

Biosynthesis of a Rare Di-N-Acetylated Sugar in the Lipopolysaccharides of both *Pseudomonas aeruginosa* and *Bordetella pertussis* Occurs via an Identical Scheme despite Different Gene Clusters[∇]

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Pseudomonas aeruginosa and *Bordetella pertussis* produce lipopolysaccharide (LPS) that contains 2,3-diacetamido-2,3-dideoxy-D-mannuronic acid (D-ManNAc3NAcA). A five-enzyme biosynthetic pathway that requires WbpA, WbpB, WbpE, WbpD, and WbpI has been proposed for the production of this sugar in *P. aeruginosa*, based on analysis of genes present in the B-band LPS biosynthesis cluster. In the analogous *B. pertussis* cluster, homologs of *wbpB* to *wbpI* were present, but a putative dehydrogenase gene was missing; therefore, the biosynthetic mechanism for UDP-D-ManNAc3NAcA was unclear. Nonpolar knockout mutants of each *P. aeruginosa* gene were constructed. Complementation analysis of the mutants demonstrated that B-band LPS production was restored to *P. aeruginosa* knockout mutants when the relevant *B. pertussis* genes were supplied in *trans*. Thus, the genes that encode the putative oxidase, transaminase, N-acetyltransferase, and epimerase enzymes in *B. pertussis* are functional homologs of those in *P. aeruginosa*. Two candidate dehydrogenase genes were located by searching the *B. pertussis* genome; these have 80% identity to *P. aeruginosa* *wbpO* (serotype O6) and 32% identity to *wbpA* (serotype O5). These genes, *wbpO*₁₆₂₉ and *wbpO*₃₁₅₀, were shown to complement a *wbpA* knockout of *P. aeruginosa*. Capillary electrophoresis was used to characterize the enzymatic activities of purified WbpO₁₆₂₉ and WbpO₃₁₅₀, and mass spectrometry analysis confirmed that the two enzymes are dehydrogenases capable of converting UDP-D-GlcNAc, UDP-D-GalNAc, to a lesser extent, and UDP-D-Glc, to a much lesser extent. Together, these results suggest that *B. pertussis* produces UDP-D-ManNAc3NAcA through the same pathway proposed for *P. aeruginosa*, despite differences in the genomic context of the genes involved.

Lipopolysaccharide (LPS) is the major component of the outer membranes of gram-negative bacteria. Conceptually, it can be divided into three parts, namely, lipid A, core oligosaccharide, and the O antigen. In *Pseudomonas aeruginosa*, the following two forms of O antigen are produced: A-band O antigen, which is common to most strains (10, 27); and B-band O antigen, which is variable and forms the basis of the International Antigenic Typing Scheme (28). The B-band O antigen of *P. aeruginosa* PAO1 (serotype O5) contains a repeating trisaccharide of 2-acetamido-3-acetamidino-2,3-dideoxy-D-mannuronic acid (D-ManNAc3NAmA), 2,3-diacetamido-2,3-dideoxy-D-mannuronic acid (D-ManNAc3NAcA), and 2-acetamido-2,6-dideoxy-D-galactose (N-acetyl-D-fucosamine [D-FucNAc]) (23). *P. aeruginosa* serotypes O2, O16, O18, and O20 have very similar repeat units; for this reason, these serotypes are collectively called *P. aeruginosa* serogroup O2. In contrast, the B-band O antigen of *P. aeruginosa* serotype O6 is made of repeating tetramer units of L-rhamnose (L-Rha), N-acetyl-D-galactosaminuronic acid (D-GalNAcA), 2-deoxy-2-formamido-D-galacturonic acid (D-GalNFoA), and 2-acetamido-2,6-dideoxy-D-glucose (D-QuiNAc) (22).

Nomenclature for the conceptual division of LPS differs between different organisms. In *Bordetella pertussis*, an obligate hu-

man pathogen and the causative agent of whooping cough, the core oligosaccharide is linked to a structure called the band A trisaccharide. The band A trisaccharide from *B. pertussis* 1414 is composed of N-acetyl-D-glucosamine (D-GlcNAc), 2,3-diacetamido-2,3-dideoxy-D-mannuronic acid (D-ManNAc3NAcA), and 2-acetamido-4-methylaminofucose (FucNAc4NMe) (6). The related species *Bordetella bronchiseptica*, a pathogen of animals, and *Bordetella parapertussis*, a pathogen of both humans and animals, also have D-ManNAc3NAcA present in the LPS (33). *B. bronchiseptica* and *B. parapertussis* LPS also contains a repeating polysaccharide known as the O antigen (12). The O antigen contains 2,3-diacetamido-2,3-dideoxy-L-galactosamine (L-GalNAc3NAcA) (33), which is thought to be synthesized from UDP-D-ManNAc3NAcA by the enzymes of the *wbm* gene cluster (21). In *B. pertussis*, the LPS is not capped with the O antigen due to the deletion of the *wbm* cluster (33).

It is intriguing that the rare di-N-acetylated mannuronic acid sugar residue D-ManNAc3NAcA is present in both *P. aeruginosa* serogroup O2 and in the LPS of *B. pertussis*. LPS is an important virulence factor of both *P. aeruginosa* and *B. pertussis*: in *P. aeruginosa*, the O antigen has been shown to be involved in protection from phagocytosis (13) and serum-mediated killing (11), and it also plays a role in eliciting a high level of immune response in the host (9). In addition, mutants of *P. aeruginosa* that lack the O antigen have a 50% lethal dose that is 1,000-fold higher than that of the wild-type organism in an animal model (9). In *B. pertussis*, the band A trisaccharide prevents clearance of the organism by host surfactant protein

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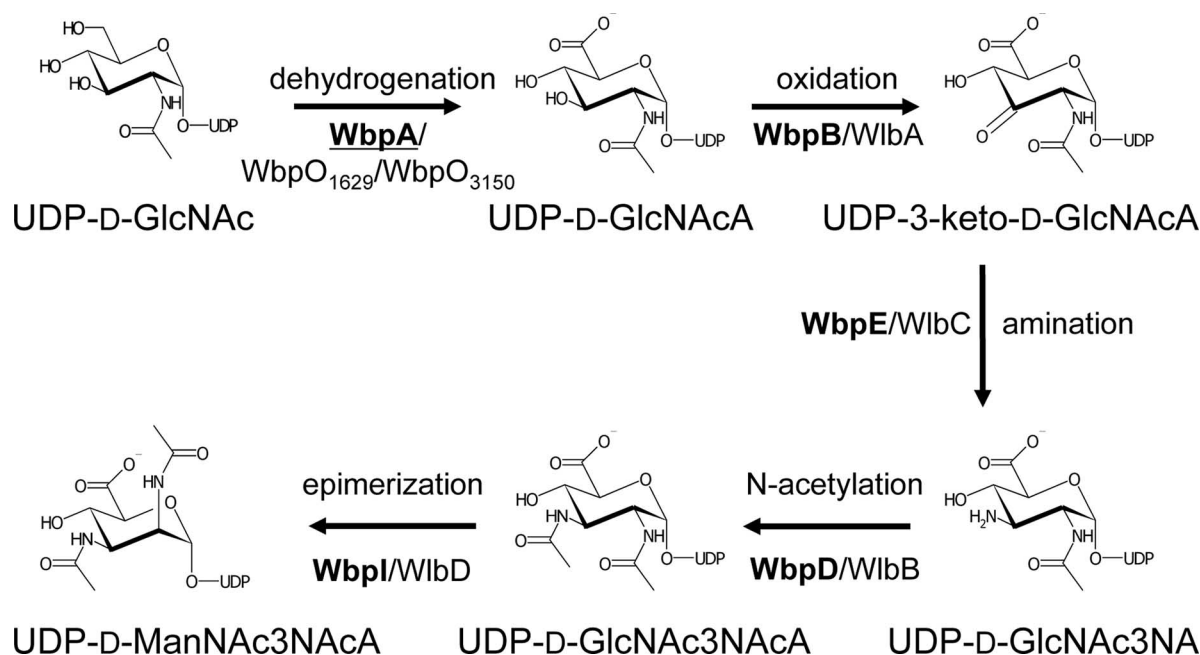


FIG. 1. Proposed biosynthetic pathway for UDP-D-ManNAc3NAcA in *P. aeruginosa* serogroup O2 and *B. pertussis*. Protein names are indicated in bold for *P. aeruginosa* and in normal text for *B. pertussis*. WbpA is underlined to show that it lacks a *B. pertussis* homolog within the band A trisaccharide biosynthesis cluster.

