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Characterizing NtrC-like Activators Affecting Myxococcus xanthus Development

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Abstract:

Myxococcus xanthus is a soil bacterium that is a member of a group of organisms known as the myxobacteria. *M. xanthus* cells live in biofilms and feed on other bacteria to obtain nutrients. During times of inadequate nourishment, *M. xanthus* cells aggregate, build fruiting bodies, and fruiting body cells differentiate into spores that are highly resistant. In order to form the fruiting bodies, *M. xanthus* cells must be able to move across solid surfaces. There are two motility systems, the adventurous system (A-motility) and the social system (S-motility), used by *M. xanthus* cells to navigate across surfaces. The adventurous system controls movement of individual cells, while the social system controls the movement of multi-cellular groups. In addition to surface motility, the development of fruiting bodies relies on large-scale changes in gene expression that are coordinated by the production of cell-cell signals. There are five known signals involved in *M. xanthus* fruiting body formation and the two cell-cell signals that have been characterized in the most detail are A-signal and C-signal. A-signal acts very early in development and it functions as diffusible cell density signal. C-signal acts after A-signal and it is important for aggregation and sporulation. C-signal controls the positioning of densely packed cells and requires cell-to-cell contacts to function properly. These signals help *M. xanthus* cells coordinate transcription of developmentally regulated genes. In *M. xanthus*, expression of many developmentally regulated genes is controlled by σ^{54} promoters and NtrC-like activator proteins. Transcription from σ^{54}-promoter elements is dependent upon the transcription factor σ^{54}, which directs RNA polymerase to promoter recognition sites. However, σ^{54}-RNA polymerase is trapped in a closed promoter complex and is, therefore, unable to activate transcription. In order to form an open promoter complex and begin transcription, σ^{54}-RNA polymerase utilizes enhancer binding proteins as activators. These enhancer binding proteins are called NtrC-like activators. NtrC-like activators bind DNA sequences located upstream of σ^{54}-RNA polymerase binding sites and use the energy from ATP hydrolysis to help σ^{54}-RNA polymerase activate transcription. These proteins are important components in the machinery that regulates transcription of *M. xanthus* developmental genes. Fifty three *M. xanthus* genes code for NtrC-like activator proteins and 16 of these genes are known to be important for the developmental process. Five uncharacterized genes have been inactivated to test for defects in motility and development. Mutations in three activator genes caused defects in surface motility and fruiting body development. One gene, MXAN 3656, was further characterized to determine its role in development and place it on the *M. xanthus* developmental pathway.

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Acknowledgments

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Introduction

Myxococcus xanthus is a Gram-negative, rod-shaped soil bacterium and member of the myxobacteria, a group of bacteria characterized by their social lifestyles and highly coordinated multi-cellular developmental cycle. *M. xanthus* cells live in vegetative swarms and are able to develop into large, highly organized communities known as biofilms. Biofilms contain structured groups of cells attached to a surface that undergo large-scale changes in gene expression triggered by environmental signals and coordinated by extracellular signals (O'Toole, *et al.*, 2000). *M. xanthus* cells obtain nutrients by preying on other bacteria, but when amino acid nutrients are scarce, coordinated groups of cells move into nodes, or aggregate, and develop into fruiting bodies. Cells within the fruiting body structure differentiate into metabolically dormant spores that are resistant to stresses such as heat and UV light. Cells outside fruiting bodies often differentiate into peripheral rods, a cell type that distinct from spherical spores the rod-shaped vegetative cells (O'Connor and Zusman, 19901a and 1991b). The development of spore-filled fruiting body structures allows *M. xanthus* cells to endure periods of limited nutrients and environmental conditions that are not optimal for normal survival.

The formation of fruiting bodies is dependent on *M. xanthus* cells' ability to move across solid surfaces. *M. xanthus* moves via gliding motility using two genetically distinct systems. The motility systems are the adventurous motility (A-motility) system, and the social motility (S-motility) system. Each

system controls movement of the cells in a different manner. The adventurous system coordinates the movement of single cells, while the social system controls the movement of multi-cellular swarms of cells (Spormann, 1999). Therefore, cells that lack A-motility tend to move in groups, those that lack S-motility tend to move individually, and those cells that lack both A-motility and S-motility are non-motile.

Cellular motility is not solely responsible for the formation of fruiting bodies. The development of fruiting bodies also relies on large-scale changes in gene expression that are coordinated by the production of cell-cell signals. There are five known signals involved in *M. xanthus* fruiting body formation, A signal, B signal, C signal, D signal, and E signal (for a review, see Kaiser, 2004). The two cell-cell signals that have been characterized in the most detail are A-signal and C-signal. The function of A-signal is to assess the state of nutrition and it is a mixture of amino acids and peptides (Kuspa *et al*., 1992a and 1992b). A-signal acts first in development, and it functions as an indicator of cell density. The diffusible A-signal has three loci on the genome that are responsible for its production, *asgA*, *asgB*, and *asgC* (Kuspa and Kaiser, 1989). The *asg* genes encode regulatory proteins thought to be important for expression of genes required for A-signal production (Plamann *et al*., 1994; Davis *et al*., 1995; Plamann *et al*., 1995). A-signal is produced as a result of starvation and the action of proteases to initiate the development of fruiting bodies. The signal that acts last in development is C-signal. This signal coordinates aggregation and the formation of spores, or sporulation

(Kim and Kaiser, 1990a and 1990b; Kim and Kaiser, 1991; Li *et al*., 1992). C-signal controls the positioning of densely packed cells and requires motility and cell-to-cell contact to function properly (Dworkin, 1996). While A-signal is a diffusible extracellular signal, C-signal is cell-bound so *M. xanthus* cells must be able to move and come in contact with one another to exchange C-signal. The development of fruiting bodies is dependent upon these signals; they coordinate the expression of developmentally regulated genes.

