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Genetic and Phenotypic Diversity of Quorum-Sensing Systems in Clinical and Environmental Isolates of *Vibrio cholerae*

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***Vibrio cholerae* is the causative agent of cholera, a severe and devastating diarrheal disease. *V. cholerae* lives naturally in various aquatic habitats during interepidemic periods. Recent studies reveal that quorum-sensing systems, which exist in many bacteria and help them monitor their population densities and regulate various cellular functions, control *V. cholerae* pathogenesis, biofilm formation, and protease production. In this study we surveyed quorum-sensing systems in 16 geographically diverse *V. cholerae* strains from epidemic-causing O1 and O139 strains as well as non-O1/non-O139 and environmental isolates and discovered an unexpectedly high rate of dysfunctional components. We also found that a functional quorum-sensing system conferred a survival advantage on bacteria in biofilms when the bacteria were exposed to seawater, though quorum sensing was less important to survival in a planktonic state under the same conditions. These findings suggest that variations in quorum-sensing systems are due to environmental selective pressures and might be beneficial to *V. cholerae*'s fitness under certain conditions found in its natural reservoirs.**

The gram-negative bacterium *Vibrio cholerae* is the causative agent of cholera, a severe diarrheal disease that affects millions of people and causes well over 100,000 deaths on an annual basis (8). *V. cholerae* communicates by monitoring its population density and alters its gene expression accordingly. This phenomenon, known as quorum sensing, is highly important for the *V. cholerae* infectious cycle in humans, controlling phenotypic factors critical to pathogenesis, such as virulence factors, biofilms, and protease production (19, 26, 32). It has been postulated that biofilms aid *V. cholerae* in the passage through the gastric barrier of the stomach, while quorum-sensing-controlled detachment from biofilms in the small intestine aids in the colonization of the intestinal epithelium and eventual infection of new hosts (32). In addition, quorum sensing and biofilms have also been implicated in the long-term survival of *V. cholerae* in its natural marine environment (19, 26).

Quorum sensing in *V. cholerae* is controlled by two central regulators, LuxO and HapR. LuxO responds to accumulated molecules in the environment called autoinducers via membrane receptors CqsS and LuxPQ. Two autoinducer molecules are important for quorum sensing in *V. cholerae*. These two molecules are cholera autoinducer 1 (CAI-1), a molecule of undetermined structure, and autoinducer 2 (AI-2), a furanosyl borate diester also produced by *Vibrio harveyi* and many other bacteria. At high cell densities and autoinducer concentrations, this system causes LuxO to turn off its repression of *hapR* (23, 25). HapR can then repress the expression of *aphA*, whose gene products are key activators of virulence regulons (22). HapR also represses the *vps* (*Vibrio* polysaccharide synthesis) operon, thus negatively regulating biofilm formation (15, 31, 32). In addition, HapR directly up-regulates the expression of *hapA*, which produces a secreted hemagglutinin (HA)/protease responsible for the detachment of vibrios from the intestinal epithelium (11, 18).

Although *V. cholerae* has over 200 known serotypes, only strains with O1 and O139 antigens are known to cause epidemic disease (26). The O1 serogroup is further divided into O1 classical and El Tor biotypes on the basis of other physiological properties. O1 classical strains were responsible for six pandemics prior to 1961, when the first El Tor strain associated with a major epidemic appeared in Indonesia, which subsequently took over as the major disease-causing O1 biotype (9). Pathogenic O139 strains are relatively new, presumably having acquired virulence genes via mobile genetic elements. They were first isolated in 1992 from an epidemic outbreak of cholera in southern Asia (10). Both O1 El Tor and O139 *V. cholerae* strains are causes of annual outbreaks today. Some environmental and non-O1/non-O139 strains have also been shown to cause symptoms of cholera, although it is generally accepted that these strains lack the capability to cause major epidemic disease. Recent comparative genomic microarray analysis of pathogenic non-O1/non-O139 strains indicates that these strains carry a type III secretion system that may be involved in virulence and environmental fitness (5).

In this study we investigated the *V. cholerae* quorum-sensing system in 16 diverse strains and its relationship to virulence factors, biofilm, and protease regulation. The examined strains belong to the epidemic-causing serotypes of O1 and O139 antigen groups, as well as non-O1/non-O139 and environmental strains lacking the CTX phage encoding cholera toxin. We found a wide variety of active and defective quorum-sensing controls in these strains, raising questions as to the importance and role of this system in infection, environmental survival, and ultimately, of the evolution of the quorum-sensing system itself.

