JOURNAL OF BACTERIOLOGY, Oct. 1991, p. 6428–6431
0021-9193/91/206428-04$02.00/0

Vol. 173, No. 20

Effect of *rfaH* (*sfrB*) and Temperature on Expression of *rfa* Genes of *Escherichia coli* K-12

ELIZABETH PRADEL AND CARL A. SCHNAITMAN*

Department of Microbiology, Arizona State University, Tempe, Arizona 85287-2701

Received 7 June 1991/Accepted 3 August 1991

In order to study the regulation of a large block of contiguous genes at the *rfa* locus of *Escherichia coli* K-12 which are involved in synthesis and modification of the lipopolysaccharide core, the transposon Tn*lacZ* was used to generate in-frame *lacZ* fusions to the coding regions of five genes (*rfaQ*, *-G*, *-P*, *-B* and *-J*) within this block. The β-galactosidase activity of strains in which these fusions had been crossed into the chromosomal *rfa* locus was significantly decreased when the *rfaH11* (*sfrB11*) allele was introduced and was restored to wild-type levels when these strains were lysogenized with a λ phage carrying wild-type *rfaH*. This indicates that the positive regulatory function encoded by *rfaH* is required throughout this block of genes. In addition, expression of the *lacZ* fusion to *rfaJ* was reduced by growth at 42°C, and this correlated with a temperature-induced change in the electrophoretic profile of the core lipopolysaccharide.

The major *rfa* locus at 81 min of the *Escherichia coli* chromosome (3) is a cluster of 16 or 17 genes (19) involved in the synthesis of the lipopolysaccharide (LPS) core. This includes a series of 10 contiguous genes, beginning with *rfaQ*, which are transcribed in the leftward (counterclockwise) direction in reference to the linkage map of *E. coli* K-12 (3). Studies of insertion mutations indicate that many if not all of these 10 genes are organized into a complex operon which may also contain internal promoters or regulatory elements (1). This block of genes includes *rfaG*, *-B*, *-I*, and *-J*, which encode glycosyl transferases involved in synthesis of the hexose region of the core; *rfaP* and *-K*, which are involved in modification or decoration of the core; and at least four additional genes (*rfaQ*, *-Y*, *-Z*, and a 38-kDa open reading frame between *rfaP* and *-B*) whose functions are unknown (19). The order of these genes is *rfaQGP*(38 kDa)*BIJYZK*, and a portion of this region is shown in Fig. 1. Genes which are part of the *rfa* cluster but which lie outside of this block include genes *rfaC*, *-D*, and *-F*, which are involved in synthesis of the heptose region of the inner core; *rfaL*, which is required for attachment of O antigen; *kdtA*, which is involved in attachment of the first two 2-keto-3-deoxyoctulosonic acid residues to lipid A; and one or two genes of unknown function.

The gene *rfaH* (formerly *sfrB* in *E. coli*), located at 87 min on the *E. coli* map outside of the major *rfa* locus (3), encodes a positive regulator of genes in this block. Beutin and Achtman (4) first described *sfrB* mutants of *E. coli* as being defective in the function of the *tra* genes of F and altered in sensitivity to LPS-specific phages. A subsequent study by Beutin et al. (5) indicated that the *sfrB* product functioned as an antiterminator in the *tra* operon. *Salmonella typhimurium rfaH* mutants were first detected as rough mutants producing a type Rc LPS core (lacking all hexoses except for the first glucose residue), although additional studies indicated the production of a more heterogeneous population of core structures (8). Sanderson and Stocker (17) showed that *rfaH* mutants were defective in expression of *tra* genes as well, indicating that *rfaH* and *sfrB* were identical, and this has been confirmed by cross-complementation studies with the cloned genes. The *sfrB* locus has been renamed *rfaH* in the most recent edition of the *E. coli* K-12 map (3). Creeger et al. (6) have presented evidence that *sfrB* affects the levels of both galactosyl transferase and glucosyltransferase II in extracts of *E. coli*. A broader role for *sfrB* as an antiterminator is suggested by the observation of Ralling and Linn (14) that the product of *sfrB* decreases termination frequency in the *rplL-rpoB* intercistronic region.

