9-2006

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JOURNAL OF BACTERIOLOGY, Sept. 2006, p. 6680–6687 Vol. 188, No. 18
0021-9193/06/$08.00+0 doi:10.1128/JB.00605-06

Expression of the Primary Carbohydrate Component of the *Bordetella bronchiseptica* Biofilm Matrix Is Dependent on Growth Phase but Independent of Bvg Regulation†

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Received 28 April 2006/Accepted 2 July 2006

We previously showed that the Bvg virulence control system regulates biofilm formation in *Bordetella bronchiseptica* (Y. Irie, S. Mattoo, and M. H. Yuk, J. Bacteriol. 186:5692–5698, 2004). Analyses of the extracellular components of *B. bronchiseptica* biofilm matrix revealed that the major sugar component in the matrix was xylose, and linkage analysis indicated a majority of it to be in a 4-linked polymeric form. The production of xylose was independent of Bvg regulation but instead was dependent on bacterial growth phase. In addition, *N*-acetyl-glucosamine in the matrix was found to be important for the initial development of the biofilm. These results suggest that *B. bronchiseptica* biofilm formation is growth phase dependent in addition to being regulated by the Bvg virulence system.

A majority of bacterial species can grow in a multicellular surface-associated community, widely referred to as a biofilm, and these communities may be more representative of microbial growths in their natural settings (11). Virtually all biofilms studied have an extracellular matrix encasing the bacterial cells, and the regulation of matrix formation appears to be critical in the development of biofilms (3). Biofilm matrices have been found to contain various organic materials, including DNA (43), lipids (40), dead cells, and other debris (42), but the key components are proteins and polysaccharides (40). The importance of extracellular polysaccharides and their components have been extensively analyzed in several bacterial species.

Pseudomonas aeruginosa biofilm matrix has been extensively characterized. Early studies showed that many *P. aeruginosa* strains isolated from cystic fibrosis patients conferred a mucoidy phenotype on agar plates, and this was the result of the overproduction of alginate (9). Alginate was hypothesized to be a major component of *P. aeruginosa* biofilm matrix. However, biofilms of nonmucoid *P. aeruginosa* strains did not contain alginate, and mutations in the genes required for alginate biosynthesis had little or no effect on in vitro biofilm formation (46). Instead, the *pel* and *psl* loci were recently characterized to play major roles in the biosynthesis of extracellular polysaccharides in the biofilms of this species (10, 17, 26). Poly-*N*-acetyl-glucosamine polymers are found in biofilm matrices in a large variety of bacterial species, including *Staphylococcus* species and *Escherichia coli* (24, 25, 41), and their biosynthetic pathways are highly conserved. Cellulose is also an important component of *E. coli* and *Salmonella enterica* serovar Typhimurium biofilm matrix (48). These various studies also demonstrate the complexity of the carbohydrate composition of the various biofilm matrices, which almost always contain a number of different sugar residues. Different environmental conditions for different species evidently affect biofilm formation, and matrix contents are also likely to be equally dynamic. The exact roles of polysaccharides in the development and function of biofilm matrices are also speculative, although it is generally thought that they can increase the adhesive properties of the cells to aid in the surface-associated growth (3). Once biofilm development has matured, the matrix may be important in preserving the structural integrity. It has also been proposed that the presence of the matrix may be important in achieving pH homeostasis (40). The matrix may also provide a strong barrier to such insults as antibiotics, resulting in a common observation that bacterial biofilms are more resistant to most antibiotics than planktonic cells (8).

Bordetella bronchiseptica is a gram-negative bacterial species that chronically infects the respiratory tract of a wide range of mammals. A majority of its virulence determinants are controlled by a two-component signal transduction system, BvgAS (for *Bordetella* virulence gene). In the virulent Bvg^+ phase, the response regulator BvgA becomes highly phosphorylated, and the transcription of various Bvg-activated genes confers on the bacterium its virulent phenotype. Virulence factors expressed in the Bvg^+ phase include adhesins such as filamentous hemagglutinin (FHA) and fimbriae as well as toxins/toxin delivery systems such as adenylate cyclase/hemolysin (CyaA) and type III secretion system. Unphosphorylated BvgA in the avirulent Bvg^- phase is unable to transcriptionally activate such genes (4). In between the transition of Bvg^+ and Bvg^- phases there appears to be at least one other distinct Bvg phase, called Bvg intermediate (Bvg^i) phase. There are specific genes that are only expressed in the Bvg^i phase (7), such as *bipA* (38). Moreover, adhesins such as FHA and fimbriae are also expressed in the Bvg^i phase in addition to the Bvg^+ phase. However, toxins such as CyaA are expressed in the Bvg^+ phase but not the Bvg^i

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† Supplemental material for this article may be found at http://jb.asm.org/.

