

**INVESTIGATING THE ROLES OF THE *STK* LOCUS IN
DEVELOPMENT, MOTILITY AND EXOPOLYSACCHARIDE
PRODUCTION IN *MYXOCOCCUS XANTHUS***

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fruiting body development

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(ABSTRACT)

Myxococcus xanthus, a Gram-negative bacterium with a developmental cycle, displays a type IV pili (TFP) mediated surface motility known as social (S) gliding. Beside the polarly localized TFP, the fibril or extracellular polysaccharide (EPS) is also required for S-motility to function. It is proposed that S-motility, along with the related bacterial twitching motility in other species, is powered by TFP retraction. EPS is proposed to anchor and trigger such retractions in *M. xanthus*. EPS production is known to be regulated by TFP and the Dif signal transduction pathway. Two genetic screens were performed previously to identify additional genes important for EPS production. The first was for the isolation of *pilA* suppressors, the second for the identification of mutants underproducing EPS in a *difA* suppressor background. Both screens identified transposon insertions at the *stk* locus. In particular, StkA, a DnaK homolog, was identified as a possible negative regulator of EPS production by a *stkA* transposon insertion that suppressed a *pilA* mutation. A *stkB* transposon insertion was found to have diminished EPS production in a *difA* suppressor background.

In this study, in-frame deletion mutants of the five genes at the *stk* locus, *stkY*, *stkZ*, *stkA*, *stkB* and *stkC*, were constructed and examined. In addition, mutations of *rbp* and *bskL*, two genes downstream of the *stk* locus, were constructed. Like transposon

insertions, the *stkA* in-frame deletion resulted in overproduction of EPS. The *stkB* and to a less extent the *stkC* mutants underproduced EPS. Mutations in the other genes had no obvious effects on EPS production. Genetic epistasis suggests that StkA functions downstream of TFP and upstream of the Dif sensory proteins in EPS regulation in *M. xanthus*. Epistasis analysis involving *stkB* was inconclusive. It is unresolved whether StkB plays a role in the biosynthesis or the regulation of EPS production in *M. xanthus*.

ATTRIBUTION

Zhou Li, Qian Xu and Dr. Wes Black were current graduate students in my lab during this research study. They have created some of the strains that were utilized in the experiments found in Chapter 2. Zhou Li created the *stkA*, *stkB* and *difD* in-frame deletion plasmids and strains. He also created the *stkAB* in-frame deletion plasmid. Qian Xu created the strains NafA/*difA* and the *aglU* in-frame deletion. He also created the NafA plasmid. Dr. Wes Black created multiple in-frame deletion strains that were also used in this study. These included *difG*, *difDdifG*, *pilA*, and *difA*.

DEDICATION

I dedicate this dissertation to my families for their love and support over the years. To mom and dad, thank you for convincing me to stay, I am glad to have finished. To Eli, my husband, I would have been lost without you.

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Chapter 1 : Literature Review

Myxococcus xanthus is a Gram-negative, rod shaped bacterium that has two motility systems – Adventurous (A) and Social (S). Social motility requires two extracellular components: the Type IV Pili (TFP) and exopolysaccharides (EPS). In *M. xanthus*, a gene encoding a DnaK homologue is one of the many genes involved in the regulation of EPS production. This literature review examines motility and chemotaxis systems, the functions of DnaK (an Hsp70 homologue), myxobacteria, and the roles of EPS in a variety of organisms.

I. Motility and Chemotaxis

A. Review of Motility

Prokaryotes may move by multiple forms of motility including swimming, swarming, twitching and gliding. Bacteria achieve a motile state through the biosynthesis of motility structures which are species-specific and environmentally regulated. In this section, brief reviews are provided for swimming, swarming, twitching and gliding motility.

i. Swimming and Swarming

Flagella are used by prokaryotes to either swim or swarm. The action of swimming can be seen with individual cells while swarming describes a collection of bacterial cells moving across a surface (30). Swarming bacteria need more flagella than swimming bacteria to move. A possible explanation for this characteristic is that there is a higher surface tension on agar surfaces than in liquid media. Bacteria that swim and swarm either have distinct polar and peritrichous flagella for each form of motility or just peritrichous flagella. As of yet, no single gene disruption in multiple types of flagellated

organisms has caused both flagella to cease functioning (4). Some examples of bacteria that have two distinct types of flagella are *Vibrio parahaemolyticus*, *Vibrio alginolyticus*, *Rhodospirillum centenum* and *Azospirillum* while *Proteus*, *Serratia*, *Salmonella*, *Yersinia*, and *Escherichia* use peritrichous flagella to swim and swarm (30, 31, 57, 58). *Pseudomonas* is an exception with multiple polar flagella that are employed only for swimming (30, 45).

Spirochetes also have flagella but their flagella are actually located in the periplasmic space between the outer membrane sheath and the inner membrane instead of outside of the cell as is the case of most bacteria. The spirochetes' flagella are driven by the proton motive force and the bacteria only move forward when flagella at the two ends of the cell are rotating in opposite directions (4).

a. Assembly of Flagella

In Gram-negative bacteria, flagella are composed of three different components, the basal body, the hook and the filament. The filament is composed of multiple copies of the flagellin protein with a cap at the top. The hook, composed of only one type of protein, connects the basal body to the filament. The basal body, the largest complex, is composed of a rod, three rings, the Mot proteins, the switch complex, and a flagellin-specific export apparatus (Figure 1-1) (4).

Flagellar basal bodies form from the bottom to the top. The MS ring is formed first and then the other basal anchoring proteins are added. After the anchoring proteins have been synthesized, the hook is formed and finally the filament (4). The flagellum is constructed when flagellin monomers flow through the hollow center of the hook and the

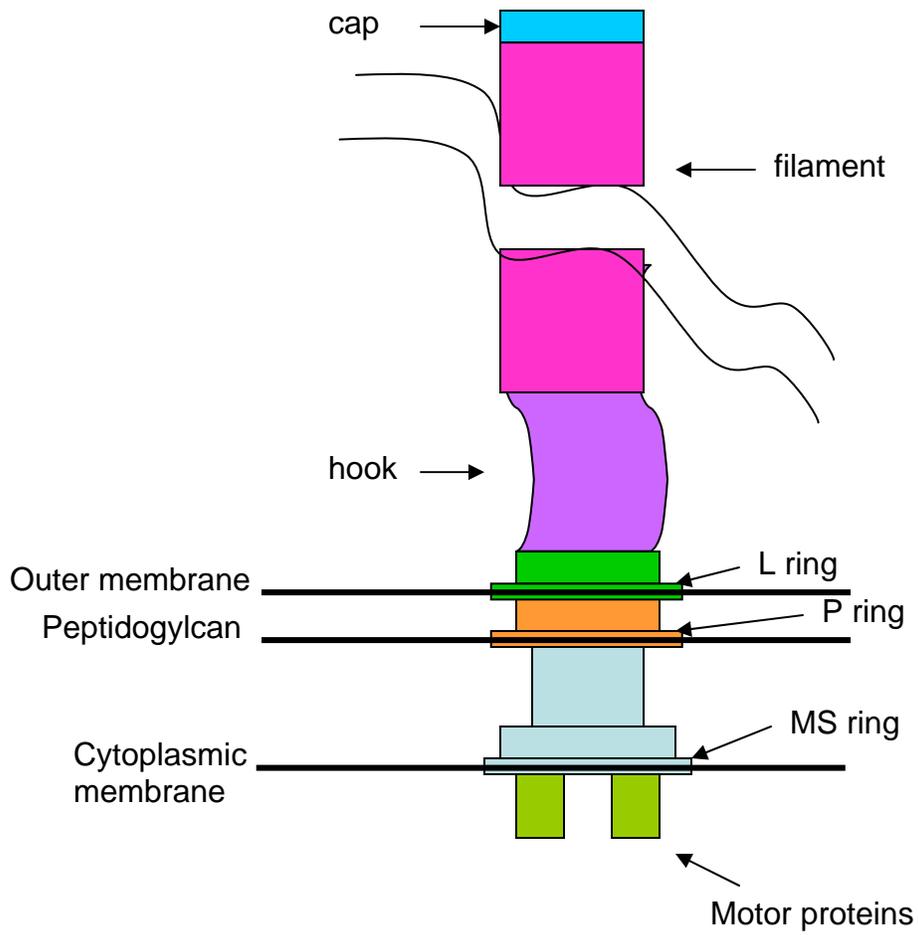


Figure 1-1: Structure of the bacterial flagellum

flagellum to assemble at the top of the filament. The monomers are stopped by the cap so they do not escape into the environment (4).

b. Functions of Flagella Proteins

Each component of the basal body functions to help prokaryotes swim or swarm. The functions of the three rings (MS, L and P) in the basal body are to anchor the flagella to different components of the bacterial cell. The MS ring anchors the flagellum to the cytoplasmic membrane, the P ring anchors to the peptidoglycan and the L ring anchors the flagellum to the outer membrane. There are no P or L rings in the Gram-positive bacteria. The switch proteins, which are controlled by chemotaxis proteins, allow the flagellum to change rotational directions. The Mot proteins form the motor and they power the rotation of the flagella by forming a channel through which protons flow (4).

ii. Gliding and Twitching Motility

Gliding motility may be viewed to encompass two types of surface movement, twitching and single cell gliding. Twitching is described as moving in a jerking manner (54) while gliding motility is defined as movement of a non-flagellated cell across a surface (77). It was originally assumed that all bacteria that exhibited gliding motility utilized the same motility structure. However, as research continued, this assumption has been proven incorrect. There are at least four different motility structures involved in bacterial gliding: Type IV Pili (TFP), the junctional pore complex, the ratchet structure and membrane attachments (55).

The myxobacteria, along with *Pseudomonas*, *Neisseria* and others, can use TFP to move over surfaces (54). The TFP may reach out and attach to a biotic or abiotic surface. The bacteria then retracts the pilus pulling itself towards the original point of attachment

(55). Pilus retraction was visualized in *P. aeruginosa* when TFP were stained with Cy3 fluorescent dye and observed by total internal reflection microscopy (73).

The cyanobacteria tend to migrate towards sources of light using two types of gliding motility structures. Some cyanobacteria such as *Synechocystis* PCC6803 move utilizing TFP in a similar fashion as described above while others move by excretion of slime. The latter may have nozzle-like junctional pore complexes that allow slime to be expelled. The release of slime from these bacteria may push it forward. When switching directions, it is proposed that the slime is extruded from the opposite side of the initial excretion through a different set of nozzles (4, 36, 55).

The third gliding motility structure exhibited by bacteria is associated with the *Cytophaga – Flavobacterium* groups. They have motility proteins that are anchored in the cytoplasm and outer membrane. These proteins are proposed to cause the bacteria to move by a ratchet like motion. The outer membrane proteins interact with the inner membrane proteins in order to move cells forward. The ratchet-like function was demonstrated when a latex bead was added to the outside of *Cytophaga*. This type of movement is driven by the proton motive force (4, 55).

Mycoplasmas also glide (32). Since they lack peptidoglycan, they do not move by the previously mentioned methods. They are proposed to move by attachment organelles that extend from the membrane of the necks of the bacteria and attach to the surface. More research needs to be done to confirm this method of movement (32).

B. Chemotaxis

Although structures such as flagella and TFP are important in the physical act of motility, the direction of cell movement is controlled by chemotaxis pathways.

Chemotaxis is the ability of bacteria to move in a concentration gradient towards higher concentrations of attractants or away from a higher concentration of repellents. The response to attractants is known as positive chemotaxis and that to repellents is known as negative chemotaxis. The best studied and perhaps the simplest type of chemotaxis was found in peritrichous enteric bacteria such as *E. coli* and *Salmonella typhimurium*. (91).

i. Chemotaxis in *E. coli* and *S. typhimurium*

E. coli along with other bacteria swim in a path and then randomly tumble which permits them to swim in a different direction. Chemoeffectors will affect the pattern of movement by causing a bacterium to tumble less in the presence of attractants but more in the presence of repellants (13). Examples of attractants for *E. coli* include aspartic acid, serine, and dipeptides. *E. coli* repellents include but are not limited to leucine and nickel ions (11, 91). *E. coli* and *S. typhimurium* both have similar chemotaxis pathways and move using flagella. There are six to eight peritrichous flagella that form a helical bundle when the bacterium is swimming. When flagella rotate counterclockwise (CCW) in a helix, the bacterium runs (or swims), but when flagella are in a clockwise direction (CW) the bacterium will tumble. Once flagella are in a CW direction, the bacterium can not swim again until flagella become realigned in the CCW direction. The bacterium will continue movement but in a different direction once realignment occurs (6, 53, 91).

Eventually, bacteria become adapted to chemoeffectors and revert back to swimming and tumbling randomly again. Tumbling frequently will no longer be suppressed in the presence of the adapted concentration of attractants or elevated in the presence of the adapted concentration of repellents. This process of chemotaxis in *E. coli*

and *S. typhimurium* is mostly controlled by six chemotaxis genes and a few others encoding chemoreceptors known as methyl accepting chemotaxis proteins (MCPs).

ii. Proteins Involved in Chemotaxis

The six chemotaxis genes are *cheA*, *cheB*, *cheR*, *cheW*, *cheY* and *cheZ*. Their products interact with chemoreceptors or MCPs (Figure 1-2). In *E. coli* and *S. typhimurium*, the signal is transduced from the MCPs to CheW to CheA and to CheY. CheW couples CheA to the MCPs such that the autophosphorylation activity of CheA is responsive to the receptors (34). In the active state, CheA autophosphorylates and transfers the phosphates to two response regulators—CheY and CheB.

Phosphorylated CheY binds to the flagella motor switch protein FliM to induce CW rotation. The CW rotation causes the bacterial cell to tumble. The system will return back to an intermediate CW-CCW motor basis when CheZ dephosphorylates CheY (3, 11, 32, 91). The second response regulator CheB removes methyl groups from MCPs when it becomes phosphorylated. CheR, a methyltransferase, adds methyl groups to MCPs. This process of adding and removing methyl groups is significant in the adaptation to chemoeffectors and allows the cell to return to normal tumbling and swimming behavior (11, 32, 91).

The chemotaxis genes are just one of the many genes required for motility. In *E. coli*, a deletion in *dnaK* causes the cell to become non-motile due to the lack of flagella (70). The DnaK chaperone system is also involved in the heat shock response along with other cellular processes.

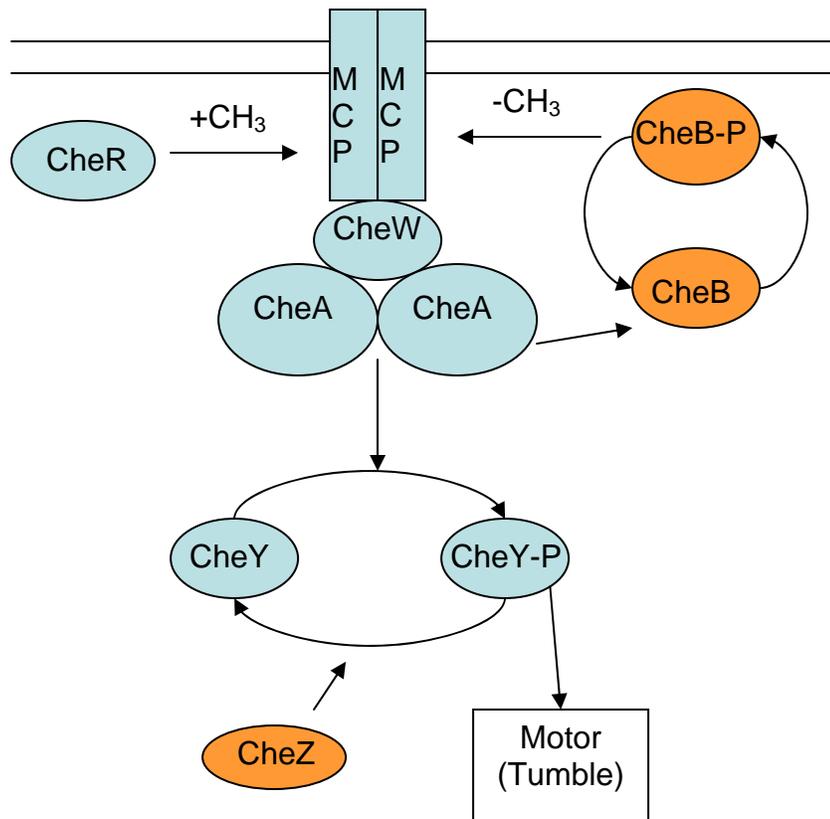


Figure 1-2: Chemotaxis system of *E. coli*

The proteins labeled in blue promote the motor to tumble while those marked in orange suppress tumbling. Figure is based on Rao, *et. al* (64).

