A *Vibrio cholerae* autoinducer–receptor pair that controls biofilm formation

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**Quorum sensing (QS)** is a cell–cell communication process that enables bacteria to track cell population density and orchestrate collective behaviors. QS relies on the production and detection of, and the response to, extracellular signal molecules called autoinducers. In *Vibrio cholerae*, multiple QS circuits control pathogenesis and biofilm formation. Here, we identify and characterize a new QS autoinducer–receptor pair. The autoinducer is 3,5-dimethylpyrazin-2-ol (DPO). DPO is made from threonine and alanine, and its synthesis depends on threonine dehydrogenase (Tdh). DPO binds to and activates a transcription factor, VqmA. The VqmA–DPO complex activates expression of *vqmR*, which encodes a small regulatory RNA. VqmR represses genes required for biofilm formation and toxin production. We propose that DPO allows *V. cholerae* to regulate collective behaviors to, among other possible roles, diversify its QS output during colonization of the human host.

Quorum sensing (QS) is a cell–cell communication process that allows bacteria to synchronize behavior in response to changes in cell population density and local species composition1. QS involves the production, release, and subsequent detection of extracellular signal molecules called autoinducers, which accumulate with increasing cell density. Once a threshold autoinducer concentration is achieved, global changes in gene expression are elicited. Processes controlled by QS are ones that require many cells acting in concert to be effective; for example, biofilm formation, and the exchange of DNA. Thus, QS is a mechanism that allows bacteria to function like multicellular organisms.

In the major human pathogen *Vibrio cholerae*, multiple QS pathways converge to control virulence and biofilm formation2–4. The two identified *V. cholerae* autoinducers are CAI-1 ((S)-3-hydroxytridecan-4-one), produced by the CqsA enzyme, and AI-2 (4,5-dihydroxy-2,3-pentanedione), synthesized by LuxS2,6,7. CqsA is conserved in all *Vibrio* species, supporting a role for CAI-1 in intragenus communication8. By contrast, homologs of luxS exist in hundreds of bacterial species, indicating a role for AI-2 in interspecies communication9–11. Using two autoinducers presumably allows *V. cholerae* to track the number of other *Vibrios* and the total number of bacteria in the vicinity. We note that not all AI-2-producing bacterial species encode an identifiable AI-2 receptor, so in some cases it is not clear how AI-2 is detected. In *V. cholerae*, CAI-1 and AI-2 are detected by the membrane-bound receptors CqsS and LuxPQ, respectively4. At low cell density, when autoinducers are scarce, CqsS and LuxPQ function as kinases, channeling phosphate to the phosphotransfer protein LuxU, which passes it to the response regulator LuxO12,13. Phosphorylated LuxO, in concert with the alternative sigma factor σ54, activates the expression of genes encoding four homologous regulatory small RNAs (sRNAs) called Qrr1–4 (refs. 14,15). The Qrr sRNAs activate translation of the low-cell-density master regulator AphA14 and destabilize the mRNA that encodes the master high-cell-density regulator HapR14,17. Thus, at low cell density, AphA is produced whereas HapR is not. AphA activates expression of genes required for biofilm formation and for pathogenicity. As *V. cholerae* cells grow to high cell density, autoinducers accumulate. Binding of the autoinducer to the receptors converts them from kinases to phosphatases, resulting in dephosphorylation and inactivation of LuxO, and cessation of *qrr* expression. This event terminates Qrr activation of *aphA* translation. *hapR* mRNA is stabilized, leading to HapR production. Thus, at high cell density, HapR is made and AphA is not14. HapR controls a large regulon, including genes underpinning collective behaviors18. Importantly for the present work, HapR represses *vpsT*, which encodes a transcriptional activator that is crucial for biofilm formation19,20. Simultaneously, HapR activates genes encoding proteases that enable *V. cholerae* to disperse from the host to return to the environment21.

New studies suggest that additional *V. cholerae* QS systems exist1,22. Indeed, our recent global transcriptome analyses revealed a *V. cholerae* QS system that depends on the transcription factor VqmA, a so-called ‘LuxR-solo’2,4,22. These orphan LuxR-type proteins are thought to play roles in QS by binding self-produced autoinducers that remain unidentified or heterologous autoinducers made by other organisms in the environment. In the latter case, LuxR-solos could expand the range of chemicals to which a bacterium can ‘tune in’ and use in QS context taking23–28. At high cell density, VqmA activates expression of *vqmR*, which encodes the VqmR sRNA. In concert with the chaperone Hfq, VqmR post transcriptionally controls target genes, including *vpsT* and *rts*, a key regulator of biofilm formation and a major *V. cholerae* toxin, respectively24,25. VqmA has recently been implicated in the control of *V. cholerae* pathogenicity via modulation of interactions with particular members of the human microbiota25. No cognate ligand for VqmA has been identified.

Here, we discover that VqmA is activated by an extracellular small molecule that accumulates at high cell density, i.e., an autoinducer. The autoinducer controls VqmA activity—not VqmA levels—and, furthermore, acts directly by binding VqmA. Isolation of VqmA bound to the ligand enabled us to capture, purify, and identify the autoinducer as 3,5-dimethylpyrazin-2-ol (DPO; 1); a new type of autoinducer and a new molecule to biology. We show that DPO is produced from threonine and alanine and requires the ubiquitous threonine dehydrogenase (Tdh) enzyme for its synthesis.

