

Regulation of gene expression during flocculation in *Azospirillum brasilense*

By

Ryan Hammond

Dr. Gladys Alexandre (Advisor)

Biochemistry Senior Honors Thesis

The University of Tennessee, Knoxville

Biochemistry, Cellular, and Molecular Biology

Department

Chancellor's Honors Program

Spring 2014

Table of Contents

1.

Abstract.....

.....3

2.

Introduction.....

.....4

3. Materials and

Methods.....5

4.

Results.....

.....15

5.

Discussion.....

.....19

6.

References.....

.....22

Regulation of gene expression during flocculation in Azospirillum brasilense

ABSTRACT:

In the alpha-proteobacterium *Azospirillum brasilense*, flocculation is a cell differentiation event whereby motile cells form large aggregates of non-motile cells encased in a dense extracellular matrix of polysaccharides. Flocculated cells are resistant to various environmental insults, a feature that is desirable for the production of *A. brasilense*-containing biofertilizers used in agricultural applications. To date, a regulatory gene (FlcA) is the only known genetic element specifically implicated in differentiation of *A. brasilense* into flocculated cells. The purpose of the research presented here was to gain insight into the genetic regulation of flocculation. The pattern of expression and potential genetic link with FlcA of three genes (referred to as PM21, PM29, and *ybgC*), previously identified in a mutant screen, were determined using promoter reporter fusions. A Beta-glucuronidase (GusA) promoter assay was carried out with fusions between the putative promoter regions of PM21, PM29 and *ybgC* and a promoterless *gusA* expressed from a broad host range plasmid, in *A. brasilense* wild type strain (Sp7) and its *flcA*::TN5 mutant derivative, in which FlcA is inactive. Results obtained suggest that the contribution of *ybgC* to flocculation is probably indirect while both PM21 and PM29 specifically function

in this differentiation process. Data also suggest that PM21 and PM29 are under FlcA regulation.

INTRODUCTION:

The goal of this project is to characterize promoter activity of a set of genes previously identified as being involved in regulating flocculation, a cell differentiation event in the alphaproteobacterium, *Azospirillum brasilense* (Mishra, 2012). This rhizospheric bacterial species is known to positively influence plant growth when present at high cell densities on a host plant's root system. The genetic regulation of flocculation, or the tendency to clump by cell-to-cell interaction and to form stable "flocs" which correspond to the aggregation of cells in a dense extracellular polysaccharide matrix, is relevant not only to *A. brasilense*'s lifestyle in the soil but also to the production of stable biofertilizers in agronomical applications (Jayaraj et al., 2004).

This research involved constructing fusions between promoter regions of genes to be analyzed (named PM21, PM29 and *ybgC*) and a promoterless *gusA* gene cloned on a plasmid vector (pFus). These genes were chosen because they have been identified in a screen for mutants impaired in flocculation and thus are

likely contributing to this behavior (Mishra, 2012). While all the genes affected have no apparent effect on cell growth and viability under any condition, the contribution of the candidate gene products to flocculation could be direct, (i.e. their function is flocculation-specific) or indirect. The aim of the gene expression analyses is to determine the pattern of gene expression during flocculation and to determine whether these expression patterns are regulated by a known transcription factor (FlcA) that functions in flocculation (Pereg-Gerk *et al.*, 1998). Two different strains of *A. brasilense* are used in these experiments: strain Sp7, the wild type for flocculation and a *flcA*::TN5 mutant derivative strain which does not flocculate (Pereg-Gerk *et al.*, 1998). As can be determined by the name, *flcA*::TN5 is the only gene that has been implicated in the regulation of flocculation in *A. brasilense* (Pereg-Gerk *et al.*, 1998). *gusA* was utilized as a reporter gene so a quantitative assay for analysis of promoter activity could be used. The *gusA* gene is encoded as a promoterless region within the pFus vector. The GusA protein cleaves 4-Methylumbelliferyl β -D-glucuronide, the final working reagent of the assay, into 4-Methylumbelliferone, which exhibits fluorescence, and thus can be detected with high sensitivity (Reeve *et al.*, 1999). Cloning putative promoter regions of the genes of interest upstream from the *gusA* gene allows one to follow promoter activity as a function of fluorescence. In order to determine the promoter activity, the constructs were being subjected to

a β -glucuronidase (the product of the *gusA* gene) assay with a microplate reader, using a recently published method (Ramsay et al., 2011).

