The Protein BpsB Is a Poly-β-1,6-N-acetyl-D-glucosamine Deacetylase Required for Biofilm Formation in Bordetella bronchiseptica*

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Background: The Bordetella polysaccharide (Bps) is involved in Bordetella biofilm formation.

Results: BpsB is a periplasmic metal-dependent poly-β-1,6-N-acetyl-D-glucosamine (PNAG) deacetylase that has unique structural and functional features from known PNAG deacytases.

Conclusion: BpsB-dependent deacetylation of Bps is required for Bordetella bronchiseptica biofilm formation.

Significance: Deacetylated Bps is a key component for the structural complexity of Bordetella biofilms.

Bordetella pertussis and Bordetella bronchiseptica are the causative agents of whooping cough in humans and a variety of respiratory diseases in animals, respectively. Bordetella species produce an exopolysaccharide, known as the Bordetella polysaccharide (Bps), which is encoded by the bpsABCD operon. Bps is required for Bordetella biofilm formation, colonization of the respiratory tract, and confers protection from complement-mediated killing. In this report, we have investigated the role of BpsB in the biosynthesis of Bps and biofilm formation by B. bronchiseptica. BpsB is a two-domain protein that localizes to the periplasm and outer membrane. BpsB displays metal- and length-dependent deacetylation on poly-β-1,6-N-acetyl-D-glucosamine (PNAG) oligomers, supporting previous immunogenic data that suggests Bps is a PNAG polymer. BpsB can use a variety of divalent metal cations for deacetylase activity and showed highest activity in the presence of Ni²⁺ and Co²⁺. The structure of the BpsB deacetylase domain is similar to the PNAG deacytases PgaB and IcaB and contains the same circularly permuted family four carbohydrate esterase motifs. Unlike PgaB from Escherichia coli, BpsB is not required for polymer export and has unique structural differences that allow the N-terminal deacetylase domain to be active when purified in isolation from the C-terminal domain. Our enzymatic characterizations highlight the importance of conserved active site residues in PNAG deacetylation and demonstrate that the C-terminal domain is required for maximal deacetylation of longer PNAG oligomers. Furthermore, we show that BpsB is critical for the formation and complex architecture of B. bronchiseptica biofilms.

Bacteria belonging to the genus Bordetella are Gram-negative coccobacilli that cause respiratory symptoms and illnesses in humans, animals, and birds (1, 2). Currently, nine species belong to this genus, three of which have been studied in detail as follows: Bordetella pertussis, Bordetella parapertussis, and Bordetella bronchiseptica. B. pertussis and B. parapertussis are the causative agents of whooping cough in humans. In contrast, B. bronchiseptica has a broad host range, colonizing and causing disease in a wide variety of animals. B. bronchiseptica can also infect immunocompromised and healthy humans thereby demonstrating zoonotic transmission (2, 3).

Despite high levels of vaccination coverage, the incidence of pertussis is increasing steadily in the United States and other developed countries, leading the Centers for Disease Control to classify pertussis as a re-emerging disease (4–6). Similarly, although multiple vaccines are used with variable success for B. bronchiseptica, animals continue to be carriers and frequently shed bacteria resulting in outbreaks among herds (2). This has generated renewed interest in identifying and understanding the role of new virulence determinants, and in uncovering mechanisms employed by these bacteria for host survival and persistence.

It has been proposed that the colonization and persistence of Bordetella are enhanced by the formation of biofilms in the respiratory tract. Several studies have shown that Bordetella spp. form biofilms on artificial surfaces (3, 7–12) and that both B. pertussis and B. bronchiseptica form biofilms in the mouse...
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Experimental Procedures

Bacterial strains, plasmids, and oligonucleotide primers used in this study are described in Table 1. The purified BpsB constructs used for crystallization and enzymatic assays carry the C48S mutation to prevent cross-linking and to avoid the use of reducing agents.

Cloning, Expression, and Purification of BpsB Constructs—The mature form of BpsB encoding residues 27–701 was cloned into the pET28a expression vector using B. bronchiseptica RB50 genomic DNA and PCR with primers 27 Fwd and 701 Rev that contain an Ndel and HindIII site, respectively. The resulting plasmid pET28-BpsB(27–701) encodes a thrombin-cleavable N-terminal hexahistidine tag fused to BpsB lacking its predicted signal sequence. The N-terminal domain of BpsB encoding either residues 27–311 or 35–311 was cloned into the pET28a expression vector in the same manner as BpsB(27–701) using pET28-BpsB(27–701)-2 as template and primers 27 Fwd or 35 Fwd and 311 Rev that contain an Ndel and HindIII site, respectively. The following variants were all cloned using the QuikChange Lightning site-directed mutagenesis kit. Plasmid pET28-BpsB(27–701)-1 was generated using pET28-BpsB(27–701) as template and primers C48S Fwd and C48S Rev. Plasmids pET28-BpsB(27–701)-2 H49A, pET28-BpsB(27–701)-2 D113A, pET28-BpsB(27–701)-2 D113N, pET28-BpsB(27–701)-2 D114A, pET28-BpsB(27–701)-2 H184A, and pET28-BpsB(27–701)-2 H189A were generated using pET28-BpsB(27–701)-2 as a template and primers H49A Fwd and H49A Rev, D113A Fwd and D113A Rev, D113N Fwd and D113N Rev, D114A Fwd and D114A Rev, H184A Fwd and H184A Rev, and H189A Fwd and H189A Rev, respectively. Plasmid pET28-BpsB(35–307) was generated by replacing Glu-308 with a stop codon using pET28-BpsB(35–311) as a template and primers 308Stop Fwd and 308Stop Rev. Plasmid pET28-BpsB(35–307) SER was generated using pET28-BpsB(35–307) as a template and primers SER Fwd and SER Rev. Plasmid pET28-BpsB(35–307) MMS was generated using pET28-BpsB(35–307) SER as a template and primers MMS Fwd and MMS Rev.

The following protocol was used to express and purify all the BpsB constructs. E. coli BL21-CodonPlus cells transformed with the appropriate plasmid were grown in Luria-Bertani (LB) broth plus 50 μg/ml kanamycin at 37 °C with shaking at 200 rpm to an A600 of ~0.4–0.5 and moved to 18 °C. After 20 min, the cultures reached an A600 of ~0.6–0.7, and protein expression was induced by the addition of isopropyl β-D-thiogalactoside to a final concentration of 1 mM. The cells were incubated overnight at 18 °C, harvested by centrifugation at 5000 × g for 20 min, and frozen on dry ice. Frozen cell pellets with several distinct structural differences. BpsB displays length-dependent PNAG deacetylation activity with the highest rates measured when Ni2+ is present. The N-terminal domain of BpsB shows ~46% of the deacetylation activity observed in the full-length protein, and mutational analysis identifies key catalytic residues. Studies in vivo show that a ΔbpsB mutant strain is still able to export Bps to the cell surface but displays a biofilm-defective phenotype and significantly altered biofilm architecture.
were thawed and resuspended in 40 ml of lysis buffer (50 mM HEPES, pH 8.0, 0.5 M NaCl, 10 mM imidazole, 5% (v/v) glycerol, and one complete mini-protease inhibitor mixture tablet (Roche Applied Science)). Resuspended cells were disrupted with three passes through an Emulsiflex-c3 (Avestin) at 15,000 p.s.i., and cell debris was removed by centrifugation at 31,000 g for 30 min. The resulting supernatant was passed over a gravity column containing 4 ml of Ni-NTA resin (Qiagen) that was pre-equilibrated with buffer A (20 mM HEPES, pH 8.0, 0.3 M NaCl, 10 mM imidazole). Bound protein was washed with 10 column volumes of buffer A, 3 column volumes of buffer A with 20 mM imidazole, and eluted with 5 column volumes of buffer A with 250 mM imidazole. For BpsB constructs used in crystallization trials, the hexahistidine tag was removed. The Ni-NTA elution fraction was dialyzed against 1 liter of buffer A without imidazole for 16 h at 4 °C. The dialyzed protein was incubated at 20 °C for 1–2 h with 1 unit of thrombin (Novagen) per 5 mg of protein. Untagged protein was separated from tagged protein by size exclusion chromatography with Superdex 200 prep grade resin (GE Healthcare). The protein was concentrated to 10–20 mg/ml by ultrafiltration with Amicon centrifugal filters (Millipore) and used for crystallization trials.

