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CHARACTERIZATION OF REGULATORY MECHANISMS OF CELL-CELL INTERACTION-DEPENDENT GENES OF *MYXOCOCCUS XANTHUS*

By

Tong Hao

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ABSTRACT

CHARACTERIZATION OF REGULATORY MECHANISMS OF CELL-CELL INTERACTION-DEPENDENT GENES OF *MYXOCOCCUS XANTHUS*

By

Tong Hao

Myxococcus xanthus is a gram-negative, rod-shaped soil bacterium that undergoes multicellular development in response to nutrient limitation. Upon starvation, many thousands of cells move in a coordinated fashion into aggregation centers and construct multicellular structures known as fruiting bodies, in which individual cells undergo morphological and physiological changes to form dormant ovoid-shaped spores. The progression through fruiting body formation and sporulation is highly coordinated and involves at least five extracellular signaling interactions. These five signaling interactions are also needed for the proper expression of developmental genes.

One of the extracellular signals is C-signaling, which is required for aggregation and sporulation. C-signaling mutants initiate development normally but fail to form compact fruiting bodies and spores. Of the genes that have been examined, almost every one expressed at six hours or later in development exhibits reduced or abolished expression in C-signaling mutant cells.

To understand how extracellular C-signaling regulates gene expression during development, the regulatory regions of several C-signal-dependent genes have been characterized. Like these genes, the operon identified by Tn*5* *lac* Ω4514 is expressed at

about the same time during development, but its expression does not depend on C-signaling. The developmental regulation of this operon has been investigated. Based on sequence similarity, the Ω4514 operon may encode an enzyme involved in glutamate fermentation and a transcriptional regulator. The promoter of the operon shares similarity with promoters that are expressed during growth and the Ω4514 promoter can be transcribed *in vitro* by the major vegetative RNA polymerase. Developmental regulation of Ω4514 expression is complex, involving negative regulation by the product of the first gene in the operon and positive regulation by one or more transcriptional activators.

Developmental expression of the Ω4403 gene is absolutely dependent on C-signaling and on DNA sequences extending to –80 relative to the start site of transcription. A search for regulatory proteins that interact with the upstream region has been carried out. No protein likely to mediate C-signal-dependent expression of Ω4403 was identified.

To my husband, Enoch, my parents, and my son, Calvin, with love.

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LIST OF ABBREVIATIONS

Ap	ampicillin
ATP	adenosine-5′-triphosphate
bp	base pair
CoA	coenzyme A
dGTP	deoxyguanosine-5′-triphosphate
DNA	deoxyribonucleic acid
DTT	dithiothreitol
ECF	extracytoplasmic function
EDTA	(ethylenedinitriol)tetraacetic acid
HEPES	N-(2-hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid)
IPTG	isopropyl β-D thiogalactopyranoside
kb	kilobases
kDa	kilodalton
Km	kanamycin
LB	Luria-Bertan:
mRNA	messenger ribonucleic acid
NAD^+	nicotinamide adenine dinucleotide (oxidized form)
NADH	nicotinamide adenine dinucleotide (reduced form)
ONPG	*o*-nitrophenol-β-D-galactoside
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction

(p)ppGpp	guanosine tetra- or penta-phosphate
PMSF	phenylmethylsulfonyl fluoride
RNAP	RNA polymerase
RNase	ribonuclease
SDS	sodium dodecylsulfate
Tc	tetracycline
TDT	terminal deoxynucleotide transferase
Tris	tris(hydroxymethyl)aminomethane
tRNA	transfer ribonucleic acid

INTRODUCTION

The gram-negative soil bacterium *Myxococcus xanthus* displays remarkable social behavior upon nutrient depletion, as cells move coordinately to aggregation centers and form fruiting bodies. Inside each fruiting body, some of the rod-shaped cells lyse, whereas others differentiate into ovoid-shaped spores. This morphological change is accompanied by a highly ordered program of developmental gene expression. At least five extracellular signaling interactions regulate the developmental process. To begin to understand how cell-cell signaling interactions regulate developmental gene expression, the regulatory mechanisms of two developmental genes have been investigated.

Chapter one presents a review of cell-cell interactions involved in *Myxococcus xanthus* growth and development, as well as transcriptional regulation in *M. xanthus*.

Chapter two describes the investigation of the regulatory mechanism of a C-signal-independent gene identified by Tn*5* *lac* Ω4514. Ω4514 is expressed during development with similar timing as several C-signal-dependent genes. However, its promoter resembles vegetative promoters in sequence and can be transcribed by the major vegetative *M. xanthus* RNA polymerase *in vitro*. Regulation of the Ω4514 operon is complex, involving negative autoregulation by one of its own gene products and positive regulation by one or more transcriptional activators. The content of this chapter will be submitted to the *Journal of Bacteriology* with Dvora Biran and Gregory Velicer as second and third authors, respectively. Dvora Biran was responsible for the *in vitro* transcription experiments. Gregory Velicer constructed the *orf1* disruption strain and

tested developmental activity from the small Ω4514 promoter in *orf1* mutants. He also constructed strain MGV03 and tested it for promoter activity.

Chapter three describes the search for a regulatory protein(s) that mediates developmental expression of Tn*5* *lac* Ω4403, a C-signal-dependent gene. DNA between –80 and –72 is crucial for C-signal-dependent expression of Ω4403. No protein that interacts specifically with this region was identified.

The Summary and Perspectives section contains the conclusions and significance of these results, and proposals for future research directions. Both Ω4514 and Ω4403 require upstream regulatory elements for expression; however, no similar sequence has been identified that is shared between the two genes. It is very likely that different transcriptional activator proteins increase expression of these genes in response to developmental signals.

Chapter I

Literature Review

Cell-cell interactions play a critical role in cellular differentiation in multicellular organisms. *Myxococcus xanthus*, a gram-negative rod-shaped soil bacterium, exhibits multiple forms of cell interactions by feeding, moving and developing cooperatively. Some of the signals have been identified chemically, other signaling interactions can be inferred from the behavior of cells. Cell-cell interactions are important for *M. xanthus* cells to search for nutrients by moving in large groups known as swarms. Growth of *M. xanthus* cells is also cell interaction-dependent, showing density dependency, which is attributed to the increased concentration of nutrients available for growth that results from the increased concentration of extracellular hydrolytic enzymes secreted by cells at high cell density (Rosenberg et al., 1977). Cell-cell interactions are also manifested during development when multicellular structures known as fruiting bodies are formed. Since *M. xanthus* is genetically simple and readily amenable to genetic and biochemical approaches, it provides a good experimental system for investigating how cell-cell interactions control cellular differentiation during multicellular events, such as development.

Motility

Myxococcus xanthus cells glide on a variety of different surfaces, forming swarms that secrete lytic enzymes to digest prey bacteria. Within the swarm, movement of a small group of cells is known as social motility (S). Occasionally, individual cells briefly move outside of a swarm before returning, and this is known as adventurous (A) motility. Cell motility is also indispensable for multicellular development. Nonmotile mutants are unable to aggregate and form fruiting bodies and spores (Kroos et al., 1988; McBride, 1993). At least five classes of mutations that affect gliding motility have been identified

and characterized. These mutations have been grouped into five gene classes: A (adventurous gliding), S (social gliding), *mgl* (mutual gliding), *frz* and *dif* (chemotaxis).

A mutation in any of the A genes results in cells that are able to move in groups, but cannot glide as individuals. Conversely, a mutation in any of the S genes results in cells that are able to move only as individuals (Hodgkin and Kaiser, 1979a). The A and S systems work additively (*i.e.*, strains with defects in both systems (A^-S^-) are nonmotile). There are at least 37 loci required for A motility (Hodgkin and Kaiser, 1979a; MacNeil et al., 1994). One subset of A genes likely encodes proteins that are localized to the cell envelope or extracellular matrix (Kalos and Zissler, 1990; Rodriguez and Spormann, 1999). The S system includes a minimum of 19 loci (Hodgkin and Kaiser, 1979b; MacNeil et al., 1994) and S motility is operative only when cells are in close proximity to each other (Kaiser, 1979; Kaiser and Crosby, 1983). S motility is correlated with pili, which are long, thin fibers found in tufts at one pole of the cell. A motility, on the other hand, is unrelated to pili. Components of the S system appear to be homologs of genes involved in the biosynthesis, assembly and function of type IV pili in *Pseudomonas aeruginosa* (Wu and Kaiser, 1995; Wu and Kaiser, 1997a).

