

# Characterization of the Poly- $\beta$ -1,6-*N*-Acetylglucosamine Polysaccharide Component of *Burkholderia* Biofilms<sup>∇</sup>

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**We demonstrated the production of poly- $\beta$ -1,6-*N*-acetylglucosamine (PNAG) polysaccharide in the biofilms of *Burkholderia multivorans*, *Burkholderia vietnamiensis*, *Burkholderia ambifaria*, *Burkholderia cepacia*, and *Burkholderia cenocepacia* using an immunoblot assay for PNAG. These results were confirmed by further studies, which showed that the PNAG hydrolase, dispersin B, eliminated immunoreactivity of extracts from the species that were tested (*B. cenocepacia* and *B. multivorans*). Dispersin B also inhibited biofilm formation and dispersed preformed biofilms of *Burkholderia* species. These results imply a role for PNAG in the maintenance of *Burkholderia* biofilm integrity. While PNAG was present in biofilms of all of the wild-type test organisms, a  $\Delta$ pgaBC mutant of *B. multivorans* (Mu5) produced no detectable PNAG, indicating that these genes are needed for *Burkholderia* PNAG formation. Furthermore, restoration of PNAG production in PNAG negative *E. coli* TRXWGM $\Delta$ C ( $\Delta$ pgaC) by complementation with *B. multivorans* pgaBCD confirmed the involvement of these genes in *Burkholderia* PNAG production. While the confocal scanning laser microscopy of untreated wild-type *B. multivorans* showed thick, multilayered biofilm, Mu5 and dispersin B-treated wild-type biofilms were thin, poorly developed, and disrupted, confirming the involvement of PNAG in *B. multivorans* biofilm formation. Thus, PNAG appears to be an important component of *Burkholderia* biofilms, potentially contributing to its resistance to multiple antibiotics and persistence during chronic infections, including cystic fibrosis-associated infection.**

Bacteria of the *Burkholderia cepacia* complex (Bcc) have emerged as opportunistic pathogens in patients with cystic fibrosis (CF) and immunocompromised individuals (20). Approximately 80% of the Bcc isolates recovered from the sputum of CF patients produce large amounts of exopolysaccharides (EPS), suggesting a possible role for this EPS in Bcc pathogenesis (5). Most of the Bcc strains investigated produced only one type of EPS, consisting of a highly branched heptasaccharide repeating unit called cepacian (27). Several studies have pointed to cepacian as a virulence factor contributing to the overall pathogenicity of Bcc members and thus to their success as pathogens. In addition, Bcc species are capable of forming biofilms *in vitro* and *in vivo*, which plays an important role in virulence and significantly increases antibiotic resistance (26). Studies with cepacian-defective mutants have demonstrated that, although not required for the initiation of biofilm formation, cepacian is required for the formation of thick and mature biofilms (6). Although cepacian is often produced along with other EPS molecules, only a small percentage of Bcc strains examined produced EPS completely different from cepacian (18).

In addition to cepacian, *Burkholderia* spp. are believed to produce other types of EPS, such as poly- $\beta$ -1,6-*N*-acetylglu-

cosamine (PNAG; also called polysaccharide intercellular adhesin [PIA] and poly- $\beta$ -1,6-*N*-acetyl-D-glucosamine [PGA]), which is a major component of biofilms in staphylococci (18, 19, 21). PNAG is an important virulence factor, as it protects bacteria against innate host defenses (17, 28). In staphylococci, the *icaADBC* operon encodes the proteins involved in the synthesis of PNAG (10). Furthermore, the *pgaABCD* operon of *Escherichia coli* was shown to promote the synthesis of PNAG (29). Functionally and genetically related loci occur in other Gram-negative bacteria, including *Klebsiella pneumoniae*, *Yersinia* spp., *Bordetella* spp., *Pseudomonas fluorescens*, *Actinobacillus pleuropneumoniae*, *Burkholderia cepacia*, and *Aggregatibacter actinomycetemcomitans* (12, 13, 22, 29). The production of PNAG in *E. coli* (29), *A. pleuropneumoniae* (13), *Acinetobacter baumannii* (4), *Bordetella* spp. (22), *A. actinomycetemcomitans* (14), and *Yersinia pestis* (2) has been biochemically and/or immunologically confirmed. A BLASTP search in the NCBI nonredundant protein database using *pgaABCD*, which encodes proteins involved in PNAG biosynthesis and export in *E. coli*, revealed the presence of a four-gene locus in *Burkholderia* spp. that shares a high degree of similarity with the genetic locus encoding PNAG-biosynthetic proteins in other Gram-negative bacteria.

