figure 17b, lane 2) as do rfaH mutants (lane 3 in Figures 17a and b). Different chemotypes may be distinguished by their mobility on these gels, the shorter core regions showing faster migration (for example, Figure 18a, lanes 7-9 contain LPS from strains of genotype rfaG (chemotype Rd_1), rfaL (chemotype Ra), and rfaI (chemotype Rb_3), respectively) (Palva and Makela, 1980; Goldman and Lieve, 1980; Hitchcock and Brown, 1983).

As predicted by phage sensitivities, some rfaH⁻ strains carrying the pKZ25-based plasmids with Tn1000 insertions produced LPS whose mobility was characteristic of rfaH⁻ strains while others showed a restoration of the complete core of E. coli K-12 or the ladder typical of wild-type S. typhimurium LPS. In both species, the strains carrying plasmids with the Tn1000 insertions labelled "zhj" were fully complemented (rfaH⁺) (Figure 17a, lanes 6, 10; Figure 17b, lanes 7, 10, 11; Figure 18a, lane 4; Figure 18b, lanes 6-8). In E. coli all of the rfaH⁻ strains carrying plasmids with Tn1000 insertions labelled rfaH showed LPS typical of that of rfaH mutants (Figure 17a, lanes 7-9; Figure 17b, lanes 6,8,9) except rfaH3122::Tn1000 (Figure 17b, lane 7) which does show LPS complementation. This is consistent with the phage sensitivities seen in the E. coli strains carrying these plasmids (Table 5). In S. typhimurium this plasmid (Figure 18b, lane 5), along with those containing rfaH3119::Tn1000, rfaH3120::Tn1000, and rfaH3120::Tn1000 (Figure 18a, lanes 3,5,6) shows partial complementation (growth of the core beyond that seen in the rfaH⁻ control (Figure 18a, lane 1; Figure 18b, lane 9). S. typhimurium strains carrying these plasmids were resistant to smooth-specific phages (Table 6) and do not show a complete side chain (Figure 18). In both species, the addition of the pKZ25 plasmid carrying the insertion rfaH3118::Tn1000 resulted in no complementation (rfaH⁻) (Figure 17a, lane 9; Figure 18a, lane 2).

D. Sequencing of the rfaH-complementing region of pKZ25

Double-stranded sequencing was begun using primers made by the Regional DNA Synthesis Laboratory to the gamma and delta ends of the Tn1000. This method did not give consistently satisfactory results, possibly due to the large size of the pKZ25::Tn1000 plasmids (12.5 kb) and sequencing of the gene could not be completed in this way. In order to obtain more reliable sequence data across the region, single-stranded sequencing was required. For this the plasmids pKZ87 and pKZ91 were created as a source of single-stranded DNA (Figure 19).

1. Production of pKZ87 and pKZ91

It was determined from Tn1000 insertions (Figure 15) that all rfaH-inactivating insertions fall within the 1.03 kb fragment between the pBR322 SalI site and the insert SacI site (Figures 11,15). This fragment was isolated following double digestion by the GENECLEAN method and ligated into the multiple cloning sites of pBluescript KS+ and KS- (Figure 19) which were previously cut with SacI and SalI to insure the orientation of the inserts. The ligation mixtures were transformed into XL1Blue by electroporation with selection for ampicillin resistance.

In both cases, three sizes of plasmid were seen from isolations of ampicillin resistant colonies. Plasmid identities were confirmed by restriction analysis and Figure 20 shows the results of these digestions for pKZ87. Colonies carrying the smallest of the plasmids (2.9 kb) gave a blue colour in the presence of IPTG and X-gal and showed a restriction pattern consistent with that of the pBluescript plasmids (Figure 20, lanes 10-16).

The other two plasmids gave white, ampicillin resistant colonies on selective media. The larger of the two (6.5 kb) was determined to be pKZ25 (Figure 20, lanes 2-9) and the remaining, 4.2 kb plasmids were the desired subclones (Figure 20, lanes 17-23) and were designated pKZ87 (the fragment carried in pBluescript KS-) and pKZ91 (carried in pBluescript KS+).

rfaH activity was confirmed in these subclones by showing their ability to complement *rfaH*$^-$ mutants, since both restored C21 resistance and U3 susceptability in *rfaH*$^-$ *E. coli* and the smooth-specific phage pattern in *rfaH*$^-$ *S. typhimurium* (data not shown). DNA sequencing was begun using these two plasmids (Figure 21).

2. Production of pKZ157 and pKZ158

pKZ157 consists of the SacI-HindIII fragment of pKZ25 (Figure 11) inserted into the SacI and HindIII sites of the multiple cloning site of pBluescript KS+

(Figure 19). pKZ158 is a PstI-SnaBI deletion of pKZ91 (Figure 19). As expected from Tn1000 insertion data, these plasmids do not complement rfaH defects as shown by phage patterns (data not shown).

3. Construction of primers

With the help of the program PCFOLD, new template sites were chosen. To complete the sequence of rfaH, primers were made, by the Regional DNA Synthesis Laboratory, to areas located partway through the insert regions of pKZ87 and pKZ91 (Table 2, Figure 14). The 17-base primers were complementary to regions with minimal secondary structure.

E. Sequence of the E. coli rfaH gene

The sequence of the rfaH-complementing region of pKZ25 from the insert SacI site to the Sau3A/BamHI junction where the pBR322 vector sequence begins, is shown in Figure 14. Of the possible open reading frames (ORF) seen, only two begin with a methionine (ATG) - the preferred initiation codon - and end with a termination site (TER). These are also the only ORFs larger than seventy nucleotides. The larger ORF is 483 nucleotides and is one of the two beginning with a methionine (ATG) codon (nucleotide 293) - the other is contained within it (393 nucleotides, codon beginning

at nucleotide 383). The larger ORF codes for a 160 amino acid protein product with a predicted molecular weight of 17.9 kDa. Based on the size of the protein product and the position of the ORF, this is believed to represent the coding region of the rfaH gene.

The -35 consensus region, seen at nucleotide 202 has the sequence CTCTGACGGTAT. Seventeen nucleotides downstream from the last position of this region -35 is the first position of a 6 base -10 consensus sequence, TATAAT. It is separated from the probable Shine-Dalgarno sequence at nucleotide 285 (GAG) by a stretch of 51 bases. Double-stranded sequencing allowed for location of the Tn1000 insertions within the gene and it was found that four of the five non-complementing insertions fell within a single, 12 bp, region within the proposed ORF (Figure 14).

The 17.9 kDa protein product predicted by this sequence has a pI of 7.81 and is neither strongly hydrophobic nor hydrophilic (Figures 14, 22). This protein product was not found to show significant homology with any other recorded proteins, including the products of the N gene, NusA, and NusB as determined with the MicroGenie database (Queen and Korn, 1984).

F. Production of the rfaH gene product using the T7 promoter

Assuming that the ORF postulated from the DNA sequence shown in Figure 14 is correct, pKZ87 and pKZ91 contain the rfaH coding region in the correct orientation for expression from the T7 promoter. mRNA for the protein was transcribed from this promoter by the method of Tabor and Richardson (1985) and labelled with ^{35}S. The resulting autoradiogram (Figure 23) shows labelled bands of approximately 19 kDa - in agreement with the predicted size of the protein product from DNA sequence analysis as well as with earlier data (Rehemtulla, 1984).

DISCUSSION

A. Southern blotting results

Digestions of the chromosomes of *rfa*$^+$ strains of *E. coli* K-12 and *S. typhimurium* LT2 were probed using two fragments from the insert region of pKZ25 (Figure 12a, b). Based on hydroxyapatite chromatography, these two species show 46% reassociation under standard conditions; therefore, considerable divergence in nucleic acid sequence is expected (Sanderson, 1976). The PstI-NarI fragment contains part of the proposed *rfaH* coding region while the PvuII-SacI fragment is a 0.5 kb region mapping 0.25 kb from this area (Figure 11). The sequence of the PvuII-SacI fragment has not been determined. Under stringent hybridization conditions, the pKZ25 probes hybridized strongly with the *E. coli* K-12 chromosome but not with *S. typhimurium* LT2 indicating that the plasmid contains *E. coli* DNA but no detectable components of the *S. typhimurium* chromosome.

pKZ17, which carries the *E. coli rfaH* gene, has a restriction pattern similar to pKZ25, however, it lacks the SacI site and includes a BamHI site mapping between HindIII and the SacI seen in pKZ25 (Figure 11) (Rehemtulla, 1984). The differences in restriction sites are consistent with some of the results obtained by Rehemtulla in probing the *S. typhimurium* F′-*ilv*-*met* plasmid pDU450 prior to creating pKZ25 (Rehemtulla, 1984). The 0.9 kb PvuII-PstI fragment of pKZ17, which includes

sites for HindIII and BamHI, was used. As expected from the pKZ25 restriction data (Figure 11), two hybridizing bands were seen when pDU450 was cut with HindIII or SalI and only one with BamHI or EcoRI. Also two bands were seen with PstI, for which no explanation is offered.

The chance of accidentally cloning the E. coli rfaH gene during pKZ25 construction is remote. pDU450 is a Salmonella F′ factor maintained in E. coli and spontaneous recombinational events between these species are vanishingly rare due to differences in nucleotide sequence. One exception might be recombination between rrn genes - which are highly conserved - but, since pDU450 has only one such site, there is little chance of an exchange of rfaH-carrying fragments between it and the chromosome. Exchange of sequence as a result of recombination between insertion sequences is also unlikely since E. coli and S. typhimurium do not share any of the same common insertion sequences (Deonier, 1987). Finally, pDU450 was isolated by CsCl dye-buoyant density centrifugation (Rehemtulla, 1984), virtually eliminating the possibility of carryover of E. coli chromosomal sequences to the ligation mix.

B. Tn1000 insertion mutagenesis

1. Location of insertions

As shown in Figure 15, insertions were not seen in areas around 4 kb, 5 kb, and between 5.5-6.5 kb. The lack of insertions in the 4 kb region is not readily explained but may indicate a region of low AT content, as compared with the area at 4.5 kb (Liu et al, 1988). The tendency of Tn1000 insertions to cluster in areas of high AT content is also noted in the rfaH sequence (Figure 14). In the sequence, a cluster of insertions is found in the AT-rich region around nucleotide 725.

The insertion-free area at 5 kb represents the origin of replication for the plasmid. Since insertions in this area would interfere with plasmid replication, they could not be recovered in this experiment. At 5.5-6.5 kb is the pBR322 ampicillin resistance gene. Although it is possible that transposons may insert in this region, the selection process used would prohibit their recovery.

2. Phage pattern analysis

As shown in Table 5, Tn1000 insertions into pKZ25 caused varying degrees of loss of rfaH-complementing activity in E. coli. In two cases, rfaH3118::Tn1000 and rfaH3124::Tn1000, the loss was complete with no sensitivity to male or smooth specific phages (the

strains were LPS$^-$ Tra$^-$). Others (all of genotypes zhj and pKZ25) restored rfa$^+$ sensitivities. A third group of insertions, all RfaH$^-$, showed partial losses of complementation with reductions in sensitivity to C21, M13, and f2.

Selected pKZ25::Tn1000 plasmids were transformed into an rfaH$^-$ S. typhimurium to determine complementation ability (Table 6). Here, also, those strains designated zhj completely restored sensitivity to smooth and male specific phages while those designated RfaH showed varying degrees of loss of this sensitivity. Only rfaH3118::Tn1000 and rfaH3121::Tn1000 had a complete loss of sensitivity to M13 and f2 but all of the E. coli rfaH-inactivating inserts were resistant to Salmonella smooth-specific phages.

All insertions causing reduction in complementation by phage pattern sensitivity (all inserts designated rfaH) mapped within a small area of the pKZ25 insert region (Figures 11,15) indicating that this area contains the structural gene for rfaH.

3. Donor efficiency in S. typhimurium strains carrying selected Tn1000 insertions

Table 7 shows the efficiency of F-mediated transfer functions in S. typhimurium strains carrying pKZ25::Tn1000 plasmids. Although a definite reduction in donor efficiency is seen in the rfaH$^-$ control strain

(Table 7, line 2) over the rfaH$^+$ (Table 7, line 1), it is not as drastic as that seen by Sanderson and Stocker (1981) in E. coli rfaH mutants. The increase in recombinants seen may be a result of a longer incubation period (by 15 minutes) or, more likely, of variations between rfaH alleles. The latter possibility is supported by the observations of Creeger et al (1984) who saw a similar degree of variation between S. typhimurium strains carrying different rfaH$^-$ alleles. Since all of the strains are capable of acting as donors in conjugation to some extent, some F-pili must be present; however, the proportion of cells with F-pili is less than 100% (cells without pili cannot act as donors in conjugation) and is apparently not high enough to allow visible lysis by phages M13 and f2 (Table 6).

Two of the insertions, rfaH3122::Tn1000 and rfaH3123::Tn1000, did not show a reduction in donor efficiency (Table 7, lines 10,11). S. typhimurium strains carrying these insertions showed no reduction in M13 and f2 sensitivity. An E. coli strain carrying rfaH3122::Tn1000 also exhibited tra functions (Table 5, line 14) and a second E. coli with rfaH3123::Tn1000 had reduced sensitivity to f2 only. It appears that these two insertions do not effect the restoration of the tra functions although they are still deficient in LPS synthesis.

4. Analysis of LPS by SDS-PAGE

Silver-stained gels containing LPS from rfaH⁻ strains of E. coli and S. typhimurium carrying pKZ25::Tn1000 plasmids are shown in Figures 17 and 18. Plasmids which restored sensitivity to smooth-specific phages also restored the complete LPS molecule. In both species, the presence of the tra functions (Table 5,7) has no detectable effect on LPS structure giving additional evidence that the two functions are not interdependent.

C. The sequence of rfaH

1. The promoter region

The -35 consensus sequence, seen at nucleotide 201 has the sequence CTCTGACGGTAT. Although this sequence does not contain the highly conserved TTG or the less conserved ACA (Figure 14), it is very similar to the -35 consensus sequence, TCCTGACTGGTAT seen in the phage fdv (Rosenberg and Court, 1979). 16 nucleotides separate the last position of this region from the first position of a 6 base -10 consensus sequence containing the highly conserved TATAAT. This -10 has the T in position six, and the TA in positions one and two - the two most common features of the RNA polymerase binding site. It is separated from probable Shine-Dalgarno sites at positions 279 (GGA) and 285 (GAG) by a stretch

of 45 bases. Within the intervening area is the HindIII site reported by Rehemtulla (1984) to interrupt the gene based on the loss of complementation seen in HindIII deletions of pLC14-28. Although this site is not within the coding region of the gene, such a deletion could inactivate the gene by removing the promoter region. However, a CAT cartridge insertion into the HindIII site, giving chloramphenicol resistance, did not cause loss of rfaH activity (P.R. MacLachlan, personal communication). PstI deletions were also previously reported to cause loss of rfaH complementation (Rehemtulla, 1984) and this site does fall within the predicted coding region (Figure 14). According to the rules for transcription initiation proposed by Rosenberg and Court (1979), mRNA transcription most likely begins at the A residue at position 241 or the G at 242.

The coding region is preceded by two possible ribosome-binding sites (GGA and GAG). Both of these are separated from the ATG start codon (underlined) by between five and thirteen nucleotides - optimal spacing for translation initiation (Gold, 1988). The GGA is easily trapped in secondary structure (Figure 25). The GAG which follows it, however, is more likely to remain free to interact with the polymerase (Figure 25). Although the ribosome attachment site is short, the existence of a transcription "window" (the size of transcript in which the Shine-Dalgarno sequence is not trapped in secondary structure) of more than 50 bases indi-

cates that the protein may be highly transcribed, since it remains available to RNA polymerase for a long period (Gold, 1988).

2. Codon usage

Preference for certain codons in E. coli DNA is related to the abundance of appropriate tRNA species available for translation. Genes which are not highly translated tend to show preference for the less abundant tRNA forms, perhaps allowing more vital products to be more highly expressed through lack of competition for tRNA (Ikemura, 1981). The arrangement of codons in rfaH, in terms of preferred codon use is shown in Figure 25.

Of the 143 amino acids in the rfaH protein product for which a codon preference is known (Ikemura, 1982), 63 are non-preferred types while a small majority (80 codons, or 56%) are preferred. The mix of preferred and non-preferred codons would indicate a protein which is moderately expressed and probably not necessary in large amounts for survival.

3. The rfaH gene product

The amino acid sequence of the rfaH gene product, as predicted from nucleic acid sequence, is shown in Figure 14. Protein structure was predicted based on

the calculations of Chou and Fasman (1974). The results of these calculations, as performed by the program SEQAID, are shown in Figure 26. These calculations allow predictions of the locations of alpha-helix and beta-sheet structures in the protein based on the probability of certain amino acid appearing.

These calculations predict a beta-sheet region extended from the first residue (MET) to residue nine (CYS). A possible helix begins at the lysine (LYS) residue twenty-one, since proline (PRO) cannot appear at the N terminal end of a helix (Chou and Fasman, 1974), and extends to residue twenty-nine, where it is broken by PRO. The next four residues may form a sheet but the structure is immediately broken by the glutamine (GLU) at position 34 (Chou and Fasman, 1974). A second helix begins at position 52 and terminates at 57. A third sheet structure begins at residue 62 and continues to 68 and a fourth begins at position 101 and continues, for seven residues to position 107 (Figure 26).

From the serine (SER) at position 83 to the valine (VAL) at 97, the calculations predict that the region could form either structure with equal probability. A larger region of ambiguous structure occurs between the glycine (GLY) at 112 and the GLU at 131. The entire region forms a helix but two interior regions, from 113 to 118 and from 124 to 130, are also capable of sheet formation. A second large region of ambiguous structure is found near the C-terminal end of the protein, between residues 138 and 157. Again, the

entire region may form a helix while a smaller internal area, from 138 to 150 may also form a sheet (Figure 26).

A hydrophobicity plot of the protein, also created by SEQAID, is shown in Figure 22. Overall, the protein is somewhat hydrophilic but varies along its length (Figure 22), indicating possible folding patterns. There are no long hydrophobic portions suggesting membrane-spanning regions or any suggestion that the protein is membrane bound. This is expected since rfaH is believed to be cytoplasmic, acting on the rfa and tra genes during transcription, rather than on their (possibly membrane-associated) protein products.

D. Expression of rfaH from the T7 promoter

pKZ87 and pKZ91 are forced clones, that is, the fragment was ligated between a SacI and a SalI site in the vector multiple cloning site so that the insert orientation is known (Figure 19). Sequence analysis showed (Figure 14) that the orientation was such that the genes could be transcribed from the Bluescript T7 promoter using the T7-polymerase promoter system described by Tabor and Richardson (1985). Analysis of the resulting autoradiogram (Figure 23) confirmed the expected size of the rfaH protein product (17.9 kDa) based on the ORF. This also agrees with the size predicted from minicells (Rehemtulla, 1984) for both pKZ17 and pKZ25.

