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Post-assembly Modification of *Bordetella bronchiseptica* O Polysaccharide by a Novel Periplasmic Enzyme Encoded by *wbmE*[*] [S]

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Bordetella bronchiseptica **is a pathogen of humans and animals that colonizes the respiratory tract. It produces a lipopolysaccharide O antigen that contains a homopolymer of 2,3-dideoxy-2,3-diacetamido-L-galacturonic acid (L-GalNAc3NAcA). Some of these sugars are found in the uronamide form (L-GalNAc3NAcAN), and there is no discernible pattern in the distribution of amides along the chain. A *B. bronchiseptica wbmE* mutant expresses an O polysaccharide unusually rich in uronamides. The WbmE protein localizes to the periplasm and catalyzes the deamidation of uronamide-rich O chains in lipopolysaccharide purified from the mutant, to attain a wild-type uronamide/uronic acid ratio. WbmE is a member of the papain-like transglutaminase superfamily, and this categorization is consistent with a deamidase role. The periplasmic location of WbmE and its acceptance of complete lipopolysaccharide as substrate indicate that it operates at a late stage in lipopolysaccharide biosynthesis, after polymerization and export of the O chain from the cytoplasm. This is the first report of such a modification of O antigen after assembly. The expression of *wbmE* is controlled by the *Bordetella* virulence gene two-component regulatory system, BvgAS, suggesting that this deamidation is a novel mechanism by which these bacteria modify their cell surface charge in response to environmental stimuli.**

Bordetella bronchiseptica is a Gram-negative coccobacillus, which colonizes the mammalian respiratory tract. It has a broad host range and is commonly associated with atrophic rhinitis in pigs (1) and infectious tracheobronchitis (kennel cough) in dogs (2). Most of the genes implicated in host colonization and virulence are under the transcriptional control of the two-component regulatory system BvgAS (reviewed in Ref. 3), being expressed maximally in the Bvg^+ phase. Transcription of some other genes, for example the flagellin gene *flaA*, is up-regulated in Bvg^- conditions (4) and an intermediate expression pattern (Bvg^i) has also been described (5). *In vitro* the Bvg^- phase can be induced by culturing *Bordetella* with millimolar concentrations of magnesium sulfate (5, 6) among other stimuli.

One of the Bvg-regulated bacterial structures is lipopolysaccharide (LPS).[3] LPS is the major component of the outer leaflet of the outer membrane. LPS has three domains: first, Lipid A is the lipophilic domain that anchors LPS into the outer membrane; second, a complex, branched-chain oligosaccharide known as core is attached to lipid A and in *Bordetella* the lipid A-core structure is known as B-band LPS; third, a domain distal to the membrane consisting of saccharide repeats may be present, which is commonly called O antigen. In a proportion of *B. bronchisepica* LPS molecules the lipid A core is substituted with a trisaccharide, and this species is known as A-band LPS. The O polysaccharide consists of a homopolymer of 2,3-dideoxy-2,3-diacetamido-L-galacturonic acid (L-GalNAc3NAcA) (7) capped at the nonreducing terminus with a complex 2,3,4-trideoxy-2,3,4-triamino galacturonamide (GalN3N4NAN) derivative (8), and in *B. bronchiseptica* is attached to the A-band trisaccharide via a pentasaccharide linker (Fig. 1) (9). Expression of O antigen by *B. bronchiseptica* is required for full virulence in animal models of infection and for resistance to complement-mediated killing (10).

A proportion of the O polysaccharide repeating units are present as the uronamide (L-GalNAc3NAcAN) (9). In Bvg^- *B. bronchiseptica* RB50, the uronamides make up ~17% of the O polysaccharide residues and the positions of these along the chain appears to be random (9). Uronamide sugars are uncommon in bacteria, but are present in the O polysaccharides of *Shigella dysenteriae* (11), *Francisella* spp (12, 13), and in *Pseudomonas aeruginosa*.

O antigen synthesis is encoded in *B. bronchiseptica* by the *wbm* locus that contains 24 coding sequences including putative genes for the *wzm* and *wzt* components of an ATP-binding cassette (ABC) O antigen transporter (14). The presence of ABC transporter genes suggests that this O antigen is probably

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. S1 and Table S1.

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[3] The abbreviations used are: LPS, lipopolysaccharide; a.m.u., atomic mass units; GalNAc, 2-acetamido-2-deoxy galactose; L-GalNAc3NAcA, 2,3-diacetamido-2,3-dideoxy-L-galacturonic acid; L-GalNAc3NAcAN, 2,3-diacetamido-2,3-dideoxy-L-galacturonamide; ManNAc3NAcAN, 2,3-diacetamido-2,3-dideoxy mannuronamide; PBS, phosphate-buffered saline; UDP-L-GalNAc3NAcA, UDP-2,3-diacetamido-2,3-dideoxy-L-galacturonic acid.

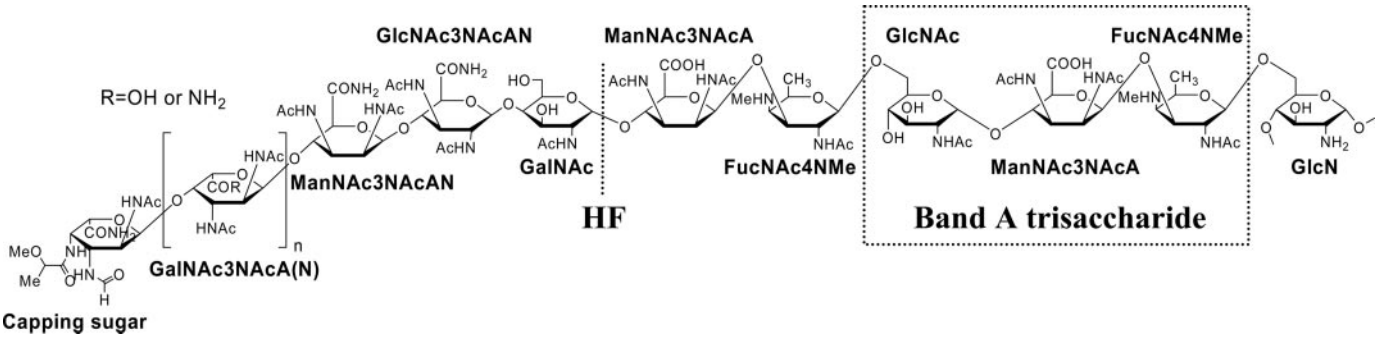

FIGURE 1. **The structure of the membrane-distal domain of O antigen-containing *B. bronchiseptica* LPS.** The homopolymeric O polysaccharide consists of repeating 2,3-diacetamido-2,3-dideoxy-L-galacturonic acid (GalNAc3NAcA; R = OH) residues, a proportion of which are present as the uronamide (GalNAc3NAcAN; R = NH_2). The chain is terminated by an unusual capping sugar, and the reducing end is attached to the Band-A trisaccharide via a linking pentasaccharide. Band-A trisaccharide attaches to the glucosamine (GlcN) of the core oligosaccharide (9). The position of hydrogen fluoride (HF)-induced cleavage of the O polysaccharide from reducing terminal fragments is indicated. The absolute configurations of Band-A trisaccharide and linking pentasaccharide sugars have not been determined; this diagram shows the D-forms.

