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UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

STUDIES ON THE REGULATION OF DEVELOPMENTAL GENE EXPRESSION AND POLYSACCHARIDE PRODUCTION IN *Myxococcus xanthus*

A Dissertation

SUBMITTED TO THE GRADUATE FACULTY

in partial fulfillment of the requirements for the

degree of

Doctor of Philosophy

By

SANG-HOON KIM
Norman, Oklahoma
1997

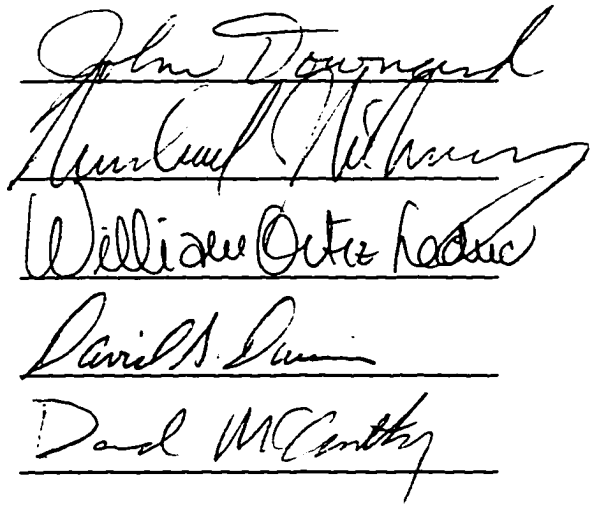
STUDIES ON THE REGULATION OF

DEVELOPMENTAL GENE EXPRESSION AND

POLYSACCHARIDE PRODUCTION IN *Myxococcus xanthus*

A Dissertation APPROVED FOR THE
DEPARTMENT OF BOTANY AND MICROBIOLOGY

BY



ABSTRACT

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The highly homologous *tps* and *ops* genes are expressed during *Myxococcus xanthus* development. Activation of *tps* occurs early in development (5h) while *ops* activation starts after 40h. Within the region of homology (from -50 to ATG codon), there exist 9 mismatched sequences. We investigated the differences in DNA sequence for their roles in differential regulation. The conclusions drawn from this study are; The *ops* -35 region contains an inverted repeat sequence and may mediate repression. The *tps* sequence at -46 and -39 was shown to be involved in an early developmental *tps* induction after 9h. Difference in the -10 region did not affect the differential regulation but affected transcription efficiency. Difference in the leader sequence apparently made *tps* mRNA weaker in translation, possibly due to the formation of a more stable secondary structure. Late developmental induction of *ops* appeared to require the region between -131 to -50.

Fibrils, an extracellular appendage, are composed of polysaccharide and protein. Regulation of fibril production in *M. xanthus* was studied. In wild-type cells, fibril production was induced upon the stationary phase and the Ca^{2+} addition. Such induction was not observed in the fibril-deficient social motility mutants and *Cds* mutants. However, normal levels of the phosphoenolpyruvate

carboxykinase (Pck) activity in these mutants suggested that the block in fibril biosynthesis in these mutants occurs in the later stages of the gluconeogenesis pathway. Ca^{2+}-mediated polysaccharide induction was also observed in developmental submerged culture conditions, resulting in cellular aggregation and fruiting body formation. Sr^{2+} but not Mg^{2+}, Mn^{2+}, Li^{2+}, or Rb^{2+} could substitute for Ca^{2}. The *stk* mutant showed constitutively high levels of polysaccharide and Pck. The double mutants with mutations in the *stk* locus and in social-motility genes or in the *cds* loci showed constitutively elevated polysaccharide and Pck activity levels. Two exceptions (*stk-dsp* and *stk-SR200* strains) suggested that the *stk* allele is hypostatic to the *dsp* or *SR200* allele. DNA sequence analysis of the 3.6-kbp KpnI-EcoRI DNA fragment which complemented the *stk-1907* insertion allele identified two ORFs, ORF673 and ORF89. Beccause of the incomplete amino acid sequence of the ORF673 within the fragment, the ORF89 appeared to be a strong candidate for the *stk* gene.

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CHAPTER III

ABSTRACT

Regulation of developmental activities in the bacterium, *Myxococcus xanthus* is an essential feature of the complex process of forming multicellular fruiting bodies. The studies described in this dissertation focus on the regulation of gene expression and on the regulation of fibril polysaccharide production.

The highly homologous and closely linked *ops* and *tps* genes are strongly expressed during *M. xanthus* development, but the regulation patterns of the two genes are different. Activation of *tps* expression occurs early in development (5h into development) while *ops* activation does not occur until about 40h. Regulated gene expression is achieved with *tps* DNA upstream to -95 (relative to transcription initiation), and *ops* DNA upstream to - 131. Both *ops* and *tps* contain Upstream Activation Sequences (UAS) for an optimal level of gene expression. The UASs function interchangeably and are contained between -311 to -131 of *ops* and between -375 to -173 of *tps*. The transcription initiation regions of *ops* and *tps* are highly homologous extending 100 bases upstream from the ATG codon, with a position -50 as its upstream end. However, there exist mismatched sequences within the region of homology which can be divided into four groups: the distal end (two bases at -45 and -38 of *ops*), the "-35 hexamer" (three bases from -30 to -28 of *ops*), the "TATA box" (additional T residue in *tps*), and the leader (three bases at +28, +37, and +45). In this study, we investigated the differences in DNA sequence for their potential role in differential regulation of the two genes. Mutations and deletions were made on the parental *ops-lacZ* and *tps-lacZ* gene fusion plasmids which

contained *ops* DNA with its upstream end to -311, and *tps* DNA to -95, respectively. The mutated gene fusions were measured and compared for β-galactosidase activity in vegetative and developmental *M. xanthus* cells.

The *ops* -35 region contains an inverted repeat sequence which may mediate repression during pre-sporulation period. Disruption of the sequence by changing the *ops* sequence TCC (-30 to -28) to the *tps* sequence ATT (D3 mutation) caused a dramatic increase in *ops* expression. Conversely, creation of the inverted repeat sequence in the *tps* -35 region by changing ATT to TCC (D2 mutation) resulted in a disrupted *tps* expression.

The *tps* distal end of the homologous region defined by two mismatched bases (two A residues at -46 and -39 of *tps*) was shown to be an important *cis*-acting region for an early developmental *tps* induction which occurred after 9h into development. Acquisition of these bases in *ops* (D6 mutation) resulted in the same temporal induction which is absent in wild-type *ops*, indicating that the two A residues are crucial in association with a putative *tps*-specific transcriptional activator which functions about 9h into development.

The results of deletion (T residue at -8) in *tps* and of insertional mutation (AGCT between -7 and -8) in *ops* indicated that the sequence difference in the TATA boxes of *ops* and *tps* does not affect the differential regulation but influences transcriptional efficiency.

Differences in the leader sequence apparently reduce the translational efficiency of *tps* mRNA, possibly due to the formation of a more complex hairpin structure within the *tps* leader mRNA.

Late developmental induction of *ops* is likely to require the region flanked by the *ops* UAS and the *ops* promoter region (e.g. -131 to -50), since the *tps-lacZ* fusion plasmids which contained both *ops* UAS and *ops* promoter sequence but not the flanking *ops* sequence were not induced.

From the previous results and this study, we propose a model in which the *tps* gene contains at least three upstream *cis* acting regions for early developmental gene expression; the *tps* UAS (-375 to -173) which is responsible for an optimal level of *tps* expression, the primary activating site (-110 to -82) which binds a transcriptional activator causing an induction of *tps* at 5h to 6h into development, and the secondary activating site defined by mutation at -46 and -39 which mediates a secondary induction of *tps* at 9h into development.

The *ops* gene may be under control of a repressor at the -35 region during pre-sporulation period, which appears to negate the transcriptional activation by the *ops* UAS-protein complex. Induction of *ops* may be initiated by derepression which would involve binding of (a) regulatory factor(s) to the region between -131 and -50 at the onset of sporulation. The late developmental regulatory factor(s) could facilitate binding of the RNA polymerase to the *ops* promoter thus allowing the upstream UAS-protein complex to activate the transcription of *ops*.

M. xanthus synthesizes a copious amount of extracellular polysaccharide as a constituent for extracellular fibrils and possibly for other structures relating to the motility and fruiting body

formation. Besides its role as a structural component, the extracellular polysaccharide has been implicated in various biological events including cell cohesion, group motility, and cell-cell interaction. Fibrils, an extracellular appendage, have been associated with the ability of *M. xanthus* cells to agglutinate and are made up of polysaccharide and protein. While mutation in the recently identified locus, *stk*, causes overproduction of the fibrils and strong cell cohesion, mutations in *dsp* and *cds* loci confer severe fibril deficiency to *M. xanthus*. In this study, fibril production was investigated in various *M. xanthus* mutant strains including *stk-cds* double mutant strains under selected physiological conditions, and a key gluconeogenic enzyme phosphoenolpyruvate carboxykinase (EC 4.1.1.49) (Pck) was used to investigate the relationship between gluconeogenesis and polysaccharide formation.

In wild-type cells, fibril production was induced in response to the stationary phase of growth and the addition of Ca^{2+} to cells. When social motility mutants and *Cds* mutants, both of which are deficient in fibril production, were tested for stationary phase induction of polysaccharide production, all were defective. However, in all of these mutants, there was an increase in the activity phosphoenolpyruvate carboxykinase (Pck) similar to that observed with the wild-type, suggesting that the block in fibril polysaccharide biosynthesis in these mutant strains occurs in the later stages of the gluconeogenesis pathway.

Ca^{2+} was shown to induce polysaccharide production in wild-type and *esg* cells growing in rich medium; induction was not seen in the

social motilty mutants or in *SR53* (a *cds* mutant). Stimulation of polysaccharide production in the wild-type by Ca^{2+} was also observed in developmental submerged culture conditions, resulting in cellular aggregation and fruiting body formation. Sr^{2+}, but not other divalent cations such as Mg^{2+}, Mn^{2+}, Li^{2+}, or Rb^{2+}, was able to substitute for Ca^{2+} in supporting *M. xanthus* development in submerged culture.

The *stk* mutant showed a constitutively high level of polysaccharide, and its Pck level was uniformly high during log or stationary phase. Also, the Pck level of the *stk* mutant was not induced by a gluconeogenic medium or a high osmolarity condition in which the wild-type cells were strongly induced. Most of the double mutants with mutations in the *stk* locus and in social-motility genes or in the *cds* loci showed constitutively elevated polysaccharide and Pck activity levels. Two exceptions to this pattern were found with the *stk-dsp* and *stk-SR200* double mutants, which displayed uninducible polysaccharide production and activation of Pck during stationary phase, suggesting that the *stk* allele is hypostatic to the *dsp* or *SR200* allele. The stk gene appears to be required for the regulation of Pck activity.

DNA sequence analysis of the wild-type KpnI-EcoRI DNA fragment which was previously shown to complement the *stk-1907* insertion allele identified two ORFs, ORF673 and ORF89. Although the Tn5 insertion (*ΩDK1907*) was within the upstream ORF673, it is likely that the KpnI-EcoRI fragments only complemented the loss of ORF89 since ORF673 lacked N-terminal amino acids and the two ORFs are likely to be cotranscribed. The predicted amino acid sequence from ORF89 was found to contain a leucine zipper motif which is commonly associated with the dimerization

of regulator proteins that bind to DNA. This observation is consistent with the possibility that Stk acts as a *trans*-acting repressor and limits the synthesis of (a) protein(s) required for limiting fibril biosynthesis.

CHAPTER I

INTRODUCTION

DEVELOPMENT IN BACTERIA

Development is "genetically directed changes in a single cell, or groups of cells, assumed in a programmed fashion over time (17)." Development encompasses both differentiation and morphogenesis. Differentiation can be briefly defined as the development of function, while morphogenesis refers to the development of shape. Some organisms undergo irreversible but cyclic development, while others are only capable of linear development. For example, certain bacteria and lower eukaryotes such as yeast differentiate into resting spores which subsequently germinate into the vegetative organism. By contrast, all "mortal" multicellular organisms pass through a linear program of differentiation, never cycling back to an earlier state.

The strategy of development allows a microorganism the advantage of specialization to cope with a continually changing environment (17). This specialization may be (1)temporal - as in the case of the formation and germination of resistant resting cells (e.g. *Bacillus* endospores, *Myxococcus* myxospores), (2)spatial - as in the case of heterogeneous filaments of cells (*Anabaena* heterocysts), (3)or both - in the case of

Caulobacter. Brief descriptions on the developmental cell cycles of the selected organisms will follow.

Under unfavorable conditions for growth, part of an individual *Bacillus* cell is converted to a spore which is metabolically dormant, optically refractile, and highly resistant to harsh environmental conditions. The remainder of the cell is doomed to lyse leaving a free spore. When conditions are favorable for germination, each spore will germinate and become a vegetative cell.

A genus of the cyanobacteria, *Anabaena* forms heterocysts along the filament at regularly spaced intervals when nitrogen source is depleted. Precursor cells, proheterocysts, are morphologically and physiologically intermediate between vegetative cells and heterocysts. The process of proheterocyst development is reversible. Concentration of a diffusible inhibitor which is originally present and evenly distributed in vegetative cells controls the process. Upon depletion of fixed nitrogen, the level of inhibitor would fall, a heterocyst would be formed, and it would then serve as a source of inhibitor, generating a one dimensional gradient along the filament. At the point where the inhibitor concentration drops below a certain level, a new heterocyst is formed, and so on.

In *Caulobacter*, there are two coexisting, functionally distinct cell types in the population, the swarmer cell and the stalked cell. The motile swarmer cell, at some point during development, loses its flagellum and starts to produce the stalk at the same site. The stalked cell becomes sessile via an adhesive holdfast at the distal end

of the stalk. The stalked cell grows vegetatively and repeatedly produces one stalked cell and one swarmer cell.

Many bacteria exist as organized populations and manifest multicellularity. Examples are the formation of fruiting bodies by the myxobacteria, heterogeneous filaments and aggregates of cyanobacteria, and the mycelia of actinomycetes. In this type of morphogenesis, differentiation of individual cells is coordinated in response to environmental stimuli. This process involves tactic, developmental movement, the exchange of chemical signals between cells, the coordinated construction of complex, multicellular structures, and most likely, cell-surface interactions between cells.

Development, like all other biological processes, is the result of a sequence of metabolic events. It involves a temporal program for the sequential activation of groups of developmental genes. This temporal program is initiated in response to environmental conditions. Thus the factors involved in the initiation of temporal regulation of the developmental program of gene expression are of central importance.

REGULATION OF GENE EXPRESSION IN BACTERIA

Prokaryotic gene expression can be controlled at several levels. One level of regulation involves the control of the synthesis of mRNA (transcriptional control). Also, control may occur at the translational level (e.g. anti-sense RNA mediated control). Finally, there is control over the activity of the enzymes. These different

levels of control are not mutually exclusive but most prokaryotic genes are controlled at the transcriptional level.

Transcription requires RNA polymerase. The core enzyme consists of three subunits, α, β and β' in the ratio of 2:1:1. Other polypeptides, sigma (σ) and rho (ρ) may associate with the core enzyme temporarily during the initiation and termination of transcription. σ-mediates initiation of RNA synthesis at (a) specific site(s) (transcription start site) upstream from a gene. A promoter is a site that is recognized by RNA polymerase and supports the initiation of RNA synthesis. The promoter consists of two functionally important regions. One of the regions, the Pribnow box, (or TATA box) is centered at 10 nucleotide upstream of the transcription start site and has the consensus sequence TATAAT. The second is 25 nucleotide further upstream with the consensus sequence TTGACA and is referred to as the -35 box or the recognition site, where RNA polymerase is bound to the promoter. Termination of transcription also occurs at specific sites containing a run of dA residues preceded by a pair of inverted repeats which can form a stem and loop structure when transcribed. The stem and loop structure may cause RNA polymerase to pause and aid its dissociation from the DNA template (ρ-independent termination). Some transcripts require a termination factor ρ (ρ-dependent termination). Where it is used, the DNA sequence at the termination site is not usually conserved. ρ is a hexamer that possesses a distinct RNA binding site and also has ATPase and helicase activities. The molecular details of its involvement in termination are not yet known.

Translation of mRNA starts while it is being transcribed. mRNAs contain a ribosome binding site, the Shine-Dalgano box (or AGGA box), which is located a few bases upstream from the initiation codon (usually AUG) and has the consensus sequence AGGAGGU. This sequence plays an important role in positioning the mRNA correctly on the ribosome.

Positive regulation at the transcriptional level can result in an increase of the basal strength of a promoter by one of several mechanisms; interaction with (a) protein activator(s), use of a new sigma factor for the RNA polymerase holoenzyme, alteration of DNA conformation or the methylation state of the DNA, or the interaction of small molecules with the promoter or with RNA polymerase (45). Protein activators bound either in the region near the -35 region or DNA sequences far upstream of a promoter region increase the rate or extent of RNA polymerase open complex formation at the promoter. The mechanisms of negative regulation are similar to this in that any protein can be a repressor if the DNA sequence to which it binds is in a position to interfere with binding of (an) activator(s) or RNA polymerase.

The *lac* operon of *Escerichia coli* has been studied intensively and is of classical importance since its examination led Jacob and Monod (29) to develop the operon model of gene expression. The *lac* operon consists of three structural genes, *lacZ*, *lacY*, and *lacA*, which are involved in the uptake and catabolism of lactose. The *lacI* gene located next to the *lac* operon, encodes a repressor protein which can bind to the operator located between the promoter and *lacZ* blocking transcription. Induction of the *lac* operon occurs when lactose is

available. Allolactose converted from lactose by the action of innate low level of β-galactosidase, a gene product of *lacZ*, is the actual inducer by binding to the repressor and removing it from the operator. Transcription of *lacZYA* can proceed.

The *lac* operon has an additional, positive regulatory control system. Efficient expression of the *lac* operon requires binding of CRP-cAMP complex to the CRP binding site on the *lac* promoter. CRP is the cAMP receptor protein. The bound complex is thought to promote destabilization of downstream DNA helix facilitating RNA polymerase binding and thus increasing expression of the *lac* operon (19). The role of cAMP in *lac* regulation and in other catabolite utilization operons is to serve as an indicator of glucose levels. When cells are grown on glucose, intracellular cAMP levels decrease, hence less CRP-cAMP complex forms. When, however, cells are grown on alternative carbon source (such as lactose, maltose etc.), cAMP levels increase and so does the amount of the CRP-cAMP complex. Thus the *lac* operon serves as a paradigm for studies of both positive and negative forms of gene regulation in other systems.

It is now well established that the prokaryotic transcriptional machinery has a complex system of multiple RNA polymerase holoenzymes consisting of several different sigma (σ) factors that interact with a common core enzyme. Each holoenzyme form can interact specifically with a cognate set of promoters which has a unique consensus sequence. At least six sigma factors encoded by the *E. coli* genome have been identified including σ^{70}, the major sigma factor. σ^{S} is required for induction of growth-phase regulated genes and expression of stationary

phenotypes (39). σ^{32} and σ^{E} confer recognition of heat shock promoters (22). σ^{54} is required for expression of genes involved in nitrogen utilization and infection by filamentous phages (24, 61). σ^{F} controls transcription of flagellar and chemotaxis genes. The five minor sigma factors appear to control genes that are generally nonessential and that evolved to allow the cell to respond to various environmental stresses.

The other major role of minor sigma factors is to express genes in a sequential and segregated fashion during developmental processes in prokaryotic cells (51). For example, in *Bacillus subtilis*, the earliest-acting sigma factor in the sporulation sequence is σ^{H} whose concentration increases several-fold shortly after the onset of sporulation. The genes encoding σ^{E} and σ^{F} are expressed soon after the initiation of sporulation before the formation of the spore septum that separates the prespore and mother cell compartments. However, σ^{E} and σ^{F} direct transcription only after septation and control genes that are expressed in a specific cell-type, those of σ^{F} in the prespore, and those of σ^{E} in the mother cell (18). The other two, σ^{G} and σ^{K}, are synthesized during the later stages of sporulation and act specifically in the prespore and mother cell, respectively.

Gene expression can also be controlled through the action of proteins that bind to DNA sequences far away from a promoter region. The regulatory proteins that bind to them activate or inhibit transcription. Enhancers, which were first described in eukaryotic genes, are binding sites for regulatory proteins and lie at a distance

from a promoter, either upstream or downstream (42). Enhancer-like sequences (or UAS Upstream Activator Sequence) are also found in prokaryotes. The *glnA-ntrBC* operon in enterobacteria is the best known operon for its UAS-mediated gene regulation. The operon encodes proteins involved in nitrogen assimilation, glutamine synthetase and two regulatory proteins, NtrB and NtrC. The *glnA* gene (encoding glutamine synthetase) is transcribed from two promoters, *glnAp1* and *glnAp2* which are recognized by RNA polymerase with different sigma factor, $E\sigma^{70}$ and $E\sigma^{54}$, respectively. Under condition of excess ammonia, the weak glnAp1 promoter provides a basal level of glutamine synthetase. Under condition of ammonia limitation, the NtrC protein is phosphorylated by the action of NtrB, and the phosphorylated NtrC activates the RNA polymerase from the remote NtrC DNA binding sites (as far as 2 kb). By occupying five NtrC binding sites located approximately 100 bp upstream from *glnAp2*, the NtrC protein not only activates transcription from *glnAp2* but also represses transcription from *glnAp1* (43). The activation event is the melting of DNA around the transcriptional startpoint by the σ^{54} RNA polymerase. NtrC appears to function by touching the bound RNA polymerase, with the intervening DNA being looped out (52, 60).

REGULATION OF EXOPOLYSACCHARIDE PRODUCTION IN BACTERIA

Bacteria produce polysaccharides as components of their cell wall structures and, in many species, as extracellular macromolecules (56, 62). Examples of the cell-wall components are bacterial teichoic and

teichuronic acids, lipopolysaccharides and peptidoglycan which are unique in type and confined to a limited range of bacteria. The term exopolysaccharides is applied to polysaccharides either associated with other surface macromolecules or totally dissociated from the bacterial cell. Capsule and slime are good examples that fit into these categories. These are extremely diverse in their chemical composition and structure.

The biosynthesis of most exopolysaccharides (EPS) closely resembles the process by which peptidogycan and lipopolysaccharide (LPS) are formed. The initial step involves (1)biosynthesis of activated precursors such as ADP-D-glucose and UDP-D-glucose in the cytoplasm, followed by (2)formation of repeating units, undecaprenol-linked oligosaccharides, at the cytoplasmic membrane (3)polymerization of repeating units, and (4)export of polysaccharides to the cell surface. Biosynthetic genes for exopolysaccharide are arranged in clusters in most bacteria and regulatory genes tend not to be linked to clusters of biosynthetic genes.

