DEMONSTRATION OF INTERACTIONS AMONG DIF PROTEINS AND THE IDENTIFICATION OF KAPB AS A REGULATOR OF EXOPOLYSACCHARIDE IN *MYXOCOCCUS XANTHUS*

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ABSTRACT

Myxococcus xanthus Dif proteins are chemotaxis homologues that regulate exopolysaccharide (EPS) biogenesis. Previous genetic studies suggested that Dif protein might interact with one another as do the chemotaxis proteins in enterics. The interactions among Dif proteins were since investigated with the yeast two-hybrid (Y2H) system. The results indicate that DifC interacts with both DifA and DifE. Using a modified Y2H system, DifC was shown to be able to bring DifA and DifE into a protein complex. Further Y2H experiments demonstrated that the different conserved domains of DifE likely function as their counterparts of CheA-type kinases because the putative P2 domain of DifE interacts with DifD, P5 with DifC and the dimerization domain P3 with itself. Similarly, DifA can interact with itself through its C-terminal region. In addition, DifG was found to interact with the CheY homologue DifD. These findings support the notion that Dif proteins constitute a unique chemotaxis-like signal transduction pathway in *M. xanthus*.

In addition, KapB, a TPR (Tetratricopeptide repeats) protein, was identified as an interacting partner of DifE byY2H library screening. Further analysis demonstrated that the N-terminal half of KapB interacted with the putative P2 domain of DifE. KapB had been previously reported to interact with several Serine/Threonine (Ser/Thr) kinase pathways including the Pkn4-Pfk pathway. This pathway is implicated in glycogen metabolism in *M.*

xanthus by a previous report. In this study, *kapB* as well as *pfkn* deletion mutants were found to overproduce EPS. It was also found that the Dif pathway is involved in glycogen metabolism because the glycogen level is altered in *dif* mutants. These results indicate EPS biogenesis and glycogen metabolism may be coordinately regulated. This coordination of the Dif-regulated EPS production and the Pkn4-regulated glycogen metabolism appears to involve KapB. This is the first example of a TPR protein mediating the interplays of a histidine kinase pathway and a Ser/Thr kinase pathway.

DEDICATION

I dedicate this work to my parents, Guiting Li and Jingmei Zhang, my sisters Bo Li and Ning Li and all of my friends. I cannot imagine a life without your love and support!

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ATTRIBUTION

Dr. Zhaomin Yang is my primary advisor for the research presented in this thesis. He provided the original ideas and suggestions through out the research. I did all of the experiments under his directions.

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CHAPTER 1

Introduction and Review of Literature

A. Myxobacteria

The myxobacteria are rod-shaped, Gram-negative bacteria (31) and best known for their complicated social behaviors. As non-flagellated bacteria, myxobacteria can move by gliding over a solid surface (29, 31). When incubated in the laboratory condition, myxobacteria can move over substrates like agar to form a thin, film-like colony. The colony spreads over the agar and may cover the entire plate in a couple of days (31). In nature, on soil particles, surfaces of leaf, barks or rocks, gliding motility of myxobacteria allows coordinated predation of other microbes such as bacteria and fungi (28). This predation involves the production and secretion of many secondary metabolites and hydrolytic enzymes for the killing and digestion of their prey microbes (32, 41, 96). Their feeding is referred as “microbial wolf-packs” to reflect the fact that many cells coordinate their movement and feed as groups to optimize the utilization of secreted hydrolytic enzymes (28, 54). Most notably, myxobacteria can undergo a form of multicellular development once nutrients become limited. Hundreds of thousands of vegetative cells aggregate to assemble a multicellular structure known as a fruiting body which is large enough to be seen by unaided eyes (28, 54). Inside the fruiting body, the rod-shaped vegetative cells develop into myxospores that are resistant to many environmental stresses such as extreme temperatures, irradiation and desiccation (29, 31). When nutrients become available, the myxospores can germinate and reenter the vegetative life cycle. Based upon these complex and unique social behaviors, myxobacteria have become one of the model bacteria for studies such as cell-cell communication and evolution of a multicellular

life-style (29, 31, 89).

B. *Myxococcus xanthus*

Myxococcus xanthus, the most studied myxobacteria, has a 9.2 Mb genome which is among the biggest bacterial genomes known (83). This places *M. xanthus* among the most complicated eubacteria sequenced so far. The size of the genome likely reflects that *M. xanthus* requires a wide variety of signaling pathways to support its complicated social behaviors such as gliding motility and fruiting body formation. The following sections will be focused on the development and gliding motility of *M. xanthus*.

1. Developmental life cycle of *M. xanthus*.

One of the most intriguing features of the myxobacteria is their ability to form multicellular fruiting bodies (29, 31). In nutrient-rich condition, *M. xanthus* cells undergo a vegetative life cycle as other bacteria. In addition, the cells can undergo a developmental life cycle when nutrients become limited. During the developmental cycle, ~100,000 of vegetative cells coordinate their movement to aggregate into mound-shaped fruiting bodies (29, 31). Within these multicellular fruiting bodies, individual vegetative cells eventually differentiate into stress resistant myxospores which are also metabolically dormant. These spores can germinate and reenter the vegetative cell cycle under conditions favorable for growth. Myxospores thus render the organism the ability to survive under unfavorable environmental conditions. The

formation of a fruiting body may ensure that a new cycle is started by a population rather than individual cells (29, 31). Being able to form fruiting body makes *M. xanthus* one of the best model systems for the study of development.

The successful fruiting body formation of *M. xanthus* requires both intracellular and intercellular signaling. As mentioned earlier, the starting point for fruiting body formation is starvation. It was discovered that before developmental cell-cell signaling happens, *M. xanthus* first senses changes in its living condition and produce an intracellular signal to initiate the developmental program. Like the "stringent response" mechanism found in many other bacteria, *M. xanthus* can produce and accumulate guanosine tetra- and penta- phosphate or (p)ppGpp in response to nutrient deprivation (52). This intracellular signal is required to initiate the development and regulatory cascade involving other intercellular signals and pathways (40).

Five intercellular signals, A, B, C, D and E, have been identified through complementation studies (30, 31, 52, 86). The A signal is an extracellular mixture of six amino acids (tryptophan, proline, phenylalanine, tyrosine, leucine, isoleucine) and small peptides with these amino acids (30, 52, 89). The A signal was proposed to be functional as concentration-dependent quorum sensing signal (30, 52, 89). The B signal is a homologue of *E. coli lon* protease in *M. xanthus*, the mechanism of B-signaling is unknown (30, 52, 86). The C signal is the best studied among these five signals. CsgA protein is required for the C signal dependent gene expression. This protein has two forms, the full length 25Kd (p25) and a 17 Kd partial form (p17) (30,

52, 86). It has not been fully understood which form is the real C signal and how p17 is produced. The D signal appears to be a translation initiation factor 3 and the E signals are subunits of a branched-chain keto dehydrogenase. The mechanisms of D and E signaling remain unknown (30, 52, 86). Production and exchanging of these five signals are essential for successful *M. xanthus* fruiting body development (52).

2. Gliding motility of *M. xanthus*.

Myxococcus xanthus cells glide on solid surfaces in the direction of the long cell axis (31). Gliding motility is important for both life cycles of *M. xanthus*. During vegetative growth, *M. xanthus* cells move over surfaces with gliding motility to search for favorable environments. Since *M. xanthus* secrets hydrolytic enzymes to prey on other organisms, utilizing its gliding motility to move as large cell groups will enable it produce enough antimicrobial and degradative activity to more effectively kill its prey (32, 41, 96). In another word, the optimal utilization of these enzymes depends on its social behavior. During fruiting body formation, the aggregation of *M. xanthus* cells is also achieved through the gliding motility.

Genetic analysis revealed that *M. xanthus* has two nearly separate motility systems, the adventurous (A) and the social (S) gliding systems (46, 47). The A-motility enables cells to move as well-isolated cells while the S-motility system enables cell movement as large groups (46, 47). Studies indicate that these two systems function independently because cells with a mutation in most genes of one particular system are still motile due to the activities of the other system (46, 47). A

mutant becomes nonmotile if it is $A^{-}S^{-}$, which normally requires two mutations. The only exception is *mgl* (mutual gliding) gene. Mutations in this single gene can completely abolish motility in *M. xanthus* so *mgl* is involved in both A- and S-motility (46, 47). It is still not understood how *M. xanthus* cells coordinate the two motility systems to move during their life cycles.

The mechanism for A-motility remains a mystery although more than 30 A-motility genes have been identified and sequenced (46, 47, 117). Some of these genes encode homologues of the TolR, TolB and TolQ transport proteins, suggesting that A-motility may depend on biopolymer transport (117). One recent hypothesis is that nozzle-like structures in *M. xanthus* can secrete a propellant or "slime" that swells as it hydrates to push the cell move forward (109, 110). This mechanism is similar to that described for the gliding of certain cyanobacteria (110). However, some concerns were raised since nozzle-like structures were also found in certain A-motility mutants (109). So far, it is still not understood whether the slime secretion is the mechanism for *M. xanthus* A-motility.

As an important factor in *M. xanthus* developmental life cycle, S motility has been the focus of many studies over the years. Experimental evidence has revealed a strong correlation between S motility and fruiting body formation as all mutants of the S-motility system are defective in development to various degrees (47, 67). Mechanistically, S-motility is believed to be similar to twitching motility of many Gram-negative bacteria such as *Pseudomonas* and *Neisseria*. Both twitching and

gliding require the presence of type IV pili and close cell proximity (51, 71, 97). It is proposed that both forms of motility are achieved by the pilus extension, attachment, and retraction to pull the cell forward (51, 71, 90, 97).

C. Bacterial chemotaxis

Bacterial chemotaxis is the tactic response of bacteria to a gradient of chemical attractants or repellents (1). Bacteria chemotaxis has been studied most extensively in *E. coli*. Chemotaxis relies on controlling the frequency of directional switches of flagellar rotation, which results in the alteration of smoothing swimming and tumbles. Chemotactic bacteria are able to detect changes in concentrations of certain chemicals, such as sugars and amino acids (1). When an increasing concentration of an attractant (e.g. sugar) or decreasing concentration of a repellant (e.g. fatty acid) is detected, the bacterium will decrease the frequency at which it tumbles and will thus swim for longer periods (2). This enables bacteria to swim into an area where there is a higher concentration of an attractant or a lower concentration of a repellent. Conversely, detection of decreasing concentrations of attractants or increasing concentrations of repellants causes bacteria to increase their tumbling frequencies and shorten the smooth swimming period (2).

Studies in *E. coli* demonstrated that the chemotaxis is achieved through a signaling pathway which involves many proteins. Detection of attractants and repellants were due to certain sensory proteins termed chemoreceptors, also known as methyl-accepting chemotaxis proteins (MCPs). Once these chemoreceptors have

detected an attractant or a repellant and bound it, they undergo a conformational change that starts an intracellular signaling cascade. In *E. coli*, six proteins are involved in this pathway that send a message from the chemoreceptor to the flagellum, signaling it to rotate one way or the other; those proteins include CheB, CheR, CheA, CheW, CheY and CheZ (74). This cascade is actually responsible for the tumbling of bacteria, i.e. the clockwise (CW) rotation of the flagella. When not stimulated, the flagella rotate counter clockwise (CCW) and swimming occurs. Increasing concentrations of attractant and decreasing concentrations of repellant causes the chemoreceptors to be less active. The signal transduction pathway is not stimulated as frequently and the bacteria swim longer. If, for example, there is an increasing concentration of repellant and thus more binding to the chemoreceptors, this signaling pathway will be stimulated.

Studies have showed the biochemical mechanisms underlying the chemotaxis signaling pathway. CheA can autophosphorylate on a conserved histidine residue, using ATP as the phosphodonor upon stimulation (18). The rate of autophosphorylation is controlled by sensory stimuli. The phosphorylated CheA transfers a phosphate on CheA to the CheY response regulator protein which then binds to the flagellum motor causing it to switch rotation from CCW to CW (10, 108), and thus tumbling. If the binding of attractants decrease, CheA will autophosphorylate to stimulate the cascade (74). CheB is a methylesterase involved in chemosensory adaptation together with CheR, a methyltransferase. CheB can also be phosphorylated by CheA and its activity increases upon phosphorylation. Both CheY and CheB have

autophosphatase activity, which allows signal termination (66). In *E. coli*, the rate of CheY dephosphorylation is enhanced by CheZ (42). Mutants that lack CheZ tumble more often than those with the protein (74).

One of the most interesting aspects of chemotactic bacteria is their ability to adapt to a new chemical environment. Once in a high concentration of attractant, the bacteria need to stay there. They also must adapt so that they can respond to any subsequent changes. When an attractant binds an MCP, a conformational change takes place that decreases the ability of the receptor to activate the CheA kinase. CheR and CheB are responsible for the methylation and demethylation of MCPs respectively. MCPs have multiple conserved glutamate residues which can be methylated by CheR (6); the more that are methylated, the less the MCPs' activity to activate the CheA kinase (81). CheB has a response regulator domain and a methylesterase domain and is also part of a feedback mechanism. The increase of the CheA kinase activity will lead to the phosphorylation of CheB. Phosphorylated CheB is then activated and will demethylate MCPs (74). The result of the adaptation is that the system will be reset back to the original state or prestimulus state. CheA, therefore, controls both the signaling cascade by phosphorylating CheY and the level of chemoreceptor methylation, i.e. the level of adaptation. This adaptation mechanism is important for the bacteria to memorize the condition of their environment and adapt whenever the concentration of chemicals become stable.

D. Type IV pili and Exopolysaccharide (EPS) are required for *M. Xanthus* S-motility.

Type IV pili, which are thin protein filaments at cell poles, have been showed to be required for a functional S-motility in *M. xanthus* (53). Experiments have showed that mutations in *pilA*, which encodes the pilus subunit pilin, eliminated *M. xanthus* S-motility (53). In *M. xanthus*, EPS (9, 13, 87, 107, 116) was also demonstrated to be essential for S-motility (63, 87, 88). Previously, it was shown that EPS-deficient mutants fail to bind calcofluor white (76) and are defective in cellular agglutination, S-motility and fruiting body formation (84, 87). In comparison with polarly localized type IV pili, EPS are located all over the surface of the cell. The peritrichous EPS constitute a matrix that connects adjacent cells to contribute to the ability of cells to move as large cell groups. The EPS is comprised of the monosaccharides such as mannose, galactosamine, galactose, glucosamine, *N*-acetylated-amine sugars, glucose, rhamnose and xylose (11), but how it is assembled and arranged structurally. It is believed that *M. xanthus* S-motility may function through the extension and retraction of type IV pili as in the twitching motility of *Pseudomonas* and *Neisseria*. EPS can provide physical connections among neighboring cells (8, 12) and may serve as the anchor and trigger for type IV pili retraction in *M. xanthus* (63).

E. Glycogen metabolism in *M. xanthus*

Glycogen is a principal polysaccharide storage of glucose in both eukaryotes and prokaryotes (75). It is a highly branched polymer with glucose linked by α-1,4 and

α-1,6 glycosidic bond at the long chain and at the branching point respectively (75). Glycogen is accumulated in many bacteria when growth is limited by some factors other than carbon and energy source (such as nitrogen and phosphate) (75).

In *Bacillus* and *Streptomyces*, accumulation and degradation of glycogen are known to be important for morphological differentiation of cells. It was found that glycogen accumulate at the end of the log phase and during the early stage of sporulation in *Bacillus* (58, 75). In *Streptomyces*, glycogen was also accumulated at the stationary phase and the early stage of sporulation but not in the mature spores (20, 21, 69, 77).

