Quorum-sensing autoinducers resuscitate dormant *Vibrio cholerae* in environmental water samples

S. M. Nayeemul Bari^a, M. Kamruzzaman Roky^a, M. Mohiuddin^a, M. Kamruzzaman^a, John J. Mekalanos^{b,1}, and Shah M. Faruque^{a,1}

^aCentre for Food and Water Borne Diseases, International Centre for Diarrhoeal Disease Research, Bangladesh, Dhaka-1212, Bangladesh; and ^bDepartment of Microbiology and Immunobiology, Harvard Medical School, Boston, MA 02115

Contributed by John J. Mekalanos, April 26, 2013 (sent for review March 27, 2013)

Cholera epidemics have long been known to spread through water contaminated with human fecal material containing the toxigenic bacterium *Vibrio cholerae*. However, detection of *V. cholerae* in water is complicated by the existence of a dormant state in which the organism remains viable, but resists cultivation on routine bacteriological media. Growth in the mammalian intestine has been reported to trigger “resuscitation” of such dormant cells, and these studies have prompted the search for resuscitation factors. Although some positive reports have emerged from these investigations, the precise molecular signals that activate dormant *V. cholerae* have remained elusive. Quorum-sensing autoinducers are small molecules that ordinarily regulate bacterial gene expression in response to cell density or interspecies bacterial interactions. We have found that isolation of pathogenic clones of *V. cholerae* from surface waters in Bangladesh is dramatically improved by using enrichment media containing autoinducers either expressed from cloned synthase genes or prepared by chemical synthesis. These results may contribute to averting future disasters by providing a strategy for early detection of *V. cholerae* in surface waters that have been contaminated with the stools of cholera patients or asymptomatic infected human carriers.

biofilm formation | CVEC | transmissibility

The natural habitats of the species *Vibrio cholerae* are estuarine or fresh water aquatic environments (1–3). In cholera endemic areas such as Bangladesh, viable *V. cholerae* can be readily detected in water during seasonal cholera epidemics; however, as disease incidence decreases, the isolation of viable *V. cholerae* becomes dramatically more difficult perhaps in part due to the influence of lytic bacteriophages (4, 5). However, during the interepidemic period, the organism can also occasionally be found in a viable but dormant state, which has been alternately referred to as the viable but nonculturable (VBNC) cells (1), conditionally viable environmental cells (CVEC) (6), or active but nonculturable (ABNC) (7). Recently we have documented the existence of CVEC in surface waters of Bangladesh by using fluorescent antibody based microscopy, which revealed clumps of *V. cholerae* O1 cells in water, which were often negative for *V. cholerae* O1 by conventional culture. To detect possible presence of relatively small number of culturable cells in such water we also used enrichment and selection approaches that depend on antibiotic resistance profiles displayed by the strains that have caused the preceding cholera epidemic. This approach referred to as antibiotic selection technique (AST) (8) allowed enhanced detection of *V. cholerae* by suppressing growth of other environmental bacteria that would otherwise mask the small number of *V. cholerae* colonies.

In our previous studies, CVEC were found to be organized as aggregates of cells embedded in extracellular material, presumably *Vibrio* extracellular polysaccharide (VPS) (6). The genes responsible for VPS production are controlled by a regulatory pathway that includes two autoinducers (AIs) called cholera autoinducer-1 (CAI-1) and autoinducer-2 (AI-2), their cognate receptors CqsS and LuxP, and a signal transduction cascade that involves

phosphorylation, dephosphorylation, noncoding small RNAs, RNA chaperones, as well as several transcriptional regulatory proteins (9–13). Because autoinducers are small molecules that typically regulate gene expression under conditions of high bacterial cell density (9–17), we reasoned that molecules might signal to dormant cells that conditions have become favorable for growth. Specifically we hypothesized that CAI-1 and AI-2 could therefore enhance CVEC resuscitation. Here we show that addition of biologically synthesized or synthetic AIs to enrichment medium dramatically improves resuscitation of pathogenic *V. cholerae* CVEC present in environmental water samples collected from a geographic locale that regularly suffers from seasonal cholera epidemics.