FIGURE 2. **Transglutaminase core motifs of WbmE, smart00460, and pfam01841.** The sequences from human coagulation factor XIII (1FIE) and human transglutaminase 3 (1L9M) represent the smart00460 conserved domain, and Red Sea bream transglutaminase (1G0D) represents pfam01841. The catalytic triad of factor XIII is indicated by *black arrows*. Two other common features of animal transglutaminases are indicated by *gray arrows*: a glycine two places upstream of the catalytic cysteine; and the aromatic side-chain upstream of active site aspartate (16). Figures in *brackets* denote excluded residues that lie between the three motifs shown; the numbers at the start and end of each row represent the residue numbers for the first and last amino acids shown.

assembled according to the ABC transporter-dependent model (reviewed in Ref. 15) in which the polymer is assembled and terminated on the cytoplasmic face of the inner membrane, then exported across this membrane to the periplasmic face where the O chain is transferred to lipid A core.

In 1999, Makarova *et al.* (16) identified the *B. bronchiseptica* gene product WbmE as a member of the papain-like transglutaminase superfamily although in their report, *wbmE* was mistakenly identified as a *B. pertussis* sequence. Alignment of WbmE with transglutaminase conserved domains (17, 18) indicates that WbmE residues Cys-165, His-201, and Asp-216 probably constitute a conserved transglutaminase-type catalytic triad (19) (Fig. 2). Transglutaminase activity is defined as bridge formation between peptide chains by an acyl transfer reaction between a glutamine γ-carboxamide and a lysine ϵ-amine, but transglutaminase enzymes also catalyze a range of other chemical reaction types, all of which involve either the formation, or breaking of amide bonds (reviewed in Ref. 20). To date, the only functionally characterized microbial members of this family, PeiP and PeiW, are peptidases (21).

In this report, we describe the characterization of the *wbmE* gene and its protein product. WbmE catalyzes deamidation of complete O chains, and this is the first report of such a late O antigen modification. Furthermore, given that *wbmE* expression is regulated by the BvgAS system (22), this enzyme probably constitutes a novel mechanism by which the *B. bronchisepica* cell surface is modified in response to environmental stimuli.

EXPERIMENTAL PROCEDURES

Bacterial Strains, Plasmids, and Culture Conditions—Bacterial strains used in this study are described in the supplemental Table S1. *B. bronchiseptica* was grown on Bordet-Gengou agar (Difco) supplemented with 10% defibrinated horse blood (TCS Cellworks Ltd). *Escherichia coli* was cultured in Luria-Bertani (LB) broth or on LB agar. All strains were incubated at 37 °C and ampicillin (100 $\mu g\ ml^{-1}$), kanamycin (50 $\mu g\ ml^{-1}$), tetracycline (10 $\mu g\ ml^{-1}$ for *E. coli*, 5 $\mu g\ ml^{-1}$ for *B. bronchiseptica*) or streptomycin (200 $\mu g\ ml^{-1}$) were added where required. Suicide plasmids were based on the host-restricted pEX100T backbone (23) and broad host-range shuttle vectors were based on a kanamycin resistant derivative of pBBR1MCS (24). The *phoA* reporter fusion was derived from pRMCD28 (25). For preparation of LPS, *B. bronchiseptica* was grown in tryptone soya broth (Oxoid) supplemented with 50 mM $MgSO_4$ as this maximizes O antigen expression in RB50 by modulating the phase to Bvg^- (supplemental Fig. S1).

DNA Methods—Standard methods were used for DNA manipulations. Oligonucleotides were supplied by Sigma-Genosys. PCR was performed with template from boiled bacteria (26) and TaqDNA polymerase (Promega) or KOD Hot Start DNA Polymerase (Novagen).

Generation of wbmE Mutants—The *wbmE* mutant allele was obtained by *in vitro* transposon-mediated mutagenesis of the *wbm* locus-containing cosmid, BbLPS1 (14) (GenBank™ accession number AJ007747) using an EZ-Tn5™ <Tet-1> insertion kit (Epicenter). The <Tet-1> transposon, plus flanking *wbmE* DNA, was cut out by partial digestion with AluI and ligated into SmaI-cut pEX100T. Allelic exchange constructs were transferred to *B. bronchiseptica* by conjugation with *E. coli* SM10λpir as donor (27). Loss of the plasmid-encoded *sacB* gene in allelic exchange mutagenesis of *B. bronchiseptica* was selected for by growth on LB agar with reduced salt supplemented with 10% (w/v) sucrose (28). Double recombination was confirmed by Southern blotting (not shown). Multiple mutant clones were obtained in independent mating experiments, and were confirmed to have the same phenotype as the representative *wbmE* strain, RBE3c (not shown).

Complementation of wbmE Mutation—The *B. bronchiseptica flaA* promoter was amplified using primers 5′-GCTCTAGATAGGCGCATGCCATGGCC-3′ (XbaI site underlined) and 5′-AAGGATCCCATATGGAGGCTCCCAAGAGAGAA-3′ (BamHI and NdeI sites underlined), and cloned into the XbaI and BamHI sites in pBBR1MCS-kan to generate the vector pCompEmpty. *wbmE* was amplified using primers 5′-AAAAAAACATATGATTCGCAAGAGCTAC-3′ (NdeI site underlined) and 5′- AAAAAGCTTAGATCTCCACATAGAGCAGATGTC-3′ (HindIII site underlined) and topoisomerase-cloned into pCR2.1-TOPO, and the insert was verified by sequencing. *wbmE* was then excised and cloned into pCompEmpty using NdeI and HindIII restriction sites to generate the *wbmE* complementation vector pCompE. For complementation of the *wbmE* mutation by expressing WbmE with a C-terminal His_6 tag, the pCR2.1-TOPO vector containing *wbmE* was used as PCR template with the primers 5′-CCCGGTTTGAAGAAGCCTTTCTC-3′ and 5′-AAAGCTTCA*GTGATGATGATGATGATG*GTTCGGGGCGCTGGCGCG-3′ (HindIII site underlined, reverse complement of His_6 codons italicized). *wbmE-his*$_6$ was then cloned into pCompEmpty using NdeI and HindIII, generating pCompETag. Shuttle vectors were moved into *B. bronchiseptica* by conjugation with *E. coli* CC118 as donor (29), with *trans*-acting transfer functions provided by *E. coli* S17-1 pNJ5000 as helper (27, 30).

SDS-PAGE Analysis of LPS—LPS for SDS-PAGE analysis was obtained from *B. bronchiseptica* using a modification of the method of Hitchcock and Brown (31) as has been described (32). SDS-PAGE of LPS was performed using Novex precast 16% Tricine gels (Invitrogen). LPS was oxidized in-gel with periodic acid (33) and visualized with the Silver Stain Plus kit (Bio-Rad).