In *M. xanthus*, it has been reported that polysaccharide granules were observed in cells growing in low-phosphate medium (105). Recently, it was demonstrated that *M. xanthus* cells accumulate glycogen at the early stationary phase during vegetative growth and at the early stage of development on a low-nutrient (CF and TM) agar plate (44). As sonication-resistant myxo spores appear, the amount of glycogen detected in the myxospores decreased to 10% of that in the early stationary phase cells (44).

The glycogen metabolism in *M. xanthus* was found to be affected by 6-phosphofructokinase (PFK) and a Ser/Thr kinase Pkn4 encoded by the *pfk* and *pkn4* genes, respectively (43). PFK uses ATP to phosphorylate fructose 6-phosphate to fructose 1, 6-bisphosphate and is the key regulator of glycolysis (75). It was showed that more glycogen accumulated in the *pfk–pkn4* deletion strain (Δ *pfkn*) than the wild

type strain DZF1 at the early stage of development, and glycogen in the mutant remained at the elevated level during the middle and late stages of development (44). The Δ *pfkn* strain produced only 3.4% of the spores of the wild type (44). Biochemical studies also showed that *M. xanthus* PFK, like eukaryotic PFKs, is regulated post-translationally by phosphorylation on a threonine residue by Pkn4 (43). It was proposed that *M. xanthus* accumulates glycogen and uses it as a carbon and energy sources during development to produce high yield of myxospores (44, 45).

F. The *dif* locus

EPS biogenesis in *M. xanthus* is regulated by a few genetic loci including the *dif* (*d*efective *i*n *f*ruiting/*f*ibrils) locus (16, 114, 116). It was discovered that the *dif* locus encodes several homologues of bacterial chemotaxis proteins (16, 114, 116). In primary sequence, DifA is homologous to a MCP, DifC to CheW, DifD to CheY, and DifE to CheA. DifG is homologous to CheC, a chemotaxis protein present in *Bacillus subtilis* and some other bacteria, but absent in the enterics (16, 114). DifB shows homology to a family of conserved hypothetical bacterial proteins with unknown function (16, 114) (Fig. 1-1). Previous studies showed that *difA*, *difC*, and *difE* mutants produced no detectable levels of EPS and were defective in S-motility and fruiting body formation (114, 116). However, *difD* and the *difG* mutants overproduce EPS in comparison with the wild-type. This indicates that DifD and DifG are negative regulators of EPS production. These phenotypes of *dif* mutants suggest that the Dif proteins define a signaling pathway that regulates the EPS production in *M. xanthus*

and it was hypothesized that the Dif proteins may interact with one another similarly as do chemotaxis proteins in other bacteria.

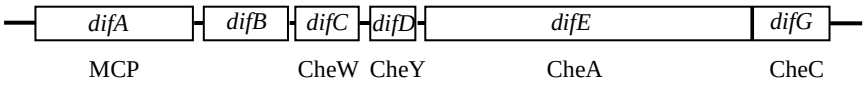

Figure 1-1. *M. xanthus dif* locus and its homology with bacterial chemotaxis proteins. Picture is drawn to scale.

DifA, the MCP homolog, is likely to mediate signal input to the Dif pathway. Recently, a NarX-DifA (NafA) chimera from the *Escherichia coli* sensory protein NarX and DifA was created to characterize the signaling properties of DifA. NafA was found to be able to restore fruiting body formation, EPS production, and S-motility in *difA* mutants when stimulated by nitrate. It was also showed that NafA requires the downstream Dif proteins to function. Although DifA does not have a periplasmic domain as found in many other MCPs, it seems to have similar transmembrane signaling mechanisms (113) and to play an important role in regulation of EPS biogenesis in *M. xanthus*.

Although bacterial chemotaxis achieves adaptation by reversible methylation on conserved sites in MCP, it is unclear whether DifA, a MCP homolog, can modulate the activity of the Dif pathway through methylation. Recent genetic studies were carried out to try to elucidate the possibility of methylation of DifA. Site-specific mutagenesis studies on potential methylation residues in DifA showed that mutations on these residues did not affect DifA mobility in gel electrophoresis, suggesting a lack of DifA methylation. It was further shown that NafA had no band shift after nitrate

exposure. These results suggest that the signaling of DifA is possibly methylation-independent (Q. Xu and Z. Yang, unpublished results).

It was not known what input signal is detected by the Dif pathway to regulate EPS biogenesis until recent studies. It was found that mutations in *dif* genes are epistatic to those in *pil* genes when using various double and triple mutants. It was thus proposed that type IV pili (Tfp) function upstream of the Dif proteins in the regulation of EPS production. Extracellular complementation experiments between various *pil* and *dif* mutants also suggests that Tfp may function as a remote sensor or part of the sensor to mediate signal input into the Dif pathway (15).

In addition, the *dif* genes have been implicated in chemotaxis responses of *M. xanthus* to phosphatidylethanolamine (PE). PE was the first known chemical attractant for *M. xanthus* or any other surface-gliding bacterium and the first lipid attractant for any bacterium (56). In *M. xanthus* two PE species, dilauroyl and dioleoyl PE, have been shown to suppress the reversal frequency of *M. xanthus* gliding motility. Previous studies showed that a functional *dif* locus is required to elicit an excitation response to certain PE species (55). Specifically, it was shown that DifA and DifE were required for excitation to dilauroyl PE and that DifE was required for response to dioleoyl PE. Due to this finding, it was suggested that multiple attractant sensing receptors might converge downstream into one common chemotaxis pathway (17).

G. Research Objectives.

There are two questions that were addressed by my research. Homology of Dif

proteins with other chemotaxis proteins and the phenotypes of *dif* mutants (16, 114) suggest they likely define a chemotaxis-like pathway to regulate the EPS biogenesis and may interact with one another as do the chemotaxis proteins found in other bacteria. The first objective was to examine the physical interactions among Dif proteins using the yeast two-hybrid system.

It was hypothesized that if DifA, DifC and DifE proteins define a chemotaxis-like pathway, there may be another protein or other proteins that function downstream of DifE to positively regulate the EPS production. The second objective was to identify additional EPS regulators using DifE as the "bait" to screen three *M. xanthus* genomic libraries in yeast two-hybrid vectors (100).

CHAPTER 2: Demonstration of interactions among *Myxococcus xanthus* Dif chemotaxis-like proteins by the yeast two-hybrid system

Archives of Microbiology published by Springer. May. 2005. 183(4): 243-252. Zhaomin Yang and Zhuo Li. Demonstration of interactions among *Myxococcus xanthus* Dif chemotaxis-like proteins by the yeast two-hybrid system.

ABSTRACT

The *Myxococcus xanthus dif* locus encodes several bacterial chemotaxis homologues that are crucial for fibril exopolysaccharide (EPS) production, social gliding motility, and fruiting body development. In primary sequence, DifA is homologous to methyl-accepting chemotaxis protein, DifC to CheW, DifD to CheY, DifE to CheA, and DifG to CheC. In this study, the interactions among the Dif chemotaxis-like proteins were investigated using the yeast two-hybrid (Y2H) system. DifC was found to interact with both DifA and DifE. Using a modified Y2H or a "three-hybrid" system, it was demonstrated that DifC is capable of mediating the formation of DifA, DifC, and DifE ternary protein complexes. The conserved domains of DifE, based on sequence analysis, likely reflect functional conservations of CheA-type kinases, because its P2 domain interacts with DifD, P5 with DifC, and the P3 domain appears to dimerize. Similarly, C-terminal regions of DifA appear to dimerize as well. In addition, DifG was found to interact with DifD, which is consistent with the hypothesis that DifG is a phosphatase of DifD-phosphate. These findings support the models in which Dif proteins constitute a unique chemotaxis-like signal transduction pathway with central functions in regulating EPS production in *M. xanthus*.

Keywords *Myxococcus xanthus;*, Fibril exopolysaccharide; Dif; Chemotaxis; Yeast two-hybrid; Protein interaction

INTRODUCTION

Myxococcus xanthus is a rod-shaped, gram-negative soil bacterium with a complex life cycle (29, 85). Under growth permissive conditions, *M. xanthus* grows vegetatively like other bacteria. Under nutrient limitation, *M. xanthus* can initiate a well-tuned developmental process and form multicellular structures known as fruiting bodies. During the developmental cycle, hundreds of thousands of cells aggregate on solid surfaces to form the mound-like fruiting bodies. Within the fruiting body, rod-shaped vegetative cells eventually differentiate into spherical myxospores, which are metabolically dormant and environmentally resistant to stress such as heat and desiccation (29, 85).

The aggregation of *M. xanthus* cells during fruiting body formation is achieved through their surface gliding motility, which is governed by two genetically defined systems (93). The adventurous motility enables the movement of well-isolated cells whereas the social (S) motility refers to the movement of multicellular groups (46, 47). S motility is more crucial to development, because all known S-motility mutants are defective in fruiting body formation to various extents (47, 67). *M. xanthus* S motility requires at least two types of cell surface components, the polarly localized type IV pili (53, 111) and the fibril exopolysaccharide (EPS) (9, 13, 87, 107, 116). It has been proposed that *M. xanthus* S motility may be powered by the retraction of type IV pili as is the twitching motility of *Pseudomonas* and *Neisseria* (50, 71, 90, 97). Recent

studies suggested that fibril EPS might interact with type IV pili and mediate pilus retraction in *M. xanthus* (63).

Fibril EPS production in *M. xanthus* is regulated by a few genetic loci including the *dif* defective in fruiting/fibrils locus (13, 16, 26, 65, 107, 116). Sequence analysis showed that the *dif* locus encodes several homologues of bacterial chemotaxis proteins (16, 114). DifA is homologous to methyl-accepting chemotaxis protein (MCP), DifC to CheW, DifD to CheY, and DifE to CheA. DifG shares sequence similarity with the *Bacillus subtilis* chemotaxis protein CheC (16, 60, 78, 99, 114). It has been shown previously that *difA*, *difC*, and *difE* mutants produce no detectable levels of fibril EPS, whereas *difD* and *difG* mutants overproduce EPS (13, 16, 114, 116). Based on the homology of deduced *dif* gene products to chemotaxis proteins and the phenotypes of *dif* mutants, it was proposed that Dif proteins define a signaling pathway that regulates EPS production in *M. xanthus* (13, 16, 114, 116), and they may interact with one another similarly as bacterial chemotaxis proteins.

Here, we report the examination of physical interactions among Dif proteins using the yeast two-hybrid (Y2H) system. Both full-length Dif proteins and different domains or segments of DifE and DifA were used in our studies. The results suggested a pattern of interactions among Dif proteins similar to those in bacterial chemotaxis pathways. DifA interacted with DifC, and DifE with both DifC and DifD. Using a “three-hybrid” system, we also showed that DifC could mediate the interactions between DifA and DifE to likely form ternary protein complexes. The

results in this report are consistent with a model in which Dif proteins interact with one another to form a chemotaxis-like pathway to regulate fibril EPS production in *M. xanthus*.

MATERIALS AND METHODS

Strains, media, and growth conditions

Escherichia coli stain XL1-Blue (23) and standard molecular cloning procedures (80) were used for routine plasmid constructions. Luria-Bertani (LB) (80) liquid medium and LB plates (1.5% agar) were used for the growth and maintenance of *E. coli*. Ampicillin was supplemented at 100 μg/ml to LB media for the selection of plasmids when applicable. The *Saccharomyces cerevisiae* strain used in Y2H studies was PJ69-4A (*MAT*a *trp1-901, leu2-3, 112 ura3-52 his3-200 gal4 Δgal80* Δ*LYS2*:: *GAL1-HIS3 GAL2-ADE2 met2*:: *GAL7-lacZ*), which has three reporter genes for a positive Y2H interaction: *HIS3* driven by *GAL1* promoter, *ADE2* by the *GAL2* promoter, and *lacZ* by the *GAL7* promoter (48). Growth media for *S. cerevisiae* were YEPD (1% yeast extract, 2% peptone, 2% dextrose, pH 6.0) and synthetic complete (SC) liquid medium and plates (1.5% agar) (79). Certain components were omitted or dropped out from SC media when indicated. All the SC histidine (SC-His) dropout plates used in this study were supplemented with 2 mM 3-amino-1,2,4,-triazole (3-AT), a competitive inhibitor of *S. cerevisiae* His3p enzymatic activity (95). *E. coli* was grown at 37°C and *S. cerevisiae* at 30°C.

Sequence alignment and domain analysis

Sequence alignment was performed using Clustal X and Clustal W (49, 101) from the BCM Search Launcher (91). Domain analysis was performed using the Conserved Domain Architecture Retrieval Tool (35) and searches of the CDD database (68) using the basic local alignment search tool (3). Because *Thermotoga aritime* CheA was used to determine the crystal structure for the bulk of a CheA kinase (14), it was used as the primary template for the analysis of DifE domain structure.

Construction of plasmids

Plasmids used in this study are listed in Table 1. The pGAD-C1, 2, 3 and the pGBD-C1, 2, 3 series of plasmids used as Y2H vectors contain the GAL4 transcription activation domain (GAL4AD) and GAL4 DNA-binding domain (GAL4BD), respectively (48). In all of the Y2H constructs, GAL4AD and GAL4BD were fused at their C-terminus to full-length or truncated Dif proteins. Restriction enzymes, T4 DNA polymerase, and T4 DNA ligase used for plasmid construction were from New England Biolabs (Beverly, Mass., USA). For the construction involving polymerase chain reaction (PCR), *Pfu* Ultra DNA polymerase (Stratagene, La Jolla, Calif., USA) with 3'–5' nuclease activity for proofreading was used. The primers used for PCR amplification for plasmid constructions are listed in Table 2. Plasmids with various DNA fragments from the *dif* locus were used as the templates for PCR reactions. The sequence of PCR fragments in all the plasmid constructs were verified by DNA sequencing.

Table 2-1. Plasmids used in this study

Plasmids	Features/description[a]	References
pGBD-C1, 2, 3	GAL4BD vectors; *TRP1*	(48)
pGAD-C1, 2, 3	GAL4AD vectors; *LEU2*	(48)
p426GPD	Yeast expression vector; *GPD* promoter; *URA3*	(73)
pDF100	GAL4AD-DifD	This study
pDF101	GAL4BD-DifD	This study
pLZ170	GAL4BD-DifG	This study
pLZ180	GAL4AD-DifG	This study
pLZ201	GAL4BD-DifE	This study
pLZ211	GAL4AD-DifA$_{54-413}$[b]	This study
pLZ214	GAL4BD-DifA$_{137-360}$[b]	This study
pLZ215	GAL4AD-DifA$_{137-360}$[b]	This study
pLZ218	GAL4BD-DifA$_{256-413}$[b]	This study
pLZ219	GAL4AD-DifA$_{256-413}$[b]	This study
pLZ221	GAL4AD-DifC	This study
pLZ222	GAL4BD-DifC	This study
pLZ224	GAL4BD-DifE$_{321-470}$[b]	This study
pLZ228	GAL4BD-DifE$_{472-540}$[b]	This study
pLZ229	GAL4AD-DifE$_{472-540}$	This study
pLZ230	GAL4BD-DifE$_{722-855}$[b]	This study
pLZ232	DifC in p426GPD	This study
pLZ401	GAL4AD-DifE	This study
pMY100	6-His-tagged DifD	This study

[a]All plasmids confer ampicillin resistance

[b]Numbers in *subscript* indicate the amino acid residues contained in the construct

Table 2-2. Primers used in this study.

