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# Identification and Characterization of the dTDP-Rhamnose Biosynthesis and Transfer Genes of the Lipopolysaccharide-Related *rfb* Locus in *Leptospira interrogans* Serovar Copenhageni

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**Immunity to leptospirosis is principally humorally mediated and involves opsonization of leptospires for phagocytosis by macrophages and neutrophils. The only protective antigen identified to date is the leptospiral lipopolysaccharide (LPS), which biochemically resembles typical gram-negative LPS but has greatly reduced endotoxic activity. Little is known about the structure of leptospiral LPS. A 2.1-kb *Eco*RI fragment from the chromosome of serovar Copenhageni was cloned in pUC18 in *Escherichia coli*, after which flanking regions were cloned from a genomic library constructed in bacteriophage λ GEM12. Sequence analysis identified four open reading frames which showed similarity to the *rfbC*, *rfbD*, *rfbB*, and *rfbA* genes, transcribed in that order, which encode the four enzymes involved in the biosynthesis of dTDP-rhamnose for the assembly of LPS in *Salmonella enterica*, *E. coli*, and *Shigella flexneri*. An additional open reading frame downstream of the *rfbCDBA* locus showed similarity with the rhamnosyltransferase genes of *Shigella* and *Yersinia enterocolitica* but not *Salmonella*. Comparison of deduced amino acid sequences showed up to 85% similarity of the leptospiral proteins with those of other gram-negative bacteria. Polyacrylamide gel electrophoresis of recombinant clones identified the putative RfbCDBA proteins, while reverse transcriptase-mediated PCR analysis indicated that the *rfbCDBA* gene cluster was expressed in *Leptospira*. Moreover, it could restore normal LPS phenotype to a defined *rfbB*::Tn*5* mutant of *S. flexneri* which was deficient in all four genes, thereby confirming the functional identification of a part of the leptospiral *rfb* locus.**

Leptospirosis is a worldwide zoonosis caused by infection with one of over 200 serovars of *Leptospira*. Immunity following infection is restricted to antigenically related serovars (7) and is mediated principally by circulating antibodies which opsonize leptospires for phagocytosis by both macrophages and neutrophils (6, 24, 38). The only known target antigen for both agglutinating and opsonic antibodies is the leptospiral lipopolysaccharide (LPS), which resembles typical gram-negative LPS biochemically and serologically but has greatly reduced endotoxic activity (8, 10, 13, 39). The lack of a classical LPS ladder when leptospiral LPS is analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) suggests that it probably contains a limited number of O-side chain units (40, 41). Monoclonal antibodies reacting with LPS epitopes are agglutinating (1), opsonic (9), and protective (17, 34), and active immunization with LPS or LPS-derived polysaccharides can protect against infection (18, 25). Genes encoding outer membrane proteins of *Leptospira* have been described (11, 35), but the role of these proteins in immunity has not yet been elucidated.

Despite the critical role of leptospiral LPS in immunity, almost nothing is known of its detailed structure. Phosphorylated mannose is a component of opsonic epitopes of *Leptospira interrogans* serovar Hardjo (40), while a trisaccharide derived from the LPS of serovar Pomona could elicit the production of agglutinating, opsonic antibodies in mice (26). However, further detailed analysis has been hampered by the difficulty and expense of growing large numbers of leptospires.

In gram-negative bacteria, the genes required for the synthesis and assembly of sugars comprising the O-antigenic side chain repeating unit are carried on the *rfb* locus (23). In particular, the synthesis of activated rhamnose from glucose-1-phosphate is achieved by the products of the *rfbABCD* genes, which encode the enzymes glucose-1-phosphate transferase, dTDP-glucose-4,6-dehydratase, dTDP-4-keto-L-rhamnose-3,5-epimerase, and dTDP-L-rhamnose synthase, respectively. Because rhamnose is a major component of the LPS of serovar Copenhageni (39) and the *rfb* genes are frequently conserved between different bacterial species (32), we surmised that it should be possible to identify the dTDP-rhamnose biosynthetic genes of the *rfb* locus in *Leptospira*. Accordingly, in this paper we report the cloning, sequencing, and functional analysis of the dTDP-rhamnose biosynthesis genes from serovar Copenhageni. This is the first molecular analysis of LPS biosynthetic genes in any spirochete.

