ARTICLE

ACS chemical biology

Cross Species Quorum Quenching Using a Native AI-2 Processing Enzyme

Varnika Roy[†,‡,§], Rohan Fernandes[‡,§], Chen-Yu Tsao[‡,§], and William E. Bentley[†,‡,§,*]

[†]Department of Molecular and Cell Biology, University of Maryland, College Park, Maryland 20742, [‡]Center for Biosystems Research, University of Maryland Biotechnology Institute, College Park, Maryland 20742, and [§]Fischell Department of Bioengineering, University of Maryland, College Park, Maryland 20742

ABSTRACT Bacterial quorum sensing (QS) is a cell−cell communication process, mediated by signaling molecules, that alters various phenotypes including pathogenicity. Methods to interrupt these communication networks are being pursued as next generation antimicrobials. We present a technique for interrupting communication among bacteria that exploits their native and highly specific machinery for processing the signaling molecules themselves. Specifically, our approach is to bring native intracellular signal processing mechanisms to the extracellular surroundings and "quench" crosstalk among a variety of strains. In this study, the QS system based on the interspecies signaling molecule autoinducer-2 (AI-2) is targeted because of its prevalence among prokaryotes (it functions in over 80 bacterial species). We demonstrate that the *Escherichia coli* AI-2 kinase, LsrK, can phosphorylate AI-2 *in vitro*, and when LsrK-treated AI-2 is added *ex vivo* to *E. coli* populations, the native QS response is significantly reduced. Further, LsrK-mediated degradation of AI-2 attenuates the QS response among *Salmonella typhimurium* and *Vibrio harveyi* even though the AI-2 signal transduction mechanisms and the phenotypic responses are species-specific. Analogous results are obtained from a synthetic ecosystem where three species of bacteria (enteric and marine) are co-cultured. Finally, the addition of LsrK and ATP to growing co-cultures of *E. coli* and *S. typhimurium* exhibits significantly reduced native "cross-talk" that ordinarily exists among and between species in an ecosystem. We believe this nature-inspired enzymatic approach for quenching QS systems will spawn new methods for controlling cell phenotype and potentially open new avenues for controlling bacterial pathogenicity.

Signal molecules mediate information transfer between cells and drive cell function. Intra- and intercellular "signaling" or "communication" guides the establishment of bacterial infections and mediates their virulence (*1, 2*). Interrupting these signaling processes can limit or halt disease progression (*3, 4*), potentially without adversely affecting bacterial growth (*4*). Such innovative approaches that are neither bactericidal nor bacteriostatic should minimize the emergence of antibiotic-resistant strains (*5*) and lead to entirely new approaches for guiding bacterial phenotype. Naturally, components of signal transduction pathways are key targets for these approaches. Signal transduction in bacteria occurs by cell−cell communication *via* small signaling molecules, in a process called quorum sensing (QS) (*6*). In QS pathways, the approach of interfering with or destroying the QS signal is referred to as "quorum quenching" (*7*). Strategies for quorum quenching include outcompeting the native signaling processes with signal molecule analogues and limiting signal generation with synthase-specific inhibitors (as reviewed by Rasmussen and Giskov (*8*)). A third method, involving sequestering native signal molecules *via* molecular traps or degradative processes, has been proposed (*9*) and is investigated here.

Bacterial autoinduction (AI) is ripe for all of these quenching strategies since the modes of signal generation and perception vary widely. Early strategies targeted intracellular communication processes typified by the acylated homoserine lactone (AHL) signal molecules, also known as autoinducer-1 (AI-1), which is specifically secreted and recognized by a variety of Gram-negative pathogens (*10, 11*), while recent findings suggest these molecules could even mediate intercellular signaling events (*12*). Most reported quorum quenching techniques have concentrated on inactivation of the AHL

*Corresponding author, bentley@umd.edu.

Received for review November 2, 2009 and accepted December 21, 2009.

Published online December 21, 2009

10.1021/cb9002738

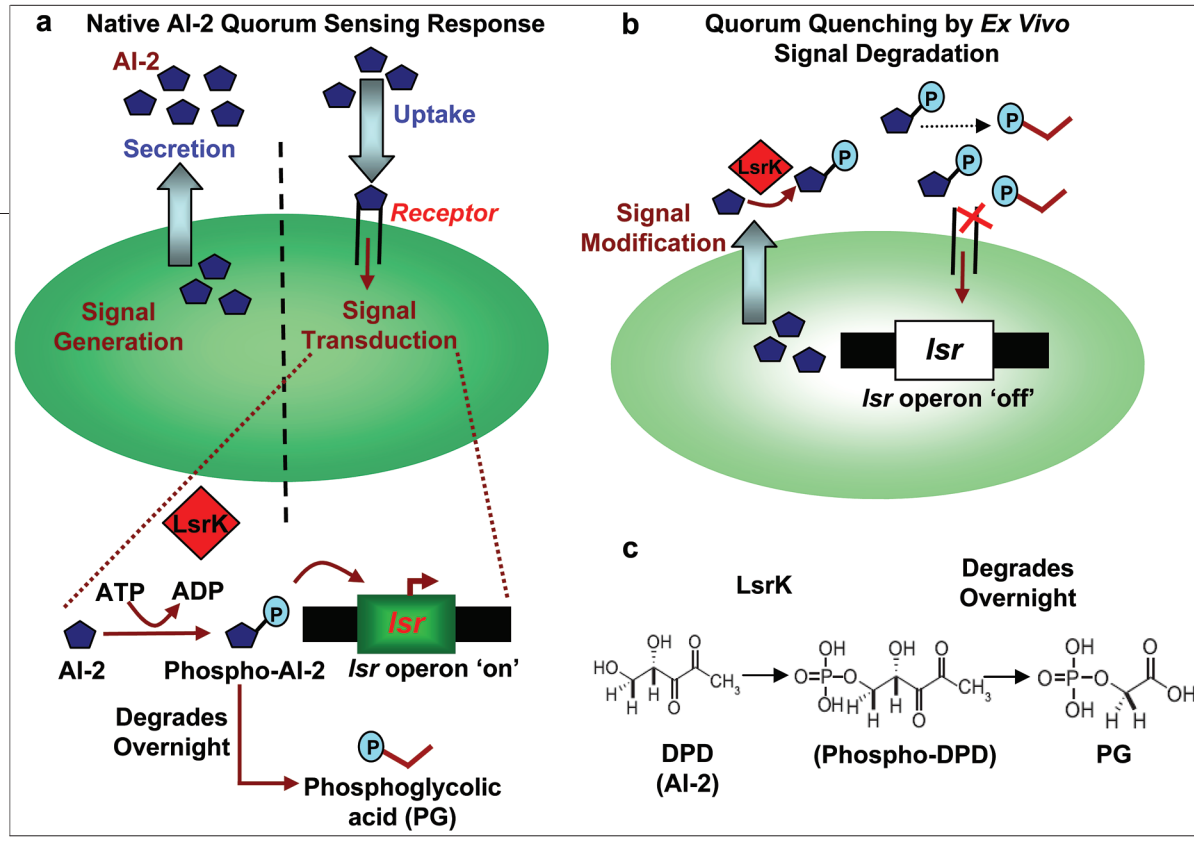

Figure 1. Schematic of LsrK-mediated quorum quenching. a) Native quorum sensing is mediated by the generation, secretion, and uptake of autoinducer-2 (AI-2) followed by signal transduction indicated by *lsr* gene expression; phospho-AI-2 degrades overnight to 2-phosphoglycolic acid (PG). b) LsrK and ATP delivered outside the cell phosphorylate AI-2; phospho-AI-2 is presumably prevented from being transported into the cells and is degraded to PG. In this scenario, the quorum sensing response is quenched. c) LsrK phosphorylates DPD (AI-2 precursor); phospho-DPD degrades to PG overnight *in vitro*.

