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Functional Analysis of Genes in the *rfb* Locus of *Leptospira borgpetersenii* Serovar Hardjo Subtype Hardjobovis

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Lipopolysaccharide (LPS) is a key antigen in immunity to leptospirosis. Its biosynthesis requires enzymes for the biosynthesis and polymerization of nucleotide sugars and the transport through and attachment to the bacterial membrane. The genes encoding these functions are commonly clustered into loci; for *Leptospira borgpetersenii* serovar Hardjo subtype Hardjobovis, this locus, named *rfb*, spans 36.7 kb and contains 31 open reading frames, of which 28 have been assigned putative functions on the basis of sequence similarity. Characterization of the function of these genes is hindered by the fact that it is not possible to construct isogenic mutant strains in *Leptospira*. We used two approaches to circumvent this problem. The first was to clone the entire locus into a heterologous host system and determine if a "recombinant" LPS or polysaccharide was synthesized in the new host. The second approach used putative functions to identify mutants in other bacterial species whose mutations might be complemented by genes on the leptospiral *rfb* locus. This approach was used to investigate the function of three genes in the leptospiral *rfb* locus and demonstrated function for *orfH10*, which complemented a *wbpM* strain of *Pseudomonas aeruginosa*, and *orfH13*, which complemented an *rfbW* strain of *Vibrio cholerae*. However, despite the similarity of OrfH11 to WecC, a *wecC* strain of *E. coli* was not complemented by *orfH11*. The predicted protein encoded by *orfH8* is similar to GalE from a number of organisms. A *Salmonella enterica* serovar Typhimurium strain producing no GalE was used as a background in which *orfH8* produced detectable GalE enzyme activity.

Leptospirosis is a zoonosis of worldwide importance caused by infection with spirochetes of the genus *Leptospira*, which is divided into 12 species based on overall genomic DNA similarity. In contrast, a serological scheme of classification divides *Leptospira* into more than 200 serovars (6). The predominant antigen to which agglutinating, opsonic antibodies bind is lipopolysaccharide (LPS) (8, 14). Despite the importance of this antigen in immunity to leptospirosis and in serological classification, relatively little is known about the biosynthesis of LPS in *Leptospira*.

Recently, the *rfb* locus encoding proteins involved in the biosynthesis of LPS in *Leptospira borgpetersenii* serovar Hardjo subtype Hardjobovis was identified and shown to span 36.7 kb and contain 31 open reading frames (ORFs). The locus was defined on the basis of the identification of putative proteins involved in the biosynthesis or polymerization of nucleotide sugars (15).

In the absence of methods that enable the production of isogenic strains in *Leptospira*, the approach to the characterization of the function of the genes contained on the locus has been restricted to analysis of function in heterologous host systems. In this study, we have pursued two approaches. Firstly, the locus identified by similarity analysis was cloned and introduced into *Escherichia coli*. The second approach utilized specific genes on the *rfb* locus to complement mutated genes in heterologous hosts to confirm function identified by similarity analysis.

MATERIALS AND METHODS

Bacterial strains and plasmids. The strains and plasmids used in this study are listed in Table 1. The inserts in plasmids containing single Hardjobovis ORFs were subcloned from pLBA577 or pLBA589 (15), with the subcloned inserts oriented such that the expression of the ORF was driven by the vector-carried *lac* promoter.

Bacterial culture and preparation of competent cells. *Leptospira* was cultured in EMJH medium (13). *E. coli*, *Salmonella enterica* serovar Typhimurium, *Vibrio cholerae*, and *Pseudomonas aeruginosa* were cultured in Luria-Bertani broth. Where necessary, ampicillin (100 μg/ml) or kanamycin (50 μg/ml) was added to media. Electrocompetent *E. coli* and *V. cholerae* cells were prepared as described previously (7, 24), as were chemically derived competent *S. enterica* and *P. aeruginosa* cells (5, 10).

