Treatment with the *Pseudomonas aeruginosa* Glycoside Hydrolase PslG Combats Wound Infection by Improving Antibiotic Efficacy and Host Innate Immune Activity

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ABSTRACT *Pseudomonas aeruginosa* is an opportunistic, nosocomial bacterial pathogen that forms persistent infections due to the formation of protective communities, known as biofilms. Once the biofilm is formed, the bacteria embedded within it are recalcitrant to antimicrobial treatment and host immune defenses. Moreover, the presence of biofilms in wounds is correlated with chronic infection and delayed healing. The current standard of care for chronic wound infections typically involves physical disruption of the biofilm via debridement and subsequent antimicrobial treatment. The glycoside hydrolases PelA_h and PslG_h have been demonstrated *in vitro* to disrupt biofilm integrity through degradation of the key biofilm matrix exopolysaccharides Pel and Psl, respectively. Herein, we demonstrate that PslG_h hydrolase therapy is a promising strategy for controlling *P. aeruginosa* wound infections. Hydrolase treatment of *P. aeruginosa* biofilms resulted in increased antibiotic efficacy and penetration into the biofilm. PslG_h treatment of *P. aeruginosa* biofilms also improved innate immune activity leading to greater complement deposition, neutrophil phagocytosis, and neutrophil reactive oxygen species production. Furthermore, when *P. aeruginosa*-infected wounds were treated with a combination of PslG_h and tobramycin, we observed an additive effect leading to greater bacterial clearance than treatments of tobramycin or PslG_h alone. This study demonstrates that PelA_h and PslG_h have promising therapeutic potential and that PslG_h may aid in the treatment of *P. aeruginosa* wound infections.

KEYWORDS *Pseudomonas aeruginosa*, Psl, biofilm, exopolysaccharide, glycoside hydrolase, therapeutic, wound infection

Pseudomonas aeruginosa is a Gram-negative opportunistic pathogen, which can cause devastating infections in immunocompromised individuals. This bacterium is one of the most common causes of nosocomial infections and is frequently isolated from acute and chronic wound infections, respiratory infections, and the surface of medical devices (1–3). Furthermore, *P. aeruginosa* frequently exhibits recalcitrance toward conventional antimicrobials, due to its ability to form protective communities, known as biofilms (2, 4). Once encased within the biofilm matrix, *P. aeruginosa* becomes more tolerant to environmental stress, host immune factors, and antimicrobial treatment (5). Thus, it is unsurprising that biofilm development during infection results in chronicity and increased hospitalization costs.

The incidence of chronic wound infections is increasing worldwide as antimicrobial

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resistance and diseases associated with chronic wound risk, such as obesity and diabetes, increase in prevalence (6, 7). While the origin of chronic wounds can vary, recurrent infection and subsequent host inflammation delay healing (8). Although the number of chronic wound cases continues to rise, wound treatment strategies are becoming increasingly effective (9). The improvement in treatment outcomes can largely be attributed to better molecular diagnostic techniques, which allow for identification of the wound microbiota followed by targeted antimicrobial therapy. Currently, *P. aeruginosa* infections are treated with a combination of β -lactams, fluoroquinolones, and aminoglycosides (10). However, the incidence of infection with multidrug-resistant *P. aeruginosa* strains continues to increase (11). As *P. aeruginosa* is one of the most common organisms isolated from chronic wound infections (12), it has become increasingly important to identify targeted therapies that will enhance the effectiveness of traditional antibiotics. The connection between biofilms, chronic infection, and drug resistance is well established across bacterial species and disease states, making the biofilm a promising target for combating chronic infection and reducing the emergence of resistance. To date, various studies have determined that biofilm disruption in the wound, either mechanically via debridement or chemically via drug treatment, results in a limited window of increased antibiotic susceptibility (13–15). Therefore, in this study we sought to identify new methods for disrupting the biofilm in order to improve antibiotic efficacy.

P. aeruginosa biofilm formation is a complex process that involves the production and secretion of a variety of components, including proteins, extracellular DNA (eDNA), and exopolysaccharides (EPS). Pel and Psl are EPS that have critical roles in the structure and initiation of biofilm development, as they promote surface attachment and cell-cell adhesion (16, 17). Furthermore, Pel and Psl have direct roles in enhancing antibiotic tolerance and host immune evasion by either binding or blocking immune components and antimicrobial drugs before they reach cells within the biofilm (5, 18–20). Many *P. aeruginosa* strains, including PAO1, are genetically capable of producing both Pel and Psl but predominantly produce one EPS at a time. While the relative amount of each EPS produced varies by strain, Pel and Psl often serve similar functions in the biofilm (21). Therefore, Pel and Psl represent appealing antibiofilm targets for novel therapeutics.

