Perspective

Israel Journal of Chemistry
www.ijc.wiley-vch.de

doi.org/10.1002/ijch.202300024

Can Non-Toxigenic *Vibrio cholerae* Reduce a Cholera Infection?

R. Sela,[a] B. K. Hammer,[b, c] and M. Halpern*[a, d]

This paper is a contribution to the Special Issue of the Israel Journal of Chemistry honoring Bonnie Bassler and her receipt of the Wolf Prize.

Abstract: *Vibrio cholerae*, is the causative agent of cholera, that infects millions, annually. Chironomids are aquatic insects that host *V. cholerae*. Toxigenic strains produce cholera toxin (CT) which is the main virulence factor that causes cholera symptoms. In contrast to other bacterial pathogens, *V. cholerae* produces CT when at low cell densities while hemagglutinin/protease (HAP) is a high cell density-controlled gene. When *V. cholerae* behavior was examined on chironomids, we showed that high cell densities of non-toxigenic strains, increased HAP production in a toxigenic strain, conditions which could also potentially reduce CT production. Here we propose the value of studies that could support the potential of *V. cholerae* non-toxigenic strains to repress virulence gene expression in cholera-infected humans. High cell densities of a non-toxigenic strain present in an infected individual, may down-regulate CT expression, reducing cholera symptoms. To further test the hypothesis supported by a chironomid model, additional experiments in animal models are first needed.

Keywords: *Vibrio cholerae* · cholera · chironomid · Quorum-Sensing (QS) · Host-bacterial-interaction

Introduction

Vibrio cholerae is the causative agent of cholera, an acute diarrheal disease that infects millions worldwide and kills at least 95,000 annually.[1] Strains of *V. cholerae* belonging to serogroups O1 and O139 produce the cholera toxin (CT), which causes the severe cholera diarrheal symptoms in humans.[2] While the majority of *V. cholerae* O1 and O139 strains carry CT genes, the majority of *V. cholerae* non-O1/O139 strains do not possess these genes. The species *V. cholerae* is ubiquitous in aquatic environments, including fresh and marine waters, and is associated with zooplankton, mainly copepods[3] and chironomids.[4] Recently, we have demonstrated that chironomids are natural reservoirs of both toxigenic O1 and O139 serogroups as well as of non-toxigenic *V. cholerae* strains (non-O1/O139 serogroups).[5]

The major virulence factor contributing to human disease is CT, which is encoded in the *ctxAB* operon. This operon is located within the CTX genetic element integrated into the *V. cholerae* genome by the CTX phage.[2] CT is a protein complex consisting of two subunits (A and B) responsible for the massive, watery diarrhea characteristic of cholera infection. Subunit B binds to the epithelial cells of the human small intestine and aids in intercellular delivery of subunit A that permanently activates intercellular G protein by blocking its GTPase activity. This results in continuous activation within intestinal epithelial cells of the adenylyl cyclase that synthesizes cAMP. Chronically high concentrations of cAMP trigger the enhanced secretion of chloride, bicarbonate, and water into the intestinal lumen. These result in dehydration and electrolyte loss, which are the major pathologies of cholera.[2]

[a] *R. Sela, M. Halpern*
Department of Evolutionary and Environmental Biology,
University of Haifa,
Haifa 3498838, Israel
Tel: +972 [4] 9838978
E-mail: mhalpern@research.haifa.ac.il
[b] *B. K. Hammer*
School of Biological Science
Georgia Institute of Technology
Atlanta, GA 30332, USA.
[c] *B. K. Hammer*
Center for Microbial Dynamics and Infection,
Georgia Institute of Technology,
Atlanta, GA 30332, USA.
[d] *M. Halpern*
Department of Biology and Environment,
Faculty of Natural Sciences,
University of Haifa at Oranim,
Tivon 3600600, Israel

V. cholerae and Quorum Sensing

Cells of *V. cholerae*, like numerous other bacterial species, release into the environment chemical signal molecules called autoinducers (AIs) to communicate with each other by quorum sensing (QS).[6] Typically, bacteria at low cell density (LCD) lead a solitary lifestyle when AI levels are low. In response to elevated AI levels at high cell density (HCD), bacteria reprogram gene expression that is tailored to life in groups. *V. cholerae* produces two major AIs: the intra-species cholera autoinducer-1 (CAI-1) signal, and an inter-species signal (AI-2) synthesized by the product of the *luxS* gene, which is also encoded in the genomes of many bacterial species.[6] Recognition of each AI by its cognate membrane-bound receptor reciprocally controls HapR and an opposing transcription factor AphA. Thus, at low cell density, AphA levels are high when HapR is not produced; at high cell density, AphA levels are low when HapR is made (Figure 1). Genetic evidence supports a model that two other receptors respond to AIs, which have yet to be identified, but participate with CAI-1 and AI-2.[7] The cytoplasmic VqmR receptor and its cognate AI, DPO, were recently identified and also shown to modulate AphA.[8] Together, this lexicon of QS helps AIs orchestrate precise timing of CT and other virulence factors.

Chironomids and *V. cholerae*

Chironomids *(Diptera*; *Chironomidae)*, are aquatic insects, ubiquitous in fresh and marine water. They undergo a complete metamorphosis of egg mass, larva, pupa, and adult. All four life stages were found to be hosts of both toxigenic and nontoxigenic *V. cholerae* strains.[4,5] Chironomid egg masses are comprised of glycoproteins and chitin.[9,10] *V. cholerae* produces the extracellular enzyme hemagglutinin/protease (HAP), which can degrade chironomid egg masses and prevents eggs from hatching.[9] HAP is encoded by the *hap* gene that is transcriptionally activated by HapR in response to the accumulation of QS signals.[6]

In contrast to other bacterial pathogens, at LCD, the absence of QS AIs results in elevated AphA and repression of HapR, leading the expression of virulence genes, like *ctxA* and

Rotem Sela received her B.S. in Biology (with honors) and her Ph.D. in Microbiology (2022) from the Faculty of Natural Sciences, University of Haifa, Israel. During her Ph.D., Rotem received the University Presidents' Scholarship. In her research (under the supervision of Prof. Malka Halpern), Rotem focused on the role of quorum sensing signals in the interactions between *Vibrio cholerae* and the microbiota of chironomids. She found that members of the chironomid bacterial consortium produce external chemical cues that, like AI-2, induce expression of the *hapA* gene in *V. cholerae*. By utilizing the nutrients generated by HAP production, the egg masses' microbiota control *V. cholerae* numbers and contribute to the equilibrium between *V. cholerae* and its chironomid host. Currently, Rotem is working as a Team Manager of Bacteriology Northern Laboratory (Megalab) in the Maccabi Health care Services, Israel.