In *M. xanthus*, expression of many developmentally regulated genes is controlled by σ^{54} promoters (Kroos and Kaiser, 1987; Romeo and Zusman, 1991; Keseler and Kaiser, 1995; Garza *et al*., 1998; Garza *et al*., 2000b; Licking *et al*., 2000; Sun and Shi, 2001a and 2000c). The σ^{54} protein is a unique σ factor in that it can be activated from a distance and it recognizes DNA sequences located 12 and 24 base pairs upstream of the transcription start site. The σ^{54} protein is responsible for directing RNA polymerase to the -12 and -24 promoter regions, and once associated with the promoter, σ^{54}-RNA polymerase forms a closed, inactive complex. An NtrC-like protein must associate with σ^{54}-RNA polymerase in order to activate it (Kustu *et al.*, 1989 and Morett and Segovia, 1993). NtrC-like proteins induce a conformational change in σ^{54}–RNA polymerase, which in turn allows it to form an active, open promoter complex and begin transcription.

In *M. xanthus*, NtrC-like activator (Nla) proteins are used to control expression of developmental genes. Nla proteins are similar to the Nitrogen regulatory protein C (NtrC) found in *E. coli*. NtrC proteins control nitrogen

assimilation genes that act in response to nitrogen limiting conditions (Ow *et al*, 1983), much like the developmental genes in *M. xanthus* that are activated upon starvation. When NtrC is phosphorylated by its histidine kinase sensor partner, it promotes oligomerization and cooperative binding at the enhancer binding sites of the σ^{54} promoter (Wiess *et al*, 1992). NtrCs recognize enhancer-binding sequences that are located 70 to 150 base pairs upstream of the -12 and -24 regions of σ^{54} promoter elements.

Through sequencing the *M. xanthus* genome, 53 genes were found to code for Nla proteins. Forty eight of the 53 *nla* genes have been inactivated and characterized for their affect on development. Sixteen of these 48 genes were shown to be important for the developmental process (Wu and Kaiser, 1997; Gorski and Kaiser, 1998; Guo *et al*., 2000**;** Hager *et al*., 2001; Sun and Shi, 2001a; Caberoy *et al*., 2003; Kirby and Zusman, 2003; Jakobsen *et al*., 2004**;** Jelsbak *et al*., 2005). The five uncharacterized *nla* genes were inactivated via a single homologous recombination event (Figure 1), and the resulting mutant strains were tested for defects in motility and development. Mutations in three activator genes caused defects in surface motility and fruiting body development. One gene, MXAN 3656, was further characterized to determine its role in development and place it on the *M. xanthus* developmental pathway.

Material and Methods

Creating Activator Gene Fragment Plasmids: A 500 base pair (bp) segment of the central domain of the gene to be studied was amplified via PCR and

cloned into the pCR II TOPO Vector (Invitrogen). The vector was then electroporated into electrocompetent *E. coli* cells, recovered in 250 µl of S.O.C. Medium (Invitrogen) and incubated at 37° C with gentle agitation for 1 hour. Aliquots were plated on LB agar plates containing kanamycin (40 µg/ml) and X-Gal (40 µl of 40µg/µl). The plates were incubated at 37° C over night. The white colonies were selected, inoculated in 4 ml of LB Broth containing 40 µg/ml kanamycin, and incubated overnight at 37° C with gentle agitation. The plasmids were recovered following the procedure in the QIAGEN Plasmid MiniPrep Kit. To ensure the presence of the gene fragment, an EcoRI digestion was performed. The plasmid and digestion were confirmed using agarose gel electrophoresis.

Creating Activator Mutants: The plasmids with the desired gene fragments was electroporated into the *M. xanthus* wild-type strain DK1622. After electroporation, the cells were recovered in 3 ml CTTYE broth and incubated overnight with gentle agitation at 32° C. Aliquots in 4 ml of CTT Soft Agar were plated on CTTYE plates supplemented with 40 µg/ml of kanamycin (CTTYE-Kan) and the plates were incubated at 32° C. Colonies were selected and patched on to CTTYE-Kan plates and incubated at 32° C to confirm that electroporants were resistant to kanamycin. The cells that grew were inoculated into 10 ml CTTYE-Kan broth until they reached a density of 100 Klett ($5x10^8$ cells/ml). This was used to make permanent cultures of 1400 µl stock bacteria in 600 µl 50% glycerol.

Creating Motility Mutants with Inactivated MXAN 3656: The plasmid with the MXAN 3656 gene fragment was electroporated into DK 1253 (A+S-) and DK 1217 (A-S+), and electroporated cells were allowed to recover in 3 ml of CTTYE. The cells were diluted, plated on CTTYE-Kan agar plates, and the plates were incubated at 32° C. Colonies were patched onto CTTYE-Kan agar plates and the plates were incubated at 32° C. The resulting cells were inoculated and grown to 100 Klett and used to make permanent cultures.

