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Creation of a viable *csrA* mutant in *Vibrio cholerae*

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Creation of a viable *csrA* mutant in *Vibrio cholerae*

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Report

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Creation of a viable *csrA* mutant in *Vibrio cholerae*

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Vibrio cholerae, the causative agent of cholera, has been a lethal enteric pathogen to humans for most of recorded history. Even though it is well studied, it still kills many people every year due to rapid and severe dehydrations from diarrhea. Part of what makes *V. cholerae* such an effective pathogen is its ability to control virulence factors depending on its environment. ToxR is a major virulence protein that has upstream control of most of the virulence genes that are turned on when in a human host. Two of the most critical virulence factors, toxin coregulated pilus and cholera toxin are controlled by ToxR.

CsrA is a protein that regulates many cellular functions in *V. cholerae*, including glycogen synthesis, motility, and biofilm production. Preliminary data suggests a link between CsrA and the regulation of ToxR. In order to study CsrA as it relates to ToxR regulation, a *csrA* mutant must be generated in *V. cholerae*. CsrA plays such an important role in glycogen metabolism that a *csrA* mutant is not viable due to excessive glycogen levels. In order to make a viable *csrA* mutant, glycogen synthesis has to be turned off. In this research, I attempt to make a viable *V. cholerae csrA* mutant by deleting *csrA* in a strain that is deficient for glycogen synthesis (*glg*). Normally without CsrA, glycogen in

the cell would increase to a detrimental level. Since a *glg*$^{-}$ *csrA*$^{-}$ mutant lacks the ability to make glycogen, the levels never reach a lethal level, allowing the mutant to survive without functional CsrA. Such a glg- csrA- double mutant's ToxR regulation can be studied by growth in various media by measuring OmpU and OmpT expression.

Using PCR, restriction enzymes, and DNA ligase, a suicide plasmid was created containing sequences that flank the *csrA* gene but instead of the *csrA* gene, a chloramphenicol resistance cassette was inserted. Through bacterial conjugation this plasmid was introduced into three *V. cholerae glg*$^{-}$ strains. Allelic exchange was carried out utilizing the homology between the DNA flanking wild type *csrA* and the *csrA* deletion with chloramphenicol cassette. This first crossover event was initiated with the requirement of the π protein for the plasmid to replicate. Without the *pir* gene to create π protein, selection for antibiotic resistance required that the plasmid integrate into the genome. This was selected based on the plasmid encoded ampicillin resistance. After the second crossover event, there were two possible outcomes of excision: reverting to wild type *csrA* or retention of the *csrA* mutation. The *csrA* mutant was selected based on its sucrose and chloramphenicol resistance and ampicillin sensitivity.

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Chapter 1: Introduction

Vibrio cholerae, the causative agent of cholera, has been a lethal enteric pathogen to humans for most of recorded history. Even though it is well studied, it remains a significant problem and still kills many people every year due to rapid and severe dehydration from diarrhea. Part of what makes *V. cholerae* such an effective pathogen is its ability to control virulence factors. *V. cholerae* can turn on and off the genes depending on its environment (Kaper, Morris, & Levine, 1995). By better understanding the genetic control of virulence, someday, deaths due to cholera will be greatly reduced or disappear.

Vibrio cholerae successfully inhabits two different habitats: aquatically in association with copepods and shellfish, and within human hosts, where transmission between hosts occurs via contaminated food or water (Matson, Withey, & DiRita, 2007). Many genes have to be controlled and changed based on these two habitats. Genes for virulence should not be expressed until the bacteria are inside the small intestine of a human host and at a high density. *V. cholerae* communicate with quorum sensing, a form of chemical communication between bacteria that involves the secretion of chemicals called autoinducers. When the concentrations of the autoinducers are high enough in a bacterial community, bacteria in that community control certain genes in unison (Miller & Bassler, 2001). One of the main ways *V. cholerae* controls virulence is through ToxR (Skorupski & Taylor, 1997). ToxR along with ToxS, controls numerous genes required

for virulence, including genes coding for the toxin coregulated pilus (TCP), cholera toxin (CT), and the outer membrane porin OmpU (Miller & Mekalanos, 1988).

CsrA (carbon storage regulator) is a global regulator that controls many different processes in multiple species of bacteria, including *E. coli* and *V. cholerae*. CsrA regulates numerous cellular functions including glycogen synthesis, motility, and biofilm production. One major job of CsrA is to control the intracellular carbon flux by upregulating glycolysis and turning off gluconeogenesis and glycogen synthesis. In *E.coli* without functional CsrA, glycogen levels increase to the point that the cells are no longer viable in rich media such as LB (Romeo, Gong, Liu, & Brun-Zinkernagel, 1993). *E. coli* double mutants in glycogen synthesis and *csrA* were able to grow on LB medium (Timmermans & Melderen, 2009). It is presumed that without glycogen synthesis, the levels of glycogen will never reach a lethal level and thus *csrA* mutants can be cultured and studied on LB media.

In order to be able to study *csrA* mutants of *V. cholerae*, the bacteria need to able to grow under normal lab conditions in LB media. The purpose of this project was to create a double mutant *V. cholerae* strain containing a mutation in glycogen synthesis and also in *csrA*. With viable *csrA* mutants, the regulation of CsrA can be studied in relation to the major virulence regulator ToxR.

Chapter 2: Review of the Literature

Preface

Vibrio cholerae is a species of curved Gram-negative rod bacteria that causes a severe and sometimes lethal diarrheal disease called cholera in humans. The characteristic 'rice-water' stool quickly causes humans to become dehydrated. Without proper medical attention and rehydration, death can occur in less than a day after infection (Ritchie & Waldor, 2009). Even though this disease has been infecting humans throughout most of history and has been studied since the dawn of epidemiology and microbiology, cholera still affects about 5 million people a year. *Vibrio cholerae* has a unique and complex set of factors that enable it to quickly and efficiently cause disease and spread rapidly through contaminated water (Kaper et al., 1995).

Virulence factors

A defining feature of all *Vibrio* species is two circular chromosomes (Trucksis & Michalski, 1998). The second chromosome contains fewer essential genes than the larger first chromosome. It is likely that the second chromosome was originally acquired as a megaplasmid. After the precursor *Vibrio* acquired the plasmid, it gained essential genes that made it indispensible for *Vibrio* (Trucksis & Michalski, 1998).

The evolution of *V. cholerae* to be the virulent bacterium it is today is largely due to lateral gene transfer. The two most critical virulence factors, toxin co-regulated pilus (TCP) and cholera toxin (CT), were picked up through two lateral gene transfers (Waldor & Mekalanos, 1996). It is presumed that the acquisition of both TCP and CT were two

separate steps in the evolution of virulent *V. cholerae*: first, a precursor strain, through unknown means, gained the TCP pathogenicity island, and secondly, the precursor became infected with a bacteriophage CTXΦ, which enabled the precursor to make CT. Virulence factors are found on both plasmid and phage mobile genetic elements (Waldor & Mekalanos, 1996).

TCP is encoded by *tcpA-tcpG* genes and is a bundle forming type IV pilus and is the best characterized colonization factor for *V. cholerae* (Cotter & DiRita, 2000). *tcpA* codes for the major pilin subunit of TCP. Except for *tcpF*, the remainder of *tcp* genes code for components of the apparatus for TCP biogenesis (Krebs & Taylor, 2011). There are three main functions of TCP: mediation of human intestinal colonization and microcolony formation, secretion of the colonization factor TcpF, and it is the receptor for CTXΦ (Krebs & Taylor, 2011). Volunteers given a mutant strain of *V. cholerae* without functional TCP did not exhibit diarrhea, and, subsequently, no vibrios were recovered from their stool. TCP is required for colonization in human intestine (Herrington, Hall, Losonsky, Mekalanos, & Taylor, 1988). TCP enhances bacterial interactions, thereby facilitating microcolony formation in the host's intestinal epithelium. The microcolonies help to protect the bacteria from the host's defenses (Kirn & Taylor, 2005, Krebs & Taylor, 2011). TcpF is a soluble protein that is found within the TCP biogenesis apparatus and plays a vital role in colonization of host intestine. Strains of *V. cholerae* that are deficient in TcpF were defective in colonization (Kirn & Taylor, 2005). These *tcpF* mutants were able to adhere to intestinal epithelial cells and still produced microcolonies, but were severely impaired in colonization of infant mouse

intestine (Kirn & Taylor, 2005). Krebs and Taylor (2011), found a fourth function of TCP. TCP also protects the bacterium by forming matrices that engulf the bacterial cells later in infection. These TCP matrices gave the bacterium better survival against bile and other host antibacterial compounds (Krebs & Taylor, 2011).

CTXΦ is a filamentous lysogenic bacteriophage that carries with it the genes *ctxAB* which code for CT (Cotter & DiRita, 2000). CT is an A-B type exotoxin that causes secretory diarrhea associated with cholera. CT contains one A subunit and five identical B subunits. The A subunit gets translated as a single protein. A protease later cleaves it into two peptides, A_1 and A_2, held together by a disulfide bond. The five B subunits form a homopentamer with a pore in the center, where the A subunit attaches (Sanchez & Holmgren, 2008). The B subunit is what enables CT to bind to the intestinal epithelial cell via the ganglioside G_{M1} (Spangler, 1992). Once attached to the intestinal epithelial cells, the A subunit enters the cell. There is a lag of 15 to 60 minutes before the effects of CT are observed. The lag is needed to allow the A_1 protein to translocate through the cell membrane to the basolateral membrane of the host intestinal epithelial cells. The A_1 peptide travels through the host cell in an endosome and eventually comes into contact with a host cell G protein (Gill & Meren, 1978). The G protein regulates the activity of adenylate cyclase; adenylate cyclase mediates the transformation of ATP to cyclic AMP (cAMP) (Cabrera-Vera et al., 2003). The A1 peptide ADP-ribosylates a G protein which leads to an increased level of cAMP in the epithelial cell (Gill & Meren, 1978). This consequently leads to secretion of chloride and water into the lumen of the

host's intestine. The chloride and water secretions lead to the massive watery diarrhea observed in patients with cholera (Matson et al., 2007).

Waldor and Mekalanos (1996) have shown that CTXΦ can convert *V. cholera* that have TCP but no CTXΦ (TCP $^{+}$ but CTXΦ $^{-}$) into carriers of both TCP and CTXΦ (TCP^{+} and CTXΦ^{+}) in the gastrointestinal tract of mice. This finding also indicates that avirulent *V. cholerae* strains can acquire CTXΦ in the host intestinal tract and can then become virulent causing severe diarrhea. This finding also leads to an issue of using a live attenuated vaccine. If the vaccine was made of CTXΦ deleted strains, the vaccine strain could pick up CTXΦ while in the host's gut and become virulent (Waldor, 1996, as reviewed in Cotter, 2000).

