

Attenuation of Quorum Sensing in the Pathogen *Acinetobacter baumannii* Using Non-native *N*-Acyl Homoserine Lactones

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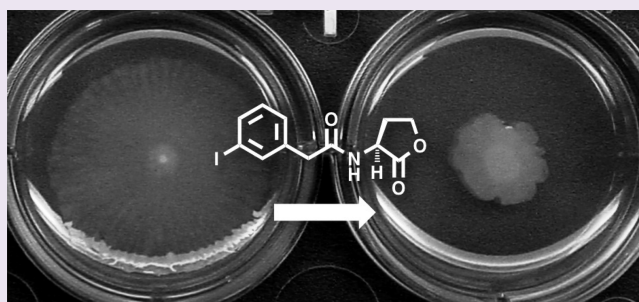
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S Supporting Information

ABSTRACT: Many bacterial pathogens use quorum sensing (QS) to control virulence. As a result, the development of methods to intercept QS has attracted significant interest as a potential anti-infective therapy. *Acinetobacter baumannii* has emerged as a pan-drug-resistant pathogen and displays a remarkable ability to persist in hospital settings despite desiccation and antimicrobial treatment. Recent studies have shown that *A. baumannii* QS mutants have limited motility and fail to form mature biofilms; these phenotypes are linked to its ability to persist on biotic and abiotic surfaces and increase its pathogenicity. *A. baumannii* uses *N*-(3-hydroxydodecanoyl)-*L*-homoserine lactone (OH-dDHL) and its putative cognate receptor, AbaR, for QS. We sought to identify non-native ligands capable of blocking or promoting AbaR activity in *A. baumannii* for use as chemical probes to modulate QS phenotypes in this pathogen. We screened a focused library of synthetic, non-native *N*-acyl homoserine lactones (AHLs) to identify such compounds, and several highly potent antagonists and agonists were uncovered, with IC_{50} and EC_{50} values in the low micromolar range, respectively. The strongest AbaR antagonists largely contained aromatic acyl groups, whereas the AbaR agonists closely resembled OH-dDHL. Notably, the 10 most potent AbaR antagonists also strongly inhibited *A. baumannii* motility, and five antagonists reduced biofilm formation in *A. baumannii* by up to 40%. The discovery of these compounds is significant, as they represent, to our knowledge, the first non-native modulators of QS in *A. baumannii* to be reported and could find utility as new tools to study the role and timing of QS phenotypes in *A. baumannii* infections.



Bacteria use small molecule or peptidic signals to assess their local population densities in a process termed quorum sensing (QS).^{1–3} The concentration of QS signals in a given environment can be correlated with cell density. Once the bacteria reach a sufficiently large population, they will shift from a unicellular to a largely multicellular existence and modify gene expression levels to initiate a broad range of group behaviors that benefit the growing community.⁴ These QS phenotypes include biofilm formation, virulence factor production, swarming, sporulation, conjugation, and bioluminescence and often play a critical role in mediating pathogenic or symbiotic relationships with a eukaryotic host.^{5–7} For instance, many pathogens will become virulent only after perceiving that a “quorum” of cells has been reached, thus increasing the probability that the bacterial population can survive the host immune response.^{6,8} Copious questions remain about QS signaling mechanisms and their roles in host–bacteria interactions, especially in infections, as several of the most deadly human pathogens use QS to control virulence (e.g., *Pseudomonas aeruginosa* and *Staphylococcus aureus*). As such, methods to block bacterial QS would be valuable to study this

signaling process at a fundamental level and could provide a route to the development of novel anti-infective strategies.^{9–11}

QS in Gram-negative bacteria has been studied extensively over the past 25 years and is typically mediated by diffusible *N*-acyl *L*-homoserine lactone (AHLs) signals (Figure 1A).^{12,13} The

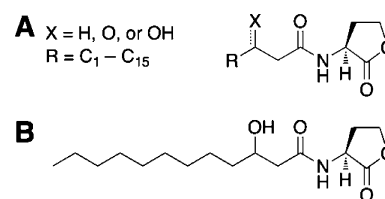


Figure 1. Structures of *N*-acyl *L*-homoserine lactones (AHLs). (A) Generic native AHL structures. (B) The structure of *N*-(3'-hydroxydodecanoyl)-*L*-HL (OH-dDHL), the predominant autoinducer produced by *A. baumannii*. Stereochemistry at the 3'-postion is shown as undefined.

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AHLs are generated *via* LuxI-type synthases and perceived by intracellular LuxR-type receptors that behave as transcriptional activators. The AHL signals are produced at low basal levels, and a sufficient density of cells is required to generate an adequate concentration of AHL for productive LuxR-type receptor binding. The AHL:LuxR-type receptor complexes then typically dimerize, bind to various QS promoters in the bacterial genome, and activate the transcription of genes linked to QS phenotypes. The LuxI/LuxR circuit constitutes the minimal system necessary for QS in Gram-negative bacteria. Several bacteria regulate QS *via* the complex interplay of multiple LuxI/LuxR circuits, however, and the pathogen *P. aeruginosa* is a prominent example of such.^{14,15}

Interception of LuxI/LuxR-type QS circuits represents one approach to attenuate QS phenotypes in Gram-negative bacteria, and strategies aimed at blocking AHL:LuxR-type receptor binding with either small molecules or macromolecules have attracted considerable attention.^{10,16–18} Our laboratory and others have recently uncovered a series of non-native AHLs and AHL analogues that are capable of strongly antagonizing or agonizing LuxR-type receptors in a range of Gram-negative bacteria.^{16,18–32} These compounds represent useful chemical probes for the study of QS phenotypes in both pathogens and symbionts and can provide methods for the spatial and temporal control of QS in a range of environments that are challenging using traditional genetic techniques. One of our major goals is to utilize these molecules to examine the role of QS in host–pathogen interactions,^{33,34} and we have recently shown that several non-native AHLs can strongly block virulence in the plant pathogens *Pectobacterium carotovora* and *Pseudomonas syringae* on their native plant hosts.³⁵ We now seek to extend these studies to mammalian systems and are focused on identifying potent QS modulators in Gram-negative human and animal pathogens. In the current study, we report our investigations of the activity of non-native AHLs in *Acinetobacter baumannii*, which has recently emerged as a major nosocomial pathogen and uses QS to control phenotypes linked to virulence.

