5-2015

Influence of tlp5, che1P, tlp4A, and che4STAS Promoters on Chemotaxis in Azospirillum brasilense

Brian W. Connor
University of Tennessee - Knoxville, bconnor@vols.utk.edu

Influence of *tlp5*, *che1P, tlp4A,* and *che4STAS* Promoters on Chemotaxis in *Azospirillum brasilense*

Brian W. Connor

Dr. Gladys Alexandre (Advisor)

The University of Tennessee, Knoxville

Biochemistry, Cellular and Molecular Biology

Introduction

Chemotaxis is the mechanism by which bacteria, as well as other single and multicellular organisms, regulate their movement based on the various chemical signals in the environment. The goal of this research project is to determine the levels of gene expression of several chemotaxis genes encoded in the genome of the alphaproteobacterium *Azospirillum brasilense* by analyzing the activity of their promoters. The four genes of interest are *tlp5P, che1P, tlp4AP*, and *che4STASP*. The products of these genes function in chemotaxis, and their relative contribution to the overall chmeotaxis response will depend on their expression level. The goal of the research conducted here is to determine the level of expression of these genes by probing the activity of their promoter regions. In order to measure promoter activity, 5' DNA regions upstream of each gene containing putative promoters were first engineered in front of a promoter-less reporter gene (gusA), which is cloned on a broad host range vector named pFUS (Reeve et. al, 1999). The product of the *gusA* gene is a beta-glucuronidase enzyme, which is able to cleave beta-glucuronides. By using commercial modified glucuronides which are colorless before cleavage by beta-glucuronidase but yield a colored or fluorescent product upon cleavage by GusA, the promoter activity can be quantitatively determined: the greater the concentration of GusA produced from the promoter cloned upstream of the *gusA* gene, the greater the kinetic of cleavage of the β-glucuronide substrate by the GusA enzyme. Using this method, the differential activities of these promoters were determined under different growth conditions**.**

Procedures and Methods

This lab employs many basic molecular biology concepts and procedures including PCR, Gel electrophoresis, bacterial cloning, mating, transformation, and restriction enzyme digestion to analyze the molecular basis of chemotaxis in *Azospirillum brasilense*. Initially, a polymerase chain reaction (PCR) is utilized to amplify the DNA sequence of interest. This reaction is carried out by placing approximately 25 microliters of reaction mixture containing *A. brasilense* wild type strain Sp7 genomic DNA, one set of the aforementioned primers of interest at a stock concentration of 50mM, water, and Promega Mastermix (containing enzyme, dNTPs and buffer for DNA synthesis) in a thermal cycler, under the conditions listed below.

Table 1. Conditions used for PCR amplification of promoter regions

Reactants	**Amount (μL)**
Sp7 genomic DNA	1
Primer (forward)	0.5
Primer (reverse)	0.5
Deionized Water	10.5
Promega Mastermix	12.5

Table 2: Thermal Cycling Conditions used in this study[#]

Initial denaturation step: 95° C	3 minutes
Denaturation: 95°C	1 minute*
Annealing: 65°C	20 seconds*
Elongation: 72°C	1 minute*
Elongation: 72°C	5 minutes

[#] The conditions listed above were used for all sets of primers

*Indicates a step that is repeated for 35 cycles

The presence of the DNA sequence of interest after the PCR was confirmed by checking its size in base pairs through gel electrophoresis. This process separates DNA bands on the basis of size, as smaller bands, containing fewer base pairs, migrate further down the gel than larger bands and the negative net charge of DNA causes the bands to travel from the negative to positive terminus of the gel electrophoresis apparatus (down the gel) when an electric current is applied. Once the correct or anticipated band size was confirmed through the gel, a gel purification of the DNA fragment generated by PCR was performed using a commercial kit (QIAquick PCR purification, Qiagen) according to the manufacturer's guidelines. The PCR fragments corresponding to the promoter sequences were then cloned into a TOPO vector (Invitrogen, Carlsbad, CA), following the guidelines laid out by the Invitrogen TOPO Cloning

User Manual (Invitrogen; Appendix I) and then transformed into competent *Escherichia coli* Top^{10} cells.

Colony PCR was used to screen potential colonies harboring a TOPO vector in which the expected promoter region was cloned. Eight different colonies were typically tested using the following conditions.