The objectives of this study were to (i) test for the production of PNAG and its dependence upon the *pgaABCD* locus in *Burkholderia* spp., (ii) probe for the PNAG polysaccharide component in the biofilms of *Burkholderia* species such as *Burkholderia multivorans*, *Burkholderia vietnamiensis*, *Burk-*

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TABLE 1. Bacterial stains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Source or reference
<b>Strains</b>		
<i>B. multivorans</i> C5393	Clinical isolate, wild type	S. T. Cardona
<i>B. vietnamiensis</i> LM618835	Wild type	S. T. Cardona
<i>B. cepacia</i> ATCC25416	Wild type	ATCC <sup>a</sup>
<i>B. cenocepacia</i> J2315	Wild type	S. T. Cardona
<i>B. ambifaria</i> LM619467	Wild type, cystic fibrosis isolate	S. T. Cardona
<i>B. multivorans</i> MU5	<i>B. multivorans</i> C5393 $\Delta$ pgaBC	This study
<i>E. coli</i> XL1-Blue	<i>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac</i> [F' <i>proAB lacI<sup>q</sup>ZΔM15 Tn10</i> (Tet <sup>r</sup> )]	Stratagene
<i>E. coli</i> TRMG1655	<i>E. coli</i> MG1655 <i>csrA::kan</i>	23
<i>E. coli</i> TRXWGMΔC	<i>E. coli</i> TRMG $\Delta$ pgaC	29
<b>Plasmids</b>		
pwFRT-Tp <sup>rc</sup>	Tp <sup>r</sup> Ap <sup>r</sup> ; Tp <sup>r</sup> cassette flanked by wild-type FRT <sup>b</sup>	1
pBSKII(-)	Ap <sup>r</sup>	Stratagene
pMCS5	Ap <sup>r</sup>	Mo Bi Tec
pBCMu	Ap <sup>r</sup> Tp <sup>r</sup> ; gene replacement vector	This study
pMCSpgaBCD	Ap <sup>r</sup> ; <i>B. multivorans</i> pgaBCD	This study

<sup>a</sup> ATCC, American Type Culture Collection.

<sup>b</sup> FRT, Flp recombination target.

*holderia ambifaria*, *Burkholderia cepacia*, and *Burkholderia cenocepacia*, and (iii) examine the role of PNAG in *Burkholderia* sp. biofilm formation using biochemical, genetic methods, and confocal microscopy.

## MATERIALS AND METHODS

**Chemicals, bacteria, and culture conditions.** All chemicals (including media ingredients) were of analytical grade. Proteinase K, DNase I, RNase A,  $\alpha$ -amylase, Western blocker solution, horseradish peroxidase-conjugated goat anti-mouse immunoglobulin M (IgM), and 3,3',5,5'-tetramethylbenzidine (TMB) were purchased from Sigma-Aldrich (St. Louis, MO). Immun-Blot polyvinylidene difluoride (PVDF) membranes were from Bio-Rad Laboratories (Hercules, CA). Restriction endonucleases and *Taq* DNA polymerases were purchased from Fermentas (Burlington, ON, Canada). T4 DNA ligase and shrimp alkaline phosphatase were from New England BioLabs (Mississauga, ON, Canada) and Roche Diagnostics (Laval, QC, Canada), respectively. Synthetic oligonucleotides were obtained from Sigma Genosys (Oakville, ON, Canada). The enzyme dispersin B was purified from a recombinant *E. coli* strain as previously described (16). The enzyme had a specific activity of  $\sim 10^3$  units  $\text{mg}^{-1}$  of protein. Bacterial strains used in this study are shown in Table 1. All strains were maintained at  $-80^\circ\text{C}$  in 15% glycerol, recovered in tryptic soy broth (TSB) or brain heart infusion broth (BHI), incubated at  $37^\circ\text{C}$  for 48 h. For exopolysaccharide extraction, *Burkholderia* and *E. coli* strains were cultured in 50 ml BHI and Luria Bertani (LB) broth, respectively, at  $37^\circ\text{C}$  for 3 days under static conditions (i.e., conditions which did not change). *E. coli* XL1-Blue was used for cloning and plasmid maintenance.

**Immunoblot analysis.** *Burkholderia* biofilm-associated PNAG was extracted by following the method for the isolation of polysaccharide intercellular adhesion (PIA) from *Staphylococcus epidermidis* with some modifications (28). Cells were harvested by centrifugation, resuspended in 0.5 M EDTA, and incubated at  $100^\circ\text{C}$  for 5 min and at  $85^\circ\text{C}$  for 30 min. After centrifugation, the clarified supernatant was first dialyzed against deionized water and then against 50 mM Tris-HCl (pH 8.0) and 20 mM  $\text{MgCl}_2$ . The crude polysaccharide preparation was treated with 100  $\mu\text{g}/\text{ml}$   $\alpha$ -amylase, 500  $\mu\text{g}/\text{ml}$  lysozyme, 250  $\mu\text{g}/\text{ml}$  DNase I, and 100  $\mu\text{g}/\text{ml}$  RNase A at  $37^\circ\text{C}$  for 2 h followed by 2 mg/ml proteinase K for 16 h at  $55^\circ\text{C}$  in the presence of 1 mM  $\text{CaCl}_2$  and 0.5% sodium dodecyl sulfate. The samples were incubated at  $85^\circ\text{C}$  for 1 h to inactivate proteinase K and dialyzed against deionized water. The polysaccharide preparations were lyophilized and dissolved in 50  $\mu\text{l}$  phosphate-buffered saline (PBS), and a 15- $\mu\text{l}$  aliquot was spotted onto an Immun-Blot PVDF membrane. The blot was blocked with Western blocker solution and probed with a 1:2,000 dilution of murine IgM

monoclonal antibody (MAb) raised against *E. coli* PGA (11) as the primary antibody. Horseradish peroxidase-conjugated goat anti-mouse IgM at a dilution of 1:10,000 was used as the secondary antibody and detected with TMB.