The *pel* and *psl* synthesis operons have been well defined and both encode proteins PelA and PslG, respectively, that contain a glycoside hydrolase domain (22, 23). PelA is a multidomain protein whose deacetylation activity is required for Pel production (24). The role of the PelA and PslG hydrolytic activity remains unclear. We have previously reported that soluble recombinant forms of PelA_n and PslG_n are capable of hydrolyzing their respective EPS leading to disruption of established *in vitro*-grown biofilms (22, 25). Additionally, we determined that these enzymes increase *P. aeruginosa* susceptibility to colistin and HL-60 neutrophil phagocytosis (22). In the current study, we sought to determine how these enzymes improve antibiotic activity and host clearance. Herein, we provide further evidence that combined PelA_n and PslG_n treatment enhances antibiotic efficacy against biofilms and that this results from improved antibiotic penetration into the biofilm. PslG_n treatment of *P. aeruginosa* also ameliorates host innate immunity by improving complement deposition, neutrophil phagocytosis, and neutrophil reactive oxygen species (ROS) production. However, PelA_n treatment of *P. aeruginosa* did not appear to affect innate immunity. Furthermore, using a murine punch biopsy model, we demonstrate that PslG_n potentiates antibiotic killing in the wound leading to reduced bacterial burden compared with wounds treated with only tobramycin.

RESULTS

PelA_n and PslG_n treatment increases *P. aeruginosa* biofilm susceptibility to antibiotic killing. The biofilm exopolysaccharides Psl and Pel provide protection from colistin and tobramycin, which are commonly used to treat *P. aeruginosa* infections (18, 19, 26). Prior studies suggest that ionic interactions between EPS-associated matrix

FIG 1 Combined PelA_h and PslG_h treatment of PAO1 biofilms improves antibiotic efficacy. PAO1 biofilms were treated as indicated followed by antibiotic treatment with (A) tobramycin, (B) polymyxin, (C) colistin, and (D) neomycin. Bacterial survival was determined by CFU quantification. Bars indicate mean $\pm$ SEM. Statistical significance was determined by one-way ANOVA followed by Bonferroni's *post hoc* tests. ***, $P < 0.001$; ****, $P < 0.0001$.

components and antibiotics results in their sequestration, preventing the compounds from reaching the bacteria within the biofilm (18, 19). Therefore, we hypothesized that disrupting the integrity of the biofilm matrix with PelA_h and PslG_h would increase *P. aeruginosa* antibiotic susceptibility. As we have previously reported, PelA_h or PslG_h treatment of static cultures which overproduced Pel or Psl led to increased colistin sensitivity (22). In this study, we sought to determine if hydrolase treatment improves killing activity with a variety of antibiotics, in a strain producing native levels of the exopolysaccharides. Additionally, this assay allows for the exclusion of planktonic cells, which provides a more specific measurement of the hydrolase effect on the biofilm.

PAO1 biofilms were grown on polystyrene pegs immersed in liquid medium and treated with an antibiotic with or without combined PelA_h and PslG_h treatment. Following the combined treatment with PelA_h and PslG_h, *P. aeruginosa* was significantly more susceptible to killing by tobramycin, polymyxin, colistin, and neomycin (Fig. 1A to D). While PelA_h was included in our biofilm treatments, PAO1 produces a predominantly Psl matrix (21). Therefore, the increased antibiotic efficacy observed here is likely due to the activity of PslG_h rather than PelA_h. Additionally, it is worth noting that

FIG 2 PslG_h treatment improves tobramycin penetration into the biofilm. Four-day-old biofilms grown under flow conditions were untreated (A) or treated with 100 nM PslG_h for 5 min (B) followed by application of Syto9- and Cy5-labeled tobramycin for 30 min. Biofilm structures were imaged with confocal microscopy ($n > 9$). The intensity of the tobramycin fluorescence signal (purple line) and bacterial population (green line) was measured across the surface of biofilm microcolonies to determine the location of tobramycin (bottom).

hydrolase treatment without antibiotics reduced bacterial numbers. As the recombinant hydrolases do not exhibit bactericidal activity (22), the decrease in CFUs is likely due to biofilm disruption and cell removal during the washing steps.

PslG_h treatment improves antibiotic penetration into the biofilm. We next sought to investigate how hydrolase treatment improves antibiotic susceptibility. A previous study demonstrated that penetration of tobramycin into the *P. aeruginosa* biofilm is impaired (19). Considering PAO1 predominantly produces Psl (21), we measured the effect of PslG_h hydrolase treatment on tobramycin biofilm penetration. Mature PAO1 biofilms were stained with Syto9 (green) and then treated with Cy5-labeled tobramycin (purple) with or without PslG_h pretreatment. The intensity of Cy5 signal was measured across the surface of microcolony biofilm structures in order to determine how well tobramycin could penetrate into the biofilm. As previously demonstrated (19), we observed tobramycin localized to the periphery of the biofilm structure when untreated (Fig. 2A). However, when the biofilm was treated with PslG_h for 5 minutes prior to antibiotic exposure for 30 minutes, tobramycin was located throughout the structure with less antibiotic at the periphery (Fig. 2B). This finding suggests that PslG_h treatment improves antibiotic penetration into the biofilm, which likely contributes to hydrolase-mediated antibiotic potentiation.

