Utilizing glycoside hydrolases to improve the quantitation and visualization of biofilm bacteria

Derek Fleming [a,1], Whitni Redman [a,1], Garrett S. Welch [a], Nontokozo V. Mdluli [b], Candace N. Rouchon [b], Kristi L. Frank [b], Kendra P. Rumbaugh [a,*]

[a] Departments of Surgery, Immunology and Molecular Microbiology, and the Burn Center of Research Excellence, Texas Tech University Health Sciences Center, Lubbock, TX, USA
[b] Department of Microbiology and Immunology, Uniformed Services University of the Health Sciences, Bethesda, MD, USA

ABSTRACT

The complexity of microbial biofilms offers several challenges to the use of traditional means of microbial research. In particular, it can be difficult to calculate accurate numbers of biofilm bacteria, because even after thorough homogenization or sonication, small pieces of the biofilm remain, which contain numerous bacterial cells and result in inaccurately low colony forming units (CFU). In addition, imaging of infected tissue *ex vivo* often results in a disparity between the CFU and the number of bacterial cells observed under the microscope. We hypothesized that this phenomenon is due to the biofilm extracellular polymeric substance decreasing the accessibility of stains and antibodies to the embedded bacterial cells. In this study, we describe incorporating EPS-degrading glycoside hydrolases for CFU determination to obtain a more accurate estimation of the viable cells and for immunohistochemistry to disrupt the biofilm matrix and increase primary antibody binding to the bacterial cells.

Introduction

The vast majority of chronic infections are biofilm-associated [2]. Biofilms are communities of one or more species of microorganisms living within the protection of an extracellular matrix composed of polysaccharides, proteins, DNA, lipids, and other molecules, collectively termed the extracellular polymeric substance (EPS). The biofilm mode of microbial life confers increased tolerances to both antimicrobials and host defenses [12,19,22], and these tolerances are responsible for both the chronicity and recalcitrance of biofilm-associated infections.

In addition to the clinical challenges inherent in biofilm-associated infections, many of the traditional methods of microbiological research often fall short when investigating complex biofilms. One area in which these difficulties is most apparent is during general staining or immunohistochemistry on fixed samples of infected tissue. A disparity often exists between the number of colony forming units (CFU) quantified from infected tissue versus the number of bacterial cells that can be visualized using 2D light, 2D epifluorescence, or 3D confocal microscopy on samples from the same infection site.

Previously, we showed that a 1:1 solution of two glycoside hydrolases, α-amylase and cellulase, can disrupt mature biofilms formed by *Staphylococcus aureus* and *Pseudomonas aeruginosa*, both in mono- and dual-species infections, in both *in vitro* and *in vivo* models of chronic wound infection [9,10]. Glycoside hydrolases (GH) act by hydrolyzing the glycosidic linkages of polysaccharides, many of which are often present in the biofilm EPS [9,11,18]. In these studies, a key data point across all infection model types was percent dispersal, which was calculated by determining the quotient of the dispersed CFU divided by the total CFU in the sample (the sum of the dispersed bacteria and the bacteria remaining in the biofilm after treatment). Over the course of these studies, we noticed an interesting phenomenon: the samples treated with GH showed consistently higher total CFU than those treated with the vehicle control (Phosphate Buffered Saline; PBS). It was demonstrated in the early days of biofilm research that insufficient separation of bacterial aggregates can lead to underestimated cell counts [5]. Today, rigorous mechanical homogenization and/or sonication of established biofilms for the resuspension and quantification of the bacterial load are widely used strategies [28]. However, we hypothesize that microscopic fragments of biofilm often remain, even after thorough homogenization of the samples. When plated onto agar plates for CFU determination, either a single cell or an aggregate of cells will grow into a single colony, and thereby be counted as one CFU. Based on our observations, GH treatment appears to help break up these remaining fragments.

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* Corresponding author.
  E-mail address: Kendra.rumbaugh@ttuhsc.edu (K.P. Rumbaugh).
[1] Co-first authors.

In this study, we investigated how treating *S. aureus, P. aeruginosa,* and *Enterococcus faecalis* multi-well plate biofilms with various GH affected CFU recovery in dispersal assays. We then examined the effects of GH treatment on biofilms formed in an established wound microcosm *in vitro* model [9,10,24] and in our previously described mouse chronic wound infection model [3,7,9,10,23,29]. We found that adding a GH treatment step to our existing CFU and IHC quantification protocols enhanced our ability to accurately determine biofilm CFU and visualize bacteria by microscopy.

