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Molecular Analysis of the *rfb* O Antigen Gene Cluster of *Salmonella enterica* Serogroup O:6,14 and Development of a Serogroup-Specific PCR Assay

Collette Fitzgerald,* Rachel Sherwood, Linda L. Gheesling, Frances W. Brenner, and Patricia I. Fields

Foodborne and Diarrheal Diseases Branch, Division of Bacterial and Mycotic Diseases, National Center for Infectious Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia 30333

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The Kauffmann-White scheme for serotyping *Salmonella* recognizes 46 somatic (O) antigen groups, which together with detection of the flagellar (H) antigens form the basis for serotype identification. Although serotyping has become an invaluable typing method for epidemiological investigations of *Salmonella*, it does have some practical limitations. We have been characterizing the genes required for O and H antigen biosynthesis with the goal of developing a DNA-based system for the determination of serotype in *Salmonella*. The majority of the enzymes involved in O antigen biosynthesis are encoded by the *rfb* gene cluster. We report the sequencing of the *rfb* region from *S. enterica* serotype Sundsvall (serogroup O:6,14). The *S. enterica* serotype Sundsvall *rfb* region is 8.4 kb in length and comprises six open reading frames. When compared with other previously characterized *rfb* regions, the serogroup O:6,14 sequence is most related to serogroup C_1. On the basis of DNA sequence similarity, we identified two genes from the mannose biosynthetic pathway, two mannosyl transferase genes, the O unit flippase gene and, possibly, the O antigen polymerase. The whole cluster is derived from a low-G+C-content organism. Comparative sequencing of an additional serogroup O:6,14 isolate (*S. enterica* serotype Carrau) revealed a highly homologous sequence, suggesting that O antigen factors O:24 and O:25 (additional O factors associated with serogroup O:6,14) are encoded outside the *rfb* gene cluster. We developed a serogroup O:6,14-specific PCR assay based on a region of the putative *wzx* (O antigen flippase) gene. This provides the basis for a sensitive and specific test for the rapid identification of *Salmonella* serogroup O:6,14.

An estimated 1.4 million cases of salmonellosis and 556 (31%) estimated food-related deaths are attributed to *Salmonella* each year in the United States (24). *Salmonella* isolates are serotyped according to the Kaufmann-White scheme by using somatic (O) and flagellar (H) antigens, with new serotypes registered in annual updates of the scheme as they are detected (29). Since the establishment of this scheme in the early 1930s (33), the public health value of this phenotypic typing method has been well proven (28). State public health laboratories in the United States began reporting the results of *Salmonella* serotype determination to the Centers for Disease Control and Prevention (CDC) in 1963, and this surveillance system has been critical for improving prevention. It has allowed not only the rapid detection, identification of sources, and control of outbreaks, but also identification of emerging serotypes and new mechanisms of transmission (40).

The 2,523 different serotypes currently described in the Kaufmann-White scheme (29) are derived from the considerable number of permutations of 46 O serogroups, 11 additional O antigens, and 119 H antigens (3). The O antigen is the outermost component of lipopolysaccharide (LPS), an immunogenic glycolipid that is a major component of the outer membrane in gram-negative bacteria (31, 35). A considerable amount of diversity is seen within *Salmonella* O antigens, which are composed of multiple repeats of an oligosaccharide unit (O unit), and they contribute major antigenic diversity to the cell surface, which is used to serotype *Salmonella* isolates. The basis of the variation in O antigen structure is represented by differences in sugar composition, arrangement of the sugars in the O unit, the specific linkages between the O units, and the addition of branch sugars and modifying side groups.

Much of the *Salmonella* O antigen variation is a consequence of the extensive genetic diversity within *rfb* (O antigen) gene clusters, which encode many of the enzymes involved in O antigen biosynthesis and assembly. Typically, three different types of genes are seen within *rfb* gene clusters: (i) genes that encode enzymes involved in the synthesis of the sugars that form the O subunit; (ii) genes that encode transferases, which assemble sugar substituents into the O subunit; and (iii) genes that encode proteins involved in processing and assembly steps to build the O antigen from the O subunit, such as the O antigen transporter (*wzx*) and O antigen polymerase (*wzy*).

Wzx proteins are involved in the transport of completed O antigen subunits across the cytoplasmic membrane (21). The mechanism of O antigen export in *Salmonella enterica* has been shown to be Wzx dependent, and all of the *Salmonella rfb* gene clusters sequenced to date have a *wzx* gene. The function of the O antigen polymerase is in the polymerization of the O units to form the O antigen, which is encoded by the *wzy* gene (formerly *rfc*). While most *S. enterica rfb* gene clusters contain a

* Corresponding author. Mailing address: National *Salmonella* Reference Laboratory, Foodborne and Diarrheal Diseases Branch, Mailstop CO3, Division of Bacterial and Mycotic Diseases, National Center for Infectious Diseases, Centers for Disease Control and Prevention, Atlanta, GA 30333. Phone: (404) 639-0838. Fax: (404) 639-3333. E-mail: chf3@cdc.gov.

wzy homolog, serogroups B and D have a *wzy* gene elsewhere in the genome.

