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Altering the Communication Networks of Multispecies Microbial Systems Using a Diverse Toolbox of AI-2 Analogues

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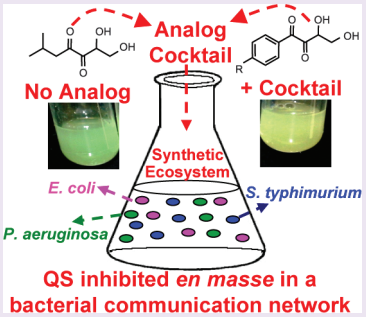
Supporting Information

ABSTRACT: There have been intensive efforts to find small molecule antagonists for bacterial quorum sensing (QS) mediated by the "universal" QS autoinducer, AI-2. Previous work has shown that linear and branched acyl analogues of AI-2 can selectively modulate AI-2 signaling in bacteria. Additionally, LsrK-dependent phosphorylated analogues have been implicated as the active inhibitory form against AI-2 signaling. We used these observations to synthesize an expanded and diverse array of AI-2 analogues, which included aromatic as well as cyclic C-1-alkyl analogues. Species-specific analogues that disrupted AI-2 signaling in *Escherichia coli* and *Salmonella typhimurium* were identified. Similarly, analogues that disrupted QS behaviors in *Pseudomonas aeruginosa* were found. Moreover, we observed a strong correlation between LsrK-dependent phosphorylation of these acyl analogues and their ability to suppress QS. Significantly, we demonstrate that these analogues can selectively antagonize QS in single bacterial strains in a physiologically relevant polymicrobial culture.



Bacteria communicate through a phenomenon known as quorum sensing (QS), whereby neighboring cells secrete and detect chemical signals known as autoinducers.[1] Once a predetermined (threshold) concentration of autoinducer is detected (typically in accordance with a threshold cell number density, or quorum), bacteria can coordinate their gene expression and perform both pathogenic and symbiotic processes such as biofilm formation, virulence factor production, and bioluminescence induction.[2,3] Recently there have been intensive efforts by several groups to find small molecules that can interrupt the communication among bacteria.[4−10] It is believed that disrupting bacterial communication and hence virulence factor production would not put substantial evolutionary pressure on bacteria to develop resistance.[11,12] The majority of studies, which investigated the inhibition of QS *via* autoinducer analogues (AHL or oligopeptide), were performed using monocultures.[13−17] Of the three major autoinducer classes (acyl homoserine lactones, AHLs, or AI-1's for Gram-negative bacteria, oligopeptides for Gram-positive bacteria, and autoinducer-2 (AI-2), which is found in among Gram-negative and Gram-positive bacteria), signaling *via* AI-2[18] is of particular interest for the development of antiquorum sensing agents because 4,5-dihydroxypentane-2,3-dione (DPD) is produced or recognized by over 70 species.[19] The key role of AI-2 in *Vibrio harveyi* and the homologous QS systems of *Escherichia coli* and *Salmonella typhimurium* have received significant attention.[20−23] Two receptors of AI-2 have been identified: LuxP in *V. harveyi*[24] and LsrB in *S. typhimurium*.[25] In *V. harveyi,* a cyclic borate form of AI-2 was found to co-crystallize with the periplasmic protein LuxP.[24] Besides LuxP, the detection of AI-2 by *V. harveyi* also requires another protein, LuxQ. LuxP has been identified as the primary AI-2 receptor in *V. harveyi* and is a member of a large family of periplasmic binding proteins whose members bind diverse ligands. When AI-2 binds LuxPQ, a phospho-relay is triggered and bioluminescence is induced.[24] Another characterized receptor of AI-2 is LsrB, found in *S. typhimurium*.[25] The crystal structure of a non-boronated chemically distinct form of AI-2 bound to LsrB has been reported. In *S. typhimurium* and *E. coli*, the internalization of AI-2 occurs as a non-borated cyclic form.[25] Once inside the cell, AI-2 is phosphorylated by the kinase LsrK. This phosphorylated AI-2 then binds and derepresses LsrR, the transcriptional regulator of QS genes in enteric bacteria (Figure 6, panel a).

Because of the ubiquitous nature of AI-2, it is believed that antagonists of AI-2 signaling would provide broader spectrum anti-QS activities than molecules that inhibit the species-specific AI-1 signaling. The caveat, however, is that many bacteria in humans play beneficial roles and these bacteria also signal with AI-2. Therefore, finding anti-AI-2 agents that display some level of selectivity could also be desired. If the receptor proteins for AI-2 in different bacteria harbor the same active site architecture, then finding selective small molecules that perturb AI-2 signaling in some bacteria and not others could be challenging.