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VqmA, in complex with DPO, activates expression of the vqmR gene encoding the VqmR sRNA, which represses genes required for V. cholerae biofilm formation. We find that this new QS autoinducer–receptor pathway operates in parallel to the canonical V. cholerae QS pathways. Notably, the canonical QS circuits and the DPO–VqmA circuit control some of the same genes. This network arrangement provides V. cholerae with a mechanism to, when necessary, uncouple expression of a particular subset of QS-regulated genes from expression of the remainder of the QS-controlled regulon.

**RESULTS**

**VqmA is activated by an extracellular factor**

We recently discovered the VqmR sRNA and showed that expression of vqmR is activated by the transcription factor VqmA. We demonstrated that VqmA and VqmR repress biofilm formation in V. cholerae	extsuperscript{35}. To characterize the mechanism of VqmA–controlled activation of vqmR, we analyzed the production of both factors throughout bacterial growth using western and northern blotting analyses, respectively (Fig. 1a). To detect VqmA, we used a C-terminal 3xFLAG fusion expressed from the native vqmR chromosomal locus. VqmA levels were constant at all stages of growth. By contrast, and consistent with our previous data	extsuperscript{34}, minimal VqmR production occurred at low cell densities, and VqmR production increased with cell growth. These results indicate that VqmA activity, not VqmA levels, must change in the course of growth (Fig. 1a), which in turn promotes cell-density-dependent changes in VqmR production.

VqmA harbors a conserved PAS domain (Per–ARNT–Sim	extsuperscript{37,39}) at its N terminus and a DNA-binding domain at the C terminus	extsuperscript{40}. PAS domains are ubiquitous sensors of intra- and extracellular ligands and are frequently involved in signal transduction processes in prokaryotes and eukaryotes	extsuperscript{41}. The presence of a PAS domain, coupled with our finding of increased VqmA activity at high cell density (Fig. 1a), inspired us to investigate the possibility that an extracellular molecule functions together with VqmA to activate VqmR production. To test this idea, we added cell-free spent culture fluids prepared from high-cell-density V. cholerae cells and assessed VqmR production. Indeed, addition of 25% (final concentration) conditioned medium caused VqmR production to increase ~3-fold (Fig. 1b; compare the first and second lanes). To exclude the possibility that the known V. cholerae autoinducers CAI-1 and AI-2 were responsible, we likewise tested culture fluids from ΔcqsA (i.e., CAI-1–, ΔluxS (i.e., AI-2–), and ΔcqsA ΔluxS (i.e., CAI-1– and AI-2–) strains for VqmR induction. In all three cases, VqmR levels increased exactly as they did following supplementation with wild-type culture fluids (Fig. 1b).

We also examined culture fluids from a V. cholerae ΔhapR mutant, which is completely deficient in all known QS capabilities. Full induction of VqmR production occurred following administration of the ΔhapR fluids (Fig. 1b). Thus, VqmA requires an extracellular factor to induce vqmR expression, and neither CAI-1, AI-2, nor any function controlled by the known QS cascade is involved.

**Genome-wide screen for factors affecting vqmR expression**

To discover the extracellular factor impinging on the VqmA–R circuit, we fused the vqmR promoter to the mKate2 gene	extsuperscript{31} and integrated this construct onto the V. cholerae chromosome at the lacZ locus. We randomly mutagenized this strain using Tn5. We arrayed ~40,000 mutant colonies in 96-well plates containing selective growth medium and screened them for mKate2 production following 12 h of incubation. Our rationale for separating colonies was to avoid cross-feeding from diffusion of the putative autoinducer between mutant colonies. We identified two transposon insertion mutants that displayed ~4-fold reduced fluorescence compared to the wild type. Both insertions resided in the tdh (vca0885) gene (Fig. 2a).

To verify this result, we generated an in-frame deletion of tdh in V. cholerae carrying the chromosomal PvqmR::mKate2 reporter.

The Δtdh mutant also produced ~4-fold less mKate2 than the parent (Fig. 2b; compare bars 1 and 2). Introduction of a plasmid carrying tdh fully rescued the mutant phenotype, whereas introduction of the tdh-containing plasmid into the Δtdh–wild-type strain had no effect on PvqmR::mKate2 expression (Fig. 2b).

tdh encodes Tdh, which is ubiquitous among bacteria, archaea, and eukaryota	extsuperscript{42}. Tdh converts l-threonine to 2-amino-3-ketobutyric acid (AKB), which then undergoes one of two fates: either spontaneously decomposes to aminooxyacetic acid and CO₂ or is acted upon by the enzyme Kbl to generate glycine and acetyl-CoA	extsuperscript{43}. Consistent with the predicted function of Tdh, MS analysis showed that purified V. cholerae Tdh protein produces aminoacetone (Supplementary Results, Supplementary Fig. 1). Previous studies of Tdh in Escherichia coli revealed two critical amino acid residues required for catalytic activity: Cys38 and His90 (ref. 34). To test if Tdh catalytic activity is necessary for production of the molecule required for VqmR-directed activation of PvqmR::mKate2 expression, we mutated the corresponding residues in the V. cholerae Tdh enzyme to alanine (C40A) and arginine (H92R), respectively. In both cases, the plasmids expressing the mutant tdh alleles failed to complement mKate2 production in the Δtdh strain (Fig. 2b). Production of the Tdh C40A variant had no effect in wild-type cells (Fig. 2b), whereas Tdh H92R caused a ~2-fold reduction in PvqmR::mKate2 production, indicating potential interference with endogenously produced wild-type Tdh (Fig. 2b). Tdh is a tetramer in E. coli	extsuperscript{44}, so one possible explanation for this interference is formation of partially functional or inactive heterotetramers composed of wild-type and Tdh H92R protomers. tdh is the second gene in the kbl–tdh operon	extsuperscript{23,26}. Consistent with the results from our in-frame Δtdh mutant analysis, deletion of kbl from wild-type V. cholerae did not alter VqmR production (Supplementary Fig. 2a), and transformation of the Δtdh mutant with a plasmid expressing kbl did not restore PvqmR::mKate2 expression (Fig. 2b). Overproduction of kbl in wild-type V. cholerae modestly repressed PvqmR::mKate2 expression (Fig. 2b), suggesting that increased Kbl activity drains a substrate required to produce the molecule necessary for VqmA activation.

