IDENTIFICATION AND CHARACTERIZATION OF GENES REQUIRED FOR AGGREGATION AND FRUITING BODY FORMATION IN *MYXOCOCCUS XANTHUS*

by

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(Under the direction of Lawrence J. Shimkets)

ABSTRACT

Transposon insertion mutants defective in *M. xanthus* fruiting body formation were characterized. Mutant strains RC277 and RC280 could not form fruiting bodies and sporulate while LS423 produced irregular fruiting bodies. The insertion in RC277 disrupted a gene homologous to a subtilisin gene. RC280 contained an insertion in a gene homologous to a histidine kinase and response regulator gene. LS423 contained a disruption in a gene homologous to a sugar kinase gene. Extracellular C-signal, produced by *csg*A, mediates aggregation and sporulation. Signaling mutants, such as *csg*A, can be rescued by codevelopment with wild type cells. The mutants failed to rescue *csg*A when codeveloped but Western blots indicated production of CsgA proteins. Strains RC277 and RC80 inhibited wild type development when mixed with wild type.

INDEX WORDS: *Myxococcus xanthus*, Fruiting bodies, CsgA

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CHAPTER 1

INTRODUCTION

Myxobacteria and their unique life cycle were first described by Roland Thaxter in 1892 (Thaxter, 1892). Myxobacteria are long, rod-shaped, gram-negative bacteria commonly found in the soil and decaying plant material all over the world and in a variety of climates (for review see Reichenbach, 1993). The microorganisms are mesophilic, aerobic chemoorganotrophs that are predatory on other microbes. All myxobacteria form a phylogenetically coherent group within the delta subdivision of the purple bacteria (Shimkets and Woese, 1992). 16S ribosomal RNA sequencing indicates a close taxonomic relationship between myxobacteria, *Bdellovibrio*, and sulfate-respirers such as *Desulfovibrio* (Oyaizu and Woese, 1985). The species *Myxococcus xanthus* is the model organism for the study of myxobacteria development.

Growth. Myxobacteria exhibit social predation when feeding. Thousands of cells move in swarms toward prey in the soil (Rosenberg and Varon, 1984). As myxobacteria glide over soil particles, they release polysaccharides, antibiotics, bacteriocins, and lytic enzymes (Rosenberg et al., 1973). The lytic enzymes degrade microbial cell walls, lipids, nucleic acids, polysaccharides and proteins (Rosenberg et al., 1973; Sudo and Dworkin, 1972). *M. xanthus* uses amino acids released from the degradation of microbes and protein as a carbon,

nitrogen, and energy source for growth. Sugars are not catabolized (Dworkin, 1962).

Motility. Gliding motility is described as smooth translocation of cells over a surface by an active process that requires the expenditure of energy (McBride, 2001). Gliding bacteria move actively over surfaces without the aid of flagella, and cell movement generally follows the long axis of the cell (McBride, 2001). Several different types of gliding motors allow movement over surfaces (McBride, 2001).

Through analysis of *M. xanthus* motility mutants, Hodgkin and Kaiser described genetic evidence for two independent gliding systems known as A and S motility (Hodgkin and Kaiser, 1979). Adventurous or A motility, is responsible for cells gliding as individuals over relatively dry surfaces. Adventurous gliding does not require pili and may be due to polysaccharide secretion (Wolgemuth et al., 2002). Social or S motility is responsible for cells gliding in swarms (Hodgkin and Kaiser, 1979). S motility is correlated with the presence of type IV pili on one cell pole (Kaiser, 1979). Cells must be in close proximity for the S motility system to function properly (Kaiser and Crosby, 1983). S motility is similar to twitching motility and relies on type IV pilus extension and retraction for cell movement over moist surfaces (Sun et al., 2000).

Other genes required for gliding motility are *mglA* and the *frz* genes. MglA is a 22-kDa cytoplasmic G-protein, which is the only gene required for both A and S motility (Hartzell and Kaiser, 1991). Mutations in *mglA* eliminate adventurous and social motility simultaneously, resulting in a non-motile phenotype.

The *frz* system controls the cell reversal frequency of gliding cells as well as directional movement (Shi et al., 2000). Frz mutants aggregate into frizzy filaments instead of mounds (Zusman, 1982). Six *frz* genes are clustered on a 7.5 kbp segment of the chromosome and are designated *frzA- frzG* (Blackhart and Zusman, 1985). Frz genes are expressed during vegetative growth but transcription increases about 12 to 18 hours after starvation, the time of early mound formation (Weinberg and Zusman, 1989). The genes of the Frz sensory transduction pathway are homologous to chemotaxis genes (Che) of the enteric bacteria (McBride et al., 1989). Mutations in *frz* genes, except for *frzD,* which hyper reverses, result in cells that infrequently reverse direction (Weinberg and Zusman, 1989).

Development

M. xanthus cells on a solid surface and at high cell density undergo fruiting body development in response to amino acid limitation (figure 1). ppGpp and pppGpp [(p)ppGpp] mediate the stringent response, which involves changes in metabolism including inhibition of rRNA synthesis. RelA catalyzes the pyrophosphate transfer from ATP to GTP, forming pppGpp, when translation stalls due to lack of a charged tRNA. (p)ppGpp accumulates during *M. xanthus* development in response to amino acid starvation (Manoil and Kaiser, 1980). Ectopic expression of the *E. coli relA* gene in *M. xanthus* cells resulted in overproduction of (p)ppGpp and early developmental gene expression despite the presence of nutrients (Singer and Kaiser, 1995). Inactivation of the *M. xanthus relA* homolog prevents accumulation of (p)ppGpp and development

(Harris et al., 1998). Signaling occurs immediately after the initiation of starvation.

Directed movement. Directed movement towards an aggregation center is the first step in fruiting body formation. Directed movement establishes contacts between adjacent cells, which are required for efficient intercellular signal transmission (Kim and Kaiser, 1990). Directed movement has been observed towards phospholipid extracts from vegetative and developing cells. *M. xanthus* is chemotactic to chemically synthesized dilauroyl and dioleoyl phosphatidylethanolamine (PE) (Kearns and Shimkets, 1998). However, the only attractant active at physiological concentrations is PE containing the fatty acid 16:1ω5c (Kearns et al., 2001). In the presence of chemoattractant, cells suppress directional reversals to achieve longer runs toward the lipid, and exhibit adaptation (Kearns and Shimkets, 1998).

Fibrils, extracellular appendages that extend away from the cell surface, are essential for stimulation by phospholipid attractants (Kearns et al., 2000). Fibrils may act as extracellular signal receptors for detecting insoluble chemical signals (Kearns et al., 2000). Fibrils are required for excitation by dilauroyl PE and PE containing 16:1ω5c, but not by dioleoyl PE, demonstrating that extracellular fibrils are not required for all chemotactic responses (Kearns et al., 2000).

The C-signal, a cell surface associated intercellular signal necessary for development after 6 hours of starvation, also affects cell behavior and organized cell movement. The C-signal has been shown to stimulate methylation of FrzCD,

which results in decreased cell reversals (Sogaard-Andersen and Kaiser, 1996). C-signal has also been shown to stimulate individual cells to move with higher transient gliding speeds in longer gliding intervals during development (Jelsbak and Sogaard-Andersen, 1999).

Fruiting body formation. During development, cells proceed through a series of partially overlapping but morphologically distinct stages known as rippling, aggregation and sporulation (Shimkets, 1990). Early in aggregation, within 6-12 hours after the initiation of starvation, parallel ridge-like accumulations of cells move coordinately and rhythmically over a substrate like ripples on a water surface (Shimkets and Kaiser, 1982). Rippling is found during early stages of development but is not necessary for fruiting body formation. Cells leave ripples and stream into aggregates. As more and more cells move toward aggregation centers, mounds form. Mounds become multicellular, hemispherical structures called fruiting bodies consisting of 10^5 cells (Reichenbach, 1993). As starvation continues, cells within the fruiting body develop into myxospores.

The developing fruiting body consists of two domains, each with different cell densities, cell arrangements, and cell movements that play different roles in spore differentiation (Sager and Kaiser, 1993). The hemispherical outer domain is composed of densely packed and ordered cells. Single cells in the outer domain move in a bi-directional stream, orbiting the fruiting body throughout development. The inner domain consists of nonmoving cells at a lower density.

In the inner domain, cells are less ordered and at 3-fold lower density (Sager and Kaiser, 1993).

Sporulation and Germination. Rod-shaped vegetative cells differentiate into spherical, environmentally resistant, metabolically dormant myxospores (Sudo and Dworkin, 1969). Spores are heat and sonication resistant and can survive hostile environmental conditions for many years. The spherical myxospores are surrounded by a thick spore coat whose major component is protein S (Dworkin, 1993). Protein S, encoded by the *tps* gene, begins being synthesized in developing cells 6 hours after the initiation of starvation and is the best characterized developmental protein in *M. xanthus*.

Spores germinate when nutrients become available by shedding their spore coat and elongating into rods. Specific nutrients or chemicals that trigger germination are unknown. Myxospore germination occurs in at least two steps, the loss of refractility and the increase of metabolic activities (Otani et al., 1995). Otani examined the germination of myxospores from fruiting bodies by monitoring RNA synthesis (Otani et al., 1995). RNA synthesis began two hours after the spores were placed in a nutrient rich environment indicating the initiation of germination. RNA synthesis was inhibited in the presence of a chymotrypsin-like protease inhibitor suggesting that a chymotrypsin-like serine protease is involved in germination. The serine protease may be similar to a Ca^{2+} dependent, spore-specific protease found in *B. megaterium*, which degrades spore-specific proteins (Otani et al., 1995).

Extracellular complementation

Intercellular signaling refers to the ability of one cell to regulate the physiology of other cells through the use of a soluble or contact-mediated signal (Shimkets, 1999). *M. xanthus* uses intercellular signaling to communicate and to direct the stages fruiting body development. Hagen identified four classes of nonautonomous developmental mutants. These mutants were identified by allowing a mutagenized culture to form fruiting bodies. The heat and sonication resistant myxospores from these fruiting bodies were placed on nutrient rich media and allowed to germinate. The mutant spores that germinated, but did not fruit when starved again, were then mixed in pairwise combinations. The mutants fell into four complementation groups: A, B, C, and D. Mixtures of members of any two groups induced the production of myxospores. The synergism or complementation groups are arrested at different stages of development. From this data, Hagen concluded that intercellular signals coordinate sporulation with fruiting body formation and that the different synergism groups are defective in producing the signals (Hagen et al., 1978). Extracellular signaling genes: *asg, bsg, csg, dsg*, and *esg* (discovered later), encode the A, B, C, D, and E complementation groups. *M. xanthus* cells with mutations in these signaling genes have defects in transcriptional regulation of developmental genes (Kuspa et al., 1986).

Signaling. (p)ppGpp initiates the production of the A-signal, one of the first stages of development (Harris et al., 1998). There are five *asg* loci: *asgA*, *asgB*, *asgC*, *asgD* and *asgE*. The *asgA* gene encodes a putative histidine

protein kinase that may function within a phosphorelay to regulate expression of genes required for A-signal production (Plamann et al., 1995). The *asgB* gene encodes a putative transcription factor (Plamann et al., 1994). The *asgC* gene encodes the major vegetative sigma factor (Davis et al., 1995). The *asgD* gene is part of a two-component regulatory system involved in sensing nutritionally limiting conditions (Cho and Zusman, 1999). The *asgE* gene encodes a protein that has similarity to amidohydrolase proteins in *Pseudomonas* (Garza et al., 2000). The A-signal consists of a mixture of amino acids, generated by extracellular proteolysis which acts as a cell density signal (Kuspa et al., 1992). A combination of the amino acids tyrosine, proline, tryptophan, phenylalanine, tyrosine, leucine and isoleucine rescued the development of *asgB* and *asgC* mutants (Kuspa et al., 1992).

