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EXPERIMENTAL THERAPEUTICS

Glycoside Hydrolases Degrade Polymicrobial Bacterial Biofilms in Wounds

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ABSTRACT The persistent nature of chronic wounds leaves them highly susceptible to invasion by a variety of pathogens that have the ability to construct an extracellular polymeric substance (EPS). This EPS makes the bacterial population, or biofilm, up to 1,000-fold more antibiotic tolerant than planktonic cells and makes wound healing extremely difficult. Thus, compounds which have the ability to degrade biofilms, but not host tissue components, are highly sought after for clinical applications. In this study, we examined the efficacy of two glycoside hydrolases, α-amylase and cellulase, which break down complex polysaccharides, to effectively disrupt *Staphylococcus aureus* and *Pseudomonas aeruginosa* monoculture and coculture biofilms. We hypothesized that glycoside hydrolase therapy would significantly reduce EPS biomass and convert bacteria to their planktonic state, leaving them more susceptible to conventional antimicrobials. Treatment of *S. aureus* and *P. aeruginosa* biofilms, grown *in vitro* and *in vivo*, with solutions of α-amylase and cellulase resulted in significant reductions in biomass, dissolution of the biofilm, and an increase in the effectiveness of subsequent antibiotic treatments. These data suggest that glycoside hydrolase therapy represents a potential safe, effective, and new avenue of treatment for biofilm-related infections.

KEYWORDS *Pseudomonas aeruginosa*, *Staphylococcus aureus*, biofilms, chronic wounds, dispersal, glycoside hydrolase

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Chronic wound bacterial biofilm infections (CWIs), which include pressure, diabetic, venous, and arterial ulcers, are a major clinical and economic burden worldwide. In fact, chronically infected diabetic foot ulcers are considered the most significant wound care problem in the world (1). Between 5 and 7 million Americans are treated for chronic wounds annually, at an estimated cost of 10 to 20 billion dollars per year, an expense which is expected to increase as the prevalence of risk factors, such as obesity and diabetes, grows (2). CWIs largely owe their chronicity to the inability of the host to clear a biofilm, with or without clinical intervention. Biofilms are communities of microorganisms protected by a self-synthesized layer of complex polymers represented mainly by polysaccharides, proteins, and extracellular DNA (eDNA), also called the extracellular polymeric substance (EPS). Biofilms form when a primary, planktonic bacterium irreversibly attaches itself to a surface and commences rapid division and recruitment of other microorganisms by providing more diverse adhesion sites to the substrate (3). Under the protection of the EPS, such polymicrobial infections thrive, making wound healing difficult.

Several mechanisms have been proposed to explain how biofilms can contribute to the chronicity of wounds, including acting as a mechanical barrier against both host-derived and exogenous antimicrobial agents, as well as impeding the reepithelialization process. This leads to a perpetual state of inflammation that delays wound healing (4, 5). It is estimated that more than 90% of all chronic wounds contain bacteria

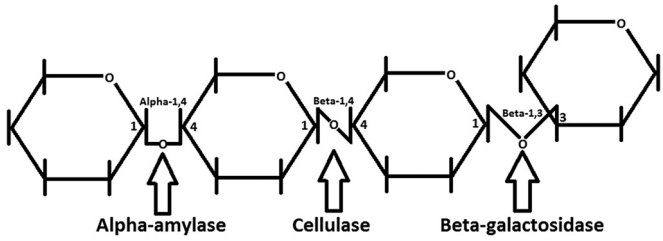

FIG 1 Examples of various glycosidic linkages found within the exopolysaccharides of biofilm EPS and the enzymes that hydrolyze them.

that are biofilm associated (6), making them up to 1,000-fold more tolerant to antibiotics and the host immune response (7). Thus, taking into account the alarming increase of antibiotic-resistant bacteria, the added ability of pathogens to reside within the protection of the biofilm matrix all too often makes effective treatment of these infections impossible.