**Threonine dehydrogenase is required for VqmA activation**

We considered three possible mechanisms that could account for reduced VqmR production in the V. cholerae Δtdh mutant: first, that Tdh is required for VqmA production; second, that Tdh is required to detect the extracellular autoinducer that stimulates VqmA activity; and third, that Tdh is required for production of the extracellular autoinducer that activates VqmA. To test the first possibility, we
compared VqmA and VqmR production in the wild-type to that in the Δtdh strain. While reduced VqmR production occurred in the Δtdh mutant, VqmA levels remained constant (Fig. 3a). These data indicate that Tdh does not influence VqmR production, eliminating the first possibility.

To determine whether Δtdh cells are unable to detect or unable to produce the autoinducer that stimulates VqmR, we grew the Δtdh V. cholerae strain with cell-free spent culture fluids prepared from wild-type and from Δtdh cells. Subsequently, we measured VqmR production by northern blotting analysis. We predicted that if Tdh was required for autoinducer production, Δtdh mutants would be complemented for VqmR production by cell-free culture fluids. By contrast, if Tdh was required for detection of the autoinducer, no complementation would occur. Figure 3b shows that conditioned medium from wild-type cells induced VqmR production in the Δtdh mutant, whereas Δtdh-derived culture fluids failed to induce VqmR production under the same conditions. We also added cell-free spent culture fluids from each of the 3,156 mutants in an ordered V. cholerae library7 to the Δtdh strain and scored PqvmR::mkate2 expression. In every case except that with culture fluids from the tdh::Tn5 mutant, mKate2 production was induced (Supplementary Fig. 2b). The tdh::Tn5 mutant culture fluids caused ~3-fold less fluorescence compared to wild-type culture fluids. Together, these results demonstrate that Tdh is required to produce, but not detect, the VqmA-activating autoinducer. As noted, the tdh gene is highly conserved, and, indeed, conditioned medium from E. coli was also capable of inducing VqmR expression in V. cholerae to a level comparable to that from fluids prepared from V. cholerae. Moreover, E. coli cells lacking tdh failed to activate expression of vqmR (Fig. 3b). This result suggests that Tdh synthesizes an autoinducer that is released by V. cholerae and E. coli and, likely, other prokaryotes.

**Threonine catabolism permits autoinducer production**

In enteric bacteria, threonine catabolism provides one route to glycine and serine biosynthesis. Threonine can also be used to synthesize isoceuline via 2-oxobutanoate34. The connections between these amino acid biosynthetic pathways prompted us to investigate which amino acids are capable of yielding the autoinducer that activates VqmA-dependent vqmR transcription. We grew wild-type V. cholerae in minimal medium supplemented with each of the 20 amino acids and measured PqvmR::mkate2 activity. Only l-threonine induced mKate2 production (~6-fold compared to medium lacking amino acids). All other amino acids either had no effect or reduced reporter activity (isoceuline, ly sine, leucine, histidine, methionine, and arginine) (Fig. 3c). Therefore, the only relevant pathway for autoinducer production is through threonine and Tdh, and autoinducer synthesis is independent of other downstream reactions mediated by enzymes such as Kbl (see Supplementary Fig. 2a).

To investigate the effect of threonine on VqmR activity, we performed chromatin co-immunoprecipitation using VqmA::3×FLAG protein with V. cholerae grown in the presence or absence of threonine. While threonine did not change VqmA production (Supplementary Fig. 3a), quantitative real-time PCR experiments showed that ~5 times more VqmA occupied the vqmR promoter when l-threonine was added to the medium than when the medium lacked threonine (Fig. 3d). Comparison of these results to a control V. cholerae strain containing VqmA but lacking the FLAG epitope furthermore showed that, in the absence of threonine, little to no VqmA is present at the vqmR promoter. This result suggests that not only is the threonine-derived autoinducer required for VqmA activity, it is also required to promote protein–DNA interaction.
To examine whether l-threonine is the VqmA autoinducer, we studied the kinetics of VqmR induction (Supplementary Fig. 3b). Specifically, we grew Δtdh V. cholerae in minimal medium to early stationary phase (optical density at 600 nm (OD600) = 1.0) and divided the culture into three aliquots. The first aliquot served as a control, supplemented with fresh minimal medium. To the second aliquot, we added cell-free spent culture fluids from wild-type V. cholerae grown with supplemental L-threonine (0.4 mM final concentration). The third aliquot was supplemented with fresh minimal medium containing excess L-threonine (0.4 mM final concentration). Total RNA samples were collected 1, 3, 5, and 10 min after treatment and assessed for VqmR production by northern blot analysis. Each experiment was performed in duplicate.