Control of virulence factors: ToxR

It is crucial for *V. cholerae* to be able to turn on and off virulence factors depending on environmental cues. Specific virulence factors have to be transcribed at the right time to ensure intestine colonization and CT production (Lee, Butler, & Camilli, 2001). One regulatory protein that is essential for controlling *V. cholerae* virulence factors is ToxR. ToxR directly and indirectly controls at least 17 genes, many of which are involved in virulence (Skorupski &Taylor, 1997). ToxS is another regulatory protein that ToxR needs in order to moderate many virulence factors. Both ToxR and ToxS are coded are found in the original ancestral chromosome (not acquired through lateral gene transfer) (Waldor & Mekalanos, 1996).

ToxR and ToxS are located in the cytoplasmic membrane. ToxR has a cytoplasmically located DNA-binding domain that causes transcription activation but the perplasmic domain has unknown function. Since ToxR controls virulence genes in response to environmental changes like pH, temperature, amino acids, and osmolarity it has been proposed that the periplasmic domain senses environmental conditions (DiRita & Mekalanos, 1991, Matson et al., 2007). ToxS is also embedded in the cytoplasmic membrane, but it is located mostly in the periplasm. It is hypothesized that the periplasm is where ToxR and ToxS interact. The function of ToxS is unclear, but it seems to be an effector of ToxR by either increasing stability (protection from proteolysis) or enhancing dimerization of ToxR (Miller, DiRita, & Mekalanos, 1989).

ToxR directly regulates the production of outer membrane porins OmpU and OmpT by binding to their promoter regions (Miller & Mekalanos, 1988). ToxR causes an increased transcription of *ompU* and conversely represses transcription of *ompT.* OmpU may not just function as a porin but also as an adhesion during pathogenesis (Provenzano & Klose, 2000). ToxR binds to the *ompU* promoter region first at a site -238 to -139, which then promotes binding at two other sites within the promoter region at -116 to -58 and -53 to -24. It is unknown if this is due to recognition of the actual sequences or secondary structure in the DNA at those locations (Crawford, Kaper, & DiRita, 1998).

ToxR inhibits OmpT production by inhibiting the binding of cyclic AMP receptor protein (CRP). CRP binds to three regions of the *ompT* promoter: one distal and two proximal sites. If CRP binds to all three sites, it acts as a positive regulator of *ompT*. If CRP only binds to the proximal sites, it acts as a negative regulator of *ompT* (Li, Merrell,

Camilli, & Kaper, 2002). OmpU and OmpT switching is essential for *V. cholerae* to be able to survive in human host intestine.

ToxR also activates ToxT, a regulatory protein that in turn activates various virulence factors, including TCP and CT. ToxR doesn't regulate ToxT production alone, it acts in association with TcpP. TcpP is also a transmembrane protein with a cytoplasmic DNA-binding domain and a periplasmic domain. TcpP requires another membrane bound protein, TcpH. Just like ToxR and ToxS, it is thought that the periplasmic regions of TcpP and TcpH interact (Matson et al., 2007). TcpP requires TcpH for protection from proteolysis from protease YaeL (Cotter & DiRita, 2000).Genes for TcpP and TcpH are located on the mobile genetic element that also contains TCP. Once activated by ToxR/ToxS and TcpP/TcpH, ToxT in turn activates the genes involved in TCP and CT production (*tcpA-F* and *ctxA/ctxB* respectfully) (Cotter & DiRita, 2000).

It is unusual that within *V. cholerae* ancestral chromosome are sequences that code for ToxR and ToxS, which affect both both mobile genetic elements. To complicate the evolution of *V. cholerae*'s virulence regulation, TcpP and TcpH are located on the large pathogenicity island that codes for TCP (Cotter & DiRita, 2000). In order for the TCP and CT to be activated (via ToxT), regulatory genes from both the ancestral genome (ToxR/ToxS) and transferred mobile genetic elements (TcpP/TcpH) are required. ToxR and ToxS have been thought to control outer membrane proteins synthesis in an ancestral *V. cholerae* and only relatively recently gained transcriptional control over factors including TCP, CT, and ToxT on mobile genetic elements (Cotter & DiRita, 2000,

Provenzano, Schuhmacher, Barker, & Klose, 2000). See figure 1 for a summary of ToxR genetic regulation.

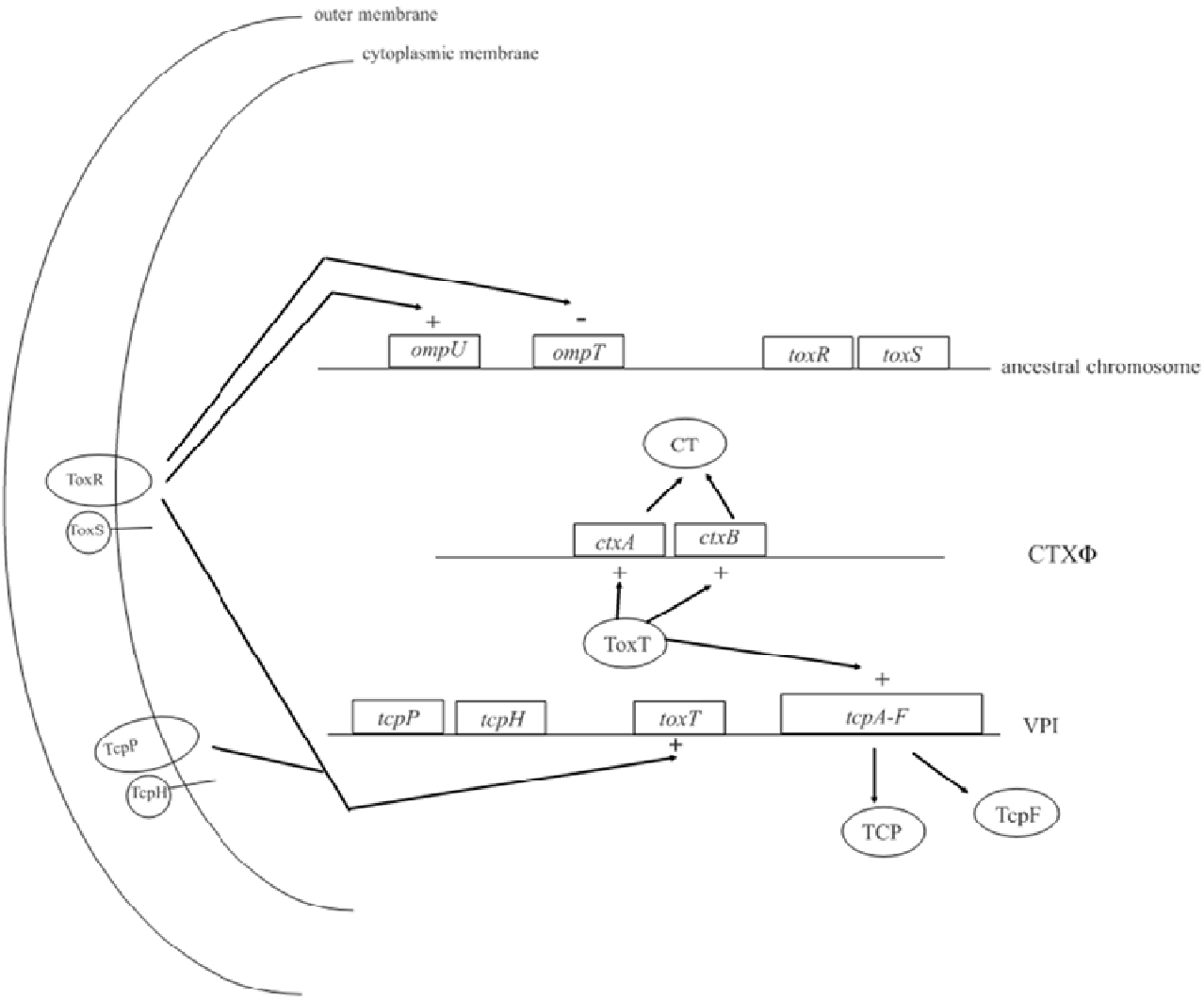

Fig. 1: The general genetic control of virulence factors by ToxR. *ToxR* and *toxS* are both located on the ancestral chromosome and regulate genes on both mobile genetic elements.

Two distinct habitats

V. cholerae inhabits two distinct habitats: aquatic environments and human small bowel. *V. cholerae* is usually associated with copepods and shellfish when living

aquatically and are transmitted to humans via contaminated food or water (Matson et al., 2007). These two environments differ in many ways. In order to successfully live in both environments, *V. cholerae* has to respond and quickly change according to both environments. One way *V. cholerae* accomplishes the rapid switch is by altering the proteins in its outer membrane (Provenzano & Klose, 2000). *V. cholerae* has about 10 major outer membrane proteins; at least three of these, OmpT, OmpU, and OmpS function as porins, which are proteins that allow diffusion of molecules through the outer membrane (Kelley & Parker, 1981). The proportion of outer membrane porins are influenced by variables such as carbon sources, pH, osmolarity, and the presence of bile (Simonet, Basle, Klose, & Delcourt, 2003). The two distinct environments have to be sensed and the outer membrane proteins need to be adjusted to the two very different environments.

Aquatic environments have lower concentrations of nutrients and osmolytes; the human small intestine contains bile salts, antimicrobial peptides, and normal intestinal flora which can inhibit pathogenic bacteria. OmpT has a greater permeability to nutrients than OmpU, which is ideal in aquatic environments with sparse nutrients. Even though OmpU has a marginally larger pore size, it is the optimal porin for the environment inside the human intestine (Duret & Delcour, 2010). The human small intestine exposes *V. cholerae* to many toxic substances such as bile salts and antimicrobial peptides. OmpU confers greater resistance to both bile salts and antimicrobial peptides (Simonet et al., 2003).

Amino acid supplementation affects virulence genes

How does *V. cholerae* alter gene expression when changing between two such different environments? In order to survive, *V. cholerae* must be able to change its regulatory protein production in response to changes it can sense in the environment. The timing of expression of virulence genes is crucial for colonizing and successfully living in the host intestine. In order to understand the mechanisms involved in virulence of *V. cholerae*, we need to understand what environmental cues make the bacteria switch on genes for virulence. One big change between aquatic and human environments is the levels of amino acids.