B-group mutants are blocked early in development (Gill and Bornemann, 1988). The *bsgA* gene encodes a 90 kDa cytoplasmic protein that is homologous to *E. coli* and *Bacillus brevis lon* genes (Gill et al., 1993). The *lon* gene in *E. coli* encodes protease La, an ATP-dependent protease that couples the degradation of protein substrates to the hydrolysis of ATP (Gill et al., 1993). The actual B-signal is unknown.

The D-group contains a mutation in *dsgA*, which encodes translation initiation factor IF3, which is essential for choosing the initiation codon (Cheng et al., 1994). *dsgA* is required for cell viability because Tn*5* insertions in the gene are lethal, but certain *dsgA* point mutants are viable and defective in development (Cheng and Kaiser, 1989). The *dsgA* gene complemented *E. coli*

IF3 mutants, and the DsgA protein product binds anti-IF3 antibodies (Kalman et al., 1994). *dsg* may be involved in regulating translation during development (Cheng et al., 1994). The D-signal is unknown.

The E-group controls *tps* gene expression, which encodes the spore coat S protein (Downard et al., 1993). The *esg* locus contains two genes that encode E1α and E1β subunits of a branched-chain keto acid dehydrogenase (Toal et al., 1995). Branched-chain keto acid dehydrogenase converts branched-chain keto acids derived from leucine, isoleucine, and valine to coenzyme A derivatives. Consequently, *esg* mutants have reduced levels of branched-chain fatty acids in their phospholipids and much higher levels of unsaturated fatty acids (Toal et al., 1995). Addition of the 2-keto acid isovalerate, a precursor of the major fatty acid C15:0, restores development of *esg* cells (Toal et al., 1995). The E-signal is unknown.

A sixth class of non-autonomous mutants has been identified. The *dsp* class is unable to synthesize extracellular fibrils, which are required for S motility, suggesting that the developmental defects in this class of non-autonomous mutants are caused by a motility defect (Arnold and Shimkets, 1988). *dsp* mutants have a complex phenotype with abnormal cell cohesion, social motility, and development (Shimkets, 1986). All three defects are the result of a single mutation in the *dsp* locus, also called *dif*, which encodes a set of chemotaxis proteins (Yang et al., 1998).

dif genes are required for construction of extracellular matrix fibrils (Yang et al., 2000). The extracellular matrix of fibrils, located over the entire bacterial

cell body, is needed for cell adhesion (Arnold and Shimkets, 1988), and to mediate intercellular coordination (Sun et al., 1999). Purified, isolated fibrils rescue cohesion and development in *dsp* mutants (Chang and Dworkin, 1994), and restore excitation to dilauroyl PE (Kearns et al., 2000).

CsgA and the C-signal

The C-signaling group is the most well studied complementation group. C-signal mutants fail to ripple, form fruiting bodies, sporulate, and are blocked in development after 6 hours (Kroos and Kaiser, 1987). The *csgA* gene was shown to encode two proteins of 25 kDa and about 17 kDa (Kruse et al., 2001). However, it is not yet understood if the 25 kDa full-length form of CsgA encodes an enzyme that produces the C-signal, or if the 17 kDa species of CsgA is the actual C-signal. Conflicting data has been generated and models have been described to support each hypothesis.

csgA encodes a 25 kDa protein that shares amino acid identity with members of the short-chain alcohol dehydrogenase family (Baker, 1994; Lee and Shimkets, 1994). These enzymes use NAD(H) or NADP(H) to catalyze the interconversion of secondary alcohols and ketones (Persson et al., 1991). A Mal-E-CsgA fusion protein containing the entire CsgA coding region was produced in *E. coli* and purified. The fusion protein induced fruiting body development in *csgA*-deficient cells in submerged culture (Lee et al., 1995). The predicted secondary structure of 25 kDa CsgA suggests that the amino terminal portion of the protein contains the $NAD(P)^+$ binding pocket. Several lines of evidence suggest that $NAD(P)^+$ binding is essential for 25 kDa CsgA activity (Lee

et al., 1995). Strains with *csgA* alleles encoding amino acid substitutions in the binding pocket failed to develop. Furthermore, exogenous MalE-CsgA with amino acid substitutions in the binding pocket failed to rescue development of *csgA*. These data suggest that $NAD(P)^+$ binding is necessary for C-signaling (Lee et al., 1995).

In a separate experiment, sporulating pseudorevertants were isolated from *csgA* mutants (Lee and Shimkets, 1994). Two strains were identified which had transposon insertions in the *socABC* operon. SocA is member of the short chain alcohol dehydrogenase family like CsgA (Lee and Shimkets, 1996). When *soc csgA* mutants were mixed with *csgA* cells, development of *csgA* cells was fully restored suggesting that C-signaling is restored (Lee and Shimkets, 1996). SocB is a putative membrane anchoring protein. SocC is a putative DNA-binding protein and a negative regulator of *socABC* operon expression. A model has emerged in which the inactivation of SocC results in the overproduction of SocA, which restores C-signaling (Lee and Shimkets, 1996). SocA is proposed to have overlapping substrate specificity with CsgA. However, the substrates of SocA or CsgA are unknown. Together these data support a model in which CsgA is an enzyme which manufactures the C-signal (Lee and Shimkets, 1996).

Another model suggests that the 17 kDa CsgA species is the active C-signal. A bioassay was constructed in which the 17 kDa form was purified based solely on its ability to rescue development of *csgA* mutants (Kim and Kaiser, 1990). When added to a submerged biofilm of *csgA* cells at a concentration of 1 to 2 nM, the 17 kDa form restored normal development to *csgA* mutant cells.

Purified 17 kDa C-factor also stimulated expression of β-galactosidase from developmentally controlled *lac*Z fusions (Kim and Kaiser, 1990). According to partial amino acid sequence, the purified 17 kDa form of C-factor is encoded by the *csgA* gene (Kim and Kaiser, 1990). It has been proposed that the full length 25 kDa form undergoes proteolytic cleavage to produce the 17 kDa species, which is thought to be the active C-signal (Kruse et al., 2001). It has not been shown that the 25 kDa species does not have activity without the 17 kDa species.

CsgA and motility. Shimkets and Rafiee demonstrated that anti-CsgA antibodies associated with the extracellular matrix and cell surface (Shimkets and Rafiee, 1990). While *csgA* mutants can be rescued by codevelopment with wild type cells, separation of wild type and *csg*A mutant cells by 0.45 μm pore size membrane filter prevents rescue of development suggesting that C-signaling requires cell-cell contact for transmission (Kim and Kaiser, 1990).

Motility is also required for C-signal transmission. Even though non-motile *mglA* cells produce wild type levels of CsgA, *mglA* cells were unable to rescue *csgA* cells with the extracellular complementation assay suggesting that donor and responder cell motility is required for C-factor transmission (Kim and Kaiser, 1990). Non-motile cells were artificially aligned in parallel groups by applying cells to a microscopic grooved surface and allowing them to settle in the grooves (Kim and Kaiser, 1990). Cells that settled into the grooves were oriented with their long axes parallel to the axis of the groove. Aligned non-motile cells sporulated and expressed a C-factor-dependent *lacZ* fusion gene (Kim and

Kaiser, 1990). These data supported the hypothesis that motility establishes a spatial pattern of cells crucial for subsequent C-factor transmission, and that C-signaling occurs between directly adjacent cells (Kim and Kaiser, 1990). The method used however, did not distinguish whether end-to-end, side-to-side, or both types of cell contacts are required for C-signaling.

CsgA and timing of development. C-factor has distinct aggregation and sporulation thresholds during development (Kim and Kaiser, 1991). C-factor added to *csgA* mutant cells at subnanomolar concentrations induces the expression of early C-dependent genes and aggregation (Kim and Kaiser, 1991). Aggregation, the expression of both late and early C-dependent genes, and sporulation were observed at a higher concentration of C-factor. By measuring *csgA* transcription and net C-factor production, Kim and Kaiser concluded that C-factor stimulates its own synthesis, increasing the C-factor concentration through a positive feedback mechanism (Kim and Kaiser, 1991).

Li et al. demonstrated that rippling, aggregation, and sporulation have different thresholds for induction by CsgA. A low concentration of C-signal is required for rippling, an intermediate concentration is required for aggregation, and a high concentration is required for sporulation and late C-signal-dependent gene expression (Li et al., 1992). Nested deletions were constructed upstream of the *csgA* gene, reducing *csgA* expression, which in turn terminated development at successively earlier stages (Li et al., 1992). *csgA* expression was artificially induced by placing it under the control of a different promoter. The ectopic induction of *csgA* transcription resulted in marked stimulation of

development. By manipulating the transcription levels of *csgA*, Li et al. proposed that gradual ordered increases in *csgA* expression over the course of development initiates the morphological stages of development (Li et al., 1992).

Kruse et al. assayed CsgA by Western blotting in overproducing cells (Kruse et al., 2001). CsgA was overproduced in wild type cells by introducing multiple copies of the *csgA* gene on the chromosome. Overproduction of CsgA resulted in bypass of rippling, early and abnormal aggregation, formation of many small fruiting bodies, and premature sporulation (Kruse et al., 2001). Overproduction of CsgA caused formation of spores inside as well as outside of the fruiting bodies, indicating that aggregation and sporulation were uncoupled (Kruse et al., 2001). When a single copy the *csgA* gene was introduced into *csgA* cells in a region of the chromosome that causes gene silencing, the strain produced reduced levels of the CsgA resulting in delayed aggregation and reduced sporulation (Kruse et al., 2001). An ordered increase in the level of C-signaling during development is essential for the spatial coordination of aggregation and sporulation (Kruse et al., 2001).

Developmental gene expression. To understand how development is regulated, a genetic marker was constructed that could be linked to genes expressed in cells during multicellular development. Kuner and Kaiser demonstrated that transposon Tn*5*, which carries a gene for kanamycin resistance, could be introduced into *M. xanthus* from *E. coli* by specialized transducing phage P1::Tn*5* (Kuner and Kaiser, 1981). Kroos and Kaiser constructed a transposable promoter-probe which makes transcriptional fusions

to the *lac*Z gene of *E. coli*. A promoterless *trp-lac* fusion fragment was inserted near the left end of Tn*5* (Jorgensen et al., 1979). The *lac*Z gene product, β-galactosidase, was synthesized in *M. xanthus* and was used as a reporter for gene expression (Kroos and Kaiser, 1984). The new transposon, Tn*5 lac*, was transduced into wild type cells to identify developmentally regulated genes. Cells containing random insertions of Tn*5 lac* were screened for expression of β-galactosidase during development (Kroos et al., 1986). The insertion mutants that did not have developmental defects were characterized with respect to the timing and amount of β-galactosidase activity. The developmentally regulated genes were later used to examine extracellular complementation of A, B, and C group mutants. Of 21 developmentally regulated genes examined, 18 failed to express β-galactosidase in A-signaling mutants (Kuspa et al., 1986). These 18 genes were A-signal dependent genes. It was determined that A-signaling mutants have a developmental block at 1-2 hours (Kuspa et al., 1986). In contrast, C-dependent genes are activated later than A-signaling genes, at about 6 hours of development, and B-dependent genes are active earlier than A-signal dependent genes (Kroos and Kaiser, 1987).

C-signal transduction pathway

The C-signal uses a branched signal transduction pathway to induce morphogenesis and gene expression (Sogaard-Andersen et al., 1996). A model to describe the pathway has been constructed from genetic analyses of mutants deficient in C-signal dependent activities (figure 2).

FruA is a putative response regulator (Ogawa et al., 1996), and serves as a control point for the temporal coordination of intercellular signals during development (Ellehauge et al., 1998). FruA mutants aggregate to form translucent mounds, but are defective in fruiting body formation and sporulation (Ogawa et al., 1996). FruA expression is initiated after six hours of development (Ogawa et al., 1996) and is A- and E-signal dependent (Ellehauge et al., 1998). It is hypothesized that the C-signal controls FruA activity post-translationally by activating the cognate histidine protein kinase. After becoming activated, FruA interacts with downstream targets in the response pathway on two branches (Sogaard-Andersen and Kaiser, 1996). One of the branches leads to rippling and aggregation, and the other branch leads to sporulation.

