The *Pseudomonas aeruginosa* lectin LecB binds to the exopolysaccharide Psl and stabilizes the biofilm matrix

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*Pseudomonas aeruginosa* biofilms are composed of exopolysaccharides (EPS), exogenous DNA, and proteins that hold these communities together. *P. aeruginosa* produces lectins LecA and LecB, which possess affinities towards sugars found in matrix EPS and mediate adherence of *P. aeruginosa* to target host cells. Here, we demonstrate that LecB binds to Psl, a key matrix EPS, and this leads to increased retention of both cells and EPS in a growing biofilm. This interaction is predicted to occur between the lectin and the branched side chains present on Psl. Finally, we show that LecB coordinates Psl localization in the biofilm. This constitutes a unique function for LecB and identifies it as a matrix protein that contributes to biofilm structure through EPS interactions.

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Biofilms are multicellular aggregates of microbes that are enclosed in a matrix composed of exopolysaccharides (EPS), exogenous DNA (eDNA), and proteins. The extracellular matrix is thought to hold these communities together as well as contribute to bacterial persistence at infection sites by protecting against the host immune system and antimicrobial stresses.

The biofilm matrix produced by non-mucoid Pseudomonas aeruginosa strains primarily contains two EPS, Pel, and Psl, which form a scaffold that maintains biofilm structure and function. Pel was recently described as an N-acetyl glucosamine (GlcNAc)- and N-acetyl galactosamine (GalNAc)-rich polysaccharide that is charged under slightly acidic pH and interacts with eDNA in the matrix. Psl is composed of a neutral pentasaccharide subunit that contains mannose, rhamnose, and glucose in a 3:1:1 ratio. The levels of these polysaccharides within the matrix and their relevance for aggregate structural stability varies across P. aeruginosa strains. Furthermore, these EPS can be found as both cell-associated and secreted forms.

Less is known concerning the identity and function of P. aeruginosa biofilm matrix proteins. Proteins that interact with these EPS can contribute to biofilm structural integrity and maintenance. To date, the best described matrix protein is the extracellular adhesin CdrA, which promotes aggregate formation through Psl interactions under planktonic conditions, and helps to stabilize the matrix and maintain aggregate structural integrity. It was recently shown that CdrA can also promote bacterial aggregation in the absence of EPS. Outside of CdrA, no other matrix proteins that play a role in biofilm structural stability have been identified.

P. aeruginosa produces two small soluble lectins, LecA and LecB (also named PAI-L and PAII-L, respectively) that interact with specific sugars. Crystal structures have been solved for both, and binding affinity experiments showed that LecA binds to galactose and its derivatives, while LecB binds to fucose, mannose, and mannose-containing oligosaccharides. In addition, it is important to note that functional LecB is a homotetramer consisting of four 114 amino acid LecB monomers which require two divalent calcium ions and has been shown to be associated with the outer membrane. The primary functional roles attributed to these lectins is to mediate attachment to the host during infection. In particular, it was shown that LecB is involved in host cell invasion and cytotoxicity, while LecB reduces ciliary beating of airway epithelium. Both lectins also are linked to biofilm formation on abiotic surfaces, although the underlying mechanism behind these observations are unknown. Culturing P. aeruginosa in the presence of the monosaccharides that are the binding partners for these lectins inhibits biofilm maturation. This discovery led to the development of putative therapeutic approaches using glycomimetics that disrupt LecB-sugar interactions.

Interestingly, PsI contains mannose, a target monosaccharide for LecB. In this study, we demonstrate that LecB binds to Psl. We then show that LecB positions Psl within the matrix and that this interaction is crucial for aggregate formation. We find that, unlike biotic surfaces, LecB is not important for adhesion to abiotic surfaces, but its presence leads to increased retention of cells and EPS in the biofilm. This study identifies LecB as a P. aeruginosa biofilm matrix protein that binds to Psl and promotes cell retention.

Results

LecB binds to the PsI exopolysaccharide. Pel and Psl are biofilm matrix EPS that are crucial for P. aeruginosa biofilm formation and structural integrity. LecB binds the monosaccharides fucose and mannose. Since mannose residues are present in the pentasaccharide subunit of Psl, we hypothesized that LecB binds to Psl and that this might influence P. aeruginosa biofilm structure.

