INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

UMI

A Bell & Howell Information Company
300 North Zeeb Road, Ann Arbor MI 48106-1346 USA
313/761-4700 800/521-0600

UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

STUDY OF THE ROLE OF THE *M. XANTHUS ESG* LOCUS IN DEVELOPMENT AND LIPID BIOSYNTHESIS

A DISSERTATION

SUBMITTED TO THE GRADUATE FACULTY

in partial fulfillment of the requirements for the

degree of

DOCTOR OF PHILOSOPHY

BY

Geoffrey A. Bartholomeusz

Norman, Oklahoma

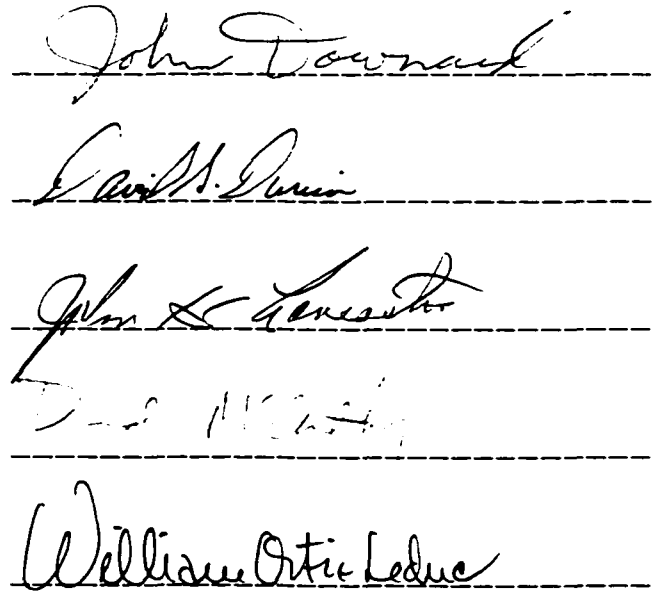
1998

STUDY OF THE ROLE OF THE *M. XANTHUS ESG* LOCUS IN DEVELOPMENT AND LIPID BIOSYNTHESIS

A DISSERTATION APPROVED FOR THE

DEPARTMENT OF BOTANY AND MICROBIOLOGY

BY



DEDICATION

To my wife Chandra and my parents Frederick and Edith Bartholomeusz

ACKNOWLEDGMENT

To begin with I record my deep appreciation and thanks to my advisor, Dr. John Downard for his unfailing courtesy and generosity with which he extended to me the benefits of his guidance, his experience, his advice and his extensive store of knowledge. Dr. Downard's approach to graduate education was both unique and refreshing.

I also owe my thanks to Dr. John Lancaster, Dr. David McCarthy, Dr. David Durica and Dr. William Ortiz-Leduc for their time and support as my graduate committee members.

I owe a special expression of thanks to Dr. Ralph Tanner, Dr. Mike McInerney and Dr. William Ortiz-Leduc for their unfailing willingness in granting me access to equipment in their labs.

May I also record my special thanks to my dear friends Gamini and Kushi Dharmasena for their unfailing support and encouragement.

My wife Chandra has always been a great source of support and inspiration to me. I thank her for always having confidence in me for she is the wind beneath my wings.

Finally to my parents I say God Bless you both for always being there for me and for your unfailing faith in my abilities. This day I owe to the two of you.

TABLE OF CONTENTS

LIST OF TABLES

TABLE

LIST OF ILLUSTRATIONS

FIGURE

Abstract

Analysis of the pattern of expression of developmentally regulated genes in the *esg* mutant background, suggests that E-signaling dependent events of development occur after 3 to 5 hours of development. It has been predicted that iso-15:0 may function as a signal molecule based on the observation that *esg* mutants do not develop and synthesize low levels of iso-15:0. By growing wild-type and *esg* mutant cells of *M. xanthus* with H^3-labeled branched-chain fatty acids we showed that *esg* mutant cells incorporated labeled leucine into lipids at no more than 11% of the wild-type cells. Levels of iso-15:0 incorporated into the phospholipids of the *esg* mutant cells were observed to be more than three times lower than those of wild-type cells. These results confirmed that the reduced synthesis of iso-15:0 in the *esg* mutant may be responsible for its developmental defect. If iso-15:0 is a signal molecule we predict that its levels would increase during the early stages of development. Analysis of leucine labeled free fatty acid fractions purified from developing wild-type cells showed that iso-15:0 is not released as a free fatty acid but is a

component of a larger labeled species whose level remained relatively constant during the first ten hours of development. These labeled lipid species were biologically active and rescued development of an *esg* mutant. Iso-15:0 by itself was also biologically active. We propose that fatty acids or a combination of fatty acids and other lipid compounds synthesized during vegetative growth in the *esg*-dependent pathway may function as signal molecules during development of *M. xanthus*. A nutritional dependent fatty acid response occurs in *M. xanthus*. We observed that *esg* gene expression and BCKAD activity were under nutritional regulation and was responsible for the changing fatty acid composition of the cell. *Esg* mutants failed to respond to nutritional changes. We conclude that the BCKAD is required for the nutrition dependent fatty acid response in *M. xanthus*.

CHAPTER 1

Myxobacteria: The Unicellular Prokaryote Exhibiting a Primitive Multicellular Life Cycle.

1.1 Introduction

Myxobacteria, a group of Gram negative bacteria that have been in existence for over 2 billion years (46), were first described by Roland Thaxter in 1892 (85). These bacteria have adopted a unique multicellular life cycle. This multicellular behavior has enabled these bacteria to be very competitive, enabling them to optimize feeding and minimize the loss of their hydrolytic enzymes by diffusion.These enzymes are used by the bacteria to lyse their prey and break down macromolecules into smaller consumable compounds. The life cycle of myxobacteria is therefore geared towards maintaining a high cell density at all times.

Myxobacteria have, to a large extent, been successfully cultured in laboratories affording scientists the opportunity to examine the mechanisms of cell interactions and cell communications in an organism that is experimentally convenient and is amenable to modern

biochemical and genetic approaches. Although a large number of myxobacterial species have been identified, most of our current knowledge of myxobacterial biology has come from extensive studies of one species in particular, *Myxococcus xanthus*. The life cycle of *M. xanthus* is typical of myxobacteria and consists of two alternative phases. In the presence of adequate nutrients, the cells enter the growth phase (vegetative phase). When the food supply is depleted, the cells adapt by forming multicellular fruiting bodies containing spores (developmental phase).

1.2 Vegetative Growth Phase

The vegetative growth phase of individual *M. xanthus* cells is typical of most bacteria. The gram negative rods grow in length and divide by binary transverse fission. However, unlike most other unicellular bacteria, *M. xanthus* cells display a remarkable degree of social behavior. This social behavior enables the bacteria to forage as a multicellular swarm of bacteria referred to as a "microbial wolf pack " (25). Cell motility on solid surfaces is essential for swarming. This type of motility is called gliding. The bacteria secrete a large amount of extracellular polysaccharide material and it is assumed that the bacteria

utilize this extracellular material to slide over a solid surface. Gliding motility is associated with individual cells and with masses of cells which move as a cooperative multicellular unit. Characterization of *M. xanthus* mutants that are defective in gliding motility suggests that gliding motility is controlled by two independent motility systems, the A and the S systems. The S (social) system directs group movement, while theA (adventurous) system, controls the movement of individual cells (42). Both systems have to be active for efficient motility. For example, A^+S^- cells fail to move as a group while A^-S^+ mutants fail to demonstrate individual cell motility. Mutants defective in both systems (A^-S^-) are nonmotile. The S and A systems are in turn controlled by a single locus called the *mgl* (mutual function for gliding) locus, since motility is abolished in these mutants.

The *M. xanthus* frizzy mutants have served to define a set of genes called the *frz* genes (94). These genes control bacterial movement by regulating the frequency at which the bacteria reverse their direction of motility. The *frz* locus comprises seven genes named *frz* A, B, CD, E, F, G, and Z (5) (94). Collectively, these genes show striking similarities to the signal transducing systems used to control chemotaxis in *E. coli* (94). It appears logical to assume that the

direction of movement in *M. xanthus* is governed by a chemotactic response. After all, chemotaxis has been shown to play an important role in the feeding behavior of most bacteria as well as in the social interactions of the multicellular eukaryote *Dictyostelium* (22) (46).

Recent work in *M. xanthus* has failed to demonstrate chemotaxis (42) (102). Dworkin and Eide (24), have proposed that myxobacteria do not utilize a chemotactic mechanism in their motility. They observed that *M. xanthus* failed to respond chemotactically to moderate gradients of a wide variety of defined chemicals. Shi et al (96), have disputed this finding. Under their experimental conditions, they were able to show that a colony of cells moved towards increasing concentrations of casitone and yeast extract and moved away from repellents such as isoamyl alcohol. They interpreted this behavior as a chemotactic response.

M. xanthus contains two surface appendages, pili and fibrils that play an important role in group motility (23) (47). The fibrils, contain equal amounts of protein and carbohydrate (3). They are important in cell-cell interactions (23) and supply the cohesive forces that enable cells to manifest cohesion, social motility and fruiting body formation (1). The pili of *M. xanthus* are polarly located and have been

shown to play a role in social motility (21). However, the precise role of the pili remains unresolved.

1.3 Developmental Life Cycle

As vegetative cells perceive nutrient depletion, they initiate a program of timed events involving the initiation of development, aggregation, the construction of a multicellular fruiting body, and finally the conversion of the vegetative rod shaped cells into oval myxospores within thisfruiting body.

INITIATION:- Limiting concentrations of amino acids in a growth medium elicit the starvation response in many microorganisms, including *M. xanthus* (26) (40) (74). Since amino acids are used by bacteria as a source of carbon and energy and for protein synthesis, it is necessary that bacteria be able to conserve their internal concentrations of amino acids during starvation conditions. This requirement is provided by the stringent response mechanism which has been studied in great detail in *E.coli* and *Salmonella.* The stringent response enables bacteria to respond to low concentrations of amino acids by increasing their intracellular concentrations of guanosine 3'-di-

5'(tri)di-phcsphate nucleotides [(p)ppGpp] (103). The (p)ppGpp appears to couple rRNA synthesis and the rate of cell growth to the capacity of the cell for protein synthesis. Intracellular levels of (p)ppGpp have been observed to increase significantly during the first 30 minutes of *M. xanthus* development and then decrease over a period of 2 to 4 hours (73) (103). To determine whether this early increase in (p)ppGpp levels serves to initiate development in *M. xanthus,* Singer and Kaiser (103) introduced the *relA* gene from *E. coli* into *M. xanthus.* The *relA* gene was expressed in a light dependent fashion using the light-inducible promoter *carQRS.* The *relA* gene of *E. coli* codes for (p)ppGpp synthetase 1 which catalyzes pyrophosphate transfer from ATP to GTP forming (p)ppGpp (103). The stringent response is usually observed in vegetative cells growing in an environment containing depleted amounts of amino acids. Singer and Kaiser (103), showed that an elevation in the intracellular concentration of [p]ppGpp stimulated early developmental gene expression recorded over the first 4 hours of development. Although (p)ppGpp is synthesized from GTP, observed levels of GTP remained unaltered during the period of their study, suggesting that GTP may not play a role in the initiation of development. Reduced intracellular concentrations of GTP have been

shown to play a role in the initiation of sporulation in *Bacillus subtilis* (70). Similar reduced levels of intracellular GTP in *M. xanthus* failed to induce development (103) suggesting that it is the increase in levels of (p)ppGpp and not the reduced levels of GTP that is the significant process in the initiation of development in *M. xanthus* .

Singer and Kaiser (103) have proposed a two level model to help explain the most probable mechanism governing the initiation of development in *M. xanthus.* In this model, the (p)ppGpp serves as an intracellular signal of starvation permitting the individual cell to assess its own nutritional status. This individual response is then followed by the population response controlled by the A-signal amino acids (see A-signal), which accumulate in the media one hour after the initiation of development and rise to a steady state somewhere between 2 to 4 hours of development.

AGGREGATION :- At the onset of aggregation cells move in a fixed direction towards aggregation loci. They accumulate at these loci stacking up one against the other until they reach a threshold cell density. It is only when this cell density has been reached that the cells can initiate the next sequence of steps that lead to fruiting body

formation and myxosporulation. Unidirectional movement towards aggregation loci is an important feature of aggregation. Developmental aggregation has also been observed in the simple eukaryote *Dictyostelium discoideum* (46). In this amoebae, the signal cAMP, secreted by selected cells, acts as a long range signal eliciting a chemotactic response from the nonproducing cells. The cells receiving the cAMP pulse move towards the source of the signal. This behavior serves to assemble dispersed and often spatially separate amoebae at a particular locus (22) (46) (47). Myxobacteria, on the other hand, are not dispersed but are in close contact with each other within the multicellular unit. This close proximity of the cells eliminates the need for a long range signal. Since both *M. xanthus* and *Dictyostelium* demonstrate unidirectional movement towards aggregation loci, it was thought that cAMP might play a similar role in *M. xanthus*. However, studies have shown that *M. xanthus* does not produce cAMP (41) (116).

Cell-cell contact is favored over chemotaxis as the mechanism responsible for driving *M. xanthus* cells towards aggregation centers (21). It has been proposed that at the onset of development, head to tail contact between cells of *M. xanthus* activates C-signaling, which in turn induces the methylation of the *Frz* CD protein. The result of

this methylation is a reduction of the reversal frequency of gliding. Being unable to change directions the cells form unidirectional trails moving towards aggregation centers. This theory is supported by the work of Shi et al (95) who observed that cells stained with tetrazolium dye had a lower reversal frequency when developing in large groups as compared to small groups. They also observed that the reduced cellular reversal frequency was correlated with the methylation of *FrzCD* [a member of the methyl accepting chemotaxis protein (MCP) family]. The signals that initiate these changes remain unknown.

FRUITING BODY FORMATION/MYXOSPORULATION:- Cells assembling at aggregation centers have to reach a threshold cell density of between 10^5 and 10^6 cells in order to continue development. Cells will not aggregate or form fruiting bodies in an environment with a concentration less than the critical cell density (101). To enable the aggregating cells to monitor their cell density, they secrete molecules that serve as intercellular signals whose concentration is directly proportional to cell density. One such signal molecule has been identified as adenosine, although there is no information about the source of this adenosine nor about its receptors (101). There is also evidence that the concentrations of amino acids secreted by the cells

as part of the A-signal system (discussed in the next section) are important sensors of cell density.

The movement of cells at aggregation centers is not random but highly ordered. Using scanning electron micrographs of developing cells of *M. xanthus*, O'Connor and Zusman (80), demonstrated that cells within aggregates move in an orderly spiral fashion. Cells at the base of the aggregate move in spirals that are parallel to the substratum, while the cells in the upper areas of the aggregate move in spirals that are perpendicular to the substratum. Once the cells at the aggregation centers have reached their critical cell density, development enters its committed stage.

From this point onwards, the stages of development are under the control of five major signal systems that regulate the temporal expression of developmental genes enabling all cells to undergo development in a synchronous manner leading to the formation of multicellular fruiting bodies. Each fruiting body contains a large number of cells which undergo a morphological change to form myxospores. Conducting three dimensional analysis of fruiting bodies of *M. xanthus* using confocal fluorescence microscopy, Sager and Kaiser (92) showed that the fruiting body is divided into two concentric hemispherical

domains. The outer domain is made up of densely packed rod shaped cells that tend to move in concentric clockwise and counterclockwise streams. The inner domain contains the less densely packed non-motile spores.

To further characterize the relationship between the types of cells in both domains, Sager and Kaiser (91) studied the spatial expression patterns of 80 *M. xanthus* developmental gene fusions with the *E.coli lacZ* gene within a maturing fruiting body. Eight of these fusions demonstrated expression restricted to the inner domain. One fusion showed initial patchy expression in the outer domain coinciding with spore precursors that became visible under bright field microscopy. As the fruiting body matured, the expression of the gene and the patches expanded inwards, eventually filling both the inner and outer domains. From these observations, Sager and Kaiser (91) proposed that myxospore formation occurs at the periphery and that the movement of these myxospores into the interior of the fruiting body is a result of their passive transport by the mobile rod shaped cells that have not undergone morphogenesis.

Developmental autolysis occurring during the stages of aggregation and early mound formation is responsible for the fact that

only 10% to 20% of the *M. xanthus* cells entering development complete fruiting body formation and eventually convert to myxospores (114) (115). Autolysis is not unique to *M. xanthus* but appears to be a common event in multicellular development. During the developmental life cycle of *Dictyostelium discoideum*, approximately one third of the 10^5 cells in a fruiting body are committed to death via stalk cell formation (109). During conditions of nutrient depletion *Streptomyces* initiate the formation of aerial mycelia as specialized branches of the substrate mycelium. Cells at the end of some of these aerial mycelia differentiate into a chain of spores. During the formation of the aerial mycelium and spores the substrate mycelia have been observed to undergo massive lysis (22). Whether autolysis in *M. xanthus* is analogous to programmed cell death (apoptosis) remains an unanswered question. There are, however, some possible explanations for the importance of developmental autolysis. For instance, lysed cells could provide the much needed nutrients to ensure that the survivors are able to successfully complete the formation of viable myxospores (97). Protein S is a spore coat protein synthesized within the developing cell. There is no evidence that this protein is a secretory protein and the question as to how this protein reaches the outer coat of the spore remains

unresolved. One theory is that the lysis of the vegetative cells releases protein S which is then free to self assemble on the myxospore surface of nonlyzed cells (107).

1.4 The signaling systems identified in *Myxococcus xanthus*

Development in *M. xanthus* is the culmination of a series of temporal events set in motion by nutrient depletion. Therefore, in order that a population of single cells may accomplish this morphogenetic event synchronously as a multicellular unit, these cells have to communicate with each other via intercellular signals. Five signaling mutant classes have been identified in *M. xanthus*. (18) (38). It has been observed that when each of these signalmutants is co-developed with equal amounts of wild-type cells the developmentally deficient mutant cells regain their ability to undergo development. This process is referred to as extracellular complementation. If the wild-type cells are able to rescue development in the mutant, this would suggest that the wild-type cells supplies the signal molecules missing in the mutant, enabling the mutant to develop. The signaling mutant

groups were also observed to be able to extracellularly complement each other indicating that the mutant groups do not belong to the same complementation group. The extracellular complementation experiments led to the characterization of five signaling systems known as the A-, B-, C-, D-, and E-signaling systems. The pattern of developmental gene expression in each mutant was also different, leading to the conclusion that the mutants block development at slightly different points in the developmental program (57) [chapter2]. These patterns of developmentally regulated gene expression were used to position the signaling systems in an ordered developmental program.

A-Signaling System

A-signaling mutants fail to develop and express the developmentally regulated *lacZ* transcriptional fusions dependent on the A signal (57). The A signal activity has been primarily linked to a mixture of 6 amino acids identified in media conditioned by developing wild-type cells (62) (63) (64) (65). These amino acids (tyr, pro, phe, trp, leu, ile) are released into the medium by the action of proteases (82). The A-signaling system is said to require the function of the protease and the amino acids since it has been demonstrated that development of an A-

signal deficient mutant is rescued either by the amino acids or by proteases (62) (83). The proteases have A-signal activity because they cleave extracellular proteins, releasing the amino acids.

The activity of all five signaling systems is required for the initiation of the pre-aggregation stage of development. The observation that the developmental program in A-signaling mutants is arrested 1 to 2 hours after the initiation of development (65) (76) and that cells release the amino acids 1 to 2 hours into development (63) suggests that the A-signaling system is required for the initiation of the early events of the pre-aggregation stage of development. As the individual cells encounter starvation conditions, they respond by releasing the amino acids. This release is proportional to the cell density of the aggregating cells (62). The released amino acids reach their threshold concentration when the aggregating cells reach a cell density of approximately 10^5 cells (62). At this cell density the A-signal amino acids are at a concentration that is high enough to induce the expression of a set of developmental genes (A-signal dependent genes). This results in the stimulation of the population of cells to initiate aggregation. The aggregation of a large number of cells at one aggregation center ensures that a sufficient number of cells will be available to build a

fruiting body so that an adequate number of viable spores will germinate under nutritionally favorable conditions giving rise to a high density population (45) (62). It is most likely, therefore, that the A-signal allows individual cells to monitor cell density and falls into the category of quorum sensing signals that were initially described for the luminescent bacteria, but that have now been shown to exist in a variety of other organisms (21).

A-signal production is controlled by three genetic loci referred to as the *asgA*, *asg B*, and *asgC* loci (64). These loci have been fairly well characterized. The *asgA* gene contains two domains, one of which has been shown to be homologous to the transmitter domain of a histidine protein kinase, while the other is homologous to the receiver domain of response regulators (69) (81). The *asgB* gene encodes a putative DNA-binding protein with a helix-turn-helix motif near its C-terminus (82). The sequence of the *asgB* locus is very similar to the segment of the major sigma factor that recognizes and binds to the -35 region of promoters. This sequence, however, does not have any sequence similarity to the highly conserved regions of the major sigma factors that interact with core RNA polymerase. The AsgB protein is most probably not a sigma factor. It is thought to function as a transcription activator (82). The

asgC locus encodes the major sigma factor of the RNA polymerase holoenzyme (15).

The current knowledge gained about the A-signaling system has been summarized in terms of a simple model (21). This model states that the starvation signal is transduced by the action of AsgA, a response regulator, and of histidine kinase which functions within a phosphorelay system. The AsgB, a DNA-binding protein, acts as a transcription factor, perhaps activating the gene coding for the extracellular proteases, while AsgC functions as a major sigma factor. The combined actions of the AsgA, B, and C-coded molecules results in the release of protease which generate the A-signal amino acids. The concentration of the amino acids acts as a sensor for the detection of cell density which is an important prerequisite for normal development.

B- Signaling System

The B-signaling mutant phenotype is the result of a mutation in a single locus called the *bsgA* locus(34)(35)(36). This mutant fails to develop and express all the developmentally regulated *lacZ* transcriptional fusions identified by Kroos et al (57). Since the expression of these fusions occurs from 0 to 24 hours of development, it is very

likely that the B-signaling system is established at the onset of development. The *bsgA* gene has been cloned and sequenced and found to be homologous to the lon genes of *E. coli* and *Bacillus brevis* (33). The lon gene of *E. coli* codes for a 90 kD protein called the La protein that functions as a protease. The BsgA protein has been partially purified and identified as a 90.4 kD protein (33). Due to the similarity between the La protein of *E. coli* and the BsgA protein, it is suggested that the *bsgA* gene probably codes for an ATP-dependent protease that is related to the La protease of *E. coli* whose function is to destroy a repressor protein controlling the expression of the developmental genes (33).

C- Signaling System

Once *M. xanthus* begins to aggregate it requires the C-signaling system to complete fruiting body morphogenesis and sporulation. C-signaling mutants fail to demonstrate developmentally regulated *lacZ* transcriptional fusions expressed after 6 hours of development. This suggests that the C-signaling system is required at about 6 to 7 hours of development (57). Mutations preventing C-signaling map to a single locus known as *csg* A (99) (100). A 17 kD CsgA protein was purified using

the capacity of the protein to rescue fruiting body formation, sporulation and the expression of C-dependent genes in a *csgA* mutant (56). Amino acid sequence analysis confirmed that the identified protein was the product of the *csgA* gene (37) (56) (98). Using an immunological probe, CsgA antibody linked to immunogold, Skimkets (98) demonstrated that the C-signal is localized in the extracellular matrix of developing *M. xanthus* cells.

Cell contact is an important factor for the transmission of C-signaling. Signal transmission is only possible if the cells are motile and can orient themselves to permit maximum cell-cell contact. Cell alignment, rather than motility alone, has been shown to be the key factor in this process by mechanically forcing nonmotile cells into a closely packed arrangement (55). Nonmotile *mgl* mutants produce C-signal but fail to respond to the signal that is present. However, when these cells were aligned lengthwise by settling into narrow grooves etched on an agar surface, the mechanically aligned cells in these troughs were found to activate C-signal dependent gene expression. It has recently been observed that end to end collision between cells may be important for C-signal transmission instead of the earlier mentioned lengthwise cell alignment (90) (105). End to end collision has also been

observed to reduce reversal frequency in *M. xanthus* permitting the cells to move in one direction (105). Thus end to end cell collision may permit C-signaling and cause *frz* gene dependent unidirectional movement of cells towards aggregation centers.

The transcription of the *csgA* locus measured by the expression of a *lacZ* reporter was observed to increase steadily during development suggesting that changing concentrations of CsgA protein may be an important factor in the activation of the different stages of development (37). This assumption was confirmed by the observation that C-signaling mutants could be rescued at various stages of development depending on the concentration of the added CsgA protein (54) indicating that the C-signal may act as a development timer.

It has been recently suggested that the CsgA protein may not function directly as a signal. It has also been proposed that the CsgA protein may be a short chain alcohol dehydrogenase (SCAD) (67) (68) which is involved in the generation of the C-signal. The CsgA protein was observed to have amino acid sequence homology with members of the SCAD family (21). Based on the amino acid sequence, Lee et al (66) proposed that CsgA contains an $NAD(P)^+$ binding site. They were also able to demonstrate by mutational studies that this NAD pocket is

necessary for CsgA activity. In spite of these encouraging results, no substrate has yet been found for the dehydrogenase nor has it been demonstrated biochemically that the C-signal has dehydrogenase activity (66). It has been shown, however, that the addition of NAD^+ or $NAD(P)^+$ together with the CsgA protein improves the ability of this protein to rescue development of a *csg A* mutant, whereas the addition of NADH or NADPH inhibits such rescue (66). While there is a considerable body of information about the C-signaling system, the precise mechanism of action of C-signaling in the mediation of cell-cell communication still remains unresolved.

D- Signaling System

The genetic defect in a D-signaling mutant maps to the *dsgA* gene. This gene is essential for growth, but a particular *dsg* mutant allele is able to grow but fails to form fruiting bodies. The requirement of the *dsg* A gene for growth is unique among *the M. xanthus* signaling genes. This mutant is able to sporulate, although the extent of the sporulation is substantially reduced and aggregation is abnormal and delayed (11) (12). Characterization of the *dsg* gene has shown that this gene bears a 50% amino acid identity to the *E. coli* translation

initiation factor IF3 that helps the ribosome select the initiation codon on the mRNA (13) (48). The DsgA protein, which is present at relatively constant levels during vegetative growth and development has been proposed to act as a translation initiation factor of *M. xanthus*. The precise mode of action of the D-signaling system in *M. xanthus* development or the chemical structure of the D-signal molecule have not as yet been identified.

E- Signaling System

An E-signaling mutant was identified by Tn5 insertion mutations of *M. xanthus* which caused a defect in the ability of this mutant to express the developmentally regulated *tps* gene (18) (43). The expression of the *tps* gene commences at about 5 hours of development reaching a peak at about 18 to 20 hours (19) (20). *Tps* expression in developing E-signaling mutant cells ranged from 1% or less of wild-type activity within the first 24 hours to about 11% within 66 hours (18). These mutant cells failed to form well-defined fruiting bodies when spotted on the agar surface under developmental conditions and formed approximately 10^4 fewer spores than wild-type cells after 4 days of development. Wild-type cells were able to

complement the E-signaling mutant defect resulting in essentially wild-type levels of sporulation by the *esg* mutant. This observation suggests that wild-type cells supplied an extracellular signal molecule that the E-signaling mutant failed to produce, but retained the ability to respond to, thus regaining the capacity for cell-cell communication which is a necessary requirement for normal development. Extracellular complementation has been demonstrated between the A-, B-, C-, and D-signaling mutant and the E-signaling mutant suggesting that the E-signaling mutant defines a fifth extracellular complementation group. Likewise, since the four other signaling mutant classes successfully complement the E-signaling mutant it can be concluded that they too produced the signal absent in the E-signaling mutant. These results indicated that the E-signaling system defined a new signaling system of *M. xanthus* (18).

