PeLA and PeLB proteins form a modification and secretion complex essential for PeL polysaccharide-dependent biofilm formation in Pseudomonas aeruginosa

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This article contains supplemental Fig. S1 and Tables S1 and S2.

The atomic coordinates and structure factors (code SWFT) have been deposited in the Protein Data Bank (http://wwpdb.org/).

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Pseudomonas aeruginosa is a Gram-negative bacterium that can grow in a variety of host-associated and environmental niches due in part to its ability to form multicellular aggregates known as biofilms. Secreted polysaccharides are an essential component of P. aeruginosa biofilms, and this bacterium is capable of producing three distinct polysaccharides: alginate, the polysaccharide synthesis locus (PSL),6 and pellicle (PEL) polysaccharides (1–4). Strains of P. aeruginosa isolated from cystic fibrosis patients have been found to overproduce each of these polysaccharides, whereas environmental isolates secrete PeL and/or PSL as the primary biofilm matrix exopolysaccharide (5–7).

PEL operons are widely distributed across Proteobacteria (8). This polymer is an important structural component of the biofilm, required for initiating and maintaining cell-to-cell interactions as well as forming interactions with other matrix components like extracellular DNA (9, 10). PEL production also mediates tolerance of P. aeruginosa to aminoglycoside antibiotics (9). Sugar composition analysis indicates that secreted PEL is rich in N-acetylgalactosamine (GalNAc) and N-acetylgalactosamine (GalNAc) (10). Partial deacetylation renders PEL cationic, a reaction catalyzed by the multidomain protein PeLA, whose domain architecture and deacetylase activity have been described previously (11). Mutation of PeLA residues involved in catalysis abrogate biofilm formation, suggesting that deacetylation of PEL is an essential modification.

Our recent characterization of the outer membrane lipoprotein PeLC revealed that this protein forms a dodecameric ring that positions a negatively charged surface toward the periplasm. We proposed that PeLC functions as an electronegative funnel that guides cationic PEL toward the export channel formed by the outer membrane protein PeLB (8). PeLB is a 135-kDa multidomain protein with properties suggestive of a transmembrane β-barrel at its C terminus, which we hypothesized is required for polysaccharide export (8). The N terminus of PeLB is thought to contain a large periplasmic tetraoctrapeptide repeat...
PeLA–PeB complex is required for PEL biosynthesis

(TPR)-containing domain connected to the C-terminal porin by an ~120-amino acid linker with poorly predicted secondary structure (8, 12). Although our characterization of PeIC has begun to provide insight into the translocation of PEL across the periplasmic space, important questions remain, including whether polysaccharide modification enzymes such as PeLA are recruited to the secretion apparatus and, if so, how this process is facilitated.

TPR motifs adopt a helix-turn-helix arrangement comprising ~34 residues of alternating small and large amino acids. Multiple repeats are frequently arranged in tandem arrays, resulting in a series of antiparallel α-helices. In proteins possessing four or more tandem repeats, the TPR-containing domain adopts a right-handed superhelix (13, 14). TPR motifs are found in proteins from all domains of life and function as protein–protein interaction modules, acting as scaffolds for large protein complexes involved in diverse cellular processes (15). In bacteria, these include the maintenance of outer membrane integrity (16), chaperone activity in the type III secretion system (17), and assembly of the type IV pilus (18).

Given the tendency of TPR-containing proteins to be involved in protein–protein interactions, we hypothesized that PeB may interact with other components of the PEL biosynthetic machinery and thus serve as a protein scaffold within the periplasm. Here, we describe an interaction between the TPR-containing domain of PeB and the multidomain enzyme PeLA. Using specific TPR deletions, we found that when repeat 9, 10, 11, 12, 13, or 14 is deleted, the interaction between PeA and PeB is abrogated and PEL-dependent biofilm formation is abolished. Copurification experiments demonstrate a direct interaction between these two proteins and allowed us to assess whether the enzymatic activities of PeLA were influenced by this interaction. We found that PeA–PeB complex formation increases the polysaccharide deacetylation activity of PeA, whereas its glycoside hydrolase activity is attenuated. Together, our results suggest that the TPR-containing domain of PeB acts as a scaffold, localizing PeA within the periplasm to the export machinery to increase polysaccharide deacetylation during PEL biosynthesis and biofilm development.

Results

PeLA interacts with PeB in P. aeruginosa

PeLA is a periplasmically localized protein (11) essential for PEL biosynthesis. Given that this protein modifies the PeL polymer, we sought to determine whether this protein associates with any other PeL proteins with domains localized to the periplasm. For this and subsequent experiments, we used the P. aeruginosa PAO1 ΔwspF Δpsl ΔpelA (parental), which has been engineered to enable L-arabinose-inducible expression of PEL (8, 11). To investigate a potential interaction between PeA and PeB, we generated a complementation vector to be used in a PAO1 ΔwspF Δpsl ΔpelA ΔpelA strain (11). Multisite recombination was used to assemble a mini-Tn7-based vector in which araC-PBAD was fused directly to the pelA open reading frame. The ΔwspF Δpsl PBADpel ΔpelA strain was then complemented with either wild-type pelA (wild type) or pelA encoding a hexahistidine tag at the C terminus (PeLA-C-His6). Addition of the hexahistidine tag did not affect PeLA stability, and this strain displayed pellicle formation and surface attachment phenotypes comparable with a strain expressing wild-type PeLA (Fig. 1A). Next, we used nickel-affinity chromatography to purify PeLA-C-His6 and analyzed the eluate using Western blot analysis and mass spectrometry. Western blotting with PeB-specific antibodies revealed a band in the PeLA-C-His6 pulldown eluate, but not the PeLA eluate, ~40 kDa less than the expected molecular mass of PeB (Fig. 1B). Mass spectrometry analysis of the eluates confirmed the enrichment of both PeLA and PeB in the PeLA-C-His6 pulldown compared with the untagged control (Fig. 1C). The identified peptides almost exclusively belonged to the TPR domain of PeB. We hypothesize that the porin domain was perhaps destabilized and degraded during the purification process. These data also indicate that, under the conditions tested, PeLA and PeB form a binary complex that does not strongly associate with other Pel proteins as PeC, PeD, and PeF were not significantly enriched, and peptides of PeE and PeG were not detected.