Both A and S motility is abolished by a single mutation in the *mgl* locus, which contains two genes, *mglA* and *mglB* (Stephens and Kaiser, 1987; Stephens et al., 1988). Mutations in *mglA* render cells completely nonmotile. It appears that MglA protein does not function as a component of the gliding motor (Hartzell and Kaiser, 1991a), nor does it regulate the transcription of A and S genes (MacNeil et al., 1994). Rather, MglA shares amino acid similarity to GTP-binding proteins, suggesting a possible role of MglA in signal transduction involved in motility (Hartzell and Kaiser, 1991b). Mutations in *mglB*

result in cells with substantially reduced motility and reduced levels of the MglA protein. The *mglB* gene is co-transcribed with *mglA* and a normal amount of *mglBA* transcript is produced in *mglB* mutants (Hartzell and Kaiser, 1991b). Because the transcription of *mglA* is not affected by *mglB* mutations, the reduced level of MglA could be due to reduced stability of the MglA protein in the absence of MglB. Part of the predicted amino acid sequence of MglB shows similarity to a calcium-binding site of yeast calmodulin, but the function of MglB is still unclear (Hartzell and Kaiser, 1991b).

Bacterial chemotaxis-like sensory transduction systems control reversal of gliding motility in *M. xanthus*. Two sensory processing systems, *frz* and *dif*, homologous to the Che chemotaxis system of *E. coli*, have been identified by sequence comparison and biochemical characterization (Shi and Zusman, 1995; Yang et al., 1998). The *frz* genes appear to regulate gliding movement of both individual cells and cells in groups. The *dif* genes, in contrast, affect only S system-dependent gliding.

Fruiting body formation and sporulation

Myxococcus xanthus cells undergo a multicellular developmental process in response to nutrient limitation (Shimkets, 1990). Within 4 to 8 hours after the onset of starvation, the cells begin to aggregate by gliding to form mound-shaped structures known as fruiting bodies. About 10^5 cells participate in the formation of a single fruiting body. Within a fruiting body, some of the cells lyse, whereas by 24 hours, a small population of motile, rod –shaped cells differentiate into non-motile, spherical spores that are resistant to harsh conditions such as heat, freezing, desiccation and UV irradiation. Aggregation and sporulation are temporally separated and sporulation in wild-type cells occurs only after cell movements have led to the formation of fruiting bodies. The earliest

stages of aggregation are often accompanied by a form of organized cell movement called rippling. During rippling, a series of equally spaced ridges of cells move in a coordinated fashion like traveling waves (Shimkets and Kaiser, 1982). Rippling is not absolutely required for the construction of fruiting bodies or sporulation, but normally accompanies fruiting body formation (Shimkets, 1990).

Multicellular development in *Myxococcus xanthus* depends on at least five extracellular signals known as the A-, B-, C-, D-, and E-signals. Signaling mutants were identified by isolating conditional nonsporulating mutants, whose sporulation can be rescued by codevelopment with wild-type cells or mutants of a different signaling group (Hagen et al., 1978). The signaling mutants are defective in producing a signal, but not in perceiving that signal. Mutants unable to produce any one of the five signals are arrested in development at a particular stage. By examining the expression of developmental reporters in signaling defective mutants, it has been shown that the A- and B-signals are required at the onset of development, the D- and E-signals are required at 3 to 5 hours after the onset of development, and C-signaling is required at about 6 hours into development (Downard et al., 1993; Kaiser and Kroos, 1993; Downard and Toal, 1995). Of the five signals, only the A-signal has been defined biochemically.

Fruiting body morphogenesis and sporulation are accompanied by the coordinated expression of many developmentally regulated genes and the synthesis of new proteins. More than 30 proteins, identified as bands separated by gel electrophoresis, as well as several new cell surface antigens are associated with developing cells (Inouye et al., 1979b; Gill and Dworkin, 1986; Gill et al., 1987). Among the most abundant proteins expressed during development are proteins S (a spore coat protein) and S-1 (a protein

found inside spores) (Inouye et al., 1979a; Teintze et al., 1985). These two proteins are encoded by *tps* and *ops*, respectively, and these genes are located next to each other, separated by a 1.4 kb DNA segment. Proteins S and S-1 are 90% identical, but they are produced at different times during development (Inouye et al., 1983a; Inouye et al., 1983b). At 5 hours after starvation, *tps* begins to be expressed and later the protein S produced self-assembles the outermost spore coat. The *ops* gene is not expressed until 24 hours into development (Downard et al., 1984; Downard and Zusman, 1985). Another protein produced during development is myxobacterial hemagglutinin, a protein with lectin activity, which appears at 10 hours (Romeo and Zusman, 1987).

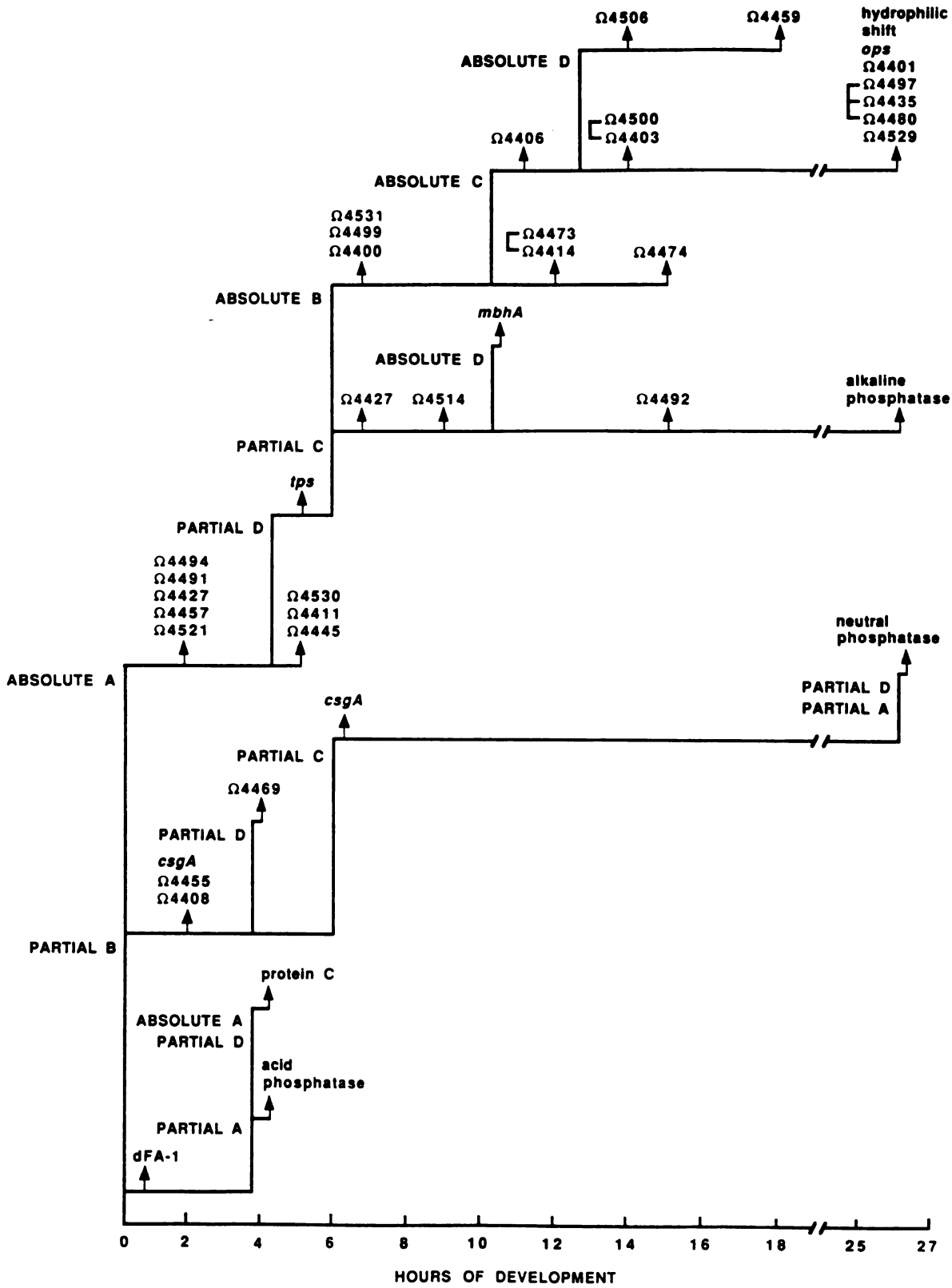
Many developmentally regulated genes have been identified by genomic insertion of Tn*5 lac*, a transposon containing a promoterless *lacZ* that generates transcriptional fusions to promoters in the chromosome (Kroos and Kaiser, 1984). Among 2374 Tn*5 lac* insertions, 29 insertions showed increased β-galactosidase activity upon starvation, implying that about 1% of the genome is up-regulated during fruiting body development (Kroos et al., 1986). Only a small portion of the 29 Tn*5 lac* insertions showed developmental defects that impair aggregation or sporulation (Kroos et al., 1990). A similar low frequency has been observed in the eukaryotic slime mold *Dictyostelium discoideum*, which has a multicellular development like that of *Myxococcus xanthus* (Kuspa and Loomis, 1992). Possible explanations are that subtle changes in morphology might escape notice and/or that many genes are at least partially redundant in their functions. β-galactosidase activities from the 29 developmentally regulated fusions have been assayed in wild-type cells as well as in cells that are defective in an extracellular signaling interaction (Kroos and Kaiser, 1987; Downard et al., 1993; Kaiser and Kroos,

