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Genetic Analysis of Lipopolysaccharide Core Biosynthesis by *Escherichia coli* K-12: Insertion Mutagenesis of the *rfa* Locus

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Tn*10* insertions were selected on the basis of resistance to the lipopolysaccharide (LPS)-specific bacteriophage U3. The majority of these were located in a 2-kilobase region within the *rfa* locus, a gene cluster of about 18 kb that contains genes for LPS core biosynthesis. The *rfa*::Tn*10* insertions all exhibited a deep rough phenotype that included hypersensitivity to hydrophobic antibiotics, a reduction in major outer membrane proteins, and production of truncated LPS. These mutations were complemented by a Clarke-Carbon plasmid known to complement *rfa* mutations of *Salmonella typhimurium*, and analysis of the insert from this plasmid showed that it contained genes for at least six polypeptides which appear to be arranged in the form of a complex operon. Defects in two of these genes were specifically implicated as the cause of the deep rough phenotype. One of these appeared to be *rfaG*, which encodes a function required for attachment of the first glucose residue to the heptose region of the core. The other gene did not appear to be directly involved in determination of the sugar composition of the core. We speculate that the product of this gene is involved in the attachment of phosphate or phosphorylethanolamine to the core and that it is the lack of one of these substituents which results in the deep rough phenotype.

The lipopolysaccharide (LPS) of enteric bacteria is a complex heterogeneous set of molecules that have a dual function as integral building blocks of the outer membrane and as important determinants in the interaction of the organism with its environment. A large number of genes are involved in the synthesis of these molecules. The first unique steps in the LPS biosynthetic pathway are those of lipid A synthesis; the genes for these early steps are scattered and in some cases associated with genes involved in other kinds of macromolecular synthesis (12). Presumably this is to allow coupling of LPS synthesis to cell growth. Genes for the later steps in which the complex carbohydrate attached to lipid A is built up are organized into large gene clusters which correspond roughly to functional units of the molecules (24), perhaps to allow cells to manage the complexity of the biosynthetic pathway while allowing interchangeability of different structures to generate surface diversity.

The first of these gene clusters is the *rfa* locus, located between *cysE* and *pyrE* at about 81 min on the linkage map of *Escherichia coli* K-12 and at a comparable site in *Salmonella typhimurium* (22, 24). This region is involved in biosynthesis of the LPS core. Genes corresponding to 10 distinct biochemical functions have been described in *S. typhimurium* (24), and most of these genes have been ordered by genetic and physical mapping (22, 24). Although *rfa* mutants have been widely used to study LPS biosynthesis, none of the mutations in these strains have been characterized, and there is only indirect evidence that the genes which have been described encode sugar transferases or other biosynthetic enzymes. In *E. coli*, information about the *rfa* locus is even more fragmentary, being confined to limited genetic characterization of a few mutants and demonstration that several Clarke-Carbon plasmids are capable of phenotypic complementation of *Salmonella* mutants (8, 10).

The interest of our laboratory has been in understanding the deep rough phenotype, which is exhibited by a subset of *rfa* mutants. This phenotype is highly pleotropic; it includes a dramatic decrease in outer membrane protein, an increase in outer membrane phospholipid, hypersensitivity to hydrophobic antibiotics and detergents, accessibility of phospholipid to extracellular reagents, which indicates redistribution of phospholipid into the outer leaflet of the outer membrane, and resistance to LPS-specific and some protein-specific bacteriophage (29, 34). In *S. typhimurium* (24), strains exhibiting this phenotype are mutant in *rfaC*, *-D*, *-E* and *-F*, which are defective in synthesis or attachment of L-glycero-D-*manno*-heptose, *rfaG*, which lacks the sugar transferase for attaching the first glucose to the heptose region, *rfaP*, which is involved in the phorphorylation of heptose, and *rfaH* (called *sfrB* in *E. coli*), which is not part of the *rfa* locus but which encodes a positive regulator that affects expression of *rfa* genes as well as genes in the *tra* operon of plasmid F (11). Genes encoding functions required for synthesis or addition of sugars distal to the first glucose, such as *rfaI*, *-J*, and *-K* and *galE*, do not exhibit the deep rough phenotype, so this phenotype is not simply due to truncation of the outer core.

To define the primary defect leading to the deep rough phenotype more precisely, we isolated and characterized a series of *rfa*::Tn*10* insertions that exhibited the deep rough phenotype. The polarity of these mutations has allowed us not only to determine the genetic basis of the deep rough phenotype but also to begin to study the regulation of core biosynthesis.

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MATERIALS AND METHODS

Strains and culture conditions. CS180 (2) was used for mutant isolation and as the background strain for all experiments. Strains constructed by moving various mutations into CS180 by P1 transduction are as follows: CS1858, carrying the *sfrB11* allele of M1170, from L. Rothfield; CS1776, carrying *galE*::Tn*10* from SG2501, from S. Gottesman; and CS1801, carrying Δ*galOPE*::Cml from SA2700, from S. Adhya. *S. typhimurium rfa* mutants used as LPS standards were from K. Sanderson. Phage λgt4.0 (13) was from R. Davis, and λ1098 was from N. Kleckner (35).

Unless otherwise stated, all cultures were grown on LB broth or agar (25). Since most experiments involved strains lysogenic for λgt4.0 or its derivatives, cultures for analysis of protein or LPS were grown at 33°C.

Isolation of Tn*10* insertion mutations. A late-log-phase culture of CS180 grown in LB broth containing 0.2% maltose was mixed with an equal volume of λ1098 (35) containing 7×10^9 phage per ml. After the culture was allowed to stand at room temperature for 45 min, the volume was doubled by the addition of fresh broth. Multiple 1-ml samples were removed and incubated for 3 h with shaking at 37°C to permit expression. To each tube was added 0.1 ml containing ca. 10^{10} phage U3, which were allowed to adsorb for 20 min at room temperature. Dry LB agar plates containing 20 μg of tetracycline per ml and 2.5 mM sodium pyrophosphate were spread with 0.5 ml of this mixture. Mutants were picked after 24 h, restreaked, and tested for P1 cotransduction of U3 resistance ($U3^r$) and tetracycline resistance (Tet^r). Only one mutant was picked from each sample to prevent selection of siblings.

Genetic techniques. P1 transduction and preparation of transducing lysates were done according to Miller (25). Since the *rfa*::Tn*10* insertion mutants were partially P1 resistant, it was necessary to use more concentrated cell suspensions when these mutations were present either in the donor or in the recipient. Complementation by cloned fragments inserted into λgt4.0 was carried out by first lysogenizing CS180 with the phage and then transducing in the *rfa*::Tn*10* insertions. This was done because we encountered difficulty in obtaining lysogens of strains that already carried *rfa*::Tn*10* insertions.

This problem and the fact that the deep rough mutants were partially defective as recipients for conjugation and P1 transduction made it impractical to use recombination-deficient strains for complementation assays. Therefore, complementation was done in Rec^+ strains, and it was necessary to verify that restoration of a wild-type phenotype was due to complementation and not recombination. In the case of complementing phage, this was done by curing out the phage and demonstrating that the cured strain regained the mutant phenotype ($U3^r$) and retained Tet^r. Curing was done by growing an LB culture to a density of 35 Klett units at 33°C, shifting the culture to 43°C for 5 min to induce excision of the prophage, and then returning the culture for 33°C for 30 min to allow curing. The culture was then serially diluted in LB containing 1.25 mM sodium pyrophosphate and plated on LB agar containing 1.25 mM sodium pyrophosphate. The plates were incubated at 42°C to select cured cells. Complementation by the phage insert was considered valid when all cured colonies were Tet^r and $U3^r$. Curing of complementing plasmids was done by selecting for $U3^r$ by plating 0.2 ml of an overnight culture on a plate seeded with U3 and then screening for colonies that also became ampicillin sensitive (Amp^s). Complementation by the plasmid was considered valid when all of the Amp^s $U3^r$ colonies were Tet^r.

DNA techniques and constructions. DNA manipulation techniques were carried out as described previously (5) except as noted. Southern hybridization analysis of chromosomal DNA was carried out by the method of Silhavy et al. (33) except that Gene Screen Plus (Dupont, NEN Research Products, Boston, Mass.) was used according to the protocol recommended by the manufacturer. To create blunt ends from 5′ overhangs, Klenow fragment of DNA polymerase was used in a fill-in reaction; to create blunt ends from 3′ ends, the procedure of Henikoff (16) was used. Linkers were ligated to these blunt ends to create necessary sites.

The original sources of all cloned DNA fragments were the Clarke-Carbon plasmids pLC10-7 and pLC17-24; Fig. 1 shows the scheme by which various chromosomal fragments were obtained by cloning fragments from the inserts of these plasmids. Although the exact endpoints of the inserts of the Clarke-Carbon plasmids are unknown, approximate boundaries of their inserts are shown in Fig. 2. Plasmid pKZ14 was constructed in the laboratory of K. Sanderson and consisted of a *Bgl*II fragment of ca. 5.1 kilobases (kb) from plasmid pLC10-7 cloned into the *Bam*HI site of pBR322. The copy of pLC10-7 from which this insert was subcloned carried an insertion of extraneous DNA (presumably an insertion sequence element) located adjacent and to the right of the *Bam*HI site as it appears in Fig. 1. This was indicated by the presence of sites to the right of the *Bam*HI site of pLC10-7 which were not from pColE1 or from the *rfa-pyrE* region of the chromosome (data not shown). Since it was not known whether this extraneous DNA extended across the *Bam*HI site, the rfa7.0 fragment was constructed by using a *Nco*I-*Bam*HI fragment derived from pLC17-24 which had an insert covering the region from *rfa* to *pyrE* (Fig. 2) and did not contain extraneous DNA.

