

# **BIOSYNTHESIS OF NUCLEOTIDE SUGAR MONOMERS FOR EXOPOLYSACCHARIDE PRODUCTION IN *MYXOCOCCUS XANTHUS***

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(ABSTRACT)

*Myxococcus xanthus* displays social (S) motility, a form of surface motility that is key to the multicellular behaviors of this organism. S motility requires two cellular structures: type IV pili (TFP) and exopolysaccharides (EPS). Previous studies have shown that *M. xanthus* does not use glucose or any other sugar as a primary carbon source. However, eight monosaccharides, namely glucose, mannose, arabinose, galactose, xylose, rhamnose, N-acetyl-glucosamine, and N-acetyl-mannosamine, are found in *M. xanthus* EPS. In this study, pathways that *M. xanthus* could use to produce the activated sugar monomers to form EPS are proposed based on genomic data. Of the eight sugars, pathways for seven were disrupted by mutation and their effects on the EPS-dependent behaviors were analyzed. The results indicate that disruption of the two pathways leading to the production of activated rhamnose (GDP- and TDP-rhamnose) affected fruiting body formation (GDP form only) and dye binding ability (both forms) but not S motility. Disruptions of the xylose, mannose, and glucose pathways caused *M. xanthus* to lose S motility, fruiting body formation, and dye binding abilities. An interruption in the pathway for galactose production created a mutant with properties similar to a lipopolysaccharide (LPS) deficient strain. This discovery led us to study the

phenotypes of all mutant strains for LPS production. The results suggest that all mutants may synthesize defective LPS configurations. Disruption of the UDP-N-acetyl-mannosamine pathway resulted in a wild type phenotype.

In addition, it was discovered that interruption of the pathway for N-acetylglucosamine production was possible only by supplementing this amino-sugar in the growth medium. In an attempt to determine if other mutants could be recovered by sugar supplementation, it was discovered that the  $\Delta pgi$  mutant can be rescued by glucose supplementation. The Dif chemotaxis-like pathway is known to regulate EPS production in *M. xanthus*. DifA is the upstream sensor of the pathway. Previous studies had created a NarX-DifA chimeric protein, NafA, that enables the activation of the Dif pathway by nitrate, the signal for NarX. In this study, we constructed a  $\Delta pgi difA$  double mutant containing NafA. This strain was then subjected to various incubations with glucose and/or nitrate to determine whether the point of EPS regulation by the Dif pathway is down- or up-stream of the step catalyzed by Pgi (phosphoglucose isomerase). Preliminary results from this study are inconclusive.

## **DEDICATION**

I would like to dedicate this thesis to my entire family but especially to my little sister Rachel for helping me learn patience, to my dad Ken for teaching me that there is always a way, and my mom Pebbles for making me stubborn enough to follow it once I found it. And also to John, for always being there with encouragement (or a giant shove) to get me moving in the right direction.

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## **LIST OF ABBREVIATIONS**

1. S motility – social motility
2. EPS – exopolysaccharides
3. LPS – lipopolysaccharides
4. TFP – type IV pili
5. GDP – guanosine diphosphate
6. TDP – thymidine diphosphate
7. UDP – uridine diphosphate

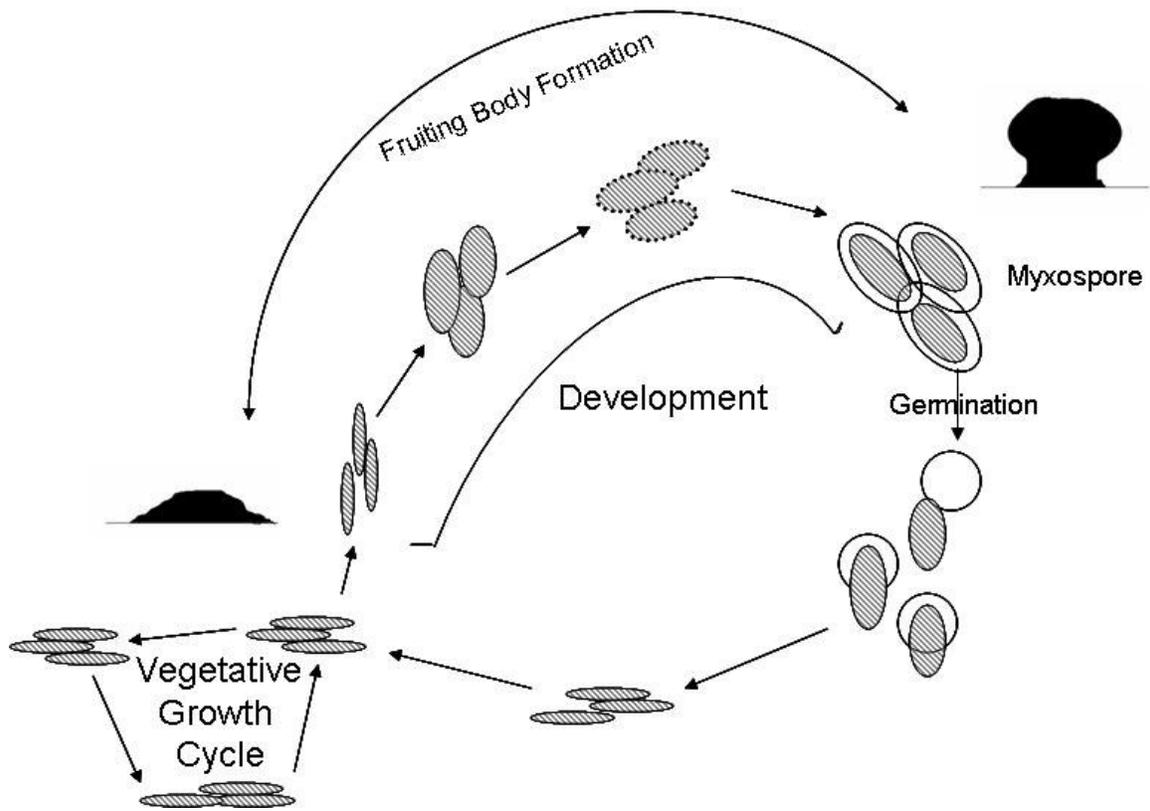
## **Chapter 1: Introduction and Review of Literature**

## **A. Myxobacteria**

Myxobacteria are a group of Gram negative bacteria that display several unique properties and behaviors. These soil bacteria develop multicellular structures known as fruiting bodies under starvation conditions (16, 58). When nutrients are scarce, groups of cells will aggregate on solid surfaces and form fruiting bodies that range in design from small mounds to large branched structures. This is made possible by extensive cell-cell signaling and a type of motility known as gliding (16, 58). The characteristics of one species of myxobacteria, *Myxococcus xanthus*, are discussed in detail.

## **B. *Myxococcus xanthus***

**I. Life-cycle.** *Myxococcus xanthus*, the model organism of myxobacteria, is a rod-shaped bacterium with a distinct life cycle (Figure 1-1). When nutrients or prey organisms are abundant, *M. xanthus* cells will remain in a vegetative state. Under these conditions, cells often display a wolf-pack-like feeding behavior wherein they act cooperatively to “hunt” and feed on prey organisms (35). When nutrients become sparse, *M. xanthus* cells enter a developmental cycle in which groups of approximately one hundred thousand cells aggregate to form fruiting bodies (13, 16). The shapes of fruiting bodies are species-specific and those of *M. xanthus* resemble raised mounds (39). Within these fruiting bodies, cells differentiate into myxospores which are dormant and resistant to many forms of environmental stress. Upon the introduction of nutrients, myxospores germinate and return to the vegetative state.



**Figure 1- 1. Life cycle of *M. xanthus*.** Displayed above is the life cycle of *M. xanthus* as described by Dworkin (14). Cells will remain in the vegetative growth phase until nutrients are unavailable. The cells will then aggregate to form fruiting bodies and develop into environmentally resistant myxospores. Upon the return of nutrients in the environment, myxospores will germinate and return to a vegetative state.

*M. xanthus* displays multicellular behaviors not commonly found in bacteria. Most notable is the wolf-pack-like feeding behavior displayed during the vegetative stage. Large groups of cells work cooperatively to produce the digestive enzymes needed to degrade macromolecules upon which the cells feed (15). For example, the capacity of *M. xanthus* to degrade casein has been shown to be cell density dependent. This cooperative feeding behavior of *M. xanthus* is likely an adaptive survival mechanism as indicated by the fact that growth on several media does not occur if cell densities are insufficiently low (51).

**II. Motility.** *M. xanthus* displays gliding motility which is controlled by two distinct genetic systems (16, 29, 30, 56). These two systems are adventurous (A) motility which supports the movement of isolated cells and social (S) motility which is involved in the movement of groups of cells (30). S motility is important for the behaviors observed during all stages of the *M. xanthus* life cycle which involve group cell movement (13, 35).

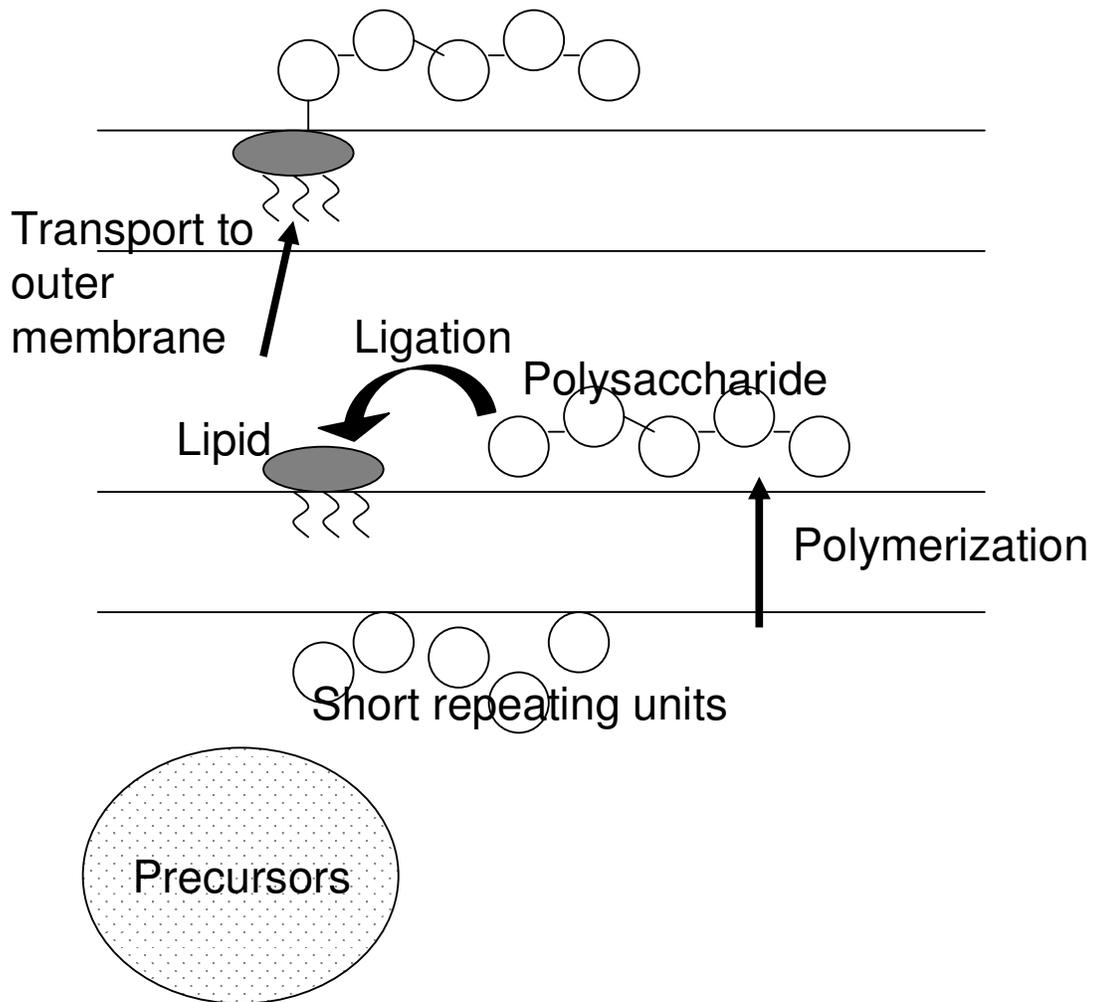
Two cellular structures are important for the manifestation of S motility, type IV pili and extracellular polysaccharides (EPS) (35). Pili are polarly located while EPS cover the exterior of the cell. Type IV pili appear to function by tethering to a surface or another cell and “pulling” cells toward the point of the tether by retraction (61). The tether point for type IV pili in *M. xanthus* is possibly EPS (41). Also, type IV pili have been shown to be upstream of the Dif pathway in the regulation of EPS production (4).

## **C. EPS in bacteria.**

**I. Functions of EPS in other bacteria.** Exopolysaccharides (EPS) are produced by many bacteria including *Rhizobium meliloti* (23, 49) and *Escherichia coli* (48, 68, 69). EPS have been shown to play a role in many important behaviors of these bacteria. In *R. meliloti*, a nitrogen-fixing bacteria that lives symbiotically, EPS is critical for the formation of nodules on its plant host (19, 60). The EPS of *E. coli* have been shown to be an important factor in determining the ability of this organism to infect a host (69).

The biosynthesis and export mechanisms used by bacteria in the production of EPS have been characterized. In general, EPS in most Gram negative bacteria are synthesized using similar steps. Synthesis begins with the production of nucleotide sugars which are then used to form short oligosaccharides in the cytosol (62, 64, 70). These oligosaccharides are then transported across the cytoplasmic membrane while being polymerized into longer polysaccharides attached to a lipid. The entire structure is then transported to the exterior of the cell where the lipid is embedded in the outer membrane and the polysaccharide is covalently attached to this lipid anchor (Figure 1-2).