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MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. *V. cholerae* strains used in this study are listed in Table 1 and were propagated in LB containing appropriate antibiotics at 37°C, unless otherwise noted. The plasmid containing a constitu-

TABLE 1. Quorum-sensing components and quorum-regulated phenotypes in various *V. cholerae* strains

Strain (reference)	Place and yr of isolation	Biotype and serogroup	Quorum-sensing component		Quorum-sensing regulation				
			hapR sequence	HapR production[a]	*lux* expression[b]	Mean CT production (μg/ml/OD_{600}) (SD)[c]	Biofilm formation in LB/SW (OD_{570})[d]	Mean HA/protease production (azocasein units) (SD)[e]	Mean protease recovery (azocasein units) (SD)[f]
N16961 (4)	Bangladesh, 1971	El Tor, O1	Frameshift	−	−	0.86 (0.07)	0.38/0.54	3.3 (1.6)	30.1 (1.7)
C6706 (4)	Peru, 1991	El Tor, O1	+[g]	+	+	2.97 (0.03)	1.03/0.59	22.9 (4.3)	NA
HK1 (4)	Hong Kong, 1961	El Tor, O1	+	−	−	2.86 (0.92)	2.45/1.16	2.9 (0.3)	27.1 (0.5)
NCTC8457 (4)	Saudi Arabia, 1910	El Tor, O1	+	+	+	0.08 (0.08)	1.79/0.84	27.6 (7.3)	NA
MAK757 (4)	Celebes Islands, 1937	El Tor, O1	+	+	−	0.31 (0.18)	0.20/0.18	1.3 (1.2)	12.1 (0.8)
857[h]	Bangladesh, 1996	El Tor, O1, environmental	+	+	−	NA	0.20/0.79	5.5 (3.5)	45.1 (0.1)
2740-80 (4)	Gulf Coast, 1980	El Tor, O1, environmental	+	+	+	NA	1.25/0.76	58.1 (1.9)	NA
O395 (4)	India, 1965	Classical, O1	Frameshift	−	−	8.87 (0.97)	0.15/0.29	3.0 (0.1)	23.6 (1.3)
CA401 (12, 16)	India, 1953	Classical, O1	+	+	Constitutive	4.46 (1.40)	0.15/0.29	51.5 (4.7)	NA
MO10 (4)	India, 1992	El Tor, O139	+	+	−	0.69 (0.26)	0.69/0.97	0.7 (0.0)	26.4 (0.3)
MDO14 (6, 17, 27)	India, 1992	El Tor, O139	+	+	+	1.42 (0.36)	0.16/0.58	48.7 (2.6)	NA
MDO14-T (6, 17, 27)	India, 1992	El Tor, O139	No *hapR*	−	−	0.69 (0.26)	0.70/1.04	3.8 (0.1)	38.6 (1.9)
SG21 (14)	India, 1992	El Tor, O139	C-terminal deletion	Short	Low	0.70 (0.11)	0.85/0.74	3.3 (0.8)	24.5 (1.2)
SG38-2 (14)	India, 1992	El Tor, O139	+	+	Constitutive	0.06 (0.05)	1.07/1.50	56.2 (0.2)	NA
MZO-2 (5)	Bangladesh, 2001	Non-O1/non-O139	+	+	+	NA	0.94/0.83	24.4 (4.2)	NA
AM15622 (5)	Bangladesh, 2001	Non-O1/non-O139	+	+	+	NA	0.27/0.52	52.2 (0.6)	NA

[a] Results summarized from the Western blot analysis of HapR protein (Fig. 2). −, not produced; +, produced.
[b] Results summarized from strains carrying pBB1 plasmid (Fig. 1). −, not expressed; +, expressed.
[c] Classical strains were grown in LB (pH 6.5) at 30°C. Others were grown under AKI conditions. NA (not applicable) refers to CTX^- strains.
[d] Strains were grown in LB or sea water (SW) on a glass surface, and biofilm mass was quantified using dimethyl sulfoxide. The standard deviations were ≤10%.
[e] Cell-free supernatants from cultures grown at 37°C for 7 h were collected and measured for protease activity as described previously.
[f] A plasmid carrying P_{tac}-*hapR* was introduced into these strains, and protease activities were then measured. NA, not applicable.
[g] + the *hapR* sequence encodes intact HapR protein.
[h] From the strain collection of John Mekalanos.