In the majority of biofilms, microorganisms make up less than 10% of the dry mass, while the EPS represents more than 90%, with polysaccharides often being a major constituent (8). These polysaccharides provide a variety of functions crucial to the formation and integrity of the biofilm, including, but not limited to, initial surface adhesion, aggregation of bacterial cells, water retention, mechanical stability, sorption of nutrients and ions, nutrient storage, and binding of enzymes, and serve as a protective barrier against antimicrobial agents and environmental stressors (8). Thus, active degradation of polysaccharides may prove to be a promising, universally applicable approach to clinically addressing biofilm infections. Glycoside hydrolases (GHs) are enzymes that act by hydrolyzing the glycosidic linkages between two or more carbohydrates (9). They can be individually characterized by the specific type of linkage that they cleave, such as α-1,4 bond hydrolysis by α-amylase, β-1,4 bond hydrolysis by cellulase, or β-1,3 bond hydrolysis by β-1,3 galactosidase (Fig. 1) (10, 11). Considering the important contribution of polysaccharides to the biofilm architecture, it has been hypothesized that hydrolyzing the glycosidic linkages that hold them together will lead to degradation of the EPS. Indeed, microorganisms themselves utilize specific glycoside hydrolases to initiate dispersal events. For example, dispersin B is a β-hexosaminidase produced by the Gram-negative bacterium *Aggregatibacter actinomycetemcomitans* in order to disperse adherent cells from a mature biofilm (12). It has been shown that exogenous addition of dispersin B is capable of preventing and disrupting biofilms *in vitro* and *in vivo* (13–17).

The polysaccharide composition of multiple, biofilm-producing bacterial pathogens has been elucidated (18–20). One glycosidic linkage commonly seen within the exopolysaccharides secreted by a wide range of pathogens is the β-1,4 bond, such as that present in cellulose, an exopolysaccharide produced by many strains of *Escherichia coli*, *Salmonella*, *Citrobacter*, *Enterobacter*, *Pseudomonas*, and other bacteria (21). Cellulase is a commercially available enzyme that hydrolyzes these β-1,4 linkages (22) and thus could theoretically serve to break up a host of biofilm exopolysaccharides into simple sugars. It has been shown that cellulase inhibits biofilm growth by *Burkholderia cepacia* and *Pseudomonas aeruginosa* on various abiotic surfaces commonly used in medical devices (22, 23). Similarly, α-amylase, a GH that acts by cleaving the α-1,4 straight-chain linkage, has been previously shown to both inhibit biofilm formation and disrupt preformed biofilms of *Vibrio cholerae*, *Staphylococcus aureus* and *P. aeruginosa in vitro* (24–26). In this study, we aimed to hydrolyze the polysaccharides produced by *S. aureus* and *P. aeruginosa* in dual-species polymicrobial biofilms by targeting a pair of highly conserved glycosidic linkages. We focused on *S. aureus* and *P. aeruginosa* because they are the two most commonly isolated bacterial species in CWIs and are often found together in polymicrobial infections (27, 28). We found that α-amylase and cellulase were able to disrupt *S. aureus* and *P. aeruginosa* biofilms, leading to increased dispersal and antibiotic efficacy.