1993; Downard and Toal, 1995). Dependency of each fusion on extracellular signaling is depicted in Figure 1.1. Expression of most fusions is dependent upon A- and B- signaling. Fusions expressed around 4 hours require D-signaling and nearly all genes that begin to be expressed after 6 hours into development appear to be at least partially C-dependent. Expression of *tps*, which normally begins around 5 hours, is reduced in E-signaling mutant cells, indicating that the E-signaling interaction affects the pattern of gene expression by 5 hours into development (Downard et al., 1993).

A-signaling

A-signaling mutants are arrested early in development, prior to aggregation. The A-signal itself has been purified from media conditioned by developing cells by monitoring the expression of Tn*5* *lac* fusion Ω4521, whose expression requires A-signaling and therefore could serve as an assay to identify fractions containing A-signal activity. The A-signal is composed of a subset of amino acids that can function either individually or in combination at a concentration greater than 10 μM (Kuspa et al., 1992). Fifteen amino acids have A-factor activity. The six amino acids with the highest specific activities are tyrosine, proline, tryptophan, phenylalanine, leucine and isoleucine. Peptides also have activity (Plamann et al., 1992). Peptides most likely generate A signal as they are degraded to their constituent amino acids by extracellular and periplasmic proteinases, because the activity of each peptide tested is approximately equal to the sum of the activities of its amino acids. It appears that proteinases, released early during development, are involved in generating the A-signal by degrading surface proteins to their constituent amino acids and peptides. All of the proteinases tested have the ability to

Figure 1.1 Dependence of developmental markers on A, B, C, and D factors. Each marker is placed above an arrow positioned along the x axis according to the time at which its expression normally begins during development. Position along the y axis indicates the marker's dependence on cell-cell interactions, as determined by measuring its expression in *asg*, *bsg*, *csg* and *dsg* mutants. For example, a marker whose expression is reduced in an *asg* mutant exhibits partial dependence on A factor (abbreviated "partial A"). Similarly, a marker whose expression is abolished in a *bsg* mutant exhibits absolute dependence on B factor (abbreviated "absolute B"). Dependencies are cumulative, so a higher position along the y axis indicates greater dependence on cell-cell interactions. Tn5 *lac* insertions are indicated by Ω followed by a four digit number, and brackets connect insertions that probably lie in the same transcription unit. Reprinted with permission from authors. Kaiser, D. and Kroos, L. (1993) In *Intercellular signaling*. (Dworkin, M. and Kaiser, D., eds) 257-283, American Society for Microbiology.


Ω4506
Ω4459
hydrophilic shift
ops
Ω4401
Ω4497
Ω4435
Ω4480
Ω4529
ABSOLUTE D
Ω4500
Ω4403
Ω4406
ABSOLUTE C
Ω4531
Ω4499
Ω4400
Ω4473
Ω4414
Ω4474
ABSOLUTE B
mbhA
ABSOLUTE D
Ω4427
Ω4514
Ω4492
alkaline phosphatase
PARTIAL C
tps
PARTIAL D
Ω4494
Ω4491
Ω4427
Ω4457
Ω4521
Ω4530
Ω4411
Ω4445
neutral phosphatase
ABSOLUTE A
PARTIAL D
PARTIAL A
csgA
PARTIAL C
Ω4469
PARTIAL D
csgA
Ω4455
Ω4408
PARTIAL B
protein C
ABSOLUTE A
PARTIAL D
acid phosphatase
PARTIAL A
dFA-1
0
2
4
6
8
10
12
14
16
18
25
27
HOURS OF DEVELOPMENT

generate A-signal activity and at least two different proteinases can be isolated from developing cell extracts (Plamann et al., 1992).

The generation of extracellular A-signal requires the products of at least five *asg* genes. These include *asgA*, *asgB*, *asgC*, and two newly characterized genes, *asgD* and *asgE* (Kuspa and Kaiser, 1989; Cho and Zusman, 1999; Garza et al., 2000). A mutation in any of these genes renders cells defective in generating A-signal, but the cells maintain their ability to respond to extracellular A-signal. The *asgA* gene encodes a histidine protein kinase that resembles the type found in sensor proteins of two-component signal transduction systems (Plamann et al., 1995). The AsgA protein is unusual in that it also contains a receiver domain typically found in the response regulator protein of two-component systems. It has been proposed that AsgA may function in a multicomponent phosphorelay-type signal transduction mechanism that senses starvation and responds by altering the expression of genes required for production of A-signal (Plamann et al., 1995).

The *asgB* gene encodes a putative DNA-binding protein with a helix-turn-helix (HTH) motif near its C-terminus (Plamann et al., 1994). The predicted HTH motif in AsgB is highly similar to the HTH found in region 4.2 of major sigma factors, which recognizes and interacts with the -35 region of promoters. It has been proposed that AsgB may act as a transcriptional activator or repressor (Plamann et al., 1994).

The *asgC* gene encodes the major sigma factor of RNA polymerase holoenzyme, σ^A (Davis et al., 1995). The mutant allele, *asgC767*, results in a glutamate to lysine substitution in region 3.1. This region is suspected to be involved in (p)ppGpp-mediated gene regulation in *E. coli* (Harris et al., 1998). One possible explanation for the A-

signaling defect of *asgC767* cells is that the mutated σ^A fails to respond to the changing levels of (p)ppGpp and the expression of (p)ppGpp-dependent genes is impaired.

The AsgD protein, like AsgA, is predicted to be a component of a signal transduction system because it has a receiver domain located at its N-terminus and a histidine protein kinase at its C-terminus (Cho and Zusman, 1999). Fruiting body formation and sporulation are arrested when *asgD* mutant cells are plated on CF medium, a low-nutrient medium that induces development upon starvation. However, when the mutant is plated on stringent starvation medium rather than CF, cells are able to form fruiting bodies, suggesting that AsgD is directly or indirectly involved in sensing nutritionally limiting conditions.

Another gene involved in generating A-factor is *asgE* (Garza et al., 2000). A-factor consists of a mixture of amino acids, which is generated by extracellular proteases that degrade cell surface proteins into constituent amino acids. The *asgE* mutant yields about 10-fold less extracellular protease activity and about twofold less A-factor than wild-type cells during development (Garza et al., 2000). These results suggest that the primary defect of *asgE* cells is in the production or release of extracellular proteases.

Insight into how cells respond to A-signaling was obtained by studying mutants that result in constitutive expression of the A-signal-dependent reporter gene, Ω4521, in *asg*$^-$ cells. Ω4521 was identified as a developmentally regulated Tn*5* *lac* insertion and its expression is abolished in any of the A-signal mutants. Genetic suppressors that allow A-signal-independent expression of Ω4521 in the *asgB* mutant background have been identified. Three loci have been characterized so far, known as *sasA*, *sasN*, and *sasS*. The *sasA* locus is required for biosynthesis of the O-antigen of *M. xanthus* lipopolysaccharide

(Guo et al., 1995). The connection between the absence of O-antigen and A-signal-independent expression of Ω4521 is unclear. The *sasN* locus encodes a novel protein with no homologue in the databases (Xu et al., 1998). The N-terminus of SasN appears to contain a highly hydrophobic region and a leucine zipper motif. The *sasN*-null mutant expressed Ω4521 at a higher level during both growth and development, as compared with wild-type cells, suggesting that SasN negatively regulates Ω4521 expression during growth and development. The *sasS* locus appears to encode a sensor histidine protein kinase of a two-component system (Yang and Kaplan, 1997). The C-terminus of SasS contains all of the conserved residues found in sensor histidine protein kinases, and the N-terminus of SasS contains two putative transmembrane domains that might localize this protein to the cytoplasmic membrane. The *sasS*-null mutants are defective in fruiting body formation and sporulation and express Ω4521 at a basal level. These data suggest that SasS may sense extracellular A-signal and positively regulate Ω4521 expression and expression of other genes induced during *M. xanthus* development. Two other putative positive regulators of A-signaling responses, SasR, an NtrC-like response regulator, and SasP, have been identified by a separate approach (Guo et al., 2000). Disruption in *sasR* or *sasP* abolished Ω4521 expression during development in the presence or absence of lipopolysaccharide O-antigen.