Figure 4 | Identification and proposed biosynthesis of DPO. (a) The V. cholerae Δtdh mutant was grown in M9 medium to an OD600 of 1.0 and treated with 0.25% or 25% (final concentration) of wild-type V. cholerae cell-free culture fluids. Alternatively, the cells were treated with the ‘molecule’ released from the 6×HIS::VqmA protein (0.025%, 0.05%, and 0.25% final concentration). Ten minutes after addition of each preparation, VqmR levels were assessed by northern blot. Pretreatment, collected before treatment; mock, no addition. SS rRNA served as loading control. Source files with blots showing the full data can be found in Supplementary Figure 8c. (b) HPLC–MS analysis of compounds released from purified VqmA (red trace) and purified LuxO control (blue trace). Shown are elution profiles monitored at 280 nm, with the peak observed in the VqmA sample corresponding to DPO (bottom). The H and 13C chemical shifts (top) and the numbering scheme (bottom) for DPO are shown. Our 1H-NMR data are consistent with the enol, rather than the ketone tautomeric form of DPO. (c) Mass spectra of DPO purified from wild-type V. cholerae grown with supplemental l-Thr, uniformly labeled [12C6]-Thr, l-Ala, and uniformly labeled [13C3]-l-Ala. The presence of a low level of [13C6]DPO following supplementation with [13C3]-l-Ala indicates that residual [13C3]Ala, generated from the carbon-source glucose (via pyruvate), is incorporated into DPO. Representative results from two similar experiments are shown. (d) Proposed biosynthesis of DPO. Tdh catalyzes the oxidation of Thr to 2-amino-3-ketobutyric acid (AKB; i), which spontaneously decarboxylates to aminooacetone (ii). Aila, activated by an unidentified enzyme (R = phosphoryl, adenyllyl, or CoA group; iii), is condensed with aminooacetone (iv), followed by intramolecular cyclization (v), dehydration (vi), spontaneous tautomeration (taut.) and oxidation (ox.), to yield DPO. We name DPO compound 1 and N-alanyl-aminooacetone compound 2 in the online chemical compound information file.
purified the 6×HIS::VqmA protein from *E. coli* (Supplementary Fig. 4b) and released the bound autoinducer through thermal denaturation. We tested the isolated autoinducer for induction of VqmR production and found that we could enrich the specific activity by ~100- to 1,000-fold (Fig. 4a and Supplementary Fig. 4c). To confirm that our isolated compound was specific for VqmA, we carried out the identical procedure with 6×HIS::LuxO. Preparations from thermally denatured 6×HIS::LuxO were incapable of 
PvqmR::mkate2 activation (Supplementary Fig. 4c) (Fig. 4b). Furthermore, unlike the wild type, a *V. cholerae* Δtdh mutant failed to produce this compound (Supplementary Fig. 4d). 1D and 2D NMR analyses allowed us to elucidate the structure of the autoinducer (Fig. 4c).

We subjected the molecules released from 6×HIS::VqmA and 6×HIS::LuxO (control) proteins to differential high-performance liquid chromatography–quadrupole time-of-flight–mass spectrometry (HPLC–Qtof–MS) analysis to characterize the active autoinducer. Absorbance at 280 nm showed a single peak in the VqmA sample, with an [M+H]+ ion mass of 125.0776, and this peak was absent from the LuxO control (Fig. 4b). Furthermore, unlike the wild type, a *V. cholerae* Δtdh mutant failed to produce this compound (Supplementary Fig. 4d). 1D and 2D NMR analyses allowed us to elucidate the structure of the autoinducer (Fig. 4c).

The 1H NMR data showed three distinct peaks, corresponding to two methyl groups and an aromatic proton (Supplementary Fig. 5a–d; Supplementary Table 1). HSQC and HMBC data identified three quaternary carbons arranged in a pyrazine scaffold, consistent with high-resolution mass spectral (HR–MS) data, which suggested the presence of two nitrogen atoms (C₇H₅N₃O; [M+H]+ calc. = 125.0715; Δp.p.m. = 6.4). The combination of UV, HR–MS, and NMR data allowed us to propose that the active component is 3,5-dimethylpyrazin-2-ol (DPO; Fig. 4c). The NMR spectra of commercially available DPO were identical to those of our isolated compound, thus confirming our structural assignment (Supplementary Fig. 5e–h; Supplementary Table 2).

**Biosynthesis of DPO**

DPO is a new bacterial natural product and thus has no known biosynthetic pathway. To gain insight into its production, we carried out isolate feeding experiments. Inclusion of uniformly ¹³C-labeled l-Thr ([¹³C]₅-l-Thr) resulted in a 3-Da shift in DPO mass, suggesting that, upon reaction with Tdh, the AKB product is decaxylation to aminoacetone, which subsequently serves as the substrate for DPO biosynthesis (Fig. 4d). Taking this into account, a retrobiosynthetic analysis of DPO suggested that alanine could serve as the other DPO precursor (Supplementary Fig. 3). ¹H NMR data showed three distinct peaks, corresponding to two methyl groups and an aromatic proton (Supplementary Fig. 5a–d; Supplementary Table 1). HSQC and HMBC data identified three quaternary carbons arranged in a pyrazine scaffold, consistent with high-resolution mass spectral (HR–MS) data, which suggested the presence of two nitrogen atoms (C₇H₅N₃O; [M+H]+ calc. = 125.0715; Δp.p.m. = 6.4). The combination of UV, HR–MS, and NMR data allowed us to propose that the active component is 3,5-dimethylpyrazin-2-ol (DPO; Fig. 4c). The NMR spectra of commercially available DPO were identical to those of our isolated compound, thus confirming our structural assignment (Supplementary Fig. 5e–h; Supplementary Table 2).

