Published in final edited form as:
Mol Microbiol. 2014 February ; 91(4): 821–833. doi:10.1111/mmi.12502.

CqsA-CqsS quorum-sensing signal-receptor specificity in *Photobacterium angustum*

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Summary

Quorum sensing (QS) is a process of bacterial cell-cell communication that relies on the production, detection, and population-wide response to extracellular signal molecules called autoinducers. The QS system commonly found in vibrios and photobacteria consists of the CqsA synthase/CqsS receptor pair. *Vibrio cholerae* CqsA/S synthesizes and detects (*S*)-3-hydroxytridecan-4-one (C10-CAI-1), whereas *Vibrio harveyi* produces and detects a distinct but similar molecule, (*Z*)-3-aminoundec-2-en-4-one (Ea-C8-CAI-1). To understand the signaling properties of the larger family of CqsA-CqsS pairs, here, we characterize the *Photobacterium angustum* CqsA/S system. Many photobacterial *cqsA* genes harbor a conserved frameshift mutation that abolishes CAI-1 production. By contrast, their *cqsS* genes are intact. Correcting the *P. angustum cqsA* reading frame restores production of a mixture of CAI-1 moieties, including C8-CAI-1, C10-CAI-1, Ea-C8-CAI-1 and Ea-C10-CAI-1. This signal production profile matches the *P. angustum* CqsS receptor ligand-detection capability. The receptor exhibits a preference for molecules with 10-carbon tails, and the CqsS Ser^{168} residue governs this preference. *P. angustum* can overcome the *cqsA* frameshift to produce CAI-1 under particular limiting growth conditions presumably through a ribosome slippage mechanism. Thus, we propose that *P. angustum* uses CAI-1 signaling for adaptation to stressful environments.

Keywords

quorum sensing; sensor kinase; signal transduction; gene-regulation

Introduction

Bacteria coordinate group behaviors by producing, detecting, and collectively responding to extracellular signaling molecules called autoinducers. This process is called quorum sensing (QS). Bacteria living in heterogeneous populations presumably encounter mixtures of autoinducers produced by themselves and their unrelated neighbors. Therefore, perceiving and integrating the information contained in autoinducer blends could enable bacteria to facilitate intraspecies, intragenus, and interspecies communication, ultimately controlling

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Conflict of Interest

The authors declare that they have no conflict of interest.

Supporting information

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niche-specific behaviors (Fuqua and Greenberg, 2002; Novick and Geisinger, 2008; Ng and Bassler, 2009; Rutherford and Bassler, 2012).

The *Vibrio harveyi* QS circuit is studied as a model to define the mechanisms that allow bacteria to interpret the information encoded in mixtures of autoinducers. *V. harveyi* has three QS systems and each is composed of an autoinducer synthase and a two-component receptor (Henke and Bassler, 2004) (Fig. 1). The LuxM synthase produces Autoinducer-1 (AI-1) (Cao and Meighen, 1989; Bassler *et al.*, 1993), an acyl homoserine lactone (AHL) used for intraspecies communication. LuxS synthesizes Autoinducer-2 (AI-2) (Surette *et al.*, 1999; Schauder *et al.*, 2001; Chen *et al.*, 2002), a furanosyl borate diester involved in interspecies communication. CqsA produces (*Z*)-3-aminoundec-2-en-4-one (Ea-C8-CAI-1) that is used for intragenus communication (Ng *et al.*, 2011). AI-1, AI-2, and Ea-C8-CAI-1 bind to the LuxN, LuxPQ, and CqsS receptors, respectively (Henke and Bassler, 2004).

When *V. harveyi* is at low cell density (LCD), there is little autoinducer present and thus, the receptors are unliganded. Under this condition, the receptors' autokinase activities dominate (Fig. 1, top). Phosphorelay to LuxU occurs (Freeman and Bassler, 1999a), followed by phospho-transfer to the response regulator, LuxO (Freeman and Bassler, 1999b). LuxO~P activates transcription of genes encoding five regulatory small RNAs (Qrr sRNAs) (Lenz *et al.*, 2004), which post-transcriptionally activate expression of the LCD transcription factor AphA (Rutherford *et al.*, 2011) and repress production of LuxR (Pompeani *et al.*, 2008), the master high cell density (HCD) transcription factor. Thus, at LCD (i.e., AphA present, LuxR absent), AphA controls the regulon of genes underpinning individual behaviors.

At HCD (Fig. 1, bottom), autoinducers accumulate and bind to their cognate receptors. Binding inhibits the receptors' autokinase activities (Freeman *et al.*, 2000). This step reverses the phospho-flow and consequently drains phosphate from LuxO. Dephosphorylated LuxO cannot activate transcription of the *qrr* genes, which reduces production of AphA and allows LuxR to be translated. LuxR regulates hundreds of genes required for group behaviors (van Kessel *et al.*, 2013). For example, the luciferase operon is activated by LuxR in *V. harveyi*. Therefore, cells are dark at LCD and bright at HCD. Luciferase, due to its large dynamic range and ease of measurement, has been used as the canonical QS readout in *V. harveyi*.

V. harveyi CqsA ($CqsA_{Vh}$) exclusively produces Ea-C8-CAI-1 and $CqsS_{Vh}$ appears to detect only that molecule (Ng *et al.*, 2011). By contrast, *V. cholerae* CqsA ($CqsA_{Vc}$) produces C10-CAI-1, and Ea-C8-CAI-1 is produced as a minor product (Higgins *et al.*, 2007; Kelly *et al.*, 2009; Ng *et al.*, 2011). $CqsS_{Vc}$ detects CAI-1 molecules harboring both 10-carbon and 8-carbon tails, with C10-CAI-1 being the preferred ligand. To understand how bacteria decode the information contained in structurally similar molecules, here, we continue our investigation of the signaling properties of CqsA-CqsS pairs.

In addition to vibrios, the closely-related photobacteria have *cqsA* and *cqsS* genes. Indeed, these genes are present in four of the five sequenced photobacterial genomes, (the exception is *Photobacterium damselae*). *Legionellacea*, *Burkholderiacea*, and *Chlorobiaceae*, also contain CqsA-CqsS homologs (Tiaden *et al.*, 2010). Only the vibrio CqsA enzyme catalysis mechanism has been investigated (Higgins *et al.*, 2007; Kelly *et al.*, 2009; Ng *et al.*, 2011). In vibrios, the sixth trans-membrane (TM) helices of CqsS receptors play crucial roles in ligand recognition (Ng *et al.*, 2010). Strikingly, however, the amino acid sequences of the sixth TM helicies in photobacterial CqsS receptors differ significantly from those of vibrio CqsS receptors (Fig. S1). Additionally, a crucial Cys^{170}/Phe^{175} residue is present in vibrio CqsS receptors, and it specifies the ligand chain length (Ng *et al.*, 2011). Most photobacterial CqsS receptors (the exception is *Photobacterium profundum* CqsS) contain a

Ser in place of the Cys/Phe (Fig. S1). These differences suggest distinct CAI-1 detection possibilities for photobacteria.

Here we focus on *Photobacterium angustum*, sometimes referred to as *Vibrio angustum*, to examine CqsA signal production and CqsS signal detection. *P. angustum* is a copiotrophic bacterium that has been reported to use QS to regulate the carbon starvation response (Srinivasan *et al.*, 1998 and hereto). Consistent with this notion, cell-free extracts prepared from carbon-starved *P. angustum* upregulated carbon-starvation-induced proteins and re-activated growth. The *P. angustum* genome contains a hypothetical *luxMN* operon, but no AHL autoinducer has been detected. AI-2 signaling has been suggested to be involved in *P. angustum* stress adaptation because culture fluids from stressed cells induced bioluminescence in a *V. harveyi* AI-2 detector strain (McDougald *et al.*, 2003). However, no obvious LuxPQ receptor exists. By contrast, both *cqsA* and *cqsS* are present in the *P. angustum* genome. To our knowledge, they have not yet been studied. The *P. angustum* CqsA-CqsS system could possess QS signaling capacity, and thus, could provide the link to the observed starvation responses. To investigate these possibilities, here, we examine ligand detection by CqsS, identify the *P. angustum* CAI-1 molecule, and evaluate its production by CqsA. No genetic tools exist that enable examination of Cqs function in photobacterium. Thus, we define the Cqs QS signaling properties using a heterogeneous vibrio system.

