PelX is a UDP-N-acetylglucosamine C4-epimerase involved in Pel polysaccharide–dependent biofilm formation

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Pel is a GalNAc-rich bacterial polysaccharide that contributes to the structure and function of Pseudomonas aeruginosa biofilms. The pelABCDEFG operon is highly conserved among diverse bacterial species, and Pel may therefore be a widespread biofilm determinant. Previous annotation of pel gene clusters has helped us identify an additional gene, pelX, that is present adjacent to pelABCDEFG in >100 different bacterial species. The pelX gene is predicted to encode a member of the short-chain dehydrogenase/reductase (SDR) superfamily, but its potential role in Pel-dependent biofilm formation is unknown. Herein, we have used Pseudomonas protegens Pf-5 as a model to elucidate PelX function as Pseudomonas aeruginosa lacks a pelX homologue in its pel gene cluster. We found that P. protegens forms Pel-dependent biofilms; however, despite expression of pelX under these conditions, biofilm formation was unaffected in a ΔpelX strain. This observation led us to identify a pelX parologue, PFL_5533, which we designate here PgpE, that appears to be functionally redundant to pelX. In line with this, a ΔpelX ΔpgpE double mutant was substantially impaired in its ability to form Pel-dependent biofilms. To understand the molecular basis for this observation, we determined the structure of PelX to 2.1 Å resolution. The structure revealed that PelX resembles UDP-GlcNAc C4-epimerases. Using 1H NMR analysis, we show that PelX catalyzes the epimerization between UDP-GlcNAc and UDP-GalNAc. Our results indicate that Pel-dependent biofilm formation requires a UDP-GlcNAc C4-epimerase that generates the UDP-GalNAc precursors required by the Pel synthase machinery for polymer production.

Exopolysaccharides are a critical component of bacterial biofilms. The opportunistic pathogen Pseudomonas aeruginosa is a model bacterium for studying the contribution of exopolysaccharides to biofilm architecture because biofilms formed by this organism use exopolysaccharides as a structural scaffold (1). P. aeruginosa synthesizes the exopolysaccharides alginate, Psl, and Pel, and each have been shown to contribute structural and protective properties to the biofilm matrix under various conditions (2). Although these polysaccharides differ in their chemical composition and net charge, the synthesis of all three polymers requires sugar-nucleotide precursors. Genes encoding enzymes required for precursor generation are often found within or adjacent to the gene cluster responsible for the production of their associated polysaccharide. For example, Psl requires GDP-mannose precursors, which are generated from mannose-1-phosphate by the enzyme PsIB (3). Similarly, alginate requires the precursor GDP-mannuronic acid and the alg locus encodes two of the three enzymes, AlgA and AlgD, required to synthesize this activated sugar (4, 5). The third enzyme, AlgC, is not found within the alg operon and is also involved in synthesizing precursors for Psl and B-band lipo-polysaccharide (6).

In Gram-negative bacteria, the pelABCDEFG operon encodes seven gene products that are required for pellicle (Pel) biofilm formation (7). These biofilms form at the air–liquid interface of standing P. aeruginosa cultures (8). In contrast to the Psl and alginate gene clusters, none of the P. aeruginosa pel genes are predicted to be involved in sugar-nucleotide precursor production, indicating that, like AlgC, these functions are encoded by genes elsewhere on the chromosome. Analyses of Pel have demonstrated that it is a cationic polysaccharide rich in GalNAc residues and that the putative Pel polymerase, PelF, preferentially interacts with the nucleotide UDP (9). Additionally, functional characterization of PelA has demonstrated that it is a bifunctional enzyme with both polysaccharide deacetylase and α-1,4-N-acetylglactosaminidase activities, which further supports the hypothesis that the precursor required for the biosynthesis of Pel is an acetylated sugar (10, 11). Together, these data suggest that a key sugar-nucleotide precursor involved in Pel biosynthesis is UDP-GalNAc, the high-energy precursor needed for the biosynthesis of GalNAc-containing glycans.

We recently made the observation that many bacteria possess an additional ORF in their pel biosynthetic gene clusters that is predicted to encode a member of the short-chain dehydrogenase/reductase (SDR) enzyme superfamily (12, 13). The SDR superfamily is an ancient enzyme family whose members share a common structural architecture and are involved in the

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EDITORS’ PICK: Pel biosynthesis requires a UDP-GlcNAc C4-epimerase

Figure 1. A pelX homologous gene is found adjacent to pel biosynthetic gene clusters in three different arrangements. Each gene is shown as an arrow, where each arrow indicates the direction of transcription. The predicted function of each gene is indicated by its color as per the legend (bottom). One representative bacterial species is shown per gene arrangement. The total number of species identified with each arrangement is indicated (right). The full list of species with pelX-containing pel loci can be found in Dataset S1.

The synthesis of numerous metabolites, including sugar-nucleotide precursors used for the generation of bacterial cell surface glycans (14). In several species of bacteria, such as the plant-protective pseudomonad *Pseudomonas protegens*, the SDR encoding gene *pelX* is found directly upstream of the *pel* genes, whereas other bacteria such as *P. aeruginosa* lack this gene within their *pel* locus.

Plant root colonization by *P. protegens* Pf-5 requires the formation of biofilms. This process has been shown to require the biofilm adhesin LapA (15). In addition to LapA, biofilms produced by this strain also contain undefined exopolysaccharides (16, 17). Besides Pel, *P. protegens* Pf-5 has the genetic capacity to synthesize the exopolysaccharides Psl, alginate, and polysaccharides which play in the biosynthesis of the Pel polysaccharide.

In the present study, we show that *P. protegens* Pf-5 forms Pel-dependent biofilms at air–liquid interfaces, and using *P. protegens* PelX as a representative Pel polysaccharide-linked SDR enzyme, we find that this enzyme functions as a UDP-GlcNAc C4-epimerase. We find that the *pelX* gene is not essential for Pel polysaccharide–dependent biofilm formation because *P. protegens* possesses a paralogue of this gene, PFL_5533. Deletion of both of these genes was found to substantially impair Pel-dependent biofilm formation. Based on our analyses we designate PFL_5533 as a polysaccharide UDP-GlcNAc epimerase (pgnE) and propose that the production of UDP-GalNAc by UDP-GlcNAc C4-epimerases is a critical step in the biosynthesis of the Pel polysaccharide.

Results

Identification of an SDR family enzyme associated with pel gene clusters

In a previous study, we used the sequence of PelC, a protein required for Pel polysaccharide export, to identify *pel* biosynthetic loci in a wide range of Proteobacteria (12). In addition to the conserved *pelABCDEFG* genes, several of these loci contained an additional ORF. We observed several genomic arrangements containing this gene (Fig. 1). In 70% of these genomes, the additional gene is located directly upstream of *pelA* and may be transcribed together with the *pel* genes. In 24% of cases, the gene is located upstream of *pelA* but is divergently transcribed, whereas 5% of the time the gene is encoded downstream of *pelG* (Fig. 1). Sequence and structure-based analyses of the protein product of this gene, PelX, using BLAST and Phyre² suggest that it likely encodes an SDR family enzyme (18, 19). In total, we identified 136 *pel* loci containing a *pelX* gene (Fig. 1 and Dataset S1).