Methods

Glycoside hydrolase dispersal assays of multi-well plate biofilm

P. aeruginosa PAO1 [15] and *S. aureus* SA31 [26] biofilms were cultivated in 24-well non-tissue culture-treated plates (Falcon) for 48 h at 37 °C with shaking at 80 rpm. Individual wells were inoculated with 10^5 CFU (in 800 μL). Following incubation, the supernatant was removed, and each well was gently rinsed with 1 mL PBS to dislodge any non-adhered cells. Subsequently, wells were treated with 1 mL of enzyme solution or PBS (vehicle control) for 2 h at 37 °C with shaking at 80 rpm. All enzymes were added to PBS and incubated at their activation temperature (37 °C for α-amylase, cellulase, and xylanase and 60 °C for invertase) for 30 min prior to treatment. Following treatment, the supernatant was removed and serially diluted in PBS, and then spot plated for CFU enumeration. 1 mL of PBS was added to the remaining biofilms, which were broken up by 30 min of sonication, then serially diluted and spot plated for CFU enumeration to determine the 'biofilm' counts. All CFU were quantified by 10 μL spot plating on *Staphylococcus* medium 110 (Difco) or *Pseudomonas* isolation agar (Difco).

E. faecalis OG1RF biofilms were cultivated for 20 h in 96-well microtiter plates as described previously [13]. Biofilms were washed three times with 10 mM potassium phosphate-buffered saline (KPBS, pH 7.4) and then treated with 100 μl of GH solution for 30 min (α-amylase) or 1 h (cellulase, xylanase, invertase) at 37 °C. Control wells were exposed to 10 mM KPBS under the same treatment conditions. The following GH solutions were used to treat *E. faecalis* biofilms: 1) 0.25% α-amylase (MP Biomedicals: 100447), w/v, prepared in 10 ml KPBS, 2) 0.5% cellulase (MP Biomedicals: 150583), w/v, prepared in 10 ml KPBS, 3) 500 U/ml xylanase prepared by adding 2 g of ≥2500 U/g xylanase stock powder (Sigma Aldrich: X2753) to 10 ml of KPBS, and 4) 1500 U/ml invertase prepared by adding 50 mg of ≥300 U/mg invertase (Sigma Aldrich: I4504) to 10 ml KPBS. Each enzyme solution was activated at 37 °C for 30 min immediately prior to treatment. Following treatment, supernatants were collected into 1.5 ml microcentrifuge tubes, and the remaining adherent biofilm was washed three times with KPBS. To harvest biofilms, 100 μl of KPBS was dispensed into each well, and biofilm cells were dislodged with a pipette tip into the buffer and collected into 1.5 ml microcentrifuge tubes. The collected supernatant and biofilm samples were sonicated for 2 min in a sonicating water bath. The sonicated samples were serially diluted in KPBS and plated on BHI agar plates for CFU enumeration. Colonies were counted after 24 h of incubation at 37 °C.

To determine if GH treatment is more effective than sonication to enumerate CFU, PAO1 and SA31 biofilms were cultivated in 24-well non-tissue culture-treated plates (Falcon) for 48 h at 37 °C with shaking at 80 rpm. Individual wells were inoculated with 10^5 CFU (in 800 μL). OG1RF biofilms were cultivated for 20 h in 96-well microtiter plates at 37 °C as described previously [13]. After incubation, the non-adhered cells in the supernatant were removed without disturbing the remaining biofilm on the bottom of the wells. Any remaining non-adherent cells were washed away with either 1 mL of PBS (PAO1 and SA31) or 100 μL of KPBS (OG1RF). The biofilms were suspended by scraping the samples in either 1 mL PBS (PAO1 and SA31) or 100 μL of KPBS (OG1RF) and placed into individual 1.5 mL Eppendorf tubes. The samples were sonicated in a water bath for 5 min followed by centrifugation at 5000 rpm for 10 min.

The supernatant was removed and the pellets were re-suspended in their respective treatment for 1 h. PAO1 and SA31 were treated at 37 °C with either PBS (control) or 500 units/mL of α-amylase, cellulase, xylanase or invertase. OG1RF biofilms were treated with KPBS (control), 500 units/mL xylanase, or 5% cellulase. After treatment, the supernatant was immediately serially diluted and spot plated on either PIA, MSA, or BHI agar plates to enumerate the CFU of PAO1, SA31, and OG1RF, respectively.