Results

Autoinducers CAI-1 and AI-2 Enhance Resuscitation of CVEC. To examine whether AIs such as CAI-1 and AI-2 also acted as resuscitation factors for environmental CVEC, we tested water samples collected in aquatic sites in Dhaka, Bangladesh that have previously yielded evidence of CVEC (6). To exclude samples that might contain a small proportion of culturable *V. cholerae* cells, aliquots of water were initially tested by AST enrichment and culture for the presence of *V. cholerae* (8). Only samples that were negative in this initial culture even after 6 h of preenrichment were selected for resuscitation assays with various spent media from *Escherichia coli* or *V. cholerae* expressing or not expressing the AIs CAI-1 and AI-2. We found that addition of 50% (vol/vol) spent media from isopropylthio- β -galactoside (IPTG)-induced *E. coli* cultures or *V. cholerae* expressing cloned CAI-1 synthase gene resuscitated dormant *V. cholerae* O1 cells in many of the water samples within only a few hours of enrichment (Table 1). Similarly, resuscitation of *V. cholerae* O1 was noticed in the presence of AI-2 expressed from a recombinant plasmid *pluxS* in strain C6706 *luxS::TnFGL3 Δ cqsA* a derivative of strain C6706 in which its indigenous CAI-1 and AI-2 synthase genes were inactivated (Table S1). Aliquots of these same water samples were mostly negative for culturable *V. cholerae* when enriched in an identical manner but exposed to 50% (vol/vol) spent media from control cultures including (i) *E. coli* DH5 α carrying the empty cloning vector pTAC, (ii) *E. coli* carrying *cqsA* that were not induced with IPTG, and (iii) *V. cholerae* C6706 with deletion of its indigenous AI synthase genes. Furthermore, characterization of culturable *V. cholerae* strains derived from these enrichments confirmed their close similarity to clones of *V. cholerae* that had recently caused local cholera epidemics in Dhaka.

To understand the parameters that allow *V. cholerae* to produce its own resuscitation factors, we tested whether *V. cholerae*

Author contributions: J.J.M. and S.M.F. designed research; S.M.N.B., M.K.R., M.M., M.K., and S.M.F. performed research; M.K., J.J.M., and S.M.F. analyzed data; and J.J.M. and S.M.F. wrote the paper.

The authors declare no conflict of interest.

¹To whom correspondence may be addressed. E-mail: john_mekalanos@hms.harvard.edu or faruque@icddr.org.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1307697110/-DCSupplemental.

Table 2. Recovery of *Vibrio cholerae* O1 CVEC in environmental water by culturing under different conditions

Date of sampling	No. of samples tested	No. of samples found positive for <i>V. cholerae</i> O1 after enrichment in LB containing filter-sterilized spent medium of different derivatives of strain C6706						
		(A) LB medium	(B) C6706	(C) C6706 Δ cqsA Δ luxS	(D) C6706 Δ cqsA Δ luxS (pcqsA)	(E) C6706 Δ cqsA Δ luxS (pluxS)	(F) C6706 (pcqsA)	(G) C6706 (pluxS)
April 2011	27	2	1	0	15	12	18	10
May 2011	25	0	2	3	11	12	15	9
June 2011	24	1	1	0	18	9	20	12
June 2012	16	0	3	1	9	10	12	12
July 2012	12	0	0	0	5	3	4	3
August 2012	9	0	0	1	6	5	7	4
November 2012	18	2	3	2	11	7	2	3
November 2012	10	0	1	1	7	3	5	6
December 2012	21	2	1	1	15	4	16	10
Total	162	7	12	9	96	65	99	69
% of total	100	4.3	7.4	5.5	59.2	40.1	61.1	42.5

The composition of enrichment media are shown. Samples were inoculated into the indicated LB media and then incubated for 6 h at 37 °C, before plating aliquots of the enrichment culture on TTGA plates containing streptomycin and sulfamethoxazole-trimethoprim (SXT). Only CVEC positive surface water samples are shown. pcqsA and pluxS are plasmids carrying the cloned cqsA and luxS genes, respectively. The observed differences in recovery of culturable cells when enriched under different conditions were statistically significant ($P < 0.001$) when compared between columns: C vs. D, C vs. E, C vs. F, C vs. G, D vs. E, and F vs. G.