## MATERIALS AND METHODS

**Bacterial strains, plasmids, and growth media.** The bacterial strains and plasmids used in this study are shown in Table 1. *Escherichia coli* strains were grown on LB medium (3) containing 100 μg of ampicillin per ml as appropriate. Selection for pUCTp-based plasmids in *Shigella flexneri* was achieved on Mueller-Hinton medium containing 0.35% glucose and 12.5 μg of trimethoprim per ml. *L. interrogans* serovar Copenhageni M20 was grown in EMJH medium with added pyruvate (16).

**Molecular biology techniques.** Chromosomal DNA was prepared by the cetyltrimethylammonium bromide method, while plasmid DNA was prepared with a modified alkaline lysis method (3). Restriction endonucleases and other DNA enzymes were obtained from Boehringer (Mannheim, Germany) and used according to the manufacturer's instructions. Standard molecular biology protocols for cloning, mapping, and induction were used throughout (3). Plasmid genomic libraries were prepared by using standard methods; a partial *Sau*3AI library was prepared in bacteriophage λ GEM12 (Promega, Madison, Wis.), and phage DNA was isolated as recommended by the manufacturer and screened by plaque hybridization (3). Probes for Southern (36) and plaque hybridization were excised from agarose gels and purified with a Geneclean kit (Bio 101, La Jolla, Calif.). DNA was labeled with digoxigenin-dUTP, and Southern hybridization was performed as specified by the manufacturer (Boehringer). Hybridization

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TABLE 1. Strains and plasmids used

| Bacterial strain or plasmid | Relevant characteristic(s) | Reference or source |
|---|---|---|
| Bacterial strains | | |
| *E. coli* DH5α | F⁻ ϕ80 Δ*lacZ* M15 *recA1* | Bethesda Research Laboratories |
| *L. interrogans* sv. Copenhageni | Wild type, virulent strain M20 | 4 |
| *S. flexneri* 2a YSH6000 | Wild type, virulent strain with normal LPS | 33 |
| *S. flexneri* 2a N1308 | *rfbB*::Tn*5* LPS mutant of YSH6000 with short O-side chain | 31 |
| Plasmids | | |
| pUC18 and -19 | High-copy-number cloning vectors, Ap$^r$, Δ*lacZ* | 42 |
| pUC18Tp and -19Tp | pUC18/19 derivatives with 0.7-kb Tp$^r$ gene at *Ssp*I site | 31 |
| pTTQ18 | IPTG-inducible $P_{tac}$ expression vector | Amersham |
| pPR341 | 11-kb *Eco*RI-*Hin*dIII fragment carrying the *S. enterica* sv. Typhimurium LT2 *rfbA* and *rfbB* genes in pUC18 | 5 |
| pSBA146 | pUC18Tp carrying the *S. flexneri rfbBDAC* genes | 31 |
| pLBA54 | Primary genomic clone containing 2.1-kb *Eco*RI (*rfbAB*) fragment of *L. interrogans* sv. Copenhageni | This study |
| pLBA416 | 4.9-kb *Xba*I-*Hin*dIII fragment containing leptospiral *rfbCDBA* genes in pUC18Tp | This study |
| pLBA418 | Entire insert from pLBA416 in pTTQ18 | This study |
| pLBA420 | 2.2-kb *Xba*I-*Hin*dIII fragment (*rfbCD*) in pTTQ18 | This study |
| pLBA421 | 2.1-kb *Eco*RI fragment (*rfbA*) in pTTQ18 | This study |

under low-stringency conditions was performed overnight at 55°C followed by washing at room temperature in 0.1% SDS–2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) (3), while high-stringency hybridization was performed at 68°C with washes in 0.1% SDS–0.1× SSC. Total RNA was prepared with Trisol (Gibco BRL, Gaithersburg, Md.), using the procedure recommended by the manufacturer. Reverse transcriptase (RT)-mediated PCR (RT-PCR) PCR was then performed by first reverse transcribing RNA with 0.5 U of RT (Boehringer) per μl at 42°C for 60 min and then amplifying the resultant cDNA by using *Taq* polymerase (Boehringer) and a program of 30 cycles of 1 min at 50, 72, and 94°C for annealing, extension, and denaturation, respectively. Nucleotide sequencing was performed by using a PRISM Ready Reaction DyeDeoxy terminator cycle sequencing kit (Applied Biosystems) and an Applied Biosystems 373A automated sequencer. Sequence data were analyzed with Sequencher 3.0 (Gene Codes Corp., Ann Arbor, Mich.), while DNA and protein database comparisons were made by using the BLAST program of Altschul et al. (2). Comparison scores (expressed in SD) were calculated by using the RDF2 program (30) with 20 shuffles. An SD value of >9 is considered to indicate homology.