MATERIALS AND METHODS:

Construction of Promoter Reporter Fusions:

The promoter regions to be tested (TABLE 1) for expression were first cloned into the pFUS reporter plasmid (Reeve et al., 1999) as EcoRI- XhoI fragments obtained after PCR amplification using the *A. brasilense* Sp7 genomic DNA as a template.

Table 1: Genes identified in the mutagenesis screen and for which a promoter activity analysis is performed in this study.

Promoter Region	Length (bp)
PM21	442
PM29	517
YbgC	418

The primers used to amplify the promoter regions of PM21, PM29, and YbgC are listed in Table 2.

Table 2: Primer sequences used in this study.

Forward Primer	Reverse Primer
pFUSPM21pXho-F 5' CCC CTC GAG CGC CGC CTA CCG ACT 3'	pFUSPM21pEcoR1-R 5' CCC GAA TTC CTC GCC GTG CAG 3'
pFUSPM29pXho-F 5' CCC CTC GAG GCC CAT GGA GGA GCC 3'	pFUSPM29ppEcoR1-R 5' CCC GAA TTC CGG CAG CAC CTG AAG 3'
pFUSYbgCpXho1-F 5' GGC CTC GAG GTC CGC GAC TTC GCC 3'	pFUSYbgCpEcoR1-R 5' CCC GAA TTC CCT GTG CTG CGC GTT 3'

The PCR reactions were conducted using the Promega Mastermix kit (Promega, FischerScientific) according to manufacturer's instructions and using the following reaction conditions (Table 3).

Table 3: PCR reaction conditions used in this study.

# of cycles	Temperature	Duration
1 cycle	98°C	1 minute
30 cycles	98°C	1 minute
30 cycles	65°C	45 seconds
30 cycles	72°C	1 minute
1 cycle	72°C	5 minutes
1 cycle	4°C	∞

PCR amplification was then analyzed by gel electrophoresis to ensure specificity and quality (Figure 1).

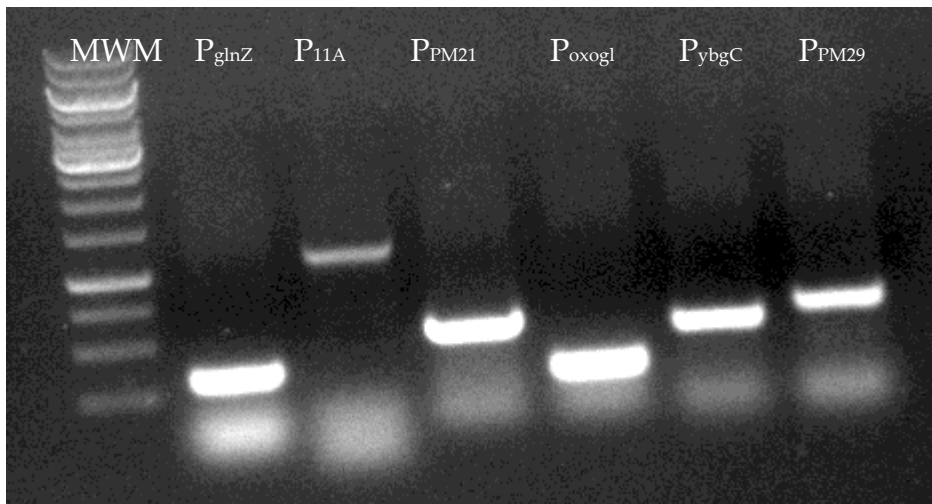


Figure 1: Agarose gel electrophoresis analysis of DNA fragments obtained following PCR under the conditions detailed in the text. MWM: molecular weight marker (O' gene ruler 1 kb, Promega). Promoter

regions from several genes were amplified and are listed above each lane. Promoters for genes coding for glnZ, 11A and oxoglutarate were initially used but not pursued further in this study.

