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Disruption of Biofilm Formation by the Human Pathogen *Acinetobacter baumannii* Using Engineered Quorum-Quenching Lactonases

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***Acinetobacter baumannii* is a major human pathogen associated with multidrug-resistant nosocomial infections; its virulence is attributed to quorum-sensing-mediated biofilm formation, and disruption of biofilm formation is an attractive antivirulence strategy. Here, we report the first successful demonstration of biofilm disruption in a clinical isolate of *A. baumannii* S1, using a quorum-quenching lactonase obtained by directed evolution; this engineered lactonase significantly reduced the biomass of *A. baumannii*-associated biofilms, demonstrating the utility of this antivirulence strategy.**

Acinetobacter baumannii is a Gram-negative bacterium that has gained global notoriety due to its rapid emergence as an opportunistic pathogen in nosocomial or hospital-acquired infections (1). The high morbidity rate associated with *A. baumannii*-mediated infections has earned the bacterial pathogen the moniker of the Gram-negative methicillin-resistant *Staphylococcus aureus* (MRSA) (2). Bacterial transmission between patients in hospitals has been associated with the use of indwelling medical devices, such as catheters and implants (3, 4). The situation is exacerbated by the emergence of a number of *A. baumannii* isolates that were found to be resistant to carbapenem, an antibiotic used for the treatment of infections caused by *A. baumannii* (5). The persistency of *A. baumannii* in hospital-acquired infections has been associated with biofilm formation by the bacteria; the biofilm provides protection for the bacteria against host immune systems and antibiotic treatment (6, 7).

The process of biofilm formation in many bacteria is mediated through quorum-sensing pathways. In *A. baumannii*, biofilm is formed upon the activation of a typical LuxI/LuxR-type quorum-sensing network that involves an *abaI* synthase and *abaR* receptor (8, 9). Although various forms of *N*-acyl-homoserine lactones (AHLs) were found to be present in various *Acinetobacter* spp., a study demonstrated that 3-hydroxy-dodecanoyl-L-homoserine lactone (3-OH-C_{12}-HSL) is the major quorum signal that is produced by the M2 strain of *A. baumannii* (9, 10). Use of AHL analogues to inhibit the quorum-sensing pathway of *A. baumannii* has been proven to be a valid strategy in the attenuation of biofilm formation in this bacterium (11). This antivirulence strategy is therapeutically attractive since it targets the virulence of the bacteria and hence minimizes the chance for the selection of resistant strains.

Quorum quenching can also be achieved through the enzymatic degradation of the quorum signal by an AHL lactonase (AHLase) (12, 13). Numerous attempts have been made to extend the application of these enzymes in the attenuation of bacterial virulence in human pathogens. Although it had been demonstrated that the expression level of virulence factors in *Pseudomonas aeruginosa* can be attenuated by AHLases (14), there is currently no evidence to suggest the effective use of quorum-quenching enzymes in the disruption of biofilm formation in bacterial pathogens. Recently, we reported on the directed evolution of a thermostable quorum-quenching lactonase from *Geobacillus kaustophilus* (GKL); a thermostable engineered mutant of the quorum-quenching enzyme was obtained with enhanced catalytic activity and broadened substrate range against AHLs (15). This enzyme belongs to the phosphotriesterase-like lactonase (PLL) family of the amidohydrolase superfamily and possesses the commonly encountered $(\beta/\alpha)_8$-barrel fold (16). Here, we report the use of this catalytically enhanced mutant enzyme in the disruption of biofilm formation by *A. baumannii*. With its inherent thermostability and molecular tractability (modulability in activity and substrate range through choice mutations in the enzyme scaffold [17]), we envision the further development of this enzyme (and other quorum-quenching enzyme scaffolds) for use as antivirulence therapeutics against *A. baumannii*-mediated infections; this demonstration also illustrates the utility of quorum-quenching enzymes in addressing the increasing therapeutic needs of our generation.

Our previous efforts in enhancing the catalytic activity (and broadening the substrate range) of a thermostable AHL lactonase resulted in the development of a number of GKL mutants with enhanced catalytic efficiency (k_{cat}/K_m) against various forms of AHLs (15). Although a large panel of AHLs was previously tested for reactivity, past unavailability of C-3 hydroxyl-substituted AHLs prevented an assessment of the lactonase activities of our engineered enzymes against these quorum molecules. In fact, C-3-hydroxylated AHLs were rarely tested as the substrates for AHL lactonases, and hence very little information is available with regard to the effect of hydroxylation at the C-3 position of the acyl chains (of the lactone substrates) on the catalytic efficiency of these enzymes (16, 18, 19).

