Video Article

Anti-virulent Disruption of Pathogenic Biofilms using Engineered Quorum-quenching Lactonases

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Abstract

The rapid emergence of multi-drug resistant bacteria has accelerated the need for novel therapeutic approaches to counter life-threatening infections. The persistence of bacterial infection is often associated with quorum-sensing-mediated biofilm formation. Thus, the disruption of this signaling circuit presents an attractive anti-virulence strategy. Quorum-quenching lactonases have been reported to be effective disrupters of quorum-sensing circuits. However, there have been very few reports of the effective use of these enzymes in disrupting bacterial biofilm formation. This protocol describes a method to disrupt biofilm formation in a clinically relevant *A. baumannii* S1 strain through the use of an engineered quorum-quenching lactonase. *Acinetobacter baumannii* is a major human pathogen implicated in serious hospital-acquired infections globally and its virulence is attributed predominantly to its biofilm's tenacity. The engineered lactonase treatment achieved significant *A. baumannii* S1 biofilm reduction. This study also showed the possibility of using engineered quorum-quenching enzymes in future treatment of biofilm-mediated bacterial diseases. Lastly, the method may be used to evaluate the competency of promising quorum-quenching enzymes.

Video Link

The video component of this article can be found at https://www.jove.com/video/53243/

Introduction

Treatment options for infectious diseases have been complicated by the rapid increase in multidrug-resistant bacteria that are immune to a wide range of antibiotic drugs[1]. With high morbidity and mortality rates from resistant bacteria-mediated infections, there is a need to escalate drug development processes and/or explore better anti-bacterial alternatives to improve therapeutic options. Lately, the anti-virulence approach is gaining interest given its potential in preventing virulence via non-bactericidal methods, hence mitigating the risks of resistance mechanisms[2].

Quorum-sensing is a 'master switch' in bacterial virulence and disruption of this signaling phenomenon is a promising anti-virulence method against pathogenesis[3]. The onset of virulence requires the accumulation of quorum molecules in the extracellular environment after a critical bacterial population density is reached. As quorum molecules diffuse back into the intracellular matrix, binding with their cognate receptors leads to the activation of virulence factors as well as genes associated with antibiotic resistance and biofilm formation[4]. In general, quorum-sensing disruption involves inhibiting quorum molecule and receptor interaction without affecting primary metabolic pathways. Hence, it does not have any direct implication on cellular growth. Since fitness is not compromised, there is minimal selection pressure for bacteria to evolve and gain resistance against such treatments[5]. In addition, quorum-sensing disruption can interfere with inherent bacterial protective mechanisms, as in the case of biofilm formation, which provides protection from anti-bacterial agents and host immune responses.

It is estimated that 99% of microbes on Earth exist in complex biofilm-like matrices, conferring crucial survival advantages to the microorganisms living within these structures[6]. More importantly, formation of these sessile domains is the cause of most persistent and chronic hospital-acquired infections[7]. *Acinetobacter baumannii* is one of the major human pathogens that is associated with global hospital-acquired infections and its virulence is largely attributed to quorum-sensing-mediated biofilm formation[8]. Quorum-quenching enzymes have been used successfully in disrupting quorum-mediated signal transduction by targeting a group of compounds known as *N*-acyl homoserine lactones (AHLs) that are produced by Gram-negative bacteria[9]. Several studies have also expanded upon the use of these enzymes to block bacterial pathogenesis through the reduction of virulence factor expression and cell numbers in biofilms[10,11]. Unfortunately, there remains a lack of palpable demonstration of the effective use of quorum-quenching enzymes against biofilm formation by bacterial pathogens. There have been attempts to use quorum inhibitors (AHL analogues), instead of quorum-quenching enzymes, to disrupt *A. baumannii* biofilm formation[12]. Although this method of using small molecules inhibitors is a valid approach, sustaining its bioavailability in translational uses can be a challenge. On the contrary, the use of catalytic quorum-quenching enzymes could circumvent the bioavailability issue as enzymes are more amenable towards immobilization on surfaces of biomedical devices for therapeutic effects.

