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Bdellovibrio bacteriovorus Inhibits *Staphylococcus aureus* Biofilm Formation and Invasion into Human Epithelial Cells

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Bdellovibrio bacteriovorus **HD100 is a predatory bacterium that attacks many Gram-negative human pathogens. A serious drawback of this strain, however, is its ineffectiveness against Gram-positive strains, such as the human pathogen *Staphylococcus aureus*. Here we demonstrate that the extracellular proteases produced by a host-independent *B. bacteriovorus* (HIB) effectively degrade/inhibit the formation of *S. aureus* biofilms and reduce its virulence. A 10% addition of HIB supernatant caused a 75% or greater reduction in *S. aureus* biofilm formation as well as 75% dispersal of pre-formed biofilms. LC-MS-MS analyses identified various *B. bacteriovorus* proteases within the supernatant, including the serine proteases Bd2269 and Bd2321. Tests with AEBSF confirmed that serine proteases were active in the supernatant and that they impacted *S. aureus* biofilm formation. The supernatant also possessed a slight DNAse activity. Furthermore, treatment of planktonic *S. aureus* with the supernatant diminished its ability to invade MCF-10a epithelial cells by 5-fold but did not affect the MCF-10a viability. In conclusion, this study illustrates the hitherto unknown ability of *B. bacteriovorus* to disperse Gram-positive pathogenic biofilms and mitigate their virulence.**

Multidrug resistance in human pathogens is a growing concern as the number of patients infected with these bacteria is increasing[1–3]. This resistive facet is further exacerbated as many chronic human diseases are biofilm associated[4]. This poses a serious threat to human health since bacteria present within biofilms are naturally more resistant to antibiotic treatments[5], even without the requisite genetic markers for antibiotic resistance.

Recent studies have shown that *Bdellovibrio bacteriovorus* and other similar organisms, collectively referred to as BALOs (*Bdellovibrio*-and-like-organisms)[6,7], predate upon Gram-negative human pathogens[8,9], including multidrug resistant *Acinetobacter baumannii*[1]. *B. bacteriovorus* was also shown to be a very promising tool for combating biofilms[10–12]. This was attributed to the ability of this bacterium to penetrate deeply inside prey biofilms and effectively destroy them; a characteristic which distinguishes them from other biological tools such as bacteriophages and protists[12]. A major limitation of BALOs, however, is their inability to attack or predate upon Gram-positive strains[8,10,13], a category that comprises numerous human pathogens[14,15], including *Staphylococcus aureus*, one of the most frequent nosocomial infection-associated multidrug resistant pathogens isolated from patients[16,17].

S. aureus commonly colonizes the skin or within the nasal passage of humans[18], but is also able to form biofilms on a variety of abiotic surfaces, including medical equipment, catheters, implants and prosthetics[19–21]. Biofilms formed by *S. aureus*, as with all bacteria, are composed of bacterial cells embedded within a matrix called the extracellular polymeric substances (EPS)[22]. This EPS matrix is formed using extracellular DNA, polysaccharides and proteins and anchors the cells to the surface, making it difficult to eradicate the organism once it establishes itself. To address this need with *S. aureus*, several groups have evaluated the use of hydrolytic enzymes, including proteases and DNases, to degrade the EPS and, thus, remove and disperse the unwanted biofilms[16,18,23].

As a predator, *B. bacteriovorus* HD100 produces numerous hydrolytic enzymes that are needed for it to effectively hydrolyze its prey's macromolecules, including a cache of 150 proteases/peptidases[24] and numerous other hydrolases. With this extensive arsenal in its genome, *B. bacteriovorus* HD100 is thought to have the highest number of protease genes per unit genome of all reported bacterial strains[24]. Whereas the production of these proteins is expected to occur during its intraperiplasmic stage of predation inside the prey cell, several studies found that cultures of host independent (HI) mutants of *B. bacteriovorus* possess a strong extracellular protease

activity[25,26]. Consequently, this study aimed at evaluating and utilizing the strong hydrolytic arsenal of *B. bacteriovorus* against *S. aureus* biofilms.

Results

Host-independent *Bdellovibrio bacteriovorus* HD100 release proteases into the media. A host-independent mutant of *B. bacteriovorus* (HIB) isolated in our lab was cultivated axenically in PYE media. This strain was selected since similarly developed host-independent isolates from other groups are known to secrete proteolytic enzymes[25]. When the cell-free supernatant from our isolate at mid-log phase (OD_{600} 0.5) was likewise assayed, we found that it harboured a proteolytic activity corresponding to 13.7 ± 7.4 ng/ml proteinase K.

***Bdellovibrio bacteriovorus* supernatant significantly inhibits *S. aureus* biofilm formation.** Wild-type *B. bacteriovorus* HD100 is capable of attacking many different Gram-negative pathogenic bacterial strains, as shown in Fig. 1A. The optical densities from each of the species tested, including a variety of *Yersinia* strains, were significantly mitigated by predation. Growth of *Staphylococcus aureus*, the causative agent of many skin infections and the most common multidrug resistant organism related with nosocomial infections[27], however, was not affected by this predatory bacterium. This was not surprising as this organism is Gram-positive and not attacked by *B. bacteriovorus* HD100.

S. aureus biofilm formation, however, is susceptible to proteinase K[28]. With HI *B. bacteriovorus* HD100 cultures releasing proteases into the media, we were intrigued by the idea that this predator and its secreted hydrolytic enzymes may block biofilm formation by *S. aureus*. This was the case as shown in Fig. 1B where 10% addition of the HIB cell-free spent media was able to prevent *S. aureus* biofilm formation by 80 to 90%. An image of the stained biofilms is provided in Supp. Fig. S1. Although the commonly reported proteinase K concentrations used in preventing and removing *S. aureus* biofilms ranges from about 1 or 2 μg/ml[28,29] to 100 μg/ml[30], this study found that proteinase K at a concentration of 1 ng/ml still led to an average inhibition of 63%. These results are in agreement with our findings with the HIB supernatants and their low proteolytic activities, which are comparable to 1.4 ng/ml proteinase K after 10% dilution. It is