Ω4521 expression depends on cell density during starvation (Kuspa et al., 1992). At cell densities below $2x10^8$ cells/ml, Ω4521 is expressed at 25% or less of the maximum level. The expression of Ω4521 increases drastically from about $5x10^8$ cells/ml, reaching its maximum level at about 10^9 cells/ml. The low expression at low cell density appears to be due to less A-signal activity since Ω4521 expression is restored to

the maximum level when A-signal is added to cells at low cell density. These results suggest that the A-signal level is proportionate to the cell density and that A-signaling monitors whether a sufficient density of starved cells is present to make a fruiting body.

To arrest growth and progress into development, *M. xanthus* cells must sense nutrient limitation as well as high cell density. In *E. coli*, when nutrient is limited, uncharged tRNA binds to the accepter site of ribosomes and the highly phosphorylated guanosine tetra- or penta-phosphate, (p)ppGpp, is formed (Cashel et al., 1996). Because (p)ppGpp is made by ribosomes when they stall due to the lack of any amino acid, generation of ppGpp is rich with nutritional information. A similar scenario is seen during *M. xanthus* development. Within 30 minutes of starvation, the level of (p)ppGpp is elevated in response to amino acid limitation (Singer and Kaiser, 1995). Singer *et al.* expressed the *E. coli relA* gene encoding (p)ppGpp synthetase I in *M. xanthus* and found that development-specific *lacZ* reporters are induced without starvation (Singer and Kaiser, 1995). This result suggests that (p)ppGpp alone is sufficient to initiate developmental gene expression. A mutant that lost the ability to accumulate (p)ppGpp in response to starvation failed to form fruiting bodies and spores (Manoil and Kaiser, 1980), and these defects were rescued by providing (p)ppGpp (Harris et al., 1998). The *M. xanthus relA* homolog has been cloned and this gene rescued the developmental defect of the above mutant (Harris et al., 1998). Disruption of the *M. xanthus relA* gene rendered cells unable to accumulate (p)ppGpp and prevented fruiting body development. These results support the idea that elevated (p)ppGpp is sufficient to initiate the developmental program. Harris *et al.* also showed that the accumulation of (p)ppGpp was necessary and sufficient to induce A-signal production (Harris et al., 1998). A-signal is not produced in

the *M. xanthus relA*-null mutant that fails to accumulate (p)ppGpp, and expression of the *E. coli relA* gene in *M. xanthus* restores production of A-signal. It has been proposed that nutrient limitation results in (p)ppGpp production, which triggers A-signal production, and the level of A-signal serves as a cell density indicator to determine whether sufficient cells are present to undergo development.

B-signaling

Another signaling interaction required early in development is B-signaling. Expression of nearly all of the developmental markers examined is either reduced or abolished by B-signaling mutations, suggesting that B signal acts early, within 2 hours after the onset of development. B-signaling mutants fail to form fruiting bodies and spores (Gill and Bornemann, 1988). A subset of the B-signaling mutations map to the *bsgA* gene, which encodes an ATP-dependent protease with a high degree of amino acid similarity to protease La (Lon) of *E. coli* and *Bacillus brevis* (Gill et al., 1993). BsgA is a cytoplasmic protein. Its role in generating the B-signal and the mechanism of extracellular complementation of B-signaling mutants are unknown. It has been proposed that the defect in intracellular proteolysis in *bsgA* mutants is involved in generating the B-signal, but the chemical nature of the B-signal is unknown. Proteolysis is involved in the activation of some regulatory proteins, such as the development-specific sigma factors σ^E and σ^K of *B. subtilis* (Kroos and Cutting, 1994). BsgA may affect a regulatory protein and/or the B-signal itself through proteolysis.

D-signaling

D-signaling mutants arrest development around the stage of aggregation. Under nutrient limitation, the mutants form larger, less compact aggregates than wild-type cells

and sporulation is delayed and substantially reduced. The mutants are defective in β-galactosidase expression from Tn*5* *lac* fusions whose expression normally begins about 5 to 6 hours into development (Cheng and Kaiser, 1989b). Two *dsg* point mutations map to the same locus, *dsgA* (Cheng and Kaiser, 1989b). The amino acid sequence of DsgA has 50% identity to the sequence of translation initiation factor IF3 of *E. coli* and *Bacillus stearothermophilus*, a protein which enables the ribosome to select the initiation codon (Cheng et al., 1994). It has also been shown that DsgA functions like translation initiation factor IF3 of *E. coli* in *M. xanthus* (Kalman et al., 1994). Although cells with *dsgA* point mutations are defective in development, they grow at the same rate as wild-type cells. Complete disruption of the *dsgA* gene by Tn*5* insertion is lethal (Cheng and Kaiser, 1989a). This suggests that the modified proteins encoded by the *dsgA* point mutants retain the functions of IF3 required for growth. The developmental defect of these mutants is not due to less DsgA protein during development since the level of DsgA is uniform throughout growth and development (Kalman et al., 1994). Rather, the developmental defect must result from the altered activity of the mutant DsgA proteins.

The *dsgA* gene product is probably not the signal itself. Rather, as part of the translational machinery, it may be involved in generating the signal. Rosenbluh and Rosenberg have shown that a mixture of fatty acids rescues the fruiting body formation and sporulation of *dsgA* mutants (Rosenbluh and Rosenberg, 1989), suggesting a possible role of fatty acids in D-signaling. Alternatively, the rescue could be explained by the idea that fatty acids alter the permeability of the cell membrane and allow the transfer of another molecule(s), such as D-signal.

E-signaling

Another class of signaling mutants was identified in the study of regulation of the *tps* gene, which encodes spore protein S (Downard et al., 1993). The development of E-signaling mutants is arrested at about 3 to 5 hours after the onset of starvation. The mutants are defective in expression of *tps* and in aggregation, fruiting body formation and sporulation. The defects in gene regulation and sporulation were rescued by mixing with wild-type cells, but the defect in fruiting body formation was not corrected. Mutations affecting E-signaling mapped to the *esg* locus. Two open reading frames have been identified at this locus, which share sequence similarity with the E1a and E1b subunits of a multienzyme complex, branched-chain keto acid dehydrogenase (BCKAD). BCKAD is involved in the synthesis of short branched-chain fatty acids (BCFAs) from branched-chain amino acids (BCAAs) (Toal et al., 1995). These BCKAD products are precursors in the biosynthesis of long branched-chain fatty acids, which are incorporated into phospholipids during vegetative growth. Functional analyses using an *esg*::Tn*5* insertion supported the results of sequence comparisons because the mutant had increased BCAAs due to a lack of BCKAD activity. Furthermore, when the mutant cells were grown vegetatively in the presence of short BCFAs, the defects in development were corrected, suggesting that short BCFAs allowed cells to bypass the metabolic block caused by the *esg* mutation. This result also suggests that BCFAs synthesized during vegetative growth affect the developmental process later, either directly or by effecting production of other molecules. Since a null mutation in *esg* reduced but did not abolish the production of short BCFAs, an alternative pathway to generate these compounds must also exist.

What is the nature of E-signal? A model has been proposed that the BCFAs, synthesized during growth, are released from cellular phospholipid by a developmentally

regulated phospholipase during fruiting body formation (Downard and Toal, 1995), and that one or more of the released BCFAs constitutes E-signal. Alternatively, fatty acids may not serve as the signal directly. Rather, they may act through modifying other molecules, such as proteins, to participate in generating the E-signal.

C-signaling

Among the five identified signaling interactions, C-signaling affects development at the latest stage based on the developmental phenotype and the effect on developmental gene expression. Upon starvation, C-signaling mutant cells are arrested early in aggregation. C-signaling mutants fail to form ripples, tight mounds, or spores. Rather, they form diffuse and flat mounds around 18 hours into development, while tight mounds are formed around 12 h by wild-type cells. Sporulation is severely impaired in C-signaling mutants and the expression of genes normally induced at six hours or later in development is reduced or completely abolished (Hagen et al., 1978; Shimkets and Kaiser, 1982; Kroos and Kaiser, 1987).