Figure 1. PeLA interacts with PeB in P. aeruginosa. A, addition of a C-terminal hexahistidine tag (C-His6) to PeLA does not affect surface attachment (top panel), biofilm formation (middle panel), or protein levels (bottom panel) compared with wild type. Error bars represent the standard error of the means of two independent experiments performed in triplicate. The arrow indicates location of the pellicle. B, Western blot analysis of solubilized membranes (input (in)) and elutions (E) from nickel-affinity pulldowns from untagged PeLA (wild type) or His-tagged PeLA-C-His6 (C-His6) strains. C, table summarizing spectral counts seen for each of the PeL proteins. The values represent an average of two biological replicates. PeL and PeLA were not detected in either experiment. D, subcellular fractionation of the cytoplasmic (C), inner membrane (IM), periplasmic (P), and outer membrane (OM) components of PAO1 ΔwspF Δpsl PBADpel strain (parental) and associated deletion and complementation strains as indicated. Fractions were probed using protein-specific antibodies with PeP and PeI serving as inner and outer membrane controls, respectively. Molecular mass markers are indicated in kDa with the exception of PilQ, which is detected in the stacking gel.
Localization of PelA is dependent on PelB

Previously, we demonstrated that PelB is localized to the outer membrane (8). Having identified an interaction between PelA and PelB, we hypothesized that PelA should localize to the outer membrane in a PelB-dependent manner. To investigate this, we used subcellular fractionation to determine the cellular location of the proteins. Following fractionation of the parental strain, we found that PelA was enriched in the outer membrane fraction compared with the periplasm (Fig. 1D). As described previously, some PelA and PelB are detected in the cytoplasmic and inner membrane compartments likely due to overexpression of the pel operon and saturation of the general protein secretion pathway (8). Consistent with our interaction data, in a strain lacking pelB, PelA no longer associated with the outer membrane. Complementation of ΔpelB with a wild-type pelB at the attTn7 site restored the localization of PelA to the outer membrane. These data suggest that PelB recruits PelA to the outer membrane.

PelB(319–436) contains multiple TPRs

Our previous bioinformatics analyses suggest that PelB contains multiple TPR motifs between residues 69 and 728 (12). We predicted that PelB has 16 non-contiguous TPRs. Secondary structure prediction algorithms suggest regions 355–387 and 524–592 will be α-helical, but fold prediction algorithms fail to identify TPR motifs in these amino acid stretches (19, 20). Proteins with TPRs are typically found in an uninterrupted parallel arrangement and are not defined by a consensus sequence but rather by a pattern of large and small amino acids at specific positions. Thus, we hypothesize that although residues 355–387 and 524–592 are not predicted to form TPRs, these predicted α-helical regions of PelB will be part of a superhelical arrangement and that PelB contains a total of 19 TPRs (R1–R19). To gain further insight into PelB function, we undertook structural studies of the TPR domain of PelB. As constructs encompassing the full-length TPR domain failed to crystallize, several different truncations of the TPR domain excluding the predicted signal sequence were pursued. A construct encompassing residues 248–625 of PelB crystallized, and the structure was solved using selenomethionine incorporation and the single-wavelength anomalous dispersion technique (Table 1). The crystals belong to space group \( P6_122 \) with one protein molecule in the asymmetric unit. Examination of the density only allowed for residues 319–436, encompassing R8 to the first 15 residues of R11, to be modeled, and this structure is depicted in Fig. 2. SDS-PAGE of native PelB crystals confirmed that the predominant species migrated at a molecular mass corresponding to the residues modeled (supplemental Fig. S1B), demonstrating that degradation had occurred during the crystallization process. The all-helical structure forms two complete TPR motifs (Fig. 2, cyan and indigo) that correspond to R9 and R10. R9 (residues 355–387; Fig. 2, cyan) was not initially predicted to form a TPR (12). The first helices of R9 and R10 superimpose well; however, a distortion in the second helix of R10 is apparent (Fig. 2). We hypothesize that the conformation of R8 observed is not biologically relevant and that this repeat was likely distorted during the crystallization process. The C-termini are likely distorted during the crystallization process. The C-terminus (Fig. 2). We hypothesize that the conformation of R8 observed is not biologically relevant and that this repeat was likely distorted during the crystallization process. The C-terminus is missing the remaining 19 residues to complete R11. Our structure reveals the TPRs of PelB, including residues 355–387 (R9), which was previously not predicted to adopt this fold. Our structural and bioinformatics analyses suggest that PelB contains 19 TPRs. We hypothesize that PelA will be positioned accordingly.