Results

Wild-type *P. angustum* produces no CAI-1

It is not possible to know the structures of small molecule autoinducers from the sequences of the genes encoding their synthases. Typically, autoinducers have been identified *via* extraction from cell-free culture fluids followed by structure determination by NMR, mass spectrometry, or crystallography. To begin our analysis of *P. angustum* CAI-1 signal production, we constructed a *V. harveyi* reporter strain to monitor CAI-1 activity. The reporter strain lacks all three endogenous QS receptors (Δ*luxN* Δ*luxPQ* Δ$cqsS_{Vh}$), so it is incapable of autoinducer detection. It also lacks *cqsA* (Δ$cqsA_{Vh}$) so it produces no CAI-1. This reporter strain produces light constitutively because the lack of QS receptors eliminates phosphorylation of LuxO, so LuxR is constitutively produced, and it activates expression of the luciferase operon (See Fig. 1 bottom, Fig. 2 Panel I). Consistent with this circuitry, addition of wild-type *V. harveyi* or *P. angustum* cell-free culture fluids does not alter light production in this strain. Introduction of $cqsS_{Vh}$ on a vector renders the strain dark because $CqsS_{Vh}$ functions as a kinase in the absence of autoinducer, leading to repression of the luciferase operon (Fig. 2 Panel I). Addition of nothing, DMSO or cell-free culture fluid from a *V. harveyi* Δ*cqsA* mutant (Vh Δ*cqsA*) does not induce light production in the reporter strain carrying $cqsS_{Vh}$ (Fig.2 compare Panel I to Panel II). However, when the reporter strain carrying $cqsS_{Vh}$ is provided cell-free culture fluid prepared from wild-type *V. harveyi* (Vh WT), which contains Ea-C8-CAI-1, the reporter produces light. This result shows that the *V. harveyi* CqsS receptor is capable of switching from kinase to phosphatase mode when bound by Ea-C8-CAI-1. The reporter strain carrying $cqsS_{Vh}$ also produced light when synthetic Ea-C8-CAI-1 but not C10-CAI-1 is added which is consistent with the known $CqsS_{Vh}$ detection specificity (Fig. 2 Panel II). When we introduced $cqsS_{Pa}$ instead of $cqsS_{Vh}$, the reporter strain became dark showing that $CqsS_{Pa}$ functions as a kinase in the absence of ligand (Fig. 2 Panel I). Addition of DMSO or culture fluid from the Δ*cqsA V. harveyi* strain did not induce light production (Fig. 2 compare Panel I to Panel III). Addition of WT *V. harveyi* culture fluid or synthetic Ea-C8-CAI-I induced modest increases in light production (~10 to 100-fold), whereas addition of synthetic C10-CAI-1 induced maximal light production (~1000-fold) (Fig. 2 Panel III). The results show that the kinase activity of $CqsS_{Pa}$ is

inhibited in the presence of ligand, and also that $CqsS_{Pa}$ detects the *V. harveyi* and *V. cholerae* CAI-1 autoinducers. We return to the basis for the different levels of detection below. Cell-free culture fluid collected from wild-type *P. angustum* (Pa WT) failed to induce light production in the reporter strain carrying *cqsS*$_{Vh}$, *cqsS*$_{Pa}$ or *cqsS*$_{Vc}$ (Fig. 2, Pa WT Panel II and III, and data not shown). These data indicate that, under our conditions, wild-type *P. angustum* is either incapable of producing CAI-1 or the *P. angustum* autoinducer cannot be detected by any of the CqsS receptors examined.

$CqsS_{Pa}$ preferentially detects CAI-1 molecules with C10 tails

To examine $CqsS_{Pa}$ ligand detection specificity, we measured dose-dependent responses of $CqsS_{Vh}$, $CqsS_{Vc}$, and $CqsS_{Pa}$ to a set of CAI-1-type molecules (C8-CAI-1, Ea-C8-CAI-1, C10-CAI-1 and Ea-C10-CAI-1) using the above reporter strain expressing either $CqsS_{Vh}$, $CqsS_{Pa}$, or $CqsS_{Vc}$ (Figs 3 and S2). Consistent with our understanding of CqsS signaling, the $CqsS_{Vh}$ receptor is stringent in ligand detection and only responds to the cognate *V. harveyi* autoinducer, Ea-C8-CAI-1 (EC_{50} = 100 nM). The $CqsS_{Vc}$ reporter responds maximally to Ea-C10-CAI-1 (EC_{50} = 150 nM), and efficiently detects C10-CAI-1 and Ea-C8-CAI-1 (EC_{50} of 400 nM and 1 μM, respectively), showing that it has relaxed specificity. The $CqsS_{Pa}$ receptor exhibits intermediate ligand stringency. Similar to $CqsS_{Vc}$, it responds robustly to Ea-C10-CAI-1 and C10-CAI-1 (EC_{50} = 4 nM and 12 nM, respectively), but it shows a modest response to Ea-C8-CAI-1 (Figs 3 and S2). Sub-maximal light is produced in response to molecules with C8 tails, and so EC_{50} values could not be reliably determined. These data indicate that $CqsS_{Pa}$ strongly prefers CAI-1 molecules carrying C10 tails over those carrying C8 tails.

Ser^{168} defines the $CqsS_{Pa}$ ligand detection preference

Previous analyses of $CqsS_{Vh}$ and $CqsS_{Vc}$ show that Phe^{175} restricts ligand detection in $CqsS_{Vh}$ to CAI-1 moieties carrying C8 tails, whereas the smaller Cys^{170} residue in the analogous position in $CqsS_{Vc}$ relaxes stringency, enabling detection of CAI-1-type molecules carrying C8 and C10 tails (Fig. S1). We wondered if Ser^{168} at the corresponding position in $CqsS_{Pa}$ plays the crucial role in determining chain-length preference. To examine this possibility, we exchanged the residues in the different CqsS receptors and measured their responses to CAI-1 molecules (Fig. S2). Consistent with previous results, when Phe^{175} was changed to Cys or Ser ($CqsS_{Vh}^{F175C}$ and $CqsS_{Vh}^{F175S}$), the $CqsS_{Vh}$ receptor gained the ability to detect CAI-1 moieties carrying 10-carbons. $CqsS_{Vc}^{C170S}$ detected CAI-1 molecules with both C8 and C10 tails, whereas $CqsS_{Vc}^{C170F}$ had greatly reduced detection of CAI-1 molecules with C10 tails.

We compared the above results to those for $CqsS_{Pa}$ carrying analogous substitutions. $CqsS_{Pa}^{S168C}$ retained the ability to detect Ea-C10-CAI-1 and C10-CAI-1, but did not gain any ability to detect CAI-1 molecules with C8 tails (Fig. S2). Replacement with other small amino acids such as Gly, Ala, Thr resulted in the same phenotype. The $CqsS_{Pa}^{S168F}$ and $CqsS_{Pa}^{S168V}$ variants with Ser^{168} replaced with bulky hydrophobic residues showed no response to any CAI-1-type molecule in our collection (Fig. S2). These experiments demonstrate that Ser^{168} is important for CAI-1 detection by $CqsS_{Pa}$, but this residue alone does not specify the chain-length preference. We reason that in $CqsS_{Pa}$, ligand specificity is determined by Ser^{168} and residues that do not exist in $CqsS_{Vh}$ or $CqsS_{Vc}$. To investigate this region further, we replaced the stretch of seven amino acids surrounding Ser^{168} (TGIA**S**HY) in the $CqsS_{Pa}$ receptor with the corresponding residues from the $CqsS_{Vh}$ receptor (FGNL**F**YF) or the $CqsS_{Vc}$ receptor (FGNL**C**FF). Neither of these chimeric mutant receptors was functional.

A two-nucleotide deletion causes a frameshift mutation in the *P. angustum* cqsA gene

The above analyses show that $CqsS_{Pa}$ is capable of detecting specific CAI-1-type molecules, however, curiously, CAI-1 activity is absent in *P. angustum* culture fluids. We wondered if the *P. angustum cqsA* gene ($cqsA_{Pa}$) was defective. Genomic analysis revealed extensive homology between $cqsA_{Pa}$ and vibrio *cqsA* genes. However, the annotated $cqsA_{Pa}$ ORF encodes a protein of 313 amino acids, significantly shorter than vibrio CqsA proteins that contain approximately 390 amino acids (see Met^{78} in Fig. 4A). This $CqsA_{Pa}$ protein sequence, beginning with Met^{78}, aligns well with an internal portion of vibrio CqsA proteins. We name this shorter ORF $cqsA_{Met78}$. The DNA sequence homology between $cqsA_{Pa}$ and vibrio *cqsA* genes extends well upstream of the annotated start site of $cqsA_{Met78}$ (Fig. 4B). We can identify a putative translation start site for $cqsA_{Pa}$ in this extended upstream region and this start codon corresponds closely to those of vibrio *cqsA* genes. We assign this putative upstream $CqsA_{Pa}$ start site as Met^{01} (Fig. 4A and B). Additionally, a predicted RBS is located upstream of Met^{01} (Fig. 4C; RBS). We hypothesize that the Met^{01} start site, if used, could enable translation of a functional CqsA protein of 390 amino acids; the same length as other CqsA proteins. We name the ORF starting at Met^{01} $cqsA_{Met01}$. However, in this putative gene, two consecutive nucleotides are missing at +39 relative to Met^{01}, which corresponds to a TC in $cqsA_{Vc}$ and a TG in $cqsA_{Vh}$ gene (Fig. 4B; FS). Restoration of a TC would introduce a conserved Gln at the 14^{th} residue, whereas a TG would introduce a Glu (Fig. 4A). To simplify our nomenclature, we assume the deleted dinucleotide to be TC in $cqsA_{Pa}$, and we name the frameshift site Gln^{14}. The naturally occurring deletion causes a frameshift at Gln^{14} and introduces a stop codon at position +49 relative to Met^{01} (Fig. 4B; Stop). Thus, even if Met^{01} is used, this frameshift would make the $CqsA_{Pa}$ protein only 16 amino acids long, ending with Lys^{14}-Lys^{15}-Asn^{16}. Introduction of TC at the frameshift would restore the reading frame to generate an ORF encoding a protein of 390 amino acids. We call this corrected ORF $cqsA_{Met01}{}^{+TC}$. We engineered $cqsA_{Pa}$ constructs carrying the various features described above (Fig. 5A), and measured the resulting CAI-1 activity using the *V. harveyi* reporter strain harboring $CqsS_{Pa}$ (Fig. 5B).