Plasmid	Primers[a]	Enzyme[b]
pLZ170/pLZ180	GTGT GGATCCTGGACGTGCC	*Bam*HI
	CCCGTCTAG GTCGACACGGG	*Sal*I
pLZ201/pLZ401	GC ATCGAT**G**ACGATGGACATGTCCCG	*Cla*I
	CT CTGCAGGGGCTGGC**TCA**CGCGGAC	*Pst*I
pLZ211	GTCACCCGCGTGAAGGTGCTCAGC	N/A
	CAGCTTCGC ATCGATGTTGTCCGA	*Cla*I
pLZ214/pLZ215	CGGCTCGTCGATGGAGAAGATT	N/A
	CGCCACCAG ATCGATTTCCTCGAT	*Cla*I
pLZ218/pLZ219	CGCGCGGGT GAATTCGGCCGCGGC	*Eco*RI
	CAGCTTCGC ATCGATGTTGTCCGA	*Cla*I
pLZ221/pLZ222	AGTGGATAG ATCGATACTCCT**GTG**	*Cla*I
	AATCAC CTGCAGGAGCGCGGACAC	*Pst*I
pLZ224	ACCGACGCC GGATCCGCGGCTCCA	*Bam*HI
	GACGCCGTG ATCGATGACGCTCGG	*Cla*I
pLZ228/pLZ229	CCGAGCGTC ATCGATCACGGCGTC	*Cla*I
	TGATGAGGG AGATCTGCGTCATGC	*Bgl*II
pLZ230	TCACCCTGC GAATTCCACTGACGG	*Eco*RI
	CT CTGCAGGGGCTGGC**TCA**CGCGGAC	*Pst*I
pLZ232[c]	TAG AAGCTTACTCCT**ATG**CGGCAC	*Hin*dIII
	CCAGGG GTCGAC**TCA**CTTGGAATG	*Sal*I
pMY100	GG AGTACTTCAC**ATG**GCTAAGCGGGT	*Sca*I
	GG AAGCTTGGACAGCCCGCGCCAGG	*Hin*dIII

[a]Sequences of primers are written from 5' to 3'. Restriction sites in primers are underlined. Start and stop codons for relevant genes are in boldface when applicable

[b]Restriction enzymes for which recognition sites were introduced in primers. *N/A* Not applicable

[c]The *difC* start codon GTG was changed to ATG for expression in *Saccharomyces cerevisiae.*

There are five plasmids (pLZ211, pLZ214, pLZ215, pLZ218, and pLZ219) with different portions of the *difA* gene in Y2H vectors (Table 2-1, Fig. 2-1a). For the construction of pLZ211, a PCR fragment amplified using the indicated primers (Table 2-2) was digested with *Cla*I and ligated into pGAD-C1 digested with *Sma*I and *Cla*I. For the construction of pLZ214 and pLZ215, the relevant PCR fragment (Table 2-2) was digested with *Cla*I and ligated into pGBD-C3 and pGAD-C3 digested with *Sma*I and *Cla*I. pLZ218 and pLZ219 were constructed by digesting the PCR fragment amplified with the indicated primers (Table 2-2) with *Eco*RI and *Cla*I and cloning it into similarly restricted pGBD-C3 and pGAD-C3, respectively.

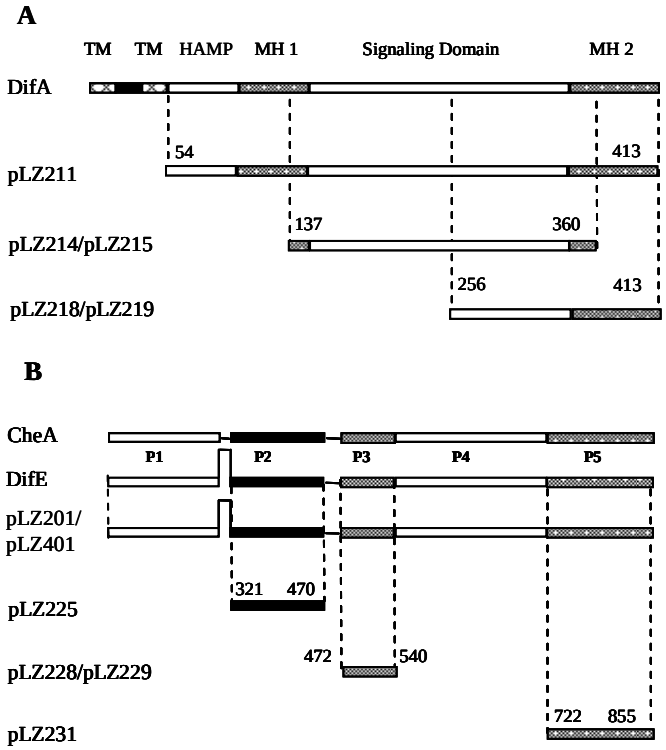

Figure 2-1. Domains of DifA and DifE proteins and their yeast two-hybrid (Y2H) constructs. Predicted domains of DifA and DifE are indicated by different patterned boxes. DifA and DifE are predicted to contain 413 and 855 amino acids, respectively. The lengths of the domains are drawn approximately to scale relative to the sizes of the open reading frames. Indicated on the left are the Y2H plasmids containing various domains of DifA and DifE. The initial and final Dif residues in each plasmid are indicated above each fragment. **A.** DifA domains and DifA-containing Y2H constructs. TM: Transmembrane domain, HAMP: linker region, MH: methylation helix. **B.** DifE domains and DifE-containing Y2H constructs. DifE was aligned with *Thermotoga aritime* CheA with the five domains (P1 through P5) indicated. Lines between the P1 and the P2 domains indicate an insertion from residues 155-340 in DifE that shares no homology with any known proteins.

Six plasmids with either full-length (pLZ201 and pLZ401) or part (pLZ224, pLZ228/229 and pLZ230) of *difE* in Y2H vectors were used in this study (Table 2-2;

Fig. 2-1b). For pLZ201 and pLZ401, full-length *difE* was amplified by PCR (Table 2-1), digested with *Cla*I and *Pst*I, and ligated into the same sites in pGBD-C3 and pGAD-C3, respectively. pLZ224 was constructed by digesting a PCR fragment (Table 2-2) with *Bam*HI and *Cla*I and ligating it into the corresponding sites in pGBD-C1. To generate pLZ228 and pLZ229, the relevant PCR fragment of *difE* (Table 2-2) was digested with *Cla*I and *Bgl*II and ligated into the same sites of pGBD-C3 and pGAD-C3. For the construction of pLZ230, a *difE* PCR fragment (Table 2-2) was digested with *Eco*RI and *Pst*I and ligated into the corresponding sites in pGBD-C1.

Full-length constructs were made for DifC (pLZ221/222), DifD (pDF100/101), and DifG (pLZ170/180) in Y2H vectors (Table 2-1) for this study. To construct pLZ221 and pLZ222, *difC* was PCR-amplified (Table 2-2), digested with *Cla*I and *Pst*I, and ligated into pGAD-C1 and pGBD-C1 digested with the same enzymes. pDF100 and pDF101 were constructed by cloning a *difD*-containing fragment from pMY100 into pGAD-C1 and pGBD-C1. Full-length *difD* was PCR-amplified (Table 2-2), digested with *Sca*I and *Hin*dIII, and cloned into the *Sma*I and *Hin*dIII sites of pQE30 vector (Qiagen, Valencia, Calif., USA) to generate pMY100 first. pMY100 was then digested with *Hin*dIII, treated with T4 DNA polymerase, and followed by *Bam*HI digestion. The resulting *difD*-containing fragment was cloned into Y2H vectors digested with *Pst*I, treated with T4 DNA polymerase, and further digested with *Bam*HI to generate pDF101 and pDF100. For the construction of pLZ170 and pLZ180, a PCR fragment (Table 2-2) with full-length *difG* was digested

with *Bam*HI and *Sal*I and ligated into pGBD-C3 and pGAD-C3 digested with the same enzymes.

pLZ232 was constructed to constitutively express DifC in *S. cerevisiae*. Full-length *difC* was first amplified using primers that converted the original GTG start codon of *difC* to ATG (Table 2-2). This PCR fragment was digested with *Hin*dIII and *Sal*I and ligated into the corresponding sites in p426GPD, which provides the strong and constitutively active GPD promoter (73).

Examination of protein–protein interactions with the Y2H system

For examination of interactions using the Y2H system, the yeast strain PJ69-4A was co-transformed with appropriate GAL4BD and GAL4AD fusion plasmids using the LiAc-PEG method (37) and plated onto SC plates lacking leucine and tryptophan. Purified transformants were examined for growth on SC plates lacking leucine, tryptophan, and histidine with 2 mM 3-AT and SC plates lacking leucine, tryptophan, and adenine (SC-Ade). Growth on plates was examined daily and documented after 4 days of incubation at 30°C. In the case of examining ternary complex formation by DifA, DifC, and DifE, yeast transformants with three plasmids were selected on SC plates without leucine, tryptophan, and uracil. Transformants were then examined for growth on SC-His and on SC-Ade without uracil. Growth on histidine and adenine dropout plates was examined either by streak plates or by spotting plates with serial dilutions of cell suspension (103). For the spot assays, cells in exponential growth were harvested and resuspended at 4×10^6 cells per milliliter. A volume of 5 μl of this

cell suspension and its 5× serial dilutions were spotted on plates with decreasing concentrations from left to right. β-Galactosidase activity of yeast transformants was examined using the method described by Kippert (59) and expressed in Miller units (72).

RESULTS

MCP homologue DifA interacts with CheW homologue DifC

In classical enteric chemotaxis, MCPs directly interact with CheW to transmit signals to the CheA kinase (7, 19, 22, 33). The *M. xanthus* MCP homologue DifA is predicted to be a multi-domain protein as shown in Fig. 1a. Starting from the N-terminus, there are two predicted transmembrane domains encompassing a short periplasmic stretch, followed by a linker or HAMP domain. The highly conserved cytoplasmic MCP signaling domain is flanked by two predicted methylation helices (MHs). To examine the interactions between DifA and DifC, we initially constructed pLZ211 (Fig. 2-1a), which contains amino acids 54–413 of DifA ($DifA_{54-413}$) fused to the C-terminus of GAL4AD. This construct essentially included the entire predicted cytoplasmic domains of DifA: the HAMP domain, the MHs, and the signaling domain. Plasmids with full-length DifC fused to GAL4 (pLZ221 and 222) were also constructed as described in Materials and methodws. These DifA and DifC constructs were transformed into *S. cerevisiae* Y2H reporter strain PJ69-4A to examine interactions by growth on SC-His and SC-Ade plates and by analysis of β-galactosidase activity. As indicated in Materials and methods, all the histidine

dropout plates used in this study were supplemented with 2 mM 3-AT, a His3p inhibitor (95), because it was discovered early on during this study that growth on histidine dropout plates without 3-AT is not a reliable indicator of positive interactions with PJ69-4A. We observed that most of our negative controls grew on SC-His dropout plates, but supplementation of 2 mM 3-AT was sufficient to eliminate these false positives. The leakiness of the *HIS3* reporter gene in the Y2H system was also observed previously (70).

Transformants containing the GAL4AD-DifA$_{54-413}$ and the GAL4BD-DifC constructs showed growth on SC-His but not on SC-Ade plates (Fig. 2-2a). This perhaps is not surprising, because it is known that growth on SC-Ade is a more stringent indicator of interactions in the Y2H system than growth on SC-His (48). Consistent with the growth phenotype, the third reporter gene *lac*Z is expressed at relatively low levels in these transformants as indicated by β-galactosidase activity (Fig. 2-2a). Yeast transformants with either of these two constructs and an empty Y2H vector showed no growth on SC-His or SC-Ade plates (Fig. 2-2a), nor did those with any of the other GAL4-Dif constructs and a vector (data not shown). These observations suggest that DifA can interact with DifC although the interaction is either weak or unstable in the Y2H system with these constructs.

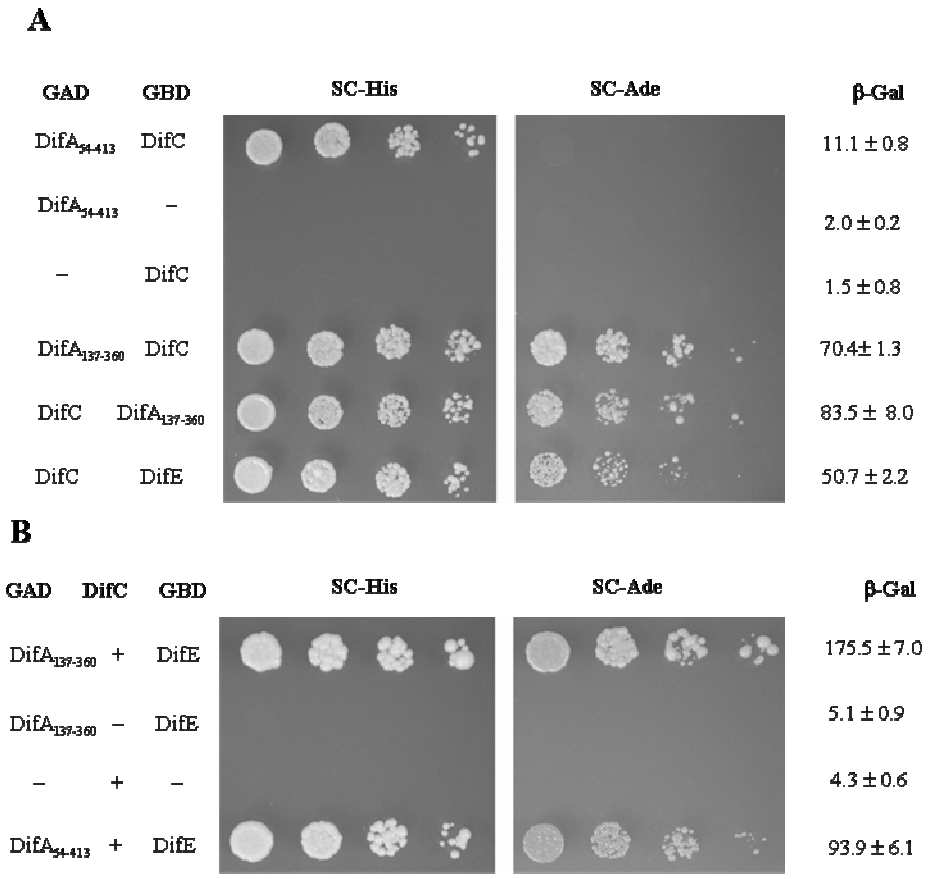

Figure 2-2. Examination of interactions among DifA, DifC, and DifE. PJ69-4A transformants were analyzed for β-galactosidase (β-Gal) activity and by spot assays (see "Materials and methods") on histidine dropout synthetic complete (SC-His) plates with 3-amino-1,2,4,-triazole (3-AT) and SC plates lacking adenine (SC-Ade) plates. For the spot assays, the total numbers of yeast cell initially placed in the four columns from left to right are 2×10^4, 4×10^3, 8×10^2, and 1.6×10^2, respectively. **A.** Pairwise interactions between DifA and DifC and between DifC and DifE. The columns labeled *GAD* and *GBD* show fusions of either GAL4AD or GAL4BD with indicated Dif proteins or their fragments. The minus signs indicate vector (pGAD-C1 or pGBD-C1) with no insert. As shown in the last two rows, transformants with pairs of $DifA_{137-360}$ and DifC in reciprocal vector sets showed similar results. For the other pairs, similar results were also observed when the constructs were made in the reciprocal set of Y2H plasmids (data not shown). **B.** Ternary complex formation by DifA, DifC, and DifE. Experimental conditions and labeling are as described for **a**, except that the histidine and adenine dropout plates used here lacked uracil to maintain the DifC expression plasmid (pLZ232) or the corresponding cloning vector (p426GPD). For the column labeled DifC, plus signs indicate the presence of pLZ232, and minus signs indicate the presence of p426GPD. Similar results were observed with DifA and DifE in the reciprocal set of Y2H vectors.