Wound microcosm samples

PAO1 and SA31 biofilms were established in an *in vitro* assay that mimics the wound environment. Briefly, sterile, glass 1 mL test tubes were inoculated with 460.5 μL total volume of 45% Bolton broth, 50% bovine plasma, 5% laked horse red blood cells, and 10^6 total bacteria. Cultures were grown for 48 h at 37 °C with shaking at 80 rpm. Following incubation, the established biofilms were transferred to 1.5 mL Eppendorf tubes and rinsed with 1 mL of PBS prior to treatment. The concentration of enzymes used was based on our previous determinations of high efficacy with no observable bactericidal activity [9,10]. The GH treatment solution was prepared by first weighing and adding 5% w/v α-amylase (MP Biomedicals: 100447) to 90% of the final volume of PBS vehicle (for example, if 10 ml of solution is desired, add the α-amylase powder to 9 ml of PBS). 5% w/v cellulase (MP Biomedicals: 150583) was then added to the α-amylase solution, and the volume was brought up to 100% (i.e. 10 ml) and incubated at 37 °C for 20 min prior to treatment. Harvested biofilms were bisected, and each half was added to a pre-weighed, 2 ml hard tissue homogenization tube containing 1 mL of PBS. The tubes containing the biofilms were then reweighed to determine biofilm mass. Samples were then homogenized in a FastPrep 24 homogenizer (MP Biomedicals: 116,004,500) at 4 m/s for 60 s, after which the bacterial cells were pelleted via centrifugation at 5000×*g* for 10 min. Supernatants were then removed, and the pellets were re-suspended in 1 ml of either PBS or 10% GH, and incubated for 2 h, with shaking (80 rpm), at 37 °C. Following incubation, the resulting solutions were serially diluted in PBS and spotted on selective agar for CFU enumeration.

Ex vivo murine chronic wound samples

Our murine chronic wound model has been previously described [3,7,9,23,29]. 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 100 μL of 10^4 of *P. aeruginosa* was injected into the wound bed. At 48 h post-infection, the mice were euthanized and the wound beds were harvested for *ex vivo* GH treatments. The harvested wound tissue was cut into 2-sections, weighed, and added to FisherScientific™ 2 mL Pre-Filled Bead Tubes with 1 mL of PBS. The wound beds were homogenized at 4 m/s for 60 s using a FastPrep-24™ MP Biomedical Benchtop Homogenizer then centrifuged at 5000 g × 10 min. The PBS was removed and the biofilm homogenates were re-suspended in either PBS or 10% GH for 2 h at 37 °C with shaking at 80 rpm. Afterwards, the supernatant containing the dispersed cells was removed, serially diluted and spot plated for CFU enumeration. 1 mL of PBS was added to the remaining tissue then serially diluted and spot plated. Percent dispersal was calculated by dividing the dispersed CFU by the total CFU (biofilm-associated plus dispersed). 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 (# 07044) was approved by the Institutional Animal Care and Use Committee of Texas Tech University Health Sciences Center.
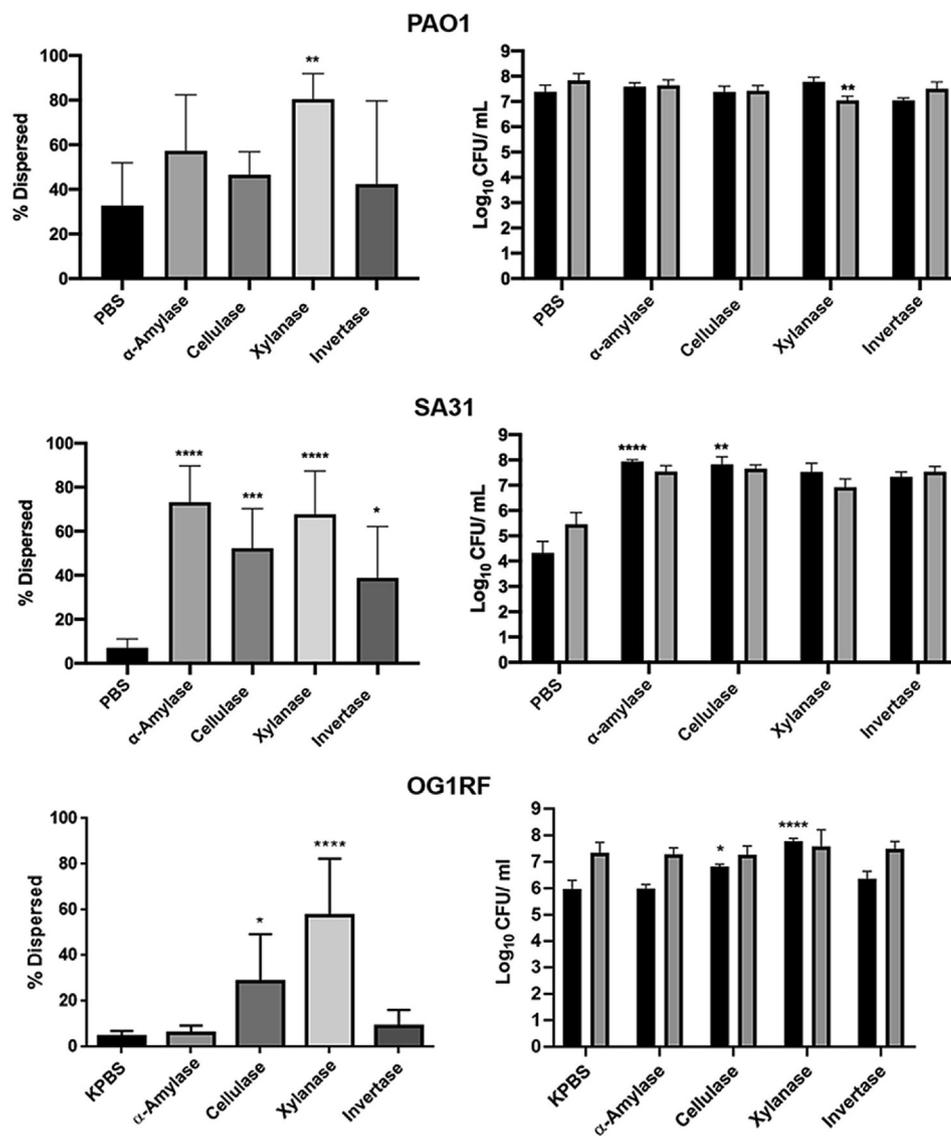
Fig. 1. Several glycoside hydrolases disperse *P. aeruginosa* PAO1, *S. aureus* SA31 and *E. faecalis* OG1RF biofilms and increase the CFU enumerated. Biofilms were grown in multi-well plates and treated with 0.25% α-amylase, 0.5% cellulase, 500 U/mL xylanase, or 1500 U/mL invertase (OG1RF) or 500 U/mL of all enzymes (PAO1 and SA31), and a vehicle control (PBS or KPBS). Then the CFU were enumerated from both the supernatant and remaining biofilm. Percent dispersal is shown on the left, and CFU is shown on the right (black bars are supernatant CFU, gray bars are biofilm CFU). N = 5 biological replicates for *P. aeruginosa* and *S aureus*. N = 3 biological replicates for *E. faecalis*, two-way ANOVA with Tukey-Kramer post-hoc test comparing treated sample to KPBS and PBS controls: * = p-value $\leq$ 0.05; ** = p-value $\leq$ 0.01; *** = p-value $\leq$ 0.001; **** = p-value $\leq$ 0.0001.