DNA manipulations. Genomic DNA was prepared from 100-ml stationary-phase leptospiral cultures using a procedure similar to the cetyltrimethylammonium bromide (CTAB) precipitation method (2), while plasmid DNA was prepared as described previously (3). Restriction endonuclease digestions, ligation reactions, and the analysis of DNA by agarose gel electrophoresis were performed using standard methods (2, 23). Southern hybridization was performed using probes labeled with digoxigenin-dUTP; the conditions used for hybridization were as specified by the manufacturer (Roche). Hybridization under high-stringency conditions was performed overnight at 68°C followed by washing at 68°C in 0.1% sodium dodecyl sulfate (SDS)–0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) (2). Nucleotide sequencing was performed using the BigDye DyeDeoxy terminator cycle-sequencing kit (PE Biosystems) and an Applied Biosystems 373A automated sequencer. Sequence data were analyzed with Sequencher 3.1 (GeneCodes), while DNA and protein database comparisons were made by using the BLAST program of Altschul et al. (1).

Cosmid library. Hardjobovis genomic DNA was partially digested with *Bgl*II. The enzyme concentration and time of digestion were varied to maximize the generation of digestion products in the range 35 to 50 kb. The partially digested genomic DNA was dephosphorylated using calf intestine alkaline phosphatase (Roche) and ligated into cosmid arms prepared by digesting pPR691 with *Bam*HI and *Pvu*II. DNA for transfection was prepared using in vitro packaging extracts (Gigapack III XL-4; Stratagene). Transfection of *E. coli* strain VCS257 resulted in a library of approximately 1,000 colonies.

Preparation of extracts. (i) LPS extract. LPS was prepared using a method modified from that of Westphal and Jann (31). The cells from a 500-ml overnight culture were pelleted and resuspended in 10 ml of phosphate-buffered saline. An equal volume of 90% (wt/vol) phenol was added to the bacterial suspension and incubated at 68°C for 15 min with stirring. The suspension was cooled in an ice bath to approximately 10°C and centrifuged at 1,500 × *g* for 10 min at room temperature. The upper, aqueous phase was transferred to a fresh tube, and absolute ethanol was added to 50% (vol/vol). A few pellets of sodium acetate were added, and the solution was stirred overnight at 4°C. Precipitated material

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TABLE 1. Bacterial strains and plasmids used in this study

Plasmid or strain	Characteristics	Reference or source
Plasmids		
pBluescript II KS	High-copy-number cloning vector; Ap^r	Stratagene
pWSK129	Low-copy-number cloning vector; Km^r	29
pWSK29	Low-copy-number cloning vector; Ap^r	29
pUCP19	*E. coli-P. aeruginosa* shuttle vector; Ap^r	30
pPR691	Low-copy-number cosmid vector, pSC101 replicon, and specifies resistance to Kn^r, $Spec^r$, and $Strep^r$	12
pLBA577	4.0-kb *Bam*HI insert in pBluescript II KS	15
pLBA589	7.1-kb *Hin*dIII insert in pBluescript II KS	15
pLBA591	1.65-kb *Hin*dIII-*Eco*RV fragment from pLBA589 cloned into pBluescript II KS (*orfH8* complete)	This study
pLBA593	1.70-kb *Hin*dIII-*Bam*HI fragment from pLBA589 cloned into pBluescript II KS (*orfH8* complete)	This study
pLBA655	Cosmid; insert containing *orfH1* through to *orfH31* from the Hardjobovis *rfb* locus	This study
pLBA658	*galE* from the *C. jejuni* 81116 *rfb* locus in pBluescript II KS	B. Fry[a]
pLBA661	2.04-kb *Bam*HI-*Eco*RV fragment from pLBA589 cloned into pBluescript II KS (*orfH10* complete)	This study
pLBA662	2.61-kb *Xba*I-*Bam*HI fragment from pLBA589 cloned into pWSK129 (*orfH11* complete)	This study
pLBA663	1.64-kb *Bam*HI-*Hind*III fragment from pLBA577 cloned into pBluescript II SK (*orfH13* complete)	This study
pLBA664	1.64-kb *Bam*HI-*Hin*dIII fragment from pLBA577 cloned into pWSK29 (*orfH13* complete)	This study
pLBA665	2.61-kb *Xba*I-*Bam*HI fragment from pLBA589 cloned into pBluescript II KS (*orfH11* complete)	This study
Bacterial strains		
L. borgpertersenii		
L171	Serovar Hardjo subtype Hardjobovis	15
S. enterica serovar Typhimurium		
SL696	Wild type	32
SL761	*galE* strain	25
E. coli		
VCS257	DP50 *sup* F[*supE44 supF58 hsd53*(r_B m_B) *dapD8 lacY1 glnV44* Δ(*gal-uvrB*)*47 tyrT58 gyrA29 tonA53* Δ(*thyA57*)]	Stratagene
DH5∝	F^- *endA1 hsdR17* (m_K^+ r_K^-) *supE44 thi-1 recA1 gyrA96 relA1* (ϕ80d*lacZ*ΔM15)	10
AB1133	K-12 *thr-1 leu*B6 Δ(*gpt-pro*A)*66 hisG4 argE3 thi-1 rfbD1 lacY1 ara-14 gal*K2 *xyl-5 mgl-51 rpsL31 supE44*	20
EC21566	As AB1133 but *rff*::Tn*10-66*	20
V. cholerae		
O17	Ol El Tor Ogawa	7
V1217	Strain O17 with Kn^r insertion in *rfbW*; rough phenotype	7
P. aeruginosa		
PAO1	Band B+; serotype O5	4
*wbp*M-2	Strain PAO1 with *wbpM* gentamicin resistance cassette inserted at *Nru*I; LPS B-band negative phenotype	4

