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Parallel quorum sensing signaling pathways in *Vibrio cholerae*

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Abstract

Quorum sensing (QS) is a microbial signaling process for monitoring population density and complexity. Communication among bacterial cells via QS relies on the production, secretion, and detection of small molecules called autoinducers. Many bacteria have evolved their QS systems with different network architectures to incorporate information from multiple signals. In the human pathogen *Vibrio cholerae*, at least four parallel signaling pathways converge to control the activity of a single regulator to modulate its QS response. By integrating multiple signal inputs, it is believed that *Vibrio* species can survey intra-species, intra-genus, and inter-species populations and program their gene expression accordingly. Our recent studies suggest that this “many-to-one” circuitry is also important for maintaining the integrity of the input-output relationship of the system and minimizes premature commitment to QS due to signal perturbation. Here we discuss the implications of this specific parallel network setup for *V. cholerae* intercellular communication and how this system arrangement affects our approach to manipulate the QS response of this clinically-important pathogen.

Keywords

Quorum Sensing; Signal Transduction; Gene Regulation; Pathogenesis

Introduction

Like many social organisms, bacterial cells interact with one another to carry out group behaviors. To accomplish these collective tasks, an individual bacterium in the group needs to communicate and coordinate with its neighbors or else its own effort would be obsolete. One of the most characterized microbial intercellular signaling processes is called quorum sensing (QS), which involves the production, secretion, and detection of cell density-dependent chemical signals, known as autoinducers (AIs) (Reviewed in (Ng and Bassler 2009, Rutherford and Bassler 2012, Schuster, et al. 2013, Williams, et al. 2007)). At low cell density, when AI concentration is low, there is little communication amongst individual bacterial cells. When bacteria grow and population cell number increases, AI concentration

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increases proportionally. Once AI concentration reaches a detection threshold, all cells respond to the signal and change their gene expression profile in unison. Thus, QS enables bacteria to act as a multi-cellular, synchronous group. QS regulates collective behaviors such as bioluminescence, biofilm formation, virulence factor production, secondary metabolite production, competence induction, and plasmid transfer.

Quorum Sensing and the *Vibrio cholerae* life cycle

Similar to many bacterial pathogens, *Vibrio cholerae*, the causative agent of cholera, depends on QS to regulate essential cellular processes for survival and adaptation inside and outside of its hosts (Antonova and Hammer 2015, Beyhan, et al. 2007, Blokesch 2012, Hammer and Bassler 2003, Shao and Bassler 2014, Shikuma, et al. 2009, Suckow, et al. 2011, Yildiz, et al. 2004, Zheng, et al. 2010, Zhu and Mekalanos 2003, Zhu, et al. 2002). Since its discovery, the *V. cholerae* QS system has served as a model to understand how pathogens employ QS for precise temporal control of virulence factor production (Jung, et al. 2015, Liu, et al. 2006, Liu, et al. 2008, Miller, et al. 2002, Tsou and Zhu 2010, Zhu and Mekalanos 2003, Zhu, et al. 2002). The current model suggests that when *V. cholerae* cells initially enter inside the host small intestine, the population cell number is relatively low (Angelichio, et al. 1999) and thus the QS response is not induced at this low cell density (LCD) state. Specifically, the master QS regulator HapR, which represses *V. cholerae* virulence genes, is not expressed at this particular stage of infection (Kovacikova and Skorupski 2002, Lin, et al. 2005, Lin, et al. 2007, Liu, et al. 2008, Miller, et al. 2002, Zhu, et al. 2002). As a consequence, expression of virulence factors, including Toxin-Coregulated Pili (TCP) and Cholera Toxin (CTX), are derepressed and timely produced. Production of these factors enables the pathogen to colonize inside the host small intestine and induce the watery diarrhea that is characteristic of the disease. Once *V. cholerae* colonization has established inside the host, population density increases to high cell density (HCD) and the QS response is induced. As a result, HapR is produced and virulence factor production is repressed. Some of the genes induced by QS at HCD are thought to help the *V. cholerae* population exit the host (e.g., Hap protease, (Finkelstein, et al. 1992, Silva, et al. 2003)) or gain competitive advantage over other micro-organisms (such as Type VI secretion (Shao and Bassler 2014, Zheng, et al. 2010)). Consistent with this model, *V. cholerae* mutants locked at a HCD state do not produce TCP and CTX, and are incapable of host colonization (Jung, et al. 2015, Miller, et al. 2002, Zhu, et al. 2002).

