The role of CheC and McpB in the chemotaxis system of *Bacillus subtilis*

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Honors Research Thesis

Sarah Paglioni

Advisor: John Kirby, Ph.D.

Abstract

Chemotaxis is one of many two-component signal transduction (TCST) systems found in cells. *B. subtilis* shares a very similar chemotaxis system to many other bacteria and archaea and thus studies of *B. subtilis* can be extrapolated to the systems in other organisms. To further elucidate the current model of chemotaxis in *B. subtilis* we used the yeast-two-hybrid and β-galactosidase assays to determine the interactions between CheC and CheA and also between McpB and CheY. Our results reveal the interaction of CheC with the HPT domain of the histidine kinase, CheA. Interestingly, the introduction of a mutation near the phosphatase region of CheC completely disrupts the interaction with CheA. The interaction between CheA and CheC may serve as yet another level of regulation to the system. Furthermore, we explore the possibility of the receptor itself interacting with the response regulator to also aid in the adaptation of the signal.

Introduction

The majority of motile organisms possess a way to direct their motility so as to interact with other species, to find more favorable conditions, and find more resources. Bacteria are no different. Bacteria possess sensory systems that perceive chemical cues and transmit this information to a motility apparatus which can then regulate the direction of the cell either towards or away this cue (11, 14). The action of responding to chemicals is referred to as chemotaxis; chemotaxis is one of many two-component signal transductions systems. Studies on chemotaxis are integral to deciphering how bacteria can process signals from the environment. Knowledge of how bacteria interact with the environment will generate advancements in understanding bacterial biofilm formation, virulence factors, antibiotic resistance and improved sanitation standards.

Chemotaxis systems are a part of a larger group of regulatory systems termed two-component signal transduction systems; two-component signal transduction systems are characterized by the presence of a kinase and a response regulator. The chemotaxis system of *Escherichia coli* is very well characterized. *E. coli* has five receptors, called Mcp's (methyl accepting chemotaxis proteins) which bind certain ligands (figure 1). Once a receptor binds a ligand, a conformational change is transmitted to CheA, the kinase; CheA then phosphorylates CheY, the response regulator, which binds to the flagellar motor and induces a clockwise rotation (12,23). A clockwise rotation results in what is called "tumbling" behavior or simply random movement. In the presence of an attractant, such as aspartate, CheA autophosphorylation is inhibited and the level of

phosphorylated CheY decreases, thus the flagellar motor defaults to counterclockwise rotation, or smooth swimming (2). Several proteins exist within the chemotaxis system which allow for adaptation of that system in the presence of chemical gradients so that the cell can respond differently to a wide range of concentrations of attractants or repellents (figure 1).

Although *E.coli's* chemotaxis system has been well characterized, it deviates from the system present in many other bacteria, including *Bacillus subtilis.* The system utilized by *B. subtilis,* a gram positive soil bacterium, is one that is thought to be much more similar to those systems found in the majority of bacteria and archaea (8). Due to the similarities between the chemotaxis systems, it is ideal to use the *B. subtilis* system, which can be extrapolated to other bacterial species, in studies of two-component signal transduction systems (figure 2).

Through analysis of the differences and similarities between the system present in *E. coli* and *B. subtilis,* a model has been developed for the chemotaxis system in *B. subtilis* (figure 3). There are ten known chemosensory receptor types present in *B. subtilis*, however only two of these receptors have known ligands. McpC is the receptor that responds to all twenty amino acids except asparagine; McpB is the only known receptor that responds to asparagine (13). Between these two receptors, McpB is the most well studied. Upon stimulation by asparagine, a conformational change occurs in McpB that is transmitted from CheW and CheA to CheY; CheY interacts with the flagellar motor to induce a smooth swimming event (10). In the *B. subtilis* model, adaptation of asparagine

stimulation is believed to occur by a multifaceted mechanism. Adaptation is essential for allowing attenuation of the signal. As in *E. coli*. CheB is phosphorylated by CheA and demethylates the receptor; CheR methylates the receptor; however the mechanism of methylation and demethylation differs between *E.coli* and *B. subtilis*. As part of the adaptation signal, CheC and CheD interact to regulate the level of phosphorylated CheY; by dephosphorylating CheY, CheC allows the flagellar motor to convert back to default. Also involved in adaptation is deamidation of the receptor by CheD; evidence now suggests that CheD-dependent deamidation allows for more efficient receptor modifications by CheR and CheB (4). Interestingly, *E.coli* lacks a homolog to CheC and CheD; these proteins are utilized by the majority of bacteria and archaea and are essential to the adaptation signal in *B.subtilis* (22, figure 2). The presence of CheC and CheD in *B.subtilis* makes it an ideal choice for analyzing chemotaxis.

Previous studies have shown an interaction between CheC and CheA, however these interactions have never been pursued to any great extent (8). This study is intended to elucidate the CheC-CheA interaction. The structure of CheA contains five domains. An ATP molecule binds to the HATPase domain; a phosphoryl group of ATP is then transferred to a histidine residue on the HPT domain. The phosphoryl group is transferred from the HPT domain to CheY which interacts with the P2 domain of CheA. The CheW domain is the site of interaction between CheA and CheW, and the H-kinase domain is believed to be a site of dimerization (3, 15, figure 4).