In the present study, we have examined the effect of *rfaH* (*sfrB*) on the expression of in-frame *lac* fusions within the coding regions of *rfa* genes in the contiguous leftward block. We have also examined the effect of temperature on these fusions, since preliminary results showed changes in gel profiles of LPS from strains grown at high temperature. An additional impetus to study the effect of temperature was the recent finding by Raina and Georgeopoulos (13) that *rfaD*, a gene involved in heptose biosynthesis, is a heat shock gene.

MATERIALS AND METHODS

Construction of *lac* fusions. Fusions were isolated in plasmid pGEM3, carrying a 9.0-kb *Bgl*II-*Sal*I *rfa* fragment (formerly designated rfa8.5) derived from Clarke-Carbon plasmid pLC10-7 (1), which is shown in Fig. 1. This plasmid was introduced into strain CC170 from C. Manoil, which carries a chromosomal Tn*lacZ*, and transposition of Tn*LacZ* to the plasmid was selected by high-level kanamycin resistance. A pool of plasmid DNA from such colonies was used to transform a Lac$^-$ strain, and blue colonies were selected on an LB plate with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) containing kanamycin. These procedures were carried out as described by Manoil (9). Plasmids were isolated from these transformants, and the sites of the insertions were determined by restriction mapping. Those which were in regions of interest were stabilized by removing the transposase by digestion with *Xho*I and religated. The precise fusion joint was determined by sequencing from the −40 primer hybridized to the beginning of the *lacZ* gene. Approximately half of the fusions turned out to be in frame within the coding region of *rfa* genes.

To introduce the fusions into the *rfa* region of the *E. coli* K-12 chromosome, the plasmids were linearized by cleavage with *Sca*I, which cuts in the ampicillin resistance gene, and

* Corresponding author.

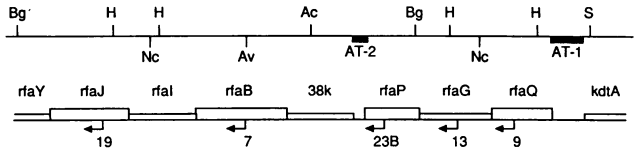

FIG. 1. Sites of insertion of in-frame *lacZ* fusions. The top portion shows a partial restriction map of the *rfa* region. The bars marked AT-1 and AT-2 indicate very AT-rich noncoding regions which could contain promoters or regulatory elements. The lower portion shows blocks indicating the sizes of the coding regions of the *rfa* genes (11, 19) and arrows indicating the insertion sites of the fusions. All of the genes shown are transcribed leftward except *kdtA*. Restriction sites: Bg, *Bgl*II; H, *Hin*dIII; Nc, *Nco*I; Av, *Ava*I; S, *Sal*I. 38k, 38-kDa protein coding region.

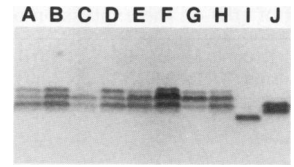

FIG. 2. Silver-stained tricine-SDS gel showing the effect of temperature and of mutations in *rfaI* and *-J* on core LPS. Lanes A, B, C, and D show the *rfa*$^+$ VJS803 background strain, and lanes E, F, G, and H show the *rfa*$^+$ Δ*lac* CS180 background strain. Lanes A, D, E, and H are strains grown at 30°C; lanes B, F, I, and J are strains grown at 37°C; and lanes C and G are strains grown at 42°C. Lanes I and J are an out-of-frame insertion of Tn*lacZ* into *rfaI* and in-frame fusion 19 to *rfaJ*, respectively.

transformed as described by Winans et al. (20) into the *recB recC sbcB* strain VJS803 from V. Stewart, which is like JC7623 (20) except it is Δ(*argF-lac*)*U169* Δ(*trpEA*)*2*. Kanamycin-resistant transformants were screened for sensitivity to ampicillin, LPS phenotype by polyacrylamide gel electrophoresis, phage and novobiocin sensitivity (1), and blue color on LB-X-Gal plates. The insertions into *rfaB* and *rfaJ* were additionally transduced by P1 into a Δ*lac* derivative of CS180 (1), while the insertions into *rfaP* and *-G*, which had a deep rough phenotype, and *rfaQ*, which had a partial deep rough phenotype and were difficult to transduce, were analyzed in the VJS803 background.