**PAGE.** Whole-cell lysates were electrophoresed on SDS–15% polyacrylamide discontinuous gels (20) and stained either with Coomassie blue to visualize protein bands or with silver nitrate to visualize LPS (12), in which case they were treated with proteinase K prior to electrophoresis (40).

**Nucleotide sequence accession number.** Sequence data have been deposited in the GenBank database under the accession number U61226.

## RESULTS

**Cloning of the leptospiral *rfbCDBA* genes.** A DNA probe derived from plasmid pPR341 (Table 1), which covered the *rfbA* and *rfbB* genes of *Salmonella enterica* serotype Typhimurium LT2, was used in Southern blot analysis under low-stringency conditions to probe serovar Copenhageni genomic DNA digested with a variety of restriction endonucleases. The probe hybridized weakly to *Eco*RI bands of 2.1 and 2.7 kb. Accordingly, genomic DNA was digested with *Eco*RI, and bands ranging in size from 2 to 3 kb were excised from the agarose gel and ligated into *Eco*RI-digested, dephosphorylated pUC18. The library thus obtained was screened by Southern blotting whereby *Eco*RI-digested, pooled plasmid preparations from six transformants per lane were probed under low-stringency conditions with the pPR341 *rfbAB* probe described above. From a total of 768 transformants screened, 2 were found to harbor a 2.1-kb insert which hybridized with the *rfbAB* probe. Partial DNA sequence analysis of one of these, designated pLBA54 (Fig. 1), showed that it had significant similarity to the *rfbB* gene of *S. enterica.*

Next, a partial *Sau*3AI library constructed in λ GEM12 was screened by plaque hybridization under high-stringency conditions, using as a probe the 2.1-kb insert from pLBA54. Two positive plaques were detected. One of these, designated λP1, contained an insert of approximately 12 kb. Preliminary sequencing of internal fragments showed similarity to the *rfb* genes of *S. enterica*. A central 4.9-kb fragment flanked by *Xba*I

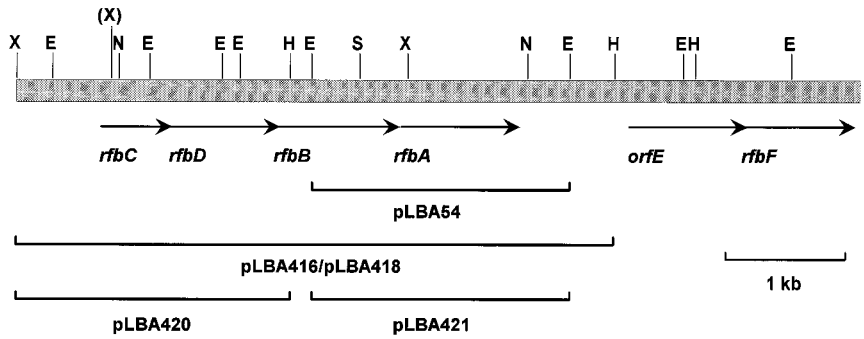


FIG. 1. Partial *rfb* region of *L. interrogans* serovar Copenhageni M20. The top bar represents the map of the 7,000-bp region whose sequence was determined. ORFs with their assigned gene names are shown under the map. Plasmid subclones used for complementation (pLBA416) and protein expression studies (pLBA418, pLBA420, and pLBA421) are indicated. pLBA54 shows the original fragment cloned by screening a genomic library by Southern hybridization (see Results). Abbreviations for selected restriction endonuclease sites: E, *Eco*RI; N, *Nsi*I; X, *Xba*I; H, *Hin*dIII; P, *Pst*I; S, *Sma*I. (X) indicates an *Xba*I site which was resistant to digestion.

