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Genes Involved in the Synthesis and Degradation of Matrix Polysaccharide in *Actinobacillus actinomycetemcomitans* and *Actinobacillus pleuropneumoniae* Biofilms

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Biofilms are composed of bacterial cells embedded in an extracellular polysaccharide matrix. A major component of the *Escherichia coli* biofilm matrix is PGA, a linear polymer of *N*-acetyl-D-glucosamine residues in β(1,6) linkage. PGA mediates intercellular adhesion and attachment of cells to abiotic surfaces. In this report, we present genetic and biochemical evidence that PGA is also a major matrix component of biofilms produced by the human periodontopathogen *Actinobacillus actinomycetemcomitans* and the porcine respiratory pathogen *Actinobacillus pleuropneumoniae*. We also show that PGA is a substrate for dispersin B, a biofilm-releasing glycosyl hydrolase produced by *A. actinomycetemcomitans*, and that an orthologous dispersin B enzyme is produced by *A. pleuropneumoniae*. We further show that *A. actinomycetemcomitans* PGA cross-reacts with antiserum raised against polysaccharide intercellular adhesin, a staphylococcal biofilm matrix polysaccharide that is genetically and structurally related to PGA. Our findings confirm that PGA functions as a biofilm matrix polysaccharide in phylogenetically diverse bacterial species and suggest that PGA may play a role in intercellular adhesion and cellular detachment and dispersal in *A. actinomycetemcomitans* and *A. pleuropneumoniae* biofilms.

Bacterial cells in a biofilm are surrounded by a self-synthesized, three-dimensional matrix that holds the cells together in a mass and firmly attaches the bacterial mass to the underlying surface (3). This matrix, referred to as the slime layer, glycocalyx, or extracellular polymeric substance (EPS) matrix, can comprise up to 90% of the biofilm biomass (8). In addition to its structural role, the EPS matrix provides biofilm cells with a protected microenvironment containing dissolved nutrients and secreted enzymes, as well as other biological molecules and abiotic substances originating from outside the biofilm. The EPS matrix may also contribute to the increased resistance to antibiotics and host defenses exhibited by biofilm cells (27, 32).

Polysaccharide is a major component of the EPS matrix in most bacterial biofilms (34). One of the best-characterized matrix polysaccharides is a hexosamine-containing polymer produced by several staphylococcal species, including *Staphylococcus epidermidis* and *Staphylococcus aureus*, and by *Escherichia coli*. This polymer, called PIA (also PNAG, PS/A, or SAA) in *Staphylococcus* spp. (2, 24, 28–30) and PGA in *E. coli* (36), consists of a linear chain of *N*-acetyl-D-glucosamine (GlcNAc) residues in β(1,6) linkage. Various forms of this GlcNAc polymer appear to differ in molecular weight, in the degree of deacetylation of the GlcNAc residues, and in the presence of *O*-acetyl substituents (24, 28, 30). PIA and PGA have been shown to play roles in abiotic surface attachment and intercellular adhesion (13, 30, 37), and PIA has been shown to protect *S. epidermidis* biofilm cells from host innate defenses, including phagocytosis and antimicrobial peptides (36).

In *S. epidermidis*, the production of PIA is encoded by a cluster of four tightly linked genes named *icaADBC* (10, 13, 30). IcaA is a transmembrane glycosyltransferase that synthesizes the GlcNAc polymer backbone (10). Sole expression of *icaA* induces only low enzymatic activity, but coexpression of *icaA* with *icaD*, a small gene that is translationally coupled to *icaA*, leads to a significant increase in enzymatic activity and is related to full phenotypic expression of PIA (10). IcaB is a secreted protein that is homologous to several polysaccharide *N*-deacetylases, including rhizobial NodB chito-oligosaccharide deacetylase, peptidoglycan GlcNAc deacetylase, and chitin deacetylase, suggesting that IcaB may be a deacetylase. IcaC is a predicted transmembrane protein with unknown function. In *E. coli*, the production of PGA is encoded by a cluster of four tightly linked genes named *pgaABCD* (37). PgaC exhibits 40% amino acid identity to *S. epidermidis* IcaA, suggesting that PgaC is also a glycosyltransferase. Although PgaD exhibits no primary amino acid sequence similarity to IcaD, both proteins are small (137 and 101 amino acid residues, respectively), and both contain two predicted transmembrane segments, which suggests that PgaD and IcaD may be functionally related. PgaA and PgaB are predicted to be outer membrane or secreted proteins (37). The N-terminal 300 amino acid residues of PgaB exhibit 25% identity to IcaB, suggesting that PgaB may also be a deacetylase. PgaA and the C-terminal 370 amino acid residues of PgaB are not homologous to any proteins in the National Center for Biotechnology Information (NCBI) public database.