Swarm Expansion Assays: M. xanthus wild-type (DK1622) and mutant strains were inoculated in CTTYE and CTTYE-Kan broth, respectively, grown to 100 Klett, and concentrated 10-fold in TPM buffer. Aliquots of 3 μl were spotted onto 1.5% and 0.4% CTTYE (wild-type cells) or CTTYE-Kan (activator mutant cells) agar plates and the plates were incubated at 32° C. The diameters of the mutant swarms were measured over 4 days and compared to DK 1622 swarm diameters

Sporulation Assay: M. xanthus wild-type and activator mutant cells were inoculated into CTTYE and CTTYE-Kan broth, respectively, grown to 100 Klett, and concentrated 10-fold in TPM buffer. Aliquots of 20 μl were spotted onto TPM agar plates and incubated at 32° C for 5 days. The cells were scraped into 500 μl of TPM buffer, sonicated at setting 4 in 10 second bursts, and placed in a 50° C heat block for 2 hours. Dilutions were plated on CTTYE or CTTYE-Kan agar plates and the number of spores that yielded

colonies were counted. Mutant spore counts were then compared to wild-type spore counts.

Glycerol Sporulation Assay: Wild-type and MXAN 3656 mutant cells were inoculated into CTTYE and CTTYE-Kan broth, respectively, and grown to a density of 100 Klett. Glycerol was added to a concentration of 0.5 M to induce sporulation and the cultures were incubated at 32° C with gentle agitation for 24 hours. Spore numbers were determined as described above.

Creating a Tet^r Plasmid: The Kan^r pCR II TOPO vector containing the MXAN 3656 gene fragment and the oxytetracycline resistant (Tet^r) plasmid pSWU22 were subjected to an EcoRI digestion. The purified MXAN 3656 gene fragment was ligated to EcoRI digested-pSWU22 plasmid using T4 DNA Ligase. One µl of the ligation reaction was electroporated into electrocompetent *E. coli* cells and the cells were allowed to recover in 250 µl S.O.C. Medium. The cells were incubated for 1 hour, aliquots of cells were plated on LB agar plates containing oxytetracyline (12 µg/ml), and the plates were incubated at 37° C overnight. To ensure the presence of the MXAN 3656 gene fragment, colonies were selected for PCR analysis. Plasmids with the appropriate composition were electroporated into Tn*5*l*acZ* reporter fusion strains as described above.

Protein Concentration Assay: Reporter fusion strains with an inactivated MXAN 3656 gene were inoculated in CTTYE broth containing oxytetracyline (12 μg/ml), and grown to a density of 100 Klett. Three sets of 1 ml aliquots were removed, centrifuged, and placed in a -20° C freezer for the zero time point. The remaining cells were concentrated 10-fold in TPM. Twenty μl aliquots were spotted on TPM agar plates. At 2, 6, 12, and 24 hours the cells were scraped into 500 μl of TPM buffer, quick frozen, and quick-frozen cells were transferred to a -20° C freezer. The cells were thawed, placed on ice, and then sonicated at setting 1.5 in three 10-second bursts. One ml of Bradford Protein Reagent (1 part dye, 4 parts water) was added to 20 μl of sample. The optical density of samples at 595 nm was taken using a spectrophotometer.

β-*Galactosidase Assay:* Reporter fusion strains with the inactivated MXAN 3656 gene were inoculated in CTTYE broth containing oxytetracyline (12 μg/ml), and grown to a density of 100 Klett. Three sets of 1 ml aliquots were removed, centrifuged, and placed in a -20° C freezer for the zero time point. The remaining cells were concentrated 10-fold in TPM. Twenty μl aliquots were spotted on TPM agar plates. At 2, 6, 12, and 24 hours the cells were scraped into 500 μl of TPM buffer, quick frozen, and quick-frozen cells were transferred to a -20° C freezer. The cells were thawed, placed on ice, and sonicated at setting 1.5 in 3 10-second bursts. Four hundred μl of O-nitrophenyl-β-D-galactoside (ONPG) was added to 100 μl of the samples.

The samples were put in a 37° C heat block until the samples were sufficiently yellow. Five hundred µl of Na_2CO_3 was added to the samples to terminate the assays and the time was noted. The optical density of samples at 420 nm was taken using a spectrophotometer.

Results

Five hundred-bp PCR products containing the central regions of activator genes were successfully inserted into the pCR II TOPO vector. The plasmids were electroporated into *Myxococcus xanthus* wild-type strain DK 1622. The gene fragments in the plasmids underwent single homologous recombination events with the activator gene loci in the *M. xanthus* chromosome. As a result of these events, truncated copies of the activator genes with Kan-resistant plasmids in between were created (Figure 1). Five *nla* mutants containing activator gene disruptions were created in this manner.

The *nla* mutant strains were tested for defects in gliding motility and development. Since surface-based gliding motility is necessary for development to occur, a swarm expansion assay was performed on each of the 5 activator mutants. Two types of agar were used because A-motility has been found to be favored on firm, dry 1.5% agar surfaces and S-motility favored on soft, moist 0.4% agar surfaces (Shi and Zusman, 1993). The diameters of the activator mutant swarms were measured and compared to wild-type swarm diameters during a four-day period. The MXAN 1757 and MXAN 4346 mutant strains were found not to be defective for gliding

motility, while the MXAN 5565, MXAN 2786, and MXAN 3656 mutants were found to be defective for gliding motility on both types of agar (Table 1). The MXAN 2756 mutant shows a similar defect on both 1.5% agar and 0.4% agar. The MXAN 5565 and MXAN 3656 mutants have greater defects on 0.4% agar than they do on 1.5% agar.

A sporulation assay was next performed to determine whether the activator mutants are defective for fruiting body development. Mutant and wild-type cells underwent development for five days and then they were subjected to sonication and heat. The spores formed by the activator mutants were counted and compared to wild-type spore counts. The results in Table 2 show that the MXAN 3656, MXAN 5565 and MXAN 2756 mutant strains have severe sporulation defects, whereas the MXAN 1757 and MXAN 4346 mutant strains have relatively minor sporulation defects.

