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Sporulation mutants of *Myzococcus xanthus*

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Case Western Reserve University (Health Sciences), 1994
SPORULATION MUTANTS OF *Myxococcus xanthus*

by

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Submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy

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SPORULATION MUTANTS OF Myxococcus xanthus

Abstract

by

RICHARD C. CARDAMAN

Myxococcus xanthus strain DZ1 was mutagenized with transposon Tn5. Twenty-two hundred eighty-eight mutant colonies were screened for their ability to make functional spores in response to starvation. Eighty-nine sporulation mutants were identified and characterized further. Forty-seven of these mutants fruited normally, or nearly so, and thus identify functions required primarily for sporulation. These mutants arrested development with terminal cell morphology ranging from rod to sphere; one arrested as an ovoid. This group of mutations mapped to at least eight chromosomal loci, and with one exception these loci are distinct from sites of physically mapped developmental mutants. All 47 mutants sporulated normally when exposed to glycerol, as judged by both visual and functional criteria. These observations challenge the conventional assumption that glycerol and starvation induce substantially overlapping sets of sporulation functions.
The spo 77 mutation blocks morphogenesis at the normal ovoid stage. This phenotype is observed in either a DZF1 or DK 1622 strain background. The spo 77 phenotype cannot be rescued by the addition of exogenous glucosamine. A clone carrying the Ω77 insertion site was isolated from RC 77. A probe created from this clone was used to identify potential wild-type clones of the spo 77 locus. Several wild-type cosmid clones were isolated, but only one was capable of complementation in a partial diploid, created by tandem duplication. Subsequent attempts to identify the boundaries of the spo 77 locus were unsuccessful. These attempts relied on the spo 77 gene being functional at a site distant from its normal chromosomal location. We believe that the spo 77 gene may be context sensitive and must be near its normal location in order to function.
DEDICATION

For my daughters...

Jessica Rachel

and

Ashley Marlene
ACKNOWLEDGEMENTS

I would like to thank Russ Maurer for his help and guidance. His patience and understanding, while I was trying to juggle grad school and raise a family, made life much more tolerable. I especially enjoyed his Ben and Becca stories, they made me laugh on many occasions. I would like to thank the rest of the Maurer lab, past and present, for their help and suggestions over the years. I also would like to thank Marion Grier for all the plates she has poured for me over the years. I hate to think of how many. Thanks go to Larry Shimkets, Dale Kaiser, Ron Gill, David Zusman, and all the other "myxo" people out there for all the strains and other materials they have sent me, along with their help with some of the more troublesome aspects of my project.

My wife Vicky deserves special credit for putting up with my bizarre schedule the past several years. She has worked hard to help raise our children and maintain our household. I know it hasn't been easy for her.

Thanks go to all my friends and family for their support and faith in me. They have stood by me during difficult times when I needed them most. I would like to thank Ed Keirnan in particular for his help when I was bogged down with work and needed to get my figures done.
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Chapter I

INTRODUCTION TO SPORULATION IN *Myxococcus xanthus*

A. DORMANCY: It is a recurring theme that organisms must find ways to cope with environmental adversity, or perish. Adaptation mechanisms range greatly: from behavioral to physiological, from minor to extreme. One of the more extreme forms of adaption is dormancy, the ability of an organism to suspend most metabolic activity until environmental conditions improve. The use of dormancy as an adaptive mechanism can be observed almost universally. Examples of dormancy can be found among the bacteria, fungi, plants, and animals (Storey and Storey, 1990). Studies of dormancy in a wide variety of organisms suggest that many aspects of the mechanisms involved have been evolutionarily conserved (Storey and Storey, 1990). Furthermore, a comparison and contrast of these various systems serves to provide a backdrop for the understanding of any one specific system.
Every organism has the ability to respond to changes in the environment. Examples of this are many and diverse, from induction of the lac operon in *E. coli* in response to lactose, to seasonal hibernation in mammals. What characterizes dormancy as a unique, definable response? Virtually all examples of dormancy have three elements in common: 1.) induction by an environmental trigger, 2.) a means of metabolic rate reduction, and 3.) isolation from environmental conditions (Storey and Storey, 1990). The first of these means that dormancy must be triggered by a change in external, or environmental, conditions. To qualify as dormancy the trigger must generally be something directly or indirectly detrimental to the organism. In many instances this is lack of food (starvation), but it can also be factors such as day length or temperature (Storey and Storey, 1990). The second element of dormancy is the most important of the three, and key to the concept of dormancy. It is the existence of an active mechanism for the reversible reduction of metabolism. This is in contrast to the passive reduction of metabolism, such as that observed when poikilotherms are exposed to low temperature (Storey and Storey, 1990). The third element required is an active mechanism to provide for isolation (protection) from the environment (Storey and Storey,
This can be behavioral, such as burrowing into mud, or physiological, such as creating an impervious spore coat. The point is that the organism takes measures to insure that it is not harmed by environmental conditions while it is dormant.

Even among organisms that use the dormancy strategy there is considerable diversity. Higher animals have evolved several dormancy mechanisms, including facultative anaerobiosis (freshwater turtle), hibernation (ground squirrel), estivation (lungfish), and daily torpor (small birds) (Storey and Storey, 1990). The extent of metabolic reduction, as measured by caloric consumption, ranges from 26-95% for daily torpor to 80-90% for estivation (Storey and Storey, 1990). Probably the most extreme is the 96-99% reduction observed in hibernating bats (Storey and Storey, 1990). There are also differences in the dormancy mechanisms of single-celled organisms including yeast, slime molds, and bacteria (Losick and Shapiro, 1984). Generally, starvation is the environmental trigger in these systems, but the response to starvation, and the means of metabolic reduction vary widely (Losick and Shapiro, 1984). Isolation from environmental conditions is also achieved differently in each instance, although most often it involves production of an impervious outer coat
(Losick and Shapiro, 1984). A more detailed discussion on the manner in which each of these elements is implemented will be elaborated later in this introduction.

Among the procaryotes there are many species that have adopted dormancy as a survival stratagem, and even among these "simple" organisms, there are different means to reach the same end. Among the best studied of these systems are those of B. subtilis, azotobacter, and the myxobacteria. The common goal for all of these is to produce an encapsulated, environmentally resistant, metabolically inert cell, generally referred to as a spore or cyst.

B. THE MYXOBACTERIA

1. General Background: The myxobacteria are gram-negative, elongated rods, closely related to the enteric bacteria based upon 16s ribosomal RNA studies (Ludwig et al., 1983). They are aerobic, mesophilic organisms with almost universal distribution (Janssen et al., 1977), (McCurdy, 1969), (Peterson, 1969). The majority of myxobacteria are soil organisms, but they can also be found in rotting wood, herbivore dung, and bodies of water (McCurdy, 1969), (Peterson, 1969), (Singh, 1947). The myxobacteria grow at a much slower rate than the
enteric bacteria. Under optimal growth conditions, at log phase, the doubling time of *M. xanthus* is approximately 5 hours (Peterson, 1969). DNA replication occurs at a rate comparable to that of *E. coli*, but since cell division takes considerably longer, there is a gap between rounds of chromosomal replication (Kimchi and Rosenberg, 1976), (Rosenberg et al., 1967). The DNA base composition of the myxobacteria is extremely G+C rich, ranging from 67 to 71% G+C (Mandel and Leadbetter, 1965), (McCurdy and Wolf, 1967). The genome size of *M. xanthus* has been calculated by a number of methods, the most accurate being pulsed-field electrophoresis of large restriction fragments (Chen et al.; 1990). The genome size is substantially larger than that of *E. coli*, in the range of 9,500 kbp (Chen et al., 1990). Individual cells move by a gliding motility that has been extensively studied.

2. **Nutrition:** The nutritional requirements of the myxobacteria have been well defined. Their preferred substrate is amino acids, some of which are absolutely required for growth, and others which serve as a carbon and nitrogen source (Bretscher and Kaiser, 1978), (Peterson, 1969). The amino acids methionine, leucine, isoleucine, and valine are essential for growth of *M.*
xanthus and form the basis of minimal defined media (Bretscher and Kaiser, 1978). Amino acids, pyruvate, and acetate are the most efficient sources for carbon and energy (Bretscher and Kaiser, 1978). The efficiency of nitrogen sources is more difficult to assess because of the requirement for essential amino acids, but it has been observed that ammonium sulfate, and to a lesser extent asparagine and spermidine, stimulate growth in the presence of minimal amino acids (Bretscher and Kaiser, 1978). Carbohydrates are not generally used as carbon or energy sources and are not efficiently absorbed by the cells (Bretscher and Kaiser, 1978), (Peterson, 1969).

The myxobacteria rely on the extracellular degradation of macromolecules for their nutritional requirements. Consequently they are able to synthesize and secrete a wide variety of hydrolytic enzymes, such as proteases, peptidases, muramidases, lipases, and nucleasees (Gnosspeilus, 1978), (Sudo and Dworkin, 1972). Much of their food is obtained by preying upon other bacteria, both gram negative and gram positive (Peterson, 1969). How the myxobacteria avoid destruction by their own enzymes has not been determined. This means of feeding imposes a severe constraint upon the myxobacteria, which is the need for high local enzyme concentrations. It has been observed in the laboratory
that growth of *M. xanthus* on insoluble protein is density dependent (Rosenberg et al., 1977). Furthermore, there is a minimal cell density of $10^3$ cells/ml required to establish growth on such media (Rosenberg et al., 1977). In contrast to this, growth on hydrolysed media does not require a minimum cell density and a single cell can give rise to a colony (Rosenberg et al., 1977). Under natural conditions the myxobacteria do not encounter pre-hydrolysed protein as a food source, and consequently cell density is a very real determinant of survival. This concept of minimal cell density will also have relevance to the discussion of sporulation which follows.

3. **Extracellular Structures:** Two types of extracellular appendages have been observed in *M. xanthus*, pili (fimbriae) and fibrils. Pili are 6-8 nm diameter structures located at the cell poles and extending up to a 5 μm in length (MacRae et al., 1977). They are linear structures composed of protein with a subunit size of approximately 8 kDa (MacRae et al., 1977). Pili appear to be expressed constitutively and independent of cell density (Kaiser, 1979).

Fibrils are branched structures approximately 30-50 nm in diameter (Behmlander and Dworkin, 1991), (Dobson and McCurdy, 1979). In contrast to pili, which are
localized at the cell poles, fibrils are located randomly on the cell surface (Behmlander and Dworkin, 1991), (Dobson and McCurdy, 1979). In addition, fibril production is not constitutive; rather, it is dependent on cell density (Arnold and Shimkets, 1988), (Behmlander and Dworkin, 1991). The higher the cell density, the more fibrils. The functions of these two extracellular organelles are not clearly understood, but there is evidence that they both play a role in cell motility (see below) and that fibrils also play a role in cell cohesion (Arnold and Shimkets, 1988a), (Arnold and Shimkets, 1988b). Fibril production can be blocked by the dye congo red, and a mutant (dsp) has been isolated which produces aberrant fibrils (Arnold and Shimkets, 1988a). In both instances, normal cell-to-cell cohesiveness disappears (Arnold and Shimkets, 1988a), (Arnold and Shimkets, 1988b).

4. Motility: The myxobacteria move by a gliding motility (Kaiser and Crosby, 1983). When gliding, cells move along a surface in a direction parallel to their long axes. Often, cell movement is accompanied by considerable flexing and bending of the cell (Kaiser and Crosby, 1983). M. xanthus glides at a rate of 1-13 μm/minute, but the rate of gliding is not constant
(Kaiser and Crosby, 1983). Cells appear to accelerate, decelerate, and reverse direction continually (Kaiser and Crosby, 1983). It has been observed in *M. xanthus* that gliding requires calcium ions in a concentration of 0.1–0.3 mM (Womack et al., 1989). The mechanism of gliding is unknown and no gliding-specific organelle has yet been identified. Wild type myxobacteria have the capacity to glide as individual cells or in dense swarms of closely associated cells (Hodgkin and Kaiser, 1979). In the latter case, cell movement is coordinated so that the swarm moves as a unit (Kaiser and Crosby, 1983), (Hodgkin and Kaiser, 1979). These two types of gliding behavior have been termed adventurous and social (Hodgkin and Kaiser, 1979), (Burchard, 1970). Both types of gliding behavior can be observed at the colony periphery in wild type strains. Adventurous gliding is typified by lone cells which leave the swarm transiently and then return. It is believed that adventurous gliding is the primary system for colonizing new areas. This is based on the observation that colonies of adventurous-motility mutants do not spread out across an agar plate as efficiently as wild type (Burchard, 1970), (Kaiser and Crosby, 1983). In addition to single cells, there are also "rafts" of cells which break off from the main colony and move as a
unit. These represent social gliding behavior (Kaiser and Crosby, 1983), (Hodgkin and Kaiser, 1979).

Gliding motility in *M. xanthus* is under the control of two nearly independent gene families (Hodgkin and Kaiser, 1979). This was discovered when transductional analysis of nonmotile mutants revealed that nearly all of them were actually double mutants (Hodgkin and Kaiser, 1979). Genetic testing through the use of double mutants revealed two distinct groups of motility genes, which correspond to the two different motilities observed in wt cells (Hodgkin and Kaiser, 1979). The integrity of either group was sufficient for some degree of motility (Hodgkin and Kaiser, 1979). The A (for adventurous) genes comprise approximately 22 loci that are required for cell-autonomous movement (Hodgkin and Kaiser, 1979). It was observed that adventurous gliding does not require cell-to-cell contact (Hodgkin and Kaiser, 1979). The S (for social) system on the other hand, requires continuous cell-to-cell contact for gliding to take place (Hodgkin and Kaiser, 1979). If cells lose contact with one another they become transiently non-motile until contact is restored (Hodgkin and Kaiser, 1979). Social motility is required for aggregation during development (Hodgkin and Kaiser, 1979). There are approximately 10 loci in the S gene family (Hodgkin and Kaiser, 1979).
The exact cell-to-cell interactions required for the S system are not understood, but it appears that pili and fibrils play a role in mediating social motility (Kaiser, 1979), (Kaiser and Crosby, 1983). Mechanical removal of pili renders cells transiently S, as does blocking fibril production using congo red (Arnold and Shimkets, 1988a), (Rosenbluh et al., 1989). Protein synthesis appears to be required for S motility, but not for A motility. This was determined by sensitivity to chloramphenicol and erythromycin, both of which block S motility exclusively. The requirement of the S system for protein synthesis is not understood, although it has been speculated that some cellular component may be consumed or lost in the process of cell movement.

While the two motility systems are nearly independent, a single mutation in the mgl locus will render cells completely non-motile, suggesting that it is required for both systems (Hodgkin and Kaiser, 1979), (Stephens et al., 1989). It was originally thought that the mgl locus might code for some part of the gliding machinery, but subsequent cloning and sequence analysis has shown otherwise (Hartzell and Kaiser, 1991a), (Hartzell and Kaiser, 1991b). The mglA gene codes for a 22 kD protein which exhibits 32% amino acid homology with sarl of S. cerevisiae (Hartzell and Kaiser, 1991a). The
Sarl protein belongs to a class of small GDP/GTP binding proteins (Nakano and Muramatsu, 1989). Further sequence comparisons with other proteins in this group demonstrated the presence in mgLA of 3 conserved sequence motifs characteristic of this type of protein (Hartzell and Kaiser, 1991a). Additional studies, using polyclonal antibodies, localized MglA to the cytoplasmic fraction of the cell, an unlikely location for the motility machinery (Hartzell and Kaiser, 1991a). On the basis of these properties, it is believed that mgLA plays a role in some signal transduction mechanism required for activating cell movement (Hartzell and Kaiser, 1991a), (Hartzell and Kaiser, 1991b).