Brian K. Hammer has been a faculty member at Georgia Tech in Atlanta GA since 2008 where he is currently a member of the Center for Microbial Dynamics and Infection, and an associate professor in Biological Sciences. Brian received his PhD from the University of Michigan Medical School in 2001 followed by his postdoc with Bonnie Bassler where he studied the role of quorum sensing in biofilm formation. At Georgia Tech he leads a research program studying mechanisms of cooperation and conflict in bacteria, with a focus on *Vibrio cholerae*. Brian was a Distinguished Lecturer for the American Society for Microbiology in 2018–2020, a recipient of an NSF CAREER award, and has received multiple teaching awards.

Malka Halpern received her Ph.D. from the University of Haifa, Israel in 2002. This was followed by a postdoc study with Prof. Eugene Rosenberg at Tel-Aviv University on the VBNC state of *Vibrio cholerae* in chironomids. In 2003, she became a tenure track faculty member (full Professor, 2019) at the Department of Biology and Environment, University of Haifa, campus Oranim. Malka leads a research group that is studying, among other subjects, the ecology of waterborne pathogens (*V. cholerae, Aeromonas* and *Legionella*). A main scientific contribution of her group is the discovery that chironomids are natural reservoirs of *V. cholerae*, including the toxigenic serogroups O1 and O139. Another important contribution of her laboratory (in collaboration with Prof. Ido Izhaki, University of Haifa), is the finding that different fish species are inhabited by *V. cholerae* in their guts and that *V. cholerae* may be globally distributed between continents by waterbirds.

Israel Journal of Chemistry

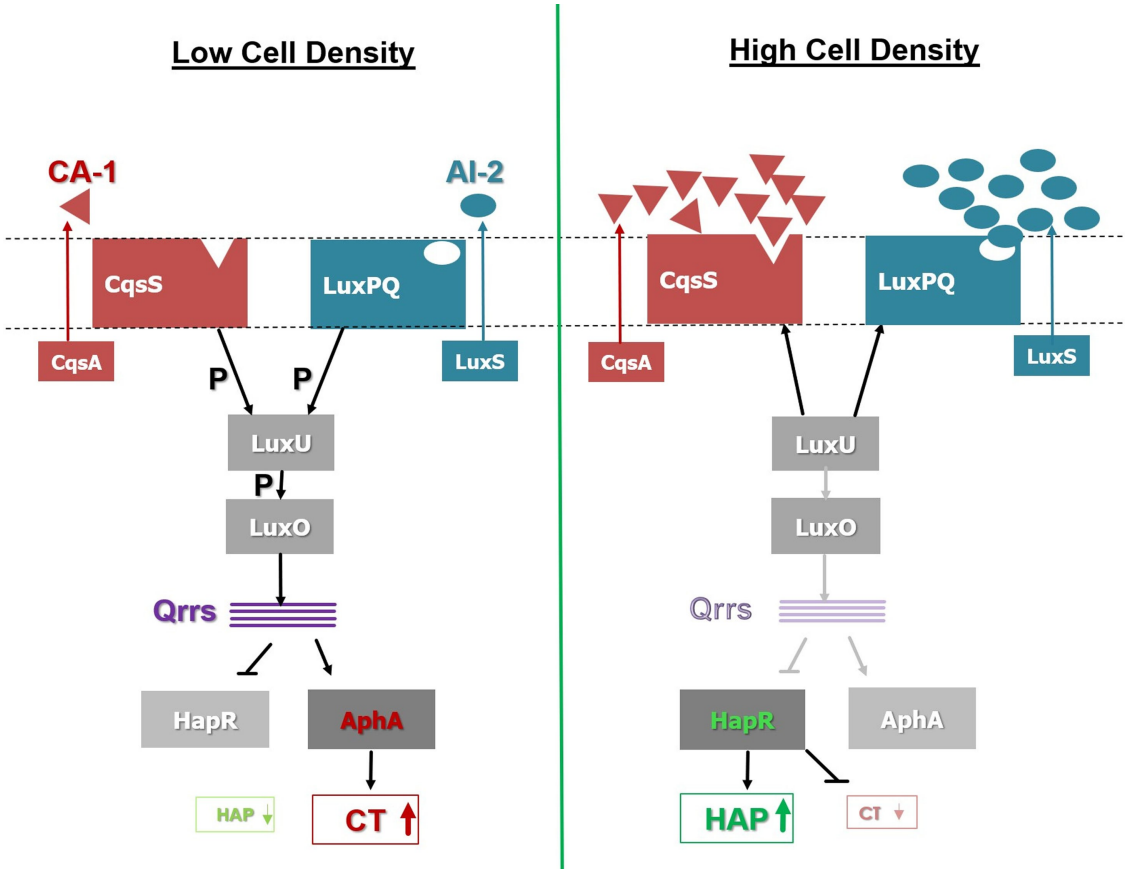


Figure 1. A schematic model of the QS circuit of *V. cholerae*. CqsA and LuxS synthesize the autoinducers CAI-1 and AI-2, respectively. At low cell density (LCD) in the absence of autoinducers, their receptors CqsS and LuxPO, respectively, act as kinases to transfer phosphate to LuxO through LuxU. Phospho-LuxO activates transcription of multiple small RNAs (Qrrs). By base pairing, the Qrrs alter translation by negatively regulating *hapR* and positively regulating *aphA*. As a result, at low cell density CT is produced but not HAP. At high cell density (HCD), AIs reach sufficient levels to bind their cognate receptors and convert them to phosphatases. Inactive LuxO halts transcription of the Qrrs, leading to production of HAP but not CT, as reviewed in Herzog et al.[8] The difference in the size of the boxes for HAP and CT indicates altered production levels.

