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UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

Identification and Characterization of *Myxococcus xanthus* Mutants Deficient in Calcofluor White Binding

A Dissertation

SUBMITTED TO THE GRADUATE FACULTY

in partial fulfillment of the requirements for the

degree of

DOCTOR OF PHILOSOPHY

By

SRINIVAS RAMASWAMY

Norman, Oklahoma

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Identification and Characterization of *Myxococcus xanthus* Mutants Deficient in Calcofluor White Binding

A DISSERTATION APPROVED FOR
THE DEPARTMENT OF BOTANY AND MICROBIOLOGY

BY

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Dedicated to my wife and parents

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Abstract

The *esg* locus of *Myxococcus xanthus* was analyzed and shown to consist of two genetic complementation groups. In an attempt to characterize the defects in *esg*, it was found that the mutant bound the dye calcofluor white poorly than wild-type cells. Unlike S-motility mutants that share this phenotype, *esg* exhibited S-motility. This led to the identification of nine new transposon insertion mutants, designated Cds (calcofluor white binding deficient, S-motile), which exhibited a phenotype similar to the *esg* strain. The Cds mutants were also found to be defective in cell-cell agglutination, polysaccharide production and developmental aggregation. The degree of polysaccharide deficiency in the Cds mutants correlated with the degree of loss of agglutination and dye binding as well as with the severity of the developmental aggregation defect. Extracellular matrix fibrils composed of roughly equal amounts of polysaccharide and protein have been shown to be involved in agglutination, and electron microscopic examination showed that *esg* and the other Cds mutants lack the wild-type level of fibrils. Preliminary genetic characterization demonstrated that the transposon insertion mutations in three of the Cds mutants (SR53, SR171, and SR200) were loosely linked.

In an attempt to further characterize the Cds phenotypes, it was

found that SR171 (a Cds mutant) drastically reduced developmentally regulated *tps* gene expression similar to *esg*. Total fatty acid analysis revealed that SR171 is deficient in branched-chain fatty acids (BCFA). Addition of IVA to growing cells rescued fruiting body formation and increased sporulation and *tps* gene expression by several folds. SR171 differed from *esg* in pigmentation and sporulation. Extracellular complementation was observed when SR171 was mixed with wild-type and *esg* mutant. A 6kbp DNA fragment cloned from the wild-type cosmid library was able to partially complement genetically, the aggregation defect in SR171. Restriction analysis revealed that the SR171 locus was different from *esg* locus. The *esg* locus encodes the E1α and E1β subunits of a branched-chain keto acid dehydrogenase (BCKAD) involved in the production of BCFA. The results suggest that SR171 locus appears to either encode for additional components of the BCKAD or components of a second enzyme involved in BCFA synthesis.

CHAPTER I

Myxococcus : A prokaryotic model system to study multicellular behavioural and developmental phenomena.

INTRODUCTION

Myxobacteria are gram-negative, strictly aerobic, gliding bacteria found in top soil all over the world. They are distinguished from other bacteria by their remarkable ability to form multicellular fruiting bodies containing dormant myxospores (21, 72). They have been found in many different types of soil (42), mainly in the pH range of 5-8. Another typical habitat of myxobacteria is decaying plant material, and the dung of herbivorous animals (59, 76). These organisms are found in all climate zones, vegetation belts and altitudes, although, not everywhere with the same frequency (60). Most myxobacterial strains are typical mesophiles. However, true psychrophilic myxobacteria have been isolated from samples found in Antarctica (15, 64). Although myxobacteria are prevalent in soil systems, their contribution to soil ecology is not well understood. Myxobacteria are microbial predators and it is believed that they play a role in controlling the population size of certain soil microorganisms. Myxobacteria secrete a variety of materials into their immediate environment which includes antibiotics,

polysaccharides, bacteriocins as well as enzymes that degrade microbial cell walls, lipids, proteins and nucleic acids (10, 25, 27, 61, 69). Roland Thaxter was the first to recognize their prokaryotic nature in 1892 (80). The G+C composition of all myxobacterial species is very high, about 67-71% (46, 52). Comparison of 16s rRNA molecules from a variety of myxobacteria indicates that they are related to bdellovibrios and sulfate-reducing bacteria and thus are phylogenetically classified as belonging to the ∂ branch of proteobacteria (74). The taxonomy of myxobacteria is based on morphological characteristics, such as shapes of vegetative cells and fruiting body structures. Approximately 40 species of myxobacteria in 12 genera can presently be distinguished morphologically. Much of our knowledge of the biology of myxobacteria has been limited to two species - *Myxococcus xanthus* mainly and to a lesser extent, *Stigmatella aurantiaca.* Most of the recent research on the genetics of myxobacterial development and social behaviour has been done on *M.xanthus* which has served as a model myxobacterium. The *M.xanthus* system is amenable to analysis by a variety of genetic and biochemical approaches. One of the fundamental problems in developmental biology is to understand how macroscopic biological structures are built from individual cells. *M.xanthus* is a unique prokaryotic system in which one can study the

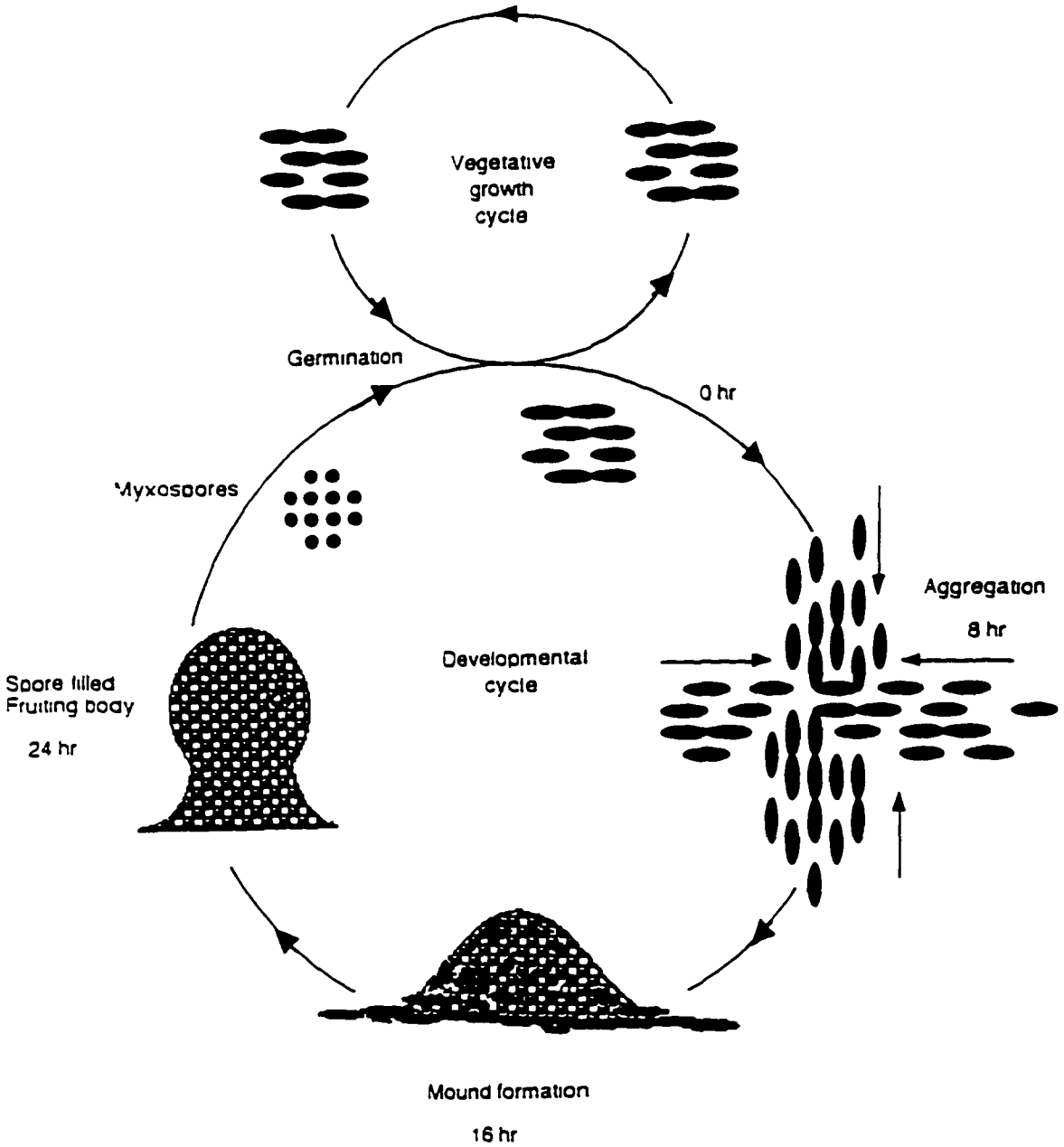

Figure 1. Life cycle of *Myxococcus xanthus*

formation of a fruiting body structure built from an interaction of tens of thousands of individual cells. This developmental process clearly requires cell-cell communication and thus it offers a simple model system to isolate and study biological signals, their mode of reception, signal transduction and developmental gene expression.

Life-cycle of *Myxococcus xanthus*

The life-cycle of *M.xanthus* can be seen as two separate but interlocking cycles of growth and development (Figure 1). Vegetative cells can be grown in liquid culture on media containing hydrolyzed protein (i.e., Casitone), with a generation time of roughly four hours. In the presence of adequate nutrients, vegetative cells grow and divide indefinitely by transverse binary fission. During the vegetative phase, the cells exhibit co-operative growth in which the growth rate on macromolecules is dependent on cell density. Rosenberg et al., (62) showed that cells that live in a dense swarm accumulate a high local concentration of hydrolytic enzymes like proteases, which in turn provide the swarm with more nutrients by hydrolyzing macromolecules, thus supporting a faster growth rate. This type of communal feeding on organic debris has been likened to a wolf-pack effect (20). The co-operative use of secreted hydrolytic enzymes is thought to have provided the selective pressure for the evolution of multicellular behaviour (social behaviour) in

myxobacteria.

M.xanthus enters the developmental phase of its life-cycle if three conditions are met: (a) nutrient depletion (b) presence of a solid surface and (c) sufficiently high cell density (19, 40, 47, 82). When these conditions are met, tens of thousands of cells move towards a discrete aggregation focus where they pile on top of each other forming a mound. The mound matures into a fruiting body within which the rod-shaped cells get converted to spherical spores. The myxospores are dormant and resistant to heat, desiccation, uv light or freezing conditions that would normally kill vegetative cells (78). When nutrients become available, the spores shed the spore coat and germinate into rod-shaped vegetative cells (23). The fruiting body formed by *M.xanthus* is one of the simplest structures built amongst myxobacteria. *S.aurantiaca* forms an elaborate treelike structure and the myxospores are contained in sac like structures referred to as sporangioles that are borne on top of a large stalk (51).

Development in *M.xanthus*

The mechanism by which *M.xanthus* cells are attracted to an aggregation center to form a fruiting body is not known. The eukaryotic slime-mold, *Dictyostelium discoideum* has been shown to aggregate in response to cAMP which acts as a long-range chemoattractant (43). Shi and Zusman (67) have shown that

chemotaxis also plays an important role in aggregating *M.xanthus* cells. They have identified mutants called *frz* that produce poorly organized fruiting bodies. Several *frz* genes have been cloned and sequenced (6, 7). The *frz* genes have been shown to encode proteins that have functional homology with chemotaxis proteins of enteric bacteria. These genes control the rate of reversal of cells gliding on solid surfaces. The wild-type cells reverse their direction of cell movement every 6-8 minutes. Many *frz* mutants rarely reverse, and some show hyperreversal. The reversal frequency has been correlated with the methylation of FrzCD, a methyl-accepting chemotaxis protein (49). Cells collected from fruiting bodies contain highly methylated FrzCD. Shi and Zusman (67) have suggested that aggregating cells somehow regulate reversal frequency by responding to some chemical in the aggregating field and that is important for fruiting body formation. However, no chemoattractant has yet been identified.

Initiation of development is correlated with an increase in the intracellular levels of guanosine penta and tetraphosphate ([p]ppGpp) (47). Manoil and Kaiser (48) have shown that *M.xanthus* cells can monitor availability of nutrients to bring about changes in the [p]ppGpp levels. Singer and Kaiser (75) have shown that if the levels of [p]ppGpp are increased by the expression of *E.coli relA* gene,

developmental gene expression and early stages of development are induced in *M.xanthus*. They have proposed that these highly phosphorylated nucleotides are synthesized in response to nutrient limitation which in turn activates several developmental genes.

Kroos et al., (38, 39) have used Tn*5-lac* fusions to identify genes that are transcriptionally activated during development. They have estimated that 8% of the genome increases its transcriptional activity during development and only 0.3-0.6% of the genome is essential for development (39). Using pulsed-field gel electrophoresis, Chen et al. (13) have estimated the genome size of *M.xanthus* to be 9454 kbp. Assuming an average gene size of 1 kbp, about 750 genes are expected to increase expression during development, and only about 55 of those genes are essential for development. Kroos et al. (39) showed that 29 genes increased their activities according to a temporal program of developmental gene expression. The expression times ranged from the onset of starvation to the onset of sporulation. Since different genes are expressed at different times during development, the developmental gene expression serves as stage specific markers in the study of fruiting body formation. As is the case with other developmental systems, the program of fruiting body formation in *M.xanthus* combines differential gene expression with morphological changes.

Sporulation begins prior to the completion of fruiting body. The spores form in the interior region of the mound first. O'Connor and Zusman (53, 54, 55) have reported that 1-2% of the population of developing cells do not sporulate and that they remain in the periphery of the developing fruiting bodies. They refer to these cells as peripheral cells. Using Confocal microscopy and Tn*5-lac* reporter genes, Sager and Kaiser (65, 66) showed that the developing fruiting body is divided into two concentric, hemispherical domains. The outer domain is densely packed with rod-shaped cells that move in concentric streams and the inner domain is less densely packed with differentiated, non-motile myxospores. As the fruiting body matures, the inner domain gets packed with myxospores. They suggest that the movement of the myxospore precursor cells to the interior of the fruiting body is a result of their passive transport by the peripheral, motile rod-shaped cells that have not undergone differentiation.

Intercellular Signaling

Cell-cell interactions are essential for the developmental process because conditional mutants have been isolated that cannot sporulate alone, but can be rescued for sporulation by mixing the mutants with wild-type cells. The germinated *M.xanthus* mutant spores from such pairwise mixtures of cells have been shown to

possess the original mutant phenotype indicating that genetic exchange had not taken place. Such a phenotypic rescue is referred to as extracellular complementation. Five classes of mutants designated Asg, Bsg, Csg, Dsg, and Esg (for A signal etc.) have been identified (17, 28). These mutants cannot sporulate alone, but their sporulation can be rescued by codevelopment with wild-type cells or cells that belong to a different mutant class. Each class of mutants behave as if they are deficient in the production of a different developmental signal (A, B, C, D and E signals) but have retained the capacity to respond to the signal which they fail to produce. Each class of mutants exhibits a characteristic pattern of developmental gene expression. Analysis of gene expression has indicated that *asg*, *bsg*, and *dsg* mutants are blocked very early in development (within 2h). The *esg* mutants are blocked about 5h into development. *csg* mutants synthesize developmental markers that are expressed before 6h of development normally, but expression is reduced or abolished for markers that begin to be expressed after 6h of development (17, 35). These signaling mutants are defective in steps leading to initiation of sporulation, but not in sporulation process itself, since sporulation can be rescued by extracellular complementation. The fact that these signaling mutants are blocked during aggregation and are also defective in sporulation suggests

that the formation of cell-cell signaling steps may be needed to synchronize aggregation with sporulation both temporally and spatially within the fruiting body.

Motility

Aggregation and fruiting body formation requires that cells move. *M. xanthus* exhibits a form of motility called gliding motility. Gliding motility is much slower than flagellar driven swimming motility (36). *M.xanthus* moves at an average rate of 1μm/min compared to 50-60μm/min in flagellated bacteria. The rate of gliding is not constant. The cells move in the direction of the long axis of the cell. Cells accelerate, decelerate, stop, and frequently reverse direction. All gliding bacteria produce extracellular slime consisting mainly of polysaccharides and also require contact with a surface in order to move (79). There is no specific organelle identified that is required for the propulsion of cells. The mechanism of gliding motility is not known, but on the basis of the properties of gliding cells, a variety of models have been proposed to explain gliding motility. They include (a) directed extrusion of slime (b) intracellular microfilament contraction-based gliding (c) interaction of rotary motors in the cell envelope with the substratum or extruded slime, and (d) cell generated surfactant gradient (9, 22, 37, 57, 58). None of these models have been confirmed by genetic or biochemical data.

However, many mutants of *M.xanthus* defective in motility have been isolated. Genetic analysis of these mutants has revealed two systems of motility in *M.xanthus* : A (Adventurous motility) and S (Social motility). The A system includes a minimum of 37 genetic loci and S-system requires a minimum of 19 loci (29). The A and S systems contribute additively to wild-type gliding phenotype. A^+S^+ (Wild type) cells exhibit single cell movement (A-motility) as well as group movement (S-motility). Cells with a single mutation in the A-system (A^-S^+) can move only as groups whereas mutations in the S system (A^+S^-) leads to absence of certain patterns of coordinated group motility and they glide primarily as individual cells. Strains with mutations in each system (A^-S^-) are non-motile (31, 32, 44). Shi and Zusman (68) have shown that A-motility and S-motility can functionally be separated by plating cells on rich medium containing different amounts of agar. They have shown that A-motility allows cells to move better than S-motility on relatively firm and dry surfaces (1.5% agar) while S-motility allows cells to move better on relatively soft and wet surfaces (0.3% agar). In addition to A and S motility genes, there are other genetic loci that contribute to motility. A single mutation at the *mgl* locus abolishes both individual and group motility in *M.xanthus* (77). Several genes

found at the *frz* locus have been shown to be involved in chemotaxis and also to control the reversal frequencies of cells (7). Mutations at this locus do not abolish motility. Dual motility systems seen in *M.xanthus* are also common among flagellated bacteria. Several species of *Vibrio* and *Proteus* are able to produce two types of flagella under different conditions. For example, when the bacterium is grown in liquid, *V. parahaemoiyticus* produces a single sheathed polar flagellum that is used for swimming, but when grown on plates, it produces numerous unsheathed lateral flagellae that are used for swarming over the solid surface (50). Based on these findings, Shi and Zusman have suggested that the dual motility systems found in *M.xanthus* allow cells to adapt to a variety of physiological and ecological environments.