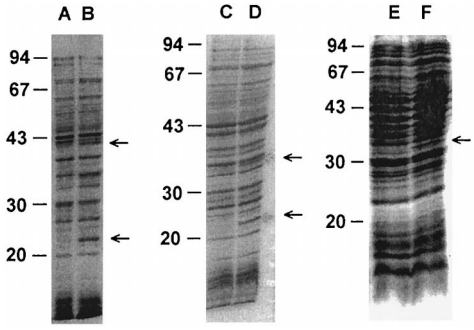


FIG. 2. Coomassie blue-stained SDS-polyacrylamide gel analysis showing expression of the four leptospiral rhamnose biosynthetic proteins in *E. coli* DH5α harboring pTTQ18-based plasmids pLBA418 (*rfbCDBA*) (lanes A and B), pLBA420 (*rfbCD*) (lanes C and D), and pLBA421 (*rfbA*) (lanes E and F) and either induced with 5 mM IPTG (lanes B, D, and F) or uninduced (lanes A, C, and E). The arrows indicate the RfbB and RfbC (lane B), RfbD and RfbC (lane D), and RfbA (lane F) proteins. The positions of standard molecular mass markers (in kilodaltons) are shown on the left. The original was scanned with an Epson GT-8000 scanner and the Corel PhotoPaint 3.0 program.

and *Hin*dIII was subcloned into pUC18Tp to generate plasmid pLBA416, whose restriction map is shown in Fig. 1.

**Sequence analysis of the leptospiral *rfbCDBA* locus.** The sequence of the 4,957-bp fragment which comprised the insert in pLBA416 was determined on both DNA strands; all restriction sites were crossed. Analysis revealed the presence of four complete open reading frames (ORFs) and indicated transcription of all four in the same direction from left to right (Fig. 1). The deduced molecular masses of the protein products were 21.5, 34.8, 40.3, and 32.8 kDa. Comparison of the deduced amino acid sequences of the four ORFs showed levels of similarity to RfbC, RfbD, RfbB, and RfbA of *S. enterica* serotype Typhimurium of 55, 47, 70, and 85%, respectively. Similar levels of similarity to the corresponding gene products of *E. coli* and *S. flexneri* were observed. The G+C contents of the four genes were 36.7, 37.1, 36.9, and 37.3%, compared with 34 to 36% for the *L. interrogans* genome (43). As seen in both *Salmonella* and *Shigella*, the intergenic regions were small, with spacings of only 7, -1, and 4 bp, respectively.

**Expression of cloned protein products in *E. coli*.** The 4,957-bp fragment (flanked by *Xba*I and *Hin*dIII sites) from pLBA416 was cloned into the expression vector pTTQ18 such that transcription was driven by the vector $P_{tac}$ promoter to generate plasmid pLBA418 (Fig. 1; Table 1). Following introduction into *E. coli* DH5α, cultures were grown, induced with 5 mM isopropylthiogalactopyranoside (IPTG), and subjected to SDS-PAGE analysis. Three protein bands were observed only in the induced culture (Fig. 2, lane B). Two of these bands had apparent molecular masses of 40.0 and 21.4 kDa, which were close to those predicted for RfbB and RfbC from their respective nucleotide sequences. A unique band had an apparent molecular mass of 32.6 kDa, close to those predicted for both RfbA and RfbD. To identify the remaining two proteins, plasmid pLBA420, containing the leftmost 2.2-kb *Xba*I-*Hin*dIII fragment (Fig. 1) which carried only the intact *rfbC* and *rfbD* genes, was constructed, and the strain was induced and analyzed as before. Bands corresponding to RfbC and RfbD (35.0 kDa) were observed (Fig. 2, lane D). Finally, the 2.1-kb *Eco*RI fragment (Fig. 1) carrying only the intact *rfbA* gene was cloned into pTTQ18, generating plasmid pLBA421. Induction and analysis revealed the presence of a unique band with an apparent molecular mass of 32 kDa (Fig. 2, lane F), again close to the predicted molecular mass of 32.8 kDa for RfbA.

**Complementation of an *S. flexneri rfbB*::Tn*5* mutant.** Because the *rfbB* gene is the 5′-most gene in the *S. flexneri* locus, *rfbB*::Tn*5* mutant N1308, which could not express any of the four genes (31), was used for complementation analysis. Plasmid pLBA416 was transformed into N1308 by selecting for trimethoprim resistance. Plasmid pSBA146, which carried the *S. flexneri rfbBDAC* genes, served as a positive control. Analysis of whole-cell lysates on silver-stained SDS-polyacrylamide gels revealed that the leptospiral *rfbCDBA* genes carried on pLBA416 could restore normal LPS side chain phenotype to the *S. flexneri* mutant almost as well as the homologous *S. flexneri* locus (Fig. 3). The complemented mutant was also restored to agglutinability by *Shigella* O-3,4 antiserum. When the 4,957-bp insert from pLBA416 was cloned into pUC19Tp such that the direction of the leptospiral *rfbCDBA* genes was in the opposite direction from the vector $P_{lac}$ promoter, no complementation of N1308 was observed.