**II. *M. xanthus* EPS composition.** The *M. xanthus* extracellular matrix is composed of proteins and EPS in a 1:1.2 ratio. Glucose, rhamnose, mannose, N-acetylglucosamine, N-acetyl-mannosamine, galactose, xylose, and arabinose constitute the eight monosaccharides that have currently been isolated from the EPS extracted from *M. xanthus* (2, 54). Interestingly, despite the amount of polysaccharide produced by *M. xanthus*, this strictly aerobic species does not use any of the monosaccharides in the



**Figure 1- 2. EPS production in Gram negative bacteria.** This process follows a basic route from the building blocks to the final product. The diagram above touches on the major steps involved in the process which include the formation of precursors, short repeating units, the polymerization of these units as they are transported across the inner membrane, the attachment of the longer polysaccharides to a lipid, and finally the transportation across the outer membrane.

environment as a source of carbon and energy (8, 12, 27, 35, 67). Given the fact that *M. xanthus* does not use glucose as a carbon and energy source, the presence of some of the key enzymes used in gluconeogenesis (pyruvate carboxylase and fructose-1,6-bisphosphatase) suggests that this pathway may be used to produce at least some of the sugars found in EPS.

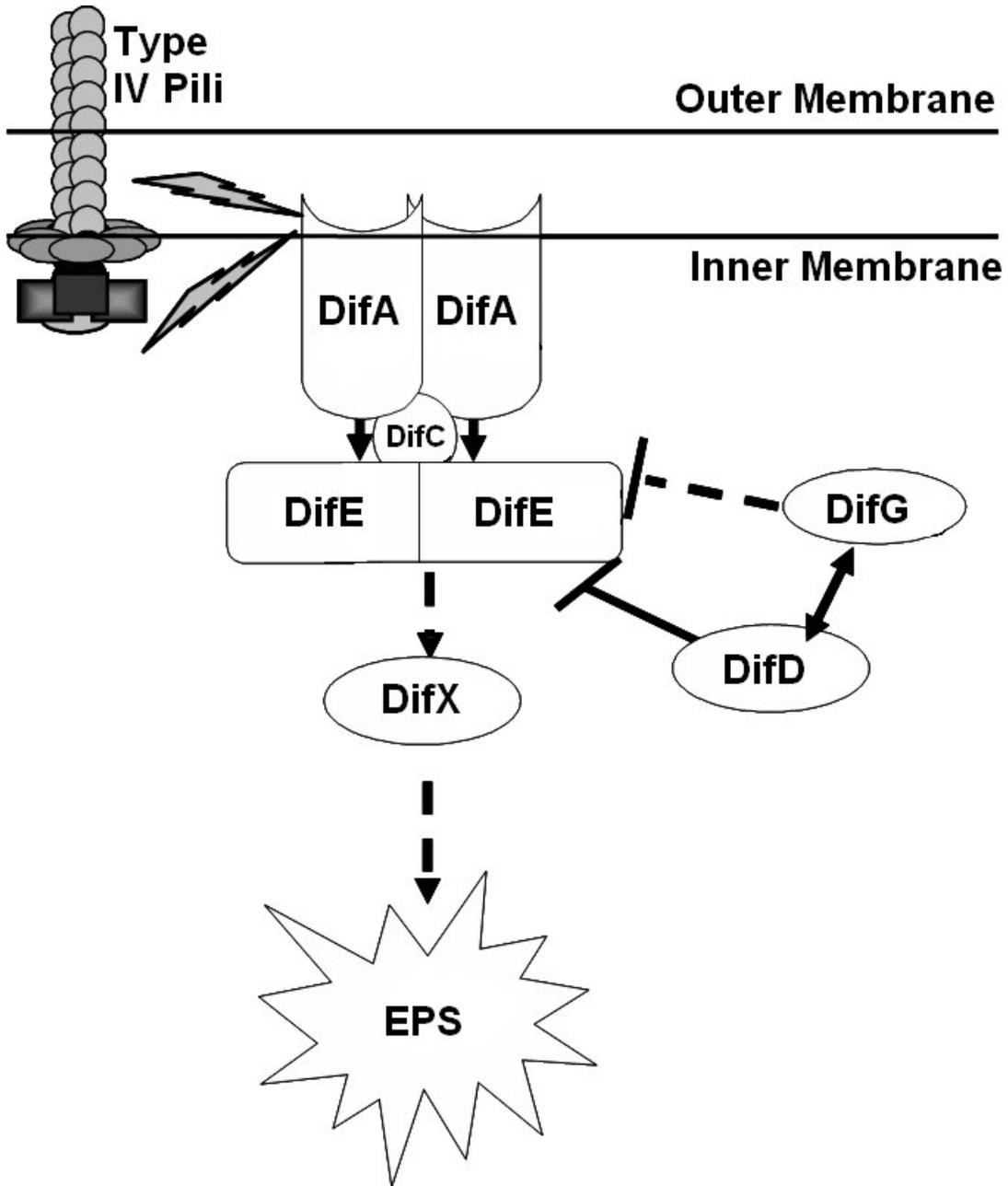
**III. *M. xanthus* EPS production.** EPS production in general involves many steps including the production of nucleotide sugar monomers, the synthesis of short repeating units, polymerization, and export (Figure 1-2)(70). Recently, a 37.2 kb region dubbed the *eps* locus in *M. xanthus* was found to be involved in EPS production. This region encodes many open reading frames with homology to genes known to be involved in the production of EPS in other species (42). *epsA*, *epsB*, *epsC*, and *epsD* encode products similar to an UDP-N-acetyl-mannosamine transferase, an endoglucanase, a serine acetyltransferase, and a glycosyltransferase, respectively. Mutations in these four genes led to defects in EPS production. Other *eps* gene products also show homology to proteins important for EPS production including glycosyltransferases, a polysaccharide transporter, and regulatory proteins (42).

A recent study was conducted to identify additional genes important for EPS production in *M. xanthus*. Although the importance of the *pil* (involved in the production of pili) genes as well as the *dif* genes for EPS production has already been established (4, 57), it is likely that there are additional genes important for the production of EPS. As part of efforts to identify additional genes involved in EPS production, a *difA* suppressor was isolated (3). To identify the suppressor mutation, the suppressor strain (YZ101) was

mutagenized with the transposon *magellan4*, which contains a kanamycin resistance cassette, an origin of replication, and the sequences necessary for transposition (52).

Mutants were screened using plates with Congo red dye; mutants that failed to bind the dye were considered EPS negative and purified for further study. Of approximately twenty thousand colonies screened, about seventy were found to be EPS negative. The insertions in the seventy mutants were identified by cloning and sequencing the surrounding genomic region from the transposon. Three insertions were found to be in genes that are likely involved in the biosynthesis of sugar monomers in *M. xanthus* EPS. These open reading frames, ORF07425, ORF05483, and ORF02674, are homologous to glucose-6-phosphate isomerase, UDP-glucose 6-dehydrogenase, and a putative UDP-glucuronic acid decarboxylase, respectively (3).

**IV. *M. xanthus* EPS regulation.** In *M. xanthus*, EPS production is regulated by the *dif* (defective in fruiting/fibril) locus (74, 77). The proteins encoded by this locus are known to form a chemotaxis-like signaling pathway in the cell (76). Sequence comparisons have shown that several of the Dif proteins are homologous to chemotaxis proteins which commonly regulate motility in other bacteria (5, 74). Specifically, DifA is homologous to a methyl-accepting chemotaxis protein (MCP), DifC to CheW, DifD to CheY, and DifE to CheA and DifG shows some similarity to CheC, a chemotaxis protein from *Bacillus subtilis* (5, 37, 50, 63). A yeast-two hybrid system has been used to elucidate the interactions of the Dif proteins with one another (76). The proposed Dif pathway is shown in Figure 1-3 (4). Although the Dif pathway is known to regulate EPS production, the exact point of regulation has not yet been found.



**Figure 1- 3. Model for Dif regulation of EPS production.** Type IV pili (TFP) relay an unknown signal to the dimerized DifA proteins which then pass the signal through DifC and DifE. DifX is an unknown protein or proteins downstream of DifE. DifD and DifG are negative regulators of the pathway and also display an as yet uncharacterized interaction with each other.

DifA is the sensory protein for the pathway; however the sensory mechanism has not yet been elucidated though pili activate the system (4). Recently, a chimeric protein was produced which incorporates the signaling module of DifA from *M. xanthus* and the sensory module of NarX from *E. coli* (73). The Nar system is a two component sensory system in which NarX senses the presence of nitrate and activates the system (40). This transmembrane protein shares similarity to DifA. *M. xanthus* growth and development cycles have not been shown to be affected by nitrate so the chimeric protein could be used to learn more about how the Dif pathway controls EPS production. Experiments in strains with a *difA* deletion background containing the chimeric protein showed that the cells were able to produce EPS in response to the presence of nitrate (73).

#### **D. LPS.**

**I. LPS structure.** Lipopolysaccharides (LPS) are another important component of the outer surface structure of Gram negative bacteria, including *M. xanthus*. LPS consist of a lipid moiety called lipid A, a core monosaccharide chain, and an O-antigen consisting of short repetitive monosaccharide subunits (9). Lipid A is the region of the LPS embedded in the outer membrane and consists of fatty acids and two glucosamines (17, 43). Attached to these glucosamines is a chain of monosaccharides known as the core. At the distal end of the core, a variable number of repeating monosaccharide units that are strain-specific form the O-antigen.

**II. Comparison of LPS and EPS.** There are several differences and similarities between LPS and EPS. One major difference involves the O-antigen which is found only on LPS. This unique feature of LPS is responsible for the immunospecificity of bacterial cells (9). A second difference between EPS and LPS involves the way in which these molecules are bound to the cell surface. Many species release EPS into the environment. When EPS is retained by the cell, it is covalently bound to the cell surface via unknown phospholipids or ionic interactions with surface proteins (70). As discussed above, LPS is attached to cells via the portion of the structure known as lipid A which is embedded in the membrane. While differences do exist between LPS and EPS, these two structures contain many of the same monosaccharides. For LPS, these monosaccharides are found in the core and the O-antigen while monosaccharide chains are the main component of EPS.

**III. *Myxococcus xanthus* LPS.** When genes important for LPS production in *M. xanthus* are interrupted, several distinct colony behaviors are affected. Namely, when genes leading to the production of the O-antigen are interrupted, social motility and developmental defects are seen (6, 75). Mutant strains lacking the ability to produce the O-antigen also displayed defects in adventurous motility.

#### **E. Research Questions.**

EPS is essential for S motility in *M. xanthus* (35) as discussed earlier. Given the fact that EPS is so important for one of the main means of locomotion in *M. xanthus*,

learning more about every aspect of the production of this component is important. The present study focuses on the following questions.

**I.** Since *M. xanthus* does not use glucose as a primary carbon source, how does this organism produce the sugars found in EPS?

**II.** Where exactly does the Dif pathway regulate the production of EPS?

**Chapter 2: Biosynthesis of Sugar Nucleotides Found in the  
Exopolysaccharide of *Myxococcus xanthus***

## ABSTRACT

*Myxococcus xanthus*, a Gram negative soil bacterium, aggregates on solid surfaces to form fruiting bodies and undergoes cellular differentiation under starvation conditions. Within these multicellular fruiting structures, cells develop into environmentally resistant myxospores. Social motility, one of two motility systems in *M. xanthus*, is required for aggregation. Two main cell surface structures essential for social motility are type IV pili and extracellular polysaccharides (EPS). Pathways leading to the biosynthesis of monosaccharides are important for the production of EPS because, unlike many other bacteria, *M. xanthus* does not use glucose or any other sugar as a carbon or energy source. Amino acids, which enter metabolism through the TCA cycle, provide the bulk of *M. xanthus*'s carbon source. *M. xanthus* cells likely use gluconeogenesis as the main means of monosaccharide production. In this study, pathways leading to the production of the monosaccharides in EPS are proposed. The proposed pathways are tested using a genetic approach. Interruptions of pathways leading to seven of the eight known EPS monosaccharides have resulted in varying effects upon the production of EPS. In all cases, LPS appear to be affected as well. Test results show that phenotypes are affected in all mutants with a range from complete lack of detectable EPS and LPS to retention of EPS but loss of some LPS properties.

## INTRODUCTION

*Myxococcus xanthus* is a Gram negative soil bacterium which displays multicellular cooperative behaviors in its vegetative and developmental cell cycles (14, 16, 34). During the developmental cycle, groups of approximately one hundred thousand cells aggregate and then differentiate into environmentally resistant myxospores. These myxospores form a structure known as a fruiting body (38). Social motility, one of two motility systems in *M. xanthus*, is required for the movement of large cell groups (30).

Two structures required for social motility are type IV pili and extracellular polysaccharides (EPS) (34). An extracellular matrix covering the exterior of the *M. xanthus* cell is composed of protein and EPS in approximately a 1 to 1.2 ratio (2). EPS contains at least eight monosaccharides: glucose (34.1%), mannose (16%), rhamnose (21.9%), N-acetyl-glucosamine (12%), N-acetyl-mannosamine (5.6%), galactose (4.3%), xylose (4.7%), and arabinose (1.4%) (2, 54) (Table 2-1). Despite the production of EPS, *M. xanthus* does not use glucose or other monosaccharides as a primary carbon source (8, 12, 27, 35, 67). The presence of enzymes known to participate in gluconeogenesis suggests that *M. xanthus* may use this mechanism to produce the monosaccharides that are found in EPS (35, 67).

In this study, mutant strains discovered during a mutagenesis study are used as the starting point for examining the biochemical pathways used by *M. xanthus* to synthesize the eight monosaccharides found in EPS. These pathways are then tested by genetically interrupting key enzymes in each pathway and performing phenotypic testing to ascertain the presence or absence of EPS in each mutant strain. Each monosaccharide is of varying

**Table 2- 1. Monosaccharides in *M. xanthus* EPS.\***

<b>Monosaccharide</b>	<b>Mole Percent of Total Carbohydrate</b>
<b>Glucose</b>	34.1
<b>Rhamnose</b>	21.9
<b>Mannose</b>	16.0
<b>N-Ac-Glucosamine</b>	12.0
<b>N-Ac-Mannosamine</b>	5.6
<b>Xylose</b>	4.7
<b>Galactose</b>	4.3
<b>Arabinose</b>	1.4

\*The monosaccharides listed above have been found in the EPS of *M. xanthus* (2, 54). Monosaccharides are listed decreasing by the mole percentage they are found in the total carbohydrate of EPS.

importance to the structure and function of EPS. However, all mutations seemed to affect the structure and function of LPS as can be seen not only in phenotypic testing but also in polyacrylamide gels used to test for the presence of LPS.