To determine whether *pelX* plays a role in Pel polysaccharide–dependent biofilm formation, we set out to characterize *pelX* in a species of bacteria for which the regulation of *pel* gene expression has been studied. In *P. protegens*, which contains a *pelX* gene upstream of *pelA*, the *pel* gene cluster is under the control of the same Gac/Rsm global regulatory cascade as in *P. aeruginosa* (17). In addition, two putative recognition sequences for the enhancer-binding protein FleQ are found upstream of *pelX* (PFL_2971), not *pelA*, suggesting that in contrast to *P. aeruginosa*, *pelX* may be the first gene of the *pel* operon in this species (20). Given that this operon is likely regulated in a similar manner to the *pel* locus of *P. aeruginosa* and that these two species are closely related, we used *P. protegens* to characterize the role of PelX in biofilm formation.

*P. protegens* forms pel-dependent biofilms that are enhanced by elevated levels of c-di-GMP

In addition to the *pel* genes, *psl* gene expression has been shown to be regulated by the Gac/Rsm pathway in *P. protegens* and this regulatory cascade is required for *P. protegens* biofilm formation (17). Interestingly, some strains of *P. aeruginosa*, including PAO1, use Psl as their predominant biofilm matrix exopolysaccharide whereas others, such as PA14, use Pel (21). Therefore, to determine whether *P. protegens* biofilms are dependent on Pel and/or Psl, we generated strains lacking *pelF* or *pslA*, genes previously shown to be required for Pel- and Psl-dependent biofilm formation, respectively, and examined whether these strains could form biofilms (8, 22). After 5 days
of static growth in liquid culture, we found that WT and ΔpslA strains of *P. protegens* adhered similarly to a polystyrene surface, whereas a strain lacking *pelF* displayed a marked reduction in surface attachment (Fig. 2A). The level of surface adherence of a ΔpelF ΔpslA double mutant was comparable to that of the ΔpelF strain. Based on these data, we conclude that the *Pel* polysaccharide is a critical component of *P. protegens* Pf-5 biofilms.

Previous analysis of the region upstream of *P. protegens* *pelX* identified a FleQ consensus binding sequence (20). FleQ is a bis-(3',5')-cyclic dimeric GMP (c-di-GMP)–responsive transcription factor that binds to specific sequences upstream of the *pel* operon in *P. aeruginosa*, blocking their transcription (23). When the intracellular concentration of c-di-GMP is high, FleQ switches to an activator and up-regulates transcription of the *pel* genes (24). Based on these observations, we reasoned that expression of the *P. protegens* *pel* operon is likely up-regulated in the presence of elevated levels of c-di-GMP (23). To test this hypothesis, we expressed the well-characterized diguanylate cyclase WspR of *P. aeruginosa* from an IPTG-inducible plasmid in *P. protegens* (25). Because WspR activity can be inhibited by c-di-GMP binding to an allosteric site of the enzyme, we inactivated this autoinhibitory site by introducing a previously characterized R242A point mutation into the sequence of the protein (WspR<sup>R242A</sup>) (26). Upon induction of WspR<sup>R242A</sup> expression, ~2.3-fold more *P. protegens* adhered to polystyrene surfaces compared with a vector control strain (Fig. 2B). Taken together, our data suggest that *P. protegens* Pel-dependent biofilm formation is enhanced in response to elevated intracellular c-di-GMP levels.

**pelX is expressed under biofilm-promoting conditions but is functionally redundant with PFL_5533**

Because Pel-dependent biofilm formation is enhanced in the presence of c-di-GMP and FleQ is predicted to bind upstream of the *pelX* gene, we reasoned that *pelX* is most likely expressed in a c-di-GMP–dependent manner along with the rest of the *pel* genes. To test this, we probed for the expression of PelX by fusing a vesicular stomatitis virus glycoprotein (VSV-G) tag to its C terminus at the native *pelX* locus on the *P. protegens* chromosome. To examine expression of the *pel* operon, a VSV-G tag was similarly added to the C terminus of the putative Pel synthase subunit, PelF (13). Strains expressing either WspR<sup>R242A</sup> or a vector control were grown under biofilm-conducive conditions and analyzed by Western blotting. In strains lacking WspR<sup>R242A</sup>, neither PelX nor PelF could be detected; however, in the WspR<sup>R242A</sup>-expressing strains, both PelX and PelF were detected at their expected molecular mass of 34 and 58 kDa, respectively (Fig. 3A). These data suggest that *pelX* and *pelF* expression are positively regulated by c-di-GMP in *P. protegens* and that PelX is expressed under conditions where the *Pel* polysaccharide is produced. How...
ever, when we deleted pelX, we found that P. protegens bio-
film biomass was unaffected, indicating that PelX is not
essential for Pel-dependent biofilm formation (Fig. 3B). These
findings led us to hypothesize that the P. protegens genome
might encode a second SDR enzyme that renders PelX function-
ally redundant. We queried the PelX amino acid sequence
against the P. protegens PF-5 proteome using BLASTP to identify
similar proteins (18). This search identified several proteins
from the SDR superfamily (Table 1); however, one protein in
particular, PFL_5533, stood out because it shares 68% sequence
identity with PelX. To determine whether PFL_5533 is ex-
dressed during P. protegens biofilm formation, we fused a C-
terminal VSV-G tag to PFL_5533 at its native chromosomal locus
and examined its expression in the presence and absence of
WspR^{R242A}. We detected similar levels of VSV-G tagged
PFL_5533 strains at levels comparable to WT, whereas no Pel polysaccharide was
detected in the ΔpelX ΔPFL_5533 double mutant. Taken to-
gether, these data indicate that pelX and PFL_5533 have geneti-
cally redundant functions in biofilm formation under our
experimental conditions, and that the activity of a predicted
SDR family enzyme is essential for Pel polysaccharide biosyn-
thesis and Pel-dependent biofilm formation by P. protegens.