Table 3: Conditions used for colony PCR reactions

Reactant	**Amount (in μL)**
Promega Mastermix	52
Primer (froward0	4
Primer (reverse)	4
Deionized Water	40

Table 4: Thermo-cycler parameters used for colony PCR

Conditions	**Time**
Initial denaturation step: 98°C	10 minutes
Denaturation: 98°C	1 minute*
Annealing: 65°C	45 Seconds*
Elongation: 72°C	1 minute*
Elongation: 72°C	5 minutes
Storage: 4°C	∞

* Indicates a step that will be repeated for 35 cycles

After the colony PCR was performed, the DNA sequences cloned into the TOPO vectors were verified by DNA sequencing at the Molecular Biology Core Facility, The University of Tennessee. Next, the promoter region was cloned on a broad host range plasmid vector, pFUS, upstream of a promoterless *gusA* gene To this end, two restriction enzyme digestions (EcoRI and HindIII) were performed in parallel, according to the manufacturer's protocol (New England Biolabs, Ipswich, MA), with one set of restriction digestion used to specifically cut the pFUS vector and another to isolate the PCR fragment corresponding to the promoter sequenced to be cloned into the digested pFUS. The restriction digested pFUS vector and DNA sequences were then ligated under conditions described in Table 5.

Table 5: pFUS Ligation Reaction

Reactant	Amount (in μL)
Digested pFUS vector (With EcoR1 and HindIII Restriction Sites)	2
Purified insert from TOPO vector (With EcoR1 and HindIII Restriction Sites)	8
10x Buffer T4 Ligase	2
T4 Ligase Enzyme	0.5
Deionized Water	7.5

Following ligation, competent *E.coli* Top10 cells were transformed and colonies carrying expected fragments into the pFUS vector were identified by PCR as described above. Permanent glycerol stocks were made of the following strains: Top^{10} [pFUS-*tlp5P*], Top^{10} [pFUS-*tlp4AP*],

and Top[10] [pFUS- *che1PP].* The pFUS vectors with cloned promoter regions were also before transferred into competent *E. coli* S17-1 cells, which is a strain suitable for biparental mating between *E. coli* and *A. brasilense*. Permanent glycerol stocks were also made for the following strains: S17-1 (pFUS-*tlp5P*), S17-1 (pFUS-*tlp4A*P), S17-1 (pFUS-*che1P).* Following mating to transfer the vectors from *E. coli* S17-1 to *A. brasilense* sp7, colonies of *A. brasilense* carrying the pFUS vectors with the promoter of interest, were purified by several rounds of streaking on minimal media plates supplemented with the appropriate antibiotics, and then used to determine promoter activity. Promoter activity was assayed under two growth conditions, growth in rich medium (TY) and growth in a minimal medium (MMAB-N+C). TY medium was prepared using the following compounds: tryptone (10g), yeast extract (5g) in a final volume of 1000mL of deionized water. MMAB-N+C was prepared using the following compounds: dipotassium phosphate (3g), monosodium phosphate (1g), potassium chloride (0.15g), sodium molybdate (a "pinch). malate (5g) in a final volume of 1000ml of deionized water. To make solid media, 15 grams of agar was added per liter of medium. To induce growth under nitrogen fixation, the cells were first grown in TY, an aliquot of the culture was then pelleted, washed and re-suspended in MMAB-N+C followed by overnight incubation without shaking (to minimize aeration and diffusion of oxygen, which inhibits nitrogen fixation). To test promoter activity in these different environmental conditions, a fluorescence assay for the *gusA* gene in *A. brasilense* cells carrying pFUS-*tlp5P*, pFUS-*tlp4A*P or pFUS-*che1P* was performed using a high-throughput β-glucuronidase assay performed in microplates. Upon the second testing of the activity of these promoters (see results), suspicion arose regarding the loss of the *gusA* reporter gene. Thus, the reagent X-Gluc (5-bromo-4-chloro-3-indolyl- β-D-glucuronic acid) (Sigma) was added to plates to test bacterial colonies for presence and activity of GusA.

Results and Discussion

GusA enzymatic activity is an indication of expression levels of the promoters in the pFUS vector because the amount of GusA protein produced directly results from the activity of the promoter cloned to direct transcription of *gusA*. DNA sequencing confirmed that the correct DNA sequences for the promoter regions targeted were cloned in the TOPO vectors and then pFUS vectors. Initial assays for β-glucuronidase activity demonstrated that the promoters for the *che1* operon, the *tlp4A* and the *tlp5* genes were expressed, albeit at different levels depending on the growth conditions (Fig 1-3)

