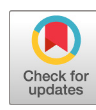
RESEARCH ARTICLE



Interbacterial Antagonism Mediated by a Released Polysaccharide

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ABSTRACT *Pseudomonas aeruginosa* and *Staphylococcus aureus* are two common pathogens causing chronic infections in the lungs of people with cystic fibrosis (CF) and in wounds, suggesting that these two organisms coexist *in vivo*. However, *P. aeruginosa* utilizes various mechanisms to antagonize *S. aureus* when these organisms are grown together *in vitro*. Here, we suggest a novel role for Psl in antagonizing *S. aureus* growth. Psl is an exopolysaccharide that exists in both cell-associated and cell-free forms and is important for biofilm formation in *P. aeruginosa*. When grown in planktonic coculture with a *P. aeruginosa psl* mutant, *S. aureus* had increased survival compared to when it was grown with wild-type *P. aeruginosa*. We found that cell-free Psl was critical for the killing, as purified cell-free Psl was sufficient to kill *S. aureus*. Transmission electron microscopy of *S. aureus* treated with Psl revealed disrupted cell envelopes, suggesting that Psl causes *S. aureus* cell lysis. This was independent of known mechanisms used by *P. aeruginosa* to antagonize *S. aureus*. Cell-free Psl could also promote *S. aureus* killing during growth in *in vivo*-like conditions. We also found that Psl production in *P. aeruginosa* CF clinical isolates positively correlated with the ability to kill *S. aureus*. This could be a result of *P. aeruginosa* coevolution with *S. aureus* in CF lungs. In conclusion, this study defines a novel role for *P. aeruginosa* Psl in killing *S. aureus*, potentially impacting the coexistence of these two opportunistic pathogens *in vivo*.

IMPORTANCE *Pseudomonas aeruginosa* and *Staphylococcus aureus* are two important opportunistic human pathogens commonly coisolated from clinical samples. However, *P. aeruginosa* can utilize various mechanisms to antagonize *S. aureus in vitro*. Here, we investigated the interactions between these two organisms and report a novel role for *P. aeruginosa* exopolysaccharide Psl in killing *S. aureus*. We found that cell-free Psl could kill *S. aureus in vitro*, possibly by inducing cell lysis. This was also observed in conditions reflective of *in vivo* scenarios. In accord with this, Psl production in *P. aeruginosa* clinical isolates positively correlated with their ability to kill *S. aureus*. Together, our data suggest a role for Psl in affecting the coexistence of *P. aeruginosa* and *S. aureus in vivo*.

KEYWORDS *Pseudomonas aeruginosa*, *Staphylococcus aureus*, exopolysaccharide, Psl, polymicrobial, cystic fibrosis, cell lysis, wound

Cystic fibrosis (CF) is a genetic disease that results in an abnormally thick mucus lining in the lung, which causes chronic pulmonary infections and reduced lung function, leading to high morbidity and mortality in this cohort (1, 2). *Pseudomonas aeruginosa* and *Staphylococcus aureus* are two dominant microorganisms colonizing CF airways (3, 4). These organisms are commonly coisolated from CF sputum samples, with one-third of people with CF being coinfected with both pathogens (5). *P. aeruginosa* and

Editor Mohamed Y. El-Naggar, University of Southern California

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The authors declare no conflict of interest.

Received 25 February 2022
Accepted 6 April 2022
Published 21 April 2022

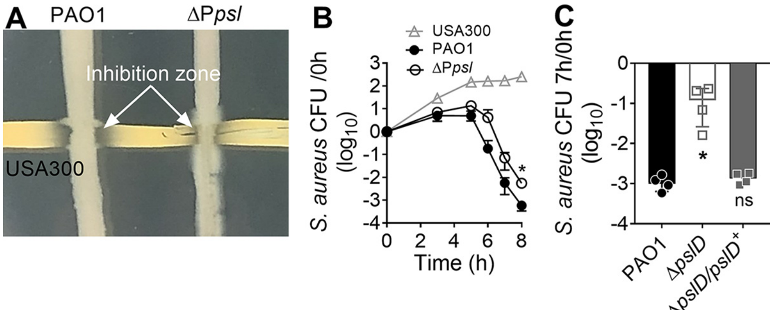


FIG 1 *P. aeruginosa* Psl production antagonizes growth of *S. aureus*. (A) *S. aureus* USA300 was grown in coculture with either *P. aeruginosa* PAO1 or ΔP_{psl} on solidified medium using a cross-streak assay. Arrows point to the inhibition zones of *S. aureus* growth that were observed after overnight incubation. (B) *S. aureus* survival over the course of 8 h when grown alone or cocultured with PAO1 or ΔP_{psl}. Data are means and standard deviations (SD); individual points indicate the means for biological replicates ($N = 5$; $n = 3$). Significance was determined using Student's *t* test. *, $P < 0.05$ compared to PAO1. (C) *S. aureus* survival when cocultured for 7 h with PAO1, Δ*pslD*, or the complemented Δ*pslD/pslD*$^+$ strain. $N = 4$; $n = 3$. Data are means and SD; individual points indicate biological replicates. *S. aureus* survival (B and C) is presented as CFU normalized to the starting CFU at 0 h. Significance was determined using a one-way ANOVA. *, $P < 0.05$ compared to PAO1.

S. aureus are also coisolated from chronic wound environments (6). Coinfection with both pathogens correlates with increased disease severity (7–9).

Although they coexist *in vivo*, *P. aeruginosa* antagonizes *S. aureus* growth *in vitro*. The *P. aeruginosa* quorum-sensing system PQS (*Pseudomonas* quinolone signal) (10), protease LasA (11), iron sequestering pyoverdine (12), and rhamnolipid (13) all inhibit the planktonic growth of *S. aureus in vitro*.

Psl (polysaccharide synthesis locus) is a *P. aeruginosa* exopolysaccharide consisting of a pentasaccharide repeat including D-glucose$_1$, D-mannose$_3$, and L-rhamnose$_1$ (14). There are two forms of Psl, a cell-associated form that is associated with the outer membrane and a cell-free form that is released into the extracellular environment. The molecular determinants for Psl localization are, however, unknown. The amount of Psl produced varies between *P. aeruginosa* strains and clinical isolates (15–17). Psl functions in early biofilm formation and maintaining biofilm structure (18). There have been several reports that Psl or other *P. aeruginosa* polysaccharides may be involved in interactions with different bacterial species, specifically, *Staphylococcus* spp. *Staphylococcus* protein A (SpA) can bind to *P. aeruginosa* clinical isolates via Psl and type IV pili (19). *P. aeruginosa* exopolysaccharides, including Psl and Pel, can disrupt established *Staphylococcus epidermidis* biofilms (20). It has also been reported that Psl does not affect the relative abundance of *P. aeruginosa* and *S. aureus* in a mature dual-species biofilm but does reduce *S. aureus* aggregate formation in the early stages of biofilm formation (21).