molecules. These methods employ a number of enzymes, such as AHL lactonases (*13*) and AHL acylases (*14*) that cleave the lactone ring or acyl chain of the AHL molecule, respectively, as well as various paraoxonases (PONs) (*15*) that have AHL hydrolytic activity. Besides AHL degradation enzymes, there are structural mimics of the QS signal, such as halogenated furanones (*16*) (*17*) and synthetic autoinducing peptides (*18*) that typically interfere with signal binding to the receptor. Approaches that exploit molecular evolution of the cognate transcriptional regulators have increased the diversity and scope of candidates (*19*) and, in turn, led to new technologies for guiding phenotype (*20*). The autoinducer-2 (AI-2) signal transduction process is thought to be "universal" in that its principal synthase, *luxS*, is found in over 80 genera and AI-2 itself is actually a family of isomers, each potentially representing a different mode of perception (*21*). Unlike the AI-1 system, molecular evolution approaches to enhance diversity of AI-2 recognition have not appeared, perhaps due to the still unresolved mechanism of its signal transduction processes.

Approaches that target AI-2 processes are conceptually fertile in that candidate molecules could conceivably "quench" the communication among multiple species simultaneously. Brominated furanones (*22*) from the alga *Delisea pulchra*, fatty acids derived from ground beef (*23*) and poultry meat (*24*), and various synthetic (*25, 26*) and transition state (*27, 28*) AI-2 analogues represent AI-2 inhibition schemes in development. Our work aims to quench AI-2-mediated communication by exploiting the native signal processing machinery, specifically, the 4,5-dihydroxy-2,3-pentadione (DPD) kinase LsrK from *E. coli* (Figure 1, panel a). DPD is the precursor of AI-2 and exists in equilibrium with several interconvertible isoforms in solution, all of which are referred to as the family of AI-2 signaling molecules (*21*). The QS machinery in *E. coli* consists of various *luxS*-regulated (*lsr*) genes (*2*), LsrK being the putative kinase that functions intracellularly in the native system to phosphorylate DPD (the single linear form of the AI-2 signaling molecules) to phospho-DPD (also referred to as phospho-AI-2). This molecule, in turn, is pivotal as it is the antirepressor of the QS system. That is, by binding to transcriptional repressor LsrR, phospho-AI-2 induces transcription of the *lsr* genes (Figure 1, panel a) (*29*). Phospho-DPD itself has been shown to be unstable and degrades in 16 h (overnight) to form 2-phosphoglycolic acid (PG) (*30*) (Figure 1, panel c).

In this study we add *E. coli* QS kinase LsrK *ex vivo* (outside the cell, in the extracellular medium) to phosphorylate AI-2 to phospho-AI-2, thus conferring a negative

charge on AI-2 and thereby restricting its transport into the cell *via* binding the Lsr transporter. PG that is subsequently produced is not predicted to affect *lsr*-mediated processes. Thus, LsrK targets AI-2 for degradation and quenches the quorum sensing response. We deliver LsrK *ex vivo* to *E. coli*, *S. typhimurium*, and *V. harveyi* for the selective modification of AI-2 and cessation of AI-2 signaling in these cell types (Figure 1, panel b). The signal molecule in this method is transformed outside the cell, and thus unlike analogue-based competitive inhibition agents there is no compulsory cell membrane barrier to overcome.

We demonstrate the effects of LsrK-treated AI-2 on phenotypic responses such as *lsr* expression in gut commensal bacteria such as *E. coli* and *S. typhimurium* and bioluminescence in the marine bacterium *V. harveyi* in both pure cultures and also in a synthetic ecosystem, where all three are co-cultured. These bacteria are chosen as they represent different modes of signal recognition and transduction. To demonstrate utility of our scheme in interrupting bacterial cross-talk in nature where one bacterium responds to the AI-2 produced by another, we co-cultured *E. coli* and *S. typhimurium*, which are known to co-exist in the gut during *S. typhimurium* infections. In this co-culture, the *E. coli* produces AI-2, which is sensed by *S. typhimurium*. The addition of LsrK and ATP *ex vivo* to this co-culture is monitored. By capturing the signal molecule in one state (linear DPD, *via* the native processing enzyme, LsrK) and sequestering it from its equilibrium isoforms, this approach has the potential of silencing QS activity in broad based populations and preventing associated pathogenic responses, such as the formation of biofilms.

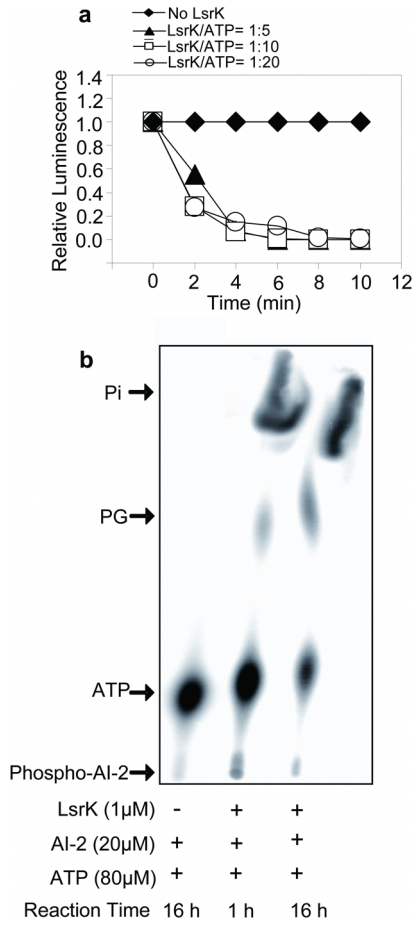

Figure 2. *In vitro* LsrK is active. a) ATP consumption assay for LsrK-mediated AI-2 phosphorylation. ATP consumption for varying LsrK/ATP ratios (1:5−1:20) in the presence of AI-2. b) Thin layer chromatography (TLC) analysis of the LsrK-mediated reaction. Lane 1: AI-2, ATP incubated for 16 h. Lanes 2, 3: LsrK, AI-2, ATP incubated for 1 and 16 h, respectively.

