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Lipopolysaccharide Biosynthesis in *Leptospira*

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Abstract

Lipopolysaccharide is the major surface antigen of *Leptospira*. Variation in LPS structure is the basis for the more than 200 serovars that have been identified. Despite the importance of this antigen in immunity and diagnostics, there is relatively little known about the genetics and chemistry of leptospiral LPS, as compared to some members of the *Enterobacteriaceae*. The nucleotide sequence of the locus encoding enzymes for the biosynthesis of the O-antigen component of leptospiral LPS (*rfb* locus) has been determined for three serovars namely, *L. interrogans* serovar Pomona, *L. interrogans* serovar Hardjo subtype Hardjoprajitno and *L. borgpetersenii* serovar Hardjo subtype Hardjobovis. In the absence of data relating to the chemical structure or genetic tools to construct isogenic mutants in *Leptospira*, similarity analysis has been used to provide insight into the mechanisms by which the leptospiral O-antigen is assembled by comparison with characterized systems from other bacteria. In addition, comparison of the gene layout in each of the serovars provides an indication of the genetic basis for serovar diversity.

Introduction

The spirochetes are a diverse group of bacteria, as illustrated by the significant differences in the composition of the outer-membranes of these organisms. Lipopolysaccharide (LPS) is the dominant antigen on the surface of the *Leptospira* and *Brachyspira*, in complete contrast to *Treponema pallidum* and *Borrelia burgdorferi*, which have no LPS. Analysis of the *T. pallidum* and *B. burgdorferi* genomes has revealed that there appears to be no genetic capacity to produce the polysaccharide component of LPS (Fraser *et al*., 1997; Fraser *et al*., 1998). Nor are these organisms likely to be able to produce the Lipid A component as indicated by the absence of an *lpxA* homolog in either of their genomes. LpxA is an UDP-*N*-acetylglucosamine-3-*O*-acetyltransferase which catalyzes the first committed step in lipid A biosynthesis (Dotson *et al*., 1998).

In *Leptospira*, LPS is the principal antigen against which the humoral immune response is directed during infection. The role of this antigen in immunity has been demonstrated by studies which showed that monoclonal antibodies directed against LPS determinants can protect against challenge with pathogenic *Leptospira* with the same determinant (Jost *et al*., 1989). In addition, anti-LPS monoclonal antibodies have been shown to be opsonic (Farrelly *et al*., 1987).

Serovar identity is attributable to differences in LPS structure. More than 200 pathogenic serovars of *Leptospira* are currently recognized. The basis for the identification of serovars is the demonstration that a particular strain has some novel antigenic characteristics; this is determined by a process of absorbing antisera raised to a candidate strain with antigenically related serovars, using the microscopic-agglutination test (MAT). A detailed explanation of this process can be found in Faine *et al*. (1999). The net effect of the substantial serological cross-reactivity between related serovars and the difficulty in creating a truly standardized MAT is that the serovar is to some degree a subjective taxon. The serovar has been a useful taxon in the diagnostic context. However, from a research perspective, and specifically with respect to the genetics of LPS biosynthesis, this taxon may not represent a group of strains with an identical LPS structure. Thus, while the list of serovars provides ample evidence of a significant diversity in LPS structure between leptospires, it is difficult to establish more than rudimentary relationships between serologically related serovars. Comprehensive testing of serovars with existing monoclonal antibodies may provide a more detailed insight into the relationships between serovars.

Clearly, the most precise way to describe the diversity of LPS structure in *Leptospira* would be through the comparison of the polysaccharide structures; however no structural data are available. The chemical composition of the polysaccharide component of leptospiral LPS has been examined in a few strains. In each instance the major component sugars were identified as rhamnose, galactose, arabinose and xylose. The proportions of these sugars varied between strains (Faine *et al*., 1974; Vinh *et al*., 1986; Vinh *et al*., 1989).