To test this hypothesis, we quantified the binding of purified LecB to Pel and Psl. For this binding assay, we used purified Psl as well as crude polysaccharide preparations from the following strains: ΔwspF (overproduces Pel and Psl), ΔwspF Δpel (overproduces Psl only), and ΔwspF Δpsl (overproduces Pel only). Fluorescently labeled LecB (LecB-FITC) was used in a fluorophore-linked lectin assay (FLLA), in which LecB-FITC was tested for binding to microtiter plate wells that were coated with exopolysaccharide preparations. We observed that LecB bound to wells coated with purified Psl as well as those coated with EPS preparations from ΔwspF and ΔwspF Δpel but not ΔwspF Δpsl (Fig. 1a). As expected, LecB-FITC also bound to wells coated with the positive controls mann (a mannose-rich polysaccharide) and mucin (highly glycosylated proteins, decorated with fucose residues). LecB-FITC did not interact with wells coated with microtiter plate wells that were coated with exopolysaccharide preparations.

Fig. 1 LecB binds to the exopolysaccharide Psl. a FLLA assay shows that LecB is able to bind to fucose/mannose-containing polysaccharides immobilized on the wells of microtiter plates. FITC-conjugated LecB does not bind to uncoated or glucose-rich polysaccharide alginate, and Pel-rich materials (ΔwspF Δpsl). However, it binds to mannans, fucose-rich proteins (mucin), Psl-rich materials (ΔwspF and ΔwspF Δpel), and to purified PsI. **p < 0.0001, t-test, n > 3. b LecB western blot showing coimmunoprecipitation of LecB and Psl. Lane 1 was loaded with purified LecB. PtenG Dynabeads coated with anti-Psl antibodies were incubated with LecB (lane 2) or purified Psl and LecB (lane 3). Eluted material was immunoblotted using anti-LecB antibodies. c Psl blot showing coimmunoprecipitation of LecB and Psl. Protein G Dynabeads coated with anti-LecB antibodies were incubated with purified LecB (lane 1) or with LecB and Psl (lane 2). After wash steps, eluted material was immunoblotted using anti-Psl antibodies. Lane 3 was loaded with purified Psl.
coated with the negative control alginate, which does not contain either mannose or fucose.

As a complementary approach to investigate LecB–Psl interactions, we performed co-immunoprecipitations (Co-IP) using beads coated with either anti-Psl or anti-LecB antibodies. When anti-Psl coated beads were incubated with purified Psl and purified LecB, we detected LecB in the eluted fraction only when both Psl and LecB were present in the input fraction (Fig. 1b). Previously, we had shown that the type VI secretion effector Tse1 preferentially bind to Psl and LecB were present in the input fraction (Fig.1c). As expected the Tse1 negative control did not bind Psl (data not shown). Together, these results support that LecB binds to Psl.

**LecB binds to a branched mannose residue on the side chain of Psl.** We sought to examine the specific nature of LecB–Psl interactions. Toward this end, we quantified the binding of purified LecB to relevant disaccharides by isothermal titration calorimetry (ITC). We hypothesized that LecB would interact with one or more of the mannose residues found in Psl. There are three linked mannose residues present in the repeating pentasaccharide unit of Psl. Two mannose residues are in the linear polysaccharide chain and are linked via β-1,3′ glycosidic bonds. The third mannose residue branches from the linear chain and is connected by α-1,2′ linkage to a mannose residue in the linear chain (Fig. 2d).

For ITC experiments we purified LecB overexpressed in *P. aeruginosa* using affinity chromatography as previously described. For each ligand, the binding constants (Kd) and thermodynamics were determined from three independent experiments (Table 1, Supplementary Fig. 8). LecB bound to α-1,2′ mannobiose with a Kd of approximately 27 µM (Fig. 2a), which is comparable to the values reported in the literature for LecB-mannose interactions. However, LecB did not bind to β-1,3′mannobiose (Fig. 2c). The α-1,2′mannobiose disaccharide is present in the Psl side chain, while β-1,3′mannobiose is part of the linear polysaccharide chain. Interestingly, we found that LecB binds to α-1,3′mannobiose with a similar binding affinity (25 µM) as to α-1,2′mannobiose (Fig. 2b). This indicates that the β-linkage and not the C3 linkage is impeding interactions between LecB and the non-reducing mannose. As expected, the negative control maltose (α-1,4′glucobiose) did not interact with LecB (Table 1).

