

**NATURAL COMPETENCE AND TYPE VI SECRETION
IN *VIBRIO CHOLERAE***

A Dissertation
Presented to
The Academic Faculty

By

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In Partial Fulfillment
Of the Requirements for the Degree
Doctor of Philosophy in Biology

Georgia Institute of Technology

August, 2016

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**NATURAL COMPETENCE AND TYPE VI SECRETION
IN *VIBRIO CHOLERAE***

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This is for you, Adam, Mom and Dad.
Thanks for always keeping me smiling.

ACKNOWLEDGEMENTS

Firstly, to my advisor Brian Hammer, who is both the heart and brains of the lab, without whom I would not have made it to this point in my PhD. You are truly the best scientist I have ever met and your constant flow of ideas and enthusiasm helped push me forward even when I didn't think it was possible. You not only trained me as a scientist, but helped mold me into a passionate and effective speaker. If I become half the scientist and teacher you are I will be extremely lucky.

To my recent collaborators, Will Ratcliff and Peter Yunker, for opening my eyes to the amazing world of ecology, evolution, physics, and mathematical modeling. I have learned so much this past year thanks to your intelligence, patience, hard work, passion, and direction. Thank you so much for making my last year so memorable, exciting, and full of fun and amazing science.

To my other committee members, both past and present, Cheryl Tarr, Frank Stewart, Thomas DiChristina, and Patrick McGrath for the time you spent guiding me, all the encouraging words, and the helpful criticisms. Thank you all so much for your kind support.

To all of my professors, who either taught me in class or mentored me in a teaching assistantship, you have helped more than you realize. I truly appreciate every minute you spent teaching me how best to teach others and how to think myself.

To all of my labmates, you made lab fun every day. Each of you helped me become a better scientist, and I am so grateful for your help and advice throughout the years.

To my mom and dad, you are the most supportive and wonderful parents anyone could ask for. I am so lucky to have you both in my life. You are my cheerleaders, mentors, role models, and show me never-ending love and support. Thank you for everything.

To the rest of my family (both Bernardy and Raymond) and friends, thank you for your constant support, understanding, and encouragement. I would not have made it without you.

To my best friend, fiancé, and personal comedian, Adam, thank you for your unwavering support, understanding, and ability to constantly keep me optimistic. I appreciate you every day.

Finally, to everyone who was important to the culmination of my research leading to this thesis. I apologize I couldn't name each and every one of you by name, but I appreciate all of you.

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LIST OF SYMBOLS AND ABBREVIATIONS

AI	autoinducer
AI-2	autoinducer-2
cAMP	cyclic adenosine monophosphate
ASW	artificial sea water
BLAST	Basic Local Alignment Search Tool
bp	base pair
BPMM	Bayesian phylogenetic mixed model
C	clinical
CAI-1	cholera autoinducer-1
CBP	chitin binding protein
CCR	carbon catabolite repression
CDC	Centers for Disease Control and Prevention
cfu	colony-forming unit
Cm	chloramphenicol
CRE	competence regulatory element
CRP	catabolic repressor protein
CT	cholera toxin
CTX	cholera toxin prophage
CytR	cytidine repressor protein
DNA	deoxyribonucleic acid
DUS	DNA uptake sequence
E	environmental
GlcNAc	β -1,4-linked <i>N</i> -acetylglucosamine
c-di-GMP	Bis-(3'-5')-cyclic dimeric guanosine monophosphate
h	hours
HCD	high cell density
HGT	horizontal gene transfer
IBM	individual based simulation model
IM	inner membrane
IPTG	isopropyl- β -D-thiogalactopyranoside
Kan	kanamycin
LCD	low cell density
LB	Luria-Bertani broth
M	molar
μ g	micrograms
mg	milligrams
mM	millimolar
μ L	microliter
mL	milliliter
μ m	micrometer
NAg	nonagglutinating
N.S.	not significant

OD	optical density
OM	outer membrane
PCA	principal component analysis
PCR	polymerase chain reaction
PDE	partial differential equation
Qrr	quorum regulatory RNA
QS	Quorum sensing
RLU	relative light units
RNA	ribonucleic acid
mRNA	messenger ribonucleic acid
sRNA	small ribonucleic acid
RNAP	RNA polymerase
Str	streptomycin
TF	transformation frequency
T6SS	type VI secretion system
T6S	type VI secretion
UTR	untranslated region
WT	wild type

SUMMARY

The waterborne bacterium *Vibrio cholerae*, responsible for epidemics of cholera diarrhea, associates with the human gut and with chitinous surfaces in aquatic reservoirs. Prior studies of two clinical *V. cholerae* isolates revealed that natural competence for genetic transformation, a horizontal gene transfer mechanism, requires the chitin-induced TfoX regulator, and quorum sensing transcription factor HapR made at high cell density. To further understand this regulation, I helped identify, in a genetic screen, CytR, a new positive regulator required for competence gene expression and natural transformation. Recently, this complex regulatory network in *V. cholerae* was shown to also control a type VI secretion system (T6SS) that allows contact-dependent killing of other bacteria by injecting toxic proteins. I characterized a diverse set of sequenced *V. cholerae* isolates, revealing that transformation was rare in all isolates, while constitutive type VI killing was common among environmental but not clinical isolates. These latter results were consistent with a “pathoadaptive” model that tight regulation is beneficial in a host, while constitutive killing is advantageous in the environment. We hypothesized that two sequenced *V. cholerae* isolates with distinct T6SSs could generate structured populations from initially well-mixed conditions by killing competitors, but not kin. Indeed, when both isolates were rendered T6SS⁻, a well-mixed population was observed via fluorescence microscopy. In contrast, mutual killing generated clonal patches with each isolate segregating into distinct groups. Structural dynamics were recapitulated with three mathematical models and a cooperation model developed supports that this assortment promotes cooperation among kin. My work in *V. cholerae* has helped elucidate a

complex regulatory network controlling multiple important phenotypes, diversity of these phenotypes among species members, and ecological consequences of antagonistic microbial interactions in the environment.

CHAPTER 1

NATURAL COMPETENCE AND TYPE VI SECRETION IN *VIBRIOS*

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1.1. Introduction

The *Vibrionaceae* family consists of a remarkably diverse set of Gram-negative bacteria. In general, *Vibrionaceae* members are readily isolated from aqueous environments ranging from freshwater to marine conditions and are easily cultured. Pathogenic *Vibrionaceae* members, particularly *Vibrio cholerae*, have significantly impacted human health, both historically and currently, and, as a result, have garnered a great deal of attention from the biomedical community (146, 171). However, numerous ecological studies of this diverse family of bacteria have revealed that they often form non-pathogenic, and in many cases beneficial relationships with eukaryotes (111, 192, 228).

Many bacterial species can become competent to take up DNA from the environment. While DNA transported across the cytoplasmic membrane by the membrane-spanning apparatus may serve as a source of nutrients or as a template for repairing chromosomal damage, it can also be incorporated onto the chromosome by homologous recombination. This latter phenomenon, referred to as natural transformation, is a prime example of horizontal gene transfer, which, along with conjugation and transduction, can result in the emergence of new traits (49). Recently, various members of the *Vibrionaceae* family, including the human pathogen *Vibrio cholerae*, have been shown to be naturally competent to take up DNA.

Many features of the competence machinery and its regulation in *Vibrionaceae* members are similar to those archetypical systems described for Gram-negative bacteria. In general, uptake of environmental DNA requires a complex apparatus that first binds the DNA at the cell surface and then delivers it through the membrane to the cytoplasm (70, 191). In *Neisseria* species, such as *Neisseria meningitidis* and *Neisseria gonorrhoeae*, the outer membrane secretin pore PilQ allows double-stranded DNA to enter into the periplasm (124). With the help of a pseudopilus encoded in part by *pilA*, the periplasmic protein ComEA binds the DNA and directs it to the inner-membrane channel ComEC (Fig. 1.1) (242). The minor pilin ComP also contributes to natural transformation by serving as a DNA receptor that recognizes species-specific DNA uptake sequences (DUS) (1, 46). One strand of the DNA enters the cytoplasm through ComEC, while the complement strand is degraded by nucleases (Fig. 1.1) (49). Once inside the cytoplasm, this DNA may be integrated into the chromosome through homologous recombination (Fig. 1.1). In *Haemophilus influenzae*, another Gram-negative bacterium that serves as a model system for natural transformation, the secretin that allows the entry of double-stranded DNA is called ComE; the crucial subunit of the pseudopilus involved in DNA uptake is PilA; and the counterparts of ComEA and ComEC are ComE1 and Rec2 respectively (50, 142, 149). *Vibrionaceae* members such as *V. cholerae* possess homologs of PilQ, PilA, ComEA and ComEC, which play crucial roles in the uptake of exogenous DNA (Fig. 1.1) (136). No homolog of ComP has been reported for *H. influenzae* or members of *Vibrionaceae*. In addition, for most Gram-negative bacteria, whether DNA uptake is achieved by a type IV pilus or a pseudopilus remains generally unknown.

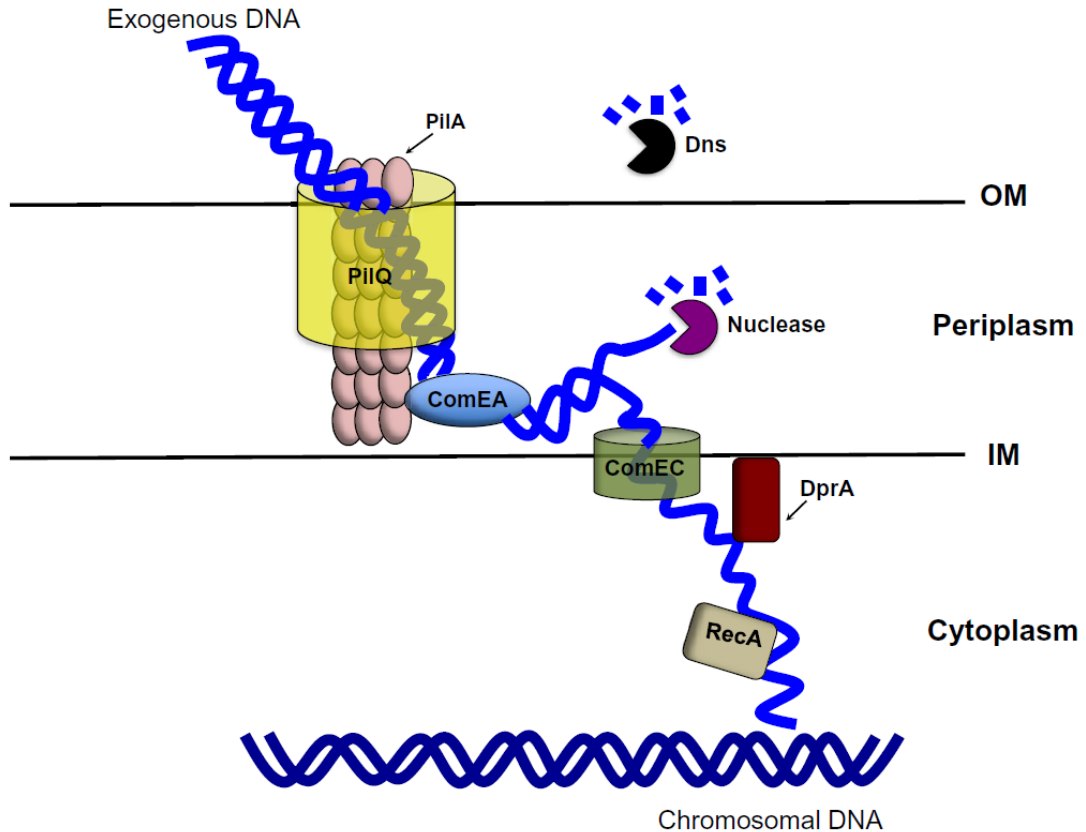


Figure 1.1. The natural transformation machinery in *Vibrio cholerae*. Double-stranded DNA enters the periplasm by means of the secretin pore PilQ located within the outer membrane. The pseudopilus (PilA represents a subunit) helps the DNA bind to the periplasmic protein ComEA, which directs the DNA to the inner-membrane channel ComEC. Other components of a type IV pili system (not shown) may contribute to this process, but their involvement in natural transformation remains unknown. The extracellular DNA that fails to enter the periplasm is degraded by extracellular deoxyribonuclease Dns. One strand of the DNA enters the cytoplasm through ComEC, while the complement strand is degraded. The internalized single-stranded DNA is shielded from nuclease attack by the DNA protecting protein DprA and incorporated into chromosome by the recombinase RecA. IM, inner membrane; OM, outer membrane.

The Type 6 secretion system (T6SS) is a mechanism found in ~25% of all sequenced gram-negative bacteria and used to attack neighboring cells in a contact-dependent manner (37, 107). It directly injects toxic effector proteins into prokaryotic or eukaryotic neighboring cells via an apparatus structurally analogous to an inverted bacteriophage tail spike (107, 223). This antagonistic behavior was originally discovered

in 2006 in an environmental isolate of *V. cholerae*, V52 (178), and has been highly studied since in not only *V. cholerae*, but also *Pseudomonas aeruginosa*.

In *V. cholerae*, the T6SS is encoded by three gene clusters (Fig 1.2A) (37, 155, 178). One large cluster (VCA0107 – VCA0124) encodes mainly structural genes, a regulatory gene (*vasH*) that activates expression of the two auxiliary clusters, and one effector-immunity pair (*vgrG-3*, *tsiV3*) that act as a part of a toxin-antitoxin like system (155). The smaller auxiliary clusters (VCA0017 – VCA0021 and VC1415 – VC1421) encode the other proteins to be secreted, including *hcp*, *vgrG1-2*, and two other effector-immunity pairs (Fig 1.2A) (155). As seen in Figure 1.2B, the apparatus consists of an outer sheath made up of VipA and VipB, an inner Hcp tube, and a spike made up of VgrG proteins. When the outer sheath contracts, the Hcp and spike are expelled through the outer membrane and into the neighboring cell (107). The VgrG spike is loaded with toxic effector proteins, shown in purple, that may cause lysis or inhibition of growth in the target cell (5, 155, 239). These toxic effectors are thought to be neutralized by their cognate immunity proteins, however the mechanism behind this is poorly understood. In some cases, protein-protein interactions have been demonstrated, presumably blocking the active site of the effector (5, 138). Immunity genes are constitutively expressed by an internal promoter in the upstream effector (Fig 1.2A), allowing for protection from toxic effectors regardless of expression of the T6SS apparatus (155). Therefore, two strains with the same set of effector-immunity pairs are not able to kill one another (143, 155, 224).

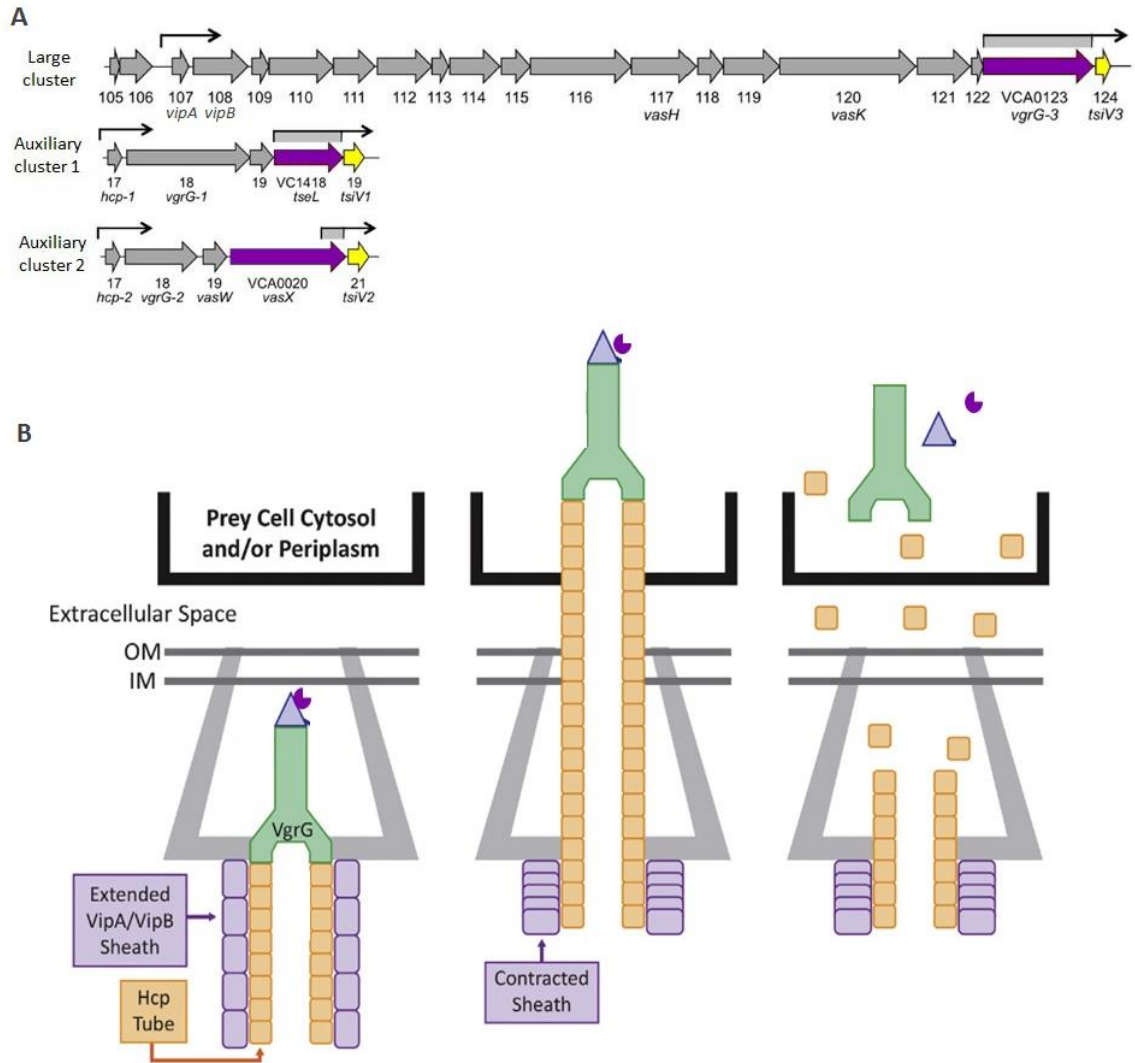


Figure 1.2. T6SS gene clusters and apparatus in *Vibrio cholerae*. (A) One large gene cluster and two auxiliary clusters encode one T6SS. Each cluster has one toxic effector (purple) and immunity (yellow) protein pair. (B) The T6SS apparatus resembles an inverted bacteriophage tail spike. The VipA/VipB outer sheath contracts, expelling the inner Hcp tube out of the cell allowing the VgrG trimer spike to puncture a neighboring cell and deliver toxic effectors (purple sector). IM, inner membrane; OM, outer membrane.

While the primary components of the competence machinery and T6SS are conserved among bacteria, the regulatory networks that govern their expression vary tremendously to accommodate differences in lifestyles. For instance, *Streptococcus*

pneumoniae and *Bacillus subtilis*, which have served as model organisms for natural transformation in Gram-positive bacteria, use the cell–cell form of communication known as quorum sensing to regulate the expression of competence genes (204). In *H. influenzae*, nutrient deprivation, rather than quorum sensing, controls competence (47, 69). Finally, both *N. meningitidis* and *N. gonorrhoeae* are constitutively competent (29).

Regulation of T6SSs are thought to be customized for the niche occupied by the bacterium (61). In *V. cholerae*, although the T6SS genes are well conserved, regulation is different. Environmental isolates have been shown to constitutively kill via their T6SS while clinical pandemic strains, defined as those with serogroup O1 or O139, do not express T6S in standard laboratory conditions and must be induced with chitin (24, 34, 178, 223). In *P. aeruginosa*, one of the many inducers of T6S is surface perturbations by cell contact, which allows these bacteria to utilize their T6SS in retaliation to attack (20, 51). Although T6SSs were discovered in 2006, most studies have focused on structure rather than regulation of this toxin delivery system. Therefore, the regulation described in this review is representative of the current literature, but many questions are left to be answered.

The complex regulatory network that controls competence and T6S in *Vibrionaceae* members exhibits some features that are reminiscent of the model systems listed above. We begin this literature review with a description of the environmental signals and regulators that stimulate competence and T6S in *V. cholerae*. We then describe the role each regulator plays in controlling these phenotypes in *Vibrionaceae*. Finally, we discuss the implications for natural transformation and T6S in the environment and corresponding impacts on human health.

1.2. Environmental inputs

Numerous studies have shown that various environmental and physiological factors impact natural transformation and T6S in *V. cholerae* (7, 8, 30, 34, 136, 147, 235). The current model of the regulatory network governing competence, T6S, and chitinase expression in *V. cholerae* consists of three stimuli and associated transcriptional regulators: chitin (TfoX), quorum sensing (HapR and QstR), and availability of extracellular nucleosides (CytR) (Fig. 1.3).

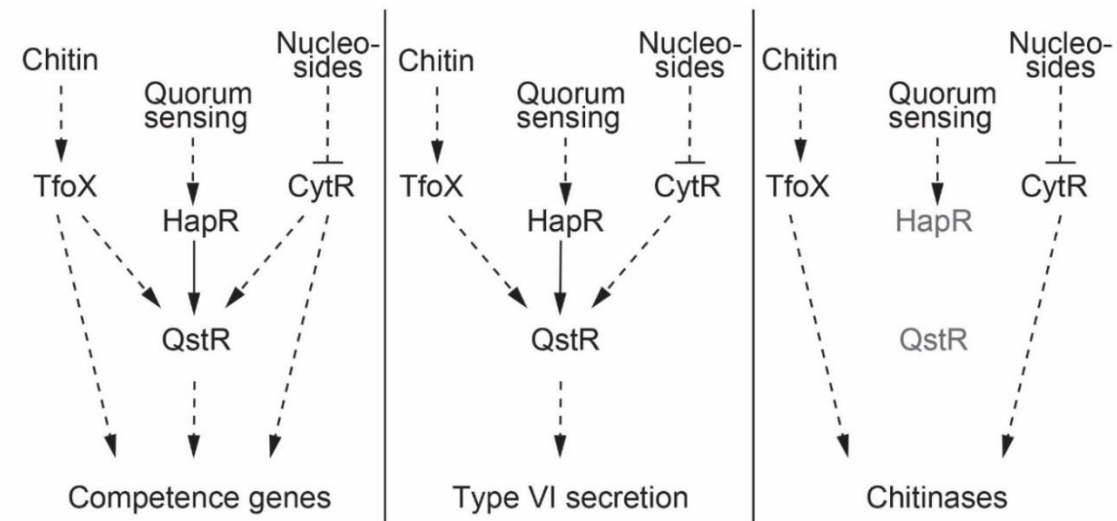


Figure 1.3. The current model of the regulatory network controlling competence, T6S, and chitinases in *V. cholerae*. The three known environmental stimuli are chitin, quorum sensing, and the availability of extracellular nucleosides. Dashed lines connecting components of the signaling cascade indicate indirect interaction, while solid lines indicate direct interaction.

1.2.1. Chitin

In 2005, it was first reported that *V. cholerae* becomes competent for natural transformation in the presence of chitin (147). This result was independent of whether the

chitin source was synthetically derived or in its natural state as crustacean exoskeletons (e.g. crabshell tiles). More recently, chitin was also shown to induce T6SS gene expression and bacterial killing in *V. cholerae* clinical isolates A1552 and C6706; however, chitin is not needed for T6S induction in environmental isolates (34, 235). To our knowledge, chitin has never been directly tested for its involvement in natural transformation in *H. influenzae*, *N. gonorrhoeae* or *N. meningitides*, or for T6S in other Gram-negative bacteria such as *P. aeruginosa* (29, 202). However, given the absence of chitin in the natural habitats of these bacterial species, chitin is unlikely to play any significant role in their gene expression.

Chitin, which is composed of chains of β -1,4-linked *N*-acetylglucosamine (GlcNAc) residues, is the second most abundant biopolymer found in nature (110, 120). Chitin can be found throughout all kingdoms, and its presence is widespread in marine environments, ranging from the cell walls of certain green algae to the exoskeletons of crustaceans (110, 120). The majority of chitin in the aquatic biosphere is recycled by chitinolytic bacteria, including members of the *Vibrionaceae* family (110, 120, 148). Chitinolytic bacteria use chitin as a source of both carbon and nitrogen, through a complex process that involves the initial detection of chitin, attachment to a chitinous surface, and degradation of chitin (110, 120, 131). For example, in *V. cholerae*, chitinase expression is activated by TfoX in the presence of chitin, as well as CytR in low levels of nucleosides (Fig 1.3). By means of extracellular secreted chitinases, chitin is cleaved into oligosaccharides fragments, (GlcNAc)_n, which translocate into the periplasm and bind to a high-affinity, chitin oligosaccharide-binding protein (CBP) (Fig. 1.4). Binding of (GlcNAc)_n to CBP activates a two-component sensor kinase named ChiS (Fig. 1.4).

Signaling by ChiS, in turn, leads to production of chitinolytic enzymes, which breakdown (GlcNAc)_n into monomers that are channeled into the central metabolism as fructose-6-phosphate, acetate, and ammonium (110, 131). In addition to providing a source of carbon and nitrogen, chitin also influences many aspects of *Vibrio* physiology, including chemotaxis, biofilm formation, and pathogenicity (6, 22, 121, 144, 176, 183, 233). Consistent with these effects on physiology, growth in chitin results in significant, global changes in gene expression (148).

One intriguing possibility is that chitin, and its oligosaccharide derivatives, signal the presence of a nearby host, as seen with *Vibrio fischeri* and its squid host *Euprymna scolopes* (144). Within this context, T6SS⁺ cells may lyse neighboring bacterial cells allowing for competent individuals to acquire genetic elements for host interaction. In this manner, the chitin signal would result not only in enhanced genetic diversity but also the acquisition of host specific factors. Consistent with this model, chitin is highly abundant in zooplankton and exoskeletons of shellfish, which are two animal reservoirs that play crucial roles in transmission of *V. cholerae* (185). Another possibility is that chitin functions as a signal for *Vibrionaceae* to access an alternative nutrient source in particular environments, as previously implicated in the genetic competence-induction program in *V. cholerae* (147). The resulting DNA uptake, in turn, may provide the *Vibrionaceae* members with an extra nutrient resource. Interestingly, chitin, which is crucial for natural transformation and T6S in *V. cholerae* (147), is also one of the substrates on which *V. cholerae* develops biofilms (233), enabling *V. cholerae* to survive stressful environments (3). In addition, exogenous DNA released during lysis possibly via T6SS-mediated killing provides genetic material for natural transformation and is also

prevalent in biofilm formation (193). Future studies that focus on chitin signaling in *V. cholerae* are required to elucidate its link to natural transformation and the T6SS.

1.2.1.1. TfoX: an integral regulator

The *tfoX* gene was originally identified among other *V. cholerae* genes that display elevated expression levels in response to chitin (148). TfoX was annotated as a transcription factor and found to be required for chitin-dependent natural transformation in *V. cholerae* (147). When constitutively expressed, TfoX can induce genetic competence in the absence of chitin (147). A homolog of TfoX named Sxy plays a central role in controlling genetic competence in *H. influenzae* (182, 240, 253), *Aggregatibacter actinomycetemcomitans* (26), *Actinobacillus pleuropneumoniae* (35) and *Actinobacillus suis* (198). *E. coli* also possesses a homolog of Sxy, but expression of *E. coli* Sxy is insufficient to induce natural transformation in this organism, despite activating expression of homologs of competence genes that are used by other bacterial species (45, 197, 199).

Chitin, in the form of either crab shells or oligosaccharides, enhances the production of TfoX, at both transcriptional and post-transcriptional levels (Fig. 1.4) (246). Genetic studies in *V. cholerae* have shown that chitin sensed by the hybrid sensor kinase ChiS, promotes production of a small regulatory RNA named TfoR (Fig. 1.4) (131, 136, 148, 246). The signaling pathway initiated by ChiS that leads to *tfoR* expression remains unclear. TfoR is predicted to base pair with the 5'-untranslated region (5'-UTR) of the *tfoX* transcript, thus preventing formation of an inhibitory secondary structure that sequesters the predicted Shine–Dalgarno sequence for *tfoX* (244).

Translational activation of *tfoX* by TfoR also requires the RNA chaperone Hfq (Fig. 1.4), which is often a crucial player in trans-encoded small RNA-mediated regulation (229, 244). A transcriptional repressor element was also identified within the *tfoX* promoter in *V. cholerae* (246). However, in contrast to the strong translational regulation described above, the transcriptional derepression of *tfoX* by chitin and (GlcNAc)_n is moderate, and the transcription factor involved in this process remains unknown (246). In *H. influenzae*, which does not possess a TfoR homolog, transcription of *sxy* is strongly induced by the CRP–cAMP complex, a global regulator activated in response to sugar starvation (253). CRP–cAMP was recently shown to also directly induce *tfoX* transcription in *V. cholerae*, suggesting conserved regulation among these Gram-negative bacteria (243). Future studies of *tfoX* regulation will greatly help our understanding of how the *Vibrionaceae* process the external chitin signal to turn on complex phenotypes like competence and T6S.

TfoX activates the expression of many genes involved in chitin degradation and chitin-induced competence in *V. cholerae* (147). Members of the chitin-dependent TfoX regulon include the *pilQ*, *pilA*, *comEA*, and *comEC* genes (Fig. 1.4), whose gene products play essential roles in the uptake and transport of exogenous DNA (Fig. 1.1) (49, 59, 84, 95, 136, 148). In addition, expression of a gene encoding a homolog of DprA, which plays a crucial role in the integration of exogenous DNA, was reported to be elevated in response to induction of *tfoX* expression (136). In *H. influenzae*, Sxy is proposed to direct the CRP–cAMP complex to a non-canonical CRP site TGCGA-N₆-TCGCA, denoted CRP-S, within the promoter region of competence genes (44, 45). These studies suggested that Sxy may serve as an accessory factor to CRP, rather than a primary

transcription factor, in promoting expression of competence genes (45, 182, 199). This model is consistent with the lack of an obvious DNA-binding domain in Sxy. The demonstration that cAMP, presumably in complex formation with CRP, is required for chitin-dependent induction of *comEA* and *pilA* in *V. cholerae* further highlights that TfoX may also serve as an accessory factor (Fig. 1.4) (136). Consistent with this hypothesis, potential CRP-S sites are located within the promoter regions of *V. cholerae comEA* and *pilA* (7). A common motif ACTCG(A/C)AA was identified in most of the 19 TfoX-induced promoter regions by means of a phylogenetic footprint analysis (45). At this time, whether TfoX binds DNA in complex with CRP has not yet been determined by experimental approaches.