**Table 1**

| PFL number | Annotation                      | Predicted function               | % Identity to PelX |
|------------|---------------------------------|----------------------------------|--------------------|
| PFL_2971   | PeIX                            | NAD-dependent epimerase/dehydratase | 100.00             |
| PFL_5533   | NAD-dependent epimerase/dehydratase | 67.97                           |
| PFL_3079   | LspL                            | UDP-glucuronate 5'-epimerase      | 30.54              |
| PFL_5405   | GalE                            | UDP-Glc 4-epimerase              | 29.88              |
| PFL_0305   | rbB                             | dTDP-glucose 4,6-dehydratase      | 33.60              |
| PFL_5490   | NAD-dependent epimerase/dehydratase | 30.87                          |
| PFL_4822   | 3β-hydroxysteroid dehydrogenase/isomerase | 29.92                        |
| PFL_6133   | NAD-dependent epimerase/dehydratase | 29.77                          |
| PFL_4307   | WbpV                            | UDP-Glc 4-epimerase/WbpV          | 29.41              |
| PFL_3045   | armA                            | Tribhifunctional UDP-glucuronic acid decarboxylase/UDP-4-amino-4-deoxy-1-arabinose formyltransferase | 25.98 |
| PFL_4587   | NAD dependent epimerase/dehydratase | 34.09                          |
| PFL_4375   | NAD dependent epimerase/dehydratase | 28.78                          |
| PFL_5491   | Gmd                             | GDP-mannose 4,6-dehydratase       | 28.07              |
| PFL_5106   | WbjB                            | Trifunctional UDP-β-GlcNAc 4,6-dehydratase/5-epimerase/3-epimerase WbjB | 24.46 |
| PFL_3633   | NAD-dependent epimerase/dehydratase | 22.34                          |

* Predicted function based on Pfam analysis (53).

**PelX is a UDP-GlcNAc C4-epimerase that preferentially epimerizes N-acetylated UDP-hexoses**

To gain further insight into PelX function, we initiated struc-
tural and functional studies on recombinant PelX protein. Ini-
tial efforts to purify His_{6}-tagged PelX overexpressed in Esche-
richia coli yielded two species consistent with a monomer and
dimer of PelX when analyzed by SDS-PAGE. Addition of
reducing agent significantly lowered the abundance of the puta-
tive PelX dimer, suggesting that this higher molecular weight
species likely arose from the formation of an intermolecular dis-
sulfide bond. This intermolecular disulfide bond is likely not
biologically relevant given that the bacterial cytoplasm is a
reducing environment. As sample heterogeneity can be prob-
lematic for both the interpretation of biochemical data and pro-
tein crystallization, we generated a PelX variant in which the
cysteine residue presumed to be involved in disulfide bond for-
mation (Cys-232) was mutated to serine (PelX^{C232S}). This
PelX^{C232S} variant appeared as a monomer on SDS-PAGE and
its purification to homogeneity was straightforward. When
examined by size-exclusion chromatography, PelX^{C232S} had
an apparent molecular mass of 64 kDa compared with its expected
monomeric molecular mass of 35 kDa, suggesting that like
other characterized SDR enzymes, PelX forms noncovalent,
SDS-sensitive dimers in solution (Fig. S1) (28).

The SDR superfamily of enzymes is known to catalyze
numerous chemical reactions, including dehydration, reduc-
tion, isomerization, epimerization, dehalogenation, and decar-
boxylation (14). We hypothesized that PelX likely functions as
an epimerase because UDP-GlcNAc, the putative precursor for
Pel, is typically generated from UDP-GlcNAc by SDR epimer-
ase–catalyzed stereochemical inversion at the C4 position of
the hexose ring. Characterized SDR C4-epimerases are classi-
fied into three groups based on their substrate preference (29).
Group 1 epimerases preferentially interconvert nonacetylated
UDP-hexoses, group 2 epimerases are equally able to interconvert nonacetylated and N-acetylated UDP-hexoses, whereas group 3 epimerases preferentially interconvert N-acetylated UDP-hexoses. Given that the Pel polysaccharide is GalNAc rich, we hypothesized that PelX likely functions as either a group 2 or group 3 epimerase. To examine the potential epimerase activity of PelX, we used $^1$H NMR to monitor the stereochemistry of UDP-GlcNAc, UDP-GalNAc, UDP-Glc, or UDP-galactose (UDP-Gal) in the presence or absence of purified PelXC232S. Two $^1$H NMR resonances with characteristic multiplicities in the 5.4–5.7 ppm H-1 region allow for the differentiation of UDP-GalNAc/UDP-Gal from UDP-GlcNAc/UDP-Glc, respectively (Fig. 4A and B). Using these resonances, we found that PelX$^{C232S}$ readily converts UDP-GlcNAc to UDP-GalNAc and vice versa (Fig. 4A and C). PelX$^{C232S}$ also converted a minor amount of UDP-Gal to UDP-Glc, however, we did not observe significant conversion of UDP-Glc to UDP-Gal (Fig. 4B). Collectively, these data define PelX as a group 3 UDP-hexose C4-epimerase.

To corroborate our biochemical data, we next performed absolute quantification of cellular GalNAc and GlcNAc levels in our WspR R242A-expressing P. protegens WT, ΔpelX, ΔPFL_5533, and ΔpelX ΔPFL_5533 strains. Although GalNAc levels were below the limit of our detection methods, we found that GlcNAc levels were significantly elevated in the epimerase-deficient background compared with both WT and the individual epimerase mutant strains (Fig. 4D). Taken together with our $^1$H NMR results, these data suggest that PelX and its homologue PFL_5533 function to generate pools of UDP-GalNAc precursors for polymerization into Pel polysaccharide.

**PelX resembles members of the SDR enzyme superfamily**

Having established that PelX is a UDP-GlcNAc C4-epimerase, we next sought to determine its structure to obtain further insight into substrate recognition by this enzyme. Despite its straightforward purification and homogenous oligomeric state, we found PelX$^{C232S}$ to be recalcitrant to crystallization. We next attempted to crystallize PelX$^{C232S}$ in complex with its confirmed substrate UDP-GlcNAc. Crystals of PelX$^{C232S}$ incubated with UDP-GlcNAc appeared within 3 days and the structure of the complex was solved to 2.1 Å resolution using molecular replacement with the SDR family member WbpP (PDB ID: 1SB8) as the search model (28). PelX crystallized in space group $P2_12_12_1$ and contains a dimer in the asymmetric unit, an arrangement observed for many other structurally characterized SDR family members (Fig. 5A) (30). The dimer interface of PelX$^{C232S}$ is similar to that observed in the WbpP crystal structure where each protomer contributes two $\alpha$-helices to a four-helix bundle.