Mey, Craig, & Payne (2012) were able to alter the growth medum of *V .cholerae* with various amino acids to determine which amino acids signaled a transcriptional change in the bacteria, specifically alterations in the Omp profiles (Mey et al., 2012). Normally in rich medium, *V. cholerae* has high levels of OmpU as its outer membrane porin; in minimal medium OmpT is the predominant porin. When minimal media was supplemented with a mixture of asparagine, arginine, glutamic acid, and serine (NRES) there was a switch from OmpT to OmpU (Miller & Melalanos, 1988). This is the same porin switch *V. cholerae* undergo when switching from an aquatic environment to their human host intestine (Provenzano & Klose, 2000).

Switching from minimal medium to minimal medium with NRES also caused an increase in the growth rate of the bacteria. To show that the Omp switching observed was not simply due to an increase in growth rate, the bacteria were grown in minimal medium supplemented with aspartate (not in NRES). The aspartate caused an increased growth

rate but not a change in Omp profile. Of the four amino acids in NRES, asparagine had the largest effect on Omp profiles, but still not as large an effect as NRES (Mey et al., 2012).

The OmpT to OmpU switch is due to an increase in ToxR levels. ToxR represses *ompT* expression and causes an increase in *ompU* expression (Provenzano & Klose, 2000). ToxR protein levels increased with NRES supplementation (Mey et al., 2012). It has been shown that ToxR needs ToxS to control virulence factors (Miller et al., 1989). When grown in NRES minimal media, *V. cholerae* had increased levels of *toxR* mRNA, indicating that NRES increases transcriptional activation of *toxR.* The control of virulence genes relies on control at the transcriptional level (Mey et al., 2012). Since Omp switching is controlled by ToxR, studying the phenotypic proportion of OmpU and OmpT in *V. cholerae* is a way to study ToxR levels in cells.

Resistance to bile salts

One big environmental change *V. cholerae* has to be able to withstand inside host intestine is the presence of bile. Bile is a common chemical found in human intestines. Bile is inhibitory to many enteric pathogens and resistance to bile is required to colonize. Bile is made up of bile salts, which are anionic detergents that help to emulsify or break down ingested fats. Since bile salts have can dissolve membranes, they are bacteriocidal (Provenzano et al., 2000). Gram negative bacteria have some natural protection from bile salts due to hiding their cytoplasmic membranes beneath an outer membrane. Extra resistance can be obtained through lipopolysaccharide, outer membrane porins, and even

efflux pumps that remove bile from the periplasmic space (24). Due to *V. cholerae*'s resistance to bile, the medium commonly used to differential it from normal enteric flora is thiosulfate-citrate-bile-sucrose (TCBS) media.

A study conducted by Provenzano, et al. (2000) showed that resistance to bile was a regulated by ToxR independently of ToxT. A mutant *toxR* strain failed to grow in the presence of bile salts, showing that ToxR is required for bile resistance. ToxR caused transcription of *ompU* without the addition of bile, but when bile was added to the growth media, *ompU* transcription increased. (Provenzano et al., 2000). Since ToxR is located on the ancestral chromosome, this suggests that the ancestral role of ToxR involved outer membrane protein control. At some point in the evolution of *V. cholerae*, ToxR gained the ability to control virulence factors located on the mobile genetic elements (pathogenicity island and CTXΦ). Perhaps *V. cholerae* evolved to become an enteric pathogen when it gained bile resistance with OmpU which enabled it survive in intestine. This could also explain why OmpU and OmpT are the only ToxR-dependent and ToxT-independent factors in *V. cholerae* (Provenzano et al., 2000).

It is possible that the environmental switch from a bile-free aquatic location to the human intestine with high levels of bile could be the environmental cue that turns on *V. cholera* resistance. In the Mey et al. 2012 study, when *V. cholerae* was grown in medium supplemented with two bile acids cholate and deoxycholate, Omp profiles switched from OmpT to OmpU just as with amino acid supplementation with NRES. The bile acids caused an increase in *ompU* mRNA. Since a *toxR* mutant did not produce OmpU when exposed to bile acids, ToxR must be present in order for bile acids to promote Omp

switching (Mey et al., 2012). Interestingly, ToxR levels decreased in the bile acid supplemented bacteria; this was in contrast to the ToxR level increase observed with NRES supplementation. The pathway by which bile controls Omp production must be different than that of NRES (Mey et al., 2012).

Quorum sensing

An essential ability for *V. cholerae* to be able to colonize and cause infection in humans is quorum sensing. Quorum sensing is a form of chemical communication between bacteria that involves the secretion of chemicals called autoinducers. When the concentrations of the autoinducers are high enough in a bacterial community, bacteria in that community control their genes in unison. Many activities are controlled by quorum sensing: DNA exchange, biofilm formation, and virulence factors are a few (Miller & Bassler, 2001).

There are three quorum-sensing systems that act together in *V. cholerae*. System 1 and system 2 have been well characterized and both feed into creating active LuxU, which in turn leads to an increase in LuxO (Miller, Skorupski, Lenz, Taylot, & Bassler, 2002). LuxO then upregulates the expression of four small RNAs called Qrr sRNAs (quorum regulatory RNA). These sRNAs bind to *hapR* mRNA, creating a decrease in HapR levels. HapR is a protein that regulates many quorum sensing activities. HapR turns off genes that code for virulence (like the ToxR regulon) and biofilms (Zhu et al., 2002). The third quorum sensing system was predicted by Miller et al. in 2002 but

wasn't characterized until 2005 by Lenz, Miller, Zhu, Kulkarni, and Bassler. The third quorum sensing system involves a protein called CsrA.

CsrA is a global regulator in *E. coli*

CsrA (carbon storage regulator) is a global regulator that controls many different systems in *E. coli* including glycogen synthesis, motility, and biofilm production. It is 61 amino acids in length. One major function of CsrA is controlling the intracellular carbon flux by upregulating glycolysis and turning off gluconeogenesis and glycogen synthesis. Without CsrA, glycogen levels rise in *E. coli* (Romeo et al., 1993).

One method of studying the effects of CsrA on gene regulation is to mutate or inactivate *csrA* and study the mutants as they regulate genes in various environments. A problem arises with *csrA* deletion mutants of *E. coli*: glycogen levels increase to the point that the cells are no longer viable on LB medium (Timmermans & Melderen, 2009). Timmermans and Melderen, in 2009, were able to make a viable *csrA* mutant in *E. coli* using two different rationales.

Timmermans and Meldren were able to grow viable *E. coli* with a *csrA* deletion by introduction a mutation in *glgCAP*, which is responsible for the first two steps in the glycogen synthesis pathway. Without functional glycogen synthesis, glycogen levels never increase to a lethal level. The *glgCAP* mutation enabled the *E. coli* to grow on glycolytic carbon sources in LB media.

A second method Timmermans and Melderen used involved altering the carbon source in the medium. When pyruvate was added to growth medium, the *E. coli csrA*

deletion mutants grew. This indicates that the Krebs cycle is still functioning in the *crsA* deletion mutant of *E. coli* (Timmermans & Melderen, 2009). Either by supplementation with different carbon sources or knocking out glycogen synthesis pathways, *E. coli csrA* mutants can grow in culture media (Timmermans & Melderen, 2009).

CsrA is part of the third quorum sensing system in *V. cholerae*

CsrA is also found in *V. cholerae* and has been shown to have similar global control as in *E. coli* (Lenz et al., 2005). In the study by Lenz, et al. 2005 it was shown that there was a third quorum sensing system and that it is controlled by CsrA along with three small RNAs called CsrB, C, and D. This system is initiated by VarS and VarA. VarS activates VarA. Active VarA causes the transcription of three sRNAs called CsrB, CsrC, and CsrD. These sRNAs bind to and inactivate CsrA protein. Lenz et al. (2005) proposed that through an unknown mechanism CsrA can act on LuxO and activate the quorum sensing cascade similar to the previously described systems 1 and 2 (Lenz et al., 2005).

General overview of rationale behind experiment

The overall experimental plan proposed for this research was to create a *csrA* mutation in a strain of *V. cholerae* that was already deficient in glycogen synthesis and to study the ToxR and Omp profiles in different growth conditions.

The glycogen synthesis deficient *V. cholerae* mutants came from a collection of mutants created by Cameron, Urbach, and Mekalanos in 2008 in strain C6706 from R. A.

Finkelstein. Alexandra Mey, in her previous work, created a suicide plasmid with a *csrA* deletion and a kanamycin cassette insertion using a SmaI restriction site. Since the *V. cholerae* mutants already expressed kanamycin resistance, the cassette was switched to a chloramphenicol cassette. From there I cloned the csrAΔ::cam insert from the plasmid pWSK30 into the suicide plasmid pHM5 that requires the π protein for replication. The suicide vector with the *csrA* and chloramphenicol insert was transformed into a strain of *E. coli* that produces the π protein required for plasmid replication of this plasmid.

Bacterial conjugation was completed to transfer the plasmid into the glycogen synthesis deficient *V. cholerae* strains. Allelic exchange involves two crossover events that must occur in order to transfer the mutation into the *V. cholerae* chromosome. Once inside *V. cholerae*, the plasmid cannot replicate independently due to the lack of π protein. The first crossover event will incorporate the plasmid into the genome of the *V. cholerae*. Without incorporating, the plasmid cannot replicate. Next, due to the homology between the genomic *csrA* and the sequences flanking the *csrA* deletion with the chloramphenicol cassette, the DNA can align again, and a second crossover event takes place with multiple outcomes depending on the location of the crossover. The desired allelic exchange product can be selected for with chloramphenicol and sucrose resistance and ampicillin sensitivity.