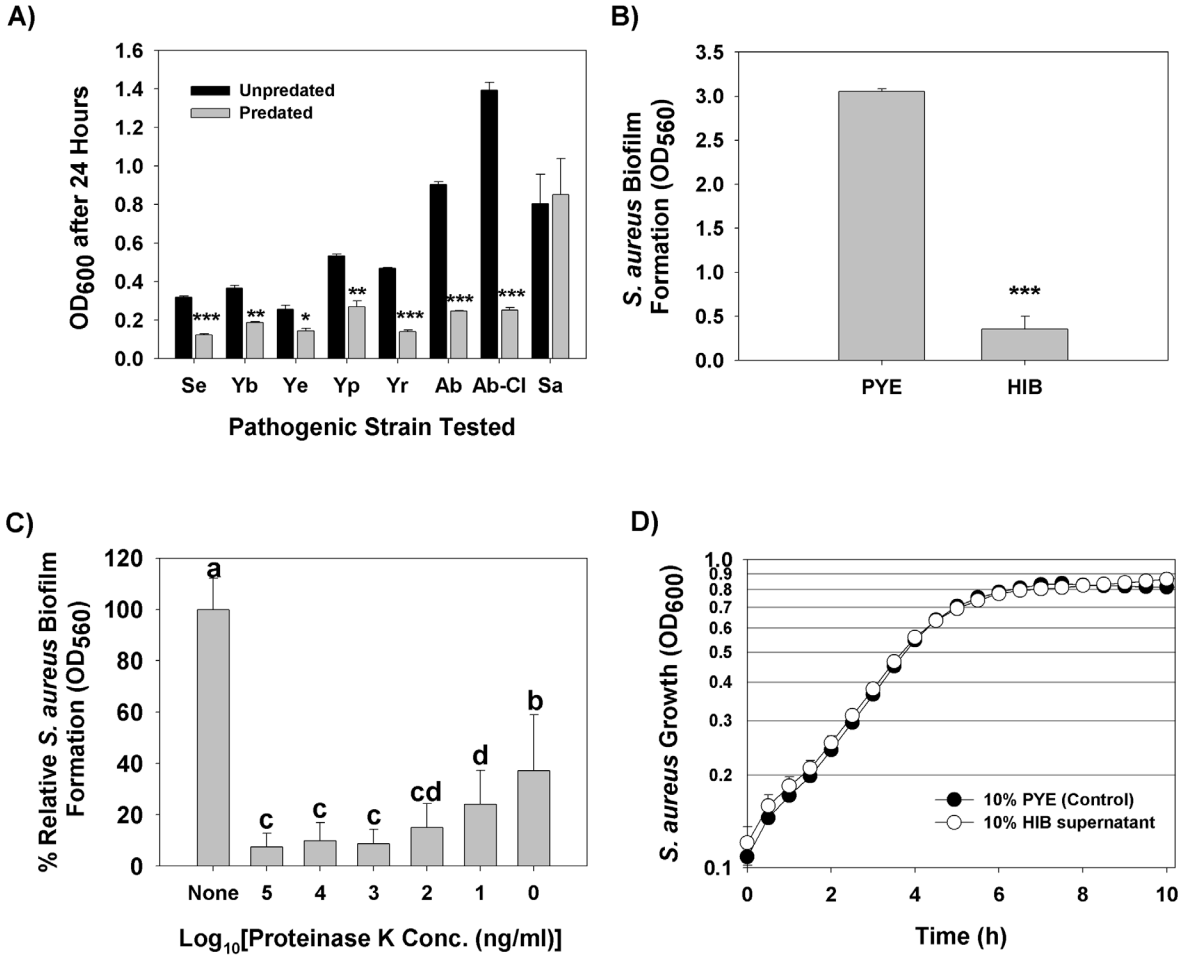

Figure 1 | (A) The prey spectrum of *B. bacteriovorus* HD100. Each prey was incubated in DNB medium in the presence or absence of the predator and the OD_{600} was measured after 24 h. A decreased OD_{600} in the presence of the predator was indicative that this prey was predated upon. The pathogenic prey evaluated are *S. enterica* (Se), *Y. bercovieri* (Yb), *Y. enterocolitica* (Ye), *Y. pseudotuberculosis* (Yp), *Y. rohdei* (Yr), *A. baumannii* (Ab), a clinical isolate of *A. baumannii* (Ab-CI) and *S. aureus* (Sa) (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). The initial OD_{600} value of each culture was: 0.18, 0.17, 0.17, 0.18, 0.15, 0.25, 0.25 and 0.4, respectively. (B) Prevention of *S. aureus* biofilm formation using culture supernatant from host-independent *B. bacteriovorus* HD100 (HIB). The supernatant was added (10%) to the *S. aureus* culture in 96-well plates. For the control wells, 10% fresh PYE medium was added (*** = $P < 0.001$). (C) Effect of different concentrations of Proteinase K on *S. aureus*-biofilm formation in 96 well plates. Proteinase K was serially diluted in PYE medium and added (10%) to the *S. aureus* culture in 96-well plates (a, b, c, and d = $P < 0.05$). (D) HIB culture supernatants have no effect on *S. aureus* growth. Fresh *S. aureus* cultures were diluted 100-fold in TSB medium supplemented with 10% fresh PYE or HIB supernatant in 96 well plates. The plate was incubated at 37°C with frequent shaking and *S. aureus* growth (OD_{600}) was monitored over 12 h.

Table 1 | Effect of different protease inhibitors on the proteolytic activity of HIB supernatant

Inhibitors	Concentration (μM)	Relative % remaining protease activity of the HIB supernatant
ABESF	5000	7.99 (±1.36)
EDTA	500	54.76 (±4.52)
EDTA	5000	32.64 (±2.86)
E-64	50	117.98 (±20.09)
Bestatin	500	64.11 (±5.67)
Pepstatin A	75	88.74 (±12.18)

worthy to note, though, that even with the significant loss in *S. aureus*' biofilm formation, the HIB spent media had no obvious effect on the growth of this pathogen (Fig. 1D) showing that the anti-biofilm activity did not results from this supernatant being toxic to this *S. aureus*.

Extracellular serine proteases contribute to *S. aureus* biofilm formation inhibition. Use of a protease assay and several different protease inhibitors, we found that treating the supernatant with AEBSF reduced the proteolytic activity by more than 90% (Table 1). This indicates that serine proteases account for a majority of the proteolytic activity seen in the HIB supernatant. Interestingly, bestatin, an aminopeptidases inhibitor, also caused a decrease in the proteolytic activity (~35%), indicating that aminopeptidases are also present. The nearly 50% inhibition brought by the addition of EDTA, a metaloprotease inhibitor, is probably due to its effect on both metaloproteases and other proteases as previous studies showed EDTA can also deactivate some serine proteases[31].

