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Establishing a Quantitative Definition of Quorum Sensing Provides Insight into the Information Content of the Autoinducer Signals in *Vibrio harveyi* and *Escherichia coli*[†]

Jessica R. Gooding, Amanda L. May, Kathryn R. Hilliard, and Shawn R. Campagna*

Department of Chemistry, University of Tennessee, 618 Buehler Hall, Knoxville, Tennessee 37996-1600

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ABSTRACT: Extracellular autoinducer concentrations in cultures of *Vibrio harveyi* and *Escherichia coli* were monitored by liquid chromatography−tandem mass spectrometry to test whether a quantitative definition of quorum sensing could help decipher the information content of these signals. Although *V. harveyi* was able to keep the autoinducer-2 to cell number ratio constant, the ratio of signal to cell number for *V. harveyi* autoinducer-1 and *E. coli* autoinducer-2 varied as the cultures grew. These data indicate that *V. harveyi* uses autoinducer-2 for quorum sensing, while the other molecules may be used to transmit different information or are influenced by metabolic noise.

Quorum sensing provides bacteria and other microorganisms with the ability to regulate behaviors on the basis of the number of cells present in the environment (*1*). The ability to socialize allows bacteria to behave as pseudomulticellular organisms, and these circuits may be crucial mediators during the establishment of bacterial colonies, formation of biofilms, and the onset of infectious diseases (*1*, *2*). Examples of intraspecies, intragenus, and interspecies signals have been identified (*1*). This work focuses on the proposed interspecies signal, autoinducer-2 (AI-2),[1] and the interaction of this molecule with the *Vibrio harveyi* intraspecies signal, autoinducer-1 (HAI-1) (*3*). Herein, we propose that a quantitative definition of quorum sensing may be sufficient to determine the information content of these signals.

Although the topic of much research, AI-2 has not been proven to function as a signal in all species that contain LuxS, the enzyme responsible for (*S*)-4,5-dihydroxy-2,3-pentanedione (DPD) production, and the widespread inclusion of *luxS* in bacterial genomes could be due to quorum sensing or to the fact that LuxS is necessary for the recycling of methionine from the activated methyl cycle. The linkage of AI-2 production to metabolism, coupled with the observation that very few, if any, genes other than those necessary for DPD metabolism are regulated by this molecule in some species, has left its role in question (*4*).

Species that produce acylhomoserine lactone signals, such as HAI-1, have a further link between signaling and metabolism because these molecules are also derived from the activated methyl cycle (Figure S1 of the Supporting Information) (*1*). For *V. harveyi*, HAI-1 and AI-2 are known to be signals, and several behaviors, e.g., bioluminescence, are under quorum sensing control (*5*). These two signals use shared regulatory components that act as coincidence detectors to mediate identical behaviors. Previous work has been unable to determine how *V. harveyi* distinguishes between the two, although it has been proposed that the timing of autoinducer production could differentiate the two signals (*6*).

We sought to determine whether autoinducers could be used to transmit information concerning cell density in two species, *V. harveyi* and *Escherichia coli*, by quantitating the DPD concentration present in the supernatants for both as well as the HAI-1 concentration for *V. harveyi*. These bacteria were chosen because the pathways involved in signal production and utilization are characterized for both (*7*, *8*). DPD gives rise to AI-2 activity (*9*). Because of this, the DPD concentration is directly correlated with signaling ability. Methods to quantitate the DPD (as a stable quinoxaline derivative) and HAI-1 concentrations from a variety of media using LC−MS/MS and stable isotope internal standards were critical to this work (*10*, *11*) (Supporting Information). Prior to this technology, the most widely used technique for quantitating DPD was the *V. harveyi* luminescence assay (*3*). However, this bioassay suffers from a small linear range (*12*) and is prone to interference from other molecules, such as glucose (*13*).

At a phenotypic level, a quorum sensing signal is one that allows cells to differentiate between low and high cell density (*1*). This could be achieved in several ways. For instance, the bacteria could produce little quorum sensing activity at low cell density and then turn on high-density behaviors like a switch after a burst of quorum sensing activity, or they could gradually turn on the response in a graded manner (*6*). We sought to provide a quantitative definition of a quorum sensing signal and hypothesized that if the primary information content is cell density, then the amount of extracellular signal must be held constant to the number of cells in the medium. Maintaining this relationship would allow information concerning cell density to be transmitted without the need to deconvolute conflicting metabolic or other cues from the signal. This leads to the equation:

$$\text{signal concentration}/\text{cell density} = \text{constant}$$

To test this hypothesis, DPD concentrations in supernatants of *E. coli* BW25113 (*14*) and DPD and HAI-1 concentrations present in the supernatants of *V. harveyi* BB120 (*3*) were measured as the cultures progressed through the exponential to the stationary growth phase. Each species was grown in appropriate media with one of four added glucose concentrations, 0.0, 0.08, 0.14, or 0.20% (w/v). With increased nutritional input and metabolic activity, the maximum DPD concentrations in the *E. coli* supernatants increased as more glucose was added to the

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*To whom correspondence should be addressed. Telephone: (865) 974-7337. Fax: (865) 974-9332. E-mail: campagna@ion.chem.utk.edu.