Combined PelA_h and PslG_h treatment leads to increased complement protein C3 deposition. The complement system, which consists of a collection of circulating proteins in the serum and tissues, is an important component of the innate immune response. Complement proteins provide the host with a number of protective functions to enhance pathogen recognition and phagocytosis (27, 28). Some complement factors, such as C3b, function as opsonins that bind to the surface of bacterial cells. Furthermore, complement factors can initiate a direct killing process by recruiting additional factors to the bacterial cell surface leading to cell lysis (29, 30). Psl inhibits the deposition of the complement components C3, C5, and C7 resulting in a significant

FIG 3 PelA_h plus PslG_h treatment improves C3 surface deposition on PAO1 and CF127. Bacterial cultures untreated (white bars) or treated (blue bars) with PelA and PslG_h were exposed to pooled human serum. C3 bound to the surface of bacterial cells was then measured using flow cytometry. Bars indicate mean $\pm$ SEM. Statistical significance was determined by Student's *t* test (*, $P < 0.05$; **, $P < 0.01$).

fitness advantage over Psl-deficient strains during infection (20). We hypothesized that hydrolase-mediated degradation of Psl from *P. aeruginosa* cells would improve complement deposition. Following hydrolase treatment, PAO1 was exposed to pooled human serum and bound C3 was quantified. For each strain tested, C3 binding values were normalized to the average of the same strain when untreated. We observed that hydrolase treatment significantly improves complement deposition onto PAO1 (Fig. 3). As predicted, hydrolase treatment of PAO1 cells lacking both Psl and Pel did not alter C3 deposition, indicating the observed effect on PAO1 is due to degradation of the EPS.

While PAO1 predominantly produces Psl, there are common clinical *P. aeruginosa* variants that produce Pel or high levels of Pel and Psl (21). Although no interaction between Pel and complement has been reported, we investigated the effect of hydrolase treatment on two clinical strains, namely, PA14 and CF127, which produce only Pel or high basal levels of both Pel and Psl, respectively. Hydrolase treatment of PA14 did not appear to have any significant impact on C3 deposition, while we observed significantly greater C3 deposition onto CF127 when treated with PelA_h and PslG_h (Fig. 3). Although the temperature conditions tested here are similar to the human body, PA14 transcription of the *pel* synthesis operon is low at 37°C (31). Thus, PelA_h treatment of PA14 may not improve C3 deposition because Pel was not produced under the conditions tested. Therefore, we conclude that hydrolase-aided complement deposition under these conditions may be limited specifically to PslG_h removing surface-associated Psl (Fig. 3).

PelA_h and PslG_h potentiate the neutrophil response to *P. aeruginosa*. Neutrophils are crucial for the control and clearance of *P. aeruginosa* infections (32), and they utilize a variety of methods, including phagocytosis and the release of antimicrobials, to remove bacteria from the host. In order to elucidate how hydrolase treatment promotes neutrophil killing of *P. aeruginosa*, we assessed the effect of hydrolase treatment on phagocytosis and ROS production, an important group of antimicrobial compounds. We observed a significant increase in uptake of PAO1 by human neutrophils following combined treatment with PelA_h and PslG_h (Fig. 4A). Treatment of PAO1 and CF127 with the hydrolases prior to neutrophil exposure also results in 2- to 3-fold greater neutrophil ROS production (Fig. 4B). As expected, treatment of PAO1Δ*pel*Δ*psl* did not have an effect on neutrophil phagocytosis or ROS production, indicating the response to PAO1 and CF127 is specific to EPS hydrolysis. Increased production of ROS suggests increased bacterial recognition, phagocytosis, and antimicrobial activity (29). One pathway that leads to neutrophil phagocytosis and subsequent ROS production is when the neutrophil complement receptor binds to complement components bound to an antigen (29, 33). Therefore, elevated ROS production in response to treated Psl-producing strains is likely a result of increased complement deposition.

FIG 4 Combined PelA_h and PslG_h treatment enhances neutrophil function against *P. aeruginosa*. (A) Combined PelA_h and PslG_h treatment increased neutrophil phagocytosis. (B) PelA_h plus PslG_h treatment promoted neutrophil ROS production during infection. Bars indicate mean $\pm$ SEM. Statistical significance was determined by Student's *t* test. (*, $P < 0.05$; **, $P < 0.01$).

As previously demonstrated, differences in the structure of lipopolysaccharides (LPS) produced by PA14 result in increased sensitivity to a variety of factors, relative to PAO1 (34). Due to increased serum sensitivity, we were unable to determine the effect of hydrolase treatment on the phagocytosis of PA14 and CF127, but we were able to measure the ROS response. Similar to our complement deposition studies, hydrolase treatment of PA14 did not affect neutrophil ROS production (Fig. 4B), which could again be the result of low levels of Pel production under the conditions tested. Thus, the activity observed here for CF127 and PAO1 is likely specific to PslG_h and the Psl EPS produced by these strains. Collectively, our results suggest that PslG_h-mediated removal of Psl improves bacterial recognition and killing.