First, we overexpressed $cqsA_{Met78}$ and $cqsA_{Met01}$ in *E. coli*. No CAI-1 activity was produced as judged by the lack of induction of light production by the $CqsS_{Pa}$ detector strain following treatment with cell-free culture fluids from these strains (Fig. 5B). Analogous overexpression of the $cqsA_{Vh}$ or $cqsA_{Vc}$ ORF in *E. coli* resulted in CAI-1 production (Kelly *et al.*, 2009; Ng *et al.*, 2011). $cqsA_{Met01}{}^{+TC}$ with the frameshift repaired, when overexpressed in *E. coli*, produced CAI-1 that could induce light production in the reporter strain (Fig. 5A and B). Restoring the reading frame and simultaneously replacing the annotated Met^{78} with a Thr codon (denoted $cqsA_{Met01}{}^{+TC,\ M78T}$) maintained CAI-1 production, indicating that Met^{78} is not employed as the start site (Fig. S3). However, CAI-1 activity depended on translation from Met^{01}, because introduction of a nonsense mutation at the Ser^{08} codon between Met^{01} and Met^{78} ($cqsA_{Met01}{}^{+TC,\ S08*}$) abolished CAI-1 activity (Fig. S3). Together, these results show that the naturally occurring frameshift inactivates $cqsA_{Pa}$, and restoring both the length and the reading frame of the *cqsA* gene is necessary for CAI-1 production.

Intriguingly, a stretch of nine consecutive adenosine nucleotides (A-repeats) is located immediately downstream of the Gln_{14} (+39) frameshift in $cqsA_{Pa}$ (Fig. 4B). Insertion or deletion of adenosines could occur when errors happen during replication or transcription. If so, the $cqsA_{Pa}$ reading frame could be restored. Indeed, insertion of two additional adenosine nucleotides into $cqsA_{Met01}$ to replace the deleted TC (denoted $cqsA_{Met01}{}^{+AA}$), which introduces a Lys at the Gln^{14} site, resulted in CAI-1 production (Fig. S3). However, deletion of one adenosine nucleotide in the A-repeats (denoted $cqsA_{Met01}{}^{\Delta A}$), while restoring the reading frame, makes a CqsA protein that lacks an amino acid at the conserved Gln^{14}

position. This alteration did not result in CAI-1 activity (Fig. S3). Thus, having a residue at the Gln^{14} site is necessary for CqsA function. Together, our results support the idea that Met^{01} is the correct start site, and a correct reading frame is essential for CqsA enzymatic activity. We infer that wild-type *P. angustum* and other photobacteria are *cqsA* null mutants, due to the acquisition of dinucleotide deletions. We discuss the biological implications of this finding below.

P. angustum CqsA synthesizes a mixture of CAI-1-type molecules

The above results show that correcting the reading frame of the *P. angustum* $cqsA_{Pa}$ gene is sufficient to produce a functional CqsA protein. Thus, the $cqsA_{Pa}{}^{+TC}$ construct provides us a means to identify which CAI-1 moieties the *P. angustum* CqsA enzyme produces. Culture fluid was prepared from the *E. coli* strain overexpressing $cqsA_{Met01}{}^{+TC}$. Preparations were extracted with dichloromethane and analyzed by triple quadrupole gas-chromatography mass spectrometry (GC-MS). We identified molecules with masses corresponding to known CAI-1 molecules (Fig. 5C). Quantification with a C9-CAI-1 standard showed that C8-CAI-1 and C10-CAI-1 are the predominant $CqsA_{Pa}$ products and were present at 5 μM and 4 μM, respectively. Ea-C8-CAI-1 and Ea-C10-CAI-1 were also present, each at around 1 μM. The composition of CAI-1-type molecules in the preparations strongly tracked with the ability of the preparations to induce bioluminescence in the *V. harveyi* reporters carrying CqsS receptor variants that preferentially detect particular CAI-1 family molecules (Fig. 5B and S4). No CAI-1 molecules were detected by GC-MS or via activation of the bioluminescent reporter strain in preparations made from *E. coli* carrying the empty vector or the $cqsA_{Met01}$ construct harboring the 2-nucleotide deletion (Figs 5B, C, and S4). Together, these results demonstrate that the repaired $cqsA_{Pa}$ gene enables production of CAI-1-like moieties. Although unlikely, we cannot exclude the possibility that other CAI-1-like molecules are also produced that do not match our synthetic GC-MS standards or induce our set of reporter strains.

Regulation of *P. angustum* CAI-1 production

Given that $CqsS_{Pa}$ functions, it is curious then that a dinucleotide frameshift exists in the *P. angustum cqsA* gene that eliminates CAI-1 production. The dinucleotide deletion and the A-repeats exist in the *cqsA* locus of two other *P. angustum* isolates that we have sequenced (S14 and B70) and genomic database analyses show that this exact frameshift coupled with the A-repeats is also present in *Photobacterium sp.* SKA34 and *Photobacterium leiognathi*, but absent in *Photobacterium profundum* (Fig. 4B). Several polymorphisms exist in the sequences of the photobacterial *cqsA* genes, but no detrimental mutations other than the dinucleotide deletion appear to be present. The other components of the QS circuit, *luxU*, *luxO*, *qrr*1, *luxR*, and *aphA* are present in all sequenced *P. angustum* strains and appear to be intact. These findings suggest that there could be some selective advantage to maintain a DNA sequence that can be capable of producing full-length CqsA.

To examine regulation and possible reactivation of $cqsA_{Pa}$, we first focused on the 5′ UTR. The 5′ UTRs of photobacterial *cqsA* genes are highly similar, and in all cases, the conserved ribosome binding site is centered at the -10 position relative to Met^{01} (Fig. 4C, D). The sequences surrounding the Shine-Dalgarno motif are AT-rich and possess dyad symmetry, potentially forming a stem loop that occludes the ribosome (Fig. 4D; denoted SL). The analogous 5′ UTR sequences of vibrio *cqsA* genes are not similar to the *P. angustum* 5′UTR sequence, and they do not appear capable of forming extended stem loops. We wondered if the putative structure in the $cqsA_{Pa}$ 5′UTR influences regulation. To test this idea, we engineered a *P. angustum cqsA* construct harboring the native 41-nucleotide 5′ UTR region that is capable of forming a stem-loop (denoted SL-$cqsA_{Met01}$ in Fig. 5A). Overexpression of SL-$cqsA_{Met01}$ did not result in CAI-1 activity (Fig. 5B). Mutating this region to eliminate

stem-loop formation (denoted SL^{mut}-$cqsA_{Met01}$) partially restored CAI-1 production, as indicated by light production from the *V. harveyi* $CqsS_{Pa}$ reporter (Fig. 5A and B). Rather than a structural motif, the palindromic sequence in the $cqsA_{Pa}$ 5′UTR could encode a repressor DNA binding site, and if so, the mutation in SL^{mut}-$cqsA_{Met01}$ could have eliminated repressor binding and derepressed $cqsA_{Pa}$ expression. We do not favor this possibility because there are less than two-fold differences in transcript levels between the $cqsA_{Met01}$, SL-$cqsA_{Met01}$, and SL^{mut}-$cqsA_{Met01}$ constructs according to qRT-PCR (Fig. S5). We therefore conclude that the differences in CAI-1 activity produced by the various constructs are primarily a consequence of post-transcriptional regulation.

