Antigenic Variation among Bordetella

BORDETELLA BRONCHISEPTICA STRAIN MO149 EXPRESSES A NOVEL O CHAIN THAT IS POORLY IMMUNOGENIC

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The O chain polysaccharide (O PS) of Bordetella bronchiseptica and Bordetella parapertussis lipopolysaccharide is a homopolymer of 2,3-diacetamido-2,3-dideoxygalacturonic acid (GalNAc3NAcA) in which some of the sugars are present as uronamides. The terminal residue contains several unusual modifications. To date, two types of modification have been characterized, and a survey of numerous strains demonstrated that each contained one of these two modification types. Host antibody responses against the O PS are directed against the terminal residue modifications, and there is little cross-reactivity between the two types. This suggests that Bordetella O PS modifications represent a means of antigenic variation. Here we report the characterization of the O PS of B. bronchiseptica strain MO149. It consists of a novel two-sugar repeating unit and a novel terminal residue modification, with the structure Me-4→α-L-GalNAc3NAcA-(4→β-D-GlcNAc3NAcA-4→α-L-GalNAc3NAcA)β,6→, which we propose be defined as the B. bronchiseptica O3 PS. We show that the O3 PS is very poorly immunogenic and that the MO149 strain contains a novel wbm (O PS biosynthesis) locus. Thus, there is greater diversity among Bordetella O PSs than previously recognized, which is likely to be a result of selection pressure from host immunity. We also determine experimentally, for the first time, the absolute configuration of the diacetimido-uronic acid sugars in Bordetella O PS.

The genus Bordetella currently comprises nine species of Gram-negative bacteria. The most extensively studied of these are the pathogens Bordetella pertussis, Bordetella parapertussis, and Bordetella bronchiseptica. B. pertussis infects only humans and is the causative agent of whooping cough in infants and persistent respiratory infections in adults (1). B. parapertussis exists as two separate lineages. One is adapted to the human host and causes whooping cough; the other is adapted to the ovine host, in which it can cause chronic pneumonia (2, 3). In contrast, B. bronchiseptica colonizes the respiratory tract of a large number of animals, and although it causes respiratory diseases in some farm, companion, and wild animals, most B. bronchiseptica infections are asymptomatic and chronic (4). B. bronchiseptica is occasionally isolated from the respiratory tract of humans and is probably acquired through contact with infected animals (5).

Bordetella lipopolysaccharide (LPS) plays a number of different roles in Bordetella biology, including during infection (e.g. see Refs. 6–9). We and others have defined the structures of the LPS of these Bordetella (Fig. 1). All three synthesize a very similar LPS core. In B. bronchiseptica and B. pertussis, the core can be further substituted by a trisaccharide to produce the structure referred to as Band A LPS (10, 11). B. parapertussis and B. bronchiseptica, but not B. pertussis, synthesize O PSs.2 Initially, the O PSs of both species were reported to be identical and composed of linear polymers of 1,4-linked GalNAc3NAcA (12), but later it was discovered that the terminal residue contained a number of modifications, that these modifications can vary between strains (13), and that the O PS polymer is linked to the rest of the LPS molecule via an unusual 5-sugar linker (14). To date, two types of modification, O1 and O2, have been described (13, 15). O1 and O2 PSs both contain 2,3,4-triamino-2,3,4-trideoxy-α-galacturonamide derivatives at their non-reducing ends. In O1 PS, this residue is formylated at positions 3 and 4 and has N-formyl-1-alanylamino1-1-alanyl substituents at N-2. In O2 PS, the amino group at position 2 is acetylated, at position 3 it is formylated, and at position 4 it is modified with a 2-methoxypropionyl substituent. Importantly, antibody responses to O PS appear to be wholly directed against the modified terminal residue because antisera raised against O1 type O PS did not recognize O2 type O PSs and vice versa, and in mice, immunization with LPS of one type did not confer protection against strains with the other type (15). This suggests that Bordetella O PSs display antigenic variation in order to help evade cross-reaction with existing immunity within hosts.