Sugars

In nature sugars are used in an array of biological functions ranging from the biosynthesis and degradation of storage and structural polysaccharides to key host-pathogen interactions. These biological functions may represent adaptions of four fundamental cell processes, namely the biosynthesis of sugars (and degradation of sugars), the polymerization of sugars (glycosyltransferases), the degradation of sugar polymers (glycoside hydrolases) and the transport of sugars across membranes. These functions may well be as fundamental to the cell as the ability to translate RNA into protein.

There are examples of bacteria where some of these carbohydrate, metabolic processes are not present; eg. some Archaebacteria are able to polymerize sugars but are unable to degrade these polymers as indicated by the absence of ORFs encoding proteins with similarity to any

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of the known glycoside hydrolases (Coutinho and Henrissat, 1999). *Leptospira* is unusual in that it is unable to use exogenous carbohydrate as a carbon source (Faine *et al*., 1999). A consequence of this metabolic peculiarity is that all sugars that are incorporated into leptospiral LPS must be synthesized *de novo*. In an evolutionary context, an unresolved question is whether these peculiarities in sugar metabolism represent "lost ability" or abilities that never existed. If we contend that these are part of a set of fundamental cellular functions, then these abilities have been lost and this loss may be related to the process of adaption to unusual environmental niches, for instance the adaption that has enabled *Leptospira* to grow in renal tubules.

A fundamental feature links the synthesis of structural polysaccharides such as O-antigen, peptidoglycan and some capsule polysaccharides; in each instance the polysaccharide is synthesized on undecaprenyl phosphate (Whitfield *et al*., 1995). The assembly of surface polysaccharide structures is complex but highly ordered, reproducibly producing identical structures in all cells. The process is controlled by a series of cytoplasmic enzymes and membrane proteins that catalyse the biosynthesis of sugars and the assembly of sugar polymers and the eventual surface location and anchoring of these structures to the cell surface (Whitfield *et al*., 1995). Comparison of the enzymes encoded on the locus for the biosynthesis of LPS in *Leptospira* with enzymes from other more characterized Gram-negative LPS biosynthesis systems is an essential exercise prior to the functional analysis of leptospiral enzymes so as to develop a model for LPS biosynthesis in *Leptospira*.

LPS Biosynthesis

The assembly of sugars into polymers in LPS biosynthesis is often a process where enzymes act sequentially; *i.e.* there is a dependence on enzymes acting and sequentially modifying a product. Therefore, the effect of changes to the function of enzymes involved in this will not be necessarily limited to the enzyme itself, but has the potential to disrupt all subsequent steps in the assembly process. Interestingly, this may be a contributing factor to the limited repertoire of sugars that are attached onto the lipid carrier. The transfer of a sugar onto undecaprenyl phosphate is the first step in O-antigen subunit biosynthesis and the sugars incorporated is limited to either UDP-galactose or UDP-NAc-glucosamine (Whitfield *et al*., 1995). Despite these constraints due to sequential assembly, genetic drift leading to changes in enzymatic function can result in a substantial change in the LPS phenotype. This was illustrated in an investigation of the genetic basis for the *Escherichia coli* O9 and O9a serotypes. In this instance a change in the processivity of a mannosyltranferase was identified as the basis for the difference in serotype (Kido and Kobayashi, 2000).

Another factor which constrains the types of changes that are possible to the structure of LPS is the importance of LPS to the integrity of the outer membrane. Where genetic changes result in truncated LPS, possible consequences would be reduced viability, such as that seen in the deeper-core mutants of *Salmonella* (Schnaitman and Klena, 1993). Given that there are no rough leptospiral strains documented, substantial truncation of the polysaccharide component of LPS may be lethal to *Leptospira*.

Leptospiral *rfb* Loci

A comparison of the genetic organization of the O-antigen biosynthesis loci (*rfb*) from three leptospiral strains is shown in Figure 1. Strain L170 is classified as *L. interrogans* serovar Pomona (Pomona), while strain Hardjoprajitno is classified as *L. interrogans* serovar Hardjo, subtype Hardjoprajitno (Hardjoprajitno). Strain L171 is classified as *L. borgpetersenii* serovar Hardjo subtype Hardjobovis (Hardjobovis). Notably, the Hardjo subtypes are serologically indistinguishable, yet they belong to different species.