ctxB for causing human disease. By contrast, at HCD when AIs are at high levels, the expression of these genes for human virulence are repressed by HapR accumulation.[6] It is proposed that a role of HAP in human infection is to release *V. cholerae* cells from the human intestine.[9] HAP also plays a role in controlling the number of chironomids in the environment, and thus from the perspective of chironomids, plays a role consistent with an insect virulence factor (Figure 2) .[11]

Because chironomids are natural reservoirs of *V. cholerae*, we suggest that they can serve as a model organism for investigating this bacterium. Using *V. cholerae* O1 mutants proficient and deficient for QS CAI-1 and AI-2 production, we have recently shown that the egg masses represent a niche where QS is maintained to induce HAP production via HapR regulation.[10] Members of the chironomids' endogenous microbiota community produce molecules, that generate a response like AI-2. These signals mimic high cell density conditions that lead to HAP production even at low cell densities of toxigenic *V. cholerae*.[10] The HAP protease, produced within the microbiota consortium by *V. cholerae* in the egg masses, supports the growth of both itself and other community members, by degrading the egg masses.[10] Understanding *V. cholerae* QS in the chironomid system may provide insights into how AI signaling by the gut microflora modulates *V. cholerae* fitness in a human host.

Non-toxigenic *V. cholerae* Against Toxigenic *V. cholerae*

Here we postulate that non-toxigenic strains of *V. cholerae* may be capable of repressing virulence genes expression (i.e. *ctx*) in cholera-infected humans. By using chironomids, we found (unpublished study, Figure 3) that non-toxigenic *V. cholerae* strains produce QS signals that increase HAP production in a toxigenic *V. cholerae* O1 strain (C6706) and thus, potentially also reduce CT production. We assessed the ability of toxigenic *V. cholerae* O1 wild type (WT) and two isogenic QS-deficient mutants to induce HAP production in a

Israel Journal of Chemistry

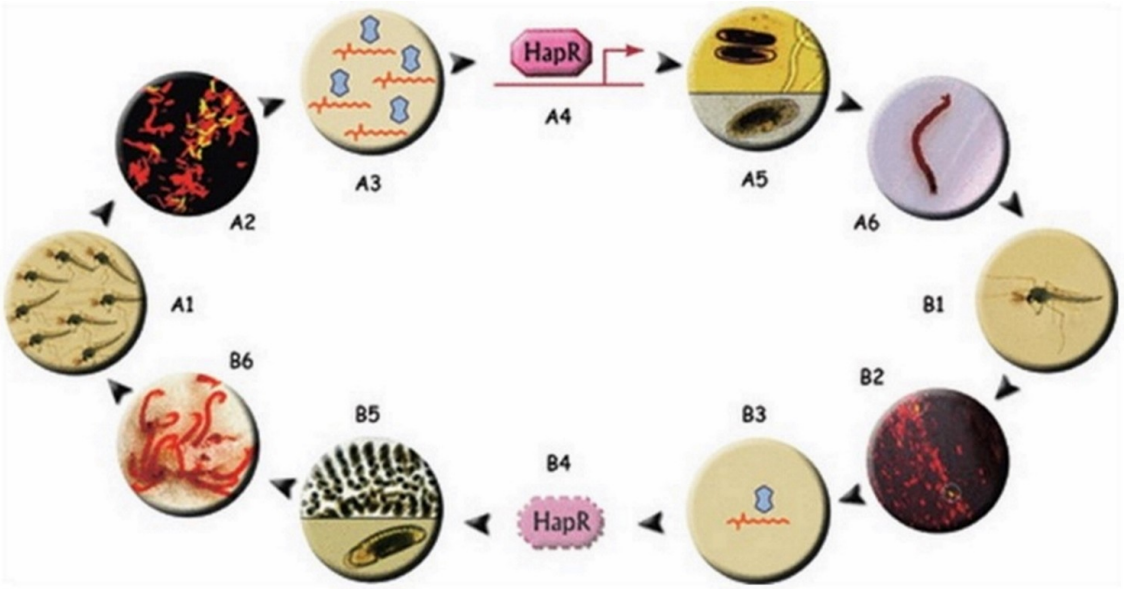


Figure 2. The effect of *V. cholerae* HAP production on chironomid population dynamics. A1–A6; At high chironomid population densities, *V. cholerae* is also present in relatively high cell densities and produces HapR due to elevated QS AI signals. As a result, hemagglutinin/protease (HAP) is produced, chironomid egg masses are degraded, and the larvae do not hatch which cause a decrease in chironomid population size. B1–B6 depict the opposite course of action. Chironomids and *V. cholerae* are at low cell densities and low QS AIs lead to a lack of HapR. As a result, HAP is down-regulated, egg masses are not damaged, larvae hatch, and the chironomid population size increases. A1. chironomid adults (high concentration). A2. *V. cholerae* are at a relatively high cell density on an egg mass [total bacteria stained with universal probe EUB 338 (red); *V. cholerae* stained with a specific probe MAL2 (yellow)]. A3. QS AIs (CAI-1 and AI-2) are up-regulated. A4. HapR is produced and up-regulates the production of the HAP enzyme. A5. HAP degrades the egg mass (upper picture) and prevents the eggs from hatching (a damaged egg, lower part of the picture). A6. Only a few larvae hatch. The result of hatching prevention is a decrease in chironomid populations. B1. Low chironomid population density. B2. Low number of *V. cholerae* cells per egg mass. B3. Low concentration of QS signals. B4. HapR and HAP are not produced. B5. The egg mass is not degraded (upper picture), and eggs develop normally (lower part of the picture). B6. The larvae hatch from the eggs. The result is an increase in chironomid populations. Adopted with permission from Halpern, 2010.[11]