**Identification of the downstream rhamnosyltransferase gene.** Because the genes encoding sugar transferase enzymes are often found close to the genes encoding the respective sugar biosynthetic enzymes (14, 27), we determined the nucleotide sequence of an additional 2,043 bp downstream of the sequence covered by pLBA416 (Fig. 1). Sequence analysis revealed the presence of two ORFs in the same direction as *rfbCDBA*. The first of these, designated *orfE*, was sufficient to encode a protein of 324 amino acids (37.7 kDa) which showed 19% similarity to the product of *orf264*, a gene of unascribed function located within the *rfb* region of *E. coli* (21); similarity rose to 49% over the 142-amino-acid stretch in which similarity was observed. A second ORF was located 1,963 bp downstream of the end of *rfbA* and was sufficient to encode a protein of 303 amino acids (35.3 kDa). Comparison of its deduced amino acid sequence indicated similarity to rhamnosyltransferase enzymes from other gram-negative bacteria (Table 2). The ORF was thus designated *rfbF*. Based on comparison scores, the leptospiral RfbF did not show any similarity to the RfbN rhamnosyltransferase of *Salmonella* (14) or to the second reported rhamnosyltransferase, RfbG, of *S. flexneri* (27).

**Evidence for expression of the *rfb* locus in *Leptospira*.** Despite extensive efforts over a wide range of conditions, we were unable to detect any mRNA in Northern blots of leptospiral RNA probed with the 3,387-bp *Nsi*I DNA fragment which covered almost the entire *rfbCDBA* locus (Fig. 1). Accordingly, an RT-PCR assay was used to demonstrate transcription from the *rfbDCBA* locus. The reverse transcription of total RNA was

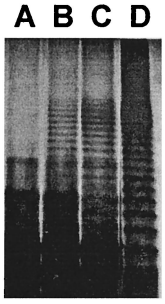


FIG. 3. Silver-stained polyacrylamide gel of proteinase K-treated whole-cell lysates of *S. flexneri* strains. Lane A, *rfbB*::Tn*5* mutant N1308 carrying pUC18Tp (control); lane B, N1308 carrying pLBA 416 (Copenhageni *rfbCDBA*); lane C, N1308 carrying pSBA146 (*S. flexneri rfbBDAC*); lane D, YSH6000 (wild-type control). The original was scanned with an Epson GT-8000 scanner and the Corel PhotoPaint 3.0 program.

TABLE 2. Similarity of the leptospiral RfbF protein with the products of *rfb* genes of other bacteria

| Gene | Organism | Function | % Similarity | Comparison score (SD)[a] |
|---|---|---|---|---|
| *rfbQ* | *S. dysenteriae* | Rhamnosyltransferase I | 48 | 20 |
| *rfbC* | *Y. enterocolitica* | Unassigned | 44 | 27 |
| *rfbF* | *S. flexneri* | Rhamnosyltransferase | 38 | 22 |
| *rfbR* | *S. dysenteriae* | Rhamnosyltransferase II | 33 | 21 |

[a] An SD score of >9 indicates homology.

primed by using a 20-mer oligonucleotide with the sequence 5′-CCGAAGGATGCAACTCGTCG-3′, corresponding to bases 2938 to 2919 at the 3′ end of *rfbB*. The product was used as a template for PCR primed with a pair of oligonucleotides corresponding to the 3′ end of *rfbC* (bases 855 to 872, 5′-TA GTCCGAGTAGTGAGAG-3′) and to the 3′ end of *rfbD* (bases 1895 to 1878, 5′-CGTACCAGCTCGCAACTC-3′). The resultant 1-kb product (Fig. 4, lane C) was the same size as the PCR product amplified from genomic DNA (Fig. 4, lane D). No product was observed in PCRs from total RNA preparations which were not first reverse transcribed (Fig. 4, lane B), indicating the presence of a transcript extending from at least the 3′ end of *rfbC* to the 3′ end of *rfbB*.