C. THE DEVELOPMENTAL CYCLE

Under ideal conditions M. xanthus undergoes a repeating vegetative cycle of growth and cell division. If, however, nutrients become limiting, M. xanthus initiates a developmental pathway which leads to formation of dormant, environmentally resistant cells (figure 1). The developmental pathway can be divided into three stages; initiation, aggregation, and sporulation. With reference to figure 1, initiation is the point where cells make the transition from the vegetative growth cycle to the developmental cycle. Once
Figure 1. Life cycle of *Myxococcus xanthus*. This is a schematic representation of the *M. xanthus* life cycle reprinted from Shimkets, 1990. This figure shows that the transition from the vegetative cycle to the developmental cycle has both multicellular (fruiting body formation) and cell-autonomous (myxospore formation) components. In addition, chemical induction uncouples the two processes and allows spore formation in the absence of fruiting bodies.
the cells are committed to the developmental cycle, aggregation and sporulation occur as parallel processes as represented in figure 1. Aggregation refers to the events which involve cooperative interaction between many cells and which lead to formation of a fruiting body. In contrast, sporulation refers to the transformation which cells undergo as individuals within the fruiting body. Each of these stages will be discussed in more detail below.

1. **Initiation:** Implementation of the developmental pathway requires three conditions: 1.) starvation, 2.) adequate cell density, and 3.) a solid support (Zusman, 1984).

Partial starvation for any of the essential amino acids triggers development (Dworkin, 1963), (Manoil and Kaiser, 1980a). In addition, starvation for some nonessential amino acids, as well as carbon and energy sources, can also induce development (Dworkin, 1963), (Manoil and Kaiser, 1980a). The first most likely represents a response to the quality of nutrition, and the second to quantity of nutrition. The aspartate family of amino acids (aspartate, asparagine, threonine, lysine, isoleucine, and methionine) appears to play a fundamental role in determining starvation (Filer et al.,
1973), (Rosenberg et al., 1973). Slight imbalances in the concentrations of these amino acids have dramatic effects, both positive and negative, on the initiation of development (Filer et al., 1973), (Rosenberg et al., 1973). It is believed that these amino acids exert their effect by feedback mechanisms that influence the activity of the enzyme aspartokinase (Filer et al., 1973), (Rosenberg et al., 1973). Isozymes of aspartokinase catalyze the committed step in the biosynthesis of these amino acids (Filer et al., 1973), (Rosenberg et al., 1973). For example, elevated levels of threonine induce development in non-starved cells (Filer et al., 1973). Threonine is a feedback inhibitor of aspartokinase and may cause the cells to starve for methionine and lysine, even under conditions of excess carbon and energy (Filer et al., 1973). In another example, methionine and isoleucine, which stimulate aspartokinase, prevent initiation of development even under starvation conditions (Filer et al., 1973). How the myxobacteria translate starvation into an intracellular signal or if such a signal even exists has not been determined. In *E. coli* and *B. subtilis* it is known that ppGpp plays a role in alerting the cell to stringent conditions (Errington, 1993). Myxobacteria have also been found to produce ppGpp, but the use of this compound as a signal for
starvation is not likely (Manoil and Kaiser, 1980a), (Manoil and Kaiser, 1980b). The evidence against ppGpp being such a signal is threefold. First, vegetative cells make high levels of this compound (Manoil and Kaiser, 1980a), (Manoil and Kaiser, 1980b). Second, during induction the ratio of ppGpp concentration to the total GTP pool remains low (Manoil and Kaiser, 1980b). In contrast, ppGpp production in *B. subtilis* results in a depletion of the GTP pool and it is this depletion which initiates sporulation (Errington, 1993). Finally, mutant strains of myxobacteria unable to synthesize ppGpp can be induced to sporulate (Zusman, 1984).

Adequate cell density is required for the multicellular aspects of development, and it will become a recurring theme that *M. xanthus* relies on several mechanisms for ensuring adequate cell densities at crucial stages in development (Shimkets and Dworkin, 1981), (Kuspa et al., 1992). The first check point for adequate cell density occurs at the initiation stage and is mediated by extracellular adenosine concentrations (Shimkets and Dworkin, 1981). The evidence for this is as follows: the myxobacteria excrete adenosine into the surrounding media; development can be induced at low cell densities by the addition of adenosine; the addition of Norit (which binds purines) blocks initiation and can be
reversed by adding adenosine; and hadacidin, an inhibitor of de novo AMP synthesis, inhibits development (Shimkets and Dworkin, 1981). This suggests that extracellular adenosine concentration provides the means by which myxobacteria monitor cell density. However, once again the actual mechanism by which adenosine concentration is sensed and interpreted is not known.

The third condition, a solid support, is believed to be simply a mechanical requirement, as the cells require a surface to glide upon (Zusman, 1984).

2. Aggregation: The need for a minimum cell density to ensure survival places an additional constraint upon the sporulation of myxobacteria. Not only must the cells become spores, but they must ensure, that upon germination, sufficient numbers of cells are present to establish a viable colony (Rosenberg et al., 1977). The myxobacteria have solved this problem by creating multicellular structures called fruiting bodies. Different species of the myxobacteria produce characteristic fruiting bodies, ranging from extremely simple to quite complex in structure, but in all cases these structures ensure that the colony does not become dispersed while it is dormant. Figure 2, panel A shows a typical M. xanthus fruiting body as viewed by scanning
Figure 2. *M. xanthus* fruiting body and spores. Photographs of a single fruiting body (panel A, x350) and spores (panel B, x1500) of wild type *M. xanthus* taken by scanning electron microscopy.
electron microscopy. Estimates vary, but each fruiting
body is believed to contain $10^6$-10$^6$ spores, as shown in
the higher magnification in panel B (Shimkets, 1990).
The formation of a fruiting body requires cooperative
interaction between cells and some means of intercellular
signalling to coordinate the process (Hagen et al.,
1978), (LaRossa et al., 1983). Successful completion of
a fruiting body requires that the vegetative cells from
which it is derived be motile to carry out the
necessary aggregation (Kaiser and Crosby, 1983).

3. **Sporulation:**

**Glycerol spores.** Historically, biochemical and genetic
studies of myxospores were hampered by a number of
technical problems. It was difficult to create
synchronized, homogeneous cell populations that could be
easily harvested (Dworkin and Gibson, 1964). The
discovery that glycerol and certain other related
compounds could bypass fruiting body formation and induce
cell-autonomous sporulation in a broth culture led to a
large volume of research in this area (Dworkin and
Gibson, 1964). It was initially believed that glycerol-
induced spores were replicas of fruiting body spores, and
a wealth of data was generated about the metabolism and
biochemistry of glycerol-induced spores (Orlowski et al.,
1972), (Watson and Dworkin, 1968). Later, a more detailed structural analysis of the two spore types revealed striking differences (figure 3) (Inouye et al., 1979). This more detailed appreciation of spore differences has inevitably called into question the notion that starvation and glycerol represent alternative methods to engage the identical (or substantially so) developmental pathway. Unfortunately, most of the available biochemical data about myxospores pertains to glycerol spores and may not be completely applicable to fruiting body spores.

**Biochemistry.** It is known that the level of over twenty proteins change during sporulation (Inouye et al., 1979). Among the most dramatic of these is protein S, which at its peak production accounts for nearly 80% of newly synthesized protein (Downard et al., 1984). Much of the accumulation of protein S can be attributed to the exceptionally long half-life of its mRNA (38 minutes) (Nelson and Zusman, 1983). Protein S (no relationship to S motility) is the product of the developmentally-regulated tps gene (Downard, 1987). Expression of tps is initiated approximately 5 hours after the onset of starvation and continues into the sporulation stage (Downard et al., 1984), (Downard and Zusman, 1985).
Figure 3. Ultrastructure Comparison of Glycerol and Starvation Spores. Transmission electron micrographs comparing thin sections of starvation (A) and glycerol (B) spores. The abbreviations are as follows: N, nucleoid; IM, inner membrane; OM, outer membrane; CX, cortex; IC, intermediate coat; SC surface coat; GC, glycerol spore coat. Bar, 0.5 μm. (From Inouye et al, 1979)
Extensive studies of the tps gene have shown it to have a large regulatory region, containing both positive and negative control elements (Downard et al., 1988), (Kil et al., 1990). Gel shift studies of developmentally induced cells have revealed development-specific proteins which bind to these control regions, but the identity of these proteins has not yet been determined (Downard et al., 1988).

Myxobacterial hemagglutinin (MBHA) and protein U are two other developmentally expressed proteins that have been examined. Protein U is a spore coat protein induced late in development (Gollop et al., 1991). It has been cloned and sequenced (Gollop et al., 1991). Protein U is composed of 179 amino acids, 25 of which form a typical signal peptide at the amino-terminal end (Gollop et al., 1991). A database comparison of the amino acid sequence revealed no homology to known proteins (Gollop et al., 1991). The sequence evidence suggests that protein U is secreted by developing cells, but how it assembles on the spore coat and its role there are unknown. MBHA is a developmentally regulated protein with lectin-like properties (Cumsky and Zusman, 1979), (Cumsky and Zusman, 1981), (Cumsky and Zusman, 1981). Expression of MBHA begins around 6-8 hours of development and reaches a peak around 26-36 hours, which coincides with the completion
of aggregation (Cumsky and Zusman, 1979). It is believed by Zusman that MBHA plays a role in the aggregation process, but a null mutation of the mbhA gene does not block aggregation, suggesting that MBHA is not essential (Romeo and Zusman, 1987).

In addition to changes in protein synthesis, there is also extensive synthesis of novel spore envelope polysaccharides and the storage molecule trehalose (McBride and Zusman, 1989). Coupled with these change is a general redirection of the cell's metabolism towards gluconeogenesis (Bacon et al., 1975), (Filer et al., 1977).

**Morphogenesis.** Sporulation in *M. xanthus* triggers two sequential morphogenic events. First, there is a dramatic reshaping of the cell, followed by the deposition of a thick, multilayered, spore coat (Dworkin and Sadler, 1966), (Filer et al., 1977).

As with other gram-negative bacteria, the shape of an *M. xanthus* cell is determined by the structure of its peptidoglycan envelope (White et al., 1968). The conversion of an elongated rod to a sphere requires significant remodeling of the peptidoglycan. The peptidoglycan of *M. xanthus* is chemically similar to that of other gram negative bacteria, including *E.coli* (Park,
1987). The actual arrangement of the peptidoglycan however, differs somewhat from the conventional model. In *E. coli*, for example, the glycan chains of alternating N-acetylglucosamine and N-acetylmuramic acid residues are crosslinked by short peptide chains (Park, 1987). The chains are crosslinked at regular intervals, resulting in a uniform peptidoglycan envelope (Park, 1987). Instead of a uniform envelope, the myxobacteria have more of a "patch-work quilt" arrangement (White et al., 1968). The peptidoglycan subunits are assembled into larger units via the conventional, trypsin-resistant, DAP linkages (White et al., 1968). These units are then crosslinked to one another by a different, trypsin-sensitive, peptide linkage (White et al., 1968). The principal difference between this and the arrangement in *E. coli* is thus a reduction in the overall degree of peptidoglycan crosslinking (White et al., 1968). The net result of this arrangement is a more flexible murein envelope, which White and others believe may be a requirement for gliding motility (White et al., 1968).

The surface area of myxospores has been calculated to be approximately one-third that of vegetative cells (White et al., 1968). However, the total amount of peptidoglycan per cell is the same for both (White et al., 1968). Radiolabel studies of glycerol spores have
been done to determine the rate of peptidoglycan turnover during sporulation (Dawson and Jones, 1979). The release of labeled diaminopimelate increased slightly in cells undergoing sporulation, indicating that the overall rate of turnover does increase during sporulation (Dawson and Jones, 1979). Further analysis of glycerol spores revealed an 11% increase in the degree of peptidoglycan crosslinking as measured by the number of diaminopimelic acid-to-alanine linkages (Dawson and Jones, 1979), (Johnson and White, 1972). A series of experiments was also done, using selective antibiotic resistance, to determine if the enzymes responsible for peptidoglycan synthesis changed during sporulation (White et al., 1968). It was observed that penicillin-G sensitivity was reduced for a period of time while the cells were ovoid in shape (White et al., 1968). This has been interpreted as indirect evidence for sporulation-specific peptidoglycan synthesis (White et al., 1968).

The spore coat of myxospores is resistant to a wide variety of chemical and enzymatic agents, including detergents, proteases, and glycosidases (Kottle et al., 1975). It is also very hygroscopic, which may aid in desiccation resistance (Kottle et al., 1975). Chemical analysis of the spore coat has revealed that it is approximately 75% polysaccharide by weight (Filer et al.,
1977). This is consistent with the observed increase in gluconeogenesis during sporulation (Bacon et al., 1975), (Filer et al., 1977). The outermost layer of the spore coat is composed of protein S (Inouye et al., 1979).

Deposition of the spore coat begins after the cells have reached a spherical shape (Filer et al., 1977), (Kottle et al., 1975). The mechanism for spore coat formation is unknown, but studies of protein S have shown that, in the presence of calcium, it is capable of self-assembly on the spore surface (Inouye et al., 1979). Detailed analysis of protein S has revealed two high affinity calcium-binding domains, with amino acid homology to those found in calmodulin (Inouye et al., 1983), (Inouye and Inouye, 1987). Alteration of these calcium-binding domains, by site directed mutagenesis, blocks the self-assembly of protein S (Teintze et al., 1988).

D. DEVELOPMENTAL MUTANTS: The multicellular aspect to sporulation sets the myxobacteria apart from the other sporulating prokaryotes. The current model of M. xanthus development holds that the multicellular pathway (fruited body formation) and the cellular pathway (sporulation) are largely independent of one another,
with some degree of overlap. The strongest evidence to support this model comes from the study of developmental mutants, some of which affect a single aspect of development (sporulation or aggregation), and others of which affect both.

1. **Aggregation Mutants:** A number of aggregation mutants have been reported which are unable to form fruiting bodies and yet are able to produce normal numbers of functional spores (Morrison and Zusman, 1979). These mutants are specific to the aggregation pathway of development, since sporulation is unaffected (Morrison and Zusman, 1979). In addition, these mutants are fully S motile, indicating that the defect is development-specific and not related to a general motility problem (Morrison and Zusman, 1979).