II. DnaK

DnaK, the prokaryotic equivalent of the Hsp70 protein in eukaryotes, is a molecular chaperone (33). Molecular chaperones exist for several reasons, including prevention of protein aggregation, refolding of denatured proteins, and maintaining proteins in their unfolded state while being transported to their final destination (52). Not all molecular chaperones are involved in every process, but each chaperone is essential in keeping the cell working properly (82).

The term molecular chaperones describes approximately 25 different families of proteins (33). The molecular chaperone of interest in motility is DnaK. DnaK in many cases is a key component in the heat shock response, but it is also involved in many different functions in various prokaryotes including EPS production, putisolvin production and pathogenesis.

A. Role of DnaK and Other Cofactors in *E. coli*

In *E. coli*, the importance of DnaK in the heat shock response was demonstrated when a deletion of *dnaK* led to a temperature-sensitive phenotype and multiple growth defects as well as the lack of flagella (70). The same phenotype was also observed when *dnaJ* was deleted (52). More phenotypic testing showed three proteins, DnaK, DnaJ and GrpE, compose the DnaK chaperone system which is mainly involved in the heat shock response. The major signals for induction of the heat shock response include elevated temperature, exposure to ethanol, oxidants and the presence of unfolded proteins within the cell along with starvation of nutrients (15, 52).

Trigger factors are proteins that are associated with ribosomes. They were discovered to interact with DnaK when a double deletion of *dnaK* and the gene coding

the trigger factor in *E. coli* was found to be lethal above 30°C (24). The deletion mutant grew at lower temperatures because less protein aggregation occurred. These results led to the conclusion that trigger factors cooperate with DnaK in folding newly synthesized proteins, but neither are essential for the process (24).

The major function of DnaK along with DnaJ is to bind nascent peptides on the ribosome and to prevent premature aggregation or misfolding (14, 15, 52, 74). DnaK is also involved in stress management either directly, by binding to unfolded protein substrates, or indirectly, through regulation of other chaperone proteins (25). Each protein, DnaK, DnaJ, GrpE, and Trigger factors, has a multitude of functions in a cell to allow it to survive adverse conditions (86).

B. Differences in the Role of DnaK in the Heat Shock Response

The function of DnaK in the heat shock response is not as crucial in many other bacteria as in *E. coli*. *Bacillus subtilis*, *dnaK* mutants have a much less severe phenotype than in *E. coli* (52, 68). Shultz *et. al* created insertions upstream of the *dnaK*, *grpE* and *dnaJ* operon and in the *dnaK* gene in *B. subtilis*. When the *dnaK* operon is mutated, *B. subtilis* colonies still formed at temperatures from 16 – 52°C whereas *dnaK* mutants in *E. coli* only grew below 30-35°C. At temperatures above 52°C, *B. subtilis dnaK* mutants stopped growing and cells formed filaments and lost motility (68). These results suggest that although DnaK is a conserved protein, its functions can vary in different organisms.

C. Functions of DnaK in Other Processes

DnaK is involved in cellular functions other than heat shock. These include putisolvin production in *Pseudomonas putida* PCL1445 and pathogenesis in *Salmonella*

enterica Serovar Typhimurium. In *M. xanthus*, two DnaK homologues are involved in EPS production but not in the heat shock response.

i. DnaK is Involved in Putisolvin Biosynthesis in *P. putida*

The DnaK chaperone system in *P. putida* PCL1445 is involved in the heat shock response along with putisolvin biosynthesis (26). *P. putida* PCL1445 produces two surface-active cyclic lipopeptides, putisolvin I and putisolvin II. Putisolvins provide a reduction in the surface tension of the medium, an increase in the formation of an emulsion with toluene, a stimulation of swarming motility, an inhibition of biofilm formation and a degradation of existing biofilms (26, 46). The deletion of *P. putida* *dnaK*, *dnaJ* and *grpE* caused a significant decrease in or complete elimination of the amount of putisolvin I and II being produced. The results suggest that the *dnaK*, *dnaJ* and *grpE* genes are part of a positive regulation system that directly or indirectly stimulates putisolvin production at the transcriptional level (26).

ii. DnaK is Involved in Pathogenic Functions

DnaK and DnaJ are involved in the heat shock response along with pathogenic functions in *S. enterica* Serovar Typhimurium. When *dnaK* and *dnaJ* were interrupted, the mutants could not grow above 39°C, indicating their involvement in the heat shock response. Since pathogenicity could not be studied with the interrupted mutants, a *dnaK* suppressor strain was isolated, which allowed experiments to be performed at temperatures above 39°C. The results suggested that DnaK and DnaJ affect pathogenic functions including invasion of epithelial cells, survival within macrophages and the ability to cause a systemic infection in the host (82). The DnaK/DnaJ chaperone system is also essential for the expression, stabilization, and/or secretion of the invasion proteins.

Motility is also affected by the DnaK/DnaJ chaperone system in *S. enterica* because it is utilized by the flagellar proteins (82). These results led to the conclusion that cellular requirements for the DnaK/DnaJ chaperone machinery to grow at a high temperature may not be identical to the cellular requirements for the machinery in the pathogenesis of *S. enterica* (82).

iii. DnaK is Involved in EPS Production in *M. xanthus*

In *M. xanthus*, the DnaK homologues SglK and Stk have been found to be important in the production of EPS but not in the heat shock response. *sglK* mutants are defective in EPS production, S motility, and development (90, 95). Since *sglK* mutants are not defective in cell division, cell growth, single-cell motility, heat shock response or Mx4 phage infection, *sglK* is probably not involved in general cellular functions. Since *sglK* encodes a homolog of a molecular chaperone, it was proposed that SglK may be involved in the assembly of some apparatus required for *M. xanthus* S motility (95). The *stk* locus was identified by transposon mutagenesis in 1993 and was found likely to regulate EPS production negatively (21).

III. Myxobacteria

The majority of myxobacteria have been found in soil environments such as dung of herbivorous animals, decaying plant material and tree bark, but some have been found in aquatic habitats (23, 37). Myxobacteria are unusual because they possess two life cycles. During periods of accessible nutrients, the bacteria are vegetative cells but form fruiting bodies during periods of starvation. *M. xanthus* is the most studied myxobacteria. While in the vegetative cell life cycle, *M. xanthus* exhibits gliding motility. Gliding motility in *M. xanthus* is possible by two different mechanisms, the

social and the adventurous gliding motility. The major focus of my research has been on the social motility of *M. xanthus*.

A. Life Cycle of *M. xanthus*

M. xanthus is a rod-shaped, Gram negative bacterium that exhibits complex social interactions. Vegetative cells move as a group and expand to new territories when food is abundant. Given periods of starvation, the *M. xanthus* cells can aggregate to form fruiting bodies (40). Once in fruiting bodies the vegetative cells eventually convert to myxospores which can resist environmental stresses better than vegetative cells (27).

B. Motility in *M. xanthus*

M. xanthus moves in swarms because more nutrients can be obtained with less energy. As the group swarms, it secretes enzymes to lyse other bacteria (77). The vegetative cells of *M. xanthus* have two genetically and morphologically distinct systems of surface motility for gliding movements: social (S) and adventurous (A) (35). A-motility describes the movement of individual cells while S-motility describes the movement of cell groups.

i. A-motility

There are two different known subclasses of genes involved in A-motility, *agl* and *cgl*. There have been 32 *agl* (adventurous gliding) and five *cgl* (contact or conditional gliding) mutants identified (77). The *cgl* mutants can be rescued to resume A-motility when they come in contact with wild-type cells but this is not the case for *agl* mutants (77). The mechanism of A-motility is not completely understood. The newest model suggests that there is an internal motor that moves along a helical tract that forces the bacteria cell forward (43, 62).

ii. S-motility

S-motility has been studied more extensively than A-motility. It is known that the polarly localized TFP as well as the peritrichous EPS are critical for S-motility to function. It is believed that the retraction of TFP powers S-motility of *M. xanthus* and twitching motility of other bacteria (1, 9, 72, 93). EPS is believed to mediate the retraction of TFP by providing an anchor and trigger for TFP retraction *M. xanthus* (50).

C. Type IV Pili

Type IV Pili are found in a multitude of bacteria, the most studied of which include *Pseudomonas*, *Neisseria* and *Myxococcus* species. TFP are involved in different functions including surface motility (20, 61), microcolony and biofilm formation (20, 63), adhesion (20), immune evasion (20), cell signaling (20), genetic exchange (20, 92, 99), activation of cells responses (55, 60), cytotoxicity (19, 55) and phage attachment (20, 41).

TFP was initially characterized in *P. aeruginosa*. There are at least 34 proteins in *P. aeruginosa* that are involved in the formation of the pilus (22). These can be divided into four groups: transcriptional regulators *pilS*, *R*, *fimS*, *algR*, and *rpoN*, che-like genes *pilG*, *H*, *I*, *J*, *K*, *L*, *chpA* and *chpB*, TFP biogenesis genes *pilA*, *B*, *C*, *D*, *E*, *F*, *M*, *N*, *O*, *P*, *Q*, *V*, *X*, *Y1*, *Y2*, *Z*, *fimT* and *U*, and pilus function genes *pilT* and *pilU* (22, 80, 88). The structure of a TFP is shown in Figure 1-3.

Most of the genes in *M. xanthus* were named after the *P. aeruginosa* genes and may have similar functions. The exceptions are the *M. xanthus pilG*, *H*, and *I* which encode a transporter possibly involved in protein translocation for pilus biogenesis (4, 55).

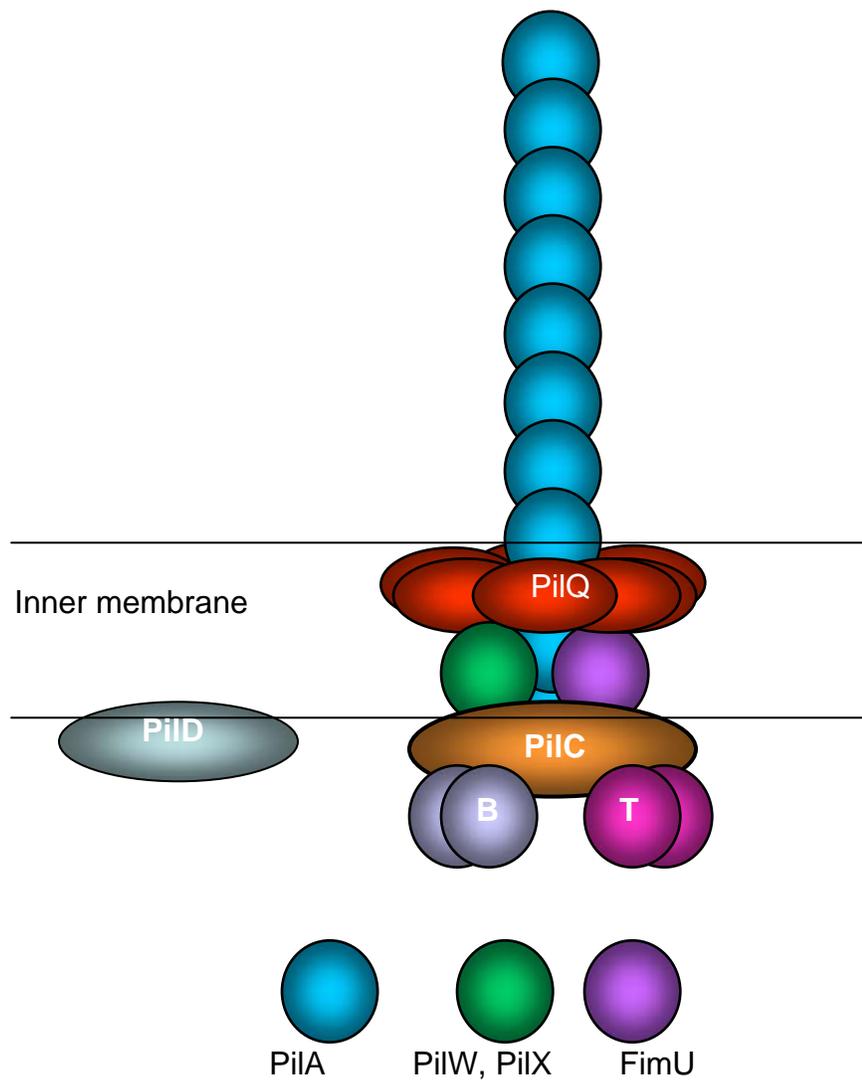


Figure 1-3: Type IV Pili

This figure is based on Mattick (54). The structures labeled B and T stand for PilB and PilT.

D. Exopolysaccharides in Bacteria

The term EPS is used very loosely to describe polysaccharides released by different bacteria or associated with bacterial surfaces. EPS are found to be associated with the cell wall to form a capsule, released as extracellular slime for motility as previously mentioned, or are used to colonize surfaces, for example in biofilm formations. Bacterial EPS is also involved in gene regulation, cell-cell interactions, symbiosis and pathogenesis (49).

i. Functions of Polysaccharide Capsules

In Gram-negative bacteria, capsules are found on the outside of the outer membrane. Capsules may mediate adherence to a surface and provide protection from desiccation. Capsules are also important because they act as virulence factors and protect the bacteria from phagocytosis. Some examples of bacteria that have reduced virulence when decapsulated are *Vibro vulnificus* (75), *Streptococcus* (76), *Staphylococcus aureus* (83), *Actinobacillus pleuropneumoniae* (89), *Haemophilus influenzae* (81), *Klebsiella pneumoniae* (28), *Cryptococcus neoformans* (18), and *Pasterurella multocida* (12). There is still a lot to be learned about capsular polysaccharides because each strain of bacteria has multiple serotypes (or classes) of capsular polysaccharides that could be involved in pathogenesis and various other functions (12).

ii. Exopolysaccharides are Involved in Biofilm Formation

Formation of biofilms is an active area of research due to the ubiquitous nature of biofilms in the environment. The functions of EPS in biofilms have been classified into five groups: structure and architecture, protection, attachment, pathogenesis and symbiosis. Although the first is the least understood function of EPS, there have been

some preliminary studies done in *E. coli* and *V. cholerae* on structure and architecture (79).

The roles of EPS in pathogenesis and attachment to a host surface impact humans the most. The EPS produced by *Staphylococcus epidermis* and *S. aureus* are responsible for agglutination of human erythrocytes and intercellular adhesion of the bacteria during infections (79). *Burkholderia cenocepacia* along with *P. aeruginosa* form biofilms in the lungs of patients with cystic fibrosis or chronic granulomatous disease. EPS produced by *P. aeruginosa* is required for the initial attachment to the lungs and the EPS produced by *B. cenocepacia* is responsible for disrupting the function of neutrophil phagocytosis of the bacteria within biofilms (16).