The above results suggest that eliminating the stem-loop reveals the native *cqsA* RBS and partially overcomes the frameshift mutation. Possibly, the lack of a stem loop enables some ribosome recruitment and transit through the gene enabling modest CqsA production even when the frameshift mutation is present. To examine this possibility, we engineered a $cqsA_{Pa}$ construct harboring the region of the 5′ UTR that preserves the native RBS, but prevents stem-loop formation because the distal, complementary sequence is not included in the construct (denoted RBS-$cqsA_{Met01}$ in Fig. 5A). Overexpression of this construct in *E. coli* indeed resulted in CAI-1 activity consistent with the idea that the presence of the native RBS is sufficient to override the frameshift mutation (Fig. 5B). We discuss possible mechanisms by which this could occur below. Mutations in the RBS abolished CAI-1 activity (Fig. S3; RBS^{mut}-$cqsA_{Met01}$) and CAI-1 production also depended on translation from Met^{01}, because no activity was produced when the $Met^{01}\rightarrow$Thr mutation was present (denoted RBS-$cqsA_{Met01}^{M01T}$, Fig. S3). By contrast, alteration of Met^{78} to Thr (RBS-$cqsA_{Met01}^{M78T}$, Fig. S3) maintained CAI-1 activity. GC-MS analysis demonstrated that the mixture of CAI-1 molecules present in the cell-free culture fluids prepared from the *E. coli* strain overexpressing RBS-$cqsA_{Met01}$ was similar to that produced by the $cqsA_{Met01}^{+TC}$ strain (Fig. 5C). The concentrations of CAI-1 moieties were, however, only half as high as those present in culture fluids prepared from the $cqsA_{Met01}^{+TC}$ strain harboring the restored reading frame. Introduction of the stem-loop upstream of the repaired $cqsA_{Met01}^{+TC}$ did not reduce CAI-1 production (SL-$cqsA_{Met01}^{+TC}$; Fig. 5A and B). We reason that, in this construct, the RBS that is supplied by the P*tac* promoter is sufficient for $cqsA_{Pa}$ expression when the frameshift is restored. Repairing the frameshift in the context of disruption of the stem-loop (denoted SL^{mut}-$cqsA_{Met01}^{+TC}$) also resulted in full bioluminescence induction and CAI-1 production (Fig. 5A, B and C). All four CAI-1-type molecules were produced (Fig. 5C). We note, however, that overexpression of this construct impaired *E. coli* growth by three fold, suggesting that *E. coli* lacked the capacity to produce as much CAI-1 as the construct could support. Thus, if we adjust for cell growth, we expect the CqsA production per cell in *E. coli* carrying the SL^{mut}-$cqsA_{Met01}^{+TC}$ construct is higher than that in the *E. coli* strain carrying the SL-$cqsA_{Met01}^{+TC}$ construct. We provide proof for this assertion in the next section. Collectively, our results show that disruption of the native stem-loop combined with restoration of the $cqsA_{Pa}$ ORF fully de-repressed *cqsA* expression, and furthermore, the stem-loop functions in post-transcriptional control.

To verify the above results, we measured CqsA protein in the above *E. coli* strains using Western blotting with an antibody targeting a 6x-His tag fused to the C-terminus of CqsA. Our strategy ensured that only fully translated CqsA protein is detected (Fig. 5D). No CqsA protein was present in lysates of *E. coli* strains harboring the empty vector, $cqsA_{Met78}$, or the $cqsA_{Met01}$ construct lacking the native 5′ UTR but carrying the frameshift, or the SL-$cqsA_{Met01}$ construct containing the stem-loop and the frameshift mutation. Low levels of CqsA protein were produced by *E. coli* expressing the SL^{mut}-$cqsA_{Met01}$ construct with the stem-loop disrupted and by *E. coli* harboring the RBS-$cqsA_{Met01}$ construct with the truncated 5′UTR (Figs. 5D and S6A). When the reading frame was restored, however, (see $cqsA_{Met01}^{+TC}$) the construct lacking the native 5′UTR produced about ten-fold higher CqsA

protein. Introducing the 5′ stem-loop upstream of *cqsA*$^{+TC}$ (see SL-*cqsA*$_{Met01}$$^{+TC}$) did not significantly alter CqsA protein production, which matches the bioluminescence assay results and suggests the RBS supplied by the vector is sufficient. However, 500-fold more CqsA protein was produced when the stem-loop was disrupted and the frameshift was simultaneously repaired (SL^{mut}-*cqsA*$_{Met01}$$^{+TC}$) compared to the construct with the restored frameshift but carrying the stem-loop (SL-*cqsA*$_{Met01}$$^{+TC}$) (Figs. 5D and S6B). These results show that disrupting the 5′UTR stem-loop structure or restoring the frameshift allows CqsA protein production, but combining these two features results in maximal CqsA protein production. Indeed *E. coli* carrying the SL^{mut}-*cqsA*$_{Met01}$$^{+TC}$ construct displayed a severe growth defect due to the high level production of CqsA. This growth defect accounts for why less CAI-1 activity is present in cell-free culture fluids than would be predicted (Fig. 5C) given that the Western shows that this strain produces the highest amount of CqsA protein per-cell (Fig. 5D). We note that the different assays we use to track *cqsA* expression and CAI-1 activity have different sensitivities and different dynamic ranges. The assays in Fig. 5 measure stages along a signal transduction cascade (protein levels → CAI-1 production → response to CAI-1). The outputs of each step are not linearly related so one cannot make a one to one comparison of the different graphs. In every case, the *cqsA* expression and the CAI-1 activity from each construct track with one another. Our results indicate that both the stem-loop that inhibits ribosome binding and the frameshift need to be overcome to achieve maximal *cqsA* expression. We assume the stem-loop can be overcome by a sRNA or other RNA regulatory process. Regarding the frameshift, one possibility is that ribosome slippage, in which the ribosome slips back and forth to alter the register, could occur, enabling reuse or bypass of nucleotides. Repeats often facilitate ribosome slippage, and in the context of *cqsA*$_{Pa}$, a conserved stretch of A-repeats immediately follows the two-nucleotide deletion (Fig. 4B and D). To test whether a slippage mechanism is plausible, we introduced an AT dinucleotide upstream of the stop codon at the +49 site in the *cqsA*$_{Met01}$ construct to mimic the outcome of two-nucleotide ribosome slippage to the left, with translation resuming with the correct reading-frame after Asn^{16}. This construct (denoted *cqsA*$_{Met01}$$^{FS+AT}$) encodes a protein that differs from the native CqsA$_{Pa}$ (encoded by *cqsA*$_{Met01}$$^{+TC}$) by two amino acids (IKKNID instead of IQKKID) (Fig. 5A). Importantly, this construct does not possess the putative stem-loop in the *cqsA*$_{Pa}$ 5′ UTR. CAI-1 activity was detected in cell-free culture fluid prepared from the *E. coli* carrying this construct (Fig. 5B), and CqsA protein was present at levels comparable to that found in *E. coli* lysates carrying *cqsA*$_{Met01}$$^{+TC}$ with the restored reading frame (Fig. 5D). We assume that the CAI-1 produced from the RBS-*cqsA*$_{Met01}$ construct occurred through a similar ribosome slippage mechanism, which would depend on continuous adenosines in the A-repeat region. Indeed, when we engineered synonymous mutations in the wobble nucleotides of the codons in the A-repeat region to prevent ribosome slippage, CAI-1 activity and CqsA protein production were abolished (RBS-*cqsA*$_{Met01}$^{A*}, Fig. 5A, B and D). By contrast, this mutation did not affect CAI-1 production or CqsA levels when the frameshift was repaired (*cqsA*$_{Met01}$$^{+TC, A*}$) (Fig. 5A, B and D). Our data suggest that a ribosome slippage model is a plausible mechanism for overriding the frameshift mutation present in the *P. angustum cqsA* gene.

P. angustum produces CAI-1 activity under stress conditions

Collectively, our results show that the *cqsA*$_{Pa}$ gene harbors the potential to be functional even in the face of a putative mRNA stem loop that occludes the RBS and a conserved dinucleotide frameshift that inserts a stop codon at position +49. However, Fig. 2 shows that stationary phase cultures of *P. angustum* do not contain any CAI-1 activity, suggesting environmental factors that are not mimicked under laboratory conditions, could be involved in enabling *cqsA*$_{Pa}$ expression presumably through melting of the 5′ stem loop and/or ribosome slippage. Previous studies suggest that QS controls the *P. angustum* response to

carbon starvation and stress adaption (Srinivasan *et al.*, 1998). During carbon and energy starvation, a *relA*-dependent surge in ppGpp synthesis occurs in *P. angustum* (Flärdh *et al.*, 1994). Ribosome slippage in *E. coli* is induced by amino acid limitation and is regulated by RelA (Masucci *et al.*, 2002). To test if carbon starvation influences ribosome slippage in *P. angustum*, we grew *P. angustum* in Biolog plates containing IF-0a medium supplemented with different carbon sources. CAI-1 activity was measured using the *V. harveyi* bioluminescent reporter supplied with the *P. angustum* Biolog culture suspension. Thirty out of the ninety-five different carbon sources tested supported more than two bacterial doublings compared to a no carbon control (Fig. S7A). As expected, many sugars were efficiently used by *P. angustum* for growth with D-galactose supporting the highest growth, to an OD of 1.3 (Fig. S7A). Several amino acids, central carbon metabolites, nucleosides and dipeptides, were also sufficient as sole carbon sources (Fig S7A).