Construction of an *sfrB*$^+$ transducing phage. The *sfrB*-containing *Bam*HI-*Sal*I fragment from plasmid pKZ17 (15) from K. Sanderson was inserted into *Bam*HI-*Sal*I-cleaved pGEM4 (Promega, Inc., Madison, Wis.). Into the *Bam*HI site of the resulting plasmid was inserted a spectinomycin-streptomycin Ω resistance cassette from pHP45Ω (12). The *Sal*I site of this plasmid was cleaved, filled in, and converted to an *Eco*RI site by insertion of a linker. An *Eco*RI fragment from the resulting plasmid, carrying both the Ω cassette and *sfrB*, was ligated into *Eco*RI-cleaved λgt4.0 DNA, the resulting DNA was transformed into *E. coli*, and lysogens carrying the recombinant *sfrB*$^+$ phage were selected by spectinomycin resistance and screened for ability to complement the *sfrB11* allele. The resulting phage was designated λSfrB$^+$. The streptomycin or spectinomycin resistance phenotype of this phage is useful for the selection of lysogens in different strain backgrounds.

Construction of *sfrB11* strains. To facilitate moving the *sfrB11* allele, the *sfrB11* strain M1170 from L. Rothfield was transduced to tetracycline resistance (Tet^r) with P1 on RK4919 (from V. Stewart), which carries Tn*10* 98% linked to *chlB*. Phage P1 grown on the resulting strain, CS1856, transduced *sfrB11* linked 50 to 60% to Tet^r. Since *sfrB11* strains are resistant to U3 and sensitive to novobiocin, transductants into strains carrying fusions in *rfaQ*, *rfaB*, and *rfaJ*, which are sensitive to U3 and resistant to novobiocin, could be scored for coinheritance of *sfrB11*. Strains with insertions into *rfaP* and *-G* are already resistant to U3 and sensitive to novobiocin, so these were scored for coinheritance by plating transductants on LB-tetracycline plates containing X-Gal. These plates gave a mixture of blue and light blue colonies, and the light blue colonies were assumed to have coinherited the *sfrB11* allele. This was confirmed by complementation of the light blue phenotype by lysogenization with λSfrB$^+$.

Biochemical assays. For measurement of expression of *lacZ* fusions, cultures were grown to mid-log phase (ca. 1.0 OD_{600}) in LB at the temperatures specified in the text, and β-galactosidase activity was determined exactly as described by Miller (10). LPS gels of protease-digested outer membrane fractions were analyzed in a tricine-sodium dodecyl sulfate (SDS) buffer system (7). The separating gel contained 18% acrylamide, 0.36% bisacrylamide, 0.1% SDS, and 10% glycerol in 1 M Tris adjusted to pH 8.5 with HCl, and the stacking gel contained 0.3% SDS, 4.5% acrylamide, and 0.08% bisacrylamide in 0.75 M Tris (pH 8.5) with HCl. The top buffer was 0.1 M Tris–0.1 M tricine–0.1% SDS, pH 8.25, and the bottom buffer was 0.2 M Tris adjusted to pH 8.9 with HCl. The sample buffer, sample preparation, and silver staining were as described previously (1).