These results led us to hypothesize that LecB would preferentially bind to the mannose present on the side chain of Psl. To explore this idea, we performed molecular docking simulations using as protein receptor template the crystal structure of LecB in complex with D-mannose and the theoretical model, based on NMR data, of the pentasaccharide repeating unit of Psl. Minimal manual rebuilding of the model of LecB–Psl complex were needed in order to properly dock the Psl (Fig. 3a) side chain (α-1,2′mannobiose) to the binding site of LecB (Fig. 3b). In contrast, major structural changes were required to prevent major steric clashes between LecB and Psl.
when the linear chain segment (β-1,3’ mannobiose) was tentatively docked into LecB binding site.

A major feature of the Psl-binding site of LecB is a pair of calcium ions. The α-D-Manp sugar side chain, adopts the lowest energy $^4C_1$ conformation, and via its hydroxyl groups interacts with either (O2 2.7 Å), (O4 2.8 Å) or both (O3 2.5 Å; 2.6 Å) the calcium ions (Supplementary Fig. 1). The protein residues involved in the coordination of the two calcium ions, 3.7 Å apart from each other, belong to a single PAO1 LecB loop encompassing residues Asn95-Asp104 and comprises also residue Asn21 (Fig. 3a). The bound Psl α-D-Manp sugar side chain interacts via hydrogen bonds with PAO1 LecB Ser22 (2.2 Å) and Ser23 (3.1 Å, 3.3 Å) (Supplementary Fig. 1).

The role of bridging water and hydrogen bonding as key determinants of non-covalent protein-carbohydrate recognition has been recently reviewed. Indeed, two water molecules are engaged at the PAO1 LecB–Psl interface (Supplementary Fig. 1). The first, besides interacting with the second water molecule (2.9 Å) and the O4 hydroxyl of the α-D-Manp sugar side chain (2.7 Å) also interacts with the PAO1 LecB residues Thr98 (2.9 Å) and Asp99 (2.4 Å; 3.1 Å). The second water molecule does not get involved in protein contacts, but interacts with the O5 oxygen (2.9 Å) of the pyranose ring and with the methyl hydroxyl O6 (3.0 Å) of the β-D-Mannp sugar side chain.

It is worthy to note that the predicted PAO1 LecB Psl docking nicely superimpose (Supplementary Fig. 2) on the previously reported crystal structure of the PA14 LecB in complex with the D-Manp-α-1,3-D-Manp disaccharide (PDBID: 5A6Y). The protein residues involved at the Psl-binding site are highly conserved and only differ at positions 23 (PA14 LecB Ser; PAO1 LecB Ala) and 97 (PA14 LecB Ser; PAO1 LecB Gly) with an overall r.m.s.d. (23 Cα atoms) of 0.11 Å. The binding orientation of D-Manp-α-1,2-D-Manp fragment of the Psl pentasaccharide in the computed complex with PAO1 LecB closely resembles that of the D-Manp-α-1,3-D-Manp disaccharide in complex with PA14 LecB, validating the previously reported IC50 binding affinities of either disaccharides as well as the reported ITC thermodynamic signatures in our present work. Collectively, these results indicate that binding to the linear chain of Psl is unlikely, and Psl structure heavily favors binding of PAO1 LecB to its side chain.

**Table 1 ITC analysis for binding of LecB to different disaccharide**

| Disaccharide        | $K_d$ (µM) | $-\Delta G$ (kJ/mol) | $-\Delta H$ (kJ/mol) | $-T\Delta S$ (kJ/mol) | $n$   |
|---------------------|-----------|----------------------|----------------------|-----------------------|-------|
| α-1,2′ mannobiose   | 26.60 ± 2.61 | 26.09 ± 0.21 | 20.71 ± 2.59 | 5.38 ± 2.75 | 0.62 ± 0.01 |
| α-1,3′ mannobiose   | 33.63 ± 7.18 | 25.58 ± 0.57 | 20.54 ± 1.57 | 5.05 ± 2.12 | 0.61 ± 0.03 |
| β-1,3′ mannobiose   | n.d.       | n.d.                | n.d.                | n.d.                  | n.d.  |
| Methyl-α-D-mannoside| 71         | 23.7                | 17.8                | 5.9                   | 0.94  |
| Maltose             | n.d.       | n.d.                | n.d.                | n.d.                  | n.d.  |

Values are mean ± S.D. from three separate titrations. n.d. indicates that with ligand concentrations used were not enough to determine these values.