A. baumannii was classified as a relatively low-grade, opportunist pathogen up until 30 years ago and was often ignored in clinical settings. The recent development of multi- and pan-drug-resistant strains of *A. baumannii* in hospitals, however, has brought renewed attention to this pathogen. *A. baumannii* infections cause serious diseases in immunocompromised human hosts, including ventilator-associated pneumonia, septicemia, and urinary tract and wound infections. Further attention has been drawn to these infections due to their incidence in injured U.S. military personnel returning from the Middle East over the past decade. Indeed, *A. baumannii* has been coined the “Gram-negative MRSA” due to its prevalence and rapid resistance development. The ability of clinical strains to survive desiccation and nutrient starvation in hospital settings makes *A. baumannii* yet more problematic, allowing it to persist on surfaces for extended periods of time and contributing to frequent infection outbreaks in ICUs.^{36,37} For example, certain *A. baumannii* strains can survive desiccated environments with a mean survival time of 27 days.^{38,39} The ability of *A. baumannii* to persist has been attributed to its capacity to form sessile biofilms on medically relevant biotic and abiotic surfaces,⁴⁰ and recently biofilm formation in *A. baumannii* has been linked to its LuxI/LuxR-type QS system (AbaI/AbaR, see below). Surface motility, which can play a role in biofilm formation, is also under the control of QS in *A.*

baumannii.⁴¹ This connection between virulence-associated phenotypes in *A. baumannii* and QS motivated us to examine the activity of our non-native AHLs as QS modulators in this treacherous pathogen.

The only regulatory QS proteins identified in *A. baumannii* to date are a LuxI-type synthase, AbaI, and a putative LuxR-type receptor, AbaR.^{42,43} AbaI produces *N*-(3'-hydroxydodecanoyl)-*L*-HL (OH-dDHL, Figure 1B), which serves as *A. baumannii*'s primary QS signal. To date, the native stereochemistry at the 3'-OH position of OH-dDHL (*R* or *S*) is yet to be assigned. The synthase AbaI has been isolated and characterized, and the activity of AbaR has been inferred from its homology to other LuxR-type proteins and *abaR* being adjacent to *abaI* in the *A. baumannii* genome. Notably, *A. baumannii* (Δ *abaI*) mutants show both reduced biofilm production and limited surface motility, and these traits can only be restored upon addition of OH-dDHL.^{41,42} We hypothesized that, similar to other Gram-negative bacteria with LuxI/LuxR-type QS circuits, non-native AHLs could potentially be utilized to intercept AbaR:OH-dDHL binding and modulate QS phenotypes in *A. baumannii*.

Herein, we report our investigations into the activity of non-native AHLs as AbaR modulators in *A. baumannii*. These studies motivated us to determine the structure of the native AHL signal for this pathogen. We synthesized and tested both diastereoisomers of OH-dDHL as AbaR activators and obtained results suggesting that (*R*)-OH-dDHL is likely the native QS signal in *A. baumannii*, in contrast to the recent report of Garner *et al.*⁴⁴ We utilized (*R*)-OH-dDHL as our positive control to screen a 77-member library of synthetic, non-native AHLs to identify AbaR agonists and antagonists using an *A. baumannii* AbaR reporter strain. Several highly potent compounds were uncovered, with IC₅₀ and EC₅₀ values in the low micromolar range. The strongest AbaR antagonists largely contained aromatic acyl groups, whereas the AbaR agonists closely resembled (*R*)-OH-dDHL. The 10 most potent AbaR antagonists also strongly inhibited *A. baumannii* motility in a swarming-type assay, and five of these compounds reduced biofilm formation in *A. baumannii* by up to 40%. The discovery of these compounds is significant, as they represent the first non-native modulators of QS in *A. baumannii* to be reported and can serve as new tools to study the role and timing of QS phenotypes in *A. baumannii* infections. Further, these compounds provide a first set of SAR data from which to initiate the development of chemical-based, anti-infective strategies against this lethal pathogen.

RESULTS AND DISCUSSION

Natural Ligand Stereochemistry. To perform AbaR agonism and antagonism screens in *A. baumannii*, we required the native OH-dDHL signal for use as a control compound. As the stereochemistry of this signal was undefined, we undertook synthetic studies to determine the active stereoisomer of OH-dDHL. The AHL lactone core is derived biosynthetically from *S*-adenosyl-*L*-methionine (see below),^{45–48} and numerous reports have established that the HL stereocenters in native AHLs are *L*;^{18,49,50} the *D*-enantiomers of these AHLs have markedly reduced activity. We therefore reasoned that OH-dDHL also contained a *D*-HL and focused our attention on assigning the 3'-OH stereocenter.

Of the ~25 known native AHLs, only a few contain 3'-OH groups, and the effects of stereochemistry at this position on activity have not been carefully scrutinized.⁵¹ However, analogous to the HL stereocenter, the biosynthetic pathway

for AHLs also provides some guidance in assigning the 3'-stereocenter in OH-dDHL *a priori*. In AHL biosynthesis, LuxI-type synthases are known to couple S-adenosyl-L-methionine and acyl-acyl carrier protein (acyl-ACP) substrates to produce AHL products.^{45–48} Acyl-ACPs, the sources of the acyl chain segment of AHLs, are intermediates in fatty acid biosynthesis. One essential step in this cyclical pathway is the reduction of β -ketoacyl-ACP to β -hydroxyacyl-ACP by the enzyme FabG,⁵² and FabG is known to produce (*R*)-3-hydroxyacyl-ACP stereoselectively.⁵³ If AbaI, like all other LuxI-type synthases, preferentially selects fatty acid biosynthesis intermediates as substrates, the 3'-stereocenter of OH-dDHL should be (*R*). In support of this hypothesis, the known native AHLs with 3'-OH groups isolated from *Vibrio harveyi*, *Aeromonas culicicola*, *Phaebacter gallaeciensis*, and *Rhizobium leguminosarum* (shown in Figure 2) have all been reported to have (*R*) stereochemistry.^{54–56}