In most bacteria, regulatory strategies are directed to modulating exopolysaccharide synthesis in response to appropriate environmental cues. A two-component regulatory system, one of the most popular strategies, is employed by *Escherichia coli* K-12 for the production of a slime polysaccharide, colanic acid (50). Colanic acid production can be activated by conditions including growth in the chemically defined media with high concentration of phosphate and high carbon:nitrogen ratios, and low incubation temperatures below 25°C (41). Under these conditions, a transmembrane protein RcsC and a cytoplasmic protein RcsF

modulate activation of RcsB protein (50). RcsB, when activated, forms a dimer with DNA-binding protein RcsA, and plays a central role in transcription activation of *cps* (capsular-polysaccharide synthesis) genes. The RcsB and RcsC proteins compose the two-component regulatory system in which RcsC is the transmembrane sensor and RcsB is the response regulator.

Other types of regulation mechanism include changes in local DNA topology which is critical in expression of environmentally regulated genes, positive regulation mediated by CRP-like (cAMP receptor protein) protein, allosteric activation as seen in bacterial cellulose synthetase, protein-protein interactions, and regulation of enzyme activity in precursor formation. Production of precursors provides a convenient point for regulation of polymer synthesis, for example ADP-glucose for glycogen and UDP-glucose for LPS and CPS. Also, an *IS* element and DNA rearrangement have been implicated in instability of exopolysaccharide synthesis of *Haemophilus influenzae* type b and *Pseudomonas aeruginosa*, respectively (34, 63).

Myxococcus xanthus AND ITS LIFE CYCLE

Myxococcus xanthus is a gram-negative, gliding bacterium with a complex life cycle (16, 30, 31, 49). In nature, they inhabit damp soils rich in decaying organic matter. *M. xanthus* cells utilize organic matter as carbon and energy sources but they also can prey on other microorganisms. They cannot engulf other microorganisms but rely instead on extracellular antibiotics which kill or immobilize their

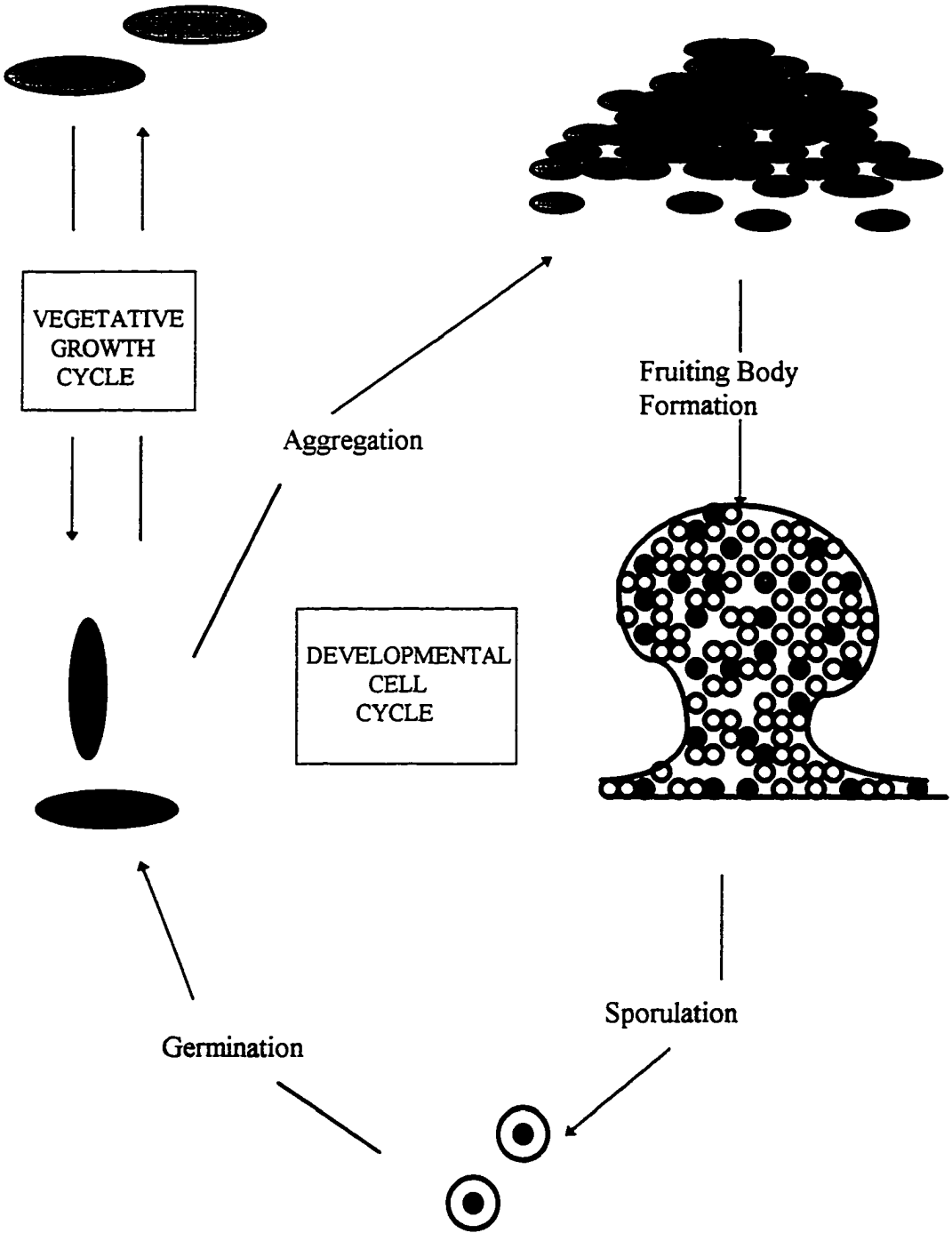
prey, and on other numerous extracellular hydrolytic enzymes to digest prey macromolecules. The vegetative cells tend to aggregate into large masses or swarms, and move in "hunting groups". The reliance on extracellular bacteriolytic and degradative activities may be responsible for the strong social interactions exhibited by these organisms.

Starvation brings about an aggregation of masses of cells into mounds (fruiting bodies) and the differentiation of individual rod-shaped bacteria into spherical or ovoid myxospores within the fruiting bodies (30). This dramatic response to nutrient depletion is referred to as "development" (Fig.1). During development, the outward migration of swarming cells stops and the cells begin to migrate, in wave- or ripple-like fashion. The aggregation centers gradually enlarge into mounds of cells which may be constricted at the base. Myxospores are formed within the fruiting aggregates to produce mature fruiting bodies. The entire process takes 2-3 days. A mature fruiting body may contain as many as 10^6 spores. Myxospores are resistant to heat, UV irradiation, dessication, and sonication. Myxospores are also metabolically dormant, enabling them to withstand prolonged periods of nutrient deprivation. The germination of myxospores occurs upon provision of the appropriate growth conditions to produce vegetative cells and complete the life cycle. This elaborate developmental strategy ensures that a highly localized population of spores can respond to favorable nutritional conditions as a multicellular, predatory colony.

Fig. 1 The life cycle of *Myxococcus xanthus*

The life cycle of *M. xanthus* can be divided into two phases. During vegetative growth, cells grow exponentially and divide by transverse fission. When conditions are unfavorable for vegetative growth (e.g. depletion of nutrient and high cell density, etc.), hundred thousands of cells move to aggregation centers where they assemble multicellular structures called fruiting bodies. Within fruiting bodies, individual cells differentiate into round, refractile, and dormant myxospores. Germination occurs when sufficient nutrient is available, and the cells recycle back to the vegetative growth phase.

Fig. 1


VEGETATIVE GROWTH CYCLE
Aggregation
Fruiting Body Formation
DEVELOPMENTAL CELL CYCLE
Germination
Sporulation

CELL-CELL COMMUNICATION OF *M. xanthus*

M. xanthus development is a social behavior involving many thousands of cells. Other types of social behavior include cooperative growth, group motility, and periodic rippling (16). These synchronized social behaviors require a high level of coordination which must involve cell-cell communications. *M. xanthus* exhibits two modes of cell-cell communication; the exchange of extracellular signals and the contact-mediated interactions.

Contact-mediated cell-cell interactions are mediated by two cell-surface appendages, pili and fibrils. The pili of *M. xanthus* are polarly located, less than 10 nm thick, and up to 10 μm long. The pili have been implicated in social motility (S-motility or group movement) which, together with adventurous motility (A-motility or individual cell movement), comprises *M. xanthus* motility (47, 64). Fibrils of *M. xanthus* are lateral, branching appendages and are composed of carbohydrate and protein in a 1.2:1.0 ratio (4). Fibrils may be as long as 50 μm and two predominant forms, with diameters of 15 or 30 nm, have been observed. They play a role in cell-cell association and social behavior in *M. xanthus*. Many fibril-deficient mutants have been isolated. These include a class of mutants termed *dsp* which are unable to show cohesion, social motility, and fruiting body formation. Arnold and Shimkets (2, 3) proposed that the fibrils supply the cohesive force that allows the cells to exhibit several social and developmental behaviors.

Five extracellular signals (A-, B-, C-, D-, and E-signal) are believed to exist which appear to be required for normal development of

M. xanthus. Hagen et al. (23) isolated conditional sporulation mutants (groups A, B, C, and D) which cannot sporulate alone, but can be rescued for sporulation by co-development with wild-type cells or with cells of a different mutant group. The rescue does not involve genetic exchange between cells, but rather occurs extracellularly. Apparently each mutant class is defective in producing a different intercellular signal required for normal development but is able to respond to signals from wild-type cells. Each mutant class is arrested at a different stage of development, prior to aggregation.

All of the *asg* (for A-signal) mutations map to one of three genetic loci, *asgA*, *asgB*, or *asgC* (36), which act early in development, at about 1 to 2 hour, during the preaggregation period. A-signal is a mixture of amino acids and peptides that is generated in turn by a mixture of proteases (37, 44). Development by the *asg* mutants can be restored by the addition of the mixture of amino acids and peptides. It has been proposed that the A-signal is used by *M. xanthus* to specify the minimum cell density required for the initiation of development.

Several of the *bsg* (for B-signal) mutations map to a gene known as *bsgA* (21). The *bsgA* gene has substantial homology to the *lon* genes of *Escherichia coli* and *Bacillus brevis* (20) suggesting that the *bsgA* phenotype may be the result of a defect in intracellular proteolysis. The nature of B-signaling is not known.

All *csg* (for C-signal) mutants contain a mutation in the same gene, *csgA* (48). The C signal acts at about 6 to 7h into development. The gene product of *csg*A (C factor) is a 17-kDa membrane-associated protein which forms a dimer in vivo (33). The *csg* mutants respond

differently to different levels of added C factor. Addition of low levels of C factor induces aggregation and early developmental gene expression, whereas higher levels are required to induce sporulation and the expression of late developmental genes, suggesting that C factor may act as an extracellular developmental timer. Although initially it was believed that Csg protein was the C-signal, it has been recently postulated that the *csg*A gene product is a short-chain alcohol dehydrogenase(SCAD) which may be involved in generation of the C signal itself (40).

In *dsg* mutants, their development is only partially impaired. Sporulation is substantially reduced and aggregation is abnormal and delayed. The *dsg* mutations map to a gene, *dsg*A (7, 8) which shows 50% sequence homology to the translation initiation factor IF3 of *E. coli*. There is however little information about the nature of D signaling.

Recently, the *esg* locus was discovered in our lab which controls the expression of the *tps* gene, an early developmental gene encoding a spore coat protein (13). *Tn5* insertion in this locus dramatically lowered the developmental *tps* expression. This mutant was also defective in developmental aggregation and sporulation. The defects in *tps* gene expression and sporulation can be significantly corrected by mixing the mutant with wild-type and other groups of signaling mutants (A-D). The *esg* gene shows substantial homology with the E1 decarboxylase of the branched-chain keto-acid dehydrogenase (BCKAD) complex (59). The *esg* mutants appear to be defective in their ability to use the branched-chain amino acids leucine, isoleucine, and valine to produce branched-chain fatty acids which are found in *M. xanthus*. It

has been proposed that the E-signal may consist of one or more of the branched-chain fatty acid, or a related compound (14).

THE *tps* AND *ops* GENES IN *M. xanthus*

The regulation of gene expression is an important aspect of development in *M. xanthus*. A number of genes have been identified which are developmentally regulated. Two of these genes, the *tps* and *ops* genes, are highly homologous (about 90%) at the DNA sequence level and they are directly repeated and closely linked (1.4 kb apart) on the *M. xanthus* chromosome (27, 28). However, the two genes are transcribed independently, and their expression is differentially regulated. The expression of the *tps* gene is activated early (about 5 to 6h) in development before cell aggregates or myxospores have formed. In contrast, the *ops* gene is expressed beginning late (about 40h) in development as spores are forming within cell aggregates (12, 15)(Fig. 2B). Differential gene expression is also observed in different culture conditions. The *tps* gene is expressed when cells are suspended in starvation liquid medium CF; the *ops* gene is not expressed. The *ops* gene, in turn, is expressed in added glycerol culture. The *tps* gene is not expressed under these conditions (12, 15).

The *tps* and *ops* genes encode two closely related proteins, S and S1, respectively. While protein S is the most abundant protein produced during development and is a component of the outer surface of the myxospore, protein S1 is found within the myxospore and is found at only about 1/10 of the level of protein S (57, 58). These proteins are apparently not essential for *M. xanthus* development. Production of

protein S1 appears to be controlled by a developmental specific sigma factor SigB which is encoded by the sigB gene. A deletion mutant of *sigB* displays normal fruiting body formation and sporulation, but production of protein S1 is blocked (1).

There are at least five extracellular signaling systems (A-E) which participate stage-specifically in the execution of development in *M. xanthus*. The expression of the *tps* gene depends upon the A, B, and E signaling systems but not on the C signaling system, whereas *ops* expression depends on all four systems. The *tps* and *ops* genes, as targets of this control, are attractive subjects for studies of the signal transduction circuits involved in the regulation of developmental gene expression.

The putative regulatory DNA sequences of the two genes are greater than 90% related for about 100-bp upstream from the translational initiation codon. Homology between the genes ends abruptly at that point. The 5′ ends of both *tps* and *ops* mRNA has been mapped to 50-bp upstream from the *trans*lational start of each gene (10) and DNA sequences having similarity to the -10 and -35 promoter consensus sequence have been identified in each gene. An interesting feature of this system is that comparison of the DNA sequences in the regulatory regions of the *tps* and *ops* genes may be helpful in developing an understanding of how differential regulation is achieved and how the regulatory systems which control gene expression may be related.

The regulatory regions of the *tps* and *ops* genes consist of at least two types of sites; promoters for RNA polymerase binding and upstream activation sites (UAS) for binding of transcription activators

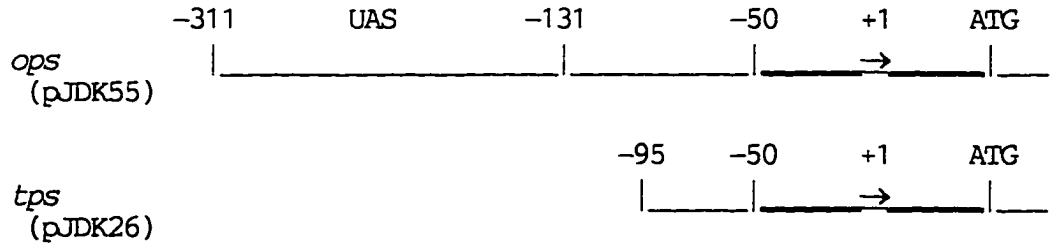
that may stimulate transcription initiation. The UASs are located within a 202-bp segment of *tps* (-375 to -173 relative to the transcription start) and a 180-bp segment of *ops* DNA (-311 to -131) (Fig. 2A), and they function interchangeably when located upstream of the UAS-deleted *ops* or *tps* gene (32). Foot printing experiments identified a 22-bp sequence within the *ops* UAS (between -269 and -290) which was bound by vegetative and early developmental binding activities (5). The *ops* UAS was shown to activate the *tps* promoter as far as -1.5 kb and as near as upstream *tps* position -66. An additional important upstream *cis*-acting site is present in *tps* which is located between -110 to -82. This site was shown to be bound by an early developmental *trans*-acting factor which is not present in the B-signaling defective mutant (the *bsg* mutant). However, deletion analysis indicated that a *tps* DNA segment extending upstream to -95 is sufficient for regulated *tps* gene expression (11) (Fig. 2B). Similarly, an *ops* DNA segment extending upstream to -131 is sufficient for regulated expression of that gene. However, the normal high levels of *tps* and *ops* gene expression require the UASs.

Presumably these facts are, at least in part, responsible for the differential regulation of the two genes. However, DNA sequence differences also exist within the upstream homologous regions (about 100-bp extending from -50 to ATG codon) of the two genes, and these sequences may also play a role in differential regulation. These are two mismatched bases at -45 and -38 of *ops*, three consecutive mismatched bases from -30 to -28 of *ops*, additional T residue at -8 in *tps*, and three mismatched bases at +28, +37, and +45 in the leader sequence. In

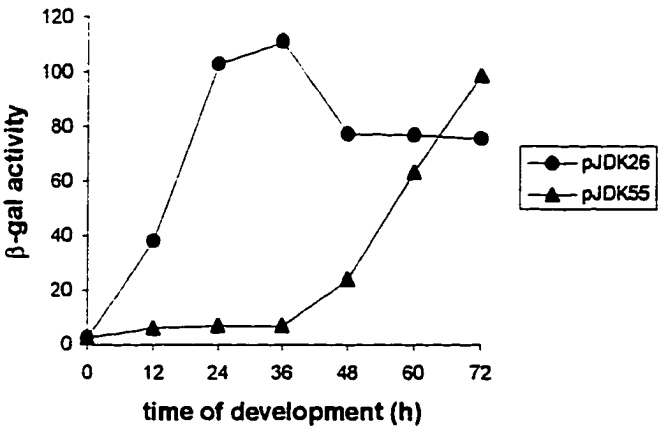
part of this study, the significance of these differences was investigated by constructing mutations which interconvert the *ops* or *tps* sequence at the mismatched sites.

Fig. 2 Developmental gene expression of *ops* and *tps*.

A.



B.



A. pJDK55 contains a 417-bp NciI-Sau3A (-311 to +106 relative to transcriptional start site) *ops* DNA fragment including the upstream activating sequence (UAS) which extends -311 to -131. pJDK26 contains a 201-bp HinfI-Sau3A (-95 to +106) *tps* DNA fragment. *ops* and *tps* upstream DNA required for the regulated gene expression is indicated. The site for transcriptional initiation is indicated by arrows. The upstream homologous region of the two genes is bold-lined.

B. Time course expression of the pJDK55 and pJDK26 gene fusion plasmids is shown. *M. xanthus* cells harboring the fusion plasmids were harvested from developmental agar plates at the indicated times and assayed for β-galactosidase activity (nmole per min per mg protein). Development of *M. xanthus* on agar plates was obtained by spotting concentrated cell suspensions (3000 Klett units with the red filter, about 7 X 10^9 cells/plate) in 10mM-Tris.HCl (pH 7.4), 10mM-$MgSO_4$ on CF agar.

EXOPOLYSACCHARIDES IN *M. xanthus*

Myxobacteria characteristically produce extracellular polysaccharide (slime) during growth and development. Vegetative cells produce and excrete slime either in liquid culture or on the surface of agar plates. It can be also isolated from fruiting bodies of *M. xanthus* amounting almost 20% of the dry weight of the cells. The major monosaccharide components of the hydrolyzed slime in vegetative cells were found to be glucose, galactose, mannose, and glucosamine (54, 55). The fruiting-body polysaccharide is composed of the same sugars with additional traces of galactosamine. However, the function or the nature of slime has not yet been characterized.

It has been suggested from the chemical and morphological analysis of fibrils that the extracellular polysaccharide (or slime) of *M. xanthus* is arranged as fibrils (4). The fibrils , which are branching appendages with a diameter of 30 nm, extend only when cells are closely associated. They are composed of protein and carbohydrate in a 1.0:1.2 ratio and are constructed as polysaccharide backbones with associated proteins. Arnold and Shimkets (2, 3) showed that the *dsp* mutant, which is defective in cell cohesion, development, and social motility, also lacked fibrils. It was also demonstrated that addition of purified fibrils to the *dsp* mutant fully restores development and cohesion, indicating that the fibrils play an integral role in the social behavior of *M. xanthus*. However, recently isolated fbd mutants, which are a set of secondary *dsp* mutants, presented an apparent contradiction (6). Although the fbd mutants still lack fibrils like the parent *dsp* mutant, they had nevertheless regained group motility and partial development.

In addition, our lab recently isolated a group of mutants called *Cds* mutants which are fibril-deficient but retain a substantial capacity for social motility. These mutants were identified based on defects in the ability to bind calcofluor white (a flourescent dye which binds exopolysaccharide) and based on capability of showing group movement on the motility agar surface. Thus, these observations are consistent with a role for fibrils in aggregation and cell cohesion but not directly in S-motility.

In contrast to the *dsp* allele, the *stk-1907* allele which contains *Tn5* insertion ΩDK1907 caused wild-type and S-motility mutant cells to exhibit increased production of fibrils and cell cohesion, and enabled the S-motility mutant cells to regain social motility (9). The colonies of the *stk* mutant have a dry, wrinkled surface, and cells adhere tightly to each other and to the agar surface. Studies with merodiploids suggest that the *stk-1907* allele is recessive and that the *stk* gene product acts as a negative regulator of fibril synthesis. The phenotype of the *stk* mutant was shown to be complemented by a 3.6 kb KpnI-EcoRI wild-type DNA fragment (9), suggesting that the *stk* gene is contained on this fragment.

OBJECTIVES OF DISSERTATION

The first part of my dissertation (Chapter II) is focused on the mechanisms by which the *ops* and *tps* genes exhibit differentially regulated gene expression during *M. xanthus* development. The regulatory systems of both *tps* and *ops* genes include the upstream UAS segments for optimum levels of developmental gene expression and the

further downstream unidentified *cis*-acting sites which are responsible for the differential timing of activation of gene expression. Although early developmental induction of *tps* has been partly attributed to the additinal *cis*-acting region between -110 and -82, little is known about the mechanism involved in late developmental induction of *ops*. The objective of this research is, therefore, to identify such *cis*-acting regions and determine their roles in developmental regulation of the two genes. The primary targets for this study are located within the 100-bp highly homologous region where nine mismatched and unmatched bases are found in *tps* and *ops*. Those regions contain the respective promoters and possibly other regulatory sites essential for developmental gene regulation. Because of the apparent importance of their localizations, these bases were altered by site-directed mutagenesis and the effects of the changes were analyzed by constructing various gene fusions with the promoterless *lacZ* as a reporter gene. Attempts were made from previous and current studies to construct models of how the two genes are regulated.