### Table 1

| X-ray data collection and refinement statistics | Se-SAD |
|-----------------------------------------------|--------|
| Data collection                               |        |
| Wavelength (Å)                                | 0.9786 |
| Space group                                   | \( P6_122 \) |
| Cell dimensions                               | \( a, b, c \) Å | 61.1, 61.1, 217.7 |
| Resolution (Å)                                | 50.00–2.82 (2.95–2.82) |
| Total no. of reflections                      | 210,764 |
| Total no. of unique reflections               | 10,963 |
| \( R_{	ext{merge}} \) (%)                    | 7.2 (87.5) |
| \( I/\sigma(I) \) (%)                        | 15.6 (2.4) |
| Completeness (%)                              | 99.8 (100.0) |
| Redundancy                                    | 19.2   |

| Refinement                                   |        |
|----------------------------------------------|--------|
| \( R_{	ext{merge}}/R_{	ext{free}} \) (%) | 21.5/27.2 |
| No. atoms                                    | 945    |
| Protein                                      | 29.5   |
| Average B-factors (Å²)                       |        |
| Bond lengths (Å)                             | 0.008  |
| Bond angles (°)                              | 1.094  |
| Ramachandran plot (%)                        |        |
| Total favored                                | 94.83  |
| Total allowed                                | 4.31   |
| Coordinate error (Å)                         | 0.35   |

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**PelA–PelB complex is required for PEL biosynthesis**

![PelB is a TPR-containing protein.](image)

**Figure 2. PelB is a TPR-containing protein.** A ribbon representation of the structure of the PelB TPR, residues 319–436, with transparent surface representation (gray) is shown. N and C indicate the N and C termini, respectively. Individual TPRs are indicated by color. Only R9 and R10 are complete (cyan and indigo, respectively). The purple, light blue, and pink helices are incomplete TPRs.
close to the outer membrane with the deacetylase domain in proximity to the negatively charged surface of PelC to facilitate the export of cationic polymer. Therefore, to determine whether a specific region of the periplasmic domain of PelB was required for interaction with PelA, we systematically deleted individual TPRs starting from the C-terminal end of this domain (residue 728; R19; Fig. 3A). We used a similar genetic approach as described above for PelA.

To determine whether deleting a single repeat resulted in a disruption of the PelA–PelB interaction, we used subcellular fractionation to characterize the enrichment of PelA in the outer membrane compared with the periplasmic fraction as an indirect measure of the interaction. We found that for PelB ΔR19, ΔR18, ΔR17, and ΔR16, PelA was still enriched in the outer membrane compared with the periplasmic fraction, whereas for ΔR14, ΔR13, ΔR12, ΔR11, ΔR10, and ΔR9, PelA was found almost entirely in the periplasmic fraction, suggesting that each deletion significantly perturbed the interaction between PelA and PelB. In ΔR15 and ΔR8, although some PelA was detected in the periplasmic fraction, PelA was enriched in the outer membrane fraction; therefore we propose that these repeats indicate the C- and N-terminal boundaries, respectively, of the TPRs required for the PelA–PelB interaction (Fig. 3B).

Having established which repeats are required to localize PelA to the outer membrane, we next tested whether the mislocalization of PelA affects PEL-dependent biofilm formation at the air–liquid interface (Fig. 3C, bottom). We found that ΔR14 to ΔR19 formed biofilms similar to the wild-type strain, whereas no biofilm was observed from ΔR9 to ΔR13 (Fig. 3C, bottom). These data correspond well with our subcellular fractionation data with the exception of ΔR14 as, although the interaction is significantly
perturbed, it appears that the residual amount of PelA localized to the outer membrane is sufficient for biofilm formation. ΔR8 appeared to form a biofilm comparable with wild type, again delineating the boundary for the interaction.

As the standing biofilm assay is qualitative and subject to interpretation, we quantified these observations by evaluating the surface attachment of these strains in a modified crystal violet assay. Surface attachment decreased from ΔR9 to ΔR14 and was completely abolished from ΔR13 to ΔR9. Surface attachment of ΔR8 is reduced compared with wild type (Fig. 3C, top), and this correlates with the subcellular fractionation data where some PelA is still found in the periplasm. PelB protein levels in each of the TPR deletion strains were comparable with wild type, suggesting that the TPR deletion does not affect protein stability and that biofilm abrogation is not due to a reduction in the levels of PelB (Fig. 3D). Together, these data suggest that a large surface area spanning multiple TPRs of PelB, specifically R9–R14, is required for the outer membrane localization of PelA and that an interaction between PelA and PelB is required for PEL-dependent biofilm formation.

The TPR domain of PelB interacts directly with full-length PelA but not its isolated hydrolase domain

Because deletion of individual PelB TPRs R9–R14 alters the association of PelA with the outer membrane, we hypothesized that this region of the protein might directly interact with PelA. To test this, we next characterized the interaction between PelA and PelB in vitro by copurification using size-exclusion chromatography. For these studies, we used a construct encoding mature PelA without its native signal sequence (PelA(Δ46)) (11) and a construct designed to express a PelB fragment encompassing TPRs R9–R15 (PelB(351–588)). The retention volumes of purified PelA or PelB were determined by analytical gel filtration and apparent molecular masses were calculated by interpolation from a standard curve. PelA(Δ46) eluted at a calculated molecular mass of 101.3 kDa compared with its expected molecular mass of 102.4 kDa, whereas the calculated molecular mass for PelB(351–588) was 39.4 kDa, larger than its expected molecular mass of 30.1 kDa (Fig. 4A). PelB likely has a higher apparent molecular mass due to an increased Stokes’ radius resulting from the elongated nature of TPR-containing domains. Combining PelA with a slight molar excess of PelB, we found that these two proteins comigrate and elute at a volume that corresponds to their combined calculated molecular mass of 139 kDa (Fig. 4A, blue). SDS-PAGE analysis confirmed the coelution of PelA(Δ46) and PelB(351–588) (Fig. 4B, blue). As PelB(351–588) and PelA(Δ46) comigrate at a combined molecular mass, this demonstrates that these proteins interact directly.

PelA is a large multidomain protein with many potential sites of interaction. Therefore, to gain insight into which region of PelA may interact with PelB, we generated a construct encoding the N-terminal hydrolase domain of PelA (PelA(47–303)) (21)). Attempts to generate a soluble construct of the C-terminal deacetylase domain have so far proven unsuccessful. PelA(47–303) and PelB(351–588) have similar molecular masses, 30.5 and 30.1 kDa, respectively. PelB eluted at a molecular mass of 39.5 kDa, whereas PelA eluted at a calculated molecular mass of 32.0 kDa (Fig. 4, C and D). No peak was observed to elute at a volume corresponding to the combined molecular mass of PelA(47–303) and PelB(351–588) (70 kDa), indicating that no interaction occurs between the isolated hydrolase domain of PelA and the TPR of PelB.