**DPO inhibits V. cholerae biofilm formation**

To verify that DPO is the autoinducer that activates VqmA, we added increasing amounts of synthetic DPO to the Δtdh *V. cholerae* strain carrying the PvqmR::mkate2 reporter (Supplementary Fig. 6a). High levels of DPO (1 mM) triggered strong mKate2 production, substantially above that produced by wild-type cells. One-thousand-fold less synthetic DPO (1 μM) promoted mKate2 production...
to the levels produced by wild-type \textit{V. cholerae} responding to endogenously produced DPO. Finally, treatment with 60 nM synthetic DPO elicited a doubling in \textit{PvqmR::mkate2} fluorescence. Comparison of the mKate2 production elicited by cell-free culture fluids from \textit{V. cholerae} wild-type cells with known amounts of synthetic DPO suggests that DPO accumulates to ~1 μM by stationary phase (Supplementary Fig. 6b). This concentration in line with the concentrations of CAI-1 and AI-2, estimated to be ~0.3 μM and ~2 μM, respectively.

The time-course analysis of VqmA activation shown in Supplementary Figure 3b indicated that cell-free culture fluids elicit rapid VqmR induction. We tested whether synthetic DPO could produce similar response dynamics. Indeed, the \textit{V. cholerae} Δtdh strain responded to DPO (100 μM final concentration) as rapidly as it responded to cell-free culture fluids (Fig. 5a). In both cases, a substantial increase in VqmR could be detected as early as 2 min after administration, supporting our arguments that DPO is the authentic autoinducer that activates VqmA.

To explore the physiological consequences of DPO signal transduction, we focused on biofilms. Our previous work showed that one target of VqmR is \textit{vpsT}\(^2\), a positive regulator of genes required for biofilm formation. VpsT, upon binding the second messenger molecule cyclic-di-GMP, activates expression of multiple genes, including \textit{vpsL}\(^2\), the first gene in the \textit{vpsI} operon that directs the synthesis of polysaccharides, which are a major component of the \textit{V. cholerae} biofilm matrix. Disruption of either \textit{vpsT} or \textit{vpsL} dramatically impairs \textit{V. cholerae} biofilm formation\(^{3-5}\). Exogenous DPO repressed expression of a \textit{vpsL–lux} reporter fusion (Fig. 5b), and DPO also inhibited \textit{V. cholerae} biofilm formation (Fig. 5c). Notably, we also show that exogenous DPO activates expression of \textit{PvqmR::mkate2} in cells inhibited for biofilm formation confirming that the pathway is activated (Fig. 5c). DPO repression is VqmA dependent and HapR independent (Fig. 5b). Thus, DPO and the canonical QS autoinducers signal through parallel pathways to control \textit{V. cholerae} biofilm formation.

**DISCUSSION**

In this study, we identify DPO, a new type of QS autoinducer and a molecule new to biology. In \textit{V. cholerae}, DPO is detected by the cytoplasmic transcription factor VqmA, which upon binding DPO activates expression of \textit{vqmR}, which encodes the VqmR regulatory RNA that controls a downstream regulon of 17 genes\(^4\). Importantly, VqmR represses \textit{vpsT}, which is required for biofilm formation. Our discovery of DPO and its VqmA-mediated link to VqmR allowed us to demonstrate that synthetic DPO inhibits biofilm formation in \textit{V. cholerae} (Fig. 5b,c).

DPO could also be relevant to the colonization of the small intestine by \textit{V. cholerae}. A previous study reported that the commensal bacterium \textit{Ruminococcus obeum} limits the severity of \textit{V. cholerae} infection by impairing colonization. The resistance conferred by \textit{R. obeum} was eliminated when animals were infected with a \textit{V. cholerae} \textit{vqmA} mutant\(^2\). We speculate that \textit{R. obeum}–mediated resistance to \textit{V. cholerae} colonization could be a consequence of high-level DPO production in the small intestine. Specifically, DPO could be made by \textit{V. cholerae} and/or commensal bacteria, activating VqmA. VqmA promotes transcription of \textit{vqmR}, and VqmR represses biofilm formation, causing \textit{V. cholerae} to disperse back into the environment. Because the \textit{V. cholerae} \textit{vqmA} mutant does not respond to DPO, it is not subject to DPO-mediated limitation. Using RNA sequencing (RNA-seq), the authors of the previous work reported that expression of \textit{V. cholerae} \textit{vqmA} was activated in the presence of \textit{R. obeum}\(^4\). By contrast, we find that VqmA levels remain constant during growth of \textit{V. cholerae}, whereas VqmR levels increase substantially (Figs. 1a and 3a). One possible explanation for this discrepancy stems from misannotation of \textit{vqmA} in several \textit{V. cholerae} genomes. Indeed, \textit{vqmR} and \textit{vqmA} are frequently annotated as a single gene. Thus, RNA-seq reads that mapped to the \textit{vqmR} locus would have been incorrectly attributed to the \textit{vqmA} gene\(^4\).

Two QS autoinducer–receptor pathways have previously been characterized in \textit{V. cholerae}. They involve the autoinducers CAI-1 (ref. 2) and AI-2 (ref. 6) and their cognate receptors CqsS and LuxPQ, respectively (Fig. 6). Detection of CAI-1 and AI-2 at high cell density results in production of HapR. HapR represses genes required for biofilm formation and virulence factor production\(^1\). DPO signaling could provide a mechanism to uncouple regulation of biofilm formation from regulation of virulence factor production in \textit{V. cholerae}. Specifically, when HapR is not active, detection of DPO via VqmA–R and subsequent repression of biofilm formation through post-transcriptional regulation of \textit{vpsT} could occur while virulence factors continue to be produced. This bypass mechanism could diversify \textit{V. cholerae} gene regulatory programs to optimize QS functions under particular conditions such as during host colonization.