Immunohistochemistry

This method was used for *P. aeruginosa* biofilms grown in the *in vivo* mouse chronic wound model for 10 days [3,7,9,10,23,29]. The infected tissues were harvested, fixed in 10% formalin, embedded in paraffin, and cut into 10 μm sections. Sections were de-paraffinized via 3 changes of xylenes for 5 min each wash, followed by two, 3- minute washes of absolute ethanol and a single, 3-min wash each in 95% and 70% ethanol. Sections were then washed in PBS for 5 min, and the slides were immersed in antigen retrieval buffer (25 mM Tris–HCl (Tris (hydroxymethyl) amino-methane), pH 8.5, 10 mM EDTA, 0.5% (w/v) SDS). NOTE: the use of the antigen-retrieval buffer is based on the protocol by Syrbu and Cohen [25]; in order for the GH solution to act on the polysaccharides in the fixed sample, rigorous degradation of the formalin cross-linkages is necessary at 97 $\pm$ 1 °C for 1 h. With approximately 30 min left in the antigen retrieval step, the GH treatment solution was prepared by first weighing and adding 2.5% w/v α-amylase to 90% of the final volume of PBS vehicle (for example, if 10 ml of solution is desired, add the α-amylase powder to 9 ml of PBS). After mixing, 2.5% w/v cellulase was added to the α-amylase solution, and the solution was incubated at 37 °C until use. NOTE: The percent w/v of the GH solution, and treatment time, will depend on a variety of factors, including polysaccharide composition, infection time, and section thickness. For these

P. aeruginosa-infected, 10-day mouse chronic wound infections cut at 10 μm, we found that treatment with a total enzyme concentration of 5% w/v for 30 min to be optimal. Following the antigen retrieval step, samples were cooled on the benchtop for 10 min at room temperature, and then washed in PBS for 3 min. Samples were then air-dried and treated with 5% GH for 30 min at 37 °C. Following treatments, the slides containing the samples were gently rinsed by dropwise dH$_2$O for a total of 20 drops, taking care not to drop directly onto the samples, and then washed in PBS for 3 min. From this point forward, all steps were performed in the dark. 3 μl of primary antibody (Chicken *anti-P. aeruginosa*; Abcam, PLC: ab74980) in 1 ml PBS +0.04 g dried milk was added directly to the samples (200 μl per sample), and the slides were placed in a humid chamber at 4 °C overnight. Following incubation with the primary antibody, samples were washed in 3 changes of 1X PBST (1XPBS with 0.1% Tween 20). 2.5 μl of 2°antibody (Goat anti-chicken IgY H&L; Abcam, PLC: ab150176) in 1 ml 1X PBST was added directly to each sample (200 μl per sample) and incubated for 1 h in a humid chamber at room temperature. Following incubation with the secondary antibody, samples were washed in 3 changes of 1X PBST for 10 min each. Finally, the samples were air dried, and coverslips were mounted with DAPI (ProLong® Diamond Antifade Mountant with DAPI (ThermoFisher: P36962) for immediate imaging with a Nikon Eclipse 80i microscope. For uninfected tissue sections, the same protocol was used, omitting the

antibody treatments.