V. cholerae ΔcqsA ΔluxS reporter strain. *cqsA* and *luxS* encode the genes for CAI-1 and AI-2 production, respectively. Thus, the reporter strain does not produce any AIs. Then, the ability of environmental non-toxigenic *V. cholerae* isolates (from chironomid egg masses), to induce HAP production in the same *ΔcqsA ΔluxS* reporter strain was also assessed and compared to *V. cholerae* O1 wild type (Figure 3). The experimental setup was described by Sela et al. (2021).[10] Briefly, the reporter strain was diluted 1 : 100 into sterile supernatant from an overnight culture of each tested strain that was diluted to 40% with sterile LB medium (a 2 : 3 ratio of sterile supernatant:medium). Bioluminescence results of the reporter strain after the addition of cell-free supernatant from non-O1/non-O139 *V. cholerae* chironomid isolates were compared to the bioluminescence results of the reporter strain with the addition of cell-free supernatant from (i) *V. cholerae* O1 (WT), (ii) a *△cqsA* single mutant, and (iii) a *△cqsA △luxS* double mutant. No significant differences in *lux* expression levels were observed between the cell-free supernatant from the toxigenic WT (C6706) and the supernatants from the non-toxigenic strains (Mann-Whitney: $p = 0.19$). This demonstrates that the impact of QS signals on *V. cholerae* HAP production in this experimental system is similar whether the AI signals are produced by the non-toxigenic or the toxigenic strains (Figure 3). Thus, we can conclude that high cell densities of *V. cholerae* non-toxigenic strains upregulate HapR expression and subsequent HAP production by a toxigenic *V. cholerae* strain and might also downregulate CT production and prevent cholera disease symptoms (Figure 4). Hence, the presence of non-toxigenic strains in an individual infected with a toxigenic *V. cholerae* strain may have the potential to diminish virulence gene expression in the human gut and improve disease outcome. Such an approach aligns with long-standing efforts to develop cholera vaccines.

Early attempts at developing cholera vaccines administered by non-oral means began over one hundred years ago but offer protection of only a short duration.[12] Today, the World Health Organization's strategy for *Ending Cholera – A Global Roadmap to 2030*, includes oral cholera vaccination as its cornerstone (https://www.gtfcc.org/about-cholera/roadmap-2030/). Contemporary, sustained efforts to develop oral cholera vaccinations with heat- or formalin-killed toxigenic strains, such as Dukoral, show promise. (see recent reviews[13,14]). However, protective efficacy of killed oral

Israel Journal of Chemistry

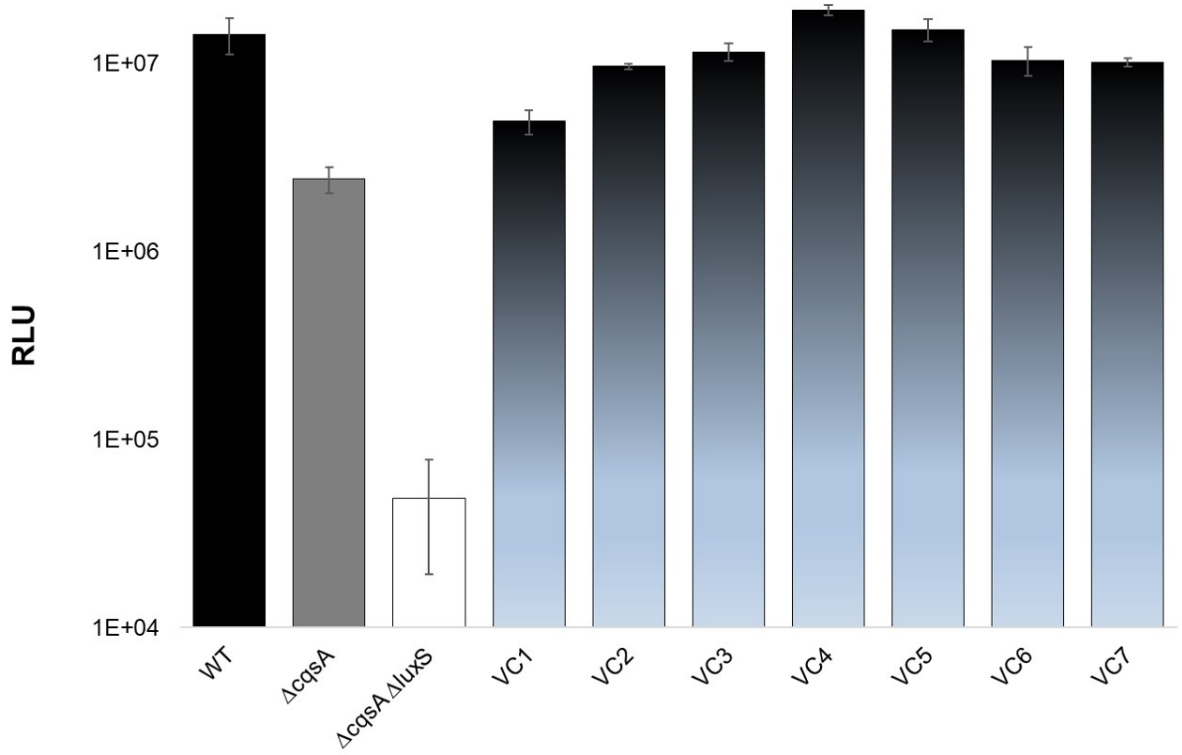


Figure 3. Bioluminescence expression of a *V. cholerae ΔcqsA ΔluxS* reporter, exposed to cell free supernatants from overnight cultures of non-toxigenic *V. cholerae* bacterial isolates. Bioluminescence from the canonical pBBR*lux-hap* reporter was measured 6 h after an overnight culture of the reporter strain (△*cqsA* △*luxS*) was diluted 1:100 with a mixture of 60% sterile Luria Broth medium and 40% sterile supernatant obtained from either a toxigenic *V. cholerae* strain (WT), a single mutant (△*cqsA*), a double mutant (△*cqsA* △*luxS*) or non-toxigenic *V. cholerae* isolates (VC1 - VC7; isolated from chironomids). Bioluminescence/OD600 is plotted as Relative Light Units (RLU). Results are presented as the mean ± standard deviation (n = 5).

vaccines remains moderate and wanes by three years, with limited protection in children five years or younger, perhaps due to the destruction of bacterial epitopes by killing. More recent efforts aim to develop live attenuated vaccines, such as HaitiV,[15] a derivative of a strain isolated from the 2010 cholera outbreak in Haiti and engineered to lack the cholera toxin genes (*ctxAB*). HaitiV demonstrates probiotic activity by preventing the intestinal colonization of infant rabbits by a wild type *V. cholerae* strain, supporting consideration of 'non-toxigenic" live strains as treatment.

