ABSTRACT

Title of Thesis: THE ROLE OF CHEV IN *S. TYPHIMURIUM* CHEMOTAXIS

Megan Elizabeth Dougherty, Master of Science, 2006

Directed By: Dr. Richard Stewart, Department of Cell Biology and Molecular Genetics

The chemotaxis systems of *Escherichia coli* and *Salmonella typhimurium* are thought to be virtually identical. However, recently a putative chemotaxis gene, *cheV*, was found to be present in *S. typhimurium* but not in *E. coli*. Sequence analysis shows that the CheV protein shares sequence similarity to both CheW and CheY. My thesis research investigated whether *cheV* does play a role in *S. typhimurium* chemotaxis. My results show that disruption of the *cheV* gene had no effect on *S. typhimurium*'s swarming ability and only a minor effect on the ability of *S. typhimurium* to sense/respond to serine and its ability to accomplish surface motility. My results also indicate that overexpression of the *cheV* gene disrupts *S. typhimurium*'s swarming ability, as well as, *S. typhimurium*'s ability to sense/respond to serine and *S. typhimurium*'s ability to accomplish surface motility. Overall, these results suggest that CheV may be involved in *S. typhimurium* chemotaxis.

THE ROLE OF CHEV IN *S. TYPHIMURIUM* CHEMOTAXIS

By

Megan Elizabeth Dougherty

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Advisory Committee:
Dr. Richard Stewart, Chair
Dr. Steven Hutcheson
Dr. Caren Chang

Dedication

To my loving husband, Jason

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I want to thank my advisor Dr. Stewart for all his help and advice along the way. He provided much needed guidance to someone who did not have a lot of laboratory experience before entering graduate school.

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Of course I need to thank my family for always being there for me. Their encouragement, support, and laughter were there whenever I needed it.

Lastly, I want to thank my husband Jason for being so understanding and supportive. He was always there to listen and cheer me up after a long day in the lab.

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List of Abbreviations

β-ME	Beta-mercaptoethanol
CFU	Colony forming unit
CW	Clockwise
CCW	Counter-clockwise
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetate
IPTG	Isopropyl β-D-thiogalactopyranoside
kDa	Kilodalton
MCP	Methyl-accepting chemotaxis protein
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction
RCR	Relative chemotactic ratio
SDS	Sodium dodecyl sulfate
SPI-1	*Salmonella* Pathogenicity Island-1

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Chapter 1: Introduction

The major goal of my thesis research was to determine if the CheV protein is a component of the chemotaxis system of *S. typhimurium*. To understand the possible role of CheV in chemotaxis it is necessary to understand how the chemotaxis system of *S. typhimurium* operates and to know the roles of the other components in this system. This introductory chapter will begin with an overview of chemotaxis and surface motility of *S. typhimurium*. Then the receptors and cytoplasmic signaling components of the chemotaxis system will be discussed before considering possible roles for CheV.

OVERVIEW OF CHEMOTAXIS

The chemotaxis systems of *E. coli* and *S. typhimurium* are very similar and have been used as model systems for understanding two-component signal transduction pathways (Reviewed in {Stock and Surette 1996}) (Figure 1). Other two-component signal transduction pathways are involved in many different aspects of prokaryotic physiology as well as response pathways in a limited number of eukaryotes (Reviewed in {Chang and Stewart 1998}).

The chemotaxis system consists of several proteins that allow communication between the cell surface receptors and the flagellar motors. This allows bacteria to move towards higher concentration of attractants, such as sugars and amino acids (Adler 1966b), and away from higher concentrations of repellents, such as metal ions and alcohols, by controlling the rotational bias of the flagellar motor. Clockwise

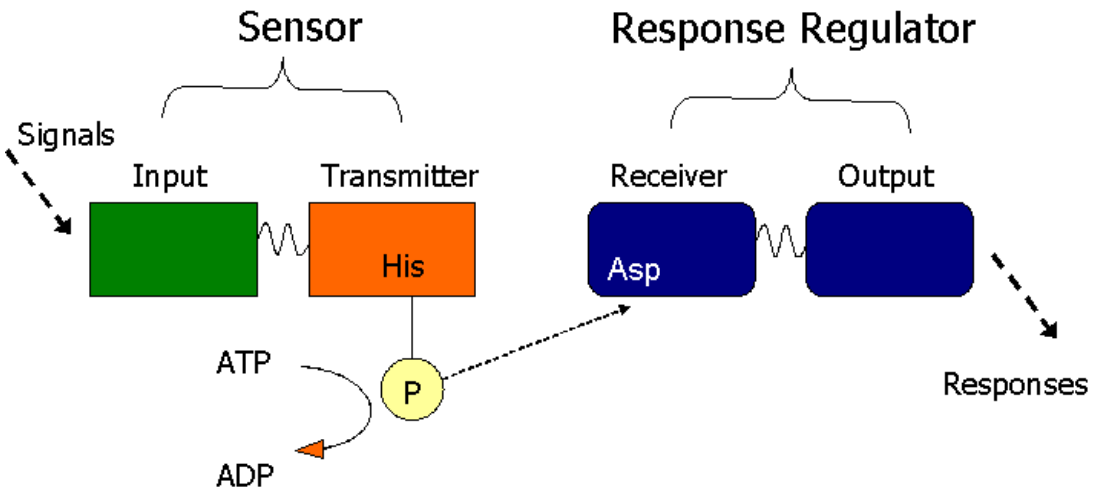

Figure 1. Two-Component Signal Transduction Pathways. This is a schematic of a generalized two-component signaling pathway. Two-component signaling pathways consist of a histidine kinase and a response regulator. The histidine kinase receives signals and this effects transfer of the phosphate to the response regulator. Upon phosphorylation, the response regulator can then elicit responses. (Adapted from Parkinson 1995)

flagellar rotation results in a tumbly behavior that changes the swimming direction of the cell (Macnab 1996). Counterclockwise rotation results in a smooth swimming behavior. A swimming S. typhimurium cell alternates frequently between smooth swimming 'runs' and brief episodes of tumbling that serve to randomly reorient the cell. By adjusting the frequency of tumbles, a cell can either extend its smooth swimming in a direction that takes it along an increasing gradient of attractant, or it can cut short a smooth swimming 'run' if the cell finds itself moving in unfavorable or neutral directions. The chemotaxis system only takes 50-100 milliseconds to detect and respond to stimuli (Segall 1986). In the absence of attractants and repellents, runs last about 1-2 seconds and tumbling episodes last about 0.1 seconds (Berg and Brown 1972). By alternately running and tumbling, a swimming cell moves along a path that has been described as a random walk.

Instead of sensing absolute concentrations of attractants and repellents, the receptors use temporal sensing to monitor changes in attractant/repellent concentrations (Macnab and Koshland 1972), i.e. the chemotaxis system compares the present concentrations to those it encountered in the recent past. The bacteria can sense changes in attractant/repellent concentrations that are less than 10% over a range of six orders of magnitude. For example, the threshold value for sensing serine is 2×10^{-7} M with a maximal response at 10^{-3} M (Adler 1969).

SURFACE MOTILITY

The preceding description of chemotaxis discussed the ability of the bacteria to swim through a liquid environment. Certain flagellated bacteria are also capable of swarming on a surface. To move in this manner, swarmer cells require slime, a mixture of

polysaccharides, surfactants and proteins that are secreted by the bacteria. These "swarmer cells" are generally longer and have more flagella than cells grown in liquid culture (Toguchi 2000). It has also been shown that expression of several outer membrane porins is decreased in swarmer cells (Kim 2004). The chemotaxis systems of *E. coli* and *S. typhimurium* play a role in swarm cell differentiation and motility. For example, it was recently shown that surface motility requires CheY binding to FliM (a component of the 'switch' that determines the direction of flagellar rotation) (Mariconda 2006). Moreover, deleting any of the *che* genes results in loss of surface motility in *S. typhimurium* and *E. coli* (Harshey and Matsuyama 1994). However, this effect may reflect the extreme CW or CCW flagellar rotation bias of these mutants. Eliminating individual chemoreceptor genes (mutations that do not have severe impacts on rotation bias) do not affect surface motility, leading researchers to conclude that chemotaxis itself (i.e., sensing attractants and repellents) is not necessary for surface movement (Burkart 1998).

CHEMOTAXIS AND PATHOGENICITY

Several decades ago it was proposed that chemotaxis contributes to the virulence of some motile pathogenic bacteria (Freter 1981a,b). However, there was not much additional research on the relationship between chemotaxis and pathogenesis in the following years. Only recently has there been a renewed interest in the relationship between chemotaxis and virulence.

Experiments performed on a wide variety of motile bacteria show that chemotaxis is important for virulence. Studies on the human pathogens *V. cholerae*, *H. pylori*, *C.*

jejuni, and *L. monocytogenes* all show that the chemotaxis system is necessary to colonize and establish infection in the host successfully (Reviewed in {Lux and Shi 2004}, Dons 2004). It has been further shown that non-chemotactic mutants of *V. cholerae* have lower toxin production *in vivo* (Lee 2001). Studies on the fish pathogen *Vibrio anguillarum* showed that a non-chemotactic mutant was less virulent than the wildtype strain (O'Toole 1996). Studies on the plant pathogens *Agrobacterium tumefacians* and *Ralstonia solanacearum* have also shown that chemotaxis is essential for pathogenesis (Hawes and Smith 1989, Yao and Allen 2006).

Some recent studies on *S. typhimurium* have also indicated that chemotaxis is involved in pathogenesis. Studies in animal models have shown that chemotaxis mutants are defective in pathogenesis. For instance, one study showed that a *cheY* deletion mutant could not compete with wildtype cells during an infection of streptomycin-pretreated mice (Stecher 2004). Another study showed that a *cheR* mutant failed to colonize the intestine of a gnotobiotic pig (Lovell and Barrow 1999). Additional evidence suggests that chemotaxis gene expression may be co-regulated with invasion gene expression. For example, one study found that *hilA* (a transcriptional regulator of SPI-1) and invasion gene expression are regulated by *fliZ* (Lucas 2000). *fliZ* is a gene with an unknown function that is located in an operon with *fliA*. FliA is a sigma factor that is required for transcription of the *mot* and *che* genes. This result raises the possibility that motility and invasion gene expression are co-regulated.

It has been proposed that surface motility can be used as a model for the initial stages of pathogenesis (Wang 2004). This idea stems from a microarray study that indicated iron metabolism genes in SPI-1 (*Salmonella* pathogenicity island -1) are

upregulated when the cells were grown on semi-solid agar compared to when the cells were grown in broth. This suggests that surface motility (and by extension, chemotaxis) may play a role in controlling gene expression in ways that promote pathogenesis. Further evidence that chemotaxis is involved in surface motility and potentially pathogenesis was provided by the gene expression profile of a *cheY* mutant swarming on the surface of agar. The gene expression profile revealed that motility related genes, including other *che* genes, and some SPI-1 genes were expressed at significantly lower levels than in a wildtype strain (Wang 2005). It was found that deletion of *flgM* restored expression of the motility genes, as well as, the virulence genes in the *cheY* mutant strain. FlgM is known to inhibit transcription of class 3 genes (including *che* genes) until the base of the flagellar motor is built and FlgM is exported out of the cell. This study indicates that FlgM affects virulence gene expression as well, raising the possibility that chemotaxis gene expression and virulence gene expression are co-regulated (Wang 2005).

COMPONENTS OF THE E.COLI/S.TYPHIMURIUM CHEMOTAXIS SYSTEMS

Overall, the roles of the essential components of the S. *typhimurium* and *E. coli* chemotaxis systems are depicted in Figure 2. In short, the signal transduction pathway involves receptor proteins that communicate with the flagellar motors by means of a cytoplasmic signaling cascade in which the protein kinase CheA directs phosphorylation of the response regulator CheY which, in turn, interacts with switching components (e.g. FliM) at the flagellar motors.

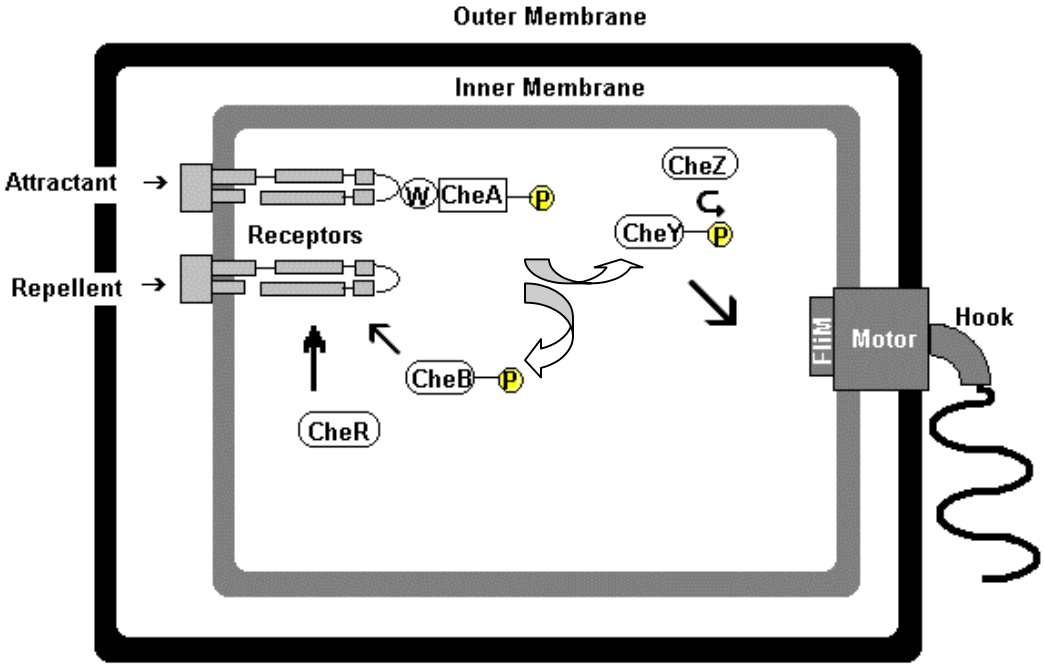

Figure 2. Schematic Overview of the Chemotaxis Systems of *E. coli* and *S. typhimurium*. The receptors are found in the inner membrane and are complexed to CheW and CheA. The binding of attractants or repellents affects the autophosphorylation ability of CheA. CheA can then pass its phosphate to CheB or CheY. P-CheY binds to FliM at the flagellar motor causing a change in the direction of rotation of the flagella. CheB is a methylesterase that, along with CheR, is part of the adaptation system. (Adapted from Levit and Lui 1998)

Signaling by the Receptors

E. coli and *S. typhimurium* cells sense their external chemical environments through transmembrane receptors. These receptors of the chemotaxis system include: Tar (taxis to aspartate and repellents), Tsr (taxis to serine and repellents), Trg (taxis to ribose and galactose), and Aer (taxis to O_2) (Clarke and Koshland 1979, Adler and Hazelbauer 1973). In *E. coli* there is the additional receptor Tap (taxis to dipeptides) that is not found in *S. typhimurium*, and in *S. typhimurium* there is the additional receptor Tcp (taxis to citrate and phenol) not present in *E. coli* (Manson 1986, Yamamoto and Iame 1993). Tar and Tsr are the most abundant receptors with approximately 5000 copies of each per cell (Parkinson 2004). Some attractants and repellents bind directly to the receptors, while the binding of others is mediated through binding proteins that reside in the periplasm. Binding of an attractant or repellent causes a conformational change in the receptor proteins that affects CheA activity. The receptors are also referred to as MCPs (methyl-accepting chemotaxis proteins) because their activity is regulated through methylation/demethylation.

All the chemotaxis receptors except Aer have similar amino acid sequence and structure. The receptors have a short N-terminal cytoplasmic extension, followed by a transmembrane sequence, TM1; then a periplasmic ligand binding domain; another transmembrane sequence, TM2; a linker region; a methylated helix, MH1; a signaling domain; another methylated helix, MH2; and a variable C-terminal domain (Wang and Koshland 1980). Figure 3 is a schematic representation of the receptor structure. The methylated helix regions contain four sites subject to methylation and demethylation reactions. These sites are encoded as either glutamate or glutamine residues. The

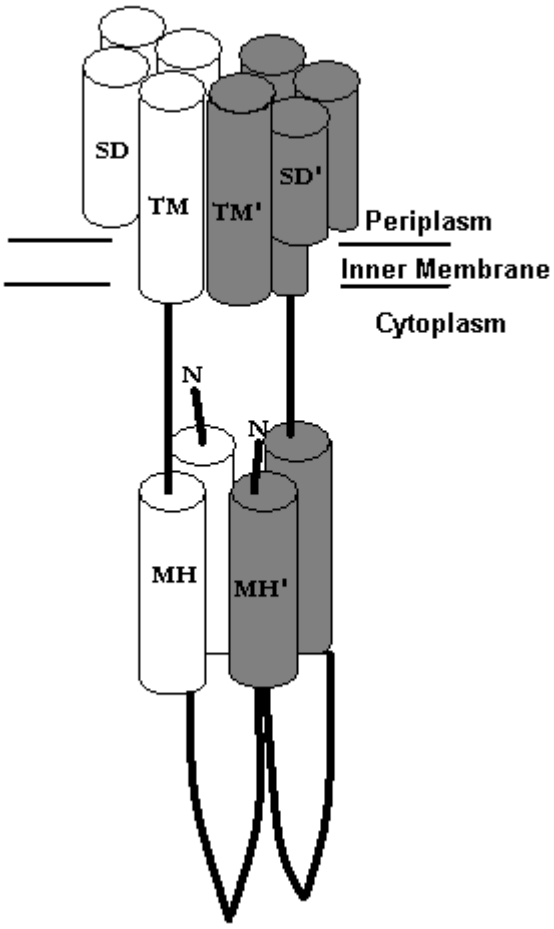

Figure 3. Schematic of a receptor dimer. One receptor subunit is shown in white and the other in grey. The transmembrane domains (TM) span the inner membrane. The attractant and repellent ligands bind at the sensory domain (SD). The loops in the cytoplasm form the signaling domain. The methylated helices (MH) are the site of adaptation (Adapted from Stock and Surette 1996.)

glutamine residues are deamidated by CheB converting them into glutamate residues which are then subject to methylation/demethylation (Sherris and Parkinson 1981).

