**Effects of biofilm production on horizontal gene transfer to *Vibrio cholerae***

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**EFFECTS OF BIOFILM PRODUCTION ON HORIZONTAL GENE TRANSFER**

**TO *VIBRIO CHOLERAE***

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To the students of the Georgia Institute of Technology

# ACKNOWLEDGEMENTS

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# NOMENCLATURE

| | |
|---|---|
| HGT | Horizontal Gene Transfer |
| QS | Quorum Sensing |
| AI | Autoinducer |
| LCD | Low Cell Density |
| HCD | High Cell Density |
| VPS | Vibrio Polysaccharide |
| DGC | Diguanylate Cyclase |
| LB | Luria Broth |
| WT | Wild Type |

# SUMMARY

The waterborne bacterial pathogen *Vibrio cholerae* utilizes a cell-cell communication system called quorum sensing to coordinate group behavior in both a human host and in aquatic environments. Virulence genes like the cholera toxin, biofilm genes for sticky secreted attachment factors, and competence genes for DNA uptake are all regulated by this population density-dependent system. In a human host, both virulence and biofilm genes are repressed at high cell densities that occur late in infection, presumably to promote transmission upon exhausting the host's resources. However, in the natural environment, regulation is more complex. Namely, at high cell densities, repression of biofilm production is coordinated with activation of competence genes that can promote horizontal gene transfer (HGT). Based on this model, it was proposed that accumulation of biofilm material on bacterial cells could hinder the uptake of extracellular DNA in aquatic settings. In support of this hypothesis, significant decreases were detected in DNA uptake by *V. cholerae* strains engineered to overproduce biofilm. However, reductions in DNA uptake were also observed in strains that produced no biofilms. These results suggest that proper timing of biofilm formation plays an important role in the capacity of *V. cholerae* to engage in HGT, one mechanism thought to allow this pathogen to rapidly evolve in changing environments.

# CHAPTER 1

# INTRODUCTION

Many bacteria use a form of chemical communication, termed quorum sensing (QS), to regulate gene expression in response to population density. This is accomplished because the bacteria make and secrete chemical signals called autoinducers (AIs). When the population reaches a high enough density, or a quorum, the bacteria respond to the accumulation of AIs and initiate a signal transduction pathway to activate and repress genes controlling numerous behaviors (Ng and Bassler, 2009). In many disease-causing bacteria, QS is thought to enable the cells to collectively activate virulence factors within a host in a timed manner. In the waterborne, human pathogen *Vibrio cholerae*, responsible for the fatal cholera diarrheal disease, QS controls numerous genes that are critical for interaction with a human host, and in a marine environment, which is the focus of this thesis. However, quorum sensing alone is not sufficient for *V. cholerae* to regulate certain genes in aquatic settings. Specifically, to express genes to become naturally competent and take up extracellular DNA, one mechanism of horizontal gene transfer (HGT), *V. cholerae* requires both QS AIs and the presence of chitin, which is a polymer of the sugar N-acetylglucosamine and the material that comprises the shells of many aquatic organisms (Sun *et al*, 2013).

HGT is the process by which bacteria are able to incorporate foreign genetic material into their chromosome and acquire new traits to better adapt to their environment. The DNA acquired could include genes for antibiotic resistance, virulence factors, or novel metabolic capabilities that are particularly useful for surviving harsh environments (Sun *et al*, 2013). QS not only controls natural competence for DNA uptake in *V. cholerae*, but also many other phenotypes including virulence and biofilm

formation (Zhu *et al* 2002; Hammer and Bassler, 2003). While numerous genes are activated and repressed by QS in response to cell density (Ng and Bassler, 2009), it remains poorly understood how this timing contributes to many QS-controlled behaviors.

Previous studies (reviewed in Ng and Bassler 2009) have shown that at low cell density (LCD), when AI levels are low, the QS pathway results in the expression of virulence genes, such as the *ctx* gene for the cholera toxin, as well as *vps* genes, which synthesize Vibrio polysaccharides crucial in the production of biofilm (Zhu *et al* 2002; Hammer and Bassler 2003). Conversely, at high cell density (HCD) in the presence of chitin, when autoinducer levels are high and virulence and biofilm genes are repressed, genes coding for factors comprising an apparatus for taking up extracellular DNA are activated. These genes are regulated by HapR, which acts as both an activator and a repressor of gene expression (Bardill *et al,* 2011). The proposed explanation for why *V. cholerae* uses quorum sensing to turn off virulence genes when in a group, in contrast to other pathogens like *Pseudomonas aeruginosa* that turn on similar genes when at high densities, is that *V. cholerae* is a transient pathogen that thrives by entering a human host, replicating, and exiting into the aquatic environment to find a new host (Hammer and Bassler, 2003; Srivastava *et al,* 2011). Therefore, instead of coordinating virulence expression at HCD in the host, as in other bacteria, the downregulation of biofilms and virulence at HCD is proposed to allow for a timely exit by *V. cholerae* after maximally utilizing the resources within the human host. These virulence factors are responsible for the diarrheal disease seen in infected hosts; however, human genetic and nutritional factors allow for many hosts to be asymptomatic despite harboring virulent *V. cholerae* cells (Nelson *et al,* 2009).