Given the above-cited reports that implicate Psl in *P. aeruginosa* and *Staphylococcus* sp. interactions, we decided to investigate the potential roles of Psl in mediating interactions with *S. aureus*. During the course of our study, we found that the cell-free form of Psl could induce *S. aureus* lysis. This is the first study to investigate the role of Psl in killing *S. aureus*.

RESULTS

***P. aeruginosa* Psl production antagonizes the growth of *S. aureus*.** To investigate the impact of Psl on *S. aureus* growth, we first performed a cross-streak assay (22). *S. aureus* strain USA300 was inoculated horizontally on solidified medium, and the *P. aeruginosa* strain was streaked vertically, perpendicularly intersecting the *S. aureus* streak (Fig. 1A). When cocultured with wild-type *P. aeruginosa* PAO1 in this assay, an inhibition zone of *S. aureus* growth was observed in the region neighboring the intersection. However, when cocultured with an isogenic *psl* promoter deletion (ΔP_{psl}) mutant that does not produce Psl, this inhibition zone was reduced, and *S. aureus* was able to

grow almost to the point of intersection with *P. aeruginosa* (Fig. 1A). This suggests that *S. aureus* survival is negatively affected by *P. aeruginosa* Psl production.

We therefore hypothesized that Psl may have a role in antagonizing *S. aureus*. To further investigate this, *S. aureus* was grown in planktonic coculture with either *P. aeruginosa* PAO1 or the ΔP_{psl} mutant (23). *S. aureus* survival was quantified by enumerating CFU. After 5 h, *S. aureus* levels decreased when cocultured with both PAO1 and the ΔP_{psl} mutant, compared to when grown alone. However, when cocultured with the ΔP_{psl} mutant, *S. aureus* had increased survival (10- to 20-fold) compared to coculture with PAO1 (Fig. 1B). Similarly, *S. aureus* had increased survival when cocultured with a *P. aeruginosa* Δ*pslD* mutant, compared to the coculture with PAO1 (Fig. 1C). Introducing the wild-type *pslD* allele in *trans* (Δ*pslD*/*pslD*$^+$) restored the activity of antagonizing *S. aureus* to that of PAO1 in coculture (Fig. 1C). Together, these data indicate that Psl production antagonizes *S. aureus*. The ΔP_{psl} mutant retained some antagonism toward *S. aureus*, likely due to other known killing *P. aeruginosa* mechanisms (10–13).

Cell-free Psl antagonizes the growth of multiple *S. aureus* isolates. *P. aeruginosa* produces both cell-associated and cell-free Psl (24). The mechanism underlying Psl localization are, however, unknown. Prior studies with three classes of monoclonal antibodies directed against Psl suggested that one of the most functionally active anti-Psl antibodies (Cam-003) failed to bind to synthetic Psl oligosaccharides (25, 26). The authors suggested that Cam-003 bound to a Psl isoform that was found in the native polymer but not in the synthetic forms. Furthermore, mild alkaline treatment during carbohydrate purification eliminated Cam-003 binding, suggesting that Psl may contain lipid moieties that were not identified in the reported Psl structure (24, 25). To investigate the potential for lipid modification of Psl, we performed a limited genetic screen of transposon mutants (27) in nonessential genes annotated to be involved in lipid A biogenesis, acyl-transferases, or acyl carrier proteins. The goal was to identify mutants with altered Psl localization, aiming to uncover mechanisms involved in Psl association with the cell. Using an immunoblot assay, we found that wild-type PAO1 had an even distribution of Psl between the two forms, while many of the mutants screened had increased ratios of cell-free Psl to cell-associated Psl (Fig. 2A; also, see Table S2 in the supplemental material). Interestingly, several mutants that impacted Psl distribution were related to lipid A synthesis and modification, warranting future investigations of lipid A in mediating the association of Psl with the cell outer membrane.

To determine if a specific form of Psl was responsible for mediating the antagonism toward *S. aureus*, we used one of the mutants identified from the screen, a *pagP* transposon mutant (*pagP*::Tn PA1343::IS*lacZ/hah*), as it produces predominately cell-free Psl (Fig. 2A; Table S2). PagP is a palmitoyltransferase which transfers palmitate from outer membrane phospholipids to lipid A (28). We grew *S. aureus* in planktonic coculture with either *P. aeruginosa* PAO1 or the ΔP_{psl} or the *pagP*::Tn strain (Fig. 2B). As previously observed (Fig. 1B), coculture with the ΔP_{psl} mutant led to a significant increase in *S. aureus* survival, compared to coculture with PAO1. In contrast, when grown in coculture with the *pagP*::Tn mutant, *S. aureus* survival was significantly reduced (more than 10-fold) compared to that in coculture with PAO1 (Fig. 2B). To determine if this increased antagonism is dependent on Psl, a *pslBCD* deletion was created in the *pagP*::Tn background (*pagP*::TnΔ*psl*). We found that *S. aureus* survival when cocultured with the *pagP*::TnΔ*psl* strain was comparable to that seen with the ΔP_{psl} mutant (Fig. 2B). This suggests that the enhanced antagonism toward *S. aureus* from the *pagP*::Tn strain is dependent on Psl production. We also observed the same trends when other *S. aureus* strains, including LAC and a methicillin-sensitive *S. aureus* (MSSA) strain, were cocultured with either *P. aeruginosa* PAO1, ΔP_{psl}, or *pagP*::Tn (Fig. S1). The above data suggest an important role for cell-free Psl in antagonizing the growth of *S. aureus* during planktonic coculture.

Since cell-free Psl is secreted and appears to be the form that mediates *S. aureus* antagonism, we tested the ability of *P. aeruginosa* spent medium to antagonize *S. aureus*