A (38) and confers protection to the bacterium from complement-mediated cell lysis (18). Mutant strains of *B. pertussis* that lack the band A trisaccharide were shown to be defective in colonization of the mouse trachea and nasal cavity (18). Mutants of *B. bronchiseptica* lacking wild-type LPS showed reduced resistance to oxidative stress and antimicrobial peptides (2, 44).

The biosynthesis of UDP-D-ManNAc3NAcA in *P. aeruginosa* has been studied by use of genetic and biochemical techniques. A five-step biosynthesis pathway involving the sequential catalytic activities of WbpA, WbpB, WbpE, WbpD, and WbpI has been proposed (Fig. 1). Genetic evidence has already been given that the initial enzyme and the last enzymes (encoded by *wbpA*, *wbpD*, and *wbpI*) are essential for B-band O antigen biosynthesis (5, 43). The initial enzyme of the pathway, WbpA, is a 6-dehydrogenase that converts UDP-D-GlcNAc to UDP-N-acetylglucosaminuronic acid (UDP-D-GlcNAcA) (31). Another 6-dehydrogenase, WbpO of *P. aeruginosa* serotype O6, has 53% similarity to WbpA and has been shown to convert UDP-D-GalNAc to UDP-D-GalNAcA for use in the O antigen but is also capable of converting UDP-D-GlcNAc to UDP-D-GlcNAcA (30, 48). In *P. aeruginosa* PAK (serotype O6), the WbpO enzyme is required for both O antigen biosynthesis and flagellin glycosylation (30). The B-band O antigen biosynthesis cluster of *P. aeruginosa* serotype O6 contains *wbpO* followed by *wbpP* (3), which encodes a 4-epimerase that can catalyze the reversible conversion of UDP-D-GlcNAc to UDP-D-GalNAc or UDP-D-GlcNAcA to UDP-D-GalNAcA (8, 30). Despite both enzymes being bifunctional, data from kinetic analysis of WbpO and equilibrium analysis of WbpP suggested a preference in vivo for WbpO to work first, converting UDP-D-GlcNAc to UDP-D-GlcNAcA, followed by WbpP, converting UDP-D-GlcNAcA to UDP-D-GalNAcA (30). Thus, homologs of either WbpA

or WbpO are theoretically capable of providing the required 6-dehydrogenation of UDP-D-GlcNAc to initiate the UDP-D-ManNAc3NAcA biosynthesis pathway.

The intermediate three steps in the UDP-D-ManNAc3NAcA biosynthesis pathway have been proposed, but functional evidence for the role of the enzymes has yet to be provided. UDP-D-GlcNAcA is thought to be used in an oxidation reaction catalyzed by WbpB, forming UDP-2-acetamido-2-deoxy-D-ribo-hex-3-ulosonic acid (UDP-3-keto-D-GlcNAcA), which in turn is the substrate for transamination by WbpE, yielding UDP-2-acetamido-3-amino-2,3-dideoxy-D-glucuronic acid (UDP-D-GlcNAc3NA). This residue is thought to be the substrate for WbpD, a putative N-acetyltransferase, which synthesizes UDP-2,3-diacetamido-2,3-dideoxy-D-glucuronic acid (UDP-D-GlcNAc3NAcA), utilizing acetyl-coenzyme A as a cofactor (43). WbpI is the final enzyme in the pathway and is known to be a UDP-D-GlcNAc3NAcA 2-epimerase that produces UDP-D-ManNAc3NAcA for incorporation into B-band LPS (46). Each of the required enzymes is encoded in the B-band O-antigen biosynthesis cluster (37).

The analogous band A trisaccharide biosynthesis cluster in *B. pertussis* contains 12 genes, which include homologs of the second to fifth genes encoding enzymes involved in UDP-D-ManNAc3NAcA synthesis in *P. aeruginosa* (Fig. 2). The missing gene in this cluster is an open reading frame (ORF) encoding a putative UDP-D-GlcNAc 6-dehydrogenase, required for the first step in the pathway. Thus, it was unclear whether synthesis of UDP-D-ManNAc3NAcA in *B. pertussis* could follow the same pathway as that in *P. aeruginosa*. Further analysis of the *B. pertussis* genome sequence led to the identification of two putative dehydrogenases, WbpO₁₆₂₉ and WbpO₃₁₅₀, which were named based on the existing annotation and genomic positions. In this study, we used genetic and biochemical ap-

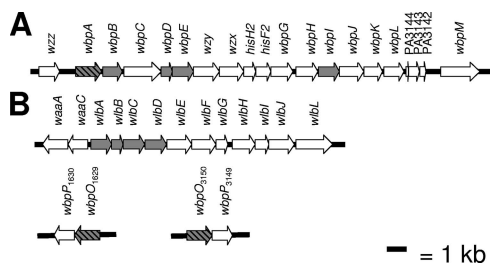


FIG. 2. Comparison of LPS biosynthetic loci from *P. aeruginosa* and *B. pertussis* (32, 42). (A) B-band O antigen gene cluster from *P. aeruginosa* PAO1 (serotype O5). (B) Band A trisaccharide gene cluster from *B. pertussis* Tohama I, also known as the *wlb* locus. Initial proposed functions for the genes in these clusters were provided from sequence and mutational analysis of *P. aeruginosa* and *B. pertussis*, respectively (4, 35). Genes known or predicted to be involved in the biosynthesis of UDP-D-ManNAc3NAcA are shaded gray; genes known or predicted to encode dehydrogenases are shaded gray with black hatching.

proaches to determine if either or both of the identified *wbpO* homologs may participate in UDP-D-ManNAc3NAcA biosynthesis in *Bordetella pertussis*.

This report documents the complementation of knockout mutants of *wbpA* (encoding a 6-dehydrogenase), *wbpB* (encoding a putative oxidase), *wbpE* (encoding a putative transaminase), *wbpD* (encoding a putative *N*-acetyltransferase), and *wbpI* (encoding a 2-epimerase) derived from *P. aeruginosa* PAO1 with *wbpO*₁₆₂₉ and *wbpO*₃₁₅₀, *wlbA*, *wlbC*, *wlbB*, and *wlbD*, respectively, of *B. pertussis* BP536. Each gene was able to restore B-band LPS production to the respective knockout mutant, indicating that each pair has the same function in vivo. The *B. pertussis* enzymes WbpO₁₆₂₉ and WbpO₃₁₅₀ have not been studied previously, so both were overexpressed and purified for use in reactions with a panel of possible substrates. Both WbpO₁₆₂₉ and WbpO₃₁₅₀ were shown to be 6-dehydrogenases that utilize UDP-D-GlcNAc and UDP-D-GalNAc, like the 6-dehydrogenase of *P. aeruginosa* serotype O6 (WbpO), but the enzymes of *B. pertussis* were shown to also have a novel ability to use UDP-D-Glc. Collectively, these results suggest that *B. pertussis* produces UDP-D-ManNAc3NAcA through the same biochemical pathway proposed for *P. aeruginosa*, despite differences in the genomic context of the genes involved.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 1. Bacterial strains were routinely propagated at 37°C in Luria-Bertani (LB) broth (Invitrogen Canada Inc., Burlington, Ontario, Canada). *Pseudomonas* isolation agar (Difco Becton, Sparks, MD) was used for selecting transconjugants from the mating experiments. The antibiotics in the selection media were used at the following concentrations for *Escherichia coli* strains: ampicillin, 100 µg/ml; kanamycin, 50 µg/ml; chloramphenicol, 34 µg/ml; gentamicin (Gm), 15 µg/ml; and tetracycline, 15 µg/ml. For *P. aeruginosa* strains, the following antibiotics were used: carbenicillin, 500 µg/ml; Gm, 300 µg/ml; and tetracycline, 60 µg/ml. Isopropyl-β-D-thiogalactopyranoside (IPTG) was used at 0.4 mM for expression studies. Antibiotics and IPTG were obtained from Sigma-Aldrich (Oakville, Ontario, Canada).