A glycosyl hydrolase that degrades *S. epidermidis* PIA was

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TABLE 1.. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Source or reference
Strains		
A. actinomycetemcomitans IDH781	Clinical isolate (serotype d)	12
A. actinomycetemcomitans IDH781N	Spontaneous Nal^r variant of IDH781	M. Bhattacharjee and D. Figurski
A. actinomycetemcomitans JK1048	IDH781N *pgaC*::R6Kγori/KAN; Km^r	This study
A. actinomycetemcomitans JK1049	IDH781N *pgaC*::R6Kγori/KAN; Km^r	This study
A. actinomycetemcomitans JK1047	IDH781N *flp-1*::Tn*903*ϕ*kan*; Km^r	J. B. Kaplan, unpublished data
A. actinomycetemcomitans CU1000	Clinical isolate (serotype f)	6
A. pleuropneumoniae IA1	Clinical isolate (serotype 1)	Iowa State University
A. pleuropneumoniae IA5	Clinical isolate (serotype 5)	Iowa State University
S. epidermidis NJ9709	Clinical isolate	21
S. epidermidis 1457	Clinical isolate	26
S. epidermidis 1457-M11	1457 *icaA*::Tn*917*	25
E. coli DH5α	Used for *pgaCD* and *icaAD* expression	New England Biolabs
E. coli TRXWEC	*pgaC*::Tn*10Cam*: Cm^r; used for expression of *E. coli pgaABCD*	37
E. coli BL21(DE3)	Used for overexpression of dispersin B	Novagen
Plasmids		
LITMUS28	*E. coli* cloning vector; Ap^r	New England Biolabs
pVK80	LITMUS28 containing *A. actinomycetemcomitans pgaCD*	This study
pVK81-1	pVK80 *pgaC*::R6Kγori/KAN	This study
pVK81-5	pVK80 *pgaC*::R6Kγori/KAN	This study
pJAK16	Broad-host-range expression vector; Cm^r	35
pVK82	pJAK16 containing *A. actinomycetemcomitans pgaCD* (Fig. 1)	This study
pVK91	pJAK16 containing *S. epidermidis icaAD*	This study
pVK93	pJAK16 containing *A. pleuropneumoniae pgaCD* (Fig. 1)	This study
pRK21761	Use to mobilize plasmids from *E. coli* to *A. actinomycetemcomitans*	35
pET-28a	Used for overexpression of dispersin B	Novagen
pRC2	pET-28a containing *A. pleuropneumoniae dspB*	This study
pUC19	*E. coli* cloning vector; Ap^r	New England Biolabs
pPGA372	pUC19 containing *E. coli pgaABCD*	37

[a] Nal^r, nalidixic acid resistant; Km^r, kanamycin resistant; Cm^r, chloramphenicol resistant; Ap^r, ampicillin resistant.

recently identified (21). This enzyme, named dispersin B (DspB), is produced by the gram-negative oral bacterium *Actinobacillus actinomycetemcomitans*, the causative agent of a severe form of periodontal disease that affects adolescents (38). Treatment of *S. epidermidis* biofilms with recombinant *A. actinomycetemcomitans* dispersin B protein causes dissolution of the EPS matrix and detachment of biofilm cells from the surface (21). Treatment of *A. actinomycetemcomitans* biofilms with dispersin B also causes the detachment of cells from the surface (20), suggesting that *A. actinomycetemcomitans* biofilms contain a matrix polysaccharide that is structurally related to PIA. In this report, we present evidence that a cluster of four *A. actinomycetemcomitans* genes that is homologous to *E. coli pgaABCD* (19, 38, 40, and 12% amino acid identity, respectively) encodes the production of a hexosamine-containing exopolysaccharide that is structurally related to *E. coli* PGA and that this polysaccharide is a substrate for dispersin B. We also show that the *A. actinomycetemcomitans* PGA-like polysaccharide is not required for the attachment of cells to surfaces but may play a role in the aggregation of cells inside the biofilm colony and the detachment of cells from the colony. We further show that a similar hexosamine-containing polysaccharide constitutes a biofilm matrix component in the closely related bacterium *Actinobacillus pleuropneumoniae*, the causative agent of porcine pleuropneumonia (11).

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. The bacterial strains used in this study are listed in Table 1. *A. actinomycetemcomitans* and *S. epidermidis* strains were grown in Trypticase soy broth (Becton Dickinson) supplemented with 6 g of yeast extract and 8 g of glucose per liter. Media were supplemented with 3 μg of chloramphenicol/ml, 40 μg of kanamycin/ml, 20 μg of nalidixic acid/ml, 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), 0.01% Congo red dye, or 1.5% agar when appropriate. *A. pleuropneumoniae* strains, which were isolated from the lungs of pigs with porcine pleuropneumonia, were cultured in Mueller-Hinton broth supplemented with 6 g of yeast extract, 8 g of glucose, and 5 mg of NAD per liter. *E. coli* strains were grown in Luria-Bertani broth supplemented with ampicillin or chloramphenicol (50 μg/ml each) or 1 mM IPTG when appropriate. *E. coli* cultures were incubated in tubes with agitation. All other bacteria were incubated statically. *A. actinomycetemcomitans* was incubated in the presence of 10% CO_2. All cultures were incubated at 37°C.

Targeted mutagenesis of *A. actinomycetemcomitans pgaC*. The *pgaCD* region of *A. actinomycetemcomitans* strain CU1000 was amplified by PCR using the forward primer 5′-TGACCGGATCCTCAAGCAGGTAAACCATAG-3′, which introduced a BamHI site (underlined) 27 bp upstream from the *pgaC* start codon, and the reverse primer 5′-TGACCGCTGCAGttaTTTTTTCTTTTTTCTCC-3′, which introduced a PstI site (underlined) immediately downstream from the *pgaD* stop codon (lowercase). PCRs were carried out as previously described (19). The PCR product was digested with BamHI and PstI and ligated into the BamHI/PstI sites of plasmid LITMUS28, resulting in plasmid pVK80 (Table 1). The DNA sequence of the insert from pVK80 (1,532 bp) was 99.3% identical (with six silent changes and five replacement changes) to that of the *pgaCD* region from strain HK1651 (Actinobacillus Genome Sequencing Project [http://www.genome.ou.edu/act.html]). Plasmid pVK80 was mutagenized by random in vitro insertion of the kanamycin resistance transposon R6Kγori/KAN by using an EZ-TN transposon insertion kit (Epicentre). Two mutant plasmids, pVK81-1 and pVK81-5, which contained transposon insertions in the middle and at the end of *pgaC*, respectively, were used to transform strain IDH781N to kanamycin resistance using a natural transformation protocol provided by Mrinal Bhattacharjee and David Figurski (Columbia University). Recombination of the transposons into the chromosomes of the transformants (designated JK1048 and JK1049) (Fig. 1) was confirmed by PCR using the forward primer 5′-GACGGTGATGCGGTATTGG-3′ and the reverse primer 5′-CCTCTATCCGGGCTGGTCCATAC-3′ (corresponding to bp 412 to 430 and 1207 to 1229 in the sequence with GenBank accession no. AY438014, respectively), which flanked the transposon insertion sites, and by DNA sequence analysis of the transposon junctions.