**Fig. 3** Modeling of LecB-Psl interactions favors binding to the side chain of Psl. a Overall conformation of the docked Psl pentasaccharide is represented as α-D-mannopyranose in cyan, β-D-mannopyranose in gray, α-L-rhamnopyranose in orange, and β-D-glucopyranose in yellow. Oxygen atoms are labeled in red. b Structural model of Psl docking in LecB binding site is favored to occur in the α-1,2′ mannobiose. LecB is depicted in white, water molecules are the red spheres, the green ones are Ca$^{12}$ ions, and Psl follows the same color code from (a).

**LecB binds in situ to Psl in the biofilm matrix.** Upon discovering that LecB binds Psl, we hypothesized that the lectin could also bind to Psl found in the context of the biofilm matrix. Since Psl is already known to interact with other matrix components, we were unsure if LecB binding sites would be accessible. To test our hypothesis, we applied fluorescently labeled LecB-FITC along with HHA-TRITC, a plant lectin known to specifically stain Psl 28,29 to 4-day-old biofilm matrix aggregates of PAO1. LecB-FITC staining was observed in the same Psl-rich regions. As shown in Fig. 4a, we observed the characteristic Psl staining pattern present at the periphery of biofilm aggregates of PAO1. LecB-FITC staining was observed in the same Psl-rich regions. As expected in strains PAO1 Δpsl and PA14 (Fig. 4b, c, respectively), which are unable to produce Psl, we did not observe staining with either HHA-TRITC or LecB-FITC. These results suggest that LecB is able to interact with Psl that is present in the biofilm matrix environment.
LecB coordinates Psl localization within the matrix. We next sought to determine the impact of a lecB mutation on biofilm structure under constant flow conditions. When biofilms were cultured in a dilute complex growth medium (NB 1.4% v/v), the wild type formed biofilms characterized by numerous cellular aggregates that extended >50 microns above the attachment surface (Fig. 5a), while the ΔlecB strain produced a monolayer of cells punctuated infrequently by small mounds of cells that did not usually extend more than 25 microns above the surface (Fig. 5b). To quantify this phenotypic difference, we applied the image analysis software COMSTAT. Surface roughness is a key measurement made by COMSTAT that illustrates the numerous aggregates present in the wild type, but are absent from the mutant. COMSTAT analysis revealed that wild-type biofilms were considerably rougher than the lecB mutant (Supplementary Fig. 3). Complementation of the ΔlecB mutation in trans largely restored the wild-type biofilm phenotype (Fig. 5c and Supplementary Fig. 3). These results suggest that LecB plays a role in producing and maintaining biofilm aggregates.

Following our discovery that LecB binds Psl, we hypothesized that the lecB mutant strain might exhibit mislocalization of Psl in the matrix. Past work with the Psl-binding matrix protein CdrA showed that aberrant Psl localization in the matrix can accompany observed biofilm structural defects. To address this possibility, we monitored Psl localization patterns in biofilms formed by the LecB conditional expression strain, ΔlecB pJNLecB. In the absence of lecB expression, we observed that biofilms failed to produce large aggregates and also had no distinct pattern in Psl localization (Fig. 6a). Semi-quantification of Psl levels across the biofilm aggregate indicated that its distribution was random and uneven (Fig. 6b). The same strain, complemented by expressing lecB, produced large cellular aggregates that exhibited peripheral Psl localization (Fig. 6c, d), similar to what is seen in the aggregates of wild-type biofilms, although the amount of Psl made in both the presence and absence of arabinose appeared to be comparable.