Previous phosphorylation assays have indicated that high levels of CheC correlate to lower levels of phosphorylated-CheY. This data has since been interpreted as CheC acting as a phosphatase on CheY (5). While we are not arguing that this is incorrect, we believe that this data, along with data that indicates an interaction between CheC and CheA, supports the idea that CheC may be acting as a phosphatase on CheA (8).

Interestingly, the phosphatase sequence found in CheC and many other known phosphatases is also found in McpB (8, figure 4). We therefore also hypothesize that McpB may be acting as a phosphatase and the target of such phosphatase action could be CheY. We believe that this interaction could be occurring because CheY is a diffusible protein and although FliY has been shown to act as a phosphatase on CheY, FliY is localized to the flagellar motor. The localization of FliY and the low ratio of FliY to CheY make it difficult to be the sole phosphatase on CheY.

The putative phosphatase role of both CheC and McpB would indicate another level of adaptation to the chemotaxis system. Adaptation is an essential control mechanism that allows an individual cell to respond to a broader range of attractants and repellants, and analysis of adaptation mechanisms will lead to insights on the nature of signal transduction.

Materials and Methods

Plasmid construction.

DNA templates for the chemotaxis genes, CheC, CheD, CheY, CheA, and McpB, were constructed using PCR amplification of chromosomal DNA from *B.subtilis* strain 1085. EcoRI and BamHI restriction sites were positioned upsteam and downstream respectively to the following genes: CheC, CheD, CheY, and CheA. BamHI and ClaI were positioned upstream and downsteam of McpB. All PCR products were ligated into the polylinker region of pGAD and pGBDU (7). The plasmids were then transformed into DH5α and the transformants were selected for by using ampicillin resistance. The isolated plasmids were sequenced and verified wild-type or the appropriate mutant.

Yeast-two-hybrid.

The yeast-two-hybrid assay was performed essentially as described (7). *S. cerevisiae,* MATa loci, was transformed with the plasmids containing the protein interactions to be tested. Single yeast colonies were picked from SD plates plus 0.04mg/ml methionine, adenine, histidine, and tryptophan (MAHW) and patched onto the same type of plate. After two days of growth these transformants were replica-plated onto plates selective for adenine and histidine production; transformants were also replica-plated to a YPD, rich media, plate. The plates were incubated at 30° C for a total of 9 days, and growth was recorded every three days.

β-galactosidase Assay.

Yeast transformants were picked from YPD plates and cultured overnight until log phase. Samples of 10 to 200 ul were transferred to a 1.5 ml centrifuge tube and used directly or frozen (-80 C). Z buffer (60 mM Na2HPO4, 40 mM NaH2PO4, 10 mM KCL, 1mM Mg2SO4, 50 mM β-mercaptoethanol and 0.2% sarcosyl) was added to give a final volume of 600 ul. The tube was incubated for thirty minutes at 30° C. After incubation, 150 ul of 2-Nitrophenyl-β-D-galactopyranoside (ONPG) (4 mg/ml) were added and incubated for a period of twenty to forty minutes (time was recorded). The reaction was stopped by addition of 400 ul of 1.5 M Na_2CO_3. The sample was centrifuged for thirty seconds at 16000 G and the absorbance of the supernatant read at 420 nm. Units are arbitrary, so a negative control is necessary.

Protein Isolation and β-galactosidase specific activity.

Yeast transformants were grown in SD + MAHW until log phase. The cells were centrifuged and the pellet was collected. A volume of lysis buffer (25mM Tri, 100 mM NaCl, 10 mM EDTA, 2 mM PMSF) equal to the cell pellet volume was added and the cells were vortexed with glass beads 9x20 sec. The cellular debris was then pelleted and the supernatant retained. The supernatant contained the cellular protein; OD of the supernatant was read at 595 and compared to a set of protein standards to calculate the mg protein per ml of lysate. Once the protein concentration was determined, 10, 30 or 60 ug was used in the β-galactosidase assay. Using Beer's law A=eCL, the nMol of o-nitrophenol (ONP) produced/min/mg protein lysate was determined (16).

Results

Yeast-Two-Hybrid analysis.

The results of the yeast-two-hybrid assay are indicated in Table 1. Positive interactions between proteins were identified by the ability of transformants to grow in the absence of histidine and adenine.

CheA interacts with CheC.

Previously, CheA has been shown to interact with CheC through the yeast-two-hybrid system (6). This experiment was first replicated and results obtained agree with the previous data. Further investigations through β-galactosidase assay revealed a very strong interaction between CheA and CheC (figure 6). A positive interaction in the yeast-two-hybrid system leads to the production of a functional β-galactosidase enzyme. The presence of this interaction can be measured by looking at the change in absorbance at 420 nm that occurs when the functional β-galactosidase enzyme converts the lactose analog, ONPG substrate, to its yellow ONP product.

The CheA and CheC interaction was interrupted upon introduction of a mutation (D149K) near the phosphatase region of CheC. The $CheC_{wt}$-CheA interaction showed the production of about 45 nMol ONP per minute per mg of protein. In contrast, the

$CheC_{d149k}$-CheA interaction showed only about 5 nMol ONP produced per minute per mg of protein, which was substantially lower than the $CheC_{wt}$-CheA interaction.