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**Figure 4. PelX is a Class 3 UDP-GlcNAc C4-epimerase.** A and B, $^1$H NMR focused on the anomeric H-1 region of the spectra (red line) for (A) UDP-GlcNAc incubated without enzyme (top), UDP-GlcNAc with PelX (middle), and UDP-GalNAc with PelX (bottom) and (B) UDP-Glc incubated without enzyme (top), UDP-Glc with PelX (middle), and UDP-Gal with PelX (bottom). C, PelX catalyzes the epimerization of UDP-GlcNAc to UDP-GalNAc by inversion of the hydroxyl group at position C4. D, LC-MS/MS quantification of GlcNAc from cell extracts of the indicated strains. Error bars represent the S.E. of the mean of six independent biological replicates. Statistical significance was evaluated using unpaired t test. *, p < 0.05.
The overall structure of PelX<sup>C232S</sup> shows that it possesses the characteristic domains associated with the SDR family, which includes an N-terminal NAD<sup>+</sup>-binding Rossmann fold (residues 1–172 and 218–243) and a C-terminal α/β-domain involved in substrate-binding (residues 173–217 and 244–310) (Fig. 5A). PelX<sup>C232S</sup> contains the GXXGXXG motif required for binding NAD<sup>+</sup> that is found in all SDR family members as well as the active site catalytic triad SX<sub>24</sub>YX<sub>3</sub>K (31). Although NAD<sup>+</sup> was not exogenously supplied in the purification or crystallization buffers, electron density for this cofactor was clearly observed, suggesting it was acquired during PelX<sup>C232S</sup> overexpression in <i>E. coli</i>. Although the addition of UDP-GlcNAc was essential for the formation of crystals, we were unable to model the GlcNAc moiety of this molecule because of the poor quality of the electron density (Fig. S3). We speculate that the sugar moiety may be disordered because PelX<sup>C232S</sup> is catalytically active and converting a portion of the UDP-GlcNAc to UDP-GalNAc. Modeling UDP alone rather than UDP-GlcNAc improved the refinement statistics of the overall model and resulted in ligand B-factors comparable to the surrounding protein atoms (Table 2).

Previous studies on a catalytically inactive variant of the UDP-Gal 4-epimerase GalE from <i>E. coli</i> allowed for the co-crystallization and modeling of UDP-Glc and UDP-Gal in the active site of this enzyme (32). In their study, these authors targeted the serine and tyrosine residues of the consensus SX<sub>24</sub>YX<sub>3</sub>K active site motif. Guided by this approach, we generated a variant of PelX<sup>C232S</sup> with S121A and Y146F mutations and confirmed that this variant is catalytically inactive (Fig. S2). PelX<sup>C232S/S121A/Y146F</sup> crystallized readily with either UDP-GlcNAc or UDP-GalNAc, and both structures were solved to a resolution of 2.1 Å using molecular replacement (Table 2). The final models of PelX<sup>C232S/S121A/Y146F</sup> in complex with UDP-GlcNAc or UDP-GalNAc were both refined to an <i>R</i><sub>work</sub>/<i>R</i><sub>free</sub> of 15.6%/19.5% (Table 2). In these structures, the electron density for the sugar moieties was well-defined compared with the PelX<sup>C232S</sup>–UDP-GlcNAc co-crystal structure and allowed for the unambiguous modeling of the expected sugar-nucleotides (Fig. S3). Given that both structures showed improved ligand density for their respective substrates, these structures substantiate our biochemical data showing that UDP-GlcNAc and UDP-GalNAc are substrates for PelX. Examination of the active site of our PelX<sup>C232S/S121A/Y146F</sup>–substrate complexes did not show any significant differences in the positions of active site residues, suggesting that both sugar-nucleotides are recognized by the enzyme in a similar manner (Fig. 5B). We next compared our substrate-bound PelX<sup>C232S/S121A/Y146F</sup>–UDP-GlcNAc–bound structure of the aforementioned UDP-hexose C4-epimerase WbpP from <i>P. aeruginosa</i>. WbpP shares 32% sequence identity to PelX and also catalyzes the epimerization of UDP-GlcNAc to UDP-GalNAc (28). The overall structure of WbpP is highly similar to PelX<sup>C232S/S121A/Y146F</sup> (PDB code 1SB8, root mean square deviation 1.9 Å over 306 C<sub>a</sub>) except that WbpP possesses an additional N-terminal α-helix.
not found in PelX. The active site residues identified as being important for sugar-nucleotide interaction in WbpP are invariant in PelX (Fig. 5C) with the exception of Ala-81, Ala-122 and Gly-189 in PelX, which correspond to residues Gly-102, Ser-143 and Ala-209 in WbpP, respectively (28). These differences are not predicted to impair specificity toward the UDP-GlcNac/GalNac substrate. Rather, Demendi et al. (33) found that bulkier residues (G102K, A209N), and mutation of S143A actually displayed en-

Discussion

In this study, we report the characterization of the Pel polysaccharide precursor-generating enzyme PelX. Using P. prote-
gen Psf-5 as a model bacterium, we found that pelX is required for Pel polysaccharide—dependent biofilm formation in a strain that also lacks the pelX paralogue PFL_5533. Guided by our 1H NMR analyses and multiple crystal structures, we have shown that PelX functions as a UDP-GlcNac C4-epimerase and that it preferentially interconverts UDP-GlcNac/UDP-GalNac over UDP-Glc/UDP-Gal, defining it as a group 3 UDP-

Table 2

Data collection and refinement statistics

|                      | PelX^{C232S} + UDP-GlcNac<sup>a</sup> | PelX^{C232S/Y146F/S121A} + UDP-GlcNac | PelX^{C232S/Y146F/S121A} + UDP-GalNac |
|----------------------|----------------------------------------|----------------------------------------|----------------------------------------|
| **Data Collection**  |                                        |                                        |                                        |
| Wavelength (Å)       | 1.075                                  | 1.075                                  | 1.075                                  |
| Space group          | P2<sub>1</sub>,2                        | P2<sub>1</sub>,2                        | P2<sub>1</sub>,2                        |
| Cell dimensions      |                                        |                                        |                                        |
| a, b, c (Å)          | 123.0, 75.5, 79.2                      | 124.2, 75.5, 79.3                      | 123.6, 75.3, 79.3                      |
| a, b, γ (%)          | 90.0, 90.0, 90.0                       | 90.0, 90.0, 90.0                       | 90.0, 90.0, 90.0                       |
| Resolution (Å)       | 50.00 - 2.1 (2.18-2.10)                | 50.00 - 2.1 (2.18-2.10)                | 50.00 - 2.1 (2.18-2.10)                |
| Total no. of reflec-
| tion               | 67600                                 | 598870                                 | 589555                                 |
| Total no. of unique reflec-
| tion               | 43495                                 | 43637                                  | 44099                                  |
| R<sub>merge</sub> (%) | 8.0 (58.0)                             | 12.2 (72.2)                            | 10.6 (78.7)                            |
| Completeness (%)     | 38.8 (5.3)                             | 23.6 (3.6)                             | 26.5 (3.1)                             |
| Redundancy           | 11.4 (14.6)<sup>e</sup>               | 7.6 (12.5)                             | 7.6 (12.2)                             |
| **Refinement**       |                                        |                                        |                                        |
| R<sub>work</sub> R<sub>free</sub> (%)<sup>d</sup> | 16.7/19.7                             | 15.6/19.5                              | 15.6/19.5                              |
| Average B-factors (Å<sup>2</sup>) | 41.29                                 | 31.23                                  | 35.80                                  |
| Protein              | 32.25                                  | 21.64                                  | 25.42                                  |
| NAD                  | 40.82                                  | 33.13                                  | 33.19                                  |
| UDP-sugar            | 41.52                                  | 37.69                                  | 40.39                                  |
| Water                | 0.006                                  | 0.006                                  | 0.006                                  |
| r.m.s. deviations    |                                        |                                        |                                        |
| Bond lengths (Å)     | 0.881                                  | 0.863                                  | 0.897                                  |
| Bond angles (°)      |                                        |                                        |                                        |
| Ramachandran plot (%) | 97.87                                  | 97.55                                  | 97.71                                  |
| Total allowed        | 2.13                                  | 2.13                                  | 2.13                                  |
| Total allowed        | 123.6, 75.3, 79.3                      | 124.2, 75.5, 79.3                      | 123.6, 75.3, 79.3                      |
| Coordinate error (Å<sup>2</sup>) | 0.21                                     | 0.2                                     | 0.22                                     |
| **PDB code**         | 6W1B                                   | 6W19                                   | 6WIA                                   |

