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Published on Web 07/26/2010

Synthetic Analogs Tailor Native AI-2 Signaling Across Bacterial Species

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Abstract: The widespread use of antibiotics and the emergence of resistant strains call for new approaches to treat bacterial infection. Bacterial cell−cell communication or "quorum sensing" (QS) is mediated by "signatures" of small molecules that represent targets for "quenching" communication and avoiding virulent phenotypes. Only a handful of small molecules that antagonize the action of the "universal" autoinducer, AI-2, have been reported. The biological basis of antagonism, as well as the targets for these select few AI-2 antagonists, have not been clearly defined. We have developed C-1 alkyl analogs of AI-2 that quench the QS response in multiple bacterial species simultaneously. We also demonstrate the biological basis for this action. Like AI-2, the analogs are activated by the bacterial kinase, LsrK, and modulate AI-2 specific gene transcription through the transcriptional regulator, LsrR. Interestingly, addition of a single carbon to the C1-alkyl chain of the analog plays a crucial role in determining the effect of the analog on the QS response. While an ethyl modified analog is an agonist, propyl becomes an antagonist of the QS circuit. In a trispecies synthetic ecosystem comprised of *E. coli*, *S. typhimurium*, and *V. harveyi* we discovered both cross-species and species-specific anti-AI-2 QS activities. Our results suggest entirely new modalities for interrupting or tailoring the network of communication among bacteria.

Introduction

Quorum sensing (QS) is a bacterial cell−cell communication system that regulates diverse phenotypes including motility, attachment, biofilm formation, and pathogenicity by the secretion and perception of small signal molecules. QS receptors, transporters, regulators, and the signals themselves (also known as autoinducers) represent a vast reservoir of targets that can be manipulated to interrupt communication.[1−3] QS effector molecules that function to eliminate pathogenicity but otherwise remain innocuous to the cells have received significant attention. They presumably pose less evolutionary pressure on bacteria than current bacteriostatic or bacteriocidal antimicrobials that drive mutation and the emergence of drug resistant strains.[3−6] Anti-QS agents, used in combination with antibiotics, have already shown promise in clearing recalcitrant *Pseudomonas aeruginosa* biofilms.[7] Additional approaches that "quench" QS communication will build on the emerging understanding of QS circuitry, its signal transduction process, and the sender−receiver relationship that connects one bacterium to another or to a population.

The autoinducer signaling molecules are organized into families based on their structure and mode of action. Autoinducers-1 (AI-1) such as acyl homoserine lactones (AHLs) mediate *intra*-species communication,[8] whereas a family of cyclic furanones, collectively termed autoinducer-2 (AI-2), are utilized in *inter*species bacterial communication.[9] Interestingly, both AI-1 and AI-2 families of autoinducers are derived from *S*-adenosylmethionine (SAM),[10] as part of bacterial 1-carbon metabolism. The AI-2 precursor 4,5-dihydroxy-2,3-pentanedione (DPD) is synthesized from *S*-adenosylhomocysteine (SAH) via a two-step enzymatic process catalyzed by methylthioadenosine (MTA) nucleosidase (Pfs) and *S*-ribosylhomocysteinase (LuxS). Synthase inhibitors can potentially alter both AI-1 and AI-2 signaling by disrupting signal generation.[11] Also, a large and diverse population of bacteria could be addressed with AI-2

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inhibitors as LuxS is present in over 70 bacterial species.[12] Correspondingly an alternative and equally important strategy is to disrupt AI-2 QS signaling by interfering with the signal reception and transduction processes.

Thus far, the majority of these quorum "quenching" strategies have focused on developing inhibitors of AI-1 (AHL) signaling,[13,14] which are mostly species specific. The ubiquitous nature of AI-2, however, opens avenues for broadly inhibiting or modulating QS communication as AI-2 modulates the behavior of many microbes including *Bacillus anthracis*,[15] *V. cholerae*,[16] and *E. coli* O157[17,18] and many others. Few small molecule antagonists of AI-2 quorum sensing have been reported, however the molecular targets for these have not been clearly defined. Identifying these targets will lead to new inhibitor families and new strategies for treating QS modulated disease. In enteric bacteria, including pathogenic *E. coli* O157 and *S. enterica* Serovar Typhimurium, a small number of AI-2-regulated genes and proteins are known; they are denoted LuxS-regulated (*lsr*). AI-2 is transported into these cells by an ABC cassette-like transporter (Lsr),[19] followed by phosphorylation via a kinase LsrK.[20,21] The phosphorylated AI-2 binds to and derepresses the transcriptional regulator (LsrR), releasing it from the *lsr* (*luxS* regulated) operator, allowing transcription of the genes encoding the aforementioned AI-2 processing proteins.[22] LsrR has been shown to play an important role in *E. coli* biofilm maturation,[23] as well as regulating the expression of over 68 proteins in *E. coli*, including important virulence determinants.[24] LsrR-like proteins are therefore important targets for the development of small molecule quorum sensing modulators.[25] By targeting AI-2/Lsr interactions we have attempted to modulate QS circuitry in a variety of bacterial species.

Herein we identify, through focused chemical library design and biological and biochemical studies, the key structural components of AI-2 that are important for the development of a potent, broad-spectrum antiquorum sensing agent. We synthesized C1-alkyl analogs of 4,5-dihydroxy-2,3-pentanedione (DPD), which is the precursor of the universal autoinducer AI-2. DPD is the linear form of AI-2, and it exists in equilibrium with several interconvertible isoforms in solution, all of which are known as the family of AI-2 signaling molecules. This panel of C1-alkyl AI-2 analogs permits detailed investigation of uptake, phosphorylation, and altered AI-2 mediated gene transcription in several bacteria. Our work suggests that the antagonistic activity of C1-alkyl analogs of AI-2 is likely due to competitive binding to the LsrR transcriptional regulator. We demonstrate that, unlike other bacterial kinases,[26,27] LsrK from *E. coli* has broad substrate specificity and phosphorylates C1-alkyl AI-2 analogs of different shapes and sizes. In *E. coli*, the majority of the phosphorylated AI-2 analogs compete with phospho-AI-2 for binding to LsrR. Interestingly, lower alkyl chain AI-2 analogs (C1 and C2) destabilize the LsrR−DNA complex and promote *lsr* transcription whereas higher alkyl chain AI-2 analogs (C3−C7) stabilize the Lsr−DNA complex and inhibit *lsr* transcription. The Lsr proteins of *S. typhimurium* and *E. coli* share high sequence and predicted structural homologies, yet the majority of AI-2 analogs that inhibited *lsr* expression in *E. coli* failed to do so in *S. typhimurium*, demonstrating both flexibility and specificity in QS circuitry. Thus our analogs differentiate between highly homologous quorum responses and represent tools for controlling QS systems with an additional level of selectivity and finesse. This current work therefore demonstrates for the first time that the nature of the C1-alkyl chain of AI-2 analogs may be used to quench QS in either a variety of bacteria (broad-spectrum anti-QS) or in selected or targeted[28,29] bacteria. The ability to modulate QS *en masse* in a trispecies synthetic ecosystem or selectively has important clinical implications. For example, in the gut, the microflora is composed of various nonpathogenic and mutualistic bacteria that also utilize AI-2 signaling for nonpathogenic processes.[30,31] Therefore, specifically targeting pathogenic bacterial species such as enterohemorrhagic *E. coli*, but not the symbionts, will allow the useful bacteria to coordinate their behavior in an efficient manner, whereas the targeted pathogenic species will be out-of-sync and hence engage in "out-of-quorum" behaviors that might be evolutionarily disadvantageous.