All of the C-signaling mutations map to a single gene, *csgA* (Shimkets and Asher, 1988). The *csgA* gene encodes a 25-kD protein with sequence similarity to the members of the short chain alcohol dehydrogenase (SCAD) family (Lee et al., 1995). These enzymes use NAD(H) or NADP(H) to catalyze the interconversion of secondary alcohols and ketones or to mediate decarboxylation. They have a wide variety of substrates and are involved in the production of many different signaling molecules including steroids, prostaglandins, and nodulation factors in plants (Persson et al., 1991; Baker, 1994). CsgA has a NAD(P) binding motif at its N-terminus. Disruption of this motif by amino acid substitution abolished its ability to rescue *csg*$^-$ mutant cells (Lee et al., 1995). The activity

is also abolished by a substitution at the putative catalytic site of the enzyme (Lee et al., 1995). Furthermore, addition of NAD(P) to a mixture of MalE-CsgA and *csgA* mutant cells increased sporulation of the *csgA* cells, while adding NAD(P)H to the mixture reduce sporulation efficiency (Lee et al., 1995). These results suggest that enzymatic activity of CsgA is necessary for C-signaling. CsgA appears to be unique among members of the SCAD family in that it functions extracellularly. The substrate(s) for CsgA is unknown.

A 17 kD protein, named C-factor, has been purified from wild-type developing cells that is able to rescue the developmental defects of *csgA* mutant cells after mixture (Kim and Kaiser, 1990c; Kim and Kaiser, 1990d). C-factor has been proposed to be encoded by *csgA* based on the following findings. First, wild-type cells produce C-factor, but *csgA* mutant cells do not. Secondly, a stretch of amino acid sequence in the middle of C-factor protein was obtained and it corresponds exactly with a deduced amino acid sequence in the middle of CsgA. Thirdly, polyclonal antibodies raised against a *lacZ-csgA* fusion protein and purified by binding to the fusion protein react with the 17 kD C-factor. These findings argue that C-factor is encoded by *csgA*. However, the relationship between the 17 kD C-factor and the 25 kD CsgA is unknown. One possibility is that C-factor is a proteolytic product of CsgA.

The aggregation, sporulation and developmental gene expression defects in *csgA* mutant cells can be rescued by codevelopment with wild-type cells or by the addition of purified C-factor (Hagen et al., 1978; Kim and Kaiser, 1990c; Kim and Kaiser, 1990d). Anti-CsgA antibodies inhibited both fruiting body morphogenesis and sporulation of wild-type cells unless they were first neutralized with purified C-factor (Shimkets and

Rafiee, 1990). Furthermore, the antibodies also localized CsgA at the cell surface and in the extracelluar matrix. These results indicate that CsgA is a cell-surface-associated protein and nondiffusible.

Rescue of *csgA* cells by wild-type cells requires direct cell-cell contact and both the donor and receptor cells have to be motile for effective C-signaling (Kroos et al., 1988; Kim and Kaiser, 1990b; Kim and Kaiser, 1990c). The extracellular complementation does not occur when wild-type and *csgA* mutant cells are separated by a membrane (Kim and Kaiser, 1990c). Nonmotile *mgl* mutant cells exhibit characteristics of the C-signaling mutant phenotype despite the finding that the *mgl* mutant produces normal amounts of C-signal. Addition of purified C-factor to the nonmotile cells induces sporulation without restoring motility and the aggregation defect (Kim and Kaiser, 1991). Manually aligning nonmotile cells by creating narrow grooves on an agar surface restored C-signaling (Kim and Kaiser, 1990a). These observations suggested that motility is required to establish the proper cell alignment needed for efficient C-signal transmission (Kim and Kaiser, 1990a). Specifically, the alignment of cells may be important to promote end-to-end contacts between cells (Sager and Kaiser, 1994).

There is evidence that C-signal induces changes in the motility behavior of individual *M. xanthus* cells. Addition of C-factor to *csgA* cells causes increased gliding speeds, increased duration of the mean gliding interval, and decreased stop frequency (Jelsbak and Sogaard-Anderson, 1999). These changes in cell motility are thought to help move cells into aggregation centers efficiently during fruiting body formation. C-signal-induced stimulation of motility depends on the cytoplasmic Frz signal transduction system. Elements of the Frz system have sequence homology to components of the signal

transduction system for chemotaxis in *E. coli*. Frz proteins control the frequency of gliding reversal, orienting the direction of *M. xanthus* cell movement in response to chemical stimuli (McBride et al., 1992; Shi et al., 1993). FrzCD, one of the proteins in the Frz system, is homologous to Tar, a methyl-accepting chemotaxis protein of *E. coli* (Dworkin, 1996). Exposure of developing *csgA* mutant cells to purified C-factor increases the ratio of methylated to non-methylated FrzCD protein (Sogaard-Anderson and Kaiser, 1996). Methylation depends on the methyltransferase FrzF, which is a *cheR* homolog. C-factor-induced methylation also depends on FruA, a DNA-binding response regulator that is necessary *in vivo* for all known responses to C-factor.

csgA mutant cells respond differently to different levels of added C-factor. C-factor at a lower concentration rescues aggregation and expression of a gene partially dependent on C-signaling, whereas a higher concentration of C-factor is needed to rescue sporulation and expression of a gene absolutely dependent on C-signaling (Kim and Kaiser, 1991). These observations suggested that C-signaling induces and coordinates the temporally separated morphogenetic processes of aggregation and sporulation during fruiting body formation. The *csgA* gene has a long upstream sequence and full expression of *csgA* depends on multiple factors including CsgA itself (Kim and Kaiser, 1991). Positive autoregulation of *csgA* leads to an increased level of CsgA later in development. Not only does *csgA* expression increase in response to C-signaling, the C-signaling interaction between cells also increases due to close and ordered packing of cells in the nascent fruiting body. Therefore, CsgA appears to act as an extracellular developmental timer to bring about the appropriate order of the developmental process, first aggregation, then sporulation.

A model has been proposed for the C-signal transduction pathway based on the results of genetic analyses of mutants deficient in C-signal-dependent activities (Figure 1.2). In this model, the signaling event is the interaction between C-signal on one cell end and a hypothetical sensor on a second cell end. This interaction activates the C-signaling pathway in the second cell. The first known component downstream of the signaling event, *fruA*, is required for all four C-signal-dependent activities: rippling, aggregation, sporulation and developmental gene expression (Ogawa et al., 1996; Sogaard-Andersen et al., 1996; Ellehauge et al., 1998). FruA is a DNA-binding response regulator that is expressed at 3 to 6 hours after the onset of development (Ellehauge et al., 1998). The expression of *fruA* is independent of C-signal, but depends on the earlier signals A and E. However, genetic experiments suggest that C-signaling controls FruA activity post-translationally by phosphorylation. Downstream of *fruA*, the pathway branches, with one branch leading to the motility responses to C-signaling, *i.e.*, rippling and aggregation. The *frz* signal transduction system is required for this branch. The other branch leads to sporulation. The products of the *devRS* locus are involved in this branch. Mutants with defects in *devRS* ripple and aggregate normally, but have impaired sporulation. Five genes are present at this locus, *orf1*, *orf2*, *devT*, *devR* and *devS*, none of which share sequence similarity with known proteins in the databases (Kaiser, 1999). Expression of the *dev* operon is first detected after 6 hours of development and is under strong negative autoregulation. *devS* is essential for this repression (Kaiser, 1999). Cytometric experiments have shown that *dev* is expressed in a bimodal pattern among cells, *i.e.*, some cells are fully on and some cells are fully off, unlike some other developmentally regulated genes (Russo-Marie et al., 1993). Fluorescence experiments indicate that in a

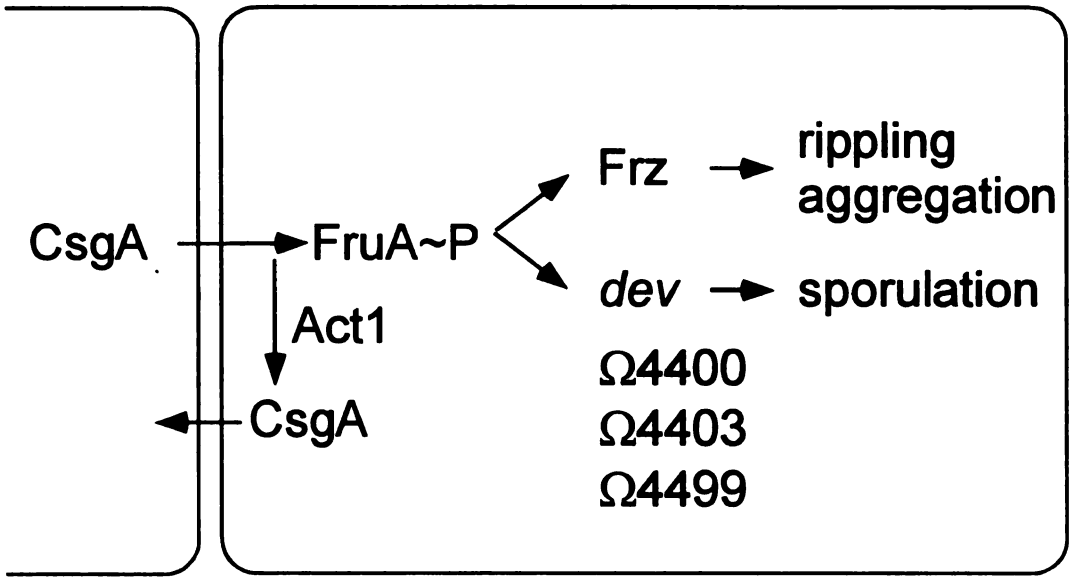


Figure 1.2. Model depicting cellular responses to C signaling.