However, the administration of non-toxigenic *V. cholerae* to alter cholera infection raises reasonable concerns. For example, some non-toxigenic (non-O1/non-O139) *V. cholerae* strains that lack cholera toxin (CT) and the toxin coregulated pili (TCP) can still cause more mild, intestinal infections and diarrhea. These strains may cause infection in humans through auxiliary virulence factors like the heat-stable toxin (NAG-ST)[16], hemolysin (Hly) and a type III secretion system (TTSS),[17] and cholix toxin (Chx) which is an ADP-ribosylating factor that can stop protein synthesis in eukaryotic cells.[18] Any non-toxigenic strain to be administered to a human as a potential therapeutic would first have to be determined as safe and tolerated in animal models before any studies in humans.

The timing of administration of a non-toxigenic *V. cholerae* strain would also require careful consideration. While treating symptomatic cholera patients infected with toxigenic *V. cholerae* with the addition of a non-toxigenic strain is unlikely to enhance symptoms, it remains unclear whether the introduced strain would be capable of establishing in a GI tract already infected with toxigenic *V. cholerae*. Further study of competitive exclusion is warranted.

V. cholerae isolates from cholera patients have been shown to possess a dysfunctional QS system, resulting in low QS output, typically due to decreased HapR function.[19] Though a small number of strains tested by Joelsson et al. (2006)[19] were QS deficient, it remains unclear whether the mutations

Israel Journal of Chemistry

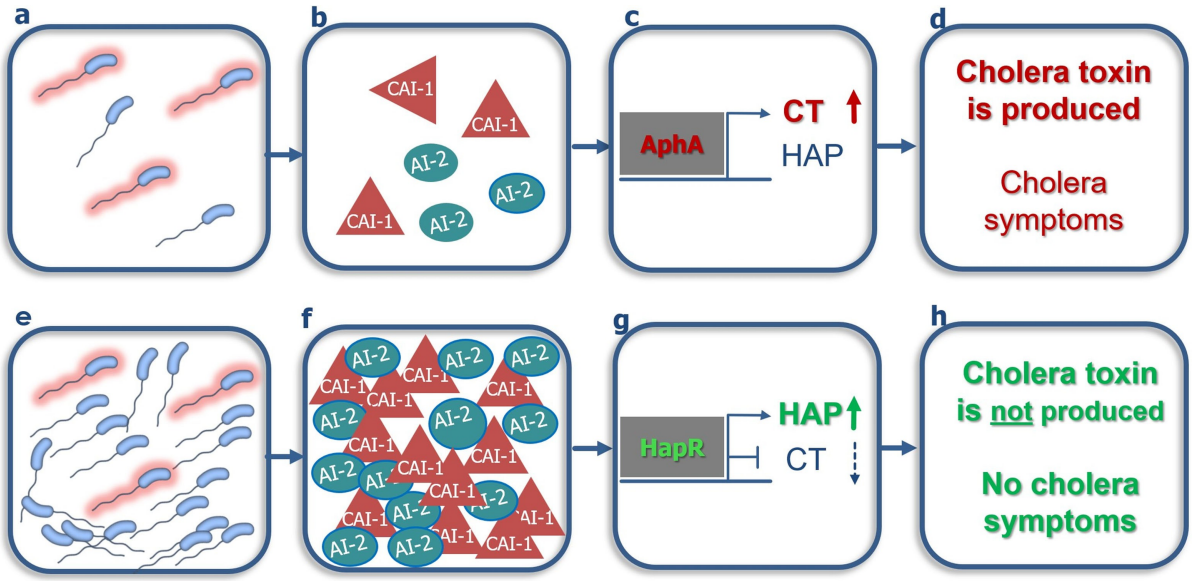


Figure 4. A model of gene regulation of toxigenic *V. cholerae* O1, in the absence or presence of non-toxigenic *V. cholerae* strains. Upper row: a. Low cell densities (LCD) of toxigenic *V. cholerae* (blue cells with a red outline) and LCD of non-toxigenic *V. cholerae* strains (blue) in the human intestine. b. Low concentrations of QS signals (CAI-1 and AI-2) are produced c. The regulatory protein AphA is upregulated d. Cholera toxin (CT) is produced and causes cholera symptoms. **Lower row:** e. LCD of toxigenic *V. cholerae* (blue surrounded with red) and high cell densities (HCD) of non-toxigenic *V. cholerae* strains (blue) in the human intestine f. High concentrations of QS signals (CAI-1 and AI-2) are produced by the high number of non-toxigenic *V. cholerae* cells. g. The regulatory protein HapR is upregulated. h. HAP is produced, while CT is not. No cholera symptoms. Note that HapR and AphA have opposing activities on expression of downstream factors under their control.

observed in QS components like *hapR* or *luxO* occur prior to isolation or as a consequence of isolation and growth in nutrient-rich laboratory conditions, which are distinct from conditions in aquatic settings.[20] Despite evidence of QS-deficient isolates, there remains an interest in modulating QS-controlled virulence gene expression in diverse pathogens as a potential therapeutic avenue.[21]

Using Non-Toxigenic Strains Against Toxigenic Strains in Other Bacterial Infections