Chapter 3: Methods

Bacterial strains, plasmids, and growth media

Bacterial strains and plasmids used are listed on Table 1. All strains were maintained at -80°C in tryptic soy broth (TSB) plus 20% glycerol. Strains were routinely grown at 37°C in Luria-Bertani (LB) broth (1% tryptone, 0.5%, yeast extract, 1% NaCl) with appropriate antibiotics. Antibiotic concentrations are listed in Table 2. Since *V. cholerae* is extremely sensitive to chloramphenicol, the concentration needed to separate resistant strains was much lower than that for *E. coli.*

Table 1: Bacterial strains and plasmids

Strain or plasmid	Description	Source or reference(s)
V. cholerae strains		
C6706	*V. cholerae* El Tor biotype	R. A. Finkelstein
CglgA	C6706 *glgA*::kan	Cameron, Urbach, & Mekalanos, 2008
CglgB	C6706 *glgB*::kan	Cameron, Urbach, & Mekalanos, 2008
CglgC2	C6706 *glgC2*::kan	Cameron, Urbach, & Mekalanos, 2008
E. coli strains		
DH5αλpir	Cloning strain; host strain for pScsrA derivatives	J. Kaper
SM10λpir	Mobilizing strain for pScsrA derivatives	Miller & Mekelanos, 1988
Plasmids		
pCVD442N	Suicide vector pGP704 carrying *sacB* and a NotI linker; Amp^r Suc^s	Wyckoff, Mey, Leimbach, Fisher & Payne, 2006
pHM5	Suicide vector pGP704 carrying *sacB*; Amp^r Suc^s	Runyen-Janecky, Hong, & Payne, 1999
pWKS30	Low-copy-no. cloning vector; Amp^r	Wang & Kushner, 1991
pScsrAΔ::cam	pHM5carrying csrAΔ::cam	This study

Table 2: Antibiotic Concentrations

Antibiotic	**Concentration (µg/ml)**
Ampicillin	35-50
Carbenicillin	250
Chloramphenicol	30 (5-7.5 with *V. cholerae*)
Kanamycin	50
Polymyxin B	20

Plasmid Miniprep (Qiagen kit and Sigma GenElute Plasmid kit)

Plasmid minipreps were used to isolate plasmid DNA from intact bacterial cells. Two kits were used, Qiagen and Sigma, both according to the manufacturer's instructions. Both the Qiagen and Sigma kits utilize alkaline lysis with sodium dodecyl sulfate (SDS)**.** When bacterial cells are exposed to a highly basic solution, the cell walls open, the chromosome is denatured, proteins are broken down, and plasmid DNA is released into the solution. Since plasmid DNA is so small and intertwined, the high pH doesn't cause it to denature like the chromosomal DNA (Sambrook & Russell, 2001). The denatured chromosomal DNA and cellular proteins get coated with SDS and are separated out by centrifugation. The plasmid is still in the supernatant and can be filtered out using column chromatography (Sambrook & Russell, 2001).

Restriction Digest

Restriction enzymes cut DNA at specific sequences called recognition sites. Some cut both DNA strands at the same point, creating a blunt end while others create

staggered cuts so that one strand is longer than the other, creating sticky ends that can bind with other strands with complementary sticky ends. There are many known restriction enzymes that can cut at various restriction sites (Sambrook & Russell, 2001). In order to complete a restriction digest of DNA, we added plasmid to be digested, restriction enzyme, appropriate buffer, and BSA. The amounts used in the initial digest of the two initial vectors used in this experiment are shown below:

DNA (pHM5 or pWcarAΔ::cam)	41 µl
10x Buffer 1 (for SacI)	5.5 µl
10x BSA	5.5 µl
SacI restriction enzyme	3.0 µl
Total	55.0 µl

All components were mixed and incubated in a 37°C water bath for 2 hours.

Nanodrop Spectrophotometer

The nanodrop spectrophotometer is a method of determining the concentration of very small volumes of things like RNA and DNA. First the spectrophotometer was cleaned by running 1 µl of deionized water. To start, a blank was loaded and read (water or buffer depending on the sample). Next, 1 µl of each sample was loaded and read. The Nanodrop Spectrophotometer measures the concentration of DNA in ng/µl.

Gel Extraction (Sigma GenElute)

After electrophoresis, DNA was removed using gel extraction. The band was cut out of the agarose gel and solubilized. The DNA was removed from the solution by

column chromatography. The gel extraction was carried out using the manufacturer's procedure.

Preparing agarose gel

To make a 1% agarose gel: 1 gram of agarose was added to 100 mL of buffer in a sterile bottle. The lid was loosened and the agarose was microwaved at 30% power until the agarose was dissolved. As the agarose cooled, the gel mold was taped and a comb was inserted. Ethidium bromide was added to the molten agarose (5 µl for every 100 mL of melted agarose). The agrose was gently poured into the mold. Once the gel solidified, it was removed from the mold and samples were loaded. Electrophoresis conditions were 80 or 90 volts for about 30 minutes or until the loading dye reached the end of the gel. The DNA ladder used was HindIII digest of lambda DNA unless otherwise indicated. Table 3 lists the fragments and concentration of lambda DNA after a HindIII digest.

Table 3: HindIII digest of lambda DNA ladder

Length of fragments (kb) Total: 48.502 kb	Approximate concentration (ng/µL) Total: 250 ng/µL
23.130	119.25
9.416	48.5
6.557	34
4.361	22.5
2.322	12
2.027	10
0.564	3
0.125	0.65

Ligation

Ligations are used to join fragments of DNA by forming a phosphodiester bond between bases (Sambrook & Russell, 2001). DNA ligase was used after a restriction digest to insert DNA fragments of DNA into cloning vectors. In this experiment, T4 DNA ligase was used to combine the suicide plasmid vector pHM5 with the insert created (csrAΔ::cam). First, the vector and insert were prepared. For a sticky end ligation, use a 3:1 molar ratio of insert to vector was used. Next the reaction was set up using the 10X ligase buffer provided with the enzyme and 1 µl T4 DNA ligase enzyme per 10 µl reaction. The samples were incubated at 16°C overnight using a PCR machine set at 16°C (Sambrook & Russell, 2001).

Making $CaCl_2$ competent cells

In order to introduce plasmid DNA into bacteria, bacteria first need to be competent. For DNA uptake their cell membranes need to be weakened. One common way to make competent cells is with a calcium chloride solution (with the concentration of 100mM $CaCl_2$, 10 mM Pipes buffer, and 15% glycerol) (Sambrook & Russell, 2001). Overnight culture of *E. coli* strain to be transformed was diluted 1:100 into 25 ml LB and grown to mid-log phase (OD_{650} 0.4-0.6) at 37°C for about 2-3 hours. The culture was centrifuged at 4°C for 3-5 min at 7000 rpm and resuspend in 20 ml ice-cold $CaCl_2$ solution. After 30 minutes on ice, the cells were centrifuged for 10 min at 4000 rpm. The

pellet was resuspended in 1.5 ml fresh $CaCl_2$ solution and used immediately or stored over night at 4°C.

Transformation

Bacteria are capable of absorbing foreign DNA from their environment. To do this, competent cells are incubated with the foreign plasmid DNA and heat shocked. The process is to further weaken the cell membrane to aid the plasmid's entry into the cell. Once inside the cell, the plasmid will be expressed and replicated (Sambrook & Russell, 2001).

To initiate a transformation, the ligation product or plasmid prep (a few µl of supercoiled plasmid prep is sufficient) is added to about 100 µl $CaCl_2$ competent cells, mix gently and leave on ice for 45 minutes. Heat shock for 2 min at 42°C then leave on ice for another 2 minutes. Add cells to 1.7 ml LB, incubate for 1 hour at 37°C, with shaking. This incubation step is to allow the cells to express the plasmid which conatins antibiotic resistance. Spin down the culture in sterile 2 ml tube, take off supernatant but leave about 100 µl. Resuspend the cells in the 100 µl. Plate the transformed cells on 3 LB plates with appropriate antibiotics and incubate at 37°C overnight. The only cells that will grow will be the ones expressing the antibiotic resistance on the plasmid (Sambrook & Russell, 2001).

Bacterial Conjugation

SM10λpir has the ability to conjugate without a helper strain. Start with overnight cultures of the donor and recipient strain. To remove any antibiotics present, centrifuge 1 ml of each and discard supernatant. Resuspend in 1 ml of fresh LB. Centrifuge again and resuspend in only 100 µl of LB. For each mating, add together: 25 µl donor (SM10λpir/pScsrAΔ::cam), and 20 µl recipient (three different *V. cholerae* mutants: CglgA, CglgB, and CglgC2). Mixed cultures were spotted on an LB plate and incubated at 37°C for 6-8 hours. After 6-8 hours the mixture of bacteria was inoculated onto appropriate selective media that selected for the recipient and transferred plasmid (LB/amp/polymyxin B in this case).

Triparental Mating

A triparental mating is a method of gene transfer between two strains of bacteria that do not themselves readily undergo conjugation. A helper strain, which does form a pilus and can undergo conjugation, aids in the gene transfer between a donor and a recipient strain. Start with three overnight cultures: the donor, a helper, and a recipient. To remove any antibiotics present, 1 ml of each culture was centrifuged and the supernatant was discarded. Each was resuspend in 1 mL of fresh LB. The sampled were centrifuged again and resuspend in only 100 µl of LB. For each mating, 25 µl donor (αλpir/pScsrAΔ::cam), 25 µl helper (MM294/pRK2013), and 20 µl recipient (three different *V. cholerae* mutants: CglgA, CglgB, CglgC2) were combined, spotted on an LB plate, and incubate at 37°C for 6-8 hours. The mixed cultures were then inoculated onto

appropriate selective media that selects for the recipient and transferred plasmid (LB/amp/polymyxin B in this case).

Plasmid integration using suicide plasmid vectors

Suicide plasmids are conditional replicons; certain conditions have to be in place in order for the plasmid to replicate. When in a cell that lacks the condition, selection for a property of the plasmid (like antibiotic resistance) gives isolates that have integrated the plasmid into its genomic DNA via homology between the plasmid and its complementary region in the genome (Maloy, Stewartm & Taylor, 1996).

In this experiment the suicide vector pHM5 was utilized. The plasmid pHM5 contains plenty of markers used for selection like ampicillin resistance and the *sacB* gene that creates sucrose sensitivity. To replicate, pHM5 requires the π protein (encoded by λ *pir*). This plasmid can only replicate in bacterial strains that contain λ *pir* (like *E. coli* DH5αλpir and SM10λpir used in this experiment). Once this plasmid is inserted into a bacterial strain lacking *pir*, the plasmid must integrate into the genome in order to replicate (see Fig. 2 for an image of plasmid integration). When the pScsrAΔ::cam is mated into *V. cholerae* and cultured in the presence of chloramphenicol, the only bacteria that can grow are the ones that have successfully integrated the plasmid into their genome (Maloy et al., 1996, Mey & Payne, 2001).