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Several laboratories have investigated AI-2 analogues as anti-QS agents in different organisms.[10,26−28] For example, Janda *et al.* have revealed that linear acyl analogues of AI-2 could inhibit AI-2 signaling in *S. typhimurium*.[27] We found branched acyl analogues with antagonist activity and further demonstrated that different bacteria have different susceptibilities to acyl AI-2 analogues. We also demonstrated the potential for species selectivity, noted above.[10] Importantly, LsrK phosphorylates these acyl analogues, and it is the phosphorylated analogues that are likely the inhibitors of phospho-AI-2 signaling. Interestingly, the longer chain acyl groups confer structural similarity to the AI-1 family of autoinducers (acylated homoserine lactones) and can transverse cell membranes *via* diffusion.[29] Our initial results have prompted us to expand our study to include a more diverse set of acyl AI-2 analogues, with the expectation that increasing the diversity of the shapes and sizes of the C1-substituents could shed light on the specificities and promiscuities of AI-2 based signaling proteins and could potentially also lead to the identification of more selective AI-2 modulators in diverse bacterial species. Of interest would be the ability to selectively modulate AI-2 signaling among specific bacteria in a polymicrobial system. Herein, we describe the synthesis and biological evaluation of this expanded set of AI-2 analogues, both biochemically and as interrupters of cross talk in a polymicrobial system consisting of *E. coli*, *S. typhimurium*, and *P. aeruginosa* (Table 1).

Table 1. List of Strains and Cells Used in This Study

strain, plasmid, or primer	relevant genotype and/or property	reference
	Escherichia coli strains	
W3110	wild type	laboratory stock
LW7	W3110 Δ*lacU160-tna2* Δ*luxS*:: Kan	30
ZK126	W3110 Δ*lacU169-tna2*	31
LW8	ZK126 Δ*lsrR*::Kan	30
	Salmonella typhimurium strains	
MET715	*rpsl putRA*:: Kan-*lsr-lacZYA luxS*:: T-POP	20
MET708	*rpsl putRA*:: Kan-*lsr-lacZYA*	20
	P. aeruginosa strains	
PAO1	wild type	laboratory stock
	plasmids	
pLW11	*galK'-lacZYA* transcriptional fusion vector, containing *lsrACDBFG* promoter region, Amp^r	21
pCT6	pFZY1 derivative, containing *lsrR* and *lsrR* promoter region fused with *T7RPol*, Ap^r	32
pET-dsRED	pET200 derivative, containing RFP, Km^r	32

RESULTS AND DISCUSSION

Synthesis of Cyclic and Aromatic AI-2 Analogues. C-1 alkyl AI-2 analogues (Figure 1, panel a) were synthesized

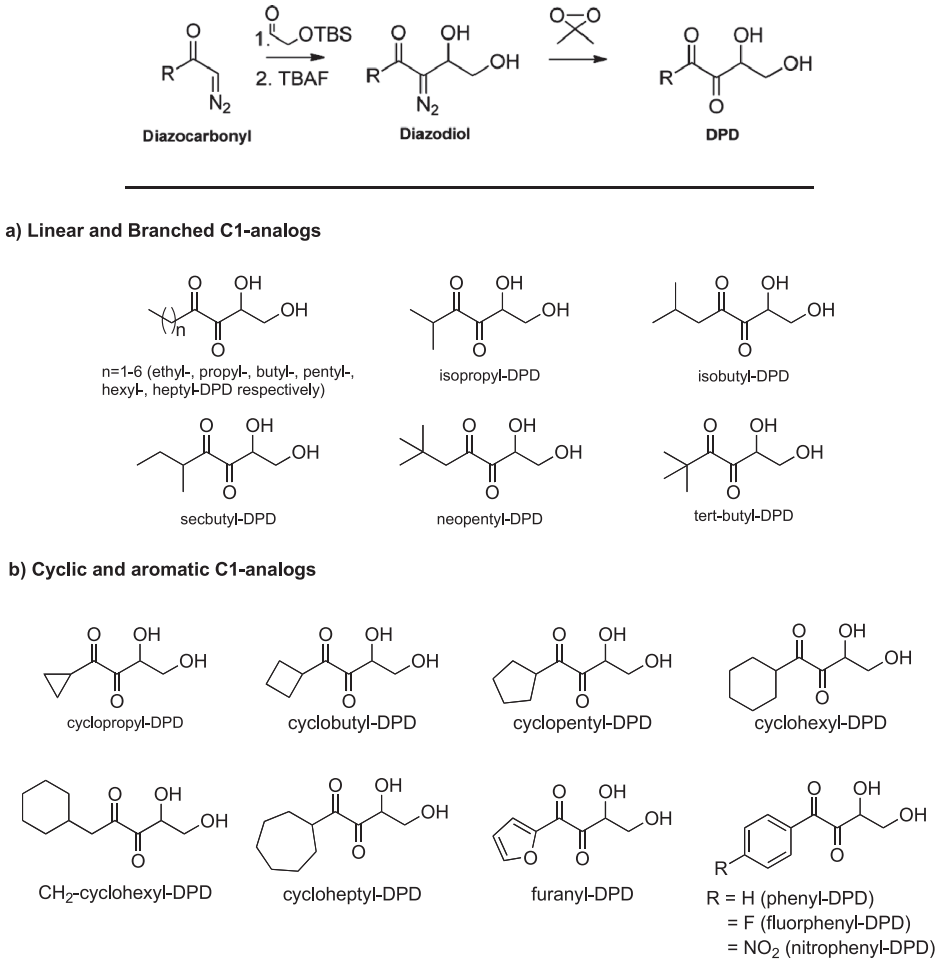

Figure 1. Analogue synthesis scheme and analogue structures. (a) Previously synthesized linear and branched analogues reported in refs 10 and 33, respectively. (b) Newly synthesized cyclic and aromatic analogues (cyclopropyl- and cyclohexyl-DPD).