Activated FruA may interact with Frz, the branch of the C-signaling pathway that is responsible for control of directed cell movement, rippling and aggregation. FrzCD is a methyl-accepting chemotaxis protein found in the cytoplasm (McBride et al., 1989). *csgA* cells have reduce methylation of FrzCD (McBride and Zusman, 1993). When exogenous CsgA or a MalE-CsgA fusion protein was added to *csgA* cells after six hours of starvation, FrzCD methylation increased, demonstrating that CsgA stimulates FrzCD methylation (Sogaard-Andersen and Kaiser, 1996). CsgA did not induce an increase in FrzCD methylation in *fruA* mutants or *fruA csgA* mutants (Sogaard-Andersen and Kaiser, 1996). This data suggests that *fruA* acts upstream of FrzCD proteins and is involved in C-signal dependent stimulation of FrzCD methylation (Sogaard-Andersen and Kaiser, 1996). After analyzing *fruA* levels in *csgA* and wild type

cells, it was determined that FruA synthesis is independent of C-signaling. A model has been proposed in which C-signaling activates FruA posttranslationally by activating a histidine kinase which phosphorylates an aspartate residue in FruA (Ellehauge et al., 1998). DevT is also required for C-signal-dependent methylation of FrzCD (Boysen et al., 2002).

In the second branch of the C-signaling pathway, C-signal may activate FruA, which promotes transcription of *dev* genes. Expression of the *dev* operon is initiated when C-signaling rises to its highest threshold, and activates genes involved in sporulation (Julien and Kaiser, 2000). Cells of nascent fruiting bodies express the *dev* operon while vegetative rods do not. *dev* mutants are defective in sporulation (Julien and Kaiser, 2000). *devRS* encodes a regulatory element and components specific for the sporulation branch, and is required at about six hours after the initiation of starvation (Thony-Meyer and Kaiser, 1993) (Ellehauge et al., 1998). DevT stimulates the transcription of *fruA* leading to a possible positive feedback loop for FruA and Dev synthesis (Boysen et al., 2002).

The third branch of the C-signal transduction pathway is located upstream of FruA and leads to *csgA* expression. The *act* operon may control the level and timing of C-signal production (Gronewold and Kaiser, 2001). *actA* and *actB* regulate the level of *csgA* expression. CsgA levels in these mutants never rise above one-quarter of the maximum wild type level. Consequently, *actA* and *actB* mutants aggregate but do not sporulate and have prolonged rippling. The *actA* gene encodes a response regulator and *actB* encodes a sigma-54 activator protein. The *actC* and *actD* genes regulate the timing of *csgA* expression. ActC

delays whereas ActD advances *csgA* expression (Gronewold and Kaiser, 2001). *actC* encodes a protein similar to members of a family of N-acetyltransferases while *actD* has no known relatives in the GenBank database (Gronewold and Kaiser, 2001).

Purpose

Myxococcus xanthus provides a unique system for studying the molecular basis of development and social interactions (Dworkin, 1991). The isolation and characterization of mutants with altered phenotypes are the basis for a genetic analysis of myxobacteria (Gill and Shimkets, 1993). Understanding these mutants can help to reveal the regulatory networks that coordinate social behavior in *M. xanthus*. The steps involved in the regulation of aggregation and fruiting body formation are still unclear. Discerning the process at the molecular level will be useful in trying to understand the complex developmental process of this unique prokaryotic organism. Mutations that alter aggregation, fruiting body formation and sporulation have been identified by screening for cells with a developmental defect. Transposon mutagenesis is a useful technique for generating loss-of-function mutations.

In this work, transposon insertion mutants defective in aggregation, fruiting body development, and sporulation were studied. By characterizing the mutants and cloning the genes disrupted by the transposon insertions, I identified three developmental genes. The results of this study suggest that genes encoding a histidine kinase and serine protease are essential for cellular aggregation, fruiting

body formation, and sporulation, and a gene encoding a sugar kinase is helpful for aggregation.

CHAPTER 2

MATERIALS AND METHODS

Bacterial strains and culture conditions. The *Myxococcus xanthus* strains used in this study are listed in table 1. The developmental mutants RC272, RC273, RC274, RC275, RC276, RC277, RC280, and RC281 were constructed by insertion of Tn*5*-132 into wild type strain DK1622. Strains were produced and supplied by Richard Cardeman. Strain LS423 was constructed by insertion of Tn*5 lac* into DK1622. Restriction analysis and Southern hybridization were previously used to map the transposon insertion in the circular genome (Chen et al., 1991).

Cells were grown in CYE broth [1% Casitone (Difco), 0.5% yeast extract (Difco), 0.1% $MgSO_4 \cdot 7H_2O$, 10 mM 3-N-morpholinopropanesulfonic acid, pH 7.6] with vigorous shaking at 32°C. Kanamycin was used at a concentration of 40 μg/ml and tetracycline was used at a concentration of 12.5 μg/ml.

Fruiting body formation and sporulation assay. Exponential-phase cells grown in CYE broth were pelleted and resuspended in CYE broth to a density of 5×10^9 cells/ml. Aliquots of 200 μl were spread onto TPM agar (10 mM Tris HCl, pH 8.0, 8 mM $MgSO_4$, 1 mM K_2HPO_4 , 1.5% Difco agar) and incubated at 32°C for 96 hours. Plates were observed for aggregation and fruiting body formation. Fruiting bodies were observed using a Wild Heerbrugg dissecting microscope and photographed using a Spot Insight digital camera

(Spot Diagnostic Instruments, Inc.). Fruiting bodies were removed from TPM agar with a razor blade and resuspended in 1 ml of TPM buffer. The cell suspension was incubated at 50°C for 2 hours to assess heat resistance, and spores were dispersed by sonication (Heat Systems-Ultrasonics, Inc.) for 30 seconds at 4.5 W power output. Spores were quantified using a Leitz Wetzlar Laborlux phase contrast microscope at 400X in a Petroff-Hausser counting chamber. Viable spores were quantified by diluting spore suspensions in TPM buffer, mixing the spore suspensions in CYE soft agar (CYE broth plus 0.7% Difco agar), and plating these on to CYE agar to allow spore germination. Plates were incubated at 32°C for 4-5 days before colonies were counted.

Extracellular complementation. Each strain was cultured in CYE broth to exponential phase, harvested, and resuspended in CYE broth to 5×10^9 cells/ml. Then 100 μl of a mutant cell type was mixed with 100 μl of *csgA* or wild type cells and spread on TPM agar. Cells were incubated at 32°C for 96 hours. Plates were observed for aggregation and fruiting body formation. Spores were quantified in a Petroff-Hausser counting chamber. Spore suspensions were diluted in TPM broth and plated on CYE plates with a CYE soft agar overlay. Spores were allowed to germinate and counted after 4-5 days of incubation at 32°C. One hundred colonies were picked onto CYE plates containing kanamycin or tetracycline. The colonies were counted to determine the number of kanamycin or tetracycline resistant spores.

DNA isolation and manipulations. *M. xanthus* genomic DNA was isolated using Easy DNA kit (Invitrogen). DNA was quantified using a Hoefer

DyNA Quant 200 fluorometer. DNA was visualized using agarose gel electrophoresis. Chromosomal DNA was digested with *Nar*I (New England Biolabs) overnight at 37°C. Remaining *Nar*I was inactivated at 65°C for 20 minutes. DNA was ligated using T4 DNA ligase (Stratagene) at 16°C overnight and the remaining ligase was inactivated at 65°C for 20 minutes.

Inverse PCR. The DNA sequence flanking the transposon insertion was isolated using the inverse polymerase chain reaction (PCR) (Ochman et al., 1988). PCR primers (Gibco BRL Life Technologies) specific to IS50 within Tn*5* were used to amplify the region flanking the Tn*5* insertion in two rounds of nested PCR. The first round used primer 1 (5'-CCGCACGATGAAGAGCAGAAG-3') and primer 2 (5'-CAGAGGGTAGTGAAGCCATGC-3') which are complementary to Tn*5*. The second round of PCR used primer 3 (5'-GGTTCCGTTCAGGACGCTACT-3') and primer 4 (5'-ACGTTTCTGCCGAGGCGATCA-3'), which are complementary to Tn*5* sequence amplified in the first round. Ready-To-Go PCR beads (Amersham Pharmacia Biotech) were used according to manufacturers instructions. The reactions contained 1.5 units of *Taq* DNA polymerase, 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 μM $MgCl_2$, 200 μM of each dNTP, bovine serum albumin, 10 pmol of each primer, and 50 pg of template DNA in a total volume of 25 μL. DNA was amplified using the PTC-150 Mini Cycler (MJ Research, Inc.). The first round reaction conditions were: 95°C for 2 minutes, and thirty cycles of 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 1.5 minutes. The second round conditions were performed as described for the first round except that 1 μL of the

first round PCR product was used as the template DNA. The PCR product was cleaned between rounds with the QIAquick PCR purification kit (Qiagen, Inc.). The inverse PCR product was purified from agarose using the Ultraclean DNA 15 Purification kit (Mobio Laboratories, Inc.). DNA was sequenced using an ABI 373 automated sequencer at the Molecular Genetics Instrumentation Facility at the University of Georgia.

Sequence analysis. The DNA sequence was examined for the location of the IS50 junction. The *M. xanthus* portion of the sequence was compared with the Cereon LLT Microbial Sequence Database to identify the entire gene. FramePlot (National Institutes of Health) was used to identify and translate putative protein coding regions based on third position codon bias (Bibb et al., 1984; Ishikawa and Hotta, 1999) http://www.nih.go.jp/~jun/cgi-bin/frameplot.pl. BlastX was used to identify homologues at the nucleotide level (Altschul et al., 1990). BlastP was used to identify amino acid homologues in the NCBI protein database (Altschul et al., 1990). Predicted amino acid sequence alignment was performed by Clustal W (Thompson et al., 1994). Bestfit was used to obtain optimal alignments of segments between sequences, and Boxshade was used to shade sequences aligned by Pileup, all with the University of Wisconsin Genetics Computer Group (GCG) software package.

Western blot assays. Western blot assays were conducted to quantify CsgA production in the mutants. Cells were allowed to develop on TPM agar plates for 16 hours. Cells were lysed and boiled for 10 minutes in sodium dodecyl sulfate (SDS) loading buffer. Equal amounts of protein were loaded on a

15% SDS polyacrylamide gel (Sambrook et al., 1989). The separated proteins were transferred to a nitrocellulose membrane (0.45 μm) using a Trans Blot Cell electrophoretic transfer apparatus. The blots were probed with rabbit anti-CsgA antibody (kindly provided by L. Sogaard-Andersen) at a 1:8000 dilution. Horseradish peroxidase-conjugated goat-anti-rabbit immunoglobulin G (heavy and light chain) (Cell Signaling Technology) was used as a secondary antibody at a 1:5000 dilution. Blots were developed with ECL Western blotting detection reagents (Amersham Pharmacia Biotech) and bands were visualized by autoradiography.

CHAPTER 3

RESULTS

Developmental phenotypes of mutants. Richard Cardeman used P1 specialized transduction to insert Tn*5* into wild type *M. xanthus*, DK1622. Eight mutants with altered phenotypes were isolated: RC272, RC273, RC274, RC275, RC276, RC277, RC280, and RC281. Strain LS423 was constructed by insertion of Tn*5 lac* into DK1622 (Shimkets, unpublished data). Strains RC277, RC280 and LS423 were selected for further analysis.

Mutants were starved for 96 hours to observe their developmental phenotypes (figure 3). DK1622 (wild type) cells rippled after 16 hours of development and formed symmetric, darkened fruiting bodies by 96 hours. RC277 cells had a severe aggregation defect, forming loose, highly irregular translucent mounds. RC280 cells had a severe aggregation defect, forming irregularly shaped translucent mounds. Strain LS423 had a slight aggregation defect and formed darkened fruiting bodies as well as irregularly shaped mounds containing spores. LS423 also appeared to form spores outside of fruiting bodies. These data suggest that the genes disrupted in RC277 and RC280 are essential for development and the gene disrupted in LS423 is important for the timing of sporulation. Table 2 summarizes the results for the other strains.