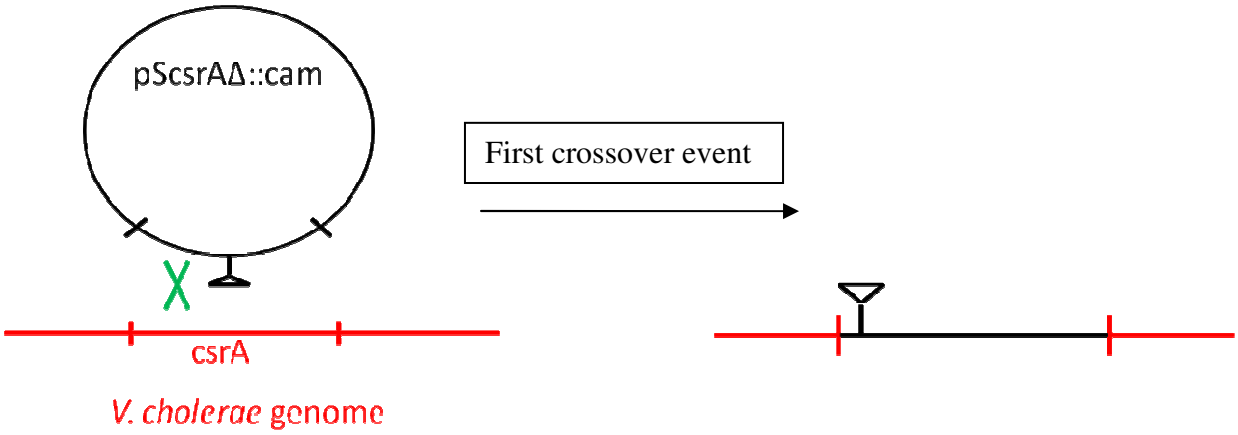

Fig.2: Integration of the λ pir dependent suicide plasmid pScsrAΔ::cam into the *V. cholerae genome.*

PCR to confirm the presence of *V. cholerae* containing integrated pScsrAΔ::cam

Polymerase chain reactions (PCR) are used to amplify specific DNA sequences. There are three basic steps to PCR reactions: the denaturation of DNA into single strands, annealing of primers to the DNA, and elongation of the primers using DNA polymerase (Sambrook & Russell, 2001).

To confirm the presence of the pScsrAΔ::cam insert within the *V. cholerae* genome, two PCR reactions were run with two different sets of primers. Both utilize one primer outside the desired sequence and one within. A wild type genome was run as a control. The wild type will only contain *csrA* without any deletion or chloramphenicol insert. Upon electrophoresis of the PCR products, the wild type segments can be compared with those of the conjugation to find which conjugation *V. cholerae* actually contain the integrated pScsrAΔ::cam. PCR products containing pScsrAΔ::cam will be

about 1.2 kb larger than the wild type PCR products (Sambrook & Russell, 2001). See figure 3 for a diagram of the two possible insertion outcomes.

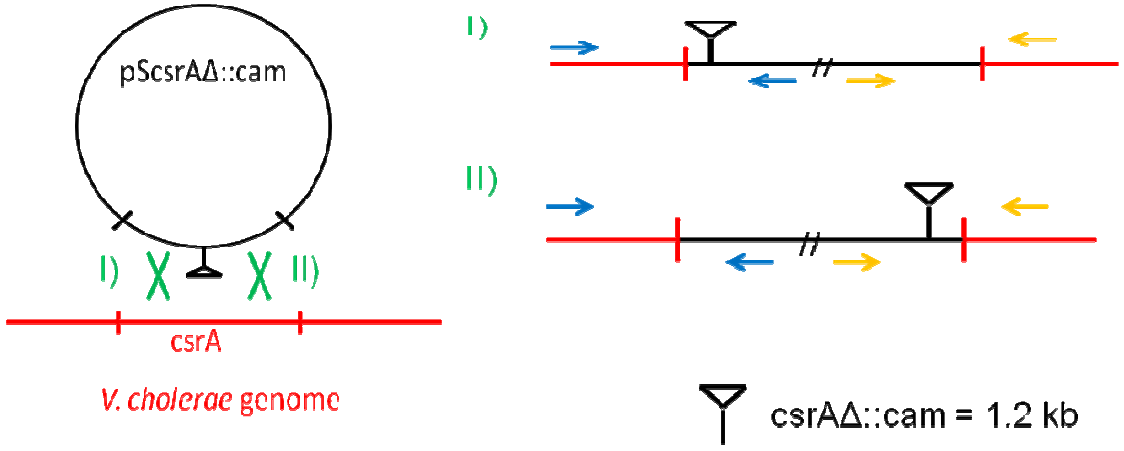

Fig. 3: The two possible outcomes of insertion of the plasmid. The csrAΔ::cam will introduce an additional 1.2 kb to the wild type. The arrows represent the two sets of primers used in the PCR reaction. Only one of the two PCR reactions will yield the extra 1.2 kb due to the presence of the insert.

PCR was run using Taq polymerase (Biolabs) and four different primers (Sigma) in two PCR reactions for each sample. The control used was DNA from *V. cholerae* strain C6706. Temperatures and times were run based on manufacturers' specifications. The wild type band lengths for the PCR reactions were about 1.4 and 1.3 kb and with the insertion 2.6 and 2.5 kb. See Table 4 for the PCR reaction settings.

Table 4: PCR Reaction Settings

Step	Temperature	Time
Denaturation	95 °C	30 seconds
Annealing	55 °C	30 seconds
Elongation	72 °C	3 minutes

Selection for the plasmid excision event retaining csrAΔ::cam

As bacteria replicate with the plasmid inserted, homologous recombination events take place along the duplicated sequence (around *csrA*). One copy of the duplicated sequence is removed (Maloy et al., 1996). Multiple excision events are possible depending on where the crossing over occurs. Either the bacteria will remove the wild type *csrA* and keep the mutated version that was originally in the plasmid or the mutated *csrA* will be removed and the bacterium will retain the wild type *csrA* (Mey & Payne, 2001). See Figure 4 for a diagram of the second crossover event.

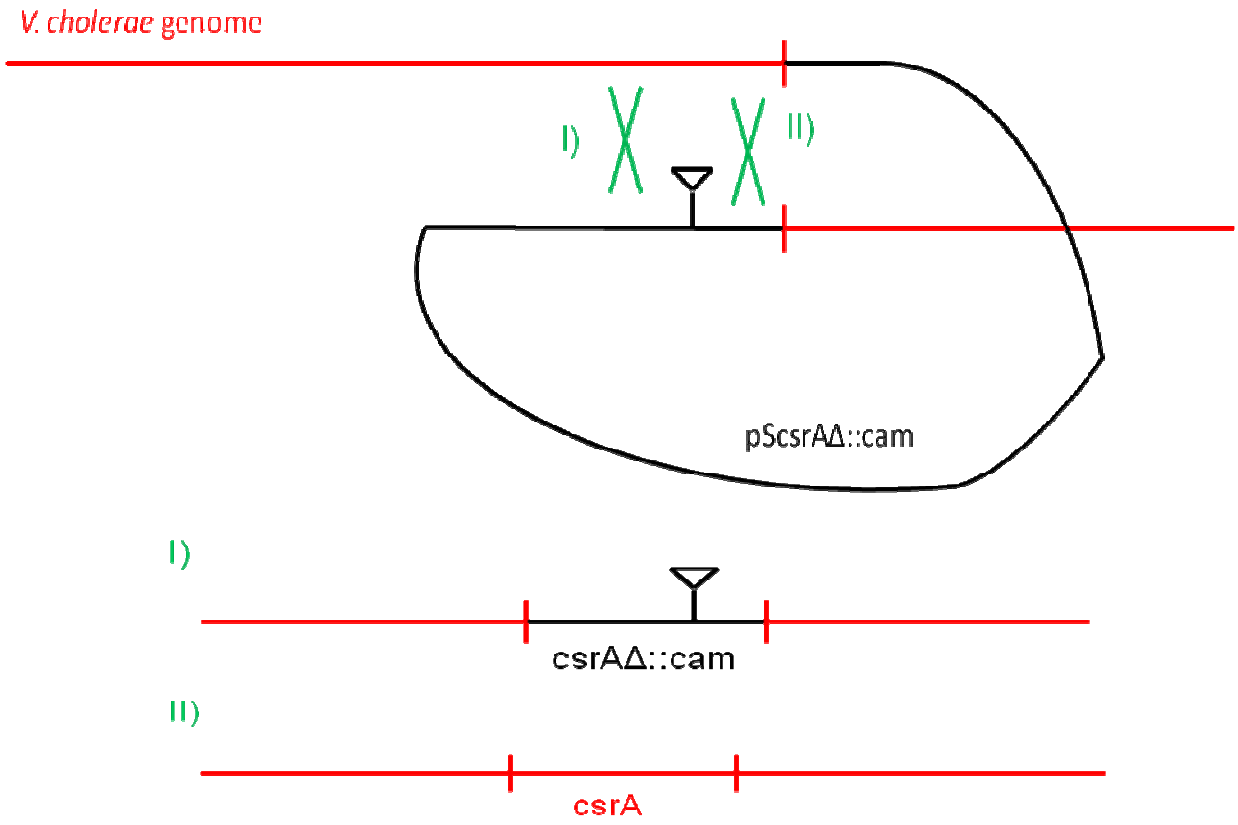

Fig 4: The second crossover event. The second crossover event will either result in a wild type csrA or retention of the csrAΔ::cam mutation depending on where the homologous crossover takes place.

To select for bacteria that have undergone crossing over and excision of the plasmid, the gene *sacB* was utilized. SacB is a protein called levan sucrase that creates polymers of sucrose that can be lethal to gram negative bacteria (Maloy et al., 1996). The pHM5 suicide plasmid contains the *sacB* gene; any bacteria that still contain the whole pScsrAΔ::cam plasmid are sensitive to sucrose and can be identified by growing the bacteria on media containing sucrose. Sucrose resistance can also be created if the bacteria simply alter *sacB* instead of by excision. To verify that the resistance is due to

excision and not a mutation of *sacB*, ampicillin sensitivity was utilized since bacteria that have excised the plasmid will be sensitive and subsequently not grow in the presence of ampicillin. Mutants were confirmed by using PCR (Mey & Payne, 2001).

In summary, the desired outcome for this allelic exchange is a strain of *V. cholerae* that has had the pScsrAΔ::cam plasmid inserted, followed by a double crossover evet due to the homologous sequences around the *csrA* gene. The desired mutant will have exchanged its *csrA* gene with the sequence in the plasmid which contains the csrA deletion and chloramphenicol cassette instead of wild type *csrA*. Selection for the desired mutant was done based on its chloramphenicol and sucrose resistance, and ampicillin sensitivity.

Chapter 4: Results and Data Analysis

Creation of suicide plasmid containing *csrA* deletion and chloramphenicol cassette insert

In order to create a suicide plasmid containing the DNA fragment with a chloramphenicol cassette inserted within the sequence for *csrA*, two liquid cultures were incubated overnight from frozen stocks. One was an *E. coli* strain containing a pWSK30 plasmid with a chloramphenicol cassette inserted within the sequence for *csrA* (pWcsrAΔ::cam) and the second culture was of an *E. coli* stain containing the suicide vector pHM5. The next day both plasmids were isolated using Qiagen's Plasmid Miniprep kit.