### TABLE 1

| Strain, plasmid, or primer | Description or characteristics | Source or reference |
|----------------------------|--------------------------------|--------------------|
| **Strains** | | |
| RB50 | Wild-type (WT) strain of *B. bronchiseptica* | 64 |
| ΔbpsB | RB50 derivative containing an in-frame deletion of the *bpsB* gene | This study |
| ΔbpsABCD | RB50 derivative containing an in-frame deletion of the *bpsABCD* locus | 8 |
| DH5α | *E. coli* laboratory strain | Stratagene |
| BL21 CodonPlus | *E. coli* laboratory expression strain; F' ompT hsdS (r− m−) dcm− Tet+ galλ (DE3) endA [argU proL Cam^R] | Stratagene |
| SM10Apir | *E. coli* strain for pIR dependent plasmids | 65 |
| **Plasmids** | | |
| pET28a | Expression vector | Novagen |
| pET28-BpsB(27–701) | BpsB (27–701) expression plasmid | This study |
| pET28-BpsB(27–701)-2 | C85 expression plasmid | This study |
| pET28-BpsB(27–311) | C85 expression plasmid | This study |
| pET28-BpsB(35–311) | C85 expression plasmid | This study |
| pET28-BpsB(35–307) | C85 expression plasmid | This study |
| pET28-BpsB(35–307)SER | C85 expression plasmid | This study |
| pET28-BpsB(35–307)Δbpsb | C85 K128A/K129A expression plasmid | This study |
| pET28-BpsB(27–701)-2 H49A | C85 H49A expression plasmid | This study |
| pET28-BpsB(27–701)-2 D113A | C85 D113A expression plasmid | This study |
| pET28-BpsB(27–701)-2 D113N | C85 D113N expression plasmid | This study |
| pET28-BpsB(27–701)-2 H184A | C85 H184A expression plasmid | This study |
| pET28-BpsB(27–701)-2 H189A | C85 H189A expression plasmid | This study |
| pSM8 | *bpsB* complementation plasmid | This study |
| pSM11 | *bpsA* deletion plasmid | This study |
| pSM27 | BpsB FLAG-tag expression plasmid | This study |
| pRE112 | Allelic exchange vector | 66 |
| pTac-GFP | Constitutive GFP expression vector: pBBR1MCS with the tac promoter cloned upstream of the gfp cassette | 8 |
| pBBR1MCS | Broad host range plasmid | 67 |
| **Primers** | | |
| 2′Fwd | GGGCATATGTACAAGTGGAATTCGTCCTCC | This study |
| 35Fwd | GGGCAATTCCTATGCTAGCCGGATCCGGCTT | This study |
| 311 Rev | GGAAAGTCTACAGCTTATGCTAGCCGGATCCGGCTT | This study |
| 701 Rev | GGGGAACCTTACATCTCGATGCCCCTGCGGCT | This study |
| 308Stop Rev | CTCAGCTGACGGCGCTAGCGCATGGCGGGG | This study |
| C85 Fwd | GACATCTCCGAGGCTGTCATTACGACAGCTGGCGGCG | This study |
| C85 Rev | CGGCGACCTGCCATGCGCTAGCGCATGGCGGGG | This study |
| SER Fwd | GTTCCCCCTTCGGCGCGCATACATCGCGCC | This study |
| SER Rev | CGGCGATATGTTGCGGGATCCGCGATGCGCG | This study |
| MMS Fwd | GACATCTCCGAGGCTGTCATTACGACAGCTGGCGGCG | This study |
| MMS Rev | GCCGCGATATGTTGCGGGATCCGCGATGCGCG | This study |
| H49A Fwd | GTGCGTCTCAAGTCGACAGTGGCGGAC | This study |
| H49A Rev | GTGGCGACAGTGCAGGCTAGGACAC | This study |
| D113A Rev | GCCGCGACAGTGCAGGCTAGGACAC | This study |
| D113N Fwd | CCCATCCTGCTGACCTTACAGCGCCGCT | This study |
| D114A Rev | CGTACCTCGACGCCGCGTCACTCGAC | This study |
| D114A Fwd | GCTGCGTACGCGCGCGGCAGCGCGG | This study |
| H184A Fwd | GGACTTTGCGCCGAGGGCGGCGGCGG | This study |
| H184A Rev | CGCCCAACATGGCCGCGGCGGCGG | This study |
| H189A Fwd | CGCCCAACATGGCCGCGGCGGCGG | This study |
| H189A Rev | CGCCCAACATGGCCGCGGCGGCGG | This study |
| DISP | CAGGAAGTCTACAGCTTATGCTAGCCGGATCCGGCTT | This study |
| SM27 | CGCGCGACAGTGCAGGCTAGGACAC | This study |
| SM28 | CGCCGAACAGTGCAGGCTAGGACAC | This study |
| SM29 | CGCCGAACAGTGCAGGCTAGGACAC | This study |
| SM30 | CGCCGAACAGTGCAGGCTAGGACAC | This study |
| SM31 | CGCCGAACAGTGCAGGCTAGGACAC | This study |
| SM32 | CGCCGAACAGTGCAGGCTAGGACAC | This study |
| SM33 | CGCCGAACAGTGCAGGCTAGGACAC | This study |
| SM34 | CGCCGAACAGTGCAGGCTAGGACAC | This study |
| SM35 | CGCCGAACAGTGCAGGCTAGGACAC | This study |
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by a second round of Ni-NTA purification, where the flow-through and 3 column volume washes using 20, 40, and 60 mM imidazole were collected and analyzed by SDS-PAGE, and the appropriate fractions were pooled. The His-tagged elution fraction or pooled untagged protein fractions were concentrated using an ultrafiltration device and further purified and buffer exchanged into buffer B (20 mM HEPES, pH 8.0, and 0.15 M NaCl) by size exclusion chromatography using a HiLoad 16/60 Superdex 200 prep-grade gel filtration column (GE Healthcare). The purified BpsB constructs were >95% pure as judged by SDS-PAGE and stable for ~2 weeks at 4 °C.