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following our previously reported diazocarbonyl aldol methodology.[10] This methodology was adapted to make a variety of cyclic and aromatic AI-2 analogues, starting from the requisite acid chlorides, which are commercially available. Briefly, a DBU-catalyzed addition of (OTBS)-acetaldehyde to diazocarbonyls (readily generated from the addition of diazomethane to acid chlorides) afforded the protected diazo intermediates. Without isolation of these products, TBAF-deprotection was performed to afford diazodiol intermediates. Oxidation of these diazodiols into DPD proceeded quantitatively with excess DMDO, and the excess reagent was evaporated to give the desired cyclic and aromatic DPD analogues. NMR characterization of newly synthesized analogues is in Supporting Information. Figure 1 (panel b) depicts eight new AI-2 analogues; we tested their effects on three different bacteria: *E. coli*, *S. typhimurium*, and *P. aeruginosa*.

Effect of Cyclic and Aromatic DPD Analogues on β-Galactosidase Production in *E. coli* and *S. typhimurium*. Cyclic and aromatic analogues were tested for their modulation of QS in *E. coli* and *S. typhimurium*. Both *E. coli* LW7 (*luxS*$^-$) and *S. typhimurium* MET715 (*luxS*$^-$) synthesize β-galactosidase in response to AI-2 by derepressing *lsr* transcription. The addition of analogues to these cells yielded no increased LacZ activity relative that of to mock addition controls, and hence they had no *lsr* agonist activity (not shown). Results in Figure 2 depict responses to analogues (20 μM) added with 20 μM AI-2 (synthetic DPD). The "Methyl (DPD)" data are a control, demonstrating the elicitation of *lsr* gene expression (QS response) due to exogenously added chemically synthesized AI-2; the "Cells Only" control depicts the background level of β-galactosidase without addition of either analogue or AI-2 (leaky transcription). In *E. coli* cultures, simultaneous addition of 20 μM DPD (eliciting response) and 20 μM cyclopentyl-DPD (quenching response) resulted in over 60% reduction in *lsr* promoter activity. Interestingly, nearly all of the cyclic analogues tested (with the exception of cyclopropyl-DPD) attenuated the QS response in *E. coli*. The aromatic analogues exhibited minimal (furanosyl-DPD) or no (phenyl, fluorophenyl, and nitrophenyl) effect. In *S. typhimurium*, which has significant homology in operon structure, the effects were strikingly different, particularly for the cyclic analogues. Cyclopentyl-DPD and cyclohexyl-DPD elicited increased expression, suggesting synergistic agonist activity due to the presence of both DPD and analogue. Effects of the other cyclic compounds when added to *Salmonella* with DPD were no different than DPD alone; this was distinctly different than *E. coli*. On the other hand, with the exception of nitrophenyl-DPD, results for the aromatic C-1 analogues were identical to those of *E. coli*, exhibiting no apparent effect on the QS response. Nitrophenyl-DPD addition resulted in increased QS response in *Salmonella*.

Earlier work from our laboratories, using a variety of linear or branched chain C1 DPD analogues, had indicated that of the analogues affecting *E. coli* and *S. typhimurium* QS activity (e.g., isobutyl-DPD, Figure 2a) all worked through the native AI-2 signal processing system involving the kinase, LsrK, and the transcriptional regulator, LsrR. We concluded that because the analogues contained C1 chains that were dissimilar to the native DPD, both *E. coli* LsrK and LsrR were somehow promiscuous.[10] Our new data with the C1 cyclic alkyl analogues of AI-2 reveal that these *E. coli* AI-2 processing/binding proteins are not entirely promiscuous since only two cyclic analogues exhibited an effect. Similarly, the aromatic analogues were largely ineffective. When coupled with our earlier work,[10]

