

## Three Parallel Quorum-Sensing Systems Regulate Gene Expression in *Vibrio harveyi*†

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**In a process called quorum sensing, bacteria communicate using extracellular signal molecules termed autoinducers. Two parallel quorum-sensing systems have been identified in the marine bacterium *Vibrio harveyi*. System 1 consists of the LuxM-dependent autoinducer HAI-1 and the HAI-1 sensor, LuxN. System 2 consists of the LuxS-dependent autoinducer AI-2 and the AI-2 detector, LuxPQ. The related bacterium, *Vibrio cholerae*, a human pathogen, possesses System 2 (LuxS, AI-2, and LuxPQ) but does not have obvious homologues of *V. harveyi* System 1. Rather, System 1 of *V. cholerae* is made up of the CqsA-dependent autoinducer CAI-1 and a sensor called CqsS. Using a *V. cholerae* CAI-1 reporter strain we show that many other marine bacteria, including *V. harveyi*, produce CAI-1 activity. Genetic analysis of *V. harveyi* reveals *cqsA* and *cqsS*, and phenotypic analysis of *V. harveyi* *cqsA* and *cqsS* mutants shows that these functions comprise a third *V. harveyi* quorum-sensing system that acts in parallel to Systems 1 and 2. Together these communication systems act as a three-way coincidence detector in the regulation of a variety of genes, including those responsible for bioluminescence, type III secretion, and metalloprotease production.**

Many bacteria coordinate gene expression using a process of cell-cell communication termed quorum sensing. Quorum sensing involves the production of, detection of, and response to extracellular signaling molecules known as autoinducers. Quorum-sensing systems allow bacteria to monitor their population density and to induce or repress particular genes in response to changes in cell number. Specifically, as a bacterial population grows, the extracellular concentration of autoinducer increases. When a critical autoinducer concentration is achieved, the bacteria respond as a group to coordinately alter target gene expression. Quorum-sensing systems have been identified in both gram-negative and gram-positive species of bacteria, and they are used to regulate diverse functions, such as bioluminescence, conjugation, virulence, biofilm formation, and antibiotic production (reviewed in reference 24).

*Vibrio harveyi* is a bioluminescent marine bacterium that lives in several milieus: it can exist free swimming in seawater, adhered to abiotic surfaces, as a constituent of biofilm consortia in marine animals, and in pathogenic associations with marine hosts (28). Two quorum-sensing systems have been identified in *V. harveyi*, and together they control functions such as bioluminescence (2, 3), type III secretion (TTS) (18), and siderophore (21), polysaccharide (21), and metalloprotease production (26) (Fig. 1A). The System 1 autoinducer, *N*-(3-hydroxybutanoyl) homoserine lactone (HSL) (denoted HAI-1 for *harveyi* autoinducer 1), is produced by LuxM and is detected by LuxN (2, 6). The System 2 autoinducer, called AI-2, is 3A-methyl-5,6-dihydro-furo(2,3-*D*)(1,3,2)dioxaborole-2,2,6,6A-tetraol, and the unborated precursor is made by LuxS

(7, 31, 35). Two proteins, LuxP and LuxQ, function together as the AI-2 sensor (3). LuxP is similar to periplasmic ribose binding proteins. Both LuxN and LuxQ are hybrid two-component proteins that contain sensor kinase and response regulator domains. The remainder of the signaling cascade is shared by System 1 and System 2 (Fig. 1A).

In *V. harveyi*, at low cell density (i.e., in the absence of the autoinducers), LuxN and LuxQ autophosphorylate and transfer phosphate to the shared phosphotransferase protein LuxU, which in turn passes it to the response regulator, LuxO (12–14). Phosphorylated LuxO is active and, in conjunction with  $\sigma^{54}$ , activates the expression of genes encoding small regulatory RNAs (sRNAs) that, together with the RNA chaperone Hfq, destabilize the mRNA encoding the activator protein LuxR (20, 21). LuxR is required for expression of the target genes in the quorum-sensing regulon (Fig. 1A) (18, 20, 22, 32). Because LuxR is required for transcription of *luxCDABE* (encoding luciferase), no light is produced under this condition. At high cell density, (i.e., in the presence of autoinducers), LuxN and LuxQ switch from kinases to phosphatases (14). Phosphate flow through the system is reversed, draining LuxO of phosphate (12). Unphosphorylated LuxO is inactive and thus cannot promote the expression of the sRNAs, leading to translation of *luxR* mRNA (20). LuxR binds the *luxCDABE* promoter and activates its expression, and *V. harveyi* produces light. Many other genes are controlled by quorum sensing in *V. harveyi*. While all require LuxR for their regulation, some of them, like *lux*, are induced at high cell densities, while others are repressed at high cell densities (Fig. 1A) (18, 26).

Miller et al. identified and characterized two two-component quorum-sensing circuits controlling quorum sensing in the human pathogen *Vibrio cholerae* (Fig. 1B) (25). Analysis of the completed genome of *V. cholerae* revealed that the *V. harveyi*-like System 2 (*luxS* and *luxPQ*) was present as well as *luxU* and *luxO*, and a genetic screen identified a novel autoinducer synthase named CqsA (for *cholerae* quorum-sensing autoinducer)

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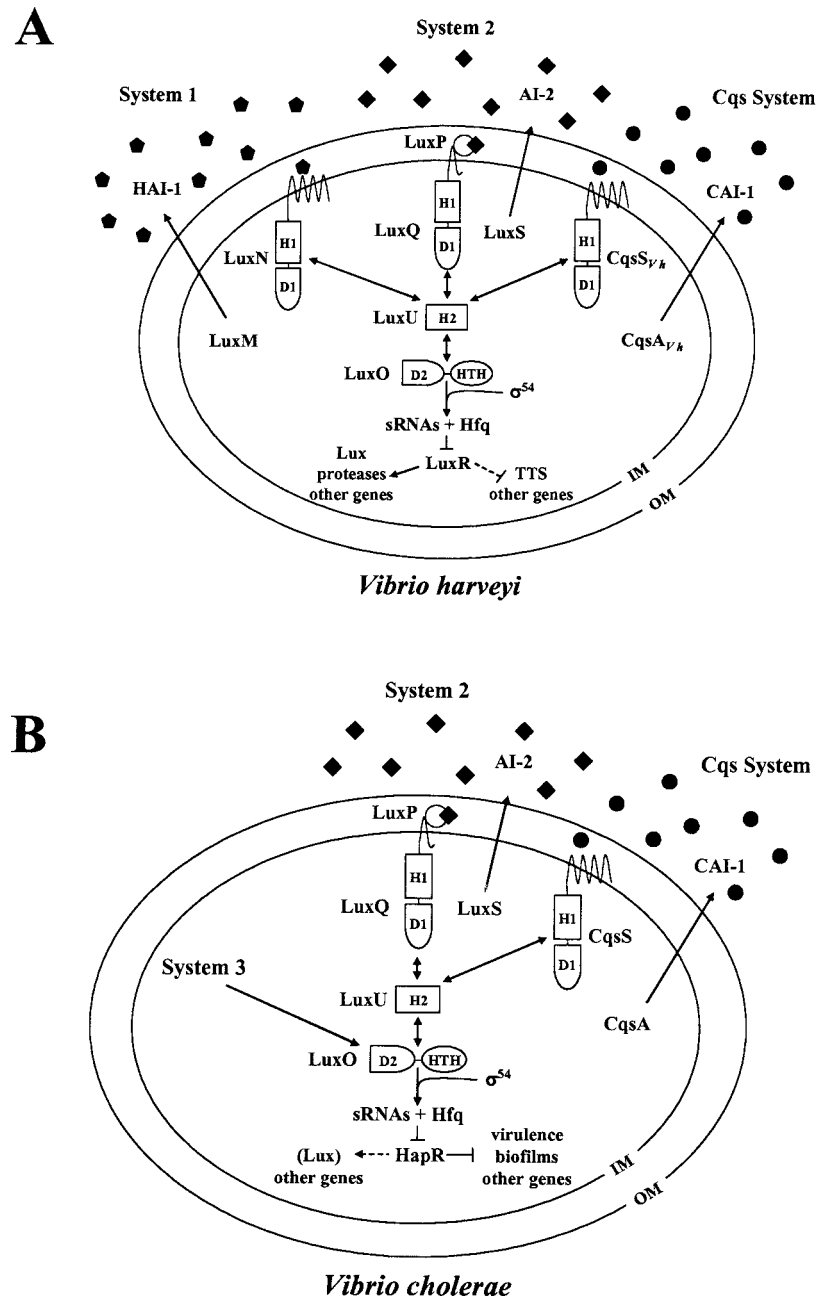


FIG. 1. Models of the *V. harveyi* and *V. cholerae* quorum-sensing systems. (A) *V. harveyi* has three parallel quorum-sensing systems that regulate the genes encoding bioluminescence (Lux), TTS, a secreted metalloprotease (VhpA), and other quorum-sensing-activated and -repressed genes. (B) *V. cholerae* possesses the CAI-1–CqsS system and the AI-2–LuxPQ system. *V. cholerae* also has an unidentified third circuit (System 3) that acts through LuxO. Symbols: pentagons, HAI-1; diamonds, AI-2; circles, CAI-1. Dotted lines denote targets for which regulation has not been shown to be direct. In both panels, phosphate flows toward LuxO at low cell density, and the flow of phosphate is reversed at high cell density (double-headed arrows). Details of the phosphorelay mechanism are provided in the text. Abbreviations: H, histidine; D, aspartate; HTH, helix-turn-helix; IM, inner membrane; OM, outer membrane.

and a cognate sensor named CqsS (for *cholerae* quorum-sensing sensor). The CqsA-dependent autoinducer is called CAI-1 (for *cholerae* autoinducer 1), and its structure has not been determined. The *V. cholerae* quorum-sensing cascade functions analogously to that of *V. harveyi*, and the downstream regulators LuxU, LuxO, sRNA, Hfq, and HapR (which is homolo-

gous to LuxR of *V. harveyi*) function identically to those of *V. harveyi* (Fig. 1B) (20, 25). However, many of the functions controlled by quorum sensing in *V. cholerae* (for example, those specifying virulence determinants) are not identical to those regulated by quorum sensing in *V. harveyi* (for example, bioluminescence and type III secretion).