When compared to the Coomassie blue-stained gel, the labelled protein falls into a region containing many proteins of similar size. There was no indication of greater intensity of stain in the lanes containing extracts from strains carrying the plasmids than that of the plasmid-less control strain. This made removal of the protein band for possible amino-acid sequencing and antibody production impossible. This difficulty might be overcome in future by creating a lacZ-fusion product in pKZ87 and pKZ91 which would increase the size of the protein product and move it out of the high-background area. Alternatively, the T7-polymerase promoter system could be used to produce the truncated protein product coded by pKZ157, which contains only 42 amino acids from the N-terminal end (Figure 19) and thus could be used for amino acid sequencing.

The predicted amino acid sequence could also be used to produce synthetic polypeptides which could be used to raise antibodies against the rfaH protein. Precipitation with these antibodies might then allow for concentration and isolation of the protein itself.

E. General observations

The plasmid pKZ25 carries the rfaH gene of E. coli K-12 and not S. typhimurium as previously reported (Rehemtulla, 1984). The source of this gene is, however, the S. typhimurium F′ factor pDU450 based on Southern analysis of that plasmid (Rehemtulla, 1984).

The sequence of the rfaH-coding region matches exactly that determined for the E. coli rfaH gene derived from the Clarke and Carbon plasmid pLC14-28 and carried on pKZ17 (P.R. MacLachlan and P. Thirukkumaran, unpublished); however, two restriction sites located upstream of the coding region differ between the two plasmids. The BamHI site seen in pKZ17 (and, according to Southern hybridizations, in the E. coli chromosome (Figure 12)) is not found in pKZ25. Analysis of the sequence in its expected position shows GGCGTCC rather than the BamHI site, GGATCC, indicating a substitution of GC for the A in the BamHI site. As no sequence has been determined upstream of BamHI in pKZ17, the difference at the SacI site (seen only in pKZ25) is not yet known. The origin of the E. coli gene in pDU450 is therefore unknown.

The ability of this gene (and also the rfaH gene of pKZ17) to complement rfaH defects in S. typhimurium is remarkable in that it suggests a lack of dependence on host factors, which would differ between species. The E. coli bacteriophage lambda N gene does not function in S. typhimurium due to its specific requirement for the E. coli Nus factors. rfaH does not appear to have this difficulty since the E. coli gene will function in S. typhimurium. The lambda Q gene product also functions in the absence of NusA (although its effect is enhanced in its presence), indicating that the requirement for specific host factors might not be a universal trait of antiterminators.

S. typhimurium strains with core mutations are not necessarily deficient in transfer functions (Sanderson and Stocker, 1981) and some of the tra-inactivating Tn1000 insertions reported here do not have a rough LPS (are tra$^-$, rfa$^+$). One of the insertions, rfaH3121::Tn1000 complements the LPS defect in E. coli only (Table 5, line 15), an rfaH$^-$ S. typhimurium carrying pKZ25 with this insertion remains rfa$^-$ (Table 6, line 4). This suggests that the binding sites in the two groups of genes (tra and rfa) may be different or that the rfa sites are more rigid in their structural requirements, so that an insertion which does not affect antitermination in the tra operon might still inhibit it in the rfa cluster. The differences seen with rfaH3121::Tn1000 suggest that the E. coli protein was altered enough by this insertion that it was no longer able to act in the other species but not enough to stop its function in E. coli.

The structure of the C-terminal end of the protein product may be more important for its function since the insertion removing the majority of this end, rfaH3118::Tn1000, (Figure 14, insertion b) has a strong effect both on LPS (Figure 17a, lane 9; Figure 18a, lane 2) and tra functions (Table 7, line 7; Table 6, line 5) (strains carrying this plasmid are tra$^-$,rfa$^-$). Insertions closer to the C-terminal end might have a weaker effect, allowing possible function in the tra operon. The four insertions which map close together (Figure 14, insertions c-f) have different effects on rfaH-complementation. Phage pattern sensitivities for

three of these show that two, rfaH3119::Tn1000 and rfaH3123::Tn1000 (Table 6, lines 6,8), are resistant to the smooth-specific phages (rfa$^-$) but give visible lysis with the male-specific phages M13 an f2 (tra$^+$) while the third, rfaH3121::Tn1000, is resistant to both smooth and male specific phages (rfa$^-$, tra$^-$) in S. typhimurium (Table 6, line 3) and complements only the LPS defect in E. coli (Table 5, line 15). The fourth insertion, rfaH3120::Tn1000, has defective LPS in both strains (Figure 17a, lane 7; Figure 18a, lane 5) and shows some lysis with M13 (Table 5, line 16), indicating some tra function.

Examination of the strains' abilities to act as donors in conjugation (Table 7), also indicates that rfaH3123::Tn1000 is tra$^+$ (Table 7, line 10) but rfaH3119::Tn1000 (Table 7, line 8) does show reduced donor efficiency as does rfaH3121::Tn1000 (Table 7, line 5). It is unexpected that four insertions so close together have different effects on gene function, but this variability may indicate that structural variation in this region of the protein has a milder effect on its function. The orientation of these four insertions does not seem to affect their function. rfaH3119::Tn1000 and rfaH3120::Tn1000 are in the gamma-delta orientation with respect to the pKZ25 EcoRI site while rfaH3121::Tn1000 and rfaH3123::Tn1000 are delta-gamma. No other insertions were found mapping in the N-terminal portion of the ORF (Figure 14), there-

fore, it is not possible to support this suggestion further at this time.

F. Directions of further research

Before any further work can be done with the rfaH gene carried on pKZ25, it will be necessary to determine its origin. Southern hybridizations and sequence comparison have shown that the gene is from E. coli (see RESULTS) despite originating from a putative S. typhimurium F′ factor (Rehemtulla, 1984; Blazey and Burns, 1983). This F′ factor, pDU450, was isolated from a cross of a S. typhimurium Hfr strain with an E. coli F^- strain. Selection was for the ilv^+ gene carried by the Salmonella donor. It is postulated that the formation of the factor was by recombination between rrn sequences in S. typhimurium, probably between rrnC and rrnA (Blazey and Burns, 1983). Therefore, as mentioned earlier (DISCUSSION, part A), the plasmid pDU450 contains only one such operon, making it unlikely that a piece of chromosome was exchanged with the E. coli host through recombination at these sites. A second possibility is that such recombination has occurred between insertion sequences in the E. coli chromosome and the F′ factor. The insertions in pDU450 may have originated in its S. typhimurium source or have inserted from the host chromosome after transformation.

The E. coli rfaH-carrying plasmid pKZ17 has already been shown to hybridize with pDU450 (Rehemtulla, 1984), however, the stringency and other conditions of this hybridization are not known. To confirm the origin of the pKZ25 insert region on this plasmid, pDU450 could be transferred by conjugation into an ilv⁻ S. typhimurium strain. The auxotrophy is necessary, since pDU450 carries no antibiotic resistance marker. Probes derived from pKZ25 do not hybridize with this strain (Figure 12). Hybridization with the plasmid could then be confirmed in this background. The probes created from the coding and flanking regions of rfaH carried on pKZ25 should also be used to probe the appropriate members of the lambda library created by Kohara et al (1987). This is currently being done and could confirm the predicted position of the rfaH gene (Figure 13).

Further sequencing of pKZ17 and restriction digestion of the appropriate lambda clones will also help to determine the existence of the SacI site seen in pKZ25 but not pKZ17. Currently, no sequence is known in pKZ17 upstream of BamHI but this site, which is not seen in pKZ25, does appear to exist in the E. coli chromosome (Figures 12, 13). The loss of the BamHI site in pKZ25 is the result of the exchange of GC for the A residue in the site (see RESULTS) and the loss or gain of the SacI site may involve a similar event.

To date, only the E. coli rfaH gene has been cloned for study. Cloning and sequencing of the S. typhimurium gene would also be useful - providing informa-

tion on sequence homology and the ability of the S. typhimurium gene to complement E. coli defects.

Table 1: List of selected strains used in this study[a]

Strain number	Genotype	Source/ Reference
E. coli K-12		
C600	*thr-1 leu-6 thi-1 supE44 lacY tonA21*	CGSC[b]
DLT111	HfrC lambda *srl*::Tn10 *recA56* / pGP1-2 (cI857 lambda p_L, T7 gene 1)	J.H. Weiner
JM109	*recA1 endA1 gyrA96 thi hsdR17 supE44 relA1* lambda$^-$ (del)*lac-proAB* / F'*traD36 proAB lacI^{q}Z*(del)M15	Yanisch-Perron et al, 1985
MG1063	*thi recA56* F^+	Guyer, 1978
NK5992	F^- *argA81*::Tn*10* lambda$^-$ IN(*rrnD-rrnE*)-1	CGSC[b]
SAB2562	*sfrB14 trp lys* (del)*lacX74 gal rpsL thi recA56* (cryptic lambda)	Rehemtulla et al, 1986
SAB2845	*sfrB14 trp lys lac* X74 *gal rpsL thi* (cryptic lambda) *recA56* / pKZ25 *sfrB*$^+$	Rehemtulla, 1984
SAB2885	*sfrB14 trp lys* (del)*lacX74 gal rpsL thi recA56* (cryptic lambda) / F'42*lac*	Rehemtulla, 1984

Table 1 (continued)

Strain number	Genotype	Source/ Reference
SAB2888	sfrB14 trp lys (del)lacX74 gal rpsL thi recA56 (cryptic lambda) / F'42lac / pKZ25 (pBR322-rfaH$^+$)	Rehemtulla, 1984
SAB2994	(del)pro(pro-lac)-41 glnV42 supE42 his-53 xyl-14 cycB2 cycA1 lambda$^-$ rfaG215	SGSC[c]
SAB2995	(del)pro(pro-lac)-41 glnV42 supE42 his-53 xyl-14 cycB2 cycA1 lambda$^-$ rfaJ216	SGSC[c]
SAB3000	(del)pro(pro-lac)-41 glnV42 supE42 his-53 xyl-14 cycB2 cycA1 lambda$^-$ rfaI221	SGSC[c]
SAB3074	F$^+$ trpE5 thr leu recA / ColE1-rfaG$^+$	Creeger and Rothfield, 1979
SAB3275	recA1 endA1 gyrA96 thi hsdR17 supE44 relA1 lambda$^-$ (del)lac-proAB / F'traD36 proAB lacI^{q}Z$^-$(del)M15 / pBluescript KS+	SGSC[c]

Table 1 (continued)

Strain number	Genotype	Source/ Reference
SAB3276	recA1 endA1 gyrA96 thi hsdR17 supE44 relA1 lambda$^-$ (del)lac-proAB / F'traD36 proAB lacI^{q}Z$^-$(del)M15 / pBluescript KS(-)	SGSC[c]
SAB3488	thr-1 leu-6 thi-1 supE44 lacY tonA21 / pKZ25 rfaH$^+$	this study[d]
SAB3490	thi recA50 F$^+$ / pKZ25 rfaH$^+$	this study[e]
SAB3491	thr-1 leu-6 thi-1 supE44 lacY tonA21 / pKZ25 rfaH$^+$ / F'42 finP301 lac$^+$	this study[f]
SAB3598	sfrB14 trp lys (del)lacX74 gal rpsL thi recA56 (cryptic lambda) / pKZ87 rfaH$^+$	this study[g]
SAB3659	recA1 endA1 gyrA96 thi hsdR17 supE44 relA1 lambda$^-$ (del)lac-proAB / F'proAB lacI^{q}Z(del)M15 Tn10 / pKZ91 rfaH$^+$	this study[h]

Table 1 (continued)

Strain number	Genotype	Source/ Reference
SAB3696	recA1 endA1 gyrA96 thi hsdR17 supE44 relA1 lambda$^-$ (del)lac-proAB / F'traD36 proAB lacI^{q}Z(del)M15 / pKZ87 rfaH$^+$	this study[i]
XL1Blue	recA1 endA1 gyrA96 thi hsdR17 supE44 relA1 lambda$^-$ (del)lac-proAB / F'proAB lacI^{q}Z(del)M15 Tn10	Stratagene Cloning Systems
S. typhimurium LT2		
SA2197	purC7 / F'42 finP301 lac$^+$	Sanderson et al, 1983
SA2887	metA22 trpC2 fla-66 rpsL120 H1-b H2-e,n,x "cured of Fels 2" xyl1404 hsdL6 hsdA29 hsdSB rfaH3074 F'42 finP301 lac$^+$	SGSC[c]
SA3444	metA22 metE551 trpC2 H1-b H2-e,n,x "cured of Fels 2" flaA66 rpsL120 xyl-404 hsdL6 hsdSA29 hsdSB ilv leu rfaH3074 / F'42 finP301 lac$^{\pm}$	SGSC[c]

Table 1 (continued)

Strain number	Genotype	Source/ Reference
SA3695	metA22 metE551 trpC2 fla-66 rpsL120 H1-b H2-e,n,x "cured of Fels 2" xyl-404 hsdL6 hsdSA29 hsdSB rfaH3074 / F'42 finP301 lac$^+$ / pKZ87 rfaH$^+$	this study[j]
SA3707	purA80 ara-9	SGSC[c]
SA3797	metA22 metE551 trpC2 fla-66 rpsL120 H1-b H2-e,n,x "cured of Fels 2" xyl-404 hsdL6 hsdSA29 hsdSB rfaH3074 / F'42 lac / recA1 srl-202::Tn10	this study[k]
SL3748	pyrE$^+$ rfa(R-res-2)432 = rfaI432	Roantree et al, 1977
SL3749	pyrE$^+$ rfaL446	Roantree et al, 1977
SL3769	pyrE$^+$ rfaG471	Roantree et al, 1977
SL3789	pyrE$^+$ rfaF511	Roantree et al, 1977

Table 1 (continued)

[a] Genotypes of all the parent strains used are listed here.

[b] Available from B.J. Bachmann, E. coli Genetic Stock Center, Department of Biology 255 OML, Yale University, P.O. Box 6666, New Haven, CT 06511-7444 USA

[c] Available from K.E. Sanderson, Salmonella Genetic Stock Center, Department of Biological Sciences, University of Calgary, Calgary, AB T2N 1N4 Canada

[d] pKZ25, isolated from SAB2845, was used to transform C600 with selection for ampicillin resistance.

[e] pKZ25, isolated from SAB2845, was used to transform MG1063 with selection for ampicillin resistance.

[f] SA2197 was used as a donor in conjugation with SAB3488. Selection was for ampicillin resistance and lactose utilization.

[g] pKZ25, isolated from SAB2845, was used to transform SAB2562 with selection for ampicillin resistance.

[h] pKZ91 was created as described (MATERIALS AND METHODS). XL1Blue was transformed with the ligation mix with selection for ampicillin resistance and loss of the blue color on IPTG and X-gal. The plasmid identity was confirmed by restriction analysis (RESULTS).

[i] pKZ87 was created as described (MATERIALS AND METHODS). XL1Blue was transformed with the ligation mix with selection for ampicillin resistance and loss of the blue color on IPTG and X-gal. The plasmid identity was confirmed by restriction analysis (RESULTS).

[j] pKZ25, isolated from SAB2845, was used to transform SA3444 with selection for ampicillin resistance.

[k] SA3444 was made RecA$^-$ by insertion of Tn10 into the recA locus. The RecA phenotype was confirmed by increased sensitivity to uv light

Table 2: Primers used for DNA sequencing

Primer	Sequence	Annealing Temp.	Source[a]
Tn1000:gamma	TCAATAAGTTATACCAT	37°C	RDSL
Tn1000:delta	GAATTATCTCCTTAACG	41°C	RDSL
T3	GAAATCACTCCCAATTA	41°C	Stratagene
M13-20	GTAAAACGACGGCCAGT	47°C	Stratagene
BamHI cc	ATGCGTCCGGCGTAGA	37°C	N.E.Biolabs
pKZ87-centre	AACTCGGTATTCTTCAC	43°C	RDSL
pKZ91-centre	AACACCTCGAAAGACAG	49°C	RDSL

[a] "RDSL" is the Regional DNA Synthesis Laboratory in Calgary, AB, Canada. "Stratagene" is Stratagene Cloning Systems, La Jolla, CA, USA.

Table 3: *rfaH* complementation by phage sensitivity patterns

			Phages[b]									
				Rough							Inferred	
			core	specific		Male specific					phenotype[c]	
	Strain	Genotype[a]	U3	Br60	C21	M13	f2				Tra	LPS
E. coli K-12												
1	C600	*rfa*$^+$,*sfrB*$^+$	+	−	−	−	−				na	+
2	SAB2562	*sfrB14* (*rfaH*$^-$)	−	+	+	−	−				−	−
3	SAB2845	SAB2562/pKZ25 (*rfaH*$^+$)	+	−	−	−	−				na	+
4	SAB2885	*sfrB14*,F'42*lac*+	−	−	+	−	−				−	−
5	SAB2888	SAB2885/pKZ25 (*rfaH*$^+$)	+	−	−	+	+				+	+

			Phages									
			Smooth specific					Rough specific		Male specific		
			P22 .*cl*	KB1 *int*	P22h .*cl*	9NA	FO	Br60	C21	M13	f2	Tra	LPS
	S. typhimurium LT2												
6	SL3770	*rfa*$^+$	+	+	+	+	+	−	−	−	−	na	+

Table 3 (continued)

			Phages										
			Smooth specific					Rough specific		Male specific			
			P22 .c1	KB1 int	P22h .c1	9NA	FO	Br60	C21	M13	f2	Tra	LPS
	S. typhimurium LT2												
7	SA2197	rfa$^+$,F'42lac$^+$	+	+	+	+	+	−	−	+	+	+	+
8	SA2887	rfaH3074,F'42lac+	−	−	−	−	+	−	+	−	−	−	−
9	SA3444	SA2887/pKZ25 (rfaH$^+$)	+	+	+	+	+	−	−	+	+	+	+
10	SL3748	rfaI432	−	−	−	−	−	+	−	−	−	na	−
11	SL3749	rfaL446	−	−	−	−	+	+	+	−	−	na	−
12	SL3769	rfaG471	−	−	−	−	−	+	+	−	−	na	−
13	SL3789	rfaF511	−	−	−	−	−	+	−	−	−	na	−

[a] The genotypes listed are partial only. For complete genotypes, see Table 1. E. coli strains have mutations in the homologous sfrB locus (therefore SAB2562, which carries sfrB14 is rfaH$^-$).