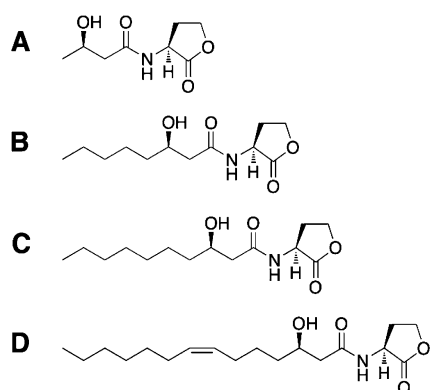
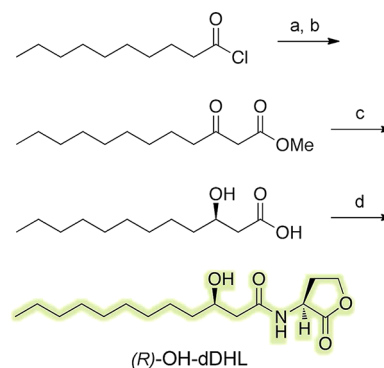


Figure 2. Structures of naturally occurring 3'-OH AHLs with defined stereochemistry at the 3'-position. (A) (2*S*,3'*R*)-*N*-(3'-Hydroxyhexanoyl)-HL from *Vibrio harveyi*. (B) (2*S*,3'*R*)-*N*-(3'-Hydroxyoctanoyl)-HL from *Aeromonas culicicola*. (C) (2*S*,3'*R*)-*N*-(3'-Hydroxydecanoyl)-HL from *Phaebacter gallaeciensis*. (D) (2*S*,3'*R*,7'*Z*)-*N*-(3'-Hydroxy-7'-tetradecenoyl)-HL from *Rhizobium leguminosarum*.

To experimentally test this hypothesis in *A. baumannii*, we synthesized the (*R*) and (*S*) diastereoisomers of OH-dDHL, producing the key 3'-OH stereocenter via a Noyori asymmetric hydrogenation (Scheme 1).⁵⁷ The compounds were purified to homogeneity using RP-HPLC, with diastereomeric excesses (de) of >99% (see Supporting Information). We tested each diastereomer for its ability to activate the AbaR receptor over a range of concentrations in an *A. baumannii* reporter strain (Δ abaI) that lacks a functioning AbaI synthase and reports AbaR activity via β -galactosidase production (see Methods). These assays revealed that (*R*)-OH-dDHL was ~40-fold more active than (*S*)-OH-dDHL, with EC_{50} values of 0.70 and 29 μ M, respectively. We note that our data conflict with a recent study of Garner *et al.*,⁴⁴ who reported that the two diastereomers were of approximately equal activity in the identical *A. baumannii* reporter strain (EC_{50} values of 0.67 and 0.82 μ M for (*R*)-OH-dDHL and (*S*)-OH-dDHL, respectively). While the EC_{50} values for (*R*)-OH-dDHL are identical between these two studies, the reasons for the disparate EC_{50} values for (*S*)-OH-dDHL are currently unclear. Nevertheless, the biological data for (*R*)-OH-dDHL and (*S*)-OH-dDHL reported herein, in concert with the known biosynthetic pathway to 3'-OH AHLs outlined above, support that (*R*)-OH-dDHL is likely the primary AHL signal in *A. baumannii*. We therefore used

Scheme 1. Synthetic Route to (*R*)-OH-dDHL^a



^aThe same route was used to synthesize (*S*)-OH-dDHL, except that (*S*)-(-)-BINAP was used in step c. Reagents and conditions: (a) bis(TMS) malonate, *n*-BuLi, Et₂O, -60 °C, 1 h, then warm to -10 °C, 5 h; (b) (CH₃)₃SiCHN₂ (2 M in Et₂O), benzene/CH₃OH (4:1), 0 °C, 10 min, then warm to 25 °C, 1 h; (c) (i) [(*R*)-(+)-BINAP]-RuBr₂(CH₃OH)₂, H₂ (g), MeOH, 50 °C, 5 h, (ii) NaOH, MeOH, 25 °C, 14 h; (d) L-homoserine lactone-HBr, TEA, EDC•HCl, CH₃CN/H₂O (1:2), 25 °C, 14 h. Abbreviations: BINAP = (1,1'-binaphthalene-2,2'-diyl)bis(diphenylphosphine), TEA = triethylamine, EDC = 1-ethyl-3-(3-dimethyl aminopropyl)carbodiimide.

(*R*)-OH-dDHL as our native AHL control throughout the remainder of this study.

Selection of Synthetic AHL Library. We next screened a set of synthetic AHLs for their ability to modulate the activity of AbaR, and thereby QS, in *A. baumannii*. Our laboratory has previously reported several libraries of native and non-native AHLs,^{19–22,24,25} and from this larger set we selected 77 AHLs for study in *A. baumannii*. These compounds had varied acyl chain structures and displayed a range of activities as LuxR-type protein agonists and antagonists in other bacteria. To simplify their analysis here, we divided this set of AHLs into five sublibraries (termed the control and A–D libraries; see Supporting Information for full set of structures).

The control library (1–9) contained the native AHL ligands from other Gram-negative bacteria (*e.g.*, *P. aeruginosa*, *V. fischeri*, and *Agrobacterium tumefaciens*) and some of the first synthetic LuxR-type protein antagonists that we and others have identified in these species.^{20,30,58} The A library (A1–A8) contained AHLs with unbranched aliphatic acyl groups of differing lengths (4–16 carbons) and with different oxidation states at the 3'-position. The B library (B1–B14) consisted of AHLs with cycloalkyl or aromatic acyl groups, and one-carbon longer or shorter homologues or D-HL analogues of the control library AHLs. The C library (C1–C25) was made up of phenylacetanoyl HLs (PHLs) with substituents on the phenyl ring of varying electronics and size; we have previously shown that PHLs are capable of strongly agonizing and antagonizing a range of LuxR-type receptors, and subtle changes in phenyl group substituents can have striking effects on their activities.^{19,20,22} Lastly, the D library (D1–D21) consisted of AHLs with a set of structurally diverse acyl substituents to more dramatically probe the effects of acyl chain composition on AHL activity in AbaR.

Non-native AHLs That Antagonize AbaR. To identify antagonists of AbaR activity, we screened the AHL libraries at 100 μ M against (*R*)-OH-dDHL at its EC_{50} value (0.70 μ M) in the *A. baumannii* (Δ abaI) reporter strain (see Methods). Performing the primary screen at this ~100:1 non-native:native

ligand ratio revealed numerous strong AbaR antagonists, with 22 compounds exhibiting greater than 70% AbaR inhibition (see Supplementary Table S-1 for full screening data). To better compare the relative antagonistic activities of these AHLs, we conducted dose–response analyses in the *A. baumannii* (Δ *abaI*) reporter strain to determine their IC_{50} values. The extent of AbaR antagonism by six of the 22 AHLs rapidly diminished at concentrations less than 100 μ M; however, we were able to determine accurate IC_{50} values for the other 16 AHLs. The IC_{50} values and primary screening data for these compounds are listed in Table 1.