LuxS and AI-2 production are widespread in the bacterial kingdom, and AI-2 is proposed to be used for interspecies communication (1). In contrast, LuxM and HAI-1 activity appear to be restricted to *V. harveyi* and the closely related bacterium *Vibrio parahaemolyticus*, indicating that this signal molecule is relatively species specific (1). The newly identified *V. cholerae* CAI-1 synthase, CqsA, is not similar to LuxM nor any other known autoinducer synthase. Additionally, LuxN does not recognize CAI-1 (M. B. Miller and B. L. Bassler, unpublished data). These findings led us to speculate that CAI-1 is a novel autoinducer. We wondered if other bacteria produce CAI-1 or if this signal is unique to *V. cholerae*. In this report we show that several vibrios, including *V. harveyi*, produce CAI-1 activity. Genetic analysis in *V. harveyi* reveals that the Cqs system is present and acts as a third quorum-sensing system that, in parallel with Systems 1 and 2, controls the quorum-sensing target genes. All three autoinducers display synergistic activity, and thus the *V. harveyi* circuit is a three-way coincidence detector for the signals HAI-1, AI-2, and CAI-1. However, the *V. harveyi* CAI-1–CqsS system appears to differ from Systems 1 and 2 by responding to autoinducer at much lower cell densities.

#### MATERIALS AND METHODS

**Bacterial strains and media.** The relevant genotypes of all *V. harveyi* and *V. cholerae* strains and plasmids are listed in Table 1. Other species of bacteria and their strain names are listed in Table 2. *Escherichia coli* strain DH5 $\alpha$  (F<sup>-</sup>  $\phi$ 80dlacZ $\Delta$ M15  $\Delta$ (lacZYA-argF)U169 *deoR recA1 endA1 hsdR17*(r<sub>K</sub><sup>-</sup> m<sub>K</sub><sup>+</sup>) *phoA supE44*  $\lambda$ <sup>-</sup> *thi-1 gyrA96 relA1*) (Promega) was used for cloning. *E. coli* strains were grown at 37°C with aeration. The *V. cholerae* bioreporter MM920 was grown in Luria-Bertani (LB) medium with tetracycline (TET) at 30°C with shaking. Tetracycline was added to all cultures with pLAFR for cosmid maintenance. *V. harveyi* cultures were grown in Luria-Marine (LM) medium for all genetic experiments and conjugations. Cell-free culture (14- to 16-h) fluids were prepared as described (2). Thirty-percent LM cell-free culture fluids were added to MM920, and 10% autoinducer bioassay (AB) cell-free culture fluids were added to *V. harveyi* strains JMH670 and JMH674. AB medium was used for bioluminescence and  $\beta$ -galactosidase ( $\beta$ -Gal) assays. The recipes for AB medium and LM medium have been reported previously (16). Cells were plated on LM agar plates for CFU determinations. Antibiotic (Sigma) concentrations used were as follows: ampicillin (AMP), gentamicin (GM), and kanamycin (KAN), 100 mg/liter; chloramphenicol (CM), and TET, 10 mg/liter; streptomycin (SM), 1,000 mg/liter; polymyxin B (PB), 50 U/liter.

**DNA manipulations.** All DNA manipulations were performed using standard procedures (30). Oligonucleotides used for PCR and sequencing reactions can be found in the supplemental material (see Table S1). At the National Center for Biotechnology Information, sequencing data for the *V. harveyi* Cqs locus can be found under the accession number AY625893, and sequencing data for the *vhpA* locus is under the accession number AYG30354.

**CAI-1 activity assay.** *V. harveyi* overnight cultures were diluted and grown to an optical density at 600 nm (OD<sub>600</sub>) of 1. Cell-free culture fluids were prepared from these cultures as previously described. A dense overnight culture of *V. cholerae* MM920 ( $\Delta$ cqsA  $\Delta$ luxQ pBB1) was diluted 1:10 in LB with TET. Thirty microliters of cell-free culture fluid was added to 70  $\mu$ l of diluted *V. cholerae* reporter MM920 in a sterile 96-well microtiter dish (Corning) and incubated at 30°C with shaking (225 rpm). Luminescence from the reporter strain was measured with a Wallac microbeta multiplate scintillation counter every half hour until the luminescence of the control (reporter with medium alone) increased. The fold induction of the reporter was measured as counts per second (cps) of reporter plus culture fluid divided by cps of the control MM920 reporter with medium alone. For the measurement of *V. harveyi* CAI-1 accumulation over time, an overnight culture of *V. harveyi* strain BB120 was diluted 1:1,000 in fresh LM medium and grown in a flask at 30°C for 22 h. Aliquots were removed over time, the OD<sub>600</sub> was measured, and cell-free culture fluids were prepared. A standard CAI-1 (MM920) activity assay was performed on all the cell-free culture fluids after 22 h.

**Screen for *V. harveyi* CAI-1 synthase.** A *V. harveyi* genomic library carried in the *E. coli* luxS mutant strain DH5 $\alpha$  (2) was grown overnight in 96-well microtiter

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant features	Reference or source
<i>V. harveyi</i> strains		
BB120	Wild type	1
BB170	<i>luxN::Tn5</i>	2
BB721	<i>luxO::Tn5</i>	4
BB886	<i>luxPQ::Tn5</i>	3
JAF375	<i>luxN::Cm<sup>r</sup> luxQ::Kan<sup>r</sup></i>	12
JAF483	<i>luxOD47A</i> linked to Kan <sup>r</sup>	12
JAF536	<i>luxU::Tn5</i>	13
JAF548	<i>luxOD47E</i> linked to Kan <sup>r</sup>	12
JAF633	$\Delta$ luxM linked to Kan <sup>r</sup>	14
KM387	$\Delta$ luxS	This study
KM357	<i>luxR::Tn5</i>	18
KM413	$\Delta$ luxS $\Delta$ luxM	This study
JMH597	<i>luxN::Tn5 cqsS::Cm<sup>r</sup></i>	This study
JMH598	<i>cqsS::Cm<sup>r</sup></i>	This study
JMH603	<i>cqsA::Cm<sup>r</sup></i>	This study
JMH605	$\Delta$ luxM linked to Kan <sup>r</sup> ; <i>cqsA::Cm<sup>r</sup></i>	This study
JMH606	$\Delta$ luxS <i>cqsA::Cm<sup>r</sup></i>	This study
JMH612	<i>luxPQ::Tn5 cqsS::Cm<sup>r</sup></i>	This study
JMH621	<i>luxU::Tn5 cqsA::Cm<sup>r</sup></i>	This study
JMH625	$\Delta$ luxN <i>luxQ::Tn5</i>	This study
JMH626	$\Delta$ luxN <i>luxQ::Tn5 cqsA::Cm<sup>r</sup></i>	This study
JMH628	$\Delta$ luxN <i>luxQ::Tn5 cqsS::Cm<sup>r</sup></i>	This study
JMH634	$\Delta$ luxM $\Delta$ luxS <i>cqsA::Cm<sup>r</sup></i>	This study
JMH670	$\Delta$ luxM $\Delta$ luxS <i>cqsA::Cm<sup>r</sup> vhpA::Tn5lacZ</i>	This study
JMH674	$\Delta$ luxM $\Delta$ luxS <i>cqsA::Cm<sup>r</sup> vcrD::Tn5lacZ</i>	This study
JMH763	<i>cqsAS::Cm<sup>r</sup></i>	This study
JMH774	<i>luxOD47E cqsA::Cm<sup>r</sup></i>	This study
<i>V. cholerae</i> strains		
KSK1052	$\Delta$ luxS	25
MM893	$\Delta$ cqsA	25
Plasmids		
pLAFR2	Broad-host-range cosmid; <i>mob</i> , Tet <sup>r</sup>	15
pRK2013	Broad host range, <i>tra</i> , Kan <sup>r</sup>	9
pPHI1	Broad host range, <i>tra</i> , <i>mob</i> , Gm <sup>r</sup>	5
pGEM-T	Cloning vector; Amp <sup>r</sup>	Promega
pALTER Amp	Amp <sup>r</sup>	Promega
pALTER Tet	Cloning vector; Tet <sup>r</sup>	Promega
pBluescript SK	Cloning vector; Amp <sup>r</sup>	Stratagene
pKD3	PCR template for Wanner deletions; Cm <sup>r</sup>	8
pCP20	Ts FLP recombinase plasmid; Amp <sup>r</sup> , Cm <sup>r</sup>	8
pKAS46	R6K replication origin; Sm <sup>r</sup>	33
pBB1	pLAFR with <i>V. harveyi luxCDABE</i> locus	
pBB154	pLAFR with <i>luxM</i> and <i>luxN</i> locus	2
pMM467	Tet <sup>r</sup> pALTER with <i>cqsA</i>	This study
pKM556	pLAFR2 with $\Delta$ luxM	26
pKM755	pLAFR2 with <i>vhpA::Tn5lacZ</i> Kan <sup>r</sup>	This study
pJMH17F7	pLAFR2 with <i>cqsA<sub>Vh</sub></i> and <i>cqsS<sub>Vh</sub></i> locus; Tet <sup>r</sup>	This study
pJM25	pLAFR2 with type III secretion locus; Tet <sup>r</sup>	18
pJMH159	pJMH17F7 <i>cqsA<sub>Vh</sub>::Tn5lacZ</i> Kan <sup>r</sup>	This study
pJMH163	pJMH17F7 <i>cqsA<sub>Vh</sub>::Tn5lacZ</i> Kan <sup>r</sup>	This study
pJMH164	pJMH17F7 <i>cqsA<sub>Vh</sub>::Tn5lacZ</i> Kan <sup>r</sup>	This study
pJMH240	pJMH17F7 with <i>cqsA<sub>Vh</sub>::Cm<sup>r</sup></i>	This study
pJMH244	pJMH17F7 with <i>cqsS<sub>Vh</sub>::Cm<sup>r</sup></i>	This study
pJMH253	pGEM-T with <i>cqsA<sub>Vh</sub></i>	This study
pJMH273	pLAFR with <i>cqsA<sub>Vh</sub></i>	This study
pJMH280	pGEM-T with <i>cqsS<sub>Vh</sub></i>	This study
pJMH282	pLAFR with <i>cqsS<sub>Vh</sub></i>	This study
pJMH300	pBB154 with $\Delta$ luxN	This study
pJMH325	pJMH25 with <i>vcrD::Tn5lacZ</i> Kan <sup>r</sup>	This study
pJMH422	pJMH17F7 with <i>cqsA<sub>Vh</sub>::Cm<sup>r</sup></i>	This study

dishes in 150  $\mu$ l of LB with TET. An overnight culture of MM920 was diluted 1:50 in fresh LB with TET. *E. coli* overnight cultures were added to 96-well microtiter dishes containing 100  $\mu$ l of the diluted MM920 *V. cholerae* reporter by stamping. These cells were coincubated for approximately 5 h at 30°C with shaking, and the resulting bioluminescence from each well was measured using the Wallac microbeta multiplate scintillation counter. MM1467 was used as a positive control for CAI-1 activity. *E. coli* clones that caused 500- to 1,000-fold induction of bioluminescence in the MM920 reporter were chosen for further