Cross-linking studies in *S. typhimurium* showed that purified Tar exists as a homodimer (Milligan and Koshland 1988). The ligand binding sites are located at the interface between the two subunits of a dimer. The receptors show negative cooperativity in that binding of one aspartate molecule inhibits binding of a second aspartate molecule to the receptor dimer (Biemann and Koshland 1994).

For receptors located in membranes, the receptor dimers appear to associate in larger complexes. For example, some evidence supports formation of trimers of receptor dimers (Ames 2002). In living cells, even larger complexes appear to form. These larger receptor complexes cluster at one or both poles of the cell (Gestwicki 2000). There is evidence to suggest that a trimer of receptor dimers forms the smallest signaling unit within the cluster. Originally it was believed that all three dimers in the trimer were made up of the same receptor protein (e.g. that distinct Tsr and Tar trimers of dimers existed: $(Tsr)_2$:$(Tsr)_2$:$(Tsr)_2$ and $(Tar)_2$:$(Tar)_2$:$(Tar)_2$). However, recent evidence from cross-linking studies suggests that the receptors form mixed trimers of dimers (Studdert and Parkinson 2004). A mixed trimer of dimers means that dimers made up of different receptor proteins form the trimer (e.g. a trimer of dimers might be $(Tsr)_2$:$(Tsr)_2$:$(Tar)_2$ and/or $(Tsr)_2$:$(Tar)_2$:$(Tar)_2$). It has been also been shown that the receptors in mixed trimers of dimers cooperate to regulate CheA activity (Lai 2005).

Chemotaxis Signaling Proteins

The chemotaxis signaling proteins (CheA, CheY, CheW, CheZ, CheR, and CheB) reside in the cytoplasm of *E. coli* and *S. typhimurium,* often in close association with the

cytoplasmic segments of the chemotaxis receptor proteins (Sourjik and Berg 2000). The *S. typhimurium* and *E. coli* versions of these proteins share high sequence similarity. Indeed, *S. typhimurium che* genes can complement (restore chemotaxis ability to) *E. coli che* deletion mutants (DeFranco 1979).

CheA

CheA is a 71 kDa cytoplasmic protein (Kofoid and Parkinson 1991) (671 amino acids in *S. typhimurium*; 655 amino acids in *E. coli*). It autophosphorylates at the histidine-48 residue using ATP as a phosphodonor (Hess 1988a). Phosphorylated CheA can donate its phosphate to CheY or CheB (Hess 1988b). Purified CheA exists as a homodimer with one subunit trans-phosphorylating the other (Surette 1996). CheA forms complexes with receptors and CheW, and signal transduction in the chemotaxis system is thought to involve a ternary complex of receptors plus CheW plus CheA.

CheA is a member of the histidine kinase superfamily of proteins that operate/function in two-component signal transduction pathways (Stock 1988). The chemotaxis receptors regulate CheA autokinase activity in response to the availability of attractant and repellent concentrations. This, in turn, determines how much phosphorylated CheY is present. CheY is a response regulator protein that, when phosphorylated, can bind to FliM at the flagellar motor. Binding of P-CheY to FliM causes a change in the direction of rotation of the flagella from CCW to CW resulting in the cell tumbling (Barak and Eisenbach 1992). In the absence of P-CheY the flagellar motors spin CCW and the cell continues swimming in a relatively straight direction termed smooth-swimming. The binding of attractants causes a decrease in the

autophosphorylation rate of CheA within the receptor-CheA-CheW complexes, while the binding of repellents causes an increase in the autophosphorylation rate of CheA (Borkovich 1989). This regulation of CheA activity allows cells to move towards higher concentrations of attractants and away from higher concentrations of repellents.

Both deletion and overexpression of *cheA* result in a chemotaxis defect due to a smooth swimming bias (Oosawa 1988, Conley 1989).

CheY

CheY is a member of the superfamily of response regulator proteins. This family of proteins regulates outputs in response to sensory inputs from their cognate histidine kinases (Stock 1985). Response regulators share several conserved residues, including two aspartate residues that coordinate magnesium, an aspartate residue that is phosphorylated, a threonine residue that is involved in the conformational change, and a lysine found in the active site adjacent to the phosphorylation site (Lukat 1991). In CheY, these conserved positions are aspartate 12 and 13 (coordinate magnesium), aspartate 57 (phosphorylation site), threonine 87, and lysine 109. These positions will come up again in the discussion of CheV.

CheY is a 14 kDa protein that is 129 amino acids in length. Its structure has been determined by x-ray crystallography (Stock 1993). The structure shows a doubly wound α/β protein consisting of five alpha-helices surrounded by a five-stranded parallel beta-sheet.

CheY binds to CheA with a K_d of 1 to 2 μM (Schuster 1993). CheA then phosphorylates CheY at aspartate-57 (Sanders 1989). Upon phosphorylation, CheY is released from CheA. Phosphorylation induces a change in the structure of CheY that

allows it to bind to FliM at the flagellar motor and promote CW flagellar rotation (Welch 1993).

Deletion of the *cheY* gene results in a severe chemotaxis defect and a smooth-swimming bias (Parkinson 1978). Overexpression of the *cheY* gene results in a tumbly phenotype (Clegg and Koshland 1984).

CheW

CheW is an adapter protein. It is an 18 kDa protein that is 167 amino acids in length. It serves to complex CheA to the receptors (Gegner 1992). Valine-36 of CheW mediates contact with Tar and glycine-57 mediates contact with CheA (Boukhvalova 2002). These positions will come up again in the discussion of CheV.

Both deletion and overexpression of the *cheW* gene result in a chemotaxis defect and a smooth-swimming bias (Parkinson 1978, Sanders 1989).

CheZ

CheZ regulates the rate of CheY dephosphorylation (Hess 1988b). The phosphorylated form of CheY binds with high affinity to CheZ and, upon dephosphorylation, it is released from CheZ (Blat and Eisenbach 1994).

Deletion of *cheZ* results in a chemotaxis defect due to a tumbly bias (Parkinson 1988). Overexpression of *cheZ* also results in a chemotaxis defect but it is due to a smooth swimming bias (Stewart 1988).

CheR and CheB

CheR and CheB are part of the sensory adaptation system of chemotaxis. These proteins function to return the cell to pre-stimulus levels of signaling. CheB is a methylesterase that participates in sensory adaptation by hydrolyzing methyl ester groups attached to specific glutamate residues of the receptors (Stock 1985). CheR is a methyltransferase that catalyzes the transfer of methyl groups from AdoMet to these same glutamate residues of the receptors (Simms 1987). Increased levels of methylation of receptors causes the rate of CheA autophosphorylation to increase, and decreased levels of methylation of receptors causes the rate of CheA autophosphorylation to decrease (Ninfa 1991). The rates of methylation and demethylation are controlled by phosphorylation of CheB and conformational changes of the receptors that affect the ability of CheR and CheB to bind to them.

Deletion of *cheR* results in a smooth swimming bias (Springer and Koshland 1977), while overexpression of *cheR* results in a tumbly bias (Stewart 1988). Deletion and overexpression of *cheB* have the opposite effects. Deletion of *cheB* results in a tumbly bias (Stock 1991), while overexpression of *cheB* results in a smooth swimming bias (Stewart 1988).

CheV

Discovery of CheV

CheV was first identified in the chemotaxis system of *B. subtilis* (Fredrick and Helmann 1994). It was shown that disruption of the *cheV* gene affected the cell's ability

to accomplish chemotaxis as measured in a swarm plate assay. Analysis of the *S. typhimurium* genome by our lab (using *B. subtilis cheV* as the query sequence) suggested that there is an additional signaling component (CheV) not found in *E. coli*. The Harshey lab and the Hughes lab also identified a putative CheV protein in *S. typhimurium* by performing microarray analysis (Wang 2004, Frye 2006).

The role of CheV in other bacteria

The chemotaxis systems of many bacteria are more complex than the chemotaxis systems of *E. coli* and *S. typhimurium* in that they contain more cytoplasmic signaling proteins. In order to gain a better understanding of what role CheV might play in *S. typhimurium*, it is important to know how other chemotaxis systems operate and how CheV fits into those systems.

The chemotaxis system of *Vibrio cholerae* contains 22 *che* gene homologs. These include three *cheA* homologs, two *cheB* homologs, one *cheD* homolog, three *cheR* homologs, four *cheV* homologs, four *cheW* homologs, four *cheY* homologs, and one *cheZ* homolog (Rao 2004). Most of the *che* genes are found in three distinct clusters. Interestingly, all of the *cheV* homologs are found in separate locations and not in any of the clusters (Reviewed in {Boin 2004}). This is similar to the situation in *S. typhimurium* in which *cheV* is not clustered with any of the other *che* genes. Evidence so far indicates that the *che* homologs in cluster II are important in *V. cholerae* chemotaxis, and the roles of the other *che* genes have not been determined yet (Reviewed in {Boin 2004}). If not all the *che* gene homologs are necessary for chemotaxis, it raises the possibility that some of the *che* gene homologs are part of some other sensory response system.

In *Helicobacter pylori* there are several paralogs of CheV. These are designated CheV1, CheV2, and CheV3. Pittman et. al. (2001) used mutants to study the effects of removing the different paralogs. It was discovered that a *cheV1* mutant had impaired chemotaxis, while the *cheV2* and *cheV3* mutants behaved the same as wild type. Pittman also separately expressed *cheV2* and *cheV3* in wild type *E. coli.* Expression of either of these genes inhibited chemotaxis just like overexpression of any of the *E. coli* or *S. typhimurium che* genes in a wildtype background inhibits chemotaxis (Parkinson 1978). Pittman et. al. performed further experiments supporting the idea that at least CheV2 is capable of being phosphorylated. A later study showed that all three paralogs are phosphorylated in the presence of CheA (Jimenez-Pearson 2005). The chemotaxis system of *H. pylori* has no CheB, CheR, or CheZ homologs (Rao 2004). Therefore, it is possible that the CheV paralogs act as a phosphate sink that could contribute to adaptation or to shutting off the tumble signal. In *H. pylori* such contributions might be necessary since it appears to lack a CheY phosphatase (i.e. no CheZ) and has no methylation/demethylation system (i.e. no CheR or CheB).

In *Bacillus subtilis* expression of the *cheV* gene is controlled by a σ^D promoter element (Frederick and Helmann 1994). This sigma factor activates the expression of several operons containing motility and chemotaxis genes. In *E. coli* and *S. typhimurium* expression of the *che* genes is controlled by a σ^{28} promoter element, a homolog of σ^D in *B. subtilis*. It has now been shown that *S. typhimurium cheV* is under the control of the σ^{28} promoter element (Frye 2006). Rosario et. al. (1994) investigated *B. subtilis* CheV in relation to CheW by examining *cheV* and *cheW* mutant strains. The single mutants showed reduced chemotaxis ability. This result indicates that CheV and CheW have

distinct roles. A double mutant strain (*cheVcheW)* showed severely diminished ability to accomplish chemotaxis, a more severe phenotype than for either of the single mutants. In a further test it was determined that the double mutant could not respond to an attractant and showed a severe tumble bias. Karatan et al. (2001) investigated CheV phosphorylation in *B. subtilis*. They used point mutations to show that aspartate-235 is the phosphorylation site. In addition, they showed that a truncated protein containing only the C-terminal CheY-like domain is capable of being phosphorylated. Their experiments demonstrated that the rate of phosphate transfer from CheA-P to CheV is slow compared to CheA-P to CheY phosphate transfer, but that CheV-P is considerably more long-lived than P-CheY. Presumably this latter difference reflects CheY's ability to catalyze its own dephosphorylation at a high rate while CheV is less capable of accomplishing this. This study also showed that CheV is necessary for complete adaptation to asparagine in *B. subtilis* (Karatan 2001).

Gene expression studies

A microarray study on *S. typhimurium* showed that *cheV* has the same expression pattern as Class 3 *fla*/*che* genes (Wang 2004). The flagellar biosynthesis genes are divided into three different classes based on when the genes are transcribed. Class 1 genes are transcribed first and promote transcription of Class 2 genes. Class 2 genes promote transcription of Class 3 genes once the base structure of the flagella is complete. Class 3 genes include the *mot* and *che* genes. Another study showed that expression of *cheV* is *flhDC* dependent as well as σ^{28} dependent (Frye 2006). *flhDC* is a Class 1 gene encoding a protein that activates transcription of Class 2 genes, and σ^{28} is encoded by a

Class 2 gene. σ^{28} recognizes a promoter element known to activate transcription of Class 3 genes. *flhDC* and σ^{28} are required for expression of all Class 3 *fla*/*che* genes. So with regard to its expression pattern and requirements, *cheV* appears to be very similar to other *che* genes.

Sequence Analysis of *S. typhimurium* CheV

A BLASTP search of the NCBI database using *S. typhimurium* CheV as the query sequence reveals hits (sharing both CheV domains) in a wide variety of bacteria and one archeon. Figure 4 shows the sequence alignment of CheV of some representative species to CheV of *S. typhimurium*. *S. typhimurium* CheV shares amino acid sequence similarity to putative CheV ORFs in species of *Yersinia*, *Ralstonia*, *Thiobacillus*, *Rhodoferax*, *Colwellia*, *Aeromonas*, *Campylobacter*, *Clostridium*, *Listeria*, other serovars of *S. enterica,* and the archeon *Methanospirillum* as well. BLASTP searches indicate that none of the fully sequenced strains of *E. coli* has a *cheV* ortholog.

Despite the fact that CheV is found in numerous prokaryotes, not much is known about the function of this protein. Sequence analysis shows that CheV has an N-terminal domain with sequence similarity to CheW (Figure 5) and a C-terminal domain with sequence similarity to CheY (Figure 6). CheW serves as an adapter protein to complex CheA to the receptors. Since CheV shares sequence similarity

with CheW, it is possible that CheV can also complex CheA to the receptors. There have been several studies that have identified residues that mediate contact of CheW with CheA and the receptors (Boukhvalova 2002 and Griswold 2002). There are ten residues that have been shown to mediate contact of CheW with CheA. These

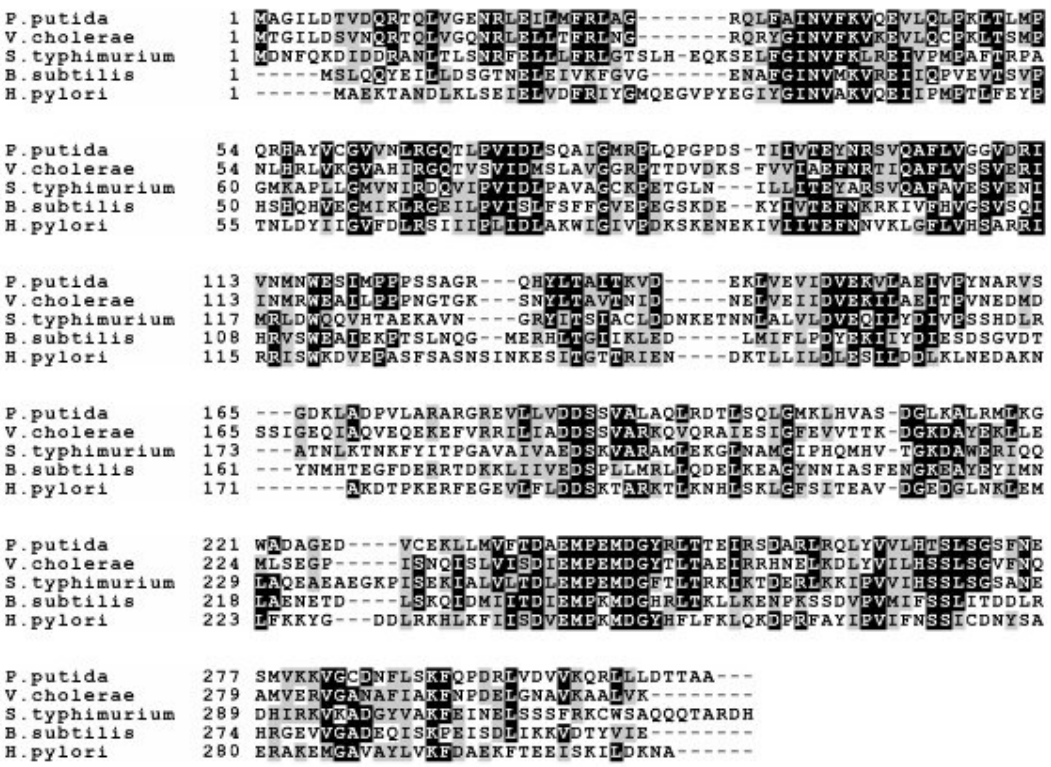

Figure 4. Sequence alignment of the CheV protein from several bacteria. The sequence alignment was done using the ClustalW multiple sequence alignment program available on the BCM Search Launcher website of the CheV protein from *P. putida*, *V. cholerae*, *S. typhimurium*, *B. subtilis*, and *H. pylori*. The e values for the alignment of the different CheV homologs with *S. typhimurium* CheV were calculated by using the BLAST program available at the NCBI website. The e value for the alignment with *P. putida* is $1X10^{-44}$, for *V. cholerae* is $1X10^{-42}$, for *B. subtilis* is $2X10^{-23}$, and for *H. pylori* is $2X10^{-26}$. The Boxshade program was used for the highlighting. The identical amino acids are highlighted in black boxes. The conservative substitutions are highlighted in grey boxes.