We have also studied the genetics of O PS biosynthesis (14, 16). B. bronchiseptica and B. parapertussis each contain a locus, wbm, containing 24 genes that is responsible for biosynthesis of the linker, polymer repeat, modifications of the terminal residue, and export of the O PS from the cytoplasm. We proposed that the central region of the wbm locus contains the genes that direct biosynthesis of the terminal residue modifications, and

2 The abbreviations used are: O PS, O chain polysaccharide; GalNAc3NAcA, 2,3-diacetamido-2,3-dideoxygalacturonic acid; HF, hydrofluoric acid; ManNAc3NAcA, 2,3-diacetamido-2,3-dideoxymannuronic acid; GlcNAc3NAcA, 2,3-diacetamido-2,3-dideoxy-glucuronic acid; SDR, short chain dehydrogenase/reductase; aa, amino acid(s); Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl)glycine.
supporting this, the gene content of this region correlates with the modification type (14). A number of studies have used post-genomic technologies to investigate genetic diversity among the Bordetella (17, 18). From these, the wbm locus emerged as one of the very few loci that exhibited clear variability among strains. In particular, these studies described a group of B. bronchiseptica strains (named Complex IV) that appeared more closely related to the human-restricted pathogen B. pertussis than other B. bronchiseptica. A majority of these B. bronchiseptica strains were isolated from cases of disease in humans and are thought to represent a lineage of human-adapted B. bronchiseptica (18). Using comparative genomic hybridization analyses, Complex IV strains appeared to contain polymorphic wbm loci (18). By these analyses, MO149 was identified as a B. bronchiseptica strain that expressed an O PS but contained homologues of just three previously characterized wbm genes, suggesting that it contained a novel wbm locus.

In this report, we describe a novel O PS structure for strain MO149, confirm that it contains a novel wbm locus, and demonstrate that its O PS is very poorly immunogenic compared with type O1 or O2 O Ps. Our data support proposals that the wbm locus is one of the few regions of the Bordetella genome that undergoes extensive recombination and/or lateral gene transfer (15, 18), that a greater number of O PS structures exist in the Bordetella population than was previously thought, and suggest that escape from host immunity might be a driver for the generation of this diversity.

**EXPERIMENTAL PROCEDURES**

**Strains**—B. bronchiseptica MO149 was obtained from Prof. Frits Mooi and has been described previously (18). B. bronchiseptica 512, 1289, and RB50 and Bordetella hinzii ATCC 51730 have been described previously (13, 15). RBwbm and 1289wbm are isogenic mutants of the respective WT strains in which the entire wbm locus has been deleted and replaced by a kanamycin resistance cassette.3 Cells were grown and LPS was isolated as described (14).

**NMR Spectroscopy**—NMR spectra were recorded at 35 °C in D2O on a Varian UNITY INOVA 500 instrument, using acetone as a reference for proton (2.225 ppm) and carbon (31.5 ppm) spectra. Varian standard programs correlation spectroscopy, nuclear Overhauser effect spectroscopy (mixing time of 200 ms), total correlation spectroscopy (spin lock time of 120 ms), heteronuclear single quantum coherence, and gradient heteronuclear multiple bond connectivity (long range transfer delay of 100 ms) were used.

**Mild Hydrolysis of LPS**—LPS (20 mg) was heated at 100 °C for 4 h in 2% acetic acid (4 ml), centrifuged at 12,000 × g, and separated on a Sephadex G50 SF gel (Amersham Biosciences) column (2.5 × 80 cm) using pyridine-acetic acid buffer (4 ml of pyridine and 10 ml of acetic acid in 1 liter of water) to give polysaccharide and core fractions.

**HF Solvolysis of LPS**—LPS (30 mg) was dissolved in anhydrous HF (about 7 ml) and kept for 24 h in a closed polypropylene tube (50-ml volume) and then transferred to a plastic Petri dish in a fume hood for evaporation. After the HF had evaporated, the product was dissolved in water, neutralized by the addition of 24% ammonia, clarified by centrifugation, and separated on a Sephadex G50 SF gel to give polysaccharide (5 mg). It was further purified by reverse phase chromatography on a Phenomenex Aqua column in 0.1% TFA for 20 min, using a linear gradient to 90% methlycyanide, with UV detection at 220 nm.

**Determination of the Absolute Configuration of O PS Sugars**—This was performed as described previously (19).

**Generation of Immune Sera**—Groups of five C57/Bl6 mice (Jackson Laboratory, Bar Harbor, ME) lightly sedated with isoflurane (IsoFlo, Abbott) were infected by intranasal instillation of 5 × 10^7 cfu of B. bronchiseptica RB50 or MO149 or 10^4 cfu of 1289 in 50 μl of sterile PBS (EMD Biosciences, Gibbstown, NJ). All of the animal procedures were approved by the Pennsylvania State University Institutional Animal Care and Use Committee. Mice were sacrificed on day 28 postinfection, and blood was collected by cardiac puncture. Pooled serum was stored at −70 °C until use.