[1]Abbreviations: AI-2, autoinducer-2; DPD, (*S*)-4,5-dihydroxy-2,3-pentanedione; HAI-1, *V. harveyi* autoinducer-1; LC−MS/MS, liquid chromatography−tandem mass spectrometry; LuxS, (*S*)-ribosylhomocysteinase; OD_{600}, optical density measured at 600 nm.

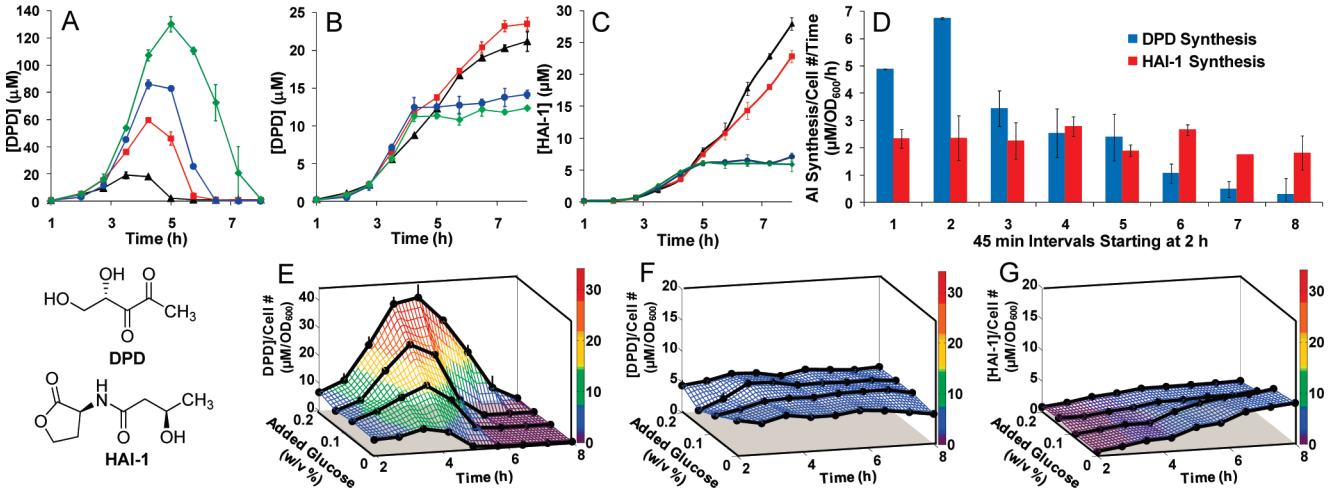

FIGURE 1: Production of DPD and HAI-1, calculated autoinducer synthesis rates, and landscapes showing the signal concentration/cell density ratios with varying glucose concentrations. Data for each culture grown with 0.00 (black triangles), 0.08 (red squares), 0.14 (blue circles), and 0.20% (w/v) (green diamonds) added glucose. (A and B) Measured DPD concentrations ([DPD]) (μM) in the supernatants of *E. coli* and *V. harveyi*, respectively. (C) Measured HAI-1 concentrations ([HAI-1]) (μM) in the supernatants of *V. harveyi*. (D) Graph of the rate of autoinducer appearance (micromolar per OD_{600} per hour) over 45 min intervals. (E) *E. coli* is unable to regulate the DPD concentration/cell density ratio in either the nutrient or the temporal dimension. (F) Conversely, *V. harveyi* maintains this ratio in both dimensions for DPD. (G) However, the HAI-1 concentration/cell density ratio increases as the culture grows. All measurements were performed in duplicate, and the error bars express the range of the data. The 1 h time points were not used in panels E, F, and G as the measured OD_{600} was out of the linear range.

