Pel is a cationic exopolysaccharide that cross-links extracellular DNA in the *Pseudomonas aeruginosa* biofilm matrix

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Edited by Margaret J. McFall-Ngai, University of Hawaii at Manoa, Honolulu, HI, and approved July 17, 2015 (received for review March 16, 2015)

Biofilm formation is a complex, ordered process. In the opportunistic pathogen *Pseudomonas aeruginosa*, Psl and Pel exopolysaccharides and extracellular DNA (eDNA) serve as structural components of the biofilm matrix. Despite intensive study, Pel's chemical structure and spatial localization within mature biofilms remain unknown. Using specialized carbohydrate chemical analyses, we unexpectedly found that Pel is a positively charged exopolysaccharide composed of partially acetylated 1→4 glycosidic linkages of *N*-acetylgalactosamine and *N*-acetylglucosamine. Guided by the knowledge of Pel's sugar composition, we developed a tool for the direct visualization of Pel in biofilms by combining Pel-specific *Wisteria floribunda* lectin staining with confocal microscopy. The results indicate that Pel cross-links eDNA in the biofilm stalk via ionic interactions. Our data demonstrate that the cationic charge of Pel is distinct from that of other known *P. aeruginosa* exopolysaccharides and is instrumental in its ability to interact with other key biofilm matrix components.

biofilms | exopolysaccharide | extracellular DNA | Pel | Psl

Biofilm infections are inherently difficult to eradicate, owing to increased resistance to antimicrobials and host defenses (1–3). Biofilms are microbial communities embedded in an extracellular matrix (4), composed of exopolysaccharides, extracellular DNA (eDNA), and proteins (5–7). *Pseudomonas aeruginosa* is an opportunistic pathogen that causes chronic biofilm infections (1) and has the capacity to synthesize three exopolysaccharides implicated in biofilm formation: alginate, Psl, and Pel (8, 9). Psl is a neutral polysaccharide consisting of a pentasaccharide repeat containing glucose, mannose, and rhamnose (10), and alginate is a negatively charged polymer of guluronic and mannuronic acid (8). Although previous reports suggest that glucose may be the primary component of Pel, its structure is unknown.

The role of Pel in biofilm formation was first identified in a screen for mutants deficient in pellicle formation (i.e., biofilms forming at the air–liquid interface of standing cultures) (11). Pel was later shown to be important for initiating and maintaining cell–cell interactions in biofilms (12, 13). In some circumstances, Pel also can play a role in adherence of cells to a surface (14). Finally, Pel affords biofilms protection against certain aminoglycoside antibiotics (12).

The importance of Pel in biofilm formation is strain-dependent. Nonmucoid strains (i.e., strains that make little alginate), use Pel and/or Psl as the primary structural scaffold. In the common laboratory strain PAO1, Psl is the primary exopolysaccharide produced in the biofilm matrix, although some Pel is produced as well (13, 15, 16). Deletion of *pel* genes in PAO1 does not have a significant impact on biofilm development (16). In contrast, Pel is the primary biofilm matrix exopolysaccharide in another commonly used laboratory strain, PA14, which is incapable of Psl production (16). Some strains appear to rely on both exopolysaccharides. Strains characterized by hyperadherence and hyperaggregation, called rugose small colony variants (RSCVs), are frequently isolated from in vivo and in vitro biofilms (17, 18). RSCVs harbor mutations (such as in *wspF*) that result in constitutive overexpression of both Pel and Psl (19, 20).

Biofilm formation is an ordered and sequential process that often begins with adherence of cells to a surface, followed by formation of large aggregates or microcolonies (21, 22). Polymers within the biofilm matrix exhibit spatial localization that is coordinated with the stages of biofilm development. In mature biofilms, controlled cell death and lysis occur in the interior of the microcolony, releasing eDNA that contributes to the stability of the biofilm structure (23–27). eDNA and Psl are spatially separated in mature microcolonies, with eDNA localized primarily to the base of the microcolony (27) and Psl located at the periphery of the microcolony (25).

Current data relating to the structure of Pel are limited and conflicting. It has been reported that PA14 pellicles were glucose-rich compared with a *pel* deletion mutant (11); however, the

Significance

Exopolysaccharides and extracellular DNA are important structural components that contribute to the self-assembly of large aggregates or microcolonies that are characteristic of biofilms. *Pseudomonas aeruginosa* is capable of producing multiple exopolysaccharides, including alginate, Psl, and Pel. At present, little is known about Pel's chemical structure and its role in microcolony formation. Our results demonstrate that Pel is composed of cationic amino sugars. Using this knowledge, we have developed a Pel-specific lectin stain to directly visualize Pel in biofilms. We show that the positive charge on Pel facilitates its binding to extracellular DNA in the biofilm stalk, and that Pel can compensate for lack of Psl in the biofilm periphery.