Pili

When a colony of A^-S^+ cells is observed under a phase contrast microscope, flare-like projections of cells can be seen at the edges in which the cells never appear to separate from one another (30). Burchard (8) has suggested that cell-cell interactions are required for S-motility since A^-S^+ cells separated by more than one cell length become non-motile. Kaiser and Crosby (34) have found that when cells are in contact with each other, the rate of A^-S^+ cell

movement away from the colony increases with cell density. Several lines of evidence indicate that pili are involved in cell-cell interactions that effect social motility : (a) several A^+S^- mutants that belong to *sgl* and *tgl* loci have been shown to lack pili (33), (b) some *tgl* mutants (A^+S^-) can regain S-motility if they are placed within one cell length of a tgl^+ cell (33), (c) tgl^- cells acquire the ability to synthesize pili when they are close to tgl^+ cells (33), (d) mechanical removal of pili in wild-type cells results in loss of S-motility but has no effect on A-motility (63), and (e) Wu and Kaiser (83) have shown that a mutation in the *sglB* locus results in loss of S-motility as well as pilus biosynthesis. Pili in *M.xanthus* were first reported by MacRae and McCurdy (45). They are proteinaceous structures, 5-10μm in length, about 10nm thick and are polarly located. Typically, 4-10 pili are observed per cell pole. Mutants lacking pili are generally defective in fruiting body formation. Sequencing of the *sglB* locus has revealed open reading frames that have significant homology to three genes involved in pilus biosynthesis in *Pseudomonas aeruginosa* and other gram negative bacteria with Type IV pili (56). How do pili bring about S-motility? The precise role of pili in S-motility is not clear at the present time. It was previously suggested that pili could bind cells together

(16), constrain their movement and lead to social motility instead of adventurous motility. Wu and Kaiser (83) argue that if S-motility were merely a form of A-motility constrained by binding of pili, then mutations that inactivate A-motility should also inactivate S-motility. This is not the case since none of the 37 different mutations in the A-system have any effect on S-motility. So, they suggest that the Type IV pili are either part of a postulated gliding motor that is used in S-motility or are part of a sensory apparatus which activates the gliding motor.

When the colony of A^+S^- mutants are observed under a phase contrast microscope, single cells can be seen moving to and fro from the edge of the colony. Although A^+ cells can move as individuals, they normally tend to remain in close proximity of the main swarm. Those cells that do leave the swarm as individuals, soon reverse their direction and reenter the swarm. Kaiser and Crosby (34) have shown that the rate of A-motility is dependent on cell density. Kaiser (33) has shown that A^+S^- cells lack pili suggesting that pili are not involved in A-motility. The mechanism of A-motility is also not known at the present time. It is speculated that the individual cells periodically return to the swarm in order to replenish a putative motility-enhancing factor (34). If that is the case, then it

would guarantee that individual cells would never be separated from the main swarm by a large distance.

Fibrils

M. xanthus cells produce extracellular organelles called fibrils which are also involved in contact-mediated cell-cell interactions. Unlike pili, the fibrils are peritrichously arranged on the surface of wild-type cells. They are about 50μm in length and the average diameter as determined by scanning electron microscopy is about 30nm. The fibrils were first identified by Fluegel (24), who observed the branching, extracellular filaments attached to hydrated cells stained with India ink. Arnold and Shimkets (1, 2) were the first to suggest that the fibrils played a role in cell-cell cohesion and fruiting body formation. They showed that the appearance of fibrils on the cell surface could be prevented by treating the wild-type cells with congo red. Such treated cells were found to be less cohesive and failed to make fruiting bodies. Congo red is a dye that is known to bind bacterial extracellular polysaccharide which suggests that fibrils are composed of polysaccharide. The mechanism by which congo red inhibits fibril formation and agglutination is not known. Wild-type cells of *M.xanthus* are cohesive and agglutinate in the presence of divalent cations like calcium and magnesium. The rate of agglutination can be quantitated

by means of an agglutination assay which is performed in a buffer containing calcium (2, 14). When wild-type cells are used in this assay, cells that are dispersed at the beginning of the assay will begin to attach to each other forming small clumps. These clumps will coalesce to form larger clumps which will eventually settle out of suspension. In *S.aurantiaca*, there are at least two types of cohesion systems - class A cohesion system is constitutively expressed and energy independent while class B cohesion system is induced by calcium and inhibited by respiratory poisons (26). In *M.xanthus*, agglutination is dependent on calcium, magnesium and an energy source. Shimkets (70) showed that agglutination can be inhibited by energy poisons that block electron transport (cyanide and azide), uncouple oxidative phosphorylation [(carbonyl cyanide-4-(trifluoromethoxy)phenylhydrozone (FCCP)], or inhibit membrane-bound ATPase [N,N'dicyclohexylcarbodiimide (DCCD)]. He suggested that energy may be required for the synthesis or secretion of cell surface components necessary for agglutination. He also showed that transcription and translation was not required for agglutination since growth inhibiting concentrations of rifampin and chloramphenicol did not block agglutination. The social motility mutants (A^+S^-) are generally found to be defective in agglutination (14, 33). Dana and Shimkets (14) have shown that a mutation at the

stk locus results in enhanced agglutination and fibril production in wild-type cells. The *stk* mutation can also enhance agglutination in several S-motility mutants. Mutations in the *asgA*, *asgB*, and *asgC* loci reduce, but do not completely eliminate agglutination (41). All three mutants could bind congo red with a K_a similar to that of wild-type. An unknown, heat-stable factor obtained from developing wild-type cells could restore agglutination in an *asgB* mutant. These results suggest that in addition to fibrils, there are several other factors in *M. xanthus* that play a role in cell-cell agglutination.

A class of S-motility mutants termed *dsp* (for dispersed growing) was shown to be severely deficient in agglutination and fruiting body formation (71). The *dsp* mutant also lacks fibrils suggesting that fibrils play a role in agglutination and fruiting body formation. Arnold and Shimkets (1,2) observed fibrils on wild-type cells prepared from the agglutination buffer by means of transmission and scanning electron microscopy. Behmlander and Dworkin (3) used low-voltage scanning electron microscopy and showed that fibrils are expressed in wild-type cells exhibiting group motility but not in cells exhibiting adventurous motility. This was done to prove that the fibrils observed by other techniques was not a result of dehydration artifacts of electron microscopy. They have conclusively shown that fibrils are a major structural component in *M.xanthus*.

They also purified fibrils from wild-type cells and showed that they are composed of protein and carbohydrate in a 1.0:1.2 weight ratio. Fibrils were found to compose up to 10% of the dry weight of cells. The polysaccharide portion of the fibrils was shown to be composed of five different monosaccharides - galactose, glucose, glucosamine, rhamnose and xylose. By treating isolated fibrils with periodic acid or protease, they found that the fibrils appeared to consist of a carbohydrate backbone with associated proteins (4). Using a monoclonal antibody (MAb 2105) directed against a cell surface antigen, a protein antigen designated IFP-1 (integral fibril protein) was localized to the fibrils (5). IFP-1 antigen actually is comprised of five major proteins whose molecular sizes range from 14 to 66kDa. All the five proteins have been purified from isolated fibrils by preparative SDS-PAGE. Western blot analysis of the proteins indicate that the different molecular sizes of the proteins may result from covalently associated multimers of a single small molecular size subunit. The N-terminal amino acid sequence analysis of one of the proteins (IFP-1:31) revealed no significant homology to any other known sequences. The ratio of the different multimers changes during development with the 14kDa subunit increasing the most during early stages of development. IFP-1 is expressed at extremely low levels in the *dsp* mutant. Chang and Dworkin (11)

added purified fibrils from wild-type cells to *dsp* mutant and showed that the development and agglutination defects in the mutant could be corrected, indicating that fibrils play an integral role in the social behaviors of *M. xanthus*. They suggested that fibrils interact with the cells by means of a ligand-receptor interaction. Recently, they isolated several secondary mutants of *dsp* which could not be rescued for cohesion and development by the addition of isolated fibrils (12). They have argued that these mutants *(fbd)* lack a putative fibril receptor. The *fbd* mutants lack fibrils, but unlike *dsp*, have regained social motility. From all of the studies mentioned above, several ideas have emerged as to how fibrils mediate cell-cell interactions. Fibrils may establish physical bridges between cells and thus bring about agglutination. They may serve as antennae to sense nearby cells or particulate substrates. Shimkets and Rafiee (73) have shown that the C-signal is located in the extracellular matrix suggesting that the fibrils may also serve as physical structures for the presentation of intercellular signals required for the completion of development. While it is apparent that extracellular appendages of *M.xanthus* mediate social and developmental behaviours like motility, agglutination and fruiting body formation, the mechanism of how they are accomplished remains to be seen.

OBJECTIVES OF DISSERTATION

As part of a study to further the knowledge on intercellular signaling, Downard et al., (17) identified a signaling mutant called *esg*. *esg* was deficient in developmental aggregation, sporulation and *tps* gene expression. These phenotypes could be substantially corrected by extracellular complementation with wild-type and the other signaling mutants. The *esg* locus was shown to reside within a 2.5kb region of *M.xanthus* chromosome. Chapter IV describes the genetic analysis of the *esg* locus and shows that the locus contains two complementation groups.

In an effort to understand other phenotypic defects in *esg*, it was found that *esg* mutants bound calcofluor white dye poorly. Calcofluor white is a dye similar to congo red (14). This result suggested that *esg* was probably deficient in the production of extracellular fibrils and behaved like S-motility mutants. When its motility was tested, *esg* exhibited group motility which was dependent on the genetic background. This phenotype, referred to as Cds, has not been described and thus led me to identify other insertional mutants that bound calcofluor white poorly while still exhibiting group motility. Chapter II describes the identification and characterization of those mutants.

Toal et al., (81) sequenced the *esg* locus and found that it encodes the E1α and E1β subunits of a branched-chain keto acid dehydrogenase (BCKAD) which is involved in branched-chain amino acid metabolism. They show that the *esg* -encoded BCKAD is involved in the synthesis of long branched-chain fatty acids, since *esg* mutants showed reduced levels of this class of compounds. The synthesis and functions of these branched-chain fatty acids in *M.xanthus* are not well understood (18). Chapter III describes the phenotypic and genetic characterization of one of the newly identified Cds mutants (SR171). The results show that this mutant is also deficient in the production of long branched-chain fatty acids. Some of the possible roles of SR171 in branched-chain amino acid metabolism will be discussed.

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CHAPTER 2

Identification and Characterization of *Myxococcus xanthus* Mutants Deficient in Calcofluor White Binding

Abstract

Calcofluor white is a fluorescent dye that binds to the extracellular polysaccharide in *Myxococcus xanthus* and many other bacteria. We observed that an *esg* mutant showed less binding to calcofluor white than wild-type cells. Unlike S-motility mutants that share this phenotype, *esg* exhibited S-motility. This led us to identify a collection of nine new transposon insertion mutants, designated Cds (calcofluor white binding deficient, S-motile), which exhibited a phenotype similar to the *esg* strain. The Cds phenotype was relatively common (0.6%) among the random insertion mutants screened. The Cds mutants were also found to be defective in cell-cell agglutination and developmental aggregation. Extracellular matrix fibrils composed of roughly equal amounts of polysaccharide and protein have been shown to be involved in agglutination, and electron microscopic examination showed that *esg* and the other Cds mutants lack the wild-type level of fibrils. Analysis of total *M. xanthus* carbohydrate demonstrated that polysaccharide content increased by about 50% when wild-type cells entered stationary phase. This induction was reduced or eliminated in all of the Cds

mutants. The degree of polysaccharide deficiency in the Cds mutants correlated with the degree of loss of agglutination and dye binding as well as with the severity of the developmental aggregation defect. Preliminary genetic characterization demonstrated that the transposon insertion mutations in three of the Cds mutants (SR53, SR171, and SR200) were loosely linked. The results of this study suggest that many genes are involved in the production of calcofluor white binding polysaccharide material found in the extracellular matrix and that the polysaccharide is fibrillar. These results are also consistent with the findings of earlier studies which indicated that fibrils function to join agglutinating cells and in the formation of multicellular fruiting aggregates.

Introduction

The myxobacteria display a wide range of social adaptations during their life cycle (14). These social behaviors, which include coordinated movements and multicellular development, are being intensively studied in the gram-negative soil bacterium *Myxococcus xanthus*. Multicellular behaviors in *M. xanthus* involve thousands of cells and are dependent on gliding motility and cell-cell contact mediated interactions (32). The most notable multicellular behavior and the defining characteristic of the myxobacteria is fruiting body formation (development). Under conditions of high cell density and nutrient depletion on a solid surface, vegetative cells move toward discrete foci and form multicellular mounds that develop into fruiting bodies (31). The individual rod-shaped cells are converted to spherical, environmentally resistant myxospores within the fruiting body. It is believed that *M. xanthus* requires at least five extracellular signals (A, B, C, D and E signals) to coordinate this multicellular developmental process (14).

Movement on a solid surface by *M. xanthus* is accomplished by gliding (17). The mechanism of gliding motility is not understood. However, genetic analysis has shown that gliding is controlled by two distinct multigene systems known as A (for adventurous gliding) and S (for social gliding) (19). A^+S^+ cells glide individually and as large cell

masses. Cells with a mutation in the A system (A^-S^+ or S-motile cells) move primarily in groups and they swarm very well on low percentage agar surfaces (30). S-motility requires that cells be in close apposition (21). Cells with a mutation in the S system (A^+S^- or A-motile cells) have lost communal motility and glide primarily as individuals; they do not swarm well on a low percentage agar surface. S-motility mutants are generally defective in fruiting body formation (19,28).

It is believed that extracellular appendages mediate a variety of cell-cell interactions in *M. xanthus* (14). Pili and fibrils are the two types of extracellular appendages that have been described. Pili are proteinaceous structures, less than 10nm in diameter, that are invariably found at cell poles. Pili have been associated with a functional S-motility system since many A^+S^- mutants have been shown to lack pili and a cluster of S-motility genes has been shown to encode a type IV pilus biosynthetic system (20,35). Fibrils, on the other hand, are made of equal amounts of protein and carbohydrate, and they display a diameter of roughly 15-30 nm when viewed by scanning electron microscopy (2,4,5). These structures are peritrichously arranged on the cell surface. Arnold and Shimkets (1) were the first to suggest that extracellular fibrils play a role in

cell-cell cohesion (agglutination) and development. This conclusion was based on experiments with a simple agglutination test and the dye Congo red which has affinity for bacterial extracellular polysaccharide. Initially, they were able to show that agglutination and fruiting body formation were inhibited in wild-type cells by Congo red. Several S-motility mutants were shown to be defective in agglutination and to bind Congo red poorly (2,8). Besides lacking pili, these mutants also failed to produce fibrils on the cell surface. One group of S-motility mutants, with defects at the *dsp* locus, was the most agglutination defective; but these mutants retained pili (2,9). Consistent with the hypothesis that fibrils are important mediators of agglutination and fruiting body formation, Chang and Dworkin were able to demonstrate that the addition of fibrils isolated from wild-type cells restores agglutination and development in the fibril deficient *dsp* mutant (7).

The *esg* locus is essential for *M. xanthus* fruiting body formation but not for vegetative growth (11). Nevertheless, growing *esg* mutant cells differ phenotypically in a number of ways from wild-type cells. It was previously reported that an *esg* mutant displays lowered *tps* gene expression during vegetative growth (11), lowered growth yields in a chemically defined medium (34), and much less yellow pigmentation than wild-type cells in the same defined

medium (34). More recently we have noted a distinct colony morphology difference between *esg* and wild-type in the DK1622 (A^+S^+ motility) genetic background (12). This colony morphology difference was not evident in the DZF1 (A^+S^-) background. The *esg* locus has been shown to encode components of a branched-chain keto acid dehydrogenase which is involved in metabolism of the branched-chain amino acids (BCAA) (34). In *M. xanthus*, this enzyme is involved in the pathway for branched-chain fatty acid biosynthesis from the BCAA. The branched-chain fatty acids (BCFA) play a structural role in *M. xanthus* cells as components of cellular phospholipid but analysis of *esg* mutants has suggested that BCFA also play a regulatory role in a fatty acid-mediated cell-cell communication system (E-signaling) and/or as components of a signal transduction system controlling gene expression (13). While attempting to characterize other defects in *esg*, we observed that this mutant resembled S-motility mutants in that *esg* was deficient in agglutination and showed reduced binding to calcofluor white (a fluorescent dye which binds to bacterial exopolysaccharide in a manner similar to Congo red). However, the *esg* strain exhibited S-motility. This phenotype was novel and, in this report, we describe the isolation and properties of several additional transposon insertion mutants that fail to bind wild-type levels of calcofluor

white but retain S-motility. These mutants were found to lack fibrils. The properties of these mutants support the hypothesis that fibrils mediate cell-cell agglutination (cohesion) and play an essential role in developmental aggregation.

Materials and methods

Bacterial strains and growth conditions. *M. xanthus* was grown in buffered casitone medium (CTT) (18) or casitone-yeast extract medium (CYE) (6) at 30°C with vigorous shaking at 250rpm. *Escherichia coli* strains were grown in L broth or on L agar (L broth with 1.5% agar) at 37°C (29). Kanamycin was used at a concentration of 50μg/ml. The wild-type *M. xanthus* strain used in this study was DK1622 (19). JD300 (*esg*::Tn*5*) (34) and DK3468 (*dsp-1680*) (9) are DK1622 derivatives. DZF1 (*sglA1*), which is equivalent to DK101 (9), is a strain that agglutinates poorly and has a defect in S-motility.

Isolation of transposon insertion mutants. (a) Transduction. DK1622 was grown in CTT broth to $5x10^8$ cells/ml and an aliquot was infected with P1:Tn*5-lac* (24) for mutagenesis. Since phage P1 does not replicate in *M. xanthus*, kanamycin resistant transductants result from the transposition of Tn*5-lac* from the viral to the bacterial chromosome. After infection, cells containing Tn*5-lac* insertions were identified by plating the infected cells on CTT medium containing kanamycin .

(b) Electroporation. DK1622 cells were collected by centrifugation at 8000 x g for 10 minutes. The cells were washed with cold water twice and 40μl of a concentrated cell suspension ($5X10^{10}$ cells/ml)

were mixed with 0.5-1.0 μg of pBR322:Tn*5* DNA. The DNA was introduced into cells by electroporation (400Ω, 25μF, and 0.65KV) in a 0.1cm cuvette at room temperature (22). The cells were immediately diluted into 1 ml of CTT broth and allowed to grow at 30°C for 4 hr. The cells were then mixed with top agar and plated on CTT containing kanamycin (50μg/ml). After 18 hr, additional kanamycin was added in top agar to bring the final concentration to 70μg/ml. The additional kanamycin helped to reduce background growth of cells which did not contain a Tn*5* insertion. The plates were incubated at 30°C for 7 days to select kanamycin resistant colonies.

Calcofluor white binding and S-motility assays. The transposon insertion strains were grown to approximately $5x10^8$ cells/ml in 5 ml of CTT broth and collected by centrifugation at 8000xg for 10 min at 4°C. The cells were then washed once in TM buffer (10mM Tris pH7.6, 1mM $MgSO_4$) and resuspended in TM to a calculated cell density of $5x10^9$ cells/ml. To test for calcofluor white binding, a 10 μl portion of each cell suspension was spotted onto a CYE plate containing calcofluor white (50μg/ml) (9). The cells were incubated for 6 days at 30°C and dye binding was qualitatively determined by observing the colonies under a hand-held

long wavelength UV light source.