It has been shown in other bacteria that it is the signaling domain of MCP that is responsible for direct interactions with CheW (4, 34, 64, 82, 98). To examine whether DifA interacts with DifC in a similar manner, a plasmid (pLZ215) with the signaling domain of DifA ($DifA_{137-360}$) fused to GAL4AD was constructed (Fig. 2-1a). Yeast transformants containing pLZ215 and the GAL4BD-DifC construct grew on both the SC-His and the SC-Ade plates (Fig. 2-2a). Similar results were observed with the reciprocal constructs GAL4BD-$DifA_{137-360}$ (pLZ214) and GAL4AD-DifC (pLZ221) (Fig. 2-2a). Consistent with their growth phenotype on both SC-His and SC-Ade plates, these transformants also displayed about six- to sevenfold higher β-galactosidase activity than that with the $DifA_{54-413}$ (Fig. 2-2a). None of these GAL4 constructs alone and an empty Y2H vector enabled yeast transformants to grow on either SC-His or SC-Ade plates. These results suggest that DifA can interact with DifC through its conserved signaling domain and the MHs may somehow inhibit or destabilize such interactions at least in the Y2H system.

DifC interacts with the CheA homologue DifE

To examine whether the CheA homologue DifE interacts with DifC, DifE was fused to the C-termini of GAL4BD and GAL4AD, respectively (see Materials and methods). The GAL4AD-DifC (pLZ221) and GAL4BD-DifE (pLZ201) fusion plasmids (Table 2-1 and Fig. 2-1b) were co-transformed into the yeast strain PJ69-4A. Yeast transformants containing the DifE and the DifC constructs were able to grow on both the SC-His and the SC-Ade plates (Fig. 2-2a). None of these constructs with an

empty vector enabled growth on these plates. Analysis of *lac*Z expression by β-galactosidase activity confirmed the observation with the plate growth assays (Fig. 2-2a). These results suggest that DifE can directly interact with DifC.

DifA, DifC, and DifE form a ternary complex

The interactions of DifC with DifA and DifE suggested that DifC may interact with both proteins simultaneously to form a ternary signaling complex analogous to their counterparts in bacterial chemotaxis signal transduction (7, 19, 22, 33). To examine whether DifC can mediate the formation of such complexes, pLZ232 was constructed (see Materials and methods), which has DifC under the control of the constitutive GPD promoter of *S. cerevisiae* (73). Negative controls with relevant vectors and Dif constructs showed no growth on SC-His and SC-Ade plates and displayed minimum levels of β-galactosidase activity (Fig. 2-2b). On the other hand, the transformants containing the $DifA_{137-360}$ and the DifE Y2H constructs along with the DifC expression plasmid grew on both histidine and adenine dropout plates. Additionally, the yeast transformant containing GAL4AD-$DifA_{54-413}$ and GAL4BD-DifC constructs did not grow on SC-Ade plates (Fig. 2-2a); yet, yeast transformants with the same $DifA_{54-413}$ construct, the DifC expression plasmid, and a GAL4BD-DifE construct grew on both the histidine and the adenine dropout plates (Fig. 2-2b). Transformants containing DifA, DifC, and DifE constructs all displayed significantly higher β-galactosidase activity (Fig. 2-2b) than the transformants only with pairwise interacting partners (Fig. 2-2a). These results strongly suggest that DifA,

DifC, and DifE can form ternary complexes, and that the interactions among these proteins in a ternary complex are probably more stable and/or stronger than the pairwise interactions between DifA and DifC and between DifC and DifE.

Putative kinase DifE interacts with response regulator DifD

Besides its interactions with CheW, CheA is known to interact with the CheY response regulator (7, 19, 22, 33). It was proposed that DifE might interact similarly with the CheY homologue DifD (16, 114, 116). To examine whether DifE can interact with DifD, Y2H constructs with full-length DifD (pDF100 and pDF101) were generated as described in Materials and methods and co-transformed with DifE constructs into yeast PJ69-4A. The transformants grew on both the SC-His and the SC-Ade plates, and they expressed *lac*Z, as indicated by β-galactosidase activity (Fig. 2-3). Transformants with each of these constructs alone and an empty Y2H vector showed no growth on the histidine and adenine dropout plates and negligible levels of β-galactosidase activity. These results suggest that DifE directly interacts with DifD as previously proposed.

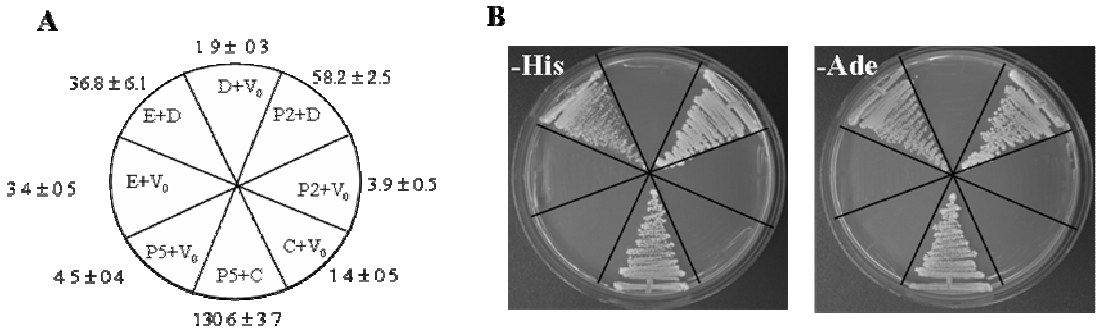

Figure 2-3. Interactions of conserved DifE domains. Yeast transformants containing Y2H constructs and/or vectors as indicated in **A** were examined for growth by streaking on selective plates as shown in **B**. V_0:Y2H vectors without inserts, C: DifC-containing plasmid pLZ221, D: DifD-containing pDF100, E DifE-containing pLZ201, P2: DifE P2-containing pLZ224, P5: DifE P5-containing pLZ230 (see Table 1 and Fig. 1 for more information). The growth of the transformants after 4 days of incubation at 30°C was documented and shown in **B**, with the 3-AT-supplemented SC-His plate on the left and the SC-Ade plate on the right. β-Galactosidase activity of these transformants was analyzed and indicated in **A** next to each sector with the corresponding transformants. Similar results were observed with Dif proteins fused to GAL4 in the reciprocal set of Y2H vectors.

DifE interacts with both DifC and DifD through conserved domains

The domains of the CheA-type kinase and their functions have been well-defined (14). A typical bacterial CheA monomer has five domains, named P1 through P5, from the N-terminus to the C-terminus (Fig. 2-1b). P1 is the histidine phospho-transfer domain, which is phosphorylated on a conserved histidine residue by the P4 kinase catalytic domain. CheA kinases function as dimeric enzymes, and the dimerization is mediated by the P3 domain. P2 and P5 provide the interacting surfaces for CheY and CheW, respectively. Sequence alignment showed that DifE has similar domain organization as CheA (Fig. 2-1b; (114)). The only exception is that

between the predicted P1 and P2 domains of DifE, there is an insertion of about 185 amino acids (Fig. 2-1b), which shares no significant homology with any CheA kinases or known proteins. To examine whether DifE interacts with DifD and DifC through the putative P2 and P5 domains respectively, Y2H plasmids with the P2 and the P5 domains of DifE were constructed (Fig. 2-1b; Materials and methods). Yeast transformants containing both the P2 and the DifD constructs grew on both SC-His and SC-Ade plates, as did the transformants containing both the P5 and the DifC constructs (Fig. 2-3b). The results of β-galactosidase analysis are consistent with the growth phenotypes of these yeast transformants (Fig. 2-3a). These results suggest that DifE interacts with DifD and DifC via its conserved P2 and P5 domains, respectively.

Both DifE and DifA may form homodimers As discussed above, CheA kinases form homodimers through their P3 domains (14). To test whether the P3 domain of DifE had similar functions, it was fused to both GAL4AD and GAL4BD in Y2H vectors (Fig. 2-1b). Yeast transformants containing the P3 domain in both vectors grew on SC-His and SC-Ade plates and gave the highest β-galactosidase activity among all the transformants in this study (Table 2-3; Figs. 2-2, 2-3, 2-4). These results indicate that DifE may form stable homodimers through its P3 domain. MCPs are also known to form homodimers (7, 19, 22, 33). To test whether DifA could form homodimers, various DifA fragments (Fig. 2-1) were examined for interactions with themselves in Y2H vectors. Among them, only the $DifA_{256-413}$ constructs enabled growth of yeast transformants on SC-His plates (Table 2-3; data not shown). None of the DifA constructs conferred growth to transformants on SC-Ade plates.

β-Galactosidase activity (~28 units) of the transformants with these $DifA_{256-413}$ constructs was also lower than the ones capable of growth on SC-Ade (Table 2-3; Figs. 2-2, 2-3, 2-4). These results suggest that DifA could potentially form homodimers as other MCPs although the interactions among the DifA fragments we examined appear weak or unstable in the Y2H system (98).

Table 2-3. Examination of DifA and DifE dimerization. SC-His Synthetic complete-lacking histidine liquid medium plates, SC-Ade synthetic complete-lacking adenine liquid medium plates.

GAD/GBD[a]	**SC-His**[b]	**SC-Ade**[b]	**β-Galactosidase**[c]
DifE-P3/DifE-P3	+	+	210.1 ± 10.4
DifE-P3/pGBDC1	–	–	4.5 ± 0.6
pGADC1/DifE-P3	–	–	7.1 ± 0.9
$DifA_{256-413}$/$DifA_{256-413}$	+	–	27.9 ± 1.3
$DifA_{256-413}$/pGBDC1	–	–	8.4 ± 2.1
pGADC1/$DifA_{256-413}$	–	–	5.4 ± 0.5

[a] *GAD* and *GBD* indicate fusions with GAL4AD and GAL4BD, respectively.

[b]Growth on these plates is indicated by + and no growth by –.

[c]β-Galactosidase activity is expressed as Miller units.

The CheC homologue DifG interacts with DifD

DifG is another member of the Dif pathway. It shares approximately 27% sequence identity with the *B. subtilis* chemotaxis protein CheC. In *B. subtilis* CheC was shown to interact with MCP and CheA in Y2H experiments (60). To examine the interactions of DifG with other Dif proteins, GAL4BD-DifG and GAL4AD-DifG fusions were constructed (see Materials and methods) and used in Y2H studies. Somewhat surprisingly, yeast transformants containing a DifG fusion construct in combination with any of the available DifA, DifC, and DifE Y2H constructs showed no growth on either the SC-Ade or SC-His plates (Table 2-1; Fig. 2-1; data not shown). The only transformants that grew on these plates are the ones with both DifG and DifD constructs (Fig. 2-4). Analysis of β-galactosidase activity also indicated the expression of the *lac*Z reporter in the transformants with DifD and DifG constructs (Fig. 2-4). It was reported recently that *B. subtilis* CheC, to which DifG is homologous, might function as a phosphatase of CheY-P (99). Our results here may also be explained if DifG functions as a phosphatase of DifD-P in *M. xanthus* similarly as CheC in *B. subtilis* (99).

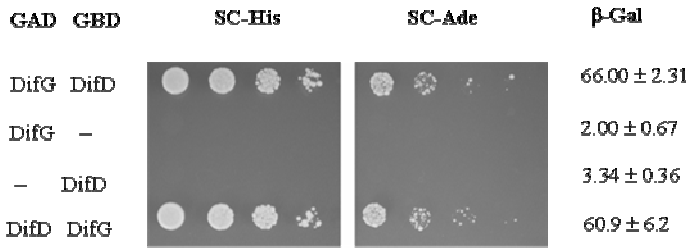

Fig. 2-4. Interactions between DifD and DifG. Transformants of yeast PJ69-4A with the DifD and the DifG Y2H constructs were examined for growth by the spot assay as described for Fig. 2a. Growth on SC-His with 2 mM 3-AT and SC-Ade was documented after 4 days of incubation. Shown on the right is β-galactosidase (β-gal) activity of the transformants. Similar results were observed when reciprocal sets of plasmids were used. β-Galactosidase activities were examined as described in Materials and methods.

DifB shows no interaction with other Dif proteins

DifB, which is downstream of DifA and upstream of DifC, is predicted to be a positively charged protein with some homology to a conserved bacterial protein family with unknown functions. Previous studies showed that an in-frame deletion of *difB* does not affect EPS production, S motility or development under the conditions examined. Although the role of DifB remains unclear, the clustering of *difB* with the other *dif* genes may nevertheless suggest interactions between DifB and the other Dif proteins. A GAL4BD-DifB full-length fusion was therefore constructed and co-transformed with other GAL4AD-Dif constructs into the Y2H reporter strain PJ69-4A. None of the resulting yeast transformants showed any growth on either SC-His plates or SC-Ade plates (data not shown). These results suggest that DifB may not interact directly with the other Dif proteins, at least not in a form detectable by the Y2H system.

DISCUSSION

It was proposed previously that the *M. xanthus* Dif proteins constitute a chemotaxis-like signal transduction pathway with crucial regulatory function in EPS production (13, 16, 114, 116). In the present study, we provided evidence for direct interactions among the Dif chemotaxis homologues using the Y2H system. DifC (CheW-like) showed pairwise interactions with both DifA (MCP-like) and DifE (CheA-like) (Fig. 2-2a). Using a modified Y2H system in which GAL4-DifA and GAL4-DifE fusions were transformed into yeast along with a construct that permits constitutive expression of DifC, we showed that DifC can interact with both DifA and DifE to form a ternary complex (Fig. 2-2b). We have additionally demonstrated that the interactions between DifC and DifE are mediated through the predicted P5 domain of DifE as expected for chemotaxis-like interactions (Fig. 2-3). These results support the proposed formation of a ternary signaling complex in which DifA functions as the equivalent of a chemoreceptor and interacts with the CheW homologue DifC, which in turn propagates signals by its interaction with the CheA-like kinase DifE.

Somewhat surprising was the finding that the reporter gene *lacZ* and *ADE2* were expressed at significantly higher level in the three-hybrid setup (Fig. 2-2b) than in the two-hybrid experiments with DifA–DifC and DifC–DifE pairwise interactions (Fig. 2-2a). There are at least two plausible explanations for this observation. It can be argued that the interactions among the three proteins in the three-hybrid system may not be any stronger or more stable than the individual pairwise interactions. The

increases in reporter gene expression may merely reflect increased stability or expression of these proteins when co-expressed in *S. cerevisiae*. However, there has been no report of protein stability issues with Y2H systems as far as we are aware, and the *ADH1* promoter (48) for the expression of the fusion proteins is unlikely affected by the co-expression of these three proteins. Alternatively, the increased expression of reporter genes in the three-hybrid setup may truly reflect stronger or more stable interactions in the DifA–DifC–DifE ternary complex. Future studies of the Dif proteins are necessary to fully understand the nature of the interactions among the Dif proteins.

The interaction between DifE and DifD were also demonstrated using the Y2H system. This intermolecular interaction occurs between DifD and the predicted P2 domain of DifE. We also demonstrated the interaction between the P3 domain of DifE with itself, suggesting that DifE may form dimers through this domain. Because it is the P2 domain of CheA that interacts with CheY in bacterial chemotaxis, and because it is the P3 domain that mediates CheA dimerization (14), these interactions are consistent with DifE being a CheA-type histidine kinase. One distinct feature of DifE is the presence of some extra 185 amino acids inserted between its P1 and P2 domains. Although P2 alone interacted with DifD, the insertion and P2 of DifE together failed to display any interaction with DifD in the Y2H system (data not shown). The biological significance of this insertion in DifE is unknown, and whether the insertion plays a role in regulating the interactions between DifE and DifD in *M. xanthus* remains to be determined.