EPS production by bacteria also affects humans because they are important in plant pathogenesis. EPS negatively affects plants by infection, but it also positively affects some plants via symbiosis. *Erwinia amylovora* causes fire blight in apples and pears. Its EPS negatively affects plants because the EPS is believed to play a role in maintaining hydration and nutrient availability of the bacteria while masking the host response and infiltrating and destroying the plant tissue (79). One well-studied example of symbiosis is the relationship of *Rhizobium* with legumes root nodules. EPS is crucial during the early stages of nodule formation after the rhizobia infect the host plant (78). In summery, biofilm formation mediated by EPS can be both harmful and helpful to host organisms.

E. Chemotaxis in *M. xanthus*

In *M. xanthus* there are two characterized chemotaxis operons *frz* and *dif* that are involved in the regulation of chemotaxis and motility (56, 98). In addition, the Che4

pathway appears to be involved in regulating gliding on solid surfaces when adventurous motility is not functioning. The Che4 pathway has 6 genes, *cheW4A*, *cheR4*, *mcp4*, *cheY4*, *cheW4B* and *cheA4*. Deletion of *cheY4* enhanced, while deletion of *mcp4* diminished vegetative swarming in an A-motility deficient background (85).

The *frz* genes are required for proper control of the frequency of reversal of cell movement as well as wild-type colony swarming and fruiting body formation. FrzA has homology to CheW, FrzE to CheA and CheY, FrzF to CheR, FrzG to CheB and FrzCD to MCPs (10, 56, 59, 69). It is generally accepted that the Frz chemotaxis system plays the most critical role in the regulation of chemotaxis in *M. xanthus*.

F. Regulation of EPS in *M. xanthus*

EPS is essential for S motility in *M. xanthus*. The production of EPS is regulated by a few genetic loci including *pil* (39), *tgl* (21), *stk* (21, 44), *sglK* (90, 95), *eps* and *eas* (51), *nla24* (47), and *dif* (5, 9, 94, 96-98). The *pil* genes are required because pilus likely sense the signal for the production of EPS. The *tgl* gene is required for pilus biogenesis. The *eps* and *eas* are believed to be involved in the biosynthesis of EPS. *nla24* mutants are unusual because they are deficient in both A and S motility. The roles of the *stk* and *sglK* genes are mentioned in other sections.

The *dif* (defective in fruiting) locus encodes both positive and negative regulators of EPS production. DifA, DifC and DifE positively regulate EPS production while DifD and DifG do so negatively (5, 9, 96). Five of the six genes are homologous to bacterial chemotaxis proteins. DifA is homologous to methyl-accepting chemoreceptor proteins (MCPs), DifC to CheW, DifD to CheY, DifE to CheA, and DifG to *Bacillus subtilis*

CheC. DifB shows homology to a conserved family of hypothetical proteins with unknown functions (9).

DifA is believed to receive signals from the TFP which are then transmitted through DifC to DifE to regulate EPS production (94). This was demonstrated to some extent when a NarX-DifA (NafA) chimera was constructed to activate the *M. xanthus* Dif pathway. Transmembrane signaling mechanisms are conserved among bacterial MCPs and sensor kinases (94). NarX is an *E. coli* sensor kinase that detects nitrate or nitrate and passes the signal through a phosphorelay system (48). The NafA chimera was used because nitrate had no obvious effects on growth and development of wild-type *M. xanthus* at concentrations of up to 1 mM. Results with NafA suggest that the N-terminus of DifA mediates signal perception and the C-terminus is sufficient for the interactions with the downstream components of the pathway in EPS regulation (94).

The known components of the Dif pathway are shown in Figure 1-4. As discussed previously mentioned the signal travels from DifA through DifC to DifE (94). DifA, DifC and DifE all positively regulate EPS production. DifD and DifG are both negative regulators of EPS production (8, 9). Yeast two-hybrid studies showed that DifD interacts with DifE (97). Genetic epistatic studies also have shown that the TFP function upstream DifA. DifX symbolizes an unknown component downstream of DifE (8).

IV. Purpose and Specific Aims

It is believed that TFP precieves the signal for the Dif pathway in the regulation of EPS production, but what functions between the TFP and the Dif pathway is unknown. It is also not known what functions downstream of the Dif pathway (Figure 1-4) (8). Two separate genetic screens were performed to identify genes that are involved in the

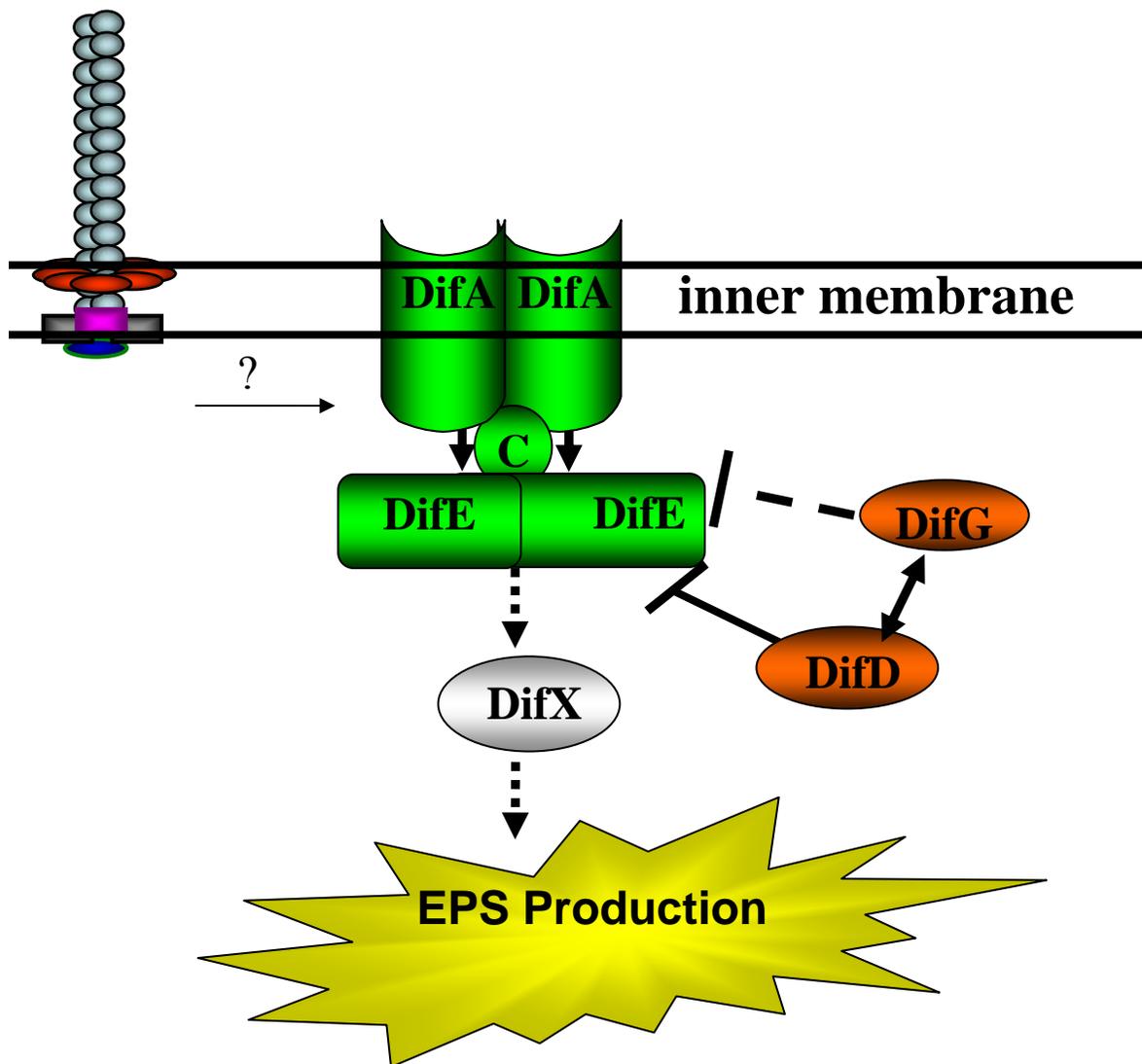


Figure 1-4: The Dif Pathway

TFP function upstream of the Dif pathway in the regulatory pathway of EPS production but it is not known what functions in between TFP and the Dif pathway. DifX is an unknown protein(s) that is located between the known Dif pathway and EPS production. Figure is based on Black (2005) (7).

regulation and production of EPS. The first genetic screen identified suppressors of *pilA* while the second identified *difA* suppressors. In both genetic screens, insertions were identified at the *stk* locus. This study was initiated in order to: (1) characterize the involvement of the *stk* locus in EPS production, motility and development, and (2) determine the functional position of StkA and StkB in the regulatory pathway of EPS production.

Chapter 2 : Investigating the Roles of the *stk* locus in Development, Motility and Exopolysaccharide Production in *Myxococcus xanthus*

This chapter is a draft for a manuscript with Wesley Black, Zhuo Li and Zhaomin Yang as co-authors.

Abstract

Myxococcus xanthus, a Gram-negative bacterium with a developmental cycle, displays a type IV pili (TFP) mediated surface motility known as social (S) gliding. Besides the polarly localized TFP, the fibril or extracellular polysaccharide (EPS) is also required for proper S-motility. It is proposed that S-motility, along with the related bacterial twitching motility in other species, is powered by TFP retraction. EPS is proposed to anchor and trigger such retractions in *M. xanthus*. EPS production is known to be regulated by TFP and the Dif signal transduction pathway. Two genetic screens using transposon mutagenesis were performed to identify additional genes important for EPS production. The first was the isolation of *pilA* suppressors, the other of mutants underproducing EPS in a *difA* suppressor background. Both screens identified transposon insertions at the *stk* locus which contains five genes, *stkY*, *stkZ*, *stkA*, *stkB*, and *stkC*, possibly in one operon. In particular, an insertion in *stkA* suppressed a *pilA* mutation and a *stkB* insertion resulted in reduced EPS production in a *difA* suppressor strain. In-frame deletions of the five *stk* genes were constructed and examined. The results indicated that the *stkA* deletion mutant overproduced EPS, while *stkB* and *stkC* mutants somewhat underproduced EPS. In addition, motility and development were affected by the *stkA* and *stkB* mutants. The *stkY* and *stkZ* mutations had no detectable effects on EPS production, or development, and motility. Genetic epistasis suggests that StkA, a known DnaK homolog, likely functions downstream of TFP and upstream of the Dif sensory proteins in EPS regulation in *M. xanthus*. It is speculated that StkB may play a role in EPS biosynthesis instead of regulation.

Introduction

Myxococcus xanthus is a rod-shaped gram negative bacterium that exhibits complex social interactions. When conditions are conducive for growth, *M. xanthus* cells grow and divide in a vegetative cell cycle but given periods of starvation, cells will aggregate to form fruiting bodies (40). Once in fruiting body formation, vegetative cells eventually convert to myxospores which can better resist environmental stresses than vegetative cells (27). Vegetative cells move on surfaces by their gliding motility which includes a social component that allows cells to move in groups. The moving or swarming groups may contain thousands of cells that feed together by secreting enzymes into the environment that lyse prey cells (65, 77). Myxobacteria, as typified by *M. xanthus*, are known as social bacteria because of their extensive social interactions in both their vegetative and developmental cycles.

M. xanthus is also unusual because not only does it have two life cycles, it also has two different types of surface motility. The two genetically and morphologically distinct systems of surface locomotion in *M. xanthus* are known as adventurous (A) and social (S) gliding motility (35). A-motility describes the movement of individual cells while S-motility describes the movement of cell groups. The polarly localized type IV Pili (TFP) and exopolysaccharides (EPS) are critical for S-motility to function. EPS mediates the retraction of TFP which powers S-motility and twitching, a related bacterial surface motility (1, 9, 50, 72, 93).

Since EPS is crucial for S-motility, the regulation of EPS production has been a topic of intensive investigation. The production of EPS is regulated by a few genetic loci including *pil* (39), *tgl* (21), *stk* (21, 44), *sglK* (90, 95), *eps* and *eas* (51), *nla24* (47), and

dif (5, 9, 94, 96-98). The *pil* genes are required because pilus likely sense the signal for the production of EPS. The *tgl* gene is required for pilus biogenesis. The *eps* and *eas* genes are believed to be involved in the biosynthesis of EPS. *nla24* mutants are unusual because they are deficient in both A and S motility.

In *M. xanthus*, two DnaK homologues, SglK and Stk, which are not regulated by heat shock, have been found to be significant in the production of EPS. Mutations in *sglK* are imilar to those in *difA*, *difC* and *difE* in that they led to defective EPS production, S-motility, and development which is s (5, 9, 95, 98). StkA was identified by transposon mutagenesis in the early 1990s and found to be a negative regulator of EPS production (21). The description of the *stk-1907* transposon insertion mutant stated that the mutant displayed more group movement and less movement of single cells (21, 44).

In this study, insertions at the *stk* locus were identified in two separate genetic screens for mutants overproducing and underproducing EPS, respectively. In-frame deletions were created to examine the roles of the individual genes in the *stk* locus in the regulation of EPS production. The results indicate that StkA is a negative regulator while StkB and StkC are possibly positive regulators of EPS production or biosynthetic enzymes. In addition, genetic epistasis showed that StkA is downstream of TFP but upstream of the Dif pathway. The results from epistatic testing with StkB were inconclusive.

Materials and Methods

Bacterial strains and growth conditions. *Escherichia coli* strain DH5 α was used for plasmid constructions while DH5 α *ypir* was used to sequence the transposon insertion mutants. *E. coli* was grown and maintained on Luria Bertani (LB) agar plates or

in LB liquid media (67). *M. xanthus* strains used in this study are listed in Table 2-1. They were grown and maintained on Casitone Yeast Extract (CYE) (17) and Casitone Tris (CTT) agar plates (39) or in CYE and CTT liquid medium at 32°C on a rotary shaker at 300 rpm (17). Clone-fruited (CF) agar plates were used as the nutrient-limiting medium for the examination of fruiting body development (29). Agar plates for general use contained 1.5% agar while soft agar plates, which were used for the examination of S-motility contained 0.4% agar (71). Whenever necessary, kanamycin and oxytetracycline were supplemented to media at 100 µg/ml and 15 mg/ml, respectively (5, 9).

Identifying, Cloning and Sequencing Transposon Insertion Mutants.

Transposon mutagenesis was performed using the *mariner*-based transposon *magellan4* (66). Approximately 500 ng of pMycoMar (containing *magellan4*) was transformed into YZ101 (*difA cheW7-1*) and DK10407 (*pilA* insertion mutation). The cells were allowed to recover for 4 hours at 32°C and plated on CYE plates with Congo Red and kanamycin. Genomic DNA (~ 1 µg) from the potential mutants was cut with SacII (New England Biolabs) and then allowed to self ligate. 5 µl of the ligation was transformed into *E. coli* DH5α *γpir*. Two primers, MarR1 and/or MarL1 were used to sequence the plasmids recovered from the transformants (100). Genomic DNA from the transposon studies were used to transfer the *magellan4* insertion mutations into YZ101, DK1622 or DK10407 to confirm phenotypic results (7).

Construction of plasmids. Plasmids used in this study are listed in Table 2-2. In-frame deletions were constructed using a two-step overlap PCR procedure (67). Restriction enzymes and T4 DNA ligase for cloning were from New England Biolabs.