Table 1. Primary Antagonism and Agonism Assay Data and IC_{50} and EC_{50} Values for the Most Active AbaR Modulators Identified in *A. baumannii* (Δ *abaI*)^a

compound	inhibition [%] ^b	IC_{50} (μ M) ^c
1	73	2.91
4	75	13.7
8	71	7.83
B10	72	11.0
C5	78	16.7
C6	83	5.47
C7	83	18.5
C8	90	5.06
C9	97	17.5
C10	91	7.06
C11	91	2.32
C12	80	6.13
C14	70	8.00
C20	91	9.37
D3	72	19.9
D15	72	5.75
	activation [%] ^d	EC_{50} (μ M) ^e
(R)-OH-dDHL	100	0.699
(S)-OH-dDHL	90	28.9
2	62	7.57
D18	31	4.89

^aSee Methods for details of reporter strain. See Supporting Information for plots of dose–response curves. All assays performed in triplicate. ^bAHLs evaluated at 100 μ M against (R)-OH-dDHL at 0.70 μ M. Error $\leq \pm 10\%$. ^c IC_{50} values determined by testing AHLs over a range of concentrations (10 nM to 100 μ M) against (R)-OH-dDHL at 0.70 μ M. ^dAHLs evaluated at 100 μ M and normalized to (R)-OH-dDHL at 100 μ M (set to 100%). Error $\leq \pm 10\%$. ^e EC_{50} values determined by testing AHLs over a range of concentrations (200 pM to 200 μ M).

In general, the strongest AbaR antagonists contained aromatic acyl groups, with IC_{50} values less than 20 μ M. The only exceptions were the control AHLs 3-oxo-octanoyl-HL (1) and heptanoyl-HL (4). Notably, 4-iodo PHL (C10) and 4-bromo PHL (8), previously identified as moderate to strong inhibitors of numerous other LuxR-type receptors (e.g., TraR, LasR, LuxR, ExpR1, and ExpR2),^{20,22,23,35} were also potent AbaR antagonists (91% and 71%, respectively), further broadening the spectrum of bacterial species in which these ligands display inhibitory activity. In contrast, 2-iodo-PHL (C12) strongly antagonized AbaR (80%) yet is only a modest antagonist of TraR, LasR, LuxR, and other LuxR-type receptors.^{20,22,23,35} This selective inhibition suggests that PHL C12 could prove useful in experiments requiring specific targeting of AbaR in mixed microbial milieu.

A closer examination of the primary screening data allows for the generation of several SARs for AbaR antagonism by AHLs, and we provide a synopsis of the major trends for the five AHL libraries here. For simple aliphatic and 3-oxo AHLs, maximal AbaR antagonism was observed for AHLs with acyl chains of eight carbons (A3 and 1, 60% and 73%, respectively). This activity diminished for AHLs that contained acyl chains shorter or longer than eight carbons (e.g., AHLs butanoyl HL (A1; 11%) or dodecanoyl HL (A5; 2%)). Interestingly, the most potent AbaR antagonist of this class, 3-oxo-octanoyl-HL (1; 73%; $IC_{50} = 2.91 \mu$ M), is also the natural AHL ligand for TraR in *A. tumefaciens*. We have previously shown that 1 is also a strong inhibitor of the LasR (50%, $IC_{50} = 0.11 \mu$ M).^{20,22} The non-3-oxo analogue of 1, octanoyl-HL (A3), was less active than 1 (60% v. 73%), suggesting that the 3-oxo group is important for AbaR inhibition by aliphatic AHLs. However, heptanoyl-HL (4) was also a strong AbaR antagonist (75%; $IC_{50} = 13.7 \mu$ M), indicating that acyl chain length can have a larger impact on ligand activity than oxidation state for aliphatic AHLs. Heptanoyl-HL (4) was first reported as a strong TraR inhibitor by Zhu *et al.*,⁵⁸ and we have demonstrated in a series of recent studies that this AHL is a strong inhibitor of almost all of the LuxR-type receptors evaluated in our laboratory,^{20–22} similar to PHLs 8 and C10.

Several B library members were strong AbaR inhibitors in the primary screen (B4, 68%; B5, 78%; B9, 74%; B10, 72%; B11, 69%). However, their extent of antagonism rapidly diminished at concentrations less than 100 μ M, which permitted us to determine an accurate IC_{50} value for only the most active AHL, phenylpropanoyl HL B10 ($IC_{50} = 11.0 \mu$ M). One interesting stereochemical SAR for AbaR antagonism was determined from library B. We have previously shown that two of the D-AHLs in this library, B4 and B5, are only modest inhibitors of other LuxR-type receptors (i.e., TraR, LasR, LuxR, and QscR) relative to their L-HL analogues (8 and 9, ~2- to 10-fold less active), presumably due to their inverted HL stereochemistry.^{16,20,22,29} Against AbaR, however, B4 was as strong an AbaR antagonist as its L-HL analogue (8, 68% vs 71% inhibition, respectively). Moreover, B5 was actually *more* active than its L-HL analogue in AbaR (9, 78% vs 48% inhibition, respectively). These data suggest that AHL-derived AbaR antagonists do not need to have native HL stereochemistry and contrast strikingly with our and other previous findings for AHL-based antagonists.^{20,22}

The C library contained some of the strongest AbaR antagonists identified in this study. This result further showcases the PHL as a valuable and general scaffold for the inhibition of LuxR-type receptors.^{20,26} Intriguingly, the PHLs displayed a pattern of activity and SARs in AbaR that were largely analogous to those that we have previously observed for PHLs in LasR from *P. aeruginosa*.^{20,22} Among the halogenated PHLs, antagonistic activity increased with the increasing size and decreasing electronegativity of the halogen. For example, fluoro PHLs (C2–C4) were weakly inhibitory, chloro (C5–C7) and bromo (10, C8, and C9) PHLs were moderately to strongly inhibitory, and iodo PHLs (C10–C12) were strongly inhibitory. Also, nitro PHLs (C13–C15) were less active than chloro PHLs (C14, $IC_{50} = 8.0 \mu$ M vs C6, $IC_{50} = 5.47 \mu$ M), yet more active than fluoro PHLs (e.g., C2, $IC_{50} > 25 \mu$ M). In general, placement of a substituent at the 3-position of the PHL aromatic ring led to stronger AbaR inhibition in all cases; antagonistic activity was diminished for substituents in the 2- and 4-positions. Again, this activity trend is congruent with that

observed with these PHLs against LasR, suggesting that the mode of inhibition by this class of non-native AHLs in AbaR and LasR could be similar. As these two receptors share an almost identical native ligand (**2** (often termed OdDHL) for LasR versus (*R*)-OH-dDHL for AbaR), only differing in oxidation at the AHL 3'-position, such analogous SARs for inhibition are perhaps not unexpected. We return to this point below.

