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The Prevalence of Functional Quorum-Sensing Systems in Recently Emerged *Vibrio cholerae* Toxigenic Strains

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Summary

Vibrio cholerae live in aquatic environments and cause cholera disease. Like many other bacteria, *V. cholerae* use quorum-sensing (QS) systems to control various cellular functions, such as pathogenesis and biofilm formation. However, some *V. cholerae* strains are naturally QS-defective, including defective mutations in the quorum sensing master regulator HapR. Here we examined the QS functionality of 602 *V. cholerae* clinical and environmental strains isolated in China from 1960–2007, by measuring QS-regulated gene expression. We found that a greater percentage of the toxigenic strains (*ctxAB*⁺) had functional QS as compared to the non-toxigenic strains (*ctxAB*[−]), and that this trend increased significantly over time. We hypothesize that QS provides adaptive value in *V. cholerae* pathogenic settings.

Introduction

Vibrio cholerae is a gram-negative bacterium responsible for the pandemic and epidemic diarrheal disease cholera. Between epidemics, this facultative pathogen resides predominantly in a variety of aqueous environments (Faruque et al., 1998). To successfully navigate the transitions between its human host and the environmental reservoir, *V. cholerae* employs a number of coordinated transcriptional regulatory events (Faruque et al., 1998). One of these regulatory systems is quorum sensing (QS), which is used by both Gram-negative and Gram-positive bacteria to sense and respond to their own population densities (Ng and Bassler, 2009). *V. cholerae* uses quorum sensing to control phenotypic factors critical to environmental survival, pathogenesis and transmission. For example, quorum sensing in *V. cholerae* negatively regulates expression of virulence genes (such as *ctxAB*, which encodes cholera toxin) and biofilm formation, and activates protease production at high cell density, suggesting that quorum sensing is important for bacteria entering and exiting the host (Zhu et al., 2002; Zhu and Mekalanos, 2003; Nielsen et al., 2006). It has also been reported that quorum sensing plays a critical role in enhancing stress response, predator grazing resistance, natural competence, and regulating a number of other cellular functions

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(Meibom et al., 2005; Vaitkevicius et al., 2006; Joelsson et al., 2007; Tsou et al., 2009), all of which may be advantageous for survival in the aquatic environment.

Quorum sensing relies on the secretion and detection of signaling molecules called autoinducers. CAI-1 and AI-2 are two autoinducers secreted by *V. cholerae*, and accumulation of these molecules at high cell density results in a series of signal transduction events, which lead to the expression of the quorum sensing master regulator HapR (Miller et al., 2002; Lenz et al., 2004; Higgins et al., 2007). Although *V. cholerae* is capable of regulating some quorum sensing-controlled gene expression using a HapR-independent pathway (Hammer and Bassler, 2007), HapR is nevertheless responsible for directly regulating a number of target genes (Tsou et al., 2009). It is, therefore, the production of HapR that is the key for quorum sensing regulated genes. Intriguingly, loss-of-function HapR mutations occur naturally in some *V. cholerae* isolates at a rather high frequency (Joelsson et al., 2006; Talyzina et al., 2009). Recently, Talyzina *et al.* analyzed *hapR* nucleotide sequences in 37 strains and found a point mutation which is strongly associated with *V. cholerae* pandemic strains (Talyzina et al., 2009). However, in that study *hapR* sequence was not correlated with HapR function and the relationship between functional HapR-dependent quorum-sensing systems and the pathogenic ability of *V. cholerae* was not determined.

In this study we investigated quorum-sensing systems in 602 strains isolated in China from 1960 to 2007. We found that a greater percentage of the toxigenic strains (*ctxAB*⁺) had functional quorum-sensing systems as compared to the non-toxigenic strains (*ctxAB*⁻), and that this trend increased significantly over time, suggesting a positive adaptive role played by quorum sensing.

Results and discussion

Quorum sensing functionality in *V. cholerae* clinical and environmental isolates

It has been shown that mutations often accumulate in *V. cholerae* isolates that lead to loss of quorum sensing functions (Joelsson et al., 2006), but due to the sample size (16 strains), the relationship between *V. cholerae* isolates that contain functional quorum sensing systems and *V. cholerae* environmental survival and/or pathogenesis is unknown. We therefore examined 602 *V. cholerae* strains isolated from different regions of China from the 1960s to the present. The strains were isolated from cholera patient stool samples and environmental water samples using standard *V. cholerae* isolation techniques and stored at -80°C. Informed consent was obtained from patients or their parents or guardians when clinical samples were collected. These strains have not been extensively propagated in laboratories; therefore the chances to have artificially-induced mutations are low. The serogroups of these strains have been determined as either O1 or O139 by using different *V. cholerae* antisera generated from typed strains. To examine the functionality of their quorum-sensing systems, we introduced a cosmid containing the *luxCDABE* operon from *V. harveyi* (pBB1) (Bassler et al., 1993) into each of these strains by conjugation or electroporation, and measured the luminescence as a function of cell growth. If the quorum-sensing system is functional (QS⁺), *V. cholerae* is able to activate the *luxCDABE* operon and produce a U-shaped Lux curve (Miller et al., 2002). Strains that harbor dysfunctional QS systems may display a constitutively expressed luminescence curve (QS^c, suggesting loss of cell-density-dependent expression of *hapR*) or a low, non-induced luminescence curve (QS⁻, possibly due to a non-functional HapR) (Fig. 1A).

