Post-assembly Modification of *Bordetella bronchiseptica* O Polysaccharide by a Novel Periplasmic Enzyme Encoded by *wbmE*<sup>§</sup>

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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 localization 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.

*B. 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<sup>−</sup> phase. Transcription of some other genes, for example the flagellin gene *flaA*, is up-regulated in Bvg<sup>+</sup> conditions (4) and an intermediate expression pattern (Bvg<sup>+</sup>) has also been described (5). In *vitro* the Bvg<sup>−</sup> 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).<sup>3</sup> 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. bronchiseptica* 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 galac-turonamide (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<sup>−</sup> *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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Capping sugar

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 (GlcNAc3NAcA; R = OH) residues, a proportion of which are present as the uronamide
(GalNAc3NAcAN; R = NH2). 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 α-forms.

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

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-amide, 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. bronchiseptica 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 µg ml⁻¹), kanamycin (50 µg ml⁻¹), tetracycline (10 µg ml⁻¹) for E. coli, 5 µg ml⁻¹ for B. bronchiseptica) or streptomycin (200 µg 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 MgSO₄ 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) (GenBankTM 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 Alul and ligated into Smal-cut PEx100T. Allelic exchange constructs were transferred to B. bronchiseptica by conjugation with E. coli SM10Apir 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′-GCTCTAGATGATCGGCGATGGCGCC-3′ (XbaI site underlined) and 5′-AAGGTATTGATTCGCAAGCTAC-3′ (Ndel site underlined) and cloned into pCR2.1-TOPO vector containing wbmE-his6 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-His6 expression from this vector was induced by supplementing the medium with 50 mM MgSO4.

WbmE Assay using Whole Cell Lysates—For analysis by SDS-PAGE: B. bronchiseptica 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 the enzyme is probably expressed from the plasmid as well as from the chromosomal copy of the wbmE gene fusion under the control of the promoter for the B. bronchiseptica flaA promoter. For complementation of the wbmE mutation by expressing WbmE with a C-terminal His6 tag, the pCR2.1-TOPO vector containing wbmE was used as PCR template with the primers 5′-AAAAACATATGGATATCAGGAGCTAC-3′ (Ndel site underlined) and 5′-AAAAACTGATCTCAGATCCG-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 Ndel and HindIII restriction sites and topoisomerase-cloned into pCR2.1-TOPO, and the insert was verified by sequencing. wbmE was then expressed using pET15b and topoisomerase-cloned into pCR2.1-TOPO, and the insert was verified by sequencing. wbmE was then excised and cloned into pCompEmpty using Ndel and HindIII restriction sites and topoisomerase-cloned into pCR2.1-TOPO, and the insert was verified by sequencing. wbmE was then excised and cloned into pCompEmpty using Ndel and HindIII restriction sites and topoisomerase-cloned into pCR2.1-TOPO, and the insert was verified by sequencing. wbmE was then excised and cloned into pCompEmpty using Ndel and HindIII restriction sites and topoisomerase-cloned into pCR2.1-TOPO, and the insert was verified by sequencing. wbmE was then excised and cloned into pCompEmpty using Ndel and HindIII restriction sites and topoisomerase-cloned into pCR2.1-TOPO, and the insert was verified by sequencing. wbmE was then excised and cloned into pCompEmpty using Ndel and HindIII restriction sites and topoisomerase-cloned into pCR2.1-TOPO, and the insert was verified by sequencing. wbmE was then excised and cloned into pCompEmpty using Ndel and HindIII restriction sites and topoisomerase-cloned into pCR2.1-TOPO, and the insert was verified by sequencing. wbmE was then excised and cloned into pCompEmpty using Ndel and HindIII restriction sites and topoisomerase-cloned into pCR2.1-TOPO, and the insert was verified by sequencing. wbmE was then excised and cloned into pCompEmpty using Ndel and HindIII restriction sites and topoisomerase-cloned into pCR2.1-TOPO, and the insert was verified by sequencing. wbmE was then excised and cloned into pCompEmpty using Ndel and HindIII restriction sites and topoisomerase-cloned into pCR2.1-TOPO, and the insert was verified by sequencing.
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-His6 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-His6 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-His6-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 His6 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 (GalNAc3NaeA) 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.