TABLE 2. Induction of bioluminescence in the *V. cholerae* CAI-1 reporter strain MM920 by cell-free culture fluids prepared from other bacteria

Species and strain	CAI-1 activity <sup>a</sup>
<i>Vibrio cholerae</i> CG706.....	11,531 ± 1,558
<i>Vibrio harveyi</i> BB120.....	3,707 ± 298
<i>Vibrio anguillarum</i> 19264.....	2,198 ± 165
<i>Vibrio alginolyticus</i> 118.....	1,723 ± 206
<i>Vibrio parahaemolyticus</i> BB22.....	1,417 ± 36
<i>Vibrio furnissii</i> 1514.....	1,417 ± 76
<i>Vibrio angustum</i> 70.....	288 ± 18
<i>Photobacterium leiognathi</i> 721.....	46 ± 6
<i>Photobacterium phosphoreum</i> NZ-11-D.....	43 ± 3
<i>Vibrio proteolyticus</i> 145.....	10 ± 2
<i>Vibrio vulnificus</i> JJ077.....	3.6 ± 0.8
<i>Vibrio natriegens</i> 77.....	1.5 ± 0.9
<i>Vibrio fischeri</i> MJ1.....	1.1 ± 0.1
<i>Vibrio logei</i> SR6.....	1.0 ± 0.1
<i>Escherichia coli</i> MG1655.....	1.1 ± 0.2
<i>Salmonella typhimurium</i> PS1.....	0.9 ± 0.03

<sup>a</sup> CAI-1 activity is defined as the fold increase in induction of bioluminescence that occurs in *V. cholerae* MM920 in the presence of 30% cell-free culture fluid over that that occurs when medium alone is added.

analysis. To identify the CAI-1 synthase gene,  $\lambda$ Tn5lacZ mutagenesis was performed on cosmid pJMH17F7 as described previously (2). Mutagenized cosmid pools were transformed into *E. coli* DH5 $\alpha$ , and recombinants were selected with KAN and TET. Recombinants were arrayed in microtiter dishes and were assayed for loss of CAI-1 activity, indicated by the inability to induce luminescence in MM920, as described above.

**Cloning and sequencing the *V. harveyi* *cqsA* and *cqsS* genes.** Tn5lacZ mutagenized pJMH17F7 cosmids were isolated from *E. coli* strains lacking CAI-1 activity and subsequently digested with EcoRI and shotgun cloned into the pBluescript SK and pALTER Amp<sup>r</sup> vectors, selecting for resistance to AMP and KAN, respectively. Positive clones were sequenced using an outwardly directed primer (Tn50ISR) that hybridized to the arm of Tn5lacZ. The locus was also cloned by digesting cosmid pJMH17F7 with EcoRI, HindIII, or PstI. Fragments were shotgun cloned into pBluescript SK and transformed into *E. coli* DH5 $\alpha$ . Transformants were arrayed in microtiter dishes, stamped into MM920 reporter diluted 1:50, and screened for CAI-1 activity. The inserts in plasmids from transformants that were able to induce luminescence in the MM920 reporter were sequenced. DNA sequence data were analyzed and aligned using the Sequencher 4.1 program (Gene Codes) and the National Center for Biotechnology Information's blastx program.

***V. harveyi* *cqsA* and *cqsS* deletion constructions.** Deletions of *cqsA*<sub>Vh</sub> (*cqsA* from *V. harveyi*), *cqsS*<sub>Vh</sub>, (*cqsS* from *V. harveyi*), and the double deletion of the *cqsA*<sub>Vh</sub> and *cqsS*<sub>Vh</sub> open reading frames (ORFs) in cosmid pJMH17F7 were constructed by the method of Datsenko and Wanner (8). The Cm<sup>r</sup> cassette from vector pKD3 was amplified with primers CqsAf-1 and CqsAr-2 for the *cqsA*<sub>Vh</sub> ORF deletion and CqsSf1 and CqsSr-2 for the *cqsS*<sub>Vh</sub> deletion, and primers CqsAf-1 and VhCqsSAWanner were used to construct the deletion of *cqsAS*<sub>Vh</sub> in pJMH17F7. The resulting pJMH17F7 deletion cosmids pJMH240 ( $\Delta$ *cqsA*<sub>Vh</sub>::Cm<sup>r</sup>), pJMH244 ( $\Delta$ *cqsS*<sub>Vh</sub>::Cm<sup>r</sup>), and pJMH422 ( $\Delta$ *cqsAS*<sub>Vh</sub>::Cm<sup>r</sup>) were conjugated into *V. harveyi* strains by using a triparental mating procedure as previously reported. The Cm<sup>r</sup>-marked deletions were subsequently transferred to the *V. harveyi* chromosome by an allelic exchange protocol (2). The deletions were verified by PCR using the primers *cqsSA*flankf and *cqsSA*flankr and, in the case of *cqsA*<sub>Vh</sub> deletions, by observation of loss of CAI-1 activity in the MM920 reporter assay.

**Cloning of *cqsA*<sub>Vh</sub> and *cqsS*<sub>Vh</sub>.** *cqsS*<sub>Vh</sub> and *cqsA*<sub>Vh</sub> were PCR amplified from the chromosome of wild-type (WT) *V. harveyi* BB120 using the following primer pairs: fCqsSXbaI and rCqsSXbaI for *cqsS*<sub>Vh</sub>, and fCqsAXbaI and rCqsAXbaI for *cqsA*<sub>Vh</sub>. Both products were subcloned into the cloning vector pGEM-T (Promega) at the XbaI site and sequenced. The resulting vectors pJMH253 and pJMH280 were digested with XbaI, and the inserts were cloned into the broad-host-range cosmid pLAFR. These constructions were transformed into *E. coli* DH5 $\alpha$  to produce cosmids pJMH273 and pJMH282, respectively.

***V. harveyi* strain construction.** An unmarked *luxN* deletion in cosmid pBB1154 was constructed by the method of Datsenko and Wanner using the oligonucleotides WannerluxNf and WannerluxNr (8). Deletions were confirmed with the flanking primers LuxNflankf and LuxNflankr. The unmarked *luxN* deletion cos-

mid pJMH300 was used for an allelic replacement of the Cm<sup>r</sup> marker harbored in *luxN* in *V. harveyi* JAF375 (*luxN*::Cm<sup>r</sup> *luxQ*::Tn5 Kan<sup>r</sup>) to make strain JMH625 ( $\Delta$ *luxN* *luxQ*::Tn5 Kan<sup>r</sup>). KM387 ( $\Delta$ *luxS*) was constructed by the method of Skorupski and Taylor (33) using PCR primer pairs LuxS1-KpnI-LuxS2-NotI and LuxS3-NotI-LuxS4-EcoRI. Both PCR products were ligated simultaneously into pKAS46, and the deletion was moved onto the chromosome of a spontaneous streptomycin-resistant strain of WT *V. harveyi* to construct KM387 ( $\Delta$ *luxS*). We moved the unmarked *luxM* deletion from cosmid pKM556 onto the chromosome of KM387 and screened for dark mutants in microtiter dishes to make KM413 ( $\Delta$ *luxM*  $\Delta$ *luxS*). The Cm<sup>r</sup> marker from the cosmids pJMH244 (*cqsS*<sub>Vh</sub>::Cm<sup>r</sup>) and pJMH1240 (*cqsA*<sub>Vh</sub>::Cm<sup>r</sup>) was introduced onto the chromosome of JMH625 by allelic replacement to construct the triple mutants JMH628 (*luxN* *luxQ* *cqsS*<sub>Vh</sub>) and JMH626 (*luxN* *luxQ* *cqsA*<sub>Vh</sub>), respectively. The Cm<sup>r</sup> markers from pJMH240 (*cqsA*<sub>Vh</sub>::Cm<sup>r</sup>) and pJMH244 (*cqsS*<sub>Vh</sub>::Cm<sup>r</sup>) were subsequently transferred to the chromosome of KM413 ( $\Delta$ *luxM*  $\Delta$ *luxS*) to make the triple mutant JMH634 ( $\Delta$ *luxM*  $\Delta$ *luxS* *cqsA*<sub>Vh</sub>::Cm<sup>r</sup>). The *verD*::Tn5 Kan<sup>r</sup> construct pJMH325 and the *vhpA*::Tn5 Kan<sup>r</sup> construct pKM755 were mobilized onto the chromosome of the triple synthase mutant JMH634 ( $\Delta$ *luxM*  $\Delta$ *luxS* *cqsA*<sub>Vh</sub>::Cm<sup>r</sup>) by allelic exchange with selection for Kan<sup>r</sup> to produce *V. harveyi* strains JMH674 ( $\Delta$ *luxM*  $\Delta$ *luxS* *cqsA*<sub>Vh</sub> *verD*::Tn5 Kan<sup>r</sup>) and JMH670 ( $\Delta$ *luxM*  $\Delta$ *luxS* *cqsA*<sub>Vh</sub> *vhpA*::Tn5 Kan<sup>r</sup>), respectively.

