JOURNAL OF BACTERIOLOGY, June 1993, p. 3408–3413
0021-9193/93/113408-06$02.00/0

Vol. 175, No. 11

Glycosyl Transferases of O-Antigen Biosynthesis in *Salmonella enterica*: Identification and Characterization of Transferase Genes of Groups B, C2, and E1

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Received 6 November 1992/Accepted 11 March 1993

In *Salmonella enterica*, there is a great variety of O antigens, each consisting of a short oligosaccharide (the repeating unit) repeated many times. The O antigens differ in their sugar composition and glycosidic linkages. The genetic determinants of the O antigen are located in an *rfb* gene cluster, and some, including those of *S. enterica* O serogroups B, C2, and E1, have been cloned and sequenced. In this study of the glycosyltransferases which form the glycosidic linkages, we identify and characterize the four mannosyl and three rhamnosyl transferase genes of the three *rfb* gene clusters.

Lipopolysaccharide (LPS) is an important cell wall antigen in gram-negative bacteria. It consists of three structural domains: lipid A, the oligosaccharide core, and the polysaccharide O antigen. The immunodominant portion of the molecule is the O antigen, which consists of oligosaccharide repeat units (repeating units) of three to six sugars responsible for serological O specificity of bacteria. The immunogenic properties of the O antigen depend entirely on the sugar composition and the linkages between monosaccharides and between repeat units. The basic structures of the repeating units of *Salmonella enterica* O serogroups B and E1 have almost the same backbones (mannose-rhamnose-galactose), the only differences being the linkage between mannose and rhamnose and the addition of an abequose in group B (Fig. 1) (10, 25). The basic structure of the O antigen of *S. enterica* group C2 has the same sugar components as that of group B, but the arrangement of the sugars is very different (Fig. 1) (9).

The mechanisms involved in the assembly of LPS have been studied intensively (17, 18, 22). Each repeating unit is assembled on the cytoplasmic face of the membrane in a sequential series of glycosyl transferase reactions that involves undecaprenol phosphate as an antigen carrier lipid (22). The repeating units are translocated to the periplasmic side, polymerized, and transferred to core-lipid A. Finally, the complete LPS is translocated to the outer membrane of the bacterial cell.

The *rfb* gene cluster encodes the nucleotide sugar biosynthetic pathways and the transferases necessary for assembly of the repeating unit (15). The *rfb* gene clusters of *S. enterica* O groups B, C2, and E1 have been sequenced and analyzed in detail (Fig. 2) (4, 11, 27). Comparison of these *rfb* gene clusters indicated that they all have dTDP-rhamnose and GDP-mannose biosynthetic pathway genes, but abequose biosynthetic pathway genes were found in groups B and C2 only (Fig. 2). The *rfbP* gene, encoding the galactosyl transferase which transfers galactose from UDP-galactose to undecaprenol phosphate, has been identified in all three groups. There is little known about other transferase genes in these *rfb* gene clusters.

In this study, we report the identification of seven transferase genes in *S. enterica* O serogroups B, C2, and E1 and the characterization of the transferase reactions in vitro and discuss the involvement of transferase genes in the evolution of the *rfb* gene cluster.

MATERIALS AND METHODS

Media, growth conditions, bacterial strains, and plasmids. Bacteria were grown in L broth (constituents from Difco) at 37°C with ampicillin (25 μg ml^{-1}) or streptomycin (40 μg ml^{-1}) as required. The bacterial strains and plasmids used in this study are listed in Table 1.

Chemicals, enzymes, and antisera. Restriction enzymes, Klenow polymerase, and T4 DNA ligase were purchased from Boehringer Mannheim or New England Biolabs, Inc. UDP-galactose, GDP-mannose, and decaprenol phosphate (DP) were obtained from Sigma. UDP-D-[U-^{14}C]galactose (200 mCi $mmol^{-1}$) and GDP-D-[U-^{14}C]mannose (100 to 200 mCi $mmol^{-1}$) were obtained from Amersham. Other chemicals were purchased from Sigma or Ajax. *S. enterica* O3 and O10 antisera were obtained from Wellcome Diagnostics.

