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Construction of CheA4 Mutant in *Azospirillum brasilense*

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INTRODUCTION

Chemotaxis describes the directed movement of motile bacteria. Bacteria move by monitoring chemical changes in their environment and adjusting their swimming behavior accordingly. The flagellum is the bacterial structure that provides cells with motility. Normally the flagella move in a counterclockwise rotation to propel the bacterium towards favorable conditions and will briefly switch to clockwise rotation to rapidly reorient the cells in a new direction and to move it away from harmful environments (Armitage 1999). The swimming motion of *Azospirillum brasilense*, a free living diazotroph, is due to detection of chemical attractants or repellents in the environment. Some examples of chemical attractants are nutrients and oxygen (Elmerich 1987). From the study of chemotaxis in *Escherichia coli* and *Salmonella typhimurium*, since the 1960s, it is clear that the signal to change direction in the bacterium is relayed by CheA, a histidine kinase (Stock 1987, Morrison 1994). This also has been confirmed in other bacteria and *Archaea* (Armitage 1999)

The CheA protein will autophosphorylate in the presence of ATP and then phosphorylate CheY, a flagellar switch protein. The phosphorylated CheY then diffuses and attaches to the flagellar motor, resulting in a change of flagella rotation (Zhulin 2001). Activation of the CheA protein results in its autophosphorylation. However, when a chemical attractant binds to the receptors of the bacterium, the CheA activity is inhibited. These membrane receptors for CheA are known as methyl-accepting Chemotaxis proteins or MCPs and CheA needs to interact with CheW to associate with the MCPs (Morrison 1994).

In *A. brasilense* four different CheA proteins have been discovered and named CheA1, CheA2, CheA3, and CheA4. Thus, the bacteria possesses several copies of the CheA histidine kinase for various functions. The exact function of three of the kinases is unknown. So far only the function of one of the CheA proteins has been determined (Hauwaerts 2001). However, the focus of this experiment is to determine the importance of CheA4 in chemotaxis of *Azospirillum brasilense*.

The CheA4 protein exhibits many characteristics associated with the main control of chemotaxis and motility. Genomic analysis and comparison shows that CheA4 is similar to proteins in other species, such as *E. coli*, that control chemotaxis. The DNA sequence has many characteristics that are necessary for the main chemotaxis control. This find is very significant, but genomic analysis alone is not conclusive. The CheA4 protein may be very similar to the main chemotaxis control protein in *E. coli*, but chemotaxis evolved so rapidly that CheA4 may provide a completely different function in *A. brasilense*.

To confirm CheA4 as the main control of chemotaxis and motility it must be tested experimentally. This will be done by creating a mutant of the cheA4 gene using the genomic information available. When bacteria with the mutant variety of cheA4 are created, the organism should, in accordance of this hypothesis, be unable to control chemotaxis in response to chemical triggers.

MATERIAL AND METHODS

To create the mutants, we used the known DNA sequence of the cheA4 gene, from an ongoing sequencing project and constructed primers (Zhulin, unpublished). The Up

fragment primers incorporated an *Xba*I and *Bam*HI site, while the primers for the Down fragments included a *Hind*III and *Bam*HI site (Figure 1). Polymerase chain reactions (PCRs) allowed for replication of the *cheA4* gene using *A. brasilense* Sp7, where mutants are available making it ideal for study (Elmerich 1987). Using a FailSafe PCR Kit, Buffer D, and enzyme mix the PCR was completed separately for the up and down fragments as illustrated in Figure 2 (Epicentre).

Figure 1: DNA Primers

CheA4-Up- <i>Xba</i> I-Forward
5'TCT AGA AAG GAG ACG GGC GGT GAC3'
CheA4-Up- <i>Bam</i> HI-Reverse
5'GGA TCC GAT GGC GTT GAC CTC GTC G3'
CheA4-Do- <i>Bam</i> HI-Forward
5'GGA TCC GAC CCC ACC CGC GCG CTG3'
CheA4-Do- <i>Hind</i> III-Reverse
5'AAG CTT AGG GCC TTG GTC GCC GAG3'

Figure 2: PCR Conditions

Steps in order	Temperature (°C)	Duration
Initial		
denaturing	95	3 min
Denaturing*	95	45 sec
Annealing*	61 for Up	30 sec
	65.8 for Down	30 sec
Elongation*	72	1 min
Final		
elongation	72	5 min
*These three steps were repeated 30 times in order before the final elongation.		