PelA_h and PslG_h are nontoxic to host cells and do not impede the host immune response. Prior to using these hydrolases *in vivo*, we sought to evaluate the safety of introducing these enzymes into the host environment. We have previously reported no negative effects following exposure of IMR-90 lung fibroblasts to PelA_h and PslG_h (22). To expand on this study, we next evaluated whether the enzymes have any toxic effect on primary human cells. Red blood cells (RBCs) were isolated from healthy human blood and treated with PelA_h and PslG_h, either alone or in combination. We observed no significant loss of intact RBCs, suggesting no cell death was occurring at the concentration of protein used (Fig. 5A). To assess any impact on neutrophil function, human neutrophils were exposed to PelA_h and PslG_h, either alone or in combination for 1 hour with or without the ROS-inducing stimulant phorbol myristate acetate. The presence of either hydrolase did not impede the ability of neutrophils to produce ROS, and the enzymes themselves did not stimulate an ROS response in neutrophils. These data further indicate that the hydrolases have no negative effects on host cells (Fig. 5B). The lack of neutrophil response when incubated with PelA_h and PslG_h also suggests that the purified hydrolases lack significant amounts of any contaminating agonist (e.g., LPS, flagellin, and peptidoglycan).

Since we observed no cytotoxicity or impairment of immune cell function *in vitro*, we next used a porcine burn wound model to examine the effects of PslG_h *in vivo*. The porcine wound closely resembles a human wound, due to anatomical similarity and a comparable reepithelization process (35, 36). These factors make the porcine wound model ideal for identifying any negative host response to the hydrolases. As PslG_h activity appears primarily responsible for improving the immune response, we limited our wound studies to treatment with this enzyme alone. Treatment of burn wounds with PslG_h had no significant effect on wound size, tissue necrosis, or inflammation (Fig. 5C to E). Collectively, these results suggest that PslG_h treatment has no negative effects for the host.

Loss of Psl and Pel production attenuates *P. aeruginosa* wound infection. Prior studies have demonstrated that Pel and Psl protect *P. aeruginosa* from antimicrobial

FIG 5 PeIA_h and PslG_h are not toxic to host cells and do not impede wound healing. (A) Primary human red blood cell viability is unaffected by PeIA_h or PslG_h treatment. (B) Human neutrophil activity is unaffected by PeIA_h and PslG_h treatment. (C, D, and E) Treatment of porcine burn wounds with up to 1 mg/ml of PslG_h had no negative effects on wound healing (D) and did not exacerbate necrosis or inflammation (D, E). Arrows indicate the epithelial tongue, and asterisks indicate large populations of neutrophils (E). Bars indicate mean $\pm$ SEM. Statistical significance was determined using two-way ANOVA followed by Bonferroni's *post hoc* tests. ns, not significant.

treatment and host clearance, leading to more severe infection (18, 19, 26, 37). However, it remains unclear if EPS production contributes to virulence *in vivo*. Here, we utilized a murine punch biopsy wound infection model, which allowed us to monitor the progression of infection in real time. Biopsy wounds were infected with either PAO1-*lux* or PAO1 Δ *pel* Δ *psl*-*lux*. Forty-eight and 72 hours postinfection (hpi), the bacterial burden was nearly 1 log lower in PAO1 Δ *pel* Δ *psl*-infected wounds than in PAO1-infected wounds (Fig. 6). However, at 96 hpi, there was no longer a significant difference in bacterial burden between PAO1 and PAO1 Δ *pel* Δ *psl* (Fig. 6B). These results suggest that the loss of EPS impacts initial colonization, but this may not necessarily affect the long-term outcome of an infection.

Topical wound treatment with PslG_h improves bacterial clearance and tobramycin efficacy. Currently, the most effective method for treating chronic wound

FIG 6 Loss of Psl and Pel production attenuates *P. aeruginosa* wound infection. (A) Representative IVIS images of luminescent signal in PAO1-*lux*- and PAO1 $\Delta pel \Delta psl$ -*lux*-infected wounds 2 days postinfection. (B) Wound bacterial burden was quantified by measuring the emitted light from *P. aeruginosa*-infected mouse wounds. Bars indicate mean $\pm$ SEM. At least five mice were included in each treatment group, and statistical significance was determined using Student's *t* test. (*, $P < 0.05$; **, $P < 0.01$).

infections involves regular debridement followed by antimicrobial treatment (9, 38). The process of debridement removes dead tissue and bacteria, and the physical disruption of biofilm integrity leads to a short period of increased antimicrobial susceptibility (14). Therefore, we hypothesized that disruption of a wound infection with hydrolases would improve *P. aeruginosa* susceptibility to topical antibiotic treatment. While tobramycin is predominantly administered orally, there is evidence that suggests topical application of tobramycin combats infection and helps reduce the risk of developing antibiotic resistance (38–40). We tested multiple concentrations of the antibiotic on PAO1-*lux*-infected wounds. A total of 30 $\mu\text{g}/\text{kg}$ and 3 $\mu\text{g}/\text{kg}$ of tobramycin drastically reduced bacterial wound burden, while 0.3 $\mu\text{g}/\text{kg}$ of tobramycin was sufficient to lower bacterial burden without fully eliminating the luciferase signal from the wound (see Fig. S1 in the supplemental material). Therefore, we chose to use 0.3 $\mu\text{g}/\text{kg}$ for our combined treatment experiments to avoid masking a detectable effect from hydrolase treatment.

