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Genetic Basis for Lipopolysaccharide O-Antigen Biosynthesis in Bordetellae

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***Bordetella bronchiseptica* and *Bordetella parapertussis* express a surface polysaccharide, attached to a lipopolysaccharide, which has been called O antigen. This structure is absent from *Bordetella pertussis*. We report the identification of a large genetic locus in *B. bronchiseptica* and *B. parapertussis* that is required for O-antigen biosynthesis. The locus is replaced by an insertion sequence in *B. pertussis*, explaining the lack of O-antigen biosynthesis in this species. The DNA sequence of the *B. bronchiseptica* locus has been determined and the presence of 21 open reading frames has been revealed. We have ascribed putative functions to many of these open reading frames based on database searches. Mutations in the locus in *B. bronchiseptica* and *B. parapertussis* prevent O-antigen biosynthesis and provide tools for the study of the role of O antigen in infections caused by these bacteria.**

Lipopolysaccharide (LPS) is the major glycolipid molecule present on the cell surface of gram-negative bacteria. Most of our understanding of LPS has come from early studies of the *Enterobacteriaceae* (15, 35), in which the molecule has usually been described as having three domains: lipid A, core, and O antigen. Lipid A is often linked through 2-keto-3-deoxyoctulosonic acid to the core oligosaccharide, which consists of heptoses and hexoses. Linked to the core is the O-antigen polysaccharide, which consists of repeats of oligosaccharide units that in turn consist of one or more sugars.

The genus *Bordetella* contains several species, some of which are respiratory tract pathogens. *B. pertussis* and *B. parapertussis* cause whooping cough (6, 7, 12, 16, 17), and *B. parapertussis* is also found in ovine species (18–20). *B. bronchiseptica* infects many species of animals and is commonly associated with atrophic rhinitis in pigs, snuffles in rabbits, and kennel cough in dogs (3, 24, 30, 31). *B. bronchiseptica* has also been occasionally described as a respiratory tract pathogen in humans (10, 23, 29). All three pathogens are very closely related in terms of multilocus enzyme electrophoresis, DNA hybridization, and DNA sequence analyses (27, 34). The mechanistic bases for their different host ranges and pathogenicities are unknown but are likely to depend on differences in surface structures between the three pathogens.

The LPS molecules from the three bordetellae share basic structural features in that they each have a lipid A domain and a branched-chain core oligosaccharide (4, 5, 13), but there are also substantial differences. One of the most striking of these is that *B. bronchiseptica* and *B. parapertussis* synthesize a long-chain polysaccharide structure consisting of a homopolymer of 2,3-dideoxy-2,3-di*N*-acetylgalactosaminuronic acid (2,3-diNAcGalA), known as O antigen, whereas *B. pertussis* does not (9). This structural difference between the LPS molecules of the three main pathogenic bordetellae is substantial and likely to confer quite different surface properties on the different species. The genetic basis for this difference is unknown.

We report the identification of a DNA locus that is present in *B. bronchiseptica* and *B. parapertussis* but is absent from *B. pertussis*. Mutation of this locus results in the loss of O-antigen expression. We thus propose that this locus contains genes required for O-antigen biosynthesis and/or assembly and that its absence from *B. pertussis* explains the absence of O antigen in this species. The generation of defined O-antigen-deficient mutants will allow the investigation of the role of this domain of the LPS molecule in the pathogenesis of infections due to *B. bronchiseptica* and *B. parapertussis*.

MATERIALS AND METHODS

Bacterial strains and plasmids. *B. pertussis* BP536, *B. bronchiseptica* CN7635E, and *B. parapertussis* CN2591 have been described (1). *B. bronchiseptica* RB50 was from Jeff Miller, University of California, Los Angeles (8). *Escherichia coli* XL1-Blue (Stratagene, Cambridge, United Kingdom) was used for the cloning and maintenance of pUC plasmids, and *E. coli* SM10λ*pir* was used as the donor in conjugations with suicide vectors.

pUC18 was used as a general cloning vector and pEX100T (26) was used to generate allelic-exchange mutants in *B. parapertussis* and *B. bronchiseptica*. This vector is a ColE1 replicon and thus is unable to replicate in bordetellae. It also contains *oriT* and can thus be mobilized by *E. coli* SM10λ*pir*. Conjugations were performed as previously described (1) on Bordet-Gengou plates supplemented with 15% defibrinated horse blood (Department of Clinical Veterinary Medicine, University of Cambridge) and 10 mM $MgCl_2$; *Bordetella* strains RB50 and CN2591 were recipients and *E. coli* SM10λ*pir*, carrying the appropriate plasmid, was the donor.