Gram stain

20 μL of the *ex vivo* biofilm homogenates were placed onto pre-cleaned, plain Fisherbrand™ microscope slides before and after the 2 h treatment. The biofilm homogenates were fixed to the slide by adding 15–20 μL of methanol and air-dried. Upon drying, the Reme™ Gram Crystal Violet staining kit was utilized as previously described [4]. Briefly, slides were flooded with crystal violet for 1 min, followed by a water rinse. Next, slides were flooded with Gram's Iodine for 1 min and rinsed with a decolorizing agent and a water rinse. Lastly, the slides were stained with the safranin counterstain for 30 min and rinsed with water. The slides were allowed to air dry before being fixed with Permount® Mounting Media and covered with a Fisherbrand™ glass cover slip. After 24 h of drying at room temperature, the stained specimens were imaged with a Nikon Eclipse 80i microscope.

Results

Treatment of multi-well plate biofilms with various glycoside hydrolases increases CFU enumerated from dispersal assays

It has long been appreciated that CFU enumeration of biofilm samples frequently underestimate the number of bacterial cells present in a sample [5]. Consequently, many protocols include extensive homogenization and/or sonication steps as an attempt to break up aggregates and obtain more accurate CFU numbers [28]. As we, and others, have previously shown that GH breakup biofilms and disperse bacterial cells [1,6,9,10,16,17,27], we sought to test whether GH could be used to more accurately enumerate biofilm CFU. We screened several GH in dispersal assays on mono-species *S. aureus* SA31 [26], *P. aeruginosa* PAO1 [15], and *E. faecalis* OG1RF [13] biofilms. Collectively, Fig. 1 demonstrates that use of a range of GH from many biological sources as dispersing agents in multi-well plate biofilm dispersal assays leads to increased percent dispersal. However, the effect is not universal for each species/GH combination. While we saw a significant increase in the dispersal of *S. aureus* for all of the GH tested, only xylanase significantly dispersed *P. aeruginosa* over the PBS control, and only xylanase and cellulase significantly dispersed *E. faecalis* over the PBS control.

We also enumerated significantly more *S. aureus* CFU after treatment with α-amylase and cellulase relative to the PBS controls. Strikingly, the number of CFU enumerated was close to a 4-log increase and higher for both the biofilm and supernatant portions. This was seen with *E. faecalis* supernatants treated with cellulase and xylanase as well, albeit to a lesser extent.

Interestingly, these data also indicate that treatment with PBS alone was able to disperse approximately 30% of the cells present in *P. aeruginosa* biofilms, but only 5–10% of *S. aureus* and *E. faecalis* biofilms. This could indicate that *P. aeruginosa* biofilms are simply less strongly adhered in this model. We tested whether these observations were consistent with other common laboratory strains of *P. aeruginosa* that produce varied exopolysaccharides in their EPS. We saw that, while GH significantly enhanced the dispersal of PA14 over the PBS control, PA103 biofilms were very easily dispersed just with PBS (Supplemental Fig. 1). These data suggest a high degree of variation in the susceptibility to GH amongst *P. aeruginosa* strains. Consequently, the utility of GH treatment to enhance CFU detection will also vary between strains.

We also compared CFU enumeration after GH treatment versus sonication, which is a standard strategy used for breaking up biofilm cells. In this experiment, PAO1, SA31 or OG1RF biofilm cells were sonicated, and then treated with PBS, cellulase or xylanase (two GH that demonstrated efficacy in earlier experiments). While there was no significant difference in the numbers of PAO1 or SA31 cells enumerated after treatment by the different methods, we did see a significant increase in the number of OG1RF cells detected after treatment with sonication plus GH

(Supplemental Fig. 2). This again suggests that depending upon the bacterial species and strain, adding a GH treatment may or may not provide a more sensitive method to quantitate biofilm cell numbers.