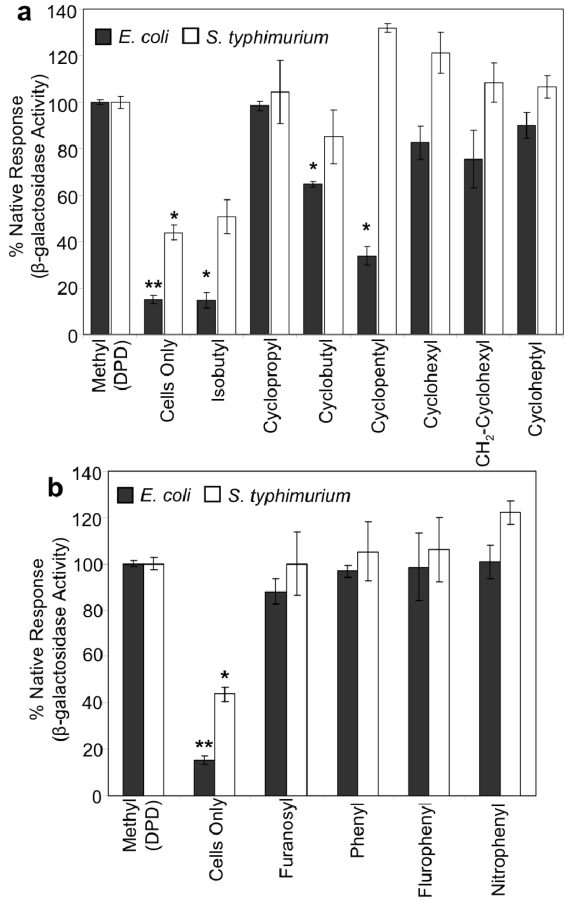

Figure 2. Competitive inhibition of QS signaling by analogues in the presence of stoichiometric amounts of DPD in *E. coli* and *S. typhimurium*; AI-2 dependent β-galactosidase production in *E. coli* LW7 pLW11 and *S. typhimurium* MET715 (both *luxS*$^-$). (a) Cyclic analogues (20 μM). (b) Aromatic analogues (20 μM) (normalized to the native *E. coli* (LW7) response (1304 Miller units) and the *S. typhimurium* (MET715) response (3480 Miller units), respectively, following 20 μM DPD addition. Controls denoted "Cells Only" represent the response of the *luxS*$^-$ cells when no compounds were added; controls denoted "Methyl (DPD)" indicate the response of *luxS*$^-$ cells to exogenously added AI-2 (DPD). (* indicates $p < 0.05$ and ** indicates $p < 0.01$ for an unpaired *t* test of the particular analogue as compared to the "Methyl (DPD)" response).

our new data suggests that C1 acyl DPD analogues (both linear and branched chain) may fit into an active site pocket of the AI-2 processing/binding proteins, whereas some of the cyclic analogues do not have this capability. We investigated whether the cyclic and aromatic analogues were also processed *via* LsrR and LsrK.

Biological Evaluations of DPD Analogues on *E. coli* AI-2 Processing Enzymes (LsrK) and AI-2 Receptor (LsrR). We have shown previously that analogues most effective in inhibiting *lsr* expression in *E. coli* were also phosphorylated by LsrK.[10] The ability of *E. coli* LsrK to phosphorylate the cyclic analogues (both alkyl and aromatic) was tested by incubation with LsrK *in vitro* for 1 h using a method adapted from Xavier *et al.*[34] Cyclobutyl-DPD and cyclopentyl-DPD, which were significantly phosphorylated (Figure 3, panel a), also inhibit *lsr* expression in *E. coli* (Figure 2, panel a). Cyclohexyl-DPD and CH_2-cyclohexyl-DPD were weakly phosphorylated

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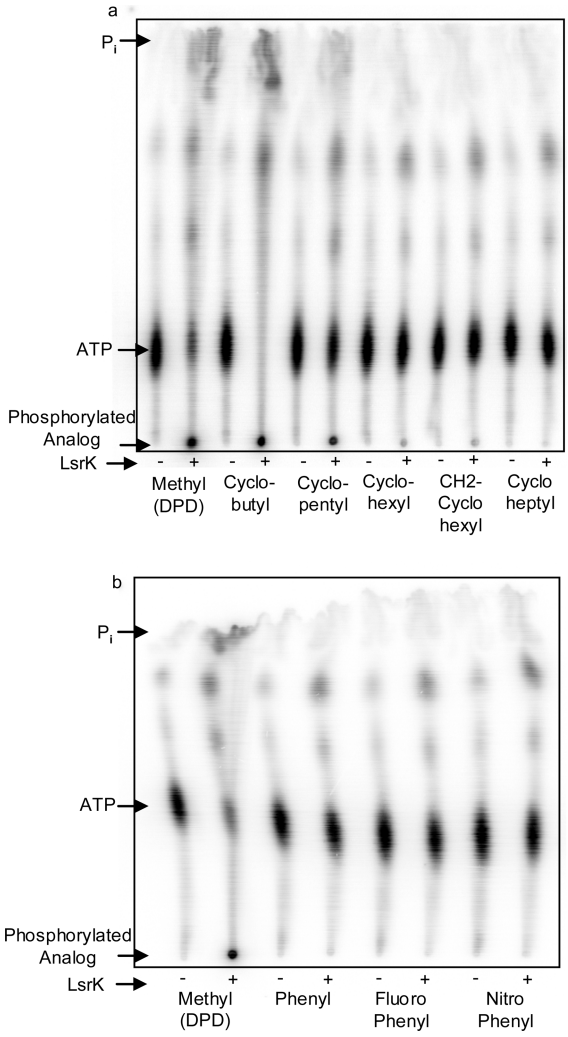

Figure 3. *In vitro* phosphorylation of analogues by LsrK. Thin layer chromatography (TLC) analysis of LsrK mediated analogue phosphorylation. (a) Cyclic analogues: methyl (DPD), cyclobutyl-DPD, cyclopentyl-DPD, cyclohexyl-DPD, CH_2-cyclohexyl-DPD, cycloheptyl-DPD. (b) Aromatic phenyl-, fluorophenyl-, and nitrophenyl-DPD treated with LsrK.