Purification of LPS—B. bronchiseptica RB50 and RBE3c LPS were extracted from 4-liter cultures using a modification of the hot aqueous phenol extraction method of Johnson and Perry (34) as has been described (26).

Analysis of LPS Structures—O polysaccharides were cleaved from LPS molecules by 24-h solvolysis with anhydrous HF and subsequently purified as described (9). Electrospray ionization MS spectra were obtained on a Micromass Quattro spectrometer, with samples dissolved in 50% MeCN, 0.2% HCOOH, and delivered by direct injection at a flow rate of 15 μl min^{-1}. Capillary electrophoresis-mass spectrometry (CE-MS) experiments were performed as described previously (35).

Generation of the phoA Fusion and Alkaline Phosphatase Assay—The first 200 bases of *wbmE* were PCR-amplified using the primers 5′-AAAAAAAGATCTCCGCGGAAGGAGGATATACATATGATTCGCAAGAGCTACATCATCG-3′ (SacII site underlined) and 5′-AAAAAAAAGCTTTTAAAGGATCCAGAATTCCAGAAGGCGTAGCAAGTCCGGC-3′ (HindIII site underlined). The PCR product was digested with HindIII and SacII, and cloned into similarly digested pRMCD28 (25), placing the *wbmE* 5′-end in-frame with the *phoA* fragment on the vector. The reporter plasmid was transformed into the *phoA E. coli* strain LMG194.

For alkaline phosphatase assays, 5 μl of stationary phase liquid culture was spotted onto low-phosphate solid medium (120 mM Tris-HCl, pH 7.4, 40 mM NaCl, 20 mM KCl, 40 mM NH_4Cl, 20 mM Na_2SO_4, 1 mM $MgCl_2$, 0.2 mM $CaCl_2$, 0.004 mM $ZnCl_2$, 0.002 mM $FeCl_3$, 0.1 mM KH_2PO_4, 0.4% (w/v) glycerol, 1.5% (w/v) agar) supplemented with 40 μg ml^{-1} 5-bromo-4-chloro-3-indolyl-phosphate (BCIP), 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG), 5 μg ml^{-1} thiamine, 0.5% (w/v) casamino acids, and ampicillin) and incubated for 16 h at 37 °C.

Expression of WbmE-His$_6$ *in B. bronchiseptica*—The plasmid pCompETag has the *wbmE-his*$_6$ gene fusion under the control of the promoter for the *B. bronchiseptica* flagellin gene *flaA*. As *flaA* is expressed in the Bvg$^-$ phase (36), WbmE-His_6 expression from this vector was induced by supplementing the medium with 50 mM $MgSO_4$.

WbmE Assay using Whole Cell Lysates—For analysis by SDS-PAGE: *B. bronchisepica* was harvested from 75-ml liquid cultures at an absorbance of 0.2 (at 595 nm). Cells were washed with 37.5 ml of phosphate-buffered saline at pH 6.5 (PBS), and suspended in 5 ml of PBS. One-tenth and one-hundredth dilutions were made of these suspensions. 1:1 mixtures were made of these neat, one-tenth, and one-hundredth cell suspensions with a 0.4 mg ml^{-1} solution of purified RBE3c LPS dissolved in PBS. WbmE was then released from the cells by sonicating the mixtures on ice for 15 s, using a VibraCell™, sonicator (Sonics and Materials Inc.), fitted with a microtip, at 40% power. They were then incubated at 37 °C for 3, 30, or 300 min with shaking. Samples were centrifuged at 15,000 × *g* for 5 min at 4 °C, and 100 μl of the supernatant was boiled with 50 μl of buffer 1 (0.1875 M Tris-HCl, pH 6.8, 6% (w/v) SDS, 30% (w/v) glycerol), and then incubated overnight at 55 °C after addition of 225 μl of buffer 2 (10 mg ml^{-1} proteinase K, 0.0625 M Tris-HCl, pH 6.8, 0.1% (w/v) SDS, 10% (w/v) glycerol, 0.1% (w/v) bromphenol blue). Samples were boiled prior to loading 10 μl per lane on Tricine SDS-PAGE gels.

In this assay, whole cell lysates are used as a source of enzyme. So that no O antigen derived from the cells themselves could interfere with analysis of the substrate after incubation, we used a *B. bronchisepica* strain, RBB1a, as the *wbmE*$^+$ cells. RBB1a is a *B. bronchiseptica* RB50-derived mutant in the putative glycosyl transferase *wbmB*, which does not produce O antigen.[4] To maximize the amount of WbmE in the lysates, the vector pCompETag was also maintained in these RBB1a cells so that the enzyme is probably expressed from the plasmid as well as from the chromosomal copy of the *wbmE* gene. For the negative control, whole cell lysates were obtained from the *wbmE* mutant strain, RBE3c, containing the empty vector pCompEmpty.

For CE-MS characterization of the transformed O antigen, incubations were performed in the same proportions, but at larger scale and for 16 h, with 15 mg of RBE3c LPS in each sample and using neat *wbmE*$^+$ or *wbmE*$^-$ cell suspensions.

Purification of WbmE-His$_6$—WbmE-His_6 was expressed in *B. bronchisepica* from the plasmid pCompETag as before. Cells were pelleted (10 min at 10,000 × *g*) from 1 liter of culture and frozen (−20 °C). The pellet was thawed and suspended in 20 ml of 50 mM Tris, pH 8.5, 300 mM NaCl. Cells were broken with ultrasound (40% power, macro-tip, 3 min, in 5-s bursts, on ice),

[4] J. D. King, A. Preston, and D. J. Maskell, unpublished results.

and cell debris was pelleted (20 min at 20,000 × *g*). Membranes were removed by ultracentrifugation (1 h at 100,000 × *g*), and the supernatant was incubated with 3 ml of nickel-nitrilotriacetic acid (Ni-NTA) slurry (Qiagen) for 1 h at 4 °C with gentle agitation. The nickel affinity resin was loaded into a column, washed with 25 ml of 50 mM Tris, pH 8.5, 300 mM NaCl, 50 mM imidazole, and then WbmE-His_6 was eluted with small fractions of 50 mM Tris, pH 8.5, 300 mM NaCl, 200 mM imidazole. 1.6 ml of eluent containing most of the eluted protein was diluted one-sixth with water and loaded onto a 5-ml Econo-Pac High-Q anion exchange column (Bio-Rad). The column was washed with 50 ml of 20 mM Tris, pH 8.5, 50 mM NaCl. The column was eluted with a linear gradient of 50–1000 mM NaCl in 20 mM Tris, pH 8.5 over 50 ml. WbmE-His_6 eluted into five 1-ml fractions at ~200 mM NaCl. The activity of each fraction was tested by incubating overnight (37 °C) with an equal volume of 0.4 mg ml^{-1} RBE3c LPS dissolved in 50 mM Tris, pH 8.5, 300 mM NaCl. After incubation, the LPS was processed for analysis by SDS-PAGE as described above. WbmE-His_6-containing fractions were then pooled, glycerol was added to 25% (v/v), and the protein was stored at −20 °C.