Synthesis of dTDP-rhamnose. α-D-[U-^{14}C]glucose-1-phosphate (Du Pont Scandinavia AB, Stockholm, Sweden) was enzymatically converted to dTDP-[U-^{14}C]glucose by using glucose-1-phosphate thymidyltransferase, which was from a culture of strain P9254 and was purified to homogeneity in three steps (14). The incubation mixture contained the following chemicals in 0.05 ml: α-D-[U-^{14}C]glucose-1-phosphate (0.48 μmol, 50 μCi); dTTP (0.65 μmol); Tris buffer, pH 8.0 (2.0 μmol); $MgCl_2$ (0.5 μmol); 0.07 U of glucose-1-phosphate thymidyltransferase; and 0.2 U of inorganic pyrophosphatase. The mixture was incubated at 37°C for 90 min. dTDP-L-[U-^{14}C]rhamnose was synthesized from dTDP-D-[U-^{14}C]glucose essentially by a method of Marumo et al. (16). The final reaction volume was 0.1 ml. Control reaction mixtures which contained only cold reagents were run in parallel and analyzed by high-pressure liquid chromatography (HPLC) at different stages of synthesis. The dTDP-L-[U-^{14}C]rhamnose was purified by HPLC on an anion-ex-

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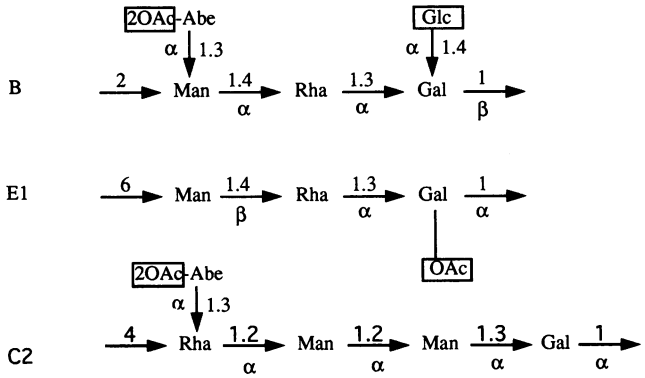

FIG. 1. Basic structure of the *S. enterica* O-antigen repeat unit of serogroups B (10), E1 (25), and C2 (9). The O-acetyl and glucose residues of group B (boxed) are added after assembly and polymerization of the basic structure, are not always present, and are determined by genes outside *rfb* (15). The other O-acetyl residues in groups E1 and C2 (also boxed) probably fit into the same category. Abbreviations: Abe, abequose; Gal, galactose; Glu, glucose; Man, mannose; OAc, O-acetyl residue; Rha, rhamnose.

change column (0.46 by 25 cm) (Supelcosil LC-SAX; Supelco, Inc., Bellefonte, Pa.). The purified dTDP-L-[U-^{14}C]rhamnose (0.30 μmol) contained 30 μCi of radioactivity. dTDP-L-rhamnose was synthesized enzymatically as described earlier (16).

Preparation of membrane fraction. The membrane fraction was prepared as described by Osborn et al. (21). Briefly, 100 ml of culture was grown to mid-log phase (optical density at 530 nm, 0.4) and the cells were collected, washed once with saline, and resuspended in buffer A (50 mM Tris-acetate [pH 8.5], 1 mM EDTA). The cell suspension was subjected to sonication or French press, the unbroken cells were removed by centrifugation at 5,000 rpm for 15 min, and the membranes were sedimented at 20,000 rpm for 1 h in a Beckman centrifuge with a JA20 rotor. The membrane pellet was washed once in buffer A, centrifuged again as described above, and resuspended in 0.3 to 0.5 ml of buffer A.