Interaction with PelB modulates the enzymatic activity of PelA

PelA is a multidomain protein essential for PEL biosynthesis. This protein has been demonstrated to have glycoside hydrolase (21) and polysaccharide deacetylase activity (11). The role of the hydrolase activity in polysaccharide biosynthesis has not yet been determined, but deacetylase activity is essential for biofilm formation (11). Because protein–protein interactions involving enzymes often modulate their activities (22), we sought to determine whether the interaction between PelA and PelB alters the enzymatic activities of PelA. We were unable to directly assay PelA activity on the Pel polysaccharide as the polymer is insoluble. Our inability to purify longer polymer also precluded the determination of the anomeric configuration of the polysaccharide and therefore the chemical synthesis of shorter Pel oligosaccharides. To experimentally address the effects of the interaction on PelA hydrolase activity, we therefore used a biofilm disruption assay. This indirect assay has been used previously as a measure of the glycoside hydrolase activity of PelA (21). Exogenous addition of PelA to preformed PEL-dependent biofilms in a microtiter dish has been demonstrated to remove the biomass attached to the surface of the wells (21). To determine whether PelB has any effect on the hydrolase activity of PelA, we performed the disruption assay using PelA(Δ46) because we have shown above that the hydrolase...
PeLA–PeB complex is required for PEL biosynthesis

Figure 5. Interaction with PeB modulates PeLA enzyme activity. A, dose-response curves examining the disruption of PA01 ΔwspF ΔpelA Δpel biofilm biomass in the presence of PeLA, PeLA + PeB, or PeB. Error bars represent the standard error of the means of three independent trials performed in triplicate. B, specific activity of PeLA, PeLA + PeB, or PeB hydrolysis of p-nitrophenyl acetate. Statistical significance was calculated using one-way analysis of variance with Bonferroni correction. Error bars represent the standard error of the means of four independent trials performed in triplicate. **, p < 0.001.

domain alone does not interact with PeB. To better approximate the physiological interaction, a construct encoding the full TPR-containing domain was used (PeB(47–880)). The EC$_{50}$ value obtained for PeLA (30.0 ± 1.4 nM) is comparable with that reported previously for the isolated PeLA hydrolase domain (21), whereas the EC$_{50}$ of PeA–PeB displays a 4.2-fold decrease (125.3 ± 4.2 nM) (Fig. 5A). This suggests that PeB may attenuate the hydrolase activity of PeLA during PEL biosynthesis.

As PeLA(Δ46) has previously been shown to deacetylate the pseudosubstrate p-nitrophenyl (pNP) acetate (11), we used this assay to examine whether the PeLA–PeB interaction affected deacetylase activity. We found a 1.7-fold increase in specific activity when PeLA(Δ46) was in complex with PeB(47–880) compared with PeLA(Δ46) alone (p < 0.001; Fig. 5B). This modest but statistically significant change suggests that the interaction with PeB may regulate the level of deacetylation during periplasmic transit.

Discussion

In this study, we report the detailed characterization of an interaction between the periplasmic exopolysaccharide-modifying enzyme PeLA and the TPR-containing domain of PeB. Using systematic TPR-deletion studies and copurification experiments, we found that R9–R14 of the PeB TPR-containing domain are required for this interaction. Furthermore, deletion of any one of the R9–R14 motifs completely abrogated biofilm formation. Because deletion of a single TPR motif did not affect the stability or localization of PeB, this suggests that the PeB-mediated localization of PeLA is required for PeL polysaccharide biosynthesis and secretion. Although we demonstrated that the hydrolase domain of PeLA does not interact with PeB, we have been unable to determine whether the TPR interact specifically with its deacetylase domain or across multiple domains in the full-length protein as the deacetylase domain is unstable when expressed in isolation.

We observed a change in the enzymatic activities of PeLA upon complex formation. A modest but statistically significant increase in deacetylase activity suggests that the interaction with PeB positions PeLA in a more stable conformation to accept the substrate. Importantly, the substrate used here was pNP-acetate, which is not fully representative of the native polysaccharide and may explain the modest change in activity. Although deacetylase activity is increased, we detected a decrease in biofilm disruption activity. The role of PeLA’s hydrolase activity in biosynthesis is unknown, although it has been proposed to be involved in chain-length regulation or biofilm disassembly (11). Our data show that the interaction between PeLA and PeB is required for assembly of the polysaccharide, which coupled with the observed reduction in hydrolase activity suggests that a lower rate of hydrolysis is required during this process. It is attractive to speculate that the presence of PeB induces a conformational change in PeLA that allows for efficient deacylation of PEL. Ultimately, structural characterization of both PeLA and the PeLA–PeB complex is required to better understand the modulation of PeLA enzymatic activity by PeB.

Consistent with our data, we propose that the discrete region of the TPR-containing domain of PeB that we identified as being essential for biofilm formation, R9–R14, facilitates the localization of PeLA within the periplasm (Fig. 6). This is important because the outer membrane lipoprotein PelC has been proposed to multimerize around the region that connects the TPR and porin domains of PeB (Fig. 6). Thus, the TPRs C-terminal to the interaction site (R15–R19) may act as “spacer” repeats where individual deletion of any of these TPRs is not sufficient to cause any steric clashing between PelA and PelC or affect biofilm formation. When PeLA is recruited by PeB, a conformational change in PeLA induced by its interaction with PeB may result in more efficient deacylation of the polymer and reduce the ability of the hydrolase to interfere with biosynthesis. Because it has been shown that PeLA deacylation activity is essential for biofilm formation, it is possible that the disruption to the interaction, caused by our systematic TPR deletions, precludes deacylation, which in turn interrupts polysaccharide biosynthesis.