RESULTS

Analysis of the wbmE LPS Phenotype by SDS-PAGE—To characterize the role of *wbmE* in O antigen expression it was mutated by insertion of a tetracycline resistance gene cassette into the coding sequence. Disruption of *wbmE* does not alter the A- or B-band LPS but does change the appearance of O band LPS on SDS-PAGE (Fig. 3). The mutant O band has reduced electrophoretic mobility and has a more clearly resolved banding pattern. Individual wild-type O antigen-containing species are not so clearly resolved, the O band appearing as a smear on the gel. Complementation of the *wbmE* mutation by expression of the wild-type allele from a plasmid restores the wild-type electrophoretic mobility of O band (Fig. 3). The mutation can also be complemented by a vector in which codons for a His_6 tag are fused to the 3′-end of the *wbmE* gene (Fig. 3).

wbmE Mutant LPS Differs from Wild-type by Having a Greater Number of Uronamides in the O Antigen—Altered electrophoretic mobility in a polymeric molecule such as O antigen often indicates that the chain length has altered (37), but can also reflect a change in the electrostatic charge on O antigen sugars (38). To determine the cause of the SDS-PAGE band shift in this case, we purified LPS from the wild-type and *wbmE* mutant and analyzed their O chains by mass spectrometry. Prior to analysis, O antigen was cleaved from the rest of the LPS molecule by solvolysis with anhydrous hydrogen fluoride, which cleaves the polysaccharide chain at the GalNAc position in the O antigen linker region (Fig. 1) (9). Electrospray mass spectra of wild-type and *wbmE* O polysaccharides both show a series of peaks separated by ~258 atomic mass units (a.m.u.) (Fig. 4). Because 258 a.m.u. is approximately the mass of an O antigen GalNAc3NAcA(N) repeating unit, these series represent the variation in O chain lengths expressed by the bacteria. There was no difference in the gross distribution of chain lengths between the two samples demonstrating that the reduction in electrophoretic mobility of the mutant O antigen-containing LPS is not due to increased O chain length. Furthermore, each of the peaks in the spectrum derived from the *wbmE* mutant sample is shifted downwards by 4–6 a.m.u compared with corresponding peaks in the spectrum of wild-type O chain. MS and NMR analysis of wild-type O antigen has established that the O repeating units are present as both uronic acids (GalNAc3NAcA) and uronamides (GalNAc3NAcAN) (9). We hypothesized, therefore, that the difference in mass between the *wbmE* mutant and wild-type O polysaccharide species was due to the *wbmE* mutant producing a greater proportion of uronamide residues (between four and six additional uronamides per LPS molecule). An implication of this hypothesis is that the mutant will have 4–6 fewer negative charges per O antigen molecule, and this is consistent with slower migration toward the anode in SDS-PAGE.

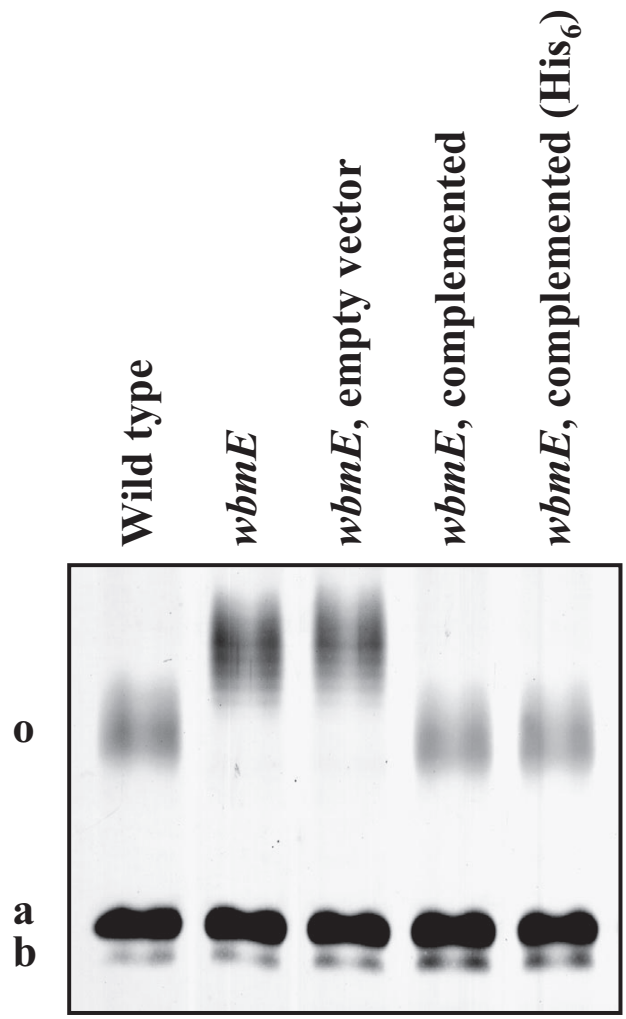

FIGURE 3. **Silver-stained SDS-PAGE analysis of LPS from *B. bronchiseptica* strains.** Analysis of LPS from wild-type *B. bronchiseptica* (RB50), a *wbmE* mutant (RBE3c), and the *wbmE* mutant harboring complementation plasmids. The positions of the O antigen-containing O band (*o*), A-band (*a*), and B-band (*b*) LPS in the wild-type are indicated. Mutation of *wbmE* reduces the electrophoretic mobility, and results in a clearer banded (*i.e.* striped) appearance, of O band LPS. The wild-type LPS profile is restored in the *wbmE* mutant by expression of wild-type or C-terminally His_6-tagged WbmE *in trans* (from plasmids pCompE and pCompETag, respectively).

The molecular weights measured for particular peaks in the *wbmE* O antigen mass spectrum suggested that while this mutant expresses a more uronamide-rich O polysaccharide