Crystallization, Data Collection, and Structure Solution—

Purified BpsB(35–307) C48S/K128A/K129A/D235H/E239H (abbreviated BpsB(35–307)MMMS for metal-mediated symmetrization) was concentrated to ~9–10 mg/ml and incubated with 2 M eq of either NiCl2, CuCl2, or CdCl2. The protein solutions were then screened for crystallization conditions using the MCSG 1–4 sparse matrix suites (Microlytic) at 20 °C using sitting-drop vapor diffusion in 96-well 3-drop Intelli-Plates (Art Robbins Instruments) with a 1-µl drop of an equal volume of reservoir and protein. After a week of incubation, needle crystals were obtained in condition 72 from the MCSG-1 suite (30% (w/v) polyethylene glycol 2000 monomethyl ether and 0.1 M potassium thiocyanate) with BpsB(35–307)MMMS incubated with NiCl2. The crystals were directly harvested from the initial screen and cryoprotected for 5 s in reservoir solution supplemented with 20% (v/v) ethylene glycol prior to vitrification in liquid nitrogen. Diffraction data were collected at wavelengths of 0.979 and 1.485 Å (nickel-absorption edge) on beam line 08ID-1 at the Canadian Light Source (Table 2). The data were indexed, integrated, and scaled using HKL2000 (36). The structure was determined by molecular replacement with PHENIX AutoMR (37) using a modified version of the N-terminal domain of PgaB(42–655) (Protein Data Bank code 4F9D) as a search model (residues 310–646 were removed from the model). The resulting electron density map enabled PHENIX AutoBuild (37) to build ~90% of the protein. The remaining residues were built manually in COOT (38) and alternated with refinement using PHENIX.REFINE (37). Translation/Libration/Screw groups were used during refinement and determined automatically using the TLSMD web server (39, 40). Structure figures were generated using PyMOL Molecular Graphics System (DeLano Scientific), and quantitative electrostatics were calculated using PDB2PQR (41, 42) and APBS (43). Programs used for crystallographic data processing and analysis were accessed through SBGrid (44).

Preparation of PNAG Oligomers—β-1,6-GlcNAc oligomers were prepared and purified, and their identities were confirmed as outlined previously (45). The β-1,6-GlcNAc oligomers were stored as lyophilized powders at room temperature and dissolved in deionized water for use in assays. Accurate oligomer concentrations were determined by 1H NMR using dimethylformamide as an internal standard.

Fluorescamine Enzyme Activity Assays—Comparison of the deacetylation activity of BpsB(27–311) C48S, BpsB(27–701) C48S, and the various BpsB(27–701) C48S active site variants and the PNAG oligomer length dependence were determined using a fluorescamine-based assay (46), and performed as described previously (47) with the following modifications: BRAND black 96-well plates were used for fluorescence measurements using a SpectraMax M2 plate reader from Molecular Devices (Sunnyvale, CA); protein concentration was 40 μM; PNAG oligomer concentration was 40 mM; 40 μM NiCl2 was used as the metal solution; and the assay was conducted in 50 mM HEPES, pH 8.0, at 26 °C. For the metal-preference assay, BpsB(27–701) C48S was reincubated in the presence of various divalent metals as chloride salt solutions (40 μM) or metal chelators (1 mM) for 30 min prior to the addition of substrate. For the length dependence assay, GlcNac to β-1,6-(GlcNac)5 were used as substrates, and BpsB(27–701) C48S or BpsB(27–311) C48S was reincubated for 30 min with 40 μM NiCl2. The amount of deacetylation was quantified using a glucosamine standard curve.

B. bronchiseptica Growth Conditions—Strains were maintained on Bordet-Gengou (BG) agar supplemented with 7.5% (v/v) defibrinated sheep’s blood and 50 μg/ml streptomycin. B. bronchiseptica strains were grown overnight at 37 °C in Stainer-Scholte (SS) broth supplemented with 40 mg/liter l-cysteine, 10 mg/liter FeSO4·4 H2O, 4 mg/liter niacin, 150 mg/liter glutathione, and 400 mg/liter ascorbic acid. The overnight culture was used to inoculate fresh medium at a 1:100 dilution and grown at 37 °C to an A600 = 1 (corresponding to 1 × 108 cells). For plasmid

### TABLE 2

Summary of data collection and refinement statistics

| BpsB(35–307)MMMS | Data collection | Refinement |
|------------------|----------------|------------|
|                 | Beamline       | CLS 08ID-1 |
| Wavelength (Å)   | 0.979          |            |
| Space group P2_1 |               |            |
| Unit-cell parameters (Å,°) | a = 47.8, b = 77.7, c = 76.8 β = 108.0 | |
| Resolution (Å)   | 50.00–1.95 (2.02–1.95) | |
| Total no. of reflections | 135,831 | |
| No. of unique reflections | 39,033 | |
| Redundancy       | 3.5 (3.6)      |            |
| Completeness (%) | 98.6 (97.7)    |            |
| Average I/σ(I)   | 33.0 (3.2)     |            |
| Rmerge (%)       | 7.0 (54.6)     |            |

Rmerge = \sum_i \sum_k \sum_j (I(k) – \langle I(k) \rangle) / I(k), where I(k) and \langle I(k) \rangle represent the diffraction intensity values of the individual measurements and the corresponding mean values. The summation is over all unique measurements.

Rwork = \sum_i \sum_k (\langle F_{\text{calc}}(i,k) \rangle / F_{\text{obs}}(i,k)), where F_{\text{calc}} and F_{\text{obs}} are the observed and calculated structure factors, respectively.

Rmerge is the sum extended over a subset of reflections (5%) excluded from all stages of the refinement.

Data were calculated using Molprobity (68).

Maximum-likelihood based coordinate error, as determined by PHENIX (37).

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|-----------------|------------|
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Maximum-likelihood based coordinate error, as determined by PHENIX (37).
maintenance, 50 μg/ml chloramphenicol was included in the media for strains harboring the GFP-expressing and complementation (ΔbpsB<sup>ompw</sup>) or vector (ΔbpsB<sup>vec</sup>) plasmids.

Chromosomal Deletion of bpsB—An in-frame nonpolar bpsB deletion in *B. bronchiseptica* RB50 was constructed using allelic exchange as described previously for the *bps* locus (8). This plasmid was constructed by ligating the regions flanking 5′ of *bpsB* and 3′ of *bpsC* for allelic exchange. The 584-bp XbaI-HindIII upstream fragment, including the first 16 bpsB codons, was amplified from *B. bronchiseptica* RB50 genomic DNA using primers SMP31 and SMP32. The 528-bp HindIII-KpnI downstream fragment, including the last 19 codons of the *bpsB* reading frame, was similarly amplified using primers SMP33 and SMP34. Using the XbaI-KpnI-digested allelic exchange vector pRE112 together with the XbaI-HindIII and HindIII-KpnI-digested fragments, a three-way ligation was performed resulting in plasmid pSM11. This plasmid was transformed into *E. coli* strain SM10Apir and subsequently conjugated into *B. bronchiseptica* RB50. Exconjugants were selected on BG agar containing 50 μg/ml chloramphenicol and streptomycin to select for single and double cross-over recombinants. Double cross-over recombinants were selected on LB agar containing 7.5% (w/v) sucrose. Sucrose-resistant colonies that were streptomycin-resistant but chloramphenicol-sensitive were putative *bpsB* deletions. The genotype of the ΔbpsB strain was confirmed by PCR and sequencing.

Genetic Complementation of bpsB—The *bpsB* gene plus 23 bp upstream of the translational start site and 33 bp downstream of the termination codon were amplified from *B. bronchiseptica* RB50 genomic DNA using primers SMP3 and SMP4. The resulting PCR fragment was confirmed by sequencing and contained flanking KpnI and HindIII restriction sites that were used to subclone the *bpsB* gene into the vector pBBR1MCS. This created the complementation plasmid pSM8 that was transformed into the *B. bronchiseptica* RB50 ΔbpsB strain.