To test for S-motility, 2 μl portions of the same cell suspensions in TM buffer were applied to CYE plates containing 0.3% agar (30). The cells were incubated for 72 hr at 30°C. Under these conditions *M. xanthus* strains exhibiting S-motility (group motility) are able to glide on the agar surface to form large colonies. S-motility mutants, on the other hand, grow to form small colonies with the cells heaped on top of each other. Spots showing a diameter of 1.5 cm (approximately 50% of the wild-type rate of colony expansion) or greater were scored as exhibiting group motility and those spots that had a diameter of less than 0.8 cm (approximately 20% of the wild-type rate of colony expansion) were scored as group motility mutants.

Agglutination assay. The agglutination assay was performed as has been described previously (31). Cells were grown to $5x10^8$ cells/ml in CTT broth, collected by centrifugation at 8000 x g for 5 min at 4°C, washed with 10mM MOPS (pH 6.8) and resuspended in MCM buffer (10mM MOPS pH6.8, 1mM $CaCl_2$, 10mM $MgCl_2$). The A_{625} of the cell suspension was measured in a Beckman DU-40 spectrophotometer over a 2 hr time interval. These data were used to calculate the relative agglutination value, a number relating the rate of agglutination for each of the the Cds mutant strains to the

wild-type. The relative agglutination values were determined by first dividing the A_{625} of the cell suspensions at the end of the 2 hr incubation by the initial A_{625}. The quotient obtained for DK1622 (wild-type) was then divided by the quotient for each mutant strain (9).

Development assay. Fruiting body formation by the Cds mutant strains was analyzed in submerged culture by a modification of the method described previously (25). Cells were grown in CYE broth to $5x10^8$ cells/ml and harvested by centrifugation. The cell pellets were washed once with 10 mM MOPS buffer (pH 7.0) and resuspended at a concentration of $1.25x10^9$ cells/ml in MC7 buffer (10mM MOPS, 1mM $CaCl_2$, pH 7.0). The cell suspensions were subjected to four two-fold serial dilutions and 0.5 ml of each dilution was placed in the well of a 24-well tissue culture plate and incubated at 30°C. Fruiting bodies were observed and photographed after 96 hrs.

Transmission electron microscopy. Transmission electron microscopy (TEM) of negatively stained specimens was used to observe pili and fibril material. For the observation of pili, *M. xanthus* cells were grown in CTT broth to $5x10^8$ cells/ml, collected by centrifugation, and suspended at the same cell density in 0.1M Tris (pH 7.6). One drop of the cell suspension was placed on a

formvar coated copper grid and the cells were allowed to settle onto the grid for 3 min. The drop was blotted and the cells were washed twice in a solution containing the negative stain uranyl acetate (1%) and the spreading agent bacitracin (30μg/ml) (20). This solution was blotted and the dried grid was observed in a JEOL 2000 electron microscope. The levels of piliation in the mutant strains were estimated as has been described (20). For observation of fibrils, cells were suspended in MCM agglutination buffer for about 1 hr (agglutination conditions) and samples of these cells were taken from the center of the cuvette. These cells were placed on the TEM grid and stained as described above before observation on the electron microscope.

Total carbohydrate assay. *M .xanthus* cells were grown to $4x10^8$ cell/ml in CYE broth, collected by centrifugation and resuspended at $2x10^9$ cells/ml in fresh CYE. These high cell density cell suspensions were allowed to gently shake for 6 hr at 30°C; conditions under which there is little growth of the *M. xanthus* cells. Under these conditions (high cell density induction) production of a high-molecular weight viscous polysaccharide is induced in wild-type cells just as it is when cells enter stationary phase during normal growth in liquid culture, but high cell density induction results in a more rapid induction of polysaccharide making it a more

convenient method for these studies (23). Samples were collected from the cell suspensions at the beginning and end of the 6 hr incubation; samples containing growing cells and stationary phase cells, respectively. The cells were washed in 0.85% NaCl and the sample was suspended in 2 ml of water. The samples were sonicated and aliquots were taken for protein and carbohydrate analysis. The total protein concentrations were determined as equivalents of bovine serum albumin by using the BCA Protein Assay Reagent Kit (Pierce). The total carbohydrate concentrations were determined as glucose equivalents by the anthrone-sulfuric acid method (16). The total specific carbohydrate content of cell lysates is reported as μg carbohydrate / μg protein. Most of the carbohydrate produced during the induction was easily removed from sonicated lysates by low speed centrifugation and was ethanol precipitable, and is therefore likely to be in the form of polysaccharide.

Cloning of Cds loci. The *M. xanthus* DNA flanking three of the Cds transposon insertions was cloned in *E.coli* using the transposon associated kanamycin resistance gene as a selectable marker. The Cds locus defined by the insertion (Ω53) in strain SR53 was cloned as a 14-kb *Hind*III fragment in the pGEM7Zf+ vector (Promega) to produce pSR53. The 14-kb insert in pSR53 contained about 11-kb

from Tn*5-lac* and 3-kb of *M. xanthus* DNA flanking one side of the insertion. DNA from one side of the insertion (Ω171) in strain SR171 was cloned as a 4.1-kb *Bam*HI fragment in the pGEM3Zf+ vector (Promega) to produce pSR171. This plasmid has 1.1-kb of *M .xanthus* DNA adjacent to Tn*5*. The DNA cloned from SR200 in the vector pGEM7Zf+ was an 18-kb *Bam*HI fragment. The pSR200 plasmid has about 15-kb of *M .xanthus* DNA adjacent to Tn*5* insertion Ω200. *M. xanthus* DNA was prepared by the method of Avery and Kaiser (3). Plasmid DNA isolation and transformation in *E. coli* was performed by standard techniques (29). Restriction enzymes, DNA Polymerase I (Klenow fragment) and T4 DNA ligase were all used as recommended by the manufacturer. To prepare probes for Southern analysis, DNA fragments generated by restriction enzyme digestion of the cloned DNAs were eluted from agarose gels using Qiagen's gel extraction kit. DNA fragments were radiolabelled with [α-^{32}P]dCTP (Amersham) based on the method developed by Feinberg and Vogelstein (15). The procedure for Southern blot hybridization analysis of DK1622 DNA using the labeled Cds probes has been described (29). Hybond nylon filters (Amersham) were employed as the blotting membranes. Membrane filters containing an *M. xanthus* library of cosmid-cloned chromosomal DNA, generously provided by Dr. Ron Gill, were screened by hybridization with the Cds probes.

Positive clones were also tested by Southern analysis of purified cosmid DNAs.

Results

Calcofluor white binding and motility by *esg* cells. Besides obvious developmental defects, *esg* mutants displayed a wide range of phenotypic alterations during the vegetative growth phase of the life cycle. Perhaps the most obvious alterations were that *esg* mutants showed less clumping than wild-type cells during growth in complex media and that the *esg* colony morphology differed from the wild-type. These alterations were dependent on the genetic background. The differences between wild-type and *esg* were most pronounced in the DK1622 (A^+S^+) background and were almost undetectable in the DZF1 (A^+S^-) background. More specifically, we observed that an *esg* mutant in the DK1622 background resembled the DZF1 strain which is an S-motility mutant. Previously, it was shown that a major difference between the DK1622 and DZF1 strains is that DZF1 (like other S-motility mutants) lacks fibrils (9). Fibril deficient *M. xanthus* mutants also are less cohesive than DK1622 and bind less calcofluor white, a fluorescent dye which binds to extracellular polysaccharide. Calcofluor white is likely to bind primarily to fibril polysaccharide since Dana and Shimkets were able to demonstrate a strong correlation between the loss of fibrils and calcofluor white binding in *M. xanthus* mutant strains (9). It has

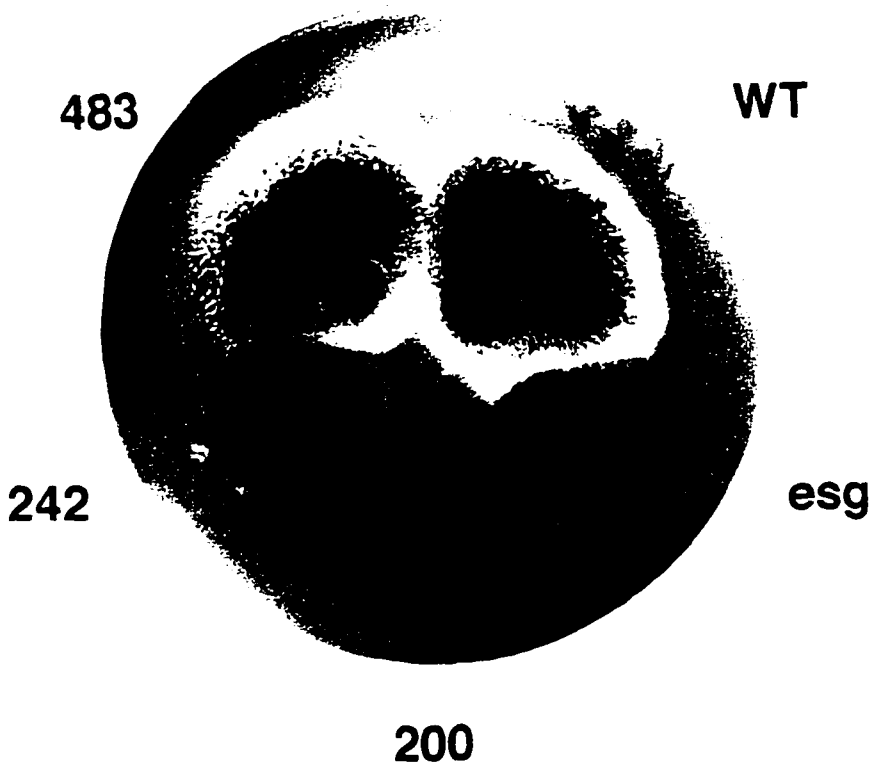

Figure 1. Calcofluor white binding by wild-type and Cds mutants. *M. xanthus* strains were tested for calcofluor white binding by plating concentrated cell suspensions on CYE agar containing the fluorescent dye. The strains shown in this photograph are DK1622 (WT), JD300 (esg), SR200 (200), SR242 (242), and SR483 (483). Although the fluorescence exhibited by DK1622 and SR483 appear similar in the photograph, the difference was readily apparent by direct visual examination of the plate.

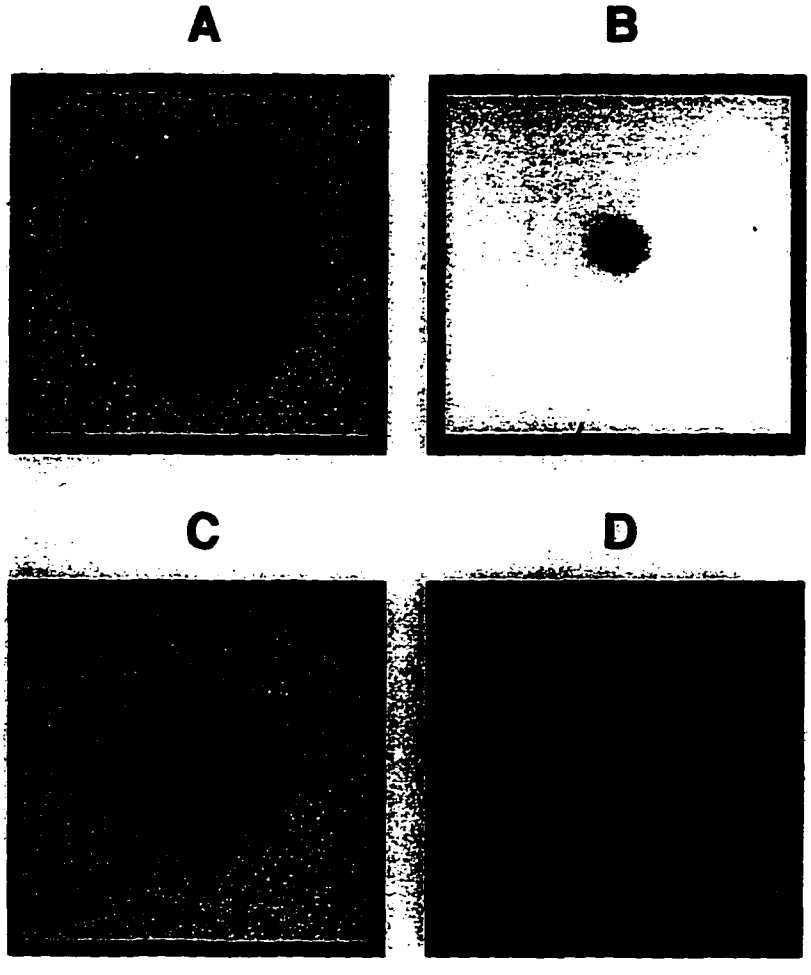

Figure 2. S-motility by wild-type, an S-motility mutant and Cds mutant *M. xanthus* strains. S-motility was assayed by plating concentrated cell suspensions on CYE containing 0.3% agar. S-motility is shown by the ability of masses of cells to swarm laterally over the surface of the agar. The strains shown in the photograph are DK1622 (S-motile) (A), DZF1 (S-nonmotile) (B), SR171 (C), and SR483 (D).

also been demonstrated that Congo red, another dye which binds extracellular polysaccharide, binds to fibrils isolated from wild-type cells (4). Bacterial colonies which bind calcofluor white can be easily identified because they become fluorescent when illuminated with long-wave UV light. To determine if an *esg* mutant resembled S-motility mutants and failed to bind calcofluor white, *esg* cells were spotted on calcofluor white plates and examined for fluorescence. The result was that the *esg* cells bound the dye poorly compared to wild-type cells (Figure 1) and in this respect resembled the S-motility mutants. However, observation of *esg* cells at the edges of colonies growing on agar plates indicated that these cells retained the ability to form swirls of cells characteristic of S-motility (group motility) (data not shown). We also tested the ability of the *esg*::Tn*5* mutant colonies to spread on a low percentage agar surface. Shi and Zusman (30) have previously shown that S-motility mutant colonies do not spread well on a 0.3% agar surface. *esg* cells were able to spread well on the low agar content surface although the rate of expansion of the colony edge was reduced to about 70% of the parental wild-type level (data not shown). In contrast, the rate of expansion of an S-motility mutant, DZF1, was only about 15% of the wild-type (Figure 2). The phenotype exhibited by *esg*, poor calcofluor white binding with a relatively high level of

AGGLUTINATION ASSAY

Strain	Relative Agglutination[a]	Fluorescence[b]
DK1622(WT)	1.00	+++
JD300(esg)	0.17	-
SR16	0.22	+
SR53	0.15	-
SR171	0.17	-
SR200	0.15	-
SR233	0.16	-
SR234	0.26	++
SR242	0.16	-
SR263	0.22	+
SR483	0.30	++

Table 1. [a] Relative agglutination was determined by dividing the absorbance of the cell suspension after 2 hr of incubation at room temperature by its initial absorbance. The quotient obtained for DK1622 (WT), was then divided by the quotient for each mutant strain, yielding the relative agglutination value for that strain.

[b] The level of calcofluor white dye binding was qualitatively assessed with DK1622 as the reference strain. (-) indicates no observable binding, (+) indicates slight binding and (++) indicates moderate binding.

S-motility, has not been previously described and it was designated Cds (calcofluor white binding deficient, S-motile) .

Isolation of transposon insertion Cds mutants. Since it is relatively simple to screen *M. xanthus* mutant colonies for calcofluor white binding, this approach was employed as the first step in identifying additional mutants with the Cds phenotype. Wild-type DK1622 cells were first subjected to random transposon insertion mutagenesis using Tn*5* or Tn*5lac*. The insertion mutants were then screened for calcofluor white binding on plates. Out of a total 1400 insertion mutant colonies, 12 strains were obtained that showed some degree of reduced calcofluor white binding. Nine of those 12 mutants exhibited high levels of S-motility (>50% of the wild-type level) on 0.3% agar plates and were therefore designated Cds mutants. The other three mutants were strongly deficient in S-motility (<20% of the wild-type level) and resembled the S-motility mutants that have been described previously. The Cds strains were relatively common since 0.6% of the total transposon insertion strain collection shared the phenotype. Six of the Cds strains have Tn*5* insertions and the other three strains have Tn*5lac* insertions (Table 1). Several strains did not bind detectable levels of calcofluor white, however three strains (SR16, SR234 and SR483) did exhibit some dye binding (Table 1). The level of dye binding by

three of the newly isolated Cds mutants (SR200, SR242, and SR483) is shown in comparison with the wild-type and *esg* strains in Figure 1. All Cds mutants, including *esg*, could be distinguished from each other based on colony pigmentation, texture and morphology.

Agglutination assays. A previous study has shown that loss of calcofluor white binding by mutant strains is highly correlated with reduced agglutination (cohesiveness) by *M. xanthus* cells (9). The agglutination assay was used to quantify the degree of cohesiveness in *esg* and the other Cds mutants. This assay measures the decrease in the optical density of a cell suspension over time (1,9,31). The decrease in optical density is due to the falling of clumped (cohesive) cells from suspension. The relative agglutination of the Cds mutants with respect to the wild-type strain is shown in Table 1. *esg* and all the other mutants showed a reduction in the rate of agglutination. Furthermore, the relative agglutination value was roughly correlated with the degree of calcofluor white binding in the mutant strains. Strains that had a relative agglutination value of 0.17 or less showed no fluorescence on the calcofluor white plates. Those strains that had a relative agglutination value of 0.22 or greater exhibited readily detectable levels of fluorescence. The loss of agglutination in association with reduced calcofluor white binding suggested that the mutants may be defective in the

ANALYSIS OF FIBRILS

STRAIN	FIBRILS
DK1622(WT)	++
JD300(esg)	-
SR16	+
SR53	-
SR171	-
SR200	-
SR233	-
SR234	+
SR242	+
SR263	+
SR483	+

Table 2. Fibrils were observed by transmission electron microscopy (TEM). Cells were suspended in agglutination buffer for an hour and samples were collected from the center of the cuvette and placed on formvar coated copper grids. The grids were then washed twice in the negative stain uranyl acetate (1%). The presence of fibrils was qualitatively assessed with (-) indicating no observable fibrils and (+) indicating a reduced amount of fibril material with respect to DK1622.

production of extracellular matrix fibrils.

Examination of Cds mutants for fibrils and pili. To determine if reduced calcofluor white binding and loss of cohesion found in the mutants correlated with a reduction of extracellular fibrils, the mutants were observed with negative staining by transmission electron microscopy (TEM). *esg* and the other Cds mutants examined by TEM were all found to have reduced levels of fibril material. Detectable levels of this material were observed only in mutant strains SR16, SR234, SR242, SR263, and SR483 (Table 2). Several of the Cds mutants were also subjected to high-resolution observation of the cell surface by field emission scanning electron microscopy (FESEM). Many thanks goes to Martin Dworkin at the University of Minnesota for performing the FESEM analysis. For this analysis, cells were deposited on glass chips and incubated under high cell density conditions previously shown to be favorable for fibril visualization (5). FESEM examination of *esg* and three other Cds mutants (SR171, SR200, and SR233) indicated that they lack fibrils.