Toal (108) sequenced the *esg* locus and identified its gene products as the E1α and E1β subunits of the branched-chain keto acid dehydrogenase (BCKAD). The results of the DNA sequence confirmed the earlier predictions that the *esg* locus differed from the *asg*, *bsg*, *csg* and *dsg* loci. This enzyme is a part of a pathway responsible for the metabolism of the branched-chain amino acids leucine, isoleucine and

valine (50) (Fig.1). It catalyzes the oxidative decarboxylation of the branched-chain α-keto acids (produced by the deamination of the three branched-chain amino acids) to CoA derivatives of the short branched-chain fatty acids isovaleryl-CoA (from leucine), α-methylbutyryl-CoA (from isoleucine), and isobutyryl-Co (from valine). The CoA esters serve as primers for the synthesis of the long branched-chain fatty acids (50). The primary defect of the *esg* mutant is therefore its inability to metabolize the branched-chain amino acids via the *esg* BCKAD resulting in a reduced synthesis of long branched-chain fatty acids. (108). The above results suggested that the branched-chain fatty acids play an important part in *M. xanthus* development. It is known from studies conducted in *Bacillus subtilis* (113) (50) that if the short branched-chain fatty acids isovalerate, methylbutryate and isobutyrate are added to the growth medium, these fatty acids will be taken up by the cells, converted to their CoA derivatives and will then be used for the synthesis of the long branched-chain fatty acids. These CoA derivatives of the short branched-chain fatty acids enter the pathway beyond the point where the BCKAD acts (Fig.1).

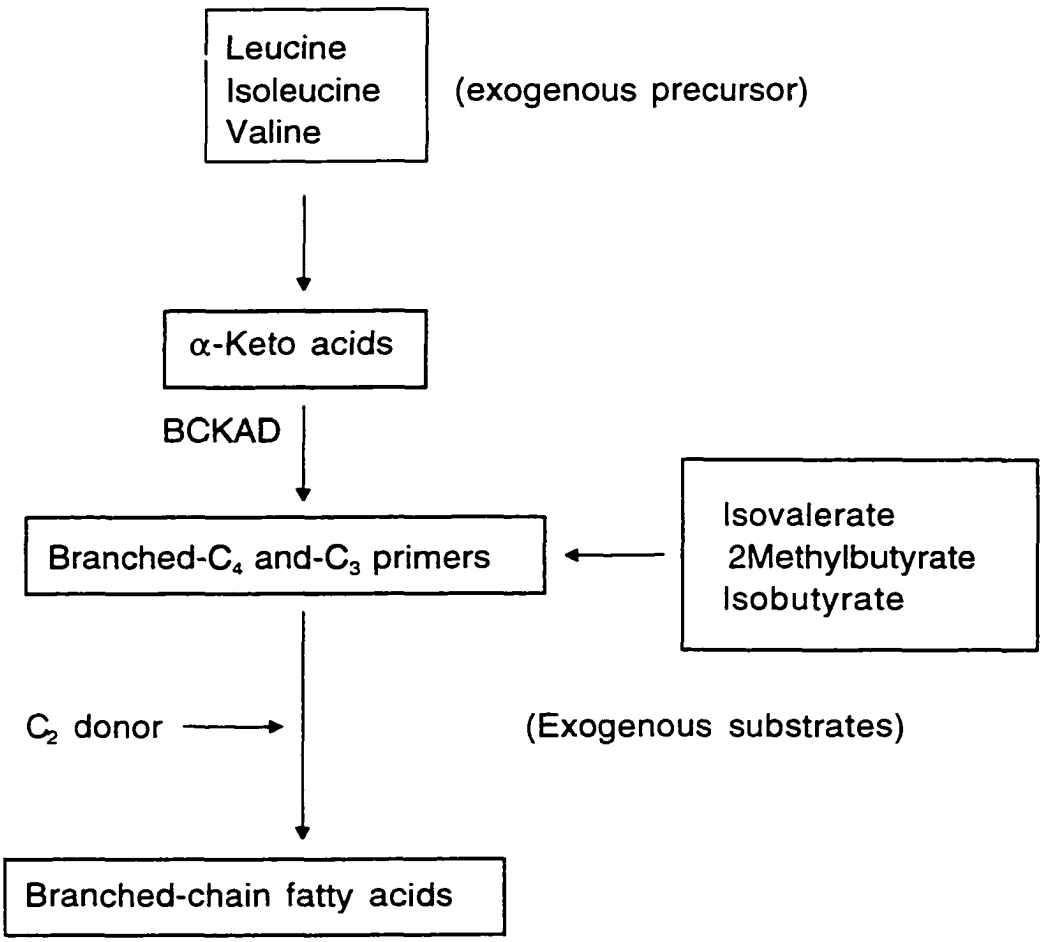


Figure.1 Pathway for the synthesis of the branched-chain fatty acids as determined from the studies on *Bacillus subtilis* (53).

Using this information, Toal et al (108) grew *esg* mutant cells in growth medium supplemented with IVA. By doing this, they were able to bypass the metabolic block caused by the *esg* mutation and enable the mutant cells to synthesize the long branched-chain fatty acids. *Esg* mutant cells grown under these conditions were able to undergo normal development when placed in a developmental media lacking these short branched-chain fatty acids. These results implied that branched-chain fatty acids or related compounds synthesized during growth are required for developdment.

This is not the first time that a role has been proposed for fatty acids in *M. xanthus* development. Fatty acids have already been shown to be responsible for developmental autolysis, a process occurring during the early stage of fruiting body formation in which as much as 80% of the cells within the fruiting body undergo lysis (87) (114) (115). The autosides have been isolated by ethanol extraction and found to belong to two major groups. The AMI autoside, comprise a mixture of fatty acids (110) (111), and the AMV autosides contain phospholipids (32). Studies have shown that AMI and not AMV is primarily responsible for inducing developmental autolysis and it has been proposed that AMI fatty acids are liberated from AMV by the action of a developmentally

regulated phospholipase (32). Mueller and Dworkin (78) have detected increases in phospholipase activity from 6 to 12 hours of development. The increase in phospholipase activity is found to occur prior to the liberation of the autosides AMI. This result is consistent with the notion that the fatty acids released from phospholipids during development of *M. xanthus* are a result of developmental phospholipase activity. There is no evidence at the present time as to whether the action of the phospholipase is under developmental regulation. The AMI autosides have also been shown to be capable of rescuing development of a *dsg* mutant (88) as well as developmental mutants deficient in the synthesis of AMI (86). These results suggest that the fatty acids may have a more significant role in development besides causing cell lysis. One current theory is that fatty acids may alter membrane permeability enabling the developing signals to pass across the cell membrane (86).

The results from the above studies have made it possible to propose a simple model to explain the role of the branched-chain fatty acids in the E-signaling system (17) (Fig. 2). In this model the BCKAD is involved in the synthesis of long branched-chain fatty acids (eg the iso-15:0 species) from branched-chain amino acids during vegetative growth. The observation that isovalerate added to developing mutant

cells failed to rescue development suggests that any esg-dependent fatty acid synthesis occurring during development is not essential for fruiting body formation (17) (108). The fatty acids synthesized during vegetative growth are then incorporated into membrane phospholipids. It is proposed that during the early stages of development a developmentally induced phospholipase activity results in the liberation of the branched-chain fatty acid signal. Efficient extracellular complementation of the *tps* gene-expression defect in the *esg* mutant was found to require extensive cell-cell contact with wild-type cells. This suggests that the E-signal is cell associated requiring cell-cell contact for transmission and therefore operates, like the CsgA protein, as a short range signal (18). The liberation of free branched-chain fatty acids results in the activation of a set of developmentally regulated genes expressed after about 3 to 5 hours of development.

1.5 Summary of Signaling

Individual cells respond to a nutritional shift down by initially synthesizing (p)ppGpp and producing the A-signal. The (p)ppGpp activates the stringent response of the individual cells while the A-signaling system seems to function primarily as a diffusible

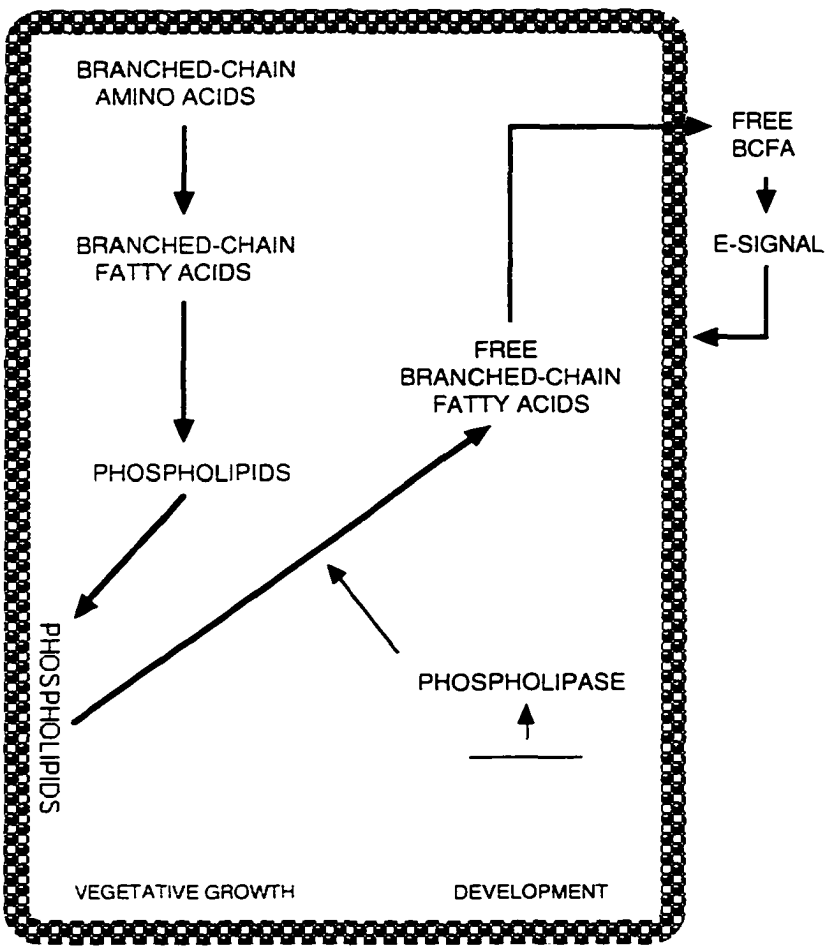


Figure 2. Model of the E-signal system

monitor of cell density. The production of the A-signal is developmentally regulated and proportional to cell density. Once the concentration of the released A-signal reaches a threshold, the next series of developmental events is permitted. An A-signal molecule concentration below the threshold level implies that the cell density is too low and that conditions are not appropriate for multicellular developmental events to be initiated. The cells then continue growing until a cell density sufficient for development is achieved. At this point development enters the committed stage. The high cell density ensures adequate cell-cell contact required for the activation of the C- and E-signals, suggesting that these signal molecules are cell bound and may play a role in controlling the movement of groups of cells into aggregation centers. The high cell density also ensures sufficient cell numbers to build fruiting bodies. The last known signal to be produced is the C-signal which plays a central role in development by activating the expression of more than half of the developmentally regulated genes. The C-signal is considered to serve as an extracellular timer inducing successive developmental stages at progressively increasing threshold concentrations.

1.6 Objective of Dissertation

It is very evident from the extensive research of Rosenbluh and Rosenberg (86), Mueller and Dworkin (78) and Downard and Toal (17) (108) that fatty acids play an important role in developmental signaling. The isolation of the *esg* mutant in Downard's laboratory (18) and the initial characterization of the *esg* locus (108) provided strong evidence that the branched-chain fatty acids are not merely structural components of the cell, but that they are also involved in signal production early in development. The work conducted in Downard's lab for the first time suggests the importance of the branched-chain free fatty acids in cell-cell communication. This work also associated developmental regulation to a single class of fatty acids namely, the branched-chain fatty acids.

Earlier studies identified the E-signaling system as a fifth signaling system of *M. xanthus* (18). In chapter 2, we determined the point in time at which this developmental signal is first produced by studying the expression patterns of a collection of developmentally regulated genes in an *esg* mutant background. The times of expression of these genes range from minutes after the induction of starvation to 16 hours of development. We also compared the expression patterns of this set of genes in the *esg* mutant obtained from this study with patterns

obtained from earlier studies using the other signal mutant backgrounds. The studies described in chapter 3 were formulated with the objective of testing the predictions of our proposed model. In this study, labeled branched-chain amino acids were added to vegetatively growing wild-type and *esg* mutant cells. By following the distribution of the label within wild-type and mutant cells, we hoped to demonstrate: i.) that branched-chain fatty acids are synthesized during vegetative growth of *M. xanthus* and incorporated into membrane phospholipids, ii.) the synthesis of the branched-chain fatty acids is an *esg* dependent event, and iii) the branched-chain fatty acids are released from the phospholipids during the early stages of development. The final part of the same chapter focused on work designed to test the prediction that the released fatty acids serve as the signal molecules of the E-signal system. Finally, in chapter 4 we demonstrated that the branched-chain fatty acid content of *M. xanthus* cells is altered in response to the composition of the growth medium and that the *esg* locus plays a role in the alteration of cellular fatty acid content.

CHAPTER 2

Esg -Dependent Regulation of Developmental Gene Expression in *Myxococcus xanthus*

2.1 Introduction

The life cycle of *M. xanthus* consists of a vegetative growth phase and a developmental phase. The bacteria enter the developmental phase when faced with nutrient depletion and undergo a series of morphological changes culminating in the formation of myxospores contained within the fruiting body (see chapter 1). In order to successfully complete development, the cells within each multicellular unit have to function in a synchronous manner. This coordinated behavior of the *M. xanthus* cells during development has been documented by Hagen et al (38). Using scanning electron microscopy, he observed that during development the cells undergo morphological changes in a reproducible sequence of stages. All the events of *M.*

xanthus development are under the control of five major signaling systems termed the A, B, C, D, and E, systems (38). (also see chapter 1)

In an attempt to identify the developmentally regulated genes controlled by the signaling systems, Kroos et al (58), transduced wild-type cells of *M. xanthus* with the transposable promoter probe Tn5*lac* and identified 36 genes in which there was an increase in expression during development. Tn5*lac* fuses the transcription of *lac Z* which encodes β-galactosidase to the promoter of any transcription unit into which the transposon inserts in the correct orientation. The expression of these developmentally regulated genes differed both in their β-galactosidase specific activity and time of expression (58). The expression times of these genes ranged from minutes after starvation to 30 hours of development, which is about the time of sporulation.

The identification of the developmentally regulated genes made it possible to determine the temporal order in which the major signaling systems were activated during development of *M. xanthus*. Kroos, Chang and Kaiser (11) (57) studied the expression of the developmentally regulated genes in the *asg*, *bsg*, *csg*, and *dsg* signaling mutants. They observed that all the developmentally regulated genes failed to be expressed in the *bsg* mutant suggesting that the B-signal is required

immediately after the initiation of development for gene regulation. Genes expressed after 2 hours of development were affected in an *asg* mutant suggesting that the A-signal is required for the regulation of developmental events commencing at about 2 hours. Similarly, genes expressed after 3 hours were affected in the *dsg* mutant while genes expressed after 6 hours were affected in a *csg* mutant. Thus, the D-signal is required for the regulation of developmental events occurring after 3 hours and the C-signal for the regulation of developmental events occurring after 6 hours. Based on these observations, it was concluded that the B-signaling system is the first to be activated during development followed by the A-, D-, and finally the C-signaling systems (Fig.3). Kroos et al (57), have proposed, that the A-, B-, C-, and D-signal systems lie on the same dependent pathway. The E-signaling system was first identified by the isolation of a Tn5 transposon insertional mutant of *M. xanthus* that demonstrated a significant reduction in the levels of *tps* expression (18). The *tps* gene, which starts to be Expressed by about 5 to 6 hours of development, has been studied in some detail. This gene codes for protein S, an abundant protein which has been estimated to contribute about 15% *of M. xanthus*

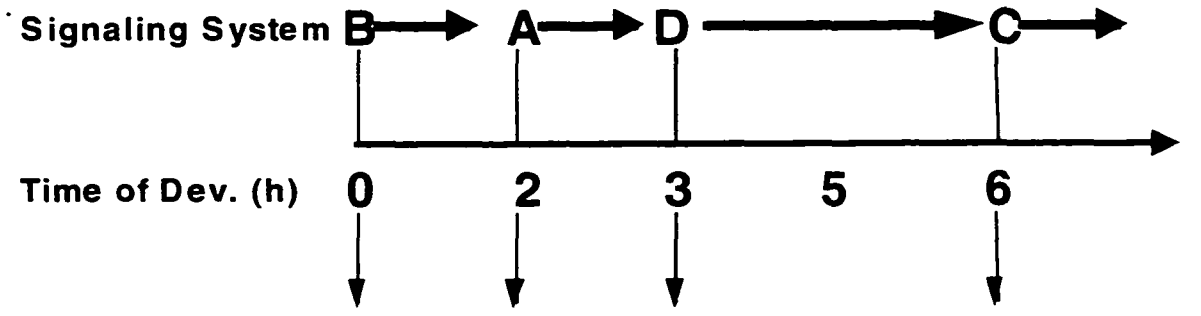


Figure 3. Sequence of signal activation in *Myxococcus xanthus*

Developmental protein synthesis (20) (43). These initial results suggested that the E-signal influences development earlier than the C-signal since it has been demonstrated that the *tps* gene is expressed normally in a *csg* mutant (57). *Tps* expression is lowered in the *asg* (65), *bsg* (57) and *dsg* (11) mutants. While the same study indicated that E-signaling

occurs by five hours in development, it did not indicate precisely when the E-signal is first required during *M. xanthus* development. At this point we are unable to position the E-signal pathway in the overall signal dependent pathway proposed by Kroos et al (57).

The objective of this study was to more accurately determine the time when E-signaling is first required for the regulation of *M. xanthus* development. This was achieved by comparing the pattern of expression of a set of developmentally regulated genes in the *esg* mutant background with expression patterns of the same set of genes in the wild-type background. The set of genes selected for this study were those whose times of expression ranged from a few minutes to sixteen hours after the initiation of development. This time range was selected since it is known from earlier work (18) that the E-signal is required to regulate developmental events commencing by 5 hours of development. We were therefore confident that the expression times of the developmentally regulated genes selected for this study would be sufficient to enable us to determine the position of the E-signaling system with respect to the other signaling systems in the signaling pathway proposed by Kroos et al (57).

2.2 Materials and Methods

2.2.1 Bacterial Strains

The wild-type strains of *M xanthus* used in the present study were DK1622 (42) and DZF1 (18). The *esg* mutant strains, JD275 and JD258 used in this study, were derivatives of DZF1. JD275 contains a Tn5 insertion in the *esg* locus while JD258 contains a *tps-lacZ* fusion and a Tn5 insertion in the *esg* locus (18). The Tn5*lac* insertion strains DK5207, DK4300, DK4521, DK4494, DK5206, DK4292, DK5279, DK4294, and DK4506 were all derived from the wild-type strain DK1622. Each strain contained a Tn5 *lac* insertion in a distinct developmentally regulated gene. The set of developmentally regulated genes chosen for this study were expressed at different times during development ranging from 0 hours to 14 hours. The Tn5*lac* insertion strains were a gift from Dale Kaiser's laboratory. *E. coli* MC1000 P1:: Tn5-32 lysogen was used as the source of the Tn5-132, a modified form of Tn5 encoding resistance to tetracycline (Tc) instead of kanamycin (Km) (18).

2.2.2 Construction of *esg* Tn5*lacZ* Strains

The Tn5 and Tn5*lac* transposons contain the gene for kanamycin phosphotransferase which catalyzes inactivation of the antibiotic kanamycin (85). Cells that have Tn5 or Tn5*lac* insertions are consequently highly resistant to kanamycin. Tn5-132 encoding tetracycline resistance and not kanamycin resistance was used to modify Tn5 insertion mutations of the *esg* locus. This served to identify double mutants containing a Tn5*lac* insertion in the developmentally regulated gene and a Tn5 insertion in the *esg* gene (61). These double mutants were selected for their ability to grow on media containing both tetracycline and kanamycin. The sequence of steps employed for the construction of the double mutants was a) the production of a phage P1::Tn5-132 lysate, b) the replacement of Tn5 (Km^r) in the *M. xanthus* strain JD275 with Tn5-132 (Tc^R), c) the purification of the *esg* : :Tn5-132 (Tc^R) MX4 lysate and d) the replacement of the *esg* locus of the wild-type Tn5*lac* insertion strains by the mutant *esg* locus.

a) Production of a P1::Tn5-132 lysate

The P1::Tn5-132 phage was carried in the *E.coli* lysogen MC1000. The P1::Tn5-132 lysate was produced as follows. Two

hundred microliters of an overnight culture of *E.coli* lysogen MC1000 P1::Tn5-132 (Tc^R) (37), grown in LB broth [10 µg/mL tetracycline (Tc)], was subcultured into 25 mL of fresh LB broth containing no antibiotic. The cells were first incubated at 30°C until they reached a cell density of approximately 50 Klett units [measured using a Klett-Summerson Photoelectric colorimeter with a red filter], and then further incubated at 42°C in order to induce the lytic cycle of the P1 phage. Cell lysis was first observed after half an hour of incubation at 42°C. This was followed by a rapid decrease in the cell density from approximately 150 Klett units to 35 Klett units. Half a milliliter of chloroform was added to the infected bacterial culture in order to lyse the remaining cells not infected by the P1 phage. The cells were then centrifuged at 8,000 rpm for 10 minutes at 4°C and the clear supernatant containing the purified P1::Tn5132 (Tc^R) lysate stored at 4°C until needed.

b) Replacement of Tn5 (Km^R) with Tn5-132 (Tc^R) in the host strain JD275

The objective of this step was to replace the antibiotic resistant gene associated with the *esg* transposon insertion with a Tn5-132 insertion. The strains containing the altered Tn5 inserted into the *esg*

locus were selected for their ability to grow on media containing tetracycline. JD275 [*esg* ::Tn5 Km^R,Tc^S] cells were grown in CYE (50 μg/mL Km) to a cell density of approximately 50 Klett units and harvested by centrifugation at 8,000 rpm for 10 minutes. The pellet was resuspended in 2.5 mL of CYE (without Km) containing 2.5 mM calcium chloride resulting in a bacterial suspension with a cell density of approximately 200 Klett units. One hundred microliters of the P1::Tn5-132 lysate was mixed with 100 μL of the concentrated cell suspension to obtain a phage multiplicity of approximately 2 phages per cell. The phage infected cells were incubated without shaking at room temperature for 30 minutes. Two and a half mL of CYE soft agar [no Km] was added to the transduction mix, vortexed briefly and overlaid on CYE agar containing 2.2 μg/mL tetracycline. The cells were incubated at 30°C. After 24 hours of incubation, 2.5 mL of soft agar containing tetracycline was overlaid on the agar surface. This was done to raise the concentration of the tetracycline in the agar to approximately 12 μg/mL. Colonies were observed growing on the plate after about a week of incubation. To confirm that the cells had successfully undergone the antibiotic resistance gene replacement, 15 colonies were spot inoculated on 2 sets of CYE agar plates. One set contained kanamycin

(25 μg/mL) while the other contained oxytetracycline (12 μg/mL). After five days of incubation at 30°C bacterial growth was observed only on the plate containing oxytetracycline. These results confirmed that the kanamycin phosphotransferase gene within Tn5 in JD275 had been replaced by the tetracycline gene from Tn5-132 giving rise to $Km^{S}Tc^{R}$ daughter cells.

c) Production of the *esg* : :Tn5-132 (Tc^{R}) MX4 Lysate

Once the desired *esg* mutant strain was constructed the next step was to produce a myxophage MX4 transducing lysate that could be used to transfer the insertion allele into strains carrying Tn5-*lac* insertions in developmentally regulated genes. The method described by Campos et al was followed to prepare the MX4 lysate (10). One hundred microliters of the MX4 phage (~10^{4} virus particles/mL) was mixed with 200 μL of the *esg* mutant donor JD275 cells [*esg* ::Tn5-132 (Tc^{R})] which had been grown to a cell density of approximately 10^{8} cells/mL. After incubation at room temperature for 10 minutes, 2.5 mL of MX4 medium top agar [1% BBL Tripticase, 0.1% $MgSO_4.7H_2O$, 0.6% Difco agar] was added to the transduction mix, the mixture vortexed briefly

and overlaid on MX4 agar [1% BBL Tripticase, 0.1% $MgSO_4.7H_2O$, 1.5% Difco agar]. The plates were incubated at 30°C. Plaques were observed on the plates after 4 days of incubation. The phage suspension was prepared by adding 10 mL of TM buffer [10 mM Tris-Hcl (pH 7.6), 10 mM $MgSO_4$] onto the agar surface containing the plaques. The plates were left standing at room temperature for about half an hour to provide sufficient time for the phage particles to diffuse into the medium. The TM buffer was then transferred from the surface of the plates into a clean tube and mixed with1/2 mL of chloroform in order to lyse the remaining viable cells. The mixture was centrifuged at 8,000 rpm for 10 minutes in order to pellet the cellular debris. The supernatant containing the MX4 lysate was transferred into a fresh tube and stored at -20°C until needed.

d) Replacement of the *esg* locus of the wild-type Tn5 *lac* insertion strains by the mutant *esg* locus

The wild-type DK1622 strains containing the Tn5*lac* insertions in the developmental genes were grown in CYE (50 µg/mL Km) to a cell density of approximately 65 to 95 Klett units. Incubation was stopped by centrifuging the cells at 8000 rpm for10 minutes. The pellet was

resuspended in CYE (no Km) to a final density of approximately 200 Klett units. One hundred microliters of the MX4 lysate was mixed with 200 μL of the concentrated cell suspension. The tube containing the MX4 [*esg* ::Tn5-132 (Tc^R)] lysate was placed at 47°C for 30 minutes prior to mixing to evaporate the chloroform. The transduction mix was incubated at room temperature for 30 minutes, mixed into 2.5 mL of CYE top agar, and overlaid on CYE agar (Km 50 μg/mL and oxyTc 2.2 μg/mL). An additional 2.5 mL of CYE top agar containing oxytetracycline was overlaid on the CYE agar after 14 hours of incubation at 30°C. This was done in order to raise the concentration of oxytetracycline in the agar to approximately 12.2 μg/mL. Between 15 to 30 transductants were observed to be growing on the plates after 5 days of incubation. Ten colonies were selected at random from each set and subcultured onto fresh CYE agar plates [Km 50 μg/mL and oxyTc 12 μg/mL].