Construction of the *B. bronchiseptica* ΔbpsB GFP Strain—The GFP plasmid, pTac-GFP described previously (8), was transformed into the *B. bronchiseptica* RB50 ΔbpsB strain, and transformants were selected on BG agar containing 50 μg/ml chloramphenicol and streptomycin. Randomly picked colonies containing pTac-GFP were grown in SS broth with 50 μg/ml chloramphenicol and were analyzed for GFP expression utilizing a Nikon Eclipse TE300 inverted microscope, and one of the GFP-expressing clones was chosen for experimental analysis. Comparison of the *B. bronchiseptica* RB50 ΔbpsB GFP-expressing strain with respective parental strains not containing the GFP plasmid revealed no differences in growth in batch cultures or colony morphology on BG agar containing blood.

Subcellular Localization of BpsB—The *bpsB* gene starting 23 bp upstream of the translational start site was amplified from *B. bronchiseptica* RB50 genomic DNA using primers SMP67 and SMP68. The resulting PCR fragment was confirmed by sequencing and containing flanking XbaI and SacI restriction sites that were used to subclone into the vector pBBR1MCS. This produced the plasmid pSM27 that encodes *bpsB* with a C-terminal FLAG tag. Plasmid pSM27 was then transformed into the *B. bronchiseptica* ΔbpsB strain. Bacteria were grown in SS medium supplemented with 50 μg/ml chloramphenicol at 37 °C until the *A<sub>600</sub> ~1.5. Approximately 30 ml of cells at an *A<sub>600</sub> ~1.5 were harvested by centrifugation (30 min, 3000 × g, 4 °C), and pellets were resuspended in 1.25 ml of 10 mM Tris-HCl, pH 8.0, 20% (w/v) sucrose, 1 mM EDTA, and 0.1 mg/ml lysozyme. Following incubation on ice for 10 min, another 1.25 ml of 10 mM Tris-HCl, pH 8.0, was added. The resuspension was then subjected to a freeze/thaw cycle and was further lysed by sonication. The cell lysate was clarified by centrifugation (10 min, 20,000 × g, 4 °C), and the supernatant was further centrifuged (60 min, 150,000 × g, 4 °C) to separate the bacterial membranes from the soluble cytoplasmic and periplasmic proteins. The soluble protein fraction was carefully withdrawn with a syringe and stored at −80 °C for further analysis. The membrane pellet was washed once with 1 ml of 10 mM Tris-HCl, pH 8.0, and then centrifuged (45 min, 100,000 × g, 4 °C), and the supernatant discarded. The membrane pellet was resuspended in 1 ml of 50 mM Tris pH 8.0, 2% (v/v) Triton X-100, 20 mM MgCl<sub>2</sub>, and 1 mM PMSF. The resuspended membranes were incubated for 30 min on ice and then centrifuged (60 min, 100,000 × g, 4 °C) to separate the inner and outer membranes. The supernatant containing the solubilized inner membranes was isolated and stored at −80 °C. The outer membrane pellet was resuspended in 500 μl of 50 mM Tris-HCl, pH 8.0, and stored at −80 °C. The isolated protein fractions were analyzed by SDS-PAGE and Western blot analysis using an α-FLAG antibody.

Purification and Immunoblot Detection of Bps Poly saccharide—*B. bronchiseptica* RB50, ΔbpsABCD, and ΔbpsB strains were grown to stationary phase (*A<sub>600</sub> ~3) and harvested by centrifugation (20 min, 3000 × g, 4 °C). The cell pellet was resuspended in 30 μl of 0.5 mM EDTA, boiled for 10 min, and centrifuged (5 min, 12,000 × g, 25 °C) to remove cell debris. The supernatant was transferred to a new tube and treated with 1 ml of phenol/chloroform to remove proteins. The aqueous phase was extracted with an equal volume of chloroform, precipitated using 100% ethanol overnight at −20 °C, and then centrifuged (5 min, 12,000 × g, 25 °C). The pellet was washed with 70% ethanol and resuspended in one-tenth volume of water (~100–250 μl). The solution was then treated with DNase I (0.1 mg/ml) and RNase (0.1 mg/ml) (2 h at 37 °C) to remove nucleic acids, followed by proteinase K treatment (2 h, 65 °C) to degrade any remaining proteins. A final phenol/chloroform extraction and ethanol precipitation were performed before the pellet was resuspended in water and stored at −20 °C. Purified Bps polysaccharide from the *B. bronchiseptica* RB50, ΔbpsABCD, and ΔbpsB strains were spotted as 10-μl samples on a nitrocellulose membrane and allowed to dry overnight. The samples were then probed using a goat antibody raised against *S. aureus* deacetylated PNAG conjugated to diphtheria toxoid (8, 48).

Biofilm Formation on Glass Coverslides—Log phase cultures of RB50 and mutant strains (*A<sub>600</sub> ~0.05) were inoculated into a 50-ml conical tube (GeneMate), containing a sterile vertically submerged glass coverslip (rectangular, 22 × 60, Fisher) and 15 ml of SS medium. Conical tubes were incubated at 37 °C under static growth for 96 h, with medium being changed every 24 h. At specific time points, coverslips were taken out, and unattached bacteria were removed by three washes with sterile phosphate-buffered saline (PBS). The coverslips were then...
transferred into a new 50-ml conical tube containing 10 ml of PBS and vortexed for 2 min at high speed, resulting in detachment of bacteria from the glass surface. Detached bacteria were diluted and plated on BG blood agar plates containing streptomycin.

**Scanning Electron Microscopy (SEM) of B. bronchiseptica** Biofilms—Three-chambered biofilm flow cells were obtained as sterile units from Stovall. Exponentially grown strains containing a GFP plasmid were inoculated into the chambers. Bacteria were allowed to attach to the chamber without outflow for 2 h at 37 °C. After attachment, the chamber was inverted, and medium flow was initiated using SS broth containing 50 μg/ml chloramphenicol at a rate of 0.5 ml/min over a time course of 96 h. Biofilms were observed every 24 h using a Zeiss LSM 510 confocal scanning laser microscope as described previously (8). Biofilm images were displayed and analyzed using the LSM Image Browser software. The COMSTAT computer software package (49) was used to calculate the average and maximum thickness of the biofilms.

**Continuous Flow Confocal Microscopy**—Continuous flow biofilm cell cultures were obtained as sterile units from Stovall. Exponentially grown strains containing a GFP plasmid were inoculated into the chambers. Bacteria were allowed to attach to the chamber without outflow for 2 h at 37 °C. After attachment, the chamber was inverted, and medium flow was initiated using SS broth containing 50 μg/ml chloramphenicol at a rate of 0.5 ml/min over a time course of 96 h. Biofilms were observed every 24 h using a Zeiss LSM 510 confocal scanning laser microscope as described previously (8). Biofilm images were displayed and analyzed using the LSM Image Browser software. The COMSTAT computer software package (49) was used to calculate the average and maximum thickness of the biofilms.

*Detection of Bps by ELISA*—Bacterial strains (10^7 cfu) resuspended in 100 μl of PBS were added into the wells of a 96-well enzyme-linked immunosorbent assay (ELISA) plate (Nunc). Samples were incubated at 4 °C overnight. The samples were then aspirated, and the plate was blocked with 5% (w/v) skim milk for 1 h and then washed three times with PBS/Tween 20 (PBST). Indicated dilutions of the human serum (15) that had been repeatedly absorbed against human serum (15) were used as the primary antibody diluted in PBST with 5% skim milk for 1 h. The plate was then washed five times with PBST before addition of the goat anti-human horseradish peroxidase detection antibody at a 1:1000 dilution in PBST for 1 h at room temperature, followed by five washes with PBST. Finally, 100 μl of tetramethylbenzidine (Sigma) was added to the wells and incubated for 15 min in the dark before the addition of 50 μl of 1.0 M H₂SO₄. The plate was then read at 450 nm using a plate reader.