Recently, TfoX was shown to positively regulate the expression of two transcription factors: CytR and QstR (Fig. 1.3) (137, 147). CytR is the nucleoside scavenging cytidine repressor that activates expression of *comEA*, *pilA* and *chiA-1* in *V. cholerae* (Fig. 1.6) (7). TfoX also activates transcription of *qstR* (Fig. 1.4), which encodes a transcription factor essential for *comEA*, *comEC*, and T6SS gene expression (Fig. 1.5) (137, 235). Activation of QstR also requires the quorum sensing major regulator, HapR, discussed in detail in the following section (Fig. 1.5) (137), which accounts for the subset of TfoX-regulated genes that are also controlled by quorum sensing (136). Many questions are still left to be answered about the regulatory network controlling T6S in *V. cholerae*. Although it is known that T6S gene expression and killing are induced with chitin (34), the chitin controlled transcription factors directly binding to T6SS and natural competence promoters are unknown. Together, these studies

underscore the complex roles of TfoX in coordinating expression of the genes required for competence and T6S in *V. cholerae*.

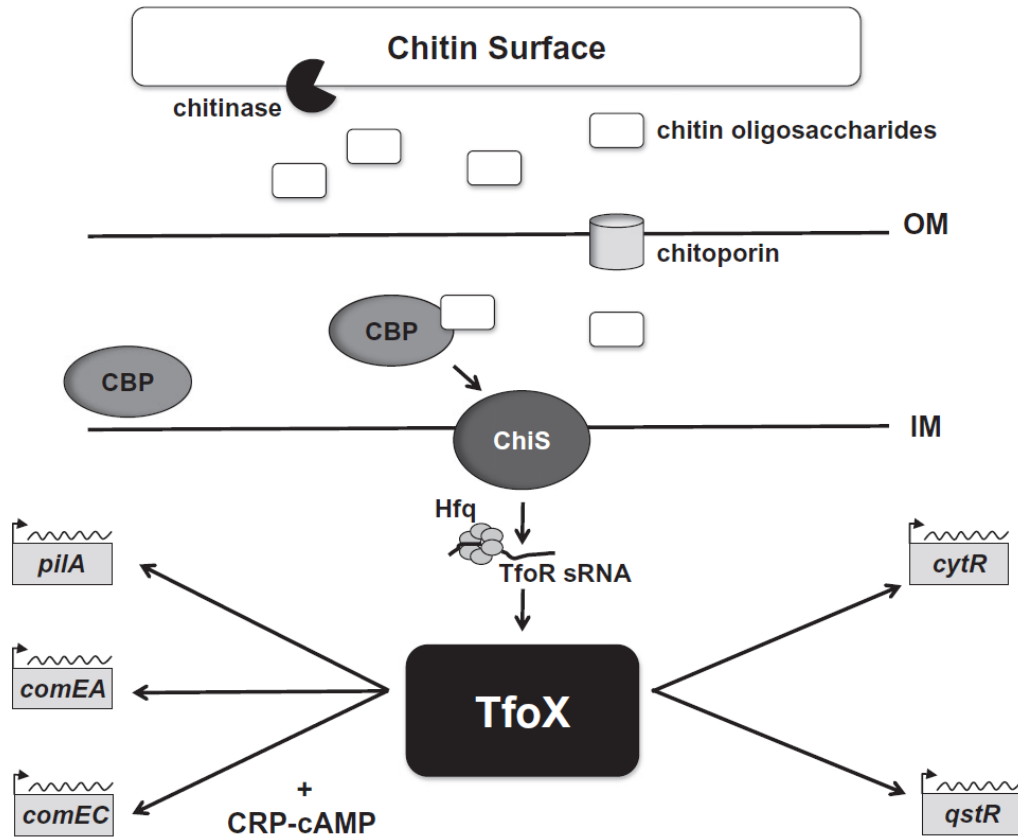


Figure 1.4. Chitin-dependent signaling pathways for natural transformation and T6S in *V. cholerae*. Chitin is degraded by extracellular chitinases into oligosaccharide fragments, which enter the periplasm through a chitoporin. With the help of CBP (chitin oligosaccharides binding protein), the sensor kinase ChiS senses the presence of chitin oligosaccharides and activates the TfoR sRNA via the RNA chaperone Hfq. TfoR then initiates translation of TfoX, a master regulator in *Vibrionaceae*. Consequently, TfoX upregulates the expression of numerous competence genes (e.g. *pilA*, *comEA* and *comEC*) and of two genes encoding transcription factors (*cytR* and *qstR*) that are also crucial for expression of competence and T6S. Regulation of the competence genes by TfoX also requires the cAMP–CRP complex. The lines connecting components of the signaling cascade represent positive regulation, but not direct interaction. IM, inner membrane; OM, outer membrane.

1.2.2. Quorum sensing

A second regulatory system controlling competence and T6S in *V. cholerae* is quorum sensing, which is a process of cell–cell communication that allows bacteria to coordinate gene expression according to population density (164). All *Vibrionaceae* members produce and detect chemical signaling molecules called autoinducers (AIs). *V. cholerae* produces two AIs: CAI-1, which is restricted to certain *Vibrionaceae* members, and AI-2, an interspecies autoinducer produced by many bacteria (23, 250). At low cell density, when CAI-1 and AI-2 levels are low, their unbound cognate receptors CqsS and LuxP/Q, respectively, behave as kinases and initiate a phosphorylation cascade via LuxU that phosphorylates the response regulator LuxO (Fig. 1.5). Phosphorylated LuxO activates the transcription of four small RNAs, called Qrrs (quorum regulatory RNAs), which via the RNA chaperone Hfq, post-transcriptionally repress HapR, the master regulator of quorum sensing (15, 16, 128). In contrast, at high cell density, when CAI-1 and AI-2 levels are high, binding of the AIs to their cognate receptors reverses the phosphate flow in the LuxU–LuxO phosphorelay, resulting in production of HapR. The role of HapR in controlling virulence and surface-attachment genes important in vivo during association with a human host has been well described (99, 251).

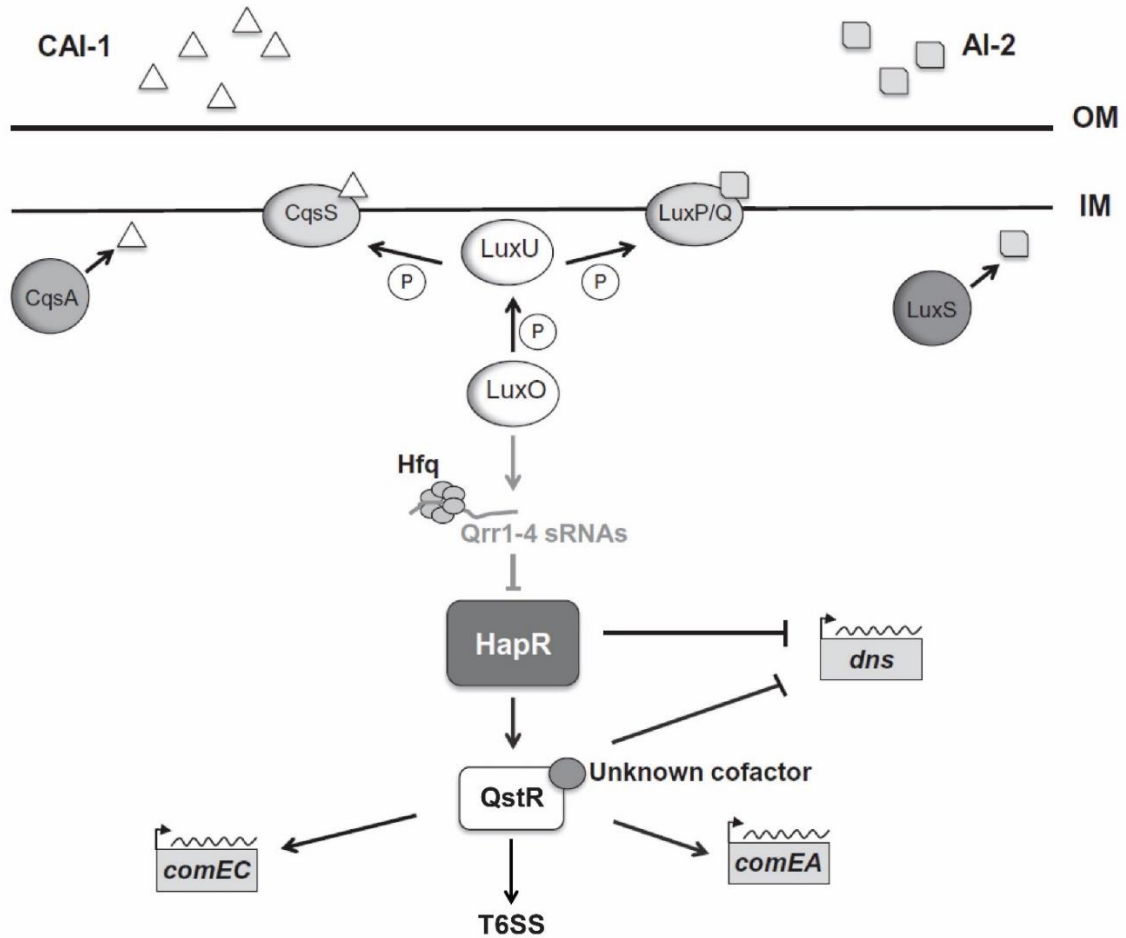


Figure 1.5. Quorum sensing-dependent pathways for natural transformation and T6S in *V. cholerae*. At high cell density (depicted here), CAI-1 and AI-2 autoinducers bind to their cognate histidine kinase receptors (CqsS and LuxP/Q respectively), which shuttle phosphate from the response regulator LuxO. In the unphosphorylated form, LuxO is unable to transcribe the Qrr1–4 sRNAs relieving repression of *hapR* translation. Therefore, the quorum-sensing master regulator HapR is produced. HapR represses transcription of *dns*. By upregulating expression of the transcription factor QstR, HapR also indirectly exerts positive effect on transcription of *comEA* and *comEC* as well as activation of the T6SS. An unknown cofactor was also implicated in the transcriptional regulation by QstR. The lines connecting components of the signaling cascade represent positive or negative regulation, but not necessarily direct interaction. Grey lines and factors indicate inactive pathways and factors not present at high cell density. IM, inner membrane; OM, outer membrane.

HapR plays a crucial role in modulating chitin-induced natural competence in *V. cholerae* (Fig. 1.5). Deletion of *hapR*, which abolishes many phenotypes controlled by

quorum sensing, also eliminates transformation and T6S. Both phenotypes can be restored by introducing *hapR* in trans, suggesting that HapR is required for this process (147). T6S can also be restored by introducing *qstR* in trans in a $\Delta hapR$ mutant, as it was shown that HapR directly activated QstR transcription. Accordingly this suggests that HapR works through QstR in regulation of T6S (235) (Fig 1.3). Both transformation frequency and *comEA* expression are affected by AI levels, with CAI-1 eliciting a stronger response than AI-2 (8, 210). In addition, within a mixed-species biofilm, *V. cholerae* cells can become competent in response to AIs that are produced from other *Vibrio* spp. located within the biofilm, suggesting that quorum sensing may facilitate DNA exchange among members of the same genus (8). Such interspecies HGT between *Vibrios* has yet to be demonstrated under laboratory conditions, and detecting low-frequency events is difficult. Interestingly, a homolog of ComP, which dictates DNA sequence specificity in *N. meningitidis* (46), is absent from the genomes of *Vibrio* spp. In addition, *Vibrio* spp. do not use a typical generalized DNA uptake sequence (DUS) to recognize species-specific DNA during natural transformation, which is contrary to the case in *H. influenzae*, which also lacks a ComP homolog but still relies on DUS for sequence specificity (149, 210). Quorum sensing, which does not regulate competence in *Neisseria* spp., may provide *Vibrionaceae* members with a ComP/DUS-independent, yet species-specific mechanism to prevent the general uptake and genomic incorporation of exogenous DNA from unrelated bacterial species (210).

Progress has been made in elucidating the mechanism by which HapR controls competence. At high cell density, HapR repression of *dns* (Fig. 1.5), which encodes an extracellular nuclease (Fig. 1.1), is believed to allow for sufficient single-stranded DNA

in the periplasm for transport into the cytoplasm (31, 147). Transcript abundance of *dns* is higher in a $\Delta hapR$ mutant than in a $\Delta luxO$ mutant that constitutively expresses *hapR* (31, 136), and a Δdns mutant is ‘hyper-transformable’, with transformation frequencies two orders of magnitude higher than a wild-type strain (31). Therefore, it was suggested that the non-transformability of a $\Delta hapR$ mutant is partly due to the failure to repress *dns*, causing constant degradation of extracellular DNA, removing the opportunity for uptake. Consistent with this model, the transformation frequency of a $\Delta hapR$ mutant can be partially restored by deleting *dns* (31). Finally, HapR appears to directly bind to the *dns* promoter to prevent transcription (137), despite the lack of a conventional HapR binding site (220). These studies suggest that quorum signaling permits Dns-mediated degradation of extracellular DNA at low cell density but represses Dns at high cell density, thereby preserving DNA for natural transformation.

HapR also positively regulates expression of *comEA* and *comEC* (Fig. 1.5), which encode components of the competence machinery important for DNA uptake (Fig. 1.1), further highlighting the important role that HapR plays in regulating competence (137). However, regulation of these genes by HapR appears indirect, as HapR does not directly bind to the promoters of *comEA* or *comEC* (137). Instead, the LuxR-type transcription regulator QstR, whose expression requires HapR (Fig. 1.5), was recently reported to be involved in the intermediate step (137).

However, the role of quorum sensing in T6S regulation is less understood. As stated above, in 2012, a new transcription factor named QstR, activated by both HapR and TfoX, is required for competence gene expression (137). More recently, T6S gene expression was also shown to be activated by HapR, TfoX, and QstR, as well as CytR

(34, 235); but QstR overexpression bypasses the requirement for the three other transcription factors in clinical isolate C6706, suggesting that those transcription factors function in activating the T6SS through QstR alone (235) (Fig 1.3).

Studies of additional *V. cholerae* clinical and environmental isolates are required to determine the general role of HapR, QstR, and quorum sensing in natural transformation and T6S. Since HapR also regulates many target genes via synthesis of the intracellular second messenger cyclic-di-GMP (85, 232), the contribution of HapR likely includes direct and indirect control of gene expression.

1.2.3. Extracellular nucleosides

The intricate response to nutrient starvation typically involves many regulatory factors that enable bacteria to utilize not only various sugars but also nucleic acids. In *E. coli*, many genes responsible for scavenging and metabolizing extracellular pyrimidine nucleosides are negatively regulated by the cytidine repressor CytR (Fig. 1.6) (225). When free nucleosides are scarce, CytR binds with the global regulator CRP and acts as an ‘anti-activator’ of the promoters of specific CRP-activated genes involved in nucleoside transport and breakdown. However, in the presence of pyrimidines, cytidine enters the cell and binds allosterically to CytR. Conformational changes in CytR, when in complex with cytidine, hamper association with CRP and, as a result, eliminate CytR-dependent anti-activation of CRP regulated genes. CRP can then activate expression of these promoters by recruiting RNA polymerase (14). Thus, genes required for nucleoside uptake and metabolism are only expressed in the presence of the nucleosides themselves.

Availability of nucleosides such as purines was also shown to play an important role in regulating natural competence in *H. influenzae* (142, 198). However, the *H. influenzae* genome lacks a CytR homolog, and a functionally similar protein, PurR, was found not to be responsible for purine-mediated repression of competence (198). Interestingly, *cytR* was originally identified as a *V. cholerae* gene regulated by TfoX (Fig. 1.3) (147). Nevertheless, its specific role in natural competence and T6S was revealed only recently, when a Δ *cytR* mutant was shown to be non-transformable and displayed decreased expression of *comEA*, *chiA-I*, and *pilA* (Fig. 1.6), as well as decreasing both T6SS gene expression and killing ability (7, 235). The presence of nucleosides, namely cytidine, also inhibited both transformation and *comEA* expression directly, suggesting a regulatory mechanism similar to that proposed in *H. influenzae* (7). Mutants containing CytR variants unable to bind to CRP were impaired for transformation, suggesting that the CytR–CRP protein–protein interactions important for nucleoside scavenging in *E. coli* are also vital to activating natural competence genes in *V. cholerae* (7). Although the exact role of nucleoside scavenging in natural competence and T6S has yet to be determined, the results described above provide evidence that these phenotypes in the *V. cholerae* may also be used for nutrient acquisition.

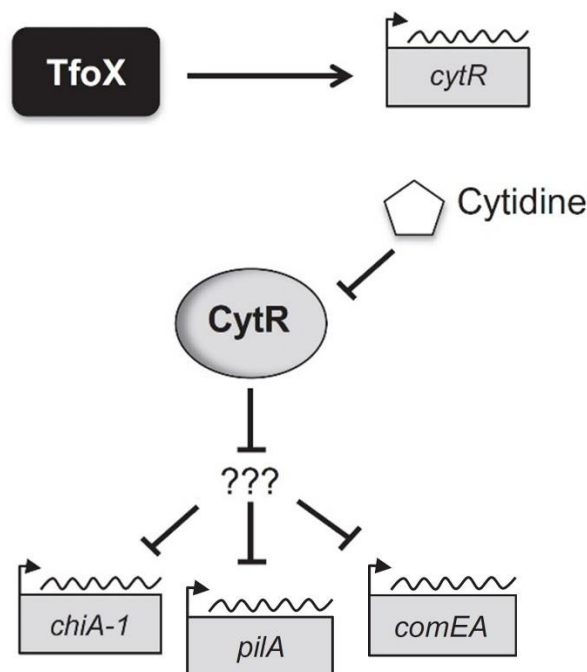


Figure 1.6. Impact of nucleosides on regulatory pathways in *V. cholerae*. When pyrimidine levels are low, the nucleoside scavenging cytidine repressor CytR anti-activates putative repressors of *chiA-1*, *pilA*, and *comEA*, resulting in upregulation of these genes. The model is over-simplified, and it remains unknown whether *comEA*, *pilA* and *chiA-1* share a common repressor that is regulated by CytR–CRP. The lines connecting components of the signaling cascade represent positive or negative regulation, but not direct interaction.

1.3. Natural transformation in other *Vibrio* species

Recently, homologs of TfoX were identified in all sequenced *Vibrionaceae* members (173). This observation, combined with the highly conserved ability of *Vibrionaceae* members to utilize chitin as a nutrient (24, 110), strongly support the notion that chitin-induced natural transformation is a shared trait among the *Vibrionaceae* family. While the presence of chitin and chitin-derived oligosaccharides does induce

natural competence in various *Vibrio* spp., differences between these organisms have been detected and are highlighted here.

1.3.1. *Vibrio fischeri*

Vibrio fischeri is a bioluminescent bacterium commonly found in free-living form within marine environments, as well as in symbiotic relationships with marine animals, such as the Hawaiian bobtail squid *Euprymna scolopes* (153, 228). When grown in the presence of chitohexaose (GlcNAc)₆, *V. fischeri* can incorporate exogenous DNA into its chromosome via natural transformation (173). *V. fischeri* also possesses *tfoX*, which is a homolog of the *V. cholerae* *tfoX* gene and similarly required for the (GlcNAc)₆-dependent natural transformation in *V. fischeri*.

When multiple copies of *tfoX* are present (i.e. on a plasmid), *tfoX* is capable of inducing genetic competence in *V. fischeri* independently of (GlcNAc)₆ (173). A paralog of *tfoX*, named *tfoY*, was also identified in the *V. fischeri* genome and shown to play a role in (GlcNAc)₆-dependent natural transformation. Intriguingly, *tfoX* in trans restores (GlcNAc)₆-induced genetic competence in a *tfoY* mutant, while *tfoY* in multi-copy fails to compensate for the loss of *tfoX* or result in (GlcNAc)₆-dependent transformation, suggesting functional differences between the two homologs (173). In the simplest model to explain these observations, TfoY serves to positively regulate TfoX in a pathway of chitin-induced natural competence. Since TfoY orthologs were also identified in other *Vibrio* spp., including *V. cholerae* (173), studying the regulation of *tfoY* expression in response to chitin as well as its functions in chitin-induced natural competence or T6S will add valuable insights to our understanding of the gene transfer processes. In fact, it

was recently shown that TfoY, in environmental isolates of *V. cholerae* like V52, activates T6S genes and bactericidal activity independent of TfoX, and is sufficient for chitin-independent constitutive T6SS expression (150).

Remarkably, polymeric chitin in the form of crab-shell tiles, which can induce natural competence in *V. cholerae* (7, 8, 147), fails to do so in *V. fischeri* (173). Even in the presence of (GlcNAc)₆, *V. fischeri* exhibits significantly lower levels of natural transformation than *V. cholerae* (173). The underlying causes of these discrepancies are unknown. Perhaps in *V. fischeri*, the chitin-dependent competence cascade (e.g. production or activities of TfoX) is under more stringent regulatory control. Alternatively, *V. fischeri* may have higher extracellular DNase activity than *V. cholerae*. These scenarios may not be mutually exclusive and additional investigation is required.

Recently, in *V. fischeri*, the N-acetyl-D-glucosamine (GlcNAc) transcriptional repressor NagC was shown to negatively regulate the expression of numerous chitin and GlcNAc utilization genes, including *VF_2139*, which is predicted to encode the chitin oligosaccharide binding protein (CBP) (152). CBP is known to play an essential part in the ChiS-mediated chitin-sensing pathways in *V. cholerae*, which eventually leads to chitin-dependent natural transformation (131, 147). It is therefore conceivable that NagC may also be involved in regulating genetic competence in *V. fischeri*. On the other hand, NagC-mediated gene regulation responds to extracellular GlcNAc (and its phosphorylated form N-acetyl-D-glucosamine-6-phosphate) (152), which does not induce natural competence in *V. fischeri* or *V. cholerae* (173, 246). Taken together, these observations suggest that any potential role of NagC in chitin-responsive natural transformation in *V. fischeri* is likely to be complex. Intriguingly, chitin oligosaccharides

produced by *E. scolopes* also serve as chemotactic signals for *V. fischeri* to facilitate host colonization (144). Whether the level of chitin oligosaccharides encountered by *V. fischeri* cells during colonization is sufficient to induce the natural competence pathway has yet to be determined.

1.3.2. *Vibrio vulnificus*

Vibrio vulnificus is another member of the *Vibrionaceae* family present in marine environments, such as estuaries, and is an opportunistic human pathogen that causes primary septicemia and wound infection (117). Like *V. cholerae*, *V. vulnificus* becomes naturally competent while growing on chitin and can both take up and incorporate exogenous DNA (91). Chitin disaccharides (GlcNAc)₂, but not GlcNAc, can also induce natural competence in *V. vulnificus* (163). The frequency of chitin-induced transformation varies among different strains. Interestingly, transformation frequency can be significantly improved by exposing biofilms comprised of different isolates to strain-specific lytic phage, which presumably leads to increased amount of extracellular DNA from cell lysis (163). In this study, *V. vulnificus* exposed to chitin took up and incorporated within its chromosome exogenous genomic DNA containing a complete *cps* loci that encodes capsular polysaccharide, which is a virulence factor. As a result of this transformation, the tested *V. vulnificus* strain underwent carbotype (capsule type) conversion. Diversity of carbotypes among *V. vulnificus* strains is thought to help this organism evade a host's immune system. Based on these results, it was proposed that chitin-induced transformation could potentially play an important role in the evolution of *V. vulnificus* (163).

1.3.3. *Vibrio parahaemolyticus*

Vibrio parahaemolyticus is a halophilic bacterium found in brackish seawater and can cause gastrointestinal illness in humans (54). In 1990, a *V. parahaemolyticus* strain was experimentally verified to pick up exogenous plasmid DNA (82). Additionally, a more recent study reported that chitin (in the form of crab shells) enables *V. parahaemolyticus* to pick up and incorporate exogenous linear DNA, which has been adapted as a genetic tool to generate specific mutations (53).

1.4. T6SS in other species

T6SS genes are present in approximately 25% of Gram-negative bacteria, but has only been experimentally investigated in a small fraction of those bacteria. Despite genes being present in many other members of the *Vibrionaceae* family, studies have been focused on *V. cholerae* (discussed in this review) with few experimental investigations with *Vibrio parahaemolyticus*, *Vibrio anguillarum*, and *Vibrio alginolyticus*. T6S has also been well studied in *Pseudomonas aeruginosa*.

1.4.1. *Vibrio parahaemolyticus*

After analyzing the genome sequence of publicly available *V. parahaemolyticus* full genomes, two gene clusters were identified, one on each chromosome, encoding two separate T6SSs, denoted T6SS1 and T6SS2 (247). All sequenced *V. parahaemolyticus* strains have T6SS2 while T6SS1 genes are primarily found in pandemic clinical strains (48, 113, 247). Both T6SSs were found to be important in adhesion to eukaryotic cells

but not cytotoxicity (247). Further studies showed that these T6SSs are differentially regulated. T6SS1 is more active in higher temperatures and salt concentrations, while T6SS2 had more expression in low salt conditions (186). T6SS1 was also found to be involved in interbacterial competition when active in warm high salt conditions, allowing *V. parahaemolyticus* to kill other Gram-negative bacteria such as *E. coli* and *V. cholerae* (186). Functions associated with T6SS2 are still left to be discovered. Overall, it appears that *V. parahaemolyticus* is capable of using its T6SSs to aid in infecting a human host, but also capable of killing neighboring bacteria in marine environments.

1.4.2. *Vibrio anguillarum*

Vibrio anguillarum is a widely distributed marine bacterium that causes hemorrhagic septicemia in fish (9). In 2009, an eight gene operon, *vtSA-H*, was identified in *V. anguillarum* and found to encode both structural (VtsE-H) and putative regulatory T6SS proteins (VtsA-D) (236). This T6SS was shown to regulate stress response and quorum sensing by activating transcription factors RpoS and VanT, rather than be involved in virulence. Specifically, the T6SS in *V. anguillarum* aided in resistance to low pH, ethanol, and hydrogen peroxide (236).

Interestingly, these T6SS genes were found highly conserved in all sequenced clinical isolates of *V. anguillarum*, however it is only expressed in non-O1 serotype strains under marine-like environmental conditions (216). This is remarkably similar to studies in *V. cholerae* showing clinical O1 serotype strain do not express T6SS in laboratory conditions, while environmental isolates are constitutive for this phenotype (24, 223). Tang, *et al.*, further showed bactericidal activity against *E. coli* and

Edwardsiella tarda, bacteria commonly found in the same environments as *V. anguillarum*, suggesting that T6S provides *V. anguillarum* with an advantage in bacterial competition (216). Therefore, it was proposed that T6S is involved in the ecology of this bacterium rather than its pathogenicity.

1.4.3. *Vibrio alginolyticus*

Vibrio alginolyticus is a known pathogen of marine animals including many fish and shellfish species (126, 127, 134, 205), but can also cause diarrhea and wound infections in humans (55). In 2012, two gene clusters putatively encoding two T6SSs were identified in *V. alginolyticus* (194). Sheng, *et al.* showed that similar to other *Vibrios*, expression of *hcp1* was controlled by the T6S internal regulator VasH, quorum sensing, and the alternative sigma factor RpoN (194). However, direct investigation of T6SS function in *V. alginolyticus* has yet to be shown.

1.4.4. *Pseudomonas aeruginosa*

As discussed above, *V. cholerae* has three T6SS gene clusters that encode one apparatus. However, *P. aeruginosa* has three gene clusters that encode three separate T6SSs, denoted H1-, H2-, and H3-T6SS, each thought to provide specific functions (65). H1-T6SS has been shown to target bacteria (108), while H2- and H3-T6SS are thought to aid in *P. aeruginosa* skin and lung infections, respectively (129, 188). Because each system has its own purpose, it is postulated that these gene clusters have been acquired horizontally (51).

Regulation of *P. aeruginosa* T6SS has been well studied and is thought to include many inputs such as quorum sensing, phosphate and iron levels, stress, and surface perturbations. More specifically, H1-T6SS is activated by membrane perturbations and is thought to act in retaliation to being attacked itself by neighboring bacteria (196). A study in 2013 showed that the T6SS of *P. aeruginosa* was selectively activated by T6SS⁺ *V. cholerae* cells that attacked *P. aeruginosa* first (19). While the T6SS attack by *V. cholerae* cells was unable to kill *P. aeruginosa*, the counterattack killed the original *V. cholerae* attacker (19). However, *P. aeruginosa* did not attack T6SS⁻ *V. cholerae* or *E. coli* cells that lack a T6SS, suggesting that *P. aeruginosa* bactericidal T6SS is activated in retaliation to attack from another cell (19).

1.5. Environmental and clinical implications

Although still under intense debate, numerous studies suggest that bacteria, including *Vibrionaceae* members, have evolved the competence pathway to aid in three major processes: nutrition, DNA repair, and horizontal gene transfer (HGT) (70, 181, 204). A particularly popular model is that natural transformation functions as a common mechanism for HGT (70, 167). Natural competence is linked to enhanced genetic diversity, improved fitness and, in some cases, increased virulence in *Vibrio* spp. Due to the abundance of the *Vibrionaceae* in a wide range of aquatic ecosystems, and their close association with various marine and freshwater plants and animals, natural competence in the *Vibrionaceae* is expected to have significant environmental, ecological and clinical implications. A previous study designed to mimic aquatic reservoirs demonstrated that, through chitin-induced natural transformation, a *V. cholerae* strain can acquire a gene

cluster (32), which effectively converts the recipient into a different serogroup (28, 157) that is known for its heightened fitness, virulence, and central role in the 1992 cholera epidemic (4). *V. cholerae* strains lacking the cholera toxin (*ctxAB*) genes required to cause cholera, can also acquire DNA encoding *ctxAB* via natural transformation (222). The potential involvement of chitin-induced natural transformation in enhancing virulence was also shown in *V. vulnificus*, as described in the previous section (163). In the latter two cases (*V. cholerae* acquiring cholera toxin genes and *V. vulnificus* obtaining virulence genes), presence of the species and strain-specific lytic phages greatly enhanced transformation frequencies. In particular, exposure of the *Vibrio* cultures to DNase exhibited negative effects on the transformation efficiency (163, 222). These results suggest that cellular DNA released by phage-mediated lysis may contribute to natural transformation. Related bacteriolytic mechanisms are used by other naturally competent bacterial species, such *S. pneumoniae* and *B. subtilis*, in the provision of donor DNA (58, 237). In contrast, *N. gonorrhoeae* uses a T6SS encoded in the gonococcal genetic island to secrete DNA outside the cells, and a mutation in this system reduces the ability of a *N. gonorrhoeae* strain to act as a donor in transformation. Together, these studies highlight the various strategies employed by bacteria to exploit exogenous DNA.