(Figure 3, panel a) and attenuated *lsr* expression in *E. coli* to a lesser extent. Neither Cycloheptyl-DPD nor the aromatic analogues were phosphorylated (Figure 3, panel a and b), nor did they affect LsrR-controlled transcription in *E. coli* (Figure 2, panel a). We evaluated IC_{50} values of all analogues; those that were significantly phosphorylated had significantly lower IC_{50} than those not phosphorylated, (*i.e.*, IC_{50} for cyclopentyl-DPD was 2.4 μM compared to phenyl-DPD, which was >40 μM). Isobutyl-DPD was the most efficient inhibitor, having an IC_{50} in the nanomolar range. These observations support our earlier proposal that the active DPD analogue species that are acting to repress *lsr* expression are the phosphorylated forms.[10]

To test whether the phosphorylated analogues work through repressor LsrR, we carried out studies in isogenic *lsrR* null mutants. Previous work showed that DPD analogues did not alter *lsr* expression in the absence of LsrR, which is the transcriptional repressor of the circuit. In this work results are similar; while there was more variability, there was no statistically significant difference between the analogues and the control, with the exception of the aromatic phenyl-DPD (Figure 4, panel a).

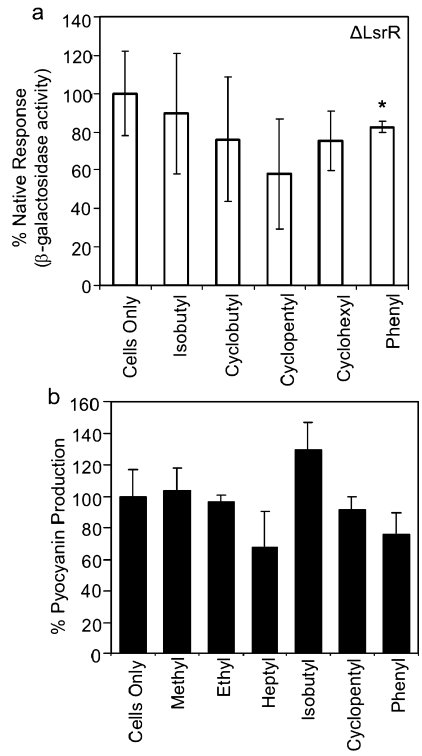

Figure 4. Effects of analogues on (a) *E. coli via* LsrR and (b) *P. aeruginosa via* production of pyocyanin. (a) AI-2 dependent β-galactosidase production in *E. coli* LW8 pLW11 (*luxS*$^+$, *lsrR*$^-$) in response to 20 μM analogue. (b) Effect of analogues (100 μM) on pyocyanin production in *P. aeruginosa* PAO1. (* indicates $p < 0.05$ for an unpaired t test of the particular analogue compared to the cells only response).

Identifying Modulators of *P. aeruginosa* QS. We screened DPD analogues (100 μM) for activity in *P. aeruginosa* PAO1 by monitoring pyocyanin production (see Figure 4b and Supplementary Figure 2). Pyocyanin is a redox active phenazine toxin produced by *P. aeruginosa* and has been shown to have antibiotic properties[35] as well as being a signal for the up-regulation of QS-controlled genes in the stationary phase.[36] Phenyl- and heptyl-DPD showed some inhibition of pyocyanin production (Figure 4, panel b and Supplementary Figure 2).

Effect of Analogues in a Trispecies Synthetic Ecosystem. Our results suggesting species-specific modulation of QS activity led us to test effects in mixed cell cultures. Isobutyl-DPD (40 μM) and phenyl-DPD (40 μM) were added to a trispecies synthetic ecosystem created by culturing *E. coli*, *S. typhimurium*, and *P. aeruginosa* in the same tubes. In order to differentiate and monitor the QS response from each organism, different and orthogonal reporter strains were used: in *E. coli*, AI-2 mediated dsRED expression; in *S. typhimurium*, AI-2 mediated β-galactosidase production; and in *P. aeruginosa*, effects of AI-2 were examined for pyocyanin production. It has previously been shown that isobutyl-DPD inhibited *E. coli* and *S. typhimurium* simultaneously, but due to its lack of inhibitory effects in *P. aeruginosa*, we suspected it would not serve well as a broad-spectrum quorum silencer in our trispecies synthetic ecosystem. Interestingly, it did inhibit all three species, but *P. aeruginosa* to a much lesser extent (Figure 5). Phenyl-DPD,