Previous studies have shown that another matrix protein, CdrA, influences biofilm aggregate formation through Psl interactions. This raises the question as to whether LecB and CdrA are functionally interchangeable? Therefore, we examined if CdrA influenced Psl distribution and aggregate formation in our system. First, we characterized a PAO1 ΔcdrA ΔlecB double mutant strain, and observed that it produced biofilms composed of a carpet of cells, which contained very little Psl (Fig. 6e, f). In the rare instances where aggregates were observed, they were more susceptible to shear stress in comparison to PAO1 wild type or ΔlecB (Movies S1, S2, and S3). Expression of lecB in trans in the double mutant background was sufficient to restore wild-type aggregate formation and Psl distribution (Fig. 6g, h). In contrast,
Fig. 6 LecB coordinates Psl positioning in the matrix. a Syto 62 (magenta) and HHA-TRITC (red) stained biofilms of ΔlecB pJNlecB grown in NB without arabinose forms a flat biofilm with b Psl distribution being fairly homogenous throughout the biomass (the x-axis, RFU, corresponds to the intensity of PSL staining.). Psl intensity profiles were generated from an average of nine independent micrographs. Inset is a representative Psl staining micrograph that was used to generate the profile. c Biofilms of ΔlecB pJNlecB grown in NB with the addition of 0.05% arabinose present a similar structure and d Psl distribution to the ones formed by PAO1. e Syto 62 (magenta) and HHA-TRITC (red) stained biofilms of ΔcdrA ΔlecB pJNlecB grown in NB without arabinose display a loose carpet of cells with f no particular Psl localization pattern. g ΔcdrA ΔlecB pJNlecB grown in NB with the addition of 0.05% arabinose rescues PAO1 phenotype and h usual Psl placement. i Biofilms of ΔcdrA ΔlecB pBADcdrAB grown in NB with the addition of 0.05% arabinose results in the formation of an undifferentiated thick layer of cells with j no particular Psl organization. Psl distribution measurements were performed in nine images from three different experiments per condition. Scale bars = 25 µm
expression of cdrA in the same background was not sufficient to restore wild-type biofilm characteristics. This strain produced a flat, thick mass devoid of aggregates (Fig. 6i), although it still retained Psl (Fig. 6j). Taken together, these results suggest that when grown in NB, CdrA can influence biofilm structure and Psl retention, but LecB is the primary matrix protein responsible for Psl localization and aggregate production.

Our previous work demonstrated a prominent role for CdrA in aggregate formation. In attempting to reconcile our current results with this past study, we noted that the main culturing difference involved the growth medium. In this study, a dilute complex medium (NB medium) was used to mimic the previous work done on LecB12. On the other hand, our past work with CdrA involved dilute LB medium. Indeed, when we compared biofilm phenotypes of PAO1 and the alecB mutant strain grown on dilute LB, we found that the mutant strain was able to produce aggregates like the wild type (Supplementary Fig. 4). Since our results suggest that LecB and CdrA can carry out similar roles in the matrix, we hypothesized that either CdrA or LecB expression levels may change significantly when P. aeruginosa is grown on NB medium as opposed to LB. The idea being that LecB may not be required under conditions where CdrA is produced at a sufficiently high level (or vice versa). To test this possibility we probed biofilm biomass harvested from the surface of silicone tubing using both CdrA and LecB antisera. We found that biofilm growth on NB produced significantly less CdrA in the matrix than growth on LB. LecB levels were observed to be roughly the same for the two growth media (Supplementary Fig. 5). These data indicate that LecB production is critical for aggregate formation under culturing conditions (e.g., growth on NB) that result in low levels of CdrA expression. The underlying cause of lower CdrA expression levels during growth on NB remains unclear and is a focus of future study.

**LecB promotes retention of Psl and cells within biofilms.** Our results indicate that LecB binds Psl and guides its localization within biofilm aggregates. Past work with CdrA demonstrated that its expression was linked to retention of cells and Psl to a growing biofilm. This led us to hypothesize that LecB might play a similar role during the course of biofilm growth. To test this hypothesis, we used the arabinose-inducible lecB expression strain and quantified the amount of cells and Psl released into the bulk liquid in the presence and absence of arabinose. To facilitate quantitation of biofilm biomass, we cultured biofilm pellets on NB medium as opposed to LB. The idea being that LecB may not be required under conditions where CdrA is produced at a sufficiently high level (or vice versa). To test this possibility we probed biofilm biomass harvested from the surface of silicone tubing using both CdrA and LecB antisera. We found that biofilm growth on NB produced significantly less CdrA in the matrix than growth on LB. LecB levels were observed to be roughly the same for the two growth media (Supplementary Fig. 5). These data indicate that LecB production is critical for aggregate formation under culturing conditions (e.g., growth on NB) that result in low levels of CdrA expression. The underlying cause of lower CdrA expression levels during growth on NB remains unclear and is a focus of future study.

**Discussion**

Lectins are found in all domains of life, and their key function is often to mediate interactions. In bacteria, lectins are nearly exclusively studied in the context of bacterium interactions with higher Eukaryotes. Indeed, these interactions have critical roles in both disease and symbioses. However, particularly in bacterial species, lectins functioning in the context of microbial communities has not been extensively explored. This is certainly the case for *P. aeruginosa*, where the roles of its two self-produced lectins are largely attributed to disease-related host interactions. Our findings suggest that lectin-mediated interactions that stabilize the biofilm matrix represent a distinct function for at least one of these lectins.