Author contributions: L.K.J., K.M.S., A.F., D.J.W., P.L.H., and M.R.P. designed research; L.K.J., K.M.S., H.E.L., C.C., L.S.M., I.S., P.R.S., B.S.T., and M.S. performed research; K.M.S. contributed new reagents/analytic tools; L.K.J., K.M.S., C.C., L.S.M., I.S., B.S.T., A.F., D.J.W., P.L.H., and M.R.P. analyzed data; and L.K.J. and M.R.P. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1503058112/-/DCSupplemental.

predominance of glucose was later attributed to cyclic glucans and not to the *pel* locus (28). In another study, analysis of extracellular material from PA14 pellicles identified lipopolysaccharide (LPS)-like material, leading to speculation that Pel is a modified form of LPS (29). We previously reported that the periplasmic PelA protein has de-*N*-acetylase activity in vitro, suggesting that some of the Pel sugars are deacetylated before being exported from the cell (30).

In the present study, we used glycosyl sugar and linkage analyses with optimized glycosidic cleavage conditions to investigate the composition of Pel. We combined lectin staining with confocal microscopy to confirm structural analyses and to directly visualize Pel in biofilms produced by distinct *P. aeruginosa* strains. These surprising results indicate that Pel is positively charged, and composed of 1→4 linked partially acetylated galactosamine and glucosamine sugars. We provide evidence that Pel cross-links eDNA in the biofilm stalk and can structurally compensate for the absence of Psl in the biofilm periphery. Thus, *P. aeruginosa* has a chemically diverse and flexible suite of matrix exopolysaccharides. Knowledge of the composition and localization of Pel is essential to delineate its functional role in biofilms, and ultimately to facilitate the development of therapeutic strategies aimed at eradicating the biofilm matrix.

Results

LPS Biosynthesis and Uridine 5′-Diphosphate–Glucose Are Not Required for Pel Production. Polysaccharide biosynthesis requires nucleotide-sugar precursors that are incorporated into the elongating polysaccharide chain (8, 31). There is precedence for a functional overlap of LPS and exopolysaccharide biosynthesis enzymes (10), and previous work suggests that Pel and LPS biosynthesis may be linked (29). Therefore, we mutated pathways involved in LPS biosynthesis and evaluated whether this impacted Pel production. These mutations impaired A band and B band O-antigen production and the LPS core. Pel immunoblots indicated that mutant strains deficient in LPS precursors reacted with the Pel antisera, whereas the corresponding mutants deficient in Pel did not (Fig. S1). Our data show that Pel production occurs even in the absence of different steps of LPS biosynthesis, demonstrating that Pel is not a modified form of LPS.

Glycosyltransferases catalyze the formation of glycosidic bonds by transferring the sugar moiety from a sugar nucleotide to a specific acceptor molecule (32). To gain insight into the activated sugar moiety that is used to generate Pel, we purified PelF, the sole predicted glycosyltransferase encoded by the *pel* operon. We used isothermal titration calorimetry (ITC) to show that PelF specifically binds uridine 5′-diphosphate (UDP) with micromolar affinity (Fig. S2), suggesting that a UDP-sugar nucleotide is required for Pel production.

Pel was previously reported as a glucose-rich polysaccharide (11). GalU is critical for the production of the nucleotide sugar UDP-glucose (Glc) (33). If Pel were a glucose-rich polysaccharide, then disrupting UDP-Glc generation should prevent Pel biosynthesis (10). Immunoblots of extracts from a *galU* mutant revealed robust Pel production relative to a Δ*pel* Δ*galU* double mutant (Fig. S1). The results demonstrate that Pel production occurs even in the absence of UDP-Glc, thus suggesting that glucose is not a primary sugar component of Pel.

Pel Is Cationic and Has a Cell-Associated and Secreted Form. We began the characterization of Pel by investigating the molecular weight and potential charge of the polysaccharide using a strain with the *pel* operon under control of the arabinose-inducible P_{BAD} promoter (PAO1 Δ*wspF* Δ*psl* P_{BAD}*pel*, herein designated P_{BAD}*pel*). This strain maximizes Pel production. Size-exclusion chromatography indicated that Pel has two forms: cell-associated and secreted. Cell-associated Pel, which was prepared by EDTA extraction of P_{BAD}*pel* cell pellets, eluted in the void volume, indicating a size >80 kDa. Secreted Pel, collected from P_{BAD}*pel* culture supernatants, eluted near the total column volume. We calculated the size of secreted Pel as 0.5 kDa by comparison with dextran standards (Fig. 1*A* and Fig. S3). In this study, we characterized the chemical composition of secreted Pel, because this form is more stable than cell-associated Pel.

To determine whether Pel carries a net charge, we performed ion-exchange chromatography on filtered supernatant of P_{BAD}*pel* and PAO1 Δ*wspF* Δ*psl* Δ*pel* (herein designated Δ*pel*) cultures. Culture supernatant was applied to a cation-exchange column preequilibrated with a pH 5.5 buffer. Examination of the cation-exchange fractions using Pel antiserum indicated that Pel bound the column and eluted at 1.25 M NaCl (Fig. S4). No binding of Pel was observed to an anion-exchange column equilibrated with a pH 7.7 buffer. These results suggest that Pel is positively charged at pH 5.5.