Since the MXAN 3656 mutant has strong defects in both motility and development, it was characterized in more detail. To determine if the MXAN 3656 mutant has a specific defect in the A- or S-motility system, the plasmid containing the MXAN 3656 gene fragment was inserted into the chromosome of strain DK1217 (A-S+) and strain DK1253 (A+S-). If the MXAN 3656 mutation affects the S-motility system and it is inserted into an A-motility mutant such as DK1217, it will produce a smooth colony edge. Conversely, if the MXAN 3656 mutation affects the A-motility system and it is introduced into strain DK1253, which is a S-motility mutant, it will produce a smooth colony edge. To inspect the colony edges, 3 μl aliquots of cells were spotted

on 1.5% CTTYE agar and incubated for 4 days. The colony edges of strains DK 1622, DK1217, DK1253, DK2161, DK1217/3656- and DK1253/3656- were examined. *M. xanthus* wild-type strain DK1622 had a rough colony edge (Figure 2A), while strain DK2161, which is an A- and S-motility mutant, had a smooth colony edge (Figure 2.D). Strain DK1217 (Figure 2 B), an A-motility mutant, and strain DK1217/3656- (Figure 2E) had rough colony edges. This finding indicates that S-motility is not greatly affected by the MXAN 3656 mutation. However, strain DK 1253/3656- produced a smooth colony edge (Figure 2F) that resembled the colony edge of strain DK2161, but not that of strain DK1253 (Figure 2 C). Thus, it appears that the MXAN 3656 mutation affects the A-motility system.

In addition to examining the colony edges, a swarm diameter assay was performed on the DK 1217/3656- and DK 1253/3656- strains. Also measured in the assay were strains DK1622, DK2161 (A-S-), and Ω4521 (Table 3), which contains a Kan^r marker. The inclusion of Ω4521 ensured that the addition of kanamycin in CTTYE agar plates did not affect motility. Strains DK2161, DK1217 and DK1253 produce swarm diameters that were about 46%, 67% and 62% of wild type, respectively. Strains DK 1217/3656- and DK 1253/3656- produced swarm diameters that were about 38% and 45% of wild type. Both strains show slower rates of growth than the parent strains that do not have the inactivated MXAN 3656 gene. Strain Ω4521 has a diameter that is 124% of wild type, indicating that the kanamycin does not affect motility.

Since the MXAN 3656 mutant displayed a defect in A-motility, its ability to aggregate was tested. MXAN 3656 and DK1622 cells were plated on TPM plates and the development of fruiting bodies was observed with phase contrast microscopy throughout the developmental period. The MXAN 3656 mutant was found to have a 24-hour aggregation delay (Figure 2). While DK1622 cells began to aggregate at 6 hours and form fruiting bodies at 24 hours, the MXAN 3656 mutant did not form fruiting bodies until 48 hours. The fruiting bodies produced by the MXAN 3656 mutant were smaller and less densely packed than those produced by wild-type cells. The aggregation delay displayed by the MXAN 3656 mutant is most likely due its defect in the A-motility system, since motility is required to aggregate.

M. xanthus cells form fruiting bodies before they sporulate. Therefore, mutations that affect aggregation may indirectly affect sporulation. To determine whether the MXAN 3656 mutation has direct effect on sporulation, a glycerol spore assay was performed. Glycerol causes *M. xanthus* cells to bypass the early events that are required for fruiting body development and activate their sporulation programs directly (Dworkin and Gibson, 1964). In the glycerol spore assay, the MXAN 3656 mutant and wild-type cells were grown to a density of 100 Klett and glycerol was added to the cultures to 0.5 M. The cells were incubated for 24 hours, subjected to sonication and heat, and plated on CTTYE agar plates. The MXAN 3656 mutant yielded no viable spores in this assay. However, MXAN 3656 mutant cells that were not subjected stress produced a sporulation count of about 42% of wild-type,

indicating that the MXAN 3656 mutation only has strong affect on the maturation of spores into stress-resistant cells. The results of assays on fruiting body spores are consistent with this result; the MXAN 3656 mutant produced a spore count of about 78% of wild type and a viable spore count of <0.0002% of wild type (Table 4). These data suggest that the MXAN 3656 mutation has a direct effect on the production of stress-resistant *M. xanthus* spores.

To determine the time at which the MXAN 3656 mutation affects the progress of fruiting body development, the MXAN 3656 gene insertion was introduced into strains carrying early-acting reporter gene fusions. *M. xanthus* development is triggered by starvation and the subsequent production of (p)ppGpp (Singer and Kaiser, 1995). After (p)ppGpp levels rise, the early developmental pathway splits into two branches, one dependent on A-signal and another that is A-signal independent. *spi* is a developmentally regulated gene on the A-signal dependent pathway (Kuspa *et al*., 1986) and *sdeK* has been found to act on the A-signal independent pathway (Garza, *et al*. 1998). The promoters of these genes were fused to the *lacZ* gene to create reporter gene fusions (Kroos *et al*., 1986).

To make the MXAN 3656 insertion in the *sdeK* and *spi* reporter strains, a plasmid with a new selectable marker was created. The pCR II TOPO vector with the MXAN 3656 gene fragment was digested with EcoRI, the fragment was purified and then ligated to the EcoRI digested pSWU22 vector (Figure 4). The resulting plasmid had an oxytetracycline resistance (Tet^r) marker and

contained the MXAN 3656 gene fragment sandwiched between two EcoRI restriction sites. The new plasmid was then electroporated into the *spi* and *sdeK* reporter fusion strains. The plasmid inserted into the reporter fusion strains genomes via a single homologous recombination event (Figure 5). Once the reporter fusion strains with the inactivated MXAN 3656 gene were created, we assayed developmental expression of *sdeK* and *spi* and compared the results to those found in wild-type cells. The results of the assays suggest that the MXAN 3656 mutation does not affect *sdeK* expression (Figure 6) but does affect *spi* expression (Figure 7). As indicated on the graph showing *spi* expression, the MXAN 3656 mutant strain has a specific activity level that is 20% of that found in the wild-type strain. These data suggest that the MXAN 3656 gene product acts on the A-signal dependent pathway (Figure 8).