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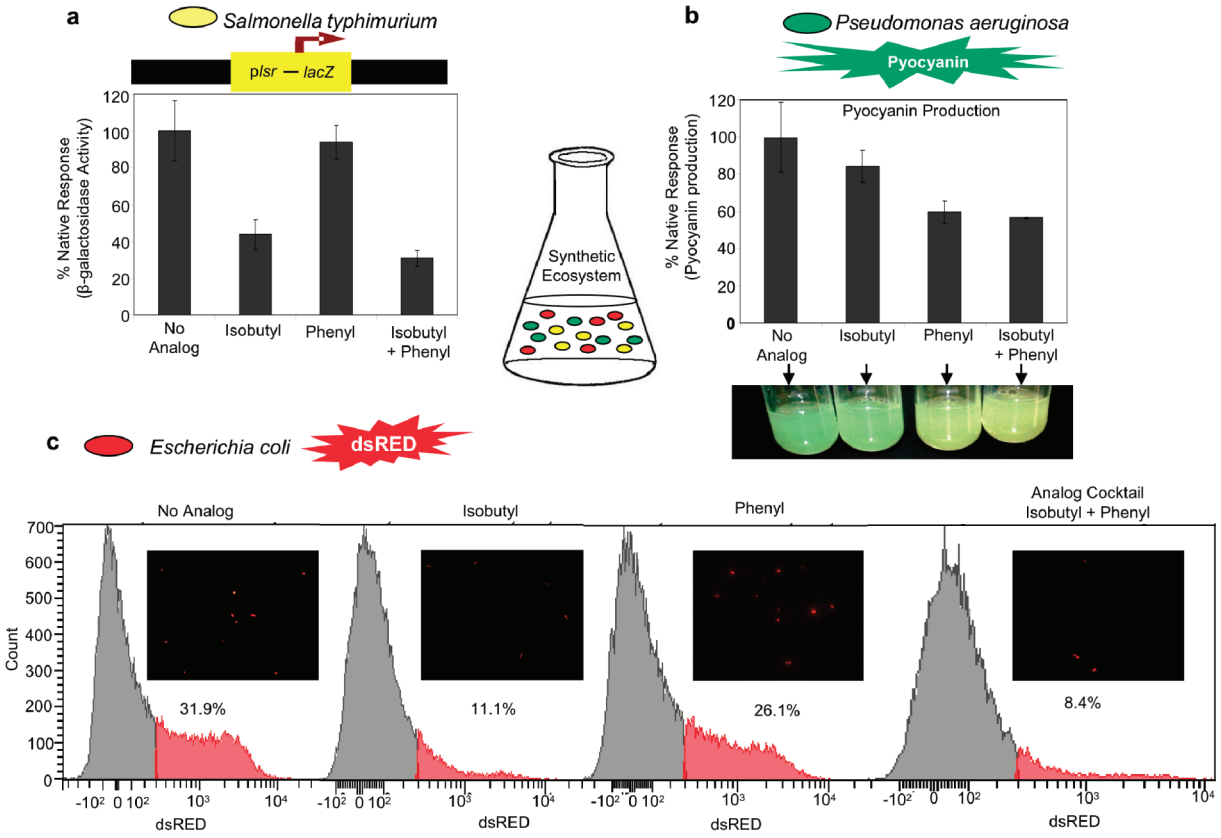

Figure 5. Effect of analogue and analogue cocktail in a trispecies synthetic ecosystem. (a) AI-2 dependent β-galactosidase production in *S. typhimurium* MET708 (normalized to native *S. typhimurium* response in trispecies culture = 475 Miller units). (b) QS related pyocyanin production in *P. aeruginosa* PAO1 (normalized to native *P. aeruginosa* response in trispecies culture = 2.5 μg/mL). (c) AI-2 dependent dsRED induction in *E. coli* W3110 pCT6 dsRED, in response to isobutyl-DPD (40 μM) and phenyl-DPD (40 μM) individually and a cocktail of both analogues. Note, in this entire system, no exogenous AI-2 was added. Instead, the enteric bacteria present in the ecosystem synthesized the AI-2 signal present.

on the other hand, was largely ineffective as a QS inhibitor for *E. coli* (Figure 5, panel c) and *S. typhimurium* (Figure 5, panel a) but retained some activity against pyocyanin production in *P. aeruginosa* (Figure 5, panel b).

While it would be tempting to state that there was a synergistic effect of one inhibitor on the other (because the responses of the cocktail were lower than the cases when each analogue was added individually to the trispecies), the statistical differences are insignificant. It can be stated, however, that there was no diminution of the effect of one analogue due to the presence of the other. That is, the effect of each analogue as a quorum quencher was undisturbed by the presence of the other analogue. A conclusion can be drawn, therefore, that a cocktail of DPD analogues consisting of isobutyl- and phenyl-DPD analogues could simultaneously disrupt QS signaling in the three different bacteria simultaneously *via* a nontoxic mechanism (see Supporting Information Figures 3, 4, 6 and 7).

Discussion. In light of the ubiquitous nature of AI-2, it has been envisioned that an AI-2 inhibitor would offer a universal quenching QS activity due to its prevalence among many bacteria in their native environments. Although this "magic bullet" may offer relief to the antibiotic resistance problem, there are many bacteria that form our microflora and are vital to our immunity and health. Ultimately anti-QS therapies must be able to offer a variety of options including both species-specific and multiorganism QS targeting.

We have developed a set of C1 analogues that enhances our understanding of the shapes and sizes of C1 substituted groups that are tolerated by AI-2 processing enzymes, such as LsrK.[33] We found C1 alkyl chains needed at least 3-carbon length for *lsr* inhibition in *E. coli*;[15] we now suggest the shape as well as the conformational flexibility of the C1 side chain is also important. For cyclic compounds there was an apparent 5-carbon maximum where analogues larger than cyclopentyl-DPD (*i.e.*, cyclohexyl, CH_2-cyclohexyl, cycloheptyl) were not phosphorylated by LsrK and were less effective on LsrR. This is in agreement with our previous studies showing that phosphorylation by *E. coli*'s LsrK was an essential checkpoint to first establish effectiveness as a modulator of the *lsr* QS circuit. IC_{50} data (Table 2) supported this conclusion in that analogues that