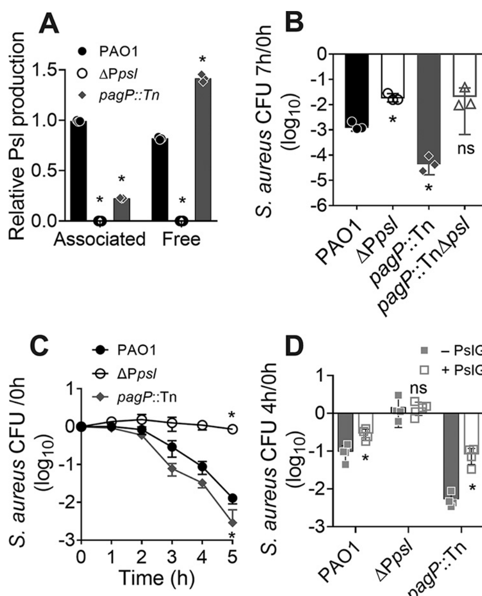


FIG 2 Cell-free Psl derived from *P. aeruginosa* spent medium antagonizes *S. aureus* growth. (A) Cell-associated (associated) and cell-free (free) Psl from *P. aeruginosa* was quantified by immunoblotting. The cell pellets and spent media of centrifuged overnight cultures were used as the source of cell-associated and cell-free Psl, respectively. Psl was quantified by densitometry of the blot and normalized to PAO1 cell-associated Psl. Data are means and SD; individual points indicate biological replicates (*N* = 3; *n* = 3). Significance was determined with one-way ANOVA. *, $P < 0.05$ compared to PAO1. (B) *S. aureus* survival when cocultured for 7 h with *P. aeruginosa* PAO1, ΔP_{psl}, *pagP*::Tn, or *pagP*::TnΔ*psl* strains. *S. aureus* survival is presented as CFU normalized to the starting CFU at 0 h. Data are means and SD; individual points indicate biological replicates (*N* = 3; *n* = 3). Significance was determined using Student's *t* test compared to PAO1. *, $P < 0.05$; ns, not significant. (C) *S. aureus* survival when grown for 5 h in spent medium of PAO1 or the ΔP_{psl} or *pagP*::Tn strain that was diluted 1:1 in fresh medium. Data are means and SD; individual points indicate means for biological replicates (*N* = 4; *n* = 3). Significance was determined using Student's *t* test. *, $P < 0.05$ compared to PAO1. (D) *S. aureus* was grown for 4 h in spent medium from PAO1, ΔP_{psl}, or *pagP*::Tn strains that had been pretreated with (+) or without (−) PslG. Data are means and SD; individual points indicate biological replicates (*N* = 4; *n* = 3). *S. aureus* survival (B and C) is presented as CFU normalized to the starting CFU at 0 h. Significance was determined using Student's *t* test, compared to the no-PslG pretreated group. *, $P < 0.05$; ns, not significant.

growth. Similar to planktonic coculture (Fig. 2B), *S. aureus* had reduced survival when grown in spent medium from PAO1 and the *pagP*::Tn strain, compared to growth in ΔP_{psl} spent medium, the latter of which did not show any changes in *S. aureus* survival when quantified by both CFU (Fig. 2C) and optical density at 600 nm (OD_{600}) (Fig. S2). Furthermore, *S. aureus* survival was significantly reduced when grown in *pagP*::Tn spent medium, compared to PAO1 spent medium (Fig. 2C; Fig. S2).

PslG is a hydrolase that cleaves the polymeric chain of Psl into monomers (29). To further support the role of cell-free Psl in antagonizing *S. aureus* growth, *P. aeruginosa* spent medium was pretreated with PslG to degrade Psl (Fig. S3). *S. aureus* was supplemented with PslG treated spent media from PAO1 or the ΔP_{psl} or *pagP*::Tn strain and grown for a further 4 h. *S. aureus* survival significantly increased by 3- and 15-fold when grown with PAO1 and *pagP*::Tn PslG-treated spent medium, respectively, compared to spent medium without PslG (Fig. 2D). As expected, PslG treatment of ΔP_{psl} spent medium resulted in no change to *S. aureus* survival, indicating that PslG does not affect *S. aureus* growth (Fig. 2D).

Psl-mediated antagonism toward *S. aureus* is independent of other *P. aeruginosa* factors that impact *S. aureus* growth. *P. aeruginosa* utilizes various mechanisms to antagonize the growth of *S. aureus*, including PQS (10), pyoverdine (12), LasA (11), and rhamnolipid (13). Alginate, another exopolysaccharide produced by *P. aeruginosa*, has been found to promote *P. aeruginosa* and *S. aureus* coexistence by downregulating some of the above mechanisms in *P. aeruginosa* (23, 30). We therefore wanted to examine if Psl-mediated antagonism toward *S. aureus* was dependent on any of these

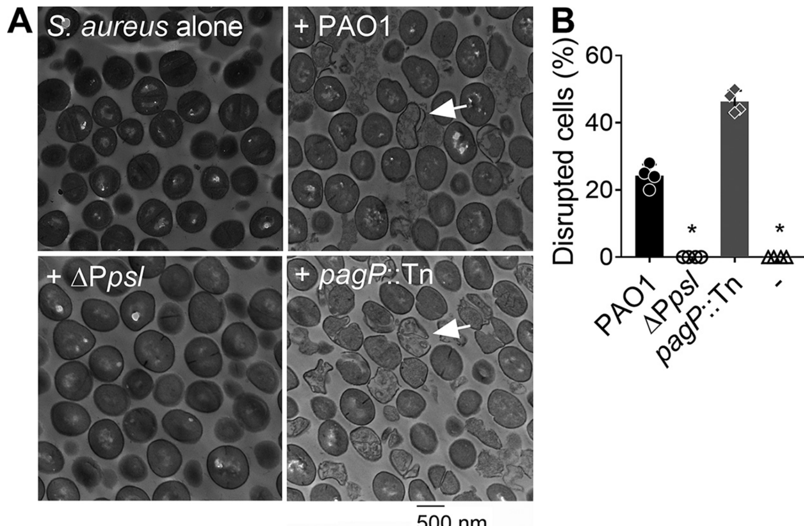


FIG 3 Cell-free Psl kills *S. aureus* by disrupting the cell envelope. (A) *S. aureus* USA300 was incubated with spent medium from *P. aeruginosa* PAO1, ΔP_{psl}, or *pagP*::Tn for 2 h. Changes in cell morphology were visualized by TEM. Arrows indicate cells with disrupted cell envelopes. (B) Total and disrupted cell counts for each group were enumerated, and the percentage of disrupted cells within each group was calculated. Data are means and SD; individual points indicate biological replicates ($N = 4$; $n = 3$). Significance was determined using a one-way ANOVA compared to PAO1. *, $P < 0.05$.

mechanisms. There were no significant differences found in rhamnolipid and LasA activity in PAO1 and the ΔP_{psl} mutant (Fig. S4A to C). A deficiency in PQS or pyoverdine did not affect the ability of *P. aeruginosa* cell-free Psl to antagonize *S. aureus* growth during coculture (Fig. S4D). As controls, corresponding mutants for each mechanism showed expected loss of these antagonistic factors (see the supplemental methods and Fig. S4A to D). Collectively, these data indicate that cell-free Psl antagonizes the growth of *S. aureus* independently of rhamnolipid, LasA, PQS, and pyoverdine.