The isoelectric point of Pel was determined by applying supernatant from a P_{BAD}*pel* culture to a cation-exchange column under acidic conditions that facilitated binding (pH 5.5), and then gradually increasing the pH until Pel eluted (Fig. 1*B*). Pel was first detected in the effluent at pH 7.0. This suggests that the isoelectric point of Pel is between 6.7 and 6.9, and thus that Pel carries a net positive charge at pH values below this range. The isoelectric point for Pel is consistent with that of a polysaccharide containing amino sugars; for example, the amino group of chitosan (poly-β-1,4-*N*-acetylglucosamine <50% acetylated) has a pK_a of 6.5.

Pel Is Rich in 1→4 Glycosidic Linkages of *N*-Acetylgalactosamine and *N*-Acetylglucosamine. We hypothesized that if Pel contains amino sugars, that detection in previous work might have been hampered by inadequate glycosidic bond cleavage (11), because the hydrolysis of glycosidic bonds connecting amino sugars requires stronger acidic conditions than needed for neutral sugars (34). To test this hypothesis, we performed glycosyl composition analysis on ethanol precipitations of P_{BAD}*pel* and Δ*pel* culture supernatants using two different methods of glycosidic bond cleavage. The first condition, which is typically used for glycosyl composition analysis, was the weaker of the two and involved no acid hydrolysis before methanolysis (Fig. 2*A*). The second and stronger cleavage condition included acid hydrolysis before methanolysis (Fig. 2*B*).

The use of strong glycosidic bond cleavage conditions revealed that P_{BAD}*pel* is rich in *N*-acetylgalactosamine (GalNAc; 72.5 ± 3.6 mol %) and *N*-acetylglucosamine (GlcNAc; 14.5 ± 2.6 mol %). In Δ*pel*, GalNAc was below detection, and GlcNAc was 1.4 ± 0.3 mol %. The ratio of GalNAc to GlcNAc was 5:1 (± 0.7) and could indicate either a minimum repeating unit or a random incorporation of GlcNAc into the polysaccharide. The sugar composition analysis

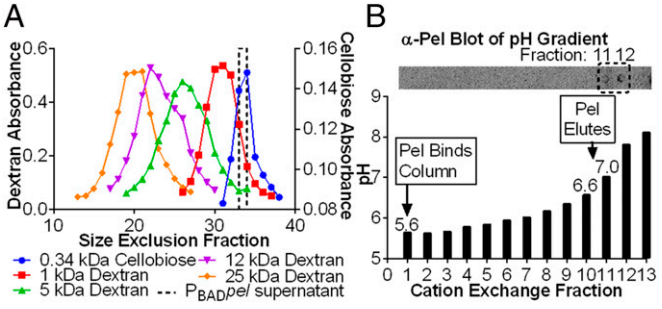

Fig. 1. Secreted Pel is a low molecular weight cation. (*A*) The average molecular weight of secreted Pel is 0.5 kDa and was determined by comparison of Pel, detected with α-Pel immunoblot in size-exclusion fractions, to dextran and cellobiose standards, detected with a colorimetric assay for neutral sugars. A single replicate is shown for clarity, but a duplicate replicate from an independent experiment behaved similarly. (*B*) A pH gradient applied to a cation column with secreted Pel bound indicated that Pel, detected with α-Pel immunoblot, had an isoelectric point of 6.7–6.9.