RESULTS AND DISCUSSION

The sites of insertion of five in-frame Tn*lacZ* fusions are shown in Fig. 1. All of these fusions gave appropriate LPS phenotypes after they were crossed into the chromosome (gel data are shown in Fig. 2 for fusions in *rfaI* and *rfaJ*). Fusion 9 to *rfaQ* remained U3 sensitive and novobiocin resistant, and on gels its LPS gave multiple bands, the fastest migrating of which comigrated with type Rd_1, produced by an *rfaG* mutant. We have observed a similar gel phenotype with mini-Tn*10* insertions 2a and 4a (1), which lie within *rfaQ* on either side of the fusion joint of fusion 9, and we attribute this phenotype to incomplete polarity. The function of *rfaQ* is not known, but its loss does not result in a deep rough phenotype (11). Fusions 13 and 23B to *rfaG* and *rfaP* exhibited a deep rough phenotype, including sensitivity to novobiocin and resistance to phage U3, and these fusions produced LPS which comigrated with that of Tn*10* insertions 2 and 8a (1), which have *rfaG* and *rfaP* phenotypes, and with the LPS of other authentic *rfaG* and *rfaP* mutants which we have recently constructed. The strain with fusion 7 to *rfaB* was U3 sensitive and novobiocin resistant and produced LPS which comigrated with the major band (type Rc) produced by a strain deleted for the *gal* operon (18). The several faint bands migrating more slowly than type Rc seen in LPS from the *gal* deletion strain were even fainter in LPS from fusion 7, presumably because the fusion is polar on *rfaI* and *-J*. Fusion 19 to *rfaJ* was also sensitive to U3 and resistant to novobiocin and produced two bands on gels (Fig. 2), the faster of which migrated more slowly than type Rc LPS or the single band produced by a strain with an out-of-frame insertion in *rfaI*.

The effect of *sfrB11* on the activity of β-galactosidase from these fusions is shown in Table 1. The expression of β-galactosidase from all of the fusions was reduced significantly by *sfrB11*, the smallest reduction being seen with the fusion

TABLE 1. Effect of *sfrB* on the expression of *rfa-lacZ* fusions

Fusion	*sfrB* allele on chromosome	Lysogenic for λSfrB+	SfrB phenotype	β-Galactosidase activity[a] (U)
9 (*rfaQ*)	+	−	+	18.2
	−	−	−	5.2
	−	+	+	16.5
13 (*rfaG*)	+	−	+	14.3
	−	−	−	3.4
	−	+	+	13.6
23B (*rfaP*)	+	−	+	8.6
	−	−	−	1.9
	−	+	+	6.7
7 (*rfaB*)	+	−	+	18.1
	−	−	−	1.0
	−	+	+	15.5
19 (*rfaJ*)	+	−	+	17.8
	−	−	−	0.4
	−	+	+	19.3

[a] Units are as described by Miller (10). Results are averages of duplicates. Strains were grown at 30°C in LB.

TABLE 2. Effect of temperature on the expression of *rfa-lacZ* fusions

Strain	β-Galactosidase activity[a] (U)		
	30°C	37°C	42°C
Controls			
VJS803	0.5	0.8	0.8
CS180 Δ*lac*	0.4	0.2	0
Chromosomal fusions			
9 (*rfaQ*)	21.6	18.0	22.0
13 (*rfaG*)	13.5	11.9	15.7
23B (*rfaP*)	9.0	8.1	5.2
7 (*rfaB*)	18.6	20.9	18.3
19 (*rfaJ*)	23.7	15.7	4.0
Multicopy plasmid fusions			
19 (*rfaJ*)	528	692	531
22B (*rfaJ*)[b]	474	683	469

[a] Units are as defined in Table 1, footnote *a*.
[b] The fusion joint of 22B is in frame, one codon 5′ to fusion 19.

to *rfaQ* and the greatest with the fusions to *rfaB* and *-J*. This suggests that the function of RfaH (SfrB) protein is necessary for expression of genes throughout this block. The data are consistent with the proposed action of RfaH protein as an antiterminator, but since only protein fusions were examined, effects on translation of the proteins cannot be ruled out.

If it is assumed that the primary effect is on transcription, these results could be explained either by the loading of RfaH protein at a single *cis*-acting site and action at multiple downstream termination sites or by loading and action both occurring at multiple sites. The genes within this block are arranged roughly in the order in which they act in completing the core oligosaccharide (8, 19). Thus, regardless of the mechanism, the multiple sites of action of RfaH protein would have an additive effect on functions encoded by the more distal genes, since there would be not only a reduction in expression of the distal gene functions but also a progressive decrease in the availability of suitable acceptor LPS molecules. It is probably this which accounts for the heterogeneity of *rfaH* LPS (8).