## **MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** The *M. xanthus* strains and plasmid constructs used in this study are listed in Table 2-2. Unless otherwise noted, all strains are grown on Casitone-yeast extract (CYE) plates or in CYE broth. Plates and liquid cultures are incubated at 32°C in a stationary or shaking incubator, respectively. XL1-Blue (Stratagene), the *Escherichia coli* strain used for plasmid construction, was grown and maintained at 37°C on Luria-Bertani agar plates or in Luria-Bertani liquid medium (44). Unless noted otherwise, agar plates contained 1.5% agar. “Soft” agar, a CYE plate with 0.4% agar, is used to examine S motility (55). Kanamycin was added to media at 100 µg/ml for selection purposes when appropriate.

**Construction of Plasmids.** For in-frame deletion plasmids, fragments upstream and downstream of the target gene were amplified using two unique sets of primers (F1+R1, F2+R2) (Table 2-3) and combined into one fragment using a two-step PCR (53). The 5' end of R1 is complimentary to F2. Where applicable, the fragments were digested using the restriction endonucleases *Hind* III and *Xba* I. This fragment was then purified via agarose gel extraction (QIAEX II Gel Extraction Kit). When a fragment could not be digested using these enzymes, it was purified and used as a blunt-ended fragment. Fragments were then ligated into the plasmid pBJ113 (31) which had been digested with

either *Hind* III and *Xba* I or *EcoR* V as appropriate. The resulting plasmids were named pLC1001 ( $\Delta$ *pgi*), pLC1007 ( $\Delta$ *glmM*), pLC1008 ( $\Delta$ *rmd*), and pLC1018 ( $\Delta$ *xylA*) (Table 2-2).

For gene insertion plasmids, an internal portion of the gene of interest was amplified via PCR using the F1 and R1 primers listed in Table 2-3. This fragment was then purified using the gel extraction method mentioned above. Cleaned fragments were ligated into the multiple cloning site of the suicide vector pZErO which had previously been digested with *EcoR* V and purified. The resulting plasmids were named pLC1002 (*mseA*::pZErO), pLC1003 (*galE*::pZErO), pLC1005 (*rfbB*::pZErO), and pLC1006 (*algC*::pZErO) (Table 2-2).

**Construction of Mutants.** In-frame deletions were constructed by a two-step homologous recombination gene replacement protocol by using the modified positive-negative kanamycin/galactose (KG) cassette (66). PCR confirmation of the deletion mutants involved a three primer PCR in which the products obtained using the DNA of the final mutant were compared to the products obtained using the DNA of the intermediate and the wild type strains. Two of the primers (F1 and R2) were the original primers located on the outside edge of the region to be deleted and the other was located inside the deleted region. The smaller product of any given pair of the three primers would out-compete any larger product during PCR thus allowing the different sizes of the products to determine correctness of the final mutant. YZ1001, YZ1008, and YZ1018 were constructed in this manner using plasmids pLC1001, pLC1008, and pLC1018 respectively.

**Table 2- 2. *M. xanthus* strains and plasmids used in this study.**

<i>M. xanthus</i> strain or plasmid	Relevant genotype or description	Source or reference
<b>Strain</b>		
DK1622	Wild Type	(33)
YZ603	$\Delta difE$	(5)
YZ1004	Wild Type w/ Kan <sup>r</sup>	This study
SW501	<i>difE::Kan<sup>r</sup> sglA<sup>+</sup></i>	(74)
HK1324	$\Delta wzt wzm wbgA$ ( $\Omega$ Kan <sup>r</sup> )	(6)
BY128	$\Delta difA cheW7-1 xylA::magellan4$	(3)
BY146	$\Delta difA cheW7-1 pgi::magellan4$	(3)
BY154	$\Delta difA cheW7-1 xlsA::magellan4$	(3)
YZ1001	$\Delta pgi$	This study
YZ1002	<i>mseA::pZErO</i>	This study
YZ1003	<i>galE::pZErO</i>	This study
YZ1005	<i>rfbB::pZErO</i>	This study
YZ1006	<i>algC::pZErO</i>	This study
YZ1007	$\Delta glmM$	This study
YZ1008	$\Delta rmd$	This study
YZ1009	$\Delta rmd rfbB::pZErO$	This study
YZ1018	$\Delta xylA$	This study
<b>Plasmid</b>		
pBJ113	Gene replacement vector with KG cassette; Kan <sup>r</sup>	(31)
pZErO	Suicide vector	(28)
pLC1001	<i>pgi</i> in-frame deletion in pBJ113	This study
pLC1002	<i>mseA</i> insertion in pZErO	This study
pLC1003	<i>galE</i> insertion in pZErO	This study
pLC1005	<i>rfbB</i> insertion in pZErO	This study
pLC1006	<i>algC</i> insertion in pZErO	This study
pLC1007	<i>glmM</i> in-frame deletion in pBJ113	This study
pLC1008	<i>rmd</i> in-frame deletion in pBJ113	This study
pLC1018	<i>xylA</i> in-frame deletion in pBJ113	This study

**Table 2- 3. Primers used in this study.\***

<b>Primer Name</b>	<b>Gene Affected</b>	<b>Sequence</b>	<b>Restriction Site<sup>o</sup></b>
<b>Deletions</b>			
ORF7425_F1	<i>pgi</i>	TCT <u>TCTAGAC</u> ACCGCTGGCGCTGCTCGG	<i>Xba</i> I
ORF7425_R1	<i>pgi</i>	GCCGCGTCTACTTCGCCTGGCGCATCC GCTCCAGGTAGT	
ORF7425_F2	<i>pgi</i>	CAGGCGAAGTAGGACGCGGC	
ORF7425_R2	<i>pgi</i>	TCC <u>AAGCTT</u> CCGCCGGGAAGAGG	<i>Hind</i> III
ORF7425_Internal	<i>pgi</i>	GAGCAGCTCGCGCGGACAT	
ORF1673_delF1	<i>glmM</i>	GG <u>AAGCTT</u> TCTCGGATGGCGGGCGC	<i>Hind</i> III
ORF1673_delR1	<i>glmM</i>	GTTTCGCAGCGGCCCTCAGACGGCCACA CCTTGTTACCGGG	
ORF1673_delF2	<i>glmM</i>	GTCTGAGGGCCGCTGCGAAC	
ORF1673_delR2	<i>glmM</i>	CCCTCTAG <u>AAGCC</u> ACCAGCACCGCGC	<i>Xba</i> I
ORF1673_F1 <sup>†</sup>	<i>glmM</i>	GCCGT <u>CATCT</u> CCGCGTCCCA	
ORF552_delF1	<i>rmd</i>	AGGACACGCCCTTCTATCCG	
ORF552_delR1	<i>rmd</i>	GCCACGGTCAGCTTCGGGACTACCAGA ATGCGCATCTACC	
ORF552_delF2	<i>rmd</i>	GTCCCGAAGCTGACCGTGGC	
ORF552_delR2	<i>rmd</i>	TCACCGTCGAGCACTGGGAG	
ORF552_F1 <sup>†</sup>	<i>rmd</i>	GCATCAACAGCAACGCCCTG	
ORF2674_delF1	<i>xylA</i>	GCG <u>AAGCTT</u> CCTCGTTGGCCCCGCGT	<i>Hind</i> III
ORF2674_delR1	<i>xylA</i>	GTTCTCTTCGTTGCCAGTGAATCTTCGC GCCGGCAAATA	
ORF2674_delF2	<i>xylA</i>	TAGTTTGGCGGCGGAAGAT	
ORF2674_delR2	<i>xylA</i>	<u>TCTAGAC</u> GTTGGCGTAGCGGAG	<i>Xba</i> I
ORF2674_Internal	<i>xylA</i>	GAAGGTTCCGCTGGAAGAGG	
<b>Insertions</b>			
ORF7894_F1	<i>algC</i>	CTTCGCCGCCAACACCTGC	
ORF7894_R1	<i>algC</i>	CCGGGCTCTCCTTCAGCACG	
ORF7894_F2	<i>algC</i>	GCTCTTACCCAGGGACACG	
ORF7894_R2	<i>algC</i>	AGTGAAGGCATCCGACACGC	
ORF5416_F1	<i>mseA</i>	CCTGCCCCGGACGTCCATCT	
ORF5416_R1	<i>mseA</i>	AGGAGGCCCGCCAGAGTCTT	
ORF5416_F2	<i>mseA</i>	TCACCCAGATGTTCGATGTCT	
ORF5416_R2	<i>mseA</i>	CTCGCAGATGACACCCCTCA	
ORF5416_R3	<i>mseA</i>	CCAGCACCAGCAGCCAGTCG	
ORF1362_F1	<i>rfbB</i>	AGTCTACGTACGCCGGCAAC	
ORF1362_R1	<i>rfbB</i>	GCCCTTCTCCAGCGCCAATAG	
ORF2671_F1	<i>galE</i>	GGGAGAACCTGGACCCGCGT	
ORF2671_R1	<i>galE</i>	TCGGCCAGCAGCGCGTAGAG	
ORF2671_F2	<i>galE</i>	CATCTTCAACACCTACGGTC	
ORF2671_R2	<i>galE</i>	CCGCACGACTGGGCCTTGA	

\*The sequence of primers used to create mutations in the indicated genes. “F” indicates a primer oriented in the forward, 5’ to 3’, direction. “R” indicates a primer is oriented in the reverse, 3’ to 5’, direction. R1 primers for deletions contain the a complementary fragment corresponding to the sequence of F2 allowing for the overlapping of the two fragments.

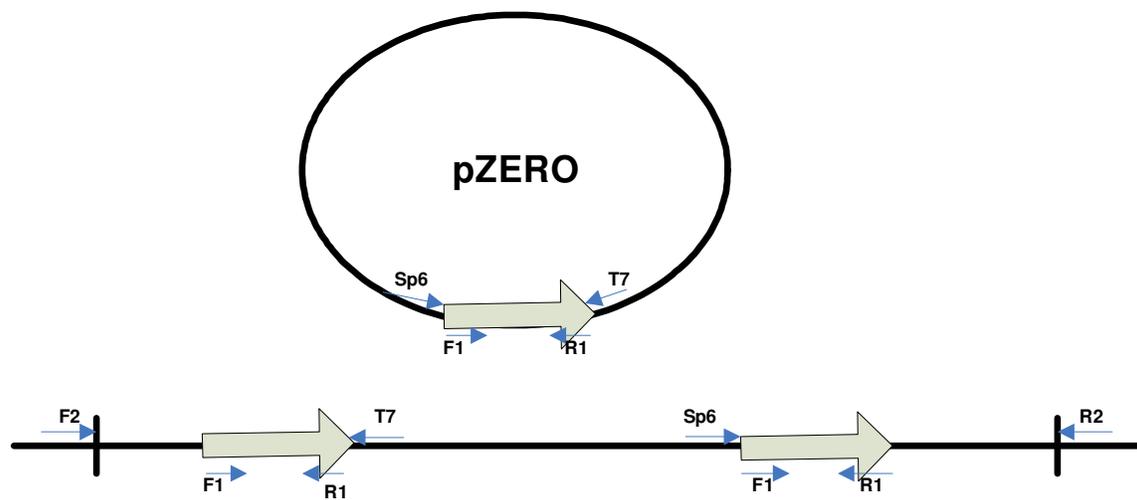
<sup>o</sup>Restriction sites are underlined in the primer.

<sup>†</sup>These primers were originally designed to construct an insertion fragment and were later used as internal primers to check corresponding in-frame deletions.

The construction of YZ1007 ( $\Delta glmM$ ) mutant differed slightly. Instead of using positive-negative selection with kanamycin and galactose, possible mutants were first grown in the presence of N-acetyl-glucosamine and kanamycin to select for the presence of the plasmid. These mutants were then transferred simultaneously to CYE-galactose plates supplemented with N-acetyl-glucosamine and plates without N-acetyl-glucosamine supplementation. Mutants which displayed growth on the supplemented media but not the unsupplemented media were tested using the PCR method described above to determine whether the genome contained the wild type gene or an in-frame deletion.

Targeted insertion mutations were constructed by integrating the pZErO-derived (28) insertion plasmid (Table 2-2) at the site of the open reading frame of interest. Plasmids were transformed into the *M. xanthus* strain DK1622 using electroporation (36). Resulting colonies were kanamycin resistant and had incorporated the plasmid into the genomic backbone. Incorporation at the target gene was confirmed using the PCR method shown (Figure 2-1). YZ1002, YZ1003, YZ1005, and YZ1006 were constructed in this manner using plasmids pLC1002, pLC1003, pLC1005, and pLC1006 respectively. YZ1009 was constructed by transforming YZ1008 with genomic DNA from YZ1005 and selecting kanamycin resistant colonies.

**Postulating Biosynthesis Pathways using Genomic Data.** Information provided by the metabolic pathway illustration distributed by Sigma-Aldrich (45) led to the construction of several pathways. Other pathways which are less well studied were proposed by examining relevant literature (7, 11, 18, 20-22, 26, 32, 46, 59, 71). The enzymes identified were compared to the *M. xanthus* genome to search for genes



**Figure 2- 1. Insertion mutation confirmation method.** PCRs were performed using the following combinations of primers: Reaction 1=F1+T7, Reaction 2=R1+Sp6, Reaction 3=F2+T7, Reaction 4=R2=Sp6. If the first two reactions produce a product of the predicted size, this shows that the plasmid is present in the strain. If the second two reactions produce a product of the predicted size, this shows that the plasmid has recombined with the intended target (namely the gene of interest).

with homology. Recently, a database of pathways for many organisms known as MetaCyc has incorporated *M. xanthus* as a searchable species (10). Using this database, the previously developed pathways were refined and a pathway for the production of arabinose was postulated.