As described in the previous section, many Gram-negative bacteria have a T6SS and all sequenced *V. cholerae* strains to date have three highly conserved T6SS gene clusters (one large and two auxiliary), suggesting the high evolutionary importance of T6S in this pathogen. This contact-dependent killing apparatus is thought to not only give these bacteria a competitive advantage in their specific environment, but is also implicated in virulence in many pathogens. For example, activating the T6SS in *V.*

cholerae clinical isolate C6706 causes increased diarrhea, inflammation, and colonization of intestinal epithelia in rabbit and mouse models (141, 248). As described above, T6S in *V. parahaemolyticus* has been shown to aid in adhesion to eukaryotic cells as well as interbacterial competition (186, 247) while T6S in *V. anguillarum* helps with the global stress response via RpoS (236). In *P. aeruginosa*, T6S is thought to be important for chronic infection in both a mouse model and patients with cystic fibrosis (159, 174, 227). The mechanisms behind this connection is unknown, but there was a clear correlation between chronic infection and T6SS active *P. aeruginosa* strains. T6S is also essential for virulence in many other Gram-negative bacteria including but not limited to *Burkholderia thailandensis* (190), *Burkholderia mallei* (189), and *Francisella tularensis* (140, 162). In several of these bacteria, the manner in which T6S alters virulence is poorly understood, but studies suggest it may be a complex interplay between adhesion, interbacterial competition, or channel formation in host cell membranes leading to cell lysis and death.

The T6SS is used by many bacteria to kill not only neighboring bacteria but also eukaryotic cells either during infection or possible grazing in the environment, providing these bacteria with a competitive edge in their occupied niche. But, since only recently discovered, there is still much to be revealed about its role in host association and environmental settings. Nevertheless, one can imagine that being able to kill neighboring cells allow bacteria to obtain and maintain territory on a surface, obtain nutrients from lysed cells, or protect itself from attack by eukaryotic grazers or immune cells.

1.6. Outlook

Natural competence and T6S in *Vibrionaceae* and its ecological and clinical impact have been attracting increasing amount of interest in the scientific community in the recent years. There are still many unanswered questions regarding the underlying molecular mechanisms regulating natural competence and T6S, as well as the physiological (both short-term and long-term) ramifications of killing neighboring cells and taking up exogenous DNA. As more environmental and genetic factors controlling the regulation of natural competence and T6S are uncovered, our understanding of these important biological phenomenon will improve and provide valuable insights into the molecular mechanisms bacteria use to adapt and respond to environmental cues.

CHAPTER 2

NATURAL COMPETENCE IN *VIBRIO CHOLERA*E IS CONTROLLED BY A NUCLEOSIDE SCAVENGING RESPONSE THAT REQUIRES CYTR-DEPENDENT ANTI-ACTIVATION

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2.1. Abstract

Competence for genetic transformation in *Vibrio cholerae* is triggered by the chitin-induced transcription factor TfoX and quorum sensing (QS) regulator HapR. Transformation requires expression of *comEA*, described as a DNA receptor in other competent bacteria. A screen for mutants that poorly expressed a *comEA*-luciferase fusion identified *cytR*, encoding the nucleoside scavenging cytidine repressor, previously shown in *V. cholerae* to be a biofilm repressor and positively regulated by TfoX, but not linked to transformation. A Δ *cytR* mutant was non-transformable and defective in expression of *comEA* and additional TfoX induced genes, including *pilA* (transformation pseudopilus) and *chiA-1* (chitinase). In *Escherichia coli*, ‘anti-activation’ of nucleoside metabolism genes, via protein–protein interactions between critical residues in CytR and CRP (cAMP receptor protein), is disrupted by exogenous cytidine. Amino acid substitutions of the corresponding *V. cholerae* CytR residues impaired expression of *comEA*, *pilA*, and *chiA-1*, and halted DNA uptake; while exogenous cytidine hampered *comEA* expression levels and prevented transformation. Our results support a speculative model that when *V. cholerae* reaches high density on chitin, CytR–CRP interactions ‘anti-activate’ multiple genes, including a possible factor that negatively controls DNA

uptake. Thus, a nucleoside scavenging mechanism couples nutrient stress and cell–cell signaling to natural transformation in *V. cholerae* as described in other bacterial pathogens.

2.2. Introduction

Vibrio cholerae is the bacterium responsible for the fatal diarrheal disease cholera, but it is also a natural inhabitant of marine and estuarine environments where it commonly forms biofilms on abiotic and biotic surfaces, such as chitinous chironomids (non-biting flies) and zooplankton molts (93, 215). Horizontal gene transfer (HGT) of traits among *Vibrios* is thought to promote rapid genetic exchange that is responsible for the mosaic genome structure of *Vibrios* revealed by recent genomic sequencing efforts (56). Competence for genetic transformation in *V. cholerae* was recently reported and represents a newly appreciated mode of HGT for this aquatic bacterium (147). *V. cholerae* natural transformation is induced by two environmental signaling pathways: a quorum sensing system and a chitin utilization system. The network connecting these two systems to natural competence remains poorly understood.

Quorum sensing (QS) enables bacterial populations to collectively control gene expression and thus coordinate behaviors presumably unproductive for individuals (164). Like many other *Vibrio* species, *V. cholerae* populations accomplish QS by producing and responding to autoinducer (AI) signal molecules, specifically two AIs, CAI-I and AI-2 (for review see (97)). At low cell density (LCD) the unbound receptors of CAI-1 and AI-2 (CqsS and LuxP/Q respectively) behave as kinases and phosphorylate response regulator LuxO, via LuxU. Phosphorylated LuxO activates transcription of four small RNAs, termed Qrr1–4 (Quorum Regulatory RNAs). In association with the RNA

chaperone Hfq, the Qrrs base-pair with the mRNAs of several target genes including *hapR*, which encodes the master regulator of QS, HapR (15, 16, 100, 128, 184, 212). At high cell density (HCD), binding of AIs to their cognate receptors switches them to phosphatases, reversing the phosphorylation cascade and inactivating LuxO. Thus, HapR is produced and activates expression of numerous genes at HCD including *hapA*, which encodes a protease that plays a role in interactions of *V. cholerae* with aquatic hosts (94), and *comEA*, which encodes a periplasmic DNA-binding protein shown to be the DNA receptor for transformation in *Bacillus subtilis* (147, 175) (Fig. 2.1). Therefore, wild-type (WT) *V. cholerae* is naturally competent at HCD, a $\Delta hapR$ mutant is non-transformable, and a $\Delta luxO$ mutant that constitutively produces HapR takes up DNA independently of cell density (31, 147). So too, a *V. cholerae* strain unable to produce either AI only expresses *comEA* and takes up DNA when provided exogenous AIs (8, 210).

Activation of *comEA* expression by QS AIs requires an additional extracellular signal, namely chitin, the most abundant carbon source in the ocean. Genetic studies (131, 148, 244, 246) support that when chitin is present, (GlcNAc)₂ binds to the CBP (chitin-binding protein) activating the ChiS sensor kinase, which in turn leads to the production of the Hfq-dependent TfoR sRNA. TfoR promotes translation of the mRNA encoding an ortholog of *Haemophilus influenzae* regulator, Sxy (called TfoX in *Vibrio* species), which appears to promote transcription of competence genes in other bacteria by direct interactions with the cAMP receptor protein, CRP (148, 182) (Fig. 2.1). In *V. cholerae*, experimental induction of *tfoX*, such as from the *tac* promoter, is sufficient to promote transformation and activate expression of *comEA* and multiple chitinase genes (including *chiA-1*) independent of chitin (8, 147, 246). In addition to *comEA* and *chiA-1*,

competence pseudopilus genes (*pilA*, *pilB*, *pilQ*) are also under TfoX control (147) (Fig. 2.1). Thus, for *V. cholerae* both the chitin responsive pathway (i.e. TfoX) and QS (i.e. HapR) are required for sufficient *comEA* expression to promote uptake of DNA, as $\Delta tfoX$ and $\Delta comEA$ mutants, like a $\Delta hapR$ mutant, are severely impaired for transformation (147).

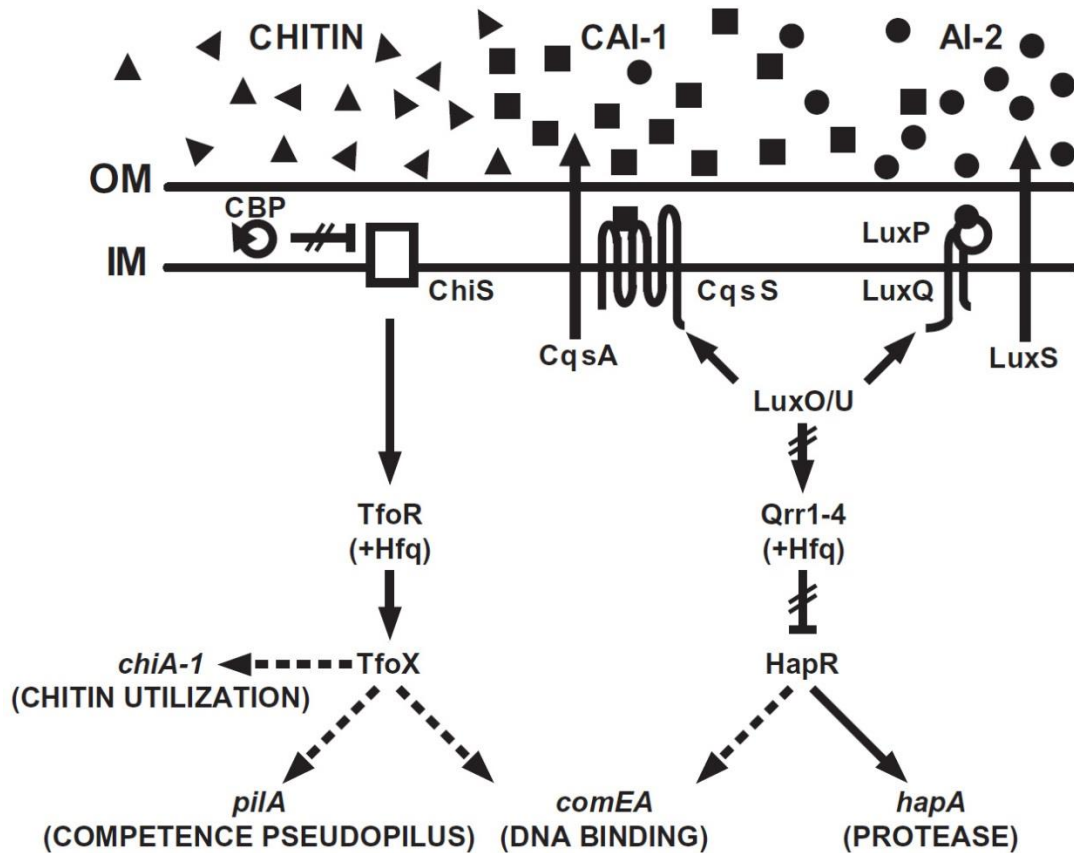


Figure 2.1. Current model for activation of TfoX- and HapR-controlled genes in response to chitin and quorum sensing signal molecules in *V. cholerae*. Chitin binding permits ChiS-dependent transcription of the TfoR sRNA that promotes TfoX translation. Quorum sensing AI accumulation at high cell density triggers dephosphorylation of LuxO (via LuxU), which prevents Qrr1-4 sRNA repression of *hapR* translation. TfoX regulates genes for chitin utilization (*chiA-1*), a competence pseudopilus (*pilA*), and DNA binding and uptake (*comEA*); while HapR positively regulates transcription of *hapA* (protease) and *comEA*. Refer to the text and (147, 164) for details of signal transduction pathways depicted. Dashed lines represent predicted network connections studied here.

DNA uptake by *V. cholerae* was demonstrated in 2005, and more recently in other marine *Vibrios*, such as *V. parahaemolyticus*, *V. fischeri* and *V. vulnificus* (52, 173, 206). Recent genome sequencing efforts, however, predict that not only the *Vibrionaceae*, but also the *Enterobacteriaceae*, encode orthologs of many DNA uptake genes, including *tfoX* and *comEA* (45). Despite the fact that many members of the *Enterobacteriaceae* are not naturally competent, it has been proposed that a common regulatory mechanism for natural competence involving both TfoX and CRP may be shared among these two families of the γ -proteobacteria (45, 182). Studies that bolster this hypothesis include demonstration that expression of natural transformation genes in *Escherichia coli* and *V. cholerae* is subjected to carbon catabolite repression (CCR) (30, 45, 147, 249). CRP, the global regulator of CCR, in Gram-negative bacteria, together with its allosteric effector cAMP, controls the expression of multiple genes involved in the utilization pathways of alternative carbon sources when glucose levels in the cell are low (39, 67). In Gram-positive bacteria, CCR also coordinates a response to low glucose levels but instead utilizes the CcpA transcription factor (231). Interestingly, a recent study demonstrating that CcpA in *Streptococcus* may induce natural competence as a consequence of CCR supports perhaps an even broader role for nutrient starvation in inducing DNA uptake (249).

The complex CRP-mediated response to nutrient stress in Gram-negative bacteria may also include participation by additional regulatory factors that enable the bacteria to utilize not only varied carbohydrates but also nucleic acids as well. In *E. coli*, for example, many proteins for scavenging of extracellular free nucleosides are encoded by genes in a regulon that is negatively controlled by the CytR regulator (225). The presence

of the nucleoside cytidine in the growth medium alleviates repression by CytR, which works in conjunction with CRP in *E. coli* as an ‘anti-activator’ of a subset of CRP-activated genes that are involved in nucleoside transport and metabolism. In *V. cholerae*, CytR has been shown to repress expression of the *udp* gene for nucleoside catabolism in nucleoside-poor environments, in addition to impairing biofilm development by unknown mechanisms (104). Moreover, several studies in *V. cholerae* demonstrated that CRP is involved in QS, biofilm formation, motility and cholera toxin production, as well as natural competence (30, 43, 80, 132, 133). While CRP may well participate in many steps of a regulatory network for DNA uptake, a molecular mechanism linking CRP to the control of natural competence genes has not been demonstrated in *V. cholerae*. Here we provide evidence that the CytR anti-activator, which requires CRP to function, coordinates a nutrient scavenging response in *V. cholerae* that controls natural competence.

2.3. Experimental procedures

2.3.1 Bacterial strains, plasmids, and culture conditions

The relevant genotypes of the *V. cholerae* strains and plasmids used in the study are listed in Table 2.1. *V. cholerae* strains were incubated at 37°C on Luria–Bertani (LB) agar, and in LB broth with shaking. AB minimal medium (57) modified to include 0.7 mM Na₂SO₄ (C.M. Waters, unpublished) was used for bioluminescence assays where noted. Artificial sea water (ASW; Instant Ocean) was used for chitin-induced natural transformation assays as described previously (8). Antibiotics (Fisher BioReagents) chloramphenicol (Cm), kanamycin (Kan), and streptomycin (Str) were used at concentrations of 10, 100, 5000 µg ml⁻¹ respectively. Expression of the *cytR* gene

encoded on p-*tac-cytR* was induced with 0.5 mM isopropyl-b-D-thiogalactopyranoside (IPTG; Fisher BioReagents). Where noted, *V. cholerae* cultures were supplemented with 100 mM cytidine or deoxycytidine (Sigma).

Table 2.1. *V. cholerae* strains and plasmids used in this study

Strains	Genotype or description	Reference
<i>V. cholerae</i> strains		
C6706str	El Tor biotype, O1; HapR ⁺	(217)
EA305	<i>tfoX</i> * (<i>tfoX</i> controlled by <i>tac</i> promoter)	This study
SLS349	$\Delta luxO$	(232)
EA281	$\Delta luxO$, <i>tfoX</i> *	This study
EA349	$\Delta luxO$, $\Delta lacZ::hapR$, <i>tfoX</i> *	This study
EA407	$\Delta luxO$, $\Delta lacZ::hapR$, <i>tfoX</i> *, <i>cytR::Tn5</i>	This study
BH1543	$\Delta hapR$	(8)
EA307	$\Delta hapR$, <i>tfoX</i> *	This study
EA408	$\Delta cytR$	This study
EA410	$\Delta cytR$, <i>tfoX</i> *	This study
EA415	$\Delta luxO$, $\Delta cytR$	This study
EA636	$\Delta luxO$, $\Delta cytR$, <i>tfoX</i> *	This study
EA517	$\Delta cytR$, $\Delta lacZ::cytR$, <i>tfoX</i> *	This study
EA605	<i>cytR-L161A</i>	This study
EA606	<i>cytR-L161A</i> , <i>tfoX</i> *	This study
EA680	<i>cytR-D273N</i>	This study
EA682	<i>cytR-D273N</i> , <i>tfoX</i> *	This study
EA577	$\Delta crp::Kan^R$	This study
EA601	$\Delta crp::Kan^R$, <i>tfoX</i> *	This study
MN171	$\Delta crp::Kan^R$, $\Delta cytR$	This study
MN173	$\Delta crp::Kan^R$, $\Delta cytR$, <i>tfoX</i> *	This study
EA090	$\Delta lacZ::Kan^R$	(8)
Plasmids	Features	Reference
pBBRlux	Cloning vector, Cm ^R	(128)
pEA209	<i>comEA-lux</i> , pBBRlux-based, Cm ^R	(8)
pEA493	<i>pilA-lux</i> , pBBRlux-based, Cm ^R	This study
pEA495	<i>chiA-1-lux</i> , pBBRlux-based, Cm ^R	This study
pEA603	<i>udp-lux</i> , pBBRlux-based, Cm ^R	This study
pBBRlux-hap	<i>hapA-lux</i> , pBBRlux-based, Cm ^R	(16)
pEA500	p- <i>tac-cytR</i> , pEVS143-based, Kan ^R	This study

2.3.2. DNA manipulations

Standard protocols were used for all DNA manipulations (187). Restriction enzymes, T4 DNA ligase (New England Biolabs) and Phusion DNA polymerase (Finnzymes) were used for cloning and PCR reactions. In-frame deletions, amino acid substitutions, and insertion mutants in *V. cholerae* were constructed by allelic exchange pKAS32-based plasmids (201). Genomic DNA marked with Kan^R gene at the *lacZ* locus (8) was extracted using a ZR Fungal/Bacterial DNA kitTM (Zymo Research) for chitin-induced natural transformation assays. Plasmids carrying the luciferase-based transcriptional reporters (*comEA-lux*, *pilA-lux*, *chiA-1-lux* and *udp-lux*) were constructed as previously described (8). Briefly, the promoter and a portion of the coding region of corresponding gene from WT *V. cholerae* was PCR-amplified with an upstream primer containing a SpeI site and a downstream primer containing a BamHI site, and then cloned into the SpeI/BamHI-digested pBBRlux vector (128). The IPTG-inducible *p-tac-cytR* plasmid was constructed by amplifying the entire coding region of *vc2677* and cloning it into the pEVS143 vector by insertion into the EcoRI and BamHI restriction sites.

2.3.3. Transposon mutagenesis of *V. cholerae*

The suicide delivery plasmid pRL27 encoding the Tn5 transposon conferring resistance to kanamycin (Kan^R) (125) was transferred by conjugation from *E. coli* S17λpir to the *V. cholerae* Δ*luxO*, *tfoX** recipient strain merodiploid for *hapR* (Table 2.1, EA349). Three independent pools of ~10⁵ Tn5 Kan^R *V. cholerae* mutants were conjugated with an *E. coli* S17λpir donor carrying the *comEA-lux* reporter plasmid to

create a library of transposon mutants. Kan^R transconjugant mutant colonies were arrayed to microtitre plates with a Genetix QPix2^{XT} colony picker followed by screening for candidates with defective *comEA-lux* expression using a BioTek multimode plate reader. The identity of candidate target genes found in the screen was determined by BLAST analysis to the *V. cholerae* C6706 genome of the DNA sequences adjacent to the Tn5 insertion, by standard methods described previously (101, 125).

2.3.4. Bioluminescence assay

Vibrio cholerae bioluminescence expression was assayed as described previously (151, 251). *V. cholerae* strains carrying a *lux*-based reporter plasmid were incubated in LB at 37°C overnight with appropriate antibiotics and IPTG where noted, and bioluminescence and absorbance were quantified thereafter. For bioluminescence measurements in the presence of cytidine, *V. cholerae* strains carrying a reporter plasmid were incubated overnight for 9–11 h at 37°C in AB minimal media containing appropriate antibiotics and supplemented with 100 mM cytidine. A chitin tile was added to each tube to provide a carbon source and for induction of *tfoX*. Bioluminescence was measured using a Wallac model 1409 liquid scintillation counter as described previously (100). The optical density of each culture was measured with a spectrophotometer. Relative Light Units (RLU) are defined as counts min⁻¹ml⁻¹/OD₆₀₀. Single-time-point experiments were performed with triplicate samples.

2.3.5. Chitin-induced natural transformation assay

Chitin-induced transformation experiments were performed as described previously (147). Two micrograms of *V. cholerae* genomic DNA marked with a Kan^R gene at the *lacZ* locus was used as an extracellular DNA source. For enumeration of transformants, cultures were plated onto LB medium containing Kan. Transformation frequency was defined as Kan^R cfu ml⁻¹/total cfu ml⁻¹. To measure transformation frequencies of *V. cholerae* strains in the presence of cytidine, ASW medium was supplemented with 100 mM cytidine or 100 mM deoxycytidine. Independent experiments were performed in triplicate.

2.4. Results

2.4.1. Identification of a competence-deficient *V. cholerae* mutant

Transcription of *comEA* depends on induction of TfoX in response to chitin, and on HapR, which is produced as a result of accumulated quorum sensing (QS) autoinducers (AIs) at HCD. *V. cholerae* strains that do not produce TfoX, or are unable to synthesize QS AIs, and thus produce no HapR, are severely impaired for *comEA* expression (8, 148, 210). To identify one or more positive regulators of the competence gene, *comEA*, in *V. cholerae*, we performed a transposon mutagenesis of El Tor *V. cholerae* strain C6706 using a well-described Tn5 system (125). We eliminated the requirement of chitin by first constructing a derivative of C6706 that expresses the *tfoX* gene from the chromosome under control of a constitutive *tac* promoter (referred to here as a *tfoX** allele). C6706 has a frameshift mutation in *lacI* and does not encode a functional LacI protein, thus *tfoX* is not LacI-repressed in this strain. Maximal expression

of a *comEA-lux* reporter that we constructed previously (8) was observed in the *tfoX** strain at HCD, when the QS response results in HapR production (Fig. 2.2A, bar 1). A deletion of *hapR* in this background results in a reduction in *comEA* expression by ~10,000-fold, as expected (Fig. 2.2A, bar 2). In contrast, a $\Delta luxO$, *tfoX** strain expresses *comEA* to levels similar to the *tfoX** strain at HCD, but does so independent of chitin and AI accumulation (data not shown). We further introduced a second copy of *hapR* at the *lacZ* locus into the $\Delta luxO$, *tfoX** strain to minimize screening of mutants with Tn5 insertion in *hapR*, or spontaneous *hapR* mutations that can occur in a $\Delta luxO$ strain (99). As expected, the merodiploid behaved in a manner indistinguishable from the isogenic $\Delta luxO$, *tfoX** strain, expressed *comEA* maximally (Fig. 2.2A, compare bar 1 and 3), and was used as the recipient for Tn5 transposon mutagenesis.

Three independent pools of ~20,000 Tn5 kanamycin^R (Kan^R) mutants were screened for defective *comEA-lux* expression, and one isolate was identified that expressed *comEA* as poorly as a $\Delta hapR$ mutant (Fig. 2.2A, bar 4). The location of the Tn5 insertion mapped to *vc2677*, which is annotated as a transcriptional repressor of the LacI family. In 2002, *vc2677* was shown to encode CytR, which represses biofilm formation in *V. cholerae* strain MO10 (104). Importantly, it was later identified by Meibom, *et al.* in a microarray study as one of ~100 TfoX-induced genes (147). Because it was predicted to participate in nucleoside metabolism and DNA uptake based on genomic comparison with other naturally competent bacteria (45), we sought to determine a role for CytR in natural competence of *V. cholerae*.

2.4.2. CytR positively regulates *comEA* expression and DNA uptake in *V. cholerae*

To confirm that the defect in *comEA* expression in the transposon-insertion mutant was indeed the result of *cytR* inactivation, we introduced an in-frame *cytR* gene deletion ($\Delta cytR$) into various genetic backgrounds of *V. cholerae* using standard methods (201), and measured expression of *comEA-lux* (Fig. 2.2A) and transformation frequency (Fig. 2.2B) of each strain. A $\Delta cytR$, *tfoX** mutant was 10,000-fold reduced for *comEA* expression compared with the WT strain carrying *tfoX** (Fig. 2.2A, compare bar 5 with bar 1), and similar to the *comEA* defect of the $\Delta hapR$, *tfoX** mutant (Fig. 2.2A, compare bar 5 with bar 2). Similarly, a $\Delta luxO$, *tfoX**, $\Delta cytR$ mutant was also deficient in *comEA* expression compared with the isogenic $\Delta luxO$, *tfoX** mutant and to the $\Delta luxO$, *lacZ::hapR*, *tfoX** mutant (data not shown, and Fig. 2.2A, compare bar 6 with bar 3). The $\Delta cytR$, *tfoX** strain was fully complemented either by inserting a copy of the *cytR* gene under control of its native promoter into the chromosome at the *lacZ* site (Fig. 2.2A, compare bar 7 with bar 1), or by introducing the same *cytR* gene on a plasmid (data not shown), as *comEA-lux* expression was restored to levels observed with the *tfoX** strain.

Transformation frequencies of corresponding *V. cholerae* strains that do not carry a *tfoX** allele, but rather require chitin for *tfoX* expression, were measured by a crab-shell microcosm system described previously (147). The results were consistent with *comEA-lux* expression (Fig. 2.2A and B). Specifically, no transformants were detected for the Tn5 mutant, $\Delta cytR$ mutant, and the $\Delta luxO$, $\Delta cytR$ double mutant (Fig. 2.2B, bars 5 and 6), similar to the $\Delta hapR$ strain. The DNA uptake level of the complemented strain was similar to WT, the $\Delta luxO$ mutant, and the $\Delta luxO$, *lacZ::hapR* mutant of *V. cholerae* (Fig.

2.2B) (8). These results confirm the role of CytR as a positive regulator of natural competence of *V. cholerae*.

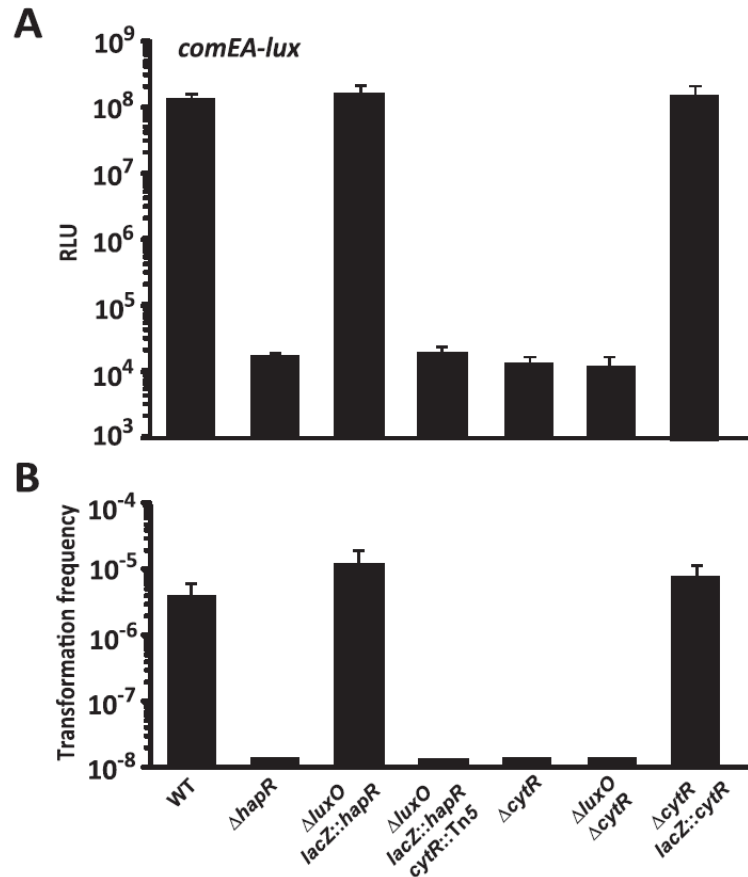


Figure 2.2. CytR regulates *comEA-lux* expression and DNA uptake in *V. cholerae*.

(A) Triplicate cultures of various *V. cholerae* strains expressing chromosomal *tfoX* gene under control of a constitutive *tac* promoter (*tfoX**) and carrying a plasmid-borne *comEA-lux* reporter were incubated overnight and analyzed for luciferase production. Bioluminescence is defined as relative light production per OD₆₀₀ (RLU). (B) Chitin-induced transformation frequency was calculated (see Material and methods) for each *V. cholerae* strain (carrying the native *tfoX* allele) incubated with extracellular DNA in triplicate wells containing crab shell tabs. The limit of detection is $<10^{-8}$. Data shown are mean values \pm standard deviation from one representative experiment of three performed.

2.4.3. CytR regulates expression of multiple TfoX-induced genes

Based on prior microarray studies, in addition to the *cytR* gene itself, three groups of genes have been shown to be under TfoX control in *V. cholerae*: DNA binding and processing genes (such as *comEA*), chitin degradation and utilization genes (such as *chiA-1*), and competence pseudopilin genes (such as *pilA*) (147). To test whether genes in each of these categories are also regulated by CytR, we constructed luciferase-based transcriptional fusions of representative genes in each group and quantified expression in Luria broth (LB medium) without chitin for *V. cholerae* WT (QS+, CytR+) and Δ *cytR* (QS+, CytR-) strains that carry the constitutive *tfoX** allele (TfoX+) or the native *tfoX* allele (TfoX-) that requires chitin for induction. Consistent with microarray studies, expression of all three genes, *comEA*, *chiA-1* and *pilA*, increased in WT *V. cholerae* in the presence of the *tfoX** allele (~115-, 55-, 15-fold respectively), which confirms that they are activated by TfoX (Fig. 2.3A–C, compare first two bars). However, expression of all three genes in a Δ *cytR* mutant was approximately as low as in the strains lacking constitutive TfoX, which suggested that not only *comEA*, but also *chiA-1*, and *pilA* are positively regulated by CytR (Fig. 2.3A–C, compare last two bars).

As a control, we also constructed a *lux*-based transcriptional fusion to the *V. cholerae* *udp* gene, encoding uridine phosphorylase, which is known to be repressed by CytR (104). As expected, the transcription pattern of *udp-lux* was reciprocal to the *comEA-lux* results (Fig. 2.3D). Namely, expression was low in the WT strain when *tfoX* was expressed (Fig. 2.3D, compare the first two bars). However, in a Δ *cytR* mutant, repression of *udp-lux* was eliminated (Fig. 2.3D, compare last two bars), consistent with studies that showed repression of the *udp* gene by CytR in *V. cholerae* (104, 252).