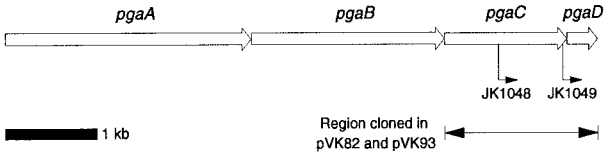

FIG. 1. Genetic map of the *pgaABCD* locus of *A. actinomycetemcomitans* and *A. pleuropneumoniae*. The open arrows indicate open reading frames and directions of transcription. Gene names are indicated above. The arrows below *pgaC* indicate the locations and directions of transcription of transposon insertions in two *A. actinomycetemcomitans* mutant strains. The double arrow indicates the region cloned into plasmids pVK82 and pVK93. Scale bar = 1 kb.

Genetic complementation of *A. actinomycetemcomitans pgaC*. The BamHI/PstI insert from pVK80 was ligated into the BamHI/PstI sites of the broad-host-range plasmid pJAK16, which placed *pgaCD* under the control of an IPTG-inducible *tac* promoter. The resulting plasmid (pVK82), or pJAK16 as a control, was mobilized into *A. actinomycetemcomitans* strain JK1048 by using the RK2 *oriT*-defective mutant plasmid pRK21761 as previously described (35). Plasmid-harboring strains were grown in media supplemented with chloramphenicol and IPTG.

Expression of *A. actinomycetemcomitans pgaCD* in *E. coli*. Plasmid pVK82, or plasmid pJAK16 as a control, was transformed into *E. coli* strain DH5α. Transformants were incubated in broth for 16 h, diluted 1:50 in 3 ml of fresh broth, and incubated for an additional 5 h until the optical density of the culture (at 590 nm) reached 0.5. IPTG was added to a final concentration of 1 mM, and the cultures were incubated for an additional 2 h. Cells were harvested by centrifugation, washed three times with phosphate-buffered saline (PBS), and resuspended in 1 ml of PBS. The cells were treated with dispersin B as described below.

Expression of *A. pleuropneumoniae pgaCD* in *E. coli*. Genomic DNA isolated from *A. pleuropneumoniae* strain IA1 was amplified by PCR using the forward primer 5′-TGACCGGATCCTCAAGCAGGTAAACCATAGGGATTGGTatg ATTCTAGAAATATTCAG-3′, which inserts a BamHI restriction site (underlined) 27 bp upstream from the *pgaC* start codon (lowercase), and the reverse primer 5′-TGACCGCTGCAGttaAGGTTTTTTATGGCGAC-3′, which inserts a PstI restriction site (underlined) downstream from the *pgaD* stop codon (lowercase). The PCR product was digested with BamHI and PstI and ligated into the BamHI/PstI sites of pJAK16, resulting in plasmid pVK93. The DNA sequence of the insert from pVK93 was 99.6 to 100% identical to *pgaCD* homologues from four other *A. pleuropneumoniae* strains (representing serotypes 1, 5, and 7) in the NCBI public database. Plasmid pVK93 was transformed into *E. coli* strain DH5α, and transformants were cultured and induced with IPTG as described above.

Expression of *S. epidermidis icaAD* in *E. coli*. Genomic DNA isolated from *S. epidermidis* strain NJ9709 was amplified by PCR using the forward primer 5′-TGACCGGATCCTCAAGCAGGTAAACCATAGGGATTGGTatgCATA TTTTTAACTTTTTAC-3′, which inserts a BamHI restriction site (underlined) 27 bp upstream from the *icaA* start codon (lowercase), and the reverse primer 5′-TGACCGCTGCAGtcaTATGTCACGACCTTTC-3′, which inserts a PstI restriction site (underlined) downstream from the *icaD* stop codon (lowercase). The PCR product was digested with BamHI and PstI and ligated into the BamHI/PstI sites of pJAK16, resulting in plasmid pVK91. Plasmid pVK91 was transformed into *E. coli* strain DH5α, and transformants were cultured and induced with IPTG as described above.

***A. actinomycetemcomitans* dispersin B purification and treatment.** *A. actinomycetemcomitans* dispersin B was purified as previously described (20). The protein concentration was determined by using a Bio-Rad Protein Assay kit. The specific activity of the enzyme was 970 U per mg of protein, where 1 U of enzyme activity was defined as the amount of enzyme needed to hydrolyze 1 μmol of 4-nitrophenyl-β-D-*N*-acetylglucosaminide to 4-nitrophenol and *N*-acetylglucosamine per min at 25°C in 50 mM sodium phosphate buffer (pH 4.5)–100 mM NaCl (20).

Bacterial cells were treated with 40 μg of dispersin B/ml in PBS for 30 min at 30°C. The cells were pelleted by centrifugation, and the supernatant was assayed for total hexosamine as described below. Crude polysaccharide samples were treated with 40 μg of dispersin B/ml in PBS for 30 min at room temperature. The samples were reprecipitated with sodium dodecyl sulfate (SDS) as described below, incubated at 100°C for 3 min, and then centrifuged. The supernatants were analyzed for total hexosamine as described below.

Expression and purification of *A. pleuropneumoniae* dispersin B. Genomic DNA isolated from *A. pleuropneumoniae* strain IA1 was amplified by PCR using the forward primer 5′-GCTAGTCCATGGACTTACCTAAAAAAGAAAGC G-3′, which inserts an NcoI restriction site (underlined) at codon 26 of *dspB*, and the reverse primer 5′-GCAGGGATCCcta*GTGGTGGTGGTGGTGGTG*ATG CGATTTCGGATCATTAG-3′, which inserts a BamHI restriction site (underlined) and six His codons (italics) flanking the *dspB* stop codon (lowercase). The PCR product was digested with NcoI and BamHI and ligated into the NcoI/BamHI sites of the T7 expression plasmid pET-28a, resulting in plasmid pRC2. The DNA sequence of the insert from pRC2 was 99.7 to 100% identical to sequences of *dspB* homologues from four other *A. pleuropneumoniae* strains in the NCBI public database. Plasmid pRC2, which encoded dispersin B (minus its leader peptide) fused to a hexahistidine metal-binding site at its C terminus, was transformed into *E. coli* strain BL21(DE3), and the fusion protein was purified by using Ni affinity chromatography as previously described (20).