The more structurally diverse D library contained only a few moderate AbaR antagonists, with **D3** ($IC_{50} = 19.9 \mu M$) and **D15** ($IC_{50} = 5.75 \mu M$) representing the most active AHLs. Previous studies have shown that **D3** is a modest QscR inhibitor and that **D15** is a strong TraR and LasR inhibitor ($IC_{50} = 0.46$ and $4.67 \mu M$, respectively),^{20,23} and our results here serve to broaden the activity of these two ligands against LuxR-type receptors. Otherwise, the D library was largely inactive as AbaR antagonists, suggesting that sterically bulky acyl groups on non-native AHLs serve to limit their inhibitory activity against AbaR.

Non-native AHLs That Agonize AbaR. We next examined the ability of the AHL libraries to agonize AbaR using the *A. baumannii* ($\Delta abal$) reporter strain. Each compound was tested for AbaR activation at $100 \mu M$ (see Methods). Relative to (*R*)-OH-dDHL at $100 \mu M$ (set to 100%), only six AHLs were capable of any level of AbaR agonism (**2**, 62%; **A8**, 9%; **C23**, 9%; **D13**, 16%; **D18**, 31%; and **D19**, 9%; see Supplementary Table S-1 for full screening data). Far fewer non-native AHL agonists of LuxR-type receptors have been identified to date relative to non-native antagonists, and thus these data in AbaR are congruent with prior findings by our lab and others.^{18,22} This set of AHLs did not have any structural homology, and only AHL **2** and **D18** (Figure 3) were active and soluble over a concentration range that enabled determination of their EC_{50} values, which were 5- to 10-fold greater than that of (*R*)-OH-dDHL (4.89 and $7.57 \mu M$, respectively (Table 1)).

Despite their moderate agonistic activities, AHLs **2** and **D18** are worthy of some further discussion in comparison to their activity in LasR. As highlighted above, AHL **2** (or OdDHL) is actually the native ligand for LasR, and is nearly identical to (*R*)-OH-dDHL. In view of this structural similarity, it is not surprising that **2** is also capable of AbaR activation. However, its lowered activity relative to (*R*)-OH-dDHL suggests that the oxidation state at the 3'-position is critical to AbaR agonism by such aliphatic AHLs. In addition, the reduced analogue of (*R*)-OH-dDHL, dodecanoyl-HL (**A5**), displayed no agonistic activity in AbaR, further supporting the requirement for the 3'-OH group. AHLs with 3-oxo acyl chains longer or shorter than 12 carbon atoms (*i.e.*, **A8**, **A7**, **3**, and **1**) displayed limited AbaR agonism, dropping rapidly from 62% (**2**) to 10% and 3% for tetradecanoyl-HL (**A8**) and decanoyl-HL (**A7**), respectively. No other chain lengths were active agonists, indicating that a 12-carbon acyl group, along with a 3-oxo group, is essential for strong AbaR agonism. With regard to **D18** ($EC_{50} = 4.89 \mu M$), this bulky AHL has previously been shown to be an agonist of LasR ($EC_{50} = 0.47 \mu M$).²⁰ This shared activity trend could suggest a similar mode of receptor activation by **D18** for the two receptors. We note that preliminary analyses of primary sequence alignments between AbaR and LasR (data not shown) indicate that residues in the putative ligand binding site of AbaR are homologous to those conserved between LasR and other LuxR-type receptors (*e.g.*, W60, D73, T75, S129, and Y56 in LasR¹⁴), suggesting that these ligand activity similarities

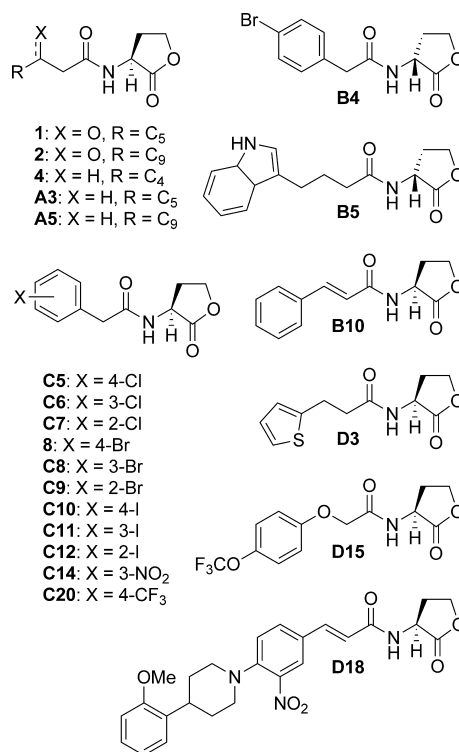


Figure 3. Selected agonists and antagonists of AbaR identified in the *A. baumannii* ($\Delta abal$) reporter strain and other AHLs of interest noted in the text. Compound numbers match the original numbering schemes from our previously reported control AHLs and AHL libraries A–D.²⁰

may be correlated to binding site similarities.⁴³ These structural similarities could then also explain the shared trends in PHL-based antagonism for AbaR and LasR discussed above (assuming that these ligands act directly *via* receptor binding). On the other hand, this structural homology obscures our understanding of AbaR's apparent sensitivity to AHL 3'-position oxidation state (as observed for (*R*)-OH-dDHL vs **2**), along with the strong inhibitory activities of certain D-AHLs in AbaR relative to other LuxR-type receptors (see above). Further analyses of sequence alignments and AHL activity trends between AbaR and LasR (along with other receptors) are clearly required, and are being pursued as part of ongoing mechanistic studies of non-native AHLs in our laboratory.