Figure 5. Sequence Alignment of CheV and *E. coli* CheW. The sequence alignment was done using the ClustalW multiple sequence alignment program found on the EMBL website. The Boxshade program was used for the highlighting. Black boxes indicate identical residues and gray boxes indicate conservative substitutions. This sequence alignment shows that the N-terminal domain of CheV shares sequence similarity with CheW based on the large number of identical or conservative residues between the proteins. In this alignment, the protein sequences are 23% identical with 50% similarity. The e value is $3X10^{-4}$. Residues that mediate contact of CheW with CheA include G41, V45, T46, T51, K56, G57, I65, G133, M156, and L158 (Alexandre 2003). Three out of the ten residues known to mediate contact of CheW with CheA are identical or conservative substitutions in CheV. Residues that mediate contact of CheW with Tar include V36, E38, I39, G41, G63, T86, V87, V88, G99, V105, V108, and G133 (Alexandre 2003). Eight out of the twelve residues that are known to mediate contact of CheW with CheA are identical or conservative substitutions in CheV.

Figure 6. Sequence alignment of CheV and *E. coli* CheY. The sequence alignment was done using the ClustalW multiple sequence alignment program available on the EMBL website. The Boxshade program was used for the highlighting. The black boxes indicate identical residues and the grey boxes indicate conservative substitutions. This alignment shows that the C-terminal domain of CheV shares sequence similarity with CheY based on the large number of identical and conservative residues between the proteins. In this alignment, the protein sequences are 22% identical with 44% similarity. The e value is 3.6. The conserved residues among the CheY family of response regulators include A12, A13, A57, T87, and K109. A57 is the site of phosphorylation in CheY; this corresponds to A250 in CheV. All the conserved residues among the CheY family of response regulators are either identical in CheV or are conservative substitutions. Residues that mediate contact of CheY with CheA include T87, A90, E93, Y106, V108, F111, T112, and E117 (Shukla 1995). Three out of the eight residues known to mediate contact of CheY with CheA are conserved in CheV. Residues that mediate contact of CheY with FliM include E27, A90, V108, F111, T112, and E117 (Shukla 1998). Five out of the six residues that are known to mediate contact of CheY with FliM are not conserved in CheV. This suggests that it is unlikely CheV is capable of binding to FliM.

include residues threonine-46, glycine-57, and isoleucine-65. These correspond to residues threonine-46, glycine-57, and valine-65 in a ClustalW alignment of CheV (Figure 5). The rest of the residues that mediate contact of CheW with CheA are not conserved in CheV. This suggests that if CheV binds to CheA it binds at a location that is distinct from the location that CheW binds to CheA. There are twelve residues that have been shown to mediate contact of CheW to Tar. These include residues valine-36, glutamate-38, isoleucine-39, valine-87-valine-88, glycine-99, valine-105, and valine-108. These correspond to residues leucine-36, glutatmate-38, isoleucine-39, isoleucine-87, leucine-88, alanine-99, valine-105, and isoleucine-108 in a ClustalW alignment of CheV (Figure 5). Since eight out of the twelve residues that are known to mediate contact of CheW with Tar are identical or conservative substitutions in CheV, it suggests that CheV might be able to bind with Tar in a manner similar to CheW.

Since the C terminal domain of CheV shares sequence similarity with CheY, it is possible that CheV can be phosphorylated by CheA as well. The site of phosphorylation in CheY is aspartate 57, which corresponds to aspartate 250 in CheV in a BLAST alignment (Figure 6). Response regulators also have two conserved aspartate residues that help coordinate magnesium, a conserved threonine that is involved in the conformational change, and a conserved lysine that is found in the active site. The two conserved aspartate residues are residues 12 and 13 in CheY. These correspond to glutamate-193 and aspartate-194 in CheV. The conserved threonine is residue 87 in CheY and this corresponds to serine-179 in CheV. The conserved lysine is residue 109 in CheY and this corresponds to lysine-302 in CheV. Overall, CheV has an identical aspartate that is the site of phosphorylation in CheY, an identical aspartate that helps coordinate magnesium

in CheY, a conservative substitution at the other position that helps coordinate magnesium in CheY, a conservative substitution at the position that is involved in the conformational change in CheY, and an identical lysine that is found in the active site in CheY. Since five out of the five residues that are conserved among response regulators are either identical or conservative substitutions in CheV, it suggests that CheV may be a response regulator.

It is also worthwhile considering the possibilities that CheV can bind to CheA and/or FliM in a matter similar to CheY. In CheY there are eight residues known to mediate contact with CheA (Shukla 1995). These include threonine-87, alanine-90, glutamate-93, tyrosine-106, valine-108, phenylalanine-111, threonine-112, and glutatmate-117. These residues correspond to serine-179, serine-182, alanine-185, tyrosine-299, alanine-301, phenylalanine-303, glutamate-304, and serine-309 of CheV in the ClustalW alignment (Figure 6). Thus, there are two identical residues and one conservative substitution between the residues known to mediate contact with CheA in CheY and the corresponding residues in CheV. Since only three out of the eight residues are conserved, it is unlikely that CheV binds to CheA at the same site that CheY binds to CheA. This does not necessarily mean that CheV binds to a different domain of CheA. Both CheY and CheB bind to the P2 domain of CheA despite sharing only six out of seventeen conserved residues that are known to mediate contact of CheY with CheA (McEvoy 1998).

There are six residues that are known to mediate contact between CheY and FliM. These include glutamate-27, alanine-90, valine-108, phenylalanine-111, threonine-112, and glutamate-117 (Shukla 1998). These residues correspond to alanine-208, serine-182,

alanine-301, phenylalanine-303, glutamate-304, and serine-309 of CheV in the ClustalW alignment (Figure 6). So, of the six residues known to mediate contact with FliM in CheY, only one is present in CheV making it unlikely that CheV is able to bind FliM.

In summary, CheV shares sequence similarity with both CheW and CheY. It is therefore possible that CheV has a similar function to CheW, CheY, or both. Upon further investigation, it seems likely that CheV may be able to act as an adapter protein in a way similar to CheW. CheV has three out of ten residues conserved that are known to mediate contact of CheW with CheA and eight out of twelve residues conserved that are known to mediate contact of CheW with Tar (Figure 4). It also seems likely that CheV is capable of being phosphorylated in a manner similar to CheY. CheV has an aspartate residue comparable to the aspartate residue that is phosphorylated in CheY (Figure 5). CheV homologs in *B. subtilis* and *H. pylori* are phosphorylated, so it seems likely that CheV in *S. typhimurium* is phosphorylated too (Pittman 2001, Karatan 2001, Jimenez-Pearson 2005). In addition, CheV contains identical residues or conservative substitutions at positions known to be conserved among response regulators (Figure 5). However, despite the overall sequence similarity of CheV to CheY, this similarity does not include the residues known to mediate contact of CheY with FliM. Therefore, it seems unlikely that CheV can bind at the flagellar motors.

Based on sequence analysis and studies of CheV homologs in other bacteria, I hypothesize that CheV is involved in *S. typhimurium* chemotaxis. Sequence analysis of *S. typhimurium* CheV shows that there is a high degree of similarity between the residues that are known to mediate contact of CheW with the receptors and the corresponding

residues in CheV (Figure 5). It therefore seems possible that CheV can bind to the receptors in a manner similar to CheW. Sequence analysis also shows that CheV has either identical residues or conservative substitutions at the positions that are known to be conserved among the response regulator family of proteins. This includes an aspartate residue that is the site of phosphorylation in other members of the response regulator family of proteins. Therefore, it seems possible that CheV can compete with CheY for the phosphate from CheA. Perhaps CheV acts as a phosphate sink. To help illustrate the role I think CheV may play in chemotaxis refer to Figure 7.

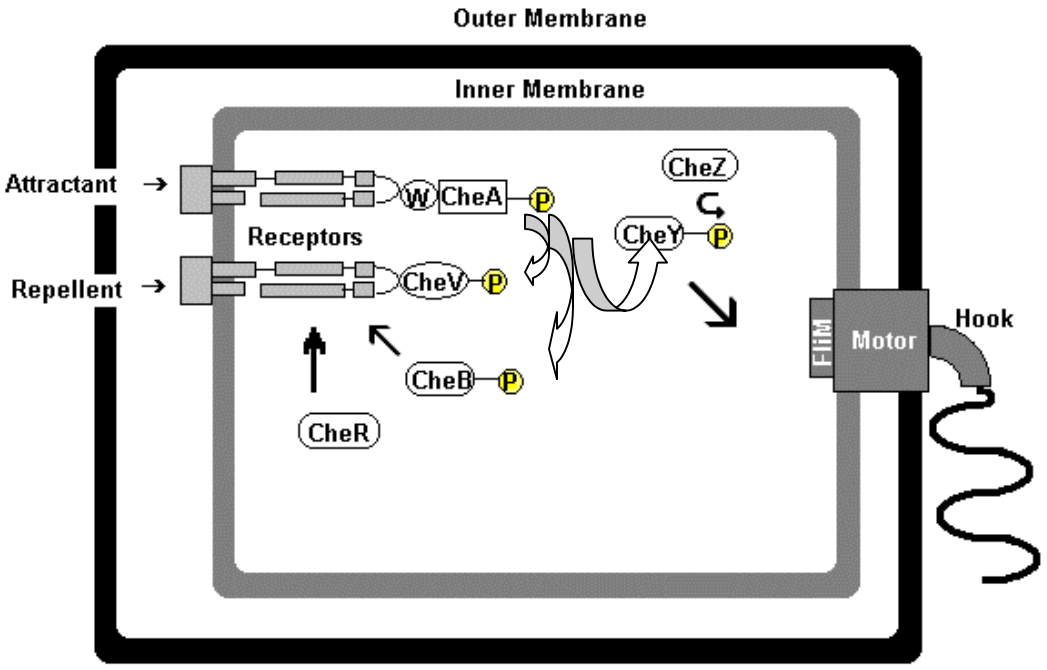

Figure 7. Schematic Diagram of Role Hypothesized for CheV in *S. typhimurium* Chemotaxis System. This schematic includes all the chemotaxis proteins known to be involved in chemotaxis, as well as, CheV. I propose that CheV can bind to the receptors and compete with CheY and CheB for the phosphate from CheA.

Chapter 2: Effects of disrupting *cheV*

INTRODUCTION

Sequence analysis shows that the CheV protein shares sequence similarity with both CheW and CheY. This raises the possibility that CheV has a similar function to either CheW or CheY, or both. CheW and CheY are both essential components of the chemotaxis system in *S. typhimurium,* and the absence of either protein results in a severe chemotaxis defect. If CheV is also involved in chemotaxis, it is likely that the absence of CheV will also result in a chemotaxis defect.

CheW is an adapter protein that serves to complex CheA to the receptors. The receptors control the rate of CheA autophosphorylation within the receptor-CheW-CheA complexes. In the absence of CheW chemotaxis is inhibited and a smooth swimming bias is observed. Since CheA is not able to form effective signaling complexes with the receptors in the absence of CheW, the receptors are unable to control the rate of CheA autophosphorylation. In the absence of P-CheA, there is no phosphate to pass to CheY and the flagella remain in the default counterclockwise rotation and the cell continues swimming in the same direction regardless of attractant/repellent concentrations.

CheY is a response regulator that is phosphorylated by CheA and then binds at the flagellar motor causing a change in the direction of flagellar rotation to clockwise. In the absence of CheY the flagella remain in the default counterclockwise rotation and the cell continues swimming in the same direction regardless of attractant/repellent concentrations.

EXPERIMENTAL METHODS

Materials – All chemicals were purchased from common sources (Fisher Scientific, Sigma, Invitrogen) and were reagent grade.

Strains and Plasmids – S. typhimurium strain ST1 (ATCC29595) was used as the wildtype strain (Tindall 2005, McClelland 2001). A chloramphenicol cassette was inserted into the *cheV* gene using the one-step inactivation method of Datsenko and Wanner (2000). The cam cassette was inserted between codons 150 and 151 creating strain ST1 *cheV::cam* (Stewart). The one-step inactivation method was also used to create strain ST1 *hisG::cam*. This strain contains an inactivated histidine gene and was used as a control. Strain RS2901 was used as a representative *S. typhimurium* chemotaxis mutant. This strain has a defective *cheZ* gene that results in a smooth swimming bias.

Swarm Assay – A colony of strain ST1 or ST1 *cheV::cam* was stabbed into the center of a tryptone swarm plate (Adler 1966a, Wolfe and Berg1989) containing 0.3% Difco Bacto Agar, 1% tryptone, 0.5% NaCl, and 34μg ml^{-1} Chloramphenicol when needed. The diameter of the outermost ring of the resulting swarm colonies was measured after incubating overnight on the bench top.

Surface Motility Assay- Liquid cultures of strain ST1 or ST1 *cheV::cam* were grown for about six hours in LB broth and 34μg ml^{-1} chloramphenicol when needed and diluted to an absorbance at 600nm of approximately 0.02. 3μL of the liquid cultures were pipetted onto surface motility plates already placed in an incubator set at 37ºC and

incubated overnight. The next day the size of the resulting surface area of the motility colony was analyzed using the ImageJ program available for download from the NIH website. The surface motility plates contained 0.6% Agar, 1% tryptone, 0.5% yeast extract, 1% NaCl, 0.5% dextrose, 34μg ml^{-1} Chloramphenicol when needed (Burkart 1998).

Capillary Assay- I followed the procedure developed by Mazumder and coworkers (Mazumder 1999). Overnight cultures of strain ST1 or ST1 *cheV::cam* were diluted into 20mL fresh LB media and 34μg ml^{-1} Chloramphenicol when needed. Cultures were grown in a rotary shaker at 37ºC until an absorbance at 600nm of 0.3 was reached. The cells were then harvested by centrifugation. The pellet was washed twice in 5mL ice cold chemotaxis buffer pH 7.0 (10^{-2}M potassium phosphate, 10^{-4}M EDTA, and 10^{-6}M L-methionine) (Adler 1969). The supernatant was removed and the pellet was resuspended in the remaining liquid. The resuspended cells were diluted to an absorbance at 600nm of 0.2. A disposable 200μL pipette tip was filled with 100μL of the bacterial suspension. A 1mL syringe attached to a 25 gauge needle was used to draw up 100μL of the recently vortexed attractant solution. Excess solution was rinsed off the needle with sterile water and dried with a Kimwipe. The needle-syringe capillary was then inserted into the pipette tip containing the bacterial suspension (Mazumder 1999). After a 45 minute incubation at room temperature the needle-syringe was removed and the outside was rinsed with sterile water and dried with a Kimwipe. The contents were then diluted into chemotaxis buffer. ST1 cells that were incubated with buffer were diluted tenfold and cells incubated with serine were diluted a hundredfold and 100μL was plated on LB

plates. ST1 *cheV::cam* cells that were incubated with buffer were diluted a tenfold and cells incubated with serine were diluted a hundredfold and 100µL was plated on LB plates containing chloramphenicol. Accumulation in the capillary was determined from the CFUs on the plates. Capillaries containing only chemotaxis buffer were used as controls. The attractant solution used was 1mM serine dissolved in chemotaxis buffer at pH 7.0.

Growth Curve – Overnight cultures of ST1 or ST1 *cheV::cam* were diluted into 20mL fresh LB media and 34µg ml^{-1} Chloramphenicol when needed. The cultures were grown at 37ºC in a rotary shaker. The absorbance at 600nm was taken every thirty minutes for a total of 420 minutes. Absorbance verses time was plotted to obtain a growth curve.