To investigate if the anti-biofilm activity of the HIB supernatant is due to these extracellular proteases, we next tested the effects the different protease inhibitors had within a *S. aureus* culture. Most of these inhibitors had no effect (Fig. 2A), but AEBSF led to a partial restoration in *S. aureus* ability to form a biofilm, *i.e.*, from 26% to 55% that of the control. When all four inhibitors were used together in a single sample, the results were identical with the AEBSF sample. In a separate experiment, when EDTA was added to the HIB supernatant at a concentration of 5 mM 30 minutes prior to HIB addition to the *S. aureus* culture, the anti-biofilm activity of HIB supernatant was still present. However, in the same experiment, EDTA at this concentration (0.5 mM) slightly reduced the biofilm formed by *S. aureus* alone in absence of HIB supernatant (Supp. Fig. 3). It is also worthy to note here that the EDTA couldn't be tested at higher concentration as its addition to the *S. aureus* culture at a final concentration of 5 mM prevented the growth of the *S. aureus* cells (Data not shown).

To evaluate this further and possibly identify the proteases active against *S. aureus*, the HIB supernatant was fractionated using several different molecular weight cut-off filters. The flow-through from each was tested to determine if it retained the ability to block *S. aureus* biofilm formation (Fig. 2B). Although there was some loss in the activity with a pore size of 50 kDa or higher, the majority of the activity was lost when the HIB was filtered through a 30 kDa pore size filter. This suggests that the majority of the proteins responsible for the HIB activity are between 30 and 50 kDa in size.

When the extracellular proteins between 30 and 70 kDa were identified by mass spectrometry, numerous serine proteases were found, including Bd2269 and Bd3857 (Table 2), reaffirming the AEBSF results in Fig. 2A. Interestingly, two additional serine proteases, Bd1444 and Bd2428, were also identified in the supernatant but were much larger than expected based upon the sizes selected for analysis. It is presumed that these proteins are secreted by the HIB into the media but subsequently cleaved by other proteolytic enzymes into smaller fragments. Aside from serine proteases, many other proteases and peptidases were also identified and may also contribute to the inhibition seen in Fig. 2A.

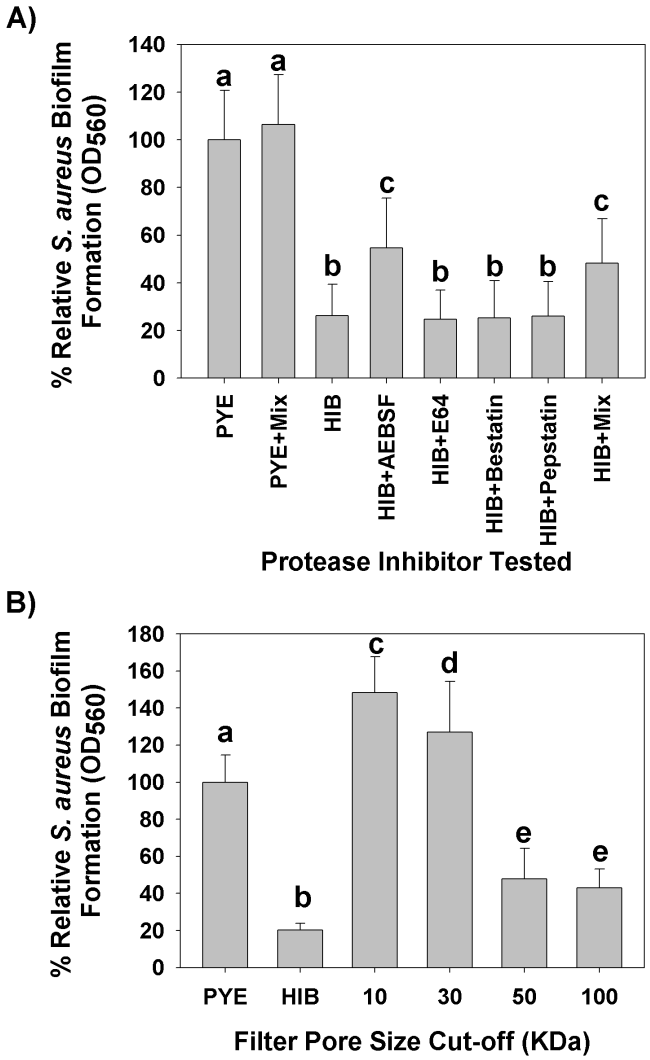

Figure 2 | (A) The effect of different protease inhibitors on the anti-biofilm activity of the HIB supernatant. The protease inhibitors AEBSF, E-64, bestatin, pepstatin A were added either separately or as a mixture to the HIB supernatant. The supernatant was then incubated for 30 min before being added at 10% to a diluted *S. aureus* culture in TSB medium within a 96-well plate. The plate was then incubated for 24 h, rinsed and CV stained (a, b, and c = $P < 0.05$). (B) Approximating the molecular mass of the active components in HIB supernatant which are responsible for *S. aureus* biofilm prevention. Samples of the HIB supernatant (500 μl) were filtered through 100, 50, 30 and 10 kDa molecular mass cut-off centrifugal filters. The filtrate from each was then tested for the anti-biofilm activity with *S. aureus* cultures (a, b, c, d and e = $P < 0.05$).

Removal of pre-formed *S. aureus* biofilms by HIB supernatants. The serine protease, proteinase K, not only reduces *S. aureus* biofilm formation but is also known to be effective at dispersing pre-formed biofilms of this strain[28,32,33]. As many of the extracellular proteases in the HIB supernatant were serine proteases, we next tested the ability of the HIB supernatants to remove pre-formed *S. aureus* biofilms. The results show that a 10% addition led to comparable losses as those seen with the well-characterized proteinase K (Fig. 3A). This was confirmed further through fluorescent microscopy and SEM imaging (Fig. 3B), providing clear evidence that the *S. aureus* biofilms are significantly dispersed by the addition of the HIB supernatant and its activity. Furthermore, the addition of AEBSF to the supernatant had only a minor effect on the removal efficiency (Fig. 3A), suggesting that other hydrolytic enzymes are also involved in the dispersal of the *S. aureus* biofilms.