Discussion

After examining 5 *M. xanthus* activator genes by mutational analysis, I found that 2 activator genes do not play significant roles in *M. xanthus* development and that 3 activators are important developmental regulatory genes. I chose to further characterize the MXAN 3656 mutant as it displayed a strong defect in motility and spore formation. I looked at the affect that the MXAN 3656 mutation has on gliding motility, aggregation, early developmental gene expression and sporulation. I found that the MXAN 3656 mutant displays a 24-hour aggregation delay that is most likely due to its defect in A-motility. The MXAN 3656 mutation also has a direct effect on the production of stress-resistant spores that is independent of its effect on motility and aggregation.

Based on a series of β-galactosidase assays, I discovered that the MXAN 3656 gene product acts on the A-signal dependent pathway, suggesting that MXAN 3656 activator is important for early developmental events.

There are many contributing factors to the regulation of the development of fruiting bodies and the study of the MXAN 3656 mutant demonstrates the complex nature of *M. xanthus* development. This activator mutant displayed a defect in motility and a delay in aggregation, yet these two factors do not appear to contribute to the lack of spore production as indicated by the glycerol-spore assays performed. Since glycerol causes *M. xanthus* cells to undergo differentiation into spores by bypassing the need for motility and aggregation, the MXAN 3656 mutation not only affects motility but directly affects the production of spores as well. This gene that I characterized beyond the primary assays most likely produces an activator protein that regulates multiple genes that are responsible for different aspects of *M. xanthus* development.

As the MXAN 3656 mutant has been shown to cause developmental defects the mutant needs to be further characterized. One test that will be performed is an assay that will determine the activator mutant's ability to produce and respond to A-signal. This assay will show if the MXAN 3656 mutant is able to produce, respond to, or both produce and respond to A-signal. This will also show how the gene acts in the A-signal dependent pathway. One of the last steps in determining how MXAN 3656 acts in development is to identify

target developmental promoters to which MXAN 3656 binds and specific binding sites within these promoters.

The remaining activator mutations that affect development will also be characterized further. The 3 activator mutations that affect development affect not only spore production but also motility. While the motility defect in the MXAN 3656 mutant does not affect spore production, it may in the other two strains, MXAN 5565- and MXAN 2756-. By performing the assays to which the MXAN 3656 mutant was subjected, the affect that motility has on aggregation and spore production can be determined. These two activator genes will also be placed on the *M. xanthus* developmental pathway. Characterization of all the activator genes will provide researchers with a better understanding of the mechanics of *M. xanthus* development and how groups of cells communicate with one another to coordinate development.

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Appendix A

Table 1: Results of the swarm expansion assay.

Strain	1.5%	0.4%
DK 1622	100 +/-2.0	100 +/-2.5
MXAN 3656-	64 +/-0.7°	44 +/-0.2
MXAN 2756-	43 +/-0.6	43 +/-1.6
MXAN 4346-	100 +/-2.5	119 +/-1.5
MXAN 1757-	103 +/-0.8	113 +/-4.7
MXAN 5565-	56 +/- 1.5	36 +/- 1.7

Mutant swarm diameters were determined on day 4 of the assays and they are presented as a percentage of the wild-type swarm diameter.
°Data is taken from day 2.

Table 2: Results of the sporulation assay.

Strain	Viable Spore Count
DK 1622	100 +/-2.0
MXAN 3656-	<0.0002
MXAN 2756-	<0.0002
MXAN 4346-	28 +/-0.1
MXAN 1757-	40 +/-0.6
MXAN 5565-	<0.0007

Sporulation efficiencies are presented as a percentage of wild type.

Table 3: The MXAN 3656- motility phenotype

Strain	% to Wild-Type
DK 1622	100 +/-2.3
DK 2161	46
DK 1217	67
DK 1217/3656-	38 +/-0.2
DK 1253	62
DK 1253/3656-	45 +/-0.1
Ω 4521	124

Mutant swarm diameters are presented as a percentage of the wild-type swarm diameter.

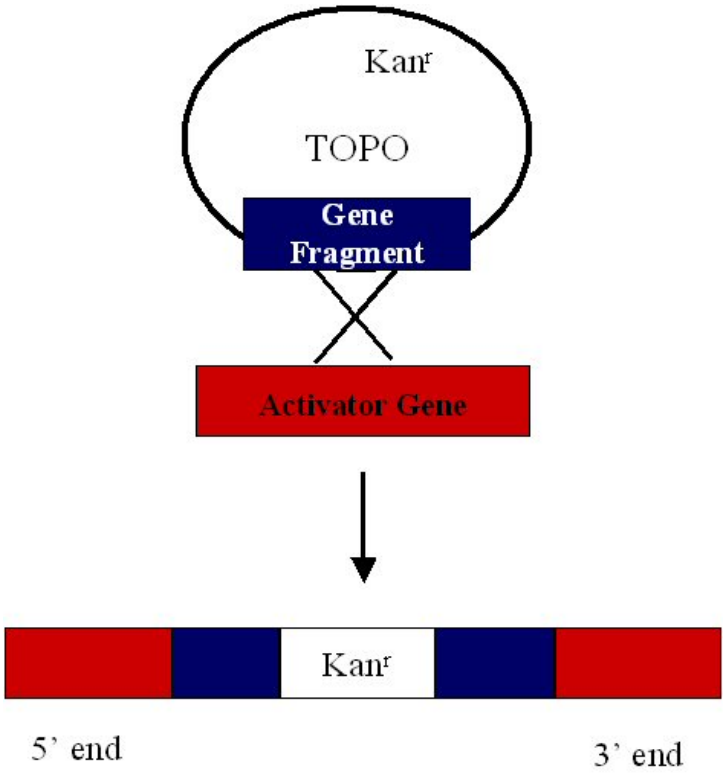
Table 4: The MXAN 3656- glycerol sporulation phenotype

