

## Gene *rfaH*, Which Affects Lipopolysaccharide Core Structure in *Salmonella typhimurium*, Is Required also for Expression of F-Factor Functions

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Mutations in gene *rfaH* of *Salmonella typhimurium* at 84 units on the linkage map make lipopolysaccharide of chemotypes Ra, Rb<sub>2</sub>, Rb<sub>3</sub>, and Rc (A. A. Lindberg and C. G. Hellerqvist, J. Gen. Microbiol. 116:25-32, 1980). F-factor expression in RfaH<sup>-</sup> strains was reduced in the following properties when compared with RfaH<sup>+</sup> strains: transfer of *Flac*, number of phage f2 infective centers, lysis by and propagation of phages f2 and M13, proportion of cells with visible F-pili, and formation of mating aggregates with F<sup>-</sup> cells. Inhibition of multiplication of Br60, a female-specific phage, was not reduced in RfaH<sup>-</sup> *Flac* strains. Plasmid transfer from RfaH<sup>-</sup> strains was reduced for Inc groups FI, FII, and T, unaffected for Inc groups β, Ia, L, N, P, and W, and increased for Inc group M when compared with plasmid transfer from RfaH<sup>+</sup> strains. Reduced F-factor function in RfaH<sup>-</sup> strains was not due to defective lipopolysaccharide since strains with mutations in other *rfa* genes were unaffected in plasmid transfer. Gene *rfaH* appears to be homologous with gene *sfrB* in *Escherichia coli* K-12, which maps at the same location, influences F-factor function, and affects synthesis of lipopolysaccharide. The gene product of *sfrB* has been proposed to be a transcription antiterminator.

The F-factor of *Escherichia coli* K-12 causes cells to act as effective donors but poor recipients in conjugation. Many *tra* cistrons in the plasmid genome are needed for these functions, which result in production of F-pili, formation of mating aggregates, and transfer of DNA (3, 7). Recently, several genes on the *E. coli* K-12 chromosome which influence F-factor function have been detected: *sfrA* (5) linked to *thr* at 0 min on the bacterial chromosome (4), perhaps identical to *fex* (16); *sfrB* at 85 min (5) linked to *metE*; *dnaB* at 91 min (14, 23); *cpxA* and *cpxB* (19) linked to *metB* at 88 min and *fabD* at 25 min, respectively; and class III ampicillin-resistant mutants (20).

The close linkage of *sfrB* to *metE* and the alterations in sensitivity of *sfrB* mutants to bacteriophages which adsorb to lipopolysaccharide (LPS) (5) suggested to us that *sfrB* of *E. coli* might be the same gene as *rfaH* of *Salmonella typhimurium* (17, 30). The class of mutants termed *rfaH* was originally defined from two mutants which show the same phage sensitivities as *galE* mutants, which lack UDPgalactose epimerase activity and thus make LPS of chemotype Rc (Fig. 1). However, *rfaH* mutants are Gal<sup>+</sup>, i.e., able to ferment galactose (33). Osborn

(21) found that extracts of these two mutants did not catalyze the transfer of labeled galactose from UDPgalactose to C-3 of glucose I of type Rc (galactose-deficient) LPS, and that, correspondingly, their LPS would accept galactose from UDPgalactose when incubated with extracts of *rfa*<sup>+</sup> bacteria. Therefore, it was assumed that the *rfaH* gene product was UDPgalactose (glycosyl) lipopolysaccharide α1,3-galactosyl transferase (trivial name, α1,3-galactosyl transferase) (10), which catalyzes the transferase reaction which forms the main-chain galactose unit, galactose I, of the LPS core. Five mutants of this phenotype were known; the mutant genes of four were closely linked to *metE* and called *rfaH*. One mutant gene, *rfa-658*, was shown not to be closely linked to *metE*, but it was otherwise unmapped (30).

In this report we show that the four *rfaH* mutants, like *sfrB*, are deficient in expression of F-factor functions. Strains carrying mutations in *rfaC*, *rfaF*, and *rfaG*, which produce defects in the inner core region of LPS, and *rfaJ* and *rfaL*, which produce defects in the outer core region of LPS (29), are not deficient in these functions.