Treatment of wound-associated biofilms with glycoside hydrolases increases enumerated CFU

We next tested whether adding a GH treatment would affect CFU determination for biofilms grown in two different wound models. We have previously described an *in vitro* wound microcosm model in which *S. aureus* coagulates the plasma-based liquid media and biofilm aggregates form within the semi-solid media [8,9]. We grew *S. aureus* (SA31) and *P. aeruginosa* (PAO1) together in this microcosm model and then treated them with a 10% GH solution, composed of 5% α-amylase and 5% cellulase. We observed that GH treatment increased the *S. aureus* CFU/g detected by 5.96×10^8 (Fig. 2) over treatment with the vehicle control; however, the number of *P. aeruginosa* detected was unchanged. We examined samples of the different homogenates under the microscope and saw that large aggregates of media, containing bacterial cells, could be seen in both the pre-treated and post-PBS treated solutions (Fig 2A and B), while no, or sparse, aggregates were seen in the post-GH treated solutions (Fig. 2C). This could indicate that the GH solution enzymatically disrupts biofilms in this model and results in a more accurate CFU determination, at least for *S. aureus*.

We performed a similar experiment for *P. aeruginosa*-infected mouse chronic wound tissue. Mice were wounded and infected with *P. aeruginosa*. 48 h after infection, tissue was excised and treated with PBS or GH *ex vivo*, as described in the Methods. We observed a 2.01×10^8 CFU/g increase over the CFU/g detected from untreated tissue (Fig. 3). Similar to our observations from the wound microcosm homogenates, we also saw large aggregates of material from untreated (Fig. 3A) and PBS-treated (Fig. 3B) murine wound homogenates. However, GH-treatment appeared to breakup this material (Fig. 3C).

Treatment of fixed, infected chronic wound tissue with GH significantly increases primary antibody binding to bacteria

We typically see that while *P. aeruginosa*-infected mouse chronic wounds contain upwards of 10^9 CFU per gram of tissue, bacteria are very difficult to locate upon histology. Immunohistochemistry (IHC) (Supplemental Fig. 3A) and hematoxylin and eosin (H&E) (Supplemental Fig. 3B) staining of PAO1 infected mouse wounds revealed bacteria only near the margins of the sample. Upon closer inspection of a thinly sectioned sample (5 μm) from a *P. aeruginosa*-infected mouse wound that was stained with H&E, it was possible to visualize bacteria throughout the sample, presumably obscured by host matrix components and possibly bacterial EPS (Supplemental Fig. 3C and D).