**Transmission Electron Microscopy (TEM) of B. bronchiseptica Cells**—TEM analysis for the presence of Bps polysaccharide on the surface of B. bronchiseptica was carried out by adsorbing 1 × 10^8 bacteria onto carbon-coated gold grids (Electron Microscopy Sciences) in a humidified chamber for 60 min. The bacteria on the grids were exposed to 10% (v/v) heat-inactivated Bps-enriched human serum for 60 min after blocking with 2% (w/v) BSA in PBS. The bound antibodies were detected with 6 nm gold-labeled anti-human monoclonal antibodies at a dilution of 1:10 in the blocking buffer. Finally, the bacteria were subjected to negative staining with 2% (w/v) phosphotungstic acid, pH 6.6, and analyzed with a Tecnai transmission electron microscope.

**Results**

**Domain Analysis and Subcellular Localization of BpsB**—Full-length BpsB from *B. bronchiseptica* is homologous to *E. coli* PgaB, and its N-terminal domain is also homologous to *S. epidermidis* IcaB with sequence identities determined using ClustalW (51) for their mature sequences of 37 and 17%, respectively. Blast searches and structural prediction using Phyre² (52) suggest BpsB contains two domains. The N-terminal domain encodes a CE4, and the C-terminal domain is predicted to be similar to GH13 family members that are primarily α-amylases (Fig. 1A) (53). Despite significant sequence homology, BpsB has three distinct differences from PgaB. First, residues 1–26 of BpsB are predicted to encode a signal sequence by SignalP (54). The protein does not contain an outer membrane lipidation site found in PgaB (26, 55). Second, the linker region between the N- and C-terminal domains is 2–3 times longer in BpsB than PgaB (~12–24 versus 7 residues). Third, there are an additional 30 residues at the C terminus of BpsB that are predicted to adopt a random coil structure. Compared with IcaB, BpsB does not contain the hydrophobic residue loop L1 that is proposed to retain the enzyme to the outer leaflet of the membrane (35), and the sequence homology is predominantly based on the five canonical CE4 motifs.

As BpsB does not have a predicted lipidation site, we used subcellular fractionation to determine the protein’s cellular localization. *B. bronchiseptica* cells were separated into three subcellular compartments, a soluble fraction, an inner and an outer membrane fraction. To ensure the efficiency of separation, the cytoplasmic protein BvgA and the outer membrane protein BcfA (56) were used as controls. The purified BcfA protein runs at a slightly higher molecular weight due to the presence of the T7 tag, whereas the BvgA protein runs at the expected weight as it is untagged and purified from inclusion bodies. Furthermore, because a BpsB-specific antibody was not available, a recombinant FLAG-tagged variant of BpsB was expressed. As shown in Fig. 1B, BpsB was localized both to the soluble and the outer membrane fractions. These results suggest that BpsB is likely a periplasmic protein that can localize to the outer membrane.

**Structure of BpsB(35–307)MMS Reveals a CE4 Domain Similar to E. coli PgaB**—To gain structural insight into the function of BpsB, crystallization trials were conducted. BpsB(27–701) produced crystals that did not diffract, so a domain isolation approach was utilized. The N-terminal deacetylation domain constructs BpsB(27–311) and BpsB(35–311) produced soluble protein but were recalcitrant to crystallization. A slightly truncated variant, BpsB(35–307), and its surface entropy mutant, K128A K129A, were also recalcitrant to crystallization. As a consequence metal-mediated synthetic crystallization was utilized (57). BpsB(35–307)MMS was incubated with 2 μ eq of NiCl₂, CuCl₂, or CdCl₂ directly before crystallization trials. BpsB(35–307)MMS produced crystals suitable for data collection in the presence of NiCl₂. Diffraction data were collected to 1.95 Å, and the structure was solved using the molecular replacement technique. BpsB(35–307)MMS crystallized in the monoclinic space group P₂₁ with two molecules in the asymmetric unit and refinement produced a final model with good geometry and R-

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**Notes:**

- **SEM** and **TEM** are used to visualize biofilms.
- **Detection of Bps by ELISA** involves antibody binds to Bps, followed by detection.
- **Transmission Electron Microscopy (TEM)** provides high-resolution images of bacterial cells.
- **Results** section discusses protein localization and structural insights into BpsB.

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FIGURE 1. Schematic representation of BpsB and subcellular localization. A, domain organization and comparison of BpsB from *B. bronchiseptica*, PgaB from *E. coli*, and IcaB from *S. epidermidis*, from N to C terminus. Residue boundaries are listed on the top of the diagram with the predicted domains labeled. The periplasmic signal sequence is abbreviated as SS and regions with no predicted regular secondary structure are depicted as thick black lines. B, localization of BpsB. Fractions containing cytoplasmic and periplasmic proteins (*lane 1*), inner membrane proteins (*lane 2*), and outer membrane proteins (*lane 3*) were separated via SDS-PAGE followed by Western blotting. The periplasmic signal sequence is abbreviated as SS, and an *α*-helix of the (β/α) barrel fold is marked in *blue*, an *α*-helix of the (β/α) barrel fold is marked in *green*, a *β*-strands arranged in a barrel of the (β/α) barrel fold is marked in *green*, and C-terminal domains of BpsB is marked in *purple*.

Factors *R*<sub>work</sub> and *R*<sub>free</sub> of 16.9 and 20.8%, respectively (Table 2). Residues 298–307 and GSHM left over from the hexahistidine tag cleavage were not included in the final model as there was no interpretable electron density present.

BpsB(35–307)<sub>MMS</sub> adopts the (β/α)<sub>7</sub> barrel fold common to the CE4 family (Fig. 2A) (58). The BpsB(35–307)<sub>MMS</sub> (β/α)<sub>7</sub> core is composed of seven parallel *β*-strands arranged in a barrel that is surrounded by only six *α*-helices. BpsB(35–307)<sub>MMS</sub> also contains three *β*-hairpin motifs (BH1–3, *green*) located on the top of the (β/α)<sub>7</sub> barrel (C termini of the core *β*-strands), and an *α*-helix (*α2, purple*) that plugs the bottom on the barrel (Fig. 2A). Both of these structural elements are commonly found within the CE4 family and are both conserved within the PNA g decacetylases PgaB and IcaB. Electrostatic surface potential analysis of BpsB(35–307)<sub>MMS</sub> reveals an electronegative face that contains the active site pocket (Fig. 2B). Interestingly, loop L1 (residues 53–70) packs against the electronegative face partially occupying the active site. The density of L1 is poor in both molecules suggesting it is flexible, and its conformation may therefore be an artifact of crystallization. Indeed, examination of interaction between molecules A and B in the asymmetric unit reveals a 2-fold rotational symmetry axis is present between residue Ile-68 of both molecules. Residue Asp-64 from molecule A hydrogen bonds with Arg-44 and Ser-291 from molecule B, and Phe-66 and Ala-67 from molecule A pack into a hydrophobic pocket in molecule B. Equivalent interactions are observed between these residues on molecule B and molecule A.