Kaiser first observed that many S-motility mutants lack pili (20) and there is increasing evidence that pili play an essential role in S-motility (35). Since the Cds mutants have been observed to retain a significant level of S-motility but, like S-motility mutants, to be

PILIATION OF STRAINS

STRAIN	% PILI[a]
DK1622(WT)	54
JD300(esg)	44
SR16	48
SR53	40
SR171	42
SR200	42
SR233	48
SR234	52
SR242	56
SR263	40
SR483	52

Table 3. Cells were grown in CTT medium, harvested in mid-log phase, washed twice and resuspended in Tris (pH7.6) buffer. A drop of the cell suspension was placed on a formvar coated copper grid and negatively stained with 1% uranyl acetate. The dried grid was then observed for piliated cells in a JEOL 2000 electron microscope. [a] The number of cell ends with pili was divided by the total number of ends scored. The fraction obtained was expressed as a percentage.

SPECIFIC CARBOHYDRATE CONTENT[a]

STRAIN	LP	SP	% WT INCREASE
	(μg Carbohydrate / μg Protein)		
DK1622(WT)	0.14	0.21	100
JD300(esg)	0.11	0.13	36
SR16	0.10	0.12	40
SR53	0.09	0.09	0
SR171	0.10	0.11	20
SR200	0.10	0.10	0
SR233	0.08	0.08	0
SR234	0.15	0.17	27
SR242	0.10	0.11	20
SR263	0.11	0.12	18
SR483	0.12	0.14	33

Table 4. [a] The specific carbohydrate content was determined as ug carbohydrate / ug of total cell protein (see Material and Methods). The samples tested were from log phase cells (LP) or stationary phase cells (SP). The increase in stationary phase carbohydrate content is expressed as a percentage relative to the increase observed for DK1622. Note that most of the Cds mutants also had a lower specific carbohydrate content than wild-type cells during log phase growth.

fibril deficient; it was of interest to examine the Cds mutants for pili. The Cds mutant cells were directly examined for pili using TEM. As shown in Table 3, all of the Cds mutants had a percentage of piliated cell poles which was similar to the wild-type. A majority of the wild-type and mutant cells had 4-8 pili at one of the two cell poles. The length of the pili was sometimes difficult to determine but did not seem to differ between wild-type and the Cds mutants (data not shown). The presence of pili in the Cds mutants is consistent with the idea that these structures are required for S-motility.

Induction of polysaccharide. We have noticed that as wild-type cells leave the exponential growth phase in a rich broth medium, there is a strong induction of viscous extracellular polysaccharide (23). This extracellular polysaccharide appears to be associated with fibrils since the induced cells agglutinate more rapidly than the uninduced cells (data not shown). A total carbohydrate assay was performed to test the Cds mutants for this response (Table 4). In the induced wild-type cells, the ratio of cell-associated carbohydrate to total cellular protein increased by about 50% over the vegetative cell value. All of the Cds mutants, including *esg*, exhibited significantly lower levels of polysaccharide induction. These values ranged from about 40% of the wild-type (*esg* and SR16)

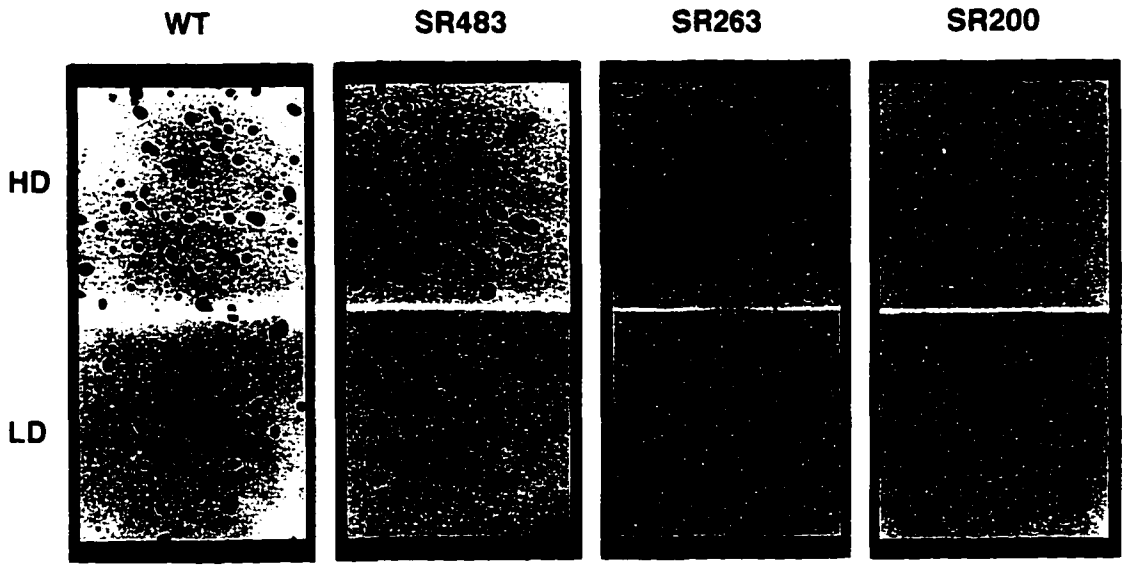

Figure 3. Developmental aggregation by *M. xanthus* strains. *M. xanthus* strains were tested for developmental aggregation in submerged culture using 24-well microtitre plates. The strains tested were DK1622 (WT); and Cds mutant strains SR483, SR263, and SR200. Developmental aggregation was examined at high cell density (HD) ($1.2x10^9$ cells per ml) and low cell density (LD) ($1.5x10^8$ cell per ml). The dark aggregates of cells observed are spore-filled fruiting bodies and lighter aggregates are incomplete with less height and fewer spores than normal *M. xanthus* fruiting bodies.

Developmental Aggregation

STRAIN	LD	HD
DK1622(WT)	+	+
JD300(esg)	-	-
SR16	-	+
SR53	-	-
SR171	-	-
SR200	-	-
SR233	-	-
SR234	-	+
SR242	-	+
SR263	-	AFB
SR483	-	+

Table 5. Fruiting body formation was tested in submerged culture under low cell density (LD) and high cell density (HD) conditions. (-) indicates no aggregation, (+) indicates wild-type like fruiting body formation and AFB indicates abnormal fruiting body formation.

to undetectable levels of induction (SR171, SR200 and SR233). Again, the degree of polysaccharide induction deficiency was generally correlated with the degree of loss of agglutination and calcofluor white binding. It should also be noted that the ratio of carbohydrate to protein in the log phase Cds mutant cells was generally lower than the wild-type level and these values varied amongst the mutants (Table 4). Presently, the significance of these results is unclear.

Developmental aggregation defects. *esg* was previously shown to be defective in developmental aggregation (11). Since the other Cds mutants shared several of the phenotypes of *esg*, we determined if they were also defective in development. Fruiting body formation was observed in sub-merged culture on a plastic surface over an 8-fold range in cell density ($1.2x10^9$ to $1.5x10^8$ cells per ml). Wild-type cells had well-developed fruiting bodies at all cell densities tested. The size and the number of fruiting bodies were less at low cell density (Figure 3, WT, LD) than at high density (Figure 3, WT, HD). All the Cds mutants were defective in developmental aggregation to some extent (Table 5). The mutant strains SR53, SR171, SR200 and SR233 failed to form normal aggregates at all cell densities tested (SR200, Figure 3). This pattern was in contrast to that observed for SR483 which aggregated normally at

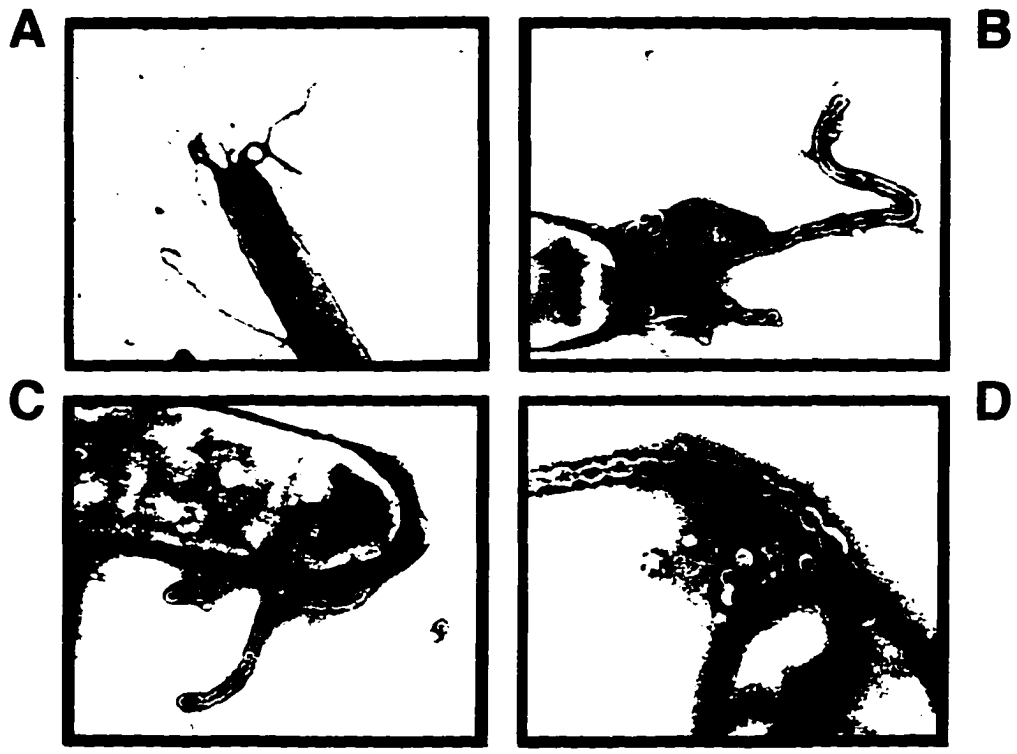

Figure 4. Transmission electron microscopy (TEM) of Cds mutant SR200. SR200 cells were negatively stained and examined by TEM. Unusual polar structures were observed primarily emanating from cell poles. These structures associated with different cell poles are shown at low (A), intermediate (B and C) and high (D) magnification (5-fold range in magnification). B and C show the two poles of the same cell. A subpolar region, which was differentially stained in these cells, should also be noted.

high cell density but formed abnormally shaped aggregates at low cell density (Figure 3). An intermediate phenotype was observed with SR263 in which abnormally-shaped aggregates were observed at high cell density but essentially no aggregation was seen at low density (Figure 3). High cell density seemed to allow several mutants to at least partially overcome the aggregation defect. There was a strong correlation between the degree of the developmental defect and the degree of loss in calcofluor white binding, cohesion and polysaccharide induction (Tables 1, 4, 5).

TEM analysis of SR200. While using TEM to examine SR200 for fibrils and pili; unusual structures were observed which were associated with the cell poles (Figure 4). These structures, which were not seen in the wild-type and in other Cds mutants, appeared to consist of flexible chains of a basic unit structure with a roughly spherical shape (rings) and a diameter of 60-70nm. This diameter was much greater than that of pili, another polar structure. More than half the cells examined on the grid surface had these remarkable structures. The number of these chains varied from 1 to 8 per cell pole. Most of the SR200 cells had these chains emanating from only one cell pole but a few cells showed these structures extending from both poles. All the cells that displayed these chains also exhibited a sub-polar region that stained intensely with uranyl

acetate (Figure 4). In all the cases, it appeared that the chains emanated from an area very close to the sub-polar structure. In some cells, both pili and chains were observed protruding from the same pole (data not shown). It is interesting to note that FESEM examination did not reveal these chain-like structures. The SR200 FESEM examination did show surface grooves which may correspond in some way with the electron dense sub-polar structures seen using TEM.

Genetic analysis. The relative ease with which new Cds mutants were identified by random transposon insertion mutagenesis suggested that many genes are involved in fibril polysaccharide biosynthesis. Also consistent with this view were the observations that all ten of the Cds mutants could be distinguished at the phenotypic level and that transduction analysis showed that none of the mutations in the newly isolated strains were at the *esg* locus. We have initiated a more in depth genetic analysis of loci exhibiting the most severe Cds and developmental phenotypes. Using transposon marked DNA from mutant loci in SR53, SR171 and SR200; the wild-type *M. xanthus* DNA copies from these loci have been cloned in *E. coli*. The cloned DNAs were used as probes for Southern analysis of *M. xanthus* chromosomal DNA and to screen a cosmid library of *M. xanthus* chromosomal DNA. The three insertions were

not tightly linked since each probe had a distinct Southern blot hybridization pattern (data not shown). This analysis suggested that each insertion was at least 10-kb from the other insertions. However, two cosmid clones were identified which hybridized to both the SR171 and the SR53 probes, and two other cosmids hybridized with the SR53 and the SR200 probes. Analysis of the hybridization results was consistent with the map order of Ω53 lying between the flanking insertions Ω171 and Ω200. Southern analysis was used to determine that one end of the chromosomal DNA in one of the cosmids was located close to the Ω171 insertion site. This cosmid also contained the Ω53 insertion site but did not contain the Ω200 site. Apparently the three insertion sites were too far apart to be cloned on a single cosmid. Since these cosmids were expected to contain approximately 40-kb inserts of *M. xanthus* DNA, our results suggested that the flanking insertions, Ω171 and Ω200, must be more than 40-kb apart.

Discussion

The Cds transposon insertion mutants were isolated based on reduced ability to bind calcofluor white, a fluorescent dye known to bind α, 1-4 linkages in polysaccharides. Exopolysaccharide deficient mutants of *Rhizobium meliloti* have been isolated using a similar calcofluor white based screening strategy (26). The Cds mutants retained a substantial level of S-motility (group motility). In an earlier study (9), reduced calcofluor white binding was shown to be associated with mutants defective in S-motility but the isolation of the Cds mutants indicates that fibril deficient mutants can exhibit a substantial level of S-motility. The first Cds mutant identified was an *esg* strain. The discovery of this phenotype in the *esg* strain led us to search for additional Cds mutants in a collection of random transposon insertion strains. Mutants with the Cds phenotype were relatively common with 0.6% (9 / 1400) of the total insertion strains exhibiting this phenotype. All the mutants were found to be subtly different from one another based on their characteristics during growth and development, and screening of this rather modest collection of insertion mutants did not result in the isolation of additional *esg* mutants. These results suggest that many genes contribute to the production of the calcofluor white binding material in *M. xanthus*.

Analysis of the Cds mutants indicated that there was a strong correlation between the degree of reduction in calcofluor white binding and the degree of reduction in agglutination (cohesiveness), polysaccharide induction, and developmental aggregation. The simplest model to explain this result is that calcofluor white is binding to extracellular polysaccharide-containing structures in *M. xanthus* called fibrils, that the fibrils are mediating agglutination and playing an important role in the formation of multicellular aggregates during development, and that the Cds mutants produce reduced levels of fibrils. There appears to be an induction of fibrils during the entry of cells into stationary phase. Fibrils have been shown to consist of roughly equal amounts of polysaccharide and protein and are known to bind Congo red, a dye that binds to polysaccharide in much the same way as calcofluor white. The Cds mutants appear to display a range in the degree of the defect in fibril production and in the associated phenotypes. Fibrils could not be detected on the Cds mutants using FESEM, a technique that has previously been used to visualize fibrils associated with intact cells (5) but some fibril material was detected in several mutants using TEM.

There is considerable evidence that fibrils mediate the agglutination of *M. xanthus* cells. First, the dye Congo red has been shown to bind

to fibrils and also to block agglutination (1,4). Second, S-motility mutants that lack detectable fibrils are deficient in agglutination (9). Third, the addition of fibrils isolated from a wild-type strain to *dsp* mutant cells which are unable to agglutinate, restores agglutination in the mutant (7). Finally, mutation of the *stk* locus, which results in enhanced fibril production, also causes an increase in agglutination (9). A *stk* mutation can also enhance agglutination in several S-motility mutants. The Cds mutants were similar to *dsp* and the other S-motility mutants in that fibrils could not be detected in these strains and they exhibited significant reduction in agglutination. The agglutination assay is generally carried out using log phase cells but fibrils have been observed only on agglutinated cells (cells removed from agglutination buffer) or on cells which have been removed from solid surfaces at high cell density (2,4,5). It may be significant that the total specific carbohydrate content of almost all of the Cds mutants was lower than in wild-type cells under growth conditions; suggesting that fibril material may be present in the growing cells as well.

There is also evidence for the role of fibrils in developmental aggregation. The strongest evidence comes again from the addition of wild-type fibrils to the fibril deficient *dsp* mutant. The *dsp* strain, like most other S-motility mutants, fails to aggregate and

form fruiting bodies. However, when fibrils were added to the *dsp* strain, the ability to form multicellular aggregates was restored (7). The Cds mutants were found to vary in their developmental aggregation capacity. Those mutants with the strongest calcofluor white binding and agglutination defects were the most defective in aggregation. This may indicate that the mutants with the strongest defects are the most deficient in fibril production but more sensitive methods need to be developed for measuring fibril protein and polysaccharide to conclusively address this point. Aggregation by these mutants was generally improved as cell density was increased.

We have noted that the induction of polysaccharide production is a characteristic of wild-type cells entering stationary phase. All of the Cds mutants were defective in this response. Although the induction of fibril production under these conditions has not been reported, certain observations suggest that *M. xanthus* polysaccharide induction may be related to fibril biosynthesis. First, those mutants with the lowest carbohydrate to cellular protein ratios and the lowest levels of polysaccharide induction were generally the most defective in fibril-related functions. Examples of such mutants are SR53 and SR200. In addition, agglutination assays with wild-type cells taken from stationary

phase cultures (induced cells) indicated that induced cells agglutinated more rapidly than log phase cells (23). Finally, Behmlander and Dworkin (4) noted that high cell density was required for the production of fibrils on solid surfaces. The production of fibrils as *M. xanthus* cells leave the vegetative growth phase of the life cycle and enter the developmental phase may be an important event in facilitating the formation of multicellular fruiting bodies.

The unusual chain-like structures formed by Cds mutant SR200 are intriguing. These structures were produced at the cell poles and appeared to arise from subpolar regions that were strongly stained with uranyl acetate. The chains of a roughly spherical unit structure were flexible and chains dissociated from cells could be seen in the electron microscope fields. The structures we have observed are somewhat similar in apppearance to the structures referred to as LPS extrusions by Dobson et al. (10), but the spherical unit structures are not seen and these authors report that the LPS extrusions were observed in wild-type *M. xanthus* cells. The SR200 polar structures do not appear to be related to the filamentous structures observed by Lunsdorf and Reichenbach in the gliding myxobacterium, *Myxococcus fulvus* (27). Further analysis will be required to determine if there is any relationship between these

various structures. SR200 was one of the Cds mutants most strongly defective in agglutination, polysaccharide induction and developmental aggregation. We are interested in investigating the hypothesis that SR200 has a fibril secretion defect which may result in the accumulation of structures which could be intermediates in the fibril production process. If this is the case, then the unusual polar structures observed in SR200 may indicate that the fibril secretion machinery is localized to the *M. xanthus* cell poles.

