Published in final edited form as:

Nature. 2005 September 29; 437(7059): 750–753.

Interference with AI-2-Mediated Bacterial Cell-Cell Communication

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Abstract

Bacteria communicate with chemical signal molecules called autoinducers. This process, called quorum-sensing, allows bacteria to count the members in the community and to synchronously alter gene expression of the population. Quorum-sensing-controlled processes are often crucial for successful bacterial-host relationships; both symbiotic and pathogenic. Most quorum-sensing autoinducers promote intra-species communication, but one autoinducer, called AI-2, is produced and detected by a wide variety of bacteria and is proposed to allow inter-species communication^{1,2}. We show here that some species of bacteria can manipulate AI-2 signalling and interfere with other species' ability to correctly assess and respond to changes in cell population density. AI-2-signalling and interference with it could have important ramifications for eukaryotes in maintaining normal microflora and in protection from pathogenic bacteria.

The bacterial signal molecule called Autoinducer-2 (AI-2) is a product of the LuxS enzyme which is broadly conserved throughout the bacterial world. LuxS enzymes synthesize 4,5-dihydroxy 2,3-pentanedione (DPD) which undergoes spontaneous rearrangements³. Importantly, DPD derivatives interconvert and exist in equilibrium. Different bacteria recognize distinct DPD derivatives, and this family of molecules is generically called AI-2⁴. The interconverting nature of these molecules presumably allows bacteria to respond to their own AI-2 and also to AI-2 produced by other bacterial species, giving rise to the idea that AI-2 represents a universal language fostering inter-species bacterial communication.

We characterized the quorum-sensing signal production and detection apparatuses in *Vibrio harveyi*, *Vibrio cholerae*, *Escherichia coli*, and *Salmonella typhimurium*^{5,8}. In *V. harveyi*, two autoinducers, AI-1 and AI-2, are detected by LuxN and LuxPQ, respectively, and control expression of genes including those for bioluminescence and Type III secretion (TTS) of virulence factors (Fig. 1a)^{5,9}. A related quorum-sensing network exists in *V. cholerae* and controls expression of virulence genes including *hapA* encoding the haemagglutinin (H/A) protease^{6,10}.

Some bacteria produce and consume AI-2. For example, *E. coli* and *S. typhimurium* release AI-2 in exponential phase, and import AI-2 at the transition into stationary phase. This occurs because one target that is activated by AI-2 is the Lsr (for **L**ux**S** **R**egulated) transporter that imports AI-2 (Fig. 1b)^{7,8}. In the absence of AI-2, LsrR represses the *lsr* operon. Following AI-2 release, low-level internalization occurs, and intracellular AI-2 is phosphorylated by LsrK^{8,11}. Phospho-AI-2 (AI-2-P) antagonizes LsrR, which leads to de-repression of *lsr* expression, assembly of the Lsr transporter, and rapid AI-2 internalization. LsrR⁻ strains are avid AI-2 consumers because the *lsr* operon is de-repressed. By contrast, LsrK⁻ strains never

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Supplementary Information accompanies the paper on www.nature.com/nature.

consume significant AI-2 due to their inability to de-repress *lsr* transcription (Fig. 1b, supplementary Fig. 1S, and 2S)^{8,11}.

In mixed-species consortia, production and consumption of AI-2 by enterics should have reciprocal effects on gene regulation in other bacterial species that communicate with AI-2. When the enterics supply AI-2, a nearby species could use the information to count the enteric cells in the mixed-species community or possibly prematurely activate the expression of quorum-sensing-regulated genes. However, when the enterics remove AI-2, a neighboring species could underestimate cell number and fail to initiate or incorrectly terminate quorum sensing. Thus, consuming AI-2 could allow enterics to interfere with AI-2-mediated communication in other bacteria.

When *E. coli* is grown in pure culture, AI-2 is required to activate *lsr* expression (Fig. 2a; compare WT to LuxS⁻). Induction of *lsr* expression also occurs when LuxS⁻ *E. coli* is mixed with WT *V. harveyi* (third bar) and depends on the AI-2 supplied by *V. harveyi* because no *lsr* activation occurs when LuxS⁻ *E. coli* is combined with LuxS⁻ *V. harveyi* (fourth bar). Thus, *E. coli* detects and responds to its own AI-2 and also to that produced by *V. harveyi*.