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
B. bronchiseptica		
RB50	Wild-type sequence strain	5
RB53	*bvgS*-C3, Bvg^+ phase-locked derivative of RB50	5
RB53i	*bvgS*-C3, *bvgS*-I1 Bvg^i phase-locked derivative of RB50	6
RB54	Δ*bvgS*, Bvg^- phase-locked derivative of RB50	5
RB58	Δ*cyaA* derivative of RB50	13
RB53i GFP	RB53i carrying pCC5	This study
RB53i YFP	RB53i carrying pCC2	This study
E. coli		
DH5α	Cloning strain	BRL, Gaithersburg, MD
SM10λpir	Conjugation donor strain	28
C. crescentus CB15N *xylX*::P_{xyl}-*gfp*	CB15N chromosomal *xylX*::*gfp* fusion	This study
Plasmids		
pCW09		44
pCC2		This study
pCC5		This study

phase. We previously showed that FHA is required for *B. bronchiseptica* biofilm formation and proposed that the expression of CyaA suppresses biofilm via its association with FHA. This results in the biofilm phenotype being predominantly expressed only when *B. bronchiseptica* is grown in the Bvg^i phase but not in the Bvg^+ nor Bvg^- phase (15).

In this study, we examined the chemical composition of the extracellular matrix of *B. bronchiseptica* biofilm, with particular focus on the carbohydrate components. We found that xylose is the major sugar residue in the matrix, a unique characteristic compared to other known bacterial biofilm matrices. While the protein factors that regulate biofilm formation in *B. bronchiseptica* (FHA and CyaA) are Bvg regulated, the production of the polysaccharides required for biofilm formation do not appear to be under Bvg control. However, the expression of xylose is primarily determined by the growth phase of the bacteria. These observations suggest that multiple levels of regulation are involved in biofilm formation in *B. bronchiseptica*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains used in this study are listed in Table 1. All *Bordetella* strains were grown on Bordet Gengou agar (Becton Dickinson) supplemented with defibrinated sheep blood and Stainer-Scholte (SS) liquid medium (37) at 37°C. *E. coli* strains were propagated in Luria-Bertani medium unless otherwise specified. *Caulobacter crescentus* strains were grown in peptone-yeast extract (PYE complex medium) at 30°C.

Construction of fluorescent *B. bronchiseptica* strains. Plasmids pCC2 and pCC5 were constructed by replacing *lacZYA* in plasmid pCW09 (44) with the genes (exclusive of their promoters) encoding yellow fluorescent protein (YFP) and green fluorescent protein (GFP), respectively. In the resulting plasmids, expressions of the YFP- and GFP-encoding genes are driven by a mutated *bipA* promoter that is expressed at very high levels under both Bvg^i- and Bvg^+-phase conditions (P. A. Cotter, unpublished data). These plasmids were introduced into RB53i by conjugation where they integrated into a nonessential region of the chromosome by homologous recombination. The genetic organization of the resulting cointegrate strains was confirmed by PCR with primers CWBpGR and CWSSHR3 (44).

Biofilm growth conditions. *B. bronchiseptica* biofilms were grown by three different methods depending on the experiments. For matrix preparations, cultures were grown in polystyrene tubes in a standard roller drum condition. For quantitative crystal violet assays and enzyme-linked lectin-sorbent assays (ELLA), biofilms were grown in a 96-well plate format as previously described (15). For microscopic analyses, biofilms were formed in non-tissue-culture-coated glass chamber slides (BD Falcon) as previously described (16).

Matrix preparations. The protocol for biofilm matrix preparation was modified from a previously described method (45). Virtually all cells of *B. bronchiseptica* grown in conditions supporting biofilm formation in polystyrene tube cultures are surface attached, leaving the liquid media completely clear (15). Biofilms were scraped out of the tubes and resuspended in 16-fold (wt/vol) sterile 0.14 M NaCl and vortexed with glass beads. Supernatants were collected upon centrifugation and filtration through sterile 0.22-μm-pore cellulose acetate membrane (Corning). Filtrates were dialyzed against deionized water and lyophilized by freeze drying.

Analyses of matrix. Glycosyl composition analyses were performed as previously described (27, 47) by combined gas chromatography/mass spectrometry (GC/MS) of the per-*O*-trimethylsilyl derivatives of the monosaccharide methyl glycosides produced from the samples by acidic methanolysis. For glycosyl linkage analyses, samples were permethylated, depolymerized, reduced, and acetylated, and the resultant partially methylated alditol acetates were analyzed by GS/MS (47). Quantification of double-stranded DNA (dsDNA) and proteins in the matrix were done with a PicoGreen dsDNA Quantitation kit (Molecular Probes) and D_C Protein Assay (Bio-Rad), respectively, according to the manufacturers' protocols.

In situ lectin staining of biofilms. Mature *B. bronchiseptica* biofilms grown for 24 h in chamber slides were washed three times with phosphate-buffered saline (PBS). Fluorescent dye-conjugated lectins (10 μg/ml; Molecular Probes) in 300 μl PBS were incubated with the samples for 30 min at room temperature. The samples were subsequently washed three times with PBS, and the top chambers were removed. Deconvolution micrographs of the stained biofilms were taken as previously described (16).