Experimental Section

Synthesis of Diazodiols. DBU (0.16−0.20 equiv) and the requisite aldehyde (2-(*tert*-butyldimethylsilyloxy) acetaldehyde or acetaldehyde) (1−1.5 equiv) were added to a solution of the diazocarbonyl in anhydrous acetonitrile (0.2 M). The reaction was stirred at room temperature under nitrogen for 4−8 h and monitored by TLC. Upon disappearance of starting material, the reaction was quenched with sodium bicarbonate. The organic layer was extracted with dichloromethane (3 × 20 mL) and dried with magnesium sulfate. The solvent was evaporated under reduced pressure. To a solution of crude product in anhydrous tetrahydrofuran (0.2 M)

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Table 1. List of Bacterial Strains and Plasmids Used in This Study

Strain, plasmid, or primer	Relevant genotype and/or property	Reference
	Escherichia coli strains	
W3110	Wild type	Laboratory Stock
BL21 *luxS*$^-$	*F′* ompT hsdS_B ($r_B^-m_B^-$) *gal dcm* Δ*luxS* :: Kan	Laboratory Stock
LW7	W3110 Δ*lacU160-tna2* Δ*luxS* :: Kan	20
ZK126	W3110 Δ*lacU169-tna2*	34
LW9	ZK126 Δ(*lsrACDBFG*) :: Kan	20
SH3	W3110 Δ*lacU160-tna2* Δ*luxS* Δ*lsrK* :: Kan; *Cm*	Laboratory Stock
LW8	ZK126 _*lsrR* :: Kan	20
	Salmonella typhimurium strains	
MET715	*rpsl putRA ::* Kan-*lsr-lacZYA luxS* :: T-POP	35
MET708	*rpsl putRA ::* Kan-*lsr-lacZYA*	35
	V. harveyi strains	
BB170	BB120 *luxN* :: Tn*5* (sensor 1$^-$, sensor 2$^+$); AI-1$^+$, AI-2$^+$	36
	Plasmids	
pLsrK	pET200 derivative, *Escherichia coli* W3110 LsrK$^+$	21
pLW11	*galK′-lacZYA* transcriptional fusion vector, containing *lsrACDBFG* promoter region, Amp^r	20
pCT6	pFZY1 derivative, containing *lsrR* and *lsrR* promoter region fused with *T7RPol*, Ap^r	37
pET-GFP	pET200 derivative, containing *gfpuv*, Km^r	37

TBAF was added (1−2 equiv) at 0 °C. The solution was allowed to warm to room temperature and stirred for 1−3 h under nitrogen. The solvent was evaporated, and the crude product was purified by column chromatography. The product eluted as a yellow oil with 1:3 to 3:2 ethyl acetate/hexane.

Synthesis of DPDs. Dioxirane (15−20 mL) in acetone was added dropwise to a solution of diazodiol (1 equiv) in acetone (1−2 mL). The reaction was allowed to stir at room temperature (1−2 h) until complete disappearance of starting material was indicated by TLC (loss of UV activity). Solvent and excess reagent were evaporated under reduced pressure. NMR was taken without further purification.

Synthesis of Quinoxaline Derivatives. 1,2-Phenylenediamine (1.5 equiv) was added to a solution of the DPD analog. The reaction was stirred at room temperature for 10 min, and then the reaction mixture was washed with (2 M) HCl. The crude mixture was purified on silica.

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 either 30 or 37 °C with vigorous shaking (250 rpm) unless otherwise noted. The *V. harveyi* strains were grown in LM medium. Antibiotics were used for the following strains: (60 or 100 μg mL^{-1}) kanamycin for *S. typhimurium* MET715, (50 μg mL^{-1}) ampicillin for *E. coli* BL21 *luxS*$^-$, (60 or 100 μg mL^{-1}) ampicillin for *E. coli* LW7 pLW11, (50 μg mL^{-1}) ampicillin and (50 μg mL^{-1}) kanamycin for *E. coli* MDAI-2 pCT6 and *E. coli* SH3 pLW11 along with (20 μg mL^{-1}) chloramphenicol for the latter and (20 μg mL^{-1}) kanamycin for *V. harveyi* BB170.

***In Vitro* Phosphorylation of DPD Analogs.** LsrK was purified from *E. coli* BL21 pET200-LsrK as described before.[21] Phosphorylated analogs 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, and (200 μM) $MgCl_2$, in (25 mM) phosphate buffer, pH 7.4 for 2 h. A 2.5 μL aliquot 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 (*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 supplemented with appropriate antibiotics as stated previously. These cells were then 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 then collected by centrifugation at 10 000 × *g* for 10 min and resuspended in 10 mM phosphate buffer. AI-2 (20 μM) and the respective analog (20 μM) were added to the *E. coli* or *S. typhimurium* suspension for 2 h at 37 °C. AI-2 dependent β-galactosidase production was quantified by the Miller assay.[32]

Measurement of the QS Response (Bioluminescence). The effect of isobutyl-DPD or isopropyl-DPD on QS associated bioluminescence production by *V. harveyi* was recorded by measuring the light production from the reporter strain, *V. harveyi* BB170. The analogs were added at concentrations of 20 or 40 μM to *V. harveyi* BB170 which was assayed as described previously.[33]

Analyzing QS Response in the Synthetic Ecosystem. The *S. typhimurium* MET708, *V. harveyi* BB170, and *E. coli* W3110 pCT6 were each cultured separately overnight in LM medium[33] supplemented with the appropriate antibiotic. *V. harveyi* BB170, *S. typhimurium* MET708, and *E. coli* MDAI-2 were diluted (1:4:8), respectively, into a single 1 mL volume of fresh LM medium without antibiotics. The coculture was supplemented with either 20 or 40 μM analog initially and again after 3 and 5 h of growth. The *V. harveyi* luminescence response was measured after 2.5 h. The *S. typhimurium lacZ* (β-galactosidase) activity was measured after 4 h. The *E. coli* response was determined after 8 h, by fixing the cells with 1:1 cold 4% paraformaldehyde and using flow cytometric analysis. Samples were analyzed by flow cytometery (FACS Canto II, BD 394 Biosciences), with 20 000 gated events analyzed per sample.

Results

Synthesis of the C1-Alkyl Analogs of DPD. A panel of linear (see Figure 1b, **1**−**7**) and nonlinear DPD analogs (see Figure 1c, **8**−**12**) was prepared via a simple two-pot DPD synthesis previously reported by us.[38] This expedient process uses diazocarbonyls as an umpolung for the dione (dicarbonyl) of DPD. Aliphatic diazocarbonyls were readily generated from acid chlorides and diazomethane. The various diazocarbonyls were then reacted with a silyl-protected oxo-aldehyde under mild, catalytic DBU conditions. Without isolation of the resulting adduct, the silyl group was deprotected with TBAF and the diazodiols were oxidized with dimethyl dioxirane to afford DPD analogs.

