Characterization of Genes Involved in *Myxococcus xanthus* Fruiting Body Formation to Understand the Formation of Biofilms

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Biofilms are highly organized communities of microbial cells attached to solid surfaces. Bacterial biofilms have been linked to numerous health problems in humans and are not treatable with typical antibiotic therapies. Therefore, scientists need different treatment options. In order to combat biofilms, researchers must understand the genetic and molecular basis of their formation. This can be achieved by identifying and characterizing genes that are believed to be involved in biofilm formation. My research addresses this need by characterizing new genes required for *Myxococcus xanthus* fruiting body development, a well-studied model system for the formation of single-species biofilms.

When deprived of nutrients, *Myxococcus xanthus* initiates a developmental program allowing groups of cells to migrate to aggregation centers and begin building multicellular fruiting bodies. Once a fruiting body is molded into its final shape, individual cells within this structure become dormant spores that are resistant to environmental stresses. Fruiting body development requires that cells be motile. Therefore, I focused on putative motility genes. Mutants containing plasmid insertions in these genes were monitored for fruiting body formation using phase-contrast microscopy and sporulation by comparing their sporulation efficiency to that of wild-type cells. Also, each mutant was tested for defects in motility using swarm expansion assays.

A majority of the mutations affected motility and fruiting body formation. Results from these studies can be expanded to identify genes involved in biofilm formation in bacterial pathogens. The practical application of this information is the potential discovery of new targets for biofilm disruption and other treatment possibilities.

Introduction

Biofilms are large communities of cells that attach to and grow on solid surfaces. They form on a variety of biotic and abiotic surfaces. Some common surfaces in which bacterial biofilms may be found include contact lenses, teeth, countertops, and medical equipment such as catheters. Bacterial biofilms have been linked to several health problems (Costerton et al., 1999) and are increasingly being implicated in chronic infections. Human biofilm infections include dental cavities, gum disease, childhood ear infections, and some infections of the heart. Lung infections occurring in people with cystic fibrosis are due to biofilms of *Pseudomonas aeruginosa*.

Health problems derived from bacterial biofilms are difficult to destroy using traditional antibiotic therapy (Mah, Pitts and Pellock). This has stimulated a lot of interest in developing new therapeutic agents to disperse pathogenic biofilms. One of the best ways to eliminate pathogenic biofilms is to gain an understanding of the genetic and molecular components of their formation. There is a need to understand more about the genes/proteins that are required to form and sustain a biofilm.

Pathogenic biofilms are often too dangerous to study in the lab; therefore we use nonpathogenic bacteria such as *Myxococcus xanthus* as models to study biofilms. When placed under starvation conditions, *Myxococcus xanthus* initiates a developmental program allowing large groups of cells to migrate to aggregation centers and begin building multicellular structures called fruiting bodies. Once a fruiting body is molded into its final shape, individual rod-shaped cells within this structure differentiate into dormant, spherical-shaped spores that are resistant to environmental stresses (for reviews, see Shimkets, 1990 and Dworkin, 1996). Striking similarities exist between *M. xanthus* fruiting body formation and biofilm formation in *P. aeruginosa* (O'Toole et al., 2000). Also, many types of genes/proteins important for biofilm are also important for fruiting body formation.

Not all genes required for fruiting body formation have been identified or characterized. Using the sequenced genome of *M. xanthus*, researchers have identified several genes that are thought to possibly play a role in fruiting body formation. My research addresses the need to find new genes involved in fruiting body formation by characterizing a subset of these identified genes. The results presented here are focused on the subset of genes that are putative motility genes, since motility is known to be required for fruiting body development. Mutations had previously been generated in these putative motility genes. In this project, the resulting mutants were characterized. Each mutant was assayed for normal or defective swarm

expansion (cell motility), aggregation (ability to form fruiting bodies), and sporulation efficiency as compared to *M. xanthus* DK1622 wild-type.

The goal of my research was to examine whether mutations in the putative motility genes cause a defect in *M. xanthus* motility and/or fruiting body development. I hypothesize that mutations in these putative motility genes will have an effect on fruiting body development.

Results from our *M. xanthus* studies on fruiting body formation can be expanded to identify additional genes involved in *P. aeruginosa* biofilm formation as well as genes involved in biofilm formation in other bacterial pathogens. The broader impact of this research is a better understanding of the genetics behind the formation of pathogenic biofilms. The practical application of this information is the potential discovery of new targets for biofilm disruption, and thus, new possibilities for treatments.