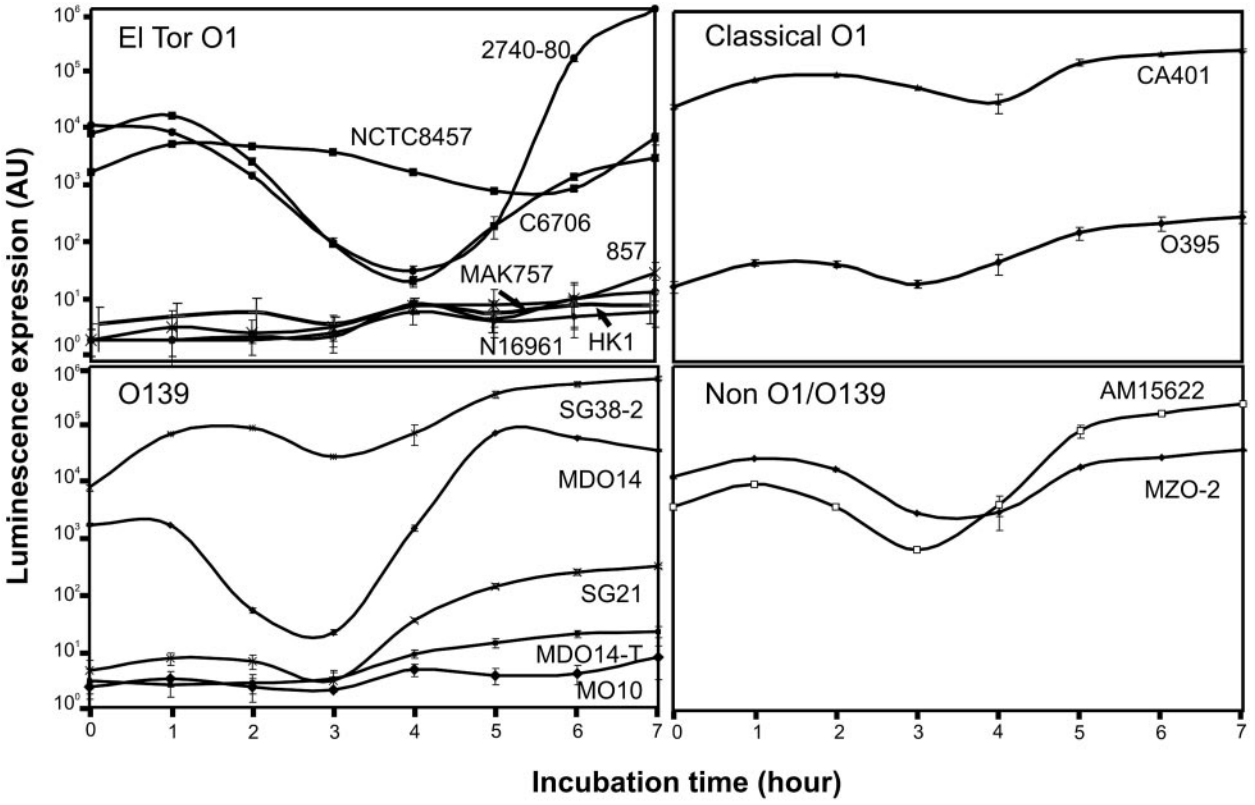

FIG. 1. Quorum-sensing-regulated luminescence expression in various *V. cholerae* strains. Overnight cultures were diluted 1:100 in LB and incubated at 30°C. Luminescence was measured at the time indicated. Lux expression curves were grouped on the basis of the strain biotypes. The values are means ± standard deviations (error bars). The results are representative of three experiments. AU, arbitrary unit.

tively expressed *hapR* was constructed by subcloning of P_{tac}-*hapR* fragment from pJZ146 (33) into pBBR1-MCS2 (20).

Luminescence assay. The pBB1 cosmid, carrying the *V. harveyi lux* operon (1) was introduced into *V. cholerae* strains by conjugation or electroporation. The resulting strains were grown in LB with appropriate antibiotics at 30°C overnight, diluted to a concentration of 1:100 in fresh LB, transferred to white opaque 96-well plates, and incubated while shaking at 30°C. Luminescence was read at 1-h intervals for 7 h, with an initial reading at time zero, using a Bio-Tek Synergy HT spectrophotometer.