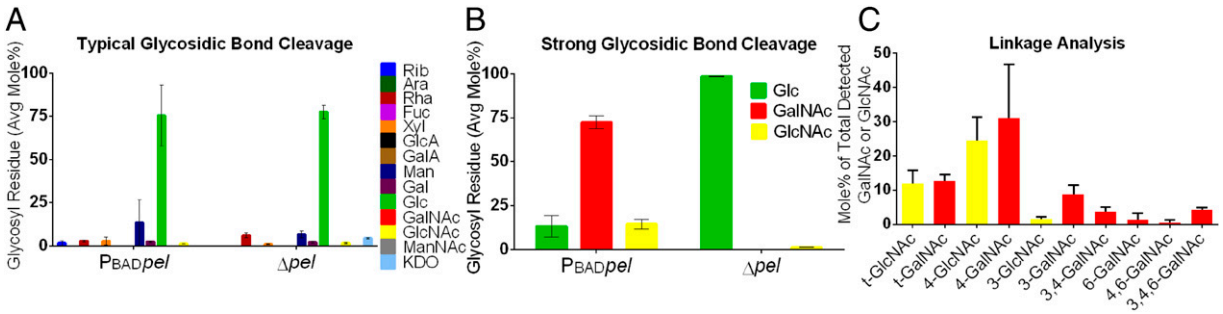

Fig. 2. Glycosyl composition and linkage analyses of P_{BAD}*pel* and Δ*pel* culture supernatant indicates that Pel is composed of 1→4 glycosidic linkages of GalNAc and GlcNAc. (*A*) Typical glycosidic cleavage conditions (no acid hydrolysis before methanolysis) were not sufficient to cleave Pel into monosaccharide components and resulted in detection of an abundance of glucose (Glc). (*B*) Strong glycosidic cleavage conditions (acid hydrolysis followed by methanolysis) enabled detection of amino sugars GalNAc and GlcNAc. (*C*) Glycosyl linkage analysis indicated that 4-linked GalNAc and GlcNAc were the most abundant residues detected of the total GalNAc and GlcNAc linkages. t, terminal. Error bars represent SD (n = 2).

involved the chemical re-*N*-acetylation of amino sugars and thus does not indicate the degree of acetylation of such sugars; however, in secreted Pel, the amino sugars cannot be 100% acetylated, because otherwise the polysaccharide would not carry a charge. The presence of partially acetylated amino sugars is consistent with the observed de-*N*-acetylase in vitro enzyme activity of PelA (30).

Glycosyl linkage analysis of ethanol precipitations of P_{BAD}*pel* supernatant revealed that among the residues detected from GalNAc and GlcNAc linkages, 4-linked GalNAc and GlcNAc were the most abundant (Fig. 2*C*). These results suggest that Pel is a linear exopolysaccharide containing 1→4 glycosidic linkages of partially acetylated GalNAc and GlcNAc. Consistent with glucosamine as a secondary component of Pel, monoclonal antibodies raised against poly-β-1,6-*N*-acetylglucosamine (PNAG) and chitosan (poly-β-1,4-*N*-acetylglucosamine) bound samples from a P_{BAD}*pel* strain (Fig. S5 *A* and *B*). Chitosanase, which catalyzes the specific cleavage of β-1,4 acetylated and nonacetylated glucosamine linkages, showed some activity on pellicle biofilms (Fig. S5*C*).

Given that GalNAc was the most abundant sugar detected, we screened lectins that recognized GalNAc sugars to identify a Pel-specific lectin. Fluorescein-labeled *Wisteria floribunda* lectin (WFL), which has specificity to GalNAc moieties, bound clusters of planktonic P_{BAD}*pel* cells, but not from *Δpel* cells (Fig. S5*D*). This finding provides evidence that in addition to secreted Pel, GalNAc is also a component of cell-associated Pel.

Pel Localization in Biofilms Is Strain-Dependent. To directly visualize Pel in developing biofilms, we coupled Pel-specific lectin staining with confocal microscopy. Biofilms were grown in continuous-flow chambers (flow cells) inoculated with strains that differ in their exopolysaccharide use/dependence. Pel localization in PA14 is similar to that previously reported for Psl localization in PAO1 (25), in that Pel was found at the periphery of cell aggregates (Fig. 3*A*). The peripheral staining pattern was enhanced in the Pel-overproducing strain PA14 Δ*wspF*; however, PAO1 and PAO1 Δ*wspF,* strains that produce significant amounts of peripherally localized Psl, exhibited minimal Pel staining at the periphery of cell aggregates (Fig. 3*B*). We hypothesized that Psl might prevent or supersede Pel production in the periphery. To test this, we generated a *psl*-deficient mutation in PAO1 Δ*wspF* (PAO1 Δ*wspF* Δ*psl*), and found a strong increase in Pel staining at the periphery of the biofilm (Fig. 3*C*).

Localization of Pel to the center of the microcolony "stalk" near the attachment surface was observed in all strains tested (Fig. 3). This staining pattern was most prominent in PAO1 Δ*wspF*, which overproduces Pel. A similar but less-intense staining pattern was observed in PAO1. Although this staining pattern was seen in most cell aggregates, it was occasionally absent in PA14 or PA14 Δ*wspF*. Minimal to no background staining was detected in biofilms produced by *pel* mutants cultured on glucose minimal media, verifying that the WFL lectin is specific to Pel (Fig. S6*A*). A PAO1 Δ*wspF* and PA14 biofilm development time course resulted in consistent localization patterns regardless of the relative age of the biofilm (Fig. S6 *B* and *C*).

Transcriptional Control Is Not Sufficient to Account for Pel Localization. One potential explanation of the observed localization of exopolysaccharides in biofilms is that only cells in the stalk and periphery of these aggregates synthesize Pel. To test this possibility, we used two strains, PAO1 Δ*wspF* Δ*pel* P_{BAD}*psl* (herein referred to as P_{BAD}*psl*) and PAO1 Δ*wspF* Δ*psl* P_{BAD}*pel*, in which either Psl or Pel expression is under the control of an arabinose-inducible promoter. The strategy was to grow biofilms in the presence of arabinose and then assess which regions were capable of exopolysaccharide production using lectin staining.