Here, we describe an assessment of the effects of engineered quorum-quenching lactonases from *Geobacillus kaustophilus* (GKL)[13] on bacterial biofilm formation, using crystal violet staining and confocal laser scanning microscopy (CLSM). This study is the first successful demonstration of biofilm disruption in a clinically relevant *A. baumannii* S1 strain using quorum-quenching enzymes. The methods described in this study are useful for assessing the efficacy of other quorum-quenching enzymes in subsequent therapeutic development efforts against pathogenic Gram-negative bacteria.

Protocol

1. Crystal Violet Quantitation of Biofilm Formation in *A. baumannii* S1

1. Grow a 5 ml culture of *A. baumannii* S1 in Lysogeny broth (LB) (tryptone 10 g/L, yeast extract 5 g/L) at 30 °C in a shaking incubator (220 rpm) for 16 hr.
2. Adjust the culture of *A. baumannii* S1 to a desired OD_{600} of 0.8. Using a 96-well plate, inoculate the bacteria culture (1:100 dilution) into fresh LB containing 10 µl of purified GKL enzyme (40 mg/ml); the new culture's final volume is 100 µl.
3. Prepare a control culture as well; this will not contain any enzyme. Repeat similar conditions to yield the desired number of replicates.
4. Cover the plate with a lid and place it into a sealed 10 L plastic container. Incubate the plate at 30 °C for 3 hr before gently removing the media.
5. Add another 100 µl of fresh LB medium to the well and incubate the plate for 21 hr at 30 °C.
6. After the second period of incubation, gently remove all the media. Wash the planktonic bacteria cell with 200 µl sterile water. Ensure that there is only minimal disturbance to the cells during washing.
7. Add 100 µl of 1% crystal violet solution to each well and incubate for 15 min at RT. Remove the crystal violet solution by washing the well with 200 µl sterile water. Repeat the wash for two more times.
8. Add 100 µl of 33% acetic acid to each well and incubate for 15 min with gentle shaking; this will dissolve the dye.
9. Quantitate the amount of biofilm formed by measuring the absorbance of crystal violet at 600 nm. The amount of crystal violet is proportional to the amount of biofilm formed.

2. Confocal Laser Scanning Microscopy of *A. baumannii* S1 Biofilm

1. Grow a 5 ml culture of *A. baumannii* S1 in LB at 30 °C in a shaking incubator (220 rpm) for 16 hr.
2. Adjust the culture of *A. baumannii* S1 to a desired OD_{600} of 0.8. Using a 35 mm glass-bottomed µ-Dish, inoculate the bacteria culture (1:100 dilution) into fresh LB containing 30 µl of purified GKL enzyme (40 mg/ml); the new culture's final volume is 1 ml.
3. Cover the µ-Dish with a lid and place it in a sealed 10 L plastic container. Incubate the µ-Dish at 30 °C for 3 hr before gently removing the media. Add 30 µl of purified GKL enzyme and fresh medium, bringing it to a total volume of 1 ml. Incubate for another 21 hr at 30 °C.
4. Repeat step 2.3 and incubate the µ-Dish for another 24 hr at 30 °C. Then, remove the media gently.
5. Add 500 µl of 5 µg/ml Alex Fluo 488-conjugated wheat germ agglutinin (WGA) dissolved in Hank's balanced salt solution (HBSS) to the µ-Dish and incubate at 37 °C for 30 min; this will stain the formed biofilm. Remove the staining solution and wash the µ-Dish with 2 ml of HBSS. Repeat the wash step one more time.
6. Add 500 µl of 3.7 % formaldehyde dissolved in HBSS and incubate at 37 °C for 30 min; this will fix the biofilm onto the µ-Dish. Wash the µ-Dish once with 2 ml of HBSS and then remove the solution completely. The µ-Dish fixed with biofilm can be stored in the dark at 4 °C prior to CLSM imaging.
7. For CLSM imaging and analysis, use 63 times magnification to generate 97 stacks per image with an interval of 0.21 µm per stack.