<sup>a</sup>Although this structure was crystallized with UDP-GlcNac, density for the sugar was not visible and therefore only UDP was modeled and refined.

<sup>b</sup>R<sub>merge</sub> = Σ[I(hk) – |ΣI(hk)|] / [I(hk)], where I(hk) and represent the diffraction intensity values of the individual measurements and the corresponding mean values. The summation is over all unique measurements.

<sup>c</sup>Values in parentheses correspond to the highest resolution shell.

<sup>d</sup>R<sub>work</sub> = Σ[I(hk) – |Σ|I(hk)|] / [I(hk)], where I(hk), and I<sub>calc</sub> are the observed and calculated structure factors, respectively. R<sub>free</sub> is the sum extended over a subset of reflections (5%) excluded from all stages of the refinement.

<sup>e</sup>As calculated using MOLPROBITY (54).

<sup>f</sup>Maximum-Likelihood Based Coordinate Error, as determined by PHENIX (52).

not found in PelX. The active site residues identified as being important for sugar-nucleotide interaction in WbpP are invariant in PelX (Fig. 5C) with the exception of Ala-81, Ala-122 and Gly-189 in PelX, which correspond to residues Gly-102, Ser-143 and Ala-209 in WbpP, respectively (28). These differences are not predicted to impair specificity toward the UDP-GlcNac/GalNac substrate. Rather, Demendi et al. (33) found that bulkier residues (G102K, A209N), and mutation of S143A actually displayed enhanced specificity toward acetylated substrates. However, while the positions of the PelX<sup>C232S/S121A/Y146F</sup> and WbpP active site residues and NAD<sup>+</sup> cofactor are highly similar, comparison of the bound UDP-GalNac substrate between the two structures reveals distinct differences in the conformations of the GalNac moiety (Fig. 5C). We suspect that this difference in conformation may be a result of the co-crystallization of UDP-GalNac with WT WbpP, whereas to observe electron density for the GalNac moiety of UDP-GalNac in complex with PelX we had to mutate two active site residues, S121A and Y146F. The residues equivalent to Ser-121 and Tyr-146 in WbpP make contact with the C4 hydroxyl group of GalNac and thus are likely involved in substrate orientation. These observations suggest that the conformation of UDP-GalNac in our mutant PelX co-crystal structure may not represent a state adopted during catalysis, but demonstrate a high degree of conformational freedom of the sugar moiety within the relatively large substrate-binding pocket. The GlcNac moiety of UDP-GlcNac in our PelX<sup>C232S/S121A/Y146F</sup> UDP-GlcNac co-crystal structure was also found in a similar ori-

Functional redundancy of sugar-nucleotide synthesizing enzymes in biofilm producing bacteria is not unprecedented. For example, in P. aeruginosa PAO1, PsIB and WbpW both catalyze the synthesis of GDP-mannose, a precursor molecule...
required for Psl polysaccharide and A-band lipopolysaccharide (LPS). Like PelX and PgnE, PslB and WbpW have been shown to be genetically redundant as a defect in Psl polysaccharide, or A-band LPS is only observed when both pslB and wbpW are deleted (22). Although P. aeruginosa PAO1 has another parologue of PslB and WbpW, AlgA, the algD promoter responsible for transcription of the algA gene is not significantly activated in nonmucoid strains such as PAO1 (34). Psl biosynthesis, like Pel, is also regulated by c-di-GMP through FleQ (23), whereas being an integral component of the P. aeruginosa outer membrane, the genes responsible for A-band LPS synthesis are constitutively expressed (35). Although at present what additional glycans PgnE may be involved in producing is unknown, it is clear that the existence of paralogous sugar-nucleotide synthesizing enzymes may be a means of keeping up with metabolic demand during the synthesis of multiple cell-surface polysaccharides.

We previously reported the isolation of Pel polysaccharide from P. aeruginosa PAO1 and carbohydrate composition analyses showed that it is rich in GalNAc (9). Therefore, the co-regulation of a UDP-GlcNAc C4-epimerase with the pel genes likely ensures that adequate quantities of UDP-GalNAc are available for Pel biosynthesis when a biofilm mode of growth is favored. In contrast to P. protegens Pf-5, P. aeruginosa PAO1 does not contain a pelX gene in its Pel biosynthetic gene cluster, yet this bacterium is also capable of producing Pel polysaccharide (36). In the PAO1 genome, the poorly characterized PA4068 gene is found in the same genomic context as pgnE whereby both genes are part of a two-gene operon, with the second gene predicted to encode a D-4-dehydrorhamnose reductase (PA4069/PFL_5534) (37). In addition, the protein encoded by PA4068 shares 76% identity with PgnE, suggesting that this gene may function analogously to pgnE and by extension pelX. A ΔPA4068 mutant was found to display a surface attachment defect during secretin-induced stress, suggesting a role for this gene in surface glycanc production (37). However, it has been established that Psl is the primary polysaccharide required for P. aeruginosa PAO1 biofilm formation even though this strain is genetically capable of synthesizing Pel (36). Consequently, studies characterizing Pel polysaccharide production by PAO1 have relied on an engineered strain that lacks the ability to produce Psl and expresses the pel genes from an arabinose-inducible promoter. It may be that only low levels of UDP-GalNAc are required to sustain Pel polysaccharide production by WT PAO1 and thus a second UDP-GlcNAc C4-epimerase that is dedicated to Pel production is not required. In contrast, Pel polysaccharide appears to be a major biofilm matrix constituent in P. protegens Pf-5 and thus the higher levels of Pel production in this organism may necessitate the need for increased synthesis of UDP-GalNAc precursors.

