JOURNAL OF BACTERIOLOGY, Nov. 1986, p. 715–721
0021-9193/86/110715-07$02.00/0

Vol. 168, No. 2

Biosynthesis of Enterobacterial Common Antigen Requires dTDPglucose Pyrophosphorylase Determined by a *Salmonella typhimurium rfb* Gene and a *Salmonella montevideo rfe* Gene

HENRY C. LEW,[1]† P. HELENA MÄKELÄ,[2] HELLA-MONIKA KUHN,[3] HUBERT MAYER,[3] AND HIROSHI NIKAIDO[1]*

Department of Microbiology and Immunology, University of California, Berkeley, California 94720[1]; National Public Health Institute, SF-00280 Helsinki, Finland[2]; and Max-Planck Institut für Immunbiologie, D-7800 Freiburg, Federal Republic of Germany[3]

Received 22 May 1986/Accepted 7 August 1986

In group C_1 salmonellae, *rfe* and *rff* genes linked to the *ilv* locus specify the synthesis of a glycolipid called the enterobacterial common antigen. In contrast, in group B salmonellae the synthesis requires in addition some of the genes in the *rfb* cluster, the main genetic determinant of the O side chains of lipopolysaccharide. In an effort to define the biochemical functions of these *rfb* genes, we looked in *Salmonella typhimurium* LT2 (group B) for *rfb* mutants in which the synthesis of both enterobacterial common antigen and the O side chains would be blocked in a manner suppressible by the wild-type *rfe* cluster of *S. montevideo*, of group C_1. We found one mutant with these characteristics. This *rfb* mutation affected the activity of dTDPglucose pyrophosphorylase (glucose-1-phosphate thymidylyltransferase, EC 2.7.7.24). Whereas the *rfe* cluster of *S. montevideo* contained a gene producing this enzyme activity, there was no evidence for the presence of such a gene in the *rfe* cluster of group B strains. These results also showed that the synthesis of dTDP-glucose is necessary for the biosynthesis of enterobacterial common antigen; this conclusion fits with the recent demonstration of 4-acetamido-4,6-dideoxy-D-galactose as a component of enterobacterial common antigen (Lugowski et al., Carbohydr. Res. 118:173–181, 1983), because the biosynthesis of the donor of this sugar, dTDP-4-acetamido-4,6-dideoxy-D-galactose, requires dTDPglucose pyrophosphorylase.

All enteric bacteria produce an acidic cell surface glycolipid called enterobacterial common antigen (ECA) (6, 12, 19). The biosynthesis of ECA is determined by *rfe* and *rff* genes close to *ilv*; in addition, a gene(s) at the *his*-linked *rfb* locus is required for ECA synthesis in certain salmonellae (11–14). The *rfe* and *rfb* gene clusters also participate in the determination of lipopolysaccharide (LPS) biosynthesis (15). The facts can be summarized as follows (Table 1).

(i) Role of *rfe*. In *Salmonella minnesota* of O group L (13) and *Salmonella montevideo* of O group C_1 (10, 11, 28), *rfe* genes are needed for the synthesis of both ECA and the O side chains of LPS; *rfe* mutants of these strains are both ECA negative and rough (i.e., they produce LPS lacking O side chains). *rfe* mutants of *S. typhimurium* are also ECA negative but smooth, suggesting that the *rfe* genes are not needed for the synthesis of O side chains in group B (28). In accordance with this, introduction of a mutant *rfe* allele from a group C_1 donor into a group B recipient, e.g., *Salmonella typhimurium*, produces an ECA-negative phenotype without any visible alterations in LPS synthesis (11).

(ii) Role of *rfb*. Some *rfb* function is necessary for the synthesis of ECA in group B, but not in group C_1; deletions in the *S. typhimurium rfb* gene cluster extending from the *his* operon to the gene *rfbA* were found to lead to the production of only traces of ECA in the presence of wild-type alleles of the group B *rfe* locus (14). These mutants were also hypersensitive to anionic detergents and accumulated secondary *rff* mutations in the region of the chromosome linked to *ilv* and *rfe* (13, 14).

(iii) Role of *rff*. The *rff* mutations completely abolished the synthesis of ECA in both groups B and C without affecting the biosynthesis of LPS in either group. Some of the *rff* genes code for enzymes needed for the synthesis of one of the building blocks of ECA, namely, *N*-acetyl-D-mannosaminuronic acid (7).

In the present study, we wanted to identify the biochemical reaction or reactions required for the synthesis of ECA and catalyzed by the products of *rfb* gene(s) in group B salmonellae. Presumably, the same function is provided by *rfe* gene(s) in group C_1 salmonellae. Based on this hypothesis, we looked in *S. typhimurium* LT2 (O group B) for *rfb* mutations that could be phenotypically suppressed by the wild-type *rfe* allele(s) of group C_1. One such mutation was found and shown to affect the gene coding for dTDPglucose pyrophosphorylase (EC 2.7.7.24). Apparently this enzyme is needed in group B salmonellae both for the biosynthesis of dTDP-L-rhamnose, a donor of rhamnosyl residues in the O side chain, and for the synthesis of dTDP-4-acetamido-4,6-dideoxy-D-galactose (17), the donor of a component recently discovered in ECA (2, 8). It appears that this enzyme is coded for by an *rfb* gene in group B salmonellae, whereas the *rfe* cluster is solely responsible for this enzyme activity in group C_1 salmonellae.