The *rfb* gene clusters from 10 *Salmonella* serogroups have been studied: A (19), B (14, 45), C_1 (18), C_2 (4), D_1 (19), D_2 (47), D_3 (6), E_1 (42), O:54 (16), and O:35 (44). The *rfb* regions from serogroups A, B, C_2, D, and E, which all have a trisaccharide O subunit containing mannose, rhamnose, and galactose, are related. The *rfb* gene cluster from serogroup C_1, whose O subunit is composed of four mannose residues, one *N*-acetylglucosamine residue, and a glucose side branch, shows little homology to them (18). The *S. enterica* O:35 *rfb* gene cluster encodes the same O antigen as *Escherichia coli* O111, and they have been shown to have closely related gene clusters (44). Synthesis of the O:54 antigen is mediated by a plasmid-encoded gene cluster (16). More recently, the genetic variations in the dDTP-L-rhamnose (*rml*) (30) and GDP-mannose (*man*) pathway genes of an additional 11 and 13 serogroups that contain rhamnose and mannose in their respective O antigen structures have been described (13).

Little is known about the *rfb* gene clusters from other *Salmonella* O serogroups. As part of a larger project to develop a DNA-based approach for serotyping *Salmonella* (7), we have sequenced the *rfb* gene cluster from two serogroup O:6,14 isolates, *Salmonella enterica* serotype Sundsvall (I 6,14,25:z:e,n,x) and *S. enterica* serotype Carrau (I 6,14,24:y:1,7), with the aim of identifying serogroup-specific DNA targets. We found that the serogroup O:6,14 *rfb* gene cluster is most related to serogroup C_1. Comparison of the *S. enterica* serotypes Sundsvall and Carrau *rfb* sequences, which differ in the expression of O factor 24 or 25, revealed essentially no difference in the *rfb* region between the two serogroup O:6,14 isolates. We targeted the *wzy* gene in a PCR detection assay and found the sequence to be specific for serogroup O:6,14.

MATERIALS AND METHODS

Strains. *S. enterica* serotype Sundsvall strain #185 and *S. enterica* serotype Carrau strain 384-90 were obtained from the *Salmonella* Reference Laboratory collection at the CDC. Serotyping was performed with somatic O and H antisera according to the Kaufmann-White scheme as previously described (2, 3). For the evaluation of the serogroup O:6,14-specific PCR assay, 443 strains were selected from the *Salmonella* Culture Collection in the National *Salmonella* Reference Laboratory at CDC. The panel included representatives of all top 100 serotypes and at least one isolate from each of the different subspecies within each of the 46 O antigen serogroups. All isolates were cultivated at 37°C overnight on blood agar base (BAB) medium under aerobic conditions.

DNA manipulation. General DNA procedures and bacterial transformations were performed as previously described (34). The *rfb* region was amplified by PCR (Expand Long PCR kit; Roche, Indianapolis, Ind.) with oligonucleotide primers corresponding to the 5′ end of *gnd* and the middle of the JUMPstart sequence as previously described (43). The 8.4-kb amplicon was doubly digested with *Eco*RI and *Cla*I (New England Biolabs), and the restricted fragments were cloned into DH5α (Gibco BRL). Three clones of different sizes were identified.

DNA sequencing. The ends of each of the fragments were sequenced with universal primers, and the sequences were extended via primer walking. Sequencing was performed on an PE Applied Biosystems 377 automated DNA sequencer with BigDye terminator cycle sequencing ready reaction mix according to the manufacturer's instructions (PE Applied Biosystems, Foster City, Calif.). DNA sequence data were processed by using Lasergene 99 (DNASTAR, Madison, Wis.) software and assembled into a contiguous sequence. The TMpred program (12) was used to predict transmembrane domains. This program uses an algorithm based on the statistical analysis of a database of naturally occurring transmembrane proteins to predict membrane-spanning regions and protein orientation. The National Center for Biotechnology Information (NCBI) BLAST network server was used to search sequence databases for sequences with homology to the open reading frames (ORFs) found in our sequence.

Serogroup O:6,14 PCR assay. Bacterial genomic DNA extracted with the Puregene DNA isolation kit (Gentra Systems, Minneapolis, Minn.) was resuspended in sterile distilled water at a concentration of 50 ng/μl. A total of 46 pools were made, with 1 to 12 samples of DNA per pool. A forward primer (5′-GTC TCCGCTAAGCTATTTCGGTTTGTA-3′) and a reverse complementary sequence primer (5′-TACCGCAATAATTCAATCACAAGGG-3′) were derived from sequence within *wzy* to generate a 501-bp PCR amplicon. PCR amplification was performed in 25-μl volumes with Ready-to-Go PCR beads (Amersham Biosciences, Piscataway, N.J.). PCR amplification was carried out in a thermal cycler (MJ Research, Waltham, Mass.) with the following cycle parameters: initial denaturation, 96°C for 2 min; followed by 25 cycles of 94°C for 30 s (denaturation), 58°C for 30 s (annealing), and 72°C for 45 s (extension); and a final extension at 72°C for 10 min. Amplification products were analyzed with a bufferless E-Gel 96 high-throughput agarose electrophoresis system (Invitrogen, Carlsbad, Calif.) and a UV transilluminator (GelDoc1000; Bio-Rad). If a positive PCR result was seen for a particular pool, all DNA samples from the pool were individually retested by PCR, and amplification products were then confirmed by conventional agarose gel elecrophoresis with 1% (wt/vol) agarose gels.

Nucleotide sequence accession number. The sequences of the rfb O antigen gene cluster determined in this study have been deposited in the GenBank database under accession no. AY 334017.