Table 2 | Proteases and peptidases identified by mass spectrometry within the HIB supernatant. The size range used for the analysis was approximately between 30 and 70 kDa

Gene	Uniprot Accession	Protein Name	Size (kDa)	Subcellular Localization
Proteases				
Bd2269	Q6MKV8	Serine protease, subtilase family	56.6	Extracellular
Bd2675	Q6MJU3	Putative membrane protein with protease subunit	33.5	Unknown
Bd2321	Q6MKR4	Subtilisin-like serine protease	74.9	Unknown
Bd2428	Q6MKG5	Serine protease	114.9	Extracellular
Bd1444	Q6MN19	Serine protease, subtilase family	111.5	Unknown
Bd2692	Q6MJS6	Protease	53	Extracellular
Bd2627	Q6MJY9	Periplasmic protease	32.3	Unknown
Bd2535	Q6MK75	Putative serine protease	28.5	Unknown
Bd3857	Q6MGR5	Alkaline serine protease subtilase family	43.2	Extracellular
Bd0449	Q6MQL6	Putative protease	57.2	Unknown
Bd2675	Q6MJU3	Putative membrane protein with protease subunit	33.5	Unknown
Peptidases				
Bd0306	Q6MQZ5	Carboxypeptidase	34	Unknown
Bd1962	Q6MLP5	Putative V8-like Glu-specific endopeptidase	31.3	Unknown
Bd3622	Q6MHC8	Aminopeptidase	45.6	Extracellular
cpt	Q6MIC9	Carboxypeptidase		N/A
dcp	Q6MII6	Peptidyl-dipeptidase		N/A
pip	Q6MHR0	Proline iminopeptidase		N/A
Bd1518	Q6MMV5	Aminopeptidase	78	Unknown

As our results show that DNAse I can also disperse the *S. aureus* biofilms (Fig. 3A), a finding corroborated by other groups[16], we tested the HIB supernatant for DNAse activity. Supp. Fig. S3 shows that the supernatant also possesses some DNAse activity. Measuring it quantitatively found it to be equivalent to ~0.07 ± 0.03 μg/ml DNAse I. This activity, however, is too low to affect *S. aureus* biofilms alone as shown in Supp. Fig. S4, where DNAse I concentrations below ~6 μg/ml didn't have a significant effect on the pre-formed *S. aureus* biofilms. Likewise, attempts to detect β-D-glucosaminidase activities within the HIB supernatant were unsuccessful (Data not shown).

Treatment of *S. aureus* with the HIB supernatant reduces its virulence and invasion. Treatment of *S. aureus* with the HIB supernatant not only reduced its ability to form or maintain biofilms but also its ability to invade human epithelial cells (Fig. 4). Fig. 4A is a confocal image showing one of the MCF-10a cells harbouring two internalized *S. aureus* bacteria. Treatment of the *S. aureus* culture with 10% HIB supernatant for 2 h reduced the number of invasion events by nearly 5-fold when compared with the untreated bacterial culture (Fig. 4B). An SDS-PAGE analysis shows that the treatment significantly reduced the presence of *S. aureus* surface proteins (Fig. 4C), a group that includes assorted virulence factors[35,36]. Furthermore, when the HIB supernatant itself was tested for its effect on the MCF-10a epithelial cells using an MTT assay, no toxicity was detected (Fig. 5) as the epithelial cell viability was quite similar in the control, HIB-treated and PYE-treated wells.

Discussion

It has been proposed that *B. bacteriovorus* HD 100 can be used as a probiotic bacterium owing to its ability to predate upon gram negative bacterial strains and their biofilms, including those composed of known human pathogens[8,37,38]. A major limitation, however, is the inability of this predator to attack gram positive strains, a grouping that includes the major nosocomial pathogen *S. aureus*. Although *B. bacteriovorus* does not predate upon this strain or affect its viability, studies have not considered *B. bacteriovorus* potential impact on the biofilm stability or virulence of *S. aureus*.

This study found that host independent (HI) mutant of *B. bacteriovorus* produces significant amount of proteolytic enzymes in its culturing medium. This agrees well with the studies done by other groups who also identified proteolytic enzymes secreted by other independently isolated HI variants of *B. bacteriovorus*[25,26]. One of these studies used 2-D gel electrophoresis to identify the proteins present approximately 100 protein spots[39]. Six of the proteases identified in their study were also found in the supernatant of our HIB variant, including serine proteases Bd1962 and Bd2269 and the carboxypeptidase Bd0306. The identification of these proteins within both of these studies through different techniques corroborates their secretion by this strain and suggests that this may be a common trait of all HIB mutants. As the biofilm EPS matrix is composed of several different macromolecules, including proteins, it is not surprising that researchers have sought to use proteases to disperse pre-formed biofilms established by *Staphylococcus aureus*[23] and other bacteria[40,41] and fungi[42], as well as those composed of multiple strains or species[43]. A recent study likewise reported that the Esp serine protease from *Staphylococcus epidermidis* is capable of both inhibiting and removing *S. aureus* biofilms[18].

The results presented here demonstrate that the HIB supernatant possesses a sufficient hydrolytic activity to significantly inhibit or disperse *S. aureus* biofilms. Furthermore, as shown in Supp. Fig. S5, the supernatant was also effective in dispersing *Staphylococcus epidermidis* biofilms. This is in contrast to a recent report that the *S. epidermidis* Esp serine protease disperses *S. aureus* biofilms, but is apparently not effective against its own biofilms under similar conditions as colonization of patients by *S. epidermidis* correlates with the absence of *S. aureus*[18]. Our results show that the HIB supernatants are effective against both of these pathogens.

Whereas a significant proportion of the anti-biofilm activity seen for the HIB supernatant comes from the serine proteases based on the experiments with the serine protease inhibitor AEBSF, our data also suggests that additional effectors are secreted as the activity is not completely blocked by AEBSF. This was particularly true in the biofilm dispersal experiments (Fig. 3). It is clear that other proteases may also be contributing as treatment with AEBSF did not completely block the proteolytic activity (Table 1). Furthermore, DNAses are also present within the supernatants and were found to have an equivalent activity as ~0.07 ± 0.03 μg/ml DNAse I. A previous study also found that *S. aureus* biofilms can be partially inhibited, although not dispersed, by the action of the Dispersin B enzyme[16], which works by degrading the PNAG (poly-N-acetylglucosamine) polymer produced in the biofilm matrix of *Staphylococci*[34]. However, our attempts to find β -D-glucosaminidase activity in the HIB supernat-