In a P_{BAD}*psl* biofilm stained with *Hippeastrum* hybrid (HHA; Psl-specific lectin) (25), Psl staining was uniform throughout the biofilm (Fig. S6*D*). This is in contrast to uninduced strains, such as PAO1 (25) and PAO1 Δ*wspF* biofilms (Fig. 3*C*), in which Psl staining occurs primarily at the surface and periphery of large aggregates. This suggests that although cells in the interior of the aggregates of PAO1 biofilms have the capacity to produce Psl, some sort of regulatory control prevents this.

Surprisingly, in a P_{BAD}*pel* biofilm stained with Pel-specific lectin, Pel was present only in the stalk and periphery of the large aggregates, leaving large unstained regions in the interior of the microcolony (Fig. S6*D*). This pattern of staining is similar to that observed in PA14 and PA14 Δ*wspF* biofilms. Staining was uniform in younger P_{BAD}*pel* microcolonies, indicating that the localization in mature microcolonies was not likely an artifact of a staining idiosyncrasy. Assuming that the *pel* transcript is expressed uniformly throughout the biofilm (which certainly appears to be the case for *psl*, as described above), these data suggest that some form of posttranscriptional control is preventing Pel production in the unstained regions of the biofilm community. We also cannot rule out the possibility that the Pel lectin is sensitive to the presence of other matrix components in the biofilm (although this appears not to be the case for the Psl lectin).

The Pel localization pattern in a 2-d P_{BAD}*pel* biofilm was such that staining of the periphery and stalk were clearly evident (Fig. 4*A*). At day 2, Pel staining in the stalk was visible as a small concentric circle in the interior of the microcolony near the substratum. By day 3, a large microcolony developed, and Pel staining material in the stalk had expanded until it was visible at the exterior of the biofilm (Fig. 4*B*). The images (Fig. 4*B* and Movie S1) illustrate that the stalk consists of intertwined fibers or columns of Pel extending from the substratum to the mushroom-shaped cap, tethering the latter in place. From these images, it is suggestive that

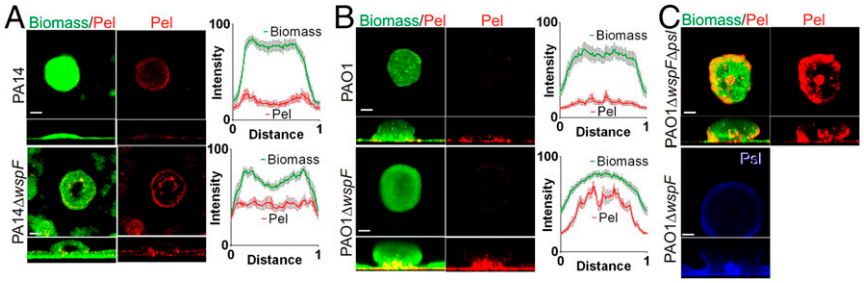

Fig. 3. Pel localization in biofilms (*A*–*C*). Representative confocal images are from biofilms cultivated for 3–4 d in flow cells and imaged with the Pel-specific lectin (WFL, red), Psl-specific lectin (HHA, blue), and/or biomass stain (Syto62, green). Horizontal optical cross-sections (large square) captured in the middle of the microcolony and side view (rectangle) are shown. (Scale bars: 30 μm.) Line profiles were quantified from a horizontal line drawn in the stalk (bottom quarter of the microcolony). The average normalized fluorescence signal intensity (100 × [intensity/maximum intensity]) from 12 to 16 microcolonies from at least two independent experiments is plotted versus normalized distance (distance/total length of the microcolony).

Pel can play a major structural role in holding different regions of the biofilm together.

Pel Colocalizes with eDNA in the Biofilm Stalk. We hypothesized that localization of Pel in biofilms could be influenced by its positive charge. Previous reports indicate that negatively charged eDNA is present in the stalk of developing biofilm aggregates (27); thus, we predict that Pel would interact with eDNA. To investigate whether Pel and eDNA staining patterns colocalized, we treated biofilms with the Pel-specific lectin and the eDNA stain propidium iodide (PI). PAO1 Δ*wspF* and P_{BAD}*pel* biofilms stained separately with PI or WFL indicate that eDNA and Pel colocalize to the stalk of the microcolony (Fig. 5*A*). When we attempted to stain Pel and eDNA in the same biofilm, PI and WFL interfered with the staining of each other (Fig. S7*A*).

We hypothesized that exogenously added DNA to the bulk liquid might directly bind to Pel in biofilms. To test this, we introduced salmon sperm DNA to mature P_{BAD}*pel* biofilms for 15 min before staining with PI. We found greater PI staining concentrated in the stalk of P_{BAD}*pel* biofilms when exogenous DNA was added, relative to the control lacking exogenously added DNA (Fig. 5*B*). PI staining in the P_{BAD}*psl* biofilm was minimal, suggesting that DNA binds something in the biofilm stalk in a Pel-dependent manner. Why exogenous DNA does not bind peripherally localized Pel is unclear, although it is possible that the pH at the exterior of the biofilm is too high to produce positively charged Pel.

