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Regulation of Flagellar Motor Movement in Azospirillum brasilense

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ABSTRACT:

Chemotaxis is the biological process in which motile cells respond to chemical signals in their environment. These chemical signals can be attractants or repellents, which entice the cells to move towards or away from the source, respectively. In *Escherichia coli*, a model to study this behavior at the molecular level, the interaction between the flagellar motor protein, FliM, and a phosphorylated intermediate, CheY~P, mediates this response, with CheY~P binding to FliM to trigger a change in the direction of flagellar rotation. A change in flagellar motor rotation from counterclockwise (CCW) to clockwise (CW) in response to CheY~P binding causes the cells to reorient their direction of movement by tumbling before resuming motility in a new direction. Many bacteria possess multiple CheY homologs, and whether all of these can bind FliM has not been elucidated in most cases.

The aim of this research was to determine if all of the CheY homologs, which are present in the alphaproteobacterium *Azospirillum brasilense*, are able to bind with the unique polar flagellum FliM protein present in the genome of this species. To this end, we created two different *A. brasilense* strains that contained FliM proteins with cyanofluorescent protein (CFP) fused to either the N- or C-terminus of FliM and optimized the design of the constructs to ensure that a functional fusion between CFP and FliM was produced. We used fluorescence microscopy to verify the localization of the tagged FliM proteins. These constructs will be used in further experimentation to analyze the relationship between the different CheY homologs and the FliM flagellar motor protein.

INTRODUCTION:

With the global population growing at an exponential rate, agriculturalists are placing more and more emphasis on the formation of a sustainable system of food production. Many different nitrogen fertilizers have been developed to expedite the rate of crop growth and amplify crop output. However, the vast majority of these fertilizers are produced by energetically costly chemical means. The widespread overuse of these chemical fertilizers takes a detrimental toll on the environment by depleting nutrients in the soil, killing aquatic organisms, and contaminating drinking water. In recent years, the contribution of bacteria to plant growth has become a topic of primary focus. Several different species of nitrogen-fixing bacteria are known to aid plant growth by converting dinitrogen (N_2) into ammonia (NH_3), a nitrogenous compound that is usable by plants (Ji, Gururani, & Chun, 2014). Many scientists believe that this uniquely bacterial metabolism could permit the development of an environmentally friendly biofertilizer.

Azospirillum brasilense is a nitrogen-fixing bacterium that colonizes plant root surfaces and directly contributes to plant growth. This species of bacterium is highly motile, due to the presence of a polar flagellum used for swimming. Swimming by the polar flagellum is not random in *A. brasilense*: it is controlled by a chemotaxis machinery that enables the bacterium to direct its movement in preferred directions. The chemotactic control of flagellar rotation affords motile *Azospirillum* the ability to navigate towards favorable nutrient conditions, including organic acids, sugars, amino acids, and aromatic compounds (Steenhoudt & Vanderleyden, 2000). In addition, the motility of these bacteria along the roots is expected to be significant to enhance colonization over time. Thus, understanding flagellar motor function has both practical and fundamental applications.

The interaction between the FliM flagellar motor protein and the chemotaxis CheY response regulator is central to the ability of the chemotaxis system to control the direction of flagellar rotation and thus cell movement. Six different CheY homologs are encoded in the *A. brasilense* genome, some of which have not yet been characterized (Wisniewski-Dyé et al., 2011). Comparative sequence analysis suggests that some of these homologs possess non-conservative amino acid substitutions in positions that were shown in the model *E.coli* to be critical for interactions with other proteins in the chemotaxis signal transduction pathway (Mukherjee T., unpublished observation), and suggests that only 3 of the homologs might interact with the polar FliM. The focus of this research is to establish an experimental approach and strategy that will eventually be used to determine which of these homologs interact with a single FliM protein on the flagellar motor. For the purpose of this work, the experimental design was optimized with one model CheY protein (CheY4; Mukherjee, Kumar, Xie & Alexandre, unpublished) for which experimental evidence supports direct interaction with the polar FliM in *A. brasilense.*