Spore assay. Sporulation assays were conducted to observe the effect of the Tn*5* insertion (figure 3). Spores were quantified by direct counts after heating

and sonication to kill vegetative cells. To determine the number of viable spores, spore suspensions were plated on CYE agar to allow spores to germinate and form colonies. RC277 formed only 1% of the number of spores formed by wild type but few of these were viable. RC280 sporulated at 45% of the level of wild type indicating that cells were capable of developing sonication resistance, but the spores were not viable. LS423 formed 44% of the number of spores formed by wild type and 43% of the number of viable spores formed by wild type.

Molecular characterization of mutants. A portion of the DNA adjacent to the Tn*5* insertion was amplified using the inverse polymerase chain reaction (PCR) (Ochman et al., 1988). The PCR product was extracted from agarose and sequenced. Each sequence was examined to locate a junction between Tn*5* and the disrupted. I attempted to obtain a PCR product from each of the nine mutants, but only four produced PCR products containing a unique band that was later sequenced: RC273, RC277, RC280, and LS423.

Sequence analysis. The transposon insertion in RC277 disrupted a serine protease-like gene. The sequence was submitted to Cereon Genomics, LLC, for comparison to the *M. xanthus* genome and was homologous to contig MYX10C836. The *M. xanthus* genome sequence is about 95% complete and consists of a series of unlinked contigs. The Tn*5* insertion site in the serine protease occurs 227 amino acids after the putative translation start site (figure 4). One hundred forty-three base pairs downstream of the protease stop codon is a putative protein coding region that encodes a sodium or non-voltage-gated

channel, which is transcribed in the opposite direction. These results suggest that the insertion is not polar on downstream genes.

The amino acid sequence was subjected to a BlastP search and is similar to the peptidase S8 subtilase family. Members of this family appear to have independently evolved an Asp/Ser/His catalytic triad like trypsin S proteases. Structures in this family consist of an alpha/beta fold containing a 7-strand parallel beta sheet. Subtilisins are extracellular serine proteases that are secreted by a wide variety of *Bacillus* species. Serine proteases carry out diverse physiological and cellular functions ranging from digestive and degradative processes to blood clotting, cellular and humoral immunity, fibrinolysis, fertilization, embryonic development, protein processing and tissue remodeling (Krem and DiCera, 2001).

The closest well characterized homolog encodes subtilisin NAT, a profibrinolytic serine protease, encoded by the *aprN* gene (Nakamura et al., 1992) from *Bacillus subtilis* var. Natto (Fujita et al., 1993). Bestfit analysis revealed that the protein has 50.6% similarity and 41.2% identity to subtilisin NAT precursor through about 200 amino acids. The *M. xanthus* putative serine protease amino acid sequence was compared with subtilisin NAT (Fujita et al., 1993) (figure 5). The gene was designated *aprM* (alkaline serine protease of *Myxococcus xanthus*) due to its homology to subtilisin NAT. AprM contains the catalytic triad of Asp 201, His 243 and Ser 423, but does not contain a calcium ion binding site conserved in subtilisins.

The transposon insertion in RC280 was located in a protein coding region with homology to a histidine kinase containing two histidine kinase motifs. The entire gene sequence was found on contig MYX10C743 in the *Myxococcus* genome (figure 6). The putative protein coding region that ends 77 base pairs upstream of the putative histidine kinase encodes a putative methyltransferase with 37.6% similarity and 29.4% identity to the *ubiE* gene of *E.* coli, which is required for synthesis of menaquinone (Lee et al., 1997). Another putative protein coding region begins 76 base pairs downstream of the stop codon making it unlikely that the insertion is polar on downstream genes. The downstream gene is a putative permease that is 45.2% identical to a peptide transporter DtpT of *Lactococcus lactis* (Hafting et al., 1994). The transposon insertion is 531 bp downstream of the predicted start site of the gene. The mutant allele was previously designated *spo-76* (Chen et al., 1991) as it is essential for sporulation and has been renamed *spoH* for sporulation histidine kinase.

Analysis of the amino acid sequence of *spoH* reveals that it is most homologous to *cyaC* (46.6% similarity; 36.4% identity). *cyaC*, an adenylate cyclase gene from *Anabaena cylindrica*, appears to be a membrane protein consisting of four distinct domains: two response regulator-like domains, one histidine kinase-like domain, and a catalytic domain of adenylate cyclase (Katayama and Ohmori, 1997). CyaC is similar to the proteins of the bacterial two-component regulatory system, and may be involved in the maintenance of the steady-state level of cellular cAMP (Kasahara and Ohmori, 1999). SpoH

does not contain an adenylate cyclase domain, but SpoH does share homology with the CyaC histidine kinase and response regulator domains.

SpoH is also homologous to TutC from *Thauera aromatica,* and TobS*,* and TodS, basic leucine zipper histidine kinases of *Pseudomonas putida* (Coschigano and Young, 1997) (Lau et al., 1997) (figure 7). TutC, TobS and TodS are involved in toluene metabolism. Hybrid sensor kinases are two-component signal transduction systems consisting of a histidine kinase motif and a response regulator motif. A conserved histidine residue, functioning as a sensor, is autophosphorylated in response to an intracellular or extracellular signal. The phosphate is transferred to an aspartate residue in the response regulator domain (West and Stock, 2001). Basic leucine zipper (bZIP) histidine kinases are unique sensory hybrid kinases. The histidine kinase contains a leucine zipper dimerization motif at the N terminus, which consists of a basic region that may contact DNA, and a heptad repeat of leucine (the leucine zipper) that mediates dimerization. The bZIP histidine kinase also contains duplicated histidine kinase motifs (Lau et al., 1997). The bZIP motif is common among transcriptional factors in fungi, plants, and mammals but rarely described in prokaryotes (Hurst, 1995). The heptide repeat of leucine is not present in SpoH.

spoH encodes a protein that consists of two histidine kinase motifs and a response regulator motif. SpoH contains a histidine residue, His 307, that is possibly autophosphorylated in response to a signal. SpoH also contains other conserved sequence motifs found in histidine kinases: an N region from Asn 420 to Ala 425, a D/F region from Asp 451 to Phe 458, and a G region from Gly 481

to Gly 498 (figure 7). These regions form the ATP-binding pocket. SpoH includes a response regulator domain homologous to CheY of *E. coli* from Pro 576 to Ile 688 with Asp 583 and Asp 625 acting as possible phosphoacceptors. An additional histidine kinase like domain is present at His 719. An N region is located at Asn 829 to Leu 835 and a G region is located from Gly 887 to Ile 906 (figure 7).

The transposon insertion in LS423 was located in a gene encoding a putative sugar kinase previously designated *spo-423* for sporulation and has been renamed *spoK* for sporulation sugar kinase (Chen et al., 1991). A portion of the gene was found on contig MYX10C1162 in the *Myxococcus* genome (figure 8). Four hundred eighty-five base pairs downstream of the putative stop codon of *spoK* is a protein coding region encoding a putative phosphoesterase. The amino acid sequence of *spoK* was homologous to a long hypothetical sugar kinase of *Aeropyrum pernix*, an aerobic hyperthermophilic archaeon (Hansen and Schonheit, 2001). The putative sugar kinase has 38.8% similarity and 31.3% identity to 2-keto-3-deoxy-gluconate kinase, KdgK from *Bacillus stearothermophilus* T-6, required for utilization of 2-keto-3-deoxygluconate, an intermediate in the degradation of pectin to galacturonate and glucuronate (Shulami et al., 1999).

The deduced amino acid sequence showed domain similarity to the phosphofructokinase B (PfkB) family of sugar kinases, which includes phosphofructokinases and ribokinases. Sugar kinases are members of the sugar kinase/ heat shock 70/ actin superfamily of enzymes (Pettigrew et al., 1998).

The amino acid sequence of the sugar kinase was compared to other members of the PfkB family of sugar kinases: KdgK, ribokinase from *D. radiodurans*, ribokinase of *M. jannaschii* (RbsK), and sugar kinase from *A. pernix* (figure 9). The sequence alignment indicates that SpoK contains the conserved AGD signature pattern of the PfkB family, which is hypothesized to form an anion hole important for phosphoryl transfer (Hansen and Schonheit, 2001).

Extracellular complementation of *csgA* to examine C-signal production. Complementation assays were used to examine extracellular signal production. Some developmental mutants that are unable to form fruiting bodies on their own, can be rescued by mixing with wild type cells suggesting a defect in signal production (Hagen et al., 1978). The extracelluar C-signal is required at 6 hours. To determine if *aprM*, *spoH* and *spoK* produce the C-signal, an extracellular complementation assay was performed by mixing each of the mutants with *csgA* deficient cells to determine whether the *csgA* developmental defect could be rescued.

Developmental mutant cells and *csgA* cells were codeveloped on TPM starvation agar for 96 hours. The *aprM* mutant mixed with *csgA* mutant cells formed irregularly shaped translucent mounds (figure 10). The *spoH* mutant mixed with *csgA* formed translucent irregularly shaped aggregates (figure 10). When *spoK* was mixed with *csgA*, fruiting bodies containing viable spores were produced, but less than 1% of the spores were produced by the *csgA* mutant. *aprM*, *spoH* and *spoK* failed to rescue the *csgA* mutant suggesting as one possibility that none of these mutants produced C-signal.

Western blot analysis to examine C-signal production. Western blot analysis was performed using antibody against CsgA to more precisely examine C-signal production. *csgA* encodes two proteins. The 25 kDa protein is the full length protein (Lee et al., 1995), and a 17 kDa form is a processed product (Kim and Kaiser, 1990). Western blot analysis was performed after 16 hours of development for maximum detection of the 17 kDa protein. Developing cell extracts from 2×10^{10} cells were separated by gel electrophoresis. The anti-CsgA polyclonal antibodies identified two proteins of 25 kDa and 17 kDa (figure 11). *csgA* expression is independent of the Tn*5* insertion mutation. As *aprM* and *spoK* produce near normal levels of both forms of CsgA, these genes are not required for production of either the 17 kDa or 25 kDa forms of the CsgA proteins. *spoH* however produced reduced levels of both forms of the protein.

Codevelopment with wild type. As the mutants produce CsgA yet fail to complement *csgA* mutant development, we wondered whether the mutants produced an inhibitor of development. To examine this point, each developmental mutant was codeveloped with an equal number of wild type cells to observe the developmental phenotype and the number of spores was quantified (figure 12). The developmental phenotypes of *aprM* and *spoH* codeveloped with wild type suggested that both strains inhibit the development of wild type cells. *aprM* codeveloped with wild type cells produced translucent, irregularly shaped mounds but no fruiting bodies (figure 12). *spoH* codeveloped with wild type produced translucent, irregularly shaped mounds and no fruiting bodies were formed (figure 12). Thus, these mutants inhibit the formation of

fruiting bodies by wild type cells. *aprM* and *spoH* inhibited the formation of spores and viable spores by wild type when codeveloped. *spoK* does not have an inhibitory effect on wild type development.

CHAPTER 4

DISCUSSION

This study describes the identification of three new genes involved in *M. xanthus* development as well as their production of the C-signal. *aprM* appears to encode a serine protease, *spoH* appears to encode a hybrid sensor kinase with two histidine kinase motifs, and *spoK* appears to encode a sugar kinase. While all the disrupted genes appear not to contain evidence of polar mutations, a weakness of this study was that the wild type genes were not used to rescue development of the mutants to demonstrate that the mutation is the cause of the phenotype.