General DNA methods. Oligonucleotide primers were prepared by the Laboratory Services Division of the University of Guelph (Guelph, Ontario, Canada) or by Sigma-Genosys Canada (Oakville, Ontario, Canada). Sequences of all primers used and a listing of PCR cycling conditions are available on request. Restriction enzymes and T4 DNA ligase were from Invitrogen (Burlington,

Ontario, Canada), except for XbaI from New England Biolabs (Pickering, Ontario, Canada). All enzymes were used according to the manufacturers' instructions. After ligation, plasmids were transformed into *E. coli* JM109 or DH5α by chemical transformation. For complementation, plasmid DNA was introduced into *P. aeruginosa* by chemical transformation according to the benchtop method (7). All constructs were verified by restriction digestion and/or nucleotide sequencing at the Laboratory Services Division of the University of Guelph.

Generation of nonpolar knockout mutants. Null mutants of *wbpA*, *wbpD*, and *wbpI* in the *P. aeruginosa* PAO1 background were previously constructed by our group (5, 43). Knockout mutants of *wbpB* and *wbpE* were constructed using the allelic replacement method described by Schweizer and Hoang (40). Briefly, knockout constructs of *wbpB* and *wbpE* were generated by insertional mutagenesis with a Gm resistance cassette. For the *wbpB* knockout, the gene was amplified from *P. aeruginosa* PAO1 genomic DNA, using primers that incorporate a BamHI site and an EcoRI site, and the resultant product was ligated into pET-28a (Novagen, Mississauga, Ontario, Canada). The *wbpB* gene was disrupted by insertion of the Gm^r cassette from PstI-digested pPS856 into the two internal PstI sites. The *wbpB*::Gm region was PCR amplified using KOD polymerase according to the manufacturer's directions (Novagen), using primers that introduced HindIII sites at each end. After PCR, the product was purified using a HighPure PCR purification kit (Roche) and digested with HindIII. The HindIII-digested *wbpB*::Gm construct was ligated into HindIII-digested pEX18Ap (20). Clones were confirmed by restriction digestion and then transformed into *E. coli* SM10 for introduction into *P. aeruginosa* PAO1 by conjugal transfer. Conjugal transfer and phenotypic selection were carried out according to the methods described by Wenzel et al. (43). For the *wbpE* knockout, *wbpE* was first cloned into an expression vector and then subcloned into a suicide vector for use in knockout construction. The *wbpE* gene from *P. aeruginosa* PAO1 was amplified from genomic DNA by PCR, using *Pwo* polymerase according to the method supplied by the manufacturer (Roche Diagnostics, Laval, Quebec, Canada). Both the *wbpE* PCR product and pET-23dr were digested with NcoI and BamHI and ligated overnight at 15°C, using T4 DNA ligase (NEB). The resulting expression construct, pWMJL085, was used as a template for a PCR using primers BADE5 and BADE3. The PCR product was then ligated into pBAD24 via XbaI and SphI sites. The Gm^r cassette from pUCGm was removed via SmaI and blunt end ligated into the MscI site of *wbpE*, generating pBAD24-*wbpE*::Gm. The disrupted *wbpE*::Gm construct was excised through digestion by XbaI and HindIII and ligated to XbaI/HindIII-digested pEX18Ap. The final construct, pEX18Ap-*wbpE*::Gm, was introduced into *P. aeruginosa* PAO1 by conjugal transfer and selection as previously described for the *wbpB* knockout. Successful knockouts of each gene were confirmed by a Gm^r Cb^s phenotype, correct PCR fragment size, silver staining of LPS, and Western blotting with the anti-B-band monoclonal antibody (MAb) MF15-4.

Assignment of *B. pertussis* gene functions. The amino acid sequences of the five enzymes of *P. aeruginosa* proposed to be involved in UDP-D-ManNAc3NAcA biosynthesis were used to query the Sanger database of predicted *B. pertussis* proteins. The top-scoring hits were considered in combination with the locations of the genes, with preference given to those in the band A trisaccharide biosynthesis cluster. Assignment of homologous pairs of enzymes from *P. aeruginosa* and *B. pertussis*, using the NCBI BLAST 2 Sequences tool (<http://blast.ncbi.nlm.nih.gov/bl2seq/wblast2.cgi>), formed the hypotheses to be tested by cross-complementation analysis. Homologs of all genes selected for analysis from *B. pertussis* are also present in *B. bronchiseptica* and *B. parapertussis*.

Construction of complementation vectors. The complementation vectors used were pUCP26 and pUCP27, which differ only in the orientation of the multiple cloning site. If available, the *P. aeruginosa* genes were subcloned from existing pET-28a or pET-23dr expression constructs.

(i) **Dehydrogenases.** Plasmid pCQW14 (31) was used to subclone His₆-WbpA via XbaI-XhoI ligated to XbaI-SalI of pUCP27 to create p27-His₆-WbpA. The WbpA gene homologs from *B. pertussis*, *wbpO*₃₁₅₀ and *wbpO*₁₆₂₉, were amplified from a boiled lysate of *B. pertussis* BP536 by use of KOD polymerase according to the manufacturer's directions (Novagen). *wbpO* PCR products purified by gel excision were digested and ligated via NdeI-EcoRI into pET-28a to yield the expression constructs. 28a-His₆-WbpO₃₁₅₀ was then digested with XbaI and EcoRI to liberate the His₆-WbpO₃₁₅₀ fragment, which was ligated into XbaI-EcoRI-digested pUCP27. 28a-His₆-WbpO₁₆₂₉ was digested with XbaI and HindIII to liberate the His₆-WbpO₁₆₂₉ fragment, which was ligated into XbaI-HindIII-digested pUCP26.

(ii) **Oxidases.** The *wbpB* gene was PCR amplified from PAO1 chromosomal DNA by using primers that incorporated BamHI and EcoRI restriction sites, using *Pwo* polymerase according to the manufacturer's instructions (Invitrogen). The *wbpB* PCR product was cleaned and ligated into pET-28a via BamHI-EcoRI sites to yield pET-His₆-WbpB, an expression vector for His₆-WbpB. For comple-