Surface attachment and biofilm formation assays. To quantitate surface attachment, 100-μl aliquots of serial twofold dilutions of inocula were transferred to the wells of a 96-well polystyrene microtiter plate (model 324662; Falcon) and incubated for 4 h. The wells were then washed with water and stained with crystal violet as previously described (16). The bound dye was solubilized in 100 μl of 100% ethanol, and the optical density of the ethanol-dye solution was measured using a Bio-Rad Benchmark microtiter plate reader set to 590 nm. To measure biofilm formation, cells were grown in 96-well microtiter plates for 16 h and then stained and quantitated as described above. Surface attachment and biofilm formation assays were performed three times with similar results.

Biofilm detachment assays. Biofilms were grown for 16 h in the wells of a 96-well microtiter plate as described above. The biofilms were washed with water and then treated for 2 h at 37°C with 10 mM sodium metaperiodate in 50 mM sodium acetate buffer (pH 4.5) or with 100 μg of proteinase K (Sigma)/ml in 20 mM Tris (pH 7.5)–100 mM NaCl. After treatment, the biofilms were washed with water, stained with crystal violet, and quantitated as described above. Biofilm detachment assays were performed three times with similar results.

Autoaggregation assay. One well of a six-well microtiter plate (model 353046; Falcon) was filled with 4 ml of fresh broth containing 10^7 CFU of bacteria/ml, and the plate was incubated for 16 h. The biofilm that formed on the surface of the well was rinsed with PBS and then scraped from the surface into 1 ml of PBS by using a cell scraper. A total of 200 μl of the cell suspension was transferred to a 0.5-ml polypropylene tube (model 6530; Corning), and the tube was vortexed at high speed for 10 s, incubated statically for 5 min, and photographed.

Immunofluorescence microscopy. Aliquots of bacterial-cell suspensions (15 μl each) were heat fixed onto printed glass microscope slides, soaked in ice-cold methanol for 2 min, and then air dried. The fixed cells were incubated with 15 μl of anti-*S. epidermidis* PIA antiserum or the corresponding preimmune serum (diluted 1:50 in PBS) for 30 min at 37°C in a wet chamber (23). The slides were washed three times for 2 min each time with PBS and then air dried. Bound antibodies were detected using a fluorescein isothiocyanate-conjugated anti-rabbit immunoglobulin G antibody (Sigma) diluted 1:80 in PBS. After incubation for 30 min at 37°C in a wet chamber, the slides were washed twice with PBS and once with double-distilled water for 2 min. The slides were then air dried and viewed under a Zeiss fluorescence microscope at a magnification of ×1,000. PIA-producing *S. epidermidis* strain 1457 and its PIA-negative *icaA* insertion mutant, 1457-M11, were used as positive and negative controls, respectively (23).

Isolation of crude polysaccharide. Confluent biofilms were grown in 100-mm-diameter tissue-culture-treated polystyrene petri dishes (model 430167; Corning) as previously described (17). The biofilms were washed with PBS, scraped from the surface into 5 ml of PBS with a cell scraper, and then transferred to a 15-ml conical centrifuge tube. The cells were pelleted by centrifugation for 10 min at 4,000 rpm in a Sorvall model RT7 centrifuge. The cell pellet was resuspended in 0.5 ml of 50 mM sodium acetate buffer (pH 5.8)–100 mM NaCl and then transferred to a 1.5-ml microcentrifuge tube. An equal volume of 10 mM Tris-HCl (pH 8.0)–5 mM EDTA–0.5% SDS was added, and the tube was vortexed briefly and incubated at 100°C for 3 min. The tube was centrifuged for 10 min at 15,000 rpm in a Sorvall MC12V microcentrifuge, and the supernatant was decanted. The pellet contained the crude polysaccharide. The yield was ~300 μg of crude polysaccharide per mg (wet weight) of cells.

SDS-polyacrylamide gel electrophoresis analysis. Polyacrylamide gels consisted of a 10-mm-long stacking gel (5% acrylamide) and a 35-mm-long running gel (15% acrylamide). The buffer system of Laemmli was employed (22). Prior to electrophoresis, crude polysaccharide samples (10 μg each) were resuspended in 25 mM Tris-HCl (pH 8.0)–5 mM β-mercaptoethanol–0.5% SDS and incubated at 100°C for 10 min. Samples were loaded directly onto the gel without centrifugation. After electrophoresis, the polysaccharides were stained with silver using a Bio-Rad Silver Stain kit.

Total hexosamine assay. After centrifugation, total hexosamine was measured in the supernatant by using the Morgan-Elson colorimetric assay (33). All assays were performed at least three times with similar results.

Nucleotide sequence accession numbers. The DNA sequence of *pgaCD* from *A. actinomycetemcomitans* strain CU1000 was deposited in GenBank under accession no. AY438014. The DNA sequences of *pgaCD* and *dspB* from *A. pleuropneumoniae* strain IA1 were deposited under accession no. AY618480 and AY618481, respectively.