Structural alignment of BpsB(35–307)<sub>MMS</sub> with the N-terminal domain of *E. coli* PgaB, residues 43–309 (Protein Data Bank code 4F9D), shows strong conservation of the CE4 domain with a root mean square deviation of 1.3 Å over 225 eq Cα atoms (Fig. 3A). BpsB(35–307)<sub>MMS</sub> contains the same circular permutation of the conserved CE4 motifs present in PgaB and IcaB (30, 35). However, two topological differences occur between BpsB(35–307)<sub>MMS</sub> and PgaB(43–309). First, the final *α*-helix of the (β/α)<sub>7</sub> barrel fold, *α*<sub>8</sub> in PgaB, is missing in BpsB(35–307)<sub>MMS</sub> (Fig. 3A). Second, loop L1 is longer in BpsB(35–307)<sub>MMS</sub> and lies across the top of the (β/α)<sub>7</sub> barrel partially occluding the active site (Fig. 2B, and 3, A and B). Removing L1 from surface representation reveals a narrow groove prior to the active site pocket (Fig. 3B). This secondary groove is not present in PgaB (Fig. 3B) and could play a role in binding PNAG if loop L1 is, as we predict, flexible in BpsB. Structural alignment of the BpsB and PgaB active site shows a strong conservation of the CE4 motifs, with the only major differences being the location of His-49 and His-55 (Fig. 3C). Our recent study of IcaB (35) suggested that His-49 is a multifunctional acid/base catalyst in the deacetylation mechanism. Interestingly, the electron density was poor for this residue in both BpsB molecules suggesting it may be inherently flexible.

*BpsB Is a Metal-dependent PNAG Decacylase*—Diffraction data collected at the nickel-absorption edge (1.485 Å) revealed a total of five nickel ions in the anomalous difference map of the asymmetric unit. Three nickel ion locations were unique. The first unique nickel, present in both molecule A and B, was coordinated by His-235 and His-239, and these were the residues engineered to produce a metal coordination site. The second unique nickel was coordinated by Asp-144 and His-240 in molecule A and formed a crystal contact with Asp-144 and His-240 from molecule B in a symmetry-related asymmetric unit. The third unique nickel, found in molecule A and B, was coordinated by Asp-114, His-184, and His-189 (Fig. 4A). The arrangement of the Asp-His-His residues at this third unique nickel ion was characteristic of the metal coordination found in the CE4 family active site. To determine whether BpsB showed metal-dependent decacylase activity on PNAG, fluorescamine assays with PNAG oligomers were conducted. BpsB(27–701) showed low levels of activity as the isolated enzyme, but ~3.8- and 7.5-fold increased activity in the presence of Co<sup>2+</sup> and Ni<sup>2+</sup>, respectively (Fig. 4B). Unlike PgaB, BpsB(27–701) activity could be abolished when incubated with 1 mM EDTA (Fig. 4B).

*BpsB Displays Length-dependent Decacylation*—The structure of BpsB(35–307)<sub>MMS</sub> revealed the absence of the final helix in the (β/α)<sub>7</sub> fold, and the length of the linker between the N- and C-terminal domains of BpsB is roughly double that of PgaB. We hypothesized that the association of the two domains may
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FIGURE 2. BpsB[35–307]MMS adopts a (β/α)5 fold with an electronegative active site. A, cartoon representation of BpsB[35–307]MMS with the β-strands (blue) and α-helices (red) of the (β/α)5 barrel labeled β1–7 and α1–7, respectively. The CE4 “capping” helix is colored purple. Additional ordered secondary structure elements are colored green; loops are colored light gray; the Ni2+ ion is a teal sphere, and the N and C termini are labeled accordingly. B, electrostatic surface representation of BpsB[35–307]MMS shown in the same orientation as A and rotated 90° to the right show an electronegative face with loop L1 occluding the active site. Quantitative electrostatics are colored from red (−5 kT/e) to blue (+5 kT/e).

FIGURE 3. Structural comparison of BpsB[35–307]MMS and PgaB(43–309). A, superposition of BpsB[35–307]MMS (blue) and PgaB(43–309) (orange) shows the differences in the canonical (β/α)5 fold. Loop L1 (magenta) in BpsB[35–307]MMS lies directly across the active site, and α8 is only present in PgaB. B, surface representation of BpsB[35–307]MMS (blue) and PgaB(43–309) (orange) reveals distinct differences in loop L1 (magenta). BpsB contains a secondary groove (yellow) that leads into the active site, which is not present in PgaB because Arg-66 plugs the groove (green). C, active site comparison reveals minor differences between BpsB and PgaB. Residue numbers refer to BpsB/PgaB. The nickel ion throughout the figure is colored teal and shown as a sphere.

be significantly weaker, and that if the C-terminal domain plays a significant role in binding PNAG (such as providing additional binding subsites) there should be a difference in length-dependent deacetylase activity. Thus, fluorescamine assays were conducted using short PNAG oligomers. Our data show a clear trend of increasing rates of deacetylation with increasing PNAG oligomer length (Fig. 5A). This suggests the C-terminal domain provides additional binding sites for PNAG, which is consistent with studies on PgaB (30). However, in contrast to PgaB, the N-terminal domain of BpsB (BpsB(27–311)) displayed ~46% activity compared with the full-length protein (Fig. 5B). The isolated N-terminal domain of PgaB does not display deacetylase activity (32). The ability of BpsB(27–311) to effectively bind PNAG and deacetylate the polymer may be due to the additional groove that precedes the nickel-active site (Fig. 3B). Furthermore, the likelihood that loop L1 is flexible could be exploited for increased substrate binding by forming an additional structural element to the active site, a component also lacking in PgaB.

Conserved Residues in BpsB Are Required for PNAG Deacetylation—Previous studies on PgaB (30, 31), IcaB (35, 47), Vibrio cholerae chitin deacetylase (VcCDA) (59), and PgdA (60) suggest BpsB likely uses a metal-assisted general acid/base mechanism common to CE4 members. Based on a number of substrate-bound structures of VcCDA, the metal ion is required for substrate binding and coordinating the 3′-hydroxyl of the GlcNAc moiety and the carbonyl of the N-acetyl group, coordinating a water molecule involved in nucleophilic attack on the N-acetyl group, and assisting in the stabilization of the tetrahedral transition state intermediate (59). In addition to a metal co-factor, the CE4 family contains five signature motifs, abbreviated MT1 to MT5 as they occur sequentially in the sequence, that are responsible for substrate binding, metal coordination, and enzymatic hydrolysis. Asp-113 and Asp-114 belong to MT1 and are involved in catalysis and metal coordination, respectively. His-184 and His-189 belong to MT2 and are involved in metal coordination. Tyr-252 and Arg-290 belong to MT3, although Arg-290 is found at the end of the CE4 domain sequence where MT5 is located, and are responsible for creating one side of the active site cavity and coordinating the catalytic base in MT1, respectively. Leu-274 belongs to MT4 and forms one side of the N-acetyl hydrophobic binding pocket. His-49 and Leu-292 belong to MT5 and are involved in catalysis and form the second side of the hydrophobic binding pocket with MT4, respectively. To probe the role of these conserved active site residues (Fig. 3C), mutagenesis studies were con-
ducted on BpsB(27–701). The D114A, H184A, and H189A variants all abolished activity showing their importance in metal coordination (Fig. 5B). The H49A and D113A variants also showed no activity suggesting their importance in the catalytic mechanism (Fig. 5B). Interestingly, the D113N mutant showed only 1% of wild-type activity (Fig. 5B). This is in contrast to what has been observed for IcaB, where the comparable variant retained 50% of wild-type activity (35). This suggests that for BpsB Asp-113 may play a significant role in catalysis along with His-49, rather than His-49 acting as a single acid/base catalyst as proposed for IcaB (35).