Pel Cross-Links DNA via an Ionic-Binding Mechanism. Gel electrophoresis of phenol extracts of PA14 standing cultures indicated the presence of a high molecular weight band that was absent from the corresponding profile of PA14Δ*pel* (Fig. S7*B*). This band stained with ethidium bromide and was DNase-sensitive (Fig. S7 *C* and *D*). These results indicate that a phenol extraction of a PA14 pellicle is enriched in DNA, possibly due to interactions with Pel.

Cross-linking can occur by ionic or covalent bonding of two polymers to each other. To investigate the mechanism of Pel/DNA cross-linking, we added DNA to supernatant from P_{BAD}*pel* 24-h planktonic cultures (Fig. 5*C*). The cross-linking of secreted Pel to exogenous DNA resulted in visible aggregates that stained with Congo red, a dye known to bind Pel (11). No aggregation was observed with Δ*pel* supernatant. A small amount of aggregation was observed in samples with P_{BAD}*pel* supernatant and no added DNA, presumably owing to endogenous eDNA.

Cross-linking of Pel to DNA was found to be pH-dependent, supporting the role of the positively charged amino groups of Pel in this process. In vitro aggregation (cross-linking) occurred at pH 6.3, where Pel is positively charged, but not above the isoelectric point of Pel at pH 7.3, where the polysaccharide carries no charge. The cross-linking of Pel to DNA was reversible. Pel /DNA aggregates could be resolubilized by increasing the pH to ∼9 or increasing the salt concentration of the solution to ∼1.4 M NaCl. Aggregates would spontaneously reform once the pH was dropped below the isoelectric point.

We reasoned that Pel could participate in the cross-linking of other anionic polymers, such as dextran sulfate, hyaluronan (an important component of the host extracellular matrix), or mucin (the primary component of mucous). Supporting this notion, positively charged Pel from P_{BAD}*pel* supernatant cross-linked and formed visible aggregates with dextran sulfate, hyaluronan, and mucin (Fig. 5*D*). Collectively, these results suggest that Pel interacts with DNA and other anionic polymers via an ionic-binding mechanism. The cross-linking of Pel to host relevant polymers, such as hyaluronan and mucin, suggests that this mechanism of adhesion could have implications in disease pathogenesis.

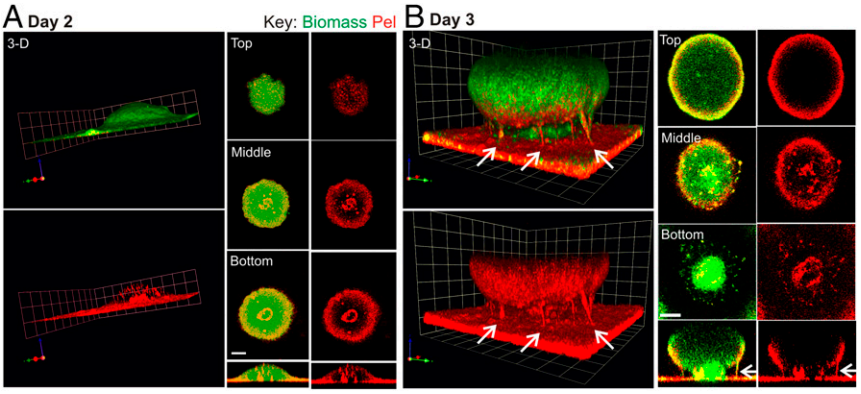

Fig. 4. Pel is localized to the periphery and stalk in a P_{BAD}*pel* biofilm and is a major structural component of the stalk. Shown are representative confocal images of flow cell biofilms cultivated for 2 d (*A*) and 3 d (*B*) before staining with biomass stain (Syto62, green) and Pel-specific lectin (WFL, red). Arrows indicate columns of Pel in the biofilm stalk. (Scale bars: 30 μm.) For 3D images, 1 U = 22.6 μm for 2 d biofilms and 1 U = 14.15 μm for 3 d biofilms. The gamma was adjusted on the red channel of the 3D images to reduce periphery staining and enhance visualization of Pel in the microcolony interior (day 2, γ = 3; day 3, γ = 2).