This led us to hypothesize that treating these tissue sections with GH may reveal bacteria that seemed to be present, but 'hidden', throughout the tissue sample. Thus, serial sections of mouse chronic wound tissue infected with *P. aeruginosa* were processed with and without GH treatment, as described in the methods section (Fig. 4). In the non-GH treated samples (Fig. 4A), *P. aeruginosa* cells were mostly seen at the periphery of the sample, similar to Supplemental Fig. 3A. However, with the addition of the GH treatment step, *P. aeruginosa* cells were seen throughout the sample (Fig. 4B). These results suggest that GH is able to 'unmask' the bacteria by degrading the biofilm EPS, allowing for increased binding of the primary antibody. However, because bacteria are not only surrounded by bacterial EPS *in vivo*, it is also possible that GH affect host extracellular matrix or tissue in a way that results in the bacteria becoming unmasked. While a full characterization of GH action on host tissue is beyond the scope of this short communication, we did examine the DAPI signal and appearance of host cells in tissue sections that were treated with either GH or a PBS control (Supplemental Fig. 4). We observed no difference in either DAPI signal or the qualitative appearance of cells between groups, suggesting that GH does not remove or
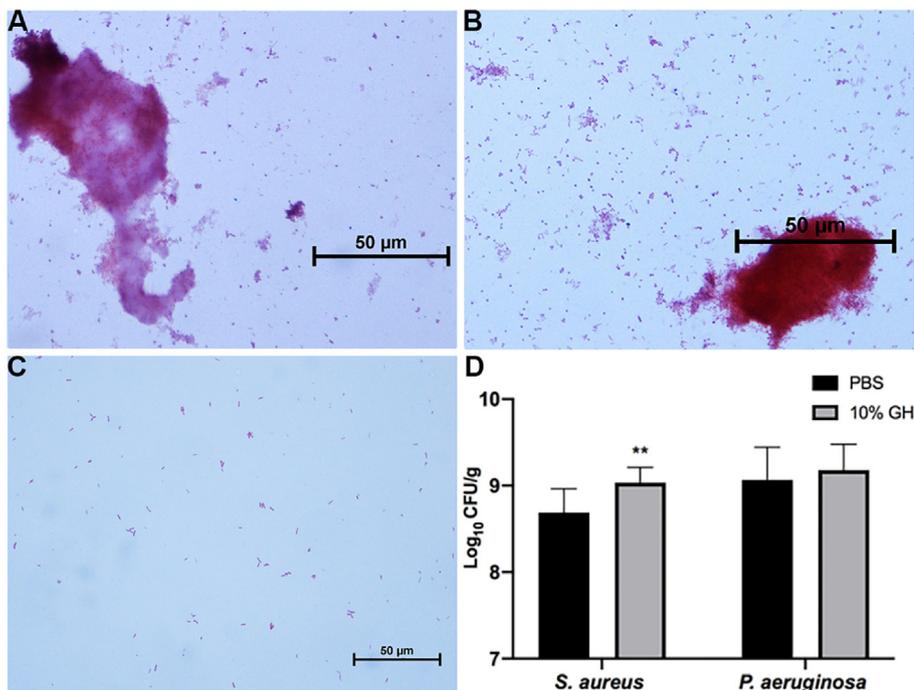
Fig. 2. GH treatment breaks up aggregates and increases CFU detection in wound microcosm model biofilms. Biofilms formed in the wound microcosm model with *S. aureus* and *P. aeruginosa* were homogenized and treated with either PBS or 10% GH (5% α-amylase + 5% cellulase) for 2 h 10 μl aliquots of homogenates pre-treatment (A), post-PBS treatment (B), and post-GH treatment (C) were spotted on microscope slides, fixed and Gram-stained (Pink = Gram Negative, Purple = Gram Positive) for imaging at 40X magnification. CFU determined for the post-GH treatment group were significantly higher for *S. aureus* (D). N = 4 per group. Paired *t*-test: ** = p ≤ 0.01. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)
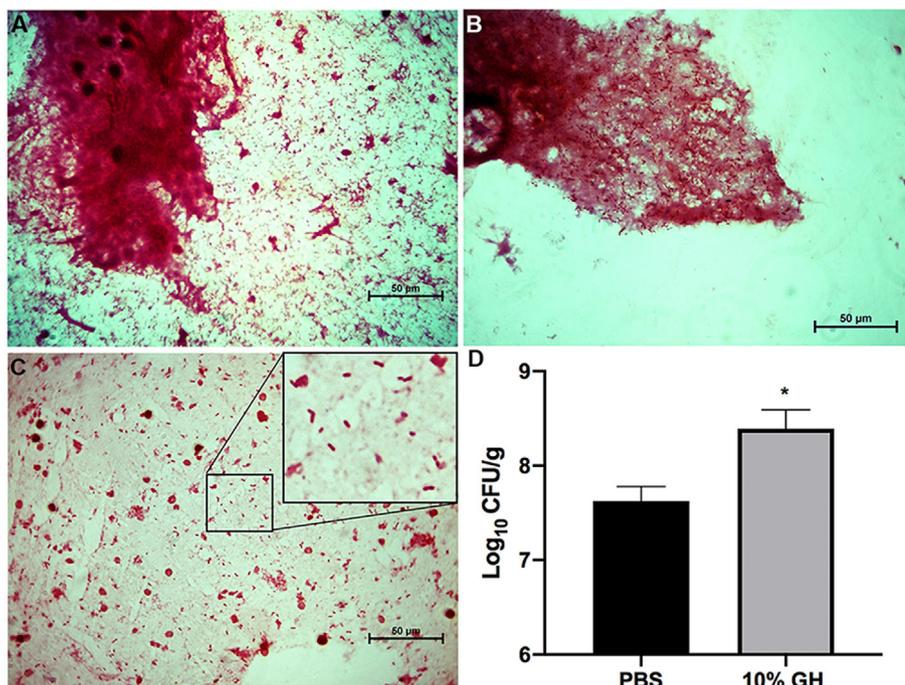
Fig. 3. GH treatment breaks up aggregates from infected tissue and increases CFU detection of *P. aeruginosa* in a mouse chronic wound model. Mouse chronic wound beds infected with *P. aeruginosa* PAO1 were homogenized and treated with either PBS or 10% GH for 2 h 10 μl aliquots of homogenates from pre-treatment (A), post-PBS treatment (B), and post-GH treatment (C) were spotted on microscope slides, fixed and Gram-stained for imaging at 40X magnification. The CFU detected were significantly higher for the post-GH treatment group (D). N = 4 per group. Paired *t*-test: * = p ≤ 0.05.

damage host cells.

Conclusions

In this work, we detail novel methodology that allows for a more accurate calculation of CFU and more effective visualization of bacterial cells in complex biofilm samples. We show that a number of GH lead to biofilm dispersal and an increase in CFU recovered from *S. aureus* and *E. faecalis* biofilms grown *in vitro* (Fig. 1). We were also able to more accurately determine biofilm CFU from a wound microcosm model and mouse chronic wound model by utilizing an EPS-degrading GH solution

(Figs. 2 and 3), and increase primary antibody binding when performing IHC microscopy on infected tissue samples (Fig. 4).