The PCR fragments corresponding to the promoter regions were then purified using the Qiagen Qiaquick PCR purification kit according to the manufacturer's protocol (Qiagen, CA). Following this purification, the DNA fragments were cloned into a TOPO vector (pCR2.1, Invitrogen) using the TOPO kit (Invitrogen). This was done by incubating 4 µl of purified PCR product, 1 µl of salt solution, and 1 µl of TOPO vector at room temp for 5 minutes. 4 µl of this reaction were then transferred to Top¹⁰ chemically competent cells. The cells were heat shocked for 45 seconds, allowing transformation to occur. 250 µl of SOC medium were added to the cells and they were grown with shaking, at 37°C, for 1 hour. The Top¹⁰ cells were then plated on plates containing LB solid medium supplemented with kanamycin and Xgal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) (to allow white/blue screening of colonies) in concentrations of 50 µg/ml and 20 mg/ml, respectively (for white/blue screening). These plates were incubated overnight at 37°C. The positive colonies, marked as white from the white/blue screen, were inoculated in Liquid LB [10g Tryptone, 5g Yeast Extract, 10g NaCl to 1000ml with dH₂O.] supplemented with kanamycin (50µg/ml) and placed on a shaker at 37°C, overnight. The TOPO vector with

cloned promoter regions was also digested with two different restriction enzymes: Xho-1 and EcoR1-HF (New England Biolabs) under conditions specified by the manufacturer (New England Biolabs). This mixture was placed at 37°C for 1 hour and then analyzed by agarose gel electrophoresis. The positive plasmids, showing two distinct bands (one corresponding to the vector and one to the insert) were sent to sequencing for confirmation (Molecular Biology Core Facility, UTK). The plasmids for which the DNA sequence was confirmed were then digested with XhoI and EcoRI and analyzed on agarose gel electrophoresis as above. The DNA bands corresponding to the inserts were extracted with the Qiagen gel extraction kit according to the manufacturer's protocol. The pFus plasmid vector, in which the promoterless *gusA* gene is cloned, was also purified using the Qiagen Plasmid preparation kit according to protocol provided by Qiagen and then digested with the same enzymes prior to ligation. The digested pFus vector was ligated with the gel extracted and purified promoter regions using the following reaction conditions (TABLE 4)

Table 4: conditions for ligation between promoter regions and pFUS vector prepared as detailed in the text.

Component	Volume
Digested pFus vector	2 μ l
Digested insert	8 μ l
10X Buffer T4 Ligase	2 μ l
T4 Ligase Enzyme	0.5 μ l
Water	7.5 μ l

These reactions were incubated at 16°C overnight to enable ligation. Next, the ligated products were transformed into competent TOP¹⁰ cells by adding 4 μ l of the ligation reaction to the competent cells. This mixture was allowed to sit on ice for 5 minutes, and was then heat shocked for 45 seconds. 250 μ l of S.O.C. medium were added, and the tubes were allowed to shake at 37°C for 1 hour. Approximately 50 μ l of these reactions were then plated on LB plates supplemented with Tetracycline (10 μ g/ml) and allowed overnight growth. Colonies grown on the LB plates were subjected to PCR and the PCR products were analyzed by agarose gel electrophoresis. The PCR conditions used were identical to those described in Table 3 and the colony PCR reactions were performed as described in Table 5.

Table 5: Colony PCR conditions.