**Western Blot Analyses**—Whole cell lysate LPS samples were prepared and resolved by SDS-PAGE as described previously (21) and transferred to polyvinylidene difluoride membranes (Immobilon-P, Millipore, Fisher). One gel was visualized by silver staining as described previously (21). Membranes were blocked with 5% nonfat dried milk in 0.1% Triton X-100 in PBS and incubated with the indicated pooled serum at a dilution of 1:3000 in blocking buffer at 4 °C overnight. Bound antigens were detected with horseradish peroxidase-conjugated goat anti-mouse immunoglobulins (1:6000; SouthernBiotech, Birmingham, AL) and visualized with SuperSignal West Pico chemiluminescent substrate (Thermo Fisher Scientific, Rockford, IL).

**RESULTS**

B. bronchiseptica Strain MO149 Synthesizes a Novel O PS—The oligosaccharide region of strain MO149 LPS was subjected to detailed structural analyses. The core fraction was analyzed by two-dimensional NMR and ESI-MS. It was found to be identical to the core fractions isolated from other strains of B. bronchiseptica (14) (data not shown).

However, the spectra derived from analyses of the O PS fraction differed from those previously derived from other strains, although they were partially interpreted by comparison with these other spectra. Signals from the linker region were identical to those derived from the linker regions characterized previously (14, 22) (data not shown), demonstrating that although the MO149 O PS and those previously described were different, they were joined to the LPS core by identical linkers.

The O-antigen was cleaved from the LPS core by hydrofluoric acid (HF) treatment of the LPS. The product was analyzed by NMR spectroscopy (Fig. 2 and Table 1). The repeating unit consisted of several disaccharide units, comprising residues of β-GlcNAc3NacA and α-GalNAc3NacA. The terminal α-GalNAc3NacA residue was methylated at the O-4 position. The O PS repeat was linked to the β-ManNac3NacA residue of the linker. Taken together, the data agreed with the structure depicted in Fig. 1. This is similar to the structure of the O-antigen of B. hinzii LPS (22, 23), which has one additional monosaccharide in the repeating unit.

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3 A. Preston, unpublished results.
Overlap of the HSQC spectra of *B. hinzii* and *B. bronchiseptica* MO149 O PSs derived by HF treatment of LPS (Fig. 2) shows complete identity of all signals of the O-antigen linker region and of the non-reducing end monosaccharide; however, signals of the O-antigen repeats differ significantly.

The ESI-MS spectrum of the HF-derived O PS shows only two major peaks at 4100 and 3585 Da (Fig. 3). The peak at 4100 corresponds to the structure HexNAc1HexNAcNAcA15Me1 (calculated average mass C159H227N31O96 for a structure with all residues containing free carboxyls = 4108.7 Da). The peak at 3585 Da corresponds to the same structure but with one disaccharide unit less. The 8-Da difference between the calculated and observed masses suggests the presence of eight amides of uronic acids, as observed in other *Bordetella* O-antigens (14, 23), three of which were located to the adjacent GalNAc3NAcA-ManNAc3NAcA-GlcNAc3NAcA residues at the linker-O PS junction (Fig. 1). The positions of other amides were not determined. Thus, in contrast to the fully amidated O-antigen repeat of *B. hinzii*, that of *B. bronchiseptica* MO149 has several free carboxyl groups. This was confirmed by anion exchange chromatography, in which the HF-derived O-antigen was retained. Minor peaks 22 Da more and 42 Da less than the major peaks were also detected. These probably represent Na+ salts and a deacteylation, respectively, although no NMR signal indicative of N-deacetylation was detected. If this occurred at low levels (e.g. one per O PS chain) or if the signal overlapped with others, it might not be detectable, and thus at present, an N-deacetylated component has not been identified.