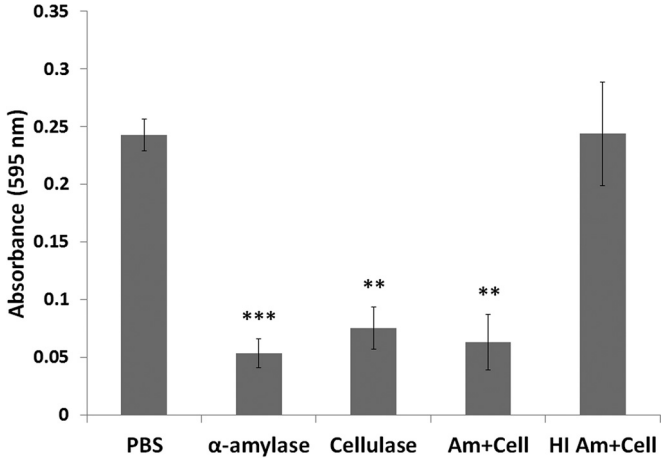

FIG 2 GHs reduce the biomass of polymicrobial biofilms. Traditional crystal violet biofilm assays (29) were performed after 48 h of coculturing *S. aureus* and *P. aeruginosa*. Planktonic cells were removed, and biofilms were treated with 0.25% GH solutions (α-amylase, cellulase, or α-amylase plus cellulase [Am+Cell]), vehicle, or heat-inactivated (HI) controls for 30 min before they were stained with 1% crystal violet. One-way analysis of variance and a Tukey-Kramer multiple-comparison test were used to test for differences between results. **, $P < 0.01$; ***, $P < 0.001$.

RESULTS

Glycoside hydrolase treatment reduces biofilm biomass and increases bacterial dispersal. We first tested the ability of α-amylase and cellulase to disrupt *S. aureus* and *P. aeruginosa* biofilms that were grown on plastic cell culture coverslips. After 48 of bacterial growth, the biofilm-coated coverslips were treated with a 0.25% GH solution for 30 min, and the biomass of the biofilms was estimated by the retention of crystal violet (CV) stain (Fig. 2). A significant reduction in biomass was observed after treatment with both GHs but not with a GH that had been heat inactivated. Significant degradation was seen at concentrations as low as 0.0025% and at treatment times as short as 2 min (see Fig. S1 in the supplemental material).

Given the degradation of biofilm biomass, we expected the biofilm-associated cells to be dispersed into their planktonic state due to the loss of EPS structure. We performed an *in vitro* well plate dispersal assay to measure total cell dispersal. *S. aureus* and *P. aeruginosa* coculture biofilms, grown in the wells of a non-tissue culture-treated plate, were treated with 5% GH solutions, and the percentage of total cells that were dispersed into the supernatant was calculated. α-Amylase, cellulase, and a 1:1 solution of both all resulted in a significant amount of dispersal compared to results with vehicle and heat-inactivated controls (Fig. 3). Solutions at concentrations of 0.25%, like those utilized in the crystal violet assays, also resulted in significant, albeit less, dispersal (Fig. S2). While the bacterial population started off with roughly equal numbers of *P. aeruginosa* and *S. aureus* bacteria, by 24 h *P. aeruginosa* represented most of the population. This is likely due to the production of several virulence factors by *P. aeruginosa* which are lethal to *S. aureus* (30). However, as we have seen before (31), when the two were cultured *in vivo* and under wound-like, *in vitro* conditions, the CFU counts for the two bacterial species were roughly equal. It should also be noted that the overall number of CFU present (in supernatant plus biofilm) in all treatment groups did not differ significantly (Fig. S3). Only the percentage of total cells present in the supernatant after GH treatment was significantly shifted, indicating that GHs do not appear to have any bactericidal activity but simply break down biofilm.

In order to determine if the results obtained *in vitro* translated to a more clinically relevant model, we next tested the ability of GH solutions to degrade the biomass of, and disperse the cells from, biofilms grown *in vivo*. A murine chronic wound infection model was utilized, and wounds were coinfected with *S. aureus* and *P. aeruginosa*. Briefly, after anesthesia, 1.5-cm by 1.5-cm full-thickness wounds were administered on