To confirm that the new strains carrying the mutated *esg* locus were not merodiploids, the development phenotype of these strains were compared to that of a known *esg* mutant (DK1622/*esg*). Ten colonies, selected from each Tn5*lac* insertion group harboring the

mutated *esg* locus, were grown in CYE liquid media [Km 50 μg/mL and oxyTc 12 μg/mL] to a cell density of approximately 100 Klett units. The cells were harvested by centrifugation at 8000 rpm for 10 minutes, washed in TM buffer, and resuspended in the same buffer to a final cell density of approximately 1000 Klett units. Five microliters of the concentrated cells were spotted on clone-fruiting (CF) agar [0.015% casitone, 0.1% sodium pyruvate, 0.2% sodium citrate, 0.02% $(NH_4)_2SO_4$] (86). After 4 days of incubation at 30°C,the phenotypes of the developing Tn5*lac* insertion strains and control DK1622/*esg* mutant strain were compared. All the colonies analyzed demonstrated the *esg* mutant phenotype. This result confirmed that the wild-type *esg* locus was replaced with the mutated copy by gene conversion and that the Tn5*lac* insertion strains harboring the mutated *esg* locus were not merodiploid strains. Merodiploid strains would have carried a wild-type and mutant copy of the *esg* locus and would therefore have demonstrated a wild-type developmental phenotype. All the colonies grew on the CYE plates confirming that the Tn5-132 had been successfully inserted into the *esg* locus of the wild-type DK1622 insertional strain by genetic recombination making the colonies resistant to both kanamycin and tetracycline. The developmental phenotypes of

TABLE 1. Tn5 *lacZ* insertion strains

Ω	Expression time in wt [h]	[a] wt DK1622	[b] *esg* Derivative
4457	0	DK5207	GB19
4408	1	DK4300	GB17
4521	2	DK4521	GB21
4494	2	DK4494	GB25
4455	3	DK5206	GB26
4273	5	[c] JD10	[d] JD258
4400	6	DK4292	GB22
4414	10	DK5279	GB18
4406	11	DK4294	GB24
4506	14	DK4506	GB23

All strains are derivatives of the wild-type *M. xanthus* strain DK1622.

[a] The parental strains (a gift from Dale Kaiser laboratory) were constructed by P1::Tn5*lac* transduction as described by Kroos et al (58)
[b] The construction of the *esg* parental strains is described in materials and methods.
[c] JD 10 - *Tps lacZ* fusion in a DZF1 wild type background (18).

[d] JD258 - JD10 with a Tn5 insertion in Ω258 (18).

the new strains, as well as their antibiotic resistance profile (Km^R, Tc^R), confirmed that we had successfully constructed a set of *M. xanthus* strains that had the selected developmentally regulated genes in the *esg* background. The names of these new strains, the strains from which they were derived and the expression times of the selected group of developmentally regulated genes are given in Table 1.

2.2.3 Conditions for Deveiopment

The cells were prepared for development as described above. Using a 30 pronged spotter, multiple 5 µL drops of the concentrated cells were deposited onto the surface of CF agar and allowed to dry at room temperature. The plates were then incubated at 30°C.

2.2.4 Harvesting Developing Cells from CF Agar

The cell aggregates developing on the agar surface were dispersed with an alcohol sterilized glass rod. The cells were resuspended in 5 mL of TM Buffer added onto the surface of the agar. The cell suspension was transferred into a centrifuge tube and

centrifuged at 8,000 rpm for 10 minutes. The dry pellet was stored at -20°C until needed.

2.2.5 β-Galactosidase Assay

The procedure for determining β-galactosidase specific activities from sonicated cell extracts has been described (10). The protein concentrations of the cell extracts were determined using the protein assay reagent kit by Pierce (Pierce Chemicals Inc). The protocol provided by the manufacturer was followed. Briefly, 2 mL of the assay reagent was introduced into a 5ml glass tube containing 100 µL of sonicated cells. The cells were well mixed into the reagent by briefly vortexing the mixture. The tubes were placed in a 60°C waterbath for 30 minutes, allowed to cool to room temperature and the optical density of the reaction mix determined at a wavelength of 562nm. A protein standard curve was determined using known concentrations of bovine serum albumin (BSA). The concentration of each assayed sample was determined from this standard curve.

2.3 Results

2.3.1 *Esg* Dependent Regulation of Early Developmentally Regulated Genes in *Myxococcus xanthus*

The effect of an *esg* mutation on developmental gene expression was investigated. The Tn5*lac* insertional strains used in this study contained a copy of the transposon Tn5*lac*, inserted into each of the developmentally regulated genes. The copy of the chosen *esg* locus was first constructed in JD275 by infecting it with a P1 phage containing the transposon Tn5-132 (Tc^R). This *esg* locus was then transferred from strain JD275 to the Tn5*lac* insertional strain using the myxophage MX4. The end result was a double mutant containing a transposon Tn5-132 (Tc^R) in the *esg* locus and a Tn5*lac* (Km^R) insertion into each of the developmentally regulated genes. These mutants were consequently capable of growth in media containing both kanamycin and tetracycline. Double mutants within each of the Tn5*lac* insertional strains that demonstrated the *esg* phenotype were selected for further analysis. Tn5*lac* contains a promoterless *lacZ* gene inserted near one end of the transposon Tn5. Accordingly, when Tn5*lac* transposes into a developmentally regulated gene in the correct orientation it generates a

transcriptional fusion with *lacZ* which brings β-galactosidase expression under the control of the promoter of that particular gene (59).

Cells were harvested at different times following the initiation of development and the β-galactosidase specific activity was determined. The expression of β-galactosidase in the group of Tn5*lac* insertional strains that contained the wild-type copy of the *esg* locus was monitored and the pattern of expression of the developmentally regulated genes in this group was compared with those in the group where the *esg* locus contained the transposon insertion (Fig.4). The results presented in figure 4 are representative of results obtained by repeating this experiment three times. The data of each individual experiment is not shown. The results revealed that the expression of the developmentally regulated genes fell into two broad groups: (1) genes whose expression is not significantly affected by the loss of the *esg* function, and (2) genes whose expression is strongly dependent on a functional *esg* locus. The former set of genes were expressed within the first 5 hours following the initiation of development. These comprised Ω4408 [1 hour], Ω4494 [2 hours], Ω4521 [2 hours] and Ω4455 [3 hours]. The latter group of genes were expressed between 6 to14 hours following the initiation of development.

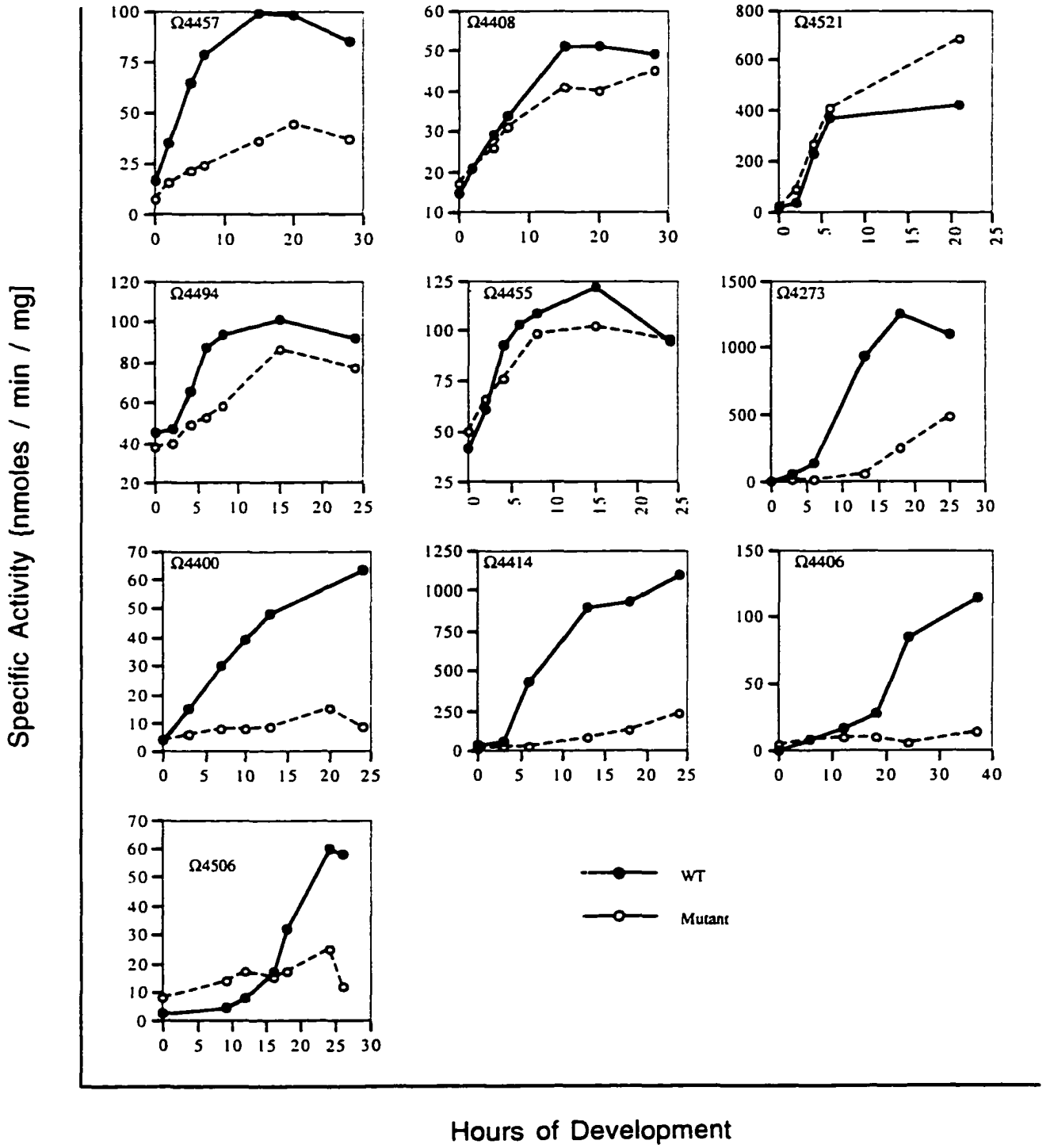


Figure 4. Developmentally regulated β-Galactosidase expression from Tn5*lac* insertions in an *esg* mutant

These comprised Ω4273, Ω4400, Ω4414, Ω4406 and Ω4506 which were expressed at 5 hours, 6 hours, 10 hours, 11 hours, and 14 hours of development respectively.

2.4 Discussion

In this study, the expression patterns of a set of developmentally regulated genes in both the wild-type (DK1622) and the *esg* mutant (DK1622/*esg*) strains of *M. xanthus* were examined and compared. This in turn led to the conclusion that the E-signal becomes active between 3 and 5 hours of development and is needed for the regulation of developmental events occurring 5 hours after the initiation of development. In this context, a comparison [displayed in Table 2.] of the expression of the set of developmentally regulated genes derived from our investigation on the *esg* mutant with those of the *asg*, *bsg*, *csg*, and *dsg* mutants obtained from the work by Cheng, Kroos and Kuspa (1) (57) (58) is of special interest.

This comparison clearly shows that the pattern of gene expression observed in the *asg* ,*bsg* and *csg* mutants differ from the pattern of gene expression observed in the *esg* mutant. The expression

pattern of Ω4494 (2 hours) and Ω4455 (3 hours) fusions indicate reduced expressions in the *asg* and *bsg* mutants but these fusions are normally expressed in the *esg* mutant. Similarly, the expression of the *tps* gene Ω4273 (5 hours) is normal in the *csg* mutant but is reduced in the *esg* mutant. The striking similarity noticed in the patterns of gene expression observed in the *dsg* and *esg* mutants called for closer attention. We observed an increased expression of Ω4521 in the *esg* mutant. However, since the increased expression of Ω4521 in the *esg* mutant was based on the final time point (Fig. 4, p.51), we hesitate to place much emphasis on this observation at the present time. All the earlier time points of Ω4521 expression in the *esg* mutant cells closely overlapped those in the wild-type cells. These results indicate that the expression pattern of Ω4521 in the wild-type and *esg* mutant cells is very similar. We therefore concluded that the expression of Ω4521 is most probably not significantly altered in the *esg* mutant strain. Based on our observation, it would appear that the E-signaling dependent events of development are initiated at approximately the same time as those events dependent on the D-signal in the major signal transduction pathway of *M. xanthus* proposed by Kroos et al (58) (Fig.5).

TABLE 2. Effect of the signaling gene mutants on developmental gene expression

Ω	Expression time (h)	β-Galactosidase Expression				
		[1]A⁻	[2]B⁻	[2]C⁻	[3]D⁻	[4]E⁻
4457	0	R	R	N	ND	R
4408	1	N	R	N	N	N
4521	2	A	R	N	N	I
4494	2	R	R	N	N	N
4455	3	R	R	N	N	N
4273	5	A	R	N	R	R
4400	6	ND	A	R	R	R
4414	10	A	A	R	R	R
4406	11	A	A	A	R	R

[1]Kroos, Kuspa and Kaiser (58).
[2]Kroos and Kaiser (57).
[3]Cheng and Kaiser (11).
[4]Present study
A - abolished, R - reduced, N - normal, I - increased
ND - not determined

Despite this high degree of similarity there is abundant evidence to suggest that the D- and E-signaling systems are in fact distinct pathways. It was observed that *dsg* mutants can extracellularly complement *esg* mutants and vice versa, implying that these mutants are not deficient in the same signal molecules (18). In addition, the protein products encoded by the *dsg* and *esg* loci appear to be involved in fundamentally different cellular processes. The *dsg* gene codes for a translation initiation factor in *M. xanthus* (13) (48), while the *esg* gene codes for the two components of the branched-chain keto acid dehydrogenase, an important enzyme in the branched-chain fatty acid biosynthetic pathway (17) (108). The levels of the branched-chain fatty acids, are significantly reduced in the *esg* mutant but not significantly reduced in the *dsg* mutant (Geoffrey Bartholomeusz, unpublished results). These results suggest that the *dsg* mutation does not affect branched-chain fatty acid synthesis. The developmental phenotypes of these two mutants also differ. The *esg* mutant displayed a pale yellow pigmentation while the *dsg* mutant displays a tan pigmentation.

Despite these observations, Rosenbluh et al (86) have shown that the fatty acids of the autosides AMI can rescue development of a *dsg* mutant. Although there is no apparent connection between this

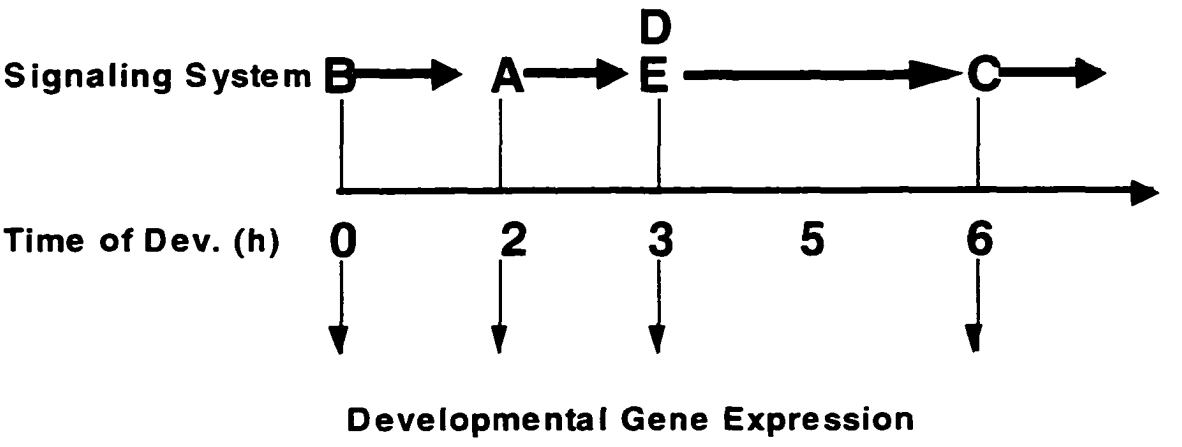


Figure 5. Predicted entry of the E-signal in the signaling pathway regulating development of *Myxococcus xanthus*

observation and the genetic defect of the *dsg* mutant, it lends support to our observation that the D- and E-signaling systems seem to be closely associated in developmental regulation of *M. xanthus*. Although our results suggest that the D- and E-signaling systems are very closely associated temporally in the regulation of the early events of

development in *M. xanthus*, our predictions remain inconclusive until the E- and D-signals are identified and the basis for extracellular complementation is known in more detail.

The expression of one of the developmentally regulated genes of *M. xanthus* Ω4457, expressed at the onset of development (0 hours) was observed to be decreased in the *esg* mutant (Fig.4, p.51). It is possible that omega 4457 is not dependent on the E-signal but on some other function that requires the *esg* locus. (eg. another signal, membrane structure or branched-chain amino acid metabolism). The possibility that the *esg* locus may be required for more than one biological process that is important for *M. xanthus* development is not surprising since the *esg* dependent BCKAD plays a central metabolic role in *M. xanthus*. We would expect cells deficient in the function of this enzyme to be impaired in function related to branched-chain amino acid metabolism.

CHAPTER 3

Analysis of the Role of the *esg* Locus in Fatty Acid Synthesis and Developmental Regulation

3.1 Introduction.

In response to adverse environmental conditions *M. xanthus* initiates a multicellular developmental process culminating in the formation of fruiting bodies. Successful fruiting body formation is dependent on the ability of the cells to develop synchronously. This is achieved by cell-cell communication or signaling. The mechanisms involved in cell-cell communication in *M. xanthus* are diverse and studies of signaling defective mutants suggest that at least five signaling systems exist (18) (38) (57). One of these signaling defective mutants is the *esg* mutant. Sequence analysis of the *esg* locus identified its gene products as the two proteins that constitute the E1 component of the branched-chain keto acid dehydrogenase (BCKAD).

The BCKAD is an important enzyme involved in the metabolism of the branched-chain amino acids (108). This enzyme, which has been

characterized from many bacterial sources (7) (79) (104), catalyzes the oxidative decarboxylation of the branched-chain α-keto acids (deamination products of the branched-chain amino acids). This results in the production of the corresponding CoA esters of short branched-chain fatty acids; isovaleryl-CoA, α-methylbutyryl-CoA and Isobutyryl-CoA. These CoA esters then serve as primers for the synthesis of the long branched-chain fatty acids that are found in phospholipids (49) (also see Fig 1, p.25).

The *esg* mutant is deficient in BCKAD activity and it was predicted that this mutant will synthesize reduced amounts of the branched-chain fatty acids. A comparison of the fatty acid profiles of wild-type and *esg* mutants showed that the mutant cells contained reduced levels of the branched-chain fatty acids (108). The greatest reduction was observed with the branched-chain fatty acid iso-15:0. This is not surprising when one considers that the branched-chain fatty acids comprise roughly 65% of the total fatty acid content of the wild-type cells of *M. xanthus* and that iso-15:0 is the most abundant single branched-chain species component of its branched-chain fatty acids (between 45% and 55% of total fatty acids) (108). The levels of the other branched-chain fatty acids were also generally reduced in the *esg* mutant. An exception to

this pattern was iso-17:0 (108). Since the *esg* mutant behaves as if it is defective in cell-cell communication and synthesizes reduced levels of the branched-chain fatty acids, it has been proposed that the branched-chain fatty acids are needed for development of *M. xanthus,* and that they or other products of the *esg* BCKAD pathway may function as signal molecules in these bacteria (17) (108).

In an attempt to demonstrate the involvement of branched-chain fatty acids in *M. xanthus* development, *esg* mutant cells were grown vegetatively in the presence of isovaleric acid (IVA), a leucine derived intermediate of fatty acid biosynthesis which enters the pathway at a point beyond the *esg* mutational block. Fatty acid analysis of the *esg* mutant cells grown vegetatively in the presence of IVA showed that these mutant cells were able to synthesize normal levels of iso-13:0 and iso-15:0 (108). The *esg* mutant fails to form fruiting bodies, but when these IVA-grown cells were transferred into a developmental medium lacking IVA they were able to undergo normal development. When IVA was added to developing *esg* mutant cells that had been grown vegetatively in the absence of the fatty acid, it failed to rescue development of the mutant. These results suggest that the branched-chain fatty acids are important for the development of *M. xanthus* and

that the fatty acid biosynthetic activity of the *esg* BCKAD is not required during development.

A simple model has been proposed, based on the studies of the role of IVA in *esg* development and on earlier observations on the release of fatty acids with autoside activity (78). This model postulates that the fatty acids that are synthesized during vegetative growth via the pathway involving the *esg* enzyme are incorporated into phospholipids. Following initiation of development, free branched-chain fatty acids are released from the phospholipids by the action of a developmentally regulated phospholipase activity (78). The free fatty acids are then passed between cells and activate a signal transduction pathway which results in the activation of E-signal dependent genes (Fig. 2 , p.29).

In the first part of this study, the incorporation of ^{3}H-labeled branched-chain amino acids into vegetatively growing wild-type cells and into *esg* mutant cells of *M. xanthus* were separately investigated. The proportion of the label incorporated into the phospholipids of the wild-type cells turned out to be high, whereas the proportion in the case of the *esg* mutant was found to be significantly less. These findings lend direct support to the proposition, in the first part of the model, that

branched-chain fatty acids are synthesized by the *esg*-dependent pathway during vegetative growth.

The model predicts that free branched-chain fatty acids will be released from the phospholipids by the action of a developmentally regulated phospholipase in the early stages of development. The second part of this study was designed to yield direct evidence of a pronounced release of fatty acids in early development. The results obtained were inconclusive. In fact, the levels of free branched-chain fatty acids in cells growing vegetatively and in cells in early development were found to be more or less the same.

In the final part of this study we demonstrated that a free branched-chain fatty acid fraction purified from developing wild-type cells was able to rescue development of an *esg* mutant. This fraction contains other compounds besides simple fatty acids that may have some activity. The results of this study lend support to that aspect of the model that predicts that the branched-chain fatty acids or related molecules could function as E-signal molecules. The results of this study will be useful in the future refinements of our model for E-signaling in *M. xanthus*.

3.2 Materials and Methods

3.2.1 Bacterial Strains

The wild-type strains of *M. xanthus* used in this study were DZF1 (26) (27) and DK1622 (42). The *esg* mutant strains were JD256, JD258 and JD300. JD256 is a derivative of DZF1 harboring a Tn5 insertion in the *esg* locus (*esg* ::Tn5) (18). JD258 is a derivative of DZF1 containing both a *tps lacZ* fusion gene and a Tn5 insertion in the *esg* locus (*esg* ::*tps-lacZ*) (18). JD300 is a derivative of DK1622 containing a Tn5 insertion in the *esg* locus (*esg* ::Tn5) (18).

3.2.2 Growth Conditions

The cells were grown in about 70 mL of CTT medium [10mM Tris-HCl (pH 7.6), 1mM KH_2PO_4-K_2HPO_4 (pH 7.6), 8 mM $MgSO_4$] (85), in a 250 mL Erlenmeyer flask with a side-arm to a cell density of 70 to 100 Klett units measured with a Klett-Summerson Photoelectric colorimeter with a red filter. Cells were prepared for growth in A1 medium containing the labeled branched-chain amino acids as described in section 3.2.3. The MCM buffer (10mM MOPS [morpholinopropanesulfonic acid], 2 mM $CaCl_2$, 4 mM $MgSO_4$ [pH 7.2] was used in this study to induce development of *M. xanthus* (89). MCM buffer was chosen since it has been shown to

support the development of *M. xanthus* and the expression of the developmentally regulated *tps* gene. Since *tps* expression is dependent on the E-signal (18) we conclude that the E-signal system was active under these developmental conditions. The use of a liquid medium to study *M. xanthus* development also affords us the opportunity to work with large volumes of cells making it easy to harvest them for analysis. *M. xanthus* cells, at a cell density of approximately 200 Klett units, were incubated in a side arm flask at 30°C in a shaker set at 150 rpm.

3.2.3 Incorporation of Labeled Leucine, Isoleucine and Valine into *Myxococcus xanthus*

To identify lipid compounds derived from the branched-chain amino acids cells were grown in A1 medium containing labeled branched-chain amino acids. The defined composition of this medium makes it a suitable tool for studying the incorporation of radio-labeled branched-chain amino acids into cellular components (6). DZF1 and JD256 were grown in CTT medium to a cell density between 60 to 70 Klett units. The cells were centrifuged at 8000 rpm for 10 minutes and the pellet washed in the chemically defined A1 medium free of spermidine and vitamin B_{12} (6). The cells were centrifuged at 8000 rpm for 10 minutes

and the pellet washed for a second time in A1 medium free of vitamin B_{12} and spermidine. The second wash was carried out by resuspending the pellet in 20 to 25 mL of A1 medium in an Erlenmeyer flask and incubating it at 30°C with continuous shaking at 250 rpm for 10 minutes. The washed cells were introduced into 50 mL of fresh A1 medium in a 250 mL Erlenmeyer side arm flask at a final cell density between 10 to 20 Klett units. The chemically defined A1 medium used in this study was slightly modified. This medium contained leucine at a concentration of 100 μg/mL instead of the normal 50 μg/mL (Table 3). We deemed this change necessary since we wanted the uptake of the H^3-labeled amino acids into the cells to provide an accurate estimation of the overall amino acids uptake (labeled and non- labeled) by the cells growing in the A1 medium. This was done to minimize any bias in the uptake of the labeled branched-chain amino acids by the *M. xanthus* cells. Preliminary tests indicated that the increase in the concentration of leucine to 100 μg/mL did not affect the growth of the cells in this modified A1 medium. After about 10 to 12 hours of growth, 25 to 30 μCi of L-[4,5-^{3}H] leucine, L-[4,5-^{3}H] isoleucine and L-[4,5-^{3}H] valine were added to the medium.

TABLE 3. Composition of the chemically defined A1 medium

Ingredient	Concentration	
	Standard[a]	Modified
L-Asparagine	100 µg/mL	100 µg/mL
L-Isoleucine	100 µg/mL	100 µg/mL
L-Leucine	50 µg/mL	100 µg/mL
L-Methionine	10 µg/mL	10 µg/mL
L-Phenylalanine	100 µg/mL	100 µg/mL
L-Valine	100 µg/mL	100 µg/mL
Vitamin B_{12}	1 µg/mL	1 µg/mL
Spermidine.3HCl	125 µg/mL	125 µg/mL
Sodium pyruvate	0.5%	0.5%
Potassium aspartate	0.5%	0.5%
Tris-HCl (pH 7.6)	10 mM	10 mM
KH_2PO_4-K_2HPO_4 (pH 7.6)	1 mM	1 mM
$MgSO_4$	8 mM	8 mM
$FeCl_3$	10 µM	10 µM
$CaCl_2$	10 µM	10 µM
$(NH_4)_2SO_4$	0.5 mg/mL	0.5 mg/mL

[a]Reference 6

The cells were grown in the presence of the labeled amino acids for about 4 generations. The concentrations of the branched-chain amino acids which are required for *M. xanthus* growth were not a limiting factor.

3.2.4 Lipid Extraction from ^{3}H-Branched-Chain Amino Acid Labeled Cells

Total lipids were extracted from the cells using a modified Bligh and Dyer technique (52). The harvested cells were centrifuged at 8000 rpm for 10 minutes and the weight of the wet pellet determined. Pellets weighing up to 40 mg were diluted in 1 mL of water and introduced into a glass vial. Methanol:chloroform [2:1 v/v (3.75 mL)] was added to this cell suspension. The samples were mixed and about 1 mL of glass beads (0.5 mm in diameter) were then introduced into the glass vial. The cells and glass beads were incubated in a sonicating water bath for half an hour. The cell extract was then transferred to a glass centrifuge tube and centrifuged at 8000 rpm for 10 minutes. The supernatant obtained after the first centrifugation was saved. The pellet was resuspended in 3.75 mL methanol:chloroform:water (2:1:0.8), and centrifuged a second time at 8000 rpm for 10 minutes. The pooled supernatant fractions

were separated into a lower chloroform phase and an upper methanol/water phase by adding 2.5 mL of chloroform followed by 2.5 mL of water. The separation of the phases was accelerated by centrifuging the mixture at 8000 rpm for 10 minutes. The chloroform phase was carefully extracted from the tube using a glass Pasteur pipette and transferred to a clean glass vial. The sample was dried under nitrogen. The dried lipid extract was yellow and viscous. The lipid extract was resuspended in 2 to 5 mL of a hexane:tertiary butyl methyl ether (MTBE) [200:3 v/v] solvent. To determine the amount of ^{3}H-label incorporated into the lipid fraction 10 µL of this extract was spotted on a Whatman 2.4 cm circular glass microfilter which was then introduced into a scintillation vial containing 5 mL of a scintillation cocktail. Samples were counted in a scintillation counter for 10 minutes.