nascent fruiting body, the oval cells undergoing development in the center of the fruiting body express *dev*, whereas the rod-shaped peripheral cells do not express *dev* (Kaiser, 1999). These results suggest that *dev* expression behaves like a switch at the cellular level. Based on the facts that CsgA synthesis is similar in *dev* mutant cells, in *fruA* mutant cells, and in wild-type cells, and that *csgA* autoregulates, a third branch leading to *csgA* expression must also exist (Kaiser, 1999). A newly identified regulator Act1 is involved in this pathway (Gorski et al., 2000). The *act1* mutant cells produce substantially less CsgA than normal. They aggregate normally, but fail to sporulate and expression of the *dev* locus is severely impaired in the mutant. These results suggest that the reduced amount of CsgA in the *act1* mutant is adequate for aggregation, but not for sporulation. It has been proposed that Act1 responds to C-signal reception by increasing the expression of the *csgA* gene, creating a positive feedback loop that boosts the level of CsgA for later developmental events.

Suppressors of *csgA* (*soc*) that restore sporulation of *csg* mutant cells also bring insight about the mechanism of C-signaling. Several suppressors have been identified. Two transposon mutations in the *socABC* operon restore development to *csgA* null mutants (Lee and Shimkets, 1996). Mixing of these *socABC* and *csgA* double mutants with *csg* cells stimulates the development of the *csgA* cells, suggesting that the suppressor cells are able to produce C-signal or a similar molecule via a *csgA*-independent pathway. Three genes are encoded in this operon: *socA*, like *csgA*, encodes a protein similar to members of the SCAD family; *socB* encodes a putative membrane anchoring protein; and *socC* has no homolog in the databases. SocA protein shares 28% identity and 51% similarity in amino acid sequence with CsgA. Both of the transposon suppressor

mutations inactivate *socC*, leading to a 30- to 100- fold increase in *socA* transcription, suggesting that *socC* may encode a negative autoregulator of the *socABC* operon. It has been proposed that the mutations cause derepression of *socA* and the overproduced SocA substitutes for CsgA.

Another suppressor mutation is named *socD*. The *socD* gene encodes a histidine protein kinase and the suppressor mutation restores sporulation to *csgA* cells under normal developmental conditions at 32°C (Rhie and Shimkets, 1991; Shimkets, 1999). This mutation also induces sporulation of otherwise wild-type cells and *csgA* cells at 15°C under conditions that normally support growth. These results suggest that SocD might be involved in nutrient sensing.

Investigation of another suppressor mutation, *socE*, has revealed a new role of CsgA in growth and development. Disruption of *socE* by a transposon restores development to *csgA* mutants (Crawford and Shimkets, 2000). SocE is a vegetatively expressed, highly basic protein with no homolog in the databases. SocE is essential for vegetative growth in *csgA*+ cells since attempts to transfer the *socE* null allele to *csgA*+ cells failed. Results from experiments in which *socE* was placed under the control of a light-inducible promoter have shown that *socE* depletion arrests growth and induces sporulation under normal growth conditions in an otherwise wild-type background. SocE depletion also curtails DNA and RNA synthesis, inhibits cell elongation, and induces accumulation of the stringent nucleotides [p]ppGpp. Amino acid substitutions in the CsgA coenzyme-binding pocket or catalytic site eliminated growth arrest. A *relA* mutation also eliminated growth arrest. Pseudorevertants selected for growth following *socE* depletion have mutations in *csgA* or *relA*. These results suggest that SocE works in

opposition to CsgA to regulate growth arrest through *relA*, i.e., SocE inhibits the stringent response and CsgA induces it. This is supported by the fact that *csgA* cells have a very low [p]ppGpp level, whereas *socE csgA* cells have a high [p]ppGpp level throughout development, as do wild-type cells. CsgA shares sequence similarity with members of the SCAD family. Although its enzymatic activity appears to be necessary for growth arrest of SocE-depleted cells, extracellular C-signaling is unlikely to be involved under the conditions tested because the cells are dispersed at low cell density in growth medium, which prevents contact-dependent exchange of C-signal.

Regulation of gene expression

Regulation of gene expression in response to various environmental cues is mediated by a variety of mechanisms in prokaryotes. Much of the regulation occurs at the transcriptional level. Many bacteria possess multiple sigma factors that combine with core RNA polymerase to produce holoenzymes that express different sets of genes in response to a wide variety of signals. A well-studied example of this is the sporulation process of *B. subtilis* (c.f. Haldenwang, 1995), which involves a cascade of sigma factors, activated at different stages in development and in two different compartments, to regulate genes spatially and temporally. Multiple sigma factors have also been identified in *M. xanthus*. The major sigma factor in vegetatively growing cells, designated σ^A, was identified by using the *E. coli* σ^{70} gene as a hybridization probe (Inouye, 1990) and RNA polymerase containing σ^A has been purified from vegetative cells (Biran and Kroos, 1997). σ^A has sequence similarity to σ^{70} of *E. coli* and σ^{43} of *B. subtilis*. Partially purified σ^A RNA polymerase or holoenzyme reconstituted from σ^A and *M. xanthus* core RNAP transcribed both vegetatively-expressed *M. xanthus* genes that were tested.

The promoters of one vegetative gene, *pilA* (Wu and Kaiser, 1997b), and three developmental genes, *mbhA* (Romeo and Zusman, 1991), *sdeK* (Garza et al., 1998), and Ω4521 (Keseler and Kaiser, 1995) resemble in the –24 and –12 regions of promoters that are recognized by σ^{54} in other bacteria. Mutational analysis of the Ω4521 promoter region has shown that DNA base pairs shared with the consensus σ^{54} promoter sequence are essential for Ω4521 promoter activity, suggesting that it is likely to be transcribed by σ^{54} RNA polymerase *in vivo* (Keseler and Kaiser, 1995). An *rpoN* gene encoding a σ^{54} homolog has been cloned from *M. xanthus* and attempts to make a null mutant have failed, suggesting that the gene is essential for *M. xanthus* growth, unlike in some other organisms where σ^{54} is nonessential (Keseler and Kaiser, 1997).

CarQ, a sigma factor of the extracytoplasmic function (ECF) subfamily, is responsible for light-induced synthesis of membrane-associated carotenoids in *M. xanthus* through the activation of its own *carQRS* promoter and the *carC* promoter. CarC encodes for phytoene dehydrogenase that converts colorless phytoene into colored carotenoids (Hodgson and Murillo, 1993; Gorham et al., 1996). The ECF sigma factors are a subfamily of σ^{70} factors that typically regulate genes encoding proteins with extracytoplasmic functions (Lonetto et al., 1994). These sigma factors are shorter than other known sigma factors and show significant divergence from other σ^{70} family members, most noticeably in regions 2.4 and 3. The activity of CarQ is negatively regulated by an inner membrane protein, CarR, which is also encoded in the *carQRS* operon (Gorham et al., 1996). CarR is unstable in the light and is essential for the inactivation of the *carQRS* promoter in the dark. It has been proposed that the membrane-

associated CarR sequesters CarQ to the membrane in the dark to prevent it from functioning, whereas in the light, loss of CarR leads to release of the sigma factor.