**Bioluminescence assays.** All bioluminescence assays were performed by using a previously reported protocol (2). Data are reported as relative light units (RLU) (counts min<sup>-1</sup> ml<sup>-1</sup> × 1,000/CFU ml<sup>-1</sup>). To measure bioluminescence at very low cell density, the overnight cultures were diluted 1:500,000 at the start of the assay, and both luminescence and cell density were measured every hour for 14 h. For single-time-point assays, overnight cultures were diluted 1:500 in AB medium and grown for approximately 8 h. At this time, the light and cell density of the cultures were measured. All single-time-point experiments were performed in triplicate.

**$\beta$ -Galactosidase assays.** *V. harveyi* strains were grown overnight in the presence of 10% cell-free culture fluids containing various combinations of autoinducers. A 1.5-ml sample of culture was resuspended in 1 ml of Z buffer, and  $\beta$ -Gal assays were performed in triplicate by a previously described method (34).  $\beta$ -Gal units are reported as (V<sub>max</sub> × dilution factor)/OD<sub>600</sub>.

## RESULTS

***V. harveyi* produces CAI-1 activity.** To test for production of CAI-1 in different species of bacteria, we used a bioluminescent reporter strain of *V. cholerae*. This strain, named MM920, is  $\Delta$ *cqsA* and  $\Delta$ *luxQ* and harbors a vector containing the *V. harveyi* luciferase operon. MM920, while unable to produce CAI-1 or respond to AI-2, can detect and respond to exogenously added CAI-1 because CqsS is intact (25). To demonstrate that the reporter acts as expected, we show that addition of cell-free culture fluids harvested from high-cell-density *V. cholerae*  $\Delta$ *luxS* (AI-2<sup>-</sup>) stimulates a 7,000-fold increase in light production, while cell-free fluids prepared from a *V. cholerae*  $\Delta$ *cqsA* (CAI-1<sup>-</sup>) strain fail to induce light production in MM920 (Fig. 2A) (25).

A variety of bacterial species were grown to stationary phase, cell-free culture fluids from these species were harvested and added to MM920, and light production was measured. The preparations from *V. cholerae*, *V. harveyi*, *Vibrio anguillarum*, *Vibrio alginolyticus*, *V. parahaemolyticus*, and *Vibrio furnissii*, induced an over 1,000-fold increase in bioluminescence in MM920, suggesting that these vibrios produce CAI-1 activity (Table 2 and Fig. 2A), while other bacteria tested either produced an activity that induced bioluminescence in the reporter to a much lesser extent or did not produce any activity (Table 2).

To show that, at least in the case of *V. harveyi*, the known autoinducers are not responsible for stimulation of the reporter strain, we assayed cell-free culture fluids from the *V. harveyi* WT and single (*luxM* or *luxS*) and double (*luxM* *luxS*)

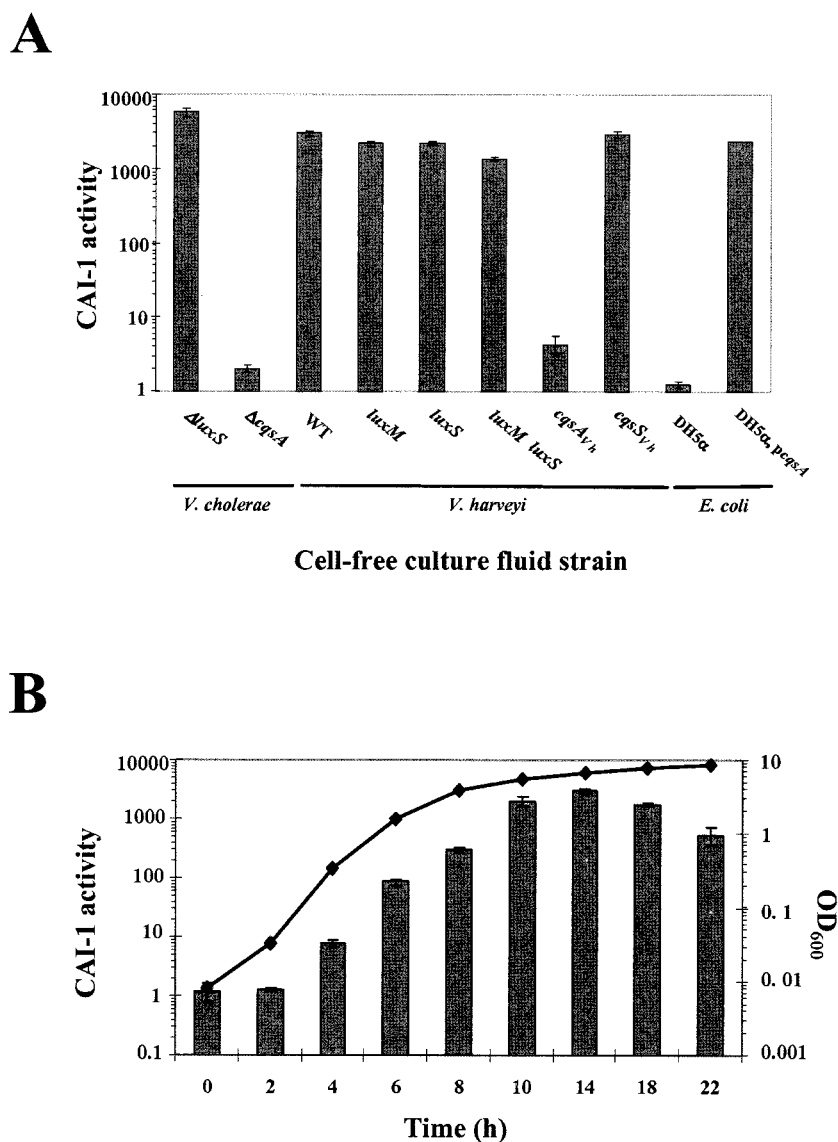


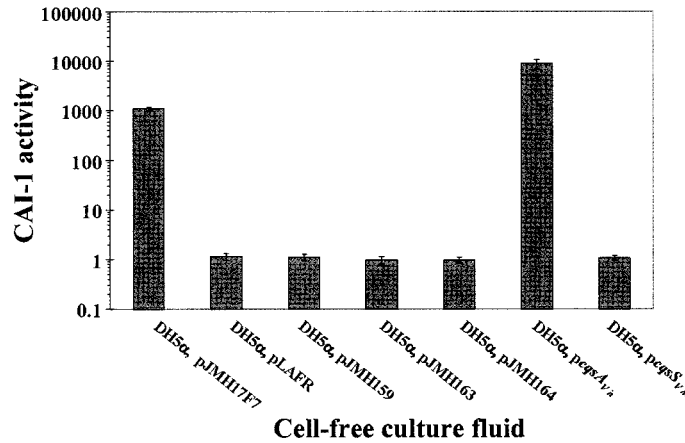
FIG. 2. Analysis of cell-free culture fluids for CAI-1 activity. (A) Cell-free culture fluids were prepared from the strains shown and added at 30% (vol/vol) to *V. cholerae* MM920. Light production by MM920 was measured. Strain names are as follows (left to right): KSK1052, MM893, BB120, JAF633, KM387, KM413, JMH603, JMH598, DH5 $\alpha$ , MM1467. (B) CAI-1 production over the growth curve is shown for WT *V. harveyi* strain BB120. Samples were collected every 2 h, the OD<sub>600</sub>s were measured, and cell-free culture fluids were prepared and assayed for CAI-1 activity using MM920. CAI-1 activity is reported as the fold induction of light production in MM920.

autoinducer synthase mutants in the *V. cholerae* CAI-1 reporter assay. All three strains produce a high level of activity capable of inducing light production in MM920 (Fig. 2A). Neither HAI-1 nor AI-2 prepared by in vitro procedures induces light production in MM920 (Miller and Bassler, data not shown). Together, these results suggest that an additional autoinducer synthase exists in *V. harveyi* and is responsible for producing the CAI-1 activity. Interestingly, we noticed that while the double *luxM luxS* mutant produces the CAI-1 activity, it consistently produces approximately fivefold less of this activity than does either the single *luxM* mutant or the single *luxS* mutant. Figure 2B shows that similar to most other autoinducers, in *V. harveyi*, CAI-1 activity accumulates in the culture fluids over time, and maximal CAI-1 activity is observed in early stationary phase. Finally, *V. harveyi* consistently produces

less CAI-1 activity than does *V. cholerae* (Table 2 and Fig. 2). We do not know whether *V. harveyi* produces a CAI-1 molecule that differs from and is detected less efficiently by *V. cholerae* than the endogenous CAI-1 or if *V. harveyi* simply produces less of the identical CAI-1 molecule that *V. cholerae* produces.

**Identification of the *V. harveyi* CAI-1 synthase gene.** To devise a genetic screen to identify the *V. harveyi* gene encoding the putative CAI-1 synthase, we made use of the following two observations. First, cell-free culture fluids prepared from *E. coli* strain DH5 $\alpha$  do not induce luminescence in the MM920 reporter, indicating that they lack CAI-1 activity (Fig. 2A, bar labeled DH5 $\alpha$ ). Second, culture fluids prepared from *E. coli* DH5 $\alpha$  carrying the cloned *V. cholerae* CAI-1 synthase gene *cqsA* induce bioluminescence in the reporter, indicating that

**A**



**B**

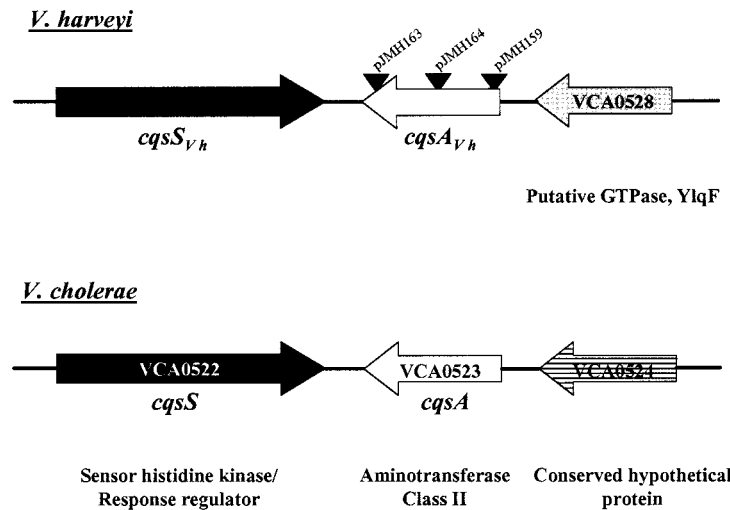


FIG. 3. The *cqs* locus in *V. harveyi*. (A) CAI-1 activity is shown for recombinant *E. coli* DH5α strains carrying either the cosmid pLAFR or pLAFR containing vibrio DNA. pJM17F7 carries 25 kb of *V. harveyi* genomic DNA containing the gene responsible for CAI-1 activity. pJM159, pJM163, and pJM164 contain Tn5lacZ insertions in pJM17F7. pJM273 carries only the *cqsA<sub>vh</sub>* gene, and pJM282 carries only the *cqsS<sub>vh</sub>* gene. (B) Map of the *cqs* locus of *V. harveyi* and *V. cholerae*. Black triangles indicate the sites of Tn5lacZ insertions in pJM163, pJM164 and pJM159. The *cqsS* and *cqsA* genes are shown in black and white, respectively. The downstream ORF in *V. harveyi* has amino acid homology to VCA0528 and is shown in gray. This gene was not fully sequenced. The downstream ORF in *V. cholerae*, denoted VCA0524, is shown in stripes.