MATERIALS AND METHODS

Bacterial strains. The construction of strains is shown in Fig. 1. We first introduced the *rfe-3853* allele of a salmonella group C_1 strain from the HfrH14 donor SH3862 (28) into the *S. typhimurium* recipient SA841, *metE338 ilv-401 his-5406 rpsL* (27), by conjugation and selection for the closely linked $metE^+$ ilv^+ alleles. The *rfe-3853* recombinant was, as expected (28), ECA negative but smooth. An *ilv* mutant

* Corresponding author.
† Present address: Department of Medical Microbiology, Stanford University School of Medicine, Stanford, CA 94305.

TABLE 1. Main gene clusters that participate in the biosynthesis of ECA or LPS in salmonellae (15).

Gene cluster	Synthesis of:	Required for LPS:		Required for ECA:	
		In group C_1	In group B	In group C_1	In group B
rfa	LPS core	+	+	−	−
rfb	O side chains	+	+	−	+
rfe	O and ECA	+	−	+	+
rff	ECA	−	−	+	+

(SH4773) was then isolated and further mutagenized with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (1). Mutants resistant to phage P22c2 were selected and tested for their sensitivity to smooth- and rough-specific phages (31). Eight mutants with the phage sensitivity pattern typical of *rfb* mutants were test crossed by conjugation with the group C_1 donor strain, SW829 (F^+) (10), and ilv^+ recombinants, many of which would also have received the closely linked intact group C_1 *rfe* locus (C-rfe^+) were selected. All such ilv^+ recombinants from seven of the mutants were still rough, but most recombinants from SH4865 were smooth (for example, strain SH4892). Thus, the synthesis of a group B-type O polysaccharide in this mutant (*rf.-4020*) appeared to require a function that could be provided by the *rfe* locus of group C_1. For further analysis of the *rf.-4020* mutation a derivative (SH4925) carrying the intact *rfe* cluster of group B (B-rfe^+) was isolated as an ilv^+ recombinant with the HfrK10 donor (27).

Other strains used in the genetic analysis were a prototype *S. typhimurium* LT2 strain and two donor *S. typhimurium* strains, HfrK6 and HfrK10 (27).

LPS characterization by serology and phage sensitivity. Slide agglutination was performed with 4% NaCl (which agglutinates rough but not smooth bacteria) and suitable dilutions of group B and group C_1-specific anti-O (OB and OC, respectively) sera (4). The presence of group B-type O antigen was, when indicated, confirmed by testing the ability of a heated (100°C, 30 min) suspension of the bacteria to remove anti-O agglutinating activity from the OB serum; the serum was tested before and after absorption in twofold dilutions by tube agglutination with a similarly heated suspension of wild-type LT2 bacteria (4). Smooth- and rough-specific phages were received from B. A. D. Stocker (Stanford University, Stanford, Calif.) and used as described previously (31).

ECA determination. The ECA character of the strains was initially determined by indirect hemagglutination, in rabbit anti-ECA sera (anti-*E. coli* O14), of erythrocytes sensitized with heated bacterial supernatants (13). For quantitative determination we used an enzyme immunoassay (EIA) with monoclonal mouse immunoglobulin G2a (IgG2a) anti-ECA antibody mAb898 (25). The wells of Falcon 3911 microtiter plates were coated with antigen by overnight incubation at 37°C with the antigen. The antigen was either ECA isolated from *S. montevideo* SH94 (16) or the supernatant of bacteria grown on nutrient agar plates, harvested in 0.9% saline, lyophilized, suspended at 1% in 0.01 M phosphate buffer (pH 7.3), heated in a boiling water bath for 1 h, and centrifuged (5,000 × *g*, 20 min). The second antibody was peroxidase-labeled heavy- and light-chain-specific goat anti-mouse IgG (Dianova, Hamburg, Federal Republic of Germany), and the substrate was 2,2'-azino-di-(3-ethylbenzthiozolin sulfonate) (6) from Boehringer GmbH, Mannheim, Federal Republic of Germany. For inhibition of EIA, 50 μl of the antigen was added to 40 μl of a suitable mAb898 dilution in wells of microtiter plates precoated with 2% bovine serum albumin

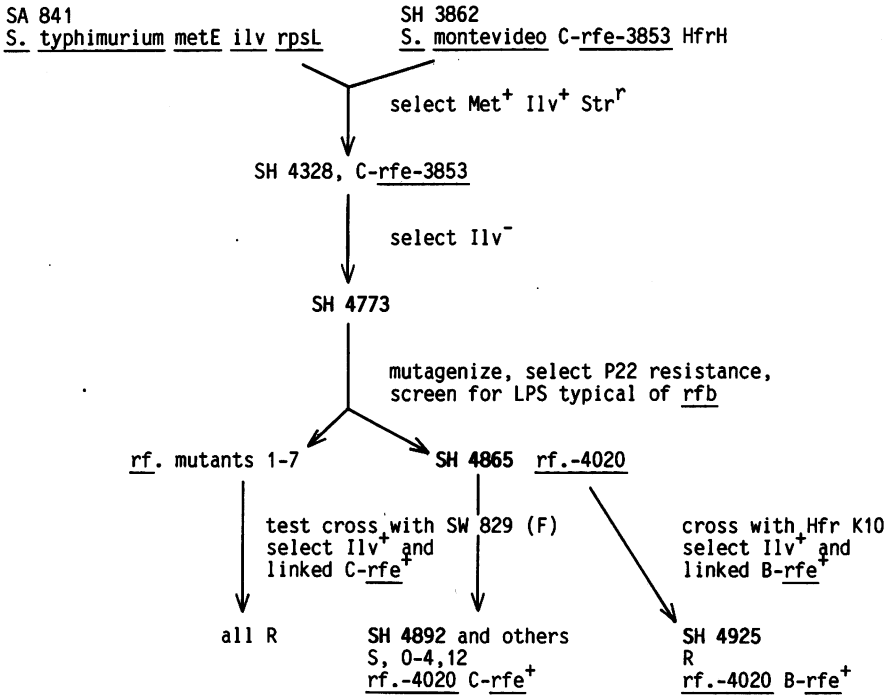

FIG. 1. Isolation and relevant properties of the strains used in this study. *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine (1) was used to mutagenize strain SH4773. Smooth (S)- and rough (R)-specific phages (31) and anti-O sera (4) were used for preliminary characterization of the LPSs of the bacteria.