DifA as an MCP homologue is predicted to be a multi-domain protein. Our results suggest that it is the highly conserved signaling domain of DifA that interacts with DifC, and that the very C-terminus of DifA may interact with itself for DifA dimerization. The interaction of DifA signaling domain with DifC was expected, because it had been well established that MCPs interact with CheW through this domain (4, 34, 64, 82, 98). Not expected was the finding that the inclusion of the flanking MHs perhaps weakened this interaction. It is known from studies of bacterial chemotaxis that the methylation of chemoreceptors modulates CheA kinase activity to bring about adaptation (7, 19, 22, 33). It has been proposed that the modulation of CheA activity is through conformational changes of MCPs, which are then transmitted through CheW to CheA. However, it is now well-established that the interactions among chemotaxis proteins and among the arrays of chemotaxis signaling complexes are very fluid and dynamic (19, 25, 27, 36, 92, 94). MHs may simply influence the conformation or structure of the DifA signaling domain to modulate the strength of DifA–DifC interactions in the *M. xanthus* Dif system.

DifG is a homologue of *B. subtilis* CheC. It was reported previously that *B. subtilis* CheC interacted strongly with the *B. subtilis* chemotaxis proteins CheD and the chemoreceptor McpB and weakly with CheA in Y2H systems (60). There is no CheD homologue encoded by the *dif* locus (114) or by the *M. xanthus* genome for that matter (http://www.tigr.org). We failed to detect any interactions of DifG with either DifA or DifE using the Y2H system (data not shown). Our results showed, however, that DifG does interact with DifD in Y2H systems (Fig. 2-4). It was reported recently

that *B. subtilis* CheC can function as a phosphatase in vitro to hydrolyze CheY-P (99), indicating direct interactions between *B. subtilis* CheC and CheY. The results here demonstrate that similar interactions also occur between CheC and CheY homologues in gram-negative bacteria.

Although much work is necessary to fully understand the Dif pathway and its regulatory roles in *M. xanthus* EPS production, S motility, and development, the Y2H studies presented here provide important insights into the architecture of the Dif pathway. The proposed formation of chemotaxis-like signaling complexes by DifA, DifC, and DifE are supported by the results in this study. The results with DifD and DifG support a prediction by the published report of CheC being a phosphatase of CheY-P in *B. subtilis* (99). Both DifD and DifG are negative regulators of EPS production in *M. xanthus*. If DifG functions as a phosphatase of DifD-P, the current available data could still be explained by the two models in either a linear or a branched pathway. The differentiation of the two models requires additional experiments. Other unresolved issues include how the Dif pathway regulates both phosphatidylethanolamine taxis (55, 57) and EPS production and how the Dif pathway may interact with other chemotaxis or chemotaxis-like pathways in *M. xanthus* (61, 104, 106). Preliminary Y2H experiments detected no interactions between Dif and Frz proteins (Z. Li and Z. Yang, unpublished results).

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CHAPTER 3

KapB, a Shared Modulator of a Ser/Thr Kinase Pathway and a Two-component Pathway

ABSTRACT

Myxococcus xanthus Dif proteins, which regulate exopolysaccharide (EPS) production, are homologous to chemotaxis proteins. DifE, the CheA histidine kinase homologue, was used as a bait to screen *M. xanthus* genomic libraries in the yeast two-hybrid (Y2H) system. KapB, a TPR (Tetratricopeptide repeat) protein, was identified as an interacting partner of DifE using the Y2H system. Further Y2H analysis demonstrated that it was the N-terminal half of KapB that interacted with the P2 domain of DifE. KapB had been previously reported to interact with components of several Serine/Threonine kinase pathways including the Pkn4-Pfk pathway, which is implicated in glycogen metabolism in *M. xanthus*. Deletion mutants of *kapB* as well as of the *pfk-pkn4* operon (*pfkn*) were found to overproduce EPS in this study. It was also found that the Dif pathway is involved in glycogen metabolism through the analysis of glycogen level in *dif* mutants. These results indicate EPS biogenesis and glycogen metabolism may be coordinately regulated in *M. xanthus*. This coordination of the Dif-regulated EPS production and the Pkn4-regulated glycogen metabolism appears to be accomplished at least partially through KapB. This is the first example of a TPR protein mediating the interplays of a histidine kinase pathway and a Ser/Thr kinase pathway.

INTRODUCTION

Myxococcus xanthus, a Gram-negative rod-shaped soil bacterium, exhibits complex social behaviors best represented by fruiting body formation (31). Under nutrient limitation, about 100,000 cells can aggregate to form the multicellular structure called a fruiting body (29, 31). During fruiting body formation, *M. xanthus* cells utilize two gliding motility systems, the adventurous (A) and the social (S) motility, to move over solid surfaces. A-motility enables *M. xanthus* to move as well-isolated cells and S-motility functions when cells are close to one another. S-motility is more crucial to fruiting body formation as all mutants of the S-motility system are defective in development to various extent (47, 67).

Exopolysaccharide (EPS) is one of the structures required for a functional *M. xanthus* S-motility (9, 13, 87, 107, 116). It was suggested that *M. xanthus* S-motility is powered by the extension and retraction of type IV pili as in the twitching motility of *Pseudomonas* and *Neisseria* (51, 71, 90, 97). Previous studies suggested EPS may serve as the anchor and trigger for type IV pili retraction in *M. xanthus* (63). EPS may also provide physical connections among neighboring cells (8, 12) as it is located all over the cell surface.

EPS biogenesis in *M. xanthus* is regulated in part by the *dif* (*d*efective *i*n *f*ruiting/*f*ibrils) locus (13, 16, 114, 116). Sequence analysis demonstrated that the *dif* locus encodes several bacterial chemotaxis protein homologues. DifA is homologous to MCP (Methyl-accepting Chemotaxis Protein), DifC to CheW, DifD to CheY, DifE to CheA (13, 114), and DifG to CheC (99). DifB belongs to a conserved family of

hypothetical bacterial proteins with unknown functions. Genetic studies showed that *difA*, *difC*, and *difE* mutants produce undetectable level of EPS (13, 114, 116) while *difD* and the *difG* mutants overproduced EPS in comparison with the wild type (16). Previous studies showed that Dif proteins interact with one another similarly as do the chemotaxis proteins, suggesting that the Dif proteins may form a chemotaxis-like pathway that regulates EPS biogenesis (115). However, genetic epistatic studies showed that DifD, the CheY homolog, does not function downstream of DifE. It was hypothesized that there are other proteins that function downstream of DifE to regulate EPS production (16). Recent studies demonstrated that type IV pili, the S-motility motor, likely functions as the sensor or part of the sensor to mediate the input signal into the Dif pathway (15). The signal then is transmitted to a DifA-DifC-DifE signaling complex to regulate the activity of the downstream components in EPS production (15).

In order to find additional EPS regulators, DifE was used as a “bait” to screen the *M. xanthus* genomic libraries in a Y2H system (100). Here we report the identification of KapB as an interacting partner of DifE and a negative regulator of EPS production. KapB was previously reported to be a regulator of the Pkn4-Pfk pathway that regulates glycogen metabolism in *M. xanthus* (45). Isolation and characterization of glycogen level from vegetative cells and spores from *dif* mutants showed that the Dif pathway is likely involved in glycogen metabolism as is KapB. We propose that KapB possibly functions as a protein that bridges the Dif and the Pkn4-Pfk pathways to coordinate EPS biogenesis and glycogen metabolism in *M. xanthus*.

MATERIALS AND METHODS

Strains, media and growth conditions. The bacteria and yeast strains used in this study are listed in table 3-1. *Escherichia coli* strains were grown in Luria-Bertani (LB) (80) liquid medium or on LB plates at 37°C. *M. xanthus* strains were grown on casitone-yeast extract (CYE) (24) plates or in CYE liquid medium at 32°C. *Saccharomyces cerevisiae* strain PJ69-4A was used as the yeast two-hybrid (Y2H) reporter strain; it has three reporter genes, *HIS3*, *ADE2* and *lacZ*, for a positive interaction (48). *S. cerevisiae* was grown at 30°C in YEPD (1% yeast extract, 2% peptone, 2% dextrose, pH 6.0) (79) and synthetic complete (SC) (37) liquid medium and plates. Plates contain 1.5% agar unless otherwise noted. Ampicillin (100 μg/μl) and kanamycin (100 μg/μl) were used for selection whenever applicable.

Construction of plasmids. The pGADC1 and pGBDC1 plasmids, which contain the GAL4 transcription activation domain (GAL4AD) and GAL4 DNA binding domain (GAL4BD) respectively (48), were used to construct plasmids for the Y2H experiments. GAL4AD and GAL4BD were fused at the C-terminus to full-length or truncated KapB or truncated DifE proteins. Four constructs were generated for Y2H studies. To construct pLZ226, a fragment encoding amino acids 121-320 of DifE was amplified by PCR, digested by *Eco*RI and *Bam*HI and ligated into pGBDC1 digested with the same enzymes. To construct pLZ450, full length KapB was PCR-amplified, digested with *Sma*I and *Sal*I and ligated into pGADC1 treated with the same restriction enzymes. To construct pLZ491, DNA encoding amino acids 148-271 of KapB was PCR-amplified, digested by *Sal*I and ligated into pGADC1 treated with

*Sal*I and *Sma*I. To construct pLZ492, DNA encoding amino acids 1-147 of KapB was PCR-amplified, digested with *Cla*I and ligated into the *Cla*I and *Sma*I sites of pGADC1.

For construction of *difD* and *kapB* in-frame deletion, fragments which cover 650 bp upstream and 650 bp downstream of *difD* and *kapB* were PCR-amplified respectively. The upstream and downstream fragments for *difD* and *kapB* were then PCR-jointed respectively. These two 1.3kb PCR-jointed fragment were cloned into pBJ113 (50) digested by S*ma*I respectively to generate plasmid pLZ405 and pLZ431. Restriction enzymes, T4 DNA ligase and T4 DNA polymerase (for creating blunt end) were purchased from New England Biolabs. *PfuUltra* DNA polymerase from Stratagene (La Jolla, CA) was used in PCR.

Construction of *M. xanthus* deletion strains. Four in-frame deletion strains were constructed in this study by a modified “positive-negative KG cassette” two step selection method (102). To construct *kapB* deletion mutants, pLZ431 was transformed into DK1622 and YZ603 (16) respectively to generate YZ818 (*kapB*) and YZ822 (*kapB difE*). To construct the *pfk-pkn4* (*pfkn*) double deletion in wild type and *difE* deletion background, plasmid pΔPFKN (44) was transformed into DK1622 and YZ603 respectively to give rise to YZ823 (*pfkn*) and YZ824 (*pfkn difE*). All deletions were verified by PCR.

Table 3-1. Strains and plasmids used in this study.

Strains or plasmids	Description/genotype	References
E. coli strain		
DH5α	Recipient strain. F^- *endA*1 *glnV*44 *thi-1 recA*1 *relA*1 *gyrA*96 *deoR nupG* Φ80*dlacZ*ΔM15 Δ(*lacZYA-argF*)U169, *hsdR*17(r_K^- m_K^+), λ–	(39)
HB101	Strain for *leuB6* complementation. F^- m*crB mrr hsdS20*(r_B^- m_B^-) *recA13 leuB6 ara-14 proA2 lacY1 galK2 xyl-5 mtl-1 rpsL20*(Sm^R) *glnV44* λ^-	(80)
S. cerevisiae strain		
PJ69-4A	*MATa trp1-901 leu2-3, 112 ura3-52 his3-200 gal4*Δ *gal80*Δ *LYS2::GAL1–HIS3 GAL2–ADE2 met2::GAL7–lacZ*	(48)
M. xanthus strains		
DK1622	Wild type	(53)
DK10407	*ΔpilA*	(112)
YZ603	Δ*difE*	(16)
YZ801	Δ*difD*	This study
YZ818	Δ*kapB*	This study
YZ822	Δ*kapB* Δ*difE*	This study
YZ823	Δ*pfkn*	This study
YZ824	Δ*pfkn* Δ*difE*	This study
Plasmids		
pBJ113	Gene replacement vector with KG cassette; Kan^r	(50)
pGADC1	GAL4AD vector; *LEU2*	(48)
pLZ201	GAL4BD-DifE	(115)
pLZ224	GAL4BD-$DifE_{321-470}$	(115)
pLZ226	GAL4BD-$DifE_{121-320}$	This study
pLZ228	GAL4BD-$DifE_{472-540}$	(115)
pLZ230	GAL4BD-$DifE_{722-855}$	(115)
pLZ405	*difD* in-frame deletion in pBJ113	This study
pLZ431	*kapB* in-frame deletion in pBJ113	This study
pLZ450	GAL4AD-KapB	This study
pLZ491	GAL4AD-$KapB_{148-271}$	This study
pLZ492	GAL4AD-$KapB_{1-147}$	This study
pLZ499	GAL4AD-$KapB_{1-244}$	This study
pΔPFKN	*Pfk-pkn4* in-frame deletion construct	(44)

Table 3-2. Primers used in this study.

Plasmid	Primers[a]	Enzyme[b]
pLZ226	CTCCTGACGGAATTCAGCCAGCGC	*Eco*RI
	TGGAGCCGCGGATCCGGCGTCGGT	*Bam*HI
pLZ405	CGGTCGAAGCGGTAGCGAATCGGG	N/A
	GGGCGCAGCCGCTCACGTCTCCTTAGC**CAT**GTGAAGAACTCC	N/A
	GAGACG**TGA**GCGGCTGCGCCC	N/A
pLZ431	CGCCGTTCATCAGATTGGGGCTGG	N/A
	GGTGACGATGGCCGGGCGCCGGGC	N/A
	TTCGTTGCCCATGGCTCAGAACAAGCGGGC**CAT**GAGCGCGCGGGCAAT	N/A
	TTGTTC**TGA**GCCATGGGCAACGAA	N/A
	TGCGCCATCTCCAGCGCGCGCTCC	N/A
pLZ450	ATTGCCCGGGCGCTC**ATG**GCCCGC	*Sma*I
	TTCGTCGACCATGGC**TCA**GAACAA	*Sal*I
pLZ491	CTGGACTCCCAGGATGCCTTCCCC	N/A
	TTCGTCGACCATGGC**TCA**GAACAA	*Sal*I
pLZ492	ATTGCCCGGGCGCTC**ATG**GCCCGC	N/A
	GGAGTCCAGATCGATGGCCGTCTG	*Cla*I

[a]Sequences of primers are written from 5' to 3'. Restriction sites in primers are underlined. Start and stop codons for relevant genes are in boldface when applicable

[b]Restriction enzymes for which recognition sites were introduced in primers. *N/A* Not applicable

Library screening using the yeast two-hybrid system. GAL4BD-DifE fusion plasmid pLZ201 (115) was used as a "bait" to screen three *M. xanthus* genomic libraries in pGAD vectors (100). The bait plasmid was transformed into yeast strain PJ-694A (48) first by the LiAc-PEG method (37) and plated onto SC plate lacking tryptophan. After the yeast transfromants that contain pLZ201 grew on the above

plates, they were transformed by the *M. xanthus* genomic libraries in pGAD vectors and plated onto SC plates lacking leucine, tryptophan and histidine with 2 mM 3-amino-1,2,4,-triazole (3-AT) (SC-His). Yeast transformants were then replica-plated onto SC-His plates and SC plates lacking leucine, tryptophan and adenine (SC-Ade). Plasmids from the yeast isolates that grew on both SC-His and SC-Ade plates were then transformed into *E. coli* strain HB101 (39). This *E. coli* strain harbors the *leuB6* mutation for facilitating the selection of pGAD derived constructs which has a *LEU2* marker. Plasmid which has *M. xanthus* genomic DNA fused at the 3' end of gene encoding GAL4 transcription activation domain in *E. coli* can complement the *leuB6* mutation and enable the cells to grow on M9 plates (80). "Fish" plasmids were isolated from these *E. coli* cells and confirmed by PCR using pGAD vector specific primers. The confirmed "fish" plasmids were then transformed back into the "bait" strain containing the GAL4BD-DifE fusion plasmid pLZ201. Growth was confirmed on SC-His and on SC-Ade plates to eliminate any false positives. Confirmed positive "fish" plasmid were then sequenced and studied further. β-Galactosidase activity of yeast transformants was examined using the method described by Kippert (59) and expressed in Miller units (72).