introduction of the *cqsA* gene into *E. coli* is sufficient for CAI-1 production and release (Fig. 2A, bar labeled DH5α, pcqsA<sub>vh</sub>). We reasoned that we could identify the *V. harveyi* CAI-1 synthase gene by introducing a *V. harveyi* genomic cosmid library into *E. coli* DH5α and assaying cell-free culture fluids from the transformants for the ability to induce luminescence in the CAI-1 reporter MM920.

One thousand *E. coli* DH5α transformants, each containing a cosmid carrying ~25 kb of *V. harveyi* genomic DNA, were tested for activation of light production in *V. cholerae* MM920.

Six positive transformants were identified, and restriction mapping and Southern blot analyses revealed that each of the six contained a set of overlapping fragments of *V. harveyi* genomic DNA (data not shown). One of these cosmids, pJM17F7, was chosen for further analysis. Cell-free culture fluids from *E. coli* DH5α carrying pJM17F7 induce luminescence 1,120-fold in MM920 (Fig. 3A), whereas preparations from *E. coli* DH5α carrying the vector alone do not (Fig. 3A, bar labeled DH5α, pLAFR). The amount of CAI-1 activity produced by DH5α carrying pJM17F7 is comparable to that made by *E. coli*

DH5 $\alpha$  carrying the *V. cholerae* *cqsA* gene in *trans* (over 1,000-fold stimulation of the reporter) (Fig. 2A).

The cosmid pJMH17F7 was subjected to Tn5lacZ mutagenesis to identify the locus responsible for producing the CAI-1 activity. Pools of transposon-mutagenized pJMH17F7 cosmid DNA were introduced into *E. coli* DH5 $\alpha$ . Transformants were arrayed in 96-well plates and coincubated with the *V. cholerae* reporter strain MM920. Subsequently, light production by MM920 was quantitated. Three *E. coli* strains that did not stimulate light production in the reporter were identified, indicating that the cosmids they carried (pJMH159, pJMH163, and pJMH164) had acquired Tn5lacZ insertions that inactivated some function required for CAI-1 production (Fig. 3A).

Sequencing of the DNA flanking the Tn5lacZ insertion sites in pJMH159, pJMH163, and pJMH164 revealed that all three transposon insertions were located in the same ORF (Fig. 3B). This ORF encodes a protein with 57% identity to *V. cholerae* VCA0523, recently designated *cqsA* (25), and so we designate this ORF *cqsA<sub>Vh</sub>*. Our sequence analysis showed that a gene encoding a two-component hybrid sensor kinase with 48% identity to *V. cholerae* VCA0522 (designated *cqsS*) was situated 3' to *cqsA<sub>Vh</sub>* with the same orientation as in *V. cholerae* (Fig. 3B). We designate this second gene *cqsS<sub>Vh</sub>*. The loci adjacent to the 5' termini of the *V. harveyi* and *V. cholerae* *cqsA* genes are not similar and encode a conserved hypothetical protein (*V. cholerae*) and a putative GTPase annotated as Y1qF (*V. harveyi*). Database analysis shows that *cqsA<sub>Vh</sub>* and *cqsS<sub>Vh</sub>* share 82 and 78% identity with VPA0711 and VPA0710 in *V. parahaemolyticus*. This latter result is not surprising given that *V. parahaemolyticus* also produces CAI-1 activity (Table 2).

The *cqsA<sub>Vh</sub>* promoter and ORF were amplified from the chromosome of WT *V. harveyi* and cloned onto the low-copy-number pLAFR cosmid. This construct was introduced into *E. coli* DH5 $\alpha$ , and the strain was tested for CAI-1 production (Fig. 3A, bar labeled DH5 $\alpha$  *pcqsA<sub>Vh</sub>*). The results show that the *V. harveyi* *cqsA* gene confers the ability to produce CAI-1 to *E. coli*. Not surprisingly, introduction of *cqsS<sub>Vh</sub>* (encoding the putative CAI-1 sensor) into *E. coli* does not promote CAI-1 production (Fig. 3A, bar labeled DH5 $\alpha$ , *pcqsS<sub>Vh</sub>*). Interestingly, the cosmid carrying the *cqsA<sub>Vh</sub>* gene without any flanking *V. harveyi* genomic DNA produces substantially greater activity than does the parent cosmid carrying the *cqsA<sub>Vh</sub>* gene on a 25-kb genomic insert. This result suggests that a repressor of *cqsA<sub>Vh</sub>* expression or CAI-1 production could be linked to the *cqsA<sub>Vh</sub>* gene.

Although the *V. harveyi* genome sequence is not available, in the closely related bacterium *V. parahaemolyticus*, two putative transcriptional regulators, VPA0716 and VPA0717, are located five genes downstream of the *cqsA* homologue (annotated as VPA0711). VPA0716 is annotated as a putative transcriptional regulator protein sharing homology with AraC regulators. VPA0717 is annotated as a LysR-like transcriptional regulator. These loci, if present on the *V. harveyi* genomic cosmid carrying *cqsA<sub>Vh</sub>*, could be involved directly or indirectly in negatively regulating CAI-1 production. We are investigating this possibility now.

To examine the *in vivo* roles of *cqsA<sub>Vh</sub>* and *cqsS<sub>Vh</sub>* in CAI-1 production, each gene was deleted from the chromosome of *V. harveyi*, and subsequently CAI-1 production was measured

by using the *V. cholerae* reporter MM920. *V. harveyi* with a deletion of *cqsA<sub>Vh</sub>* does not make CAI-1, whereas *V. harveyi* with a deletion of *cqsS<sub>Vh</sub>* produces WT levels of CAI-1 (Fig. 2A, bars labeled *cqsA<sub>Vh</sub>* and *cqsS<sub>Vh</sub>*). The function of CqsS<sub>Vh</sub> is examined further in a subsequent section.

**CAI-1 acts synergistically with HAI-1 and AI-2 to control quorum-sensing genes in *V. harveyi*.** The above results show that *cqsA<sub>Vh</sub>* encodes the *V. harveyi* CAI-1 synthase, and based on our earlier findings in *V. cholerae*, we predict that CAI-1 controls quorum-sensing target gene expression in *V. harveyi*. To test this prediction, we constructed the three *V. harveyi* single autoinducer synthase deletions, all the combinations of double synthase mutants, and the triple autoinducer synthase mutant and assayed light production in each. In this experiment, we are using bioluminescence at high cell density as the representative readout for the quorum-sensing regulon.

Deletion of any single autoinducer synthase severely decreases light production in *V. harveyi* (Fig. 4A). Specifically, Fig. 4 shows that the *cqsA<sub>Vh</sub>* mutant (CAI-1<sup>-</sup>) makes 17%, the *luxS* mutant (AI-2<sup>-</sup>) produces 4%, and the *luxM* mutant (HAI-1<sup>-</sup>) produces only 0.1% of the light produced by the WT (note the log scale). The double and triple synthase mutants produce less light than the single mutants. The levels of light measured here are at the limit of detection in our assay, and so we do not know if the small differences in light production by the double and triple synthase mutants are meaningful. Nonetheless, we make three conclusions from this experiment. First, the CAI-1 autoinducer is a signal that is critical for regulation of bioluminescence expression in *V. harveyi*. Second, under these experimental conditions, the influence of HAI-1 on light production is stronger than that of AI-2, which is stronger than that of CAI-1. Third, the three autoinducers act synergistically because elimination of even the weakest signal (CAI-1) results in an 83% reduction in light production. Thus, by the logic presented previously (26), the *V. harveyi* Lux circuit appears to act as a three-way coincidence detector.

The absence of the CAI-1 signal is responsible for the reduction in light production observed in the *cqsA<sub>Vh</sub>* mutant because exogenous addition of CAI-1 complements the defect. Specifically, addition of cell-free culture fluids prepared from a *luxM luxS V. harveyi* strain (i.e., HAI-1<sup>-</sup> AI-2<sup>-</sup> CAI-1<sup>+</sup>) or a *luxS V. cholerae* strain (i.e., AI-2<sup>-</sup> CAI-1<sup>+</sup>) restores WT bioluminescence to the *cqsA<sub>Vh</sub>* (i.e., CAI-1<sup>-</sup>) mutant (Fig. 4A). We have shown previously that exogenous addition of HAI-1 and AI-2 restores light production in the *luxM* and *luxS* mutants, respectively (2, 3).