Strain	Spore Count	Viable Spore Count	Glycerol Spore Count	Viable Spore Count
DK 1622	100 +/-9.8	100 +/-9.8	100 +/-5.4	100 +/-11.0
MXAN 3656-	78 +/-6.2	<0.0002	42.36 +/-10.4	<0.0003

Sporulation efficiencies are presented as a percentage of wild type.

Appendix B

Figure 1. Insertion of the kanamycin resistance plasmids containing activator gene fragments occurs via a single homologous recombination event.



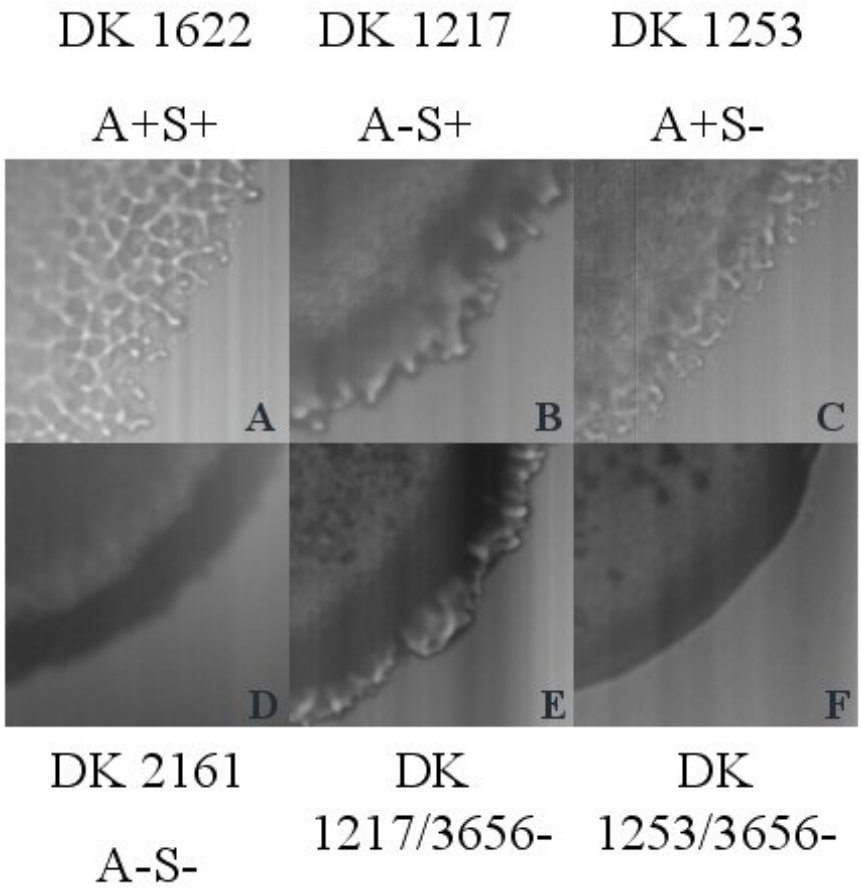


Figure 2. The edges of *M. xanthus* colonies. (A) DK1622 cells are shown to move both as multi-cellular groups and individually. (B) DK1217 cells move only as groups and (C) DK1253 cells move as individuals. (D) DK2161 cells show a smooth colony edge. The MXAN 3656- mutation affects the A motility system as the (E) DK1217/3656- colony edge is similar to that of DK1217, while the (F) DK1253/3656- strain has a smooth colony edge.

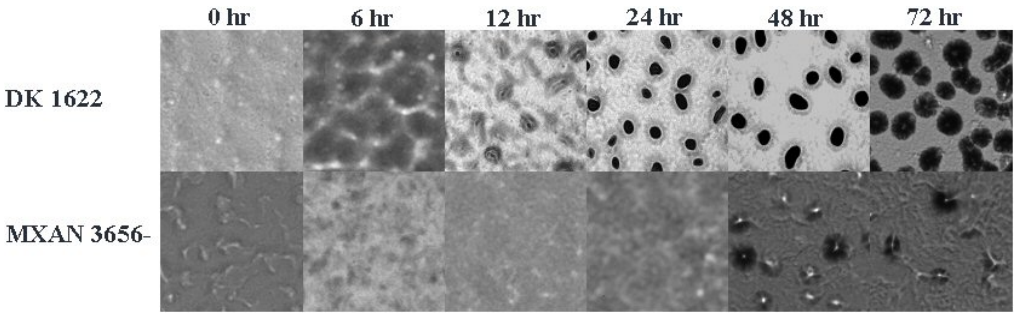


Figure 3. An overhead view of *M. xanthus* fruiting bodies. When compared to DK1622 wild-type, MXAN 3656- has a 24 hour aggregation delay. At 12 hours, DK 1622 has begun to form fruiting bodies and has complete their formation at 24 hours. MXAN 3656- does not form fruiting bodies until 48 hours and these are smaller and more spread out than wild-type fruiting bodies.