TPRs are a versatile fold responsible for protein–protein interactions found across all domains of life. When found associated with polysaccharide biosynthetic systems, the domain
The 10/11003 MgSO4/H1003 Vögel-Bonner minimal medium (VBMM) was prepared as a growth media and antibiotic selection.

Experimental procedures

Growth media and antibiotic selection

Lysogeny broth (LB) contained, per liter of ultrapure water, 10.0 g of tryptone, 5.0 g of yeast extract, and 5.0 g of NaCl. Vogel-Bonner minimal medium (VBMM) was prepared as a 10× stock, which contained, per liter of ultrapure water, 2.0 g of MgSO4·7H2O, 20 g of citric acid, 100 g of K2HPO4, and 35 g of NaNH4PO4·4H2O and was adjusted to pH 7.0 and filter-sterilized. The 10× VBMM solution was diluted as needed in sterile, ultrapure water. Semisolid media were prepared by adding 1.0% (w/v) noble agar to VBMM and 1.5% (w/v) agar to LB.

Antibiotic selection was added to growth media where appropriate as follows: for E. coli, ampicillin at 100 μg/ml, gentamicin (Gen) at 10 μg/ml, and kanamycin at 50 μg/ml; for P. aeruginosa, carbencillin at 300 μg/ml and Gen at 30 or 60 μg/ml depending on the application.

Standard molecular methods

All basic molecular and microbiological techniques were executed according to standard protocols (29). Genomic DNA (gDNA) isolation, plasmid preparation, and DNA gel extraction were performed using nucleotide purification kits purchased from Qiagen or BioBasic. All restriction enzymes, T4 DNA ligase, and Antarctic phosphatase were purchased from New England Biolabs. All primers were obtained from Integrated DNA Technologies. Transformations of P. aeruginosa were carried out using established protocols for electroporation (30). Site-directed mutagenesis of plasmids was carried out using the QuickChange II XL site-directed mutagenesis kit (Agilent).

Construction of P. aeruginosa chromosomal mutations

An in-frame, unmarked P. aeruginosa PAO1 pelB gene deletion was constructed using an established allelic replacement strategy (31). Briefly, flanking upstream and downstream regions of the pelB open reading frame (ORF) was amplified and joined by splicing-by-overlap extension PCR (primers are listed in supplemental Table S2). The upstream forward and downstream reverse primers were tagged with KpnI and HindIII restriction site sequences, respectively. The PCR product was gel-purified, digested, and ligated into pEX18Gm, and the resulting construct was identified and sequenced as described above. The pelA gene deletion was described previously (11).

Deletion alleles were introduced into P. aeruginosa PAO1 Δ wspE Δpsl PBADpel via biparental mating with donor strain E. coli SM10 or S17.1 (λpir) (32). Merodiploids were selected on VBMM containing 60 μg/ml Gen. SacB-mediated counterselection was carried out by selecting for double-crossover mutations on no-salt lysogeny broth (NSLB) agar containing 15% (w/v) sucrose. Unmarked gene deletions were identified by PCR with primers targeting the outside, flanking regions of pelB (supplemental Table S2). These PCR products were Sanger sequenced using the same primers to confirm the correct deletion.

Construction of miniTn7 vectors

The Gateway-compatible destination vector pUC18-miniTn7T2.1-Gm-GW (supplemental Table S1) has been described previously for the purpose of making single-copy chromosomal insertions with divergently transcribed ORFs (33). Using a recombineering strategy based on multisite Gateway technology (Invitrogen) established previously by our groups, we assembled a pUC18-miniTn7T2.1-Gm-GW vector in which araC-PBAD was fused to the pelA ORF (8, 34, 35). To begin, the pelA ORF was cloned by PCR from P. aeruginosa PAO1 gDNA using the primers ojH1554 and ojH1607, which introduced a synthetic ribosomal binding site at the 5′-end of the ORF as well as attB5 and attB2 sequences at the 5′- and 3′-ends of the PCR product, respectively.
*PelA–PelB complex is required for PEL biosynthesis*

This PCR product was then recombined with pDONR221P5P2 using BP Clonase II. The resulting entry vector, pCAS1, was isolated on LB agar containing 50 µg/ml kanamycin, identified by colony PCR, and verified by Sanger sequencing using M13 forward and reverse primers. Subsequently, pCAS1 was recombined with pUC18-miniTn7T2.1-Gm-GW and the entry vector pJH187 (supplemental Table S1), which contains araC-P\textsubscript{BAD} on pDONR221P5P2, using LR Clonase II Plus Enzyme Mix (Invitrogen) (34). The resulting vector, pCAS5 (bearing araC-P\textsubscript{BAD}:PelA on miniTn7), was isolated on LB agar containing 50 µg/ml carbenicillin and 10 µg/ml Gen, identified by colony PCR, and verified by Sanger sequencing using the sequencing primers oJJH1695 and oJJH1696.

Because pelB was recalcitrant to the Gateway recombination techniques described above (8), we built the restriction-compatible vector pUC18T-miniTn7T2.1-Gm (supplemental Table S1) for the purpose of making single-copy chromosomal insertions with divergently transcribed ORFs. To begin, a DNA fragment containing tandem T4 terminators and overhangs compatible with the KpnI restriction digestion site was created in vitro. To do this, complementary 5’-phosphorylated single-stranded DNA oligomers containing T4 terminator sequences (oJH510 and oJH511; supplemental Table S2) were combined in equimolar ratios, boiled for 15 min at 100 °C, and then cooled in stepwise increments of 2 °C/3 min until the mixture reached 22 °C. The assembled dsDNA fragment was then ligated into KpnI-digested pUC18T-miniTn7T2.1-Gm (supplemental Table S1). Ligated plasmids were transformed into *E. coli* DH5α, and clones harboring the correct insert were identified by colony PCR and sequenced using the primers oJH506 and oJH507 (supplemental Table S2). Similar to pUC18-miniTn7T2.1-Gm-GW, the resulting pUC18T-miniTn7T2.1-Gm vector has terminator sites at both ends of the miniTn7 transposon.