**The Absolute Configuration of the Diacetimidouronic Acids in Bordetella O PS**—To date, these have not been confirmed experimentally. To do this, they were converted into methyl esters, which were converted into chiral 2-butylglycosides, acetylated, and analyzed by GC-MS (Fig. 4). Standards were prepared from synthetic methyl 2,3-diacetamido-2,3-dideoxy-D-hexopyranosides using the same approach. The diastereomers of gluco- and galacto-2,3-diaminouronic acids gave good separation and revealed that GlcNAc3NAcA has the D-configuration in *B. hinzii*, *B. bronchiseptica* 512, and *B. bronchiseptica* MO149 LPS. L-GalNAc3NAcA acid gave only a small peak from all of the analyzed LPSs, probably due to incomplete conversion of amidated sugars into methyl esters. Acetylated (S)- and (R)-2-butyl glycosides of ManNAc3NAcA could not be separated on the DB-17 column used in the current work; however, trimethylsilylated derivatives showed some separation. The difference between elution times were very small, but coinjection of the standards with the analyzed compounds revealed that ManNAc3NAcA in *Bordetella* O PS has the D-configuration (data not shown). Thus, for the first time, the abso-

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**TABLE 1**

NMR data generated by analysis of MO149 O PS (D2O, 35 °C)

| Unit | Nucleus | H/C 1 | H/C 2 | H/C 3 | H/C 4 | H/C 5 | H/C 6 |
|------|---------|------|-------|-------|-------|-------|-------|
| A α-GalNAc3NAcAN4Me | H | 5.12 | 4.14 | 4.20 | 3.94 | 4.23 |
| C | 99.0 | 48.2 | 49.2 | 78.9 | 71.8 | 173.8–174.5 |
| B β-GlcNAc3NAcAN | H | 4.56 | 3.80 | 4.06 | 4.01 | 4.06 |
| C | 102.8 | 55.4 | 54.6 | 77.2 | 76.9 | 172.2 |
| C α-GalNAc3NAcAN | H | 5.09 | 4.12 | 4.19 | 4.36 | 4.23 |
| C | 99.0 | 48.3 | 48.3 | 76.0 | 71.8 | 174.0 |
| Z α-GalNAc3NAcAN | H | 5.01 | 4.12 | 4.23 | 4.35 | 4.28 |
| C | 99.5 | 48.3 | 48.3 | 76.0 | 71.9 | 174.0 |
| MM β-ManNAc3NAcAN | H | 4.76 | 4.37 | 4.17 | 3.83 | 4.08 |
| C | 100.9 | 51.9 | 53.4 | 79.4 | 77.8 | 172.5 |
| X β-GlcNAc3NAcAN | H | 4.87 | 3.86 | 4.01 | 3.88 | 3.98 |
| C | 103.1 | 54.5 | 54.5 | 75.4 | 75.6 | 173.6 |
| α-Y α-GalNAc | H | 5.16 | 4.02 | 4.01 | 4.12 | 4.07 |
| C | 92.4 | 51.6 | 68.8 | 77.0 | 71.3 | 62.3 |
| β-Y β-GalNAc | H | 4.59 | 3.79 | 3.79 | 4.05 | 3.65 |
| C | 96.8 | 54.9 | 72.6 | 76.2 | 75.6 | 62.3 |

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**FIGURE 1. Schematic of Bordetella LPS structure.** In *B. bronchiseptica*, most strains appear to share a common core structure. Most core molecules are substituted with the band A trisaccharide. Some LPS molecules also contain O PS, which consists of a 5-sugar linker that joins an O PS repeat region to the core. R represents the variably modified terminal GalNAc3NAcA residue that determines the O1 or O2 type O PS (14, 15). FucNAc4N, 2-acetamido-4-amino-2,4,6-trideoxy-galactose; Hep, L-glycero-D-manno-heptose; Kdo, 3-deoxy-D-manno-octulosonic acid. *, non-stoichiometric substituents.
Novel O PS of B. bronchiseptica M0149

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FIGURE 2. Fragment of overlapped 1H-13C HSQC spectra of the HF-released B. bronchiseptica MO149 (gray) and B. hinzii (black) O polysaccharides. Non-labeled black signals belong to the extra β-GlcNac3NAc residue in B. hinzii O polysaccharide. Signal labels correspond to the residues in Table 1.

FIGURE 3. Negative mode ESI mass spectrum of the HF-released B. bronchiseptica 2494 polysaccharide. Two major peaks corresponding to O PS containing 4 (3585 Da) or 5 (4100 Da) disaccharide units were detected. Peaks corresponding to 22 Da more and 42 Da less than these probably derive from Na+ adducts and an uncharacterized N-deacetylation event, respectively.

Novel terminal residue modification, different from either the O1 or O2 type modifications that appear to be widespread among B. bronchiseptica and B. parapertussis strains (15).