Table 2. Inhibitory Concentrations (IC_{50}) of Select Analogues[a]

analogue	IC_{50} of analogue (nM) to inhibit QS in *E. coli*	standard error (log IC_{50}) *E. coli*	IC_{50} of analogue (nM) to inhibit QS in *S. typhimurium*	standard error (log IC_{50}) *S. typhimurium*
isobutyl-DPD	54	181	20000	116
butyl-DPD	2967	193	>40000	ND
cyclopentyl-DPD	2443	146	>40000	ND
heptyl-DPD	15940	244	>40000	ND
phenyl-DPD	>40000	ND	>40000	ND

[a] IC_{50} values reflect the concentration of analogues required to reduce lsr expression (β-galactosidase production) to 50% in the presence of 20 μM exogenously added synthetic DPD. β-Galactosidase production in the absence of analogue (only 20 μM DPD) was set as the 100% response.

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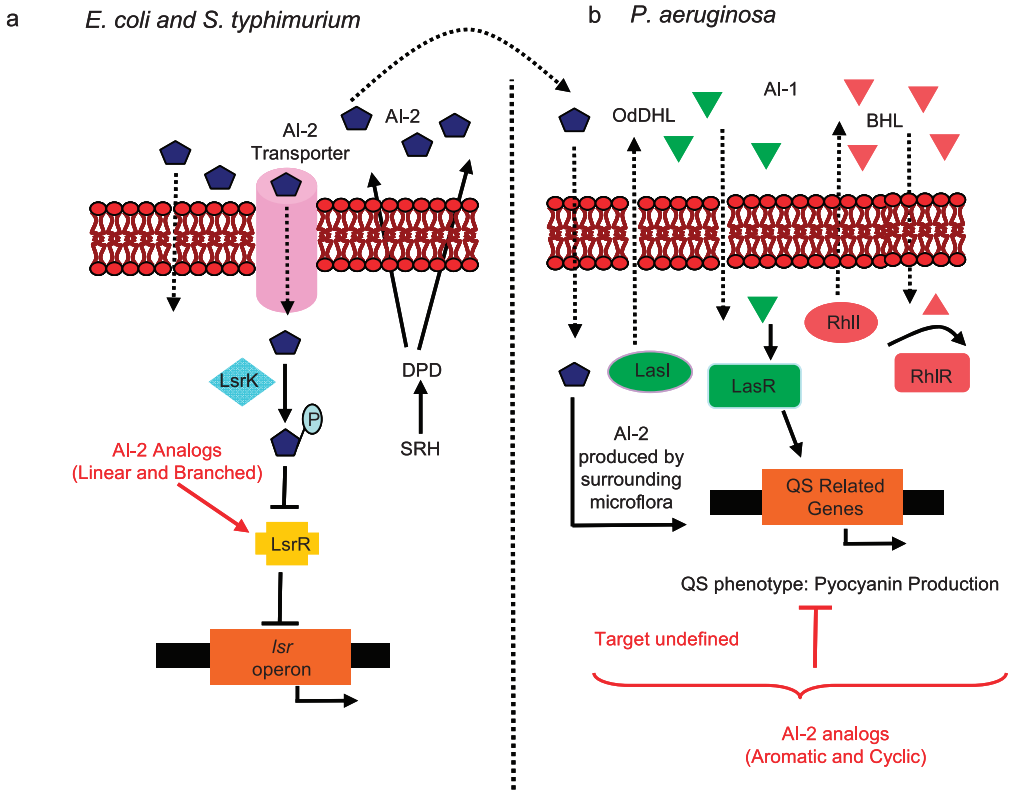

Figure 6. Quorum sensing pathways in *E. coli*, *S. typhimurium* and *P. aeruginosa* and AI-2 analogue points of action. (a) AI-2 based QS signaling circuit in *E. coli* and *S. typhimurium*; effective analogues are phosphorylated by LsrK and work *via* altering repressor LsrR activity. (b) The two AI-1 based LuxI/LuxR QS signaling circuits in *P. aeruginosa*; LasI/R and RhlI/R that produce different AHL autoinducers, *N*-(3-oxododecanoyl)-HSL (OdDHL) and *N*-butyryl-HSL (BHL), respectively, to control QS associated virulence factors. There remain no identified effectors modulated by AI-2 or analogues, just phenomenological observation.

are not readily phosphorylated require higher concentrations in order to obtain 50% *lsr* repression. In *P. aeruginosa*, where the phosphorylation pathway is not involved, phenyl-DPD inhibited QS related pyocyanin production *via* unknown mechanisms. The inability of phenyl-DPD to completely inhibit pyocyanin production is probably due to the fact that other pathways, such as the AI-1 pathway, also control pyocyanin production.