TABLE 1. Strains and plasmids used for this study

Strain or plasmid	Relevant characteristics	Reference or source
Strains		
<i>E. coli</i> strains		
JM109	General use cloning strain	47
DH5 α	General use cloning strain	Life Technologies, Inc.
SM10	Mating donation strain	41
<i>B. pertussis</i> strains		
BP536	Sm ^r derivative of Tohama I	36
<i>P. aeruginosa</i> strains		
PAO1	Serotype O5; <i>P. aeruginosa</i> type strain	17
KEP-A3	<i>wbpA</i> knockout in PAO1; Gm ^r	5
ELW-B1	<i>wbpB</i> knockout in PAO1; Gm ^r	This study
ELW-E100	<i>wbpE</i> knockout in PAO1; Gm ^r	This study
CQW47	<i>wbpD</i> knockout in PAO1; Gm ^r	43
KEP-I9	<i>wbpI</i> knockout in PAO1; Gm ^r	5
Plasmids		
pPS856	FRT cassette vector including Gm ^r determinant	20
pUCP26	pUC18-derived broad-host-range cloning vector	45
pUCP27	pUC19-derived broad-host-range cloning vector (opposite multiple cloning site)	45
pCR-Blunt II-TOPO	Efficient one-step ligase-free cloning vector	Invitrogen, Inc.
pCQW14	His ₆ -WbpA in pET28a	31
p27-His ₆ -WbpA	His ₆ -WbpA in pUCP26 (XbaI/XhoI fragment from pCQW14 subcloned into XbaI/SalI sites of pUCP27)	This study
pET-His ₆ -WbpB	His ₆ -WbpB in pET28a (BamHI/EcoRI fragment from PCR)	This study
pTOPO-WbpB-His ₆	<i>wbpB</i> without stop codon, cloned into pCR-Blunt II-TOPO after PCR with NcoI/XhoI sites in primers	This study
pET-WbpB-His ₆	<i>wbpB</i> without stop codon, NcoI/XhoI fragment from TOPO-WbpB-His ₆ subcloned into pET28a	This study
p27-His ₆ -WbpB	His ₆ -WbpB in pUCP27 (XbaI/HindIII fragment from pET-His ₆ -WbpB)	This study
p27-WbpB-His ₆	WbpB-His ₆ in pUCP27 (PCR on pET-WbpB-His ₆ with XbaI/HindIII sites in primers, product ligated to XbaI/SalI sites of pUCP27)	This study
pWMJL085	His ₆ -WbpE in pET23dr (NcoI/BamHI fragment from PCR)	Wayne L. Miller (unpublished work)
p26-His ₆ -WbpE	His ₆ -WbpE in pUCP26 (XbaI/BamHI fragment from pWMJL085)	This study
pCQW13	His ₆ -WbpD in pUCP26	43
pWMJL072	His ₆ -WbpI in pET23dr (AflIII/BamHI fragment from PCR ligated to NcoI/BamHI sites of pET23dr)	46
p26-His ₆ -WbpI	His ₆ -WbpI in pUCP26 (XbaI/BamHI fragment from pET23dr-wbpI)	This study
p27-WlbC	<i>wlbC</i> in pUCP27 (BamHI/EcoRI fragment from PCR)	This study
p26-WlbB	<i>wlbB</i> in pUCP26 (EcoRI/BamHI fragment from PCR)	This study
pEWJL201	His ₆ -WlbD in pET23dr (NcoI/BamHI fragment from PCR)	46
p26-His ₆ -WlbD	His ₆ -WlbD in pUCP26 (XbaI/BamHI fragment from pEWJL201)	This study
BbLPS1	<i>Bordetella bronchiseptica</i> cosmid containing <i>wlb</i> cluster	33
p26-WlbA	<i>wlbA</i> in pUCP26 (EcoRI/BamHI fragment from PCR on BbLPS1)	This study
pET-His ₆ -WbpO ₁₆₂₉	His ₆ -WbpO ₁₆₂₉ in pET28a (NdeI/EcoRI fragment from PCR)	This study
p27-His ₆ -WbpO ₁₆₂₉	His ₆ -WbpO ₁₆₂₉ in pUCP27 (XbaI/EcoRI fragment from pET-His ₆ -WbpO ₁₆₂₉)	This study
pET-His ₆ -WbpO ₃₁₅₀	His ₆ -WbpO ₃₁₅₀ in pET28a (NdeI/EcoRI fragment from PCR)	This study
p27-His ₆ -WbpO ₃₁₅₀	His ₆ -WbpO ₃₁₅₀ in pUCP27 (XbaI/EcoRI fragment from pET-His ₆ -WbpO ₃₁₅₀)	This study

mentation, pET-His₆-WbpB was digested with XbaI and HindIII, and the liberated fragment was ligated into pUCP27. A C-terminally polyhistidine-tagged construct was also generated by amplification of *wbpB* without a stop codon from chromosomal DNA, using KOD polymerase according to the manufacturer's directions (Novagen). The PCR product was then TOPO cloned with a pCR-Blunt-II-TOPO kit (Invitrogen) according to the manufacturer's protocol. *wbpB* was then subcloned via NcoI and XhoI sites in the primers and ligated into pET-28a, generating pET-WbpB-His₆. For the complementation construct, PCR was performed on pET-WbpB-His₆ with XbaI and HindIII sites in the primers. KOD polymerase was used according to the manufacturer's directions (Novagen). The digested PCR product was ligated to XbaI-SalI-digested pUCP27. The WbpB gene homolog from *B. pertussis*, *wlbA*, was amplified from the cosmid BbLPS1, which contains the band A trisaccharide biosynthesis cluster from *B. bronchiseptica* (32, 33). Use of BbLPS1 allowed chromosomal PCR problems to be resolved, and the sequence of *wlbA* from *B. pertussis* was identical to that from *B. bronchiseptica*. KOD polymerase was used according to the manufacturer's directions (Novagen), and after PCR, the *wlbA* band was purified by gel excision, using a HighPure PCR purification kit (Roche). The purified *wlbA* PCR product

was digested using the EcoRI and BamHI restriction sites on the primers and then ligated into pUCP26. Clones were confirmed by DNA sequencing.

(iii) **Transaminases.** pWMJL085 was digested with XbaI and BamHI to liberate the His₆-WbpE insert; this was then ligated into pUCP26. The WbpE gene homolog from *B. pertussis*, *wlbC*, was amplified from a boiled lysate of *B. pertussis* BP536 by use of KOD polymerase according to the manufacturer's directions (Novagen). After PCR, the *wlbC* band was purified by gel excision, and the purified *wlbC* PCR product was ligated into pUCP27 via BamHI and EcoRI sites.

(iv) **N-Acetyltransferases.** pCQW13, which encodes His₆-WbpD in pUCP26, has been described previously (43). The homologous gene *wlbB* was amplified from a boiled lysate of *B. pertussis* BP536 by use of KOD polymerase according to the manufacturer's directions (Novagen). Using the EcoRI and BamHI restriction sites on the primers, *wlbB* was digested and ligated into pUCP26.

(v) **Epimerases.** p26-His₆-WbpI and p26-His₆-WlbD were subcloned from previously described expression constructs in pET-23dr (46). The affinity-tagged genes were liberated, ligated to pUCP26 via XbaI and BamHI sites, and verified by restriction digestion.

Preparation and analysis of LPS. Small-scale LPS preparation was performed by the method of Hitchcock and Brown (19). LPS was separated by using 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (25) or 4 to 12% Bis-Tris NuPage gels (Invitrogen) and was visualized by an ultrafast silver staining method (14). Western immunoblotting was performed according to a standard procedure used in our laboratory, and the blots were probed using MAb MF15-4, specific for B-band O antigen (26). The secondary antibody was alkaline phosphatase-conjugated goat anti-mouse immunoglobulin G (Jackson ImmunoResearch). Blots were developed according to standard protocols for colorimetric detection (Qiagen, Mississauga, Ontario, Canada).

Protein expression and purification. Expression clones were transformed into *E. coli* BL21(DE3) and induced using 1 mM IPTG for 3 h at 37°C. Cultures (250 ml) were harvested by centrifugation at $5,000 \times g$ for 10 min in an Avanti J25I centrifuge (Beckman Coulter Canada, Mississauga, Ontario, Canada), and purification was carried out according to the protocol established for His₆-WbpA (31). Proteins separated by SDS-PAGE were visualized by staining with Coomassie brilliant blue R-250 (Sigma-Aldrich). Proteins were quantitated by spectrophotometric assay, using the formula $A_{280} = \epsilon Cl$, where ϵ is $37,820 \text{ M}^{-1}\text{cm}^{-1}$, as predicted by ProtParam software (15), and Cl is molar concentration and path length (in cm).

