The Pel polysaccharide is predominantly composed of a dimeric repeat of $\alpha$-1,4 linked galactosamine and $N$-acetylgalactosamine

François Le Mauff, Erum Razvi, Courtney Reichhardt, Piyanka Sivarajah, Matthew R. Parsek, P. Lynne Howell & Donald C. Sheppard

The genetic capacity to synthesize the biofilm matrix exopolysaccharide Pel is widespread among Gram-negative and Gram-positive bacteria. However, its exact chemical structure has been challenging to determine. Using a *Pseudomonas aeruginosa* strain engineered to overproduce Pel, improvements to the isolation procedure, and selective hydrolysis with the glycoside hydrolase PelAh, we demonstrate that Pel is a partially de-$N$-acetylated linear polymer of $\alpha$-1,4-$N$-acetylgalactosamine comprised predominantly of dimeric repeats of galactosamine and $N$-acetylgalactosamine.
Biofilms are aggregates of microorganisms attached to each other and/or to a surface encased in a self-produced extracellular matrix. This matrix, composed of proteins, extracellular DNA, lipids, and polysaccharides, creates a unique microenvironment and enables the biofilm community to have emergent properties that are different from planktonic bacteria. Exopolysaccharides are vital to the biofilm matrix and contribute to adhesion, cell-to-cell interactions, and protection from antimicrobials and host immune responses. Exopolysaccharides produced by microorganisms are structurally very diverse with linear and branched homo- and heteropolymers composed of distinct monosaccharides that are produced by unique biosynthetic pathways. These polymers can also be further modified post-polymerization through the action of various carbohydrate-active enzymes (CAZymes).

*Pseudomonas aeruginosa* is a Gram-negative bacterium that has been the focus of intense research due to its prominent role in disease and ability to form biofilms on medical devices and human tissues. *P. aeruginosa* is recognized as a primary pathogen for individuals with cystic fibrosis (CF), who become chronically infected with the bacterium. The failure to clear the infection from CF airways leads to inflammation and lung disease, and *P. aeruginosa* remains one of the leading causes of morbidity and mortality in this patient population. *P. aeruginosa* is genetically capable of producing at least three distinct exopolysaccharides: alginate, Psl, and Pel. Alginate is primarily associated with chronic infections and used for biofilm formation by mucoid *P. aeruginosa* strains. Aggregates of non-mucoid clinical isolates in CF airways have recently been shown to express both Pel and Psl.

Pel-dependent biofilm formation in *P. aeruginosa* requires the activity of a seven gene operon, *pelABCDDEFG*, whose products mediate sugar polymerization and transport across the cytoplasm (PelDEFG), modification by hyaluronidase and deacetylation activities (PelA), and export (PelBC) of the polymer into the extracellular milieu. We recently identified a variant form of this gene cluster, *pelDEA21FG*, in numerous Gram-positive bacterial species and have shown that this locus is required for biofilm formation in *Bacillus cereus* ATCC 10987. Our bioinformatics analyses have now identified that more than 1400 Gram-positive and Gram-negative bacterial species have the genetic capacity to synthesize Pel, making the Pel biosynthetic locus one of the most phylogenetically widespread biofilm matrix determinants in bacteria. Determination of the role of Pel in the formation, maintenance, and properties of biofilms by this wide range of organisms has been hampered by our incomplete knowledge of its chemical structure.