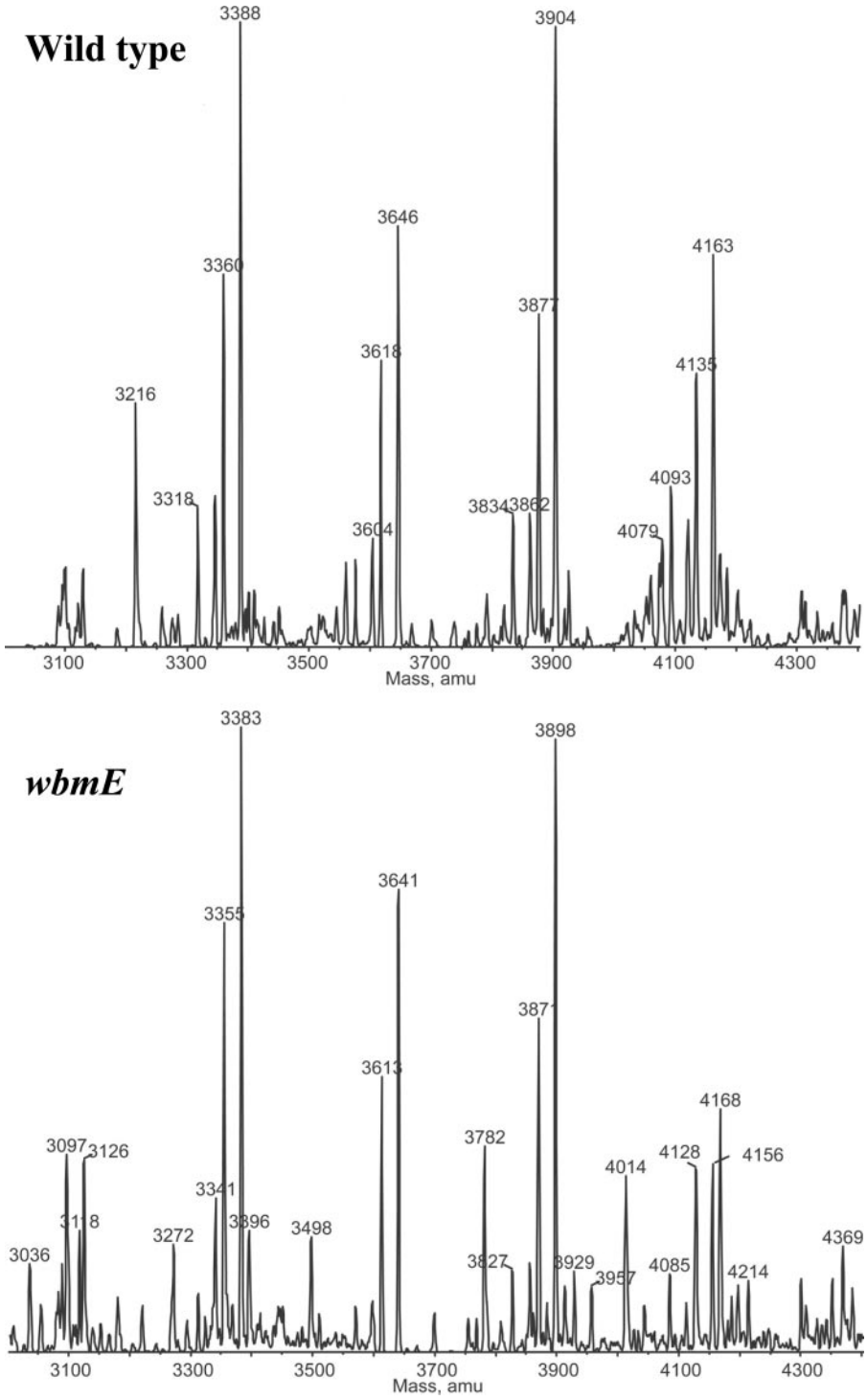

FIGURE 4. **Negative ion electrospray MS analysis of HF-cleaved O polysaccharides from wild-type *B. bronchiseptica* and the *wbmE* mutant.** After HF solvolysis, the O polysaccharides were purified by C18 reversed phase chromatography and then analyzed by electrospray MS. Each sample produced a series of peaks separated by 258/259 intervals, representing O polysaccharides of different chain lengths differing by the mass of a single GalNAc3NAcA(N) residue. While the mass distributions are similar in the two samples, the *wbmE* peaks are shifted downwards by 4–6 a.m.u. relative to corresponding peaks in the analysis of wild-type O polysaccharide.

than its parental strain, the *wbmE* O antigen still contains a mixture of uronic acid and uronamide residues. For example the peak at 3383 corresponds with the predicted molecular weight of an HF-cleaved polysaccharide containing five GalNAc3NAcAN residues, four GalNAc3NAcA residues, the capping sugar, and the ManNAc3NAcAN-GalNAc3NAcAN-GalNAc portion of the linker (calculated MW = 3383.1).

To confirm our interpretation of the electrospray MS data, HF-cleaved O polysaccharide was subjected to fragmentation in a capillary electrophoresis mass spectrometry (CE-MS) experiment. The pseudo-tandem mass spectra of wild-type and *wbmE* O polysaccharides show peaks corresponding to mono-, di-, tri-, and tetrasaccharide fragments derived from the polymers (Fig. 5, *A* and *B*). Interpretation of these spectra is complicated by the fact that true M+1 peaks due to additional uronic acids overlap with isotopic peaks, but comparison of the two spectra indicates that *wbmE* O antigen fractures to give a greater proportion of all-uronamide fragments, but the distribution of masses still indicates the presence of uronic acid residues in the mutant O chain.

WbmE Is Localized in the Periplasm—Analysis of the structure of the LPS synthesized by the *wbmE* mutant suggested that WbmE plays a role in converting uronamide residues to uronic acids. Conceivably this could occur either by WbmE acting on completed O chain, or WbmE could operate at an earlier stage, and catalyze the deamidation of a sugar-nucleotide O antigen precursor. The stage at which LPS biosynthetic enzymes operate is dictated by their cellular localization: sugar-nucleotides are soluble, cytoplasmic metabolites, and according to the ABC transporter-dependent model of O antigen biosynthesis, the O polysaccharide will be completed before it is transported across the inner membrane. Analysis of the WbmE sequence using the LipoP 1.0 signal peptide prediction server (39, 40) predicts a signal peptidase I cleavage signal (log-odds score >10) including a predicted transmembrane helix close to the N terminus (Ile-7 to Gln-26) with the peptide bond targeted for cleavage probably one of those between amino acids Gly-21 and Ala-32. WbmE is therefore highly likely to be secreted from the cytoplasm.