TABLE 2. ECA- and LPS-related characteristics of the *rfb-4020* mutant and related strains

Strain	Derivation	Relevant genotype[a]		ECA[b]			Phage pattern[c]	Reaction with[d]:			O-specific sugars (mol/2 mol of heptose)		Relative specific enzyme activities[e]				
		rfe	*rfb*	IHA	EIA	I-EIA (%)		OB	OC	4% NaCl	Rhamnose	Abequose	UDPG PPase	RHA-1[f]	RHA-2	RHA-3	TA
LT2	Parent	B+	B+	+	1.36	84	S	+	−	−	4.5	1.9	100	100	100	100	100
SH4773	*rfe* recombinant	C-3853	B+	−	0.12	−4	S	+	−	−	4.8	ND[g]	80	80	120	ND	130
SH4865	Rough mutant	C-3853	B-4020	−	0.16	3	Ra	−	−	+	0.03	<0.01	80	11 (4)	150	50	120
SH4892	C-*rfe*$^+$ recombinant	C+	B-4020	+	1.46	90	Ra modif.	+	−	−	0.1	0.02	70	28 (23)	140	ND	130
SH4925	B-*rfe*$^+$ recombinant	B+	B-4020	Trace	0.68	44	Ra	−	−	+	ND	ND	ND	12 (5)	ND	ND	ND

[a] Genes derived from group C_1 have a prefix C, and those derived from group B a prefix B; the wild type is indicated by +.

[b] Determined by indirect hemagglutination (IHA), EIA (optical density at 490 nm; Fig. 2), or inhibition of EIA (I-EIA) with purified ECA as the antigen, (extent of inhibition expressed as percentage of the noninhibited optical density); the values are means of eight separate determinations.

[c] Sensitivity pattern when assayed with a number of smooth- and rough-specific phages: S, pattern typical of bacteria with complete, smooth type of LPS; Ra, pattern typical of bacteria with the complete LPS core, no O side chains; Ra modif., pattern like Ra but resistant to one of the core-binding phages (6SR).

[d] The reaction was assayed by slide agglutination and the ability of the bacteria to absorb the specific antibodies from the OB serum; all assays gave concurrent results (+ or −); OB, antiserum specific to O-4,12 of group B; OC, antiserum specific to O-6,7 of group C_1.

[e] Assays were carried out for all of the following enzymes in strains LT2 and SH4865, and for many of them also in SH 4773 and SH4892, but no significant differences were found: phosphomannomutase, GDPmannose pyrophosphorylase, CDPglucose pyrophosphorylase, CDPglucose oxidoreductase, CDPabequose synthetase, UDPgalactose:undecaprenol-phosphate galactose-1-phosphate transferase, dTDPrhamnose:galactosyl-lipid rhamnosyl transferase, and GDPmannose:rhamnosyl-galactosyl-lipid mannosyl transferase. See Fig. 3 for reactions catalyzed by some of these enzymes. Abbreviations: UDPG PPase, UDPglucose pyrophosphorylase; RHA-1, dTDPglucose pyrophosphorylase; RHA-2, dTDPglucose oxidoreductase; RHA-3, dTDPrhamnose synthetase; TA, L-glutamate:dTDP-4-keto-6-deoxy-D-glucose transaminase;

[f] Values in parentheses are believed to represent the true activity of this enzyme, obtained by correction for the contribution of a cross-reactive UDPglucose pyrophosphorylase (21, 23). These values are averages of four separate experiments.

[g] ND, Not determined.

and incubated overnight at 37°C; the mixture was then used in place of antibody in the EIA on plates coated with 1 μg of isolated ECA.

Biochemical analysis. Cells were grown in L broth (10 g of tryptone [Difco Laboratories, Detroit, Mich.], 10 g of yeast extract [Difco], and 5 g of NaCl per liter) at 37°C with aeration by shaking and were harvested at the late exponential phase. The cells were washed once in cold water, resuspended in 1/100 of the original volume of 40 mM Tris chloride buffer (pH 7.5) containing 10 mM $MgCl_2$ and 1 mM EDTA, and disrupted by passage (twice) through a French pressure cell at 10,000 lb/in^2. After centrifugation at 1,500 × *g* for 10 min to remove unbroken cells, the supernatant fraction was centrifuged at 100,000 × *g* for 1 h. The sediment was suspended in 50 mM Tris chloride–10 mM $MgCl_2$–1 mM EDTA (pH 7.5) and used for assays of sugar transferases (32) after being kept frozen at −70°C. Similarly, the supernatant solution was kept at −70°C as small working samples and used as the soluble enzyme fraction for the assay of enzymes of nucleotide-sugar synthesis (23, 24).

LPS was extracted from whole cells by the phenol-water procedure (30), and the sugar composition was determined as described previously (20).

RESULTS

Characterization of the *rf.-4020* mutation. (i) Genetic analysis of the rough mutant strain SH4865. The phage sensitivity pattern and lack of reaction in anti-O sera of the *rf.-4020* mutant SH4865 were typical of bacteria with a complete LPS core without O side chains. This suggested that the mutation was either in the *rfb* cluster, close to *his*, or in *rfaL*, between *cysE* and *pyrE* (15). To localize the mutation, the strain was crossed with Hfr donors of O group B (27). In a cross with HfrK10 all 20, and in another cross with HfrK6 18 of 20, *his*$^+$ recombinants had become smooth, placing the mutation at *rfb*. It was therefore termed *rfb-4020*.