Four other putative *M. xanthus* sigma factors have been described, but no promoters have been identified that are recognized by these sigma factors. SigB, a sigma factor expressed later in sporulation, may be responsible for the expression of late developmental genes (Apelian and Inouye, 1990). Expression of one such gene, *ops*, which is expressed around 24 hours into development, was blocked in a *sigB* deletion strain. However, no evidence of direct transcriptional regulation by SigB has been described. *sigB* is not essential for development since *sigB* mutant cells exhibit normal fruiting body formation and sporulation. However, *sigB* mutant spores show severe defects in stability and viability, suggesting a role for SigB in maturation of myxospores. SigC, another developmental sigma factor, is expressed immediately after cells enter development and its expression is reduced significantly later, at the onset of sporulation (Apelian and Inouye, 1993). *sigC* mutant cells develop normally, but *sigC* cells are capable of forming fruiting bodies and myxospores on semi-rich media. This result suggests that *sigC* may be involved in negatively regulating the initiation of fruiting body formation. SigD is a sigma factor expressed during the stationary phase of vegetative growth and early in development (Ueki and Inouye, 1998). *sigD* mutant cells exhibit growth defects during the late-log phase and stationary phase as well as an altered pattern of protein synthesis. The deletion mutant also showed a significant delay in fruiting body formation and sporulation and yielded fewer spores than wild-type cells. Additionally, another sigma factor of the ECF subfamily, RpoE1, has been identified that may play a

role in transcriptional regulation of genes involved in motility behavior during both vegetative growth and development (Ward et al., 1998).

Transcriptional activator proteins are involved in transcriptional regulation of many genes in *M. xanthus*. In all systems investigated, σ^{54} promoters require the binding of an activator protein to an upstream sequence. Likewise, the *M. xanthus* Ω4521 and *mbhA* genes require DNA upstream of the core promoter elements for developmental expression (Romeo and Zusman, 1991; Keseler and Kaiser, 1995). Despite strong similarity in the core promoter regions, expression of these two promoters differs significantly in terms of the timing of expression during development and the requirements for a solid surface and extracellular signals, suggesting the involvement of different activator proteins.

Another example of transcriptional regulation by activator proteins comes from the study of *tps* and *ops* gene expression (Kil et al., 1990). These two genes are separated by 1.4 kb on the *M. xanthus* chromosome and are 90% identical in DNA sequence. The *tps* gene encodes the abundant spore coat protein S and its expression is activated about 5 hours after the initiation of development, whereas *ops* encodes a protein found inside myxospores and is not expressed until much later in development, around the stage of sporulation. A DNA segment between 131 and 311 bp upstream from the *ops* transcriptional start site regulates not only the expression of *ops*, but also the expression of *tps* located about 2 kb downstream. The activation is orientation independent and the UAS is still functional when the DNA segment is moved farther upstream. The need for an activator protein for *tps* expression is supported by the finding that a protein-DNA complex was observed when an upstream DNA segment was incubated with extracts of

developing *M. xanthus* cells. Also, expression of *tps* requires two upstream sequences centered at -90 and -270 bp relative to its transcriptional start site.

The regulation of *csgA* gene expression provides another example of the likely involvement of a transcriptional activator protein(s). Responding to environmental cues such as nutrient level, peptidoglycan and B signal, *csgA* expression and the resulting increase in CsgA level during the developmental process is thought to entrain the order of morphological events, *i.e.*, ripping, aggregation, then sporulation (Li et al., 1992). Hence, *csgA* expression must be tightly controlled. Indeed, the expression of *csgA* requires DNA upstream of the core promoter. DNA extending to –400 bp appears to be needed for optimal *csgA* expression under starvation condition, whereas another 500 bp upstream (up to –930 bp) is necessary for full expression under low nutrient level conditions.

C-signal-dependent Tn*5* *lac* fusions Ω4403 (Fisseha et al., 1996), Ω4400 (Brandner and Kroos, 1998) and Ω4499 (Fisseha et al., 1999) all require DNA upstream of the region typically recognized by RNA polymerase for full developmental expression. In the following chapter, I show that full expression of Ω4514 also requires DNA upstream of its core promoter sequence, suggesting the involvement of a transcriptional activator protein.

I chose to study Ω4514 regulation because it is expressed with similar timing during development as the other Tn*5* *lac* fusions just mentioned, but unlike the other fusions, Ω4514 expression does not depend on C-signaling. The promoters of the C-signal-dependent genes are not similar in the –10 and –35 regions, and they do not resemble the consensus sequence for σ^{70} or σ^{54} promoters. However, a C-rich sequence CATCCCT has been identified in the Ω4403 and Ω4400 promoters centered at –49.

Similar sequences have also been identified in the Ω4499 promoter at position –55, and in another C-signal-dependent promoter, *csgA*, at position –63. A degenerate consensus 5′-CAYYCCY-3′ (Y means pyrimidine), known as the C box, has been deduced from these sequences and it has been proposed that C-box sequences are *cis*-acting regulatory elements important for C-signal-dependent gene expression (Fisseha et al., 1999). The Ω4514 promoter DNA does not contain a region similar to the C box sequence around –50. Additionally, unlike C-signal-dependent promoters, the Ω4514 promoter resembles the promoters transcribed by σ^{70} RNAP and it can be transcribed by σ^{A} RNAP isolated from vegetative *M. xanthus* cells. These results suggest that Ω4514 expression is regulated by a different mechanism than C-signal-dependent genes. My investigation revealed a new aspect of gene regulation during *M. xanthus* development, thus contributing to our overall understanding of developmental gene regulation in response to cell-cell interactions.

Chapter II

Characterization of regulatory mechanism of a cell interaction-dependent gene in *Myxococcus xanthus*

Abstract

Ω4514 is a Tn*5 lac* insertion in the *Myxococcus xanthus* genome that fuses *lacZ* expression to a developmentally regulated promoter. I cloned DNA upstream of the Ω4514 insertion site and localized the promoter. The promoter, which is induced during development, resembles vegetative promoters in sequence. σ^A RNA polymerase, the major form of RNA polymerase in growing *M. xanthus*, initiated transcription from this promoter *in vitro.* Two complete open reading frames were identified downstream of the promoter and before the Ω4514 insertion. The first gene product (ORF1) has a putative helix-turn-helix DNA-binding motif and shows sequence similarity to several transcriptional regulators. Our results show that the product of *orf1* exerts a negative regulatory effect on expression of its own operon. However, we were unable to demonstrate specific binding of ORF1 to the Ω4514 promoter region. The second gene (*orf2*) is most similar to subunit A of glutaconate CoA-transferase which is involved in the glutamate fermentation pathway. Mild sporulation defects were observed in *orf1* and *orf2* disruption mutants, whereas the Tn*5 lac* Ω4514 insertion did not cause a developmental defect. Although ORF1 negatively regulates Ω4514 expression, developmental induction of this operon does not result primarily from a loss of ORF1. Rather, transcriptional activation appears to be involved and multiple upstream DNA elements are necessary for full developmental expression. Ω4514 is the first example of a developmentally expressed *M. xanthus* operon that is transcribed by the major vegetative RNA polymerase, and its regulation appears to involve both negative autoregulation by ORF1 and positive regulation by one or more transcriptional activators.

Introduction

Myxococcus xanthus is a gram-negative soil bacterium that undergoes multicellular development (Dworkin and Kaiser, 1993). When starved at high cell density on a solid surface, cells move in a coordinated fashion into aggregation centers, where they form mound-shaped fruiting bodies that each contain approximately 10^5 cells. Within the fruiting bodies, some of the rod-shaped cells differentiate into dormant, ovoid spores that are heat and desiccation resistant.

Cell-cell interactions play a critical role in this multicellular developmental process. At least five extracellular signals, known as the A-, B-, C-, D-, and E-signals, appear to be involved. Mutants defective in the production of any one of these signals are arrested in development at a particular stage, but are rescued by codevelopment with wild type cells or cells that are defective in the production of a different signal (Hagen et al., 1978; LaRossa et al., 1983; Downard et al., 1984).

To study the role of cell-cell interactions in controlling gene expression during *M. xanthus* development, Tn*5 lac*, a transposon containing a promoterless *E. coli lacZ* gene, has been used to identify developmentally regulated genes (Kroos and Kaiser, 1984). Transposition of Tn*5 lac* into the *M. xanthus* chromosome can generate a transcriptional fusion between *lacZ* and an *M. xanthus* promoter. Among 2374 Tn*5 lac* insertions, 29 of them were activated during *M. xanthus* development (Kroos et al., 1986). The dependence of developmental gene expression on cell-cell interactions was examined by monitoring β-galactosidase expression of the *lacZ* fusions in cell interaction mutants (Kroos et al., 1986). A- and B-signaling are required for normal developmental gene

expression at the onset of development (Gill and Cull, 1986; Kuspa et al., 1986; Kroos and Kaiser, 1987), D- and E-signaling are required at 3 to 5 hr into development (Cheng and Kaiser, 1989b; Downard et al., 1993), and C-signaling is required at about 6 hr into development (Kroos and Kaiser, 1987; Li and Shimkets, 1993).