Cell-free Psl kills *S. aureus* by disrupting the cell envelope. Exopolysaccharides isolated from bacteria and fungi can disrupt bacterial cell envelopes (31–33). We therefore hypothesized that Psl similarly caused *S. aureus* cell lysis. To test this, *S. aureus* was incubated with diluted spent medium from *P. aeruginosa* PAO1, ΔP_{psl}, or *pagP*::Tn for 2 h and processed for transmission electron microscopy (TEM). This time point was selected as *S. aureus* was still viable after 2 h of incubation with *P. aeruginosa* spent medium (Fig. 2C). TEM imaging revealed that *S. aureus* incubated with either PAO1 or *pagP*::Tn spent medium had disrupted cell membranes, while *S. aureus* incubated with ΔP_{psl} spent medium showed little evidence of cellular damage, similar to the no-treatment control (Fig. 3A; Fig. S5). Image analysis, which quantified the percentage of cells with membrane damage relative to the untreated control, revealed that approximately 25% of *S. aureus* cells incubated with PAO1 spent medium had disrupted cell envelopes (Fig. 3B). However, when incubated with spent medium from the *pagP*::Tn strain, the number of *S. aureus* cells with membrane damage increased to approximately 50% (Fig. 3B). This is consistent with our previous results which showed decreases in *S. aureus* cell numbers (Fig. 2C) and OD_{600} (Fig. S2) when the organism was grown in medium supplemented with *P. aeruginosa* spent medium, suggestive of cell lysis. The data indicate that cell-free Psl likely kills *S. aureus* by disrupting the cell envelope.

Psl promotes *S. aureus* killing in conditions that mimic *in vivo* growth. The above-described experiments were performed using planktonic cultures grown in a rich medium. We wanted to further determine if cell-free Psl could mediate *S. aureus* killing under *in vivo*-like conditions. Biofilm formation is one of the key factors contributing to *P. aeruginosa* and *S. aureus* chronic infections (34, 35). As such, we next examined if

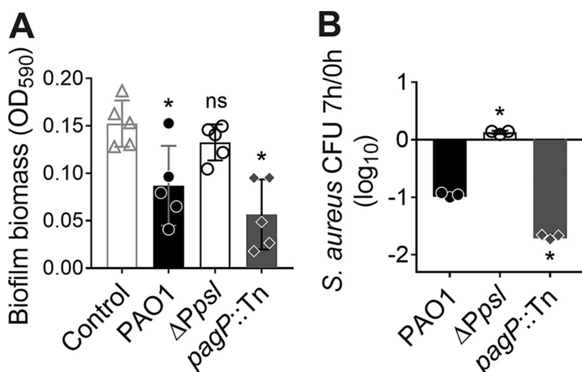
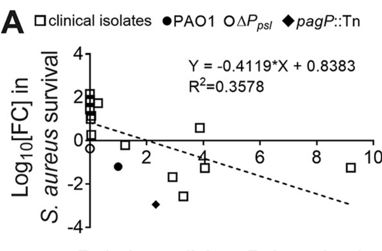


FIG 4 Cell-free Psl promotes *S. aureus* killing under *in vivo*-like conditions. (A) Twenty-four-hour *S. aureus* USA300 biofilms were incubated with or without *P. aeruginosa* PAO1, ΔP_{psl}, or *pagP*::Tn spent medium for 5 h. Biofilm biomass was quantified by crystal violet staining. Data are means and SD; individual points indicate biological replicates ($N = 5$; $n = 3$). Significance was determined using one-way ANOVA. *, $P < 0.05$, and ns, not significant, compared to the medium-only control. (B) *S. aureus* survival when cocultured with *P. aeruginosa* PAO1, ΔP_{psl}, or *pagP*::Tn in SCFM2 for 7 h. Data are means and SD; individual points indicate biological replicates ($N = 3$; $n = 3$). Significance was determined using Student's *t* test. *, $P < 0.05$ compared to PAO1.

cell-free Psl could kill *S. aureus* grown in biofilms (Fig. 4A). Pre-established *S. aureus* biofilms were incubated for 5 h with spent medium from *P. aeruginosa*. Biofilm biomass was then quantified by crystal violet staining (36). Compared to the control group with no *P. aeruginosa* spent medium added, *S. aureus* biofilm biomass was significantly decreased when treated with both PAO1 and *pagP*::Tn spent media. No significant difference was found between the control and ΔP_{psl} spent media. This suggests that cell-free Psl also promotes killing of *S. aureus* in biofilms.

Synthetic CF sputum medium (SCFM2) mimics the CF sputum composition and has been used to culture both *P. aeruginosa* and *S. aureus* (23, 37). We quantified *S. aureus* survival when cocultured with *P. aeruginosa* PAO1, ΔP_{psl}, or *pagP*::Tn in SCFM2 for 8 h (Fig. 4B). Like our previous results in rich media (Fig. 2B), *S. aureus* survival significantly increased by 10-fold when cocultured with the ΔP_{psl} mutant, compared to coculture with PAO1 (Fig. 4B). Furthermore, *S. aureus* survival significantly decreased by 5-fold in coculture with the *pagP*::Tn mutant compared to coculture with PAO1 (Fig. 4B). This suggests that cell-free Psl may contribute to *S. aureus* killing in nutritional environments like a CF lung. Together, these data suggest that Psl-mediated killing of *S. aureus* occurs in conditions that are reflective of *in vivo* scenarios.

Increased cell-free Psl production in *P. aeruginosa* clinical isolates promotes *S. aureus* killing. Some *P. aeruginosa* clinical isolates lack or have reduced Psl production (15–17). Given that cell-free Psl appears to kill *S. aureus*, it is possible that reduced Psl production in *P. aeruginosa* occurs during coevolution with *S. aureus*, facilitating polymicrobial infections. To test if there is a correlation between Psl production and *S. aureus* killing among *P. aeruginosa* clinical isolates, 16 *P. aeruginosa* isolates derived from CF sputum samples were collected from the Cure CF Columbus Translational Core at Nationwide Children's Hospital (Fig. 5). Since mucoid *P. aeruginosa* can coexist with *S. aureus*, due to the overproduction of alginate (23), only nonmucoid *P. aeruginosa* isolates were evaluated. Psl production of each nonmucoid *P. aeruginosa* isolate was quantified by immunoblot assay. *S. aureus* USA300 was grown in planktonic coculture with each CF isolate, as well as PAO1 or the ΔP_{psl} or *pagP*::Tn mutant, and *S. aureus* survival was quantified. A correlation ($R^2 = 0.3578$) was found between the cell-free Psl production of the *P. aeruginosa* strains and the cocultured *S. aureus* survival (Fig. 5A). Furthermore, 6 of the 16 *P. aeruginosa* CF isolates produced no Psl, and each had reduced *S. aureus* killing activity compared to PAO1 (Fig. 5B). This suggests that low Psl production in *P. aeruginosa* clinical isolates can result in enhanced survival when in



