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# Poly-*N*-acetylglucosamine mediates biofilm formation and antibiotic resistance in *Actinobacillus pleuropneumoniae*

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**Abstract**

Most field isolates of the swine pathogen *Actinobacillus pleuropneumoniae* form tenacious biofilms on abiotic surfaces in vitro. We purified matrix polysaccharides from biofilms produced by *A. pleuropneumoniae* field isolates IA1 and IA5 (serotypes 1 and 5, respectively), and determined their chemical structures by using NMR spectroscopy. Both strains produced matrix polysaccharides consisting of linear chains of *N*-acetyl-D-glucosamine (GlcNAc) residues in $\beta$(1,6) linkage (poly-$\beta$-1,6-GlcNAc or PGA). A small percentage of the GlcNAc residues in each polysaccharide were *N*-deacetylated. These structures were nearly identical to those of biofilm matrix polysaccharides produced by *Escherichia coli*, *Staphylococcus aureus* and *Staphylococcus epidermidis*. PCR analyses indicated that a gene encoding the PGA-specific glycoside transferase enzyme PgaC was present on the chromosome of 15 out of 15 *A. pleuropneumoniae* reference strains (serotypes 1–12) and 76 out of 77 *A. pleuropneumoniae* field isolates (serotypes 1, 5 and 7). A *pgaC* mutant of strain IA5 failed to form biofilms in vitro, as did wild-type strains IA1 and IA5 when grown in broth supplemented with the PGA-hydrolyzing enzyme dispersin B. Treatment of IA5 biofilms with dispersin B rendered them more sensitive to killing by ampicillin. Our findings suggest that PGA functions as a major biofilm adhesin in *A. pleuropneumoniae*. Biofilm formation may have relevance to the colonization and pathogenesis of *A. pleuropneumoniae* in pigs.


*Keywords:* Crystal violet; Dispersin B; *dspB*; *pgaABCD*

## 1. Introduction

Surface-associated colonies of bacteria known as biofilms play a role in the pathogenesis of many chronic infections [1]. Bacterial cells in a biofilm are encased in a self-synthesized, extracellular hydrogel matrix that holds the cells together in a mass and firmly attaches the bacterial mass to the underlying surface [2]. This matrix, also referred to as the slime layer, glycocalyx, or extracellular polymeric substance (EPS) matrix, can comprise up to 90% of the biofilm biomass [3]. In addition to its structural role, the EPS matrix provides biofilm cells with a protected microenvironment containing dissolved nutrients, secreted enzymes, DNA, and bacteriophages. The EPS matrix also contributes to the increased resistance to antibiotics and host defenses exhibited by biofilm cells [4]. Polysaccharide is a major component of the EPS matrix in most bacterial biofilms [2].

*Abbreviations:* BHI, brain heart infusion; EPS, extracellular polymeric substance; GlcNAc, *N*-acetyl-D-glucosamine; $GlcNH_2$, glucosamine; MHB, Mueller–Hinton broth; PGA, poly-$\beta$-1, 6-*N*-acetyl-D-glucosamine; PBS, phosphate buffered saline; TSA, Tryptic Soy agar

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*Actinobacillus pleuropneumoniae* is a member of the *Pasteurellaceae*, a family of Gram-negative bacteria that includes many important human and animal pathogens. *A. pleuropneumoniae* colonizes the lungs of pigs and causes the severe and contagious respiratory disease swine pleuropneumonia [5]. Most field isolates of *A. pleuropneumoniae* form tenacious biofilms on abiotic surfaces in vitro [6]. *A. pleuropneumoniae* biofilms contain a hexosamine-rich polysaccharide that is functionally and genetically related to extracellular polysaccharide adhesins produced by *Escherichia coli*, *Staphylococcus aureus* and *Staphylococcus epidermidis* [7]. These polysaccharides, usually referred to as PGA, PNAG or polysaccharide intercellular adhesin (PIA), consist of linear chains of *N*-acetyl-D-glucosamine (GlcNAc) residues in $\beta$(1,6) linkage (hereafter referred to as PGA). Various forms of PGA appear to differ in their molecular weight, in the degree of *N*-deacetylation of the GlcNAc residues, and in the presence of *O*-succinate substituents [8–11]. PGA has been shown to play a role in abiotic surface attachment and intercellular adhesion [12–15], protection from host innate defenses including phagocytosis and antimicrobial peptides [16], and virulence [17]. PGA appears to be essential for *A. pleuropneumoniae* biofilm formation in vitro because biofilms treated with a PGA-hydrolyzing enzyme were efficiently detached from surfaces [7].