Component	Amount
MasterMix (Promega)	6.5 μ l
Forward Primer	0.3 μ l
Reverse Primer	0.3 μ l
dH ₂ O	5.4 μ l
Colony from Plate	biological material from the top of 1 colony lightly touched with a pipette tip

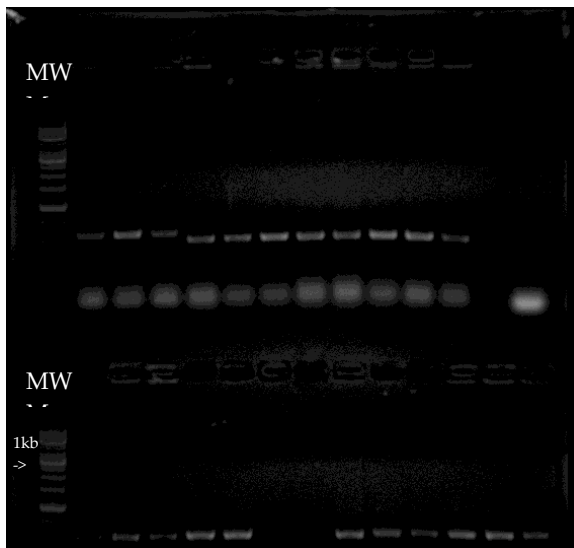


Figure 2: A typical colony PCR result showing bands corresponding to expected size of the DNA region cloned into the pFUS vectors. The bottom row contains 8 replicates of the pFUS-PM21 construct, and 5

replicates of the pFUS-PM29 construct from left to right. The top row contains 3 more replicates of pFUS-PM29 on the left followed by 8 results of the pFUS-ybgC construct.

Positive PCR products showed a single band corresponding to the length of the putative DNA region cloned into the pFUS vector. Permanent glycerol stocks were made of cultures of positive colonies and included Top¹⁰[pFUS-PM21], Top¹⁰[pFUS-PM29], and Top¹⁰[pFUS-YbgC]. The positive pFUS vectors with inserts were purified as above and transformed into *E. coli* S17-1 competent cells and plated on LB plates supplemented with tetracycline (10 µg/ml). After transformation, colonies that showed growth on the plates were placed in overnight LB liquid cultures. The following day, 500 µl of the overnight culture were inoculated in fresh LB liquid medium and placed on the shaker at 37°C, overnight. The *E. coli* S17-1 cells carrying the pFUS vectors with cloned promoter regions were mated with *A. brasilense* strain Sp7 and *flcA*::TN5, according to laboratory protocols. Briefly, the cultures were first washed three times with 0.8% KCl by centrifugation. 3 ml of an overnight culture of the Sp7 or *flcA*::TN5 strain in TY medium and 1 ml of the *E. coli* S17-1 cells carrying the pFUS reporter constructs of interest were mixed and placed as a drop on a D-medium (8 g Nutrient Broth, 0.25g MgSO₄•7H₂O, 1 g KCl, 0.01g MnCl₂, and dH₂O to 1000 ml) plate for overnight incubation at 28°C, to allow sufficient time for plasmid

transfer. The mixed cultures were incubated in a moist environment consisting of a Styrofoam box with wet paper towels in the base. The next day, the cells were streaked off of the plate and plated on a selective media for *A. brasilense* cells that had incorporated the pFUS with insert plasmids and consisting of MMAB, a minimal medium, with no nitrogen source and with malate as a carbon source (3g K₂HPO₄, 1 g NAH₂PO₄, 0.15g KCl, a 'pinch' of Na₂MoO₄, 5 g Malate, 5 ml MgSO₄, 500 µl CaCl₂, 250 µl FeSO₄, and dH₂O to 1000ml) as well as tetracycline (10 µg/ml) and placed at 28°C to incubate for 3 days. At least 5 colonies from each mating that had grown on the selective plates were re-streaked on the same medium three times to completely eliminate the donor *E. coli* S17-1 cells. The *A. brasilense* strains containing the pFUS with inserts were then transferred to TY (10 g Tryptone, 5 g Yeast Extract, dH₂O to 1000 ml) plates supplemented with tetracycline (10 µg/ml).