Media, chemicals, and reagents. *Bordetella* strains were grown on Bordet-Gengou agar supplemented with 15% defibrinated horse blood. *E. coli* strains were grown on Luria-Bertani agar or in Luria-Bertani broth. All media were purchased from Difco or Oxoid. Antibiotic resistance was selected by using ampicillin at 100 μg/ml, chloramphenicol at 10 μg/ml (*Bordetella*) or 30 μg/ml (*E. coli*), and streptomycin at 200 μg/ml. Antibiotics and standard chemicals were purchased from Sigma. DNA restriction endonucleases and other modifying enzymes were bought from Boehringer Mannheim (Lewes, United Kingdom).

DNA preparations. Plasmid DNA was purified using a plasmid DNA preparation kit (Qiagen, Crawley, United Kingdom). Chromosomal DNA was prepared in agarose blocks as described previously (25).

Southern hybridizations. Southern hybridizations were performed using a digoxigenin hybridization and detection system from Boehringer Mannheim according to the manufacturer's instructions.

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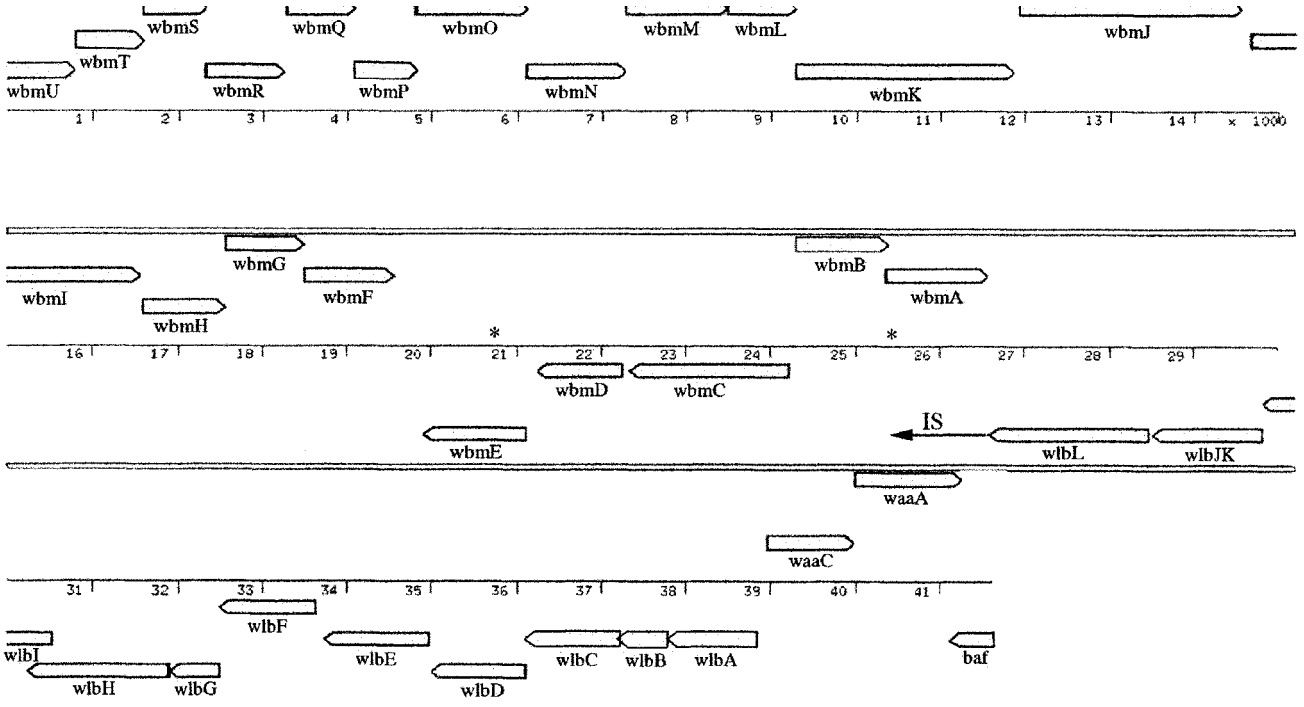