and at regular intervals during an AI resuscitation experiment (Fig. 1). In CAI-1-exposed cultures, extracellular material associated with coccoid CVEC cellular aggregates was found to disappear with time and cells began to disperse within 3 h of incubation. However, the cells became culturable only after 4 h, and by 6 h these aggregates changed from a coccoid to more vibrio and rod-like shapes. These morphological changes corresponded to the dramatic recovery of viable cells after 6 h of exposure to CAI-1 preparation. Mechanical disruption of such water samples to disperse CVEC by vortexing vigorously with silica beads at earlier time points (e.g., 3 h and less) during the resuscitation process failed to produce detectable viable counts, indicating that CAI-1-induced resuscitation is not solely related to physical breakdown of the extracellular matrix in which CVEC are embedded and subsequent dispersion of culturable cells within this matrix. Nonetheless, these changes in individual cell morphology and the disappearance of the extracellular matrix correlate with the detection of culturable cells after exposure to CAI-1-containing media. Similar morphological changes and dispersion of cells were also noticed, albeit at a slower pace when AI-2 was used in the enrichment culture.

Discussion

The data presented here provide several unique insights into the ecological and epidemiological behavior of *V. cholerae*. We have

shown that quiescent forms of pathogenic *V. cholerae* that exist in aquatic reservoirs can be readily converted to culturable cells by exposure to either of two AI types. Because these AI molecules can be produced by multiple bacterial species, it follows that viable cells of pathogenic *V. cholerae* can suddenly emerge from any environment that is replete in such AI molecules as well as a nutrient source. Because CAI-1 is narrowly distributed in *Vibrio* species (15), whereas AI-2 is produced by numerous bacterial species (15–17), our data suggest that resuscitation of *V. cholerae* CVEC could occur either outside or inside a human host as a result of exposure to a high concentration of AIs. Thus, CVEC could be exposed to AI molecules produced by environmental bacterial species or by the normal intestinal bacterial microbiota present in aquatic animals, which include plankton, crustaceans, insects, mollusks, or fish. This concept could explain aspects of cholera seasonality in geographical areas like the Ganges Delta. In brief, if seasonal parameters (temperature, salinity, carbon content, the presence of aquatic animal hosts, etc.) give rise to environmental blooms of heterologous bacterial organisms that produce AI signals, then nearby dormant *V. cholerae* might resuscitate and more readily cause human disease during this period (Fig. 2). Alternative models could include a role for the human host in the resuscitation process. For example, resuscitation might also occur in a human that ingested CVEC through the influence of AI molecules produced by normal human

Table 3. Effect of synthetic autoinducers CAI-1 and AI-2 added to enrichment cultures on the recovery of *V. cholerae* O1 CVEC in environmental water samples

Date of sampling	No. of samples tested	No. of samples found positive for <i>V. cholerae</i> O1 after enrichment in LB containing filter-sterilized spent medium of C6706 or C6706 Δ cqsA Δ luxS supplemented with synthetic cholera autoinducer CAI-1 or AI-2 as indicated						
		(A) LB	(B) LB + CAI-1	(C) C6706	(D) C6706 Δ cqsA Δ luxS	(F) C6706 Δ cqsA Δ luxS + CAI-1	(G) C6706 Δ cqsA Δ luxS + AI-2	(H) C6706 Δ cqsA Δ luxS + CAI-1 + AI-2
June 2012	7	0	4	0	1	5	2	4
November 2012	15	0	5	0	1	4	3	7
November 2012	10	1	7	2	0	8	5	8
Total	32	1	16	2	2	17	11	19
% of total	100	3.1	50	6.2	6.2	53.1	34.3	59.3

The composition of enrichment media is shown. Samples were inoculated into the indicated LB media and then incubated for 6 h at 37 °C, before plating aliquots of the enrichment culture on TTGA plates containing streptomycin and SXT. The observed differences in recovery of culturable cells when enriched under different conditions were statistically significant ($P < 0.001$) when compared between columns: A vs. B, and D vs. F.