In the second part of dissertation (Chapter III), preliminary studies on the regulation of polysaccharide production in *M. xanthus* are described. Conditions such as growth phase, medium composition, and addition of metal cations were tested for polysaccharide production. The activity of a key guluconeogenic enzyme (phosphoenolpyruvate carboxykinase, Pck) was also measured under different conditions in order to obtain insight on how these two parameters are related and how such relationships affect biological aspects of *M. xanthus*. A variety of fibril-deficient mutants were also tested for polysaccharide

production and Pck activity to try to understand the nature of the genetic defects in these strains. Finally, a 3.6 kb KpnI-EcoRI DNA fragment which complements the *stk-1907* allele was sequenced, and a putative *stk* ORF was determined. A model for the genetic control of fibril production was proposed to help explain the results presented in this study and to focus future studies on the molecular mechanisms of fibril biosynthesis and regulation.

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CHAPTER II

MUTATIONAL ANALYSIS OF THE PUTATIVE PROMOTER REGIONS OF THE *ops* AND *tps* GENES IN *Myxococcus xanthus*.

INTRODUCTION

Myxococcus xanthus is a gram-negative soil bacterium which utilizes organic matter for carbon and energy sources but is also a predator preying on other microorganisms and simple eucaryotes such as nematodes. The vegetative cells tend to aggregate into large masses or swarms, and move in "hunting groups". *M. xanthus* has a complex life cycle and undergoes development in response to nutritional downshift. Development involves aggregation of numerous cells into mounds, formation of fruiting bodies, and differentiation of rod-shaped vegetative cells into spherical or ovoid myxospores (17, 29, 32). Myxospores are formed within the fruiting bodies in 2 to 3 days on the solid surface (18, 29, 31, 32). Myxospores are resistant to heat, UV irradiation, dessication, and sonication. Myxospores are also metabolically dormant, enabling them to withstand prolonged periods of nutrient deprivation, and germinate upon provision of the appropriate growth conditions. Sporulation can also be induced by the addition of glycerol (0.5 M) to the vegetative cell culture (16). Glycerol-induced

myxospores form rapidly (3 to 5 h), but they differ in ultrastructure from fruiting body spores (12).

Intercellular signaling has been implicated in the control of *M. xanthus* development. Hagen et. al. (14) found that developmentally defective mutants could be classified into four sets based on the extracellular complementation, and concluded that wild-type cells exchange at least four developmental signals and that each set of mutants is defective in the production of one such signal. At present, at least five extracellular signals have been recognized; A-, B-, C-, D-, and E-signal. Two signals, A and C, have been chemically characterized. A-signal acts early in development (1h to 2h) and is a mixture of amino acids and peptides produced, at least, proteolytically. C-signal is a dimer of a 17kDa protein and exerts its effect at about 6h to 7h into development. E-signal may act before the C signal and has been proposed to be branched-chain fatty acids such as iso-15:0. The nature of the B- and D-signals is unknown.

The *ops* and *tps* genes are closely linked and are separated by 1.4 kb on the *M. xanthus* chromosome. These genes are about 90% identical at the DNA sequence level (15, 16). However, the temporal expression patterns of the two genes are different. The expression of the *tps* gene begins about 5h after the initiation of development, and reaches its peak level at about 30h into development. The *ops* gene is not expressed until much later in development, after myxospores have formed within multicellular mounds (7, 8, 11). The *tps* and *ops* genes encode two closely related proteins, S and S1, respectively. While protein S is the most abundant protein produced during development and is a

component of the outer surface of the myxospore, protein S1 is found within the myxospore and is produced about 1/10 of the amount of protein S (35, 36).

The expression of all recognized *M. xanthus* developmentally regulated genes, including *ops* and *tps*, is controlled by the intercellular signaling. The expression of the *tps* gene depends upon the A-, B-, and E-signaling systems (12, 37) but not on the C-signaling system, whereas *ops* expression depends on all four systems.

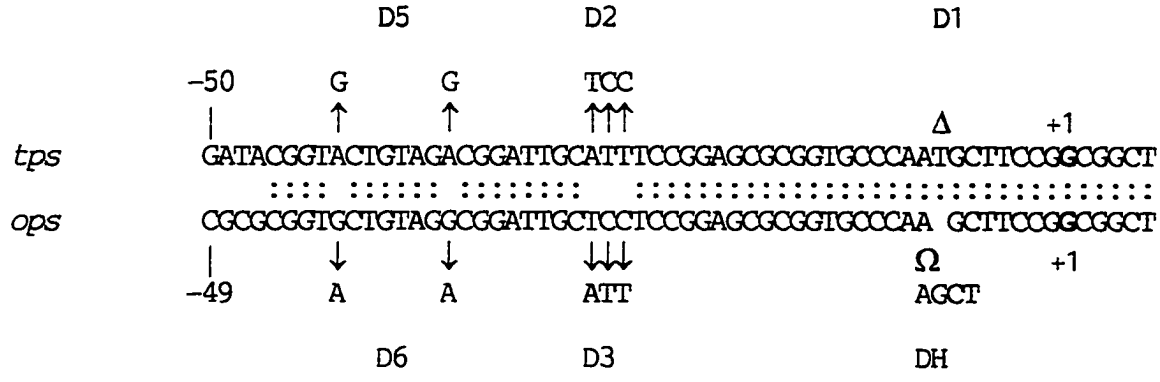
The proteins involved directly in *tps* and *ops* regulation have not been identified. However, in *M. xanthus*, the genes for three sigma factors have been recognized, and one of them, sigB, was found to be expressed during the onset of sporulation through late development (1, 2). Deletion of sigB was shown to produce spores lacking protein S1, the gene product of *ops*.

The *tps* and *ops* genes are regulated at the transcriptional level and DNA sequences involved in the specific transcription of the two genes have been identified. The high degree of homology between *ops* and *tps* continues upstream about 100 bases from the translational start (upstream homologous region), and transcription of both genes is initiated within the homologous region about 50 bases upstream from the translational start (9). Previous deletion studies indicated that the *tps* and *ops* genes both contain the Upstream Activation Sequences (UAS) for an optimal level of gene expression during *M. xanthus* development (20, 21). A 202-bp segment of *tps* (-375 to -173 relative to the transcriptional start) and a 180-bp segment of *ops* DNA (-311 to -131) have been shown to enhance gene transcription by several fold and

function interchangeably when located upstream of the *ops* and *tps* genes. Foot printing experiments identified a 22-bp sequence within the *ops* UAS (between -269 and -290) which was bound by vegetative and early developmental binding activities (5). The *ops* UAS at either orientation was shown to activate the *tps* promoter as far as -1.5 kb and as near as upstream *tps* position -66. The UASs, however, were not necessary for the temporal activation of gene expression which can be achieved with DNA sequences close to the transcription start sites of the two genes.

The cis-acting regulatory regions of both *tps* and *ops* genes extend upstream from the homologous region (upstream from -50). Developmentally regulated *tps* gene expression is achieved with *tps* DNA upstream to position -95, and an *ops* DNA segment extending upstream to -131 is sufficient for regulated *ops* expression (10). Presumably this observation accounts, at least in part, for the differential regulation of the genes. However, DNA sequence differences also exist within the upstream homologous regions (-50 to the transcription start) of the two genes, and these sequences may also play a role in differential regulation. Four groups of mismatched sequences are noted in this region (Fig. 1), which are at the distal end (two bases at -45 and -38 of *ops*), the "-35 hexamer" (three bases from -30 to -28 of *ops*), the "TATA box" (additional T residue at -8 in *tps*), and the leader sequence (three bases at +28, +37, and +45). The significance of these differences was investigated by constructing mutations which interconvert the *ops* or *tps* sequence at the mismatched sites (Fig. 1).

Fig. 1 Sites of mismatched sequences and mutations in the upstream homologous region of the *ops* and *tps* genes.



Identity between the two sequences is indicated by vertical dashed lines, and the converted bases are indicated above the aligned sequences. Delta (Δ) and omega (Ω) represent deletion and insertion respectively at that site. Numbers indicate base positions in relation with the transcription initiation site (+1). Each mutation is described in detail in the text. Three mismatched bases in the leader region are not shown.

MATERIALS AND METHODS

Bacterial strains and culture conditions

M. xanthus cultures were grown vegetatively in casitone-yeast extract (CYE) medium (6). Development of *M. xanthus* on agar plates was obtained by spotting concentrated cell suspensions (3000 Klett units with the red filter, about 7 X 10^9 cells/plate) in TM buffer (10mM Tris-HCl pH 7.4, 10mM-$MgSO_4$) on clone-fruiting (CF) agar (25). Development in liquid shaker culture was initiated by suspending vegetatively grown cells in clone-fruiting (CF) medium at a density of 2 X 10^8 to 4 X 10^8 cells per ml and incubating on a rotary shaker (250 rpm) at 30°C. *M. xanthus* DZF1 and M380 (13) strains were used as the wild-type and *bsgA* strains, respectively. *E. coli* strain MC1000 was used for cloning, and JM109 was used as the host for M13 phages (23).

Plasmids

For all *tps* and *ops* gene fusion plasmids, the *M. xanthus* DNA was joined to the *lacZ* DNA of pMLB1034 (33) at a Sau3A site which is found within the *tps* and *ops* structural DNA sequence, so that the N-terminal 18 amino acids of the *tps* and *ops* protein products are at the N terminus of the fusion protein (7, 8). The gene fusion plasmids used in this study are listed in Table 1.

Construction of *tps-lacZ* gene fusion plasmids

The Amp^r *tps-lacZ* fusion plasmid, pJDK24, contains the 201-bp HinfI-Sau3A *tps* DNA fragment (-95 to +106) inserted into the SmaI-BamHI-cleaved pMLB1034. Insertion of Tn*5* into the Amp^r gene of the pJDK24 plasmid generated the Kan^r pJDK26. pJDK26-D1, pJDK26-D2, pJDK26-D5, pJDK26-D12, and pJDK26-D125, were all derived from pJDK26, and contain the same amount of upstream *tps* DNA (-95 as their upstream end). The detailed methods to create these fusion plasmids are described below. pJDK68 and pJDK69 were constructed by fill-in ligation of the 180-bp (-311 to -131) NciI-PvuII *ops* fragment containing the *ops* UAS and the EcoR1-cut pJDK26-D125 fusion plasmid. They differ in the orientation of the *ops* UAS relative to *tps* gene polarity, with the UAS in pJDK68 being in the normal orientation. To construct ampicillin-resistant pSK14-D1 and pSK14-D12, an AccI site (-41) within the *tps* promoter was used to delete an EcoRI-AccI *tps* upstream DNA fragment from pJDK26-D1A and pJDK26-D12A which lack Tn*5* of pJDK26-D1 and pJDK26-D12, respectively.

Construction of *ops-lacZ* gene fusion plasmids

The wild-type *ops-lacZ* fusion plasmid, pJDK51, contains the 417-bp NciI-Sau3A *ops* DNA fragment (-311 to +106) inserted into the SmaI-BamHI-cleaved pMLB1034. pJDK55 was constructed by inserting Tn*5* into the Amp^r gene of pJDK51. pJDK55-D3, pJDK55-D6, and pJDK55-D36, were all derived from pJDK55, and contain the same amount of upstream *ops* DNA (-311 as their upstream end) including the 180-bp *ops* UAS (-311 to -131). The unique HindIII site within the *ops* promoter was used to construct insertional mutations (mutation DH) by cutting and filling in the

protruding ends created by HindIII digestion. Thus 4bp (AGCT) was inserted into the position between -8 and -9 within the *ops* promoter of pJDK55 creating the pJDK55-DH fusion plasmid. To construct ampicillin-resistant pSK14-D6 and pSK14-D36, the AccI site (-41) within the *ops* promoter of pJDK55-D6A and pJDK55-D36A which lack Tn*5* of pJDK55-D6 and pJDK55-D36 was used to delete the EcoRI-AccI *ops* upstream DNA fragment. The 180-bp *ops* upstream DNA (-311 to -131), which contains the *ops* UAS, was deleted using NciI-PvuII double digestion from pJDK55, pJDK55-D3, pJDK55-D6,and pJDK55-D36, generating pJDK55-DP, pJDK55-D3P, pJDK55-D6P, and pJDK55-D36P, respectively.

Oligonucleotide site-directed mutagenesis.

Base substitution mutations were introduced into the *tps* DNA by site-directed oligonucleotide mutagenesis. To obtain the *tps* DNA template, the HinfI (filled-in)-Sau3A fragment from the *tps* gene (the same fragment used to construct pJDK26) was inserted into M13mp19 (26) which had been cleaved with BamHI and SmaI contained in the polylinker region. A kit from Amersham, Inc., based on the procedure of Taylor et al. (34) was used for site directed mutagenesis and enrichment for M13 phage containing mutant DNA sequences. Alterations in the DNA sequence were confirmed by DNA sequence analysis (30). The oligonucleotides used were:

5′-GAGCGCGGTGCCCAAGCTATTTCCGGCGGCTTC-3′ (for D1 mutation),

5′-AGACGGATTGCTCCTCCGGAGCGCG-3′ (for D2 mutation)

and 5′-CGGACGGATACGGTACTGTAGACGGATTGC-3′ (for D5 mutation).

These were synthesized on an Applied Biosystems DNA synthesizer in the laboratory of Dr. Roe (University of Oklahoma). The changes introduced into the *tps* DNA sequence were the deletion of a T residue at position -8 (D1 mutation; see Fig. 1), the substitution of the sequence TCC for ATT at positions -30 to -28 (D2 mutation), and the simultaneous switch of the two A residues at -39 and -46 to two G residues (D5 mutation). D125 mutation was constructed by three sequential mutations (D1, D2 and D5). The correct changes in DNA sequences were confirmed by dideoxynucleotide DNA sequencing. The mutagenized DNA segments were retrieved by EcoRI-ClaI double digestion from the recombinant dsM13 phage DNA, and were ligated back to the EcoRI-ClaI-cut pJDK26.

The same protocols were used to mutagenize the *ops* DNA. The EcoRI-HindIII *ops* fragment from pJDK55 was inserted into M13mp18, and the site directed mutagenesis was carried out on the recombinant single-stranded M13mp18 using the mutagenic oligonucleotides;
5′-AGGCGGATTGCATTTCCGGAGCGCG-3′ (for D3 mutation),
and 5′-GCCGCCCGCGCGGTGCTGTAGGCGGATTGC-3′ (for D6 mutation). The changes in the *ops* DNA were the substitution of the sequence ATT for TCC at positions -29 to -27 (D3 mutation), the simultaneous conversion of two G residues to two A residues at positions -45 and -38. D36 mutation was obtained by incorporating D6 oligonucleotide on the D3-mutated ssDNA template. The mutant *ops* DNA segments were joined to the EcoR1-HindIII-cut pJDK55.

TABLE 1. Plasmids

Plasmids designation	Vector	Comments/Source
tps-lacZ fusion plasmids		
pJDK26	pMLB1034	Tn*5* insertion in Amp^r gene and a 201-bp HinfI-Sau3A fragment of *tps* DNA in pMLB1034 (Silhavy 1984)
pJDK26-D1	"	same as pJDK26 except a deletion of T at -8
-D2	"	same as pJDK26 except substitution of TCC for ATT from -28 to -30
-D5	"	same as pJDK26 except substitution of A residues at -39 and -46 for G
-D125	"	same as pJDK26 except containing D1, D2 and D5 mutations
pJDK68	"	180-bp *qps* UAS DNA inserted into *tps* DNA upstream of pJDK26-D125 in correct orientation
pJDK69	"	180-bp *qps* UAS DNA inserted into *tps* DNA upstream of pJDK26-D125 in opposite orientation
pSK14-D1	"	*tps-lacZ* fusion plasmid with D1-mutated *tps* DNA up to -41, absent of Tn*5* insertion
pSK14-D12	"	*tps-lacZ* fusion plasmid with D12-mutated *tps* DNA up to -41, absent of Tn*5* insertion
qps-lacZ fusion plasmids		
pJDK55	pMLB1034	Tn*5* insertion in Amp^r gene and a 417-bp NciI-Sau3A fragment of *qps* DNA in pMLB1034
pJDK55-D3	"	same as pJDK55 except substitution of ATT for TCC from -27 to -29
-D6	"	same as pJDK55 except substitution of G residues at -38 and -45 for A residues
-DH	"	same as pJDK55 except additiona insertion of AGCT between -7 and -8
-D3P	"	a 180-bp *qps* DNA NciI-PvuII fragment deleted from pJDK55-D3
-D6P	"	a 180-bp *qps* DNA NciI-PvuII fragment deleted from pJDK55-D6
pSK14-D6	"	deletion plasmid derived from pJDK55-D6 with *qps* DNA up to -41, absent of Tn*5* insertion
-D36	"	deletion plasmid derived from pJDK55-D36 with *qps* DNA up to -41, absent of Tn*5* insertion

Bacteriophage P1 transduction

The *ops* and *tps* gene fusion plasmids were transferred from *E. coli* to *M. xanthus* DZF1 by *E. coli* phage P1-mediated transduction as has been described (27). The recipient wild-type DZF1 cells were concentrated to approximately 4 X 10^9 cells per ml in CYE medium before phage infection. Transduction was carried out by mixing *M. xanthus* cells with varying amounts of P1 phage lysate at room temperature for 30 min. Melted CYE top agar (45°C) was mixed with the phage-cell mixture and poured onto the selective CYE plates. Transductants were selected for kanamycin resistance (50 ug/ml). The presence of gene fusion DNA in Kan^r transductants was confirmed by colony hybridization (7) by using pMLB1034 plasmid as a ^{32}P- labeled probe.

β-galactosidase assays.

M. xanthus cells were harvested from vegetative or developmental liquid (CF) cultures, and were sonicated to obtain cell extracts as has been described (8). For disruption of myxospores, developmental cells incubated on CF agar plate (diameter, 100 mm) were concentrated in 1 ml of Z buffer (24), to which zirconium beads (diameter, 0.15 mm) were added and agitated vigorously in a Mini-Beadbeater (Biospec Products) with 5 X 1-min bursts for a total of 5 min. The beads and cell debris were removed by centrifugation before assaying β-galactosidase activity. The assay has been described elsewhere (8, 24).

Myxophage MX4 transduction

MX4 Phage (50 ul) propagated on the mutant *bsgA* strain M380 (Tet^r), was added to 0.2 ml of recipient cells (*ops-lacZ* and *tps-lacZ* fusion strains, Kan^r) at a multiplicity of infection of 0.5. Recipient cells were prepared by centrifuging exponentially growing cells ($5X10^8$ to $10X10^8$ cells/ml) and resuspending them in a buffer which contained 0.01M-Tris buffer (pH7.5) and the salts mixture used in the 17P medium (6). The infected cells were incubated for 8 to 12h with aeration at 32°C. The phage-cell mixtures were plated in 2.5 ml CT top agar on CT bottom agar containing both tetracycline and kanamycin. Plates were incubated for 10 days at 32°C to collect transductants.

RESULTS

To investigate the regulatory roles of DNA sequences located close to the transcriptional start sites of the *tps* and *ops* genes, site-specific changes were made to make the DNA sequence of one gene more like the other. We expected that if those sequences were involved in the differential regulation, then changes in one regulatory region might result in a change in the temporal pattern of gene expression. More specifically, to investigate possible roles of differences within the homologous region (downstream from -50) of *tps* and *ops*, we used two parental gene fusion plasmids (pJDK26 and pJDK55) to perform mutational analysis. A *tps-lacZ* gene fusion plasmid, pJDK26, contained a 201-bp segment of *tps* DNA (-95 to +106) and an *ops-lacZ* fusion plasmid, pJDK55, contained a 417-bp of *ops* DNA (-311 to +106), which were fused to the *E. coli lacZ* structural gene. The pJDK55 also contained the *ops* UAS (-311 to -131) to facilitate the quantitation of *ops* gene expression (Fig. 2A CHAPTER I). Mutations were made using oligonucleotide-directed mutagenesis on the recombinant ss-M13 phage DNA and transferred to the gene fusion plasmids. The mutant gene fusions were introduced into the *M. xanthus* chromosome through coliphage P1-mediated transduction. The effect of the mutations was analyzed by assaying the β-galactosidase activity produced from the *M. xanthus* fusion strains.

The *ops* promoter is under control of repression mediated by an inverted repeat sequence.

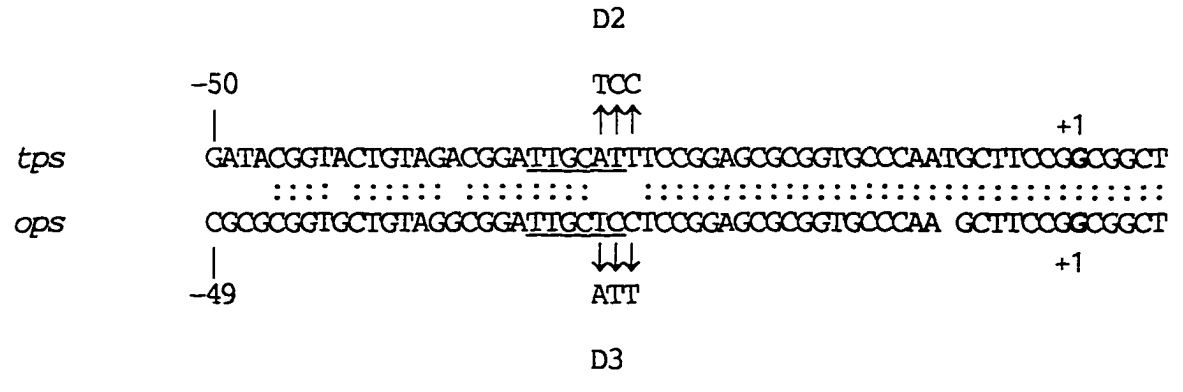
The three consecutive mismatched bases found in the *tps* and *ops* promoters appear to be important in differential regulation of the two genes since this region overlaps their putative -35 hexamer which would be recognized and bound by an RNA polymerase sigma factor (Fig. 2A). The *ops* promoter contains an inverted repeat sequence, GCTCCTCCGGAGC which is not present in the *tps* promoter due to the 3-bp difference. D2 mutation which converts the three *tps* DNA bases (ATT -30 to -28) to the corresponding *ops* DNA bases (TCC), thus creating the inverted repeat sequence, resulted in a severe reduction in *tps* expression (Fig. 2B). About 5-fold reduction was observed in 24-h developmental cells (105 U VS. 19 U). Disruption of the inverted repeat by D3 mutation (conversion of TCC in *ops* to ATT), however, caused a surprisingly high level of *ops* gene expression (Fig. 2C). More than a 10-fold increase was observed in both vegetative and developmental cells. Removal of the *ops* UAS from the D3 fusion caused a decrease not only in the level of late developmental *ops* expression but also in early developmental *ops* expression (D3 and D3P in Fig. 2C and 3A) indicating that the *ops* UAS could activate the D3 mutated *ops* promoter during early development. However, such activation was not present in wild-type *ops* gene fusions (pJDK55 and DP in Fig. 2C and 3A).