Culture conditions:

Flocculation occurs when visible "flocs" are formed when *A. brasilense* is grown in a liquid medium limited in nitrogen source, under high aeration conditions. Flocculation is best and most consistently observed with cultures being grown in MMAB supplemented with nitrate at 0.5 mM as the nitrogen source and fructose (8mM) as the carbon source (so called "Floc" medium). In

order to determine gene expression during flocculation, it was important to examine the activity of the promoters to be tested in both *A. brasilense* wild type and *flcA::TN5* mutant strains, under various conditions, including flocculation. For flocculation, overnight cultures in liquid TY supplemented with tetracycline (10 µg/ml) (plasmid maintenance) and ampicillin (200 µg/ml) (*A. brasilense* are naturally resistant to ampicillin) were grown at 28° C with shaking. 200 µl of these cultures were transferred into Floc medium and incubated with shaking for up to 24 hours. Under these conditions, cultures are fully flocculated after 24 hours post-inoculation, so samples were taken at this time for analysis. In addition, another set of cultures grown under flocculation conditions were grown and samples were collected after 12 hours post-inoculation. As controls for gene expression under non-flocculating conditions, cultures were prepared in liquid TY and samples were collected after 24 hours growth at 28°C, with shaking. Next, promoter activity in these cultures was determined.

Beta-glucuronidase assay:

After all of the cultures above were successfully grown and collected, the optical densities of each culture were determined at 600 nm using a non-diluted and a 10 times diluted sample. 100 µl of each sample were then placed in a 96-well microplate. This microplate was frozen overnight at -80°C. The following

day, the microplate was de-frosted in the 37°C incubator. Next, 10 µl of each sample were added into a new microplate which was then incubated at -80°C for 15 minutes. The microplate reader was pre-warmed to 37°C during this time. The final working reagent was prepared according to protocol (Ramsay et al., 2011). It consisted of a 200X stock of 4-Methyumbelliferyl Beta-D-glucuronide prepared by dilution in DMSO at a 50 µg/ml final concentration. This stock solution was diluted to 1x in PBS buffer that contained 2mg/ml of lysozyme (Ramsay et al., 2011). 100 µl of this reagent were immediately added to each well containing samples in the microplate. The microplate was immediately placed in the reader and the program was started. The program was setup to record fluorescence every 46 seconds for a 15 minute time span. The excitation wavelength was set at 365 nm, while the emission wavelength was set at 445 nm. After completion of the assay, the data was extracted and saved to Microsoft Excel, where the evolution of fluorescence over time, which represents the enzymatic activity of GusA and thus correlates with promoter activity was calculated. The rate of each reaction were determined by calculating the slopes of fluorescent evolution over time and normalized to the optical cell density to express the results in Miller Units.

RESULTS:

After completion of the promoter assay, data were collected and analyzed using Microsoft Excel. Two replicates were initially performed for each construct and three distinct growth conditions were used: rich medium (TY), early flocculation conditions (12 hour post-inoculation in flocculation medium) and late flocculation (24 hour post-inoculation in flocculation medium). The final data were expressed in Miller units by dividing the slopes (rate of reaction) by the optical density of the culture (an estimate of the amount of cells). The optical density (OD 600nm) values of some of the cultures exceeded 1 and these had to be diluted 10 times to ensure they remained in a reliable range. All data expressed as Miller units shown in the accompanying figures have been adjusted to remove the background (subtracting the values obtained for Sp7[pFUS], which expresses a vector in which *gusA* is promoterless). Due to time constraints and suspected contamination of the cultures used in the initial sets, the final results shown below correspond to a single assay, using cultures that have been extensively purified.