Examination of EPS production. Two methods were used to examine EPS production. One was a plate assay (26, 76) to examine the binding of calcofluor white, an EPS-specific fluorescent dye. Cells were harvested and resuspended in MOPS buffer (10 mM MOPS [pH 7.6], 2 mM $MgSO_4$) (38) at 5×10^9 cells/ml. 5 μl of this cell suspension were put on the CYE agar plates with calcofluor white (50 μg/ml).

Plates were examined under UV light (365 nm) after 7 days of incubation at 32°C. Another was a liquid assay to quantitatively measure EPS level by the amount of binding of Trypan blue to the EPS on cell surface (9). Cells were harvested at OD_{600} value at about 0.7 and resuspended in MOPS buffer to 2.8×10^8 cells/ml. 1350 μl of these cell suspensions were mixed with 150 μl Trypan blue (50 μg/ml) and incubated for 30 minutes with shaking at room temperature. These mixtures were centrifuged and OD_{585} values of the supernatants were measured. Absorbance of each sample was divided by the absorbance of control to give the percentage of dye binding and the relative amount of EPS produced.

Examination of development. For the examination of development, cells of various strains were resuspended to 10, 5 and 2.5 at OD_{600} respectively with MOPS buffer. 5 μl cell suspension were put onto CF plates (62). To document fruiting body development, pictures were taken after 3, 6, 9, 12, 24, 36 and 48 hours of incubation. To examine heat resistant spores, various strains were grow to OD_{600} about 2 (early stationary phase) and cells were resuspended to OD_{600} 10. 10 μl (5×10^6 cells) aliquots were put onto CF plates and incubated at 32°C for 2 days. These plates were then incubated at 52°C for 2 hours. Fruiting bodies were collected with a razor blade and resuspended in TM buffer (10 mM Tris-HCL, 8 mM $MgSO_4$, pH 7.6) (44). Samples were sonicated for 1 minute, and serial dilutions were plated onto CYE plates. Colonies numbers were counted after 7 days of incubation at 32°C.

Glycogen assay. Isolation and analysis of glycogen were carried out as described by Nariya and Inouye (44) from 1×10^{10} vegetative cells at middle log phase (OD_{600}

1), early stationary phase (OD_{600} 2), and myxo spores which were initiated by 1×10^{10} cells of early stationary cells. Myxo spores were collected with razor blades from TM plates after 24 hours, 30 hours and 48 hours of incubation at 32°C respectively. Cells or spores were washed twice with 1.5 ml of TM buffer. They were then boiled with 0.5 ml 33 % KOH for 30 minutes and cooled down to room temperature. After centrifugation at 10, 000 x g for 10 minutes, cell debris were removed. The pellets were mixed with 1ml ethanol and boiled for 5 minutes and then immediately cooled on ice for 5 minutes. White pellets were collected after centrifugation at 10, 000 x g for 10 minutes at 4°C. These pellets were dissolved in 0.5 ml of H_2O and boiled for 5 minutes. After cooled down to room temperature, 1 ml of -20°C ethanol was added into the tube and the mixture was incubated on ice for 5 minutes. After centrifugation at 10, 000 x g for 10 minutes at 4°C, pellets were washed by 1.5 ml -20°C ethanol and Acetone respectively. The pellets were air-dried and then dissolve in 0.2 ml H_2O. 1.3 ml Iodine-iodide solution (44) were added to each tube and mixed by vertex. OD_{510} of each sample was then recorded.

RESULTS

DifE interacts with KapB, a TPR protein

To identify additional EPS regulators, the GAL4BD-DifE fusion plasmid pLZ201 was used as a "bait" to screen three *M. xanthus* genomic libraries in pGAD vectors. 24 yeast transformants were found to grow on SC-His plates and 2 of these 24 transformants also grew on SC-Ade plates (data not shown). Sequencing results showed that one of these two potential interacting partners is KapB (kinase associated protein B). The other one is a PilF homologue, a deletion of this gene did not cause any defect in motility, EPS production and fruiting body formation (data not shown). The KapB protein that was pulled out from the library lacks 27 amino acids at the carboxyl terminus. The "fish" plasmid containing GAL4AD-KapB$_{1-244}$ was named pLZ499. As shown in Fig. 3-1, yeast transformants containing pLZ201 and pLZ499 were able to grow on both SC-His and SC-Ade plates and expressed high level of β-galactosidase activities. The relevant controls did not show any growth on the selective plates and expressed negligible level of β-galactosidase activity.

Plasmid pLZ450 was generated by fusing full-length KapB with GAL4AD and this construct was used to examine the interaction of KapB with DifE. Yeast transformants containing GAL4BD-DifE and GAL4AD-KapB were able to grow on both SC-His and SC-Ade plates (data not shown). To further confirm the interactions between KapB and DifE, reciprocal sets of above constructs were used in Y2H studies. We observed that yeast transformants were also able to grow on both SC-His and

SC-Ade plates (data not shown). These results suggest that KapB can interact with DifE in the Y2H system.

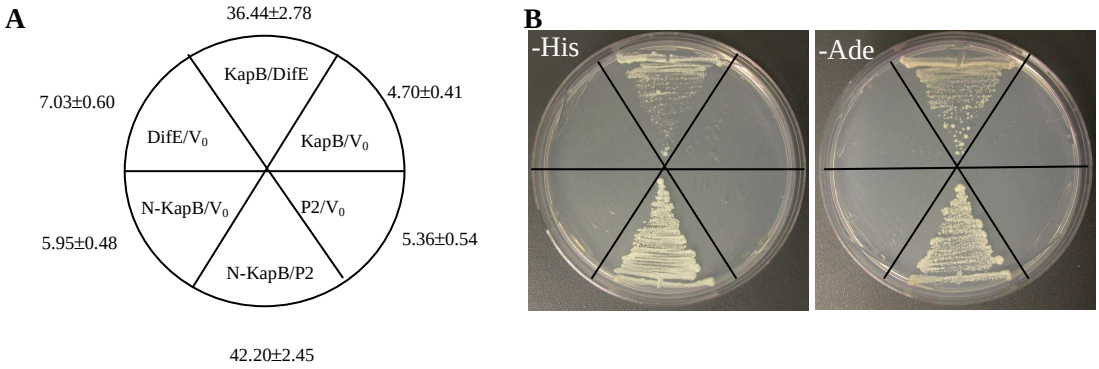

Figure 3-1. Interactions of KapB with DifE. Yeast transformants containing Y2H constructs and/or vectors as demonstrated in panel A were examined for growth on selective plates as shown in panel B. V_0: Y2H vectors without inserts. KapB is a truncated form lacking 27 amino acids of the C-terminus. N-KapB: the N-terminal half (containing amino acids 1-147) of KapB. P2: the putative substrate-binding domain of DifE. Pictures were taken after 5 days of incubation at 30°C. –His plate was supplemented with 2mM 3-AT. β-galactosidase activities were analyzed and are shown in panel A next to each corresponding section. Plasmids used are pLZ201 (GAL4BD-DifE), pLZ499 (GAL4AD-$KapB_{1-244}$), pLZ224 (GAL4BD-$DifE_{321-470}$), pLZ492 (GAL4AD-$KapB_{1-147}$) and empty Y2H vectors pGAD-C1 and pGBD-C1.

KapB is a protein containing 8 TPR (Tetratricopeptide repeats) domains with 4 in each half of the protein (45). In order to characterize which individual domain of DifE interacts with KapB, plasmids pLZ224 (GAL4BD-$DifE_{321-470}$), pLZ228 (GAL4BD-$DifE_{472-540}$), pLZ230 (GAL4BD-$DifE_{722-855}$) and pLZ226 (GAL4BD-$DifE_{121-320}$) were used with plasmid pLZ450 in Y2H experiments. Surprisingly, no interaction was identified in this experiment (Table 3-3). pLZ491 and pLZ492, each containing the C-terminal half and the N-terminal half of KapB respectively, were constructed (each has four TPR domains) and used in Y2H studies. It was found that the yeast transformants containing the N-terminal part of

KapB with either the full-length DifE (Table 3-3) or the P2 domain of DifE ($DifE_{321-470}$) were able to grow on SC-His and SC-Ade plates and expressed high level of β-galactosidase activity (Fig. 3-1 and Table 3-3). We also observed the growth of yeast transformants on SC-His and SC-Ade plates when reciprocal sets of Y2H vectors were used (data not shown). These results indicate that KapB possibly interacts with the P2 domain of DifE via the mediation of its N-terminal half in Y2H system.

Table 3-3. Interactions among various domains of DifE and KapB

GAL4AD \ GAL4BD	DifE	$DifE_{121-320}$	$DifE_{321-470}$	$DifE_{472-540}$	$DifE_{722-855}$
KapB	+[a]	–[a]	–	–	–
$KapB_{1-147}$	+	–	+	–	–
$KapB_{148-271}$	–	–	–	–	–

[a] "plus" or "minus" stand for growth or no growth on SC-His and SC-Ade plates respectively. Plasmids used are pLZ201 (GAL4BD-DifE), pLZ226 (GAL4BD-$DifE_{121-320}$), pLZ224 (GAL4BD-$DifE_{321-470}$), pLZ228 (GAL4BD-$DifE_{472-540}$), pLZ230 (GAL4BD-$DifE_{722-855}$), pLZ450 (GAL4AD-KapB), pLZ491 (GAL4AD-$KapB_{148-271}$) and pLZ492 (GAL4AD-$KapB_{1-147}$).

KapB negatively regulates EPS production in *M. xanthus*

The interaction between KapB and DifE may indicate that KapB is involved in the regulation of EPS biogenesis as Dif proteins in *M. xanthus*. A *kapB* deletion strain was constructed and the EPS production of this mutant was examined as described in Materials and Methods. This mutant was first examined by a plate assay using the fluorescent dye calcofluor white. When exposed under UV light, it was found that the

colony of *kapB* deletion mutant fluoresced more than that of the wild type (Fig. 3-2). We further examined the EPS amount produced by this mutant with a quantitative assay. As shown in figure 3-3, the amount of EPS produced by *kapB* mutant is about 1.7 times that of the wild type. These results suggest that KapB is involved in the regulation of EPS biogenesis in *M. xanthus* and possibly serves as a negative regulator.

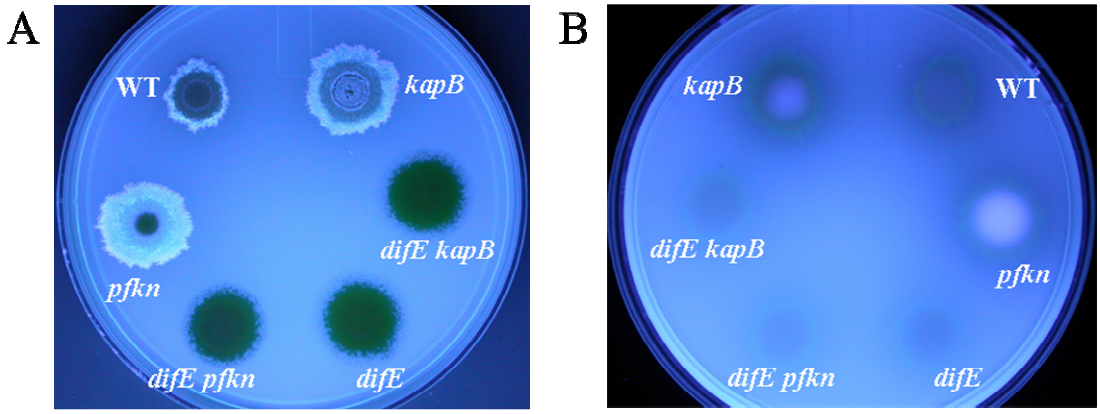

Figure 3-2. Binding of calcofluor white by various *M. xanthus* strains. 5 μl of cell suspensions at 5×10^9 cells/ml were spotted onto CYE plates containing calcofluor white at 50 μg/ml. After 7 days of incubation at 32°C., the plates were examined under 365-nm-wavelength UV light on both top (panel A) and bottom (panel B) side. Strains used are DK1622 (WT), YZ603 (*difE*), YZ818 (*kapB*), YZ822 (*difE kapB*), YZ823 (*pfkn*) and YZ824 (*difE pfkn*).

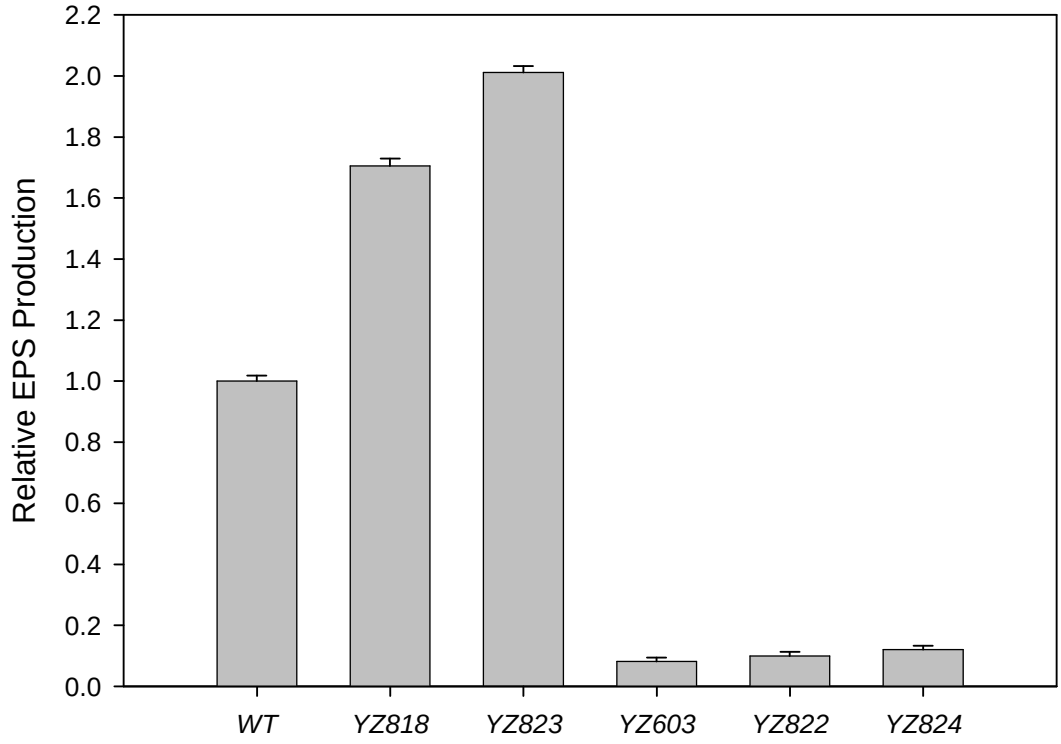

Figure 3-3. Quantitative examination of EPS production. Relative EPS production is the absorbance of various strains divided by the absorbance of controls (see Materials and Methods). The relative EPS production of wild type was normalized to 1. The experiment was repeated three times with samples in triplicate. Strains used are DK1622 (WT), YZ603 (*difE*), YZ818 (*kapB*), YZ822 (*difE kapB*), YZ823 (*pfkn*) and YZ824 (*difE pfkn*).

Pkn4-Pfk pathway is involved in EPS production.