**DNA manipulations.** All enzymatic reactions were performed according to the manufacturers' instructions. *E. coli* cells were transformed by heat shock using frozen competent cells prepared by using the calcium chloride method described in *Molecular Cloning* (24). *Burkholderia* strains were transformed by the electrotransformation method (7). Plasmid construction and plasmid DNA extraction were carried out according to standard molecular biology techniques (25). Genomic DNA was extracted following the phenol-chloroform method (8). PCR was carried out using a P<sub>x</sub>2 thermal cycler (Thermo Electron Corporation, Milford, MA).

**pBCMu construction.** The genomic sequence containing putative genes responsible for PNAG synthesis, deacetylation, and export were isolated from *B. multivorans* C5393 genomic DNA by PCR using primers based on the sequence information of the genomic region containing the BMULJ\_04028, BMULJ\_04027, BMULJ\_04026, and BMULJ\_04025 genes of *B. multivorans* ATCC 17616, which are homologous to *E. coli* pgaA, pgaB, pgaC, and pgaD, respectively. For construction of pBCMu, the 826-bp fragment (F1) that includes 778 bp of the 3' coding region of pgaA and 33 bp of the 5' coding region of pgaB and an 851-bp fragment (F2) spanning 24 bp of the 3' coding region of pgaC, the complete pgaD gene, and 329 bp of the downstream region were isolated using the primer pairs P1F (5' TAATATTCTAGAAGCTTGCAACCGTTTCGGCGCAACTG 3')-P1R (5' TAATATGGATCCGCATCCGCACATGAAGGTC CGTC 3') and P2F (5' TAATATGGATCCATGAAGAACGCGCCGATT ATTG 3')-P2R (5' TAATATCTCGAGAAGCTTGGCGCGCCGTGCGTCCG CCAG 3'), respectively. The PCR mixture contained 0.25  $\mu\text{M}$  (each) forward and reverse primers, 200  $\mu\text{M}$  (each) deoxynucleoside triphosphates, 100 ng template DNA, 75 mM Tris-HCl (pH 8.8), 20 mM  $(\text{NH}_4)_2\text{SO}_4$ , 2.5 mM  $\text{MgCl}_2$ , 0.01% Triton X-100, 5% dimethyl sulfoxide, and 1.5 units of *Taq* DNA polymerase. The thermal program consisted of 1 cycle of  $94^\circ\text{C}$  for 2 min, 25 cycles of  $94^\circ\text{C}$  for 30 s,  $58^\circ\text{C}$  for 30 s, and  $72^\circ\text{C}$  for 2 min followed by 5 min at  $72^\circ\text{C}$ . First, the PCR fragments F1 and F2 were cloned between the XbaI and HindIII sites of pBSKII(-), and then the trimethoprim-resistant cassette excised from pwFRT-Tp<sup>rc</sup> was introduced into the BamHI site to flank F1 to F2 and yield the mutagenesis vector pBCMu.

**pMCSpgaBCD construction.** A 558-bp DNA fragment containing the PC-s12 promoter of pwFRT-Tp<sup>rc</sup> and a 669-bp genomic DNA fragment containing a 5' segment of pgaB of *B. multivorans* C5393 were amplified by PCR, as described above, using the primer pairs PCS12-F (5' TATAATCTCGAGTCTAGATGATTCCCTTTGTCAACAGCAATGG 3')-PCS12-R (5' TATAATAAGCTTGTC GAATCTTCTTGTAATCTATTATGGCG 3') and P4F (5' TATAATAAG CTTCATGCAATCCAGACGGACCTTC 3')-P4R (5' GTTGCGCACGCGT GCGTGAATTCCTCGTC 3'), respectively, and cloned between the XhoI and EcoRI sites of pBSKII(-) to produce the vector pPCS12pgaB5'. A 3,351-bp genomic fragment containing the 1,419 bp at the 3' end of pgaB and pgaCD of *B. multivorans* C5393 was amplified by PCR, as described above, as three fragments using the primer pairs P5F (5' GACGAGGAATCCACGCACGCGTCCG CAAC 3')-P5R (5' TCAGATCCTCGTAGATCTCTCT 3'), P6F (5' TCGAGG AGATCTACGAGGATCTG 3')-P6R (5' CGACGGGTACCAGACGGTGT CAAG 3'), and P7F (5' ACACCGTCTGGTACCCGTCGC 3')-P7R (5' TAA TATAAGCTTAGTCTGATCCGCGATACTCGAG 3') and assembled between the EcoRI and XhoI sites of pBSKII(-) with several subcloning steps to produce pPgaBCD. The EcoRI-XbaI fragment of plasmid pPCS12pgaB5 containing PC-s12 promoter and 5' region of pgaB gene and EcoRI-HindIII fragment of plasmid pPgaBCD containing 3' region of pgaB gene and pgaCD was cloned in between XbaI-HindIII sites of the vector pMCS5 to produce the final complementation plasmid, pMCSpgaBCD.