The yeast-two-hybrid assay was used to analyze the interaction between CheC and truncated portions of CheA. First, the CheA domains were each deleted from the C-terminus giving progressively shorter versions of the CheA protein (figure 7). The $CheA_{P1-P4}$ yeast isolate used in the β-galactosidase assay did not grow on any media and is not presently included in β-galactosidase results. The CheC-CheA interaction was apparent when all domains of CheA, except the HPT domain, were deleted (figure 7, 8, table 1,2). Interestingly the $CheA_{P1-P2}$, which includes the HPT and P2 domain, displayed the strongest interaction with CheC when compared to all CheA lengths. CheA mutants were than created by deleting domains from the N-terminus of the protein (figure 9). All of these mutants lacked at least the HPT domain and showed no interaction with the $CheC_{wt}$ protein using the yeast-two-hybrid assay (Table 3).

Discussion

The interaction between CheC and CheA could indicate yet another level of adaptation in the chemotaxis system. Presently the adaptation proteins CheD, CheR, and CheB modify the receptor in a way that either inhibits or promotes transduction of a signal and ultimately affects the rotation of the flagellar motor (4). Increased levels of CheC have previously been correlated to a decrease in the amount of phosphorylated CheY present, which serves to turn off the flagellar motor and produce a tumbling event (5,8).

However, this correlation was observed in the presence of CheA, and the study was never performed in a CheA-minus mutant. In the future we intend to perform such phosphorylation studies in the presence and absence of CheA to determine if CheC is indeed acting as a phosphatase on CheY or CheA. If CheC acts as a phosphatase on CheA it would in turn inhibit CheA's ability to phosphorylate CheY (in agreement with previous phosphorylation assays), thus inhibiting the production of a smooth swimming event; in effect the interaction between CheA and CheC would inhibit transduction of the signal. It is perhaps the situation that CheC plays a role in inhibiting signal transduction in the presence of a repellant so that the cell no longer produces a smooth swimming event and can use tumbling behavior to find a more favorable environment.

The β-galactosidase assay revealed that the CheC-CheA interaction could occur when only the HPT domain of CheA is present; this data is supported by the lack of CheC-CheA interaction when the HPT domain is not present. The HPT domain possesses a phosphate group on a histidine residue when CheA is activated by CheW, and thus is a conceivable site of dephosphorylation. Because the HPT domain appears to be the location of interaction between CheC and CheA it is still a valid possibility that CheC is acting as a phosphatase on the HPT domain of CheA. However, the recent studies have shown that the $CheC_{d149k}$ mutant has no effect on phosphatase activity *in vitro*. The CheC aspartate 149 residue is one of four residues that is shown to interact with CheD, and these residues are modeled to be near the phosphatase region of CheC (5). Evidence from this study supports that the CheC-CheA interaction is also occurring around this same area as a mutation in residue 149 completely disrupts the CheC-CheA interaction.

Thus, while it is still the possibility that CheC is acting as a phosphatase on CheA it is also possible that CheC is interacting with CheA with a function other than that of a phosphatase.

The idea of a non-enzymatic interaction between CheC and CheA may be supported by the data that shows the CheC-CheA interaction enhanced upon addition of the P2 domain to the CheA mutant. The P2 domain is the site of CheY binding so that the transfer of the phosphoryl group to CheY can occur. There are many possible explanations for this unexpected result. It may be the case that CheC can interact with both the HPT and the P2 domain, but the P2 domain may be the preferred site of interaction. It may also be the case that the HPT domain is the site of interaction, but the P2 domain adds extra stabilization to the interaction. Further analysis reveals the possibility that both CheC and CheY could simultaneously dock onto CheA. We hypothesize that CheC interacts with the HPT domain of CheA and simultaneously CheY docks onto the P2 domain of CheA so that the CheC phosphatase activity on CheY is enhanced. In effect, CheA may act as a stabilizing dock to enhance the interaction between CheC and CheY. If CheC must be present in order for CheY to bind to CheA then it would explain the lack of interaction between CheY and CheA in the yeast-two-hybrid system. Whether or not the lack of interaction between CheY and CheA is an artifact of the yeast-two-hybrid system or is truly representative of the chemotaxis pathway will need to be investigated in the future.

To further explicate the interaction between CheC and CheA many more future experiments need to be performed. First and foremost the production of $CheC_{d149k}$ protein must be determined because it is possible that the results were caused by the low or absent production of the mutant protein. In order to do this, an antibody must be generated against CheC and a western blot performed.

Yeast-two-hybrid and B-galactosidase assay revealed that McpB and CheY do not interact. Interestingly CheA and CheY, two proteins that are known to interact, also produced a negative yeast-two-hybrid and B-galactosidase result. This indicates that the yeast-two-hybrid system may not be sensitive enough to detect very transient interactions. Further investigations of the McpB-CheY interaction will ensue. Presently it is still believed that McpB may be acting as a phosphatase on CheY, this being the case the interaction between McpB and CheY may only occur when CheY is in its phosphorylated state. To determine this, a series of mutations will be made in the CheY phosphate domain to produce a mutant CheY that resembles the phosophorylated form of CheY.