Assay of transferase activity. The incubation mixture contained 6 nmol of DP (or its intermediate; see below), 50 mM Tris-acetate (pH 8.5), 10 mM $MgCl_2$, 1 mM EDTA, 20 μl of membrane fraction, and 0.33 μM ^{14}C-nucleotide sugar (15,000 cpm) in a final volume of 0.25 ml. Other cold nucleotide sugars or intermediates (see below) were added to the reaction mixture as required to a final concentration of 0.1 mM. The mixture was incubated at 37°C for 15 min, and the reactions were stopped by combining the reaction mixture with 4 ml of chloroform-methanol (2:1). The chloroform-methanol phase was extracted three times with 0.8 ml of pure solvent upper phase (21) and dried by evaporation. The product was dissolved in 4 ml of aqueous scintillant (ACS II from Amersham), and the radioactivity was counted by using a Packard liquid scintillation counter. Note that the DP in the reaction mixture acts as a carrier lipid.

Preparation of intermediate by *n*-butanol extraction. When the product of a transfer reaction was required for a subsequent assay, the reaction was carried out with cold nucleotide sugar(s) (0.1 mM) and the reaction mixture was extracted twice with an equal volume of *n*-butanol. The extracts were combined and washed twice with an equal volume of water (12). The *n*-butanol phase was then mixed with an equal volume of water and freeze-dried in order to remove the butanol and concentrate the intermediate. The prepared intermediate could be used immediately or stored at −20°C.

DNA methods and computer analysis. DNA purification, restriction enzyme digestion, ligation, and transformation were as described by Sambrook et al. (26). The deduced amino acid sequences were analyzed by using the following programs in the Genetics Computer Group (University of Wisconsin) package: Bestfit (6) for comparing the sequences and Pepplot (5) and Peptidestructure (5, 8) for the prediction of the secondary structures. The potential transmembrane segments were predicted by using programs based on the methods of Eisenberg et al. (7) and the program ALOM (NIH programs; M. Kanehisa, National Institutes of Health) by the method of Klein et al. (13).

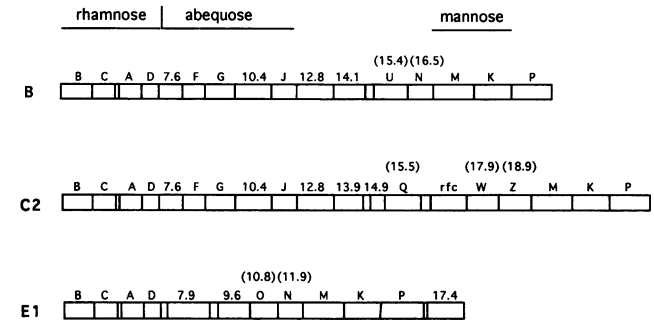

FIG. 2. *rfb* gene clusters of *S. enterica* O serogroups B (11), E1 (27), and C2 (4). The genes for biosynthesis of the nucleotide sugars dTDP-rhamnose, CDP-abequose, and GDP-mannose are shown. UDP-galactose is synthesized by housekeeping genes outside *rfb*. The letters indicate the genes that have been identified. The identification of pathway genes and *rfbP* was described previously (11). Transferase genes *rfbN*, *rfbU*, *rfbW*, *rfbZ*, *rfbQ*, and *rfbO* are identified in this study. The numbers, which correspond to the start position of each open reading frame in the sequences, indicate the open reading frames for which a function has not yet been identified.

RESULTS

Identification of the transferase genes. The mannosyl and rhamnosyl transferase genes of *S. enterica* group B strain LT2 were identified by enzyme assay of membrane fractions from the cells carrying cloned genes. The plasmid pPR686 (11), carrying the *rfbP* gene, was transferred to strain P9031, which lacks all the putative transferase genes, and the resulting strain is referred to as P9297. Galactose transferase activity could be detected in this strain (Fig. 3). After plasmid pPR1208, containing only *orf16.5*, was transferred into strain P9297, incorporation of [^{14}C]rhamnose in the presence of UDP-Gal could be detected (Fig. 3), indicating rhamnosyl transferase activity. Membranes from P9297/pPR1303 (carrying *orf15.4* and *orf16.5*) showed mannosyl transferase activity in the presence of UDP-Gal, dTDP-Rha, and [^{14}C]GDP-Man (Fig. 3). These results indicate that *orf16.5* is the rhamnosyl transferase gene named *rfbN* and *orf15.4* is the mannosyl transferase gene, now named *rfbU*, of *S. enterica* LT2.