### MATERIALS AND METHODS

**Bacterial strains and phage.** The strains used are listed in Table 1. The *rfaH* strains used, SL1277,

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night unshaken broth cultures of the donor and the recipient strains grown in CM67 broth in 13-mm tubes were back-diluted 1/20 into tubes of broth, and grown unshaken for about 3 h to about  $3 \times 10^8$  cells per ml. A 0.1-ml amount of the donor and 2.0 ml of the recipient were mixed in a 125-ml Erlenmeyer flask; after 15 min of mating in unshaken culture 7.9 ml of prewarmed broth was added. After another 45 min in unshaken culture, the cells were diluted in broth, and 0.1-ml amounts were added to 2.5 ml of saline-soft agar (0.85% NaCl plus 0.7% agar) and overlaid on plates of selective medium. At the start of mating, the donor cells were diluted and plated on MacConkey lactose agar to permit determination of total and Lac<sup>+</sup> donor cells.

**Testing for infective centers (modified from reference 11).** At the time the mating was initiated, 1.0 ml of the donor strain was centrifuged and resuspended in LCa broth; the suspension was then placed in a tube at 37°C, and 0.1 ml of f2 phage at a titer of  $5 \times 10^{10}$  PFU/ml was added to give a multiplicity of infection of about 20; after 10 min of adsorption, anti-f2 serum was added in an amount which reduced the titer of phage by  $10^4$  in 10 min. Twenty minutes after starting the test, the cells were placed on a Millipore filter; 10 ml of LCa broth was added; the cells were sucked almost to dryness, and then washed through the filter with two more batches of 10 ml of the same broth. The filter was then transferred to 10 ml of broth in a tube, and the cells were resuspended by vigorous shaking. The released bacterial suspension was then immediately assayed for infective centers by plating in a soft agar layer on LCa agar with an indicator strain, usually SA2197. The total time from infection of the cells to plating was not more than 25 min.

**Electron microscopy.** Bacterial cells were grown to mid-log phase in CM67 broth, mixed with f2 bacteriophage at a multiplicity of infection of 100, incubated for 5 min, and centrifuged. The supernatant fluid was then poured off, and the cell pellet was suspended in 10 mM Tris-hydrochloride, pH 7.6. The cell suspension was placed on a carbon-coated electron microscope grid, stained with uranyl acetate according to Kaiser (15), and then observed in a Phillips electron microscope.

**Microscopic observation of mating aggregates.** Mixtures of 1 ml of each of the donor and the recipient cells were made in 13-mm tubes at the start of matings, and kept at 37°C without agitation for 15 to 30 min. Then they were placed on a microscope slide and observed under a cover slip at  $\times 400$  to  $\times 800$  magnification in a phase-contrast microscope. Motility and cell aggregation were observed in the mating mixtures and in separate cultures of donor and recipient cells (1, 2, 22).

## RESULTS

**Preliminary tests of rfa<sup>+</sup>/Flac and rfaH/Flac strains.** Preliminary experiments were done with F128lac, but to collect consistent and adequate data, two modifications in the strains were required. First, the repression of the F-factor by MP10, the cryptic plasmid of *S. typhi-*

*murium* (27, 28), was overcome by using a de-repressed mutant, F42finP301 lac, originally isolated in *E. coli* K-12 (12, 13), which gave up to 100% of *S. typhimurium* cells with F-pili, high frequency plasmid transfer, and visible lysis by male-specific phages (unpublished data). Second, the plasmid transfer experiments gave higher transfer values when cells lacking O (somatic)-side chains on the LPS (rough mutants) were used as recipients (31; J. Walton and B. A. D. Stocker, unpublished data). In all experiments reported below, unless stated otherwise, the donor strain carried F42finP301 lac (hereafter called Flac), and the recipient was a rough mutant lacking the O-side chains on the LPS.