Plasmids used for complementation studies and for analysis of coding capacity by in vitro transcription-translation were constructed by insertion of the cloned fragments into the polylinker regions of either pGEM3 or pGEM4 (Promega Biotec, Madison, Wis.), depending on the orientation desired with respect to the SP6 and T7 promoters which flank these polylinker regions. To generate recombinant phage for single-copy complementation assays, *Eco*RI fragments flanked by sites from the polylinker or sites generated by blunt-end ligation of *Eco*RI linkers into chromosomal restriction sites were ligated into *Eco*RI-digested λgt4.0, and recombinant phage were identified by plaque hybridization of transfectants (4).

Gel electrophoretic analysis of proteins and LPS. Analysis of protein products of in vitro transcription-translation of plasmids was done by using a procaryotic DNA-directed translation kit from Amersham Corp. (Arlington Heights, Ill.) as described previously (5). For analysis of outer membrane proteins, the outer membrane fraction was isolated by a simplified differential centrifugation procedure (32), and gel loadings were normalized by using an internal radioactive standard so that each well contained protein from the same number of cells (5). This was necessitated by the fact that the deep rough mutations affected the total amount of outer membrane protein per cell. The protein gels systems are those described previously (32).

Silver-stained LPS gels were done by a modification of the protease digestion procedure of Hitchcock and Brown (17). The modifications were as follows. Instead of whole cells, an outer membrane fraction identical to that used for protein gels was used. Glycerol was omitted from the lysing buffer,

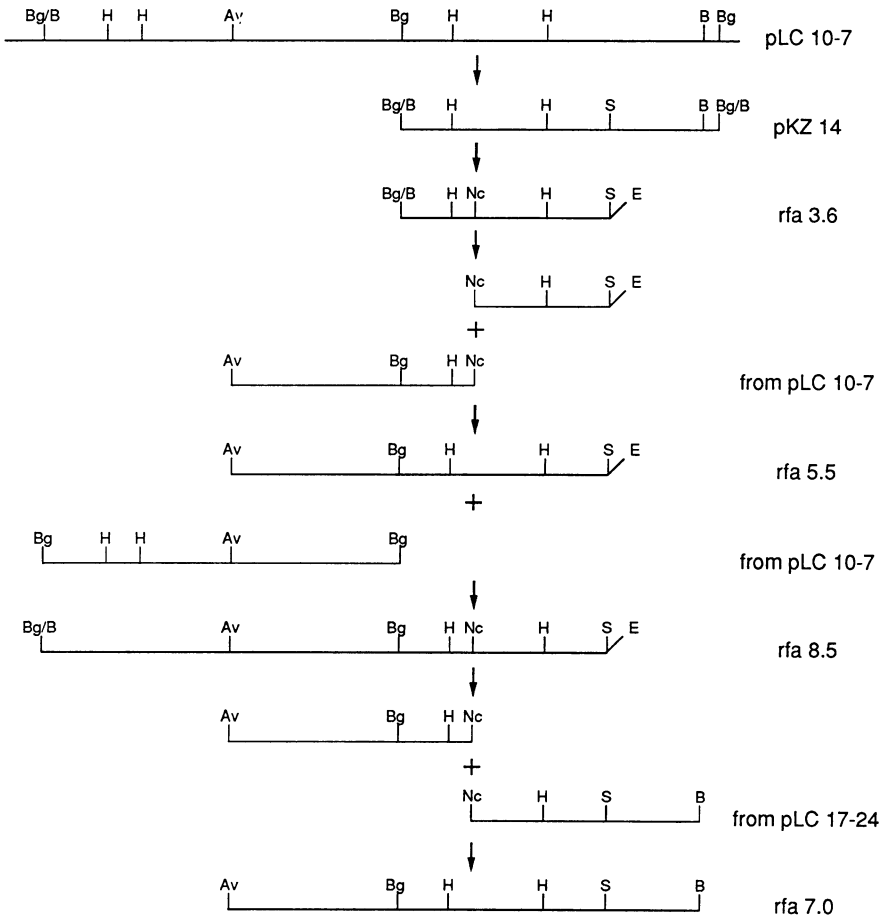

FIG. 1. Plasmid constructions. The scheme shows the origin of the various cloned *rfa* fragments derived from Clarke-Carbon plasmids pLC10-7 and pLC17-24. Only partial restriction maps are shown. Restriction sites: Av, *Ava*I; B, *Bam*HI; Bg, *Bgl*II; Bg/B, hybrid site from cloning *Bgl*II into *Bam*HI; E, *Eco*RI; H, *Hin*dIII; Nc, *Nco*I; S, *Sal*I.

and 10% Ficoll was used for increasing the density of the samples layered in the gel wells. An 18% acrylamide gel with a ratio of acrylamide to bisacrylamide of 37.5 was used, and staining was carried out exactly as described by those authors. The boiling and proteinase K digestion were omitted when phenol-chloroform-petroleum ether (PCP)-extracted LPS preparations were analyzed.

Purification and chemical analysis of LPS. LPS was isolated by a modification of the PCP procedure of Galanos et al. (15). Bacteria from 12 liters of culture grown to late log phase in LB in shake flasks at 33°C were harvested by centrifugation and washed once by suspension in 300 ml of cold deionized water, followed by centrifugation. The pellets were suspended in a small amount of deionized water to make a thick paste, to which was added 180 ml of cold methanol. This mixture was homogenized briefly in a laboratory blender and centrifuged. The pellet was suspended in 180 ml of cold acetone, and homogenized as described above, and centrifuged in a glass bottle. After all of the acetone was drained off, the mouth of the bottle was covered with a tissue secured with a rubber band and dried in vacuo in a lyophilizer jar for several hours. The dried bacteria (ca. 4 g) were placed in the bowl of a small blender and homogenized with 50 ml of PCP prepared exactly as described by Galanos et al. (15). After centrifugation and removal of the PCP, the gummy pellet was again homogenized with 50 ml of PCP and centrifuged. The pooled PCP supernatants were filtered through Whatman no. 1 paper and placed in a small beaker placed on a warm (about 50°C) metal heating block. The material was concentrated under a stream of N_2 to about 15 ml. During this concentration step, LPS from some of the mutants began to precipitate on the walls of the beaker. After the preparation cooled to room temperature, precipitation was completed by adding, dropwise with stirring, about 1 to 2 ml of water. The suspension was transferred to a Corex tube and centrifuged. Depending on the strain, the LPS formed a pellet or floated on the surface as a waxy cake. This and any LPS remaining on the walls of the beaker were suspended in 20 ml of methanol and centrifuged. The pellet was suspended in 10 ml of methanol and centrifuged; this step was repeated once. The final pellet was drained and dried in vacuo in a lyophilizer for 1 h. The dried material was suspended with a Potter homogenizer in 24 ml of water containing 0.1 mM $MgCl_2$ and 0.1 mg each of pancreatic RNase and DNase. The suspension was centrifuged at 200,000 × *g* for 4 h. The pellet, which ranged from opalescent to completely transparent, was suspended in water and lyophilized. The yield was 80 to 140 mg, and the product was essentially free of detectable nucleic acid or protein.

Neutral sugars were quantitated by gas chromatographic analysis of their alditol acetates as described by York et al. (36). Glucosamine was determined by hydrolyzing the LPS in 4 M HCl at 100°C for 16 h, followed by N acetylation with acetic anhydride and pyridine as described by Kozulic et al.

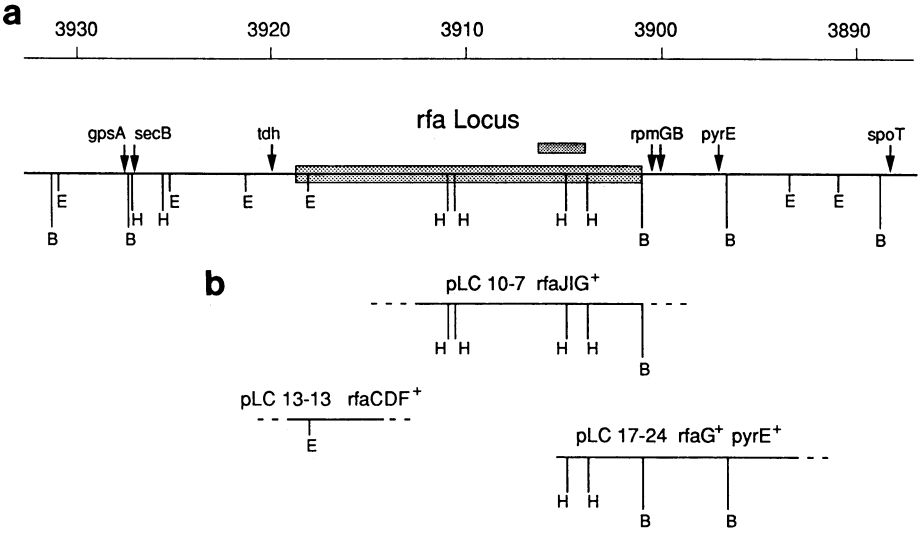

FIG. 2. (a) Physical map of the 81-min region of the *E. coli* K-12 chromosome. The top line shows the coordinates (in kilobases) of the physical map of Kohara et al. (19). The second line shows a partial restriction map including genes in the region and the approximate size of the *rfa* locus (long shaded bar). The short shaded bar shows the region in which deep rough insertion mutations were located. (b) Inserts from three Clarke-Carbon plasmids and the mutations in *S. typhimurium* that these plasmids complement. Dashed lines indicate uncertainty about the junctions between plasmid and vector sequence.

(20) and gas chromatographic analysis on a 30-m fused silica column.

RESULTS AND DISCUSSION

Physical structure of the *rfa* locus. The structure of the 81-min region of the *E. coli* chromosome, which includes *rfa*, is shown in Fig. 2. This map was compiled from several kinds of observations. Tn*10* insertion mutations selected on the basis of resistance to the LPS-specific phage U3 which mapped to the *cysE-pyrE* region were characterized by Southern blot hybridization, using a probe derived from plasmid pRT29 containing sequence from the *tet* element of Tn*10* and probes derived from flanking regions of the chromosome. All of the insertion mutations were located within a 2-kb region (shaded bar, Fig. 2) in the middle of an approximately 24-kb *Eco*RI fragment and near one end of an approximately 26-kb *Bam*HI fragment. A probe consisting of the 3.7-kb *Bam*HI fragment from plasmid pJSL4 which had sequence adjacent to the *rpmGB* gene cluster (23) hybridized to both the *Bam*HI and the *Eco*RI fragments, but a probe consisting of plasmid pCK1 which carried the *secB* gene (21) hybridized only to the *Bam*HI fragment.

We observed that the U3^r phenotype of these *rfa* insertion mutations was complemented by restriction fragments subcloned from Clarke-Carbon plasmid pLC10-7; this finding indicated that probes derived from the insert of this plasmid could be used for additional characterization of the 81-min region. The restriction map shown in Fig. 2 was generated by combining chromosomal restriction site data obtained by Southern blots using these probes with restriction maps of the inserts of plasmids pLC10-7 and pLC17-24 determined in our laboratory and published restriction site data for the flanking *rpmGB-pyrE* and *tdh* regions (1, 23). This restriction map was in good agreement with the map of Kohara et al. (19) of the *E. coli* chromosome and allowed unambiguous placement of the inserts of plasmids pLC10-7 and pLC17-24 with respect to the map coordinates shown at the top of Fig. 2. One minor difference which should be noted is that the pair of closely spaced *Hin*dIII sites shown at coordinate 3911 are shown as a single site in the map of Kohara et al. (19).

Plasmid pLC13-13 complements mutations in the *rfaC*, *-D*, and *-F* genes of *S. typhimurium* but does not overlap with pLC10-7 and pLC17-24 (8; K. Sanderson, personal communication). We do not have data that would permit unambiguous placement of the insert of this plasmid. However, the observation of an *Eco*RI site in the insert (W. Coleman, personal communication) allows provisional placement adjacent to the *Eco*RI fragment containing the *tdh* gene (1) as shown in Fig. 2, which is in agreement with genetic data placing these genes at the *cysE* end of the *rfa* locus (18, 22).

Plasmid pLC10-7 complements mutations in genes *rfaG*, *-I* and *-J* of *S. typhimurium*, whereas pLC17-24 complements mutations in *rfaG* and *pyrE* (9; Sanderson, personal communication). In combination with the physical map data in Fig. 1, this indicates that *rfaG* must lie at the right end of the pLC10-7 insert, whereas the *rfaIJ*-complementing activity must lie at the left end.

A possible source of confusion in interpretation of cross-species complementation data lies in the difference in core structure between *E. coli* K-12 and *S. typhimurium*. The backbone structure of the *Salmonella* core oligosaccharide is glucose I (GlcII)–galactose (Gal)–GlcI–heptose (Hep)–Hep-2-keto-3-deoxyoctulosonic acid (KDO), whereas that of *E. coli* K-12 is GlcIII-GlcII-GlcI-Hep-Hep-KDO (10, 31). The sugar transferase specified by *rfaG*, which adds GlcI to Hep, should be present in both species, whereas those specified by *rfaI*, which adds Gal to GlcI, and *rfaJ*, which adds GlcII to Gal, should be present only in *S. typhimurium*. Observing that pLC10-7 complemented the *Salmonella rfaI* gene (10), Creeger and Rothfield suggested that *E. coli* had both (i) an *rfaI* gene specifying a UDP-galactose:(glucosyl) LPS α-1,3-galactosyltransferase that complemented the *Salmonella* defect but was otherwise of unknown function and (ii) a gene which they termed *rfaM* that specified an analogous UDP-glucose:(glucosyl) LPS α-1,3-glucosyltransferase involved in synthesis of the *E. coli* core backbone. Since pLC10-7 complements both *rfaI* and *rfaJ* mutants, a more plausible hypothesis (suggested by Sanderson [personal communication]) is that pLC10-7 contains genes for the three glycosyltransferases necessary for synthesis of the *E. coli* core

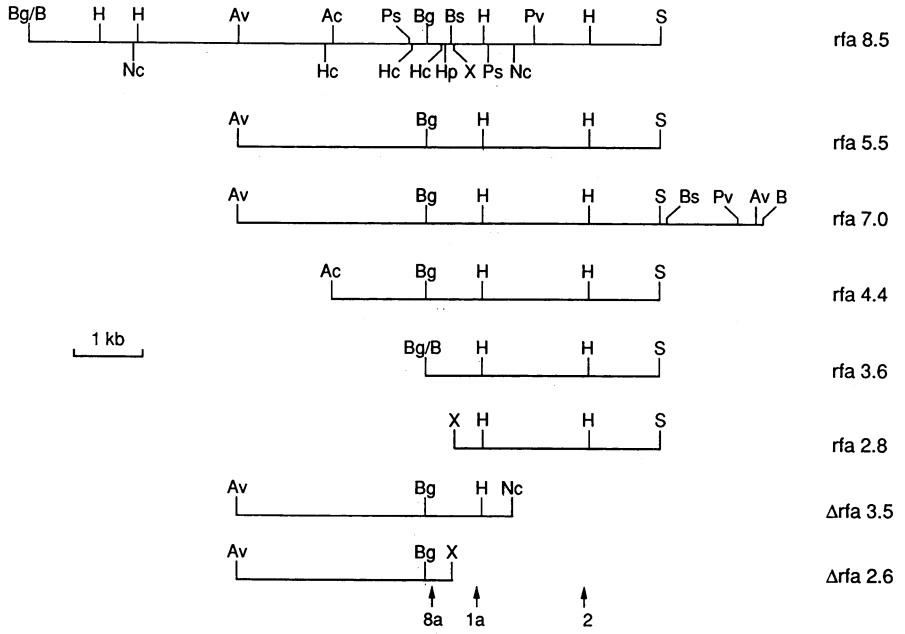

FIG. 3. Restriction fragments used in gene localization and complementation studies. Complete restriction maps are given only for rfa8.5 and the right end of rfa7.0. Ac, *Acc*I; Bs, *Bst*EII; Hc, *Hinc*II; Hp, *Hpa*I; Ps, *Pst*I; Pv, *Pvu*II; X, *Xba*I. Other restriction sites are as in Fig. 1.

backbone and that *Salmonella rfaI* and *rfaJ* mutants complemented by this plasmid actually synthesize a core of the *E. coli* type containing three glucose residues to which the *Salmonella* O antigen is then added. In other words, the plasmid contains, in addition to *rfaG*, the gene *rfaM*, which is analogous to *rfaI* of *S. typhimurium* and encodes the glucosyltransferase adding GlcII to GlcI, and a third gene for which we propose the name *rfaN*, which is analogous to *rfaJ* of *S. typhimurium* and encodes the UDP-glucose:(glucosyl) LPS α-1,2-glucosyltransferase adding GlcIII to GlcII.

The results presented above indicate that the order of genes in the *rfa* region is *cysE*-(*rfaC rfaD rfaF*)-(*rfaM rfaN*)-*rfaG-pyrE*. This order is in agreement with the map order of the analogous genes in *S. typhimurium* determined by transductional crosses by Kuo and Stocker (22) and by physical mapping of cloned genes by Kadam et al. (18). This agreement suggests a similarity in the organization of the *rfa* locus between *E. coli* K-12 and *S. typhimurium*.

To determine the location of genes within the cloned inserts in pLC10-7 and pLC17-24, a series of subcloned fragments was constructed (Fig. 3). To determine the location and direction of transcription of genes within this region, these fragments were inserted into the polylinker regions of plasmids pGEM-3 and pGEM-4 which are flanked by SP6 and T7 promoters. These plasmids were used as templates for coupled transcription and translation in an in vitro system containing rifampin to inhibit transcription from *E. coli* promoters and either SP6 or T7 RNA polymerase to provide unidirectional transcription. Translation products were labeled by incorporation of [^{35}S]methionine and visualized by autoradiography (Fig. 4 and 5).

Six polypeptides with apparent sizes of 26, 35, 38, 39, 42, and 44 kilodaltons (kDa) were detected in reactions primed with the rfa8.5 fragment, which is the entire insert of pLC10-7 (Fig. 4, lane D). These polypeptides were made from transcripts that extended leftward with respect to the chromosomal orientation shown in Fig. 2; no polypeptides were detected from transcripts in the reverse direction (compare lanes C and F in Fig. 4).

The 44-kDa polypeptide was absent in reactions primed with the rfa5.5 fragment (Fig. 4, lane H), indicating that its coding region extends to the left of the *Ava*I site. We also observed a polypeptide of 30 kDa that was present in

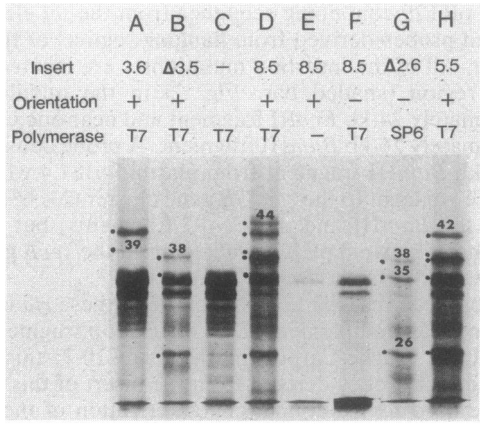

FIG. 4. Identification of polypeptides encoded by restriction fragments derived from the insert of plasmid pLC10-7. Shown is a radioautograph of an SDS-gel of ^{35}S-labeled polypeptides synthesized in an in vitro system programmed with pGEM plasmids carrying restriction fragments shown in Fig. 3. The + orientation indicates that fragments shown in Fig. 3 have been inserted into the polylinker sites of the pGEM vectors with the T7 promoter on the right side of the insert and the SP6 promoter on the left; the – orientation is the reverse. In both pGEM vectors, the β-lactamase gene is oriented so that it is transcribed from the T7 promoter. Lanes C and E are controls of no insert and no polymerase, respectively. The dots indicate polypeptides that are unique to plasmids containing *rfa* inserts; the numbers indicate their apparent molecular masses in kilodaltons.

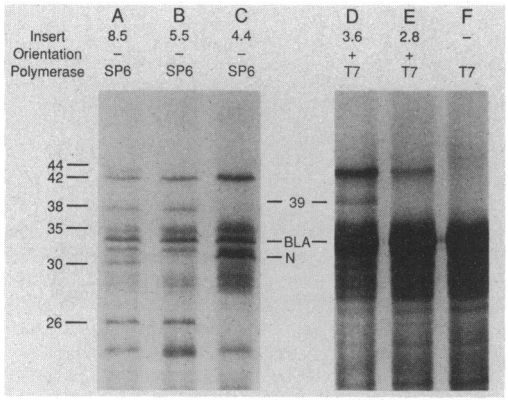

FIG. 5. Ordering of polypeptides encoded by restriction fragments. Shown is a radioautograph of a gel similar to that shown in Fig. 4 except that different fragments were used to permit more detailed ordering of the polypeptide-coding regions. BLA, Major β-lactamase fragment; N, a new fragment that is presumed to be a truncated form of the 38-kDa polypeptide.

reactions primed with the rfa8.5 fragment but not in reactions primed with the rfa5.5 fragment (compare lanes A and B in Fig. 5), but we do not have data to rule out the possibility that this was a breakdown product of the 44-kDa polypeptide.

Three polypeptides, 26, 35, and 38 kDa, are encoded by the central *Ava*I-*Bgl*II fragment, as indicated by their absence in reactions primed with rfa3.6 and their presence in reactions primed with Δrfa2.6 and Δrfa3.5. The 35-kDa polypeptide is partially obscured by β-lactamase bands when these are present but can be seen clearly when transcription of the plasmid is in the opposite direction and the β-lactamase bands are absent. The 26- and 38-kDa polypeptides were absent from reactions primed with rfa4.4, and two new bands, both larger than 30 kDa, were observed (Fig. 5, lane C). These new bands must be truncated forms or breakdown products of the 38-kDa polypeptide, indicating that the *Acc*I site at the end of rfa4.4 must lie within the 38-kDa coding region. This result indicates that the order of the three polypeptide-coding regions must be 5′-35 kDa-38 kDa-26 kDa-3′.

Similarly, reactions primed with the rfa3.6 fragment produced 39- and 42-kDa polypeptides, whereas the rfa2.8 fragment produced only the 42-kDa polypeptide (Fig. 5, lanes D and E). This result indicates that the order of the coding regions for these two polypeptides is 5′-42 kDa-39 kDa-3′. A summary of the arrangement of the polypeptides in relation to the restriction map of the insert of pLC10-7 is shown in Fig. 6.

No additional polypeptides were observed when reactions primed with rfa7.0 oriented so that transcription was in the leftward direction were compared with reactions primed with rfa5.5 in the same orientation (data not shown). However, we recently observed preliminary evidence for a polypeptide of about 40 kDa produced in reactions primed with rfa7.0 oriented so that transcription was in the rightward direction (data not shown). This finding suggests the possibility of divergent transcription of genes from a promoter located near the rightward *Hin*dIII site of rfa7.0. Complementation studies described below provide evidence for a promoter in this region. The *Hin*dIII-*Bam*HI fragment adjacent to *rpmGB* contains enough information for one or two genes, and it is possible that this is the rightward margin of the *rfa* locus. If it is assumed that the leftward margin of the *rfa* locus is near the *tdh* locus, the result is an overall size of the *rfa* locus of about 18 kb, shown as a hatched area in Fig. 2. Assuming an average size of *rfa* gene products of about 35 kDa, this region would accommodate about twice as many genes at the *rfa* locus as are presently known.

Insertion mutagenesis of *rfa*. Selection and characterization of *rfa* insertion mutations was facilitated by mutagenesis with phage λ1098, which is nonintegrating and nonreplicating and has a segment containing a 3.1-kb mini-*tet* transposon adjacent to a transposase driven from a *tac* promoter (35). The advantages of this mutagenesis system are that the insertions are stable since the transposable element has no transposase, the element is easily mapped since it is small and has several unique restriction sites, and transposition to the chromosome occurs at a very high frequency as compared with wild-type Tn*10*. This latter advantage was particularly important in mutagenizing the *rfa* locus since the rate of spontaneous mutation was high.

Mutants were selected for resistance to tetracycline and, after a brief period of outgrowth, for resistance to the

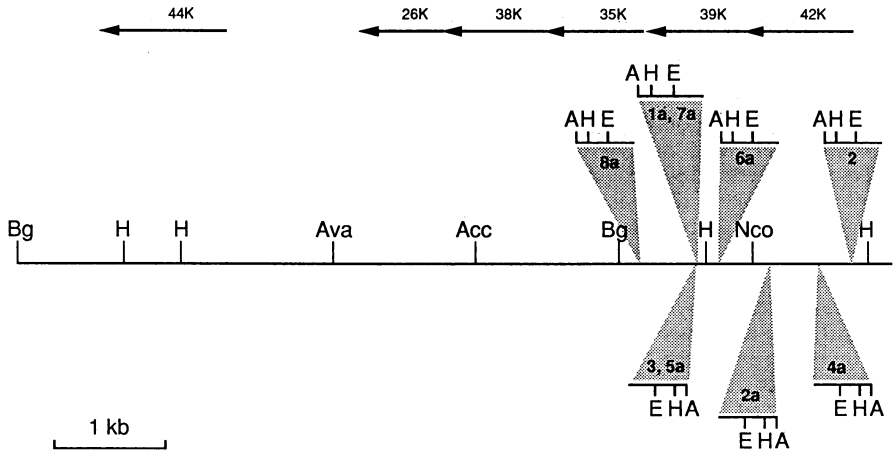

FIG. 6. Location of the mini-Tn*10* insertions with respect to the physical map of the *rfa* locus and the location of protein-coding regions. The restriction map is as shown in Fig. 3; the points of the shaded triangles indicate the sites of insertion. The orientation of the insertions is indicated by the letters A, H, and E, which represent the single *Ava*I, *Hin*dIII, and *Eco*RI sites in the insertion. The arrows represent, approximately to scale, coding regions for the polypeptides identified in Fig. 4 and 5.

LPS-specific phage U3. In the majority of mutants obtained, resistance to U3 was shown by transduction to be 100% linked to tetracycline resistance, indicating that the frequency of transposition was higher than the spontaneous mutation frequency.

Ten independently selected mutants that exhibited linkage of Tet^r and U3^r were characterized in detail. In nine of these mutants, the Tn*10* insertions contransduced with *mtl* at a frequency ranging from 36 to 52% and were subsequently shown by Southern blot analysis to be clustered in a small region at the *rfa* locus. One mutation, from isolate 3a, showed no linkage to *mtl*, *cysE*, or *pyrE*. Conjugational mapping by gradient of transfer placed the mutation at ca. 65 min, and the mutation exhibited 21% cotransduction with *metC* and 57% cotransduction with *rpoD*. The mutant was sensitive to deoxycholate, novobiocin, colicin E2, and colicin E3 but resistant to colicin E1. The outer membrane protein profile of this mutant showed a reduction in OmpF protein but otherwise showed no change in protein composition (data not shown). On the basis of these observations, we concluded that this isolate had an insertion into *tolC*. We subsequently examined other alleles of *tolC* and found that these were also resistant to phage U3. This result is interesting in that *tolC* encodes a minor outer membrane protein (26) yet affects sensitivity to an LPS-specific phage.

The phenotypes of the original isolates carrying the nine insertion mutations that mapped to *rfa* were not identical with respect to colonial morphology and sensitivity to novobiocin. These differences were apparently due to secondary mutations, since the insertion mutations all exhibited an identical deep rough phenotype (30) after transduction into a fresh background. The growth rate and colonial morphology of these transductants were nearly identical to those of the wild-type background strain. All of the *rfa* mutants exhibited hypersensitivity to hydrophobic agents. On gradient plates containing 0 to 40 μg of novobiocin per ml, the mutants grew only at the edge with the lowest concentration, whereas CS180 grew on the entire plate. The mutants would not grow on eosin-methylene blue plates or on plates containing 0.1% deoxycholate. The mutants did not exhibit sensitivity to EDTA, nor was the sensitivity to hydrophobic agents affected by the addition of divalent cations. All of the mutants exhibited an identical reduction in major outer membrane proteins. A representative example is shown in Fig. 7. Densitometry of stained gels indicated that the reduction of OmpF and OmpC porins was more than 90%, whereas reduction of OmpA protein was about 50%. We estimate the reduction in total protein content of the outer membrane to be about 50 to 60%. Another aspect of the deep rough phenotype is an increase in the phospholipid content of the outer membrane (30), and preliminary analyses (data not shown) indicated that this was also true of these *rfa* insertion mutants. Figure 8 shows the dramatic decrease in buoyant density of the outer membrane of a representative *rfa*::Tn*10* insertion mutant, isolate 2, which was presumably due to the combined decrease in protein and compensatory increase in phospholipid. It is interesting to note that no significant difference in buoyant density was observed between the wild type and the *tolC*::Tn*10* insertion 3a (Fig. 8), even though the sensitivity to hydrophobic agents of *tolC*::Tn*10* insertion 3a was identical to that of *rfa*::Tn*10* insertion 2. This result indicates that detergent hypersensitivity is not invariably linked to gross compositional changes in the outer membrane such as those observed in deep rough mutants.

All of the *rfa*::Tn*10* insertion mutants became resistant to phage K20 as well as to phage U3. This finding is in agreement with results of Silverman and Benson (34), who reported that K20 requires LPS as well as protein as its receptor. The insertion mutants remained completely sensitive to the OmpC-specific phages SS-4 and Hy2 and the OmpA-specific phage K3. We now know that our previous results indicating that some deep rough mutants became simultaneously resistant to phages U3 and K3 (2) were incorrect because those mutants contained mutations in *ompA* as well as *rfa* (J. Graves and C. Schnaitman, unpublished data).

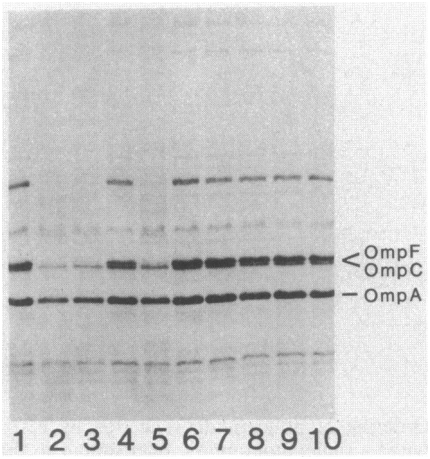

FIG. 7. Complementation of the outer membrane protein defect of deep rough *rfa* mutants. Shown is a Coomassie blue-stained SDS-gel of the outer membrane protein fraction of the wild type and two *rfa* mutants complemented in single copy by various fragments cloned into λ. The sample loading is normalized (5) so that each lane represents protein from the same number of cells. Lanes: 1 and 10, wild type; 2, *rfa-2*; 3, *rfa-8a*; 4, *rfa-2* plus λrfa3.6; 5, *rfa-8a* plus λrfa3.6; 6, *rfa-2* plus λrfa5.5; 7, *rfa-8a* plus λrfa5.5; 8, *rfa-2* plus λrfa8.5; 9, *rfa-8a* plus λrfa8.5.

Figure 6 shows the sites of insertion of the nine *rfa*::Tn*10* mutations in relation to polypeptide-coding regions identified by transcription and translation of cloned *rfa* DNA. Mutant

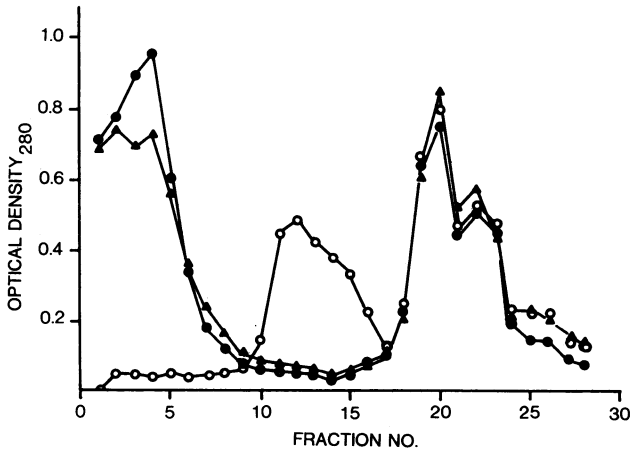

FIG. 8. Density gradient centrifugation of the envelope fraction from wild-type (●), *rfa-2* (○), and *tolC3a* (▲). Centrifugation was for 36 h on a continuous gradient of 35 to 55% (wt/wt) sucrose. The direction of sedimentation is from right to left, so the peak fraction of the wild-type outer membrane is fraction 4 and that of the cytoplasmic membrane is fraction 20.

TABLE 1. Single-copy complementation of insertion mutations by *rfa* fragments cloned into λ

Mutation	Sensitivity to[a]:					
	Novobiocin			U3		
	No λ	λrfa3.6	λrfa4.4	No λ	λrfa3.6	λrfa4.4
Wild type	R	—	—	S	—	—
rfa-2	S	R	R	R	S	S
rfa-1a	S	R	R	R	S	S
rfa-3	S	R	—	R	S	—
rfa-5a	S	R	—	R	S	—
rfa-6a	S	R	—	R	S	—
rfa-7	S	R	—	R	S	—
rfa-8a	S	S	R	R	R	S
rfa-2a	S	—	—	R	V	—
rfa-4a	S	—	—	R	V	—
tolC3a	S	—	—	R	—	—

[a] S, Sensitive; R, resistant; V, variable among different clones; —, not determined.

2 appears to lie adjacent to the 5′ end of the coding region of this cluster of proteins, possibly in the promoter region. Insertions 2a and 6a lie within the coding region of the 42-kDa protein gene. Insertions 1a, 7a, 3, 5a, and 6a lie within the coding region of the 39-kDa protein gene, and four of these appear to be at the same site, suggesting a hot spot for insertion. Insertion 8a lies within or immediately adjacent to the 5′ end of the coding region of the 35-kDa protein gene.

Complementation of insertion mutations. Single-copy complementation analysis was carried out by inserting the subcloned fragments shown in Fig. 3 into the integration-proficient vector λgt4.0 and then transducing the insertion mutations into strains lysogenic for these phage. For simplicity, the phage are named after the fragments that they carry. Complementation of the novobiocin sensitivity and U3 resistance phenotypes of the insertion mutations is shown in Table 1. Figure 7 illustrates complementation of the protein phenotype of some of the mutants. In all of the cases that we have examined, complementation of novobiocin sensitivity has been accompanied by a restoration of wild-type levels of major outer membrane proteins as seen in Fig. 7.

Mutations 2a and 4a lie within the first gene of the cluster shown in Fig. 6 and exhibit complex complementation characteristics. When they were transduced into a strain lysogenic for λrfa3.6, the transductants exhibited a mixture of $U3^s$ and $U3^r$ phenotypes, with about half remaining $U3^r$. When P1 grown on either $U3^s$ or $U3^r$ transductants was used to transduce one of these mutations into a fresh lysogenic background, a mixture of both phenotypes was again obtained. This result indicates an apparent phase variation affecting the $U3^r$ phenotype. The basis for this variation is unknown. Because of the phenotypic instability of these mutations, we have not characterized them further.

Mutations 1a, 3, 5a, 6a, and 7a, which lie within the second gene in the cluster, and mutation 2, which lies at the 5′ end of this cluster, exhibited identical, stable complementation of both novobiocin sensitivity and U3 resistance by λrfa3.6. The complemented mutants produced LPS of identical migration on gels (data not shown). Mutation 2 was not complemented by a phage carrying the rfa2.8 fragment, which includes only the promoter region and the gene for the 42-kDa protein. We interpret these data as indicating that mutation 2 lies in the 5′ region of an operon that includes, at minimum, the genes for the 42- and 39-kDa proteins.

TABLE 2. Complementation of U3 resistance by *rfa* fragments cloned into λ or a multicopy pGEM plasmid

Mutation	Phage U3 sensitivity[a]						
	None	λrfa2.8	λrfa5.5	Δ2.6		Δ3.5	
				In λ	In pGEM	In λ	In pGEM
rfa-2	R	R	S	R	R	R	R
rfa-1a	R	R	S	R	R	R	R
rfa-8a	R	—	S	R	S	R	S

[a] S, Sensitive; R, resistant; —, not determined.

The deep rough phenotype of mutation 8a was not complemented by λrfa3.6 although its site of insertion lay within the *Bgl*II site at the end of the rfa3.6 fragment. This result indicated that sequence distal to the *Bgl*II site was also required, and this was confirmed by the complementation of 8a by λrfa4.4. This finding indicates that the gene for the 35-kDa protein is also part of the minimal set of genes required to avoid a deep rough phenotype. The observation that the deep rough phenotypes of mutations such as 1a and 2, which lie proximal to this gene, were complemented by the rfa3.6 fragment indicates that the 35-kDa protein gene must be expressed from a different promoter than that which is used for the 42- and 39-kDa protein genes. The evidence for a second promoter is strongest in the case of mutation 2, in which the lack of complementation of this mutation by the rfa2.8 fragment indicates clearly that it is polar on the gene for the 39-kDa protein. This finding argues against expression of the gene for the 35-kDa protein from a promoter located within Tn*10* such as that described by Ciampi et al. (6).

To test for a second promoter, the ability of fragments that were deleted for upstream genes to complement mutation 8a was examined by using both a single-copy λ vector and a multicopy plasmid vector. The results shown in Table 2 indicate a complex situation. The lack of complementation by λΔrfa2.6 and λΔrfa3.6 shows that the putative promoter for the 35-kDa protein gene cannot function when it is separated from some element lying upstream in the chromosome. Moreover, this element must be located some distance upstream from the promoter, since λΔrfa3.6 includes almost all of the coding region of the 39-kDa protein gene and covers the sites of insertion mutations such as 1a which are complemented by the rfa3.6 fragment and are not polar on genes upstream. The fact that mutation 8a is complemented by the Δrfa2.6 and Δrfa3.6 fragments when they are present in a multicopy plasmid can be interpreted in one of two ways: either that the 35-kDa protein gene is expressed from a promoter in the plasmid vector or that there is a promoter present in these fragments which is too weak to provide complementation except when present at high copy number.

Nature of the lesion in deep rough mutants. The studies described above define two genes, those for the 39- and 35-kDa proteins, whose function is critical in terms of the deep rough phenotype. To define more precisely the biochemical nature of the deep rough lesion, we compared LPS from mutants in which these genes were inactivated and from a strain in which the mutation was complemented with the shortest possible cloned fragment.

Figure 9 shows the migration of LPS on a sodium dodecyl sulfate (SDS)-gel as visualized by silver staining. The LPS in lanes A to C was isolated by proteolytic digestion of the

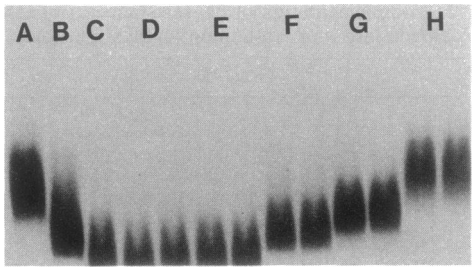

FIG. 9. Silver-stained gel of purified LPS. Lanes A to C are single lanes showing, respectively, LPS from the outer membrane fraction of wild type, *rfa-8a*, and *rfa-2*. LPS from the outer membrane fractions was prepared by the protease digestion procedure of Hitchcock and Brown (17). Lanes D through H (*rfa-2*, *rfa-1a*, *rfa-8a*, *rfa-8a* plus λrfa4.4, and wild type, respectively) are double lanes showing purified LPS extracted by the PCP procedure of Galanos et al. (15) which was not protease treated before electrophoresis.

whole outer membrane fraction by a modification of the procedure of Hitchcock and Brown (17); the remaining lanes show purified LPS isolated from the same cultures by a slight modification of the PCP procedure of Galanos et al. (15). We observed identical migration of samples prepared by the two procedures, indicating that the PCP-extracted sample was representative of the LPS present in the outer membrane.

Mutants 2 and 1a produced an identical, rapidly migrating LPS, whereas mutant 8a produced a slower-migrating LPS. Complementation of 8a with λrfa4.4, which carries no intact genes distal to the 35-kDa protein gene, resulted in an additional shift to lower mobility, but this LPS still migrated much more rapidly than that of the Rfa^+ parent. Electrophoresis of these samples with LPS of known chemotype from *S. typhimurium* mutants (data not shown) indicated approximate comigration of LPS from mutants 2 and 1a with chemotype Rd1, of 8a with chemotype Rc, and of 8a complemented with λrfa4.4 with chemotype Rb3. Compositional analysis of the PCP-extracted LPS from these strains is given in Table 3.

Figure 10 is our working model for the structure of the LPS molecules. The figure is based on several considerations: (i) the relative migration of the LPS species on gels in comparison with known chemotypes; (ii) compositional analysis; (iii) the established location of *rfaG*- and *rfaJ*-complementing activity in the cloned fragment from which λrfa4.4 was derived; and (iv) present knowledge of the LPS biosynthetic pathway in enteric bacteria (31).

The sugar backbone structure shown in Fig. 10 is consistent with the following evidence. Compositional analysis of LPS from mutants 1a and 2 indicated no glucose and about two molecules of heptose. No defect in heptose was anticipated in the mutants, since the cluster of genes involved in the biosynthesis and addition of heptose is located at the opposite end of the *rfa* locus. The third branch heptose is thought to be added after the first glucose; in Fig. 10 this was added to the structure for 8a LPS because more heptose was detected in this LPS and because the difference in mobility of 8a and 1a LPS on gels in comparison with differences between known chemotype standards was more consistent with a two-sugar addition than with a one-sugar addition. The addition of glucose to 8a LPS was indicated by the compositional analysis and is consistent with *S. typhimurium* complementation data mentioned previously implicating one of the first genes in the *rfa* cluster as *rfaG*. Moreover, the identical RfaG phenotypes of insertion mutations 1a and 2 strongly suggest that *rfaG* is the gene for the 39-kDa protein.

The most definitive difference in the composition of LPS of 8a when it is complemented by λrfa4.4 is the presence of galactose. The decrease in electrophoretic mobility when 8a is complemented by λrfa4.4 is about half as much as the difference between LPS from mutants 1a and 8a and is consistent with what would be expected for addition of a single sugar. On the basis of these observations, it could be proposed that the 35-kDa protein is a galactosyltransferase (the *rfaB* product) and that the deep rough phenotype results from failure to add galactose to the LPS. We tested this hypothesis by examination of two well-characterized *galE* null mutants, one of which was *galE*::Tn*10* (CS1776) and the other of which was Δ*galOPE* (CS1801). These strains were grown in defined medium without exogenous galactose.

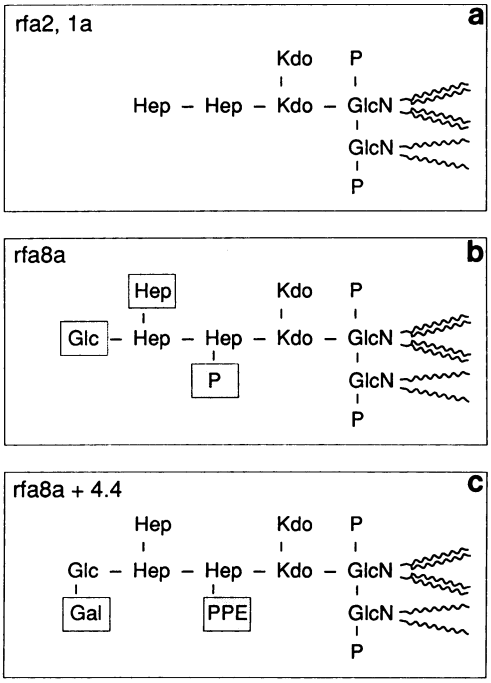

FIG. 10. Schematic representation of the hypothetical structure of the LPS produced by the strains shown in Fig. 9. Hep, Heptose; Kdo, 2-keto-3-deoxyoctulosonic acid; GlcN, glucosamine; Glc, glucose; Gal, galactose; P, phosphate; PPE, pyrophosphorylethanolamine. ~, Acyl groups.

TABLE 3. Compositional analysis of purified LPS samples

Sample	Molar ratio of LPS constituents[a]				
	Galactose	Glucose	Heptose	Glucosamine	Phosphate
Mutant 2	0	0	1.3 (0.52)	2.0 (0.84)	2.3 (0.94)
Mutant 1a	0	0	1.9 (0.77)	2.0 (0.78)	2.2 (0.85)
Mutant 8a	0	1.3 (0.37)	2.5 (0.72)	2.0 (0.55)	4.9 (1.36)
Mutant 8a, λrfa 4.4	0.54 (0.17)	1.1 (0.20)	1.1 (0.35)	2.0 (0.61)	2.7 (0.85)
Wild type	1.1 (0.22)	3.0 (0.61)	3.3 (0.67)	3.9 (0.78)	8.9 (1.80)

[a] Expressed relative to arbitrary values set for glucosamine or glucose, as indicated by underlining. Glucose was chosen in the case of wild-type LPS since the extent of glucosamine substitution is not stoichiometric. The numbers in the parentheses indicate the actual amount of each constituent in micromoles per milligram of LPS.

Under these growth conditions, the *galE* mutants remained sensitive to U3 and completely resistant to novobiocin. The predominant LPS species produced by the *galE* mutants appeared to be of the Rc chemotype, since it comigrated with LPS from mutant 8a (data not shown). These results are in complete agreement with those of Nikaido and Nakai (29), who found that permeability toward hydrophobic compounds was normal in *S. typhimurium* producing LPS of the Rc chemotype. Thus, it seems very unlikely that the gene for the 35-kDa protein is *rfaB*, and the presence of galactose in the LPS from mutant 8a complemented by the rfa4.4 fragment must reflect a change elsewhere in the molecule which permits it to become an acceptor for galactose.

In addition to indifference toward the presence of galactose, the deep rough phenotype shows a similar lack of correlation with the presence of the first glucose residue. Although mutants whose LPS lacks this glucose seem invariably to have a deep rough phenotype (30; this study), mutant 8a is still deep rough even though its LPS contains glucose. In sum, these results suggest that the deep rough phenotype is caused not by the lack of a core sugar but by loss or alteration of a substituent attached to one or more of the sugars.

The pyrophosphorylethanolamine attached to the 4 position of the first heptose is a likely candidate for this critical substituent. This residue is probably attached by a two-step process (summarized in reference 31 and Fig. 10). The first step, which requires *rfaP*, is transfer of the γ phosphate from ATP to the 4 position of the heptose (27, 28). Although heptose phosphate-deficient Rd_1 LPS (Fig. 10a) is an acceptor for this phosphate, the preferred substrate is phosphate-deficient Rc LPS (shown, with heptose phosphate added, in Fig. 10b). The second step, for which genes have not yet been identified, is the proposed transfer of the phosphorylethanolamine head group from phosphatidylethanolamine to this heptose phosphate (14).

Since *rfaP* mutants exhibit a deep rough phenotype in *Salmonella minnesota* (28) and presumably also in *E. coli* K-12 (2), it is possible that the gene for the 35-kDa protein absent in mutant 8a is *rfaP*. However, the increased phosphate content of the LPS from mutant 8a in comparison with that of LPS from mutants 2 and 1a (Table 3) would argue that the *rfaP*-dependent phosphorylation has already occurred. If this is the case, an alternative possibility is that the 35-kDa protein is not the product of *rfaP* but is involved in the second step, the transfer of the phosphorylethanolamine from phosphatidylethanolamine. It is this scheme which is shown in Fig. 10. It should be pointed out that final conclusions regarding substituents such as pyrophosphorylethanolamine that are found in nonstoichiometric amounts will require structural as well as compositional analysis.

Our working hypothesis to explain the deep rough phenotype is that substituents such as pyrophosphorylethanolamine are sites for recognition of LPS by major outer membrane proteins during the terminal steps of protein secretion and that such recognition may be necessary to allow secreted proteins to fold properly or to be released from the translocation machinery. Failure to do so would lead to jamming of the secretion pathway and an inhibition of the translation of outer membrane proteins such as that which is observed when secretion is jammed by the *ompC*td mutant protein (5) or by overproduction of a major protein (7). However, this alone cannot account for all of the manifestations of the deep rough phenotype, since neither the *ompC*td mutant nor multiple major protein-defective mutants (such as *ompA ompC ompF* triple mutants) exhibit significant sensitivity to hydrophobic compounds such as novobiocin in comparison with that exhibited by deep rough mutants. Therefore, we feel it likely that LPS substituents play an additional role in facilitating strong lateral interaction between LPS molecules and that it is in fact the simultaneous loss of this lateral interaction and a reduction in outer membrane protein synthesis which allow the well-documented formation of domains of phospholipid in the outer leaflet of the outer membrane (30).

Evidence for a complex *rfa* operon. On the basis of the clustering of genes for core biosynthesis at *rfa*, Makela and Stocker (24) questioned whether these genes might be organized into an operon. As noted previously, complementation of U3 resistance indicated a complex situation in which there were two promoters in the region of the chromosome covered by the rfa3.6 fragment, but one of these did not appear to be able to function independently when moved to another site in the chromosome. This finding suggested the possibility that the genes for the hexose region of the core were functionally arranged in an operon even though there appeared to be more than one promoter for these genes.

To provide additional evidence for this type of coordinate regulation, the LPS from strains in which insertion mutations were complemented in single copy by cloned fragments of different lengths were compared on silver-stained gels. The rationale for these experiments is as follows. From what is known of the arrangement of genes for the hexose region of the core, all of the genes appear to be transcribed in the same direction and arranged roughly in the same order as steps in the biosynthetic pathway. If these genes are functionally an operon, complementation of polar mutations by a series of fragments that carry the promoter plus an increasing number of downstream genes should result in the production of LPS molecules which appear progressively larger on gels. This analysis depends on the assumption that LPS molecules which are larger or more complex will migrate more slowly on SDS-gels, but this assumption is well supported (17).

The results from this series of experiments are presented both as photographs of stained gels and as cartoons of gels drawn to illustrate hypothetical situations. For example, Fig. 11 illustrates two distinctly different patterns that might be predicted if (i) the genes each had independent promoters or (ii) the genes were organized in a classical operon. The observed gel profiles (Fig. 11a and 12) did not agree with either of these predictions. The leading edge of the LPS from the complemented mutants, indicative of the smallest molecules in the population, increased in size as the number of genes supplied on the complementing fragment increased. This would be expected if the genes were part of an operon expressed from a single promoter. However, as shown in lanes C, E, and F of Fig. 12 and the corresponding lanes in Fig. 11a, the trailing edge which contained the largest molecules in the population was nearly identical to wild-type LPS. As in the case of complementation of the deep rough phenotype, this result indicates that there must be one or more additional promoters which provide expression of genes distal to the 3′ ends of the complementing fragments.

The migration of LPS from insertion 2 complemented by the rfa4.4 fragment (Fig. 11; Fig. 12, lane C) does not fit the analysis above, since the trailing edge migrates more rapidly than wild-type LPS. One interpretation of this result is that the second promoter in the operon described above may exhibit on or off states, depending upon the nature of the strain. This hypothesis is illustrated schematically in Fig. 13.

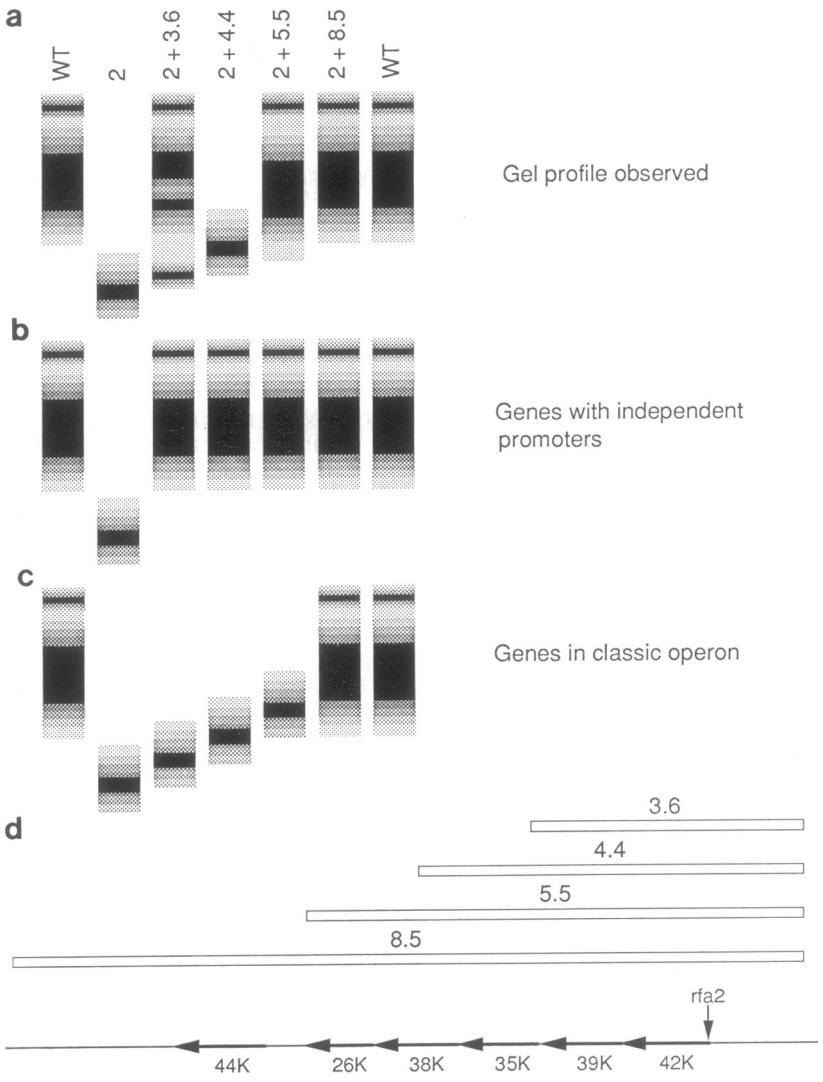

FIG. 11. Schematic illustration of how a gel of LPS from *rfa-2* complemented with fragments of increasing length might appear if the mutation were completely polar and the genes on the fragments were arranged in an operon. (a) Representation of the pattern observed (see Fig. 12); (b and c) predicted patterns in two hypothetical situations; (d) protein-coding regions and lengths of the complementing fragments.

The promoter is presumably located 5′ to the gene for the 35-kDa protein and is designated P_2.

In this context, it should be noted that P_2 may also be the site of action of the positive regulator encoded by *sfrB* (3, 9, 11). We obtained *sfrB* mutants from two different sources and observed that they were somewhat variable in U3 plating efficiency and produced LPS that was heterogeneous on gels. However, when *sfrB11*, the allele that seemed to exhibit the most consistent $U3^r$ phenotype, was transduced into a fresh strain background, the resulting strain (CS1859) was completely U3 resistant and produced a homogeneous LPS that comigrated with LPS from strains carrying *rfa* insertion mutation 8a (data not shown).

The migration of LPS from mutant 8a complemented by the various fragments (Fig. 14) indicates that there may be a third promoter located in the vicinity of the gene for the 26-kDa protein. The evidence for this, summarized schematically in Fig. 15, is that the trailing edge of LPS from mutant 8a complemented with rfa5.5 comigrated with wild-type LPS (Fig. 14, lane E). Since LPS from 8a complemented by rfa4.4

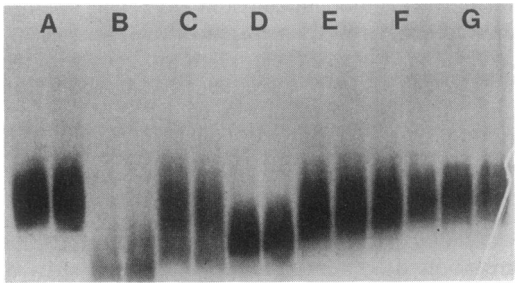

FIG. 12. Silver-stained gel showing the LPS from *rfa-2* complemented with fragments of increasing length. All lanes are double. Lanes: A and G, wild type; B, *rfa-2*; C to F, *rfa-2* complemented by λrfa3.6, λrfa4.4, λrfa5.5, and λrfa8.5, respectively. All lanes show LPS from the outer membrane fraction prepared by protease digestion.

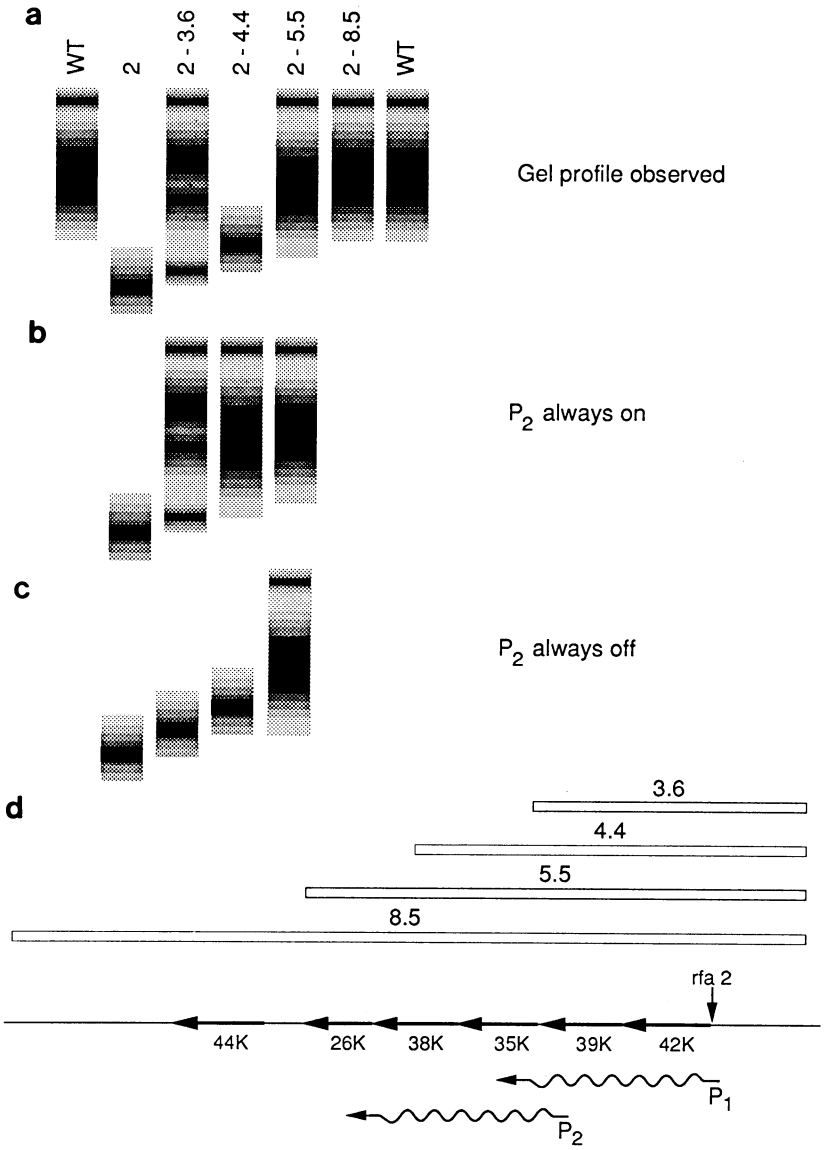

FIG. 13. Schematic illustration of the evidence that promoter P_2 has stable off and on states. (a) Representation of the gel shown in Fig. 12; (b and c) predicted patterns in two hypothetical situations; (d) possible arrangement of transcription from promoters P_1 and P_2 in relation to the coding regions and complementing fragments.

did not have such a trailing edge (Fig. 14, lane D), it appears that 8a is polar and prevents transcription of distal genes from P_2. Thus, it is necessary to invoke the presence of the third promoter designated in Fig. 15 as P_3.

In summary, complementation analysis indicates a high degree of complexity in the organization of expression of the *rfa* genes involved in biosynthesis of the hexose region of the LPS core. One arrangement that would fit the available data is a series of overlapping transcription units arranged much like shingles on a roof, in which each unit has its own independently regulated promoter but in addition is dependent on a master element P_1 located at the 5′ end of the gene cluster. Such an arrangement might provide for some level of coordinate control of these genes while allowing for diversity in the LPS core which is produced either by a single organism or within a population of organisms.

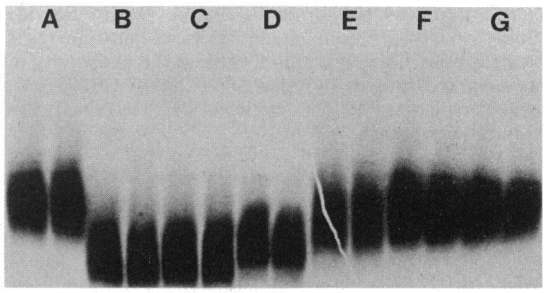

FIG. 14. Silver-stained gel showing the LPS from *rfa-8a* complemented with fragments of increasing length. Details are as in the legend to Fig. 12. Lanes: A and G, wild type; B, *rfa-8a*; C to F, *rfa-8a* complemented by λrfa3.6, λrfa4.4, λrfa5.5, and λrfa8.5, respectively.

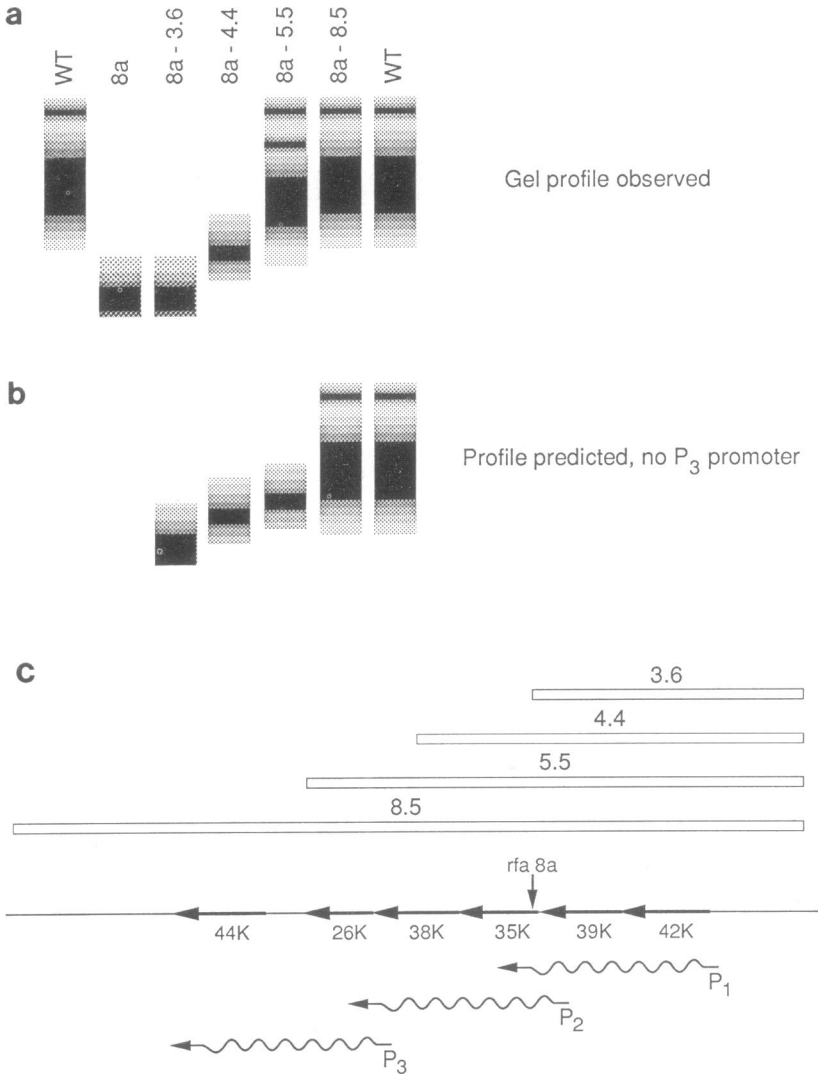

FIG. 15. Schematic illustration of the evidence for a third promoter in the *rfa* gene cluster. (a) Representation of the gel shown in Fig. 14; (b) profile that would be predicted if there were no third promoter; (c) possible arrangement of transcription from the three promoters.

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