RESULTS

DNA sequence of the *rfb* region from *S. enterica* serotypes Sundsvall and Carrau. Primers located in the middle of the JUMPstart sequence and the 5′ end of the *gnd* gene, which flank the *rfb* region, were used to amplify the entire *rfb* gene cluster from *S. enterica* serotypes Sundsvall and Carrau, and the nucleotide sequence was determined. Analysis of the DNA sequence revealed 8.4 kb flanked by JUMPstart and *gnd* and the presence of six ORFs (Fig. 1 and Table 1). Alignment of the DNA sequences from *S. enterica* serotypes Sundsvall (O:6,14,25) and Carrau (O:6,14,24) revealed only 3 nucleotide differences between the two sequences. One of these base differences occurred within ORF 3.72, and one occurred within ORF 5.33: they were silent substitutions. The third nucleotide difference was in noncoding sequence.

The ORFs of the DNA coding strand all have the same transcriptional direction from JUMPstart to *gnd*. All ORFs started with an ATG codon. Three ORFs (1.12, 2.33, and 3.72) ended with a TAA codon, two ORFs (5.33 and 6.67) ended with a TGA codon, and one ORF (0.13) ended with a TAG codon. The sites for putative translation starts and potential Shine-Dalgarno ribosomal binding sites are shown in Table 2. Noncoding sequences of 1, 4, and 155 bp were found preceding the start codons of ORFs 2.33, 3.72, and 5.33, respectively. All of the other ORF junctions have overlapping termination and initiation codons. There is a 509-bp region between the end of ORF 6.67, the last gene in the serogroup O:6,14 *rfb* gene cluster, and the start codon of *gnd*. The size of the intergenic region between the last *rfb* gene and *gnd* in different *S. enterica* serogroups is shown in Table 3. Putative gene designations were made based on knowledge of O antigen structure homology and to previously characterized genes. The similarities in the sequences of the deduced polypeptides of the encoded phosphomannomutase, guanosine diphosphomannose (GDP-man) pyrophophorylase, and mannosyl transferases are shown in Tables 4 and 5.

PCR identification of serogroup O:6,14. Primers within the coding sequence of *wzx* were designed. PCR was performed on each of the 46 DNA pools corresponding to the 46 O serogroups. A 501-bp amplicon was generated with only one pool; the one containing serogroup O:6,14 DNA. All other pools,

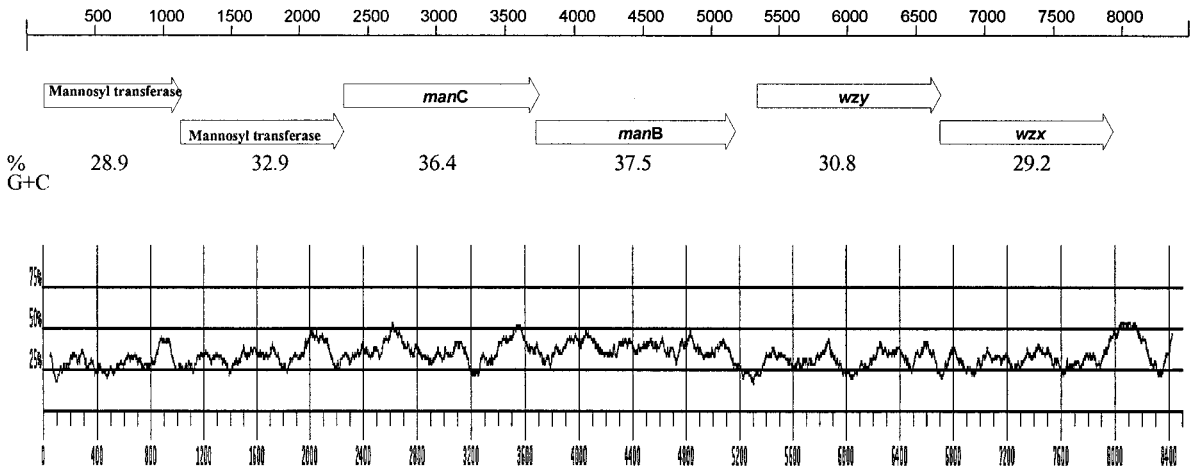


FIG. 1. O antigen gene cluster of *S. enterica* serogroup O:6,14. Putative ORFs are represented by arrows, with the corresponding assignment of gene name. The percentage G+C ratios were calculated and plotted for each 100 bases.

containing DNA from 408 strains representing the other 45 serogroups, were negative in the PCR assay. DNA from all 12 of the strains in the O:6,14 pool were individually tested by PCR, and all generated a PCR product of the expected size (Fig. 2). An additional 21 O:6,14 strains were tested, and all generated the expected 501-bp PCR product. The 33 serogroup O:6,14 strains tested represented all of the five subspecies within this serogroup.

DISCUSSION

We have sequenced the *rfb* gene cluster from two *S. enterica* serogroup O:6,14 isolates. Of the previously characterized *rfb* regions, the serogroup O:6,14 *rfb* region was most similar to the C_1 *rfb* region. Both of these serogroups have O6 and O14 in common. On the basis of DNA sequence similarity, we have putatively identified all of the six ORFs.

Mannose biosynthetic genes. *manB*, encoding phosphomannomutase, and *manC*, encoding mannose-1-phosphate guanylytransferase, are responsible for the biosynthesis of GDP-mannose from mannose-6-phosphate (13). Mannose is present in the O antigen side chain of serogroup O:6,14 (22), and the *rfb* gene cluster of this serogroup contained two genes with sufficient similarity to identify them as *manB* and *manC*, respectively. At the amino acid level, serogroup O:6,14 *manC* (ORF 2.33) had 58% identity to *manC* from serogroup C_1 and 57% identity to *manC* from serogroup B. Serogroup O:6,14 *manB* (ORF 3.72) had 58 and 57% amino acid identity to the *manB* genes from serogroups C_2 and B, respectively, with much lower homology to *manB* from serogroup C_1 (14.9%). Both genes from the *S. enterica* serotype Carrau strain had 100% identity to a previously published sequence of *manC* and *manB* from *S. enterica* serotype Carrau (13). Two genes from the *cps* gene cluster, *cpsB* and *cpsG*, are considered isogenes to *manC* and *manB* (37). The serogroup O:6,14 *manC* and *manB* genes share a similar level of identity to the respective isogenes of serogroup B, as previously reported for LT2 (37).