Zusman has reported three groups of such mutants, rough, swirl, and frizzy (Morrison and Zusman, 1979), (Zusman, 1982). Of the three, only the frizzy mutants have been extensively studied (Blackhart and Zusman, 1985), (Blackhart and Zusman, 1986). Frizzy mutants are unable to make normal, compact mounds, but instead produce diffuse, tangled aggregates of cells, which have a "frizzy" appearance (Zusman, 1982). All of the frizzy mutants identified thus far cluster to an approximately
7.5 kb region of the chromosome (Blackhart and Zusman, 1985). Genetic analysis revealed 6 distinct loci, 5 of which were contiguous, and one that was separated from the others by 1.4 kb (Blackhart and Zusman, 1985). These loci have been designated frzA, frzB, frzCD, frzE, frzF, and frzG (Blackhart and Zusman, 1985). The frizzy genes bear sequence homology to chemotaxis genes in Salmonella (McBride et al., 1989). The FrzA protein has 28.1% amino acid homology to the CheW protein of *S. typhimurium* (McBride et al., 1989). FrzE has sequence similarities to CheA and CheY, and FrzF has homology to methytransferase CheR (McCleary and Zusman, 1990). FrzCD possesses a region highly homologous to the C-terminal regions of methyl-accepting chemotaxis proteins (MCPs) (McNab, 1987). MCPs are transmembrane proteins which mediate response to chemotactic substances by binding them in the periplasm and stimulating the motility machinery (McNab, 1987). FrzCD has been demonstrated to be modified by methylation, which lends support to the idea it acts as an MCP in *M. xanthus* (McBride et al., 1992), (McCleary et al., 1990).

Fruiting body formation requires the coordinated movements of millions of cells. Intercellular communication is vital to the process, as demonstrated by the identification of signal mutants (discussed in
section 2). *M. xanthus* aggregation does not rely upon a chemoattractant gradient such as the cellular slime molds use (Dworkin and Eide, 1983), (Tomchik and Devreotes, 1981). Instead, cell-to-cell contact and proper cell alignment play a crucial role in the aggregation process (discussed below). It is not unreasonable to think that a chemotaxis type mechanism could be utilized to coordinate cell movement to achieve proper cell-to-cell contact. The frz genes appear to play such a role. Genes A-E are organized as a developmentally regulated operon (Weinberg and Zusman, 1989). Peak expression occurs around the time of mound formation (Weinberg and Zusman, 1989). It has been observed that the frz genes affect the frequency at which a gliding cell reverses its direction (Blackhart and Zusman, 1985). In wild type, changing the frequency of reversal results in net movement in a particular direction (Kaiser and Crosby, 1983). This mechanism is analogous to the swim/tumble mechanism observed in enteric bacteria during chemotaxis (McNab, 1987). Instead of CW or CCW flagellar rotation controlling directional movement, the myxobacteria use forward-backward gliding. By changing direction in response to a signal the cells could align themselves in the proper manner for successful development.
2. **Signal Mutants:** Among the most thoroughly studied developmental mutants are the signal or sg mutants (Hagen et al., 1978), (LaRossa et al., 1983). The sg mutants are defective for both aggregation (fructing body formation) and sporulation (Hagen et al., 1978), (LaRossa et al., 1983). Thus, they identify common elements of the two pathways. Both the aggregation and sporulation defects of these mutants can be rescued by development in the presence of wild-type cells (Hagen et al., 1978), (LaRossa et al., 1983). It is thought that the mutants are unable to produce an essential extracellular signal compound, but can respond to signal compound produced by the wild type (Hagen et al., 1978), (LaRossa et al., 1983). Sg mutations have been assigned to four groups Asg, Bsg, Csg, and Dsg, on the basis of rescue in pairwise mixtures of mutants (Hagen et al., 1978), (LaRossa et al., 1983). Although these experiments formally resemble classical genetic complementation, in this case the unit of function is the signal, rather than the gene. These experiments thus reveal the existence of four distinct signalling events during *M. xanthus* development and engender the obvious questions about each signal: what is the signalling compound, how is it produced, how is it received, how does the receiving cell respond, what is the purpose of the signal, what is the
timing of the signals, and do the signals function independently? These questions are beginning to be addressed.

All of the Asg mutants map to 3 loci (Kuspa and Kaiser, 1989). Expression of the asg loci is induced at the onset of development and peaks at about 2 hrs (Kuspa et al., 1986). An extracellular substance, capable of restoring aggregation and sporulation to asg mutants, was isolated from media in which wild type cells were grown (Kuspa et al., 1992), (Planmann et al., 1992). This "A-factor" was subsequently shown to have two components (Kuspa et al., 1992), (Planmann et al., 1992). The first, a heat stable, dialyzable substance, was determined to be a mixture of amino acids and peptides (Kuspa et al., 1992). The second component of A-factor was found to be a 27 kDa protein with a proteolytic activity similar to that of trypsin (Planmann et al., 1992). Furthermore, trypsin has been demonstrated to have A-factor activity, suggesting an important role of proteolysis in production of the A signal (Planmann et al., 1992). It turns out that A factor is a cell density signal specific for aggregation (Kuspa et al., 1992). The amount of A factor released into the surrounding medium is directly proportional to the number of cells present (Kuspa et al., 1992). The critical cell density,
to produce adequate A factor, is $3 \times 10^6 - 5 \times 10^8$ cell/ml (Kuspa et al., 1992). When the A signal is induced (1-2 hours after initiation), there must be this number of cells present in order for aggregation to begin (Kuspa et al., 1992). In this way, the A signal provides a means to ensure that sufficient cells are present to form a fruiting body (Kuspa et al., 1992). An extragenic suppressor of asgA has been isolated (Kaplan et al., 1191). A sasA mutation restores fruiting ability to asgA mutants, but the mechanism of sasA action is not known (Kaplan et al., 1191).

The bsg locus has been sequenced and a 90.4 kDa protein predicted on this basis (Gill and Bornemann, 1988), (Gill et al., 1988). A comparison to known sequences revealed a 45% homology with the lon gene of E. coli and 48% homology with the lon gene of Bacillus brevis (Gill et al., 1993). The bsg locus has been cloned and overexpressed in E. coli (Gill et al., 1993), (Gill and Bornemann, 1988), (Gill et al., 1988). A partially purified protein has been shown to have ATP-dependent protease activity (Gill et al., 1993). Antibody studies have revealed that the location of the bsg gene product is intracellular and therefore it is not likely to be the actual B signal (Gill et al., 1993).
The C signal mutants all map to the csgA locus which produces a 17.7 KDa extracellular protein essential to complete aggregation and sporulation (Hagen and Shimkets, 1990), (Kim and Kaiser, 1990). The csgA gene is weakly expressed in vegetative cells but its expression increases throughout development until the protein accumulates to about 3-fold the vegetative level, with peak expression occurring around the time of sporulation (Li et al., 1992). Studies indicate a biphasic response to C factor (Kim and Kaiser, 1991). Fruiting body formation requires and occurs at approximately 30% of maximal levels (Kim and Kaiser, 1991). Sporulation on the other hand, requires approximately 82% of maximal levels (Kim and Kaiser, 1991). Detailed analysis of csgA expression has revealed that the upstream region from -400 to -930 plays an important role in sensing nutrient levels and peptidoglycan components, which in turn have an effect on C signal production (Shimkets and Kaiser, 1982). In contrast to the A signal, which is diffusible, C signal requires cell-to-cell contact for its transmission (Kim and Kaiser, 1990), (Kim and Kaiser, 1990). The ability to transmit and respond to the C signal is influenced by proper cell alignment and cell density (Kim and Kaiser, 1990). In an elegant experiment by Kim and Kaiser, sporulation potential could be
restored to nonmotile csg\(^+\) cells by plating under the proper conditions (Kim and Kaiser, 1990). Normally, nonmotile csg\(^+\) cells cannot sporulate, presumably because motility is required to obtain proper cell alignment (Kim and Kaiser, 1990). Lack of motility was compensated for by plating the cells on a CF plate which had been prescored with fine emery cloth (Kim and Kaiser, 1990). The parallel scratches in the agar surface served to align the cells properly so that sporulation could proceed, presumably by restoring transmission of the C signal (Kim and Kaiser, 1990). As with the A signal, C signal appears to play a role in sensing cell density (Kim and Kaiser, 1990), (Li et al., 1992). Beyond this, the biphasic effect of C signal upon aggregation and sporulation provides a mechanism to coordinate the two processes with a single gene product. Increasing levels of C signal transmission may serve as a feedback mechanism to monitor cell packing. At the 30% threshold C signal stimulates further aggregation, and tighter cell contact. When the 83% threshold is reached transmission of the C signal signifies that aggregation is complete, and that the tightly packed cells are ready to sporulate (Kim and Kaiser, 1990). It is believed that the C signal activates late sporulation functions, which lead to the
final stages of cell differentiation (Kaiser and Losick, 1993), (Kim and Kaiser, 1990), (Li et al., 1992).

Two of the Dsg mutants have been mapped to the dsgA locus (Cheng and Kaiser, 1989). Point mutations in this locus result in arrested development; null mutations, generated by transposon insertion, however are lethal (Cheng and Kaiser, 1989). This evidence suggests that the dsgA gene is an essential gene (Cheng and Kaiser, 1989). Dsg mutants can be rescued by the addition of autocide AMI to the media (Rosenbluh and Rosenberg, 1989). AMI is a mixture of saturated and unsaturated fatty acids produced during normal vegetative growth (Rosenbluh and Rosenberg, 1989). The mechanism by which AMI rescues Dsg mutants isn't known and there is no evidence to suggest that AMI itself is the D signal.

The interrelationship of the four groups of signal mutants has been extensively studied (Hagen et al., 1978), (LaRossa et al., 1983), (Kuspa et al., 1986). Each of the four groups has been tested for its effect on the expression of 26 developmentally-regulated lac fusions (Kroos and Kaiser, 1987), (Kuspa et al., 1986). β-galactosidase activity can be detected from these fusions, in Sg+ cells, at times ranging from a few minutes to as late as 30 hours after development is initiated (Kroos et al., 1986), (Kroos and Kaiser, 1987).
Figure 4. Relationship of the Signal-Defective mutants. The horizontal bars represent the period of time prior to an observable developmental defect. The columns to the right indicate the aggregation and sporulation phenotypes of the respective mutants. (From Kaiser and Losick, 1993)
These experiments provide a means for resolving the arrested state of gene expression for each group. From these data, and the morphological data, Kaiser has developed a model of sequential gene regulation, as shown in figure 4 (Kaiser and Losick, 1993). In this model each signal relies upon the normal expression of all those prior to it. The B signal is required earliest, almost immediately after initiation of development (Kroos and Kaiser, 1987). All of the 26 fusions examined require B signal for their expression (Kroos and Kaiser, 1987). The A signal is required by approximately 3 hours of development; 3 fusions expressed prior to this time do not depend upon A signal for their expression (Kroos and Kaiser, 1987), (Kuspa et al., 1986). Neither asg or bsg mutants are able to aggregate or sporulate (Hagen et al., 1978). Phenotypic effects of a dsg mutation appear around 5 hours, about the time that the first D signal-dependent promoters are expressed (Hagen et al., 1978). Promoters dependent upon C signal are expressed as early as 6 hours, but the phenotypic effects of a csg mutation are not evident until approximately 11 hours (Hagen et al., 1978). As summarized in figure 4, dsg and csg mutants exhibit some degree of abnormal aggregation, and are unable to sporulate (Kaiser and Losick, 1993), (Hagen et al., 1978).
3. **Sporulation Mutants:** Only a handful of sporulation-specific mutants have been reported and these for the most part have not been extensively investigated (Hagen et al., 1978), (Morrison and Zusman, 1979). A systematic approach to isolating a large group of sporulation-specific mutants is reported in Chapter II and will be discussed briefly at the end of this section.

The translucent mound (tm) mutants reported by Zusman are proficient for aggregation, but temperature-sensitive for spore formation (Morrison and Zusman, 1979). Temperature shift experiments defined the critical time periods specific for each of the four mutants examined (Morrison and Zusman, 1979). Mutant strains were tested two ways. They were incubated at either the permissive temperature and shifted up to the non-permissive temperature at 3 hour intervals, or incubated at the non-permissive temperature and shifted down at 3 hour intervals. The former treatment defines the endpoint of the temperature-sensitive period and the latter defines a time point after which the cells cannot recover. The earliest temperature-sensitive period was over at 9 hours, and the latest at 30 hours (Morrison and Zusman, 1979). In these studies, visible cellular morphogenesis begins around 28 hours of development, suggesting that required components of the sporulation
pathway must be present nearly 19 hours prior to the onset of morphogenesis (Morrison and Zusman, 1979). Other than the timing and duration of temperature-sensitive periods, little else has been reported about the tm mutants.

In another study, insertions of a Tn5lac transposon were characterized in a global search for developmentally-regulated promoters (Kroos and Kaiser, 1984), (Kroos et al., 1986). Of 2,374 Tn5lac strains examined, only 36 had increased β-galactosidase activity during development (Kroos et al., 1986). Subsequent testing revealed that 7 of the 36 developmentally regulated fusions demonstrated a defect in sporulation (Kroos et al., 1986). Five additional spo' lac fusions were identified, but they were not developmentally regulated (Kroos et al., 1986). All but one of the spo' mutants were also severely defective in aggregation, indicating that the defect was not specific to sporulation (Kroos et al., 1986), (Kroos et al., 1990). The exception to this is the devRS locus reported by Kaiser (Kroos et al., 1986), (Kroos et al., 1990).

A Tn5 lac insertion into devR results in a reduction of sporulation by approximately 1000-fold (Kroos et al., 1986). Whereas devR does not appear to be strictly sporulation-specific, since aggregation is reduced, its
relative effect on sporulation is great enough for it to be considered here, especially since insertions in or near this locus were recovered in our mutant hunt (Chapter II) (Thony-Meyer and Kaiser, 1994). Expression of devR begins around 6 hours of development, and is partially dependent upon csgA expression (Kroos and Kaiser, 1987). Cell density also appears to play a role in controlling devR, with a correlation between high cell density and reduced expression (Thony-Meyer and Kaiser, 1994). Complementation studies with the wild-type locus have revealed that proper devR function is dependent upon gene position and length of the adjacent upstream region (Thony-Meyer and Kaiser, 1994). Partial diploids created by tandem duplication are able to efficiently rescue sporulation with as little as 2kb of flanking DNA (Thony-Meyer and Kaiser, 1994). However, if the second copy of devR, with 2 kb flanking, is inserted at a distant prophage integration site, sporulation is not rescued (Thony-Meyer and Kaiser, 1994). Increasing the upstream flanking sequence at the distant site to 4 kb results in partial rescue of sporulation (to 10% of wild type) (Thony-Meyer and Kaiser, 1994). This result is in stark contrast to that obtained with csgA, which is able to complement locally or at a distance (Li and Shimkets, 1988). An intriguing aspect of the devR studies is the
preliminary evidence for possible autoregulation. The observation is that a wt/devR::lac partial diploid exhibits reduced B-galactosidase activity compared to the haploid mutant strain (Thony-Meyer and Kaiser, 1994). Comparison of different tandem duplications revealed that the extent of B-galactosidase reduction depends upon the structure of the duplication (Thony-Meyer and Kaiser, 1994). If the wild-type copy is upstream of the mutant copy, and fused to an authentic dev+ upstream region, then B-galactosidase activity is reduced by 98% (Thony-Meyer and Kaiser, 1994). If the wild-type copy is located downstream of the mutant copy, and has 2 kb of flanking upstream DNA, then the reduction is about 48% (Thony-Meyer and Kaiser, 1994). These results imply that whereas 2 kb of flanking upstream DNA may be sufficient to restore sporulation, there are other aspects to devR control which may be influenced by even more distant elements (Thony-Meyer and Kaiser, 1994). While the possibility of autoregulation is an interesting concept, the evidence currently is not compelling. That devR expression is regulated is clear; that DevR is the direct regulatory factor is unproven. Hopefully, future experiments will serve to clarify this issue.