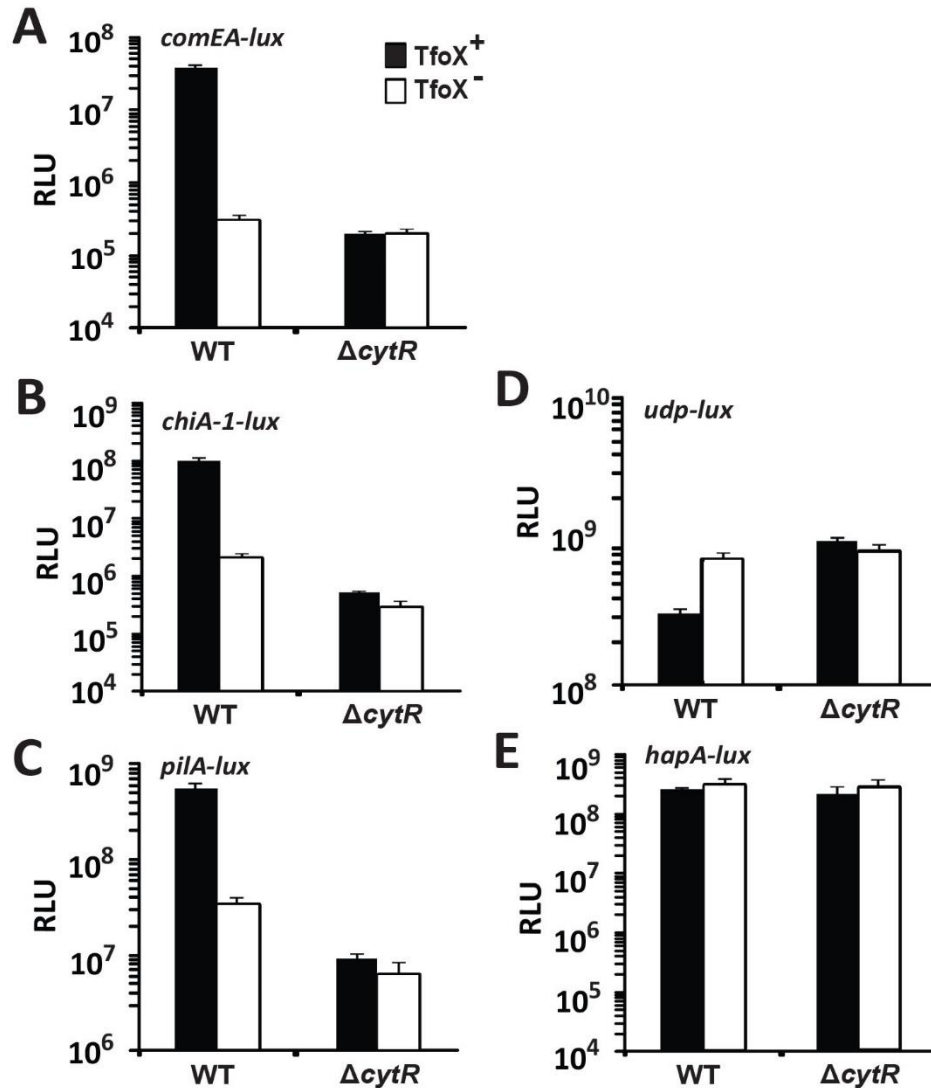


Figure 2.3. CytR positively regulates multiple genes for DNA uptake and chitin utilization. *V. cholerae* strains carrying chromosomally-encoded *tfoX* under control of a constitutive *tac* promoter (TfoX⁺, black bar) or under its native promoter (TfoX⁻, white bar) were analyzed for expression of bioluminescence from plasmid-borne *comEA-lux* (A), *chiA-1-lux* (B), *pilA-lux* (C), *udp-lux* (D), and *hapA-lux* (E) transcriptional fusions. Bioluminescence is defined as relative light production per OD₆₀₀ (RLU). Data shown are mean values ± standard deviation for the triplicate cultures from one representative experiment of three performed.

As an additional control, we measured the expression pattern of the *hapA* gene, which encodes a haemagglutinin protease that is positively regulated by HapR (16, 99),

but not predicted to be under control of either TfoX or CytR. The HapR-dependent expression pattern of *hapA-lux* was described prior (99). The maximal transcription of *hapA* was observed in both WT and Δ *cytR* mutants as predicted, independent of TfoX and CytR (Fig. 2.3E). These results confirm that regulation of *hapA* is CytR-independent. Taken together, these data indicate that CytR positively regulates genes controlling the natural competence and chitin utilization network in *V. cholerae*, and as it was shown previously, negatively regulates the *udp* gene involved in nucleoside metabolism.

2.4.4. *V. cholerae* CytR behaves like a CRP-dependent anti-activator

Extensive studies in *E. coli* have demonstrated that CytR associates with the global cAMP receptor protein, CRP, to inhibit transcription of a subset of promoters activated by CRP (for review see (225)). For example, the CRP-dependent promoter of *udp* in *E. coli* contains both a distal (CRP-2) and a proximal (CRP-1) binding site allowing activation of *udp* transcription by CRP in the absence of glucose when intracellular cAMP levels are high (38, 252). Specifically, binding of a CRP dimer at the two-fold symmetric CRP-2 site and at the CRP-1 site, which overlaps the -35 region, positions each CRP dimer to recruit RNA polymerase (RNAP) (Fig. 2.4A, top). The CRP consensus site in *E. coli* is depicted in Fig. 2.4B. Optimal spacing of 52–53bp between the CRP-2 and CRP-1 sites of several CRP-dependent promoters, including *udp*, ensures that a CytR dimer inhibits initiation of transcription of such genes by protein–protein interactions with each CRP dimer. Interaction of CytR with a highly degenerate operator site between the two CRP sites plays a minor role (41), and indeed overexpression of *E. coli* CytR that lacks a DNA-binding domain can still repress the *deoP* promoter, which is

also under CytR–CRP control (170, 203). Thus, CytR–CRP interactions are necessary for ‘anti-activation’ of genes in *E. coli*, including *udp*, which are involved in nucleoside scavenging in the absence of preferred carbon sources (Fig. 2.4A, bottom) (225). As a result, we sought to test whether a CRP-dependent CytR anti-activation mechanism may control natural competence in *V. cholerae*, and if such a mechanism was consistent with prior observations that a Δcrp mutant is defective in transformation and that the presence of glucose inhibits chitin-induced DNA uptake (30, 147).

To determine whether the CytR–CRP protein–protein interactions important for the *E. coli* nucleoside scavenging response play a role in *V. cholerae* natural competence, we first examined the degree of similarity between the regulatory proteins involved. It has been shown previously that *V. cholerae cytR* complements an *E. coli cytR* mutant, confirming that it is a functional homolog (104). Protein alignments indicated that *V. cholerae* CytR and CRP are 81% and 98% similar (65% and 95% identical), respectively, to their *E. coli* homologs (Fig. 2.4C). Importantly, alignment revealed that specific residues of *E. coli* CytR, notably residues L169 and F173 (corresponding to L161 and F165 in *V. cholerae*) that form a patch on the surface of CytR crucial for the CytR–CRP interactions in *E. coli* (118), are conserved in *V. cholerae* CytR (Fig. 2.4C). As expected, residues that are components of a corresponding patch on the surface of CRP are also conserved between these nearly identical proteins (not shown). Thus, to determine whether a CytR–CRP anti-activation mechanism is conserved and responsible for natural competence in *V. cholerae*, we measured expression of our *lux*-based gene fusions in a WT *V. cholerae* control strain expressing *tfoX** and compared each to expression levels in an isogenic strain with an in-frame *cytR* gene deletion ($\Delta cytR$), or with an L161A

amino acid substitution in CytR (*cytR*^{L161A}), which corresponds to the *E. coli* L169A CytR mutation that abolishes CytR-dependent anti-activation (118). Based on the results of the genetic screen with *comEA* (Fig. 2.2), the expression pattern of CytR-dependent reporters, *comEA*, *chiA-1* and *pilA* in the control strains was as expected. Namely, the maximal transcription of each gene was observed in the WT strain carrying the *tfoX** allele, with minimal expression of each gene fusion in an isogenic Δ *cytR*, *tfoX** strain (Figs 2.3 and 2.4D–F; compare bar 1 with bar 2). We reasoned that the *V. cholerae* *tfoX** strain expressing *cytR*^{L161A} would behave like a Δ *cytR* mutant, because a L161A amino acid substitution in CytR prevents CytR–CRP protein–protein interaction. As predicted, a *tfoX**, *cytR*^{L161A} mutant was severely impaired for expression of *comEA* and *chiA-1*, like the isogenic Δ *cytR* strain (Fig. 2.4D and E, compare bar 3 to bar 2). Expression of *pilA* was slightly higher (threefold) in the *tfoX**, *cytR*^{L161A} double mutant compared with the Δ *cytR* strain, but was still over 10-fold lower than the *tfoX** mutant that expresses *pilA* maximally (Fig. 2.4F; compare bar 3 with bars 2 and 1). We also measured expression levels of *comEA*, *chiA-1* and *pilA* in a *cytR*^{F165A}, *tfoX** strain and observed similar alterations in expression (data not shown), consistent with *E. coli* studies (118).

Previous studies predicted and also documented a role for carbon catabolite repression (CCR) in regulating transformation by *V. cholerae*, and a requirement of CRP for this process (30, 45, 147). We identified CytR as a critical regulator for transformation and expression of multiple competence genes in *V. cholerae* (Figs 2.2 and 2.3). The impact of the loss of CytR depends on CRP in *E. coli*, thus, to test whether CRP is also required for the observed effects of CytR in *V. cholerae*, we measured *comEA*, *chiA-1* and *pilA* expression in a *V. cholerae* *tfoX**, Δ *crp* mutant that constitutively

expressed *tfoX*, but carried an in-frame deletion in *crp*. It is important to note that the *V. cholerae* strain used does not encode a functional LacI, and the *tfoX** allele is controlled here by the *tac* promoter, which is also insensitive to catabolite repression. The Δcrp , *tfoX** mutant showed a level of expression intermediate between the WT (*tfoX**) and $\Delta cytR$, *tfoX** strains for all three gene fusions tested (Fig. 2.4D–F, bar 4).

To confirm that CytR function in *V. cholerae* requires CRP, we designed an epistasis experiment test to provide additional evidence that competence gene expression and DNA uptake require CytR–CRP interaction. Specifically, we predicted that the Δcrp mutation, which resulted in intermediate levels of competence gene expression would be epistatic to the $\Delta cytR$ mutation which produced minimal levels of expression for *comEA*, *pilA* and *chiA-1*. We constructed a $\Delta cytR$, Δcrp , *tfoX** mutant and measured expression of each of these gene fusions. Indeed, the $\Delta cytR$, Δcrp , *tfoX** mutant displayed intermediate levels of expression for each of the three gene fusions; like the Δcrp , *tfoX** mutant (Fig. 2.4D–F, compare bar 5 with bar 4), and unlike the minimum values observed in the $\Delta cytR$, *tfoX** mutant (Fig. 2.4D–F, compare bar 5 with bar 2).

While the genetic analysis supported a potential model that three TfoX-activated genes previously described (147) are positively controlled by CytR–CRP regulation, this regulation is likely indirect since CytR–CRP is known to anti-activate, or effectively represses, promoters under their direct control (see Discussion). In contrast, the *udp* gene in *V. cholerae* is directly controlled by CytR, like in *E. coli* (104, 252). Indeed, upstream of the *V. cholerae* *udp* gene is a well-conserved distal CRP-2 site, centered at -154, separated by 52 bp from a proximal CRP-1 binding site, centered at -102, relative to the ATG start codon (Fig. 2.4B) (252). Thus, we predicted that the expression pattern of a

udp-lux gene fusion would be the reciprocal to that observed for the competence genes in *V. cholerae*; namely, *udp-lux* expression would be higher in a *cytR*^{L161A} mutant, as it is in a Δ *cytR* mutant. As already shown in Fig. 2.3, *udp* expression was lowest in the *tfoX** strain, and maximal (although only approximately fivefold higher) in a *tfoX**, Δ *cytR* strain. As predicted, the *tfoX**, *cytR*^{L161A} mutant had a *udp* expression level similar to the Δ *cytR* mutant and still higher than the *tfoX** strain (Fig. 2.4G). Surprisingly, the Δ *crp* mutants did not appear to have an intermediate level of expression, perhaps due to the difficulty of resolving difference of less than threefold with *lux*-based gene fusions as described prior (98). While the fold differences in *udp-lux* expression observed here in *V. cholerae* were not identical to observations by Zolotukhina, *et al.* with a *lacZ* transcriptional fusion of *V. cholerae udp* measured in *E. coli* (252), the pattern in each case is consistent with negative regulation of *V. cholerae udp* by CytR.

Productive CytR–CRP interactions yield maximal expression of the three competence and chitin utilization genes tested (Fig. 2.4D–F), thus we predicted that DNA uptake would likely follow a similar pattern in transformation assays with corresponding *V. cholerae* strains that expressed *tfoX* in the presence of chitin, rather than in response to a constitutive *tfoX** allele. Indeed, transformation frequencies were consistent to the expression of CytR-dependent reporters (Fig. 2.4H). Compared with the WT strain that has a transformation frequency of $\sim 10^{-5}$, Δ *cytR* and *cytR*^{L169A} mutants were non-transformable; as were the Δ *crp* and Δ *cytR*, Δ *crp* mutants (Fig. 2.4H). These observations are consistent with previous studies (30, 147), and confirm the essential role of CytR–CRP interactions in natural competence of *V. cholerae*. Specifically, they suggest that productive protein–protein interaction between these two regulators ensures the

Figure 2.4. (continued) protein-protein interactions via L161 and F165 with each CRP dimer blocking CRP-dependent activation of transcription at the promoter via an “anti-activation” mechanism. Arrows at each CRP binding site highlight that the sequence is an inverted repeat (adapted from (41)). (B) The *E. coli* consensus binding site for one CRP dimer, and the predicted distal (CRP-2) and proximal (CRP-1) binding sites in the *udp* promoter region of *V. cholerae*. Arrows highlight the inverted repeat sequence, and underlined nucleotides indicate the critical nucleotides of the 5'-TGTGA(N₆)TCACA-3' consensus. (C) Alignment of the amino acid sequences of *V. cholerae* and *E. coli* CytR. Boxed amino acids indicate important residues for CytR-CRP interaction (L161 and F165) and for cytidine induction (D273) as described in text. (D-G) Expression of *comEA-lux* (D), *chiA-1-lux* (E), *pilA-lux* (F), and *udp-lux* (G) transcriptional fusions in *V. cholerae* strains carrying chromosomal *tfoX* under control of a constitutive *tac* promoter (*tfoX**). Bioluminescence is defined as relative light production per OD₆₀₀ (RLU). Data shown are mean values ± standard deviation for the triplicate cultures from one representative experiment of three performed. (H) Transformation frequency for *V. cholerae* mutants described in D-G.

2.4.5. CytR overexpression is not sufficient for maximal *comEA* expression

Previously, Meibom, *et al.* demonstrated that not only were *comEA*, *pilA* and *chiA-1* positively regulated by TfoX, but also CytR as well (147). Because we had identified *cytR* in a screen with a strain that expressed TfoX and HapR constitutively (Fig. 2.2), we sought to determine whether CytR may fit in a regulatory pathway downstream of TfoX, or possibly HapR. To test this we first constructed a *cytR-lux* fusion; however, we observed no change in expression when we compared levels in a WT strain to strains carrying a *tfoX** allele, or $\Delta tfoX$ and $\Delta hapR$ strains (data not shown). As an additional test to determine whether CytR may be under control of TfoX, we constructed a plasmid (p-*tac-cytR*) to control transcription of the *cytR* gene by the IPTG-inducible, *tac* promoter. We reasoned that if the only role of TfoX, when induced by chitin (mimicked by the *tfoX** allele), was to activate *cytR* transcription, then a strain carrying p-*tac-cytR* (+ IPTG) would express *comEA* to maximal levels sufficient for DNA uptake, even in a $\Delta tfoX$ strain. Alternatively, if CytR acts on competence gene

expression in a manner that is independent of TfoX, then *tfoX** would still be required for maximal *comEA* expression in strains overexpressing *cytR* from the *p-tac-cytR* plasmid. As Fig. 2.5 shows, in the absence of chitin, WT *V. cholerae* (with the native *tfoX* allele) carrying the *p-tac-cytR* plasmid expresses *comEA* minimally in the absence of IPTG, and reaches an intermediate level of *comEA* expression upon IPTG induction of *cytR* (Fig. 2.5, first set of bars). In a Δ *cytR* mutant, and a Δ *tfoX* mutant, a similar modest increase in *comEA* expression (10-fold) was also observed upon *cytR* expression by IPTG from *p-tac-cytR* (Fig. 2.5, second and third set of bars). However, the 100-fold increase in *comEA* expression was only achieved in strains carrying *tfoX** strain (Fig. 2.5, fourth and fifth sets of bars). Consistent with the epistasis test shown in Fig. 2.4, the Δ *cytR*, Δ *crp* and Δ *cytR*, Δ *crp*, *tfoX** strains express intermediate levels of *comEA* that are not altered by CytR overexpression (Fig. 2.5, sixth and seven sets of bars). These results suggest that *cytR* has a positive impact on competence gene expression in the absence of TfoX; however, an independent contribution by TfoX is required to achieve the levels of competence gene expression necessary for DNA uptake.

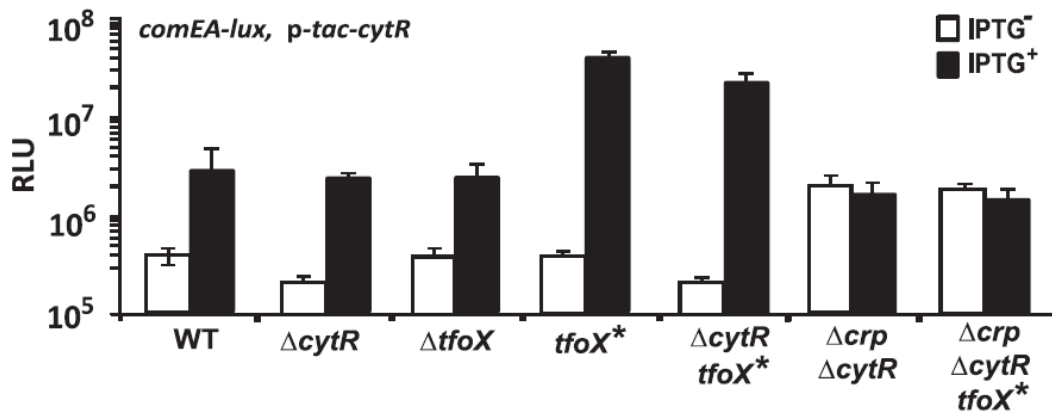


Figure 2.5. CytR overexpression is not sufficient for maximal *comEA* expression. Triplicate cultures of indicated *V. cholerae* strains expressing *cytR* gene under control of

Figure 2.5. (continued) an IPTG-inducible *tac* promoter (p-*tac-cytR*) and carrying a plasmid-borne *comEA-lux* reporter were incubated overnight without and with IPTG (white and black bars respectively) and analyzed for luciferase production. Bioluminescence is defined as relative light production per OD₆₀₀ (RLU). Data shown are mean values \pm standard deviation for the triplicate cultures from one representative experiment of three performed.

2.4.6. Cytidine is a repressor of natural competence

In *E. coli*, protein–protein interactions between CytR and CRP result in anti-activation of numerous CRP-activated metabolism genes, including *udp* and *cytR* itself (225). CytR is termed the Cytidine Repressor because the accumulation of cytidine is thought to induce conformational changes in CytR that weaken contact with CRP. Prior studies in *E. coli* have shown that cytidine relieves CytR-dependent anti-activation (repression) and permits CRP-dependent activation of nucleoside scavenging genes including *udp* in minimal media lacking glucose (13). Thus, we next tested the role of cytidine scavenging in CytR anti-activation of genes controlling natural transformation in *V. cholerae*. We measured levels of *comEA-lux* expression in *V. cholerae* strains incubated in AB minimal media that was supplemented with chitin to activate *tfoX* expression and to provide the bacteria with a carbon source. Under these conditions where induction of *tfoX* by chitin promotes competence, the presence of 100 mM cytidine reduced transcription of *comEA-lux* in WT *V. cholerae* >100-fold, to levels comparable to a Δ *cytR* mutant, which was unresponsive to cytidine addition (Fig. 2.6A).

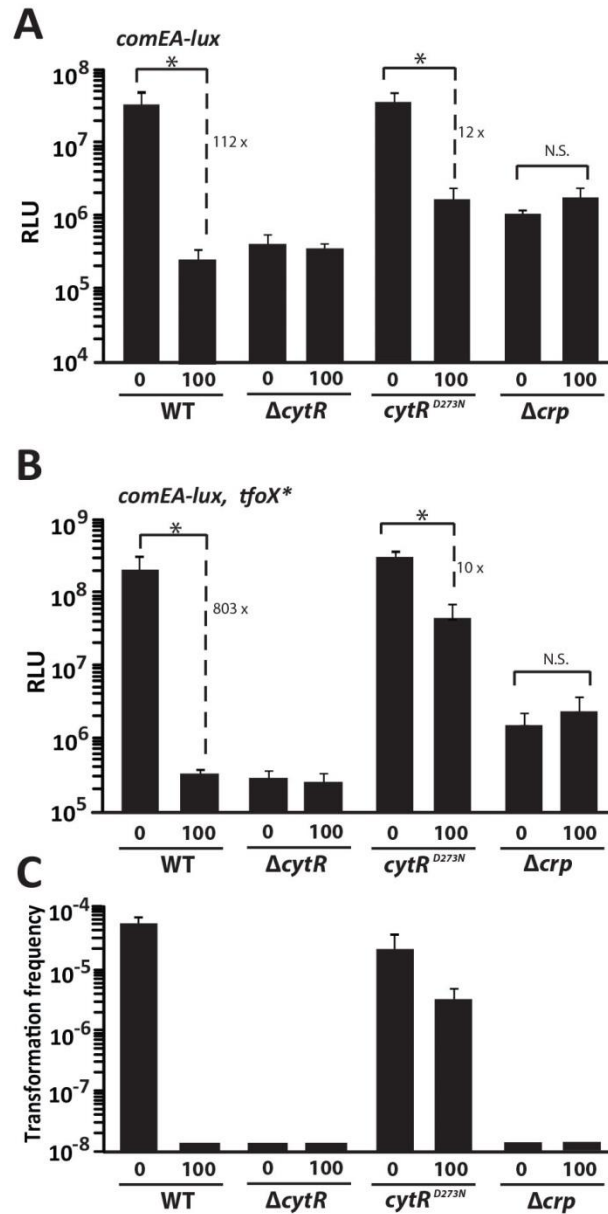


Figure 2.6. Scavenging of cytidine prevents CytR-dependent expression of *comEA-lux*. *V. cholerae* strains carrying *comEA-lux* on a plasmid were incubated overnight in AB minimal media containing a crab shell tile, and supplemented with 100 mM cytidine where indicated. **(A)** Induction of the native *tfoX* allele occurred in response to the chitin tile that also served as a carbon source. **(B)** Constitutive expression of the chromosomal *tfoX* gene controlled by the constitutive *tac* promoter uncoupled *tfoX* induction from the chitin tab provided. Bioluminescence is defined as relative light production per OD₆₀₀ (RLU). **(C)** Chitin-induced transformation frequency was calculated for each *V. cholerae* strain incubated with extracellular DNA and 100 mM cytidine where indicated in triplicate wells carrying crab shell tabs. The limit of detection is $< 10^{-8}$. Data shown are mean values \pm standard deviation from one representative experiment of three performed. * $P < 0.001$, N.S. $P > 0.05$ (*t*-test).

Escherichia coli CytR with amino acid substitution D281N (corresponding to D273N in *V. cholerae*) (Fig. 2.4C) binds cytidine with three orders of magnitude lower affinity than native CytR and this mutation severely curtails cytidine-mediated disruption of CytR–CRP interactions (13, 14). We constructed a *V. cholerae* strain that carries the corresponding *cytR*^{D273N} allele and compared *comEA* expression in this mutant to the isogenic WT control strain carrying the native *cytR* allele in the presence or absence of 100 mM cytidine. The expression levels of *comEA–lux* in the *cytR*^{D273N} mutant were similar to WT when incubated in AB minimal medium with chitin but lacking exogenous cytidine. However, unlike the WT strain that experienced a >100-fold reduction in *comEA* in response to cytidine, the *cytR*^{D273N} mutant showed only a slight (12-fold) reduction in *comEA–lux* expression (Fig. 2.6A), indicating that CytR mediates the cytidine response observed in this assay. Expression of the *comEA* reporter in a Δ *crp* mutant, which was already ~10-fold decreased relative to WT strain incubated without cytidine (as in Fig. 2.4D), remained unresponsive to cytidine (Fig. 2.6A), because the absence of CRP is epistatic to both CytR- and cytidine-mediated effects.

To uncouple the role of chitin as a nutrient source and inducer of *tfoX* in our assay, we performed a complementary experiment with strains that expressed the constitutive *tfoX** allele. The expression patterns in the presence of cytidine were similar, even under conditions where *tfoX* was no longer under control of its native promoter (Fig. 2.6B). The effect of cytidine on *comEA* expression in the WT strain carrying *tfoX** was enhanced, resulting in an ~800-fold reduction in the presence of cytidine. The remaining *tfoX** strains displayed a similar pattern as in Fig. 2.5A, with no effect of cytidine on the Δ *cytR* and Δ *crp* mutant, and a minor (10-fold) reduction in the *cytR*^{D273N} mutant.

Finally, to test whether the presence of exogenous cytidine not only halted *comEA* expression in a CytR-dependent manner, but also prevented DNA uptake, we measured transformation efficiency of corresponding strains carrying the native *tfoX* allele but incubated on chitin. Unlike the WT strain that is highly transformable, the WT strain incubated on chitin with 100 mM cytidine showed an ~10,000-fold defect in transformation, with no DNA uptake recorded, similar to the ΔcytR mutant (Fig. 2.6C), which was not transformable without and with 100 mM cytidine. In contrast, transformation frequency of the *cytR*^{D273N} mutant was similar to WT grown without cytidine, and only slightly decreased (10-fold) in the presence of cytidine (Fig. 2.6C). The Δcrp mutant that expressed intermediate *comEA* levels that were unresponsive to cytidine addition, was not transformable as expected (Fig. 2.6C). Deoxycytidine also severely impaired CytR-dependent transformation in a manner similar to cytidine (Fig. 2.7), suggesting that DNA uptake in *V. cholerae* is responsive to the presence of extracellular nucleosides and deoxynucleosides. Thus, a cytidine-responsive CytR-dependent nucleoside scavenging mechanism, described in *E. coli* (14), appears to be a critical component of a regulatory network controlling natural competence in *V. cholerae*.

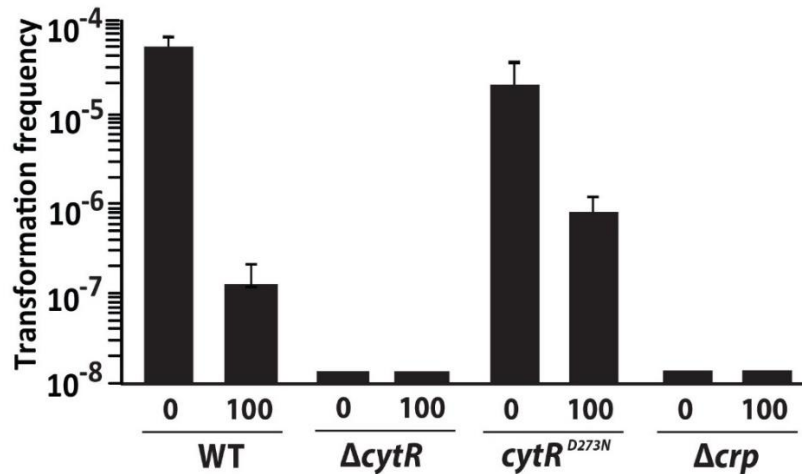


Figure 2.7. Scavenging of deoxycytidine impairs CytR-dependent natural transformation. Chitin-induced transformation frequency was calculated for each *V. cholerae* strain with extracellular DNA and 100mM of 2'-deoxycytidine where indicated in triplicate wells carrying crab shell tabs. The limit of detection is $<10^{-8}$.

2.5. Discussion

Why bacteria become naturally competent to take up DNA is a matter of controversy that has persisted since the pioneering studies of the 'transforming principle' by Griffith and later Avery, McLeod, and McCarthy who established *Streptococcus pneumoniae* as a model organism for studying natural competence for DNA uptake (10, 89). Numerous studies support the hypotheses that natural competence evolved in bacteria to aid in three major processes: DNA repair, HGT, and nutrition (for reviews see (70, 181, 204)). However, it is acknowledged that DNA taken up by bacteria may not be used exclusively for one function or another, since extracellular DNA scavenged as nutrients may also be available for recombination onto the chromosome when of sufficient sequence identity (70). Indeed, the nutrition hypothesis has been viewed with particular skepticism as the sole explanation for maintenance of competence systems in bacteria because *Neisseria gonorrhoeae* and *H. influenzae* exclude DNA that is not

species-specific, and many bacteria including *B. subtilis* can secrete exoenzymes to degrade extracellular DNA and then utilize nucleoside scavenging transporters for acquisition of the extracellular bases (59, 60, 70, 158). Our results presented here begin to define components of a nucleoside scavenging system in *V. cholerae* that, along with chitin and quorum sensing signaling, alters expression of transformation gene expression. Indeed, features of this emerging network suggest that regulation of *V. cholerae* natural competence has characteristics of pathways described below for both Gram-negative and Gram-positive bacteria.

Despite the unique features and regulatory components of the chitin- and QS-induced natural competence system described in *Vibrios*, the general architecture of this regulatory network shares some features with other naturally competent bacteria. In particular, Gram-positive *S. pneumoniae* and *B. subtilis* require a peptide-based QS system to regulate a phosphorylation cascade, which induces a regulator (sigma factor) that controls genes for the uptake of DNA without sequence preference (60). In contrast, Gram-negative *N. gonorrhoeae* and *H. influenzae* only take up DNA carrying species-specific uptake sequences; yet do not appear to use QS to mediate this process (50). Interestingly, it has been proposed (70, 218) that *S. pneumoniae* and *B. subtilis* regulate competence in response to species specific QS AIs to limit competence induction to HCD conditions that may favor acquisition of ‘self’ and not ‘foreign’ DNA. It is believed that *N. gonorrhoeae* and *H. influenzae*, in contrast, utilize a sequence-based mechanism rather than QS to ensure ‘sexual isolation’. However, such a model is insufficient to explain the *V. cholerae* competence network elucidated here. First, unlike the other Gram-negative bacteria that regulate competence without QS system input, *Vibrios* appear to be notable

exceptions to this generalization. Second, *V. cholerae* lacks uptake sequences and instead appears to take up DNA broadly (210), in contrast to the archetypal Gram-negative *N. gonorrhoeae* and *H. influenzae*. Finally, the competence network in *Vibrios* shares additional features with Gram-positive systems by not only utilizing QS signaling but also a regulatory circuit for monitoring nutrient stress. A complex regulatory circuitry in *B. subtilis* coordinates competence and sporulation in response to nutrient cues (90). So too, the CcpA regulator in *Streptococcus gordonii*, which orchestrates a CCR similar to that in Gram-negative bacteria, controls both biofilm formation and natural competence as a response to nutrient deprivation (249).