CAI-1 is an α-hydroxyketone produced by the CqsA enzyme. AI-2 is a furanosyl borate diester synthesized by LuxS (Fig. 6). DPO, the new autoinducer identified here, is a pyrazine unrelated to CAI-1 and AI-2. Although pyrazines are common in nature, few biological roles have been ascribed to them\(^4\). Consistent with our findings, pyrazines are known to be efficiently produced by bacteria using L-threonine as the main carbon source\(^4\), suggesting that the Tdh pathway could be a general source for pyrazines in organisms beyond \textit{V. cholerae}. Indeed, TdhS are conserved among bacteria, archaea, and eukarya\(^2\), and cell-free culture fluids from \textit{E. coli} substantially induce \textit{vqmR} expression (Fig. 3b). Together, these findings indicate that DPO production, and possibly its role in cell–cell communication, could be relevant in other bacterial organisms including pathogens.

In contrast to the universality of Tdh, homologs of the VqmA receptor are limited to species in the \textit{Vibrio} genus. In all cases, VqmA proteins possess both PAS- and DNA-binding domains (Supplementary Fig. 7). PAS domains facilitate protein–protein and protein–small molecule interactions\(^6\). Three other QS receptors harbor PAS domains: TraR of \textit{Agrobacterium tumefaciens}, which binds the autoinducer N-3-oxooctanoyl-homoserine lactone\(^2\), \textit{Vibrio} LuxQ, which detects AI-2 via interaction with the periplasmic binding protein LuxP\(^3,4\), and RpfR from \textit{Xanthomonas campes-}

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**METHODS**

Methods, including statements of data availability and any associated accession codes and references, are available in the online version of the paper.

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ONLINE METHODS

Bacterial strains, plasmids and growth. Strains, plasmids, and oligonucleotides used in this study are listed in Supplementary Tables 4–6, respectively. V. cholerae and E. coli were grown aerobically in LB or M9 medium (0.4% glucose, final concentration) at 37 °C. Growth at 30 °C was used for biofilm assays. When indicated, M9 medium was supplemented with casamino acids (0.4% final concentration). Antibiotics were used at the following concentrations: 50 μg ml⁻¹ polymyxin B, 200 μg ml⁻¹ ampicillin, 100 μg ml⁻¹ kanamycin, 5,000 μg ml⁻¹ streptomycin, and 20 μg ml⁻¹ chloramphenicol.

Construction of Vibrio cholerae strains. A list of all strains used in this study is provided in Supplementary Table 4. V. cholerae C6706 was the wild-type strain. All V. cholerae mutants were generated using established cloning strategies²¹,²². Plasmids were introduced into V. cholerae by mating with the E. coli S17-1pir strain carrying the plasmid of interest on LB agar plates for 6–8 h. Subsequently, the mating mixtures of V. cholerae and E. coli were collected and streaked on LB agar plates containing the antibiotic that selects for the plasmid and polymyxin B to eliminate E. coli.

Plasmid construction. A complete list of all plasmids used in this study is shown in Supplementary Table 5. E. coli S17-1pir was used for cloning purposes. Restriction enzymes, T4 DNA ligase, and Antarctic phosphatase were purchased from New England Biolabs. cDNA polymerase (Bio-Rad) was used for all PCR reactions. For plasmids pKP-426 and pKP-427, the vca0885 and vca0886 genes were PCR-amplified using V. cholerae genomic DNA with oligonucleotide pairs KPO-866x878 and KPO-909x9201, respectively. The PCR products were ligated into plasmid pKP-418 (ref. 24) using KpnI restriction sites. Plasmid pKP-426 served as the template to generate plasmids pKP-429 and pKP-430 using oligonucleotides KPO-906x907 and KPO-910x911, respectively. To amplify the gene, the flanking regions of vca0885 were amplified with KPO-888x889 and KPO-890x891 and the fused PCR product was amplified using KPO-892x893. The PCR products were ligated into pKAS32 using AvrII/KpnI restriction sites. The plasmid was conjugated into V. cholerae, and exconjugants were selected with ampicillin. Single colonies were transferred to new plates with streptomycin. Lastly, cells were tested for possessing the correct mutation using PCR. For plasmid pKP-418, the 3×FLAG epitope was introduced downstream of vqmA (vca1078) by PCR amplification using oligonucleotides KPO-618x619 and KPO-620x621. Oligonucleotides KPO-642 and KPO-623 were used to amplify the fused PCR product, which was subsequently cloned into pKAS32. For plasmid pKP-483, gene synthesis was used to fuse the V. cholerae micX promoter to the superfolder gfp gene and the promoter of vqmR to the mKate2 (Evrogen) open reading frame. Both sequences were integrated into the lacZ locus of V. cholerae in opposite genomic orientations. For plasmids pKP-443 and pKP-445, the V. cholerae vca0885 and vqmR genes were PCR-amplified using oligonucleotides KPO-953x954 and KPO-935x972, respectively. Both fragments were ligated into pET15b (Novagen) using Ncol/BamHI and Ndel/BamHI restriction sites, respectively. For plasmid pLES24, full-length vqmR and an origin of transfer were PCR-amplified from pKP-363 (a vector harboring vqmR and the p15a origin of replication) using primer pair ISO-103/104 generating a KpnI site at the oriT end of the PCR product. The insert was subsequently digested with KpnI and ligated to pKP-337 (ref. 24) that was PCR amplified with ISO-109/110 and digested with KpnI.