Enzyme-linked lectin-sorbent assays (ELLA). ELLA procedures were carried out as previously described (23), using Vaccu-Pette/96 (Scienceware) for the wash steps. The experiments were done in sextuplicate, and the error bars represent the standard deviations of the collected data.

Dispersin B digestion. *B. bronchiseptica* biofilms were treated with poly-*N*-acetyl-glucosaminidase from *Actinobacillus actinomycetemcomitans* (dispersin B, 1,000 U/mg) (18, 19) by two methods, with nearly identical results. Wells of microtiter plates were pretreated with 50 μg/ml dispersin B in 150 μl SS medium at room temperature for 1 h, and the solution was removed by inverting the plate before *B. bronchiseptica* inoculation. Alternatively, 50 μg/ml dispersin B was added simultaneously upon bacterial inoculation. Biofilms were allowed to form for only 6 h before crystal violet quantitation. Dispersin B was inactivated by heat killing at 95°C for 40 min.

Xylose bioassay. A *C. crescentus* xylose bioassay strain was grown in PYE plus 5 μg/ml kanamycin at 30°C with aeration overnight prior to the assay. Sugar solutions were filtered through sterile 0.22-μm-pore polyvinylidene difluoride (PVDF) membranes (Millipore) and serially diluted. Likewise, *B. bronchiseptica* cultures were centrifuged, and the supernatants were filtered through the PVDF

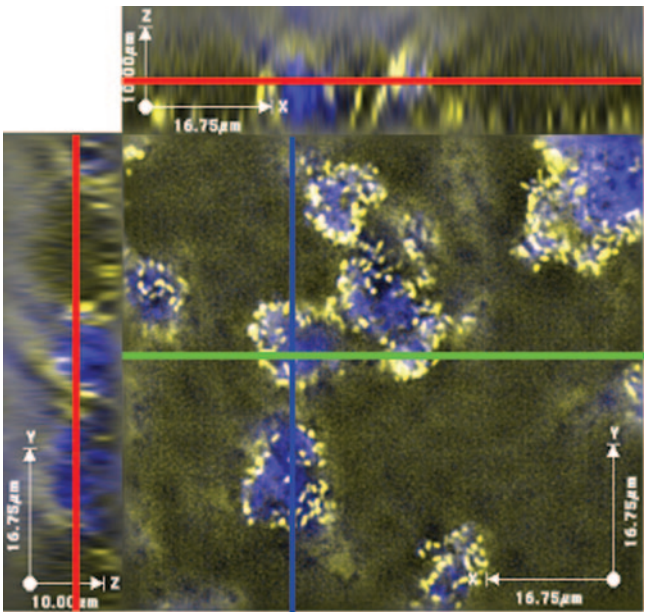

FIG. 1. Deconvolution micrograph of *Bordetella bronchiseptica* biofilm grown on a glass chamber slide stained with Alexa Fluor 350-conjugated lectin wheat germ agglutinin (WGA). Recombinant *B. bronchiseptica* expressing YFP is shown in yellow, and WGA is shown in blue. The central picture shows a horizontal section while two flanking pictures show vertical sections. The blue line and green line indicate positions of the *y-z* section and *x-z* section, respectively. Red lines indicate positions of the horizontal section.

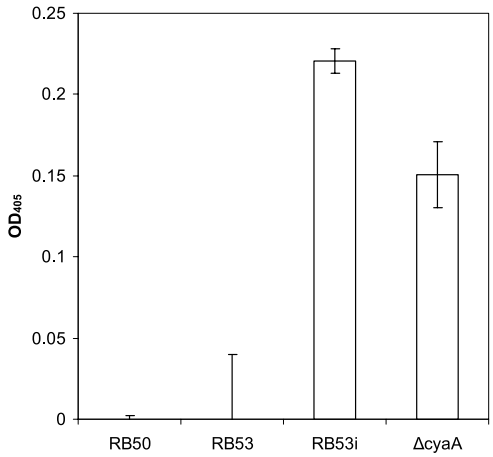

FIG. 2. Quantitative analysis of *Bordetella bronchiseptica* biofilm matrix with WGA ELLA. Bacteria were grown in 96-well plates for 24 h, and ELLA was performed as described in Materials and Methods. The Bvg^i phase-locked strain (RB53i) and the Δ*cyaA* mutant form biofilms, while the wild-type bacteria grown in Bvg^+ phase (RB50) and Bvg^+ phase-locked (RB53) strains do not (15). The strong binding of WGA to the wells containing the biofilm-forming strains indicates the presence of surface-associated *N*-acetyl-glucosamine residues within the biofilm matrix. The absence of WGA binding to the samples containing RB50 and RB53 indicates the absence of matrix material. OD_{405}, optical density at 405 nm.