Western immunoblots of HapR. Strains were grown in LB overnight and inoculated at 1:100 into 3 ml LB broth and grown for 7 h to late log phase. Cell pellets were size fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, and detected with affinity-purified polyclonal anti-HapR rabbit antiserum.

Detection of CT, HA/protease, and autoinducers. GM1 ganglioside enzyme-linked immunosorbent cholera toxin (CT) assays (13) were carried out following overnight incubations of *V. cholerae* strains with shaking at 37°C in AKI medium (for strains other than classical biotypes) or 30°C in LB (pH 6.5) (for classical strains) (17a).

HA/protease azocasein assays were performed as described previously (2). Briefly, strains were grown in 1 ml LB overnight with shaking and at 37°C. One hundred microliters of cell-free supernatant was combined with 100 μl azocasein (MP Biomedicals) in 100 mM Tris-Cl (pH 8) and incubated at 37°C for 1 h. After incubation, 400 μl 10% trichloroacetic acid was added and mixed, followed by a 15-min centrifuge step to pellet resulting precipitate. Three hundred microliters of this supernatant was transferred to 350 μl NaOH (525 mM), and the optical density at 442 nm (OD_{442}) was taken. Results are reported as azocasein units calculated as follows: (OD unit − background) × 100.

The AI-2 bioassay using the *V. harveyi* reporter strain BB170, sensitive only to AI-2, has been reported previously (29). CAI-1 production was assayed as previously published using *V. cholerae* bioassay strain MM920 (25). Assays were performed with an opaque 96-well plate, and luminescence was read at 4 h postinoculation with a Bio-Tek Synergy HT spectrophotometer. Results were calculated as log of the change in the level from the background level (blank LB).

Biofilm formation assays. Strains were suspended in 1 ml LB broth from growth on LB plates to an OD_{600} of 0.6. One milliliter of LB broth or seawater supplemented with 0.1% casein amino acids was inoculated at 1:100 in duplicate in borosilicate glass tubes (10 × 75 mm) and incubated at room temperature for 24 h. Biofilms were stained using crystal violet, and biofilm mass was measured as previously reported (32).

Biofilm and planktonic fitness. Biofilms were grown for 24 h in 1 ml LB inoculated 1:100 from cultures of C6706 and C6706 *hapR* mutant at an OD_{600} of 0.6. After incubation, culture broth was removed and resuspended in 1.5 ml seawater (sampled from New Jersey coastline, filtered using Whatman paper, and sterilized) for planktonic survival assay. Biofilms were washed and then submerged in 1.5 ml seawater. Test tubes were placed in 4°C. After 5-day incubation, CFU (colony formation unit) was quantified from both planktonic and biofilm samples on LB plates.

RESULTS

Induced luminescence shows quorum-sensing variability across *V. cholerae* biotypes and serogroups. As previously shown, quorum-sensing systems in *V. cholerae* are able to activate the *V. harveyi luxCDABE* operon (23, 25). On the basis of luminescence levels, a functional quorum-sensing system generates a U-shaped curve on a plot of luminescence versus time of incubation (1). To study quorum-sensing regulation in various *V. cholerae* strains, the pBB1 cosmid containing the *V. harveyi luxCDABE* operon was introduced into 16 clinical or environmental *V. cholerae* isolates, and luminescence was measured as a function of cell growth. The strains were grown at 30°C and displayed similar growth rates. Using this assay, three patterns of *lux* expression were observed (Fig. 1 and Table 1). The first

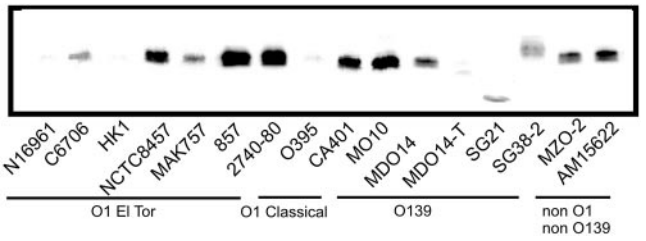

FIG. 2. Production of quorum-sensing regulator HapR in 16 *V. cholerae* strains. Equal amounts of total protein (10 μg) of late-log cultures were size fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, and detected with affinity-purified polyclonal anti-HapR rabbit antiserum (23).