Dehydrogenase reactions and CE analyses. Dehydrogenase reactions were similar to those used for His₆-WbpA (31), with mixtures consisting of 2.5 mM NAD⁺, 100 mM Tris-HCl buffer, pH 7.5, 100 mM ammonium sulfate, 1 mM substrate, and 10.5 μg of enzyme in a 35-μl volume. UDP-D-GlcNAc, UDP-D-GalNAc, UDP-D-Glc, and UDP-D-Gal were obtained from Sigma-Aldrich and tested individually as potential substrates. TDP-D-Glc was produced from TTP and glucose-1-phosphate through reaction with RmlA and RmlB and then verified by mass spectrometry (MS) analysis (unpublished data). Reactions were allowed to proceed for 4 h at 37°C and were then stopped by freezing at -20°C until capillary electrophoresis (CE) analysis. For CE analysis, 35 μl of water was added to each reaction mix, and the samples were centrifuged for 5 min at $12,000 \times g$ at room temperature before 60-μl aliquots were loaded into a P/ACE MDQ glycoprotein system (Beckman Coulter). The capillary was bare silica (75 μm by 60 cm, with a detector at 50 cm), and the running buffer was 25 mM sodium tetraborate, pH 9.5. The capillary was conditioned at the start of each run with 1 M sodium hydroxide for 2 min, followed by running buffer for 2 min. Samples were introduced by pressure injection for 16 s, and separation was performed at 22 kV. Detection was done by measuring UV absorbance, monitored at 254 nm. Electropherograms were analyzed using Beckman 32 Karat software and Sigma-Plot. Reactions were performed in triplicate, and results were averaged to calculate the percent conversion for each enzyme with each substrate. For comparison to theoretical reaction products, known standards were obtained from Sigma-Aldrich (UDP-D-GlcA) or through enzymatic synthesis using His₆-WbpA or His₆-WbpO according to established methods (UDP-D-GlcNAcA and UDP-D-GalNAcA) (30).

MS. For MS, unpurified dehydrogenase reaction mixtures were mixed directly with matrix solution (2 mg of 3,4-dihydroxybenzoic acid in 20% ethanol) at an analyte-to-matrix ratio of 1:2 (vol/vol), and 1 μl was spotted on the matrix-assisted laser desorption/ionization (MALDI) sample target and allowed to dry at room temperature. In some cases, good crystal formation was not achieved and the spots on the MALDI plate looked shiny; in these cases, the sample spots were covered by an additional 1 μl of matrix solution. After the samples were dried at room temperature, crystals were forming on the edges of the sample spots. Proteins were present in the reaction mixtures with UDP-D-Glc and UDP-D-GlcNAc but were removed by filtration using a Microcon YM-10 centrifugal filter device (Millipore Corporation, Bedford, MA) for the UDP-D-GalNAc sample. Analysis of the total reaction mixtures was performed using a MALDI-time-of-flight MS instrument (model Reflex III; Bruker, Germany) equipped with a 337-nm nitrogen laser (Biological Mass Spectrometry Facility, University of Guelph). Samples were analyzed in reflectron and negative-ion modes, scanning from 0 to 1,000 m/z and using ion suppression of up to 150 m/z . For all experiments, ion sources 1 and 2 were held at 20 kV and 16.35 kV, respectively, and guiding lens voltage was held at 9.75 kV. The reflector detection gain was set up at 5.3, with pulsed ion extraction at 200 ns. The nitrogen laser power was set to the minimum level necessary to generate a reasonable signal and to avoid possible degradation of analytes. Typically, 15 to 25% of laser energy was used. Two-point external calibration was performed, using the $[M - H]^-$ (223.06 Da) and $[2M - H]^-$ (447.12 Da) peaks of sinapinic acid (SA; Sigma-Aldrich) prepared in acetonitrile-water solution (1 mg SA in 100 μl of H₂O-CH₃CN [1:1]). The mass accuracy with external calibration using SA was estimated to be below 5 ppm.

RESULTS

Functional prediction of *B. pertussis* genes required for UDP-D-ManNAc3NAcA biosynthesis. Based on the similarities of the predicted amino acid sequences to those of *P. aeruginosa* sequences, six ORFs were assigned putative functions. The *wlb* locus, known to be involved in band A trisaccharide biosynthesis in *Bordetella* (1), encodes the putative oxidase (*wlbA*), *N*-acetyltransferase (*wlbB*), and transaminase (*wlbC*) as well as the previously characterized UDP-D-GlcNAc3NAcA 2-epimerase (*wlbD*) (46). WlbA has 40% similarity to WbpB of *P. aeruginosa*, WlbB shares 85% similarity with WbpD, WlbC has 74% similarity to WbpE, and WlbD shares 70% similarity with WbpI. ORFs encoding proteins with similarity to WbpO and WbpA dehydrogenases from *P. aeruginosa* were not found inside the band A trisaccharide biosynthesis cluster but, rather, elsewhere in the chromosome (Fig. 2). Two ORFs, BP1629 and BP3150, were found in separate loci that may be involved in polysaccharide biosynthesis; BP1629 is in a putative capsule locus, while BP3150 is part of a cryptic locus. Both ORFs are adjacent to genes encoding putative homologs of WbpP, the 4-epimerase that works with the WbpO dehydrogenase in *P. aeruginosa* serotype O6. BP1630 and BP3149, the putative *wbpP* genes from *B. pertussis*, encode proteins that are 100% identical to each other and 80% similar to WbpP of *P. aeruginosa* serotype O6. The putative dehydrogenases, BP1629 and BP3150, share 90% similarity to WbpO of *P. aeruginosa* (WbpO_{O6}) and 54% similarity to WbpA, contain conserved domains of UDP-glucose/GDP-mannose dehydrogenase families, and are 99% identical to each other. For convenience, the genes were therefore called *wbpO*₁₆₂₉ and *wbpO*₃₁₅₀. The sequence differences between WbpO₁₆₂₉ and WbpO₃₁₅₀ are restricted to the N terminus of the proteins: WbpO₁₆₂₉ has five additional amino acids relative to WbpO₃₁₅₀, but after the initial 63 bp, the remaining sequences were identical even at the DNA level. Although the predicted Rossmann fold for predicted NAD(P) binding is close to the N terminus, the GXGXXG motif is located at amino acids 17 to 23 of WbpO₁₆₂₉ and 12 to 17 of WbpO₃₁₅₀, so the differences between the two proteins do not seem to affect any structural features. Both WbpO₁₆₂₉ and WbpO₃₁₅₀ were targeted for further characterization to demonstrate that they are dehydrogenases and to investigate if their functional properties are more like those of WbpO of *P. aeruginosa* serotype O6 or WbpA of *P. aeruginosa* serotype O5.

Complementation of *P. aeruginosa* knockout constructs with corresponding wild-type genes. Knockouts of *wbpB* and *wbpE* were transformed with plasmids encoding His₆-WbpB, WbpB-His₆, or His₆-WbpE. B-band O antigen was detected in each strain after transformation, but the empty vector alone could not restore B-band production (data not shown). A *wbpA* knockout strain was previously complemented by *wbpA* (5) and was complemented here with plasmid-encoded His₆-WbpA (Fig. 3). Complementation of *wbpD* and *wbpI* knockouts with *wbpD* and *wbpI* was also previously reported (5, 43, 46), and these strains can also be complemented with N-terminally histidine-tagged fusions (data not shown).