The purpose of the present study was to gain better insight into the structural and functional role of PGA in *A. pleuropneumoniae* biofilm formation. We purified PGA polysaccharides from two biofilm-producing field isolates of *A. pleuropneumoniae* and determined their chemical structures by using NMR spectroscopy. We also investigated the phylogenetic distribution of PGA biosynthetic genes among 92 *A. pleuropneumoniae* reference strains and field isolates by using a PCR assay. Finally, we investigated the role of PGA in *A. pleuropneumoniae* biofilm formation in vitro by using both a PGA mutant strain and a PGA-degrading enzyme. In this report, we present the structure of *A. pleuropneumoniae* PGA along with evidence that PGA mediates intercellular adhesion, biofilm formation and antibiotic resistance in phylogenetically diverse *A. pleuropneumoniae* strains.

## 2. Results

### 2.1. Purification of A. pleuropneumoniae PGA

PGA was purified from two biofilm-positive *A. pleuropneumoniae* field isolates, IA1 and IA5 (serotypes 1 and 5, respectively). Extraction of IA1 and IA5 biofilms with saline was not sufficient to release PGA from the cells. Sonication, however, liberated large amounts of PGA, which eluted as a single peak on a gel filtration column (Fig. 1). Based on the elution profile, the molecular weight of *A. pleuropneumoniae* PGA was similar to that of staphylococcal PGA (>20 kDa; [10]). *A. pleuropneumoniae* PGA was partially soluble in water. Complete solubility of PGA was achieved by solubilizing the samples in a small volume of 5 M HCl. GLC analysis revealed that glucosamine was the only monosaccharide component of *A. pleuropneumoniae* PGA.

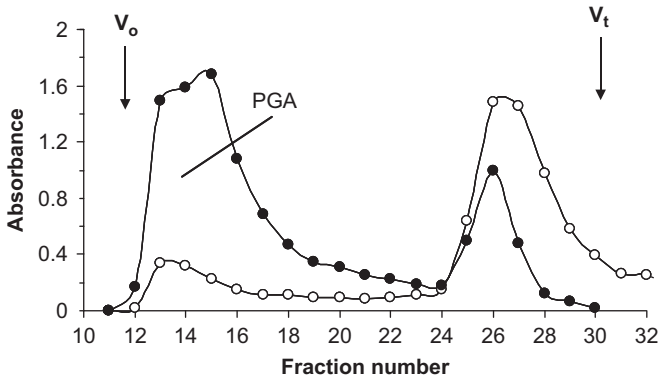


Fig. 1. Elution profile of a crude extracellular extract of *A. pleuropneumoniae* IA1 biofilm cells on a Sephacryl S-300 column irrigated with water. Aliquots (200 µL) of each 5-mL fraction were assayed for neutral sugars (○, $A_{485}$) and aminosugars (●, $A_{530}$). Void ($V_o$) and total ($V_t$) volumes of the column are indicated with arrows.

### 2.2. A. pleuropneumoniae PGA is a β(1,6)-linked GlcNAc polymer

Purified PGA polysaccharides were analyzed by $^1$H-NMR spectroscopy (Fig. 2). The $^1$H-NMR spectra of PGA from *A. pleuropneumoniae* IA1 and IA5 (Fig. 2, spectra b and a, respectively) were nearly identical to each other and to that of PGA purified from *S. epidermidis* strain RP62A (Fig. 2, spectrum c). In addition, all of the major signals of the $\beta$(1,6)-linked GlcNAc residues had shift assignments that were nearly identical to those reported for $^1$H-NMR spectra of PGA isolated from various other staphylococcal strains and from *E. coli* [8–11,15,18]. The presence of minor peaks, including one at 2.7 ppm (H2 of $GlcNH_2$), may be due to partial *N*-deacetylation of GlcNAc residues [8,9]. These results indicate that *A. pleuropneumoniae* PGA is a linear polymer with the structure →6)-$\beta$-GlcNAc-(1→.

The chemical structure of *A. pleuropneumoniae* PGA was confirmed by 2D-NMR spectroscopy (Fig. 3). $^1$H and $^{13}$C chemical shifts of both $GlcNH_2$ and GlcNAc residues (Table 1) closely corresponded to those reported for PGA purified from *S. aureus* strain MN8m [8]. PGA from strain IA1 showed more heterogeneity than PGA from IA5 due to a higher degree of *N*-deacetylation, which was evident by the relative intensity of the peak at 2.7 ppm. According to the NMR data, ~25% of the total glucosamine residues in this IA1 PGA preparation were *N*-deacetylated. This result was in good agreement with the value obtained colorimetrically using the Smith and Gilkerson method (22±2%). The degree of *N*-deacetylation in PGA from strain IA5 varied from 1% to 16%, depending on the PGA preparation. No evidence for partial *O*-succinate substitution of *A. pleuropneumoniae* PGA was found.