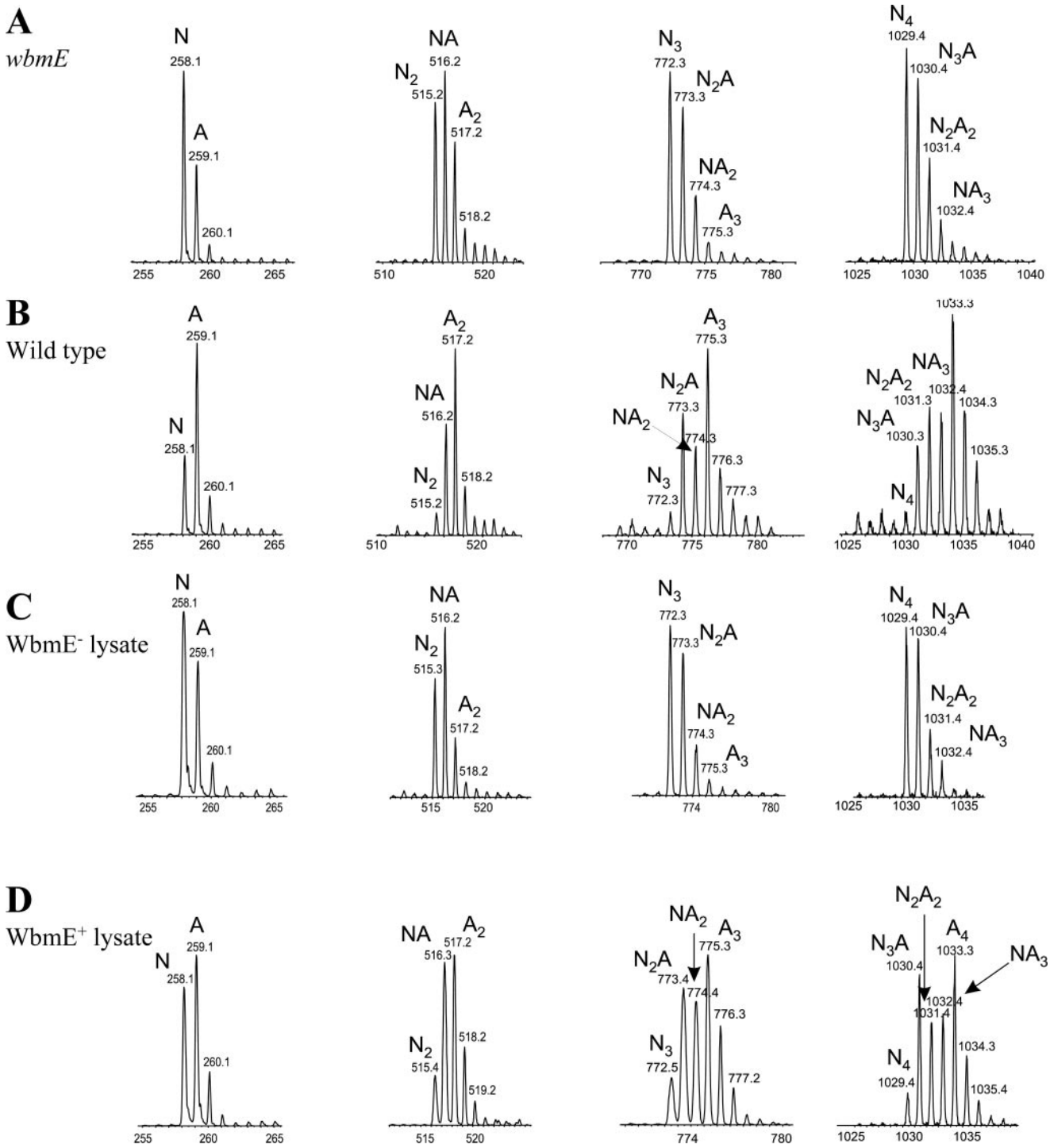

FIGURE 5. **Capillary electrophoresis-mass spectrometry (CE-MS) analysis of HF-cleaved O polysaccharides.** The pseudo-tandem mass spectra shown represent analyses of samples from the *B. bronchiseptica wbmE* mutant RBE3c (*A*) wild-type *B. bronchiseptica*, RB50 (*B*), RBE3c LPS incubated with lysed *B. bronchiseptica* cells that lack *wbmE* (*C*) and RBE3c LPS incubated with lysed *B. bronchiseptica* cells expressing the *wbmE* gene (*D*). Fragmentation of the oligosaccharide samples in this experiment produces mono-, di-, tri-, and tetrasaccharides of HexNAc3NAcA(N). Peaks are labeled according to the uronamide/uronic acid composition for which the exact mass matches that of the peak. *N*, uronamide, HexNAc3NAcAN; *A*, uronic acid, HexNAc3NAcA. Mutation of *wbmE* increases the relative proportions of amide-rich oligosaccharide fragments. Treatment of *wbmE* LPS with the extract of *wbmE*$^+$ cells restores the wild-type uronamide/uronic acid balance.

To verify the function of the predicted signal peptide, and localize WbmE, C-terminally His_6-tagged WbmE (WbmE-His_6) expression was induced in *B. bronchiseptica* from the vector previously used to test the ability of WbmE-His_6 to complement the *wbmE* mutation. The His_6 tag was used to capture Ni^{2+}-binding proteins from different cellular fractions, and to detect them in SDS-PAGE. WbmE-His_6 was not detected in culture supernatant, but was released from cells by sonication. The N-terminal sequence of mature WbmE-His_6 expressed in *B. bronchisepica* was determined to be ATPAATDATA, which

FIGURE 6. **The WbmE signal peptide targets alkaline phosphatase to the periplasm.** 5 μl of stationary phase liquid culture was spotted onto solid media containing the alkaline phosphatase substrate BCIP, and the plate was incubated for 16 h at 37 °C. The development of blue color is indicative of alkaline phosphatase-catalyzed cleavage of BCIP. Alkaline phosphatase is only active when exported to the periplasm. *1*, the *phoA E. coli* strain LMG194 harboring the plasmid-born *wbmE-'phoA* fusion. *2*, *E. coli* LMG194 harboring the parental vector pRMCD28 that contains *'phoA*, but lacks the *wbmE* 5′-end. The *'phoA* fragment in pRMCD28 lacks its native periplasmic signal sequence, therefore no color change is observed. *3*, the *phoA*$^+$ *E. coli* strain JM109, harboring pRMCD28.

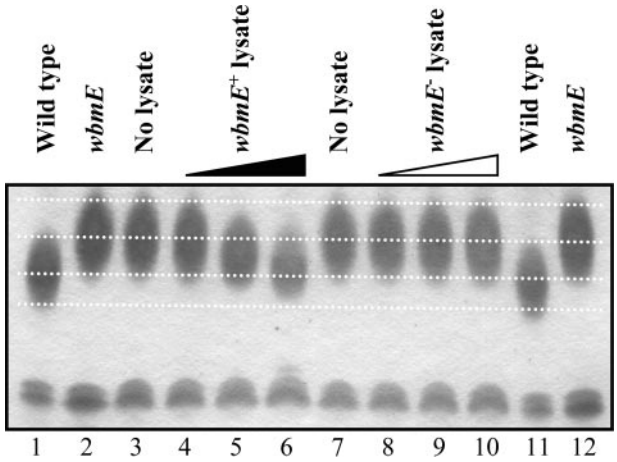

FIGURE 7. **WbmE assay with whole cell lysates used as a source of enzyme.** Purified LPS from the *wbmE* mutant, RBE3c, was mixed with a neat suspension of cells or a dilution thereof, prior to sonication and incubation at 37 °C. A *wbmE*-dependent transformation of the LPS substrate results in increased electrophoretic mobility of the O band LPS. *Lanes 1* and *11* contain untreated, wild-type *B. bronchisepica* LPS. *Lanes 2* and *12* contain untreated *wbmE* LPS. *Lanes 3* and *7* contain *wbmE* LPS that was incubated with buffer. The filled wedge (*lanes 4–6*) indicates that *wbmE* LPS was incubated with increasing concentrations of *wbmE*$^+$ cell lysates. The open wedge (*lanes 8–10*) indicates that *wbmE* LPS was incubated with increasing concentrations of *wbmE*$^-$ cell lysates.

corresponds to *wbmE* codons 31–40. This is consistent with secretion of the protein and subsequent cleavage of the signal peptide by signal peptidase I as predicted *in silico*. This cleavage of the N terminus, including the predicted transmembrane helix is also indicated by the fact that protein remained in the supernatant after ultracentrifugation at 100,000 × *g* demonstrating that it is not associated with membranes. Finally, we tested the function of the WbmE signal peptide in *E. coli* by cloning the N-terminal 66 codons of *wbmE* into the vector pRMCD28 (25) to create a reporter fusion of the 5′-end of *wbmE* with the plasmid-encoded alkaline phosphatase gene fragment *'phoA*. Alkaline phosphatase is inactive unless exported to the periplasm (41). Growth of *E. coli* cells harboring this reporter plasmid on solid media containing the alkaline phosphatase substrate, 5-bromo-4-chloro-3-indolyl-phosphate (BCIP), resulted in blue colonies (Fig. 6), indicating that the WbmE signal peptide functions to target the alkaline phosphatase enzyme for secretion across the inner membrane in *E. coli*. *phoA* fusions have been shown to exhibit the same alkaline phosphatase phenotypes in *Bordetella* as in *E. coli* (42).