Cross-complementation of putative *B. pertussis* UDP-D-GlcNAc dehydrogenases. SDS-PAGE was used to separate the LPS and was allowed to run for an extended period to improve the

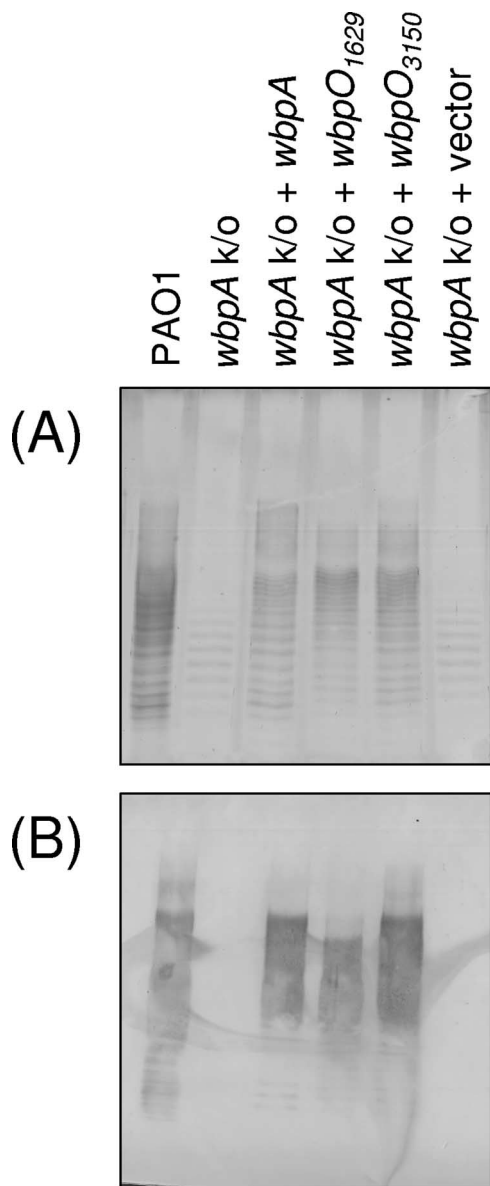


FIG. 3. Cross-complementation of *wbpA* knockouts in *P. aeruginosa* PAO1 with plasmid-encoded His₆-WbpA or either His₆-WbpO₁₆₂₉ or His₆-WbpO₃₁₅₀ from *B. pertussis*. (A) Silver-stained SDS-PAGE gel; (B) Western blot with anti-B-band O antigen MABs. B-band O antigen was not detected in the *wbpA* knockout strain or the knockout transformed with empty vector; B-band O antigen was detected when the knockout strains were complemented with plasmid-encoded His₆-WbpA, His₆-WbpO₁₆₂₉, or His₆-WbpO₃₁₅₀.

resolution of the B-band O antigen bands. The control *P. aeruginosa* PAO1 LPS revealed a ladder-like banding pattern that is known to represent individual LPS species with different numbers of O antigen repeats (Fig. 3). Because of the extended running time for SDS-PAGE, the LPS core bands are not visible on the gel. The *wbpA* knockout of *P. aeruginosa* PAO1 is devoid of B-band O antigen but still produces A-band O antigen in a variety of lengths. A-band O antigen bands were present on the silver-stained SDS-PAGE gel but were not reactive with the B-band-specific MAb MF15-4 in a Western

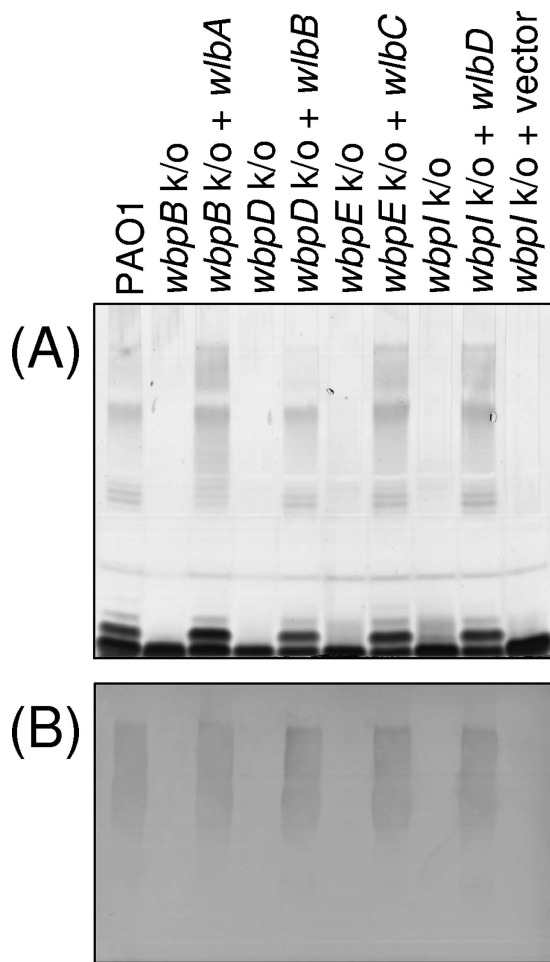


FIG. 4. Cross-complementation of various *wbp* knockouts in *P. aeruginosa* PAO1 by homologs from the *wlb* locus of *B. pertussis*. (A) Silver-stained SDS-PAGE gel; (B) Western blot with anti-B-band O antigen MABs. B-band O antigen was not detected in the knockout strains or knockout strains transformed with an empty vector; B-band O antigen was detected when the knockout strains were complemented with the relevant *B. pertussis* gene.

blot (Fig. 3). B-band O antigen was restored when His₆-WbpA was expressed in *trans*, as tested by LPS silver staining and Western blotting with MAb MF15-4 (Fig. 3). No differences were detected in the modality of the A-band or B-band O antigens. The hexahistidine tag did not interfere with the *in vivo* function of His₆-WbpO₁₆₂₉ or His₆-WbpO₃₁₅₀, since the plasmid-carried His-tagged genes were capable of restoring B-band O antigen production in the *wbpA* mutant (Fig. 3). As expected, the empty vector control was not able to complement the B-band O antigen deficiency in the *wbpA* mutant (Fig. 3).

Cross-complementation of *B. pertussis* band A biosynthesis cluster members. Silver-stained SDS-PAGE analysis of wild-type PAO1 LPS showed two dark bands of low molecular weight (Fig. 4). These are known as the core and core-plus-one O-antigen repeat unit, based on previous findings of Western blotting with anti-core and anti-core-plus-one MABs (data not shown). In addition, a range of high-molecular-weight molecules are present, which are the LPS bands containing A- or B-band O antigen polymers. Knockouts of the *P. aeruginosa*

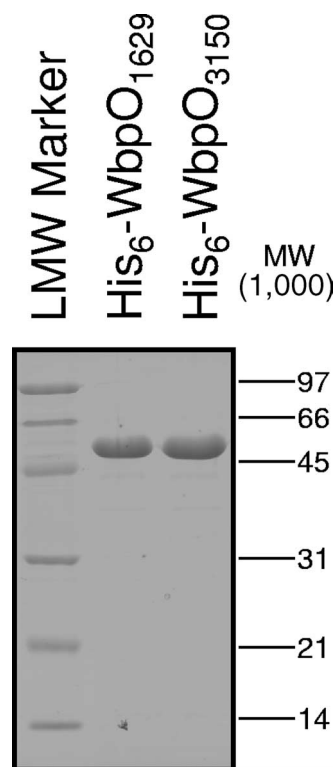


FIG. 5. SDS-PAGE analysis of purified dehydrogenase proteins after Ni²⁺-affinity chromatography and buffer exchange according to the work of Miller et al. (31). Proteins were expressed with a hexahistidine tag from pET-28a. All proteins were free of contamination.

PAO1 genes *wbpB*, *wbpE*, *wbpD*, and *wbpI* were expected to abrogate the functions of the oxidase, transaminase, *N*-acetyltransferase, and epimerase, respectively. In comparison to strain PAO1, each individual knockout construct lacked B-band O antigen, as determined by the lack of reactivity to MAb MF15-4 (B band specific) in the Western immunoblot (Fig. 4). In addition, the core-plus-one O antigen band was absent in the LPS of each of the knockout strains (Fig. 4). As expected, an empty vector control did not alter the LPS profile (Fig. 4). When copies of the putative oxidase gene (*wlbA*), putative transaminase gene (*wlbC*), putative *N*-acetyltransferase gene (*wlbB*), and established 2-epimerase gene (*wlbD*) from *B. pertussis* were individually provided on plasmids to *P. aeruginosa* oxidase (*wbpB*), transaminase (*wbpE*), *N*-acetyltransferase (*wbpD*), and epimerase (*wbpI*) knockout strains, respectively, the production of B-band O antigen was restored (Fig. 4).