RESULTS AND DISCUSSION

LsrK Phosphorylates AI-2 *in Vitro*. The uptake of AI-2 and its subsequent processing has been described in various bacteria (*31*). The enzyme LsrK, a kinase component of the AI-2 processing machinery in *E. coli* (*2*) and *S. typhimurium* (*32*), phosphorylates AI-2 upon uptake into the cell, using ATP. Purified LsrK (YdeV) was derived from *E. coli* W3110 and assayed for activity using *in vitro* synthesized AI-2 as a substrate. An ATP luminescence assay that monitors the residual ATP present in the sample (measured *via* luminescence) following phosphorylation of AI-2 by LsrK is used to indicate activity (see Methods). In this system, luminescence was dependent on the ATP concentration. The luminescence decreased rapidly over time for all tested ATP concentrations in the presence of LsrK, relative to controls as shown in Figure 2, panel a. These results indicate that the *in vitro* purified *E. coli* LsrK was active (depleted ATP), and observed trends were in agreement with previous studies where the homologous *S. typhimurium* LsrK was observed to phosphorylate AI-2 within 10 min (*30*).

The product of the phosphorylation reaction is phospho-AI-2, which breaks down over time to PG (*30*).

TABLE 1. Bacterial strains, plasmids and primers 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	*29*
MDAI2	W3110 *luxS*::Tc^r W3110-derived *luxS* mutant strain	*35*
ZK126	W3110 Δ*lacU169-tna2*	*43*
	Salmonella typhimurium strains	
MET715	*rpsl putRA*::Kan-*lsr-lacZYA luxS*::T-POP	*32*
	Vibrio harveyi strains	
BB170	BB120 *luxN*::Tn*5* (sensor 1$^-$, sensor 2$^+$); AI-1$^+$, AI-2$^+$	*44*
MM30	BB120 *luxS*::Tn5 (sensor-1$^+$, sensor-2$^+$); AI-1$^+$, AI-2$^-$	*38*
	Plasmids	
pLsrK	pET200 derivative, *Escherichia coli* W3110 LsrK$^+$	This study
pLW11	*galK'-lacZYA* transcriptional fusion vector, containing *E. coli lsrACDBFG* promoter region, Amp^r	*29*
pCT6	pFZY1 derivative, containing *E. coli lsrR* and *lsrR* promoter region fused with *T7RPol*, Ap^r	*35*
pET-GFP	pET200 derivative, containing *gfpuv*, Km^r	

Oligonucleotide primers

Name	Sequence	Relevant property
LsrKFrwd	5′-CAC CAT GGC TCG ACT CTT TAC CCT TTC-3′	Upstream primer to amplify LsrK sequence from whole genomic DNA W3110. (This study)
LsrKRev	5′-CTA TAA CCC AGG CGC TTT CCA TAA C-3′	Downstream primer to amplify LsrK sequence from whole genomic DNA W3110. (This study)

To ascertain the relative proportion of phospho-AI-2 and PG as a function of time, samples containing AI-2 and ATP were treated with LsrK for either 1 or 16 h (overnight) and spotted on a thin layer chromatography (TLC) plate. A large amount of AI-2 was converted to phospho-AI-2 after 1 h, which appears at the bottom of the TLC plate (Figure 2, panel b). Samples left for 16 h showed a near-complete conversion of phospho-AI-2 to PG.

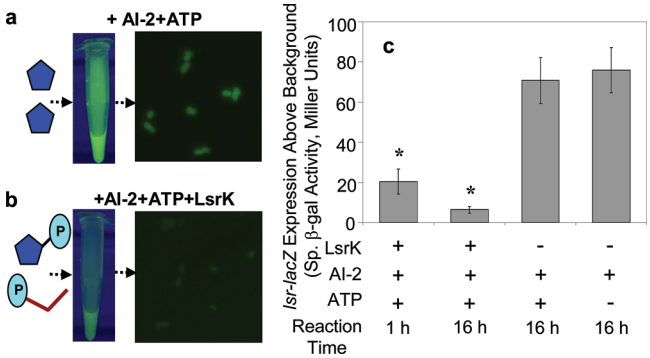

Figure 3. LsrK-treated AI-2 quenches QS response in *E. coli*. AI-2-dependent β-galactosidase production in *E. coli* LW7 pLW11 in response to (a) AI-2 and ATP and (b) AI-2, ATP, and LsrK recorded *via* a fluorescent reporter, fluorescein di-β-D-galactopyranoside (FDG). c) AI-2-dependent β-galactosidase production in the same reporter in response to combinations of AI-2, ATP. and LsrK and differing reaction times, quantified by colorimetric reporter *o*-nitrophenyl-β-D-galactoside (ONPG) (* indicates $p < 0.05$ for an unpaired *t* test of the experimental sample compared to the native response with AI-2 and ATP).

Image analysis of the TLC results indicated that the 16 h phospho-AI-2 levels were 4-fold lower than those of the 1 h sample, while the net increase in PG in the 16 h sample was 2-fold that of the 1 h sample. Our results demonstrate that *E. coli* derived LsrK-treated AI-2 formed both phospho-AI-2 and PG and that the relative ratio of phospho-AI-2 to PG after LsrK treatment decreased as a function of reaction time. Since the LsrK reaction rate is rapid and there is a 4-fold excess of ATP in the reaction samples (Figure 2, panel b), we expect that there was nearly complete conversion of AI-2 to phospho-AI-2 in 1 h. This is consistent with investigations involving *S. typhimurium* LsrK (*30*). Subsequent experiments for AI-2 activity were performed using both the 1 h and the 16 h mixtures described above.

LsrK-Treated AI-2 Precludes *lsr* Expression. Inside the cells, LsrK phosphorylated AI-2 normally derepresses gene expression in the *lsr* operon by binding LsrR (*2*) (Figure 1, panel a). The effect of addition of LsrK-treated AI-2 outside the cells on the QS response of *lsr* gene expression was observed using the reporter strain *E. coli* LW7 pLW11 which is a *luxS* null mutant and cannot produce AI-2 (See Table 1). Plasmid pLW11 encodes the *lacZ* gene under the control of the *E. coli lsr* promoter so that *E. coli* LW7 pLW11 synthesizes β-galactosidase in response to added AI-2. *E. coli* LW7 pLW11 was incubated with fluorescein di-β-D-galactopyranoside (FDG) and either AI-2 or LsrK-treated

AI-2. 2-fold higher β-galactosidase activity was found in cells incubated with AI-2 relative to those with LsrK-treated AI-2 (Figure 3, panel a and b). The β-galactosidase activity of the latter was similar to controls (without AI-2; data not shown).

To more accurately quantify the cell response, β-galactosidase activity was assayed and was reduced from 60−70 Miller units in the presence of AI-2 to 10−20 Miller units in the 1 h LsrK-treated AI-2 samples and to only 7 Miller units for the 16 h samples (Figure 3, panel c). The results confirm that the *lsr* expression is significantly higher when AI-2 is transported into the cell and phosphorylated internally by LsrK (*33, 34*). In the case when LsrK is external to the cell and provided ATP, the resultant phospho-AI-2 is unable to stimulate the QS response (*lsr* gene expression). This is presumably due to the prevention of AI-2 transport into the cell after phosphorylation.

Quorum Quenching in *E. coli* Populations. In order to investigate the effect of LsrK-treated AI-2 on the *lsr* expression in a cell population, LsrK-treated AI-2 was added to *E. coli* MDAI2 pCT6 pET-GFP (Table 1) and assayed using flow cytometry. This strain is a *luxS* mutant and produces GFP in response to AI-2 (*35*). Experiments were performed analogously to those in Figure 3. Ten hours after AI-2 addition, 33% of the cell population was found to be GFP positive (Figure 4, panel a). Conversely, addition of LsrK-treated AI-2 showed that only 1% of the population expressed GFP (Figure 4, panel b). These findings demonstrate that the QS response was quenched in the entire population. By extension, LsrK-treated AI-2 enables a phenotypic "switching" behavior in the population in that an individual cell's response could be turned "off" from "on".

Cross Species Quenching of *lsr* Expression. Since AI-2 is presumed to be a "universal" signaling molecule (*31*), it is hypothesized that the degradation of AI-2 should quench QS expression across species and among species in mixed populations. A study was conducted to investigate the effect of LsrK-treated AI-2 on two other species of QS bacteria: *S. typhimurium* and *V. harveyi*. *S. typhimurium* was selected as its AI-2 uptake and processing mechanisms are similar to those of *E. coli* (*2*), while *V. harveyi* is the most commonly used AI-2 reporter. Also, the AI-2 uptake and processing machinery in *V. harveyi* is significantly different from that of *E. coli* and *S. typhimurium* in that the signal is transduced

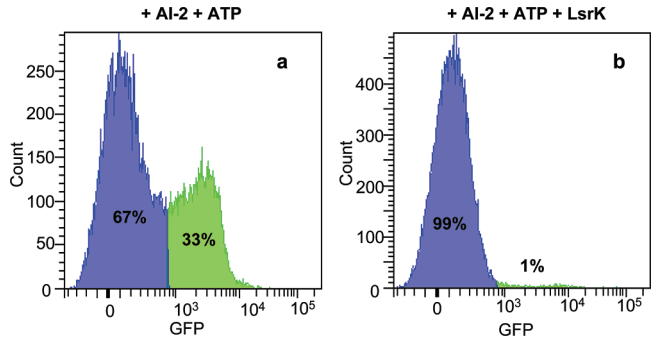

Figure 4. LsrK-treated AI-2 quenches the population-wide QS response in *E. coli*. AI-2 dependent GFP production in *E. coli* MDAI-2 pCT6 in response to (a) AI-2 and ATP or (b) AI-2, ATP, and LsrK, measured by flow cytometry.

through a protein kinase cascade and an RNA chaperone to a luminescence reporter (*6*).

In vitro AI-2 is catalyzed using *E. coli* AI-2 synthases (*40*), so the effects on *S. typhimurium* and *V. harveyi* are in response to *E. coli* derived AI-2. The *S. typhimurium* MET715 strain used in the study (Table 1) is a *luxS* mutant containing *lacZ* expressed from the *lsr* promoter. Analogous to *E. coli* experiments (Figure 3, panel c) LsrK-treated AI-2 (1 and 16 h) was incubated with pure cultures of *S. typhimurium* MET715. We observed a 17- and 100-fold reduction in response in the

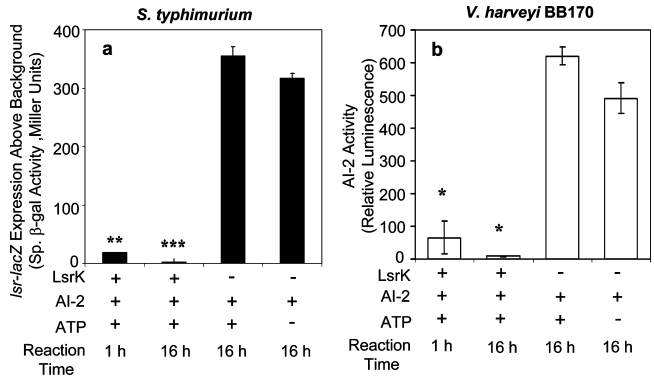

Figure 5. LsrK-treated AI-2 quenches the QS response in *V. harveyi* and *S. typhimurium*. a) AI-2-dependent β-galactosidase production in *S. typhimurium* MET715 in response to *in vitro* AI-2 treated with ATP and LsrK (indicates $p < 0.01$ and *** indicates $p < 0.001$ for an unpaired *t* test as compared to the native response with AI-2 and ATP). b) AI-2-dependent bioluminescence production in *V. harveyi* BB170 in response to *in vitro* AI-2 treated with ATP and LsrK (* indicates $p < 0.05$ for an unpaired *t* test as compared to the native response with AI-2 and ATP).**

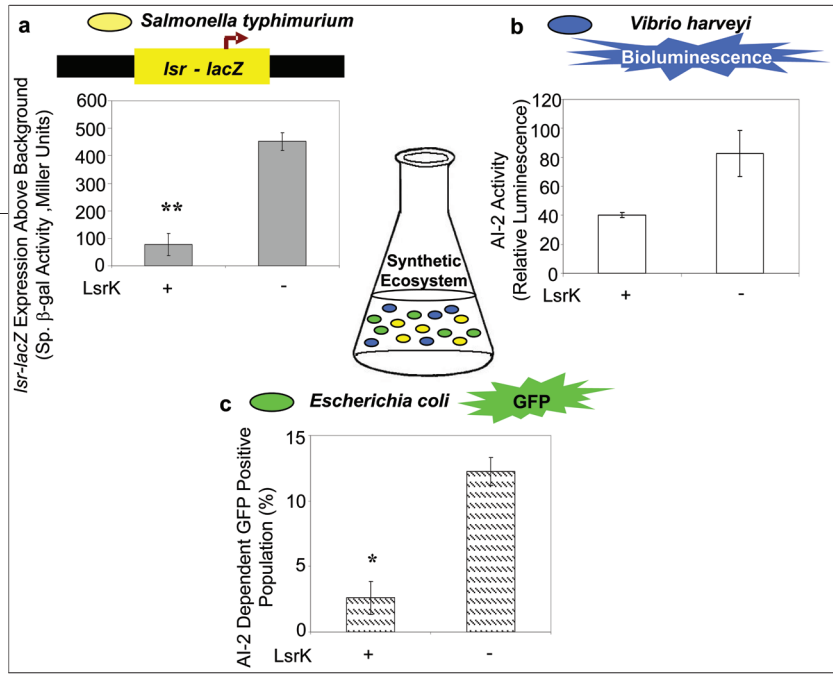

Figure 6. LsrK-treated AI-2 quenches the QS response in a trispecies synthetic ecosystem. a) AI-2-dependent β-galactosidase production in *S. typhimurium* MET715 (indicates $p < 0.01$ for an unpaired *t* test as compared to the native response with AI-2 and ATP). b) AI-2-dependent bioluminescence production in *V. harveyi* MM30. c) AI-2-dependent GFP induction in *E. coli* MDAI-2 pCT6 (* indicates $p < 0.05$ for an unpaired *t* test as compared to the native response with AI-2 and ATP) in response to LsrK-treated AI-2 in a co-culture of the three species.**

1 and 16 h samples, respectively, relative to the controls (Figure 5, panel a). The reduction in *S. typhimurium lsr* response was far more dramatic than that observed in *E. coli*. We speculate that in addition to restricted transport of phospho-AI-2, this could be due to sequestration of the linearized form of the AI-2 isomers and, correspondingly, the complete elimination of the identified "active" form for *S. typhimurium*. That is, phospho-AI-2 is derived from phosphorylation of the terminal hydroxyl group on DPD (*30*) so that DPD cannot cyclize to (2*R*,4*S*)-2-methyl-2,3,3,4 tetrahydroxytetrahydrofuran (R-THMF), the confirmed signaling molecule transduced by *S. typhimurium (21)*. Also, the distinct chemical form of the signal molecule that binds to the *E. coli* AI-2 transporter (or otherwise is transported into the cells) has not been reported, and thus there is ambiguity in the forms of DPD that can enter *E. coli* through the AI-2 transporter. Similar decreases in AI-2-based response (bioluminescence) were observed for *V. harveyi* incubated with LsrK-treated AI-2 (Figure 5, panel b). Unlike *S. typhimurium* or *E. coli* used in our study, *V. harveyi* BB170 (Table 1) is *luxS*$^+$; however, the observed luminescence represents a response elicited by the *ex vivo* addition of the *in vitro* AI-2 or phospho-AI-2 as BB170 cells typically produce their own AI-2 at a later time, typically after 4 h of growth. The phospho-DPD, as explained above in the case of *S. typhimurium*, is also prevented from cyclizing into its known boronated AI-2 signaling molecule (*36*) .

Quorum Quenching in a Synthetic Ecosystem. Bacteria rarely grow in isolated, pure cultures in a host organism or a natural environment. Therefore, to investigate the effects of LsrK-treated AI-2 in a mixed bacterial environment, we assembled a synthetic ecosystem composed of three bacterial populations. Specifically, *S. typhimurium* MET715, *V. harveyi* MM30 and *E. coli* MDAI-2 (pCT6), each *luxS*$^-$, were co-cultured in the same tubes. Under these conditions, the native and quenched responses were entirely due to *in vitro* addition of AI-2 to the cells. As each species contained a different QS reporter, β-galactosidase (MET715), bioluminescence (MM30), and GFP (MDAI-2), their responses could be differentiated.

S. typhimurium β-galactosidase activity decreased 10-fold, whereas *V. harveyi* luminescence dropped 2-fold (Figure 6, panels a and b), as compared to untreated AI-2 controls. The *E. coli* GFP response in co-culture was analyzed by flow cytometry, and 12% of the *E. coli* population was GFP positive in the presence of untreated AI-2, whereas only 3% of the population was GFP positive in response to LsrK-treated AI-2 (Figure 6, panel c). The GFP positive population in co-culture was lower than that seen in the pure culture (12% *vs* 33%). Direct correlation of co-culture results to pure culture is not straightforward because of many factors including different growth media (LM (*37*) vs LB), specific uptake of AI-2 at different rates by *S. typhimurium* and *V. harveyi*, and other potentially nonspecific effectors secreted or taken up by these cells. Our results clearly demonstrate that LsrK-treated AI-2 resulted in an attenuated QS response from all three bacteria in the co-cultured synthetic ecosystem. We note, however, that the reduction was less dramatic in the synthetic ecosystem than in the pure cultures. In the case of *V. harveyi,* we used MM30 in the ecosystem and BB170 in the pure cultures; MM30 responds to endogenously synthesized AI-1 (*38*), whereas BB170 responds to its own AI-2, both by bioluminescence. Hence any reduction in the *V. harveyi* response was viewed as significant.

Quenching Bacterial Cross-Talk. The results demonstrated thus far show that quorum quenching was facilitated by *in vitro* LsrK phosphorylation of AI-2 on various *luxS*$^-$ bacterial species. In order to better simulate the natural condition, the native QS response of a wild-type

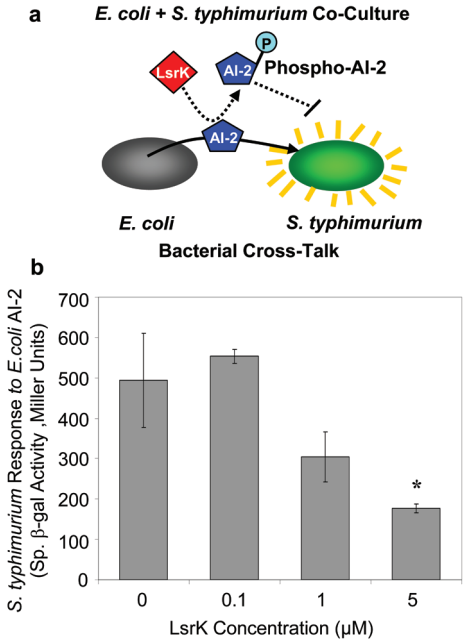

Figure 7. Addition of LsrK and ATP quenches AI-2 signal generated by *E. coli* and detected by *S. typhimurium* in a co-culture. a) *S. typhimurium* produces β-galactosidase in response to AI-2 secreted by *E. coli* in the environment; LsrK sequesters the AI-2 in a phosphorylated form and prevents the *S. typhimurium* response. b) AI-2-dependent β-galactosidase production in *S. typhimurium* MET715 in response to increasing concentrations of LsrK added to the co-culture of 1:1 *E. coli* and *S. typhimurium* (* indicates $p < 0.05$ for an unpaired *t* test as compared to the control with no LsrK).

(*luxS*$^+$) mixed bacterial population was quenched by LsrK and ATP added *in vitro*. In order to test bacterial cross-talk where one bacterial species responds to AI-2 produced by another species, gut consortia *E. coli* ZK126 (*luxS*$^+$, *lacZ*$^-$) and *S. typhimurium* MET715 (*luxS*$^-$, *lacZ*$^+$) were co-cultured and assayed (Table 1). The presence of *lacZ* expression indicates a positive response in *S. typhimurium* to AI-2 produced by *E. coli* (Figure 7, panel a). As shown in Figure 7, panel b, *S. typhimurium* exhibited significant β-galactosidase activity in response to *E. coli* AI-2, and the response decreases significantly with increasing concentrations of LsrK added *ex vivo* to the bacterial populations (see Methods). That is, addition of 5 μM LsrK quenched the native QS response by 3-fold relative to the control (no LsrK).

As demonstrated by our results for LsrK phosphorylation, we hypothesize that LsrK phosphorylated AI-2 that had been secreted from *E. coli* and the product of this reaction (phospho-DPD) degraded to PG or was otherwise unable to enter the *S. typhimurium* cells and produce a response. Since QS responses are dependent on AI-2 in the environment after having reached a "threshold" concentration, this study suggests that the addition of LsrK sufficiently sequesters the native AI-2 so that the threshold concentration is not achieved. Hence, a potentially pathogenic phenotype may be prevented.

Conclusion. This investigation provides the basis for AI-2 quorum quenching using the native bacterial phosphorylation machinery. LsrK is typically viewed as the QS kinase for phospho-AI-2 generation, which in turn is required to switch "on" *lsr*-regulated gene expression in *E. coli* and *S. typhimurium* QS circuits. Instead, we provide an alternative view of LsrK in which it functions outside of cells to switch "off" *lsr*-regulated gene expression. LsrK-based quorum quenching modulates responses in pure cultures of *E. coli*, *S. typhimurium*, and *V. harveyi*, as well as in a trispecies synthetic ecosystem. Moreover, LsrK-based quorum quenching was observed in a simulated *in vivo* environment wherein LsrK (and ATP) were added to co-cultures of AI-2 signal generating *E. coli* and AI-2 detecting *S. typhimurium*. Thus, we have demonstrated intraspecies and interspecies quenching. This suggests the utility of LsrK as a broad range quorum quencher in a variety of applications. For example, we anticipate this advance will enable a more mechanistic understanding of cell−cell communication within mixed cultures where competing signals from a variety of bacteria are synthesized and perceived in an otherwise undefined network. That is, specific interruption of one signal molecule within a network of signaling stimuli will help to elucidate its role in relation to others as the population "phenotype" is being determined. Since the levels of DPD needed to affect the biofilm phenotype in natural mixed culture communities is significantly lower (0.08−8 nM) (*39*) than the levels tested here, one might assume this approach would be feasible and result in "inactivated" DPD. Therefore anticipated biomedical applications may include its incorporation into an antimicrobial therapy by encapsulation with ATP and delivery to sites of infection and its use as a modulator of phenotype in environments prone to biofilm formation.

METHODS

Bacterial Strains and Growth Conditions. Table 1 lists 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 AB 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, 50 μg mL^{-1} ampicillin and 50 μg mL^{-1} kanamycin for *E. coli* MDAI-2 pCT6, and 20 μg mL^{-1} kanamycin for *V. harveyi* BB170.

Construction of *E. coli* LsrK Plasmid. To construct plasmid pET200-LsrK, *E. coli lsrK* (*ydeV*) was amplified with primers listed in Table 1 from *E. coli* W3110 whole genomic DNA using Vent DNA polymerase (New England Biolabs; NEB). The 1593 bp fragment was isolated and purified *via* the QIAquick gel extraction kit (Qiagen). This blunt ended fragment was then inserted into pET200/D-TOPO (Invitrogen), which has a hexa histidine tag at the N-terminus (see Supplementary Figure 1, panel a). The sequence of pET200-LsrK was confirmed by sequencing.

Overexpression and Purification of *E. coli* LsrK. The *E. coli* pET200-LsrK expression vector (Supplementary Figure 1, panel a) was transformed into *E. coli* BL21 *luxS*$^-$ and was cultured in LB medium supplemented with 50 μg mL^{-1} kanamycin at 37 °C and 250 rpm. LsrK protein expression was induced by IPTG addition and later purified as previously described (*40*). Purified LsrK (61,670 Da) was confirmed by loading 1 μg protein on an SDS−PAGE gel (Biorad) (see Supplementary Figure 1, panel b).

LsrK Activity. AI-2 was synthesized *in vitro* using synthases LuxS and Pfs (*40*). LsrK activity was estimated using an ATP bioluminescence assay kit (CLS II, Roche Scientific). One micromolar LsrK and 300 μM AI-2 were incubated with varying concentrations of ATP (5, 10, and 20 μM). A 100 μL aliquot was mixed with 100 μL of the luciferase reagent, and light production was recorded at 2 min intervals over a span of 30 min using a luminometer (EG&G Berthold). Values depicted were 10 s running averages. The concentrations of AI-2 (300 μM) and LsrK (1 μM) for the ATP assay were selected after trying a range of concentrations of AI-2 from 50 to 400 μM and from 0.5 to 4 μM LsrK, to determine the combinations at which maximum ATP was consumed.

***In Vitro* Phosphorylation of AI-2.** LsrK-treated AI-2 was synthesized by incubating LsrK (1 μM) with 80 μM ATP (Roche), 20 μM AI-2, 200 μM $MgCl_2$, in 25 mM phosphate buffer, pH 7.4 for either 1 or 16 h.

Thin Layer Chromatography (TLC). LsrK-treated AI-2 (1 or 16 h) samples (as described above) were incubated with 0.2 Ci of [γ-^{32}P] ATP (Perkin-Elmer) after which 5 μL of the sample was 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 and *S. typhimurium* MET715 overnight in LB medium supplemented with suitable antibiotics as stated previously. These were diluted into fresh LB medium supplemented with the antibiotic 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. LsrK-treated AI-2 (1 h; 16 h) or untreated AI-2 (500 μL; 20 μM) was added to 500 μL of either the *E. coli* LW7 or *S. typhimurium* MET715 suspension for 2 h at 37 °C. AI-2 dependent β-galactosidase production was quantified by the Miller assay (*41*) or Fluorescein di-β-D-galactopyranoside (FDG) (*42*).

Flow Cytometry. *E. coli* MDAI-2 pCT6 was cultured overnight in LB medium, diluted in fresh LB medium, and grown at 30 °C, 250 rpm for 8 h. Either the 16 h LsrK-treated AI-2 or untreated AI-2 (final concentrations, 10 μM) were added at time 0 and then added again after 2.5 h. After 7.5 h, the cells were fixed overnight in a 1:1 ratio of cold 4% paraformaldehyde. All samples were analyzed by flow cytometery (FACS Canto II, BD 394 Biosciences), with 20,000 gated events analyzed per sample.

Measurement of QS Response (Bioluminescence). The effect of LsrK degraded AI-2 on QS associated bioluminescence production by *V. harveyi* was recorded by measuring the light production using reporter strain, *V. harveyi* BB170. AI-2 treated with LsrK for 16 h or untreated AI-2 both at a concentration of (2 μM) was added to *V. harveyi* and assayed as previously described (*38*).

Analyzing QS Response in the Synthetic Ecosystem. The *S. typhimurium* MET715, *V. harveyi* MM30, and *E. coli* MDAI-2 pCT6 were each cultured separately overnight in LM medium (*37*) supplemented with the appropriate antibiotic. The cultures of MM30, MET715, and MDAI-2 were diluted (1:4:8) respectively, into a single volume of 1 mL of fresh LM medium without antibiotics. The co-culture was supplemented with LsrK-treated AI-2 or untreated AI-2 (final concentrations, 10 μM) 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 10 h, by fixing the cells with 1:1 cold 4% paraformaldehyde and using flow cytometric analysis.

Analyzing Bacterial Cross-Talk in a Natural System. The *S. typhimurium* MET715 and *E. coli* ZK126 were each cultured overnight in LB medium with the appropriate antibiotic. The samples were grown to OD_{600} 0.6−0.8 at 30 °C, 250 rpm and collected by centrifugation at 10,000 × *g* for 10 min. The pellets were then resuspended in 5 mL of LB. A 1:1 mixture of *E. coli* and *S. typhimurium* was prepared in fresh microcentrifuge tubes with increasing concentrations of LsrK (0.1 μM, 1 μM and 5 μM). Negative controls were also prepared without LsrK. ATP (80 μM) was added to all samples and incubated in a 30 °C water bath for 2 h. AI-2-dependent β-galactosidase production was quantified by the Miller assay (*41*).

Acknowledgment: The authors acknowledge the advice provided by H. Sintim in understanding the significance of the mixed culture studies, B. Bassler for generously providing *S. typhimurium* MET715, and T. Dunn and H. Wu for their help in conducting the flow cytometry studies. Funding for this work was provided by the National Science Foundation and the R. W. Deutsch Foundation.

Supporting Information Available: This material is available free of charge *via* the Internet at http://pubs.acs.org.

REFERENCES

1. Vendeville, A., Winzer, K., Heurlier, K., Tang, C. M., and Hardie, K. R. (2005) Making 'sense' of metabolism: autoinducer-2, LuxS and pathogenic bacteria, *Nat. Rev. Microbiol. 3*, 383–396.
2. Xavier, K. B., and Bassler, B. L. (2005) Regulation of uptake and processing of the quorum-sensing autoinducer AI-2 in *Escherichia coli*, *J. Bacteriol. 187*, 238–248.
3. Higgins, D. A., Pomianek, M. E., Kraml, C. M., Taylor, R. K., Semmelhack, M. F., and Bassler, B. L. (2007) The major *Vibrio cholerae* autoinducer and its role in virulence factor production, *Nature 450*, 883–886.

4. Rasko, D. A., Moreira, C. G., Li de, R., Reading, N. C., Ritchie, J. M., Waldor, M. K., Williams, N., Taussig, R., Wei, S., Roth, M., Hughes, D. T., Huntley, J. F., Fina, M. W., Falck, J. R., and Sperandio, V. (2008) Targeting QseC signaling and virulence for antibiotic development, *Science 321*, 1078–1080.
5. Rasmussen, T. B., and Givskov, M. (2006) Quorum sensing inhibitors: a bargain of effects, *Microbiology 152*, 895–904.
6. Fuqua, W. C., Winans, S. C., and Greenberg, E. P. (1994) Quorum sensing in bacteria: the LuxR-LuxI family of cell density-responsive transcriptional regulators, *J. Bacteriol. 176*, 269–275.
7. Raina, S., Vizio, D. D., Odell, M., Clements, M., Vanhulle, S., and Keshavarz, T. (2009) Microbial quorum sensing: a tool or a target for antimicrobial therapy? *Biotechnol. Appl. Biochem. 54*, 65–84.
8. Rasmussen, T. B., and Givskov, M. (2006) Quorum-sensing inhibitors as anti-pathogenic drugs, *Int. J. Med. Microbiol. 296*, 149–161.
9. Dong, Y. H., and Zhang, L. H. (2005) Quorum sensing and quorum-quenching enzymes, *J. Microbiol. 43*, 101–109.
10. Singh, P. K., Schaefer, A. L., Parsek, M. R., Moninger, T. O., Welsh, M. J., and Greenberg, E. P. (2000) Quorum-sensing signals indicate that cystic fibrosis lungs are infected with bacterial biofilms, *Nature 407*, 762–764.
11. Greenberg, E. P. (2003) Bacterial communication: tiny teamwork, *Nature 424*, 134.
12. Schaefer, A. L., Greenberg, E. P., Oliver, C. M., Oda, Y., Huang, J. J., Bittan-Banin, G., Peres, C. M., Schmidt, S., Juhaszova, K., Sufrin, J. R., and Harwood, C. S. (2008) A new class of homoserine lactone quorum-sensing signals, *Nature 454*, 595–599.
13. Dong, Y. H., Wang, L. H., Xu, J. L., Zhang, H. B., Zhang, X. F., and Zhang, L. H. (2001) Quenching quorum-sensing-dependent bacterial infection by an N-acyl homoserine lactonase, *Nature 411*, 813–817.
14. Leadbetter, J. R., and Greenberg, E. P. (2000) Metabolism of acyl-homoserine lactone quorum-sensing signals by *Variovorax paradoxus*, *J. Bacteriol. 182*, 6921–6926.
15. Yang, F., Wang, L. H., Wang, J., Dong, Y. H., Hu, J. Y., and Zhang, L. H. (2005) Quorum quenching enzyme activity is widely conserved in the sera of mammalian species, *FEBS Lett. 579*, 3713–3717.
16. Manefield, M., Rasmussen, T. B., Henzter, M., Andersen, J. B., Steinberg, P., Kjelleberg, S., and Givskov, M. (2002) Halogenated furanones inhibit quorum sensing through accelerated LuxR turnover, *Microbiology 148*, 1119–1127.
17. Hentzer, M., Wu, H., Andersen, J. B., Riedel, K., Rasmussen, T. B., Bagge, N., Kumar, N., Schembri, M. A., Song, Z., Kristoffersen, P., Manefield, M., Costerton, J. W., Molin, S., Eberl, L., Steinberg, P., Kjelleberg, S., Hoiby, N., and Givskov, M. (2003) Attenuation of *Pseudomonas aeruginosa* virulence by quorum sensing inhibitors, *EMBO J. 22*, 3803–3815.
18. Lyon, G. J., Mayville, P., Muir, T. W., and Novick, R. P. (2000) Rational design of a global inhibitor of the virulence response in *Staphylococcus aureus*, based in part on localization of the site of inhibition to the receptor-histidine kinase, AgrC, *Proc. Natl. Acad. Sci. U.S.A. 97*, 13330–13335.
19. Collins, C. H., Leadbetter, J. R., and Arnold, F. H. (2006) Dual selection enhances the signaling specificity of a variant of the quorum-sensing transcriptional activator LuxR, *Nat. Biotechnol. 24*, 708–712.
20. Basu, S., Gerchman, Y., Collins, C. H., Arnold, F. H., and Weiss, R. (2005) A synthetic multicellular system for programmed pattern formation, *Nature 434*, 1130–1134.
21. Miller, S. T., Xavier, K. B., Campagna, S. R., Taga, M. E., Semmelhack, M. F., Bassler, B. L., and Hughson, F. M. (2004) *Salmonella typhimurium* recognizes a chemically distinct form of the bacterial quorum-sensing signal AI-2, *Mol. Cell 15*, 677–687.
22. Ren, D., Bedzyk, L. A., Ye, R. W., Thomas, S. M., and Wood, T. K. (2004) Differential gene expression shows natural brominated furanones interfere with the autoinducer-2 bacterial signaling system of *Escherichia coli*, *Biotechnol. Bioeng. 88*, 630–642.
23. Soni, K. A., Jesudhasan, P., Cepeda, M., Widmer, K., Jayaprakasha, G. K., Patil, B. S., Hume, M. E., and Pillai, S. D. (2008) Identification of ground beef-derived fatty acid inhibitors of autoinducer-2-based cell signaling, *J. Food Prot. 71*, 134–138.
24. Widmer, K. W., Soni, K. A., Hume, M. E., Beier, R. C., Jesudhasan, P., and Pillai, S. D. (2007) Identification of poultry meat-derived fatty acids functioning as quorum sensing signal inhibitors to autoinducer-2 (AI-2), *J. Food Sci. 72*, M363–368.
25. Lowery, C. A., Park, J., Kaufmann, G. F., and Janda, K. D. (2008) An unexpected switch in the modulation of AI-2-based quorum sensing discovered through synthetic 4,5-dihydroxy-2,3-pentanedione analogues, *J. Am. Chem. Soc. 130*, 9200–9201.
26. Smith, J. A., Wang, J., Nguyen-Mau, S. M., Lee, V., and Sintim, H. O. (2009) Biological screening of a diverse set of AI-2 analogues in *Vibrio harveyi* suggests that receptors which are involved in synergistic agonism of AI-2 and analogues are promiscuous, *Chem. Commun. (Cambridge)* 7033–7035.
27. Schramm, V. L., Gutierrez, J. A., Cordovano, G., Basu, I., Guha, C., Belbin, T. J., Evans, G. B., Tyler, P. C., and Furneaux, R. H. (2008) Transition state analogues in quorum sensing and SAM recycling, *Nucleic Acids Symp. Ser.* 75–76.
28. Kapadnis, P. B., Hall, E., Ramstedt, M., Galloway, W. R., Welch, M., and Spring, D. R. (2009) Towards quorum-quenching catalytic antibodies, *Chem. Commun. (Cambridge)* 538–540.
29. Wang, L., Hashimoto, Y., Tsao, C. Y., Valdes, J. J., and Bentley, W. E. (2005) Cyclic AMP (cAMP) and cAMP receptor protein influence both synthesis and uptake of extracellular autoinducer 2 in *Escherichia coli*, *J. Bacteriol. 187* 2066–2076.
30. Xavier, K. B., Miller, S. T., Lu, W., Kim, J. H., Rabinowitz, J., Pelczer, I., Semmelhack, M. F., and Bassler, B. L. (2007) Phosphorylation and processing of the quorum-sensing molecule autoinducer-2 in enteric bacteria, *ACS Chem. Biol. 2*, 128–136.
31. Waters, C. M., and Bassler, B. L. (2005) Quorum sensing: cell-to-cell communication in bacteria, *Annu. Rev. Cell Dev. Biol. 21*, 319–346.
32. Taga, M. E., Miller, S. T., and Bassler, B. L. (2003) Lsr-mediated transport and processing of AI-2 in *Salmonella typhimurium*, *Mol. Microbiol. 50*, 1411–1427.
33. Wang, L., Li, J., March, J. C., Valdes, J. J., and Bentley, W. E. (2005) luxS-dependent gene regulation in *Escherichia coli* K-12 revealed by genomic expression profiling, *J. Bacteriol. 187*, 8350–8360.
34. Xavier, K. B., and Bassler, B. L. (2005) Interference with AI-2-mediated bacterial cell-cell communication, *Nature 437*, 750–753.
35. Shi, X. W., Tsao, C. Y., Yang, X., Liu, Y., Dykstra, P., Rubloff, G. W., Ghodssi, R., Bentley, W. E., and Payne, G. F. (2009) Electroaddressing of cell populations by co-deposition with calcium alginate hydrogels, *Adv. Funct. Mater. 19*, 2074–2080.
36. Chen, X., Schauder, S., Potier, N., Van Dorsselaer, A., Pelczer, I., Bassler, B. L., and Hughson, F. M. (2002) Structural identification of a bacterial quorum-sensing signal containing boron, *Nature 415*, 545–549.
37. Bassler, B. L., Wright, M., and Silverman, M. R. (1994) Multiple signalling systems controlling expression of luminescence in *Vibrio harveyi*: sequence and function of genes encoding a second sensory pathway, *Mol. Microbiol. 13*, 273–286.
38. Surette, M. G., Miller, M. B., and Bassler, B. L. (1999) Quorum sensing in *Escherichia coli*, *Salmonella typhimurium*, and *Vibrio harveyi*: a new family of genes responsible for autoinducer production, *Proc. Natl. Acad. Sci. U.S.A. 96*, 1639–1644.
39. Rickard, A. H., Palmer, R. J., Jr., Blehert, D. S., Campagna, S. R., Semmelhack, M. F., Egland, P. G., Bassler, B. L., and Kolenbrander, P. E. (2006) Autoinducer 2: a concentration-dependent signal for mutualistic bacterial biofilm growth, *Mol. Microbiol. 60*, 1446–1456.

40. Fernandes, R., and Bentley, W. E. (2009) AI-2 biosynthesis module in a magnetic nanofactory alters bacterial response via localized synthesis and delivery, *Biotechnol. Bioeng. 102*, 390–399.
41. Miller, J. (1972) *Experiments in Molecular Genetics*, Cold Spring Harbor Laboratory, New York
42. Plovins, A., Alvarez, A. M., Ibanez, M., Molina, M., and Nombela, C. (1994) Use of fluorescein-di-β-D-galactopyranoside (FDG) and C12-FDG as substrates for β-galactosidase detection by flow cytometry in animal, bacterial, and yeast cells, *Appl. Environ. Microbiol. 60*, 4638–4641.
43. Connell, N., Han, Z., Moreno, F., and Kolter, R. (1987) An *E. coli* promoter induced by the cessation of growth, *Mol. Microbiol. 1*, 195–201.
44. Bassler, B. L., Wright, M., Showalter, R. E., and Silverman, M. R. (1993) Intercellular signalling in *Vibrio harveyi*: sequence and function of genes regulating expression of luminescence, *Mol. Microbiol. 9*, 773–786.