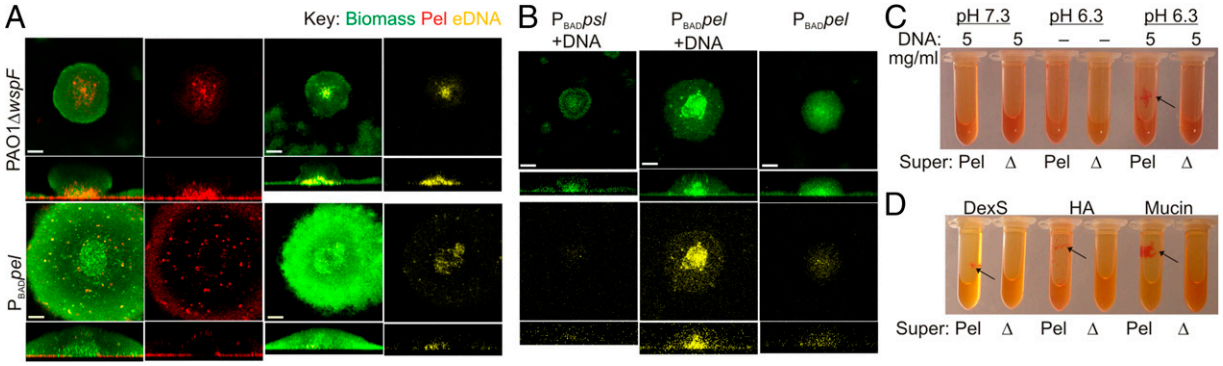

Fig. 5. Pel cross-links eDNA in the biofilm stalk via an ionic binding mechanism. (*A* and *B*) Representative confocal images of flow cell biofilms before staining with Pel-specific lectin (WFL, red), biomass stain (Syto62, green), and/or eDNA stain (PI, yellow). Horizontal cross-sectional views were captured in the biofilm stalk (lower quarter of the microcolony). (Scale bars: 30 μm.) (*A*) Pel and eDNA colocalized in PAO1 Δ*wspF* and P_{BAD}*pel* biofilms. (*B*) Salmon sperm DNA incubated for 15 min with mature P_{BAD}*pel* biofilms concentrated in the stalk compared with P_{BAD}*pel* without added DNA. Exogenous DNA was not localized in P_{BAD}*psl* biofilms. (*C*) Cross-linking of secreted Pel from P_{BAD}*pel* supernatant (Pel) to salmon sperm DNA resulted in visible aggregates that bind Congo red (arrow). (*D*) Aggregation of the anionic polymers dextran sulfate (DexS), hyaluronan (HA), and mucin with positively charged Pel from P_{BAD}*pel* supernatant (Pel) at pH 6.3 (arrows). Aggregation was not observed in Δ*pel* supernatant (Δ).

Discussion

Herein we provide multiple complementary lines of data indicating that Pel is composed of positively charged amino sugars. More specifically, our data suggest that Pel is a cationic exopolysaccharide consisting of partially acetylated GalNAc and GlcNAc. The positive charge imparts important functional characteristics on Pel, as it is capable of cross-linking eDNA in the biofilm matrix. Finally, our findings allowed us to devise an approach to directly visualize Pel in biofilms with the WFL lectin. Our data illustrate that (*i*) Pel binds eDNA in the biofilm stalk region and (*ii*) Pel can compensate for the lack of Psl in the biofilm periphery.

It is known that *P. aeruginosa* can produce the neutral and anionic exopolysaccharides Psl and alginate, respectively. In this study, we have shown that Pel is positively charged under neutral to mildly acidic conditions. The ability to produce three exopolysaccharides, each with a different charge at physiological pH, may afford *P. aeruginosa* biofilms increased flexibility to maintain biofilm structure and/or protect cells from antimicrobials under different conditions. We imagine gaining further insight into this question as we start to unravel how environmental conditions regulate the production of these polymers. We predict that the positive charge comes from the partial de-*N*-acetylation of GalNAc and GlcNAc sugars. In *Staphylococcus* species, partial deacetylation (10–20%) of the amino groups from the exopolysaccharide PNAG introduces a net positive charge (35). Nondeacetylated PNAG does not attach to the cell surface, presumably due to loss of cationic charge, and thus strains in which the PNAG deacetylation machinery is impaired are deficient for biofilm formation (36).

There is precedence for exopolysaccharides (e.g., Psl) to have a secreted and cell-associated form that can differ slightly in structure and modifications (10). NMR structural analysis of Pel likely would indicate whether cell-associated Pel is chemically distinct from secreted Pel, but attempts to obtain an NMR spectrum of Pel have thus far been unsuccessful. NMR also would indicate whether there is a small glucose component to Pel, or whether the cellulase sensitivity of pellicles (11) and use of UDP-Glc as a substrate by PelF (37) is related to nonspecific enzyme activity.

Cell death and the corresponding eDNA release is a coordinated process in the biofilm lifecycle (24). Cell lysis occurs in the central region of microcolonies, releasing abundant amounts of eDNA that concentrate primarily in the stalk (25, 27). Although eDNA can act as a structural component of biofilms (23, 26, 27), the idea that it might directly bind to other matrix polysaccharides was never clearly appreciated. In the present study, we have demonstrated that secreted Pel cross-links DNA via an ionic-binding mechanism. pH gradients in *P. aeruginosa* biofilms, reported to range from pH 5.5 at the center of the microcolony to pH 7 near the bulk fluid (38), indicate that Pel likely is positively charged in the biofilm stalk, facilitating ionic interactions. We propose that Pel localization to the stalk is partially driven by the presence of eDNA, and that Pel/eDNA interactions form the structural core of the biofilm stalk. Research investigating the correlation between Pel-dependent strains and the importance of eDNA is currently underway.