To determine if PslG_n potentiates antibiotic activity, PAO1-*lux*-infected wounds were treated with tobramycin and PslG_n, either alone or in combination, as indicated (Fig. 7). Since Psl appears particularly important at the early stages of infection and biofilm development (Fig. 6) (16, 20), we chose to treat wounds with PslG_n at the time of inoculation. Using an *in vivo* imaging system (IVIS) and CFU quantification, at 72 hpi, we found the bacterial burden was significantly reduced in wounds treated with PslG_n and tobramycin compared with that of wounds treated with only tobramycin (Fig. 7). Although we observed improved innate immune activity following treatment with

FIG 7 PslG_h enhances the effectiveness of tobramycin during wound treatment. (A) Representative IVIS images of luminescent signal in PAO1-*lux*-infected wounds 72 hpi following daily treatments with PslG_h and/or tobramycin as indicated. (B) The wound bacterial burden was quantified at the indicated time points by measuring light emission. (C) The 72 hpi bacterial burden was quantified by homogenizing wound biopsy specimens and quantifying tissue CFUs. Bars indicate mean $\pm$ SEM. At least seven mice were included in each treatment group, and statistical significance was determined using two-way (B) and one-way ANOVA (C) followed by Bonferroni's *post hoc* tests. (*, $P < 0.05$; **, $P < 0.01$; ns, no significance).

PslG_h (Fig. 3 and 4), under the conditions tested, the impact of PslG_h activity alone was not sufficient to improve bacterial clearance *in vivo* (Fig. 7). While innate immunity may contribute to clearance in the PslG_h- and tobramycin-treated wounds, it is likely that the additive killing effect we observed here is due to the potentiation of antibiotic activity.

Combined, these results suggest that PslG_h treatment effectively reduces wound bacterial burden when combined with antibiotic therapy.

DISCUSSION

As chronic *P. aeruginosa* wound infection and antibiotic resistance become more prevalent, it is crucial that new methods are developed to improve treatment. Disruption of the biofilm through physical or chemical means improves antibiotic susceptibility in chronically infected wounds (13–15). There is growing evidence to suggest that glycoside hydrolases can be utilized to disrupt *P. aeruginosa* biofilms (22). Similarly, it has been demonstrated that treatment with various other glycoside hydrolases improves antibiotic efficacy against *Aspergillus fumigatus*, *Staphylococcus epidermidis*, and *Escherichia coli* biofilms (41, 42). Collectively, these studies suggest glycoside hydrolases may represent a new class of therapy, which could be used in treating biofilm infections in a wide range of microbial species. In the current study, we sought to gain further insight into the efficacy of PelA_h and PslG_h therapy. We demonstrate that PelA_h and PslG_h treatment enhances antibiotic efficacy against biofilms and that PslG improves antibiotic penetration into the biofilm (Fig. 1 and 2). While PelA_h does not appear to impact innate immune activity, PslG_h treatment improves complement deposition and neutrophil killing activity against Psl-producing *P. aeruginosa* strains (Fig. 3 and 4). Collectively, these functions likely contribute to improving bacterial clearance. While our investigation was limited to neutrophils, many immune cells, such as monocytes and dendritic cells, also utilize complement factors during phagocytosis (27). It is possible that enhanced complement deposition following glycoside hydrolase treatment could broadly improve the antimicrobial activity of other immune cells; however, additional studies will be necessary to determine the extent of these effects.

We further demonstrate that PslG_h treatment potentiates tobramycin antimicrobial activity leading to reduced bacterial burden in infected wounds (Fig. 7). Although we do not observe complete bacterial clearance from the wound 72 hpi, we expect that bacterial load would continue to decrease over time as the wound heals. Future studies that look specifically at the progression of infection and wound healing during treatment will be necessary to determine the optimal timing and dosing conditions for glycoside hydrolase treatments.

We demonstrated that treatment of PAO1 biofilms *in vitro* with PelA_h and PslG_h improves the efficacy of tobramycin, polymyxin, colistin, and neomycin leading to nearly 1 log greater bacterial killing than antibiotic treatment alone (Fig. 1A to D). Tobramycin and colistin are commonly used to treat *P. aeruginosa* infection, and the concentrations used here are similar to levels predicted to be in the wound fluid following intravenous antibiotic administration (43). These antibiotics also utilize different mechanisms to kill bacteria, suggesting hydrolase antibiotic potentiation is not specific to a single antibiotic mode of action. We propose that the additive effect between PslG_h and antibiotics may be due to improved penetration of the antibiotic into the biofilm. Tseng et al. determined that Psl inhibits tobramycin penetration into the biofilm and proposed that antibiotic sequestration to the periphery of the biofilm could protect metabolically active cells within the biofilm interior (19). In agreement with this hypothesis, we observe that PslG_h treatment increases tobramycin penetration into PAO1 biofilm structures (Fig. 2).

