AAC Accepts, published online ahead of print on 30 December 2013
Antimicrob. Agents Chemother. doi:10.1128/AAC.02410-13
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Disruption of Biofilm Formation by the Human Pathogen *Acinetobacter baumannii* using Engineered Quorum-quenching Lactonases

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Running Title: AHLase disruption of *Acinetobacter baumannii* biofilm.

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ABSTRACT

Acinetobacter baumannii is a major human pathogen associated with multi-drug resistant nosocomial infections; its virulence is attributed to quorum-sensing-mediated biofilm formation, and disruption of biofilm formation is an attractive anti-virulence 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 anti-virulence strategy.

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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-C12-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 bacteria (11) . This anti-virulence 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

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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 kaustropilus* (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 anti-virulence 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 C3 hydroxyl-substituted AHLs prevented an assessment of the lactonase activities of our engineered enzymes against these quorum molecules. In fact, C3-hydroxylated AHLs were rarely tested as substrates for AHL-lactonases, and hence, very little information is available

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with regards to the effect of hydroxylation at the C3 position of the acyl chains (of the lactone substrates) on the catalytic efficiency of these enzymes (16,18,19).

Although it has been reported that the M2 strain of *A. baumannii* uses 3-OH-C12-HSL as the major quorum molecule, we found that a clinical isolate, *A. baumannii* S1, uses 3-hydroxy-decanoyl-L-homoserine lactone (3-OH-C10-HSL) as the major quorum signaling molecule (Table S1 of the Supplemental Material). Since the lactonase activity of the wild-type and E101G/R230C mutant of GKL against both 3-OH-C10-HSL and 3-OH-C12-HSL, respectively, were 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-C12-HSL; the unevolved enzyme hydrolyzed 3-OH-C10-HSL with a $k_{cat} \leq 0.011$ s^{-1} (Table S2 of the Supplemental Material). Using the evolved mutant GKL enzyme (E101G/R230C), reasonable kinetic parameters against 3-OH-C10-HSL and 3-OH-C12-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 C10-HSL and C12-HSL revealed that the catalytic rates of the wild-type and mutant GKL varied with chain length and substitution of the AHLs.

Wild-type and AHL-synthase-deficient (ΔabaI) mutant of *A. baumannii* were cultured in a low salt medium at 30 ^{O}C and biofilm formation was determined using crystal violet staining. Within expectation, a deletion of the AHL-synthase (ΔabaI) from the genome of *A. baumannii* resulted in a reduction in the amount of biofilm formed by the mutant bacteria (Figure 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

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104 controls to rule out any sequestration effects exhibited by the quorum-quenching lactonases.
105 Significant reduction in the amount of biofilm formed was observed in the presence of the
106 engineered mutant enzyme (Figures 1 and 2).

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

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

124 The complexity of bacterial quorum-signaling systems has limited the success of
125 using quorum-quenching enzymes for biofilm disruption of bacterial pathogens: 1)
126 recombinant AiiA (a type of AHL-lactonase belonging to the metallo-β-lactamase
127 superfamily) was used to reduce the amount of planktonic cells residing within *P.*
128 *aeruginosa*-mediated biofilm structures (20); 2) immobilized SsoPox, an orthologue of GKL,

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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 C3-hydroxylated AHLs (3-OH-C10-HSL and 3-OH-C12-HSL, respectively) by quorum-quenching lactonases. The rates observed with these substrates revealed that modification *via* hydroxylation of the C3 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-C10-HSL instead of the previously identified 3-OH-C12-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 (Figure S5); 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 (Figure S6). The efficacy of biofilm disruption was also demonstrated against multiple strains of *A. baumannii* (S2 and R2 strains, respectively, Figures S7 and S8), and against pre-formed biofilm (Figure 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 bio-mass 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 towards antibiotic treatments. We believe that future development

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of quorum-quenching enzymes will be critical in translating the utility of this therapeutic route in the treatment of biofilm-mediated bacterial diseases.

ACKNOWLEDGEMENTS

We would like to thank Otsuka Pharmaceuticals for the kind gift of 3-oxo-C12-HSL. We also thank Dr. Lai Lai Yap for her technical assistance in confocal scanning laser microscopy. This work was supported by grants from the Academic Research Fund of the Ministry of Education, and the National Medical Research Council and the National Research Foundation, Singapore.