One area of the sporulation pathway that remains unresolved is the relationship between glycerol-induced
sporulation and starvation-induced sporulation. In the conventional view the two pathways overlap, and it is supposed that the genes involved in glycerol sporulation represent a subset of those involved in starvation. Dworkin believes that glycerol may act as an endogenous sporulation signal (Mueller and Dworkin, 1991). His evidence for this comes from a series of experiments in which exogenous glucosamine was able to rescue sporulation in a csgA mutant (Janssen and Dworkin, 1985). Further examination of the glucosamine effect suggests that glucosamine may increase phospholipase activity, which then results in release of glycerol from phospholipid (Mueller and Dworkin, 1991). Dworkin has been able to measure intracellular glycerol concentrations and show a correlation with exposure to glucosamine (Mueller and Dworkin, 1991).

If this were true, then one might expect to find a number of sporulation mutants that are defective for both types of spores. In reality, such mutants are the exception rather than the rule, and the conventional view may reflect more than anything else a disbelief in the possibility of dual sporulation pathways.

Among mutants defective for fruiting body (starvation) spores, only the translucent mound mutants of Zusman are affected for glycerol sporulation as well
(Morrison and Zusman, 1979). In contrast, all of the lac fusion mutants reported by Kaiser, including devRS, are proficient for glycerol sporulation (Kroos et al., 1986), (Kroos et al., 1990). In addition, the 47 sporulation mutants presented in chapter II are also able to sporulate in glycerol. Of course, the obvious question is: are there glycerol sporulation mutants defective for the starvation pathway? Burchard attempted to answer this by isolating 117 UV-induced glycerol (glc) mutants (Burchard and Parish, 1975). Upon further testing, 109 of the glc mutants were wild-type for starvation sporulation (Burchard and Parish, 1975). Of the remaining 8 strains, all were defective for aggregation, but only one demonstrated a sporulation defect when induced by starvation (Burchard and Parish, 1975). These results would only make sense if 1.) there was not substantial overlap of the two pathways, or 2.) the selection was biased towards mutants specific to the unique aspects of the glycerol pathway. There is a strong possibility that this is the case for Burchard's mutants since the selection process requires continued cell growth in the presence of glycerol. This requirement of the selection would suggest a bias towards mutants that cannot be induced, i.e. blocked in the initial step(s) of the glycerol pathway. Any mutant
blocked subsequent to the initiation step, most likely would be "stuck" and unable to continue growing, so that no visible colony would be formed.

From the above discussion it should be evident that very few sporulation-specific mutants have been reported, and aside from devR none have been well characterized. In chapter II we describe a global search for sporulation-specific mutants of M. xanthus. Previous mutant searches in other laboratories relied upon either visual examination of fruiting defects, or expression from promoter-lacZ fusions, to identify sporulation mutants. Each approach has its limitations and neither has been very successful in identifying mutations specific for the cell-autonomous functions of sporulation. Our approach was quite different and (as described in chapter II) relied upon a functional assessment of the ability to produce mature spores. In chapter II we will describe the isolation and characterization of 47 sporulation-specific mutants.

E. COMPARATIVE STUDIES

1. Phosphorylation: The role of protein kinases and subsequent protein phosphorylation has been studied intensively in higher eukaryotes and in bacteria. It comes as no surprise that second messenger/protein kinase
systems appear to play a significant role in the sporulation of lower eukaryotes and in prokaryotes. It is known that in *Saccharomyces cerevisiae* a reduction of cAMP levels triggers sporulation by activating several meiosis-specific genes (Losick and Shapiro, 1984). Furthermore, the gene IMEZ is believed to encode a protein kinase which acts as a positive regulator of the sporulation genes (Su and Mitchell, 1993). Spore formation in Dictyostelium is also tied to protein phosphorylation. Inhibition of cAMP-dependent protein kinase has been demonstrated to block the differentiation of pre-spore cells (Hopper et al., 1993).

Probably the best understood of these systems is that of *B. subtilis*. This system recognizes and integrates environmental signals to initiate sporulation via a series of phosphorylation steps. Key to this process is the phosphorylation of Spo0F by one of two protein kinases (Frach, Hoch, 1993). Spo0F then mediates the phosphorylation of Spo0A, a positive regulator of sporulation genes, including Spo0F (Strauch et al., 1993). Phosphorylation of the N terminal domain of Spo0A increases the DNA-binding affinity of the C terminal domain, which mediates the protein's regulatory effect (Strauch et al., 1993). Phosphorylated Spo0A activates expression of a number of sporulation genes including
SpoIIIG which encodes the sigma E factor that initiates a sigma factor cascade (Errington, 1993).

The role of protein phosphorylation is not known in M. xanthus sporulation, but a protein serine/threonine kinase has been identified (Munoz-Dorado et al., 1991). The pkn1 gene encodes a protein of 693 amino acids with sequence similarity to several known protein kinases (Munoz-Dorado et al., 1991). Expression of pkn1 is developmentally regulated and is expressed near the time of sporulation (Munoz-Dorado et al., 1991). Deletion of the pkn1 gene results in premature cell differentiation, suggesting that pkn1 plays a role in the timing of sporulation events (Munoz-Dorado et al., 1991).

2. **Sigma Factors:** In B. subtilis the most significant changes in gene expression during sporulation are a direct result of sigma factors (Errington, 1993). Most sigma factors recognize promoters through conserved sequences that are located at about -10 and -35 with respect to the transcription start site. Alternative sigma factors confer new promoter specificities on the core RNA polymerase. B. subtilis possesses one vegetative sigma factor; sigma A, and five minor sigma factors which play a role in sporulation (Errington, 1993). These sporulation-specific sigma factors are
expressed sequentially in a regulated "sigma cascade" and
serve to coordinate the proper timing and location of
developmental events (Errington, 1993).

In *M. xanthus* three sigma factors have been
identified. Sig A is the vegetative sigma factor,
alogous to sigma A in *B. subtilis* and sigma 70 in *E.
coli* (Inouye, 1990). In addition to Sig A, two other
sigma factors have been described; Sig B, and Sig C
Sig B is expressed during middle to late development
based upon studies with a lacZ fusion (Apelian and
Inouye, 1990). A deletion mutant of Sig B is capable of
normal fruiting body formation, but does not synthesize
protein S. The only observable phenotype for this
deletion mutant is a reduction of long term spore
viability (Apelian and Inouye, 1990). This suggests a
role for Sig B in events which influence spore maturation
and environmental resistance (Apelian and Inouye, 1990).

Sig C, in contrast, is expressed immediately after the
onset of development (aggregation) and is dramatically
reduced at the onset of cellular morphogenesis (Apelian
and Inouye, 1993). As with Sig B, a Sig C deletion
mutant is capable of fruiting body formation and
sporulation (Apelian and Inouye, 1993). The only
apparent difference between a Sig C deletion mutant and
wild type is the ability of the mutant to develop on semirich media (Apeleian and Inouye, 1993). Inouye offers the possible explanation that Sig C is involved with negative regulation of the initiation of fruiting body formation.

3. **Post Translational Modification**: Sporulation in *B. subtilis* requires post-translational modification of the pro-sigma E by a protease expressed in the same operon. Two of the genes required for *M. xanthus* development, asgA and bsgA, encode proteins with demonstrated protease activity (Planmann et al., 1992), (Gill et al., 1993). In the case of asgA, the sole function of this protease activity appears to be the production of peptides and amino acids required for the A signal, but the function of the bsgA protease has not been determined (Planmann et al., 1992). It is quite possible that this protease activity is required for post-translational modification in the course of normal development.

4. **Environmental Resistance**: Myxospores are resistant to harsh environmental conditions such as desiccation, UV light, and high temperature. The genes required for acquisition of these characteristics, and their mechanism of action, are not known. Studies of other sporulation
systems have identified specific genes associated with environmental resistance. In *B. subtilis*, the diaminopimelate operon contains a locus designated orfX which appears to play a role in the heat resistance of endospores (Chen et al., 1993). Disruption of orfX has no effect on vegetative growth, however it results in the formation of heat-sensitive spores (Chen et al., 1993). Furthermore, primer extension studies have revealed that orfX is only transcribed during late stages of sporulation (Chen, et al, 1993). Another example is the spi A gene of *D. discoideum* which affects environmental resistance and spore stability (Richardson and Loomis, 1992). Spores produced from a spiA mutan are morphologically indistinguishable from wild type, but are extremely sensitive to environmental conditions (Richardson, et al, 1992). Antibody labeling studies have revealed that in wild type the spiA gene product is closely associated with the inner face of the spore coat (Richardson, et al, 1992). Yet another example is the sprl gene of *S. cerevisiae*. The sprl gene has extensive DNA and protein sequence homology with the exgl gene which encodes an exo-1,3-beta-glucanase (Muthukumar et al., 1993). Mutants of sprl have reduced thermal resistance compared to wild type, and it is believed that sprl encodes a sporulation-specific exo-1,3-beta-
glucanase required for establishing thermal resistance (Muthokumar, et al., 1993).

5. **Morphogenesis**: In other sporulation systems, such as *B. subtilis*, much information has come from mutants blocked in morphogenesis. Unfortunately, in *M. xanthus* few cell-autonomous mutants have been described, let alone any blocked in morphogenesis. Of the few blocked mutants, all have been blocked prior to the initiation of morphogenesis. One exception to this is the spo77 mutant described in chapters II and III. Mutants at this locus arrest as ovoids. Presently, little is known about this locus, other than the fact that it seems to be required for the completion of morphogenesis.

In *B. subtilis*, several genes have been identified which affect the morphogenesis of forespore engulfment. All of these genes are sporulation-specific and play a role in shifting the normal division septum (Levin et al., 1992), (Henriques et al., 1992). The spoVE locus has amino acid sequence homology to the *E. coli* morphogénés, ftsW and rodA, located near murD and murG which are involved in peptidoglycan synthesis (Henriques et al., 1992).
Chapter II

Isolation of Sporulation Mutants

A. INTRODUCTION: *Myxococcus xanthus*, a gliding gram-negative soil bacterium, must survive periods of famine which occur in its natural environment. Myxobacteria respond to starvation by initiating a developmental program of cell differentiation which culminates in the formation of dormant, morphologically distinct spores exhibiting elevated resistance to common soil conditions such as dehydration or exposure to UV light (Sudo and Dworkin, 1969). Sporulating bacteria manifest altered patterns of gene expression (Kroos et al., 1986), protein synthesis (Inouye et al., 1979), and metabolic activity, including increased accumulation of trehalose (a carbohydrate storage molecule) (McBride and Zusman, 1989), increased activity in enzymes associated with gluconeogenesis (Bacon et al., 1975), (Filer et al., 1977), synthesis of spore-specific coat polysaccharides (Sutherland and Thomson, 1975), (Filer et al., 1977), and alterations in the synthesis and cross-linking of peptidoglycan (implicated in cellular morphogenesis) (White et al., 1968). The levels of at least 20 major proteins have been observed to change during sporulation (Inouye et al., 1979). One of these, protein S (a spore
coat protein), constitutes up to 15% of the total protein synthesized during sporulation (Inouye et al., 1979). Concomitant with the extensive redirection of metabolism and cellular morphogenesis, sporulating Myxobacteria engage in cooperative interactions involving millions of cells leading to the formation of a distinctive structure called a fruiting body in which the spores are housed. Physiological tests using temperature-sensitive mutants defective in fruiting or sporulation reveal critical time periods for each of these pathways early in development (Morrison and Zusman, 1979). Although the two pathways can function independently in mutants, there is clear genetic and biochemical evidence that the pathways are coupled in multiple ways in wild-type cells (Hagen et al., 1978), (Morrison and Zusman, 1979). Most significantly, signal (sg) mutants, which are deficient in cell-to-cell communication during development, are impaired in both fruiting and sporulation (Hagen et al., 1978). The recently purified C factor has been shown to rescue both fruiting and sporulation in Csg A mutants (Kim and Kaiser, 1991). The A-signal factor has also been isolated and shown to rescue asgA mutants (Kuspa et al., 1992).

Sporulation can be uncoupled from fruiting by agents such as glycerol. Addition of glycerol to a broth
culture results in rapid, synchronous, cell-autonomous conversion of vegetative cells to spores (Dworkin and Gibson, 1964). Glycerol spores appear grossly identical to starvation spores but in ultrastructural detail they are rather different (Inouye et al., 1979). Among other differences, glycerol spores contain no protein S (Inouye et al., 1979). The precise relationship between the starvation-induced and glycerol-induced sporulation pathways has not been defined. It has been proposed that glycerol (produced from phospholipid) is an endogenous inducer during starvation-induced sporulation (Mueller and Dworkin, 1991). On the other hand, almost all known sporulation mutants affect starvation or glycerol spores uniquely (Hagen et al., 1978), (Kroos et al., 1986), (Burchard and Parish, 1975).

Sporulation can also be uncoupled from fruiting genetically. Among known fruiting mutants, many exhibit normal or near-normal levels of sporulation, as judged by morphological examination and in some cases also by functional (germination) tests (Morrison and Zusman, 1979). On the other hand, there have been no systematic searches for mutants with a primary defect in starvation-induced sporulation. Sporulation deficiencies have been discovered as a secondary phenotype of mutants identified initially on other grounds (Hagen et al., 1978), (Kroos
et al., 1986), (Morrison and Zusman, 1979). Given the extensive changes in gene expression and metabolism during starvation-induced sporulation, it seemed inevitable that there should exist many undiscovered genes required specifically for sporulation. We report here the results of our search for, and characterization of, such genes.

B. MATERIALS AND METHODS:

1. Strains: All M. xanthus strains were derived from DZF1, a wild-type strain obtained from David Zusman. Cells were grown vegetatively in CTT broth (1% casitone, 10 mM Tris HCl, 8 mM MgSO₄, 1mM KPO₄; pH 7.5) or on CTT agar (CTT broth containing 1.5% Bacto Agar). Fruiting was done on CF agar plates (Bretscher and Kaiser, 1978). Cell densities in liquid culture were determined using a Klett-Summerson colorimeter with a 620 nm red filter, subtracting the absorbance of a media blank to obtain the net value (=Δklett). E. coli strain RM 990 was grown in LB thy broth (Silhavy et al., 1984) and used for propagation of the phages P1::Tn5 and P1::Tn5-132.

2. Phage Lysates: P1::Tn5 and P1::Tn5-132 lysates were prepared using standard methods for preparation of P1 lysates (Silhavy et al., 1984).
Mx4 lysates were prepared as follows. Donor strains were grown to ΔKlett=25 and infected with Mx4 at an M.O.I. of 0.01. The cultures were then incubated for 12-16 hours at 32°C, on a tube roller, until lysis took place. Chloroform (1-2 drops) was added to the cultures after which they were centrifuged at 12,000 X g for 10 min. at 4°C. The supernatants were then transferred to a clean tube and additional chloroform (1-2 drops) was added prior to storage at 4°C. Prior to being used for mapping experiments, each lysate was U.V. irradiated with a G.E. germicidal lamp at 2000 μW/cm² for 60 seconds. UV irradiation reduced the titer of virulent phage and also enhanced recombination.