Distinct from a human host, in aquatic settings where *V. cholerae* is commonly found, this model for the timing of virulence factors is no longer relevant. *V. cholerae*

communities in the environment typically attach to chitinous surfaces such as crab shells or zooplankton molts (Meibom *et al*, 2005). The insoluble chitin can be digested by *Vibrio*-derived chitinases into soluble oligomers including $(GlcNAc)_2$, which serves as a signal to induce natural competence for DNA uptake. Natural transformation is one mechanism of HGT. HGT can also be accomplished by transduction via viral infection or by conjugation where direct contact between two bacterial cells allows for passage of genetic material via a sex pilus (Sun *et al,* 2013).

Until recently, natural competence by *V. cholerae* was predicted based on genome sequence, but had not been demonstrated (Heidelberg, *et al*, 2000). By studying the natural environment in which *V. cholerae* commonly resides, it was discovered by the Schoolnik group that chitin itself serves as a signal to induce the natural competence genes of *V. cholerae* required to take up DNA (Meibom *et al,* 2005). The presence of $(GlcNAc)_2$ induces the expression of the TfoX regulator and, as discussed, QS promotes production of HapR. Both of these regulators have been shown to be required for activation of numerous DNA uptake genes including *comEA*, which encodes a component of a DNA uptake apparatus (Antonova and Hammer, 2011). The impact of biofilms on transformation in *V. cholerae* is not yet understood, though production of biofilm appears deeply intertwined with both pathways leading to competence.

Additionally, bacteria often produce many diguanylate cyclase and phosphodiesterase enzymes which work to produce and degrade c-di-GMP, an intracellular second messenger molecule that promotes the expression of attachment factors and biofilm formation, (Massie *et al*, 2012; Zhao *et al*, 2013). HapR accumulation at HCD inhibits c-di-GMP production and the loss of this small molecule prevents the transcriptional activators VpsT and VpsR from inducing expression of the *vps* genes (Waters *et al*, 2008; Srivastava *et al*, 2011). Thus HapR accumulation at HCD leads to

repression of biofilm formation concomitant with activation of competence genes for DNA uptake.

The levels of c-di-GMP in the cell can be experimentally controlled independently of QS by genetic modification to *V. cholerae*, resulting in alterations in the amount of biofilm produced (Srivastava and Waters, 2012). In this study such manipulations are exploited to test whether inappropriate biofilm formation can alter the capacity of *V. cholerae* to engage in transformation, and whether upregulation of competence genes with the downregulation of biofilm production by the signaling network (Figure 1) maximizes the potential for DNA uptake. Therefore, unlike wildtype *V. cholerae* strains that produce biofilms at LCD (Figure 1A) and then stop expressing them at HCD (Figure 1B), *V. cholerae* mutant strains that either make no biofilms or make biofilms constitutively at both LCD and HCD were engineered.

Inappropriate formation of biofilm was tested to determine whether it can hinder uptake of extracellular DNA and whether the production of biofilm is carefully regulated to maximize the potential for DNA uptake in the natural environment while still allowing for the stability that a biofilm can afford at LCD (Berk *et al*, 2012). The extent to which biofilm formation is regulated through quorum sensing suggests a deeper involvement of biofilms in the ability of *V. cholerae* to accomplish HGT, which is thought to play an important role in the survival of bacteria in changing environments. This study contributes to our understanding of how the levels and regulation of biofilms can impact HGT by *V. cholerae* in marine environments.

# CHAPTER 2

# MATERIALS AND METHODS

### Bacterial strains and culture conditions

The *V. cholerae* strains used in this study are all derivatives of the C6706 El Tor WT strain and *Escherichia coli* S17-λpir strains were used for cloning and conjugation to *V. cholerae*. All strains were grown in LB or on LB agar at 37°C with the appropriate antibiotics added if needed.