[b] The following symbols are used: '+' indicates that the strain is sensitive to the phage (lysis occurs), '−' indicates that the strain is resistant to the phage (no lysis).

Table 3 (continued)

[c] The following symbols are used: '+' indicates that the *tra* and/or *rfa* functions are completely restored according to the phage patterns. '-' indicates that the functions are not complemented. 'na' indicates that complementation of the *tra* functions can not be determined since the strain is F^-.

Table 4: Restriction enzymes used to map pKZ25

Enzyme	Cuts in pBR322[a]	Cuts in pKZ25[b]	Cuts in insert[c]
AatII	1	1	0
ApaI	0	0	0
Asp718	0	0	0
AvaI	1	1	0
BamHI[d]	1	0	0
BglI	0	0	0
BsmI	1	2	1
BstXI	0	0	0
ClaI	1	1	0
Eco0109	0	0	0
EcoRI	1	1	0
EcoRV	1	1	0
HindIII	1	2	1
KpnI	0	0	0
MluI	0	0	0
MstII	0	0	0
NcoI	0	1	1
NotI	0	0	0
NruI	1	1	0
NsiI	0	0	0
PstI	1	2	1
PvuI	1	1	0
PvuII	1	2	1
RsrII	0	0	0
SacI	0	1	1
SacII	0	2	2
SalI	1	1	0
ScaI	1	1	0
SmaI	0	0	0
SnaBI	0	0	1
SpeI	0	0	0
XbaI	0	0	0
XcyI	0	1	1
XhoI	0	0	0

[a] Taken from published maps of pBR322 (Maniatis et al, 1972; BRL and Pharmacia catalogues).

[b] Fragments determined from digestion of pKZ25 in this study.

[c] Predicted sites in the 2.2 kb insert determined as the difference between the other two columns.

[d] The vector BamHI site was lost in the creation of pKZ25 (Figure 9).

Table 5: Phage sensitivities of an *rfaH*$^-$ *E. coli* strain carrying pKZ25::Tn*1000* insertions

			Phages[b]					Inferred complementation[c]	
			Rough specific		Smooth specific	Male specific			
	Strain	Genotype[a]	Br60	C21	U3	M13	f2	Tra	LPS
1	C600	*rfa*$^+$, *tra*$^+$	-	-	+	+	+	na	na
2	SAB2885	*rfaH*$^-$, F$^+$	-	+	-	-	-	-	-
3	SAB3762	*zhj-1449*::Tn*1000*	-	-	+	+	+	+	+
4	SAB3754	*zhj-1448*::Tn*1000*	-	-	+	+	+	+	+
5	SAB3765	*zhj-1447*::Tn*1000*	-	-	+	+	+	+	+
6		*zhj-1446*::Tn*1000*	-	-	+	+	+	+	+
7	SAB3759	*zhj-1445*::Tn*1000*	-	-	+	+	+	+	+
8		*zhj-1444*::Tn*1000*	-	-	+	+	+	+	+

Table 5 (continued)

			Phages[b]						
			Rough specific		Smooth specific	Male specific		Inferred complementation[c]	
	Strain	Genotype[a]	Br60	C21	U3	M13	f2	Tra	LPS
9		zhj-1443::Tn1000	-	-	+	+	+	+	+
10		zhj-1442::Tn1000	-	-	+	+	+	+	+
11		zhj-1441::Tn1000	-	-	+	+	+	+	+
12	SAB3763	rfaH3124::Tn1000	-	+	-	-	-	-	-
13	SAB3766	rfaH3123::Tn1000	-	+	-	+/-	-	-	-
14	SAB3767	rfaH3122::Tn1000	-	-	+	+	+	+	+
15	SAB3761	rfaH3121::Tn1000	-	-	+	-	-	-	+
16		rfaH3120::Tn1000	-	+	-	+	-	+/-	-
17	SAB3764	rfaH3119::Tn1000	-	+	-	+	-	+/-	-
18		rfaH3118::Tn1000	-	+	-	-	-	-	-
19		pKZ25-9::Tn1000	-	-	+	+	+	+	+
20		pKZ25-7::Tn1000	-	-	+	+	+	+	+

Table 5 (continued)

			Phages[b]						
			Rough specific		Smooth specific	Male specific		Inferred complementation[c]	
	Strain	Genotype[a]	Br60	C21	U3	M13	f2	Tra	LPS
21	SAB3760	pKZ25-5::Tn1000	-	-	+	+	+	+	+
22	SAB3758	pKZ25-3::Tn1000	-	-	+	+	+	+	+
23	SAB3756	pKZ25-2::Tn1000	-	-	+	+	+	+	+
24		pKZ25-16::Tn1000	-	-	+	+	+	+	+
25	SAB3757	pKZ25-15::Tn1000	-	-	+	+	+	+	+
26		pKZ25-14::Tn1000	-	-	+	+	+	+	+
27		pKZ25-13::Tn1000	-	-	+	+	+	+	+
28		pKZ25-12::Tn1000	-	-	+	+	+	+	+
29		pKZ25-11::Tn1000	-	-	+	+	+	+	+
30		pKZ25-10::Tn1000	-	-	+	+	+	+	+
31	SAB3755	pKZ25-1::Tn1000	-	-	+	+	+	+	+

Table 5 (continued)

[a] The genotypes are partial only. For complete genotypes of C600 and SAB2885, see Table 1. Lines 3-31 of this table are phage sensitivities for SAB2885 carrying pKZ25 plasmids with the indicated Tn*1000* insertions.

[b] The following symbols are used: '+' indicates that the strain is sensitive to the phage (lysis occurs). '-' indicates that the strain is resistant to the phage (no lysis). '+/-' means that lysis occurs but not as strongly as '+'. '-/+' indicates that very little lysis occurs.

[c] The following symbols are used: '+' indicates that complementation of the *tra* and/or *rfa* functions is seen. '-' indicates loss of complementation. '+/-' indicates partial complementation. The 'na' listed for C600 indicates that the strain is not complemented as it is *rfa*$^+$.

Table 6: Phage patterns of *Salmonella* strains carrying selected Tn*1000* insertions

			Phages[b]										
			Smooth specific					Rough specific		Male specific		Inferred phenotype[c]	
	Strain	Genotype[a]	P22 .c1	KB1 int	P22h .c1	9NA	FO	Br60	C21	M13	f2	tra	LPS
1	SA2197	*rfa*$^+$	+	+	+	+	+	−	−	+	+	+	+
2	SA3797	*rfaH3074*	−	−	−	−	+	−	+	−	−	−	−
3	SA3798	*zhj-1448*::Tn*1000*	+	+	+	+	+	−	−	+	+	+	+
4	SA3800	*rfaH3121*::Tn*1000*	−	−	−	−	+	−	−	−	−	−	−
5	SA3801	*zhj-1449*::Tn*1000*	+	+	+	+	+	−	−	+	+	+	+
6	SA3802	*rfaH3118*::Tn*1000*	−	−	−	−	+	−	−	−	−	−	−
7	SA3803	*rfaH3119*::Tn*1000*	−	−	−	−	+	−	−	+	−	+/−	−
8	SA3804	*zhj-1447*::Tn*1000*	+	+	+	+	+	−	−	+	+	+	+
9	SA3805	*rfaH3123*::Tn*1000*	−	−	−	−	+	−	−	+	+	+	−
10	SA3813	*rfaH3122*::Tn*1000*	−	−	−	−	+	−	−	+	+	+	−

Table 6 (continued)

[a] Genotypes are partial only. For the complete genotype of SA3797, see Table 1. All other strains are SA3797 carrying the pKZ25-based plasmid with the listed insertion. For example, SA3798 (line 3) is rfaH3074/pKZ25 zhj-1448::Tn1000 and therefore has the $RfaH^+$ phenotype.

[b] The following symbols are used: '+' indicates sensitivity to the phage (lysis occurs), '-' indicates resistance to the phage (no lysis).

[c] The following symbols are used: '+' indicates complementation as determined by phage sensitivity, '-' indicates no complementation, '+/-' indicates that the complementation is not complete.

Table 7: Efficiency of F-mediated transfer functions in *Salmonella typhimurium* strains carrying selected Tn*1000* insertions

	Strains	Genotype[a]	Recombinants[b] per donor cell	F-transfer[c] functions	Tra$^+$ based on phage sensitivity[d]
1	SA2197	*tra*$^+$	2.14	+	
2	SA3797	*rfaH3074* F'42*1ac*	0.29	-	-
3	SA3798	*zhj-1448*::Tn*1000*	1.6	+	+
4	SA3799	*zhj-1445*::Tn*1000*	1.3	+	+
5	SA3800	*rfaH3121*::Tn*1000*	0.25	-	-
6	SA3801	*zhj-1449*::Tn*1000*	1.6	+	+
7	SA3802	*rfaH3118*::Tn*1000*	0.26	-	-
8	SA3803	*rfaH3119*::Tn*1000*	0.34	-	+/-
9	SA3804	*zhj-1447*::Tn*1000*	1.5	+	+
10	SA3805	*rfaH3123*::Tn*1000*	2.4	+	+/-
11	SA3813	*rfaH3122*::Tn*1000*	2.1	+	+

Table 7 (continued)

[a] Genotypes are partial only. For the complete genotypes of SA2197 and SA3797, see Table 1. Strains listed in lines 3-11 consist of SA3797 containing the pKZ25 plasmid with the indicated insertions, therefore, all are F'42 finP301 lac$^+$, rfaH3074.

[b] Procedures are given in MATERIALS AND METHODS. The indicated strains were used as donors in broth matings with a lac$^-$, rfaL strain (SL3749). Selection of recombinants was for growth on minimal agar with lactose.

[c] The following symbols are used: '+' indicates that the ability to act as a donor in F-mediated conjugation has been restored. '-' indicates that F-mediated conjugation is occurring at a greatly reduced efficiency.

[d] From Table 6, based on sensitivity to the male specific bacteriophages M13 and f2.

Figure 1: The Gram-negative Outer Membrane

The structure of the bacterial gram-negative outer membrane is shown. The arrangement of phospholipids; proteins including structural proteins, porins, and other transport proteins; and LPS molecules is indicated (Nikaido and Vaara, 1987).

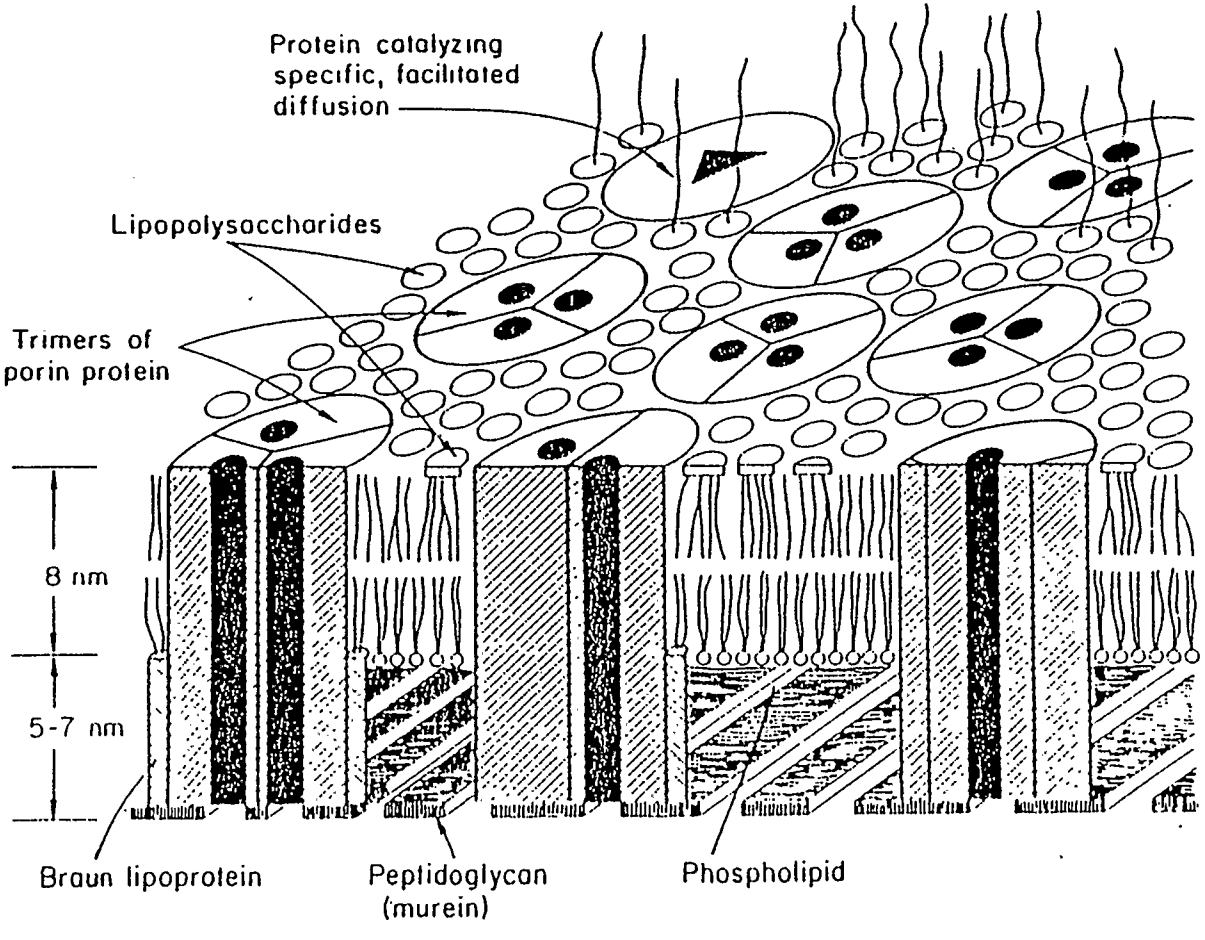

Protein catalyzing specific, facilitated diffusion
Lipopolysaccharides
Trimers of porin protein
8 nm
5-7 nm
Braun lipoprotein
Peptidoglycan (murein)
Phospholipid

Figure 2: The structure of LPS from Salmonella typhimurium and Escherichia coli K-12.

The organization of sugar residues comprising complete Salmonella typhimurium (A) (reviewed in Sirisena, 1990) and Escherichia coli K-12 (B) (Jannson et al, 1981; Austin et al, 1990) LPS chains is shown here. Linkages shown with a broken line indicate that the groups are not added in all cases. Lipid A is anchored within the outer membrane and KDO represents a structural bridge between this hydrophobic portion and the first heptose residue of the core region. Where known, the genes involved in specific steps in LPS synthesis are shown (italics) associated with their point of action. The resulting termination sites leading to chemotypes Ra-Re (indicated below the genes) in S. typhimurium are indicated by broken lines. rfaH mutants show a mixed chemotype consisting of Ra, Rb_1, Rb_2, Rb_3 and Rc.

The core shown in A is believed to be invariant among Salmonella while E. coli cores are of a few different types (Figure 3).

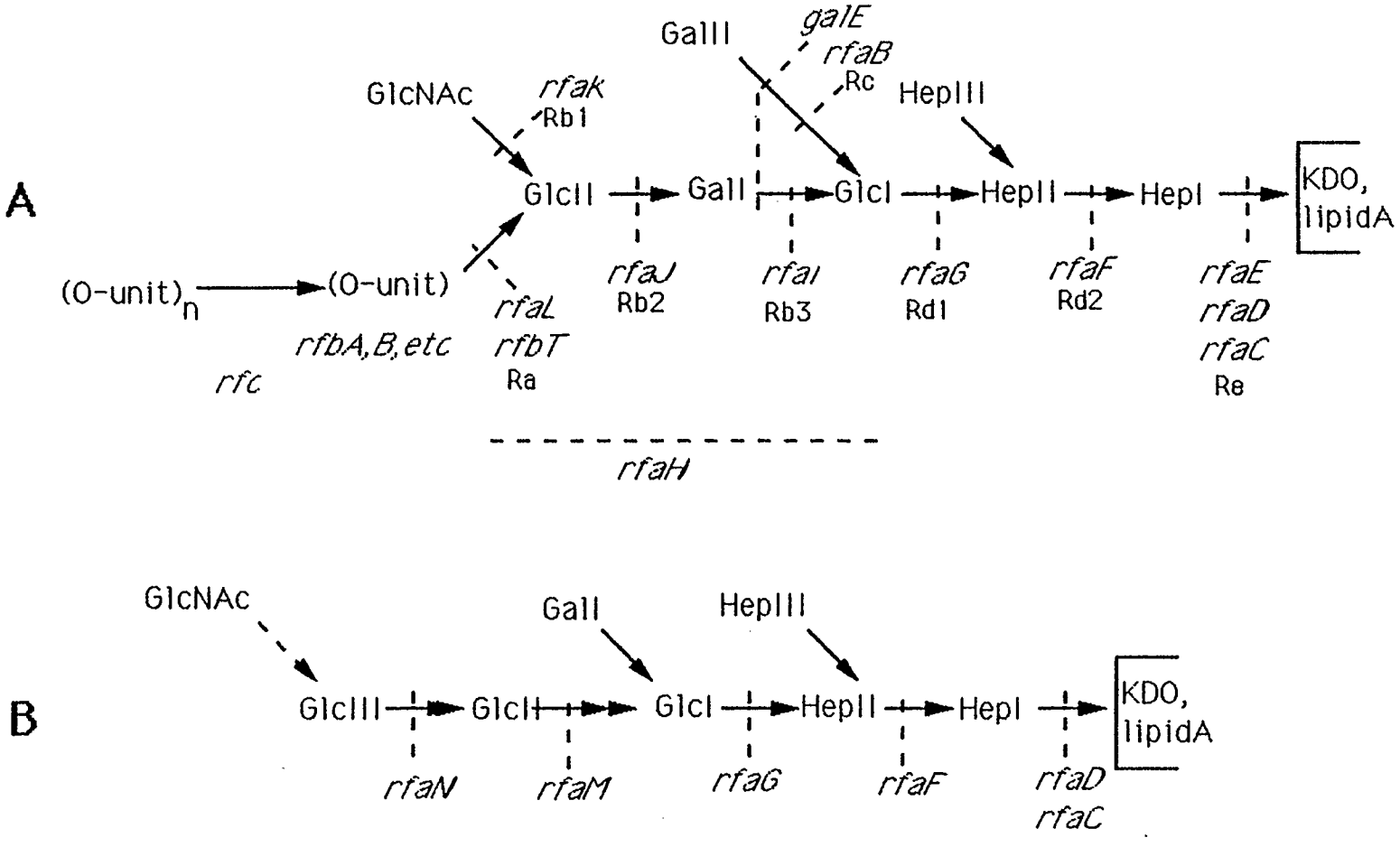

A
GalII
galE
rfaB
Rc
GlcNAc
rfaK
Rb1
HepIII
GlcII
GalI
GlcI
HepII
HepI
KDO,
lipidA
(O-unit)n
(O-unit)
rfaL
rfbT
Ra
rfaJ
Rb2
rfaI
Rb3
rfaG
Rd1
rfaF
Rd2
rfaE
rfaD
rfaC
Re
rfc
rfbA,B,etc
rfaH
B
GlcNAc
GalI
HepIII
GlcIII
GlcII
GlcI
HepII
HepI
KDO,
lipidA
rfaN
rfaM
rfaG
rfaF
rfaD
rfaC

Figure 3: Other E. coli core structures

Unlike Salmonella, the LPS core in E. coli strains may contain one of six different structures. In all of these, the lipid A and KDO regions remain unchanged and are identical to those of Salmonella (Figure 2). Five of the E. coli structures are shown here, the sixth (K-12) is shown in Figure 2. The cores shown are A) E. coli R_1, B) E. coli R_2, C) E. coli R_3, D) E. coli R_4, and E) E. coli B (Jannson et al, 1981).