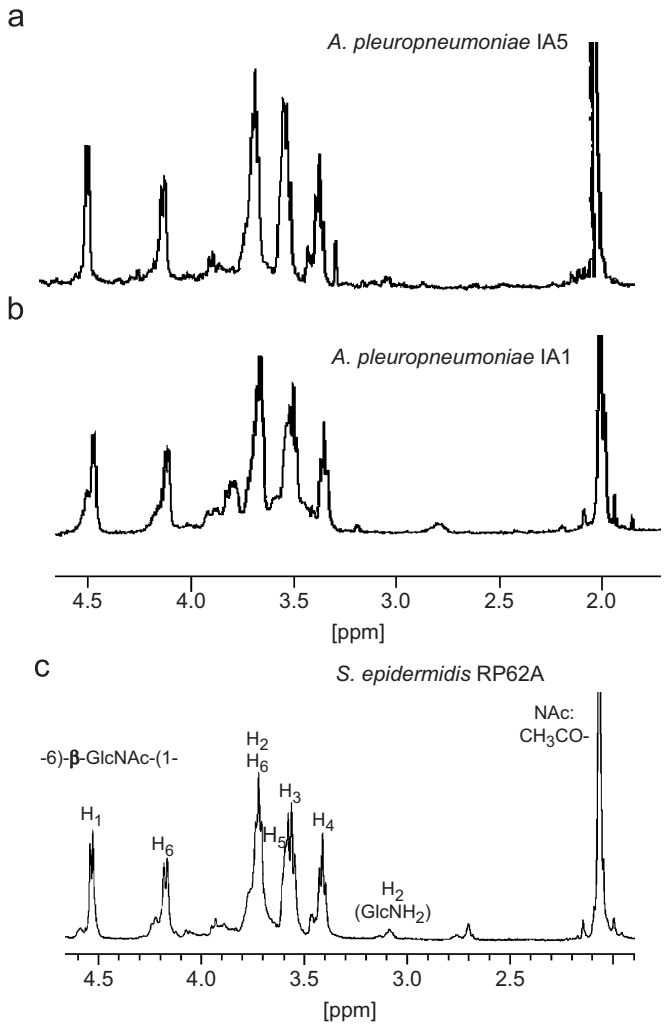


Fig. 2. $^{1}$H NMR spectra of PGA from of *A. pleuropneumoniae* strains IA5 (a), *A. pleuropneumoniae* strain IA1 (b), and PGA from *S. epidermidis* RP62A (c). The spectrum in panel (c) is from [18]. Peak assignments are according to Mack et al. [9].

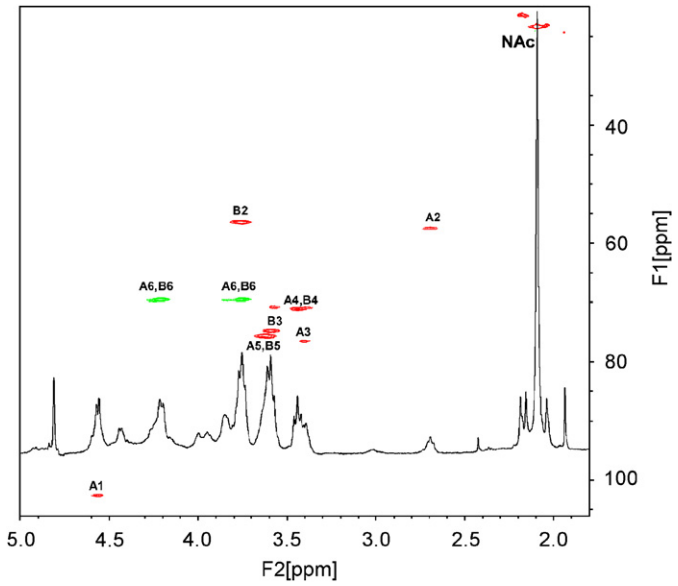


Fig. 3. Partial 500-MHz heteronuclear $^{1}$H-$^{13}$C chemical shift correlation (HSQC) spectrum of *A. pleuropneumoniae* IA1 PGA in $D_2O$ at 25 °C. Correlation peaks are labeled with residue identification of $\beta$-GlcNH$_2$ (A) and $\beta$-GlcNAc (B), as in [8].

### 2.3. PGA biosynthetic genes are widespread among A. pleuropneumoniae strains

The *A. pleuropneumoniae pgaC* gene encodes an integral membrane glycoside transferase enzyme (PgaC) that catalyzes the polymerization of PGA from UDP-GlcNAc monomers [7]. We used a PCR assay to identify *pgaC* in 15 *A. pleuropneumoniae* reference strains and 77 *A. pleuropneumoniae* field isolates (Table 2). Genomic DNA isolated from all 15 reference strains (serotypes 1–12) and from 76 out of 77 field isolates (serotypes 1, 5 and 7) produced PCR products of the expected size when amplified with *pgaC*-specific PCR primers (data not shown).