AprM may be a cytoplasmic protease according to PSORT (Nakai and Kanehisa, 1991). PSORT predicts the presence of signal sequences by considering the N-terminal basic region and central hydrophobic region of signal sequences as well as a consensus pattern around cleavage sites (Nakai and Kanehisa, 1991). N-terminal signal sequences indicative of secreted serine proteases were not observed, suggesting that it is not secreted through a type II secretion system. Further experimentation could be conducted to determine whether the addition of an extracellular protease, such as chymotrypsin, would restore development of the *aprM* mutant. Based on the results of Western blot assays, we concluded that *aprM* cells produce both forms of CsgA at wild types levels.

Other proteases involved in *M. xanthus* development include the A-signal, which consists of extracellular proteases required for early development and the B-signal, an ATP-dependent protease that shares 45% amino acid identity with the *E. coli* Lon protease (Gill et al., 1993). The BsgA protease involved in making the B-signal is intracellular and the substrate is unknown. A chymotrypsin-like protease involved in spore germination has been described but not identified. Proteolytic activity may be involved in processing the full length 25 kDa form of CsgA to the 17 kDa form, however, the protease has not been identified.

SpoH is homologous to a hybrid sensor kinase, containing two histidine kinase motifs separated by a response regulator motif homologous to CheY. *spoH* produced high levels of sonication resistant rod-shaped spores, but the spores were not viable (figure 3). The *spoH* mutation resulted in reduced levels of CsgA production suggesting that full CsgA expression is dependent on *spoH.*

spoK encodes a sugar kinase. *spoK* produces CsgA in levels equal to that of wild type, therefore *spoK* functions downstream or independently of C-signaling. *spoK* mutants ripple, form irregular fruiting bodies and reduced levels of sonication and heat resistant spores. *spoK* formed spores outside of fruiting bodies suggesting that the mutation caused an uncoupling of aggregation and sporulation (figure 3). SpoK has close homology to an ATP-dependent 6-phophofructokinase of *Aeropyrum pernix* (Hansen and Schonheit, 2001). The sugar kinase of *A. pernix* phosphorylates fructose-6-phosphate, glucose-6-phosphate, adenosine, fructose, ribose 5-phosphate, and ribose and is highly

thermostable (Hansen and Schonheit, 2001). SpoK is also homologous to 2-keto-3deoxygluconate kinase (KdgK) of *Bacillus stearothermophilus* T-6 and *Erwinia chrysanthemi*, which is required for pectin degradation (Hugouvieux-Cotte-Pattat et al., 1994; Shulami et al., 1999). The sugar kinase *spoK* may play a role in the anabolic process of gluconeogenesis and not in a degradative pathway, since enzymes required for glycolysis in *M. xanthus* function mainly in gluconeogenesis (Shimkets, 1984).

Both *aprM* and *spoH* strains were unable to rescue the development of *csgA* cells even though they produced CsgA (figures 10 and 11). Both strains exhibited a dominant negative phenotype by inhibiting the development of wild type cells. When examining synergism between morphogenic mutants, Hagen et al. identified two strains that inhibited fruiting body development of wild type cells, DK631 and DK652 (Hagen et al., 1978). DK753, an A-signaling mutant, somewhat inhibited the sporulation of wild type (Hagen et al., 1978).

Another example of an *M. xanthus* developmental mutant producing an inhibitor is strain MxH1171*, a stable derivative of a *gidA* mutant (White et al., 2001). MxH1171* cannot aggregate or form fruiting bodies, but does form heat-resistant rods which germinate in rich media to form colonies. MxH1171* inhibits the formation of fruiting bodies by wild type during codevelopment. The inhibitory effect of MxH1171* was decreased when mixed with an excess of wild type cells indicating that a minimum concentration of inhibitor is required and cell-cell contact may be necessary (White et al., 2001). Monitoring the level of developmental gene expression using Tn*5-lac* insertions, White et al. determined

that the inhibitor affects the transcription of genes expressed in the early stages of development (White et al., 2001). Vegetative MxH1171* cells do not produce the inhibitor. Wild type cells were resuspended in aliquots of buffer from MxH1171* cells at different time periods during development and placed on starvation agar. Aliquots taken after 8 hours prevented wild type fruiting. Thus, the inhibitor is secreted 8 hours after the onset of starvation and peaks at about 18 hours. By passing the buffer through filters of various sizes, it was determined that the inhibitor is a molecule of about 500 kDa. The partially purified inhibitor blocked fruiting body formation by wild type even after treatment with proteinase K and incubation at high temperatures (White et al., 2001). The inhibitor has not been identified nor has its role in development been defined.

Gierer and Meinhardt described a theory of biological pattern formation based on a local self-enhancing reaction coupled with a longer range antagonistic reaction acting together to control pattern formation (Gierer and Meinhardt, 1972). An activator carries out the self-enhancing reaction, and acts within a short range. The activator is autocatalytic, stimulating its own production and the production of an inhibitor. The inhibitor rapidly diffuses and suppresses the self-enhancing reaction in an extended region (Gierer and Meinhardt, 1972). The balance between the local activator and the dilution of the inhibitor generates a spatial concentration of patterns.

The Gierer and Meinhardt model has been used to describe fruiting body formation in the slime mold *Dictyostelium discoideum* (MacWilliams and Bonner, 1979). The evidence from Hagen et al., White et al., and this work, which shows

that mutations in *spo*H and *aprM* disrupt the spatial arrangement of *M. xanthus* fruiting bodies, suggest that fruiting body formation may be controlled by diffusible activators and inhibitors similar to the Gierer and Meinhardt model. Identifying the inhibitors produced by *aprM* and *spoH* would be of great interest.

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Table 1. *Myxococcus xanthus* strains used in experiments.

Strain	Genotype	Construction	Reference
M xanthus			
DK1622	wild type	transduction of DK1217	Kaiser, D. (1979)
LS205	*csgA*205	P1(pLJS21) × DK1622 → Km^r, Spo^-	Shimkets and Asher (1988)
LS523	*csgA*205	P1Tn*5*-132 × LS205 → Km^s, Tcr	Shimkets and Asher (1998)
LS423	*spo*-423	P1Tn*5* lac^+ × DK1622 → Km^r, lac^+	this study
RC272	ΩRC124	P1Tn*5*-132 replacements, Tc^r	unpublished
RC273	*spo*-75	P1Tn*5*-132 replacements, Tc^r	Chen et al. (1991)
RC274	*spo*-133	P1Tn*5*-132 replacements, Tc^r	Chen et al. (1991)
RC275	*spo*-147	P1Tn*5*-132 replacements, Tc^r	Chen et al. (1991)
RC276	two insertions	P1Tn*5*-132 replacements, Tc^r	unpublished
RC277	*aprM*	P1Tn*5*-132 replacements, Tc^r	this study
RC280	*spo*-76	P1Tn*5*-132 replacements, Tc^r	this study
RC281	*spo*-87	P1Tn*5*-132 replacements, Tc^r	Chen et al. (1991)

Table 2. Phenotypic characterization of developmental mutants.

Strain	Phenotype	Viable Spores	Insertion Site
RC272	cannot rescue development of *csg*A mutant	52% of wild type	unknown
RC273	aggregation defect; cannot rescue development of *csgA* mutant	32% of wild type	23S rRNA gene
RC274	severe aggregation defect; cannot rescue development of *csgA* mutant	0.0% of wild type	unknown
RC275	aggregation defect; cannot rescue development of *csgA* mutant	0.22% of wild type	unknown
RC276	aggregation defect; cannot rescue development of *csgA* mutant	0.11% of wild type	unknown
RC281	aggregation defect; cannot rescue development of *csgA* mutant	0.0001% of wild type	unknown

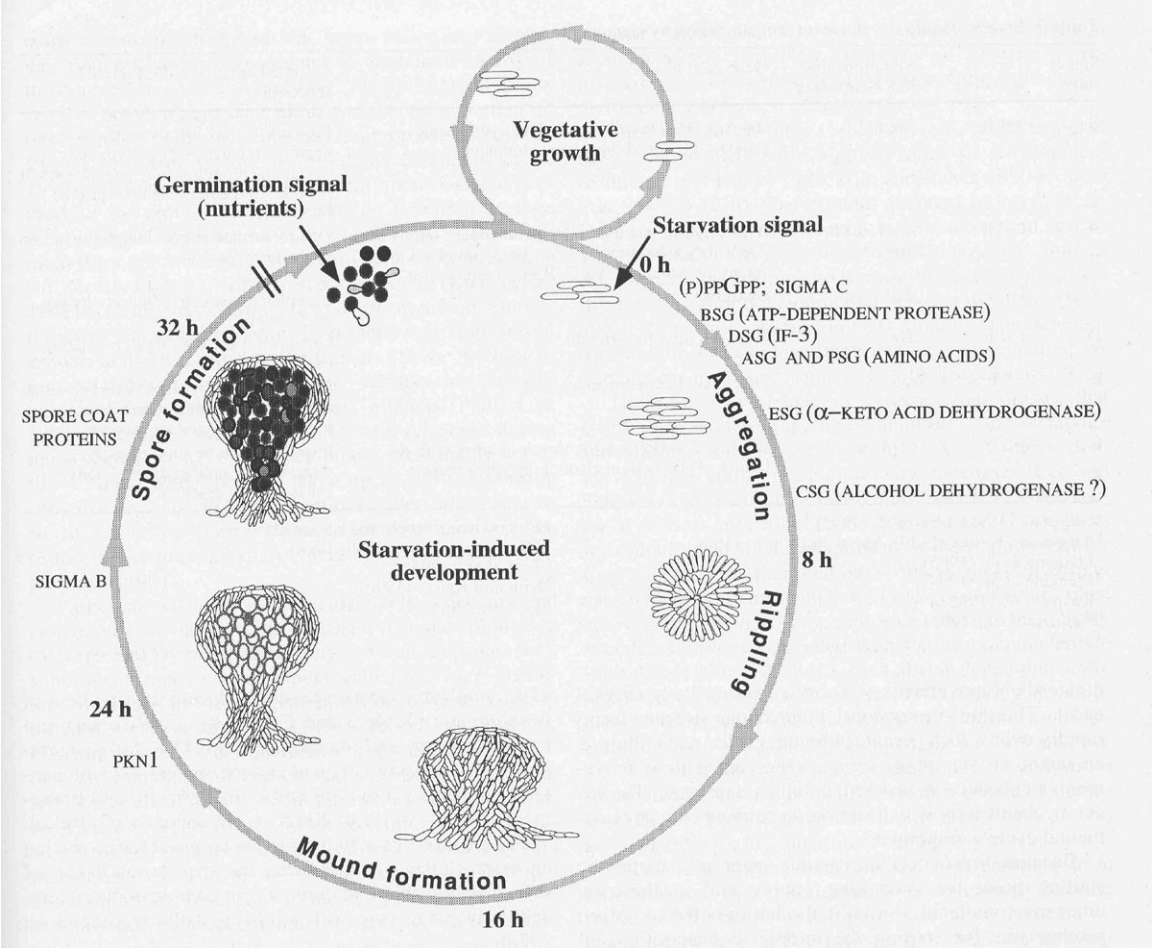


Figure 1. The life cycle of *Myxococcus xanthus*. When nutrients are available, rod-shaped *M. xanthus* cells grow vegetatively and divide by binary fission. If starved for nutrients, new sets of genes, which control fruiting body morphogenesis and sporogenesis, are activated. Several events that are critical for normal fruiting body development and spore formation are shown according to their proposed time of occurrence (clockwise along the developmental cycle) (Hartzell and Youderian, 1995).