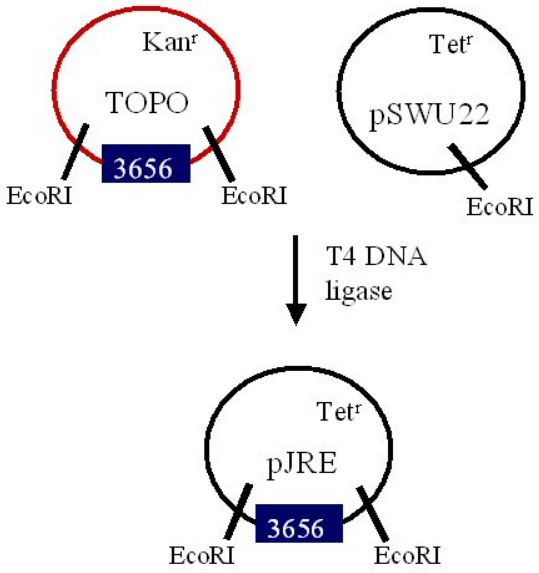


Figure 4. The kanamycin resistance (Kan^r) plasmid containing the MXAN 3656 gene fragment and the oxytetracycline resistance (Tet^r) pSWU22 vector were subjected to EcoRI digestions to release the MXAN 3656 gene fragment and open the pSWU22 vector. The MXAN 3656 gene fragment and pSWU22 vector were ligated together with T4 DNA ligase to create a Tet^r plasmid containing the MXAN 3656 gene fragment.

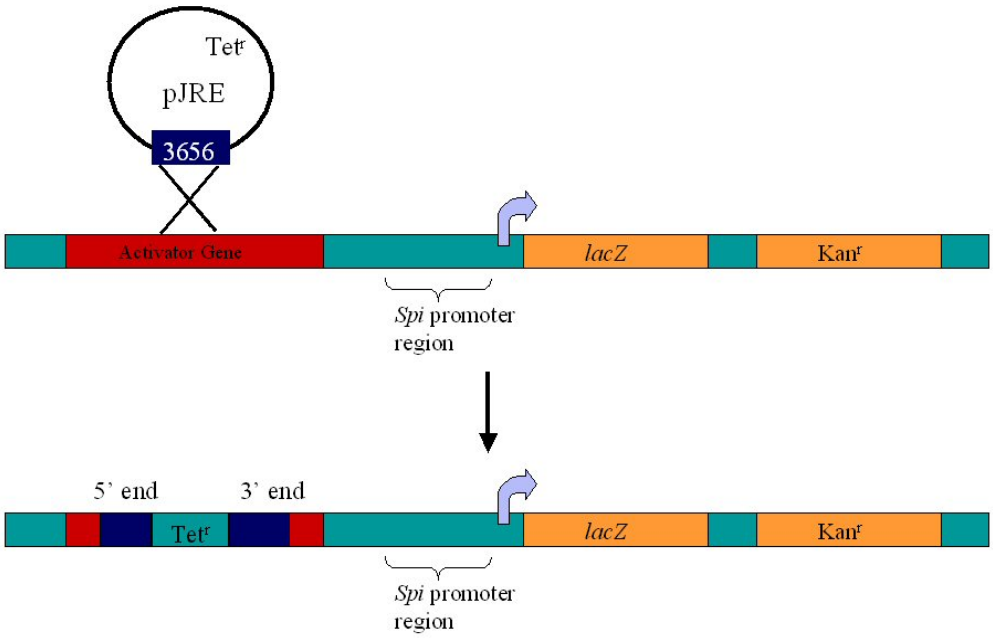


Figure 5. The insertion of the oxytetracycline resistance plasmid containing the MXAN 3656 gene fragment into the chromosome of a Tn5*lacz* reporter fusion strain occurs by a single homologous recombination event.