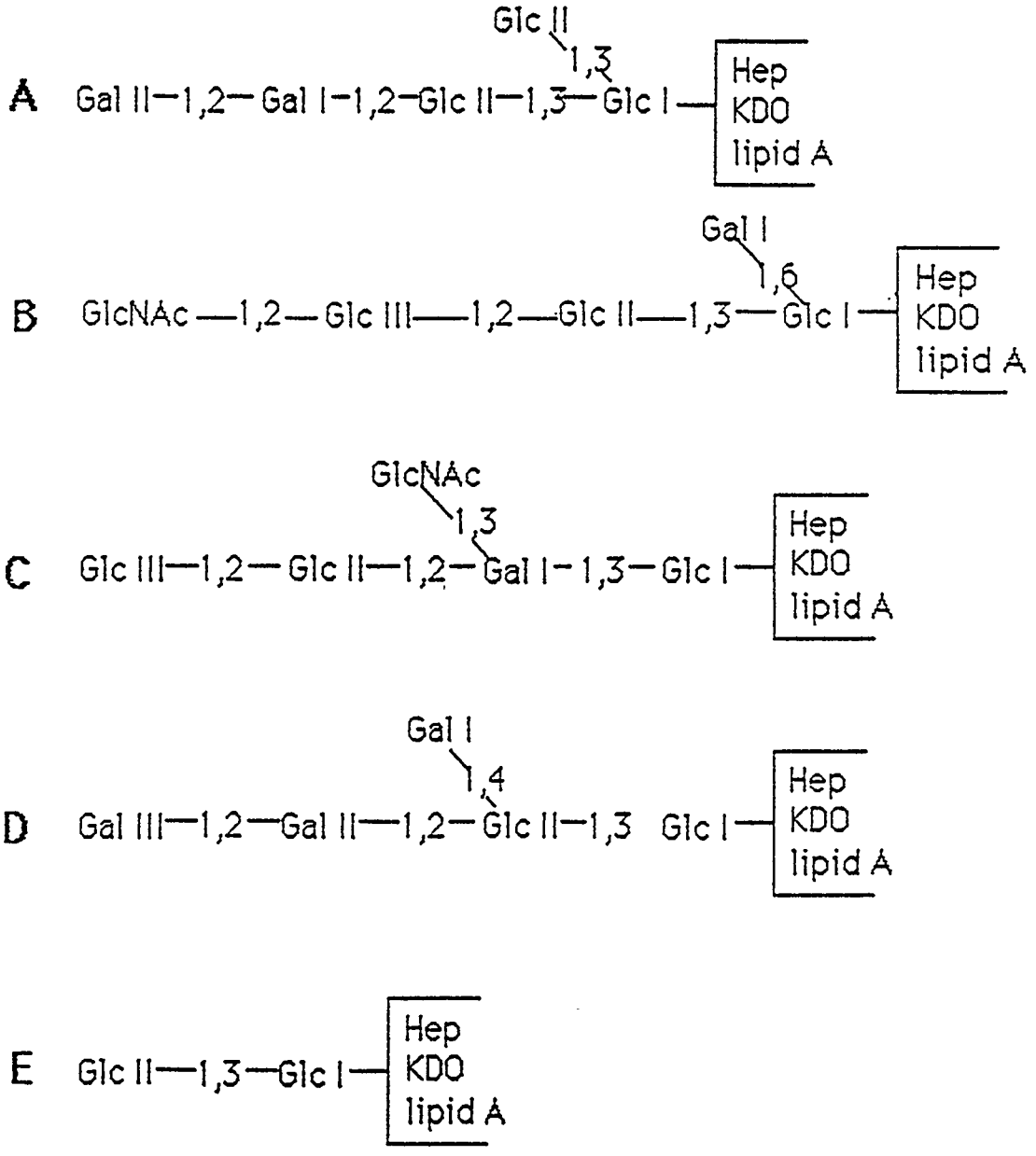
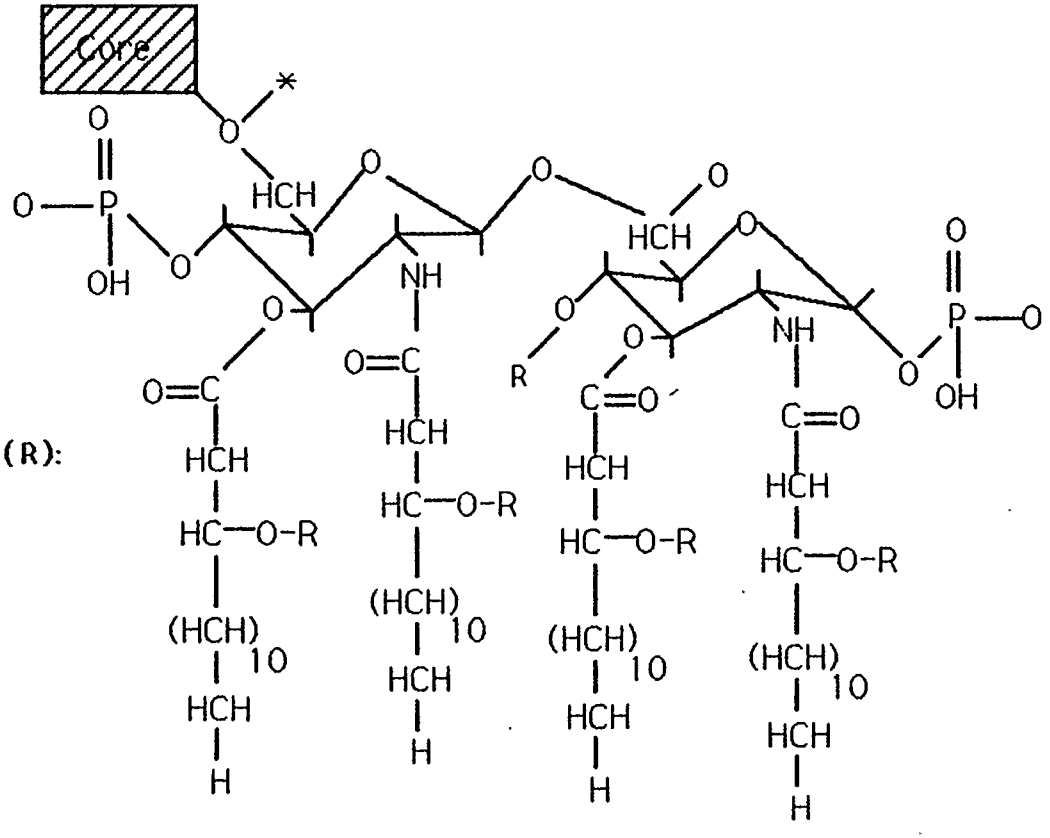

A
Gal II—1,2—Gal I—1,2—Glc II—1,3—Glc I
Glc II
1,3
Hep
KDO
lipid A
B
GlcNAc—1,2—Glc III—1,2—Glc II—1,3—Glc I
Gal I
1,6
Hep
KDO
lipid A
C
Glc III—1,2—Glc II—1,2—Gal I—1,3—Glc I
GlcNAc
1,3
Hep
KDO
lipid A
D
Gal III—1,2—Gal II—1,2—Glc II—1,3 Glc I
Gal I
1,4
Hep
KDO
lipid A
E
Glc II—1,3—Glc I
Hep
KDO
lipid A

Figure 4: The structure of lipid A

The structure of the lipid A region of the LPS, shown here, is believed to be invariant among enteric bacteria. Microheterogeneity is seen, however, in the amount of acylation of the diglucosamine backbone. The attachment of KDO, and hence the rest of the core, is to the 6′ carbon of the backbone (Takayama et al, 1983, Jiao et al, 1989).



ESTER-LINKED ACYL RESIDUES (R):

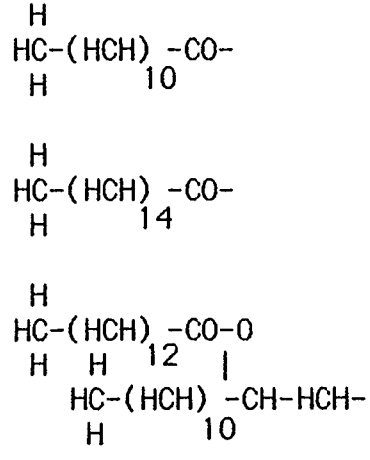


Figure 5: The KDO region

This structure is believed to be identical in S. typhimurium and E. coli. In the synthesis of this area, KDO III is not added until after the incorporation of Heptose I, therefore, Re mutants (Figure 2) do not include this residue. The phosphorylethanolamine is nonstoichiometrically substituted on KDO II, since these Re mutants also show its presence (Ghalambor et al, 1976; Brade et al, 1983a,b)

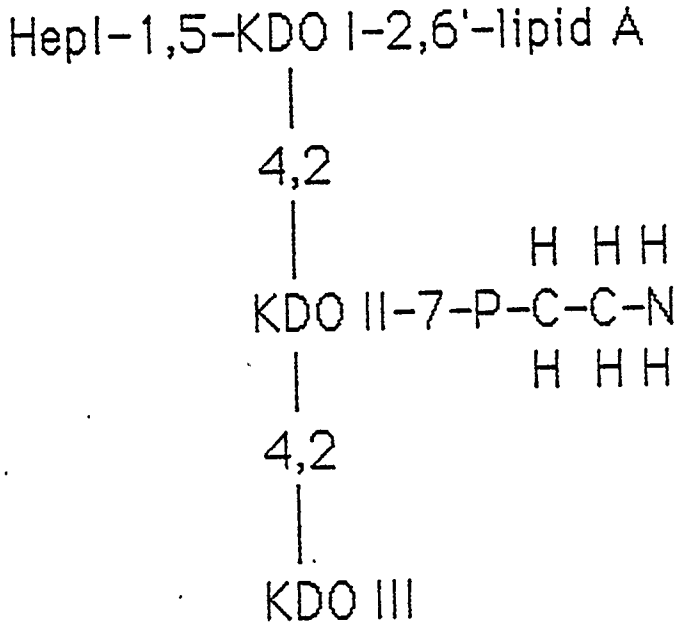


Figure 6: The rfa cluster

The known orders of genes in the rfa regions of E. coli K-12 and S. typhimurium are shown here. Parenthesis indicate that the exact order of the genes is not known. The Salmonella cluster has been more extensively studied than that of E. coli, consequently, more genes have been located. The location of rfaP in the E. coli cluster is not known. Its presence has recently been reported in S. typhimurium but its location is not yet known. The chemotypes of S. typhimurium LPS cores resulting from mutants in these genes are indicated in Figure 2.

E. coli

cysE---(rfaD rfaF rfaC)-(rfaP)-(rfaM rfaN)-rfaG---pyrE

Austin et al, 1990

Creeger and Rothfield, 1979

S. typhimurium

cysE--rfaD-rfaF-rfaC-rfaL-rfaK-rfaJ-rfaI-rfaB-rfaG--pyrE

summarized in Sirisena, 1990

Figure 7: Termination sites within the tra operon

The termination sites were determined by Gaffney et al (1983). sfrB mutants are deficient in F-mediated transfer function due to premature termination of transcription in this operon. The genes of the operon are indicated below the line showing EcoRI restriction sites. The open boxes below the genes indicate the sizes of the open reading frames and the open boxes above the line indicate the two regions in which transcription terminates in the absence of rfaH. The sites of termination, as determined by lengths of mRNA message produced (adapted from Gaffney et al, 1983) are shown here.

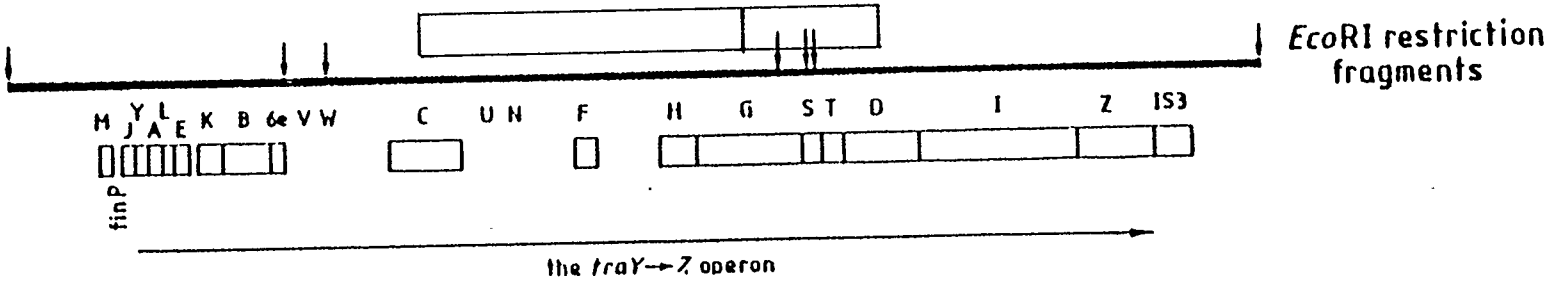

EcoRI restriction fragments
M
Y
J
A
L
E
K
B
6e
V
W
C
U
N
F
H
G
S
T
D
I
Z
IS3
finP
the traY→Z operon

Figure 8: The bacteriophage lambda and the lambda lytic cascade

The order of genes on the bacteriophage lambda is shown here. In the life-cycle of the phage, transcription begins from the p_L and p_R promoters and, in the absence of gpN, ends at the termination sites just beyond N and cro (transcript indicated by solid box). When sufficient gpN has accumulated, transcription extends into the delayed early genes (transcript indicated by open box). For the lytic life-cycle, gpQ allows transcription of the late genes for head and tail proteins as well as lysozyme (gene R) (transcript indicated by hatched box) (adapted from Birge, 1981).

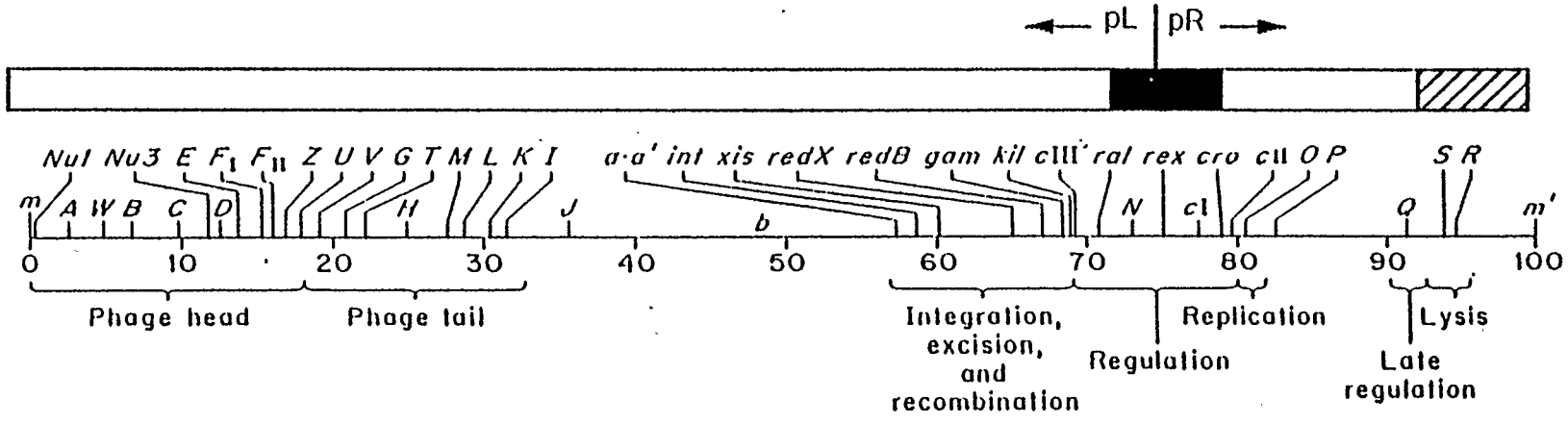

pL
pR
Nul Nu3 E F1 FII Z U V G T M L K I
a·a' int xis redX redB gam kil cIII' ral rex cro cII O P
S R
m
A W B C D H J
b
N cI Q m'
0 10 20 30 40 50 60 70 80 90 100
Phage head
Phage tail
Integration, excision, and recombination
Regulation
Replication
Late regulation
Lysis

Figure 9: The construction of pKZ25

pKZ25, the source of the E. coli rfaH gene for this study, was created by shotgun cloning of 2-4 kb fragments of the rfaH-complementing F'-ilv-met factor pDU450 (Rehemtulla, 1984). The plasmid is ampicillin resistant, from a vector gene, but sensitive to tetracycline, since the insertion is within the coding region of the pBR322 tetracycline resistance gene. Although, pDU450 is a putative Salmonella F', pKZ25 has been shown to carry the E. coli rfaH gene (see Results and Discussion).