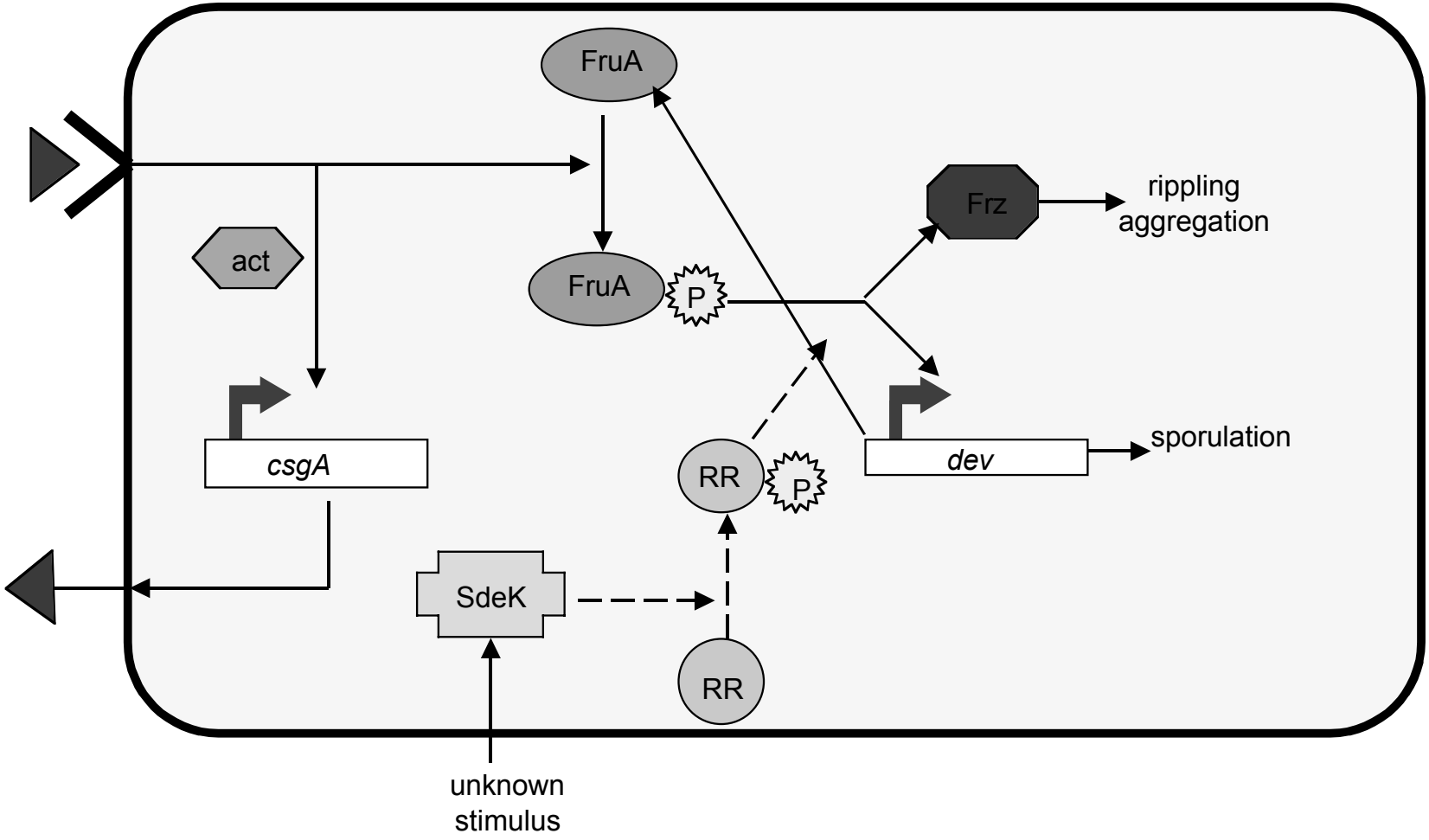
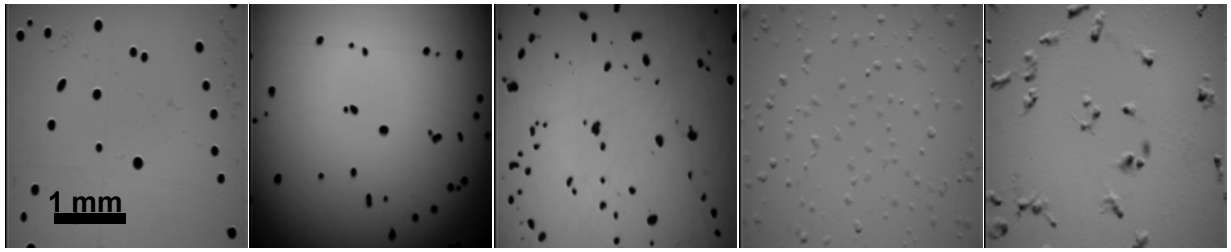
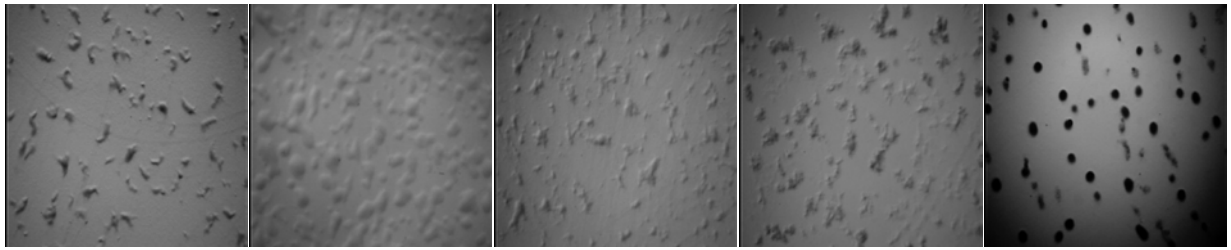


Figure 2. C-signal response pathway. Details described in text.



Strain	DK1622	RC272	RC273	RC274	RC275
Spores	100%	52.0%	33.0%	0.30%	2.00%
Viable Spores	100%	51.0%	32.0%	0.01%	0.22%



Strain	RC276	RC277	RC280	RC281	LS423
Spores	1.70%	1.00%	45.0%	59.0%	44.0%
Viable Spores	0.11%	<0.001%	<0.0001%	0.0001%	43.0%

Figure 3. Aggregation and fruiting body phenotypes of wild type and developmental mutants. DK1622 and mutant cells were placed on TPM agar for 96 hrs. Bar 1.0 mm. Fruiting bodies were viewed with Wild Heerbrugg dissecting microscope. Spore numbers were quantified by direct counts. Viable spores were quantified by germination on CYE agar.

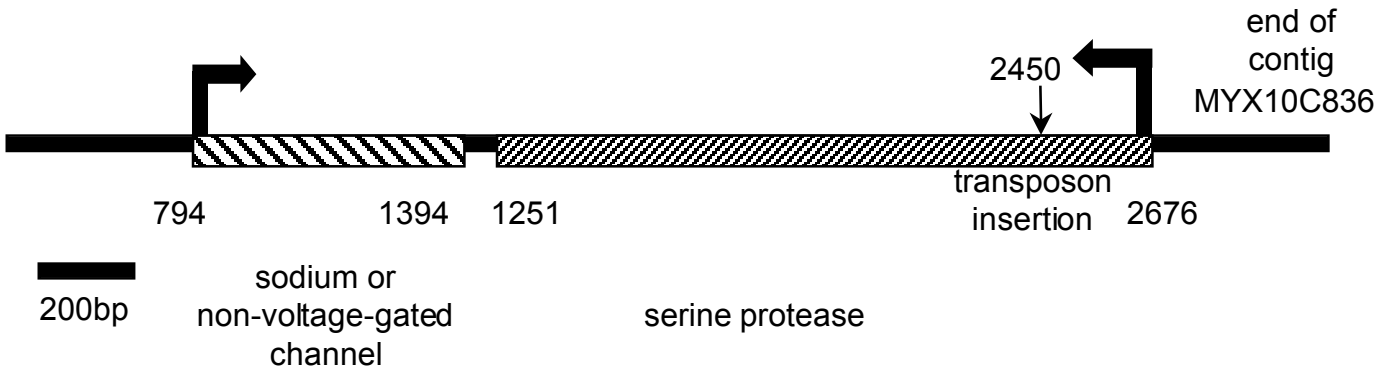


Figure 4. Physical map of the *aprM* locus. Bold arrows indicate the direction of transcription. Vertical arrow indicates the site of Tn*5* insertion and the number refers to the distance in bp from the left end of the contig. Numbers below the map refer to locations of start and stop codons in bp from the left end of the contig.

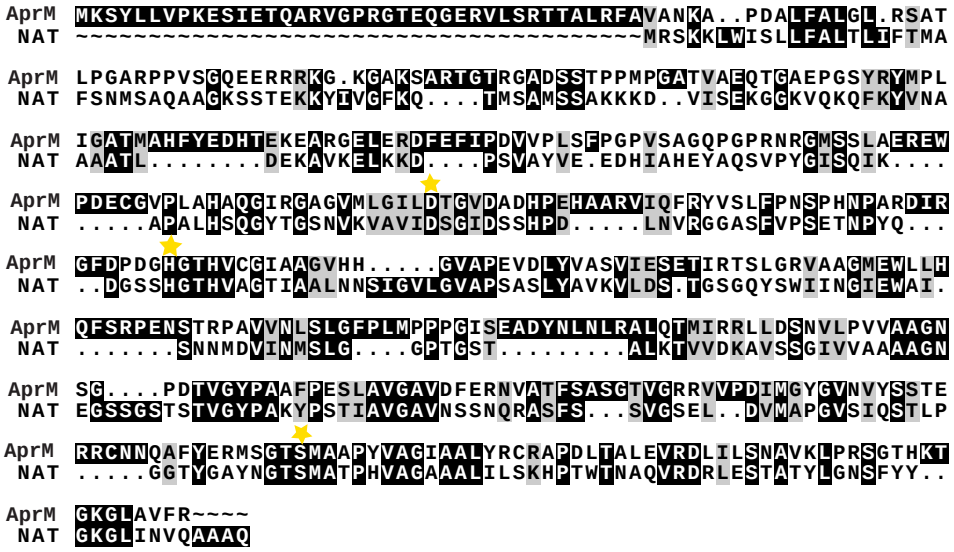


Figure 5. Alignment of AprM with known serine protease subtilisin NAT precursor from *Bacillus subtilis* var. natto (GenBank accession number p35835). Stars indicate the amino acids that form the catalytic triad conserved in subtilisins, Asp/His/Ser.