An engineered Pel overexpression strain, PAO1 Δ*p wspF Δpsl Δ*pel*, has been used extensively in the literature to study Pel. In this strain a polar mutation in the *psl* operon and the replacement of the native promoter region of *pelA* with the ara-C-Δ*p*BAD promoter on the PAO1 chromosome allows for the arabinose-dependent expression of the *pel* operon specifically. The in-frame mutation in *wspF*, a negative regulator of the diguanylate cyclase WspR, results in a high bis-(3’-5’)–cyclic dimeric guanosine monophosphate (c-di-GMP) background which transcriptionally and post-translationally activates Pel biosynthesis. *P. aeruginosa* allows for robust, inducible expression of Pel and was used to show that the Pel polymer is de-N-acetylated, conferring a cationic charge at physiological pH that facilitates interactions with anionic host polymers such as DNA, and increases antimicrobial tolerance. Subsequent studies using *P. aeruginosa* have identified the *Wisteria floribunda* (WFL) lectin as being Pel specific, suggesting that the polymer contains terminal N-acetylgalactosamine (GalNAc) moieties. Previous glycosyl composition, linkage analysis, and solid-state NMR of Pel from *P. aeruginosa* have reported that secreted Pel is a cationic polymer of 50% de-N-acetylated 1–4 linked GalNAc and N-acetylgalactosamine (GlcNAc) in a 5:1 ratio.

Attempts to fully characterize the anionic configuration and structure of Pel have been hampered in the past by the insolubility of the polymer. Using a modified isolation procedure and hydrolysis with PelAΔN, the recombinantly expressed glycoside hydrolase domain of the Pel modification enzyme, PelAΔN, we overcame these challenges and herein present the chemical structure of Pel. We determined that Pel does not contain GlcNAc and is a linear homopolymer of partially de-N-acetylated α-1,4-GalNAc comprised predominantly of dimeric repeats of galactosamine (Gal) and GalNAc.

**Results and discussion**

Pel is a polymer of partially de-N-acetylated 1,4 linked N-acetylgalactosamine. Secreted Pel was isolated from the supernatants of the genetically engineered *P. aeruginosa* strain and the isogenic Pel-deficient PAO1 Δ*p wspF Δpsl Δpel* strain as a negative control. The monosaccharide composition of Pel was analyzed via gas chromatography coupled to mass spectrometry (GC–MS) following reductive amination of the polymer heparosamines, polymer hydrolysis, re-N-acetylation and trimethylsilyl derivatization of the monosaccharides. GlcNAc was found to be the main monosaccharide present in Pel with an average relative abundance of 66 ± 2.9% (Fig. 1). The rest of the sample was predominantly composed of GalNAc (33 ± 2.9%, Fig. 1). Partially methylated alditol acetate derivation of chemically re-N-acetylated Pel was used to determine the linkages between GalNAc/GlcNAc residues of secreted Pel. Only 4-linked GalNAc and GalN residues were found (Fig. 1). Unlike previous studies, GlcNAc was not detected in our GC–MS analyses. We hypothesize that the 4-GlcNAc previously reported in secreted Pel likely originated from contaminating peptidoglycan, fragments of which may have been released into the culture supernatant following cell lysis or outer membrane vesicle release. Consistent with this hypothesis, the previous analysis of Pel composition also reported the presence of GlcNAc and high amounts of glucose in the secreted polysaccharide isolated from the Pel-deficient Δ*pel*.

![Fig. 1 Pel is a partially de-N-acetylated polymer of 1,4-N-acetylgalactosamine.](https://example.com/fig1.png)
Pel is a polysaccharide composed of α-linked monosaccharides.

The determination of the anomeric configuration of the monosaccharides in Pel was performed using $^1$H NMR spectroscopy. Prior to the spectral acquisition, solubilization of Pel was achieved through re-N-acetylation and enzymatic hydrolysis of the re-N-acetylated Pel with the recombinantly expressed hydroxylase domain of PelA (PelAh). This treatment released short soluble acetylated Pel with the recombinantly expressed hydrolase eliminating these contaminating sugars. Taken as a whole, these findings reveal that secreted Pel is a linear polymer of partially de-N-acetylated 1,4 linked GalNAc.