orf11.9 of *S. enterica* group E1 is homologous to *orf16.5* (*rfbN*) of group B, and the two genes have 75.2% identity and 84.9% similarity at the amino acid level. The function of *orf11.9* was confirmed by rhamnose incorporation using membrane from P9297/pPR1135 (Fig. 3), indicating that *orf11.9* is *rfbN*, encoding rhamnosyl transferase in group E1. Plasmid pPR1330, carrying *orf9.6*, *orf10.8*, and *rfbN* of

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Lab stock no.	Characteristics	Source or reference
E. coli K-12 strain			
Delete4	P3127	*trpC recA rpsL* Δ*4(galK-attL)*	1
Salmonella LT2 strains[a]			
SL1654	P9003	*hsdL6 trpC2 fliB flaA66 rpsL120 xylT404 ilvE452 metE551 metA22 hsdSA29*	20
	P9029	Δ*(hisO-metG)388*	19
	P9031	Δ*(flaA-rfbH)801*	19
	P9254	P9029 carrying pPR945	This study
	P9297	P9031 carrying pPR686	This study
Plasmids			
pPR686		*rfbK*, *rfbP*, and part of *rfbM* of *S. enterica* group B in vector pGB2	11
pPR945		Derivative of pPR645 (2) with 8.5-kb insert from positions 2.9 to 11.4, which carries first 7 genes of *rfb* gene cluster of group B, including rhamnose pathway, in vector pKO1	This study
pPR1135		pT7T3 with 2.7-kb *Bgl*II-*Bam*HI fragment from pPR966 (27) from positions 11.2 to 13.9, carrying *orf11.9* of *S. enterica* group E1	This study
pPR1208		pT7-7 with 1-kb PCR[b] product from positions 16.4 to 17.4, carrying *orf16.5* of *S. enterica* group B	This study
pPR1295		Derivative of pPR984 (3) with 8.8-kb insert from positions 16.3 to 25.1, which carries *orf16.7*, *orf17.9*, *orf18.9*, *rfbM*, *rfbK*, and *rfbP* of *S. enterica* group C2	This study
pPR1299		Derivative of pPR984 (3) with 6.4-kb insert from positions 18.7 to 25.1, which carries *orf18.9*, *rfbM*, *rfbK*, and *rfbP* of *S. enterica* group C2	This study
pPR1303		pUC18 with 2.5-kb *Hin*dIII fragment from pPR281 (2) from positions 15.3 to 17.8, carrying *orf15.4* and *orf16.5* of *S. enterica* group B	This study
pPR1328		Derivative of pPR984 (3) with 8.3-kb insert from positions 16.8 to 25.1, which carries *orf17.9*, *orf18.9*, *rfbM*, *rfbK*, and *rfbP* of *S. enterica* group C2	This study
pPR1329		Derivative of pPR984 (3) with 9.7-kb insert from positions 15.4 to 25.1, which carries *orf15.6*, *rfc*, *orf17.9*, *orf18.9*, *rfbM*, *rfbK*, and *rfbP* of *S. enterica* group C2	This study
pPR1330		pUC18 with 4.2-kb *Eco*RI-*Bam*HI fragment from pPR966 (27) from positions 9.7 to 13.9, carrying *orf9.6*, *orf10.8*, and *orf11.9* of *S. enterica* group E1	This study
pPR1332		Derivative of pPR1330 which has a point mutation at *Nco*I site at position 11.0 in *orf10.8*	This study
pPR1426		Derivative of pPR1328. A frameshift mutation was introduced in *orf18.9* by *Bsp*E1 digestion at position 19.3, end-filling, and religation.	This study

[a] Serovar typhimurium group B.
[b] PCR, polymerase chain reaction.

group E1, was transferred into P9297. The resulting strain expressed *Salmonella* group E1 O antigen (factors O3 and O10), as detected by agglutination. This suggested that either *orf9.6* or *orf10.8* encodes the mannosyl transferase. A frameshift mutation was introduced into pPR1330 at the unique *Nco*I site in the proximal half of *orf10.8* by *Nco*I digestion, end-filling with Klenow polymerase, and blunt-end ligation. The resulting plasmid, named pPR1332, carries only *orf9.6* and *rfbN*, and when transferred into P9297, it did not confer the mannosyl transferase function (Fig. 3), showing that *orf10.8* is the mannosyl transferase gene (*rfbO*) in group E1.