To show that SH4865 still retained its *rfe* mutation it was test crossed with a group C_1 donor (SW829 F$^+$), and *his*$^+$ recombinants, the majority of which would also have received the group C_1-*rfb* cluster, were selected. All such recombinants remained rough, consistent with the fact that the synthesis of group C_1-type O polysaccharide requires the function of intact *rfe* genes.

For further analysis of the suppression of *rfb-4020* by *rfe* genes, we constructed derivatives of SH4865 that had the intact *rfe* cluster from either a group C_1 or a group B donor [strains SH4892 (C-*rfe*$^+$) and SH4925 (B-*rfe*$^+$)]. These were isolated as *ilv*$^+$ recombinants in the appropriate crosses (Fig. 1); the majority of the selected recombinants had the expected phenotypes described in detail below.

(ii) ECA in strains with the *rfb-4020* mutation. Indirect hemagglutination gave a presumptive identification of the strains as ECA$^-$ (all strains that had the *rfe-3853* mutation), ECA$^+$ (strains that had wild-type C-*rfe*$^+$ genes or both *rfe*$^+$ and *rfb*$^+$ genes from group B), or ECA^{trace} (strain SH4925, in which *rfb-4020* was combined with B-*rfe*$^+$) (Table 2). Since the ECA^{trace} phenotype was not well characterized we wanted to obtain a more quantitative estimation of the ECA content of these strains. This was now possible by using a monoclonal anti-ECA antibody (25) in an EIA. When the microtiter wells were coated with different dilutions of supernatants derived from a standard amount of bacteria, the ECA^{trace} phenotype appeared to be intermediate between ECA$^+$ and ECA$^-$ (Fig. 2). To make a clear cut separation between the three phenotypes, we selected 0.25 μl of the supernatant to be used as antigen (Fig. 2). The readings with this standard assay were then determined for the strains with various combinations of *rfb-4020* or *rfb*$^+$ with *rfe-3853* or *rfe*$^+$ from either group B or group C_1 (Table 2). Very similar results were obtained when the same supernatants were used to inhibit the EIA, with purified ECA as the coating antigen (Table 2).

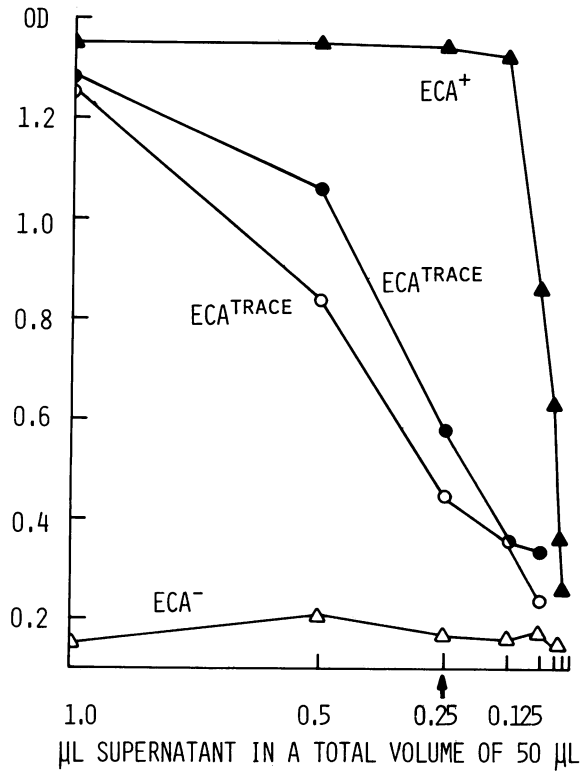

FIG. 2. EIA with the monoclonal anti-ECA antibody mAb898 diluted 1:1,000; the microtiter wells were coated with different dilutions of bacterial supernatants. The points shown are means of duplicate determinations of optical density (OD) at 490 nm. The arrow indicates the dilution used in the standard EIA assays, from which the data in Table 2 are derived. The bacteria were classified as ECA$^+$ (SH4892), ECA$^-$ (SH4773), or ECA^{trace} (SH4925, two separate batches) by indirect hemagglutination.

The data thus show that the *rfb-4020* mutant in fact affected ECA, reducing its apparent content in strain SH4925, with the homologous group B-*rfe*, to a trace level. The wild-type C-*rfe*$^+$ allele could suppress this effect (strain SH4892), whereas the mutant *rfe-3853* allele, as expected, completely abolished ECA.

(iii) O-antigen in strains with the *rfb-4020* mutation. The suppression exerted by the C-*rfe*$^+$ on O polysaccharide synthesis in the *rfb-4020* mutant strain SH4892 was first indicated by its specific agglutination in OB serum but not in OC serum or 4% NaCl. The agglutinates were large clumps typical of smooth bacteria. The presence of group B O-antigenic determinants was further confirmed by the ability of the C-*rfe*$^+$ strain SH4892, but not the *rfe-3853* strain SH4865, to remove anti-O antibodies from the OB serum (Table 2). B-*rfe*$^+$ could not suppress *rfb-4020*: strain SH4925 did not react with the OB serum (Table 2).