Biofilm formation usually involves the production of EPS and proteins that lend structural integrity to the matrix. One of the better characterized systems demonstrating these principles involves *Vibrio cholerae*. Three matrix proteins were identified in *V. cholerae* that contribute to biofilm stability. After the production of the main exopolysaccharide VPS, a protein involved in cell–cell and cell–surface adhesion (RmbA) accumulates at the cell surface. Next, Bap1 is secreted and is thought to crosslink unknown matrix components and cells to ensure matrix integrity, as well as to contribute to the hydrophobicity of the pellicle. Last, RmbC accumulates at discrete sites and is crucial for retaining VPS throughout the biofilm. These principles appear to be conserved in *P. aeruginosa*, with Pel and Psl performing the role of VPS and CdrA and LecB emulating RmbA, Bap1, and RmbC functions.

Biofilm aggregates likely serve some key functions. For biofilms growing at a liquid–solid interface, aggregates protrude out of the boundary layer found at the surface and into the flow stream overlying bulk liquid. One consequence of this is that cells positioned toward the top of the aggregate have favorable access to the overlying nutrients. Indeed, this point is described in a number of laboratory and computational studies of biofilms. Aggregates also harbor the most antibiotic tolerant subpopulations of biofilm cells. Thus, aggregates may represent the most protective structures present in a surface-associated community. Therefore, the ability of biofilm communities to produce aggregates may be critical for obtaining the maximal fitness benefits of this growth state.

When CdrA was first described, its function within the biofilm was to maintain the structural integrity of aggregates, in part, by promoting Psl localization to the aggregate periphery. In this study, we show a very similar role for LecB. Both CdrA and LecB are tethered to the outer membrane (CdrA through its outer membrane pore CdrB and LecB is thought to occur at OprF) and bind Psl. Otherwise, they are quite distinct from one another, with LecB being a much smaller protein with a clearly defined EPS binding site (no clear EPS binding domain is present in CdrA). In addition, we know that a secreted, extracellular form of CdrA is capable of binding to Psl in the matrix and promoting matrix stability. The simplest interpretation may be that having functionally redundant (or partially redundant) matrix proteins ensures that deleterious mutations targeting either of their genes do not impair the production of aggregates. Redundancy for critical functions is certainly a common theme encountered in *P. aeruginosa* and could partially explain our results.

However, our observations in NB medium indicate that it might not be that simple. In a ΔlecB ΔcdrA double mutant strain, bacteria remain as a monolayer of cells that largely fail to retain Psl. Complementation with lecB restores wild-type biofilm formation, with large aggregates and Psl retained at the aggregate periphery of these aggregates. Curiously, complementation of the double mutant strain with cdrA failed to restore production of wild-type aggregates. Psl was retained in the biofilm, but the...
biofilm was a thick homogenous mat of cells (Fig. 6i, j). This result suggests that under some instances, simple expression of LecB or CdrA is not sufficient to support aggregate production. Why is it unclear? Perhaps, the two proteins differ in their stability under changing environmental conditions. Finally, we cannot rule out that yet unidentified matrix proteins can also contribute to matrix stability in the absence of either LecB or CdrA.

Another point of interest is that two clades of LecB have been proposed, one that groups with PAO1 and another that groups with PA14. Although PA14 cannot synthesize Psl, other members of the PA14 clade can. Whether LecB can serve a similar role in Psl-binding and biofilm structure for other Psl-producing members of the PA14 clade remains to be determined.

We propose the following model to explain our experimental observations. Surface attachment is similar for both strains, resulting in the production of matrix components (EPS and CdrA). At this stage, cells proliferate and produce both CdrA (at low levels) and LecB which leads to the retention of Psl at the base of the biofilm. In the wild-type strain, aggregates continue to grow and biofilm biomass begins to extend beyond the boundary layer and into the overlying bulk fluid. At this stage, lecB mutant strains are swept away by the shear stress resulting from fluid flow due to their inability to stabilize the aggregate through Psl interactions. While we are able to determine the consequences of the lack of LecB for biofilm maturation, our data do not explain why lecB and Psl begin to co-localize at the aggregates periphery. Does CdrA affect LecB expression? Does CdrA affects why LecB and Psl begin to co-localize at the aggregates periphery. Why is it unclear? Perhaps, the two proteins differ in their stability controlled.