It was previously reported that KapB served as a modulator of the Pkn4-Pfk pathway in the regulation of glycogen metabolism and physically interacted with the Ser/Thr kinase Pkn4 (45). Since KapB is involved in EPS biogenesis, the role of Pkn4-Pfk pathway in EPS biogenesis was thus investigated. An in-frame deletion of the *pfk-pkn4* (*pfkn*) operon was made in a DK1622 background and EPS production of this mutant (YZ823) was examined. It was found that under UV light, the colony of YZ823 fluoresced more than the wild type (Fig. 3-2). When examined with a liquid

assay, we observed that the amount of EPS produced by the YZ823 is about 2 times of that produced by the wild type (Fig. 3-3). These results demonstrated that deletion of the *pfk-pkn4* (*pfkn*) operon can cause elevated level of EPS production.

kapB and *pfkn* mutations did not suppress *difE*

In order to examine the epistatic relationship between the Dif pathway with KapB and with the Pfkn pathway, a *kapB difE* deletion strain YZ822 and a *pfkn difE* deletion strain YZ824 were constructed as described in Materials and Methods. The EPS production of these two strains was then examined by both plate assay and liquid assay. Colonies of YZ822 and YZ824 emitted similar level of fluorescence under UV light as that of the *difE* mutant YZ603 (Fig. 3-2). In the quantitative assay, YZ822 and YZ824 produced slightly more EPS than that of YZ603 but much less than the EPS produced by YZ818 and YZ823 (Fig. 3-3). In addition, both YZ822 and YZ824 showed similar swarming pattern as YZ603 when S-motility was checked on soft agar (0.4%) plate (data not shown). These results indicate that neither the *kapB* nor the *pfkn* mutation suppressed the *difE* mutation in EPS production and S-motility.

Dif pathway is involved in glycogen metabolism

Previous studies demonstrated that the Pkn4-Pfk pathway is required for glycogen consumption in *M. xanthus* (44). It was shown that *M. xanthus* accumulate glycogen in early stationary phase and the glycogen is consumed during development (44). Biochemical studies showed Pfk can be phosphorylated and activated by Pkn4.

Phosphorylated Pfk is the key enzyme to break down glycogen to provide energy for *M. xanthus* cells to finish development (43, 44). Since DifE interacts with KapB, the regulator of glycogen metabolism, we examined whether Dif pathway is involved in glycogen accumulation. Glycogen from vegetative cells and spores of different developmental stages of various strains were isolated and assayed as described in the Materials and Methods. As shown in Fig. 3-4, wild type cells accumulate glycogen at early stationary phase and the level gradually decreased when development progressed. The *difE* mutant YZ603 and *pilA* mutant DK10407 showed similar patterns and levels of glycogen as wild type at different stages. However, the *difD* mutant YZ801 and *pkfn* mutant YZ823 had different patterns. Although the glycogen level gradually decreased as that of wild type after the development was initiated, these two mutants accumulated much higher level of glycogen at every stage in comparison with the wild type. The level of glycogen at each stage in YZ823 is the highest among all the strains examined. The *kapB* mutant YZ818 also accumulated glycogen at the early stationary phase and the amount gradually decreased through development. The glycogen level at each developmental stage in YZ818 is lower than that of the wild type (Fig. 3-4). The results of *kapB* and *pfkn* mutations in glycogen accumulation are consistent with the previous studies (44, 45). The results also showed that the Dif pathway is likely involved in glycogen metabolism as deletion of *difD* can cause higher level of glycogen accumulation in *M. xanthus*.

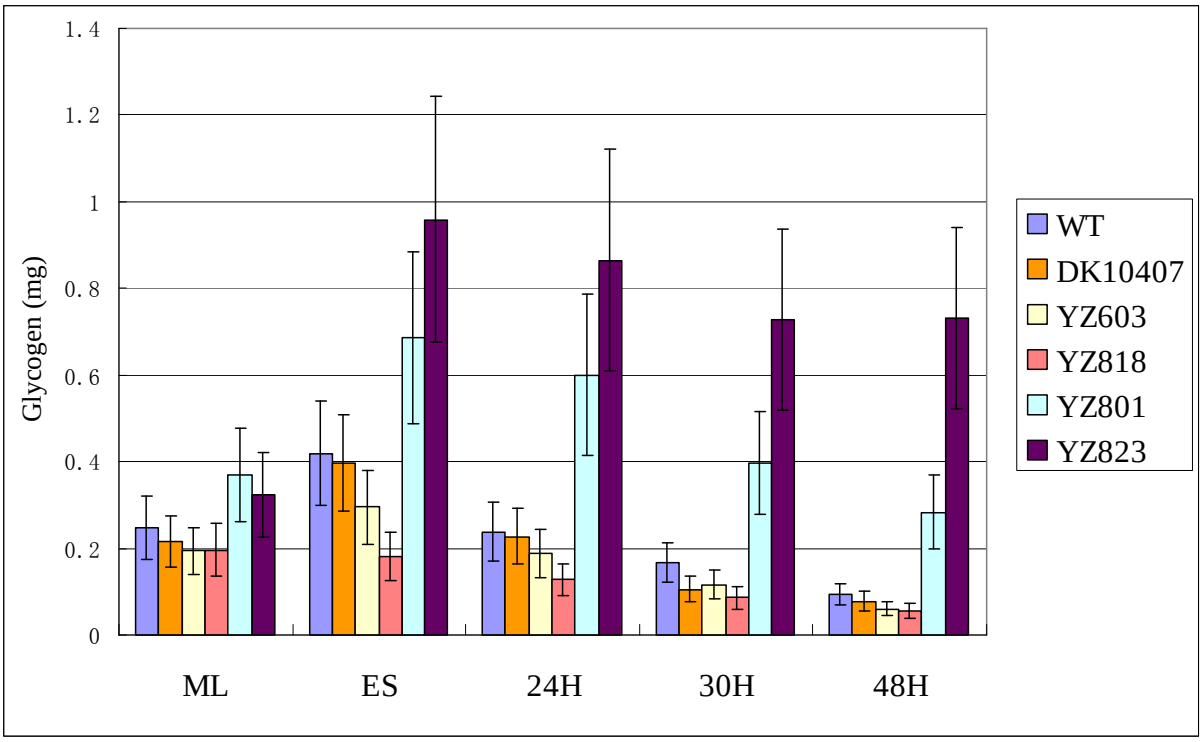

Figure 3-4. Glycogen level at various stages of *M. xanthus*. Glycogen was isolated and characterized as described in materials and methods. 1×10^{10} cells from middle log (ML) phase and early stationary (ES) phase and myxo spores initiated by 1×10^{10} early stationary cells after 24 h, 30 h and 48 h of development were collected respectively. Experiments were performed three times with triplicate. Strains used are DK1622 (WT), DK10407 (*pilA*), YZ603 (*difE*), YZ818 (*kapB*), YZ801 (*difD*), and YZ823 (*pfkn*).

Examination of development of *kapB* and *pfkn* mutants.

Since the Dif pathway controls fruiting body formation, it was examined whether the KapB and Pkn4-Pfk pathway are involved in the same process. Development of YZ818 and YZ823 was examined in a time course. It was found that both mutants developed faster than wild type. As shown in Fig. 3-5, in all three different cell densities, there was more aggregation at 6 hour of development for the mutants than that for wild type. After 24 hour of development, the aggregation appears to reach the peak for both wild type and mutants. The final fruiting bodies formed by both mutants

are irregular in comparison with that of wild type. We next examined the heat-resistant spore yield by both *kapB* and *pfkn* mutants. As described in Materials and Methods, the heat-resistant spores from both mutants and wild type were examined by colony forming units. The results showed that both mutants are defective in spore yield. Spore yield of the *kapB* and *pfkn* mutant is about 5.09% and 3.63% of that of wild type, respectively (Table 3-4). The spore yields in our results are little bit higher than that in previous studies (44, 45). This is probably because the CF plate which we used in the experiments has little more nutrients than TM plates.

Table 3-4. Spore yield of *M. xanthus* strains.

Strains	Spore Yield (%)[a]
WT	100±11.95%
YZ818	5.09±0.42%
YZ823	3.63±0.21%

[a] spore yield after 48 hours of development on CF plates were calculated as described in Materials and Methods. The spore yield of wild type was normalized to 100%. Strains used are DK1622 (WT), YZ818 (*kapB*) and YZ823 (*pfkn*).

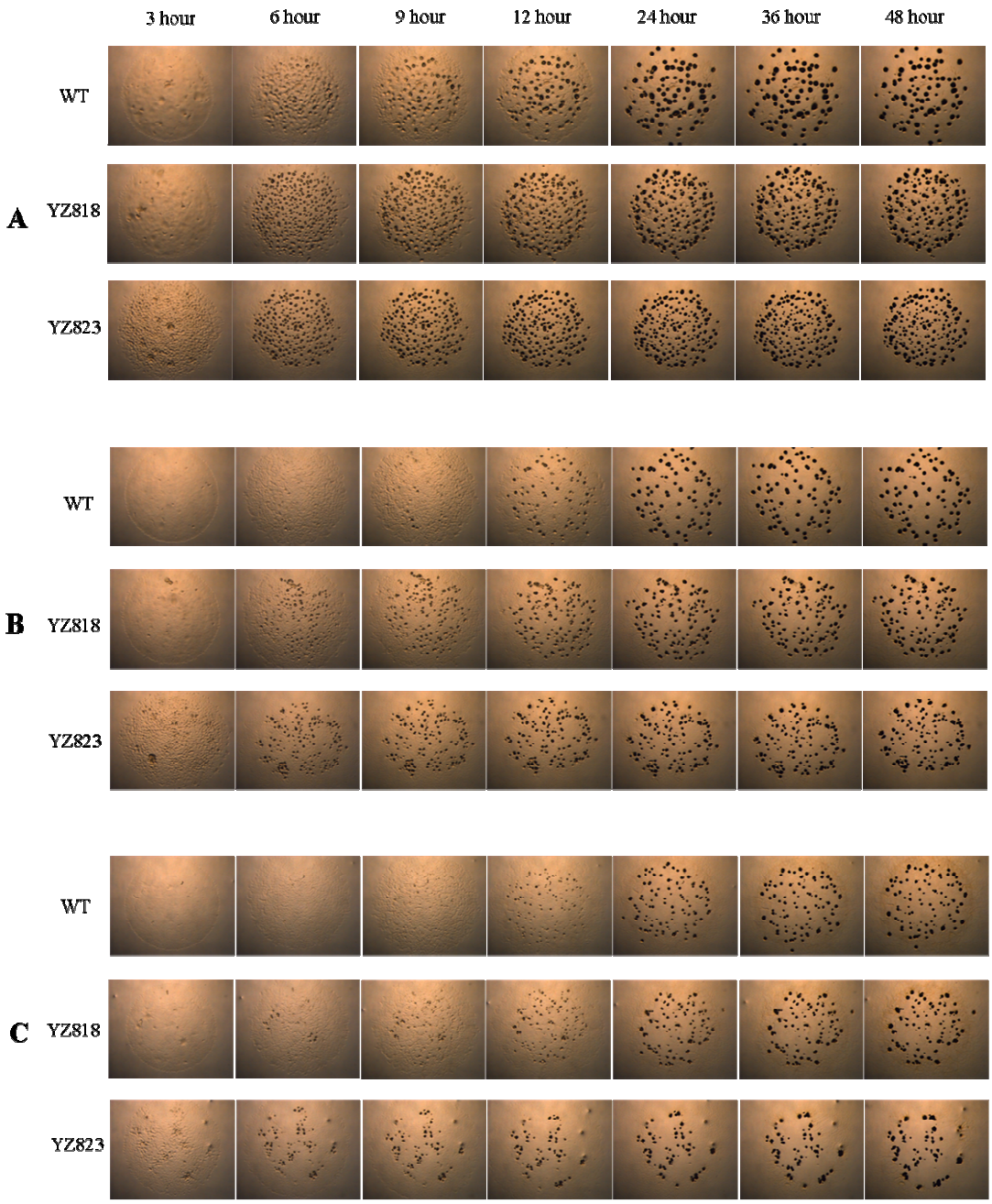

Figure 3-5. Examination of development of *M. xanthus* strains in a time course. Various strains were resuspended to different concentrations and put on CF plates. Pictures were taken after various time points after incubation at 32°C. A. OD_{600} 10. B. OD_{600} 5. C. OD_{600} 2.5. Strains used are DK1622 (WT), YZ818 (*kapB*) and YZ823 (*pfkn*).

DISCUSSION

EPS is important for *M. xanthus* cells to maintain physical contact (8, 12) as well as to for signaling with one another. Cells that fail to produce EPS are defective in S-motility and fruiting body formation (9, 13, 87, 107, 116). It was proposed that EPS functions as an anchor and trigger for pili of neighboring cells to attach and to retract (63). The Dif pathway is among several pathways to control EPS biogenesis in *M. xanthus* (13, 16, 114, 116). Sequence homology and protein-protein interaction studies using Y2H or a yeast "three-hybrid" system indicate DifE possibly functions similarly as CheA in other bacteria (115). It is possible that DifE can autophosphorylate and transfer the phosphate to a downstream regulator to regulate EPS biogenesis in *M. xanthus*.

In this study, KapB was identified as an interacting partner of DifE using a Y2H system. KapB is a protein with 8 TPR domains as previously reported (45). TPR domains are typically found in proteins that serve as scaffolds to mediate protein-protein interactions (5). In the case of KapB, it appears it interacts with the Ser/Thr kinase Pkn4 and the putative histidine kinase DifE. Further Y2H studies showed that the N-terminal half of KapB interacts with the putative P2 domain (the putative response regulator-binding domain) of DifE (Fig. 3-1 and Table 3-3). Genetic studies showed that KapB negatively regulates EPS biogenesis since *kapB* mutant overproduce EPS (Fig. 3-2). Epistatic studies showed that KapB unlikely functions downstream of DifE regarding EPS production as the *kapB* mutation did not suppress the *difE* mutation (Fig. 3-2 and Fig. 3-3). This could indicate that KapB may function

as a phosphate sink for DifE or an inhibitor of the kinase activity of DifE. It is also possible that KapB regulates a parallel pathway that is influenced by the activity of DifE. Further studies are needed to elucidate the biochemical feature of interactions between KapB and DifE.

KapB was previously reported to be a regulator of the Pkn4-Pfk Serine/Threonine kinase pathway involved in glycogen metabolism (45). As demonstrated by Nariya and Inouye, *M. xanthus* cells accumulate glycogen at the early stationary phase and glycogen level gradually decreases when development progresses 49. It was also shown that effective sporulation requires glycogen consumption through the key enzyme 6-phosphofructokinase (Pfk). Pfk can phosphorylate fructose-6-phosphate to fructose 1, 6-bisphosphate which can be further catabolized to possibly provide energy for cells to successfully finish the developmental life cycle (45). A *pfk-pkn4 (pfkn)* deletion mutant accumulated glycogen at high level through out the whole developmental process and showed very poor spore yield (43, 44). Biochemical studies showed Pfk can be activated by Pkn4 through phosphorylation at Thr-226. KapB was later found to interact with the regulatory domain of Pkn4 and can be phosphorylated by Pkn4. Phosphorylated KapB remains association with Pkn4 and thus inhibit the phosphorylation of Pfk by Pkn4 as well (43). The *kapB* mutant does not accumulate glycogen presumably because the activity of Pfk remains high and produce more spores than *pfkn* mutant (43).

Glycogen is a highly branched polymer of glucose residues with α-1, 4-linkage and α-1, 6 glycosidic bonds at the branch points. It accumulates in many bacteria

when growth is limited by factors other than carbon and energy (75). Since glucose is one of the major monosaccharides in *M. xanthus* EPS (11), it is likely that cells can effectively coordinate the glucose resource and utilize it in both EPS biogenesis and glycogen metabolism. In the *kapB* mutant, Pfk activity is not inhibited and continue to break down glycogen to release glucose, part of the glucose could be incorporated into EPS biogenesis pathway which may explain why this mutant overproduce EPS (Fig. 3-2 and Fig. 3-3). Phosphorylated KapB by Pkn4 may become active and thus inhibit the kinase activity of DifE which may explain why EPS is overproduced in the *pfkn* mutant (Fig. 3-2 and Fig. 3-3) since KapB is not activated. Another possibility for the overproduction of EPS in this mutant could be that accumulated fructose-6-phosphate is incorporated into EPS by some enzymes involved in EPS biosynthesis.