The pWcsrAΔ::cam was sequenced using two different primers: a universal primer and the csrA1 forward primer to find appropriate restriction enzymes to use to remove the csrAΔ::cam insert from the pWSK30 plasmid. The direction of the insert ran in the plasmid was determined. A SacI restriction digest of both the pWcsrAΔ::cam and pHM5 were completed. Electrophoresis of the digest was performed to ensure the reaction took place appropriately and to isolate the desired fragment from the pWcsrAΔ::cam plasmid that contained csrAΔ::cam insert (estimated to be about 3.2 kb). The pHM5 had only one SacI site so the entire 5.6 kb plasmid is one band. To get the DNA fragments from the agarose gel, a gel extraction was completed using the Sigma GenElute kit. The eluate from the extraction was checked for purity and concentration.

See figure 5 for a picture of desired products of the SacI restriction digest of pWcsrAΔ::cam and pHM5.

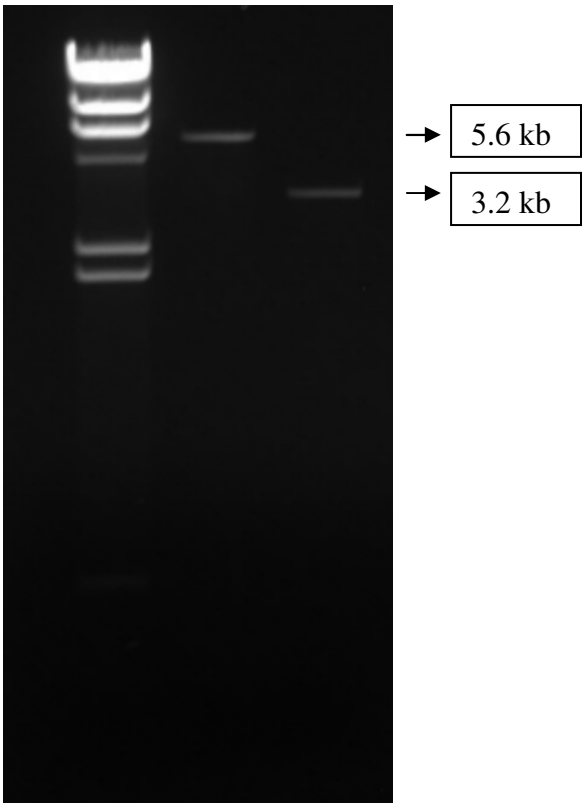

Fig. 5:Desired products of the SacI restriction digest of pWcsrAΔ::cam and pHM5. Well 2 contains the 5.6 kb fragments of the SacI digest of pHM5 Estimated to be about 6 ng/µl. Well 3 contains the 3.2 kbfragment of pWcsrAΔ::cam estimated to be about 4-5 ng/µl.

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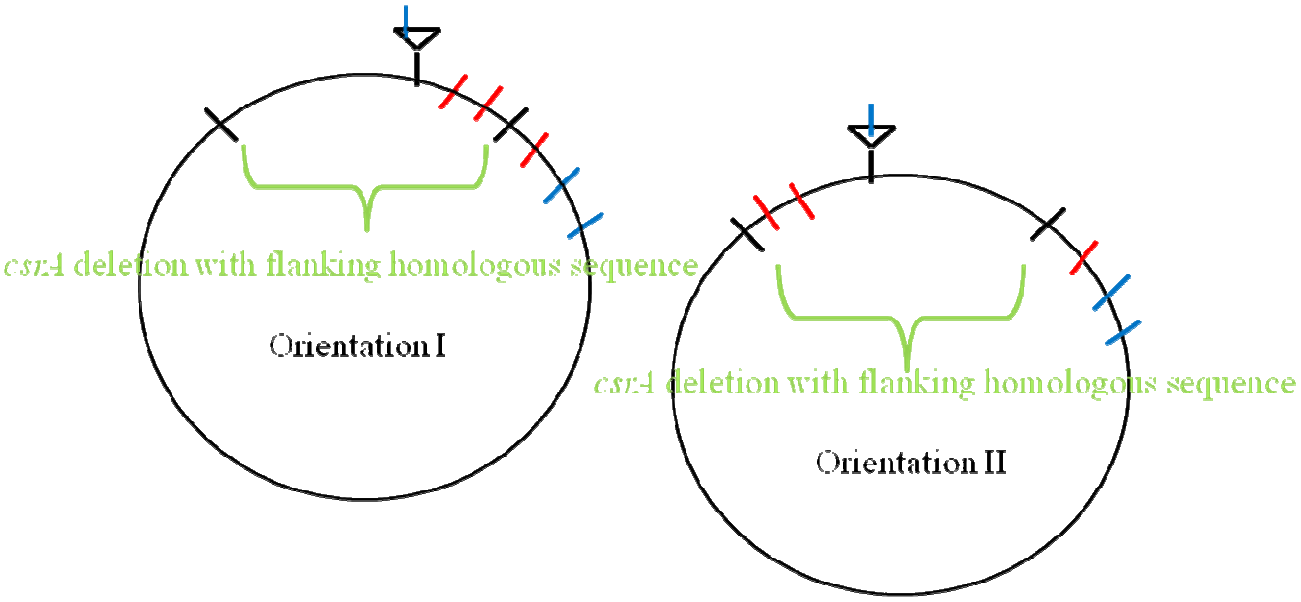
The eluted DNA fragments were ligated together using T4 DNA ligase. The ligation product (pScrsAΔ::cam) was transformed into $CaCl_2$ competent *E. coli* DH5αλpir and plated on 3 plates containing LB agar supplemented with chloramphenicol. Each of the three plates grew colonies. Since the medium contained chloramphenicol, the pScrsΔA::cam plasmid must be present and expressed. Six isolated colonies were streaked on fresh LB/chloramphenicol plates in a 3-phase pattern for

isolation. A colony from each plate was grown overnight in liquid medium containing LB and chloramphenicol.

Plasmids from each of the 6 cultures were extracted using Sigma's GenElute Plasmid Miniprep kit. Two restriction digests of the plasmids were conducted using EcoRI and XbaI , the digests were subsequently electrophoresed in order to analyze the products. This was done to check for the presence of the pScrsΔA::cam and the orientation of the csrAΔ::cam insert within the plasmid. See figure 6 for the two possible orientations of the csrAΔ::cam insert. See figure 7 for a picture of the electrophoresis of the EcoRI and XbaI digests.



XbaI digest:	EcoRI digest:	XbaI digest:	EcoRI digest:
8.2 kb	5.1 kb	5.6 kb	5.5 kb
0.3 kb	0.7 kb	2.9 kb	2.6 kb
0.3 kb	3.0 kb	0.3 kb	0.7 kb

Fig. 6: Two possible orientations of the csrAΔ::cam insert. Red lines represent XbaI and blue represent EcoRI.

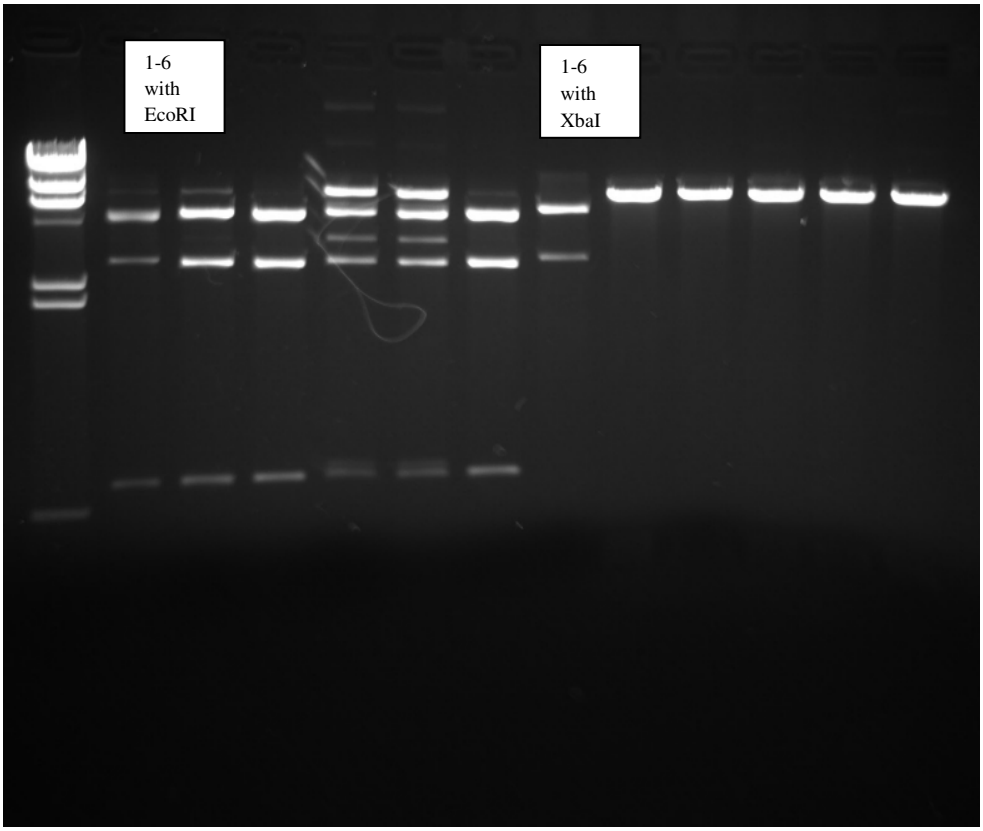


Fig 7: Restriction digest of DH5αλpir transformed with pScrsAΔ::cam. Wells 2-7 represent the EcoRI digest of the 6 plasmid preps and wells 8-13 represent the XbaI digest of the same 6 plasmid preps. Notice samples 2-6 have orientation I while sample 1 has orientation II of the insert

The third sample was chosen for future use because the insert was in the prevalent orientation (samples 2-6 has the same orientation with only sample 1 being flipped) and also sample 3 also gave the clearest bands of the 6 samples analyzed.

Insertion of plasmid into *V. cholerae* using triparental mating

Three triparental matings were set up using DH5αλpir/pS*crsA*Δ::cam as the donor, MM294/pRK2013 as the helper strain, and one of three different glycogen synthesis deficient strains of *V. cholerae*: CglgA, CglgB, and CglgC2 (Cameron et al., 2008). The three matings were then streaked onto 2 LB agar plates supplemented with ampicillin to select for the plasmid and polymyxin B to select for *Vibrio*. All 6 plates had overnight growth, although there were no isolated colonies. The growth was plated again on LB/ampicillin/polymyxin B medium for more isolated growth without success.

A second attempt at the triparental mating (DH5αλpir/pScrsAΔ::cam, helper strain MM294/pRK2013, and the three *V. cholerae* strains) was unsuccessful. The inefficiency of the triparental mating could be due to the C6706 *V. cholerae*'s nuclease activity degrading the plasmid DNA. The bacterial conjugation without the helper strain is more efficient so the odds of obtaining *V. cholerae* with the plasmid integrated are greater.