### 2.4. An A. pleuropneumoniae pgaC mutant is deficient in PGA production and biofilm formation

We used natural transformation to construct an isogenic mutant of *A. pleuropneumoniae* IA5N that contained *Haemophilus ducreyi nadV* inserted into the middle of *pgaC* on the chromosome. This mutant strain (named EI1001) produced white colonies on Congo red agar, as opposed to the red colonies produced by wild-type strain IA5. Congo red binding has been correlated with PGA production in other bacteria [7,19]. EI1001 cells also released significantly less total hexosamine when treated with the PGA-hydrolyzing enzyme dispersin B compared to the amount released by wild-type cells ($<1$ μmol of total hexosamine per mg of wet cells for *pgaC* mutant IA1001 versus ~100 μmol/mg for wild-type strain IA5). Strain EI1001 was also completely deficient in biofilm formation when tested in a microtiter plate assay (Fig. 4a). We attempted to genetically complement the *pgaC* mutation in EI1001 with pVK93, a broad-host-range plasmid that contains the *A. pleuropneumoniae pgaCD* genes located downstream from an IPTG-inducible *tac* promoter [7]. Plasmid pVK93 was previously shown to complement *pgaC* mutations in *A. actinomycetemcomitans* and *E. coli* [7]. Although pVK93 replicated in *A. pleuropneumoniae*, it did not restore the ability of strain EI1001 to synthesize PGA or to produce biofilms. This result could be due to poor expression of the pVK93 *tac* promoter in *A. pleuropneumoniae* [20]. To confirm that the biofilm-negative phenotype in strain EI1001 was due to the *nadV* insertion and not to mutations in other locations on the chromosome, we isolated genomic DNA from EI1001 and used it to transform *A. pleuropneumoniae* IA5N to NAD-independence. The resulting transformant (EI1005) was also deficient in biofilm formation (Fig. 4a). These results are consistent with the hypothesis that *pgaC* expression is essential for PGA production and biofilm formation in *A. pleuropneumoniae*.

Table 1
$^{1}H$ and $^{13}C$-NMR chemical shifts of *A. pleuropneumoniae* IA1 PGA. NAc:$CH_3$ signals were at 2.09 ($^{1}H$) and 23.3 ($^{13}C$) ppm

| Residue | Nucleus | 1 | 2 | 3 | 4 | 5 | 6a | 6b |
|---|---|---|---|---|---|---|---|---|
| $\beta$-Glc$NH_2$ A | $^{1}H$ | 4.54 | 2.69 | 3.40 | 3.39 | 3.62 | 3.75 | 4.21 |
| | $^{13}C$ | 102.3 | 57.4 | 76.5 | 70.8 | 75.6 | 69.4 | |
| $\beta$-GlcNAc B | $^{1}H$ | 4.54 | 3.76 | 3.58 | 3.44 | 3.62 | 3.75 | 4.21 |
| | $^{13}C$ | 102.3 | 56.4 | 74.7 | 71.0 | 75.6 | 69.4 | |

Table 2
Biofilm phenotypes and *pga* genotypes of *A. pleuropneumoniae* strains

| Strain | Relevant characteristics[a] | Biofilm phenotype[b] | *pgaC* genotype[b] | Source or reference[c] |
|---|---|---|---|---|
| *Reference strains* | | | | |
| 27088 | Serotype 1A | – | + | ATCC |
| ISU158 | Serotype 1B | – | + | [35] |
| 27089 | Serotype 2 | – | + | ATCC |
| 27080 | Serotype 3 | – | + | ATCC |
| 33378 | Serotype 4 | – | + | ATCC |
| ISU178 | Serotype 5 | – | + | [35] |
| K-17 | Serotype 5A | – | + | [35] |
| L20 | Serotype 5B | + | + | [35] |
| 33590 | Serotype 6 | – | + | ATCC |
| ISU63 | Serotype 7 | – | + | [35] |
| 405 | Serotype 8 | – | + | [35] |
| CVJ1326 | Serotype 9 | – | + | [35] |
| 13038 | Serotype 10 | – | + | [35] |
| 56513 | Serotype 11 | + | + | [35] |
| 1096 | Serotype 12 | – | + | [35] |
| *Field isolates* | | | | |
| IA1 | Serotype 1 | + | + | [7] |
| IA5 | Serotype 5 | + | + | [7] |
| Strain collection | 77 field isolates[d] | (53) | (99) | [6] |
| *Mutant strains* | | | | |
| IA5N | Spontaneous Nal$^{r}$ mutant of IA5 | + | + | This study |
| EI1001 | IA5N (*pgaC*::*nadV*) | – | – | This study |
| EI1005 | IA5N (*pgaC*::*nadV*) | – | – | This study |

[a] Nal$^{r}$, nalidixic acid resistant.
[b] Numbers in parentheses indicate the percentage of positive strains. Biofilm phenotypes of reference strains and field isolates are from [6].
[c] ATCC, American Type Culture Collection.
[d] Serotypes 1, 5 and 7.