However, when SH4892 was tested for sensitivity to a series of rough- and smooth-specific phages, it gave a pattern grossly similar to its rough mutant parent, SH4865 (sensitive only to core-binding phages FO, Br2, Ffm, Br60, and P221). The only exception was its resistance to phage 6SR, which binds to the complete LPS core (Table 2). This combination of LPS characteristics, the presence of O antigen as detected by reaction with specific antisera and concomitant exposure of core structures as detected by rough-specific phages, is typical of partially rough strains, in which defective synthesis of O antigen leads to incomplete coverage of the core (15). Such partial defects can result from a leaky mutation but also from less than complete suppression by a suppressor mutation or exogenous supplementation (5, 9). We conclude that the presence of C-*rfe*$^+$ genes in the recombinants partially alleviated the defect of O antigen synthesis caused by the *rfb-4020* mutation.

(iv) Composition of LPS. The chemical analysis of LPS composition fully confirmed the results of the serological tests (Table 2). Thus the introduction of the *rfe-3853* allele did not alter the composition of LPS of the group B strain SH4773, as compared with the parent, LT2. The *rfb-4020* mutation, on the other hand, almost completely abolished the synthesis of O polysaccharide as seen in the very low values of the O-specific sugars rhamnose and abequose in SH4865. The introduction of the *rfe*$^+$ genes from a group B donor did not affect the O-chain synthesis (SH4925), but the *rfe*$^+$ region of group C_1 partially restored the biosynthesis of O chains (SH4892) (Table 2).

Enzymatic basis of the phenotype in *rfb-4020* mutants. (i) Enzymes involved in nucleotide sugar synthesis. Many enzymes are required for synthesizing the various nucleotide sugars, which act as donors of saccharide residues in the biosynthesis of LPS and ECA (Fig. 3). When the levels of these enzymes were determined by using the soluble enzyme fractions, SH4865, containing the *rfb-4020* mutation, showed significantly lower dTDPglucose pyrophosphorylase activity. Although the residual activity appeared to be about 10% of the wild-type level, much of this was due to the cross-reaction catalyzed by UDPglucose pyrophosphorylase (21, 23), and we believe that the mutation nearly completely abolished the activity of dTDPglucose pyrophosphorylase proper, as seen in values corrected for this contribution (in parentheses in Table 2). The activity was not elevated by the introduction of B-*rfe*$^+$ in SH4925, but was slightly but reproducibly elevated in strain SH4892, which had received the wild-type *rfe* region from a group C_1 strain (Table 2).

(ii) Activity of sugar transferases. Because the *rfb* region is known to code for enzymes that are responsible for the transfer of galactose 1-phosphate, L-rhamnose, D-mannose, and abequose (15, 22), we also tested some of these transferase activities by using membrane fractions from strains LT2, SH4773, SH4865, and SH4892. The specific activity of UDPgalactose: undecaprenol-phosphate galactose 1-phosphate transferase in these strains was between 14 and 25 nmol/mg of protein per h, and we could not find any consistent differences between them. Similarly, all strains listed above showed significant activity of L-rhamnosyl and D-mannosyl transferases.

(iii) Activity of dTDP-4-keto-6-deoxy-D-glucose transaminase. The C-*rfe*$^+$ mutation could thus partially restore the activity of dTDPglucose pyrophosphorylase in SH4892; it also made the strain ECA positive. This finding suggested to us that dTDP-glucose or its derivatives may be serving as a precursor of ECA. Since serotypes such as C_1 do not contain significant enzyme activity for dTDPrhamnose synthetase (23) yet synthesize ECA, it is unlikely that rhamnose is a component of ECA. However, another product derived from dTDP-glucose, dTDP-4-acetamido-4,6-dideoxy-D-galactose, is known to be widely distributed among members of the family *Enterobacteriaceae* (17, 18). In view of this observation, the key enzyme for the biosynthesis of this compound, L-glutamate:dTDP-4-keto-6-deoxy-D-glucose transaminase (18), was assayed in the soluble enzyme fraction of various strains. The specific activity was at a similar level regardless of the presence or absence of *rfb-4020* and *rfe-3853* mutations (Table 2).