V. cholerae integrates multiple signal inputs to control its QS response

Two QS pathways have been established previously in *V. cholerae* (Miller, et al. 2002). The first system is composed of the signal CAI-1, which is synthesized by the enzyme CqsA, and the cognate receptor CqsS, a transmembrane histidine kinase (Higgins, et al. 2007, Miller, et al. 2002, Ng, et al. 2011, Wei, et al. 2012, Wei, et al. 2011). The second system is composed of the signal AI-2, which is synthesized by the enzyme LuxS, and another histidine kinase receptor LuxPQ (Chen, et al. 2002, Miller, et al. 2002, Schauder, et al. 2001). The kinase activity of CqsS and LuxPQ is inversely proportional to CAI-1 and AI-2 concentrations; therefore, the total receptor kinase activity is high at LCD and low at HCD. Together, these signaling pathways converge and determine the direction of a series of phosphor-transfer

reactions that ultimately regulate the *V. cholerae* QS response (Fig. 1). Surprisingly, disruption of these two canonical pathways is insufficient to abolish *V. cholerae* QS communication. In other words, double sensory mutants are capable of regulating gene expression according to population density and they timely express virulence factors at LCD, suggesting that additional means are adopted by *V. cholerae* for intercellular communication (Jung, et al. 2015, Miller, et al. 2002). In a recent study from our lab, we proposed that *V. cholerae* detects at least four different signals including CAI-1 and AI-2 using four parallel receptors CqsS, LuxPQ, VpsS, and CqsR (VC1831) to regulate a common response regulator, LuxO, and modulate its QS response (Fig. 1, (Jung, et al. 2015)). Interestingly, *V. cholerae* is proficient in cell density-dependent QS gene regulation as long as at least one of these four signaling pathways is functional. Moreover, the loss of three of the four pathways seems to have only a minor, if any, effect on virulence of this pathogen. In contrast, mutants lacking all four receptors have a disrupted QS circuit and, therefore, are avirulent (Jung, et al. 2015).

Bacteria are multilingual to adapt to their changing surroundings

Why does *V. cholerae* perceive multiple parallel sensory inputs, even if a single pathway appears to be capable of supporting effective communication? Indeed, bacterial species have evolved independently to have differential contributions from multiple signaling inputs to control particular behaviors, which depend on specific environmental niches (e.g., in *V. cholerae*, biofilm formation in the aquatic environment and virulence factor production inside the host, both of which are controlled by QS). *V. cholerae* is just one of several QS bacterial species that utilizes a multi-signal network configuration to accomplish specific goals (Cornforth, et al. 2014, Drees, et al. 2014). Other research has shown that there is an association between variable environmental challenges and multi-signal complexity for bacteria that have multiple niches, like *V. cholerae*, *Pseudomonas aeruginosa*, *Burkholderia thailandensis*, and some *Bacillus* species which have niches both inside and outside of human hosts (Cornforth, et al. 2014, Pollak, et al. 2015, Schuster, et al. 2013, Williams, et al. 2007, Yang and Lan 2015). Consequently, fine tuning multiple signal inputs from specific molecules may provide greater resolution and, therefore, a more robust response, where gene expression profiles are matched with social and physical alterations (Cornforth, et al. 2014).