Table 1

| ***V. cholerae* strains** | **Features** | **Source** |
|---|---|---|
| C6706str | El Tor wild type | Antonova and Hammer, 2011 |
| SLS349 | Δ*luxO* | Waters *et al*, 2008 |
| EA305 | *tfoX** | Antonova, Bernardy, and Hammer, 2012 |
| EA281 | Δ*luxO, tfoX** | Antonova, Bernardy, and Hammer, 2012 |
| SKW045 | Δ*vpsR* | This study |
| SKW035 | *tfoX**, Δ*vpsR* | This study |
| SKW037 | Δ*luxO,* Δ*vpsR* | This study |
| SKW041 | Δ*luxO, tfoX**, Δ*vpsR* | This study |

| **Plasmids** | **Features** | **Source** |
|---|---|---|
| pEVS141 | control vector | Dunn *et al*, 2006 |
| pCMW75 | DGC vector with kanR | Waters *et al*, 2008 |
| pCMW75-cmR | DGC vector with cmR | This study |

## Genetic engineering of mutant *V. cholerae* strains

To uncouple upregulation of competence genes and downregulation of biofilm genes by QS (Figure 1), using standard "allelic exchange" methods, a *V. cholerae vps* deletion mutant was created that is unable to form biofilms (*Δvps*) (Thelin and Taylor, 1996; Hammer and Bassler, 2003). A constitutive biofilm-producing strain (Δ*hapR*) was also engineered that makes biofilms at LCD and HCD, irrespective of QS function (Hammer and Bassler, 2003). Specifically, plasmid pCMW75 described previously (Waters *et al*, 2008) was used, which can be induced with IPTG to express a diguanylate cyclase previously shown to activate *vps* transcription and promote biofilm formation, independent of QS. The pCMW75 plasmid (labeled as "DGC vector" in Figure 2) was introduced by conjugation from an *Escherichia coli* donor into various *V. cholerae* strains tested (Table 1). To utilize this plasmid in more backgrounds, a version was constructed with a chloramphenicol resistance (cmR) marker instead of the original kanamycin resistant (kanR) marker. The pCMW75 vector was digested at BamH1 and Xho1 sites to cut out the KanR marker. A BamH1/Xho1 fragment from plasmid pSLS3 that carried the cmR gene was ligated to pCMW75. The resulting pCMW75-cmR plasmid was electroporated into *Escherichia coli* S17-λpir cells which were then used to mate the plasmid into various *V. cholerae* strains (Table 1). Empty vector pEVS141, which does not carry the diguanylate cyclase gene for c-di-GMP production was used as a control. Biofilm production was measured by minimal biofilm-eliminating concentration (MBEC) crystal violet biofilm assay (Massie *et al*, 2012; Hammer and Bassler, 2003). These strains and methods were used to determine how the different genotypes affect formation of biofilms.

## DNA uptake efficiency

The standard chitin-induced transformation assay used to quantify the efficiency of DNA uptake by *V. cholerae* involves incubating the bacterial cultures in artificial

seawater on a chitinous crab shell chip to induce natural competence (Meibom *et al*, 2005). The bacteria are then incubated for an additional 24 hours with exogenous DNA (eDNA) marked with the KanR gene. Finally, *V. cholerae* cells are removed from the crab shells by vortexing and plated onto LB medium containing or lacking antibiotic. The efficiency of DNA uptake is calculated as the number of colonies that grow on the selective plates (LBkan) divided by the number of colonies on permissive plates (LB). This assay quantifies how effectively bacteria take up DNA, and was used to determine whether deficiency or proficiency in biofilm formation alters this process.

*V. cholerae* utilizes QS to coordinate its virulence genes (Figure 1A) to effectively infect and then be expelled from its human hosts into aquatic environments. But QS also plays an important role in aquatic settings where attachment to zooplankton molts and other material in the water column may aid in transmission back into humans. All of the described techniques above were utilized to determine whether the repression of biofilms at HCD along with the expression of competence genes in *V. cholerae* is a synchronized switch to maximize DNA uptake in the natural environment.

## Statistical Methods

Throughout this study, statistical significance was determined through the use of T tests, which allows for a comparison of two conditions with multiple trials of each. The standard $p < 0.05$ was used to determine significance, but in all cases, the p-value was less than 0.001.

# CHAPTER 3

# RESULTS

## Biofilm Assays

MBEC biofilm assays were performed to quantify the average amount of biofilm accumulation in each strain of *V. cholerae* tested (Figure 2). Lab wildtype (WT) strain C6706 produced reasonable biofilms as described prior (Hammer and Bassler, 2003), and the addition of IPTG did not effect biofilm levels (Fig. 2, bars 1-2). There was a decrease in biofilms for the WT strain with the control vector, but this is likely an effect of the addition of the antibiotic required to maintain the vector; and addition of IPTG to this strain had no effect (Fig. 2, bars 3-4). The WT strain carrying the DGC vector (pCMW75) was similar to WT when not induced with IPTG, but showed a significant increase in biofilm when IPTG was added ($p<0.0001$) (Fig. 2, bars 5-6). These results confirm that biofilm formation is enhanced by induction of a DGC enzyme that produces c-di-GMP (Waters *et al*, 2008).