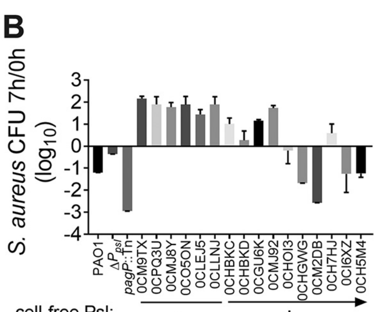


FIG 5 Psl production in *P. aeruginosa* clinical isolates positively correlates with the ability to kill *S. aureus*. *S. aureus* USA300 was cocultured with each of the 16 *P. aeruginosa* CF isolates, PAO1, and the ΔP_{psl} and *pagP*::Tn strains. The number of CFU at 7 h was divided by that at 0 h to quantify survival. (A) Linear regression analysis was performed to determine any correlation between the two parameters indicated. Cell-free Psl production by the designated strains was measured by immunoblot assay using Psl antibody and then normalized to PAO1. Individual points indicate biological replicates (N = 3; n = 3). (B) *P. aeruginosa* clinical isolates with low Psl production showed reduced *S. aureus* killing activity. Six of the CF isolates produced no cell-free Psl (−), while the other 10 produced variable amounts of cell-free Psl (+; the arrow indicates the increasing production of Psl), compared with that produced by PAO1. The number of CFU of *S. aureus* USA300 cocultured with each of the isolates, PAO1, and the ΔP_{psl} and *pagP*::Tn strains for 7 h was divided by that at 0 h to quantify *S. aureus* survival. Data are means and SD (N = 3; n = 3).

coculture with *S. aureus* and that Psl production may be one factor involved in their coexistence during infections.

Purified cell-free Psl is sufficient to kill *S. aureus*. Exopolysaccharides produced by some bacteria and fungi have antimicrobial activities (31–33). Our results demonstrate that cell-free Psl mediates the killing of *S. aureus*. However, this was determined from planktonic coculture of *P. aeruginosa* and *S. aureus* (Fig. 1 and 2A and B) or growth of *S. aureus* in *P. aeruginosa* spent medium (Fig. 2C and D), both conditions which could contain Psl-independent antagonists of *S. aureus* growth. We therefore tested if cell-free Psl purified from PAO1 and the *pagP*::Tn mutant could directly inhibit *S. aureus* growth. *S. aureus* survival was quantified by measuring the OD_{600} after 16 h of growth in medium supplemented with increasing concentrations of purified cell-free Psl (0 to 100 μg/mL). *S. aureus* grown alone, without the addition of cell-free Psl (untreated), was used as the control. *S. aureus* survival decreased as the concentration of purified Psl increased (Fig. 6A). The ΔP_{psl} strain also underwent the identical purification process, and the resulted extraction had no effect on *S. aureus* survival. In addition, we found that purified Psl originating from Δ*pqsA* and Δ*pvdA* mutants showed activity similar to that of Psl from the parent strains in killing *S. aureus* (Fig. S4E). This demonstrates that purified cell-free Psl, in a concentration-dependent manner, is sufficient to mediate the killing of *S. aureus*.

Cell-free Psl consists of D-mannose, D-glucose, and L-rhamnose, at a ratio of 3:1:1, respectively (24). The influence of these individual monosaccharides on *S. aureus* survival was also evaluated. There was no change in survival of *S. aureus* grown in medium supplemented with either each monosaccharide separately or a combination (Fig. 6B). This indicates that an integral structure of Psl is critical for the *S. aureus* killing property.

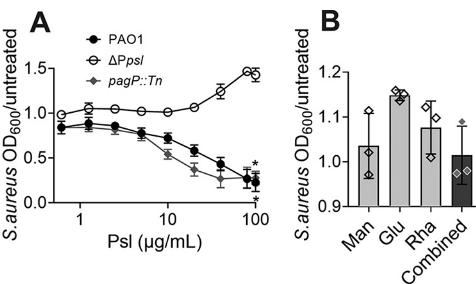


FIG 6 Purified cell-free Psl is sufficient to kill *S. aureus*. Survival of *S. aureus* grown in media supplemented with (A) increasing concentrations of purified cell-free Psl from PAO1 or the *pagP*::Tn mutant or cell-free products from the ΔP_{psl} mutant or (B) 60 μg/mL of D-mannose (Man), 20 μg/mL of D-glucose (Glu), 20 μg/mL of L-rhamnose (Rha), or a combination of the three monosaccharides (Combined). *S. aureus* grown without cell-free Psl or monosaccharides (untreated) was used as a control. OD_{600} was measured after 16 h. *S. aureus* survival is presented as OD_{600} normalized to that of the control. (A) The *x* axis is a log scale. Data are means and SD; individual points indicate the mean of biological replicates ($N = 3$; $n = 3$). Significance was determined using Student's *t* test compared to the ΔP_{psl} mutant. *, $P < 0.05$.

DISCUSSION

P. aeruginosa and *S. aureus* often cause chronic coinfections that can lead to more severe disease outcomes (7–9). Understanding the dynamic between the two pathogens is key to successfully treating and eliminating such coinfections. Here, we demonstrate a novel role for *P. aeruginosa* exopolysaccharide Psl in killing *S. aureus in vitro* (Fig. 1). Specifically, we found that purified cell-free Psl mediated *S. aureus* killing (Fig. 6), likely by disrupting the cell envelope and causing cell lysis (Fig. 3). This antagonistic relationship would likely impact the coexistence of these two opportunistic pathogens *in vivo*.

Furthermore, we demonstrate that cell-free Psl can kill *S. aureus* in mature biofilms (Fig. 4A), suggesting that the antimicrobial properties of Psl are not limited to highly metabolically active, planktonic *S. aureus*. In support of this, *P. aeruginosa* extracellular Psl from spent medium can disrupt established *S. epidermidis* biofilms. Interestingly, this was independent of any bactericidal effect, as spent media of wild-type *P. aeruginosa* and a *psl* mutant were able to comparably inhibit the growth of planktonic *S. epidermidis* (20). This suggests that cell-free Psl may have different killing mechanisms for *S. aureus* and *S. epidermidis*. It is also possible that Psl-mediated killing of planktonic *S. aureus* and disruption of biofilms occur through distinct mechanisms. Conversely, *Staphylococcus* protein A (SpA) secreted by *S. aureus* can inhibit *P. aeruginosa* biofilm formation, and this can be prevented by Psl binding to SpA (19). However, we found that a *spa* transposon mutant (*spa*::Tn) was as susceptible as wild-type *S. aureus* to killing by PAO1 and *pagP*::Tn when grown in planktonic coculture (Fig. S6). This suggests that SpA does not play a role in the cell-free Psl-mediated killing of *S. aureus* described here.