### 2.5. Depolymerization of *A. pleuropneumoniae* PGA inhibits biofilm formation and increases antibiotic sensitivity

To confirm that PGA mediates *A. pleuropneumoniae* biofilm formation, we grew wild-type strains IA1 and IA5 in 96-well microtiter plates in broth containing 0 or 20 μg/mL of dispersin B. The presence of dispersin B in the broth completely inhibited biofilm formation by both strains (Fig. 2b). Concentrations of 2 and 0.2 μg/mL of dispersin B also completely inhibited *A. pleuropneumoniae* biofilm formation (data not shown). Fig. 2c shows live, 24-h old biofilm colonies of strain IA5 that were treated with dispersin B. The biofilm colonies began to dissolve almost immediately after addition of the enzyme and were completely detached by gentle rinsing after 2 min of treatment. Control experiments showed that dispersin B dispersed IA5 biofilm into uniformly turbid cell suspensions but did not lyse the cells (data not shown). This phenotype was similar to the detachment phenotype exhibited by *E. coli* and *S. epidermidis* biofilms treated with dispersin B [21,22].

We also measured the effect of PGA depolymerization on the sensitivity of IA5 biofilms to killing by ampicillin (Fig. 5). Biofilms were grown in polystyrene microcentrifuge tubes for 18 h. The broth was then removed and replaced with fresh broth containing 0, 10 or 100 μg/mL of ampicillin and 0 or 20 μg/mL of dispersin B. After 4 h the number of CFUs per tube was determined. Biofilms treated with ampicillin exhibited a significant dose-dependent decrease in the number of CFUs/tube when compared to

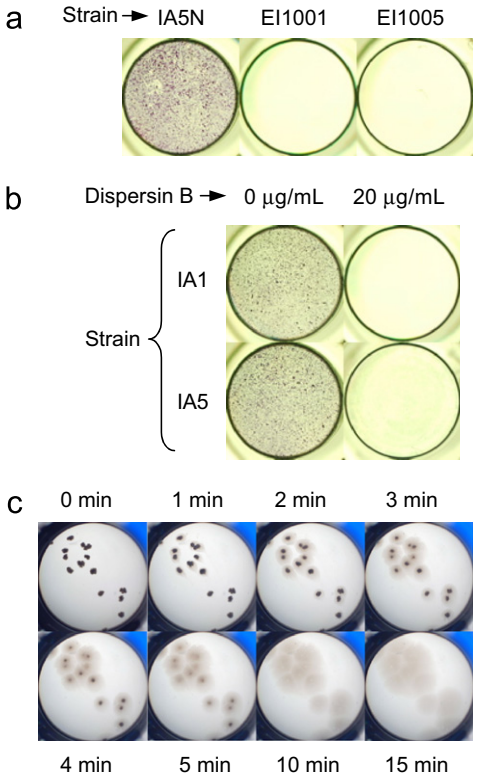


Fig. 4. Growth and detachment of *A. pleuropneumoniae* biofilms in 96-well microtiter plates: (a) Growth of biofilm-forming strain IA5N and isogenic *pgaC* mutants EI1001 and EI1005 in MHB. (b) Growth of wild-type strains IA1 and IA5 in MHB containing 0 or 20 μg/mL of dispersin B. Biofilms in panels A and B were stained with crystal violet. (c) Detachment of IA5 biofilm colonies by dispersin B. A well containing a small number of biofilm colonies (dark spots) was aspirated and filled with 100 μL of dispersin B (20 μg/mL in PBS) and photographed at the indicated times.

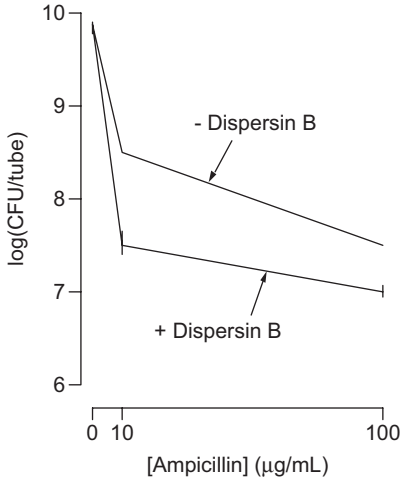


Fig. 5. Killing of *A. pleuropneumoniae* biofilms by ampicillin. Biofilms were grown for 18 h in MHB and then for 4 additional h in MHB with 0, 10 or 100 μg/mL of ampicillin and 0 or 20 μg/mL of dispersin B. CFUs were determined by dilution plating. Values indicate the mean number of CFUs/tube. Error bars indicate range.

biofilms treated with unsupplemented broth ($P<0.02$). Also, biofilms treated with ampicillin plus dispersin B exhibited a significant decrease in the numbers of CFUs/tube when compared to biofilms treated with ampicillin alone ($P<0.02$). There was not a significant difference between the CFUs/tube in tubes treated with 100 μg/mL of ampicillin and in tubes treated with 10 μg/mL of ampicillin plus dispersin B, indicating that *A. pleuropneumoniae* biofilm cells were resistant to approximately 10-fold higher concentrations of ampicillin than were planktonic cells.