**Construction of the pgaBC mutant strain.** *Burkholderia multivorans* C5393 was transformed with pBCMu by electroporation, and the transformants were plated on solid TSB medium containing 100 mg/liter trimethoprim. Genomic DNA was extracted from the trimethoprim-resistant colonies and analyzed by PCR to confirm replacement of pgaBC by the trimethoprim resistance marker carried on the plasmid. The PCR was carried out with the primers P3F (5' GCTGGCCG ACAACAGCTTC 3'), which anneals with the pgaA genomic sequence upstream of the pgaA sequence carried on the transformed plasmid, and P3R (5' TGAA GCTAATTCGAGCTCGGTAC 3'), which anneals with the trimethoprim resistance cassette.

**Genetic complementation.** The PNAG-negative *E. coli* strain TRXWGMΔC ( $\Delta$ pgaC) was transformed with plasmid pMCSpgaBCD. Crude exopolysaccharide was extracted from both *E. coli* TRMGΔCpgaBCD and *E. coli* TRXWGMΔC, which had been cultured under the same conditions, and examined for the presence of PNAG by immunoblot analysis.

**Biofilm assay.** Biofilms were assayed by crystal violet staining, as described previously (15). The overnight-grown cultures were diluted 100 times in TSB or BHI medium in the presence and absence of dispersin B at 100  $\mu\text{g/ml}$  concentrations. Aliquots of cells were transferred to the wells of a 96-well microtiter plate (Corning Inc., New York, NY), and the plate was incubated at 37°C for 48 h. Biofilms were washed with water and then stained for 15 min with 200  $\mu\text{l}$  crystal violet. Stained biofilms were rinsed with water, and the amount of biomass was quantified by destaining the biofilm with 200  $\mu\text{l}$  of 33% acetic acid and then measuring the absorbance at 630 nm. To determine the effect of dispersin B on biofilm dispersal, 200  $\mu\text{l}$  of diluted overnight culture was added to each well of a 96-well microtiter plate, and the plates were incubated at 37°C for 18 h. After incubation, the planktonic cells were removed, and 200  $\mu\text{l}$  of dispersin B (100  $\mu\text{g/ml}$ ) or water (controls) was added to the wells. The plates were further incubated at 37°C for 6 h. The biofilm dispersal was determined by measuring the absorbance of crystal violet-stained biofilm at 630 nm. At least six replicates were carried out for each sample, and each experiment was performed at least three times. The results are presented as averages and standard deviations from three or more experiments. Statistical analysis was performed using Student's *t* test. *P* values of  $\leq 0.01$  were considered statistically significant.

**Biofilm killing assay.** Biofilms were grown in 1.5-ml polypropylene microcentrifuge tubes as described previously (13). Tubes were filled with 200  $\mu\text{l}$  of inoculum (diluted 100 times in fresh TSB). After 16 h of incubation at 37°C, the broth was aspirated and replaced with fresh broth containing 0 and 250  $\mu\text{g/ml}$  tobramycin (4 times the MIC). After 3 h, the cells were vortexed, pelleted, and rinsed with sterile saline three times to remove tobramycin. Cell pellets were resuspended in saline by vortexing, and the number of CFU/ml was determined by serial dilution plating.

**Confocal microscopy.** Biofilm staining and confocal scanning laser microscopy (CSLM) was performed as described previously (3). For biofilm growth, plastic coverslips were individually placed in each well of a 12-well tissue culture plate, 2 ml inoculated broth was added to each well, and the plate was incubated at 37°C for 16 h. Biofilms were grown in the presence and absence of 200  $\mu\text{g/ml}$  dispersin B. Biofilm growth on plastic coverslips was stained with wheat germ agglutinin–Alexa Fluor 488 conjugate, which selectively binds to *N*-acetylglucosamine and gives green fluorescence. The biofilm was observed by using an Olympus IX-70 with an argon laser for excitation at 488 nm (green fluorescence). Images were captured and thickness was determined by using Fluoview software and Image Pro Plus software.

## RESULTS

***pgaABCD* locus and PNAG polysaccharide production.** By performing a BLAST search, we identified four open reading frames in *B. cenocepacia* J2315 and *B. multivorans* ATCC 17616 that encode proteins with high levels of similarity to the *E. coli* proteins PgaA, PgaB, PgaC, and PgaD. Percents similarity and identity were calculated by using EMBOSS, an online pairwise sequence alignment tool from EMBL-EBI (<http://www.ebi.ac.uk>). Based on the similarity and identity of this locus with *E. coli pgaABCD* and the genetic as well as biochemical data below, we believe that this locus is *pgaABCD* and is responsible for the synthesis of PNAG in *Burkholderia* spp. We constructed *B. multivorans* Mu5  $\Delta\text{pgaBC}$ , an isogenic mutant in which *pgaBC* is replaced with a trimethoprim resistance cassette, in order to confirm that the production of PNAG polysaccharide requires these genes in *B. multivorans*. *pgaB* encodes a predicted 695-amino-acid protein with a putative polysaccharide deacetylase domain. *B. multivorans* PgaB shares 56% similarity and 38% identity with *E. coli* PgaB. *E. coli* PgaB is predicted to be a periplasmic lipoprotein that, along with PgaA, is necessary for PNAG export (11). *pgaC* is predicted to encode a 423-amino-acid *N*-glycosyltransferase, which is thought to catalyze the first committed step in the synthesis of PNAG. *B. multivorans* PgaC shares 64% similarity and 45% identity with *E. coli* PgaC. While the immunoblot analysis of polysaccharide extract from wild-type *B. multivorans* biofilm showed the presence of PNAG, there was no detect-