media, and the maximum DPD concentrations ranged from 19 ± 2.4 μM with no added glucose to 130 ± 5.6 μM when 0.20% (w/v) glucose was added. For *V. harveyi*, the maximum DPD and HAI-1 concentrations were 24 ± 0.9 and 27.9 ± 1.0 μM, respectively, at lower glucose concentrations [0 and 0.08% (w/v)], and DPD and HAI-1 concentrations were 14 ± 0.5 and 7.1 ± 1.1 μM, respectively, at higher glucose concentrations [0.14 and 0.20% (w/v)] (Figure 1B,C). Neither *E. coli* nor *V. harveyi* exhibited significantly increased cell density with the increased availability of nutrients, and the growth kinetics for both species were nearly unchanged during the exponential growth phase at all glucose concentrations. However, growth of *V. harveyi* during the stationary phase was slower in the cultures containing 0.14 and 0.20% (w/v) added glucose, although the cells were still viable upon plating (Figure S2A−C of the Supporting Information). The glucose concentration in each culture was monitored via a colorimetric glucose oxidase assay (*15*) to determine how quickly the bacteria were using this nutrient and to ensure that the lack of increased growth was not due to an inability to metabolize the sugar. All *E. coli* cultures depleted the glucose in the media at ~3.5 h, corresponding to entry into the stationary phase, and the *V. harveyi* cultures were able to take up all of the glucose by the onset of the stationary phase at ~4.25 h, except cultures grown with 0.20% (w/v) added glucose in which the sugar persisted into the stationary phase (Figure S2D,E of the Supporting Information).

With these data in hand, our quantitative definition of quorum sensing was tested in these two species. After the DPD concentration/cell density ratio had been calculated for the *E. coli* cultures, it was apparent that AI-2 was not simply a quorum sensing signal in this bacterium. It is known that *E. coli* imports AI-2 at early to midstationary phase and that this behavior can be suppressed by the addition of glucose (*1*, *7*). However, previous work did not eliminate the possibility that AI-2 is a quorum sensing signal during the exponential phase for this species. The observation of large variations (4.8 ± 0.5 to 39 ± 4.8 μM/OD_{600}) in the DPD concentration/cell density ratio indicates that AI-2 cannot transmit cell density information in *E. coli* without the need to filter metabolic noise (Figure 1E).

For AI-2 in *V. harveyi*, the opposite was observed, and the DPD concentration/cell density ratio was nearly constant under all nutrient conditions (Figure 1F). The average ratio was 5.5 ± 2.9 μM/OD_{600} if all points displayed in Figure 1F are used in the calculation. If only the time point for 0.14% (w/v) added glucose at 2 h is removed from this analysis, the error is considerably smaller, giving a value of 5.5 ± 1.8 μM/OD_{600}. These data quantitatively show that *V. harveyi* can maintain the cell density information content of the AI-2 signal from the exponential to midstationary phase even if nutrient availability fluctuates. However, the HAI-1 concentration/cell density ratio was not constant and increased from 0.7 ± 0.2 to 6.6 ± 0.4 μM/OD_{600} as the cultures grew (Figure 1G); however, HAI-1 concentrations did not differ greatly between cultures with varying glucose concentrations during the exponential phase. We believe this indicates that the information content of HAI-1 is not cell density, but rather metabolic in nature. These hypotheses are strengthened by the observation that *V. harveyi* lowers the per cell DPD synthesis rate from a maximum of 6.8 ± 0.1 to 0.03 ± 0.6 μM OD_{600}^{-1} h^{-1} as the culture grows, as would be needed to maintain the DPD concentration/cell density ratio in the absence of degradation, while the per cell HAI-1 synthesis rate was nearly constant at 2.2 ± 0.4 μM OD_{600}^{-1} h^{-1} for healthy cultures (Figure 1D).

With regard to the aspects of signal production and integration, neither of these signals seems to be produced in a switchlike manner, and these data support previous work (*6*, *16*) indicating that signal reception and gene regulation in *V. harveyi* are able to give a graded response. Further, these data show that the appearance of HAI-1 lags behind that of AI-2 by ~45 min, although both are present at cell densities below which high-density behaviors are usually observed (*17*), and the ratio of these two signals may allow *V. harveyi* to determine both cell density and growth phase.