When investigated the role of Dif pathway in glycogen metabolism, we found that the glycogen accumulation pattern of the *difE* and *pilA* strains is similar to that of wild type (Fig. 3-4). To our surprise, the glycogen accumulation pattern of the *difD* mutant is quite different as this mutant accumulates much more glycogen in both vegetative cells and spores (Fig. 3-4). *difD* mutant overproduces EPS as reported in previous studies (16). It is possible that EPS in this mutant could be transformed into glycogen by yet unknown mechanism. The transformation might be through the interplay of Dif pathway and Pkn4 pathway via KapB.

In addition, the development of the *kapB* and *pfkn* mutants was examined. It was found that both mutants aggregate faster than wild type and formed irregular fruiting

bodies (Fig. 3-5). Further studies also showed both mutants are defective in producing heat-resistant spores (Table 3-4) which is consistent with previous studies (45). The reason that these mutants develop faster than wild type remains unknown. As mentioned earlier, these two mutants overproduce EPS. The overproduction of EPS may contribute to the faster aggregation of cells during fruiting body formation.

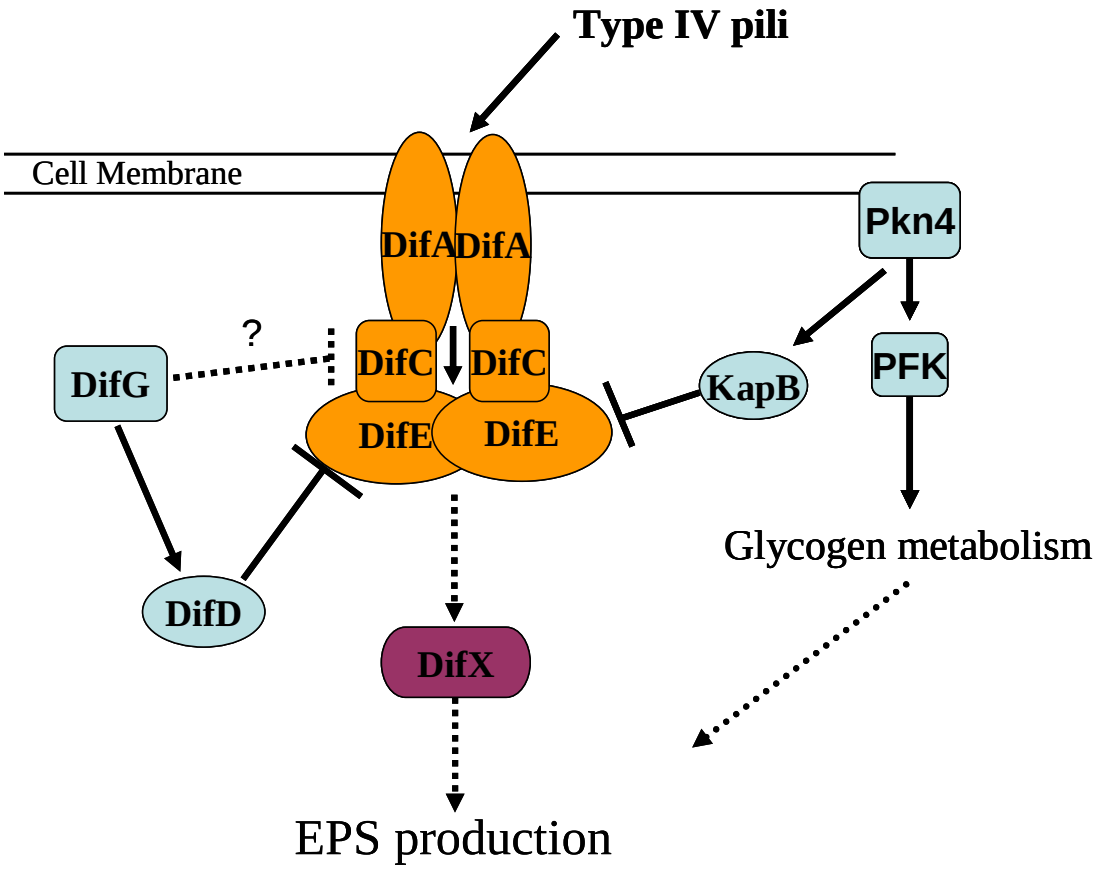

Figure 3-6. Proposed model of interaction between the Dif pathway and the Pkn4 pathway. KapB is proposed to be a phosphate sink of DifE or an inhibitor of the autokinase activity of DifE. DifX is/are the hypothetical downstream component(s) of the Dif pathway that regulate the EPS biogenesis.

Based on the results of this study, we propose that *M. xanthus* utilizes a Ser/Thr pathway and a two-component pathway to coordinate glycogen consumption with

EPS biogenesis through a shared modulator KapB (Fig. 3-6). These two pathways may also interact with other pathways to form a complex network to regulate the utilization of carbon source in *M. xanthus*. Phosphorylation studies using purified DifE and KapB proteins may provide insights on how these two proteins interact with each other to regulate these processes.

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Appendix: Plasmids and strain lists

Table 4-1. Plasmids constructed.

Plasmids	Features	References
pLZ101	DifC in pQE31	This study
pLZ170	GAL4BD-DifG	Chapter 2
pLZ180	GAL4AD-DifG	Chapter 2
pLZ201	GAL4BD-DifE	Chapter 2
pLZ210	GAL4BD-DifA$_{54-413}$	Chapter 2
pLZ211	GAL4AD-DifA$_{54-413}$	Chapter 2
pLZ212	GAL4BD-DifA.$_{137-413}$	Chapter 2
pLZ213	GAL4BD-DifA$_{137-413}$	Chapter 2
pLZ214	GAL4BD-DifA$_{137-360}$	Chapter 2
pLZ215	GAL4AD-DifA$_{137-360}$	Chapter 2
pLZ216	GAL4BD-DifA$_{256-360}$	Chapter 2
pLZ217	GAL4AD-DifA$_{256-360}$	Chapter 2
pLZ218	GAL4BD-DifA$_{256-413}$	Chapter 2
pLZ219	GAL4AD-DifA$_{256-413}$	Chapter 2
pLZ220	GAL4BD-DifB	Chapter 2
pLZ221	GAL4AD-DifB	Chapter 2
pLZ222	GAL4BD-DifC	Chapter 2
pLZ223	GAL4AD-DifC	Chapter 2
pLZ224	GAL4BD-DifE321-470	Chapter 2
pLZ225	GAL4AD-DifE321-470	This study
pLZ226	GAL4BD-DifE121-320	Chapter 3
pLZ227	GAL4BD-DifE121-470	This study
pLZ228	GAL4BD-DifE472-540	Chapter 2
pLZ229	GAL4AD-DifE472-540	Chapter 2
pLZ230	GAL4BD-DifE 722-855	Chapter 2
pLZ231	GAL4AD-DifE 722-855	This study
pLZ232	DifC in p426	Chapter 2
pLZ233	DifD in pWB200	This study

Table 4-1. Plasmids constructed.

Plasmids	Features	References
pLZ234	GAL4BD-EpsW	This study
pLZ235	GAL4AD- EpsW	This study
pLZ236	pWB200 without BamHI	This study
pLZ237	DifE and DifG in pWB200	This study
pLZ238	DifD in pYC274	This study
pLZ239	DifE and DifG in pYC274	This study
pLZ401	GALAD-DifE	This study
pLZ405	*difD* in-frame deletion in pBJ113	Chapter 3
pLZ406	*sglK* in-frame deletion in pBJ113	This study
pLZ407	*stK* in-frame deletion in pBJ113	This study
pLZ408	GALAD-CHP	This study
pLZ409	CHP in pWB350	This study
pLZ410	DifD in pXQ724	This study
pLZ411	DifE and DifG in pXQ724	This study
pLZ412	pLZ411 without XhoI	This study
pLZ413	DifD (D10K) in pXQ724	This study
pLZ414	DifD (D54N) in pXQ724	This study
pLZ415	DifD (D54A) in pXQ724	This study
pLZ416	DifE (E87D) in pXQ724	This study
pLZ417	EpsW (D13K) in pXQ724	This study
pLZ418	DifE (P550L) pXQ724	This study
pLZ419	DifE (P550S) pXQ724	This study
pLZ421	GAL4BD-EpsW (D13K)	This study
pLZ422	GAL4BD-DifE (P550L)	This study
pLZ423	GAL4BD-DifE (P550S)	This study
pLZ424	EpsW (D58A) in pXQ724	This study
pLZ425	DifD (D10K, D54N) in pXQ724	This study
pLZ426	EpsW (D13K, D58A) in pXQ724	This study

Table 4-1. Plasmids constructed.

Plasmids	Features	References
pLZ427	DifE$\Delta_{165\text{-}336}$ in pYC274	This study
pLZ428	*chp* in-frame deletion in pBJ113	This study
pLZ429	*stkB* in-frame deletion in pBJ113	This study
pLZ430	DifE (H45Q, P550S) in pYC274	This study
pLZ431	*kapB* in-frame deletion in pBJ113	Chapter 3
pLZ432	SglK in pYC274	This study
pLZ433	StK in pYC274	This study
pLZ434	Eps*W* (D13K) in pYC274	This study
pLZ445	Eps*W* (D13K) in pYC274 not in frame with ATG codon of *difA*	This study
pLZ436	DifE in pYC274	This study
pLZ437	Stk (G217D) in pYC274	This study
pLZ438	GAL4BD-DifA	This study
pLZ439	*stkA* and *stkB* in-frame deletion in pBJ113	This study
pLZ440	SglK and upstream in pYC274	This study
pLZ441	GAL4AD-SglK	This study
pLZ442	GAL4AD-StK	This study
pLZ443	GAL4BD-SglK	This study
pLZ444	GAL4BD-StK	This study
pLZ445	GAL4BD-DifE$_{1\text{-}470}$	This study
pLZ446	DifB in pQE30	This study
pLZ447	DifC in pQE30	This study
pLZ450	GAL4AD-KapB	Chapter 3
pLZ451	KapB in pWB425	This study
pLZ452	KapB in pQE31	This study
pLZ453	DifE$_{165\text{-}336}$ in-frame deletion in pBJ113	This study
pLZ454	ORF00270 in-frame deletion in pBJ113	This study
pLZ455	ORF01034 in-frame deletion in pBJ113	This study
pLZ456	:: putative glycogen phosphorylase in pZERO	This study

Table 4-1. Plasmids constructed.

Plasmids	Features	References
pLZ457	:: putative glycogen synthase in pZERO	This study
pLZ476	pQE32 without BamHI	This study
pLZ477	pQE32 without BamHI and XhoI	This study
pLZ478	DifE in pLZ476	This study
pLZ479	DifE (without Met) in pLZ478	This study
pLZ480	DifC (without Met) in pOE30	This study
pLZ481	DifD (without Met) in pOE30	This study
pLZ482	N-terminal DifE in pLZ477	This study
pLZ483	$DifE_{1-722}$ in pOE30	This study
pLZ484	$DifE\Delta_{165-336}$ in pOE30	This study
pLZ485	DifG in pOE30	This study
pLZ490	GAL4BD-$KapB_{1-147}$	This study
pLZ491	GAL4AD-$KapB_{147-271}$	Chapter 3
pLZ492	GAL4AD-$KapB_{1-147}$	Chapter 3
pLZ495	GAL4BD- $KapB_{147-271}$	This study
pLZ496	::downstream gene of *kapB* in pZERO	This study
pLZ497	*pfk* in-frame deletion in pBJ113	This study
pLZ498	*pkn4* in-frame deletion in pBJ113	This study
pLZ499	GAL4AD-$KapB_{1-244}$	Chapter 3

Table 4-2. *M. xanthus* strains constructed.

Strains	Features	References
YZ801	*difD* in-frame deletion	Chapter 3
YZ802	pLZ410 (wild type difD) in YZ801, Kan resistant	This study
YZ803	pLZ413 (DifD D10K) in YZ801, Kan resistant	This study
YZ804	pLZ414 (DifD D54N) in YZ801, Kan resistant	This study
YZ805	pLZ415 (DifD D54A) in YZ801, Kan resistant	This study
YZ806	pLZ416 (DifD E87D) in YZ801, Kan resistant	This study
YZ807	pLZ425 (DifD D10K, D54N) in YZ801, Kan resistant	This study
YZ808	pLZ427 (DifE$\Delta_{165\text{-}336}$ and DifG) in YZ603, Kan resistant.	This study
YZ809	pLZ433 (wild type *stkA*) in YZ812, Kan resistant	This study
YZ810	*chp* in-frame deletion	This study
YZ811	*sglK* in-frame deletion	This study
YZ812	*stkA* in-frame deletion	This study
YZ813	*stkB* in-frame deletion	This study
YZ814	:: *pilA* Δ *sglK* double mutant, Tet resistant	This study
YZ815	*pilT stkB* double in-frame deletion	This study
YZ816	*pilT stkA* double in-frame deletion	This study
YZ817	pLZ432 (wild type *sglK*) in YZ811, Kan resistant	This study
YZ818	*kapB* in-frame deletion	Chapter 3
YZ819	pLZ412 (wild type *difE, difG*) in YZ603, Kan resistant	This study
YZ820	pLZ436 (wild type *difE*) in YZ603, Kan resistant	This study
YZ821	pLZ448 (DifE$\Delta_{165\text{-}336}$) in YZ603, Kan resistant	This study
YZ822	*kapB difE* double in-frame deletion	Chapter 3y
YZ823	*pfkn* in-frame deletion, Kan resistant	Chapter 3
YZ824	*pfkn difE* in-frame deletion, Kan resistant	Chapter 3
YZ825	*pfk* in-frame deletion	This study
YZ826	*pfk kapB* double in-frame deletion	This study
YZ827	:: *pilA* Δ *kapB* double mutant, Tet resistant	This study
YZ828	:: *pilA* Δ *pfkn* triple mutant, Tet and Kan resistant	This study

Table 4-2. *M. xanthus* strains constructed.

Strains	Features	References
YZ829	*kapB difE difD* in-frame deletion	This study
YZ830	*pfkn difE difD* in-frame deletion, Kan resistant	This study
YZ831	:: *pilA* Δ *difD* Δ *kapB* mutant, Tet resistant	This study
YZ832	:: *pilA* Δ *difG* Δ *KapB* mutant, Tet resistant	This study
YZ833	:: *pilA* Δ *difD* Δ *pfkn* mutant, Tet and Kan resistant	This study
YZ834	:: *pilA* Δ *difG* Δ *pfkn* mutant, Tet and Kan resistant	This study
YZ835	pLZ425 (DifD D10K, D54N) in YZ801, Kan resistant	This study
YZ836	:: *aglU* Δ *KapB* mutant, Tet resistant.	This study
YZ837	DifE$\Delta_{165\text{-}336}$ in-frame deletion.	This study
YZ838	:: putative glycogen synthase, Kan resistant	This study
YZ839	:: putative glycogen phosphorylase, Kan resistant	This study
YZ840	*sglK kapB* double in-frame deletion	This study
YZ841	*difA kapB* double in-frame deletion	This study
YZ842	ORF00270 (first gene downstream of *kapB*) in-frame deletion.	This study
YZ843	ORF01034 (2nd gene downstream of *kapB*) in-frame deletion.	This study
YZ844	*pfkn kapB* in-frame deletion, Kan resistant	This study