The limits of the *rfb* locus in each instance were determined by sequence analysis, specifically sequence similarity to proteins involved in sugar biosynthesis, polymerization of the activated sugars or the processing of these polymerized sugars to the surface of the bacterium. Using these criteria, the Pomona, Hardjobovis and Hardjoprajitno loci are 34.7 kb, 37.6 kb and 37.9 kb in length respectively.

In order to facilitate the comparison of the loci and to indicate that the functions of the proteins encoded on the loci are in the majority assigned on the basis of sequence similarity we have adopted an ORF numbering system, although eventually it is anticipated that the w* system of nomenclature will be adopted (Reeves *et al*., 1996). In our temporary system, ORFs with sequence similarity are assigned the same number and a letter indicates the originating strain (P=Pomona, J=Hardjoprajitno and H=Hardjobovis). In our view the assignment of a formal nomenclature could prejudice the assignment of function if homologs were assigned the same letter code. This may be best illustrated by the recent demonstration that the processivity of a mannosyltransferase was affected by a single changed residue (Kido and Kobayashi, 2000).

The Hardjobovis *rfb* Locus

The Hardjobovis *rfb* locus comprises 31 ORFs, each of which is encoded on the same strand. The intergenic region between *orfH14* and *orfH15* contains an IS element orientated such that the transposase is encoded on the opposite strand to the ORFs in the *rfb* locus. The features of this element indicate that it is a member of the IS*5*-family (Mahillon and Chandler, 1998). The right terminal repeat and part of the 3' end of the transposase gene of this IS*5*-family element is duplicated in a tandem fashion beyond the genetic remnant of another, unrelated transposase. This mosaic of elements is 1.8 kb in size. Two IS*1533* elements flank the *rfb* locus. One is located downstream of *orfH31* and the other is located approximately 8 kb upstream of *orfH1* (Kalambaheti *et al*., 1999). It is tempting to speculate that these IS*1533* elements may have been involved in the acquisition of all or part of the locus by horizontal transmission, but there is no evidence yet for such a proposal.

Nine ORFs encode proteins with similarity to glycosyltransferases. Using the classification scheme of Campbell *et al.* (1997), OrfH1, OrfH21, OrfH22, OrfH23, OrfH28, OrfH29 and OrfH30 are included in Family 2. OrfH5 is included in Family 4, while OrfH12 and OrfH13 have not