We also examined the effects of *E. coli* AI-2 production and consumption on *V. harveyi* using bioluminescence as the readout. When incubated with WT *E. coli*, WT *V. harveyi* at high cell density produces only 18% of the light it produces in pure culture (Fig. 2b; left two black bars). Thus, *E. coli* consumes both its and *V. harveyi*'s AI-2 causing reduced light output by *V. harveyi*. Co-culture of WT *V. harveyi* with LsrR⁻ *E. coli* (constitutive AI-2 importer) causes a greater reduction in *V. harveyi* bioluminescence (third black bar). Diminution of light is due exclusively to Lsr-mediated transport of AI-2 into *E. coli* because no reduction in light production occurs when *V. harveyi* is co-cultured with the LsrK⁻ *E. coli* mutant that does not internalize AI-2 (fourth black bar).

In addition to AI-2, *V. harveyi* produces an autoinducer called AI-1 and responds to it via the LuxN sensor (Fig. 1a)⁵. *V. harveyi* LuxN⁻ strains produce less light than WT because LuxN⁻ strains induce bioluminescence only in response to AI-2 whereas WT responds to AI-1 and AI-2 (Fig. 2b; first gray bar). Growth of LuxN⁻ *V. harveyi* with WT and LsrR⁻ *E. coli* reduces light output to 2% and <1%, respectively (second and third gray bars). *E. coli* has a stronger effect on LuxN⁻ *V. harveyi* than on WT *V. harveyi* because WT *V. harveyi* retains its response to AI-1, the levels of which are not altered by *E. coli* consumption of AI-2. When the LuxN⁻ *V. harveyi* is co-cultured with the LsrK⁻ *E. coli* mutant, increased light production occurs in response to the additional AI-2 provided by the internalization-defective *E. coli* (fourth gray bar). Finally, these AI-2 effects on *V. harveyi* require the LuxQ sensor (Fig. 1a) because manipulating extracellular AI-2 levels by co-culture with *E. coli* does not alter light production by the LuxQ⁻ *V. harveyi* (Fig. 2b; white bars).

Dilution of *V. harveyi* in pure culture causes autoinducer levels to fall below that required for detection, and bioluminescence expression terminates at low cell densities. During subsequent growth, autoinducers are released. Upon achieving a threshold concentration, they are detected, and the cells respond by inducing a rapid increase in light production. Our premise is that in mixed cultures, early production and later consumption of AI-2 by *E. coli* should inversely affect the *V. harveyi* quorum-sensing response. To test this idea, we measured *V. harveyi* bioluminescence during growth in co-culture with *E. coli*. Both species were diluted to low cell density and combined under conditions allowing each to grow exponentially (Fig. 3a). *V. harveyi* growing with the non-AI-2-producing, non-AI-2-importing *E. coli* LuxS⁻, LsrK⁻ strain shows the characteristic initial decline in bioluminescence followed by the rapid increase to the maximal, pre-dilution level (Fig. 3b; ×). In the presence of all AI-2⁺ *E. coli* strains tested, the initial decrease in bioluminescence is significantly reduced because *V. harveyi* rapidly

initiates quorum sensing in response to the AI-2 supplied by *E. coli* (■,+,▲). At later times, corresponding to post-*lsr* induction, all *E. coli* strains that have LsrK, and are therefore capable of transporting AI-2, consume the AI-2 (■,+,○). This has the consequence of decreasing *V. harveyi* light output by almost 100-fold compared to when *V. harveyi* is grown in the presence of LsrK⁻ strains that are unable to internalize AI-2 (▲,×). Mixing *V. harveyi* with the *E. coli* LsrR⁻ strain results in the most pronounced reduction in light production presumably due to the constitutive removal of AI-2 (+).

A more dramatic effect on *V. harveyi* quorum sensing occurs when exponential phase *V. harveyi* encounters *E. coli* in stationary phase (Fig. 3c). In this case, at the time of mixing, WT *E. coli* is induced for *lsr* expression, and the cells are actively internalizing AI-2. An almost immediate and continuous decrease in *V. harveyi* bioluminescence occurs (Fig. 3d; ■), which is further exaggerated in the mixture containing the LsrR⁻ *E. coli* (+). In stark contrast, in co-culture with stationary phase LsrK⁻ *E. coli*, *V. harveyi* steadily maintains maximal light production (▲) due to the presence of AI-2 produced by the transport deficient *E. coli* mutant. As expected, in the presence of the LsrK⁻, LuxS⁻ *E. coli*, *V. harveyi* displays density dependent bioluminescence (×). Mixing *V. harveyi* with LuxS⁻ *E. coli* initially has no effect on *V. harveyi* quorum sensing because LuxS⁻ *E. coli* has not been exposed to AI-2 prior to the addition of *V. harveyi*, so the Lsr transporter is repressed (○). During co-incubation, the *V. harveyi*-produced AI-2 induces LuxS⁻ *E. coli* to express the Lsr transporter. *E. coli* consumes AI-2, causing a reduction in *V. harveyi* light production. Therefore, at late times, light output from the *V. harveyi* strain mixed with the *E. coli* LuxS⁻ strain falls below that of *V. harveyi* co-incubated with the *E. coli* LsrK⁻, LuxS⁻ strain.