Several studies showcase the use of non-toxigenic bacterial strains to repress or reduce the effect of toxigenic strains on infected humans. *Clostridioides* (*Clostridium*) *difficile* is an anaerobic spore-forming bacterium, and the causative agent of common and deadly health care-associated infections.[22,23] Toxin A and toxin B produced by toxigenic *C. difficile* strains trigger diarrhea and colitis in patients. Gerding et al. (2015),[24] explored the concept of treating *C. difficile* infected patients with endospores of non-toxigenic *C. difficile* strains. Non-toxigenic *C. difficile* strain M3 colonized the gastrointestinal tract of patients that had toxigenic *C. difficile* infection and significantly reduced the disease recurrence. Conclusions were later supported by observations that nontoxigenic *C. difficile* endospores were safe and efficient in preventing chronic *C. difficile* infections, despite a clear understanding of the mechanism of protection.[25]

Similarly, enterotoxigenic *Bacteroides fragilis* strains secrete the *B. fragilis* toxin (BFT) and cause colonic inflammation and tumor development in mice[26] and in humans.[27] Treatment with a non-toxigenic *B. fragilis* strain reduced bacteria-driven chronic colitis and tumor development in mice.[28] Moreover, it has been demonstrated that colonization of a mouse model with non-toxigenic *B. fragilis* that possessed type VI secretion system modulated the composition of the host microbiota, blocking enterotoxigenic *B. fragilis* colonization of the host and preventing colitis.[29] Thus, a therapeutic role for non-toxigenic bacteria in altering disease by their toxic counterparts is an area of active investigation.

The Effect of Gut Commensal Bacteria on Cholera Symptoms In Situ

Previous studies have explored the importance of commensal bacteria for inhibiting cholera symptoms. Midani et al. (2018),[29] compared the gut microbiota in cholera uninfected and infected individuals and found that ~100 bacterial taxa differentiated between the two groups, demonstrating linkage between gut microbiota structure and cholera clinical outcome.[21] Metagenomics studies of the gut microbiomes of symptomatic and asymptomatic cholera patients and healthy individuals also identified bacterial species and functions that may affect the ability of *V. cholerae* to colonize the gut. For example, species from the genera *Bifidobacterium* and

Prevotella are associated with asymptomatic cholera infection.[31] In a mouse model study, animals transplanted with dysbiotic human microbiomes were also more susceptible to *V. cholerae* colonization and less resistant to the pathogen. By contrast, non-dysbiotic human microbiomes transplanted to mice were less susceptible to *V. cholerae* colonization.[32] A contribution of QS in these studies was not explored.

A study using an infant mouse model found that commensal bacterial AI production can cause a reduction in CT levels and alter the outcome of cholera infection.[33] Mice pretreated with engineered bacteria that expressed CAI-1 before they were infected with *V. cholerae* showed a survival increase of 77%.[33] Indeed, it is appreciated that QS modulation by resident microbiota is also an important consideration in the development of new live oral cholera vaccines.[13] A more recent study collected fecal samples from patients in a region of Bangladesh where cholera is endemic, during severe diarrhea and recovery phases of cholera.[34] By studying the human fecal microbiota using metagenomic tools, the authors discovered that *Ruminicoccus obeum* (later reclassified as *Blautia obeum*[35]) prevalence increased when *V. cholerae* was present. Similarly, in a mouse model with an artificial gut community containing *B. obeum*, accumulation of *B. obeum* AI-2 resulted in repression of *V. cholerae* virulence factor expression.[34] The study also reported that in some cases, when families sharing the same water and food sources were exposed to toxigenic *V. cholerae*, only a subset of family members became symptomatic for cholera.[34] Such observations support the emerging appreciation for gut microbiome composition in altering disease susceptibility and outcome.

Polymicrobial Infections

The phenomenon of polymicrobial infections has been reported for some pathogens. A study of *Legionella pneumophila*, which causes the often fatal Legionnaires' disease pneumonia, demonstrated that patients are commonly infected by a community of *Legionella* species and also by a population of *L. pneumophila* strains.[36,37] Using sequence-based typing and whole genome sequencing, Coscollá et al. (2014)[36] found potential evidence of a mixture of *Legionella* profiles in uncultured respiratory samples of patients. Furthermore, using 16S rRNA gene sequencing, Mizrahi et al. (2017)[38] demonstrated that *Legionella* patients may be infected by several *L. pneumophila* strains rather than by only one strain. A similar phenomenon was documented in wild waterfowl species intestines where a mixture of toxigenic *V. cholerae* O1 and several non-O1 serogroups were identified from the same individual bird.[39] Specifically, four different *V. cholerae* non-toxigenic serogroups (O13, O16, O36, and O128) and the toxigenic O1 serogroup were identified from the same individual bird (little egret, *Egretta garzetta*).[39] These studies support a hypothesis that the development and severity of disease symptoms in some individuals but not others may be a consequence of a polymicrobial colonization by toxigenic and non-toxigenic species of the same microbe.

In the case of Legionnaires' disease, individuals become infected by inhalation of *Legionella* in contaminated aerosols from water droplets from environmental sources or the built environment. Similarly, the source of cholera is also the environment (contaminated water or food). If there is a population of *V. cholerae* strains introduced into a host from the environment, as was found in birds,[39] chironomids[5] and *L. pneumophila*,[38] the outcome of *V. cholerae* infection may vary. Specifically, we hypothesize that a population of different *V. cholerae* serogroups in a person, may contribute to the phenomenon of asymptomatic carriage in individuals positive for toxigenic *V. cholerae.*[31] Thus, patients infected with a population enriched with non-toxigenic *V. cholerae* strains, may display modest or no symptoms, while those infected with predominately toxigenic strains, could display more acute diarrheal cholera symptoms. Recent work proposes the use of predatory bacteria against infectious diseases as an alternative to antibiotics.[40] We propose that instead of using predatory bacteria against toxigenic *V. cholerae*, non-toxigenic *V. cholerae* may tip the balance of disease from symptomatic to asymptomatic cholera.