B. bronchiseptica MO149 Contains a Novel wbm Locus—The genome of strain MO149 is currently being sequenced as part of a second-generation Bordetella genome sequencing project (see the Wellcome Trust Sanger Institute Web site). Analysis of preliminary genome sequence data identified the wbm locus of this strain (Fig. 5 and Table 2). Like the other wbm loci characterized to date, MO149 wbm is flanked at one end by the band A trisaccharide LPS biosynthesis locus wlb and at the other by the conserved hypothetical protein BB0120. The locus is present at the same chromosomal location as in other strains.

Highly Conserved Genes—Strain MO149 contains orthologues of wbmA to -C (Table 2). Previously, we proposed that some wbm genes contribute to the synthesis of the linker region (14). The almost perfect conservation of wbmA to -C in strain MO149 that synthesizes an identical linker to other strains supports the idea that these genes encode linker biosynthesis functions.

Moderately Conserved Genes—In MO149, downstream of wbmC, there is a homologue of wbmD, but it displays only 50% identity at the amino acid level to WbmD from other strains (Table 2). Furthermore, MO149 WbmD is just 282 amino acids compared with the 340 of other WbmD proteins. It is missing the N-terminal 42 amino acids of the other proteins, containing a novel 8-amino acid sequence in their place, and also contains a 10-amino acid deletion compared with the other proteins.
The function of WbmD is unknown. Two other *Bordetella* hypothetical proteins are the only ones in the public data bases identified as containing homology to WbmD by BlastP analysis and WbmD does not contain any conserved domains. A *B. bronchiseptica* wbmD mutant synthesizes LPS in which the O PS linker is attached directly to the core, suggesting that WbmD is required for assembly of the wild type LPS molecule. The difference between MO149 WbmD and previously characterized WbmD proteins might suggest that they interact with the region of the O PS that differs between these strains.

**MO149 O-antigen Chain Termination and Export**—The MO149 wbm locus contains genes that putatively encode the integral membrane (Wzm) and ATP-hydrolyzing (Wzt) components of an O-antigen export ABC (ATP-binding cassette) transporter. The presence of these genes is a good indication that the MO149 O PS is synthesized according to the ABC transporter-dependent model (24, 25). These genes have homologues in the *B. bronchiseptica* RB50 wbm locus and in the O-antigen biosynthesis clusters of *Escherichia coli* O8 and O9a, both of which produce homopolymeric mannan O PSs. *E. coli* WztO8 and WztO9a were the first-characterized members of a subclass of ABC transporter Wzt components, which have an extended C-terminal domain. This domain binds the non-reducing terminus of the O polysaccharide transport substrate, recognizing strain-specific modifications of the terminal sugar (26, 27). In *E. coli* O8, the terminal sugar is methylated, and in *E. coli* O9a, the sugar is phosphorylated and methylated. The addition of these methyl and phosphate substituents is a prerequisite for transport of the completed O polysaccharide (28). The WztMO149 is 487 amino acids long, placing it in the same subfamily as the *E. coli* O8 and O9a Wzt enzymes. A third iteration PSI-BLAST aligns WztMO149 with each of the *E. coli* sequences over the whole protein length. Most of the conserved amino acids are found in the N-terminal domains. These observations lead us to hypothesize that the O-antigen ABC transporter in this *B. bronchiseptica* strain recognizes the methylation present at the terminus of the O chain, as a molecular signal of the completion of O polysaccharide synthesis and readiness for export. The C termini are poorly conserved between WztMO149 and the *E. coli* proteins, and this is consistent with the difference between the methylated sugars produced by *B. bronchiseptica* MO149 and the *E. coli* strains.

The addition of methyl and phosphate substituents to the non-reducing terminal sugars is catalyzed in both *E. coli* O8 and *E. coli* O9a by WbdD (28). The *wbdD* genes reside immediately downstream of *wzt* on the O8 and O9a chromosomes.

Interestingly, WbdD interacts with WbdA, and this appears to coordinate chain elongation and chain termination (29). Immediately downstream of *wzt* in the MO149 *wbm* cluster is a putative methyltransferase-encoding gene (*wbmOO*). This gene encodes a homologue of WbdD, sharing 29% amino acid identity over 108 amino acids. This is a level of sequence conservation similar to that shared by WbdD and WbdD, 34% amino acid identity over 113 residues (28). Therefore, we suggest that this *B. bronchiseptica* enzyme catalyzes the terminal methylation of the O-antigen chain, which is putatively recognized by the ABC transporter.