A complementation vector for pelB was made in a two-step restriction cloning process. To begin, araC-P\textsubscript{BAD} was cloned from pJJH187 via PCR with the primers oJDR92 and oJDR93 (supplemental Table S2). The resulting PCR product, which was flanked with PstI and embedded Hpal restriction sites, was gel-purified, digested with PstI-HF, and ligated into the PstI site of pUC18T-miniTn7T2.1-Gm. This yielded the vector pJDR10, which was verified by colony PCR and sequenced using the primers oJH1695 and oJJH1696. Next, the pelB ORF was cloned from PAO1 gDNA by PCR with the primers oJDR95 and oJH511, which contained Hpal and HindIII restriction sites, respectively. oJDR95 also contained a synthetic ribosome-binding site at the 3’-end of the primer (supplemental Table S2). The resulting PCR product was gel-purified, double digested with Hpal and HindIII, and ligated into pJDR10, yielding the vector pJDR11 that encodes araC-P\textsubscript{BAD}:pelB on miniTn7. pJDR11 was sequence-verified using the primers oJDR33, oJDR34, and oJJH1696.

*Mutagenesis of *P. aeruginosa* with miniTn7*

Single-copy insertion of miniTn7 vectors at the neutral attTn7 of the chromosome was carried out by electroporation of *P. aeruginosa* with the recombinant miniTn7 vector and a helper plasmid, pTNS2, as described previously (30). Transformant clones were verified by colony PCR and sequenced using the primers oJJH1695 and oJJH1696.

*Generation of pelA-His\textsubscript{6}*

Incorporation of the PelA His tag was generated using the QuickChange site-directed mutagenesis protocol (Stratagene). To incorporate the tag, a set of divergent 5’-phosphorylated primers (PelA F and PelA R; supplemental Table S2) was generated. The forward primer annealed to the PelA stop codon and the reverse primer annealed to 20 bp directly upstream of the stop codon and on the coding strand (3’–5’). Both primers each have a 5’-overhang encoding three His residues to encode a hexahistidine tag. This PCR amplified an 8.6-kb fragment. Following the PCR, the template DNA was digested by DpnI for 1 h at 37 °C, and the fragment was treated with 1 unit of T4 DNA ligase overnight at room temperature to self-ligate the blunt ends and recircularize the vector. The construct was verified by DNA sequencing (TCAG DNA Technologies Corp., Toronto, Canada). Plasmids were transferred to PAO1 ΔwspF Δpsl P\textsubscript{BAD}-pel ΔpelA by electroporation as described above.

*Sample preparation for Western blotting*

To verify protein levels, overnight cultures of the appropriate strains were grown in 5 ml of LB with 0.5% (w/v) L-(-)-arabinose and 30 µg/ml Gen where required at 37 °C with shaking. The following day cultures were normalized using optical density (A\textsubscript{600}), and samples were probed using Western blot analysis and protein-specific antibodies (8, 9, 11, 36).

*Western blotting*

0.2-µm polyvinylidene difluoride (PVDF) membranes were prewetted with methanol and soaked in transfer buffer (25 mM Tris, 192 mM glycine, 20% (v/v) methanol) prior to transfer of proteins via wet blotting (25 mV, 1.5 h). The membranes were blocked for 30 min in TBST + 5% (w/v) skim milk powder at room temperature with gentle agitation. The membranes were placed in primary antibody (1:500 α-PelB; 1:2000 α-PelA; 1:1000 α-PilQ, α-PilP, α-PilF, and α-alkaline phosphatase) in TBST + 1% (w/v) skim milk powder) for 1 h. Membranes were then washed three times for 10 min in TBST prior to the addition of a 1:3000 dilution of secondary antibody (Bio-Rad enzyme immunoassay-grade affinity-purified goat α-rabbit IgG-HRP conjugate) in TBST + 1% (w/v) skim milk powder. Membranes were incubated in secondary antibody for 1 h and then washed three times with 15 ml of TBST. Membranes were then immersed in SuperSignal West Pico chemiluminescent substrate (Pierce) for 2 min, and developed using a Bio-Rad ChemiDoc imaging system.

*In vivo affinity pulldown*

One liter of each PAO1 ΔwspF Δpsl P\textsubscript{BAD}-pel ΔpelA complemented with pelA or pelA-His\textsubscript{6} inserted into the attTn7 site were grown overnight with shaking at 37 °C with 30 µg/ml Gen and 0.5% L-arabinose to induce expression. Cells were collected and resuspended in Buffer A (20 mM Tris, pH 8, 150 mM NaCl, 2% (v/v) glycerol, one protease inhibitor tablet (Roche Applied Science)) and lysed by homogenization using an Emulsiflex-C3.
(Avestin, Inc.) at a pressure between 15,000 and 20,000 p.s.i. until the resuspension appeared translucent. Insoluble cell lysate was removed by centrifugation for 25 min at 25,000 × g at 4 °C. The supernatant containing crude membranes was centrifuged for 1 h at 130,000 × g. Crude membranes were then resuspended in Buffer A containing 1% (w/v) lauryldimethylamine–N-oxide and left overnight at 4 °C to solubilize the membranes. The samples were then centrifuged for 1 h at 130,000 × g to remove any insoluble membranes. The supernatant was incubated with Ni²⁺-nitrilotriacetic acid resin (Qiagen) preincubated with Buffer B (20 mM Tris, pH 8, 150 mM NaCl, 2% (v/v) glycerol, 0.065% (w/v) lauryldimethylamine–N-oxide) and 5 mM imidazole for 30 min at 4 °C with gentle rocking. The purification was performed using a batch method, and centrifugationsteps were performed for 10 min at 700 × g to sediment the resin. Flow-through was removed, the resin was washed three times with 5 ml of Buffer B containing 20 mM imidazole, and a final wash of 1 ml was collected. A second wash of Buffer B containing 30 mM imidazole was performed in the same manner. The proteins were eluted in 200 μl of Buffer B (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 5% (v/v) glycerol) containing 250 mM imidazole. Samples were resuspended in 2× Laemmli sample buffer and immunoblotted using Western blot analysis.