Pel is predominantly constituted of a dimer repeat (GalN-GalNAc). To study Pel monosaccharide arrangement within the polymer, Pel was partially hydrolyzed with hydrochloric acid. The analysis of the released oligosaccharides by MALDI-TOF mass spectrometry revealed that ~50% of the oligosaccharide population was comprised of an equal number of hexosamine (HexN) and N-acetylated hexosamine (HexNAc) residues (Fig. 3a and b). MSMS fragmentation of these ions revealed that the oligosaccharides were composed of a dimeric repeat of HexN-HexNAc (Fig. 3c). The remaining 50% of ions found in the spectra were identified as homo-α-1,4-HexNAc with different degrees of de-N-acetylation (Fig. 3b). No de-N-acetylation was observed for ~18% of the ions, partial de-N-acetylation (less than 50%) was observed for ~19% of the ions, and about 14% of the ions exhibited more than 50% de-N-acetylation (Fig. 3b). Combined, these data indicate that Pel is comprised predominantly of GalN-GalNAc repeating units.

Enzymatic confirmation of Pel structure. To further validate our findings, we used MALDI-TOF enzyme fingerprinting with a panel of recently characterized α-1,4-GalNAc specific CAZymes (Figs. 4 and 5, Supplementary Fig. 1). Using native or re-N-acetylated Pel as substrates, we found that the endo α-1,4-galactosaminidase Ega3 cleaved native Pel, producing a specific HexN-MS fingerprint at the reducing end of dimer repeats of HexN-HexNAc$^27$ (Fig. 4a). As anticipated, the re-N-acetylated polymer was more resistant to Ega3 degradation (Fig. 4b). Conversely, while the endo-α-1,4-α-acetylglactosaminidases Sph3 and PelAh, both degraded re-N-acetylated Pel, only PelAh was able to release small amounts of GalN-GalNAc oligosaccharides from the native polymer (Fig. 4c-f). This is consistent with previous findings revealing that despite sharing the same enzymatic activity, differences in active site architecture result in Sph3 being specific for poly-GalNAc, while PelAh is able to tolerate the presence of GalN within the target polymer.$^25$ No oligosaccharide products were detected in native Pel and re-N-acetylated Pel samples incubated in PBS, confirming that the oligosaccharides detected by MALDI-TOF were the product of the action of the respective CAZymes (Supplemental Fig. 2).

Lastly, Agd3, an α-(1-4)-N-acetylglactosamine deacetylase was able to de-N-acetylate re-N-acetylated Pel (Fig. 5)$^{28}$, Collectively, these enzymatic studies add further evidence that Pel is a partially de-N-acetylated polymer of α-1,4-GalNAc.

Discussion

Although secreted P. aeruginosa Pel has previously been characterized as a cationic polymer of 50% de-N-acetylated 1-4 linked
GalNAC and GlcNAC in a 5:1 ratio\(^9,20\) determination of the anomeric configuration and structure of Pel have been hindered by its insolubility. Using PBAD\(_{pel}\), a P. aeruginosa strain engineered to overproduce Pel, improvements to the isolation procedure, recombinantly expressed PelA\(_p\), and a combination of MS and NMR analyses, we have determined that Pel is a linear homopolymer of partially de-N-acetylated \(\alpha\)-1,4-linked GalNAc that is comprised predominantly of dimeric repeats of GalN and GalNAc. Notably, we found that Pel does not contain GlcNAc\(^20\).

The PBAD\(_{pel}\) strain was used to determine the structure of Pel due to its ability to produce sufficient quantities of the polymer for structural studies. This strain has been used to characterize Pel-dependent phenotypes and has been extensively compared to P. aeruginosa PA14, an intrinsically Pel-producing strain in which the pel operon was the first identified\(^10\). Both PBAD\(_{pel}\) and PA14 require PelA deacetylase activity for Pel-dependent biofilm formation, have the same phenotypes in crystal violet assays measuring biofilm adherence, and wrinkly colony morphologies on Congo red plates indicative of exopolysaccharide production\(^12\). PBAD\(_{pel}\) and PA14 both form flow cell biofilms that have signals with fluorescein-labeled WFT\(^20,29\). In addition, biofilms formed by PBAD\(_{pel}\) and PA14 can be disrupted by the exogynous addition of PelA\(_p\), an endo-\(\alpha\)-1,4-N-acetylgalactosaminidase with a deep electronegative active site able to bind and cleave cationic partially de-N-acetylated GalNAc oligosaccharides\(^25,30\). Given the corresponding phenotypes, we anticipate the chemical composition of secreted Pel produced by PBAD\(_{pel}\) and PA14 will be equivalent.