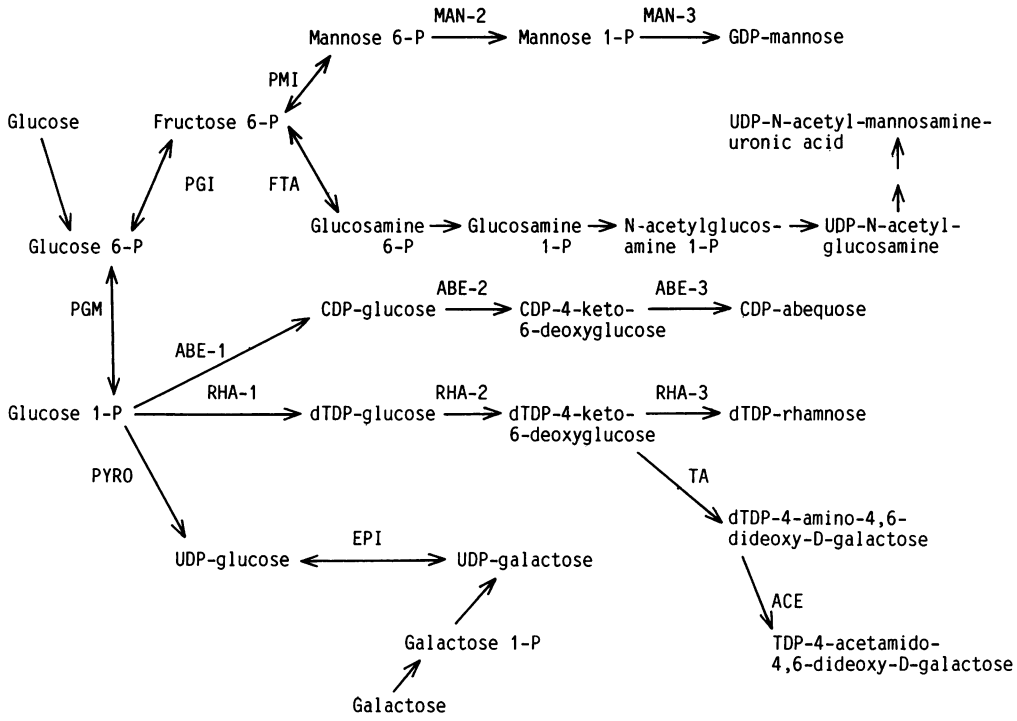

FIG. 3. Pathways of sugar nucleotide synthesis in *S. typhimurium*. The enzymes catalyzing these reactions are abbreviated as follows: ACE, transacetylase; EPI, UDPglucose-4-epimerase; FTA, fructose-6-phosphate-transaminase; PGI, phosphoglucoisomerase; PGM, phosphoglucomutase; PMI, phosphomannoisomerase; PYRO, UDPglucose pyrophosphorylase; MAN-2, phosphomannomutase; MAN-3, GDPmannose pyrophosphorylase; ABE-1, CDPglucose pyrophosphorylase; ABE-2, CDPglucose oxidoreductase; ABE-3, CDPabequose synthetase.

(iv) Other enzymes involved in ECA synthesis. We reported earlier than UDP-*N*-acetyl-D-glucosamine is converted to UDP-*N*-acetyl-D-mannosaminuronic acid, the donor of a component of ECA, and that the activities of the two enzymes responsible for this conversion were often missing in *rff* mutants, which are unable to synthesize ECA but can synthesize the O side chains of the group C_1 LPS (7). The activities of these enzymes were, however, in the normal range, as judged by the overall rate of conversion of UDP-*N*-acetylglucosamine into UDP-*N*-acetylmannosaminuronic acid (data not shown).

DISCUSSION

In this study, we found that wild-type alleles of the *rfe* genes from a group C_1 salmonella, *S. montevideo*, could partially restore the dTDP-glucose pyrophosphorylase activity apparently missing in the *rfb-4020* mutant of *S. typhimurium* and at the same time allowed the synthesis of both ECA and the O-antigenic polysaccharide of the group B LPS. Such phenotypic suppression was not exerted by the wild-type alleles of the *rfe* region from group B salmonellae. The simplest explanation of these results is as follows. (i) The enzyme dTDPglucose pyrophosphorylase is needed for the synthesis of ECA, because a product derived from dTDP-glucose, dTDP-4-acetamido-4,6-dideoxy-D-galactose, acts as the donor for a sugar in ECA. This prediction (H. C. Lew, Ph.D. thesis, University of California, Berkeley, 1978) was recently confirmed by the discovery by Lugowski et al. (8) of 4-acetamido-4,6-dideoxy-D-galactose in ECA. (ii) The O side chains of group C_1 salmonellae contain no component derived from dTDP-glucose (3). Thus apparently the sole function of dTDPglucose pyrophosphorylase in group C_1 is to produce a component of ECA, and the only gene that expresses a low activity of this enzyme (24) is located in the *rfe* gene cluster. (iii) In group B salmonellae, a higher activity of dTDPglucose pyrophosphorylase and dTDPglucose oxidoreductase is needed for O-antigen synthesis (Fig. 3). The *rfb* gene cluster of group B strains, mainly responsible for O side chain synthesis, contains the structural genes for these enzymes (23), and possibly this redundancy could have led to the weakening or loss of the dTDPglucose pyrophosphorylase gene of the *rfe* cluster during the evolution of the group B salmonellae.

These assumptions can explain most of the data in this study. According to them, the *rfb-4020* mutation in strain SH4865 inactivated the *rfbA* gene specifying dTDPglucose pyrophosphorylase, and in absence of this enzyme the mutant was unable to synthesize any of the metabolites generated from dTDP-glucose. The resulting lack of rhamnose led to a defect in the synthesis of the O polysaccharide of the group B-type LPS, and the LPS of the mutant was rough, as demonstrated by its lack of not only rhamnose but also abequose, another sugar specific to the O side chain. The lack of dTDP-glucose would also prevent the synthesis of 4-acetamido-4,6-dideoxy-D-galactose and its incorporation into ECA. This could not be directly verified in SH4865 because of its *rfe-3853* mutation, which already abolishes ECA synthesis. When a wild-type copy of the group C_1 *rfe* gene presumably coding for this enzyme (we propose to call this gene *rfeA*) was brought in, this partially restored the enzyme activity, O antigen synthesis, and ECA synthesis. However, the introduction of wild-type copies of the *rfe* region genes from a group B donor could not restore the

enzyme activity or the synthesis of O antigen (SH4925 in Table 1), presumably because the *rfe* cluster of group B strains does not contain the gene coding for dTDPglucose pyrophosphorylase. A partial restoration of ECA synthesis (to trace levels) took place, however, perhaps by utilizing the low-degree cross-reaction catalyzed by UDPglucose pyrophosphorylase, which was intact in all of these strains (Table 2).