Fig. 1. Fluorescent antibody-based microscopic visualization of the resuscitation process after exposure of aliquots of environmental water to CAI-1 preparations. *V. cholerae* O1 CVEC cells observed at $T = 0, 3$, and 6 h after exposure to CAI-1. Multiple aliquots of water samples were incubated in LB medium supplemented with equal volumes of cell-free spent culture supernatant of C6706 deleted of its indigenous autoinducer synthase genes and supplemented with a plasmid clone of the *cqsA* gene, C6706 Δ *cqsA* Δ *luxS* (*pcqsA*). In parallel, multiple control assays were run in which spent culture supernatants of different bacteria including C6706 Δ *cqsA* Δ *luxS*(*pUC18*) carrying the empty cloning vector and all were negative for viability. Water samples were monitored at regular time intervals using fluorescent antibody-based microscopy. Morphology of *V. cholerae* in two sets of samples exposed to CAI-1, which were found positive for *V. cholerae* O1 at different time intervals are shown (A–C). In the CAI-1-exposed cultures, extracellular material appeared to be progressively degraded with time and the cellular morphology changed from a coccoid shape to a more rod-like shape and these cellular changes corresponded to the dramatic recovery of viable cells at 6 h. Mechanical disruption by vortexing with silica beads did not produce viable counts at $T = 0$ or 3 h and 6 h without CAI-1 treatment. Thus, resuscitation is not related to dispersion of cells alone but requires additional metabolic activities.

gastrointestinal microbiota. However, this alone cannot explain cholera seasonality unless the human microbiome undergoes seasonal changes as well. It is also possible that environmental conditions linked to seasonal cholera epidemics might also favor the aquatic survival of normal human intestinal bacteria (e.g., fecal coliforms) that could trigger *V. cholerae* CVEC resuscitation by producing AIs in environmental water that was contaminated with normal human stools. In this model, one might further speculate that environmental blooms of phages that kill normal fecal coliforms could indirectly affect CVEC resuscitation by suppressing AI production in such contaminated water settings. In sum, it seems remarkably logical that AIs are a signal that CVEC use to regulate their resuscitation because these molecules reliably report the presence of bacteria that have grown to high density and therefore also environmental conditions that may be permissive for *V. cholerae* growth as well.

Clearly, the possibility that seasonality of cholera might ultimately depend on bacterial interspecies communication sets the stage for ecological studies that could provide further evidence for these conclusions as well as novel means for cholera surveillance, prevention, and control. Furthermore, other pathogenic bacteria, such as *E. coli*, *Vibrio vulnificus*, *Salmonella enteritidis*, *Shigella sonnei*, *Shigella flexneri*, *Campylobacter jejuni*, and *Legionella pneumophila*, have been shown to enter the dormant state after exposure to adverse environmental stresses (3, 18, 19). If AI molecules play a role in the resuscitation of dormant cells of these species, our results may have significant implications in understanding the epidemiology of bacterial disease beyond cholera per se.

Materials and Methods

Bacterial Strains and Culture Condition. The bacterial strains used in this study were routinely cultured in Luria–Bertini broth (LB) or LB–agar containing

appropriate antibiotics at 37°C , unless otherwise stated. The transposon insertion mutants were obtained from a TnFGL3 insertion library of strain C6706 described previously (20). Other genetically marked strains or mutants used were either obtained from our collection or were constructed during the present study.

Construction of Deletion Mutants. For construction of deletion mutants, appropriate primers were used to amplify two short DNA segments flanking the two ends of the targeted gene. These fragments were ligated to form a fusion product that was amplified further by PCR using forward primer of the left flanking fragment and the reverse primer of the right flanking fragment. The PCR product was cloned into the *Sma* site of the suicide vector pRE112 and transformed into *E. coli* SM10 λ pir. Plasmid DNA isolated from selected colonies was used to electroporate appropriate recipients and was plated on LB agar plates containing antibiotic ($20\text{ }\mu\text{g/mL}$ of chloramphenicol). The selected colonies were grown in LB with 5% (wt/vol) sucrose for 3 – 4 h and plated on LB agar plates without antibiotic. Colonies that lost the resistance marker and hence the suicide vector were further purified on LB– 5% sucrose and their genotypes were confirmed by PCR. Bacterial strains and plasmids used in this study are summarized in Table S2.