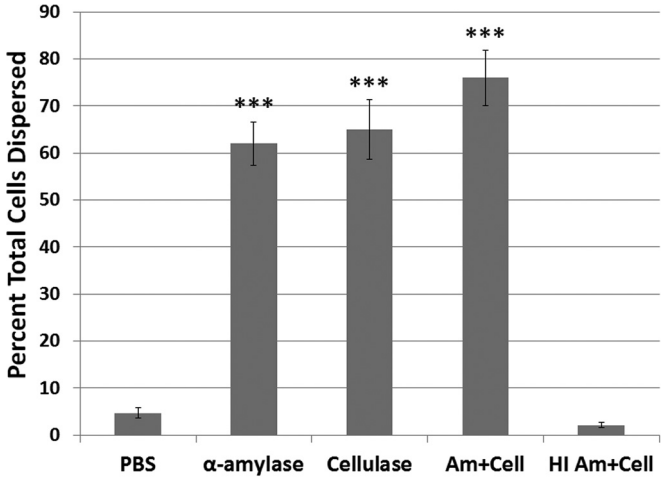

FIG 3 GHs disperse bacterial cells *in vitro*. *S. aureus* and *P. aeruginosa* biofilms at 48 h were treated with 5% GH solutions (α-amylase, cellulase, or amylase plus cellulase [Am+Cell]), vehicle, or heat-inactivated controls (HI) for 30 min. Percent dispersal was calculated as follows: (number of CFU in the supernatant)/(number of CFU in the supernatant + number of CFU remaining in the biofilm). One-way analysis of variance and a Tukey-Kramer multiple-comparison test were used to test for differences between results. Note that the result for amylase plus cellulase were not significantly greater than the result for either α-amylase or cellulase alone. ***, $P < 0.001$.

the dorsal surface of mice and covered with a transparent, adhesive bandage under which the bacteria were injected. At 3 days postinfection, we extracted the biofilms from the wound beds and treated them with GHs. For analysis of biomass degradation, we measured the weights of the extracted wound beds before and after treatment with GHs and compared the percent reduction in weight to that of biofilms treated with heat-inactivated GH. We found that α-amylase and cellulase, both alone and in a 1:1 mixture, were able to degrade *S. aureus* and *P. aeruginosa* polymicrobial biofilms harvested from murine chronic wounds (Fig. 4A). It should be noted that biomass loss in the heat-inactivated control is likely due to osmosis-powered diffusion into the distilled water over the 1 h of treatment time. To test whether GHs had any degradative effects on tissue alone, we performed the same GH treatment on uninfected connective tissue extracted from the wound beds of mice and saw no reduction in the tissue weight due to treatment (Fig. 4A). This indicates that GH treatment causes the disso-