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...
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.
To verify the function of the predicted signal peptide, and localize WbmE, C-terminally His\textsubscript{6}-tagged WbmE (WbmE-His\textsubscript{6}) expression was induced in \textit{B. bronchiseptica} from the vector previously used to test the ability of WbmE-His\textsubscript{6} to complement the \textit{wbmE} mutation. The His\textsubscript{6} tag was used to capture Ni\textsuperscript{2+}-binding proteins from different cellular fractions, and to detect them in SDS-PAGE. WbmE-His\textsubscript{6} was not detected in culture supernatant, but was released from cells by sonication. The N-terminal sequence of mature WbmE-His\textsubscript{6} expressed in \textit{B. bronchiseptica} was determined to be ATPAATDATA, which

![Capillary electrophoresis-mass spectrometry (CE-MS) analysis of HF-cleaved O polysaccharides.](image)

**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 \textit{B. bronchiseptica} \textit{wbmE} mutant RBE3c (A) wild-type \textit{B. bronchiseptica}, RB50 (B), RBE3c LPS incubated with lysed \textit{B. bronchiseptica} cells that lack \textit{wbmE} (C) and RBE3c LPS incubated with lysed \textit{B. bronchiseptica} cells expressing the \textit{wbmE} gene (D). Fragmentation of the oligosaccharide samples in this experiment produces mono-, di-, tri-, and tetrasaccharides of HexNAc\textsubscript{3}NacAl(N). Peaks are labeled according to the uronamide/uronic acid composition for which the exact mass matches that of the peak. N, uronamide, HexNAc\textsubscript{3}NacAN; A, uronic acid, HexNAc\textsubscript{3}NacA. Mutation of \textit{wbmE} increases the relative proportions of amide-rich oligosaccharide fragments. Treatment of \textit{wbmE} LPS with the extract of \textit{wbmE}\textsuperscript{+} cells restores the wild-type uronamide/uronic acid balance.
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 the 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 deamination 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. 5D). By contrast, the O polysaccharide fragments from an incubation with wbme− cells are richer in uronamides (Fig. 5C), resembling the unincubated wbme mutant LPS (Fig. 5A).

Purified WbmE-His6 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. bronchiseptica O antigen. To establish whether WbmE is sufficient to catalyze the deamidation of B. bronchiseptica O antigen, we expressed the His6-tagged protein in B. bronchiseptica cells, and purified it in two steps. WbmE-His6 eluted from the ion exchange column as the only band visible in SimplyBlue™ SafeStained SDS-PAGE analysis (Fig. 8A). Incubation of wbme mutant LPS with the purified protein caused the same band shift observed in incubations with whole cell lysates (Fig. 8B). 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
be completely complemented by a His6-tagged gene fusion amidation of the O chain. The fact that the protein.

eluted into five 1-ml fractions in the anion exchange purification of the pro-

FIGURE 8.
The incubation with WbmE-His6 also progressively obliterates the banded tions, the greater the increase in electrophoretic mobility of the O band LPS.

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 His6-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 deami-
dase which catalyzes the conversion of a proportion of 1-GalNAc3NACAN uronamides in the O polysaccharide to 1-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 glycosyltransferases encoded by sero-type-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 glycosylation 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, alginic 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 AlgI) (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 deami-
dated 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-His6 (Fig. 8B), supports the idea that this unres-
solved smear is due to the activity of WbmE.

O antigen polymers are initially assembled on an undecapre-
nyl-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 pres-
ent 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.
WbmE Deamidates B. bronchiseptica O Antigen

Within the wbm locus there are three candidate genes encoding the formation of the C-6 primary amide on the \(1\)-GalNac3NacA uronamide repeating unit. These are \(wbnC\), \(wbnl\), and \(wbnz\), 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 \(1\)-GalNac3NacA and \(1\)-GalNac3NacA3A in the \(wbmE\) mutant O antigen suggests that this amidotransfer is the last step in O antigen 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 \(B.\ bronchiseptica\) (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 \(wbnA\)) are more conservatively retained within the genomes of diverse \(Borrelia\) 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 hydrophobicity 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 Bvg\(^-\) 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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