It is possible that the cross-linking of eDNA to other cationic exopolysaccharides (e.g., PNAG) may be a general mechanism underlying the structural integrity of biofilms. Furthermore, the cross-linking of Pel to host polymers (i.e., hyaluronan and mucin) may have implications in disease pathogenesis. Hyaluronan and mucin are abundant at sites of chronic infection, such as the lungs of patients with the genetic disorder cystic fibrosis. *P. aeruginosa's* capacity to produce a polysaccharide that binds diverse host polymers may be a key mechanism by which the bacterial pathogen initiates and sustains cystic fibrosis lung infections.

Both Psl and Pel can serve as key structural components of the biofilm matrix (16). In PAO1, Psl is localized to the periphery of the microcolonies (25). In this study, we have shown that either Pel or Psl can localize to the periphery of microcolonies. In the absence of Psl, Pel production increases in the periphery, suggesting that Pel can compensate for the lack of Psl. We propose that in general, the structural integrity of a biofilm microcolony depends on the presence of an exopolysaccharide at the aggregate's periphery. Psl appears to be the dominant matrix component suited to fill this role, because in strains that have the genetic capability to produce both exopolysaccharides, only Psl is observed at the periphery. The ability of Pel to compensate for Psl is consistent with previous findings demonstrating increased Pel production in pellicles in the absence of Psl (15). The regulatory mechanism that ensures that Pel compensates for the absence of Psl in the periphery is unknown, but it is tempting to speculate that the Psl polysaccharide is the environmental signal that down-regulates Pel production in the biofilm periphery.

In conclusion, our study demonstrates that the repertoire of exopolysaccharide types that can be used by *P. aeruginosa* is surprisingly diverse in its chemistries. We hypothesize that a primary role of Pel is to provide structural stability to the interior/core of biofilm microcolonies. Our data also suggest that peripherally localized exopolysaccharide may be an important general feature of biofilm microcolonies. Knowledge of the spatial localization of conserved biofilm matrix components may impact treatment regimens for biofilm infections.

Materials and Methods

More detailed information is provided in *SI Materials and Methods*.

Culturing and Purification of Pel Polysaccharide. Bacterial strains are listed in Table S1. Planktonic cultures were maintained on Jensen's defined medium (pH 7.3). Biofilms were grown in continuous-flow cell chambers on glucose minimal media (pH 7). Cell-associated Pel was extracted from P_{BAD}*pel* and Δ*pel* cell pellets with EDTA. Secreted Pel was collected from culture supernatants and in some cases purified further using cation-exchange or ethanol precipitation.

Glycosyl Composition and Linkage Analyses. Carbohydrate analyses were conducted at the University of Georgia's Complex Carbohydrate Research Center (CCRC) on ethanol precipitated supernatant from P_{BAD}*pel* and Δ*pel*. Samples for composition analysis were subjected to (*i*) typical glycosidic cleavage (no acid hydrolysis before methanolysis) or (*ii*) strong glycosidic cleavage (hydrolysis with TFA and hydrochloric acid, followed by methanolysis). GC/MS was used to analyze per-*O*-trimethylsilyl methyl glycosides for composition analysis and partially methylated alditol acetates for linkage analysis.

Lectin Staining and Confocal Microscopy. Flow cell biofilms were stained and then imaged on a confocal microscope. To determine whether exogenous DNA bound Pel in biofilms, salmon sperm DNA was added to mature biofilms and incubated statically for 15 min, and then rinsed, stained with Syto62 and PI, rinsed again, and finally imaged on the confocal microscope.

In Vitro Pel Cross-Linking Experiments. P_{BAD}*pel* and Δ*pel* supernatant were mixed with anionic polymers including DNA, dextran sulfate, hyaluronan, and mucin until dissolved. The pH of the polymer solution was adjusted to 7.3 or 6.3. Congo red was added, and aggregates resulting from the cross-linking of the Pel and anionic polymers were visualized.

ACKNOWLEDGMENTS. We thank T. Romeo and S. Schillberg for antibodies, and M. Vedadi and G. Senisterra (Structural Genomics Consortium, Toronto) for ITC assistance. This work was supported by National Institutes of Health Grants 2R01AI077628 (to M.R.P.) and R01AI097511 (to D.J.W.), and Canadian Institutes of Health Research Operating Grant 13337 (to P.L.H.). L.K.J. is the recipient of an American Heart Association Postdoctoral Fellowship (14POST20130017). P.R.S. and B.S.T. are recipients of Cystic Fibrosis Foundation Postdoctoral Fellowships. L.S.M. is supported by graduate scholarships from the Natural Sciences and Engineering Research Council of Canada, the Ontario Graduate Scholarship Program, and the Hospital for Sick Children Foundation Student Scholarship Program. P.L.H is the recipient of a Canada Research Chair.

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