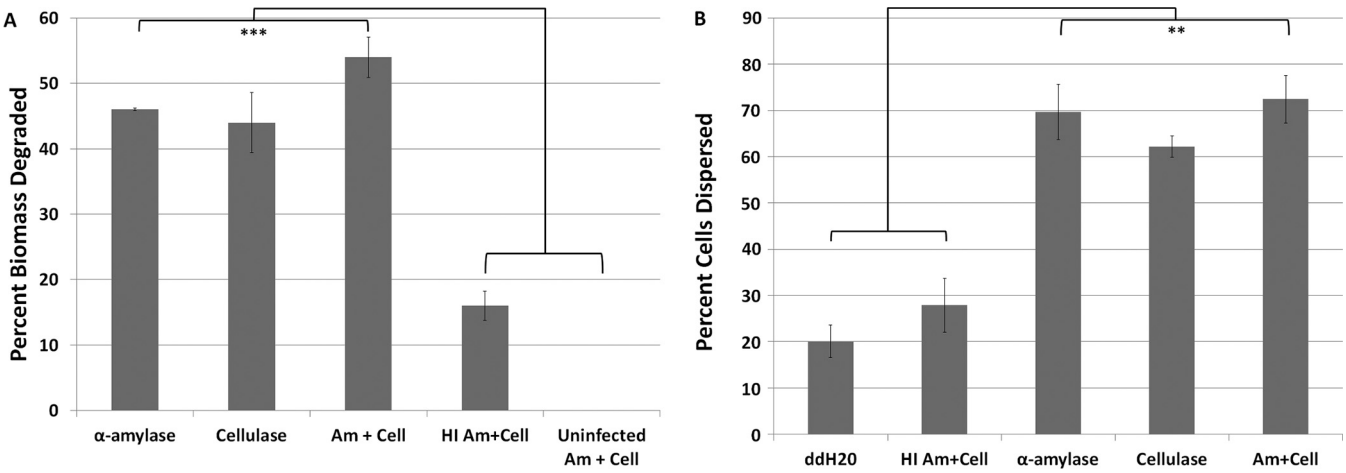

FIG 4 GHs degrade and disperse bacterial cells *ex vivo*. Tissue was extracted from the wounds of mice coinfected with *S. aureus* and *P. aeruginosa* and treated with α-amylase, cellulase, or both (Am+Cell) and compared to treatment with heat-inactivated (HI) and/or uninfected controls. Extracted tissue was suspended in 5% enzyme solutions for 1 h. (A) Percent biomass degraded was calculated as follows: (posttreatment weight/pretreatment weight). (B) Percent dispersal was calculated as follows: (number of CFU in the supernatant)/(number of CFU in supernatant + number of CFU remaining wound tissue associated). One-way analysis of variance and a Tukey-Kramer multiple-comparison test were used to test for differences between results. **, $P < 0.01$; ***, $P < 0.001$.

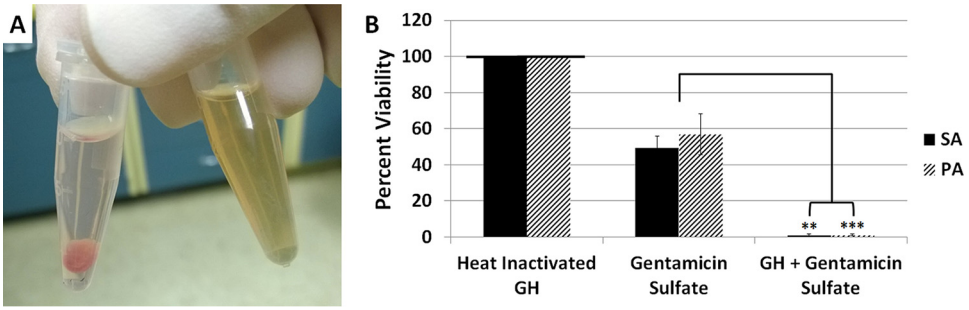

FIG 5 GHs degrade biofilms and improve the efficacy of antibiotics *in vitro*. (A) One hour of treatment with 10% cellulase (shown) and α-amylase completely disassociates 24-h-old polymicrobial biofilms (*S. aureus* plus *P. aeruginosa*) cultured in wound-like medium. (B) Treatment of wound-like biofilms with 5% α-amylase plus 5% cellulase plus 200 μg/ml gentamicin sulfate was more effective at killing *S. aureus* (SA) and *P. aeruginosa* (PA) than treatment with gentamicin alone. One-way analysis of variance and a Tukey-Kramer multiple-comparison test were used to test for differences between results. **, $P < 0.01$; ***, $P < 0.001$.

lution of up to half of the weight of the material present in the wound beds of infected (but not uninfected) mice. To determine whether this reduction in biomass correlated with bacterial cell dispersal, we also calculated the numbers of viable bacteria that were located in the treatment solution versus the number in the remaining biofilm after treatment with active or heat-inactivated GH. We found that α-amylase, cellulase, and a 1:1 solution of α-amylase and cellulase resulted in significantly more total cell dispersal than treatment with vehicle and heat-inactivated controls (Fig. 4B). Cell dispersal into the control solutions was likely due to osmosis over the 1-h treatment time, as mentioned above for biomass degradation.

Taken together, these results indicate that hydrolysis of glycosidic linkages of EPS exopolysaccharides by α-amylase and cellulase leads to degradation of mature biofilms grown *in vitro* and *in vivo* and that this degradation leads to the dispersal, or planktonic release, of biofilm-resident bacterial cells.