## 3. Discussion

PGA polysaccharides were isolated from biofilms produced by two field isolates of *A. pleuropneumoniae*, IA1 and IA5. Structural analyses indicated that *A. pleuropneumoniae* PGA is a linear polymer of GlcNAc residues in $\beta(1,6)$ linkage (Figs. 2 and 3). Approximately, 1–25% of the GlcNAc residues were *N*-deacetylated depending on the strain and PGA preparation. This structure was identical to that reported for staphylococcal PGA [9], except that *A. pleuropneumoniae* PGA did not contain *O*-succinate substitutions. Varying degrees of *N*-deacetylation in different staphylococcal PGA preparations have been reported: 20% in *S. epidermidis* RP62A [9]; 43% in *S. aureus* MN8m [8]; and 2–20% in clinical staphylococcal strains [11]. Although *E. coli* PGA was shown to be a linear polymer of $\beta(1,6)$-linked GlcNAc residues [15], no NMR or chemical evidence for partial *N*-deacetylation of *E. coli* PGA was reported.

In *S. epidermidis*, *N*-deacetylation of PGA is catalyzed by IcaB, a protein encoded by the staphylococcal PGA biosynthetic operon (*icaADBC*) [23]. A *S. epidermidis icaB* mutant that produced fully acetylated PGA exhibited reduced biofilm formation, immune evasion, and virulence, which suggests that *N*-deacetylation is crucial for PGA function [24]. In addition, *N*-deacetylated PGA was shown to be the major epitope in the human antibody response to *S. aureus* in cystic fibrosis patients [25], and antiserum raised against *N*-deacetylated PGA, but not against fully acetylated PGA, was effective at clearing *S. aureus* in a mouse bacteremia model [26]. Interestingly, the PGA biosynthetic operon of *A. pleuropneumoniae* (*pgaABCD*) encodes a protein (PgaB) that exhibits homology to IcaB and to several other polysaccharide *N*-deacetylases [7]. The *N*-terminal half of the PgaB polypeptide comprises the deacetylase domain and the C-terminal half comprises a novel domain of unknown function. These observations suggest that PgaB may be a bifunctional PGA-specific *N*-deacetylase, and that *N*-deacetylation may be required for biological activity of PGA in *A. pleuropneumoniae*.

We found that the PGA biosynthetic gene *pgaC* was present in 15 out of 15 *A. pleuropneumoniae* reference strains and 76 out of 77 *A. pleuropneumoniae* field isolates. Previous studies showed that only 2 of these 15 reference strains and 41 of these 77 field isolates exhibited biofilm formation when tested in polystyrene tube and microtiter plate assays [6]. One explanation for this discrepancy may involve the fact that *A. pleuropneumoniae* strains can undergo a spontaneous and irreversible transition from

a biofilm-positive to a biofilm-negative phenotype upon subculture in the laboratory [6]. The genetic mechanism of this phenotypic switch is unknown. However, the closely related bacterium *A. actinomycetemcomitans* undergoes a similar irreversible switch from a biofilm-positive to a biofilm-negative phenotype upon continuous subculture [27], and this phenotypic switch in *A. actinomycetemcomitans* involves the down-regulation of PGA production (J.B. Kaplan, unpublished). *A. pleuropneumoniae* strains that exhibit a biofilm-negative phenotype may have undergone a similar down-regulation of PGA production. This hypothesis is supported by the fact that the frequency of the biofilm-negative phenotype is higher among reference strains, which are likely to have been subcultured more frequently than field isolates.

Our findings suggest that PGA functions as a major biofilm matrix adhesin in *A. pleuropneumoniae*. PGA appears to be essential for *A. pleuropneumoniae* biofilm formation because PGA mutants were completely deficient in biofilm formation (Fig. 4a), and depolymerization of PGA by dispersin B inhibited biofilm formation by wild-type strains (Fig. 4b) and caused the rapid detachment and disaggregation of preformed wild-type biofilms (Fig. 4c). Pretreatment of *A. pleuropneumoniae* biofilms with dispersin B rendered them more sensitive to killing by ampicillin (Fig. 5), indicating that *A. pleuropneumoniae* biofilm cells exhibit increased resistance to antibiotics compared to the resistance exhibited by planktonic cells.

## 4. Materials and methods

### 4.1. Bacterial strains, media and growth conditions

The *A. pleuropneumoniae* strains used in this study are listed in Table 2. Strain IA5N was isolated as a spontaneous nalidixic acid-resistant mutant of strain IA5 after plating of dense suspensions of cell on TSA containing nalidixic acid. Strain IA5N exhibits the same PGA production and biofilm formation phenotypes as strain IA5. Bacteria were grown in Mueller-Hinton broth (MHB) or on Trypticase Soy agar (TSA) or brain heart infusion (BHI) agar. MHB and TSA were supplemented with 6 g of yeast extract and 8 g of glucose per liter. Except where indicated, all media were also supplemented with 10 mg of NAD per liter. For Congo red binding assays, TSA plates were further supplemented with 0.1% (w/v) of Congo red dye. Strains were passaged twice weekly on TSA. All cultures were incubated statically at 37 °C.