FIG. 1. Arrangement of genes in the insert in pBgl-br and the different frames of the coding sequences. *wbmA* to *wbmU* are novel, while the remaining genes have been previously described (1), including *wlb* (LPS band A biosynthesis locus), *waaC* (heptosyltransferase), *waaA* (2-keto-3-deoxyoctulosonic acid transferase), and *baf* (Bvg accessory factor). The DNA to the left of *wlbL* is present in *B. bronchiseptica*. The region to the left of *wlbL* extending to *wbmH* is also present in *B. parapertussis*. DNA sequence information beyond this region is not available although whole-genome DNA sequencing of *B. parapertussis* is under way. In *B. pertussis*, the *wbm* locus is replaced by an IS (arrow). The positions of the *Bst*EII restriction sites used for the construction of deletion mutants are indicated by asterisks. *wbm* contains at least three different transcriptional units, suggested by the direction of the genes. The coding sequences of *wbmA* and *wbmB*, *wbmH* to *wbmF*, *wbmL* and *wbmK*, *wbmO* to *wbmM*, *wbmP* and *wbmQ*, and *wbmR* to *wbmU* may comprise translationally linked units; the coding sequences of adjacent genes within these units overlap. Position numbers are in thousands.

LPS preparation, SDS-PAGE, and Western blot. LPS was prepared as described previously (21) and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using the Tricine gel buffer system as described previously (14), followed by silver staining (32). Western blotting was performed as previously described (1).

DNA sequencing and sequence analysis. *B. bronchiseptica* DNA was sequenced as a preliminary to the *Bordetella* genome sequencing project at the Sanger Centre, Hinxton, United Kingdom. *B. parapertussis* DNA was sequenced with an automated sequencer at the Department of Biochemistry, University of Cambridge. DNA sequence was analyzed with the Genetics Computer Group software package (Wisconsin Package, version 9.1; Genetics Computer Group, Madison, Wis.) and the annotation tool DIANA (2a), which allows the integration of the results of BLASTX, BLASTN, BLASTP, and FASTA searches against the EMBL, TREMBL, and SwissProt databases with Prosite, Pfam, and other protein motif searches. Gene prediction was based on codon-specific positional base preference and amino acid bias.

Nucleotide sequence accession numbers. The DNA sequence and detailed annotations are available in the EMBL DNA sequence database under accession no. AJ007747.

RESULTS

Cloning of the O-antigen biosynthesis locus. The identification and characterization of the *wlb* loci (previously *bpl*), required for LPS biosynthesis in the bordetellae, were described previously (2). Cosmid clones were isolated from *B. bronchiseptica* (pBgl-br) and *B. parapertussis* (pBgl-pa) that were apparently the same, each containing a 41,624-bp *Bgl*II fragment containing *wlb* as well as an extra 20 to 25 kb of DNA. It was noted that the DNA sequence of the 3′ end of *wlb* in *B. bronchiseptica* and *B. parapertussis* differed from that in *B. pertussis*. In place of the insertion sequence (IS) found at the 3′ end of the *B. pertussis* locus, *B. bronchiseptica* and *B. parapertussis* had DNA which we speculated may be required for O-antigen biosynthesis. We have now determined the DNA sequence of the entire 42-kb insert in pBgl-br, which contains both the *B. bronchiseptica wlb* locus and a putative O-antigen biosynthesis locus. One end of the insert (right end in Fig. 1) contained the *wlb* locus as previously reported whereas the other end of the insert encoded 21 additional open reading frames (ORFs) (Fig. 1 and Table 1), named *wbm* according to the proposed new nomenclature for LPS biosynthesis genes (22). In addition, 9.5 kb of DNA from the putative O-antigen locus from *B. parapertussis* was sequenced and was 99.6% identical to the analogous *B. bronchiseptica* region. The *B. bronchiseptica wlb* locus was highly homologous to the previously reported *B. pertussis wlb* locus. However, a significant difference in the two loci is the fact that *wlbJ* and *wlbK*, which are separate genes in *B. pertussis*, are fused into a single ORF in *B. bronchiseptica*. This may indicate that these genes have different functions in the two bordetellae, and we are currently investigating this by mutating these genes in the different bordetellae.