In addition to Psl, *P. aeruginosa* produces two other exopolysaccharides, alginate and Pel (38, 39). In contrast to the antimicrobial properties of Psl toward *S. aureus* observed here, alginate mediates *P. aeruginosa* and *S. aureus* coexistence (23, 30). Alginate overproduction, resulting in a mucoid phenotype, downregulates known antagonistic mechanisms in *P. aeruginosa*, including rhamnolipid, pyoverdine, and HQNO (2-heptyl-4-hydroxyquinoline *N*-oxide) (23). Interestingly, alginate and Psl share the same precursor, mannose-1-phosphate (40), leading to reduced Psl production when alginate is overproduced (41, 42). This is in accord with our finding that Psl plays the opposite role of alginate, by promoting *S. aureus* killing. This study focused exclusively on *S. aureus* antagonism mediated by nonmucoid *P. aeruginosa*. Prior studies showed that some nonmucoid clinical isolates harbor secondary mutations in *algTU* (43, 44), and we did not determine if isolates used in this study indeed harbored these mutations. Jones et al. (42), on the other hand, found that functional Psl, albeit at reduced levels, is still produced in some mucoid *P. aeruginosa* isolates, warranting further investigation of *S. aureus* killing by *P. aeruginosa* producing both

Psl and alginate. Pel also contributes to *P. aeruginosa* and *S. aureus* polymicrobial interactions. It can reduce cross-linking within the biofilm matrix, enabling the expansion of *P. aeruginosa* in a dual-species biofilm with *S. aureus* (21). Secreted Pel can also remove established *S. epidermidis* biofilms (20). However, our data suggest that Pel is not directly involved in killing *S. aureus* (Fig. S7).

Our data also suggest that the structure of Psl may affect its antimicrobial activity. PslG-treated PAO1 and *pagP*::Tn spent media had no detectable Psl, similar to the ΔP_{psl} mutant (Fig. S3). However, while they both showed reduced *S. aureus* killing compared to untreated spent medium (Fig. 2D), *S. aureus* survival was still significantly reduced compared to that with ΔP_{psl} spent medium (Fig. 2D). This suggests that PslG-treated PAO1 and *pagP*::Tn spent media demonstrate intermediate *S. aureus* antimicrobial properties. It is possible that a higher-molecular-weight (MW) structure is needed for the antimicrobial property of cell-free Psl. One interpretation of these data is that PslG may partially disrupt Psl polymers such that it cannot be recognized by Psl antibodies, but these forms can have reduced, although some, *S. aureus* killing activity. Consistent with this, Maalej et al. found that a high-molecular-weight polysaccharide produced by *Pseudomonas stutzeri* showed antimicrobial activity toward *S. aureus* and other bacteria (31). This polysaccharide consists of a trisaccharide backbone chain containing glucose, mannose, and lactyl rhamnose, similar to *P. aeruginosa* Psl. It is likely that these two polysaccharides, both isolated from *Pseudomonas* species, share similar properties in killing *S. aureus*. Maalej et al. also compared antimicrobial abilities of the *P. stutzeri* polysaccharide in different MWs and found that higher-MW polysaccharides had increased Gram-positive bacterium-killing activity. In addition, we found that the monosaccharides making up cell-free Psl showed no killing of *S. aureus* (Fig. 6B). Together, these observations suggest that the structure of cell-free Psl may contribute to its antimicrobial activity.

From our TEM analysis, we propose that cell-free Psl damages *S. aureus* cell envelopes, causing cell lysis (Fig. 5). Our observations contribute to the growing evidence that exopolysaccharides produced by bacteria and fungi can have antimicrobial properties by disrupting cell envelopes (31–33). A polysaccharide isolated from *Streptomyces virginiae*, consisting of mannose, glucose, and galactose, also inhibits *S. aureus* growth (33). Interestingly, this polysaccharide has a sugar composition similar to that of Psl and a bactericidal concentration comparable to what we determined for cell-free Psl (Fig. 6). As mentioned above, the polysaccharide produced by *P. stutzeri* is antimicrobial toward *S. aureus* (31). It was speculated that the amphiphilicity of the polymer may provide structural affinity between the *S. aureus* cell wall and polysaccharide, presumably leading to lysis. Metal chelation was suggested as another potential mechanism of *P. stutzeri* polysaccharide permeabilization of the *S. aureus* cell envelope (31). This might also apply to the Psl-mediated killing of *S. aureus*, as Psl can sequester and store iron (45). Despite determining that cell-free Psl causes *S. aureus* cell lysis, we have yet to elucidate the mechanism. However, we observed no difference in PAO1 and ΔP_{psl} spent media in lysing heat-killed *S. aureus* (Fig. S4C). This suggests that killing requires active *S. aureus* growth/metabolism. Future investigations will focus on the mechanism(s) involved in Psl-mediated killing.

It is also possible that Psl can antagonize *S. aureus* by other mechanisms, in addition to cell lysis. The loss of cell-associated Psl leads to reduced aggregation (46). Cell aggregation promotes *P. aeruginosa* pyoverdine (47) and quorum sensing signal production, including PQS (48), both of which are involved in killing *S. aureus*. In addition, purified cell-associated Psl can act as a signal to stimulate cyclic di-GMP production in *P. aeruginosa* (49). It is therefore possible that Psl could affect the production of known *S. aureus*-killing factors by promoting cell aggregation and/or acting as a signal molecule in *P. aeruginosa*. However, this is not supported by our finding that purified Psl is sufficient to promote *S. aureus* killing and that *pqs* and *pvdA* mutants still showed Psl-dependent killing.

Psl is important for *P. aeruginosa* biofilm formation and contributes to chronic infections (18). However, several *P. aeruginosa* clinical isolates produce little or no Psl (15–17). Consistent with this, we found that 6 of the 16 examined CF isolates produced

no Psl. A positive correlation was observed between Psl production and *S. aureus* killing activity among the isolates (Fig. 5), suggesting a role for Psl in affecting the coexistence of the two pathogens in CF lungs. Coexistence with *S. aureus* can be beneficial to *P. aeruginosa*. For example, *S. aureus* is predominant in early dual-species biofilms and can promote *P. aeruginosa* attachment and biofilm formation (50). We speculate that *P. aeruginosa* may undergo adaptation to reduce its antagonistic weapons, like Psl, to better coexist with *S. aureus in vivo*.

Taken together, our findings demonstrate that cell-free Psl produced by *P. aeruginosa* can disrupt the *S. aureus* cell envelope, leading to *S. aureus* cell death. Psl-mediated killing also occurs under *in vivo*-like conditions, including in SCFM2 and *S. aureus* biofilms. Consistent with this, the amount of Psl produced in *P. aeruginosa* clinical isolates positively correlates with their antistaphylococcal activity. This study demonstrates a novel role for exopolysaccharides in polymicrobial infections and highlights the need for future investigations in polymicrobial coevolution during chronic infections.