### 4.2. Preparation of inocula

A 100-mm-diam tissue-culture-treated polystyrene Petri dish (Corning no. 430167) containing 20 mL of broth was inoculated with a single colony from a TSA plate and incubated for 24 h. The broth was discarded and the biofilm was rinsed with fresh broth, scraped from the surface with a cell scraper, and resuspended in 3 mL of fresh broth. The cells were vortexed for 15 s and then incubated statically for 5–10 min to allow the clumps to settle. The upper layer was transferred to a new tube and vortexed briefly. The resulting inoculum contained $>10^8$ CFU/mL.

### 4.3. Large-scale biofilm cultures

Bacteria were grown in 150-mm-diam tissue-culture-treated polystyrene Petri dishes (Corning no. 430199) or in 3 L borosilicate glass (Pyrex) Erlenmeyer flasks containing MHB (60 mL for dishes and 400 mL for flasks). For each purification, 10 dishes or 3 flasks were employed. Culture vessels were inoculated with a 0.2% volume of inoculum and incubated 18–24 h. Biofilms were washed with 0.9% (w/v) NaCl, detached from the surface using a cell scraper (for dishes) or 6-mm-diam glass beads (for flasks), and harvested by centrifugation (2200*g*, 4 °C, 15 min). Cell pellets were processed immediately after harvesting.

### 4.4. Purification of A. pleuropneumoniae PGA

The cell pellet (approximately 1.5 g of wet cells) was resuspended in 40 mL of 0.9% NaCl. The suspension was sonicated twice for 30 s on ice using an Ikasonic sonicator (IKA Labortechnik) set to 30% amplitude and 50% duty cycle. The sonicate was clarified twice by centrifugation (2200*g*, 4 °C, 15 min, then 8700*g*, 4 °C, 10 min), and the supernatant was concentrated using an Amicon 10-kDa cutoff ultrafiltration cell. TCA was added to a concentration of 5% and the precipitate was removed by centrifugation (8700*g*, 4 °C, 10 min). The deproteinated extract was fractionated on a Sephacryl S-300 column (1-cm × 90-cm; Pharmacia) irrigated with water. Fractions (5 mL) were assayed colorimetrically for aldoses [28] and amino sugars [29]. Amino sugar-rich fractions corresponding to PGA were pooled and lyophilized. The average yield was approximately 4 mg of PGA/g of wet cells.

### 4.5. Structural analyses

For NMR analysis, 3 mg of lyophilized PGA was dissolved in 30 μL of 20% (w/v) DCl/$D_2O$ and the total volume was adjusted to 1 mL with $D_2O$. NMR spectra were recorded at 25 °C in $D_2O$ with a Varian Unity Inova 500 MHz spectrometer using acetone as internal reference ($^1H$, $\delta$ 2.225 ppm, $^{13}C$, $\delta$ 31.5 ppm). Varian standard programs COSY and HSQC were used. The degree of *N*-acetylation of PGA was estimated from the ratio of GlcNAc/$GlcNH_2$ CH-2 signal intensities in the HSQC spectrum. *N*-deacetylation of PGA was also assessed colorimetrically by dissolving lyophilized PGA samples in 5 M HCl, adjusting the concentration to 0.5 M HCl, and then measuring the amount of total hexosamine and nonacetylated hexosamine as described by Smith and Gilkerson [30], with and without heating at 110 °C for 2 h, respectively. Absorbance ($A_{260}$, $A_{485}$, $A_{530}$ and $A_{650}$)

was measured using 1-cm semi-micro cuvettes (Kartell) and a Helios $\beta$ spectrophotometer (Unicam).

### 4.6. Other carbohydrate techniques

Polysaccharides were converted to alditol acetates by conventional methods following hydrolysis with 4 M trifluoroacetic acid at 120 °C for 2 h. Monosaccharides were identified by GLC with a Shimatzu GC-14 gas chromatograph equipped with a flame ionization detector and a Zebron ZB-5 capillary column (30-m × 0.25-mm, Phenomenex) with hydrogen as a carrier gas and an initial programmed temperature of 170 °C for 3 min followed by an increase to 260 °C at a rate of 5 °C/min. The amount of total hexosamine released from dispersin B-treated cells was measured using the Morgan-Elson assay as previously described [7].

### 4.7. PGA-specific PCR assay

A small loopful of cells from a 24-h-old BHI agar plate (approximately 5 mg) was transferred to a microcentrifuge tube containing 100 μL of sterile water. Cells were disrupted by vortex agitation and then incubated at 100 °C for 10 min to lyse the cells. Tubes were centrifuged for 5 min to pellet cell debris and the supernatant was transferred to a new tube. A total of 5 μL of lysate was amplified by PCR using primers 5-TTTATTGGGCTT TTGCA-3 and 5-CTCCAATACAGCCAAGG-3, which hybridize to bp 234–256 and 876–896, respectively, in the *A. pleuropneumoniae pgaC* coding region (bp 1-1236; GenBank accession no. AY618480). The PCR conditions and the sequences of 16 S rRNA-specific PCR primers used as a control were described previously [31]. PCR products were electrophoresed on agarose gels and visualized by staining with ethidium bromide. The expected sizes of the PCR products were 663 bp for the *pgaC*-specific primers and 478 bp for the 16 S rRNA-specific primers.