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was removed by centrifuging at 15,000 × *g* for 15 min at 4°C; further ethanol was then added to a final concentration of 90% (vol/vol). A few pellets of sodium acetate were added, and the solution was stirred overnight at 4°C. The LPS was pelleted by centrifuging at 15,000 × *g* for 15 min at 4°C and resuspended in deionized water.

(ii) Cell envelope preparation. The method for cell envelope preparation was based on previously published methods (11, 19). Bacterial cells from a 100-ml culture were pelleted at 8,000 × *g* for 10 min at 4°C and washed once with sterile phosphate-buffered saline. The pellet was then resuspended in 4 ml of solution I (50 mM Tris-HCl, 2 mM EDTA [pH 8.5]). The cell suspension was then subjected to sonication using a Branson B12 sonifier (four 30-s bursts at 80 W, with incubation on ice for 2 min between bursts). The remaining intact cells were removed by centrifuging at 1,200 × *g* for 20 min at 4°C. The supernatant was then transferred to a fresh microcentrifuge tube and centrifuged at 13,000 × *g* and 4°C for 1 h. The cell envelope pellet was suspended in 100 μl of solution II (2 mM Tris-HCl [pH 7.8])–100 μl of 2× sample buffer (23) and boiled for 10 min. The solution was cooled, 40 μg of proteinase K (in solution) were added, and the mixture was incubated at 56°C for 1 h. Between 10 and 20 μl of the preparation was analyzed by SDS-polyacrylamide gel electrophoresis (PAGE).

SDS-PAGE. A Bio-Rad Mini-PROTEAN II apparatus was used with 12.5% resolving gels and a 4.5% stacking gel buffered with Tricine (18). LPS was stained using the silver-staining method of Tsai and Frasch (26).

Colorimetric assay for UDP-glucose 4-epimerase activity. The colorimetric assay was performed as described previously (21).

RESULTS

Cloning of the *rfb* locus. Partially *Bgl*II-digested Hardjobovis genomic DNA was mixed with cosmid arms and ligated. After packaging and transfection into *E. coli* strain VCS257, a library

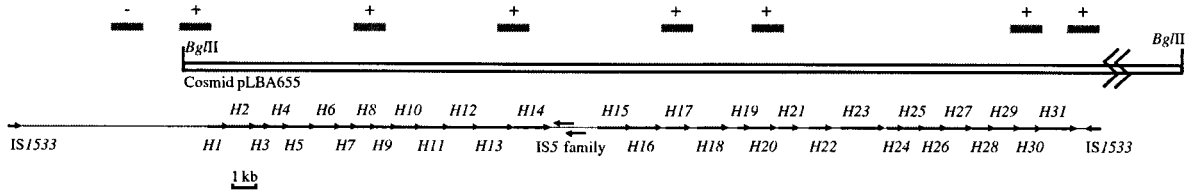

FIG. 1. The insert from cosmid pLBA655 aligned with the Hardjobovis *rfb* locus. The *Bgl*II sites bounding the cosmid insert are shown, and the bars indicate the locations of probes used in Southern hybridization to confirm the cosmid insert (+, hybridization; −, no hybridization). Arrows indicate ORFs included in the *rfb* locus or transposase genes from putative IS elements.