3.2.5 **Fractionation of the Lipid Extract.**

The total lipid extract was separated into neutral lipid [NL], free fatty acid [FFA] and phospholipid [PL] fractions using the Supelclean LC-Si SPE 500 mg minicolumn. The method for the extraction of these lipid fractions from the column has been previously described (39) and was used in a previous study of *M. xanthus* by Mueller and Dworkin (78).

The lipid extract resuspended in the hexane:MTBE [200:3 v/v] solvent was carefully loaded onto the column. The column was then washed with 10 mL of the first solvent containing hexane:MTBE [200:3 v/v]. The column wash was collected and designated the neutral lipid [NL] fraction. The NL fraction is not a pure fraction but contains neutral lipids and other lipids that do not bind to the matrix (39). The column was next acidified with a 12 mL wash of the second solvent containing hexane:acetic acid[100:0.2 v/v]. This was followed by a third wash of 12 mL of a hexane:MTBE:acetic acid solvent mixture [200:2:0.2]. The fraction eluted from the column with this solvent wash was designated the free fatty acid [FFA] fraction. The FFA fraction is itself not a pure fraction. It contains free fatty acids and other lipid compounds soluble in this solvent (39). It has been shown that approximately 97% of the free fatty acids bound to the matrix of the column are eluted in this wash (39) (78). A fraction containing phospholipids [PL] was eluted from the column with 10 mL of methanol. The three lipid fractions were dried under nitrogen and the NL and FFA fractions resuspended in 50 to 100 μL of 200% ethanol while the PL fraction was resuspended in 400 to 800 μL of methanol:chloroform [1:1 v/v]. A 10 μL sample from each

fraction was spotted onto a Whatman 2.4 cm circular glass microfilter to determine the amount of ^{3}H-label.

3.2.6 **Alkaline Hydrolysis of the Lipid Fractions**

The procedure for hydrolysis of phospholipids into free fatty acids has been described (32). Briefly, the lipid fractions are dried under nitrogen, and the dried extract resuspended in 1.5 mL of 0.5 M KOH. The resuspended extracts were incubated at 50°C for 30 minutes, and cooled to room temperature. The pH of the cooled mixture was adjusted to between 3 and 4 by the addition of 1M HCl. The mixture was extracted 3 times in 2 mL of hexane. The extracts were pooled, dried under nitrogen, and resuspended in 100 % ethanol.

3.2.7 **Thin Layer Chromatography [TLC]**

The species contained in each of the lipid fractions were fractionated by TLC using silica gel pre-coated 2x20 cm glass plates. Samples of dissolved lipid fractions (8 to 15 µL) were spotted approximately 0.5 inches from one end of the TLC plate and dried at room temperature. The dried plates were then introduced into a solvent-saturated incubation jar and developed with an ascending mobile phase

composed of petroleum ether, diethylether and acetic acid (70:30:2). The plates were incubated until the mobile ascending solvent had almost reached the top of the plate. (3 to 5 mm from the top). This took approximately 10 to 15 minutes. The plates were air dried for about an hour before being used for flourography.

3.2.8 **Fluorography**

The lipid species on the TLC plate were detected by fluorography. EH^{3}Hance was finely sprayed on the entire surface of the air dried TLC plates. The plates were then wrapped in saran wrap, placed in an autoradiogram cartridge together with the X-ray film and exposed at -76°C for between 6 to 14 days.

3.2.9 **Biological Assays**

3.2.9.1 *Tps* Developmental Expression Rescue Assay

The objective of this assay is to identify a compound(s) from wild-type cells that would restore *tps* gene expression in *esg* mutant cells. The assumption inherent in this approach is that such a compound may be the E-signal which the *esg* mutant fails to produce. Rescue of

the *tps* gene defect in an *esg* mutant was used as an assay to test fatty acid and lipid fractions for E-signaling activity.

The *M. xanthus* strain used in the assay was JD258 (see Bacterial Strains, p.63). This strain contains a Tn5-*lacZ* fusion gene inserted into the *tps* gene such that the expression of β-galactosidase is under the control of the promoter of the *tps* gene. This enables us to monitor *tps* expression by determining the expression of β-galactosidase using the convenient β-galactosidase activity assay. JD258 was grown in 50 mL of CTT medium contained in a 250 mL side-armed Erlenmeyer flask, to a cell density of 80 to 100 Klett units. The cells were harvested by centrifugation at 8000 rpm for 10 minutes. The pellet was washed in MCM buffer and centrifuged a second time at 8000 rpm for 10 minutes. The washed pellet was resuspended in MCM medium and grown to a cell density of 100 Klett units. Nine hundred microliters of the concentrated cell suspension was then introduced into 25 mL glass tubes containing 600 μL of MCM mixed with 8 μL of the fatty acid sample, bringing the final cell density to approximately 60 Klett units. The tubes were incubated for 24 hours at 30°C. At the end of the incubation period the cells were transferred into 1.5 mL Eppendorf tubes and centrifuged for

5 minutes. The pellets obtained were stored at -20°C until they were used in the β-galactosidase activity assay.

The assay for β-galactosidase activity has been described (77). One and a half mL of developing cells were collected into Eppendorf tubes and centrifuged for 5 minutes. The pellet was resuspended in assay buffer (Z-buffer) and the cells were disrupted by sonication to produce a cell extract. The protein concentration was determined using the protein assay reagent kit by Pierce Pierce Inc. The protocol was provided by the manufacturer. Briefly, 2 mL of the protein assay reagent was introduced into a 5 mL glass tube containing 100 μL of sonicated cell extract. The cell extract was then mixed with the reagent by briefly vortexing the tube. The tubes were placed in a 60°C water bath for 30 minutes and then allowed to cool to room temperature. The optical density of the reaction solution was then determined at a wave length of 562nm. A standard curve relating BSA concentration to absorbance was determined and used to calculate the protein concentration of the *M. xanthus* extract.

formation in developmentally defective mutants since it is a cohesive strain adhering to the plastic surface of the well and undergoing fruiting body formation in a submerged culture. In this assay, vegetatively grown cells are resuspended in the starvation buffer and introduced into each well of a 24-well culture plate. The culture plates are incubated without agitation at 32°C and the cells settle to the bottom of the plate at a fairly high cell density. In submerged cultures, the wild-type strain of DK1622 settles at the bottom of the well and forms a layer of cells that adhere to the surface. These submerged cells at a high cell density on a solid surface undergo normal development to form rounded fruiting bodies easily observed by an inverted microscope as dark rounded structures at the base of the well. The morphology of submerged fruiting bodies resemble those on an agar surface (60). The DK1622/*esg* cells, on the other hand, fail to undergo normal development. Analysis of the submerged mutant cells with an inverted microscope reveals a homogeneous layer of cells showing signs of cell aggregation but lacking well developed fruiting bodies. Fruiting bodies, when present, are few in number and immature.

The wild-type strain (DK1622) and the *esg* mutant strain (JD300) were grown in 50 mL of CTT medium in a 250 mL side-armed

well developed fruiting bodies. Fruiting bodies, when present, are few in number and immature.

The wild-type strain (DK1622) and the *esg* mutant strain (JD300) were grown in 50 mL of CTT medium in a 250 mL side-armed Erlenmeyer flask to a cell density of 80 to 100 Klett units. The cells were centrifuged at 8000 rpm for 10 minutes. The pellet was washed in MC7 buffer and centrifuged a second time. The washed pellet was resuspended in MC7 buffer and allowed to grow to a cell density of 60 Klett units. Five hundred microliters of this cell suspension and 8 μL of the fatty acid sample were then introduced into a 1.5 mL Eppendorf tube and mixed thoroughly by vortexing. The 500 μL of the cells suspension was then introduced into each well of a 24-well microtitre plate, and the plates were incubated undisturbed at 30°C for between 3 to 4 days. At the end of this period, the cells developing in each well were analyzed microscopically for the presence of fruiting bodies. The morphology and number of fruiting bodies observed in the wells containing *esg* mutant cells developed in the presence of the fatty acids was compared with those observed in the wells containing developing wild-type cells (positive control) and *esg* mutant cells which were free of added fatty acid (negative control).

3.3 Results

3.3.1 Incorporation of the Labeled Branched-Chain Amino Acids into Wild-Type and *esg* Mutant Lipids

It was shown in previous studies (109) that the *esg* mutant contains reduced levels of most of the branched-chain fatty acids. The BCKAD is needed for the synthesis of branched-chain fatty acids from branched-chain amino acids (49) (51) (113). Direct evidence was sought for the notion that the *esg* BCKAD is used to produce branched-chain fatty acids and related lipids from the branched-chain amino acid. ^{3}H-labeled branched-chain amino acids were used to determine if the branched-chain amino acids are substrates for the synthesis of lipids. A1 medium was used as the vegetative growth medium in this study since it contains fairly low concentrations of the three essential branched-chain amino acids. The concentrations of the labeled branched-chain amino acids were sufficiently high to ensure that a good proportion of the labeled amino acids would be taken up into the cells. The concentration of leucine present in the original A1 medium was raised to 100 μg/ml to match the concentrations of isoleucine and valine [section 3.2.3, p. 64 and Table 3, p. 66].

Wild-type cells grown under these conditions incorporated the labeled amino acids into their lipids. This result provides direct evidence that the branched-chain amino acids in the medium were used as substrates during the synthesis of the branched-chain fatty acid component of the lipids. Most of the label in the lipid fraction was derived from leucine (42%) followed by valine (19%) and finally isoleucine (10%) [Table 4]. The greater utilization of leucine is probably due to the fact that most of the branched-chain fatty acids in *M. xanthus* are iso-fatty acids with an odd number of carbons, fatty acids expected to be produced using leucine (109) (112).

The incorporation of the labeled branched-chain amino acids into the lipid fraction of the *esg* mutant cells grown under our conditions was significantly lower than those observed in the wild-type cells. In these cells, most of the label in the lipid fraction was also derived from leucine (11%), followed by valine (3%), and finally isoleucine (2%) [Table 4]. We found that the levels of the labeled branched chain amino acids incorporated into the lipid fraction of the *esg* mutant are approximately 11% that of the levels of the labeled branched-chain amino acids incorporated into the lipid fraction of the wild-type cells. These results provide direct evidence of the fact that the *esg* BCKAD is

an important member of the metabolic pathway used for the synthesis of branched-chain fatty acids from their branched-chain amino acid substrates. It is evident from these findings that the reduced levels of the branched-chain fatty acids in the *esg* mutant are directly related to the inability of the mutant cells to synthesize branched-chain fatty acids using the *esg* BCKAD enzyme. The results of this study provide direct evidence in support of the prediction that branched-chain fatty acids can be synthesized from branched-chain amino acids during vegetative growth of *M. xanthus* by an *esg* BCKAD dependent pathway. In the light of the facts that a) the branched-chain amino acid leucine is utilized by the cells for the synthesis of lipids in far greater amounts than the other two branch-chain amino acids isoleucine and valine as revealed by this study and b) the branched-chain fatty acid iso-15:0, a leucine derivative, is the most abundant *M. xanthus* fatty acid (109), we decided to focus the rest of this study on identifying labeled iso-15:0 and monitoring its distribution in developing cells in order to determine whether or not iso-15:0 fits the pattern of an E-signal molecule as predicted by the model.

TABLE 4. Incorporation of labeled branched-chain amino acids into wild-type and *esg* mutant cells of *Myxococcus xanthus*

STRAIN	SUP[A] [cpm]	PEL[B] [cpm]	LIPID EXT [cpm]	INCOR[C] [%]	MUT AS % OF WT[D]
WT:leu	915,000	364,000	152,000	42	11
esg :leu	1,000,000	160,000	17,000	11	
WT:isoleu	885,000	93,000	9,000	10	11
esg :Isoleu	1,000,000	52,000	1,000	2	
WT:val	961,000	82,000	16,000	19	12.5
esg :val	1,000,000	62,000	2,000	3	

The preparation of the samples has been described under Materials and Methods. In determining the counts per minute[cpm] of the incorporated label for each sample was analyzed in a scintillation counter for a period of 10 minutes. The results presented in this table have been adjusted to 1 mL of cell culture that had been standardized to a uniform wet weight.

[A]label present in the growth media at the start of incubation

[B] label present in the cell pellet at the end of incubation

[C]counts incorporated into wild-type and *esg* mutant lipid extracts divided by the counts incorporated into wild-type and *esg* mutant cell pellets (b) X100

[D]counts incorporated into *esg* mutant lipid extract divided by the counts incorporated into wild-type lipid extract X 100

3.3.2 **Incorporation of Leucine Labeled iso-15:0 into *Myxococcus xanthus* Phospholipids**

In accordance with the model, the branched-chain fatty acids are supposed to be synthesized and incorporated into phospholipids during the vegetative growth phase of *M. xanthus.* In our case, this would imply that iso-15:0 is synthesized into phospholipids during the vegetative growth phase of *M. xanthus.* To identify the leucine derived fatty acids incorporated into phospholipids, the phospholipid fraction was first purified (section 3.2.5, p.68) and then hydrolyzed to release all its fatty acids (section 3.2.6, p.70). The released fatty acids were then separated by thin layer chromatography (TLC).

TLC analysis of the alkaline hydrolyzed PL fraction purified from vegetatively grown wild-type cells of *M. xanthus* revealed a number of ^{3}H-labeled fatty acid species (Fig 6A). One species in particular was found to be strongly labeled. This particular species was observed to co-migrate with authentic iso-15:0 on a TLC plate (data not shown). Since an earlier GC analysis identified iso-15:0 as the major branched-chain fatty acid of *M. xanthus* (108) (112), we concluded that this intensively labeled band was the branched-chain fatty acid iso-15:0. As was anticipated, the lack of a functional *esg* B CKAD enzyme in the mutant

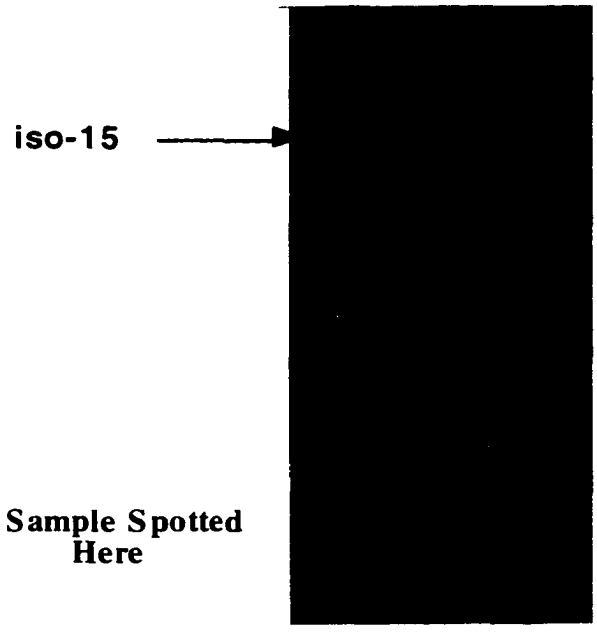


Figure 6. Autoradiogram of the detectable labeled fatty acid species in the hydrolyzed phospholipid fraction of the wild-type [A], and *esg* mutant [C] cells of *Myxococcus xanthus* and 1/3 concentration of the hydrolyzed phospholipid fraction of the wild-type cells [B]

Dry extracts of the phospholipid fraction were hydrolyzed as described in Materials and Methods. Approximately 25 μg of each labeled phospholipid fraction was separated by TLC using silica gel pre-coated 5X20 cm glass plates as described in materials and methods. The radioactive count of the wild-type phospholipid fraction was approximately 26,000 cpm while that of the *esg* mutant was approximately 5,800 cpm.

resulted in a reduced synthesis of iso-15:0 (Fig.6C). These results are consistent with the hypothesis that most of the branched-chain fatty acids are synthesized from branched-chain amino acids via an *esg* BCKAD dependent pathway.

Fatty acid analysis of vegetatively growing wild-type and *esg* mutant cells show that the *esg* mutant cells contain approximately 1/3 the wild-type levels of iso-15:0 (108). It was therefore expected that a 1/3 dilution of our wild-type PL fraction would contain labeled iso-15:0 at approximately the same intensity as that found to be present in the *esg* PL fraction. Our results show that even at a 1/3 dilution, the labeled iso-15:0 band in the wild-type hydrolyzed phospholipid was more prominent than the iso-15:0 band in the *esg* hydrolyzed phospholipid (Fig.6B).

3.3.3 **Leucine Labeled Lipid Species in a Free Fatty Acid Fraction from Wild-Type and *esg* Mutant Cells of *Myxococcus xanthus***

Wild-type and *esg* mutant cells of *M. xanthus* were grown vegetatively in A1 medium containing ^{3}H-leucine and were then transferred to MCM buffer to induce the early stages of development.

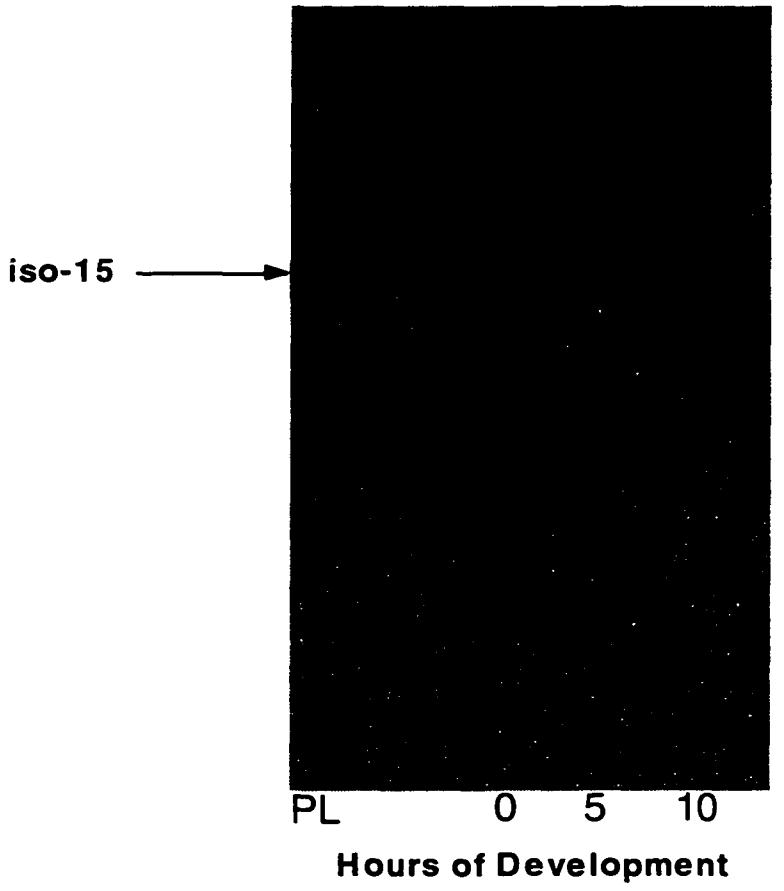


Figure 7. Autoradiogram of the detectable labeled fatty acid species in the free fatty acid fraction of developing wild-type cells of *Myxococcus xanthus*. Approximately 60 μg of the labeled free fatty acid fractions were separated by TLC using silica gel pre- coated 5X20 cm glass plates as described in Materials and Methods. The radioactive count of each sample was approximately 14,800 cpm. The sample of hydrolyzed phospholipid (PL) was used as the control for labeled iso-15:0. The fluorogram was analyzed after 2 weeks of incubation at -76 °C.

TLC analysis of the FFA fraction obtained from vegetatively growing cells and cells that had been incubated for 5 and 10 hours showed that the FFA fraction of the wild-type cells contained several labeled species which were derived from leucine (Fig 7). The three most intensively labeled species did not have a mobility corresponding to iso-15:0, the major branched-chain fatty acid of *M. xanthus.* It came somewhat as a surprise that iso-15:0 was not observed in these free fatty acid fractions despite the detection of a substantial amount of this fatty acid by GC analysis of the FFA fractions obtained from developing cells during the first 14 hours (Table 5).

TABLE 5. Percentage distribution of iso-15:0 in the alkaline hydrolyzed free fatty acid fractions of developing wild-type cells of *Myxococcus xanthus.*

Fatty acid	Time of Development					
	0	1	3	5	7	14
iso-15:0	50.81	58.77	50.88	42.19	58.40	42.15

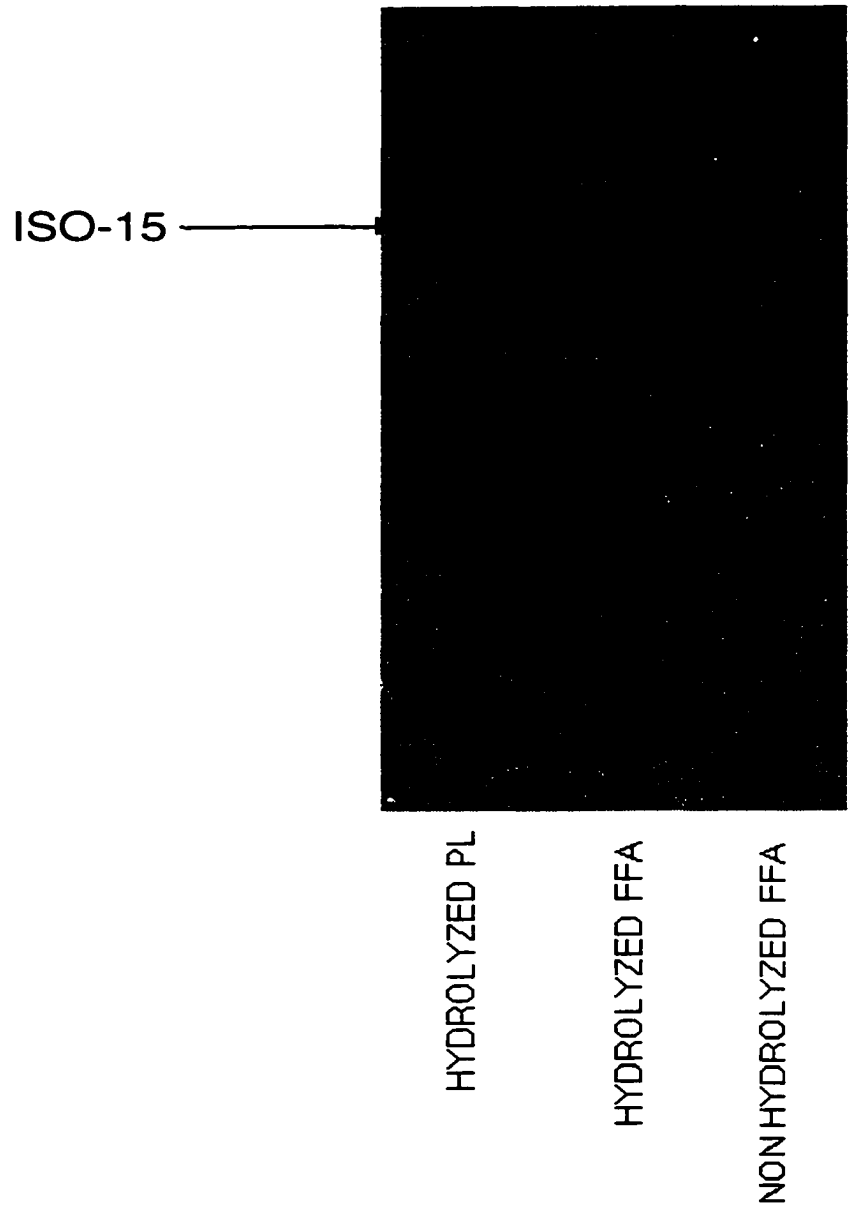


Figure 8. Appearance of the iso-15:0 fatty acid following alkaline hydrolysis of the leucine labeled free fatty acid fraction produced by wild-type cells.

Since alkaline hydrolysis of the FFA fraction was performed prior to GC analysis, one explanation for the apparent contradiction is that iso-15:0 in the FFA fraction is a component of a larger lipid molecule. Consistent with this interpretation we subjected the FFA fraction to alkaline hydrolysis and observed that a labeled species with the motility of iso-15:0 was produced (Fig 8). We propose that one or more of the heavily labeled species observed in the free fatty acid fraction is not a simple fatty acid but is instead a larger lipid complex composed in part of iso-15:0. These labeled lipid species were significantly reduced in the *esg* mutant (Fig.9), suggesting that the major labeled lipid species in this fraction are produced using the *esg* BCKAD. If one or more of these labeled species was a signal molecule, we might expect the amount of that compound to increase during the first 5 hours of development of wild-type cells. This has been shown to be the period when the E-signal dependent events of development commence (see chapter 2). Wild-type cells were grown to mid-log phase in A1 medium containing labeled leucine. A portion of these cells was used for fatty acid analysis while the remainder were developed in MCM buffer. Samples of developing cells were collected for fatty acid analysis at 5 hours and 10 hours after the initiation of development.

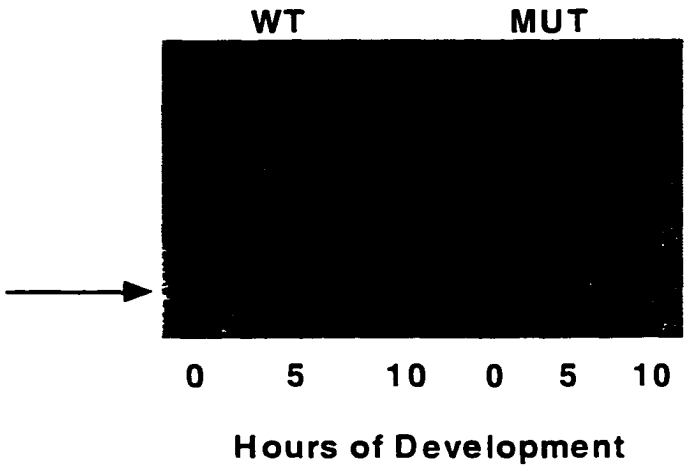


Figure 9. Autoradiogram of the detectable labeled species in the free fatty acid fractions of wild-type and *esg* mutant cells of *M. xanthus*

The cell density in each sample was adjusted to be approximately equal to make sure that a similar weight of cell pellet was used for the fatty acid analysis. We observed that labeled species contained in the FFA fraction obtained from the vegetatively growing cells were no different in intensity than those contained in the FFA fractions obtained from cells that had been developed for 5 and 10 hours (Fig.7, p.83).

Our observations suggest, contrary to the predictions of the model, that these labeled species are most probably not released from phospholipids during the first five hours of development. It was noticed however, that one of the minor species that did not correspond to iso-15:0 and was not observed in the FFA fraction of vegetatively growing cells (0 hours) reached a maximum concentration by 5 hours of development and then decreased in concentration by 10 hours of development (Fig.9, p 87 see arrow).