Conclusions

We propose that non-toxigenic strains of *V. cholerae* may have the potential to repress expression of virulence associated genes in cholera-infected humans (Figure 4). We have begun developing this hypothesis by studying chironomids as a model organism. Our results support a model that at low cell densities of a toxigenic strain cultured alone, the concentration of the AIs is low, AphA is upregulated and CT is produced, causing cholera symptoms in a human host (Figure 4a–d). However, when the toxigenic *V. cholerae* is at low cell density and in the presence of high cell densities of non-toxigenic strains, high concentrations of QS AI signals released by non-toxigenic *V. cholerae* upregulate HapR production in toxigenic *V. cholerae.* If similar conditions are achievable in humans, it is plausible that cholera toxin production could be reduced or halted, reducing or eliminating cholera symptoms (Figure 4e–h). Specifically, non-toxigenic *V. cholerae* strains that produce QS AI signals, which increase HapR in a toxigenic *V. cholerae* O1 strain (Figure 4), could enhance HAP production and decrease CT production, with positive health outcomes.

Here we suggest that further studies are needed in animal models to determine the capacity of non-toxigenic *V. cholerae* strains to repress virulence genes expression in vivo. We propose this has the potential to be a novel and unique approach for treating the often-fatal disease cholera.

Future Perspectives

There may be concerns of CTX-phage transfer from toxigenic O1 to non-O1 *V. cholerae* in the gut, as it is known in the literature that non-toxigenic strains without CTX may carry the *Vibrio* pathogenicity island encoding the *tcp* locus, and acquire the CTX phage, which uses TCP as a receptor.[41] Thus, it is possible that co-localization of non-toxigenic and toxigenic *V. cholerae* in the gut may generate more toxigenic variants. However, even during *in vitro* experiments, the frequency of such transduction is very low.[41] This phenomenon should be further examined both in a chironomid as well as in relevant animal models.

To further test our hypothesis, additional research is needed to understand the factors responsible for *V. cholerae* susceptibility. Firstly, the hypothesis that non-toxigenic *Vibrios* could act as a therapeutic agent against cholera and prevent CT production, should be examined in a relevant animal model examining co-infection by toxigenic and non-toxigenic *V. cholerae* WT and mutant strains. Secondly, studies to evaluate the relative abundance of non-toxigenic *V. cholerae* strains in severe and moderate cholera patients, in comparison to its abundance in patients' household members that do not show any disease symptoms, would also be informative. This novel approach for treating cholera may have the potential to reduce the burden of cholera cases in areas where *V. cholerae* is endemic.

Author Contributions

RS and MH conceived and designed the experiments. MH and BH contributed reagents, materials, and analysis tools. RS performed the experiments and analyzed the data. RS and MH wrote the paper. RS, BH and MH discussed the results.

Acknowledgements

This study was supported by the United States-Israel Binational Science Foundation (BSF, grant no. 2015103).