Mannosyl transferase genes. ORF 0.13 of serogroup O:6,14 shared 47% identity with *wbaC* (ORF 6.17) from *S. enterica* serotype Choleraesuis (serogroup C_1). Both ORFs have a low G+C content; 29% for serogroup O:6,14 and 30% for serogroup C_1, respectively. ORF 1.12 shared 49% identity with *wbaD* (ORF 7.17) in serogroup C_1 (*S. enterica* serotype Choleraesuis), and again they have a low G+C content: 33% for serogroup O:6,14 and 31% for serogroup C_1. The *wbaC* and *wbaD* genes from the *rfb* gene cluster of serogroup C_1 are thought to encode the mannosyl transferases that assemble the serogroup C_1 O antigen subunit (18). Until now, however, the sequences of these genes had shown little similarities to the *rfb* region of other *Salmonella.* Although mannosyl transferases have been identifed in *rfb* regions from serogroups A, B, D, C_2, and E_1 (19, 20), they share little homology to *wbaC* and *wbaD* (Table 5), suggesting that different specific functions are performed by these transferases from the different serogroups.

TABLE 1. Characteristics of each ORF, including gene identity and length, G+C content of each gene, and *P* values for the corrected average G+C contents for codon positions 1, 2, and 3

ORF	Gene or gene product	Length (bp)	G+C content (%)	*P* value for codon position		
				P1	P2	P3
0.13	Mannosyl transferase	1,005	28.9	38.8	28.6	19.5
1.12	Mannosyl transferase	1,203	32.9	43.1	31.1	24.7
2.33	*manC*	1,416	36.4	51.2	34.9	23.3
3.72	*manB*	1,428	37.5	48.0	39.6	25.0
5.33	*wzy*	1,344	30.8	36.1	30.7	25.7
6.67	*wzx*	1,254	29.2	35.6	27.7	24.4

TABLE 2. Features of the initiation regions of the ORFs of *S. enterica* serotype Sundsvall strain #185[a]

ORF	Initiation region sequence
0.13	TTTAAGAGTAAAT**ATG**
1.12	GAATTGAGAAAAATTT**ATG**AGTAGTC
2.33	ATGGAGAGTGTTAAATAAA**ATG**
3.72	GTTGGAGAAAAATAAAATT**ATG**
5.33	TATATTTGTTGATAAAA**ATG**
6.67	CAGTAGAATCAAA**ATG**ATCAA

[a] Start codons are shown in boldface. Potential Shine-Dalgarno sequences are underlined.

TABLE 3. Comparison of the size of the intergenic region between the last gene in the *rfb* gene cluster and the start of *gnd* for a number of *Salmonella* serogroups

Salmonella serogroup (serotype)	Last gene in *rfb* cluster	Distance (bp) from last gene to start of *gnd*
B (Typhimurium)	*wbaP*	163
E_1 (Anatum)	*wbaE*	251
C_1 (Montevideo)	*wzx*	136
O:35 (Adelaide)	*wbdM*	180
O:6,14 (Sundsvall)	*wzx*	509

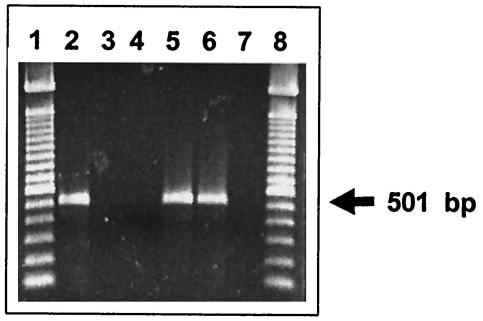


FIG. 2. PCR amplification products with serogroup O:6,14-specific primers using template DNA from different *S. enterica* serogroups. Lanes 1 and 8 contain a 100-bp ladder (Invitrogen, Carlsbad, Calif.). Lanes 2, 5, and 6 represent serogroup H strains; lane 3 represents a serogroup B strain, lane 4 represents a serogroup C_2 strain, and lane 7 represents the negative control.

O antigen transport and polymerase genes. Both ORFs 5.33 and 6.67 had no significant homology to any sequences in GenBank. Proteins Wzx and Wzy from different O antigen gene clusters have little sequence homology even at the amino acid level; however, their predicted structural homology (multiple transmembrane domains) has been used to putatively identify them. Analysis with the TMpred program (12) showed that ORFs 5.33 and 6.67 contained 13 and 12 transmembrane helices, respectively (Fig. 3A and B). As is characteristic of flippases and O antigen polymerases, both of these predicted proteins are hydrophobic.

The serogroup O:6,14 Wzx was most homologous to other *wzx* gene products from *S. enterica* serogroups A, B, and C_2 and *E. coli* (09a:k30, O111, K12), *Shigella sonnei*, and related organisms when a PSI-blast search (1) was performed. The predicted protein also shared some homology to the conserved motif found near the amino-terminal end of *wzx* gene products (38). We believe that ORF 6.67 is the *wzx* gene, and as in *Salmonella* serogroup C_1, this gene is at the distal end of the *rfb* gene cluster.