Studies by Bassler and others[39,40] have indicated that phosphorylated DPD, and not the unphosphorylated form, binds to LsrR to destabilize the LsrR/DNA complex. To verify that unphosphorylated byproducts of LsrK-mediated phosphorylation are not antagonists, we synthesized DPD analogs (see Figure 1d, **13**−**14**) that lacked the primary hydroxyl unit (site of phosphorylation). As these deoxy-DPD analogs cannot be

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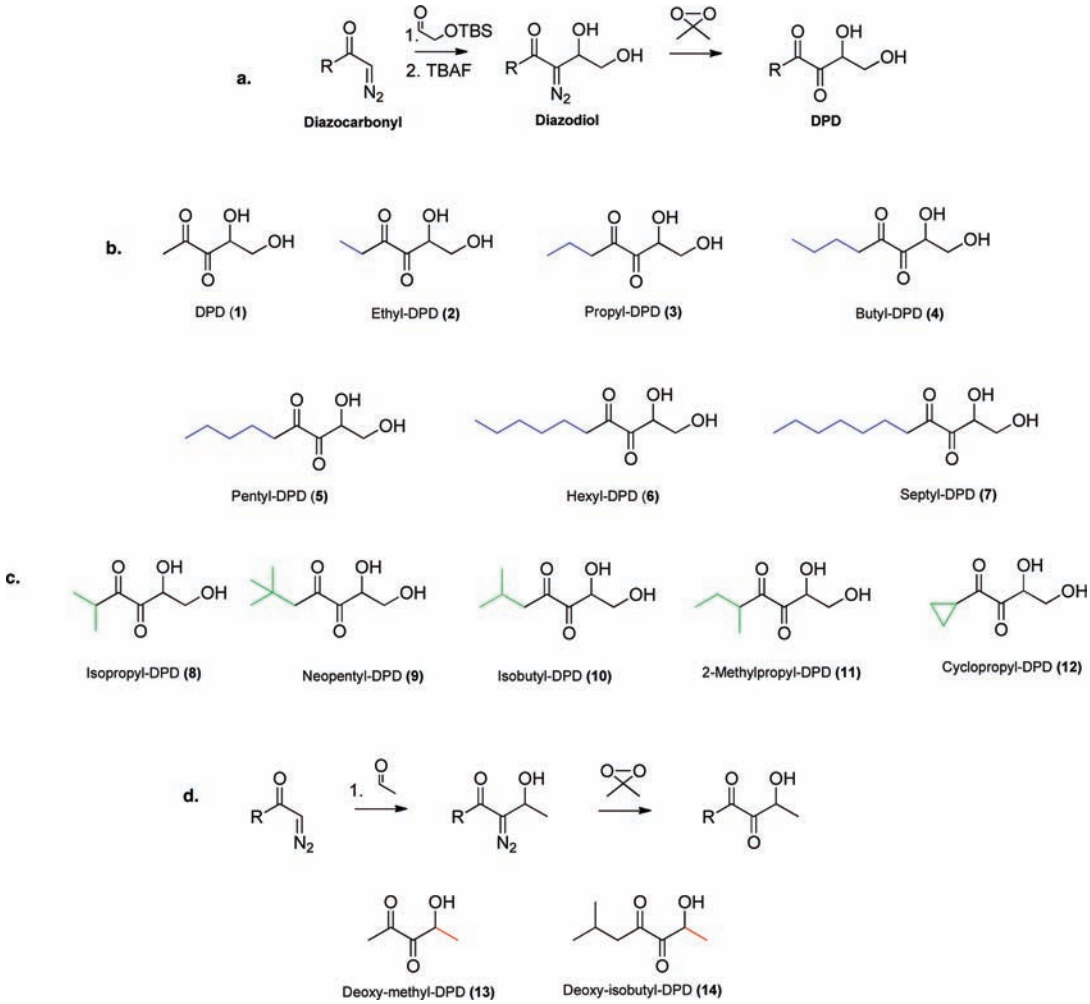

Figure 1. Library of 14 C1-alkyl analogs. (a) Synthesis strategy for linear and branched DPD analogs. (b) Structures of the seven linear DPD analogs. (c) Structures of the five branched DPD analogs. (d) Synthesis strategy and structures for the deoxy-DPD analogs.

phosphorylated, they serve to test whether the phosphate moiety of AI-2 analogs is important for *lsr* antagonism.

Identifying Quorum Quenchers in Enteric Bacteria. The panel of C1-alkyl analogs was screened to identify QS modulators in the enteric bacteria *E. coli* and *S. typhimurium*. We monitored transcription of the QS associated *lsr* operon, by using *lsr-lacZ* reporter strains (Table 1) which produce β-galactosidase in response to added AI-2. To first establish if the analogs were agonists or antagonists in the QS circuit, the analogs were incubated with *E. coli* LW7 pLW11 and *S. typhimurium* MET715 (see Supporting Information Figure S1a−b) both of which are *luxS*$^-$. None of the analogs, except ethyl-DPD, behaved as agonists for *lsr* expression. Previous studies investigating AI-2 antagonism in *P. aeruginosa*[41] or in *S. typhimurium*[42] were carried out using chemically synthesized AI-2 added to *luxS* $^-$ cells. To simulate the natural scenario where wild-type cells produce and detect their own AI-2, our panel of C1-alkyl analogs were tested on *luxS*$^+$ cells *E. coli* ZK126 pLW11 and *S. typhimurium* MET708 (Table 1).

We observed that a minimum of three carbons in the C1 alkyl chain of DPD was required for QS antagonism; propyl-DPD and all larger linear alkyl chain analogs tested (see Figure 2a) caused a significant knockdown in native *lsr* expression in *E. coli*. However, for the linear analogs, only butyl-DPD caused a considerable reduction in *lsr* expression in *S. typhimurium*. In the panel of nonlinear DPD analogs (see Figure 2b), neopentyl-DPD, isopropyl-DPD, and 2-methylpropyl-DPD caused substantial reduction of the *E. coli* QS response, but only isobutyl-DPD caused substantial reduction of the wild type *lsr* expression in both *E. coli* and *S. typhimurium* (see Figure 2b). The neopentyl, 2-methylpropyl, and cyclopropyl QS analogs attenuated expression in *S. typhimurium*, but minimally. The deoxy-C1-analogs did not substantially reduce the QS response in either *E. coli* or *S. typhimurium* (see Figure 2b). The same trends were obtained by adding a 1:1 ratio of enzymatically synthesized AI-2 and DPD analogs to the *luxS*$^-$ strains *E. coli* LW7 pLW11 and *S. typhimurium* MET715 (see Supporting Information Figure S2a−b). None of our tested DPD analogs were bacteriocidal or bacteriostatic as the growth of *E. coli* ZK126 pLW11 and *S. typhimurium* MET708 growth in the presence and absence of the analogs were similar (see Supporting Information Figure S3a−b).