3.3.4 **Biological Assay for Fatty Acids and Lipid Fractions for E-Signaling Activity**

We have explored the use of another approach for the identification of the E-signal. This approach utilizes biological assays to identify lipid compounds capable of restoring developmental function in an *esg* mutant.

The design of the first assay enabled us to study development in a liquid developmental media (MCM). This study employed the induction of *tps* expression in *esg* mutant cells developing in liquid media to identify lipids capable of restoring developmental function in the *esg* mutant. The *tps* gene was used as a reporter gene in this liquid assay

for these reasons: a) the *tps* gene is expressed in low nutrient or starvation shaker cultures in the absence of the extensive cell-cell contact which occurs during development on a solid surface (53), b) the expression of the *tps* gene is greatly reduced in an *esg* mutant (18), and c) the *tps* gene which is expressed at low levels during vegetative growth is strongly expressed commencing at about 5 to 6 hours of development. Therefore a change in the expression of the *tps* gene would be easy to detect (19) (20).

Wild-type and *esg* mutant cells were grown in CTT medium to mid-log phase and then transferred to MCM medium at a cell density of between 20 and 40 Klett units. The developing *esg* mutant cells were divided into two groups. One group was incubated under developmental conditions in the presence of the FFA fraction while the other was incubated without the FFA fraction. The FFA fractions tested in this assay were obtained from developing wild-type and *esg* mutant cells. The times ranged from 1 to 10 hours after the initiation of development. The wild-type cells were used as a control to monitor the developmental conditions of the assay, while the *esg* mutant cells developing in the absence of the FFA fraction enabled us to rule out background expression in the mutant strain.

The results of our *tps* developmental expression rescue assay show that the complex lipids present in the FFA fraction purified from developing wild-type cells had a strong effect in inducing *tps* expression in an *esg* mutant while the similar fraction obtained from developing *esg* mutant cells exhibited about a five fold weaker response (Fig.10). These results suggest that *esg* dependent compounds may serve as the signal components missing in the *esg* mutant. This assay is designed to detect E-signaling activity (*tps* expression) but we cannot rule out the possibility that other molecules may have activity in this assay. It is reasonable to speculate that these compounds may be related in structure to the E-signal but that these are not the actual compound that is used in signaling.

Studies have shown that commercially purified branched-chain fatty acids are able to rescue *tps* activity in an *esg* mutant (Downard, data not shown). The strongest response was observed with iso-15:0. We compared the ability of purified iso-15:0 and the FFA fraction obtained from developing wild-type cells to rescue *tps* expression in an *esg* mutant and showed that they were closely comparable in their ability to rescue *tps* expression (Fig.11). This result suggests that iso-15:0 may function as one of the E-signal molecules. The submerged

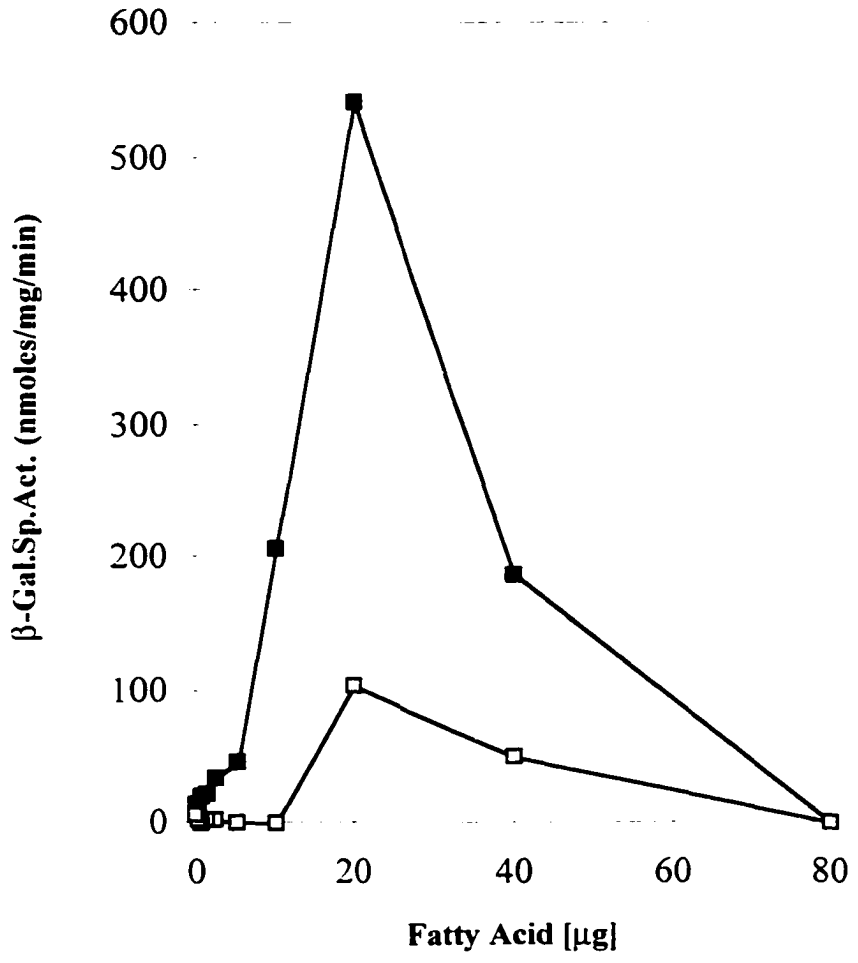

600
500
400
300
200
100
0
β-Gal.Sp.Act. (nmoles/mg/min)
0
20
40
60
80
Fatty Acid [µg]

Figure 10. The ability of free fatty acids fraction to rescue *tps* expression in an *esg* mutant.
Fatty acid fractions purified from wild-type and *esg* mutant cells that had been developed for 8 h were used in the assay.
8 µL of the free fatty acids at concentrations varying from approximately 80 µg to 0.156 µg was introduced into 1500 µL of JD10258 resuspended in MCM buffer at a cell density of 60 Klett units. The fatty acids purified from developing wild-type cells rescued *tps* expression in the *esg* mutant after 24 hours of development (■). The strongest response in regard to ability to rescue *tps* expression in the *esg* mutant was seen with fatty acid concentrations of approximately 20 µg. Fatty acids purified from developing *esg* mutant cells on the other hand demonstrated a weak response in the same regard (□).

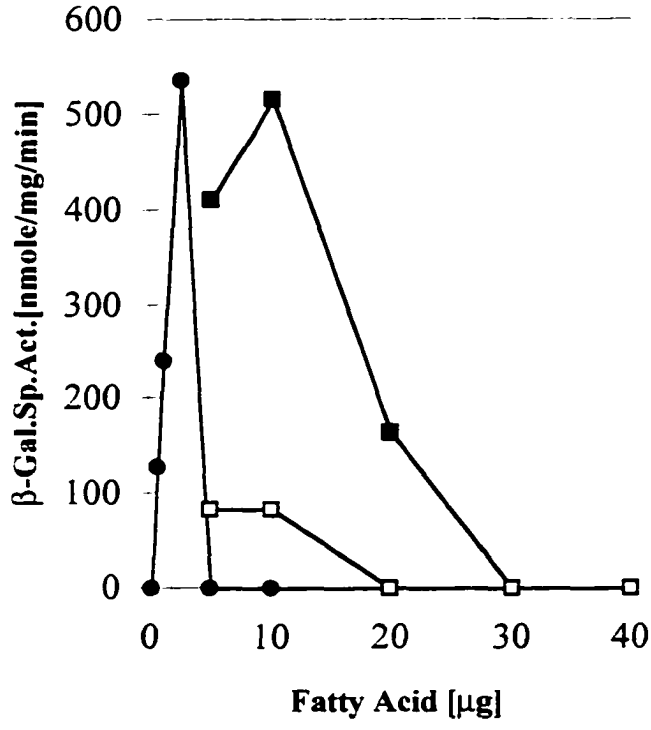


Figure 11. The ability of fatty acids to rescue *tps* expression in an *esg* mutant.
Free fatty acid fractions obtained from developing wild-type cells (■), *esg* mutant cells (□), and purified iso-15:0(●), were used in the liquid culture assay. The concentration of purified iso-15:0 tested in the assay ranged from 40 μg to approximately 0.3 μg while that of the wild-type and mutant fatty acid fractions ranged from 40 μg to 5 μg. Since saturation with the purified iso-15:0 was reached by a concentration of 10μg readings of β-Galactosidase activity above this concentration were omitted from the graph.Eight μL of the fatty acid samples were mixed with 1500 μL of JD258 in MCM medium at a cell density of 60 klett units. The cells were developed for 24 hours. *Tps* expression was determined by assaying for β-galactosidase activity.

culture fruiting body formation assay was utilized to further test the ability of iso-15:0 to rescue fruiting body formation of the *esg* mutant (section 3.2.9.2., p.73). In this assay, *esg* mutant cells were incubated in MC7 developmental buffer containing varying concentrations of iso-15:0. If iso-15:0 has the potential to function as a signal molecule, one would expect the test cells to respond to the iso-15:0 by forming fruiting bodies. We observed that the *esg* mutant cells were able to form fruiting bodies after 3 days of development (Fig.12). These fruiting bodies were smaller and less dense compared to the fruiting bodies produced by the wild-type strain of *M. xanthus*. In the absence of iso-15:0, *esg* mutant cells failed to undergo normal development. These results suggest that iso-15:0 rescued fruiting body formation despite the fact that the rescued *esg* fruiting bodies were somewhat different in appearance than wild-type fruiting bodies.

At this point we have clearly shown that the FFA fraction obtained from developing wild-type cells contains complex lipids and that this fraction can rescue a *tps* expression defect in an *esg* mutant. We have also shown by alkaline hydrolysis that one or more of these lipid complexes contain iso-15:0. Exogenously supplied iso-15:0 has also

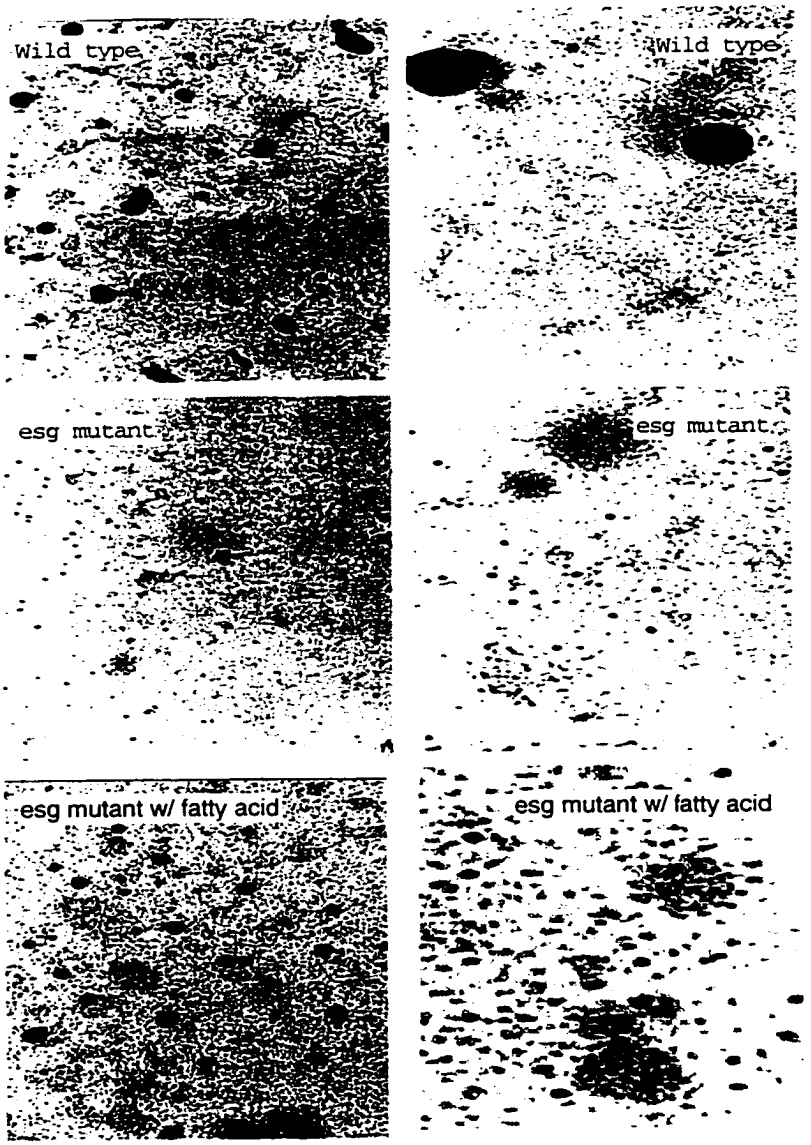

Wild type
Wild type
esg mutant
esg mutant
esg mutant w/ fatty acid
esg mutant w/ fatty acid

Figure 12. Fruiting body formation in submerged cultures of wild-type and *esg* mutant cells.
The cells were grown vegetatively in CTT medium to a cell density of between 70 to 90 Klett units. Development was initiated in MC7 buffer as described in materials and methods.The photographs were taken after 3 days of development at low(10X) and high magnification(40X).The DK1622 wild-type and DK1622/*esg* mutant were developed in MC7 buffer without the addition of iso-15:0 and served as the positive and negative controls respectively. Iso-15:0 concentrations ranging from 0.3 μg to approximately 40 μg were tested in the assay. Iso-15:0 at concentrations between 10 μg and 40 μg had the ability to rescue development of the *esg* mutant, although the strongest response was observed at iso-15:0 concentrations of 40 μg. Fruiting bodies were not formed by the *esg* mutant cells developed without iso-15:0 but it does appear to be a part of the signaling molecules missing in the *esg* mutant. The free fatty acid fractions obtained from developing wild-type cells also rescued development of an *esg* mutant in the submerged culture assay. The response was observed to be significantly stronger than that produced by the free fatty acid fraction purified from developing *esg* mutant cells (data not shown).

been shown to rescue development of the *esg* mutant. Since free iso-15:0 as well as lipid complexes containing iso-15:0 were both able to rescue *tps* expression in an *esg* mutant, we decided to determine whether or not there was a significant difference in biological activity between hydrolyzed and non hydrolyzed FFA fractions. In the former, iso-15:0 is present in a free form while in the latter iso-15:0 is tied in with other lipid compounds (Fig.8, p.85). Our results demonstrate that the hydrolyzed and nonhydrolyzed FFA fractions purified from developing wild-type cells were able to rescue *tps* expression in an *esg* mutant tested in the *tps* developmental expression rescue assay. However, the biological activity of the hydrolyzed fraction was markedly reduced in comparison with that of the nonhydrolyzed fraction (Fig.13).

In this project we have initiated studies designed to identify the *esg* signal. This signal is proposed to be a lipid molecule produced using the biosynthetic pathway involving the *esg* BCKAD. Branched-chain fatty acids constitute the most abundant fatty acid species (65%) of *M. xanthus* and are synthesized and incorporated into membrane phospholipids during the vegetative growth cycle of the bacterium (108). The incorporation of the branched-chain fatty acids into the

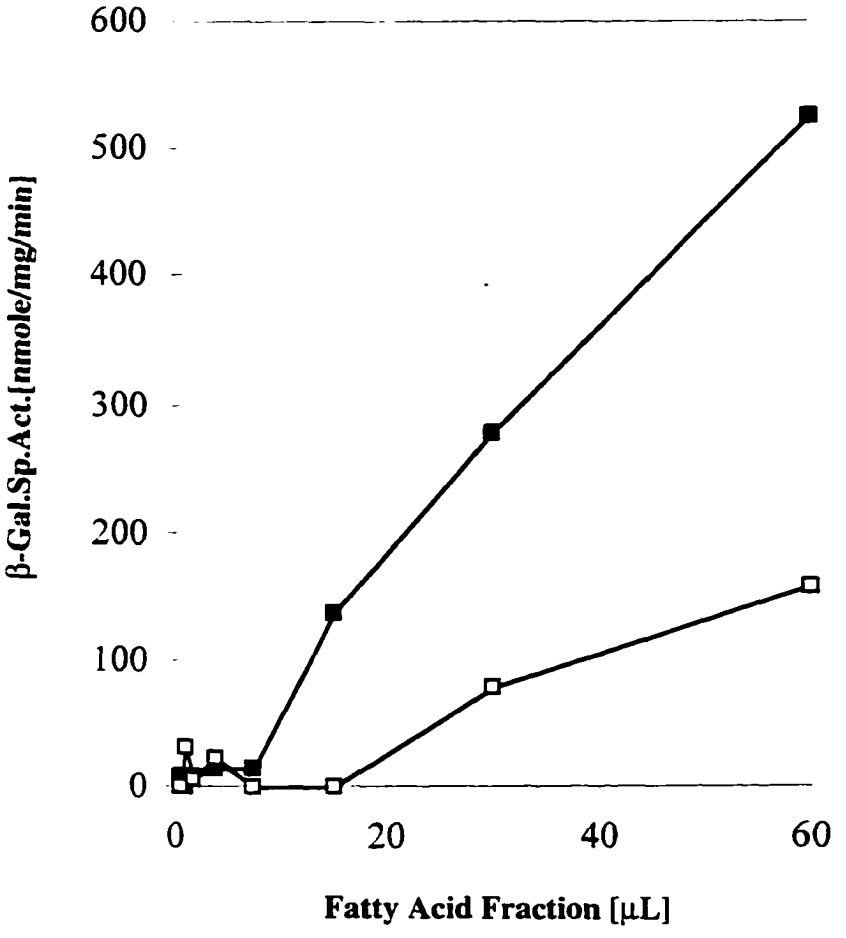


Figure 13. Rescue of *tps* expression in an *esg* mutant by the lipid fractions containing non-hydrolyzed (■) and hydrolyzed (□) fatty acids.
1.5 mL of cells were incubated with 8 μL of varying concentrations of the fatty acids. *Tps* gene expression was determined by the assay for β-Galactosidase activity using mutant cells that had been developed in MC7 buffer at 60 K.U. for 24 hours. Hydrolysis of the fatty acid fraction resulted in a reduced biological activity of the fatty acid fraction. Saturation levels were not reached with these samples despite the earlier observation (Fig.11 p.93that similer samples did reach saturation levels. The most probable explanation for this difference is that the range of fatty acid concentration used in this experiment was too low to reach saturation levels.

phospholipid occurs at varying proportions with iso-15:0 accounting for approximately 45% of these fatty acids (108) (112).

3.4 Discussion

Results presented in an earlier study suggested that the *esg* BCKAD is part of an important pathway for the production of branched-chain fatty acids from branched-chain amino acids. Much of the branched-chain fatty acid found in *M.xanthus* is likely to be derived from leucine. Our results complement the earlier observations in that we observed that ^{3}H-labeled leucine was taken up into the cell and incorporated into lipids at a much higher level than either isoleucine or valine (Table 4 p.79). Approximately 42% of the labeled leucine incorporated into the wild-type cell was utilized for lipid synthesis compared with 11% of the isoleucine and 19% of the valine. The *esg* mutant cells have less BCKAD enzyme and as a result synthesize reduced levels of the branched-chain fatty acids (108). In accordance with this decrease in BCKAD activity, we observed that the incorporation of the three labeled branched-chain amino acids into lipids of the *esg* mutant was about 11% of wild-type levels (Table 4). It would appear from these results that the synthesis of an important lipid

component that is derived from the exogenously supplied branched-chain amino acids is dependent on the *esg* BCKAD.

The *esg* mutant fails to undergo normal development, and it has thus been proposed that the branched-chain fatty acids may function as E-signal molecules required for the regulation of *M. xanthus* development. When the release of leucine labeled branched-chain fatty acids from phospholipids was investigated in vegetatively grown wild-type *M. xanthus* cells as well as during the first 10 hours of development, we failed to detect a release of free labeled species corresponding to the major leucine derived branched-chain fatty acids such as iso-13:0, iso-15:0 or iso-17:0 (Fig.7, p.83). We did, however, observe that the FFA fraction from the wild-type cells did contain other strongly labeled species that were missing or found at low levels in the FFA fractions from the *esg* mutant cells (Fig.9 p.87). These results suggest that the synthesis of the unidentified labeled species is dependent on the *esg* BCKAD and raises the possibility that these lipid species may in fact function as the E-signal molecules. If this were the case, it would follow from predictions in our model and from results obtained in chapter 2 that concentrations of one or all of the signal

molecules will be observed to increase during the first 3 hours of development.

The results obtained show that these labeled lipid species are synthesized during vegetative growth with scant evidence of any detectable increase in their concentration during the first 10 hours of development (Fig.7, p.83). Therefore the pattern of synthesis of the labeled lipid species does not conform to the criteria for an E-signal molecule. Nevertheless, the observed decreased synthesis in the *esg* mutant can be taken as evidence that the synthesis of these labeled lipid species is dependent on the *esg* BCKAD. To determine whether the lipid species could function as probable E-signal molecules, we tested their ability to rescue the expression of *esg* -dependent developmental gene expression using the *tps* gene whose expression has been shown to be dependent on the *esg* BCKAD.

We have shown that the FFA fraction obtained from developing wild-type cells is able to induce *tps* expression in an *esg* mutant while similar FFA fractions obtained from developing *esg* mutant cells have low activity (Fig.10, p.91). These results support our hypothesis that the E-signal molecules are lipid species. We have also shown that some of these labeled lipid species are complex base-labile lipids and at least

one of these species appears to contain the iso-15:0 fatty acid as a component (Fig.8, p.85). Iso-15:0 by itself is biologically active, having the capabilities of inducing *tps* expression in an *esg* mutant and rescuing its ability to develop (Fig.11, p.93 and Fig.12, p.95). However, free iso-15:0 was not detected in our investigation. Base hydrolysis of the FFA fraction obtained from developing wild-type cells was found to release free iso-15:0 from one or more of the complex labeled lipid species (Fig.8, p.85); at the same time, the hydrolyzed FFA fraction was observed to lose some of its ability to induce *tps* expression in an *esg* mutant (Fig.13 p.98). This data is consistent with the interpretation that the labeled lipid complexes are biologically active and changes in their structure reduced this biological activity. We also propose that while iso-15:0 could function as the E-signal, other lipid(s) with iso 15:0 as a component part must be considered as possible E-signaling molecules. At this point it is clear that none of the lipid species tested satisfy all the predictions which would help us identify an E-signal candidate. However, none are completely eliminated from consideration either.

We also provide evidence to suggest that the *esg* BCKAD pathway is not the only pathway responsible for the synthesis of

branched-chain fatty acids in *M. xanthus*. The *esg* BCKAD is defective in the *esg* mutant and we would expect this mutant to be completely unable to incorporate the labeled branched-chain amino acids into lipids. Contrary to this expectation, it was observed that the *esg* mutant was able to incorporate a low level of H^3-labeled leucine into the lipid components, and at least some of the labeled lipid was in the form of iso 15:0 (Table 4, p.79 and Fig.6, p.81). Our results are in agreement with earlier observations that the *esg* mutant is capable of synthesizing branched-chain fatty acids, albeit at a much lower level as compared to the wild-type cells (108). These results strongly suggest that there may be a second minor BCKAD present in *M. xanthus* which may be utilized by the cell in the event that the major *esg* BCKAD is inactivated. The possibility of containing two separate BCKAD is not unique to *M. xanthus*. Two gene clusters situated 12 kb apart and coding for two separate BCKAD enzymes have been identified in *Streptomyces avermitilis* (16). One of the multienzyme complex is thought to be dominant being responsible for the major BCKAD activity of the bacterium. Our results provide direct evidence that the *esg* mutant is able to synthesize labeled iso-15:0, indicating thereby that the

predicted second BCKAD also utilizes the exogenous leucine as the substrate to synthesize the iso-15:0.

Fatty acid analysis of the unlabeled fatty acids showed that the relative levels of iso-15:0 in the *esg* mutant were approximately 1/3 those of wild-type levels (55% to about 16%) (17) (108). Our result (see Fig. 6, p.81), shows that the ratio of iso-15:0 synthesized from the labeled leucine in the wild-type and *esg* mutant cells differs from that of the unlabeled iso-15:0. Labeled iso-15:0 was synthesized in the *esg* mutant to a much smaller extent than its unlabeled counterpart. The detection of a larger amount of unlabeled iso-15:0 as compared to labeled iso-15:0 raises the possibility that there may be a leucine independent pathway in *M. xanthus* that is being utilized for the synthesis of iso-15:0. If this were indeed the case, it would lead to the conclusion that three separate pathways are present in *M. xanthus* for the synthesis of branched-chain fatty acids. Two of these pathways are leucine dependent pathways requiring either the *esg* BCKAD or a minor BCKAD while the third is a leucine independent pathway. This proposal is highly speculative at the present time and calls for further experimental confirmation.

CHAPTER 4

Growth Medium Dependent Regulation of the *Myxococcus xanthus* Fatty Acid Content is Controlled by the *esg* Locus

4.1 Introduction

The fruiting myxobacteria are unusual among gram negative bacteria in that branched-chain fatty acids constitute the majority of the cellular fatty acids (112). Most Gram negative bacteria have been found to contain straight chain saturated and monounsaturated fatty acids (93). In *M. xanthus*, the most extensively studied myxobacterium, the branched-chain fatty acids have been reported to make up about 65% of the total fatty acid content, with iso-15:0 being the single most abundant species (45% to 55%) (108) (112). Branched-chain fatty acids are also often found to be the predominant fatty acid components in most species among the gram positive genera such as *Bacillus*, *Micrococcus*, and *Sarcina* (28) (93). Our current understanding of the biosynthesis of branched-chain fatty acids (BCFA) is based primarily on

work carried out on the Bacillus species (28) (49) (50). The pathway for branched-chain fatty acid synthesis begins with the three branched-chain amino acids (BCAA) leucine, isoleucine, and valine (Fig.1, p.25). These amino acids are deaminated and decarboxylated to produce short branched-chain fatty acid derivatives which serve as primers in the fatty acid elongation reaction. Elongation occurs by the addition of two carbon atoms in steps analogous to those used in straight chain fatty acid synthesis.

An evolutionary conserved multienzyme complex, the branched-chain keto acid dehydrogenase (BCKAD), is responsible for decarboxylation of the three branched-chain keto acids producing CoA derivatives of the three short branched-chain fatty acids. The BCKAD multienzyme complexes are composed of four polypeptide chains referred to as E1α, E1β, E2 and E3. In *M. xanthus* the *esg* locus encodes the E1α and E1β BCKAD proteins (108). This conclusion is based on the following observations: (a) the predicted amino acid sequence of the *esg* proteins are very similar to the amino acid sequences of proteins belonging to these conserved protein families and (b) *esg* transposon insertion mutants have reduced levels of the branched-chain fatty acids and reduced BCKAD enzyme activity. It is significant that the *esg*

mutant retains a reduced capacity for branched-chain fatty acid synthesis and a low level of enzyme activity. This points to the existence of an *esg*-independent pathway(s) for the production of these compounds.

As part of a project designed to confirm that lipid species are produced from BCAA via the *esg* -dependent pathway [chapter 3], the total fatty acid content was determined for wild-type and *esg* mutant cells of *M. xanthus* grown in a complex medium (CTT) (112) and the chemically defined A1 medium (6). We found that cells grown in CTT had significantly higher proportions of the most abundant branched-chain fatty acid iso-15:0, together with several other less abundant branched-chain species. *Esg* gene expression was also observed to be regulated by the nutritional content of the growth medium. This regulation determined the fatty acid profile of the vegetatively growing cells of *M. xanthus*. These results are seen to be consistent with a model in which the composition of the growth medium is involved in the transcriptional regulation of the level of BCKAD activity which in turn is responsible, at least in part, for controlling the relative levels of the branched-chain fatty acids found in the *M. xanthus*. Regulation of *esg* transcription

appears to play an important part in controlling the cellular levels of BCKAD.