Similar tests were performed with a *V. cholerae* strain engineered to be unable to produce biofilms, to confirm that DGC induction by IPTG could not enhance biofilm formation in a strain carrying a deletion of a *vps* biosynthesis gene (Hammer and Bassler, 2003). Biofilm levels were minimal in a *V. cholerae* Δ*vpsR* mutant, and were unaltered when the strain carryied vectors, or when IPTG was added (Fig. 2, bars 7-12). Biofilm could not be recovered for Δ*vpsR* strains induced with IPTG to produce the DGC as no polysaccharides are able to be produced. These control strains served as an additional measure to ensure there were no other contributors to biofilm growth other than the *vps* genes.

Finally, a *V. cholerae* strain was tested that carries a deletion in the gene for the QS regulator HapR, and is "locked" at LCD and constitutively produces biofilms due to the inability to repress VpsT and VpsR (Hammer and Bassler, 2003; Srivastava and Waters, 2012). As expected, biofilm levels were significantly higher in the Δ*hapR* strain than in WT ($p<0.0001$) and remained at these levels with the addition of IPTG (Figure 2, bars 13-14). There was a decrease in biofilms seen with the addition of the control vector, but again this is likely an effect of the needed antibiotic (Figure 2, bars 15-16). The Δ*hapR* strain with the DGC vector also had significantly higher biofilm levels than WT, but addition of IPTG did not increase levels higher than the Δ*hapR* strains without the vector (Figure 2, bars 17-18). These results confirm that the constitutive biofilm producers always had significantly higher biofilm growth regardless of whether they overexpressed the DGC vector or not. This test confirmed that the vectors behaved as expected, and were consistent with previous studies showing that the vector does not inhibit cell growth or viability in the process (Waters *et al*, 2008).

**DNA Uptake Assays**

DNA uptake assays on chitin were performed with triplicate samples of each strain tested above, using established methods (Meibom *et al*, 2005). The WT C6706 *V. cholerae* strain had a transformation frequency of ~ 1.0E-5, consistent with previous studies (Antonova and Hammer, 2011) (Figure 3, bar 1). The control vector did not significantly change the transformation frequency of the WT strain, as expected (Figure 3, bar 2). The WT strain carrying the DGC vector and induced with IPTG had a transformation frequency that was significantly decreased relative to the WT strain (Figure 3, bar 3, $p<0.0001$). The same pattern was seen in the *V. cholerae* Δ*luxO* strain, which has a mutation that results in constitutive expression of HCD genes (Figure 3, bar 6-8). Because a Δ*luxO* strain is effectively constitutive for QS, it displays a higher transformation frequency than WT (Figure 3, bar 6), as shown prior (Meibom *et al*,

2005). However, like WT, the DNA uptake by the Δ*luxO* mutant was not changed by the control vector (Figure 3, bar 7), and was significantly lowered when the DGC expression vector was present (Figure 3, bar 8, p = 0.0002).

Strains of *V. cholerae* (*tfoX**) can be genetically engineered to express the TfoX regulator (Figure 1B) irrespective of the presence of chitin, by expressing the *tfoX* gene from a constitutive promoter. It has already been documented that *tfoX** strains have higher transformation frequencies than WT (Meibom *et al*, 2005) and similar results were observed here in an otherwise WT (Figure 3, compare bars 4 and 1) or Δ*luxO* background (Figure 3, compare bars 9 and 6). Production of c-di-GMP from the DGC vector by IPTG induction in these two strains led to a modest (Figure 3, bar 4 versus 5) to negligible (Figure 3, bar 9 versus 10) change in transformation frequency compared to the control vector. These results showed that the overexpression of biofilm production decreases the transformation frequency of *V. cholerae* strains in WT and ΔluxO strains but had little to no effect in the *tfoX** strains from these backgrounds, respectively.

In the DNA uptake assay in which a biofilm-defective *V. cholerae* Δ*vpsR* strain was tested alongside the WT C6706 strain, the presence of a constitutive *tfoX** allele significantly enhanced transformation frequencies in all backgrounds (Figure 4, bars 2, 4, 6, and 8). However, in the wild type strain lacking *tfoX**, a significant decrease in transformation frequency was seen when the *vpsR* gene was deleted (Figure 4, bars 1 and 3, $p<0.0001$). However, the transformation frequency seen in either the *tfoX**, Δ*vpsR* or the Δ*luxO*, Δ*vpsR* double mutants was unaffected by deletion of *vpsR* (Figure 4, compare bars 4 and 2, and bars 8 and 6). These results indicate that the absence of a biofilm lowers transformation in strains where *tfoX* expression is chitin-induced, but not in *tfoX** strains, in which *tfoX* is overexpressed irrespective of chitin.