MATERIALS AND METHODS

Bacterial strains, primers, and growth conditions. All bacterial strains and plasmids are listed in Table 1. All primers used in this study are listed in Table S1. Gene deletion constructs were incorporated into the *P. aeruginosa* genome using homologous recombination as previously described (51). *S. aureus* was grown in lysogeny broth (LB; 10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl). *P. aeruginosa* was grown in LB with no salt (LBNS). Overnight cultures were grown at 37°C with 200 rpm shaking for 16 h. Antibiotic concentrations were 10 μg/mL gentamicin and 100 μg/mL ampicillin for selection of *Escherichia coli* and 300 μg/mL carbenicillin for *P. aeruginosa*.

***P. aeruginosa* and *S. aureus* coculture.** Cross-streak and planktonic cocultures were performed as previously described with modifications (23). For the cross-streak coculture, overnight cultures of *P. aeruginosa* strains and *S. aureus* USA300 were normalized to an OD_{600} of 1.5. A loopful of *S. aureus* culture was streaked horizontally on lysogeny agar with no salt (LANS) followed by a vertical streak of *P. aeruginosa*. The cross-streaked plate was incubated overnight at 37°C. For the planktonic coculture, overnight cultures of *P. aeruginosa* and *S. aureus* were diluted to an OD_{600} of 0.05 and combined at a ratio of 1:1 in 2 mL of either fresh LBNS or SCFM2 (52). The coculture was incubated at 37°C with shaking at 200 rpm for up to 8 h. Aliquots were taken at 0, 3, 5, 6, 7, and 8 h, serially diluted to plate on Difco *Pseudomonas* isolation agar (PIA) and BBL mannitol salt agar (MSA) to enumerate CFU of *P. aeruginosa* and *S. aureus*, respectively. *S. aureus* survival at each time point was normalized to the number of CFU at 0 h.

Crude extraction of cell-free and cell-associated exopolysaccharide. *P. aeruginosa* strains were grown overnight at 37°C and 200 rpm in 5 mL of LBNS and normalized to an OD_{600} of 2. Crude exopolysaccharide was isolated using a modified protocol (53). Briefly, the bacteria were centrifuged at 8,000 $\times$ *g* for 10 min at room temperature to harvest the spent medium and pellet. For cell-free exopolysaccharide, the spent medium after centrifugation was removed and passed through a 0.22-μm filter to remove any residual bacteria. For cell-associated exopolysaccharide, the bacterial pellet was suspended in 2 mL of 1.5 M NaCl, vortexed vigorously, and placed on a platform rocker at room temperature for 15 min. After extraction, the sample was centrifuged at 5,000 $\times$ *g* for 10 min at room temperature. The supernatant was removed and passed through a 0.22-μm filter to remove any residual bacteria.

Psl immunoblot assay. Two microliters of the crude exopolysaccharide extracts and Psl standards (500 μg/mL, 250 μg/mL, 100 μg/mL, 50 μg/mL, and 10 μg/mL) were spotted onto a nitrocellulose membrane (0.2 μm; Bio-Rad) and incubated at room temperature with 5% skim milk for 1 h, Psl antibody (AstraZeneca; 1:3,000 dilution) for 1 h, and secondary antibody (goat anti-human IgG; 1:5,000 dilution; Abcam) for 1 h sequentially. Western blotting detection reagent (ECL) was added to the membrane for visualization via the ChemiDoc imaging system (Bio-Rad). Densitometry analysis was performed with ImageJ software, and results were compared to a standard curve of pure Psl.

***S. aureus* killing assay using *P. aeruginosa* spent medium.** Overnight cultures of *P. aeruginosa* and *S. aureus* were diluted to an OD_{600} of 0.05 and incubated at 37°C with shaking at 200 rpm for 7 h and 4 h, respectively. Psl-mediated antagonism was observed when *S. aureus* was grown in coculture with *P. aeruginosa* for 7 h, so we selected this time point to harvest *P. aeruginosa* spent medium. A *P. aeruginosa* culture was filter sterilized using a 0.22-μm filter (Fisherbrand) and diluted 1:1 in fresh LBNS. Four milliliters of stationary-phase *S. aureus* (OD_{600} = 1.5) was centrifuged, resuspended in 4 mL of diluted *P. aeruginosa* spent medium, and incubated at 37°C with shaking at 200 rpm for up to 4 h. Aliquots were taken every hour, and either absorbance (OD_{600}) was measured using a spectrophotometer (Thermo Fisher), or the sample was serially diluted and CFUs were enumerated. For experiments with PslG-treated spent media, filter-sterilized *P. aeruginosa* spent medium was treated with 100 μM PslG for 1 h at 37°C to degrade cell-free Psl, prior to the addition of *S. aureus*. *S. aureus* survival at each time point was normalized to the CFU or OD_{600} at 0 h.

Cell-free Psl purification. Psl was isolated by following an established protocol (24, 49) with modifications. Briefly, concentrated spent medium of *P. aeruginosa* was subjected to 3 rounds of ethanol precipitation, treatment with 0.1 mg/mL of DNase I, RNase A, and proteinase K, and 3 rounds of dialysis (Slide-A-Lyzer dialysis cassette; molecular weight cutoff [MWCO] of 3,500). Concentration and carbohydrate content of the resulting purified cell-free Psl were determined by immunoblotting and phenol-sulfuric acid assay (54), respectively.