Phusion polymerase for the PCR reactions was from Finnzymes. The primers used for PCR amplification for plasmid construction are listed in Table 2-3. The construction of pXQ730, pXQ719 and pWB116 was described elsewhere (94).

PCR products of the in-frame deletions *stkA*, *stkAB* and *stkC* were blunt-end ligated into pBJ113 (38) cut with *SmaI* to create pLZ407, pLZ439 and pAM108 respectively. The PCR product for the *stkB* in-frame deletion was blunt-end ligated into pBJ114 cut with *SmaI* to create pLZ429. The *stkY* in-frame deletion was created by cutting both the PCR fragment and the vector pBJ113 with *EcoRI* and ligated together to create pAM104. The *stkZ* in-frame deletion plasmid pAM106 was constructed by cutting both the PCR product and pBJ113 with *SalI* and *PstI*. The F1 primer had a *PstI* site engineered into it, while the R2 primer used did not have a restriction site engineered into it because a *SalI* site was present 80 bp downstream of where the primer started.

Construction of *M. xanthus* strains. *M. xanthus* strains used in this study are listed in Table 2-1. A two-step screening process was performed, first kanamycin resistant mutants were identified after 5 – 7 days and then the resistant colonies were plated on 1% galactose for another 5 – 7 days. Potential mutants were screened for a galactose “resistant”, kanamycin-sensitive phenotype (84). The mutants were confirmed by triple primer PCR utilizing HotStarTaq from Qiagen. Triple primer PCR requires a primer that hybridizes to the 5' region upstream of the gene, 3' region downstream of the gene and to an internal region of the gene being deleted. The construction of strains YZ101 (*difA* suppressor mutation), YZ604 (*difG*), YZ603 (*difE*), and YZ601 (*difA*) has been previously reported (7, 9).

Table 2-1: *M. xanthus* strains used in this study

<i>M. xanthus</i> strain	Relevant genotype or disruption	Source or reference
DK1622	Wild-type	Kaiser 1979 (39)
DK10407	$\Delta pilA$	Wall and Kaiser 1998 (87)
BY1801	<i>stkA</i> insertion	this study
BY801	<i>pilA</i> <i>stkA</i> insertion	this study
YZ101	$\Delta difA$ suppressor strain	Black 2005 (7)
BY129	<i>stkB</i> insertion in YZ101	this study
BY1129	<i>stkB</i> insertion in wild-type	this study
YZ812	$\Delta stkA$	this study
YZ813	$\Delta stkB$	this study
YZ910	$\Delta stkC$	this study
YZ903	$\Delta stkY$	this study
YZ904	$\Delta stkZ$	this study
YZ905	$\Delta stkAB$	this study
YZ603	$\Delta difE$	Black and Yang 2004 (9)
YZ690	$\Delta pilA$	this study
YZ901	$\Delta pilA$ <i>stkA</i>	this study
YZ601	$\Delta difA$	Xu, 2005 (94)
YZ932	$\Delta difA$ <i>stkA</i>	this study
YZ604	$\Delta difG$	Black and Yang 2004 (9)
YZ801	$\Delta difD$	this study
YZ929	$\Delta difG$ <i>stkB</i>	this study
YZ931	$\Delta difD$ <i>stkB</i>	this study
YZ935	$\Delta difD$ <i>difG</i> <i>stkB</i>	this study
YZ927	$\Delta stkB$ in YZ101	this study
YZ933	$\Delta difA$ <i>stkB</i>	this study
YZ934	<i>difA</i> <i>stkB</i> / <i>Pdif-nafA</i> ; Kan ^r	this study
YZ908	<i>stkB</i> / <i>Pdif-nafA</i> ; Kan ^r	this study
YZ724	<i>difA</i> / <i>Pdif-nafA</i> ; Kan ^r	Xu, 2005 (94)
YZ923	<i>pilA</i> insertion in $\Delta stkA$	this study
YZ916	<i>pilA</i> insertion in $\Delta stkB$	this study
YZ917	<i>pilA</i> insertion in $\Delta stkC$	this study
YZ918	<i>pilA</i> insertion in $\Delta stkY$	this study
YZ919	<i>pilA</i> insertion in $\Delta stkZ$	this study
YZ723	<i>aglU</i> insertion in wild-type	Xu, 2005 (94)
YZ925	<i>aglU</i> insertion in $\Delta stkA$	this study
YZ926	<i>aglU</i> insertion in $\Delta stkB$	this study
YZ920	<i>aglU</i> insertion in $\Delta stkC$	this study
YZ921	<i>aglU</i> insertion in $\Delta stkY$	this study
YZ922	<i>aglU</i> insertion in $\Delta stkZ$	this study

Table 2-2: Plasmids used in this study

Plasmid	Genotype or description	Source or reference
pBJ113	Gene replacement vector with KG cassette, Kan ^r	Julien, 2000 (38)
pBJ114	Gene replacement vector with KG cassette, Kan ^r	Julien, 2000 (38)
pLZ407	<i>stkA</i> inframe deletion in pBJ113	This study
pLZ429	<i>stkB</i> inframe deletion in pBJ114	This study
pAM108	<i>stkC</i> inframe deletion in pBJ113	This study
pAM104	<i>stkY</i> inframe deletion in pBJ113	This study
pAM106	<i>stkZ</i> inframe deletion in pBJ113	This study
pLZ439	<i>stkAB</i> inframe deletion in pBJ113	This study
pXQ730	<i>aglU</i> insertion, tet ^r	Xu, 2005 (94)
pXQ719	<i>Pdif-nafA</i> in pWB200, Kan ^r	Xu, 2005 (94)
pMycoMar	<i>magellan4</i> mutagenesis vector	Rubin, 1999 (66)
pWB119	Δ <i>difD</i> in frame deletion in pBJ113	Xu, 2005 (94)

Table 2-3: Primers used in plasmid construction

Plasmid ^b	Primers ^a	Enzyme ^c
pLZ407	CGGCTGACCCAGCCGGAGGCGTCC	N/A
	TCGCC ATCT GGACTCCTAGCCCCTGTCTATCACGTCGCG CAC AGG	N/A
	AGGGGCT AG GAGTCCAGATGGCGA	N/A
	AGGGCCTTGA ACT CGCTGCGGGGC	N/A
pLZ429	CAGCCGGGTGCTGGCGGAGAAGGG	N/A
	CATGGGGTCTCTCGCTCAGGGCTCCTTCGCCATCTGGACTCCTAG	N/A
	GAGCCCT GAG CGAGAGACCCCATG	N/A
	AGGTGTCCCTGTCGGT CAT CGCCG	N/A
pLZ439	CGGCTGACCCAGCCGGAGGCGTCC	N/A
	CATGGGGTCTCTCGCTCAGGGCTCGTCTATCACGTCGCG CAC AGG	N/A
	GAGCCCT GAG CGAGAGACCCCATG	N/A
	AGGTGTCCCTGTCGGT CAT CGCCG	N/A
pAM108	CTTAAGCCTCACCGAGGAGT	N/A
	TTCG ACT ACAGCGCGCCGAGGTCGTCCTTGATTCC CAT GG	N/A
	CTCGGCGCGCTG TAG TCGAA	N/A
	CCGTCTAGAACGGCCGCAAG	N/A
pAM104	<u>CGGGAATTC</u> CAGGTT CAT GC	<i>EcoRI</i>
	CCGGAT CAG GAACCGGCTTGTTTTTTGACGAC CAC AGCTT	N/A
	AAGCCGGTTC CTG ATCCGG	N/A
	TGCGAATTCACGTCCGGGTG	<i>EcoRI</i>
pAM106	ATC CTG CAGCGCTGGGTGCA	<i>PstI</i>
	CGGTG TCAG TCCACCGGTTCCGCGCCGCAAT CAC AGCA	N/A
	GACCCGGTGG ACTG ACCCG	N/A
	GGGCAGGTCGATGACGACGT	N/A

^aThe sequences of primers are written from 5' to 3'. Restriction sites in primers are underlined. Start and stop codons for genes are in **boldface** when applicable.

^bIn-frame deletions were created by amplifying the upstream and the downstream fragments and then overlap PCR was used to join the two fragments. The primers are listed in the order of Forward 1, Reverse 1, Forward 2 and Reverse 2. The first two primers were to amplify the upstream fragments while the last two primers were to amplify the downstream fragments. The “F2s” are complementary to the 5' of the R1.

^cN/A means Not applicable.

To construct *stkA* deletion mutants, pLZ407 was transformed into DK1622 (wild-type), YZ690 (*pilA*) and YZ601 (*difA*) to create strains YZ812 (*stkA*), YZ901 (*pilA stkA*) and YZ932 (*difA stkA*). The *stkB* deletion mutants were constructed by transformation by electroporation of pLZ429 into DK1622 (wild-type), YZ601 (*difA*), YZ801 (*difD*), and YZ604 (*difG*) to create strains YZ813 (*stkB*), YZ933 (*difA stkB*), YZ931 (*difD stkB*) and YZ929 (*difG stkB*), respectively. Plasmids pAM108, pAM104, and pAM105 were transformed by electroporation into DK1622 (wild-type) to create in-frame deletion mutants YZ910 (*stkC*), YZ902 (*stkY*), and YZ904 (*stkZ*), respectively. Plasmid pWB116 (*difG*) was transformed into YZ931 (*difD stkB*) to create YZ935 (*difD difG stkB*) (42).

Genomic DNA of insertion mutants YZ724 (*aglU*) (94) or DK10407 (*pilA*) (from Dale Kaiser) was transformed into each of the *stk* deletion mutants by selection for oxytetracycline resistance to create double motility mutants YZ923 (*pilA stkA*), YZ916 (*pilA stkB*), YZ917 (*pilA stkC*), YZ918 (*pilA stkY*), YZ919 (*pilA stkZ*), YZ925 (*aglU stkA*), YZ926 (*aglU stkB*), YZ920 (*aglU stkC*), YZ921 (*aglU stkY*) and YZ922 (*aglU stkZ*).

Mutants YZ813 (*stkB*) and YZ933 (*difA stkB*) were transformed with pXQ719 by electroporation to produce *nafA*-carrying strains YZ908 (*stkB/Pdif-nafA*) and YZ934 (*difA stkB/Pdif-nafA*) by selection for kanamycin resistance (94).

Examination of Cellular Cohesion. The agglutination assay described by Wu *et al* (93) was used to determine cellular cohesion of various *M. xanthus* stains. *M. xanthus* cells grown overnight were harvested and resuspended to approximately 2.5×10^8 cells/ml in CYE medium. The optical density (OD) at 600 nm was recorded every 10 minutes for a total of 2 hours. Agglutination is expressed as relative absorbance for each

time point, which was calculated by dividing the OD at each time point by the initial OD of the cell suspension (9, 84).

Examination of EPS Production. EPS production was examined by two different assays, one qualitative and one quantitative. The qualitative one utilized plates with calcofluor white (CW), a fluorescent dye that bound to EPS (9, 21). Cells from overnight cultures were pelleted and resuspended in MOPS (morpholinepropanesulfonic acid) buffer (10 mM MOPS [pH 7.6], 2 mM MgSO₄) at approximately 5×10^9 cells/ml. 5 μ ls of the suspension was spotted onto CYE plates with 50 μ g/ml of calcofluor white (CW) and incubated at 32°C for 6 days. Strains with NafA were observed by plating the cells on CTT plates supplemented with calcofluor white at 50 μ g/ μ l and KNO₃ at 0, 35 or 100 μ M. Fluorescence was detected and documented under the illumination of a handheld long-wavelength (365 nm) UV light source (1, 2, 9, 21).

The second assay incorporated the binding of trypan blue in a liquid assay (9). Cultures were grown overnight in CYE then harvested at approximately 3.5×10^8 cells/ml, washed and resuspended to approximately 2.8×10^8 cells/ml in MOPS buffer with 5 μ g/ml trypan blue. The control samples contained trypan blue in MOPS buffer without cells. The samples were vortexed and incubated with shaking at 300 rpm at 25°C for 30 minutes. The absorbance of the supernatants collected after centrifugation for 10 minutes were measured at 585 nm. EPS production of all strains was normalized to that of the wild-type strain which was arbitrarily set as 1 (21).

Examination of Motility. Motility was examined by spotting 5 μ l of the cell suspension containing 5×10^9 cells/ml onto the center of a standard (1.5% agar) or soft (0.4% agar) CYE plate. Standard agar plates were utilized when evaluating the overall

colony morphology. Colony expansion and colony edge morphology were examined both macroscopically and microscopically after 2 days of incubation at 32°C. Soft agar plates were incubated at 32°C for 5 days before observation and documentation (9, 71).

Examination of Fruiting Body Formation. For the examination of fruiting body formation, cells were grown overnight and resuspended in MOPS at 5×10^9 cells/ml. Five microliters of the suspension was placed onto CF agar plates and development was observed after 5 days of incubation at 32°C using a dissection microscope.

Nucleotide sequence accession numbers. The nucleotide sequences of the genes studied here have been deposited in Genbank under the accession numbers EF524192 (*stkC*), EF524193 (*stkB*), EF524194 (*stkA*), EF524195 (*stkZ*), and EF524196 (*stkY*).

Results

Two transposon insertions at the *stk* locus altered EPS production in *M. xanthus*. To identify genes involved in EPS biogenesis, two genetic screens were carried out using transposon mutagenesis. The first transposon mutagenesis study used the mycomar transposon in a *pilA* insertion background (DK10407) to identify suppressors of *pilA*. The second transposon mutagenesis study used the mycomar transposon in YZ101 (a *difA* suppressor strain) to look for potential underproducers of EPS. The identifications of two mutants from these screens are shown in Figure 2-1. The insertion mutant BY801 (*stkA*) overproduced EPS in a *pilA* insertion mutant. The insertion mutant BY129 (*stkB*) underproduced EPS in a *difA* suppressor strain background. The sites of insertion by the transposon were identified by cloning and sequencing as described in Materials and Methods.

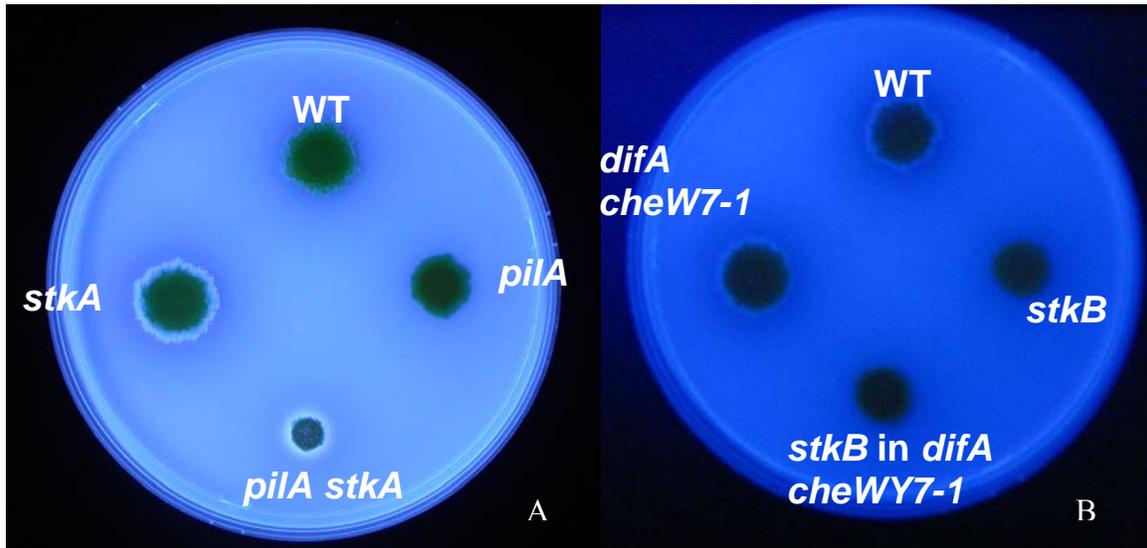


Figure 2-1: Two insertions in the *stk* locus were identified during two separate transposon mutagenesis studies.