There are a wide range of wound management strategies utilized in the clinical setting. Currently, the most effective treatment strategy for chronic wound infections involves periodic wound debridement and subsequent administration of topical, oral, and/or intravenous (i.v.) antimicrobials (14). As next-generation sequencing diagnostic testing becomes routine, specialized treatments are becoming more common for effectively treating these infections (9, 38). Selecting appropriate antimicrobials that specifically target the organisms present greatly reduces the risk of developing antibiotic resistance and reduces the length of hospitalization required for treatment (10). Targeted wound treatments have already had a noticeable impact on reducing health care costs (9), which emphasizes the importance of developing therapeutics that

specifically target common wound pathogens, such as *P. aeruginosa*. Here, we demonstrate that Psl functions as an important virulence factor in the wound environment. At 48 and 72 hpi, the bacterial burden of PAO1-infected wounds was nearly 1 log greater than PAO1 Δ psl/ Δ pel-infected wounds. (Fig. 6A and B). Interestingly, the difference in wound burden between strains becomes less significant 96 hpi (Fig. 6B), which indicates EPS production is particularly important during the early stages of infection. Pel and Psl have important roles in surface attachment and initiating biofilm formation (16, 17, 44), so it is possible that the loss of EPS primarily impedes the initial establishment of a *P. aeruginosa* infection. While further studies will be required to determine the optimal timing for targeting EPS, our data suggest that impeding Psl formation with hydrolases or other therapeutics can attenuate *P. aeruginosa* infection.

While it is now well established that disrupting the EPS matrix improves antibiotic efficacy, a recent study suggests biofilm disruption may also promote bacterial dissemination (15). Fleming and Rumbaugh observed increased rates of septicemia when treating *P. aeruginosa*-infected wounds with the hydrolases α -amylase and cellulase (15). However, when paired with an antibiotic, the hydrolases reduced bacterial wound burden. Although we did not directly evaluate bacterial dissemination, we did not observe changes in clinical signs associated with sepsis following PslG_h treatment. One possibility for these observed differences could be the specificity of PslG_h for Psl (22). Cellulase and α -amylase have a broad range of targets and could affect other factors in the host environment. Additionally, Fleming and Rumbaugh report that smaller wound sizes reduced the risk of septicemia (15). They did not observe septicemia in mice with a single 0.5-cm wound, but nearly 60% of the mice died with a single 1.0-cm wound. In our study, the mice were administered two 0.6-cm wounds, and we observed no difference in mouse survival rate following PslG_h treatment. It remains unclear how wound size impacts dissemination rates, but it is possible that the size of an individual wound is important for determining the systemic spread of an infection. A third factor that may contribute to bacterial dissemination could be the concentration of hydrolase administered. Our study used a lower hydrolase dose, and it is possible that high concentrations of hydrolase cause rapid dissociation of the biofilm leading to dissemination. Additional studies that evaluate these risk factors will be crucial for determining the safest and most effective way of utilizing biofilm-disrupting agents therapeutically.

In conclusion, PelA_h and PslG_h appear to be promising new therapeutics for treating *P. aeruginosa* biofilm infections. We demonstrate here that PelA_h and PslG_h improves antibiotic efficacy and determined that PslG_h potentiates antimicrobial activity during wound infection. We anticipate biofilm disruption will become a necessary aspect of managing *P. aeruginosa* infections and PslG_h hydrolase therapy may be an effective method to address this challenge.

MATERIALS AND METHODS

Ethics statement. All animal procedures performed for this study were preapproved by The Ohio State University Institutional Animal Care and Use Committee (IACUC) under the protocol numbers 2017A00000028 (pig) and 2017A00000033 (mouse). All blood was obtained from healthy human adults. Informed written consent was obtained from all donors, and The Ohio State University Institutional Review Board (IRB) protocol number 2015H0162 approved all procedures.

Bacterial growth and media. In all experiments, excluding antibiotic susceptibility assays, *P. aeruginosa* strains were grown overnight rotating at 37°C in lysogeny broth (LB) without sodium chloride (LBNS), and when appropriate, 300 μ g/ml of carbenicillin was added to maintain plasmids. Log-phase cultures were used in all experiments (optical density at 600 nm [OD₆₀₀], 0.5 to 0.8), which were prepared by inoculating 100 to 200 μ l of overnight cultures into LBNS.

Recombinant protein production. PslG_h and PelA_h were cloned into an expression plasmid as described previously (22, 25). Clearcoli cells were transformed with the expression plasmid and grown in autoinduction medium or terrific broth (TB) for PslG_h and PelA_h, respectively, with 50 μ g/ml kanamycin. For PslG_h, the autoinduction medium inoculated with the Clearcoli cells was incubated at 37°C overnight. For PelA_h, bacteria were grown in TB and induced with 0.5 mM isopropyl β -D-1-thiogalactopyranoside (IPTG) when the cells reached an OD₆₀₀ of 1.2 to 1.4. The cells were incubated postinduction overnight at 18°C with shaking at 200 rpm before being harvested by centrifugation at 5,000 $\times$ g for 30 min at 4°C. Both proteins were purified using nickel-affinity chromatography and, subsequently, buffer was exchanged into 20 mM Tris (pH 8.0), 150 mM NaCl, and 10% (vol/vol) glycerol or 20 mM Tris (pH 7.5), 150 mM NaCl, and 2% (vol/vol) glycerol for PelA_h and PslG_h, respectively, and stored at -20°C until required.