Synthetic AbaR Antagonists Limit *A. baumannii* Motility. We sought to determine whether the AbaR antagonists identified above in reporter gene assays were also able to inhibit a phenotype linked to QS in *A. baumannii*, and we selected motility as our first phenotype for testing. Although *A. baumannii* has previously been classified as nonmotile, it is capable of a rapid form of motility on low agar (0.3%) that is straightforward to quantify by measuring bacterial migration distances after fixed time intervals.⁴¹ This motility appears to be due to a combination of twitching motions mediated by type-IV pili and sliding motility. Motility is severely reduced in the *A. baumannii* ($\Delta abal$) mutant (75% reduction relative to wild-type M2), but is restored to wild-type levels when agar plates are supplemented with the native QS signal OH-dDHL.⁴¹ We thus tested the 10 most potent AbaR antagonists uncovered above (AHLs **1**, **B10**, **C6**, **C8**, **C10–C12**, **C14**, **C20**, and **D15**) for their ability to inhibit (*R*)-OH-dDHL mediated rescue of

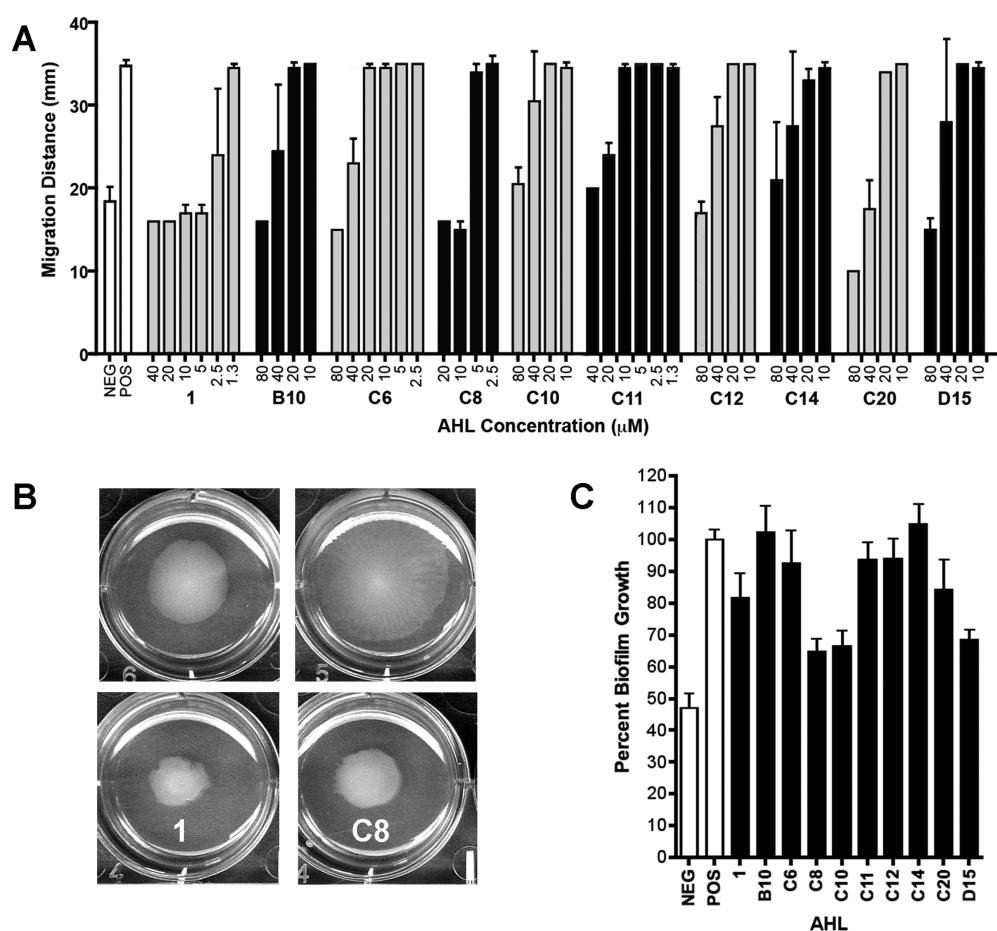


Figure 4. (A) *A. baumannii* (Δ abaI) motility inhibition assay data for selected AbaR antagonists. Migration distance reports colony mobility over 6–7 h on 0.3% (w/v) Eiken soft agar at 30 °C. Positive control (POS) = (*R*)-OH-dDHL supplemented agar. Negative control (NEG) = agar alone. See Methods. (B) Image of motility assay. Top left, *A. baumannii* (Δ abaI). Top right, *A. baumannii* (Δ abaI) on (*R*)-OH-dDHL supplemented agar. Bottom left, *A. baumannii* (Δ abaI) on (*R*)-OH-dDHL and **1** (5 μ M) supplemented agar. Bottom right, *A. baumannii* (Δ abaI) on (*R*)-OH-dDHL and **C8** (10 μ M) supplemented agar. (C) *A. baumannii* (Δ abaI) biofilm inhibition assay data for selected AbaR antagonists at 100 μ M. Percent biofilm growth reports crystal violet quantification (A_{590}) after 24 h growth at 37 °C. Negative control (NEG) = DMSO alone; positive control (POS) = 250 nM (*R*)-OH-dDHL in DMSO. See Methods.

motility in the *A. baumannii* (Δ abaI) mutant (see Methods for assay details).

We were pleased to observe that three of the AbaR antagonists (**1**, **C8**, and **C11**) were potent inhibitors of *A. baumannii* motility, preventing migration at concentrations less than 20 μ M that were comparable to the *A. baumannii* (Δ abaI) mutant (Figure 4A). These compounds represent, to our knowledge, the first motility inhibitors to be reported in this pathogen. The two strongest AbaR antagonists in the β -galactosidase reporter assays, AHLs **1** and **C11** (Table 1), were also the two strongest motility inhibitors (see Figure 4A and B). In addition, SAR trends for the 10 AHLs tested in the motility assay paralleled the SAR observed in the reporter assays. Among the halogenated PHLs, the 3-chloro PHL (**C6**, IC_{50} value = \sim 40 μ M) was more active than 3-nitro PHL (**C14**, \sim 70 μ M), yet less active than the 3-iodo PHL (**C11**, \sim 15 μ M), and the 3-bromo PHL (**C8**, \sim 7 μ M) was the most active of this series. Further, placement of the iodo substituent at the 3-position (as in PHL **C11**) led to the strongest motility inhibition, and its placement at the 2-position (**C12**, \sim 40 μ M) or 4-position (**C10**, \sim 70 μ M) led to diminished inhibition, in accord with the reporter assay data. Overall, these results indicate that chemical inhibition of AbaR in *A. baumannii* using

non-native AHLs results in motility inhibition and are in accord with the genetic model that AbaR regulates this phenotype in *A. baumannii*.