4.2 Materials and Methods

4.2.1 Bacterial Strains

The wild-type strain used in this study was DZF1 (26) (27). The two *esg* mutant strains used were JD256 and JD306. JD256 is a DZF1 derivative containing a Tn5 insertion in the *esg* locus and JD306 is a DZF1 merodiploid containing both a wild-type copy and a mutant copy of the *esg* locus. The mutation was created by a Tn5*lac* insertion (18). This merodiploid strain has a wild-type phenotype.

4.2.2 Vegetative Growth Conditions

To determine the effect of the growth media on fatty acid synthesis in *M. xanthus*, DZF1 and JD256 were grown in the nutritionally rich CTT medium (1% casitone, 10mM Tris-hydrochloride (pH7.6), 1mM $KHPO_4$, 8mM $MgSO_4$) (85), or CYE medium (1% casitone, 0.5% yeast extract, 0.1% $MgSO_4.7H_2O$; pH 7.0) (10), and the chemically defined A1 medium (6) (also see Table 3 , p.66). The primary carbon and energy

sources in A1 medium are pyruvate and aspartate, and *M. xanthus* grows much more slowly in A1 than in CTT (6). The generation times in A1 and CTT are 24 hours and 4 to 5 hours respectively (6). The cells were grown to 70 to 100 Klett units and harvested by centrifugation at 8,000 rpm for 10 minutes. The cell pellets were frozen in dry ice until required for fatty acid analysis. Whole-cell fatty acid analysis was performed by Microcheck. Inc, Burlington, Vermont on 45 mg samples of *M. xanthus* cell (wet cell weight).

To determine the effect of growth media on the expression of the *esg* locus, JD306 cells were grown in the chemically defined A1 medium (6) for approximately 6 generations. These cells were then centrifuged and the pellet resuspended in CTT medium at a starting cell density of approximately 5 Klett units. The cells were incubated at 32°C with shaking at 200 rpm. To determine the effect of amino acids on *esg* expression, JD306 cells were grown in CTT medium to a cell density between 70 to 100 Klett units and were harvested by centrifugation at 8,000 rpm for 10 minutes and prepared for growth in A1 medium described earlier. The washed cells were subcultured in an A1 medium containing various combinations of amino acids (Table 6).

TABLE 6. Modified version of A1 growth medium containing varying concentrations of amino acids

[1]A1
A1+ 1% [2]casitone
A1+ 0.1% casitone
A1 without the branched-chain amino acids + 1% casitone
A1 without the branched-chain amino acids + 0.1 % casitone
A1+125µg/mL leucine
A1+250µg/mL leucine
A1+250µg/mL isoleucine
A1+500µg/mL isoleucine
A1+250µg/mL valine
A1+500µg/mL valine
A1 +75µg/mL leucine, 150 µg/mL each of isoleucine and valine
A1 +125µg/mL leucine, 250 µg/mL each of isoleucine and valine
A1 +250µg/mL leucine, 500 µg/mL each of isoleucine and valine

[1] basic A1 medium contains 50µg/mL leucine, 100 µg/mL each of isoleucine and valine (see Table 3 p.72)
[2] see appendix for amino acid content of casitone

4.2.3 Developmental Conditions

The DZF1 wild-type cells of *M. xanthus* were grown vegetatively in CTT or in A1 medium to 80 to100 Klett units. These cells were harvested by centrifugation at 8000 rpm for a period of 10 minutes. The pellets obtained were washed in TM buffer (10 mM Tris-HCl (pH7.6), 10mM $MgSO_4$) and resuspended in fresh TM buffer to a final cell density of approximately 1000 Klett units. The concentrated cell suspension (5 μL) was spotted on plates of clone fruiting (CF) agar (0.015% casitone, 0.1% sodium pyruvate, 0.2% sodium citrate, 0.02% $(NH_4)_2SO_4$, 8mM $MgSO_4$, 1mM KH_2PO_4-K_2HPO_4, 10mM Tris-HCL (pH 7.6), 1.5% agar) (85), and the plates were incubated at 30°C for 4 days. The fruiting bodies were photographed using a Nikon camera at regular intervals over a 60 hour period of development.

4.2.4 2-Keto Acid Dehydrogenase Assay

This assay has been already described (109). Crude cell-free extracts were used to assay for 2-Keto acid dehydrogenase activity. Vegetative cells were grown in 400 mL of CTT and A1 medium to a cell density of approximately 100 Klett units. The cells were harvested by centrifugation at 1000 rpm for 15 minutes. The resulting cell pellets

were resuspended in a 50mM potassium phosphate buffer (pH 7.6) {KH_2PO_4 [86%] and K_2HPO_4 [14%]} and centrifuged at 8000 rpm for 10 minutes. These pellets were then washed and resuspended in 5 mL of the assay buffer [50mM potassium phosphate buffer (pH 7.6), 1mM EDTA, 0.5mM thiamine pyrophosphate (TPP)]. The cells were disrupted with a Branson Sonifier (two 60s bursts at a power setting of 2.5), and the cell debris was removed by centrifuging the disrupted cells at 8000 rpm for 10 minutes. The supernatant obtained was introduced into an ultracentrifugation tube and spun at 90,000 rpm for 1 hour. The pellets obtained after ultracentrifugation were resuspended in 400 μL of assay buffer (pH 7.6). The protein concentration of the sample was determined at this point by the BSA Protein Assay Reagent Kit supplied by Pierce. Approximately 0.5 mg of the blue dye 2,6,-dichlorophenol-indophenol was resuspended in 1mL of water and added in microliter amounts to the reaction mix (100 mM potassium phosphate buffer, 1 mM $MgCl_2$ and 100 μM TPP) until the OD_{600} of the reaction mix was approximately 0.700. The reaction was initiated by the addition of the appropriate substrate into the reaction mix and the dehydrogenase activity was determined by following the reduction of 2,6-dichlorophenol-indophenol in a spectrophotometer at 600nm. Alpha-

ketoglutarate (4 mM) was added as a substrate to assay for α-ketogluterate dehydrogenase activity, pyruvate (4 mM) was added to assay for pyruvate dehydrogenase activity and finally 4 mM of one of the following branched-chain keto acids, 2-ketoisovalerate [KIV], 2-keto-3-methylvalerate[KMV], and 2-ketoisocaproate [KIC] was added to assay for branched-chain keto acid dehydrogenase activity .

One unit of dehydrogenase activity was defined as the amount of enzyme required to reduce 1μ mole of dichlorophenol indophenol (NAD analogue) per minute using 6250 $M^{-1}c\,m^{-1}$ extinction coefficient of dichlorophenol indophenol. Initial reaction rates were found to be linear for the first minutes under the prevailing conditions. All the necessary blank determinations were implemented and these were subtracted from the values obtained in the test sample in order to obtain an accurate assessment of the enzyme activity.

4.2.5 β-Galactosidase Activity Assay

At each time point, a 1 mL sample of the culture was removed in order to determine the β-galactosidase specific activity by procedures described earlier (see chapter 2, p.48). The protein concentration was

determined using the BCA Protein Assay Kit by Pierce. The protocol was provided by the manufacturer.

4.3 Results

4.3.1 Growth Media Dependent Regulation of the *Esg* Locus

4.3.1.1 Influence of the Growth Media on the Fatty Acid Profile of *M. xanthus*

The relationship between nutritional control of *esg* expression and *M. xanthus* fatty acid content was investigated. Wild-type cells growing in the chemically defined media were found to synthesize lower amounts of the branched-chain fatty acids iso-15:0 and iso-13:0 than was the case with wild-type cells growing in the nutritionally rich CTT medium (Table 7). On the other hand, the levels of the straight chain fatty acids 16:0 and 16:1 w5c were found to be higher among the wild-type cells grown in the chemically defined medium. Just one of the branched-chain fatty acids, iso-14:0 3OH, was found in greater quantities in A1 grown cells. In the *M. xanthus* wild-type cells, the fatty acid content varied widely depending on the growth media.

TABLE 7. Fatty acid profiles of wild-type and *esg* mutant cells grown in CTT and A1 medium

Fatty Acid[a]	Wild-type (%)[b]		*esg* (%)	
	CTT	A1	CTT	A1
iso-13:0	0.8	nd[c]	nd	nd
iso-15:0	45.3	26.7	16.2	20.8
iso-14:0 3OH	5.5	8.4	1.1	3.5
16:1 w5c	16.4	20.6	39.8	40.5
16:0	3.9	22.3	8.2	11.6
iso-17:0	7.2	5.7	4.2	8.5
iso-17:0 3OH	2.2	0.6	nd	1.4

[a] The relative contents of the most abundant fatty acid species found in *M. xanthus* DFZ1 (wild-type) and an *esg* mutant JD257 (18) is shown in Table 5. The pattern of growth of the cells and the determinations of their fatty acid profiles is described in materials and methods

[b] (%) - area of individual fatty acid peak as a percentage of the total area of the identified fatty acid peaks.

[c] nd, not detected

Clearly, fatty acid synthesis in the wild-type cells of *M. xanthus* is greatly influenced by the nutritional content of the growth medium.

In contrast, the fatty acid content of the *esg* mutant cells grown in the two media were observed to change very little. In fact, the levels of their branched-chain fatty acid components iso-15:0 and iso-13:0 and the straight chain fatty acid components 16:0 and 16:1 w5c increase marginally when mutant cells were grown in A1 medium (Table 7). These results indicate that the mutation of the *esg* locus diminished the medium-dependent alteration in the *M. xanthus* fatty acid content found in wild-type cells.

4.3.1.2 Regulation of the *esg* Locus by the Growth Medium

One explanation for the observed *esg* dependent variation of fatty acid content in the *M. xanthus* cells is that the expression of the *esg* locus is regulated by the growth medium. In this case, it is to be expected that the level of *esg* expression will be found to be low in A1 medium and high in CTT medium. Such a pattern of gene expression would be anticipated to result in a low level of BCKAD activity with a correspondingly low production of the branched-chain fatty acids in A1

grown cells compared to high levels of BCKAD activity with high production of branched-chain fatty acids in CTT grown cells.

To investigate *esg* expression, use was made of a *esg* ::Tn5 *lacZ* fusion in which the production of β-galactosidase was placed under *esg* transcriptional control. The cells used to monitor *esg* expression were first grown for several generations in A1 medium before being transferred to fresh CTT medium. Samples of the CTT grown cells at room temperature were removed for assay of β-galactosidase (Fig.14). Expression of the *esg* locus was found to be low in the A1 grown cells, whereas after a lapse of about 5 hours expression increased dramatically in CTT. Peak *esg* driven β- galactosidase activity recorded in CTT was 550 units while the activity in A1 medium stood at around 30 units. The level of β-galactosidase activity plateaued in the mid-log phase of growth after about three generations of growth in the rich media (Fig.14) and were found not to increase when cells entered their stationary phase (data not shown). A very similar pattern of *esg* expression was observed when the same experiment was performed using another complex growth medium (casitone-yeast extract medium {CYE}) instead of CTT (data not shown).

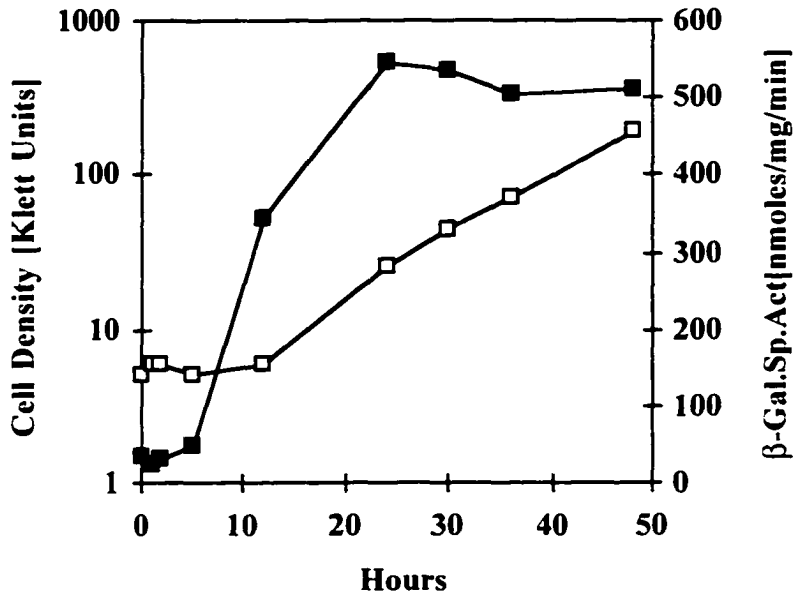


Figure 14. Cell density [□] and expression of the *esg* locus[■] following transfer of *M.yxococcus xanthus* cells from a chemically defined to a complex growth media. Expression of the *esg* locus was monitored using *M. xanthus* JD306 as described in materials and methods. At each time point a 1mL sample of the culture was removed in order to determine the β-galactosidase specific activity as described by Miller (77). The protein concentration was determined using the BCA Protein Assay Kit by Pierce.

4.3.1.3 Growth Media Dependent Regulation of BCKAD Activity

To ascertain whether an increase in *esg* expression in the CTT medium was accompanied by an increase in the level of BCKAD activity, cell extracts were assayed for enzyme activity. Crude extracts from wild-type cells were assayed using the three branched-chain keto acid substrates (α-ketoisovaleric acid (KIV), -keto-β-methyl-n-valeric acid(KMV), and α-ketoisocaproic acid (KIC) produced by deamination of the branched-chain amino acids. Assays with all three substrates confirmed that higher levels of BCKAD activity were present in CTT grown cells than in A1 grown cells (Figure 15). The difference in activity was greatest when KIV was used as the substrate. This result agreed with an earlier result of enzyme activity in which the increased expression of *esg* observed in CTT grown cells was accompanied by a corresponding increase in BCKAD activity (109).

4.3.1.4 Correlation Between the Levels of *esg* Expression and Branched-Chain Fatty Acid Synthesis

Wild-type cells, grown for several generations in the chemically defined A1 medium, were transferred to the nutritionally rich CTT medium. Samples were removed at various times for assay of *esg*

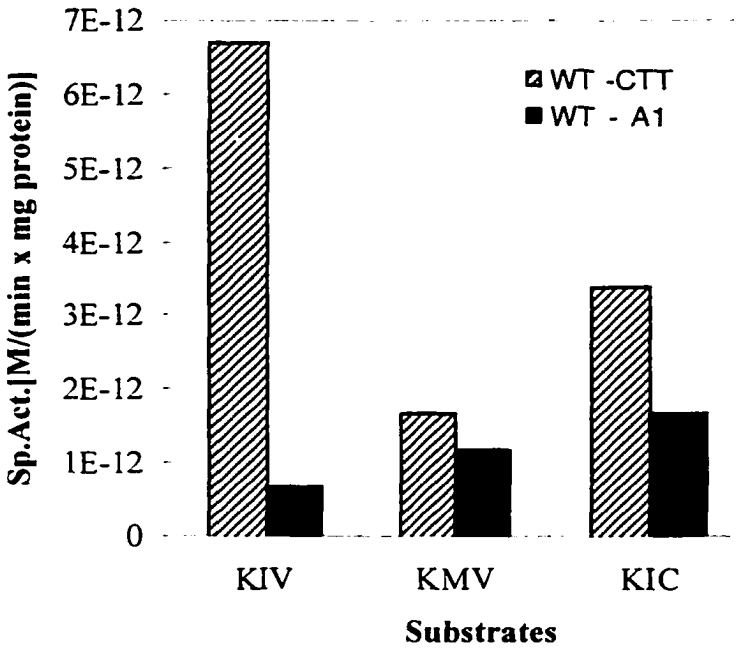


Figure 15. Branched-chain keto acid dehydrogenase activity in *Myxococcus xanthus* cells grown in A1 or CTT media.

The branched-chain keto acid dehydrogenase specific activity was determined in extracts of the wild-type *M. xanthus* strain DZF1 grown vegetatively in the complex medium CTT or in the chemically defined medium A1. The three branched-chain keto acids (α-ketoisovaleric acid (KIV),-keto-β-methyl-n-valeric acid(KMV), and α-ketoisocaproic acid(KIC) were used as substrates. Cell extracts were prepared by sonication using cells grown to a cell density of between 70 and 80 klett units. The specific enzyme activities were determined as has been described (109).

driven β-galactosidase activity and for fatty acid analysis. The changes in the relative amounts of the three most abundant fatty acids in *M. xanthus*, viz., iso-15:0, 16:0 and 16:1 w5c, were determined (Fig.16B). When cells grown in the A1 medium were transferred to the CTT medium most of the changes in fatty acid composition was found to occur during the first 10 hours of growth. There was a strong increase in *esg* expression during this interval but peak expression was not observed until about 20 hours. *Esg* expression was also strongly induced during the first 10 hours of growth in CTT (Fig.16A). An increase from 32% to 50% was recorded in the amount of iso-15:0 accompanied by a corresponding decrease in the level of 16:0 from 18% to 6%. The amount of the unsaturated fatty acid 16:0 w5c decreased only slightly. These cells exhibited a lag in growth to start with, followed by a logarithmic growth with a generation time of 4 to 5 hours (Fig.14, p.118). During the 10 to 30 hour interval, the observed increase in the iso-15:0 level was small and so also was the observed decrease in the 16:0 level (Fig.16B). The amount of *esg*-driven β-galactosidase activity increased less than 2 fold between 10 and 20 hours and declined slightly between 20 and 30 hours while the cells continuing to grow logarithmically.

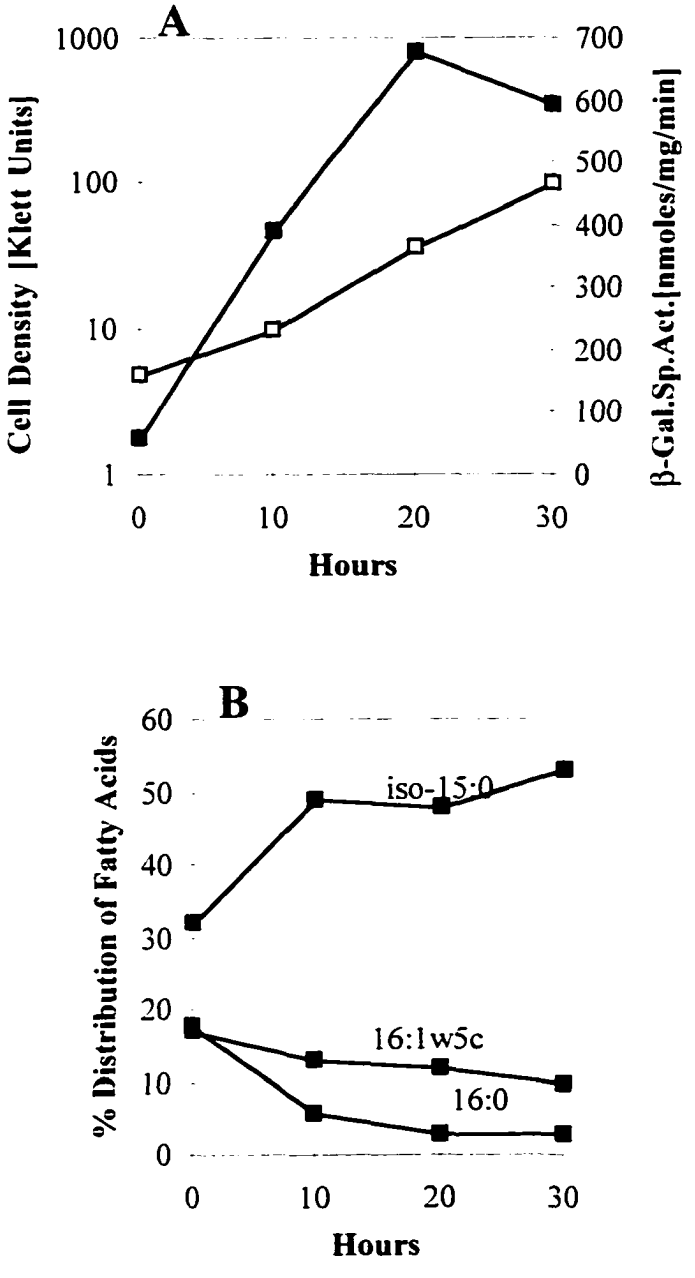


Figure 16. Time course changes in iso-15, 16:1 w5c and 16:0 in the FB306 strain of *Myxococcus xanthus* subcultured into CTT medium from A1 medium. [A] *esg* expression [■] and cell growth [□]

4.3.2. Development by A1 or CTT Grown *Myxococcus xanthus* Cells

4.3.2.1 Effect of Minimal A1 Growth Media on the Development of *Myxococcus xanthus*.

Wild-type cells of *M. xanthus* growing in A1 medium resemble developmentally defective *esg* mutant cells in that both have relatively low levels of branched-chain fatty acids. The wild-type A1 grown cells were tested to determine whether they retained the ability to form fruiting bodies. Concentrated suspensions of wild-type cells of *M. xanthus* grown in CTT or A1 media were used to initiate development. Our results show that despite a reduction in their relative amounts of the branched-chain amino acids, wild-type cells grown vegetatively in A1 medium were able to undergo normal development (Fig.17). Cells grown in A1 medium appeared to initiate development at a somewhat faster rate but this effect has not been investigated further.

4.3.3. Nutritional Regulation of *esg* Expression

Studies in *Pseudomonas putida* have shown that when BCAA are added to the growth media they induce the expression of the set of genes encoding the *Pseudomonas putida* BCKAD (71) (72). To ascertain

whether or not the concentration of branched-chain amino acids in the growth media influence *esg* locus expression we measured *esg* expression in the JD306 strain of *M. xanthus* grown vegetatively in A1 medium supplemented with branched-chain amino acids whose concentrations were varied first individually and then collectively (Table 6, p.110). In the first category of tests, the concentration of leucine was varied from 50 μg/mL to 250 μg/mL, and those of isoleucine and valine were varied from 100 μg/mL to 500 μg/mL. In the second test all 3 branched-chain amino acids in the medium were raised simultaneously from 1.5 to 5 times their normal concentrations in A1 media. No observable regulation of *esg* expression was detected in response to changes in the branched-chain amino acid concentration (data not shown).

To determine whether a component of the A1 medium acted as an inhibitor of *esg* expression, the growth of JD306 in A1 medium supplemented with 1% casitone was examined. Casitone, derived from the hydrolysis of the milk protein casein, contains many amino acids at relatively high concentrations (see appendix 1). Casitone was added into A1 medium at a concentration of 1% because CTT medium which

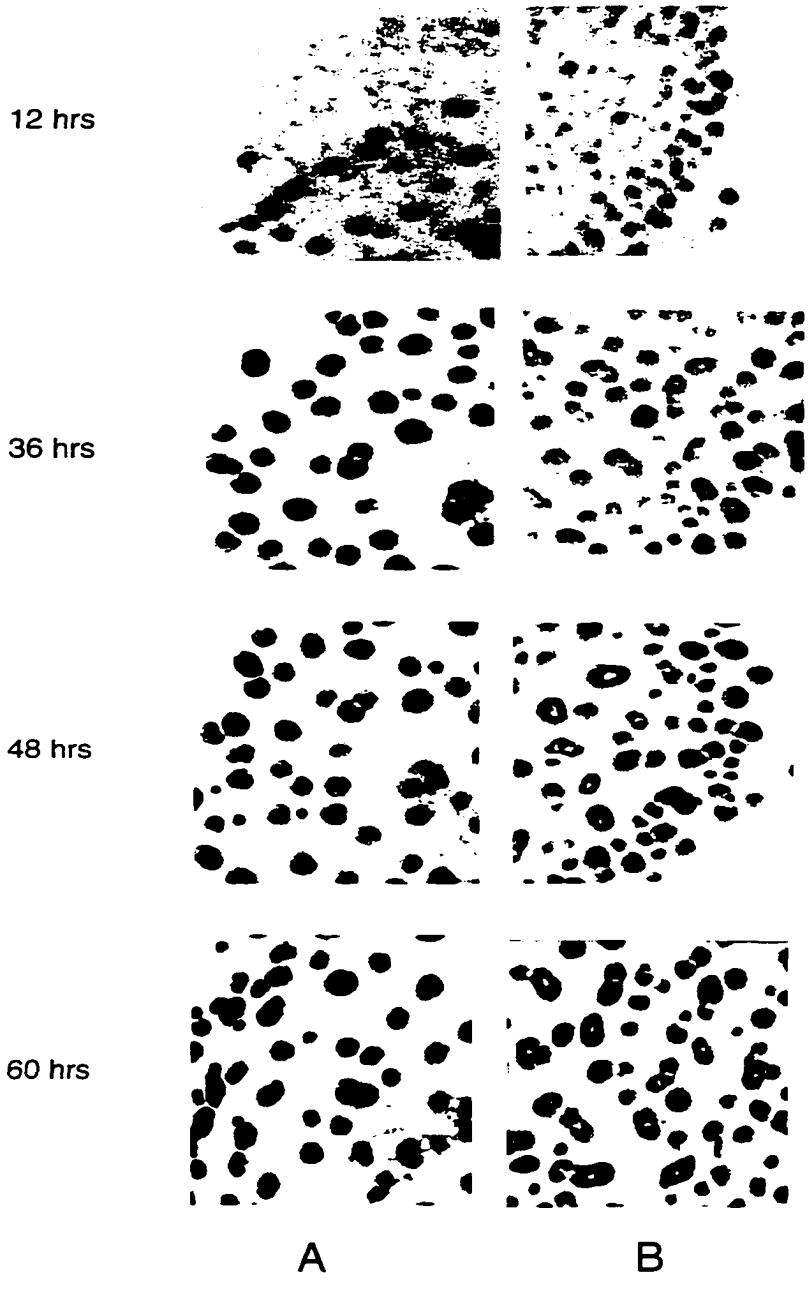
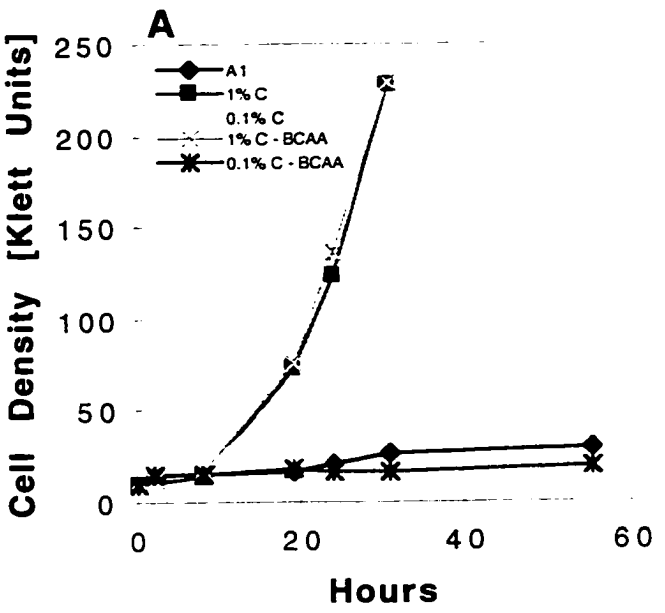


Figure 17. Development of wild-type cells grown vegetatively in the minimally defined A1 medium (A), and the nutritionally rich CTT medium (B)

stimulates *esg* expression (Fig.14 p.118),contains casitone at a 1% concentration. The results obtained show that a casitone concentration of 1% in A1 medium stimulated *esg* expression of JD306 (Fig.18 A and B). This suggests that the low nutrient content of A1 medium and not an inhibitory factor is primarily responsible for the reduction in *esg* expression. Finally, we compared the *esg* expression of JD306 growing in the following A1 media. (a) Basic A1 medium, (b) A1 medium supplemented with 1% casitone, (c) A1 media supplemented with 0.1 % casitone, (d) modified A1 medium (without the branched-chain amino acids) supplemented with 1% casitone and (e) modified A1 medium supplemented with 0.1% casitone (Fig.18 A and B). The estimated concentrations of the branched-chain amino acids in each of these instances is given in Table 8. The media (a) to (e) are all modified versions of A1. It was found that a reduction in the concentration of the amino acids contained in casein (including branched-chain amino acids) was accompanied by a reduction in *esg* expression. However, at the same time, there was a reduction in growth. At the present time, there is no conclusive evidence that *esg* expression responds directly to the branched-chain amino acids and it is unclear why complex media


A
Cell Density [Klett Units]
250
200
150
100
50
0
0
20
40
60
Hours
A1
1% C
0.1% C
1% C - BCAA
0.1% C - BCAA

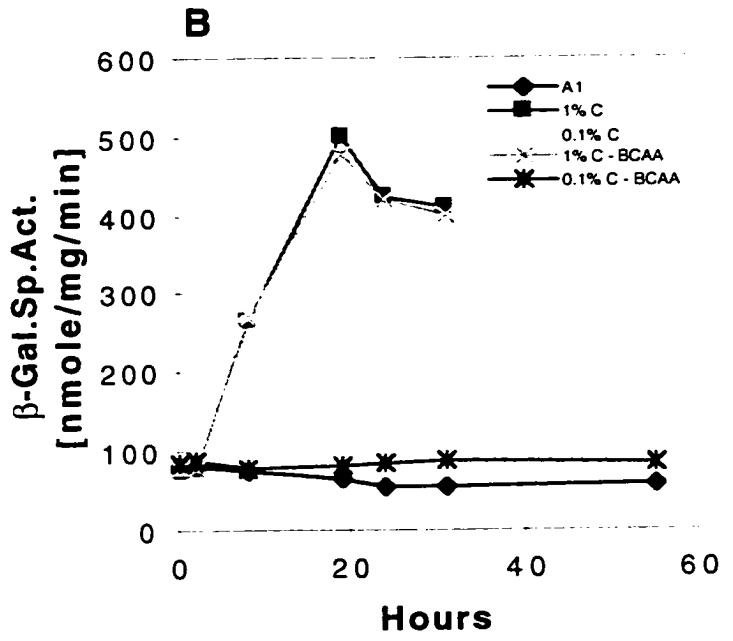

B
β-Gal.Sp.Act.
[nmole/mg/min]
600
500
400
300
200
100
0
0
20
40
60
Hours
A1
1% C
0.1% C
1% C - BCAA
0.1% C - BCAA

Figure 18. Observed effect of branched-chain amino acids on cell growth [A], and *esg* expression [B], of *Myxococcus xanthus* JD306 grown in A1 medium. FB306 was grown in CTT medium and then subcultured into the following 20 mL preparations of A1 medium. (a) Basic A1 medium (A1), (b) Basic A1 medium containing 1% casitone (1% C), (c) Basic A1 medium containing 0.1% casitone (0.1% C), (d) A1 medium without the addition of the branched-chain amino acids but supplemented with 1% casitone (1% C - BCAA), and (e) A1 medium without the addition of the branched-chain amino acids containing 0.1% casitone (0.1% C - BCAA). The cells were incubated at 30°C shaking at 200 rpm. At each time point a 1mL sample of the culture was removed in order to determine the β-galactosidase specific activity as described by Miller (68). The protein concentration was determined using the BCA Protein Assay Kit by Pierce. The growth rate of FB306 and expression of *esg* in A1 medium was the control.

containing partially hydrolyzed proteins induce high levels of *esg* expression.