O antigen polymerases share a characteristic secondary structure of multiple transmembrane regions and a large cytoplasmic loop. Serogroup H *wzy* encoded a protein predicted to have a similar secondary structure. However, there was no sequence homology between this ORF and any of the other known O antigen polymerases. When the predicted protein of ORF 5.33 was used to then search databases with PROPSEARCH (11), a program designed to find putative protein families for protein sequences with little sequence identity, 87% reliability was found, linking ORF 5.33 to the O antigen polymerases of *S. enterica* serogroup C_2, *E. coli* K-12, and *Shigella flexneri*. Other features common to Wzy-like polymerases include similar amino acid compositions and codon usage (25). The predicted protein from ORF 5.33 had a high content of leucine, isoleucine, and phenylalanine and contained a high percentage of rare or modulating codons, 12.9% (10), as seen in other *wzy* genes (25). Thus, *wzy* has many features in common with other *wzy* genes, but definitive identification requires further experimental validation.

Other features of the *rfb* region. The intergenic region between the last *rfb* gene and *gnd* was much larger in serogroup O:6,14 than in the other *Salmonella* serogroups (Table 3). A 103-base sequence starting 3 bp downstream from the end of the *wzx* gene shared 89 and 82% homology to the IS*3*-like insertion element IS*1230B* of *E. coli* (32) and *S. enterica* serotype Enteritidis (5), suggesting that it is the remnant of an IS*3*-like element. Sequences with homology to IS*3* have been previously reported in *Salmonella*, located near the *invH* gene of *S. enterica* serotype Choleraesuis (9) and the *sef* operon of *S. enterica* serotype Enteritidis (5). The occurrence of an IS*5* element located about 500 bp upstream from the *gnd* promoter

TABLE 4. Comparison of GDP-mannose pyrophosphorylases and phosphomannomutases from serogroups B, C_1, C_2, and O:6,14

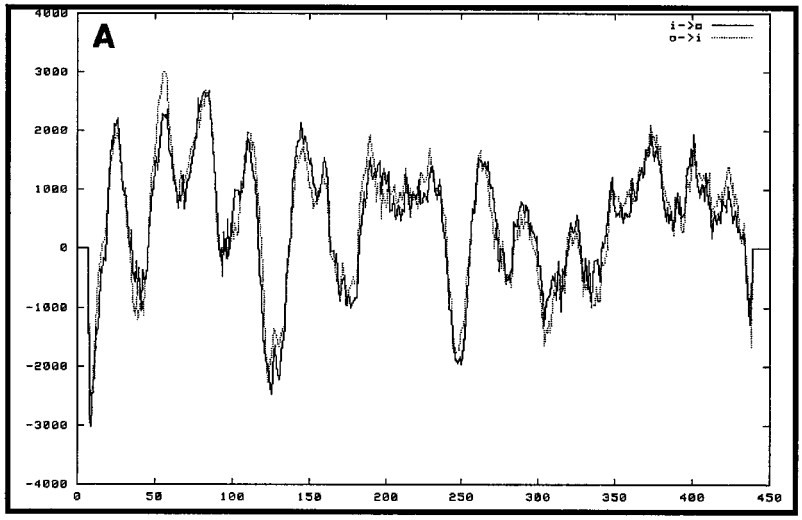
Gene	Protein size (amino acids)	% G+C content	% Amino acid identity
GDP-mannose pyrophosphorylases			
manC (H)	472	36.4	
manC (C_1)	472	39.0	58.5
manC (C_2)	474	39.0	56.4
manC (B)	480	40.3	57.0
cpsB[a] (B)	481	60.4	59.1
Phosphomannomutases			
manB (O:6,14)	476	37.4	
manB (C_1)	457	61.3	14.9
manB (C_2)	479	37.6	58.2
manB (B)	478	40.9	57.4
cpsG[a] (B)	457	61.7	14.9

[a] *cpsB* and *cpsG* are isogenes of *manC* and *manB*, respectively, from *S. enterica* serotype Typhimurium LT2, encoding enzymes that synthesize GDP-mannose (37).

TABLE 5. Amino acid sequence homologies between *Salmonella* mannosyl transferases

Serogroup	BPGN name[a]	Protein size (amino acids)	% Amino acid identity to serogroup O:6,14	
			ORF1	ORF2
O:6,14 (ORF 1)		334		9.6
O:6,14 (ORF 2)		400	9.6	
A/D_1	*wbaU*	333	7.5	10.2
B	*wbaU*	353	14.4	13.6
C_1	*wbaB*	333	11.4	10.8
C_1	*wbaC*	335	47.3	8.1
C_1	*wbaD*	399	9.0	49.1
C_2	*wbaW*	33	13.5	17.0
C_2	*wbaZ*	385	8.4	3.6
D_2	*wbaO*	103	10.7	8.7
E_1	*wbaO*	361	8.1	10.0

[a] BPGN, bacterial polysaccharide gene nomenclature (31).