Vibrios commonly form biofilms on biotic chitinous surfaces, such as chironomids and zooplankton moults (93, 215) producing chitinases that allow exploitation of this abundant GlcNAc polymer in an otherwise nutrient-poor aquatic biosphere (131). Initial studies demonstrating that chitin also induced natural competence noted that the presence of glucose suppressed DNA uptake, which prompted the suggestion that competence in *V. cholerae* was under control of carbon catabolite repression (CCR) (147). Indeed, Blokesch recently confirmed a role for CRP in DNA uptake by *V. cholerae*, although a specific mechanism for the role of CRP in competence was not validated (30).

Cameron and Redfield have proposed a model for γ -proteobacteria (including *E. coli*, *V. cholerae* and *H. influenzae*) that transcription from the promoters of *comEA* and *pilABCD* may be under Sxy (TfoX) control (45). Specifically, *H. influenzae* Sxy (TfoX) is proposed to direct CRP to interact with a competence regulatory element (CRE) sequence (TGCGA-N₆-TCGCA) in the *comE1* (*comEA*) and *pilA* promoters, although a

precise mechanism has not been revealed to fully explain how CRP and/or Sxy engage at the CRE site (which is remarkably similar to the CRP binding site: TGTGA-N₆-TCACA) (182). Inspection of the promoter region of *V. cholerae comEA* that is included in our reporter fusion indicates one potential CRE site (TGCGA-N₆-AAGCA); and the *pilA* promoter contains a potential CRP binding site or a CRE (TGAGA-N₆-TCAAA), but is not in our reporter fusion (data not shown). Thus it is possible that in *V. cholerae*, as in *H. influenzae*, CRP (via TfoX) directly promotes transcription of competence genes like *comEA*. In addition, our results here also support a role for CRP (via CytR) in indirectly regulating a similar class of genes as described below. The independent contribution of TfoX and CytR could explain why *tfoX* induction (*tfoX**) was required for maximal *comEA* expression when *cytR* was overexpressed (Fig. 2.5). Although naturally competent *H. influenzae* does not encode a CytR homolog, our data linking CytR to competence in *V. cholerae* may be useful in discovering why *Enterobacteriaceae* such as *E. coli*, *Shigella* and *Yersinia* species that encode homologs of both TfoX and CytR (182) are not naturally transformable.

In *E. coli*, which is not naturally competent, the CytR regulator has been extensively studied for its role in anti-activation of a set of CRP-dependent nucleoside scavenging genes (including *cytR*, *udp*, *deoP*, *nupG*, *cdd*, *tsx*, *cytX-rot*) that are anti-activated by CytR (for review see (225)). However, BLASTP analysis suggests that CytR–CRP regulated promoters in *V. cholerae* and *E. coli* may be different (data not shown). In *E. coli*, CytR-controlled promoters contain two CRP binding sites, with the exception of *cytR* itself, which is autorepressed and yet contains a single CRP site (169). This is likely the case for *V. cholerae cytR* as well, which appears to contain a single CRP

binding site in its promoter region (data not shown). So, while we predict that the *V. cholerae* CytR regulon includes *cytR* and *udp* (Figs 2.3 and 2.4; (104, 252)), the apparent *V. cholerae* *deoP* homolog, *deoC*, lacks two CRP sites in its promoter, and *V. cholerae* does not encode an obvious *nupG* homolog (data not shown). As a result, we are currently defining the CytR regulon by experimental and computational methods to further define whether CytR has direct or indirect effects on competence gene expression.

Our results presented here are consistent with a model that CytR–CRP interactions have a positive effect on competence gene expression and DNA uptake. However, it is likely that one or more intermediate steps exist between CytR–CRP and the competence genes described here. Only the *udp* promoter contains two CRP binding sites for direct anti-activation, while the promoter regions of *comEA*, *chiA-1* and *pilA* do not have two CRP binding sites (data not shown). While it remains possible that *V. cholerae* CytR may not act identically to its *E. coli* counterpart as an anti-activator, we favor a speculative model supported by our data that CytR and CRP, via protein–protein interactions, interact with the promoter of a putative factor X, which in turn represses *comEA*, *chiA-1* and *pilA* (Fig. 2.8). Given such a mechanism, WT *V. cholerae* would result in maximal competence gene expression, a $\Delta cytR$ mutant would maximally repress competence, and a $\Delta cytR$, Δcrp double mutant unable to activate X could result in intermediate *comEA* levels (Fig. 2.8). However, since CRP is a pleiotropic regulator, and a *V. cholerae* Δcrp mutant displays a growth defect (data not shown) (30, 201), it is also possible that the intermediate expression observed here for Δcrp mutants may be complex and result from changes in a CytR response, as well as consequence due to growth alterations or additional role(s) that CRP may play in directly regulating one or

more competence genes. Nonetheless, our epistasis results support that the CytR-mediated effects observed here do not occur in the absence of CRP, consistent with our model that CytR function in *V. cholerae* requires CRP.

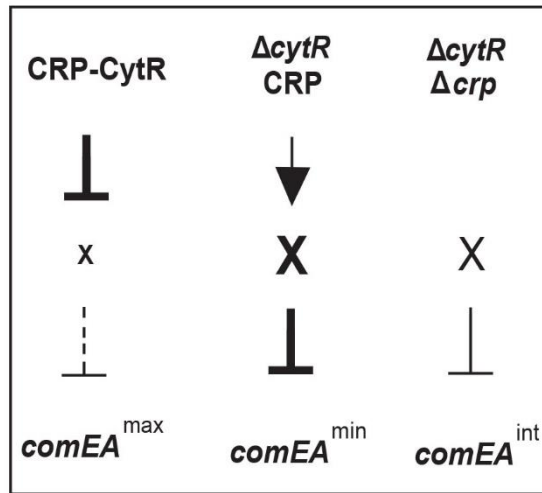


Figure 2.8. A model for the role of a putative repressor X in CytR-dependent anti-activation of the competence gene, *comEA*, in *V. cholerae*. **Left:** CytR–CRP anti-activation of the X repressor results in maximal expression of *comEA*. **Middle:** In a ΔcytR mutant, CRP activation of X results in minimal *comEA* expression. **Right:** In a ΔcytR , Δcrp mutant, the lack of CRP activation of X results in intermediate basal *comEA* expression levels.

Haugo and Watnick demonstrated that CytR represses biofilm formation in *V. cholerae* strain MO10, although a direct mechanism linking CytR to biofilm genes was not revealed (104). Recently, Garavaglia, *et al.* have also demonstrated CytR repression of *E. coli* biofilms, as a ΔcytR mutant displayed reduced expression of the *csgDEFG* operon, which controls assembly and transport of curli fibers that promote aggregation (86). Modulation of intracellular pyrimidine concentrations appears responsible for the changes in curli expression leading the authors to propose that biofilm gene expression is an indirect consequence of CytR control of nucleoside pools in the cell. It remains

possible that CytR functions in a similar indirect manner to control expression of the competence genes in *V. cholerae* and experiments are underway to test this.

In *V. cholerae* A1552, *cytR* was identified one of ~100 genes positively regulated at least 2.5-fold by *tfoX* induction (147); however, we did not observe an increase in *cytR-lux* expression in *V. cholerae* strain C6706 under similar conditions (data not shown). So too, we showed that maximal *comEA* expression required *tfoX* activation (*tfoX**), as *cytR* overexpression was not sufficient to bypass the need for TfoX (Fig. 2.5). It is possible that TfoX controls transcription of *comEA*, and other competent genes via direct interactions (at CRE sites as indicated above), while CytR plays an additional independent role. Determining the identity of a putative factor X that may be anti-activated by CytR in *V. cholerae* could indeed provide insight into how CytR–CRP mediates its effects on competence. Complementary biochemical, bioinformatics, and genomic methods are being developed to identify the set of CytR-regulated targets.

The manner in which HapR influences *V. cholerae* competence genes is likely to be complex. Rather than direct interaction of HapR with each promoter (or a unique upstream regulator for each gene), it is likely that HapR may also impinge on this network by directly controlling a single factor that in turn regulates multiple competence genes. Induction of *cytR* by HapR was not observed in prior studies or in this current study (data not shown) (147). In accordance with this, a strain carrying a constitutive *tfoX** allele still requires *hapR* for *comEA* expression and DNA uptake (Fig. 2.2A and B); thus like CytR, HapR likely regulates competence in a manner that is TfoX-independent. QS in *V. cholerae* C6706 controls many genes including several that alter levels of the intracellular second messenger molecule, cyclic dimeric GMP (c-di-GMP), which acts on

many targets in the cell (98, 232). HapR-mediated effects on competence may also be an indirect consequence of alterations in this pool of di-nucleotides in response to HCD conditions. Interplay between c-di-GMP and levels of intracellular and extracellular nucleic acids would suggest complex metabolic changes while *V. cholerae* is in the naturally competent state.

A similar QS system to that described in *V. cholerae* C6706 is used by many members of the *Vibrio* genus (164); and chitin-induced DNA uptake has been demonstrated for several *Vibrios* including *V. parahaemolyticus*, *V. fischeri* and *V. vulnificus* (52, 91, 173). Counterparts to the competence genes and *cytR* are also present in other *Vibrios* (data not shown) (18); however, a more complete understanding of the network connections between these systems in *Vibrios* is obviously required. Curiously, *Vibrio harveyi* does not appear to be naturally competent under the assay conditions described here for *V. cholerae* (8), but has been proposed to use CytR to control a regulon important for pathogenesis of marine hosts (180). Naturally competent *V. fischeri* encodes a CytR protein with an amino acid substitution at a position corresponding to F165 in *V. cholerae* (data not shown), suggesting that additional contacts may mediate CytR–CRP interaction in some *Vibrios*.

As we have proposed previously, the reliance of *V. cholerae* on a genus-wide QS system to control DNA uptake may be a contributing factor sculpting the genome of *V. cholerae*, which has undergone rampant HGT (8, 56). As nucleotide scavenging, via CytR–CRP, appears to halt DNA uptake, as shown here, it may be that the evolutionary role of competence in the *Vibrios* includes HGT as well as nutrient acquisition. Determining whether extracellular DNA and nucleosides can support growth of *V.*

cholerae, as shown for *E. coli* (77), is an important next step. Uncovering the complex network connections linking TfoX, HapR, and CytR to competence will likely contribute to our knowledge of signaling in other naturally competent *Vibrios*, as well as elucidate an expanding role for CytR-based regulation. Defining the CytR role in *Vibrios* may also be applied to understanding *Enterobacteria*, like *E. coli*, that encode CytR and many other competence genes and appear to have genetic differences that limit DNA uptake (182, 200).

2.6. Acknowledgements

We thank B. Duke and M. Nellesson for assistance with luciferase assays. V. Rajan and M. Borodovsky provided support for genome analysis. This study was funded by National Science Foundation grants (MCB-0919821 and MCB-MCB-1149925) to B.K.H.

CHAPTER 3

DIVERSITY OF CLINICAL AND ENVIRONMENTAL ISOLATES OF *VIBRIO CHOLERAE* IN NATURAL TRANSFORMATION AND CONTACT-DEPENDENT BACTERIAL KILLING INDICATIVE OF TYPE VI SECRETION SYSTEM ACTIVITY

Reproduced in part with permission from Bernardy E.E., Turnsek M.A., Wilson S.K., Tarr C.L., and Hammer B.K. Diversity of clinical and environmental isolates of *Vibrio cholerae* in natural transformation and contact-dependent bacterial killing indicative of type VI secretion system activity. *Appl Environ Microbiol* 82:2933-2842. Copyright 2016, American Society for Microbiology.

3.1. Abstract

The bacterial pathogen *Vibrio cholerae* can occupy both the human gut and aquatic reservoirs, where it may colonize chitinous surfaces that induce the expression of factors for three phenotypes: chitin utilization, DNA uptake by natural transformation, and contact-dependent bacterial killing via a type VI secretion system (T6SS). In this study, we surveyed a diverse set of 53 isolates from different geographic locales collected over the past century from human clinical and environmental specimens for each phenotype outlined above. The set included pandemic isolates of serogroup O1, as well as several serogroup O139 and non-O1/non-O139 strains. We found that while chitin utilization was common, only 22.6% of the isolates tested were proficient at chitin-induced natural transformation, suggesting that transformation is expendable. Constitutive contact-dependent killing of *Escherichia coli* prey, which is indicative of a functional T6SS, was rare among clinical isolates (only 4 of 29) but common among environmental isolates (22 of 24). These results bolster the pathoadaptive model in which tight regulation of T6SS-mediated bacterial killing is beneficial in a human host, whereas constitutive killing by environmental isolates may give a competitive advantage in

natural settings. Future sequence analysis of this set of diverse isolates may identify previously unknown regulators and structural components for both natural transformation and T6SS.

3.2. Introduction

Vibrio cholerae, the bacterium responsible for the diarrheal disease cholera, can occupy a range of freshwater and marine environments, where it commonly associates with abiotic chitinous material and the biotic surfaces of algae, invertebrates, plants, and fish (213). When water carrying *V. cholerae* is ingested, cells that survive passage through the acidic stomach may gain access to the small intestine and bind to its mucus layer. Isolates carrying the CTX prophage can secrete cholera toxin (CT), which is responsible for the potentially fatal diarrhea that also aids in transmission from the host.

Over 200 O serogroups have been described, with each defined as a group of bacteria that share a surface antigen. Although only the O1 and O139 serogroups carrying the CTX prophage are responsible for major cholera epidemics, other serogroups may be associated with isolated cases of gastroenteritis, but so far have not been shown to spread globally (71). The pandemic O1 CTX⁺ isolates are further divided into two biotypes, Classical and El Tor, on the basis of several biochemical and phage susceptibility tests (17, 78, 103, 160). Seven cholera pandemics have been described. The O1 Classical biotype was responsible for the sixth and likely prior pandemics, but was displaced by the O1 El Tor biotype in the seventh pandemic which began in Southeast Asia in 1961 (75). In 1992, an El Tor mutant, serotype O139, became responsible for some regional cholera outbreaks, and continues to coexist with O1 El Tor, although in a minor capacity (76, 116, 179).

When inhabiting aquatic environments, *V. cholerae* can degrade the chitinous surfaces of copepods, zooplankton, and crabs to soluble (GlcNAc)_n oligosaccharides that can be imported and utilized as a carbon source (148). Liberated chitin oligosaccharides (GlcNAc₂₋₆) may also act as an extracellular signal recognized by membrane bound receptors that triggers a signaling cascade for the expression of genes encoding a DNA uptake apparatus for natural transformation (62, 131, 147, 244-246). Transformation is one mode of horizontal gene transfer that can promote rapid gene exchange, allowing bacteria to quickly adapt to their ever-changing environment, but this DNA can also be used for repair and nutrition (211). Although successful natural transformation has been studied extensively in a small number of *V. cholerae* reference strains, little is known regarding the broader prevalence of transformation ability among members of the species. In fact, contemporary *V. cholerae* isolates from the recent Haiti outbreak were impaired for natural transformation (63, 119), highlighting the need for a more comprehensive understanding of the transformation proficiency of *V. cholerae*.

It was recently discovered that growth on chitin also induces the expression of a type VI secretion system (T6SS) in *V. cholerae* (34, 235). The type VI apparatus, which is structurally analogous to a phage tail spike, can penetrate adjacent cells and deliver toxic effectors that cause contact-dependent lysis (107). These toxic effectors can be used to target either prokaryotic or eukaryotic prey cells (223). Liberated DNA from lysed prey cells may then serve as the genetic material for natural transformation (34) or as an alternative nutrient source. T6SS was originally discovered in 2006 in a non-O1/non-O139 environmental isolate of *V. cholerae*, V52, in an attempt to understand virulence mechanisms in non-pandemic CTX⁻ strains that cause isolated cases of gastroenteritis

(178). Subsequently, genomic analyses of sequenced genomes of *V. cholerae* have identified three major T6SS gene clusters that encode one T6SS (37, 155, 178). It has been proposed that contact-dependent T6SS-mediated killing ability provides a competitive advantage allowing *V. cholerae* to persist in both the human gut and the environment. However, despite extensive analyses documenting the presence of T6SS genes in all sequenced *V. cholerae* genomes (37, 154), a broader survey of contact-dependent killing has not been performed.

In *V. cholerae* clinical O1 El Tor isolates C6706 and A1552, genes involved in chitin utilization, natural transformation, and the T6SS are under the control of several positive regulatory factors. TfoX, induced by growth on chitin (147), and the transcription factor CytR, controlled by nucleoside starvation, act as positive regulators of all three phenotypes in *V. cholerae* C6706 (235). Quorum sensing (QS) also controls natural transformation and type VI secretion (T6S) in *V. cholerae* by upregulating the expression of transcription factor HapR in response to the accumulation of secreted autoinducer signals at high cell density (8, 34, 147, 209). HapR directly activates the transcription of the gene encoding the transcriptional regulator QstR (137). Expression of TfoX and QstR by chitin at high cell density or from a heterologous promoter is sufficient to induce transformation and T6SS-mediated killing (34, 137, 235). Although DNA uptake and T6SS are both controlled in A1552 and C6706 by the presence of chitin, suggesting coupling of these phenotypes, it is not known whether these activities are also coordinately regulated in other members of the species.

In this study, we analyzed a set of 53 patient-derived and non-patient-derived *V. cholerae* isolates collected between 1910 and 2011. Each isolate was characterized for

chitinase activity, natural transformation, and constitutive contact-dependent killing of *Escherichia coli* consistent with a functional T6SS. We designated patient-derived isolates “clinical” and non-patient-derived isolates “environmental”, which are represented by the letters C and E, respectively, in Table 3.3. These isolates include serogroups O1 (both Classical and El Tor biotypes), O139, and non-O1/non-O139, which were further differentiated by CTX⁺ or CTX⁻ status. The majority of the isolates we tested possessed chitinase activity, while >50% were deficient in chitin-induced natural transformation. Clinical isolates were largely unable to engage in constitutive contact-dependent killing of *E. coli* prey cells in a standard killing assay, but nearly all of the environmental isolates tested displayed constitutive bacterial killing. The diversity of the set of isolates tested in transformation proficiency and bacterial killing suggests that transformation may be dispensable in various settings. In contrast, constitutive contact-dependent antagonism, like that mediated by a T6SS, appears to be valuable in environmental habitats distinct from a human host.

3.3. Materials and Methods

3.3.1. Bacterial strains and growth conditions

The bacterial strains used in this study were obtained from various laboratories (see Table 3.1. for detailed descriptions). Isolates were previously characterized in the respective laboratories to determine serogroup and the presence or absence of the CT gene (*ctxA*). The methods used at the Centers for Disease Control and Prevention (CDC) were summarized by Talkington, *et al* (115, 119, 214). The *V. cholerae* clinical and environmental isolates were collected from a variety of locations between 1910 and 2011.

Chloramphenicol resistant (Cm^R) *E. coli* MG1655 was used as the prey in bacterial killing assays. All strains were grown in LB liquid medium or on LB agar at 37°C with the appropriate antibiotics added at the following concentrations when needed: kanamycin (kan) at 50 µg/ml and chloramphenicol (Cm) at 10 µg/ml for *V. cholerae* and 25 µg/ml for *E. coli*. Specific assay conditions are described below.

Table 3.1. List of strains used in this study and the lab from which they were acquired.

Strains used in this study	Source
C6706	Lab stocks
C6706 TfoX*	
C6706 CytR ⁻	
C6706 TfoX* HapR* QstR* CytR ⁺ (C6706 maximum killer)	
C6706 TfoX* HapR* QstR* CytR ⁺ $\Delta vasK$ (C6706 T6SS ⁻)	
*denotes constitutive expression of corresponding gene	
2010EL-1749, 2012V-1001, 3582-05, 3566-08, 3541-04, 3558-04, 2011V-1043, 3500-05, 3546-06, 2011EL-1141, 3554-08, 2009V-1085, 2009V-1096, 2011EL-1137, 2009V-1046, 2011EL-1938, Nepal 25, 2010EL-1786, Nepal 14, 2010EL-1941, 2010V-1014, 2011EL-1939, 2633-78, 2631-78, E8498, 2479-86, 3223-74, 3272-78, 1074-78, 2559-78, 2512-86, 3225-74, 692-79, 1496-86, 2497-86, 2523-87	C. Tarr and C. Bopp, Centers for Disease Control and Prevention
NCTC8457, MZO-2, MAK757, CA401, O395, MO10, 857, 2740-80	J. Zhu, University of Pennsylvania
VC56, VC22, VC53, VC48	A. DePaulo, Food and Drug Administration
SIO, TP	D. Bartlett, Scripps Institution of Oceanography
HE46	R. Colwell, University of Maryland

3.3.2. Construction of a plasmid for heterologous *qstR* and *tfoX* expression

A previously constructed plasmid containing a kanamycin resistance gene and the *tfoX* gene under the control of the *ptac* promoter (8) was digested at the BamHI restriction enzyme site located 5' of the *tfoX* gene. A fragment that carries the *qstR* gene, including its native ribosome binding site was amplified from the C6706 chromosome with primers pQT_1 and pQT_2 (Table 3.2). Gibson assembly (New England BioLabs) was used to introduce this PCR product into the BamHI-digested plasmid, so that both *tfoX* and *qstR* were under the control of the same isopropyl- β -d-thiogalactopyranoside (IPTG)-inducible *ptac* promoter. The resulting plasmid, designated pQT, was confirmed for the *qstR* insertion by sequencing with primers pQT_3 and pQT_4 (Table 3.2). *Escherichia coli* S17- λ pir cells transformed by electroporation with pQT were used to introduce the plasmid into *V. cholerae* strains of interest by conjugation.

3.3.3. Construction of suicide vector used in allelic exchange to delete *vasK* in isolate 692-79

The suicide vector, pRE118, containing an R6K origin of replication, a kanamycin resistance gene, and the *sacB* gene conferring sucrose sensitivity, was used for allelic exchange (72). Plasmid pRE118 was digested at the KpnI and XhoI restriction enzyme sites. The genome sequence of 692-79 was used to design primers to PCR amplify 500 bp upstream (*vasK*_1 and *vasK*_2) and downstream (*vasK*_3 and *vasK*_4) of the *vasK* gene (Table 3.2). Gibson assembly (New England BioLabs) was used to combine these two PCR products and the digested plasmid. The resulting plasmid was verified via sequencing with flanking *vasK*_1 and *vasK*_4 primers (Table 3.2). *E. coli*

S17- λ pir cells transformed by electroporation with the plasmid were used to introduce the suicide vector into isolate 692-79 via conjugation. LB agar plates containing kanamycin were used to screen for transconjugants containing the chromosomally-integrated plasmid. These colonies were then streaked onto LB agar plates containing 10% sucrose to select for candidates that had lost the plasmid by homologous recombination. Isolated colonies unable to grow when restreaked onto LB agar plates with kanamycin were selected as presumptive *vasK* deletion mutants. Deletion of *vasK* was confirmed by PCR with internal *vasK_5* and *vasK_6* primers (Table 3.2). The resulting 692-79 Δ *vasK* mutant was used to verify the necessity of T6SS in contact-dependent bacterial killing.

Table 3.2. Sequences of primers used in cloning experiments during this study.

Primer	Sequence, 5' \rightarrow 3'
pQT_1	AGTTGTCAGCAGCGTTAAGTTAAAGGATCCAGGAGCTAAGGAA GCTAAAATGCAACGAGCCAACCTATGC
pQT_2	ATAAAGCTTGCTCAATCAATCACCTTACAGTAAATTTTGATTAA CCCAAGC
pQT_3	TTGACAATTAATCATCGGCTCG
pQT_4	TTTTTTCACAAAACGGTTTACAAGC
vasK_1	AATTCCCGGGAGAGCTCGATATCGGGATGAGTTACACCATTGCC
vasK_2	AAACTCGCTCTGGCAACCTAGAATTGTGTCCTTGTTTACTCTG
vasK_3	CAGAGTAAACAAGGACACAATTCTAGGTTGCCAGAGCGAGTTTT
vasK_4	CAGGTCGACGGATCCCAAGCCAGCTGATGTAACCGCTCG
vasK_5	CCGAGTTTAGTGGAACAAATTC
vasK_6	CAACTTCGTCCTAAAAAGCCAG

3.3.4. Chitinase plate assay

Colloidal chitin was prepared from practical grade chitin (Sigma) derived from shrimp shells as previously described (27, 109). Colloidal chitin plates were prepared by mixing 2% (wt/vol) colloidal chitin with LB medium buffered to pH 7.0 with 0.1 M phosphate buffer. Strains were incubated overnight at 37°C in LB broth and diluted to an

optical density at 600 nm (OD₆₀₀) of 1.0, and 10 µl of each suspension was plated onto the colloidal chitin agar. After incubation at 37°C for 96 h, the presence or absence of a zone of chitin clearing for each colony was recorded by comparison to positive (C6706) and negative (C6706 CytR⁻) controls.

3.3.5. Natural transformation chitin assay

The standard chitin-induced transformation assay was used to quantify the transformation frequency (TF) of *V. cholerae* as described previously in detail (147, 234). Briefly, cells grown in the presence of chitin (crab shell fragments) were exposed for 24 h to genomic DNA carrying a kanamycin resistance cassette and then plated on selective and non-selective media to determine the TF (234). Three independent experiments were performed with each isolate in triplicate, and the mean TF ± the standard deviation for one representative experiment were reported.

3.3.6. Bacterial killing assay

The killing assay (143) was modified as described previously in detail (235). Briefly, *V. cholerae* predators and Cm^R *E. coli* prey strains were grown overnight in LB medium with shaking at 37°C to an OD₆₀₀ of ~1.0. Predator *V. cholerae* (clinical and environmental isolates) and prey (Cm^R *E. coli*) cells were mixed at a ratio of 10:1, and 50 µl of each suspension was spotted onto sterile Whatman cellulose gridded filters (GE Healthcare) placed on LB plates. For *qstR*, *tfoX* inducible killing assays with isolates carrying pQT, IPTG (Fisher BioReagents) was added to a final concentration of 200 µM to each 50-µl suspension before samples were spotted onto filters. A prey-alone control

was also prepared in the same ratio with fresh LB medium to determine total prey counts in the absence of a predator. After incubation at 37°C for 3 h, cells were removed from filters by vortexing in LB medium, serially diluted, and plated on LB agar supplemented with chloramphenicol to determine the number of CFU per milliliter of surviving *E. coli* prey.

3.3.7. Contact dependence assay

The contact dependence assay described in (143) was modified slightly to parallel the standard killing assay described above. Briefly, each *V. cholerae* isolate (predator), as well as an *E. coli* control, was plated as a lawn on LB agar plates and incubated overnight. A sterile 0.22- μ m filter (Pall Life Sciences) was placed on top of each confluent lawn, and then 50 μ l of *E. coli* prey was spotted on top of the filter, allowing the *E. coli* cells access to nutrients but keeping them physically separated from the plated *V. cholerae* predator and *E. coli* control. After incubation at 37°C for 3 h, cells were removed from filters by vortexing in LB medium, serially diluted, and dilutions were spot plated on LB agar supplemented with chloramphenicol to determine the approximate number of CFU of surviving *E. coli* prey per milliliter.

3.4. Results

3.4.1. *V. cholerae* clinical and environmental strains

Strains were chosen to include various locations, sources, years of isolation, and serogroups as outlined in detail in Table 3.3. Serogroups of clinical and environmental

isolates, as well as the presence or absence of the CT gene (*ctxA*), were obtained from previous publications (115, 119) or determined by methods described by Talkington, *et al* (214). Isolates that did not fall into major serogroups O1, O139, O75, O14, or O141 were deemed nonagglutinating and designated NAg. The majority of the 53 isolates are in the O1 serogroup and were acquired from various locations in various years. All of the isolates had comparable growth kinetics and grew to similar ODs (data not shown); therefore, any differences seen were not attributed to growth defects. Clinical O1 El Tor strain C6706 served as the reference strain in all assays (7). On the basis of the Pearson correlation matrix obtained by principal component analysis (PCA) (Table 3.6), differences in location, year, and serogroup do not appear to be correlated with differences in chitinase activity, natural transformation ability, or constitutive bacterial killing, as described below.

Table 3.3. *V. cholerae* isolates obtained from numerous locations, sources, and years with varying serogroups and CTX status. Clinical (C) and environmental (E) isolates were assigned “+” or “-” for each phenotype: chitinase activity (Chi), transformation frequency (TF), and constitutive contact-dependent bacterial killing (Killing).