Chitin-Induced Transformation. Transformation of *V. cholerae* cells in the presence of chitin was also used to create defined insertion mutants, in which DNA containing the mutated gene obtained from an appropriate mutant in the TnFGL3 transposon insertion library (20), was used to incorporate mutation into the recipient strain by marker exchange. Chitin-induced transformation was done using the method described by Meibom et al. with modifications (21, 22). Overnight cultures of the recipient *V. cholerae* strains were diluted $1:100$ -fold in LB medium and grown to an OD_{600} of ~ 0.3 . The bacteria were precipitated by centrifugation, washed, and resuspended in a 0.10 volume of filter-sterilized environmental water or 0.5% sterile sea salt solution (SS). Aliquots of a 2-mL bacterial suspension were dispensed into the wells of a 12 -well tissue culture plate containing sterile pieces of shrimp shell. After incubation at 30°C , grown statically for 24 h, the planktonic phase was removed, and fresh water or SS was added. At the same time, 1 – $2\text{ }\mu\text{g}$ of the appropriate DNA was added to the wells. After 24 h, the shrimp shells were removed from the wells, washed in SS, and vortexed in SS to release any attached bacteria. The released bacteria were then plated on LB–agar containing appropriate antibiotic. Suspected transformants were further analyzed using PCR and hybridization assays.

Preparation of Cloned CAI-1. The CAI-1 was prepared from a culture of *E. coli* DH5 α carrying a cloned *cqsA* gene (VCA0523). The recombinant construct pJZ176 kindly provided as a gift by Jun Zhu (Department of Microbiology, University of Pennsylvania School of Medicine, Philadelphia) carried the *cqsA* gene cloned in pTAC under an IPTG-inducible *lac* promoter as described previously (12). Overnight culture of *E. coli* DH5 α (pJZ176) was diluted $1:100$ times in fresh LB medium (100 mL) containing ampicillin ($50\text{ }\mu\text{g/mL}$), and grown for 3 – 4 h (until $\text{OD}_{600} = 0.5$ – 1.0). Cells were precipitated by centrifugation at $4,500 \times g$ for 15 min, the supernatant was discarded, and the pellet was resuspended in original volume of fresh prewarmed LB medium without antibiotic. IPTG solution was added to a final concentration of 0.5 mM , and the culture was incubated for another 6 – 8 h at 37°C with shaking. Aliquots of the culture were centrifuged at $6,000 \times g$ for 20 min to precipitate bacterial cells. The culture supernatant containing CAI-1 was collected and sterilized by filtration through $0.22\text{-}\mu\text{m}$ pore-sized filters.

Preparation of Autoinducer Using Mutant *V. cholerae* Strains. To prepare AI from cloned AI synthase genes in *V. cholerae*, strain C6706 Δ *cqsA* Δ *luxS* (*pcqsA*) (for CAI-1) or C6706 Δ *cqsA* Δ *luxS* (*pluxS*) (for AI-2) the relevant strain was grown in LB at 37°C for ~ 16 – 18 h with shaking. Culture supernatant which contained the AI was prepared by centrifugation of the culture to precipitate the bacterial cells. The supernatant was sterilized by filtration through $0.22\text{-}\mu\text{m}$ pore-sized filters. When appropriate, the sterile filtrate was then fractionated by using a column of Sephadex-G50 to partially purify and concentrate the AIs.

Resuscitation of *V. cholerae* O1 CVEC in Water. Environmental water samples which were found to be negative for *V. cholerae* O1 in routine enrichment cultures were selected for this assay. Three milliliters of the AI preparation was added to 3.0 mL of water along with 3.0 mL LB and grown at 37°C . Samples of the culture were withdrawn at regular intervals and plated on taurocholate tellurite gelatin agar (TTGA) (23) containing appropriate antibiotics. The cfu count on first appearance of *V. cholerae* O1 colonies in the time course growth experiment was considered as the most probable