WbmE Catalyzes the Deamidation of LPS O antigen in Vitro—If the *B. bronchiseptica* O antigen is biosynthesized according to the ABC transporter-dependent model (15), the full O chain will be assembled before it is exported from the cytoplasm (37). Therefore a periplasmic localization for WbmE implies that this enzyme catalyzes a chemical transformation of the O antigen after polymerization. We postulated, therefore, that the complete, smooth-type LPS molecule may be a substrate for WbmE. To test this hypothesis, we mixed LPS purified from the *wbmE* mutant with whole cell lysates of *B. bronchiseptica* cultures either expressing, or lacking *wbmE*.

Lysates derived from the *wbmE*$^+$ cells were able to transform the substrate LPS and increased the electrophoretic mobility of the O band LPS (Fig. 7). The magnitude of this effect depended on both the amount of lysate added (Fig. 7) and on the incubation time (data not shown). At the maximum lysate concentration and longest incubation time, the product of the WbmE-catalyzed reaction mimics the electrophoretic mobility of wild-type *B. bronchiseptica* O band. In contrast with these results, lysates derived from *wbmE*$^-$ cells did not affect the migration of the substrate LPS.

To confirm that the alteration in electrophoretic mobility observed by SDS-PAGE analysis of the products of these incubations was due to deamidation of uronamide residues in the LPS O antigen, we performed larger scale incubations and analyzed the products using CE-MS analysis of their HF-cleaved O polysaccharides. O polysaccharide fragments from the incubation with *wbmE*$^+$ cells have a similar uronic acid/uronamide composition to wild-type, with a predominance of uronic acid residues (Fig. 5*D*). By contrast, the O polysaccharide fragments from an incubation with *wbmE*$^-$ cells are richer in uronamides (Fig. 5*C*), resembling the unincubated *wbmE* mutant LPS (Fig. 5*A*).

Purified WbmE-His$_6$ Deamidates Uronamide-rich LPS O Antigen—The genetic data and results from incubations with whole cell lysates indicated that *wbmE* is necessary to attain the wild-type balance of uronamides and acids in *B. bronchisepica* O antigen. To establish whether WbmE is sufficient to catalyze the deamidation of *B. bronchisepica* O antigen, we expressed the His$_6$-tagged protein in *B. bronchisepica* cells, and purified it in two steps. WbmE-His$_6$ eluted from the ion exchange column as the only band visible in SimplyBlue™ SafeStained SDS-PAGE analysis (Fig. 8*A*). Incubation of *wbmE* mutant LPS with the purified protein caused the same band shift observed in incubations with whole cell lysates (Fig. 8*B*). Addition of more protein resulted in greater changes in the electrophoretic mobility as well as progressive loss of the banded structure of the O band on the gel. The protein could be stored at −20 °C after the addition of 25% (v/v) glycerol and retained activity for 2 weeks (data not shown).

DISCUSSION

The alteration in LPS profile, which results from mutation of *wbmE* and the restoration of the wild-type phenotype on complementation of this mutation established that *wbmE* plays a role in O antigen biosynthesis in *B. bronchiseptica*. Detailed analysis of the *wbmE* LPS structure and comparison with the

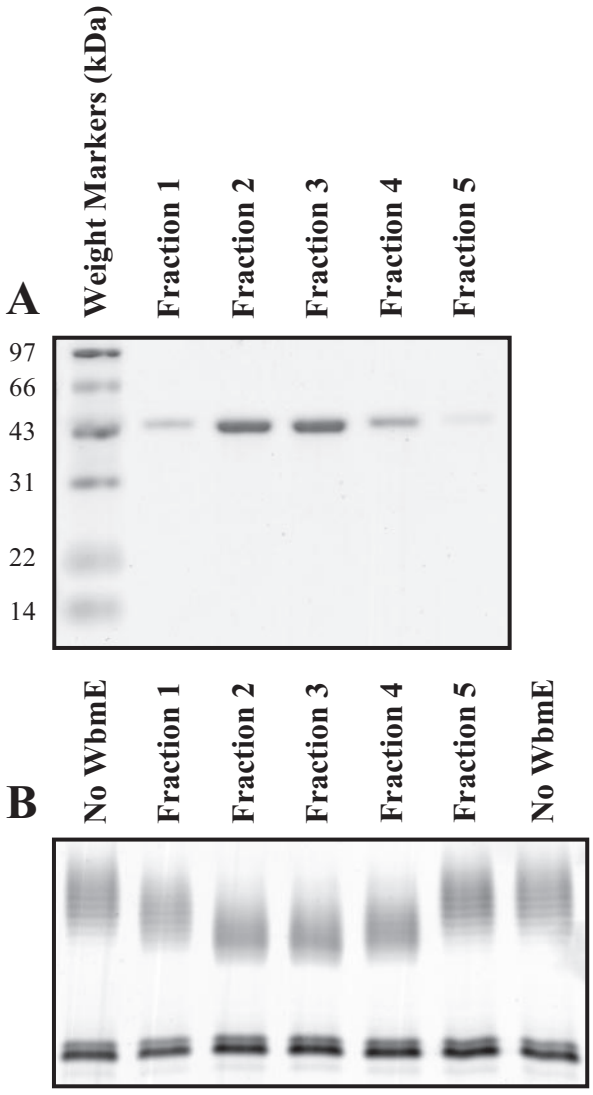

FIGURE 8. **SDS-PAGE and enzyme activity assay analyses of ion exchange chromatography fractions from the WbmE-His$_6$ purification.** WbmE eluted into five 1-ml fractions in the anion exchange purification of the protein. *A*, simplyBlue™ SafeStained SDS-PAGE analysis of these fractions. WbmE-His$_6$ is the only visible band. *B*, these fractions were incubated with purified LPS from the *wbmE* mutant and then analyzed by silver-stained SDS-PAGE. The greater the concentration of WbmE-His$_6$ in the ion exchange fractions, the greater the increase in electrophoretic mobility of the O band LPS. The incubation with WbmE-His$_6$ also progressively obliterates the banded pattern of untreated LPS; thus incubation with fractions 2 and 3 resulted in an O band smear like that of wild type.

wild-type O antigen indicates that *wbmE* reduces the extent of amidation of the O chain. The fact that the *wbmE* mutation can be completely complemented by a His$_6$-tagged gene fusion demonstrates that the introduction of this tag did not affect the function or localization of the enzyme.