One of the advantages of integrating multiple sensory systems is to allow QS bacteria to decipher distinct information contained within specific signals. For instance, *Vibrio harveyi*, a marine bacterium related to *V. cholerae*, detects three AIs HAI-1, CAI-1, and AI-2 using receptors LuxN, CqsS, and LuxPQ, respectively, to regulate its QS response. HAI-1 is made exclusively by *V. harveyi*; CAI-1 and related molecules are made by many *Vibrio* species; and AI-2 is made by a wide variety of bacteria (Bassler, et al. 1997, Bassler, et al. 1993, Bassler, et al. 1994, Cao and Meighen 1989, Chen, et al. 2002, Henke and Bassler 2004, Neiditch, et al. 2005, Neiditch, et al. 2006, Ng, et al. 2011, Schauder, et al. 2001). Therefore, these three AIs are proposed to be used for intra-species, intra-genus, and inter-species communication, respectively (Federle and Bassler 2003, Henke and Bassler 2004, Ng, et al. 2011). Taken together, these studies suggest that bacteria gather information not only from

their immediate surrounding environment that is likely dominated by its own species, but they also survey the presence of neighboring microbial species.

It is thought that the *V. harveyi* QS system detects each AI independently and functions as a “coincidence detector” (Mok, et al. 2003, Waters and Bassler 2006), allowing for a downstream QS response that is triggered by any one of the signals. Therefore, perhaps each AI-specific gene expression program is beneficial for the bacterium in different niches with varying species composition. Indeed, addition of one of the AIs, HAI-1, is sufficient for triggering a premature QS response in a growing LCD culture of wild type *V. harveyi* containing all of its functional QS pathways (Bassler, et al. 1993, Freeman, et al. 2000). However, in the case of *V. cholerae*, we found that the four parallel signaling pathways act together to prevent a premature committed response caused by signal perturbation, and the downstream QS response cannot be induced in the presence of excess amount of one of the signals (e.g., CAI-1) (Jung, et al. 2015). Indeed, the overall population QS response is not significantly impacted by signal perturbation as long as at least one or two remaining QS pathways are functional. No *V. cholerae*-specific AI has been identified as of yet, since both CAI-1 and AI-2 are produced by other species and signals detected by VpsS and CqsR are currently unknown. Given that the *V. cholerae* CqsS receptor activity could be altered with molecules that show little structural similarity to CAI-1 (Ng, et al. 2012, Ng, et al. 2011) and LuxPQ activity could be modulated by other AI-2-producing micro-organisms, this parallel network circuitry may be particularly important for *V. cholerae* fitness inside and outside the host. It is unfavorable for *V. cholerae* to switch to a HCD QS state prematurely because virulence factor production and biofilm formation are repressed (Beyhan, et al. 2007, Fong and Yildiz 2008, Hammer and Bassler 2003, Kovacicova, et al. 2003, Kovacicova and Skorupski 2002, Lin, et al. 2007, Miller, et al. 2002, Shikuma, et al. 2009, Zhu, et al. 2002). Similarly, the presence of multiple AIs enforces QS response homogeneity in *V. harveyi* (Anetzberger, et al. 2009, Anetzberger, et al. 2012a, Anetzberger, et al. 2012b, Plener, et al. 2015). Thus, functionally redundant receptors may be employed to ensure that bacteria do not commit to a population-wide gene expression pattern until all signals are accounted for. This “all-or-nothing” scheme for regulating QS behaviors could be a safety precaution that ensures that bacteria act as a cohesive population only when ready and when appropriate.

Targeting QS components for treating *V. cholerae* infections

Interruption of bacterial QS communication has been proposed to be an attractive alternative to traditional antibiotics for treating infectious diseases. It is thought that bacteria are less prone to develop resistance to QS drug targets as opposed to antimicrobials, although this notion is under debate (Garcia-Contreras, et al. 2013, Garcia-Contreras, et al. 2015, Gerdt and Blackwell 2014, Kalia, et al. 2014, Maeda, et al. 2012, Mellbye and Schuster 2011). Nonetheless, several strategies have been tested for interfering with QS in *V. cholerae* to abolish virulence factor production. All of these strategies are aimed at inducing a premature QS response, thereby repressing virulence gene expression. The earliest reported attempt to disrupt the *V. cholerae* QS circuit was through the use of native QS receptor ligands CAI-1 and AI-2. It was demonstrated that excess synthetic CAI-1 could lead to decreased TCP production *in vitro* in cells devoid of the LuxPQ receptor that senses AI-2. However, TCP production cannot be completely repressed by adding exogenous CAI-1 alone in this strain

(Higgins, et al. 2007). These results are consistent with our findings that multiple redundant signaling pathways contribute to QS modulation and virulence control in this pathogen (Jung, et al. 2015).