Interestingly, though the *lsr* operon and AI-2 processing proteins of the enteric organisms *E. coli* and *S. typhimurium* are homologous, they responded differently to the DPD analogs. The *E. coli* QS circuitry appears susceptible to silencing by a

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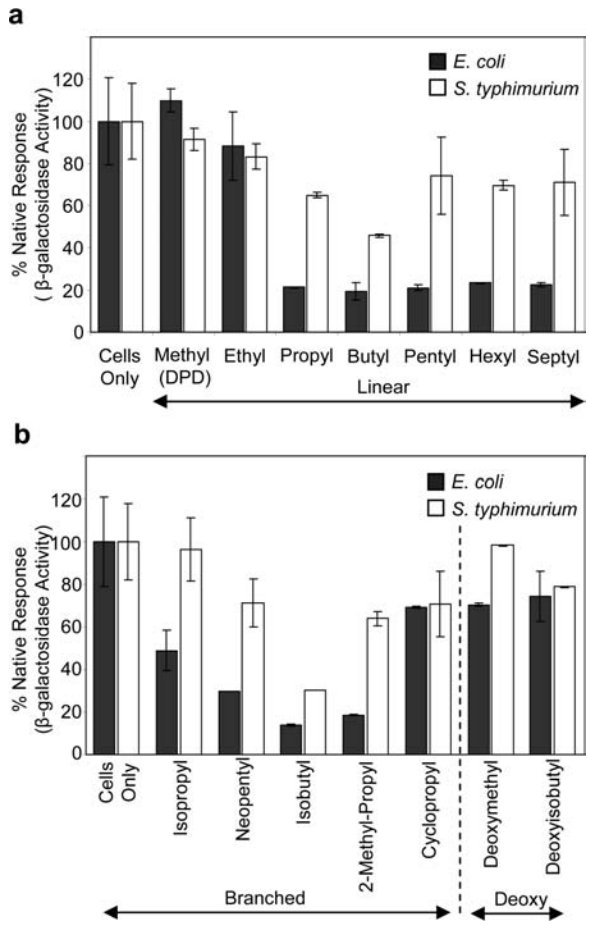

Figure 2. Analogs inhibit native signaling in *E. coli* and *S. typhimurium.* AI-2 dependent β-galactosidase production in *E. coli* ZK126 pLW11 and *S. typhimurium* MET708 both (*luxS*$^+$) in response to (a) linear analogs and (b) branched and deoxy analogs. (100% Native *E. coli* (ZK126) response = 1103 Miller units and 100% native *S. typhimurium* (MET708) response = 4478 Miller units.)

variety of DPD analogs of different shapes and sizes, whereas *S. typhimurium* was typically minimally affected (isobutyl-DPD being a noted exception). This suggests that the QS processing machinery in *E. coli* is flexible (or promiscuous) in processing different DPD analogs whereas that of *S. typhimurium* seems to be more specific. Isobutyl-DPD emerged as an effective and cross-species QS quencher, causing significant *lsr* expression knockdown in both *E. coli* and *S. typhimurium*.

Uptake and Phosphorylation of DPD Analogs by *E. coli.* The decrease of *lsr* expression caused by the linear DPD analogs in *E. coli* seemed to be dependent on the length of the alkyl chain; a minimum C3 alkyl chain was required for significant *lsr* expression knockdown. We note that the AI-1 family of autoinducers, long chain homoserine lactones, are known to diffuse freely into the cells through the membrane and directly bind to cognate transcriptional regulators. The possibility that our C3+ AI-2 alkyl analogs might freely pass through the cells and function as antagonists was intriguing. Hence, we studied whether the *lsr* AI-2 transporter[20,22] was necessary for analog transport by adding DPD analogs to *E. coli* LW9 pLW11 cells (Table 1) that lack the Lsr transporter. This strain also lacks phospho-AI-2 degradation enzymes, LsrG and LsrF. This strain is expected to be more sensitive for detecting the presence of processed AI-2 or analogs than a strain containing both LsrF and LsrG, as the intracellular level of phospho-AI-2 may be higher. Results indicated nearly identical suppression for all analogs among both the Lsr$^-$ and Lsr$^+$ strains (e.g., transporter $^-/^+$) (compare Figure 2a−b with Figure 3a−b). There was apparently no difference due to the transporter. We had previously found that enzymatically synthesized AI-2 could enter *E. coli* and activate lsr transcription independent of the *lsr* transporter.[20] Our new results demonstrate that AI-2 and synthesized analogs can enter bacteria via an alternative pathway, either through simple diffusion (e.g., enabled by "AI-1 like" alkyl chains) or as mediated by an unknown transporter. To elucidate the action pathway of the analog once inside the cells, we looked at the importance of the transcriptional repressor of the circuit LsrR and observed that the analog could not function in repressing *lsr* expression in the absence of LsrR. Thus LsrR seems to be crucial for the action of an analog as an antagonist in the cell.

If the native AI-2 processing machinery were flexible, then analogs that are sufficiently "AI-2 like" could potentially be acted upon and "activated" for signal transduction. *In vivo*, the critical step that makes AI-2 functional in derepressing the *lsr* operon is phosphorylation by the kinase, LsrK. Thus, the ability of LsrK to phosphorylate the analogs was monitored by incubating the analogs with LsrK and an excess of ATP for 2 h using a method adapted from Xavier *et al.*[39] All analogs, except the deoxy-C1-alkyl forms, were phosphorylated but to varied extents (see Figure 4 and Supporting Information Figures S4, S5). A representative thin layer chromatography (TLC) plate of DPD analog phosphorylation is shown in Figure 4, including an analog from each category (linear, branched, and deoxy-C1-alkyl-DPD) and chemically synthesized AI-2. Radio-labeled phosphorylated DPD analogs have lower mobility than radio-labeled ATP, in agreement with Xavier *et al.*[39] The deoxy-C1-analogs remained unphosphorylated as the terminal hydroxyl required for DPD phosphorylation was absent, further confirming Bassler's earlier report that LsrK phosphorylates the primary and not the secondary hydroxyl unit of DPD.[39] Interestingly, isobutyl-DPD was found to be a potent QS "quencher" and its unphosphorylated form, deoxy-isobutyl-DPD, silenced the *lsr* operon minimally as shown in Figure 2a−b. This provides circumstantial evidence that phosphorylation of an analog is most likely essential for its role in QS inhibition.

Phosphorylation of a DPD analog is, however, not the only determinant as to whether the analog can affect *lsr* expression. For example, cyclopropyl-DPD is phosphorylated by LsrK but is neither an *lsr* agonist nor antagonist. Significantly, isopropyl-DPD which has a similar size and electrostatic molecular surface to those of cyclopropyl-DPD (see Figure 5a−b) is both phosphorylated by LsrK and acts as a partial antagonist of *lsr* expression in *E. coli*. Cyclopropyl-DPD is similar in size/shape to isopropyl-DPD but the hydrogen bonding capability of the carbonyl moiety in cyclopropyl-DPD is slightly different from that of isopropyl-DPD. The cyclopropyl substituent can stabilize the carbonyl moiety better than the isopropyl alkyl group. Density functional theory (DFT) calculations (see Figure 5c−h) reveal that the highest occupied molecular orbital (HOMO) of both cyclopropyl- (HOMO-3) and isopropyl- (HOMO-4) DPD are close in energy to the lowest unoccupied molecular orbitals (LUMO) of the carbonyl moieties. However the cyclopropyl HOMO-3 overlaps better with the carbonyl LUMO as compared to the overlap between the isopropyl HOMO-4 and its carbonyl LUMO. These effects are subtle but the biological responses to these analogs are drastically different, suggesting that the