pattern of cell-density-dependent curves, indicative of the presence of functional quorum-sensing systems, was observed in six strains (C6706, NCTC8457, 2740-80, MDO14, MZO-2, and AM15622). The second pattern of low, noninduced luminescence was observed in eight strains (N16961, HK1, MAK757, 857, O395, MO10, SG21, and MDO14-T). The third pattern was observed in two strains (CA401 and SG38-2), displaying an apparent constitutive expression of luminescence. The *lux* expression patterns of the latter two categories suggest that these strains have defective quorum-sensing systems. There was no obvious correlation between the quorum-sensing regulation pattern and the biotype, serogroup, or year of isolation of *V. cholerae*.

HapR expression, *hapR* sequence, and HapR's effect on luminescence. Two key proteins, LuxO and HapR, regulate quorum sensing in *V. cholerae* (25, 33). HapR is a functional *V. harveyi* LuxR homolog, which positively regulates luminescence in these organisms, while LuxO represses *hapR* expression at low density, and thus luminescence, through a set of small RNAs (23). To determine whether the *V. cholerae* strains that were defective in quorum sensing were also deficient in their expression of *hapR*, Western blotting using HapR antiserum was performed. Surprisingly, most strains produced HapR at or above the levels of the previously studied C6706 strain (23), with a few notable exceptions (Fig. 2 and Table 1). Strains N16961, HK1, O395, and MDO14-T did not produce any detectable HapR. The Western blot of strain SG21 showed a smaller band, indicating the production of a truncated protein. Also, strain SG38-2 produced a band representing a slightly larger protein than that of the other *V. cholerae* strains. All strains lacking HapR expression, N16961, HK1, O395, and MDO14-T, exhibited very low levels of luminescence induction. Conversely, strains MAK757, MO10, and 857 all expressed HapR at levels higher than that of C6706 but were still deficient in inducing luminescence. This is indicative of a nonfunctional HapR, possibly due to a mutation. Interestingly, SG21 induced low levels of luminescence in a quorum-sensing-dependent manner, indicating a HapR partially functional in luminescence induction, even though its HapR protein is smaller than the full-length HapR.

We have reported that there is a naturally occurring frameshift mutation in the *hapR* sequence in both the sequencing strain N16961 and the widely used classical strain O395 (33). To further characterize the functionality of HapR, we sequenced *hapR* in each of the strains to identify any mutations (Fig. 3 and Table 1). Three strains, N16961, O395 and SG21, all had premature stop codons producing predicted peptides with 79, 68, and 119 amino acids, respectively, compared with the 204 amino acids of the peptide of strain C6706. These data are consistent with a lack of functional HapR expression (Fig. 2) and a lack of luminescence induction of pBB1 in strains N16961 and O395. Strain SG21, however, shows a response typical of quorum sensing and a distinct band on the Western blot assay. This indicates a possible conservation of function in this truncated protein. Point mutations found in strains MO10, 857, and MAK757, leading to one, two, and seven altered amino

FIG. 3. Alignment of HapR amino acid sequences. The well-studied HapR protein from strain C6706 was used as a reference, and all other sequences were aligned against this sequence using BLOSUM 62 scoring matrix. The amino acids that are different from those in HapR from C6706 are highlighted. Dashes indicate absence of amino acids.

acids, respectively (Fig. 3), had no effect on HapR expression, though these strains did not induce significant levels of luminescence with the introduced *lux* operon (pBB1), raising the possibility that these mutations have had a deleterious effect on HapR function. Point mutations also existed in five strains (2740-80, SG38-2, NCTC8457, MZO-2, and AM15622) though these strains had normal HapR expression and positive induction of luminescence.

We failed to PCR amplify the *hapR* region from strain MDO14-T. MDO14-T is a translucent variant of MDO14 (opalescent) that changes from an opalescent to translucent type at a rate of approximately 1% on LB plates. No hybridization was detected on a Southern blot, using C6706 *hapR* DNA fragments as probes, in MDO14-T (data not shown). This leads to the conclusion that the translucent variant of MDO14 completely lacks *hapR*. The mechanism of this deletion is currently under investigation.