3. **Mutagenesis:** Phage P1::Tn5 was used to deliver Tn5 to strain DZF1. A 0.1-ml volume of cells at ΔKlett=100 (∼2 x 10⁷/ml) was added to 0.1 mls of P1::Tn5 lysate (∼10⁸ phage) resulting in an M.O.I. of ∼5. Phage were allowed to adsorb for 20 minutes at room temperature. The mixture was then spread onto a CTT plate containing kanamycin at 10 μg/ml. Plates were incubated for 16-24 hours at 32°C and then overlayed with 2.5 mls. top agar containing enough kanamycin to bring the total plate concentration up to 40 μg/ml. Plates were then incubated an additional 4-5 days until colonies were visible.
4. Tests for Spore Function:

**Primary screen:** Kanamycin-resistant colonies were gridded onto a CTT master plate and a CF agar plate. These plates were incubated at 32°C for five days, a period which is at least 3 days longer than needed for complete development and sporulation by wt cells. The CF plates were then heated at 52°C for two hours to kill vegetative cells, cooled to room temperature and underlaid with casitone sufficiently concentrated to create a 1% concentration in the plate. The spores were allowed up to 4 days at 32°C for germination, which was 2-3 days longer than needed by the wild-type spores. Negative isolates were retrieved from the master plate.

**Spot test:** Cells were grown to mid-log phase in CTT broth and diluted back to ΔKlett=100. A 5 ml aliquot of diluted culture was centrifuged at 12,000 x G for 10 minutes at 4°C and the resulting cell pellet was resuspended in 1/10 volume of TM buffer (10 mM Tris, 8 mM MgSO₄, pH 7.5). Each CF plate received 8-10 spots (10μl each) of mutant strains as well as RC 27 (wild type) a positive control demonstrating that functional spores survived the subsequent heat treatment and germinated, and RC 54 (spo- mutant) a negative control demonstrating that vegetative cells were effectively killed by the heat
treatment. The spots were placed equidistant from the center of the plate in a circle - 1 cm from the plate edge. This geometry was important to obtain consistent responses to heating and nutrient supplementation. After incubation at 32°C for 5 days the plates were heated to 52°C and underlaid as described for the primary screen. Each spot was evaluated daily for 4-5 days. Plating was done in triplicate and only plates exhibiting the correct control responses (RC 27 - growth, RC 54 - no growth) were used to evaluate the mutant responses.

5. **Fruiting Body and Cell Morphology:** The plates just described (spot test), or equivalent ones, were examined under a dissecting microscope during fruiting to assess fruiting body morphology.

To examine cell morphology, agar plugs, supporting the fruiting bodies, were excised from the plates with a scalpel. The fruiting bodies were fixed by floating them onto the surface of a 3% glutaraldehyde solution. After fixation they were placed onto a cover slip and either critical point dried or lyophilized. Subsequent to drying, the samples were mounted on studs and sputter coated. The samples were examined and photographed with a Jeol 840 A scanning electron microscope.
6. **Genetic Mapping:** Tetracycline-resistant, kanamycin-sensitive replacement strains were created from each of the 47 spo' mutants by the method of Kaiser. These strains served as recipients for the mapping crosses. For each cross, 0.1-0.3 ml of Mx4 phage lysate (grown on a kan', spo' mutant) was added to 0.2-0.5 ml of recipient cells (∆Klett=100). After 20 minutes at room temperature to allow phage adsorption, the mixture was incubated at 35°C for 2 hours and then 40μl of Mx4 anti-sera (provided by L. Shimkets) was added. The anti-sera renders Mx4 incapable of adsorption and prevents subsequent rounds of infection. The mixture was then spread onto a CTT plate containing (20 μg/ml) of kanamycin. After overnight incubation of the plate at 35°C, top agar containing sufficient kanamycin was added to give a final overall drug concentration of 40 μg/ml. The plate was incubated an additional 5-7 days and then transductants were picked and analyzed for tetracycline resistance. Linkage is given as the ratio of tea' to total kanamycin resistant transductants.

7. **Glycerol Induction:** Log phase cultures (∆Klett of 100-160) were diluted with CTT, if necessary, to a ∆Klett= 100. Glycerol was then added to a final concentration of 0.5 M. Cultures were incubated at 32°C.
for ~16 hours on a tube roller. Glycerol induction was assessed by microscopic examination for the presence of spores and by testing for sonication resistance as follows. A two ml volume of culture was sonicated in a test tube with a microtip probe for 10 seconds and 60 seconds at a setting of 30 watts. A 0.1 ml aliquot was removed after each time point and serially diluted. The dilutions were then plated on CTT agar to determine the titer of colony forming units (c.f.u.).

8. **Library construction:** *M. xanthus* DNA was partially digested with the enzyme Sau3A and size fractionated using a sucrose gradient. Fragments in the size range of 18-28 kbp were ligated to Bam HI-digested pLAFR 3. A map of the cosmid vector pLAFR 3 is shown in figure 14. The DNA concentrations of the ligation reactions were sufficiently high to ensure concatamerization. The ligation mixtures were packaged into lambda phage particles using commercial packaging extracts from Promega.

9. **Restriction mapping:** All restriction digests and ligations were performed with commercially obtained restriction enzymes as per the manufacturer's instructions.
10. **Southern hybridisations:** DNA samples were digested and separated on 0.5-0.7% agarose gels in 1x TBE (Sambrook et al., 1989). The separated fragments were transferred to nitrocellulose membranes (Schleicher & Schuell, BA-S 83) by capillary transfer. After drying for 2 hours, at 80°C, in a vacuum oven, the membranes were sealed in a plastic "seal-a-meal" bag and incubated in prehybridization buffer (50% formamide, 6x SSC, 5x Denhardt's, 100 ng/ml denatured salmon sperm DNA) at 42°C overnight. Radiolabeled probe was prepared by the random primer method, using a random primer kit from USB, as per manufacturers instructions.

C. **RESULTS**

1. **Isolation of mutants:** Our goal in these experiments was the identification of mutants specifically affected in sporulation. In particular, we sought to avoid two prominent aspects of previous mutant searches, namely, the association of sporulation defects with aggregation defects and the use of visible rather than functional criteria for spore integrity. The scheme that we followed is outlined in Figure 5. Briefly, a total of 2288 transposon-containing mutants obtained in 11 independent experiments were allowed to fruit on CF agar. After treatment of the fruiting bodies to kill residual
Figure 5. Mutant Isolation Method. As described in the Materials and Methods, wt cells of DZF1 were infected with P1::Tn5 and kanamycin resistant colonies were selected. These were gridded onto CTT and CF plates and incubated at 32°C for 5 days. The CF plates were heat treated at 52°C for 2 hours and underlaid with concentrated casitone. Germination was assessed, and negative points on the grid were retrieved from the CTT plate for further analysis.
vegetative cells (and immature spores), germination was initiated by the addition of concentrated nutrients. One hundred twenty-nine non-germinating candidates (5%) were retrieved from a master plate. Of these, forty proved to be Spo⁺ on retesting and were not considered further (see below).

We established a standardized assay for fruiting and sporulation for detailed analysis of the mutants. This assay, which incorporated features of equal inocula and geometrically equivalent position of fruiting spots on agar plates, yielded results that were internally consistent over an extended period of time and from one plate to another (see M&M). In the standardized assay, cells were allowed to fruit for 120 hours and to germinate for up to 168 hours. The wild-type strain RC 27, under these conditions, completed fruiting by 48 hours, and germinated within 24-48 hours after nutrient addition. A spo⁻ control, RC 54, exhibited no discernable germination even after 7 days of incubation. Strains which exhibited no discernable growth for at least 48 hours after the first appearance of growth of RC 27 and no more than isolated points of growth 72 hours after the appearance of RC 27 were defined as spo⁻. A representative spot test is illustrated in figure 6. Eighty-nine strains met these criteria, and in fact the
Figure 6. Spot Test for Heat Resistance. This figure shows the results of a typical spot test for heat resistance. The time above each panel is the number of hours after the addition of casitone. The spots are labeled as follows: RC 27 (A), RC 363 (B), DK 4414 (C), and RC 362 (D). Spot A (RC 27) is wild type strain DZF1 and the others are sporulation mutants from this study, or in the case of DK 4414 from another laboratory. Spot A shows growth by 48 hours and the rest of the spots show no growth until 192 hours, when spots C and D have 1 and 3 isolated points of growth. Spot B demonstrates no growth by 192 hours.
Spot Growth After Heat Treatment

0 hours

48 hours

96 hours

192 hours
majority of the 89 mutants showed no germination whatsoever. As a consequence of the extended fruiting and germination periods, mutants which were merely delayed, rather than blocked in sporulation or germination, would have scored as spo+ and are not among our mutants.

2. **Fruiting body morphology**: Among the 89 spo mutants a full range of FB morphology was observed, from no aggregation at all to normal, darkened fruiting bodies. We set aside 42 strains that were not able to progress at least to the point of forming discrete, well-defined fruiting bodies. Among the remaining 47 strains, 22 could not be distinguished from wt on the basis of fruiting morphology, whereas the last 25 exhibited subtle alterations in FB size, shape, or refractility (as judged by fb darkness). Illustrative examples of fruiting body morphology are shown in Figure 7.

3. **Cell morphology**: Fruiting bodies of each mutant were examined by scanning electron microscopy 120 hours after initiating fruiting to determine the terminal cell morphology. The results are detailed in table II and may be summarized as follows. In the majority of the mutants, spheres were the predominant cell form observed
Figure 7. Representative Fruiting Body Morphologies. This figure compares the fruiting body morphologies of four mutant strains with wild type. Each comparison is shown at low (A) and high (B) magnification. Strain RC 27 is wild type and produces sharply defined, very dark fruiting bodies. The mutants presented represent subtle differences in size (363, 401), shape (360), and darkness (362).
at the end of development. In many cases, the spheres were indistinguishable from those of wild type (Figure 8, panel A), but in other cases the spheres appeared slightly deformed or, in one case, collapsed, presumably indicating that these spores are unusually sensitive to the conditions of preparation for microscopy (panels B, D). In strain 386 the spheres were strikingly smaller than those of wild type (mean diameter 0.7 relative to wild type). In a few cases spheres were accompanied by rods, either as a minority population (410, panel H) or as a roughly equal component (379, panel G). In strains 385 (panel E) and 377, spheres were accompanied by a few unusually large, elongated cells which we call myxoshmoos.

In three mutants morphogenesis arrested prematurely. Strain 388 (panel C) and 397 yielded predominantly rods, whereas 363 yielded ovoid forms. Strain 401 was unusual in displaying dimorphic behavior. Both large and small fruiting bodies were observed, the large ones filled with rods (panel J) at the same time as the small ones were filled with ovoids. Finally it is important to note that in about a third of the strains the fruiting bodies were covered with a layer of unidentified opaque extracellular material that interfered with visualization of the cell types, particularly rods and ovoids.
Table I. Physical mapping data. Presented here is the physical mapping data from Chen et al., 1990. The tet replacement strain numbers are given along with the insertion number in parentheses. The fragment letter refers to the chromosomal Asel fragment where the insertion is located. The fragment sizes indicate the two fragments produced from digestion of the Tn5-132 Asel I site when it is located in that particular chromosomal fragment. The fragment sizes indicate how far from one end of the chromosomal fragment the Tn5-132 is located, but do not indicate which end.
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<th>RESTRICTION FRAGMENTS</th>
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<td>F</td>
<td>30/270</td>
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<td>Ω 147</td>
<td>J</td>
<td>26/590</td>
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Figure 8. Representative Cell Morphologies. This figure compares the cell morphologies of 9 mutants with wild type (RC 27). Photographs were taken by SEM at a magnification of x4500.
4. Genetic and physical mapping: We used two-factor transductional crosses to organize 43 of the 47 mutations into 8 distinct linkage groups. All members of any one group are cotransducible by phage Mx4 (a generalized transducing phage of M. xanthus) with a single reference mutation; the eight reference mutations are unlinked to one another. The physical map locations of the reference mutations of six groups have been reported previously (Chen et al., 1990); they are widely separated, consistent with our transduction results. A summary of the physical mapping data is shown in table I. Three strains, RC 402, 409, and 410 could not be assigned with confidence to a linkage group. Strain 402 demonstrated a high percentage of linkage to more than one of the reference strains, making assignment to a linkage group impossible. Strains 409 and 410 yielded insufficient numbers of transductants (<10) for analysis after several crosses. Finally, strain RC 157 was not included in the linkage study because physical mapping determined the insertion site to be indistinguishable from that in RC 362.

5. Glycerol induction: All of the aggregation-proficient mutants were assessed for their response to glycerol
induction. After incubation in the presence of glycerol each mutant was examined visually under a microscope to check for sporulation. All 47 of the mutant strains produced myxospores in response to glycerol. Since visual examination reveals nothing about the functional integrity of glycerol spores, each mutant was also tested for sonication resistance.

After glycerol induction, cultures were sonicated for 10 seconds and 60 seconds. During glycerol induction the cells tend to clump. Ten seconds of sonication breaks up clumps of cells and allows a reference titer to be made for comparison to longer periods of sonication (60 sec). Vegetative cell numbers are reduced only slightly by 10 seconds of sonication under these conditions, whereas 60 seconds of sonication reduces the viable titer by 4-5 orders of magnitude as shown in Figure 9. Sonication of wild type glycerol spores for 60 seconds results in a 27-67% drop in C.F.U. compared to the 10 second time point (Figure 4). Each of the mutants was titered after 60 seconds of sonication and the percent change from the 10 second point was determined. These results are presented in Table II. The reduction of mutant titers was in the range of 0-80%. This degree of sonication resistance is equivalent to that displayed by wild type.
Figure 9. Relative Sonication Resistance of Vegetative Cells and Glycerol Spores. Vegetative cells and glycerol spores were prepared as described in the Materials and Methods. Samples of each were sonicated for time periods ranging from 10-60 seconds and then titered for the number of cfu. Each point represents the average value for 3 trials (spores) or 6 trials (vegetative cells). Spore samples were sonicated for 10 seconds prior to the time course, to provide an accurate initial titer.
6. **Comparison of RC 362 to DK 4414:** The strain DK 4414 carries the Ω4414 insertion described earlier. The insertion Ω4414 lies in the devR locus described in Chapter I. Since physical mapping placed the spo 147 locus at, or near the site of Ω4414 we decided to determine the relationship between the two mutations and compare their phenotypes directly.

Strain DK 4414 was fruited under our test conditions along side of RC 362 (Ω147). The strain RC 362 is the tet replacement strain of RC 147. A comparison of their fruiting bodies is shown in figure 10. DK 4414 demonstrates some aggregation, but does not form well-defined fruiting bodies. The mounds produced by DK 4414 are much more diffuse, and less darkened, than the fruiting bodies of RC 362. DK 4414 would have been excluded from our collection of sporulation-specific mutants.