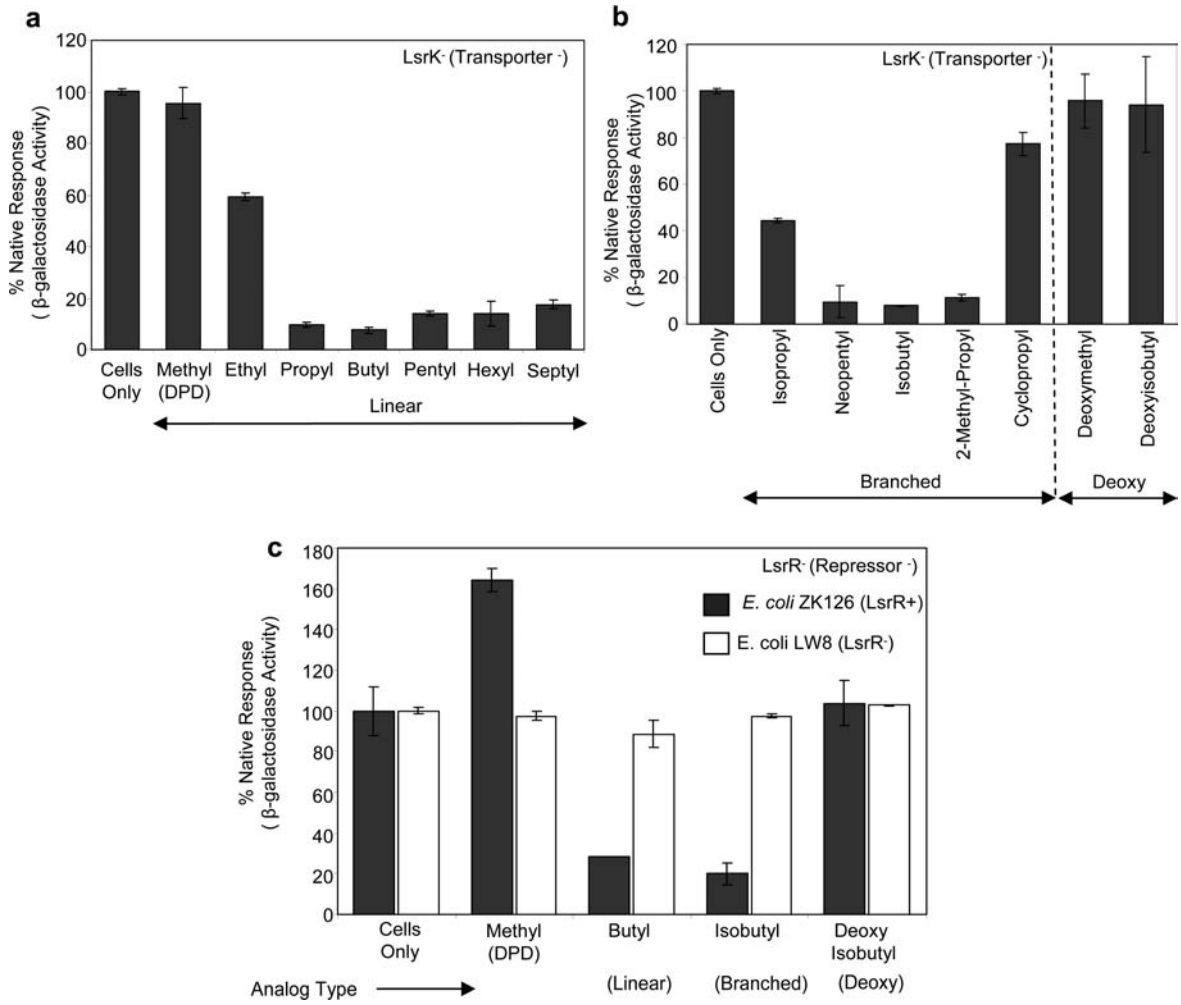

Figure 3. DPD Analogs inhibit native signaling in *E. coli* in the absence of the AI-2 transporter but only in the presence of the QS *lsr*-circuit repressor LsrR AI-2 dependent β-galactosidase production in *E. coli* LW9 pLW11 (*luxS*$^+$) in response to (a) linear analogs, (b) branched and deoxy-DPD analogs, (c) AI-2 dependent β-galactosidase production in *E. coli* ZK126 pLW11 (*luxS*$^+$, *lsrR*$^+$) and LW8 pLW11 (*luxS*$^+$, *lsrR*$^-$) in response to representative analogs from the linear branched and deoxy-analog categories (100% Native *E. coli* (LW9) response = 3716 Miller units, 100% Native *E. coli* (ZK126) response = 1507 Miller units and 100% Native (LW8) response = 2883 miller units).

carbonyl units in DPD analogs might play an important role in modulating AI-2-based QS circuitry.

Ethyl-DPD also acts as a QS circuit agonist in *E. coli* but only in the presence of *in vivo* LsrK; in a LsrK knockout strain, ethyl-DPD did not initiate *lsr* transcription (see Supporting Information Figure S6a). Neither AI-2 nor butyl or isobutyl-DPD could function in the absence of LsrK (see Supporting Information Figure S6b). This suggests that the phosphorylation of DPD analogs by LsrK inside the bacterial cell is essential for them to effect the *lsr* QS circuit, as either agonists or antagonists. The phosphate moiety in phospho-AI-2 and analogs, therefore, seems important for binding to LsrR, but it appears that the stabilization of the LsrR/DNA complex is governed by the C1 alkyl chain (C3 is the minimum requirement).

Cross-Species and Species-Specific Quorum Quenchers in a Synthetic Ecosystem. Bacteria, whether in their natural environments or during a host infection, seldom grow in isolated pure cultures. Therefore, to investigate the effect of our DPD analogs in a mixed bacterial environment, a synthetic ecosystem composed of three different bacterial populations was assembled. Specifically, *S. typhimurium* MET708, *V. harveyi* BB170, and *E. coli* W3110 (pCT6), each *luxS*$^+$ (Table 1), and each producing their native AI-2 were cocultured in the same tubes. By design, we selected each species so that a different reporter probe would indicate the specific bacterium's QS response: *S. typhimurium* (MET708) expresses β-galactosidase; *V. harveyi* (BB170) elicits bioluminescence; and *E. coli* (W3110) are engineered to synthesize GFP. In this way, their responses can be differentiated and quantified in response to AI-2.

Since isobutyl-DPD was able to suppress QS in pure cultures of *E. coli* and *S. typhimurium* and isopropyl-DPD was selective only causing *lsr* suppression in *E. coli*, these analogs were selected to monitor their specificity in the trispecies culture environment. Upon addition of isobutyl-DPD, we found *S. typhimurium*'s β-galactosidase activity sharply decreased 4-fold while isopropyl-DPD was ineffective (see Figure 6a). These trends were similar to those observed in pure culture studies. Interestingly, both isobutyl-DPD and isopropyl-DPD decreased bioluminescence in *V. harveyi* and this appeared to be concentration dependent (see Figure 6b). It has been shown that DPD analogs when added to *V. harveyi* grown in AB media actually were found to be synergistic agonists of bioluminescence.[42,38] Our LM media results, which demonstrate antagonism of bioluminescence in *V. harveyi*, could be thought of as conflicting but reinforce the context-dependent nature of QS responses.[22] That is, in LM media the analogs antagonize bioluminescence