**CqsS is the sensor for CAI-1.** CqsS is the sensor for CAI-1 in *V. cholerae* and thus acts downstream of the CAI-1 signal (25). To test if this is also the case in *V. harveyi*, we performed an epistasis analysis with single and double *cqsA<sub>Vh</sub>* and *cqsS<sub>Vh</sub>* mutants. Figure 4B shows that the *cqsA<sub>Vh</sub>* mutant produces approximately 13% as much light as the WT. In contrast, the *cqsS<sub>Vh</sub>* mutant produces nearly WT levels of light. We assume that this latter phenotype stems from Systems 1 and 2 remaining operational in the *cqsS<sub>Vh</sub>* mutant. Importantly, the *cqsA<sub>Vh</sub>* *cqsS<sub>Vh</sub>* double mutant has a phenotype similar to that of the single *cqsS<sub>Vh</sub>* mutant (i.e., it produces WT levels of light), demonstrating that CqsS acts downstream of CAI-1. Based on these results, we assert that the information supplied by CAI-1 cannot be transduced to the *lux* operon in the absence of CqsS,

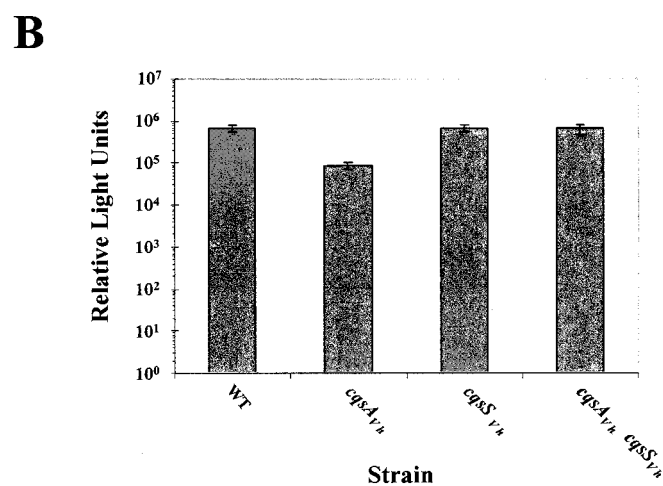
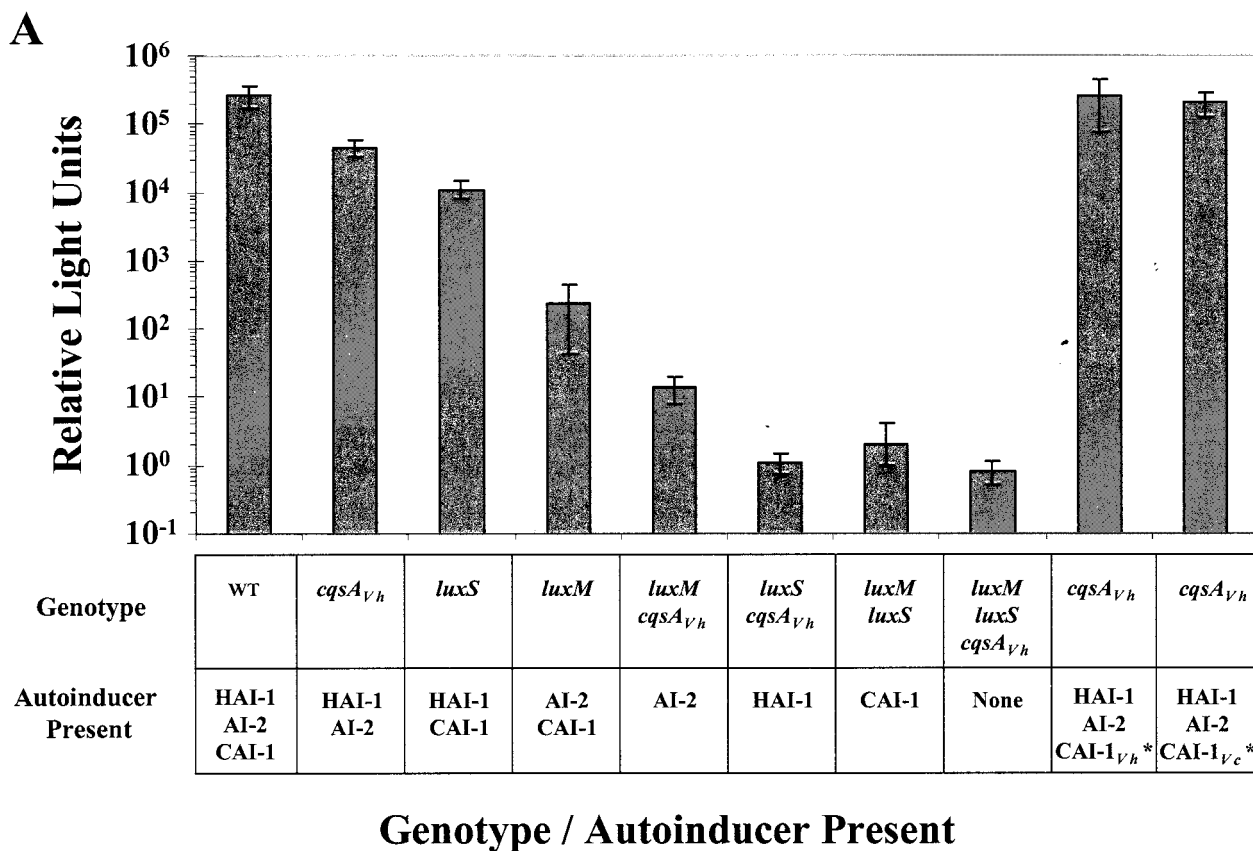


FIG. 4. *V. harveyi* single, double, and triple autoinducer synthase mutant phenotypes. (A) Light production per cell (RLU) was measured from high-cell-density *V. harveyi* cultures. The *V. harveyi* strains are (from left to right) BB120 (WT), JMH603 (*cqsA<sub>Vh</sub>*), KM387 (*luxS*), JAF633 (*luxM*), JMH605 (*luxM cqsA<sub>Vh</sub>*), JMH606 (*luxS cqsA<sub>Vh</sub>*), KM413 (*luxM luxS*), and JMH634 (*luxM luxS cqsA<sub>Vh</sub>*), and the rightmost two bars are JMH603 (*cqsA<sub>Vh</sub>*). In these latter two cases, JMH603 (*cqsA<sub>Vh</sub>*) was grown in the presence of 30% *V. harveyi* or *V. cholerae* WT culture fluids. The asterisks denote exogenously added autoinducer. (B) *CqsA<sub>Vh</sub>* and *CqsS<sub>Vh</sub>* epistasis analysis. RLU were measured at high cell density in the following strains (left to right): BB120 (WT), JMH603 (*cqsA<sub>Vh</sub>*), JMH598 (*cqsS<sub>Vh</sub>*), JMH763 (*cqsA<sub>Vh</sub> cqsS<sub>Vh</sub>*). Relative light units are defined as counts min<sup>-1</sup> ml<sup>-1</sup> × 1,000/CFU ml<sup>-1</sup>.

and therefore, *CqsS* is the sensor for the CAI-1 autoinducer in *V. harveyi*.

**LuxU and LuxO are required to transduce the CAI-1 signal in *V. harveyi*.** We wondered whether CAI-1 signaling is medi-

ated through LuxU and LuxO or through other regulatory functions. We again performed epistasis tests to examine the roles of LuxU and LuxO. In this case we constructed single and double *cqsA<sub>Vh</sub>*, *luxU*, and *luxO* null mutants and compared



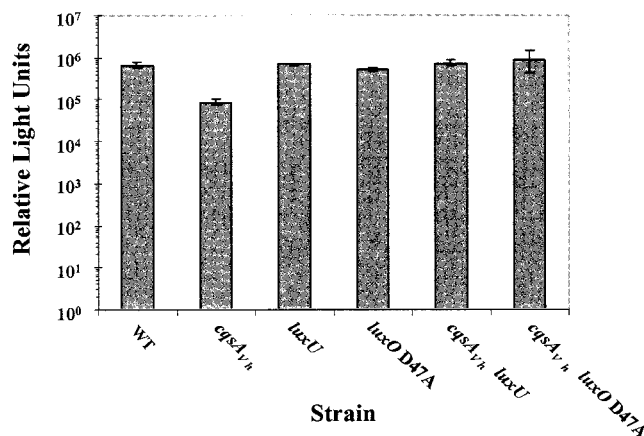


FIG. 5. LuxU and LuxO are epistatic to CqsA. RLU were measured from high-cell-density *V. harveyi* cultures of the following strains (left to right): BB120 (WT), JMH603 (*cqsA<sub>vh</sub>*), JAF536 (*luxU*), JAF483 (*luxOD47A*), JMH621 (*luxU cqsA<sub>vh</sub>*), JMH774 (*luxOD47A cqsA<sub>vh</sub>*).

their bioluminescence phenotypes. We used a transposon insertion in *luxU* to analyze the *luxU* null phenotype and used the *luxOD47A* missense mutation to test the *luxO* null phenotype. We chose this particular *luxO* allele because, while it confers the *luxO* null phenotype, it is not polar on *luxU* (*luxO* and *luxU* are in an operon) (12). Thus, this strategy allowed us to individually test the effects of *luxO* and *luxU* null mutations on CAI-1 signal transduction. Like Fig. 4, Fig. 5 shows that the *cqsA<sub>vh</sub>* single mutant produces approximately 13% of the light produced by the WT. The *luxU* null and *luxOD47A* single mutants are constitutively bright and produce WT levels of light. The epistasis tests show that the *cqsA<sub>vh</sub> luxU* and *cqsA<sub>vh</sub> luxOD47A* double mutants have phenotypes like those of the *luxU* and *luxO* single mutants (constitutive bright). Therefore, both LuxU and LuxO are required for CAI-1 signal relay, and therefore, both act downstream of CAI-1 and CqsS in the *V. harveyi* quorum-sensing circuit (Fig. 1).

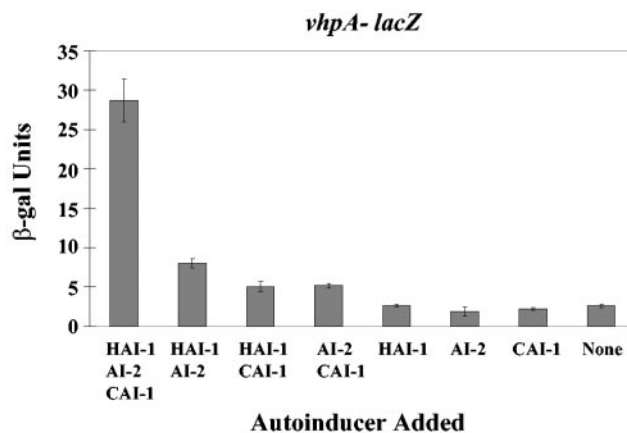
### Three autoinducers control the quorum-sensing regulon.