membranes. Log-phase growth was monitored by measuring and graphing the optical density at 600 nm read-out of the cultures as well as plating the bacteria to verify the measurements. Mid- and late stationary phases were determined at ~24 and ~48 h after inoculation, respectively. Samples were distributed in at least triplicates of 100-μl aliquots per well in a clear non-tissue-culture-coated polystyrene 96-well microtiter plate. Overnight *C. crescentus* bioassay strain culture was added at 1:20 and incubated at 30°C for 2 to 24 h. GFP expression was read by a plate reader through excitation 360 nm and emission 460 nm filters at an excitation wavelength of 485 nm and an emission wavelength of 528 nm. Standard deviations are represented by the error bars in the corresponding figures.

RESULTS

Analyses of the extracellular matrix of *Bordetella bronchiseptica* biofilm reveal that xylose is the primary carbohydrate component. In situ detection of biofilm matrix using specific lectin binding assays has been previously performed on *P. aeruginosa* grown on filter membranes to determine the presence of carbohydrates in these structures (39). We have previously shown that *B. bronchiseptica* cells can form distinct microcolony-like structures on glass surfaces that are typical of biofilms (15, 16). In this study, we used various fluorescently labeled lectins to examine the presence of carbohydrates within the *B. bronchiseptica* biofilm and also to determine the localization of the carbohydrates in relation to the bacterial cells in the biofilm structure. As shown in Fig. 1, wheat germ agglutinin (WGA) bound to the extracellular substances primarily in the interior of the biofilm microcolonies formed by recombinant *B. bronchiseptica* expressing YFP. In addition, we did not observe WGA binding to the bacterial cells, supporting the idea that the lectin is bound to a secreted matrix that is not part of the cellular structure. In situ stainings were also done with other lectins, including *Griffonia simplicifolia* isolectin IB_4 (Fig. S1 in the supplemental material). Similar staining patterns were observed where IB_4 specifically bound only to the extracellular matrix, essentially confirming the presence of the extracellular component of the biofilm that contains complex carbohydrates.

We also used enzyme-linked lectin-sorbent assay (ELLA) for the semiquantitative measurement of extracellular polysaccharide in the *B. bronchiseptica* biofilm. ELLA is similar in principle to enzyme-linked immunosorbent assay, except that labeled lectins instead of antibodies are used as probes (23). ELLAs were directly done on biofilm formed within the 96-well microtiter plate wells to measure the contents of the matrix without disrupting the biofilm. Using WGA as a probe, ELLA detected the extracellular carbohydrates in strains and growth conditions that support biofilm formation (Fig. 2). *B. bronchiseptica* biofilm forms biofilm in Bvg^i phase. However, in the absence of expression of the adenylate cyclase/hemolysin gene (*cyaA*, which normally represses biofilm formation in the Bvg^+ phase), biofilm is formed in both the Bvg^+ and Bvg^i phases (15). Figure 2 shows that the observed ELLA data are consistent with the absence of biofilms in the wild-type bacterium grown in the Bvg^+ phase (RB50) and the Bvg^+ phase-locked strain (RB53), as no surface-bound carbohydrates were detected in these samples. The Bvg^i phase-locked strain (RB53i) and Δ*cyaA* mutant strains formed biofilms as expected and correlated with the large amount of carbohydrates that was detected by ELLA. It also appears that the expression of the polysaccharides required for the formation of the biofilm matrix was not limited to the Bvg^i phase, even though biofilm formation was normally maximal in this phase in the wild-type

TABLE 2. Carbohydrate contents of *B. bronchiseptica* biofilm in Bvg^+ phase and Bvg^i phase

Biofilm matrix	Glycosyl residue	Mol%
RB53i (Bvg^i)	Xylose	69.7
	Mannose	1.9
	Glucose	12.9
	Heptose	1.5
	N-Acetyl-glucosamine	14.0
Δ*cyaA* (Bvg^+)	Xylose	82.1
	Mannose	1.7
	Glucose	8.9
	Heptose	0
	N-Acetyl-glucosamine	7.3

TABLE 3. Linkage analyses of biofilm matrix from Bvg^+ phase and Bvg^i phase

Biofilm matrix	Glycosyl residue	Mol%
RB53i (Bvg^i)	4-Linked xylofuranosyl	73.3
	4-Linked glucopyranosyl	8.5
	2,4-Linked xylofuranosyl	1.4
	Terminal glucopyranosyl	10.1
	Terminal xylofuranosyl	6.8
Δ*cyaA* (Bvg^+)	4-Linked xylofuranosyl	75.3
	4-Linked glucopyranosyl	4.5
	2,4-Linked xylofuranosyl	1.8
	Terminal glucopyranosyl	10.1
	Terminal xylofuranosyl	6.8

bacteria, since polysaccharides could be readily detected in the Bvg^+ phase in the Δ*cyaA* mutant.