**Motility Assays.** Motility was analyzed as previously described (3, 55). Briefly, 5  $\mu\text{L}$  of an approximately  $5 \times 10^9$  cells/mL morpholinepropanesulfonic acid (MOPS) buffer (10 mM MOPS [pH 7.6], 2 mM  $\text{MgSO}_4$ ) was spotted onto the center of an agar plate containing either 1.5% or 0.4% agar. The plates were then incubated at  $32^\circ\text{C}$  for two to five days respectively before being viewed both macroscopically and microscopically to assess colony spreading and morphology, both at the edge and overall.

**Fruiting Body Development.** Development of fruiting bodies was examined similarly to the motility assays mentioned above. 5  $\mu\text{L}$  of a  $5 \times 10^9$  cell/mL MOPS buffer was spotted onto the surface of Clone-fruiting (CF) agar plates (25).

**Analysis of EPS Production.** The production of EPS was examined using four methods. The first and one of the most sensitive is an agglutination assay to examine cellular cohesion. This method was similar to that used by Wu *et al* (72). Briefly, cultures were grown overnight and then resuspended to a cell density of approximately  $2.8 \times 10^8$  cells/mL in CYE broth. The optical density at 600 nm of these samples was then measured at various times which differ in various experiments. These measurements were then

plotted based on relative absorbance which was calculated by dividing the optical density of a sample at each time point by the initial optical density of that sample.

Two other methods are plate assays involving the binding of dye to EPS. Calcofluor white, a fluorescent dye, was added to plates to a final concentration of 50  $\mu\text{g}/\text{mL}$ . 5  $\mu\text{L}$  of an approximately  $5 \times 10^9$  cells/mL in MOPS buffer was spotted onto these plates and incubated at 32°C for 6 days. The plates were then photographed under a handheld UV light source (47). A similar plate assay was done using the non-flourescent dye Congo red at a concentration of 30  $\mu\text{g}/\text{mL}$ .

In addition, trypan blue was used in a liquid dye binding assay to measure the amount of EPS produced in some strains. The assay was originally adapted from Arnold and Shimkets (1) by Black (3). Briefly, cells were grown to similar culture densities, washed, and resuspended in MOPS buffer. Then 900  $\mu\text{L}$  of the cell suspension was mixed with 100  $\mu\text{L}$  of a trypan blue stock solution to give final concentrations of  $2.5 \times 10^8$  cells/mL and 10  $\mu\text{g}$  of trypan blue/mL. Each sample, including a control of MOPS buffer in place of cell suspension, was assayed in triplicate. After being briefly mixed using a vortex mixer, the suspensions were incubated at room temperature for 30 minutes after which the cells were pelleted at 16,000 x g for 5 minutes. The absorbance of each supernatant was measured at 585 nm and the percentage of bound dye was calculated relative to the wild type by first dividing the absorbance of each sample by the absorbance of the control.

**LPS Extraction and Examination.** LPS were isolated from cell cultures using the method described by Fink and Zissler (17) except that cells were harvested at a

density of approximately  $4 \times 10^8$  cells/mL, twice the density used in the original reference. Briefly, cells were lysed by sonication, incubated with RNase and DNase, and LPS were extracted from the remaining material using a phenol water separation method. The pellets obtained after dialysis against water and lyophilization were suspended in 50  $\mu$ L distilled water. 10  $\mu$ L of the suspension was boiled for 10 minutes with an equal volume of 0.1 M Tris-HCl buffer, pH 6.8, containing 2% (w/v) SDS, 20% sucrose (w/v), 1% 2-mercaptoethanol (v/v), and 0.001% bromophenol blue (w/v) (65). These samples were then loaded onto a 15% polyacrylamide gel with a final concentration of approximately 4% urea and run at 100 volts for 60 minutes or until the dye front had reached the bottom of the gel. The gels were then stained using the silver staining kit from BioRad.

## RESULTS

**Transposon mutagenesis identified sugar biosynthesis genes as important for EPS production.** To identify genes important for EPS production, YZ101, a *difA* suppressor strain, was mutagenized using the *magellan4* transposon (3) and screened for mutants deficient in EPS production. Insertion in three mutants occurred in genes important for the biosynthesis of nucleotide monosaccharides known to be found in EPS (2, 54) (Table 2-2). More specifically, BY146 has an insertion in *pgi* which encodes glucose-6-phosphate isomerase that catalyzes the reversible isomerization of fructose-6-phosphate to glucose-6-phosphate. BY154 has an insertion in ORF5483 which appears to encode a UDP-glucose 6-dehydrogenase, an enzyme which catalyzes the oxidation on UDP-glucose to UDP-glucuronic acid. BY128 contains an insertion in ORF02674 which

encodes a putative UDP-glucuronic acid decarboxylase that catalyzes the conversion of UDP-glucuronic acid to UDP-xylose. These three insertions caused a similar EPS negative phenotype in a wild type background, leading us to examine the *M. xanthus* genome for genes involved in the biosynthesis of other sugars found in EPS.

**Proposing sugar biosynthesis pathways in *Myxococcus xanthus*.** Eight sugar monomers have been found in the EPS of *M. xanthus*: glucose, rhamnose, xylose, mannose, galactose, arabinose, N-acetyl-glucosamine, and N-acetyl-mannosamine (2, 54) (Table 2-1). Using the methods described in the Materials and Methods, pathways leading to the production of the eight nucleotide monomers found in EPS were proposed (Figure 2-2, Table 2-4). All pathways diverge from fructose-6-phosphate, one of the products of gluconeogenesis. Two pathways lead to TDP- or GDP-rhamnose. All other sugars have only one pathway leading to the synthesis of the activated form (Figure 2-2). Currently, no pathway has been identified for the production of nucleotide arabinose. However, a pathway leading to arabinose-5-phosphate appears to be present in *M. xanthus*.

**Construction of mutants in each pathway.** Mutants were constructed to disrupt the proposed pathways for each nucleotide sugar as described in the Materials and Methods. In each main pathway, the objective was to remove or alter a gene product essential for the pathway. Insertions were used when the gene encodes the last open reading frame in a likely operon. Otherwise, in-frame deletions were constructed or attempted. The exception is YZ1006 (*algC::pZErO*), which actually does have the



**Table 2- 4.** Enzymes in proposed sugar biosynthesis pathways.

<b>Gene Name</b>	<b>Gene Product</b>	<b>Sugar Affected</b>	<b>Accession Number</b>	<b>Open Reading Frame<sup>†</sup>/ MXAN<sup>°</sup></b>
<i>algC</i>	Phosphomannomutase (phosphoglucomutase)	Mannose, GDP-Rhamnose	YP_634622.1	ORF07894 MXAN6499
<i>galE</i>	UDP-glucose 4-epimerase	Galactose	YP_631701.1	ORF02671 MXAN3507
<i>galU</i>	UTP-glucose-1-P uridylyltransferase	Glucose, Galactose, Xylose	YP_629682.1	ORFA00707 MXAN1425
<i>glmM</i>	phosphoglucosamine mutase	N-acetyl-Glucosamine, N-acetyl-Mannosamine	YP_632525.1	ORF01673 MXAN4352
<i>glmS1</i>	Glucosamine fructose-6-P aminotransferase, isomerizing	N-acetyl-Glucosamine, N-acetyl-Mannosamine	YP_629643.1	ORF05067 MXAN1386
<i>glmS2</i>	Glucosamine fructose-6-P aminotransferase, isomerizing	N-acetyl-Glucosamine, N-acetyl-Mannosamine	YP_628771.1	ORF06137 MXAN0501
<i>glmU</i>	UDP-N-acetylglucosamine pyrophosphorylase	N-acetyl-Glucosamine, N-acetyl-Mannosamine	YP_629642.1	ORF05068 MXAN1385
<i>gmd</i>	GDP-mannose 4,6-dehydratase	GDP-Rhamnose	YP_633477.1	ORF00554 MXAN5327
<i>gmmA*</i>	putative phosphoglucomutase/phosphomannomutase	Glucose, Galactose, Xylose, Mannose, GDP-& TDP-Rhamnose	YP_633855.1	ORF00098 MXAN5717
<i>gnd</i>	6-phosphogluconate dehydrogenase, decarboxylating	Arabinose-5-P	YP_629212.1	ORF05605 MXAN0951
<i>gutQ</i>	Arabinose-5-P isomerase	Arabinose-5-P	YP_634060.1	ORF08554 MXAN5923
<i>manA*</i>	putative phosphomannose isomerase	Mannose, GDP-Rhamnose	YP_634465.1	ORF08074 MXAN6340
<i>manC*</i>	mannose-1-phosphate guanylyltransferase	Mannose, GDP-Rhamnose	YP_634624.1	ORF07892 MXAN6501

<i>mseA</i> *	UDP-N-acetylglucosamine 2-epimerase	N-acetyl-Mannosamine	YP_629361.1	ORF05416 MXAN1101
<i>pgi</i>	glucose-6-phosphate isomerase	Glucose, Galactose, Xylose, TDP-Rhamnose, Arabinose-5-P	YP_635022.1	ORF07425 MXAN6908
<i>pgl</i>	6-phospho-Gluconolactonase	Arabinose-5-P	YP_629213.1	ORFA00784 MXAN0952
<i>pgm</i>	phosphoglucomutase	Glucose, Galactose, Xylose, TDP-Rhamnose	YP_633045.1	ORF01051 MXAN4888
<i>rfaA</i>	Glucose-1-P thymidyltransferase	TDP-Rhamnose	YP_632779.1	ORF01364 MXAN4611
<i>rfaB</i>	dTDP-glucose 4,6-dehydratase	TDP-Rhamnose	YP_632781.1	ORF01362 MXAN4613
<i>rfaC</i>	dTDP-4-dehydrorhamnose 3,5-epimerase	TDP-Rhamnose	YP_632778.1	ORFA00200 MXAN4610
<i>rmd</i>	GDP-4-dehydro-6-deoxy-D-mannose reductase	GDP-Rhamnose	YP_633478.1	ORF00552 MXAN5328
<i>rmlD</i> *	putative dTDP-4-dehydrorhamnose reductase	TDP-Rhamnose	YP_632780.1	ORF01363 MXAN4612
<i>xlsA</i> *	UDP-glucose 6-dehydrogenase	Xylose	YP_629309.1	ORF05483 MXAN1048
<i>xylA</i> *	putative UDP-glucuronic acid decarboxylase	Xylose	YP_631700.1	ORF02674 MXAN3506
<i>zwf</i>	Glucose-6-P 1-dehydrogenase	Arabinose-5-P	YP_629214.1	ORF05603 MXAN0953

\*Proposed gene name.

†Initial designation given by TIGR (<http://www.tigr.org/>)

°One designation used by XanthisBase ([http://www.xanthisbase.org/myxopedia/index.php/Main\\_Page](http://www.xanthisbase.org/myxopedia/index.php/Main_Page))

possibility of downstream affects, an in-frame deletion is being made to confirm the phenotypic results discussed here at a later date.

YZ1001 ( $\Delta pgi$ ) has the *pgi* gene deleted. The lack of Pgi (phospho-glucose isomerase) may interrupt the pathways for the production of UDP-glucose, UDP-galactose, UDP-xylose, and TDP-rhamnose (Figure 2-2). Deletion of *rmd* (YZ1008) may interrupt the production of GDP-rhamnose. The production of UDP-xylose is interrupted by the deletion of the gene *xylA* (YZ1018). Lastly, the deletion of *glmM* (YZ1007) interrupts the proposed pathway for the production of UDP-N-acetyl-mannosamine and UDP-N-acetyl-glucosamine. Since UDP-N-acetyl-glucosamine is required for cell wall synthesis, this mutant can only grow when N-acetyl-glucosamine is supplemented in the growth media.

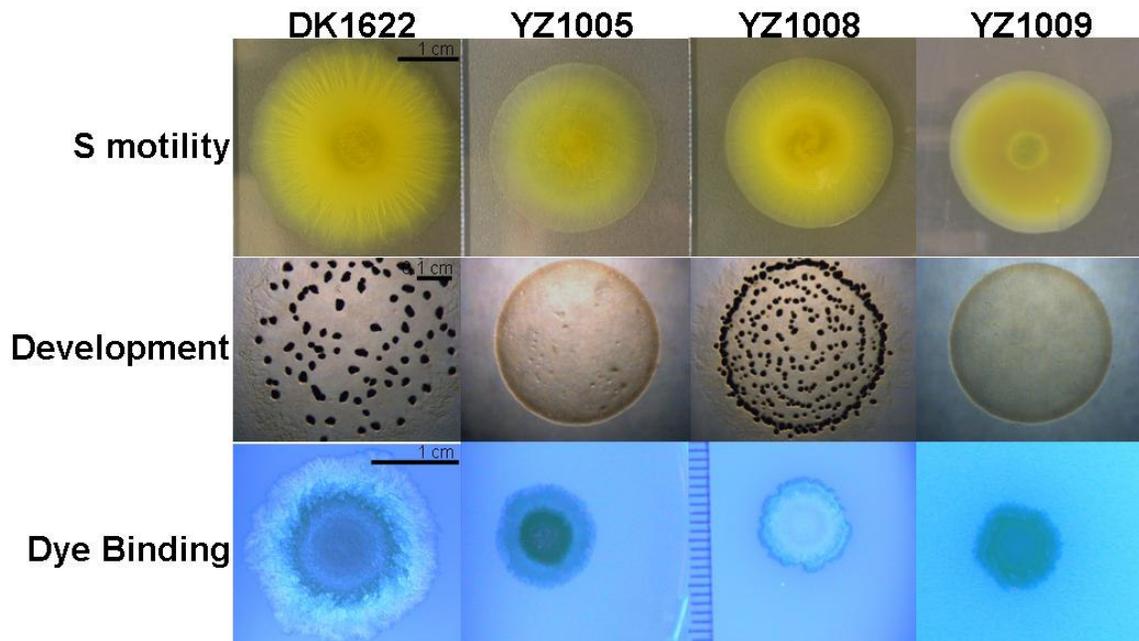
Several interruptions were made by insertion. One mutant with an insertion in *algC* (YZ1006) may not produce UDP-mannose and GDP-rhamnose. A *rfbB* insertion mutant (YZ1005) may not produce TDP-rhamnose. An insertion in *mseA* (YZ1002) may not produce N-acetyl-mannosamine. Finally, YZ1003 may not produce UDP-galactose (Figure 2-2). YZ1002 (*mseA::pZErO*) displayed no detectable defects in the assays discussed below (data not shown). The remaining mutants could be separated into several main groups based on their phenotypes as described in the following sections.