of approximately 1,000 cosmid strains was obtained. Plasmid preparations from transfected strains were screened by dot blot hybridization analysis to identify strains containing both the 5′ (probe within *orfH1*) and 3′ (probe in the intergenic region between *orfH31* and IS*1533*) ends of the leptospiral *rfb* locus (Fig. 1). Both probes were found to hybridize to cosmid pLBA655. This cosmid was further characterized by Southern analysis using the probes indicated by bars in Fig. 1. The hybridization profile derived from the cosmid digested with a series of restriction endonuclease enzymes was consistent with patterns predicted from the nucleotide sequence of the locus (data not shown). The insert in pLBA655 was thus determined to extend from a *Bgl*II site upstream of *orfH1* to a point beyond the IS*1533* element downstream of *orfH31* (Fig. 1). Western blot analysis of whole-cell preparations from strain VCS257 containing pLBA655 using anti-Hardjo immune sera from a series of patients revealed that no Hardjo polysaccharide was detectable (data not shown).

Determination of function. Similarity analysis has been used as the basis for assigning putative functions to several genes in the *rfb* locus (15). Based on their sequence similarity to genes of known function, genes from the leptospiral *rfb* locus were used to complement specific mutations in hosts other than *Leptospira*. In addition, an enzyme assay was used to investigate the function of OrfH8.

OrfH8 functional analysis. OrfH8 was proposed to be a galactose epimerase; this activity can be detected specifically by a sensitive enzyme assay (21). *S. enterica* strain SL761 (25), which lacks galactose epimerase activity, was chosen as a host for plasmids pLBA591 and pLBA593, which contained *orfH8* such that its expression was driven by the *lac* promoter. Extracts from strain SL761 containing pLBA591 or pLBA593 were shown to have 52 and 133 U of galactose epimerase activity per g of protein, respectively. These activities were lower than those detected in wild-type *S. enterica* (strain SL696) and strain SL761 transformed with the *Campylobacter jejuni galE* gene (Table 2).

Functional analysis of putative nucleotide-sugar epimerases encoded by *orfH10* and *orfH11*. Based on similarity analysis, *orfH10* and *orfH11* were proposed to encode epimerases that convert UDP-*N*-acetylglucosamine (UDP-GlcNAc) to unknown epimers. These epimerases may be involved in the biosynthesis of UDP-*N*-acetylgalactosamine (GalNAc), UDP-*N*-acetylmannosamine (UDP-ManNAc), UDP-*N*-acetylfucosamine (UDP-FucNAc), or other UDP-hexoseNAcs. OrfH10 is most closely related to *Staphylococcus aureus* Cap5E (80% similarity, 65% identity), while OrfH11 is most closely related to Cap5G (72% similarity, 53% identity) as well as to Cap5P (52% similarity, 27% identity) (15).

TABLE 2. Determination of GalE enzyme activity

Strain	Feature	Enzyme activity (mU/mg of protein)[a]
SL696	*S. enterica* wild type	318
SL761	*galE* strain	0
SL761(pBluescript II KS)	pBluescript II KS control	0
SL761(pLBA591)	*orfH8*	52
SL761(pLBA593)	*orfH8*	133
SL761(pLBA658)	*galE* from *C. jejuni*	367

[a] Unit definition: the amount of enzyme which produced 1 μmol of UDP-glucose in 1 min at 37°C.

Cap5P and Cap5O are involved in UDP-ManNAcA biosynthesis; Cap5P is a 2-epimerase which converts UDP-GlcNAc to UDP-ManNAc, and Cap5O is a dehydrogenase which converts UDP-ManNAc to UDP-ManNAcA. Encoded on the same locus, Cap5G is also proposed to be a 2-epimerase, possibly with a role in the biosynthesis of UDP-FucNAc (16). Kiser and Lee (16) used *E. coli* strain EC21566 containing a mutation in *wecC* (known to encode a protein which converts UDP-GlcNAc to UDP-ManNAc) to demonstrate that *cap5P* complemented the mutation while *cap5G* did not. We have attempted to complement the *wecC* mutation in *E. coli* strain EC21566 with *orfH11*.