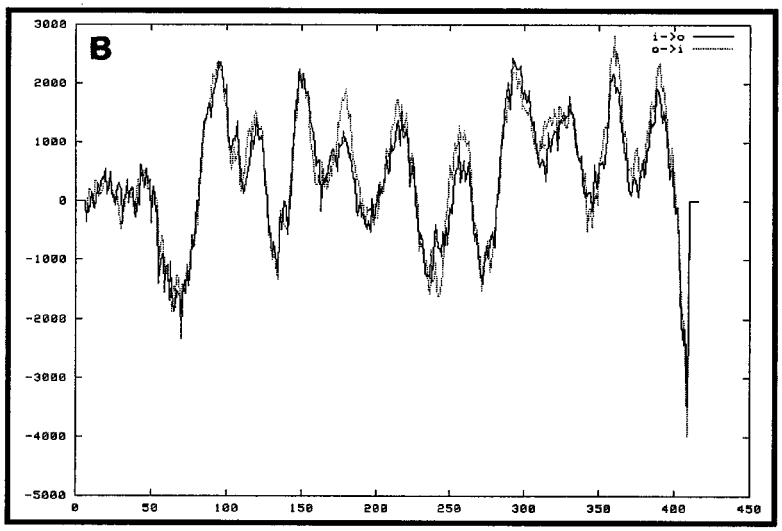


FIG. 3. Suggested transmembrane topology for Wzy (A) and Wzx (B).

in *E. coli* K-12 has been shown (15) and was thought to play a role in gene expression. Analysis of the promoter region of the *gnd* revealed that the −10 region of the *gnd* gene (AGGAG) is identical to the corresponding region of *gnd* in *S. enterica* serotype Typhimurium and *E. coli* K-12, and the 30 bp upstream of the *gnd* share between a 96 and 100% homology to the corresponding region in *S. enterica* serotypes Typhimurium and Choleraesuis and *E. coli* K-12 and O111.

G+C content. Although bacterial species display large variation in their overall G+C content, the genes within a particular bacterial species' genome are usually relatively similar in base composition (27). However, *S. enterica rfb* gene clusters that have been sequenced to date all contained segments of different G+C content, which are thought to have evolved in organisms of largely low G+C content that have been independently acquired and incorporated into the same *Salmonella* locus (31). Consistent with this, Shepard et al. (36) reported interspecies transfer of an entire O antigen gene cluster via a plasmid. All of the genes within the serogroup O:6,14 gene cluster had a low G+C content ranging from 28.9 to 37.4% (Table 1), which is significantly lower than the 51% GC content seen in the majority of *S. enterica* coding sequences, suggesting that all of the genes in the serogroup O:6,14 *rfb* gene cluster were also acquired by transfer from a different species.

Within a bacterial species, codon positions 1, 2, and 3 have a characteristic base composition, with differences due to biases in the mutation rates for the 4 bases (26, 39). Consequently, each species has a characteristic G+C content for each codon position and specific codon preferences. A positive, linear relationship exists between the genomic base composition and the G+C content for each codon position (26), with the effect greatest at position 3, where most changes are

synonymous and not under strong selective constraints. Therefore, if base composition and codon usage patterns are primarily caused by mutational biases, horizontally acquired DNA will often have unusual sequence characteristics that distinguish it from ancestral DNA. At the time of introgression, newly acquired DNA will reflect the base composition of the donor genome. However, the introgressed genes will be subject to the same mutational pressures as the recipient genome, and so over time, these sequences will change or "ameliorate" to reflect the base composition of the new genome (17). This is most evident at sites with few functional constraints, such as codon position 3. The *P* values for the corrected average G+C contents for codon positions 1, 2, and 3 (P1, -2, and -3) (39) are shown in Table 1. These properties are similar to those of the other *Salmonella* serogroups that have been sequenced, with the P3 value being the lowest. Given that the average G+C content at P3 is 58% for *E. coli* and *S. enterica* serotype Typhimurium, the observed P3 value of 23.8% for the serogroup O:6,14 *rfb* gene cluster suggests that this region is still in the process of amelioration.

Models of amelioration can been used to estimate the time of introgression of foreign genes into a chromosome. The G+C content of the third codon positions can each be back-ameliorated until the sequences conform to the Muto and Osawa relationships (25), providing estimates of both time of introgression and nucleotide composition of the donor genome (15). Unfortunately, however, *rfb* genes are not good candidates for amelioration because they have seen too many mutational contexts. Moreover, there is likely too little information to use: the amelioration algorithm becomes reliable only when the DNA sequence under scrutiny exceeds 15 to 20 kb; below that, the estimates get very large variances. Wang and Reeves (44) recently applied this algorithm to back-ameliorate the *E. coli* O111 and *S. enterica* O35 *rfb* gene clusters, which they showed to be highly conserved. They found the program did not give meaningful data and suggested the reason was because the genes of interest deviated too much from the Muto and Osawa models or incorrect parameters were used in the amelioration program.

Comparison of *S. enterica* serotypes Sundsvall and Carrau. Only minor sequence differences were identified for the *rfb* region of *S. enterica* serotypes Sundsvall (O:6,14,25) and Carrau (O:6,14,24), indicating that O antigen factors 24 and 25 are encoded outside the *rfb* gene cluster, possibly by phage or plasmid. A number of *Salmonella* O antigens have been reported to be bacteriophage encoded, such as factors O1 (41) and O34 (46). A plasmid-encoded *rfb* gene cluster has also been reported necessary for biosynthesis of the O54 antigen in *S. enterica* serotype Borreze (16).

PCR for identification of serogroup O:6,14 isolates. A PCR based on *wzx* was developed for the rapid identification of *Salmonella* serogroup O:6,14. Several serotypes of this serogroup were among the top 20 emerging serotypes in a study investigating the trend in *Salmonella* serotypes isolated from humans in the United States from 1987 to 1997 (28). Together with the serogroup PCR assays that have been previously described (23), we intend to identify additional *rfb* targets specific for other *Salmonella* serogroups (8). The overall goal is to combine this serogroup identification system with DNA targets specific for each H antigen (J. R. McQuiston, M. Ortiz-Rivera, L. Gheesling, F. Brenner, and P. I. Fields, submitted for publication) to establish a comprehensive DNA-based scheme for identification of the major *Salmonella* serotypes, without the need for serological testing (7). This will allow a rapid and convenient alternative for identification of *Salmonella* serotypes attainable by nonspecialized laboratories.