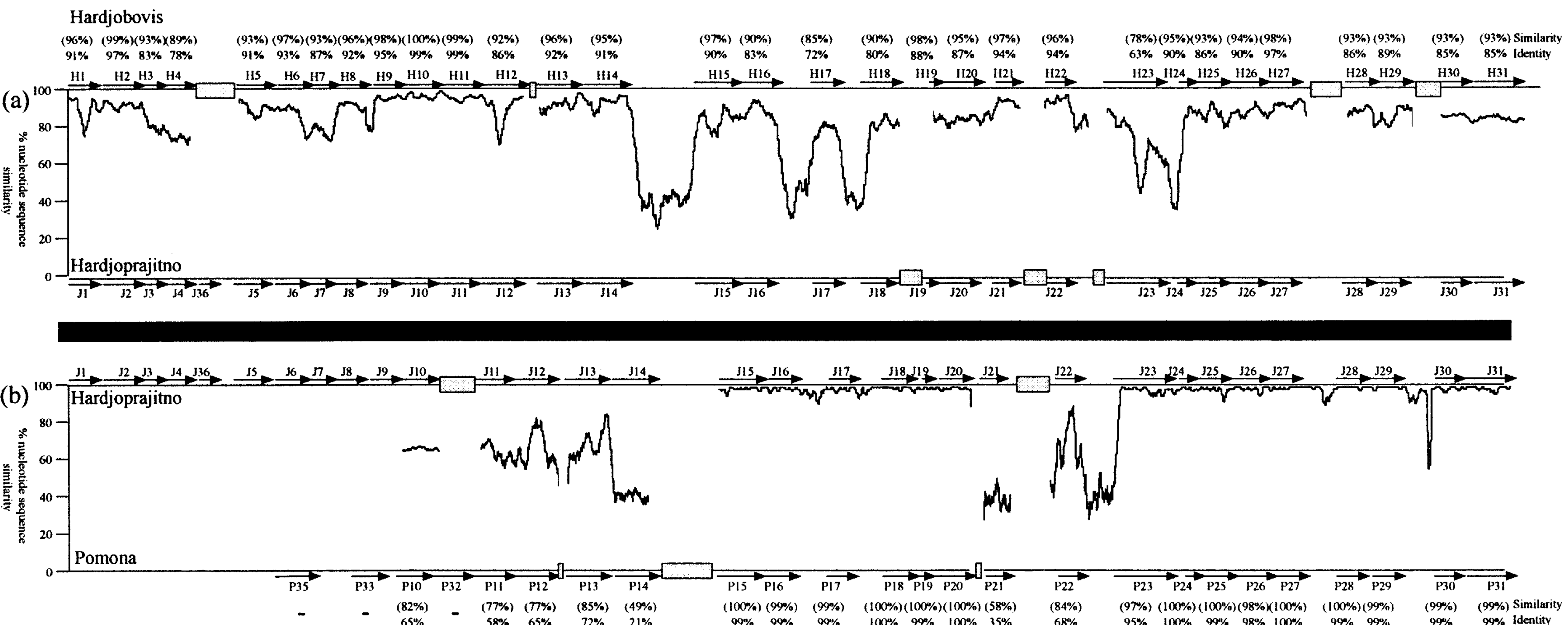

Figure 1. Graphical comparison of the organization and nucleotide sequence of the *rfb* locus of (a) Hardjoprajitno and Hardjobovis and (b) Hardjoprajitno and Pomona. For each comparison, a pairwise alignment of sequences was constructed using GAP. PLOTSIMILARITY (window size 200 bases) was used to draw the graphical relationship. Gaps of greater than 100 bases in the alignments (indicating insertions or deletions) are shown as shaded boxes. Flanking the line diagram of the Pomona and Hardjobovis *rfb* loci are sequence similarity and identity values comparing the protein sequence encoded by each ORF in the Pomona and Hardjobovis *rfb* loci with the corresponding encoded protein in the Hardjoprajitno *rfb* locus. These values were determined using BESTFIT. The programs used to construct this diagram were from the GCG suite of programs (Genetics Computer Group, 1994).

been classified. This system of classification is based on sequence similarity and does not give insight into the specificity of the glycosyltransferases. In the absence of any *in silico* method for predicting the function of glycosyltransferases, ultimately the function of these enzymes will have to be determined experimentally.

Towards this end, the function of OrfH13 has been investigated. Hydrophobicity analysis revealed that the profile of OrfH13 resembled the profiles of a number of undecaprenyl-glycosyltransferases, which transfer the first nucleotide sugar to the lipid-carrier (Kalambaheti *et al.*, 1999), and is the first step in O-antigen subunit biosynthesis. Functional analysis revealed that OrfH13 is a galactosyltransferase (Bulach *et al.*, 2000); further analysis is required to demonstrate that it is an undecaprenyl-galactosyltransferase. Notably, the identification of the first sugar incorporated into the leptospiral O-antigen subunit will facilitate studies investigating subunit assembly. This is especially relevant to the analysis of leptospiral genetic systems, where there are no systems for the construction of isogenic mutants.

Sugar biosynthesis genes are clustered in three sections of the Hardjobovis *rfb* locus. Historically, the *rfb* locus in *Leptospira* was identified by locating the rhamnose biosynthetic gene cluster. The genes were identified using low stringency hybridization and a probe which contained the *rmlA* and *rmlB* genes from *Salmonella enterica* (Mitchison *et al.*, 1997). Confirmation of the function of the rhamnose biosynthetic genes (*orf24* through to *orf27*) from *L. interrogans* serovar Copenhageni was achieved by demonstrating that these genes, supplied *in trans*, complemented a deficiency in rhamnose biosynthesis in the *Shigella flexneri* 2a strain N1308. Gas chromatography analysis has been used to show that rhamnose is a component of the LPS in each of Pomona, Hardjoprajitno and Hardjobovis (Faine *et al.*, 1974; Vinh *et al.*, 1986) and it is reasonable to assume that the function of *orf24* through to *orf27* in each of these serovars is to encode enzymes involved in the biosynthesis of rhamnose. Notably, there is almost 100% nucleotide sequence identity between serovars Copenhageni and Pomona in this region.