Preliminary experiments showed no effect of *sfrB11* on alkaline phosphatase expression from a chromosomal Tn*phoA* fusion to a transmembrane region of the protein encoded by *rfaL*, a gene which lies outside of the 10-gene block and is transcribed in the opposite direction (19). This indicates that a requirement for RfaH protein is not a general property of genes at the *rfa* locus.

Table 2 shows the effect of growth temperature on the expression of β-galactosidase from these fusions. The fusions to *rfaQ*, *-G*, and *-B* showed no change or a slight increase with increasing growth temperature. In contrast, the fusion to *rfaJ* showed a small reduction in activity at 37°C and a decrease of more than 80% at 42°C. This decrease in expression at 42°C was not observed when *rfaJ* fusion 19 and a slightly shorter *rfaJ* fusion were present in multicopy plasmids instead of in single copy on the chromosome. This suggests that the copy number or chromosomal context of the fusion was important and indicates that the reduced activity at 42°C was not due to temperature sensitivity of the hybrid fusion protein.

A smaller decrease at 42°C was seen with the fusion to *rfaP*. Since the expression of this fusion was considerably lower than that observed with the other fusions, we examined the expression of two other chromosomal *rfaP* fusions which were located at the 5′ and 3′ ends of the gene and flanked fusion 23B. These also showed a modest decrease in expression at 42°C and low activity (data not shown). The low activity of the *rfaP* fusions may reflect less efficient translation of RfaP protein and may be due to the fact that the 3′ end of the *rfaG* coding region overlaps the first two codons of *rfaP* (11).

Figure 2 shows the effect of temperature on the gel profile of LPS. The effects are shown for both *rfa*+ background strains used in this study, because we have observed that different *E. coli* K-12 strains show very reproducible differences both in the number of bands seen on tricine-buffered gels and in the relative intensity of these bands. This kind of difference is shown in Fig. 2. Both background strains showed three major LPS bands, but at 30°C these were roughly equal in the VJS803 background (lane D), while the middle band was more intense in the CS180 background (lane H). In both backgrounds, growth at 42°C resulted in a reduction in the lower band (lanes C and G), while in the VJS803 background there was also a significant reduction in the upper band (lane C).

The reason behind these strain differences is not known, but it is likely to be due at least in part to mutation of genes encoding synthesis or attachment of LPS substituents resulting from the extensive X-ray treatment of the early *E. coli* K-12 strains (2). An example is the *rfbD* mutation, which is found in many of the K-12 strains derived from the early strain Y-10 (2). The product of *rfbD* is involved in synthesis of rhamnose, a partial substituent of the 2-keto-3-deoxyoctulosonic acid region of *E. coli* K-12 LPS (16).

The *rfaJ* gene appears to lie at the first branch point involved in generating diversity of the LPS core. This is seen in lanes I and J of Fig. 2. Strains which lack *rfaI* or lack genes acting earlier in the core pathway, such as *rfaB*, *-G*, or *-C*, produce a single band of LPS, as seen in lane I of Fig. 2, while the LPS produced by an *rfaJ* mutant migrated as a doublet (lane J). Strains with mutations in genes distal to *rfaJ* produce LPS which is even more complex on gels (data not

shown). Thus, the *rfaJ* gene would appear to be an appropriate point at which to differentially regulate the LPS pathway.

ACKNOWLEDGMENTS

We thank K. Sanderson and S. Carson for supplying *sfrB* plasmids and sequence and for helpful discussion, and we are grateful to C. Manoil for the gift of Tn*lacZ*.

This research was supported by grants DMB-8820633 from the National Science Foundation and GM-39087 from the National Institutes of Health.