Northern blot analysis. Total RNA was prepared and blotted as described. Membranes were hybridized in ULTRAhyb Ultrasensitive Hybridization Buffer (Thermo Fisher) at 42 °C with 32P-end-labeled DNA oligonucleotides. Signals were visualized using a Typhoon phosphorimager (GE healthcare) and band intensities were quantified using the GelQuant software (BiochemLabSolutions). Oligonucleotides for northern blot analyses are provided in Supplementary Table 6.

Western blot analysis. Culture samples were collected over growth and subjected to centrifugation at 16,100g for 2 min at 4 °C. Cell pellets were suspended in sample loading buffer to a final concentration of 0.01 OD/μl. Following denaturation for 5 min at 95 °C, 0.1 OD₅₅₀ equivalents were separated on SDS gels. Western blot analyses probing for FLAG fusion proteins followed previously published protocols. Signals were visualized on an ImageQuant LAS 4000 imager (GE healthcare), and band intensities were quantified using the GelQuant software (BiochemLabSolutions). The FLAG antibody is available at Sigma (F3165) and the antibody that recognizes the RAPIpX loading control is available at NeoClone (WP003).

A genetic screen for factors controlling PsvqmA::mkate2 expression. Wild-type V. cholerae carrying a chromosomal copy of the PsvqmA::mkate2 reporter (strain KPS-661) was mutagenized with Tns as described. Briefly, a library of ~100,000 mutants was assembled of which ~40,000 were transferred into 96-well plates containing LB medium supplemented with kanamycin using a colony-picking robot (Molecular Devices). The plates were incubated for ~12 h at 37 °C without shaking followed by sub-culturing into fresh 96-well plates. After an additional 8 h of growth, the cells in each well were tested for mKate2 production using a plate reader (PerkinElmer). mKate2 levels were corrected for optical density (OD₅₅₀) to obtain the final fluorescence levels.

Threonine dehydrogenase (Tdh) activity assay. Tdh activity assays were carried out using the previously reported Fmoc–chloride conjugation method. Reactions contained 10 μM Tdh, 1 mM l-Thr, 0.5 mM NAD in a final volume of 0.5 ml in elution buffer (50 mM HEPES, 50 mM KCl, 250 mM imidazole, 5 mM 2-mercaptoethanol). After 1 h of incubation at room temperature, reactions were quenched by addition of 0.25 ml of a 0.8 M K2HPO4 solution (pH 10.0), followed by vigorous vortex for 1 min. Reactions were worked up and analyzed by HPLC–MS as described.

Fluorescence and bioluminescence assays. V. cholerae reporter strains were grown overnight and back-diluted 1:1,000 in M9 medium supplemented with casamino acids. At OD₅₅₀ = 0.1, 150 μl of culture was transferred to the wells of 96-well microtiter plates. Subsequently, 50 μl of cell-free culture fluids or medium was added to the wells. Mineral oil (50 μl) was layered on top of each well to prevent evaporation and the plates were incubated overnight in a 37 °C plate reader (BioTek Synergy Mx). Fluorescence intensity from PsvqmA::mKate2 was measured every 15 min along with OD₅₅₀. PvaT::lux assays were performed using the identical protocol except at 30 °C. Bioluminescence, PsvqmA::mKate2 fluorescence, and OD₅₅₀ were measured at 15 min intervals.

VqmA::3×FLAG co-immunoprecipitation. The co-immunoprecipitation protocol is based on previously published methods. Overnight cultures of V. cholerae wild-type cells with and without the chromosomal VqmA::3×FLAG were diluted 1:1,000 and cultivated in M9 medium (with or without 0.4 mM l-threonine final concentration) until cells reached OD₅₅₀ = 1.0. Fifty OD₅₅₀ units of cells were crosslinked and washed as previously described and cells were lysed in 1 ml of lysis buffer (1× protease inhibitors, Sigma), 50 μg/ml lysozyme, 1× BugBuster, 1× Triton X-100, and 1 mM phenylmethylsulfonyl fluoride (PMSF)) for 20 min at room temperature on a rotator. Following lysis, the DNA was sheared by sonication to an average size of 100 to 1,000 bp. The supernatant was clarified at 13,000 r.p.m. for 10 min at 4 °C. Immunoprecipitation reaction mixtures contained a 200-μl aliquot of input solution, 800 μl of IP buffer (50 mM HEPES–KOH, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, and 1 mM PMSF), and 40 μl EZ-view anti-FLAG agarose beads (Sigma; equilibrated in Tris-buffered saline). Immunoprecipitations were carried out for 2 h at room temperature on a rotator. Subsequently, beads were collected and washed. Immunoprecipitated complexes were eluted, and crosslinks were reversed as described. Samples were analyzed by qRT-PCR using oligonucleotides specific to the vqmR promoter sequence. Identical analyses using oligonucleotides specific to the control hfg gene were used for normalization. Data analyses followed previously established protocols.