The second sugar biosynthetic region spans *orfH6* through to *orfH11*. Based on similarity analysis, we propose that this region encodes enzymes for the biosynthesis of at least four sugars. Biochemical analysis has been used to demonstrated that OrfH8 converts UDP-glucose to UDP-galactose (Bulach *et al.*, 2000). This confirmed an earlier assignment of function, based on sequence similarity, that *orfH8* encoded a galactose epimerase (Kalambaheti *et al.*, 1999). Gas Chromatography analysis has confirmed that galactose is a component of the LPS from Pomona, Hardjoprajitno and Hardjobovis (Faine *et al.*, 1974; Vinh *et al.*, 1986). However, no *galE* homolog has been identified in the Pomona *rfb* locus. Given that *Leptospira* synthesizes all sugars *de novo*, it is certain that a *galE* homolog will be present on the Pomona genome.

Complementation of a *wbpM* mutation in strain *wbp*M-2 of *Pseudomonas aeruginosa* serotype O5 using *orfH10* supplied *in trans* confirmed that this ORF encoded a UDP-NAc-galactosamine epimerase, converting UDP-NAc-glucosamine to UDP-NAc-galactosamine (Bulach *et al.*, 2000). Based on similarity it was proposed that *orfH11* would encode an epimerase that converted UDP-NAc-glucosamine to UDP-NAc-mannosamine; however *orfH11* does not complement *E. coli* with a mutation in *wecC* (Bulach *et al.*, 2000). Interestingly, in the Pomona *rfb* locus there is an additional putative NAc-hexosamine epimerase (*orfP32*) located between *orfP10* and *orfP11*. The common substrate for each of these enzymes (*orf10*, *orf11* and *orfP32*) is possibly UDP-NAc-glucosamine. The strategy of using a common precursor as a starting point for the synthesis of a variety of related sugars is the basis for the synthesis of sugars such as tyvelose, paratose and colitose (Kido and Kobayashi, 2000) and represents interesting (and energetically economical) means of introducing diversity into LPS structure.

Similarly, the enzymes produced by *orfH6* and *orfH9*, with similarity to RmlB and RmlD respectively, may intercept precursors in the biosynthesis of rhamnose to produce another related sugar. This is the case for the biosynthesis of talose in *Actinobacillus actinomycetemcomitans*, where dTDP-4-keto-L-rhamnose, a precursor in the synthesis of rhamnose, is converted to talose by a reductase (Nakano *et al.*, 1998). Alternatively and of equal feasibility, these ORFs may be involved in the synthesis of UDP-NAc-fucosamine as we have previously speculated (Kalambaheti *et al.*, 1999).

The third sugar biosynthetic region includes *orfH18*, *orfH19* and *orfH20*. These ORFs are probably involved in the biosynthesis of an amino sugar; based on similarity *orfH19* encodes an isomerase and *orfH20* encodes an aminotransferase (Kalambaheti *et al.*, 1999).

OrfH31, OrfH14 and OrfH15 are predicted (Psort http://psort.nibb.ac.jp) to contain multiple membrane-spanning segments (14, 10 and 10 membrane spanning segments respectively). By analogy with other O-antigen biosynthetic loci, integral membrane proteins such as these may play a role in the transport and processing of the O-antigen subunit through the membrane and ligation to surface exposed structures (Whitfield *et al.*, 1995). OrfH15 is similar (31% identity and 59% similarity) to the O-antigen flippase (Wzx) of *Yersinia enterocolytica* (Kalambaheti *et al.*, 1999). The identification of OrfH15 as a putative O-antigen flippase is significant, because it indicates that the processing of the O-antigen subunit through the membrane is related to the Wzy-dependant pathway found in many Gram-negative bacteria (Whitfield *et al.*, 1995), based on the unique requirement for a flippase in this pathway.