MATERIALS AND METHODS:

Formation of DNA constructs:

DNA fragments corresponding to the open reading frame for CFP and FliM were first obtained using PCR amplification using pFa6 and genomic DNA from *A. brasilense* Sp7, respectively, as templates. The following primers were used to amplify the promoter regions (Table 1):

Table 1: Primer sequences

Forward Primer	Reverse Primer
CFPCtermFwd 5' – GGC GGG GGC ATG GRG AGC AAG GGC GAG GAG CTG TTC – 3'	*CFPCtermRev* 5' – TTA TCG ATA CCG TCG ACC TCG AGT TAA GAT CTG TAC AGC TCG TCC ATG CCG AG – 3'
FliM5XGlyFwd 5' – GGA ACA AAA GCT GGG TAC CAG GAG GAG TCC CTT ATG AGC AAC ACC GAG GAA CTG AGC GAA GAG GAA CGC – 3'	*FliM5XGlyRev* 5' – GCT CAC CAT GCC CCC GCC CCC GCC TAG CCG CAT GAC – 3'
CFP5XGlyFwd 5' – GGA ACA AAA GCT GGG TAC CAG GAG GAG TCC CTT ATG GTG AGC AAG GGC GAG – 3'	*CFP5XGlyRev* 5' – GTT GCT CAT GCC CCC GCC CCC GCC AGA TCT GTA CAG – 3'
FliMCtermFwd 5' – GGC GGG GGC ATG AGC AAC ACC GAG GAA CTG AGC GAA G – 3'	*FliMCtermRev* 5' – TTA TCG ATA CCG TCG ACC TCG AGT CAT AGC CGC ATG ACC TCC TGC TTG GG – 3'

The Promega Mastermix kit (Promega, Fischer Scientific) was used to conduct the PCR reactions according to manufacturer's protocol and under the following reaction conditions (Table 2):

Table 2: PCR conditions

Number of cycles	Temperature (ºC)	Duration
1 cycle	98 ºC	8 minutes
30 cycles	98 ºC	1 minute, 30 seconds
30 cycles	55 ºC	30 seconds
30 cycles	72 ºC	1 minute, 30 seconds
1 cycle	72 ºC	6 minutes
1 cycle	4 ºC	∞

The two PCR fragments were joined using splicing by overlap extension (SOE) PCR (Horton, Hunt, Ho, Pullen & Pease, 1989). In two separate reactions, the CFP fragment was connected to either the 5' or the 3' end of the FliM fragment. The fragments were separated with a linker consisting of DNA for five glycine residues, which was engineered in the primers. This linker was added to provide enough physical separation between the protein products to ensure proper functioning. The SOE PCR products contained 3'-adenine overhangs, which were complimentary to the 3'-thymine overhangs on the commercial pCR2.1 TOPO vector used for this purpose (Invitrogen, Carlsbad, CA).

The SOE PCR products were cloned into a TOPO vector (pCR 2.1) using the protocol for the Invitrogen TOPO kit (Invitrogen, Carlsbad, CA). Briefly, 4 µl of the A-overhang PCR product were incubated with 1 µl of salt solution and 1 µl of the linearized pCR 2.1 TOPO vector at room temperature for five minutes. 4 µl of this reaction was transferred to chemically

competent Top 10 *E. coli* cells, according to the manufacturer's protocol (Invitrogen, Carlsbad, CA).