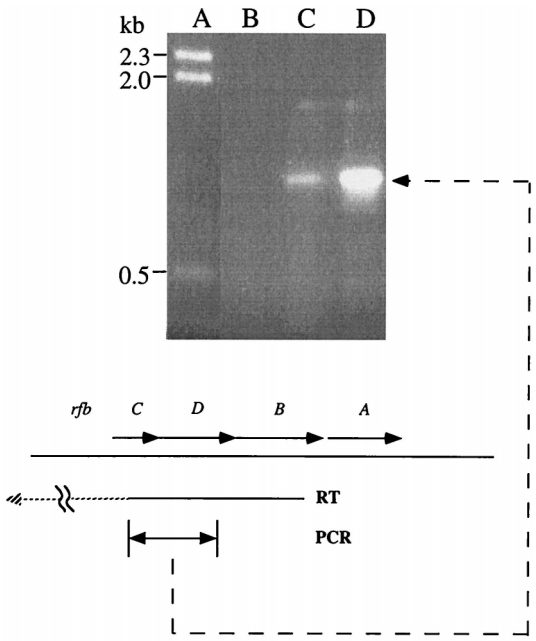


FIG. 4. Ethidium bromide-stained 0.8% agarose gel used to resolve RT-PCR products generated as shown at the bottom. The positions of the *rfbCDBA* genes are indicated. The position of the primer used to reverse transcribe RNA is indicated (RT), as is the location of the PCR product amplified from the resultant cDNA (PCR). The solid line to the left of RT indicates the minimum transcript size identified. The 5′ end of the transcript (dotted line and arrow) is unknown. Lane A, *Hin*dIII-digested λ DNA size markers; lane B, PCR product from total RNA without reverse transcription; lane C, PCR product from total RNA with reverse transcription; lane D, PCR product from genomic DNA. PCR amplification used 30 cycles of 94, 50, and 72°C for denaturation, annealing, and extension, respectively. The original was scanned with an Epson GT-8000 scanner and the Canvas 3.5.4 program with Twain Scan.

## DISCUSSION

Despite the key role of leptospiral LPS in immunity (see the introduction), nothing is known about its structure or genetics. Attempts at conventional immunochemical and physicochemical analyses were not successful because we could not obtain sufficient quantities of material. We therefore took a molecular approach in an attempt to understand the biosynthesis of leptospiral LPS. Rhamnose constitutes 45% of the monosaccharide composition of serovar Copenhageni LPS (39). The rhamnose biosynthetic pathway, which comprises four enzymes encoded by the *rfb* locus which catalyze the synthesis of dTDP-rhamnose from glucose-1-phosphate prior to its addition to the O-antigen subunit, has been identified in *S. flexneri* (22, 31), *Shigella dysenteriae* (19, 37), *S. enterica* (5), *E. coli* (21), and *Yersinia enterocolitica* (44), although in the last case the final enzyme probably leads to altrose (28).

Accordingly, in the present study we were able to use a portion of the *Salmonella rfb* locus as a probe to screen a library of leptospiral DNA in order to clone the leptospiral *rfbB* gene, which was then used as a homologous probe to clone the remainder of the dTDP-rhamnose biosynthetic locus. The four genes identified were closely linked and in fact overlapped in the case of *rfbD* and *rfbB*, as also seen in *S. enterica* serotype Typhimurium (5) and *Shigella* (19, 27, 31). However, the leptospiral gene order of *rfbCDBA* was different from the *rfbBDAC* observed in *S. flexneri*, *S. dysenteriae*, and *S. enterica*. Comparison of DNA sequences indicated a high level of similarity with the corresponding genes from other gram-negative bacteria. The G+C content of the leptospiral genes was 37% and was thus not significantly different from the *L. interrogans* genome content of 34 to 36%. In contrast, the fact that the G+C content of *rfb* loci in *S. flexneri*, *E. coli*, and *S. enterica* is substantially lower than that of the chromosome has led to the suggestion that the *rfb* genes were acquired relatively recently from some common ancestral source (31). According to 16S rRNA sequence analysis, spirochetes diverged from eubacterial groupings early in evolution (29), and it is thus tempting to speculate that the *rfb* loci may have been acquired by gram-negative bacteria from a spirochete ancestor.

A key finding of our study was the fact that the leptospiral *rfbCDBA* genes could complement an *S. flexneri rfbB*::Tn*5* mutant and restore the LPS phenotype to almost the same level as the homologous *Shigella rfbBDAC* genes (31). The slightly reduced level of complementation may have been due to a reduced level of transcription and/or translation of leptospiral genes in *Shigella*. Alternatively, the suggestion has been made that *rfb* enzymes which constitute a homologous system exhibit specificity not only for their substrates but also for each other (28). The finding that no complementation was seen when the leptospiral locus was cloned such that transcription did not occur from the vector promoter is consistent with the lack of any apparent promoter sequences upstream of the *rfbA* gene and suggests that the *rfbCDBA* genes are part of a larger operon.