Figure 1: TY Media with 10x Diluted Samples

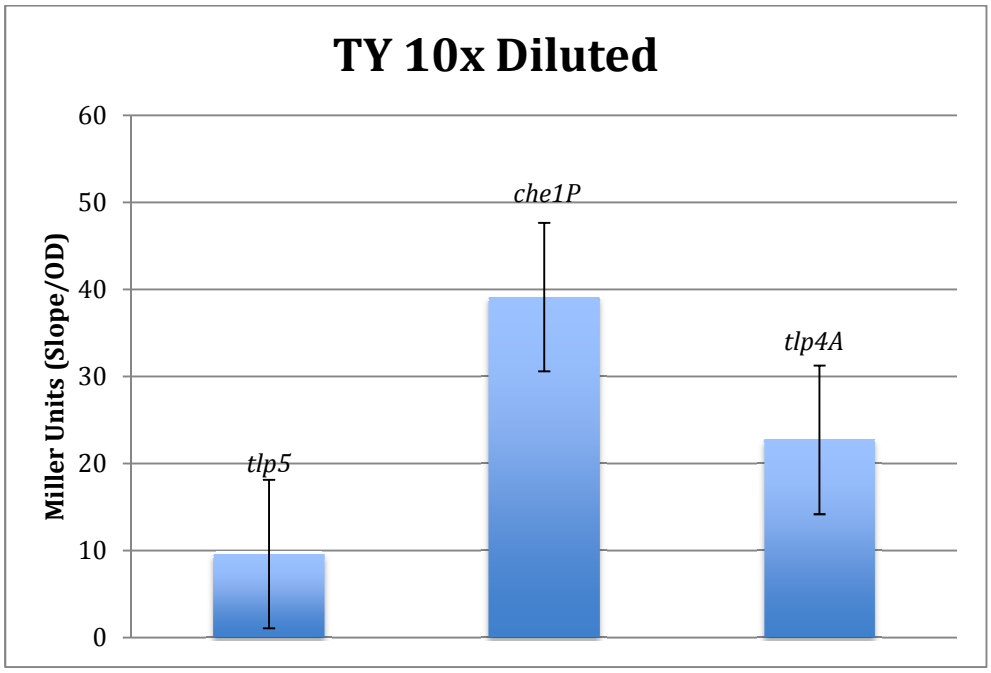


Figure 2: TY Media with Non-diluted Samples

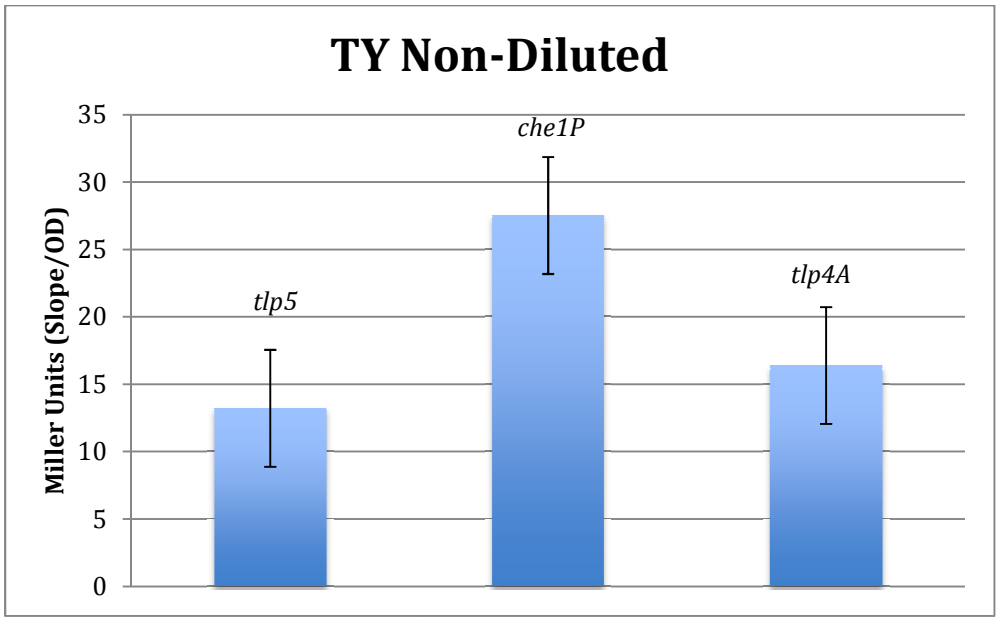
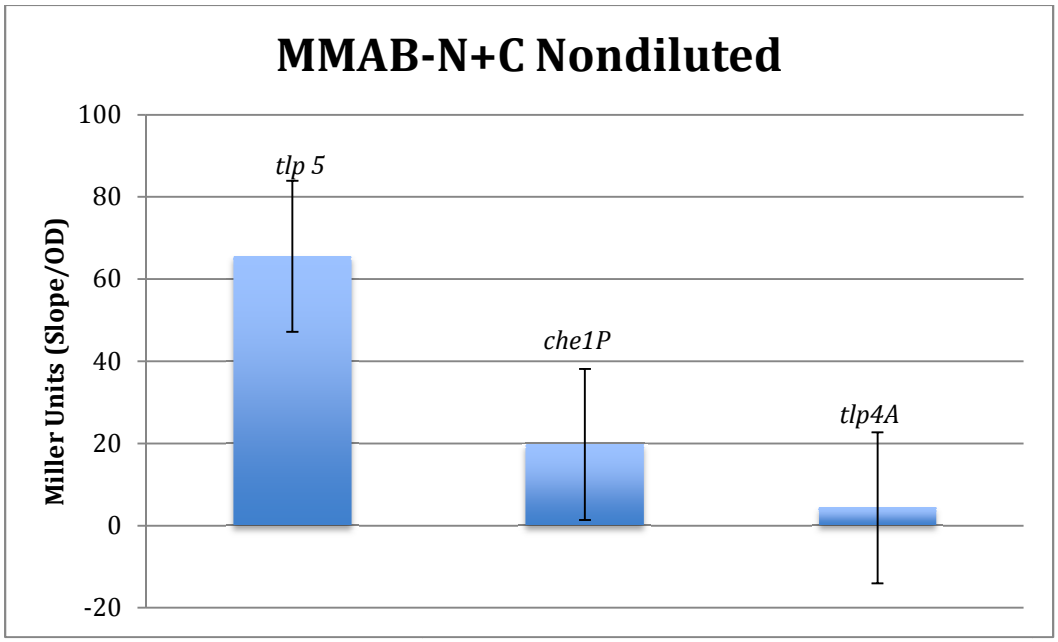