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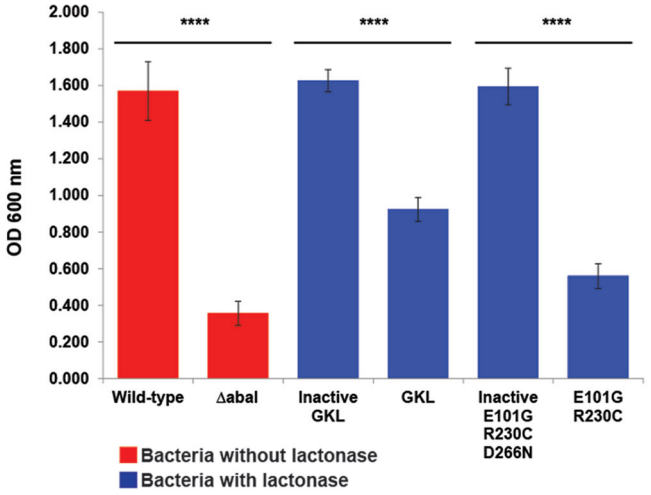

FIG 1 Biofilm disruption assay. Biofilm was quantitated by crystal violet staining. Red columns represent the amount of biofilm formed by *A. baumannii* (wild type and Δ*abaI* mutant, respectively) without the addition of AHL lactonases. Blue columns represent the amount of biofilm formed by wild-type *A. baumannii* in the presence of different GKL enzymes (inactive D266N GKL, wild-type GKL, inactive E101G/R230C/D266N GKL, and E101G/R230C GKL, respectively). ****, *P* value of ≤0.0001.

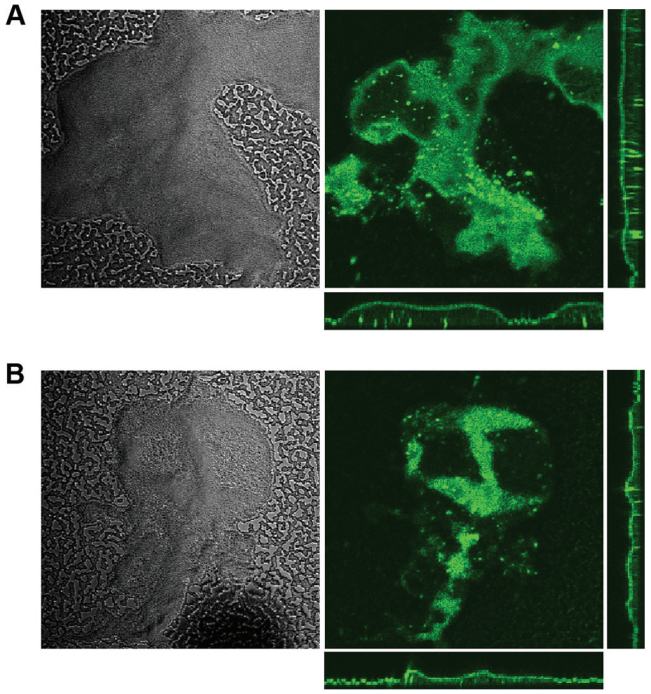

FIG 2 Representative confocal laser scanning microscopy images of *A. baumannii* biofilms. *A. baumannii* biofilms were treated with inactive E101G/R230C/D266N GKL (A) and E101G/R230C GKL (B) and stained with Alexa Fluor 488-conjugated WGA. DIC images of the biofilms (left) and fluorescence images of the biofilms (right) are shown for representative *xy* (center), *yz* (right), and *xz* (bottom) sections.