The human gut is home to more than 10^{14} bacteria that assist in the digestion of our food, immune system development, as well as providing a barrier against invading pathogens (Backhed, et al. 2005, Kau, et al. 2011, Savage 1977, Thompson, et al. 2015). Since it is believed that many bacteria in the host gut microbiota have QS systems, inter-species communication could have a significant impact on pathogen colonization inside the host. Many bacterial species produce AI-2 (Federle 2009, Schauder, et al. 2001, Waters and Bassler 2005, Xavier and Bassler 2003) and the production of this chemical signal could interfere with the *V. cholerae* QS gene expression program. For instance, it is shown that *Ruminococcus obeum*, a human commensal bacterium, increases its AI-2 production to inhibit *V. cholerae* colonization in an adult gnotobiotic mouse gut. However, this inhibition occurs through a mechanism independent of the *V. cholerae* AI-2 sensor LuxP (Hsiao, et al. 2014). In addition, it is unclear if all *V. cholerae* QS pathways are required for colonization in this particular animal model. In another study, a commensal *Escherichia coli* strain was engineered to overexpress *V. cholerae* CqsA and produce a high level of CAI-1. Therefore, this engineered strain can secrete both CAI-1 and AI-2 into its extracellular milieu. Studies showed that this engineered *E. coli* strain can cause *V. cholerae* to induce QS prematurely *in vitro* (Duan and March 2008) and reduce *V. cholerae* gut colonization in infant mice (Duan and March 2010). However, it is unclear if CAI-1 and AI-2 produced by this *E. coli* strain induce QS in *V. cholerae* via the canonical CqsS and LuxPQ pathways, or if this *E. coli* strain can also produce other unidentified signals that are perceived by VpsS, CqsR, and/or other unrelated pathways. Nonetheless, these findings suggest that a combination of native AIs could modulate *V. cholerae* virulence factor production inside the host.

Conclusions

Our work and previous findings imply that bacteria have evolved independently to integrate multiple sensory networks as a mechanism to resist sporadic perturbations encountered in their ever-changing environments, thereby eliciting robust and coordinated responses. While the basic network architecture of the *V. cholerae* QS system has been determined, many questions remain to be answered. For example, are all of the QS signals perceived by *V. cholerae* chemical in nature? Do these four pathways contribute equally, and are they functioning simultaneously and behaving the same in all niches? While QS has become a focus for therapeutics, what is the best target for this novel strategy? Is the best approach to focus on exploiting small molecule inhibitors to target downstream QS components (e.g., LuxO inhibitor, (Ng, et al. 2012))? How do commensals and the host microbiota affect our therapeutic strategy by interfering with intercellular communication? We hope that future research in this subject will give rise to more insight into these questions.

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Fig. 1.

The *Vibrio cholerae* quorum sensing system. **a** At LCD, AI levels are low and kinase activities of CqsS, LuxPQ, VpsS, and CqsR predominate. These four histidine kinase receptors, via a series of phosphor-relay reactions, activate the response regulator LuxO. Activated LuxO promotes transcription of four small RNAs called Qrr1-4, which in turn activate translation of AphA and inhibit production of HapR. These two regulators control expression of over 100 genes. The LCD QS regulon includes genes required for biofilm formation in the aquatic environment and virulence factor production in the host. **b** At HCD, AI levels are high and dephosphorylation activities of the four receptors predominate. Thus, LuxO is no longer active and Qrr transcription is repressed. HapR is made at this state while Apha is not produced. Reciprocal expression of these two regulators leads to repression of biofilm formation and virulence factor production.