Exogenous AI-2 can up-regulate genes important for *P. aeruginosa* pathogenesis.[37] *P. aeruginosa* utilizes the AI-1 controlled LuxI/LuxR QS signaling circuit (Figure 6, panel b), but because they are often found among other Gram-negative microbes capable of synthesizing AI-2, *P. aeruginosa* likely detects their AI-2 to coordinate the expression of pathogenic genes during infection.[37] Although the mechanisms by which AI-2 affects *P. aeruginosa* are unknown, this study has revealed that AI-2 analogues, such as phenyl-DPD, could become lead compounds for further development as *P. aeruginosa* antitoxin inhibitors. Interestingly, we found some evidence that the effects of phenyl-DPD were greater against *P. aeruginosa* when cultivated in the mixed culture. We have not explored, however, concentration dependence nor population dependence to see whether this is a robust finding or an experimental artifact. We do note, however, that *V. harveyi* QS signaling was dramatically attenuated when in a mixed culture with *E. coli*.[38]

In conclusion, this diverse library of AI-2 analogues has shed light on the QS systems of *E. coli*, *S. typhimurium*, and *P. aeruginosa*. In the future, these and other analogues might be tailored with molecular precision to alter QS activity among many clinically relevant species. That is, several studies have revealed that among polymicrobial infections, AI-2 increases virulence factor production.[39−41] This study also demonstrates an initial foray into "quorum quenching" among mixed cultures, and because the apparent effects of the key antagonists appear to retain their independence, it may be feasible to interrogate effector molecules, their concentrations, and differing species and their own relative proportions among consortia as a means to unravel these complex communication networks. Additional efforts on developing meaningful *in vitro* testing platforms that will enable a delineation of effects of antimicrobial concentration and response time will be helpful.

METHODS

Bacterial Strains and Growth Conditions. Table 1 lists the bacterial strains and plasmids used in this study. *S. typhimurium* and *E. coli* strains were cultured in Luria−Bertani medium (LB, Sigma) at 37 °C with shaking at (250 rpm) unless otherwise noted. Antibiotics were used for the following strains: kanamycin (60 or 100 μg/mL) for *S. typhimurium* MET715 and *S. typhimurium* MET708; ampicillin (60 or 100 μg/mL) for *E. coli* LW7 pLW11; and ampicillin (50 μg/mL) and kanamycin (50 μg/mL) for *E. coli* W3110 pCT6 dsRED .

Measurement of the QS Response (*lsr* Expression). The QS response indicated by *lsr* gene expression was analyzed in pure culture studies by culturing *E. coli* LW7 pLW11, *E. coli* ZK126 pLW11 and *S. typhimurium* MET708, *S. typhimurium* MET715 overnight in LB medium individually supplemented with appropriate antibiotics, as specified above. These cells were diluted into fresh LB medium (with antibiotics) and grown to an OD_{600} of 0.8−1.0 at 30 °C, 250 rpm. Cells were collected by centrifugation at 10,000 × g for 10 min and resuspended in 10 mM phosphate buffer. AI-2 (20 μM) and the respective analogue (20 μM) were added to the *E. coli* or

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S. typhimurium suspension for 2 h at 37 °C. AI-2 dependent β-galactosidase production was quantified by the Miller assay.[42]

***In Vitro* Phosphorylation of Analogues.** LsrK was purified from *E. coli* BL21 pET200-LsrK as described before.[9] Phosphorylated analogues were synthesized by incubating 1 μM LsrK with 40 μM ATP (Roche), 0.2 Ci of [P^{32}] ATP (Perkin-Elmer), 300 μM AI-2 or analogue, and 200 μM $MgCl_2$ in 25 mM phosphate buffer, pH 7.4 for 2 h. An aliquot (2.5 μL) was then spotted onto a cellulose TLC plate (Selecto Scientific). The plate was developed using 0.8 M LiCl as the solvent, air-dried, and developed *via* autoradiography.

Measurement of the QS Response (Pyocyanin Production). The cells were grown with the analogue at 3 mL total volume in 50 mL flasks in LB medium, with continuous shaking. Between 22 and 24 h, when the cells turned green after pyocyanin secretion, the pigment was extracted. The pyocyanin quantification assay was conducted as described.[43] Two milliliters of chloroform was added to the 3 mL culture and pipetted up and down. One milliliter of the chloroform was transferred to a separate tube, and the pyocyanin was re-extracted into 200 μL of 0.2 M HCl. The absorbance of the solution was measured at OD 520 nm. To calculate the concentration of pyocyanin extracted as μg/mL, the OD at 520 nm was multiplied by 17.07.[43]

Analyzing QS Response in the Synthetic Ecosystem. *S. typhimurium* MET708, *P. aeruginosa* PAO1, and *E. coli* W3110 pCT6 dsRED were each cultured separately overnight in LB medium supplemented with the appropriate antibiotic. *P. aeruginosa* PAO1, *S. typhimurium* MET708, and *E. coli* W3110 pCT6 dsRED were diluted (25 μL:2.5 μL:100 μL) from the overnight cultures, respectively, into a single 2 mL final volume of fresh LB medium without antibiotics. The co-culture was supplemented with the respective analogue at 40 μM concentration or analogue cocktail initially and again after 2.5, 5, 9, and 18 h of growth. The *S. typhimurium lacZ (β-galactosidase)* activity was measured after 4 h. The *E. coli* response was determined after 24 h, by fixing the cells with 1:1 cold 4% paraformaldehyde and using flow cytometric analysis. Samples were analyzed by flow cytometry (FACS Canto II, BD 394 Biosciences), with 30,000 gated events analyzed per sample. Pyocyanin was also extracted after 24 h of growth.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

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Notes

The authors declare no competing financial interest.

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