Synthetic AbaR Antagonists Inhibit *A. baumannii* Biofilm Formation. We next investigated the ability of the AbaR antagonists to inhibit biofilm formation in *A. baumannii*. Previous studies have shown that the *A. baumannii* (Δ abaI) mutant formed \sim 40% less biofilm than wild-type *A. baumannii* (as observed by crystal violet staining) and that wild-type biofilm formation can be fully restored in the mutant through the exogenous addition of OH-dDHL.⁴² We therefore tested our top 10 AbaR antagonists for their ability to inhibit biofilm recovery in *A. baumannii* (Δ abaI) (see Methods). The crystal violet biofilm quantification data is shown in Figure 4C. Five of the AHLs (**1**, **C8**, **C10**, **C20**, and **D15**) were observed to inhibit biofilm formation to significant levels. Moreover, the three most active AHLs (**C8**, **C10**, and **D15**) not only reduced biofilm recovery in *A. baumannii* (Δ abaI) by \sim 40% but also inhibited biofilm formation in wild-type *A. baumannii* by \sim 40% (see Supplementary Figure S-13). These results are important, as small molecule inhibitors of bacterial biofilm formation are rare in general,^{10,59,60} and to our knowledge, no LuxR-type receptor inhibitors that also attenuate biofilm formation in *A.*

baumannii are known.^{61,62} Notably, the most potent *A. baumannii* biofilm inhibitor (3-bromo PHL C8) was also one of the most active AbaR antagonists identified in the *A. baumannii* reporter strain and motility assays. The reasons for the somewhat lower activities of AHLs 1 and C11 in the biofilm assay, both potent inhibitors in the reporter and motility assays, relative to C8 are currently unclear. Nonetheless, these biofilm assay data for AHLs 1, C8, C10, C20, and D15 provide further support for the application of small molecule AbaR antagonists for the attenuation of QS-regulated phenotypes in *A. baumannii*.

Summary and Outlook. *A. baumannii* is an emerging human pathogen and utilizes QS to control at least a subset of phenotypes linked to virulence, including surface motility and biofilm formation. Methods to modulate QS in this bacterium with spatial and temporal control could provide valuable new insight into the role of QS in the infection process and to date are currently lacking in the field. Herein, we have outlined the first study aimed at the identification of non-native small molecules capable of intercepting a putative QS receptor in *A. baumannii*, AbaR.

There were several important outcomes of our work. First, the synthesis and testing of both (S)-OH-dDHL and (R)-OH-dDHL as AbaR activators revealed the latter molecule to be the most active diastereomer. We propose that, in view of the known AHL biosynthetic pathways and the other structurally characterized 3'-OH AHL signals, (R)-OH-dDHL is likely the native signal in *A. baumannii*. Second, using an *A. baumannii* AbaR reporter strain, we systematically screened a library of synthetic AHLs and uncovered the first known non-native AHLs capable of modulating AbaR activity. Both AbaR antagonists and agonists were identified, and several antagonists were highly potent (for example, AHLs C11 and 1, with IC_{50} values of 2.32 and 2.91 μM , respectively). Two AHLs (2 and D18) were identified as moderate AbaR agonists, with EC_{50} values that were only 5- to 10-fold greater than that of the native signal (R)-OH-dDHL (7.57 and 4.89 μM , respectively). Third, careful structural analysis of these molecules revealed important SAR for AbaR antagonism and agonism by non-native AHLs. Perhaps most strikingly, selected D-AHLs were found to be strong antagonists of AbaR and in some cases were equal or stronger antagonists than their L-AHL analogues. This result was unexpected, as D-AHLs are typically far less active than their L-AHL analogues in other LuxR-type receptors (e.g., LasR, TraR, LuxR, and QscR) as either antagonists or agonists, and suggests that AbaR may interact with these ligands in a different manner.^{16,20,22,29,43} Maximal antagonism was observed for AHLs with aliphatic acyl chains containing seven to eight carbons and a 3'-oxo group. AHLs with aromatic acyl groups, most notably the *meta*-halogenated PHLs, were some of the most potent inhibitors of AbaR, further securing the PHL as a "privileged" scaffold for LuxR-type receptor modulation. Guided by these SARs for AbaR antagonism, synthesis and screening of a focused library of *N*-(3-hydroxy-4-phenylbutanoyl)-L-HLs and related OH-AHLs is now underway, and we anticipate that these new compounds may show enhanced AbaR antagonism relative to the AHLs identified in this study through the additive effects of both halogenated aromatic and 3'-OH groups.

Lastly, a fourth and perhaps the most important outcome of this study was the identification of small molecules capable of modulating two important phenotypes in *A. baumannii* directly linked to QS and virulence. Several of the most potent AbaR

antagonists revealed by the reporter gene assays were also found to inhibit *A. baumannii* surface motility and biofilm production (e.g., AHLs 1, C8, and C11). These compounds represent new and valuable chemical tools to study the role of QS in *A. baumannii* virulence in both fundamental and applied contexts. As we identified these lead compounds from screening a relatively small set of compounds (77 AHLs), we are optimistic that next-generation abiotic ligands with enhanced activities in AbaR can be generated. Ongoing investigations in our laboratory are focused on such experiments, along with biochemical experiments to elucidate their mechanisms of action, and will be reported in due course.

METHODS

Natural Ligand Synthesis. (R)- and (S)-OH-dDHL were prepared on a 200 mg scale using reported synthetic methods.^{57,63,64} See Supporting Information for full synthetic details and compound characterization data, including ee analysis of intermediates by chiral GC.

Biological Reagents and Strain Information. All biological reagents were purchased from Fisher Scientific and used according to enclosed instructions. Luria–Bertani (LB) medium was prepared as instructed with pH = 7.0. Buffers and solutions (Z buffer, 0.1% (m/v) aqueous SDS, and phosphate buffer) for Miller absorbance assays in *A. baumannii* were prepared as described.⁶⁵ The *A. baumannii* M2 *abaI::lacZ* ($\Delta abaI$ reporter) or *A. baumannii* M2 (wild-type) strains were used for all bacteriological assays in this study.⁴² Bacterial cultures were grown in a standard laboratory incubator at 37 °C with shaking (200 rpm) unless noted otherwise. Absorbance measurements were obtained using a Biotek Synergy 2 microplate reader using Gen5 data analysis software. Bacteriological reporter and biofilm assays were performed in triplicate. Motility assays were performed in duplicate. No AHL was found to inhibit *A. baumannii* growth over the time course of the assays in this study (see Supplementary Figure S-7).