PCR – Colony PCR was used to verify the *cheV* and *hisG* insertional mutants. The reaction mixture included 2µL colony lysate in PBS, 5µL 25mM $MgCl_2$, 5µL 10X Taq buffer, 1µL dNTP mix, 2µL 10µM primer up, 10µM primer down, 32µL sterile H_2O, and 1µL Taq polymerase. The thermocycler was set to denature for 5 minutes at 94°C; 30 cycles of 30 seconds at 94°C, 30 seconds at 55°C, and 3 minutes at 72°C; a final extension of 7 minutes at 72°C; and a final hold at 10°C. Primer cheV-up is 5'-CGATAACCAAACGTGTATGGACAAGA and hybridizes to a site located approximately 1kB upstream of the start of *cheV*. Primer cheV-down is 5'-GTCTACAGCGGGAAATAACACAACC and is complementary to a site located approximately 1kB downstream of the end of *cheV*.

RESULTS

The results presented below summarize my attempts to determine whether disrupting *cheV* affects the ability of *S. typhimurium* to accomplish chemotaxis or its surface motility. Before delving into the results, it is useful to briefly review the assays I used to answer the question.

There are several useful methods for examining the chemotactic ability of bacteria. The chemotaxis movement of motile bacteria can be observed by inoculating the center of a Petri plate containing semi-solid nutrient agar. The bacteria "swarm" outwards in concentric rings formed in response to spatial gradients generated by transport and metabolism of nutrients (Wolfe 1989). The bacteria need to be able to both run and tumble, as well as regulate their movements in response to chemical gradients, to efficiently move through the semi-solid agar and generate the "rings".

A capillary assay can be used to access chemotactic ability towards certain attractants at specific concentrations. Pfeffer first used this method in the 1880's. If a capillary tube containing an attractant is placed in a liquid suspension of bacteria, the bacterial cells will start to accumulate in the capillary tube.

Certain flagellated bacteria are also capable of swarming on a surface. Swarmer cells require slime, a mixture of polysaccharides, surfactants and proteins, for movement. These cells are generally longer and have more flagella than cells grown in liquid culture (Harshey 2003, Toguchi 2000). Burkart et. al. (1998) found that the chemotaxis systems of *E. coli* and *S. typhimurium* play a role in swarm cell differentiation and motility, but chemotaxis itself is not necessary for surface movement.

Construction of a *cheV::cam* insertion mutant

A *cheV* insertional mutant was obtained by using the one-step inactivation method of Datsenko and Wanner (2000) to insert a chloramphenicol cassette into the *cheV* gene in the chromosome of ST1 (Stewart). The presence of the chloramphenicol cassette was verified by colony PCR (Figure 8). The *cheV* gene is approximately 1kB. The primers that were used in the PCR reaction were located 1kB upstream of *cheV* and 1kB downstream of *cheV*. Therefore, in a wildtype cell the resulting PCR product should be 3kB in length. The chloramphenicol cassette is approximately 1.2kB. If the chloramphenicol cassette inserted into *cheV* the resulting band would be 4.2kB in length. The first three lanes in Figure 6 have a band approximately 4kB in size indicating that the chloramphenicol cassette has inserted. The fourth lane is a ST1 *hisG::cam* mutant and shows a 3kB band indicating that the *cheV* gene is intact.

Effect of Disrupting *cheV* on swimming ability

The effect of disrupting *cheV* was analyzed by inoculating strain ST1 *cheV::cam* into the center of a Petri dish containing semi-solid agar and a complete mixture of nutrients provided by tryptone. The bacteria swarm outwards in concentric circles that can be measured. *S. typhimurium* cells lacking the *cheW* gene have a severe chemotaxis defect and barely swarm outwards from the point of inoculation (Parkinson 1978). *S. typhimurium* cells lacking the *cheY* gene also have a severe chemotaxis defect and barely swarm outwards from the point of inoculation (Parkinson 1978). I had hypothesized that eliminating the *cheV* gene would result in a chemotaxis defect and a reduced swarm size compared to wildtype. However, strain ST1 *cheV::cam* was capable of swarming just as

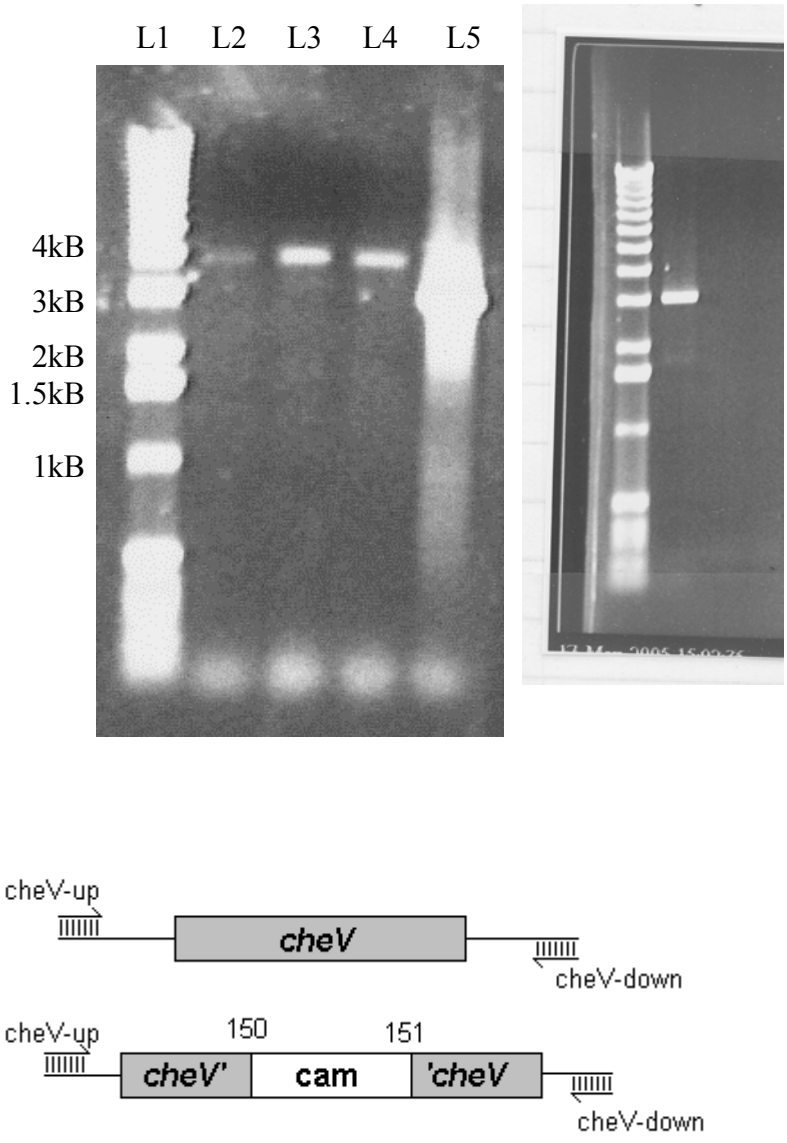

Figure 8. Colony PCR analysis of ST1 *cheV::cam* candidates. These PCR fragments were amplified using primers CheVup and CheVdown. The primers are approximately 1Kb upstream and downstream, respectively, from *cheV*. The *cheV* gene is approximately 1Kb. Lane 1 is the ladder. Lanes 2 through 4 are ST1 *cheV::cam* candidates. Lane 5 is ST1 hisG::cam. ST1 hisG::cam serves as a control because the *cheV* gene is intact and the cells were subjected to the same chromosomal integration procedure as were the *cheV::cam* candidates (but using a *hisG*-directed *cam* insert). Lanes 2-4 have the chloramphenicol cassette inserted as seen by the larger band size compared to lane 5. The second panel is wildtype ST1 amplified with the same primers. The diagram shows that the chloramphenicol cassette was inserted between codons 150 and 151.

well as wildtype (Figures 9 and 10). *S. typhimurium* strain RS2901 (which has a *che*$^-$ phenotype) was used for comparison. Strain RS2901 is a smooth swimming *cheZ* point mutant. As can be seen in Figures 9 and 10, strain RS2901 is not capable of swarming as well as wildtype.

Swarm assay results are sensitive to the growth rates of the bacteria growing in the plates. To determine whether a *cheV::cam* disruption affected the growth properties of *S. typhimurium*, I analyzed growth curves for cells growing in LB broth cultures. The resulting growth curves of two ST1 *cheV::cam* colonies are comparable to the growth curve of ST1 (Figure 11).

Effect of disrupting *cheV* on chemotaxis towards serine

The effect of eliminating *cheV* on chemotaxis towards serine was determined by the use of a capillary assay. ST1 or ST1 *cheV::cam* cells were incubated with either chemotaxis buffer or a 1mM concentration of serine and the number of cells in the capillary tube was determined (Figure 12). A two-sample t-test was used to analyze the data (Table 1). The results of the t-test showed that the number of cells in the capillary tube after incubation with buffer was not significantly different for wildtype and ST1 *cheV::cam* cells at the 95% confidence level. This result indicates that disrupting *cheV* did not have a significant effect on the overall motility of ST1. The results of the t-test also showed that the number of cells in the capillary tube after incubation with 1mM serine was significantly lower for the *cheV* mutant compared to wildtype (95% confidence level). This result indicates that disruption of *cheV* affects the ability of ST1 to sense/respond to serine. The variability of these experiments as demonstrated by the

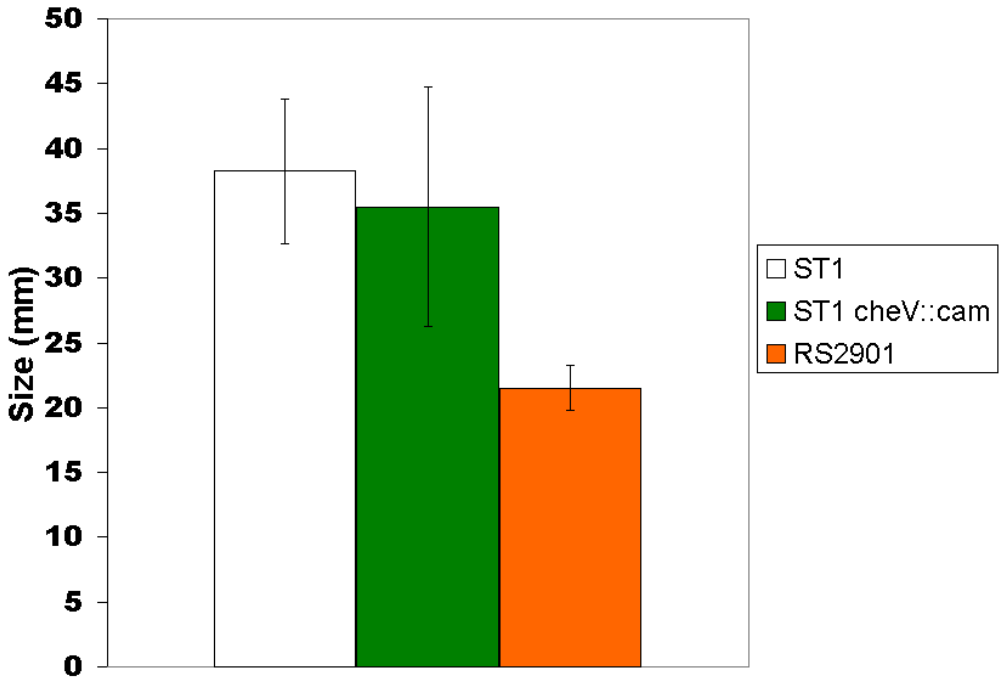

Figure 9. Effect of Disrupting *cheV* on Swarming Ability. This figure gives the average swarm colony diameter of ST1, ST1 *cheV::cam*, and RS2901 cells that were plated in duplicate in three separate trials. The error bars indicate ± one standard deviation. The averages of ST1 and ST1 *cheV::cam* are comparable as shown by the overlapping error bars. RS2901 cells have a mutant *cheZ* gene that disrupts swarming ability compared to wildtype cells.

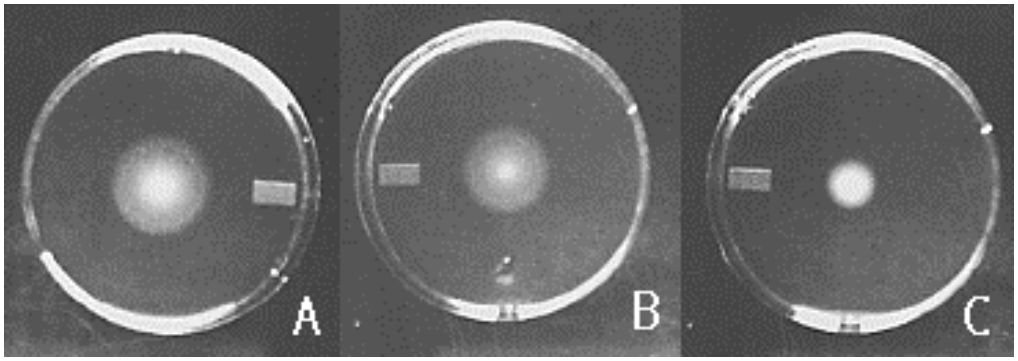

Figure 10. Swarming ability of ST1, ST1 *cheV::cam*, and RS2901. Panel A is ST1. Panel B is ST1 *cheV::cam*. Panel C is RS2901. ST1 *cheV::cam* is capable of swarming as well as ST1 as seen in the fact that the swarm colony size in panel B is comparable to the swarm colony size in panel A. RS2901 has a chemotaxis defect and cannot swarm as well as ST1 as seen in the fact that the swarm colony in panel C is smaller than the swarm colony in panel A.

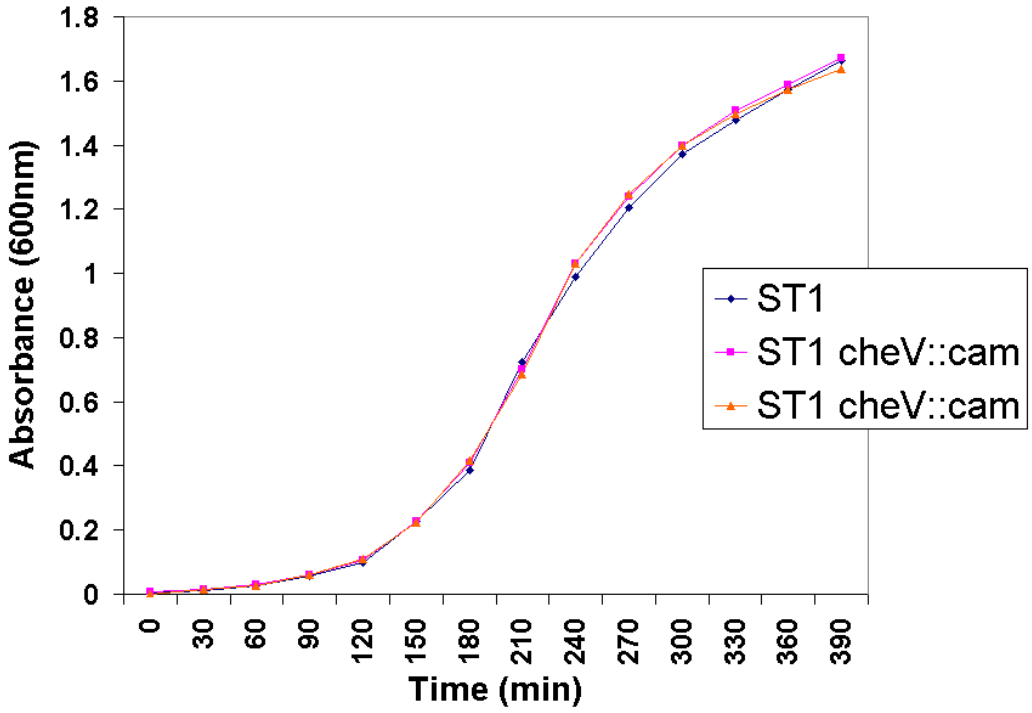

Figure 11. Growth Curves of ST1 and ST1 *cheV::cam*. This figure shows the growth curves that were determined for each of the strains growing in LB broth at 37°C by measuring absorbance at 600nm every 30 minutes. This figure shows that the growth curves of both ST1 *cheV::cam* colonies are comparable with each other as well as with the growth curve of ST1.