We have shown that WbmE is a soluble, periplasmic deamidase which catalyzes the conversion of a proportion of L-GalNAc3NAcAN uronamides in the O polysaccharide to L-GalNAc3NAcA uronic acids. This is novel in two respects. First, while other postassembly modifications of LPS have been reported (for example the palmitoyl transfer to lipid A catalyzed by PagP (26)), this is probably the first example of a post-assembly modification of O antigen. Second, as far as we are aware, this is the first report of the deamidation of a polysaccharide substrate by a member of the transglutaminase protein family, though this proposed role is entirely consistent with the chemistry catalyzed by other transglutaminase enzymes, namely the formation or breaking of amide bonds.

Modification of O antigen structure by enzymes that operate in the periplasm has been previously reported: a series of inner membrane-spanning glucosyltransferases encoded by serotype-converting, temperate bacteriophages in *Shigella flexneri* (reviewed in Ref. 43) and *Salmonella* spp (44). It is not known exactly at what stage this O antigen glucosylation occurs, but it is thought to occur prior to, or during, O antigen polymerization rather than after (45). While it is not an O antigen, alginate is an extracellular polysaccharide, which is modified in the periplasm. It is produced by several species, including the Gram-negative bacterium *Pseudomonas aeruginosa* and is first synthesized as a β-1,4-linked mannuronic acid homopolymer. A proportion of mannuronic acid residues is then epimerized at C5 to guluronic acid, then the polymer is partially *O*-acetylated. The C5-epimerase (AlgG) (46), and two proteins required for the acetylation (AlgF and AlgJ) (47) localize to the periplasm.

The function we propose for WbmE also helps to explain another feature of the *wbmE* phenotype: that compared with the smear that is seen with the wild-type LPS, the *wbmE* O band LPS has a clear banded pattern on SDS-PAGE. The banded pattern we observed in the *wbmE* mutants may reflect a more regular distribution of acids and amides along the O chain. If so, individual O band LPS molecules will be very similar to each other except in the number of repeating units. Our structural analyses did not prove such a regular distribution, but such a structure would account for the banded LPS profile. In the parental strain the pattern of acids and amides is determined in part by a stochastic process: which of the residues are deamidated is presumably determined by the probability of an encounter with WbmE during the time it takes the O polysaccharide to pass through the periplasm. Thus the wild-type O band LPS molecules differ in the number of O antigen residues, the number that have a negative charge, and in the positions of those charges in the chain. The presence of so many different, but closely related O chain structures may be the reason why wild-type O band species are not individually resolved by SDS-PAGE. The mimicry of the wild-type O band smear, seen after incubation of *wbmE* LPS with the highest concentrations of purified WbmE-His$_6$ (Fig. 8*B*), supports the idea that this unresolved smear is due to the activity of WbmE.

O antigen polymers are initially assembled on an undecaprenyl-pyrophosphoryl carrier (48), and it is in the periplasmic space that the O polysaccharide is transferred from this lipid carrier to lipid A-core (49). We have demonstrated that complete O band LPS is a substrate for WbmE *in vitro*. These molecules could also constitute the *in vivo* substrate, or this could be identified as the undecaprenyl-linked O polysaccharide. It seems likely that both molecules may in fact be substrates, since a soluble periplasmic protein may have access to the O polysaccharide before and after ligation to core.

The observation that a small number of uronic acids is present in the O chain, even in the *wbmE* mutant, indicates that WbmE-catalyzed deamidation of the O polymer is not the sole pathway by which these uronic acids can be synthesized *in vivo*.

Within the *wbm* locus there are three candidate genes encoding the formation of the C-6 primary amide on the L-GalNAc3NAcAN uronamide repeating unit. These are *wbmC*, *wbmI*, and *wbmZ*, each encoding a glutamine-dependent amidotransferase family protein. We cannot rule out the possibility that the amidotransferase substrate is a completed O polysaccharide chain, but most sugar modifications are performed prior to glycosyltransferase-catalyzed incorporation into the nascent oligosaccharide chain and so it is more likely that the amidotransferase substrate is a sugar-nucleotide. If this is the case, then the presence of both L-GalNAc3NAcA and L-GalNAc3NAcAN in the *wbmE* mutant O antigen suggests that this amidotransfer is the last step in O antigen precursor biosynthesis. Only then will both activated sugar forms be available for incorporation into the chain. This information will be useful in designing experiments to characterize the sugar-nucleotide modification pathways which lead to O antigen expression in *B. bronchiseptica*.

B. parapertussis produces a structurally identical homopolymeric O antigen to that of *B. bronchisepica* (7). Microarray-based comparative genome hybridization analysis of 32 strains indicated (on the basis of a single probe) that *wbmE* is common to many, though not all *B. bronchiseptica* and *B. parapertussis* isolates. Other genes which are presumably more fundamental to the assembly of an O antigen (for example *wbmA*) are more conservatively retained within the genomes of diverse *Bordetella* strains (50). It is possible that the ability to express more negatively charged O antigen may confer some resistance to phagocytosis in a manner analogous to negatively charged capsule (reviewed in Ref. 51). Alternatively, the increased hydrophilicity of a more charged cell surface may help to prevent desiccation when the bacterium is outside of a host organism. *B. bronchiseptica* in particular has been suggested to have an environmental reservoir and either as an anti-desiccant or by some other mechanism, *wbmE* may be a part of its adaptation to an environmental niche.

RB50 is unusual among *B. bronchiseptica* isolates in expressing very low levels of O band LPS in the Bvg^+ phase (supplemental Fig. S1). For this reason, LPS analyzed in this report was all derived exclusively from Bvg^- phase organisms. Most *B. bronchiseptica* and *B. parapertussis* strains investigated by van den Akker (6) express enough O antigen in both phases to enable detection by silver-stained SDS-PAGE. In almost every such case the Bvg^+ O band had reduced electrophoretic mobility and in some strains also exhibited a banded pattern similar to that we have observed for our Bvg^- phase *wbmE* mutant samples. This observation suggests that there may be lower WbmE expression in the Bvg^+ phase, and this correlates with microarray data (22) that identify *wbmE* as part of the Bvg regulon, being maximally expressed in the Bvg^- phase. This phase has been associated with characteristics thought to be better adapted to the environmental *B. bronchiseptica* reservoir such as motility and lower nutrient requirements (52). Even if minimally expressed in the Bvg^+ phase, we cannot rule out the possibility that WbmE-catalyzed deamidation of O antigen participates in adapting *B. bronchiseptica* to the host. It has been suggested, for instance, that intermediate Bvg (Bvg^i) phases may be important for colonization and biofilm formation in the nasopharynx (53). Therefore the cell surface properties that result from Bvg^i levels of *wbmE* expression may play a role in the infective process.

In conclusion, we have shown that *wbmE* is required for *B. bronchiseptica* to exhibit the wild-type balance of uronamides and uronic acids in LPS O antigen and that WbmE is sufficient to catalyze the deamidation of O chains when they are part of a completed LPS molecule. We have also described a novel assay for this activity. This is the first report of a mechanism by which O antigen is modified after polymerization and may constitute a means by which *B. bronchiseptica* regulates the properties of its cell surface in response to environmental stimuli.

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