# CHAPTER 4

# DISCUSSION

In the well-studied pathogen *Pseudomonas aeruginosa*, quorum sensing activates virulence factors and biofilm only when they have reached a high cell density, presumably allowing this chronic pathogen to more effectively colonize its host (Davies *et al*, 1998). However, in *V. cholerae*, the opposite is true as the bacteria express virulence factors and biofilm at low cell density (Hammer and Bassler, 2003). The proposed role of QS in human hosts is that AI accumulation allows *V. cholerae* to transit out of the host upon effectively reaching a HCD (Hammer and Bassler, 2003). This model is consistent with observations that *V. cholerae* strains "locked" at HCD make no biofilms and are avirulent in mouse models, while strains "locked" at LCD produce robust biofilms and are virulent (Zhu *et al*, 2002; Zhu and Mekalanos, 2003). Thus it has been proposed that virulence genes and biofilm producing genes are expressed at LCD when ingested bacteria are presumably entering the small intestine, and then are repressed after replicating to HCD. This concordant regulation of multiple traits through a single QS pathway is thought to provide an elegant mechanism of synchronizing the needed phenotypic changes for a timely exit from the infected host. However, *V. cholerae* spends the majority of its life in marine environments and the need for this precisely regulated system is less clear in aquatic environments. This study reveals a potential inverse relationship between biofilm production and DNA uptake.

Overproduction of biofilm through the use of an inducible DGC plasmid significantly decreases the transformation frequency of *V. cholerae* (Figure 3). However, additional work needs to be done to determine whether this result is due to the accumulation of biofilm material on the outside of the cells impeding DNA uptake or

other issues with overexpression of a diguanylate cyclase. It is possible that accumulation of c-di-GMP inside the cells affects DNA uptake in a manner that is unrelated to biofilm production. For example, it is known that c-di-GMP inside *V. cholerae* cells not only binds to VpsT and VpsR to alter biofilms, but in other bacteria also binds numerous factors including flagellar transcription factor FleQ, motility-controlling PilZ, and phase transition regulator PopA (Hengge, 2009). It is possible other direct cellular targets of c-di-GMP include components of the DNA uptake apparatus and not biofilm accumulation (Sun *et al,* 2013). One could test this by expressing the *vps* operons from a promoter that does not depend on VpsT or VpsR, which are both c-di-GMP dependent. If biofilms are unaffected and yet one saw a decrease in transformation, this would support the idea that c-di-GMP affects DNA uptake through other methods. Such experiments have not yet been done and it would be interesting to uncover direct c-di-GMP targets that alter transformation.

We show that *V. cholerae* mutants (Δ*vpsR*) that produce no biofilms have a reduced transformation frequency compared to WT C6706 (Figure 4). Therefore, it seems likely that not only does an overproduction of biofilm affect the ability of *V. cholerae* to take up extracellular DNA, but minimal biofilm also impairs competence. At HCD, V. cholerae has established a biofilm that enhances its ability to stick to a chitin food source. At this population density, it seems to be beneficial to repress the production of biofilm in order to avoid creating a physical barrier to the exchange of DNA between *V. cholerae* and its neighbors within the biofilm community. Conversely, repressing DNA uptake until HCD has been reached could ensure that the community within the newly formed biofilm is more likely to contain DNA that will have either a neutral or beneficial effect on the recipient.

Hence, this study suggests it may be beneficial for *V. cholerae* to tightly regulate the balance of biofilm formation and DNA uptake through the quorum sensing pathway. In future studies, directly testing the expression levels of biofilm genes in response to the overexpression plasmids will ensure that the observed effects are not due to alternative actions of the vector inside the cell. Such studies could include fusions of vps genes to luciferase in order to quantify the activation or repression of the genes responsible for biofilm formation in response to various conditions. A broader approach would be to run RNA-seq in order to see the overall genes that are activated or repressed in response to the activation of the biofilm production pathway. This test could expose any unwanted side effects from the overexpression DGC vector that were undetected previously. It could also shed light on new parts of the pathway that have yet to be discovered and are responsible for the phenotypic results seen in this study.