In conclusion, our study demonstrates that LecB can serve as a key structural protein in the biofilm matrix. We also demonstrated that Psl and LecB are binding partners and that this interaction impacts biofilm structure. Our findings also have implications for multi-species systems. P. aeruginosa may use LecB to adhere to mannose/fucose containing EPS or glycosylated proteins present in established biofilms of other species. We also predict that the converse may be true: P. aeruginosa biofilms containing lectins may retain planktonic bacteria of other species that are producing target EPS or capsule. Finally, we predict that lectin production within biofilms may allow P. aeruginosa to incorporate free host EPS/oligosaccharides during disease, which might serve as a way of camouflaging the biofilm aggregates from the immune system.

Methods

Bacterial strains, media, and growth conditions. Planktonic cultures were routinely grown on Lysogeny broth (LB) medium at 37 °C with constant shaking (225 rpm) unless indicated otherwise. P. aeruginosa PAO1 strains and Escherichia coli strains used for mutant construction were cultured in LB broth (Fisher Scientific) at 37 °C. For selection and maintenance of plasmids and its derivatives, gentamicin was used at 10 µg/mL for E. coli and 30–100 µg/mL for P. aeruginosa. Growth curves were performed for all the backgrounds used and no growth defect was observed. All strains are listed in Supplementary Table 1.

Plasmids, primers, and genetic techniques. Generation of mutants followed the previously published protocol. Briefly, lecB flanking regions were produced by PCR amplification from PAO1 genomic DNA using set of primers lecB mut UpF (GATCGAGCTCAGCCAGCAAGTGGATGCGAAGTATA) and lecB mut DownF (CACCTCTTGTTGTGCGATGTGG) and DownN (AACAAACCGTGTGATCAACTGCGCGTGGCA) using SOE PCR. lecB fragment was inserted into pDONRPEX18Gm using SacI/XbaI sites to produce the deletion construct. Constructs were then conjugated into PAO1 or PAO1 ΔadrA. All mutants were confirmed by sequencing using the primers UpF and DownR. For the creation of the overexpression vector, lecB was amplified using the primers lecB over Rv (GCACTAGTGAACTCCTAGCGAGCGAC) and lecB over Fw (GAATCTAGTGAACCTCCTAGGCGGAC) using SacI/XbaI sites. Insert was sequenced using the primer pBAD upstream primer.
**LecB purification.** To purify LecB, *P. aeruginosa* harboring pNLecB was grown overnight in LB containing gentamycin and arabinose. After growth, cells were centrifuged at 10,000 × g for 10 min and resuspended in 100 mM CaCl₂, 100 mM NaCl, and pH 8. Bacterial cells were lysed by sonication and centrifuged at 7000 × g for 10 min at 4 °C. The supernatant obtained after centrifugation was loaded onto a mannos e agarose column (Vector Laboratories). Psl was detected in these samples by performing Psl immunoblot as previously described. Antibodies were incubated in the wells of coated plates for 1 h at room temperature. Plates were washed three times using TBS plus 0.05% Tween 20. The coating efficiency of the different crude extracts was confirmed by titration with anti-Psl-specific antisera. Anti-Psl antibodies were incubated in the wells of coated plates for 1 h at room temperature. Plates were washed five times. Binding was detected by incubation with anti-human IgG–Fc-specific, horseshadish peroxidase conjugate. Plates were washed five times and developed with Supersignal West Pico Chemiluminescent Substrate (Thermo Scientific). Absorbance was measured using a FluorchemQ. Readings were measured against a blank of uncoated wells. All assays were carried out in triplicate. For lectin-poly saccharide interactions, a modification of the above mentioned technique was used (FLLA). After coating with crude or purified polysaccharides, plates were washed five times. Binding to coated substrate was assessed by incubating 50 µL of FITC-conjugated lectins at 100 µg/mL for 1 h at 37 °C. Plates were washed five times and fluorescent was excited with 485 nm and emission collected at 515 nm.