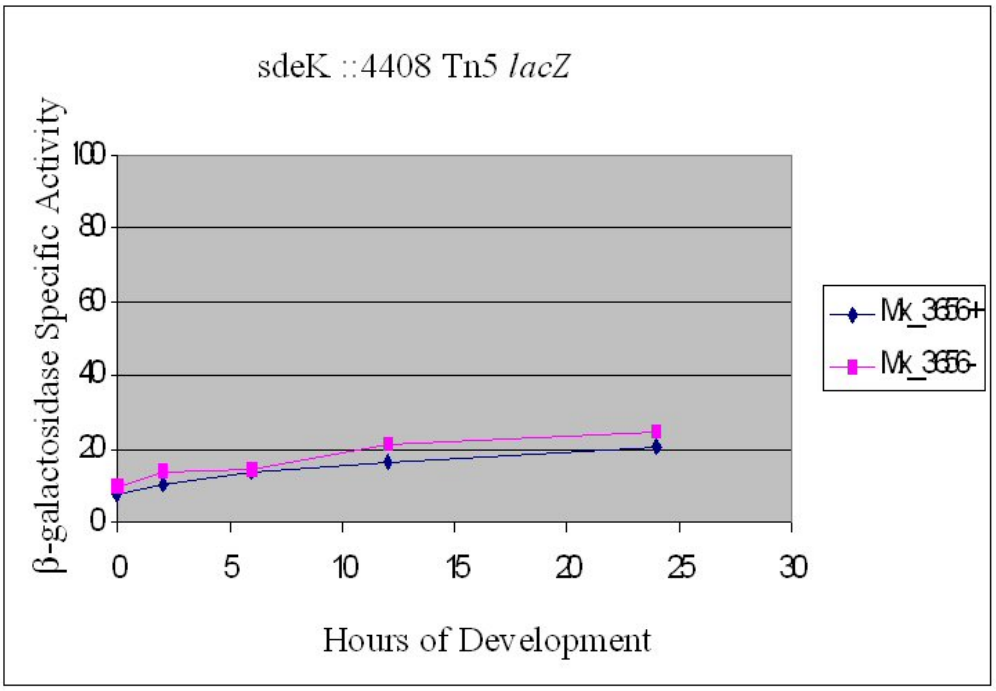


Figure 6. The plasmid containing the MXAN 3656 gene fragment was inserted into the chromosome of the *sdeK* reporter fusion strain and the strain was subjected to a β-galactosidase assay. The blue line indicates expression of *sdeK* in a wild-type strain, while the pink line shows *sdeK* expression in the MXAN 3656 mutant strain.

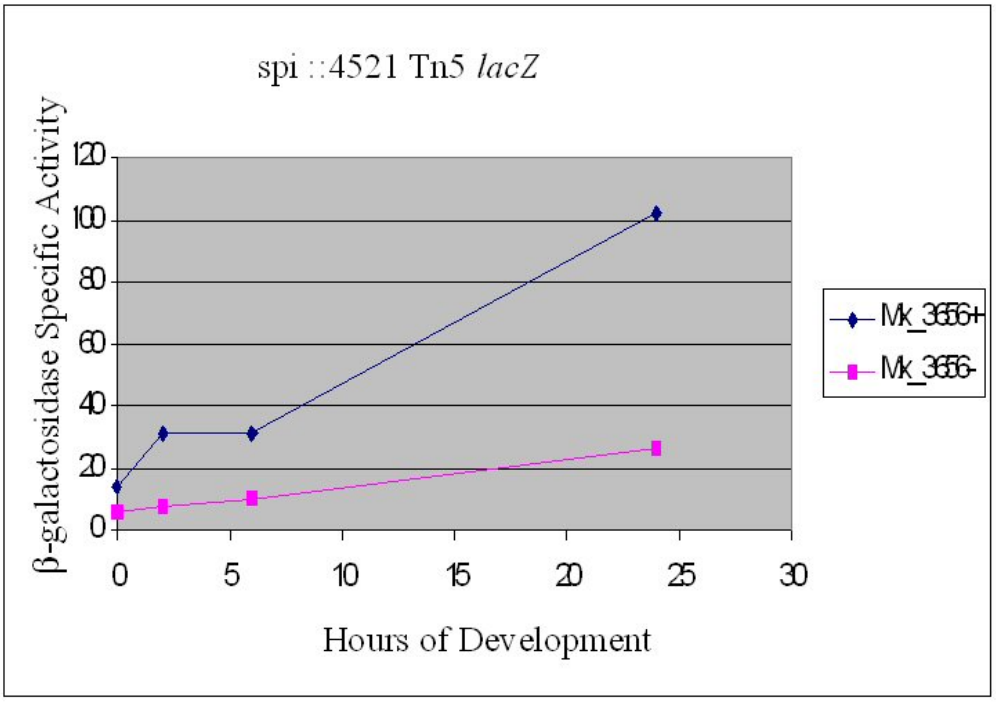


Figure 7. The plasmid containing the MXAN 3656 gene fragment was inserted into the chromosome of the *spi* reporter fusion strain and the strain was subjected to a β-galactosidase assay. The blue line indicates expression of *spi* in a wild-type strain, while the pink line shows *spi* expression in the MXAN 3656 mutant strain.

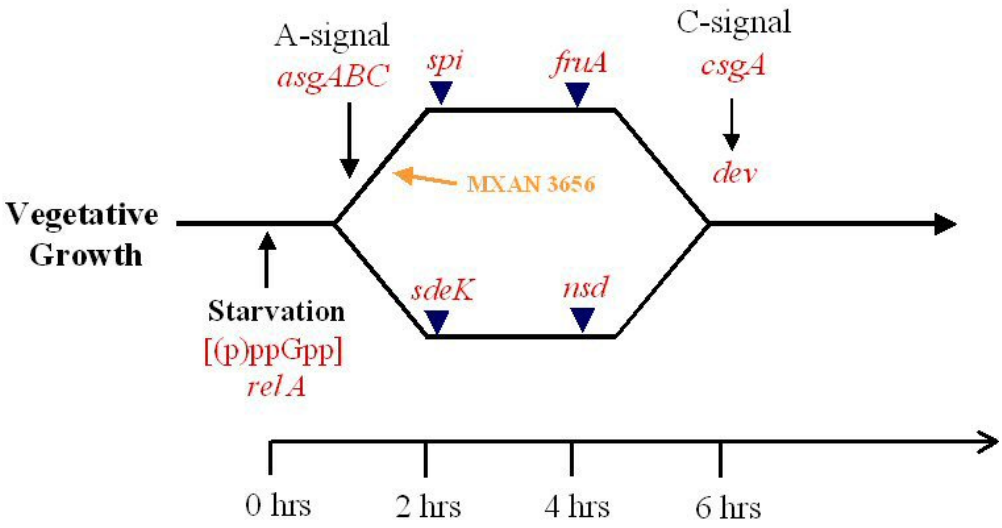


Figure 8. The *M. xanthus* early developmental pathway is triggered by starvation. It then branches into an A-signal dependent pathway and an A-signal independent pathway. The two branches come together around the time of *dev* expression. MXAN 3656 has been determined to act on the A-signal dependent pathway.

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