The presence of the *rfeA* gene in the group C_1 but not in the group B salmonellae allows us, furthermore, to understand the ECA phenotypes of *S. typhimurium* (group B) strains carrying extensive deletions of the *rfb* gene cluster. It was previously found (14) that among a series of deletions starting beyond the "right" (or *his*-proximal) end of the cluster, the loss of the genes up to *rfbF* had no effect on ECA production, but the deletion of *rfbA-rfbD* region resulted in a dramatic decrease of ECA to give the so-called ECA^{trace} phenotype, which in the light of our present results appears to be due to the loss of dTDPglucose pyrophosphorylase. The data in this paper (Fig. 2, Table 2) show that this ECA^{trace} phenotype corresponds to a quantitative decrease of ECA material. On the other hand, immunoblotting experiments with the same monoclonal antiserum have shown that the ECA in the trace strains is qualitatively similar, in both the distribution and the location of bands corresponding to increasing numbers of repeating units, to ECA in normal strains (H. M. Kuhn, unpublished data). The synthesis of small amounts of qualitatively normal ECA in the *rfb* deletion strains most probably takes place by utilizing the cross-reaction catalyzed by UDPglucose pyrophosphorylase, just as in the *rfbA* strain SH4925 discussed above.

Another property of *S. typhimurium* strains containing deletions through the *rfbA* gene is to accumulate secondary mutations in the *rff* gene cluster (14). This phenotype can also be explained on the basis of our current finding. Mutants unable to synthesize the third or the fourth sugar residue of the O repeat unit are unstable, possibly because the mutations result in the irreversible accumulation of oligosaccharide-lipid intermediates (32). Since the repeating unit of the ECA is 4-acetamido-4,6-dideoxy-D-galactosyl-*N*-acetyl-D-mannosaminuronyl-*N*-acetyl-D-glucosamine (8), and since the isolation of *N*-acetyl-D-glucosaminyl-(undecaprenyl pyrophosphate) from *S. typhimurium* (26) suggests that the first sugar of the repeating unit is *N*-acetyl-D-glucosamine, it appears that the sugar missing in these deletion mutants, 4-acetamido-4,6-dideoxy-D-galactose, corresponds to the third sugar in the biosynthesis of the ECA repeating unit. Thus the unavailability of this sugar will cause accumulation of an oligosaccharide-lipid intermediate, a condition that could be alleviated by *rff* mutations eliminating the synthesis of *N*-acetyl-D-mannosaminuronic acid, the presumed second sugar of the repeating unit (7).

Our results thus strongly suggest that one of the enzymes coded for by the *rfe* gene cluster in group C_1 salmonellae is dTDPglucose pyrophosphorylase. However, it is most likely that this cluster contains other genes coding for other functions needed for the synthesis of ECA as well as some O chains. Indeed we found that another *rfe* mutation isolated in *S. montevideo*, *rfe-3623*, affects a step(s) in the building up of the ECA polymer rather than the steps in the biosynthesis of nucleotide-sugar precursors (Lew, Ph.D. thesis), and Jann and co-workers recently suggested that the products of the *rfe* genes function mainly in supporting the orderly extension of O side chain in *Escherichia coli* serotype O9 strains (29). Indeed the simplest hypothesis on the nature of the *rfe-3853* mutation present in most of the strains studied here is that it is a polar mutation or a deletion affecting the gene for dTDPglucose pyrophosphorylase as well as other gene(s) needed for the assembly of ECA and the group C_1 O polysaccharide. Obviously the true molecular nature of the *rfe-3853* mutation as well as the identification of additional functions coded for by the *rfe* gene cluster are topics for future research.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant AI-09644 from the National Institute of Allergy and Infectious Diseases. H.C.L. was a predoctoral trainee supported by a Public Health Service training grant from the National Institutes of Health.

LITERATURE CITED

1. **Adelberg, E. A., M. Mandel, and G. C. C. Chen.** 1965. Optimal conditions for mutagenesis by *N*-methyl-*N'*nitro-*N*-nitrosoguanidine in *Escherichia coli* K12. Biochem. Biophys. Res. Commun. **18**:788–795.
2. **Dell, A., I. Oates, C. Lugowski, E. Romanowska, L. Kenne, and B. Lindberg.** 1984. The enterobacterial common antigen, a cyclic polysaccharide. Carbohydr. Res. **133**:95–104.
3. **Fuller, N. A., and A. M. Staub.** 1968. Immunochemical studies on *Salmonella*. Chemical changes appearing on the specific polysaccharide of *S. cholerae-suis* (6_2, 7) after its conversion by phage 14 (6,7). Eur. J. Biochem. **4**:286–300.
4. **Kauffmann, F.** 1966. Enterobacteriaceae. Ejnar Munksgaard, Copenhagen.
5. **Krishnapillai, V., D. G. MacPhee, and B. A. D. Stocker.** 1971. Properties of a *Salmonella typhimurium* mutant with an incomplete deficiency of uridinediphosphogalactose-4-epimerase. J. Bacteriol. **107**:155–157.
6. **Kuhn, H.-M., E. Neter, and H. Mayer.** 1983. Modification of the lipid moiety of the enterobacterial common antigen by the *Pseudomonas* factor. Infect. Immun. **40**:696–700.
7. **Lew, H. C., H. Nikaido, and P. H. Mäkelä.** 1978. Biosynthesis of uridine diphosphate-*N*-acetylmannosaminuronic acid in *rff* mutants of *Salmonella typhimurium*. J. Bacteriol. **136**:227–233.
8. **Lugowski, C., E. Romanowska, L. Kenne, and B. Lindberg.** 1983. Identification of a trisaccharide repeating-unit in the enterobacterial common antigen. Carbohydr. Res. **118**:173–181.
9. **MacPhee, D. G., and B. A. D. Stocker.** 1969. Suppression of amber and ochre mutants in *Salmonella typhimurium* by a mutant F'-1-gal factor carrying an ochre suppressor gene. J. Bacteriol. **100**:240–246.
10. **Mäkelä, P. H., M. Jahkola, and O. Lüderitz.** 1970. A new gene cluster *rfe* concerned with the biosynthesis of *Salmonella* lipopolysaccharide. J. Gen. Microbiol. **60**:91–106.
11. **Mäkelä, P. H., and H. Mayer.** 1974. Participation of lipopolysaccharide genes in the determination of the enterobacterial common antigen: analysis of *Salmonella* groups B and C. J. Bacteriol. **119**:765–770.
12. **Mäkelä, P. H., and H. Mayer.** 1976. Enterobacterial common antigen. Bacteriol. Rev. **40**:591–632.
13. **Mäkelä, P. H., H. Mayer, H. Y. Whang, and E. Neter.** 1974. Participation of lipopolysaccharide genes in the determination of the enterobacterial common antigen: analysis of R mutants of *Salmonella minnesota*. J. Bacteriol. **119**:760–764.
14. **Mäkelä, P. H., G. Schmidt, H. Mayer, H. Nikaido, H. Y. Whang, and E. Neter.** 1976. Enterobacterial common antigen in *rfb* deletion mutants of *Salmonella typhimurium*. J. Bacteriol. **127**:1141–1149.
15. **Mäkelä, P. H., and B. A. D. Stocker.** 1984. Genetics of lipopolysaccharide, p. 59–137. *In* E. T. Rietschel (ed.), Chemistry of endotoxin. Elsevier-North Holland, Amsterdam.
16. **Männel, D., and H. Mayer.** 1978. Isolation and chemical characterization of the enterobacterial common antigen. Eur. J. Biochem. **86**:361–370.
17. **Matsuhashi, M., and J. L. Strominger.** 1964. Thymidine diphosphate 4-acetamido-4,6-dideoxyhexoses. I. Enzymatic