D2 and D3 gene fusions were assayed more in detail for early developmental induction. As shown in Fig 3B, D2 mutation reduced leaky vegetative *tps* expression, and also caused disruption in early developmental induction. By contrast, D3 mutation resulted in high,

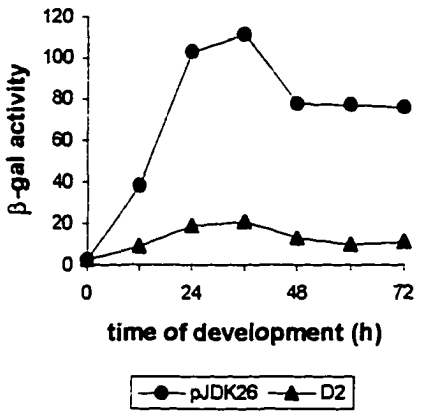
constitutive *ops* expression in vegetative cells, and a linear increase in early developmental *ops* expression. These results strongly suggest that the *ops* -35 region may be involved in repression of *ops* gene expression during vegetative and early developmental period.

Fig. 2 Mutations in the putative -35 region of the *ops* and *tps* genes.

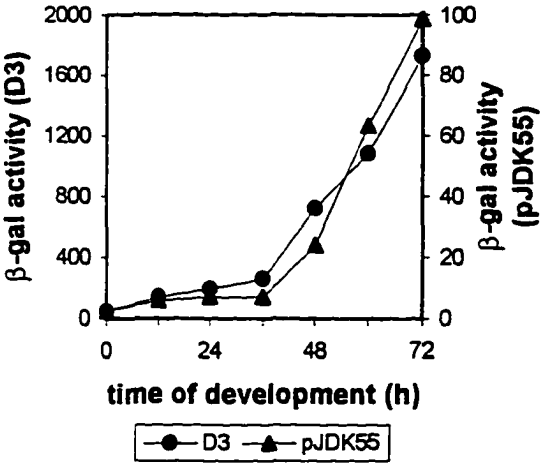
A.



B.



C.

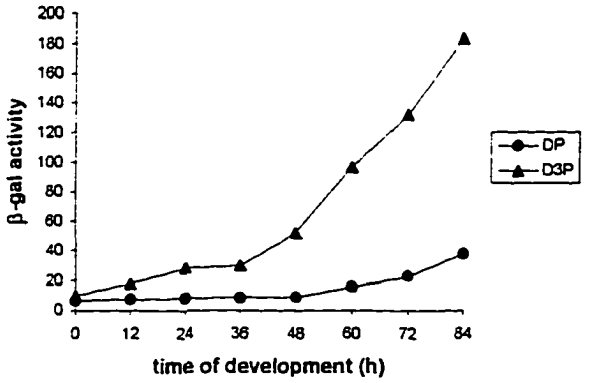


A. Sites and base changes of D2 and D3 mutations are shown. Identity between the two sequences is indicated by vertical dashed lines, and the converted bases are indicated above the aligned sequences. The putative -35 heamers are underlined. Numbers indicate base positions in relation with the transcription initiation site (+1).

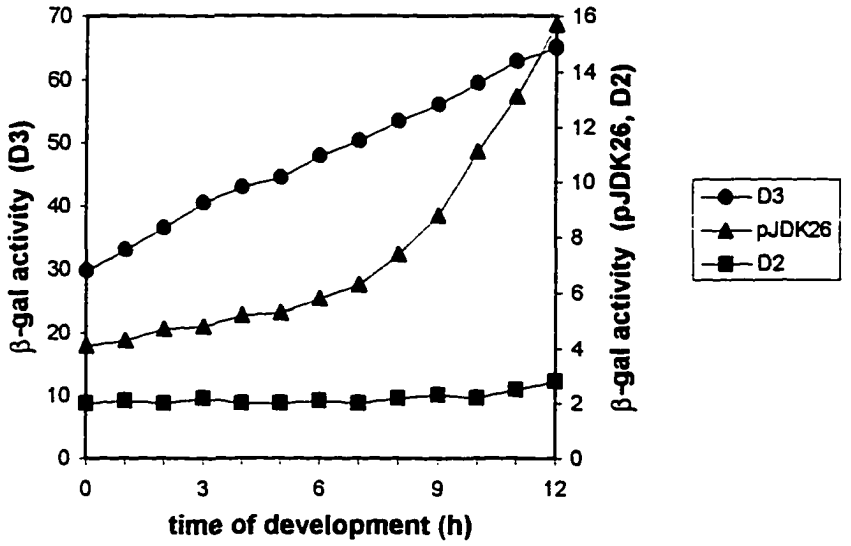
B. C. Effects of D2 and D3 mutations on the gene expression of *tps* and *ops*. *M. xanthus* strains harboring the gene fusion plasmids were harvested during the mid-log phase, concentrated (3000 Klett units), and plated on CF agar plates. During incubation, the sample cells were scraped out at the indicated times and assayed for β-galactosidase activity. pJDK26-D2, which was derived from the wild-type *tps-lacZ* fusion plasmid pJDK26, contains the D2 mutation. Similarly, pJDK55-D3 contains the D3 mutation in the wild-type *ops- lacZ* fusion plasmid, pJDK55.

Fig. 3

A.



B.



A. Effect of the *ops* UAS on the expression of the D3 gene fusion. pJDK55-DP and pJDK55-D3P were constructed by deletion of the *ops* UAS (-311 to -131) from pJDK55 and pJDK55-D3, respectively. Cells harboring the fusion plasmids were harvested from developmental agar plates at the indicated times and assayed for β-galactosidase activity (nmole/min/miligram protein).

B. Early developmental induction of the D2 and D3 gene fusions. Cells harboring the indicated plasmids were grown vegetatively and concentrated at a density of $2X10^8$/ml in CF liquid. The cultures were incubated at 28C and shaken for 12 hours. At every hour, aliquots were removed and assayed for β-galactosidase activity.

Distal part of the homologous region in *tps* is involved in early developmental induction.

There are two mismatched DNA sequences at the distal part of the upstream homologous region, positioned at -46 and -39 of *tps* (-45 and -38 of *ops*, Fig. 4A). To investigate the role of this site on the differential regulation of the two genes, mutations were made to convert two A residues to two G residues at -39 and -46 of *tps* (D5 mutation), and two G residues to two A residues at -38 and -45 of *ops* (D6 mutation). As seen in Fig. 4B, D5 mutation resulted in overall reduction in *tps* gene expression (D5 fusion or pJDK26-D5). However, its expression pattern was similar to that of wild-type. The result of D6 mutation of *ops* was of great surprise since it caused a novel *ops* induction early in development (Fig. 4C). About a 20-fold increase in *ops* expression was obtained with 24h-developmental cells, which is similar to the *tps* expression pattern. However, unlike the *tps* pattern, expression of D6 gene fusion continuously increased through late development.

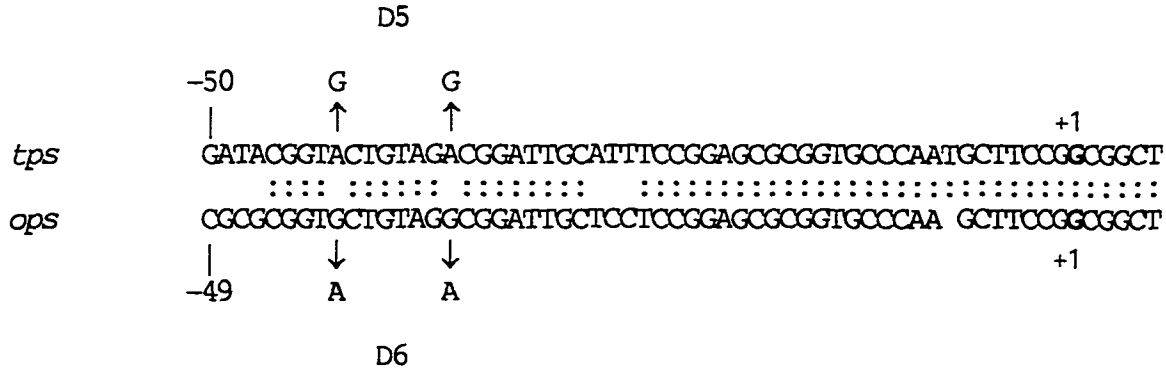
To examine temporal induction of D5 and D6 gene fusions in more detail, gene fusion activity was measured for the first 12h of development (Fig. 5B). A small increase in expression of the D5 gene fusion occurred at around 6h into development with a gradual increase in *tps* gene expression thereafter. However, the strong induction which was found with the pJDK26 fusion (wild-type) after 9h into development was not detected. In D6 gene fusion cells, a novel induction began at 9h and a substantial increase in gene expression followed (Fig. 5B). Thus D5 mutation of *tps* caused a loss of the subsequent induction which

would have normally appeared after 9h into development, whereas D6 mutation of *ops* resulted in an acquisition of such induction. This result strongly suggests that the *tps* upstream region identified by the two adenine residues at -46 and -39 is essential for the subsequent induction of *tps* expression which occurs after 9h into development.

To test if the *ops* UAS has any effect on the high level of early developmental expression displayed by the D6 gene fusion, D6P gene fusion was constructed by deleting the *ops* UAS (Fig. 5A). Removal of the *ops* UAS did not affect induction patterns of both wild-type *ops* and D6 gene fusions, however, the magnitude of reduction in the expression level was different such that removal of the UAS caused over 5 fold reduction in wild-type *ops* gene fusion (pJDK55 in Fig. 4C vs DP in Fig. 5A) whereas it caused less than 2-fold reduction in D6 gene fusion (D6 in Fig. 4C vs D6P in Fig. 5A). This result suggests that the novel early developmental induction of D6 gene fusion is due to the creation of the potential cis-acting activation site, and such activation is not dependent on the *ops* UAS.

Fig. 4 Mutations in the upstream end of the homologous region of the *ops* and *tps* genes.

A.



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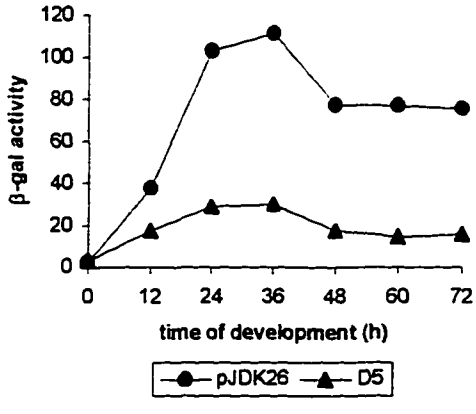
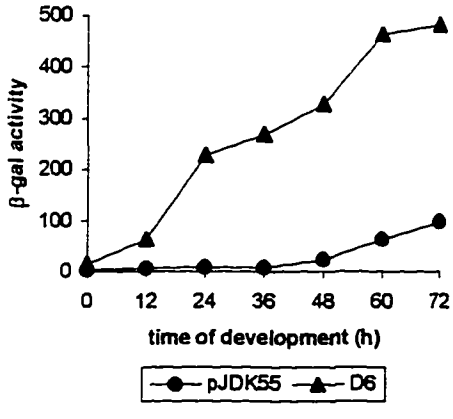


C.

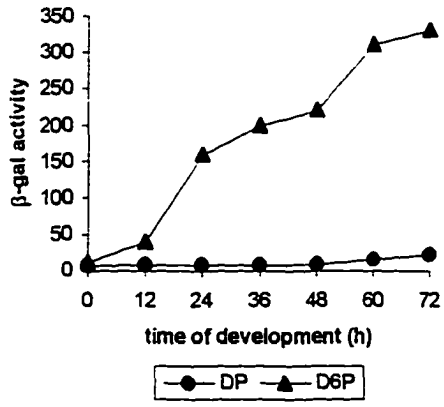


A. Sites and base changes of D5 and D6 mutations are shown. Identity between the two sequences is indicated by vertical dashed lines, and the converted bases are indicated above the aligned sequences. Numbers indicate base positions in relation with the transcription initiation site (+1).

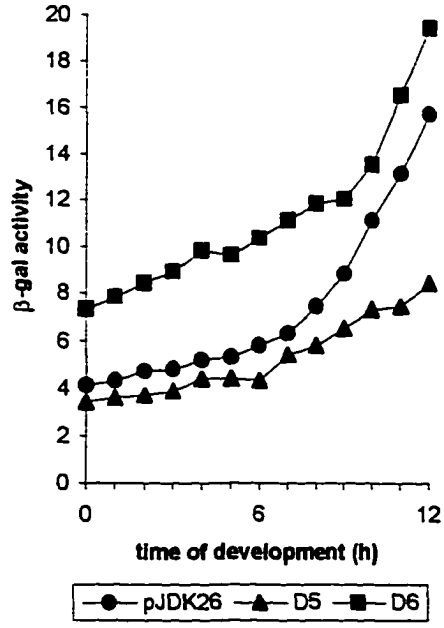
B. C. Effects of D5 and D6 mutations on the gene expression of *tps* and *ops*. pJDK26-D5, which was derived from the wild-type *tps-lacZ* fusion plasmid pJDK26, contains D5 mutation. pJDK55-D6 contains D6 mutation in the wild-type *ops-lacZ* fusion plasmid, pJDK55. *M. xanthus* strains harboring the gene fusion plasmids were harvested during the mid-log phase, concentrated (3000 Klett units), and plated on CF agar plates. During incubation, the sample cells were scraped out at the indicated times and assayed for β-galactosidase activity.

Fig. 5

A.



B.



A. Effect of the *ops* UAS on the expression of the D6 gene fusion. pJDK55-DP and pJDK55-D6P were constructed by deletion of the *ops* UAS (-311 to -131) from pJDK55 and pJDK55-D6, respectively. Cells harboring the fusion plasmids were harvested from developmental agar plates at the indicated times and assayed for β-galactosidase activity (nmole/min/miligram protein).

B. Early developmental induction of the D5 and D6 gene fusions. Induction of the wild-type *tps-lacZ* plasmid, pJDK26, is depicted for comparison. Cells harboring the indicated plasmids were grown vegetatively and concentrated at a density of $2X10^8$/ml in CF liquid. The cultures were incubated at 28C and shaken for 12 hours. At every hour, aliquots were removed and assayed for β- galactosidase activity.

Changes in DNA sequence of the TATA box do not affect the *tps* and *ops* regulatory patterns.

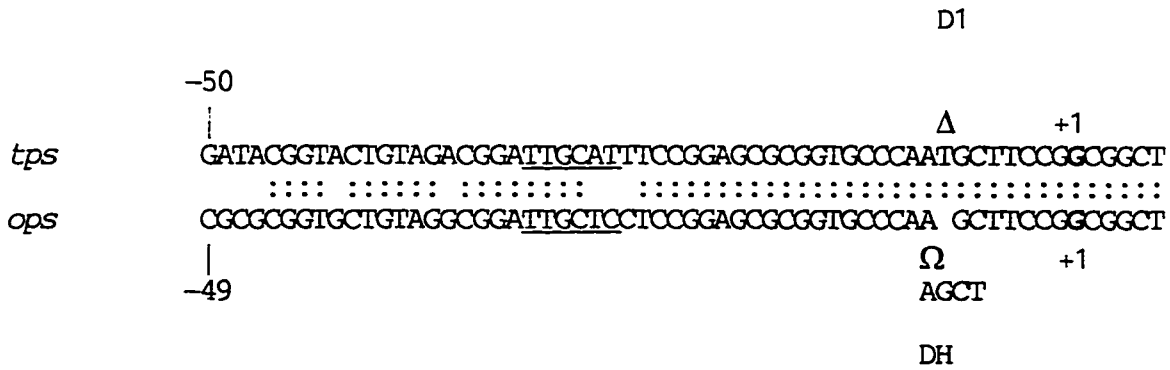
There is another difference between *ops* and *tps* DNA sequence at position -8 relative to the transcriptional initiation site, in which an additional T residue is found in the *tps* promoter (Fig. 6A). This site resides within the "TATA box" in a conventional bacterial promoter where an RNA polymerase catalyzes denaturation of duplex DNA and initiates transcription. The consensus *E. coli* "TATA box" is composed of TATAAT hexamer whereas those of *tps* and *ops* were assigned as AATGCT and AAGCTT, respectively. As shown in Fig. 6B, deletion of the T residue at -8 (D1 mutation) of the *tps* promoter caused somewhat higher levels of both vegetative and developmental *tps* gene expression. However, the pattern of developmental gene expression remained similar to that of the wild-type *tps* gene fusion, pJDK26, suggesting that the D1 mutation does not affect the regulatory machinery of *tps* gene expression but promotes the efficiency of transcription.

The putative *ops* "TATA box", AAGCTT, which lacks a T residue at position -8, when compared with that of *tps*, can be recognized by HindIII restriction enzyme (Fig. 6A). The significance of this site was investigated by inserting 4bp (AGCT) between -8 and -9 position through fill-in reaction at the HindIII restriction site (DH mutation). The changed DNA sequence, AAGCTAGCTT, has lost the sequence of AAGCTT and disrupts the order of subsequent bases. The result of DH mutation is depicted in Fig. 6B. Surprisingly, DH mutation caused a rather higher level of developmental *ops* expression. However, the pattern of gene expression exhibited by this mutated *ops* construct was similar to

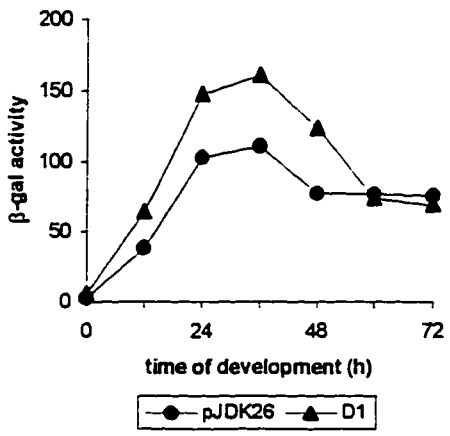
that of wild-type, suggesting that the 4bp insertion enhances the efficiency of transcription without affecting temporal regulation. We do not know why the 4bp insertion causes more *ops* gene expression. One possibility is that since insertion of the four bases (AGCT) considerably enhances the relative AT content at the transcription initiation region, it may better facilitate denaturation of the local duplex DNA and formation of open complex.

Fig. 6 Deletional and insertional mutaions in the TATA region of the *ops* and *tps* genes.

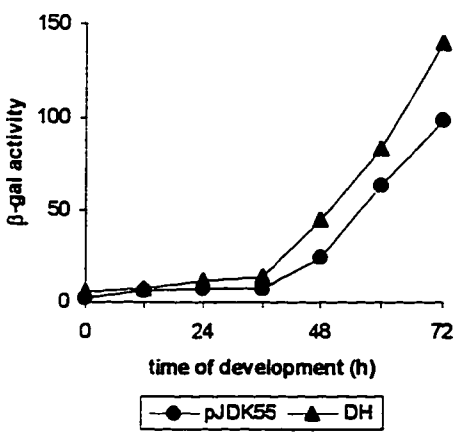
A.



B.



C.



A. Sites and base changes of D1 and DH mutations are shown. Identity between the two sequences is indicated by vertical dashed lines, and the converted bases are indicated above the aligned sequences. Delta (Δ) and omega (Ω) represent deletion and insertion respectively at that site. The putative −35 hexamers are underlined. Numbers indicate base positions in relation with the transcription initiation site (+1).

B. C. Effects of D1 and DH mutations on the gene expression of *tps* and *ops*. pJDK26-D1 derived from the wild-type *tps-lacZ* fusion plasmid pJDK26 with deletion of a T residue at position −8. pJDK55-DH contains additional 4-bp (AGCT) between −9 and −8 (DH mutation) in the wild-type *ops-lacZ* fusion plasmid, pJDK55.

An *ops* DNA segment between -131 to -50 is likely to be required for the *ops*-specific developmental induction.

To test whether the intact *ops* promoter region is sufficient for showing the *ops* specific gene expression, we constructed D125 gene fusion (pJDK26-D125) which simultaneously contains D1, D2 and D5 mutations in the wild-type *tps* gene fusion (Fig. 7A). Thus D125 mutation has converted the *tps* sequence from -50 to +27 to the corresponding *ops* sequence. As shown in Fig. 7B, D125 mutation completely disrupted developmental gene expression without showing any signs of the *ops*-like late developmental induction. Little expression was detected with D125 fusion strain grown in either CF liquid culture or glycerol developmental culture, the conditions which selectively allow *tps* or *ops* gene expression, respectively (data not shown). In addition, insertion of the *ops* UAS (-311 to -131) distal to the upstream end (-95) of D125 gene fusion (pJDK68 and pJDK69 in Fig. 7C) did not result in late developmental gene induction (*ops* pattern). These results suggest that the *ops*-specific gene expression requires the region flanked by the *ops* UAS and the *ops* promoter (e.g. -131 to -50). This region of *ops* is likely to contain a cis-acting site for activation or derepression of the downstream *ops* promoter.

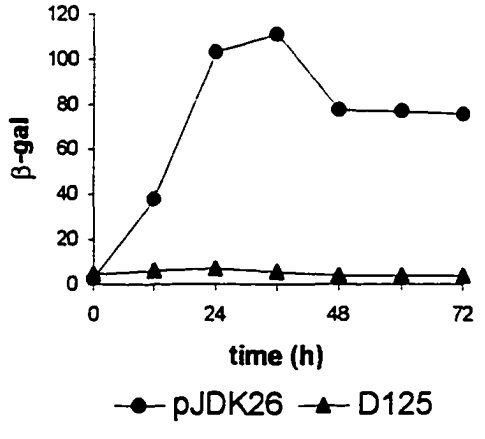
Fig. 7 Effect of *ops* DNA fragments on late developmental gene expression.

A.