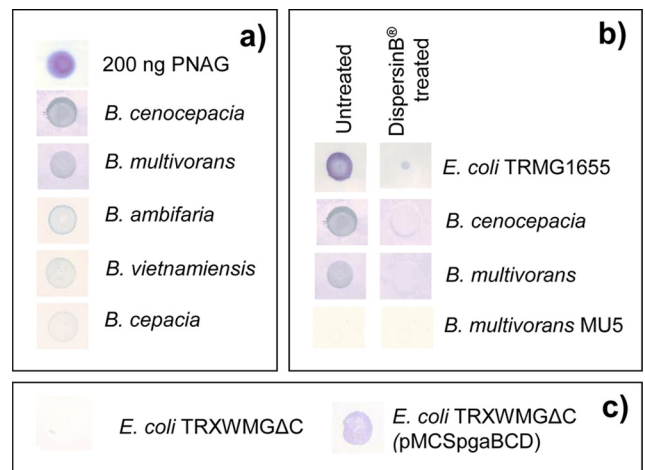


FIG. 1. Immunological detection of cell-bound PNAG. (a) Crude exopolysaccharide extracted from *Burkholderia* spp. (b) Effect of dispersin B (25  $\mu\text{g}$ ) on PNAG from crude extracts of *B. cenocepacia* and *B. multivorans*. Crude exopolysaccharide extracted from *E. coli* TRMG1655 was used as a positive control. (c) Effect of complementation of *E. coli* TRXWVG $\Delta$ C ( $\Delta\text{pgaC}$ ) with pMCSpgaBCD on PNAG in exopolysaccharide extract.

able PNAG in the polysaccharide extract of Mu5 (Fig. 1a and b). The involvement of *Burkholderia pgaABCD* genes in PNAG production was further confirmed by the observation that the restoration of PNAG production after genetic complementation of PNAG-negative *E. coli* TRXWVG $\Delta$ C ( $\Delta\text{pgaC}$ ) with plasmid-carrying genes under the expression signals of PC-s12 promoter (Fig. 1c). Our attempts to complement *B. multivorans* Mu5 ( $\Delta\text{pgaBC}$ ) with a plasmid expressing *B. multivorans* C5393 *pgaBCD* were hampered due to the very high levels of antibiotic resistance of *Burkholderia* strains (data not shown). When we compared the biofilm dispersal effect of dispersin B on wild-type *B. multivorans* biofilm with that on Mu5 using a 96-well biofilm dispersal assay, it was found that dispersin B dispersed biofilm only in the wild-type strain (Fig. 2). In addition, the sensitivity of biofilm in wild-type *B. multivorans* and Mu5 to 250  $\mu\text{g/ml}$  of tobramycin was determined in a biofilm killing assay (Table 2). Tobramycin showed 3.25 and 5.1  $\log_{10}$  reductions in biofilm-embedded cells of the wild type and Mu5, respectively. The 2- $\log_{10}$ -greater reduction in the biofilm-embedded cells of Mu5 than that of the wild type indicates an increased sensitivity of the Mu5 biofilm to antibiotics. This indirectly suggests that the *pgaBC* mutant did not produce PNAG and that PNAG is involved in forming normal biofilm, which confers antibiotic resistance.

**Detection of PNAG polysaccharide component.** To detect the PNAG that is synthesized by the *pgaABCD*-encoded proteins in *Burkholderia* spp., we used *E. coli* PNAG-specific MAb (11) for immunoblot analysis of purified polysaccharides from five *Burkholderia* spp. In this experiment, a known quantity of highly purified PNAG from *E. coli* TRMG 1655 (*csrA::kan*) was used as a positive control (11). The immunoblot signals showed the presence of PNAG in the polysaccharide extracts of all five *Burkholderia* spp. tested (Fig. 1a). The presence of PNAG was further confirmed by the observation that the positive immunoblot signals of *B. cenocepacia* and *B. multivorans*