Representative Results

In the crystal violet quantitation experiment, two quorum-quenching enzymes were used to demonstrate feasibility in disrupting biofilm formation: wild-type GKL and an improved GKL double mutant (E101G/R230C). Both enzymes have been shown to demonstrate lactonase activity against 3-hydroxy-decanoyl-L-homoserine lactone (3-OH-C_{10}-HSL), the major quorum molecule used by *A. baumannii* S1[14]. For valid assessment of biofilm disruption, their respective catalytically inactive enzymes (previously shown to not sequester AHL ligands) were also included as immediate controls (GKL D266N mutant and GKL E101G/R230C/D266N mutant). Besides using wild-type *A. baumannii* S1, the biofilm forming ability of a mutant Δ*abaI* strain (AHL synthase-deficient) was also tested. Both quorum-quenching enzymes, wild-type GKL and GKL E101G/R230C mutant were able to significantly reduce biofilm formation in pretreated *A. baumannii* S1 cultures ($n = 10$; p-value ≤ 0.0001) (**Figure 1**). Nevertheless, the extent of biofilm disruption for both enzymes is not proportional to their efficacy against 3-OH-C_{10}-HSL. The turnover rate (k_{cat}) of the GKL E101G/R230C mutant is six times faster than wild-type GKL against 3-OH-C_{10}-HSL[14]. However, the difference in the extent of biofilm disruption between the enzymes is only two-fold.

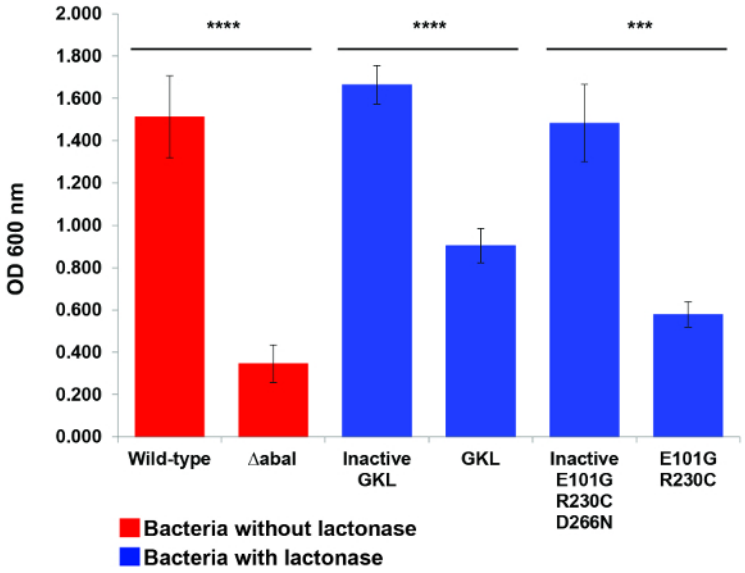

Figure 1. *A. baumannii* biofilm disruption assay. Biofilm formation was measured by crystal violet staining. Red columns represent the amount of biofilm formed by wild type *A. baumannii* and Δ*abal* mutant, without the addition of quorum-quenching lactonases. Blue columns represent the amount of biofilm formed by wild-type *A. baumannii* in the presence of four different GKL enzymes: inactive GKL D266N mutant, wild-type GKL, inactive GKL E101G/R230C/D266N mutant and GKL E101G/R230C mutant. ****, *P*-value of ≤ 0.0001. Reproduced with permission from *Antimicrobial Agents and Chemotherapy* 58, 1802-1805 (2014). Please click here to view a larger version of this figure.