To further characterize the contents of the biofilm matrix, glycosyl composition and glycosyl linkage analyses were done on purified matrix samples; the results are shown in Table 2 and Table 3, respectively. Interestingly, xylose was found to be the major component of the biofilm matrix. Linkage analysis indicated that the majority of the xylose polymers were in a 4-linked form, different from a widely characterized plant xylose polymer, xylan. There is also evidence of some branching occurring with the xylose residues. Consistent with the WGA (specific for *N*-acetyl-glucosamine) binding data, *N*-acetyl-glucosamine was also found in the matrix. However, IB_4 has binding specificity to *N*-acetyl-galactosamine end groups and terminal α-galactosyl residues (32), but neither residue was detected in significant quantities in our analyses. This suggests that the IB_4 lectin used in in situ staining may have nonspecific recognition of other sugars in the matrix.

In addition to polysaccharides, some lipids were also detected from the matrix samples by gas chromatography. Fatty acids with chain lengths of 12:0, 14:0, 16:1, 16:0, and 18:0 were the major lipid components from the purified matrix samples (data not shown). Proteins and dsDNA were quantitated from the matrix, measuring at approximately 10 mol% of protein and approximately 0.01 mol% of dsDNA per sample. Since we cannot exclude the presence of normal cellular components in the biofilm matrix samples, there is likely to be present some lipopolysaccharides (LPS) from the outer membrane. However, the major sugar residue detected is not derived from LPS, as xylose is not a component of LPS from *Bordetella* species (36).

An important observation is that the glycosyl composition (Table 2), glycosyl linkage (Table 3), and the lipid composition (data not shown) were not found to be significantly different between samples from biofilms formed in the Bvg^i phase (made by the RB53i phase-locked strain) and that from the Bvg^+ phase (made by a Δ*cyaA* mutant). This is consistent with the aforementioned idea that while overall biofilm formation is regulated by BvgAS systems via differential expression of FHA and CyaA in the Bvg^+ and Bvg^i phases (15), the matrix contents and their expression do not appear to be significantly different in the Bvg^+ and Bvg^i phases.

***N*-acetyl-glucosamine may be critical for the early biofilm development stage.** Poly-*N*-acetyl-glucosamine has been implicated to play a major role in biofilm development for various bacterial species (24, 25, 41). In *Actinobacillus actinomycetemcomitans*, poly-*N*-acetyl-glucosamine is found in the biofilm matrix but may be degraded by the enzyme dispersin B for cellular detachment and dispersal to occur (19). In order to investigate whether the *N*-acetyl-glucosamine found in the *B. bronchiseptica* matrix is important for biofilm formation, biofilms preformed in vitro on polystyrene surfaces were treated with dispersin B and quantitated by crystal violet. Mature biofilms (grown for 24 h) treated with dispersin B did not show any decrease in the amount of attachment to the plastic surface, even at concentrations that were effective for disruption of other bacterial biofilms (data not shown). However, if we pretreated the polystyrene surfaces with dispersin B before inoculation of bacterial cells, biofilm formation was significantly inhibited (Fig. 3). Dispersin B that was heat inactivated did not inhibit biofilm formation. Dispersin B did not inhibit planktonic growth of *B. bronchiseptica* (data not shown). This observation suggests that during the initial phases of biofilm formation, the formation of *N*-acetyl-glucosamine polymers in the matrix is critical to further development of the biofilm structure to its mature form. The ineffectiveness of dispersin B on mature biofilms might indicate that once the mature biofilm structure is formed, its integrity no longer depends on *N*-acetyl-glucosamine linkages or that these are no longer accessible to the enzyme in the mature biofilm structure. The latter

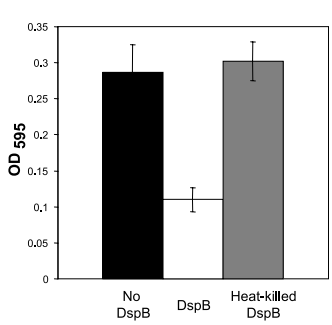

FIG. 3. Crystal violet quantitative assay of 6-h biofilm grown in 96-well plates precoated with dispersin B (DspB). Wells were precoated with 50 μg/ml of DspB and then inoculated with Bvg^i phase-locked *B. bronchiseptica* for growth for 6 h. Biofilm formation was significantly lower in the presence of DspB than the controls without the enzyme and heat-inactivated DspB. OD_{595}, optical density at 595 nm.

may be more likely, as mature biofilms treated with dispersin B demonstrated similar binding to WGA (specific for *N*-acetyl-glucosamine) compared to untreated biofilms (data not shown).