**Enzyme-linked immunosorbent assay and lectin assays.** Procedures performed here were adapted from previously published protocol. Polysaccharides were coated onto the wells of ELISA plates (Nunc) by incubation in 250 mM NaCl overnight at 37 °C (50 µl/well). Plates were sealed and incubated in a dark box. Bacterial total sugar extracts were coated at 40 µg/mL. Commercially available bacterial alginate (V-Labs), mucin (Sigma–Aldrich), mannan (Sigma–Aldrich), and purified Psl were coated at 5 mg/mL. After coating, plates were washed five times. All washes were performed with 0.05% (v/v) Tween 20. LecB was detected by incubation with anti-human IgG–Fc-specific, horseshadish peroxidase conjugate. Plates were washed five times and developed with Supersignal West Pico Chemiluminescent Substrate (Thermo Scientific). Absorbance was measured using a FluorchemQ. Readings were measured against a blank of uncoated wells. All assays were carried out in triplicate. For lectin-poly saccharide interactions, a modification of the above mentioned technique was used (FLLA). After coating with crude or purified polysaccharides, plates were washed five times. Binding to coated substrate was assessed by incubating 50 µL of FITC-conjugated lectins at 100 µg/mL for 1 h at 37 °C. Plates were washed five times and fluorescent was excited with 485 nm and emission collected at 515 nm.

**Co-immunoprecipitation of LecB and Psl.** To assay for LecB binding to Psl, Protein G Dynabeads (Life Technologies, Carlsbad, CA) were incubated with 100 µg/mL anti-Psl polyclonal antibodies (Genscript, Piscataway, NJ) following the manufacturer’s instructions, and then incubated with a mixture of purified Psl and LecB or with only purified LecB (Sigma–Aldrich, St. Louis, MO) for 1 h at 37 °C on a low-speed rotator. Beads were washed three times with TBS plus 0.05% Tween and 1 mM CaCl₂ and resuspended in Laemmli buffer (Bio-Rad Laboratories, Hercules, CA). Psl was detected in these samples by performing Psl immunoblot as described previously.

We also performed the Co-IP assay with Protein G Dynabeads conjugated with 100 µg/mL of anti-Psl monoclonal antibodies (MEDI/MUNNE). Reaction followed very similar preparation, where conjugated beads were either incubated with a mixture of purified Psl and LecB or with only purified LecB (Sigma–Aldrich, St. Louis, MO) for 1 h at 37 °C on a low-speed rotator. Beads were washed three times with TBS plus 0.05% Tween and 1 mM CaCl₂ and resuspended in Laemmli buffer (Bio-Rad Laboratories, Hercules, CA). Psl was detected in these samples by performing Psl immunoblot as described previously.

**Antibody-mediated labeling.** Antibody-mediated labeling was performed by incubating 100 µg/mL of anti-Psl polyclonal antibodies (MEDI/MUNNE) on Protein G Dynabeads (Genscript, Piscataway, NJ) following the manufacturer’s instructions and then incubated with a mixture of purified Psl and LecB or with only purified LecB (Sigma–Aldrich, St. Louis, MO) for 1 h at 37 °C on a low-speed rotator. Beads were washed three times with TBS plus 0.05% Tween and 1 mM CaCl₂ and resuspended in Laemmli buffer (Bio-Rad Laboratories, Hercules, CA). Psl was detected in these samples by performing Psl immunoblot as described previously.

The material still adhered to the tube walls was physically removed using an L-shaped loop and this material was resuspended in 500 µL of sterile 1× PBS. This fraction was named adherent and we applied the same treatment described above. To image the tube biofilms by confocal microscopy we performed the same steps of removing the tube, except that we cut the first centimeter of tubing and used the subsequent 2.4 cm of tube for confocal microscopy. The tube was opened longitudinally and carefully placed in the well of a 12-well plate containing 2 mL of sterile media. A Zeiss LSM 800 CLSM was used to image the biofilms and Velocity software (Improvement) was used for image processing. The biomass was stained with 2.5 µM Syto 62 (Molecular Probes) to visualize the entire biomass before imaging. Fluorescent lectins were used to stain the different polysaccharides and were allowed to interact with the biomass for 15 min. WFL lectin (100 µg/mL, Vector Laboratories) was used for Psl visualization, and HHA lectin (100 µg/mL; EY Laboratories) for Psl. LecB–FITC (Eliclty, Crolles, France) staining followed the same procedure used with HHA and WFL. To determine Psl localization, each condition had nine images from three different experiments analyzed using lines profile from Velocity software (Improvement).
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Author contributions
D.P.S. and M.R.P. designed the study. D.P.S. performed molecular biology, flow cell, FLLA, co-immunoprecipitations, ITC, and tube biofilm experiments. M.L.M. and D.O.T. participated in molecular biology and tube biofilm experiments. D.L. designed and executed molecular simulations. D.P.S., C.R., D.J.W., and M.R.P. contributed data interpretation. D.P.S. drafted the manuscript and all authors participated to generate the current version of the manuscript.

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