RESULTS

***A. actinomycetemcomitans pgaC* mutants are defective in exopolysaccharide production.** We constructed two isogenic mutants of *A. actinomycetemcomitans* strain IDH781N that contained transposon insertions in *pgaC* by using in vitro transposon mutagenesis, natural transformation, and homologous recombination (Fig. 1). The mutant strain JK1048, which contained a transposon insertion in the middle of *pgaC*, bound to polystyrene with the same avidity as the wild-type strain (Fig. 2A). Strain JK1047, which contains a transposon insertion in a gene (*flp-1*) required for type IV pilus production and biofilm formation (16), failed to bind to polystyrene. Unlike biofilms produced by the wild-type strain IDH781N, JK1048 biofilms were resistant to removal by the carbohydrate-modifying agent sodium metaperiodate and sensitive to detachment by proteinase K (Fig. 2B). These results are consistent with the hypotheses that JK1048 was deficient in exopolysaccharide production and that expression of *pgaC* was not required for surface attachment. Mutant JK1049, which contained a transposon insertion near the 3′ end of *pgaC* (Fig. 1), displayed the same adherence and autoaggregation phenotypes displayed by strain JK1048 (data not shown).

To test whether *pgaC* was involved in intercellular adhesion, we grew biofilms in polystyrene petri dishes and then scraped the biofilm cells from the polystyrene surface and transferred them to a tube (Fig. 2C, left). Five minutes after being vortexed, cells of the wild-type strain had settled to the bottom of the tube, whereas JK1048 cells remained in suspension. After 15 min, JK1048 cells had begun to settle, whereas cells of the *flp-1* mutant JK1047 remained in suspension for long periods of time (data not shown). A plasmid carrying the wild-type *A. actinomycetemcomitans pgaCD* genes (pVK82) restored the ability of JK1048 cells to rapidly autoaggregate (Fig. 2C, right). These findings indicate that strain JK1048 exhibited a reduced autoaggregation phenotype.

To demonstrate that the *pgaC* mutant was deficient in exopolysaccharide synthesis, we grew wild-type strain IDH781N and mutant strain JK1048 on nutrient agar containing Congo red dye, which has an affinity for bacterial exopolysaccharides (1, 9, 39). Strain IDH781N produced red colonies on Congo red agar, whereas strain JK1048 produced white colonies (Fig. 2D). Plasmid pVK82 restored the ability of JK1048 colonies to bind Congo red dye.

To confirm that the *pgaC* mutant was deficient in exopolysaccharide production, we analyzed crude polysaccharide isolated from the wild-type strain IDH781N and the mutant JK1048 by polyacrylamide gel electrophoresis (Fig. 3). Strain IDH781N produced an abundant, high-molecular-weight material that was retained in the stacking gel during electrophoresis (lane A). This material was absent from cells of strain JK1048 (lane B), and its production was restored by transformation of JK1048 with the complementary plasmid pVK82 (lanes C and D). This material was resistant to extraction by phenol (lanes E and F) and to digestion by proteinase K (data not shown), consistent with the hypothesis that it contained primarily polysaccharide. The material was degraded by dispersin B (lanes G and H) and was absent from IDH781N cells that were pretreated with dispersin B (lanes I and J). Cells of a different serotype of *A. actinomycetemcomitans* (strain CU1000) also produced the high-molecular-weight material (lane K), which was degraded by dispersin B (lane L). These

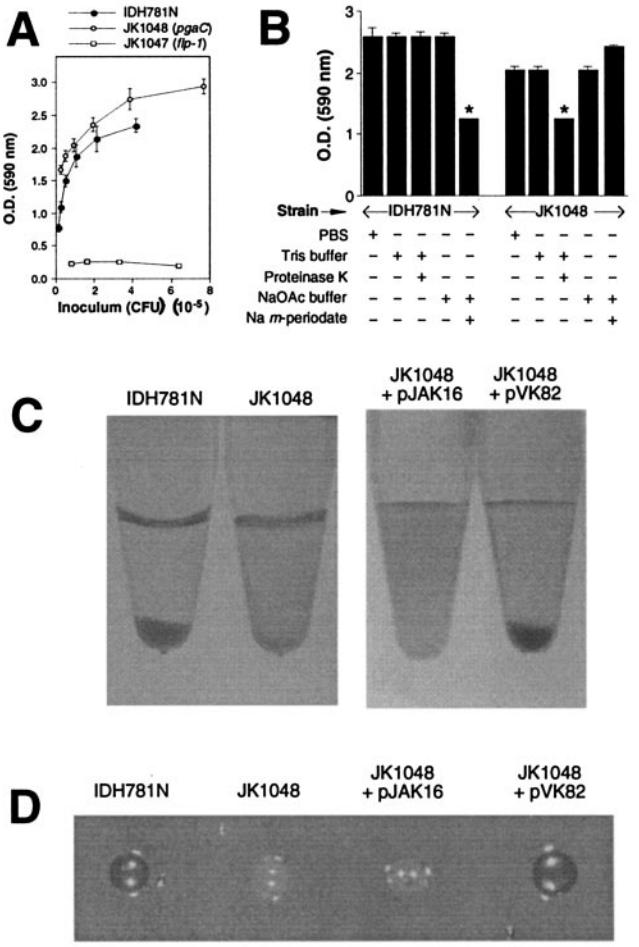

FIG. 2. Phenotypes of wild-type *A. actinomycetemcomitans* strain IDH781N and *pgaC* mutant JK1048. (A) Surface attachment was measured in 96-well microtiter plates by staining with crystal violet. The optical density (O.D.) at 590 nm is proportional to the number of attached cells in the well. Strain JK1047 contains a transposon insertion in *flp-1* which results in a complete loss of pilus production and surface attachment (16). The values indicate means (± standard deviations) for triplicate wells. (B) Detachment of preformed biofilms by proteinase K and sodium metaperiodate as measured by crystal violet staining. The values indicate means (plus standard deviations) for triplicate wells. The asterisks indicate values that were significantly less than the those of corresponding mock-treated control ($P < 0.05$; unpaired two-tailed Student *t* test). +, present; −, absent. (C) Autoaggregation of IDH781N and JK1048 (left) and of JK1048 carrying the vector plasmid pJAK16 or the complementary plasmid pVK82 (right). (D) Colonies of strains IDH781N and JK1048 and of strain JK1048 carrying the vector plasmid pJAK16 or the complementary plasmid pVK82 on Congo red agar. The dark colonies were red, and the light colonies were white. The colonies were ~3 mm in diameter.