Transposon insertions were identified using procedures listed in Materials and Methods. The genetic screen shown in A was done to identify overproducers of EPS in a *pilA* insertion background. The genetic screen shown in B was done to identify underproducers in a *difA* suppressor background. Indicated strains were spotted on CYE containing Calcofluor white.

- A. WT (DK1622), *pilA* (DK10407), *pilA stkA* (BY801), *stkA* (BY1801).
- B. WT (DK1622), *stkB* insertion in *difA* suppressor, (BY129), *stkB* insertion in wild-type (BY1129), *difA* suppressor (YZ101)

The mutant sequences were compared to the *M. xanthus* genome (TIGR) to determine the location of the insertion. The two insertion mutants of interest (BY801 and BY129) both occurred at the *stk* locus (Figure 2-1). BY801 had an insertion in *stkA* between the dinucleotides TA located at 1372 bp, whereas BY129 had an insertion at the TA site at 337 bp of the *stkB* gene. The insertion mutants were confirmed by creating single *stkA* and *stkB* in-frame deletions in wild-type. Then double deletion strains were constructed by transforming the in-frame deletion plasmid back into the initial strains used in the study, YZ901 (Δ *pilA* *stkA*) and YZ927 (Δ *stkB* in the *difA* suppressor strain).

Genes at the *stk* locus. Analysis of the recently sequenced *M. xanthus* genome indicates that the *stk* locus contains are five genes in a potential operon (Figure 2-1), *stkY*, *stkZ*, *stkA*, *stkB* and *stkC*. StkY (592 aa) was listed as a putative lipoprotein although it has the highest identity (26%) with an alpha-2-macroglobulin-like protein found in *Blastopirellula marina* DSM 3645 (Accession number ZP_01093762). *stkZ* encodes a protein of 652 aa with 48% identity to UvrD/rep helicase in *Syntrophus aciditrophicus* (Accession number YP_461520). StkA (559 aa) is listed as a DnaK homologue and has a 43% identity to a DnaK protein found in *Methylococcus capsulatus* str. Bath (Accession number YP_114293). *stkB* encodes a protein of 113 aa with 24% identity to a predicted sterol carrier lipoprotein found in *Thermoplasma volcanium* GSS1 (Accession number NP_110914) and StkC (90 aa) shares homology with unknown proteins found in myxobacteria (Figure 2-2). It is believed that these genes are in an operon because there are only 167 bp between *stkY* and *stkZ*, 36 bp between *stkZ* and *stkA*, 35 bp between *stkA* and *stkB* and 12 bp between *stkB* and *stkC*.

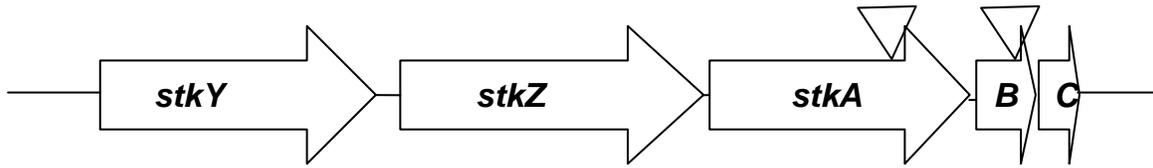


Figure 2-2: Location of genes and sites of transposon insertion in the *stk* locus.

The mycomar transposon inserted between TA at the 1372 bp in *stkA*. The transposon inserted at the TA site in the 337 bp in *stkB*. There are five genes in the *stk* locus and in-frame deletions were constructed for all five genes. The *stkY* mutant contains a deletion of DNA spanning codons 5–587, *stkZ* mutant from codons 5–647, *stkA* mutant from codons 5–554, *stkB* from codons 5–108, *stkC* from codons 5–85.

***stkA* mutant overproduces EPS whereas *stkB* and *stkC* mutants underproduce EPS.** Previously, a *stkA* transposon insertion mutant had been characterized before the genome was sequenced (21, 44). The *stk* locus was re-examined to determine if the previous results were due to polar effects on genes downstream of *stkA*. In-frame deletions of the five genes, *stkA*, *stkB*, *stkC*, *stkY* and *stkZ* were created. The deletion mutants were phenotypically examined for involvement in EPS production, motility and fruiting body formation.

EPS production is critical for cellular cohesion. An agglutination assay was performed for all five mutants to examine their cohesive properties (Figure 2-3). The strains were all re-suspended to approximately the same cell density in agglutination buffer. The OD of the strains was measured for 2 hours. Only *stkB* and *stkC* showed consistent differences in their agglutination rate compared to wild-type, suggesting possible involvement in EPS production.

EPS production was examined more directly using plates containing calcofluor white, a fluorescent dye that binds EPS, and visualized under UV light (Figure 2-4). Both the *stkB* and *stkC* mutants underproduced EPS compared to wild-type. On the other hand, the *stkA* mutant overproduced EPS which agrees with previous findings from the *stkA* transposon insertion studies.

The trypan blue assay, which utilizes a dye that binds EPS, was used to quantify the amounts of EPS present (Figure 2-5). The results confirmed that the *stkA* mutant overproduced EPS while the *stkB* and *stkC* mutants underproduced EPS compared to wild-type. The *stkY* and the *stkZ* mutants produced levels of EPS similar to those in the

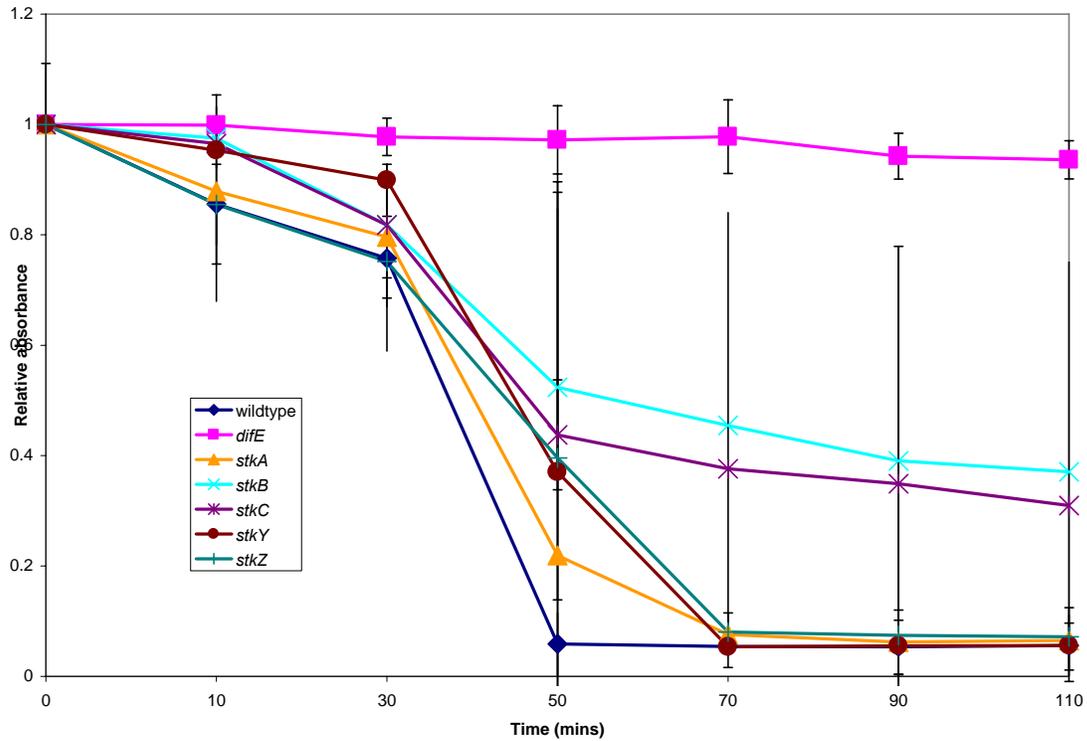


Figure 2-3: The *stkB* and *stkC* mutants agglutinated slower than wild-type.

M. xanthus cells grown overnight were harvested and resuspended to approximately 2.5×10^8 cells/ml in CYE medium. The optical density (OD) at 600 nm was recorded every 20 minutes for a total of 2 hours. Relative absorbance is calculated by dividing the OD at each time point by the OD of the strain at time zero (0). This agglutination assay was performed three different times. The average of the three experiment values was calculated and plotted. The strains listed are WT (DK1622), *stkA* (YZ812), *stkB* (YZ813), *stkC* (YZ910), *stkY* (YZ902), *stkZ* (YZ904).

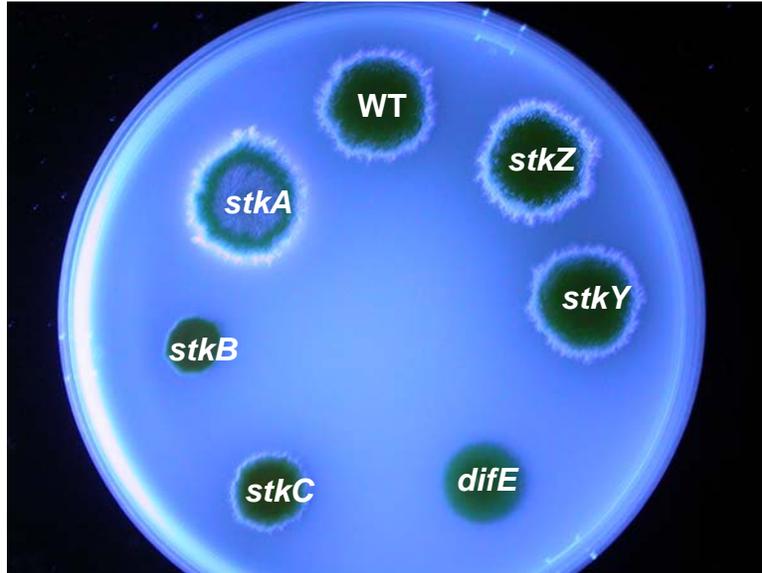


Figure 2-4: The *stkA* mutant overproduces EPS while the *stkB* and *stkC* mutants underproduce EPS.

Experimental procedures for the Calcofluor White assay are found in Materials and Methods. The indicated strains were plated on CYE supplemented with Calcofluor White. The strains listed are WT (DK1622), *stkA* (YZ812), *stkB* (YZ813), *stkC* (YZ910), *stkY* (YZ902), *stkZ* (YZ904).

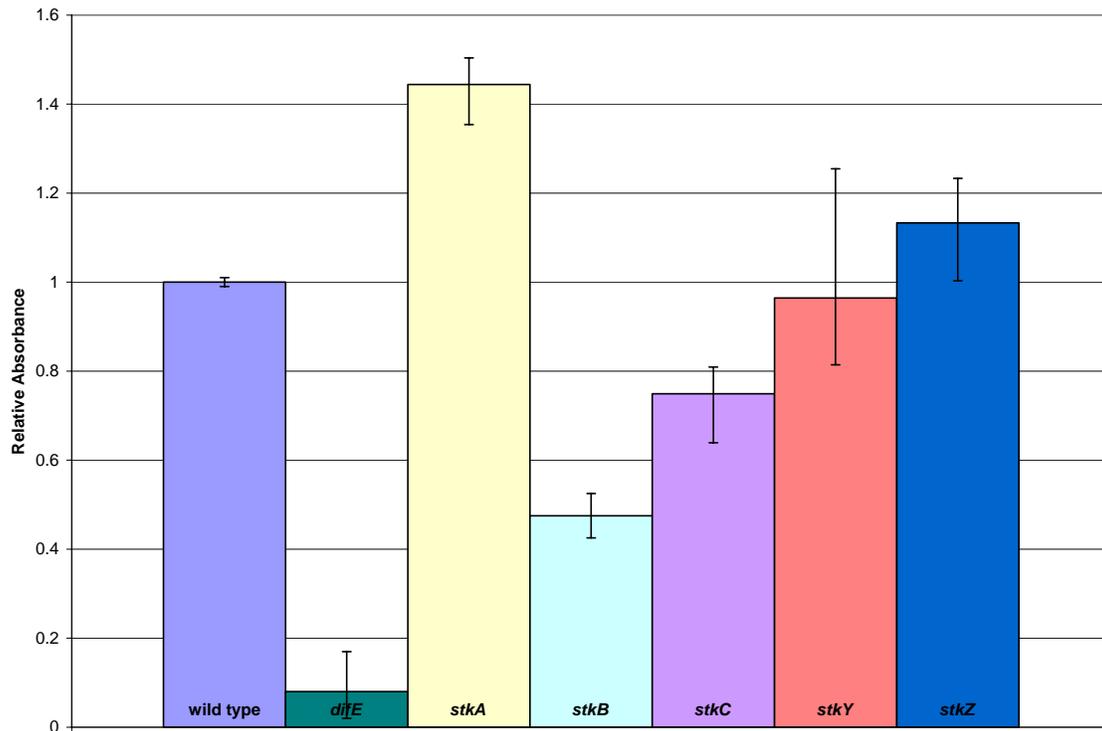


Figure 2-5: The *stkA* mutant overproduced EPS while the *stkB* and *stkC* mutants underproduce EPS.

Cultures were grown overnight in CYE, harvested at approximately 3.5×10^8 cells/ml, washed and resuspended to approximately 2.8×10^8 cells/ml in MOPS buffer. Trypan blue (50 $\mu\text{g/ml}$) was added to the cell suspension and incubated for 30 minutes at 25°C. The absorbance of the supernatant was collected after centrifugation was measured at 585 nm. EPS production of all strains was normalized to that of the wild-type strain which was arbitrarily set as 1.

wild-type in all three assays. A *difE* deletion mutant, which produces no EPS, was used as a negative control.

StkA functions downstream of PilA but upstream of the Dif Proteins. EPS production is regulated in part by the Dif pathway. It is believed that TFP perceives or transduces the signals for EPS production to the Dif pathway downstream (8). To determine the location of StkA in the regulatory pathway of EPS, two in-frame double deletion mutants were created, YZ932 (*difA stkA*) and YZ901 (*pilA stkA*). Both *pilA* and *difA* mutants produce limited amounts of EPS. Epistatic testing of the double mutants was visualized using both Calcofluor white plates (results not shown) and the trypan blue assay. The double mutant *pilA stkA* produced similar levels of EPS as *stkA* but the *difA stkA* mutant produced similar levels of EPS as *difA* (Figure 2-6). In conclusion, StkA functions between the Type IV Pili and the Dif pathway in the regulatory pathway of EPS production.

StkA and StkB are not in the same regulatory pathway. To determine where StkB is positioned in relationship to StkA, basic genetic epistasis was done by creating a *stkAB* double deletion. The EPS production of the deletion mutant was examined quantitatively using the trypan blue assay. As shown in Figure 2-7, the *stkAB* mutant produced less EPS than the *stkA* mutant but more EPS than the *stkB* mutant. Thus, StkA and StkB may not function in the same regulatory pathway of EPS production.