Insertion of plasmid into *V. cholerae* using bacterial conjugation

To set up a bacterial conjugation without the helper strain, the pScrsAΔ::cam plasmid prep was transformed into $CaCl_2$ competent *E. coli* strain SM10λpir which has the ability to conjugate and transfer DNA independent of a helper strain. The transformed SM10λpir was cultured on LB supplemented with chloramphenicol overnight to select for the pScrsAΔ::cam plasmid. There was no growth overnight. The transformation of

SM10λpir with pScrsAΔ::cam was attempted again but with special attention paid to the competent SM10λpir cells being kept cold. The second attempt was also cultured onto LB supplemented with ampicillin instead of chloramphenicol without overnight growth. This indicated that the transformation was not successful. The plasmid was not being properly maintained in the SM10λpir cells.

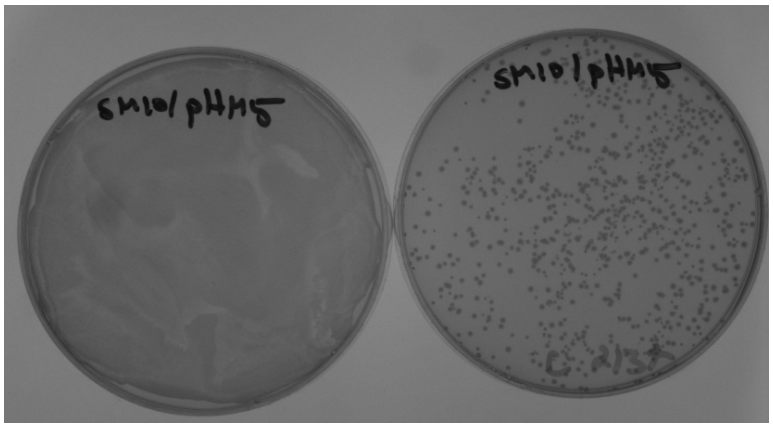
Meanwhile, a fresh plasmid prep was made from DH5αλpir/pScrsAΔ::cam sample 3 using the Qiagen Plasmid Miniprep Kit. An EcoRI restriction digest was completed on the plasmid prep and electrophoresis of the digest showed the appropriate segments for the pScrsAΔ::cam plasmid. The transformation of SM10λpir with this fresh Qiagen sample of pScrsAΔ::cam still showed no overnight growth in the presence of ampicillin.

Fresh SM10λpir $CaCl_2$ competent cells were made and another transformation was completed. This time controls were run to make sure that the SM10λpir $CaCl_2$ competent cells were viable and able to pick up plasmid. Three transformations were carried out, two plasmid controls (pHM5 and pCV0442) and pScrsAΔ::cam. All three transformations were plated onto three different media: plain LB, LB supplemented with ampicillin, and LB supplemented with carbenicillin. All plates showed growth except for the SM10λpir/ pScrsAΔ::cam grown with either antibiotic. See figure 8 for pictures of the three transformations. Thus, the SM10λpir $CaCl_2$ competent cells are indeed viable and able to be transformed. The problem with this transformation must be an incompatibility of SM10λpir with the pScrsAΔ::cam plasmid compared to the plasmid controls.

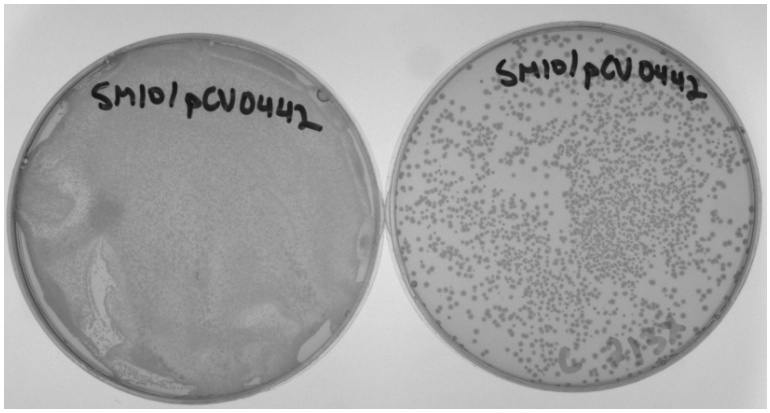
Medium:	LB	LB/carbenicillin

Cells cultured:

a. SM10/ pHm5



b. SM10/ pCV0442



c. SM10/ pScrsAΔ::cam

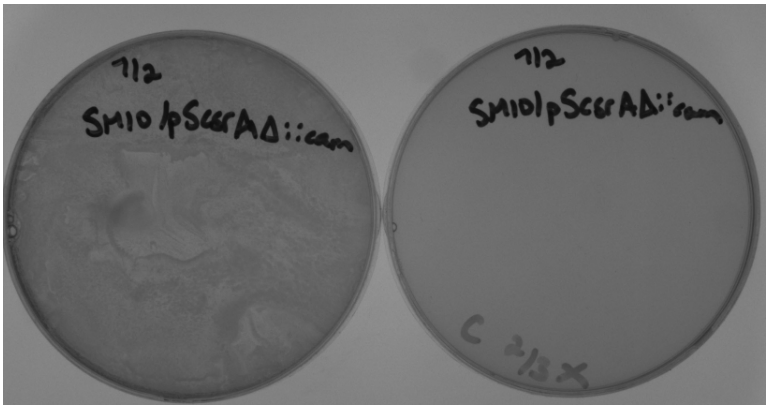


Fig. 8: The three transformations of SM10λpir. All show growth on LB. The two control plasmids have growth on the LB with antibiotic; only the transformation with pScrsAΔ::cam failed to grow on LB with antibiotic. This indicates that the SM10λpir cells are competent and able to express plasmid DNA. There must be a specific issue with the pScrsAΔ::cam plasmid in SM10λpir cells.

After the second night of growth, one round of transformation did yield a few, small colonies. Two of the colonies were isolated and plasmid was collected using the Qiagen Plasmid Miniprep kit. An EcoRI restriction digest and electrophoresis was completed on the plasmid preps. The electrophoresis showed that there is pScrsAΔ::cam plasmid present, but that it was degraded. There is a missing band at 0.7 kb. This could possibly be due to high nuclease activity within SM10λpir. See figure 9 for a picture of the EcoRI digest of the successful SM10/ pScrsAΔ::cam transformation.

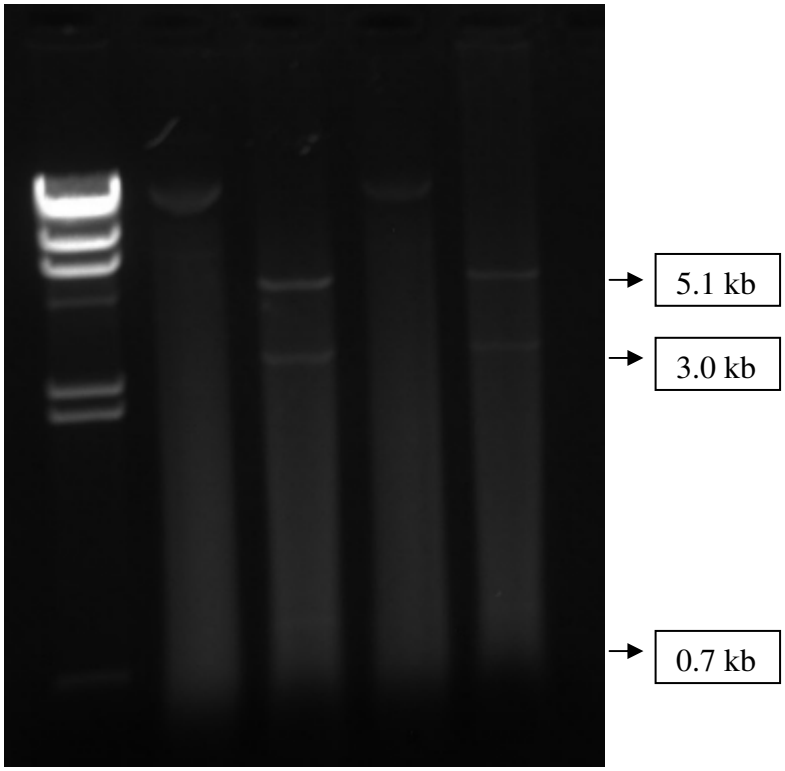


Fig. 9: EcoRI digest of the successful SM10/ pScrsAΔ::cam transformation . Lanes 3 and 5 are the plasmid preps digested with EcoRI. Notice the degraded DNA throughout all of the samples. Arrows indicate the faint bands from the EcoRI digest at about 3.0 kb and 5.1 kb and the missing band at 0.7 kb.

Selection for the first crossover event

Bacterial conjugations were set up using all six combinations of the two SM10λpir strains and the three *V. cholerae* strains. These conjugation reactions were

incubated overnight on LB supplemented with ampicillin and polymyxin B to select for *V. cholerae* that contain the integrated plasmid. Colonies grew on different combinations of SM10λpir and *V. cholerae* strains. Eight potential *V. cholerae* colonies were streaked for isolation on fresh LB/ampicillin/polymyxin B plates. All eight had growth with *V. cholerae* colony morphology. Since these *V. cholerae* colonies were growing in the presence of ampicillin, it can be assumed that they have acquired and integrated the pScrsAΔ::cam plasmid into their genome.

To confirm the presence of the integrated plasmid, two overlapping PCR reactions were run to determine the presence of the *csrA* gene (for wild type) or the chloramphenicol cassette. The wild type band lengths for the PCR reactions were about 1.4 and 1.3 kb. The chloramphenicol cassette insertion with the *csrA* deletion adds about 1.2 kb to the wild type band lengths (2.6 and 2.5 kb). Each sample should have the additional 1.2 kb from the pScsrAΔ::cam in one of the two PCR reactions depending on if the crossover was before or after the csrAΔ::cam insert. Only one of the two PCR reactions created a product. The one reaction that made product did show the additional 1.2 kb insertion in four of the eight samples. See figure 10 for an image of the successful PCR reaction showing the insertion of the csrAΔ::cam insert.