Subcellular fractionation

A cell fractionation protocol adapted from Thein et al. (37) was utilized as described previously (8).

Generation of TPR deletions

Using the miniTn7T-Gm plasmids encoding pelB, deletions of individual TPR motifs (34 amino acids, or 102 bp) were designed according to TPR predictions and structural analysis and accomplished in two successive steps using the QuikChange Lightening site-directed mutagenesis kit (Agilent Technologies). In the first step, 30-bp forward and reverse primers were designed to anneal to 15 bp upstream and 15 bp downstream of the sequence to be deleted, encoding the first half, or 51 bp, of the TPR motif (supplemental Table S1). The deletion was confirmed with DNA sequencing (TCAG DNA Technologies Corp.). The plasmids and strains used in this study are summarized in supplemental Table S1.

Expression and purification

The expression and purification of P. aeruginosa proteins PelB(47–880) and PelA(Δ46) have been described elsewhere (8, 11). The pelB gene was codon-optimized using GeneArt (Invitrogen). Primers encoding PelB(248–625) and PelB(351–588) were designed from this sequence. The PCR product encoding PelB(248–625) was digested with Ndel and Xhol restriction enzymes and subsequently cloned into a pET-28a vector (Novagen). PelB(351–588) was generated in two steps from pLSMPelBpa(47–880). Divergent primers were designed (46R and 351F) to delete base pairs encoding residues 47–350, and a similar method described above for pelA-His₆ was performed. Site-directed mutagenesis was then used to mutate alanine 589 to a stop codon. The resulting expression vectors (pLSMPelBpa(248–625) and pLSMPelBpa(351–588)) encode residues 248–625 and 351–588, respectively, of P. aeruginosa PelB fused to a cleavable N-terminal His₆ tag for purification purposes. The fidelity of the protein inserts was confirmed using DNA sequencing (TCAG DNA Technologies Corp.). The plasmids and strains used in this study are summarized in supplemental Table S1.

Analytical gel filtration

The size of the PelA–PelB complex was determined using a Superdex 200 10/300 GL column (GE Healthcare). The column was equilibrated in 20 mM Tris, pH 8.0, 150 mM NaCl, 5% (v/v) glycerol. Molecular mass standards (GE Healthcare Gel Filtration LMW Calibration kit) were applied to the column as directed. PelA(Δ46) + PelB(351–588) were each applied to the column at 12 µM, whereas for the complex, 8 µM PelA was threaded vials (Fisher Scientific) in triplicate. Vials were incubated statically for 48 h at 25 °C. Following incubation, nonattached cells were removed, and the vials were rinsed thoroughly with water. The vials were stained with 750 µl of 0.1% (w/v) crystal violet for 10 min. The vials were rinsed, adhered crystal violet was solubilized in 1 ml of 10% (v/v) glacial acetic acid for 10 min with gentle agitation, and 200 µl was subsequently transferred to a fresh microtiter plate where the absorbance was measured at 590 nm.

PelA–PelB complex is required for PEL biosynthesis

The generation of the expression vector pLSMPelBpa(47–880) and pNAPelApa(Δ46) has been described elsewhere (8, 11). The pelB gene was codon-optimized using GeneArt (Invitrogen). Primers encoding PelB(248–625) and PelB(351–588) were designed from this sequence. The PCR product encoding PelB(248–625) was digested with Ndel and Xhol restriction enzymes and subsequently cloned into a pET-28a vector (Novagen). PelB(351–588) was generated in two steps from pLSMPelBpa(47–880). Divergent primers were designed (46R and 351F) to delete base pairs encoding residues 47–350, and a similar method described above for pelA-His₆ was performed. Site-directed mutagenesis was then used to mutate alanine 589 to a stop codon. The resulting expression vectors (pLSMPelBpa(248–625) and pLSMPelBpa(351–588)) encode residues 248–625 and 351–588, respectively, of P. aeruginosa PelB fused to a cleavable N-terminal His₆ tag for purification purposes. The fidelity of the protein inserts was confirmed using DNA sequencing (TCAG DNA Technologies Corp.). The plasmids and strains used in this study are summarized in supplemental Table S1.

Pellicle assays

Assays were performed as described previously in standing cultures of 3 ml of NSLB for 48 h at 25 °C (8, 11)

Crystal violet assays

The crystal violet assay was performed as described previously with the following modifications. Overnight cultures were diluted to a final A₅₆₀ of 0.005 in 500 µl of NSLB containing 0.5% (v/v) l-arabinose in Fisherbrand™ Class A clear glass threaded vials (Fisher Scientific) in triplicate. Vials were incubated statically for 48 h at 25 °C. Following incubation, nonattached cells were removed, and the vials were rinsed thoroughly with water. The vials were stained with 750 µl of 0.1% (w/v) crystal violet for 10 min. The vials were rinsed, adhered crystal violet was solubilized in 1 ml of 10% (v/v) glacial acetic acid for 10 min with gentle agitation, and 200 µl was subsequently transferred to a fresh microtiter plate where the absorbance was measured at 590 nm.
**PelA–PelB complex is required for PEL biosynthesis**

mixed with a slight molar excess of PelB and then applied to the column (100 µl). Protein elution was monitored at 280 nm. To resolve the peaks between PelA(47–303) and PelB(351–588), proteins were applied to a Superdex 75 10/300 GL column at a concentration of 11 µM. These proteins were also mixed with a slight molar excess of PelB and applied to the column. Samples were collected from 1-ml fractions, mixed with 2× SDS loading buffer, and analyzed using SDS-PAGE and Coomassie staining.