Mutations in *dif* genes failed to suppress *stkB*. Next, attempts were made to determine if there was a clear epistatic relation between the mutations in *stkB* and the *dif* genes. The NafA plasmid was used to examine the position of StkB in relationship to DifA because in the presence of nitrate, the NafA mutants produce EPS. YZ908

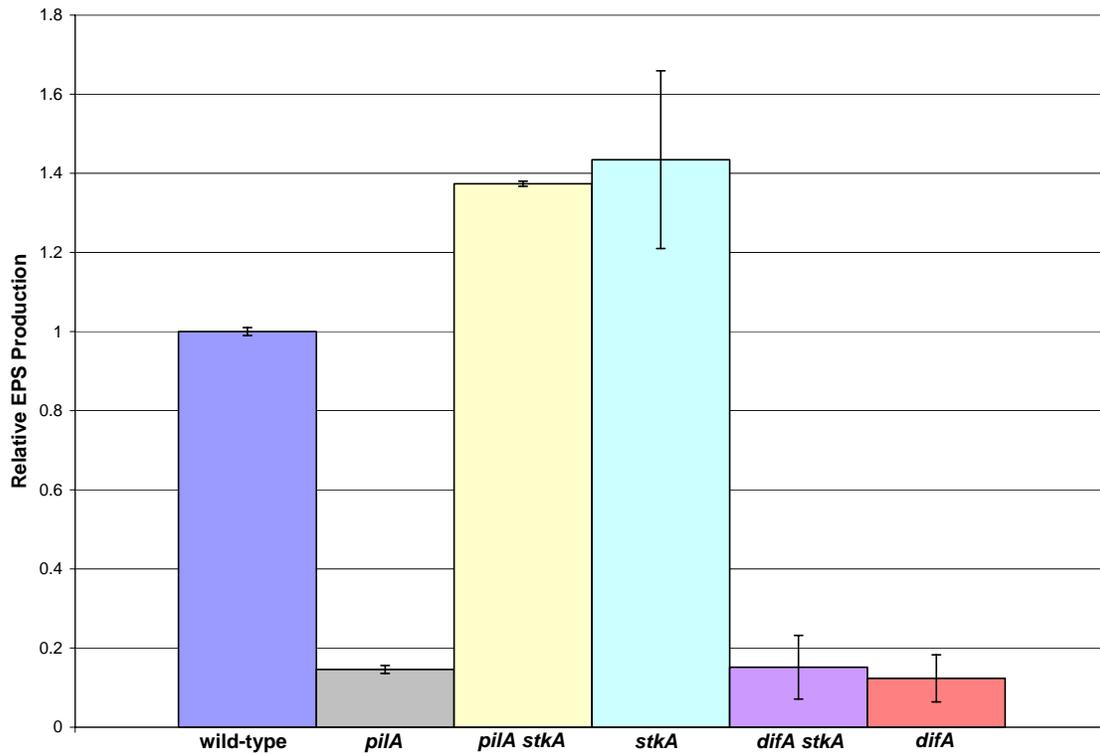


Figure 2-6: StkA functions between the Type IV Pili and the Dif Pathway in the regulatory pathway of EPS production.

The *pilA stkA* deletion mutant produced similar levels of EPS as the *stkA* deletion mutant while the *difA stkA* deletion mutant produced similar levels of EPS as the *difA* deletion mutant. This assay was performed as described for Figure 2-5. The double deletions were created by deleting *stkA* in *difA* and *pilA* deletions strains, respectively. The strains shown are WT (DK1622), $\Delta pilA$ (YZ690), $\Delta pilA stkA$ (YZ901), $\Delta stkA$ (YZ812), $\Delta difA stkA$ (YZ932), $\Delta difA$ (YZ601).

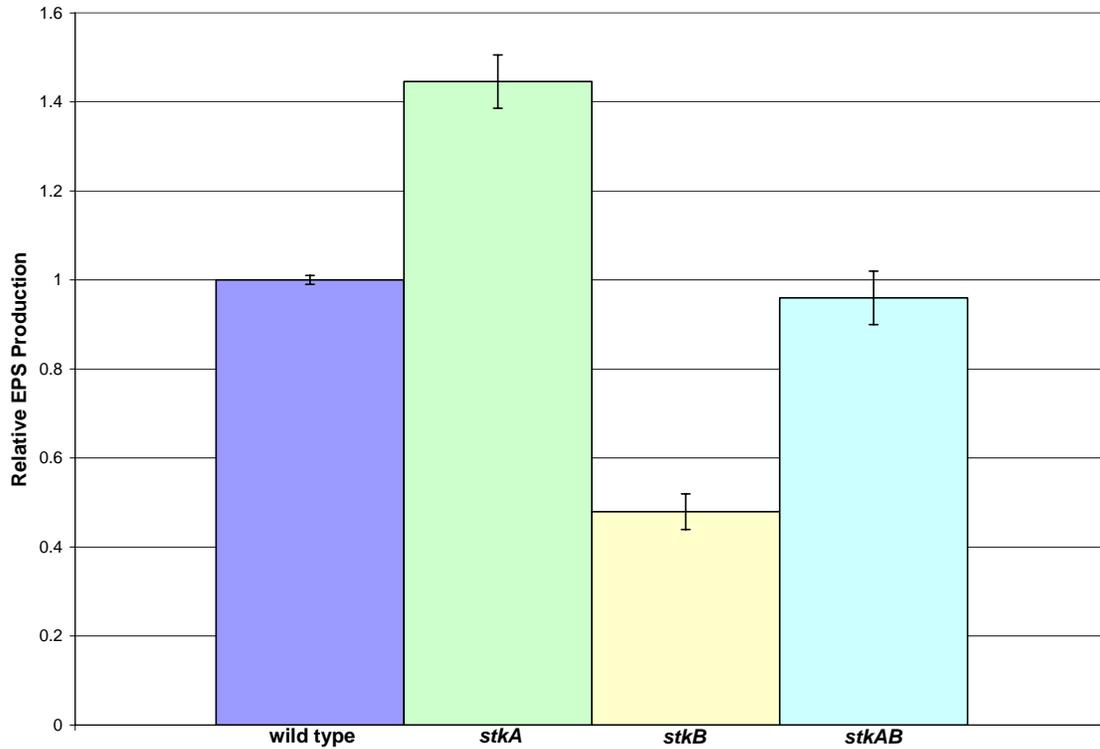


Figure 2-7: StkA and StkB are not in the same regulatory pathway of EPS production.

The *stkA stkB* deletion mutant produced more EPS than the *stkB* deletion mutant but significantly less than the *stkA* deletion mutant. The *stkA stkB* deletion contains a deletion spanning from codons 5 - 698. This assay was performed as described for Figure 2-5. The strains used were wild-type (DK1622), Δ *stkA* (YZ812), Δ *stkB* (YZ9813), Δ *stkAB* (YZ905).

(*stkB/Pdif-nafA*), and YZ934 (*difA stkB/Pdif-nafA*) and YZ724 (*difA/Pdif-nafA*) were examined on calcofluor white plates with 0, 35 and 100 μM KNO_3 added (Figure 2-8). The mutants with NafA produced EPS after nitrate was added, although the *stkB* deletion mutants produced less EPS.

In addition, three deletion strains, YZ929 (*difG stkB*) and YZ935 (*difD difG stkB*) were created. The multiple deletion mutants were examined on calcofluor white plates (results not shown) and by the trypan blue assay. The results showed that the double mutants produced similar or slightly higher levels of EPS as *stkB* (Figure 2-9), but significantly less than the single *difD* and *difG* mutants. In conclusion, StkB is affecting the Dif pathway but the position of StkB in the regulatory pathway of EPS production is still unknown.

The *stkA*, *stkB* and *stkC* mutants are deficient in motility. Since EPS is essential for S-motility and the *stkA*, *stkB* and *stkC* mutants are all altered in the regulation of EPS production, motility of the *stk* mutants were examined on hard agar after 2 days (Figure 2-10) and on soft agar after 5 days (Figure 2-11) as described in Materials and Methods. The *stkA*, *stkB* and *stkC* mutant colonies are smaller than that of the wild-type on hard agar. The *stkA* and *stkB* mutants do not swarm or spread as much as the wild-type on soft agar. The *difE* mutant was used as a negative control because it is deficient in S-motility.

M. xanthus has a unique motility system that encompasses two components, A- and S- motility. To examine the possibility that either component of motility was affected by the *stk* locus, insertions in the *pilA* (important in S-motility) and *aglU*

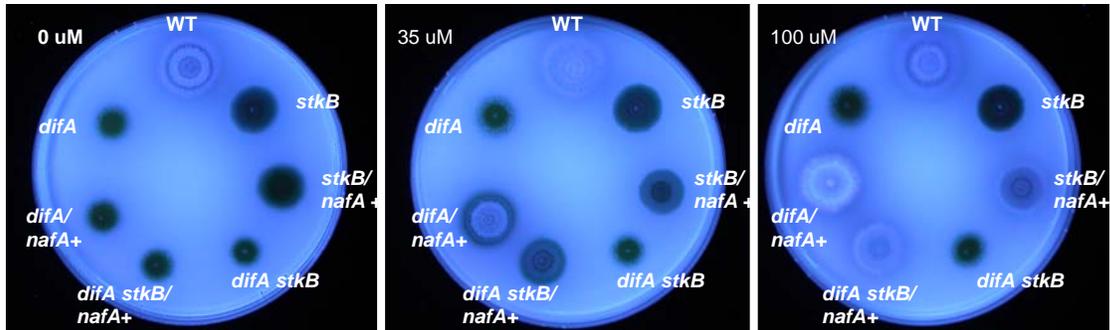


Figure 2-8: The strains with the NafA chimera produced EPS when nitrate was added

The plates were prepared as described in Materials and Methods. The strains that are plated on CYE with varying concentrations of nitrate of 0, 35 and 100 μM of KNO_3 were WT (DK1622), *difA* (YZ601), *stkB* (YZ813), *difA stkB* (YZ933), *difA/nafA+* (YZ724, a *difA* mutant expressing the NafA chimera), *stkB/nafA+* (YZ908, a *stkB* mutant expressing the NafA chimera), and *difA stkB/nafA+* (YZ934, a *difA stkB* mutant expressing the NafA chimera).

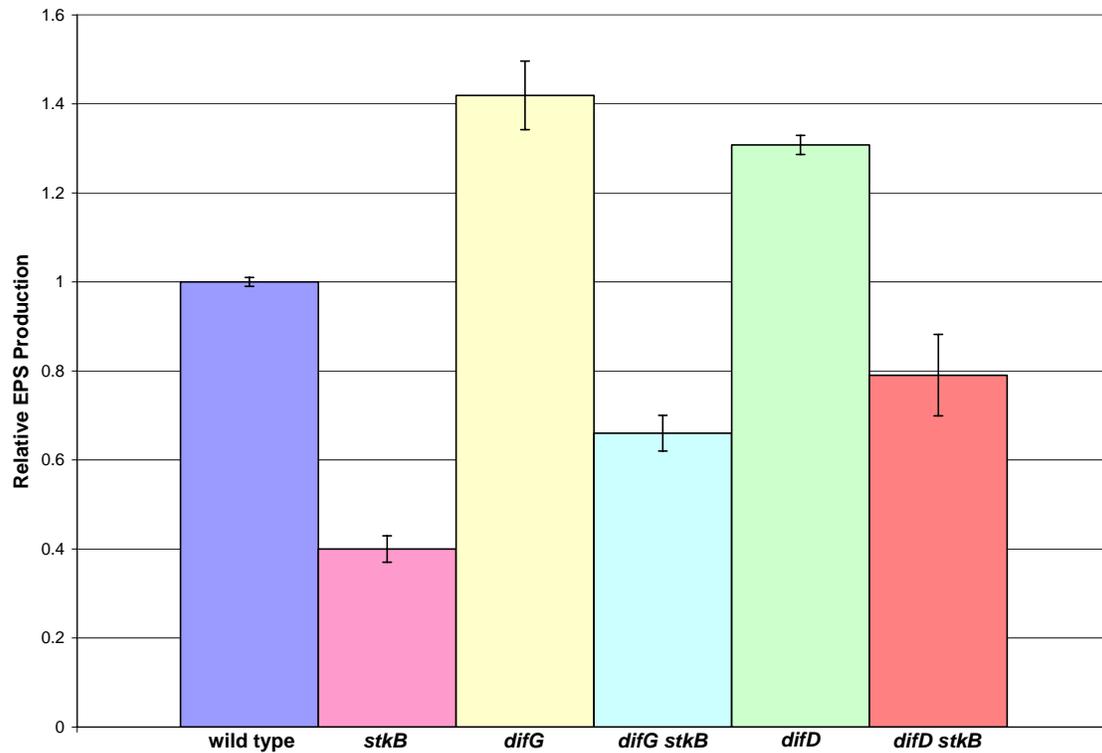


Figure 2-9: StkB interacts with DifD and DifG

The procedures for the Calcofluor White Binding Assay are found in the Materials and Methods. The strains indicated are wild-type (DK1622), *stkB* (YZ813), *difD* (YZ801), *difG* (YZ604), *difD stkB* (YZ931), and *difG stkB* (YZ929).

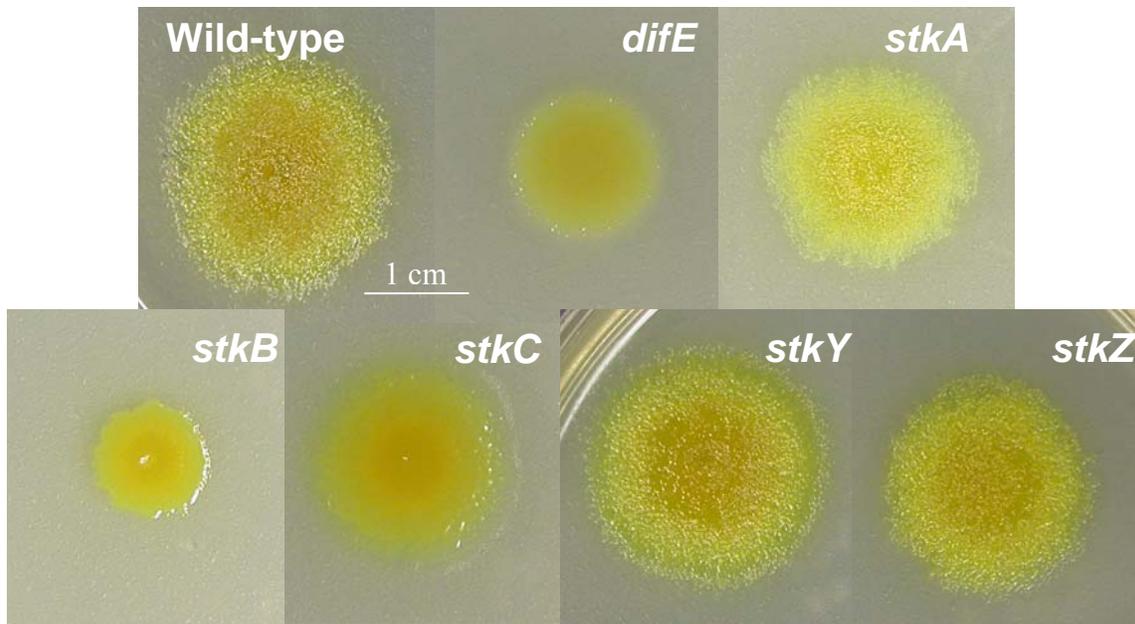


Figure 2-10: The *stkA*, *stkB* and *stkC* mutants had motility defects

The plates contain 1.5% agar. These strains were examined after 2 days of incubation. The strains indicated are wild-type (DK1622), *stkA* (YZ812), *stkB* (YZ813), *stkC* (YZ910), *stkY* (YZ902), *stkZ* (YZ904), and *difE* (YZ603). The *stkA*, *stkB* and *stkC* mutants were deficient (if only slightly) in motility.