Xylose is produced primarily in the stationary growth phase, but production is not regulated by Bvg. Although the involvement of *N*-acetyl-glucosamine may be important in building a biofilm, xylose is the major component of the *B. bronchiseptica* matrix, and understanding its roles will be critical for studying the development of *B. bronchiseptica* biofilm. There is no described or published lectin that has a binding specificity to xylose. A convenient method for detecting xylose was required, and we developed a quantitative xylose detection bioassay. A *Caulobacter crescentus* strain was constructed containing a xylose-inducible promoter controlling the expression of a *gfp* gene so that in the presence of xylose, GFP fluorescence could be quantitatively assessed. As shown in Fig. 4A, strong GFP signals were detected from this strain in a xylose concentration-dependent manner. Other sugars, such as glucose and *N*-acetyl-glucosamine, do not induce GFP expression at any concentration. Besides xylose monomers, polymers of xylose can also be detected by this *Caulobacter* strain (data not shown), presumably after degradation to monomers by enzymes from the bacteria. The *C. crescentus* bioassay strain therefore allows for a reliable xylose-specific detection method.

To determine if xylose production in *B. bronchiseptica* was regulated by Bvg, mutant strains of the bacteria locked at various specific Bvg phases were tested for xylose production (Fig. 4B). The bacteria were grown overnight into stationary phase, and all the supernatants of bacteria grown at any Bvg phase were found to contain large amounts of xylose, indicating that xylose production was Bvg independent. However, supernatants of bacteria taken at different stages of their growth phases displayed an increase in xylose content, particularly in the stationary phase (Fig. 4C). Comparison of fluorescence units normalized to CFU (Fig. 4C, bottom table) indicates that xylose production increases significantly in the stationary phase compared to the log phase, and the increase of xylose content in the medium is not simply an accumulative effect. Xylose expression also appeared to be Bvg independent, since the xylose production profiles of *B. bronchiseptica* grown in all phases were identical to each other.

DISCUSSION

We have previously shown that biofilm formation in *Bordetella bronchiseptica* is regulated by the Bvg virulence control system and that the biofilm phenotype is maximal in the Bvg^i phase (15). This is in slight variance from another report (29), but our results have been consistent and correlate well with the observation that FHA (expressed in both Bvg^i and Bvg^+ phases) is required for biofilm formation, while CyaA (expressed only in the Bvg^+ phase) represses this phenotype. The presence of extracellular polysaccharides is one of the hallmarks of all described microbial biofilms (3). In this study, we characterized the biofilm matrix of *B. bronchiseptica* and found that it primarily consists of polysaccharides, although lipids, DNA, and proteins are also present. Interestingly, the major composition of the polysaccharides was 4-linked xylose polymers.

Eukaryotes have previously been characterized to synthesize xylose by a conserved metabolic mechanism from yeast, plants, and mammals. Synthesis routes involve two-step enzymatic activities of UDP-glucose dehydrogenase, which converts UDP-glucose to UDP-glucuronic acid, and UDP-glucuronate decarboxylase, which converts UDP-glucuronic acid to UDP-xylose (2, 12, 31). BLAST searches of the *Bordetella* genome revealed no obvious homologues of either of the enzymes, suggesting prokaryotes may utilize a different metabolic pathway or functional analogues with few sequence similarities. Although small quantities of xylose have been detected in specific growth conditions of some bacteria (46), literature searches did not reveal any reports that prokaryotes can synthesize xylose de novo. *Bordetella* species are grown in Stainer-Scholte (SS) medium, which does not include any carbohydrates (37), since they cannot utilize sugars as a carbon source. The classical glycolytic pathway is nonfunctional in *Bordetella* species, as specific key enzymes in the glycolysis pathway are absent in the *Bordetella* genome, although the gluconeogenesis pathway appears to be intact (35). Casamino Acids and glutamate are the primary carbon sources for *Bordetella* grown in SS (37), and it is clear that the sugars found in the biofilm matrix are synthesized de novo by *B. bronchiseptica*, possibly via gluconeogenesis. The sugars synthesized and secreted by this organism cannot be recycled back into the cell for an energy source unless an alternative pathway, such as the pentose phosphate pathway, is utilized. It implies that the sugars we have detected in the biofilm matrix are most likely to have been synthesized for the primary purpose of biofilm matrix construction.

Although xylose appears to be the major component of the matrix, *N*-acetyl-glucosamine may be important for the early biofilm development. It is possible that the *N*-acetyl-glucosamine polymers serve as an additional "adhesive" to initiate surface association, consistent with earlier suggestions of the roles of extracellular polysaccharides in other biofilm systems (3). Furthermore, xylose secretion appears to be more pronounced in the stationary phase of growth, suggesting that the matrix glycosyl composition may change throughout biofilm development. Upon slowing of cellular replication during biofilm formation, xylose polymers may populate the matrix to reinforce the overall biofilm structure.