TABLE 1 Strains and plasmids

Strain or plasmid	Description	Source or reference
E. coli		
NEB5α		New England Biolabs
S17		
P. aeruginosa		
PAO1	WT *P. aeruginosa*	55
ΔP_{psl} mutant	*psl* production deficient; *psl* operon promoter deletion mutant	55
Δ*pslD*/V mutant	*psl* production deficient; Δ*pslD* with an empty vector pUCP18	24
Δ*pslD*/*pslD*$^+$ mutant	pUCP18::*pslD* was used to complement Δ*pslD*	24
PAO1/V	WT *P. aeruginosa* with an empty vector pUCP18	24
pagP::Tn mutant	*pagP* transposon mutant (UWGC:PW3442, PA1343::IS*lacZ*/hah)	27
htrB1::Tn mutant	*htrB1* transposon mutant (UWGC:PW1009, PA0011::IS*lacZ*/hah)	27
htrB2::Tn mutant	*htrB2* transposon mutant (UWGC:PW6435, PA3242::IS*lacZ*/hah)	27
lpxO1::Tn mutant	*lpxO1* transposon mutant (UWGC:PW8596, PA4512::IS*phoA*/hah)	27
lpxO2::Tn mutant	*lpxO2* transposon mutant (UWGC:PW2702, PA0936::IS*phoA*/hah)	27
pagL::Tn mutant	*pagL* transposon mutant (UWGC:PW8859, PA4661::IS*phoA*/hah)	27
phoP::Tn mutant	*phoP* transposon mutant (UWGC:PW3128, PA1179::IS*lacZ*/hah)	27
phoQ::Tn mutant	*phoQ* transposon mutant (UWGC:PW3131, PA1180::IS*lacZ*/hah)	27
pmrB::Tn mutant	*pmrB* transposon mutant (UWGC:PW9023, PA4777::IS*lacZ*/hah)	27
pagP::TnΔ*psl* mutant	*pagP* transposon mutant with *pslBCD* deletion	This study
Δ*pvdA* mutant	*pvdA* deletion mutant	This study
Δ*pqsA* mutant	*pqsA* deletion mutant	This study
Δ*pvdA* ΔP_{psl} mutant	*psl* promoter and *pvdA* double-deletion strain	This study
Δ*pqsA* ΔP_{psl} mutant	*psl* promoter and *pqsA* double-deletion strain	This study
rhlA::Tn mutant	*rhlA* transposon mutant (UWGC:PW6886, PA3479::IS*phoA*/hah)	27
lasA::Tn mutant	*lasA* transposon mutant (UWGC:PW4282, PA1871::IS*lacZ*/hah)	27
Δ*pelA* mutant	*pelA* deletion mutant	56
0CM2DB	CF clinical isolate	This study
0CMJ92	CF clinical isolate	This study
0CPQ3U	CF clinical isolate	This study
0CO5ON	CF clinical isolate	This study
0CMJ8Y	CF clinical isolate	This study
0CM9TX	CF clinical isolate	This study
0CHBKC	CF clinical isolate	This study
0CHBKD	CF clinical isolate	This study
0CH5M4	CF clinical isolate	This study
0CLLNJ	CF clinical isolate, coisolated with *S. aureus*	This study
0CLEJ5	CF clinical isolate, coisolated with *S. aureus*	This study
0CH7HJ	CF clinical isolate, coisolated with *S. aureus*	This study
0CHGWG	CF clinical isolate, coisolated with *S. aureus*	This study
0CGU6K	CF clinical isolate, coisolated with *S. aureus*	This study
0CI6XZ	CF clinical isolate, coisolated with *S. aureus*	This study
0CHOI3	CF clinical isolate, coisolated with *S. aureus*	This study
S. aureus		
USA300	WT	57
MSSA	ATCC 29213, methicillin sensitive	ATCC
LAC	MRSA	58
spa::Tn mutant	*spa* transposon mutant (NE286, NARSA)	57
Plasmids		
pEX18Gm	For allelic exchange in *P. aeruginosa*	
pΔ*pqsA*	For *pqsA* deletion	This study
pΔ*pvdA*	For *pvdA* deletion	This study
pMpsl-KO1	For *pslBCD* deletion	59

***S. aureus* killing assay by purified Psl.** Overnight *S. aureus* culture was diluted to an OD_{600} of 0.05 and incubated at 37°C with shaking at 200 rpm for 4 h to reach an OD_{600} of 1.5. The culture was resuspended in fresh LBNS containing increasing concentrations (0, 2.5, 5, 10, 20, 40, 80, and 100 μg/mL) of purified cell-free Psl, and 200 μL was added into each well of a 96-well plate. To test if the monosaccharides of Psl contributed to *S. aureus* killing, 20 μg/mL L-rhamnose, 20 μg/mL D-glucose, 60 μg/mL D-mannose, or a combination of the three monosaccharides was added to *S. aureus*. Plates were incubated in a

plate reader (SpectraMax i3x; Molecular Devices) at 37°C for 16 h. OD_{600} was measured every 30 min as an indication of survival and compared to that of the control (*S. aureus* grown alone). Relative *S. aureus* survival was measured by OD_{600} normalized to the control.

TEM imaging. *S. aureus* cultures were treated with *P. aeruginosa* spent medium for 2 h, centrifuged, and washed with phosphate-buffered saline (PBS). Samples were fixed in 2.5% glutaraldehyde overnight and then embedded in 2% agarose for TEM processing. Samples were postfixed with 1% osmium tetroxide, stained *en bloc* with 1% aqueous uranyl acetate, dehydrated in a graded series of ethanol, and embedded in Eponate 12 epoxy resin (Ted Pella Inc.; 18012). Ultrathin sections were cut with a Leica EM UC6 ultramicrotome (Leica Microsystems; EM FC7) and collected on copper grids. Images were acquired with an FEI Technai G2 Spirit transmission electron microscope (FEI), a Macrofire (Optronics) digital camera, and AMT image capture software. Total and disrupted cell counts were manually enumerated. All intact and disrupted cells are individually labeled in Fig. S5.

Biofilm killing assay. An overnight culture of *S. aureus* was diluted in fresh LB to an OD_{600} of 0.01. Two hundred microliters was aliquoted into each well of a 96-well plate and incubated at 37°C statically for 24 h. Biomass was washed with PBS 3 times, and the remaining attached biofilm was treated with diluted *P. aeruginosa* spent medium for 5 h. After the removal of planktonic cells and a PBS wash, the remaining biofilm was stained with 200 μL of 0.1% crystal violet for 15 min at room temperature. Biofilm was then washed with PBS 3 times, and crystal violet was extracted with 200 μL of 100% ethanol for 15 min at room temperature. OD_{590} was measured by a plate reader as an indication of biofilm mass.

Statistical analysis. Statistical significance was determined using either a one-way analysis of variance (ANOVA) with Tukey's *post hoc* test or Student's *t* test. Analyses were performed using GraphPad Prism v.7 (GraphPad Software). Statistical significance was determined using a P value of <0.05.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.5 MB.

ACKNOWLEDGMENTS

This study was supported by the National Institute of Allergy and Infectious Diseases (R01AI143916) to D.J.W., an American Heart Association Career Development Award (19CDA34630005) to E.S.G., and a Cure CF Columbus fellowship (C3LIU0219 and C3LIU0520) and a fellowship program for Advancing Research in Infection and Immunity to Y.L. This work was supported in part by the Cure CF Columbus Translational Core (C3TC). C3TC is supported by the Division of Pediatric Pulmonary Medicine, the Biopathology Center Core, and the Data Collaboration Team at Nationwide Children's Hospital. Grant support was provided by The Ohio State University Center for Clinical and Translational Science (National Center for Advancing Translational Sciences, grant UL1TR002733) and by the Cystic Fibrosis Foundation (Research Development Program, grant MCCOY19RO).

We acknowledge resources from the Campus Microscopy and Imaging Facility (CMIF) and the OSU Comprehensive Cancer Center (OSUCCC) Microscopy Shared Resource (MSR) at The Ohio State University. We thank Ken Bayles and P. Lynne Howell for generously providing the *S. aureus* LAC strain and PslG hydrolase, respectively. We also acknowledge Antonio DiGiandomenico at AstraZeneca for providing us with Psl monoclonal antibodies.

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