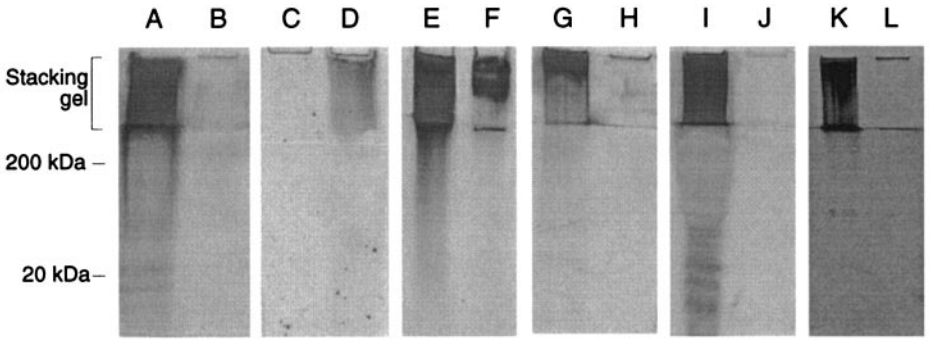

FIG. 3. SDS-polyacrylamide gel electrophoresis analysis of *A. actinomycetemcomitans* crude polysaccharides. The location of the stacking gel and the approximate positions of molecular mass standards electrophoresed in an adjacent lane are indicated on the left. Polysaccharides were stained with silver. (A and B) Polysaccharide isolated from strain IDH781N (lane A) and *pgaC* mutant JK1048 (lane B). (C and D) Polysaccharide isolated from strain JK1048 harboring plasmid vector pJAK16 (lane C) or the complementary plasmid pVK82 (lane D). (E and F) Untreated polysaccharide isolated from strain IDH781N (lane E) and the same sample extracted with phenol (lane F). (G and H) Polysaccharide isolated from strain IDH781N was mock treated (lane G) or treated with dispersin B (lane H) prior to electrophoresis. (I and J) Polysaccharide isolated from IDH781N cells that were mock treated (lane I) or pretreated with dispersin B (lane J) prior to polysaccharide purification. (K and L) Polysaccharide isolated from CU1000 cells that were mock treated (lane K) or pretreated with dispersin B (lane L) prior to polysaccharide purification.

findings indicate that *A. actinomycetemcomitans pgaC* encodes the production of a high-molecular-weight extracellular polysaccharide that is a substrate for dispersin B.

Enzymatic treatment of exopolysaccharide suggests the presence of a hexosamine-containing component. We treated *A. actinomycetemcomitans* cells with dispersin B and analyzed the cell supernatants for the presence of total hexosamine (Fig. 4A). Supernatants from mock-treated IDH781N cells contained no detectable hexosamine (<10 nmol per mg [wet weight] of cells), whereas supernatants from dispersin B-treated IDH781N contained high levels. Treatment of crude polysaccharide isolated from strain IDH781N resulted in the release of a similar amount of total hexosamine (126 ± 12 μmol per mg of polysaccharide). Cells of the *pgaC* mutant strain JK1048 released no detectable levels of total hexosamine, even after treatment with dispersin B (Fig. 4A). The complementary plasmid pVK82 restored the ability of JK1048 cells to release hexosamine after treatment with dispersin B. These data indicate that the *pgaC*-dependent exopolysaccharide produced by *A. actinomycetemcomitans* contains a hexosamine component.

Evidence that the *A. actinomycetemcomitans pgaC*-dependent polysaccharide is structurally related to *E. coli* PGA and *S. epidermidis* PIA. *E. coli* strains DH5α and TRXWEC (*pgaC* mutant) were transformed with plasmid pPGA372, which contains the *E. coli pgaABCD* locus and promoter region (37). The transformants released high levels of hexosamine when treated with dispersin B (Fig. 4B). The levels of hexosamine released by strains DH5α and TRXWEC harboring the vector plasmid pUC19 were similar, suggesting that *pgaC* is absent or not