Colonies that contained the desired fusion inserted into pCR2.1, named CFP-Gly-FliM and FliM-Gly-CFP, respectively, were identified using colony PCR and primers *CFP5XGlyFwd* and *FliMCtermRev* for the former and *FliM5XGlyFwd* and *CFPCtermRev* for the latter. These colonies were inoculated individually into LB liquid medium supplemented with kanamycin (50 µg/ml) and grown overnight in a 37ºC shaker. The plasmids with the cloned inserts were isolated using the Qiagen Miniprep kit according to the manufacturer's protocol (QIAGEN, Valencia, CA). The presence of the desired fusion cloned in the purified plasmids was verified by sequencing at the Molecular Biology Core Facility, UTK. Next, the plasmids were digested with two restriction enzymes, KpnI-HF and XhoI (New England Biolabs) per the manufacturer's recommendations to isolate the inserts that were verified by sequencing. The digested products were analyzed using gel electrophoresis, and the bands that corresponded to the inserts of interest (approximately 1100 base pairs) were purified using a Qiagen gel purification kit per the manufacturer's instructions (QIAGEN, Valencia, CA). The purified inserts were ligated with the pBBR1MCS5 plasmid vector (Landeta et al., 2011) digested with KpnI-HF and XhoI restriction enzymes, according to the manufacturer's recommendations (New England Biolabs). The ligation of digested pBBR1MCS5 with the appropriate insert (DNA fragments corresponding to CFP-Gly-FliM or FliM-Gly-CFP isolated by restriction digestion from the sequenced-verified vectors) occurred under the following reaction conditions (Table 3):

Table 3: T4 Ligation Mixture

Component	**Volume**

Digested pBBR1MCS5 vector	50 ng (0.020 pmol)
Digested insert	37.5 ng (0.060 pmol)
10X Buffer T4 Ligase	2 µl
T4 Ligase Enzyme	1 µl
dH_2O	Up to 20 µl

Different ratios (1:1, 2:1, and 3:1) of inserts to plasmid DNA were used for the ligation reactions. These reaction tubes were incubated at 16ºC overnight, which is the optimal temperature for the formation of hydrogen bonds, which stabilize the base pairing of compatible ends of fragments and thus facilitate the subsequent ligation by the ligase enzyme. 4 µl of these ligated products were added to competent Top 10 *E. coli* cells for chemical transformation. These mixtures were kept on ice for five minutes and then heat shocked at 42ºC for 45 seconds. 250 µl of SOC medium was added, and the reaction tubes were placed on the shaker at 37ºC for one hour. Both of the reaction mixtures were spread on three LB plates with Gentamycin (20 µg/ml) in volumes of 50, 100, and 150 µl. These plates were placed in a 37ºC incubator overnight to allow growth. Colony PCR was used to analyze the successful colonies. The colony PCR tubes were prepared with the components listed in Table 4.

Table 4: Colony PCR mixture used in this study

Component	**Volume**
Promega MasterMix	12.5 µl
Primer 1 (Forward)	0.5 µl
Primer 2 (Reverse)	0.5 µl

dH_2O	11.5 µl
Colony from plate	Biological material from one colony gathered with a pipette tip

The PCR products were subjected to agarose gel electrophoresis to confirm the presence of the appropriate insert (Figure 1).

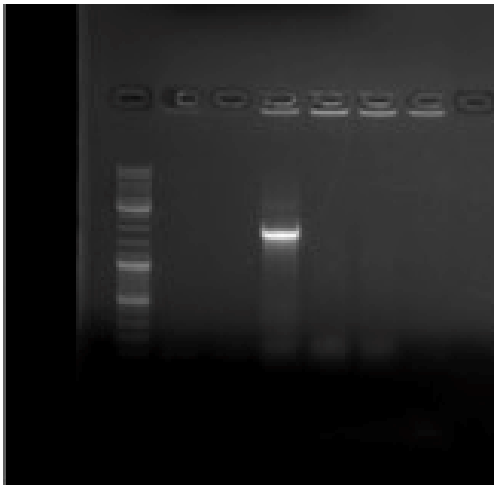

Figure 1: Agarose gel electrophoresis analysis of colony PCR product pBBR1MCS5 (FliM-Gly-CFP) obtained under the PCR conditions described in the text. Molecular weight marker (MWM): O' gene ruler 1 kb, Promega. The band shown corresponds to the size of the insert (~1.7 kb).