The *V. harveyi* quorum-sensing regulon contains many genes, and in consortia, all are presumably susceptible to effects of AI-2 production and consumption. To examine this possibility, the TTS gene *vopN* was measured in co-cultures of *E. coli* and *V. harveyi*. TTS genes are repressed by autoinducers at high cell density in *V. harveyi* (Fig. 1a). Production of AI-2 by *E. coli* incapable of importing AI-2 does not affect *vopN* expression showing that AI-2 supplied by *V. harveyi* is sufficient for maximal repression (Fig. 4a; black bars, compare LsrK⁻ to LsrK⁻, LuxS⁻). Growth with *E. coli* strains that consume AI-2 (black bars, WT and LsrR⁻) results in de-repression of *vopN*. Full de-repression only occurs in combinations in which neither *V. harveyi* nor *E. coli* produces AI-2 (striped bars) demonstrating that in the mixtures depicted by the black bars, *E. coli* has not internalized all of the AI-2. As in Fig. 2a, AI-2 production and consumption does not affect *vopN* expression if *V. harveyi* lacks LuxQ (white bars).

We recognize that *V. harveyi*, which routinely lives in mixed-species marine communities, is not likely to co-exist in environments with *E. coli*. In contrast, *V. cholerae* must co-exist with *E. coli* during pathogenic associations with humans. To determine whether *E. coli* AI-2 consumption could interfere with *V. cholerae* signalling, we measured expression of the quorum-sensing-activated gene *hapA*, encoding the H/A protease. Indeed, our results mimic those acquired with TTS in *V. harveyi* except that the pattern of regulation is reversed (Fig. 4b). Induction occurs in mixtures containing *E. coli* strains incapable of AI-2 transport (LsrK⁻ and LsrK⁻, LuxS⁻) and no induction occurs in mixtures containing *E. coli* strains that do transport AI-2 (WT, and LsrR⁻).

AI-2 consumption has a more modest effect on *vopN* and *hapA* regulation than on regulation of bioluminescence. Many environmental factors are known to control H/A protease production and TTS^{10,12}. AI-2 interference likely has more subtle effects on H/A protease and TTS than on bioluminescence because, in the former cases, additional regulators exert control over the outputs, and regulation by these factors remains unchanged in our experiments.

Our results show that AI-2 can foster two-way communication between bacterial species because, when *E. coli* and *V. harveyi* are grown in co-culture, AI-2 production by either species can regulate light production in *V. harveyi* and trigger *lsr* induction in *E. coli*. Thus, AI-2 production allows each species to include the other in the 'census'. Because *E. coli* and *V. harveyi* respond to AI-2s with different structures^{2,4}, cross-communication implies that the signal released by one species must convert into that used by the other species. Presumably, these transformations occur in the medium between the sender and receiver cells. This is especially interesting given that the vibrios detect an AI-2 molecule containing boron whereas there is no boron in the AI-2 signal recognized by enterics^{2,4}. Our results demonstrate that these drastic chemical interconversions are occurring on a time scale that promotes major effects on gene expression.

In model *V. harveyi*-*E. coli* mixtures, induction of *lsr* genes in *E. coli* results in assembly of the AI-2 transporter and subsequent consumption of AI-2. This has the consequence of inhibiting light production by *V. harveyi* demonstrating that *E. coli* interferes with AI-2-mediated communication by causing *V. harveyi* to prematurely terminate its quorum-sensing behaviors. The impact of the *E. coli* AI-2-Lsr system is not restricted to interference with activation of bioluminescence because repression of Type III secretion is also affected. These findings imply that interference with AI-2 signalling influences the expression of entire quorum-sensing regulons.