Of the 21 *wbm* genes in the putative O-antigen locus, three encode proteins that exhibit homology to sugar epimerases and dehydratases involved in O-antigen biosynthesis in other organisms (Table 1). Three others encode proteins that are similar to formyltransferases. Most interestingly, WbmL, WbmM, and WbmN are similar to proteins that comprise ATP-binding cassettes (ABC) transporter systems for the export of O antigen from various bacterial species (11, 28). These similarities provided strong evidence that this locus is indeed responsible for O-antigen biosynthesis in *B. bronchiseptica* and *B. parapertussis*.

TABLE 1. Putative functions of proteins encoded by *wbmA* to *wbmU* based on similarities to previously characterized proteins[a]

Protein	Length (no. of amino acids)	Putative function
WbmA	405	Glycosyltransferase
WbmB	361	Unknown
WbmC	628	Amidotransferase
WbmD	340	? (possible membrane protein)
WbmE	393	? (possibly secreted)
WbmF	357	Nucleotide sugar epimerase/dehydratase
WbmG	310	Nucleotide sugar epimerase/dehydratase
WbmH	313	Nucleotide sugar epimerase/dehydratase
WbmI	636	Amidotransferase
WbmJ	878	Glycosyltransferase
WbmK	866	Unknown
WbmL	263	O-antigen exporter (membrane protein)
WbmM	402	O-antigen exporter (membrane protein)
WbmN	389	O-antigen exporter (ATP binding)
WbmO	432	Unknown
WbmP	239	Unknown
WbmQ	274	Formyltransferase
WbmR	309	Formyltransferase
WbmS	239	Unknown
WbmT	262	Unknown
WbmU	>274	Formyltransferase

[a] Conceptual translations of the *B. bronchiseptica* cosmid DNA sequence were compared to sequences in the GenBank and EMBL databases as described in Materials and Methods. Full annotations for these ORFs are contained in GenBank under accession no. AJ007747.

Mutagenesis of the O-antigen locus. To confirm whether or not the locus was required for O-antigen biosynthesis, a 4,895-bp *Bst*EII restriction fragment was replaced by a chloramphenicol resistance gene cassette, deleting DNA between positions 20613 and 25507 (numbering as in the database sequence). This deletion encompassed *wbmB* to *wbmD* and the N-terminal half of *wbmE*. The mutated construct was moved into the chromosomes of *B. bronchiseptica* and *B. parapertussis* through allelic exchange as described previously (1). Chloramphenicol-resistant *B. bronchiseptica* and *B. parapertussis* organisms which contained the expected chromosomal DNA rearrangements were recovered, as was confirmed by Southern hybridization (data not shown).

LPS was prepared from several mutants and analyzed by SDS-PAGE, followed by silver staining (Fig. 2). All the mutants were devoid of O antigen, suggesting that the presence of a wild-type locus was required for O-antigen expression. The *B. parapertussis* O-antigen mutant produced an LPS structure not seen in the wild type. This structure was not recognized by a monoclonal antibody that recognizes the band A trisaccharide of *B. pertussis* and *B. bronchiseptica* LPS (Fig. 2). Previously, it was demonstrated that *B. parapertussis* is probably deficient in an enzyme required for the addition of the terminal GlcNAc to band A and thus is unable to synthesize the entire band A structure (2). It was hypothesized that wild-type *B. parapertussis* synthesizes a disaccharide band A that is inefficiently transferred to the band B acceptor structure (2). Furthermore, it was hypothesized that in *B. parapertussis*, the addition of O antigen to produce full-length molecules occurs with high efficiency. Thus, the band B–band A structure was present in very small amounts, if at all. In the O-antigen mutant, the band B–truncated band A structure did not contain further substitutions of O antigen and thus accumulated, possibly constituting the novel band observed in the *B. parapertussis* O-antigen mutant LPS.