To identify the glycosyltransferases in *S. enterica* group C2, a number of plasmids were made and transferred into P3127, an *Escherichia coli* strain which carries a deletion of the *gal* operon (1) and which cannot synthesize UDP-galactose, so that no oligosaccharide intermediate can be synthesized in vivo. The plasmids all carry *rfbM* and *rfbK* of the mannose biosynthetic pathway, which should not affect the current assay, and also *rfbP*, which is necessary for the first transfer reaction. The plasmid pPR1299 carries only *orf18.9* in addition to the genes named above, and the membranes from P3127 carrying this plasmid have mannosyl transferase activity (Fig. 3), indicating that *orf18.9* encodes the enzyme which transfers mannose to Gal-decaprenol PP_i (Gal-PP_i-D) to form the intermediate Man-Gal-PP_i-D. Thus, *orf18.9*, the first mannosyl transferase gene, now named *rfbZ*, of group C2.

The product, Man-Gal-PP_i-D, of the above reaction was extracted and used as a substrate to assay membranes from strains carrying pPR1328 or pPR1299. The strain carrying pPR1328 could add mannose to the intermediate (Man-Gal-PP_i-D) much more efficiently than the strain carrying pPR1299, presumably to form Man-Man-Gal-PP_i-D (Fig. 3). The difference between pPR1328 and pPR1299 is that

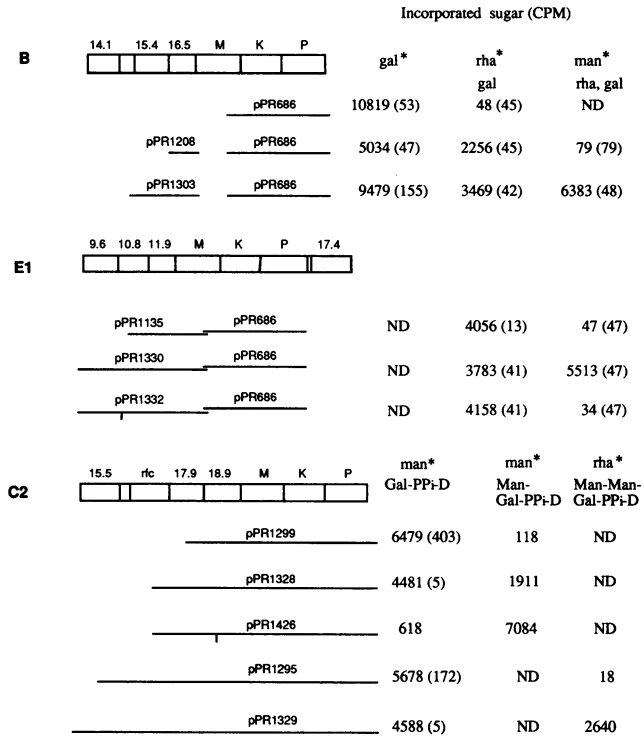

FIG. 3. Identification of transferase genes. For each O serogroup, B, E1, and C2, the boxes represent genes and open reading frames present in the relevant regions of *rfb*. The lines indicate plasmids carried by the strains used for membrane preparation for enzyme assay. Membrane from P9029 was used as a negative control for group B and group E1, and membrane from P3127 was used for group C2 (data shown in parentheses). The transferases were assayed by detection of ^{14}C-sugar transfer onto the lipid-linked intermediate in the presence of DP and the nonradioactive nucleotide sugars required for the previous steps. Asterisks indicate the radioactive sugar components. Man-Gal-PP$_i$-D and Man-Man-Gal-PP$_i$-D are the intermediates extracted from P3127 carrying pPR1299 and pPR1328. The mutation sites in pBR1332 and pPR1426 are indicated. Abbreviations: CPM, counts per minute; man, GDP-Man; rha, dTDP-Rha; gal, UDP-Gal; ND, not determined.

pPR1328 also carries *orf17.9*, which indicates that *orf17.9* is the second mannosyl transferase gene, now named *rfbW*, of group C2. Rhamnosyl transferase function could be detected in the strain carrying pPR1329 but not in the strain carrying pPR1295 when the intermediate (Man-Man-Gal-PP$_i$-D) from the strain carrying pPR1328 was used as the substrate (Fig. 3). This result indicates that *orf15.5* is the rhamnosyl transferase gene, now named *rfbQ*, of group C2.