Fig. 2. Diagrammatic representation of the formation of CVEC and their resuscitation. Stools of cholera victims contain *V. cholerae* as a heterogeneous mixture of planktonic cells, and biofilm-like clumped cells. Upon introduction of cholera stools into environmental water, a temporary loss of quorum sensing occurs due to dilution of extracellular autoinducers. As a result, quorum-mediated regulatory responses that would normally break down biofilms are blocked and the cells exist embedded in thick exopolysaccharides in a dormant form referred to as CVEC. The CVEC can provide a large dose of the pathogen when ingested by a potential victim. Presumably, the CVEC form of *V. cholerae* survives phage predation and persists in water during interepidemic time. When climatic factors lead to multiplication of diverse environmental bacteria many of which produce autoinducers, the resuscitation of CVEC is initiated, and the resulting planktonic cells seed the environment for rapid spread and amplification of the strain. Foodstuff contaminated with pathogenic *V. cholerae* in the water allows further amplification of the bacteria and consumption of the contaminated food eventually causes an index case of cholera and may eventually initiate an epidemic.

number of CVEC of *V. cholerae* O1 present in a sample. Samples without adding the AI preparation were also incubated in parallel as control assays. Parallel tests of aliquots of the same water samples by PCR and by fluorescent-labeled antibody were conducted when appropriate (24). Briefly, aliquots of samples were centrifuged to concentrate cells, and the pellets were stained using fluorescein isothiocyanate-conjugated *V. cholerae* O1-specific antiserum. Stained samples were counted under UV light, using an epifluorescence microscope connected to a digital camera recorder.

Preparation and Use of Synthetic Autoinducers in Resuscitation of CVEC. The synthetic autoinducers were produced as described previously (25–28) and were provided as a kind gift by Bonnie L. Bassler and Wai-Leung Ng, (Princeton University, Princeton, NJ and Tufts University, Medford, MA, respectively). The CAI-1 preparation was dissolved in dimethyl sulfoxide to prepare a 5-mM stock solution. The AI-2 was prepared by adding 10-fold

molar excess of boric acid to 4,5-dihydroxy-2,3-pentanedione (DPD), and the stock concentration of AI-2 was adjusted to 25 μ M. Synthetic autoinducer resuscitation mixtures consisted of 3 mL of spent culture supernatant of C6706 *luxS::TnFGL3ΔcqsA* (defective in production of both CAI-I and AI-2), 3.0 mL of environmental water, and 3.0 mL LB was spiked with 9 μ L of CAI-I or 18 μ L of AI-2 stock solution. The mixtures were incubated at 37 °C and samples of the culture were withdrawn at regular intervals and plated on TTGA containing appropriate antibiotics as described above.

ACKNOWLEDGMENTS. The authors thank Dr. Bonnie Bassler and Dr. Wai-Leung Ng for their kind gifts of synthetic CAI-1 and AI-2. This research was funded in part by National Institutes of Health Grants 2R01-GM068851-9 and AI070963-01A1, under different subagreements between the Harvard Medical School and the International Centre for Diarrhoeal Disease Research, Bangladesh, who is supported by countries and agencies that share its concern for the health problems in developing countries.