To ensure that strains were not judged to be QS⁻ simply due to a defect in luciferase substrate production, we measured hemagglutinin/protease (Hap) production [activated by HapR (Zhu et al., 2002)] on milk plates (Finkelstein et al., 1992) and performed HapR

western blots (Liu et al., 2008) on all strains that displayed QS⁻ phenotypes. We found that QS⁻ strains (defined by luminescence phenotypes) also were Hap⁻ and no HapR protein was detected (data not shown). Out of 602 strains, we found that 31.6% strains contain functional quorum sensing systems (QS⁺), 13.4% strains are QS^c and 55% are QS⁻ (Fig. 1B). Interestingly, these data are quite comparable to the quorum sensing distribution (37.5% QS⁺, 12.5% QS^c, and 50% QS⁻) found in a previous study with a much smaller sample size (16 strains) but isolated from various locations globally (Joelsson et al., 2006). These data imply that the distribution of quorum-sensing systems in the strains isolated from China we analyzed in this study may reflect the global distribution of quorum-sensing systems in different *V. cholerae* strains.

Functional quorum-sensing systems in toxigenic strains

In order to elucidate any possible correlations of quorum sensing with *V. cholerae* life styles, we analyzed the percentage of quorum sensing-positive strains in different categories of *V. cholerae* isolates. We did not detect any statistical differences in these numbers based on the point of isolation of the strain (rivers, lakes, estuaries, or seawater) (data not shown). Similarly we found no preferential correlation of QS⁺ strains with either serogroup (O1 and O139) or serotype (Ogawa, Inaba, and Hikojima) (data not shown).

We then investigated how quorum sensing functionality in these strains is related to the presence of other factors which influence *V. cholerae* pathogenesis. As cholera toxin (CT) plays a profound role in the pathogenesis of the bacterium, the presence of cholera toxin genes is the hallmark of toxigenic *V. cholerae*. Toxigenic *V. cholerae* is defined by the presence of one or more copies of CT genes (*ctxAB*) (Faruque et al., 1998). We examined the presence or absence of *ctxAB* genes in all 602 strains by using PCR amplification (Keasler and Hall, 1993), and found 403 strains to be CT⁺ and 199 to be CT⁻. In addition, we measured CT production of all these strains. Each strain was grown under AKI conditions (Ishikawa et al., 2009) and the culture supernatants were subjected to the CT ELISA assays (Gardel and Mekalanos, 1994). We found that CT production results correlated well with the presence/absence of *ctxAB* genes assayed by PCR (data not shown). Strikingly, we found that the percentage of QS⁺ strains was significantly higher in toxigenic strains than in non-toxigenic ones. As shown in Fig. 2A, 39.0% of CT⁺ strains had functional quorum sensing systems (U-shaped Lux curve, QS⁺), while only 14.5% of CT⁻ strains were QS⁺ (p value <0.001). Moreover, we observed an increase in the percentage of QS⁺ in CT⁺ strains over time, from 20.8% in the 60s to 64.6% in the 2000s (Fig. 2B, solid line), while the percentage of QS⁺ in CT⁻ strains remained virtually unchanged over the years (average of approximately 15%) (Fig. 2B, dashed line). These data suggest that having a functional quorum-sensing system may confer continuous selective advantages for toxigenic strains that are required to enter and exit hosts efficiently. We then speculated that there might be a greater percentage of QS⁺ strains among clinical isolates as compared to environmentally isolated strains. However, when we regrouped the strains based on the source of isolation, we did not observe a significant correlation between clinical isolates and QS⁺ (32% in clinical isolates vs. 23% in environmental isolates). This conclusion is complicated however, by the fact that so many of our environmentally isolated strains are CT⁺ (80%). We believe that this skews the data since there is such a positive correlation of toxigenic genes and QS⁺.