CAI-1 activity could be detected in cultures grown on some of the above carbon sources (Fig S7B). Intriguingly, the sugars (Glc, Gal, Fru, etc.) and amino acids (Ser, Ala) that enabled the highest growth resulted in the lowest CAI-1 production. This result could explain our lack of detection of CAI-1 in *P. angustum* grown in rich medium. Likewise, Asn, Glu and Gln, while stimulating high CAI-1 production, supported only a few cell doublings. The trend appears to be that slowly growing *P. angustum* cells produce high CAI-1 activity. This result is consistent with the notion that sub-optimal growth conditions, or specifically carbon starvation, could stimulate ribosome slippage and enable CqsA production. There were a few exceptions to this pattern. Thymidine and Ala-Gly supported significant bacterial growth and CAI-1 production.

Discussion

The CqsA-CqsS QS system coupled to downstream signal relay components provides photobacteria and vibrios an apparatus capable of detecting and responding to extracellular signal molecules to orchestrate collective behaviors. Vibrios use the CqsA/S QS system to regulate bioluminescence, virulence, and EPS production (Ng and Bassler, 2009; Rutherford and Bassler, 2012). While we do not understand what processes photobacteria control with CqsA-CqsS, several previous studies have related the carbon starvation response of *P. angustum* to QS regulation. Our analyses identify CqsA-CqsS as the candidate QS system to consider in this context and that CAI-1 signal production is coupled to metabolic stress. The lack of genetic techniques for manipulating photobacterium hinders our ability to define its QS outputs. Nonetheless, we could characterize $CqsA_{Pa}$ and $CqsS_{Pa}$ function via expression and analysis in heterologous systems.

$CqsS_{Pa}$ detects CAI-1-type molecules, indicating that the overall architecture of the $CqsS_{Pa}$ ligand binding pocket is similar to that of vibrio CqsS receptors, despite striking sequence differences in the key ligand-detection transmembrane domain (TM6). Vibrios discriminate between CAI-1 tail lengths by controlling the ligand binding pocket size. A Phe^{175} in $CqsS_{Vh}$ restricts detection to CAI-1 molecules with C8 tails. The smaller Cys^{170} at the corresponding position in $CqsS_{Vc}$ enables detection of CAI-1 molecules with C8 and C10 tails. By contrast, $CqsS_{Pa}$ strongly favors CAI-1 molecules with C10 tails over those with C8 tails. Our analyses suggest that $CqsS_{Pa}$ ligand discrimination is accomplished by a different mechanism than that used by vibrio receptors. While the Ser^{168} residue in $CqsS_{Pa}$ is essential for detection of C10-CAI-1, it does not account for the tail-length preference. Possibly, ligands with short, C8, tails do not make sufficient contacts with the residues lining the $CqsS_{Pa}$ ligand binding pocket, resulting in reduced binding affinity. Alternatively, C8-CAI-1 could bind $CqsS_{Pa}$ with high affinity, but be incapable of inducing the requisite conformational change to initiate signal transduction. Chemical-genetic analyses (Ng *et al.*, 2010) could distinguish between these possibilities.

$CqsS_{Pa}$ and $CqsS_{Vc}$ both detect Ea-C10-CAI-1 and C10-CAI-1, suggesting these molecules could be involved in communication between photobacteria and vibrios. It is intriguing that *P. angustum* does not produce CAI-1 under normal laboratory conditions. We attribute this inability to a frameshift mutation in the $cqsA_{Pa}$ ORF and an inhibitory regulatory stem-loop in the 5′UTR that prevents ribosome access. These same features are conserved in several photobacterial species. Retaining the capability to detect exogenous antoinducers without the intrinsic ability to produce the corresponding autoinducer could be an "eavesdropping" strategy that *P. angustum* uses to garner information about its neighbors. For example, *P. angustum* could "tune in" to vibrios producing CAI-1 and use that information to execute appropriate behaviors in heterogeneous bacterial communities.

If photobacteria exclusively relies on eavesdropping for cell-cell communication, it could simply lose the *cqsA* gene thereby ensuring that CAI-1 production never occurs. This is not what we observe from genome sequence analyses. Harboring a full-length $cqsA_{Pa}$ gene preserves the potential to produce CAI-1. An alternative explanation could be that *P. angustum* actively regulates CAI-1 production. The CqsA enzyme employs *S*-adenosylmethionine (SAM) and fatty acids for CAI-1 synthesis (Kelly *et al.*, 2009; Wei *et al.*, 2011). Possibly, it is advantageous to divert these substrates to other uses under many of the environmental circumstances encountered by photobacteria. Perhaps, relatively extreme repression mechanisms have evolved that ensure CAI-1 production remains off, albeit with the possibility of reactivation. We favor a hypothesis along these lines because our results show that CAI-1 production is restored when the frameshift is repaired and the stem-loop is disrupted. We speculate that the conserved A-repeats located near Gln^{14} in $CqsA_{Pa}$ presumably supply the necessary track to overcome the frameshift. A change could occur during transcription or translation. During transcription, insertion of two adenosine nucleotides into the A-repeat region would produce an mRNA transcript with the correct reading frame, permanently restoring CAI-1 activity. In this scenario, CAI-1 production could still be subject to negative regulation via the 5′ stem-loop. During translation, the correct reading frame could be restored by ribosome slippage and/or usage of overlapping codons. Ribosome frameshifting is enhanced in an *E. coli relA* mutant during amino acid starvation (Masucci *et al.*, 2002). Under nutrient limiting conditions, RelA and SpoT increase synthesis of (p)ppGpp, which in turn, activates the stringent response (Boutte and Crosson, 2013). In *V. cholerae*, three synthases, RelA, SpoT, and RelV, modulate (p)ppGpp metabolism depending on nutrient availability (Das *et al.*, 2009). $RelA_{Vc}$ activity increases during amino acid starvation, whereas $SpoT_{Vc}$ and $RelV_{Vc}$ synthesize (p)ppGpp during carbon and fatty acid starvation. Homologs of the three synthases exist in *P. angustum* and presumably regulate (p)ppGpp dynamics depending on growth conditions (Flärdh *et al.*, 1994). It is known that glucose upshift of carbon-starved *P. angustum* S14 causes amino acid starvation and induction of the stringent response (Flärdh and Kjelleberg, 1994). Possibly, carbon starvation conditions influence *P. angustum* (p)ppGpp levels, promote ribosome slippage in $cqsA_{Pa}$, and restore CAI-1 production. If so, this mechanism could link the stress response to QS, as suggested previously. An alternative mechanism could occur under conditions of aminoacyl-tRNA shortage, which stimulates ribosome frameshifting at corresponding codons. This mechanism enables alternative and overlapping reading frames to be used (Yelverton *et al.*, 1994; Barak *et al.*, 1996). One could imagine that when *P. angustum* is challenged with amino acid limitation, ribosome slippage occurs at a site near the conserved frameshift. In $cqsA_{Pa}$, the frameshift is located at the Gln^{14} codon and causes a stop codon following the codon specifying Asn^{16}. Asn and Gln are the amino acids that act as sentinels to reflect carbon and nitrogen availability. Nutrient starvation could cause accumulation of uncharged tRNA-Asn and promote ribosome slippage at this location.

P. angustum could use these CAI-1 restoration mechanisms to react to carbon- and nitrogen-limited situations to induce QS to initiate its stress adaptation response. Additionally,

production of the CAI-1 signal could mimic vibrio QS, and *P. angustum* could uses this tactic to "trick" neighboring vibrios into running their QS programs under inappropriate conditions. This idea is supported by our finding that *P. angustum* produces but does not detect C8-CAI-1 and Ea-C8-CAI-1, two molecules that are detected by *V. harveyi*. Such a strategy could, for example, trigger vibrios to produce extracellular enzymes that are beneficial public goods that photobacteria can exploit, enabling it to thrive at the cost of its competitors.