A 2.6-kb *Xba*I-*Bam*HI fragment containing *orfH11* was subcloned into pWSK129 and pBluescript II SK (pLBA662 and pLBA665, respectively) such that expression of *orfH11* could be driven by the *lac* promoter. Whole-cell lysates from *E. coli* strain DH5α containing pLBA665, prepared after induction overnight with 2 mM isopropyl-β-D-thiogalactopyranoside (IPTG), were shown to contain an additional band at 42 kDa compared to lysates from a control strain containing pBluescript II SK (data not shown). This corresponded to the predicted molecular mass of 41.5 kDa for OrfH11. No similar band was observed for strain DH5α containing pLBA665. pLBA662 and pLBA665 were transformed into EC21566. The phenotypes of the parental strain of EC21566 (AB1133), EC21566, and the two strains containing *orfH11* were assessed by Western blotting using monoclonal antibody 898 (20, 22). This monoclonal antibody reacted with the parental strain but not with the control or either of the strains containing *orfH11*, indicating that this ORF was not able to complement the *wecC* mutant.

Similarity analysis revealed that OrfH10 is also related to a number of GlcNAc epimerases including Cap8E from *S. aureus* (65% identity and 80% similarity) and WbpM from *P. aeruginosa* (32% identity and 58% similarity) (15). WbpM converts UDP-GlcNAc to UDP-GalNAc, the first step in the biosynthesis of UDP-FucNAc. This function is proposed to reside in the N-terminal half of the protein. Strain *wbp*M-2 contains a *wbpM* gene which has been insertionally inactivated at the 5′ end. Phenotypically, this strain has no detectable B-band LPS and the wild-type phenotype can be restored by providing *wbpM* in *trans* (4). Accordingly, we used this mutant to determine if its mutation could be complemented by *orfH10*. Plasmid pLBA661 was derived by cloning a 2-kb *Bam*HI-*Eco*RV

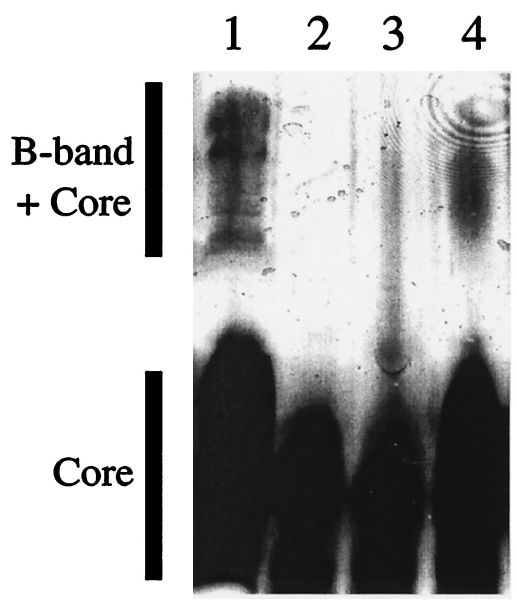

FIG. 2. Silver-stained Tricine-SDS-PAGE (12.5% polyacrylamide) analysis of LPS extracts. Lanes: 1, *P. aeruginosa* strain PAO1, wild type, serotype O5; 2, strain *wbp*M-2, B-band negative; 3, strain *wbp*M-2 (pUCP19); 4, strain *wbp*M-2 (pLBA661). Stained polysaccharide features are indicated.

fragment containing the entire *orfH10* into *Bam*HI-*Sma*I-digested pUCP19. This plasmid was transformed into strain *wbp*M-2; the B-band was partially restored in strain LBA736 compared to the situation for strain PAO1 (Fig. 2).

***orfH13* functional analysis.** Based on similarity analysis, OrfH13 was proposed to be an undecaprenyl-glycosyl-1-phosphate transferase (und-pp-glycosyltransferase) (15). Among the proteins identified as similar to OrfH13 was WbaP from *S. enterica* (55% similarity, 25% identity). Importantly, similarity extends across the entire length of the compared proteins, and