**Expression of F-factor in rfaH mutants and in other strains.** A group of strains, all of which carry Flac, was tested for F-factor functions (Table 2). Two rfaH<sup>+</sup> strains, SA2219 and SA2223, gave high frequency transfer of Flac (0.56 to 1.5 Lac<sup>+</sup> transconjugants per donor cell), visible lysis on solid medium by the male-specific phages f2 and M13, high frequency (about 1.0) of phage f2 infective centers after antibody addition to phage-bacteria mixtures, and supported multiplication of the male-specific phages f2 and M13 to  $3 \times 10^{10}$  to  $4 \times 10^{10}$  PFU/ml (Table 2). Three strains which are practically isogenic with the smooth strains except for their rfaH alleles gave low-frequency transfer efficiency ( $<0.01$  Lac<sup>+</sup> transconjugants per donor cell), were not visibly lysed by the male-specific phages, had the frequency of f2 infective centers in the presence of anti-f2 serum reduced to only 0.015 to 0.055 per cell, and supported multiplication to less than  $10^{10}$  PFU/ml of phages f2 and M13. Thus, all three rfaH mutants tested in detail gave much reduced transfer efficiency and sensitivity to male-specific phages. A fourth rfaH allele, strain SA2254 (SL1060 carrying Flac), was not lysed by the male-specific phage. Strains with mutations in rfaC, rfaF, rfaJ, rfaL, and rfaI genes, when carrying Flac, gave no detectable reduction in F-factor function.

Certain phages in *E. coli* are female specific in the sense that they adsorb to but they do not multiply normally in F<sup>+</sup> cells. Thus, *E. coli* K-12 *sfrB*<sup>+</sup> F<sup>+</sup> cells gave low efficiency of plating of  $\phi$ II compared with F<sup>-</sup> cells; some *sfrB* mutants in the presence of F-factor gave no reduction in efficiency of plating of  $\phi$ II, indicating that the functioning of F-factor was defective in those strains, whereas others behaved like *sfrB*<sup>+</sup> strains (5). Br60 is a female-specific phage (18) which lyses rough mutants, including rfaH strains (32). No plaques were observed when  $10^7$  PFU (previously titrated on F<sup>-</sup> strains) were tested on lawns of cells of rfaH<sup>+</sup>/Flac or rfaH/

TABLE 2. Phenotype of strains of *S. typhimurium* carrying *F42finP301 lac*

Strain	Lipopolysaccharide		Transfer efficiency <sup>a</sup>	Lysis with f2 and M13	f2 infective centers <sup>b</sup>	Titer of phage produced <sup>c</sup>	
	Genotype	Chemotype				f2	M13
SA2219	<i>rfa</i> <sup>+</sup>	smooth	1.5	+	1.1	3 × 10 <sup>10</sup>	3 × 10 <sup>10</sup>
SA2223	<i>rfa</i> <sup>+</sup>	smooth	0.56	+	0.84	3 × 10 <sup>10</sup>	4 × 10 <sup>10</sup>
SA2220	<i>rfaH481</i>	Rb <sub>3</sub>	0.0053	—	0.047	5 × 10 <sup>8</sup>	2 × 10 <sup>9</sup>
SA2221	<i>rfaH655</i>	Rb <sub>3</sub>	0.0053	—	0.055	2 × 10 <sup>9</sup>	1 × 10 <sup>9</sup>
SA2222	<i>rfaH659</i>	Rb <sub>3</sub>	0.0078	—	0.015	1 × 10 <sup>9</sup>	5 × 10 <sup>9</sup>
SA2254	<i>rfaH487</i>	Rb <sub>3</sub>	NT <sup>d</sup>	—			
SA2215	<i>rfaI432</i>	Rb <sub>3</sub>	1.90	+			
SA2216	<i>rfaL446</i>	Ra	0.42	+			
SA2217	<i>rfaJ417</i>	Rb <sub>2</sub>	0.87	+			
SA2233	<i>rfaJ971</i>	Rb <sub>2</sub>	0.90	+			
SA2218	<i>rfaF511</i>	Rd <sub>2</sub>	1.50	+			
SA2211	<i>rfaC630</i>	Re	NT	+			

<sup>a</sup> Ratio of number of Lac<sup>+</sup> transconjugants in a cross to SA2228 F<sup>−</sup> to the number of Lac<sup>+</sup> donor cells in the mating mixture.

<sup>b</sup> Number of f2 plaques formed per number of Lac<sup>+</sup> donor cells.

<sup>c</sup> A Wasserman tube with 4.0 ml of CM67 broth (for M13) or LCa broth (for f2) was inoculated with log-phase cells to a titer of 3 × 10<sup>6</sup> colony-forming units per ml and phage to a titer of about 10<sup>4</sup> PFU/ml and incubated overnight without shaking. Phage were then titrated in a soft agar overlay with SA2197 as indicator strain, using CM55 agar for M13 and LCa agar for f2.