**BpsB Is Not Required for Bps Synthesis or Export**—Previous studies in *E. coli* have shown that PGA is not required for the production of the PNAG polymer but is required for its modification and export (33). To determine the role of BpsB in Bps biosynthesis and export, a nonpolar in-frame deletion of *bpsB* in RB50 was constructed (∆*bpsB*). An immunoblot assay with antisera raised against deacetylated PNAG from *S. aureus* (8) was used to compare the production of Bps between RB50 (WT), ∆*bpsB*, and ∆*bps* strains. Compared with the WT strain, the ∆*bpsB* strain showed no significant differences in extracellular Bps production. As expected, the ∆*bps* strain showed a severe loss in Bps production (Fig. 6A). The residual immuno-reactive material that is observed in the ∆*bps* strain has been observed previously (8, 15) and probably represents some other material that is weakly cross-reactive with the dPNAG antibody.

As an additional means to confirm that Bps is expressed and exported to the surface of the ∆*bpsB* strain, we utilized an enzyme-linked immunosorbent-based assay with a serum that specifically detects Bps (50). At serum dilutions of 10⁻³ and 10⁻⁵, there were no statistically significant differences between
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Complementation of bpsB on a plasmid (pDR195) formed biofilms similar to that of the ΔbpsB strain. As expected, the Δbps strain where the entire bpsABCD operon has been deleted was recovered from the glass coverslips in lower numbers compared with the WT strain. Interestingly, there were no significant differences in CFU numbers between the ΔbpsB and Δbps strains recovered from the coverslips at any of the time points tested. This suggests that BpsB is required for producing the biofilm-relevant form of Bps, and its absence from B. bronchiseptica severely impairs biofilm formation to similar levels as the Δbps strain, which lacks the capacity to produce the Bps polysaccharide.

To further examine the mechanism by which BpsB contributes to biofilm formation, we utilized SEM and flow cells assays followed by confocal scanning light microscopy. SEM analysis of the biofilm coated-coverslips showed that although the ΔbpsB strain was still able to adhere to the surface, it lost the characteristic three-dimensional biofilm structure displayed by the WT strain (Fig. 6B). Quantitative assessment of the biofilms of the WT and ΔbpsB strains formed in the flow cell chamber under continuous flow was achieved by analyzing the confocal scanning light microscopy-generated images for biomass, average thickness, and maximum thickness. The COMSTAT computer program was utilized for this purpose. No significant differences were observed after 24 and 48 h between the WT and the ΔbpsB strain with respect to any of the parameters tested (Table 3). At 72 h however, the biofilms formed by the ΔbpsB strain exhibited a significantly lower maximum thickness (Table 3). At 96 h the ΔbpsB mutant formed biofilms that displayed significantly lower values for all the three parameters, biomass, average thickness, and maximum thickness compared with the WT strain (Table 3). Taken together, these results indicate that BpsB-dependent modification of Bps promotes biofilm formation in B. bronchiseptica by contributing to the extracellular matrix structure.

Discussion

Previously, we have shown that Bps is exported to the surface of B. bronchiseptica cells and is critical for biofilm formation (8). In this report, we show that the BpsB protein from B. bronchiseptica is a PNAG deacetylase, and we characterize its role in the deacetylation and export of the Bps polysaccharide and biofilm formation.

FIGURE 7. Localization of the Bps exopolysaccharide. Electron microscopy analysis for the presence of Bps on the surface of B. bronchiseptica was carried out by adsorbing $1 \times 10^8$ bacteria onto carbon-coated gold grids in a humidified chamber for 1 h, and then exposed to 10% heat-inactivated Bps-enriched human serum for 1 h after blocking with 2% BSA in PBS. The bound antibodies were detected with 6 nm gold-labeled anti-human polyclonal antibodies at a dilution of 1:10 in the blocking buffer using negative staining with 2% phosphotungstic acid, pH 6.6, and analyzed with a Tecnai transmission electron microscope. Arrows indicate the presence of gold-labeled Bps, which is present on the surface of the WT and ΔbpsB strains.

FIGURE 6. Detection of Bps by immunoblot and ELISA. A, exopolysaccharides were extracted from overnight-grown cultures of B. bronchiseptica strains using EDTA and protease K treatment. Extracted samples were spotted onto nitrocellulose membranes and probed with goat antibody raised against S. aureus deacetylated PNAG conjugated to diphtheria toxoid (8). B, B. bronchiseptica strains or PBS were added to an ELISA plate to measure the amount of Bps present on the surface of bacterial cells. The absorbed human serum at indicated dilutions was used to probe for Bps, followed by detection using goat anti-human IgG conjugated to horseradish peroxidase. Error bars are representative of the standard deviation. *** indicates a p value of < 0.0001 between Δbps and other strains, and # denotes a p value of < 0.05 between WT and Δbps strains. Samples were run in duplicate and are representative of one of two independent experiments.

The absorbance readings when the WT and ΔbpsB strains were used to coat the plates (Fig. 6B). Although at the serum dilution of $10^{-4}$, the absorbance readings of the ΔbpsB strain were slightly lower than that observed for the WT strain, it was still considerably higher than that observed when either the Δbps strain or PBS was used to coat the plates.

Finally, to ensure the presence of extracellular Bps in the ΔbpsB strain was not from periplasmic leakage during isolation, TEM on bacterial cells was conducted. Electron microscopic analysis showed abundant colloidal gold labeling of both the WT and ΔbpsB strains. As expected, no labeling of the Δbps strain was observed (Fig. 7). Taken together, the results from immunoblotting, ELISA, and TEM suggest that BpsB is not required for Bps production or export.

BpsB Is Required for Biofilm Formation and Contributes to the Biofilm Structural Complexity—Next, we aimed to determine whether BpsB had a role in biofilm formation in B. bronchiseptica. To address this, a static biofilm assay using glass coverslips was glass formed. First, to validate the assay, biofilms formed by the WT and the Bvg− phase-locked strain RB54 were compared. It is well established that the BvgAS locus is required for biofilm formation in B. bronchiseptica (9, 11), and RB54 displays a severe defect in biofilm formation compared with the WT strain. As expected, the Bvg− phase-locked strain was recovered in much lower numbers than the WT strain at all the time points tested. There were no significant differences in biofilm formation between the WT and ΔbpsB strains at 24 h. However, at 72 and 96 h, the ΔbpsB strain was recovered at significantly lower numbers compared with WT (Fig. 8A). Complementation of bpsB on a plasmid (ΔbpsBcom) restored the biofilm defect in the ΔbpsB strain, as the biofilms either exceeded (72 h) or were similar (96 h) to that formed by the WT strain.
The structure and functional characterization of BpsB reveal that the deacetylase domain belongs to the CE4 family with strong structural conservation to PgaB (Figs. 2 and 3). BpsB is an inefficient metal-dependent deacetylase of PNAG oligomers (Fig. 4) similar to PgaB and IcaB. BpsB can use a variety of divalent cations for deacetylation with highest activity in the presence of Ni$^{2+}$ and Co$^{2+}$ ions. As the PNAG deacetylases are likely expressed during nutrient-limiting conditions (i.e. when a biofilm is needed), it is not surprising that they can utilize a variety of metal cofactors for activity. What remains unknown for the PNAG deacetylase and other periplasmic CE4s is how the enzymes are loaded with their respective metal cofactor. It is possible that the enzymes can scavenging the ions directly from the outer membrane metal ion porins (such as BtuB in *E. coli*). However, metal ions in the periplasm are usually specifically transported by solute-binding proteins (such as NikA for Ni$^{2+}$ in *E. coli*) and delivered to the inner membrane ATP-binding cassette transporters for cytosolic delivery. Thus, it is more likely that the enzymes are directly loaded by the solute-binding proteins or via periplasmic metallochaperones.