4.4 Discussion

In our study, we have shown that fatty acid synthesis in the wild-type strain of *M. xanthus* was dependent on the nutritional content of the growth media. Wild-type cells grown in a nutritionally rich medium containing hydrolyzed proteins synthesized high levels of the branched-chain fatty acids. When these cells were grown in the chemically defined A1 medium they synthesized low levels of the branched-chain fatty acids but high levels of the 16 carbon straight-chain fatty acid, palmitic acid. The growth medium dependent changes in fatty acid content in the *esg* mutant was much less pronounced than that observed in the wild-type cells (Table 7 , p.115). These results suggest that the *esg* BCKAD plays a role in the growth media dependent fatty acid response in *M. xanthus.* Our studies suggest that the regulation of *esg* expression is part of the mechanism utilized by *M. xanthus* to increase the BCFA content of cells grown in rich media. We observed that the

TABLE 8. Predicted concentrations of the branched-chain amino acids in the A1 growth medium supplemented with casitone

AMINO ACID	[1]A1	A1		[2]A1-BCAA	
		1% C	0.1% C	1% C	0.1% C
Leucine	[3]50	924	137	874	87
Isoleucine	100	517	142	417	42
Valine	100	506	141	406	41

[1] A1 medium (6)
[2] A1 medium with the BCAA not included
[3] concentrations of the amino acids in the media in µg/mL

growth of *M. xanthus* cells in a complex medium resulted in a relatively high level of*esg* expression. *Esg* expression was induced in *M. xanthus* cells grown in a nutritionally rich medium but not in cells grown in a chemically defined A1 medium. This increased expression of the *esg* locus in wild-type cells grown in a nutritionally rich medium was accompanied by an increased activity of the BCKAD enzyme. These results led to the conclusion that the increased activity of the BCKAD enzyme in *M. xanthus* cells grown in nutritionally rich media is a result of an increased synthesis of the enzyme brought about by a rise in the expression of the *esg* locus. The increased activity of the BCKAD enzyme resulted in a relative increase in the percentage of branched-chain fatty acids in the bacterial membrane. The observed correlation between *esg* expression levels, BCKAD enzyme activity, and branched-chain fatty acid content suggests that the mechanism of growth media dependent fatty acid response in *M. xanthus* involves transcriptional regulation of the *esg* gene.

Most bacteria respond to at least some changes in their environment by changing the fatty acid composition of their cell membranes (84). Some of the well documented environmental factors that affect the synthesis of membrane fatty acids are nutritional

content of the growth media, growth temperature and ethanol. Studies on the effect of the growth media on the fatty acid content of *E. coli* showed that the percentage of palmitic acid increased in cells grown in glucose minimal media, whereas the percentage of the unsaturated fatty acids increased in cells grown in glucose minimal media supplemented with casamino acids or yeast extract (75). Increased growth temperatures result in an increased level of saturated fatty acids in the cell membrane while reduced growth temperatures result in an increased presence of unsaturated fatty acids in the cell membrane (29) (75). This inverse relationship between growth temperature and unsaturated fatty acid composition serves to maintain membrane fluidity (29). Short chain alcohol's such as ethanol cause a decrease in the amount of saturated fatty acids analogous to a drop in growth temperature (4) (8) (9). On the other hand, long chain alcohols induce changes analogous to a rise in growth temperature (4) (8) (9). Ethanol-induced changes in fatty acid composition in vivo arise primarily from the inhibition of saturated fatty acid synthesis (9). Among the environmental factors influencing the fatty acid content of bacterial cell membranes, the bacterial fatty acid response to temperature changes is the best understood.

Most of the studies on the mechanism of temperature regulation of fatty acid content were conducted with *E. coli.* These studies led to a proposal that the growth of *E.coli* at reduced temperatures activates the enzyme 3-ketoacyl-ACP synthase (14) (30). This enzyme catalyzes the conversion of palmitoleic acid to cis-vaccenic acid which in turn regulates the fatty acid composition by producing more cis-vaccenoyl ACP for incorporation into phospholipids (14) (30). This temperature adaptation in *E.coli* is detected within 30 seconds following a temperature shift from 42°C to 24°C (31). This response is not affected by the addition of either chloramphenicol or rifampin into the growth medium (31). Since these antibiotics inhibit protein and RNA synthesis (31), it has been concluded that the thermal response in *E. coli* does not involve the induction of the synthase enzyme or any other changes in gene expression. This enzyme has been shown to be present at all growth temperatures, but is active only when cells are grown at low temperatures (14).

Our results on the growth medium dependent fatty acid response in *M. xanthus* provide compelling evidence of a second mechanism that is utilized by bacteria to alter the proportion of their membrane fatty acids in response to changing environmental conditions. Unlike the

synthase enzyme activity of *E.coli*, which most probably involves temperature dependent changes in enzyme activity, the *esg* dependent BCKAD enzyme activity of *M. xanthus* stems from its increased synthesis brought about by the transcriptional regulation of the *esg* locus.

Branched-chain amino acids present in the growth media are thought to be inducers of BCKAD expression in *Pseudomonas putida* (71) (72). In a search for evidence of a similar role for the branched-chain amino acids in the regulation of *esg* expression, we observed that casitone added to the A1 medium at a concentrations of 1% induced *esg* expression to similar levels irrespective of whether the A1 medium contained the branched-chain amino acids or not (Fig. 18 B, p.127). On the other hand, *esg* expression was reduced in a 0.1% casitone media made up in A1 lacking the branched-chain amino acids. Based on the concentrations of the branched-chain amino acids in the media it could be proposed that branched-chain amino acids greater than 137 µg/mL may have the ability to induce the expression of the *esg* locus (Fig.18B , p.127 and Table 8 , p.130). Although these results strongly suggest that the *esg* gene could be regulated by the branched-chain amino acids, we hesitate to claim any direct role of the branched-chain amino

acids in *esg* regulation since all attempts to observe regulation of *esg* expression by altering the concentrations of the branched-chain amino acids in A1 medium were unsuccessful. Casitone is a complex medium (see appendix 1) and we conclude that it is not simply the changes in the concentration of the branched-chain amino acids alone that may be responsible for the changes of *esg* expression but perhaps the cumulative change in concentration of the branched-chain amino acids and / or any one of the other compounds contained in casitone.

In general, *esg* expression was found to be high in media which allowed a rapid rate of growth (4 to 6 hour generation times) and low in defined media which promoted slower growth (>20 hour generation times). This relationship suggest that expression of the *esg* locus responds more directly to the growth rate of cells than to the levels of the BCAA in the growth medium. However, we have not been able to regulate growth rate by controlling the nutrient content of the medium without also altering the branched-chain amino acid content.

The *esg* dependent BCKAD seems to function as one component in the nutrition dependent fatty acid response that is responsible for changes in the branched-chain fatty acid content of the membrane. We observed that straight chain fatty acids were increased in the *esg*

mutant. These results indicate the existence of a compensating system for the production of straight chain fatty acids as the rate of branched-chain fatty acid production declines. The branched-chain fatty acids and the unsaturated fatty acids are alike in their effects on membrane fluidity (26). It is therefore highly likely that the inverse relationship that has been observed between branched-chain fatty acids and straight-chain fatty acids in their responses to nutrition and the observed inverse response between saturated and unsaturated fatty acids in their reactions to temperature changes may serve to supply the optimal mixture of fatty acids for cell membrane synthesis to preserve membrane integrity and function in the face of changing environmental conditions.

It does appear from our results, that the expression of the *esg* locus is dependent on the growth rate of *M. xanthus*. There is a correlation between growth rate and *esg* expression. We observed that a decreased *esg* expression always accompanied a decrease in growth rate and vice versa. This observation raises the possibility that growth rate may have a direct bearing on the regulation of the *esg* locus.

CHAPTER 5

Summary and Future Studies

A model for the production and transmission of the *M. xanthus* E-signal has been proposed based on studies conducted over a number of years in this and several other laboratories. Studies of an *esg* mutant, a mutant unable to form fruiting bodies and apparently unable to produce the E-signal, was central to the construction of this model. According to the model, a BCKAD encoded in part by the *esg* locus is involved in the production of the branched-chain fatty acids that are incorporated into membrane phospholipids during vegetative growth. Following the initiation of development, these branched-chain fatty acids are released by the action of a developmentally regulated phospholipase. The free fatty acids can then be transmitted between cells as the E-signal. The E-signal acts, at least in part, by activating the expression of developmental genes. In the studies described in this dissertation, the roles of the *esg* locus in the regulation of developmental gene expression and in fatty acid production were investigated. These investigations have led to new information that will

help to clarify our understanding of E-signaling and its effects on developmental gene expression in *M. xanthus*.

The pattern of developmental gene expression in the *esg* mutant is displayed in Table 2 (see p.54) and compared with the known patterns of expression of the same set of genes in the other 4 signal mutants. This table exhibits points of difference in respect of patterns of developmental gene regulation and signal initiation (see Fig.4 p.51). In the comparison of the *esg* mutant to the *asg*, *bsg* and *csg* mutants, these differences are seen to be substantial. The patterns of developmental gene expression of *esg* and *dsg* mutants, on the other hand, turn out to be closely similar. A somewhat puzzling reduction in the expression of Ω4457 at 0 hours was observed in the case of the *esg* mutant; the corresponding expression in the *dsg* mutant was not available. This similarity between the patterns of developmental gene expression of *esg* and *dsg* mutants raises the possibility that the two mutants are defective in the production of the same developmental signal. However, there are distinct differences between the phenotypes of the two mutants that argue against this possibility. Perhaps most importantly, the *dsg* mutant extracellularly complements the *esg* mutant suggesting that the *dsg* mutant can produce the E-signal and

transmit the signal to the *esg* mutant (18). The *dsg* and *esg* mutants also have different developmental phenotypes (eg. the fruiting bodies differ in appearance) which indicates that they are blocked at slightly different stages in development. Finally, while the primary defect in the *esg* mutant appears to be related to the production of the branched-chain fatty acids or related lipid species, there is evidence that the *dsg* mutant produces the branched-chain fatty acids normally. Despite these observations, it is clear that the *dsg* and *esg* mutants are blocked at similar stages in development and we are just beginning to learn about the defects in these two mutants. As more is learned about the D- and E-signaling systems it will be important to consider the relationship between the two systems.

Labeling studies were employed to investigate the role of the *esg* locus in lipid biosynthesis. The branched-chain amino acids were used to label lipids during the vegetative growth of *M. xanthus*. These studies demonstrated the use of branched-chain amino acids for branched-chain fatty acids synthesis, with leucine being the most effective in this regard. This result is consistent with the observation that about 60% of the fatty acids of *M. xanthus* are branched-chain fatty acids of which about 80% are expected to be derived from

leucine. The same experiment conducted with *esg* mutant cells grown showed the same trend in the relative levels of incorporation of branched-chain amino acids into the lipid fraction. Significantly less label was incorporated into lipids of the *esg* mutant. This result demonstrated that the of the *esg* BCKAD is part of the major pathway for the synthesis of branched-chain fatty acids from branched-chain amino acids in *M. xanthus*.

Among the branched-chain fatty acids synthesized during the vegetative growth of wild-type cells of *M. xanthus*, the branched-chain fatty acid iso-15:0 has been found to be the most abundant. Chapter 3 of this dissertation presents our initial attempts to test the importance of iso:15:0, in E-signaling. In the first stage of these studies, we sought to determine if free iso-15:0 (not associated with phospholipid), could be detected in vegetative and developmental *M.xanthus* cells. Fluorography of the leucine-labeled FFA of wild-type cells revealed the presence of strongly labeled lipid species but very little labeled iso-15:0. However,GC analysis of the same fraction, subjected to base hydrolysis prior to the analysis, did detect iso-15:0. Base hydrolysis of the leucine-labeled FFA fraction resulted in the appearance of a prominent labeled band that co-migrated with iso-15:0.

These results taken together argue that most of the iso-15:0, that is found in the free fatty acid fraction and produced by the *esg* pathway, has been incorporated into a base labile lipid compound(s).

We also developed a bioassay to test fatty acids and lipid fractions for their ability to rescue development in an *esg* mutant (E-signaling activity). The outcome of the developmental rescue assay demonstrated a capability of pure iso-15:0 to rescue development of the *esg* mutant to an extent comparable to the FFA fraction purified from developing wild-type cells. However, the question as to whether or not iso-15:0 participates in this process directly or as part of another lipid species has not been resolved. To begin to address this question, base hydrolyzed wild-type FFA fractions containing free iso-15:0 were tested for developmental rescue activity against comparable non-hydrolyzed material already known to contain relatively little free iso-15:0. The somewhat surprising outcome of this experiment was that the nonhydrolyzed sample displayed greater activity in the developmental rescue assay. These results are consistent with the possibility that a base labile lipid species containing iso-15:0 or another of the branched-chain fatty acids is important in E-signaling. It should be possible to use our developmental rescue assay

in the purification and chemical characterization of cellular lipids with E-signal activity.

In chapter 4 we demonstrated that the fatty acid content of *M. xanthus* varies depending on the growth medium and that this alteration in fatty acid content is dependent on the *esg* locus. This study began with the examination of the fatty acid profiles of wild-type and *esg* mutant cells, each grown in A1 medium (low in nutritional content) and CTT medium (rich in nutritional content). Different fatty acid profiles were observed with the wild-type grown cells in the 2 media while the media had little effect on the fatty acid content in *esg* cells. Transcriptional regulation of the *esg* locus was found to be involved in the *esg*-dependent alteration of the fatty acid content. The transcription of the *esg* locus increased in CTT medium where cells have high branched-chain fatty acid content and was reduced in A1 medium where the branched-chain fatty acid content is low. The level of BCKAD activity and branched-chain fatty acid synthesis were correlated with the expression of the *esg* locus. Since the *esg* locus encodes only 2 of the 4 BCKAD protein components these results suggest that the other genes involved, encoding the E2 and E3 subunits,may be regulated similarly. These genes have not yet been identified in *M. xanthus*.

The nutritional factor controlling the levels of *esg* expression have not been identified. The obvious nutritional factor which would be expected to control the level of *esg* expression is the concentration of the branched-chain amino acid compounds used to produce the keto acid substrates for the BCKAD. However, we were unable to demonstrate changes in *esg* expression in response to changing concentrations of the branched-chain amino acids.

These studies have tested some of the predictions made in our earlier model for E-signaling (17) (108) and led us to propose some alterations in the model. Based on these new experiments a revised model is presented in figure 19. The evidence gathered in this study further supports the importance of the *esg* pathway in branched-chain fatty acid synthesis in *M. xanthus*. We have shown that in addition to its role in E-signaling this pathway plays an important role in the growth medium dependent fatty acid response. In the case of the latter, we have shown that nutritional changes of the growth medium have an effect on *esg* expression and levels of BCKAD enzyme activity. This change in BCKAD activity may be regulated by the nutrient composition of the growth medium or in response to the growth rate of cells. The regulation of the BCKAD enzyme activity, in turn, has a direct effect on

the branched-chain fatty acids present in the membranes of cells growing under the stipulated growth condition.

Regarding the role of the esg locus in development, it was proposed from earlier studies that the branched-chain fatty acids could be released from phospholipids to act as E-signal molecules (17)(108). It was concluded from these studies that the developmental defect observed in the *esg* mutant was a result of a reduced concentration of branched-chain fatty acids (signal molecules). In this study however, we observed the cells grown in A1 medium, with reduced branched-chain fatty acid content remain capable of undergoing development indicating that reduced branched-chain fatty acid content of the cells is not sufficient to prevent development. It is very likely therefore, that the *esg* pathway may be required for the production or compartmentalization of lipids that required for the regulation of development in *M. xanthus*. Our studies have suggested that not all of the branched-chain fatty acids synthesized during the vegetative growth are incorporated into phospholipids. We demonstrated that some of the branched-chain fatty acids are found incorporated into complex lipid molecules.

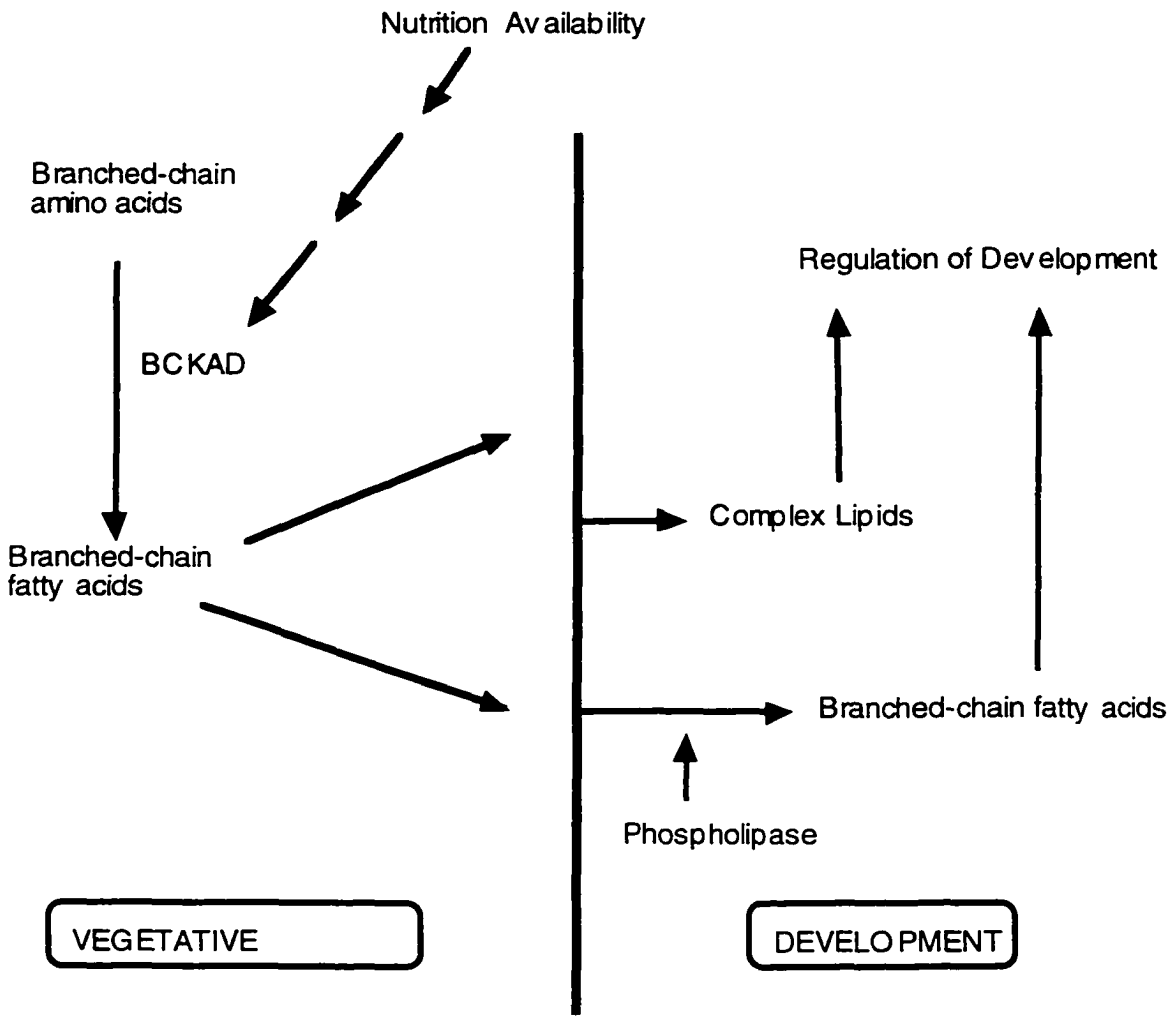


Figure 19. Updated model of the E-signaling system

A lipid fraction from wild-type cells (FFA) was able to restore development to an *esg* mutated cell (*esg* activity). This fraction contained no detectable free fatty acid derived from leucine but substantial levels of the base labile lipids that can partially restore the capability to aggregate in the *esg* mutant (E-signaling activity). In fact, the major portion of the labeled fatty acid species detected in the free fatty acid fraction (but not cells in general) were those found to be incorporated into these lipid molecules. Although we failed to detect free fatty acids produced by the *esg* pathway from exogenously supplied amino acids, it is still possible that low levels of endogenously produced fatty acids have sufficient activity to serve as signals.

In chapter 2, it was demonstrated that developmental gene expression is blocked at the 3 to 5 hours stage of development suggesting that the E-signal is produced or reaches a sufficiently high concentration beginning at that time. We have failed to identify any *esg*-derived lipids (potential E-signal compounds) that were produced in developing cells at that time. While the explanation for this result may be that the methods employed were not sufficiently sensitive to detect the presence of the E-signal. There is another explanation that should be considered. A possible explanation of this phenomenon is that the

lipids are synthesized in the inner membrane of the cell during vegetative growth and need to be transported across the periplamic space to the outer membrane in order to function as the E-signal during development. To investigate this possibility the procedure developed by Orndorff and Dworkin (80a) could be used to separate the inner and outer membrane of *M.xanthus* to determine the distribution of leucine derived lipids during vegetative growth and early development. This type of study would indicate if there are lipids which change location during the time that the E-signal is transmitted.

A detailed understanding of how the lipid molecules of the E-signaling system regulate gene expression is another promising research objective. The outcome of such a study may well provide valuable evidence relating to the role of lipids in transcriptional regulation. This research confirms the previous view that lipids are not mere structural components of the cell. In fact they have been shown to play a more prominent part in the biological activity of the cell than was previously surmised.

APPENDIX 1

Bacto CASITONE

ANALYTE	RESULTS	ANALYTE	RESULTS
Physical Characteristics		**Inorganics(%)**	
Ash (%)	7.0	Calcium	0.010
Clarity, 1% Soln(NTU)	0.6	Chloride	0.110
Filterability (g/cm^2)	1.7	Cobalt	<0.001
Loss of Drying (%)	3.7	Copper	<0.001
pH, 1% Soln	7.2	Iron	0.003
		Lead	<0.001
Carbohydrate (%)		Magnesium	0.019
Total	0.2	Manganese	<0.001
		Phosphate	2.604
Nitrogen Content (%)		Sodium	3.073
Total Nitrogen	13.3	Sulfate	0.339
Amino Nitrogen	4.7	Sulfur	0.676
AT/TN (%)	35.3	Tin	<0.001
		Zinc	0.004
Amino Acids (%)			
Alanine	3.01	**Vitamins(μg/g)**	
Arginine	0.70	Biotin	0.2
Aspartic Acid	6.61	Choline	550.0
Cystine	0.02	Cyanocobalamin	<0.1
Glutamic Acid	20.03	Folic Acid	0.8
Glycine	1.97	Inositol	980.0
Histidine	2.17	Nicotinic Acid	20.3
Isoleucine	4.16	PABA	15.9
Leucine	8.74	Pantothenic Acid	7.7
Lysine	13.62	Pyridoxine	1.3
Methionine	1.71	Riboflavin	0.4
Phenylalanine	4.02	Thiamine	<0.1
Proline	8.57	Thymidine	342.9
Serine	4.82		
Threonine	3.74	**Biological Testing (CFU/g)**	
Tryptophan	0.14	Coliform	negative
Tyrosine	2.09	Salmonella	negative
Valine	4.06	Spore Count	300
		Standard Plate Count	1850
		Thermophile Count	100

The above values are based on 100 grams of Casitone.

References

1. **Arnold, J. W., and L. J. Shimkets.** 1988. Cell surface properties correlated with cohesion in *Myxococcus xanthus*. J. Bacteriol. **170:**5771-5777.

2. **Avery, L., and D. Kaiser.** 1983. In situ transposon replacement and isolation of a spontaneous tandem genetic duplication. Mol. Gen. Genet. **191:**99 -109.