Two additional ORFs were found downstream of the *rfbCDBA* locus. OrfE showed some similarity to the product of an *rfb* gene of unspecified function in *E. coli* (21), while RfbF was most similar to the RfbQ rhamnosyltransferase I of *S. dysenteriae*. RfbQ catalyzes the addition of rhamnose to the [*N*-acetylglucosamine]-[galactose] acceptor to form the O-antigen repeat unit (19). The next highest similarity of leptospiral RfbF was to RfbC of *Y. enterocolitica* (44), which was unassigned in reference 44 but was subsequently suggested to be an altrose transferase (28). Interestingly, the leptospiral RfbF showed similarity to RfbF, but not RfbG, of *S. flexneri*, both reported to be rhamnose transferases. Both RfbF and RfbQ catalyze the addition of dTDP-rhamnose via 1→2 linkages but to different acceptors, whereas RfbG catalyzes the addition of dTDP-rham-

nose via a 1→3 linkage, suggesting the presence of the former linkage in serovar Copenhageni. Likewise, there was no similarity to RfbN of *S. enterica*, which catalyzes the transfer of rhamnose to a mannose residue (15). The lack of similarity suggests that such a mannose-rhamnose linkage is unlikely in serovar Copenhageni. Indeed, analysis of the monosaccharide composition of serovar Copenhageni H45 LPS found that mannose was only a very minor component, constituting 3.4% of the total carbohydrates (39). On the other hand, the fact that rhamnose is the major component of leptospiral LPS, and is not known to occur elsewhere in leptospiral cells, is compelling evidence that the *rfb* genes identified in this study are part of the LPS biosynthetic locus. This work thus constitutes the first genetic study of LPS biosynthetic genes in any spirochete.

In an attempt to identify possible transferase motifs, Morona et al. (28) aligned the known deoxyhexose transferase enzymes and identified three motifs close to the N terminus. The leptospiral RfbF had the T(F/Y)N sequence at amino acids 13 to 15, a conserved Q at position 26, the (V/I)X(V/I)XDXX(S/T) sequence at positions 35 to 42, and also the DNDS sequence at amino acids 90 to 93. On this basis, it is reasonable to propose that *rfbF* encodes a rhamnosyltransferase. The similarity of RfbF to rhamnosyltransferase II of *S. dysenteriae* was lower, at 33%. This enzyme adds the second rhamnose residue, and our results may thus indicate that the leptospiral RfbF may not transfer rhamnose to an existing rhamnose residue, although definitive assignment of specific transferase activity must await functional analysis of the gene products.

Interestingly, OrfE possessed the TYN sequence and also the second IXIXDXXS sequence but not the third motif. This fact together with its low-level similarity to the *E. coli* ORF264 within the *rfb* region suggests that *orfE* also encodes a transferase enzyme, although we cannot as yet assign a definitive function for it.

We were unable to detect an mRNA transcript in Northern blots, suggesting that *rfb* mRNA is either present in very low amounts or very short-lived. However, RT-PCR indicated a transcript extending from at least the 3′ end of *rfbC* to the 3′ end of *rfbB*. The tight linkage of the genes within the *rfbCDBA* locus in conjunction with the RT-PCR results is consistent with the expression of the entire locus as a single transcript, as observed with *rfb* loci in other bacteria. The inability to detect transcripts by Northern analysis thus suggests low levels of specific mRNA, as transcripts were detected in the RT-PCR assay at all stages of exponential growth (unpublished data).

Finally, preliminary analysis of sequence data obtained from the region downstream of *rfbF* indicated no similarity to any genes involved in LPS biosynthesis, thereby suggesting that the rhamnose biosynthetic and transferase genes are at the 3′ end of the leptospiral *rfb* locus. The second-most-abundant monosaccharide in Copenhageni LPS is xylose, at 25% (39). Given that *rfb* genes are usually clustered, we would expect to locate xylose biosynthetic genes as well as other genes involved in LPS biosynthesis upstream of *rfbC*. The results presented in this study will therefore provide a basis for future genetic work on leptospiral LPS.

## ACKNOWLEDGMENTS

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