The thick polysaccharide matrix encapsulating the biofilms has been suggested to have several roles. It may aid the bacteria to attach to surfaces during the early formation, eventually creating a structural foundation to preserve its architecture (3). The matrix may function as a barrier to maintain a homeostasis inside to create a more consistent microenvironment (40) and also to prevent external chemicals and environmental factors such as antibiotics from entering (8). It may also be important for pathogenic bacteria in vivo to hide antigens on bacterial surfaces normally recognized by the host immune system by encapsulating the cells with polysaccharide polymers that have lower immunogenicity. The role of biofilm formation in promoting colonization and persistent chronic infections has been documented in *P. aeruginosa* (21). We have also proposed that *B. bronchiseptica* can form biofilms in the upper respiratory tract of infected hosts (15). Our more recent studies in-

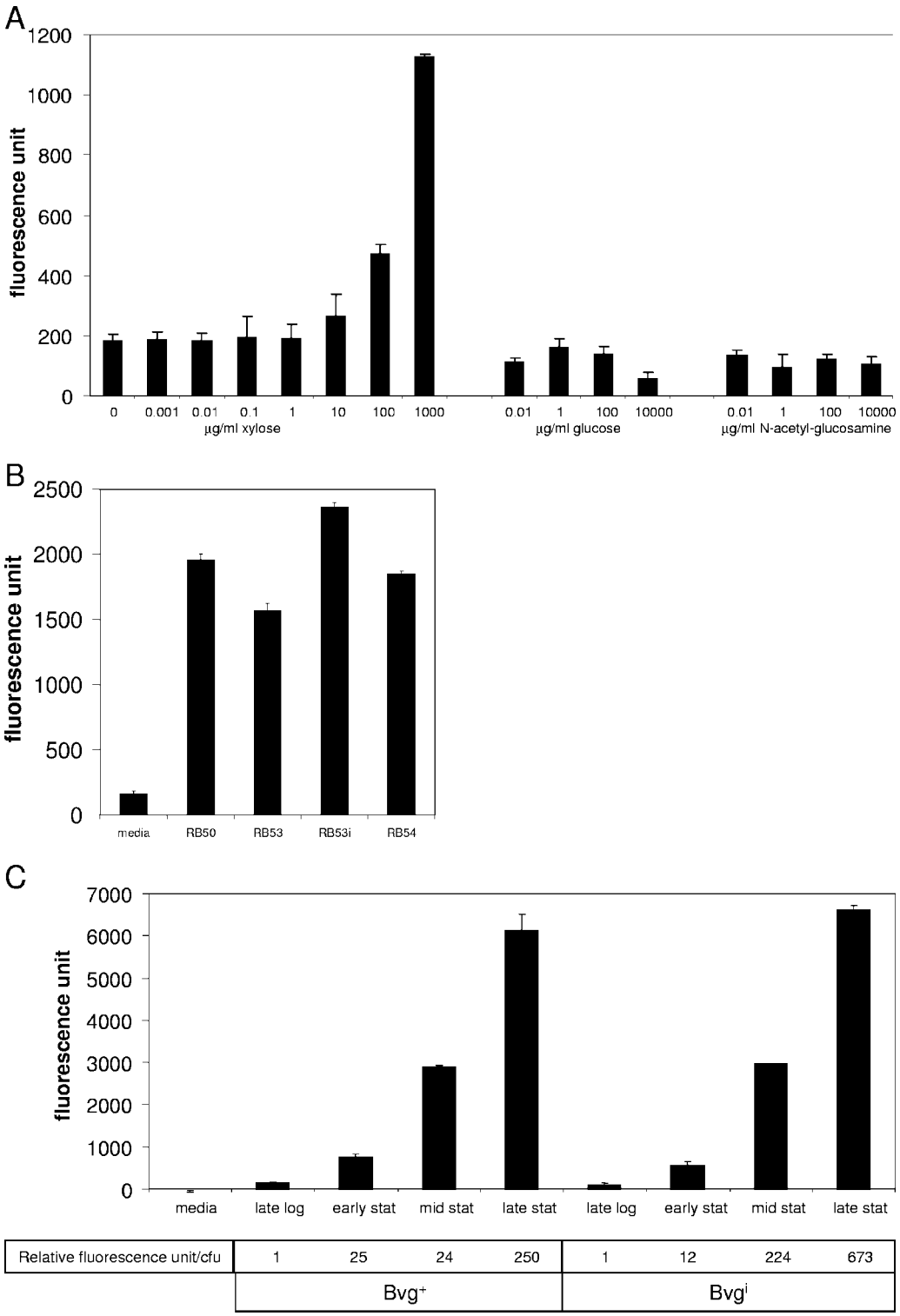

FIG. 4. *Caulobacter crescentus* xylose bioassay. (A) The *Caulobacter* xylose bioassay strain was added to serially diluted xylose, glucose, and *N*-acetyl-glucosamine solution, and the GFP fluorescence read-outs were measured by a fluorescence microplate reader. Only xylose induced GFP expression in a concentration-dependent manner. (B) Supernatants of overnight cultures of *B. bronchiseptica* strains RB50 (wild type), RB53 (Bvg^+ phase locked), RB53i (Bvg^i phase locked), and RB54 (Bvg^- phase locked) were assayed. Xylose production appears to be Bvg independent, as all supernatant induced GFP expression of the *Caulobacter* bioassay strain to similar levels. (C) Culture supernatants of RB50 (grown in Bvg^+ phase) and RB53i (Bvg^i phase locked) were collected at different growth stages and assayed for xylose. The table at the bottom shows relative fluorescence units that have been normalized with *B. bronchiseptica* CFU at the time of supernatant collection, and the reading from the late log phase was adjusted to 1 individually for Bvg^+ readings and Bvg^i readings. The relative fluorescence unit/CFU values of early stationary phase, mid-stationary phase, and late stationary phase represent the fold increases of fluorescence units/*B. bronchiseptica* CFU compared to the late log values. Most of the xylose was produced in the stationary phase.

dicate that the epithelial surfaces of the nasal cavity serve as the primary sites for biofilm formation in vivo (Y. Irie and M. H. Yuk, unpublished data).

Developmental biology of biofilm formation has been most well characterized in *Pseudomonas* species and *Vibrio cholerae* (33). The initial attachment stage is often mediated by various adhesins, such as the type IV pili in *P. aeruginosa* (34) and mannose-sensitive hemagglutinin type IV pili in *V. cholerae* (30). Stalk formation of *P. aeruginosa* microcolonies has been suggested to be formed by cells downregulating pili, while the mushroom caps have been formed by pili-motile cells climbing the stalks (20). The detachment stage of biofilm has been attributed to several factors and is not well understood. Polysaccharide lyases (1), cell death, and survival partially mediated by phages within microcolonies (42), protease activity (22), and quorum sensing affecting as yet unknown mechanisms (3) may be involved in biofilm detachment. However, in most studies of the biofilm development, the