A cosmid library was made from strain RC 147 and a clone of the Ω147 locus insertion was isolated on the basis of kanamycin resistance. The 147 clone (pRCC 12) was restriction-mapped and found to contain the entire Tn5 insertion, along with approximately 20 kb of *M. xanthus* DNA to one side and approximately 1-3 kb to the other side. The clone pRCC 12 was restriction-digested
and probed with total pLT-5 DNA. These results are shown in figure 11. The plasmid pLT-5 contains a 5 kb insert which spans the devRS region along with 2 kb of upstream-flanking DNA (see figure 12). The probe hybridized to the two large Hind III fragments. The 25-kb Hind III fragment extends from the Hind III site in Tn5 and includes a small amount of M. xanthus DNA, along with the vector pLAFR 3. The slightly smaller (22 kb) fragment extends from the other side of Tn5 and includes the majority (18 kb) of the insert. In order for pLT-5 to hybridize to both of these fragments it must span the site of the Ω147 Tn5 insertion (the vectors do not crosshybridize; see below). This is confirmed by the Bam HI digest. The large Bam HI fragment originates in Tn5 and includes the same flanking region as the 25 kb Hind III fragment, including the vector. The 10 kb Bam HI fragment includes approximately 3 kb of Tn5 DNA and 7 kb of flanking M. xanthus DNA. The probe hybridizes to both of these fragments, confirming that pLT-5 spans the insertion site. The Bam HI/Hind III lane is complicated by partial Hind III digestion. As a result, there is hybridization to the remaining 10 kb Bam HI fragment, as well as the predicted 8 kb Bam HI/Hind III fragment. The Eco RI digest was included as a control for vector cross-hybridization. There are two Eco RI sites in pLAFR 3,
one on each side of the insert. An Eco RI digest excises
the insert fragment in two pieces, producing a 22 kb
vector fragment which does not hybridize to pLT-5.

D. DISCUSSION

Several previous isolations of developmental mutants
of M. xanthus have been based on a simple visible
phenotype, namely the failure to form mature fruiting
bodies (Hagen et al., 1978), (Morrison and Zusman, 1979),
(Kroos et al., 1986). Many of these mutants exhibit
associated defects in sporulation (i.e., reduced yield of
visible spores) and have been used to argue that fruiting
and sporulation are coupled processes. Another approach
was to screen Tn5-lac insertions in M. xanthus for
developmentally regulated lacZ expression (Kroos et al.,
1986). The majority of the developmentally regulated
genes found in that study did not have a visible mutant
phenotype. In all of these studies taken together, only
a few mutants were found that exhibit a developmental
defect bearing primarily on sporulation (Morrison and
Zusman, 1979), (Kroos et al., 1986). Yet it seemed to us
that there should exist a substantial number of such
genes, given the dramatic metabolic and morphological
changes that occur as cells become functional spores.
The identification of such genes would be significant in
**Table II. Summary of Mutant Phenotype and Linkage Data.**

Mutants are organized by genetic linkage group. In the percent linkage column (r) refers to the reference strain for each group. In the terminal cell morphology column the letters in parenthesis are defined as follows: (b), see text; (c), collapsed; (d), deformed; (m), presence of myxoshmoos; (sm), small; ND, not determined. Not determined means that the cell morphology was obscured by large amounts of extracellular material and the cell morphology could not be accurately determined (see text). Glycerol spore survival is given as the percentage of survival after 60 seconds of sonication, as compared to 10 seconds.
<table>
<thead>
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<th>Genotypic Linkage Group</th>
<th>Strain (allele)</th>
<th>Percent Linkage (number)</th>
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<th>Terminal Cell Morphology</th>
<th>Glycol Spore Survival</th>
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at least two respects: their roles in cellular morphogenesis could be defined, and their regulation could be understood. In the present study we have used a method of screening random Tn5 insertions in M. xanthus specifically for a defect in sporulation without regard to fruiting behavior. Approximately half of the sporulation-defective isolates were found to have a major fruiting defect; these were set aside as many of them could have been identical to previously described fruiting mutants. We were left with 47 mutants out of approximately 2,000 colonies. A significant feature of the approach we took was that sporulation was assessed by a functional test, i.e., germination. We discovered many mutants that form morphologically normal spores, housed in normal-looking fruiting bodies, yet the spores do not germinate. Such mutants would have been overlooked in earlier screens, and their recovery may account for the higher yield of sporulation mutants in our experiment as contrasted to that of Kroos & Kaiser (Kroos et al., 1986).

As indicated below, the detailed developmental and morphological phenotypes of the mutants are heterogeneous and defy simple classification. However, genetic and physical mapping have organized 44 of the mutations into eight groups. All the members of any one group are
Figure 10. Phenotypic comparison of DK 4414 and RC 362. Strains DK 4414 and RC 362 were grown and spot tested as described in the Materials and Methods. The fruiting body morphology is shown at low (A) and high (B) magnification for each strain.
transductionally linked to a single reference mutation of
the group, and are transductionally unlinked to the
reference mutations of all other groups. Therefore, our
group of mutations embraced at least eight genes, and
probably more (see below).

The reference mutations for six of the linkage
groups were included in a large set of transposon
insertions physically mapped by Chen et al. In one case,
our group six, the site of insertion was not
distinguished from that of a developmentally regulated
insertion exhibiting a sporulation defect, Ω4414 reported
by Kaiser, it was a formal possibility that Ω147 and
Ω4414 were at opposite ends of the same 616 kb AseI
fragment. To clarify the relationship between Ω147 and
Ω4414 we compared the fruiting phenotypes of the mutants
and used Southern analysis to localize the two insertions
with respect to one another. The fruiting phenotype of
the two mutants is very different and suggests that the
mutations are in different genes. It is possible that
the phenotypic differences are due to different strain
backgrounds. The insertion Ω4414 is in a DK 1622
background, while Ω147 is in a DZF1 background. In our
experience, (with Ω77 for example) aggregation effects
are less pronounced in strain DK 1622, suggesting that
Ω147 would produce fruiting bodies just as well in such a
background and the phenotypic differences would remain. However, until the two mutations are compared in an isogenic strain background, this issue will not be resolved.

Southern analysis indicates that the two insertions are in close proximity. A 5 kb clone which spans the devRS region hybridized to DNA on either side of Ω147. The exact relationship between Ω147 and devRS is still undetermined and will have to be resolved by either more detailed Southern analysis or DNA sequencing. If it turns out that Ω147 lies within devR or devS then the answer will be clear, but if Ω147 lies outside the devRS region, we will have to consider the possibility that the spo-147 phenotype reflects altered devRS expression. Normal function of devR requires significant stretches of intact upstream-flanking sequence. Insertion of a transposon into one of these flanking regions could adversely affect devR expression without necessarily producing the same phenotype as a knockout.

The other physically mapped reference mutations are clearly distinct from developmental mutations that have been physically mapped. They lie at least 7 kb (group five) or more than 20 kb (other groups) away from the nearest physically mapped insertion regardless of phenotype (Chen et al., 1990). Since transducing phage
Mx4 can package about 60 kb of DNA (Campos et al., 1978), it is possible that some of our mutations (other than the reference mutations) could overlap previously mapped insertions; this remains to be determined. Neither the sasA mutation (Kaplan et al., 1191) nor the "translucent mound" mutations (Morrison and Zusman, 1979) have been physically mapped, but our mutants, which sporulate normally in response to glycerol, are distinct from the "translucent mound" mutants, which fail to do so. In summary, then, five of the reference insertions identify previously unknown genes. One reference insertion, Ω147 may overlap or be identical to a previously described mutation Ω4414 (devR). Two groups were not mapped physically, leaving their uniqueness uncertain.

Almost half of the mutants obtained in the study are cotransducible with the insertion in RC 147. The two-factor linkage values range from 11-99%, indicating the likely presence of several sporulation genes at this locus. The high yield of mutation here may thus be roughly a reflection of target size. Mutants within this group exhibit variety in their aggregation and cellular phenotype, but not in a way that obviously correlates with transductional linkage. For instance, mutation 149, 160, and 171 are all highly linked (89-99%) to RC 147, but they do not have identical phenotypes. Similarly,
Figure 11. Hybridization of pLT-5 to pRCC 12. The restriction map of pRCC 12 (N147) is shown with the relative position of the Tn5 insertion. Restriction data places the 2.1 and 9 kb Bam HI fragments adjacent to one another but does not provide the order. Also shown is a Southern hybridization of digested pRCC 12 probed with pLT-5. Analysis of the hybridization data places the pLT-5 probe in the approximate position shown with respect to the pRCC 12 map. See text for analysis of the hybridization data.
the large subgroup that is weakly linked (11-75%) to RC 147 includes examples of wt, misshaped, and undersized fruiting bodies, as well as normal and abnormal terminal cell morphology. The two-factor linkage values do not have sufficient resolution to accurately estimate the number of genes at this locus and complementation data are not yet available. If we assume that the insertions produce a null phenotype and that distinct phenotypes signify different mutant genes, then, combining transductional and phenotype data we can estimate that group 6 includes mutations in at least 6 genes exemplified by RC 147, 171, 146, 195, 145, and 137. Inspection of the remainder of table II on similar basis suggest the presence of 2 distinct loci in groups 1, 3, 5, 7; three loci in group 8, and 4 loci in group 4. This tentative count of genes requires experimental confirmation, and in particular, the possibility of polarity effects within operons needs to be evaluated. 

Both starvation and exposure to glycerol lead to grossly similar cellular morphogenesis (Dworkin and Gibson, 1964), but the detailed relationship between the morphogenetic programs induced by starvation and by glycerol is not clearly defined. There are some points of overlap. For example, both pathways lead to the inclusion of protein U in the spore (Gollop et al.,
1991), both types of spores are similarly resistant to sonication (Sudo and Dworkin, 1969), and both pathways of sporulation are blocked in certain mutants (Morrison and Zusman, 1979), (Hagen et al., 1978). On the other hand there are many points of dissimilarity. Some proteins (e.g. protein S) are found only in starvation spores (Inouye et al., 1979), the glycerol spores are much more heat-sensitive than starvation spores (Sudo and Dworkin, 1969), there are major differences between the two spore types apparent in ultrastructural detail (Inouye et al., 1979), and a large number of mutants are blocked uniquely in one pathway or the other (Hagen et al., 1978), (Burchard and Parish, 1975), (Kroos et al., 1986).

Despite this accumulated information suggesting more differences than similarities of the two spore types and their pathways of formation, it remained plausible that most of the "end-effector" genes of cellular morphogenesis would be shared by the two pathways. Since our mutant search was restricted to genes affecting spore function, we expected that "end-effector" genes would be among those we found. We were therefore surprised to discover that all 47 mutants formed functional glycerol spores normally.

We can offer several hypotheses to explain this surprising result. First, it may be a direct, though
unintended consequence of our experimental approach. The functional test that was applied incorporated a step of heat exposure to kill vegetative cells remaining in fruiting bodies, prior to germination of the spores. Any mutation that prevented development of heat-resistance in the spores would of course be scored as spo'. However, wt glycerol spores are much less heat-resistant than starvation spores (Sudo and Dworkin, 1969), so any mutation specifically affecting heat resistance of starvation spores would not be expected to have an effect on glycerol spores. In a similar vein, the screen trapped only those mutants with a severe down phenotype. The appearance of vegetative growth following addition of nutrients to the fruiting bodies was delayed at least 72 hours in the mutants relative to the wild type (and in most cases there was no growth at all.) Under the conditions we used, even a single viable spore would have produced a visible colony. These facts suggest that the mutant fruiting bodies contain less than 0.1% the number of viable spores relative to wild type. If end-effector mutants are not so severely affected as this, they would have been missed by our screen. Although either of these possibilities could account for the ability of some of our mutants to sporulate in glycerol, neither seems
general enough to explain glycerol sporulation in all 47 mutants.

Another view of these results is that they accurately reflect the intrinsic organization of sporulation pathways in *M. xanthus*. Even though it is apparent that all sporulating cells must remodel their peptidoglycan and diminish their metabolism (as examples), *M. xanthus* may possess substantially redundant systems for doing so, one activated in response to starvation, the other in response to glycerol. A mutant such as RC 77, in which starvation spores have a distinctive arrest morphology, would be readily explained by functional redundancy. There is some precedent for redundancy in *M. xanthus*. For example, the genes ops and tps encode highly similar proteins subject to differential regulation (Inouye et al., 1983), (Downard, 1987), (Downard et al., 1988). Evidence has also been presented for an alternative aggregation system active at low temperature (O'Connor and Zusman, 1990). Redundancy would also help to explain the general absence of a developmental phenotype in most mutants of the developmentally regulated genes identified by Kroos & Kaiser (Kroos et al., 1986). Still, it must be noted that other than ops and tps, specific redundant gene pairs have not been identified.
Chapter III

Characterization of the spo 77 locus

A. INTRODUCTION: The myxobacteria are gram negative, gliding bacteria which possess the ability to sporulate. Sporulation is normally induced by starvation and occurs in the context of cooperative, multicellular interaction. This cooperative behavior, accomplished via intercellular signalling, results in a cellular structure called a fruiting body. It is within the fruiting body that sporulation takes place.

Sporulation is a process in which the cell converts available nutrients into storage molecules, reduces its metabolic activity to undetectable levels, and restructures itself for isolation from the surrounding environment (McBride and Zusman, 1989), (Bacon et al., 1975), (Sudo and Dworkin, 1969). The extensive changes associated with sporulation result from carefully orchestrated changes in the pattern of gene expression. Numerous developmentally regulated genes have been identified using lacZ-promoter fusions (Kroos et al., 1986). Further studies have revealed that these developmental promoters are sequentially expressed and
under tight temporal control (Kroos and Kaiser, 1987), (Kuspa et al., 1986).

Biochemical data corroborate the genetic evidence for sporulation-specific gene expression. Pulse label experiments reveal an altered pattern of protein expression during development (Inouye et al., 1979). The levels of at least 20 proteins change in the course of development, some of which represent the synthesis of novel sporulation proteins (Inouye et al., 1979). These changes in gene expression and protein synthesis serve to redirect the cellular metabolism. Much of the cell's resources are utilized for the production of storage molecules, such as trehalose, and synthesis of spore coat polysaccharides (see below). Since *M. xanthus* requires monosaccharides for these two processes, and yet, incorporates sugars poorly, there is a dramatic increase in gluconeogenesis associated with the induction of sporulation (Bretscher and Kaiser, 1978), (Bacon et al., 1975), (Filer et al., 1977).

In *M. xanthus* many of the biochemical aspects of sporulation have been studied in glycerol-induced spores. Glycerol is an artificial inducer of sporulation which enables the preparation of homogeneous, synchronized cell populations (Dworkin and Gibson, 1964). There is some evidence to suggest that endogenous glycerol derived from
phospholipid may play a role in the sporulation pathway (Mueller and Dworkin, 1991). It has been observed that exogenous glucosamine can rescue sporulation in certain developmental mutants (Janssen and Dworkin, 1985). Glucosamine appears to increase the activity of phospholipase and some believe there is an increase in endogenous glycerol as a result (Mueller and Dworkin, 1991). Spores produced by glucosamine rescue resemble glycerol spores rather than starvation spores (Mueller and Dworkin, 1991). Glycerol spores are not identical to starvation spores and the data obtained from glycerol spores may not be completely applicable to starvation spores (Inouye et al., 1979).