Strain	Location	Source	C/E	Year	Serogroup	CTX	Chi	TF	Killing
C6706 WT	Peru	patient	C	1991	O1 El Tor	+	+	+	-
NCTC8457	Saudi Arabia	patient	C	1910	O1 El Tor	+	-	+	+
MZO-2	Bangladesh	patient	C	2001	O14	-	+	+	+
2010EL-1749	Cameroon	patient	C	2010	O1 El Tor	+	+	+	+
MAK757	Celebes	patient	C	1937	O1 El Tor	+	+	-	+
CA401	India	patient	C	1953	O1 Classical	+	+	-	-
O395	India	patient	C	1965	O1 Classical	+	+	-	-
MO10	India	patient	C	1992	O139 El Tor	+	+	-	-
3541-04	GA, USA	patient	C	2004	O75 CII	+	+	-	-
3558-04	AL, USA	patient	C	2004	O75 CI	+	+	-	-
3582-05	Pakistan	patient	C	2005	O1 El Tor	+	+	-	-
3500-05	India	patient	C	2005	O1 El Tor	+	+	-	-
3546-06	India	patient	C	2006	O1 El Tor	+	+	-	-
3554-08	Nepal	patient	C	2008	O1 El Tor	+	+	-	-
2011EL-1141	Afghanistan	patient	C	2008	O1 El Tor	+	+	-	-
3566-08	NJ, USA	patient	C	2008	O141	+	+	-	-
2009V-1085	Sri Lanka	patient	C	2009	O1 El Tor	+	+	-	-
2011EL-1938	Orissa, India	patient	C	2009	O1 El Tor	+	+	-	-
2009V-1096	India	patient	C	2009	O1 El Tor	+	+	-	-
2011EL-1137	South Africa	patient	C	2009	O1 El Tor	+	+	-	-
2009V-1046	Pakistan	patient	C	2009	O1 El Tor	+	+	-	-
Nepal 25	Nepal	patient	C	2010	O1 El Tor	+	+	-	-
Nepal 14	Nepal	patient	C	2010	O1 El Tor	+	+	-	-
2011EL-1941	Kolkata, India	patient	C	2010	O1 El Tor	+	+	-	-
2010V-1014	Pakistan	patient	C	2010	O1 El Tor	+	+	-	-
2010EL-1786	Artibonite, Haiti	patient	C	2010	O1 El Tor	+	+	-	-
2011EL-1939	India	patient	C	2011	O1 El Tor	+	+	-	-
2012V-1001	USA	patient	C	2011	O1 El Tor	+	+	-	-
2011V-1043	FL, USA	patient	C	2011	O75 CI	+	+	-	-
3223-74	Guam	storm drain	E	1974	O1	-	+	+	+
2631-78	LA, USA	Moore swab	E	1978	O1	-	+	+	+
2633-78	Brazil	sewage	E	1978	O1	-	+	+	+
E8498	LA, USA	water	E	1978	O75 CII	+	+	+	-
VC22	FL, USA	oyster	E	1981	O1	-	+	+	+
2479-86	LA, USA	Moore swab	E	1986	O1	-	+	+	+
TP	CA, USA	water	E	2000	NAG	-	+	+	+
VC56	AL, USA	oyster	E	2009	O1	-	-	+	+
3225-74	Guam	storm drain	E	1974	O1	-	+	-	+
3272-78	MD, USA	bay	E	1977	O1	-	+	-	+
1074-78	Brazil	sewage	E	1978	O1	-	+	-	+
2559-78	LA, USA	crab	E	1978	O1	+	+	-	+
692-79	LA, USA	Canal	E	1979	O1	-	+	-	+
2740-80	Gulf Coast, USA	water	E	1980	O1	-	+	-	+
VC48	FL, USA	oyster	E	1981	NAG	-	+	-	+
2512-86	LA, USA	Moore swab	E	1986	O1	+	+	-	+
2497-86	LA, USA	Moore swab	E	1986	O1	-	+	-	+
1496-86	LA, USA	Moore swab	E	1986	O1	-	+	-	-
2523-87	LA, USA	Moore swab	E	1987	O1	-	+	-	+
857	Bangladesh	water	E	1996	O1	-	+	-	+
SIO	CA, USA	water	E	2000	NAG	-	+	-	+
3568-07	Mexico	queso fresco	E	2007	O141	+	+	-	+
VC53	AL, USA	oyster	E	2009	NAG	-	-	-	+
HE46	Centre, Haiti	Gray water	E	2011	NAG	-	+	-	+

3.4.2. Chitinase activity is common in *V. cholerae*

In clinical O1 El Tor isolate C6706, chitin degradation, natural transformation, and T6SS-mediated killing all require chitin as an inducing signal (235). Therefore, to characterize the set of isolates, we first sought to determine qualitatively whether each isolate was defective or proficient in chitin utilization. Specifically, we observed degradation of colloidal chitin, which requires the expression of two major chitinases, *chiA-1* and *chiA-2*, that are both under TfoX and CytR control in *V. cholerae* C6706 (235). As expected, a zone of clearing was visible around a colony of C6706 on colloidal chitin agar, indicative of chitinase activity (Fig. 3.1) (27). Clearing was more pronounced for a C6706 strain that constitutively expressed the chitin-responsive TfoX regulator (TfoX*) (7), while a C6706 strain lacking the *cytR* gene (CytR⁻) was unable to degrade chitin by this method (Fig. 3.1) (7, 235). Each clinical and environmental isolate was then assessed for the ability to degrade chitin in this assay. The majority of the isolates degraded colloidal chitin and showed a visible zone of clearing. Only one clinical isolate (NCTC8457) and two environmental isolates (VC56 and VC53) were unable to produce a detectable zone of clearing, comparable to that of the C6706 CytR⁻ strain, and are designated negative in Table 3.3. However, these three isolates were capable of growing (~1.0E+07 CFU/ml) in minimal medium containing a chitin crab shell fragment under the conditions used in a transformation assay described below, though they reached a lower stationary-phase cell density than C6706 (~1.0E+09 CFU/ml). Thus, although unable to degrade colloidal chitin, each negative isolate in Table 3.3 appeared to possess chitinase activity sufficient to utilize chitin for growth.

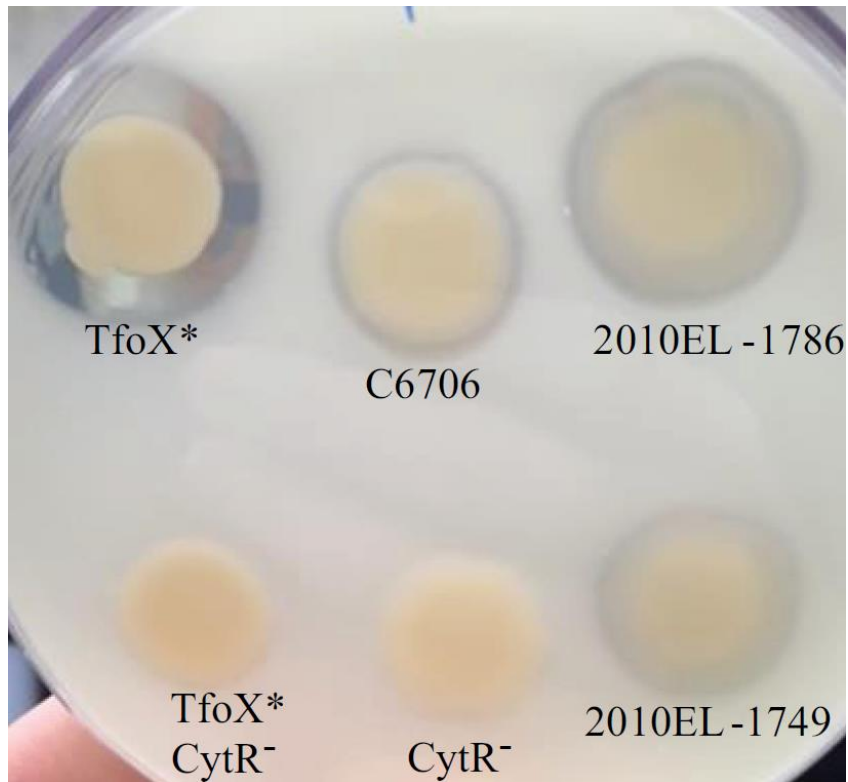


Figure 3.1. Chitin agar plate assay reveals chitinase activity by visualizing zone of clearing. *V. cholerae* isolate C6706, and isogenic derivatives constitutive for TfoX (*) and deleted for *cytR* (CytR⁻), as well as all clinical and environmental isolates were assayed for the ability to degrade chitin, which results in a visible zone of clearing on LB agar plates containing 2% colloidal chitin. Shown are C6706, the TfoX* derivative, and isolates 2010EL-1786 and 2010EL-1749, which produce a detectable zone of clearing; as well as two strains deleted for *cytR* that do not produce a zone of clearing.

3.4.3. Natural transformation proficiency in *V. cholerae* is rare

Since the discovery of natural competence and transformation in *V. cholerae* in 2005, most studies have focused on a small number of clinical strains, while less is known regarding the overall prevalence of natural transformation ability more broadly in this *Vibrio* species (147). To address this, each isolate was tested for the ability to take up DNA and recombine it onto the chromosome via chitin-induced natural transformation, by methods previously described (234). Isolates with a transformation frequency (TF) of

$\leq 1.0E-08$ were deemed severely impaired for transformation, as described previously (8), and are designated negative in Table 3.3. Proficient isolates are designated positive in Table 3.3, and the corresponding values are shown in Table 3.4.

Of the 29 clinical isolates tested, only 4 (13.8%) were proficient in natural transformation, with TFs ranging from $1.00E-05$ to $5.42E-07$ (Tables 3.3 and 3.4). As described prior (7, 147), the O1 El Tor reference strain C6706 had an expected TF of $1.0E-05$. The TF of O1 El Tor isolate NCTC8457, obtained in Saudi Arabia in 1910, was $5.42E-07$, and that of O14 MZO-2, isolated in Bangladesh in 2001, was $4.79E-06$. Contemporary clinical O1 El Tor isolate 2010EL-1749, collected in 2010 in Cameroon, was also transformable with a frequency of $4.53E-06$. Of the 24 isolates tested that were derived from environmental sources, 8 (33.3%) were proficient at natural transformation with a range spanning from $1.04E-05$ to $1.75E-07$ (Table 3.4). The set of proficient environmental isolates included samples obtained over a 30 year period from 1974 to 2009 at locations within and outside the United States and included O1 and non-O1 serogroup isolates (Tables 3.3 and 3.4). Similar results were obtained when we attempted to transform deficient isolates with their own genomic DNA carrying a kanamycin-resistance cassette (data not shown).

Table 3.4. Transformation frequencies of proficient *V. cholerae* clinical (C) and environmental (E) isolates. Data shown are mean values \pm standard deviation for biological triplicates from one representative experiment of three performed.

Strain	C/E	TF
C6706 WT	C	1.00E-05 \pm 8.50E-06
NCTC8457	C	5.42E-07 \pm 2.04E-07
MZO-2	C	4.79E-06 \pm 1.94E-06
2010EL-1749	C	4.53E-06 \pm 3.49E-07
3223-74	E	1.04E-05 \pm 3.29E-07
2631-78	E	1.77E-06 \pm 5.74E-07
2633-78	E	1.07E-06 \pm 4.56E-07
E8498	E	2.51E-06 \pm 3.17E-07
VC22	E	1.75E-07 \pm 8.19E-08
2479-86	E	7.61E-06 \pm 1.40E-06
TP	E	2.62E-06 \pm 8.91E-07
VC56	E	1.08E-07 \pm 1.18E-08

3.4.4. Constitutive contact-dependent bacterial killing is common among environmental isolates

Four environmental isolates, V52, 2740-80, DL4211, and DL4215, have been reported previously to display constitutive T6SS-mediated killing of *E. coli* under standard killing assay conditions in the absence of chitin (21, 178, 223). However, in clinical isolates C6706 and A1552, transcription of the genes encoding the T6SS requires activation by the QstR and TfoX transcription factors, which are induced at high cell density and in the presence of chitin (7, 34, 147). Chitin induction is sufficient to induce killing of prey by A1552, as observed by fluorescence microscopy (34). Nevertheless, chitin induction is insufficient for killing of *E. coli* prey above the limit of detection by A1552 and C6706 in a standard quantitative 3 h killing assay. Only genetic manipulation that places the TfoX regulator under the control of a non-native constitutive promoter allows significant bacterial killing in this assay (34, 235).

On the basis of these and other studies, it was hypothesized that clinical pandemic isolates like C6706 tightly control T6SS, while non-pandemic isolates from environmental sources may express T6SS constitutively to compete with other microbes under conditions outside a human host (223). Because this “pathoadaptive” hypothesis (112) has not been extensively tested experimentally in killing assays, we examined each of the 53 isolates for killing of *E. coli* prey in the absence of chitin to determine constitutive bacterial killing activity indicative of a T6SS. Standard killing assays were performed by exposing each isolate of *V. cholerae* to Cm^R *E. coli* to permit enumeration of surviving *E. coli* prey cells following the 3 h of exposure to the *V. cholerae* predator, as described previously (143, 235). Although bactericidal activity was not directly measured in these assays, we use the term “killing” as it was used to describe similar results in *V. cholerae* killing assays (34, 143, 177, 235).

A CytR⁺ C6706 O1 El Tor strain that was genetically manipulated to express TfoX, HapR, and QstR constitutively (here designated the C6706 maximum killer) decreased prey survival by 10,000-fold compared to a T6SS-deficient C6706 $\Delta vasK$ mutant strain (here designated C6706 T6SS⁻), consistent with previous experiments (235) (Fig. 3.2, white bars). The modest <10-fold reduction in the survival of prey in the presence of the C6706 T6SS⁻ derivative compared to that of the prey alone is most likely due to competition for nutrients and has also been observed in prior studies that used the standard killing assay (143, 235). Twenty-five of the 29 clinical isolates tested displayed little or no constitutive killing, with surviving *E. coli* counts within 10-fold of those recorded when *E. coli* was exposed to the C6706 T6SS⁻ strain (Fig. 3.2A, below negative bracket), including the original O1 El Tor C6706 isolate as expected, because of its

requirement for chitin-induction. In Table 3.3 and Fig. 3.2, a plus sign denotes a >10-fold reduction of *E. coli* prey from the level recorded when *E. coli* was exposed to C6706 T6SS⁻, and a minus sign indicates a reduction of <10-fold. A single clinical isolate, MZO-2, reduced *E. coli* survival by ~10,000-fold, similar to the C6706 maximum killer; three additional clinical isolates, NCTC8457, 2010EL-1749, and MAK757, reduced *E. coli* survival more modestly by ~100-fold (Fig. 3.2A, below positive bracket).

In sharp contrast, the environmental isolates tested covered a 100,000-fold range of constitutive *E. coli* prey-killing abilities (Fig. 3.2B). Only two environmental isolates tested, E8498 and 1496-86, displayed little or no constitutive killing of *E. coli* prey (Fig. 3.2B, negative bracket), with the majority of the isolates (22 of 24) showing >10-fold killing than that recorded with the C6706 T6SS⁻ strain (Fig. 3.2B, positive bracket). Of these 24 isolates, 14 killed *E. coli* with an efficiency within 10-fold of that of the C6706 maximum killer, providing at least 10,000-fold decrease in the prey, suggesting that these strains are efficient constitutive killers (Fig. 3.2B). Two isolates, 3225-74 and 3223-74, showed >100,000-fold killing, exceeding values obtained with the C6706 maximum killer (Fig. 3.2B). These results are consistent with previous observations, by the same standard methods described here (195), that environmental isolates, like V52 and 2740-80, constitutively kill *E. coli* cells. Thus, on the basis of the isolates tested here, constitutive killing appears to be common among *V. cholerae* environmental isolates but rare among clinical isolates.

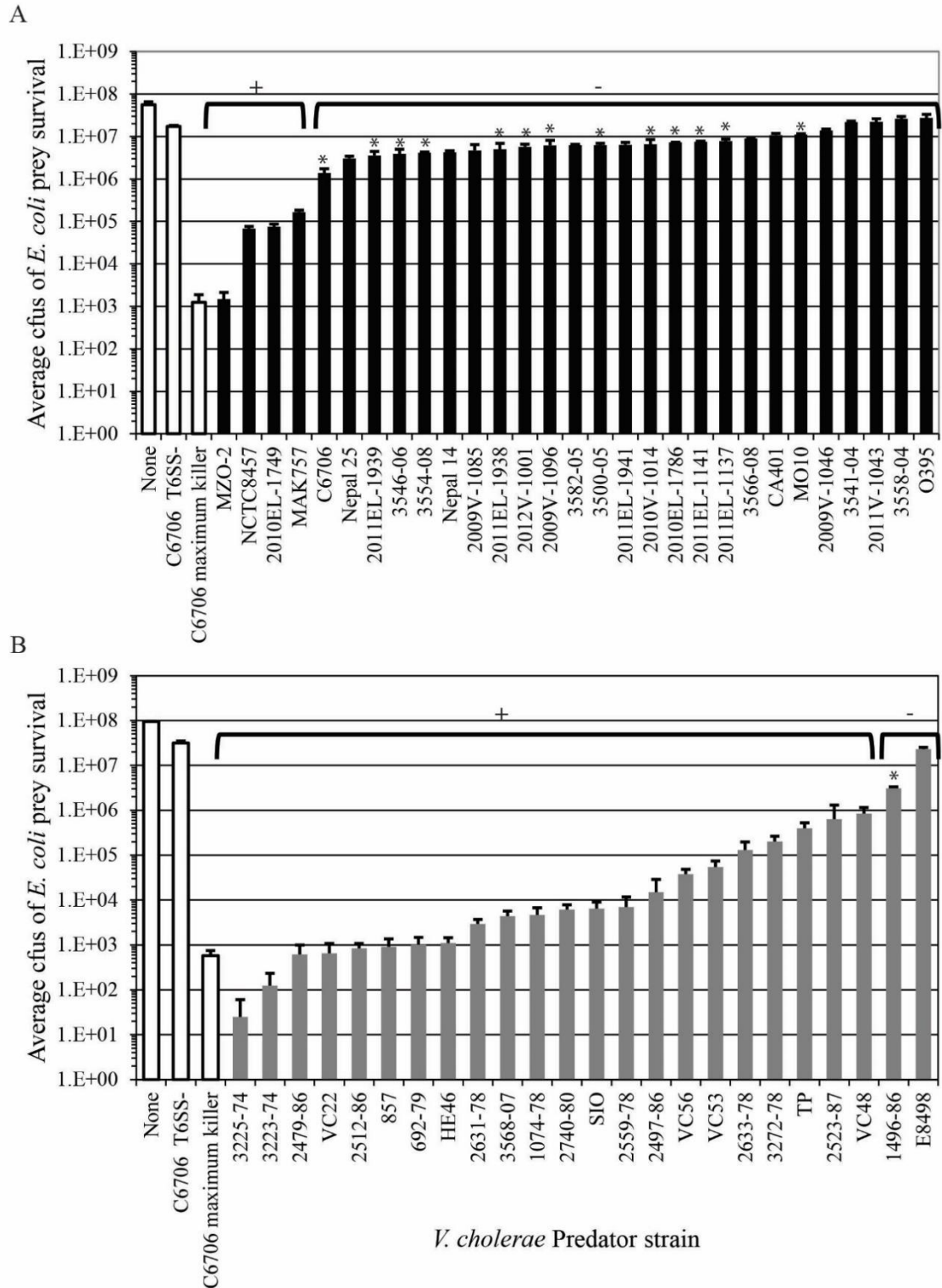


Figure 3.2. Bacterial killing assay shows that constitutive killing of *E. coli* prey is common among environmental but not clinical isolates. Chloramphenicol resistant *E. coli* prey were incubated with the indicated *V. cholerae* predator strains at a ratio of 1:10 on membrane filters on LB agar to measure bacterial killing. Prey alone (“None”) and

Figure 3.2. (continued) negative (“C6706 T6SS⁻”) and positive (“C6706 maximum killer”) controls are represented by white bars in both panels. Shown are average prey survival values \pm standard deviation after triplicate encounters of *E. coli* prey in contact with *V. cholerae* clinical isolates (Panel A, black bars) and environmental isolates (Panel B, grey bars). One representative experiment is shown of three performed. Isolates considered constitutive for bacterial killing as defined in text are placed below the “+” bracket, while non-constitutive isolates are below the “-” bracket. A * indicates isolates that demonstrated killing when inducing expression of TfoX and QstR.

Killing by the T6SS requires a physical association of *V. cholerae* predator cells with *E. coli* prey cells for the delivery of toxic effectors; thus, we tested whether the constitutive killing we observed was contact dependent. In contrast to the standard killing assay, where predator and prey cells are mixed prior to plating to ensure contact, *E. coli* cells were dispensed onto a 0.22- μ m filter that was placed on a confluent lawn of *V. cholerae* cells to physically separate the predator and prey cells, similar to a method described previously (143). *E. coli* prey levels were highest when the filter was directly placed on the agar surface, and only modest (\sim 3-fold) reductions in *E. coli* survival were observed when the agar surface was first seeded with isogenic *E. coli* or a C6706 T6SS⁻ mutant. However, when the C6706 maximum killer, 4 clinical, and 24 environmental isolates that had displayed constitutive 10- to 100,000-fold killing of *E. coli* in the standard assay (Fig. 3.2 and Table 3.3, positive isolates) were incubated under these conditions, prey survival was unaltered ($<$ 2-fold) (Fig. 3.3). The physical barrier of the filter allowed *E. coli* growth, but not killing, demonstrating that the constitutive killing documented by our standard bacterial killing assay was contact dependent.

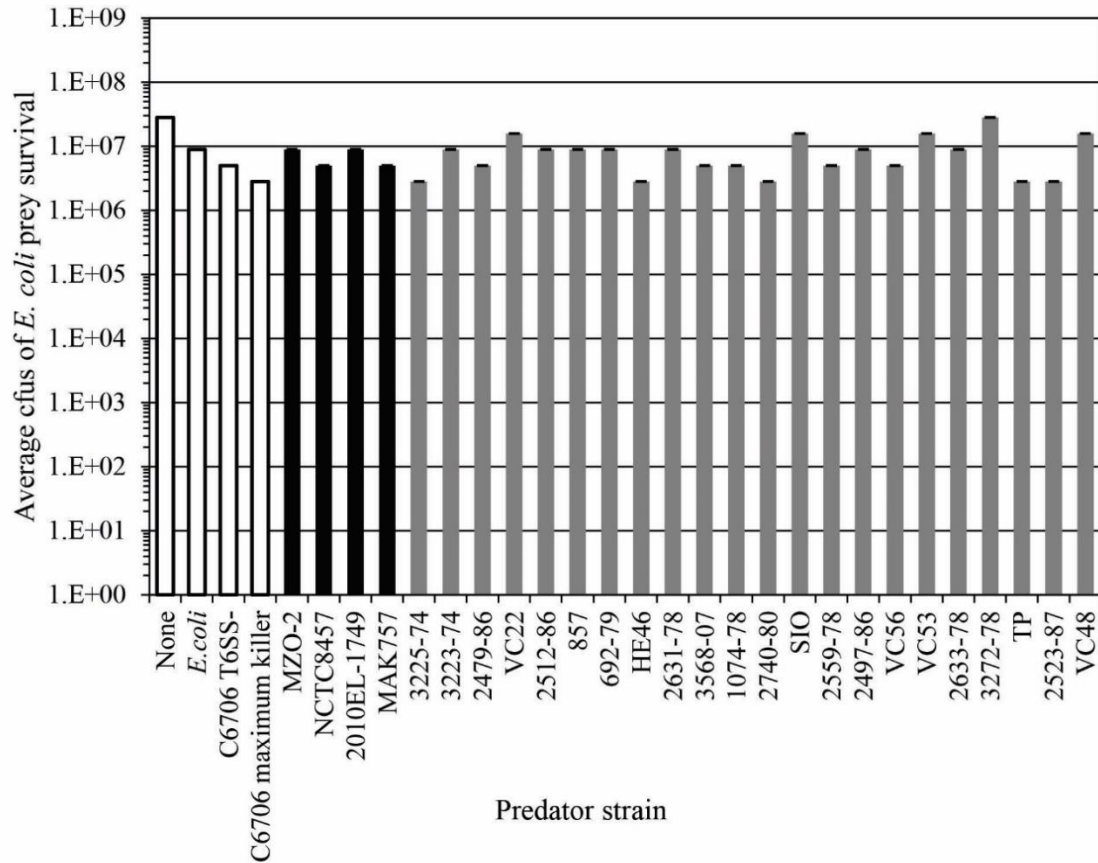


Figure 3.3. Bacterial killing requires contact. Chloramphenicol resistant *E. coli* prey were incubated with *V. cholerae* predator strains separated by a 0.22- μ m filter to measure contact dependence of killing. Prey alone (“None”), isogenic *E. coli* control, and negative (“C6706 T6SS⁻”) and positive (“C6706 maximum killer”) controls are represented by white bars. Shown are average prey survival values \pm standard deviation after triplicate encounters of *E. coli* prey with *V. cholerae* clinical isolates (black bars) and environmental isolates (grey bars) for all isolates that demonstrated constitutive killing previously. One representative experiment is shown of three performed.

To demonstrate that constitutive contact-dependent bacterial killing observed with one of the environmental isolates was T6SS-mediated, an in-frame *vasK* deletion mutant of isolate 692-79 was constructed via allelic exchange. The 692-79 isolate and the isogenic $\Delta vasK$ mutant (692-79 T6SS⁻) were tested for contact-dependent killing of *E. coli* along with the C6706 maximum killer and the isogenic $\Delta vasK$ mutant (C6706

T6SS⁻) as controls. As described previously, *E. coli* prey survival was highest in the absence of a predator, decreased ~10,000-fold in the presence of the C6706 maximum killer, and decreased <10-fold in the presence of C6706 T6SS⁻ (Fig. 3.4, white bars). Similarly, a >10,000-fold reduction in *E. coli* prey survival was observed when it was incubated with 692-79 but abolished with 692-79 T6SS⁻, confirming for this isolate that T6SS was responsible for the observed loss of *E. coli* prey viability (Fig. 3.4, gray bars).

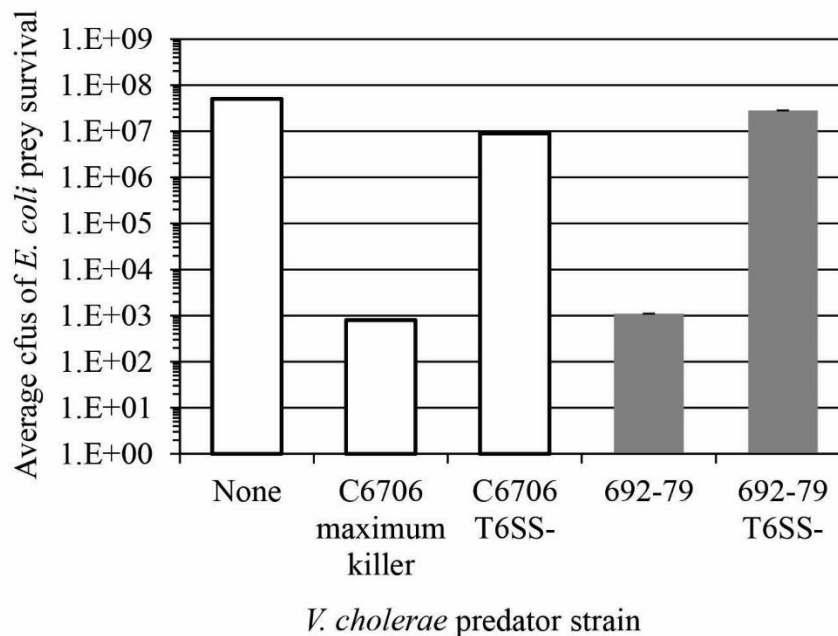


Figure 3.4. Contact-dependent bacterial killing by environmental isolate 692-79 is mediated by a T6SS. Chloramphenicol resistant *E. coli* prey were incubated with the indicated *V. cholerae* predator strains at a ratio of 1:10 on membrane filters on LB agar to measure bacterial killing. Prey alone (“None”) and positive (“C6706 maximum killer”) and negative (“C6706 T6SS⁻”) controls are represented by white bars. Isolate 692-79 and isogenic $\Delta vasK$ (692-79 T6SS⁻) are represented by grey bars. Shown are average prey survival values \pm standard deviation for triplicate predator-prey encounter. One representative experiment is shown of three performed.

3.4.5. Additional isolates are capable of killing under conditions that mimic chitin induction

Chitin induction is insufficient to induce T6SS-mediated killing of *E. coli* by C6706 above the limit of detection in a 3 h killing assay, as described above. Thus, in order to mimic chitin signaling in isolates that did not show constitutive killing (negative in Fig. 3.2 and Table 3.), a plasmid expressing both *qstR* and *tfoX* from a heterologous IPTG-inducible promoter, designated pQT, was introduced into each of these 27 isolates and killing assays were performed. This determined whether any of these isolates were indeed capable of contact-dependent bacterial killing by induction. The C6706 T6SS⁻ ($\Delta vasK$) mutant did not kill *E. coli* regardless of pQT induction and served as a negative control (Table 3.5). Relative to the T6SS⁻ control, the C6706 maximum killer showed an ~10,000-fold reduction in *E. coli* levels irrespective of pQT induction. *E. coli* prey levels were similarly reduced when C6706 was induced with pQT, with only a modest (< 10-fold) effect observed in the absence of induction, as expected. Likewise, induction with pQT was capable of restoring bacterial killing activity in C6706 $\Delta qstR$, $\Delta tfoX$, and $\Delta qstR tfoX$ mutants (data not shown). Relative to the T6SS⁻ control, 13 of 25 clinical isolates and 1 of 2 environmental isolates that were initially scored negative for constitutive contact-dependent bacterial killing (Fig. 3.2, asterisks) showed ≥ 10 -fold reductions in *E. coli* levels only after pQT induction (Table 3.5). Thus, 14 additional isolates were capable of contact-dependent bacterial killing in response to conditions that mimic chitin induction, consistent with a functional T6SS.

Table 3.5. Fold reduction in prey survival when exposed to *V. cholerae* isolates with and without pQT. Only isolates that killed over 10-fold compared to C6706 T6SS⁻ when induced are shown. Clinical (C) and environmental (E) isolates are denoted as such.

	C/E	No induction	With induction
C6706 T6SS⁻	C	1.0	1.0
C6706 maximum killer	C	13760.0	15855.0
C6706	C	9.5	8094.1
3554-08	C	4.2	557.1
1496-86	E	5.6	264.6
2011EL-1141	C	2.3	200.3
MO10	C	1.5	155.0
2012V-1001	C	3.0	124.6
3546-06	C	4.4	76.3
2011EL-1938	C	3.4	43.3
2009V-1096	C	2.8	35.6
2011EL-1137	C	2.2	31.9
2010V-1014	C	2.6	26.3
3500-05	C	2.7	20.4
2011EL-1939	C	4.8	16.5
2011EL-1786	C	2.4	11.0

3.5. Discussion

To understand the diversity present in this set of 53 environmental and clinical isolates in the absence of their genome sequences, the serogroups and CTX status of all isolates were obtained from previous publications (115, 119) or determined in this study by methods we have described previously (214). Most isolates belonged to the O1 serogroup, consistent with the context of their isolation. The majority of the clinical isolates were obtained from patients during epidemics, which are typically caused by O1 strains that carry the CT-encoding genes. Many of the environmental isolates were acquired from locations believed to be the sources of cholera outbreaks, increasing the likelihood that these isolates were also of the O1 serogroup. Principal-component analysis (PCA) of the entire data set, as well as clinical or environmental isolate data

alone, was performed by using the following variables: year of isolation, serogroup, CTX status, chitinase activity, TF, and fold reduction of *E. coli* prey in killing assays. The Pearson correlation matrix did not show any significant correlation between the serogroup, a proxy for genetic relatedness, and any of the three phenotypes studied here when clinical and environmental isolate data were tested together or separately (Table 3.6). For example, looking at isolates of serogroup O141 in Table 3.3, 3566-08 and 3568-07 are both deficient in transformation, but one is capable of constitutive contact-dependent bacterial killing while the other is not. The only significant correlation observed when testing clinical and environmental isolates together was between the clinical or environmental designation and CTX status (see Table 3.6, clinical and environmental, -0.778 bold values). This correlation is expected because clinical isolates are defined as causing disease in patients by CTX. There were no significant correlations between any variables when environmental isolates were tested alone (see Table 3.6, environmental only).

Table 3.6. Pearson correlation matrix generated from principal component analysis (PCA) on clinical and environmental data, clinical data only, or environmental data only. Each variable was assessed for its correlation with all other variables. Variables include clinical (C) or environmental (E), year of isolation, serogroup, CTX status, chitinase activity (Chi), transformation frequency (TF), and fold reduction in prey survival due to contact-dependent constitutive bacterial killing (Killing). Values in bold represent a correlation between variables that are different from 0 with a significance level $\alpha \leq 0.05$.