While there are no sequences similar to OrfH14 in the genetic databases, it has been proposed that this protein is the Hardjobovis Wzy or O-antigen polymerase. There are instances where there is no detectable similarity between characterized O-antigen polymerases (Kalambaheti *et al.*, 1999). A factor contributing to the high level of sequence divergence is specificity for O-antigen subunit. Notably, OrfJ14 and OrfP14 have significantly less sequence similarity (21% identity, 49% similarity, Figure 1) compared to other ORFs for which homologs exist (average similarity 87%). However, despite the lower similarity, the hydrophobicity profiles of these proteins are remarkably similar. OrfH31 shows similarity to a number of transporter proteins, but the role of this protein in LPS biosynthesis is unknown.

Some ORFs seem to be misplaced in the Hardjobovis *rfb* locus. *orfH3* and *orfH4* respectively encode proteins similar to the amidotransferase and cyclase for the biosynthesis of histidine. Interestingly, homologs of these same genes are found in the O-antigen biosynthetic loci of

both *P. aeruginosa* serotype O5 (Burrows *et al*., 1996) and *Campylobacter jejuni* (Parkhill *et al*., 2000). The reason for presence of these genes in the O-antigen biosynthesis loci of some bacteria is unclear, a possible explanation being that these genes are remnants from an earlier horizontal transfer event (Kalambaheti *et al*., 1999).

Three ORFs (*orfH2*, *orfH16* and *orfH17*) could not be assigned a putative function based on similarity analysis. One of the obvious biosynthetic functions not identified was the presence of genes encoding proteins involved in arabinose or xylose biosynthesis. In particular, enzymes for arabinose biosynthesis have not been identified in other bacteria for which arabinose is a component of either LPS or capsule *e.g.* the capsule of *Pasteurella multocida* M1404 (B:2) (Boyce *et al*., 2000).

Comparison of the Pomona, Hardjoprajitno and Hardjobovis *rfb* Loci

Unremarkably, the *rfb* loci from Hardjo subtypes (Hardjobovis and Hardjoprajitno) have almost identical ORF compositions. This was expected, considering that these strains produce LPS that is indistinguishable serologically. Both *rfb* loci have 31 ORFs with conserved gene order and content. The only difference in coding potential is an additional ORF between *orfJ4* and *orfJ5* in Hardjoprajitno for which similarity analysis identifies no homologs. Other differences in locus layout are restricted to intergenic regions but most notably the Hardjoprajitno *rfb* locus does not contain any IS-elements (de la Peña Moctezuma *et al*., 1999). A surprising feature of the comparison of the Pomona and Hardjoprajitno *rfb* loci is the near nucleotide sequence identity between sections of these loci, specifically from *orf15* through to *orf31*, excluding *orf21* and *orf22*. This in contrast to the average of 86% nucleotide sequence similarity between Hardjobovis and Hardjoprajitno in the corresponding region (Figure 1). It would seem likely that these sections of all three loci either encode enzymes of identical function or where there is variation in function, the structural differences in the LPS of Hardjoprajitno and Hardjobovis do not alter the antigenic epitopes available to the immune system (de la Peña Moctezuma *et al*., 1999).

Since Hardjoprajitno and Pomona are more genetically related (both *L. interrogans*) than Hardjobovis (*L. borgpetersenii*), it is interesting to observe that the sequences from the Hardjoprajitno *rfb* locus in the regions *orfJ1* through *orfJ14* and *orfJ21* though *orfJ22* are more related to the Hardjobovis sequences. Alignment of nucleotide sequences in the coding regions of these ORFs reveals that nucleotide sequence differences are largely confined to the third base of codons (de la Peña Moctezuma *et al*., 1999). We propose that the differences in sequences are the consequences of an assimilation of DNA, acquired by lateral transfer from an *L. borgpetersenii* strain, to the codon usage in the *L. interrogans* strain (de la Peña Moctezuma *et al*., 1999). The convergence of Hardjoprajitno to an antigenic phenotype which is serologically indistinguishable from Hardjobovis suggests a biological impetus. Notably, there is a well documented association between serovar Hardjo and cattle and the advantage to the Hardjoprajitno subtype may relate to an aspect of this association (de la Peña Moctezuma *et al*., 1999; Faine *et al*., 1999).