Transferase specificity. The specificities of the transferases were tested by assaying their activities with different substrates. The results showed that in group B, the rhamnosyl transferase transfers rhamnose only when UDP-Gal is present and hence not to DP alone, and the mannosyl transferase transfers mannose to Rha-Gal-PP$_i$-D only, not to Gal-PP$_i$-D or DP (Table 2). The first mannosyl transferase in group C2 (*rfbZ*) could transfer mannose only to Gal-PP$_i$-D, not to DP or Man-Gal-PP$_i$-D, while the second mannosyl transferase could transfer mannose to Man-Gal-PP$_i$-D and had only very low activity with DP or Gal-PP$_i$-D (Table 3). The group C2 rhamnosyl transferase could recognize only Man-Man-Gal-PP$_i$-D as a substrate and transferred rhamnose to it but not to any other substrate (Table 3). These

TABLE 2. Substrate requirements for sugar incorporation in group B[a]

Addition(s) to reaction mixture (nonradioactive)	Incorporation of radioactively labelled sugar (cpm)[b]		
	[^{14}C]UDP-Gal	[^{14}C]dTDP-Rha	[^{14}C]GDP-Man
DP	5,779 (227)	240 (40)	488 (35)
UDP-Gal, DP		2,611 (40)	445 (35)
dTDP-Rha, UDP-Gal, DP			6,432 (35)

[a] Membrane extracts were from P9003 (or P9029 for the negative control strain).
[b] Numbers in parentheses indicate radioactive counts for the negative control strain.

results show that the transferases act in sequence in the assembly of a particular repeating unit and cannot function until completion of the previous step. The low levels of incorporation observed when the substrate was incomplete are presumably due to incomplete substitution in the preparation of the intermediate or to the presence of an endogenous substrate.

DISCUSSION

We have identified the three rhamnosyl transferase genes and the four mannosyl transferase genes in *S. enterica* serogroups B, E1, and C2. Together with the previously identified galactosyl transferase gene (11), these constitute all the transferase genes, except the two abequose transferases expected for the basic structure of the three repeating units (Fig. 1). The program Bestfit was used to compare the deduced amino acid sequences of the transferases. The degree of identity between the transferases was found to be very low, except between the two RfbN proteins in group B and group E1 mentioned above, which both add rhamnose in α(1,3) linkage to Gal-PP$_i$-U and are similar proteins with 75% amino acid identity and no gaps in the alignment (Table 4). In order to establish the significance of the alignments, we did a shuffle test with the program Bestfit. It was found that except for RfbO, the quality of the alignments between mannosyl transferases and those between rhamnosyl transferases did exceed the mean quality of the alignments with randomly shuffled sequences by a value of five times the standard deviation (data not shown), indicating that the identity is not by chance. The alignments between different types of transferases turned out to be not significant (data not shown). Thus the three α-mannosyl transferases and the

TABLE 3. Substrate specificities for transferases of group C2[a]

Acceptor	Incorporation of radioactively labelled sugar (cpm)[b]		
	rfbZ (pPR1299)	*rfbW* (pPR1426)	*rfbQ* (pPR1329)
DP	443	791	86
Gal-PP$_i$-D	7,826	618	79
Man-Gal-PP$_i$-D	118	11,428	46
Man-Man-Gal-PP$_i$-D			2,294

[a] Membranes from strains P9297 P3127/pPR1299, and P3127/pPR1328 are used for synthesis of Gal-PP$_i$-D, Man-Gal-PP$_i$-D, and Man-Man-Gal-PP$_i$-D, respectively. Reaction mixtures contained membranes from P3127, carrying the plasmid indicated, as the source of transferases.
[b] Radioactivity incorporated from GDP-[^{14}C]Man for *rfbZ* and *rfbW* and from dTDP-[^{14}C]Rha for *rfbQ*.