Although it has been reported that the M2 strain of *A. baumannii* uses 3-OH-C_{12}-HSL as the major quorum molecule, we found that a clinical isolate, *A. baumannii* S1, uses 3-hydroxy-decanoyl-L-homoserine lactone (3-OH-C_{10}-HSL) as the major quorum signaling molecule (see Table S1 in the supplemental material). Since the lactonase activity of the wild type and the E101G/R230C mutant of GKL against both 3-OH-C_{10}-HSL and 3-OH-C_{12}-HSL, respectively, was not known, we sought to determine the hydrolytic activities of our enzymes against these *A. baumannii* quorum substrates. Using wild-type GKL, we were unable to detect lactonase activity against 3-OH-C_{12}-HSL; the unevolved enzyme hydrolyzed 3-OH-C_{10}-HSL with a k_{cat} of ≤0.011 s^{-1} (see Table S2 in the supplemental material). Using the evolved mutant GKL enzyme (E101G/R230C), reasonable kinetic parameters against 3-OH-C_{10}-HSL and 3-OH-C_{12}-HSL, respectively, were observed (k_{cat}/K_m of 180 M^{-1} s^{-1} and 150 M^{-1} s^{-1}, respectively). A more detailed comparison of the kinetic parameters with different forms of C_{10}-HSL and C_{12}-HSL revealed that the catalytic rates of the wild-type and mutant GKL varied with chain length and substitution of the AHLs.

The wild type and the AHL synthase-deficient (Δ*abaI*) mutant of *A. baumannii* were cultured in a low-salt medium at 30°C, and biofilm formation was determined using crystal violet staining. Within expectation, a deletion of the AHL synthase (Δ*abaI*) from the genome of *A. baumannii* resulted in a reduction in the amount of biofilm formed by the mutant bacteria (Fig. 1). In an attempt to determine the effect of quorum-quenching lactonases on biofilm formation, both wild-type GKL, the catalytically inactive E101G/R230C/D266N mutant of GKL, and the catalytically enhanced E101G/R230C mutant of GKL were added to a log-phase culture of wild-type *A. baumannii*. The catalytically inactive D266N mutant of GKL (and the equivalent catalytically inactive E101G/R230C/D266N mutant) served as negative controls to rule out any sequestration effects exhibited by the quorum-quenching lactonases. Significant reduction in the amount of biofilm formed was observed in the presence of the engineered mutant enzyme (Fig. 1 and 2).

Confocal laser scanning microscopy (CLSM) was used to (qualitatively and quantitatively) assess the effect of lactonase treatment on the overall morphology and architecture of the *A. baumannii* biofilm. We chose to use the engineered E101G/R230C mutant based upon the observed enhancement in lactonase activity. As shown in the differential image contrast (DIC) image, treatment with the enhanced GKL mutant caused a reduction in the size of the biofilm (Fig. 2). The biofilm was stained with Alexa Fluor 488-conjugated wheat germ agglutinin (WGA) to reveal a reduction in the biomass, thickness, and surface area of the biofilm after treatment with the engineered lactonase (Fig. 2, Table 1).

In addition, although it had been shown that AHL analogues could also prevent the formation of biofilm in *A. baumannii* (11), the bioavailability (or lack thereof) of these quorum inhibitors has limited their translational potential in the treatment of quorum-mediated diseases. The use of catalytic quorum-quenching enzymes, on the other hand, could circumvent the bioavailability problem. One could envision synergy/complementarity between these two therapeutic approaches in the treatment of quorum-mediated diseases: a bioactive, multifunctional biomaterial (involving immobilized quorum-quenching enzymes and matrix-entrapped quorum inhibitors for controlled release) could be used to functionalize the surface of catheters or implants to prevent biofilm formation.

The complexity of bacterial quorum-signaling systems has limited the success of using quorum-quenching enzymes for bio-

TABLE 1 Quantification of untreated and treated *A. baumannii* biofilm structures

Characteristic	Value ± SD[a]		
	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
Maximum thickness (μm)	11.49 ± 4.72	13.82 ± 4.17	7.75** ± 1.63
Surface area (μm)	235,920.59 ± 79,456.46	209,872.6 ± 115,094.7	115,354.9* ± 7,630.3
Surface-to-vol ratio ($\mu m^2/\mu m^3$)	2.74 ± 1.53	1.57 ± 1.06	1.90 ± 0.17
Roughness coefficient	0.58 ± 0.17	0.65 ± 0.10	0.59 ± 0.08

[a] $n = 10$ image stacks. ***, $P \leq 0.001$; **, $P \leq 0.01$; *, $P \leq 0.05$, compared with treatment with inactive E101G/R230C/D266N mutant.

film disruption of bacterial pathogens: (i) recombinant AiiA (a type of AHL lactonase belonging to the metallo-β-lactamase superfamily) was used to reduce the amount of planktonic cells residing within *P. aeruginosa*-mediated biofilm structures (20); (ii) immobilized SsoPox, an orthologue of GKL, was used to inhibit the production of various virulence factors in *P. aeruginosa* (18). However, in both studies, there was no direct evidence for the reduction of the biomass of the biofilm structures.