The remaining four strains (C6706, HK1, CA401 and MDO14) showed no alterations in their HapR sequences. El Tor strain HK1 has a C6706-type sequence of *hapR*, yet no HapR was detected on the Western blot. This might indicate a defect in another component of its quorum-sensing system, including autoinducer production. *V. cholerae* has been shown to produce at least two of these autoinducers, CAI-1 and AI-2, which synergistically modulate the activity of LuxO. We tested each strain's ability to produce such autoinducers by incubating *V. harveyi* and a *V. cholerae* reporter strain in each of the strain's culture supernatants, under conditions that induce luminescence activity only in the presence of specific inducers. We found all strains to have normal CAI-1 and AI-2 production (data not shown) except for HK1, which had a delayed production of CAI-1 based on incubation time (Fig. 4). This delay may be responsible for an elongated period of HapR repression due to the activity of LuxO, which is critical for the control of *hapR* expression. Alternatively, it is possible that other unknown quorum-sensing components are defective in HK1.

Strains CA401 and SG38-2 induced luminescence of the *lux* operon in a constitutive manner. This, again, indicates a possible dysfunction of the LuxO repressor or other signaling components in these strains, negating the effects of cell densities on HapR function. We sequenced the *luxO* locus in these strains and found that they both have C6706-type LuxO amino acid sequences, indicating that other signaling steps in the quorum-sensing cascade might be defective. With the exception of SG21, all strains that produced a quorum-sensing curve in the luminescence assay had a wild-type or functional LuxO sequence as well as strong HapR expression.

Quorum-sensing-controlled phenotypes: HA/protease, CT, and biofilms. Many of the above experiments used the nonnative Lux target as an indicator of quorum-sensing behavior. To investigate the expression of endogenous *V. cholerae* quorum-sensing targets, we examined protease production, cholera toxin, and biofilm formation in these strains. At high cell densities, HapR positively regulates protease production and negatively regulates virulence genes and biofilm production (15, 25, 33).

A variety of phenotypes related to quorum-sensing functions was observed. Protease production (Table 1) was deficient in all strains that either had a premature stop codon or deleterious mutations (as defined by a lack of luminescence induction) in the HapR protein. El Tor strain HK1 was also deficient in producing protease, which is consistent with its lack of HapR expression. Strains CA401 and SG38-2 produced high levels of protease even at low density (data not shown), reinforcing the notion that they have a possible quorum-sensing signaling cascade dysfunction that is locked in the regulatory state characteristic of high cell density. The remaining strains all induce protease activity in a density-dependent manner with wild-type HapR protein expression and luminescence induction (data not shown).

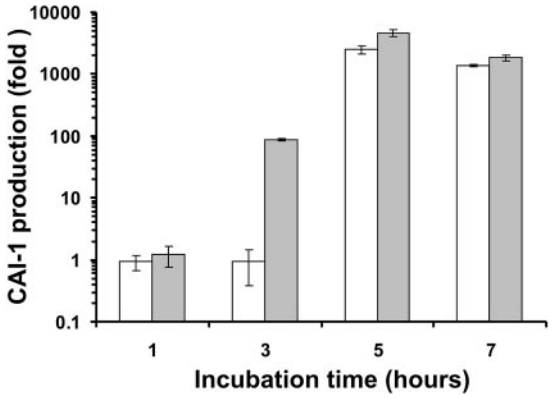

FIG. 4. *V. cholerae* autoinducer CAI-1 production in strains HK1 and C6706. Overnight cultures were diluted 1:100 in fresh LB and grown at 30°C. Cell-free supernatants were withdrawn at the time indicated and assayed for CAI-1 activity using *V. cholerae* bioassay strain MM920 (25). CAI production is represented as the change in luminescence induction from the background level in strains HK1 (open bars) and C6706 (gray bars). The values are means ± standard deviations (error bars). The results are representative of three experiments.

To see whether a constitutively expressed HapR could recover protease production, we introduced a plasmid containing P_{tac}-controlled *hapR* into the protease-deficient strains. All strains recovered normal levels of protease production using this method (Table 1), indicating that the protease-deficient phenotype results solely from a dysfunctional HapR.