To facilitate further manipulation and allow sequestering of inserts we cloned the individual fragments and then transformed the DNA into competent *E. coli* cells using a standard TOPO Cloning Kit (Invitrogen). *E. coli* are ideal for these types of transformations because they have a *lacZ* reporter gene. A reporter gene is useful in determining whether the cell has taken up the plasmid or not. In this case we recovered white colonies from plates containing 5-Bromo-4-chloro-3-indoxyl-beta-D-galactopyranoside (X-gal) and kanamycin (Km). However, to ensure the right fragment

transformed, the samples were digested with *EcoRI* enzyme. After one and a half hours we analyzed the samples by gel electrophoresis. The desired band should be approximately 850 base pairs long as seen on Figure 3.

Figure 3: Gel Electrophoresis of CheA4-Up-*EcoRI*

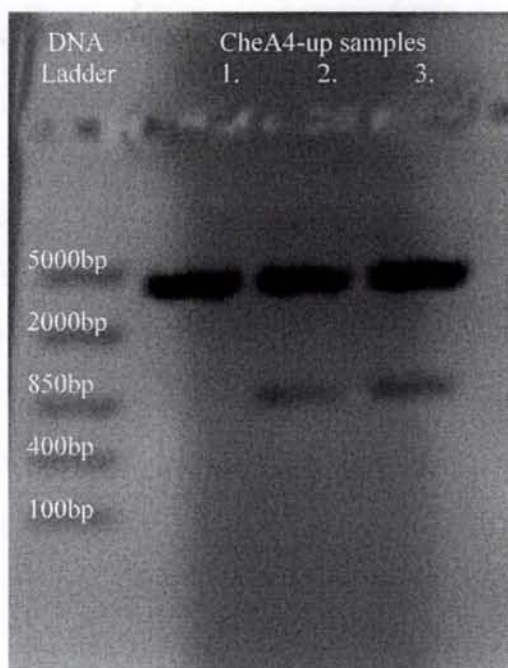


Figure 3: The three different samples of CheA4-Up were run on the gel after being digested with *EcoRI*. Samples 2 and 3 have the desired insert at approximately 800 bp.

Following the cloning, the transformed DNA was isolated using a Fast Plasmid Mini Prep kit (Eppendorf). Once the *EcoRI* test was completed the CheA4-Up portion was digested with *XbaI* using 30uL plasmid DNA, 5uL buffer 2, 2uL *XbaI* enzyme and 11uL distilled water. The CheA4-Down was digested with *HindIII* (30uL plasmid DNA, 5uL buffer 2, 2uL *HindIII* enzyme, and 11uL distilled water). All enzyme reactions were activated at 37°Celsius and ran for two hours. Then both enzyme reactions needed

to be inactivated at 65°Celsius for 20 minutes. In order to digest both samples with *BamHI* we first performed a DNA precipitation according to Figure 4 and then to re-hydrate the DNA we added 42.5uL distilled sterile water.

Figure 4: DNA Precipitation

1. add 2-3 times the volume ice cold ethanol 95%
2. add 1/10 total volume of 3M Sodium Acetate pH5.2
3. Centrifuge at 14,000rpm for 30min at 4°C
4. Decant supernatant and add 500uL ice cold ethanol 70%
5. Centrifuge for 10 min at 4°C
6. Decant supernatant and let air dry for 10-15 minutes
7. Re-hydrate with sterile distilled water

Next both the CheA4-Up-*Xba*I and the CheA4-Down-*Hind*III DNA fragments were digested with *Bam*HI at 37°C for 2 hours (42.5uL plasmid DNA, 5uL *Bam*HI buffer, 0.5uL BSA, 2uL *Bam*HI enzyme). Following the second digest, we ran the samples on a 0.7% agarose gel for gel purification of the desired fragments using a QIAquick Gel Extraction Kit (QIAGEN).

The vector used for creating the mutant is pKNOCK-Tc (Alexeyev 1999). We also digested it with *Xba*I and *Bam*HI and isolated in the same fashion as describe for CheA4-Up above (Figure 5). pKNOCK-Tc was chosen, because it is a suicide vector and therefore unable to replicate in the absence of *pyr* factors, produced by certain *E. coli* strains. It also has multiple cloning sites and tetracycline resistance making it ideal for selection and cloning (Alexeyev 1999).

The pKNOCK-Tc and CheA4-Up-*Xba*I-*Bam*HI were ligated using a Fast-Link DNA Ligation kit (Epicentre, Figure 5). Then the product was transformed into competent *E. coli* GT115 cells (InvivoGen). From there the cells needed to be tested for actual transformation. To do this we cut the samples using *Bam*HI and then ran and compared them to pKNOCK-Tc-*Xba*I-*Bam*HI on an agarose gel. The pKNOCK-Tc CheA4 fragments should be identified as being larger than the pKNOCK-Tc-*Xba*I-*Bam*HI band.