In this study, we determined the rates of hydrolysis of two biologically relevant C-3-hydroxylated AHLs (3-OH-C_{10}-HSL and 3-OH-C_{12}-HSL, respectively) by quorum-quenching lactonases. The rates observed with these substrates revealed that modification via hydroxylation of the C-3 position (in comparison to unmodified decanoyl and dodecanoyl HSLs) resulted in a decrease in catalytic efficiencies (k_{cat}/K_m); nevertheless, the broadened range of substrate specificities (promiscuities) translated to a serendipitous quenching of quorum-mediated biofilm formation, resulting in a decrease in biomass (and thickness) of the biofilm structures. Our observation of the use of an alternate AHL (3-OH-C_{10}-HSL, instead of the previously identified 3-OH-C_{12}-HSL for the M2 strain) by a clinical isolate of *A. baumannii* (the S1 strain) further highlighted the utility of engineered quorum-quenching lactonases with broad-spectrum (range) reactivities. In addition, the effect of biofilm disruption against the S1 strain was also evident in the presence of serum-like conditions (see Fig. S5 in the supplemental material); the translational potential of using the quorum-quenching enzymes to disrupt biofilm formation was further highlighted by the observed stability of the enzymes in bacterial cultures (see Fig. S6). The efficacy of biofilm disruption was also demonstrated against multiple strains of *A. baumannii* (S2 and R2 strains, respectively; see Fig. S7 and S8) and against preformed biofilm (see Fig. S9).

In summary, we have provided the first demonstration of the use of recombinant quorum-quenching enzymes in the disruption of biofilm formation (*vis a vis* reduction in biomass and thickness) by a bacterial human pathogen, *A. baumannii*. A decrease in the biomass of biofilm can translate to more effective antibiotic therapies due to the increased susceptibility of bacteria toward antibiotic treatments. We believe that future development of quorum-quenching enzymes will be critical in translating the utility of this therapeutic route in the treatment of biofilm-mediated bacterial diseases.