By enzymatically disrupting the small aggregates of biofilm that remain after homogenization or sonication with GH, a more accurate estimation of biofilm CFU can be obtained by reducing the ratio of bacterial cells to CFU. That is, instead of the biofilm fragments containing many individual bacterial cells forming a single colony on an agar plate, the bacteria are dispersed and form multiple colonies. However, it is clear from our data that the efficacy of GH will depend on the type used, the bacterial species (or even strain) targeted, and the biofilm model. We saw that when biofilms were grown in *in vitro*, GH were much more effective
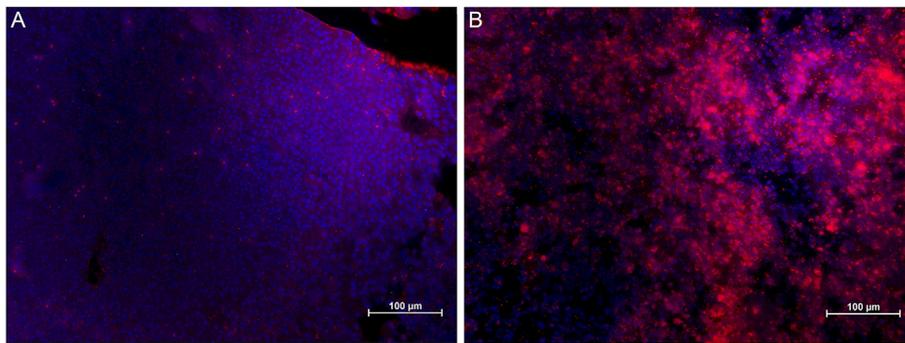
Fig. 4. GH treatment reveals *P. aeruginosa* antigens for immunohistochemistry. 10 μm sections were cut from fixed, 10-day mouse chronic wound infections (*P. aeruginosa*) for IHC microscopy. Samples were prepared either by the standard protocol (A), or with the addition of a 30-min treatment of 5% GH (B). Red = *P. aeruginosa*; Blue = DAPI. 20X magnification. Representative images from N = 6. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

on *S. aureus* than *P. aeruginosa* or *E. faecalis*. Yet, when *P. aeruginosa* was grown in mouse wounds, GH effectively broke up aggregates, increasing the CFU detected and unmasking antigenic sites for IHC. Currently we can only speculate on why GH work well in some models and not others. It is possible that bacteria alter the expression of their biofilm matrix components depending on their growth environment. Thus the amount and composition of polysaccharides present in the biofilm could differ depending on many conditions including surfaces present, nutrients available, host immune factors, etc. It is also likely that different strains of the same species will produce biofilms that will be differentially targeted by GH. For example, the *P. aeruginosa* strain PA14 was much more susceptible to GH-mediated dispersal than PAO1 or PA103 biofilms grown in well plates (Fig. 1 and Supplemental Fig. 1). Therefore, while there is clearly a difference in the strength with which these strains adhere to the plastic dish, the ability of GH to disperse cells likely depends on both the type and amount of EPS components expressed. PAO1 is capable of producing all three of the previously identified *P. aeruginosa* exopolysaccharides (alginate, Pel and Psl), but PA14 is a natural Psl mutant, and the exopolysaccharides expressed by PA103 have yet to be characterized [14,21]. Alginate and Psl both possess β- 1,4 linkages [20, 30], which should be targeted by cellulase and xylanase. Pel, which possess α- 1,4 linkages, should be targeted by amylase and invertase. Thus it is possible that depending on the *P. aeruginosa* strain and the environment in which it is grown, different bonds may need to be targeted with GH. While a more complete understanding about the composition of biofilm EPS by different bacterial species in different environments is clearly needed, the data presented here suggest we may actually be significantly underestimating bacterial numbers in samples that contain biofilms, and GH could aid in better resolving these issues.

CRediT authorship contribution statement

Derek Fleming: Conceptualization, Data curation, Formal analysis, Methodology, Writing - original draft. **Whitni Redman:** Data curation, Formal analysis, Methodology, Writing - original draft, Writing - review & editing. **Garrett S. Welch:** Data curation, Formal analysis, Methodology, Writing - original draft, Writing - review & editing. **Nontokozo V. Mdluli:** Data curation, Formal analysis, Methodology, Writing - original draft, Writing - review & editing. **Candace N. Rouchon:** Data curation, Formal analysis, Methodology, Writing - original draft, Writing - review & editing. **Kristi L. Frank:** Funding acquisition, Methodology, Project administration, Supervision, Writing - original draft, Writing - review & editing. **Kendra P. Rumbaugh:** Funding acquisition, Methodology, Project administration, Supervision, Writing - original draft, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioflm.2020.100037.

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