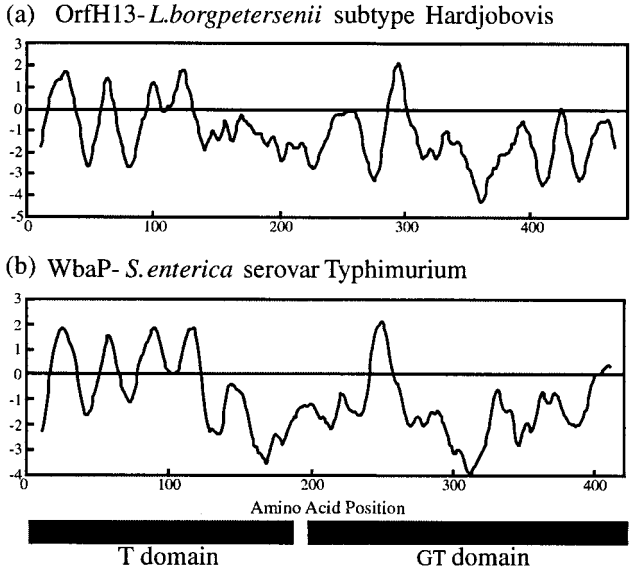

FIG. 3. Hydropathy profile of Hardjobovis OrfH13 (a) and WbaP from *S. enterica* serovar Typhimurium (b). The T and GT domains indicated are as identified by Wang et al. (28).

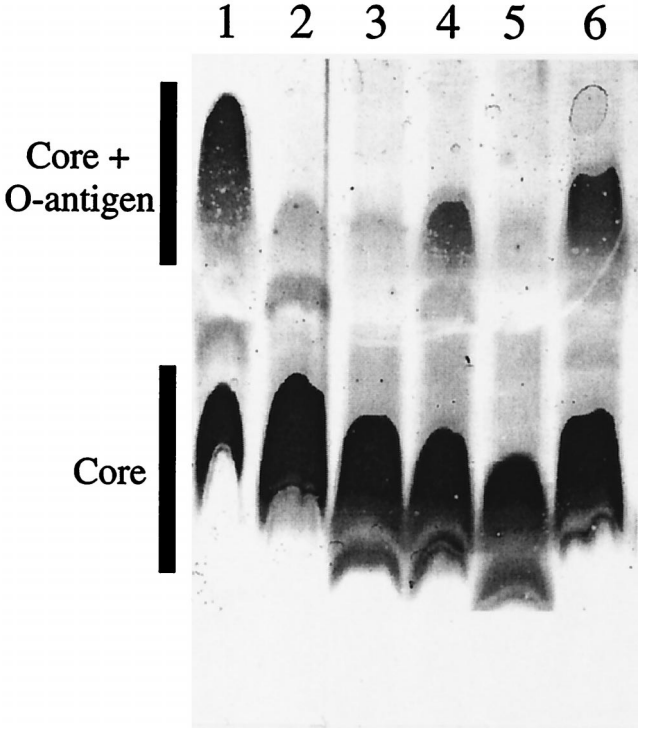

FIG. 4. Silver-stained Tricine-SDS-PAGE (12.5% polyacrylamide) analysis of proteinase K-treated cell envelope extracts. Lanes: 1, *V. cholerae* strain O17, wild type; 2, strain 1217, *rfbW* strain; 3, strain 1217 (pBluescript KS+); 4, strain 1217(pLBA663); 5, strain 1217(pWSK129); 6, strain 1217(pLBA664). Stained polysaccharide features are indicated.

hence OrfH13 may contain both the T and GT domains (N- and C-terminal halves, respectively) identified previously in WbaP (28). Furthermore, the hydropathy profiles of OrfH13 and WbaP are remarkably similar; in particular, the T domain of both OrfH13 and WbaP is predicted to contain four transmembrane domains (Fig. 3).

We attempted to examine the sugar specificity of OrfH13, the putative und-pp-glycosyltransferase of Hardjobovis. Based on the assumption that the two domains present in OrfH13 represent a fusion of two proteins (28) and that the original, independent functions of these proteins have not been destroyed by the fusion, it should be possible to identify the glycosyltransferase activity of the GT domain of OrfH13. RfbW (63% similarity and 43% identity to OrfH13) from *V. cholerae* is a galactosyltransferase. The *rfbW* gene in strain V1217 has been insertionally inactivated using a kanamycin resistance cassette, resulting in a rough phenotype (7). When *rfbW* was supplied in *trans*, the smooth phenotype was restored, as indicated by the presence of O-antigen and core bands in SDS-PAGE analysis (7). A comparison of the LPS produced by this strain with the LPS produced by strain V1217 transformed with either pLBA663 or pLBA664 (containing the entire *orfH13* coding region) is shown in Fig. 4. The restoration of the O-antigen and core bands in the complemented strains indicated that OrfH13 has galactosyltransferase activity.