The epimerization of UDP-Gal to UDP-Glc by PelX occurs much less efficiently than its N-acetylated counterpart. Creuzenet and colleagues noted a similar trend for WbpP, a UDP-GlcNAc C4-epimerase involved in P. aeruginosa PAK O-antigen biosynthesis, and hypothesized that the poor efficiency displayed by this enzyme toward nonacetylated substrates means that this reaction is unlikely to occur in vivo (38). The equilibrium of the PelX-catalyzed epimerization between UDP-GalNAc and UDP-GlcNAc in vitro is skewed toward the more thermodynamically stable UDP-GlcNAc epimer. A similar balance for this equilibrium has been documented for other epimerases (38, 39). We speculate that the continuous polymerization of UDP-GalNAc by the putative Pel polysaccharide polymerase, PelF, would keep the cellular concentration of UDP-GalNAc low and thus drive the equilibrium toward its production.

In conclusion, this work demonstrates the involvement of a Pel polysaccharide precursor generating enzyme required for biofilm formation in P. protegens. Our data linking the production of UDP-GalNAc to Pel polysaccharide production lend genetic and biochemical support to the chemical analyses that showed Pel is a GalNAc-rich carbohydrate polymer (9). Furthermore, the identification of a new Pel polysaccharide–dependent biofilm forming bacterium provides an additional model system that can be used for the characterization of this understudied polysaccharide secretion apparatus.

**Experimental Procedures**

**Bacterial strains, microbiological media, and physiological buffers**

All bacterial strains and plasmids used in this study are listed in Table S1. Jensen’s medium contained per liter of MilliQ water: 5 g NaCl, 2.51 g KH₂PO₄, 13.46 g glutamic acid, 2.81 g L-valine, 1.32 g L-phenylalanine, 0.33 g/liter MgSO₄·7H₂O, 21 mg CaCl₂·2H₂O, 1.1 mg FeSO₄·7H₂O, 2.4 mg ZnSO₄·7H₂O, and 1.25% D-glucose. Semi-solid agar medium in Petri dishes was prepared by adding 1.0% noble agar to Jensen’s medium. A 10× solution of PBS was purchased from Amresco and diluted, as required, in sterile MilliQ water. King’s B medium contained per liter of MilliQ water: 10 g proteose peptone no. 2 (DIFCO), 1.5 g anhydrous KH₂PO₄, 15 g glycerol, and 5 ml MgSO₄. Lysogeny broth (LB) contained per liter of MilliQ water: 10 g tryptone, 10 g NaCl, and 5 g yeast extract. E. coli strains were grown with shaking at 37°C. P. protegens strains were grown at 30°C. The following concentration of antibiotics were used: gentamicin 15 μg ml⁻¹ (E. coli); gentamicin 30 μg ml⁻¹ (P. protegens); kanamycin, 25 μg ml⁻¹. Plasmids were maintained in DH5α(λpir).

**Bioinformatic identification of PelX among pel gene clusters in sequenced bacterial genomes**

We previously constructed a database of genomes containing pel gene clusters using the Geneious platform (12, 13, 40). Briefly, identification of pel gene clusters was made via BLASTP (18) searching of the National Center for Biotechnology Information, Pseudomonas (41), and Burkholderia (42) databases (as of May 6, 2018) using P. aeruginosa PAO1 PelC (NP_251752.1) as the query sequence. Annotated genomes encoding PelC orthologues were downloaded from the databases and manually binned according to synteny of the pel operon. Conserved domains encoded by open reading frames (ORFs) linked to pel loci were queried by searching the Conserved Domain Database (43). Visualizations of pel gene clusters were drawn to scale using Geneious Prime 2020 and Adobe Illustrator.
Sequence analysis of PelX and PgnE orthologues

According to the Pseudomonas Genome database, PFL_2971 and PFL_5533 belong to the Pseudomonas orthologue groups (POGs) POG020331 and POG000167, respectively. Prior to this study, the POGs were unnamed; therefore, based on our observations, we have named these POGs as pelX and pgnE. PelX primary amino acid sequences were aligned using MUSCLE (44) to identify highly conserved amino acid residues. Additionally, the P. protegens PelX sequence was submitted to Phyre² to determine the predicted fold of the protein (19). The PelX and PgnE protein sequences from P. protegens Pf-5 were obtained from the Pseudomonas Genome Database (41). Comparison of the PelX structure to previously determined structures was performed using the DALI pairwise comparison server (45).

Construction of P. protegens chromosomal mutations

In-frame, unmarked pslA (PFL_4208), pelF (PFL_2977), pelX (PFL_2971), and PFL_5533 gene deletions in P. protegens Pf-5 were constructed using an established allelic replacement strategy (46). Flanking upstream and downstream regions of the ORFs were amplified and joined by splicing-by-overlap extension PCR (primers are listed in Table S1). The pslA, pelF, and pelX, alleles were generated using forward upstream and downstream reverse primers tailed with EcoRI and HindIII restriction sites, respectively (Table S1). These PCR products were digested with EcoRi and HindIII restriction endonucleases and subsequently cloned into the pPSV39 vector (Table S1). Confirmation of the correct nucleotide sequence of wspR was achieved through DNA sequencing (The Center for Applied Genomics, The Hospital for Sick Children). Arg-242 was mutated to an alanine to prevent allosteric inhibition of WspR using the QuickChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies), as described previously. The resulting expression vector (pLSM-wspR^{R242A}) encodes residues 1–347 of WspR. Introduction of the pPSV39 empty vector or pSLM-wspR^{R242A} into P. protegens was carried out by electroporation. Positive clones were selected for LB agar containing 30 μg ml⁻¹ Gen.

Crystal violet assay

Overnight cultures grown in King’s B media (KB), were diluted to a final OD of 0.005 in 1 ml of KBM in a 24-well VDX plate (Hampton Research) and left undisturbed at 30°C for 120 h. Nonattached cells were removed and the wells were washed thoroughly with water and stained with 1.5 ml 0.1% (w/v) crystal violet. After 10 min, the wells were washed again and the stain solubilized using 2 ml of 95% (v/v) ethanol for 10 min. 200 μl was transferred to a fresh 96-well polypropylene plate (Nunc) and the absorbance measured at 550 nm. For strains containing empty pPSV39 or pSLM-wspR^{R242A}, the above protocol was modified slightly. As c-di-GMP significantly up-regulated biofilm formation, crystal violet staining for these strains was performed as described previously using 96-well polypropylene plates that were incubated statically for 6 or 24 h at 30°C. All strains were grown in KBM containing 30 μg ml⁻¹ Gen and 30 μM IPTG.