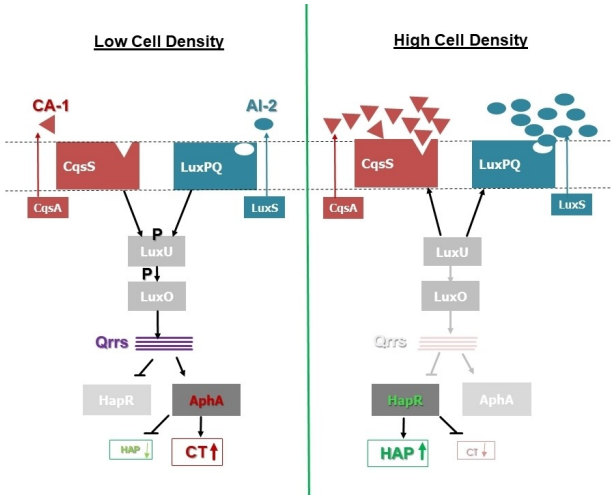
References

[1] "Cholera - *Vibrio cholerae* infection | Cholera | CDC," can be found under https://www.cdc.gov/cholera/index.html, **2021**.
[2] D. A. Sack, R. B. Sack, G. B. Nair, A. K. Siddique, *Lancet* **2004**, *363*, 223–233.
[3] A. Huq, E. B. Small, P. A. West, M. I. Huq, R. Rahman, R. R. Colwell, *Appl. Environ. Microbiol.* **1983**, *45*, 275–283.
[4] M. Broza, M. Halpern, *Nature* **2001**, *412*, 40–40.
[5] S. Laviad-Shitrit, R. Sela, L. Thorat, Y. Sharaby, I. Izhaki, B. B. Nath, M. Halpern, *PLoS Neglected Trop. Dis.* **2020**, *14*, e0008959.
[6] W.-L. Ng, B. L. Bassler, *Annu. Rev. Genet.* **2009**, *43*, 197–222.
[7] S. Watve, K. Barrasso, S. A. Jung, K. J. Davis, L. A. Hawver, A. Khataokar, R. G. Palaganas, M. B. Neiditch, L. J. Perez, W.-L. Ng, *PLoS Pathog.* **2020**, *16*, e1008313.
[8] R. Herzog, N. Peschek, K. S. Fröhlich, K. Schumacher, K. Papenfort, *Nucleic Acids Res.* **2019**, *47*, 3171–3183.
[9] M. Halpern, H. Gancz, M. Broza, Y. Kashi, *Appl. Environ. Microbiol.* **2003**, *69*, 4200–4204.
[10] R. Sela, B. K. Hammer, M. Halpern, *Mol. Ecol.* **2021**, *30*, 1736–1746.
[11] M. Halpern, *Mol. Ecol.* **2010**, *19*, 4108–4112.
[12] A. L. Lopez, M. L. A. Gonzales, J. G. Aldaba, G. B. Nair, *The. Adv. Vaccines* **2014**, *2*, 123–136.
[13] B. Sit, B. Fakoya, M. K. Waldor, *Ann. Rev. Microbiol.* **2022**, *76*, 681–702.
[14] Q. Bi, E. Ferreras, L. Pezzoli, D. Legros, L. C. Ivers, K. Date, F. Qadri, L. Digilio, D. A. Sack, M. Ali, J. Lessler, F. J. Luquero, A. S. Azman, P. Cavailler, K. Date, N. Sreenivasan, H. Matzger, F. Luquero, R. Grais, L. Wiesner, M. Ko, V. Rouzier, C. Peak, F. Qadri, J. Landegger, J. Lynch, A. Azman, D. Sack, M. Henkens, I. Ciglenecki, L. Ivers, E. Diggle, M. Weiss, A. Hinman, K. Maina, I. Mirza, G. Gimeno, M. Levine, *Lancet Infect. Dis.* **2017**, *17*, 1080–1088.
[15] B. Sit, T. Zhang, B. Fakoya, A. Akter, R. Biswas, E. T. Ryan, M. K. Waldor, *PLoS Neglected Trop. Dis.* **2019**, *13*, e0007417.
[16] K. Bagchi, P. Echeverria, J. D. Arthur, O. Sethabutr, O. Serichantalergs, C. W. Hoge, *J J. Clin. Microbiol.* **1993**, *31*, 1315–1317.
[17] S. Chatterjee, K. Ghosh, A. Raychoudhuri, G. Chowdhury, M. K. Bhattacharya, A. K. Mukhopadhyay, T. Ramamurthy, S. K. Bhattacharya, K. E. Klose, R. K. Nandy, *J. Clin. Microbiol.* **2009**, *47*, 1087–1095.
[18] A. E. Purdy, D. Balch, M. L. Lizárraga-Partida, M. S. Islam, J. Martinez-Urtaza, A. Huq, R. R. Colwell, D. H. Bartlett, *Environ. Microbiol. Rep.* **2010**, *2*, 198–207.
[19] A. Joelsson, Z. Liu, J. Zhu, *Infect. Immun.* **2006**, *74*, 1141–1147.
[20] S. Stutzmann, M. Blokesch, *MSphere* **2016**, *1*, e00098–16.
[21] B. Rémy, S. Mion, L. Plener, M. Elias, E. Chabrière, D. Daudé, *Fron. Pharmacol.* **2018**, *9*.
[22] S. Di Bella, P. Ascenzi, S. Siarakas, N. Petrosillo, A. Di Masi, *Toxin Rev.* **2016**, *8*, 134.
[23] B. K. Sandhu, S. M. McBride, *Trends Microbiol.* **2018**, *26*, 1049–1050.
[24] D. N. Gerding, T. Meyer, C. Lee, S. H. Cohen, U. K. Murthy, A. Poirier, T. C. Van Schooneveld, D. S. Pardi, A. Ramos, M. A. Barron, H. Chen, S. Villano, *Jama* **2015**, *313*, 1719–1727.
[25] D. N. Gerding, S. P. Sambol, S. Johnson, *Front. Microbiol.* **2018**, *9*.
[26] S. Rabizadeh, K.-J. Rhee, S. Wu, D. Huso, C. M. Gan, J. E. Golub, X. Wu, M. Zhang, C. L. Sears, *Inflamm. Bowel. Dis.* **2007**, *13*, 1475–1483.
[27] W. T. Cheng, H. K. Kantilal, F. Davamani, *Malays J Med. Sci.* **2020**, *27*, 9–12.
[28] J. L. Chan, S. Wu, A. L. Geis, G. V. Chan, T. A. M. Gomes, S. E. Beck, X. Wu, H. Fan, A. J. Tam, L. Chung, H. Ding, H. Wang, D. M. Pardoll, F. Housseau, C. L. Sears, *Mucosal. Immunol.* **2019**, *12*, 164–177.
[29] A. L. Hecht, B. W. Casterline, Z. M. Earley, Y. A. Goo, D. R. Goodlett, J. Bubeck Wardenburg, *EMBO Rep.* **2016**, *17*, 1281–1291.
[30] F. S. Midani, A. A. Weil, F. Chowdhury, Y. A. Begum, A. I. Khan, M. D. Debela, H. K. Durand, A. T. Reese, S. N. Nimmagadda, J. D. Silverman, C. N. Ellis, E. T. Ryan, S. B. Calderwood, J. B. Harris, F. Qadri, L. A. David, R. C. LaRocque, *J. Infec. Dis.* **2018**, *218*, 645–653.

[31] I. Levade, M. M. Saber, F. S. Midani, F. Chowdhury, A. I. Khan, Y. A. Begum, E. T. Ryan, L. A. David, S. B. Calderwood, J. B. Harris, R. C. LaRocque, F. Qadri, B. J. Shapiro, A. A. Weil, *J. Infec. Dis.* **2021**, *223*, 342–351.
[32] S. Alavi, J. D. Mitchell, J. Y. Cho, R. Liu, J. C. Macbeth, A. Hsiao, *Cell* **2020**, *181*, 1533–1546.e13.
[33] F. Duan, J. C. March, *PNAS* **2010**, *107*, 11260–11264.
[34] A. Hsiao, A. S. Ahmed, S. Subramanian, N. W. Griffin, L. L. Drewry, W. A. Petri, R. Haque, T. Ahmed, J. I. Gordon, *Nature* **2014**, *515*, 423.
[35] P. A. Lawson, S. M. Finegold, *IJESM* **2015**, *65*, 789–793.
[36] M. Coscollá, C. Fernández, J. Colomina, L. Sánchez-Busó, F. González-Candelas, *Int. J. Med. Microbiol.* **2014**, *304*, 307–313.
[37] G. Wewalka, D. Schmid, T. G. Harrison, S. A. Uldum, C. Lück, *Clin. Microbiol. Infect.* **2014**, *20*, O13–O19.
[38] H. Mizrahi, A. Peretz, R. Lesnik, Y. Aizenberg-Gershtein, S. Rodríguez-Martínez, Y. Sharaby, N. Pastukh, I. Brettar, M. G. Höfle, M. Halpern, *Sci. Rep.* **2017**, *7*, 40114.
[39] S. Laviad-Shitrit, I. Izhaki, E. Arakawa, M. Halpern, *TM and IH.* **2018**, *23*, 758–764.
[40] R. J. Atterbury, J. Tyson, *Microbiology* **2021**, *167*, DOI 10.1099/mic.0.001025.
[41] E. F. Boyd, M. K. Y. Waldor, *Microbiology* **2002**, *148*, 1655–1666.

Manuscript received: February 6, 2023
Revised manuscript received: March 28, 2023
Version of record online: ■■, ■■

PERSPECTIVE



*R. Sela, B. K. Hammer, M. Halpern**