Experiment

Monitoring Fruiting Body Formation

Note: *All glassware, micropipette tips, and microfuge tubes used in the experiments were sterilized by autoclaving at 121℃ for 30 minutes. All media used was sterilized by autoclaving at 121℃ for 40 minutes.*

Materials

- ❒ CTTYE (nutrient rich) broth (1.0% Casitone, 0.5% yeast extract, 10.0 mM Tris-HCl [pH 8.0], 1.0 mM KH_2PO_4, 8.0 mM $MgSO_4$)
- ❒ TPM (starvation) buffer (10.0 mM Tris-HCl [pH 7.6], 1.0 mM KH_2PO_4, 8.0 mM $MgSO_4$)
- ❒ TPM agar plates (10.0 mM Tris-HCl [pH 7.6], 1.0 mM KH_2PO_4, 8.0 mM $MgSO_4$, 1.5% Difco Bacto Agar)
- ❒ Kanamycin, 40 mg/ml
- ❒ Wild-type *Myxococcus xanthus* cultures (DK1622)
- ❒ Mutant *Myxococcus xanthus* cultures (MXAN_0752, MXAN_0818, MXAN_1658, MXAN_1948, MXAN_2000, MXAN_2658, MXAN_5179, MXAN_7493)
- ❒ 125 ml flasks
- ❒ Incubated shaker
- ❒ Plate incubator at 32°C
- ❒ Phase-contrast microscope with camera
- ❒ 2-20 µl miropipetter and corresponding tips
- ❒ 20-200 µl micropipetter and corresponding tips
- ❒ 200-1000 µl micropipetter and corresponding tips
- ❒ UV-visible spectrophotometer

- ❒ Spectrophotometer cuvettes
- ❒ Microcentrifuge
- ❒ Microfuge tubes

Procedures

1.) Start liquid cultures of wild-type *M. xanthus* strains by placing cells into flasks containing 10 mL of CTTYE broth and incubate at 32°C for 12 to 24 hours with vigorous agitation.
2.) For mutant strains of *M. xanthus*, repeat step one but supplement the CTTYE broth with 40 µg of kanamycin sulfate per ml.
3.) Transfer 1 ml of culture into a spectrophotometer cuvette. Also transfer 1 ml of CTTYE broth into a separate cuvette.
4.) Adjust the wavelength of the spectrophotometer to 600 nm and zero the spectrophotometer using the cuvette containing CTTYE broth.
5.) Place the cuvette containing the culture into the spectrophotometer and record the absorbance at 600 nm.
6.) If cultures are not dense enough, allow each culture to continue shaking at 32°C. Repeat steps 3-5 until each culture reaches a density of 5 X 10^8 cells/ml.
7.) After each culture reaches the specified density, pellet the cells in 1.6 ml microfuge tubes using a microcentrifuge. Remove the supernatant and resuspend the cells in TPM buffer to a density of 5 X 10^9 cells/ml.
8.) Aliquots (20 µl) of the cell suspension are spotted onto TPM agar plates using a micropipette and incubated at 32°C.
9.) Monitor the progress of the fruiting body development visually by using a phase-contrast microscope with a camera.

Monitoring Sporulation

Note: *All glassware, micropipette tips, and microfuge tubes used in the experiments were sterilized by autoclaving at 121℃ for 30 minutes. All media used was sterilized by autoclaving at 121℃ for 40 minutes.*

Materials

- ❒ CTTYE (nutrient rich) broth (1.0% Casitone, 0.5% yeast extract, 10.0 mM Tris-HCl [pH 8.0], 1.0 mM KH_2PO_4, 8.0 mM $MgSO_4$)
- ❒ TPM (starvation) buffer (10.0 mM Tris-HCl [pH 7.6], 1.0 mM KH_2PO_4, 8.0 mM $MgSO_4$)
- ❒ TPM agar plates (10.0 mM Tris-HCl [pH 7.6], 1.0 mM KH_2PO_4, 8.0 mM $MgSO_4$, 1.5% Difco Bacto Agar)
- ❒ Kanamycin, 40 mg/ml

- ❒ 15 ml conical tubes containing 3 ml CTT soft agar (1.0% Casitone, 10.0 mM Tris-HCl [pH 8.0], 1.0 mM KH_2PO_4, 8.0 mM $MgSO_4$, 0.7% Difco Bacto Agar)
- ❒ Wild-type *Myxococcus xanthus* cultures (DK1622)
- ❒ Mutant *Myxococcus xanthus* cultures (MXAN_0752, MXAN_0818, MXAN_1658, MXAN_1948, MXAN_2000, MXAN_2658, MXAN_5179, MXAN_7493)
- ❒ 125 ml flasks
- ❒ Incubated shaker
- ❒ Plate incubator at 32°C
- ❒ Phase-contrast microscope with camera
- ❒ 2-20 µl miropipetter and corresponding tips
- ❒ 20-200 µl micropipetter and corresponding tips
- ❒ 200-1000 µl micropipetter and corresponding tips
- ❒ UV-visible spectrophotometer
- ❒ Spectrophotometer cuvettes
- ❒ Microcentrifuge
- ❒ Microfuge tubes
- ❒ Vortexer
- ❒ Cell scraper