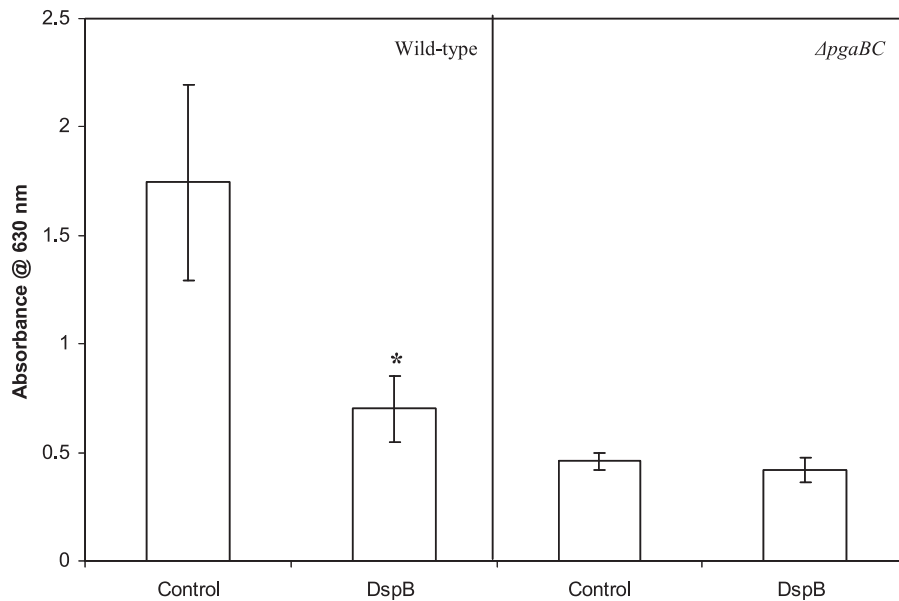


FIG. 2. Comparison of the biofilm dispersal effect of dispersin B on wild-type *B. multivorans* with that on the *pgaBC* mutant. Values are means  $\pm$  standard deviations from two experiments with six replicates per sample. \*,  $P < 0.01$  compared with untreated control biofilm.

disappeared when the polysaccharide extracts were treated with dispersin B (Fig. 1b), which specifically hydrolyzes PNAG (11). However, in all five species of *Burkholderia*, detection of PNAG was possible only from highly concentrated preparations of polysaccharide.

**Role of PNAG in *Burkholderia* biofilm formation.** We tested both the inhibitory and dispersal effects of the PNAG-hydrolyzing enzyme dispersin B on biofilm formation and preformed biofilms, respectively, in *B. multivorans*, *B. vietnamiensis*, *B. cepacia*, and *B. cenocepacia*. All of these bacteria formed substantial biofilms in the control wells of a 96-well microtiter plate under the assay conditions. Dispersin B at 100  $\mu$ g/ml showed a significant inhibitory effect ( $P < 0.01$ ) on the biofilm formation of all *Burkholderia* spp. (Fig. 3a). Additionally, there were no discernible differences in the planktonic growth of *Burkholderia* spp. tested in the presence or absence of dispersin B (data not shown). When dispersin B was tested against preformed biofilms in these organisms, the enzyme dispersed significantly more biofilm ( $P < 0.01$ ) than the control at 37°C in all the test organisms (Fig. 3b).

**CSLM analysis of wild-type *B. multivorans* and Mu5 biofilms.** CSLM was used to examine the morphological features

of biofilms formed by wild-type *B. multivorans* and Mu5 after 16 h of growth. In addition, the wild-type biofilm grown in the presence of 200  $\mu$ g/ml dispersin B was analyzed by CSLM. As shown in Fig. 4a and b, wild-type *B. multivorans* formed a multilayered biofilm which was inhibited by 200  $\mu$ g/ml dispersin B. Conversely, Mu5 biofilm adhered poorly to the coverslip and showed loosely attached as well as scattered cells across the coverslip (Fig. 4c). To confirm the CSLM observations of biofilm structure, Fluoview image analysis software was used to evaluate the thickness of the biofilm. The biofilm produced by wild-type *B. multivorans* was thicker ( $39.11 \pm 1.7 \mu$ m) than dispersin B-treated biofilm ( $22.71 \pm 1.87 \mu$ m) and the biofilm produced by Mu5 ( $15.68 \pm 0.46 \mu$ m). Note that there was no apparent difference in the growth of the wild-type and mutant strains (data not shown).

## DISCUSSION

We confirmed that *pgaABCD* locus in *Burkholderia* spp. shares sequence similarity with and is functionally related to the *pga* locus in *E. coli*, which is required for the synthesis of PNAG polysaccharide and biofilm formation. The replacement of *pgaB* and *pgaC* genes with the  $Tp^r$  cassette in wild-type *B. multivorans* resulted in a *B. multivorans* mutant, Mu5, defective for PNAG production, and PNAG production was restored in PNAG-negative *E. coli* TRXWMG $\Delta$ C ( $\Delta$ *pgaC*) after complementation with *B. multivorans pgaBCD*, as determined by immunoblotting. Compared to the strong immunoblot signal from polysaccharide extract of wild-type *B. multivorans* biofilm, Mu5 biofilm polysaccharide extract had an undetectable signal, indicating that the mutant did not produce PNAG. This confirmed that the *pgaABCD* locus is needed for the biosynthesis of PNAG polysaccharide component of *Burkholderia* biofilm. Additional confirmation that Mu5 does not produce PNAG because of the replacement of the *pgaBC* locus

TABLE 2. Sensitivity of *B. multivorans* wild-type and PNAG-negative Mu5 ( $\Delta$ *pgaBC*) biofilms to 250  $\mu$ g/ml tobramycin

Strain	Mean log <sub>10</sub> CFU of biofilm-embedded cells/ml $\pm$ SD with:		Log reduction due to tobramycin treatment <sup>a</sup>	<i>p</i> <sup>b</sup>
	No treatment (control)	Tobramycin		
Wild type	9.95 $\pm$ 0.27	6.7 $\pm$ 0.08	3.25	0.001
Mu5	8.87 $\pm$ 0.13	3.77 $\pm$ 0.16	5.1	<0.0001

<sup>a</sup> Mean log density for untreated control minus the mean log density for corresponding tobramycin treatment.