<sup>d</sup> NT, Not tested.

*Flac*; thus, inhibition of this phage by F-factor is not detectably reduced in *rfaH* cells.

**F-pili on *rfa*<sup>+</sup>/*Flac* and *rfa*/*Flac* cells.** Cells were examined for F-pili by electron microscopy after adsorption of f2 phage and negative contrast with uranyl acetate. The proportions of cells with at least one visible F-pilus with bound f2 phage were as follows: SA2197 (*rfaH*<sup>+</sup>), 87 cells with F-pili of 106 cells observed (82%); SA2219 (*rfaH*<sup>+</sup>), 55 cells with F-pili of 57 cells observed (96%); SA2220 (*rfaH*), 17 cells with F-pili of 98 cells observed (17%). In addition to the reduced proportion of cells with F-pili, *rfaH* cells with pili very seldom had more than one pilus, whereas most of the *rfaH*<sup>+</sup> cells had more than one pilus.

**Mating aggregates.** Mixtures of F<sup>+</sup> or Hfr strains of *E. coli* K-12 with F<sup>−</sup> strains formed mating aggregates with most of the cells incorporated into these aggregates; not many of the cells were in pairs (1, 2). Mixtures of broth cultures of *S. typhimurium* LT2 *rfaH*<sup>+</sup>/*Flac* with SA2228 (*rfa*-971 F<sup>−</sup>) observed in the phase-contrast microscope after 15 to 30 min of mating also show a high proportion, 57 to 66% of cells, in mating aggregates (Table 3). Donors which were *rfaH*/*Flac* produced no detectable mating aggregates with F<sup>−</sup>; there were some small clumps of cells in these mixtures, but these were also observed in the rough donor cultures. The cells of strains SA2219 to SA2223 were smaller and less motile than those of the recipient strains, and thus mating aggregates involving *Flac* and F<sup>−</sup> cells were detectable. There were

no detectable large mating aggregates with *rfaH*/*Flac* strains among many cells observed. Obviously some aggregates must be formed since a low frequency (less than one Lac<sup>+</sup> recombinant per 100 donor cells) of *Flac* transfer was detected (Table 2).

**Effect of *rfaH* on transfer of plasmids of other Inc groups.** Plasmids representing several different Inc groups were transferred into SL1265 (*rfaH*<sup>+</sup>) and SL1277 (*rfaH481*), and these strains were used as donors in crosses to F<sup>−</sup> recipient strains (Table 4). Only plasmids of the Inc groups FI, FII, and T gave significantly more transfer from *rfaH*<sup>+</sup> than from *rfaH* strains. Representatives of several groups (β, Ia, L, N, P, and W) gave about the same transfer from *rfaH*<sup>+</sup> and *rfaH* strains; surprisingly, R446b (IncM) in duplicate experiments gave about 50 times as many recombinants in crosses of *rfaH* (rough) donors as in crosses of *rfaH*<sup>+</sup> (smooth) donors to the same recipient.

## DISCUSSION

Our observations show that the *rfaH* gene of *S. typhimurium* LT2, like the *sfrB* gene of *E. coli* K-12, is required for normal expression of the F plasmid. *S. typhimurium* cells with the normal allele *rfaH*<sup>+</sup> and carrying *FfinP301 lac*, have the following properties: high frequency plasmid transfer (1.0 Lac<sup>+</sup> transconjugant per donor cell in 60 min of mating); high frequency of f2-sensitive cells (indicated by antiserum-insensitive plaque-forming centers), visible lysis on agar by male-specific phages f2 and M13, and

TABLE 3. *Mating aggregates in mixtures of Flac and F<sup>-</sup> cells*

Donor strain <sup>a</sup>	Frequency of aggregate with SA2228 F <sup>-</sup> cells <sup>b</sup>							Estimated proportion of cells in aggregates <sup>c</sup>
	No. of cells per aggregate							
	1	2	3	4	5-10	11-20	>20	
SA2197 <i>rfaH</i> <sup>+</sup>	90	3	3	2	10	2	0	0.57
SA2219 <i>rfaH</i> <sup>+</sup>	50	5	1	1	6	1	0	0.59
SA2223 <i>rfaH</i> <sup>+</sup>	70	3	4	2	5	1	2	0.66
SA2220 <i>rfaH481</i>	>100	0	0	0	0	0	0	0.00
SA2221 <i>rfaH655</i>	>100	0	0	0	0	0	0	0.00
SA2222 <i>rfaH659</i>	>100	0	0	0	0	0	0	0.00

<sup>a</sup> The full genotype of the donor strains is given in Table 1. Each strain carried *FfinP301 lac*.