**Poorly Conserved Genes**—The remainder of the locus contains genes that encode proteins that are homologous to previously characterized Wbm proteins but at much lower levels than are usually observed between *Bordetella* strains (Table 2).

*B. bronchiseptica* RB50 and *B. parapertussis* 12822 wbm loci encode three putative amidotransferases, WbmC, -I, and -Z. We propose that these enzymes catalyze the formation of the uronamide groups observed on ManNAc3NAcN, GlcNAc3NAcN, and GalNAc3NAcN in the O PSs of these strains. The need for a specific amidotransferase for each sugar is consistent with formation of the uronamide being the last step in synthesis of each of these sugars. MO149 also encodes three putative amidotransferases. MO149WbmI shares 60% identity with RB50/12822 WbmI. MO149 WbmC has 98% identity (discussed above) with RB50 WbmC. The third homologue, WbmCC, has just 33% identity with RB50 WbmC. All three contain the pfam 00733 (asparagine synthase) and 00310 (glutamine amidotransferase class II) conserved domains. Using PSI-Blast analysis, WbmCC displays similar levels of identity to WbmC (33%), WbmI (28%), and WbmZ (27%). MO149 O PS contains the same uronamide sugars as found in RB50 and *B. parapertussis* 12822 O PS. We propose that MO149 WbmCC, WbmI, and WbmZ are functional homologues of the putative RB50/12822 amidotransferase enzymes WbmC, -I, and -Z.

MO149 also contains homologues of RB50/12822 WbmF-H, WbmJ, WbmK, and WbmBB (Table 2). The WbmF-H and WbmBB proteins all contain the pfam 01370 short chain dehydrogenase/reductase (SDR) conserved domain. We have characterized RB50 WbmF, -G, and -H and proposed that they covert UDP-δ-ManNAc3NAcA to UDP-δ-GalNAc3NAcA for synthesis of the O PS repeat sugar (30, 31). The conservation between RB50 and MO149 of WbmF, -G, and -H sequences is between 57 and 73% amino acid identity (Table 2), which for SDR family proteins is of an order that implies related function. Furthermore, sequence alignments show that the MO149 proteins contain nearly the same active site residues as the RB50 proteins (data not shown), suggesting that they have the same activities. We thus propose that MO149 WbmF-H are functional homologues of RB50 WbmF-H. Like RB50/12822 WbmBB, MO149 WbmBB has a conserved catalytic triad (Ser127, Tyr151, and Lys159) typical of SDR family.

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*A. Preston, J. D. King, and E. Vinogradov, manuscript in preparation.*
enzymes, but the possible role of WbmBB in O PS biosynthesis is unclear.

The assembly of Bordetella O PS presumably requires the action of a number of glycosyltransferases. MO149 contains highly conserved homologues of WbmA and -B that we propose function in the assembly of the linker region (see above) and less conserved homologues of RB50/B. parapertussis 12822 Wbmj and -K. By BlastP analysis, both Wbmj and -K contain homology to conserved domains typical of glycosyltransferases, Wbmj in its C-terminal half and WbmK in its N-terminal half. Wbmj (878 amino acids) and WbmK (866 amino acids) are unusually long for typical glycosyltransferases and much longer than the other predicted Wbm glycosyltransferases (WbmA, 405 amino acids; WbmB, 366 amino acids; WbmO, 441 amino acids). Furthermore, Wbmj and WbmK share homology (15%) in their N-terminal regions, including that in which Wbmj contains the predicted glycosyltransferase motif. It is thus possible that Wbmj and -K each contain two glycosyltransferase domains, each of which forms a different glycosidic bond. There are precedents for this because the WbdA O PS glycosyltransferase of E. coli serotypes O8 and O9 is 815 amino acids in length and contains two glycosyltransferase domain motifs, and WbdA of Klebsiella pneumoniae serotype O5 is 1215 amino acids in length and contains three glycosyltransferase domain motifs (32, 33). We propose that MO149 Wbmj and -K are glycosyltransferases involved in assembly of the O PS.