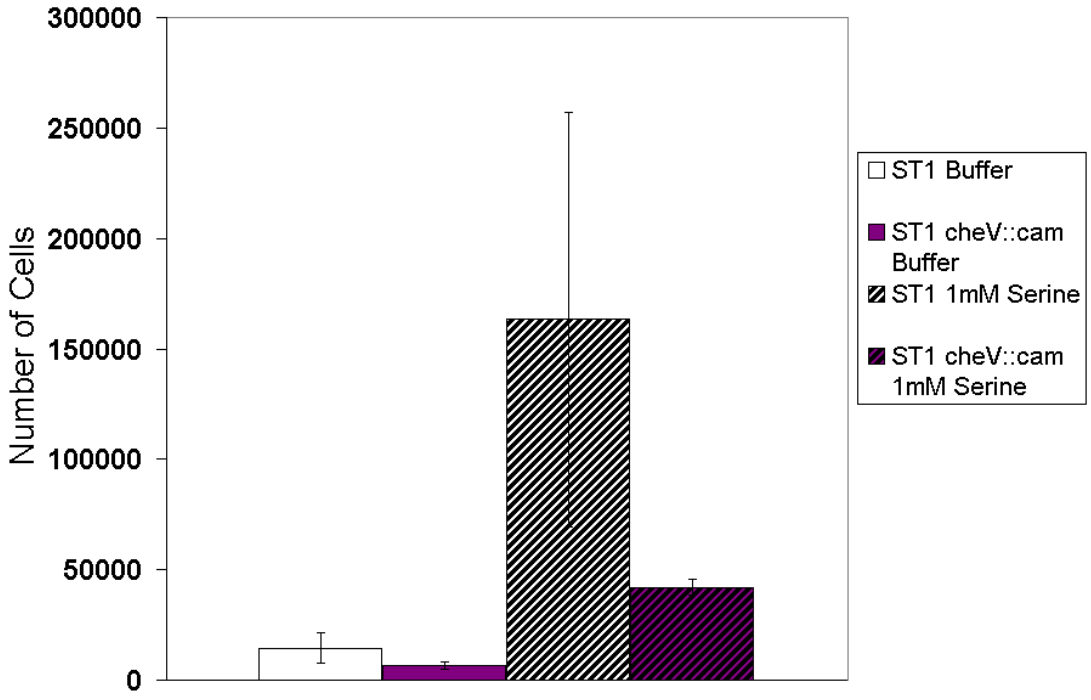

Figure 12. Effect of *cheV* disruption on ability to sense/respond to serine. This chart shows the average number of cells in the capillary tube after incubation with either buffer or serine. The average represents two trials that were plated in duplicate. The error bars represent one standard deviation.

Table 1. Analysis of the effect of disrupting *cheV* on ability to sense serine.

Two-Sample T-Test H_o $u_1=u_2$		
	Mean Number of Cells in the Capillary Tube	Standard Deviation
ST1 + buffer	14525	6809
ST1 *cheV::cam* + buffer	6775	1632
	t = 2.2, accept H_o	
ST1 + 1mM serine	163225	93798
ST1 *cheV::cam* + 1mM serine	42000	3674
	t = 2.6, reject H_o	

A two-sample t-test was used to determine if the number of cells in capillary tube after incubation with buffer and 1mM serine were significantly different between wildtype and ST1 *cheV::cam* cells. The null hypothesis (H_o) was that the number of cells in the capillary tube after incubation with buffer and 1mM serine were the same for wildtype and ST1 *cheV::cam* cells. The t values were evaluated using a chart for small samples at six degrees of freedom (Spence 1976). The t value is too low to be significant when the number of cells in the capillary tube after incubation was compared between wildtype and ST1 *cheV::cam* cells. Therefore, the null hypothesis is accepted. The t value was significant when the number of cells in the capillary tube after incubation with 1mM serine was compared between wildtype and ST1 *cheV::cam* cells. Therefore, the null hypothesis is rejected and the number of cells in the capillary tube after incubation with 1mM serine is different between wildtype and ST1 *cheV::cam* cells.

large error bars in Figure 12, suggests that disruption of *cheV* does not have a large effect on the ability of ST1 to sense/respond to serine. My results are consistent with the idea that CheV might contribute to the chemotaxis response (to serine), but that it is not essential for this response.

Effect of *cheV* disruption on surface swarming ability

I also examined the effect of disrupting *cheV* on the ability of strain ST1 *cheV::cam* to accomplish surface motility. ST1 *cheV::cam* surface motility colonies covered almost the same amount of surface as the ST1 surface motility colonies, indicating that eliminating *cheV* only has a minor effect on surface motility (Figures 13 and 14). The ImageJ program was used to calculate the amount of surface area covered by the surface motility colonies. The surface area covered by the ST1 *cheV::cam* surface motility colonies was approximately 80% of the surface area covered by the wildtype surface motility colonies (Figure 13). The growth rate of cells during exponential phase that are swarming on the surface of agar is comparable to the growth rate of cells during exponential phase that are grown in broth (Kim 2004). Since the growth rate of ST1 *cheV::cam* cells grown in broth is comparable to the growth rate of ST1 cells grown in broth (Figure 11) then the growth rate of ST1 *cheV::cam* cells swarming on the surface of agar is comparable to the growth rate of ST1 cells swarming on the surface of agar. Therefore, it is not a growth defect that is affecting the surface motility of the ST1 *cheV::cam* cells.

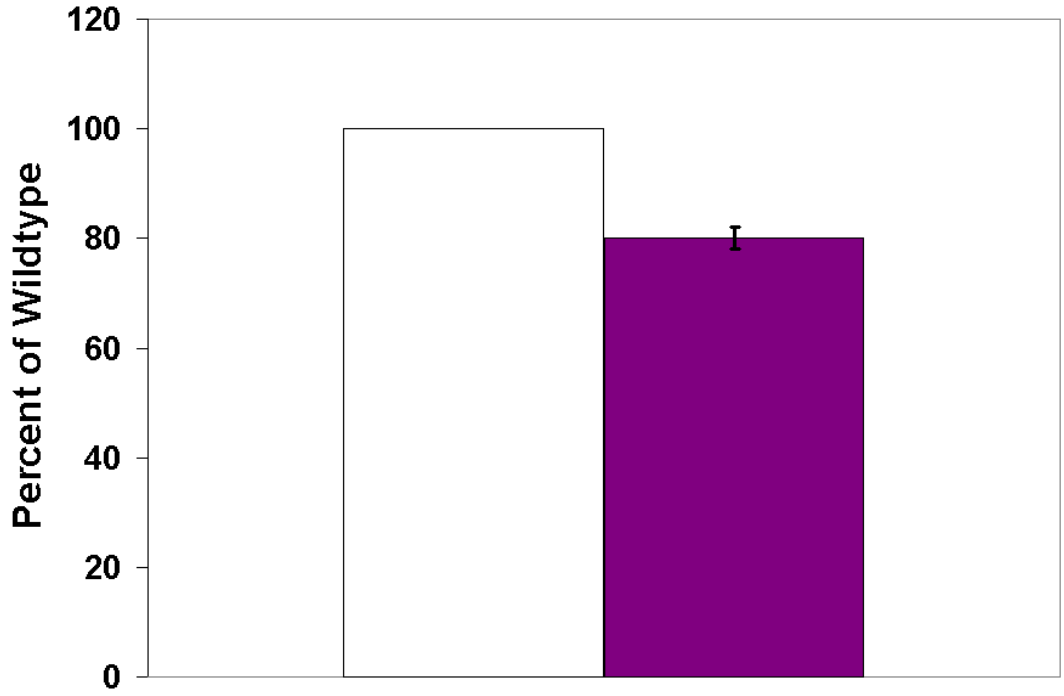

Figure 13. Effect of disrupting *cheV* on surface motility colony size. This figure shows the average size of the ST1 *cheV::cam* surface motility colony as a percentage of the size of the wildtype surface motility colony. The average percentage is represented by three trials and the error bars represent one standard deviation. Elimination of *cheV* has a minor effect on the surface swarming ability of ST1 *cheV::cam* cells compared to wildtype cells.

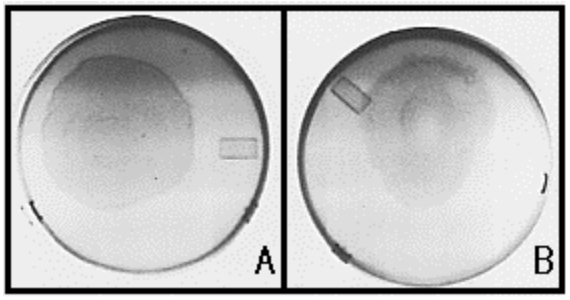

Figure 14. Surface swarming ability of ST1 and ST1 *cheV::cam*. Panel A is ST1. Panel B is ST1 *cheV::cam*. The darker areas are the swarm of cells. ST1 and ST1 *cheV::cam* are capable of spreading over almost the same amount of the plate.

DISCUSSION

Is CheV a component of the chemotaxis system of *S. typhimurium*? Some of my results do not support such a role, while other results suggest that CheV might make at least some contribution to the chemotaxis response and to surface motility. My results show that a *cheV* disruption does not have an effect on the cells' ability to accomplish chemotaxis as measured by a swarm plate assay. This result does not support my hypothesis that CheV is involved in *S. typhimurium* chemotaxis. My results also show that a *cheV* disruption has a small affect on the cells' ability to sense/respond to serine, as well as, a small effect on the cells' ability to accomplish surface motility. These results support my hypothesis that CheV plays a role in *S. typhimurium* chemotaxis.

I found that disruption of *cheV* did not affect the cells' ability to accomplish chemotaxis in a swarm plate assays. By contrast, elimination of any of the other chemotaxis genes (*cheA*, *cheW*, *cheY*, *cheZ*, *cheR*, or *cheB*) results in severe inhibition of chemotaxis that can be readily observed in a swarm plate assay (Parkinson 1978). For example, my results show that *E. coli cheW* and *cheY* null mutants have an average swarm colony diameter of only 3 millimeters (Refer to Figure A-1). If *cheV* were an essential chemotaxis gene, then disruption of *cheV* should have resulted in a Che^- phenotype on swarm plates. My result is in agreement with the study by Frye et. al. (2006), who observed a Che^+ phenotype for a *cheV*::MudJ insertion mutant.

I observed that a *cheV* disruption had a small effect on the cell's ability to sense/respond to serine. This suggests that CheV is not an essential chemotaxis protein but may be involved in chemotaxis.

I also observed that a *cheV* disruption had a small effect on the cell's ability to accomplish surface motility. By contrast, elimination of any of the other chemotaxis genes (*cheA*, *cheW*, *cheY*, *cheZ*, *cheR*, or *cheB*) results in a severe surface motility defect and the cells cannot swarm outwards from the point of inoculation (Harshey and Matsuyama 1994). If *cheV* were an essential chemotaxis gene that it should have exhibited a Che^- phenotype. Since my results did not show a Che^- phenotype, it suggests that CheV is a non-essential chemotaxis protein but might have some accessory role.

Overall, my findings support the idea that CheV is not an essential chemotaxis protein, but that it might play some accessory role or contribute under conditions that I did not mimic with my experiments.

Chapter 3: Effects of Overexpression of *cheV*

INTRODUCTION

The results presented in this chapter examine the effects of overproducing *cheV* in *S. typhimurium*.

The chemotaxis system in bacteria consists of several components that function together to relay a signal from the chemoreceptors to the flagellar motors. If any of the components are missing or are in higher concentrations than usual it disrupts the balance of the system, and the cells lose the ability to perform chemotaxis. It has been shown that overexpression of any of the chemotaxis genes in an otherwise wildtype background inhibits chemotaxis ability (Parkinson 1978). If CheV is part of the chemotaxis system, then overexpression of *cheV* should disrupt the balance of the system and inhibit chemotaxis ability.

CheV shows sequence similarity to CheW. It is therefore possible that CheV has the same function as CheW or a related function. CheW is an adapter protein that serves to complex CheA to the receptors. CheW overexpression inhibits chemotaxis ability and results in a smooth swimming bias (Sanders 1989). It is believed that overexpression of CheW results in the protein sequestering components. In other words, instead of joining CheA to the receptors, there is enough CheW to bind CheA and the receptors independently. Without formation of the receptor-CheW-CheA complexes, there is no signal generated, and the flagella continue to rotate counterclockwise moving the cell in a straight line regardless of the chemical environment encountered by the cell.

CheV also shows sequence similarity to CheY. It is therefore possible that CheV has a function similar to that of CheY. CheY is the response regulator that binds at the flagellar motor causing a change in the direction of rotation to clockwise (Welch 1993). Overexpression of CheY inhibits chemotaxis resulting in a tumbly bias (Clegg and Koshland 1984). The increase in CheY concentration results in more CheY available to bind at the flagellar motor leading to an increase in the tumble signal, such that cells tumble (continually change direction) regardless of the concentration of attractants and/or repellents.

EXPERIMENTAL PROCEDURES

Materials – All chemicals were purchased from common sources (Fisher Scientific, Sigma, Invitrogen) and were reagent grade.

Strains and Plasmids - *S. typhimurium* strain ST1 (ATCC29595) was used as the wildtype strain (Tindall 2005, McClelland 2001).

Plasmid pCW:CheV was used to overexpress *cheV* (Figure 15). Plasmid pCW:CheV is a derivative of plasmid pCW (Gegner and Dahlquist 1991). This plasmid contains the *ori* from pBR322. It is a moderate copy plasmid (15-20 copies per cell). Plasmid pCW contains the wildtype *E. coli cheW* gene under the control of the *tac* promoter; it contains the *lacI* gene and confers ampicillin resistance to host cells. Plasmid pCW:CheV was generated by digesting plasmid pCW with *Nde*I and *Xba*I to excise the *cheW* gene. *cheV* (with *Nde*I and *Xba*I sites at the 5' and 3' ends, respectively) was ligated into the cut plasmid. Plasmid pCnoW was used as a control. This plasmid is also a derivative of plasmid pCW. It lacks *cheW* but is otherwise identical to plasmid pCW. Plasmid pCnoW was generated by digesting plasmid pCW with *Sty*I and *Xba*I. The *cheW* fragment was removed and the digested ends were ligated together (Boukhvalova 2002).

Cloning – The *cheV* fragment was generated by PCR from genomic DNA of strain ST1 (obtained from ATCC). The reaction mixture contained 1μL genomic DNA, 0.5μL Taq polymerase, 10μL 10X Taq buffer, 4μL MgCl2, 2μL dNTP mix,

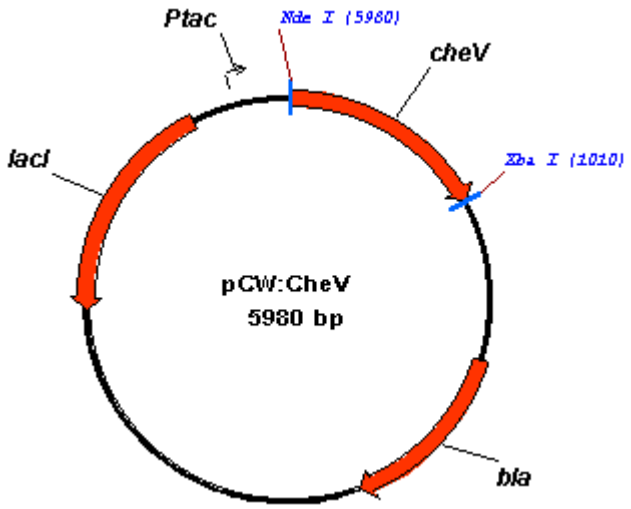

Figure 15. CheV Overexpression Plasmid. This plasmid is a derivative of pCW (Gegner and Dahlquist 1991). The *ori* is from pBR322. It is a moderate copy number plasmid (15-20 copies per cell). It confers ampicillin resistance and is inducible with the addition of IPTG.

2µL primer CheVfor, 2µL primer CheVrev, and 78.5µL sterile water. Primer CheVfor is 5'- CATATGGAGGTTAGGATGGACAATTT with the *Nde*I site underlined. Primer CheVrev is 5'- TCTAGACCTGTTACGTCAGGCGCTT with the *Xba*I site underlined. The thermocycler was set to denature for 4 minutes at 94°C; 30 cycles of 30 seconds at 94°C, 30 seconds at 55°C, and 1.5 minutes at 72°C; a final extension of 10 minutes at 72°C; and a final hold at 10°C. The PCR fragment was cloned into a TOPO blunt vector. The TOPO blunt vector was digested with *Nde*I and *Xba*I and the *cheV* fragment was isolated. The resulting fragment was ligated into plasmid pCW.

Swarm Assay – For a detailed description of this assay, refer to the methods section of Chapter 2. To maintain the pCW:CheV and pCnoW plasmids, 100µg/ml^{-1} Ampicillin was added to the media. To overexpress *cheV*, the pCW:CheV plasmid was induced with 0-200µM IPTG.

Surface Motility Assay- For a detailed description of this assay, refer to the methods section of Chapter 2. To maintain the pCW:CheV and pCnoW plasmids, 100µg/ml^{-1} Ampicillin was added to the media. To overexpress *cheV*, the pCW:CheV plasmid was induced with 0-200µM IPTG.

Capillary Assay- For a detailed description of this assay, refer to the methods section of Chapter 2. To maintain the pCW:CheV and pCnoW plasmids, 100µg/ml^{-1} Ampicillin was added to the media. To overexpress *cheV*, the pCW:CheV plasmid was

induced with 0-50μM IPTG. Cells carrying the pCW:CheV plasmid were diluted tenfold in chemotaxis buffer and then plated on LB plates containing ampicillin.