- Colwell RR, Huq A (1994) *Vibrios in the environment: Viable but non-culturable Vibrio cholerae. Vibrio cholerae and Cholera: Molecular to Global Perspectives*, eds Wachsmuth IK, Blake PA, Olsvik O (ASM Press, Washington, DC), pp 117–133.
- Faruque SM, Albert MJ, Mekalanos JJ (1998) Epidemiology, genetics and ecology of toxigenic *Vibrio cholerae*. *Microbiol Mol Biol Rev* 63:1301–1314.
- Oliver JD (2000) The public health significance of viable but nonculturable bacteria. *Nonculturable Microorganisms in the Environment*, eds Colwell RR, Grimes DJ (ASM Press, Washington, DC), pp 277–299.
- Faruque SM, et al. (2005) Seasonal epidemics of cholera inversely correlate with the prevalence of environmental cholera phages. *Proc Natl Acad Sci USA* 102(5): 1702–1707.
- Nelson EJ, Harris JB, Morris JG, Jr., Calderwood SB, Camilli A (2009) Cholera transmission: The host, pathogen and bacteriophage dynamic. *Nat Rev Microbiol* 7(10): 693–702.
- Faruque SM, et al. (2006) Transmissibility of cholera: In vivo-formed biofilms and their relationship to infectivity and persistence in the environment. *Proc Natl Acad Sci USA* 103(16):6350–6355.
- Nelson EJ, et al. (2008) Transmission of *Vibrio cholerae* is antagonized by lytic phage and entry into the aquatic environment. *PLoS Pathog* 4(10):e1000187.
- Faruque SM, et al. (2006) An improved technique for isolation of environmental *Vibrio cholerae* with epidemic potential: Monitoring the emergence of a multiple-antibiotic-resistant epidemic strain in Bangladesh. *J Infect Dis* 193(7):1029–1036.
- Zhu J, Mekalanos JJ (2003) Quorum sensing-dependent biofilms enhance colonization in *Vibrio cholerae*. *Dev Cell* 5(4):647–656.
- Lenz DH, et al. (2004) The small RNA chaperone Hfq and multiple small RNAs control quorum sensing in *Vibrio harveyi* and *Vibrio cholerae*. *Cell* 118(1):69–82.
- Hammer BK, Bassler BL (2003) Quorum sensing controls biofilm formation in *Vibrio cholerae*. *Mol Microbiol* 50(1):101–104.
- Higgins DA, et al. (2007) The major *Vibrio cholerae* autoinducer and its role in virulence factor production. *Nature* 450(7171):883–886.
- Hammer BK, Bassler BL (2007) Regulatory small RNAs circumvent the conventional quorum sensing pathway in pandemic *Vibrio cholerae*. *Proc Natl Acad Sci USA* 104(27): 11145–11149.
- Kelly RC, et al. (2009) The *Vibrio cholerae* quorum-sensing autoinducer CAI-1: analysis of the biosynthetic enzyme CqsA. *Nat Chem Biol* 5(12):891–895.
- Waters CM, Bassler BL (2006) The *Vibrio harveyi* quorum-sensing system uses shared regulatory components to discriminate between multiple autoinducers. *Genes Dev* 20(19):2754–2767.
- Bassler BL, Greenberg EP, Stevens AM (1997) Cross-species induction of luminescence in the quorum-sensing bacterium *Vibrio harveyi*. *J Bacteriol* 179(12):4043–4045.
- Xavier KB, Bassler BL (2003) LuxS quorum sensing: More than just a numbers game. *Curr Opin Microbiol* 6(2):191–197.
- Oliver JD (2010) Recent findings on the viable but nonculturable state in pathogenic bacteria. *FEMS Microbiol Rev* 34(4):415–425.
- Colwell RR, Grimes DJ (2000) Semantics and strategies. *Nonculturable Microorganisms in the Environment*, eds Colwell RR, Grimes DJ (ASM Press, Washington, DC), pp 1–6.
- Cameron DE, Urbach JM, Mekalanos JJ (2008) A defined transposon mutant library and its use in identifying motility genes in *Vibrio cholerae*. *Proc Natl Acad Sci USA* 105(25):8736–8741.

21. Meibom KL, Blokesch M, Dolganov NA, Wu CY, Schoolnik GK (2005) Chitin induces natural competence in *Vibrio cholerae*. *Science* 310(5755):1824–1827.
22. Udden SMN, et al. (2008) Acquisition of classical CTX prophage from *Vibrio cholerae* O141 by El Tor strains aided by lytic phages and chitin-induced competence. *Proc Natl Acad Sci USA* 105(33):11951–11956.
23. Monsur KA (1961) A highly selective gelatin-taurocholate-tellurite medium for the isolation of *Vibrio cholerae*. *Trans R Soc Trop Med Hyg* 55:440–442.
24. Brayton PR, Colwell RR (1987) Fluorescent antibody staining method for enumeration of viable environmental *Vibrio cholerae* O1. *J Microbiol Methods* 6:309–314.
25. Ng WL, et al. (2010) Probing bacterial transmembrane histidine kinase receptor-ligand interactions with natural and synthetic molecules. *Proc Natl Acad Sci USA* 107(12):5575–5580.
26. Wei Y, Perez LJ, Ng WL, Semmelhack MF, Bassler BL (2011) Mechanism of *Vibrio cholerae* autoinducer-1 biosynthesis. *ACS Chem Biol* 6(4):356–365.
27. Ng WL, et al. (2011) Signal production and detection specificity in *Vibrio* CqsA/CqsS quorum-sensing systems. *Mol Microbiol* 79(6):1407–1417.
28. Perez LJ, et al. (2012) Role of theCAI-1 fatty acid tail in the *Vibrio cholerae* quorum sensing response. *J Med Chem* 255:9669–9681.