REFERENCES

1. **Altschul, S. F., T. L. Madden, A. A. Schäffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman.** 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. **25:**3389–3402.
2. **Brenner, F. W., and A. C. McWhorter-Murlin.** 1998. Identification and serotyping of *Salmonella.* Centers for Disease Control and Prevention, Atlanta, Ga.
3. **Brenner, F. W.** 1998. Modified Kaufmann-White scheme. Centers for Disease Control and Prevention, Atlanta, Ga.
4. **Brown, P. K., L. K. Romana, and P. R. Reeves.** 1992. Molecular analysis of the *rfb* gene cluster of *Salmonella* serovar *muenchen* (strain M67): the genetic basis of the polymorphism between groups C_2 and B. Mol. Microbiol. **6:**1385–1394.
5. **Collighan, R. J., and M. J. Woodward.** Sequence analysis and distribution of an IS3-like insertion element isolated from *Salmonella enteritidis.* FEMS Microbiol. Lett. **154:**207–213.
6. **Curd, H., D. Liu, and P. R. Reeves.** 1998. Relationships among the O-antigen gene clusters of *Salmonella enterica* groups B, D1, D2, and D3. J. Bacteriol. **180:**1002–1007.
7. **Fields, P. I., C. Fitzgerald, and J. R. McQuiston.** 2002. Development of DNA-based methods for the determination of serotype in *Salmonella*, p. 37–41. *In* Proceedings of the International Symposium on *Salmonella* and Salmonellosis. Saint Brieuc, France.
8. **Fitzgerald, C., R. Sherwood, L. Gheesling, and P. I. Fields.** 2002. Molecular analysis of the O antigen gene clusters of *Salmonella enterica* serogroups O:17, O:18 and O:48. Development of serogroup specific PCR assays, p. 99–100. *In* Proceedings of the International Symposium on *Salmonella* and Salmonellosis. Saint Brieuc, France.
9. **Ginocchio, C. G., K. Rahn, R. C. Clarke, and J. E. Galan.** 1997. Naturally occurring deletions in the centisome 63 pathogenicity island of environmental isolates of *Salmonella* spp. Infect. Immun. **65:**1267–1272.
10. **Grosjean, H., and W. Fiers.** 1982. Preferential codon usage in prokaryotic genes: the optimal codon-anticodon interaction energy and the selective codon usage in efficiently expressed genes. Gene **18:**199–209.
11. **Hobohm, U., and C. Sander.** 1995. A sequence property approach to searching protein database. J. Mol. Biol. **251:**390–399.
12. **Hofmann, K., and W. Stoffel.** 1993. TMbase—a database of membrane spanning proteins segments. J. Biol. Chem. Hoppe-Seyler. **347:**166.
13. **Jensen, S. O., and P. R. Reeves.** 2001. Molecular evolution of the GDP-mannose pathway genes (*man*B and *man*C) in *Salmonella enterica.* Microbiology **147:**599–610.
14. **Jiang, X. M., H. N. Brahmbhatt, N. B. Quigley, and P. R. Reeves.** 1991. Structure and sequence of the *rfb* (O antigen) gene cluster of *Salmonella* serovar *typhimurium* (strain LT2). Mol. Microbiol. **5:**695–713.
15. **Jordan, B. A., R. E. Wolf, and M. S. Nasoff.** 1982. IS5 is at the end of the *his-gnd* transposon in *Escherichia coli.* Plasmid **8:**98.
16. **Keenleyside, W. J., M. Perry, L. Maclean, C. Poppe, and C. Whitfield.** 1994. A plasmid-encoded *rfb* O:54 gene cluster is required for biosynthesis of the O:54 antigen in *Salmonella enterica* serovar Borreze. Mol. Microbiol. **11:**437–448.
17. **Lawrence, J. G., and H. Ochman.** 1997. Amelioration of bacterial genomes: rates of change and exchange. J. Mol. Evol. **44:**383–397.
18. **Lee, S. J., L. K. Romana, and P. R. Reeves.** 1992. Sequences and structural analysis of the *rfb* (O antigen) gene cluster from a serogroup C1 *Salmonella enterica* strain. J. Gen. Microbiol. **138:**1843–1855.
19. **Liu, D., N. K. Verma, L. K. Romana, and P. R. Reeves.** 1991. Relationships among the *rfb* regions of *Salmonella* serovars A, B, and D. J. Bacteriol. **173:**4814–4819.
20. **Liu, D., A. M. Haase, L. Lindqvist, A. A. Lindberg, and P. R. Reeves.** 1993. Glycosyl transferases of O-antigen biosynthesis in *Salmonella enterica*: identification and characterization of transferase genes of groups B, C2, and E1. J. Bacteriol. **175:**3408–3413.
21. **Liu, D., R. A. Cole, and P. R. Reeves.** 1996 An O-antigen processing function for Wzx (RfbX): a promising candidate for O-unit flippase. J. Bacteriol. **178:**2102–2107.
22. **Lüderitz, O., A. M. Staub, and O. Westphal.** 1966. Immunochemistry of O and R antigens of *Salmonella* and related *Enterobacteriaceae.* Bacteriol. Rev. **30:**192–255.
23. **Luk, J. M., U. Kongmuang, P. R. Reeves, and A. A. Lindberg.** 1993. Selective amplification of abequose and paratose synthase genes (*rfb*) by polymerase chain reaction for identification of *Salmonella* major serogroups (A, B, C2, and D). J. Clin. Microbiol. **31:**2118–2123.