We have shown that HAI-1 and AI-2 act synergistically to regulate Lux and many other quorum-sensing targets, such as a TTS system (18) and a secreted metalloprotease (26). The results illustrated in Fig. 4A show that with respect to *lux* expression, three autoinducers contribute to this synergy. These findings, coupled with the finding that CAI-1 acts through LuxU and LuxO (Fig. 5), suggest that CAI-1 regulation is not exclusive to bioluminescence but can be extended to other members of the quorum-sensing regulon. To test this assumption, we analyzed CAI-1 regulation of the expression of two genes that we have previously shown to be jointly controlled by HAI-1 and AI-2. Here we describe our examination of one representative quorum-sensing-activated gene (encoding a metalloprotease we have named VhpA [*V. harveyi* protease A]) (26) and one representative quorum-sensing-repressed gene (encoding the TTS system protein VcrD) (18). *lacZ* transcriptional fusions to these two genes were made and introduced onto the chromosome of a *V. harveyi* triple autoinducer mutant (i.e., *luxM luxS cqsA<sub>vh</sub>*). Cell-free culture fluids containing either HAI-1, AI-2, CAI-1, or combinations of these signals were

added to the fusion strains, and LacZ activity was subsequently measured.

Induction of the *vhpA-lacZ* fusion occurs when cell-free culture fluids containing all three autoinducers are added (Fig. 6A, leftmost bar); however, much reduced expression occurs following the addition of the preparations containing any two, any one, or no autoinducers (Fig. 6A, remaining bars). The quorum-sensing-repressed target *vcrD* shows an opposite pattern of expression (Fig. 6B). In this case,  $\beta$ -Gal activity is low (20 U) when all three autoinducers are present (Fig. 6B, leftmost bar) but is high (80 to 150 U) in the presence of none or any one or two autoinducers (Fig. 6B, remaining bars). Thus, for bioluminescence, metalloprotease production, and type III secretion, all three autoinducers act in concert.

A



B

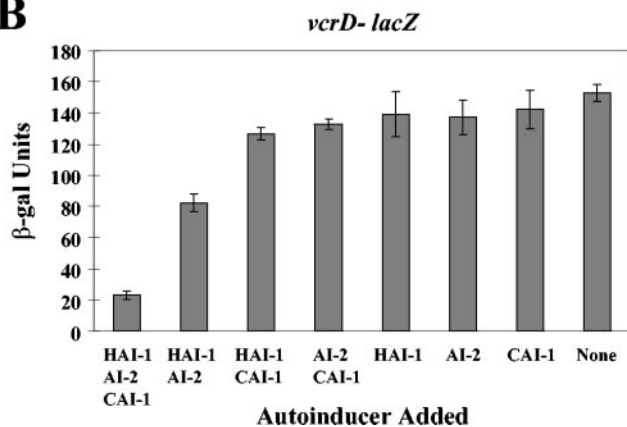


FIG. 6. All three autoinducers are required for quorum-sensing-controlled gene regulation.  $\beta$ -Galactosidase assays were performed on *V. harveyi* recipient strains (high cell density) grown overnight in the presence of 10% cell-free culture fluids containing various autoinducers. Cell-free culture fluids were prepared from the following strains (left to right): BB120 (WT), JMH603 (*cqsA<sub>vh</sub>*), KM387 (*luxS*), JAF633 (*luxM*), JMH606 (*luxS cqsA<sub>vh</sub>*), JMH605 (*luxM cqsA<sub>vh</sub>*), KM413 (*luxM luxS*), and JMH634 (*luxM luxS cqsA<sub>vh</sub>*). (A) The recipient strain was *V. harveyi* JMH670 (*vhpA::Tn5lacZ luxM luxS cqsA<sub>vh</sub>*). (B) The recipient strain was JMH674 (*vcrD::Tn5lacZ luxM luxS cqsA<sub>vh</sub>*).  $\beta$ -Galactosidase units are reported as ( $V_{\max} \times$  dilution factor)/OD<sub>600</sub>.

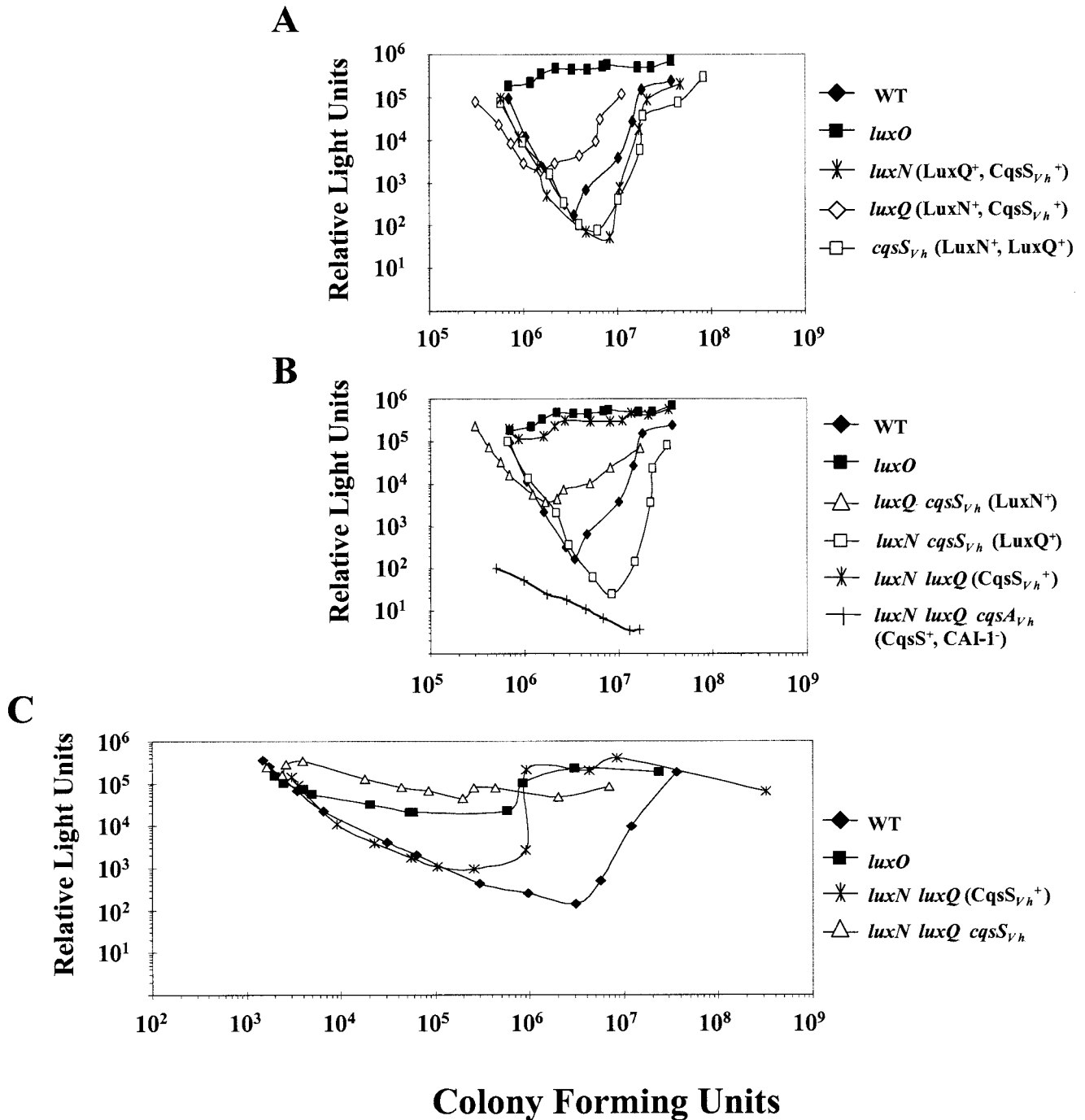


FIG. 7. *V. harveyi* single, double, and triple autoinducer sensor mutant phenotypes. Density-dependent bioluminescence assays were performed on (A) BB120 (WT), BB721 (*luxO*), BB170 (*luxN*), BB886 (*luxQ*), and JMH598 (*cqsS<sub>Vh</sub>*); (B) BB120 (WT), BB721 (*luxO*), JMH612 (*luxQ cqsS<sub>Vh</sub>*), JMH597 (*luxN cqsS<sub>Vh</sub>*), JMH625 (*luxN luxQ*), and JMH626 (*luxN luxQ cqsA<sub>Vh</sub>*); and (C) BB120 (WT), BB721 (*luxO*), JMH625 (*luxN luxQ*), and JMH628 (*luxN luxQ cqsS<sub>Vh</sub>*). Relative light units are reported as counts min<sup>-1</sup> ml<sup>-1</sup> × 1,000/CFU ml<sup>-1</sup>. In panels A and B, cells were diluted 1:5,000, and in panel C, cells were diluted 1:500,000 prior to the start of the experiment.

**The CAI-1-CqsS system functions at very low cell densities.** The results of this study demonstrate that HAI-1, AI-2, and CAI-1 act synergistically through the Lux network to control gene expression. To begin to analyze the relative contribution of each sensory system, we constructed combinations of quorum-sensing mutants and assayed their resulting biolumines-

cence phenotypes. This strategy allowed us to examine both the timing and the intensity of each of the three inputs.

Figure 7A shows the characteristic WT *V. harveyi* density-dependent bioluminescence curve. Following dilution of the culture into fresh medium, light production sharply decreases because no autoinducers are present. Under these conditions,

the sensors act as kinases and funnel phosphate to LuxO, and this results in Lux repression (12). Light production begins increasing at approximately  $2.5 \times 10^6$  cells/ml because by the time the cells have grown to this cell density, the autoinducers have accumulated to the threshold level required to induce the sensors to switch from net kinase to net phosphatase. Removal of phosphate from LuxO terminates repression and allows light production to occur (Fig. 1A and see the introduction). A *luxO* mutant is constitutively bright (Fig. 7A) because LuxO is required (indirectly) for Lux repression (21). Mutation of any one of the three sensors alters the shape of the Lux curves slightly; however, each sensor mutant (*luxN*, *luxQ*, and *cqsS<sub>Vh</sub>*) retains density-dependent expression of bioluminescence, presumably due to the input from the remaining two systems.