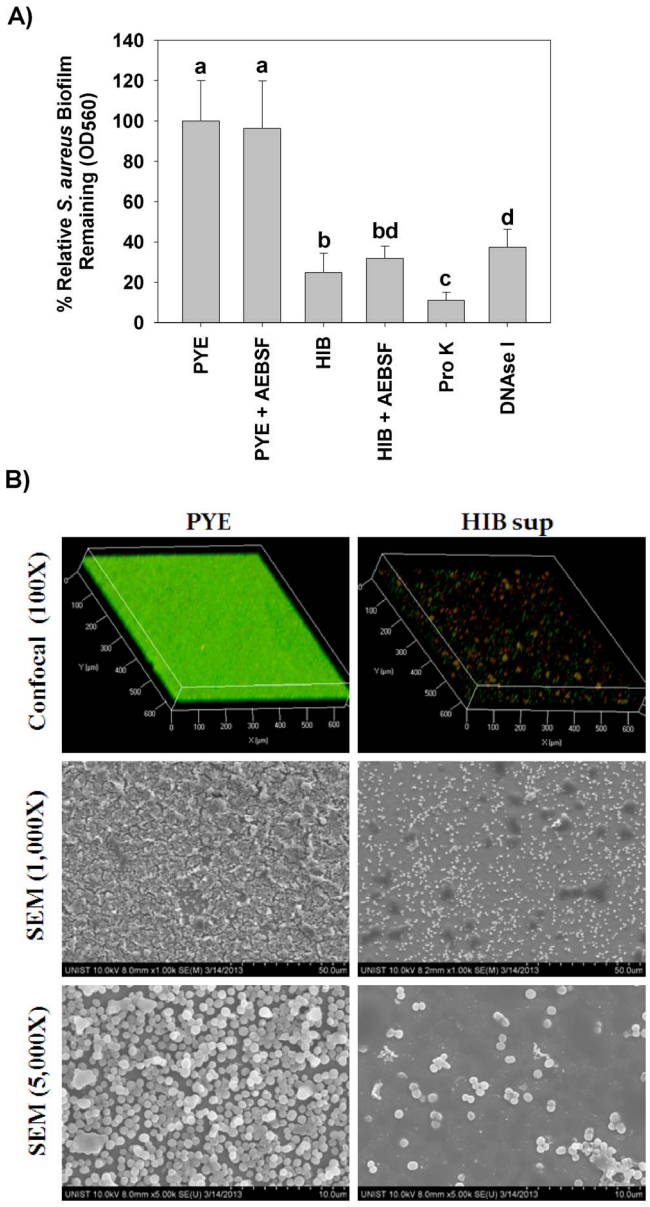

Figure 3 | (A) Removal of *S. aureus* biofilms by HIB supernatants, proteinase K and DNAse I. *S. aureus* biofilms were prepared in 96 well plates. Afterwards, the medium was removed, and the wells were filled with 200 µl of HEPES buffer supplemented with either 10% PYE medium, 10% HIB supernatant, 100 µg/ml proteinase K or 20 µg/ml DNAse I. The plates were then incubated for 24 h more, washed and CV stained (a, b, c, and d = $P < 0.05$). (B) Confocal and SEM images for the *S. aureus* biofilms on silicon chips with and without HIB supernatant treatment. *S. aureus* biofilms were formed on silicon chips, washed and incubated for 24 h with HEPES buffer supplemented with either 10% PYE medium or HIB supernatant. The chips were then washed and analyzed. For the confocal microscopy(Upper photos), the biofilms were stained with CFSE (green) for the live cells and EthD-1 (red), for the dead cells and extracllular DNA. The middle and the lower photos show the SEM images for the chips at low and high magnifications, respectively.

ant using 4-nitrophenyl-N-acetyl-β-D-glucosaminide as a substrate were unsuccessful (Data not shown). Consequently, the HIB supernatant contains a cocktail of degradative enzymes, including proteases and nucleases, and these likely work in an orchestrated manner to degrade the *S. aureus* biofilm. Individually, each hydrolytic grouping cannot account for the full activity observed and, as such, the possible synergistic impact of the various DNAses and proteases may be important and is a topic that will be studied further.

In addition to preventing and dispersing *S. aureus* biofilms, treatment of *S. aureus* with the HIB supernatant significantly reduced its virulence. A similar finding with *Listeria monocytogenes* was reported previously where hydrolysis of the surface proteins, which includes virulence factors, lowered invasion by this pathogen[40]. To ascertain that this was also happening in our study, we analysed the surface protein content of *S. aureus* after treatment with the HIB supernatant and found basically identical results; HIB supernatant treatment led to a significant reduction in the *S. aureus* surface protein number and content. However, a second possible explanation is the cleavage of receptors present on the epithelial cells necessary for *S. aureus* during colonization[44], including fibronectin[45,46], vitronectin[47] and fibrinogen[45,48]. However, this is very unlikely due to the protocol employed as the treated *S. aureus* were pelleted and washed prior to performing the invasion, thereby minimizing the transfer of HIB proteases into the epithelial cell culture. When HIB supernatants were added to MFC-10a human cell cultures and the viabilities of the cell lines were determined using the MTT assay, they were not deleteriously affected by its addition. This confirms a recent finding that the presence of *B. bacteriovorus* does not elicit a cytokine response from human cell lines[49].

In conclusion, this study shows a hitherto unknown aspect of *B. bacteriovorus* hydrolytic enzymes, especially proteases, and their impacts on Gram-positive bacterial pathogens. Based upon its mild disposition towards human epithelial cells but strongly adverse effects on *S. aureus* biofilms and virulence, HIB media may be an option for treating and removing this pathogen both *in vitro* and *in vivo*. This will be evaluated further in subsequent studies.

Methods

Bacterial strains and culturing conditions. The following strains were used in this study; *Staphylococcus aureus* KACC 10768, *Staphylococcus epidermidis* KACC 13234, *Escherichia coli* MG1655, *Salmonella enterica* KACC 11595, *Yersinia bercovieri* KACC 15319, *Yersinia enterocolitica* KACC 15320, *Yersinia pseudotuberculosis* KACC 15321, *Yersinia rohdei* KACC 15322; *Acinetobacter baumannii* KACC 12454 and a clinical isolate of *A. baumannii*. All were kept in frozen glycerol stocks at −80°C. Upon need, they were streaked on a nutrient broth (NB; Acumedia, USA) agar plate and incubated at 37°C overnight. From this plate a single colony was inoculated into 3 ml TSB broth and incubated overnight at 37°C with agitation (250 rpm). This culture was then used for biofilm experiments as described below.