Glycoside hydrolase therapy increases antibiotic efficacy. Bacterial cells residing within the protection of a biofilm are thought to exhibit greater tolerance to antibiotics due to the inability of certain drugs to penetrate the EPS and to the sessile, dormant nature that many biofilm-dwelling bacteria adopt (32). Thus, we would expect dispersed, planktonic cells resulting from GH treatment to be more susceptible to antibiotics. To begin testing this, we utilized the Lubbock wound model (LWM), a clinically relevant *in vitro* wound-like model (31, 33, 34), in which we inoculated *S. aureus* and *P. aeruginosa*. After 48 h of growth, the resulting biofilms were extracted and treated either with antibiotic alone or with antibiotic plus GH; the posttreatment CFU were enumerated, and counts were compared to those of the heat-inactivated control. We found that a 1:1 mixture of α-amylase and cellulase increased the efficacy of gentamicin sulfate against biofilm-resident bacteria compared to treatment with gentamicin sulfate alone (Fig. 5). Gentamicin sulfate was used because it is a positively charged aminoglycoside, and it has been shown that positively charged antibiotics are less able to penetrate the largely negatively charged biofilm EPS (35). This makes aminoglycosides ideal for studying changes in antibiotic efficacy due to EPS destruction. We have previously seen that approximately half of *P. aeruginosa* and *S. aureus* cells remain viable after gentamicin treatment when they are cocultured in the LWM (31), and that finding was consistent in these experiments. However, we also found that GH pretreatment significantly increased the efficacy of gentamicin (Fig. 6). Taken together, these data suggest that degradation of EPS polysaccharides with GH significantly increases the ability of antibiotics to act upon the resident bacteria by dispersing the cells from the protection of the biofilm.

DISCUSSION

The ability of pathogens to exist within the protection of a biofilm poses wide-reaching complications to our ability to successfully clear infections. In particular, CWIs,

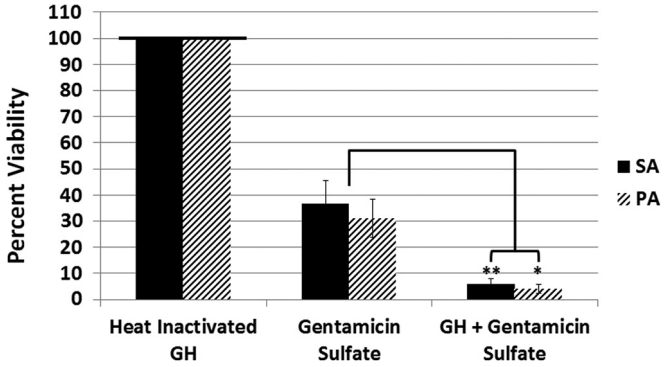

FIG 6 GHs improve the efficacy of antibiotics *ex vivo*. Treatment of murine chronic wound biofilms with 5% α-amylase plus 5% cellulase plus 200 μg/ml gentamicin sulfate was more effective at killing *S. aureus* (SA) and *P. aeruginosa* (PA) than treatment with gentamicin alone. One-way analysis of variance and a Tukey-Kramer multiple-comparison test were used to test for differences between results. *, $P < 0.05$; **, $P < 0.01$.

such as diabetic foot ulcers, are significantly more recalcitrant and recurrent when harboring a biofilm infection (6, 36–38). As exopolysaccharides represent a substantial and important constituent of many bacterial biofilms and contribute to both the physical and chemical stability of the EPS (8), their degradation should disperse bacteria into their planktonic state and may afford the host improved healing abilities by increasing the access of the host immune system and of administered antimicrobials/antibiotics to the cells.