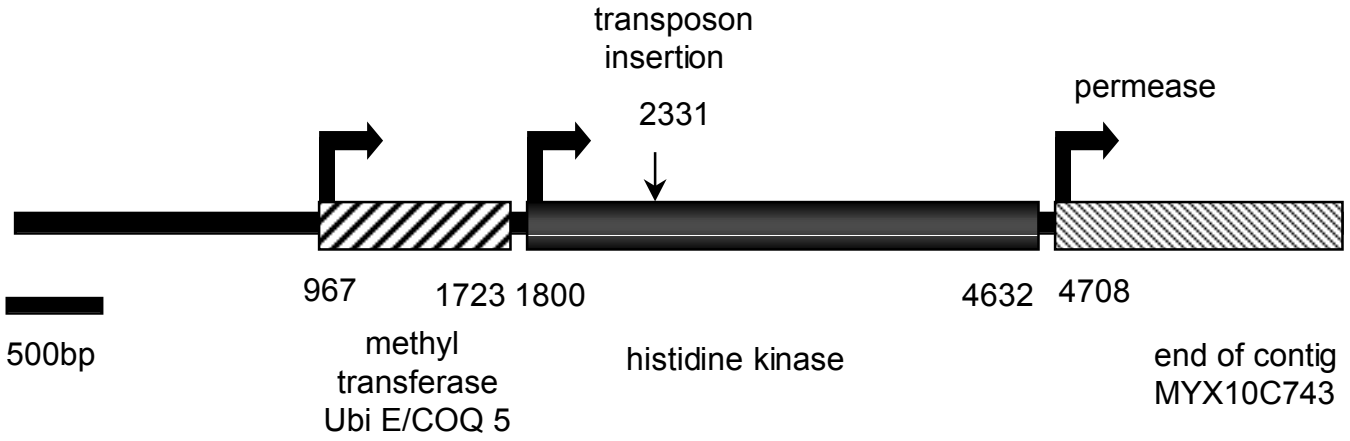


Figure 6. Physical map of the *spoH* locus. Bold arrows indicate the direction of transcription. Vertical arrow indicates the location of the Tn*5* insertion and the number refers to the distance in bp from the left end of the contig. Numbers below the map refer to locations of start and stop codons in bp from the left end of the contig.

```
TutC            ------------------------------------------------------------
TobS            ------------------------------------------------------------
SpoK            MLRSVMLYYERTYGRDRLVRVWQREELPLTLAYVETLTNFVSVGFTERLFDVLDKDSGDP 60

TutC            --MTSNNSSVSDISAV-------------------------------------LRVRDVT 21
TobS            --MSSLDRKKPQNRSK-------------------------------------NNYYNIC 21
SpoK            DFMTKAGRSIASPEAVGFLYYMMKALATPRSVYERALELDHTYNRVGGFHIDTLTDSRVQ 120
                . *:. . . ..  : .      .: ::. :  . : . . : .  ..   .:     :

TutC            LRAVDDLQTYREKLARVVLDGLYEFVGLLDAKGNTLEINQAALDGAGTRLEDIRDKPFWE 81
TobS            LKEKGSEELTCEEHARIIFDGLYEFVGLLDAHGNVLEVNQVALEGAGITLEEIRGKPFWK 81
SpoK            VRYVSRIRERDRNFCRARQGNLSAFPAIWNLPLAEVKELRCQVDGADCCEYDIRWQSLPP 180
                ::  .  .   .: .*   ..*  * .: :    ::  :  ::**.    :** :.:

TutC            ARWWQVSRETQEEQRKLIARAS---AGEFVRCDVEIYGRASG-----------EETIVV 126
TobS            ARWWQISKKTEATQKRLVETAS---SGEFVRCDVEILGKSGG-----------REVIAV 126
SpoK            LAWRYIVGGMAGMVAGLVSGTLNLAPPVFAVSSLGMVGVAAGAWLHTRAALLRKDEKLSE 240
                  *  :          *:  : . :.  *. ..: : * :.*:   : ::   . * :

TutC            DYS--ILPIRDCNGKVVFLLPEGRNITD-KKLAEAELARKNEELQHLLEKIRQLDEAKNE 183
TobS            DFS--LLPICNEEGSIVYLLAEGRNITE-KKKAEAMLALKNQELEQSVECIRKLDNAKSD 183
SpoK            QHQGLQTSLEDLQRRNDEIFAANKALEDRVAERTQELSEANTKLEASLARQQELDRLKSE 300
                :...   .: : :     ::. .: : :       *:  * :*:  :   ::**. *.:

TutC            FFANLSHELRTPLSLILGSVESLLADSGDYSGVQRVDLDVIQRNAITLLKYVNDLLDLAK 243
TobS            FFAKVSHELRTPLSLILGPLEAVMAAEAGRESPYWKQFEVIQRNAMTLLKQVNTLLDLAK 243
SpoK            FFDNVSHELRTPLTLILLTLEALAKEAESLPPELPPLVTNMERSAQRLLRLINNLLDLAQ 360
                ** ::********:*** .:*::     .        .  ::*.*  **: :* *****:

TutC            LQAEKLQLHYSRVDLAAVTRMICAHFEALAEYKCLSYVIDAPAFMEAEVDVEKYERIVLN 303
TobS            MDARQMGLSYRRANLSQLTRTISSNFEGIAQQKSITFDTKLPVQMVAEVDCEKYERIILN 303
SpoK            LESGKARLRYQPLELFGFLSTVVPPFHTMAERQGVTLRLEGATVTPVHVDHERIEIVFQN 420
                ::: :  * *   :*  .   : . *. :*: : ::   . ..   ..** *: * :. *

TutC            LLSNAFKFSPDGGRIRCSLSATGTGRILLSIQDSGPGIPADQQSEIFGRFRQGGDIKSRQ 363
TobS            LLSNAFKFTPDGGLIRCCLSLSRPNYALVTVSDSGPGIPPALRKEIFERFHQLSQEGQQA 363
SpoK            LLGNALKFTQKGG--VTVRVREDDSEVHVEVEDSGQGIAPQDIPVIFDRFSQADNSGTRR 478
                **.**:**: .**           .   : :.*** **..     ** ** * .:   :

TutC            FGGTGLGLTIVKDFVCLHGGVVVVSDAPGGGALFQIELPRNAPSG--------VYVNAVA 415
TobS            TRGTGLGLSIVKEFVELHRGTISVSDAPGGGALFQVKLPLNAPEG--------AYVASNT 415
SpoK            FGGSGIGLALVKETLELHAGGISVTSELGQGSVFHVRLPKGTAHIREDLRERRQAVMPVR 538
                  *:*:**::**: : ** * : *:.  * *::*::.** .:.   ..  .    * .

TutC            KAGELS--PTSFDISAWGLEGRSEWTSAEG---ASDRPRILIVEDNVDMRCFIGRVLIDE 470
TobS            APRRDN--PQVVDTDEYLLLAPNAENEAEVLPFQSDQPRVLIVEDNPDMRGFIKDCLSSD 473
SpoK            RERRISGAFPSLEPTGTDVLGASPPPARDHAGPGPESPRIMVVEDDPEIRSFLARLLAQH 598
                   . ..:   .:     : . .    :     .: **:::***: ::* *:   * ..


TutC            YQISVAADGEQALELITSSPPDLVITDLMMPKVSGQLLVKEMRSRGDLANVPILVLSAKA 530
TobS            YQVYVAPDGAKALELMSNMPPDLLITDLMMPVMSGDMLVHQVRKKNELSHIPIMVLSAKS 533
SpoK            YRVMEAINGDDGRQRALRERPDLILSDVMMPVMSGLQMLTALRNDPQTVDIPVILLTARQ 658
                *::  * :* .. :      ***:::*:*** :**  ::  :*.  :  .:*:::*:*:

TutC            DDGLRIKLLAESVQDYVVKPFSATELRARVRNLVTMKRARDALQRALDSQSDDLSQLTRQ 590
TobS            DAELRVKLLSESVQDFLLKPFSAHELRARVSNLVSMKVAGDALRKELSDQGDDIAILTHR 593
SpoK            EVTAKVEGLGTGANDYLGKPFSPNELLARIETQLRLREA-------------------- 697
```

```
                :   ::: *. ..:*:: ****. ** **: . : :: *

TutC            IIDNRQELQRSHDALQESESRWRAVYENSAAGIVLTNLDGLILSANQAFQKMVGYAEDEL 650
TobS            LIKSRHRLQQSNIALSASEARWKAVYENSAAGIVLTDPENRILNANPAFQRITGYGEKDL 653
SpoK            ------------AVRAAEN---------------------------------------- 704
                            *:  :*

TutC            RVIEISDLVPEHDREKIRSRVSNLISGRVDDYQVQRQCRRKDGRMMWANVRASLIPGLAN 710
TobS            EGLSMEQLTPSDESPQIKQRLANLLQGGGAEYSVERSYLCKNGSTIWANASVSLMPQRVG 713
SpoK            ------------------------------------------------------------


TutC            QSPMVVRIFDDITEKIQTEAELARAREKLTRVMRVTAMGELAASIAHELNQPLAAIVTNG 770
TobS            ESPIILQIIDDITEKKQAQENLNQLQQQLVYVSRSATMGEFAAYIAHEINQPLSAIMTNA 773
SpoK            --------------------------------ERLAAIGLLTSGFAHEVRNPLNGLMNAL 732
                                                * :::* ::: :***:.:** .::.

TutC            HASLRWLGSEPCNLLEAVEAVRRIIHDANRASEIIKRIRGFLQRGEGRRSAVDIFQVVAD 830
TobS            NAGTRWLGNEPSNIPEAKEALARIIRDSDRAAEIIRMVRSFLKRQETVLKPIDLKALVTD 833
SpoK            LPLKDMLTGGSADVELSKAMLEVVEECGQRIRHLAESLLSFTRTSESPVVLSLDSSLDST 792
                 .    * . ..::  :   :  : . .:*  .: . : .* :  *          : :

TutC            VAAIVSDMARSHCIDMRYQAVGQLSLVIADKVQLQQVILNLCINGIESIVGGNSERGELS 890
TobS            TSLILKAPSQNNSVNLDVVADDELPEIWGDGVQIQQLIINLAMNAIEAISQADCETRQLT 893
SpoK            LSVLAWKVPPGVKVERAYHCS---EPIRGNPGALNQVWLNLLDNALRAVGDKG------R 843
                 : :    . .  ::    .      : .:   ::*: :**  *.:.::   .

TutC            ITVTQSDK-RFLTVSVHDSGPGLAPGEAENVFDAFYTSKVE--GLGMGLAISRSIIEAHG 947
TobS            LSFSGNDTGDALVISVKDTGPGISERQMAQLFNAFYTTKKE--GLGMGLAICLTITEVHN 951
SpoK            VRISTANTAEEAIVTIGDDGVGIRPEDMERLFQPFFSTRAAGEGTGLGLALSRRIIIQHG 903
                : .:  :..    ::: * * *:   :  .:*:.*::::  ..* *:***:.  *   *.

TutC            GRLDVLSPSTEGGCTFCFTLPTEEMASPCAPQ-------------- 979
TobS            GKIWVECP-PAGGACFLVSIPARQGSGT----------------- 978
SpoK            GSIALSSV-PGEGTQVEVRLPLRPVAPRVTGGLPDLASEPRVGRLG 948
                * : : .  .  *  . . :* .  :   :   .. ::..  .  .
```

Figure 7. Alignment of SpoH with known histidine kinase proteins. Histidine kinase TutC from *Thauera aromatica* (GenBank accession no. AAD12184), and histidine kinase TobS from *Pseudomonas putida* (GenBank accession no. AAG09417). Asterisks indicate conserved residues.

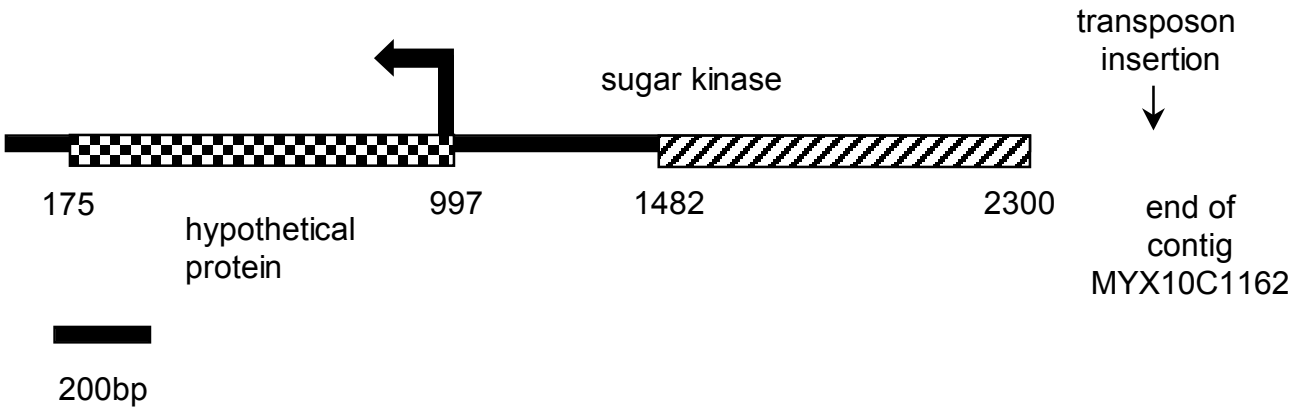


Figure 8. Physical map of the *spoK* locus. Bold arrow indicates the direction of transcription. Vertical arrow indicates the site of Tn*5* insertion. Numbers below the map refer to locations of start and stop codons in bp from the left end of the contig.