Growth Curve – For a detailed description of this assay, refer to the methods section of Chapter 2. To maintain the pCW:CheV and pCnoW plasmids, 100μg/ml^{-1} Ampicillin was added to the media. To overexpress *cheV*, the pCW:CheV plasmid was induced with 0-200μM IPTG.

RESULTS

The results presented below summarize my attempts to determine if *cheV* overexpression has an effect on *S. typhimurium'*s ability to accomplish chemotaxis or surface motility. The assays I used were the same assays used to determine the effect of disruption of *cheV* on *S. typhimurium*'s chemotactic ability and surface motility (generally described on pages 40-41).

Effect of *cheV* overexpression on swarming ability

Chemotaxis ability of cells overexpressing *cheV* was analyzed by inoculating the center of a Petri dish containing semi-solid agar. The cells swarm outwards in concentric rings and the diameter of the resulting swarm colony can be measured. It has been shown that overexpression of any of the *che* genes (*cheA*, *cheW*, *cheY*, *cheZ*, *cheR*, and *cheB*) in an otherwise wildtype background inhibits swarming ability resulting in smaller swarm colonies (Parkinson 1978). Plasmid pCW:CheV was used to overexpress *cheV* by inducing the cells with 0-200μM IPTG. I found that overexpression of *cheV* in wildtype *S. typhimurium* strain ST1 inhibits swarming ability resulting in a decrease in the size of the swarm colonies (Figures 16 and 17). At an IPTG concentration of 200μM the diameter of swarm colonies decreased to approximately 50-60% of that observed in the absence of inducer. Increasing the concentration of IPTG to 500μM had no further effect (i.e. at an IPTG concentration of 500μM the resulting swarm colony was still 50-60% the size of wildtype swarm colonies) (results not shown). Adding IPTG to ST1 cells containing plasmid pCnoW had no effect on swarming ability. To determine whether the smaller swarm size observed with *cheV* overproduction was due to slower growth

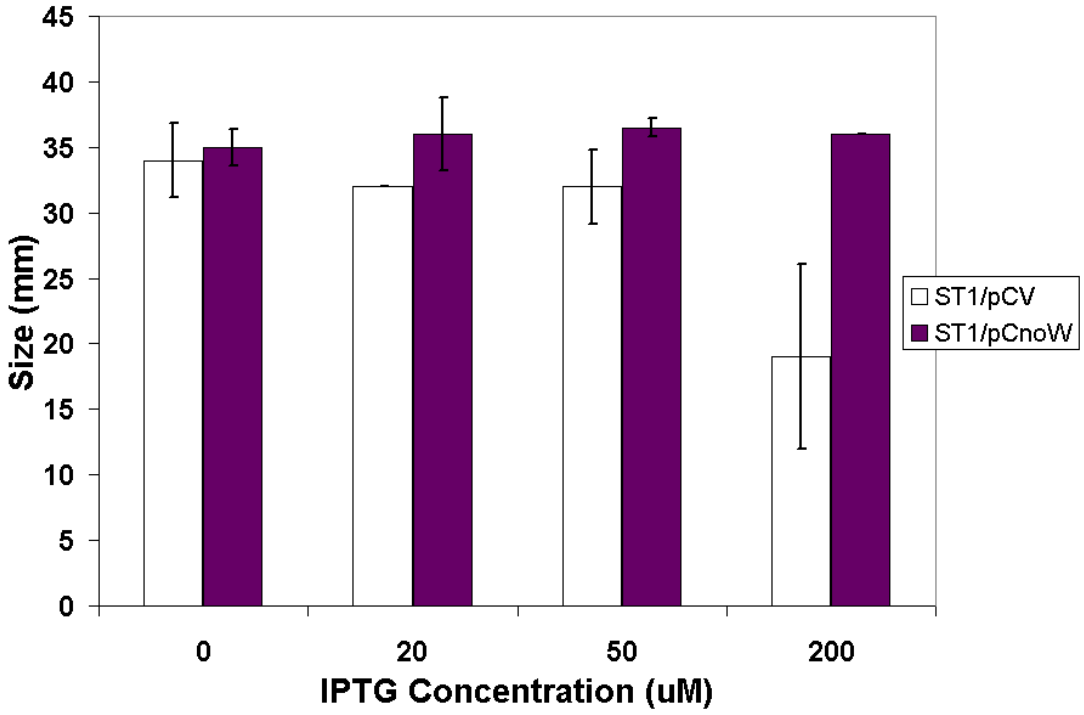

Figure 16. Effect of overexpressing *cheV* on swarming ability. This figure shows the affect of *cheV* overexpression on swarm colony size. *che*V overexpression was induced by adding increasing concentrations of IPTG to swarm plates. Adding IPTG had no effect on ST1 cells carrying plasmid pCnoW. However, increased expression of *cheV* from plasmid pCW:CheV inhibited chemotaxis ability as shown by the decrease in swarm size with increasing concentrations of IPTG.

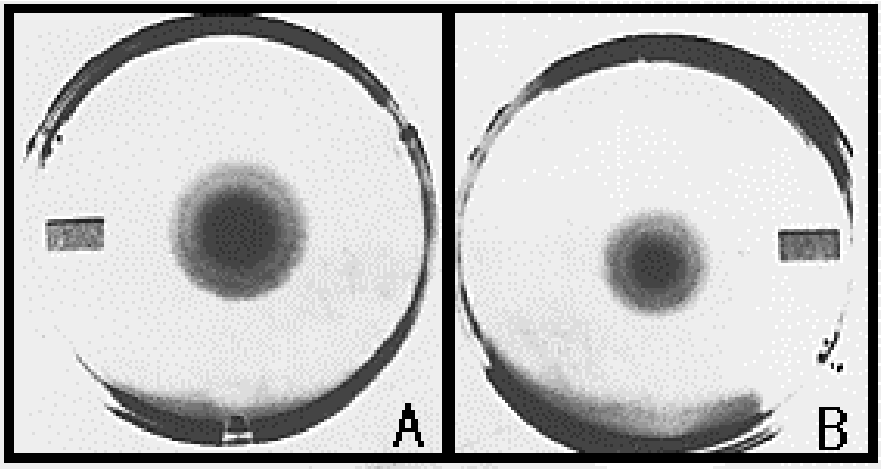

Figure 17. Swarming ability of ST1 overexpressing *cheV*. Panel A is ST1/pCnoW induced with 50μM IPTG. Panel B is ST1/pCW:CheV induced with 50μM IPTG. Overexpressing *cheV* results in a chemotaxis defect that can be seen in the fact that the swarm colony in panel B is smaller than the swarm colony in panel A.

of cells overexpressing *cheV*, I analyzed growth curves for cells growing in broth cultures. ST1 cells carrying pCW:CheV were induced with 0-200μM IPTG to overexpress *cheV*. The growth curves of the cells are comparable regardless of the amount of *cheV* overexpression (Figure 18). The doubling time of cells in logarithmic phase carrying plasmid pCW:CheV is comparable to the doubling time generated from ST1 carrying plasmid pCnoW. This indicates the reduced size of the swarm colonies in Figure 16 was not due to CheV affecting the growth rate of *S. typhimurium*.

Effect of *cheV* overexpression on chemotaxis towards serine

A capillary assay is a useful method for investigating chemotaxis towards a single attractant. I wanted to determine if overexpression of *cheV* affects the cell's chemotaxis ability toward serine. ST1 cells carrying plasmid pCW:CheV were induced with 0, 20, and 50 μM IPTG and the number of cells in the capillary tube after incubation with buffer were compared to the number of cells in the capillary tube after incubation with 1mM serine. I hypothesized that overexpression of *cheV* would disrupt the cells' ability to sense/respond to serine, and that there would be fewer cells in the serine containing capillary tubes for cells grown at higher IPTG concentrations. My results indicate that overexpression of *cheV* affects the cells' ability to sense/respond to serine (Figure 19). I analyzed the data by performing two-sample t-tests (Table 2). My results show that the number of cells in the capillary tube after incubation with 1mM serine at IPTG concentrations of 20μM and 50μM were significantly different than the number of cells in the capillary tube after incubation with 1mM serine at 0μM IPTG (95% confidence level).

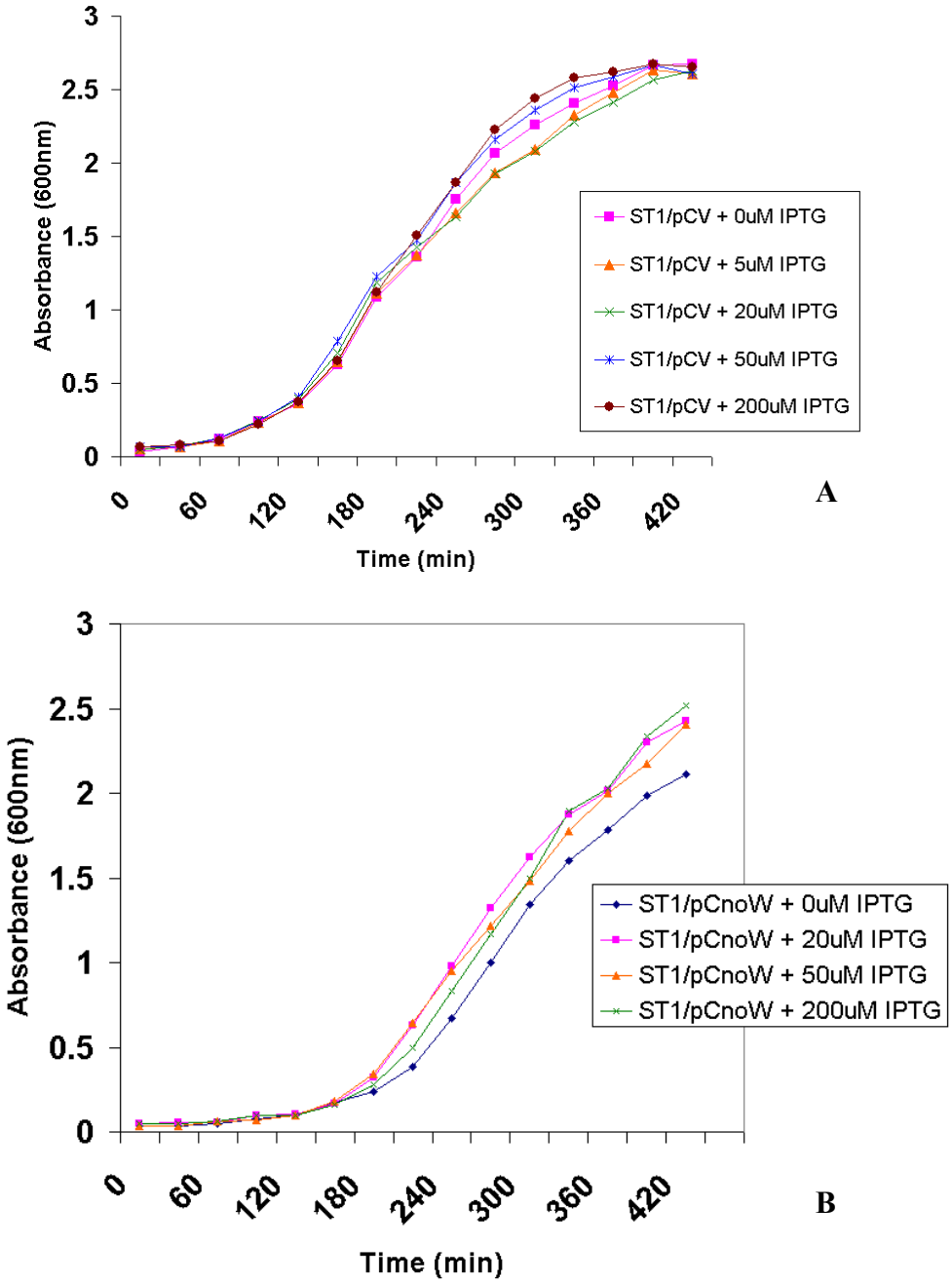

Figure 18. Growth Curves of ST1/pCnoW and ST1/pCW:CheV. Panel A shows the growth curves of ST1 cells containing plasmid pCW:CheV induced with increasing amounts of IPTG. Panel B shows the growth curves of ST1 cells containing plasmid pCnoW induced with increasing amounts of IPTG. Inducing ST1 cells containing either plasmid pCnoW or plasmid pCW:CheV had no effect on the growth rate of the cells as seen by the comparable growth curves within each panel. ST1 cells overexpressing *cheV* by induction of plasmid pCW:CheV with IPTG showed comparable doubling time to cells containing plasmid pCnoW which contains no chemotaxis genes. This can be seen by the similarity of the slopes in panel A to the slopes in panel B.

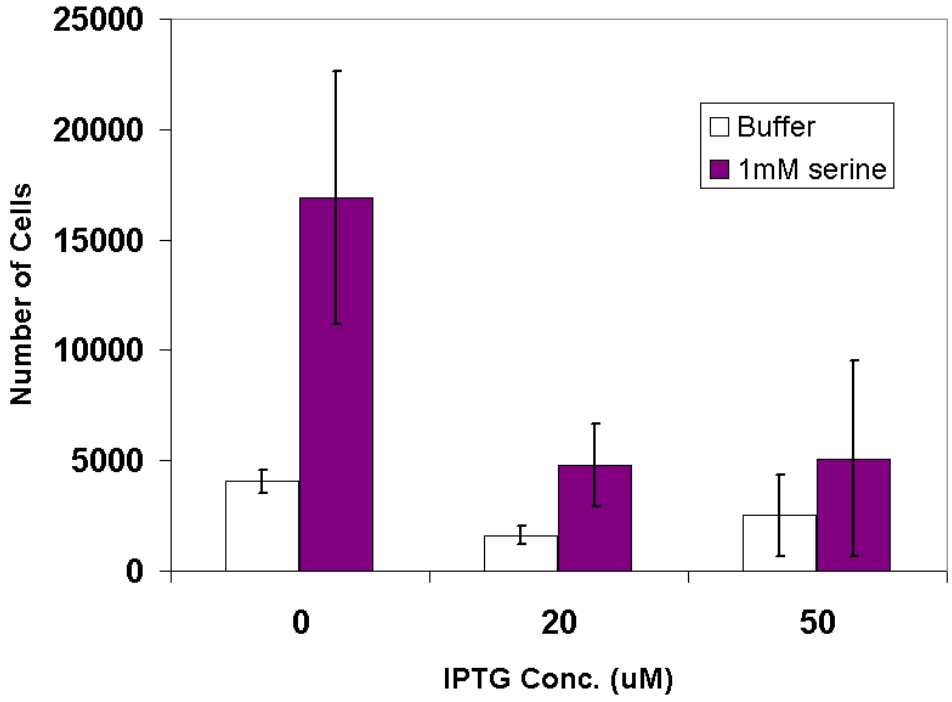

Figure 19. Effect of *cheV* overexpression on ability to sense/respond to serine. Overexpression of *cheV* was obtained by inducing ST1 cells carrying the pCW:CheV plasmid with increasing concentrations of IPTG. The bars represent the average number of cells in the capillary tube for two trials that were plated in duplicate. The error bars represent one standard deviation.

Table 2. Analysis of the effect of overexpressing *cheV* on ability to sense serine.

Two Sample T-Test H_o $u_1=u_2$				
	Buffer		1mM Serine	
	Mean Number of Cell	Standard Deviation	Mean Number of Cells	Standard Deviation
ST1/pCW:CheV + 0μM IPTG	4050	514	16925	5733
ST1/pCW:CheV + 20μM IPTG	1620	398	4775	1879
	t = 7.5, reject H_o		t = 4.0, reject H_o	
ST1/pCW:CheV + 50μM IPTG	2510	1827	5075	4429
	t = 1.6, accept H_o		t = 3.3, reject H_o	

A two-sample t-test was used to determine if there was a significant difference in the number of cells in the serine containing capillary tubes between cells that were not overexpressing *cheV* and cells that were overexpressing *cheV*. The null hypothesis (H_o) was that the number of cells in the serine containing capillary tubes was the same for cells not overexpressing *cheV* and cells overexpressing *cheV*. My results show that cells induced with 20μM and 50μM IPTG have a significantly lower number of cells in the serine containing capillary tubes than cells not induced with IPTG.

Effect of *cheV* overexpression on surface swarming ability

I also examined the effect of overexpressing *cheV* on the ability of *S. typhimurium* to accomplish surface motility. In these assays 3μL of liquid culture is pipetted onto the center of a Petri dish containing 0.6% agar and a mixture of nutrients. The cells swarm on the surface of the plate, and the coverage of the plate can be determined by using the ImageJ program. These so-called swarmer cells have more flagella and produce slime that lubricates the surface of the plate allowing the outward migration (Toguchi 2000). Overexpression of any of the *che* genes (*cheA*, *cheW*, *cheY*, *cheZ*, *cheR*, or *cheB*) inhibits swarming ability on the surface of agar (Harshey and Matsuyama 1994). ST1 cells carrying plasmid pCW:CheV were induced with 0-200μM IPTG to overexpress *cheV*. My results show that ST1 cells carrying either the pCW:CheV or pCnoW plasmid were able to spread out over the same amount of surface area at 0μM IPTG. Increasing the concentration of IPTG caused a decrease in the ability of cells carrying either plasmid to spread out over the surface of the plate. This suggests that IPTG affects the ability of ST1 cells to migrate over the surface of agar. However, the decrease in the size of the motility colony was more pronounced in cells overexpressing *cheV*, raising the possibility that the smaller swarm size is affected by *cheV* overexpression, as well as, IPTG concentration (Figures 20 and 21). The growth rates of cells growing on the surface of agar has been shown to be comparable to the growth rate of cells growing in broth (Kim 2004), which suggests that it is not a growth defect that is causing the results.