Further work using co-culture assay conditions could also provide valuable insight where extracellular DNA is provided by donor cells to test the effects of biofilm formation when *V. cholerae* is existing in nature, as opposed to an artificial environment (Meibom, 2005). In this experiment, a donor strain, constructed to be deficient for DNA uptake, would be incubated alongside the proficient recipient strain on chitin with no extracellular DNA added. Both strains would have different antibiotic markers and selective media would be used to quantify only recipients that successfully acquired and incorporated donor DNA onto its chromosome. This would likely mimic the DNA conditions seen in a natural setting where DNA is provided by lysed donor cells rather than the current manner where DNA is added artificially to determine whether the results seen in this study are reduced or exacerbated by the change. There are still many unanswered questions on how biofilm is able to affect the horizontal gene transfer of *V. cholerae*. This study is a step towards fully understanding the evolutionary advantages of

maintaining the quorum sensing system in nature which, when introduced into human hosts, regulates the virulence of *V. cholerae.*

APPENDIX A

# FIGURES

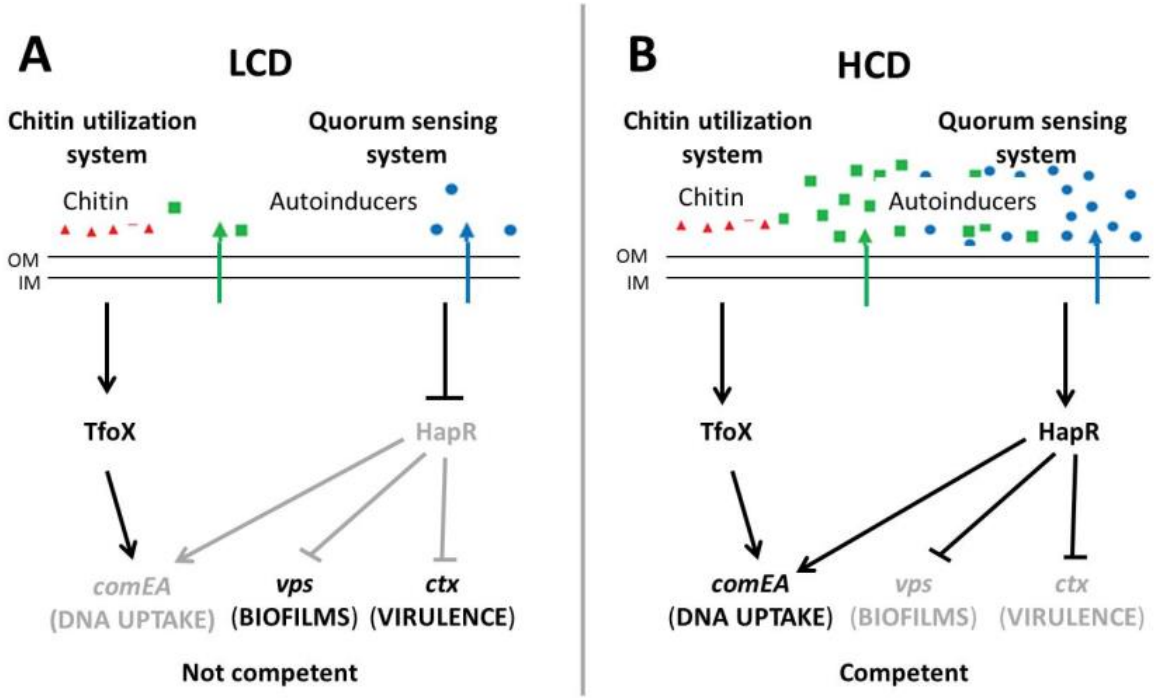


Figure 1: Model of the QS-dependent regulation of competence, biofilm, and virulence genes in *V. cholerae* at A) low and B) high cell densities.