Beyond the conserved frameshift, a putative stem-loop at the 5′UTR of $cqsA_{Pa}$ provides another layer of post-transcriptional control over CAI-1 production. This stem-loop could function *in vivo* to inhibit $cqsA_{Pa}$ translation with release under appropriate conditions. We envision several mechanisms by which the stem-loop could be regulated. One possibility is that a small regulatory RNA base pairs with and destabilizes the self-inhibitory stem-loop structure. In a variety of bacteria, regulatory RNAs (DsrA, GlmZ, RNAIII, RprA, RyhB, and Qrr1-5) pair with and disrupt inhibitory stem-loops in the 5′ regions of target mRNAs to promote translation by an 'anti-antisense mechanism' (Majdalani *et al.*, 1998; Prévost *et al.*, 2007; Hammer and Bassler, 2007; Fröhlich and Vogel, 2009). Bacterial genomes typically possess 100–200 sRNAs, any one of which in *P. angustum* could be the $cqsA_{Pa}$ activator. One obvious candidate to fulfill this role is the *P. angustum qrr*1 sRNA because there exist predicted base-pairing regions between Qrr1 and the $cqsA_{Pa}$ stem-loop. If *qrr*1 activates *cqsA* expression, CAI-1 production would occur at LCD but not at HCD, perhaps increasing sensitivity to CAI-1 at the onset of QS. We overexpressed $qrr1_{Pa}$ in *E. coli* carrying $cqsA_{Pa}$ but no activation occurred as measured by bioluminescence. We know our $qrr1_{Pa}$ construct was functional because it repressed *V. harveyi luxR* translation (Fig. S8). Alternatively, another small RNA for example, one analogous to *Caulobacter crescentus* CrfA, that is specifically induced upon carbon starvation could be involved (Landt *et al.*, 2010). A riboswitch mechanism could also be used to regulate the $cqsA_{Pa}$ stem-loop. In these cases, small molecules bind to mRNA riboswitch modules to alter mRNA structure, or changes in physical-chemical conditions, such as temperature, occur to stabilize alternative mRNA conformations. These changes lead to alterations in gene activity (Henkin, 2008). Known riboswitch-binding molecules include metabolites such as nucleotides, SAM, amino acids, sugars, and metal ions as well as larger polymers such as uncharged tRNAs (Serganov and Nudler, 2013). We predict a riboswitch regulatory mechanism would promote or repress CAI-1 production under particular metabolic regimes. A third possibility is that dedicated RNA binding regulatory proteins interact with and modulate $cqsA_{Pa}$ stem-loop folding, and such proteins could themselves be subject to environmental regulation. Our preliminary results suggest that CAI-1 production is elevated in *P. angustum* cultures grown on non-ideal carbon sources. Screening more diverse stimuli such as pH, temperature, osmolarity, or antibiotic stress could reveal specific conditions in which CAI-1 is robustly produced. Such experiments could define the environmental cues governing CAI-1 production thereby allowing us to study the *P. angustum* QS response.

Material and Methods

Bacterial strains and culture conditions

P. angustum S14 and *P. angustum* B70 were generous gifts from Edward F. DeLong. The *cqsAS* sequences from these two strains are similar, and no difference in CAI-1 activity or in CqsS response could be detected. *P. angustum* S14 genomic DNA was used to clone $cqsA_{Pa}$ and $cqsS_{Pa}$, except for in the experiment in Fig. S2, in which *P. angustum* B70 genomic DNA was used. $cqsS_{Vh}$ and $cqsS_{Vc}$ were cloned from the *V. harveyi* BB120 (Vh WT, Bassler *et al.*, 1997) and *V. cholerae* El Tor C6706 (Thelin and Taylor, 1996). *E. coli* S17-1 (λ*-pir)* and XL-10 Gold were used as recipients in all cloning procedures. *V. harveyi* strain

JMH603 (Vh Δ*cqsA*) is a Δ*cqsA::Cm*^{r} mutant that does not produce CAI-1. The *V. harveyi r*eporter strain WN1397 (Δ*cqsAS::Cm*^{r} Δ*luxPQ* Δ*luxN*) carrying pLAFR2-*cqsS* was used in bioluminescence assays, and $cqsS_{Vh}$, $cqsS_{Pa}$ and $cqsS_{Vc}$ were expressed under the native $cqsS_{Vh}$ promoter. Genotypes of strains and plasmids are provided in the Supporting information. Unless specified, *E. coli* was grown in LB medium at 37°C with shaking, and *V. harveyi* and *P. angustum* were grown in LM medium at 30°C with shaking. Antibiotic concentrations are: kanamycin, 100 mg L^{-1}; chloramphenicol and tetracycline, 10 mg L ^{1} unless otherwise specified.

DNA manipulation, site-directed mutagenesis, and mutant construction

Standard procedures (Sambrook *et al*., 1989) were used for DNA manipulation. Oligonucleotide sequences employed in PCR, site-directed mutagenesis, and sequencing reactions will be provided upon request. *cqsS* constructs were each fused to the $cqsS_{Vh}$ promoter and cloned into pLAFR2 using XbaI and BamHI. Point mutations were engineered into *cqsS* by overlap extension PCR (Ho *et al.*, 1989). Plasmids carrying wild-type and mutant *cqsS* genes were introduced into *V. harveyi* WN1397 via conjugation, and the mutant *cqsS* alleles were maintained as exogenotes. $cqsA_{Pa}$ variants ($cqsA_{Met78}$, $cqsA_{Met01}$, SL-$cqsA_{Met01}$, RBS-$cqsA_{Met01}$) were cloned into pEVS143 using AvrII and BamHI. Expression was controlled by an IPTG inducible P_{tac} promoter. All mutations engineered upstream of $cqsA_{Pa}$ were made with the QuikChange II XL Site-Directed Mutagenesis Kit (Agilent) according to the manufacturer's instructions.

Bioluminescence assay

Cell-free culture fluids from *V. harveyi* and *P. angustum* were prepared from overnight cultures unless otherwise specified. Overnight cultures of *E. coli* strains carrying $cqsA_{Pa}$ constructs were diluted 1:1000 in LB medium containing 50 mg l^{-1} kanamycin and grown at 30°C with shaking. *cqsA* expression was induced with 0.2 mM IPTG for ~8 hrs. Cell-free culture fluids were subsequently collected at OD_{600} = 1.5. For CAI-1 activity assessment in bioluminescence assays, overnight cultures of reporter strains carrying pLAFR2-*cqsS* were grown in LM medium with 5 mg l^{-1} tetracycline and diluted 50-fold with sterile medium. Cell-free culture fluids were added from 1–20% (v/v) to the diluted reporter strains. Bioluminescence and OD_{600} were measured using an Envison Multilabel Reader following 4.5 h incubation at 30°C with shaking. Synthetically prepared CAI-1 analogues were dissolved in DMSO.

Western blot analyses

E. coli strains carrying $cqsA_{Pa}$ constructs were grown as described for the bioluminescence assay. 1 ml of cells were collected and resuspended in 100 μl of Bugbuster (Novagen) with 50 ug ml^{-1} lysozyme (Sigma) and 10 U ml^{-1} Benzonase Nuclease (Novagen), and combined with 4x SDS-PAGE protein loading buffer. Samples were electrophoresed on 4–20% Mini-Protein Gels (Bio-Rad), and subsequently transferred to nitrocellulose membranes. Membranes were blotted with anti-RNA polymerase beta subunit (NeoClone) and with monoclonal anti-polyhistidine antibody produced in mice (Sigma). Following exposure, films were scanned and analyzed using ImageJ software (NIH).

Measurement of the concentration of CAI-1-type molecules in culture fluids

E. coli carrying $cqsA_{Pa}$ constructs grown in LB medium with kanamycin at 37°C overnight with shaking. Cultures were diluted 1000-fold in fresh LB medium and incubated at 30°C with shaking. At OD_{600} = 0.25, the cultures were induced with 0.2 mM IPTG and allowed to grow for an additional 6 h to an OD_{600} = ~2.0. Cells were removed by centrifugation. Synthetic C9-CAI-1 was added to 9 ml of collected cell-free fluids at 1 μM. The mixtures

were extracted into 500 μl of dichloromethane. The organic layer was separated. This extract was diluted 1:1 with dichloromethane, and samples were subjected to GC-MS analysis. Calibration curves and correction factors were obtained for each molecule by preparing samples containing known concentrations of the CAI-1 type molecule together with an internal standard (C9-CA1-1) in LB medium (Ng *et al.*, 2011). Correction factors (normalized to C9-CAI-1) were as follows: C8-CAI-1 (1.8 ± 0.7), Ea-C8-CAI-1 (0.7 ± 0.2), C10-CAI-1(1.0 ± 0.4), Ea-C10-CAI-1 (1.4 ± 0.4).