### **Production of NDP-rhamnose may not be essential for EPS and motility.**

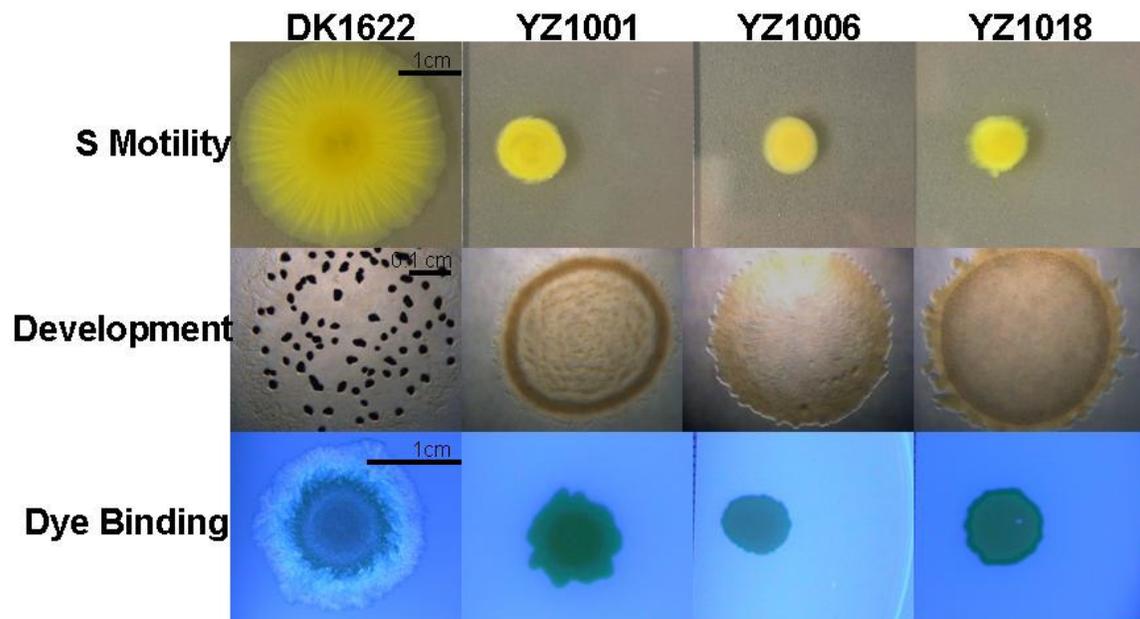
YZ1005 (*rfbB::pZErO*) and YZ1008 ( $\Delta rmd$ ) have mutations which disrupt the biosynthesis of TDP- and GDP-rhamnose respectively (Figure 2-2). Both of these mutants bind calcoflour white and Congo red dyes in plate assays indicating the

production of EPS. YZ1008 binds calcofluor white much better than YZ1005 (Figure 2-3). When examined on soft agar, their colony sizes and morphology looked similar to that of wild type (Figure 2-3), indicating the retention of S motility. Also, both of these mutants agglutinate in a manner similar to wild type when suspended in broth (Figure 2-8). Interestingly, YZ1008, but not YZ1005, was able to form fruiting bodies (Figure 2-3). A mutant (YZ1009) containing mutations in both genes behaved similarly to YZ1005. These findings indicate that disruption of the production of TDP-rhamnose appears to affect the production and/or functionality of EPS more severely than the disruption of the production of GDP-rhamnose.

**Production of NTP-glucose, mannose, and xylose are essential for EPS production.** The second group of mutants, YZ1001, YZ1006, and YZ1018, loses S motility, is unable to bind dye, and fails to form mature fruiting bodies (Figure 2-4). In YZ1001 ( $\Delta pgi$ ), production of UDP-glucose, UDP-xylose, UDP-galactose, and TDP-rhamnose may be interrupted. In YZ1006 (*algC::pZErO*), UDP-mannose and GDP-rhamnose production may be interrupted. Finally, UDP-xylose production may be interrupted in YZ1018 ( $\Delta xylA$ ) (Figure 2-2). All three mutants form colonies approximately 30% the size of wild type on soft agar (Figure 2-4). When these mutants were tested using the agglutination assay, all displayed an agglutination pattern similar to a mutant known to be EPS negative (*AdifE*) (Figure 2-8). These results indicate that UDP-xylose and UDP-mannose are essential for the production of EPS. UDP-glucose may also be important, although this is unclear because disrupting the production of UDP-glucose affects the production of UDP-xylose as well as UDP-galactose.



**Figure 2- 3. Phenotypic testing of mutants in the rhamnose pathway.** Displayed above are assays for S motility (as seen by the colony movement on soft agar), development (fruiting body formation on starvation media), and calcoflour white dye binding properties of strains with mutations affecting the production of rhamnose. (DK1622 = wild type, YZ1005 = *rfbB*::pZErO (TDP-rhamnose), YZ1008 =  $\Delta$ *rmd* (GDP-rhamnose), YZ1009 =  $\Delta$ *rmd*, *rfbB*::pZErO (TDP- and GDP-rhamnose) (Photographs by author.)

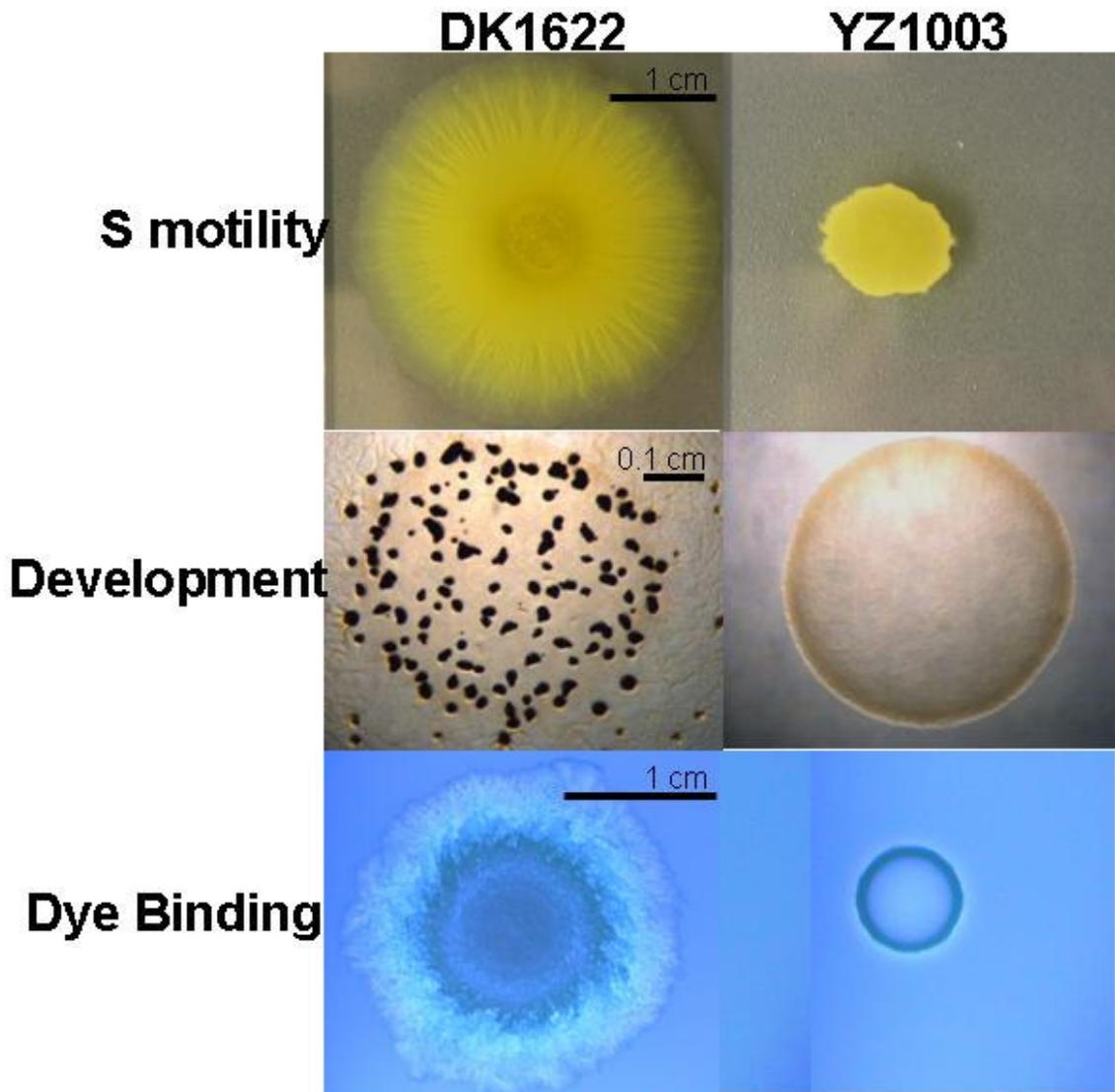


**Figure 2- 4. Phenotypic testing of mutants lacking EPS.** Assays as performed in Figure 2-3. (DK1622 = wild type, YZ1001 =  $\Delta$ *pgi* (UDP-glucose, UDP-xylose, UDP-galactose, TDP-rhamnose), YZ1006 = *algC*::pZErO (UDP-mannose and GDP-rhamnose), YZ1018 =  $\Delta$ *xylA* (UDP-xylose)) (Photographs by author.)

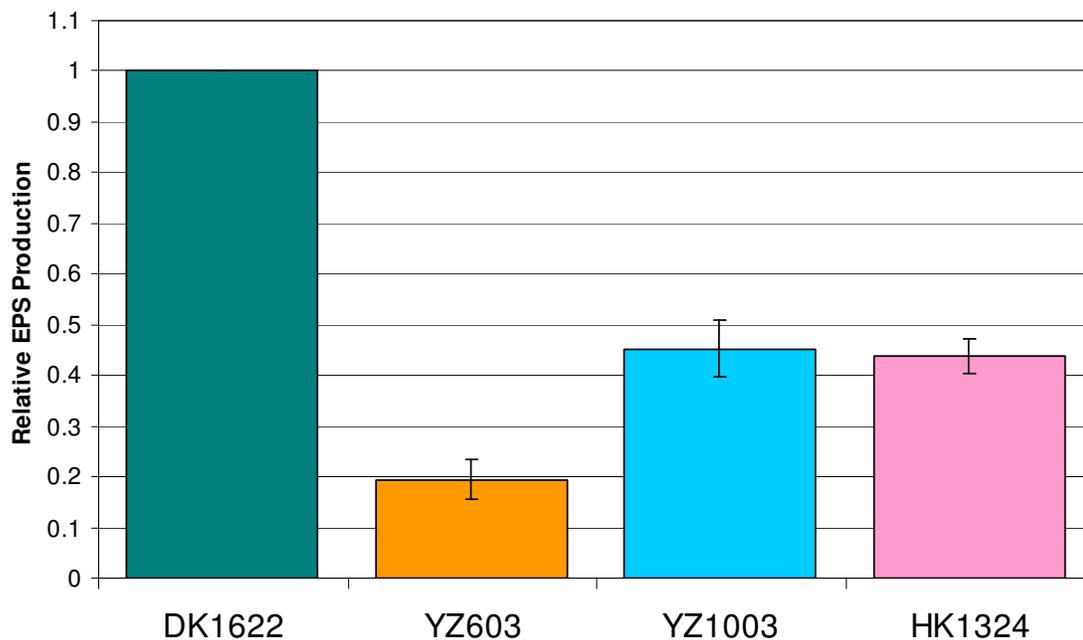
**Mutant with disrupted UDP-galactose pathway loses S motility but may retain EPS production.** One mutant, YZ1003 (*galE::pZErO*), comprises the last group. It displays S motility defects on soft (0.4%) agar and developmental deficiencies on starvation media (Figure 2-5). Surprisingly, it binds both calcoflour white and Congo red in plating assays (Figure 2-5). While the dye binding plate assay seems to indicate the presence of EPS, the agglutination assay, also used to detect EPS, has somewhat ambiguous results with YZ1003 agglutinating more than the EPS negative control (*ΔdifE*) but less than the EPS positive control (wild type) (Figure 2-8).

A liquid dye binding assay was performed to determine if the EPS produced by this mutant are attached to the cell surface or released into the environment. In this assay, trypan blue in solution binds to EPS and the solution is then centrifuged to remove any bound dye associated with the cells. The results indicate that cell-associated EPS of this strain is found in amounts similar to those of the LPS mutant control (Figure 2-6). This EPS is possibly non-functional or minimally function in terms of motility but still remains able to bind dye in this assay.