<sup>b</sup> Observation of cell aggregation was done according to the method described in the text.

<sup>c</sup> The estimated proportion of cells in aggregates was done by estimating that aggregates of 5 to 10 had 7 cells, 11 to 20 had 15 cells, and >20 cells had 30 cells.

TABLE 4. *Effect of the rfaH gene on transfer of plasmids of different Inc groups*

Donor plasmid <sup>a</sup>	Inc group	Donor marker selected	Frequency of transfer <sup>c</sup> for donor genotype <sup>d</sup>		Ratio of frequencies for <i>rfaH</i> <sup>+</sup> : <i>rfaH481</i>
			<i>rfaH</i> <sup>+</sup>	<i>rfaH481</i>	
<i>FfinP301 lac</i>	FI	Lac <sup>+</sup>	1.0	6 × 10 <sup>-3</sup>	166
R1	FII	Ap <sup>r</sup>	1.9 × 10 <sup>-2</sup>	4.6 × 10 <sup>-4</sup>	41
Rts1	T	Km <sup>r</sup>	8 × 10 <sup>-4</sup>	3.5 × 10 <sup>-5</sup>	23
R343	T		10 <sup>-2</sup>	10 <sup>-3</sup>	10
R16	β	Ap <sup>r</sup>	0.3	0.13	2.3
R64	Iα	Sm <sup>r</sup>	1.4 × 10 <sup>-2</sup>	3 × 10 <sup>-3</sup>	4.6
R471a	L	Ap <sup>r</sup>	3 × 10 <sup>-4</sup>	4 × 10 <sup>-4</sup>	0.75
pKM101	N	Ap <sup>r</sup>	10 <sup>-3</sup>	10 <sup>-3</sup>	1.0
RP4	P	Ap <sup>r</sup>	10 <sup>-3</sup>	10 <sup>-3</sup>	1.0
S-a	W	Sm <sup>r</sup>	5 × 10 <sup>-5</sup>	10 <sup>-4</sup>	0.5
R446b	M	Sm <sup>r</sup>	1.4 × 10 <sup>-4</sup>	8 × 10 <sup>-3</sup>	0.018

<sup>a</sup> The plasmids used were from the following sources: *F42finP301 lac*, from N. S. Willetts; R394 and R446b as PRC 128 and PRC 113, respectively, from E. M. Lederberg of the Plasmid Reference Center; all others were from D. Brunner.

<sup>b</sup> Selection of transconjugants was on minimal medium in which auxotrophic requirements of the recipients were supplemented. For Lac<sup>+</sup> recombinants, the carbon source was lactose. Minimal medium with glycerol was used for selection of antibiotic-resistant recombinants, with the following amounts of antibiotics (in micrograms per milliliter): ampicillin (Ap), 25; kanamycin (Km), 25; streptomycin sulfate (Sm), 100. The recipient strains used were SA2228, in which selection was for kanamycin or ampicillin resistance, or SL3749, in which selection was for streptomycin resistance.

<sup>c</sup> The tests for frequency of transfer were done as described in the text except that the mating mixture had 1.0 ml of donor cells, 2.0 ml of recipient cells, and 7.0 ml of broth, and L-broth was used instead of CM67 broth.

<sup>d</sup> The *rfaH*<sup>+</sup> donor strains were SL1265 carrying the plasmid; the *rfaH481* donor strains were SL1277 carrying the plasmid.

ability to support multiplication of these two phages in broth to titers of  $3 \times 10^{10}$  to  $4 \times 10^{10}$  PFU/ml, and a high proportion of cells (60 to 90%) with at least one F-pilus per cell, many of these cells having more than one pilus. Three independently isolated *rfaH* alleles were previously transferred by transduction to produce lines otherwise isogenic to an *rfaH*<sup>+</sup> strain (30), and these strains, carrying *Flac*, have the following properties: plasmid transfer reduced to  $10^{-2}$  to  $10^{-3}$  Lac<sup>+</sup> transconjugants per donor cell, much lower frequency of f2-sensitive cells, no visible lysis by male-specific phages, multiplication of f2 and M13 in broth to titers of only  $5 \times$