P. angustum growth and CAI-1 activity on Biolog PM Plates

P. angustum S14 was grown in LM medium with shaking overnight. Cells were collected by centrifugation at 10,000 x g. The cells were washed and resuspended in IF-0a (Biolog) containing 0.3 M NaCl and 1x Biolog redox dye mix F to reach a final OD_{600} of 0.06-0.07. The cell suspension was used to inoculate PM1 Microplates (100 μl per well for each carbon source). Absorbance at OD_{510} was measured after 36 hrs incubated at 30°C with shaking. CAI-1 activity was measured by bioluminescence assay as described using the WN1397 reporter strain carry $CqsS_{Pa}$ supplied with 50% v/v of the *P. angustum* cell suspension.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank members of the Bassler laboratory for insightful discussions and suggestions, and especially Julie S. Valastyan for critical reading of this work. We also thank the Groves lab for use of the GC-MS. This work was supported by the Howard Hughes Medical Institute, National Institutes of Health (NIH) Grant 5R01GM065859 and National Science Foundation (NSF) Grant MCB-0343821 to B.L.B. and an HHMI International Student Research fellowship to X.K.

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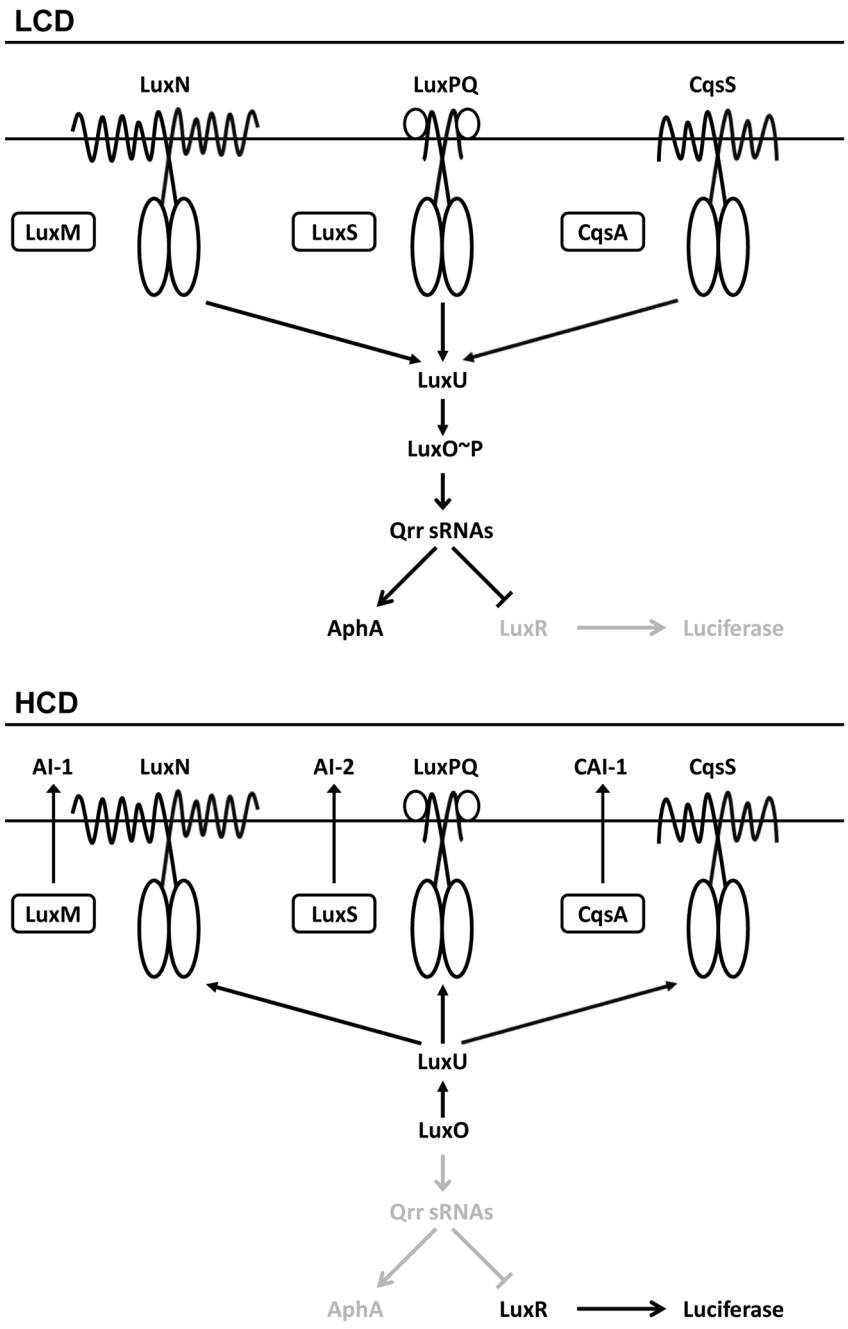

Fig. 1. The *V. harveyi* quorum-sensing system

Top, low cell density (LCD). Bottom, high cell density (HCD). See text for details of the signal relay mechanism. Important for the present work is that at HCD, LuxR activates expression of the luciferase operon.

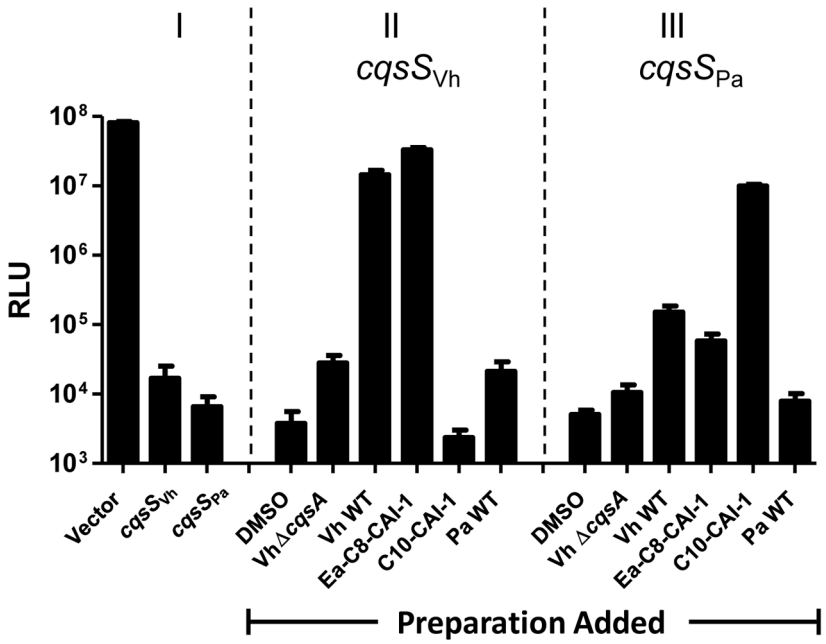

Fig. 2. *P. angustum* does not produce CAI-1 activity

A *V. harveyi* bioluminescent reporter strain (Δ*luxN* Δ*luxPQ* Δ*cqsAS*$_{Vh}$) expressing a vector, *cqsS*$_{Vh}$, or *cqsS*$_{Pa}$ was used to detect CAI-1 activity. **Panel I:** The reporter strain carrying a vector or the vector with cloned *cqsS*$_{Vh}$ or *cqsS*$_{Pa}$ when no molecules or culture fluids are added. **Panel II:** The reporter strain carrying *cqsS*$_{Vh}$. **Panel III:** The reporter strain carrying *cqsS*$_{Pa}$. In panels II and III, DMSO was used as the control, cell-free culture fluids from WT *V. harveyi*, a *V. harveyi* Δ*cqsA* mutant, or WT *P. angustum* were provided at 10% v/v and Ea-C8-CAI-1 or C10-CAI-1 were supplied at 5 μM. RLU, Relative Light Units (normalized to OD). Error bars represent standard deviations for three replicates.

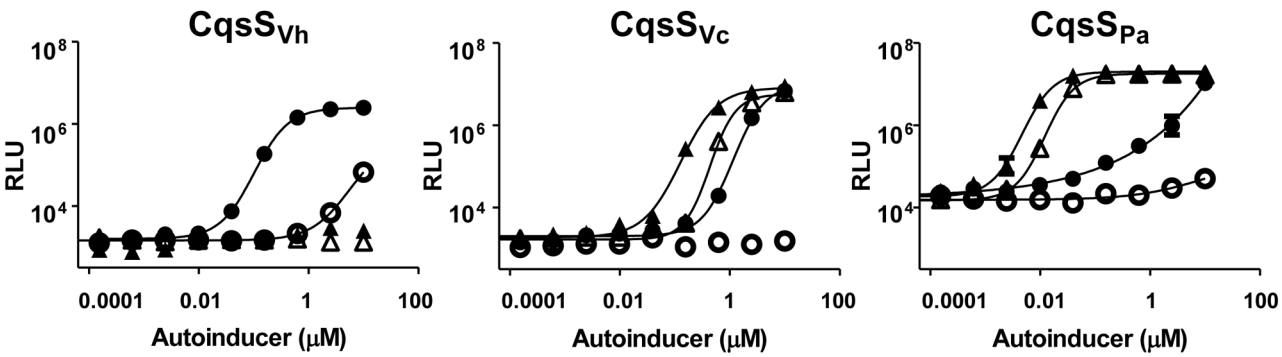

Fig. 3. *P. angustum* CqsS preferentially detects CAI-1 moieties with C10 tails

Bioluminescence from a *V. harveyi* reporter strain expressing *cqsS*$_{Vh}$, *cqsS*$_{Vc}$, or *cqsS*$_{Pa}$ was measured in response to Ea-C8-CAI-1 (closed circles); C8-CAI-1 (open circles); Ea-C10-CAI-1 (closed triangles); C10-CAI-1 (open triangles).