While evidence is accumulating for the role of fibrils in agglutination and developmental aggregation, the relationship between fibrils and S-motility is less clear. The first group of fibril deficient mutants identified were S-motility mutants, suggesting that fibrils might function in S-motility. However, a group of mutants called *fbd* were recently isolated which has argued against such a role (8). These mutants were isolated in a *dsp* genetic background and the *fbd dsp* mutants fail to make fibrils, but these cells do exhibit group motility. The Cds mutants were also found to have defects in fibril production while retaining a strong capacity for S-motility. But, it should be pointed out that despite this general characteristic of the Cds mutants, several of them were mildly impaired in the rate of S-motility on low percent agar plates.

Current evidence suggests that if fibrils are involved in S-motility, the role is a rather subtle one. Clearly there is a connection between the S-motility genes and fibril production, but this connection may be indirect since several of the S-motility genes have recently been shown to be directly involved in the production of type IV pili (35). The Cds mutants were found to produce pili; a result that is consistent with the strong correlation between S-motility and presence of these extracellular structures.

There appear to be many Cds genes and these genes seem to be required for fibril biosynthesis. *M. xanthus* fibrils have a polysaccharide backbone with several major associated proteins (4,5). It is apparent that there should be genes in *M. xanthus* for the synthesis, export, and assembly of the fibril polysaccharide, genes for the fibril-associated proteins, and genes involved in fibril regulation. Recently, the gene for a fibril protein was identified by a reverse genetics approach (33). Genetic approaches will also be important in understanding fibril structure and function. Based on epistasis studies with *stk* and S-motility mutants, Dana and Shimkets suggested that the S-motility genes fall into two classes with respect to their involvement in fibril production (9). The first class, including the *dsp* and *sgl-3119* loci, may encode components of the fibrils; and the second class, including all of the other S-

motility loci analyzed, may encode proteins controlling the activity or expression of the first class of genes. Preliminary genetic studies with the Cds mutants suggest that there may be two similar classes of Cds genes. Three of the Cds loci (SR53, SR171 and SR200) have been cloned and it has been possible to show linkage between these loci by hybridization to cosmid clones. The chromosomal region containing these three insertion sites appeared to be greater than 40-kb in size and to contain multiple Cds genes since each insertion strain had a distinctive Cds phenotype. A more detailed analysis will be required to determine the complexity of this region and if the other Cds insertions are found in the same region.

In conclusion, the isolation of the Cds mutants represents an important step in the study of *M. xanthus* fibril structure and function. The properties of these mutants are consistent with the idea that fibrils play an important role in cell-cell interactions both during vegetative growth and during development. The approach described in this study has identified a number of new genes involved in fibril production and additional genes could surely be identified in the same way. Ultimately, the identification and analysis of the genes involved in fibril production will be a major step in understanding the role of these complex and dynamic

structures in *M. xanthus* biology.

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CHAPTER 3

A *cds* genetic locus in *Myxococcus xanthus* is involved in branched chain fatty acid synthesis

I. ABSTRACT

The *cds* mutants of *Myxococcus xanthus* are S-motile but lack wild-type levels of fibrils and are to varying degrees deficient in cell-cell agglutination, extracellular polysaccharide production and development. In an attempt to further characterize the phenotypes, we found that strain SR171 drastically reduced developmentally regulated *tps* gene expression similar to *esg*. Total fatty acid analysis revealed that SR171 is deficient in branched chain fatty acids. Addition of isovaleric acid to growing cells rescued fruiting body formation and increased sporulation and *tps* gene expression by several fold. SR171 differed from *esg* in pigmentation and sporulation. Extracellular complementation was observed when SR171 was mixed with wild-type and the *esg* mutant. A 6kbp DNA fragment cloned from the wild-type cosmid library was able to partially complement genetically the aggregation defect in SR171. Restriction analysis revealed that the SR171 locus was different from the *esg* locus. The *esg* locus encodes the E1α and E1β subunits of a branched chain keto acid dehydrogenase involved in the production of branched chain fatty acids. Our results suggest that

the SR171 locus appears to either encode for additional components of the branched-chain keto acid dehydrogenase or components of a second enzyme involved in branched-chain fatty acid synthesis.

II. INTRODUCTION

Myxococcus xanthus is a gliding, gram-negative, soil bacterium that is distinguished from other procaryotes by the ability to form multicellular fruiting bodies in the developmental phase of its life cycle. Fruiting body formation is initiated on a solid surface by nutrient depletion which triggers many thousands of rod-shaped cells to move to aggregation centers to form macroscopic mounds. The mounds mature into a fruiting body within which the rod shaped cells differentiate into dormant, environmentally resistant myxospores (14, 34). Cell-cell interactions are essential for the developmental process because five groups of conditional mutants (Asg, Bsg, Csg, Dsg, and Esg) have been isolated that cannot sporulate alone, but can be rescued for sporulation when the mutant cells are mixed with wild-type cells or cells that belong to a different group (12, 14). This correction of a phenotypic defect is termed extracellular complementation and does not involve genetic exchange. Each complementation group has been shown to belong to distinct genetic loci and is believed to be deficient in the production of a different developmental signal (12, 14,). These extracellular signals are thought to regulate the sequential expression of several sets of developmental genes.

The *esg* locus of *M.xanthus* is thought to control the production of

one of the extracellular signals (E-signal) that must be transmitted between cells for the completion of development (12). Failure to produce the E-signal results in reduced expression of many developmentally regulated genes expressed after 5h of development (12). Genetic complementation and sequence analysis has revealed that the *esg* locus encodes for the α and β sub-units of the E1 component of Branched-Chain keto acid dehydrogenase (BCKAD). This is a multi-enzyme complex involved in branched-chain amino acid metabolism in many bacteria and vertebrates. BCKAD decarboxylates the branched-chain keto acids formed by transamination of branched-chain Amino acids (Leucine, Isoleucine and valine) to produce the corresponding branched chain fatty acid CoA derivatives. The short branched-chain fatty acids are isovaleric acid (IVA), methylbutyric acid (MBA) and isobutyric acid (IBA) respectively. The fatty acid CoA derivatives serve as primers in the production of long branched chain fatty acids which are then incorporated into membrane phospholipids (10, 21). The branched-chain fatty acids are abundant in *M.xanthus* cells (21, 36). Several biochemical experiments suggest that *esg* is defective in BCKAD activity. First, *esg* mutants have reduced levels of branched-chain fatty acids. Second, crude cell extract of an *esg* mutant showed reduced activity of BCKAD enzyme. Third, several phenotypic

defects in *esg* including growth, pigmentation and development could be rescued when the cells were grown in the presence of IVA. Finally, the percent distribution of long branched-chain fatty acids in *esg* were restored to Wild-type levels when IVA was exogenously added to growing cells (36). It is believed that the branched chain fatty acids produced during vegetative growth are somehow modified and used in the production of E-signal during fruiting body formation.

While attempting to characterize other defects in *esg*, the Cds phenotype was observed in an *esg* mutant which led to the isolation of nine different transposon insertion mutants sharing the Cds phenotype. The Cds mutants were identified based on their inability to bind calcofluor white to wild-type levels (35). Calcofluor white is one of the dyes that is known to bind the polysaccharide material found in extracellular matrix (8, 35). The extracellular matrix in *M.xanthus* is believed to be organized as fibrils made of roughly equal amounts of polysaccharide and protein (4, 5). Cds mutants have been shown to be deficient in extracellular polysaccharide and fibril production to varying degrees. In addition, these mutants are defective in fibril mediated functions like cell-cell agglutination and the formation of multicellular aggregates during initial phases of development (1, 2, 4, 8, 14, 35). Although these phenotypes are

known to be shared by S-motility (group) mutants (8), the Cds mutants have been shown to exhibit group motility (35). All of the Cds mutants were different from one another based on their morphological characteristics during growth and development. Four Cds mutants analyzed to date have been shown to map to distinct genetic loci. In an attempt to further describe the properties of Cds mutants, it was found that SR171 reduced *tps* gene expression significantly compared to other Cds mutants tested. *tps* is a developmentally regulated gene which requires *esg* for its expression (12). Several morphological and developmental characteristics of SR171 were found to be very similar to that of *esg*. So, SR171 was chosen for further phenotypic and genetic analysis. The results indicate that the genetic defect in SR171 is in a different genetic locus than *esg* and that this locus is also involved in the synthesis of branched-chain fatty acids.

III. MATERIALS AND METHODS

Bacteria, phages and growth media. *M.xanthus* strains were grown vegetatively in CYE or CTT broth (18). Solid media contained 1.5% Bacto-Agar (Difco Laboratories). Bacteria were routinely grown in a New Brunswick Series 25 incubation shaker at 250 rpm and 30^{o}C. Growth was monitored in a Klett-Summerson photoelectric colorimeter with a red filter. A1 medium was used as the chemically defined broth for the growth rate experiments. The broth contained six amino acids (val, ile, leu, met, phe and Asn) up to 100μg/ml, Spermidine(125μg/ml), vitamin B12(1μg/ml) and various salts and buffers. 0.5% pyruvate served as a major carbon source and cell growth was primarily dependent on pyruvate. The preparation of cells for growth in A1 medium was followed as described previously (36). Myxophage Mx4 was utilized to transduce *cds* mutations into JD10(*tps-lacZ*) strain (7). *Escherichia coli* strains were grown in L broth or on L agar (L broth with 1.5% agar) at 37^{o}C (27). Antibiotics were supplemented, when appropriate, with kanamycin (50μg/ml), ampicillin (100μg/ml) and oxytetracycline (15μg/ml).

β-galactosidase Assays. *M.xanthus* cells having *tps-lacZ* fusions were grown vegetatively in CYE broth, harvested and washed with TPM buffer (10mM Tris-HCl (pH7.6), 1mM potassium phosphate, 8mM

$MgSO_4$) and resuspended to a calculated cell density of 40 Klett units in CF broth. The cells were shaken at 150 rpm at 28^{0}C. 10ml samples were collected at appropriate times by centrifugation and the pellets were stored frozen at -20^{0}C until all of the samples were collected (11). Cells developing on CF agar were harvested in TPM buffer, washed twice and the pellets were similarly stored frozen. To determine the levels of β-galactosidase activity, the cell pellets were suspended in 1 ml of Z-buffer (0.1M sodium phosphate (pH 7.0), 0.01M KCl, 0.001M $MgSO_4$ and 0.05M β-mercaptoethanol) and sonicated for 2 min at 30 -s bursts. The samples were spun down in a table-top Beckman Microcentrifuge for 2min and 0.2ml of supernatant was used for β-galactosidase assay and for total protein determination. β-galactosidase activity was measured using the chromogenic substrate ONPG (o-nitrophenyl-β-D-galactopyranoside) as described previously (11). Total proteins were estimated as equivalents of BSA by using the bicinchonic acid method (PIERCE).

Total carbohydrate induction by calcium. *M.xanthus* cells were grown in CYE medium and harvested at mid-log phase. 4mM $CaCl_2$ was then added to one half of the culture and incubated further for 2 hours. Aliquots from both treated and untreated cells were

measured for total carbohydrate content. Total carbohydrates were determined as glucose equivalents by the anthrone-sulfuric acid method as described previously (17, 35).

Fatty acid Analysis. *M.xanthus* cells were grown on CTT agar at 30^{0}C. Whole cell fatty acid analysis was performed by Microcheck, Inc., Northfield falls, Vermont. The fatty acids were extracted and the methyl ester derivatives were analyzed by gas chromatography with MIDI microbial identification system (MIDI,Inc. Newark, Delaware). The methods for extracting fatty acid methyl esters have been described previously (28, 29, 33).

Developmental Conditions and Spore counts. Vegetatively growing *M.xanthus cells* in CTT broth (with or without IVA) were harvested in mid-log phase and the pellet was washed once with TM buffer (10mM Tris HCl (pH 7.6), 10mM $MgSO_4$) . The pellet was then resuspended in TM buffer to a calculated cell density of 2 x 10^9 cells/ml and 0.1 volume was plated on CF agar as multiple spots. The plates were incubated at 30^{0}C for 96h and the spots were scraped into 100μl of TM buffer using a clean razor blade. The cell suspension was then sonicated for 30-s twice to disperse the spores and also to kill any viable vegetative cells. Serial dilutions of the sample were then made and plated onto CTT agar and scored for growth after 72 hours. For extracellular complementation

experiments, 2 x 10^9 cells/ml of Wild-type cells were mixed with the same concentration of mutant cells before the spots were plated on CF agar. Fruiting bodies were observed and photographed after four days.

Scanning Absorbance Spectroscopy. Cells were grown to log phase in CTT medium. The cells were harvested and the pellet was washed once with water, and sonicated for a total of 60 seconds in 15-s intervals followed by cooling to break open the cell walls. The extract was then centrifuged at 8000x g for 10 minutes and the supernatant was collected. Total proteins of the cell free extract was estimated by the bicinchonic acid method (PIERCE). 550μg of protein in 1ml was taken in a cuvette and the absorbance profile of the extract was monitored using a Beckman DU-64 Spectrophotometer at wavelengths ranging between 300-500nm.

Cloning and Genetic Analysis of SR171 locus. A plasmid designated pSR171 containing *M. xanthus* DNA flanking Tn*5* in SR171 was constructed in *E.coli* using Kanamycin as selectable marker. pSR171 was cloned as a 4.1kbp *Bam*HI fragment in pGEM3Zf+ vector (promega). This plasmid contained 1.1kbp of *M.xanthus* DNA adjacent to Tn*5*. A 0.7kbp *Xho*I fragment of *M. xanthus* DNA was then purified from 0.5% agarose gel using Qiagen's gel extraction kit. This fragment was radiolabelled with [α-^{32}P]dCTP (Amersham) based

on the method developed by Feinberg and Vogelstein (15). The DNA probe was then used to hybridize a library of cosmid clones containing wild-type *M.xanthus* chromosomal DNA(27). A Southern analysis was then performed with Wild-type chromosomal DNA and DNA extracted from the cosmid 5A-G5 that hybridized to the probe (3, 27). A 6.0kbp *Bam*HI-*Sal*I fragment from the cosmid containing the Ω171 region was ligated into a *Bam*HI-*Sal*I digested pGEM3Zf+ vector to create pSR172. Restriction analysis of this plasmid revealed that the Tn*5* insertion was located roughly about 1kbp from the *Bam*HI site. pSR174 was constructed by inserting a 3.4kbp *Hind*III fragment containing the gene for Kanamycin resistance. The *Hind*III fragment was obtained from Tn*5* inserted into pBR322. Genetic complementation was performed with a recipient strain (SR172) containing Ω171 insertion with a modified form of Tn*5* called Tn*5-132*. The modified transposon encodes for tetracycline resistance instead of kanamycin (3). SR172 was constructed by in situ replacement of Tn*5* with Tn*5-132*. This was accomplished by infecting SR171 with P1:Tn*5-132* phage. Two homologous cross-overs between the transposon inverted repeats result in replacement of Tn*5*-WT by Tn*5-132*. The phage genome and Tn*5*-WT are lost. pSR174 was introduced into SR172 by electroporation (20). Merodiploid strains were collected by plating the transformants on

CTT agar containing kanamycin (50μg/ml) and oxytetracycline (15μg/ml). The ability of pSR174 to complement the developmental defect was tested in a submerged culture assay as described previously (24). Photographs of fruiting bodies were taken after 96h of incubation at 30^{0}C.

Transmission electron microscopy. *M.xanthus* strains were grown to mid-log phase in CTT broth, harvested by centrifugation and concentrated to 2x10^9 cells/ml in 10mM Tris-HCl (pH7.6). The samples were sprayed with an airbrush at 20 psi into a 5μm nylon mesh (Small Parts, Inc., Miami Lakes, FL) transfer basket submerged in liquid propane maintained at -183^{0}C (16). Liquid nitrogen was used to liquefy and maintain the temperature of propane. The air brush was equipped with a needle valve that adjusted the orifice size, and the droplet diameter size of the samples were approximately adjusted between 40-50μm. After freezing, the mesh basket containing the frozen sample was drained and transferred through anhydrous acetone rinses thrice at -85^{0}C (16). The freeze substituted cells were brought to room temperature gradually over 10h, rehydrated and post-fixed with 2% OsO_4 for 3h at 20^{0}C, rinsed with water thrice and then dehydrated in a graded acetone series. The cells were then infiltrated in EmBed 812 resin (Electron

Microscopy Sciences, Fort Washington, PA) and polymerized at 65^{0}C for 18h. The embedded samples were cut with a diamond knife to obtain silver sections on a Reichert Ultracut ultramicrotome and 5-8 sections were loaded onto formvar coated copper grids. The grids were then stained twice with saturated uranyl acetate and Reynold's lead acetate. The sections were viewed using a JEOL 2000 electron microscope at an accelerating voltage of 100kV.

IV. RESULTS

Tn*5* insertion Ω171 reduces *tps* gene expression. As part of a continuing study on characterizing Cds mutant phenotypes, several mutations were introduced into JD10, a wild type strain containing *tps-lacZ* translational fusion gene integrated into *M.xanthus* chromosome by homologous recombination (11, 12). The effects of Cds mutations on *tps* expression were monitored by measuring β-galactosidase activity in cells suspended in liquid CF medium. Previous studies have shown that the *tps* gene is highly expressed in shaker culture and that the highest activity was recorded at about 20 hours of development (11). Figure 1 shows that four of the Cds mutants tested had *tps* gene expression ranging from about 40% (SR233 and SR53) to 85% (SR200) of wild-type activity. Only about 8% of wild-type *tps* gene activity was measured in SR171. A detailed time course experiment on *tps* expression in SR171 was then undertaken (Figure 2). The peak activity of *tps* gene in Wild-type is seen around 20 hours in both liquid culture and CF plates. In SR171, there was dramatically reduced *tps* expression suggesting that this locus is essential for the regulation of *tps* gene expression during development. This drastic reduction of *tps* gene expression by Ω171 insertion was comparable to that observed with the *esg* mutant which had about 4% of the wild-type level of *tps* gene

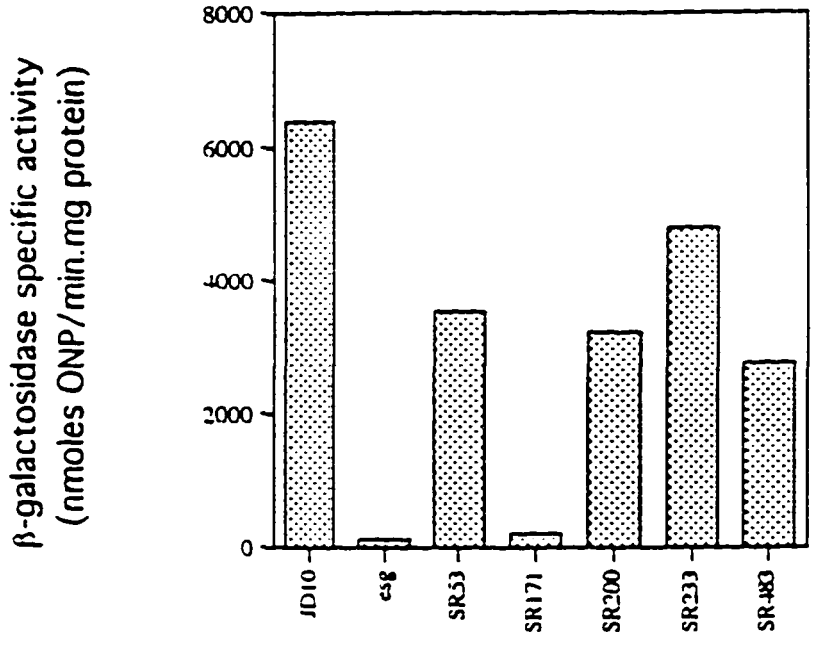


Figure 1. Effect of Cds mutations on *tps* expression. JD10(WT) has *tps-lacZ* fusion in *M.xanthus* chromosome. JD258(esg) contains Ω258 insertion in JD10. The five Cds mutations (Ω53, Ω171, Ω200, Ω233, and Ω483) are transduced into JD10. Cells were suspended in liquid CF medium and incubated at 28°C in shaker culture. Samples were collected at 0h and 20h of incubation and *tps* gene expression was measured by assaying for β-galactosidase activity.