The phenotypes of the double sensor mutants are compared to those of the WT and the constitutively bright *luxO* null strain, shown in Fig. 7B. The *luxQ cqsS<sub>Vh</sub>* double mutant that has only LuxN remaining transitions from decreasing to increasing light production at a lower cell density than in the WT. By contrast, the *luxN cqsS<sub>Vh</sub>* double mutant that has only LuxQ remaining transitions to increasing light output that is delayed relative to that of the WT. Together these first two results suggest that normally, LuxQ remains a kinase beyond the point when LuxN has switched to phosphatase. Finally, a *luxN luxQ* double mutant that has only CqsS<sub>Vh</sub> remaining is nearly constitutively bright.

Because the *luxN luxQ* double mutant phenotype is nearly identical to the *luxO* mutant phenotype (constitutive light production), we hypothesized that either CqsS<sub>Vh</sub> switches from kinase to phosphatase at a very low cell density or CqsS<sub>Vh</sub> contributes neither kinase nor phosphatase activity to the Lux circuit. To distinguish between these possibilities we examined a *luxN luxQ cqsA<sub>Vh</sub>* triple mutant. In this mutant, the only sensor present is CqsS<sub>Vh</sub>, but because no CAI-1 is produced due to the *cqsA<sub>Vh</sub>* mutation, according to our model, CqsS<sub>Vh</sub> should be locked in kinase mode. The *luxN luxQ cqsA<sub>Vh</sub>* triple mutant is completely dark (Fig. 7B), demonstrating that CqsS<sub>Vh</sub> indeed acts as a kinase that shuttles phosphate to LuxO to produce full Lux repression. We therefore conclude that CAI-1 and CqsS<sub>Vh</sub> contribute to quorum-sensing gene regulation at very low cell densities.

We suspect that the input from CqsS<sub>Vh</sub> is not apparent under our usual experimental conditions. Specifically, we hypothesize that at the cell densities at which we started the experiments, the transition of CqsS from kinase to phosphatase had already occurred. If so, this could explain why we never identified CAI-1 and CqsS<sub>Vh</sub> as components of the Lux quorum-sensing cascade. To test this possibility, we performed a density-dependent bioluminescence assay using *V. harveyi* strains diluted 1:500,000 instead of using the usual 1:5,000 dilution (Fig. 7C). At these very low cell densities, the *luxN luxQ* double mutant that has only CqsS remaining exhibits density-dependent bioluminescence and has a phenotype that is quite distinct from the *luxO* mutant. Importantly, unlike the double *luxN luxQ* sensor mutant, under this condition the triple *luxN luxQ cqsS<sub>Vh</sub>* sensor mutant is constitutively bright, showing that in WT cells, the CqsS<sub>Vh</sub> sensor switches from a kinase to a phosphatase at very low cell densities ( $\sim 1 \times 10^5$  cells/ml), and this switch occurs earlier than that of LuxN and LuxQ. The results of this experiment also show that the buildup of the

critical concentrations of the autoinducers required to switch their cognate sensors from kinase mode to phosphatase mode occurs in the following order: CAI-1, then HAI-1, then AI-2.

## DISCUSSION

This report describes the identification and analysis of a quorum-sensing system in *V. harveyi* that acts in parallel with the two previously identified quorum-sensing systems (Fig. 1A). *V. harveyi* and other closely related species produce an autoinducer activity that mimics the CAI-1 activity of *V. cholerae* (Table 2). As in *V. cholerae*, in *V. harveyi* the genes *cqsA* and *cqsS* are required for production and detection of CAI-1, respectively (Fig. 3, 4, and 7). CAI-1 acts in conjunction with the *V. harveyi* HAI-1 and AI-2 autoinducers to control a common set of target genes (Fig. 5 to 7). However, the CAI-1–CqsS system switches from kinase mode to phosphatase mode at a much lower cell density than do the HAI-1–LuxN and AI-2–LuxQ circuits (Fig. 7). While it remains possible that genes responsive to only one or only two of the three autoinducers exist in *V. harveyi*, proper regulation of the quorum-sensing targets identified thus far requires the simultaneous presence of all three autoinducers (Fig. 4 and 7), suggesting that the *V. harveyi* quorum-sensing circuit likely functions as a three-way coincidence detector.

The two major classes of autoinducers, HSLs in gram-negative bacteria and oligopeptides in gram-positive bacteria, function in intraspecies communication (reviewed in reference 24). By contrast, the autoinducer AI-2 and its synthase LuxS are broadly conserved in the bacterial kingdom, and are proposed to function in interspecies communication (reviewed in references 11 and 37). *V. harveyi* CAI-1 can control gene expression in *V. cholerae* and vice versa, demonstrating that CAI-1-mediated cell-cell communication need not be restricted to within a species. Thus, CAI-1 represents a new activity that fits under the classification of interspecies communication signal. Importantly, however, in contrast to LuxS and AI-2, CqsS and its product CAI-1 are not exceptionally widespread. Rather, our preliminary analysis suggests that they are only present in closely related marine bacteria. Thus, CAI-1, while capable of promoting communication across species boundaries, might be restricted to use exclusively within the genus *Vibrio*. Database analysis shows that while *V. cholerae* and *V. parahaemolyticus* possess *cqsA* and make CAI-1, *Vibrio vulnificus* and *Vibrio fischeri* do not. We predict the existence of a *cqsA* gene in the genomes of *V. anguillarum*, *V. alginolyticus*, and *V. furnissii* based on our ability to detect CAI-1 activity in their cell-free culture fluids (Table 2). Conceivably, the vibrio species that produce and detect CAI-1 are ones that inhabit the same niches and communicate with one another therein.

We do not know the identity of CAI-1, whether the CAI-1 molecule made by different bacteria is identical, or whether more than one molecule has CAI-1 activity, nor do we know the role of CqsA in CAI-1 production. The CqsA protein is similar to class II aminotransferases which catalyze the transamination of the terminal amino groups of basic  $\alpha$ -amino acids, diamines, and  $\omega$ -amino acids (23). Interestingly, aminotransfer reactions are also involved in HSL autoinducer production by LuxI-type autoinducer synthase enzymes. Specifically, LuxI-type enzymes couple the acyl group from acyl-acyl

carrier proteins to the amino moiety of methionine on *S*-adenosylmethionine to produce specific HSLs (17, 27, 29, 36). It is possible that enzymes in addition to CqsA are required for CAI-1 production. However, because *E. coli* containing either the *V. cholerae* or the *V. harveyi* *cqsA* gene produces high levels of CAI-1 activity (Fig. 3), either CqsA is the only protein required to make CAI-1 from substrates existing in *E. coli*, or, if several steps are required, then *E. coli* must possess the additional enzymes in the pathway.

We are presently attempting to purify CAI-1 from cell-free culture fluids of *V. cholerae* and *V. harveyi* and from recombinant *E. coli*. These studies should reveal whether *V. harveyi* and *V. cholerae* make identical CAI-1 or not. Our preliminary evidence suggests that CAI-1 is not an HSL-type autoinducer because it has no activity in the commonly used HSL reporters that detect a variety of HSL molecules, and coinoculation of cell-free culture fluids containing CAI-1 with a *Bacillus* species that secretes a nonspecific homoserine lactonase (AiiA) (10) causes no loss of CAI-1 activity (data not shown). Verification of this supposition awaits structural identification of CAI-1.

Our work demonstrates that the simultaneous presence or simultaneous absence of HAI-1, AI-2, and CAI-1 is required for maximal impact on gene expression in *V. harveyi* (Fig. 4 and 7); however, the strengths of the different autoinducer signals are not equivalent. The different signal strengths are represented in the relative kinase-to-phosphatase ratio of the corresponding receptors (LuxN > LuxQ > CqsS). In natural settings, the strength of input from each system could vary depending on the prevailing environmental conditions. Supporting this idea, we have observed that on agar surfaces, the *luxS* mutant produces WT levels of light, the *cqsA* mutant is dim, and the *luxM* mutant is dark. Thus, under this condition, the relative signal strengths must be as follows: HAI-1 > CAI-1 > AI-2. This observation implies that, unlike in liquid where the relative kinase-phosphatase inputs are greatest for LuxN, followed by LuxQ and then CqsS, on a surface the activities are greatest for LuxN, followed by CqsS and then LuxQ (data not shown). We are presently studying the regulation of the three sensors and synthases on agar surfaces to understand the molecular basis for the alteration in signal strength that occurs between liquid and surface environments.

Importantly, whereas in liquid cultures of *V. harveyi* the CqsS input is the weakest, in *V. cholerae* it is the dominant input (25). Furthermore, the CqsS switch from kinase mode to phosphatase mode occurs at a much higher cell density in *V. cholerae* than it does in *V. harveyi*. We suspect that the differences in the relative strengths and timing of these inputs in *V. harveyi* and *V. cholerae* reflect each species's fine-tuning of the signals for optimized survival in the distinct niches in which each resides. Thus, while *V. harveyi* and *V. cholerae* likely have some niches in common, they obviously have distinct niches as well. For example, *V. cholerae* colonizes the human intestinal tract (for a review, see reference 19), while *V. harveyi* does not. While we do not know the underlying molecular mechanism giving rise to the differences in the individual signal input strengths in *V. harveyi* and *V. cholerae*, an obvious possibility is that the sensors simply have different relative kinase and/or phosphatase activities or different affinities for CAI-1.

Why does *V. harveyi* use three quorum-sensing circuits that converge to control the same set of genes? Using multiple

signals in the coincidence detection scheme could protect the quorum-sensing system from noise due to the presence of molecules that are similar or identical to one of the autoinducers or from the disappearance of one of the signals due to degradation or consumption by other species of bacteria. Alternatively, if the system does not act as a coincidence detector under natural conditions, one obvious rationale for having multiple autoinducers is that three signals provide eight possible input states, whereas only four states are achievable with two signals and only two states are possible with one signal. The ability to monitor fluctuations in many signals could be critical for the survival of *V. harveyi* in different niches because the cell numbers and/or species compositions of the quorums required for commensal living, biofilm formation, and pathogenesis could vary greatly. Irrespective of the detection logic used, this careful probing of the environment could ensure that the decision to transition into or out of quorum-sensing mode is made with high fidelity.

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