The protein yield for PelA_h and PslG_h using the methods described is on average ~80 to 100 mg/liter of medium used.

Antibiotic susceptibility. Bacterial cells were scraped from Luria agar plates (LA) and transferred to Dulbecco's phosphate-buffered saline (PBS) (Gibco number 14190-144) and then diluted to an OD₆₀₀ of 0.26. Cells were further diluted 1:30 in tryptic soy broth (TSB). A total of 150 μ l of cells was added to each well of a Calgary biofilm device (CBD) plate (Innovotech) and incubated with the lid for 10 h shaking at 37°C. The polystyrene pegs of the CBD plate were rinsed twice by transferring the peg lid to a new 96-well plate filled with 200 μ l of sterile PBS and incubated for 1 min. Three pegs per condition were removed with sterile needle-nose pliers and sonicated for 10 min in a tube containing sterile PBS. CFUs were calculated by serial dilution to determine the average initial number of bacteria present on peg biofilms prior to treatment. Following peg removal, the lid and remaining pegs were inserted into a new 96-well plate containing antibiotics and/or PelA_h and PslG_h. Plates were treated for 4 h at 37°C in a humidified chamber. Pegs were washed once in PBS as described above and then transferred to a new plate containing 200 μ l of TSB. The pegs and plate were sonicated together for 10 min, and viable remaining bacteria were quantified by serial dilution. All experiments were run in triplicate in at least three independent experiments. Statistical significance was determined by one-way ANOVA followed by Bonferroni's *post hoc* tests.

Tobramycin penetration of *in vitro* biofilms treated with PslG_h. Tobramycin penetration and continuous flow cell biofilm reactors were prepared and assembled as previously described (19, 45). Log-phase cultures, grown in LB, were diluted in 1% (vol/vol) LB to a final OD₆₀₀ of 0.01. Flow cell chambers were then inoculated with these diluted cultures and incubated inverted for 1 h before initiation of flow. Biofilms, which were continuously supplied with fresh 1% (vol/vol) LB at 10 ml/h, were grown for 4 days at room temperature. A Zeiss LSM 510 confocal laser scanning microscope was used to image the biofilms, and Volocity software (Quorum Technologies) was used for compiling and quantifying images.

Four-day-old biofilms were treated with 5 μ g/ml PslG_h statically for 5 min followed by incubation with 2.5 μ M Syto 9 and 20 μ g/ml of Cy5-tobramycin for 30 min. Biofilms were then flushed for 30 min and imaged to measure antibiotic penetration. To determine the aggregate boundaries the Syto 9 (stains intact cells), the signal was analyzed and the widest z-slices were used to quantify the spatial distribution of Cy5-tobramycin in at least 9 images per condition. A graph of fluorescence intensity versus aggregate diameter was created for both Syto 9 (green) and Cy5-tobramycin (purple).

C3 deposition. Surface-bound C3 was quantified as described previously (20). Log-phase *P. aeruginosa* cultures with OD₆₀₀ of 0.5 were suspended in 1 ml of sterile PBS. Cultures were treated with 50 μ g/ml of PslG_h and 105 μ g/ml of PelA_h for 1 h at 37°C with nutation. Cells were washed once with PBS and suspended in 1 ml of 20% (vol/vol) pooled human serum (catalog number NHS; Complement Technology, Inc.) and incubated for 1 h at 37°C with nutation. The cells were then washed once in sterile PBS and fixed overnight in 4% (wt/vol) paraformaldehyde. C3 on the surface of the bacterial cells was measured by staining with a rabbit α -C3 antibody (Complement Technology, Inc.) followed by staining with an Alexa Fluor 647 goat α -rabbit secondary antibody (1:1,000). Bacterial cells that were not exposed to human serum served as a negative control. All strains were tested in at least three independent experiments. Statistical significance was determined by Student's *t* test.

Neutrophil internalization. Neutrophil internalization was measured using previously described methods (37). Neutrophils were isolated from healthy human donor blood by Ficoll-Hypaque separation followed by 1.5% (wt/vol) dextran sedimentation. The plasmid pMRP9 was transformed into PAO1 and PAO1 Δ psl Δ pel allowing for constitutive green fluorescent protein (GFP) expression. *P. aeruginosa* was treated for 1 h with 105 μ g/ml of PelA_h and 50 μ g/ml PslG_h at 37°C with nutation. The bacterial cultures were washed once in PBS and then opsonized in 20% (vol/vol) human serum for 5 min. The neutrophils were infected with log-phase *P. aeruginosa*, multiplicity of infection (MOI) of 1:50, for 30 min at 37°C with nutation. The neutrophils were washed twice in PBS by centrifugation at 2,500 $\times$ g for 2 min. Neutrophils were then fixed in 4% (wt/vol) paraformaldehyde overnight. Extracellular bacteria were stained with the α -*Pseudomonas* antibody (1:2,500) and goat α -rabbit Alexa Fluor 647 (1:1,000) to exclude any bacteria that were not fully taken up by the neutrophil. A BD FACSCanto II flow cytometer (BD Biosciences) and FlowJo 9.0 analysis software were used to calculate the neutrophil population that was GFP+/Alexa647-. All experiments were done in duplicate with neutrophils from at least three different donors. Statistical significance was determined by Student's *t* test.