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229 **FIGURE LEGENDS**

230 Figure 1. Biofilm disruption assay. Biofilm was quantitated by crystal violet staining. Red
231 columns represent amount of biofilm formed by *A. baumannii* (wild-type and ΔabaI mutant,
232 respectively) without the addition of AHL-lactonases. Blue columns represent the amount of
233 biofilm formed by wild-type *A. baumannii* in the presence of different GKL enzymes
234 (inactive D266N GKL, wild-type GKL, inactive E101G/R230C/D266N GKL, and
235 E101G/R230C GKL, respectively). ****, p value ≤ 0.0001.

236

237 Figure 2. Representative confocal laser scanning microscopy images of *A. baumannii*
238 biofilms. *A. baumannii* were treated with A) inactive E101G/R230C/D266N GKL, and B)
239 E101G/R230C GKL, and stained with Alex Fluo 488-conjugated WGA. DIC images of the
240 biofilms (left panels), and fluorescence images of the biofilms (right panels) are shown for
241 representative *xy* (center), *yz* (right) and *xz* (bottom) sections.

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243 Table 1: Quantification of untreated and treated *A. baumannii* biofilm structures.

	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
Average 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)	235920.59 ± 79456.46	209872.6 ± 115094.7	115354.9* ± 7630.3
Surface to volume 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

244 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.

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246 Figure 1

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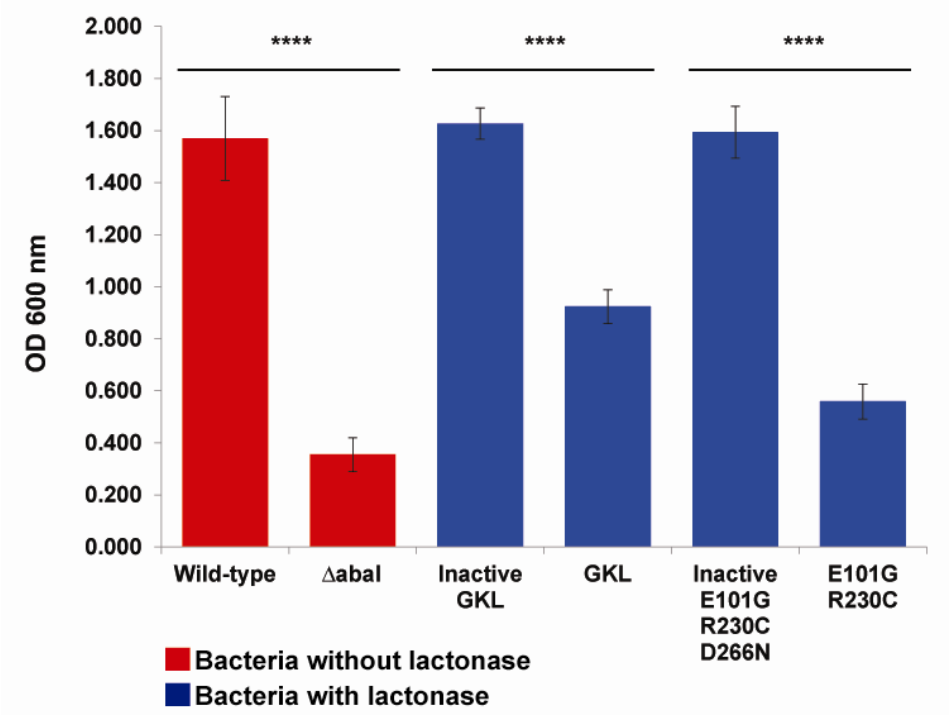