HapR has also been shown to inhibit expression of virulence genes, such as the toxin-coregulated pilus pathogenicity island and cholera toxin genes, by repressing the expression of *aphA*, which encodes a key virulence regulator in *V. cholerae* (21, 28). In the in vitro induction condition, early expression of the *hapR* gene, such as in *luxO* mutants, inhibits the virulence regulon (32). We measured cholera toxin production of these isolates studied here using a CT enzyme-linked immunosorbent assay. Environmental strains 857 and 2740-80 as well as non-O1/non-O139 strains MZO-2 and AM15622, lack the genes encoding CT and subsequently produced no toxin. This is also true for O1 El Tor strain NCTC8457, which has been shown to lack the entire CTX phage, and thus produces no detectable toxin (4). The strains with a functional HapR sequence and HapR expression showed variability in their CT expression. Of these strains, only SG38-2 produced no detectable CT under AKI-inducing conditions. SG38-2 has what seems to be a LuxO deficiency that allows for a high level of HapR expression independent of cell density, and thus, can

explain the very low levels of CT production in this strain. On the other hand, the classical strain CA401 produced high levels of CT, even though it also has a possible constitutive quorum-sensing system. Kovacikova et al. reported that the *aphA* promoter of classical strains is different from that of El Tor, and as such, HapR cannot repress *aphA* expression in classical strains (21). This may explain why CA401 still produced CT. The remaining strains produced a variable amount of CT whether they had an intact or mutated HapR, confirming previous in vitro results that mutations in *hapR* do not affect cholera toxin production (33).

Quorum sensing also negatively regulates biofilm formation in *V. cholerae*. Strains with HapR deletions have been shown to produce more robust biofilms than their wild-type counterparts (15, 30, 32). Biofilms are an important physiological characteristic of *V. cholerae* and have been implicated in many bacteria as an adaptation that provides protection against environmental stresses, such as acid shock (such as in the stomach), detergents, and antibiotics and also the predatory actions of certain protozoa (3, 24). All strains surveyed in this study produced some biofilm on a glass surface in both LB medium and seawater, but they differed greatly in the amount of biofilm produced. Strain HK1, which expressed no HapR, produced a very robust biofilm. MDO14-T, which lacks the *hapR* gene, produced more biofilm than MDO14, which has an intact *hapR* gene. NCTC8457, however, with a functional type HapR sequence and normal expression, produced a very robust biofilm, highlighting the variation of this trait in individual strains. Also, O1 classical strains produced, on average, the least biofilm mass of the serotypes and biotypes tested, also highlighting the variation of biofilm production in *V. cholerae* isolates.

Quorum sensing and *V. cholerae* environmental survival. The results of the quorum-sensing survey above indicate that *V. cholerae* quorum-sensing systems are naturally diverse. This raises an important question regarding the selection of quorum sensing in the evolution of *V. cholerae* in terms of environmental survival. *V. cholerae*, which has the capacity to adapt to changes in salinity, temperature, and availability of nutrients, can successfully occupy one or more ecological niches in aquatic habitats as free-living bacteria or in association with phytoplankton or zooplankton (7). Because the bacterium exists in these two distinct phases, we hypothesized that the frequent mutations in the quorum-sensing systems of *V. cholerae* are favorable in certain aquatic environments by allowing the bacterium to more readily respond to environmental stresses. To investigate this possibility, we tested the longevity of a well-studied wild-type strain and an isogenic *hapR* mutant strain existing solely in biofilms versus that of planktonic cells in 4°C seawater for 5 days.

First, we investigated the importance of quorum sensing in biofilm-associated cells. Both wild-type and *hapR* mutant strains were grown in LB for 24 h to produce saturated biofilms. The LB was removed, and biofilms were then exposed to cold unsupplemented seawater. This procedure models a fast switch from a nutrient-rich to a nutrient-deprived environment that *V. cholerae* might experience in its natural reservoirs. The wild-type C6706 strain survived better than the isogenic *hapR* mutant under these experimental conditions ($P < 0.05$) (Fig. 5). This indicates a possible evolutionary role of quorum-sensing systems as an adaptation for environmental survival. This is quite surprising,