Wild-type host dependant *Bdellovibrio bacteriovorus* HD100 (HDB) was purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ). Upon need, this strain was streaked from a frozen stock into DNB (dilute nutrient broth) top agar plate containing *E. coli* MG1655 as a prey as described previously[7,11]. The *B. bacteriovorus* liquid culture had an average of 1.5×10^9 PFU/ml.

Susceptibility of pathogenic strains to predation. Each bacterial strain was cultivated on plates as described above and inoculated into 3 ml NB for growth overnight at 30°C and 250 rpm. After growing, 0.5 ml of this culture was added to 4 ml DNB and cultivated once more for 24 h. For the predated culture, 0.5 ml of attack phase *B. bacteriovorus* HD100 was also added. The final OD was measured at 600 nm after 24 h using a spectrophotometer (Biophotometer +, Eppendorf, Germany).

Isolation and culturing of the Host Independent *B. bacteriovorus* (HIB). Isolation and cultivation of a host independent variant of *B. bacteriovorus* HD100 was performed as described previously[25]. A portion of this culture was then kept as frozen glycerol stocks at −80°C and, upon need, fresh cultures were prepared by streaking them out on PYE agar plate. The composition of the PYE medium was peptone (Difco, USA) 10 g/L, and yeast extract 3 g/L. After autoclaving the medium, $CaCl_2$, and $MgCl_2$ were added to it at a final concentrations of 2 and 3 mM respectively. The subcultures in PYE media were typically grown to an OD_{600} around 0.5. For biofilm experiments, the culture was centrifuged (16,000 ×g, 5 min) and filtered using a 0.22 µm syringe filter to remove all the cells.

Identification of the extracellular proteins by LC-MS/MS. To identify the extra cellular proteins located in the HIB supernatant, 10 ml of 0.5 OD_{600} HIB culture was taken and the bacteria were removed by centrifugation followed by filtration. The proteins in the filtrate were precipitated using 15% trichloroacetic acid and washed with cold acetone. The pellet was then dissolved in PBS (pH 7.4). Afterwards, the proteins were run on a 12% Tris-glycine SDS-PAGE gel (Biorad, USA). After staining with Coomassie blue, the proteins between 30 to 70 kDa were subjected to in-gel

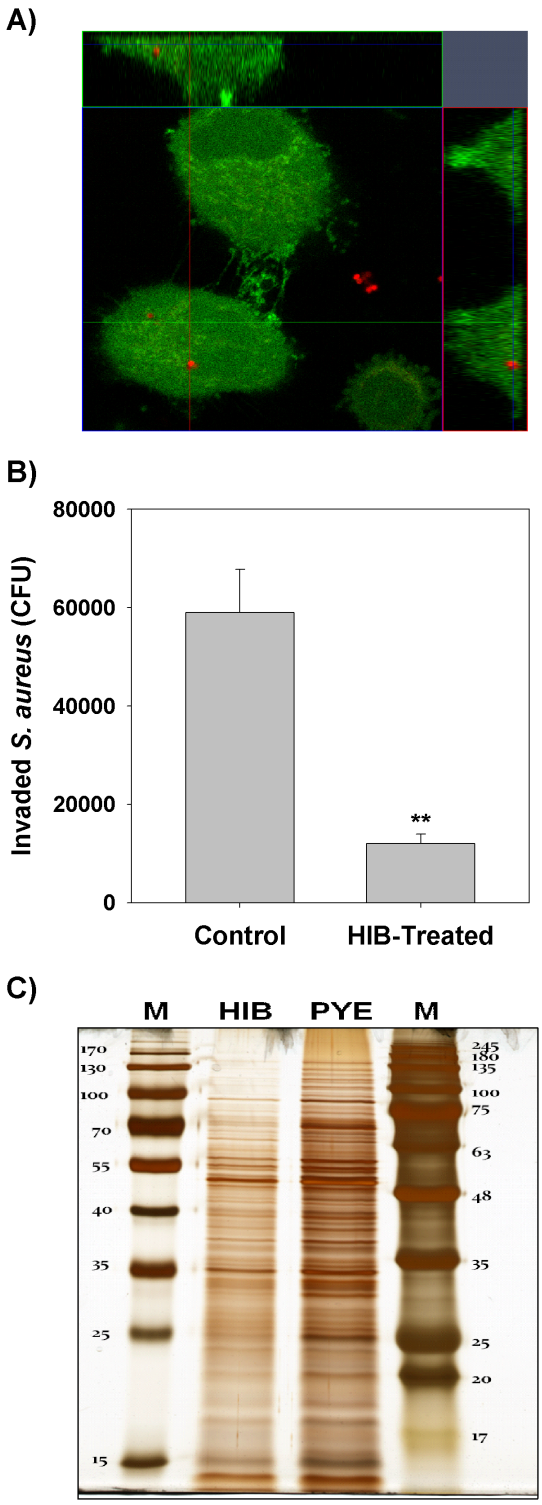

Figure 4 | (A) *S. aureus* KACC 10768 invades MCF-10a human epithelial cells. A monolayer culture of MCF-10a cells was stained with CFSE (green) and co-cultured with CellTracker™ Red CMTPX stained *S. aureus*. Invasion was carried out as described in Materials and Methods. The image was taken at 100× magnification using a confocal microscope. (B) Effect of HIB supernatant treatment on *S. aureus* invasion. *S. aureus* cells were suspended in HEPES buffer supplemented with 10% HIB supernatant or with 10% PYE media as a control for 2 h. The HIB supernatant-treated and untreated *S. aureus* cells were tested for their MCF-10a cells invasion. To enumerate the invasive *S. aureus*, the MCF-10a cells were lysed and the resulting suspension was diluted, spread on LB agar plates and the developed colonies were counted (** = $P < 0.01$). (C) SDS-PAGE analysis of the *S. aureus* surface proteins after treatment with the HIB supernatant. *S. aureus* cells were treated with HIB supernatant or with PYE media (control) as in (B). After pelleting and washing the cells, the surface proteins were purified and separated on an SDS-PAGE gel. The first and last lanes are two different commercial pre-stained protein markers. The HIB supernatant-treated cells clearly show a reduction in the surface proteins still associated with the pathogen, helping to explain its lower invasion.

tryptic digestion, as described by Shevchenko and co-workers[50]. The resulting tryptic peptides were analyzed by LC-MS/MS. All mass analyses were performed on a LTQ-Orbitrap (Thermo, Bremen, Germany) equipped with a nanoelectrospray ion source. To separate the peptide mixture we used a C18 reverse phase HPLC column (150 mm × 75 µm i.d.) using an acetonitrile/0.1% formic acid gradient of 66 min at a flow rate of 300 nl/min. For MS/MS analysis the precursor ion scan MS spectra (m/z 400–2000) were acquired in the Orbitrap at a resolution of 60,000 at m/z 400 with an internal lock mass. The three most intensive ions were isolated and fragmented in the linear ion trap by collisionally induced dissociation (CID).