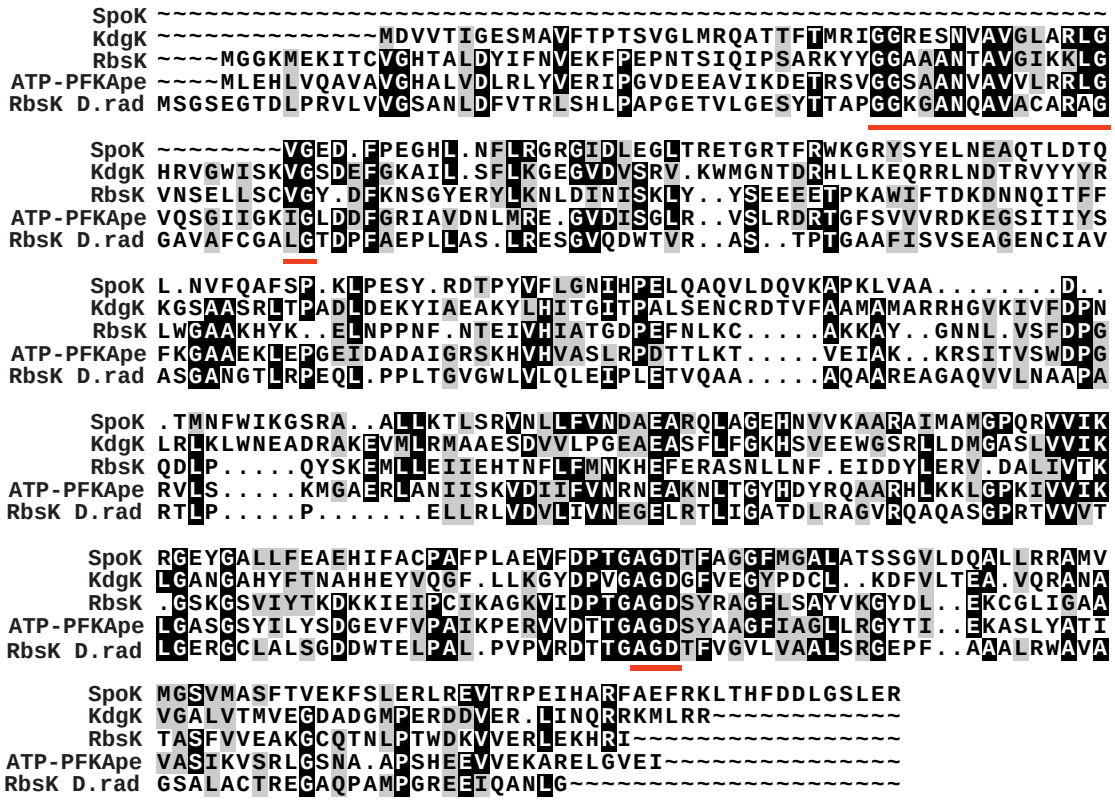
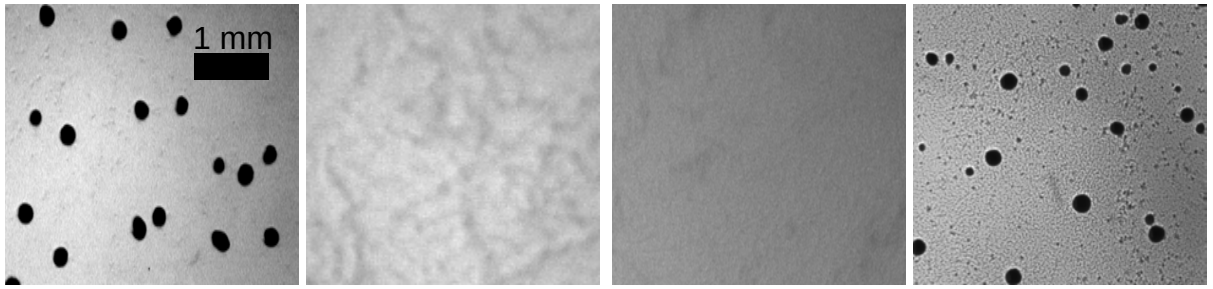
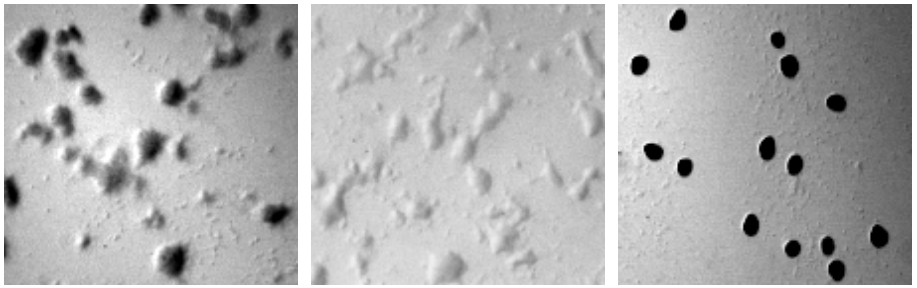


Figure 9. Alignment of SpoK with known proteins of the PFK-B family of sugar kinases. KdgK, 2-keto-3-deoxygluconate kinase from *Bacillus stearothermophilus* (GenBank accession no. AAC98130); RbsK, ribokinase from *Methanococcus jannaschii* (GenBank accession no. NP_24738); ATP-PFKApe, sugar kinase from *Aeropyrum pernix* (GenBank accession no. NP_146902); RbsK D.rad, ribokinase from *Deinococcus radiodurans* (GenBank accession no. NP_285378). Conserved signature pattern of PFK-B family sugar kinases and AGD motif are underlined.



Strain	DK1622	LS205	LS523	LS205 + DK1622
% of spores due to *csg*A mutant		0	0	32



Strain	LS205 + RC277	LS205 + RC280	LS523 + LS423
% of spores due to *csg*A mutant	2.5	<1	<1

Figure 10. Extracellular complementation of sporulation of *csg*A mutants. DK1622, wild type; LS205 *csgA*; LS523 *csgA;* RC277, *aprM;* RC280, *spoH;* LS423, *spoK*.

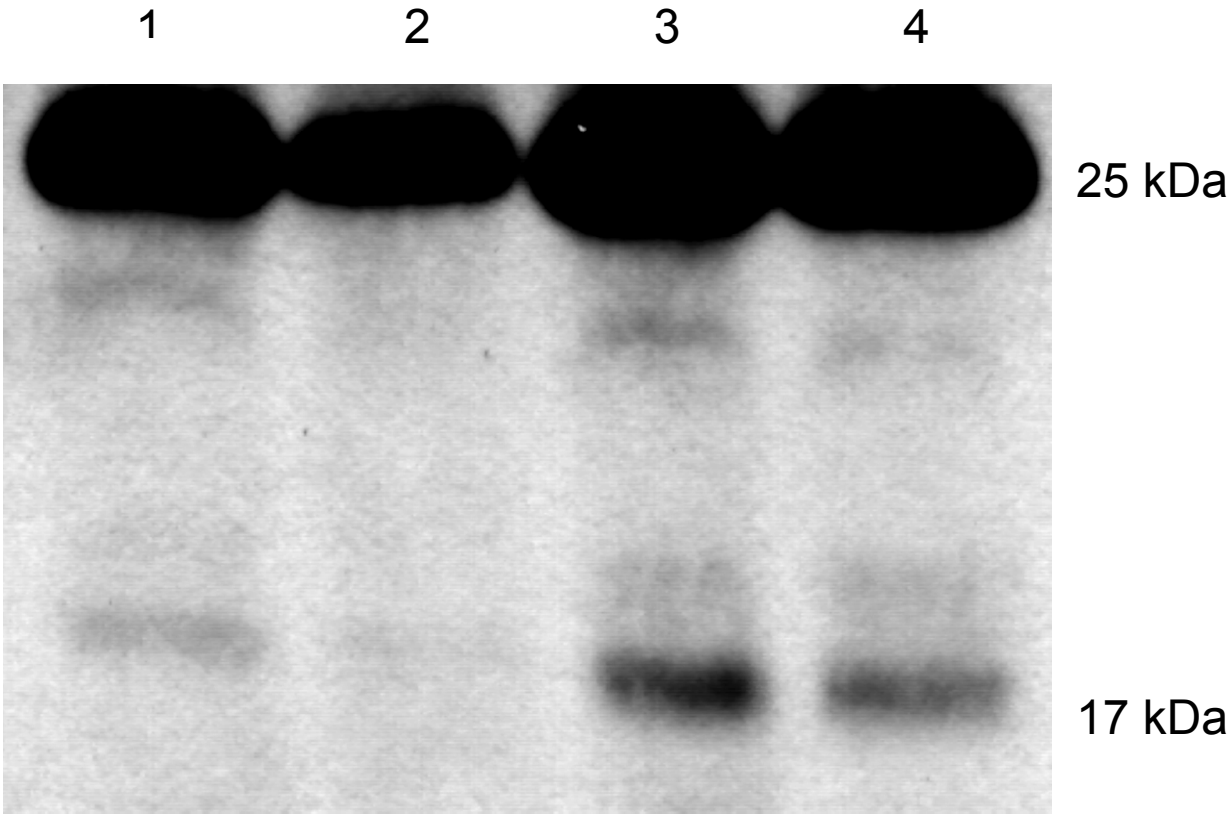
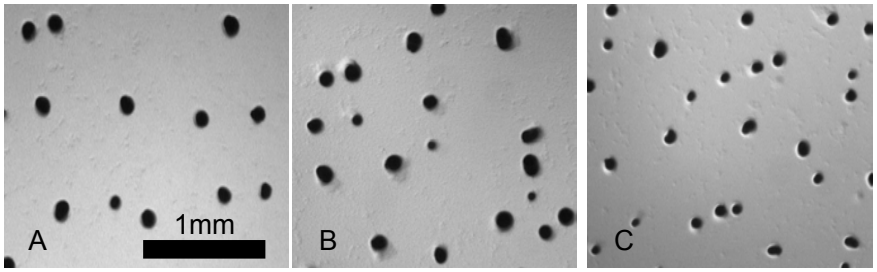
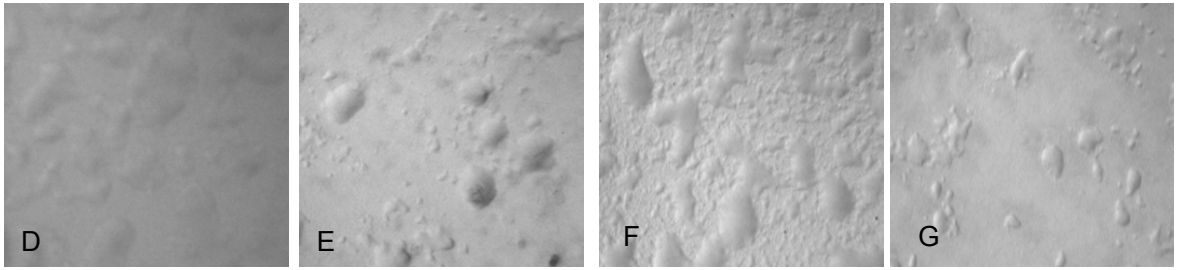


Figure 11. Immunoblot analysis of CsgA proteins in wild type and developmental mutant cells. Total cell lysates from 2.0×10^{10} cells were prepared from cells after plating onto starvation agar for 16 hrs. LS423, *spoK* (lane 1), RC280, *spoH* (lane 2), RC277, *aprM* (lane 3), and DK1622, wild type (lane 4).



Strain	DK1622	LS423	DK1622 + LS423
% Spores	100	43.4	39.5
% Viable Spores	100	26.0	35.6
% Spores due to wild type			68.0



Strain	RC277	DK1622 + RC277	RC280	DK1622 + RC280
% Spores	0	1	0.5	7
% Viable Spores	< 0.0001	0.0002	< 0.00001	0.0004
% Spores due to wild type		0		12.5

Figure 12. Developmental mutants codeveloped with wild type cells. Wild type cells were codeveloped with equal amounts of developmental mutant cells for 96 hrs on TPM agar. DK1622, wild type (A); LS423, spoK (B); DK1622 with LS423 (C); RC277, aprM (D); DK1622 with RC277 (E); RC280, spoH (F); DK1622 with RC280 (G). Bar, 1.0 mm.