Our structural and functional analysis of BpsB shows high conservation to PgaB; however, in contrast to PgaB, the isolated BpsB deacetylase domain, BpsB(27–311), is able deacetylate PNAG oligomers (Fig. 5B). The secondary groove preceding the active site in the BpsB(35–307)MMS structure in combination with a significantly altered L1 loop appear to be the key elements that allow for PNAG deacetylation in the absence of the C-terminal domain. Although not required for deacetylation, the C-terminal domain of BpsB is required for increased rates of deacetylation with oligomer length and likely increases the binding affinity for PNAG. These structural and functional differences suggest that the mechanism of periplasmic processing and outer membrane export may be different from that proposed for PgaB (32). Dot-blot assays, ELISA, and TEM analyses showing Bps secretion into the extracellular matrix in a ΔbpsB mutant (Figs. 6 and 7) further support this hypothesis as a ΔpgaB *E. coli* mutant retains PNA in the periplasm (33). These observations may also be the result of structural/functional differences between the outer membrane proteins BpsA and PgaA. PgaA and BpsA are predicted to contain a periplasmic domain that contains multiple TPR motifs involved in protein-protein interactions and a β-barrel porin. Interestingly, PgaA is 146 residues longer than BpsA, and structural prediction servers such as Phyre$^2$ (52) suggest that the additional 146 amino acids are predominantly located to the C terminus of the periplasmic TPR-motif containing domain. One possible explanation is that the smaller TPR domain in BpsA could affect the ability to form a protein-protein interaction with BpsB. However, our localization data do not support this as BpsB co-fractionates with the outer membrane suggesting it likely forms a

**TABLE 3**

COMSTAT analysis of CSLM-generated Z-series of the WT and ΔbpsB mutant strains

| Time (h) | Strain | Biomass | Avg. Thickness | Max. Thickness |
|---------|--------|---------|----------------|---------------|
|         |        | Value$^a$ | $^b$          | Value$^a$ | $^b$          | Value$^a$ | $^b$          |
| 24      | WT     | 1.21 (1.09) | 0.850 | 2.96 (2.93) | 0.677 | 21.12 (7.50) | 0.932 |
|         | ΔbpsB  | 1.31 (0.43) | 0.792 | 3.56 (1.02) | 0.744 | 21.44 (3.17) | 0.477 |
| 48      | WT     | 2.88 (1.57) | 0.072 | 8.38 (3.16) | 0.896 | 31.20 (5.25) | 0.011 |
|         | ΔbpsB  | 2.66 (0.93) | 0.098 | 7.71 (3.07) | 0.438 | 28.96 (4.21) | 0.477 |
| 72      | WT     | 4.75 (0.96) | 0.072 | 12.44 (4.96) | 0.026 | 22.56 (1.73) | 0.477 |
|         | ΔbpsB  | 5.36 (1.39) | 0.072 | 12.08 (3.19) | 0.026 | 22.56 (1.73) | 0.011 |
| 96      | WT     | 3.56 (0.70) | 0.072 | 11.22 (1.57) | 0.026 | 23.04 (1.73) | <0.001 |
|         | ΔbpsB  | 2.28 (1.20) | 0.072 | 6.30 (3.73) | 0.026 | 29.96 (4.21) | 0.477 |

$^a$ Biomass is given as μm$^2$/μm$^2$ of surface area covered by bacteria expressing GFP. 
$^b$ Values were determined using an unpaired two-tailed Student’s t test.
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complex with BpsA. Furthermore, a protein-protein interaction has been observed using cross-linking with the orthologous proteins, HmsF and HmsH, from *Yersinia pestis* (61). An alternative explanation may be that the additional residues in PgaA to the C terminus of the periplasmic TPR domain may occlude the β-barrel porin preventing dPNAG export without some conformational change. Thus, in BpsA the porin may be constitutively open and allow for PNAG transport in the absence of a protein-protein interaction driven by Bps deacetylation. Because of difficulties in isolating pure Bps from both WT and ΔbpsB knock-out strains for compositional analysis, it is still a possibility that Bps could be deacetylated nonspecifically by another unknown protein in the absence of BpsB. This in turn could also be sufficient to modulate the conformation of BpsA to allow for Bps export.

BpsB(27–701) deacetylation is inefficient, yet the conserved residues in the BpsB(35–307)_MMR active site are all required for activity (Fig. 5B). The PNAG deacetylases PgaB, BpsB, and IcaB all have low rates of deacetylation activity in vitro. Maintaining low levels of deacetylation of PNAG in vivo is likely a mechanistic feature to modify the chemical properties of the polysaccharide needed for biofilm formation. For example, partial deacetylation of PNAG from *S. epidermidis* has been shown to be crucial for adhesin properties, as deletion of the PNAG deacetylase IcaB results in shedding of the polymer from the cell surface and abolishment of biofilm formation (62). Thus, the ability to fine-tune the level of deacetylation/acetylation of exopolysaccharides appears to be an important aspect for biofilm formation.

This report is the first in-depth analysis of a Gram-negative PNAG deacetylase other than PgaB. Our results suggest that there are structural and mechanistic variations between the orthologous PNAG biosynthetic systems of *E. coli* and *B. bronchiseptica*. Although BpsB is not required for the biosynthesis and export of Bps (Figs. 6 and 7), it plays an essential role in biofilm formation (Fig. 8 and Table 3). Our data suggest that Bps or deacetylated Bps is not primarily used as a surface adhesin, as *B. bronchiseptica* Δbps or ΔbpsB strains can still effectively adhere to surfaces (Fig. 8). However, deacetylated Bps is required for the intricate three-dimensional architecture seen in later stages of biofilm development (Fig. 8B and Table 3). It may be that evolutionary differences among orthologous PNAG biosynthetic systems have occurred to cause important differences to the dPNAG polymer in vivo, such as polymer length and level of deacetylation. These variations in the structure and chemical properties of PNAG may have evolved so that bacteria can more efficiently colonize distinct microenvironments within the host. We propose that by contributing to biofilm formation in the respiratory tract, BpsB promotes long term survival within the mouse respiratory tract. The contribution of BpsB in promoting the pathogenic properties of *B. bronchiseptica* is currently under investigation.

The structural and functional characterization of BpsB presented herein provides further evidence that Bps is the same compositional homopolymer as PNAG produced by numerous Gram-negative and -positive bacteria. Understanding how the modification of Bps affects surface adhesion and biofilm formation will help guide the development of anti-biofilm agents against *Bordetella*. Identifying key links in biofilm formation and *Bordetella* infections will be pertinent for the identification of vaccine targets or drugs to combat respiratory related illness.

**Author Contributions**—D. J. L., S. M., R. D., and P. L. H. designed the study and wrote the paper. S. M. and T. G. designed and performed the *in vivo* characterizations described. N. C. B. constructed the vectors for expression of the deacetylase active site mutant proteins, purified them, and assisted in the *in vitro* characterization. D. J. L. designed and constructed expression vectors of the wild-type, truncated, and crystallization mutants, purified the proteins, determined the structure, and performed the *in vitro* enzymatic characterizations described. B. R. D. synthesized the oligosaccharides used during the *in vitro* analysis. D. J. L., S. M., T. G., N. C. B., M. N., R. D., and P. L. H. analyzed the results. All authors approved the final version of the manuscript.

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