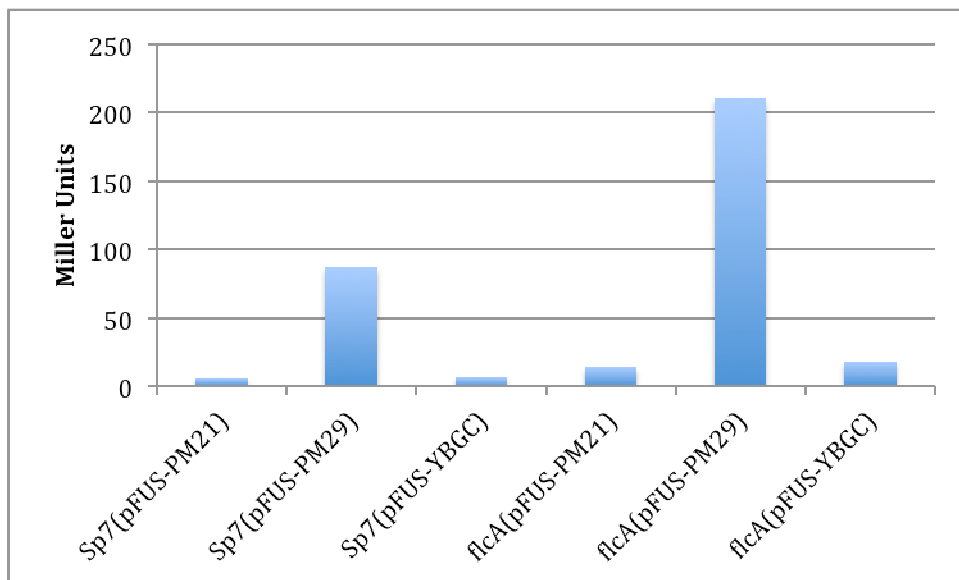


Figure 3: Promoter activity of selected genes from A. brasilense in rich medium (TY) after 24 hours of growth. Promoter activities detected via GusA reporter assays were determined in the wild type strain (Sp7) and flcA::TN5 mutant strain background.

Prior to being inoculated in flocculation medium, cells are typically grown 24 hours in TY medium. In order to determine the promoter activity of genes of interest at the time of inoculation in flocculation medium, we first determined their activity after 24 hours of growth in this rich medium (Figure 3). Under these conditions, the promoter for genes corresponding to PM21 and YbgC products are minimally active in both the Sp7 wild-type and the *flcA*::TN5 mutant strain backgrounds although a small increase was detected when the PM21 and *ybgC* promoters were tested in the *flcA*::TN5 mutant strain. The activity of the promoter driving PM29 expression is significantly higher in both

strains with a significant increase when expressed in the *flcA*::TN5 strain background. The increased expression in the *flcA*::TN5 mutant background suggests that FlcA represses all three promoters.

Upon inoculation in flocculation medium, changes in the expression of some of the genes analyzed were noted at early (12 hours post-inoculation, Figure 4) and later times (24 hours post-inoculation, Figure 5).

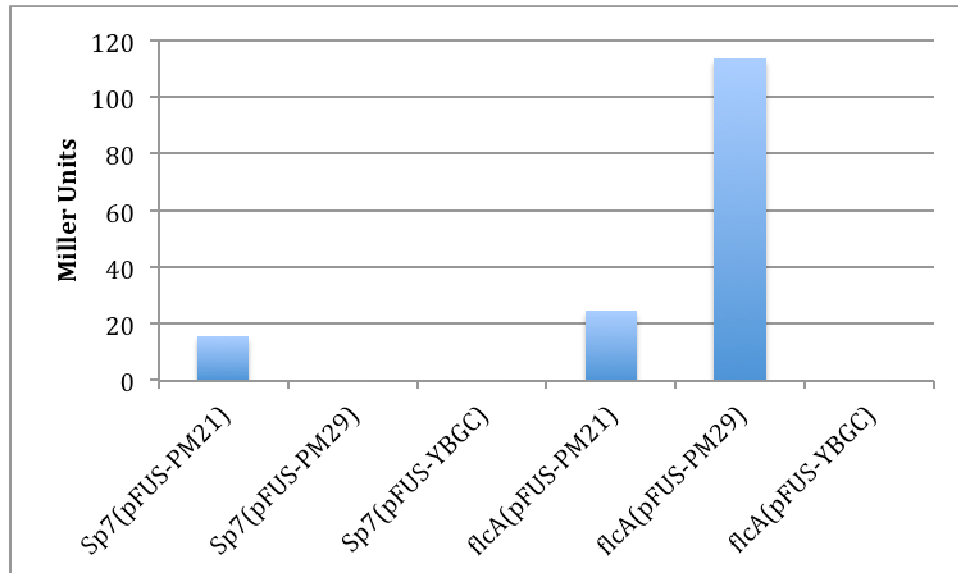


Figure 4: Promoter activity of selected genes from *A. brasilense* in Flocculation medium after 12 hours of growth. Promoter activities detected via GusA reporter assays were determined in the wild type strain (Sp7) and *flcA*::TN5 mutant strain background.