**In vitro affinity pulldown**

Equimolar amounts of untagged PelA(Δ46) and N-His₆-PelB(248–625) were mixed in a total of 1 ml and allowed to incubate at 4 °C for 30 min. The solution was then loaded onto a 1-ml Ni²⁺-nitrilotriacetic acid column. The flow-through was collected and washed through the column using 7 column volumes of Buffer C (20 mM Tris, pH 8.0, 150 mM NaCl, 5% (v/v) glycerol) containing 5 mM imidazole. The column was washed with 7 column volumes of Buffer C containing 20 mM imidazole to remove any excess protein. A final wash step of 1 column volume was collected. The complex was eluted using 7 column volumes of Buffer C containing 250 mM imidazole. As a control, untagged PelA(Δ46) alone was added to a column to ensure that PelA(Δ46) did not nonspecifically bind the resin.

**Biofilm disruption assay**

An overnight culture of PA01 Δ wspF Δ psl PBADpel was diluted to an A₅₆₀ of 0.5 in NSLB and further diluted to 1:100 in NSLB containing 0.5% (v/v) L-arabinose. 100 µl of culture was added to wells of a 96-well polypropylene plate (Nunc) and incubated statically for 24 h at room temperature. Following incubation, non-attached cells were removed, and the plate was rinsed thoroughly with water. Plates were then incubated with 150 µl of protein diluted in phosphate-buffered saline (dilutions from 1.5 µM) in triplicate for 2 h with gentle agitation. PelB(47–880) was added in 1.5× excess of PelA(Δ46). Following protein treatment, protein was removed, and wells were rinsed thoroughly with water. The remaining biomass was stained with 150 µl 0.1% (w/v) crystal violet for 10 min. The plate was rinsed, adhered crystal violet was solubilized in 150 µl of 95% (v/v) ethanol for 10 min, and the absorbance was read at A₅₉₅. EC₅₀ values were calculated using nonlinear least-square fitting to a dose-response model.

**pNP-acetate assay**

The assay was performed as described previously (11). All enzyme assays were performed at least in triplicate, in a 96-well microtiter plate, using a SpectraMax M2 from Molecular Devices (Sunnyvale, CA). Standard assays contained 2.5 mM pNP-acetate dissolved in ethanol and 2 µM PelA(Δ46) in Buffer D in a total volume of 200 µl of Buffer D at 25 °C. For reactions containing both PelA and PelB, PelB(47–880) was added to a final concentration of 3 µM or 1.5× excess of PelA(Δ46). Reactions were initiated by the addition of pNP-acetate and allowed to proceed for 10 min. Reaction progress was monitored in real time at 405 nm for the appearance of p-nitrophenyl. According to the manufacturer’s instructions, the extinction coefficient was taken to be 18,300 M⁻¹ cm⁻¹. The background hydrolysis rate was monitored and subtracted from the enzyme-catalyzed reactions.

**Crystallization and structure determination**

Commercial sparse-matrix crystal screens from Microlytic (MCSG1–4) were prepared at room temperature (22 °C) with N-His₆-PelB(248–625) at a concentration of 9 mg/ml. 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. Native crystals appeared in 0.1 M HEPES, pH 7.5, 13% (w/v) PEG 3350, 0.2 M proline after 5 days. These crystals appeared as hexagonal bipyramids that grew to maximum dimensions of 100 × 100 × 100 µm. The selenomethionine derivative crystallized in the same condition; however, after 7 days, a single crystal appeared in 0.1 M CHES, pH 9.5, 10% (w/v) PEG 3000. This crystal grew as a large hexagonal bipyramid that grew to maximum dimensions of 200 × 200 × 200 µm.

Crystals of PelB₈₆ were flash frozen and cryoprotected in the crystallization solution supplemented with 25% (v/v) glycerol prior to vitrification, and both were subsequently stored until X-ray diffraction data were collected on beamline 08B1-1 at the Canadian Light Source. A total of 602 images of 0.5° Δψ oscillation were collected on an MX300HE detector with a 315-mm crystal-to-detector distance and an exposure time of 5 s per image. The data were processed using DENOZO, and integrated intensities were scaled using SCALEPACK from the HKL-2000 program package (39). The data collection statistics are summarized in Table 1.

A total of two selenium sites in PelB₈₆ were located using HKL2MAP (40), and density-modified phases were calculated using SOLVE/RESOLVE (41). The resulting map was of good quality and allowed for manual building using Coot (42). The model was then refined against the native data using PHENIX.REFINE (43) to a final Rwork/Rfree (%) of 21.5/27.2. The refinement statistics are summarized in Table 1.

Molecular graphics and analyses, including examination of the electrostatic surface potential of PelB, were performed with the UCSF Chimera package. Chimera was developed by the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (44). All software indicated above has been curated by SBGrid (45).

**Author contributions**—L. S. M. and P. L. H. designed the research. L. S. M., J. D. R., L. B. G., C. A. S., P. Y., and J. J. H. performed the research. L. S. M., G. B. W., M. R. P., J. J. H., J. C. W., and P. L. H. analyzed the data. L. S. M. and P. L. H. wrote the paper. All authors provided feedback and revised the manuscript.

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PeLA–PelB complex is required for PEL biosynthesis
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