DISCUSSION

The initial objective of this work was to investigate the possibility of using the Hardjobovis *rfb* locus to synthesize "recombinant" leptospiral O antigen in a heterologous host such as *E. coli*. The cosmid (pLBA655) containing *orfH1* through to

orfH31 was unable to direct synthesis of Hardjobovis O antigen. Reverse transcription-PCR analysis of *E. coli* strains containing the cosmid revealed that *orfH10* through *orfH16* were not transcribed (unpublished data). A plausible explanation for the failure of the cosmid strain to produce Hardjobovis O antigen is that there was no O-antigen subunit assembly because the und-pp-glycosyltransferase (*orfH13*) was not expressed. This enzyme carries out a critical early function in O-antigen subunit assembly.

In the context of the development of a rational strategy for the expression of Hardjobovis O antigen in a heterologous host, the identification of the activity of the GT domain of OrfH13 is significant. Previously, ^{14}C-labeled nucleotide sugars have been used to detect the incorporation of sugars onto the lipid carrier, and in combination with thin-layer chromatography, this approach has been used to determine the specificities of glycosyltransferases (17, 27). Significantly, the use of this approach to determine the specificities of the glycosyltransferases in the Hardjobovis *rfb* locus is only now possible because of the identification of galactose as the first sugar transferred to the lipid carrier.

Demonstrating that the putative und-pp-galactosyltransferase, OrfH13, can complement a galactosyltransferase mutant is not consistent with the model suggested by Wang et al. (28), which proposed that the specificity for the lipid carrier was contained in the GT domain. However, a functional dependence for glycosyltransferases on the lipid carrier (perhaps by binding to the lipid carrier) may be a means by which the function of glycosyltransferases is coordinated in the cytoplasm. It is also possible to speculate that und-pp-galactosyltransferases may have arisen through a fusion of a 'T' protein and a glycosyltransferase. Gene fusions involving glycosyltransferases appear to be common (e.g., OrfH23 in the Hardjobovis locus [15] and Y4gI in *Rhizobium* [9]) and may provide an advantage by intimately linking the functions of the fused proteins, as in the case of OrfH13, ensuring that galactose is the first sugar attached to the lipid carrier.

OrfH8, a galactose epimerase, converts UDP-glucose to UDP-galactose, thereby providing the substrate for OrfH13. Notably, a homolog of OrfH13 is present in the *rfb* locus of *L. interrogans* serovars Copenhageni and Pomona, although no OrfH8 homolog is present in either of these loci (unpublished data). The high level of similarity (86% similarity and 74% identity) between the OrfH13 homologs indicates that UDP-galactose is probably the substrate for each of the homologs and therefore suggests that the *orfH8* homolog is located elsewhere on the Copenhageni and Pomona genomes.

Based on similarity analysis, we have previously proposed that the OrfH10 and OrfH11 are epimerases that have a common substrate (15). In this study, OrfH10 has been shown to convert UDP-GlcNAc to UDP-GalNAc. While it has not yet been possible to identify the function of OrfH11, it is feasible that OrfH11 converts UDP-GlcNAc to an epimer other than UDP-ManNAc. The WbpM protein is another protein that may have arisen as a result of a gene fusion. The N-terminal half of this protein is similar to OrfH10 and other UDP-GlcNAc epimerases, while the C-terminal half is similar to a number of GalE protein homologs. Notably, the C-terminal half does not play an essential role in B-band synthesis, as indicated by the unchanged phenotype in the *wbp*M-1 strain, in which the 3′ end of *wbpM* was insertionally interrupted (4).

The data presented here provide an important insight into the biosynthesis of LPS in *L. borgpetersenii* subtype Hardjobovis. The identification of OrfH13 as an und-pp-galactosyltransferase indicates that the process by which leptospiral LPS is synthesized is the same as in other bacteria (i.e., the O-antigen subunit is synthesized on a lipid carrier). This knowledge is essential for the design of effective strategies for the eventual synthesis of leptospiral LPS in heterologous host systems. In addition, the identification of the nucleotide sugars synthesized by proteins encoded on the leptospiral *rfb* locus will assist in the eventual determination of the structure of leptospiral LPS.

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