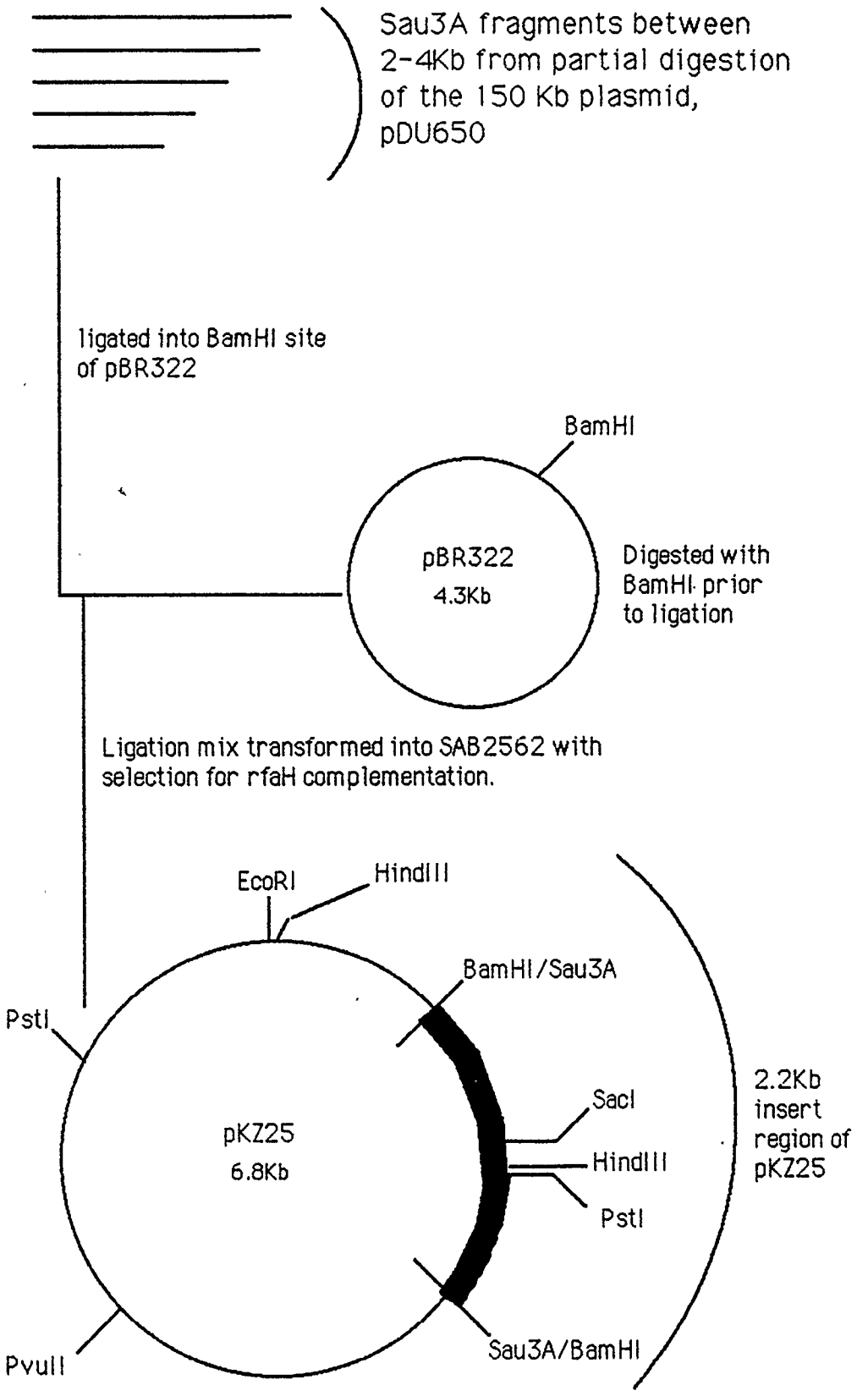

Sau3A fragments between
2-4Kb from partial digestion
of the 150 Kb plasmid,
pDU650
ligated into BamHI site
of pBR322
BamHI
pBR322
4.3Kb
Digested with
BamHI prior
to ligation
Ligation mix transformed into SAB2562 with
selection for rfaH complementation.
EcoRI
HindIII
BamHI/Sau3A
PstI
SacI
pKZ25
6.8Kb
HindIII
PstI
2.2Kb
insert
region of
pKZ25
PvuII
Sau3A/BamHI

Figure 10: The T7 RNA-polymerase promoter system

The gene of interest, under the control of the bacteriophage T7 promoter, is inserted into DTL111, which contains the structural gene for T7 RNA polymerase under the inducible control of the lambda cI857 promoter (Tabor and Richardson, 1985; Latour and Weiner, 1989). At high temperature (42°C), T7 RNA polymerase is made which transcribes the gene under control of the T7 promoter. rfaH, carried on the plasmids pKZ87 and pKZ91, was induced in this way.

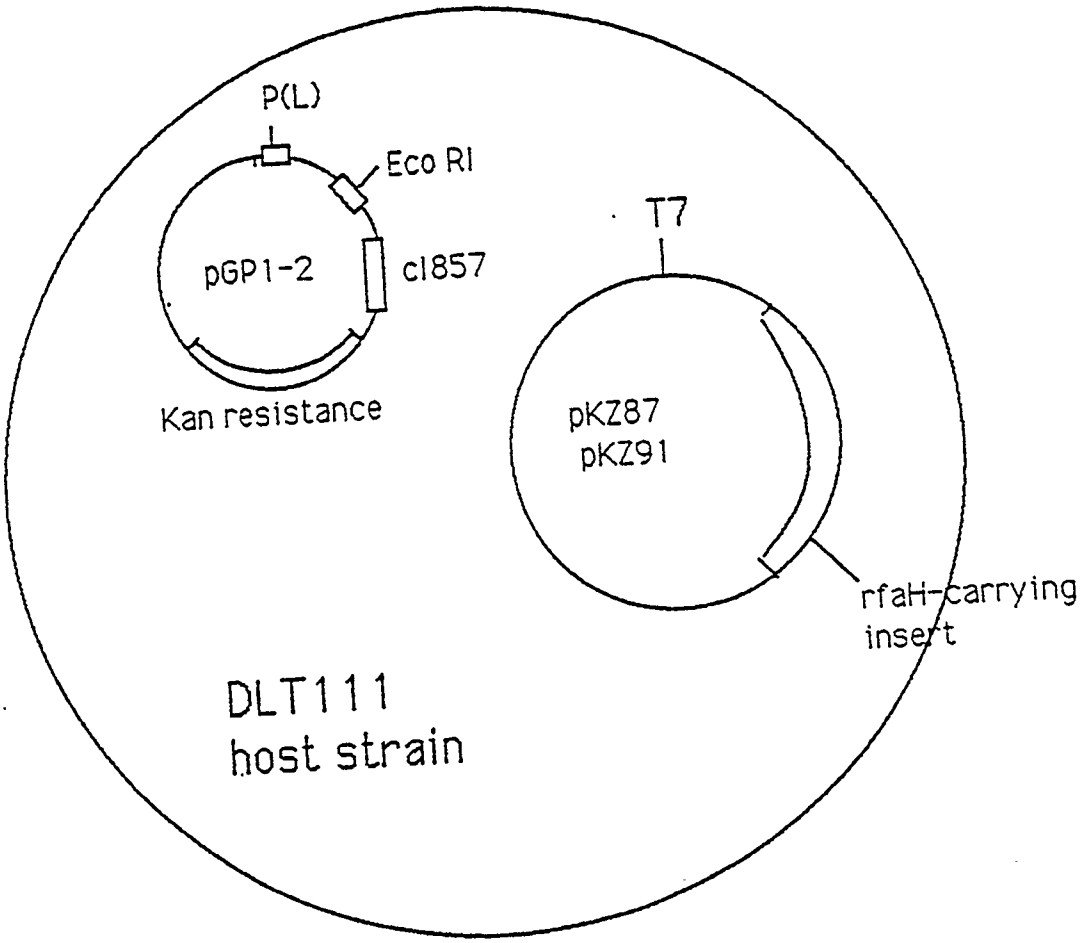
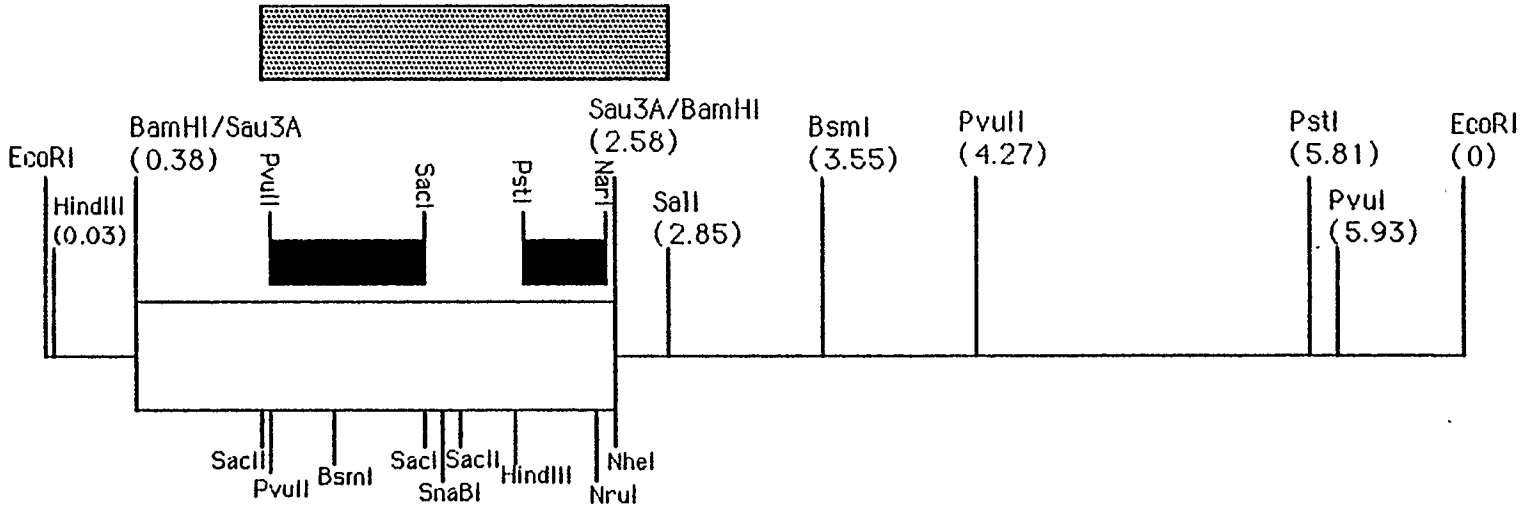


Figure 11: The restriction map of pKZ25

Restriction fragments were determined for enzymes generating one or fewer cuts in the pBR322 vector. The restriction sites for enzymes cutting within the insert region are shown here. Locations were determined by comparison to known vector sites (Maniatis et al, 1982; BRL and Pharmacia catalogues). For enzymes cutting only within the insert region, locations were determined relative to the vector HindIII and PvuII sites by the use of double digestions. The plasmid is shown as a linear map opened at the EcoRI site of the vector. The insert is shown as an open box, the vector as a single line.

Numbers in brackets indicate the locations of the vector sites in pKZ25 numbered from the EcoRI site. Numbers on the lines below indicate fragment sizes observed with the enzymes shown at the right. An asterisk indicates a fragment which was not visualized on the gel but is believed to exist based on sequence data or existing fragment sizes. The positions of the restriction sites shown here have not been corrected based on the known sequence (Figure 14).

The fragments used as probes for Southern hybridizations are indicated by solid boxes and the rfaH-complementing fragment cloned into pKZ87 and pKZ91 is indicated by the shaded box.



3.3			3.2	PvuII
2.1	4.5			HindIII
2.3		4.3		BsmI
0.3*	1.3	0.7	3.2	PvuII/BsmI
0.8	2.5		3.3	PvuII/SnaBI
1.9	2.5		3.3	PvuII/SacI
>0.1* 1.9	2.5		3.2	PvuII/SacII
1.5	1.9		3.2	PvuII/NruI
1.6	1.3		3.8	PvuII/NheI

Figure 12: Identification of rfaH-carrying fragments in the E. coli chromosome by Southern hybridization

The chromosome of E. coli NK5992 was probed with two fragments from the insert region of pKZ25 (Figure 11). The PvuII-SacI fragment contains rfaH flanking sequences (12a) while the PstI-NarI fragment contains the majority of the coding region (12b). There is strong hybridization seen with the E. coli chromosome with both probes and the fragment sizes shown match with those found in the appropriate region of the E. coli chromosome (Figure 13) (Kohara et al, 1987). In Figure 12a, lane 1 contains 25 ng pKZ25 cut with SacI and PvuII as a positive control for hybridization. Lane 1 in Figure 12b contains uncut pKZ25. The genomic DNA used was from NK5992 (E. coli K-12) and was digested as follows:

12a and 12b

lane:

2 NK5992/NarI

3 NK5992/NarI-PvuII

4 NK5992/PvuII

5 NK5992/HindIII-PvuII

6 NK5992/HindIII

7 NK5992/BamHI

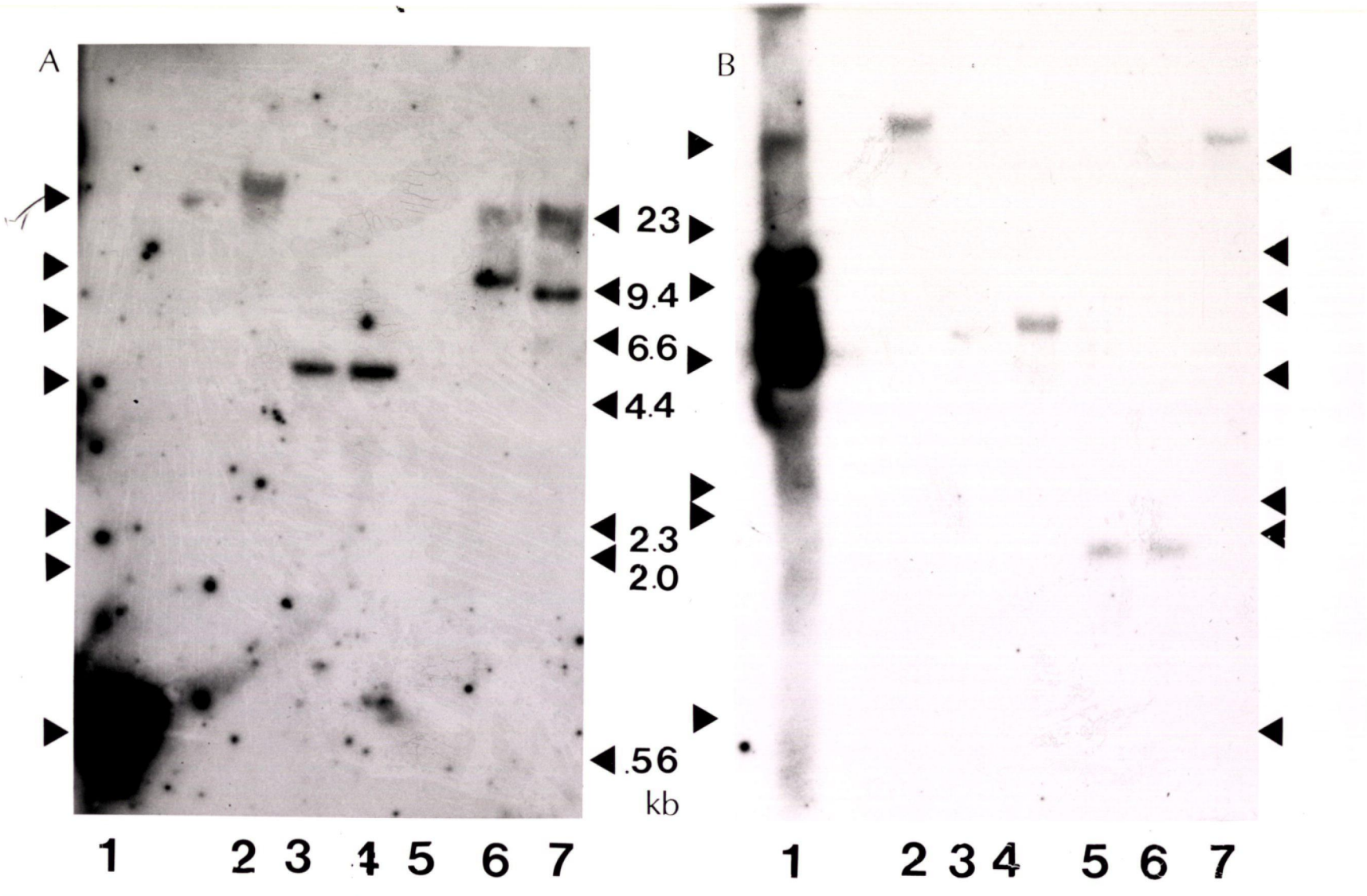

A
B
23
9.4
6.6
4.4
2.3
2.0
.56
kb
1 2 3 4 5 6 7
1 2 3 4 5 6 7

Figure 13: Location of rfaH on the E. coli chromosome

rfaH occurs at 86 minutes on the E. coli chromosome. The physical map of this area (Kohara et al, 1987) shows fragment sizes compatible with those seen from Southern hybridizations (Figure 12). The lighter shaded areas represent fragments hybridized by the SacI-PvuII probe and the striped area represents the BamHI fragment hybridized by the NarI-PstI probe. The darker shaded area is the PvuII fragment hybridized by both probes and the solid fragment is the rfaH-containing HindIII fragment hybridized by the NarI-PstI probe. The positions of the hybridizing probes are indicated by solid boxes above the map.

The observation that the two probes hybridize to different BamHI fragments although not separated by a BamHI site in pKZ25 indicates that the BamHI site seen in the E. coli rfaH gene carried on the plasmid pKZ17 (Rehemtulla, 1984) is also seen in the chromosome. SacI fragments are not included in the map (Kohara et al, 1987), so the existence of this site in E. coli is not yet determined.