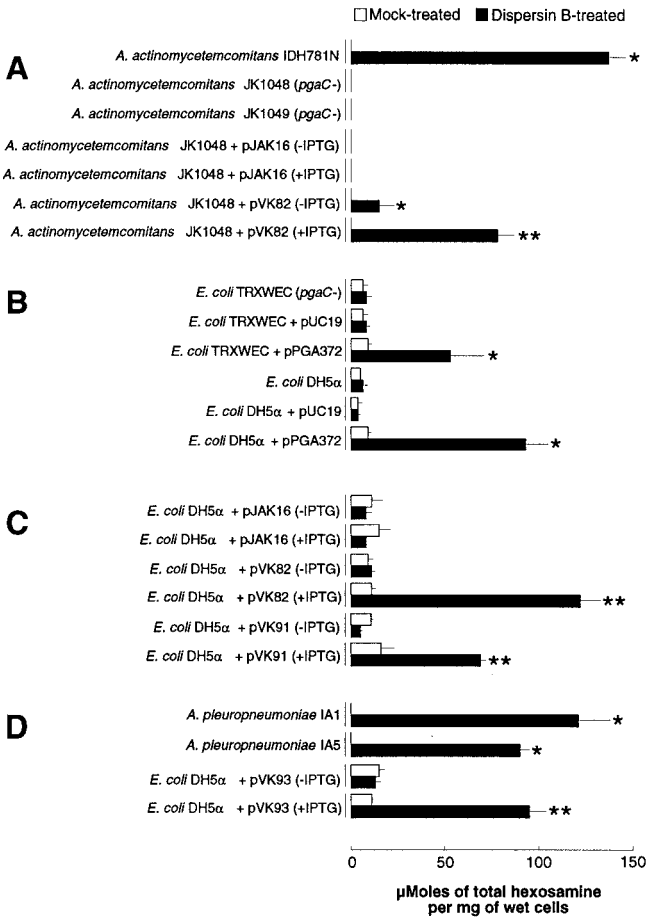

FIG. 4. Total hexosamine contents of supernatants from mock-treated and dispersin B-treated bacterial cells. (A) Wild-type *A. actinomycetemcomitans* strain IDH781N and isogenic *pgaC* mutants JK1048 and JK1049. Plasmid pVK82 contains the wild-type *A. actinomycetemcomitans pgaCD* genes. (B) Expression of *E. coli pgaABCD*(pPGA372) in two strains of *E. coli*. (C) Expression of *A. actinomycetemcomitans pgaCD*(pVK82) and *S. epidermidis icaAD*(pVK91) in *E. coli* DH5α. Values are means and ranges for duplicate samples. (D) Two wild-type strains of *A. pleuropneumoniae* and *E. coli* DH5α expressing the *A. pleuropneumoniae pgaCD* genes on a plasmid (pVK93). The asterisks indicate values that were significantly greater than the corresponding mock-treated control ($P < 0.05$; unpaired two-tailed Student *t* test). The double asterisks indicate values that were significantly greater than both the corresponding mock-treated control and the corresponding non-IPTG-induced control ($P < 0.05$).

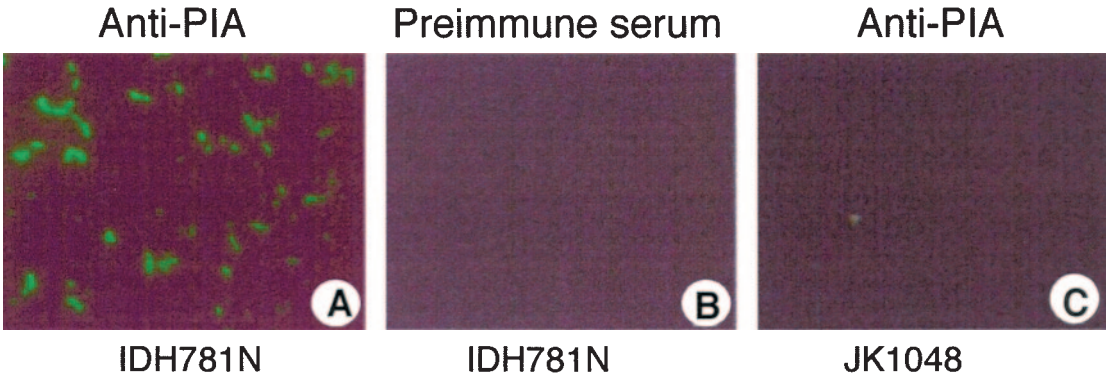

FIG. 5. Detection of exopolysaccharide by immunofluorescence microscopy. (A) Wild-type strain IDH781N with anti-PIA antiserum. (B) Strain IDH781N with preimmune serum. (C) *pgaC* mutant strain JK1048 with anti-PIA antiserum.

expressed in strain DH5α. The level of hexosamine released by DH5α harboring pPGA372 was similar to the level released by IPTG-induced DH5α cells harboring pVK82 (containing *A. actinomycetemcomitans pgaCD*) or pVK91 (containing *S. epidermidis icaAD*) (Fig. 4C). These findings suggest that *E. coli pgaC*, *A. actinomycetemcomitans pgaC*, and *S. epidermidis icaA* encode the production of similar hexosamine-containing polysaccharide substrates for dispersin B. In addition, wild-type *A. actinomycetemcomitans* IDH781N cells, but not cells of the *pgaC* mutant JK1048, cross-reacted with antiserum raised against *S. epidermidis* PIA (Fig. 5). Taken together, these data suggest that the *pgaC*-dependent polysaccharide produced by *A. actinomycetemcomitans* is structurally related to *E. coli* PGA and *S. epidermidis* PIA.

Evidence that *A. pleuropneumoniae* biofilms produce a hexosamine-containing extracellular polysaccharide. Two different wild-type strains of *A. pleuropneumoniae* released high levels of total hexosamine when treated with *A. actinomycetemcomitans* dispersin B (Fig. 4D), suggesting that *A. pleuropneumoniae* cells produce a hexosamine-containing extracellular polysaccharide similar to that produced by *A. actinomycetemcomitans*. The genome of *A. pleuropneumoniae* contains a locus that is homologous to *A. actinomycetemcomitans pgaABCD* (46, 46, 68, and 31% amino acid identity, respectively). When *E. coli* DH5α cells carrying the *A. pleuropneumoniae pgaCD* genes on a plasmid (pVK93) were treated with dispersin B, high levels of total hexosamine were released (Fig. 4D). These data suggest that the *A. pleuropneumoniae pgaCD* genes are orthologous to *E. coli* and *A. actinomycetemcomitans pgaCD*. The genome of *A. pleuropneumoniae* also contains a homologue of *A. actinomycetemcomitans dspB* (59% amino acid identity). Purified recombinant *A. pleuropneumoniae* DspB protein caused the detachment of *A. pleuropneumoniae* biofilms from surfaces (Fig. 6A). *A. actinomycetemcomitans* dispersin B also caused the detachment of *A. pleuropneumoniae* biofilms from surfaces (Fig. 6A), and *A. pleuropneumoniae* DspB caused the detachment of *A. actinomycetemcomitans* and *S. epidermidis* biofilms from surfaces (data not shown). These data suggest that *A. pleuropneumoniae* DspB exhibits the same substrate specificity as *A. actinomycetemcomitans* dispersin B. Like *A. actinomycetemcomitans* biofilms, biofilms produced by wild-type strains of *A. pleuropneumoniae* were sensitive to detachment by sodium metaperiodate and resistant to removal by proteinase K (Fig.