Lsr-mediated interference with AI-2 signalling also occurs in co-cultures of *E. coli* and *V. cholerae*, two bacteria that certainly jointly colonize the human intestine during *V. cholerae* disease. We do not expect these interactions to be restricted to enteric bacteria; indeed disappearance of AI-2 in stationary phase has been reported for diverse bacterial species indicating that, either Lsr transporters exist in these species, or additional mechanisms exist to eliminate AI-2¹³. Any species of bacteria that relies on AI-2-mediated communication and inhabits niches containing another bacterial species that produces and/or consumes AI-2 could be similarly affected. Whether quorum sensing is enhanced or inhibited will depend on the growth status of the different species when they encounter one another. Complex pro- and anti-AI-2-mediated interactions could be taking place in natural niches. Possibly, eukaryotes have capitalized on this by evolving specific associations with bacteria that use or manipulate AI-2 signalling; and these interactions could have important human consequences in the maintenance of the normal human gut microflora as well as in the prevention of bacterial diseases.

Methods

Bacterial Strains and Growth Conditions. Bioluminescence was measured in *V. harveyi* strain BB120 (WT)¹, MM30 (*luxS*)¹⁴, BB170 (*luxN*)¹⁵, and BB960 (*luxQ*)⁵. *E. coli* strains are derivatives of MG1655¹⁶. The *E. coli* strains used in Fig. 2, 3, and supplementary Fig. 1S, and 2S contain a *lacZYA* deletion and an *lsr-lacZ* fusion integrated at the *att* site. These strains are KX1123 (WT), KX1218 (*luxS*), KX1186 (*lsrK*), KX1322 (*lsrR*), and KX1372 (*lsrK*, *luxS*)⁸. To measure *vopN* expression, *V. harveyi* strains JMH385 (WT), KX1530 (*luxS*), JMH669 (*luxQ*), containing a chromosomal *vopN::mini-Mu_llacZ* insertion were used⁹. The final two strains were obtained by introduction of *luxS::Tn5*¹⁴, and *luxQ::Tn5*⁵ onto the chromosome of JMH385, respectively. *E. coli* strains used in TTS experiments are KX1102 (WT), KX1479 (*lsrK*), KX1477 (*lsrR*), and KX1526 (*luxS*, *lsrK*) and each was constructed by the method reported⁸. *V. cholerae* strains are derivatives of El Tor C6706str2, a streptomycin-resistant isolate of C6706¹⁷, and all have a *hap-luxCDABE* transcriptional fusion cloned into a plasmid containing a chloramphenicol resistance (Cm^R) marker. The *hap-luxCDABE* transcriptional fusion was constructed as reported¹⁸ except that a PCR-amplified fragment containing the promoter region of *V. cholerae hapA* was cloned into unique *SpeI* to *BamHI* sites. The *V.*

cholerae strains used are: BH1220 (WT), BH1312 (*luxS*), and BH1253 (*luxQ*). Details of their construction will be reported elsewhere (B. K. Hammer, B. L. Bassler, manuscript in preparation). *E. coli* strains used for co-culture with *V. cholerae* are identical to those described for Fig. 3 except for KX1583 (WT) contains a Cm^R marker downstream of the *luxS* gene. Addition of Cm was required in *E. coli*-*V. cholerae* co-cultures to maintain the plasmid harboring the *hapA-luxCDABE* fusion in *V. cholerae*. Co-cultures were grown in LM⁵ in experiments with *V. harveyi*, and in LB supplemented with Cm (10 mg/L) in experiments with *V. cholerae*. All cultures were incubated at 30°C with aeration.

β-Galactosidase Assays. Overnight cultures of *E. coli* and *V. harveyi* strains were diluted 1:1000 into LM and grown for 8 h after which *lsr-lacZ* expression was measured. When *vopN-lacZ* expression was measured, *V. harveyi* strains were diluted 1:5000 into LM and 1:100 dilutions of overnight cultures of *E. coli* were added. Cultures were grown for 18 h because expression of TTS genes is maximal in stationary phase. Cells from 1 ml of culture were resuspended in 1 ml of Z buffer for β-galactosidase assays¹⁹. The values shown represent the average of triplicates.

Bioluminescence Assays. Overnight cultures of *V. harveyi* strains were diluted 1:5000 into LM. In co-incubations, 1:100 dilutions of overnight cultures of *E. coli* were added. In the experiments shown in Fig 2b bioluminescence was measured after 12 h of incubation. In experiments in Fig. 3c and d the *V. harveyi* strains were added to *E. coli* cultures that had been pre-grown for 4 h in LM. At the reported times, aliquots were plated to determine cfu, and bioluminescence was measured using a liquid scintillation counter (Wallac model 1409).