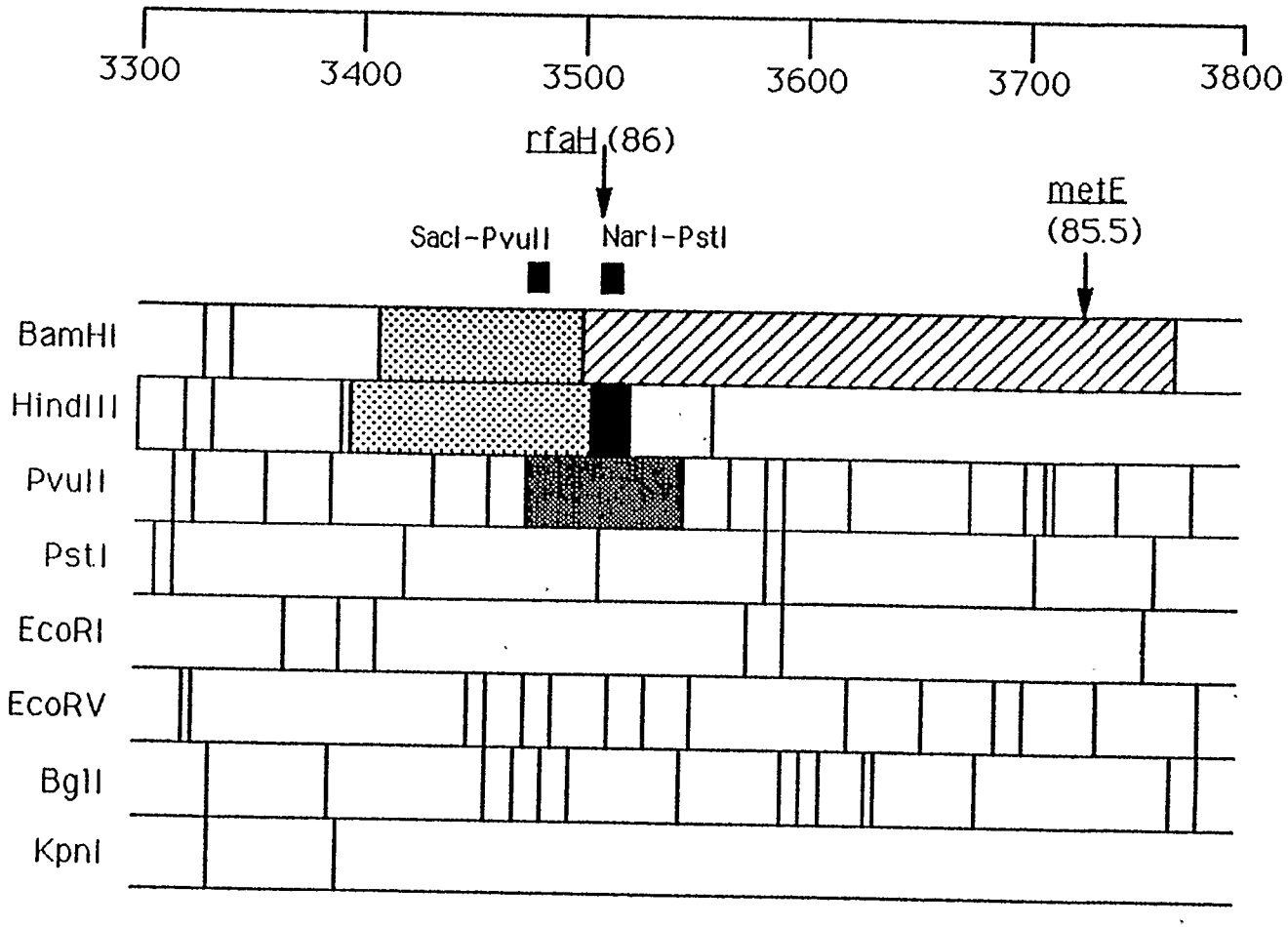

3300
3400
3500
3600
3700
3800
rfaH (86)
metE
(85.5)
SacI-PvuII
NarI-PstI
BamHI
HindIII
PvuII
PstI
EcoRI
EcoRV
BglI
KpnI

Figure 14: The sequence of the E. coli rfaH gene

The DNA sequence of the E. coli rfaH gene is shown, along with the proposed amino acid sequence for the protein product. -10 and -35 consensus sequences are indicated, as well as a possible Shine-Dalgarno sequence (overlined). The locations of the pKZ87-centre and pKZ91-centre primers are underlined and labelled (above the sequence) and the positions of the Tn1000 insertions are indicated by lower case letters where known. The letters indicating Tn1000 insertion sites represent the first position of the recognition site. The actual recognition site for the insertion contains the nucleotide below the letter as well as the following 3 residues. The Tn1000 insertions positioned in this figure are as follows: (orientations are given with respect to the pKZ25 EcoRI site at position 0)

a: zhj-1447::Tn1000 delta-gamma orientation
b: rfaH3118::Tn1000 gamma-delta orientation
c: rfaH3119::Tn1000 gamma-delta orientation
d: rfaH3121::Tn1000 gamma-delta orientation
e: rfaH3120::Tn1000 delta-gamma orientation
f: rfaH3123::Tn1000 delta-gamma orientation

```
SacI
GAGCTCTGGCAGAGTAAGGCATAAGTGGTAAATACGTACCACCGGGAGCG 50
TGATACGTTTTAGCTCACCCTGCTGTTCAAGCAGCGTCAAGAAGTCGCGT 100
AAATCGTTATATTTCATGGCGTCCATTGTAGCCTCTTAATCTCGCGCCCA 150

                                                    a
TTATACGGCGTTCATCTTTGCGATGCTGTAAATTTGTTAAATTAGCGTGA 200

 -35.........                  -10...           HindIII
ACTCTGACGGTATAACGCAAACCGGGGAATATAATTAACTTAGCGTAAAG 250

CTTTTGCTATCCTTGCGCCCCGATTAAACGGATAAGAGTCATTATGCAAT 300
                                                M  Q

                                                   pKZ91-
CCTGGTATTTACTGTACTGCAAGCGCGGCAACTTCAACGTGCCAGGAACA 350
S  W  Y  L  L  Y  C  K  R  G  N  F  N  V  P  G  T

centre primer
CCTCGAAAGACAGGCTGTGAATTGCTGGCACCGATGATCACCCTGGAAAA 400
 P  R  K  T  G  C  E  L  L  A  P  M  I  T  L  E  K

                      PstI
AATCGTGCGTGGAAAACGTACTGCAGTCAGTGAGCCATTGTTCCCCAACT 450
  I  V  R  G  K  R  T  A  V  S  E  P  L  F  P  N

                                        b
ACCTGTTTGTGGAATTTGACCCAGAAGTGATTCATACCACGACTATCAAC 500
Y  L  F  V  E  F  D  P  E  V  I  H  T  T  T  I  N

GCGACCCGCGGTGTCAGCCACTTCGTGCGCTTTGGCGCGTCGCCAGCGAT 550
 A  T  R  G  V  S  H  F  V  R  F  G  A  S  P  A  I

AGTCCCATCGGCGGTTATTCATCAGCTATCGGTATATAAACCGAAAGACA 600
  V  P  S  A  V  I  H  Q  L  S  V  Y  K  P  K  D

TTGTCGATGCGGCAACCCCTTATCCGGGTGATAAGGTGATTATTACCGAA 650
I  V  D  A  A  T  P  Y  P  G  D  K  V  I  I  T  E

GGCGCGTTCGAAGGCTTTCAGGCCATTTTCACCGAACCCGATGGTGAGGT 700
 G  A  F  E  G  F  Q  A  I  F  T  E  P  D  G  E  V

                       c d    ef                 pKZ87-
CGCTCCATGCTATTGCTTAATCTTATTAATAAAGAGATTAGCACAGTGTG 750
  A  P  C  Y  C  L  I  L  L  I  K  R  L  A  Q  C

centre primer
AAGAATACCGAGTTCCGCAACTCTAAACGCAATCCAACAGTGTTTGACAT 800
E  E  Y  R  V  P  Q  L  *

TAGCATCCGTGGTGGCAGCCAGCCATGCGGCATCTTCTCCACGCCGAGTG 850
CGCAATACGTTGCAAAATATGGGGCAGATGGGCTGGCTCGTTGCGCCGGG 900
ATGATGGCTTTGGCGTGAGATCCTCTACGCCGGACGCATCGTGGCCGGCA 950
TCACCGGCGCCACAGGTGCGGTTGCTGGCGCCTATATCGCCGACATCACC 1000
        Sau3A
GATGGGGAAGATC->pBR322 sequence
```

Figure 15: The locations of Tn*1000* insertions in pKZ25

The pKZ25 plasmid is displayed as a linear sequence opened at the EcoRI site (0 kb). Insertions marked *zhj* fall within the pKZ25 insert region but do not inactivate *rfaH*. Those marked *rfaH* do interfere with *rfaH*-complementing activity, and those labelled *pKZ25* fall within the pBR322 vector (and thus do not interfere with *rfaH*-complementation). Insertions shown on the left of the figure are in the gamma-delta orientation with respect to the pBR322 EcoRI site and those on the right are in the delta-gamma.

Six of the insertions were positioned exactly based on DNA sequence (Figure 14). These are indicated by the lower case letters a-f. The shaded bar indicates the SacI-SalI fragment which was cloned into pKZ87 and pKZ91.

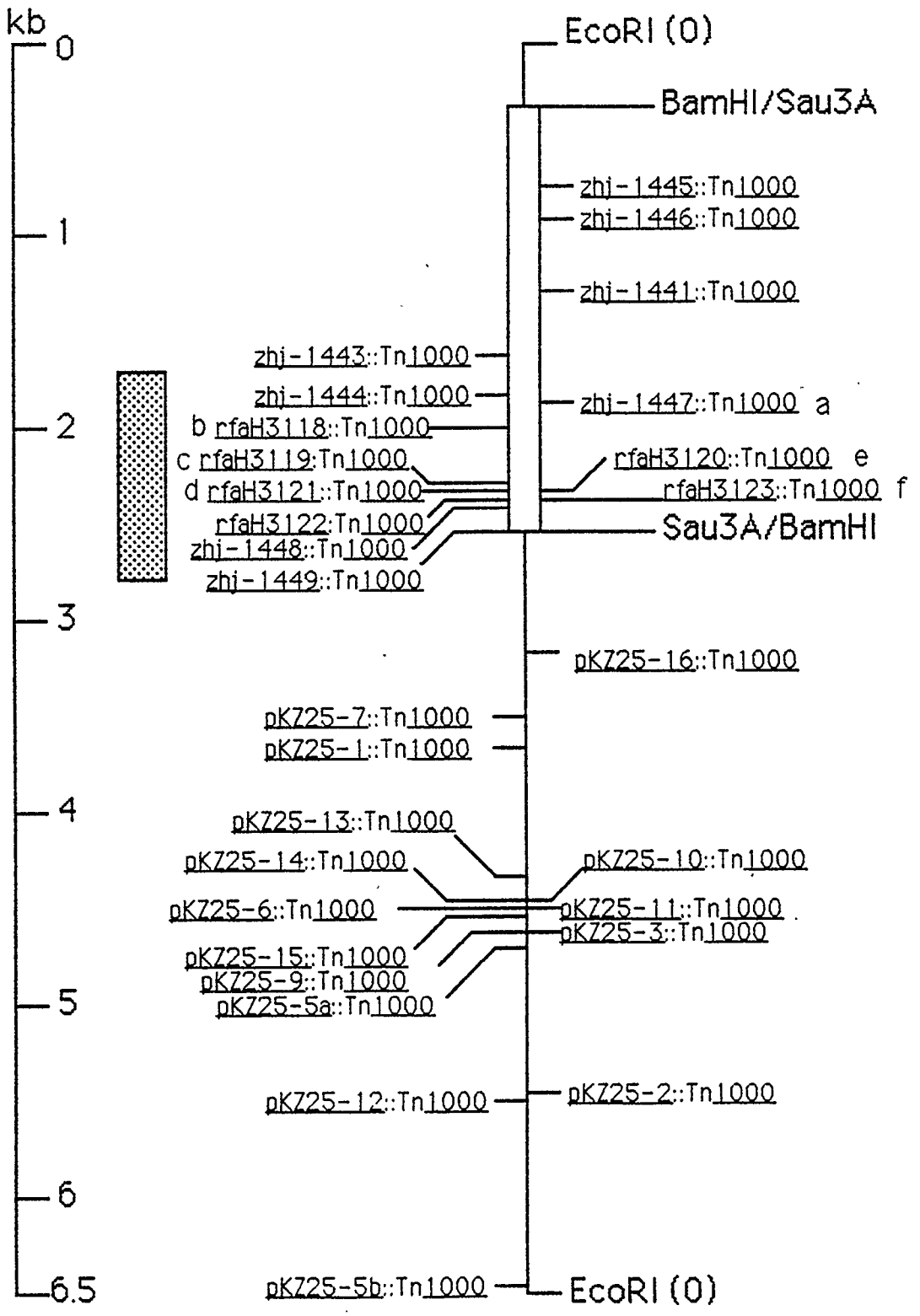

kb
0
1
2
3
4
5
6
6.5
EcoRI (0)
BamHI/Sau3A
zhj-1445::Tn1000
zhj-1446::Tn1000
zhj-1441::Tn1000
zhj-1443::Tn1000
zhj-1444::Tn1000
zhj-1447::Tn1000 a
b rfaH3118::Tn1000
c rfaH3119:Tn1000
rfaH3120::Tn1000 e
d rfaH3121::Tn1000
rfaH3123::Tn1000 f
rfaH3122:Tn1000
Sau3A/BamHI
zhj-1448::Tn1000
zhj-1449::Tn1000
pKZ25-16::Tn1000
pKZ25-7::Tn1000
pKZ25-1::Tn1000
pKZ25-13::Tn1000
pKZ25-14::Tn1000
pKZ25-10::Tn1000
pKZ25-6::Tn1000
pKZ25-11::Tn1000
pKZ25-3::Tn1000
pKZ25-15::Tn1000
pKZ25-9::Tn1000
pKZ25-5a::Tn1000
pKZ25-12::Tn1000
pKZ25-2::Tn1000
pKZ25-5b::Tn1000
EcoRI (0)

Figure 16: The restriction map of Tn1000

The restriction sites found within the Tn1000 transposon are shown here (Guyer, 1978). The transposon is a linear molecule and is shown with the gamma end at the top of the page. The EcoRI and SalI sites were used to locate the transposons within pKZ25 and determine its orientation relative to the pKZ25 EcoRI site.

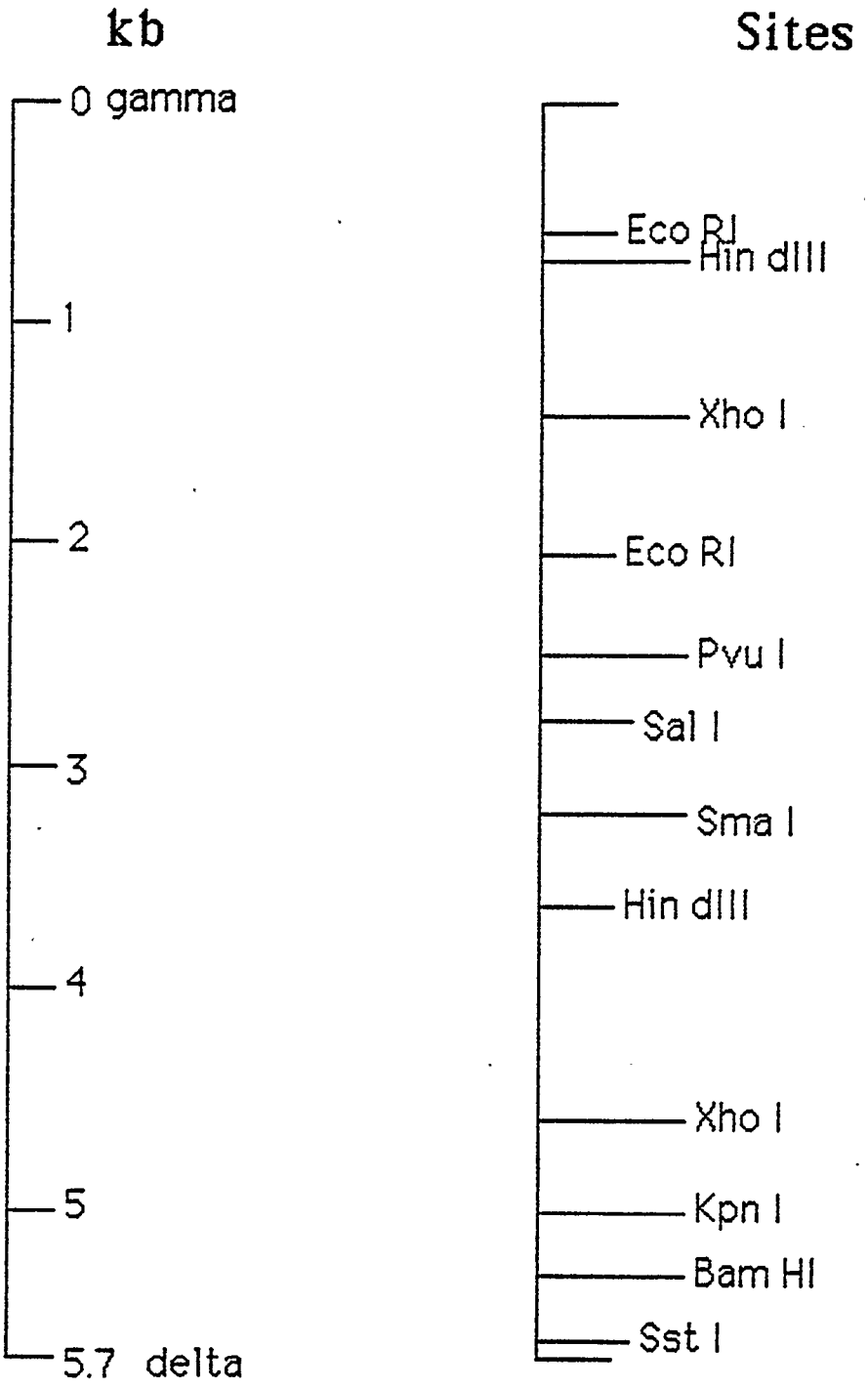

kb
Sites
0 gamma
1
2
3
4
5
5.7 delta
Eco RI
Hin dIII
Xho I
Eco RI
Pvu I
Sal I
Sma I
Hin dIII
Xho I
Kpn I
Bam HI
Sst I

Figure 17: Analysis of LPS from E. coli rfaH mutants carrying selected Tn1000 insertions

Lanes 1-5 (17a and b) contain LPS from various core mutants of E. coli and serve as controls. The complete genotypes of these strains are found in Table 1. The remaining lanes contain LPS from SA2885 carrying the pKZ25 plasmid with the indicated Tn1000 insertion (and therefore contain the mutation sfrB14). The rfaH$^-$ strains have a heterogeneous core as do the rfa$^+$ strains. Other defects migrate as a single spot representing the core region.