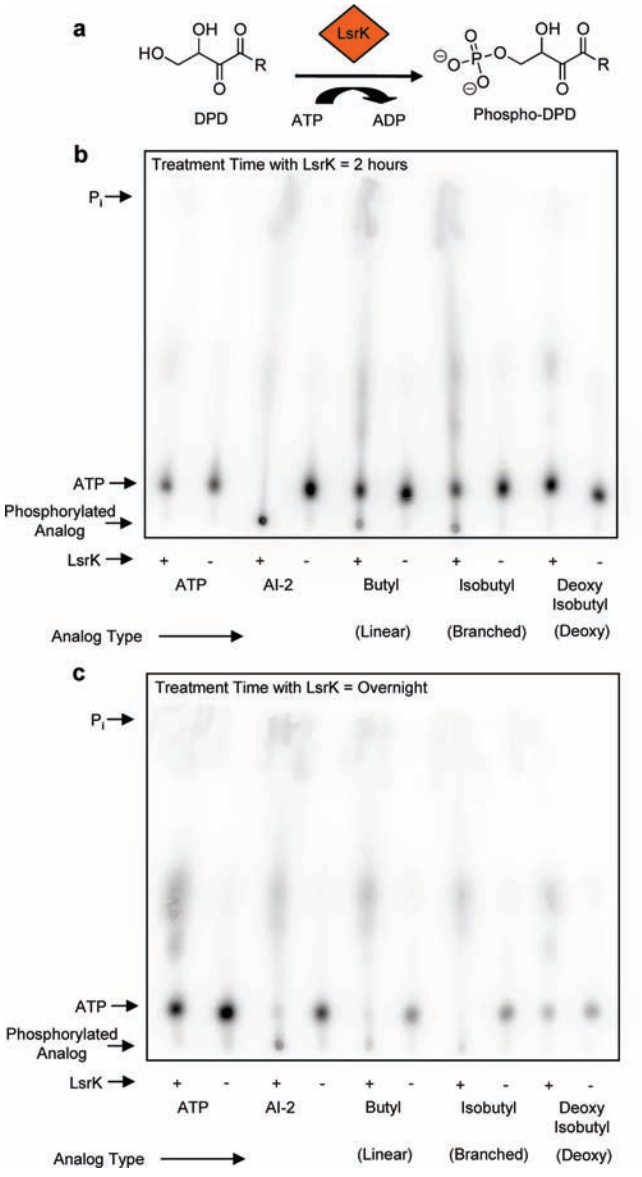

Figure 4. *In vitro* phosphorylation of analogs by LsrK. (a) Schematic of phosphorylation of DPD by LsrK in the presence of ATP. Representative thin layer chromatography (TLC) analysis of the LsrK mediated analog phosphorylation. ATP, AI-2 (methyl-DPD), butyl-DPD, isobutyl- DPD, and deoxy-isobutyl-DPD, treated with LsrK for (b) 2 h and (c) overnight.

in the synthetic ecosystem and also in pure cultures (see Supporting Information Figure S7). Hence, additional media components as demonstrated by Xavier and Bassler[22] play a pivitol role in modulating QS signaling.

It is important to note that the AI-2 uptake and processing machinery in *V. harveyi* are distinctly different from those of the enteric organisms *E. coli* and *S. typhimurium*. In *V. harveyi*, AI-2 binds to cell surface bound receptor LuxP which, in turn, recognizes a boronated form of AI-2;[43] the QS signal is transduced through phospho-relay mechanisms[44] whereas in *E. coli* or *S. typhimurium* AI-2 is first internalized and processed by *lsr* machinery before eliciting a QS response. Hence, it is not surprising that these analogs yielded different outcomes. To ascertain the QS response in *E. coli*, we monitored the AI-2 stimulated GFP expression via flow cytometry. In the absence of isobutyl-DPD, ~60% of the population was QS positive (green), and after the addition of isobutyl-DPD, QS positive cells were reduced to less than 1%. Isopropyl-DPD decreased the QS response to a lesser extent (to 21% of the population) (see Figure 6c).

Discussion

It is now generally accepted that AI-2 is a universal *bona fide* quorum sensing autoinducer in several bacteria including several clinically relevant species, although in some bacteria AI-2 is also a metabolic waste.[1] Both unmodified and "processed" AI-2 have been shown to bind to protein receptors and affect QS response in a variety of bacteria.[44,41] In some bacterial species, such as *V. harveyi* and *V. cholerae*, both AI-1 and AI-2 feed into the same phospho-relay QS circuitry.[45] In other bacteria, AI-1 and AI-2 circuits do not necessarily converge but, instead, affect the production of different factors yielding different phenotypes. Therefore, for the exquisite modulation of bacterial quorum sensing, a "tool kit" that contains molecules that can separately antagonize the actions of AI-1 or AI-2, or broad-spectrum molecules that can simultaneously quench the QS response of both AI-1 and AI-2 are desired. Although a handful of reports have demonstrated AI-2 antagonism[46] or synergism[38] using AI-2 like molecules, this is the first systematic study using an expanded set of AI-2-like molecules (14 total analogs) to identify or define the functional units of AI-2 that can be tweaked to yield agonists or antagonists. Also, another unresolved issue that this manuscript addresses is whether "AI-2-like" antagonists need to be phosphorylated in order to be active. Our data provide strong evidence that indeed phosphorylation is also important for AI-2 analogs that act as antagonists.

Our results show that, of the panel of DPD analogs, only ethyl-DPD acts as a QS circuit agonist in *E. coli* and *S. typhimurium*. A variety of DPD analogs caused inhibition of native QS signaling in *E. coli* while only butyl-DPD and isobutyl-DPD significantly inhibited signaling in the homologous QS circuit of *S. typhimurium*. Alignment studies (using protein blast[47]) reveal that both *E. coli* and *S. typhimurium* LsrK and LsrR proteins show significant homology (82% and 77% identical sequences; see Supporting Information Figures S8 and S9). The 30 bp putative LsrR binding site in *E. coli*[40] was aligned to the respective promoter region in *S. typhimurium* using Clustal W[48] and showed 83% homology (see Supporting Information Figure S10). Secondary[49] (see Supporting Information Figure S11) and tertiary structure[50] (see Supporting Information Figure S12) predictions show that LsrR from both *E. coli* and *S. typhimurium* have similar folds. We conclude from this work that although the AI-2 processing enzyme LsrK and LsrR-DNA binding sites in both *E. coli* and *S. typhimurium* share significant homology, subtle structural differences can

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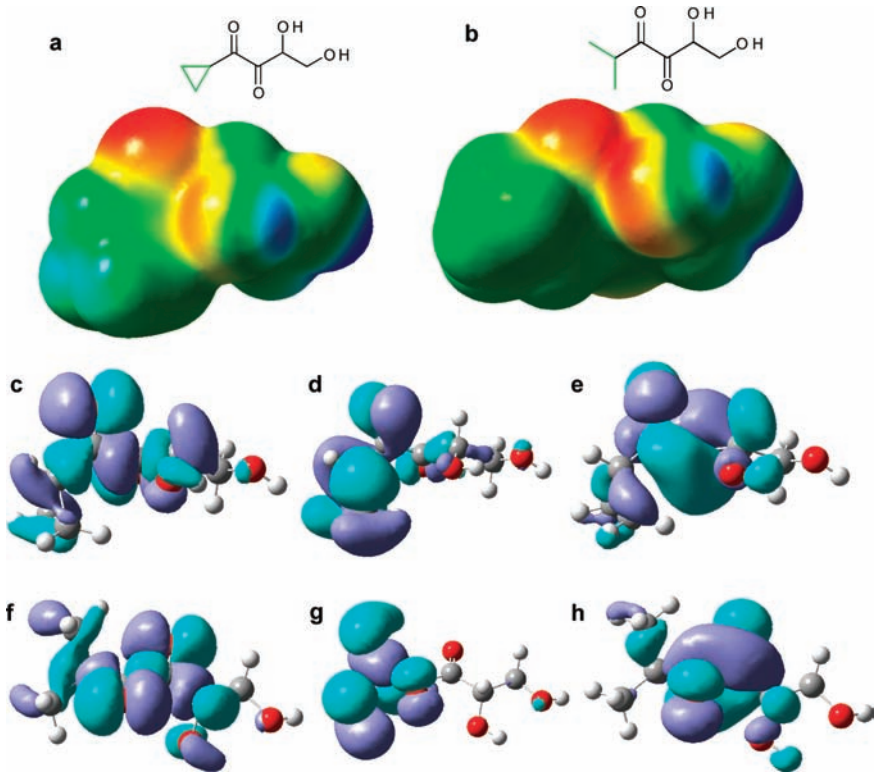