3. **Behmlander, R. M., and M. Dworkin.** 1994. Biochemical and structural analysis of the extracellular matrix fibrils of *Myxococcus xanthus*. J. Bacteriol. **176:**6295-6303.

4. **Berger, B., C.E.Carty, and L.O. Ingram**. 1980. Alcohol-induced changes in the phospholipid molecular species of *Escherichia coli*. J.Bacteriol. **142**:1040-1044.

5. **Blackhart, B. D., and D. R. Zusman.** 1985. Cloning and complementation analysis of the "Frizzy" genes of *Myxococcus xanthus*. Mol. Gen. Genet. **198:**243-245.

6. **Bretscher, A. P., and D. Kaiser.** 1978. Nutrition of *Myxococcus xanthus*, a fruiting Myxobacterium. J. Bacteriol. **133:**763-768.

7. **Burns, G., T. Brown, K. Hatter, J.M. Idriss and J.R.Sokatch** 1988. Similarity of the E1 subunits of branched-chain-oxoacid dehydrogenase from *Pseudomkonas putida* t o the corresponding subunits of mammalian branched-chain-oxoacid and pyruvate dehydrogenase. Eur. J. Biochem. **176:**311-317.

8. **Buttke, T.M. and L.O. Ingram.** 1980. Ethanol-induced changes in lipid composition of *Escherichia coli* : Inhibition of saturated fatty acid synthesis in vitro. Arch. Biochem. Biophy. **203**:565-571

9. **Buttke,T.M. and L.O.Ingram.** 1978. Mechanism of ethanol-induced changes in lipid composition of *Escherichia coli* : Inhibition of saturated fatty acid synthesis in vivo. Biochem. **17**:637-644

10. **Campos, J. M., J. Geisselsoder, and D. Zusman.** 1978. Isolation of bacteriophage MX4, a generalized transducing phage for *Myxococcus xanthus*. J. Mol. Biol. **119:**167-178.

11. **Cheng, Y., and D. Kaiser.** 1989. *dsg*, a gene required for cell-cell interaction early in *Myxococcus* development. J. Bacteriol. **171:**3719-3726.

12. **Cheng, Y., and D. Kaiser.** 1989. *dsg*, a gene required for *Myxococcus* development, is necessary for cell viability. J. Bacteriol. **171:**3727-3731.

13. **Cheng, Y. L., L. V. Kalman, and D. Kaiser.** 1994. The *dsg* gene of *Myxococcus xanthus* encodes a protein similar to translation initiating factor IF3. J. Bacteriol. **176:**1427-1433.

14. **Cronan, J.E. and C.O. Rock.** 1987. Biosynthesis of membrane lipids, vol.1 American Society for Microbiology, Washington D.C.

15. **Davis, M. D., J. Mayor, and L. Plamann.** 1995. A missense mutation in *rpo* D results in an A-signalling defect in *Myxococcus xanthus*. Mol. Microbiol. **18:**943-952.

16. **Denoya, C. D., R. W.Fedechko, E.W.Hafner, H.A.I.cArthur, M.,R.Morgenstern, D.D.Skinner, K.Stutzman-Engwall, R.G.Wax**

and W.C.Wernau 1995. A second branched-chain α-keto acid dehydrohenase gene cluster (*bdkFGH*) from *Streptomyces avermitilis* : Its relationship to avermectin biosynthesis and the construction of a *bkdF* mutant suitable for the production of novel antiparasitic avermectins. J. Bacteriol. **177:**3504-3511.

17. **Downard, J., and D. Toal.** 1995. Branched-chain fatty acids: the case for a novel form of cell-cell signalling during *Myxococcus xanthus* development. Mol. Microbiol. **16:**171-175.

18. **Downard, J., S. V. Ramaswamy, and K.-S. Kil.** 1993. Identification of *esg* , a genetic locus involved in cell-cell signaling during *Myxococcus xanthus* development. J. Bacteriol. **175:**7762-7770.

19. **Downard, J. S., and D. R. Zusman.** 1985. Differential expression of protein S genes during *Myxococcus xanthus* development. J. Bacteriol. **161:**1146-1155.

20. **Downard, J. S., D. Kupfer, and D. R. Zusman.** 1984. Gene expression during development of *Myxococcus xanthus* : analysis of the genes for protein S. J. Mol. Biol. **175:**469-492.

21. **Dworkin, M.** 1996. Recent advances in the social and developmental biology of Myxobacteria. Microbiol. Rev. **60:**70 - 102.

22. **Dworkin, M.** 1985. Developmental biology of the bacteria. The Benjamin / Cummings Publishing Company, Inc., Reading, Massachusetts.

23. **Dworkin, M.** 1983. Cell surfaces and appendages. American Society for Microbiology, Washington D.C.

24. **Dworkin, M., and D. Eide.** 1983. *Myxococcus xanthus* does not respond chemotactically to moderate concentration gradients. J. Bacteriol. **154:**437-442.

25. **Dworkin, M.** 1973. Cell-cell interaction in the Myxobacteria. Symp. Soc. Gen. Microbiol. **23:**125-147.

26. **Dworkin, M.** 1963. Nutritional regulation of morphogenesis in *Myxococcus xanthus*. J. Bacteriol. **86:**67-72.

27. **Dworkin, M.** 1962. Nutritional requirements for vegetative growth of *Myxococcus xanthus*. J.Bacteriol. **84:**250-257.

28. **Fulco, A.J.** 1983. Fatty acid metabolism in bacteria. Prog.Lipid.Res. **22:**133-160.

29. **Fulco, A.J.** 1972. The biosynthesis of unsaturated fatty acids by bacilli : Temperature-mediated control mechanisms. J. Biol. Chem. **247**:3511-3519

30. **Garvin, J.L. , A.L. Klages, and J.E. Cronan Jr.** 1980. β-ketoacyl-acyl carrier protein synthase II of *Escherichia coli*. J.Biol. Chem. **255**:3263-3265

31. **Garvin, J.L. and J.E. Cronan Jr.** 1980. Thermal modulation of fatty acid synthesis in *Escherichia coli* does not involve de novo enzyme synthesis. J.Bacteriol. **141**:1457-1459.

32. **Gelvan, I., M. Varon, and E. Rosenberg.** 1987. Cell-density-dependent killing of *Myxococcus xanthus* by autoside AMV. J.Bacteriol. **169:**844-848.

33. **Gill, R. E., M. Karlok, and D.Benton.** 1993. *Myxococcus xanthus* encodes and ATP-dependent protease which is required for developmental gene transcription and intercellular signalling. J. Bacteriol. **175:**4538-4544.

34. **Gill, R. E., and C. Bornemann.** 1988. Identification and characterization of the *Myxococcus xanthus bsgA* gene product. J. Bacteriol. **170:**5289-5297.

35. **Gill, R. E., M. G. Cull, and S. Fly.** 1988. Genetic identification and cloning of a gene required for developmental cell interactions in *Myxococcus xanthus*. J. Bacteriol. **170:**5279-5288.

36. **Gill, R. E., and M. G. Cull.** 1986. Control of development gene expression by cell-to-cell interactions in *Myxococcus xanthus*. J. Bacteriol. **168:**341-347.

37. **Hagen, T. J., and L. J. Shimkets.** 1990. Nucleotide sequence and transcriptional products of the *csg* locus of *Myxococcus xanthus*. J. Bacteriol. **172:**15-23.

38. **Hagen, D. C., A. P. Bretscher, and D. Kaiser.** 1978. Synergism between morphogenetic mutants of *Myxococcus xanthus*. Dev. Biol. **64:**284-296.

39. **Hamilton, J. G. and K. Comai.** 1988. Rapid seperation of neutral lipids, free fatty acids and polar lipids using prepacked silica Sep-Pak Columns. Lipids. **23:**1146-1149.

40. **Hemphill, H. E., and S. A. Zahler.** 1968. Nutritional induction and suppression of fruiting in *Myxococcus xanthus* FBa. J. Bacteriol. **95:**1018-1023.

41.**Ho, J., and H. D. McCurdy.** 1979. Demonstration of positive chemotaxis to cyclic GMP and 5' AMP in *Myxococcus xanthus* by means of a simple apparatus for generating practically stable concentration gradients. Canad. J. Microbiol. **25:**1214-1218.

42. **Hodgkin, J., and D. Kaiser.** 1979. Genetics of gliding motility in *Myxococcus xanthus* : two gene systems control movement. Mol. Gen. Genet. **171:**177-191.

43. **Inouye, M., S. Inouye, and D. R. Zusman.** 1979. Biosynthesis and self-assembly of protein S,a development-specific protein of *Myxococcus xanthus*. Proc.Natl.Acad.Sci. USA. **76:**209-213.

44. **Jorgensen, R. A., S. J. Rothstein, and W. S. Rezeikoff.** 1979. A restriction enzyme cleavage map of Tn5 and location of a region encoding neomycin resistance. Mol. Gen. Genet. **177:**65-72.

45. **Kaiser, D. and L. Kroos.** 1993. Intercellular signaling. American Society for Microbiology, Washington D. C.

46. **Kaiser, D.** 1986. Control of multicellular development: *Dictyostelium* and *Myxococcus*. Ann.Rev.Genet. **20:**539-566.

47. **Kaiser, D.** 1979. Social gliding is correlated with the presence of pili in *Myxococcus xanthus*. Proc. Natl. Acad. Sci. USA. **76:**5952-5956.

48. **Kalman, L. V., Y. L. Cheng, and D. Kaiser.** 1994. The *Myxococcus xanthus dsg* gene product performs functions of translation initiation factor IF3 in vivo. J. Bacteriol. **176:**1434-1442.

49. **Kaneda, T.** 1991. Iso- and anteiso-fatty acids in bacteria: biosynthesis, function, and taxonomic significance. Microbiol. Rev.**55:**288-302.

50. **Kaneda, T.** 1977. Fatty acids of the genus *Bacillus*: an example of branched-chain preference. Bacteriol. Rev. **41:**391-418.

51. **Kaneda, T.** 1966. Biosynthesis of branched-chain fatty acids IV. Factors affecting relative abundance of fatty acids produced by Bacillus subtilis. Can. J. Microbiol. **12:**501-514.

52. **Kates, M.** 1986. Techniques of lipidology: isolation, analysis and identification of lipids, 2nd ed. Elievier.

53. **Kil, K.-S., G. L. Brown, and J. S. Downard.** 1990. A segment of *Myxococcus xanthus ops* DNA functions as an upstream activation site for *tps* gene transcription. J.Bacteriol. **172:**3081-3088.

54. **Kim, S. K., and D. Kaiser.** 1991. C-factor has distinct aggregation and sporulation thresholds during *Myxococcus* development. J. Bacteriol. **173:**1722-1728.

55. **Kim, S. K., and D. Kaiser.** 1990. Cell alignment required in differentiation of *Myxococcus xanthus*. Science. **249:**926-928.

56. **Kim, S. K., and D. Kaiser.** 1990. Purification and properties of *Myxococcus xanthus* C-factor, an intercellular signaling protein. Proc. Natl. Acad. Sci. USA. **87:**3635-3639.

57. **Kroos, L., and D. Kaiser.** 1987. Expression of many developmentally regulated genes in *Myxococcus* depends on a sequence of cell interactions. Genes and Dev. **1:**840-854.

58. **Kroos, L., A. Kuspa, and D. Kaiser.** 1986. A global analysis of developmentally regulated genes in *Myxococcus xanthus*. Dev. Biol. **117:**252-266.

59. **Kroos, L., and D. Kaiser.** 1984. Construction of Tn5 *Lac*, a transposon that fuses *lacZ* expression to exogenous promoters, and its introduction into *Myxococcus xanthus*. Proc. Natl. Acad. Sci. USA. **81**:5816-5820.

60. **Kuner, J. M., and D. Kaiser.** 1982. Fruiting body morphogenesis in submerged cultures of *Myxococcus xanthus*. J. Bacteriol. **151**:458-461.

61. **Kuner, J. M., and D. Kaiser.** 1981. Introduction of transposon Tn5 into *Myxococcus* for analysis of developmental and other nonselectable mutants. Proc. Natl. Acad. Sci. USA. **78**:425-429.

62. **Kuspa, A., L. Plamann, and D. Kaiser.** 1992. A-signalling and the cell density requirement for *Myxococcus xanthus* development. J. Bacteriol. **174**:7360-7369.

63. **Kuspa, A., and D. K. L. Plamann.** 1992. Identification of heat-stable A-factor from *Myxococcus xanthus*. J. Bacteriol. **174**:3319-3326.

64. **Kuspa, A., and D. Kaiser.** 1989. Genes required for developmental signalling in *Myxococcus xanthus* : Three asg Loci. J. Bacteriol. **171**:2762-2772.

65. **Kuspa, A., L. Kroos, and D. Kaiser.** 1986. Intercellular signaling is required for developmental gene expression in *Myxococcus xanthus*. Dev. Biol. **117**:267-276.

66. **Lee, B., K. Lee, J. Mendez, and L. J. Shimkets.** 1995. A tactile sensory system of *Myxococcus xanthus* involves an extracellular $NAD(P)^+$ - containing protein. Genes Dev. **9**:2964-2973.

67. **Lee, K., and L. J. Shimkets.** 1996. Suppression of a signaling defect during *Myxococcus xanthus* development. J. Bacteriol. **178:**977-984.

68. **Lee, K., and L. J. Shimkets.** 1994. Cloning and characterization of the *socA* locus which restores development of *Myxococcus xanthus* C-signaling mutants. J. Bacteriol. **176:**2200-2209.

69. **Li, Y., and L. Plamann.** 1996. Purification an in-vitro phosphorylation of *Myxococcus xanthus asg* A protein. J. Bacteriol. **178:**289-292.

70. **Lopez, J. M., C. L. Marks., and E. Freese.** 1979. The decrease of guanine nucleotides initiates sporulation of *Bacillus subtilis*. Biochem. Biophys. Acta. **587:**238-252.

71. **Madhusudhan, K.T. , N. Huang and J.R. Sokatch.** 1995. Characterization of Bkdr-DNA binding in the expression of the *bkd* operon of *Pseudomonas putida*. J. Bacteriol. **177**:636-641.

72. **Madhusudhan, K.T. , D. Lorenz and J.R. Sokatch.** 1993. The *bkdR* gene of *Pseudomonas putida*. is required for expression of the bkd operon and encodes a protein related to Lrp of *Escherichia coli* . J.Bacteriol. **175**:3934-3940.

73. **Manoil, C., and D. Kaiser.** 1980. Accumulation of guanosine tetraphosphate and guanosine pentaphosphate in *Myxoxoccus xanthus* during starvation and myxospore formation. J. Bacteriol. **141:**279-304.

74. **Manoil, C., and D. Kaiser.** 1980. Guanosine pentaphosphate and guanosine tetraphosphate accumulation and induction of *Myxococcus xanthus* fruiting body development. J. Bacteriol. **141:**305-315.

75. **Marr, A.G. and J.L.Ingram**. 1962. Effect of temperature on the composition of fatty acids in *Escherichia coli* J. Bacteriol. **84**:1260-1267.

76. **Mayo, K. A., and D. Kaiser.** 1989. *asg* B, a gene required early for developmental signalling, aggregation, and sporulation of *Myxococcus xanthus* . Mol. Gen. Genet. **218:**409-418.

77. **Miller, J. H.** 1972. Experiments in Molecular Genetics. Cold Spring Harbor Laboratory.

78. **Mueller, C. , and M. Dworkin.** 1991. Effect of glucosamine on lysis, glycerol formation and sporulation in *Myxococcus xanthus.* J.Bacteriol. **173:**7164-7175.

79. **Namba, Y., K. Yoshizawa, A.Ejima , T. Hayashi and T. Kaneda.** 1969. Coenzyme A- and nicotinamide adenine dinucleotide-dependent branched-chain α-keto acid dehydrogenase. J.Biol.Chem. **244:**4437-4447.

80. **O'Connor, K. A., and D. R. Zusman.** 1989. Patterns of cellular interactions during fruiting body formation in *Myxococcus xanthus.* J. Bacteriol. **171:**6013-6024.

80(a). **Orndorff , P.E. and M. Dworkin.** 1980 Separation and properties of the cytoplasmic and outer membranes of vegetative cells of *Myxococcus xanthus.* J. Bacteriol. **141**:914-947

81. **Plamann, L., Y. Li, B. Cantwell, and J. Mayor.** 1995. The *Myxococcus xanthus asgA* gene encodes a novel signal transduction protein required for multicellular development. J. Bacteriol. **177:**2014-2020.

82. **Plamann, L., J. M. Davis, B. Cantwell, and J. Mayor.** 1994. Evidence that *asg* B encodes a DNA-binding protein essential for growth and development of *Myxococcus xanthus*. J. Bacteriol. **176:**2013-2020.

83. **Plamann, L., A. Kuspa, and D. Kaiser.** 1992. Proteins that rescue A-signal defective mutants of *Myxococcus xanthus*. J. bacteriol. **174:**3311-3318.

84. **Rose, A. H.** 1989. Influence of the environment on microbial lipid composition, vol. 2. Academic Press, London.

85. **Rosenberg, E.** 1984. Myxobacteria. Development and cell interactions. Springer-Verlag, New York.

86. **Rosenbluh, A., and E. Rosenberg.** 1990. Role of autoside AMI in development of *Myxococcus xanthus*. J.Bacteriol. **172:**4307-4314.

87. **Rosenbluh, A., R.Nir, E.Sahar, and E.Rosenberg.** 1989. Cell-density dependent lysis and sporulation of *Myxococcus xanthus* in agarose microbeads. J. Bacteriol. **171:**4923-4929.

88. **Rosenbluh, A., and E. Rosenberg.** 1989. Autoside AMI rescues development in *dsg* mutants of *Myxococcus xanthus* . J. Bacteriol. **171:**1513-1518.

89. **Rosenbluh, A., E. Rosenberg.** 1989. Sporulation of *Myxococcus xanthus* in liquid shake flask cultures. J. Bacteriol. **171:**4521-4524.

90. **Sager, B., and D. Kaiser.** 1994. Intercellular C-signaling and the traveling waves of *Myxococcus* . Genes and Dev. **8:**2793-2804.

91. **Sager, B., and D. Kaiser.** 1993. Spatial restriction of cellular differentiation. Genes and Dev. **8:**1645-1653.

92. **Sager, B., and D. Kaiser.** 1993. Two cell-density domains within the *Myxococcus xanthus* fruiting body. Proc. Natl. Acad. Sci. USA. **90:**3690-3694.

93. **Schweizer, E.** 1989. Biosynthesis of fatty acids and related compounds, vol. 2. Academic Press, London.

94. **Shi, W., and D. R. Zusman.** 1995. The *frz* signal transduction system controls multicellular behavior in *Myxococcus xanthus* . American Society for Microbiology, Washington D.C.

95. **Shi, W., T. Kohler., and D. R. Zusman.** 1995. Presented at the 22nd Int. Conf. Biol. Myxobacteria.

96. **Shi, W., T. Kohler, and D. R. Zusman.** 1993. Chemotaxis plays a role in the social behavior of *Myxococcus xanthus* . Mol. Microbiol. **9:**601-611.

97. **Shimkets, L. J.** 1990. Social and Developmental Biology of the Myxobacteria. Microbiol. Rev. **54:**473-501

98. **Shimkets, L. J., and H. Rafiee.** 1990. *Csg* A, an extracellular protein essential for *Myxococcus xanthus* development. J. Bacteriol. **172:**5299-5306.

99. **Shimkets, L. J., and S. J. Asher.** 1988. Use of recombination techniques to examine the structure of the *csg* locus of *Myxococcus xanthus*. Mol. Gen. Genet. **211:**63-71.

100.**Shimkets, L. J., R. E. Gill, and D. Kaiser.** 1983. Developmental cell interactions in *Myxococcus xanthus* and the *spoC* locus. Proc. Natl. Acad. Sci. USA. **93:**1406-1410.

101. **Shimkets, L., and M. Dworkin.** 1981. Excreted adenosine is a cell density signal for the initiation of fruiting body formation in *Myxococcus xanthus* . Dev. Biol. **84:**51-60.

102. **Shimkets, L. S., M. Dworkin, and K. H. Keller.** 1979. A method for establishing stable concentration gradients in agar suitable for studying chemotaxis on a solid surface. Can. J. Microbiol. **25:**1460-1467.

103. **Singer, M. , and D. Kaiser.** 1995. Ectopic production of guanosine penta- and tetraphosphate can initiate early developmental gene expression in *Myxococcus xanthus*. Genes Dev. **9:**1633-1644.

104. **Skinner, D. D., M.R. Morgenstern, R.W. Fedechko and C.D. Denoya.** 1995. Cloning and sequencing of a cluster of genes encoding branched-chain α-keto acid dehydrogenase from *Streptomyces avermitilis* and the production of a functional E1[αβ] component in *Escherichia coli*. J. Bacteriol. **177:**183-190.

105. **Sogaard-Anderson, A., F. J. Slack, H. Kimsey, and D. Kaiser.** 1996. Intercellular C-signalling in *Myxococcus xanthus* involves a branched-signal transduction pathway. Genes Dev. **10:**740-754.

106. **Strauch, M. A., D. de Mendoza, and J. A.Hoch.** 1992. cis-unsaturated fatty acids specifically inhibit a signal-transducing protein kinase required for initiation of sporulation in *Bacillus subtilis*. Mol. Microbiol. **6:**2909-2917.

107. **Teintze, M., R. Thomas, T. Furuichi, M. Inouye, and S. Inouye.** 1985. Two homologous genes coding for spore specific proteins are expressed at different times during development of *Myxococcus xanthus*. J. Bacteriol. **183:**121-125.

108. **Toal, D. R., S.W. Clifton , B.A. Roe and J. Downard.** 1995. The *esg* locus of *Myxococcus xanthus* encodes the E1α and E1β subunits of a branched-chain keto acid dehydrogenase. Mol. Microbiol. **16:**177-189.

109. **Toal, D.** 1994. Ph.D. thesis. University of Oklahoma, Norman.

110. **Varon, M., A. Tietz, and E. Rosenberg.** 1986. *Myxococcus xanthus* autoside AMI. J. Bacteriol. **167:**356-361.

111. **Varon, M., S. Cohen, and E. Rosenberg.** 1984. Autosides produced by *Myxococcus xanthus*. J. Bacteriol. **160:**1146-1150.

112. **Ware, J. C., and M. Dworkin.** 1973. Fatty acids of *Myxococcus xanthus*. J. Bacteriol. **115:**254-261.

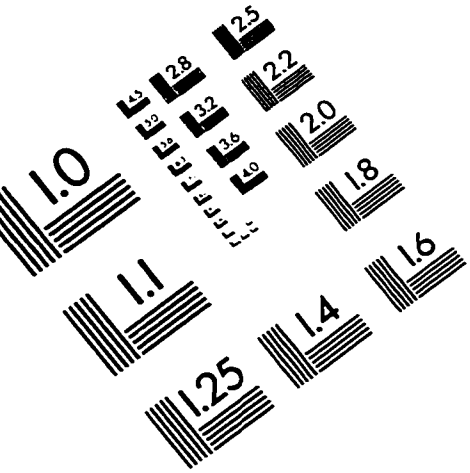
113. **Willecke, K., and A. B. Pardee.** 1971. Fatty Acid-requiring mutant of *Bacillus subtilis* defective in branched-chain α-keto acid dehydrogenase. J. Biol. Chem. **246:**5264-5272.

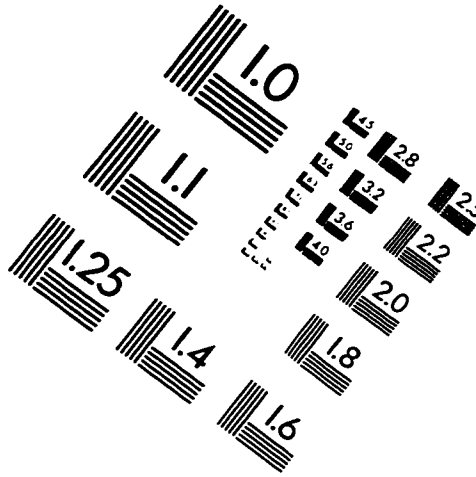
114. **Wireman, J. W., and M. Dworkin.** 1977. Developmentally induced autolysis during fruiting body formation by *Myxococcus xanthus*. J. Bacteriol. **129:**796-802.

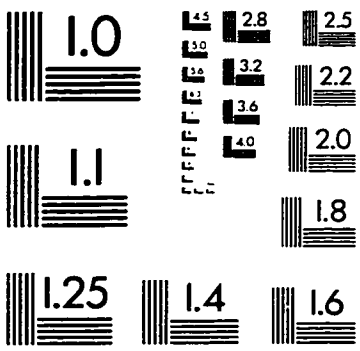
115. **Wireman, J. W., and M. Dworkin.** 1975. Morphogenesis and developmental interactions in Myxobacteria. Science. **189:**516-523.

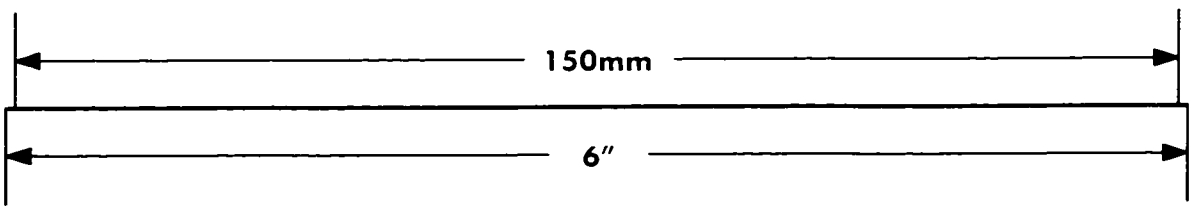
116. **Yajko, D. M., and D. R. Zusman.** 1978. Changes in cAMP levels during development of *Myxococcus xanthus* . J. Bacteriol. **133:**1540-1542.

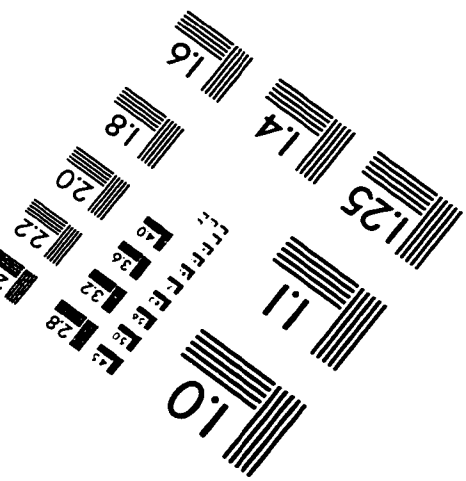
IMAGE EVALUATION
TEST TARGET (QA-3)

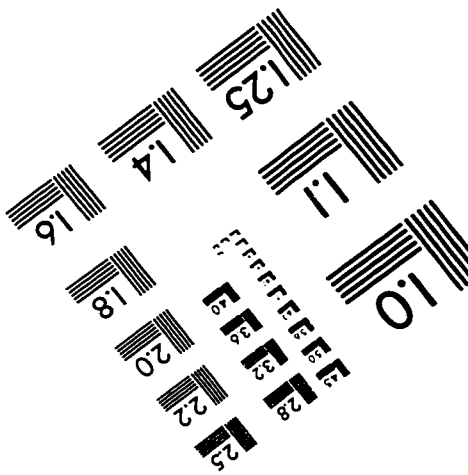












APPLIED IMAGE, Inc
1653 East Main Street
Rochester, NY 14609 USA
Phone: 716/482-0300
Fax: 716/288-5989