17a Lane:

1: C600 - rfa$^+$

2: SAB3000 - rfaI221

3: SAB2885 - sfrB14 (rfaH$^-$)

4: SAB2995 - rfaJ216

5: SAB2994 - rfaG215

6: zhj-1448::Tn1000

7: rfaH3120::Tn1000

8: rfaH3119::Tn1000

9: rfaH3118::Tn1000

10: zhj-1449::Tn1000

17b Lane:

1: SAB3000 - *rfaI221*

2: C600 - *rfa*$^{+}$

3: SAB2885 - *sfrB14*(*rfaH*$^{-}$)

4: SAB2995 - *rfaJ216*

5: SAB2994 - *rfaG215*

6: *rfaH3123*::Tn*1000*

7: *rfaH3122*::Tn*1000*

8: *rfaH3124*::Tn*1000*

9: *rfaH3120*::Tn*1000*

10:*zhj-1445*::Tn*1000*

11:*zhj-1447*::Tn*1000*

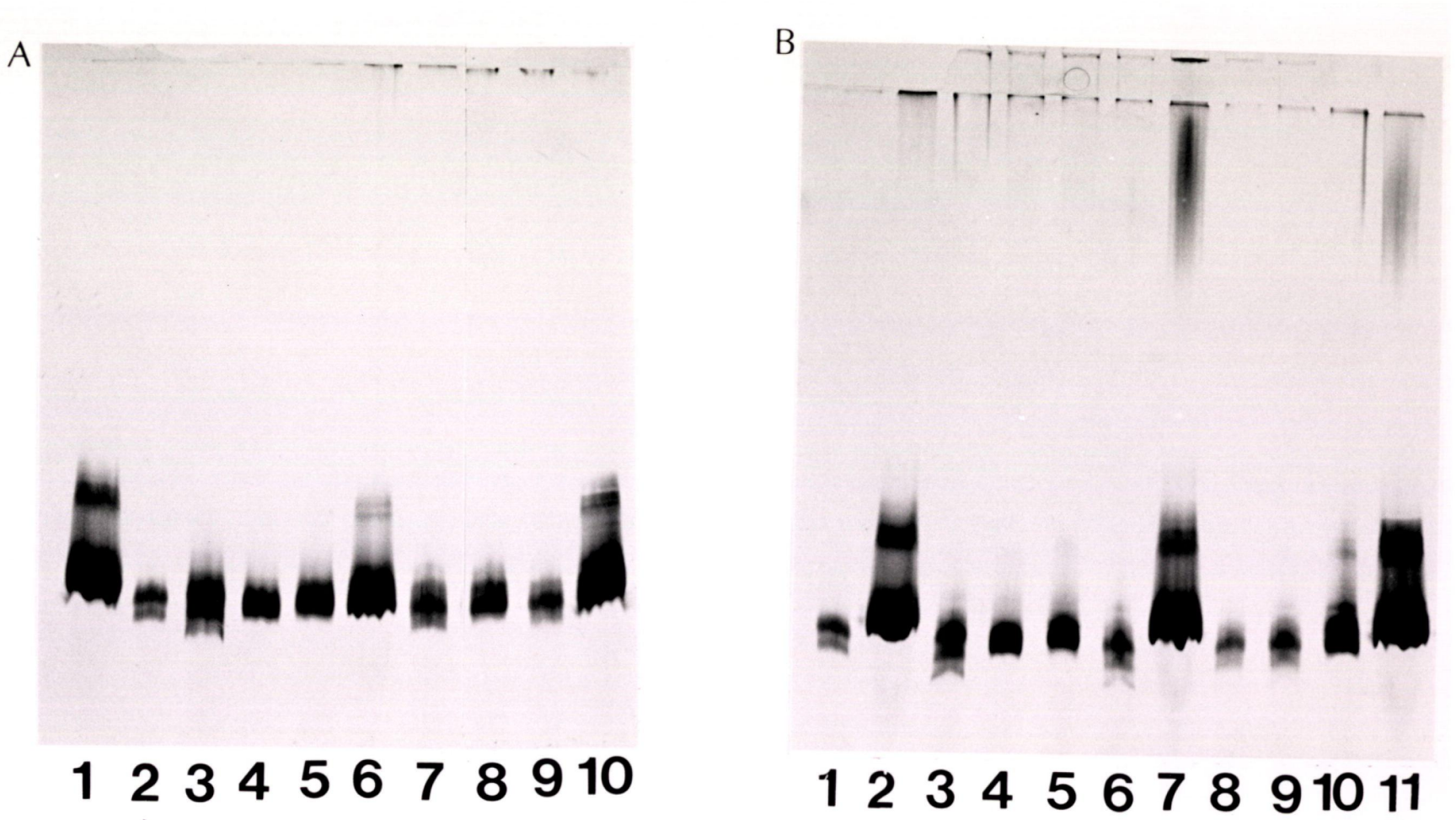

A
1 2 3 4 5 6 7 8 9 10
B
1 2 3 4 5 6 7 8 9 10 11

Figure 18: Analysis of LPS from S. typhimurium rfaH mutants carrying selected Tn1000 insertions

Figure 18a, lanes 1, 7-10, and 18b, lanes 1-4 and 9, contain LPS from various S. typhimurium core mutants. For the full genotypes of the strains in these lanes, see Table 1. The remaining lanes contain LPS from SA3444 which contains the pKZ25 plasmid carrying the indicated Tn1000 insertion. rfa$^+$ strains show a full side chain with many repeating units. Other defects migrate as a single spot representing the core region.

18a Lane:

1: SA3444 - rfaH3074

2: rfaH3118::Tn1000

3: rfaH3119::Tn1000

4: zhj-1448::Tn1000

5: rfaH3120::Tn1000

6: rfaH3121::Tn1000

7: SL3769 - rfaG471

8: SL3749 - rfaL446

9: SL3748 - rfaI432

10: SL3770 - rfa$^+$

18b Lane:

1: SL3748 - rfaI432

2: SL3770 - rfa^{+}

3: SL3749 - rfaL446

4: SL3769 - rfaG471

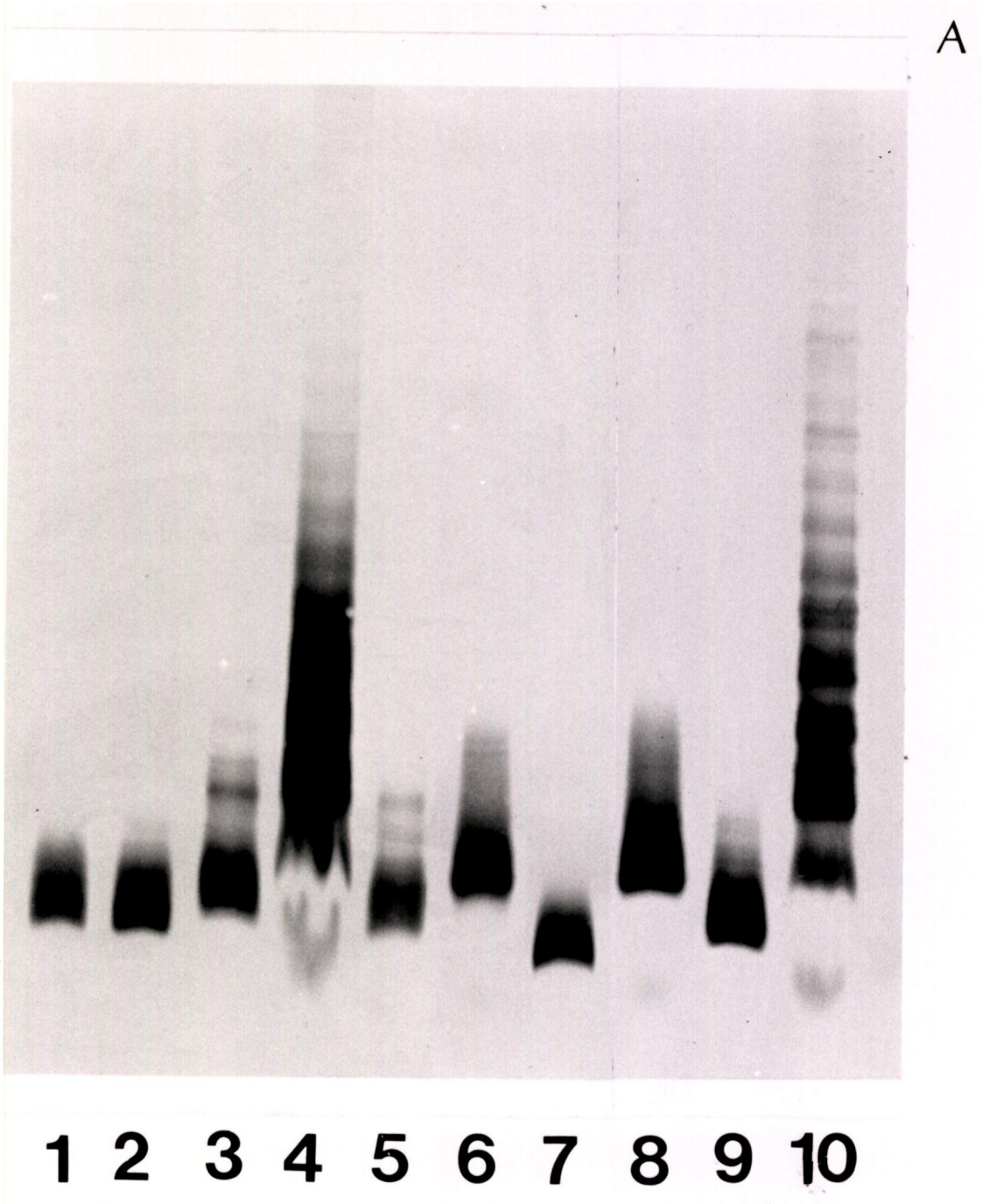
5: rfaH3122::Tn1000

6: zhj-1449::Tn1000

7: zhj-1445::Tn1000

8: zhj-1447::Tn1000

9: SA3444 - rfaH3074


A
1 2 3 4 5 6 7 8 9 10

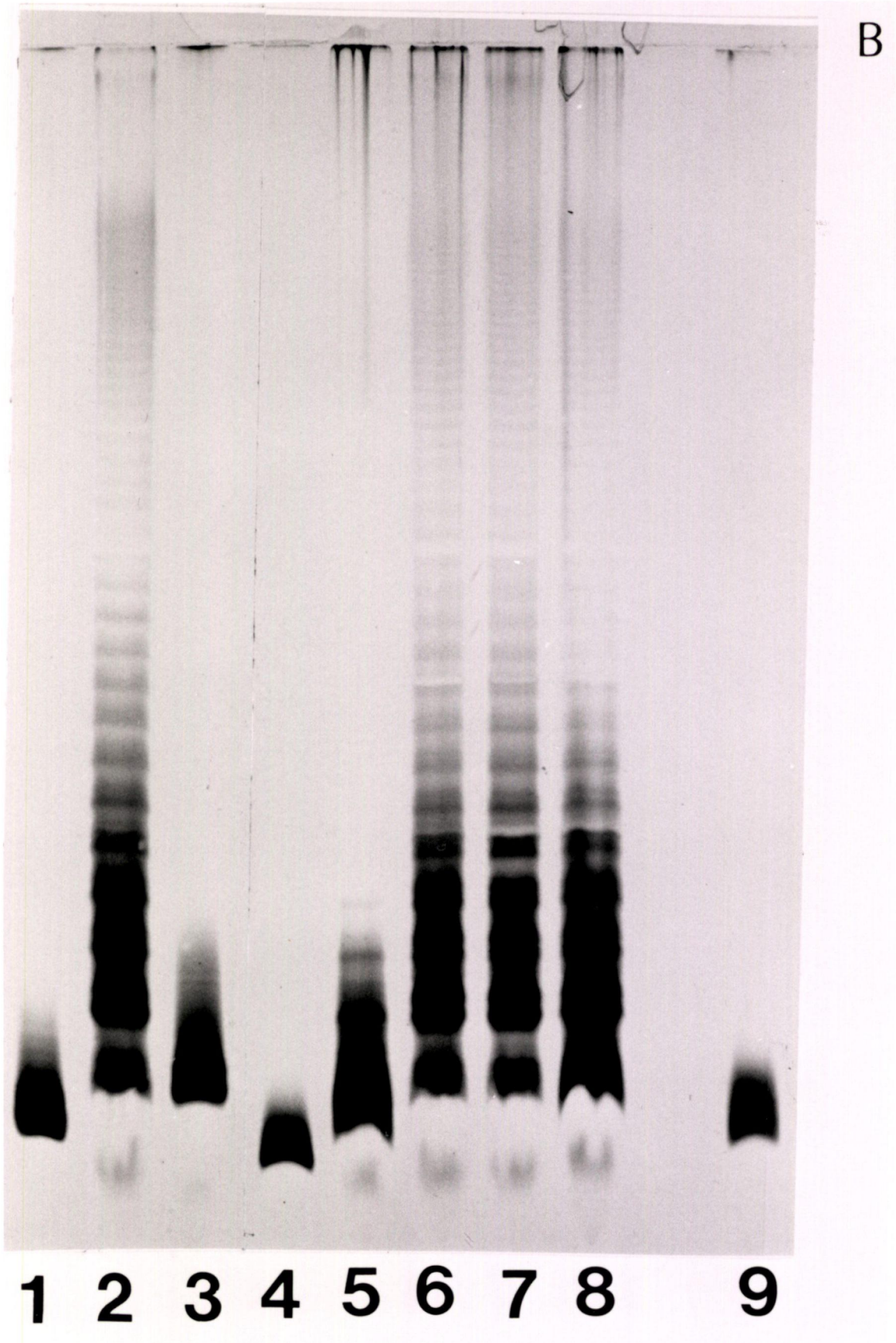
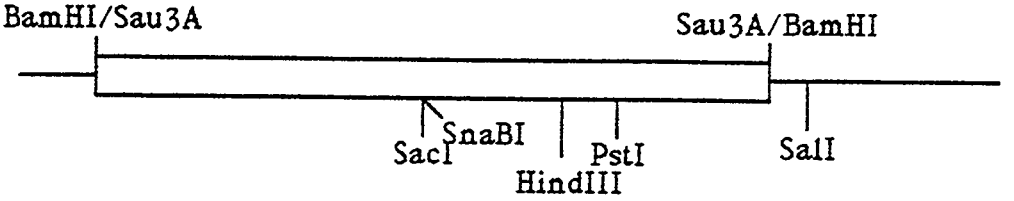

B
1 2 3 4 5 6 7 8 9

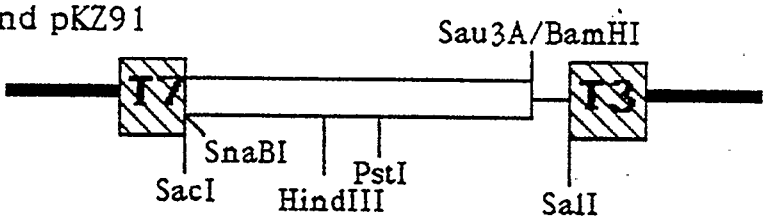
Figure 19: The subclones of rfaH

The open box at the top of the figure indicates the chromosomal insert in pKZ25. pKZ87 and pKZ91 contain the SacI-SalI fragment of pKZ25 inserted, in identical orientation with respect to the T7 and T3 promoters, into the multiple cloning sites of pBluescript KS- and pBluescript KS+, respectively. pKZ157 contains a HindIII-SacI fragment from pKZ25 inserted into pBluescript KS+. pKZ158 is a PstI-SnaBI deletion of pKZ87; the deletion is indicated by a triangle. The heavy lines indicate pBluescript vector sequences. The restriction sites in parenthesis were lost in the deletion.

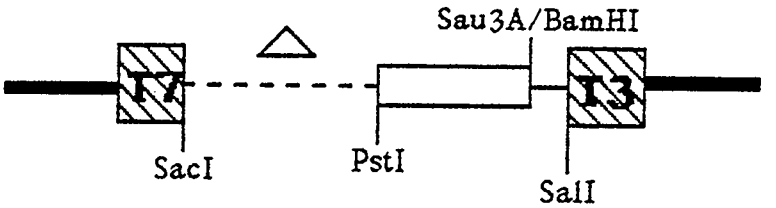
pKZ25



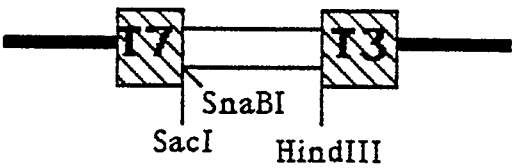
pKZ87 (KS-) and pKZ91 (KS+)



pKZ158



pKZ157



0.5 kb

Figure 20: Restriction patterns of plasmids generated from the pKZ87 ligation mix.

Three sizes of plasmid were recovered after ligation of the pKZ25 SacI-SalI fragment into pBluescript KS- and transformation in to XL1Blue. Digestion of the largest (lanes 2-9) and smallest (lanes 11-17) of these show fragment sizes consistent with those expected from 6.5 kb pKZ25 and 2.95 kb pBluescript KS- without an insert. The remaining, 3.2 kb plasmid (lanes 19-25) shows restriction fragments consistent with those predicted for the desired subclone, pKZ87. Lanes labelled "m" are lambda-HindIII molecular weight markers.

Lane

1: Marker

2: pKZ25/HindIII

3: pKZ25/PstI

4: pKZ25/PvuI

5: pKZ25/PvuII

6: pKZ25/SacI

7: pKZ25/SacII

8: pKZ25/SalI

9: pKZ25/SspI

10: KS-/HindIII

11: KS-/PstI

12: KS-/PvuI

13: KS-/PvuII

14: KS-/SacI

15: KS-/SacII

16: KS-/SspI

17: pKZ87/HindIII

18: pKZ87/PstI

19: pKZ87/PvuI

20: pKZ87/PvuII

21: pKZ87/SacI

22: pKZ87/SacII

23: pKZ87/SspI

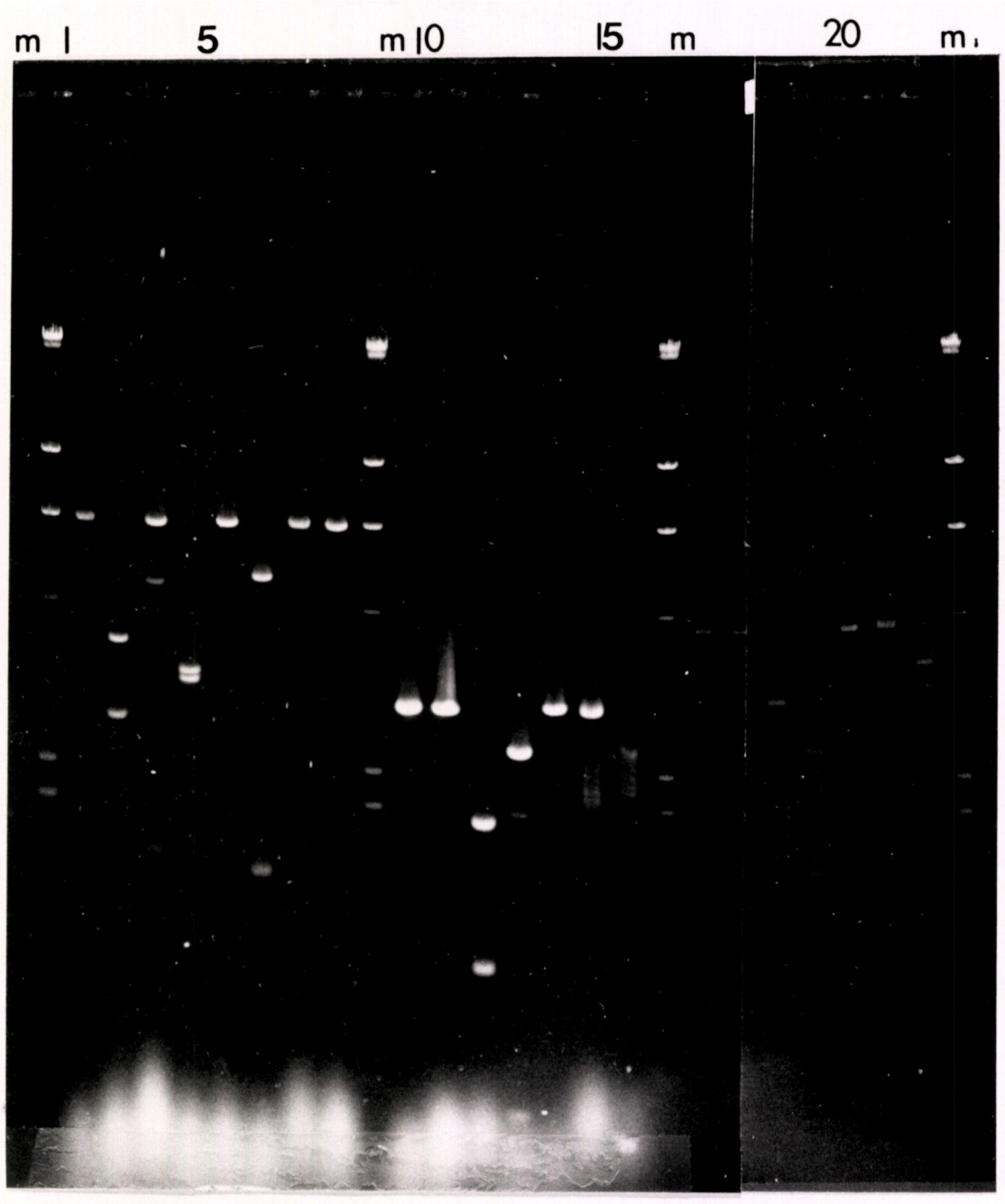

m
1
5
m
10
15
m
20
m

Figure 21: Strategy for sequencing rfaH

The overlapping sequences covering the entire rfaH-complementing region of pKZ25 are indicated here. Arrows show the sequence obtained from the subclone and primer combination indicated above. Sequence was obtained on both strands through a combination of subcloning and primer creation. The complete sequence is shown in Figure 14.