Compound Handling. Stock solutions of synthetic compounds (10 mM) were prepared in DMSO and stored at 4 °C in sealed vials. The amount of DMSO used in small molecule screens did not exceed 2% (v/v). Solvent-resistant polypropylene or polystyrene 96-well multititer plates were used when appropriate for small molecule screening. The concentrations of synthetic ligand used in the primary antagonism and agonism assays and the relative ratios of synthetic ligand to (R)-OH-dDHL (100 μM :0.70 μM) in the antagonism assays were chosen to provide the greatest dynamic range between inhibitors and activators for each bacterial reporter strain. The concentration of (R)-OH-dDHL used in the antagonism assays was equal to its EC_{50} value in the *A. baumannii* ($\Delta abaI$) reporter strain.

Reporter Gene Assay (β -Galactosidase) Protocol. For primary agonism assays, 2 μL of concentrated control or AHL stock solution (to give a final concentration of 100 μM) was added to wells in a 96-well multititer plate. An overnight culture of the *A. baumannii* ($\Delta abaI$) reporter strain ($OD_{600} = 1.2$) was diluted 1:100 with fresh LB. A 198- μL portion of the diluted culture was added to each well of the multititer plate containing AHLs. Plates were incubated statically at 37 °C for 18–24 h. The cultures were then assayed for β -galactosidase activity following the standard Miller assay method, with the exception that the enzyme was incubated with the substrate *o*-nitrophenyl- β -D-galactopyranoside (ONPG) at 55 °C.⁶⁵ Briefly, the OD_{600} of each well of the 96-well multititer plate was recorded (see Supplementary Figure S-7 for these data). Next, 50 μL aliquots from each well were transferred to a solvent-resistant 96-well multititer plate containing 200 μL of Z buffer, 8 μL of $CHCl_3$, and 4 μL of 0.1% (w/v) aqueous SDS. This suspension was mixed *via* repetitive pipetting (30 \times), after which the $CHCl_3$ was allowed to settle. A 150- μL aliquot from each well was transferred to a fresh 96-well multititer plate, 20 μL of ONPG (4 $\mu g mL^{-1}$ in phosphate buffer) was added to each well at time zero, and the plate was incubated at 55 °C for 20 min. Thereafter, the enzymatic reaction was terminated by the addition of 50 μL of 1 M Na_2CO_3 . Absorbance at 420 and 550 nm was measured for each well

using a plate reader, and Miller units were calculated according to standard methods.⁶⁵ Primary AbaR antagonism assays were performed in a similar manner except that the AHL was screened at 100 μM against 0.70 μM (R)-OH-dDHL.

Dose–Response Reporter Gene Assays. The dose–response reporter gene assays were performed according to the protocols outlined above, except that the concentrations of AHLs were varied between 1×10^{-3} and 1×10^5 nM. IC_{50} and EC_{50} values were calculated using GraphPad Prism software (v. 4.0) using a sigmoidal curve fit.

A. baumannii Motility Assay Protocol. Motility assays were performed in 6-well microtiter plates (35 mm wide well) using 0.3% (w/v) Eiken soft agar prepared either unsupplemented or supplemented with 250 μM of (R)-OH-dDHL (added when the agar temperature was approximately 50–55 °C). AHL antagonists were added to wells in 2-fold decreasing concentrations, 3 mL of agar was added to each well, and the agar was allowed to set. A 3-mL LB culture of the *A. baumannii* (ΔabaI) reporter strain was inoculated from a glycerol freezer stock and incubated overnight (to an $\text{OD}_{600} = 1.2$). A 1- μL spot of *A. baumannii* (ΔabaI) was added to the center of each agar well, and the plate was incubated at 30 °C without shaking. The plate was monitored until bacterial surface migration in the control well (250 μM (R)-OH-dDHL) reached the edge of the agar (35 mm). The migration in all other wells was then measured.

A. baumannii Biofilm Assay Protocol. A 10-mL culture of *A. baumannii* (wild-type or ΔabaI mutant) was incubated overnight (to an OD_{600} of ~ 1.35). A 1:100 dilution of this culture was prepared by centrifugation of a 100- μL aliquot of the overnight culture (3,000 rpm, 1000g, 10 min), removal of the supernatant, and resuspension of the cell pellet in 9.95 mL of 50% LB medium (1:1, LB/18 M Ω H₂O). A 50- μL aliquot of concentrated AHL stock solution was then added to give a final compound concentration of 100 μM (0.5% DMSO final). Next, 3-mL aliquots of the diluted culture with AHL were added to test tubes (16 mm \times 100 mm, borosilicate glass), and the cultures were grown statically at 30 °C for 24 h to allow for robust biofilm growth at the air–water interface. After incubation, a 200- μL aliquot was taken from the center of each tube, and the OD_{600} was measured. The remaining culture was carefully poured off and the attached biofilm was washed with H₂O (3.5 mL, 2 \times) to remove nonadherent cells. Biofilms were then stained with a crystal violet (CV) solution (0.1% CV w/v in 95:5 H₂O/EtOH) for 15 min. The stain was poured off and the biofilms were washed with H₂O (3.5 mL, 2 \times). The tubes were then dried at 37 °C for at least 8 h.

The amount of biofilm formed at the air–liquid interface in each tube was quantified by resolubilizing the CV in acetic acid (30% aq) as follows: AcOH (2.5 mL) was added to the tube, gently mixed by micropipet to wash the bottom of the tube yet avoid contact with the biofilm (to remove any residual background staining of the bottom of the tube not associated with interfacial biofilm), and this washing was removed. Then, fresh AcOH (300 μL) was added to each tube, and the biofilm ring formed at the air–water interface was dissolved by carefully rinsing the tube using micropipet. A 200- μL aliquot of the resulting solution was removed, and the amount of CV was quantified by reading absorbance at 590 nm and normalizing to cell density per tube.

■ ASSOCIATED CONTENT

■ Supporting Information

This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interest.

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