$10^8$  to  $5 \times 10^9$  PFU/ml, and only 17% of cells of *rfaH481/Flac* with F-pili, with most pilus-bearing cells having only one. Plasmid transfer, sensitivity to male-specific phages, and F-pili are all properties controlled by the *tra* operon on the F factor. This group of genes, at 62 to 94 kilobases on F, is transcribed as a unit (3). In all of these above tests, the phenotype of *rfaH* mutants of *S. typhimurium* resembled that of *sfrB* mutants of *E. coli* K-12. However, the *sfrB* mutants reduced inhibition of sensitivity to female-specific phage φII by the F factor (5), whereas no reduction in inhibition of sensitivity to female-specific phage Br60 by F was detected in *rfaH*

mutants. The reason for this difference is unknown.

The following data suggested that *rfaH* could be the structural gene for galactosyl-1 transferase: the phage sensitivity which resembles that of *galE* mutants despite the Gal<sup>+</sup> phenotype (33), the inability of extracts of *rfaH* mutants to catalyze, to a significant extent, the transfer of labeled galactose from UDPgalactose to suitable acceptor LPS, and the ability of *rfaH* LPS to accept galactose from UDPgalactose with *rfa*<sup>+</sup> extracts (21). However, the following data later cast doubt on this conclusion. First, an analysis of LPS from *rfaH* mutants by passive hemagglutination inhibition and by methylation showed that the block in LPS core biosynthesis in *rfaH* mutants is incomplete, with only 40 to 60% of the cores terminating with glucose I, while a significant number terminate instead with the core galactose I, or glucose II, or *N*-acetylglucosamine (17). Second, in *rfaH* strains carrying ColE1 plasmids with *E. coli* genes (8), which cure the LPS defect of *rfaH* mutants of *S. typhimurium*, the activity of galactosyl-1 transferase in cell extracts was restored only to the normal level, and was not "amplified" as was expected in this multicopy plasmid (9), whereas ColE1-*rfaG*<sup>+</sup> plasmids gave 10 times the amplification of glucosyltransferase (10). Third, our observation that *rfaH* strains are defective in F factor function is not easily explained by the notion that the *rfaH* gene product is  $\alpha$ 1,3-galactosyltransferase.

All of the *rfaH* mutants tested differ from the wild type in two properties. They are deficient in normal LPS, and they fail to express the donor properties of the F-factor. Our evidence indicates that changes in the cell envelope through modifications of LPS are not the basis for the failure of expression of F in *rfaH* strains since many other rough mutants with LPS defects further into the inner core, similar to the *rfaH* mutants, or in the outer core of the LPS, were tested and none was deficient in F-factor expression.

Based primarily on passive hemagglutination inhibition and methylation analysis (17), it was suggested that the *rfaH*<sup>+</sup> gene product was either a polypeptide whose presence was needed for normal action of several glycosyltransferases needed for addition of core sugar units distal to glucose I, or a positive regulatory substance, serving to turn on several *rfa* genes specifying such glycosyltransferases (17, 30). The latter hypothesis appears more plausible, especially considering our data which indicate that the function of the *tra* genes is also dependent on the *rfaH* gene product. In addition, the fact that the *rfaH* gene is at 84 units on the linkage map

of *S. typhimurium* (unlinked to the *rfa* cluster of genes at 79 units which determines the activity of several glycosyl transferases) also suggests that *rfaH* is involved in regulation of the synthesis or the activity of these transferases rather than directly determining components of the enzymes. The *sfrB* gene product in *E. coli* has been shown to be required to prevent premature transcription termination at one or more *rho*-dependent termination sites (6). In addition, *sfrB* mutations caused defects of LPS synthesis, defects of several chromosomally determined outer membrane proteins, and defects of functional flagella. Thus, the *sfrB* gene product was proposed to be an antiterminator in transcription of several operons encoding cell envelope components (6). The similarities of phenotype and of map location of *sfrB* of *E. coli* and of *rfaH* of *S. typhimurium* suggest that the *rfaH* gene product may act by the same mechanism to regulate the activity of the glycosyltransferases for LPS synthesis and of the *tra* operon of the F-factor.

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