Following PCR, colonies containing the correct ligated pBBR1MCS5-insert were identified by the presence of a band corresponding to the length of the inserts (1100 base pairs) on the agarose gel. Permanent glycerol stocks of the these *E. coli* Top 10 colonies containing positive plasmids were made following culture at 37ºC in presence of antibiotic: *E. coli* Top 10

[pBBR1MCS5 (CFP-Gly-FliM)] and *E. coli* Top 10 [pBBR1MCS5 (FliM-Gly-CFP)]. One colony from each of these strains was inoculated in LB liquid medium supplemented with Gentamycin (20 µg/ml) and placed on the 37ºC shaker overnight. The plasmids were collected with a miniprep kit and then transformed into *E. coli* S17-1 competent cells, which are suitable for biparental mating with *A. brasilense* (Broek, Lambrecht & Vanderleyden, 1998). These cells were grown overnight on the 37ºC shaker. 500 µl of the overnight culture was inoculated in fresh LB liquid medium the next day and grown overnight.

The *E. coli* S17-1 cells containing the pBBR1MCS5 vectors with the ligated inserts were mated with *A. brasilense* Sp7 strain using laboratory protocols. The cultures were washed three times with sterile 0.8% KCl, by centrifugation. 50 µl of the *E. coli* S17-1 cells was mixed with 50 µl of the Sp7 overnight culture grown in TY medium (5 g Tryptone, 2.5 g Yeast Extract, and dH_2O to 500 ml). This mixture was added as a series of drops to a D-medium plates (4 g Nutrient Broth, 0.125 g $MgSO_4 \bullet 7H_2O$, 0.5 g KCl, 0.005 g $MnCl_2$, and dH_2O to 500 ml) and placed in a Styrofoam box with wet paper towels in the bottom to provide a moist environment. The box containing the mixed cultures was placed in a 28ºC incubator overnight to allow for plasmid transfer. The next day, the cells were streaked off of the D-medium plates and plated on MMAB medium, a minimal medium, which is selective for *A. brasilense* cells with the constructed plasmids. The MMAB was made without a nitrogen source (*A. brasilense* fixes atmospheric N_2 but not *E. coli*) and with malate as a carbon source (1.5 g K_2HPO_4, 0.5 g NAH_2PO_4, 0.075 g KCl, a "pinch" of Na_2MoO_4, 2.5 g Malate, and dH_2O to 500 ml). This medium was then supplemented with salts (2.5 ml of $MgSO_4$, 250 µl of $CaCl_2$, and 125 µl of $FeSO_4$) and gentamycin (20 µg/ml). These plates were incubated at 28ºC for three days. Colonies that grew on the selective plates were then re-streaked on the same medium and

allowed to grow three times to remove the donor *E. coli* S17-1 cells. The *A. brasilense* cells that contained the pBBR1MCS5 plasmids with the constructed inserts were streaked on TY plates supplemented with ampicillin (200 µg/ml) and gentamycin (20 µg/ml).

Imaging by Fluorescence Microscopy:

The presence of a functional fusion between CFP and FliM was verified using fluorescence microscopy. Cultures of Sp7 [pBBR1MCS5 (FliM-Gly-CFP)] and Sp7 [pBBR1MCS5 (CFP-Gly-FliM)] were grown overnight at 28ºC with shaking. These cultures were grown in four different media samples – TY with or without gentamycin (20 µg/ml) and MMAB –N +C with or without gentamycin – to determine the optimum conditions for imaging. Cultures were grown to a maximum optical density of 0.3. The cells were pelleted by centrifugation at 5000 rpm for 3 minutes at room temperature. Pellets were then re-dissolved in 200-300 µl of Che Buffer (0.85 g K_2HPO_4, 0.68 g KH_2PO_4, and dH_2O to 500 ml).

Meanwhile, the microscope slides were prepared. Fisherfinest Premium Microscope Slides (Fisher Scientific) were cleaned with ethanol. A low melting agarose (Promega, Fisher Scientific) was melted, and 100 µl of the molten agar was pipetted onto each slide. Each slide was covered with a cover slip and placed in the freezer at 4ºC for 1 hour to dry. The slides were removed from the freezer, and the cover slips were removed gently, so as to avoid disturbing the solidified agar. Then, each slide was supplemented with 30 µl of a sample of the dissolved pellets, and the cover slips were added back to the agar pads. These samples were allowed to dry at room temperature for 2 hours. Finally, fluorescence was check using a Nikon 80i Fluorescent Microscope with a 100X oil immersion objective.

RESULTS:

The microscopy images obtained in this experiment show the localization of the FliM flagellar motor protein in *A. brasilense* cells. Two different microscopy techniques, differential interference contrast microscopy (Figure 2) and fluorescence microscopy (Figure 3), were used to verify correct localization. The following images correspond to the Sp7 [pBBR1MCS5 (FliM-Gly-CFP)] construct. Images were taken for the second construct, Sp7 [pBBR1MCS5 (CFP-Gly-FliM)], but more time is needed to optimize the imaging conditions for this strain.

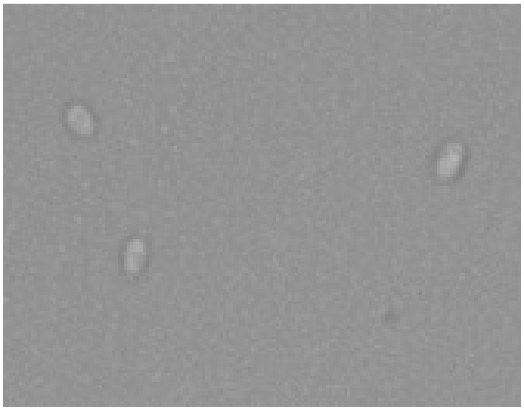

Figure 2: Differential interference contrast microscopy image of Sp7 [pBBR1MCS5 (FliM-Gly-CFP)]

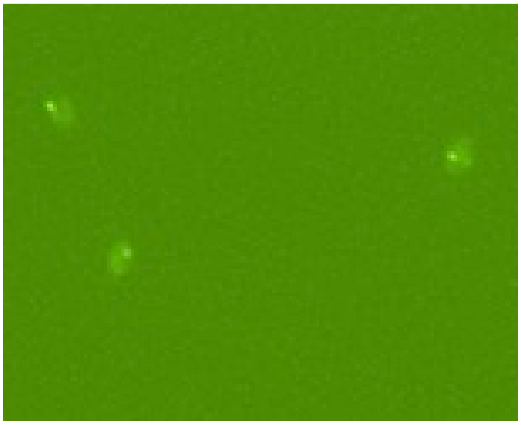

Figure 3: Fluorescence microscopy image of Sp7 [pBBR1MCS5 (FliM-Gly-CFP)]. The functional fusion created between FliM and CFP shows the FliM flagellar motor protein to be localized at the pole of the cell, as expected.

DISCUSSION:

The physiological responses of motile bacteria have been studied extensively, and similar experiments have been conducted to measure the interaction of the response regulator CheY~P with its target protein, FliM, under different environmental conditions (Sourjik & Berg, 2002). However, this experiment and its future directions take this research one step further. In future experimentation, Fluorescence Resonance Energy Transfer (FRET) will be used to test the closeness of the interaction between the FliM-Gly-CFP and CFP-Gly-FliM constructs designed in this experiment and fluorescently labeled CheY homologs encoded in the *A. brasilense* genome. The FRET approach relies on the observation that proteins in close proximity to one another can transfer energy via resonance. The energy that is transferred varies significantly with small distance changes, providing a highly accurate estimation of the closeness with which two proteins interact (Periasamy, 2001). Thus, the next steps for this experiment will be used to verify which of the six CheY homologs identified in *A. brasilense* can function in the chemotaxis response pathway by binding to a single FliM flagellar motor protein and resultantly altering the orientation of the cell. This insight into the relationship between these two proteins is essential for a comprehensive understanding of the overall chemotactic mechanisms of the cell.

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