Procedures

1.) Start liquid cultures of wild-type *M. xanthus* strains by placing cells into flasks containing 10 mL of CTTYE broth and incubate at 32°C for 12 to 24 hours with vigorous agitation.

2.) For mutant strains of *M. xanthus*, repeat step one but supplement the CTTYE broth with 40 µg of kanamycin sulfate per ml.

3.) Transfer 1 ml of culture into a spectrophotometer cuvette. Also transfer 1 ml of CTTYE broth into a separate cuvette.

4.) Adjust the wavelength of the spectrophotometer to 600 nm and zero the spectrophotometer using the cuvette containing CTTYE broth.

5.) Place the cuvette containing the culture into the spectrophotometer and record the absorbance at 600 nm.

6.) If cultures are not dense enough, allow each culture to continue shaking at 32°C. Repeat steps 3-5 until each culture reaches a density of 5×10^8 cells/ml.

7.) After each culture reaches the specified density, pellet the cells in 1.6 ml microfuge tubes using a microcentrifuge. Remove the supernatant and resuspend the cells in TPM buffer to a density of 5×10^9 cells/ml.

8.) Aliquots (15 X 20 µl) of the cell suspension are spotted onto TPM agar plates using a micropipetter and incubated at 32°C for 5 days.

9.) Scrape cells and fruiting bodies with a cell scraper.

10.) Transfer to a microfuge tube containing 500 µl TPM.
11.) Sonicate 3 times at 70%. Make sure to add ice to cup sonicator to keep the water in the cup cold.
12.) Heat the samples at 50°C for 2 hours.
13.) Make serial dilutions from 10^{-2} through 10^{-6} with TPM in microfuge tubes and vortex.
14.) Transfer each dilution to a separate 15 ml conical tube containing 3 ml CTT soft agar and vortex.
15.) Pour each onto a CTTYE agar plate and slosh soft agar around to mix. Let solidify at room temperature.
16.) Incubate at 32°C for 5 days and count colonies. Compare mutant strain numbers to wild-type numbers to determine sporulation efficiency of each mutant.

Motility Assays

Note: *All glassware, micropipette tips, and microfuge tubes used in the experiments were sterilized by autoclaving at 121℃ for 30 minutes. All media used was sterilized by autoclaving at 121℃ for 40 minutes.*

Materials

- ❒ CTTYE (nutrient rich) broth CTTYE (nutrient rich) broth (1.0% Casitone, 0.5% yeast extract, 10.0 mM Tris-HCl [pH 8.0], 1.0 mM KH_2PO_4, 8.0 mM $MgSO_4$)
- ❒ CTTYE plates containing 1.5% or 0.4% agar
- ❒ Kanamycin, 40 mg/ml
- ❒ Wild-type *Myxococcus xanthus* cultures (DK1622)
- ❒ Mutant *Myxococcus xanthus* cultures (MXAN_0752, MXAN_0818, MXAN_1658, MXAN_1948, MXAN_2000, MXAN_2658, MXAN_5179, MXAN_7493)
- ❒ 125 ml flasks
- ❒ Incubated shaker
- ❒ Plate incubator at 32°C
- ❒ Phase-contrast microscope with camera
- ❒ 2-20 µl miropipetter and corresponding tips
- ❒ 20-200 µl micropipetter and corresponding tips
- ❒ 200-1000 µl micropipetter and corresponding tips
- ❒ UV-visible spectrophotometer
- ❒ Spectrophotometer cuvettes
- ❒ Microcentrifuge
- ❒ Microfuge tubes
- ❒ Ruler
- ❒ Calculator or computer with Microsoft Excel™ program