For LPS preparations, the *B. bronchiseptica* and *B. parapertussis* strains were grown in the presence of 50 mM $MgSO_4$. This modulated the activity of the Bvg two-component regulatory system (33). Under this condition, *B. bronchiseptica* and *B. parapertussis* produced the simple LPS banding patterns seen in Fig. 2. Under nonmodulating conditions, additional LPS structures were produced in these strains (data not shown).

The O-antigen locus is absent from *B. pertussis*. These data suggest that the DNA immediately downstream of the *wlb* locus in *B. bronchiseptica* and *B. parapertussis* is required for O-antigen biosynthesis. This region is replaced by an IS in *B. pertussis*, but it was unclear whether the O-antigen locus was simply present elsewhere in the *B. pertussis* genome. To investigate this, DNA from the left end (positions 1 to 1355), the middle (positions 2617 to 7684), and the right end (positions 17941 to 22820) of the *B. parapertussis wbm* locus were used as probes in Southern hybridizations of genomic DNA from all three *Bordetella* species that had been restricted with the enzyme *Eco*RI. Hybridizing DNA fragments were present in the *B. bronchiseptica* and *B. parapertussis* genomes but were not present in the genome of *B. pertussis* (data not shown). Furthermore, the complete genome sequence of *B. pertussis* Tohama I has been initiated and is nearing completion. Searches of this sequence with the DNA sequence from the *B. bronchiseptica* O-antigen locus did not show any similarities, which substantiates the claim that the locus is absent from *B. pertussis*.

DISCUSSION

We have identified a locus from *B. bronchiseptica* and *B. parapertussis*, called *wbm*, that is required for the expression of the O-antigen domains of their LPS molecules. Mutation of this locus led to the generation of *B. bronchiseptica* and *B. parapertussis* mutants lacking O antigen. These mutants will pro-

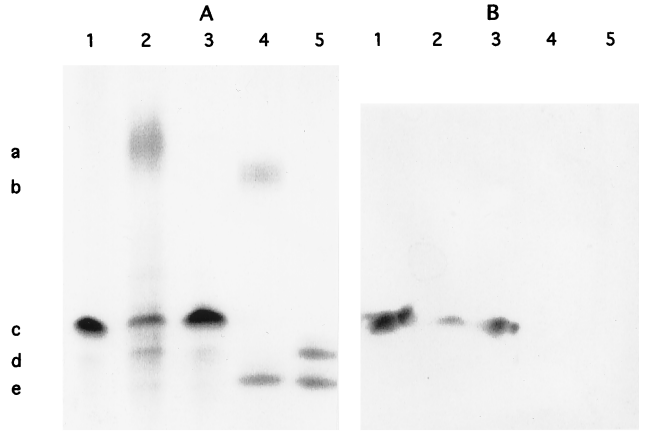

FIG. 2. (A) SDS-PAGE–silver stain analysis of LPS from *B. pertussis* Tohama I (lane 1), *B. bronchiseptica* wild type (lane 2) and mutant (lane 3), and *B. parapertussis* wild type (lane 4) and mutant (lane 5). The markers indicate the migratory positions of the O antigen of *B. bronchiseptica* (a, lane 2) and *B. parapertussis* (b, lane 4), band A of *B. pertussis* (c, lane 1) and *B. bronchiseptica* (c, lanes 2 and 3), band B of *B. pertussis* (d, lane 1) and *B. bronchiseptica* (d, lanes 2 and 3), the novel structure expressed by the *B. parapertussis* mutant (d, lane 5), and the truncated band B of *B. parapertussis* (e, lanes 4 and 5). Wild-type *B. bronchiseptica* and *B. parapertussis* both expressed O antigen whereas the mutants did not. (B) Western blot analysis of a replica of the gel shown in panel A with monoclonal antibody BL-2, which recognizes an epitope in band A. This analysis demonstrates that the *B. bronchiseptica* O-antigen mutant was not affected in band A expression and that the novel structure expressed by the *B. parapertussis* O antigen mutant was not a full band A structure.

vide excellent tools for the study of the role of O antigen in infections caused by these bacteria.