Figure 5. Electrostatic potential map; the alkyl chain of the cyclopropyl-DPD analog is slightly more electron deficient than the isopropyl-DPD analog (a) cyclopropyl-DPD and (b) isopropyl-DPD. Both cyclopropyl (HOMO-3 orbital) and *i*-Pr (HOMO-4 orbital) stabilize the adjacent carbonyl group (LUMO) via hyperconjugation. (c) HOMO of cyclopropyl-DPD (−0.23 eV); (d) HOMO-3 of cyclopropyl-DPD (−0.30 eV); (e) LUMO of cyclopropyl (−0.06 eV); (f) HOMO of *i*-Pr-DPD (−0.24 eV); (g) HOMO-4 of *i*-Pr-DPD (−0.34 eV); (h) LUMO of *i*-Pr-DPD (−0.08 eV).

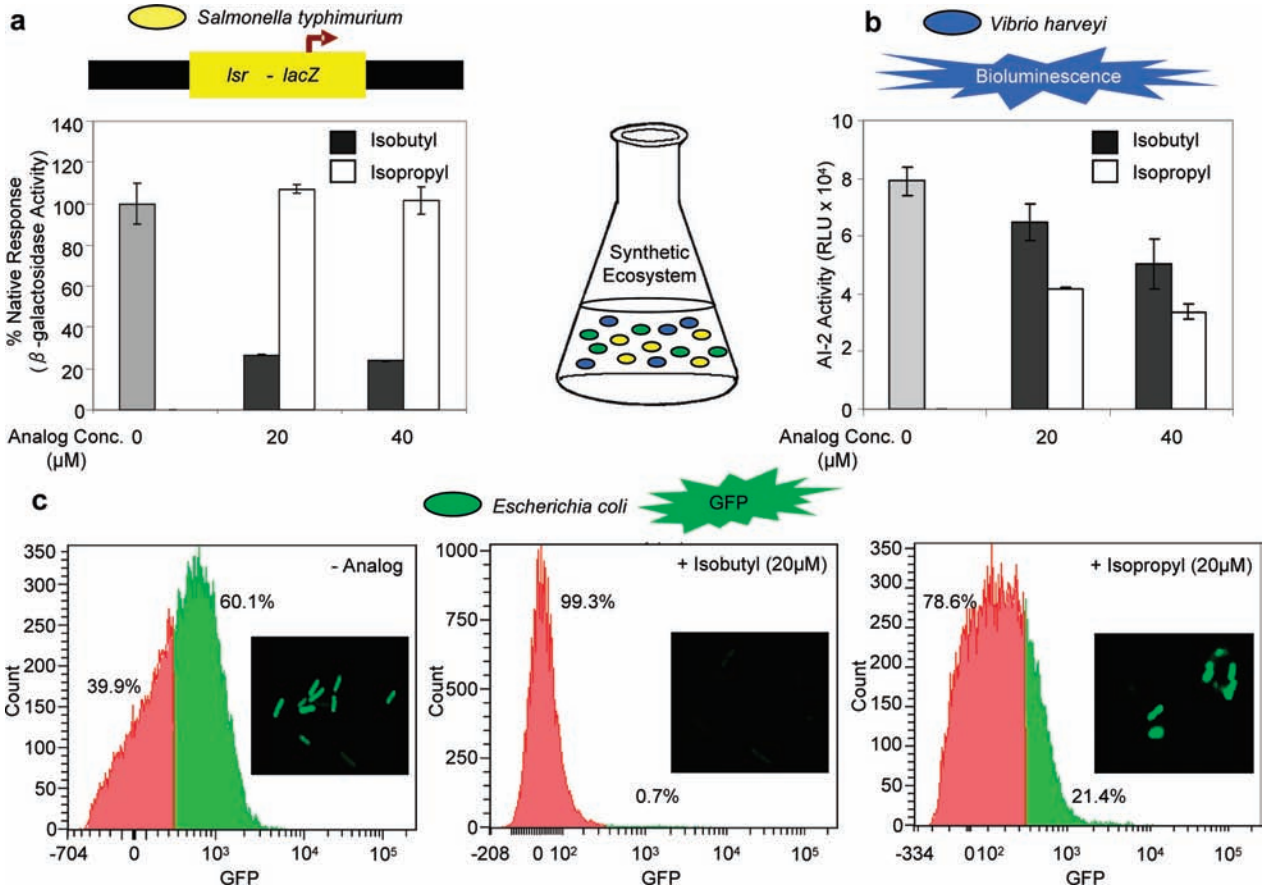

Figure 6. Analogs inhibit native signaling in a trispecies synthetic ecosystem: (a) AI-2 dependent β-galactosidase production in *S. typhimurium* MET708 (100% Native *S. typhimurium* response in trispecies culture = 1063 Miller units). (b) AI-2 dependent bioluminescence production in *V. harveyi* BB170 and (c) AI-2 dependent GFP induction in *E. coli* W3110 pCT6 (all strains are *luxS*$^+$) in response to isobutyl-DPD and isopropyl-DPD.

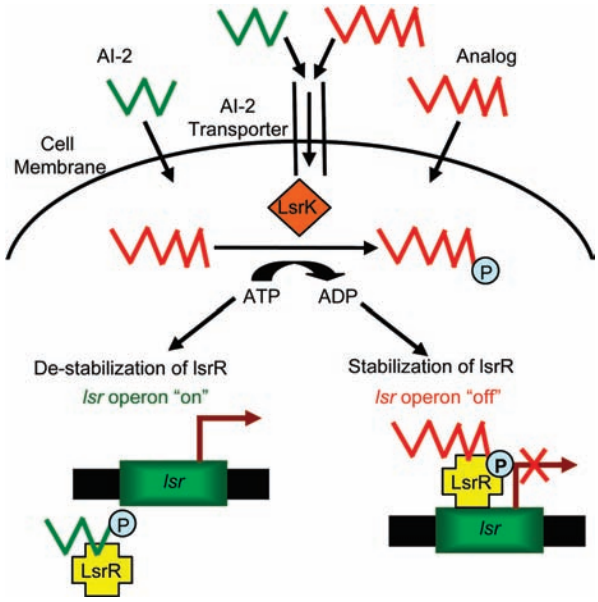

Figure 7. Suggested scheme for QS inhibition by a DPD analog in *E. coli*. Analog/AI-2 enters the cell via the Lsr transporter or diffuses into the cell independent of the transporter. Analog needs to be phosphorylated by LsrK to function as an antagonist in the QS circuit. Phospho-analog also needs to compete with phospho-AI-2 for binding to the repressor protein (LsrR) to repress *lsr* expression.

result in these systems responding differently to small molecules. The instability of LsrR which also leads to its insolubility, as observed by us and also by others,[40] has made difficult a more thorough study of LsrR binding to AI-2 analogs. Future engineering of a more stable LsrR and structural work should help shed more light on the origin of the differences in AI-2 analogs binding between the *E. coli* and *S. typhimurium* LsrR proteins.

Kinases are important enzymes for several processes in bacteria, and the substrate specificity of these enzymes can be important for maintaining the high fidelity of critical processes.[27] In this paper we have demonstrated that LsrK is promiscuous; different AI-2-like molecules with structurally diverse alkyl groups can be efficiently phosphorylated. Substrates that lack primary hydroxyl functionalities are however not accepted as substrates, but the mere presence of a primary hydroxyl group is not sufficient for kinase processing, as it has already been shown that LsrK from *S. typhimurium* phosphorylates AI-2 but not ribose or glucose, which also contain primary hydroxyl units.[35]

There are three known AI-2 processing enzymes which would be the most likely checkpoints for analog function namely LsrK, LsrR, and LsrG. As all analogs and natural AI-2 functioned irrespective of the Lsr(ACDBFG), it shows that the analogs function independent of the AI-2 transporter and LsrG. Our working model for AI-2 and analog function starts with phosphorylation of DPD analogs (see Figure 7) as an early critical checkpoint for *lsr* transcription repression; isobutyl-DPD is a potent quorum quencher while its unphosphorylated counterpart deoxy-isobutyl-DPD minimally represses *lsr* transcription (Figure 2a,b). We note, however, phosphorylation is not the only criterion for antagonism, as some analogs such as cyclopropyl-DPD which is phosphorylated by LsrK are still not able to repress *lsr* expression. Some of the DPD analogs, such as hexylDPD, are not as readily phosphorylated as is butyl DPD, yet these analogs cause substantial repression of *lsr* expression. Therefore we cannot completely rule out the possibility that for some of these analogs, such as hexylDPD, inhibition of lsr expression is due to the unphosphorylated form of the molecule. Future work should help resolve this issue.

A more downstream checkpoint is the binding to LsrR (see Figure 7) forming a stable LsrR-*lsr* DNA complex, and thus preventing derepression of the *lsr* operon by phospho-AI-2 binding. Previous work has already demonstrated that phospho AI-2 and not AI-2 bind to LsrR[40,39] Data from the aforementioned studies, coupled with our observation that DPD analogs do not inhibit *lsr* expression in LsrR mutant bacteria strongly suggest that the biological profiles of these analogs are due to the binding of phospho-DPD or phospho-analogs to LsrR. We hypothesize that noncovalent interactions (most probably, van der Waals in nature) engage the alkyl chains of DPD analogs with certain residues in the active site of LsrR, and locks the protein into a conformation that has a higher affinity for the DNA binding sequence. The observation that a minimum of C3 alkyl chain length is required for DPD-analog antagonism lends credence to the hypothesis that a hydrophobic pocket in LsrR plays a role in the distribution of the various LsrR conformations. Addition of a single carbon to the C1-alkyl chain of the analog seems to make a critical difference in stabilizing or destabilizing the interaction of the analog with LsrR; as while ethyl is an agonist, propyl becomes an antagonist of the QS circuit in both *E. coli* and *S. typhimurium*. The fact that isobutyl-DPD is a better antagonist than butyl-DPD (same number of carbons) or other DPD analogs, especially in *S. typhimurium* suggests that shape and not just "greasiness" (e.g., length) of the C1 alkyl chain is also important for antagonism.

It is important to emphasize that while the specific library of C1-alkyl analogs used here probes the Lsr AI-2 circuitry, additional AI-2 specific targets may be revealed by parallel studies focused on other AI-2 regulated genes revealed by previous microarray studies.[23] Moreover, more comprehensive libraries of DPD analogs (e.g., substitutions at other positions and further C1 forms) may lead to new insights not currently explored.

Conclusion

This work has unveiled several important findings and refined our understanding of AI-2-based QS in both *S. typhimurium* and *E. coli*. Notably, we have demonstrated that depending on the nature of the C1-alkyl chain, phosphorylated AI-2-like molecules can either stabilize or destabilize LsrR−DNA complex. Therefore, small molecules that possess phosphate-like moieties as well as C1-alkyl chains of appropriate length and shape could become potential QS modulators in bacteria that utilize LsrR-like transcriptional factors to regulate QS circuits. Second, we show that subtle differences in AI-2 processing enzymes in different bacteria allow for selective modulation of QS processes in an ecosystem. On the other hand, it is also possible to effectively modulate QS processing in a variety of bacteria that have different QS receptors or processing enzymes using a single small molecule. In the trispecies synthetic ecosystem, isopropyl-DPD could modulate QS response in *E. coli* and *V. harveyi* but not in *S. typhimurium* whereas isobutyl-DPD could modulate QS response in all three bacteria. The identification of both broad-spectrum and narrow-spectrum anti-AI-2 molecules could be important for both basic science and clinical applications whereby different scenarios might require a "conquer-all" or "conquer-selectively" approach.

Earlier observations by others revealed an unexpected switch in the modulation of AI-2-based QS in *S. typhimurium* and *V. harveyi* by AI-2 analogs.[42] This switch in AI-2 perception could be explained by differences in the structure of AI-2 receptors in *S. typhimurium* and *V. harveyi* which are significantly different and do not share high homology. Additionally, the nature of the QS molecule in the *V. harveyi* case is unphosphorylated and boronated AI-2,[43] whereas in *S. typhimurium* phosphorylated AI-2 is responsible for the QS response. In this work, however, AI-2-like molecules have been shown to selectively differentiate closely related bacterial species, such as *E. coli* and *S. typhimurium*. As both the processing enzymes and the nature of the QS molecules in both species are similar, this observation is indeed remarkable and adds an additional level of sophistication to the control of bacterial quorum sensing with small molecules. The prevalence of AI-2 in controlling virulence in various pathogens such as *V. cholerae* and *E. coli* O157 suggests an increased understanding of the AI-2 receptors, and signal transduction cascades will open new avenues for guiding bacterial phenotype and modulating pathogenicity. Indeed, the AI-2 signaling molecules themselves are garnering significant attention as agents of diversity among QS-communicating populations, much like the family of AI-1 signaling molecules that mediate intraspecies communication. Our work and others related to signal perception demonstrate that *both* the generation and the perception/transduction processes are tuned by bacteria to weave a rich tapestry of communication networks and resultant phenotypes.

Acknowledgment. The authors would like to thank Dr. Jim Culver for generously providing his laboratory's facilities to conduct radioactive experiments. This work was supported by funds from the University of Maryland GRB fellowship, R.W. Deutsch Foundation, the Defense Threat Reduction Agency, National Science Foundation Grants CHE0746446 and CHE0946988, and the National Science Foundation (EFRI Program). J.A.I.S. is a recipient of the Ministry of Education GANN fellowship.

Supporting Information Available: Figures S1−S12, Supporting Methods−NMR spectra characterization of analogs. This material is available free of charge via the Internet at http://pubs.acs.org.

JA102587W