	Variables	C/E	Year	Serogroup	CTX	Chi	TF	Killing
Clinical and Environmental	C/E	-----	-0.252	0.351	-0.778	-0.105	0.088	0.212
	Year	-0.252	-----	0.230	0.170	0.206	-0.106	-0.161
	Serogroup	0.351	0.230	-----	-0.309	-0.168	-0.002	-0.051
	CTX	-0.778	0.170	-0.309	-----	0.146	-0.157	-0.238
	Chi	-0.105	0.206	-0.168	0.146	-----	0.069	0.047
	TF	0.088	-0.106	-0.002	-0.157	0.069	-----	0.072
	Killing	0.212	-0.161	-0.051	-0.238	0.047	0.072	-----
	Clinical only	Year	N/A	-----	0.261	-0.025	0.373	-0.030
Serogroup		N/A	0.261	-----	-0.212	0.205	0.042	0.205
CTX		N/A	-0.025	-0.212	-----	-0.036	-0.366	-1.000
Chi		N/A	0.373	0.205	-0.036	-----	0.013	0.015
TF		N/A	-0.030	0.042	-0.366	0.013	-----	0.681
Killing		N/A	0.008	0.205	-1.000	0.015	0.681	-----
Environmental only	Year	N/A	-----	0.641	-0.089	-0.547	-0.231	-0.280
	Serogroup	N/A	0.641	-----	-0.005	-0.334	-0.090	-0.174
	CTX	N/A	-0.089	-0.005	-----	0.155	-0.048	-0.128
	Chi	N/A	-0.547	-0.334	0.155	-----	0.123	0.088
	TF	N/A	-0.231	-0.090	-0.048	0.123	-----	0.071
	Killing	N/A	-0.280	-0.174	-0.128	0.088	0.071	-----

Chitin is the most abundant polymer in the ocean; therefore, utilization of this carbon source is likely advantageous for the proliferation of many aquatic microorganisms (110). Consistent with this, most of the *V. cholerae* isolates tested possessed chitinase activity, determined by the production of a zone of clearing on a plate containing chitin colloid, which requires the secreted chitinases ChiA-1 and ChiA-2 (27, 235). Only three isolates were unable to produce a zone of clearing, but each was still capable of utilizing a chitin crab shell fragment during transformation assays. Indeed, two

of these three isolates, NCTC8457 and VC56, were also proficient at natural transformation, which requires chitin degradation for GlcNAc₂₋₆ signal production. The ubiquity of chitin utilization among all of the isolates tested here bolsters the argument that the use of chitin as a carbon source is critical to *V. cholerae* survival and proliferation in its natural aquatic environment.

Natural transformation has been demonstrated in only a limited set of *V. cholerae* isolates (8, 62, 147, 209, 246). Analysis of *V. cholerae* isolates obtained during the Haiti epidemic, that followed the 2010 earthquake, confirmed the source of the outbreak and also revealed that these contemporary isolates were severely impaired in transformation, in contrast to C6706, A1552, and others isolated in 1991 (119). This study prompted us to determine the prevalence of natural transformation among a larger set of *V. cholerae* isolates. Here, we showed that transformation proficiency appears to be slightly more common among isolates from environmental sources (33.3%) than among clinical isolates (13.8%). This perhaps suggests that *V. cholerae* isolates occupying aquatic reservoirs may maintain the genes necessary for transformation in order to take up DNA as an alternative nutrient source in relatively nutrient-poor environmental settings compared to the nutrient-rich gut of a human host (70, 181, 207, 211). As a whole, transformation proficiency was rare among all of the isolates tested (22.6%), but our collection of strains contained a majority of O1 isolates, suggesting that transformation may simply be uncommon within the O1 serogroup.

The inability to take up DNA and recombine it onto the chromosome may result from loss-of-function mutations in competence regulators, apparatus components, or recombination genes. Indeed, two of the transformation-deficient clinical isolates,

MAK757 and CA401, were previously shown to carry mutations in the gene encoding the QS regulator HapR, which is required for natural competence (115). These isolates were complemented for transformation with a plasmid carrying the C6706 *hapR* gene under the control of a constitutive promoter (data not shown). A third clinical isolate deficient in transformation, 2012V-1001, carries an S50Y missense mutation in the DNA binding domain of HapR (66) on the basis of the publicly available genome sequence and independent sequence analysis and its transformation ability was also restored by the same *hapR*-encoding plasmid (data not shown).

It is also possible that transformation-deficient isolates have acquired a factor that impaired DNA uptake. Recently, Dalia *et al.* showed that the transformation deficiency of clinical strains isolated during the 2010 Haiti outbreak appeared to result from of a gain of function (63). Specifically, the Haiti isolates discussed in (119) carry a large integrative and conjugative element (VchInd5) on chromosome 2 that encodes a constitutively expressed periplasmic DNase. That DNase, IdeA, can degrade extracytoplasmic DNA, reducing the opportunity for uptake and subsequent recombination onto the chromosome and causing a severe decrease in natural transformation (63). Sequence analysis of clinical isolates from Bangladesh showed an increase in the prevalence of *ideA* from 0 to 60% of that in the genomes obtained from 2001 to 2011, with the most dramatic increase after 2005, suggesting a major acquisition event (63). We identified *ideA* and 1 kb of its flanking sequences with 100% identity in the published genomes of all of the clinical isolates collected after 2005 and tested here. Interestingly, 2010EL-1749, isolated in 2010, was noteworthy in that it carries the *ideA*

gene, yet the strain remains transformation proficient. Further study may reveal differences in the expression of *ideA* in this isolate.

Genomic analysis of the T6SS of many members of the species *V. cholerae* has been conducted, but T6SS-mediated killing has been demonstrated in only a small group of isolates (21, 34, 178, 223). On the basis of results from a small number of isolates, it was proposed that constitutive T6SS activity is prevalent among environmental strains because of constant exposure to predators, while clinical pandemic strains tightly regulate T6SS (223). Consistent with this “pathoadaptive” hypothesis, we showed that only 4 of 29 clinical isolates were constitutively capable of killing *E. coli* prey in a contact-dependent manner indicative of a T6SS, while 22 of 24 environmental isolates killed prey under laboratory conditions without chitin-induction. The only clinical isolate constitutively capable of killing as effectively as the C6706 maximum-killing strain was MZO-2, which is a CTX⁻ non-pandemic O14 isolate, showing a high correlation between killing and CTX status in the PCA of clinical isolates alone (see Table 3.6, clinical only, -1.000 bold values). These results further validate the pathoadaptive hypothesis. In fact, all but one of the CTX⁻ isolates exhibited constitutive bacterial killing, but of the 39 pandemic O1 or O139 isolates, only 5 showed both CTX and constitutive killing (Table 3.3). It is possible that non-pandemic clinical isolates like MZO-2 have maintained constitutive killing activity to overcome deficiencies resulting from a lack of CT.

It is important to note that although wild-type pandemic isolate C6706 does not express T6SS under laboratory conditions in the absence of chitin, expression of T6SS genes has been described *in vivo* (83, 145). Indeed, T6SS was shown to be important in the infection of both mice and rabbits (141, 248). It was recently shown that mucin, the

main protein component of the mucus layer in the small intestine, which *V. cholerae* colonizes, are capable of causing this activation (11). Therefore, while pandemic isolates may not display constitutive contact-dependent bacterial killing, they can still upregulate this mechanism during colonization of both chitin in the environment and of the small intestine in a human host. In fact, 13 of 25 clinical isolates, including C6706, that did not show constitutive killing were induced by *qstR* and *tfoX* expression, which mimics chitin signaling (Table 3.5). These results suggest that these isolates have a fully functional, yet regulated T6SS.

Genetic manipulation of the environmental isolates characterized here remains challenging. Environmental isolate 692-79, but not an in-frame isogenic $\Delta vasK$ mutant constructed by allelic exchange, exhibited contact-dependent killing of *E. coli* (Fig. 3.4). Thus, the observed killing of *E. coli* prey by 692-79 is indeed mediated by a T6SS. Additionally, sequencing results confirm that the 24 environmental isolates characterized here, like numerous sequenced *V. cholerae* isolates described previously (37), contain conserved T6SS-encoding genes. However, limited success with genetic manipulation of the majority of the environmental isolates characterized here suggests that studies of the T6SS of these isolates will require methods beyond those used in this survey.

The molecular mechanism by which environmental strains are T6SS constitutive while clinical pandemic strains do not express T6SS constitutively is still unclear. It has been proposed (223) that constitutive T6SS-mediated killing by environmental isolates may be advantageous due to constant competition from other bacteria and potential predators in the environment, while pandemic strains utilize other virulence mechanisms for competition in the human gut and therefore may not require a constitutively active

T6SS. Our results support this hypothesis. It is intriguing to speculate that in nutrient-poor environmental settings, the DNA and nucleotides released from neighboring cells may be consumed by *V. cholerae* via T6SS activity. Perhaps constitutive expression of the T6SS is favored in bacteria that occupy niches like marine settings where DNA may be a valuable food source, but costly for enteric pathogens like pandemic *V. cholerae* that have become better adapted to living in the human gut where preferential nutrients are abundant.

As stated previously, when the complete set of data from both clinical and environmental isolates tested here was analyzed by PCA, there was no significant correlation between year, location of isolation, or serogroup and possession of each of the three phenotypes. However, by PCA of clinical isolates alone, a statistically significant correlation between constitutive bacterial killing and TF was found (see Table 3.6, clinical only, 0.681 bold values). Among the four clinical isolates that are capable of constitutive bacterial killing to some degree, three were also transformation proficient (Table 3.3). This relationship is not surprising because of our knowledge about their coordinate expression and regulation. NCTC8457 was collected >100 years ago and has retained its ability to incorporate DNA onto its chromosome and constitutively kill bacteria in a contact-dependent manner, suggesting that these phenotypes may provide an adaptive advantage. All but one of the more contemporary clinical isolates, collected in 2005 or later, have lost the ability to transform DNA and no longer constitutively kill, suggesting that they are relatively poor competitors in the environment. It is interesting to speculate that *V. cholerae* strains adapt to different niches and express genes for contact-dependent killing and transformation differently.

Chitinase activities are likely to provide a major growth advantage in aquatic environments, consistent with their prevalence in this set of isolates. On the other hand, more complex behaviors like transformation and T6SS-mediated bacterial killing are metabolically expensive; therefore, the prevalence and regulation of these phenotypes are highly variable, as described here. These survey data will inform future sequence analyses and genomewide association studies to help identify previously unknown regulators and structural components for both transformation and the T6SS.

3.6. Acknowledgements

We thank Taylor Griswold for assistance with genome analysis of the sequenced clinical isolates and Hammer lab members for discussions and critical manuscript review. We also thank Jacob Thomas for constructing the pQT plasmid and the 692-79 $\Delta vasK$ mutant. We thank Cheryl Bopp, Jun Zhu, Andy DePaulo, Douglas Bartlett, and Rita Colwell for providing us with the isolates tested in this survey. This study was funded by a grant (MCB-1149925) from the National Science Foundation to B.K.H and the Gordon and Betty Moore Foundation.

CHAPTER 4

CONTACT-DEPENDENT KILLING BY TYPE VI SECRETION DRIVES CLONAL PHASE SEPARATION AND EVOLUTION OF BACTERIAL COOPERATION

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4.1. Summary

By nature of their small size, dense growth, and frequent need for extracellular metabolism, microbes face persistent public goods dilemmas (81, 106, 123, 165, 238). Positive spatial assortment achieved by segregation into clonal patches can act as a general solution to social conflict by allowing extracellular goods to be utilized preferentially by productive genotypes (64, 79, 81). Established mechanisms that generate microbial assortment depend on physical isolation (208, 230), competitor exclusion during clonal outgrowth (73, 156, 226), and density-dependent migration (74, 135). Here we describe a novel class of self-organized pattern formation in bacterial communities. Contact-mediated killing through the Type VI secretion system (T6SS) drives the origin of highly assorted populations by generating phase separation, even in initially well-mixed populations that do not necessarily exhibit net growth. We examine these dynamics using three different classes of mathematical models and two mutually antagonistic strains of *Vibrio cholerae* co-cultured on solid media, and find that all de-mix via the same ‘Model A’ universality class of order-disorder transition. We demonstrate mathematically that T6SS-mediated killing should favor the evolution of

public goods cooperation, and empirically examine the relationship between T6SSs and potential cooperation through phylogenetic analysis. Across 26 genera of Proteobacteria and Bacteroidetes, the proportion of a strain's genome that codes for potentially-exploitable secreted proteins increases significantly with both the number of T6SSs and the number of T6SS effectors that it possesses. This work demonstrates how antagonistic traits, likely evolved for the purpose of killing competitors, can indirectly lead to the evolution of cooperation by driving genetic phase separation.

4.2. Introduction

Microbes are fundamentally social organisms. They often live in dense, surface-attached communities, and participate in a range of social behaviors mediated through the production and consumption of extracellular metabolites. Classic examples include the cooperative production of digestive enzymes (68), metal chelators (40), signaling molecules (68), and the structural components of biofilms (102). Many of these cooperative compounds are susceptible to social exploitation, in which non-producing 'cheats' gain an evolutionary advantage. If unchecked, this social exploitation can lead to the extinction of cooperative genotypes. While it is widely recognized that the spatial segregation of cooperative microbes away from cheats can solve this cooperative dilemma (81, 106), relatively little work has investigated the mechanisms generating spatial structure in bacterial communities.

Vibrio cholerae, the bacterium responsible for the often fatal diarrheal disease cholera, colonizes the gut of a human host but also thrives in the nutrient-poor environment of the ocean (213). *V. cholerae* possesses a Type VI secretion system

(T6SS) that pierces and delivers toxic effector proteins into both bacterial and eukaryotic cells (107). T6SSs are widely distributed in Proteobacteria and were initially thought to be involved in virulence, but are now considered to be more often involved in inter-bacterial antagonism (130). All sequenced *V. cholerae* strains have three T6SS gene loci encoding one T6SS; a major cluster encoding the apparatus and two auxiliary gene clusters that each encode a toxic effector protein and adjacent immunity protein to protect against self-intoxication (37, 154). The protective effect provided by this cognate immunity protein means that strains sharing effector-immunity gene sets are incapable of killing one another, while strains with different sets antagonize each other (224).

In *V. cholerae*, T6SS-mediated killing is tightly regulated or absent in clinical strains (like C6706), but commonly expressed constitutively in environmental isolates (24). These observations have fostered a “pathoadaptive” model positing that T6SS mediates inter-microbial aggression *ex vivo* (in mixed-species environmental communities), while factors like the cholera toxin that causes the diarrheal disease play a more prominent role in competition *in vivo* (the human microbiome) (223). In the clinical O1 El Tor isolates C6706 and A1552, genes involved in the T6SS are upregulated in response to nucleoside starvation, growth on chitin, and quorum sensing at high cell density but these requirements may be bypassed by constitutive expression of the transcription factor QstR (34, 137, 235). This suggests that T6SS-mediated intermicrobial aggression may be more important in the marine environment where *V. cholerae* is likely found in mixed-species biofilms. How constitutive expression of this antagonistic phenotype affects microbial communities in which *V. cholerae* is commonly found is poorly understood.

Here, using a combination of experiments, modeling, and bioinformatics, we show that bacterial killing via T6SSs among two mutually antagonistic strains, clinical isolate C6706 and environmental isolate 692-79, leads to the phase separation and assortment of *V. cholerae* strains into clonal patches. We propose that this creates an environment in which cooperators can segregate themselves from competitors and control the population of cheaters, possibly prompting the evolution of cooperation.

4.3. Experimental procedures

4.3.1. Individual based simulation model (IBM)

To simulate T6SS-mediated killing, we randomly seeded a 500x500 lattice with an equal number of red and blue cells, initially well-mixed (Fig 4.1a). Each population is assigned a growth rate and killing rate. Every time step, 5% of the cells were randomly chosen to activate their T6SS systems, killing any adjacent (eight cells surrounding the focal cell) cells of the opposite color. Similarly, 5% of the cells in the landscape were randomly chosen to attempt to reproduce, filling up to one adjacent unoccupied patch with a cell of its color. Reproduction was aborted if all neighboring patches were occupied. Within each time step, model updates were propagated sequentially across rows, starting with the first position in the upper left corner. This model was coded in Python and is available upon request. This modeling was performed by Will Ratcliff.

4.3.2. Partial differential equations model (PDE)

To ensure that our results are driven by deterministic self-organization owing to contact killing, rather than by any stochastic effects, we also developed a partial differential equation (PDE) model of T6SS-mediated killing. This model utilizes equations to determine densities of two strains (A and B) over time by both density-dependent growth and loss of density due to killing. Our PDE model also assigns particular locations to the strains and accounts for movement either through division or mobility, mimicking growth on a solid surface. The following equations were used to determine densities of strains A and B over time:

$$\frac{\partial A}{\partial t} = A(r - s(A + B) - \alpha_{AB}B) + d\Delta A$$
$$\frac{\partial B}{\partial t} = B(r - s(A + B) - \alpha_{BA}A) + d\Delta B$$

where A and B are the strain's densities, r is the strain's growth rate, s is the density dependent mortality rate, α_{AB} and α_{BA} are the rates at which strain A kills strain B and vice versa, and d is the bacterial dispersal rate where we can incorporate different levels of diffusion and mobility.

To consider the effects of T6SS-mediated phase separation on the evolution of cooperation we extend our model so that strain A produces a diffusible public good secreted at rate ρ , while strain B does not invest in its production. We assume that strain A pays a growth rate cost c for production of the secretion. We also assume that the secretion increases each strain's growth rate by amount b per unit concentration S by increasing nutrient availability (e.g. an exoenzyme digesting a substrate or siderophores

binding insoluble iron). From these assumptions we write the dynamics of the two strains (A and B) and the secretion concentration (S) as:

$$\frac{\partial A}{\partial t} = A(r - c + bS - s(A + B) - \alpha_{AB}B) + d\Delta A$$

$$\frac{\partial B}{\partial t} = B(r + bS - s(A + B) - \alpha_{BA}A) + d\Delta B$$

$$\frac{\partial S}{\partial t} = \rho A - \lambda S + D\Delta S$$

where λ is the decay rate of the secretion, D is its diffusion coefficient, and all other variables are as previously defined. We numerically explore both the non-spatial and spatial systems in the presence ($\alpha_{AB} > 0$, $\alpha_{BA} > 0$) and absence ($\alpha_{AB} = 0$, $\alpha_{BA} = 0$) of killing. This modeling was performed by Luke McNally.

4.3.3. Ising spin model

To simulate killing using the Ising spin model, we populate a square (500 x 500) array with an equal number of “up” and “down” magnetic spin entries, representing an initially well-mixed population. In this classic order parameter non-conserving simulation, there is an energetic cost to misaligned magnets, so the so-called spins change their orientation to conform with their neighbors. An algorithm is implemented to determine whether each “up” or “down” will flip to a “down” or “up”, respectively (92). This algorithm looks at the eight nearest neighbors of an entry and counts how many are in a state different from the entry in question. If the entry has four or more neighbors which do not share its configuration, it will flip to match that of these neighbors. If the entry has fewer than four of these neighbors, it will be flipped with a probability that exponentially decreases with the number of matching neighbors it has. The algorithm is

successively applied to randomly-chosen entries in the array, which moves the system forward one step in time. This modeling was performed by Ben Kalziqi.

4.3.4. Bacterial strains and culture conditions

The bacterial strains used in this study are shown in Table 4.1. *V. cholerae* fluorescence reporter constructs (161) were chromosomally-integrated; gene deletions and promoter replacements were constructed by allelic exchange as described and verified by Sanger sequencing (72, 201). *V. cholerae* was routinely grown at 30°C in LB medium supplemented with 50µg/ml of kanamycin or 150µg/ml of spectinomycin when appropriate. Bacterial killing assays were performed as previously described (24), using a C6706 strain that constitutively expresses *qstR* and is therefore T6SS active without the need for chitin-induction, and environmental isolate, 692-79, that natively expresses its T6SS constitutively (Table 4.1). In-frame *vasK* deletions were made in each isolate to make non-killing T6SS⁻ controls as described previously (24, 235). For confocal microscopy, mixed cultures were inoculated on LB agar (1.5%) pads on glass slides and incubated at 17°C, 25°C, or 30°C. C6706 and 692-79 were inoculated at a 1:6 initial ratio for optimal co-culture. A 1:8 initial ratio was also used in order to observe an intermediate level of phase separation.

Table 4.1. List of strains used in this study

<i>V. cholerae</i> strains	Genotype
C6706 red T6SS ⁺	$\Delta vc1807::ptac-mKO$ <i>ptac-qstR</i>
C6706 red T6SS ⁻	$\Delta vc1807::ptac-mKO$ <i>ptac-qstR</i> $\Delta vasK$
C6706 green T6SS ⁺	$\Delta vc1807::ptac-mTFP1$ <i>ptac-qstR</i>
C6706 green T6SS ⁻	$\Delta vc1807::ptac-mTFP1$ <i>ptac-qstR</i> $\Delta vasK$
692-79 red T6SS ⁺	<i>lacZ::ptac-mKO</i>
692-79 red T6SS ⁻	<i>lacZ::ptac-mKO</i> $\Delta vasK$
692-79 green T6SS ⁺	<i>lacZ::ptac-mTFP1</i>
692-79 green T6SS ⁻	<i>lacZ::ptac-mTFP1</i> $\Delta vasK$

4.3.5. Microscopy and image analysis

Laser fluorescence confocal microscopy was performed with a Nikon A1R confocal microscope. The filters used were FITC (for detecting mTFP1, cyan) and TRITC (for detecting mKO, orange). Full colony images were captured in one z-plane using the 20x Plan Apo objective lens and a 2x internal multiplier was applied to capture close-up images. The Galvano scanner was used to scan 2048 X 2048 pixels on all images in order to maximize resolution. For every sample, the top and bottom of the colony was located, and a plane in the middle was imaged. The images were stitched and channels were merged using NIS Elements software. To eliminate issues with red-green colorblindness, we present cyan fluorescence in images as blue.

To calculate the structure factor, $S(q)$, we start with an image from a simulation or experiment, $I(x, y)$. $S(q_x, q_y)$ is the absolute value of the Fourier Transform of $I(x, y)$ squared: $S(q_x, q_y) = |\int I(x, y) dx dy|^2$, where q_x and q_y are spatial frequencies in the x- and y- directions, respectively. We then radially average $S(q_x, q_y)$ and multiply by q : $S(q) = \int q S(q_x, q_y) d\theta$. Finally, we weight $S(q)$ by q to make changes in length scales readily apparent. These calculations were performed by Peter Yunker.

To calculate the assortment (r) of the genotype, we again start out with a binarized image from a simulation or experiment $I(x,y)$ in which we set values of the focal strain g to 1 and the competitor strain c to -1. We first convolved $I(x,y)$ with a kernel in which all positions other than the center were set to 1, and the center set to $-((2h+1)^2-1)$, generating the transformed matrix $C(x,y)$. For example, the kernel for

distance h of 1 would be

1	1	1
1	-8	1
1	1	1

Edges within distance h were trimmed. For each

interaction radius h (which ranged from 1-36), we calculated the assortment r of the focal strain g as $r_g = (1 - \frac{\overline{C(x,y)_g}}{2((2h+1)^2-1)} - \bar{g}) / (1 - \bar{g})$. r is thus the mean frequency of focal strain g within interaction radius h , relative to frequency of g in the population as a whole. r , which ranges from -1 to 1, describes the spatial association of each genotype above or below what would be expected from random associations ($r = 0$). This definition of assortment is commonly used in social evolution studies, and is conceptually analogous to Hamiltonian relatedness (166, 168, 241). Similarly, we calculated the assortment of the competitor strain c as $r_c = (1 - \frac{|\overline{C(x,y)_c}|}{2((2h+1)^2-1)} - \bar{g}) / (1 - \bar{g})$. These calculations were performed by Will Ratcliff.

4.3.6. Phylogenetic analysis

We gathered data on the presence of putative T6SSs and effectors across proteobacterial genomes from the SecReT6 database (130). We restricted our analysis to genera in which there has been experimental verification of the presence of at least one T6SS in at least one strain. This gave data for a total of 439 proteobacterial genomes from the genera *Acidovorax* (N = 5), *Acinetobacter* (N = 19), *Aeromonas* (N = 4),

Agrobacterium (N = 4), *Azoarcus* (N = 2), *Bacteroides* (N = 9), *Bordetella* (N = 10), *Burkholderia* (N = 38), *Campylobacter* (N = 24), *Citrobacter* (N = 2), *Edwardsiella* (N = 4), *Enterobacter* (N = 11), *Escherichia* (N = 59), *Flavobacterium* (N = 5), *Francisella* (N = 19), *Helicobacter* (N = 59), *Methylomonas* (N = 1), *Myxococcus* (N = 3), *Pectobacterium* (N = 5), *Proteus* (N = 2), *Pseudomonas* (N = 53), *Ralstonia* (N = 10), *Salmonella* (N = 41), *Serratia* (N = 9), *Vibrio* (N = 22), and *Yersinia* (N = 19). For each genome we also recorded the genome size and secretome size (number of genes coding for secreted proteins) from PSORTdb (168). Any T6SS effectors identified in SecReT6 were removed from secretome size counts from PSORTdb to avoid creating a spurious correlation owing to double counting of effectors. To control for the phylogenetic relationships among strains we used the SUPERFAMILY phylogeny (241), which we ultrametricized using the chronopl function in ape (166).

We used a Bayesian phylogenetic mixed model (BPMM) approach to test for an evolutionary association between T6SSs and secretome size. Analyses were implemented in R using the package MCMCglmm (92). We treated secretome size as a binomial response variable, expressing it as a proportion of genome size. In all models we included phylogeny as a random effect to control for the shared evolutionary history of strains, and also included a residual random effect to account for overdispersion. For fixed effects we used an uninformative normally distributed prior with mean 0 and variance 10^8 . For the phylogenetic and residual variances we used an uninformative inverse gamma prior with shape and scale both set to 0.001. We ran all models for 6,000,000 iterations with a burn-in of 1,000,000, and thinning interval of 1,000 iterations. We used visual inspection of traces, as well as the Gelman-Rubin test (87, 172) on 3 independent chains to assess

model convergence. In all cases the potential scale reduction factor was less than 1.03. We first fit a model including both the number of T6SSs and number of T6SS effectors as fixed effects. To test the sensitivity of our results we also fit univariate models with number of T6SSs and number of T6SS effectors as fixed effects in isolation. Finally, to control for the potential of non-linear scaling of secretome size with genome size we ran a model with number of T6SSs, number of T6SS effectors, and the log of genome size as fixed effects. In all cases both the numbers of T6SSs and T6SS effectors show significant associations with secretome size. Statistics quoted are posterior modes, 95% credible intervals, and p_{MCMC} a Bayesian equivalent to the frequentist p-value, which is set as twice whichever is smaller of the proportion of posterior samples above or below zero. This analysis was performed by Luke McNally.

4.4. Results and Discussion

Using a bacterial model system (25) and mathematical modeling, we examine the biophysical basis of novel ecological structuring created by contact-mediated bacterial killing through the Type VI secretion system (T6SS). The T6SS is a potent mechanism of intermicrobial aggression, allowing bacteria to deliver lethal doses of toxic effector proteins to adjacent competitors, while leaving clonemates with identical protective immunity proteins unscathed (33, 36).

Our system illustrates the profound effect of T6SS-mediated killing on spatial patterning of a surface attached population. Mathematical modeling suggests that an initially well-mixed population of mutual killers should rapidly undergo phase separation due to ‘selfish herd’ dynamics (96), as the cells within genetically-uniform groups no

longer risk T6SS-mediated death due to immunity from shared effector proteins used by clonemates. Indeed, we observe phase separation in three distinct classes of models, all starting with a randomly seeded population on a 2D lattice (Fig. 4.1a): an Individual Based Model (IBM; Fig. 4.1b), a Partial Differential Equation model (PDE; Fig. 4.1c), and an Ising spin model (105) (Fig. 4.1d). Similarly, initially-well mixed populations of two strains (C6706 and 692-79; Table 4.1) of *Vibrio cholerae* capable of mutual T6SS-mediated killing (Fig. 4.2) underwent phase separation (Fig. 4.1f, i, j) when grown together on a solid substrate and visualized by laser confocal microscopy. Sequencing confirms that these two strains have different sets of effector-immunity pairs, consistent with their ability to kill one another using T6S (Watve, Chande, Hammer, unpublished). Non-killing controls ($\Delta vasK$, i.e. T6SS⁻; Fig. 4.3e, g) and T6SS⁺ mutual killers cultured at low temperatures that impede T6SS activity (219) (Fig. 4.3h) remained well-mixed. These dynamics were also observed when fluorescent reporters were swapped between strains (data not shown).

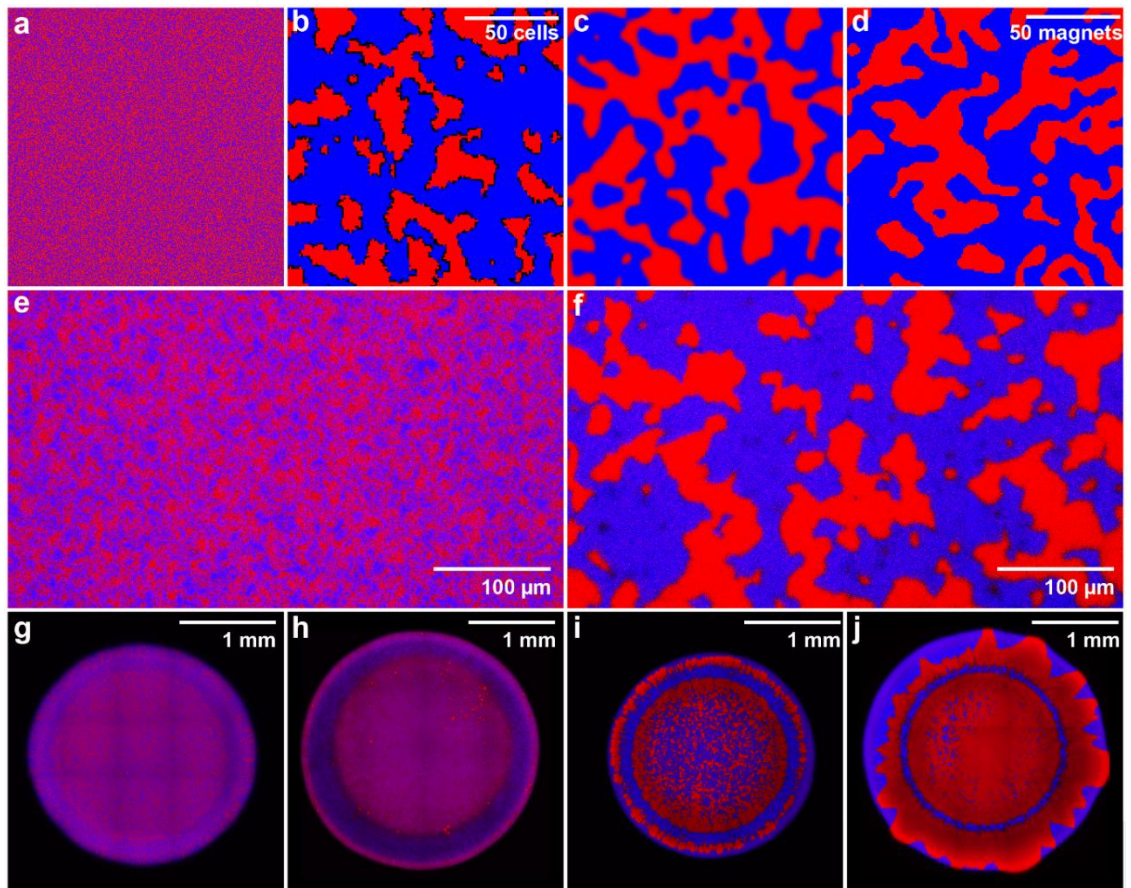


Figure 4.1. T6SS-mediated killing drives phase separation in dense bacterial populations. We modeled the dynamics of phase separation in fully-occupied, randomly seeded square lattices (a). Phase separation between red and blue bacteria capable of mutual killing occurred in an individual based model (b), in a partial differential equation model (c) and in an Ising spin model (d). No phase separation occurred between red (C6706) and blue (692-79) non-killing T6SS⁻ mutants of *Vibrio cholerae* (e), in contrast to T6SS⁺ cells (f). We varied the efficacy of T6SS while still allowing for growth by culturing *V. cholerae* at a range of temperatures: 17°C (h), 25°C (i) and 30°C (j). T6SS⁻ controls cultured at 25°C exhibited no phase separation (g). Non-killing controls at 17°C and 30°C also had no phase separation (data not shown). As expected (161), results are unaltered when performed with fluorescence reporters swapped between strains (data not shown) (Table 4.1).