Confocal imaging of *A. baumannii* S1 biofilm formation was used to provide a qualitative and quantitative measure of quorum-quenching effects on the structural morphology of these sessile domains. The improved GKL E101G/R230C mutant and its catalytically inactive enzyme were used for comparison. The differential image contrast (DIC) image showed that treatment with the improved GKL E101G/R230C mutant resulted in a decrease in biofilm size (**Figure 2**). Analysis of the fluorescence images also revealed that there was reduction in surface area, biomass and average thickness of biofilm when treated with the improved GKL E101G/R230C mutant (**Table 1**).

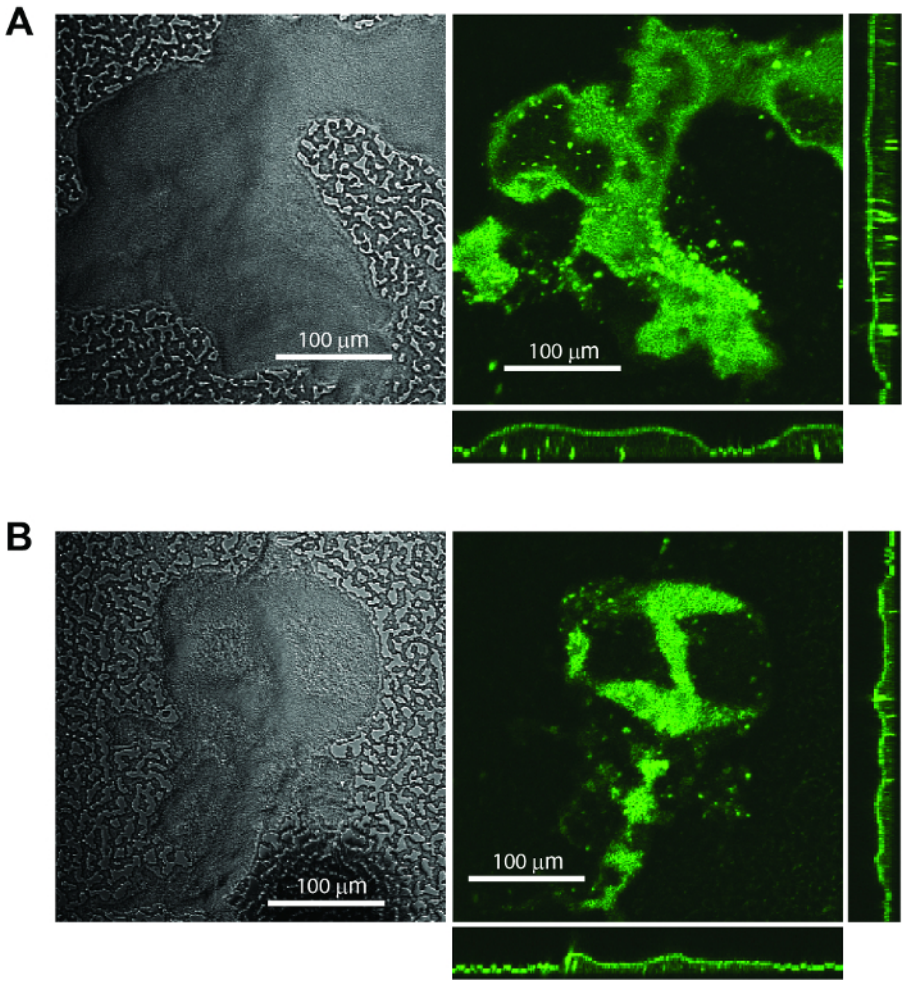

Figure 2. Representative confocal laser scanning microscopy images of *A. baumannii* biofilms. *A. baumannii* biofilms were treated with inactive GKL E101G/R230C/D266N mutant (**A**) and GKL E101G/R230C mutant (**B**) and stained with Alexa Fluor 488-conjugated WGA. DIC images of the biofilms (left) and fluorescence images of the biofilms (right) are shown for representative *xy* (center), *yz* (right), and *xz* (bottom) sections. Reproduced with permission from *Antimicrobial Agents and Chemotherapy* **58**, 1802-1805 (2014). Please click here to view a larger version of this figure.