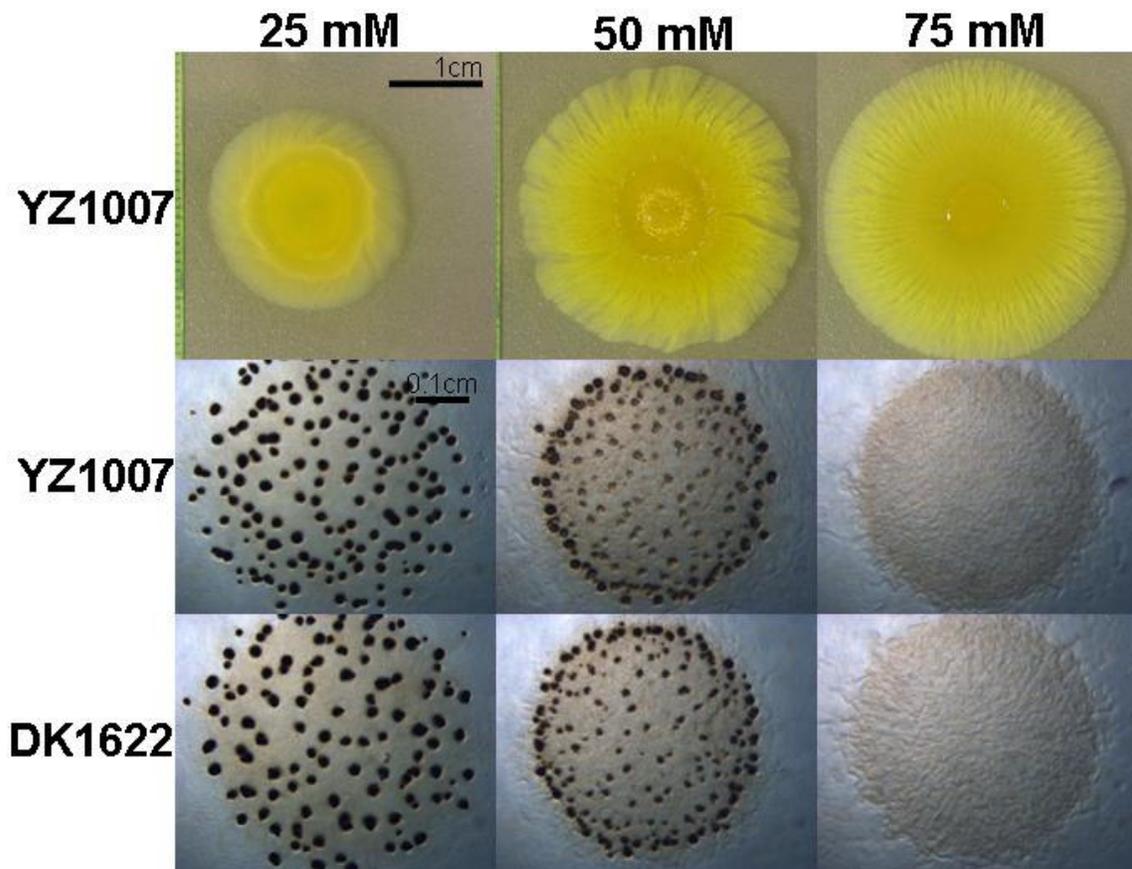
**The deletion of *glmM* requires supplementation of N-acetyl-glucosamine in the growth media.** Although N-acetyl-glucosamine is essential for the production of peptidoglycan, a gene needed for the production of this sugar can be deleted if the mutant is supplied with N-acetyl-glucosamine in the growth media. The swarming ability on soft agar of the mutant strain is directly related to the concentration of N-acetyl-glucosamine present with higher concentrations of the sugar leading to the recovery of a wild type phenotype (Figure 2-7).



**Figure 2- 5. Phenotypic testing of UDP-galactose deficient mutant.** Assays as performed in Figure 2-3. (DK1622 = wild type, YZ1003 = *galE*::pZErO (UDP-galactose)) (Photographs by author.)



**Figure 2- 6. Trypan Blue liquid dye binding assay.** After cells are incubated with the trypan blue dye, the cells are pelleted and optical densities of the supernatants at 585 nm are measured. All measurements are compared to the optical density of a blank control. The graph indicates the relative EPS production in reference to DK1622 (wild type) with an EPS negative control YZ603 (*ΔdifE*) and LPS negative control HK1324 (*Δwzt wzm wbgA*) and the mutant strain YZ1003 (*galE::pZErO*).



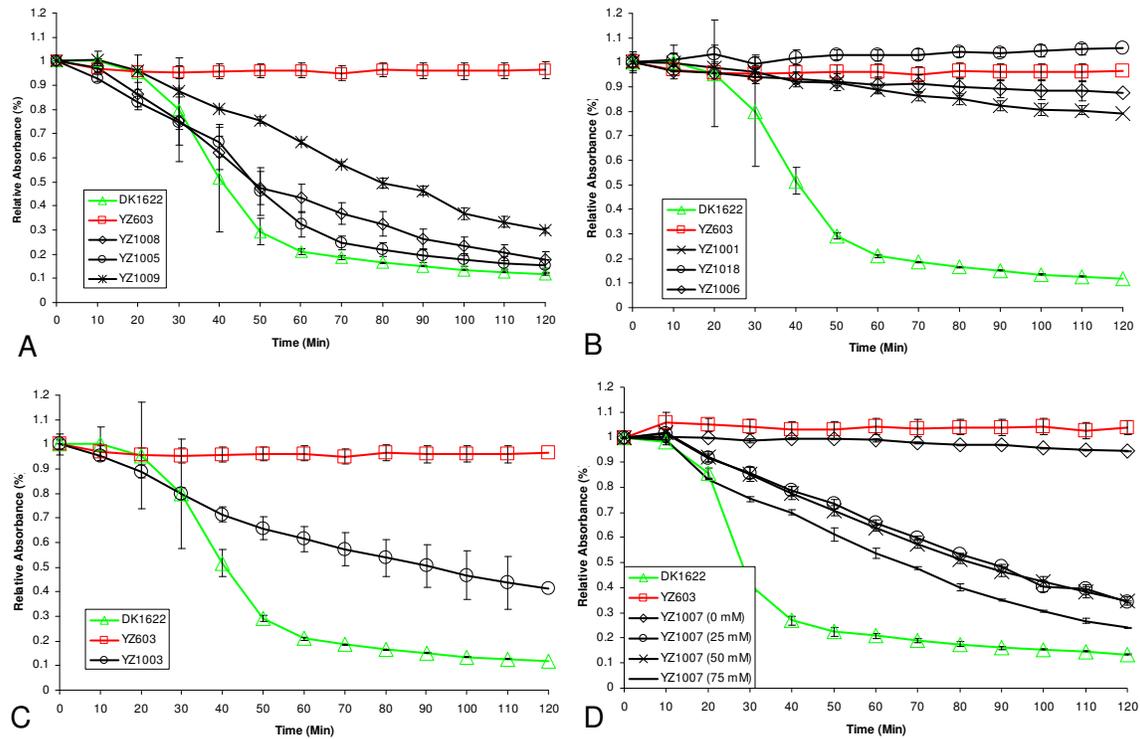
**Figure 2- 7. Recovery of YZ1007 ( $\Delta glmM$ ) in the presence of N-acetyl-glucosamine.** Assays were performed as described in Figure 2-3 except that N-acetyl-glucosamine was supplemented at the indicated concentrations. (DK1622 = wild type, YZ1007 =  $\Delta glmM$  (UDP-N-acetyl-glucosamine)) (Photographs by author.)

This mutant forms fruiting bodies similar to wild type at concentrations of 25 and 50 mM N-acetyl-glucosamine. Both the wild type and the mutant lose the ability to form fruiting bodies in the presence of 75 mM N-acetyl-glucosamine (Figure 2-7).

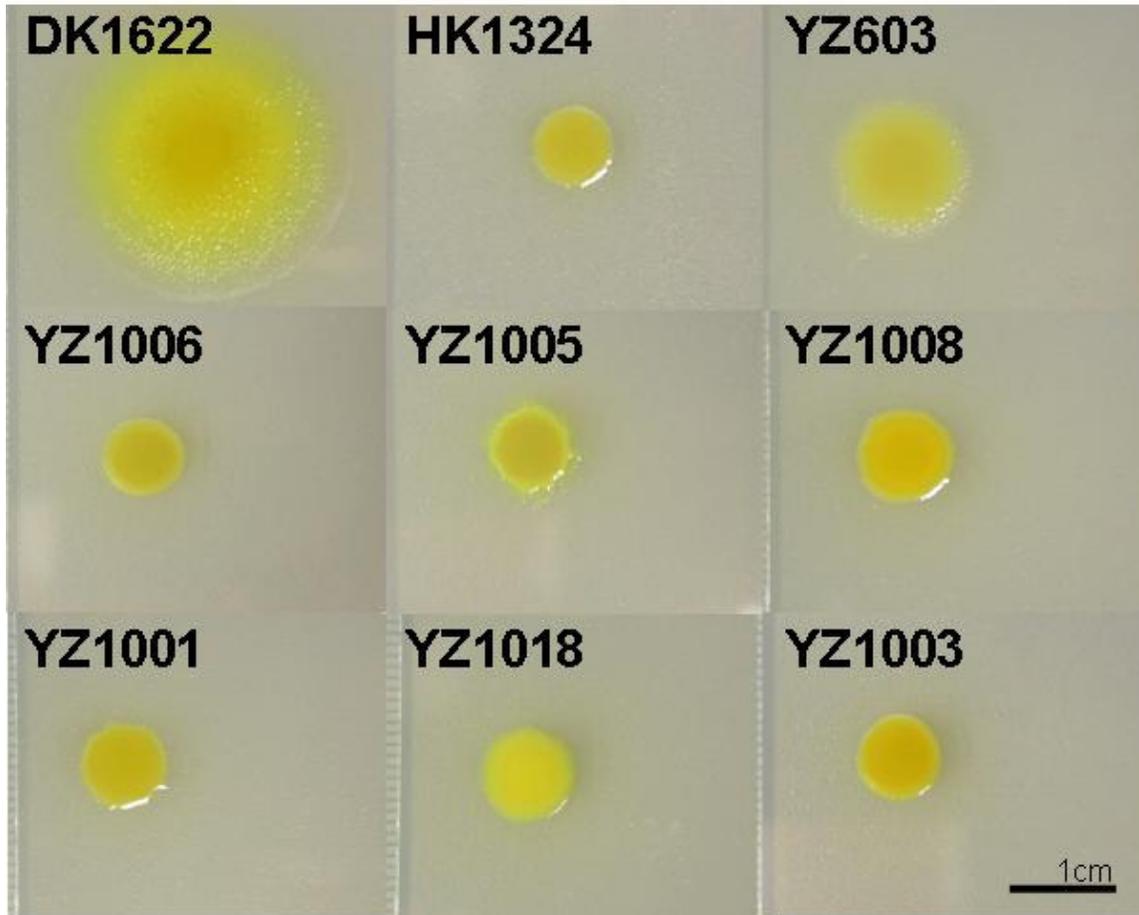
Agglutination is recovered in relation to the sugar concentration but seems to reach a threshold at which recovery is halted short of wild type characteristics (Figure 2-8). Since the phenotype of this mutant is related directly to the concentration of supplemented sugar, no further analysis of EPS production was undertaken.

**Mutants may have structural or functional abnormalities in LPS.** The colony morphology of all mutant strains was examined on hard (1.5%) agar. The colonies of all mutants appeared smooth while that of wild type appears rough (Figure 2-9). These mutant colony morphologies are similar to that of an LPS negative strain, HK1324 ( $\Delta wzt wzm wbgA$ ) (6). These results seem to indicate that in the mutants, the structure or function of LPS is affected. In order to test this possibility, the presence of LPS in each strain was tested.

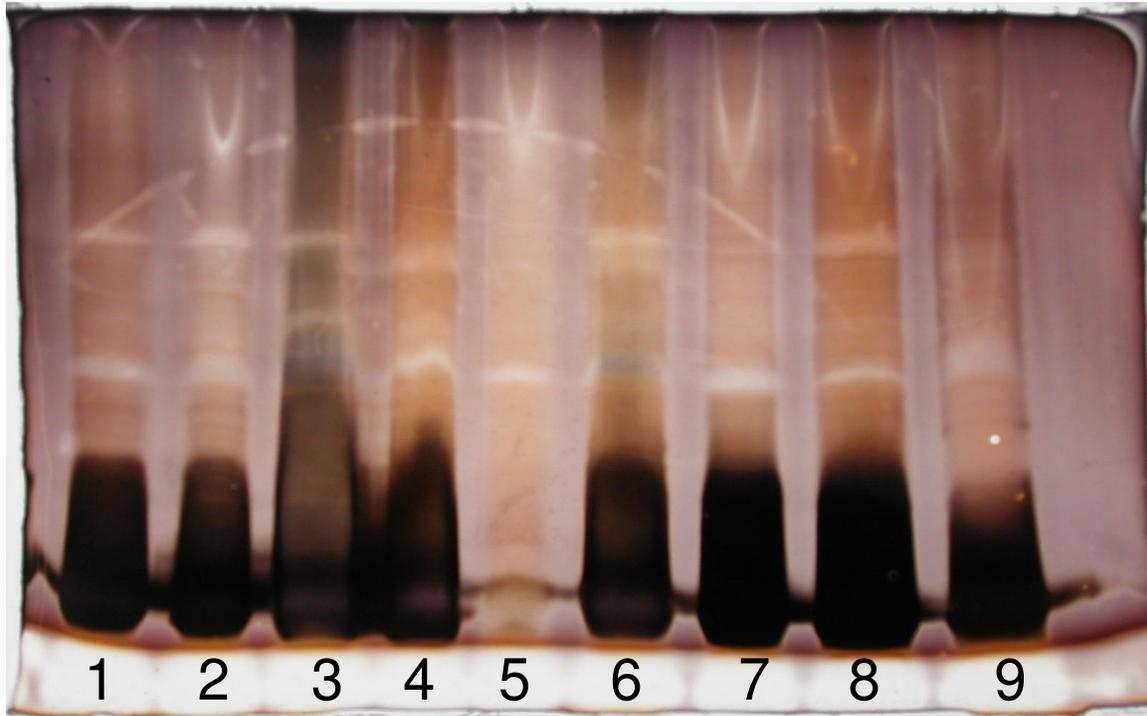
Using the method described in the Materials and Methods, LPS were extracted from all mutants as well as wild type, an EPS negative strain, YZ603 ( $\Delta difE$ ), and an LPS negative strain, HK1324 ( $\Delta wzt wzm wbgA$ ). Samples from each of these were evaluated using polyacrylamide gel electrophoresis and silver staining. Ladder-like bands are visible for all samples, including the LPS negative control, however differences can be seen in the new mutant strains, suggesting that the LPS of each may have been altered (Figure 2-10). These results are preliminary and require further confirmation.