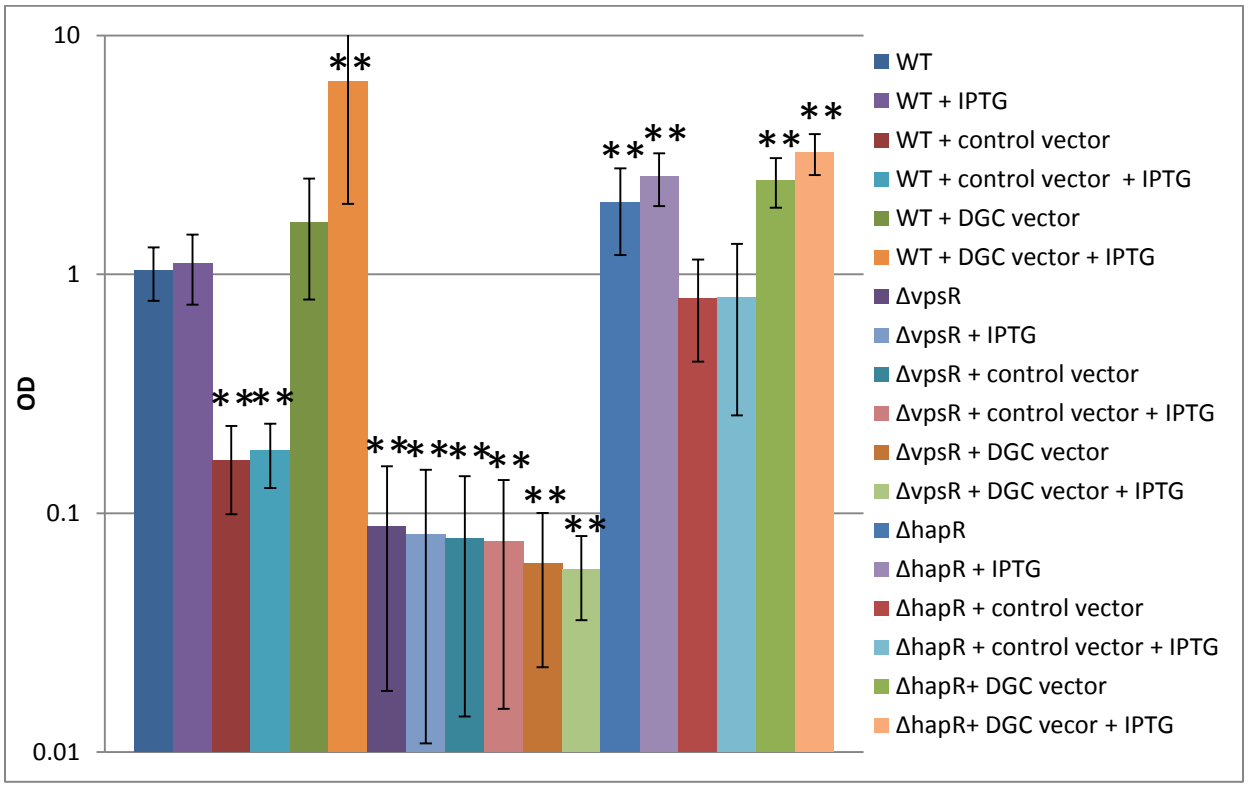


Figure 2: Biofilm assay mean optical densities +/- standard deviation of eight replicates of wild type (WT), *ΔvpsR*, and Δ*hapR* strains containing control and inducible diguanylate cyclase (DGC) overexpression vectors. ** indicates a significant ($p<0.001$) difference of transformation frequencies from the wild type (WT).