Reactive oxygen species quantification. *P. aeruginosa* cultures were grown to log phase, and 0.5 OD₆₀₀ was suspended in 1 ml of PBS. Cells were treated with 105 μ g/ml of PelA_h and 50 μ g/ml PslG_h for 1 h on a nutator at 37°C. Cells were washed once in PBS. Cells were then suspended in 1 ml of 20% (vol/vol) pooled human serum in Hanks' balanced salt solution (HBSS) and incubated for 30 min on a nutator at 37°C. Cells were washed once with PBS, and neutrophils in HBSS were infected with bacteria (MOI, 1 neutrophil:50 bacteria). ROS was measured as previously described (46), and luminescence was measured every 3 min over a 1-h period in the presence a luminol reporter (100 μ M). The area under the concentration-time curve (AUC) was measured to determine the relative ROS produced by neutrophils over the time course. Results were normalized to the neutrophil response of the ROS inducer phorbol myristate acetate to correct for variation between donors. Experiments were done in triplicate in three independent experiments using neutrophils from different donors. Statistical significance was determined by Student's *t* test.

Cell toxicity. Human red blood cells (RBCs) were isolated from healthy donor blood from three different individuals, and the cells were exposed to 1 mg/ml of PelA_h and PslG_h for 1 h. RBC viability was measured by measuring the OD₇₀₀ to quantify the remaining intact cells. A total of 1% (vol/vol) Triton

X-100 was used as a positive control to lyse cells. Statistical significance was determined using two-way ANOVA followed by Bonferroni's *post hoc* tests.

Porcine model and wound assessment. Porcine infections were carried out using a previously established model (47). A pig was burned along the dorsolateral spine/trunk in 5.08- by 5.08-cm squares, creating 6 full-thickness burn wounds. Each wound was individually wrapped with Tegaderm, and then the entire site was bandaged and left for 3 days. The delivery vehicle PF-127 is a solid at room temperature and a liquid at 4°C. PslG_H at two concentrations, namely, 0.1 mg/ml and 1.0 mg/ml, was suspended in 20% (wt/vol) PF-127 at 4°C and added to each wound 3, 4, and 7 days postwounding. Strip biopsy specimens were taken from all wounds after 7 days and fixed in 10% (vol/vol) neutral buffered formalin prior to being processed and embedded in paraffin for histological analysis. All tissue sectioning and staining was done by the Comparative Pathology Core at The Ohio State University College of Veterinary Medicine. Formalin-fixed tissues were paraffin embedded, sectioned (5 μ m), and stained with hematoxylin and eosin (H&E). The slides were scanned with an Aperio slide scanner and analyzed and measured using ImageScope Software (Leica Biosystems, Buffalo Grove, IL). Wound closure was measured by a pathologist in a blind manner and determined by measuring the distance between the leading edges of the wounds, as previously described (48). Additionally, H&E-stained tissue was used to assess necrosis and inflammatory markers for differences between vehicle-treated and PslG_H-treated tissue.

Murine wound infection. Anesthetized female BALB/c mice were wounded with a 6-mm punch biopsy tool generating two identical full-thickness dorsal wounds. Mice were given buprenorphine at the time of wounding and bandaged with an occlusive dressing (Opsite Flexifix). Mice were given 24 h to recover, and then 60 μ l of bacterial culture containing 10⁷ cells of PAO1 or PAO1 Δ pel Δ psl was inoculated under the bandage onto the surface of each wound. All bacterial strains contained a constitutively expressed luminescent (luciferase) marker. Mice in groups receiving PslG_H treatment were also administered 400 μ g of PslG_H at the time of infection. Topical treatments of 60 μ l doses of 400 μ g PslG_H and/or 0.3 mg/kg of tobramycin were administered daily to each wound as indicated. Mock-treated wounds received 60 μ l of sterile PBS. One hour after treatment, the wounds were washed with 500 μ l of sterile PBS, and the bandage was replaced daily. Additionally, 400 μ g of PslG_H was reapplied after bandage replacement to relevant mouse groups to ensure PslG_H was present in the wound throughout the time course. Wound luminescence was imaged daily with an IVIS Lumina II optical imaging system to assess bacterial burden. Three to 4 days postinfection, wound biopsy specimens were collected for CFU quantification. At least five mice were included in each treatment group, and statistical significance was determined using two-way and one-way ANOVA followed by Bonferroni's *post hoc* tests.

Statistical analysis. Prism (Graphpad v7.04 software) was used for all statistical analysis. The threshold for significance was set at a *P* value of <0.05, and the error bars in all figures indicate the standard error of the mean (SEM). Significance in each experiment was determined using the statistical test specified in each corresponding methods section.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AAC.00234-19>.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

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