Knowledge of the chemistry and structure of Pel provides more insight and substantiates previous findings regarding its biosynthesis. For example, the lack of GlcNAC in Pel correlates with the absence of multiple glycosyltransferases and an epimerase in the P. aeruginosa pel operon\(^10,16\). Multiple glycosyltransferases encoded in an operon frequently indicate that more than one type of monosaccharide is incorporated into the polymer, as seen in the psl operon, which contains the glycosyltransferases pslC, pslH, and pslL, and produces a repeating branched pentasaccharide containing D-mannose, L-rhamnose, and D-glucose\(^7,31\). In the P. aeruginosa alg operon, a C5-epimerase, AlgG, converts D-mannuronate (D-ManA) to L-guluronate (L-GulA), producing bacterial alginate, a randomly acetylated linear polysaccharide composed of \(\beta\)-1,4-linked D-ManA and varying amounts of L-GulA\(^4,32,33\). The presence of only one glycosyltransferase and the absence of an epimerase to perform post-polymerization modification in the P. aeruginosa pel operon had long suggested that Pel was a homopolymer, which the data presented herein confirms.

Similarly, our finding that Pel does not contain GlcNAC also adds support to the proposal that the key sugar-nucleotide precursor involved in Pel biosynthesis is likely, GalNAC bound to uridine 5'-diphosphate (UDP)\(^34\). Previous studies have already established that PelF, the putative Pel glycosyltransferase, binds UDP\(^20\). The genes encoding enzymes for precursor generation in P. aeruginosa for Psl and alginate are found within and/or adjacent to their associated exopolysaccharide gene clusters. However, in P. aeruginosa, the pel operon does not contain genes that encode an enzyme capable of producing the sugar-nucleotide precursor, indicating that they have to be encoded elsewhere on the chromosome\(^7,10,12,34\). We have previously identified PelX, a UDP-GlCNAC C4-epimerase in Pseudomonas protegens Pf-5 that generates UDP-GalNAC precursors\(^34\). PelX and its parologue, PgnE, were found to be critical for Pel polysaccharide production in this species. While, an active UDP-GlCNAC C4-epimerase has not been identified in P. aeruginosa Pel biosynthesis, it is likely that PA4068, which shares 76% identity with PgnE, fulfills this role\(^34\). Confirmation that the Pel polysaccharide is a homopolymer of de-N-acetylated GalNAC supports the requirement for an active UDP-GlCNAC C4-epimerase to generate UDP-GalNAC, thus warranting further investigation of the role of PA4068 in P. aeruginosa Pel biosynthesis.

The identification of the Pel structure reveals its high degree of similarity to the fungal Aspergillus fumigatus biofilm.
exopolysaccharide, galactosaminogalactan (GAG), a hetero-polysaccharide composed of α-1,4 linked Gal, GalNAc, and galNAc. Both GAG and Pel have an α-anomeric configuration, are 1,4 linked, and contain GalNAc and galNAc, thus explaining the cross-kingdom activity of the fungal and bacterial glycoside hydrolases, Ega3 and PelAh, to disrupt both A. fumigatus GAG and P. aeruginosa Pel biofilms. NMR and molecular dynamics simulations performed on synthetic (α-1,4-GalNAc)₇ to investigate the conformation and spatial presentation of the oligomer revealed that C2 acetyl substituents are on alternating sides of the longitudinal axis of the polymer. These data suggest that the dimeric GalN-GalNAc repeat present in the Pel structure, is the result of PelA de-N-acetylating one face of the polymer processively.