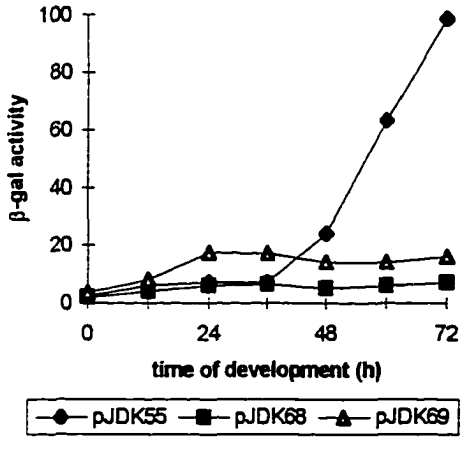
D125

```
          -50      G      G       TCC
           |       ↑      ↑       ↑↑↑                    Δ      +1
tps        GATACGGTACTGTAGACGGATTGCATTTCCGGAGCGCGGTGCCCAATGCTTCCGGCGGCT
               :::: :::::: ::::::::  ::::::::::::::::::::::::::::::::
ops        CGCGCGGTGCTGTAGGCGGATTGCTCCTCCGGAGCGCGGTGCCCAA GCTTCCGGCGGCT
           |                                                        +1
          -49
```

B.



C.



A. D125 mutaion in the *tps* promoter is a combined mutation of D1, D2, and D5. The converted bases are indicated above the aligned sequences. Delta (Δ) represent a deletion at that site. Numbers indicate base positions in relation with the transcription initiation site (+1).

B. C. The pJDK26-D125 fusion plasmid contains D125 mutation in the *tps* promoter region. pJDK68 and pJDK69 contain the 180bp *ops* UAS (-311 to -131) at the distal end (-95) of *tps* DNA of pJDK26-D125. pJDK68 and pJDK69 differ in the orientation of *ops* UAS relative to the *tps* gene polarity, with the UAS in pJDK68 being in the right orientation. PJDK26 and pJDK55 are the wild-type *tps* and *ops* gene fusion plasmids and are depicted for comparison.
M. xanthus strains harboring the gene fusion plasmids were harvested during the mid-log phase, concentrated (3000 Klett units), and plated on CF agar plates. During incubation, the sample cells were scraped out at the indicated times and assayed for β-galactosidase activity.

Effect of the difference in the leader sequences of the *ops* and *tps* genes.

Because of their location within the untranslated leader region, the three mismatched DNA sequences (at positions +28, +37 and +45, Fig. 8A) of *ops* and *tps* are likely to influence gene expression through translational efficiency. We tested this possibility in *Escherichia coli* using *tps* and *ops* fusion plasmids which were deleted to contain the same upstream end but different promoter and leader sequence. Using AccI restriction site (positioned at -41) in pJDK26-D1, -D12, pJDK55-D6, and -D36, we constructed four gene fusion deletion plasmids, pSK14-D1, -D12, -D6, and -D36. Thus pSK14-D12 and pSK14-D6 contained the same amount of *M. xanthus* DNA and identical promoter DNA sequences but they differ in the three mismatched sequences which were originated from the *tps* and *ops* leaders, respectively. When assayed for gene fusion activity, pSK14-D6 exhibited 5-fold higher expression than pSK14-D12 (Table 2). Similarly, pSK14-D36 containing the *ops* leader sequence showed more than 2-fold higher expression than its *tps* counterpart, pSK14-D1, indicating that the *ops* leader sequence confers more efficient translation. Attempts to investigate the effect of the leader sequences in *M. xanthus* were not satisfactory, since the gene fusions integrated into *M. xanthus* chromosome were not expressed consistently within each transductant. This may be due to position effects resulting from integration of gene fusions at different sites on the *M. xanthus* chromosome.

We found within the *tps* and *ops* leaders a region which could function to form a major hairpin structure which extends from position +1 to +32 (Fig. 8BC). The predicted *tps* secondary structure was more stable than that predicted for the *ops* leader region. Presumably, this more complex secondary structure of the *tps* leader may negatively affect its translational efficiency.

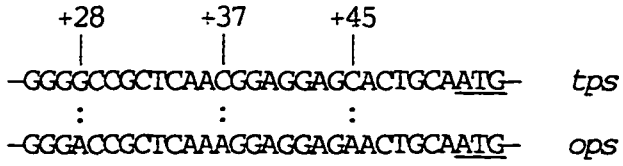
Table 2. Expression of the deleted fusion plasmids in *E. coli*.

fusion plasmid	specific activity (nmol/min/mg protein)
pSK14-D6	49
pSK14-D12	11
pSK14-D36	28
pSK14-D1	12

E. coli MC1000 containing the gene fusion plasmid was grown exponentially (100 Klett units) in LB medium containing ampicillin (50 ug/ml), and the harvested cells were washed and resuspended in Z buffer for disintegration. Assay for β-galactosidase activity is found elsewhere. All plasmids contain *M. xanthus* DNA downstream from -41. pSK14- D6 and pSK14-D36 were derived from the *ops* fusion plasmids, pJDK55- D6A and pJDK55-D36A, respectively. Similarly, pSK14-D1 and pSK14-D12 were derived from the *tps* fusion plasmids, pJDK26-D1A and pJDK26-D12A.

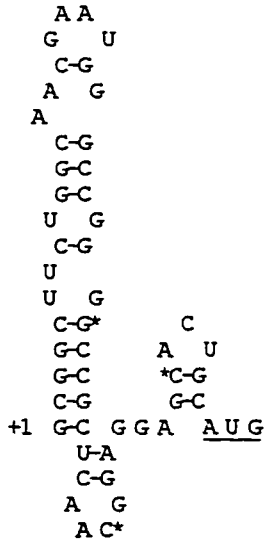
Fig. 8 Three mismatched DNA bases in the leader region of the *tps* and *ops* gene, and potential hairpin structures in the *tps* and *ops* leaders.

A.



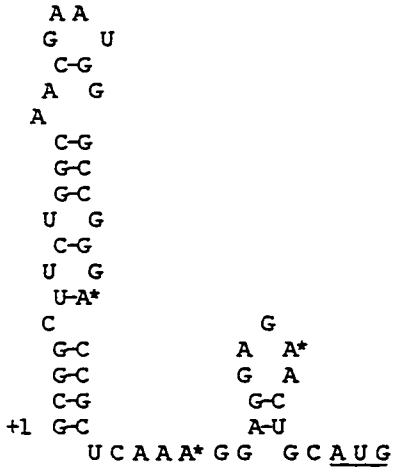
B.

tps leader



C.

ops leader



A. The three mismatched DNA sequences are indicated by vertical dashed lines, and the ATG codons are underlined. Numbers indicate base pairs downstream from the transcriptional start.

B. C. Potential hairpin structures in the leader of *tps* and *ops* mRNA are shown. Intra-strand base pairing is indicated with connecting lines and the AUG start codons are underlined. The first base of mRNA is marked with +1 and the three mismatched bases are indicated with asterisks (*).

B-signal pathway dependence of *tps* and *ops* expression

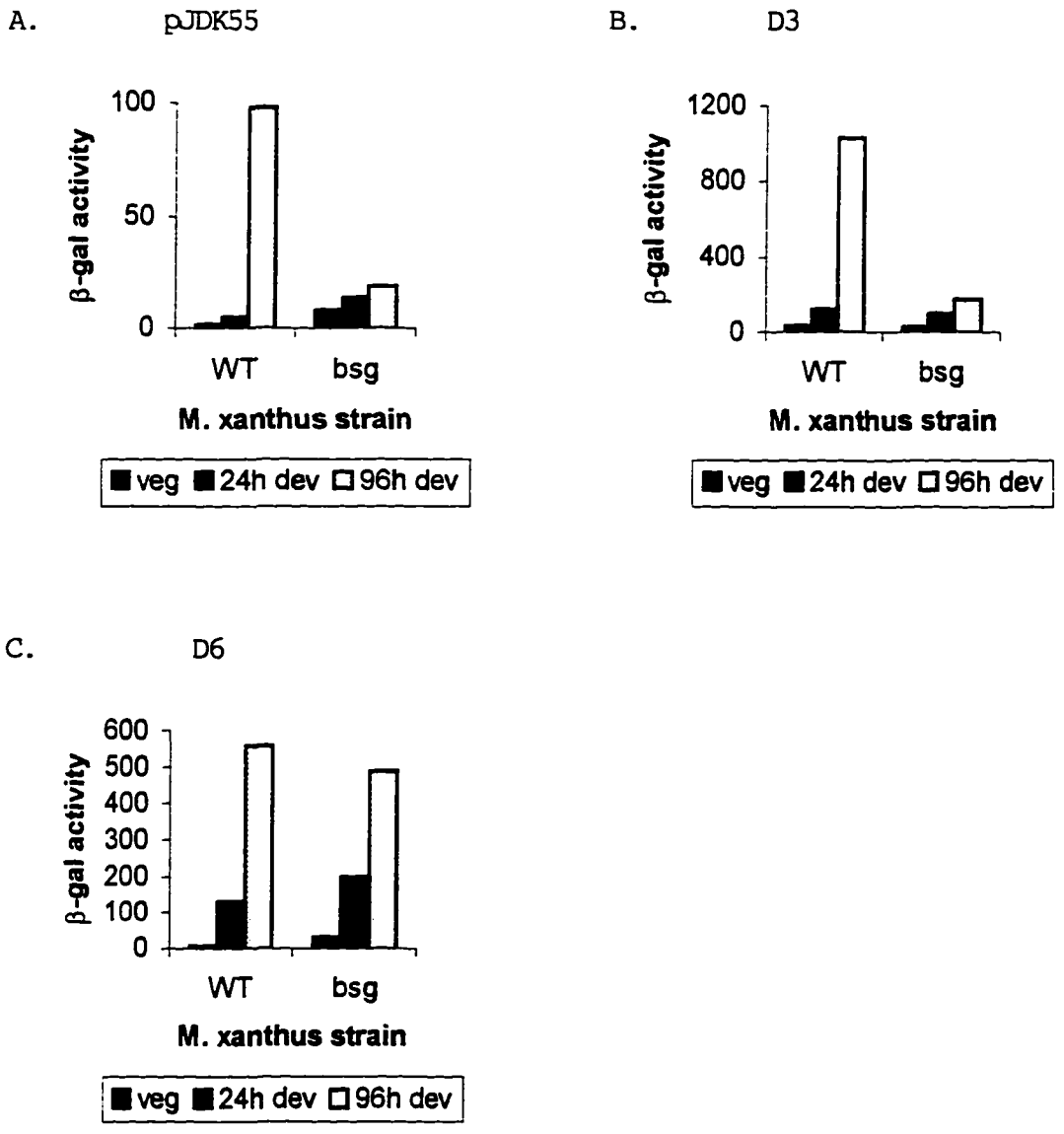
In *M. xanthus*, five groups of loci (*asg*, *bsg*, *csg*, *dsg*, and *esg*) are known to be involved in extracellular signaling (12, 14). Defect in any of these loci disables normal *M. xanthus* development and has a significant effect on the developomental-specific gene expression. The *bsg* mutant is defective in aggregation and sporulation (13) and exhibits reduction or disruption in gene expression of many developmental specific genes including *tps* and *ops*. To obtain a better understanding of *tps* and *ops* gene regulation, the mutant gene fusions of *tps* and *ops* were assayed in the *bsg* mutant for their fusion gene expression.

When pJDK55, which contains the wild-type *ops* DNA, was introduced into *bsg* strain, late (96h) developmental induction was severely impaired (Fig. 9A). The magnitude of induction upon 96h development decreased from over 40-fold to only 4-fold. D3 gene fusions also exhibited a dramatic reduction during 96h of development when placed in *bsg* strain, but similar levels of vegetative and 24h developmental gene expression were maintained (Fig. 9B). This reduction may be linked to disability of the *bsg* strain to produce a spore-specific sigma factor SigB which is expressed at the onset of sporulation through late development (1, 2). Deletion of sigB was shown to produce spores lacking the *ops* gene product, protein S1.

Surprisingly, when D6 gene fusion was introduced into *bsg* strain, no significant reduction was found (Fig. 9C). Assay of fifteen independent transductants harboring the D6 gene fusion consistently showed similar levels of gene fusion activity as in wild-type transductants. Thus, expression of D6 gene fusion appeared to be

independent of B-signal, suggesting that the potential *tps* activator functioning on the D6 promoter is not affected by the B-signaling system. In all cases, the *tps-lacZ* gene fusions harboring the wild-type or mutant promoter showed a much reduced activity in *bsg* background (data not shown) presumably because the primary transcriptional activator which binds to *tps* upstream region (-110 to -82) is under control of the B-signaling system.

Fig. 9 Expression of the *ops-lacZ* gene fusions in B signaling-defect *M. xanthus* strain, bsgA.



Wild type FB or bsgA mutant cells harboring the indicated *ops-lacZ* fusion plasmids were grown in CYE culture, concentrated, and plated on the CF agar at a density of $7X10^9$ cells/plate. Vegetative and 24h- and 96h- developmental cells were harvested and assayed for β-galactosidase activity. Depicted is the averaged result of β-galactosidase activities obtained from at least 8 independent transductants.

DISCUSSION

In this study, our attention has focused on the regulatory roles of the highly homologous region of *tps* and *ops* which extends 100-bp from ATG codon (or 50-bp from transcription initiation). Within this region, only 9 bases are different, and they were divided into four groups depending on their locations. These are: the distal end, the "-35 hexamer", the "TATA box", and the leader region. The significance of these differences on the differential gene regulation was investigated by site-directed mutagenesis. Conclusions drawn from this research are: (a) The *ops* -35 region is under control of repression during pre-sporulation period. (b) The *ops* DNA segment from -131 to -50 is required for the late developmental *ops* gene expression. (c) The *tps* distal end of the homologous region (positions -46 and -39) is implicated in early developmental induction which begins after 9h into development. (d) The *tps* rather than *ops* leader sequence confers weaker expression possibly due to the more complex secondary structure formed on the untranslated leader.

Although both "-35 hexamers" of *tps* (TTGCAT) and *ops* (TTGCTC) have 50% homology to the consensus sequence (TTGACA) of *E. coli* $E\sigma^{70}$, the *ops* -35 region contains an inverted repeat sequence GCTCCTCCGGAGC in which the TCC residues within the left box are replaced with ATT in *tps*. This region of *ops* is implicated in transcriptional repression, since conversion of *tps* sequence ATT to *ops* TCC sequence (D2 mutation), thus creating the inverted repeat sequence, impaired developmental induction and reduced the level of *tps* expression dramatically. Also, disruption of the inverted repeat sequence in the *ops* promoter (D3 mutation) caused

high levels of gene expression both during vegetative and developmental period and allowed the *ops* UAS to activate transcription. In addition, when expressed in *E. coli*, all of the gene fusion plasmids containing wild-type *ops* promoter sequence exhibited much higher expression than those with *tps* promoter sequence, suggesting that repression of the *ops* promoter is specific for *M. xanthus*. There are many instances where promoter DNA sequences containing inverted repeats or palindomes are involved in repressor binding (3, 28). It is thus possible that the inverted repeat of the *ops* promoter is recognized by a repressor protein which binds during vegetative and early developmetal stages. However, attempts to identify such repressor by a gel mobilty shift assay resulted in an inconclusive result suggesting that a more purified and defined assay system is required to detect the potential repressor.

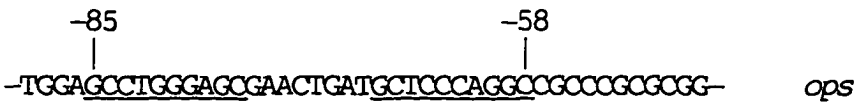
Two A residues positioned at -45 and -39 in the *tps* promoter were shown to be very important in early developmental gene expression. Changing the sequence to two G residues (D5 mutation) resulted in a marked reduction of *tps* expression due to the absence of subsequent induction after 9h into development. Whereas, replacing the two G residues of the *ops* promoter with two A residues (D6 mutation) caused a novel induction initiating after 9h into development. It is very tempting to speculate that this region of *tps* is involved in binding of a transcriptional activator which appears about 9h into development. This idea was partly supported by an earlier observation that a *tps* gene fusion with a deleted upstream sequence to -66 also showed a delayed induction, due to the absence of the primary cis-acting site (-110 to -81) which is required for initial induction of *tps* after 5h into

development. Brown showed in his gel mobility shift assay that binding activity on the primary cis-acting site (-110 to -81) was eliminated in the B-signaling defective mutant (5). Although it is premature to discuss, the putative *tps* activator (appearing at 9h) seems to be free from the B-signaling effect since the D6 gene fusion activity was similarly induced in both wild-type and *bsg* mutant strains. Also, it is likely that the cis-acting site defined by D6 mutation is also a site for interaction of the UAS-complex and RNA polymerase, because, while removal of the UAS from the wild-type *ops* or D3 gene fusion caused 5 to 8 fold reduction in gene expression, no significant change was found in the D6 gene fusion.

Conversion of the *tps* DNA sequence within the region of homology to the *ops* DNA sequence did not cause *tps* to induce late developmental expression of *ops*. Examples are the *tps* gene fusions of pJDK26-D125, pJDK68, and pJDK69, all of which contain the *ops* promoter sequence from -50 to +27 and the *tps* upstream sequence from -51 to -95, but they differ in further upstream sequence with no *ops* UAS (pJDK26-D125), the *ops* UAS (pJDK68), and the *ops* UAS in opposite direction (pJDK69). In all cases, the *ops*-like late developmental induction was not detected, suggesting that additional factor(s) other than the *ops* UAS and the *ops* promoter is (are) required for the *ops*-specific late developmental induction. It is possible that activation by the *ops* UAS requires a minimum distance to function on the downstream promoter. However, previous results indicated that the *ops* UAS could function properly just upstream from -66 of the *tps* gene (20, 21). Also, it is unlikely that the *tps* region between -51 and -95 plays an inhibitory role to the late

developmental induction since addition of the *ops* UAS to the upstream -95 of the *tps* gene strongly enhanced developmental *tps* expression (20). It is more plausible that the *ops* DNA segment flanking -131 to - 51 is required for late developmental induction which is absent in the *tps* hybrid gene fusions, pJDK26-D125, pJDK68, and pJDK69. This upstream *ops* sequence may be involved in derepression of the repressed *ops* promoter. At the present time we do not know the potential regulatory site within this region although there is one interesting site situated between -58 and -85 with a set of 100% inverted repeat sequence (10bp each) spaced by 8bp (Fig. 10). Mutational analysis of this site will be needed to test this possibility.

Fig. 10 DNA sequences of a perfect inverted repeat located upstream region of the putative *ops* promoter. The DNA sequences of the inverted repeat are underlined, and the numbers represent base pairs upstream from the *ops* transcriptional start site.



The leader sequences of *ops* and *tps* may play a significant role in achieving optimal levels of gene expression, since there is a substantial difference in gene expression caused solely by the difference in the leader sequences of *tps* and *ops*. At least three fold less expression was attributable to the *tps* leader sequence. We found in both the *tps* and *ops* leaders a potential hairpin structure, but the *tps* leader was predicted to form a more complex secondary stucture. There are instances where hairpin structures within leader sequence

affect gene expression, either by a stall of transcription (22) or by an inhibition of translational initiation (38).

From the previous results and this study, primary induction of *tps* gene expression occurs about 5h into development requiring a cis-acting region (-110 to -82) which was shown to be bound by (a) trans-acting factor(s). Secondary induction, although it is not as evident, which may begin around 9h into development is mediated by a site defined by D5 mutation (e.g. -46 and -39). However, maximal *tps* expression requires a region identified as *tps* UAS (-375 to -173).

Regulatory system of *ops* also requires a UAS (-311 to - 131) for optimal expression during late development. The *ops* promoter appears to be under control of repression during vegetative and early development although the *ops* UAS could function otherwise during this period. When spores begin to form, derepression or activation of the *ops* promoter is likely mediated by (a) cis-acting site(s) located within -131 and -50, which could allow an RNA polymerase containing spore-specific sigma factor SigB to initiate transcription and the *ops* UAS to activate transcription.

We still have many unanswered questions about the detailed molecular mechanisms of how *tps* and *ops* expression is regulated. Identification and isolation of important *trans*-acting factors, including RNA polymerase and other transcriptional activators will be required for in vitro biochemical studies to address these questions.

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CHAPTER III

REGULATION OF POLYSACCHARIDE PRODUCTION IN *Myxococcus xanthus*

INTRODUCTION

Myxococcus xanthus is a gram-negative soil bacterium which undergoes a complex life cycle. When starved, *M. xanthus* cells exhibit social behavior involving cell aggregation, fruiting body formation, and sporulation. Other types of social behavior include cooperative growth, gliding motility, and periodic rippling (22). During these social behaviors, *M. xanthus* exhibits two modes of cell-cell communication: the exchange of extracellular signals and the contact-mediated interactions. Five extracellular signals (A-, B-, C-, D-, and E-signal) have been recognized which are required for normal development of *M. xanthus*. Contact-mediated cell-cell interactions are mediated by two cell-surface appendages, pili and fibrils. While pili are polarly located and composed exclusively of protein, the fibrils of *M. xanthus* coat the entire external cell surface (Ramaswamy, unpublished observation) and are composed of carbohydrate and protein in a 1.2:1.0 ratio (4). Several lines of evidence indicated that the pili are associated with social motility (or S-motility). S-motility is one of two types of motility found in *M. xanthus*, which governs movement of

groups of cells. The other type of motility, A-motility (A for Adventurous), refers to a type of movement displayed by individual cells (31, 41, 51). Arnold and Shimkets (1, 2) demonstrated that the fibrils play a role in cell-cell cohesion and social behavior in *M. xanthus*. They showed that *dsp* mutants which lack fibrils were noncohesive, defective in S-motility, and unable to form fruiting bodies. However, more recently it was shown that a secondary *dsp* mutant, *fbd*, which still lacks fibrils, regained social motility and developmental aggregation (11). In addition, our lab recently isolated a group of mutants called Cds mutants which are fibril-deficient but retain a substantial capacity for S-motility. These mutants were identified based on defects in the ability to bind calcofluor white (a flourescent dye which binds exopolysaccharide) while maintaining the capacity for S-motility. Thus there exists an apparent contradiction: the loss of fibrils due to the *dsp* mutation is clearly correlated with the loss of cohesion, S-motility, and development, yet the *fbd* mutant which lacks fibrils, regains S-motility and ability to aggregate. In contrast to the *dsp* allele, the *stk-1907* allele which contains *Tn5* insertion *ΩDK1907* causes S-motility mutant cells to exhibit increased production of fibrils, cell cohesion, and social motility (17). The wild-type cells containing the *stk-1907* allele also showed increased levels of these properties. However, when the *stk-1907* allele was introduced, the dsp mutant was unable to show the Stk phenotype indicating that the *stk-1907* allele is hypostatic to the *dsp* allele. It has been suggested that the gene product of *stk* acts as a negative regulator of fibril synthesis.