DPO purification from recombinant 6×His::VqmA in E. coli. The active molecule was produced and purified from a total of 7 l E. coli cultures and used in NMR and MS studies for DPO identification. Production (12 l scale): The pET15b vector carrying 6×His::VqmA was transformed into BL21–Gold (DE3) E. coli cells (Agilent) and plated on LB medium plates supplemented with 200 μg/ml ampicillin (Amp). A 250 ml overnight culture was prepared from a
freshly transformed colony, back-diluted 1:50 (240 ml) into 12 L LB–Amp and divided into 16 × 21 baffled flasks, each filled to 750 ml. Cultures were grown at 37 °C with shaking (260 r.p.m.) to OD_{660} = 0.5–0.7. IPTG was added to a final concentration of 1 mM, and the cultures grown for an additional 4 h. Pellets were collected and stored at −80 °C before DPO molecule purification.

Supplementary Table 3. A 100-mM stock solution of the compound was injected iteratively onto a Thermo Scientific Hypercarb column (4.6 × 100 mm, 3 μm) operating at a flow rate of 0.7 ml/min using water and MeCN. Elution was monitored at 280 nm and initially carried out isocratically at 40% ethanol and heating the slurry to 70 °C in a water bath for 20 min with occasional vortex. The heated slurry was pelleted by centrifugation at 4,000 r.p.m. for 80 min at 4 °C. Resin was washed 5× with Wash buffer (40 mM imidazole in PBS) by centrifugation at 1,500 r.p.m. for 3 min at 4 °C, fresh Wash buffer was added, and this step was repeated. The resin was subsequently washed twice, in the same manner, with ice cold PBS to remove residual imidazole and twice more in ice cold Milli-Q water to remove salts. The DPO molecule was isolated by resuspending the final volume of resin in 40% ethanol and heating the slurry to 70 °C in a water bath for 20 min with occasional vortex. The heated slurry was pelleted by centrifugation at 4,000 r.p.m. for 10 min and the molecule was recovered in the supernatant. These supernatants were concentrated by rotovap to approximately 5 ml and stored at −80 °C before further purification. The batches were pooled, lyophilized to dryness, and resuspended in a small volume of water (~0.5 ml). DPO was subterminally collected and stored at −80 °C before DPO molecule purification. The total DPO collected is estimated to be 1 mg, corresponding to ~8 μmol DPO per 10 μmol VqmA protein.

Production of cell-free culture fluids. Cell-free culture fluids were prepared in the same fashion as unlabeled culture fluids but standard amino acids were replaced with uniformly 13C-labeled amino acids (Cambridge Isotope Laboratories). Additions to the standard M9 medium recipe were as follows:

- Unlabeled Thr: M9 + 0.4% glucose + 0.4 mM [13C]-Thr
- Unlabeled Ala: M9 + 0.4% glucose + 0.4 mM [13C]-Ala
- Labeled Thr: M9 + 0.4% glucose + 0.4 mM [13C]-Thr
- Labeled Ala: M9 + 0.4% glucose + 0.4 mM [13C]-Ala

Overnight cultures of wild-type V. cholerae were divided into aliquots and back-diluted into unlabeled medium or medium containing a labeled amino acid. Cell-free culture fluids were prepared as above and loaded onto a Thermo Scientific Hypersil Hypercarb column (100–700 mg resin) that had been washed with 5 column volumes (CV) of MeOH, 5 CV of MeCN, and equilibrated with 10 CV of water. After loading, the column was washed with 5 CV of water and eluted with 5 CV of the following: 15% MeCN, 50% MeCN, and 100% MeCN. DPO and its isotopomers typically eluted in the 15% MeCN fraction, which was subsequently dried in vacuo and resuspended in 100 μl of water. 35 μl of this solution was analyzed by HPLC–Qtof–MS as described below. Note that Strata–C18, Strata–C8, and Strata–CN Seppak resins (Phenomenex) all failed to retain DPO. Optimal retention of DPO by Hypercarb chromatography was achieved by increasing the resin size to 200–1,000 mg.

HPLC–Qtof–MS analysis. High-resolution HPLC–MS was carried out on an Agilent UHD Accurate Mass Q-tof LC–MS system equipped with a 1260 Infinity Series HPLC, an automated liquid sampler, a photodiode array detector, a JetStream ESI source, and the 6540 Series Qtof. Samples were subjected to separation on a Thermo Scientific Hypercarb column (4.6 × 100 mm, 3 μm) operating at a flow rate of 0.7 ml/min using water and MeCN. Elution was initially carried out isocratically with 8% MeCN in water for 6 min followed by a gradient of 8–50% MeCN over 15 min.

Synthesis of the ligation product of aminoacetone and L-Ala. The ligation product of aminoacetone and L-Ala (N-alamyl-aminoacetone, the product of step (iv) in Fig. 4e) was synthesized by WuXi AppTec. The manufacturer estimates >95% purity which was verified by in-house NMR analysis (Supplementary Fig. S1; Supplementary Table S3). A 100-mM stock solution of the compound was prepared in water and tested in the PqvmR::mkate2 reporter assay as described above for DPO.

Data availability. All data generated or analyzed during this study are included in this published article (and its supplementary information files) or are available from the corresponding author on reasonable request.
A *Vibrio cholerae* autoinducer-receptor pair that controls biofilm formation

Kai Papenfort, Justin E Silpe, Kelsey R Schramma, Jian-Ping Cong, Mohammad R Seyedsayamdost & Bonnie L Bassler

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In the version of this article initially published, the compound structure immediately upstream of DPO in the biosynthetic scheme in Figure 4e was redrawn incorrectly during production. The structure was missing a methyl group. The error has been corrected in the HTML and PDF versions of the article.
ERRATUM

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In the version of this article initially published, the trace shown in Figure 4b was mislabeled as the RNA VqmR instead of the protein VqmA. The error has been corrected in the HTML and PDF versions of the article.