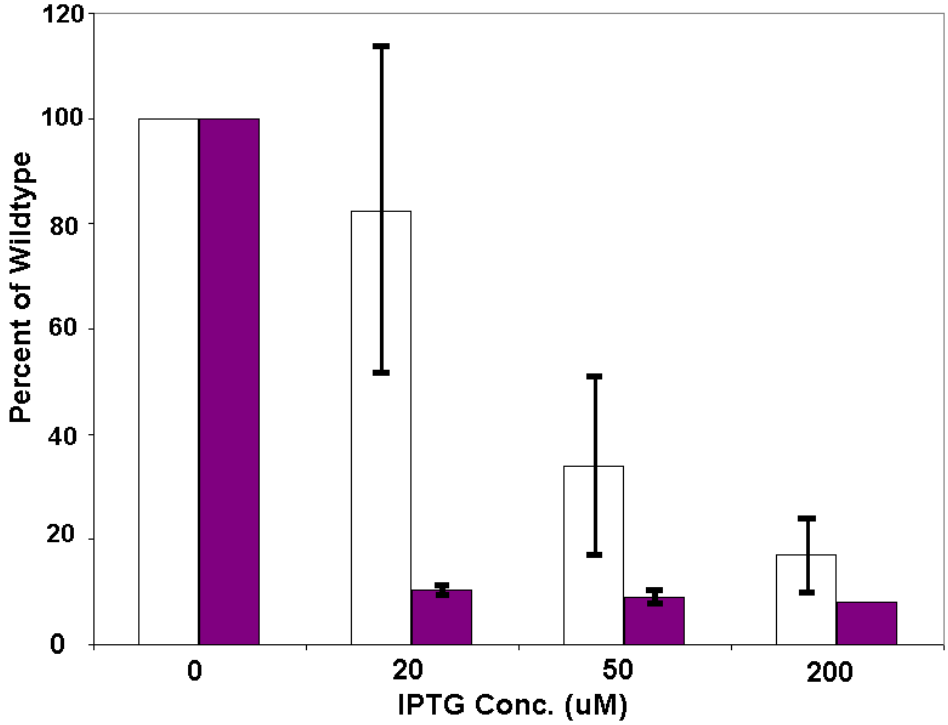

Figure 20. Effect of overexpressing *cheV* on surface motility colony size. This chart shows the average surface motility colony size of three trials as a percentage of the size of a wildtype surface motility colony. In this case wildtype is ST1 pCnoW induced with 0μM IPTG. The error bars represent one standard deviation.

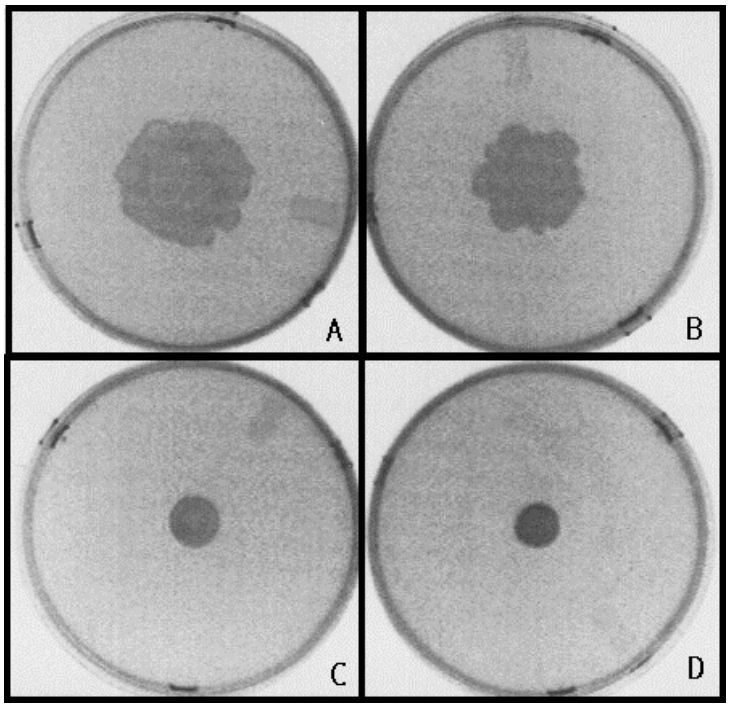

Figure 21. Effect of *cheV* Overexpression on Surface Motility. Panel A is ST1 containing plasmid pCW:CheV induced with 0μM IPTG, panel B is ST1 containing plasmid pCW:CheV induced with 5μM IPTG, panel C is ST1 containing plasmid pCW:CheV induced with 20μM IPTG, panel D is ST1 containing plasmid pCW:CheV induced with 50μM IPTG. Overexpression of *cheV* decreases the ability of ST1 cells to spread out over the surface of the plate as seen by the smaller surface coverage in panels C and D compared to panels A and B.

DISCUSSION

The major goal of this project was to determine if CheV plays a role in *S. typhimurium* chemotaxis. Demonstrating that *cheV* overexpression has an effect on chemotaxis ability would provide evidence that CheV can interact with other components of the chemotaxis machinery, at least when it is overproduced. My results show that overexpression of *cheV* has an effect on ST1's swarming ability, its ability to sense/respond to serine in a capillary assay, and its surface motility. These results suggest that CheV can interact with components of the chemotaxis system, but not necessarily under normal expression conditions.

A Western blot would have been useful to determine the amount of CheV that was being produced in wildtype cells and at the different IPTG concentrations I used to overexpress *cheV*. However, at the time I was conducting my research, there was no antibody available against *S. typhimurium* CheV. Previous work using plasmid pCW to overexpress CheW found that at an IPTG concentration of 10μM, the level of CheW was approximately 2.5 times higher than normal levels, and at an IPTG concentration of 110μM, the level of CheW was approximately 40 times higher than normal levels (Boukhvalova 2002). Cells carrying plasmid pCW that were induced with 110μM IPTG had swarm colony diameters that were approximately 25% of the size of wildtype swarm colony diameters (Boukhvalova 2002). My results show that cells carrying plasmid pCW:CheV that were induced with 500μM IPTG had swarm colony diameters that were approximately 50% of the size of wildtype swarm colony diameters (data not shown). Since a larger effect was seen in cells overexpressing *cheW*, it suggests that CheV is not an essential chemotaxis protein. If it turns out that the IPTG concentrations I used have

comparable effects to overexpression of CheW and caused small overexpression levels (e.g. 10x), it would seem reasonable to propose that the "extra CheV" is interacting with the proteins that it also interacts with at normal expression levels. For instance, at normal expression levels CheV may bind CheA to the receptors, and at higher expression levels CheV may bind CheA and the receptors independently in ways that prohibit formation of signaling complexes, causing the observed chemotaxis defect. However, if the IPTG concentrations I used caused large overexpression levels (e.g. 1000x), then it may be that I forced cells to produce enough excess CheV that it can begin to interact with proteins that it doesn't normally interact with. For example, if CheV is part of another (non-chemotaxis) signaling cascade, at sufficiently high concentrations there may be enough CheV present to outcompete CheW for binding sites on CheA and on the receptors, even if CheV binds to these sites with low affinity.

Overexpressing *cheV* had more of an effect on surface motility than on swarming ability. Inducing ST1 pCW:CheV cells with 20μM IPTG caused a noticeable decrease in surface motility colony size where as ST1 pCW:CheV cells needed to be induced with at least 200μM IPTG to see an effect on swarming ability. This raises the possibility that CheV plays a larger role in surface motility than swarming ability. The role of the chemotaxis proteins in surface motility has not been fully elucidated but research does indicate that surface motility may be linked to pathogenicity (Wang 2004). This raises the possibility that CheV may play a role in pathogenesis.

Since IPTG also had an effect on the surface motility of ST1 cells carrying plasmid pCnoW, it is important to try this experiment again with a plasmid that has a

different induction system and see if the same results are obtained with overexpression of *cheV*.

Chapter 4: Summary and Conclusion

BACKGROUND AND AIM OF THIS PROJECT

The chemotaxis systems of *E. coli* and *S. typhimurium* have been extensively studied. Until recently it was believed that the chemotaxis systems of *E. coli* and *S. typhimurium* were identical in terms of possessing the same cytoplasmic signaling proteins. However, BLAST analysis of the *S. typhimurium* genome suggests that there is a chemotaxis protein, CheV, which is not found in *E. coli*. Since the chemotaxis systems of both bacteria are thought to be so similar, it is surprising that there is a putative chemotaxis protein in *S. typhimurium* that is not found in *E. coli*. I thought it would be useful to investigate if CheV did in fact have a role in *S. typhimurium* chemotaxis.

The aim of my project was to try to examine the effects of disrupting and overexpressing *cheV* on the chemotaxis ability of *S. typhimurium*. I attempted to answer this question by using a variety of approaches that have been used to study other chemotaxis proteins, including swarm plate assays, capillary assays, and surface motility assays.

CONCLUSIONS

The main conclusions resulting from this project are:

1. CheV is not necessary for chemotaxis by *S. typhimurium*

2. CheV is not necessary for surface motility by *S. typhimurium*

3. CheV might contribute to chemotaxis signaling and surface motility by *S. typhimurium*

4. CheV may interact with components of the chemotaxis signaling pathway and surface motility machinery of *S. typhimurium*

Here I briefly summarize the evidence supporting each of the conclusions and attempt to make a critical assessment of the conclusions and their underlying experiments.

1. CheV is not necessary for chemotaxis by *S. typhimurium*

This conclusion is supported by my results comparing a *cheV::cam* disruption mutant to wildtype *S. typhimurium* in swarm plate assays and capillary assays. In both experiments, the mutant performed well, close to or equal with wildtype. It is important to remember that *cheW* and *cheY* mutants of *S. typhimurium* have a severe chemotaxis defect despite having a functional *cheV* gene present (Parkinson 1978). This makes it unlikely that CheV is functionally redundant with either CheY or CheW.

One possible weakness in this conclusion is that my interpretation of these results assumes that the *cheV::cam* disruption strain is a *cheV* null mutant. It is conceivable, but not likely, that CheV protein fragments could still be produced in the *cheV::cam* strain and that these protein fragments are capable of interacting with the chemotaxis components, a situation that would allow the mutant to perform like wildtype in my

assays. One way of eliminating such a possibility would be to generate and characterize a *cheV* deletion mutant.

2. CheV is not necessary for surface motility by S. typhimurium

This conclusion is supported by my results comparing a *cheV::cam* disruption to wildtype *S. typhimurium* in surface motility assays. The mutant was able to perform almost as well as wildtype. It is important to note that elimination of *cheW* or *cheY* causes a severe defect in surface motility despite the presence of a functional *cheV* gene (Harshey and Matsuyama 1994). As discussed above, this conclusion rests on the assumption that the *cheV::cam* disruption created a null mutation.

3. CheV might contribute to chemotaxis signaling and surface motility by *S. typhimurium*

This conclusion is supported by my results that show disruption of *cheV* had a small effect on chemotaxis ability in capillary assays and on surface motility. There is also some indirect evidence that CheV may be involved in *S. typhimurium* chemotaxis in that sequence analysis shows that CheV shares sequence similarity to CheW and CheY, both essential components of the chemotaxis system.

One criticism of this conclusion is that there are large standard deviations in the number of cells found in the capillary tubes after incubation with buffer and 1mM serine for both the mutant and wildtype strains. To improve these results, the number of replicates should be increased or perhaps a different protocol for performing capillary assays should be developed. Another criticism is that sequence similarity between proteins does not always indicate a similar function.

4. CheV may interact with components of the chemotaxis signaling pathway and surface motility machinery of *S. typhimurium*

This conclusion is supported by my results showing that overexpression of *cheV* affects swarming ability, ability to sense/respond to serine, and surface motility. Without knowing the expression levels of *cheV* obtained at the different concentrations of IPTG I used, it is uncertain whether the effects seen are due to CheV interacting with proteins it normally interacts with or if the effects are the result of CheV interacting with proteins it does not normally interact with.

One criticism of this conclusion is that the level of *cheV* overexpression in my experiments was not determined. To improve these results, a Western blot could be performed to determine the levels of CheV in wildtype cells and under overexpression conditions. If it turns out that the expression levels I was getting resulted in a small overproduction of CheV (e.g. 10x the normal level), then it would provide evidence that my data is the result of CheV interacting with the proteins it normally interacts with (i.e. the chemotaxis signaling components). Another criticism of this conclusion is that presence of IPTG affected the surface motility of ST1 cells carrying the control plasmid (although less severely than for cells carrying the *cheV* plasmid). I was not able to find any explanation for this effect but this experiment could be improved by trying a plasmid with a different induction system.

Possible interaction of CheV with CheA and/or chemoreceptors could be examined using *in vitro* binding assays, as described later in this chapter (Future Perspectives).

Since my research was not able to define a clear role for CheV, it is important to discuss why CheV might be present in *S. typhimurium*. One possibility is that CheV is necessary for chemotaxis under conditions that I didn't test. I chose to use assays that have historically shown a strong phenotype for *che* mutants. However, there are other assays that I could have chosen, such as, the tethered cell assay to monitor rotational bias after the addition or removal of attractants. Perhaps those assays would be more sensitive to the effects of disrupting *cheV*. It is also possible that there are aspects of the chemotaxis system that are not adequately tested by the currently known assays. In support of this idea, there are several Class 3 putative flagellar genes in *S. typhimurium* that, when deleted, have no phenotype on swarm plates (Frye 2006). Another example of a *che* mutant lacking a phenotype in $cheA_{short}$. In many bacteria, including *E. coli* and *S. typhimurium*, there is an alternative translational start site producing a short variant of CheA referred to as $CheA_{short}$ (Kofoid and Parkinson 1991). Elimination of $CheA_{short}$ (while maintaining $CheA_{long}$) had a very small effect in capillary assays and no effect on swarm plates (Sanatinia 1995). The researchers concluded from that data that $CheA_{short}$ was not necessary for chemotaxis under those conditions but might be necessary under conditions that were not tested.

Another possibility is that CheV is an accessory protein that improves responses/sensitivity. It would be worthwhile to test different concentrations of attractants in a capillary assay to determine if disruption of *cheV* affects the threshold or maximal response as compared to chemotactically wildtype strains. It would also be useful to perform FRET-based assays, which have been shown to be fairly sensitive

assays for assessing response times, adaptation times, and sensitivity (Sourjik and Berg 2002). In these assays, CheZ is labeled with CFP and CheY is labeled with YFP. The ratio of YFP to CFP is then measured. The YFP emission increases when the two proteins interact. Therefore, a high YFP/CFP ratio indicates a high concentration of CheY-P since CheZ interacts with the phosphorylated protein. The YFP/CFP ratio can be measured after the addition or removal of attractants or repellents. It would be useful to compare the YFP/CFP ratio obtained with wildtype cells to the YFP/CFP ratio obtained with *cheV* mutant cells to determine if there is any difference in response time, adaptation time, or sensitivity to various attractants and repellents.

It is also possible that CheV is part of a minor signaling pathway that operates in parallel with the main chemotaxis pathway. For instance, CheV may be able to compete with CheY for the phosphate from CheA. However, instead of binding to FliM like CheY, CheV may be able to interact with another downstream component.

Moreover, it is possible that CheV mediates responses of the Tcp receptor. This could explain why CheV is not present in *E. coli*, since there is no Tcp receptor in *E. coli.* The Tcp receptor senses citrate. To further investigate the possibility that CheV mediates responses of the Tcp receptor, it would useful to examine the effects of the *cheV* disruption on the cells' ability to sense/respond to citrate. This will be discussed in more detail in the future perspectives section of this chapter.

In addition, there is always the possibility that CheV is involved in another signaling pathway (i.e. other than chemotaxis). In this regard, it is interesting to note that only some of the *che* gene homologs in *V. cholerae* have been shown to be necessary for chemotaxis (Reviewed in {Boin 2004}). This raises the possibility that the other *che*

homologs are involved in distinct (non-chemotaxis) signaling pathways, at least in other bacteria. What might this pathway be for CheV? Unfortunately, the amino acid sequence of CheV doesn't provide any clues. CheV has a CheW-like domain and a receiver domain (found in response regulators), but doesn't have any sequence similarity to other proteins or functional domains. The location of *cheV* in the genome of *S. typhimurium* does not provide any clues about possible function either. *cheV* is flanked by *elaC*, a ribonuclease, and *yfbK*, a putative von Willebrand factor. Neither gene is known to be part of any defined response pathway.