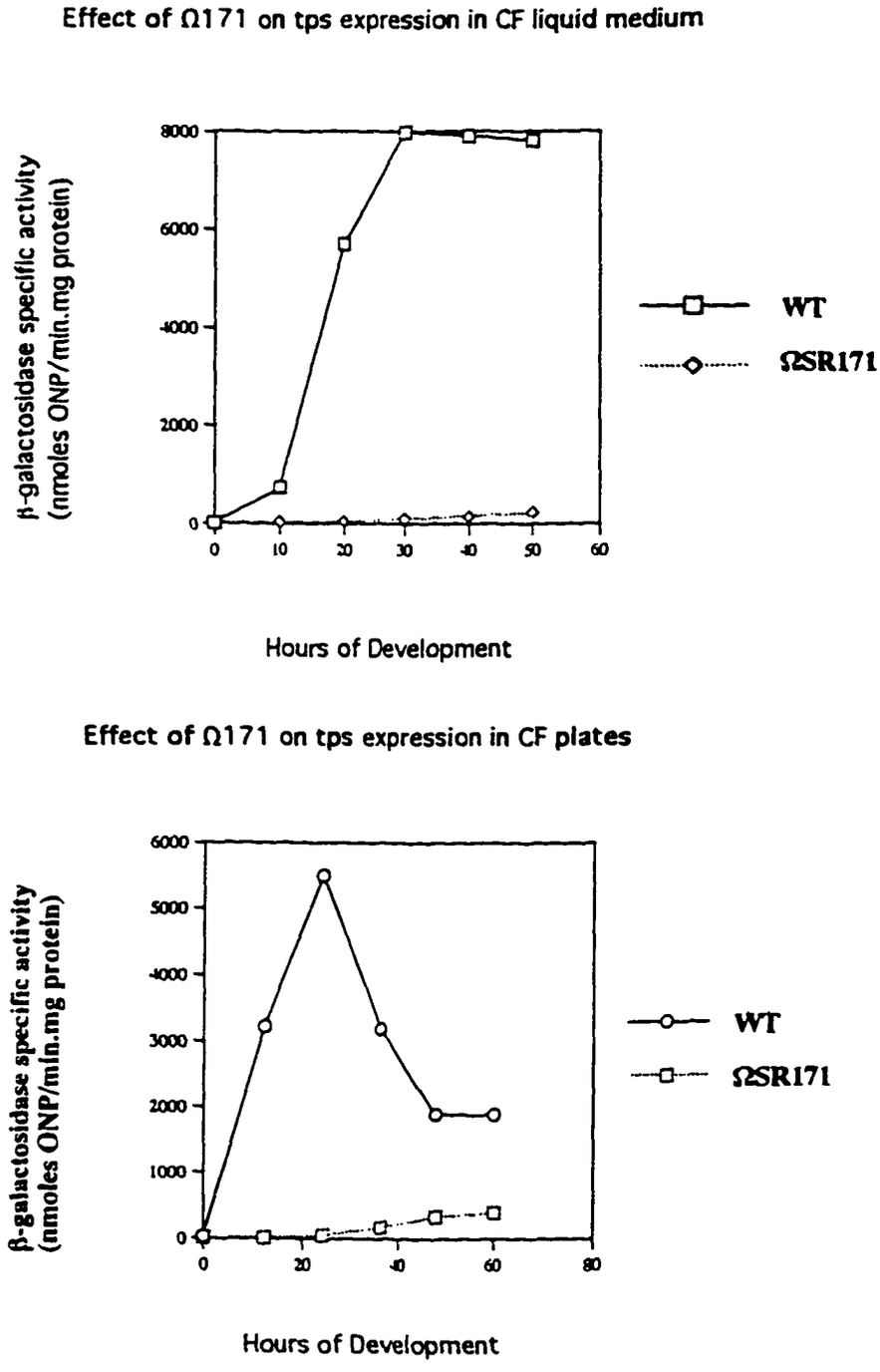


Fig 2. Expression of *tps-lacZ* during development of JD10(WT) and SR171. The Ω171 insertion locus was transduced into JD10 by Mx4 transduction. Cells were grown in CF liquid medium as well as plated on developmental (CF) agar. Samples were collected at indicated time points and assayed for β-galactosidase specific activity.

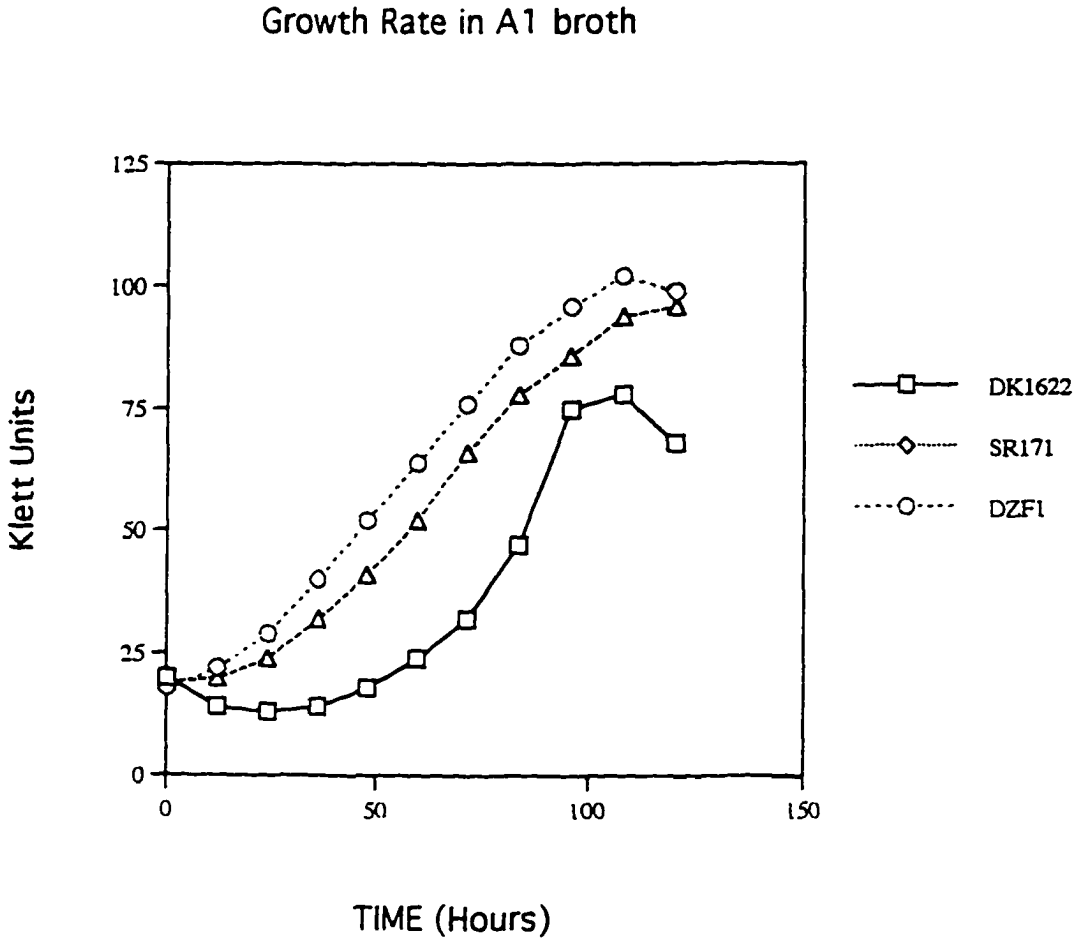


Fig 3. Growth of SR171 in chemically defined A1 medium. DK1622 and DZF1 were used as wild-type strains. DK1622 had an apparent slower growth rate because of its clumpiness and its ability to stick to glass surface. DZF1 grew in a dispersed state in the liquid medium. Cultures were incubated at 30°C and cell growth was monitored at indicated times in a Klett-Summerson photoelectric colorimeter with a red filter.

activity. Several SR171 phenotypic characteristics including agglutination, dye binding, pigmentation and aggregation defects were more similar to *esg* than to other Cds mutants (35). Based on its relatedness with *esg*, SR171 was chosen for further phenotypic and genotypic analysis.

Growth Analysis in Al broth. Previous studies had indicated that the *esg* mutant failed to achieve wild-type levels of growth in Al medium (36). SR171 grew well in complex medium containing casitone. Fig. 3 shows the growth rate of this mutant in the chemically defined Al medium. Pyruvate serves as the main source of carbon and energy in this medium. This medium also contains the branched-chain amino acids which are essential for the growth of *M.xanthus* (6, 36). DK1622 and DZF1 were used as wild-type strains because DK1622 formed clumps within the growth medium as well as on the side-arm flask surface making it difficult to measure turbidity of growth. A slow growth rate was thus observed until 50 hours for DK1622. However, when total protein was measured, a steady increase in growth was observed (data not shown). The DZF1 strain did not clump in Al medium and showed steady increasing growth till about 110 Klett units. The growth pattern of SR171 was similar to DZF1 but growth ceased at a lower cell density. There was no significant change in the pattern of the protein levels compared

to wild-type. It has been shown earlier that *esg* grew only up to 70 klett units under similar conditions (36). The results suggest that SR171 may not be defective in growth related enzymatic activities.

Calcium induced polysaccharide production. It has been previously shown that Cds mutants were defective in extracellular polysaccharide production (35). Previous studies indicated that addition of divalent ions like calcium or strontium to growing wild-type cells induces the production of exopolysaccharide and that calcium induced cells agglutinate more effectively than uninduced cells. The study also showed that SR53, a Cds mutant and several fibril deficient mutants did not induce polysaccharide synthesis with calcium unlike *esg* suggesting that calcium induced polysaccharide is genetically separable (23). It has been argued that polysaccharide induction is not only regulated by the entry of cells into stationary phase but also independently by calcium (23). So, it was of interest to see if SR171 responded to carbohydrate induction by calcium. Table 1. shows that both *esg* and SR171 were capable of inducing polysaccharide to wild-type levels when calcium was added to growing cells in log phase. The calcium induced cells showed more clumping than uninduced cells. The result shows that *esg* and SR171 have retained the capacity to make exopolysaccharide material when treated with calcium. Furthermore, this

Total Carbohydrate induction by Calcium

STRAIN	μg Carbohydrate /μg Protein		
	- $CaCl_2$	+$CaCl_2$	% INCREASE
WT	0.13	0.15	15
esg	0.12	0.14	17
SR171	0.12	0.14	17

Table 1. Cells were grown in CYE medium to mid-log phase and the cultures were divided in half. The divided cultures were further incubated for 2h with or without $CaCl_2$. The samples were assayed for total carbohydrate by anthrone-sulfuric acid method.

Branched-Chain Fatty acid analysis

	% Fatty acids		
Fatty acid	WT	esg	SR 171
11:0 ISO	0.2	- - -	---
13:0 ISO	0.7	0.2	---
14:0 ISO	- - -	0.1	---
15:0 ISO	45.1	16.9	22.4
16:0 ISO	1.1	5.9	2.0
17:0 ISO	6.3	4.1	2.8
14:0 ISO 3OH	4.7	1.1	1.5
15:0 ISO 3OH	1.7	0.6	1.2
17:0 ISO 3OH	2.0	1.1	1.2
ISO 17:1 ω10C	1.2	1.4	0.5
Total BCFA[a]	63.0	31.4	31.6

Table 2. Percent distribution of total branched-chain fatty acids in wild-type and mutant cells grown in CTT broth. The values for wild-type and *esg* cells shown were reported previously. [a] Total BCFA is the ratio of branched-chain fatty acids to total fatty acids.

exopolysaccharide material appeared to be in the form of fibrils.

SR171 is deficient in branched-chain fatty acids. The primary defect in *esg* was found to result in a reduction of long branched chain fatty acids (36). Since several phenotypes are shared by SR171 and *esg*, it was of interest to determine the branched-chain fatty acid profile in SR171. Table 2 shows the total cellular branched-chain fatty acid profile for SR171 and *esg* strains in comparison with the wild-type. The total branched-chain fatty acids is reduced in half in both *esg* and SR171 compared to wild-type levels. Except for iso-16:0, all the other branched-chain fatty acid species are reduced in the mutant cells. The results indicate that like *esg*, SR171 is defective in the production of iso-15:0 (13-methyl-tetradecanoic acid) which is the most abundant long branched-chain fatty acid found in *M.xanthus.* However, some differences between the two mutant strains were noted. For example, iso 16:0, iso 17:0 and the modified branched-chain fatty acid species, iso 17:1ω10C, were increased in *esg* whereas iso15:0 3OH was reduced. The significance of these findings is not clear at the present time.

Correction of phenotypic defects by IVA. In an earlier study, several of the phenotypic defects in the *esg* mutant were corrected by growth of cells in media containing the short branched-chain

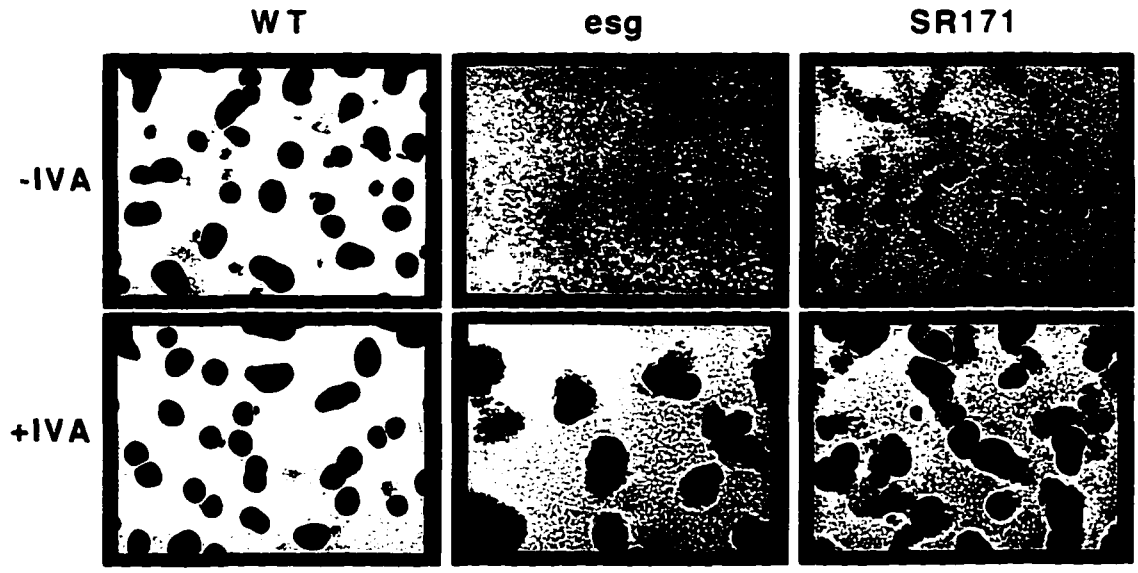


Fig 4. Effect of IVA on fruiting body formation. Wild-type and mutant cells were grown in CTT medium with and without 1mM IVA. Development was initiated by spotting cells on CF agar. Photographs were taken after four days of development. Wild-type cells was proficient in making fruiting bodies and was not affected by IVA. *esg* and SR171 did not make fruiting bodies without IVA.

EFFECT OF IVA ON SPORULATION

	No. OF SPORES		
STRAIN	- IVA	+ IVA	FOLD STIMULATION
WT	1.3×10^8	7×10^8	5.4
esg	5×10^4	3.5×10^8	7×10^3
SR171	1.5×10^4	3.3×10^8	2.2×10^4

Table 3. Wild-type and mutant cells grown in CTT medium were allowed to develop on CF agar for 96h. Spore filled fruitng bodies were scraped into TM buffer, sonicated, and dilutions of cells were plated onto CTT agar to determine the number of viable spores. The mutant cells grown in the presence of IVA sporulated to nearly wild-type levels.

Effect of IVA on tps expression

Strain	- IVA			+ IVA		
	0h	25h	Dev/Veg	0h	25h	Dev/Veg
WT	32	5500	170	89	5415	61
esg	3	20	7	18	458	25
SR171	7	49	7	14	703	52

Table 4. Cells were grown in CTT medium with or without IVA and allowed to develop on CF agar. Samples were harvested at the beginning and 25h of development and assayed for β-galactosidase specific activity. Addition of IVA significantly increased developmental gene expression in the mutants. Dev/Veg are the ratios of β-galactosidase specific activities of developing to vegetative cells.

fatty acid, IVA (36). This result could be explained by the use of IVA to bypass the *esg* mutational block and restore branched-chain fatty acid synthesis. Since SR171 was deficient in the synthesis of odd-numbered iso-fatty acids (Table 2) which are known to be derived from IVA, it was of interest to see if IVA rescued the developmental phenotypes in the mutant. The cells were grown in CTT broth containing 1mM IVA and then placed as concentrated spots on CF agar to initiate development. Fig. 4 shows that the mutant cells were not able to complete aggregation and supplementation of IVA to growing cells enhanced the fruiting body formation in both *esg* and SR171. Growth with IVA did not affect fruiting body formation in the wild-type strain. Determination of the number of spores in those developmental cultures indicated that IVA stimulated sporulation by more than four orders of magnitude to about 50% of wild-type levels in SR171 (Table 3). IVA increased sporulation by about five-fold in the wild-type. Rescue of *tps* expression with IVA was then tested by measuring the β-galactosidase activity on cells undergoing development on CF agar plates (Table 4). The results show that the wild-type cells were not affected in developmental *tps* expression by IVA, but the Dev/Veg ratio was reduced owing to a slight increase in *tps* expression in the vegetative cells. IVA stimulated developmental *tps* gene expression significantly in both

the mutants. However, the increase in gene expression did not reach wild-type levels. The significance of this result is not clear at the present time. A similar pattern of *tps* gene expression was observed in CF shaker cultures (data not shown). In summary, the results indicate that addition of IVA to vegetatively growing cells rescues developmental phenotypes such as fruiting body formation, sporulation and *tps* gene expression. It suggests that like *esg*, SR171 is likely to be defective in one of the early steps in the pathway for the synthesis of branched-chain fatty acids from the branched-chain amino acids (10, 36).