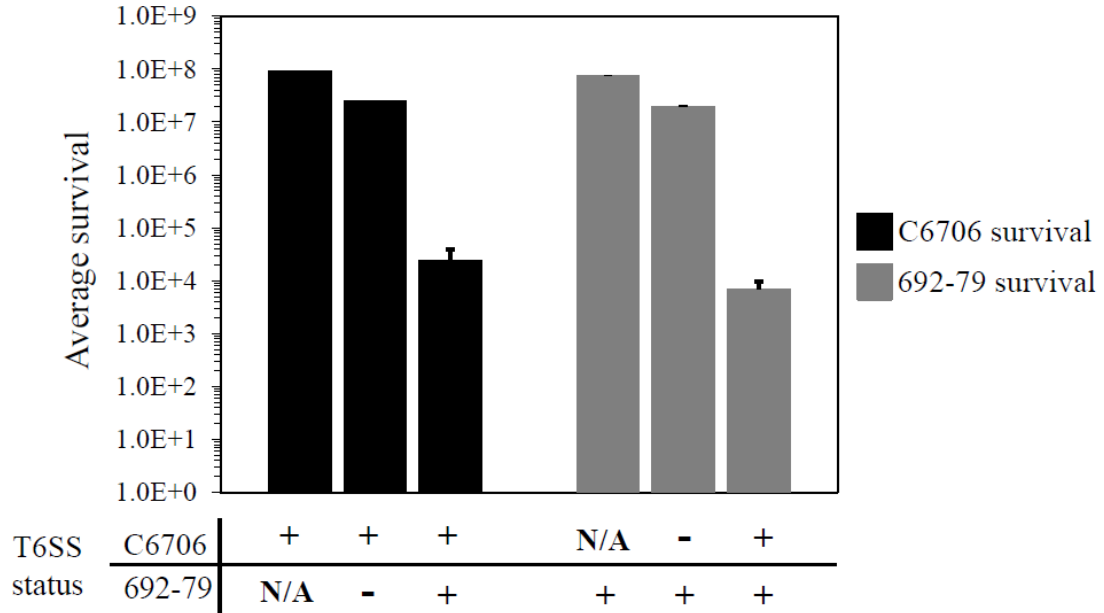


Figure 4.2. C6706 and 692-79 are mutual killers. Spectinomycin resistant C6706 survival was measured after 3 hour incubation on membrane filters on LB agar in a 1:10 ratio (24) with LB broth, or liquid cultures of 692-79 T6SS⁻ (non-killing mutant, $\Delta vasK$), or 692-79 T6SS⁺ strains and is represented by black bars. Kanamycin resistant 692-79 survival was measured similarly in a 1:10 ratio with LB broth, C6706 T6SS⁻ (non-killing mutant, $\Delta vasK$), or C6706 T6SS⁺ and is represented by gray bars. Shown are average prey survival values \pm standard deviation after triplicate encounters. One representative experiment is shown of three performed.

To determine if our models and experiments are undergoing the same type of order-disorder transition, we quantitatively examined the dynamics of phase separation in each. We first computed the Fourier-transformed structure factor, $S(q)$ (Fig. 4.3a & b). The peak in $S(q)$ represents the most common characteristic length scale of clonal groups (i.e. the size of each color patch), and the height of the peak is related to how often it occurs in the lattice. For models at early timesteps (Fig. 4.3a, red line), experimental non-killing controls (Fig. 4.3b, brown line), and T6SS⁺ strains inactivated by growth at low temperatures (Fig. 4.3b, purple line), $S(q)$ is relatively flat, as expected for a well-mixed population. T6SS-mediated killing causes a peak to appear in $S(q)$, which grows in height

and moves to smaller values of q as the population grows increasingly structured (Fig. 4.3a, moving from red to blue lines; Fig. 4.3b, red and black lines). $S(q)$ of PDE and Ising models (data not shown) show indistinguishable characteristics as those seen for IBM and experiments. Over time, the network structure (seen in Fig. 1) develops, and the peak in $S(q)$ grows in height, and moves to smaller values of q , as the network becomes thicker and more uniform, creating large clonal patches of red and blue. This progression of $S(q)$ is a hallmark of phase separation (139).

It is ambiguous how to relate simulation time to experiment time and therefore not intuitive how to compare structure factors from models and experiments over time. However, the location of the first peak in $S(q)$, denoted q_{\max} , is inversely proportional to the square root of time, while the height of the peak $S(q_{\max})$ is proportional to the square root of time. Therefore, we plot $S(q_{\max})$ versus q_{\max} (Fig. 4.3c) in order to relate $S(q)$ of models and experiments over time. Remarkably, all models (IBM, PDE and Ising) and experiments fall on the same line $S(q_{\max})/q_{\max}$ (Fig. 4.3c), showing that the size of the peak on the most common length scale behaves the same over time in all studied systems. When scaling $S(q)$ for each system, we see that the overall distribution of length scales is shared by all models and experiments (Fig. 4.3d), consistent with a universal distribution associated with Model A order-disorder phase separation process (2), described by the Allen-Cahn equation.

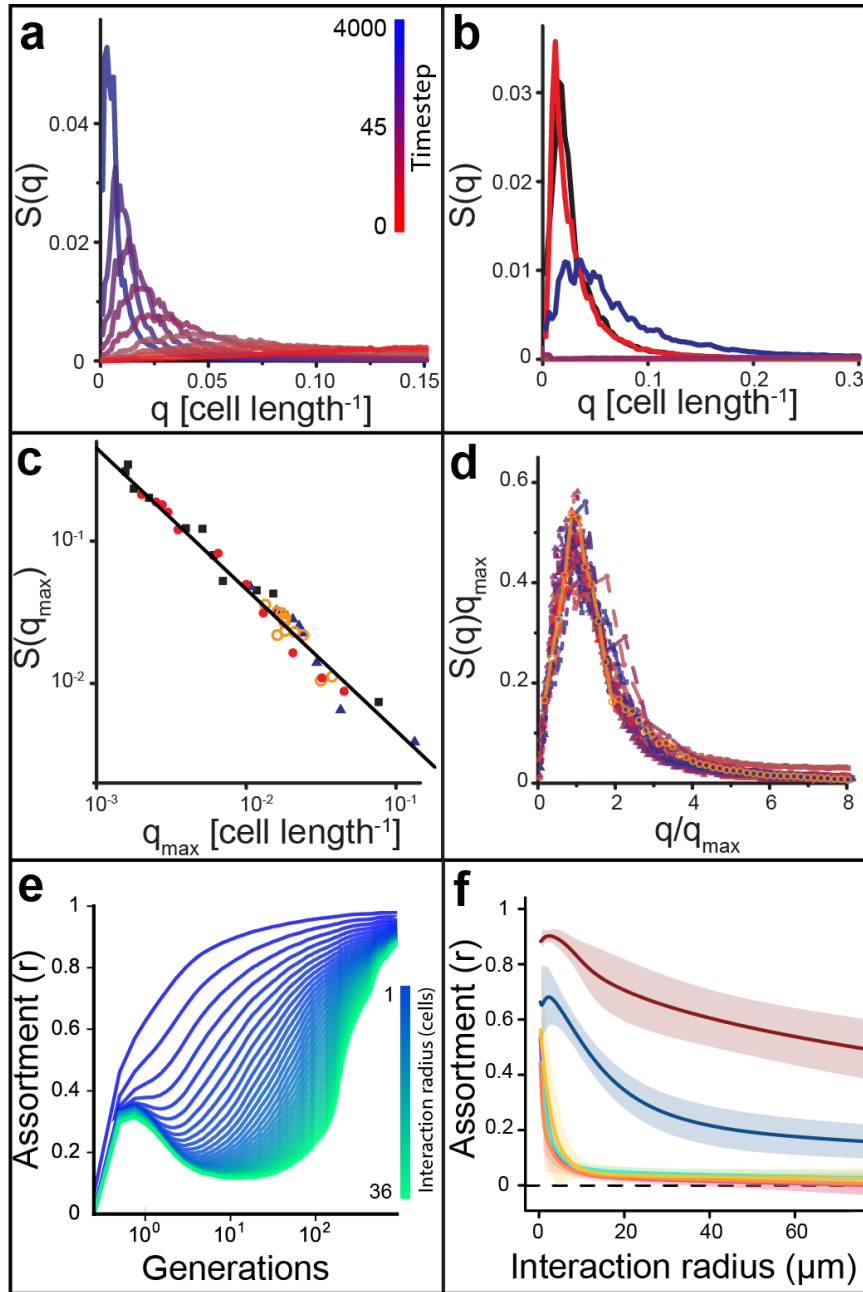


Figure 4.3. Structural analysis of models and experiments provides further evidence for killing-mediated phase separation. The static structure factor $S(q)$, plotted vs. wavenumber q for the individual based model (IBM; a) and for experiments (b). In the latter, the red and black lines depict two separate fields of view of *V. cholerae* strains C6706 and 692-79, started at an initial ratio of 1:6, while blue indicates a 1:8 initial ratio. The brown line lying flat depicts T6SS⁻ mutants, and purple indicates mutual killers grown at 17°C for 24 h (all others grown at 25°C). Mutual killing drives phase separation, increasing the peak in $S(q)$ at smaller values of q_{\max} . The relationship between $S(q_{\max})$ and q_{\max} is summarized in (c) with \circ = experimental data (25°C at a 1:6 inoculation ratio, as in panel b), \blacksquare = IBM, \bullet = PDE model ($d=0.01$), and \blacktriangle = Ising

Figure 4.3. (continued) model ($T=1$); all three models and the experiments follow a universal q_{\max}^{-1} trend. $S(q)$ curves collapse when $S(q)q_{\max}$ is plotted versus q/q_{\max} (d), indicating that all models and experiments are undergoing the same coarsening process. Color denotes model timestep, as in (a), while symbols indicate type of model or experiment, as in (c). We also examine the creation of spatial structure by calculating a biological metric, assortment (r), through time in the IBM (e) and after 24 h in experiments (f). Mutual killers were grown at 30°C (red), 25°C (blue) and 17°C (green). Non-killing controls were grown at 30°C (purple), 25°C (teal) and 17°C (orange). Plotted is the mean assortment of ≥ 3 replicate populations \pm 95% confidence intervals.

The above models assume that cells are immobile. Indeed, one might hypothesize that by increasing between-strain mixing, mobility would impede phase separation. While initial formation of clonal patches is hindered, it accelerates the process of decomposition once patches form by enhancing killing at their borders (data not shown). The universality of the Model A phase separation dynamics, even in the face of diffusive mobility, highlight the robustness of phase separation caused by T6SS-mediated killing.

To provide biological context for this process of phase separation, we calculated clonal assortment (r), for the IBM (Fig. 4.3e) and *V. cholerae* experiments (Fig. 4.3f). Assortment is a way to measure how likely a cell is to be surrounded by self, relative to its density in the population, in a chosen radius surrounding the cell. Assortment varies from -1 to 1, where -1 means the focal cell is surrounded completely by non-self, 0 represents a well-mixed population, and 1 means completely surrounded by self. Here, we calculated assortment over various length scales, from a radius of 1 μm surrounding the focal cell to 80 μm . As described previously, T6SS-mediated killing resulted in the creation of highly structured populations over long length scales, which provides high assortment (Fig. 4.3e&f). Over time, assortment increases when modeling mutual killers (Fig. 4.3e); high assortment is also observed from mutual killing experiments after 24 h

of growth (Fig. 4.3f, red and blue lines). Non-killing controls or mutual killers grown at low temperatures have some assortment when looking at small areas but that quickly declines as the interaction radius used to calculate assortment increases, as expected (Fig. 4.3f, green, purple, teal, and orange lines).

The observed high assortment created via T6SS killing mediated phase separation should protect diffusible public goods from consumption by competing strains. Therefore, to explore the effect of T6SS killing on the evolutionary stability of public goods cooperation, we introduced a cooperative good into our PDE model. We considered two competing strains: a cooperator that secretes an exoproduct into its environment at individual cost, and a non-producing cheat that, all else equal, grows faster than the cooperator, as it does not pay the cost of production. In this model, cellular growth rates depend on the local concentration of the diffusible exoproduct. In a non-spatial environment (i.e. liquid culture) or when killing is absent, there is no phase separation or assortment and cheaters dominate the population (Fig. 4.4a, yellow, red, and green lines). However, in a spatial environment (i.e. solid surface), phase separation driven by T6SS-mediated killing physically separates producers from cheaters, expanding the conditions favoring cooperation and allowing them to invade a population of cheaters from rarity (Fig. 4.4a, blue line; Fig. 4.4b).

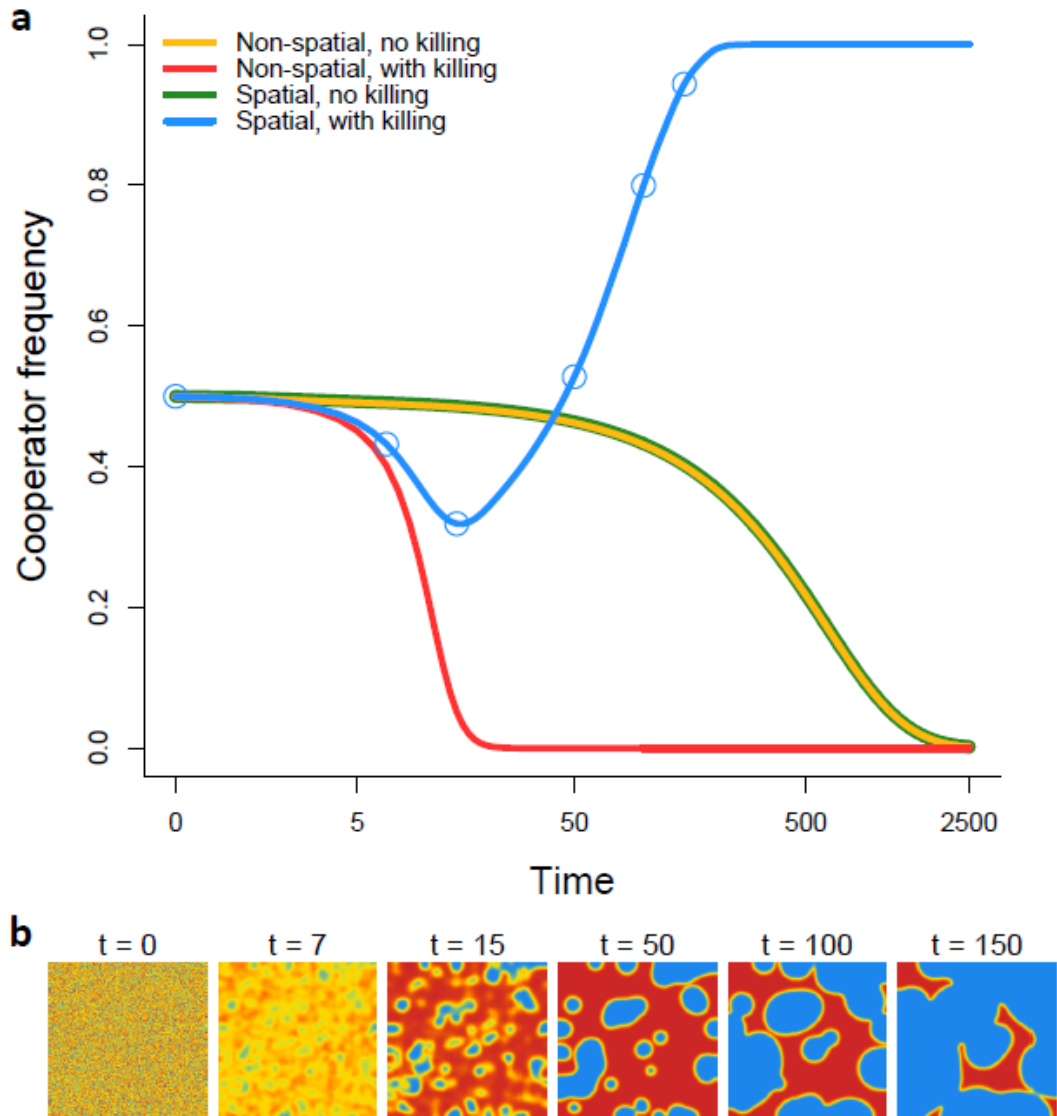


Figure 4.4. Phase separation favors the evolution of cooperation. The dynamics of competition between cooperators and cheats are shown in (a) for spatial and non-spatial (i.e., liquid culture) environments with and without reciprocal killing via T6SS. The spatial organization of cooperators and cheats on blue line during competition is shown in (b). Time-points correspond to the circles on the blue curve in (a). Cooperators are shown in blue, cheats in red, and mixtures of the two strains in yellow.

Our models and experiments demonstrate that T6SS-mediated killing rapidly structures surface-attached bacterial populations, generating favorable conditions for the evolution of public-goods cooperation (88). Does it have a similar effect in the real world, where ephemeral resources, physical disturbance, and intense competition may

impede phase separation? We approach this question phylogenetically, examining the relationship between T6SS complexity and the proportion of each genome coding for potentially-exploitable extracellular products, aka the secretome, which we used as a proxy for cooperativity. All else equal, a greater number of T6SSs and effector proteins might increase chances of killing and therefore phase separation in bacterial communities, allowing for more efficient exclusion of non-kin competitors and cheats. We constructed a Bayesian phylogenetic mixed model (BPMM) of T6SS-containing proteobacteria using 439 genomes from 26 genera (Fig. 4.5a). Secretome size is positively correlated with both the number of T6SSs (Fig. 4.5b, d) and effector proteins (Fig. 4.5c, e) present. These results are robust in univariate analyses and to the inclusion of genome size as a predictor (data not shown). We also used the BPMM to predict secretome size for each genome and show that it highly agrees with the observed secretome provided by the PSORTdb (Fig. 4.5f). As our analyses include many closely related strains (i.e., a large number of *Helicobacter pylori* strains, Fig. 4a), most (91%) of the variance in secretome size is explained by the phylogenetic relationships among strains. Nonetheless, the number of T6SSs and T6SS effectors are important predictors of secretome size, explaining 8% of the total, and 90% of the non-phylogenetic variance in secretome size. While, as with any phylogenetic analysis, alternative hypotheses cannot be ruled out entirely, these results strongly suggest that T6SS-mediated killing creates conditions that favor exoproduct evolution across a broad diversity of bacterial taxa.

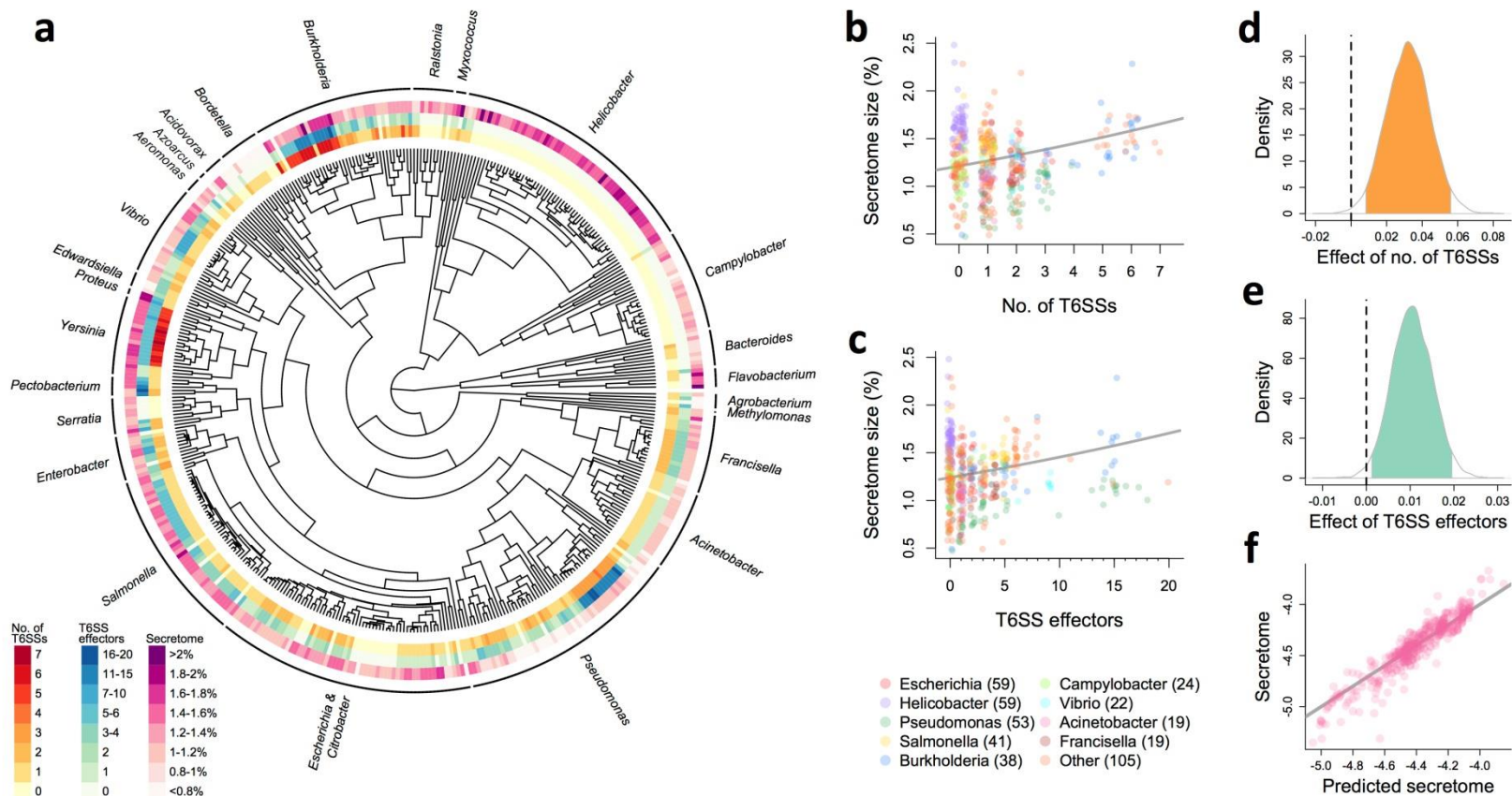


Figure 4.5. T6SS is associated with investment in other extracellular metabolites across Proteobacteria and Bacteroidetes. The phylogenetic distribution of T6SS, T6SS effectors and secretome size across the Proteobacteria and Bacteroidetes (a). Secretome size of a strain (expressed as a percentage of genome size) increases with both its number of T6SSs (b) and T6SS effectors (c). Lines are the fits of univariate BPMMs. Posterior distributions of the effects of the numbers of T6SS (d) and T6SS effectors (e) on secretome size from the multivariate BPMM. 95% credible intervals of the estimates are shaded, showing that the effects of T6SSs and effectors on secretome size are significant. Plot of observed against predicted secretome size from the multivariate BPMM (f),

Figure 4.5 (continued) including effects of the number of T6SS, number of T6SS effectors and phylogeny. The line represents a 1:1 mapping showing that the model explains observed data well.

Phase separation is a well-known driver of pattern formation in biology (135), but has mainly been investigated using either Turing activator-inhibitor feedbacks (122, 221), or positive density-dependent movement, described by the Cahn-Hilliard equation (42, 135). In this paper we describe a third general mechanism of self-organized pattern formation: targeted killing of non-kin competitors. This drives a ‘Model A’ phase separation; the kinetics of this coarsening process, described by the Allen-Cahn equation, only depend on a few small details. While we explore this process in bacteria, it is probably more general, applying to other organisms that kill adjacent non-kin (i.e., allelopathy in plants (12) and animals (114)).

In recent years, there has been a growing appreciation that many microbial behaviors requiring extracellular metabolism are susceptible to social exploitation. Here, we show how simple cell-cell aggression can, as a consequence, create a structured population favorable to cooperation. Because T6SSs are common, found in ~25% of Gram-negative bacteria (36), and microbes often live in surface-attached communities, phase-separation driven by contact-mediated killing may play a fundamental role in defining the genetic composition and ecosystem-level functionality of microbial communities globally.

4.5. Acknowledgements

We thank our colleagues for helpful discussions and critical reviews of our developing manuscript over the past year. This study was funded by a grant (MCB-1149925) from the National Science Foundation to B.K.H and the Gordon and Betty Moore Foundation.

CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS

The ability to sense and respond to environmental signals is crucial for the survival and adaptation of bacteria. In *Vibrio cholerae*, genetic competence for natural transformation and the Type VI secretion system (T6SS) used for intermicrobial aggression are regulated by three extracellular signals and associated transcriptional regulators: chitin (TfoX), quorum sensing autoinducer molecules (HapR and QstR), and extracellular nucleosides (CytR). Transformation and T6S are coordinately expressed and utilized in clinical isolates studied in laboratories; however the regulation, function, and consequences of these phenotypes are poorly understood more broadly across the species and the communities they inhabit.

The research detailed in this dissertation aimed to define novel components regulating natural competence, understand the prevalence of transformation proficiency and T6SS-mediated bacterial killing among various clinical and environmental isolates of *V. cholerae*, and investigate the evolutionary and ecological consequences of T6SS killing in bacterial communities. First, using transposon-mutagenesis of *V. cholerae* C6706, I helped identify a novel positive regulator of competence, CytR, only studied prior in *E. coli* as a regulator of nucleoside scavenging and biofilms. Second, I tested a set of environmental and clinical isolates collected over the past 100 years and determined that chitinase activity is common, transformation proficiency is rare, and constitutive T6SS killing was common among environmental but not clinical isolates. Third, I identified two strains of *V. cholerae* that mutually kill each other by their T6SSs

and observed the structural dynamics of co-culturing these bacteria experimentally via fluorescence microscopy as well as with three different mathematical models. We discovered that over time, killing competitors but not kin through T6S helps bacteria form clonal patches consistent with a universal type of phase separation called Model A. We showed that these clonal patches allow for cooperators to separate themselves from competitors and therefore increase their frequency over time, possibly allowing for the evolution of cooperative traits among those bacteria that can utilize T6SSs for bacterial killing in communities.

The results of this study suggest several areas of future research:

- Many regulatory components that activate natural transformation and type VI secretion in *V. cholerae* have been discovered, however more factors remain to be identified. TfoX, HapR, and CytR have not been shown to directly bind to gene promoters for either phenotype, thus additional factors likely exist connecting each regulatory protein to specific genes. Comparative genomics of isolates proficient or impaired for either phenotype identified in my survey followed by experimental validation could be used to define direct and indirect targets of each regulator.
- The results of my study supported a pathoadaptive hypothesis in which tight regulation of the type VI secretion system is beneficial in a human host, while constitutive killing is advantageous in the nutrient-poor environment. However, the molecular mechanism(s) conferring constitutive T6SS-mediated killing in the environmental isolates is unknown. Experimental evidence suggests that the regulatory mechanisms do not appear conserved between tightly regulated isolates

and those that are constitutive (Bernardy, Watve, Chande, Thomas, and Hammer unpublished). Full genome sequences of these environmental isolates have been obtained and are currently being analyzed by Hammer lab members to further our understanding of this regulation. Mutagenesis of these isolates can also be conducted and killing dynamics observed via fluorescence microscopy to help identify putative regulators. Mathematical models used to model T6SS killing may also be used to help determine this regulation, and suggest additional experiments to perform.

- Characterization of T6SSs of environmental isolates in this study has opened up many new areas of research to identify novel toxic effector and immunity proteins. Recent sequence analysis has shown that multiple isolates possess new effector-immunity pairs that have not been characterized before (Watve, Chande, and Hammer unpublished). Further investigation will elucidate the activity of these effectors and how they interact with their cognate immunity pair.
- Structural dynamics of *V. cholerae* mutual killers observed at 17⁰C, 25⁰C, and 30⁰C were vastly different as seen in our fluorescent microscopy images. Further studies investigating changes in gene expression (both T6SS genes and global changes) at each temperature could help explain these differences.
- Collaborations with colleagues in evolution, ecology, mathematical modeling, and physics in this study have led to the discovery of interesting consequences of T6SS activity in bacterial communities. Future studies will explain how exactly the T6SS allows these bacteria to phase separate and determine whether T6SS active bacteria may be used to manipulate a more complex community such as a gut microbiome. Current work is also being conducted to investigate cooperator/cheater dynamics to

experimentally test the cooperation model described in Chapter 4 by incorporating bacteria that are genetically engineered to be either public good cooperators or cheaters in this mutual killing system.

- The positive relationship seen between secretome size and number of T6SSs and number of effectors suggests an evolutionary relationship between T6S and cooperation. The secretome size used in this analysis included only proteins with N-terminal signal sequences for secretion outside of the cytoplasm. However, this set of proteins includes those destined for the inner and outer membranes, and the periplasm, while excluding possible cooperative products secreted from the cell that are not proteinaceous in nature, such as quorum sensing autoinducers and some antibiotics. It is of great interest to refine this analysis by more precisely defining secreted goods that could possibly be exploited by competing strains, and excluding products that are not exploitable.

The findings discussed in this dissertation have advanced the scientific understanding of processes contributing to natural transformation in *V. cholerae* as well as the prevalence and consequences of transformation proficiency and T6SS-mediated killing among various members of the species. Because transformation and T6S are important mechanisms for survival and adaptation, these studies have begun to define a role for how and why *V. cholerae* utilizes these phenotypes in their natural environment and how it may contribute to the evolution of the species as a whole. How *Vibrio cholerae* survives in nutrient-poor environments while still remaining a deadly human pathogen is poorly understood; the work described here and future studies that arise from

these findings will lead to a better understanding of how this human pathogen remains competitive in both the aquatic environment and human host.

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