B. bronchiseptica MO149 O PS Is Non-immunogenic or Poorly Immunogenic—A survey of 18 B. bronchiseptica strains identified that all contained either the RB50 (O1) type O PS or the 12822 (O2) type O PS (15). Strikingly, host antibody responses to the LPS of these strains were dominated by antibodies against the O PS terminal residue, and this resulted in inefficient cross-protection against strains of different O PS types (15). Serum was collected from mice infected with either MO149, RB50 (O1 type O PS) or B. bronchiseptica 1289 (O2 type O PS) (15) and used to probe LPS from proteinase K-treated cell lysates in Western blots (Fig. 6). As described previously, mice infected with B. bronchiseptica strains expressing O PSs with O1 or O2 type modifications generate strong antibody responses to the O PS-containing LPS, as demonstrated by the loss of reactivity to the LPS of mutants from which the entire wbm loci have been deleted (Fig. 6, lanes RBwbm and 1289wbm). The lack of cross-reactivity between anti-O1 O PS antibodies and O2 O PS and vice versa is demonstrated by the lack of recognition by anti-RB50 serum of 1289 O PS (Fig. 6) Furthermore, neither anti-RB50 or anti-1289 sera recognize MO149 O PS, suggesting that the novel O PS of this strain is not recognized by antibodies directed against the O1 or O2 type modifications. To confirm that MO149 does express O PS when grown in these conditions, proteinase-treated cell lysates were run on Tricine gels and visualized by silver staining (Fig. 6D), and O PS containing LPS is clearly visible by these methods. The O PS appears as two bands, consistent with the two major O PS species containing 7 and 8 discchardide units that were detected by MS.

Interestingly, anti-MO149 serum does not detect O PS from any of the strains, including MO149 itself. The lack of recogni-
lead to the biosynthesis of a different O PS. Our findings demonstrate that at least one other O PS type exists, that the extent of variability among wbm loci is greater than previously thought, and that variability is not confined to the terminal residue.

Previously, we described the *B. bronchiseptica* RB50 and *B. parapertussis* 12822 wbm loci as being composed of a variable central portion that contained genes involved in synthesis of the strain variable terminal residue modifications flanked by highly conserved genes involved in synthesis of the highly conserved linker-polymer region (14). *B. parapertussis* BPP5 wbm also conforms to this model, containing the unique methyltransferase gene and other medium conserved genes flanked by very highly conserved wbm homologues. All of the MO149 wbm genes display homology to RB50 and/or 12822 wbm genes except for the putative methyltransferase that we propose as the sole terminal residue modification function in this locus. However, the gene arrangement in the MO149 locus is very different from that of the 12822/RB50 loci (Fig. 5), including multiple differences in regions that we had previously described as being part of the highly conserved flanking regions of the *Bordetella* wbm locus. The MO149 locus shows nearly complete DNA identity with the RB50/12822 loci at both ends, but there are clearly visible points at which divergence begins. At one end, this is 13 bp upstream of the wbmC stop codon (Fig. 7), resulting in MO149 WbmC containing a different C-terminal 5 amino acids compared with RB50/12822 WbmC, and at the other end it is 21 bp upstream of the BB0120 homologue start codon (Fig. 7), suggesting that in MO149, the BB0120 homologue may have a different promoter compared with BB50/12822 strains.

*Bordetella* genomes have relatively high GC content genomes (Table 3). In all of the *Bordetella* strains for which sequence information is available, the GC contents of their wbm loci are considerably lower than their overall genome GC contents (Table 3). Interestingly, the variable regions have a lower GC content than the relatively conserved wbmA to -C genes, which in turn have a GC content different from either the variable region or the overall genome average. Even in the relatively distantly related *Bordetella, Bordetella avium* and *Bordetella petrii*, the wbm loci have low GC contents. These loci have not been functionally characterized but were designated as the wbm loci for these analyses on the basis that they are adjacent to the wlb locus in each strain and contain genes that encode proteins that are homologous to other O-antigen biosynthesis enzymes.

### DISCUSSION

Previous analyses suggested that many *B. bronchiseptica* strains had either O1 or O2 type O PSs (15) and that variation among *B. bronchiseptica* and *B. parapertussis* O PS occurred at the terminal residue with the polymer backbone being highly conserved among strains (13, 14). One other variant O-antigen locus had been described, for the ovine *B. parapertussis* strain BPP5 (34). Its wbm locus resembles that of the human *B. parapertussis* strain 12822 except for the presence of single nucleotide frameshift mutations in three genes, the absence of a wbmE and a wbmK homologue, and the presence of a unique methyltransferase-encoding gene. However, it is not known if this strain synthesizes an O-antigen or if these genetic differences
Novel O PS of B. bronchiseptica M0149