The current chemotaxis model for *B. subtilis* displays the main adaptation modalities as CheR and CheB, which interact with the receptor, and CheC which is believed to interact with the response regulator CheY. While this study does not address CheR and CheB, the results clearly indicate that CheC does interact with CheA which we believe aids in the adaptation process. This study supports the proposed model in which CheC acts as a regulator on the HPT domain of CheA (figure 10). The function of the CheC regulation

upon CheA will be determined in the future. Furthermore the results obtained from this study reveal a lack of interaction between CheC and CheY; whether this is due to an artifact of the yeast-two-hybrid system or truly represents the nature of the system will be investigated further.

The idea of McpB acting as a phosphatase on CheY should not be quickly discarded. This interaction may be the type of transient reaction that occurs between CheA and CheY, which also gave a negative yeast-two-hybrid result, and should be investigated in the future.

Acknowledgments

I would like to thank John Kirby and Susanne Muller for their guidance and helpful advice and Nicole Fay for her help in constructing the plasmids. I would also like to thank George Ordal for providing the $CheC_{d149k}$ mutant strain.

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Figure 1.

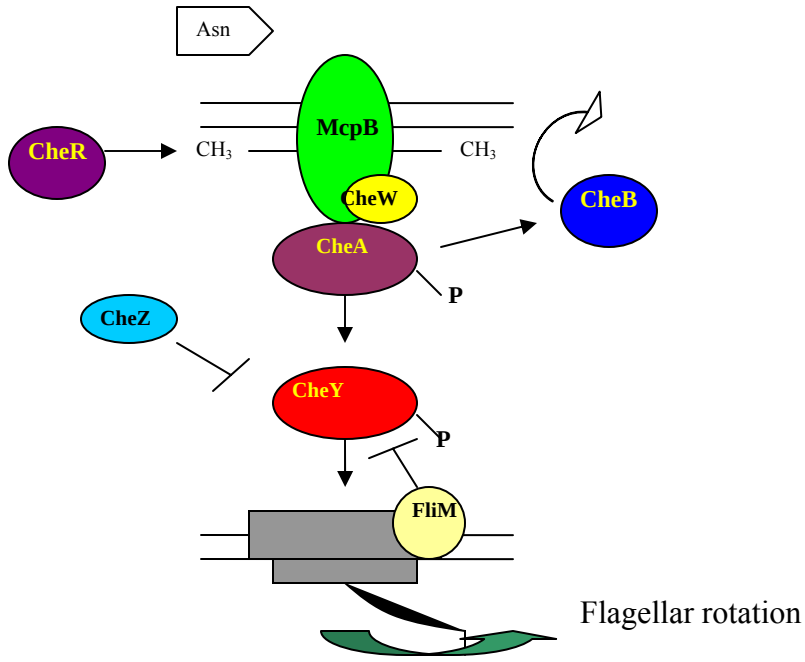
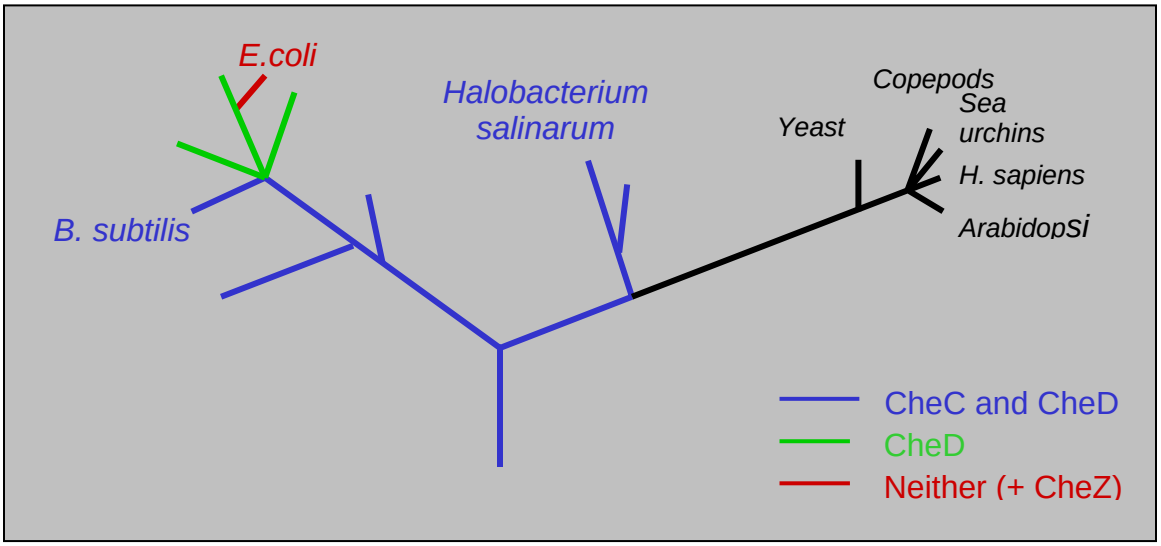


Figure 1. This model portrays the chemotaxis system present in *E.coli*. Asn (asparagine) binds to the receptor, McpB. A conformational change occurs in the receptor that is transmitted by CheW to the sensor kinase, CheA. CheA autophosphorylation decreases, thus decreasing the phosphorylation of CheY. The decrease of phosphorylated CheY leads to a counterclockwise flagellar rotation which leads to a smooth swimming event. Adaptation occurs by methylation and demethylation of the receptor by CheR and CheB respectively. CheZ and FliM are responsible for dephosphorylating CheY (13, 18).