All MS/MS spectra were analyzed by Sequest against the curated version of *B. bacteriovorus* protein sequence database (3583 entries, UniProt (http://www.uniprot.org/)) containing all the protein-coding sequences of *Bdellovibrio bacteriovorus* strain ATCC 15356, DSM 50701, NCIB 9529 and HD100 and frequently observed contaminants. Carbamidomethyl cysteine and oxidized methionine were set as a fixed modification and variable modification, respectively. A maximum of two missed cleavages were allowed. The mass tolerances allowed forth MS peaks were 10 ppm, and for the MS/MS peaks they were 0.8 Da. The search results were further filtered with the following options: minimum number of matching peptides was 2 and the false discovery rate was set to 0.01.

Quantitative protease assay. The protease activity in the samples was determined as described elsewhere[51]. Briefly, 2 ml test samples and the prepared proteinase K standards were added to the 0.5 ml prepared azocoll solution and incubated at 37°C for 2 h with shaking (250 rpm). After centrifugation (16,000×g, 10 min), 200 µl of the supernatant was transferred to a transparent 96 well plate (Costar, USA) and the absorbance measured at 550 nm (Tecan, Infinite M200). Three independent samples were used for each analysis. For experiments with the protease inhibitors, each inhibitor was added to the HIB supernatant at the indicated concentration, and the samples were incubated for 30 min at 37°C before performing the protease assay.

Size fractionation of the HIB supernatant and effect of protease inhibitors. Samples of the HIB supernatant (0.5 ml) were passed through a molecular weight cut-off filter (10, 30, 50 or 100 KDa (Ultracel, Amicon, USA)). The filtrate from each was collected and tested for activity against *S. aureus* biofilm formation. The protease inhibitors tested were AEBSF (4- (2-aminoethyl) benzenesulfonyl fluoride), E64, Bestatin, and Pepstatin A, all from Sigma-Aldrich (USA) and EDTA (ethylenediaminetetraacetic acid, Invitrogen, USA). The stock solution for each was prepared according to the suppliers instructions. Each individual protease inhibitor was mixed with the supernatant at a concentration of 5000, 50, 500, 75 or 5000 µM, respectively, for 30 min before adding the supernatant to the *S. aureus* cultures.

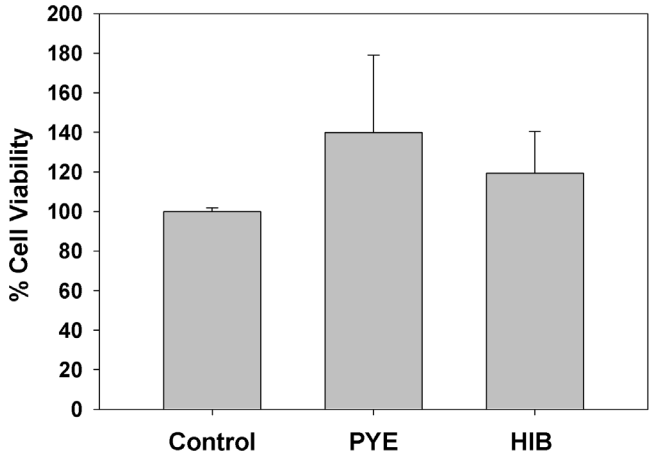

Figure 5 | **MTT assay to evaluate the effects of HIB supernatants on the viability of MCF-10a human epithelial cells.** The assay was performed using Vybrant® MTT Cell Proliferation Assay Kit in 96-well plates using a 72 h exposure. The results show that the HIB supernatant had no effect on the viability of MCF-10a cells.

Qualitative and quantitative DNAse activity assay. The DNAse activity was first qualitatively assayed using the pUC19 vector. For this, equal volumes of the HIB supernatant (or fresh PYE medium for the control) and the plasmid solution were mixed so that the final concentration of the plasmid was 20 ng/μl. After 2 h of incubation at 37°C, the samples were analysed by gel electrophoresis within a 1.2% agarose gel.

For the quantitative DNAse activity measurements, the assay was performed as described previously[52], albeit with some modifications. Briefly, double-stranded genomic DNA extracted from *E. coli* MG1655 was added to 20 μl of HIB supernatant or a DNAse I standard (prepared in PYE medium) so that the final DNA concentration was 100 μg/ml. The reaction mixtures were incubated for 2 h at 37°C. The PicoGreen dsDNA quantitation kit was then used to measure the concentration of the dsDNA remaining in the samples. The DNAse activity of the supernatant was then determined relative with the DNAse I standards.

β-N-acetylglucosaminidase assay to test for glycanase activity within the HIB supernatant. This assay was performed according to the protocol described by Kaplan et al (2003)[53]. The substrate 4-nitrophenyl-N-acetyl-β-D-glucosaminide, was purchased from Sigma-Aldrich (USA). The reaction was carried out in 1.5 ml tubes. Samples (100 μl) of the HIB supernatant or fresh PYE medium, as a control, were added to 900 μl of the reaction mixture (1 ml final volume). Aliquots (200 μl) were taken over an 18 h time period and the absorbance at OD_{405} was measured to detect any activity by *p*-nitrophenolate release.

Biofilm prevention and removal assays. To generate the biofilms, an overnight culture of *S. aureus* in trypticase soy broth (TSB) medium was diluted 100 fold into fresh TSB broth. To each well within a 96-well plate (Costar, USA), 180 μl of this culture was added, after which, 20 μl (10%) of either PYE media, HIB supernatant or other additives were added. The plate was incubated for 24 h at 37°C without shaking. After washing carefully with distilled water and drying, 250 μl of 0.1% crystal violet (CV) was added to each well. The plate was incubated for 20 minutes at room temperature, washed and dried. Subsequently, 250 μl of ethanol supplemented with 2% acetic acid was added to each well for 30 min. The absorbance of each well was then measured at 560 nm using a Glomax Multi+ (Promega, USA).