### 4.8. Construction of PGA mutant strains

Genomic DNA isolated from *A. pleuropneumoniae* IA5 was amplified by PCR using primers 5-GTCACGA ATTCTTATTGGGCTTTTGCAGGGC-3 and 5-CTCA GGGTACCTTCGGACACTTGTAAGCGTCC-3, which hybridize to bp 57–76 and 529–549, respectively, in *A. pleuropneumoniae pgaC* (accession no. AY618480). The resulting PCR product (525 bp) was digested with *Eco*RI and *Kpn*I (recognition sequences underlined) and ligated into the *Eco*RI/*Kpn*I sites of pC18KnadV, which contains the *H. ducreyi nadV* gene with a constitutive Tn*903* kanamycin promoter ligated into the *Pst*I site of pUC18 [32]. The *nadV* gene of pC18KnadV confers NAD independence in *A. pleuropneumoniae* [33]. The resulting plasmid (pVK208) contained the 5′ half of *A. pleuropneumoniae pgaC* located upstream from *nadV*. The 3′ half of *A. pleuropneumoniae pgaC* was amplified by PCR using primers 5-GACTCGCATGCCGCAATGTTTAATGG-GAACC-3 and 5-CTGAGAAGCTTCCAATACAGC-CAAGGATACC-3, which hybridize to bp 581–600 and 1118–1137, respectively, in *A. pleuropneumoniae pgaC* (accession no. AY618480). The PCR product (579 bp) was digested with *Sph*I and *Hin*dIII and ligated into the *Sph*I/*Hin*dIII sites of pVK208. The resulting plasmid (pVK215) contained the nearly complete *pgaC* coding region interrupted by a 1.6 kb *nadV* gene that was transcribed in the same orientation as *pgaC*. Plasmid pVK215 DNA (linearized with *Sca*I) was used to transform strain IA5N to NAD independence using natural transformation [34]. Transformant were selected on TSA without NAD. Six transformants were obtained, all of which contained *nadV* inserted into *pgaC* on the chromosome as determined by PCR using primers that hybridize to *nadV* and to DNA sequences outside the homologous regions in pVK215. For genetic complementation, plasmid pVK93, which contains the *A. pleuropneumoniae pgaCD* genes downstream from an IPTG-inducible *tac* promoter, was conjugated from *E. coli* strain C600 into the mutant strains using the helper plasmid pRK21761 as previously described [7]. Transconjugants were plated on TSA containing 3 μg/mL of chloramphenicol and 20 μg/mL of nalidixic acid.

### 4.9. Biofilm growth and detachment assays

Biofilms were grown in 96-well tissue-culture-treated polystyrene microtiter plates (Falcon no. 324662). Wells were filled with 100 μL of inoculum (diluted 1:1000 in MHB) and incubated for 12–16 h. For genetic complementation experiments, broth was supplemented with 3 μg/mL of chloramphenicol and 1 μg/mL of IPTG. For inhibition studies, media were supplemented with 0.2–20 μg of *A. actinomycetemcomitans* dispersin B per mL (ca. $10^3$ units per mg of protein [7]). For detachment studies, bacteria were grown in broth without dispersin B and the wells were then washed with phosphate buffered saline (PBS) and filled with 100 μL of PBS containing 0 or 20 μg/mL of dispersin B. After incubation at 37 °C for 5 min, the wells were rinsed with water and stained for 1 min with 100 μL of crystal violet solution (Fisher no. 23255960). Wells were then rinsed, dried and photographed. All assays were performed in duplicate or triplicate wells, which exhibited minimal variation. All assays were performed on at least three separate occasions with similar results.

### 4.10. Biofilm killing assay

Biofilms were grown in 1.5-mL polypropylene microcentrifuge tubes (Sarstedt). Tubes were filled with 200 μL of inoculum (diluted 1:1000 in fresh broth). After 16 h the broth was aspirated and replaced with fresh broth containing 0, 10 or 100 μg/mL of ampicillin and 0 or 20 μg/mL of dispersin B. After 4 h the cells were pelleted and rinsed with

sterile PBS three times to remove the ampicillin. Cell pellets were resuspended in 200 μL of PBS containing 20 μg/mL of dispersin B and incubated for 5 min to disaggregate the cells. Tubes were vortexed briefly and the number of CFUs/tube was determined by dilution plating. Killing assays were performed in duplicate tubes and on two separate occasions with similar results. The significance of differences between groups was determined by a one-way ANOVA test.

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