Figure 2.



Adapted from Kirby, Zhulin *et al.*, 2001

Figure 2. This phylogenetic tree displays the chemotaxis proteins present in different organisms. As can be seen, *B. subtilis* contains proteins CheC and CheD which are also present in many bacteria (left branch) and Archaea (*Halobacterium salinarum)*. (6)

Figure 3

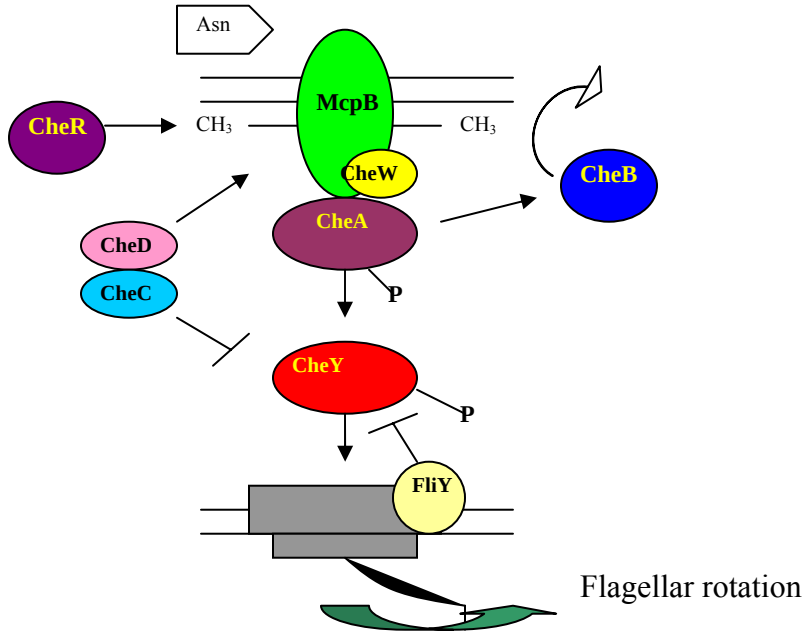


Figure 3. In the *B. subtilis* model, Asn is an attractant for McpB. Upon binding Asn, McpB goes through a conformational change which is transmitted by CheW to CheA. CheA autophosphorylates and then transfers a phosphate group to CheY as well as CheB. The phosphorylated CheY leads to counterclockwise rotation and a smooth swimming event. CheR and CheB methylate and demethylate the receptor respectively. CheC and CheD interact and regulate the function of each other. CheD permanently deamidates the receptor as a post-translational modification, and this function is inhibited by the interaction with CheC. CheD has been shown to enhance the phosphatase activity of CheC(5).

Figure 4.

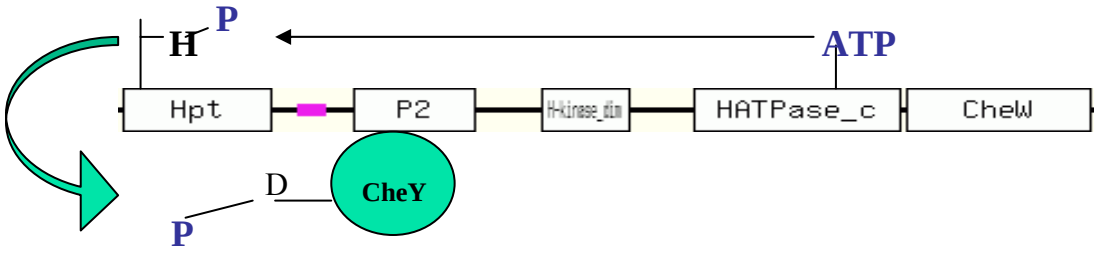


Figure 4. The structure of CheA contains the Hpt domain which receives the phosphoryl group from the ATP that binds the H-kinase domain. The phosphoryl group is then transferred from the Hpt domain to CheY, which binds the P2 domain (MIST website).

Figure 5.

626-ASAEEQLASMEEISSSATTLAQ**MAEELRDLTKQ**FKIE-662

CheZ fragment	
E. coli	137-M A Q D F Q **D** L T G **Q**-147
P. aeruginosa	137-L A Q D Y Q **D** L T G **Q**-147
S. typhimurium	137-M A Q D F Q **D** L T G **Q**-147
B. subtilis McpB *indel*	648-M A E E L R **D** L T K **Q**-658

Figure 5. This figure demonstrates the presence of a conserved phosphatase sequence present in McpB of *B. subtilis.* The topsequence in bold is the McpB putative phosphatase sequence. The chart shows known phosphatases and the conserved residues present in each of the sequences (8).

Figure 6.