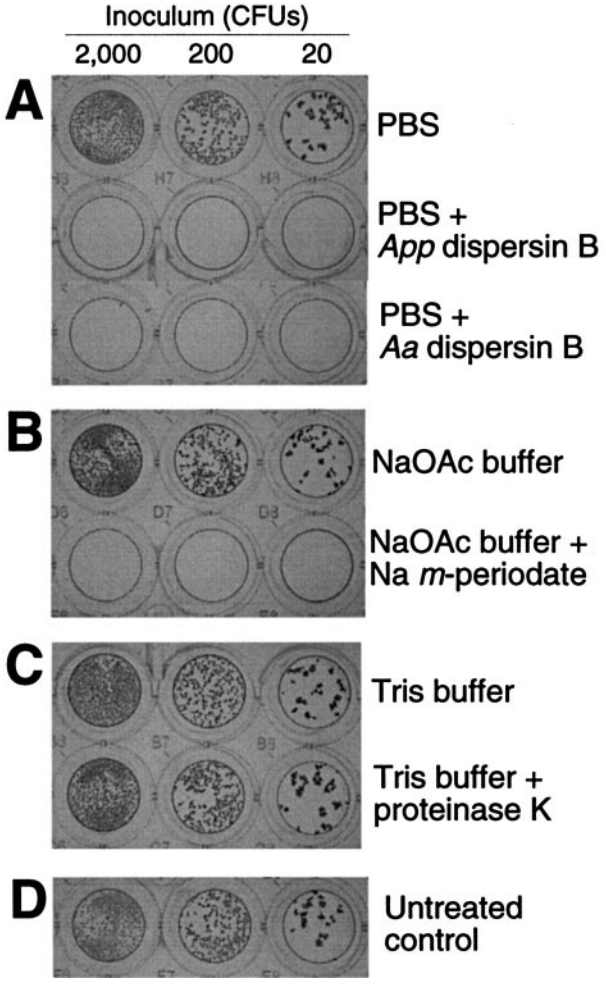

FIG. 6. Detachment of 18-h-old biofilm colonies of *A. pleuropneumoniae* strain IA5 grown in a 96-well microtiter plate. Biofilm colonies were treated with PBS with or without *A. pleuropneumoniae* (*App*) or *A. actinomycetemcomitans* (*Aa*) dispersin B (A); 50 mM sodium acetate (NaOAc) buffer, pH 4.8, with or without 10 mM sodium metaperiodate (B); 10 mM Tris-HCl (pH 7.6)–100 mM NaCl with or without 10 μg of proteinase K/ml (C). (D) Untreated controls. Biofilms were stained with crystal violet as previously described (16).

6B and C). Taken together, these data suggest that *A. pleuropneumoniae* biofilm cells produce a hexosamine-containing polysaccharide intercellular adhesin that is structurally and functionally related to the *pgaC*-dependent polysaccharide produced by *A. actinomycetemcomitans*.

DISCUSSION

Our findings demonstrate that the *pgaABCD* locus of *A. actinomycetemcomitans* encodes the production of a high-molecular-weight, hexosamine-containing extracellular polysaccharide adhesin that is a substrate for dispersin B. This polysaccharide is structurally and functionally related to *E. coli* PGA and *S. epidermidis* PIA, suggesting that it is composed of a linear polymer of GlcNAc residues in β(1,6) linkage and that this linkage is the natural substrate for dispersin B. Because of the genetic similarity between the *pgaABCD* loci of *A. actinomycetemcomitans* and those of *E. coli*, this polysaccharide is referred to here as *A. actinomycetemcomitans* PGA.

Our data indicate that PGA is a component of *A. actinomycetemcomitans* biofilm colonies. Our findings suggest that PGA may play a role in intercellular adhesion within the biofilm colony and detachment of cells from the colony (17, 18, 20). PGA does not appear to play a major role in *A. actinomycetemcomitans* surface attachment (Fig. 2A) or biofilm colony formation (J. B. Kaplan, unpublished data), phenotypes that have been shown to be mediated by proteinaceous, adhesive type IV pili (5, 6, 14–16). The fact that *pgaC* mutants were sensitive to detachment by proteinase K (Fig. 2B) suggests that PGA may also function as a physical and/or chemical barrier which prevents the access of external agents to the bacterial cells and adhesive pili within the biofilm (3). PGA may account for the increased resistance to antimicrobial agents exhibited by *A. actinomycetemcomitans* biofilm cells compared to that exhibited by cells grown in planktonic form (4). A similar protective function was ascribed to *S. epidermidis* PIA (36).

Our findings constitute the first report of biofilm formation in *A. pleuropneumoniae*. Our data suggest that the *A. pleuropneumoniae pgaCD* and *dspB* genes are orthologues of *A. actinomycetemcomitans pgaCD* and *dspB*, respectively. The similar detachment phenotypes exhibited by biofilms produced by wild-type *A. pleuropneumoniae* and *A. actinomycetemcomitans* strains (Fig. 2B and 6B and C) further suggest that *A. pleuropneumoniae* forms biofilms by mechanisms analogous to those employed by *A. actinomycetemcomitans*. Biofilm formation has been shown to play an important role in the colonization and pathogenesis of *A. actinomycetemcomitans* (7, 31). Biofilm formation may also have relevance to the colonization, pathogenesis, and transmission of *A. pleuropneumoniae*.

Homologues of the *pgaABCD* locus are present in the genomes of several other beta and gamma *Proteobacteria*, including *Bordetella pertussis*, *Bordetella parapertussis*, *Bordetella bronchiseptica*, *Burkholderia cepacia*, *Chromobacterium violaceum*, *Photobacterium profundum*, *Pseudomonas fluorescens*, *Ralstonia solanacearum*, *Xanthomonas axonopodis*, *Yersinia pestis*, *Yersinia enterocolitica*, and *Yersinia pseudotuberculosis*. Our findings support the hypothesis that these homologous loci encode the production of hexosamine-containing exopolysaccharides that stabilize biofilms in these species (37).

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