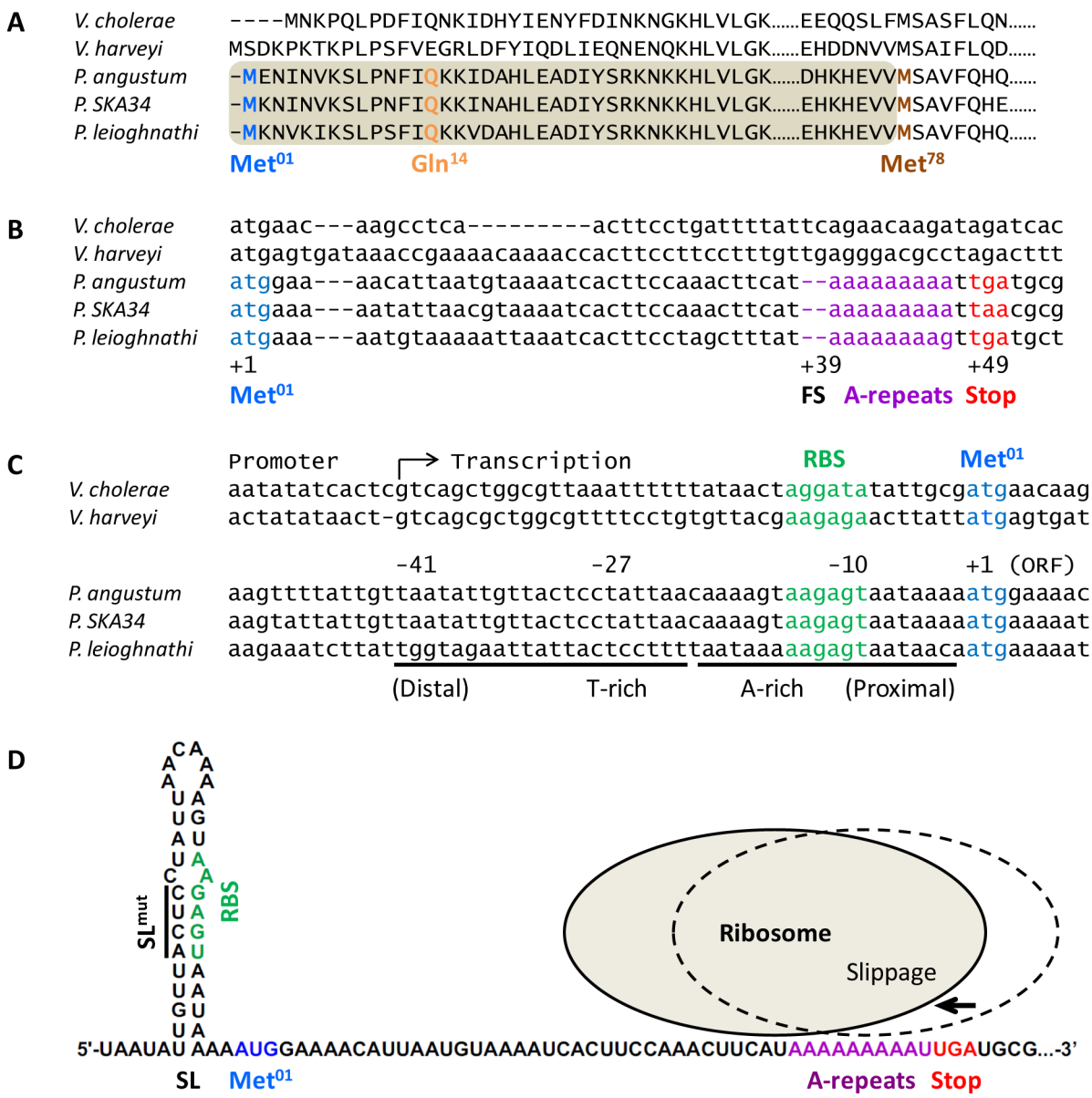

Fig. 4. The *P. angustum cqsA* gene contains a conserved frameshift mutation and a stem-loop in the 5′UTR occludes ribosome binding

A) Alignment of the $CqsA_{Pa}$ sequence with vibrio CqsA proteins. Dashes represent gaps in the alignment. Dots denote amino acids that are not shown. The annotated $cqsA_{Pa}$ ORF begins at Met^{78} (brown), and would encode a protein shorter than vibrio CqsA proteins and it would lack the amino acids in the shaded area. A protein beginning at the Met^{01} site (blue) would have a frameshift at Gln^{14} (orange). **B)** Alignment of vibrio and photobacterial *cqsA* genes. The $cqsA_{Pa}$ ORF starting at +1 (blue, Met^{01}) contains a two-nucleotide frameshift at +39 (FS), resulting in a stop codon at +49 (red, Stop) that immediately follows an adenosine-repeat (purple, A-repeats). **C)** Alignment of 5′UTRs of vibrio and photobacterial *cqsA* genes. A-rich and T-rich regions with dyad symmetry (underline) exist in $cqsA_{Pa}$, which could form a stem loop in the mRNA that blocks the conserved ribosome binding site (RBS, green). **D)** Predicted $cqsA_{Pa}$ mRNA folding. Colors are as in panel **C** and **D**. The putative stem-loop (SL) is shown. Sites of mutations engineered to disrupt the stem-loop are

shown by the line (SL^{mut}). The cartoon depicts how ribosome retraction by two nucleotides could restore *cqsA* expression.

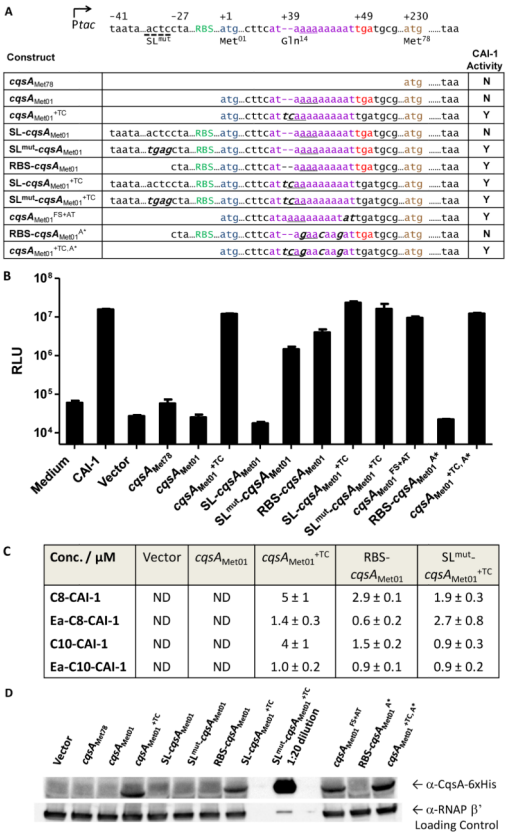

Fig. 5. CAI-1 activity is restored when the frameshift mutation is corrected and the 5′UTR stem-loop is disrupted

A) *P. angustum cqsA* constructs expressed in *E. coli*. Color designations are the same as in Fig. 4. Mutated nucleotides are noted in italics. Underlined trinucelotides represent the first codon that would be specified following the frameshift. CAI-1 activity shown in panel B is summarized as Y (yes) or N (no). **B)** *E. coli* harboring the *P. angustum cqsA* constructs in panel **A** were assayed for CAI-1 activity using the bioluminescent reporter strain carrying *cqsS*$_{Pa}$. Cell-free culture fluids were added at 1% v/v. Error bars represent standard deviations for three replicates. **C)** CAI-1–type molecules detected by GC-MS in culture fluids prepared from *E. coli* strains expressing the designated *cqsA*$_{Pa}$ constructs. ND, none detected. **D)** Western blot analysis of 6x-His tagged CqsA protein detected in lysates made from *E. coli* carrying the designated *cqsA*$_{Pa}$ constructs. The SL^{mut}-*cqsA*$_{Met01}$$^{+TC}$ sample was diluted 20-fold relative to the other samples on the gel because of the high CqsA protein production.