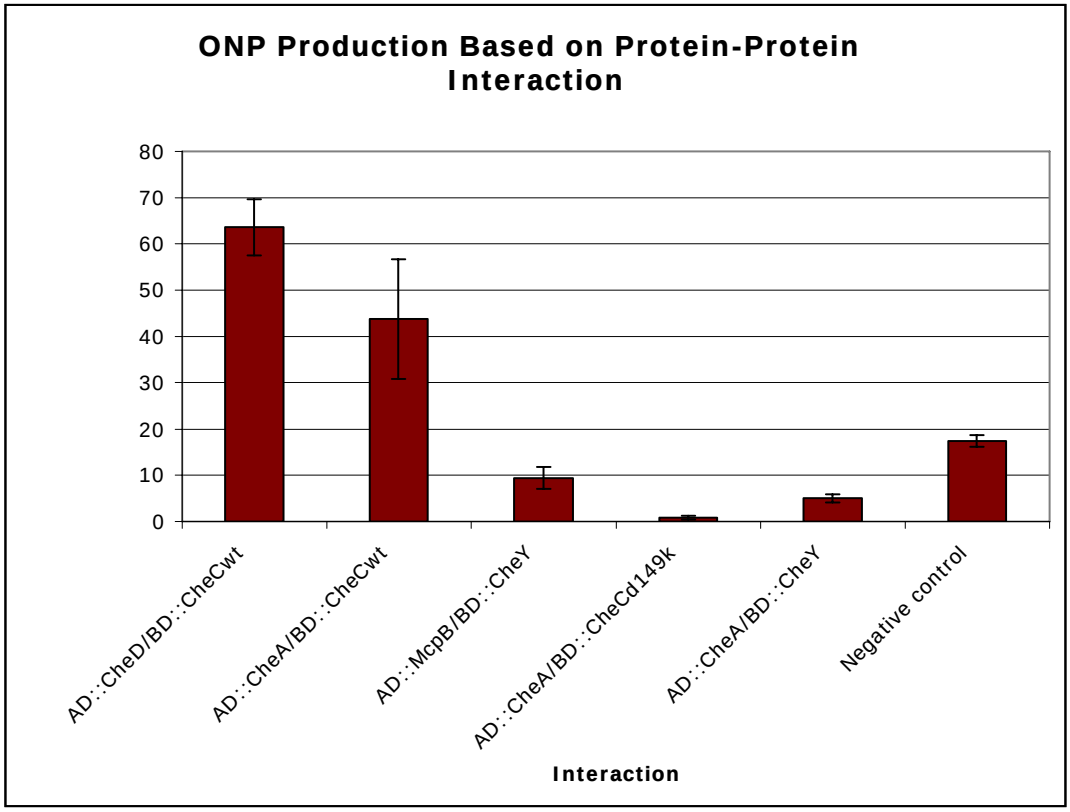


Figure 6. This graph indicates the amount of ONPG to ONP conversion per minute per mg of protein in yeast isolates. The CheA/CheC interaction had ONP production that was significantly higher than the negative control, indicating a positive interaction. The CheA/CheC419k isolate showed very little ONP production, thus indicating an absence of functional β-galactosidase enzyme and hence lack of interaction between the two proteins.

Figure 7.

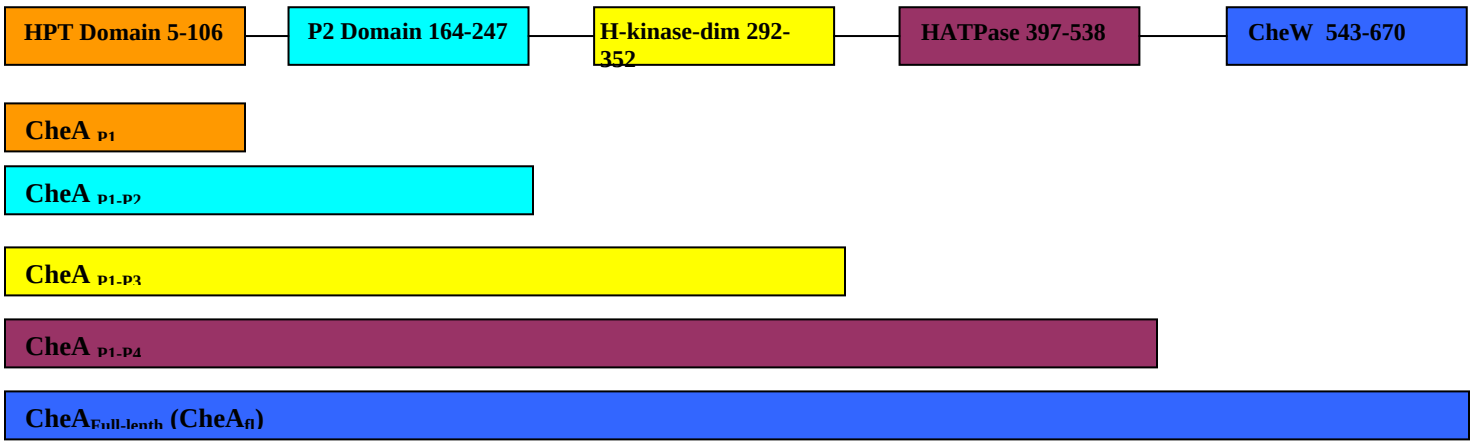


Figure 7. This represents the CheA constructs created. Domain deletions were made from the C-terminus to make progressively shorter CheA proteins.