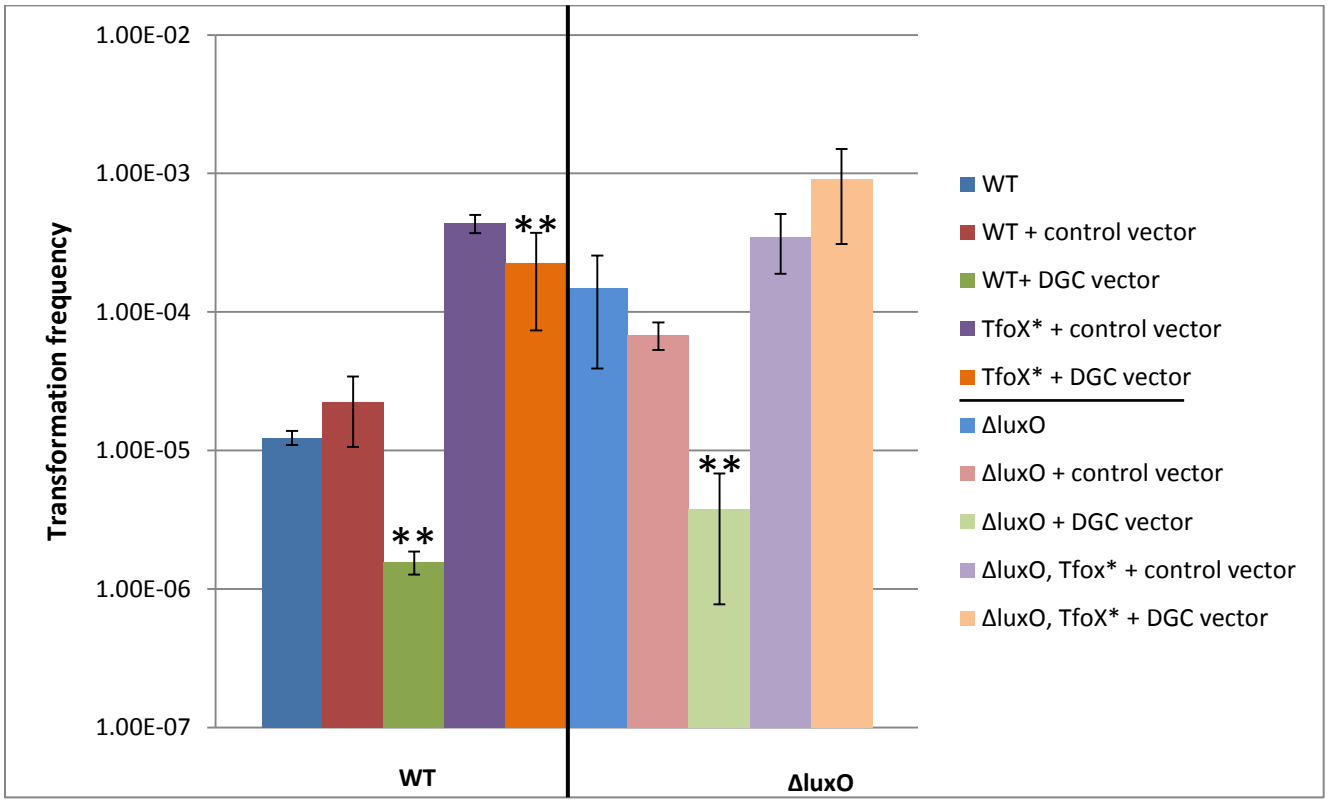


Figure 3: Chitin assay mean transformation frequencies +/- standard deviation of three replicates of wild type (WT) and *ΔluxO* strains containing control and inducible diguanylate cyclase (DGC) overexpression vectors all grown with IPTG. ** indicates a significant ($p<0.001$) difference of transformation frequencies between strains with the control vector (pEVS141) and strains with the DGC vector (pCMW75).

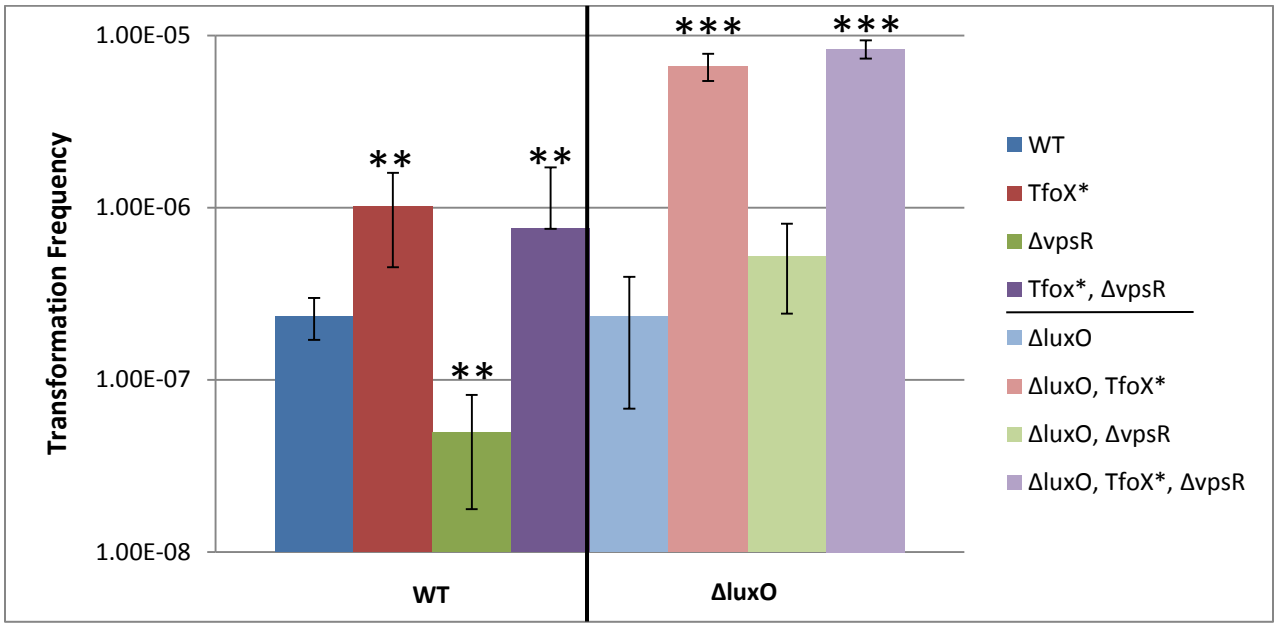


Figure 4: Chitin assay mean transformation frequencies +/- standard deviation of wild type (WT) and *ΔluxO* strains with and without the *ΔvpsR* mutation. ** indicates a significant ($p<0.001$) difference of transformation frequencies from the wild type (WT), and *** indicates a significant ($p<0.001$) difference from the Δ*luxO* strain.

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