**Figure 2- 8. Agglutination of all strains studied.** An agglutination assay examines the ability of a strain to agglutinate and “fall out” of solution. All of the mutants in this study are compared with wild type and an EPS negative control (YZ603). (A) Mutants in the rhamnose pathways (YZ1005 = *rfbB*::pZErO (TDP-rhamnose), YZ1008 =  $\Delta$ *rmd* (GDP-rhamnose), YZ1009 =  $\Delta$ *rmd rfbB*::pZErO (TDP- and GDP-rhamnose)), (B) All mutants with an EPS negative phenotype (YZ1001 =  $\Delta$ *pgi* (UDP-glucose, UDP-xylose, UDP-galactose, TDP-rhamnose), YZ1006 = *algC*::pZErO (UDP-mannose and GDP-rhamnose), YZ1018 =  $\Delta$ *xyIA* (UDP-xylose)), (C) Galactose deficient mutant (YZ1003 = *galE*::pZErO (UDP-galactose)), and (D) the recovery of the N-acetyl-glucosamine deficient strain by supplementation (YZ1007 =  $\Delta$ *glmM* at varying concentrations of N-acetyl-glucosamine).



**Figure 2- 9. Colony morphologies on hard agar plates.** This figure compares the phenotype of mutant strains on hard agar with DK1622 (wild type) and HK1324 ( $\Delta wzt wzm wbgA$ ), the LPS negative mutant, as controls. Note the smooth, glossy appearance of the LPS negative control and all of the mutant colonies and the rough colonies of wild type and EPS negative YZ603 ( $\Delta difE$ ). (YZ1005 = *rfbB*::pZErO (TDP-rhamnose), YZ1008 =  $\Delta rmd$  (GDP-rhamnose), YZ1009 =  $\Delta rmd rfbB$ ::pZErO (TDP- and GDP-rhamnose), YZ1001 =  $\Delta pgi$  (UDP-glucose, UDP-xylose, UDP-galactose, TDP-rhamnose), YZ1006 = *algC*::pZErO (UDP-mannose and GDP-rhamnose), YZ1018 =  $\Delta xylA$  (UDP-xylose), YZ1003 = *galE*::pZErO (UDP-galactose), YZ1007 =  $\Delta glmM$  (N-acetyl-glucosamine)) (Photographs by author.)



**Figure 2- 10. LPS Gel Analysis.** The SDS-PAGE gel above has been stained with silver to view the ladder-like bands displayed when LPS are extracted from cells. Lanes contain the following samples: 1) DK1622 (Wild Type), 2) YZ603 (EPS negative,  $\Delta difE$ ) 3) HK1324 (LPS negative,  $\Delta wzt wzm wbgA$ ), 4) YZ1001 ( $\Delta pgi$ , UDP-glucose, UDP-xylose, UDP-galactose, TDP-rhamnose), 5) YZ1003 (*galE*::pZERO, UDP-galactose), 6) YZ1005 (*rfbB*::pZERO, TDP-rhamnose), 7) YZ1006 (*algC*::pZERO, UDP-mannose, GDP-rhamnose), 8) YZ1008 ( $\Delta rmd$ , GDP-rhamnose), 9) YZ1018 ( $\Delta xylA$ , UDP-xylose) (Photographs by author.)

## DISCUSSION

We report here the proposed pathways by which *M. xanthus* produces the sugars in EPS. These pathways are constructed based upon the presence of genes encoding the required enzymes for each pathway in the *M. xanthus* genome (24). The presence of the enzymes required for these biosynthesis pathways, coupled with the fact that *M. xanthus* does not use glucose as a primary carbon source, supports the idea that these pathways are responsible for creating the building blocks of EPS and possibly LPS.

In order to test the importance of the pathways proposed in this study, we attempted to systematically disrupt each pathway by mutation and test their effects on EPS production. Mutants in the two pathways for the production of rhamnose displayed behaviors consistent with EPS production. Even when both mutations were introduced into one strain, the double mutant (YZ1009) showed no more phenotypic abnormalities than the single disruption in the pathway for the production of TDP-rhamnose. These results indicate that neither TDP- nor GDP-rhamnose is essential for S motility as all three mutants retain the ability to move on soft agar. While TDP-rhamnose appears important for development and both pathways are important for wild type dye binding, GDP-rhamnose seems to play a smaller role in all of these behaviors than TDP-rhamnose.

Most of the other pathways appear to play integral roles in EPS-dependent behaviors. Mutants in the pathways for the production of GDP-mannose (YZ1006), UDP-xylose (YZ1018), UDP-glucose and other sugars (YZ1001) are all defective in S motility, dye binding, and developmental. These results indicate the importance of these sugars in *M. xanthus* EPS-dependent behaviors.

YZ1007 contains an interruption in the pathway leading to the production of UDP-N-acetyl-glucosamine. Construction of YZ1007 was possible only when N-acetyl-glucosamine is supplied in the growth media. These results indicate that although *M. xanthus* does not use glucose or other sugars as a primary carbon source, it can transport at least N-acetyl-glucosamine across the membrane and use it for certain metabolic purposes within the cell.

Possibly the most unique mutant strain is YZ1003 with an interruption in the pathway leading to the production of UDP-galactose. This strain is unable to move on hard or soft agar. It also has an intermediate agglutination in comparison to the wild type and an EPS negative control. Yet it retains the ability to bind calcoflour white dye in plate assays. The trypan blue liquid dye binding assay showed that this mutant binds dye in amounts similar to an LPS mutant HK1324 ( $\Delta wzt wzm wbgA$ ). These results suggest that interrupting the proposed UDP-galactose biosynthesis pathway leads to defects in EPS and LPS biosynthesis.

Further evidence supporting a defect in the LPS of these mutants came from the colony morphology and LPS analyses. The colonies of all mutants appeared similar to an LPS defective strain with known motility defects. When we compared the LPS of the various mutants and controls, it was found that there are distinct changes in each mutant. The most significant difference was displayed by YZ1003 which appeared to lose the most LPS compared to all the others. Some of the phenotypic changes observed for the mutants may have been due to a change in the LPS, a change in the EPS, or a combination of both.

**Chapter 3: Using the NarX-DifA Chimeric Protein to Help Elucidate  
the Point of Regulation of Exopolysaccharide Production by the Dif  
Chemotaxis Pathway in *Myxococcus xanthus***

## ABSTRACT

*Myxococcus xanthus* is a Gram negative soil bacterium which does not use glucose or other sugars as a primary carbon source. However, this organism produces extracellular polysaccharides (EPS) which are essential for social motility. Social (S) motility involves the movement of large groups of cells and is required for development and movement on soft (0.4%) agar. EPS are one of the cellular structures required for this motility. Currently, the chemotaxis-like Dif (defective in fruiting/fibrils) pathway is known to regulate the production of EPS, but the exact point of regulation is unknown. In this study, we demonstrated that EPS production could be restored to the EPS negative  $\Delta pgi$  mutant by glucose supplementation. Along with the ability of another mutant strain to activate the Dif pathway in the presence of nitrate, the location of regulation by the Dif pathway was explored. The results, although not conclusive, point to the Dif pathway regulating EPS production downstream of sugar monomer production.

## INTRODUCTION

*Myxococcus xanthus* is a Gram negative bacterium commonly found in the soil (16). These bacteria display unique multicellular behaviors including the wolf-pack-hunting behavior in which large groups of cells “attack” and consume other bacteria, and development during which large groups of cells aggregate and differentiate into fruiting bodies consisting of environmentally resistant myxospores (14, 16, 35). Social (S) motility, one of two motility systems in *M. xanthus*, is required for the movement of large groups of these cells (30).

S motility requires two cellular structures: type IV pili (TFP) and extracellular polysaccharides (EPS) (35). The production of EPS is known to be regulated by the chemotaxis-like Dif (defective in fruiting/fibrils) pathway (77). However, it is unknown exactly how the Dif pathway regulates the process of EPS production. Previously, DifA, the signaling protein in the Dif pathway, was combined with NarX, a nitrate sensor (40). In a  $\Delta difA$  mutant containing the resulting chimeric protein, NafA, EPS production is regulated by the presence of nitrate in the media (73). This study also showed that DifC and DifE are essential for the propagation of the signal from DifA.

*M. xanthus* does not use glucose or other sugars as a primary carbon source (8, 12, 27, 67). Interestingly, this organism produces exopolysaccharide containing glucose and at least seven other monosaccharides (see Chapter 2). In this study we show the EPS negative  $\Delta pgi$  (phospho-glucose isomerase) mutant which lacks the enzymes required to produce glucose-6-phosphate through gluconeogenesis is able to produce EPS when grown in media containing glucose. Using the  $\Delta pgi$  mutant with NafA, an experiment to narrow the possible reactions regulated by the Dif pathway was conceived. The NafA

protein was expressed in a  $\Delta pgi difA$  double deletion mutant. Theoretically, this mutant strain (YZ1017) can only produce EPS in the presence of both nitrate and glucose. So, if the strain is introduced to these substances in sequence, it may be possible to discover whether the Dif pathway regulates EPS production upstream or downstream of monosaccharide production. The results of this experiment were unfortunately inconclusive with some indications that the Dif pathway likely does not regulate EPS production upstream of sugar biosynthesis.

## **MATERIALS AND METHODS**

**Bacterial strains and growth conditions.** Unless otherwise noted, all strains are grown on CYE plates with 1.5% agar or in CYE broth. Plates and cultures are incubated at 32°C in a stationary or shaking incubator, respectively. *M. xanthus* strains used in this study are listed in Table 3-1. Kanamycin was added to media at 100 µg/ml for selection purposes when appropriate.

**Construction of mutants.** The construction of  $\Delta pgi$  (YZ1001) is described in Chapter 2 of this document. Briefly, an in-frame deletion of the *pgi* gene was made by using PCR to amplify portions of the genome directly upstream and downstream of *pgi* and an overlapping PCR was used to fuse these pieces (53). The resulting fragment was then ligated into pBJ113 (31) and the resulting plasmid, pLC1001, was transformed into wild type *M. xanthus*, DK1622. Colonies were selected using a modified positive-negative kanamycin/galactose (KG) cassette (66) and confirmed using triple primer PCR as described in Chapter 2.

YZ1001 was transformed by electroporation with the plasmid pWB116 to produce *Δpgi difA* (YZ1011). The plasmid pXQ719 (73) was then transformed into this strain to produce YZ1017 (*Δpgi difA/Pdif-nafA*; Kan<sup>r</sup>).

**Motility Assays.** Methodology for this experiment is previously described (3, 55). Briefly, 5  $\mu$ L of an approximately  $5 \times 10^9$  cells/mL MOPS (morpholinepropanesulfonic acid) buffer (10 mM MOPS [pH 7.6], 2 mM MgSO<sub>4</sub>) solution was spotted onto the center of an agar plate containing either 1.5% or 0.4% agar. The plates were then allowed to grow at 32°C for two to five days respectively before being viewed both macroscopically and microscopically to assess colony spreading and morphology, both at the edge and overall.

**Agglutination Assay.** One of the most sensitive ways to detect EPS production is an assay to examine the cellular cohesion known as an agglutination assay. The methods were similar to those used by Wu et al (72). Briefly, cultures are grown overnight and then resuspended to a cell density of approximately  $2.8 \times 10^8$  cells/mL in Casitone-Tris (CTT) broth. The optical density at 600 nm of these samples is then measured at various time points for two hours. These measurements were then plotted based on relative absorbance which was calculated by dividing the optical density at each time point by the initial optical density for the given strain.

**Table 3- 1. *Myxococcus xanthus* strains and plasmids used in this study.**

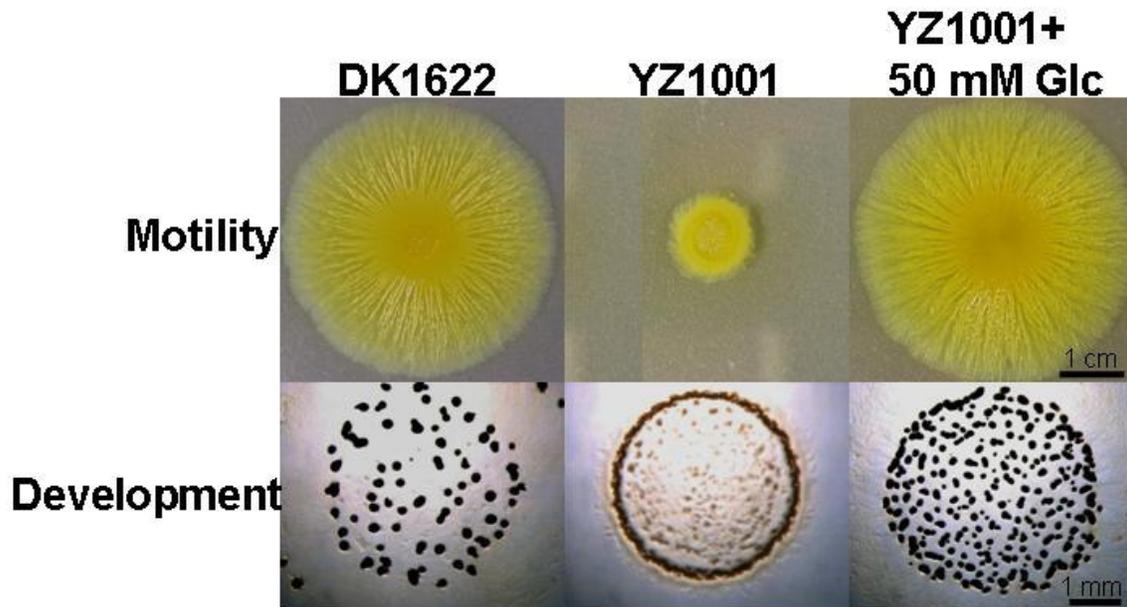
<i>M. xanthus</i> strain or plasmid	Relevant genotype or description	Source or reference
<b>Strain</b>		
YZ1001	$\Delta pgi$	This study
YZ601	$\Delta difA$	(73)
YZ1011	$\Delta pgi difA$	This study
YZ1017	$\Delta pgi difA/Pdif-nafA$ ; Kan <sup>r</sup>	This study
<b>Plasmids</b>		
pWB116	<i>difA</i> in-frame deletion on pBJ113	(73)
pXQ719	<i>Pdif-nafA</i> in pWB200; Kan <sup>r</sup>	(73)

**Modified Agglutination Assay.** Cells are grown in 5 mL CYE broth overnight shaking at 37°C. These cells are then inoculated into 50 mL CTT broth at an approximate optical density at 600 nm of 0.1 and allowed to grow overnight shaking at 37°C to an optical density at 600 nm range of 0.3 to 0.6. The cells are collected by centrifugation, washed twice with CTT and resuspended to an optical density at 600 nm of 0.5 in 5 ml CTT in tubes containing a final concentration of 100  $\mu$ M nitrate, 50 mM glucose, or nothing. These tubes are then incubated for 2 hours shaking at 37°C. At the end of 2 hours, the cells were collected, washed three times with CTT and resuspended in 5 ml of CTT with glucose, nitrate, or both. These solutions were then incubated another 2 hours at 37°C, shaking. After the final incubation period, an agglutination assay was conducted to detect the presence of EPS.