Significantly, there are obvious differences in the gene content and gene layout between Pomona *rfb* locus and the Hardjo *rfb* loci. These differences are predominantly located at the 5' end of the *rfb* loci (Figure 1). The Pomona *rfb* locus does not contain homologs of *orf1* though to *orf9*. In addition, there are three ORFs in the Pomona locus that are not found in either of the Hardjo *rfb* loci. As noted above, *orfP32* encodes a putative NAc-glucosamine epimerase and is located between *orfP10* and *orfP11*. Immediately upstream of *orfP10* there is an ORF (*orfP33*) encoding a glycosyltransferase. This glycosyltransferase is similar to several mannosyltransferases but most related to the putative mannosyltransferase from *Synechocystis* (45% similarity, 27% identity GenBank Accession: BAA18084). The intergenic region between *orfP33* and *orfP35* contains an ORF which is disrupted by two single base insertions; this ORF is intact in other serovars such as Copenhageni (de la Peña Moctezuma *et al*., unpublished). *orfP35* encodes a protein with 13 hypothetical trans-membrane segments (Psort http://psort.nibb.ac.jp) and with no homologs identified by similarity analysis. These additional proteins encoded on the Pomona *rfb* locus could potentially synthesize an epimer of possibly NAc-glucosamine (OrfP32) and add it to the O-antigen subunit (OrfP33).

In Hardjoprajitno, the apparent "acquisition" of *orfJ21* and *orfJ22*, which encode proteins that are more related to the Hardjobovis than the Pomona homologs (Figure 1) suggests that the functions of the Pomona Orf21 and Orf22 may differ from the Hardjo homologs. Notably, both Orf21 and Orf22 are glycosyltransferases.

A striking feature of the Pomona, Hardjobovis and Hardjoprajitno *rfb* loci is the conserved gene order from *orf11* through to *orf31*. As indicated earlier, the function of some of the encoded proteins may vary between serovars *e.g. orf14* (putative O-antigen polymerase) and *orf21* and *orf22* (glycosyltransferases). However, some functions seem to be conserved *e.g. orf24*, *orf25*, *orf26* and *orf27* (rhamnose biosynthesis) given that the LPS of each of these serovars is known to contain rhamnose (Faine *et al*., 1974; Vinh *et al*., 1989). Arabinose and xylose are also found in the LPS of each of these serovars and since the biosynthesis of these sugar has not been characterized in any bacterial system, it is possible that these sugars are synthesized by some of the putative sugar biosynthetic enzymes encoded by *orf16* through to *orf20*.

Overview

The differences and similarities that are observed among proteins encoded by *rfb* loci in Pomona, Hardjobovis and Hardjoprajitno are consistent with the LPS from the two Hardjo subtypes being identical (despite the obvious differences in nucleotide sequences) and different from the Pomona LPS. The surprising aspect of the comparison is the features that are common to all of the loci.

The comparison of the *rfb* loci from the Hardjo subtypes has provided insight into the types of events that may be involved in the development of novel LPS phenotypes, although indications from the comparison of the *rfb* loci from ten *L. interrogans* serovars suggest that the difference may be more subtle (de la Peña Moctezuma *et al*., unpublished), possibly more like the difference that is responsible for the structural differences between the 9

and 9a serotypes in *E. coli* (Kido and Kobayashi, 2000). However, considering the uncertainty relating to the serovar as a measure of difference in surface topography, particularly between closely related serovars, there is a requirement for a more rigorous means of distinguishing between LPS phenotypes. It would seem unlikely that sugar composition will be sufficient to provide this, thus leaving the determination of the polysaccharide structure as the only definitive way of accurately defining phenotype.

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