The *wbm* locus contains 21 ORFs, some of which are similar to sequences corresponding to previously identified O-antigen biosynthetic functions. A previous study defined the O-antigen polysaccharide from several *B. bronchiseptica* and *B. parapertussis* strains as consisting of a homopolymer of 2,3-dideoxy-2,3-di-*N*-acetylgalactosaminuronic acid (9). The authors suggested that O antigen is identical in all *B. bronchiseptica* and *B. parapertussis* strains. The mutagenesis data presented here, coupled with the putative functions assigned from similarity searches, strongly support the view that several of the ORFs in the *wbm* locus are involved in the biosynthesis of this homopolymer. Full annotations of the ORFs with the best matches to sequences in the databases are given in a GenBank entry (accession no. AJ007747). Speculative attempts to match each of the putative functions with the known structure of the O antigen indicate that there are more genes in the locus than are required to biosynthesize such a relatively simple polymer. This may suggest that other polysaccharides are present in the bordetellae, and their biosynthesis may depend on enzymes encoded by genes in the *wbm* locus.

It is unknown whether the entire *wbm* locus is required for O-antigen biosynthesis. We suggest that the ORFs from *wlb* up to and including the putative ABC transporter system (i.e., *wbmA* to *wbmN*) are highly likely to be required because (i) the ABC transporter is most similar to previously characterized polysaccharide export systems, (ii) other ORFs in this region are homologous to previously identified O-antigen biosynthesis functions, and (iii) deletion of the region encompassing *wbmB* to *wbmE* abrogates O-antigen expression. The functions of the seven ORFs outside this area are unknown. Three of them have strong similarities to formyltransferases, and the other four are not similar to any other proteins in the sequence databases. This region of DNA is, however, not present in *B. pertussis*, which may suggest that these ORFs do function in O-antigen biosynthesis. The fact that the entire *wbm* locus is absent from *B. pertussis* might suggest that there is more unique DNA in this region of the chromosome adjacent to the DNA that we have cloned. If this is so, then either this DNA is part of a much larger O-antigen biosynthesis region or the O-antigen biosynthesis locus forms part of a larger locus peculiar to *B. bronchiseptica* and *B. parapertussis* that encodes functions besides O-antigen biosynthesis.

Our data support previous hypotheses that explain the absence of band A from *B. parapertussis* LPS (2) by demonstrating that, in the absence of O-antigen transfer to the LPS molecule, a structure consistent with band B containing a truncated, disaccharide band A is synthesized. *B. parapertussis* synthesized a band B that, on SDS-PAGE, appeared to be truncated when compared to the band Bs of *B. pertussis* and *B. bronchiseptica*. This altered band B was not recognized by BL-8, a monoclonal antibody that recognizes *B. pertussis* and *B. bronchiseptica* band B (2), and thus it was not possible to confirm that the novel structure synthesized by the *B. parapertussis* O antigen mutant contained a substituted band B without detailed structural analysis.

The fact that the O-antigen biosynthesis locus is not present in *B. pertussis* provides an explanation for why this bacterium is never seen to express O antigen. Recent work suggests that *B. pertussis* and *B. bronchiseptica* evolved from a common ancestor which was more similar to *B. bronchiseptica* (34). During this evolution it appears that the O-antigen locus was lost from *B. pertussis* and replaced by an IS. The genetic rearrangement leading to this may have occurred as a consequence of the insertion of the IS followed by recombination between this copy of the IS and a second copy elsewhere in the chromosome, with concomitant genetic rearrangement. The evolutionary consequences of this event for *B. pertussis* pathogenesis and host range are unknown.

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