TABLE 4. Comparison of transferase genes at the amino acid level[a]

rfb	Group	Bond[b]	% Identity with:						
			P	U	W	Z	O	N (group B)	N (group E1)
Q	C2	Rhaα	17.5	18.8	15.9	17.5	18.1	**22.1**	**20.0**
N	E1	Rhaα	18.2	16.2	15.8	15.0	17.5	**75**	
N	B	Rhaα	19.8	17.8	15.7	17.5	19.7		
O	E1	Manβ	16.6	16.4	25.7	15.0			
Z	C2	Manα	17.6	**23.4**	**21.5**				
W	C2	Manα	19.1	**26.3**					
U	B	Manα	16.5						

[a] The sequences were compared by using the program Bestfit with a gap weight of 3 and a length weight of 0.1. Abbreviations: N, *rfbN*; O, *rfbO*; P, *rfbP*; Q, *rfbQ*; U, *rfbU*; W, *rfbW*; Z, *rfbZ*.

[b] Rhaα, Manα, and Manβ, enzyme forms α-rhamnosyl, α-mannosyl, or β-mannosyl linkage, respectively. Boldface type indicates that the quality of the alignments was significant as indicated by using randomly shuffled sequences (higher than average by 5 times the standard deviation, whereas the others range from −1.5 to +2.3 standard deviations.

three rhamnosyl transferases form two families, but any relationship between them or with the one β-mannosyl transferase is too distant to be detected by our analysis. The *rfbO* gene, which is the mannosyl transferase gene in group E1, forms β linkage and stands outside the α-mannosyl transferase family (Fig. 1).

The predicted secondary structures all consist of alternating α helices and β sheets, but conserved motifs were not found.

The galactosyl transferase has five transmembrane segments, and it has been predicted to be an integral membrane protein (11). In contrast, no significant transmembrane segments have been found in any of the other identified transferases, and the basis of their membrane association is not yet known.

The two families of transferase genes have low G+C contents (33 to 37%) (Table 5) compared with the average G+C content (51 to 54%) of *S. enterica* chromosomal DNA (24). The nucleotide sugar pathway genes have already been identified, and the transferase genes are now located to the central, highly variable regions of previously unidentified genes. It is not clear whether they evolved in situ or by gene capture.

In group B, only two genes remain to be identified, and one, *orf12.8*, is thought not to be a transferase gene (11), leaving *orf14.1* as the presumptive abequose transferase gene. In group C2, *orf12.8* is thought to be a homolog of *orf12.8* in group B and *orf14.9* is thought to encode an acetyltransferase, leaving *orf13.9* as the only remaining unidentified open reading frame and presumably the abequose transferase gene. The positively identified transferase genes other than *rfbP* are contiguous in groups B and E1, and this applies also to the presumptive abequose transferase gene of group B, but in group C2, the transferase gene cluster is interrupted by the *rfc* gene and, if our presumptions are correct, by an acetyltransferase gene. The transferase genes are also in each case in the order in which the products act, but this is presumably a coincidence. The three gene clusters are clearly related, with homologous genes in the same order (Fig. 2). The transferase genes other than *rfbP* are very divergent, and clearly the divergence of these genes is very ancient. Some of the evolutionary implications have been discussed recently (23).

TABLE 5. G+C contents of the transferase genes[a]

Gene	% G+C
rfbO	33.7
rfbN (group E1)	37.3
rfbU	33
rfbN (group B)	37.5
rfbQ	34.6
rfbW	34.3
rfbZ	36.8

[a] The features of the G+C contents of the genes of groups E1, B, and C2 are as reported by Wang et al. (27), Jiang et al. (11), and Brown et al. (4), respectively.

ACKNOWLEDGMENTS

This work was supported by grants from the Australian Research Council and the Swedish Medical Research Council (grant 16X-656).

We thank Gordon Stevenson and Wang Lei from the laboratory of the University of Sydney, who made plasmids pPR1208 and pPR1135, and we thank Peter Brown for most of the C2 plasmids.

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