	Value ± SD[a]		
Characteristic	**No treatment**	**Treatment with inactive mutant**	**Treatment with E101G/R230C mutant**
Biomass ($\mu m^3/\mu m^2$)	2.57 ± 1.65	3.39 ± 1.33	1.37** ± 0.20
Avg thickness (µm)	3.68 ± 2.51	3.41 ± 1.31	1.21** ± 0.21
Surface area (µm)	235,920.59 ± 79,456.46	209,872.6 ± 115,094.7	115,354.9* ± 7,630.3
[a] n = 10 image stacks. **, P ≤ 0.001; *, P ≤ 0.05, compared with treatment with inactive E101G/R230C/D266N mutant.			

Table 1. *A. baumannii* biofilm structural quantitation.

Discussion

In both sets of experiment, *A. baumannii* S1 was cultured in LB media without NaCl as a high salt concentration may reduce the amount of biofilm formed by the bacteria[15]. The presence of such artifact could underestimate the amount of biofilm formed, as well as the effects of quorum-quenching enzymes across different treatment conditions. The use of a catalytically inactive enzyme is important as a negative control to eliminate the possible effects of enzyme sequestration. **Figure 1** shows that even if inactive enzymes sequester quorum molecules, biofilm formation is not disrupted.

A. baumannii S1 forms a delicate ring-like biofilm structure between air and liquid interface in the well of the 96-well plate. Hence, it is crucial to avoid excessive agitation during media removal and washing steps to prevent incidental removal of bacterial biofilms. LB media was removed after each incubation to eliminate planktonic cells. In addition, the 96-well plate was placed in a 10 L plastic container to minimize perturbations

in airflow and to create a micro-anaerobic environment that favors biofilm formation. Biofilm quantitation in a 96-well plate is also dependent on the amount of crystal violet added to each well. In the event that excess crystal violet is added into a well, the number of wash steps may be increased. The crystal violet staining experiment provides a relative comparison of quorum-quenching efficiencies in biofilm disruption. Although crystal violet staining is quantitative for measuring biofilm formation, it is only semi-quantitative in terms of the catalytic efficiency of quorum-quenching enzymes. For accurate statistical comparison, sufficient sample sizes are important for the different treatment groups. Outliers should also be removed to prevent misrepresentation of results.

Confocal imaging and analysis of bacterial biofilm is a useful tool for assessing the qualitative effects of quorum-quenching enzymes. However, the process of selecting biofilm for analysis may be a potential channel for bias if the user is aware of the type of quorum-quenching enzyme administered (active or inactive). To avoid the possibility of bias, the experiment can be designed to exclude enzyme information from the user or use a random approach for selection. An unbiased selection strategy also enables better quantitative comparison of biofilm morphologies by CLSM. Nevertheless, this is the first study that describes a method for evaluating the outcome of quorum-quenching enzymes on bacterial biofilms. Crystal violet staining has demonstrated the utility of quorum-quenching enzymes in biofilm disruption and revealed that enzymes' *modi operandorum* are not limited to reducing cell numbers and virulence factor expression. Meanwhile, morphological changes determined by CLSM analysis can also provide insights into possible molecular targets of these enzymes. Although the current experiment was designed to investigate the effects of enzymes pretreatment on bacterial biofilms, the protocol may be modified to assess the enzyme's effect on preformed biofilm by adding the enzyme after bacterial growth or to examine its competency under physiological conditions through the use of serum-like conditions for culture. The protocol used to study GKL enzymes may be extended to other quorum-quenching enzymes and pathogens to investigate their relationship in biofilm disruption.

Disclosures

The authors declare that they have no competing financial interests.

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