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REFERENCES

1. **Perez F, Hujer AM, Hujer KM, Decker BK, Rather PN, Bonomo RA.** 2007. Global challenge of multidrug-resistant *Acinetobacter baumannii*. Antimicrob. Agents Chemother. **51:**3471–3484. http://dx.doi.org/10.1128/AAC.01464-06.
2. **Ho P-L, Cheng VC-C, Chu C-M.** 2009. Antibiotic resistance in community-acquired pneumonia caused by *Streptococcus pneumoniae*, methicillin-resistant *Staphylococcus aureus*, and *Acinetobacter baumannii*. Chest **136:**1119–1127. http://dx.doi.org/10.1378/chest.09-0285.
3. **Weinstein RA, Gaynes R, Edwards JR, System NNIS.** 2005. Overview of nosocomial infections caused by Gram-negative bacilli. Clin. Infect. Dis. **41:**848–854. http://dx.doi.org/10.1086/432803.
4. **Sunenshine RH, Wright MO, Maragakis LL, Harris AD, Song X, Hebden J, Cosgrove SE, Anderson A, Carnell J, Jernigan DB, Kleinbaum DG, Perl TM, Standiford HC, Srinivasan A.** 2007. Multidrug-resistant *Acinetobacter* infection mortality rate and length of hospitalization. Emerg. Infect. Dis. **13:**97–103. http://dx.doi.org/10.3201/eid1301.060716.
5. **Kempf M, Rolain JM.** 2012. Emergence of resistance to carbapenems in *Acinetobacter baumannii* in Europe: clinical impact and therapeutic options. Int. J. Antimicrob. Agents **39:**105–114. http://dx.doi.org/10.1016/j.ijantimicag.2011.10.004.
6. **Gaddy JA, Actis LA.** 2009. Regulation of *Acinetobacter baumannii* biofilm formation. Future Microbiol. **4:**273–278. http://dx.doi.org/10.2217/fmb.09.5.
7. **Thallinger B, Prasetyo EN, Nyanhongo GS, Guebitz GM.** 2013. Antimicrobial enzymes: an emerging strategy to fight microbes and microbial biofilms. Biotechnol. J. **8:**97–109. http://dx.doi.org/10.1002/biot.201200313.
8. **Clemmer KM, Bonomo RA, Rather PN.** 2011. Genetic analysis of surface motility in *Acinetobacter baumannii*. Microbiology **157:**2534–2544. http://dx.doi.org/10.1099/mic.0.049791-0.
9. **Bhargava N, Sharma P, Capalash N.** 2010. Quorum sensing in *Acinetobacter*: an emerging pathogen. Crit. Rev. Microbiol. **36:**349–360. http://dx.doi.org/10.3109/1040841X.2010.512269.
10. **Niu C, Clemmer KM, Bonomo RA, Rather PN.** 2008. Isolation and characterization of an autoinducer synthase from *Acinetobacter baumannii*. J. Bacteriol. **190:**3386–3392. http://dx.doi.org/10.1128/JB.01929-07.
11. **Stacy DM, Welsh MA, Rather PN, Blackwell HE.** 2012. Attenuation of quorum sensing in the pathogen *Acinetobacter baumannii* using non-native N-Acyl homoserine lactones. ACS Chem. Biol. **7:**1719–1728. http://dx.doi.org/10.1021/cb300351x.
12. **Dong YH, Wang LH, Xu JL, Zhang HB, Zhang XF, Zhang LH.** 2001. Quenching quorum-sensing-dependent bacterial infection by an N-acyl homoserine lactonase. Nature **411:**813–817. http://dx.doi.org/10.1038/35081101.
13. **Chow JY, Wu L, Yew WS.** 2009. Directed evolution of a quorum-quenching lactonase from *Mycobacterium avium* subsp. *paratuberculosis* K-10 in the amidohydrolase superfamily. Biochemistry **48:**4344–4353. http://dx.doi.org/10.1021/bi9004045.
14. **Ng FS, Wright DM, Seah SY.** 2011. Characterization of a phosphotriesterase-like lactonase from *Sulfolobus solfataricus* and its immobilization

for disruption of quorum sensing. Appl. Environ. Microbiol. **77:**1181–1186. http://dx.doi.org/10.1128/AEM.01642-10.

15. **Kelly RC, Bolitho ME, Higgins DA, Lu W, Ng WL, Jeffrey PD, Rabinowitz JD, Semmelhack MF, Hughson FM, Bassler BL.** 2009. The *Vibrio cholerae* quorum-sensing autoinducer CAI-1: analysis of the biosynthetic enzyme CqsA. Nat. Chem. Biol. **5:**891–895. http://dx.doi.org/10.1038/nchembio.237.
16. **Afriat L, Roodveldt C, Manco G, Tawfik DS.** 2006. The latent promiscuity of newly identified microbial lactonases is linked to a recently diverged phosphotriesterase. Biochemistry **45:**13677–13686. http://dx.doi.org/10.1021/bi061268r.
17. **Sharma Y.** 2012. Developing countries face up to synthetic biology challenges. SciDev.net, London, United Kingdom. http://www.scidev.net/global/agriculture/farming/.
18. **Hawwa R, Aikens J, Turner RJ, Santarsiero BD, Mesecar AD.** 2009. Structural basis for thermostability revealed through the identification and characterization of a highly thermostable phosphotriesterase-like lactonase from *Geobacillus stearothermophilus*. Arch. Biochem. Biophys. **488:**109–120. http://dx.doi.org/10.1016/j.abb.2009.06.005.
19. **Xiang DF, Kolb P, Fedorov AA, Meier MM, Fedorov LV, Nguyen TT, Sterner R, Almo SC, Shoichet BK, Raushel FM.** 2009. Functional annotation and three-dimensional structure of Dr0930 from *Deinococcus radiodurans*, a close relative of phosphotriesterase in the amidohydrolase superfamily. Biochemistry **48:**2237–2247. http://dx.doi.org/10.1021/bi802274f.
20. **Igarashi J, Suga H.** 2011. Custom synthesis of autoinducers and their analogues. Methods Mol. Biol. **692:**265–274. http://dx.doi.org/10.1007/978-1-60761-971-0_19.