Morphogenesis is perhaps the most striking feature of sporulation in any organism. For *M. xanthus* this is particularly true, as elongated vegetative rods convert to spherical spores (Dworkin and Sadler, 1966). The reshaping of the cell is also accompanied by formation of a thick, multilayered spore coat (Dworkin and Sadler, 1966). Very little is known about how the spore coat is synthesized or assembled. Chemical analysis indicates that it is approximately 75% polysaccharide by weight (Filer et al., 1977). Three developmentally regulated proteins, protein S, protein U, and protein C, are all associated with the spore coat, but their function is
unknown. Experiments with protein S have shown that it is capable of self-assembly on the spore surface, in the presence of calcium (Inouye et al., 1979). It is not known if the cellular reshaping occurs all at once, or in a series of stages. Observations of glycerol spores suggest that there may be discrete steps in the process (Dworkin and Sadler, 1966). Intermediate morphologies have been documented and described, but there is no definitive evidence that these represent discrete stages brought about by distinct biochemical mechanisms (Dworkin and Sadler, 1966). In other systems, such as B. subtilis, this question has been addressed by isolating mutants blocked with intermediate morphologies (Errington, 1993). RC 77 is the first such described mutant in M. xanthus. This made its spo 77 locus an ideal candidate for further analysis and the work described here.

B. MATERIALS AND METHODS

All materials and methods are the same as those described in chapter II. Only the materials and methods, or specialized conditions, unique to the experiments presented in chapter III are presented here.
1. **Bacterial strains:** All of our *M. xanthus* strains were derived from DZF1, with the exception of RC 7 and RC 250 which were derived from DK 1622. DK 1622, another wild type strain, was obtained from Dale Kaiser. Cells were grown vegetatively in CTT broth (1% casitone, 10 mM Tris HCl, 8 mM MgSO₄, 1mM KPO₄, pH 7.5) or on CTT agar (CTT broth containing 1.5% Bacto Agar). Fruiting was done on CF agar (Bretscher and Kaiser, 1978) plates. Cell densities in liquid culture were determined using a Klett-Summerson colorimeter with a 620 nm red filter, subtracting the absorbance of a media blank to obtain the net value (=Δklett) *E. coli* strain RM 990 was grown in LB thy broth (Silhavy et al., 1984) and used for propagation of the phage P1 and the described cloning vectors.

2. **Fruiting body and cell morphology:** For the time course of cell morphology, fruiting bodies were scraped from the surface of the CF plate and suspended in TM buffer on a glass slide. Slides were examined with a light microscope under oil immersion and photographed.

3. **Glucosamine test:** Cells to be tested for glucosamine response were grown to mid-log phase in CTT broth and diluted back to ΔKlett=100. Ten ml of each strain was
Figure 12. Time Course of Morphogenesis. The two strains, RC 27 (wt) and RC 77 (Ω77) were prepared and spot tested as described in the Materials and Methods. Cells were scraped from the agar plate at the times shown and suspended in TM buffer on a glass slide. Cell morphology was examined and photographed using a light microscope and under oil immersion.
pelleted and resuspended in 1.0 ml of TM buffer. Aliquots of 0.1 ml were spread onto each of 10 CF plates per strain and the plates were incubated at 32°C. At four hour intervals, starting at time zero, center plugs were cut from one plate for each strain using a Falcon #2063 6 ml plastic test tube. For a plate with a 35 ml agar volume, this removes a 0.5 ml plug, which was replaced with a mixture of glucosamine and molten CF agar. CF agar was mixed with 1.0 M glucosamine in a 9:1 ratio, resulting in a 100 mM final glucosamine concentration. A total of 10 time points were tested, ranging from 0-36 hours. Plates were incubated at 32°C for a total of 5 days, after which time the plates were heat tested for the presence of functional spores.

4. Restriction mapping and subcloning: All restriction digests and ligations were performed with commercially obtained restriction enzymes according to the manufacturer's instructions. Subcloning into the vector pLJS 49 required an unconventional approach due to the large size of the vector (see figure 13), and the size of the insert fragments. The vector, pLJS 49, and source plasmids, pRCC 43, 44, 45, 46, 47 were double-digested with Hind III and Eco RI. The insert fragments and vector were gel purified, and ligated at a high DNA
Figure 13. Comparison of RC 7 (DK 1622) and RC 250 (Ω 77). A comparison of the terminal cell morphologies between wt (RC 7) and a spo 77 mutant (RC 250) in a DK 1622 strain background.
(SEM at x4500)
concentration ( >10 μg/ml) to ensure concatamerization. Following ligation, each ligation mix was digested with Eco RI, diluted 50x, and religated. The dilute ligations were done with excess ligase for approximately 16-20 hours, at the end of which time the samples were concentrated with sec-butanol and ethanol precipitated. Precipitated DNA was resuspended in TE and electroporated into E. coli. Kanamycin-resistant colonies were then screened for the presence of the proper recombinant plasmids.

5. Electroporation: Recipient strains were pelleted at 10,000 x G for 10 minutes at 25°C and washed with distilled water. This was repeated 3-5 times. At the time of the final wash, the cells were resuspended in distilled water at 1/100 the original volume. For E. coli strains, 80 μl of cells were placed into a 0.2 cm cuvette along with 1-3 μl of DNA and electroporated using 2.50 kVolts, 200 ohms, and 25 μF. M.xanthus strains were electroporated using 0.65 kV, 400 ohms, and 25 μF. The same volumes were used for M. xanthus as for E. coli, except that all manipulations were done at room temperature.
Figure 14. Cloning Vectors. The restriction maps of pLAFR 3, pLJS 49, and pREG 511 are shown. pLAFR 3 and pREG 511 are cosmid clones and were used for library constructions. The vector pLJS 49 was used for complementation studies in M. xanthus.
Figure 15. Restriction Map of pRCC 14. The clone pRCC 14 contains a 1.2 kb Hind III fragment of Tn5 along with approximately 7 kb of flanking M. xanthus DNA. The clone is in the vector pUC 19 and the known restriction sites are as marked.
pRCC 14
6. Colony hybridizations: Colonies were lifted from the library plates by placing a nitrocellulose filter on the surface of the plate. Filters were denatured (0.5 N NaOH; 1.5 M NaCl), neutralized (1.0 M Tris HCl, pH 7.5; 1.5 M NaCl), and washed (1.0 M Tris HCl, pH 7.5). Afterwards, the filters were dried and hybridized as for Southern hybridizations.

7. Protein Expression: Each of the inserts from pRCC 45, 46, and 47 were excised from pGEM 11 by double digestion with Hind III and Eco RI. The insert fragment were gel purified and ligated into the vectors pT7-5 and pT7-6 (figure 21). The new constructs were designated as the insert number with a /5 or /6. For example, pRCC 45/5, pRCC 45/6.

   The constructs were electroporated into strain RM 3296 which possesses a lambda prophage containing T7 RNA polymerase under control of a lac promoter. For the expression assay, cells were grown in the presence of timentin and chloramphenicol to log phase at 37°. IPTG was added to a final concentration of 400 ug/ml and the cells allowed to grow an additional 30 minutes, at which time rifampicin was added to a final concentration of 200 ug/ml. Aliquots were taken from each sample at hourly intervals.
C. RESULTS

1. Time course of spo 77 sporulation: The time course of spo 77 sporulation is shown in figure 12. At 6 hours, both wild-type (RC 27) and spo 77 (RC 77) cell populations are exclusively rod-shaped. By 30 hours, shortened rods and ovoid cells begin to appear in both strains. At the 48 hour time point, wild-type cell population is almost exclusively composed of spherical cells, whereas spo 77 cells remain ovoid. A small percentage of the cells in the spo 77 population appear to be round, but these we believe (and confirmed by SEM) to be ovoids viewed on end. The 96-hour time point best illustrates the arrest of spo 77. The cells are still blocked as ovoids, in contrast to the wild-type cells which, by this time have been spherical for 48 hours.

2. Comparison of RC 7 (DK 1622) and RC 250 (Ω77). The Ω77 insertion was transduced into a DK 1622 strain background to see if the mutant phenotype compared to that observed in a DZF1 background. RC 250 (Ω77) produces fruiting bodies nearly indistinguishable from RC 7 (DK 1622 wild type) (data not shown). A comparison of wild type and mutant cell morphologies is shown in figure 13. As can be seen in the photograph, RC 250 arrests at
an ovoid stage, just as RC 363 (Ω77). RC 363 is the tet replacement version of RC 77 and has been backcrossed into an isogenic DZF1 strain background. RC 363 carries the Ω77 insertion and has been used for most of the studies described in this work.

3. **Isolation of the spo 77 locus**: A library of RC 77 was constructed using the cosmid vector LAFR 3 (see figure 14). Clones able to confer kanamycin resistance were isolated and restriction-mapped. It was determined that all of the clones carried a partial copy of Tn5 and some flanking M. xanthus DNA. The insert from cosmid clone pRCC 10 was further subcloned into pUC 19 and designated pRCC 14. The restriction map of pRCC 14 is shown in figure 15.

4. **Sequence analysis**: An 20-mer oligonucleotide complementary to the bases 11-30 of Tn5 was used as a primer for pRCC 14. Approximately 200 bp of sequence, flanking Tn5, were determined as shown in figure 16. Analysis of this sequence revealed six possible ORF's but a search of the DNA and amino-acid sequence databases revealed no homology to known sequences.
Figure 16. Sequence Data From pRCC 14. Sequence of *M. xanthus* DNA flanking one side of the Tn5 insertion. pRCC 14 DNA was sequenced using a 20-mer primer complementary to bases 11-30 of Tn5.
TCAGCCCCAA CTGCCGGCAC AATCCGGGCGC GTGACATCCG CGGCTTCGAC
CCGGACGGCC ACGGTACGCA GTGTGCGGGC ATGCGGGGGG TGTGCACCAC
GGCGTCGCCG GGGAGGGTGA CCTTCAGXTC GCCTCCGTGTA TCGAATCGGA
GACCATXCGC ACCAGCCTGG GCCCGGTTGGC CGCGGCCGATG GAGTGGCGTC
TGCAACCAGTT CAGCCGCCGG GAGAACTCGA CGCGGCCCCG GGTGGTCAAC
CTGTGCGCTCG GCTTCCCCGT GATGCCGCCG GGCATCTCGG AGGGCGACTAC
ACTCACTC
5. **Isolation of wild type clones:** A library of *M. xanthus* wild-type strain DK 1622, created in the cosmid vector pREG 511 (figure 14), was obtained from Ron Gill. The library was probed with a 1.6kb Nco I fragment from pRCC 14 (figure 15). Several filter spots hybridized to the probe and plasmid DNA was prepared from the corresponding strains. Purified wild type clones were designated RC 25, 26, and 36.

6. **Restriction mapping and subcloning:** The clones pRCC 25, 26, 36 were restriction-mapped and their relationship to one another determined by Southern analysis as shown in figures 17 and 18. The three wild-type clones were digested and probed with total pRCC 36 DNA. A Sal I digest of pRCC 25 produces three bands which hybridize to the probe: large, 8.5 kb, and around 3.8 kb. The 8.5-kb fragment is a vector fragment, which means that the large fragment and the 3.8 must overlap pRCC 36. Since they are of a different size than any of the pRCC 36 bands, they must represent the junction fragments; one terminating in pRCC 36 in one direction and the other extending beyond pRCC 36 in the other direction. The Bam HI-Sal I double digest of pRCC 25 reveals the relative positions of the large and 3.8-kb fragments since the large Sal I fragment is replaced by a 6.5 Bam HI-Sal I
fragment as predicted by the restriction map of pRCC 36. A similar analysis of pRCC 26 reveals the diagnostic junction fragment to be the 6 kb Sal I fragment. In the double digest, Bam HI cuts the 6 kb Sal I band into 4.3 and 1.5. The 1.5 Sal I-Bam HI band positions that junction fragment with respect to pRCC 36.

The site of Tn5 insertion was also determined by restriction mapping and Southern analysis (figure 19). Figure 19 also shows the relationship between pRCC 36 (wild type) and pRCC 14 (containing the Tn5 insertion). A 0.8 kb Nco I-Rsa I probe was prepared from pRCC 14. This probe extends from base 60 of Tn5 to approximately 200 bp past the Sal I site in the M. xanthus DNA. A BamHI digest of pRCC 36 produces one band, of approximately 15 kb, that hybridizes to the probe from pRCC 14. In the double digest lane, Sal I cuts the 15 kb Bam HI fragment into the predicted 8.5 and 6.5 fragments, both of which hybridize to the probe. The Sal I digest produces a 10 kb doublet, as predicted from the restriction map, and both bands hybridize to the probe. The site marked Cs in pRCC 36 is an artificial Sal I site created for cloning purposes.

The clone pRCC 36 was further subcloned into the vector pGem 11 as shown in figure 20. The subclone designated pRCC 43 is a 15 kb Bam HI fragment which spans
the Tn5 insertion site and approximately 9 kb and 6 kb of flanking DNA to either side.

The subclones pRCC 44, 45, 46, and 47 were derived from pRCC 43 and represent regions either near or spanning the Tn5 site (see figure 20). The subclone pRCC 44 is the 2kb Xho I fragment located approximately 1 kb to one side of Tn5. The adjacent 6 kb Xho I fragment spans the Tn5 site and was designated pRCC 45. The subclone pRCC 46 terminates approximately 0.5 kb to one side of the insertion site, whereas pRCC 47 spans the Tn5 site. The subclones 46 and 47 share approximately 3.5 kb of overlap with each other. pRCC 47 is a 10 kb Bam HI to Nsi I fragment of pRCC 43. It extends about 6 kb to one side and 4 kb to the other side of the insertion site. The overlapping clone, pRCC 46, is a Bam HI to Sal I fragment extending approximately 8.5 kb to the one side of the Tn5 insertion. These are all summarized in figure 20.

7. **Complementation:**

**Tandem duplication:** Wild-type clones were transduced into the mutant strain RC 363 and tested for their ability to rescue production of heat resistant spores. As described earlier RC 363 carries the Ω77 insertion. Rescue requires homologous recombination between the wild
Table III. Complementation Summary. Wild type cosmid clones were tested for their ability to complement the spo77 mutation in RC 363 (Ω77). Complementation requires formation of a tandem duplication via homologous recombination.
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<td>pRCC 25</td>
<td>10/10</td>
<td>0/10</td>
<td>0/10</td>
</tr>
<tr>
<td>pRCC 26</td>
<td>10/10</td>
<td>0/10</td>
<td>0/10</td>
</tr>
<tr>
<td>pRCC 36</td>
<td>10/10</td>
<td>3/10</td>
<td>3/10</td>
</tr>
<tr>
<td>pRCC 37</td>
<td>10/10</td>
<td>0/10</td>
<td>0/10</td>
</tr>
</tbody>
</table>
type DNA and the mutant chromosomal DNA to produce a tandem duplication. Only one wild-type clone, pRCC 36, was capable of rescuing the mutant. These results are shown in table III.