For future use, we digested pWM6 with *Bam*HI enzyme to be added to the growing DNA (Metcalf 1993, Figure 4). pWM6 is the cassette being used due to its antibiotic resistance gene for kanamycin (Km) and because its effectiveness in producing marked as well as unmarked mutations (Metcalf 1993). Also, pWM6 has been identified

as a high-copy-number plasmid, which encounters little modification when transferred between several hosts (Minas 1993).

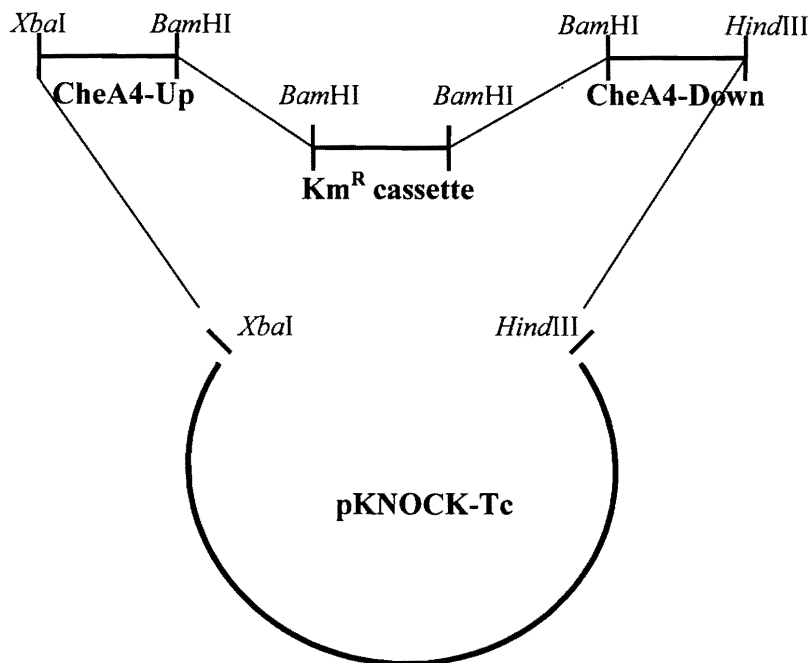


Figure 5: Construction of the mutant. pKNOCK-Tc is the suicide vector into which the CheA4-Up element will be inserted followed by the CheA4-Down. The kanamycin resistance (Km^R) cassette has two *Bam*HI restriction sites, allowing it to be inserted between the two CheA4 fragments.

DISCUSSION

The complete product will be suicide vector with a mutated copy of the *cheA4* gene (Figure 5). This vector allows direct target mutation in the *Azospirillum brasilense* genome. However, to complete the experiment a few extra steps are required. First both the Up and Down fragment must be properly inserted into pKNOCK-Tc followed by the kanamycin resistance gene from the pWM6 vector. By using the genomic data of the *CheA4* gene of *Azospirillum brasilense* any mutation taking place will be concentrated on that gene alone, thereby eliminating any possible mutations on genes other than *cheA4*.

To test the effects of the mutation, the mutagenized cells will be grown on minimum malate-salt medium with 0.325% agar to test for chemotaxis. Further analysis will involve the study of the frequency of reversal and speed of the free swimming cells using video microscopy (Hauwaerts 2001). This process will involve growing and creating large number of mutant cells and comparison to normal *Azospirillum brasilense*.

The construction of a mutant is a complex and lengthy process. Designing the appropriate primers and finding the proper PCR conditions often requires fine tuning. Many problems encountered tend to extend the time frame of this experiment. One common problem encountered included difficulties with the DNA transformations. The plasmid often would not insert into any of the cells. In one case the colonies that grew on plates containing 5-Bromo-4-chloro-3-indoxyl-beta-D-galactopyranoside (X-gal) and kanamycin were all white, but when re-streaked on to fresh plates the colonies came up blue, evidence that these cells lacked the plasmid. Currently we are encountering problems with one of the inserts, CheA4-Down. This insert has not successfully been cut from the TOPO using *Hind*III and *Bam*HI enzymes. The problem appears to be the stock solution of CheA4-Down fragment and to remedy this problem several analyses are in progress.

These hurdles are just a few examples of what complications may be expected when creating a mutant. However, progress has been made. We are not far from completing the creation of cheA4 construct for mutagenesis of *A. brasilense*. Once the mutants have been created they will be observed according to their swimming patterns and speed and such analysis will reveal if the cheA4 gene really is the main control of chemotaxis in *Azospirillum brasilense*.

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