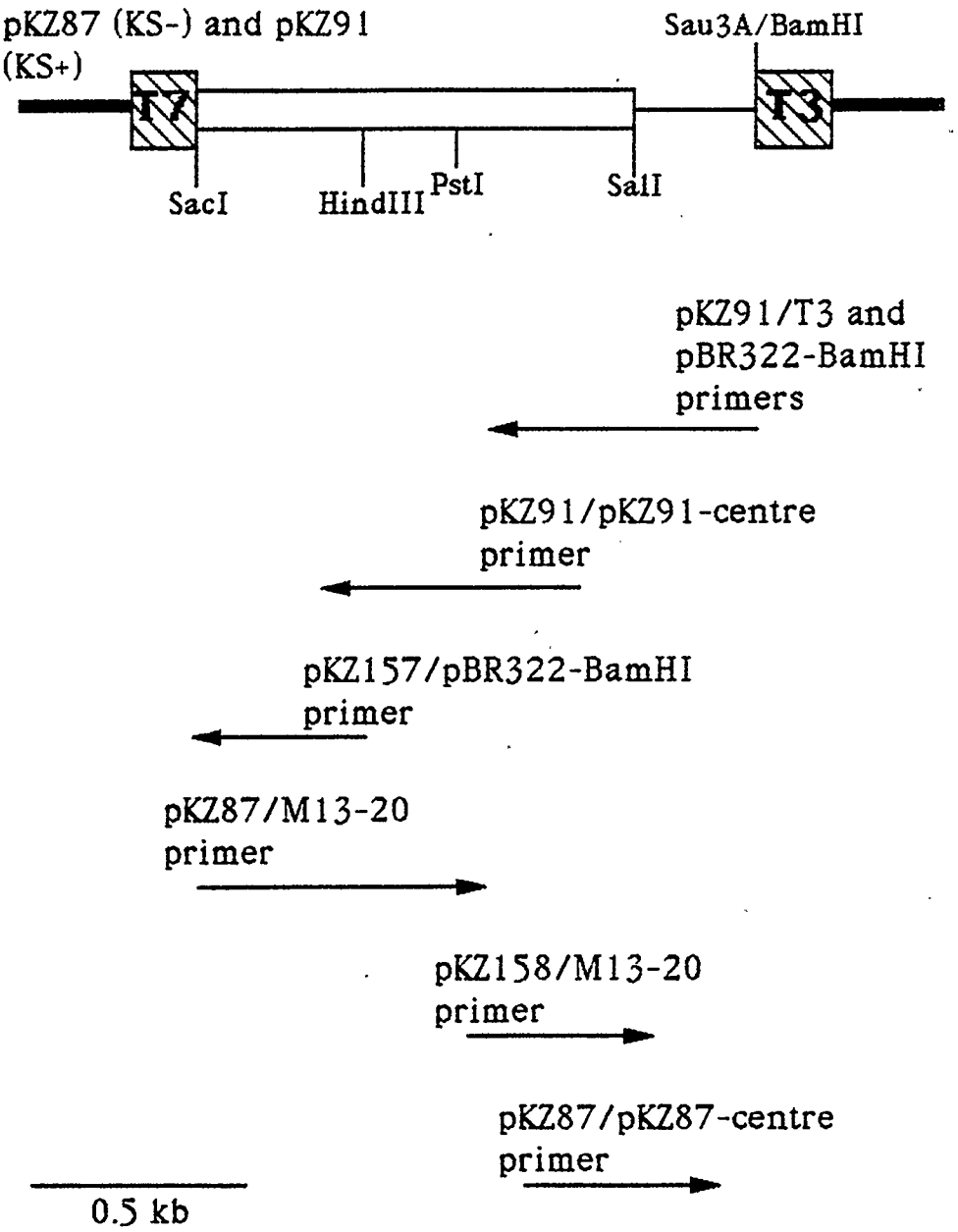

pKZ87 (KS-) and pKZ91
(KS+)
Sau3A/BamHI
T7
T3
SacI
HindIII
PstI
SalI
pKZ91/T3 and
pBR322-BamHI
primers
pKZ91/pKZ91-centre
primer
pKZ157/pBR322-BamHI
primer
pKZ87/M13-20
primer
pKZ158/M13-20
primer
pKZ87/pKZ87-centre
primer
0.5 kb

Figure 22: Hydrophobicity plot of the rfaH gene product [153]

The hydrophobicity was calculated using the SEQAID program and graphed with the aid of FRAMEWORK III (Ashton-Tate). The graph shows the hydrophobicity of the amino acid residues from the initial MET (left side) to the TER (right side). Hydrophobic residues are above the line and hydrophilic below. The overall product is neither strongly hydrophobic nor hydrophilic but it does have short regions of both types which are involved in the folding structure of the protein.

Figure 23: Analysis of the rfaH gene product using the T7 promoter

Using the T7 RNA-polymerase promoter system (Tabor and Richardson, 1985), the protein product of the rfaH genes carried on pKZ87 and pKZ91 was identified. The resulting band matches the size of 19.7 kDa predicted from the DNA sequence as well as from earlier minicell analysis of pKZ25 (Rehemtulla, 1984).

Lane:

1: SC75 (DLT111/pKZ87) - rfaH$^+$

2: SC68 (DLT111/pBluescript KS-) - rfaH$^-$

3: SC76 (DLT111/pKZ91) - rfaH$^+$

4: DS243 (DLT111/pBluescript KS+) - rfaH$^-$

5: DLT111

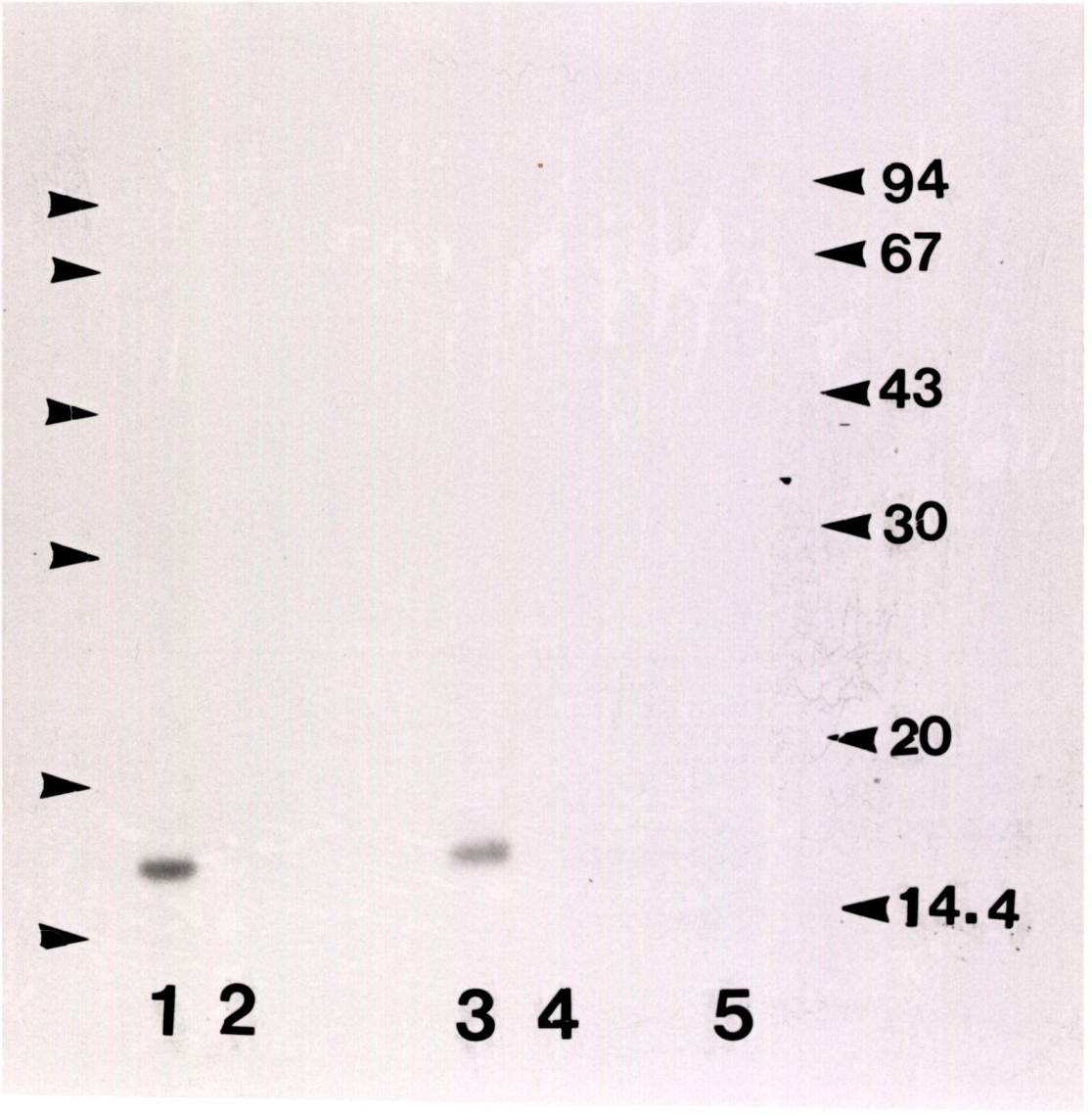
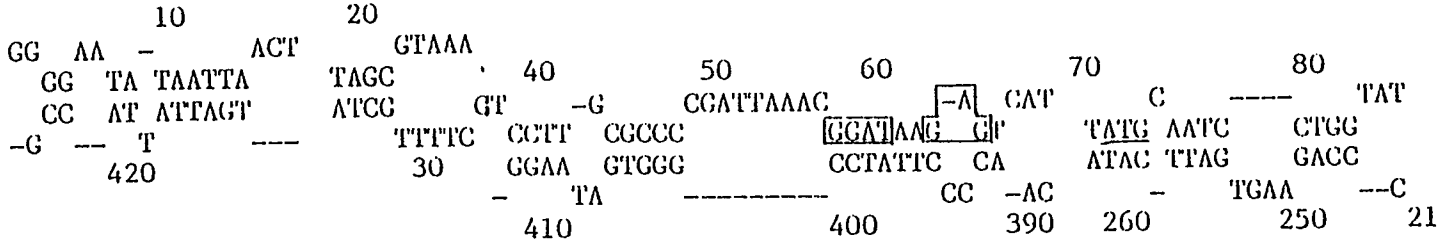

94
67
43
30
20
14.4
1 2
3 4
5

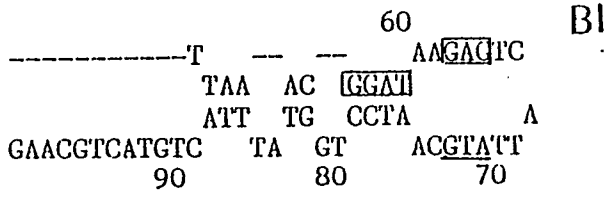
Figure 24: mRNA secondary structure in the rfaH promoter region

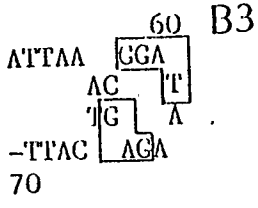
The structure was analyzed using the program PCFOLD. Two possible Shine-Dalgarno sequences (In boxes) are seen in the area preceding the MET codon (underlined). One of these (GAG) is available for association with the ribosome for some time before being trapped in secondary structure. This indicates that the protein may be highly expressed since the opportunity for translation initiation is relatively good.

A. mRNA secondary structure over 425 base region beginning at -10 sequence.

B. mRNA secondary structures in smaller regions surrounding the Shine-Dalgarno sequence.


A
10
20
40
50
60
70
80
420
30
410
400
390
260
250
21


B1
60
90
80
70


B3
60
70

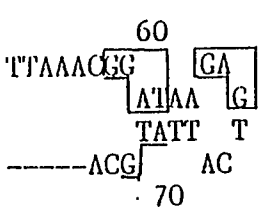

B2
60
70

Figure 25: Codon usage in the E. coli rfaH gene based on preferred codon usage in E. coli

The codons used in the E. coli rfaH gene coded for in pKZ25 are indicated below based on their frequency of usage in E. coli (Ikemura, 1982). Amino acids which are coded for by only one codon are represented by an uppercase "A". A lowercase "a" indicates an amino acid coded for by more than one codon, but the preferred codon is not known (Ikemura, 1982). "O" and "o" indicate optimal codons, with the uppercase letter being used when only one such codon exists for and amino acid. "x" indicates a non-optimal codon and "T" represents the termination codon (TAA in this case).

```
.AxxAx xOOax ooOOO oxxxx xxxoa 25
 0x0oO AOoOO OOoox Oooox xxxxO 50
 xOOOx oOxax Ooxao xoOOo oooxx 75
 aOoox ooxxo xxxxo oxaOx xoxOO 100
 Oaxxa oooxx Ooaxo xxoOo oOOox 125
 OxxOo Oxaox xoxax axOxx xxxxo 150
 OaOOO xoOxx T                 161
```

Figure 26: Predicted protein structure of the rfaH gene product

The tendency of amino acid residues to form alpha helices and beta pleated sheets was determined based on Chou and Fasman calculations (1974) using the program SEQAID. The 160 amino acid residues of the rfaH gene product are shown below their predicted structures. A lower case "a" indicates a tendency to form an alpha helix, a "b" indicates a tendency to form a beta-sheet, and "ns" indicates no structural preference. Regions predicted to form a helix or sheet are indicated by lines. In some areas, such as residue 139-150, a region within a helix-forming area may form a sheet. These regions are indicated by a broken line above the solid line indicating the larger structure.

Proline (PRO) residues disrupt protein structure (Chou and Fasman, 1974). These residues are indicated by bold type and an asterisk (*) in this figure.

```
            sheet
          _______________________
 b   b   ns  b   b   ns  ns  b   ns  ns
MET GLN SER TRP TYR LEU LEU TYR CYS LYS 10

 ns  ns  ns  ns  ns  b  *ns  ns  ns *ns
ARG GLY ASN PHE ASN VAL PRO GLY THR PRO 20

              helix
          ________________
 ns  ns  ns  ns  ns  a   a   a   a  *b
ARG LYS THR GLY CYS GLU LEU LEU ALA PRO 30

    sheet
  ___________
 b   b   ns  a   ns  ns  b   ns  ns  ns
MET ILE THR LEU GLU LYS ILE VAL ARG GLY 40

 ns  b   b   a   ns  ns  ns *ns  ns  ns
LYS ARG THR ALA VAL SER GLU PRO LEU PHE 50

        helix
      _________
*ns  a   b   a   b   ns  a   ns  a  *ns
PRO ASN TYR LEU PHE VAL GLU PHE ASP PRO 60

        sheet
      ____________
 a   b   b   ns  b   b   b   b   ns  ns
GLU VAL ILE HIS THR THR THR ILE ASN ALA 70

 b   ns  ns  b   a   ns  b   b   ns  ns
THR ARG GLY VAL SER HIS PHE VAL ARG PHE 80

                                 _____
 ns  a   ns  *b  a   b   ns *ns  a   a
GLY ALA SER PRO ALA ILE VAL PRO SER ALA 90

sheet or helix
 b   b   a   b   ns  ns  b   ns  ns *ns
VAL ILE HIS GLN LEU SER VAL TYR LYS PRO 100

          sheet
      ____________
 a   a   b   b   ns  ns  ns  ns *ns  ns
LYS ASP ILE VAL ASP ALA ALA THR PRO TYR 110

      ------sheet-----------   helix
      ____________________________
*ns  ns  ns  a   b   b   ns  ns  a   ns
PRO GLY ASP LYS VAL ILE ILE THR GLU GLY 120

              ----------sheet----------
            ____________________________
 a   ns  a   b   b   b   a   b   ns  ns
ALA PHE GLU GLY PHE GLN ALA ILE PHE THR 130

                                 -------
___                             _______
 a  *ns  a   ns  ns  b   ns  *b  b   b
GLU PRO ASP GLY GLU VAL ALA PRO CYS TYR 140

--------------------sheet-------------
 b   a   b   a   a   b   a   b   a   a
CYS LEU ILE LEU LEU ILE LYS ARG LEU ALA 150

  helix
 ____________________
 ns  ns  a   ns  b   b   b  *ns  b   a
GLN CYS GLU GLU TYR ARG VAL PRO GLN LEU 160
```

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