REFERENCES

1. **Austin, E. A., J. F. Graves, L. A. Hite, C. T. Parker, and C. A. Schnaitman.** 1990. Genetic analysis of lipopolysaccharide core biosynthesis by *Escherichia coli* K-12: insertion mutagenesis of the *rfa* locus. J. Bacteriol. **172**:5312–5325.
2. **Bachmann, B. J.** 1987. Derivations and genotypes of some mutant derivatives of *Escherichia coli* K-12, p. 1190–1219. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaecter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology, vol. 2. American Society for Microbiology, Washington, D.C.
3. **Bachmann, B. J.** 1990. Linkage map of *Escherichia coli* K-12, edition 8. Microbiol. Rev. **54**:130–197.
4. **Beutin, L., and M. Achtman.** 1979. Two *Escherichia coli* chromosomal cistrons, *sfrA* and *sfrB*, which are needed for expression of F factor *tra* functions. J. Bacteriol. **139**:730–737.
5. **Beutin, L., P. A. Manning, M. Achtman, and N. Willetts.** 1981. *sfrA* and *sfrB* products of *Escherichia coli* K-12 are transcriptional control factors. J. Bacteriol. **145**:840–844.
6. **Creeger, E. S., T. Schulte, and L. I. Rothfield.** 1984. Regulation of membrane glycosyltransferases by the *sfrB* and *rfaH* genes of *Escherichia coli* and *Salmonella typhimurium*. J. Biol. Chem. **259**:3064–3069.
7. **Lesse, A. J., A. A. Campagnari, W. E. Bittner, and M. A. Apicella.** 1990. Increased resolution of lipopolysaccharides and lipooligosaccharides utilizing tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis. J. Immunol. Methods **126**: 109–117.
8. **Makela, P. H., and B. A. D. Stocker.** 1984. Genetics of lipopolysaccharide, p. 59–137. *In* E. T. Rietschel (ed.), Handbook of endotoxin, vol. 1: chemistry of endotoxin. Elsevier, Amsterdam.
9. **Manoil, C.** 1990. Analysis of protein localization by use of gene fusions with complementary properties. J. Bacteriol. **172**:1035–1042.
10. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
11. **Parker, C., E. Pradel, N. Pearson, and C. Schnaitman.** Unpublished data.
12. **Prentki, P., and H. M. Krisch.** 1984. In vitro mutagenesis with a selectable DNA fragment. Gene **29**:303–313.
13. **Raina, S., and C. P. Georgopoulos (University of Utah Medical Center, Salt Lake City).** Personal communication.
14. **Ralling, G., and T. Linn.** 1987. Evidence that Rho and NusA are involved in termination in the *rplL-rpoB* intercistronic region. J. Bacteriol. **169**:2277–2280.
15. **Rehemtulla, A., S. K. Kadam, and K. E. Sanderson.** 1986. Cloning and analysis of the *sfrB* (sex factor repression) gene of *Escherichia coli* K-12. J. Bacteriol. **166**:651–657.
16. **Rick, P. D.** 1987. Lipopolysaccharide biosynthesis, p. 648–662. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaecter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology, vol. 1. American Society for Microbiology, Washington, D.C.
17. **Sanderson, K. E., and B. A. Stocker.** 1981. Gene *rfaH*, which affects lipopolysaccharide core structure in *Salmonella typhimurium*, is required also for expression of F-factor functions. J. Bacteriol. **146**:535–541.
18. **Schnaitman, C. A., and E. A. Austin.** 1990. Efficient incorporation of galactose into lipopolysaccharide by *Escherichia coli* K-12 strains with polar *galE* mutations. J. Bacteriol. **172**:5511–5513.
19. **Schnaitman, C. A., C. Parker, J. Klena, E. Pradel, N. Pearson, K. Sanderson, and P. R. MacLachlan.** Physical map of the *rfa* locus of *Escherichia coli* K-12 and *Salmonella typhimurium*. J. Bacteriol., in press.
20. **Winans, S. C., S. J. Elledge, J. H. Krueger, and G. C. Walker.** 1985. Site-directed insertion and deletion mutagenesis with cloned fragments in *Escherichia coli*. J. Bacteriol. **161**:1219–1221.