Generation of WspR overexpression strains

The wspR nucleotide sequence from P. aeruginosa PAO1 was obtained from the Pseudomonas Genome Database and used to design primers specific to full-length wspR (Table S1). The forward primer encodes an EcoRi restriction site and a ribosomal-binding site, whereas the reverse primer encodes a HindIII restriction site. The amplified PCR products were digested with EcoRi and HindIII restriction endonucleases and subsequently cloned into the pPSV39 vector (Table S1). Confirmation of the correct nucleotide sequence of wspR was achieved through DNA sequencing (The Center for Applied Genomics, The Hospital for Sick Children). Arg-242 was mutated to an alanine to prevent allosteric inhibition of WspR using the QuickChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies), as described previously. The resulting expression vector (pLSM-wspR^{R242A}) encodes residues 1–347 of WspR. Introduction of the pPSV39 empty vector or pSLM-wspR^{R242A} into P. protegens was carried out by electroporation. Positive clones were selected for LB agar containing 30 μg ml⁻¹ Gen.

Dot blots

Pel antisera were obtained as described in Colvin et al. (10) from P. aeruginosa PA14 pBADpel. The adsorption reaction was conducted as described by Jennings et al. (9). Culture supernatants containing secreted Pel were harvested by centrifugation (16,000 × g for 2 min) from 1 ml aliquots of P. protegens grown overnight at 30°C in LB containing 30 μg ml⁻¹ Gen and 30 μM IPTG, and treated with proteinase K (final concentration, 0.5 mg ml⁻¹) for 60 min at 60°C, followed by 30 min at 80°C to inactivate proteinase K.

Pel immunoblots were performed as described by Colvin et al. (10) and Jennings et al. (9). 5 μl of secreted Pel, prepared as described above, was pipetted onto a nitrocellulose membrane and left to air dry for 10 min. The membrane was blocked with 5% (w/v) skim milk in TBS (10 mM Tris-HCl, pH 7.5, 150 mM NaCl) containing 0.1% (v/v) Tween 20 (TBS-T) for 1 h at room temperature and probed with adsorbed α-Pel at a 1:60 dilution in 1% (w/v) skim milk in TBS-T overnight at 4°C with shaking.
Blots were washed three times for 5 min each with TBS-T, probed with goat α-rabbit HRP-conjugated secondary antibody (Bio-Rad) at a 1:2000 dilution in TBS-T for 45 min at room temperature with shaking, and washed again. All immunoblots were developed using SuperSignal West Pico (Thermo Scientific) following the manufacturer’s recommendations.

For Western blot immunochemistry, 5 μl of secreted Pel was prepared as described above, was pipetted onto a nitrocellulose membrane and left to air dry for 10 min. The membrane was blocked with 5% (w/v) BSA in TBS-T for 1 h at room temperature and probed with 10 μg/ml of WFL-HRP (EY Laboratories) in 2% (w/v) BSA in TBS-T with 0.2 g/liter CaCl₂ overnight at room temperature with shaking. Membranes were washed twice for 5 min and once for 10 min with TBS-T, then developed as described above.

Western blotting sample preparation and analysis

For analysis of protein levels from WspR<sup>B242A</sup> overexpressing strains containing VSV-G–tagged PelF, PelX, or PFL_5533, 5 ml of LB media containing 30 μM IPTG and 30 μg ml<sup>−1</sup> Gen was inoculated with the appropriate strain and allowed to grow overnight at 30°C with shaking. Culture density was normalized to an <i>A₆₆₀</i> of 1 and 1 ml of cells was centrifuged at 5000 × g for 5 min to pellet cells. The cell pellet was resuspended in 100 μl of 2× Laemmli buffer, boiled for 10 min at 95°C, and analyzed by SDS-PAGE followed by Western blotting. For Western blot analysis, a 0.2-μM PVDF membrane was wetted in methanol and soaked for 5 min in Western transfer buffer (25 mM Tris- HCl, 150 mM glycine, 20% (v/v) methanol) along with the SDS-PAGE gel to be analyzed. Protein was transferred from the SDS-PAGE gel to the PVDF membrane by wet transfer (25 mA, 2 h). The membrane was briefly washed in TBS-T before blocking in 5% (w/v) skim milk powder in TBS-T for 2 h at room temperature with gentle agitation. The membrane was briefly washed again in TBS-T before incubation overnight with α–VSV-G antibody in TBS-T with 1% (w/v) skim milk powder at 4°C. The next day, the membrane was washed four times in TBS-T for 5 min each before incubation for 1 h with secondary antibody (1:2000 dilution of Bio-Rad Affinity-purified mouse α-rabbit IgG conjugated to alkaline phosphatase) in TBS-T with 1% (w/v) skim milk powder. The membrane was then washed three times with TBS-T for 5 min each before development with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium chloride (BioShop ready-to-use BCIP/NBT solution). Developed blots were imaged using a Bio-Rad ChemiDoc imaging system.

Cloning and mutagenesis

The pelX nucleotide sequence from <i>P. protegens</i> Pf-5 (PFL_2971) was obtained from the <i>Pseudomonas</i> Genome Database (41) and used to design primers specific to full-length pelX (Table S1). The amplified PCR products were digested with NdeI and XhoI restriction endonucleases and subsequently cloned into the pET28a vector (Novagen). Confirmation of the correct nucleotide sequence of pelX was achieved through DNA sequencing (ACGT DNA Technologies Corporation). The resulting expression vector (pLSM-PelX) encodes residues 1–309 of PelX fused to a cleavable N-terminal His<sub>6</sub> tag (His<sub>6</sub>-PelX) for purification purposes (Table S2). To prevent aggregation of PelX in solution, a nonconserved cysteine (Cys-232) was mutated to a serine with the aid of the QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies) and confirmed with DNA sequencing (ACGT DNA Technologies Corporation). The PelX<sup>C232S</sup> active site mutant (S121A/Y146F) was generated analogously.

**Expression and purification of PelX**

The expression of PelX<sup>C232S</sup> was achieved through the transformation of the PelX<sup>C232S</sup> expression vector into <i>E. coli</i> BL21 (DE3) competent cells, which were then grown in 2 liters LB containing 50 μg ml<sup>−1</sup> kanamycin at 37°C. The cells were grown to an <i>A₆₆₀</i> of 0.6 whereupon IPTG was added to a final concentration of 1.0 mM to induce expression. The induced cells were incubated for 20 h at 25°C prior to being harvested via centrifugation at 6260 × g for 20 min at 4°C. The resulting cell pellet was stored at −20°C until required.