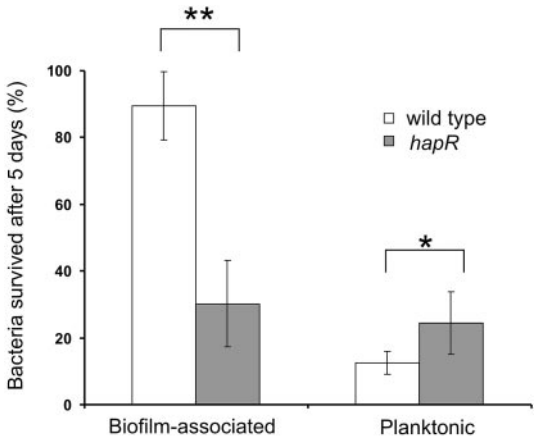

FIG. 5. Effect of quorum sensing on *V. cholerae* survival in an extreme environment. Biofilm-associated and planktonic wild-type C6706 cells and quorum-sensing-deficient mutant *hapR* cells were exposed to 4°C seawater. The number of CFU was determined after 5 days of incubation and represented as the percentage of the initial CFU. The values are means ± standard deviations (error bars). The results are representative of three experiments. Values that are significantly different from the wild-type value by the Student *t* test are indicated by asterisks (**, $P < 0.05$; *, $P < 0.5$).

considering the observed quorum-sensing dysfunctions in *V. cholerae* in this study. We also tested the survival rate of cells existing in a planktonic state under the same conditions. We found that the survival rate for planktonic *hapR* mutant cells was slightly higher than that for wild-type cells ($P < 0.5$). Although the statistical significance is low with these conditions and relatively small sample sizes, we believe these data suggest that a loss or alteration of quorum-sensing functions may actually confer a survival advantage for planktonic bacteria, which might explain why many quorum-sensing-deficient *V. cholerae* isolates exist. This possibility warrants further study.

DISCUSSION

Bacteria consistently sense and respond to environmental stimuli to modulate gene expression to adapt to changing environments. For example, many bacteria utilize quorum-sensing systems to monitor their population densities. In this study, we studied the *V. cholerae* quorum-sensing system and its regulated genes in 16 geographically diverse strains. Our data indicate three different states of quorum-sensing systems in the strains used in this study. First, six strains (C6706, NCTC8457, 2740-80, MZO-2, AM15622, and MDO14) have both functional *hapR* sequences and expression along with functional HapR-mediated regulation of protease production and *V. harveyi* luminescence. Second, eight strains (N16961, HK1, MAK757, 857, O395, MO10, MDO14-T, and SG21), have mutated HapR proteins that lack the ability to induce or induce very low levels of protease and luminescence. Third, two strains (CA401 and SG38-2) seem to have a constitutively expressed quorum-sensing system even though they have functional *hapR* and *luxO* sequences. Our subsequent data suggest that the improved stress survival of *V. cholerae* wild-type biofilm and *hapR* mutant planktonic cells provides possible explanations for the conservation and high rate of dysfunction of quorum-sensing components in *V. cholerae*, depending on what features are being positively selected for in the envi-

ronment. We speculate that *V. cholerae* has the capacity to change quorum-sensing regulatory components in response to different environmental pressures. Recent reports have shown that the frequency of quorum-sensing regulator mutations under certain laboratory conditions is relatively high (15, 30), and thus, this phenomenon might be an important survival tactic of *V. cholerae*.

Since quorum sensing in *V. cholerae* negatively regulates virulence gene expression, having a dysfunctional quorum-sensing system does not reduce *V. cholerae* virulence factor production per se, and thus in this sense is not related to a strain's ability to cause cholera symptoms. For example, *hapR* mutant strains can colonize infant mice as well as wild-type strains can (25, 33). Therefore, quorum sensing is less likely to play a role in the natural selection of pathogenic strains, but it is important in the infectious cycle due to its benefits of aiding survival between outbreaks and its role in late stages of infection.

Quorum sensing may also be important for enhancing *V. cholerae* colonization if *V. cholerae* enters hosts as a part of a biofilm. When *V. cholerae* biofilms reach the upper intestine, quorum sensing down-regulates *vps* gene expression, causing *V. cholerae* detachment from biofilm structures and thus promoting colonization of the intestinal surface (32). Furthermore, previous research also suggests that quorum sensing down-regulates virulence genes and activates the production of extracellular proteases at the late infectious stage (33). These proteases are proposed to serve as detachases, allowing the bacteria to exit the host. Finally, although this study focused on HapR-dependent quorum-sensing systems, we cannot rule out the possible existence of other distinct quorum-sensing regulatory pathways in various *V. cholerae* isolates.

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