Purification of recombinant dehydrogenases. Milligram quantities of His₆-WbpO₁₆₂₉ and His₆-WbpO₃₁₅₀ were obtained after affinity chromatography and buffer exchange, at >98% purity, as judged by SDS-PAGE and densitometry analysis (Fig. 5). His₆-WbpO₁₆₂₉ and His₆-WbpO₃₁₅₀ are predicted to have molecular weights of 49,388 and 49,007, respectively, and the apparent molecular weights are in agreement with these estimates (Fig. 5).

CE of dehydrogenase-substrate reactions. Control reaction mixtures lacking enzyme resolved three peaks in each case; the two peaks which were consistent among all traces were identified as NAD⁺ and a minor contaminant present with NAD⁺,

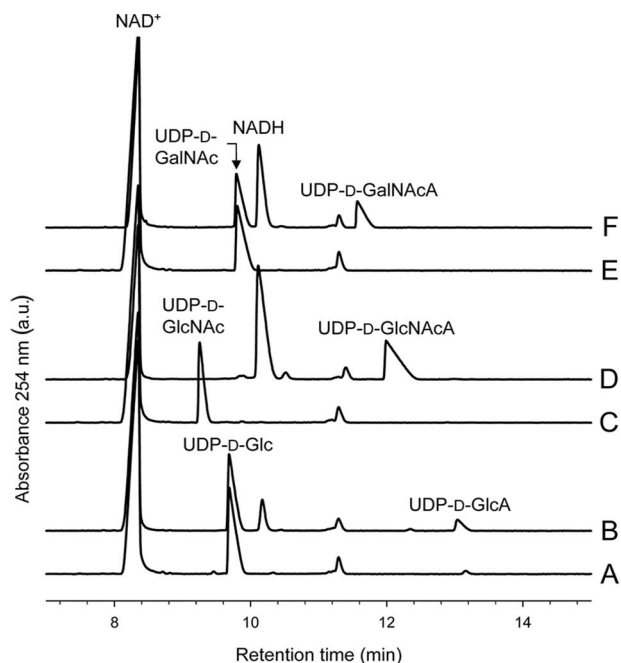


FIG. 6. Electropherograms from CE of dehydrogenase reactions. (A) Control reaction mix (no enzyme) with NAD⁺ and UDP-D-Glc. (B) Reaction with His₆-WbpO₃₁₅₀, NAD⁺, and UDP-D-Glc yielded UDP-D-GlcA. (C) Control reaction mix with NAD⁺ and UDP-D-GlcNac. (D) Reaction with His₆-WbpO₃₁₅₀, NAD⁺, and UDP-D-GlcNac yielded UDP-D-GlcNacA. The same reaction is catalyzed by His₆-WbpA, as previously shown by Miller et al. (31). (E) Control reaction mix with NAD⁺ and UDP-D-GalNac. (F) Reaction with His₆-WbpO₃₁₅₀, NAD⁺, and UDP-D-GalNac yielded UDP-D-GalNacA. The same reaction is catalyzed by *P. aeruginosa* His₆-WbpO, as previously shown by Miller et al. (30).

while the third peak was identified as the sugar-nucleotide substrate based on the use of reagent standards (Fig. 6). Reaction mixtures containing WbpO₁₆₂₉ or WbpO₃₁₅₀ and UDP-D-Glc, UDP-D-GlcNac, or UDP-D-GalNac showed two new large peaks that were not present in the controls. One of these peaks was found for the reactions with all of the substrates and was identified as NADH (Fig. 6). The remaining unique peaks found in reactions using UDP-D-Glc, UDP-D-GlcNac, or UDP-D-GalNac were identified as UDP-D-GlcA, UDP-D-GlcNacA, or UDP-D-GalNacA, respectively, after addition of the relevant UDP-hexuronic acid standard. The percent conversion of UDP-D-GlcNac or UDP-D-GalNac to UDP-D-GlcNacA or UDP-D-GalNacA, respectively, was not significantly different between WbpO₁₆₂₉ and WbpO₃₁₅₀ (*t* test; $\alpha = 0.05$). Conversely, the percent conversion of UDP-D-Glc to UDP-D-GlcA was significantly lower for WbpO₁₆₂₉ than for WbpO₃₁₅₀ (*t* test; $\alpha = 0.05$). No reaction product peak could be discerned when either TDP-D-Glc or UDP-D-Gal was used as the substrate for reaction with WbpO₁₆₂₉ or WbpO₃₁₅₀ (Table 2).

MS of dehydrogenase reactions. Reactions used for CE analysis were further analyzed by MS. Control reaction mixtures lacking enzyme were found to contain ions of either 565.39 *m/z* or 606.40 *m/z* (in negative-ion mode), consistent with the expected mass of UDP-D-Glc or UDP-D-GlcNac/UDP-D-GalNac, respectively (the epimers UDP-D-GlcNac

TABLE 2. Percent conversion from substrate to product for WbpO₁₆₂₉ and WbpO₃₁₅₀ reactions analyzed by CE

Substrate	Enzyme	% Conversion ^a
UDP-D-Glc	WbpO ₁₆₂₉	5.4 ± 1.8
	WbpO ₃₁₅₀	13.1 ± 2.1
TDP-D-Glc	WbpO ₁₆₂₉	ND
	WbpO ₃₁₅₀	ND
UDP-D-GlcNAc	WbpO ₁₆₂₉	100
	WbpO ₃₁₅₀	95.8 ± 7.3
UDP-D-Gal	WbpO ₁₆₂₉	ND
	WbpO ₃₁₅₀	ND
UDP-D-GalNAc	WbpO ₁₆₂₉	28.7 ± 2.2
	WbpO ₃₁₅₀	37.4 ± 6.0

^a ND, none detected. Data are means ± standard deviations.

and UDP-D-GalNAc have the same expected mass). Reactions with His₆-WbpO₃₁₅₀ and UDP-D-Glc present showed the presence of ions consistent with both UDP-D-Glc (665.50 *m/z*) and UDP-D-GlcA (579.48 *m/z*). Reactions with His₆-WbpO₃₁₅₀ and UDP-D-GlcNAc or UDP-D-GalNAc present showed the presence of ions consistent with the expected mass of UDP-D-GlcNAcA or UDP-D-GalNAcA (620.35 *m/z*), but unreacted substrate (606.39 *m/z*) only was detected in reactions with UDP-D-GalNAc. This indicates that the reaction with UDP-D-GlcNAc was the only one to go to completion and that His₆-WbpO₃₁₅₀ catalyzes the production of UDP-D-GlcA, UDP-D-GlcNAcA, or UDP-D-GalNAcA.

DISCUSSION

In *P. aeruginosa*, five genes are predicted to be required for the production of UDP-D-ManNAc3NAcA, including *wbpA*, *wbpB*, *wbpE*, *wbpD*, and *wbpI*. The evidence presented here demonstrates that B-band O antigen is not produced when *wbpB* or *wbpE* is knocked out in *P. aeruginosa* (Fig. 4), consistent with a deficiency in UDP-D-ManNAc3NAcA synthesis. Thus, together with prior studies, genetic evidence has been obtained to show that all five members of the proposed pathway are essential for the production of UDP-D-ManNAc3NAcA in *P. aeruginosa* PAO1. Complementation of *wbpB* and *wbpE* knockouts with His-tagged versions of the *wbpB* and *wbpE* gene products have established that His₆-WbpB and His₆-WbpE are functional (data not shown) and can be used for further biochemical studies.