- ❒ Digital camera

Procedures

1.) Start liquid cultures of wild-type *M. xanthus* strains by placing cells into flasks containing 10 mL of CTTYE broth and incubate at 32°C for 12 to 24 hours with vigorous agitation.
2.) For mutant strains of *M. xanthus*, repeat step one but supplement the CTTYE broth with 40 µg of kanamycin sulfate per ml.
3.) Transfer 1 ml of culture into a spectrophotometer cuvette. Also transfer 1 ml of CTTYE broth into a separate cuvette.
4.) Adjust the wavelength of the spectrophotometer to 600 nm and zero the spectrophotometer using the cuvette containing CTTYE broth.
5.) Place the cuvette containing the culture into the spectrophotometer and record the absorbance at 600 nm.
6.) If cultures are not dense enough, allow each culture to continue shaking at 32°C. Repeat steps 3-5 until each culture reaches a density of 5×10^8 cells/ml.
7.) After each culture reaches the specified density, pellet the cells in 1.6 ml microfuge tubes using a microcentrifuge. Remove the supernatant and resuspend the cells in CTTYE broth to a density of 5×10^9 cells/ml.
8.) Spot aliquots (3 µl) of the concentrated cells onto CTTYE plates containing 1.5% or 0.4% agar. Allow spots to dry and incubate plates at 32°C for 3 days.
9.) After 3 days, the diameters of the mutant colonies are measured and compared to the diameters of the wild-type colonies. To be classified as a motility mutant, the mean diameters of the mutant colonies have to be less than 80% of those of wild-type colonies.

Discussion

During *M. xanthus* development, cells undergo an elaborate series of morphological changes that end in the formation of a multicellular, spore-filled fruiting body. What genes does *M. xanthus* use for fruiting body development? It is thought that *M. xanthus* uses a number of genes involved in motility to build fruiting bodies. *M. xanthus* cells must be motile to aggregate into a multicellular fruiting body. In fact, mutants that are defective for motility display a variety of developmental phenotypes (Hartzell and Youderian).

Two motility systems control *M. xanthus* swarming (or gliding) motility on a solid surface, the A and S systems (Hodgkin and Kaiser, 1979a; 1979b). Mutants that are defective for either A-motility (A-S+ cells) or S-motility (A+S- cells) swarm at a reduced rate, while mutants defective for both types of motility (A-S- cells) have a nonswarming phenotype. Two different agar percentages were used in this project because A-motility appears to be favored on relatively

firm and dry surfaces (1.5%), but S-motility seems to be favored on soft and wet surfaces (0.4%) (Shi and Zusman). Hence, a particular motility defect may be more evident with one agar concentration than with the other.

Given that motility plays a role in biofilm formation and fruiting body formation, insertion mutations were made in a number of putative motility genes in *M. xanthus*. Table 1 displays the results from motility, fruiting body development, and sporulation assays for all mutants tested in this study. Of the eight mutants analyzed, five had defects in both motility and fruiting body development. Four of the mutants that failed to develop normally have S-motility defects. These results agree with earlier studies by Hodgkin and Kaiser in 1979 (Hodgkin and Kaiser). They demonstrated that a number of S-motility mutants are also defective for the formation of multicellular fruiting bodies. One of the mutants (MXAN_7493) in this study shows no noticeable defect in motility, but it is defective for fruiting body development. The majority of the mutants did not display a defect in sporulation efficiency, which is not surprising based on previous studies (Caberoy et al., 2003).

A number of genes in *M. xanthus* have not been characterized. In my project, I have characterized eight previously uncharacterized genes. Results from my project support the results from other studies. More importantly they increase the number of genes within *M. xanthus* that have been found to be important for both motility and fruiting body formation. This information could be useful for scientists as they design new therapeutic agents to destroy pathogenic biofilms.

The results of this experiment could have been affected by some uncontrollable events. There is always the threat of contamination from particles in the air, no matter how much you sterilize the utensils or glassware used. To reduce the risk of contamination, I always had a flame going while working at the lab bench. A second factor that could affect the outcome of the experiment is using different lots of media components. Different lots could vary slightly in pH or chemical concentrations. This might cause the bacteria to behave slightly different. Another factor affecting the results is the thickness of the media plates used to grow the bacteria. If plates are poured too thin, they dry out quickly causing the bacteria to not grow well. Also, humans make mistakes when pipetting which could lead to incorrect amounts of solution added at one time.

If this experiment could be repeated, I would simply increase the number of mutants assayed. This would help strengthen my results and conclusions. In the future, the studies presented here could be expanded to characterize more previously uncharacterized genes that are not motility genes, but yet are thought to possibly play a role in fruiting body development. This would allow for the identification of large sets of new genes important for fruiting body

(biofilm) development. This information would apply to the discovery of new genes required for the formation of pathogenic biofilms.