TABLE 3
The GC contents of Bordetella genomes compared with their wbm loci

| Strain          | GC content of genome | GC content of variable region of wbm locus (%) | GC content of wbmA to - C (%) |
|-----------------|----------------------|-----------------------------------------------|-------------------------------|
| B. bronchiseptica RB50 | 68.07                | 57.98                                         | 64.64                         |
| B. bronchiseptica M0149 | 68.41                | 58.21                                         | 64.85                         |
| B. bronchiseptica 253 | 67.62                | 58.64                                         | 64.57                         |
| B. parapertussi 12822 | 68.10                | 58.62                                         | 64.43                         |
| B. parapertussi BP5 | 67.81                | 59.2                                          | 64.44                         |
| B. avium 197 | 61.58                | 55% **                                       | Absent                        |
| B. pertii DSMZ12804 | 65.48                | 56.24%                                       | Absent                        |

* The genes BAY0081–BAY0089, adjacent to the B. avium wlb locus and displaying homology to other O-antigen biosynthesis genes, were used as comprising the O-antigen biosynthesis locus in this strain.

** The genes BPE4829–BPE4837, adjacent to the B. pertii wlb locus and displaying homology to other O-antigen biosynthesis genes, were used as comprising the O-antigen biosynthesis locus in this strain.

Low GC content is one of the signatures of horizontally acquired DNA. We propose that the Bordetella wbm loci are genomic islands, although they lack any obvious DNA mobility functions that are often associated with genomic islands. In the mammalian-adapted Bordetella, these islands are integrated at a highly conserved site and in all Bordetella are situated immediately adjacent to the wlb LPS biosynthesis locus. Our findings suggest that a repertoire of wbm genomic islands exists among the Bordetellae, conferring variability to the O PS of Bordetella LPS.

There is little cross-reactivity between antibody responses generated to the previously characterized O1 and O2 PS (Fig. 6) (15). This suggests that O PS diversity might represent a means of antigenic variation that allows escape from preexisting host immunity. The poor immunogenicity of MO149 PS suggests that some strains of B. bronchiseptica employ a different means by which they escape host immunity by avoiding the generation of strong anti-O PS responses. It is interesting that B. bronchiseptica MO149 is a Complex IV strain that was isolated from infection in a human. Complex IV strains are proposed to be more closely related to the human-adapted B. pertussis than they are to Complex I B. bronchiseptica strains that have been isolated mainly from non-human sources (18). B. pertussis does not express O PS because the wbm locus has been deleted from seemingly all B. pertussis strains (17). It is hypothesized that B. pertussis evolved from a human-adapted B. bronchiseptica (or B. bronchiseptica-like) ancestor and that avoidance of existing anti-B. bronchiseptica immunity in the human host was key to its emergence in this niche (18). Loss of expression of highly immunogenic O PS was probably part of this avoidance. The O PS confers protection on B. bronchiseptica against host immune mechanisms, such as complement-mediated killing (20), but B. pertussis appears to have evolved alternative protection mechanisms to compensate for the loss of O PS. Previously, analysis of the gene content of a large number of B. bronchiseptica strains identified the O1 or O2 wbm locus in all non-Complex IV strains studied. However, 4 of 13 Complex IV strains contained variant loci, including apparent deletions that abrogated expression of the Band A/O PS LPS (18). Thus, it is possible that avoidance of a strong anti-O PS host immune response, either through a poorly immunogenic O PS, such as O3, or absence of O PS expression, is required for B. bronchiseptica strains to exist in human hosts and that this generates selection pressure for the generation of O PS diversity that appears to be a feature among Complex IV B. bronchiseptica strains (18). Interestingly, we have identified the O3 wbm cluster in a number of Complex IV strains but not in any of 96 Complex I strains analyzed, suggesting that the O3 PS is specific to Complex IV and thus either advantageous for some aspect of Complex IV biology or selected against in Complex I strains.5 Interestingly, anti-MO149 recognized Band A LPS but gave a strong signal in Western blots only from strains deleted for wbm although MO149 and WT 1289 appeared to express high levels of Band A LPS. This suggests that the presence of O PS appears to reduce recognition of Band A LPS by anti-MO149 antisera. Thus, the wbm locus might modify Band A LPS in some subtle way that prevents efficient antibody binding. In turn, this could result in anti-MO149 antibodies recognizing B. pertussis that expresses high levels of Band A LPS, which would be unmodified due to the loss of wbm from B. pertussis and possibly result in cross-species immunity that would help eliminate B. pertussis from human hosts and promote survival of B. bronchiseptica strains in this niche. It is evident that the role of O PS in Bordetella infection is not yet fully understood.

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