Many bacteria are known to produce several different species of exopolysaccharide or chemically modified polysaccharide (46). *Pseudomonas* sp. NCMB 2021 produces two different exopolysaccharides at different stages of the growth cycle (15). Also, in *Pseudomonas* sp.S9, induced starvation of exponentially growing cells leads to the production of polysaccharide which is not found in the growing cells (50). Physiological and physical factors can influence the production of polysaccharide in many bacteria. For example, in strains of *E. coli* and other enterobacteria, polysaccharide production increases under conditions where growth is limited by the nitrogen, phosphorus, sulphur or potassium content of the medium (48). Nitrogen limitation in the presence of excess carbohydrate leads to high yields of polysaccharide in *Rhizobium meliloti* (20). Various ions are known to be required either for substrate uptake or as cofactors in polysaccharide synthesis. Polysaccharide production in washed suspensions of *Enterobacter aerogenes* was stimulated by Mg^{2+}, K^{+} and Ca^{2+}. In a myxobacterium, *Stigmatella aurantiaca*, Ca^{2+} promotes the synthesis of extracellular fibrils, and causes changes in the profile of the cell-surface proteins (13). Production of polysaccharides in certain microorganisms is also affected by aeration, pH, temperature, or solid surface (44).

It has been known that myxobacteria produce a large amount of extracellular matrix (or slime) which is mainly composed of polysaccharide. In many genera, the major monosaccharide components of the hydrolyzed exopolysaccharide were found to be glucose, galactose, mannose, and hexose amines (45). Chemically similar polysaccharides

were also found in the fruiting body surrounding the myxospores (45, 46).

It has been suggested from the chemical and morphological analysis of the fibrils that the extracellular matrix (or slime) of *M. xanthus* is arranged as fibrils (5). However, little is known about the regulation of polysaccharide synthesis by *M. xanthus* during development or in response to different environmental conditions.

Similarly, little is known about the regulation of gluconeogenic activity which is important in provision of hexose precursors for polysaccharide biosynthesis. Induction of polysaccharide synthesis in *M. xanthus* is likely to involve the activation of gluconeogenic enzymes since *M. xanthus* is a poor utilizer of exogenous carbohydrate substrates and uses amino acids as the main source of carbon and energy (7). Watson and Dworkin proposed that the principal function of Embden-Meyerhoff enzymes in *M. xanthus* is gluconeogenic because of the absence of hexokinase and pyruvate kinase (47). Therefore, a key gluconeogenic enzyme, phosphoenolpyruvate carboxykinase (EC 4.1.1.49) (Pck) which catalyzes decarboxylation and phosphorylation of oxaloacetate to phosphoenolpyruvate is likely to play an important role in carbon flow necessary for polysaccharide synthesis. Although the Pck activity in *M. xanthus* was first observed in 1968 (47), very little information is available concerning the regulation of this key enzyme in *M. xanthus*.

In this study, we have investigated the regulation of polysaccharide production in *M. xanthus*. Conditions such as growth phase, medium composition, and availability of divalent cations were tested for the effect on polysaccharide production, and the Pck activity

was measured to obtain an insight of how the regulation of polysaccharide production and the Pck activity are related. The genetic control of polysaccharide production and Pck activity was also investigated by analysis of mutants previously shown to have defects related to fibril production. In addition, the *stk-1907* allele was used to create secondary mutants of fibril-deficient Cds strains and changes in their phenotypes were investigated. Finally, a 3.6 kb KpnI-EcoRI DNA fragment which complements the *stk-1907* allele was sequenced. We found three ORFs within the fragment and propose that one of the ORFs, ORF89, is a strong candidate for the *stk* gene.

MATERIALS AND METHODS

M. xanthus strains and growth conditions

M. xanthus strains DK1622 (32) was used as a wild-type strain and other *M. xanthus* strains used in this study are listed in Table 1. *M. xanthus* cultures were grown vegetatively in casitone-yeast extract (CYE) medium (10). When required, kanamycin and oxytetracyclin were added at a concentration of 50ug/ml, 25ug/ml, respectively. Log phase cells were routinely grown to a cell density of 70 to 80 Klett units (red filter) in liquid shaker culture (250 rpm) at 30°C.

To prepare concentrated cell suspensions for stationary phase polysaccharide induction studies, cells were harvested in log phase, pelleted by centrifugation (8000g for 10 min), and suspended in fresh CYE to a density of 500 Klett units. The concentrated cell suspensions (1 ml) in sterilized pyrex tubes were further incubated in a water bath set at 30°C with shaking (200 rpm).

The chemically defined A1 medium for growth of *M. xanthus* was prepared as described by Bretscher and Kaiser (7). Briefly, A1 medium contains small amounts (10-100 ug/ml) of six amino acids (Val, Ile, Leu, Met, Phe, and Asn), spermidine (125 ug/ml), vitamin B_{12} (1 ug/ml), and various salts and buffers. Pyruvate (0.5%) and aspartate (0.5%) serve as major carbon sources.

Fruiting body formation was analyzed in submerged culture by a modification of the method described by Kuner and Kaiser (32). Wild-type (DK1622) cells growing exponentially in CYE were collected by centrifugation and washed with 10 mM MOPS (morpholinepropanesulfonic

acid, pH 7.2) buffer. The cells were resuspended at cell densities of 25, 50 or 100 Klett in the same buffer supplemented with $CaCl_2$, $MgCl_2$, $SrCl_2$ at 4mM concentration. These cells were placed in 24-well tissue culture plates. Cell aggregates and fruiting bodies were photographed using an inverted microscope after incubation for 96h at 30°C.

Myxophage MX4 transduction

Myxophage MX4 (10) transductions were performed by the method of Rhie and Shimkets (39) with *M. xanthus* LS1102 as the donor strain. MX4 Phage (50 ul) propagated on the mutant *stk* (LS1102) strain, was added to 0.2 ml of recipient cells (JD300, SR53, SR171, or SR200) at a multiplicity of infection of 0.5. Recipient cells were prepared by centrifuging log phase cells ($5X10^8$ to $10X10^8$ cells/ml) and resuspending them in 0.01M-Tris buffer (pH7.5), containing the salts mixture used in the 17P medium (10). The infected cells were incubated for 8 to 12h with aeration at 30°C. The phage-cell mixtures were plated in 2.5 ml CT top agar on CT bottom agar containing both oxytetracycline and kanamycin. Plates were incubated for 10 days at 30°C to collect transductants.

Carbohydrate measurement

The amount of anthrone-reactive material was determined based on the Molish test described by Dische (18) with glucose as the standard. Total carbohydrate was measured directly from disrupted cells which had been washed and sonicated in 10mM MOPS buffer (pH 7.0). Pelletable

carbohydrate was measured from the pelleted fraction of the total carbohydrate through centrifugation for 15min at 14,000g.

Preparation of cell-free extracts

Routinely, 1 ml samples of concentrated cell suspensions (500 Klett unit) were harvested by centrifugation for 10 min at 8,000g and washed with KH_2PO_4-K_2HPO_4 buffer (0.1M, pH 7.0) and resuspended in 2 ml of the same buffer. Cells were disrupted by ultrasonication on ice with a Branson sonifier (a power setting of 4.0 for 3 x 30s with alternating cooling period). The cell debris was removed by centrifugation for 15 min at 14,000g (4°C). The supernatant (crude extract) was used as the source of Pck activity. Protein concentrations were determined by the BCA Protein Assay Reagent Kit purchased from Pierce using BSA as the standard.

Assay of phosphoenolpyruvate carboxykinase (Pck)

Phosphoenolpyruvate carboxykinase (Pck) mediates the first reaction in the gluconeogenic pathway and catalizes a reversible reaction to convert oxaloacetate (OAA) to phosphoenolpyruvate (PEP). Since attempts to measure PEP-forming Pck activity were not satisfactory due to the competing reaction which utilizes OAA to form pyruvate in the crude extract, we used OAA-forming reaction to measure Pck activity. The activity of Pck was measured spectrophotometrically at room temperature by monitoring disappearance of NADH in the assay mixture with light/UV wavelength set at 340 nm. The assay mixture (2 ml) was 100 mM imidazole/HCl, pH 6.6, 50 mM $NaHCO_3$, 2.5 mM phosphoenol

pyruvate, 1.25 mM ADP, 2 mM $MnCl_2$, 2 mM glutathione, 0.25 mM NADH, 3 IU of malate dehydrogenase, and 100ul of crude cell extract. The endogenous rate of NADH oxydization in the enzyme preparation was measured without addition of ADP so that it could be substracted from the total rate of NADH oxidization (34). One unit of activity is defined as the amount of enzyme that catalyses the oxydization of 1 nmol of NADH/min/mg protein under the assay condition.

Two protocols have been widely used to measure the OAA-forming Pck activity; PEP-dependent and ADP-dependent measurements. Although the PEP-dependent enzyme assay gave a higher Pck activity in general, more consistent results were obtained from the ADP-dependent measurement, and the latter method was used exclusively in the subsequent Pck assays. Enzyme activity of phosphoenolpyruvate carboxylase (Pc) which also converts PEP to OAA but using CO_2 and ATP was not significant under our Pck assay conditions (data not shown).

Cell agglutination assay

Agglutination was measured by a modification of the method described by Shimkets (42). Cells grown in CYE to a density of 70 to 80 Klett units were centrifuged at 8,000g for 10 min at 4°C, washed once with 10mM MOPS (pH6.8), and suspended to a density of 100 Klett units in 10 mM MOPS (pH6.8) containing 1 mM $MgCl_2$ and 1 mM $CaCl_2$. The cell suspensions were incubated at room temperature without shaking, and changes in turbidity were measured in a spectrophotometer at 625 nm.

DNA and protein sequence analysis

A 3.6 kbp KpnI-EcoRI DNA fragment from pJRD2 which had been kindly provided by Dr. Shimkets was subcloned into pGem4Z+ generating pSTK. DNA sequence determination was carried out by Doris Kupfer. Plasmid pSTK was sonicated and shotgun-cloned into M13mp18. The single-stranded M13 clones were isolated using the Eperon DNA isolation procedure performed on the Biomek 1000 automated laboratory work station. The clones were sequenced using an Applied Biosystems model 373A DNA sequencer with dye-labeled M13 universal primers giving greater than 7.5X redundancy. Ambiguities were resolved using appropriate oligonucleotide primers synthesized on a Beckman Oligo 1000 DNA Synthesizer.

Identification of ORFs in the 3.6 kbp DNA sequences was performed by using an internet-accessible program, DNA sequence translation (http://alccs. med.umn.edu./webtrans.html). BLAST (Basic Local alignment Search Tool at NCBI) and CLUSTALW (multiple sequence alignments at Washington University Institute for Biomedical Computing) were used to search and align protein sequences with a high level of similarity. Analysis of protein domains and motifs in the three ORFs was carried out using PRODOM (Protein Domains Database) analyses and PROSITE search which were available through SDSC (San Diego Supercomputer Center, http://www.sdsc.edu/Res Tools/cmshp.html).

Table. 1 *M. xanthus* strains

Strain*	Genotype	Phenotype	Reference
DK1622	wild type		(31)
DZF1 (FB)	*sglA1*	S^-	(21)
JD300	*esg-258*	Esg^-	(19)
DK3468	*dsp-1680*	S^-	(42)
LS1111	*dsp-1680, stk-1907*	S^- Stk^-	(17)
DK3481	*sgl-2234*	S^-	(42)
LS1118	*sgl-2234, stk-1907*	S^- Stk^-	(17)
DK3482	*tgl-3114*	S^-	(42)
DK3088	*stk-1907, sglA1*	S^- Stk^-	(17)
SR53	Tn*5*ΩSR53	Cds^-	(S. Ramaswamy)
SR171	Tn*5*ΩSR171	Cds^-	(S. Ramaswamy)
SR200	Tn*5*ΩSR200	Cds^-	(S. Ramaswamy)
SR53-stk	Tn*5*ΩSR53, *stk*-1907	Cds^-	(in this study)
SR171-stk	Tn*5*ΩSR171, *stk-1907*	Cds^-	(in this study)
SR200-stk	Tn*5*ΩSR200, *stk-1907*	Cds^-	(in this study)
esg-stk	*esg-258, stk-1907*	Esg^- Stk^-	(in this study)

* All mutant strains except DZF1 were derived from the wild-type DK1622 strain.

RESULTS

Stationary phase polysaccharide induction

During studies of an *M. xanthus esg* mutant, we observed that *esg* stationary phase cultures differed dramatically from the wild-type (DK1622) parental strain. While cells in wild-type culture were clumped and associated with a copious amount of viscous extracellular polysaccharide material, *esg* mutant cells remained dispersed with much less extracellular material. The difference in the rheological properties was not observed until the cultures began to enter stationary phase. The morphology of aged colonies of the *esg* strain also differed greatly from the wild-type with the *esg* colonies having a much smoother appearance.

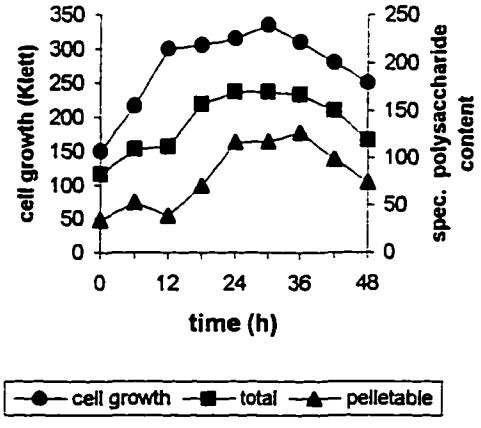
The polysaccharide content of the wild-type and *esg* strains was determined during vegetative growth using a simple carbohydrate assay. Cells were cultivated in CYE medium (a rich casitone-yeast extract based medium) and aliquots of the cells were collected at 6h intervals through the late stationary phase of growth. Total or pelletable carbohydrate content was obtained from disrupted cell material or from the pelleted fraction (at 14,000g for 15 min) of the disrupted cell material, respectively. Hence, the pelletable carbohydrate content represents a high MW polysaccharide fraction. The total carbohydrate content of the wild-type culture increased greatly as the cells entered stationary phase, rising from 75 to 160 ug carbohydrate per mg protein (Fig. 1A). Much of the carbohydrate appeared to be in the form of high MW polysaccharide since more than 80% of the carbohydrate in a sonicated

stationary cell extract (36h sample) could be removed by low speed centrifugation and about 90% of the cell extract carbohydrate could be precipitated with 67% ethanol (data not shown). The *esg* cells, which grew at a rate similar to wild-type completely failed to induce polysaccharide production (Fig. 1B). Measurement of the polysaccharide excreted into the surrounding medium did not indicate any sign of polysaccharide induction by the *esg* cells (data not shown). The *esg* cells also had a significantly lower specific polysaccharide content than wild-type cells (54 VS 82 ug/mg protein at 150 Klett) during log phase growth before the entry into stationary phase.

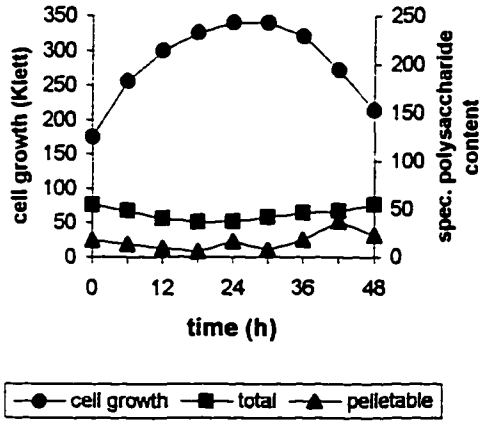
To facilitate handling several strains at once and to obtain relatively synchronous physiology of the cells to be tested, we employed a high-cell-density culture in which highly concentrated cells were forced to enter stationary phase in a short period of time. Both wild-type (DK1622) and *esg* strains were harvested from the log phase, concentrated, and further incubated at 500 Klett cell density in fresh CYE medium. Under these conditions, the maximum polysaccharide content was observed after about 6-8h incubation and the amount of polysaccharide produced was similar to that found in stationary phase cells of the normal batch culture (Fig. 2A). Under this set of conditions, once again, polysaccharide induction was not observed by the *esg* strain (Fig. 2B).

Fig. 1 Polysaccharide induction during stationary phase

A. wild-type



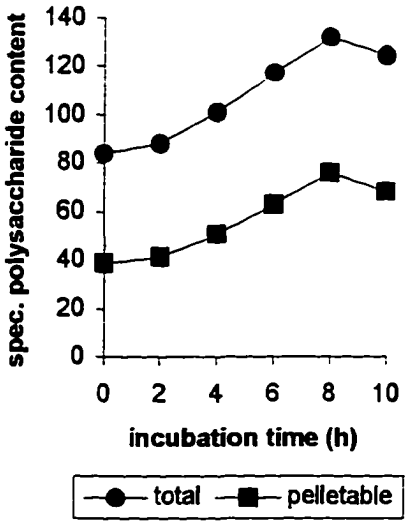
B. *esg*



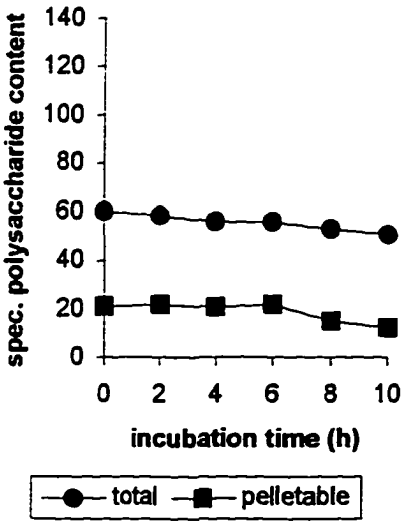
M. xanthus wild-type (DK1622) and *esg* cells were cultivated in CYE rich medium and aliquots of the cells was collected at 6h intervals until the late stationary phase. Total or pelletable carbohydrate content was obtained from the disrupted cell suspension or from the pelleted fraction (at 14,000g for 15 min) of the cell suspension, respectively. The disrupted cell suspension was obtained by ultrasonication, and was measured for its protein content in order to obtain the specific amount of carbohydrate (ug per mg protein) which was associated with the collected cells. Cell growth represents the turbidity measured in Klett-Summerson colorimeter (red filter).

Fig. 2 Exopolysaccharide production in high-cell-density culture

A. wild-type



B. *esg*

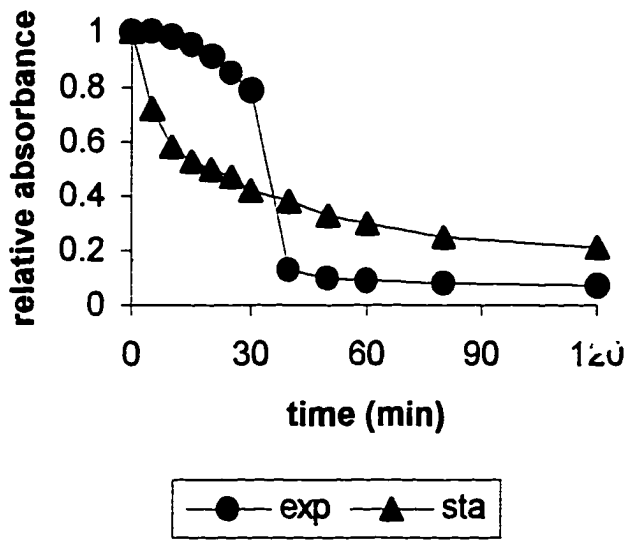


Both DK1622 and *esg* strains were harvested from the log phase CYE cultures (70 to 80 Kletts), concentrated, and further incubated at 500 Klett cell density in fresh CYE medium. Every two hours, cells were collected for total and pelletable carbohydrate content (ug carbohydrate per mg protein).

Fibrils are extracellular structures in *M. xanthus* that are composed of similar amounts of polysaccharide and protein. These structures have been associated with the ability of cells to agglutinate and settle from suspension during incubation in MOPS-Ca^{2+} buffer. Stationary phase wild-type cells which had a high polysaccharide level were compared with low polysaccharide content log phase cells in the agglutination assay (Fig. 3). The stationary phase cells agglutinated much more rapidly than the log phase cells. The agglutinated stationary phase cells formed aggregates in which the cells were less

tightly associated than the log phase cell aggregates and the absorbance of the stationary phase cells remained somewhat higher than log phase cells. The induced cells behaved functionally like they had an increased fibril content and this result suggested that the induced polysaccharide was associated with fibrils. When the polysaccharide-deficient *esg* mutant cells were tested, both log and stationary phase cell agglutinated poorly (data not shown).

Fig. 3. Agglutination by log phase and stationary phase wild-type cells



DK1622 cells harvested from exponential growth (diamond) and stationary phase (square) were washed in MOPS (10 mM, pH 6.8), and resuspended in cohesion buffer at a cell density of 100 Klett units. Cohesion buffer contained 10 mM MOPS pH. 6.8, 1 mM $CaCl_2$, 1 mM $MgCl_2$. The cell suspensions were incubated in 3ml- crystal cuvettes without shaking, and at indicated times, the turbidity was measured in a Beckman spectrophotometer at 625 nm.

Polysaccharide induction in *M. xanthus* mutants.

Several *M. xanthus* mutant strains have been reported to be deficient in fibril production (19,42). These mutants include *esg*, Cds mutants, *dsp* and other S-motility mutants. Scanning electron microscopy failed to detect fibrils in these mutants and all of these strains agglutinated poorly compared to the wild-type. Several of these mutants were tested for stationary phase induction of polysaccharide (Table 2). As was already shown, DK1622 had a strong induction of polysaccharide during stationary phase while there was no detectable induction in the *esg* strain. Four S-motility mutants (DZF1, *dsp*, *sgl*, and *tgl*) and three Cds mutants (*SR53*, SR171, and *SR200*) also failed to induce polysaccharide production. In addition, these mutant cells remained dispersed throughout stationary phase and failed to produce the viscous polysaccharide that was readily apparent in cultures of the wild-type cells.