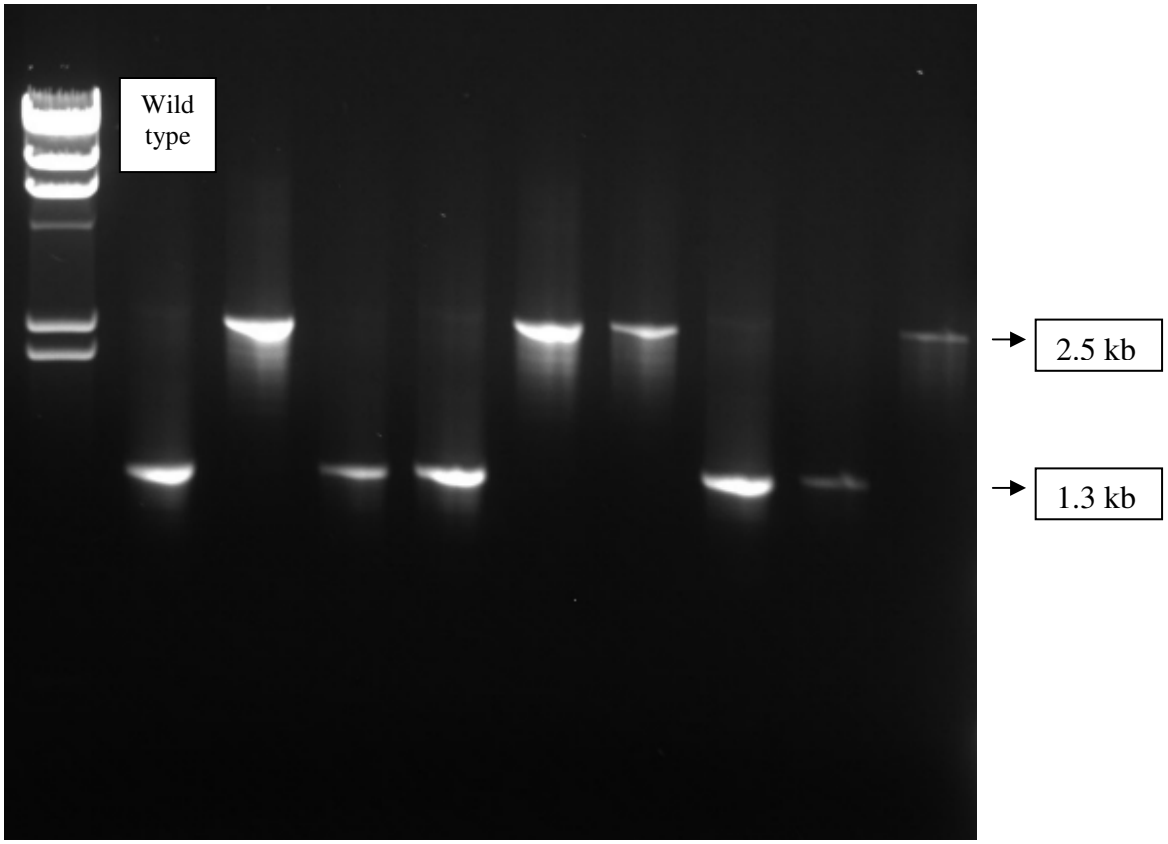


Fig. 10: PCR of the pScrsAΔ::cam integration into *V. cholerae.* Well 2 contains wild type C6706 DNA with a band at about 1.3 kb. Samples 1, 4, 5, and 8 show a band at about 2.5 kb, indicative of the csrAΔ::cam insert.

Selection for the second crossover event

Samples 1, 4, and 5 from were chosen for selection of the second crossover event so that each of the three mutations in *V. cholerae* were represented. Sample 1 is CglgC2, , sample 4 is a CglgA, and lastly, sample 5 is a CglgB. These three samples were cultured onto LB supplemented with chloramphenicol and 10% sucrose. There are two outcomes

of a second crossover event: removal of the csrAΔ::cam insert and return to wild type *csrA* or removal of wild type *csrA* and retention of the csrAΔ::cam insert. Chloramphenicol selects for bacteria that contain the csrAΔ::cam insert; any bacteria that undergo a second crossover event and retain the wild type *csrA* will not be able to survive. The sucrose in the media enabled selection for the removal of *sacB*. As long as bacteria contain the entire pScsrAΔ::cam plasmid, they have the *sacB* gene and are therefore sucrose sensitive. Resistance to both sucrose and chloramphenicol is indicative of the second crossover event with the desired product of retention of the csrAΔ::cam insert and removal of wild type *csrA*.

After two to three passages onto LB supplemented with chloramphenicol and 10% sucrose, sucrose resistant colonies were isolated for all three *V. cholerae* strains. Unfortunately, none of the sucrose resistant colonies were sensitive to ampicillin. This indicates that the wild type *csrA* was not excised. These were still colonies with the entire plasmid inserted into their genome. The *sacB* gene had probably mutated to give the colonies sucrose resistance.

Chapter 5: Conclusions

Preliminary Conclusions

The object of this study was to create a viable *csrA* deletion mutant of *V. cholerae*. This was done by allelic exchange, whereby a fragment of *V. cholerae* DNA containing chloramphenicol resistance cassette in place of the csrA gene was used to replace wild type *csrA* in the *V. cholerae* chromosome. Because *csrA* deletion mutants are non-viable unless the strain is also defective for glycogen production, the allelic exchange was attempted in strains of *V. cholerae* carrying mutations in glycogen biosynthesis genes (glg^- strains).

By using restriction enzymes and DNA ligase, a suicide plasmid was created that requires the π protein to replicate and contains csrAΔ::cam, the *sacB* gene, and an ampicillin resistance gene. Initially the plasmid was transformed into *E. coli* DH5αλpir for use in a triparental mating with the object of moving the allelic exchange construct into the *V. cholerae* glg^- recipient strains. The triparental mating was attempted twice with DH5αλpir/pScsrAΔ::cam, a helper strain, and *V. cholerae* without success. To undergo direct bacterial conjugation without the use of a helper strain, pScsrAΔ::cam was transformed into *E. coli* SM10λpir. The SM10λpir strain grew slowly with the plasmid. A restriction digest of the plasmid from SM10λpir created hazy bands which could indicate endonuclease activity that was degrading the plasmid and was thus causing the decreased growth with ampicillin.

The bacterial conjugations were set up between SM10λpir/pScsrAΔ::cam and three different *glg* mutants of *V. cholerae* C6706 (VCA0016, VCA0699, and VC1726) was successful. Through homologous recombination, the pScsrAΔ::cam was inserted into the genome of all three *glg* mutants. The insertion of the plasmid into the *V. cholerae* mutants was selected based on ampicillin and polymyxin B resistance. A second homologous recombination event led to excision of the plasmid with two possible outcomes. There are two possible outcomes of the second crossover: retention of the wild type *csrA* or retention of the csrAΔ::cam. Resistance to both sucrose and chloramphenicol is indicative of the second crossover event with the retention of the csrAΔ::cam. Unfortunately, none of the sucrose resistant colonies isolated were sensitive to ampicillin. This indicates that the allelic exchange plasmid was not excised. These were still colonies with the entire plasmid inserted into their genome. The *sacB* gene had probably mutated to give the colonies sucrose resistance.

Future Research

Very little known about the glycogen biosynthesis pathway in *V. cholerae*. *V. cholerae* genetic evolution is complex and there are many gene duplications. It is possible that there are duplications in the *glg* genes within *V. cholerae*. This would make it more difficult and complicated to inhibit glycogen synthesis in *V. cholerae* than in *E. coli* due to any functional redundancy. If there are redundant genes for glycogen synthesis, having only one copy knocked out will not actually prevent glycogen buildup in the absence of

CsrA. In order to go forward and create a viable *csrA* mutant, the glycogen biosynthesis pathway in *V. cholerae* will have to be studied and better understood.

In order to create a viable *csrA* mutant in *V. cholerae*, more needs to be understood about the glycogen biosynthesis pathway in *V. cholerae*. It is unknown what that glycogen levels were in the *glg* mutants obtained for this study (Cameron et al., 2008). Timmermans and Melderen (2009) were able to grow *csrA* deficient *E. coli* in media supplemented with pyruvate. Adding pyruvate to media of *csrA* deficient *V. cholerae* could possibly result in growth also. When a viable *csrA* mutant is successfully created in *V. cholerae*, OmpU and OmpT levels should to be studied in varying environments to determine how CsrA affects ToxR regulation (Mey et al., 2012).

Chapter 6: Applications to Practice

Applications to teaching Advanced Biotechnology

This final research summer has been invaluable to me for the Advanced Biotechnology course I teach at my high school. This course falls under the Career and Technical Education (CTE) department. These courses are designed to fit into career clusters that help prepare students for study in specific areas. For me, this means Advanced Biotechnology needs to be a course that is both hands-on and an authentic experience for these high school students in their future scientific learning.

I was the first teacher to teach this course at my school. I am still the only teacher to have ever taught this in my school. This means that there is not anyone I can ask on a daily basis about this course involving content or laboratories. A lot of the laboratory techniques I use have come from my own trial and error. The research I have conducted while pursuing my master's degree has helped tremendously with many of those daily technical laboratory questions I have had. For example, I follow the manufacturer's protocol exactly every time I reconstitute and prepare solutions for PCR (nucleotides, polymerase, primers, and buffer called a master mix). The instructions given are very specific about temperatures and times. Often the protocol says that the master mix can only be combined within 30 minutes of a PCR reaction. Unfortunately 30 minutes before my Advanced Biotechnology course is a chemistry course. I've had to juggle 30 chemistry students during lecture, labs, or activities and prepare a complicated master

mix for my biotechnology class. Turns out, as long as they are kept on ice, the master mix should be good for use for a few hours. This enables me to make the master mix outside other class times. Just being in a laboratory that is doing the technical things that I spend time doing in my own classroom doing and teaching has enabled me to learn how to be more efficient in my classroom.

Nature of science

This final research summer has given me the experience of living science. I didn't just read about it or even complete a designed experiment. I struggled and experimented with lab techniques and issues surrounding my research. I experienced the true nature of science. The nature of science encompasses the idea, theory, and driving force behind gaining new scientific knowledge about the world around us. Science teachers have a difficult time conveying the nature of science to their students. The nature of science isn't about memorizing all the steps in photosynthesis or being able to write the formulas of various chemicals. The nature of science involves understanding the interrelatedness of the world by using scientific inquiry. The nature of science is not something students can learn by memorizing things that have already been done or proven. It's the ability to study and look at what's already been done as a way to find out new things. Science involves creating new methods and procedures to study the world.

A very important thing I have gained while researching for my master's report has been that the topics and applications I teach my biotechnology students are done on a daily basis in molecular, microbiological, and biotechnology labs across the world. These techniques are also used in infinite combinations depending on what is being done. This means that my students need to not only understand the basis of the technique but also be comfortable enough with the concept behind the technique that they can use the techniques in different contexts. My students do need to understand the steps and requirements for a PCR reaction. Students need to know these steps so that they can use that technology in ways that further their research or inquiry. The research I have conducted while pursuing my master's degree is an example of manipulating proven and understood techniques in ways that further the understanding of *V. cholerae* virulence. It exemplifies the nature of science in a way that I have never experienced. This research will be invaluable for conveying what the nature of science is to my students.

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