Pigmentation Defect. Wild-type *M.xanthus* cells are intensely yellow in appearance. A non-carotenoid pigment which absorbs strongly at 379nm is primarily responsible for this coloration (32). In an earlier study, it was reported that *esg* failed to produce this pigment when grown in A1 medium (36), although cells remained yellow when grown in CTT medium. SR171 was different from *esg* and displayed very little pigmentation on developmental agar plates and appeared pale yellow even when grown in CTT medium. Fig. 5 shows a scanning spectrophotometric analysis of cell extracts. The wild-type cells exhibited a prominent peak centered about 384nm. *esg* showed less than half the absorbance of wild-type levels while the peak is completely absent in SR171 mutant extract. The results

indicate that SR171 and *esg* are both involved in the production of the intracellular pigment. Furthermore, the pigmentation defect in the two mutants were distinguishable based on the severity of the defects in different growth media.

Extracellular Complementation Analysis. It has been shown previously that the sporulation defect in *esg* could be substantially corrected by extracellular complementation when mixed with wild-type cells or other signaling mutants (12). It was of interest to see if sporulation could be rescued when SR171 was mixed with wild-type or *esg* mutant cells. Table 5 shows that SR171 is defective in sporulation and the number of spores obtained is at least seven fold less than *esg*. The result shows that in a mixture with wild-type cells, sporulation of SR171 was increased suggesting that it responds to extracellular complementation. SR171 also responded to extracellular complementation by *esg*, but the effect was less in magnitude. In a reciprocal experiment, *esg* was able to respond to extracellular complementation by SR171 only to a lesser extent. Cells resulting from the germination of SR171 spores could be differentiated from the wild-type and *esg* mutant cells on the basis of the tetracycline resistance marker present in SR171. Extracellular complementation of developmental defects has been used to define signaling mutants (12, 14, 34). However, the increase

in sporulation seen when SR171 is mixed with *esg* is not to the extent of what is measured with wild-type cells. Nevertheless, the result suggests that the phenotypes are distinguishable in the two mutants.

Cloning and Genetic Analysis. A cosmid clone was identified which contained the wild-type copy of the chromosomal DNA from the Ω171 region. Southern analysis revealed a common 6kbp *Bam*HI-*Sal*I fragment found in both the cosmid and genomic DNA, which was then cloned in *E.coli*. Restriction analysis of this fragment revealed that it is different from the *esg* locus (12, 36). The Tn*5* in *esg* is inserted within a 0.3kbp *Xho*I-*Sma*I fragment whereas Ω171 is inserted within a 0.4kbp *Eco*RI-*Pst*I fragment, located roughly about 1kbp from the *Bam*HI site. A *Hind*III site is found only in the *esg* locus whereas a *Kpn*I site is found only in the SR171 locus (Figure 6). Genetic complementation was performed by introducing pSR174 which contained wild-type DNA from the Ω171 region and a kanamycin resistance marker. The recipient strain contained a modified version of Ω171 encoding tetracycline resistance. Several merodiploid strains containing kanamycin and tetracycline resistance markers were obtained because of homologous recombination. The morphological traits of merodiploids including pigmentation, texture and spreading ability on CTT agar were like

Absorption Spectrum of cell extracts

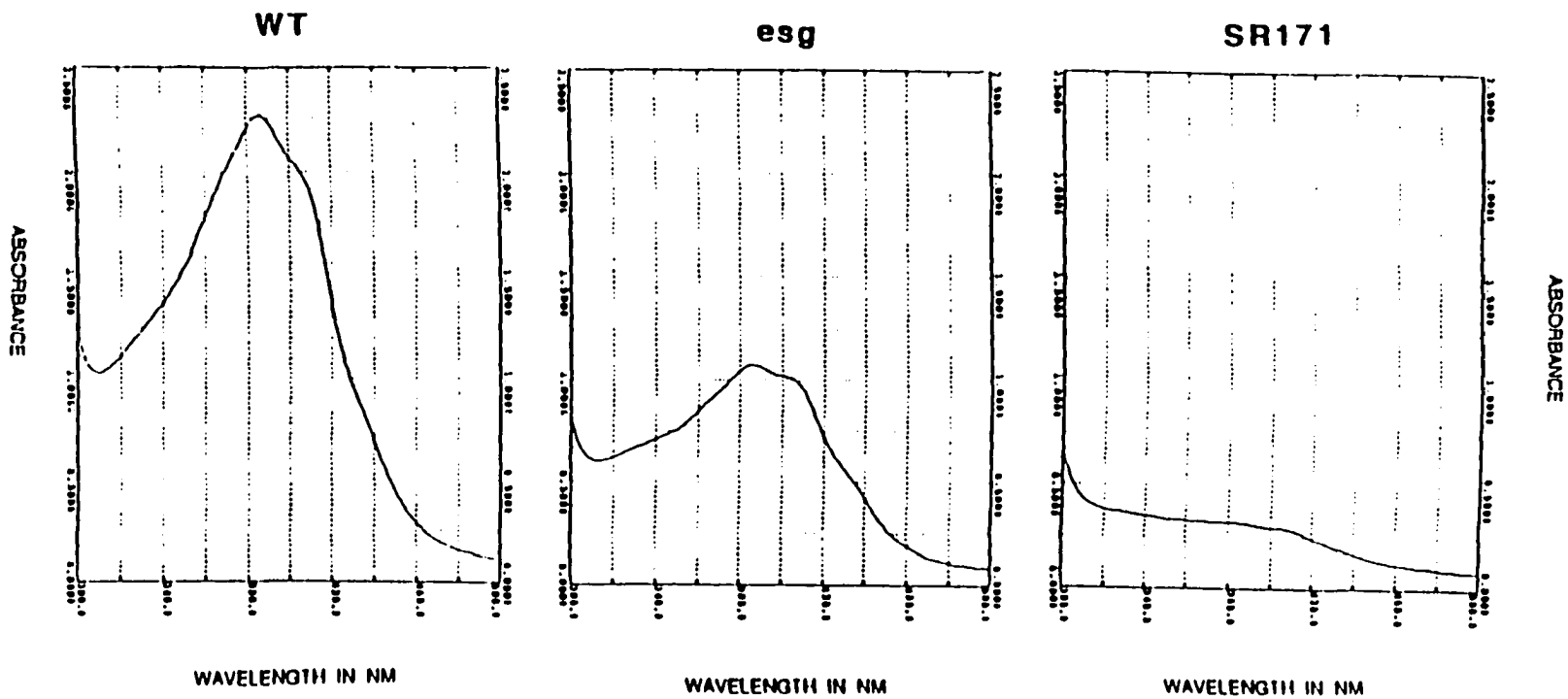


Fig . Cells were grown to mid-log phase in CTT medium. The cell extracts were prepared as described in Materials and Methods. Each extract sample contained 550μg/ml protein and the absorbance profile was taken using a Beckman DU-64 spectrophotometer. The wild-type had an absorption maxima at 384nm.

Sporulation of Strains

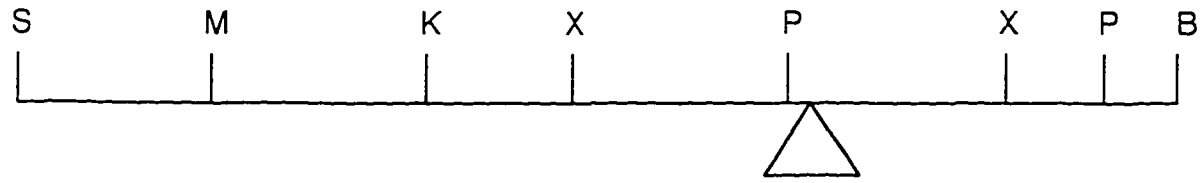
STRAIN	No. Of SPORES	% WT SPORES
WT	1.3×10^8	100
esg	9×10^4	0.07
SR171	1×10^4	0.01

EXTRACELLULAR COMPLEMENTATION

STRAIN	No. Of SPORES	FOLD INCREASE
WT+171	2.8×10^7	5.6×10^3
171+esg	4×10^5	9
esg+171	1.7×10^6	340

Table 5. Extracellular complementation of SR171 sporulation by mixing with wild-type and *esg* mutant cells. Wild-type and mutant cells grown in CTT medium were allowed to develop on CF agar for 96h. For extracellular complementation, the input ratios of the two strains was 1:1. Sporulating strains being counted in the mixing experiments are indicated by an underline. Spore filled fruitng bodies were scraped into TM buffer, sonicated, and dilutions of cells were plated onto CTT agar containing appropriate antibiotics to determine the number of viable spores.

Genetic map of SR171 locus



Genetic map of esg locus

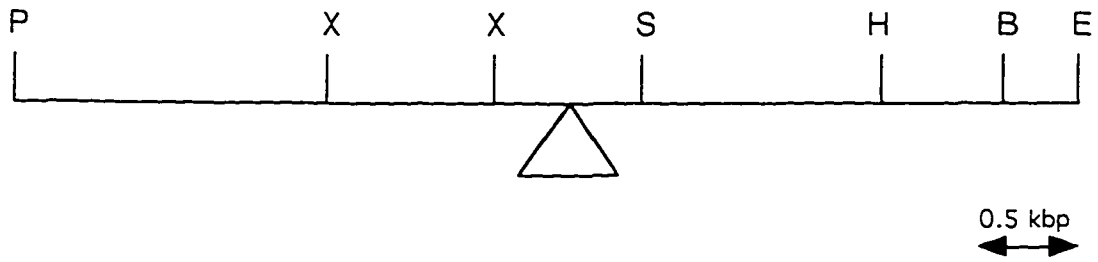


Fig . Genetic maps of *esg* and SR171 loci. A partial restriction map of SR171 locus is compared with *esg* locus. S, *Sal*I; M, *Mlu*I; K, *Kpn*I; X, *Xho*I; P, *Pst*I; B, *Bam*HI; H, *Hin*DIII; E, *ECo*RI. SR171 locus is about 6kbp and *esg* locus is about 5.5kbp. The approximate location of Tn*5* inserts are shown. The Tn*5* insertion is within 0.3kbp *Xho*I -*Sal*I fragment in *esg* and 0.4kbp *Pst*I -*ECo*RI fragment in SR171. The *Bam*HI-*Sal*I fragment of SR171 was used for genetic complementation.

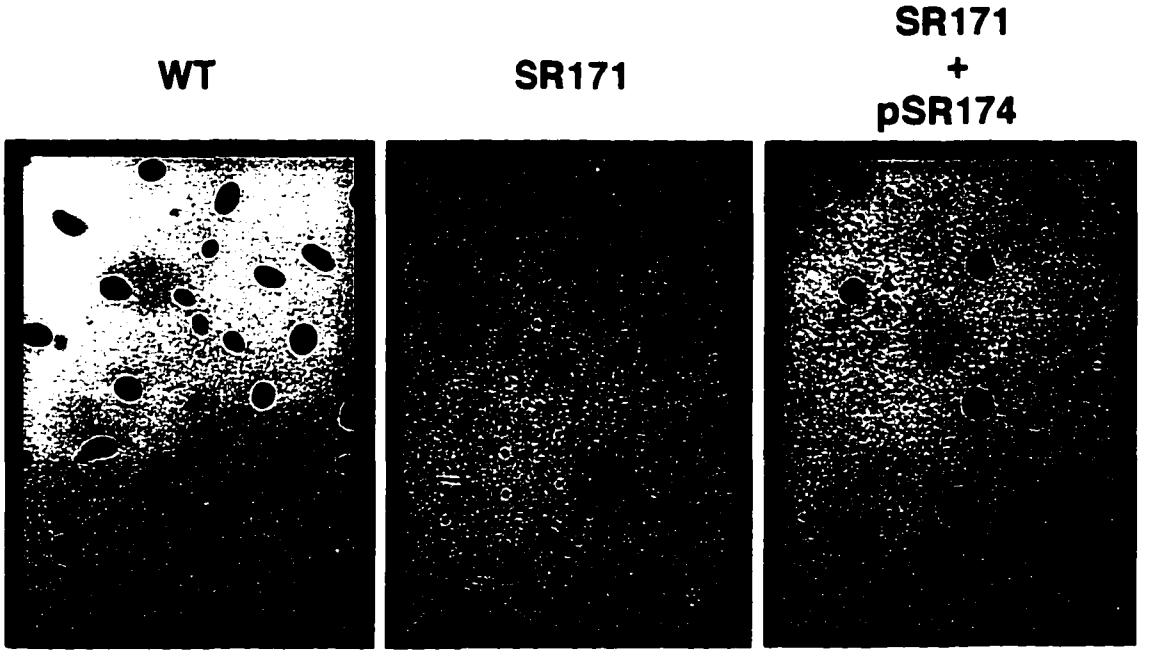
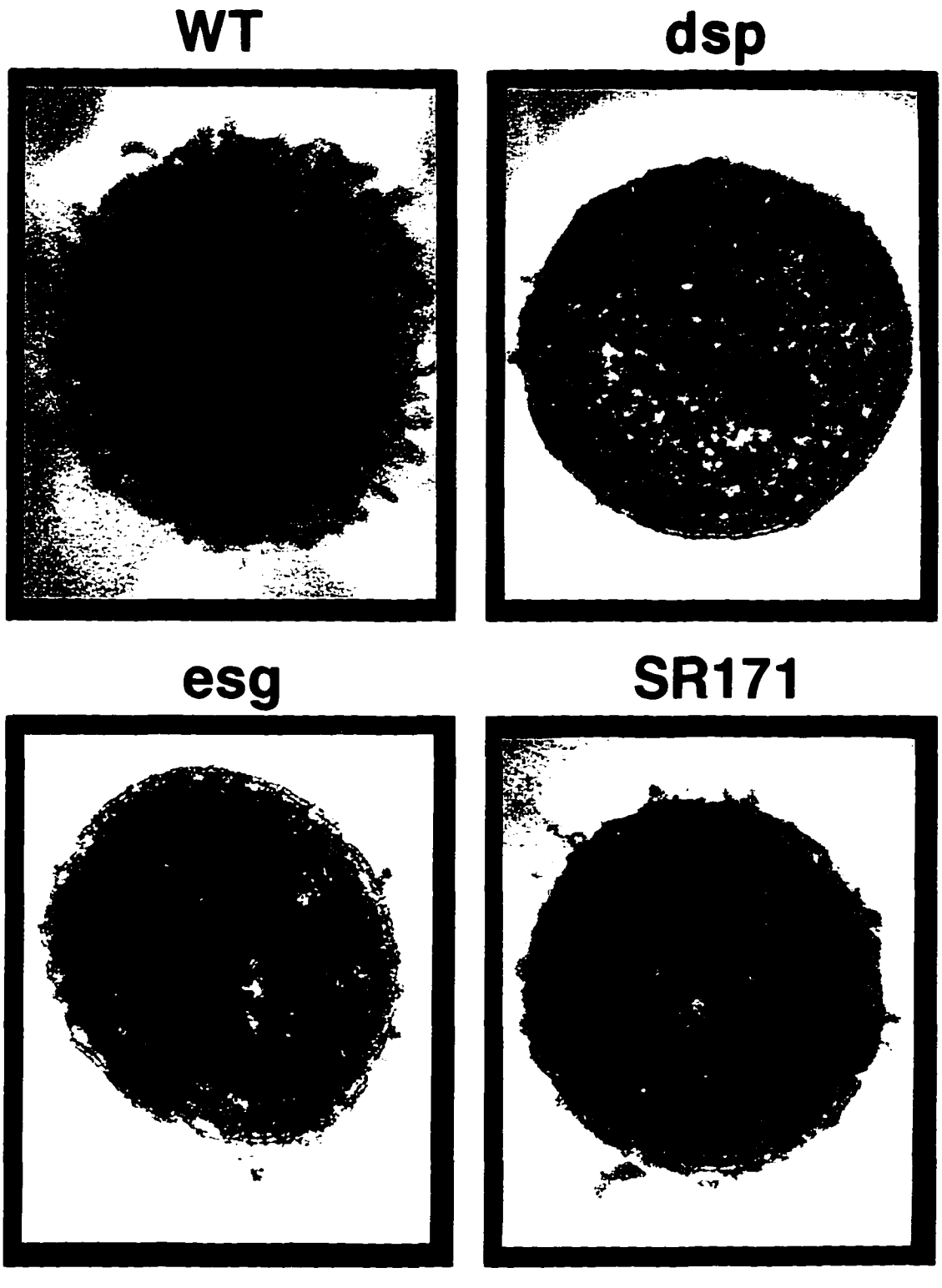


Fig 7. Fruiting body formation of Wild-type, SR171 and merodiploid (SR171 + pSR174) cells on CF agar. Cells were grown vegetatively in CTT medium and allowed to develop in submerged culture. Fruiting bodies were observed and photographed after four days.

the wild-type strain and the aggregation defect seen in SR171 was also rescued indicating genetic complementation had taken place (Figure 7).

Electron microscopic examination of wild-type and cds mutant cell surfaces. Cell surfaces of wild-type and Cds mutants were previously examined by FESEM and it was found that the mutants were deficient in fibril production (5,35). Cells were prepared for FESEM by growing on plates to high cell density. The loss of fibrils was correlated to a reduced production of carbohydrate in Cds mutants. It was also noted that the ratio of carbohydrate to protein in the exponentially growing wild-type cells was much higher than Cds mutant cells (35). In an attempt to understand the significance of this result, log-phase cells were prepared for transmission electron microscopy by ultrarapid freezing. It has been shown that biological materials prepared by ultrarapid freezing yield superior ultrastructural preservation since cellular constituents are immobilized in vitreous ice within milliseconds (22). Ultrarapid freezing of cells was achieved by spray-freezing (16,22). High quality freezing was observed in 75-80% of cells from each sample. Freezing damage was easily recognized as electron translucent patches within cells. Figure 8 shows that the surfaces of wild-type cells were coated with fibril

Fig 8. Electron microscopic examination of cell surface in wild-type and mutants. Cells were prepared for transmission electron microscopy by spray-freezing technique as described in Materials and Methods. Cross-sections of wild-type and Cds mutant cells (*esg*, SR171) are shown. *dsp* was used as a negative control for fibril production. The wild-type cells show cell associated fibrillar material whereas the mutants lack them. The fibrillar structures measured about 10nm in diameter and 60-100nm in length. The micrographs were taken from 120,000X images formed at an accelerating voltage of 100kV.


WT
dsp
esg
SR171

like structures. These structures are 10nm in diameter, 60-100nm in length and are peritrichously arranged on the cell surface. It appears that vegetatively growing wild-type cells have the ability to produce fibril like structures and the clumping of cells observed in shaker cultures are probably mediated by these structures. The cell associated fibrillar like material was absent in *dsp* and several Cds mutants tested (*esg* and SR171 shown in Figure 8). Since the log-phase Cds mutant cells have reduced levels of carbohydrate to protein ratios compared to wild-type cells, the results suggest that the fibrillar material is in part composed of carbohydrate.