Several studies have shown the ability of exogenous GHs to inhibit biofilm formation and disrupt mature biofilms. Recently, Baker et al. showed that GHs specific for the polysaccharides Pel and Psl are capable of both preventing the formation of and degrading *P. aeruginosa* biofilms *in vitro*, as well as potentiating antibiotics and increasing the ability of neutrophils to kill the bacteria (39). However, to our knowledge GHs have not until now been tested against biofilms grown *in vivo*. Ideally, GHs to be used clinically would exhibit broad efficacy against a variety of polysaccharides produced by vastly different species of pathogens, especially considering the complex polymicrobial nature of most infections (40). Therefore, it stands to reason that GHs that target highly conserved glycosidic linkages would be highly advantageous. In this way, clinicians would be able to administer the enzymes to any patient presenting with a biofilm infection, regardless of the causative microorganisms, and have a reasonable expectation that the therapy will be effective. α-Amylase and cellulase are two inexpensive, commercially available GHs that target common linkages found in the EPS made by many different species of bacteria, and multiple studies have shown that they can inhibit and disrupt the preformed *in vitro* biofilms of a variety of bacterial species (22–26).

In this study, we investigated the clinical applicability of utilizing α-amylase and/or cellulase to disrupt CWIs by utilizing the biologically relevant LWM, as well as a CV biomass assay, an *in vitro* cell dispersal assay, and an established murine chronic wound infection model. We found that α-amylase and cellulase, separately and in combination, significantly degraded established, polymicrobial biofilms formed *in vitro*, resulting in dispersal of the biofilm-resident bacteria into the supernatant. It is known that planktonic cells are more susceptible than their biofilm-resident counterparts to killing by both the immune system and antimicrobials/antibiotics (41–43) due, in part, to the higher metabolic rates and better cell surface access to free-floating cells. Antibiotic treatment of polymicrobial biofilms grown in the LWM and in our murine chronic wound model displayed increased efficacy when combined with GH, suggesting that targeting highly conserved glycosidic linkages with GHs may improve the ability of both the host and the clinician to eradicate recalcitrant biofilm infections.

Future work will be focused on optimizing the biodelivery of GHs, with and without

antibiotics, in animal wound infection models *in situ*. Before clinical applications can be explored, several questions must be addressed, such as the following: What is the most efficacious vehicle for topical administration (e.g., hydrogel or irrigation solution with or without negative pressure)? What species of bacteria and/or fungi can be successfully targeted? Can GHs be used alone to potentiate clearing of the infection by the immune system? Do GHs increase the efficacy of all antibiotics or just certain classes? What is the effect on the host when such a massive dispersal event is triggered? Also, given that there is a range of metabolic activities within the biofilm-associated bacterial cell population, from fully metabolically active to nearly dormant (i.e., persister cells) (44) cells, and that the majority of antibiotics target metabolically active, replicating cells, what happens to these dormant cells once they are dispersed from the biofilm? It is possible that freeing these cells from the protection of the biofilm will render them more visible to opsonic and nonopsonic phagocytosis and that metabolic activity will resume, making the cells more susceptible to antibiotics. Thus, while many questions need to be answered before GHs can be used clinically, they appear to be a very promising method of dispersing biofilm and making highly refractory infections susceptible to conventional treatments.

MATERIALS AND METHODS

Bacterial strains. *P. aeruginosa* strain PAO1 (45) and *S. aureus* strain SA31 (46) have been previously described. *S. aureus* and *P. aeruginosa* were grown in baffled Erlenmeyer flasks, with shaking at 200 rpm, in Luria-Bertani (LB) broth at 37°C. Planktonically grown cells were then used to initiate infection in the *in vitro* and *in vivo* models. All CFU were quantified by serial dilution and 10-μl spot plating on *Staphylococcus* medium 110 (Difco) and *Pseudomonas* isolation agar (Difco).

Glycoside hydrolases. Bacterial α-amylase (from *Bacillus subtilis*) (02100447; MP Biomedicals, LLC) and fungal cellulase (from *Aspergillus niger*) (02150583; MP Biomedicals, LLC) were utilized for these experiments. All enzymes were prepared by dissolving lyophilized powder in either double-distilled water (ddH_2O) or 1× phosphate-buffered saline (PBS) at 65°C for 5 min. Heat-inactivated controls were generated by heating the enzyme solutions at 95°C for 20 min.