Mutation of the *M. xanthus stk* locus results in cells with a higher than normal level of fibrils (17). Stk mutant cells exhibit a variety of phenotypic properties that are likely to result from fibril overproduction including the clumping of cells during growth in liquid shake culture, rapid agglutination, and the formation of colonies in which cells adhere tightly to each other and to the agar surface. Stk mutation has also been shown to restore fibril production in several fibril-deficient S-motility mutant strains. The effect of *stk* mutation on polysaccharide production was tested in the wild-type and several fibril-deficient mutants. The *stk* mutant produced a very high constitutive level of polysaccharide both during the log and stationary

phases of growth (Table 2). This level was twice the log phase value in wild-type cells and significantly greater than their induced level. When double mutants were constructed by transducing transposon insertion alleles from fibril deficient strains into the *stk* mutant, the double mutants generally displayed higher than wild-type levels of polysaccharide in the log phase and the level did not increase significantly during the stationary phase. The constitutive level of synthesis varied from strain to strain but was never quite as high as that found in the *stk* strain. The double mutant strains were also generally similar to *stk* in that many clumps of cells were observed during vegetative growth. Only the *dsp stk* and *SR200 stk* mutants failed to exhibit the phenotypic characteristics of the *stk* mutant; these strains contained reduced levels of polysaccharide and grew dispersed like the *dsp* and *SR200* strains. These observations with the *dsp stk* strain were consistent with the previous observations that *dsp stk* failed to produce fibrils (17).

Pck activity during polysaccharide induction

M. xanthus does not utilize exogenous sugars and is apparently completely dependent on gluconeogenesis for the production of hexoses for polysaccharide production. The evolutionarily conserved enzyme phosphoenolpyruvate carboxykinase (Pck) converts the TCA cycle intermediate oxaloacetate to phosphoenolpyruvate, an important step in gluconeogenesis. Pck activity was measured in log and stationary phase cell extracts prepared from wild-type and the various mutants (Table 2). In wild-type cells, Pck activity increased more than 2-fold in

stationary phase cells producing high levels of polysaccharide. Stationary phase Pck activity also increased from 2- to 4-fold in the fibril-deficient mutants that were tested. In the *stk* mutant, the level of activity found in log phase extracts was high and activity did not increase during stationary phase. The constitutive level of activity in the *stk* mutant was similar to that found in stationary phase wild-type extracts. Two patterns of activity were observed in double mutants containing the *stk* transposon insertion allele and insertions causing polysaccharide deficiency. One pattern was seen in several double mutants, including *esg stk*, which exhibited constitutive levels of Pck activity that were significantly lower than the activity found in the *stk* mutant. The other pattern of activity was observed in the *dsp stk* and *SR200 stk* strains. In these strains there was a pattern of Pck activity that was similar to that observed in the *dsp* and *SR200* strains without insertion and Pck activity was induced during stationary phase.

Table 2. Induction of polysaccharide and phosphoenolpyruvate carboxykinase (Pck) activity in mutant *M. xanthus* strains.

	Specific polysaccharide content (ug/mg protein)		Pck activity (nmole/min/mg protein)	
	Veg.	Dev.	Veg.	Dev.
Wild-type	72	127	8.1	18.1
stk	153	169	18.6	17.6
sglA1	64	60	6.3	13.6
esg	61	56	6.7	15.2
esg-stk	122	119	12.3	12.1
dsp	70	67	7.6	22.3
dsp-stk	81	74	7.6	25.2
sgl	73	70	8.3	16.8
sgl-stk	130	134	13.9	13.8
tgl	57	57	5.0	21.0
SR53	62	60	5.3	16.1
SR53-stk	134	136	14.3	14.1
SR171	76	69	7.6	18.4
SR171-stk	91	94	11.6	12.1
SR200	60	57	5.9	12.7
SR200-stk	70	68	6.5	14.2

For measurements of both carbohydrate and Pck, concentrated cell culture (500 Klett units) in CYE was used. Exponential (exp.) and stationary (sta.) cells were harvested from 0h and 6h cell cultures, respectively.

Nutritional and osmotic control of Pck activity.

Since induction of Pck activity was observed in response to the growth phase of *M. xanthus* cells, additional conditions which might induce enzyme activity were investigated. In *E. coli* and *R. meliloti*, the expression of the *pck* gene is dependent on the growth phase and the carbon source present in growth media. The onset of the stationary phase and gluconeogenic carbon sources such as succinate and arabinose were shown to induce pck expression early during log phase (26, 38). To determine whether the Pck activity of *M. xanthus* is also inducible in the gluconeogenic culture condition, we transferred wild-type cells to a chemically defined medium with pyruvate as the primary carbon and energy source. When incubated for 3h in A1 medium, wild-type cells had almost 4-fold higher activity (29.2U) than cells grown and incubated under the same conditions in CYE medium (7.7U) (Table 3). The A1-grown cells did not have an increased polysaccharide content. When *stk* mutant cells were tested in the same fashion, essentially the same levels of Pck activity were observed in A1 and CYE, and these levels were intermediate (13.9U and 14.7U) to those found in the two media with the wild-type.

The Pck activity was also determined in cells grown in CYE with 0.25M NaCl. These are conditions in *M. xanthus* under which a large amount of the disaccharide trehalose is produced (36). Trehalose accumulation is believed to function in the stabilization of cell membranes and in conferring resistance to dehydration. In wild-type cells, Pck activity increased about 2-fold after two hours in response to the NaCl addition (Table 4). The cells with elevated Pck activity

also had increased carbohydrate content, but this carbohydrate was most likely in the form of trehalose and not polysaccharide (data not shown). Pck activity was not induced in the *stk* mutant cells under these conditions.

Table 3. Induction of phosphoenolpyruvate carboxykinase (Pck) activity of *M. xanthus* strains in minimal growth medium A1.

M. xanthus strain	Pck activity of cells from:		Induction ratio
	CYE	A1	
DK1622	7.7	29.2	3.8
stk	13.9	14.7	1.1

M. xanthus strains were vegetatively cultured in CYE and washed with MOPS-Mg buffer (10mM MOPS pH 7.2 plus 8mM $MgSO_4$) for concentration to 2000 Klett per ml. The concentrated cell suspensions were diluted into fresh CYE or A1 medium to make 100 Klett per ml. Cells were collected after culturing for 3h and subject to Pck assay.

Table 4. Induction of carbohydrate production and Pck activity by high osmolarity

	amt. carbohydrate (pck activity)*	
M. xanthus strain	no treat.	NaCl (0.25M)
DK1622	71 (8.0)	128 (15.6)
stk	150 (19.0)	152 (14.0)

NaCl (0.25M) was added to the vegetatively growing *M. xanthus* cells and treated for 3 h (for carbohydrate measure) or 2h (for Pck measure). Cell pellets were washed and resuspended with MOPS buffer (10 mM pH 7.2) or phosphate buffer (0.1M pH 7.0) for carbohydrate or pck assay, respectively.

* unit: carbohydrate (ug/mg protein); Pck (nmol/min/mg protein)

Effect of Ca^{2+} on polysaccharide production.

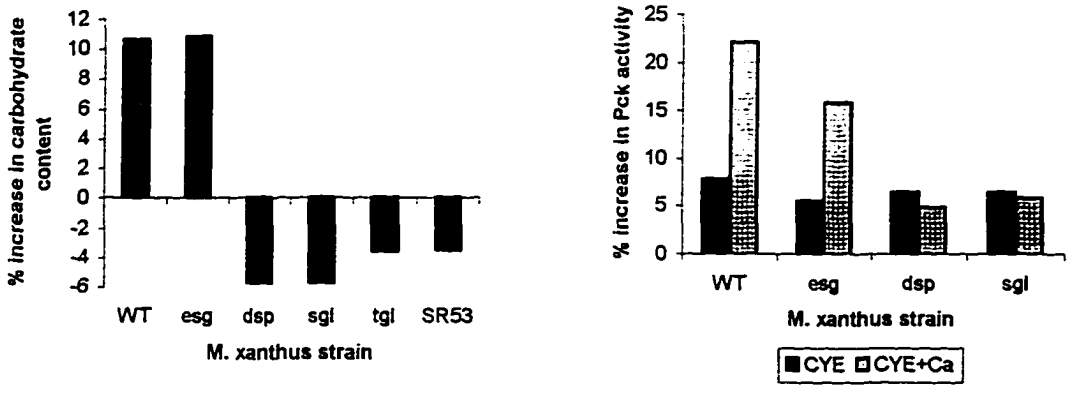
Calcium ion has been shown to stimulate the production of polysaccharide in certain microorganisms and to be involved with motility and developmental aggregation in myxobacteria (25, 43). When Ca^{2+} was added to wild-type *M. xanthus* cells growing in CYE medium, cells began to stick together forming multicellular clumps. The wild-type cells were tested for polysaccharide content and Pck activity after two hours incubation with Ca^{2+}. The Ca^{2+}-treated cells had about 10% greater polysaccharide content than untreated cells and about 3-fold higher Pck activity (Fig. 4). Several of the fibril-deficient mutant strains were also tested for Ca^{2+} induction. The polysaccharide content of *esg* cells increased by about 10%, the same amount as the

wild-type, but no induction was observed with the *dsp*, *sgl*, *tgl*, and *SR53* (Cds) mutants. Ca^{2+} addition also increased Pck activity in the wild-type and *esg* strains but had no effect on the activity found in the *dsp* and *sgl* strains. Ca^{2+}-induced wild-type, *esg*, and *dsp* cells were also tested in agglutination assay. The Ca^{2+}-induced wild-type and *esg* cells agglutinated much more rapidly than uninduced cells (Fig. 5). However, Ca^{2+}-induced *dsp* cells failed to agglutinate and behaved similarly to the uninduced cells.

Resuspension of the agglutinated cells with addition of EDTA resulted in a dispersion of a significant fraction of the wild-type cells (20 to 30%) indicating that ionic interaction on cell is also involved in cell cohesion (data not shown).

Fig. 4 Induction of carbohydrate and phosphoenolpyruvate carboxykinase activity of *M. xanthus* by Ca^{2+} ion

A. B.

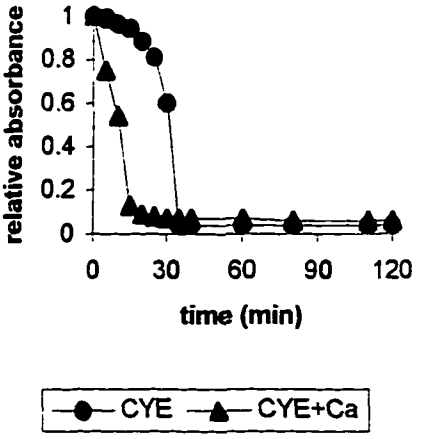


A. Carbohydrate induction by Ca^{2+} ion.
The wild-type DK1622 and mutant strains were grown exponentially in CYE, and were treated with 4 mM $CaCl_2$, incubated further for 2h, and collected for quantitation of cellular carbohydrate. Cells untreated with $CaCl_2$ were measured for carbohydrate content and it was used as a standard. The *esg* mutant is deficient in E signaling system which is required for normal development, and produces very little exopolysaccharide during the stationary phase. the *dsp*, *sgl*, and *tgl* mutants are defective in social motility and fibril production. The *SR53* mutant was isolated based on its poor ability to produce exopolysaccharide with functional social motility.

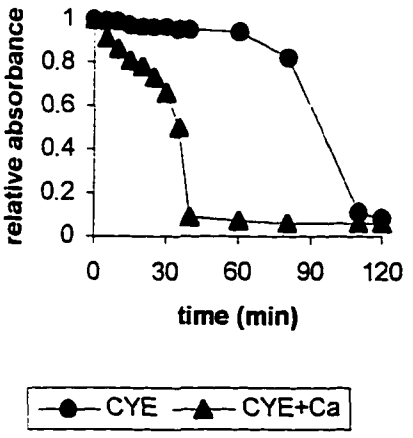
B. Induction of the Pck activity of *M. xanthus* by Ca^{2+} ion.
The *M. xanthus* strains which had been treated with 4 mM $CaCl_2$ for 2h were washed and disrupted in phosphate buffer, and their crude cell extracts after removal of cell debris were assayed for Pck activity. Dark bars and light bars represent the Pck activities of the cells untreated or treated with Ca^{2+} ion, respectively.

Fig. 5 Increased agglutination by Ca^{2+}-induced cells

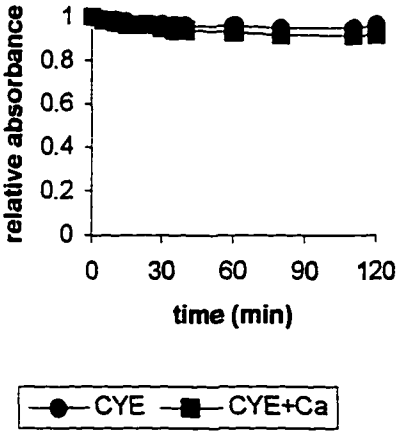
A. wild-type



B. *esg*



C. *dsp*



M. xanthus strains (DK1622, *esg*, and *dsp*) were grown exponentially in CYE (60 to 70 Klett), and each culture was devided into two subcultures; one with 4 mM $CaCl_2$ (solid square), and the other without (solid circle). The cells were further incubated for 2h and washed with MOPS buffer (10 mM, pH 6.8) and resuspended in cohesion buffer at a cell density of 100 Klett units. Cohesion buffer contained 10 mM MOPS pH. 6.8, 1 mM $CaCl_2$, 1 mM $MgCl_2$. The cell suspensions were incubated in 3ml-crystal cuvettes without shaking, and at indicated times, the turbidity was measured in a Beckman spectrophotometer at 625 nm.

Phosphate ion (PO_4^{3-}) has been known to induce exopolysaccharide production in many microorganisms when present in excess in the growing cell culture. To determine how *M. xanthus* responds to PO_4^{3-} addition, potassium phosphate (5 mM) was added to growing wild-type (DK1622) cell culture. Unlike the effect with Ca^{2+}, wild-type cells did not form macroscopic cell aggregates under these conditions but were strongly induced in cellular carbohydrate precipitable with acetone (Table 5). After 3h incubation, over 20% more production of the acetone-precipitable polysaccharide was observed with PO_4^{3-}-treated cells. A similar level of induction was also observed with sodium phosphate (5 mM, data not shown). However, unlike the effect with Ca^{2+}, the *esg* mutant cells did not respond to PO_4^{3-} (Table 5) suggesting that polysaccharide synthesis induced by Ca^{2+} and PO_4^{3-} may be differently regulated. We do not know at present whether the polysaccharide induced by PO_4^{3-} is also organized as fibrils, although cell aggregation was not detected in the culture of PO_4^{3-}-induced DK1622.

Table 5. Induced production of polysaccharide by phosphate ion

M.xanthus strain	amt. carbohydrate; ug/mg protein total (precipitable)		
	0h	3h	*3h-PO_4^{3-}
DK1622	71(60)	73(62)	89(79)
esg	58(52)	56(50)	55(50)

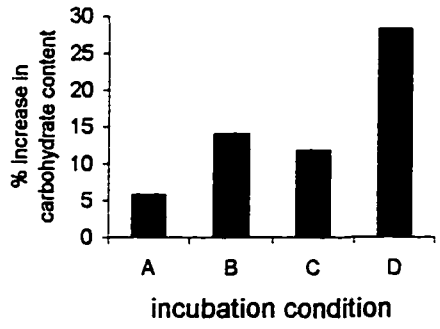
* Potassium phosphate (dibasic, pH 7.2) was added to the exponentially growing cell culture (final concentration of 5 mM) and treated for 3h. The cells were pelleted, washed and resuspended in MOPS (10mM pH 7.2) buffer for disruption by sonication. The disrupted cell suspension was used to assay both total carbohydrate content and acetone-precipitable carbohydrate fraction. Two volumes of acetone were added to the disrupted cell suspension, and the mixture was pelleted (10,000g for 10 min) after incubation at 4°C for 1h. The pellets were dried at room temperature before assayed for their carbohydrate content.

The observation that Ca^{2+} induces polysaccharide production in cells growing in a rich medium led us to investigate the effect of Ca^{2+} on cells under low nutrient conditions required for multicellular development in *M. xanthus*. Incubation of wild-type cells in agglutination buffer, a MOPS-$CaCl_2$ buffer, has been shown to result in fibril production in an energy-dependent process (41). Wild-type cells incubated in the agglutination buffer were examined for the accumulation of polysaccharide. Polysaccharide was found to increase about 5%

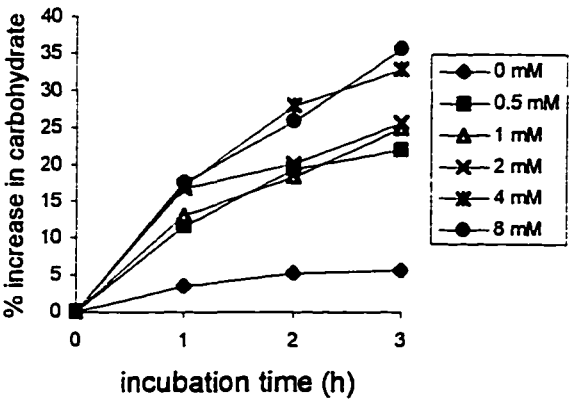
during a standard two hour incubation without agitation (Fig. 6A). By incubating the cells in the same buffer in the presence of small glassbeads or by agitating agglutinating cells, the level of polysaccharide production was found to increase to 10–15%, and incubation with both glassbeads and agitation resulted in a nearly 30% increase. The glassbeads presumably increased the surface area avilable for contact with the cells, a factor which has been implicated in fibril production. Agitation obviously increased the oxygen level of the buffer and aided in aerobic respiration, but it also acted to decrease the contact of the cells with surfaces and would seem to counteract the effect of the glassbeads. Nevertheless the combination of agitation and glassbeads greatly stimulated polysaccharide production. Under these conditions the cells responded to Ca^{2+} concentrations from 0.5–8mM with similar rates of polysaccharide production (Fig. 6B).

Fig. 6

A.



B.

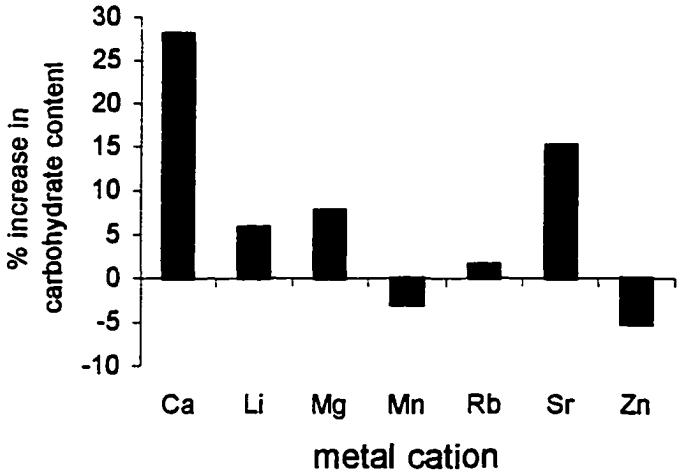


A. Effects of aeration and solid surface on the Ca^{2+}-mediated carbohydrate induction.
DK1622 cells grown exponentially in CYE were washed and suspended in Ca-MOPS (4 mM $CaCl_2$, 10 mM MOPS pH 6.8) at a cell density of 100 Klett units and dispensed equally (1 ml) into four culturing tubes; two tubes with 2-ml volume of acid-washed 0.15mm-diameter glassbeads (tubes B and D) and the other two without (tubes A and C). Within each set, a tube was incubated with shaking at 100 rpm and the other was incubated without shaking. After incubation at 30°C for 2h, each cell suspension was assayed for total carbohydrate by directly adding sulfuric acid-anthrone reagent to the cell suspensions. The averaged results of triplication are depicted. Standard deviations in all samples were within 10%.

B. Ca^{2+}-mediated carbohydrate induction in MOPS buffer
M. xanthus DK1622 cells harvested from log phase were washed in MOPS buffer (10 mM, pH 6.8) and resuspended in MOPS buffer containing various concentrations of $CaCl_2$ (0, 0.5, 1, 2, 4, 8 mM). Each cell suspensions (1 ml of 100 Klett units) were incubated in sterilized boiling tubes with aluminum foil tops at 30°C with shaking (200 rpm) . At every hour to 3h, the sample cell suspensions were retrieved and directly added with sulfuric acid-anthrone reagent for carbohydrate quantification.
Data were obtained from two independent experiments.

The ability of other divalent cations to substitute for Ca^{2+} was examined using the polysaccharide assay. Mg^{2+}, Mn^{2+}, Zn^{2+}, Li^{2+}, Sr^{2+}, and Rb^{2+} were tested. About 50% of Ca^{2+} level of polysaccharide production was achieved with Sr^{2+} (15% induction) (Fig. 7). Mg^{2+} and Li^{2+} supported similar 6–8% levels of induced polysaccharide synthesis. No significant effect was found with the remaining divalent cations, Mn^{2+}, Zn^{2+}, and Rb^{2+}.