focus has mainly been on protein factors affecting the cellular behaviors. There are few reports on the differential expression of carbohydrates in the biofilm matrix and how these processes may affect the development of biofilm. This study presents evidence that *B. bronchiseptica* produces xylose, the major component of this bacterial matrix, primarily at the stationary phase of growth. The dynamic expression patterns of carbohydrates in bacteria have been investigated (14), but the integration of the knowledge with biofilm formation has yet to be achieved.

We have shown that *B. bronchiseptica* biofilm forms primarily in the Bvg^i phase, with FHA being the primary factor that is required for biofilm formation (15). However, it can also form strong biofilms in the Bvg^+ phase in the absence of CyaA expression. Our present studies indicate that the carbohydrate profile of the *B. bronchiseptica* biofilm matrix does not show significant differences between Bvg^+ and Bvg^i phases. Therefore, the primary driver of biofilm formation appears to be the Bvg control system via its regulation of FHA and CyaA expression, but the expression of the carbohydrate components of the biofilm matrix does not appear to be Bvg regulated. Expression of specific sugars required for LPS synthesis have been shown to be Bvg regulated (7), but the primary carbohydrate components of the biofilm matrix do not constitute any part of the known *Bordetella* LPS structure. A recent microarray study of *Bordetella* species has produced a list of Bvg-regulated genes (7), but an examination of these data does not lead to any obvious candidate for gene products that are involved in polysaccharide metabolism. One factor that does affect the expression of the primary carbohydrate component of the *B. bronchiseptica* biofilm matrix is the growth phase of the organism, as xylose is maximally expressed in the stationary growth phase. Thus, a combination of Bvg regulation and growth phases appears to be the primary driver for formation of biofilm in *B. bronchiseptica*. Bvg controls the expression of primary protein factor (FHA) that is required for this phenotype, while the expression of xylose, the primary carbohydrate component of the biofilm matrix, is growth phase dependent. With the development of the bioassay for detecting xylose, we have begun to perform a genetic screen to identify specific genes that are involved in its biosynthesis, and these studies should lead to a better understanding of the mechanism and regulation of the formation of the *Bordetella* biofilm matrix.

ACKNOWLEDGMENTS

We thank Audrey Jackson and Christine Jacobs-Wagner (Yale University) for providing us with the *Caulobacter* xylose bioassay strain as a generous gift. We thank Peggy Cotter, Corinne Williams, and Christopher Cain (UC Santa Barbara) for their kind gifts of fluorescent *B. bronchiseptica* strains. We also thank Jeffrey Kaplan, Chandran Ragunath, and Narayanan Ramasubbu (UMDNJ Dental School) for providing dispersin B protein. Lipid composition analyses were done with the help of Russell Herwig (University of Washington).

Parts of the carbohydrate analyses of the matrix were performed at the University of Georgia Complex Carbohydrate Research Center (supported by DOE fund DE-FG09-93ER-20097). This work was supported in part by NIH grant AI04936 to M.H.Y. and an NSERC Discovery grant to A.P.

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