By constructing a complete set of knockouts in the genes that encode enzymes for the UDP-D-ManNAc3NAcA biosynthetic pathway, we made a unique set of tools whereby the function of the corresponding homologs from *B. pertussis* could be tested through pairwise cross-complementation. Initial assignments of *B. pertussis* genes to particular pathway steps, which was based on alignment comparison of the gene products with those encoded by the *wbp* cluster of *P. aeruginosa* PAO1, were supported by the ability of each *B. pertussis* gene to cross-complement the relevant *wbp* knockout (Fig. 3 and 4). This genetic evidence suggests that *wbpO*₁₆₂₉ and *wbpO*₃₁₅₀ encode UDP-D-GlcNAc 6-dehydrogenases, *wlbA* encodes a UDP-D-GlcNAcA 3-oxidase, *wlbC* encodes a UDP-D-3-keto-GlcNAcA 3-aminotransferase, *wlbB* encodes a UDP-D-GlcNAc3NA 3-*N*-acetyltransferase, and *wlbD* encodes a UDP-D-GlcNAc3NAcA 2-epimerase. The discovery of functionally

identical *B. pertussis* homologs to the *P. aeruginosa* UDP-D-ManNAc3NAcA biosynthesis enzymes suggests that UDP-D-ManNAc3NAcA production occurs through the same enzymatic steps in *B. pertussis*.

The presence of functional homologs encoding putative UDP-D-ManNAc3NAcA biosynthesis enzymes in *P. aeruginosa* and *B. pertussis* (Fig. 2) raises questions about the evolutionary conservation of the proposed pathway. *P. aeruginosa* and *B. pertussis* are members of the *Proteobacteria*, belonging to the *Gammaproteobacteria* and *Betaproteobacteria*, respectively. Homologs of each of these five genes can also be identified in several other proteobacteria, including *Marinomonas* sp. (*Gammaproteobacteria*), *Polaromonas naphthalenivorans* (*Betaproteobacteria*), and *Wolinella succinogenes* (*Epsilonproteobacteria*). A subgroup contains homologs to *wbpABED* but not *wbpI* (which encodes a UDP-D-GlcNAc3NAcA 2-epimerase), suggesting that UDP-D-GlcNAc3NAcA may be produced; these include *Roseovarius nubinhibens* (*Alphaproteobacteria*) and *Ralstonia eutropha* (*Betaproteobacteria*). In many cases, the genes homologous to those of the UDP-D-ManNAc3NAcA biosynthesis cluster from *P. aeruginosa* PAO1 are also found in clusters. Unfortunately, little is known about the composition of the LPS in these gram-negative bacteria at this time, although evidence has been given for the presence of mannose-derived residues in the O antigens of *W. succinogenes* and *Wolinella recta* (16, 24). Interestingly, evidence has been given for the presence of ManNAc3NAcA in the gram-positive bacterium *Geobacillus stearothermophilus* (29, 39), which belongs to the *Firmicutes*. Sequencing of *Geobacillus stearothermophilus* is in progress at the University of Oklahoma, and the use of tBLASTn searches (http://www.genome.ou.edu/blast/bstearo_blastall.html) indicates that homologs of all five UDP-D-ManNAc3NAcA genes are present [B. Roe, S. Lewis, B. Perry, F. Najar, and R. Morales-Diaz, *Bacillus* (*Geobacillus*) *stearothermophilus* genome sequencing project]. Although further work is required, we suggest that the proposed pathway for UDP-D-ManNAc3NAcA biosynthesis may be highly conserved among this broad range of bacteria. In future studies, the findings of this study can be exploited to predict the functions of the genes required for UDP-D-ManNAc3NAcA biosynthesis in organisms known to produce ManNAc3NAcA.

CE analysis showed that WbpO₁₆₂₉ and WbpO₃₁₅₀ are promiscuous 6-dehydrogenases, capable of utilizing UDP-D-GlcNAc, UDP-D-GalNAc, and UDP-D-Glc as substrates, but not TDP-D-Glc or UDP-D-Gal (Fig. 3 and Table 2). MS analysis of reactions confirmed the production of UDP-D-GlcNAcA, UDP-D-GalNAcA, and UDP-D-GlcA by use of His₆-WbpO₃₁₅₀, but MS was not used to analyze His₆-WbpO₁₆₂₉ reactions, since CE had demonstrated the products of both dehydrogenases to be identical. The two WbpO enzymes from *Bordetella* have higher similarity at the amino acid level to the UDP-D-GlcNAc/UDP-D-GalNAc 6-dehydrogenase WbpO of *P. aeruginosa* serotype O6 than to the UDP-D-GlcNAc 6-dehydrogenase WbpA of *P. aeruginosa* serotype O5; the ability of the *B. pertussis* enzymes to use both UDP-D-GlcNAc and UDP-D-GalNAc further supports the annotation with the gene name *wbpO*. WbpA acts as the first enzyme in the UDP-D-ManNAc3NAcA pathway that we have presented, and it is clear that the *B. pertussis* WbpO enzymes can provide the same activity, since they complement the

wbpA knockout and can catalyze UDP-D-GlcNAc 6-dehydrogenation in vitro. It is interesting that WbpO₁₆₂₉ and WbpO₃₁₅₀ can use UDP-D-Glc as a substrate, because neither of the homologs from *P. aeruginosa*, WbpA and WbpO, has this ability. This is a novel finding because although other UDP-D-Glc 6-dehydrogenases are known, to our knowledge these enzymes are the first that have been shown to utilize UDP-D-GlcNAc, UDP-D-GalNAc, and UDP-D-Glc as substrates. Although the percent conversion of UDP-D-Glc to UDP-D-GlcA was significantly lower for WbpO₁₆₂₉ than for WbpO₃₁₅₀, both values were <15% conversion, so the difference may not be physiologically relevant. Also of biochemical interest is the fact that TDP-D-Glc was not utilized, suggesting that both enzymes can discriminate between nucleotides, despite their somewhat loose specificity for different sugars.

A remaining question surrounds the presence of the two copies of *wbpO* in *B. pertussis*. This study has provided evidence that the products of the two *wbpO* copies can convert the same substrates. However, the distinct locations in separate putative polysaccharide biosynthesis loci suggest that each *wbpO* gene might be involved in the production of different polysaccharide molecules. Two identically functioning copies of *wbpO* might be important to meet the many demands for 6-dehydrogenation that the cell requires.

The presence of neighboring *wbpO* and *wbpP* homologs in two separate loci is conserved in the related species *B. bronchiseptica* and *B. parapertussis*. In *B. pertussis*, UDP-D-ManNAc3NAcA is used for the production of the band A trisaccharide, but in *B. bronchiseptica* and *B. parapertussis*, ManNAc3NAcA is also found in the O antigen linker (34). The O antigen linker also contains ManNAc3NAcAN, a uronamide derivative of ManNAc3NAcA, and GlcNAc3NAcAN, a uronamide derivative of an intermediate from the ManNAc3NAcA pathway (34). The *Bordetella* O antigen produced by *B. bronchiseptica* and *B. parapertussis* contains L-GalNAc3NAcA and L-GalNAc3NAcAN, believed to be derivatives of UDP-D-ManNAc3NAcA synthesized by enzymes of the *wbm* locus (21, 34). Based on our hypothesis, an active copy of either of the *wbpO* genes should be essential for the production of all these uronic acid sugars.

In conclusion, *P. aeruginosa* and *B. pertussis* produce LPS that contain the rare sugar D-ManNAc3NAcA. Cross-complementation analysis was used to demonstrate that B-band LPS production was restored to *P. aeruginosa* knockout mutants when genes encoding *B. pertussis* oxidase, transaminase, *N*-acetyltransferase, and epimerase enzymes were supplied in *trans*. Two dehydrogenases, WbpO₁₆₂₉ and WbpO₃₁₅₀, were identified in *B. pertussis* and were shown to cross-complement a UDP-D-GlcNAc 6-dehydrogenase knockout of *P. aeruginosa*. Biochemical analysis showed that both WbpO proteins have the novel ability to catalyze 6-dehydrogenation of UDP-D-GlcNAc, UDP-D-GalNAc, and UDP-D-Glc in vitro. Thus, these results suggest that *B. pertussis* and *P. aeruginosa* use the same biosynthetic pathway for the production of UDP-D-ManNAc3NAcA but that the first step in the pathway in *B. pertussis* is catalyzed by a novel promiscuous 6-dehydrogenase that is biochemically distinct from its homolog in *P. aeruginosa* PAO1.

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