FUTURE PERSPECTIVES

There is still a lot to be learned about the CheV protein. My results indicate that CheV may be involved in *S. typhimurium* chemotaxis and surface motility. To examine this connection further, future work should include using *in vitro* experiments to examine the activities of this protein, *in vivo* experiments to further examine the effects of disrupting or overexpressing *cheV*, and experiments to determine if CheV plays a role in pathogenesis.

In vitro experiments

There is a lot of information that could be gained from *in vitro* experiments with CheV. I was able to purify $(His)_6$-tagged CheV protein so the *in vitro* experiments that I am proposing would be possible. *In vitro* experiments would be useful to determine if CheV can interact with CheA and/or the receptors. An ATPase system could be used to determine if CheV affects CheA activity (Ninfa 1991). This system looks at steady-state turnover of ATP by coupling it to NADH oxidation, which is easily measured using a spectrophotometer. If CheV affects CheA activity then the steady-state turnover of ATP should increase in the presence of increasing concentrations of CheV. The ATPase system can also be used to determine if CheV promotes formation of active CheV-CheA-receptor complexes. An increase in the steady-state turnover of ATP in the presence of CheV compared to when only CheA and receptors are incubated would indicate that CheV can promote the formation of active ternary complexes. It would be useful to try this experiment with different receptors in case CheV can only bind to some of the receptors. It would be especially interesting to try this experiment with Tcp since that

receptor is not found in *E. coli*. It would also be useful to determine if CheV can compete with CheW for binding to CheA and/or the receptors. This could be determined by performing a pull down assay (Boukhvalova 2002). In these assays CheW is labeled with flourescein and mixed with either membrane bound receptors or His_6-CheA and Ni-NTA beads. After incubation the samples are centrifuged and the fluorescence intensity of the supernatant is determined. The fluorescence intensity of the supernatant represents any CheW that is not bound to receptors or CheA. If CheV affects CheW's ability to bind the receptors or CheA then the fluorescence intensity of the supernatant should increase with addition of CheV to the mixture.

Sequence analysis shows that CheV has an aspartate residue comparable to the aspartate residue in CheY that is the site of phosphorylation. The ability of CheV to be phosphorylated by CheA could be determined by an *in vitro* phosphorylation assay using ^{32}P-CheA. After incubation of ^{32}P-CheA and CheV, the sample would be run on an SDS-PAGE gel and presence of radioactivity at a band corresponding to the molecular weight of CheV would indicate that CheV is capable of being phosphorylated. If it turns out that CheV is capable of being phosphorylated by CheA, it would be important to see if CheV can compete with CheY for the phosphate from CheA. An *in vitro* phosphorylation assay could be used again and the relative amounts of ^{32}P-CheY and ^{32}P-CheV could be determined after the sample was run on an SDS-PAGE gel. To get the most accurate results the concentrations of the proteins that are found *in vivo* should be used (9μM CheA, 12μM CheY) (Li 2004). It would also be interesting to determine if CheV is capable of being phosphorylated by other histidine kinases. It is possible that CheV plays a role in another signaling pathway and determining if CheV is capable of being

phosphorylated by a histidine kinase other than CheA would provide evidence for CheV playing a role in that signaling pathway.

In vivo experiments

In vivo experiments could be used to further examine the effects of disrupting and overexpressing *cheV*. My results showed that both elimination and overexpression of *cheV* affected the cell's ability to sense serine. This raises the possibility that CheV binds to or alters the CheA-CheW-receptor complexes and affects signaling. It would be useful to perform capillary assays using different attractants (i.e. attractants that are sensed by different receptors) to see if disruption or overexpression of *cheV* had any effect on the cells' ability to sense these chemoattractants compared to wildtype cells' ability to sense these same chemoattractants. It would be especially useful to look at the effect of *cheV* disruption and overexpression on the cells' ability to sense citrate since there is no receptor that senses citrate in *E. coli* and this could explain why there is no *cheV* gene in *E. coli*. It would also be useful to vary the concentrations of the attractants to see if the mutant has a maximal response at a concentration different than wildtype cells. It is additionally important to look at the effects of eliminating *cheW* and *cheY* on the ability of *S. typhimurium* to sense attractants and comparing this to the results obtained with the *cheV* mutant. Examining the effects of a double mutant (i.e. *cheVcheW* or *cheVcheY*) could provide additional information.

It is also possible to use minimal media swarm plate assays to determine the effects of disrupting and overexpressing *cheV*. Minimal media swarm plates only contain one attractant instead of the mixture found in the tryptone swarm plates that I used. It would be useful to perform minimal media swarm plate assays using attractants that are

sensed by different receptors to see if disruption or overexpression of *cheV* has an effect on the cells' ability to swarm outwards compared to the ability of wildtype cells to swarm outwards in the minimal media. For instance, if *cheV* disruption abolished chemotaxis ability in the presence of citrate it would provide evidence that CheV mediates the response to the Tcp receptor. Varying the concentrations of the attractants could provide additional information about the effects of disrupting and overexpressing *cheV* on the cells' ability to swarm outwards.

Another way to assess chemotaxis that is more sensitive than swarm plate assays, is to monitor signaling events *in vivo* using a FRET based assay. Sourjik and Berg (2002) used a CheY-YFP fusion and a CheZ-CFP fusion to monitor regulation of CheA kinase activity by the chemotaxis system in living bacterial cells by measuring the degree to which excitation of CFP generates fluorescence of YFP. This same system could be applied to monitor regulation of CheA activity in cells with a disrupted *cheV* gene and cells overexpressing *cheV*. If regulation of kinase activity is different from wildtype cells it would indicate that CheV is involved in *S. typhimurium* chemotaxis.

Pathogenicity experiments

Furthermore, it would be interesting to determine if CheV plays a role in pathogenesis. Studies on chemotaxis mutants in a variety of bacteria including *S. typhimurium* indicate that chemotaxis may play a role in pathogenesis ({Reviewed in Lux and Shi 2004}, Dons 2004, Stecher 2004). I observed that overexpression and elimination of *cheV* affected the cell's ability to swarm on the surface of agar. If surface motility does turn out to be a model for the initial stages of pathogenesis (Wang 2004) then *cheV* could have a role in pathogenesis. Studies in mice that looked at colonization and inflammation

like those conducted by Stecher et. al. (2004) could provide evidence for *cheV* involvement in pathogenesis if they indicated deficiencies in a *cheV* null mutant. For example, if mice infected with ST1 *cheV::cam* exhibited less severe symptoms than mice infected with wildtype *S. typhimurium,* it would provide evidence for CheV playing a role in pathogenesis. Comparing the ability of ST1 *cheV::cam* cells and wildtype cells to infect cells in tissue culture would also be informative. Dons et. al. (2004) performed such experiments with *che* mutants of *L. monocytogenes*.

Appendix A

Ability of CheV to complement *E. coli cheW* and *cheY* null mutants

Aim of the experiment

CheV shares sequence similarity with both CheW and CheY. This suggests the possibility that CheV has a similar function to CheW or CheY or both. If CheV has a similar function to either CheW or CheY it should be able to complement a *cheW* or *cheY* null mutant. Both *cheW* and *cheY* null mutants have a severe chemotaxis defect and show little outward migration in a swarm assay (Clegg 1984, Sanders 1989). The ability of *cheV* to complement an *E. coli cheW* or *cheY* null mutant was investigated by determining if CheV could restore chemotaxis ability to either mutant in a swarm assay.

Experimental Procedures

Strains and Plasmids – E. coli strain RS128 was used as the wildtype. *E. coli* strain RS36 is the same as strain RS128 except it lacks a *cheW* gene. *E. coli* strain RS65 is the same as strain RS128 except it lacks a *cheY* gene.

Plasmid pCW:CheV was used to express *cheV*. For a detailed description of the plasmid refer to the methods section of Chapter 3.

Swarm Assay - A colony of strain RS128, RS36, or RS65 containing the pCW:CheV plasmid was stabbed into the center of a tryptone swarm plate (Adler 1966a, Wolfe 1989) containing 0.3% Difco Bacto Agar, 1% tryptone, 0.5% NaCl, and 100µg/mL Ampicillin. The diameter of the outermost ring of the resulting swarm colonies was measured after incubating overnight on the bench top.

Results

The plasmid pCW:CheV was used to express *cheV* in a *cheW*⁻ strain, a *cheY*⁻ strain, and in a chemotactically wildtype strain. In the absence of any *cheV* expression both *cheW* and *cheY* formed swarm colonies whose diameters were three millimeters (Figure A-1). This is due more to growth than chemotaxis ability. When *cheV* expression level was increased (by addition of 20μM IPTG, 50μM IPTG, and 200μM IPTG) the swarm colonies formed by the *cheW* and *cheY* null mutants remained at diameters of three millimeters. This indicates that *cheV* cannot complement (restore chemotaxis ability) to either a *cheW* or *cheY* null mutant. This also implies that the function of CheV is not redundant with either CheW or CheY. *cheV* was overexpressed with plasmid pCW:CheV in wildtype *E. coli* as well. Increasing concentrations of IPTG result in smaller swarm colony diameters. This is the same result as when *cheV* was overexpressed in wildtype *S. typhimurium*. Thus, high levels of *cheV* can interfere with chemotaxis signaling in both *E. coli* and *S. typhimurium*, presumably reflecting interaction of CheV with CheA, MCPs, or other components of the signaling pathway. A valid criticism of these complementation attempts is that they used *E. coli cheW* and *cheY* mutants. This experiment might be more informative if it was repeated using *S. typhimurium cheW* and *cheY* mutant strains.

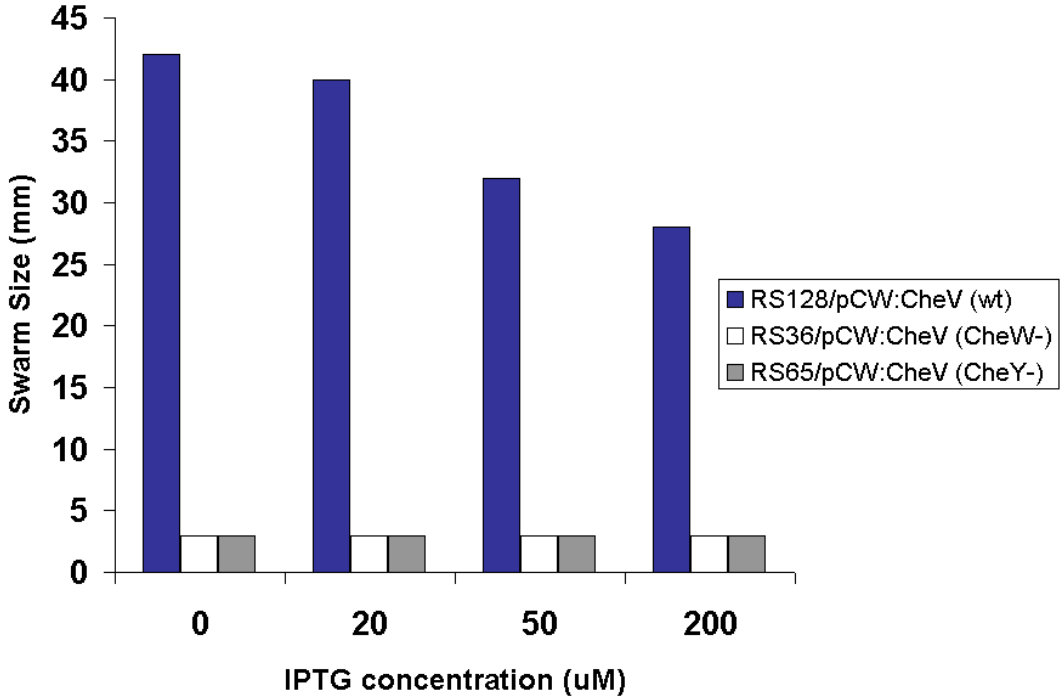

Figure A-1. Effect of *cheV* Overexpression on *E. coli* swarm plate motility. *cheV* was expressed in wildtype, *cheW*⁻, and *cheY*⁻ *E. coli* cells. *cheV* overexpression in wildtype *E. coli* cells results in a smaller swarm colony diameter. *cheV* expression in *cheW*⁻ or *cheY*⁻ *E. coli* cells has no effect on swarm colony diameter. This demonstrates that *cheV* cannot complement *E. coli cheW* or *cheY* null mutations.

Appendix B

Purification of the CheV Protein

Aim of the experiment

All the *che* gene products (CheA, CheY, CheW, CheZ, CheR, and CheB) have been purified and used in *in vitro* experiments that have provided important information about the chemotaxis system. My goal was to determine if it was also possible to purify the *cheV* gene product.

Experimental procedures

Protein Purification – CheV was purified from *E. coli* strain BL21λDE3 containing plasmid pET28a:CheV. pET28a added a his-tag to the N-terminus of the CheV protein. $(His)_6$-CheV overexpression was induced by addition of 1mM IPTG to cells in early exponential phase. 3 hours later cells were harvested by centrifugation. Cell pellets were resuspended in Buffer B pH 8.0 with BME and PMSF (50mM Na2HPO4, 50mM NaH2PO4, 300mM NaCl, and 1mM Imidazole). Cells were sonicated and spun in the ultracentrifuge to collect protein extracts. The protein extract was loaded onto a Ni-NTA chromatography column (Qiagen Inc). The column was washed with Buffer B and then the protein was eluted by washing with Buffer B fractions containing increasing concentrations of imidazole. The fractions containing protein were identified by SDS-PAGE. The fractions were dialyzed against 500mL TKMD pH 8.0 (50mM Tris, 5mM MgCl2, 50mM KCl, 0.2mM DTT, and 10% glycerol). After dialysis the fractions were concentrated using a Centrifugal Filter Device (Millipore) and the protein was stored at -80ºC.

Results

I was able to purify the CheV protein using a method similar to the methods used for purifying other *che* gene products. The CheV protein is expected to be 45 kDa based on its amino acid sequence (Figure B-1). The position of my His_6-CheV band on the SDS PAGE gel is close to the 45kDa marker, but somewhat lower. Since I was able to purify the CheV protein, it means that it is possible to perform *in vitro* experiments in the future.

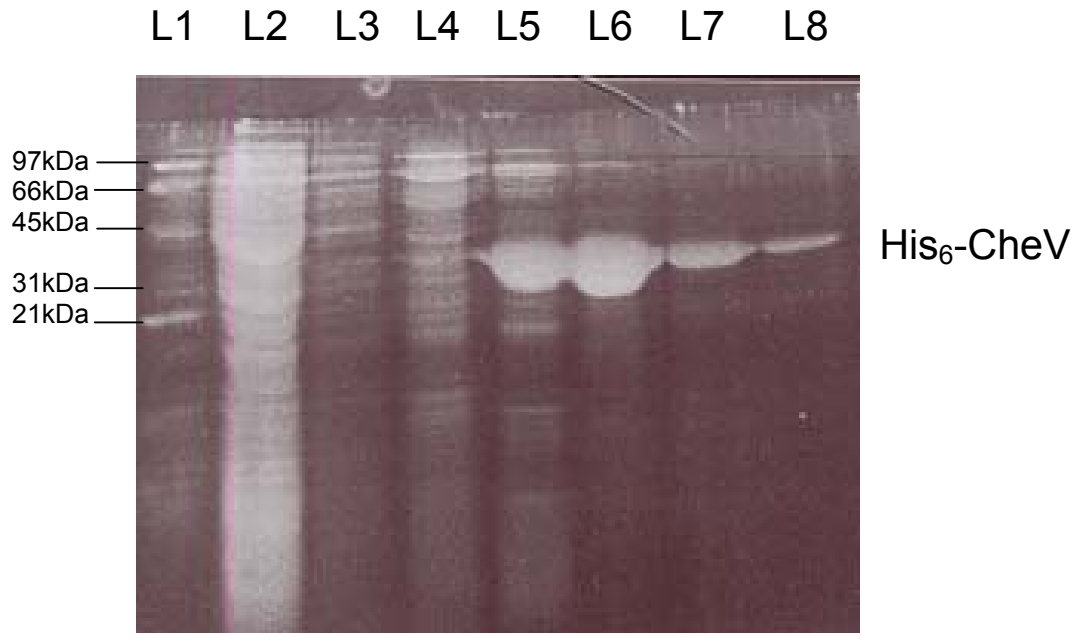

Figure B-1. Protein purification of $(His)_6$-CheV. This figure shows an inverted image of a Coomassie-Blue stained SDS-PAGE gel of Ni-NTA fractions obtained during loading, washing, and elution of protein. Lane 1 is the ladder. Lane 2 is the crude protein mixture loaded onto the column. Lane 3 is the material that comes off the column during the washing. Lanes 4-8 are aliquots of elution fractions obtained at increasing concentrations of imidazole. Lane 4 contains 20mM imidazole, lane 5 contains 40mM imidazole, lane 6 contains 60mM imidazole, lane 7 contains 80mM imidazole, and lane 8 contains 150mM imidazole. His tagged CheV is about 45 kDa.

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