Figure 3: Minimal Media with Non-diluted Samples



As depicted in Figures 1 and 2, *che1* promoter activity is greatly elevated in the rich TY medium as compared to growth in a minimal medium. Furthermore, in TY, the *che1* operon promoter is significantly more active than both the *tlp4A* and *tlp5* promoters. In minimal medium (Figure 3), however, the *tlp5* promoter is significantly more active than both the *tlp4A* and *che1P* promoters. Building off these preliminary results, further investigation will more definitively depict the pattern of expression of each of the promoters analyzed here.

After this first experimental replicate, similar results were unable to be produced. Through, the use of microscopy, it was determined that a cross-contamination event occurred. Thus, a new mating was performed to prepare newly mated *A. brasilense* colonies. Several subsequent rounds of purification were then performed. During two additional experimental replicates of this experiment, zero fluorescence was detected for each of the promoter constructs. After confirming the presence of the pFUS vector through a continuous selective resistance to tetracycline and confirming the presence of each promoter by running the products of a restriction enzyme digestion on an agarose gel and confirming the appropriate band lengths, it was hypothesized that the reporter *gusA* gene was perhaps not expressed in the newly transformed colonies of *A. brasilense.* To test this hypothesis, we used a colorimetric assay employing X-Gluc as a substrate to test for the activity of the enzyme via a blue-white screening since X-Gluc produces a by-product that form blue precipitates when acted upon by GusA. This test confirmed that while the pFUS plasmids were present, *gusA* was not expressed. Thus, retrospectively, this finding explains why a lack of expression was detected for all three promoter constructs on the third and fourth experimental replicates of the fluorescence microplate assay. Afterwards, a mating from stocks of *E.coli* S17.1 competent cells containing

the pFUS vector with each of the three promoters of interest were prepared to enable these experiments to be repeated in the future.

Of the four promoters, three have been successfully integrated into *A. brasilense* and tested for enzymatic activity using a microplate assay, all except *che4STAS*. After initial struggles correctly identifying the correct promoter sequence of the *che4STAS* gene, it has now been identified and inserted into a TOPO vector that was used to transform *E. coli*, TOP^{10} competent cells. Next, the *che4STAS* insert must be isolated and ligated into a PFUS vector, which will subsequently be used to transform *E.coli* S17-1 cells to be mated with *A. brasilense* to allow for genetic testing. Currently, permanent glycerol stocks have been made of *A. brasilense* sp7 pFUS[*tlP5, che1P, and tlp4A*]. A glycerol stock was also made of TOPO[*che4STAS*] in *E. coli*. These will be used for further experimentation in the future.

Reference

Reeve, W. G., Tiwari, R. P., Worsley, P. S., Dilworth, M. J., Glenn, A. R., & Howieson, J. G. (1999). Constructs for insertional mutagenesis, transcriptional signal localization and gene regulation studies in root nodule and other bacteria. *Microbiology-sgm*. doi:10.1099/13500872-145-6-1307