## RESULTS

**Recovery of the  $\Delta$ *pgi* mutant in the presence of glucose.** YZ1001 ( $\Delta$ *pgi*) is a mutant which displays an EPS negative phenotype. However, when this strain is grown on media containing glucose, it is able to recover a phenotype similar to that of wild type (Figure 3-1). YZ1001 becomes motile on soft agar and forms fruiting bodies on starvation media when glucose is supplemented. Both of these phenotypes indicate that when glucose is present, YZ1001 is able to produce EPS.

**Construction of a  $\Delta$ *pgi difA* mutant containing *NafA*.** A mutant with both the *pgi* and *difA* genes deleted was constructed as described in the Materials and Methods. The plasmid which contains *nafA* was then introduced into this double mutant to generate



**Figure 3- 1. Recovery of YZ1001 in the presence of glucose.** The above assays depict the recovery of wild type EPS production in YZ1001 ( $\Delta pgi$ ) when glucose is present in the growth media. The first row of panels displays colony movement on soft agar while the second row is showing fruiting body development on starvation media. (Photographs by author.)

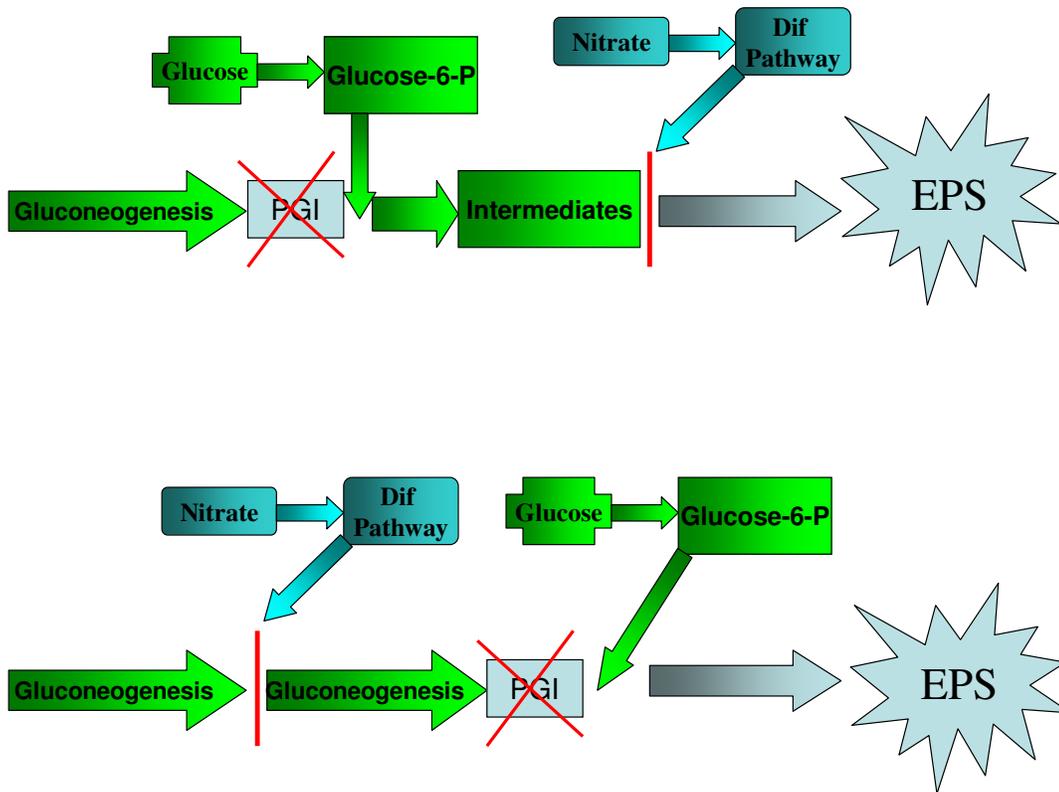
YZ1017. YZ1017 displayed phenotypes indicating that EPS was being produced only in the presence of glucose and nitrate (data not shown).

**Combining two recoverable mutations in one strain creates a unique “switch” system.** If glucose and nitrate are added to YZ1017 in sequence, nitrate followed by glucose or vice versa, the inherent mutations in the strain would allow these compounds to act as “switches” turning portions of the EPS production process on and off (Figure 3-2). An agglutination assay can then be used to determine the conditions under which the cells produce the most EPS. Since cells will theoretically stockpile intermediates to some extent, one series of exposures should produce more EPS than the other.

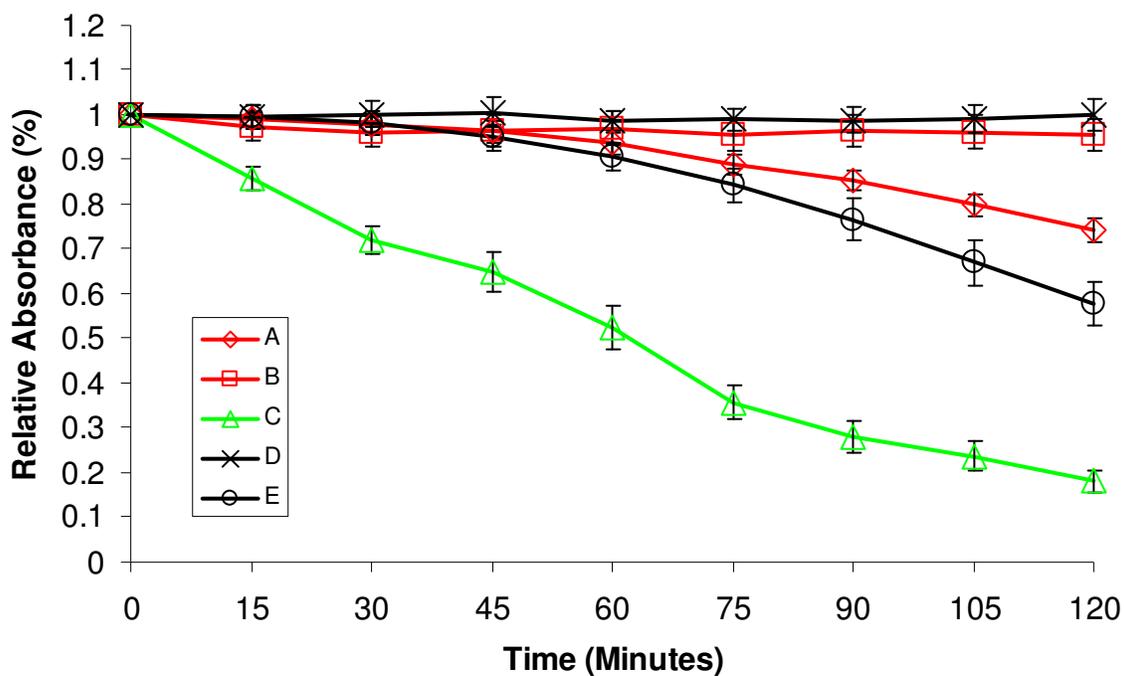
When this experiment was performed, the results were somewhat unclear. The sample which was exposed first to glucose and then to nitrate agglutinated better than the opposite sequence of exposures, nitrate followed by glucose. However, the glucose-nitrate sample did not agglutinate as well as the positive control which was exposed to both glucose and nitrate simultaneously (Figure 3-3).

## **DISCUSSION**

Tentatively, looking at sample D (nitrate followed by glucose) displayed in Figure 3-3, the lack of agglutination of this sample is preliminary evidence that the Dif pathway does not regulate EPS production upstream of glucose production and therefore all monosacchride production. On the other hand, the sample E (glucose followed by nitrate) does not agglutinate similarly to the positive control C (glucose and nitrate



**Figure 3- 2. Two possible EPS regulation schemes.** This diagram depicts how the NafA chimeric protein and the deletion of the *pgi* gene can be used as a “switch” system to determine whether the Dif pathway regulates EPS production upstream or downstream of monosaccharide production. The red Xs indicated that the enzyme is not present and production stops at this point. The red vertical lines indicate points at which intermediates would accumulate when the signal from the Dif pathway is not present. (Drawing by author.)



**Figure 3- 3. Agglutination of YZ1017 under various supplementation conditions.** Above is a graph displaying the agglutination of strain YZ1017 (*Δpgi difA/Pdif-nafA*) in varying conditions. A) Negative Control (1<sup>st</sup> incubation = CTT only, 2<sup>nd</sup> incubation = CTT + 100 μM nitrate); B) Negative Control (1<sup>st</sup> incubation = CTT only, 2<sup>nd</sup> incubation = CTT + 50 mM glucose); C) Positive Control (1<sup>st</sup> incubation = CTT only, 2<sup>nd</sup> incubation = CTT + 100 μM nitrate + 50 mM glucose); D) Test Sample (1<sup>st</sup> incubation = 100 μM nitrate, 2<sup>nd</sup> incubation = 50 mM glucose); E) Test Sample (1<sup>st</sup> incubation = 50 mM glucose, 2<sup>nd</sup> incubation = 100 μM nitrate)

simultaneously) indicating that sample E does not produce EPS in amounts similar to the positive control. Unexpectedly, sample A (nitrate only), a negative control, displayed better agglutination than samples B (glucose only), a second negative control, and D (nitrate followed by glucose).

A few scenarios to explain the effect caused by the addition of nitrate alone can be hypothesized at this point. YZ1017 ( $\Delta pgi\ difA/Pdif-nafA$ ) may not contain a completely stable mutation. Also, the “stockpiles” of intermediates formed during a given incubation may not be large enough to produce EPS at wild type levels. Lastly, *M. xanthus* could be affected by nitrate in such a way that the effect is not readily apparent on solid media but becomes a factor in liquid media. The last theory is unlikely as an  $\Delta difA/Pdif-nafA$  *M. xanthus* strain has been tested in liquid medium with nitrate without any of the effects shown here (73). The first theory is also unlikely as all of the mutations introduced into the strain are stable when present alone and are on the genome in locations well removed from one another. The theory that the cell is not “stockpiling” enough intermediates seems the most likely explanation for the reduced level of agglutination compared to wild type. Energetically this explanation is viable because the cell would not waste energy producing monosaccharides that are not being immediately used in cellular processes.

Future studies could undertake the challenge of determining a way to quantify the cell “stockpiles” after the cell has been exposed to glucose. Then how much of those “stockpiles” are used to produce EPS upon the introduction of nitrate could be determined as well. Perhaps this experiment or one similar would be able to definitively

answer the question of whether the Dif pathway regulates EPS production upstream or downstream of glucose and other monosaccharide production.

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## EDUCATION

### **Master of Science, Biological Sciences**

Concentration: Microbiology

Virginia Polytechnic Institute and State University (Virginia Tech), Blacksburg, VA

*Defense:* Fall 2007

*Thesis:* Biosynthesis of Nucleotide Sugar Monomers for Exopolysaccharide  
Production in *Myxococcus xanthus*.

Advisor: Dr. Zhaomin Yang

**Bachelor of Science, Biochemistry;** Minors: Chemistry and Biology, May 2006

Virginia Polytechnic Institute & State University (Virginia Tech), Blacksburg, VA

## HONORS/AFFILIATIONS

Cum Laude, Virginia Tech, May 2006

Virginia Tech Honors Program 2002-2007

National Society of Collegiate Scholars

## RESEARCH INTERESTS

- Genetic mutation and phenotypic effect
- Enzyme kinetics and reactions

## TEACHING EXPERIENCE

- Undergraduate introductory microbiology laboratory courses
- Private tutoring

## RESEARCH EXPERIENCE

### **Researcher**

**United States Army Medical Research Institute of Chemical Defense  
via The Oak Ridge Institute for Science and Education**

*Aberdeen Proving Ground, Edgewood Area*

- Research on native human enzymes which break down toxic agents

**Masters Candidate*****Microbiology Department, Virginia Tech***

June 2005 – Current

- Graduate research project involving the characterization of sugar biosynthesis pathways in *Myxococcus xanthus* using various genetic manipulation techniques

**Undergraduate Research*****Microbiology Department, Virginia Tech***

Aug 2003 – June 2005

- Worked to determine genetic sequence of over seventy transposon mutants
- Prepared sterile media and glassware that was used in the laboratory

**Laboratory Assistant*****Biochemistry Department, Virginia Tech***

May 2004 – Dec 2004

- Entry of sampling data into as yet untested database software
- Compositing of raw food materials for sampling purposes