The cell pellet from 2 liters of bacterial culture was thawed and resuspended in 80 ml of Buffer A (50 mM Tris- HCl pH 8.0, 300 mM NaCl, 5% (v/v) glycerol, and 1 mM tris-(2-carboxyethyl) phosphate (TCEP)) containing 1 SIGMAFAST Protease Inhibitor EDTA-free mixture tablet (Sigma). Because of the presence of two remaining cysteines in PelX<sup>C232S</sup>, TCEP was included to prevent intermolecular crosslinking of the protein. These cysteines are not predicted to be involved in disulfide bond formation given their poor conservation and the cytoplasmic localization of PelX<sup>C232S</sup>. The resuspension was then lysed by homogenization using an Emulsiflex-C3 (Avestin, Inc.) at a pressure between 15,000–20,000 psi, until the resuspension appeared translucent. Insoluble cell lysate was removed by centrifugation for 25 min at 25,000 × g at 4°C. The supernatant was loaded onto a 5 ml Ni<sup>2+</sup>–nitrilotriacetic acid column (Millipore) with a 10-kDa molecular weight cut-off. PelX<sup>C232S</sup> was purified and buffer exchanged into Buffer B (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5% (v/v) glycerol, 1 mM TCEP) by size-exclusion chromatography using a HiLoad 16/60 Superdex 200 gel-filtration column (GE Healthcare). PelX<sup>C232S</sup> eluted as a single Gaussian-shaped peak, and all PelX<sup>C232S</sup> containing fractions were pooled and concentrated by centrifugation, as above, to 8 mg ml<sup>−1</sup> and stored at 4°C. PelX<sup>C232S/Y146F/S121A</sup> was purified similarly.

**Determination of the PelX oligomerization state by gel filtration analysis**

Oligomerization of PelX<sup>C232S</sup> was determined using a Superdex 200 10/300 GL column (GE Life Sciences). The column was
equilibrated in Buffer B. Molecular weight standards (Sigma, 12–200 kDa) were applied to the column as directed. PelX<sub>C232S</sub> was applied to the column at 7.5 mg ml<sup>−1</sup> (100 µl) and protein elution was monitored at 280 nm.

**NMR activity assay**

The following method has been adapted from Wyszynski et al. (39). Enzymatic reactions were performed in 30 mM sodium phosphate, pH 8.0, with 50 µg of freshly purified PelX<sub>C232S</sub> and 10 mM UDP-GlcNAc, UDP-Glc, UDP-Gal, or 5 mM UDP-Gal-NAC in a total reaction volume of 220 µl. After incubation at 37°C for 1 h, the mixture was flash frozen and lyophilized. The resulting material was dissolved in 220 µl of D<sub>2</sub>O and analyzed by 1H NMR. As control experiments, the same procedures were applied to samples lacking PelX or UDP-GlcNAc. Data were collected on a Varian 600 MHz NMR spectrometer.

**Intracellular metabolite extraction**

P. protegens Pf-5 WT, ΔpelX, ΔPFL<sub>5533</sub>, and ΔpelX ΔPFL<sub>5533</sub> strains that had been transformed with a plasmid expressing WspR<sup>R242A</sup> (pLSM21) were streaked out twice in succession on Jensen’s agar containing 30 µg/ml gentamicin, and these first and second subcultures were grown for 48 h at 30°C. For each biological replicate, cells from the second subcultures were collected using a polyester swab and suspended into a Petri dish (60 mm x 15 mm) containing 2 ml of cold 80% (v/v) methanol extract were then stored at 4°C for 3 min. Raw data acquisition was carried out using Thermo Xcalibur 4.0.27.19 software. Data analysis was carried out using MAVEN software (49). Compound identification was achieved through matching of high-resolution accurate mass and retention time characteristics to those of authentic standards. Secondary compound confirmation was performed by matching of fragmentation profiles obtained through parallel reaction monitoring.

**Crystallography and structure determination**

Commercial sparse matrix crystal screens from Microlytic (MC501-4) were prepared at room temperature (22°C) with PelX<sub>C232S</sub> at a concentration of 8 mg ml<sup>−1</sup> (0.23 mM). UDP-GlcNAc was added exogenously to a concentration of 2 mM. Trials were set up in 48-well VDX plates (Hampton Research) by hand with 3 µl drops at a ratio of 1:1 protein to crystallization solution over a reservoir containing 200 µl of the crystallization solution. Crystal trays were stored at 22°C. The best crystals were obtained from condition 32 (0.2 M ammonium sulfate, 0.1 M sodium citrate, pH 5.6, 25% (v/v) PEG 4000) from MC501 (Microlytic). This condition yielded stacked flat crystals that took ~5 days to grow to maximum dimensions of 300 µM x 300 µM x 50 µM. PelX was unable to form crystals in the absence of UDP-GlcNAc.

Crystals of PelX<sub>C232S</sub> were cryo-protected in well solution supplemented with 20% (v/v) ethylene glycol by briefly soaking the crystal in a separate drop. Crystals were soaked for 2–3 s prior to vitrification in liquid nitrogen and subsequently stored until X-ray diffraction data were collected on beamline X29A at the National Synchrotron Light Source at Brookhaven National Laboratory. A total of 360 images of 1° oscillation were collected on an ADSC Q315 CCD detector with a 250 mm crystal-to-detector distance and an exposure time of 0.4 s per image. The data were processed using DENZO and integrated intensities were scaled using SCALPACK from the HKL-2000 program package (50). The data collection statistics are summarized in Table 2. The structure was solved by molecular replacement using WbpP as a model with PHENIX AutoMR wizard. The resulting map was of good quality and allowed manual model building using COOT (51, 52). The model was then refined using PHENIX.REFINE (52) to a final Rwork/Rfree of 16.7 and 19.7%, respectively.
**EDITORS’ PICK:** Pel biosynthesis requires a UDP-GlcNAc C4-epimerase

PelX<sub>C232S/S121A/Y146F</sub> in complex with UDP-GalNAc or UDP-GlcNAc was crystallized under the same conditions as the WT protein, and data collection and refinement were performed as described above. The corresponding statistics can be found in Table 2.

**Data availability**

All the data described are located within the manuscript and the supplemental information. The coordinates and structure factors for PelX<sub>C232S</sub> in complex with NAD<sup>+</sup> and UDP, PelX<sub>C232S/S121A/Y146F</sub> UDP-GlcNAc, and PelX<sub>C232S/S121A/Y146F</sub> UDP-GalNAc have been deposited in the PDB, ID codes 6WJB, 6WJA, and 6WJ9, respectively.

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**Conflict of interest**—The authors declare that they have no conflicts of interest with the contents of this article.

**Abbreviations**—The abbreviations used are: SDR, short-chain dehydrogenase/reductase; Pel, pellicle; c-di-GMP, cyclic dimeric GMP; IPTG, isopropyl-β-d-1-thiogalactopyranoside; VSV-G, vesicular stomatitis virus glycoprotein; WFL, Wisteria floribunda; UDP-Gal, UDP-galactose; LPS, lipopolysaccharide; LB, lysogenic broth; Gen, gentamicin; KMB, King’s B media; TBS-T, Tris-buffered saline with Tween 20; TCEP, tri(2-carboxyethyl)phosphine; Buffer A, 50 mm Tris-HCl, pH 8.0, 300 mm NaCl, 5% (v/v) glycerol, and 1 mm TCEP; Buffer B, 20 mm Tris-HCl, pH 8.0, 150 mm NaCl, 5% (v/v) glycerol, 1 mm TCEP.

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