**Site specific integration:** Although the site of Tn5 insertion had been determined, the boundaries of the spo 77 locus had not been defined. The tandem duplication assay for rescue has the limitation that the entire gene need not be present in the test clone. All that is required is a sufficient portion of the gene to bypass the lesion (marker rescue). A complementation experiment was performed using the vector pLJS 49 shown in figure 13. The subclones pRCC 44, 45, 46, and 47 were digested with Hind III and Eco RI, and their respective inserts ligated into pLJS 49. The vector pLJS 49 carries an att site from the myxophage Mx8, which enables the vector to integrate into the M. xanthus chromosome via site specific recombination (Li and Shimkets, 1988). Since integration of the test fragment occurs in a region of the chromosome distant from the mutant locus, marker rescue is not a problem. Each of the four subclones were transformed into strain RC 363 via electroporation. Ten kanamycin-resistant colonies were isolated from each transformation and tested for their retention of
tetracycline resistance. All of the isolated strains remained resistant to oxytetracycline. Twenty of the isolates (5 for each subclone) were then spotted onto clone fruiting agar and examined for fruiting body morphology prior to heat testing. None of the partially diploid strains produced wild-type fruiting bodies and furthermore, all proved to be heat-sensitive, indicating the absence of functional spores.

8. Glucosamine experiment: A concentration of 50 mM glucosamine is lethal to *M. xanthus* and concentrations below 5 mM have no observable effect on growth or development (Mueller and Dworkin, 1991). The reported rescue of sporulation mutants occurs in the range of 10-20 mM (Mueller and Dworkin, 1991). We started with an initial concentration of 100 mM in the center of each plate, which when fully diffused would give a final concentration of 1.4 mM (1:70). After the addition of glucosamine, diffusion should establish a concentration gradient in the plate, with some areas of the plate reaching the target concentration (10-20 mM) at different times. Under these conditions, the proper intersection of development time and glucosamine concentration would result in a zone of heat resistant spores if rescue took place. The glucosamine experiment demonstrated no rescue
Figure 17. Overlap of wild type clones. Wild type clones of the spo 77 locus were obtained from a DK 1622 cosmid library. The clones were restriction mapped and analyzed by Southern hybridization (see figure 18) to determine their physical relationship to one another. The site Cs designates an artificial Sal I site. The // symbol denotes a discontinuity in the M. xanthus DNA in order to maintain the figure scale and fit the figure on one page. There are additional unassigned Sal I sites in pRCC 36 to the left of the leftmost Sal I site shown.
Overlap of wild type clones
of RC 363 (spo 77). Wild type (RC 27) was able to sporulate normally at all time points whereas RC 363 was equally unable to produce viable spores at any time point.

9. Protein expression assay: Subclones pRCC 45, 46, 47 were excised from pGEM 11 by digesting with Eco RI and Hind III. The gel purified inserts were then ligated into the expression vectors pT7-5 and pT7-6 as shown in figure 21. Each of the six constructs were transformed into strain RM 3296 and tested for protein expression from the insert DNA. One construct, pRCC 45/5 appeared to synthesize two new proteins in small amounts (data not shown).

D. DISCUSSION
The spo 77 locus is clearly required for successful spore formation in M. xanthus. Disruption of the spo 77 locus results in an arrest of the morphogenic pathway prior to completion. Because of its integral role in sporulation, we set out to further characterize the spo 77 locus using a variety of methods.

Is the spo 77 mutant blocked in morphogenesis or merely delayed? A time course of sporulation revealed
Figure 18. Southern analysis of wild type clones. Wild type clones of spo 77 were digested by the restriction enzymes shown and after separation by gel electrophoresis, transferred to a nitrocellulose membrane. The membrane was probed with total pRCC 36 DNA and the pattern of hybridization is shown.
that, in wild type, ovoids start to appear around 24 hours after development is initiated. The spo 77 mutant remains ovoid for at least another 72 hours, suggesting that the mutant is truly blocked. Does the spo 77 phenotype represent a blocked morphology on the normal sporulation pathway, or an aberrant morphology? This question is more difficult to answer with certainty, for we must rely on two pieces of circumstantial evidence. First, ovoid morphology has been observed as an intermediate in wild type morphogenesis, and second, the timing of ovoid appearance in the spo 77 mutant coincides with that of wild type (Dworkin and Sadler, 1966). While this evidence is not conclusive, it does suggest that the spo 77 mutant is blocked along the normal sporulation pathway. More direct analysis of ovoids is difficult because they are sensitive to the same selective treatments as vegetative cells, and their fragility makes physical isolation of discrete cell populations nearly impossible (Kottle and White, 1974).

We were able to successfully clone the site of Ω 77 and utilize the flanking M. xanthus DNA to isolate the wild type locus. We used two methods of testing wild type clones for their ability to restore sporulation potential to the mutant strain. Unfortunately, neither method proved useful for determining the functional
Figure 19. Relationship of pRCC 14 to pRCC 36. The physical relationship of pRCC 14 and pRCC 36 was determined by restriction mapping and Southern analysis. (see text)
Comparison of Wild Type and Mutant Clones

pRCC 36

pRCC 14

Probe: 0.8 kb Nco I - Rsa I
boundaries of the spo 77 locus. We were able to identify a clone, pRCC 36, that, when integrated into the chromosome by homologous recombination, could rescue the mutant phenotype. Only 3 out of 10 integrants gave phenotypic restoration, a result that in customary circumstances would suggest that pRCC contained a truncated gene that restored function by recombining with the chromosomal mutation to create a wild-type gene. This may be so, but the peculiar way in which it was necessary to convey pRCC 36 into M. xanthus offers an alternative explanation. The phage P1 was used to introduce the test clones into the recipient cells. This technique relies on recombination between the vector pREG 511 and native P1 DNA, so that the test fragment will be packaged into a P1 phage head (O'Connor and Zusman, 1983). The vector pREG 511 carries a region of P1 DNA specifically for this purpose, but depending upon where the recombination takes place, and where packaging starts, the entire test clone may not be included in the resultant transducing particle. The net result of this is that some of the tandem duplications may have been made from truncated clones. To address this problem we analyzed the wild-type clones by restriction mapping and southern hybridization. We were able to determine that the clone pRCC 36 did indeed contain the site of Tn5
insertion, and approximately 10 kbp flanking DNA to one side and 20 kbp to the other side of this site. In addition, two wild-type clones that did not rescue the mutant strain were found to terminate in the vicinity of the insertion site. From these data we were fairly confident that, barring some extremely unusual gene arrangement, pRCC 36 quite likely contained the entire spo77 locus. The third possible explanation comes from some recent work on the devRS locus. There is evidence that some functions of devR may rely on fusion to normal upstream flanking DNA (Thony-Meyer and Kaiser, 1994). This is based upon the observation that not all tandem duplications of devR exhibit identical repression of a reporter gene (a second copy of devR fused to lacZ) (Thony-Meyer and Kaiser, 1994). Although we have no evidence to suggest that the spo77 locus is regulated in such a manner, the possibility of such effects remains a formal consideration.

Because of the potential for marker rescue, a second method of testing was used for subclones of pRCC 36. This method relies on site specific recombination to create partial diploids. The vector pLJS 49 possesses a myxophagous Mx8 att site, which enables high frequency, site-specific, integration into the M. xanthus chromosome (Li and Shimkets, 1988). This method has been used
Figure 20. Subclones of pRCC 36. A restriction map of the wild-type cosmid clone pRCC 36 is shown along with the location of the N77 insertion site. Subclones of pRCC 36 are also shown and the dashed line provides a reference for their position relative to the N77 insertion site.
Subclones of pRCC 36

pRCC 36

pRCC 43

pRCC 47

pRCC 46

pRCC 45

pRCC 44
successfully to create partial diploids for the csgA gene (Li and Shimkets, 1988). We were surprised to find that, despite successful integration, none of our subclones could restore sporulation ability to a spo 77 mutant. There are two possible explanations for this result: either none of the test fragments contained the entire spo 77 locus, or the spo 77 gene was not functional at a site distant from its normal location. There are many examples of genes being context-sensitive for proper function. More general examples include the transposase proteins encoded by IS elements, replication initiation proteins of certain plasmids and phage, and transcriptional regulators such as the anti-termination factor Q of bacteriophage lambda (Echols et al., 1976), (Francke and Ray, 1972), (Jain and Kleckner, 1993). All of these examples require that the gene in question be expressed in the immediate vicinity of its target DNA (in cis). Expression at distal sites (in trans), results in severely reduced activity or loss of activity. In M. xanthus, function of the devRS locus also appears to be context sensitive. A 7.8 kb wild-type clone of the devRS region, which includes 1.2 kb of upstream sequence, is sufficient for complementation by tandem duplication, but is unable to complement when integrated at the Mx8 att site (Thony-Meyer and Kaiser, 1994). Increasing the
amount of upstream flanking sequence to 4.9 kb improved the efficiency of complementation considerably, but did not restore wild-type function (Thony-Meyer and Kaiser, 1994).

An attempt was made to sequence out from the end of the Tn5 insertion into the flanking M. xanthus DNA. Approximately 200 bp of flanking sequence was obtained, but attempts to sequence beyond this region were unsuccessful. The sequencing approach was discontinued for two reasons, both a consequence of the high GC content of M. xanthus DNA. First of all, were technical difficulties in obtaining sequence, even with the use of dITP. Secondly, the relative low frequency of AT base pairs made identifying legitimate open reading frames (ORF) difficult. In the small amount of sequence we obtained, there were 6 possible ORFs. More recently, modified sequencing protocols for M. xanthus DNA have been worked out, as has a sequence analysis for detecting ORFs in M. xanthus DNA. As a result, sequencing may now be a viable alternative for further analysis of the spo 77 locus.

It has been reported that the amino sugar glucosamine can rescue sporulation of several csg mutants (Janssen and Dworkin, 1985). The resultant spores resemble glycerol-induced spores more than fruiting body
Figure 21. Expression Vectors. Restriction maps of the expression vectors pT7-5 and pT7-6 showing the direction of transcription from the T7 promoter.
T7 start

pT7-5
2.40 Kb

Col E1 origin

Amp

Cla I 0.02
Hind III 0.03
Pst I 0.04
Sal I 0.05
Xba I 0.06
Bam HI 0.07
Sma I 0.08
Sac I 0.09
Eco RI 0.10

Eco RI 0.03
Sac I 0.03
Sma I 0.04
Bam HI 0.05
Xba I 0.06
Sal I 0.09
Pst I 0.10
Hind III 0.11

T7 start

pT7-6
2.21 Kb

Col E1 origin

Amp
spores, and it has been hypothesized that an increase of intracellular glycerol levels may mediate this effect (Mueller and Dworkin, 1991). We thought it would be interesting to see if glucosamine could rescue spo 77 sporulation. We already knew that spo 77 can be directly induced by glycerol, and thought that if the block in the starvation pathway could be bypassed, that a functional, heat resistant spore could be made. The results of this study indicate that the spo 77 mutant is not rescued by glucosamine, but since we did not have a positive control for glucosamine rescue (a csgA mutant for example) we cannot be completely sure that our experimental conditions provided adequate levels of glucosamine for long enough time periods.

There is some evidence that the ovoid stage may be a milestone in the morphogenic process (White et al., 1968). Glycerol spores show increased resistance to penicillin G and D-cycloserine as they approach the ovoid stage (White et al., 1968). This has been interpreted as a reflection of changes in how peptidoglycan is synthesized and crosslinked (White et al., 1968). In addition, peptidoglycan-crosslinking data shows that ovoids possess more free amino groups in their diaminopimelate (DAP) residues than spheres, indicating reduced crosslinking. Finally, the transition from ovoid
to sphere results in the following: resistance to phage adsorption, resistance to detergent, and acquisition of sonication resistance (White et al., 1968).

In the absence of additional experimental data, which might provide some clues about the mechanism of morphogenesis and the role of the spo 77 locus, we are left with theoretical considerations. An excellent review on the morphogenesis of microorganisms has been written by Franklin Harold (Harold, 1990). He advances the hypothesis that nearly all morphology of microorganisms can be derived mathematically if mechanical constraints are considered. The most significant variable, according to Harold, is the manner in which new peptidoglycan is incorporated with the existing structure. In his model for a spherical cell, peptidoglycan is added in single units and at a localized growth zone. For rod shaped cells, he states that stable extension of a cylinder is possible if new material is added diffusely, rather than a localized growth zone, and as larger preassembled units.

In the case of *Myxococcus xanthus*, cells are transformed from elongated rods to spheres. This change in cell shape would be consistent with the above model if the means by which new peptidoglycan material was added changed during sporulation. If peptidoglycan synthesis
shifted from diffuse addition of subunits, to a localized growth zone it might account for the change from rod to sphere.

One prediction from Harold's model is that very few "end-effector" morphogenes would be required to reshape a cell. If this is true, it might account for the observation that most of our mutants are capable of reshaping. What Harold's model does not explain is a mutant such as spo 77, which is blocked at an intermediate stage. Presumably, shape conversion would require a single shift in how new peptidoglycan is assembled, and once this shift is made the reshaping should proceed to completion. The existence of a blocked intermediate suggests that there is at least one additional step in the process, beyond the initial shift in peptidoglycan assembly.
Most of the work on *M. xanthus* sporulation in the past 15 years has focused on a biochemical approach to identifying sporulation genes. In some instances a sporulation-specific protein was isolated (protein S for example) and the amino acid sequence used to clone the corresponding gene. In other instances *M. xanthus* genomic libraries have been screened with probes derived from known DNA sequence (sigB and sigC for example) in other organisms. These two approaches have been for the most part disappointing. While several genes have been identified, none have proved to be absolutely essential for sporulation. For this reason we decided to take a genetic approach based strictly upon loss of function.

We set out to create, isolate, and characterize sporulation-specific mutants of *Myxococcus xanthus*. Why did we target sporulation-specific mutants? Our reasons were several; very little is known about how *M. xanthus* cells transform themselves into spores, or how sporulation and aggregation interact, and few sporulation-specific mutants have been reported. It was
our hope that such mutants could be used to identify essential sporulation functions.

We were able to isolate 47 such mutants encompassing at least 8 chromosomal loci. Physical mapping data suggests that nearly all of these mutants are new. One exception, Ω147, is quite likely in the devR or devS locus described by Kaiser. Terminal cell morphologies of the mutants range from rod to sphere, with one mutant arresting as an ovoid. Comparing the mutant phenotypes with the genetic linkage data, we estimate that approximately 21 sporulation-essential genes have been identified. Of particular note is the observation that none of our mutants are affected for glycerol sporulation. There are some possible explanations for this unexpected result (see chapter II discussion) but, none are compelling, and these observations challenge the conventional assumptions about the relationship between glycerol and starvation sporulation.

We have cloned the sites for two of the Tn5 insertions, Ω77 and Ω147. The spo 77 locus (Ω77) was of particular interest to us because of its morphogenetic arrest as an ovoid. A time course experiment established that spo 77 is truly blocked at a normal stage of morphogenesis. We obtained a wild-type clone of the spo 77 locus that could rescue the mutant phenotype in a
partial diploid strain created by tandem duplication. Attempts at defining the boundaries of the spo 77 locus were unsuccessful. Once again, we took a functional approach in examining spo 77. We tested subcloned fragments of pRCC 36 for their ability to restore function in partial diploid strains. Unfortunately, in M. xanthus, gene function seems to be context-sensitive in some instances. For this reason we believe that an intact spo 77 gene cannot function properly at a site distant to its normal location. In the early stages of characterizing spo 77 an unsuccessful attempt was made to sequence the DNA flanking the Tn5 insertion. Now that advances in sequencing techniques and interpretation have been made, it seems that this should be the direction of future work on spo 77. If the boundaries of spo 77 can be determined by sequence data, the function issue can be readdressed under more defined conditions.
LITERATURE CITED:


