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# Genetic Analysis of the Genes Involved in Synthesis of the Lipopolysaccharide Core in *Escherichia coli* K-12: Three Operons in the *rfa* Locus

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**The region of the *Escherichia coli* K-12 chromosome encoding the enzymes responsible for the synthesis of the lipopolysaccharide (LPS) core has been cloned in vivo by using a mini-Mu vector. This region, formerly known as the *rfa* locus, comprises 18 kb of DNA between the markers *tdh* and *rpmBG*. Results of in vitro mutagenesis of this region with MudII1734 indicate the presence of at least 17 open reading frames or genes, a number considerably higher than expected on the basis of genetic and biochemical studies. Specific insertions in different genes have been recombined into the chromosome, and the mutations have been phenotypically characterized. Complementation analysis indicates that these genes are arranged in three different operons transcribed in opposite directions. A detailed physical map of this region has been constructed on the basis of complementation analysis, fusion protein data, and phenotypic characterizations. Additionally, the role of some genes in the synthesis of LPS has been defined by complementation analysis with known *Salmonella typhimurium* LPS mutants. The genetic organization of this locus seems to be identical in *E. coli* K-12 and *S. typhimurium*.**

The outer membrane of gram-negative bacteria is formed by at least two types of lipids, lipopolysaccharide (LPS) and phospholipids, as well as a set of characteristic proteins (reviewed in reference 24). In addition, the outer membrane of some members of the family *Enterobacteriaceae* contains a unique polysaccharide, enterobacterial common antigen.

LPS (Fig. 1) is a complex molecule that plays an important role in the structure of the outer membrane as well as in the interaction between cells and environment. LPS is composed of three parts: the proximal hydrophobic lipid A region, the distal hydrophilic O antigen that protrudes into the medium, and the core region that connects the two (Fig. 1). Its biosynthesis begins with the lipid A region, which is believed to be linked to the assembly of the outer membrane (reviewed in reference 29). The genes involved in the synthesis of the complex carbohydrate attached to lipid A are organized in rather large groups or clusters along the bacterial chromosome that roughly resemble the functional units of the molecule. This seems to be the way chosen by the bacteria to manage the complexity of this biosynthetic pathway without compromising the basis for surface diversity.

The genes for the synthesis of the LPS core are clustered, probably with the exception of those specifying 2-keto-3-deoxyoctulosonic acid (KDO) biosynthesis, in the *rfa* locus, located between *cysE* and *pyrE* (at 81 min in the linkage map of *Escherichia coli* K-12 [2]). A comparable position has been determined for the *Salmonella typhimurium rfa* cluster (33). The number of genes present in this region is still unclear; however, 10 different biochemical functions have been described for *S. typhimurium* on the basis of genetic and biochemical studies (21). Some of these genes have been cloned in *S. typhimurium*, and their order has been partially established (14, 20). In *E. coli*, the picture is less clear, since very limited information is available; most of this knowledge is based in the demonstration that some Clarke-Carbon plasmids complement *Salmonella* LPS mutants (9, 10). Very recently, the first hints of the molecular biology of the *E. coli* genes have been obtained with the cloning and partial characterization of three fragments of the *rfa* cluster (1, 8, 25, 34). However, many questions remain to be answered. How many functions are encoded? How are these genes organized? How are they regulated?

Independently of its structural role in the outer membrane, LPS acts as a receptor for a large number of phages, the LPS-specific phages. Our laboratory has been interested in the synthesis of this molecule because of its role as a receptor for phage Mu (15, 31). This phage has been widely used as a powerful genetic tool (36) in *E. coli* and related bacteria, but its short host range limits its use in other enteric bacteria. The limited host range is probably due to the lack of an appropriate receptor, since some strains that are able to propagate the phage efficiently are resistant to it. Eventually, the knowledge of LPS biosynthesis in different bacteria would help to develop a strategy for increasing the host range of Mu. The interaction of Mu and the related phages MuhP1 and D108 with their LPS receptors has recently been investigated in our laboratory by using *S. typhimurium* LPS mutants (31). Using this as starting point, we decided to determine the nature of the genes involved in the synthesis of these phages' receptors.

The genes for the synthesis of the LPS core of *E. coli* K-12 have been cloned in vivo by using mini-Mu technology. The DNA corresponding to the *rfa* cluster (approximately 18 kb) has been subcloned, and a protein map has been obtained with the help of *lacZ* fusions. The recombination of these fusions into the chromosome led us to generate a defined set

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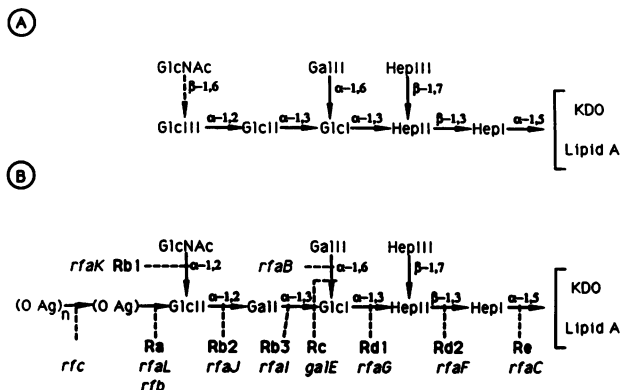

FIG. 1. Comparative representation of LPS structure in *E. coli* K-12 (A) and *S. typhimurium* (B). Arrows indicate linkage between units, with the dashed arrow indicating partial substitution. Boldface roman symbols indicate the names of different chemotypes obtained in truncated LPS, while italics indicate the name of the gene required in each step (21, 29). The lateral charged groups are not presented. Note the differences between panels A and B in GlcNAc and GlcII.

of mutants with alterations in the LPS structure. Complementation analysis has shown that the number of genes in this region is higher than expected from the genetic and biochemical data.

## MATERIALS AND METHODS

**Strains and culture conditions.** The bacterial strains, phages, and plasmids used are presented in Table 1. MC4100 was the control strain. Additionally, in the in vivo cloning experiments, Mu lysogens of BW322 and JM15 were used as recipient strains. The lysogenic phages Mu, MuhP1, D108, and P1 were obtained by thermal induction as described previously (36). All the other phages were propagated by confluent lysis (23).

All new strains were constructed by using P1 generalized transduction as described previously (23). Mu transductions were also carried out as described previously (36). All strains were routinely grown in Luria-Bertani (LB) medium (with or without agar) at 37°C, with the exception of phage lysogens (30°C). When required, antibiotics were added to the medium at 25 or 60 μg/ml for ampicillin (Ap), 20 or 50 μg/ml for kanamycin (Km), 20 or 100 μg/ml for spectinomycin (Sp), 10 μg/ml for tetracycline (Tc), and 25 μg/ml for chloramphenicol (Cm). The lower concentrations were used when resistance genes were in single copy. Minimal M63 medium was used as defined medium (23).

**DNA manipulations.** The in vivo genomic bank of Xph43 was obtained for the Mud 5924 vector as described previously (12); this bank was later used for cloning the *rfa* genes (see Results). All other DNA manipulations have been described elsewhere (32). Plasmid DNA was routinely introduced into different strains by the $CaCl_2$ method (32). However, the low efficiency of transformation of *S. typhimurium* strains made necessary the use of electroporation. Cells were grown to early logarithmic phase, collected, washed twice with ice-cold distilled water, and suspended in ice-cold 10% glycerol at a concentration of approximately $2 \times 10^{10}$ cells per ml. Forty microliters of cell suspension was subjected to electrical pulse in a cuvette with a 0.2-cm electrode gap; a Bio-Rad gel pulser with pulse controller was used at 2.5 kV, 25 μF, and 200 Ω. This method increased the efficiency of *Salmonella* transformation by 3 to 4 orders of magnitude.

**Subcloning.** A *Pvu*II fragment of pLC10-7 (Fig. 2) was subcloned into pTZ-18R (US Biochemical) to generate pCR01 (not shown); this fragment includes the DNA region shown in Fig. 2 as pCR0131, together with a small portion of the original ColE1 plasmid on the left side. Plasmid pCR01 was later subjected to controlled exonuclease III digestion to generate the set of plasmids pCR0102 to pCR0131. In addition, the large *Bam*HI-*Eco*RI fragment of pCR11 was also cloned into pTZ-18R to give pCR07, which was used as a DNA source for further subcloning. Plasmid pCR06 was made by replacing the left part of pCR0131 with a larger fragment of the same region from pCR11 (see legend to Fig. 2 and Results for details).

**Obtaining *lacZ* fusions in plasmids.** Plasmid pCR06, -09, or -010 (Fig. 2) was transformed into strain POII1734 TR or POI1734 TR (6), and stable $Ap^r$ transformants were selected. Mu growth in these strains was induced by a shift to 42°C (36), and the resultant lysates were transduced into strain MC4100. Plating on selective MacConkey media gave colonies with mini-Mu insertions ($Km^r$) in the original plasmid ($Ap^r$). In addition, red colonies indicated the presence of translational (MudII1734) or transcriptional (MudI1734) fusions. Plasmids from these colonies were isolated by the alkaline lysis method (32) and subjected to restriction mapping to determine the position of the insertion or fusion. In general, over 90% of the fusions were located in the insert. The orientation of the fusions provided an estimate of the transcriptional units present in the region. In addition, some white colonies with transcriptional insertions were also mapped.

Analysis of protein fusions (MudII1734) was performed as described previously (5). Cells harboring the plasmid with the fusion were grown in selective media until late logarithmic phase. Cells (1 ml) were collected, washed in 0.5 ml of 10 mM Tris HCl (pH 7.5)–1 mM EDTA, resuspended in 150 μl of loading buffer (18), and boiled for 5 min. After centrifugation for 6 min at 12,000 × *g*, the supernatant was collected and kept frozen until used. Protein samples were run on 7.5% polyacrylamide gels under denaturing conditions (18) and stained with Coomassie blue (Sigma). Fusion products appeared as new bands with apparent molecular masses higher than that of native β-galactosidase (116 kDa) (Fig. 3). To determine the size of the protein fusion, the migration distances of these bands were plotted against molecular weight standards; this value, used to calculate the starting point of the gene interrupted by the fusion, was calculated as follows: [(molecular weight of protein fusion − molecular weight of β-galactosidase) × 3]/111 = number of base pairs from the origin, where 3 is the number of base pairs per amino acid and 111 is the average molecular weight of an amino acid. Different fusions in the same gene gave very similar starting points, indicating the validity of the method (see Results).

**Isolation of fusions in the chromosome.** Mini-Mu and Ω insertions obtained in the plasmids were recombined into the chromosome of BN138 (35), taking advantage of the special properties of this strain (*recBC sbcB*) (reviewed in reference 17). BN138 was transformed with plasmids carrying different insertions, and the transformants were selected in LB medium supplemented with kanamycin or spectinomycin used as mini-Mu or Ω markers, respectively. In theory, only recombinants of the marker in the chromosome should grow; however, a significant background of small colonies was

TABLE 1. Strains, plasmids, and phages used

| Strain | Relevant genotype | Source or reference |
|---|---|---|
| Strains | | |
| *E. coli* | | |
| MC4100 | $F^-$ *araD139* Δ(*lacIPOZYA-argF*) | Laboratory stock |
| BN138 | Δ(*lacIPOZYA-argF*) *recB21 recC22 sbcB15* | B. Nichols (35) |
| BW322 | *rfa-210*::Tn*10 pyrE70* | EGSC[a] |
| JM15 | *cysE50* | EGSC |
| XPH43 | Δ(*lacIPOZYA-argF*) *trpE* Δ(*brnQ phoA proC phoB phoR*) | Laboratory stock |
| CR01 to CR0300 | MC4100 derivatives with MudII1734 insertions | Fig. 4 |
| CR01 to CR0300:Ω | CR strains with Ω insertions in position 1, 2, or 3 | Fig. 4 |
| *S. typhimurium* | | |
| SL3750 | *rfaJ-417* | SGSC[b] |
| SL3748 | *rfaI-432* | SGSC |
| SL4807 | *rfaB-707* | SGSC |
| SH7770 | *rfaP* | SGSC |
| SL3769 | *rfaG-471* | SGSC |
| SL3789 | *rfaF-511* | SGSC |
| SL3600 | *rfaD-657* | SGSC |
| Plasmids | | |
| pTZ-18R | pUC-18 derivative with T7 promoter | USB |
| pDΩ9 | Ω cassette ($Sp^r$) in pUC-18 | R. Haselkorn (27) |
| pCR1 to pCR50 | Mini-Mu generated plasmid of the 80- to 83-min region of the *E. coli* K-12 chromosome | Fig. 2 |
| pCR01 to 012 | Subclones of pCR11 into pTZ-18R | Fig. 2 |
| pCR0102 to pCR0131 | Exonuclease III deletions of plasmid pCR0101 | Fig. 2 |
| pCR06Φ1 to Φ99 | MudII1734 insertions into pCR06 | Fig. 4 |
| pCR09Φ100 to Φ199 | MudII1734 insertions into pCR09 | Fig. 4 |
| pCR010Φ200 to Φ299 | MudII1734 insertions into pCR010 | Fig. 4 |
| pCR06::Ω2 | Same as pCR06, with Ω insertion in position 1 | Fig. 4 |
| pCR09::Ω1 | Same as pCR09, with Ω insertion in position 2 | Fig. 4 |
| pCR06Ω3::Ω3 | Same as pCR06Φ3, with Ω insertion in position 3 | Fig. 4 |
| Phages | | |
| Mu | cts62 | Laboratory stock |
| MuhP1 | Mucts62/P1 hybrid; Mu but with host range of P1 | Laboratory stock (31) |
| D108 | cts | Laboratory stock |
| MudI1734 | LacZ transcriptional fusion mini-Mu, $Km^r$ | Laboratory stock (6) |
| MudII1734 | LacZ translational fusion mini-Mu, $Km^r$ | Laboratory stock (6) |
| Mud 5924 | Mini-Mu, T7p, colE1 Rep, $Km^r$ | Laboratory stock (12) |
| U3 | LPS-specific phage | SGSC |
| C21 | LPS-specific phage | SGSC |
| SP6 | LPS-specific phage | SGSC |
| P22-c2 and Felix-O | Smooth specific phages of *S. typhimurium* | SGSC |
| Br60 and Ffm | Rough specific phages of *S. typhimurium* | SGSC |

[a] EGSC, *E. coli* Genetic Stock Center, New Haven, Conn.
[b] SGSC, *Salmonella* Genetic Stock Center, Calgary, Alberta, Canada.

always detected because of partial plasmid replication. Transformants that clearly outgrew this background were purified and checked for the presence of $Km^r$ ($Sp^r$) and $Ap^r$ markers. $Ap^r$ $Km^r$ transformants would likely arise as a result of loop-to-loop integration, while $Km^r$ $Ap^s$ transformants, which were chosen for further study, were the result of homologous recombination. In a typical experiment, over 30% of the colonies picked were true recombinants. Whenever possible, the presence of the fusion was confirmed by plating on 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) medium. Since complementation experiments were not possible in this background, all the insertions were mobilized into MC4100 (*pyrE*) by using P1 generalized transduction and selection for prototrophy and $Lac^+$ phenotype. The positions of insertions in the MC4100 chromosome were confirmed by Southern blot analysis. Each insertion was designated by the same number that it holds in the plasmid.

**Phenotypic characterization of LPS mutants.** The characterization of altered LPS structures was carried out by two techniques: sensitivity to LPS-specific phages (21) and sensitivity to novobiocin (1). For phage typing, the cells were grown in LB (supplemented with antibiotics when necessary) until late logarithmic phase, resuspended in warm H-top agar (23), and plated in LB medium (with or without ampicillin). After the agar solidified, 5 μl of phage lysate ($10^8$ to $10^{10}$ PFU/ml) was applied onto the surface, and the plates were incubated overnight at 37°C. The strain was considered sensitive to the phage if clearing was observed. Phages Mu, MuhP1, D108, U3, C21, and SP6 were used for typing *E. coli* K-12 strains; phages Felix-O, P22-c2, Br60, and Ffm were used for typing *S. typhimurium* strains (Table 1). To test for novobiocin sensitivity, a similar protocol was followed, but the phage lysate was substituted by a solution of 10, 5, or 1 mg of novobiocin per ml. Strains were considered sensitive when a halo of lysis was observed with the 1-mg/ml solution.

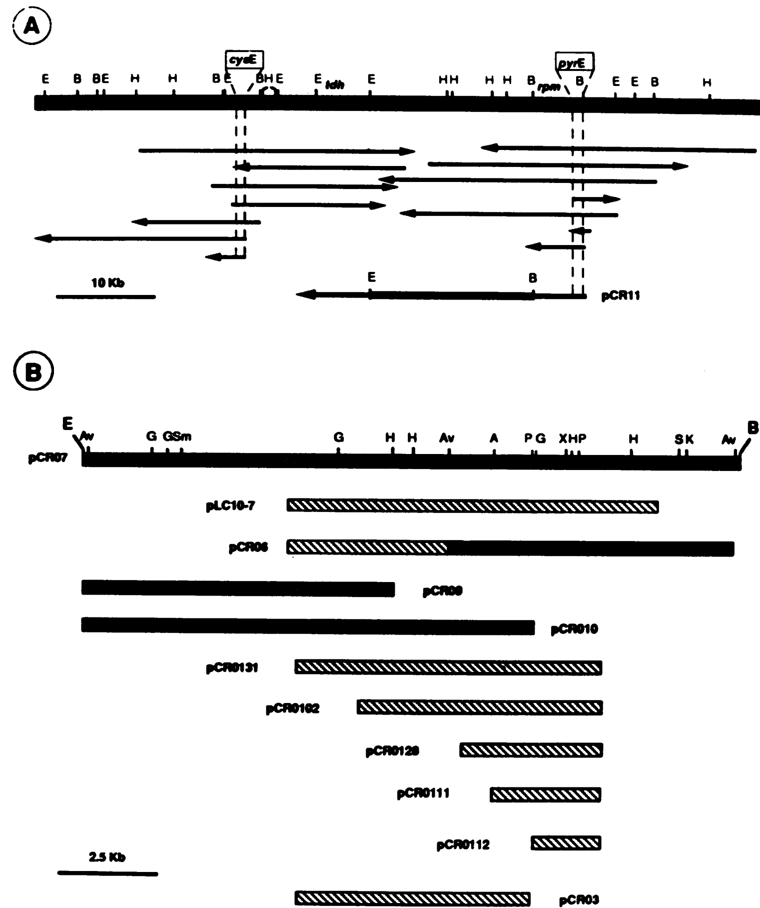


FIG. 2. Schematic representation of the plasmids generated in this work. (A) Mud 5954-generated plasmids of the 80- to 83-min region of the *E. coli* chromosome; some genetic markers in this region are indicated, and the boxed ones were used in the in vivo cloning experiments. The arrows indicate arbitrary orientation with respect to the vector. Plasmid pCR11 was used as a source to subclone the indicated *Bam*HI-*Eco*RI fragment into pTZ-18R (pCR07). (B) Detailed physical map of the *rfa* cluster and deletion plasmids obtained from it. All of the different subclones, with the exception of pLC10-7 (9), are in the poly-linker region of pTZ-18R (not represented) with the *lac* promoter going from right to left. For details, see the text. ▩, DNA from pCR11; ▩, DNA from pLC10-7. Enzymes used are as follows: A, *Acc*I; Av, *Ava*I; B, *Bam*HI; G, *Bgl*II; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; P, *Pst*I; S, *Sal*I; Sm, *Sma*I; X, *Xba*I.

**Isolation and characterization of LPS samples.** LPS samples were obtained from the outer membrane fraction by the proteinase K method, basically as described previously (1); however, cell lysis was achieved by vortexing a concentrated culture with an excess of glass beads four times for 30 s each. LPS samples were then run on 18% polyacrylamide gels and silver stained with the Bio-Rad kit. This system gave higher backgrounds because of residual protein staining but provided good LPS staining in samples obtained either by the proteinase K method or the phenol-chloroform-petroleum ether method (data not shown).

**Measurement of β-galactosidase activity.** β-Galactosidase

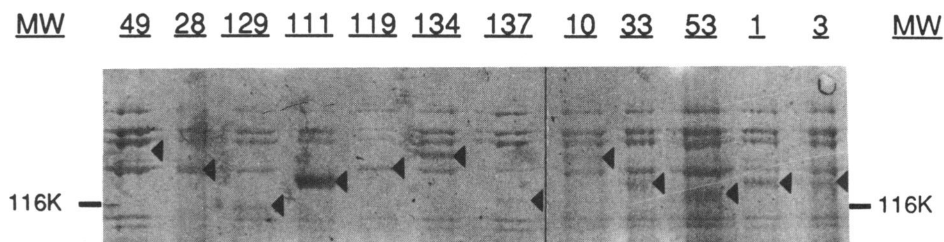


FIG. 3. Analysis of protein fusions. Crude protein extracts were obtained from cells harboring *rfa* plasmids with different insertions, run on 7.5% polyacrylamide gels, and stained with Coomassie blue (for details, see text). The columns are labeled with the number of the insertion present in the plasmid. The position of native β-galactosidase (molecular weight, 116,000 [116K]) is indicated. New bands, representing fusion proteins, are indicated by black arrowheads; note that the protein in column 129 is similar in size to the control β-galactosidase. Only the upper part of the gel is presented.

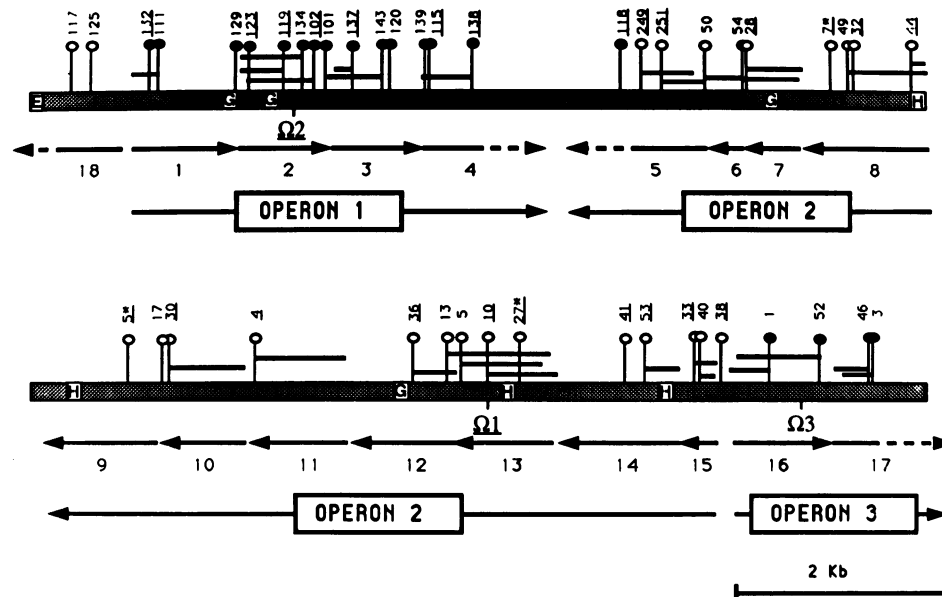


FIG. 4. Translational map of the *rfa* cluster. MudII1734 translational fusions (○ and ●) were obtained in different *rfa* plasmids (see the text), and the results are presented in the figure. The orientation of the fusions is as follows: ○, right to left; ●, left to right. The black lines indicate the extension of the protein fusion determined as described in the text. The insertions are labeled for the plasmid used as a target (see Table 1); underlined insertions have been recombined into the chromosome. Insertions 7*, 5*, and 27* are Tn*5-lacZ* (21) instead of MudII1734 (6). The Ω insertions used are also included (for details, see text). Restriction sites are also given (see Fig. 2). The lower part of the figure represents the working model for the organization of this region: proteins (arbitrarily numbered) are in the upper line, and transcriptional units are in the lower line.

activity in exponentially growing cells (optical density at 600 nm, 0.2 to 0.5) was measured by the toluene permeabilization method as described previously (23). The activity was expressed in Miller units (23).

## RESULTS

The understanding of LPS biosynthesis in gram-negative bacteria is an old goal that reflects the importance of LPS in the biology of bacteria. Although an exhaustive biochemical approach to the problem was taken in the late sixties, little is known about the genes responsible for its synthesis. This is especially true for *E. coli*, where only one mutant in this pathway has been characterized over the years. Very recently, the first genetic and molecular characterization of some of these genes has been performed (1, 8, 25, 34).

Since the approximate location of the genes responsible for LPS biosynthesis was known for *E. coli* K-12 (81 to 82 min) (reviewed in reference 2), we decided to clone in vivo this chromosomal region by using a mini-Mu vector and flanking genes as markers. Once the clones generated were characterized, they were inactivated in vitro and reintroduced into the chromosome to determine the mutant phenotype. This approach allowed us to circumvent the problems associated with the absence of phenotype in some of the mutants.

**Cloning of the *rfa* locus.** The in vivo genomic bank of *E. coli* K-12 (Xph43) obtained in Mud 5924 (12) was transduced into Mu lysogenic strains of BW322 (*pyrE*) and JM15 (*cysE*), and the transductants were selected for growth in minimal medium supplemented with kanamycin. Plasmids were isolated by the alkaline lysis method and subjected to physical mapping. The pool of plasmids obtained is partially presented in Fig. 2A and is aligned with the physical map of this region. This map is similar to the Kohara map and to previous reports of the region for all enzymes tested (*Hin*dIII, *Eco*RI, *Bam*HI, and *Pst*I), except *Eco*RV (1, 8, 16, 25). Altogether, the characterized plasmids comprise a region of the *E. coli* K-12 chromosome of approximately 2 min (80 kb). The region between the *rpm* and *tdh* genes also includes clones pLC17-24 and pLC10-7 of the Clark-Carbon collection (reference 1 and data not shown). The presence of *rfa* genes was confirmed by complementation of *E. coli* mutants deficient in LPS biosynthesis (data not shown).

Figure 2B presents some plasmids used in further experiments (see Materials and Methods for details).

**Characterization of open reading frames (ORFs) in the region.** To determine the number of genes present in this region, random mutagenesis with MudII1734 (translational vector) was carried out with plasmids pCR06, -09, and -010, and insertions were selected for the expression of β-galactosidase (see Materials and Methods). The positions of these fusions were determined by restriction analysis (Fig. 4). The orientations of the fusions define four different regions. Additionally, the direction of transcription in the two leftward regions was confirmed by mapping transcriptional (MudI1734) insertions (data not shown).

The fusion proteins were characterized by determining the molecular weights on sodium dodecyl sulfate (SDS)-polyacrylamide gels (see Materials and Methods). A representative sample of these fusions is presented in Fig. 3. In most cases, a new band is observed in the upper region of the gel (molecular mass > 116 kDa). However, in 30 to 40% of the cases, no new band was observed or, if present, it was the same size as the β-galactosidase band (Fig. 3, lane 3). The difference in molecular mass between the fusion protein and β-galactosidase allowed us to calculate the approximate start of the gene inactivated by the fusion (see Materials and

TABLE 2. Phenotypic characterization of MudII1734 insertions in the chromosome of MC4100

| Strains[a] | Phenotype | | | | | | | | Gene[e] |
|---|---|---|---|---|---|---|---|---|---|
| | Nv[b] | U3[c] | Mu | MuhP1 | D108 | C21 | SP6 | LPS[d] | |
| MC4100 | R | ++ | ++ | ++ | ++ | − | − | wt | |
| 38 | S | − | − | ± | ++ | ++ | − | ND | ? |
| 33 | S | − | − | ± | ++ | ++ | − | 6 | 15 |
| 53 | S | − | − | ± | ++ | ++ | − | 6 | 14 |
| 27* and 10 | S | − | − | ± | ++ | ++ | − | 6 | 13 |
| 36 | S | − | − | ± | ++ | ++ | − | 5 | 12 |
| 4 | R | − | − | ++ | ++ | ++ | ++ | 4 | 11 |
| 30 and 17 | R | − | − | ++ | ++ | ++ | ++ | 4 | 10 |
| 5* | R | − | − | − | − | − | ++ | 3 | 9 |
| 44 and 32 | R | − | − | ++ | ++ | ++ | − | 2 | 8 |
| 28 | R | − | ++ | ++ | ++ | ++ | ++ | 1 | 7 |
| 251 and 255 | R | − | ++ | ++ | ++ | ++ | ++ | 1 | 5 |
| 138 and 115 | R | ++ | ++ | ++ | ++ | ± | − | wt | 4 |
| 137 and 120[f] | S | − | − | − | − | − | − | 7 | 3 |
| 119 and 123[f] | S | ± | − | − | − | − | − | 7 | 2 |
| 111 and 132[f] | S | − | − | − | − | − | − | 7 | 1 |
| 1, 19, and 52[g] | | | | | | | | | 16, 17 |

[a] The number of each strain (other than MC4100) indicates the MudII1734 fusion originally obtained in a plasmid and then recombined into the chromosome (see Materials and Methods). Asterisks indicate Tn*5*-*lacZ* fusions.
[b] Sensitivity (S) or resistance (R) to novobiocin (Nv) was measured by agar diffusion tests (see Materials and Methods).
[c] Sensitivity to the phages is as follows: ++, fully sensitive (normal clearing); ±, partial clearing; −, fully resistant (not clearing even at high phage concentration).
[d] The numbers indicate the size of the LPS as defined by its molecular weight in acrylamide gels, from the largest (wild type [wt]) to the smallest (7) (see text and Fig. 5). ND, not determined.
[e] Position of the insertions as defined in Fig. 4.
[f] These strains are also temperature sensitive and poor transformants (efficiency of $10^{-4}$ when compared with other strains of similar background).
[g] These strains may have lethal insertions in the chromosome.

Methods). The derived protein-gene map for this region is presented in Fig. 4. In order to draw the map, we have assumed that the genes do not overlap (1) and have considered the phenotype of strains carrying different insertions in the chromosome. The genes or ORFs have been numbered arbitrarily starting at the left end of the region. The results also indicate that there is a region of approximately 1.2 kb (between genes 4 and 5), not covered by fusions or insertions, which could contain an additional gene(s).

**Phenotypic characterization of fusions.** A representative pool of fusions was recombined into the *E. coli* K-12 chromosome by using the special properties of *recBC sbcB* strains (for details, see Materials and Methods). Plasmids carrying the fusions were transformed into strain BN138, and the transformants were plated on low-concentration kanamycin-X-Gal plates to select for markers and fusions recombined into the chromosome. All of the insertions were transferred to MC4100 (*pyrE*) via P1 transduction. The positions of most of these insertions were confirmed by Southern blot analysis (data not shown). Single insertions into the different genes present in this region were obtained, with the exception of genes 16 and 17, for which over 100 $Km^r$ transformants were checked with negative results.

The insertion mutants were characterized by three different criteria: phage sensitivity, novobiocin sensitivity, and LPS structure. The results obtained are presented in Table 2. Mutants can be assigned to two groups on the basis of resistance to novobiocin: sensitive, a phenotype usually known as deep rough and indicative of a severe reduction in the length of LPS, and resistant. The use of LPS-specific

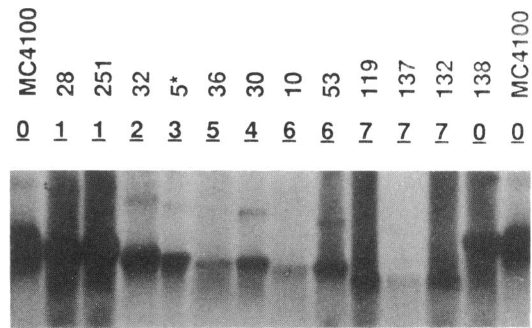


FIG. 5. Silver-stained gel showing LPS from different insertion mutants. All lanes are labeled with the number of the insertion (upper line) and with an arbitrary number that indicates the relative length of the LPS (lower line), from wild type (0) to the shortest detected (7). In all cases, the LPS samples were obtained from the outer membrane fraction by protease digestion (1).

phages allowed us to classify the insertions into seven different groups. However, this criterion is not absolute because of gaps in our knowledge of some phage receptors (31). Therefore, the LPSs of the different mutants were isolated and characterized on acrylamide gels. The results obtained are presented in Fig. 5, which shows LPSs of at least eight different lengths. Numbers have been assigned starting with the longest LPS structure; i.e., the higher the number, the shorter the LPS backbone. It is worth noting the sensitivity of LPS gels, since they allowed us to differentiate between insertions 36, 10, and 30. No phenotypic differences were observed between mutants 33, 53, and 10, since all of them showed a similar pattern of phage sensitivity (Table 2) and an LPS of type 6 (Fig. 5). Mutants 137, 119, and 132 are also phenotypically similar, with an LPS of type 7, and resistant to all the phages tested (Table 2). Additionally, mutant 138 behaved like the wild-type strain under all conditions tested.

**Genetic organization of *rfa* region: complementation analysis.** To investigate how the genes are organized, exhaustive complementation analysis was carried out. A representative set of insertions in the chromosome was complemented with different plasmids. The transformants obtained were tested for phenotype on the basis of sensitivity to novobiocin and to phages Mu and D108. In all cases, three different transformants were tested to avoid recombination problems.

In the region covering genes 5 to 15 (Table 3), the results indicate the presence of a single operon. Complementation to the wild-type phenotype was observed with plasmids carrying insertions upstream but not downstream of the insertions in the chromosome. Similar results were obtained when plasmids with downstream deletions were used. In conclusion, insertions in the chromosome show polarity, indicating the existence of a single operon covering genes 5 to 15. In addition, insertions in genes 12, 14, and 15 were complemented to Mu sensitivity, but not to novobiocin resistance, by plasmids that do not carry the corresponding genes (Table 3). These results indicate that these genes are not absolutely required for the synthesis of the backbone structure and that their phenotype is a result of polarity (see Discussion for further details).

A similar approach (Table 4) was used to characterize the other operon (genes 1 to 4). Again, mutations in the chromosome showed polarity, indicating the presence of an operon. This type of organization was also predicted by the

TABLE 3. Complementation[a] analysis of MudII1734 insertions in the *rfa* region (genes 5 to 15) of MC4100

| Plasmid | Results with strain[b]: | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 33 | | 53 | | 10 | | 36 | | 4 (Mu) | 30 (Mu) | 5* | | 44 (Mu) |
| | Mu | Nv | Mu | Nv | Mu | Nv | Mu | Nv | | | Mu | D | |
| (Control) | − | + | − | + | − | + | − | + | − | − | − | − | − |
| pCR06Φ33 | + | + | + | + | + | ND | ND | ND | + | + | + | + | + |
| pCR06Φ53 | + | + | + | + | + | ND | ND | ND | + | + | + | + | + |
| pCR06Φ10 | − | + | − | + | − | ND | + | − | + | + | + | + | + |
| pCR06Φ4 | + | ND | + | ND | + | ND | ND | ND | + | + | + | + | + |
| pCR06Φ30 | − | − | − | − | − | − | − | − | − | − | + | + | + |
| pCR0131Φ5 | ND | ND | ND | ND | ND | ND | ND | ND | − | − | − | − | + |
| pCR06Φ44 | − | − | − | − | − | ND | ND | ND | − | − | + | + | − |
| pCR0131 | + | + | + | + | + | ND | + | − | + | + | + | + | + |
| pCR0102 | − | + | − | + | − | ND | − | − | − | − | ND | ND | − |
| pCR03 | − | + | − | + | ND | ND | + | − | ND | ND | + | + | + |

[a] Different strains were transformed with *rfa* plasmids (see Fig. 2 and 4), and these transformants were tested for sensitivity (+) or resistance (−) to phage Mu (Mu), phage D108 (D), or novobiocin (Nv) as described in the text. ND, not determined.
[b] All the strains used as recipients are MC4100 derivatives with MudII1734 insertions in the *rfa* region (see Table 1), except that in strain 5*, the insertion is Tn*5*-*lacZ*.

similarity in the phenotypes of insertions 132, 119, and 137 (temperature sensitivity, D108 resistance, and same LPS; see Table 2) (31). It is especially significant that a plasmid with an insertion in gene 2 complemented the insertion in genes 1 or 2 regarding D108 sensitivity but not Mu sensitivity. In addition, complementation of insertion 132 (gene 1) to Mu sensitivity was only achieved with plasmids carrying Mud insertions downstream of gene 3. No complementation analysis of insertions in gene 4 was possible, since all mutants in this gene lack a detectable phenotype.

The results presented clearly indicate that insertions in the plasmids are not fully polar; however, the existence of partial polarity cannot be ruled out. In addition, none of the Tn*5*-*lacZ* insertions (22) (the most representative is insertion 5 in gene 9) were polar either in the chromosome or in plasmids.

The approach described above could not be used to study the organization of genes 16 and 17, since insertions in these genes failed to recombine into the chromosome. Therefore, a strong terminator (Ω casette) (27) was introduced into the unique *Kpn*I site of plasmid pCR06Φ3, a site that lies in the ORF of gene 16 (Ω3; Fig. 4). β-Galactosidase activity of strains carrying plasmids pCR06Φ3 or pCR06Φ3::Ω was measured; the Ω3 insertion led to a 15-fold decrease in β-galactosidase levels (data not shown), indicating the polarity of the insertion, even in high-copy plasmids.

Although the results presented so far strongly suggest the presence of three operons, we decided to further confirm this hypothesis. To do so, an Ω insertion ($Sp^r$) was introduced into the unique *Xba*I (gene 7, pCR06) and *Sma*I (gene 2, pCR09) sites (Fig. 4). These insertions were recombined into the chromosome of BN138 and transduced (via P1 phage) to representative mutant strains. The levels of β-galactosidase were then measured in isogenic strains with and without the insertion. From the results obtained (Table 5 and data not shown), it is clear that one Ω insertion in the *Xba*I site (Ω1; Fig. 4) abolished the expression of downstream (Φ30, Φ44, Φ28, or Φ251) but not upstream (Φ33) fusions or expression in the other operon (Φ138). Similar results were obtained in the leftmost operon, where a Ω insertion in gene 2 (Ω2) blocked the expression of downstream fusion 138 (gene 4). In all cases, at least an 18-fold decrease in activity was observed. In conclusion, genes 5 to 15 define one operon, while genes 1 to 4 define another.

It is important to point out that the levels of β-galactosidase activity in conditions of "repression" are actually similar to the levels detected for a wild-type *lac* operon in the absence of lactose (repressed). These very low levels of expression would explain the polarity effect of the MudII1734 insertions in the chromosome observed in the complementation experiments (Table 3 and 4).

TABLE 4. Complementation analysis[a] of MudII1734 insertions in the *rfa* region (genes 1 to 4) of MC4100

| Plasmid | Results with strain[b]: | | | | | |
|---|---|---|---|---|---|---|
| | 137 | | 119 | | 132 | |
| | Mu | D | Mu | D | Mu | D |
| (Control) | − | − | − | − | − | − |
| pCR09 | + | + | + | + | − | − |
| pCR09Φ138 | ND | ND | ND | ND | + | + |
| pCR09Φ137 | − | − | − | − | − | − |
| pCR09Φ119 | + | + | − | + | − | + |
| pCR09Φ132 | + | + | + | + | − | − |

[a] Different strains were transformed with *rfa* plasmids (see Fig. 2 and 4), and these transformants were tested for sensitivity (+) or resistance (−) to phage Mu (Mu) or phage D108 (D) as described in the text. ND, not determined.
[b] All the strains used as recipients are MC4100 derivatives with MudII1734 insertions in the *rfa* region (see Table 1).

**Identifying genes: complementation of *S. typhimurium* mutants.** In an attempt to identify the biochemical function of the genes previously defined, we decided to complement well-known mutants of *S. typhimurium* deficient in the synthesis of LPS (30). Different plasmids were transformed into mutant strains, and their LPS phenotypes were determined with the help of LPS-specific phages (see Materials and Methods). In all cases, we were able to detect complementation (Table 6), indicating functional similarities between *E. coli* and *S. typhimurium* genes. Additionally, the complementation pattern of different plasmids allowed us to relate the unknown *E. coli* genes to a known phenotypic defect (gene) in *S. typhimurium*. This assignment is presented in the last line of Table 6.

It is worth noting that, as in the case of insertion 132 in *E. coli*, we were unable to complement a mutation in the *rfaD* gene of *S. typhimurium* with a wild-type plasmid (pCR09);

TABLE 5. β-Galactosidase activity[a] of different fusions in the chromosome of MC4100

| Growth conditions[b] | Sp act with the following strain[c]: | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 33 | 33::Ω1 | 30 | 30::Ω1 | 28 | 28::Ω1 | 28::Ω2 | 251 | 251::Ω1 | 138 | 138::Ω1 | 138::Ω2 | 132 |
| LB, 30°C | 6.6 | | 18.7 | | 33.1 | 1.3 | | 24.9 | | 39.7 | | 2.1 | 18.7 |
| LB, 37°C | 10.2 | 6.9 | 24.9 | 0.6 | 43.6 | 2.1 | 44.0 | 33.9 | 0.9 | 43.0 | 35.5 | 2.4 | 19.2 |
| LB, 42°C | | | 13.2 | | 31.4 | | | | | 15.9 | | | 10.2 |

[a] The activity was measured for toluene-permeabilized cells as described in the text. Values are averages from at least three experiments and are represented as specific activities expressed in Miller units (23).

[b] Preinocula of cultures for the 30 or 37°C experiments were grown overnight at the indicated temperature. For the 42°C experiment, cells were grown overnight at 30°C, resuspended in fresh medium, and incubated at 30°C for 90 min, the cultures were then transferred to 42°C, incubated for another 20 min, and finally transferred to an ice-cold water bath for processing.

[c] The strains are described in Table 1 (CR01 to CR0300). For the exact positions of the insertions, see Fig. 4. In addition to the MudII insertions, some strains also have Ω insertions in the positions indicated in Fig. 4.

however, the mutation was complemented by plasmids carrying a Mud insertion downstream of gene 1 (*rfaD*).

## DISCUSSION

In this report, we present the genetic organization of the *rfa* cluster in *E. coli* K-12. The results obtained show that the synthesis of the LPS core in *E. coli* is mostly directed by genes organized into three operons, designated 1, 2, and 3 and encoding for all of the glycosyltransferases required for this synthesis. The deduced organization of this cluster, compiled by using protein data (Fig. 3), phenotypic characterization (Table 2), complementation data (Tables 3, 4, and 6), and data from previous reports (1, 8, 14, 25), is presented in Fig. 6. This map is in clear agreement with the physical map of the *rfa* locus recently reported (34), although it includes a new gene, *rfa15* (see below for further details).

Operon 1, which includes genes *rfaD* (25), *rfaF*, and *rfaC*, encodes the enzymes implicated in the synthesis of the heptose core. A fourth gene present in this operon has been tentatively designated *rfaL*. The inactivation of this gene does not show a detectable phenotype in *E. coli* K-12, as expected by its lack of O antigen. In addition, this positioning of *rfaL* would match the organization reported for the *S. typhimurium rfa* cluster (20). Complementation experiments, showing the polarity of MudII1734 insertions into chromosomal genes (Tables 3 and 4), indicate that these genes are organized into a classical operon. The complementation of insertion 119 (gene 2) by pCR09Φ119 is especially significant since the transformants were Mu resistant but D108 sensitive, indicating the presence of an LPS in the transformant of the Rd2 chemotype (Fig. 1) (31). This result confirms that gene 2 is *rfaF* and that gene 3 (*rfaC*) is expressed from plasmids because of a lack of polarity of MudII1734 insertions in high-copy-number plasmids. The polarity on gene 4 (*rfaL*) of a Ω insertion in gene 2 (*rfaF*) confirms the operon organization and the presence of gene 4 on this operon (Table 5).

One puzzling result is the lack of complementation of *rfaD* mutations in either *E. coli* (Tables 3 and 4) or *S. typhimurium* (Table 6) by plasmids carrying the whole operon; however, these mutations were complemented in both cases by plasmids carrying MudII1734 insertions downstream of the *rfaD* gene. A possible explanation for this behavior might reside in differences in the copy number of plasmids. The MudII insertion, over 10 kb, would reduce copy number and therefore lower the expression of the genes, allowing complementation. If this is indeed the case, it suggests the necessity for a delicate balance in the synthesis of these enzymes for full functionality. Although *E. coli* and *S. typhimurium* show similar results, there are significant differences; plasmid pCR09Φ119 or pCR09Φ137 complemented the *S. typhimurium rfaD* mutation but not the equivalent mutation (insertion 132) in *E. coli*. These results could reflect the intrinsic differences between the mutations in *S. typhimurium* and *E. coli*: the mutation in the *rfaD* gene of *S. typhimurium* is not polar, whereas insertion 132 in *E. coli* is.

Operon 2 could consist of 11 genes or ORFs. The complementation experiments with *S. typhimurium* (Table 6) clearly identify the role of some of these genes (*rfaG*, *rfaP*, *rfaB*, *rfaI*, and *rfaJ*) in LPS biosynthesis; however, there are still more genes of unknown function. Genes 5 (*rfaK*), 6 (*rfaZ*), and 7 (*rfaY*) (see reference 34 for nomenclature) seem to be involved in the synthesis or modification of the last part of the core, since only resistance to phage U3 was observed with mutants lacking these genes (Table 2). It is, then, likely that the last gene, gene 5, is the *E. coli* equivalent of *Salmonella rfaK* (20). Genes 14 (*rfaQ*) and 15 are expected to be involved in the modification of the backbone core rather than in its synthesis, since neither gene is absolutely required for the synthesis of a complete core (Tables 3 and 4), but both are required to confer novobiocin resistance. Mutations in gene 12 (Tables 3 and 4), *rfaP*, which is known to be involved in the addition of phosphate to the core (13), yield similar results. Although our results show that the activities of these three genes are not required for comple-

TABLE 6. Complementation[a] of *S. typhimurium* LPS mutants

| Plasmid | Presence of complementation with[b]: | | | | | | |
|---|---|---|---|---|---|---|---|
| | *rfaD* | *rfaF* | *rfaG* | *rfaP* | *rfaB* | *rfaI* | *rfaJ* |
| pCR0131 | | | + | + | + | + | + |
| pCR0128 | | | + | + | − | − | − |
| pCR0111 | | | + | + | − | ND[c] | ND |
| pCR0112 | | | + | − | − | ND | ND |
| pCR06Φ30 | | | ND | ND | − | + | + |
| pCR0131Φ5 | | | ND | ND | ND | − | + |
| pCR06Φ32 | | | + | ND | + | + | − |
| pCR09 | − | + | | | | | |
| pCR09Φ138 | + | ND | | | | | |
| pCR09Φ137 | + | ND | | | | | |
| pCR09Φ119 | + | − | | | | | |
| pCR09Φ132 | − | + | | | | | |

[a] Plasmids were transformed into the different *S. typhimurium* strains, and the transformants were checked for complementation of the "rough" phenotype by using LPS-specific phages (see Materials and Methods).

[b] On the basis of the physical map presented in Fig. 4, *E. coli* gene assignment is as follows: *rfaD*, gene 1; *rfaF*, gene 2; *rfaG*, gene 13; *rfaP*, gene 12; *rfaB*, gene 10; *rfaI*, gene 9; *rfaJ*, gene 8.

[c] ND, not determined.

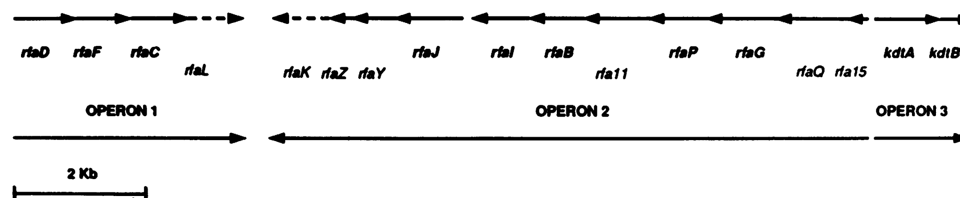


FIG. 6. Organization of the *rfa* cluster in *E. coli* K-12. The region covers most of the *Eco*RI-*Bam*HI fragment presented in Fig. 2, with the *Eco*RI site positioned to the left of the figure. The genes have been named by using the guidelines proposed previously (34). All the genes in boldface have assigned functions as defined in Table 6; other genes have been preliminarily assigned by analogy with the *S. typhimurium* genes. The genes designated with numbers are of unknown function.

tion of the backbone structure, synthesis of complete molecules of LPS could be only partial in the complementation experiments if the other transferases work under suboptimal conditions. A partial synthesis of complete LPS might explain the Mu and novobiocin sensitivity observed in these strains (Tables 3 and 4). The conclusions presented here clearly agree with the physical map of the *rfa* region recently reported (34); however, that report postulated a complex promoter region instead of the *rfa15* gene reported here. The fact that we have found two different hybrid proteins in this region (Φ33 and Φ40) and the fact that the levels of expression of Φ33 are similar to those of other fusions (Table 5) favor the existence of this necessarily short gene.

Gene 16 (*kdtA*) in operon 3 has been recently shown (8) to encode the enzyme KDO-transferase, responsible for the transfer of KDO molecules to the lipid A backbone (8). Immediately downstream of this gene, there is another ORF of unknown function (8). Our results show that an Ω insertion in the *kdtA* gene is polar into the next gene, indicating that both form part of the same transcriptional unit. All our attempts to inactivate any of these genes in the chromosome have been unsuccessful, confirming the essentiality of these genes as expected from their function (8).

The synthesis of the *E. coli* K-12 LPS core is mostly directed at the level of these operons, although the participation of other genes cannot be ruled out so far. The recent suggestion (1) about the existence of fully functional internal promoters in operon 2 is in clear disagreement with the conclusions presented here. The presence of internal promoters cannot be totally ruled out, since a small level of β-galactosidase (1 to 2 U) activity is still present even under "repression" (Ω insertions) conditions (Table 5). However, the low strength of these hypothetical internal promoters argues against any biological significance. A likely explanation for the differences between these results resides in the use of different insertions in the chromosome, mini-Tn*10* insertions (1) instead of Ω or mini-Mu in our case. Mini-Tn*10* insertions have been shown in other cases to have only partial polarity (7), which could lead to misleading interpretations. Indeed, mini-Tn*10* inserted upstream of any fusion in the operon only reduced its activity four- to sixfold (data not shown) compared with the 21- to 40-fold reduction observed with Ω insertions. Our conclusions are in clear agreement with the results recently reported for *S. typhimurium* (3).

All the results presented indicate that, while the Mu-dII1734 insertions in the chromosome are polar, they do not show polarity in high-copy-number plasmids. This effect is likely due to the amplification effect of very weak promoter sequences in high-copy plasmids; however, we don't know yet where these sequences are, since they can be in either the vector, the mini-Mu element, or the *rfa* region. These results are observed because of the absence of specific terminator sequences between the mini-Mu elements.

Although the organization of these two operons is well supported by the results presented, there are some questions that remain unanswered. We have been unable to assign a size to gene 9 (*rfaI*), although the phenotype of some new insertions (data not shown) suggests a protein of 22 to 30 kDa. The function of gene 11 is still unclear; this gene seems to be dispensable, since no detectable phenotype has been observed. However, the only fusion obtained is in the carboxy-terminal region of the gene, a fusion that could not lead to inactivation of the original protein. Additionally, there is a region of 1.2 kb, between operons 1 and 2, which is not covered by insertions and part of which is likely covered by genes 4 and 5. If we assign an average size to these genes, the gap between them is reduced to 0.5 kb, a space large enough for a small gene or, more likely, a regulatory signal such as a terminator. The presence of a terminator in this region would explain the lack of insertions in this region and the unusual orientation of the very weak 118 fusion. This fusion should be part of operon 1, but its phenotypic characterization (data not shown) indicates that it is really in gene 5; low levels of transcription through the terminator would perfectly explain the generation of this weak fusion. Additionally, a terminator between two operons in different orientations is likely to exist, and preliminary results with *S. typhimurium* (20) suggest the presence of a terminator sequence in the same region.

From the results presented here and reported elsewhere (2, 33, 34), we can conclude that the position of the *rfa* genes in *E. coli* is similar to that in *S. typhimurium*. It is very likely, then, that the *Salmonella* genes are, as in *E. coli*, arranged in single operons. Additionally, the results presented in Table 6 clearly indicate the complementation of *S. typhimurium* mutants (*rfaG*, *P*, *B*, *I*, *J*, *F*, and *D*) by their homologous genes from *E. coli* K-12. This complementation is specific for each gene (Table 6). These results are rather surprising, because *E. coli* K-12 and *S. typhimurium* have different LPS terminal structures so far thought to be due to different enzymatic activities (9, 10). The picture emerging now is that the *rfa* genes are very similar in *E. coli* K-12 and *S. typhimurium* (reference 34 and this work), although some divergences are still reported (4). It is likely, then, that the differences in LPS structure arise from minor changes affecting the specificity of the *rfaI* and *rfaJ* gene products; some of these changes, however, could also be due to other factors, such as differences in the availability of donor and/or acceptor molecules. The answer may have to wait until the biochemistry of this region can be worked out.

How are these genes regulated? The levels of β-galactosidase detected suggest that their expression is moderate to low and that all the genes are expressed at similar levels,

since no significant differences were observed. The regulation of operons 1 and 2 seems to be uncoupled, since insertions in either of them did not alter the expression of the other (Table 5). Additionally, no feedback regulation was observed, since insertions downstream of a specific fusion did not affect the levels of its β-galactosidase activities (Table 5). Finally, the expression of these genes is not under catabolic repression or carbon source regulation (data not shown), suggesting that these genes belong to the bacterial set of housekeeping genes. In agreement with this idea is the result that the expression of these genes is slightly activated by increases in growth rate (either temperature or medium dependent).

Recently, it has been shown that the expression of operon 2 is regulated by a mechanism of *rho*-dependent antitermination through the product of gene *rfaH* (11, 26); this type of regulation would suggest a possible function for the product of gene *rfa15*, which could be similar to the leader peptide of different bacterial operons (reviewed in reference 19). The use of the fusions described in this paper could help to clarify the specific mechanism of this regulation. In addition, the biological significance of the heat-shock regulation of the *rfaD* gene (28) remains unclear, since no significant differences in the expression of this gene, measured as levels of β-galactosidase activity in fusion 132, have been observed at 42°C (Table 5) or even higher temperatures (data not shown).

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