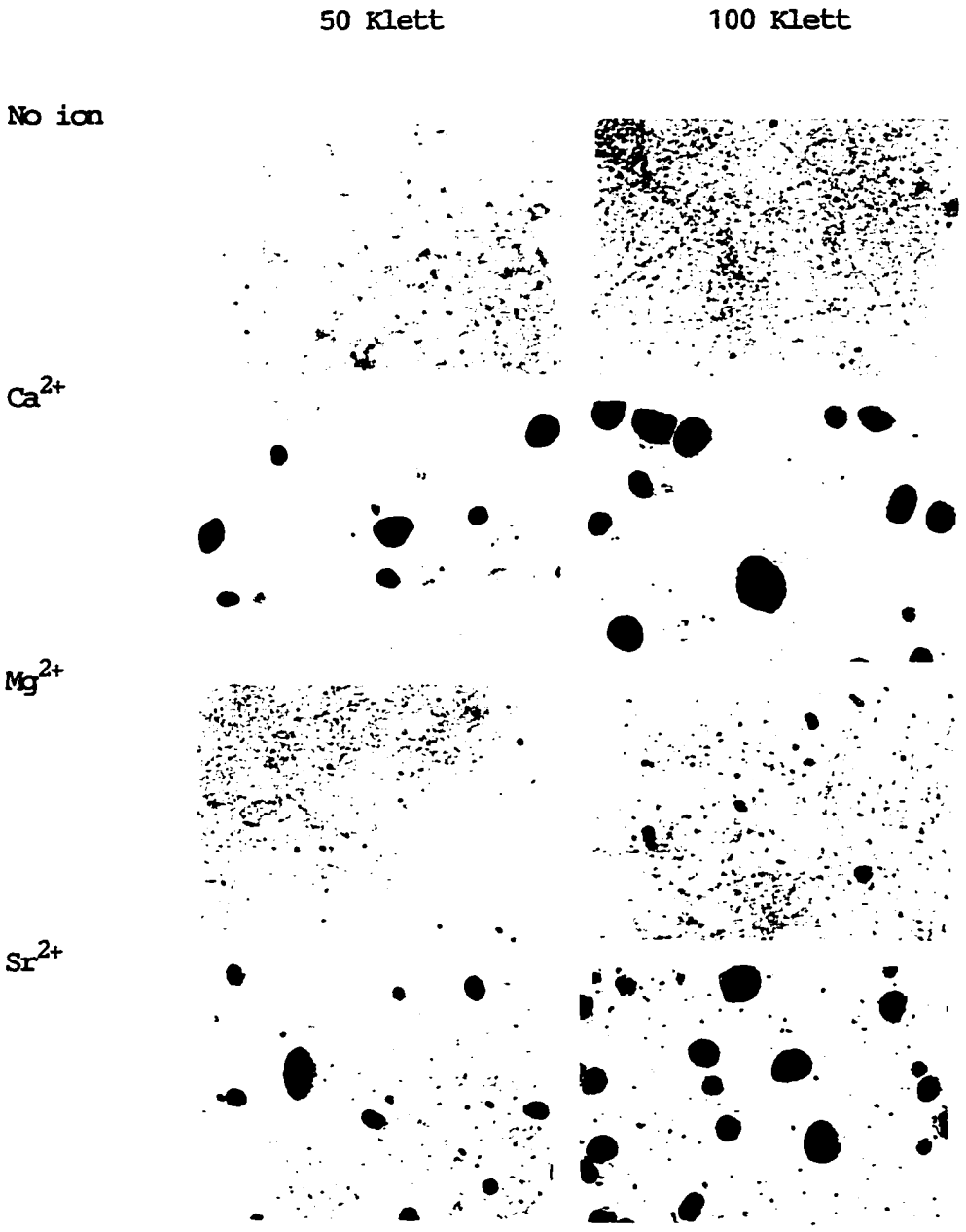
Fig. 7 Effects of metal ions on the polysaccharide synthesis



M. xanthus DK1622 cells harvested from log phase were washed in MOPS buffer (10 mM, pH 6.8) and resuspended in MOPS buffer containing 4 mM of various divalent metal ions ($CaCl_2$, $LiCl_2$, $MgCl_2$, $MnCl_2$, $RbCl_2$, $SrCl_2$, and $ZnCl_2$). Each cell suspension (1 ml of 100 Klett units) was incubated in sterilized boiling tubes with aluminum foil tops at 30C with shaking (200 rpm) . After 2h incubation, the sample cell suspensions were retrieved and directly added with sulfuric acid-anthrone reagent for carbohydrate quantification. The cell suspension at time 0h was used to obtain the base amount of carbohydrate.
The averaged results of triplication are depicted. Standard deviations in all samples were within 10%.

Ca^{2+} is required for fruiting body formation by *M. xanthus* cells incubated in buffer in the wells of microtitre plates (submerged culture development) (32). The ability of different divalent cations to substitute for Ca^{2+} in submerged culture development was also examined. Exponentially growing wild-type (DK1622) cells were washed and incubated under submerged culture conditions in 10 mM MOPS (pH 7.2) with 4 mM of selected divalent cations and were photographed for multicellular development. As shown in Fig. 8, the addition of Ca^{2+} greatly stimulated the formation of fruiting bodies by wild-type cells. Sr^{2+} also strongly stimulated fruiting body formation but Mg^{2+} had only a slight effect. These fruiting bodies were found to contain myxospores as early as 72h incubation. In general, the ability of buffers with various divalent cations to support development was correlated with the ability of the cations to support polysaccharide induction. Under the same incubation conditions, cells treated with Mn^{2+}, Rb^{2+}, or Zn^{2+} were unable to aggregate and form fruiting bodies.

Fig. 8 Cellular development in submerged culture

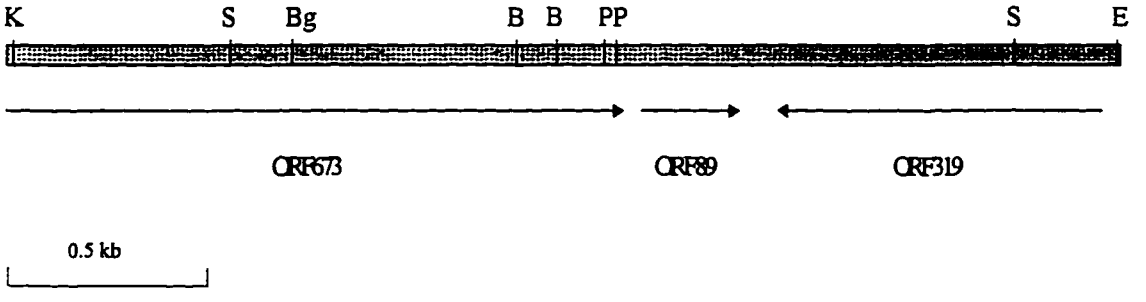


M. xanthus DK1622 cells harvested from log phase were washed in MOPS buffer (10 mM, pH 6.8) and resuspended in MOPS buffer containing 4 mM of $CaCl_2$, $MgCl_2$ or no salt at cell densities of 50, 100 Klett units. Each cell suspension was placed in a 24-well cell culture plate and incubated statically at 30°C for 96h. Cellular development was photographed using Nikon inverted microscope photo system.

Dana and Shimkets (17) determined that the Stk phenotype in the *stk-1907* insertion mutant could be complemented by a 3.6 kbp wild-type KpnI-EcoRI DNA fragment indicating that this DNA fragment contained the the gene function missing in the mutant. To initiate a more detailed analysis of the role of the *stk* locus in the regulation of fibril and polysaccharide production, the DNA sequence of the 3.6 kbp fragment was determined. This 3621-bp DNA fragment appeared to contain three ORFs which are likely to be expressed in *M. xanthus* based on the strong bias for G or C bases at the third codon position in the protein coding sequences of this high G+C% organism. (Fig. 9). ORF319 began close to the EcoRI end and extended into the central region of the fragment. ORF319 was predicted to encode a protein with about 50% identity to ORF321 of *E. coli* encoding a protein of unknown function (Acc # 1176126). The other two ORFs, ORF673 and ORF89, were oriented in the opposite direction. The predicted amino acid sequence of the product of the upstream ORF673 was found to be about 30% identical to the *DnaK* protein of *E. coli* and many other members of the highly conserved hsp70 family of proteins (35). ORF673 began at the KpnI end of the fragment and appeared to be missing the extreme N-terminus coding region. Assuming perfect alignment of the *M. xanthus* ORF673 and *E. coli* DnaK proteins at the N-terminus, the *M. xanthus* fragment may be lacking amino terminal nine codons which contain part of one of the three signature patterns for the hsp70 family of proteins (Fig. 10A). In these proteins, the first signature pattern with a conserved 8-aa sequence (signature 1) is found in the N-terminal section. The two others (14- and 15-aa sequences for signature 2 and 3) are located in the central

part of the proteins. ORF89 began 12-bp downstream from ORF673 and the close proximity of these ORFs suggests that they may be cotranscribed. The relatively small and basic (pI of 10.1) protein product of ORF89 was composed exclusively of α-helices and did not match known protein sequences in the data bases, but this protein contained a leucine zipper motif(33) at the extreme C-terminus (Fig. 10B, residues 68–89), a motif frequently associated with eucaryotic transcription factors. Insertion *Ω1907* was located within ORF673 but, if ORF673 and ORF89 are cotranscribed, this insertion might also be expected to limit transcription of the downstream ORF89. These results suggest that ORF673 and ORF89 are within the *stk* locus.

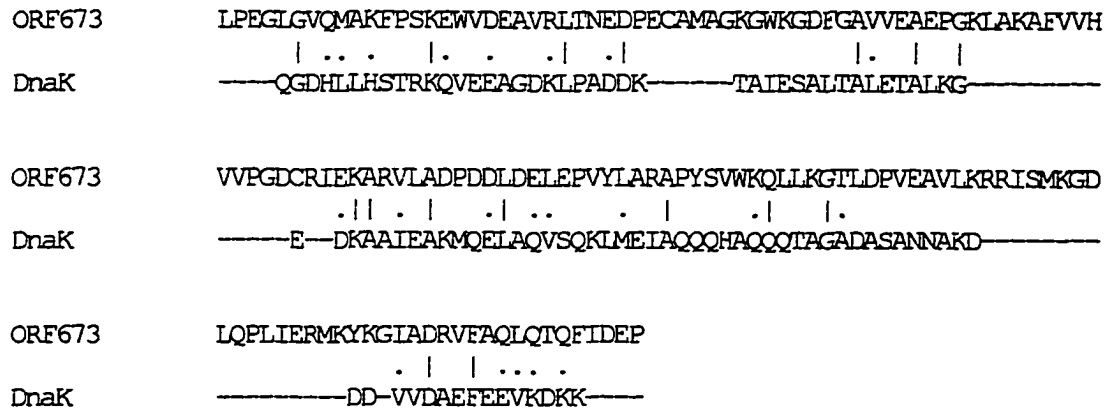
Fig. 9



Restriction map of the *stk* locus. Locations of the identified genes are indicated with arrows. B, BamHI; Bg, BglII; E, EcoRI; K, KpnI; P, PstI; S, SalI.

Fig. 10

A.



B.

ORF89

MGIKDDLKKQALNVSGKAMEKLMADDRRAMAIANAIGKAQRGKQALDRGQEELMKALNFAPRSEFKALGKQL

SGLKRRLRELDEKLGAL

A. Comparison of the deduced amino acid sequences of *M. xanthus* ORF673 and *E. coli dnaK* genes. Amino acids identical with those of *dnaK* are indicated by a vertical line, and conservative replacements are indicated by a point. Gaps, indicated by dashes, are introduced in order to obtain a maximum fit. The three signature patterns found in the hsp70 family of proteins are underlined.

B. Deduced amino acid sequence of ORF89. A leucine zipper motif is indicated with an underline, and four leucine residues which repeatedly occur at every seventh position are bold-typed.

DISCUSSION

Under appropriate conditions, myxobacteria cells produce an extracellular matrix referred to as fibrils. With scanning electron microscopy, fibrils can be observed connecting *M. xanthus* cells and these structures appear to play a central role in the multicellular activities displayed by this organism (4). For example, fibrils mediate the agglutination of cells incubated in solution and appear to be involved in the aggregation of cells, a requirement for fruiting body formation. Fibrils may also allow *M. xanthus* cells to form communal mats (biofilms) and to asssociate firmly with solid substrates. Although the structure of fibrils has not been described in detail, fibril material has been isolated and appears to consist of a polysaccharide backbone which is associated with a roughly equal amount of protein (5). Several abundant proteins are known to be associated with fibrils, and genetic and biochemical studies of these proteins are underway (5, 16, myxomeeting abstract).

In this study, we have investigated the regulation of total cellular polysaccharide production in *M. xanthus*. In wild-type cells, polysaccharide production was induced in response to the stationary phase of growth, the addition of Ca^{2+} to cells under a variety of conditions, and the addition of phosphate to growing cells. Our observations are consistent with the hypothesis that all or most of the induced polysaccharide is fibril polysaccharide. These observations include (a) stationary phase- or Ca^{2+}-induced cells agglutinated more rapidly than uninduced cells, (b) the extent of developmental

aggregation of wild-type cells in the presence of different divalent cations was correlated with the degree of induced polysaccharide production under developmental conditions, and (c) mutants previously shown to have defects in fibril production also exhibited alterations in polysaccharide induction.

Two groups of *M. xanthus* mutants have been shown to be deficient in fibril production. The S-motility mutants are one of these groups. These mutants have defects in a type of gliding motility, social motility, which involves the movement of groups of cell. Among the S-motility mutants, those with defects at the *dsp* locus have been shown to be the most severely defective in fibril production (42). The other group of fibril-deficient mutants is the Cds mutants (isolated by Downard's lab). These mutants were identified based on defects in the ability of these cells to bind calcofluor white (a flourescent dye which binds exopolysaccharide) and they retain a substantial capacity for S-motility. When members of each group were tested for stationary phase induction of polysaccharide production, all were defective. In all of these mutants there was an increase in the activity of the key gluconeogenic enzyme, phosphoenolpyruvate carboxykinase (Pck), which was similar to that observed with the wild-type. The increase in the activity of Pck presumably allows an increase in the flow of carbon from TCA cycle intermediates to the polysaccharide biosynthetic apparatus in these cells. These results suggest that the block in fibril polysaccharide biosynthesis in these mutant strains occurs in the later stages of the gluconeogenesis pathway, in the biosynthetic steps associated with the polymerization of the sugars into polysaccharide,

or in the assembly of polysaccharide and protein into fibrils. A few of these fibril-deficient mutants were also tested for Ca^{2+}-mediated induction of polysaccharide in a rich growth medium. In this case, a somewhat different pattern was observed and the different mutants were not uniform in their response. Polysaccharide induction was not observed by the *dsp*, *sgl*, *tgl* or *SR53* (a Cds mutant) strains and no increase in Pck activity was observed. However, in the *esg* mutant, a wild-type level of polysaccharide induction was observed and there was an associated increase in Pck activity. Cohesion and gliding motility of another myxobacterium *Stigmatella aurantiaca* are inducible with Ca^{2+}, and such induction is prevented by inhibitors of protein synthesis (13) and may also involve a production of polysaccharide. The differences in the response of the mutant strains to stationary phase and Ca^{2+} argue that the regulatory pathways for the activation of polysaccharide production by the two conditions are different in some important ways.

The ability of Ca^{2+} to stimulate fibril production helps explain the requirement for Ca^{2+} in agglutination and in development. As noted earlier, evidence has accumulated that shows fibrils as the mediators of the cell-cell contacts that occur during agglutination (32, 42) and as essential structural components for developmental aggregation. This evidence includes the demonstration that developmental aggregation could be rescued in the fibril-deficient *dsp* mutant by the addition of fibril material isolated from wild-type cells (12), and the observation that Cds mutants that were strongly deficient in polysaccharide production were also severely defective in fruiting body formation (Downard and Ramaswamy). The ability of Sr^{2+} to effectively substitute for Ca^{2+} in

supporting development in submerged culture could also be explained by the effectiveness of Sr^{2+} to induce fibril polysaccharide production. Other divalent cations such as Mg^{2+}, Li^{2+} or Rb^{2+} could not substitute for calcium in the developmental aggregation assay and had little or no effect on polysaccharide production.

Polysaccharide production and activation of Pck activity was also investigated in a *stk* mutant, a mutant previously shown to produce increased levels of fibril material. This mutant showed a constitutively high level of polysaccharide which did not increase in response to stationary phase, and Pck levels were uniformly higher during log or stationary phase. In wild-type cells, Pck activity was higher when cells were grown in a chemically defined medium with pyruvate as the principal carbon source or when cells were grown in a medium with NaCl, but in the *stk* mutant, activity did not increase above the level found in casitone-yeast extract medium.

Dana and Shimkets (17) demonstrated that the phenotypic effect of the *stk* mutation was epistatic to the fibril deficiency caused by several mutations in S-motility genes; that is to say that double mutants with mutations in the *stk* locus and in S-motility genes behaved like the *stk* mutant. One exception to this pattern was found with the *stk dsp* double mutant which displayed the fibril-deficient phenotype. We reexamined this genetic relationship using the polysaccharide induction and Pck activity phenotypes. The results of this analysis correlated well with the earlier study in that most of the double mutants examined showed constitutively elevated polysaccharide and Pck activity levels. The levels of polysaccharide and Pck activity were

not as high in the double mutants as in the *stk* so there were some effect of the fibril-deficiency mutation on the overall phenotype of the double mutant strains. As expected, the *stk dsp* mutant displayed a phenotype very similar to the *dsp* strain; uninducible polysaccharide production and activation of Pck during stationary phase. A second mutant, Cds mutant SR200, was discovered in this study with genetic and biochemical characteristics similar to dsp mutant. Thus, the *stk SR200* mutant also displayed a similar phenotype as the *stk dsp* mutant.

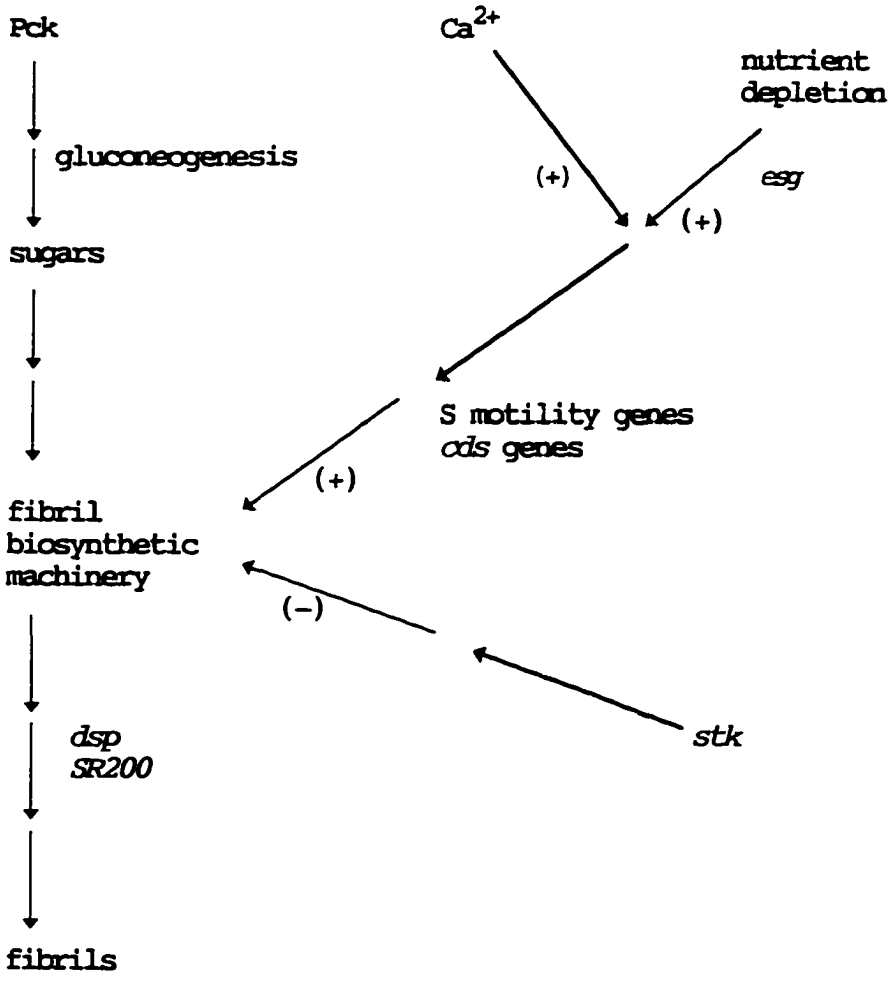
It has been proposed that the *stk* locus might encode a repressor protein that negatively regulates fibril biosynthesis (17). DNA sequence analysis of the wild-type KpnI-EcoRI DNA fragment which was previously shown to complement the *stk-1907* insertion allele identified two ORFs which constitute at least a part of the *stk* locus (17). These ORFs, ORF673 and ORF89, were separated by only 12 nucleotides and are likely to be cotranscribed. The *ΩDK1907* insertion was in the upstream ORF673 which, if the ORFs are cotranscribed, would also have a polar effect on expression of ORF89. It appears most likely that the KpnI-EcoRI fragment complements only the loss of ORF89 since ORF673 did not appear to be the complete coding sequence for a protein which was predicted to be a member of the highly conserved Hsp70 family of proteins (35). Based on alignment of the ORF673 amino acid sequence with the E.coli Hsp70 protein, *DnaK*, ORF673 was predicted to be missing the ORF sequences for the N-terminal nine amino acids. These amino acids are part of one of the three tightly conserved signature patterns for the Hsp70 family of proteins and may be essential for function. The predicted amino acid sequence from ORF89 did not match sequences

deposited in the data bases but it was found to contain the leucine zipper motif (33), a motif commonly associated with the dimerization of regulator proteins that bind to DNA. Thus, it is possible that the product of ORF89 functions as a trans-acting repressor to limit the expression of some component essential for fibril polysaccharide production. Clearly the *stk* locus plays an important role in fibril regulation in *M. xanthus*. Further analysis of the *stk* locus will be required to completely define the locus and to clarify the importance of the *stk* products in fibril production.

From the results of this and other studies it is becoming clear that fibril production in *M. xanthus* is the focus of extensive regulation. It now appears that fibril production is induced in response to the entry of cells into stationary phase and the availability of Ca^{2+} or Sr^{2+}. Phosphate ion also induces polysaccharide in *M. xanthus* which differs from fibrillar polysaccharide in the ability to mediate cell-cell cohesion. We propose a model to explain the regulation of fibril biosynthesis (Fig. 11). An important regulation for fibril biosynthesis is mediated by the product of the *stk* gene which inhibits fibril production under normal exponential cell growth. The S-motility genes and *cds* genes may be involved in positive regulation of fibril production, independent of the pathway involving the *stk* gene, since consistently less production of fibrils was observed in the double mutant strains than in the single *stk* mutant strain. These genes are subjected to the environmental signals such as Ca^{2+} ion and nutrient downshift caused by stationary-phase cell growth. The fact that the esg mutant responds to Ca^{2+} ion for fibril induction but not to stationary

phase suggests that the intact esg gene is not involved in Ca^{2+}-mediated fibril induction but affected by nutrient downshift. The gene products of *dsp* and *SR200* are likely to be involved directly in fibril biosynthetic machinery possibly as an important component for assembly of repeating units or polysaccharide polymerization since accumulation of fibrillar polysaccharide was not detected within these mutant cells. The mechanism by which Pck is induced under genetic modification (e.g. mutation in the stk gene) or different culture conditions is not at present understood. Cloning of the *M. xanthus pck* gene and its gene fusion study will be required to obtain better understanding of such mechanism. Also, further research with the fibril-deficient strains including the *esg* mutant which responds differently to Ca^{2+} and phosphate will help to elucidate the regulatory pathways in fibril biosynthesis.

Fig. 11



Model outlining the regulation pathways for fibril biosynthesis. The downward pathway comprises the gluconeogenic pathway for the production of sugar precursors and the biosynthetic pathway for fibril production. The gene product of *stk* represses fibril production under normal vegetative cell growth. The S-motility and *cds* genes may constitute another regulatory pathway which induces fibril production. These genes are under influence of environmental signals such as Ca^{2+} ion and nutrient depletion caused by stationary-phase cell growth. The *esg* gene appears to be not affected by Ca^{2+} ion but affected by nutrient depletion. The gene products of *dsp* and *SR200* are likely to be directly involved in fibril biosynthetic machinery possibly as an important component for polysaccharide polymerization. Positive and negative regulation is indicated as (+) and (-), respectively.

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