Knowledge of the Pel polysaccharide composition coupled with a structure of PelA will aid in understanding the mode of action of this CAZyme.

Our recent bioinformatics analyses found the pel operon to be widespread in a diverse array of eubacteria, suggesting that partially de-N-acetylated α-1,4-GalNAc exopolysaccharides are probably not limited to Pseudomonas spp, Bacillus cereus, and A. fumigatus biofilms. The extraordinary conservation of the pel operon across organisms suggests that other bacterial species also secrete a partially de-N-acetylated α-1,4-GalNAc exopolysaccharide and that Pel could play a greater role in virulence than...
previously appreciated. Targeting this polymer at the post-tran-
scriptional, post-translation, or post-polymerization level could 
represent a promising avenue for diagnostic and personalized 
antimicrobial treatments based on the exopolysaccharide compo-
sition determined in this work.

Methods

**Polysaccharide isolation.** The Pel overexpression strain PAO1 ΔwspF Δpel PAH52 (PasPe) and negative control strain PAO1 ΔwspF Δpel (Pel-) were cultured and Pel isolated as described previously\(^\text{20}\) with the following modifications: (i) Jensen’s medium was inoculated with 2 mL of overnight culture; (ii) after the culture supernatant precipitate was washed three times with 95–100% (v/v) ethanol, it was left to air dry before resuspension in 20 mL of buffer (1 mM CaCl\(_2\), 2 mM MgCl\(_2\), in 50 mM Tris, pH 7.5); (iii) the re-suspended precipitate was treated with 15 mg DNase I and 15 mg RNase A overnight at 37 °C, followed by 20 mg proteinase K overnight at 37 °C; (iv) after heating the enzyme-treated sample at 95 °C for 10 min it was centrifuged at 36,000 × g for 10 min at 20 °C; and (v) in the final step the sample was extensively dialyzed against water using 50 kDa molecular weight cut tubing prior to being flash-frozen and lyophilized.

**De-N-acetylation quantification by reductive amination and monosaccharide composition by Gas chromatography coupled to mass spectrometry (GC-MS).** To perform reductive amination, samples were reconstituted in a solution of 1 M sodium cyanoborohydride reconstituted in dimethylsulfoxide (DMSO): acetic acid 2 mM, reconstituted with Trimethylsilyl as reported previously\(^\text{38}\). Briefly, samples were hydroyzed with either 2 M trifluoroacetic acid (TFA) for 2 h at 110 °C, or 6 M hydrochloric acid (HCl) for 4 h at 100 °C to quantify non-amino and mono-

**Anomer conformation by nuclear magnetic resonance**

**Pel chemical re-N-acetylation.** Purified Pel samples were incubated in a solution of methanol: pyridine: acetic anhydride (10:2:3) overnight at room temperature, and then washed twice with pure methanol.

**Linkage analysis.** Partially methylated alditol acetate (PMAA) derivatives of re-N-acetylated Pel were made using a modified protocol\(^\text{28}\). Briefly, samples were first permethylated with 300 μL of iodomethane in a slurry of DMSO: sodium hydro-

**Anomer determination by nuclear magnetic resonance**

**PMAA derivatives was carried out using a solution of methanol: pyridine: acetic anhydride (10:2:3) for 1 h at room temperature. Residues were then silylated with a mix of hexamethyldisiloxane-trimethylchlorosilane: pyridine (3:1:9). Quantification of reduced hexosamines to N-acetylhexosamines was performed by using GC-MS by injecting the TMS derivatives into the Agilent Technology Ensemble 5977B GC-MS equipped with a CP-SILCB capillary column using a temperature gradient as previously reported\(^\text{25}\).

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