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260 Figure 2

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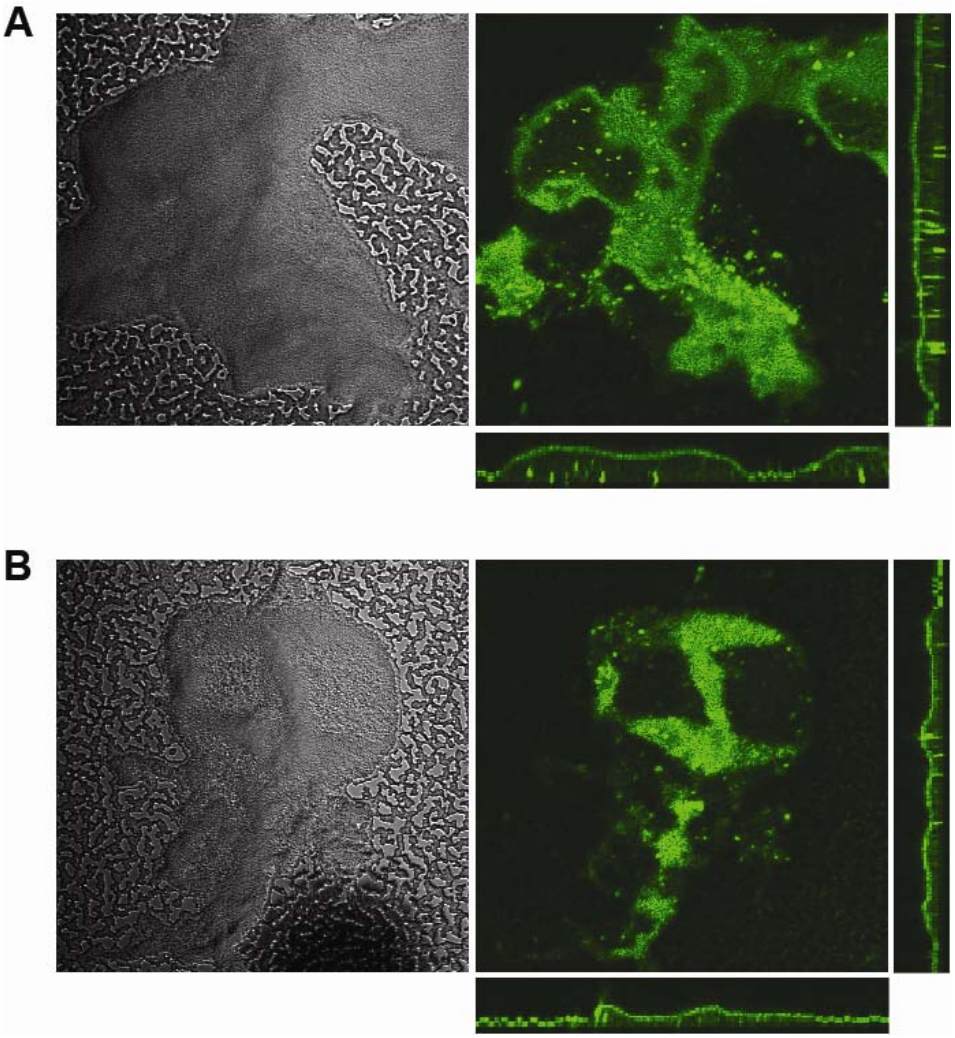

263

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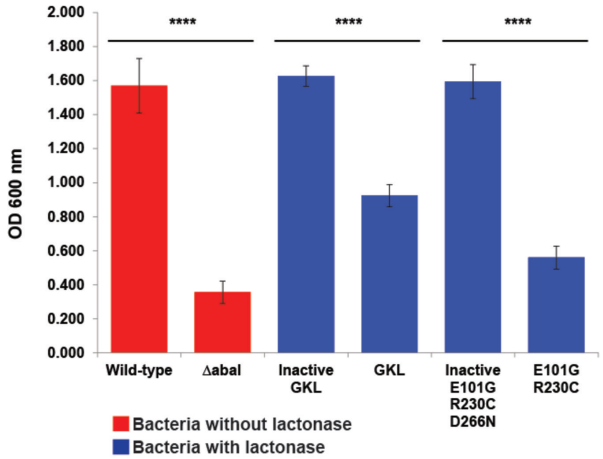

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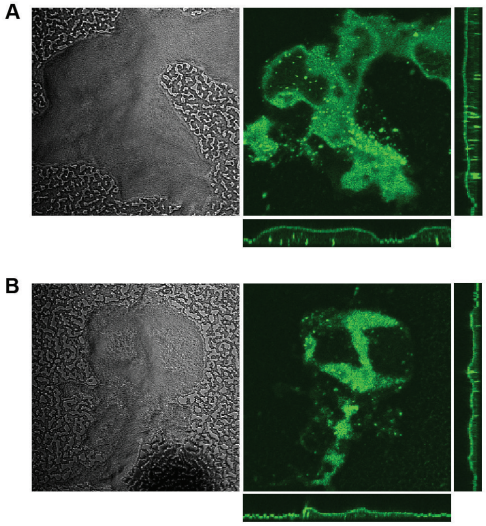

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