Table 1.

pGAD

pGBDU	CheA	CheC	CheD	CheY	McpB	$CheC_{d149k}$
CheA		-	-	-	-	
CheC	+ + +	+ + +	+ + + +	-	+ +	- - -
CheD	-	+ + + +		-	-	-
CheY	-	-	-		-	-
McpB			+ +	-		-
$CheC_{d149k}$	-		-	-	-	

Table 1. This table indicates results from the yeast-two-hybrid assay. Positive interactions are noted by the +, negative interactions are noted by the -. The number of + signs reflects the relative strength of the interaction compared to the CheC and CheD interaction. Positive interactions have been shown between CheC and CheD, and CheC and CheA. Interestingly, interactions have not been seen between CheY and McpB, CheA and CheCd149k, and CheD and CheCd149k. Although CheY and McpB have not been shown to interact, this may be a result of such a transient interaction that can not be detected with the yeast-two-hybrid system.

Table 2.

pGBDU \ pGAD	$CheA_{FL}$	$CheA_{P1}$	$CheA_{P1-P2}$	$CheA_{P1-P3}$	$CheA_{P1-P4}$	$CheC_{WT}$	$CheC_{D149K}$
$CheA_{FL}$							
$CheA_{P1}$							
$CheA_{P1-P2}$						+ + +	
$CheA_{P1-P3}$						+ + +	
$CheA_{P1-P4}$							
$CheC_{WT}$	+ + +	+ + +	+ + +	+ + +	+ + +	+ + + +	
$CheC_{D149K}$	-	-				-	

Table 2. This table indicates results from the yeast-two-hybrid assay of CheC with the various CheA's. Positive interactions are denoted by the + sign, and no interactions are denoted by the – sign. Interactions were seen between CheC and all CheA's tested.

Table 3.

pGBDU \ pGAD	$CheC_{WT}$
$CheA_{FL}$	+ + + + +
$CheA_{P5-P4}$	- - - - -
$CheA_{P5-P3}$	- - - - -
$CheA_{P5-P2}$	- - - - -

Table 3. This table indicates results from the yeast-two-hybrid assay of CheC with the various CheA's. Positive interactions are denoted by the + sign, and no interactions are denoted by the – sign.

Figure 8.

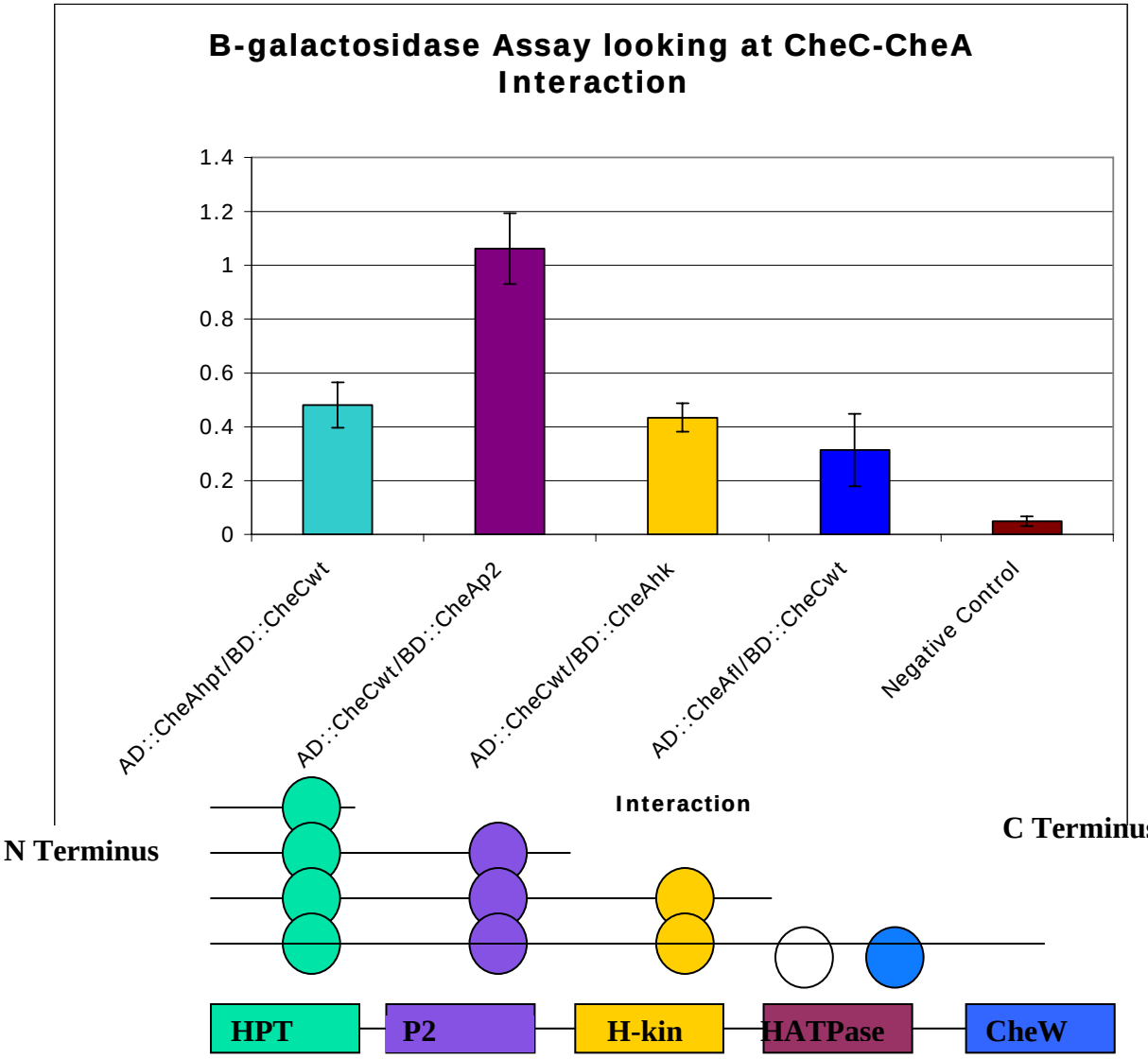


Figure 8. This graph displays the results of the β-galactosidase assay between CheC and the various CheA's. Note that this graph does not include data on the $CheA_{P1\text{-}P4}$ mutant. The results indicate that the presence of CheA's HPT domain alone is enough to confer the same level of interaction that is seen between $CheA_{FL}$ and CheC.

Figure 9.

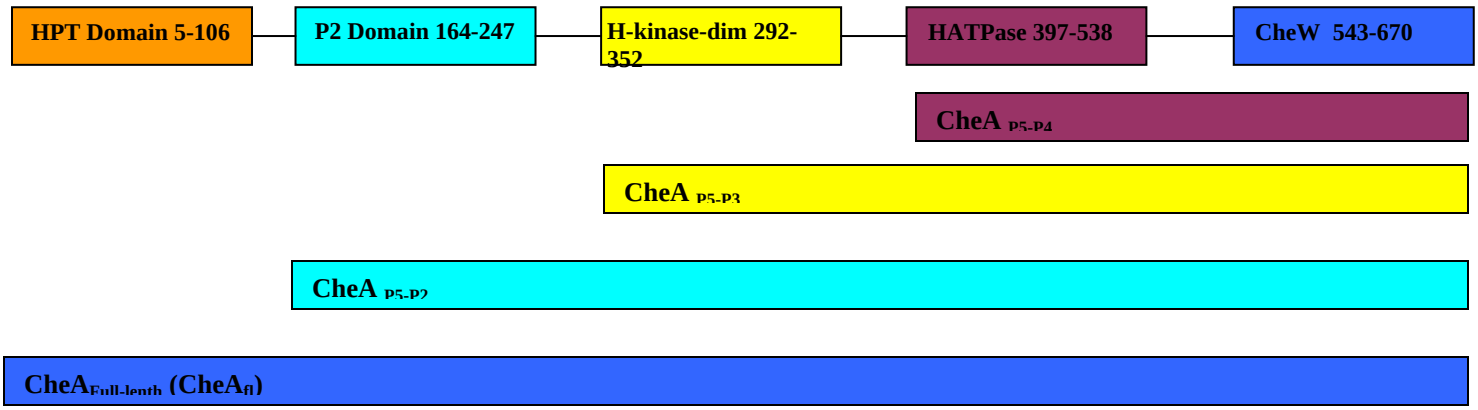

HPT Domain 5-106
P2 Domain 164-247
H-kinase-dim 292-352
HATPase 397-538
CheW 543-670
CheA p5-p4
CheA p5-p3
CheA p5-p2
CheAFull-length (CheAfl)

Figure 10.

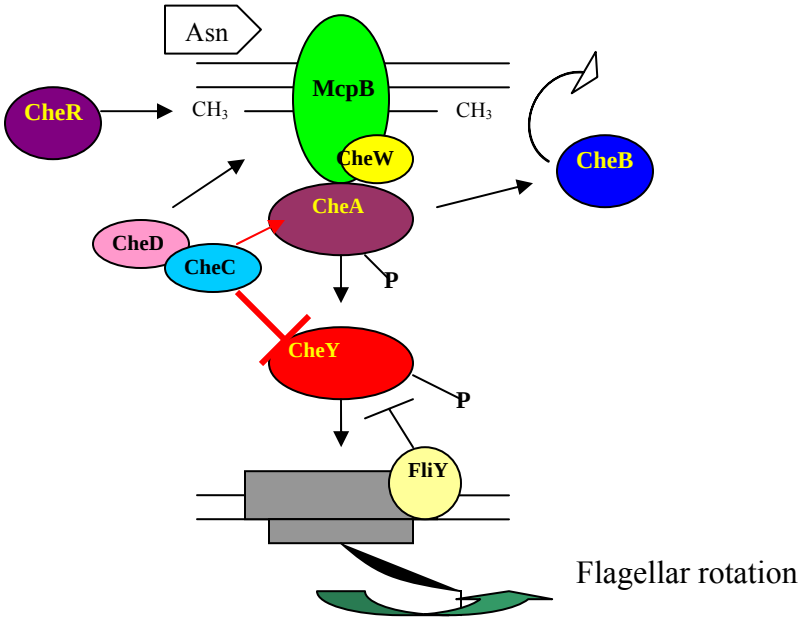


Figure 10. In the proposed *B. subtilis* chemotaxis model, CheC is acting as a regulatory protein on CheA. The interaction with CheC is hypothesized to facilitate CheC phosphatase action on CheY. Currently, all other features remain the same as shown in figure 3.

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