Analysis of *hapA-luxCDABE* Expression. Overnight cultures of *E. coli* and *V. cholerae* were diluted 1:1000 into LB supplemented with Cm and grown for 16 h. Aliquots were plated to determine cfu, and luciferase activity from the *hapA-luxCDABE* fusion was measured in a liquid scintillation counter. Values shown represent the average of triplicates.

Measurements of cell number in co-cultures. In all co-culture experiments, the mixtures of cells were plated on two different types of media. The media conditions were chosen so that they either permitted growth of only one of the two species in the mixture or the colonies of each species could be distinguished visually by morphology. This strategy allowed us to determine the cell numbers of both species in each co-culture experiment. *V. harveyi* cfu were assessed following overnight incubation at 30° on LM agar supplemented with ampicillin (100 mg/L). *V. cholerae* cfu were counted after incubation at 37°C on LB agar containing 50 U/L polymyxin B. *E. coli* cfu were counted following incubation at 37°C on LB. *V. harveyi* does not grow at 37°C and *V. cholerae* colonies are translucent under this condition whereas *E. coli* colonies are opaque.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This research was supported by NIH and NSF grants (B. L. Bassler) and a Praxis XXI postdoctoral fellowship, Portugal (K. B. Xavier). We thank the members of the Bassler lab, as well as Drs. Ned Wingreen and Frederick Hughson for insightful discussions. We are grateful to Dr. B. Hammer for *V. cholerae* strains and to Dr. J. Henke for *V. harveyi* TTS mutants.

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Xavier and Bassler Figure 1

Figure 1.

AI-2 Signalling Systems. a, *V. harveyi* quorum sensing. The autoinducers AI-1 and AI-2 are detected by LuxN and LuxPQ, respectively. Information is transduced by phosphorylation. b, The *E. coli* Lsr transporter imports AI-2. See text for details.

Xavier and Bassler Figure 2

Figure 2. *E. coli* and *V. harveyi* communicate with AI-2. a, *V. harveyi* AI-2 induces the *E. coli* *lsr* operon. β-galactosidase activities of an *E. coli* *lsr-lacZ* fusion for *E. coli* mono-cultures and co-cultures with *V. harveyi*. Abbreviations: WT (AI-2⁺), LuxS⁻ (AI-2⁻), β-gal units (Abs₄₂₀min⁻¹ × 10⁹ ml⁻¹)/(*E. coli* cfu ml⁻¹). Error bars represent standard deviations. b, *E. coli* consumes AI-2 and inhibits *V. harveyi* bioluminescence expression. Light production in *V. harveyi* WT (black), LuxN⁻ (gray), LuxQ⁻ (white) in co-culture with *E. coli* WT, LsrR⁻, LsrK⁻. *V. harveyi* bioluminescence is the percentage of WT (140,000 counts per min (cpm) ml⁻¹/(*V. harveyi* cfu ml⁻¹).

Xavier and Bassler Figure 3

Figure 3.

The *E. coli* Lsr system interferes with *V. harveyi* quorum sensing. a, Growth curves for strains in b. Solid and dotted lines denote *V. harveyi* and *E. coli* cfu, respectively. b, Light production for LuxN⁻ *V. harveyi* in co-culture with *E. coli* strains: WT (■), LsrR⁻ (+), LsrK⁻ (▲), LuxS⁻ (○), and LsrK⁻ LuxS⁻ (×). c, Growth curves for strains in d; see legend for a. d, LuxN⁻ *V. harveyi* was diluted into *E. coli* cultures entering stationary phase. *E. coli* strains are identical to those in b. Relative light units (RLU) are $\text{cpm ml}^{-1} \times 10^3 / (V. harveyi \text{ cfu ml}^{-1})$.

Xavier and Bassler Figure 4

Figure 4.

V. harveyi TTS and *V. cholerae* H/A protease production are regulated by *E. coli* Lsr-mediated AI-2 transport. a, *V. harveyi* TTS expression was measured using a *vopN-lacZ* fusion (18 h co-culture). b, *V. cholerae* *hapA* expression was measured with a *hapA-lux* fusion (16 h co-culture). Activities were measured in the following *vibrio* strains: WT, black bars; LuxS⁻, striped bars; LuxQ⁻, white bars. Each *vibrio* strain was grown in co-culture with the following *E. coli* strains: WT, LsrR⁻, LsrK⁻, and LuxS⁻, LsrK⁻ double mutant. β-gal units and RLU are as in Fig. 2 and 3. Error bars denote the standard deviations.