Mutations in quorum sensing master regulator HapR

We then went on to explore the genetic determinant(s) of the loss of quorum sensing function in the QS⁻ strains. Since HapR is the master quorum sensing regulator and naturally occurring HapR null mutations are observed in a number of strains, we determined *hapR* sequences in all of the QS⁻ strains (see the supplementary Table 1). We found that

hapR in approximately 70% of the QS⁻ strains (228 strains) contained various mutations as compared to wild type *hapR* (such as in C6706) (Joelsson et al., 2006), including frame-shift mutations. Of note, in strains containing these frame-shift mutations, the *hapR* sequence remained unchanged after the mutation, suggesting a relatively recent event. Interestingly, 73 of them contained an alanine to valine mutation at the 191st amino acid of HapR. Unlike wild type *hapR* (Zhu and Mekalanos, 2003), a constitutively expressed *hapR*^{A191V} failed to complement a *hapR* deletion in C6706, as assessed by the ability to convert rugose colonies to smooth (Fig. 3, left panel) and to activate the *V. harveyi luxCDABE* operon (Fig. 3, right panel), suggesting that this residue is critical for HapR function. Intriguingly, the percentage of the A191V mutants was significantly higher in CT⁻ strains than in CT⁺ (Fig. 4). Since it has been shown that HapR regulates a number of varieties of genes with different affinities (Tsou et al., 2009), it is possible that this mutation may confer additional, unknown functions to HapR and render *V. cholerae* strains an advantage for survival in aquatic environments. In addition, we discovered that 103 QS⁻ strains either encoded a wild type *hapR* (as in C6706) or had silent mutations within *hapR*. Whereas these strains produced wild type levels of both CAI-1 and AI-2 autoinducers (Surette and Bassler, 1998; Miller et al., 2002), no HapR protein could be detected by western blots (data not shown). Introduction of a constitutively expressed *hapR* on a plasmid (Joelsson et al., 2006) restored quorum sensing phenotypes in these strains (data not shown). These data suggest that there are other mutations in the quorum sensing regulatory pathways that prevent quorum sensing-dependent HapR expression in these strains. For example, a naturally occurred constitutively active mutation in LuxO that renders a quorum sensing defective phenotype in *V. cholerae* has been isolated previously (Vance et al., 2003). For unknown reasons, the percentage of this kind of mutation was significantly higher in CT⁺ strains (Fig. 4). It is unclear whether the two naturally occurring quorum sensing mutations of *V. cholerae* mentioned above exist outside of China.

Conclusion

Our finding may suggest a co-evolution of quorum-sensing systems and toxigenic genes which would add valuable knowledge to our understanding of how virulent strains of *V. cholerae* emerge. We conclude that the presence of a functional quorum-sensing system provides greater adaptive value in *V. cholerae* pathogenic settings. The results of the study also implies that the proposal to use *V. cholerae* quorum-sensing molecules as a therapy to prevent cholera infection (Higgins et al., 2007) may indeed hold promise as more virulent *V. cholerae* strains tend to have intact QS systems that can be interfered.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Functionality of quorum-sensing systems in *V. cholerae* strains isolated in China from 1960. (A) Representative Lux curves of QS⁺, QS^c and QS⁻ type strains. These are defined by the expression patterns of *V. harveyi luxCDABE* cosmid pBB1 introduced in these strains, as described in (Miller et al., 2002). RLU (relative Lux units) is defined by cell luminescence units normalized by OD₆₀₀. (B) The number of QS⁺, QS^c and QS⁻ type strains isolated in China from 1960s.

Fig. 2.

The distribution of different types of quorum-sensing systems in *V. cholerae* strains. (A) The percentage of different quorum-sensing systems in strains that contain cholera toxin genes (CT⁺, n=403) and in strains without cholera toxin genes (CT⁻, n=199). (B) The changes of percentage of QS⁺ isolates in either CT⁺ or CT⁻ strains. Note that 00 indicates strains isolated from 2000 to 2007. The number of CT⁺ and CT⁻ strains isolated from each decade is listed as a table below the bar graph.

Fig. 3.

The A191V HapR mutant is not functional. A vector plasmid or plasmid containing P_{tac} controlling either wild type *hapR* (from C6706) (Joelsson et al., 2006) or *hapR*^{A191V} was introduced into wild type C6706 or *hapR* in-frame deletion mutants (Zhu et al., 2002) containing a *V. harveyi luxCDABE* plasmid (Miller et al., 2002). Colony morphology was photographed after strains were streaked on LB plates and incubated at 30°C for 48 hrs (Left panel). The same strains were grown in LB in 96-well plates at 30°C overnight and photographs were taken in the dark using a LAS4010 Imager.

Fig. 4. Frequently isolated, naturally occurring quorum sensing mutations and their distribution in *V. cholerae* strains. The percentage of the A191V HapR mutation or silent HapR mutation (as compared to C6706 HapR) in either CT⁺ (n=188) or CT⁻ (n=143) strains.