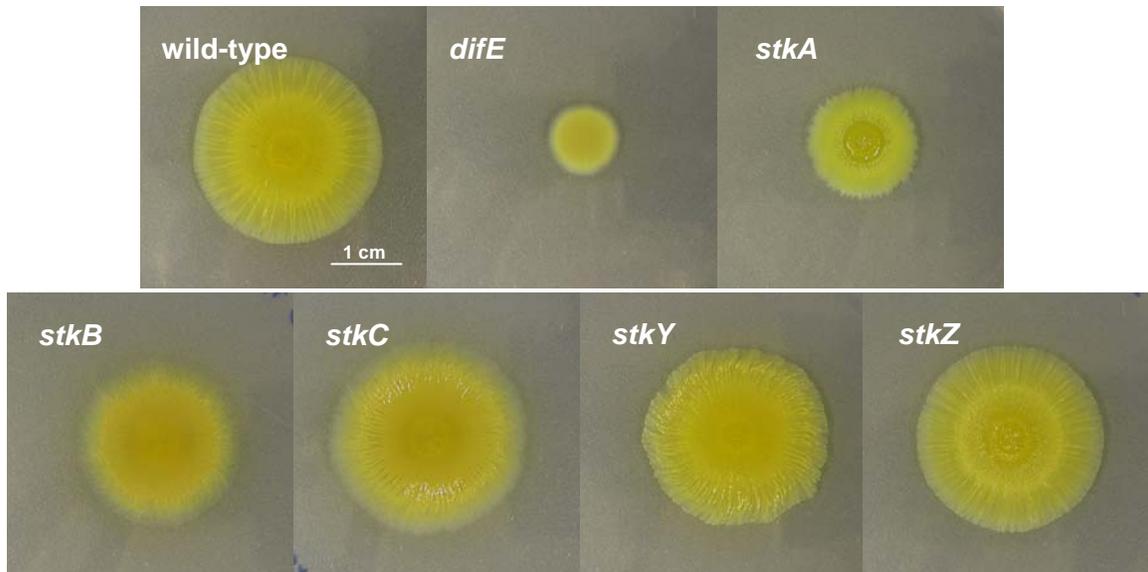


Figure 2-11: The *stkA* and *stkB* mutants exhibit S-motility defects

The plates contain 0.4% agar. These strains were examined after 5 days of incubation. The strains indicated are wild-type (DK1622), *stkA* (YZ812), *stkB* (YZ813), *stkC* (YZ910), *stkY* (YZ902), *stkZ* (YZ904), and *difE* (YZ603). The *stkA*, *stkB* and *stkC* mutants were deficient (if only slightly) in S-motility.

(important for A-motility) were introduced into the *stk* deletion mutants. The colony edge morphology of these mutants along with the parental strains was examined microscopically. Insertions in the *pilA* gene showed that A-motility did not appear to be affected in the *stk* locus mutants (Figure 2-12). The insertion in the *aglU* gene showed that S-motility is affected in the *stkA* and *stkB* mutants. The *stkA* mutant exhibited more group movement while the *stkB* mutant exhibited considerably less group movement than the wild-type. Once again, there appeared to be little to no phenotypic difference in either *stkY* or *stkZ* mutants compared to wild-type (Figure 2-12). After examination of motility it was concluded that StkA and StkB affects motility.

The *stkA*, *stkB*, and *stkC* mutants are deficient in fruiting body formation. S-motility and EPS production have been shown to be intimately associated with fruiting body formation in *M. xanthus*. The fruiting body formations of the *stk* mutants were examined on starvation media (Figure 2-13). The *stkA* and *stkB* mutants showed abnormal fruiting body morphology compared to the wild-type. The fruiting bodies of the *stkA* mutant were smaller and more scattered while the fruiting bodies of the *stkB* mutant were still smaller than wild-type but considerably larger than the *stkA* mutant. The *stkC* mutant had the most normal morphology, but the fruiting bodies appeared smaller in diameter than wild-type. The *stkY* and *stkZ* mutants have similar developmental phenotypes as the wild-type. The *difE* mutant was used as a negative control because it does not produce fruiting bodies. This study suggests that the *stkA* and possibly *stkB* and *stkC* are involved in the fruiting processes.

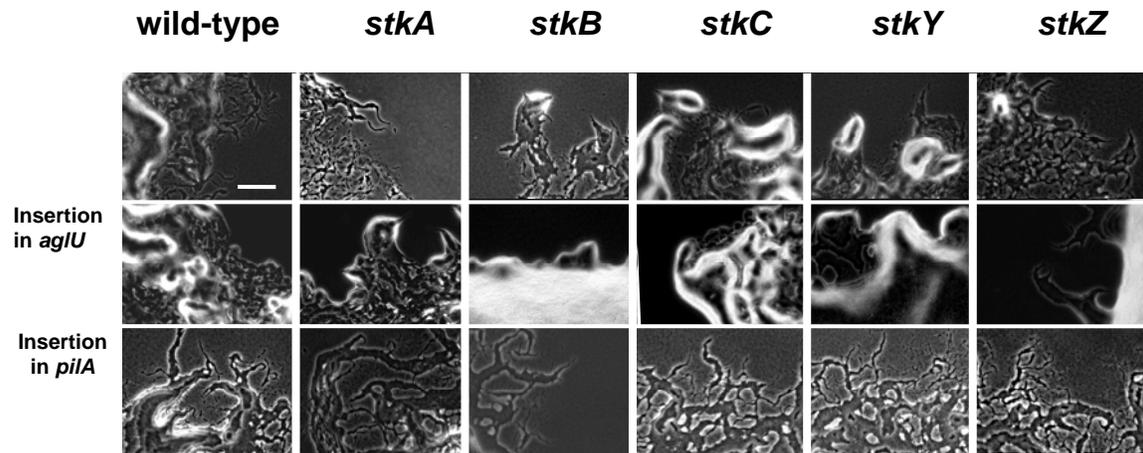


Figure 2-12: The *stkA* and *stkB* mutants had different phenotypes from wild-type when an insertion was created in an A-motility gene

The mutants were plated on CYE and examined under the microscope after 2 days. An insertion in *aglU* (A-motility gene) was used to examine S-motility while an insertion in *pilA* (S-motility gene) was used to examine A-motility. The strains indicated are wild-type (DK1622), *stkA* (YZ812), *stkB* (YZ813), *stkC* (YZ910), *stkY* (YZ903), *stkZ* (YZ904), *pilA* insertion in wild-type (DK10407), *pilA* insertion in *stkA* (YZ923), *pilA* insertion in *stkB* (YZ916), *pilA* insertion in *stkC* (YZ917), *pilA* insertion in *stkY* (YZ918), *pilA* insertion in *stkZ* (YZ919), *aglU* insertion in wild-type (YZ723), *aglU* insertion in *stkA* (YZ925), *aglU* insertion in *stkB* (YZ926), *aglU* insertion in *stkC* (YZ920), *aglU* insertion in *stkY* (YZ921), *aglU* insertion in *stkZ* (YZ922). The scale bar represents 100 μm .

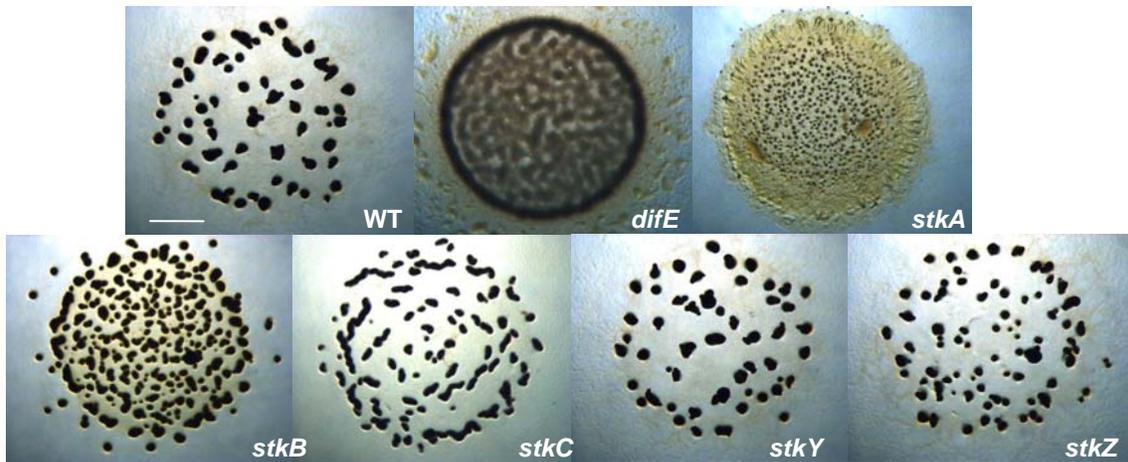


Figure 2-13: The *stkA*, *stkB* and *stkC* mutants displayed varying fruiting body phenotypes

Fruiting body formation was examined using procedures listed in Materials and Methods. DifE was used as a negative control because it lacks fruiting bodies. StkA, StkB and StkC had a different fruiting body formation from wild-type. The strains indicated are wild-type (DK1622), *stkA* (YZ812), *stkB* (YZ813), *stkC* (YZ910), *stkY* (YZ902), *stkZ* (YZ904). The scale bar represents 1 mm.

Discussion

Two different transposon mutagenesis studies were done to identify additional regulators of EPS production which isolated two insertions in the *stk* locus. In-frame deletions were created in the five possible genes located in the *stk* locus. The *stkA* mutant overproduced EPS while the *stkB* and *stkC* mutants underproduced EPS somewhat. These three mutants also had different motility and developmental phenotypes compared to the wild-type. The *stkY* and *stkZ* mutants displayed phenotypes similar to the wild-type in all assays. In the genetic epistatic studies, the *pilA stkA* double mutant produced levels of EPS similar to *stkA* while the *difA stkA* mutant produced levels of EPS similar to *difA*. The *stkAB* double mutant also produced EPS at levels similar to the wild-type. In the studies utilizing the nitrate-dependent NafA chimera, all three mutants with NafA produced EPS after the addition of nitrate, although the *stkB* mutant produced slightly less EPS than the controls. The *difD stkB* and *difG stkB* mutants produced more EPS than the *stkB* mutant but significantly less than the *difD* and *difG* single mutants.

After examination of EPS production by the *stkA* in-frame deletion mutant it was concluded that StkA is a negative regulator of EPS production. The *stkA* deletion mutant overproduced EPS significantly compared to wild-type. The phenotypes exhibited by the *stkA* transposon insertion mutant and the *stkA* in-frame deletion mutant were the same. Thus, the previously published data with a *stkA* insertion mutant was not due to polar effects. The in-frame deletion mutants of *stkB* and *stkC* affected EPS production. In conclusion, StkB and StkC are either positive regulators of EPS production or biosynthetic enzymes.

Type IV Pili is believed to perceive the signal for the Dif pathway in the regulation pathway of EPS production (8). The *dif* (defective in fruiting) locus encodes

both positive and negative regulators of EPS production. DifA, DifC and DifE positively regulate EPS production while DifD and DifG do so negatively (5, 9, 96). Five of the six genes are homologous to bacterial chemotaxis proteins. DifA is homologous to methyl-accepting chemoreceptor proteins (MCPs), DifC to CheW, DifD to CheY, DifE to CheA, and DifG to *Bacillus subtilis* CheC. DifB, which has no apparent effect on EPS, shows homology to a conserved family of hypothetical proteins with unknown functions (9).

Preliminary studies suggested that StkA was upstream of the Dif pathway in the regulatory pathway of EPS production. After examining the results shown in Figure 2-6, it was concluded that StkA functions between the TFP and the Dif proteins the regulatory pathway of EPS production (Figure 2-14).

The next logical step was to determine where StkB was positioned in the regulatory pathway of EPS. This ended up being a lot more complicated than determining the position of StkA. In the three genetic epistasis experiments, the double deletions with *stkB* mutants produced more EPS than just the single *stkB* deletion mutant, but significantly less than the overproducers of EPS. It is possible that StkB may function in a biosynthetic capacity instead of a regulatory one.

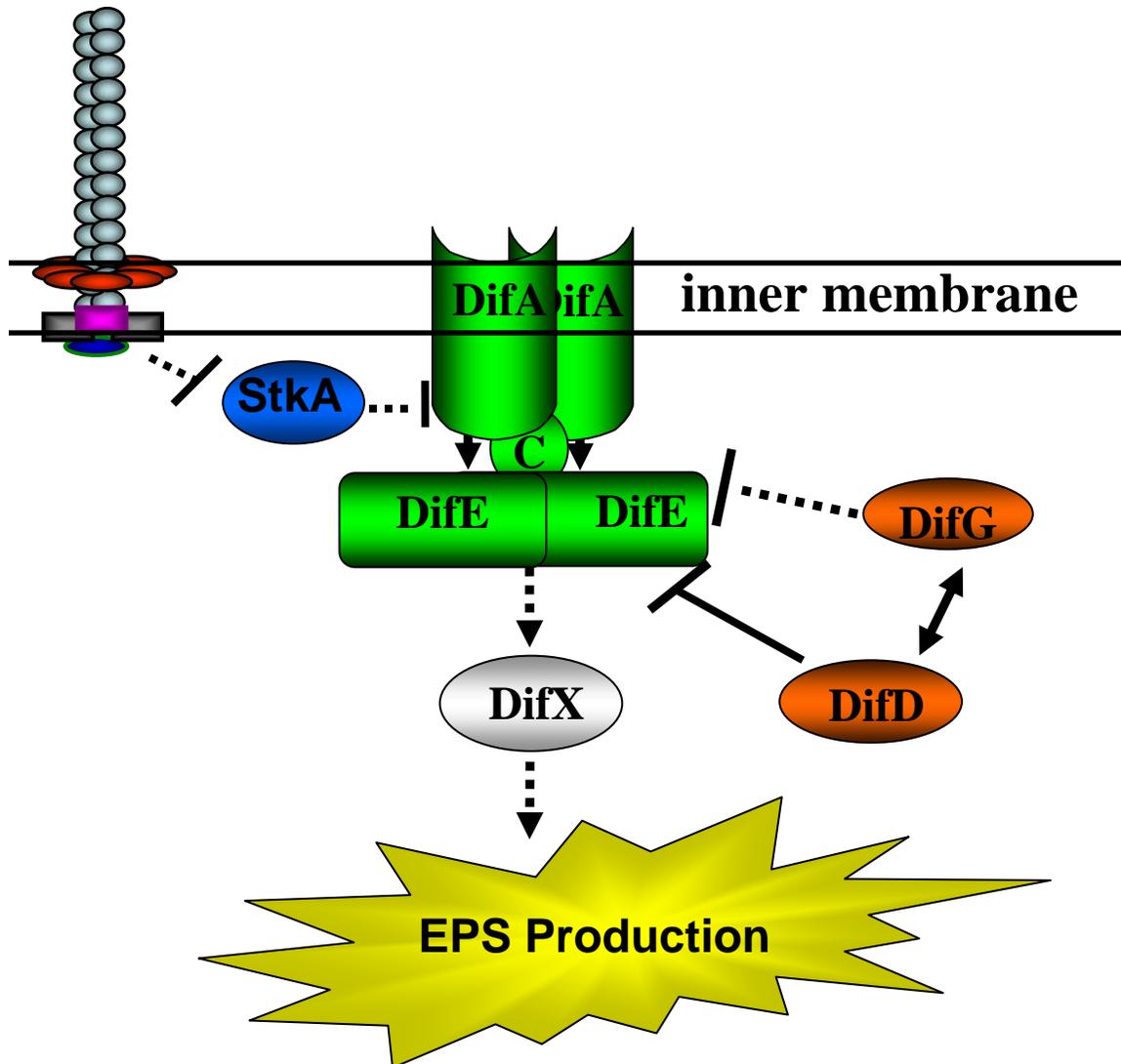


Figure 2-14: Working model of the regulation pathway of EPS production in *M. xanthus*.