Conclusions

Bacterial biofilms have received a significant amount of attention in recent years because they are linked to a variety of health problems in humans, including many infections that are resistant to traditional antibiotic therapy. Therefore, there is a need to find new therapeutic agents that disperse these biofilms. Designing these agents requires an understanding about the genes/proteins that are required to form and sustain a biofilm and the roles each gene/protein plays in this process. The goal of my research was to address this specific need in biofilm research. The project focuses on previously uncharacterized genes that play a role in *M. xanthus* motility and hence fruiting body (biofilm) formation. My results expand the number of genes known to be involved in both motility and fruiting body formation in *M. xanthus*. These results taken together with others help expand our understanding of the genetic and molecular basis for biofilm formation. This information is relevant to biofilm formation in pathogenic bacteria and hence, could possibly used in helping scientist design new therapeutic agents to destroy bacterial biofilms.

Acknowledgements

I would like to thank the laboratory of Roy Welch at Syracuse University for sending plates containing wild-type and mutant strains of *M. xanthus.* Second, I would like to thank Dr. Kimberly Murphy for supervising and mentoring me throughout my studies. I would also like to thank my mother for her support of me and my project. A big thank you goes out to all those who helped me that are not mentioned here.

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Data

MXAN	S-Motility 0.4% agar	A-Motility 1.5% agar	Fruiting Body Development	Sporulation Efficiency
MXAN_0752	-	+	-	+
MXAN_0818	-	+	-	+
MXAN_1658	+	+	+	+
MXAN_1948	+	+	+	+
MXAN_2000	-	+	-	-
MXAN_2658	-	+	-	+
MXAN_5179	+	-	-	+
MXAN_7493	+	+	-	+

Table 1: Phenotypes of M. xanthus mutants from this study. Each mutant was assayed for normal (+) or defective (-) cell motility, fruiting body development, and sporulation efficiency aggregation as compared to M. xanthus DK1622 wild type.

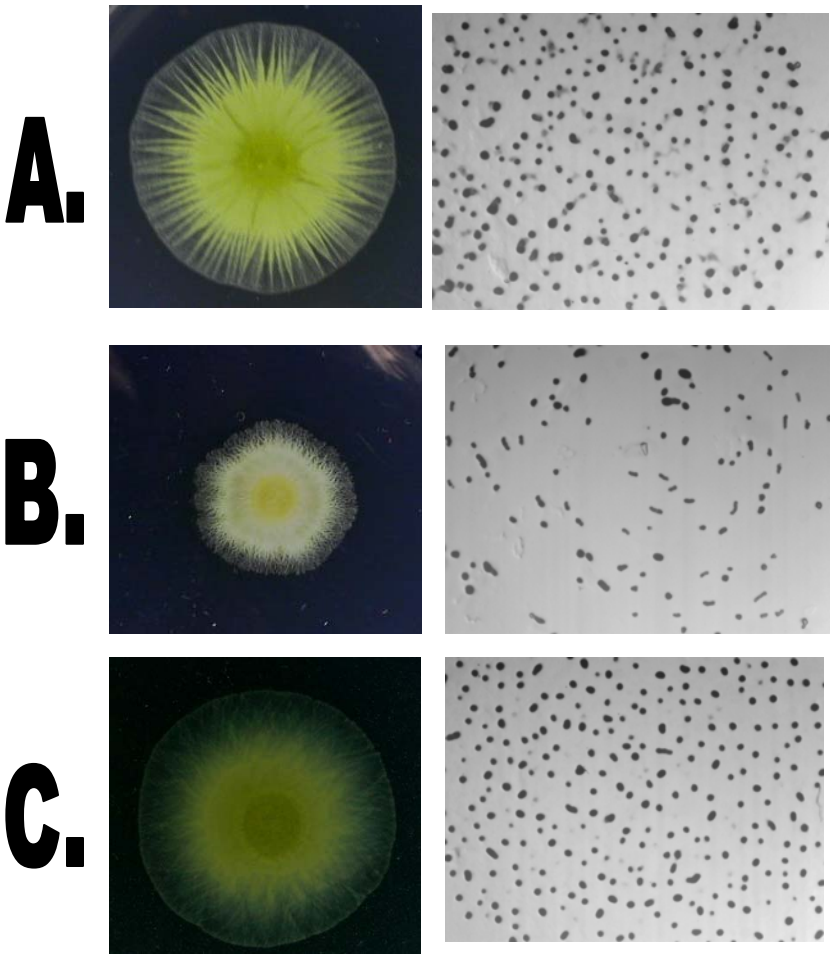

Figure 1:

A. Wild-type motility and fruiting body formation
B. Depicts example of mutants with both motility and fruiting body development defects
C. Depicts example with defect in neither motility nor fruiting body formation