For biofilm removal tests with *S. aureus* or *S. epidermidis*, their biofilms were prepared as described above. The medium was removed and the wells filled with 200 μl HEPES buffer (with 2 mM $CaCl_2$ and 3 mM $MgCl_2$) supplemented with either 10% PYE medium, 10% HIB supernatant, 100 μg/ml proteinase K (Invitrogen, USA), 20 μg/ml DNAse I (Invitrogen, USA) or other additives. The plates were then incubated for 24 h at 37°C, washed and then stained with CV. The absorbance at 560 nm was measured as described above.

Statistical analysis of the biofilm experiments. Each experiment was done using three independent *S. aureus* cultures using at least four wells for each (total of 12 wells for each experimental condition). To minimize the error due to plate-to-plate handling variations, the results for each plate were analyzed relative to the control wells for the given plate and the results from different days were then averaged together. Statistical analysis was performed using Graphpad Prism program (version 5.01). For comparing two groups, the student t-test was used and statistical significance was designated on the graphs using asterisks (*, **, or ***) for *P* values $<$ 0.05, 0.01, and 0.001, respectively. For comparing more than two sets of data, the ANOVA test was performed, followed by Tukey test. In this case, the statistically significantly different groups, at $P < 0.05$, were designated on the graphs using different letters (a, b, c, and d).

Biofilm formation on silicon chips. The silicon chips used in this study were 10 mm $\times$ 10 mm chips cut from silicon wafers (Shin-Etsu, Japan). The chips and their biofilms were prepared as described previously[10].

Confocal and scanning electron microscopy. To visualize the *S. aureus* biofilms formed on the silicon chips, the bacterial cells were stained by immersing the chip for 30 min in HEPES buffer containing CellTrace™ CFSE (1 μg/ml), and ethidium homodimer-1 (EthD-1, 0.004 mM) (Life Technologies, USA). These dyes stain live cells and dead cell/extracellular DNA, respectively. The chips were then washed gently to remove any excess dye before imaging. Confocal microscopy was performed using an LSM700 confocal microscope (Carl Zeiss) operated by ZEN 2009 software. Scanning electron microscopy imaging of these biofilms was performed as described previously[10]. The images shown are representatives for the results obtained from three different chips that were prepared and analysed independently for each case.

Visualization and enumeration of invasive *S. aureus*. MCF-10a cell monolayer was prepared in an 8-well chambered cover glass (Nunc® Lab-Tek® II, Thermo-Scientific) by inoculating 7.5×10^4 cells/well in DMEM/F12 media. After growth for 36 h, the MCF-10a cells were stained with CFSE by replacing the media with 0.5 ml PBS containing 5 μg/ml CFSE and incubating for 10 minutes at 37°C in a CO_2 incubator. The cells were washed twice with PBS and then 0.5 ml of DMEM/F12 media was added to each well. The stained cells were again incubated for 1 h at 37°C.

An overnight culture of *S. aureus* was centrifuged at 16,000×g for 2 minutes and resuspended at an OD_{600} of 1.0 in HEPES buffer supplemented with 1 μg/ml CellTracker Red CMTPX (Invitrogen, USA). After incubation for 20 minutes in a shaking incubator at 37°C, the labelled bacteria were centrifuged as above and washed three times with HEPES buffer. The cell pellet was suspended in 1 ml HEPES buffer and diluted 1 : 100 into DMEM/F12 media. For the invasion, the culture media in the well was replaced with 0.5 ml of the prepared *S. aureus* solution and the plate incubated for 1 h at 37°C. Afterwards the media was replaced with 1 ml fresh DMEM/F12 media containing 100 unit/ml penicillin G and 100 μg/ml streptomycin (penicillin-streptomycin mixture, Invitrogen, USA) and 50 μg/ml gentamicin (Sigma, USA) for 15 minutes. This mixture of antibiotics was used to ensure all extracellular bacteria, *i.e.*, those that did not invade, were killed before plating and colony enumeration as described below. The medium was then replaced with antibiotic-free DMEM/F12 media and the culture was imaged immediately using confocal microscopy.

To enumerate the invaded bacteria, the MCF-10a cells were lysed by incubating them at room temperature for 10 min in 1 ml PBS containing 0.2% triton X-100 (Sigma-Aldrich, USA.) followed by manual disruption. The resultant cell lysate was serially diluted and plated out on LB agar plates to allow the colonies to grow at 37°C for 24 h.

***S. aureus* surface protein extraction and processing.** Three different colonies of *S. aureus* were grown overnight in TSB broth. *S. aureus* cells from each tube were then suspended at an OD_{600} of 1.0 in 5 ml HEPES buffer supplemented with either HIB supernatant (10%) or PYE (10%) as a control. The six tubes were then incubated for 2 h at 37°C. Afterwards, the cells in each tube were centrifuged, and washed 3 times with PBS. The proteins were isolated and run out on an SDS-PAGE gel according to the protocol published previously[40]. For SDS-PAGE analysis, two commercial pre-stained protein markers were used - PageRuler, (Thermo Scientific, USA) and Wide-View (Wako Chemical, Japan).

Assessing the impact of the HIB supernatant on MCF-10a viability. Viability assays were performed using the Vybrant® MTT Cell Proliferation Assay Kit (Invitrogen, USA) in 96 well plates (Microtest, Falcon, USA). For this, each well was seeded with 2500 MCF-10a cells in DMEM/F12 media and incubated at 37°C inside a CO_2 incubator for 24 h. Afterwards, the medium was replaced with 100 μl of fresh culture medium supplemented with 10% of the test samples and then the plates were incubated again for 72 h at 37°C. The medium was then removed and 100 μl of fresh phenol red-free media supplemented with 10 μl of the 12 mM MTT stock solution was added to each well. After 4 h incubation at 37°C, the MTT media were removed and 100 μl of DMSO was added. The contents inside each well were mixed and the plate was incubated again at 37°C for 10 minutes. The absorbance of each well was then measured at 540 nm.

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Author contributions

A.K.M., M.D. and R.J.M. conceived and performed the experiments, J.K.S. performed the LC-MS/MS experiments and analysis, J.-H.H., A.K.M. and M.D. performed the microscopic imaging and analyses. A.K.M., M.D., J.K.S. and R.J.M. wrote the main manuscript text and A.K.M., M.D. and R.J.M. prepared the figures and tables. All authors reviewed the manuscript.

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