synthesis by strains of *Escherichia coli*. J. Biol. Chem. **239**:2454–2463.
18. **Matsuhashi, M., and J. L. Strominger.** 1965. Thymidine diphosphate 4-acetamido-4,6-dideoxyhexoses. III. Purification and properties of thymidine diphosphate 4-keto-6-deoxy-D-glucose transaminase from *Escherichia coli* strain B. J. Biol. Chem. **241**:4738–4744.
19. **Mayer, H., and G. Schmidt.** 1979. Chemistry and biology of enterobacterial common antigen (ECA). Curr. Top. Microbiol. Immunol. **85**:99–153.
20. **Naide, Y., H. Nikaido, P. H. Mäkelä, R. G. Wilkinson, and B. A. D. Stocker.** 1965. Semi-rough strains of *Salmonella*. Proc. Natl. Acad. Sci. USA **53**:147–153.
21. **Nakae, T., and H. Nikaido.** 1971. Multiple molecular forms of uridine diphosphate glucose pyrophosphorylase from *Salmonella typhimurium*. I. Catalytic properties of various forms. J. Biol. Chem. **246**:4386–4396.
22. **Nikaido, H.** 1973. Biosynthesis and assembly of lipopolysaccharide and the outer membrane layer of Gram-negative cell wall, p. 131–208. *In* L. Leive (ed.), Bacterial membranes and cell walls. Marcel Dekker, Inc., New York.
23. **Nikaido, H., M. Levinthal, K. Nikaido, and K. Nakane.** 1967. Extended deletions in the histidine-rough-B region of the Salmonella chromosome. Proc. Natl. Acad. Sci. USA **57**:1825–1832.
24. **Nikaido, H., K. Nikaido, and P. H. Mäkelä.** 1966. Genetic determination of enzymes synthesizing O-specific sugars of *Salmonella* lipopolysaccharides. J. Bacteriol. **91**:1126–1135.
25. **Peters, H., M. Jürs, B. Jann, K. Jann, K. N. Timmis, and D. Bitter-Suermann.** 1985. Monoclonal antibodies to enterobacterial common antigen and to *Escherichia coli* lipopolysaccharide outer core: demonstration of an antigenic determinant shared by enterobacterial common antigen and *E. coli* K5 capsular polysaccharide. Infect. Immun. **50**:459–466.
26. **Rick, P. D., H. Mayer, B. A. Neumeyer, S. Wolski, and D. Bitter-Suermann.** 1985. Biosynthesis of enterobacterial common antigen. J. Bacteriol. **162**:494–503.
27. **Sanderson, K. E., H. Ross, L. Ziegler, and P. H. Mäkelä.** 1972. F^+, Hfr, and F′ strains of *Salmonella typhimurium* and *Salmonella abony*. Bacteriol. Rev. **36**:608–637.
28. **Schmidt, G., H. Mayer, and P. H. Mäkelä.** 1976. Presence of *rfe* genes in *Escherichia coli*: their participation in biosynthesis of O antigen and enterobacterial common antigen. J. Bacteriol. **127**:755–762.
29. **Weisgerber, C., B. Jann, and K. Jann.** 1984. Biosynthesis of the O9 antigen of *Escherichia coli*. Core structure of *rfe* mutant as indication of assembly mechanism. Eur. J. Biochem. **140**:553–556.
30. **Westphal, O., and K. Jann.** 1965. Bacterial lipopolysaccharides: extraction with phenol-water and further applications of the procedure, p. 83. *In* R. L. Whistler (ed.), Methods in carbohydrate chemistry, vol. 5. Academic Press, Inc., New York.
31. **Wilkinson, R. G., P. Gemski, Jr., and B. A. D. Stocker.** 1972. Nonsmooth mutants of *Salmonella typhimurium*: differentiation by phage sensitivity and genetic mapping. J. Gen. Microbiol. **70**:527–554.
32. **Yuasa, R., M. Levinthal, and H. Nikaido.** 1969. Biosynthesis of cell wall lipopolysaccharide in mutants of *Salmonella*. V. A mutant of *Salmonella typhimurium* defective in the synthesis of cytidine diphosphoabequose. J. Bacteriol. **100**:433–444.