<sup>b</sup> Determined using two-tailed Student's *t* test with unequal variance.

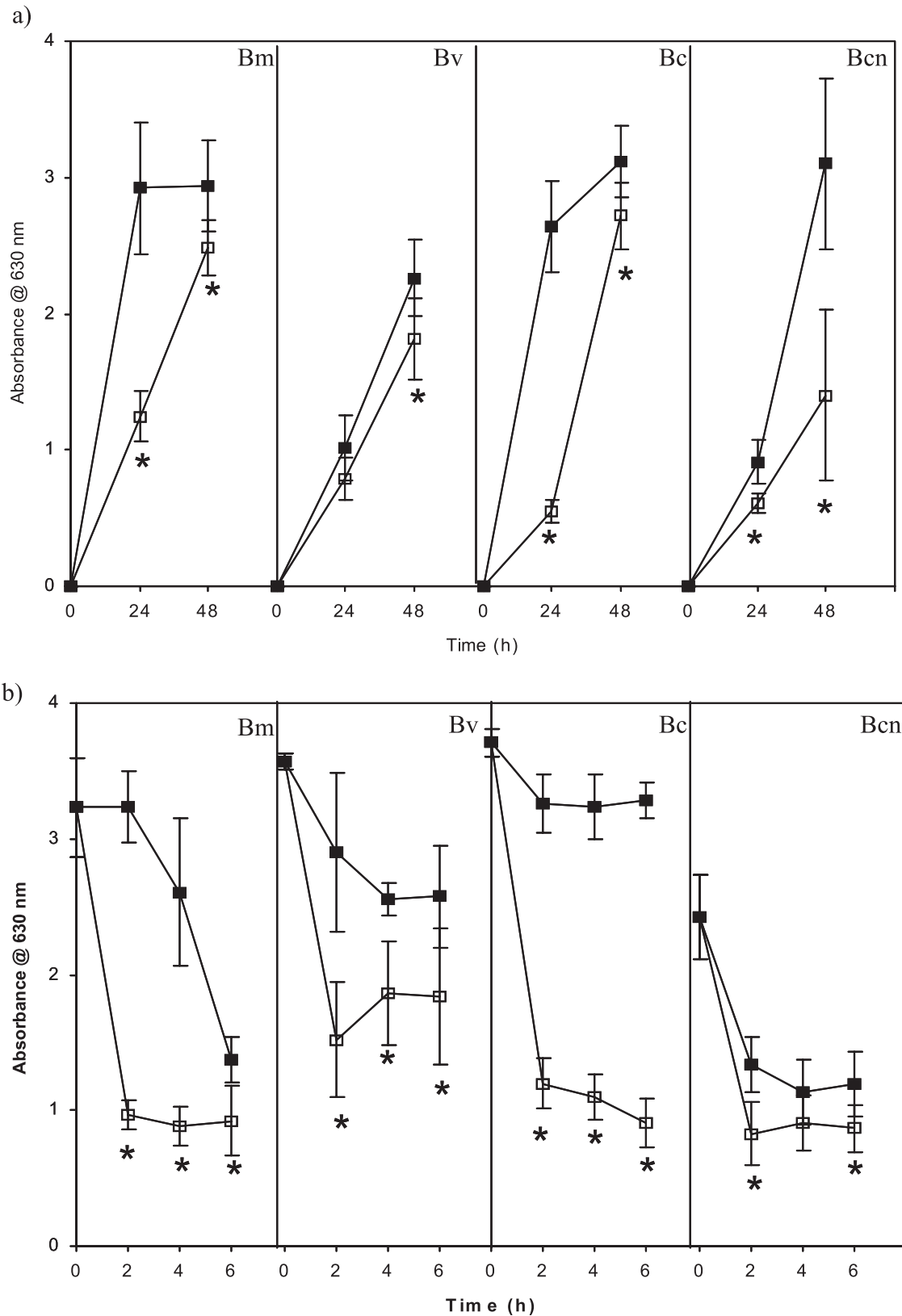


FIG. 3. Effect of untreated control (■) and dispersin B (□) on biofilm formation (a) and biofilm dispersal (b) in *B. multivorans* (Bm), *B. vietnamiensis* (Bv), *B. cepacia* (Bc), and *B. cenocepacia* (Bcn). The values are means ± standard deviations. \*,  $P < 0.01$  compared with untreated control biofilm.