V. DISCUSSION

SR171 was originally identified as one of the ten Cds mutants (35). In an earlier study, the Cds mutants were characterized phenotypically and it was found that they were defective in agglutination, polysaccharide production, fibril synthesis and development (35). The mutants retained S-motility and thus were different from S-motility mutants which shared several of the phenotypes with Cds mutants (8,35). The Cds mutants were distinguished from one another based on their subtle morphological charecteristics seen during vegetative growth and development. However, SR171 had the closest morphological resemblance to *esg* when compared to other Cds mutants.

In this study, SR171 was found to be distinguishable from other Cds mutants by its effect on *tps* gene expression. The *tps* gene is an example of a developmentally regulated gene which is normally expressed at a very low level during vegetative growth and the first five hours of development (11, 12). The *tps* gene fails to be expressed in group A, B and E signaling mutants (12). The gene encodes for a myxospore associated protein and is highly expressed after 5h of development (19). Transducing Cds mutations into JD10 which contains the *tps-lacZ* fusion, resulted in moderate to high levels of *tps* expression except for SR171 and *esg*, both of which

drastically reduced its activity. With this result, it became apparent that SR171 was more closely related to *esg* than any other Cds mutants.

SR171 and *esg* mutants were both able to induce polysaccharide synthesis when calcium was added to growing cells. However, both the mutants were deficient in polysaccharide and fibril production when grown in complex medium (35). Previous results have indicated that fibril production is regulated by Cds genes under nutrient limiting conditions (23, 35). The present results suggest that both the mutants have retained the capacity to produce fibrillar material when stimulated by calcium suggesting that fibrils are not only induced in stationary phase cultures, but also independently by calcium. It should be noted that more direct evidence is warranted to validate the fibrillar nature of calcium induced exopolysaccharide.

The results presented in this chapter strongly suggest the involvement of SR171 locus in branched-chain fatty acid synthesis. This suggestion stems from a comparative analysis made of a number of phenotypic properties between SR171 and *esg*. Table 2 shows that the total branched-chain fatty acids are reduced by half in both the mutants. The major branched-chain fatty acid found in *M.xanthus* is iso15:0 and is deficient in both the mutants. Most of the

branched-chain fatty acids in *M.xanthus* are derived from IVA and a previous study had indicated that IVA was more effective in correcting *esg* phenotypes than MBA or IBA (36). When IVA is added to vegetatively growing cells, the developmental phenotypes including aggregation, sporulation and *tps* gene expression were found to be corrected in both the mutants. It suggests that in both the mutants, IVA is able to bypass the mutational block in the branched-chain amino acid metabolic pathway. Exogenously added IVA is believed to be taken up by the mutant cells and is converted to the fatty acyl CoA derivative by a thiokinase (10). IVA-CoA is then used as a primer for the biosynthesis of long branched-chain fatty acids. The developmental defects are not corrected when IVA is added to cells undergoing development. This indicates that IVA is utilized by vegetative cells to produce essential fatty acid component(s) that are required later for the completion of development. The fact that IVA could rescue developmental phenotypes suggests that the mutation in SR171 is at the level of *esg* encoded BCKAD or at the deamination step. BCKAD is a multienzyme complex which is composed of four proteins namely E1α, E1β, E2, and E3 (31, 37). The *esg* locus encodes the E1α and E1β subunits of BCKAD (36). The E2 and E3 components of BCKAD have not yet been identified in *M.xanthus*. If the defect is in the BCKAD

enzyme, then it is likely that E2 or E3 components of BCKAD are involved since genetic analysis indicates that Ω171 insertion is in a different genetic locus than *esg*. Alternatively, the defect in SR171 could be in a regulatory gene that controls the production of BCKAD. Positive regulators of BCKAD have been identified and characterized in *E.coli* and *Pseudomonas putida* and mutations in those genes reduce BCKAD activity (26).

Analysis of growth patterns revealed that both the mutants grew like wild-type in complex media. However, in minimal defined medium, both the mutants failed to achieve wild-type levels of growth. The BCKAD deficiency in *esg* results in poor growth while Ω171 insertion does not seriously affect growth. This is probably because *esg* encoded BCKAD is not affected in SR171. Previously, it was shown that the *esg* locus encoded for a BCKAD enzyme and that cell-free extracts of *esg* were deficient in BCKAD activity (36). *esg* had no activity with KMV or KIC as substrates while reduced activity was observed with KIV. However, when crude cell-extracts of SR171 was tested, the BCKAD activity was found to be different from the *esg* pattern (data not shown). SR171 showed wild-type like activity when the three branched-chain keto acids (KIV, KMV and KIC) were used as individual substrates in the enzyme assay suggesting the two phenotypes are distinguishable. The BCKAD is a

multienzyme complex and has structural and enzymatic properties similar to those of pyruvate dehydrogenase and α-ketoglutarate dehydrogenase (21). The BCKAD activity was not affected in both the mutants when α-ketoglutarate or pyruvate were used as substrates. Many thanks go to Yanglong Zhu for providing the data on the enzyme assay.

SR171 and *esg* are both Tn*5* insertional mutants, yet, they lowered but did not eliminate branched-chain fatty acids when grown in rich medium (Table 2). One possibility is that both the mutants scavenged short branched-chain fatty acids from the complex medium and bypassed the mutational block to produce long branched-chain fatty acids. An alternative explanation is that there is a second pathway for making branched-chain fatty acids whose components are encoded by SR171 locus which functions co-operatively with *esg* encoded BCKAD. A dual purpose α-keto acid dehydrogenase complex which has both pyruvate and branched-chain dehydrogenase activities has been isolated from *Bacillus subtilis* (25). In addition, an exclusive BCKAD which is essential for branched-chain fatty acid synthesis has also been isolated from *B.subtilis* (30). Also, a second cluster of genes encoding the subunits of BCKAD complex has been cloned and characterized in *Streptomyces avermitilis*, another soil microorganism (9). In light of these findings, it will be interesting

to see if *esg*⁻SR171⁻ double mutant completely eliminates branched-chain fatty acids.

One of the major differences between SR171 and *esg* was their levels of pigmentation. *esg* retained certain amount of pigments compared to wild-type when grown in complex media while SR171 was very pale. An earlier study had indicated that *esg* was completely deficient in the production of pigments when grown in minimal medium (36). Scanning absorbance profile of cell extracts show that SR171 is more severely affected in pigment production than *esg*. The results suggest that both *esg* and SR171 are involved in the pigment production, plausibly by feeding catabolic products of branched-chain amino acid metabolism to synthesize the intracellular non-carotenoid pigment.

The SR171 locus was cloned from a wild-type cosmid that hybridized to a probe made from DNA adjacent to a Tn*5* insertion in the mutant. Restriction analysis showed that it was different from the *esg* locus. Sequencing the locus will provide additional insights as to how Ω171 insertion brings about a reduction in branched-chain fatty acid levels. An earlier study had indicated that developmental defects in *esg* could be corrected by extra-cellular complementation when mixed with wild-type cells (12). The results from this study indicate that developmental defects in SR171 are also corrected

when mixed with wild-type cells. In this regard, SR171 behaves as a signaling mutant and suggests that it is defective in the production of an essential signal required for the completion of development. Toal and Downard (10) have proposed that branched-chain fatty acids in a modified or unmodified form may constitute the E-signal. The results show that SR171, like *esg*, is required for the production of branched-chain fatty acids (Table 2). Owing to its similarities with *esg* mutant, SR171 appears to contribute to the production of fatty acid compounds during vegetative growth which are then used later on to produce the E-signal during development. However, to characterize SR171 as a new signaling mutant is premature since extracellular complementation with *esg* did not yield wild-type levels of sporulation. It remains to be seen if SR171 can be rescued for sporulation and other developmental phenotypes by other signaling mutants.

SR171 and other Cds mutants have been shown previously to be deficient in extracellular polysaccharide and fibril production (35). The log phase cells had lower levels of carbohydrate to protein ratios in the mutants compared to wild-type cells (35). It was of interest to see if this difference reflected any observable changes on the cell surface. The cells were prepared for transmission electron microscopy by spray freezing, an ultrarapid freezing

technique that is used for better preservation of biological structures (22). The results show that in wild-type, both cross-sections and full length cells, the cell surface is covered with fibril like structures that were 8-12nm in diameter and ranged between 60-100nm in length. The Cds mutants tested did not produce any cell associated fibrillar material. Fibrils, made of roughly equal amounts of protein and polysaccharide are known to be produced when wild-type cells are plated on complex medium at a high cell density (4, 5). The fibrils observed on plates were shown to have diameters ranging from 10nm to 60nm with the most common diameter being 30nm (5). Also, fibrils have been observed when wild-type cells are suspended in agglutination buffer containing calcium (2). However, *M.xanthus* cells growing vegetatively in liquid medium have not been described to produce any cell associated fibril like material. Previous studies have identified an antigen (FA-1) which is located exclusively on the extracellular fibrils (4). It will be interesting to see if this fibril specific marker can be identified by western analysis or immunogold electron microscopy on the newly described structure.

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CHAPTER 4

The *esg* locus of *Myxococcus xanthus* consists of two complementation groups

I. INTRODUCTION

The life cycle of *Myxococcus xanthus* includes the formation of multicellular structures called fruiting bodies. Fruiting body formation is initiated on a solid surface when nutrients are depleted and this process involves the movement of thousands of cells into mounds in which the individual cells differentiate into myxospores. The synchronous differentiation of a large population of rod-shaped cells into spherical spores require that cells communicate (10). Hagen et al.,(5) have isolated four groups (A-D) of conditional mutants that are defective in sporulation. Each group can sporulate well when mixed with wild-type cells or cells that belong to a different group. This type of phenotypic rescue of a developmental defect is termed extracellular complementation and does not involve genetic exchange. Each group of mutants behave as if they are deficient in the production of an essential signal during development, but are able to respond to such a signal produced by wild-type cells or cells belonging to a different group. An important function of cell-cell signaling is to control developmental gene

expression (3, 10). *tps* is a developmentally regulated gene that is expressed at a high level after 5h of development (2, 3, 4). *tps* gene expression can be monitored by measuring β-galactosidase activity produced from a *tps-lacZ* translational fusion gene integrated by homologous recombination into the *M.xanthus* chromosome (3). A transposon insertion mutant (JD258) was identified that reduced developmental *tps* gene expression (3). Other defects in JD258 included fruiting body formation and sporulation. The developmental defects could be corrected when JD258 was mixed with wild-type and other signaling mutants. The Ω258 insertion in JD258 defined a fifth extracellular complementation group (groupE) of signaling mutants and its genetic locus was referred to as *esg*. In this chapter, the genetic analysis of *esg* locus is described.

II. MATERIALS AND METHODS

Culture conditions and development. *Escherichia coli* cultures were grown in L broth or L agar (L broth with 1.5% agar). When appropriate, kanamycin was used at 50μg/ml. *M.xanthus* was grown in CYE medium. Development was initiated by plating concentrated suspensions of cells on CF agar. X-gal was used at 40μg/ml to assess β-galactosidase activity on CF agar.

DNA isolation and manipulations. *E.coli* plasmid DNA was isolated and manipulated by standard techniques (7). Restriction enzymes and T4 DNA ligase were used as recommended by the suppliers of the enzymes. Agarose gel electrophoresis was performed with 0.7% agarose gels with Tris-borate buffer. *E.coli* transformations were performed by standard methods (7). Tn*5* and Tn*5lac* transposons were introduced into plasmid pKS56 (8) which contains the *esg* locus by infecting the *E.coli* host MC1000 with λ::Tn*5* (4) or P1::Tn*5lac* (6). Plasmids containing the transposons were isolated and the locations of transposon insertions were determined by restriction mapping of plasmid DNA from the individual transformants.

Ω102 insertion was utilized to construct pSR3 which facilitated the sequencing of DNA representing complementation group 1. A 1.2kbp *Bam*HI-*Xho*I fragment was cloned in pGEM7Zf+ vector

(Promega). The *Bam*HI site is within the Tn*5lacZ* DNA and *Xho*I site is to the left of Ω258 insertion. A nested set of unidirectional double stranded deletions in pSR3 were generated using the Erase-a-Base system from Promega corporation. Single stranded DNA was then generated from several of those deletions and was utilized for sequencing by the Sanger dideoxy method (7). The sequencing reactions were manually performed by Doug Toal. Since pSR3 did not contain enough DNA from complementation group 2, the plasmid pKS56 containing the entire *esg* locus (5.5kbp) was sequenced by Sandy Clifton with the use of B. Roe's automated DNA sequencers.

Genetic analysis. The abilities of the various transposon insertions isolated in pKS56 to produce *esg* mutations were tested by transducing the plasmids into the wild-type *M.xanthus* strain, DZF1 (8). The developmental phenotypes of insertion mutant strains were determined by streaking on CF plates. β-galactosidase activity from the *esg* locus was determined qualitatively from spots of concentrated cells on CF agar or CYE agar.

III. RESULTS AND DISCUSSION

A 5.5kbp *Pst*I-*Eco*RI fragment containing the *esg* locus was subcloned from a 28kbp *Xba*I fragment to produce pKS56 (3). Figure 1 shows a partial restriction map of the *esg* locus along with the location of the Ω258 insertion in JD258. When pKS56 was introduced into JD258, genetic complementation was observed since the merodiploid strains displayed a wild-type developmental phenotype. To study the *esg* locus in more detail, several Tn*5* and Tn*5lac* insertions were isolated in the 5.5kbp *esg* fragment. When plasmids containing these insertions were introduced into a strain with a modified version of Ω258 insertion encoding tetracycline resistance (1), genetic complementation was observed in all cases except for Ω100 insertion. This suggests that the complementation group defined by Ω258 lies between the closest flanking insertions, Ω57 and Ω58, which are located about 1.5kbp apart. However, Ω58, Ω100, Ω102, and Ω106 insertions were able to generate mutant strains with an *esg* phenotype when they were introduced into wildtype *M.xanthus*. Insertions Ω56, Ω57, and Ω108 did not generate the *esg* phenotype. Since the insertions Ω58, Ω102, and Ω106 were capable of generating *esg* mutants, but were also able to genetically complement Ω258, it suggests that there are at least two complementation groups at the *esg* locus. Thus, complementation

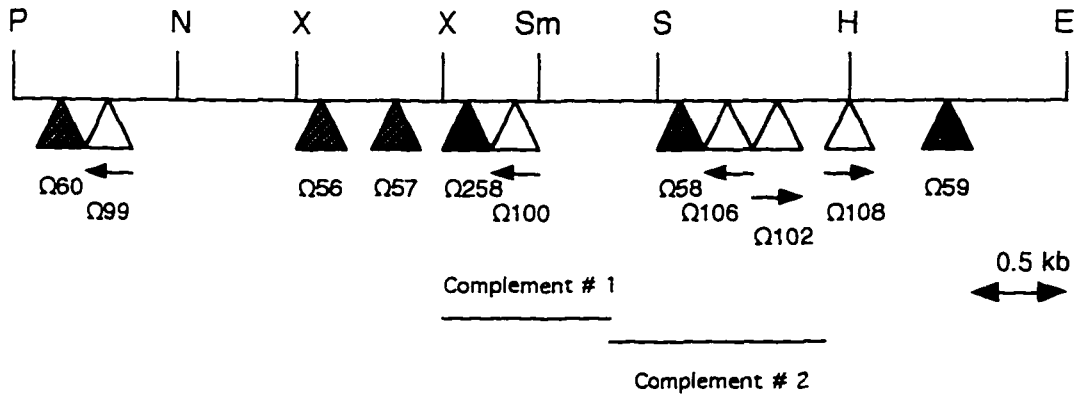

Figure 1. Genetic analysis of *esg* locus. The *esg* locus defined by Ω258 insertion (solid black triangle) is mapped within a 5.5kbp *Pst*I -*Eco*RI fragment. A partial restriction map of the locus is shown. P, *Pst* I; N, *Nru*I; X, *Xho* I; Sm, *Sma*I; S, *Sal* I; H, *Hind*III; E, *Eco*RI. Locations of additional Tn*5* (striped triangles) and Tn*5lac* insertions (open triangles) are also shown. The arrows below the Tn*5lac* insertions indicate the direction of transcription from each insertion. Genetic complementation studies identified two complementation groups and the boundaries of each group is shown as black lines labelled 'complement # 1' and 'complement # 2'.

group 1 is between Ω57 and Ω58 and complementation group 2 is between Ω100 and Ω108.

The expression of the *esg* locus was investigated utilizing the Tn*5lac* insertions that had been introduced into *M.xanthus*. Tn*5lac* is a modified transposable element that can be utilized to form transcriptional fusions with the *lacZ* gene of *E.coli* when they insert into a transcription unit in the appropriate orientation (6). Strains containing Ω99, Ω100, and Ω106 insertions had *lacZ* oriented in a right to left direction while it was reversed in Ω102 and Ω108 insertions. Tn*5lac* insertions having right to left orientations (Ω100, and Ω106) expressed low levels of β-galactosidase both during vegetative growth and development while insertion Ω102 expressed high levels of activity suggesting that *esg* locus is transcribed from left to right direction as shown in fig1. Ω108 insertion which appeared to reside outside the *esg* locus also expressed β-galactosidase both during vegetative growth and development. But, the level of expression was significantly lower than Ω102 suggesting that Ω108 lies within a different transcription unit. Ω99 insertion was located outside the *esg* locus, approximately 1.9 kbp from Ω258 and was oriented from right to left. This insertion produced significant levels of β-galactosidase both during vegetative growth and development suggesting that a

third transcription unit is present close to the *esg* locus. In conclusion, the Tn*5lac* insertions isolated in the *esg* locus has been very useful in studying the regulation of *esg* expression in response to different media conditions both during vegetative growth and development.

Ω102 insertion was used to subclone *M.xanthus* DNA from pKS56. A 1.2 kbp *Xho*I-*Bam*HI fragment containing DNA from the complementation group 1 region was cloned and utilized for sequencing. DNA sequencing analysis of the *esg* locus revealed the presence of two open reading frames which encoded for the α and β subunits of E1 component of a branched-chain keto acid dehydrogenase (BCKAD) (11). The two open reading frames (ORF1 and ORF2) corresponded to the two complementation groups identified by genetic analysis (fig 1). The direction of transcription from the *esg* locus inferred from restriction analysis of secondary Tn*5lac* insertions was also confirmed from the DNA sequence analysis. The BCKAD is a multi-subunit enzyme complex which is structurally and genetically related in a wide variety of organisms including vertebrates and bacteria (9). Purified BCKAD complexes from *Pseudomonas putida*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, and several mammals are all found to be composed of four polypeptides, E1α, E1β, E2, and E3 (9). The genes encoding the BCKAD complex in

P.putida and *B.subtilis* are found to be clustered in the sequence E1α, E1β, E2, and E3. Secondary Tn*5* insertions to the right of complementation group 2 (figure 1) did not yield the *esg* phenotype suggesting that a third complementation group did not exist. This was confirmed from DNA sequence analysis which showed no evidence of homology to E2 or E3 subunits of BCKAD downstream of ORF2 (unpublished observations). The absence of homology to E2 or E3 components within the *esg* locus may be unusual, but does not suggest that these components are not found in *M.xanthus*. Further analysis will be required to better understand the role of *esg* encoded BCKAD in both vegetative growth and development of *M.xanthus*.

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