24. **Mead, P. S., L. Slutsker, V. Dietz, F. L. McCaig, J. S. Bresee, C. Shapiro, P. M. Griffin, and R. V. Tauxe.** 1999. Food-related illness and death in the United States. Emerg. Infect. Dis. **5:**607–625.
25. **Morona, R., M. Mavris, A. Fallarino, and P. A. Manning.** 1994. Characterization of the *rfc* region of *Shigella flexneri.* J. Bacteriol. **176:**733–747.
26. **Muto, A., and S. Osawa.** 1987. The guanine and cytosine content of genomic DNA and bacterial evolution. Proc. Natl. Acad. Sci. USA **84:**166–169.
27. **Ochman, H., and J. Lawrence.** 1996. Phylogenetics and the amelioration of bacterial genomes, p. 2627–2637. *In* F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology, 2nd ed., vol. 2. ASM Press, Washington, D.C.
28. **Olsen, S. J., R. Bishop, F. W. Brenner, T. Roels, N. Bean, R. V. Tauxe, and L. Slutsker.** 2001. The changing epidemiology of *Salmonella*: trends in *Salmonella* serotypes isolated from humans in the United States, 1987–1997. J. Infect. Dis. **183:**753–761.
29. **Popoff, M. Y., J. Bockemuhl, and F. W. Brenner.** 2001. Supplement 2001 (no. 44) to the Kauffmann-White scheme. Res. Microbiol. **152:**907–909.
30. **Qun, L., and P. R. Reeves.** 2000. Genetic variation of dTDP-L-rhamnose pathway genes in *Salmonella enterica.* Microbiology **146:**2291–2307.
31. **Reeves, P. R., M. Hobbs, M. A. Valvano, M. Skurnik et al.** 1996. Bacterial polysaccharide synthesis and gene nomenclature. Trends Microbiol. **4:**495–503.
32. **Russo T. A., U. B. Carlino, A. Mong, and S. T. Jodush.** 1999. Identification of genes in an extraintestinal isolate of *Escherichia coli* with increased expression after exposure to human urine. Infect. Immun. **67:**5306–5314.
33. **Salmonella Subcommittee.** 1934. The genus *Salmonella* Lignieres, 1900. J. Hyg. **34:**333.
34. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
35. **Schnaitman, C. A., and J. D. Klena.** 1993. Genetics of lipopolysaccharide biosynthesis in enteric bacteria. Microbiol. Rev. **57:**655–682.
36. **Shepherd, J. G., L. Wang, and P. R. Reeves.** 2000. Comparison of O-antigen gene clusters of *Escherichia coli* (*Shigella*) *sonnei* and *Plesiomonas shigelloides* O17: Sonnei gained its current plasmid-borne O-antigen genes from *P. shigelloides* in a recent event. Infect. Immun. **68:**6056–6061.
37. **Stevenson, G., S. J. Lee, L. K. Romana, and P. R. Reeves.** 1991. The *cps* gene cluster of *Salmonella* strain LT2 includes a second mannose pathway: sequence of two genes and relationship to genes in the *rfb* gene cluster. Mol. Gen. Genet. **227:**173–180.
38. **Stevenson, G., K. Andrianopoulos, M. Hobbs, and P. R. Reeves.** 1996. Organization of the *Escherichia coli* K-12 gene cluster responsible for production of the extracellular polysaccharide colanic acid. J. Bacteriol. **178:**4885–4893.
39. **Sueoka, N.** 1988. Directional mutational pressure and neutral molecular evolution. Proc. Natl. Acad. Sci. USA **85:**2653–2657.
40. **Tauxe, R. V.** 1996. An update on *Salmonella.* Health Environ. Dig. **10:**1–4.
41. **Vander Byl, C., and A. M. Kropinski.** 2000. Sequence of the genome of *Salmonella* bacteriophage P22. J. Bacteriol. **182:**6472–6481.
42. **Wang, L., L. K. Romana, and P. R. Reeves.** 1992. Molecular analysis of a *Salmonella enterica* group E1 *rfb* gene cluster: O antigen and the genetic basis of the major polymorphism. Genetics **130:**429–443.
43. **Wang, L., and P. R. Reeves.** 1998. Organization of *Escherichia coli* O157 O antigen gene cluster and identification of its specific genes. Infect. Immun. **66:**3545–3551.
44. **Wang, L., and P. Reeves.** 2000. The *Escherichia coli* O111 and *Salmonella enterica* O35 gene clusters: gene clusters encoding the same colitose-containing O antigen are highly conserved. J. Bacteriol. **182:**5256–5261.
45. **Wang, L., K. Andrianopoulos, D. Liu, M. Y. Popoff, and P. R. Reeves.** 2002. Extensive variation in the O-antigen gene cluster within one *Salmonella enterica* serogroup reveals an unexpected complex history. J. Bacteriol. **184:**1669–1677.
46. **Wright, A.** 1971. Mechanism of conversion of the *Salmonella* O antigen by bacteriophage ε^{34}. J. Bacteriol. **105:**927–936.
47. **Xiang, S.-H., M. Hobbs., and P. R. Reeves.** 1994. Molecular analysis of the *rfb* gene cluster of a D2 *Salmonella enterica* strain: evidence for its origin from an insertion sequence-mediated recombination event between group E and D1 strains. J. Bacteriol. **176:**4357–4365.