CV assay. A traditional *in vitro* crystal violet (CV) biomass assay (29) was performed by inoculating the wells of a 24-well non-tissue culture-treated plate (Falcon) containing 13-mm plastic cell culture coverslips with 1:100 dilutions of overnight cultures of *S. aureus* and *P. aeruginosa* in fresh LB broth and allowing 48 h of growth at 37°C, with shaking at 80 rpm. Planktonic cells were then removed via rinsing with PBS, and the biofilm-coated coverslips were treated with enzyme solutions, vehicle, or heat-inactivated controls. After treatment, the coverslips were rinsed with ddH_2O, and the remaining biomass was stained with 1% CV in ddH_2O for 20 min. Coverslips were then rinsed once more with ddH_2O and transferred to fresh wells in which the CV was eluted in 95% ethanol for 1 h. The eluted CV from treated versus untreated samples was quantified via absorbance of 595-nm light in a Synergy H1 Hybrid Reader (Biotek).

***In vitro* cell dispersal.** To measure percent dispersal *in vitro*, the wells of a 24-well non-tissue culture-treated plate (Falcon) were inoculated with 1:100 dilutions of overnight cultures of *S. aureus* and *P. aeruginosa*, and biofilms were allowed to grow for 48 h at 37°C, with shaking at 80 rpm. Following incubation, the supernatant was removed, and each well was gently rinsed with PBS to discard any nonattached biomass. Subsequently, wells were treated with enzyme solutions, vehicle, or heat-inactivated controls. Following treatment, the supernatant was removed and centrifuged at a relative centrifugal force (RCF) of 11,000 for 10 min in order to pellet the cells. Cell pellets were then resuspended in PBS for CFU quantification. Biofilm remaining on the wells was dispersed via sonication and suspended in PBS for CFU quantification. Percent bacterial cell dispersal was calculated by finding the quotient of the total CFU (biofilm-associated plus planktonic) count divided by the planktonic CFU (in the supernatant) count.

Lubbock chronic wound biofilm model. The Lubbock chronic wound biofilm model, or Lubbock wound model (LWM), has been previously described (31, 33, 34). Briefly, wound-like medium (50% bovine plasma, 45% Bolton broth, 5% laked horse blood) was inoculated with 10^5 bacterial cells and incubated for 48 h, statically, at 37°C. Following incubation, the resulting biofilms were extracted, weighed, and treated with a vehicle control (ddH_2O), GH, or GH plus antibiotics. Percent bacterial cell dispersal was calculated by finding the quotient of the total CFU (biofilm-associated plus planktonic) count divided by the planktonic CFU (in the supernatant) count. Percent biomass degraded was calculated by rinsing samples to remove the posttreatment supernatant, weighing the remaining biomass, and finding the quotient of the posttreatment biofilm weight divided by the pretreatment biofilm weight. Percent antibiotic tolerance was obtained by treating biofilms with GH or with GH plus antibiotic and finding the quotient of the antibiotic-treated biofilm CFU count/gram divided by the GH-only treated biofilm CFU count/gram.

Murine chronic wound model. Our murine chronic wound model has been previously described (34, 36, 47, 48). Briefly, mice were anesthetized by intraperitoneal injection of sodium pentobarbital. After a surgical plane of anesthesia was reached, the backs were shaved and administered a full-thickness, dorsal, 1.5- by 1.5-cm excisional skin wound to the level of panniculus muscle with surgical scissors.

Wounds were then covered with a semipermeable polyurethane dressing (Opsite dressing; Smith and Nephew), under which 10^4 bacterial cells were injected into the wound bed. Biofilm formation was allowed to proceed for 72 h, after which the mice were euthanized, and the wound beds were harvested for *ex vivo* treatment with vehicle control, GH alone, or GH plus antibiotic. Percent dispersal, percent biomass degraded, and antibiotic tolerance were calculated as with the LWM, described above.

All animal experiments were carried out in strict accordance with the recommendations in the *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee of Texas Tech University Health Sciences Center (IACUC protocol number 07044).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AAC.01998-16.

TEXT S1, PDF file, 0.6 MB.

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