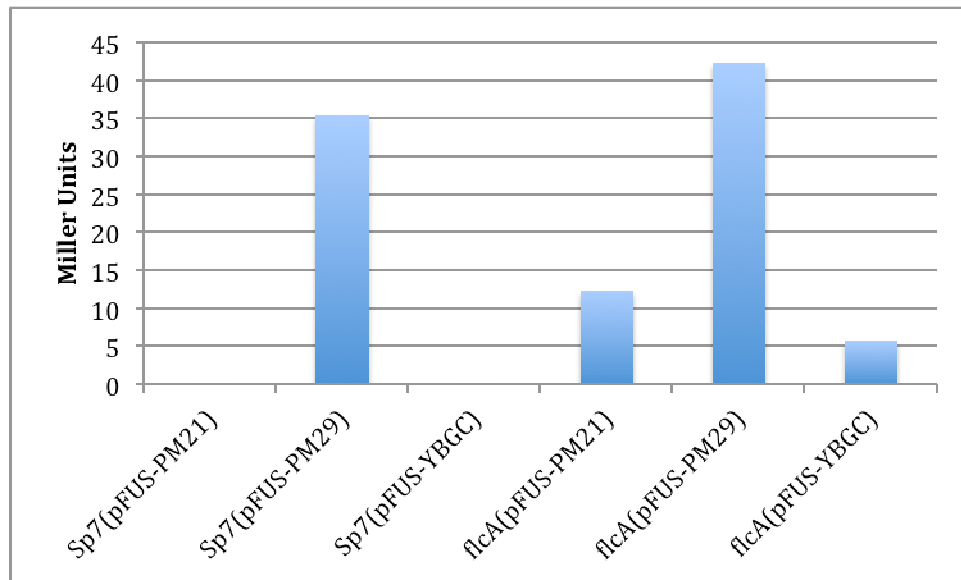


Figure 5: Promoter activity of selected genes from A. brasilense in Flocculation medium after 24 hours of growth. Promoter activities detected via gusA reporter assays were determined in the wild type strain (Sp7) and flcA::TN5 mutant strain background.

After 12 hours of growth in flocculation medium, there was no expression detected for the promoter driving YbgC production, regardless of the strain in which its activity was probed. The promoter driving PM21 expression is active in both the wild-type and mutant strains under these conditions and it also shows a slightly elevated expression level in the *flcA*::TN5 mutant compared to the wild type strain. While the gene encoding for PM29 is not expressed in the wild-type strain under these conditions, it is very highly expressed in the *flcA*::TN5 mutant strain. Together these results indicate that FlcA represses the expression of both PM21 and PM29 at different phases of flocculation. The

expression of *ybgC* may also be under the control of FlcA during the later stages of flocculation but not at later stages.

At 24 hours in flocculation medium neither PM21 nor YbgC show any expression in the wild type strain, indicating that these genes are not active under these conditions. In contrast, the promoter of PM29 is expressed at high levels in both the wild-type and mutant strains. Noticeably, the promoters for PM21 and *ybgC* are derepressed in the *flcA::TN5* mutant background. In addition, the expression of PM29 is elevated in the *flcA::TN5* mutant strain compared to its level of expression in the wild type strain. Together, these observations are consistent with FlcA being a regulator of PM21, PM29 and, perhaps YbgC.

DISCUSSION:

The genes for which promoter activity was studied here were previously isolated in a mutant screen as modulating flocculation in *A. brasilense* (Mishra, 2012). However, their exact contribution to flocculation was not known. The data obtained here clarify the contribution of these genes and suggest some

regulatory connection between some of these genes and FlcA, the transcriptional regulator for flocculation for which no regulatory target has been identified yet.