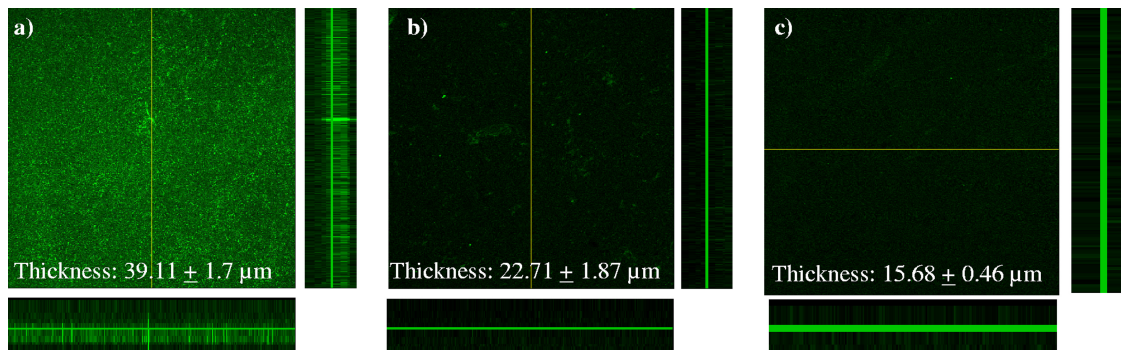


FIG. 4. Confocal image of *B. multivorans* biofilm formation on plastic coverslip. (a) Wild-type biofilm in the absence of dispersin B; (b) wild-type biofilm in the presence of 200  $\mu\text{g/ml}$  dispersin B; (c) *B. multivorans* Mu5 ( $\Delta\text{pgaBC}$ ) biofilm in the absence of dispersin B. The images are maximum projections or reconstructed confocal stacks consisting of a series of  $xy$  (center),  $yz$  (left), and  $xz$  (bottom) sections. A representative CSLM image for each sample is shown.

with the  $\text{Tp}^{\text{f}}$  cassette was obtained by showing that the PNAG-hydrolyzing enzyme dispersin B does not disperse Mu5 biofilm. Furthermore, because it lacks PNAG, Mu5 produced a weaker biofilm and was therefore significantly more sensitive to tobramycin than the wild type ( $P < 0.0001$ ), suggesting a role for PNAG in antibiotic resistance of *Burkholderia*. Furthermore, confocal microscopy of the Mu5 biofilm showed that it was poorly developed and thin compared to the multilayered thick biofilm of the wild type, suggesting that the *pgaABCD* locus is required for the synthesis of a PNAG polysaccharide component, which may play a critical role in the development of *Burkholderia* biofilms.

An immunoblot analysis of polysaccharide extracts from *Burkholderia* spp. biofilms using a PNAG-specific MAb confirmed the presence of PNAG polysaccharide, which appears to be a minor component compared to the major polysaccharide cepacian in the *Burkholderia cepacia* complex (6). The presence of PNAG in *Burkholderia* biofilms was further confirmed by treating the polysaccharide extracts with the PNAG-hydrolyzing enzyme dispersin B, which led to the disappearance of immunoblot signals. Further evidence for the presence of PNAG in *Burkholderia* biofilms was obtained by showing that dispersin B inhibited and dispersed biofilms in *Burkholderia* spp. More importantly, the inhibition of *Burkholderia* biofilm formation by the PNAG substrate-specific enzyme dispersin B provides evidence that PNAG is needed for biofilm formation in *Burkholderia* spp. Interestingly, studies performed on cepacian-defective mutants have demonstrated that, although not required for the initiation of biofilm formation, cepacian is required for the formation of thick and mature biofilms (6). Our studies seem to suggest that PNAG in *Burkholderia* is needed for initiating biofilm formation. The confocal microscopy of untreated and dispersin B-treated *B. multivorans* biofilms showed multilayered and disrupted biofilms, respectively, further demonstrating that PNAG, though a minor polysaccharide component, plays an important role in forming and maintaining the integrity of *Burkholderia* biofilms.

The ability of many pathogens to adhere to human tissues and medical devices and form biofilms is a major virulence factor that correlates with an increase in antibiotic resistance, reduced phagocytosis, and overall persistence of the bacterial population. Of all the different molecules identified as biofilm

components, PNAG is perhaps the most important and widely conserved factor (17, 28). As this surface polysaccharide is a known virulence factor in various staphylococcal infections and is a target for developing vaccines (9), PNAG production may have a role in the pathogenesis of *Burkholderia* infection. *Burkholderia* infection has been shown to be an important threat for CF patients, owing to its association with a dramatic increase in symptoms and decline in pulmonary function. This is in addition to causing a potentially fatal necrotizing pneumonitis with bacteremia called “cepacia syndrome” in infected individuals. Multidrug resistance (MDR) and patient-to-patient transmission are other possible consequences of *Burkholderia* infection, and they contribute to increased morbidity and mortality in CF patients. PNAG-specific immunotherapies or PNAG-hydrolyzing dispersin B–antibiotic combinations could provide effective approaches and tools for combating CF-associated *Burkholderia* infections, which pose a significant challenge for current antibiotic therapies.

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