After observing that *E. coli* and *V. harveyi* exhibited differences in AI-2 production, we used stable isotope flux profiling to probe the mechanism of signal production in both. Such experiments provide information about the rate and pathways used for biosynthesis and allow newly synthesized metabolites to be

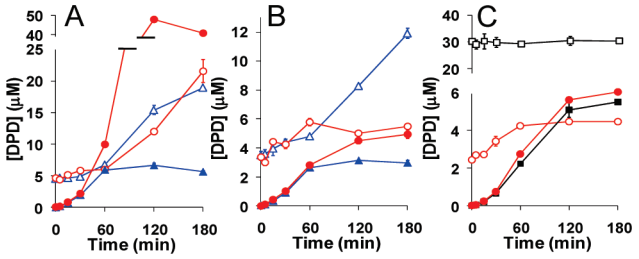

FIGURE 2: Flux profiling of DPD biosynthesis. Empty and filled symbols indicate DPD and ($^{13}C_5$)DPD concentrations, respectively. Data for cultures grown with 0.04 (blue triangles) or 0.14% (w/v) (red circles) ($^{13}C_6$)glucose or 0.14% (w/v) ($^{13}C_6$)glucose with 28 μM DPD (black squares). (A and B) DPD biosynthesis monitored at differing ($^{13}C_6$)glucose concentrations for *E. coli* and *V. harveyi*, respectively. (C) DPD biosynthesis monitored at differing exogenous DPD concentrations in *V. harveyi*. Data were collected in duplicate, and the error bars express the range of the data.

differentiated from old molecules (*18*). For these experiments, fully ^{13}C-labeled glucose (($^{13}C_6$)glucose) was added to *E. coli* or *V. harveyi* cultures in rich media after unlabeled glucose was exhausted (2.5 or 3.0 h, respectively), and the incorporation of ^{13}C into DPD was monitored for 180 min via LC−MS/MS. DPD isotopomers with two, three, or five (($^{13}C_5$)DPD) ^{13}C labels are most likely (*19*), and ($^{13}C_5$)DPD was observed to account for the majority of the labeled material in all experiments (Figures S3 and S4 of the Supporting Information).

For *E. coli*, the *lsrB*$^-$ mutant strain (JW1509-1) was used to probe the synthesis of DPD in the absence of degradation. To determine whether the higher DPD concentration observed with an increased glucose concentration was wholly due to repression of DPD degradation for this species, DPD flux profiles for cultures spiked with 0.04 and 0.14% (w/v) ($^{13}C_6$)glucose were obtained (Figure 2A). The maximal ($^{13}C_5$)DPD concentrations were 6.6 ± 0.4 and 48.1 ± 0.3 μM in the cultures with 0.04 and 0.14% (w/v) ($^{13}C_6$)glucose, respectively. Further, the production of unlabeled material continued at nearly identical rates in both cultures. These data indicate that the higher DPD concentrations observed in Figure 1A are partially from upregulated biosynthesis.

For *V. harveyi*, we sought to determine whether the extracellular DPD concentration was controlled by biosynthesis or by degrading excess signal in the medium. During flux profiling, ^{13}C-labeled DPD was detected within 15 min of addition of ($^{13}C_6$)glucose, and ^{13}C-labeled DPD was made preferentially to unlabeled DPD as long as ($^{13}C_6$)glucose was available [30 and 60 min for 0.04 and 0.14% (w/v) ($^{13}C_6$)glucose, respectively] (Figure 2B and Figure S5 of the Supporting Information). Further, the sums of the DPD concentrations for all isotopomers from cultures with ($^{13}C_6$)glucose were nearly identical to the DPD concentration observed in previous experiments over the corresponding glucose concentrations and times (Figure S5B), indicating that *V. harveyi* regulates DPD synthesis. Also, the persistence of the initial unlabeled DPD shows that the rate of degradation is negligible.

We also sought to determine whether the addition of exogenous DPD would lead to a misregulation of AI-2 signaling. This could occur via either a change in the DPD synthesis rate or triggering of DPD importation as the mechanism to correct the external DPD concentration/cell density ratio after recognition of the exogenous signal, and the ability to differentiate new versus old DPD via labeling allowed us to probe whether *V. harveyi* does either. By adding 0.14% (w/v) ($^{13}C_6$)glucose either with or without 27.7 ± 1.0 μM synthetic DPD (*20*) at 3 h and then monitoring ($^{13}C_5$)DPD production for 180 min, we found it was clear that *V. harveyi* does not alter DPD synthesis rates in response to an exogenous signal (Figure 2C). Again, the persistence of unlabeled DPD rules out significant degradation. In conclusion, we show that a quantitative investigation of quorum sensing signal production and biosynthesis can provide insight into the true utilization and information content of these molecules. In *E. coli*, the production of DPD is altered by nutritional availability making signaling prone to metabolic noise. In contrast, *V. harveyi* can regulate the DPD production to maintain the fidelity of the cell density-related information content of this molecule. However, HAI-1 is produced at a constant rate and may be used to signal metabolic fitness instead of cell density.

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SUPPORTING INFORMATION AVAILABLE

Detailed methods, tabulated data, and further discussion. This material is available free of charge via the Internet at http://pubs.acs.org.

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