Under all growth conditions tested and regardless of the strain in which it was expressed, the activity of the promoter for *ybgC* was either minimal or absent. Expression of the *ybgC* promoter was detected in cells grown in TY but not in flocculation conditions, which suggest that the role of YbgC in flocculation is not direct or specific for this process. Given that the mutants were initially screened for changes in outer membrane properties, it follows that YbgC may play a role in modulating outer membrane property in *A. brasilense* (Mishra, 2012). YbgC is part of the Tol-Pal cluster in *E. coli*, which regulates outer membrane integrity during cell constriction at division (Gerding et al., 2007). The lack of expression of *ybgC* under flocculation conditions would suggest that cells do not divide. However, the concentration of cells does increase in flocculation medium. Therefore, it may be that an alternative system is used under flocculation conditions to ensure outer membrane integrity. Alternatively, *ybgC* expression may be so low or transient that we failed to detect it under the conditions of the assay.

In previous mutant screens, it was found that the gene coding for PM29 lies just upstream of that coding for PM21 in the *A. brasilense* genome (Mishra, 2012). The identification of transposon insertions in both these genes strongly

suggests they play a more direct role in flocculation. The genomic organization of PM29 and PM21 also suggests that these genes are co-regulated. The data obtained here indicate that PM21 and PM29 are independently expressed with each gene showing a unique expression profile. PM21 and PM29 are expressed in a pattern consistent with a role in flocculation with PM21 being expressed early in flocculation while PM29 would be important at later stages of flocculation. Another finding is made when comparing PM21 and PM29 promoter activity in Sp7 and *flcA*::TN5 strains. Promoter activity of both PM21 and PM29 are always significantly higher in the *flcA*::TN5 strain compared to the wild type strain, suggesting that FlcA acts as a repressor of PM29 and PM21. The expression of YbgC also appears to partly depend on FlcA function. However, whether FlcA is a transcriptional regulator able to bind to the promoter of PM21 and PM29 and perhaps *ybgC* or if it regulates another protein that itself controls the activity of these genes remains to be determined.

While the information obtained here is significant, caution should be taken because replicates need to be performed to confirm these data. It would also be interesting to conduct studies to find out the mechanism for FlcA repression of PM29 and PM21 promoters.

REFERENCES:

- Gerding MA, Ogata Y, Pecora ND, Niki H, de Boer PA. The trans-envelope Tol-Pal complex is part of the cell division machinery and required for proper outer membrane invagination during cell constriction in *E. coli*. *Molecular Microbiology*. February, 2007.
- Jayaraj J, Muthukrishnan S, Liang GH. "Transfer of a plant chitinase gene into a nitrogen-fixing *Azospirillum* and study of its expression." *Canadian Journal of Microbiology*. July, 2004.
- Mishra, Priyanka Satish, "Genetic Basis of Flocculation in *Azospirillum brasilense*.. " Master's Thesis, University of Tennessee, 2012.
http://trace.tennessee.edu/utk_gradthes/1186
- Pereg-Gerk L., Paquelin A., Gounon P., Kennedy IR., Elmerich C., "A transcriptional Regulator of the LuxR-UhpA family, FlcA, controls flocculation and wheat Root surface colonization by *Azospirillum brasilense* Sp7." *MPMI*. March, 1998.
- Ramsay, J. P., Williamson, N. R., Spring, D. R. and Salmond, G. P. (2011). [A quorum-sensing molecule acts as a morphogen controlling gas vesicle organelle biogenesis and adaptive flotation in an enterobacterium.](#) *Proc Natl Acad Sci*

S A 108(36): 14932-14937.

Reeve WG, Tiwari RP, Worsley PS, Dilworth MJ, Glenn AR, Howieson JG.

“Constructs

for insertional mutagenesis, transcriptional signal localization and gene
regulation studies in root nodule and other bacteria.