The signal is transmitted from the TFP through StkA to the Dif Pathway. DifX is an unknown protein located between the known Dif pathway and the production of EPS. The position of StkB is still inconclusive although it interacts with DifD and DifG.

**Chapter 3 : Genes Located Downstream of the *stk* Locus and Other
Projects Attempted**

I. Genes Downstream of the *stk* Locus

Insertions were identified in genes downstream of the *stk* locus. The transposon mutagenesis study that identified the *stkB* insertion (previous chapter) in the *difA* suppressor also identified insertions in the stop codon of *rbp*. It was discovered that *rbp* is located three genes downstream of the *stk* locus after searching the *M. xanthus* genome (Figure 3-1). The two genes between *stkC* and *rbp* are annotated as *bskC* and *bskL*. The sequences were retrieved from TIGR and compared against the sequenced genomes found in BLAST. BskC (320 aa) has the highest identity (57%) with an integral membrane protein TerC found in *Herpetusiphon aurantiacus* ATCC23779 (Accession number ZP_01426499). BskL (363 aa) was listed as a lipoprotein although it has the highest identity (40%) with an unknown protein in *Trichomonas vaginalis* G3 (Accession number XP_001305425) and Rbp (536 aa) has the highest identity (40%) to a Rieske 2Fe-2s family protein found in *Mycobacterium avium* 104 (Accession number YP_879494).

Materials and Methods

Construction of Plasmids. Plasmids used in this study are listed in Table 3-2. The in-frame deletion plasmids were constructed by using a two-step overlap PCR (67). Restriction enzymes and T4 DNA ligase were from New England Biolabs, while Phusion polymerase was from Finnzymes. The primers used to construct the in-frame deletion plasmids *bskC* and *bskL* along with the primers used to construct the insertions in *rbp* are listed in Table 3-2.

The *bskL* in-frame deletion plasmid pAM109 was constructed by cutting both the PCR product and pBJ113 with *KpnI* and *PstI* followed by ligation. The plasmid pAM110 was created by amplifying a 500 bp region in the middle of the *rbp* gene and engineering

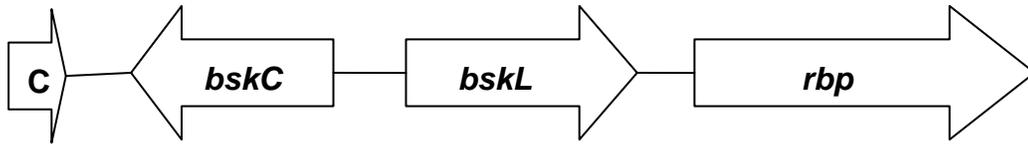


Figure 3-1: Location of the genes downstream of the *stk* locus

These are the position of the three genes downstream of the *stk* locus. The initial insertion created by the transposon was located in the stop codon of *rbp*. C stands for *stkC*.

Table 3-1: Plasmid primer list for mutants downstream of the *stk* locus

Plasmid ^b	Primers ^a	Enzyme ^c
pAM109	CTCGGT <u>ACCGGT</u> TACAGCGCG	<i>KpnI</i>
	GCGG CTCA CTGCTCCGGCGCACGATTCCGAGT CAA AATAG	N/A
	GCGCCGGAGCAGTGAGCCGC	N/A
	AGCCTGCAGTCGTTGATGTT	N/A
pAM110	AGCGAATTCGAGGCGGTGGC	<i>EcoRI</i>
	<u>AAGCTT</u> TCCTGGATGTCCG	<i>HindIII</i>
pAM112	AACGAATTC CCG AACGCGAC	<i>EcoRI</i>
	CTGA <u>AAGCTT</u> CTCCTGCTCAC	<i>HindIII</i>
pAM113	GGCGAATTC TTT CGGCTTGT	<i>EcoRI</i>
	GCGG CTC AGACCTTGGGCGCTGGGAAGGTTTCCATATGGG	N/A
	GCGCCCAAGGTCTGAGCCGC	N/A
	CGAA <u>AAGCTT</u> GAAGTACAAGGGCAT	<i>HindIII</i>

^aThe sequences of primers are written from 5' to 3'. Restriction sites in primers are underlined. Start and stop codons for genes are in **boldface** when applicable.

^bIn-frame deletions were created by amplifying the upstream and the downstream fragments and then overlap PCR was used to join the two fragments. The primers are listed as Forward 1, Reverse 1, (to amplify upstream fragments), Forward 2 and Reverse 2 (to amplify downstream fragments).

^cN/A means Not applicable.

an *EcoRI* and *HindIII* site into the primers used. The fragment was cloned into the *EcoRI* and *HindIII* sites of pBJ113. The plasmid pAM112 was created by amplifying 843 bp in the middle of the *rbp* gene using primers RBP_R3 and RBP_F2. This insertion fragment was cloned into pBJ113 restricted by *EcoRI* and *HindIII*.

Construction of *M. xanthus* strains. *M. xanthus* strains used in this portion of the study are listed in Table 3 – 2. A two step screening process occurred, first kanamycin resistant mutants were identified after 5-7 days and then the resistant colonies were plated on 1% galactose for another 5-7 days. Potential mutants were screened for a galactose “resistant”, kanamycin – sensitive phenotype (84). The in-frame deletion strain YZ911 (*bskL*) was constructed by transformation by electroporation of pAM109 into DK1622 (wild-type). An in-frame deletion of plasmid *bskC* (pAM113) was created using primers listed in Table 3-2 and the PCR fragment was integrated into pBJ113. Unfortunately, after multiple attempts, this plasmid could not be transformed into wild-type (DK1622).

Examination of EPS Production. EPS production produced by the *bskL* (Figure 3-2) and *rbp* (Figure 3-3) mutants was examined by binding of the fluorescent dye calcofluor white (CW) (9, 21). Cells from overnight cultures were pelleted and resuspended in MOPS (morpholinepropanesulfonic acid) (10 mM MOPS [pH 7.6], 2 mM MgSO₄) buffer at approximately 5×10^9 cells/ml. 5 μ l of these suspensions were spotted onto CYE plates with 50 μ g/ml of calcofluor white (CW) and incubated at 32°C for 6 days. Fluorescence was photographed under the illumination of a handheld long-wavelength (365 nm) UV light source (1, 2, 9, 21).

Table 3-2: Plasmids and other *M. xanthus* strains

Designation	Genotype or description	Source or reference
Strains		
YZ911	<i>ΔbskL</i>	This study
YZ912	<i>rbp</i> insertion mutant in YZ101	This study
YZ915	<i>rbp</i> insertion mutant in YZ101	This study
YZ924	<i>rbp</i> insertion mutant in DK1622	This study
YZ950	transposon mutagenesis mutant	This study
YZ951	transposon mutagenesis mutant	This study
YZ952	transposon mutagenesis mutant	This study
BY134	<i>rbp</i> transposon insertion mutant	This study
Plasmids		
pAM109	<i>bskL</i> inframe deletion in pBJ113	This study
pAM113	<i>bskC</i> inframe deletion in pBJ113	This study
pAM110	<i>rbp</i> insertion	This study
pAM112	<i>rbp</i> insertion	This study

Examination of Motility. Motility exhibited by the *bskL* mutant was examined by spotting 5 μ l of the cell suspension containing 5×10^9 cells/ml onto the center of a standard (1.5%) or soft (0.4%) CYE agar plate. Standard agar plates were utilized when evaluating the overall colony morphology. Colony expansion and colony edge morphology were examined both macroscopically and microscopically after 2 days of incubation at 32°C. Soft agar plates were incubated at 32°C for 5 days before observation and documentation (9, 71) (Figure 3-2).

Examination of Fruiting Body Formation. Fruiting body formation of the *bskL* mutant was observed by growing cells overnight and resuspending them in MOPS to a concentration of 5×10^9 cells/ml. Five microliters of the suspension was placed onto CF agar plates and development was observed after 5 days of incubation at 32°C (9, 29) (Figure 3-2).

Creation and evaluation of in-frame deletions of *bskL*. An in-frame deletion was created for *bskL* that spans from codons 5 – 358. The deletion mutant was evaluated on soft agar, CF plates and CYE with calcofluor white added. The procedures are listed in Materials and Methods. EPS production and motility of the *bskL* mutant looked similar to wild-type but fruiting body formation was different. This mutant was not examined further because in-frame deletions could not be created in all three genes downstream of the *stk* locus (Figure 3-2).

Creation and evaluation of insertions in *rbp*. Since the insertion was located in the stop codon of *rbp*, an insertion was created using the internal region of the gene to see if the same phenotype was observed. YZ912 (*rbp* insertion mutant) was created by transforming plasmid pAM110 into YZ101. This mutant had the same phenotype as

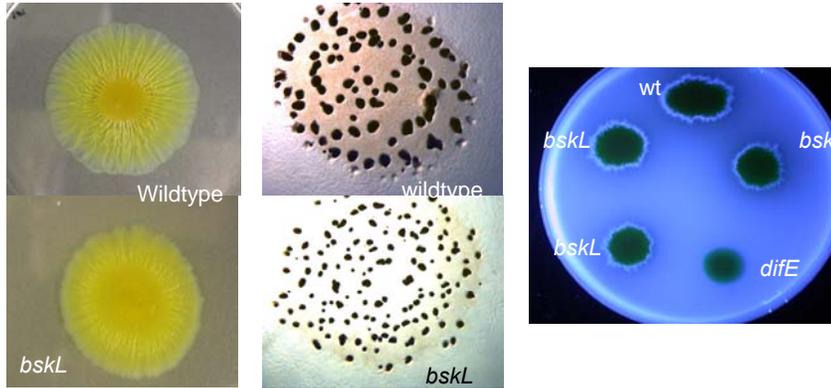


Figure 3-2: The *bskL* deletion mutant had similar EPS production as wild-type

This mutant was evaluated using protocols listed in Materials and Methods. From left to right, the different methods used are soft agar plates, CF plates and CYE plates supplemented with calcofluor white. Strains used were wild-type (DK1622), *bskL* (YZ911), *difE* (YZ603).

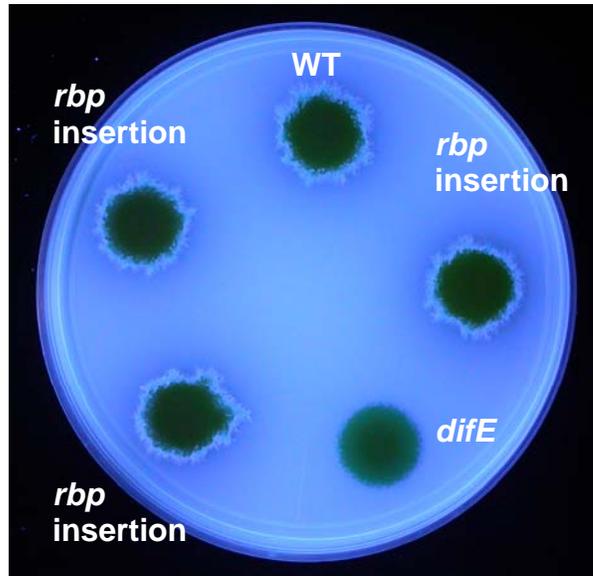


Figure 3-3: A mutation in *rbp* does not affect EPS production

The strains indicated are WT (DK1622), *difE* (YZ601), and *rbp* insertion in wild-type (YZ924). Fruiting body formation was not examined for the *rbp* insertion mutant.

They were plated using methods listed in Materials and Methods.

YZ101 (*difA* suppressor strain) (results not shown). Next, a larger insertion was created in the internal region of the gene. The insertion mutant was made in *M. xanthus* by transforming pAM112 by electroporation into YZ101 (*difA* suppressor strain) to make YZ915 and DK1622 (wild-type) to create YZ924. After phenotypic examination of YZ924 (Figure 3-3) on CYE plates with calcofluor white added, this mutant was deemed not significant in the regulation of EPS production so this project was discontinued.

II. Construction of *stkABC* and *stkBC* Deletion Mutants.

After the *stkAB* deletion mutant was created and characterized, the next logical step was to create an in-frame *stkABC* mutant and a *stkBC* double deletion. The primers were created for both mutants using existing outside primers created for the single mutants and a new internal primer to connect the upstream and downstream fragments. The primers used to create the in-frame double deletion *stkABC* mutants were the first primer listed for *stkA*, the third and fourth primers listed for *stkC* and the sequence of the intermediate primer used is

TTCGACTACAGCGCGCCGAGTCCACTGTCTATCACGTCGC. The primers used to create the in-frame double deletion mutants were the first primer listed for *stkB*, the third and fourth primers listed for *stkC* and the intermediate primer is used was

TTCGACTACAGCGCGCCGAGGACCGCTTCGTCCACCCACT. In both cases, upstream and downstream fragments were created. Unfortunately, it was not possible to obtain a clean PCR fragment that connected the upstream and downstream fragments to create an in-frame deletion. After several attempts, this project was discontinued.

III. Transposon Mutagenesis Study to Identify New *difA* Suppressors

A transposon mutagenesis study was performed to identify more *difA* suppressors by using the *mariner* – based transposon *magellan 4* (66). Approximately 500 ng of pMycoMar (containing *magellan4*) was transformed into YZ601 (*difA*). The cells were recovered for 4 hours at 32°C and plated on CYE plates with Congo Red and kanamycin (7). Approximately 20,000 colonies were screened for potential mutants. In this study, mutants that overproduced EPS were to be kept. Unfortunately, there was no *difA* suppressor mutations found in the screening process so this project was stopped. Three mutants that were beige colored instead of yellow so they were kept and are stored as YZ950, YZ951 and YZ952. These mutants were not phenotypically examined.

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- Virginia Tech, Biological Sciences, Blacksburg, Virginia.** Graduate Teaching Assistant in Microbiology Labs Fall 2004- Spring 2005
- Salisbury University Biological Sciences, Salisbury, Maryland.** Microbiology Lab Assistant, Fall 2002 – Spring 2004
Prepared media, set-up and cleaned-up labs, properly disposed of used bacteria
- University of Georgia, Athens, Georgia.** Researcher, Summer 2003
Researched two novel genes involved in flagellar function of *Helicobacter pylori*
- Salisbury University, Salisbury, Maryland.** Researcher, Fall 2002-Spring 2003
Researched the host preference of *Bdellovibrio bacteriovorus* by comparing the growth rate of several infected host species

Academic Honors:

Tri-Beta Biological Honor Society
Phi Sigma Biological Honor Society

Awards:

1st Place: Poster Presentation, Graduate Research Day, Virginia Tech, Blacksburg Virginia. February 24, 2007

Publications:

Rogosky, A.M, P.L.Moak, and E.A.B.Emmert (2006) Differential predation by *Bdellovibrio bacteriovorus* 109J *Current Microbiology* **52**:81-85

Presentations:

Poster Presentation: "The Role of a DnaK Homologue in the Regulatory Pathway of EPS Production in *Myxococcus xanthus*" Graduate Research Day, Virginia Tech, Blacksburg, VA. February 24, 2007

"Hsp70 and its Role In Signal Transduction" Departmental Microbiology Seminar, Virginia Tech, Blacksburg, VA November 1, 2005

"Are the *stk* genes involved in EPS production?" Informal Regional *Myxococcus xanthus* Meeting, University of Georgia, Athens, GA, April 2005

Service:

Social Chair (Spring 2006) and Treasurer (2006 – 2007) of Biological Graduate Student Association