Among the five extracellular signaling interactions, only the A- and C-signaling interactions are well-characterized. A-signal is a mixture of peptides and amino acids apparently generated by extracellular proteases released in response to nutrient limitation and used to determine whether cells are at a sufficiently high density to initiate multicellular development (Plamann et al., 1992; Kuspa et al., 1992; Kuspa et al., 1992). All of the C-signaling mutations map to a single gene called *csgA*, and the mutants are defective in both fruiting body formation and sporulation (Hagen et al., 1978; Shimkets and Kaiser, 1982; Shimkets and Asher, 1988). CsgA, a cell surface and/or extracellular matrix-associated protein (Shimkets and Rafiee, 1990), appears to mediate C-signaling, which both controls cell movements (Sogaard-Anderson and Kaiser, 1996; Jelsbak and Sogaard-Anderson, 1999) and is influenced by the alignment of cells that results from movement of cells into aggregates (Kim and Kaiser, 1990a; Sager and Kaiser, 1994). Close packing of cells within fruiting bodies is thought to allow efficient C-signaling, which triggers sporulation (Sager and Kaiser, 1994). Different levels of C-signaling are also required for expression of different developmental genes (Kim and Kaiser, 1991). Hence, C-signaling seems to act as a timer governing the developmental process and appears to couple morphogenesis of the fruiting body with expression of genes at the proper times.

We are interested in the regulation of developmental gene expression in response to extracellular signals. To begin to understand the mechanisms involved, the regulatory regions of several C-signal-dependent transcriptional units, identified by Tn*5 lac* insertions Ω4403, Ω4400 and Ω4499, have been characterized (Fisseha et al., 1996; Brandner and Kroos, 1998; Fisseha et al., 1999). The promoters are not similar to each other in the –10 and –35 regions and they do not resemble promoters that are transcribed by σ^{70} or σ^{54} RNAP. However, both Ω4403 and Ω4400 have the sequence 5′-CATCCCT-3′ centered at –49 bp. Similar sequences are also found in the Ω4499 promoter region and other C-signal-dependent promoters at positions near –50 bp. Changing 5′-CATCCCT-3′ in the Ω4400 promoter to 5′-ACGAAAG-3′ abolished activity of this promoter, demonstrating the importance of this sequence. Hence, a sequence with the consensus 5′-CAYYCCY-3′ (known as the C box) has been proposed to be important for C-signal-dependent gene expression.

Here, I report studies on the regulation of Ω4514, a Tn*5 lac* insertion that is expressed with similar timing during development as Ω4400, Ω4403 and Ω4499, but Ω4514 expression does not depend on C-signaling. I constructed a series of 5′ and 3′ deletions to localize the sequence required for promoter activity, determined the nucleotide sequence of the promoter region, and mapped the transcriptional start site. It appears that the Ω4514 promoter is different from other *M. xanthus* development-specific promoters that have been characterized in that it resembles σ^{70} promoters and can be transcribed by σ^{A} RNAP isolated from vegetative *M. xanthus* cells. Also, no sequence matching the C box consensus is present near –50 bp in this C-signal-independent promoter. Regulation of the Ω4514 operon is complex, involving negative autoregulation

by the product of the first gene of the operon and positive regulation mediated by upstream DNA elements. These studies provide the first example of a development-specific *M. xanthus* gene that is transcribed by the major vegetative RNAP and establish a foundation for biochemical approaches toward identifying proteins that regulate Ω4514 expression.

Materials and Methods

Bacterial strains and plasmids. Strains and plasmids used in this work are listed in Table 2.1.

Growth and development. *Escherichia coli* cells were grown at 37°C in LB medium (Sambrook et al., 1989) containing 50 μg ampicillin, 25 μg kanamycin or 10 μg of tetracycline per ml, as necessary. *M. xanthus* was grown at 32°C in CTT medium (Hodgkin and Kaiser, 1977) in liquid culture or on agar (1.5%) plates with 40 μg of kanamycin or 12.5 μg of oxytetracycline per ml when required (Kroos et al., 1986). Fruiting body development was performed on TPM (10 mM Tris-HCl, pH 8.0, 1 mM KH_2PO_4, 8 mM $MgSO_4$, final pH 7.6) agar (1.5%) plates as described previously (Kroos et al., 1986).

Molecular cloning. Recombinant DNA work was performed using standard techniques (Sambrook et al., 1989). Plasmid DNA was prepared from *E. coli* DH5α or JM83.

To clone the DNA upstream of Tn*5 lac* Ω4514, chromosomal DNA was prepared (Laue and Gill, 1994) from *M. xanthus* DK4514 and digested with *Xho*I, the fragments were ligated to *Xho*I-digested pGEM-7Zf, and the mixture was transformed into *E. coli* DH5α, selecting for both ampicillin resistance (Ap^r) of the vector and kanamycin resistance (Km^r) of the desired insert. One transformant with a plasmid bearing an insert of the expected size was characterized further. Restriction fragments of *M. xanthus* DNA from this plasmid, pTH1-3, were gel-purified and ligated into vectors as indicated in Table 2.1. In these and the subsequent subcloning steps described in Table 2.1, vectors

Table 2.1. Bacterial Strains and Plasmids

Strain or plasmid	Characteristics	Source or Reference
E. coli		
DH5α	*supE*44 Δ*lacU*169 ϕ80 Δ*lacZ* ΔM15 *hsdR*17 *recA*1 *endA*1 *gyrA thi*-1 *relA*1	Hanahan, 1983
JM83	*ara* Δ*lac-pro strA thi* ϕd*lacZ* ΔM15	Messing, 1979
BL21(DE3)	F⁻ *ompT gal [dcm] [lon] hsdS*$_B$ ($r_B^- m_B^-$;an *E. coli* B strain) with DE3, a λ prophage carrying the T7 RNA polymerase gene	Novagen
M. xanthus		
DK1622	wild type	Kaiser, 1979
DK4514	Tn5 *lac* (Km^r) Ω4514	Kroos et al., 1986
MMF1727	*attB*::pREG1727	Fisseha et al., 1996
MTH1-3	*attB*::pTH1-6	This study
MTH4-1	*attB*::pTH4-1	This study
MTH4-2	*attB*::pTH4-2	This study
MTH4-3	*attB*::pTH4-3	This study
MTH5-3	*attB*::pTH5-3	This study
MTH5-4	*attB*::pTH5-4	This study
MTH5-5	*attB*::pTH5-5	This study
MTH10-3	*attB*::pTH10-3	This study
MTH11-1	*attB*::pTH10-3 *orf2*:pTH11-1	This study

MTH11-2	*orf2*:pTH11-1	This study
MGV01	*orf1*::pBRE47Sp4514	Greg Velicer
MGV02	*attB*::pTH10-3 *orf1*::pBRE47Sp4514	Greg Velicer
MTH8-1	Tn5 *lac* (Tc^r) Ω4514	This study
MTH9-1	MTH8-1::pTH9-1, Km^r Tc^r	This study
MTH9-2	MTH8-1::pTH9-2, Km^r Tc^r	This study
MTH9-4	MTH8-1::pTH9-4, Km^r Tc^r	This study
DK5208	*csgA*::Tn*5-132* (Tc^r) Ω205	Shimkets and Asher, 1988
MTH7-1	*csgA*::Tn*5-132* (Tc^r) Ω205 *attB*::pTH1-6	This study
MTH7-2	*csgA*::Tn*5-132* (Tc^r) Ω205 *attB*::pTH4-3	This study
MTH7-3	*csgA*::Tn*5-132* (Tc^r) Ω205 *attB*::pTH4-1	This study
JPB03	*csgA*::Tn*5-132* (Tc^r) Ω205 *attB*::pREG1727	Brandner and Kroos, 1998
DK5225	*csgA*::Tn*5-132* (Tc^r) Ω205 Tn5 *lac* (Km^r) Ω4514	Kroos and Kaiser, 1987
MGV03	DK1622::p1175SmSl4514	Greg Velicer
Plasmid		
pUC19	Ap^r *lacα*	Yanisch-Perron et al., 1985
pTH4-4	Ap^r (pUC19)[a]; 1.6-kb *Sma*I-*Bam*HI fragment from pTH1-3	This study
pTH4-6	Ap^r (pUC19); 0.7-kb *Sac*I-*Bam*HI fragment from pTH4-4	This study
pTH4-7	Ap^r (pUC19); 1.3-kb *Sph*I-*Bam*HI fragment from pTH4-4	This study
pTH7-1	Ap^r (pUC19); 2.2-kb *Sal*I-*Bam*HI fragment from pTH1-3	This study

pTH7-2	Ap^r (pUC19); 4.3-kb *Xho*I-*Bam*HI fragment from pTH1-3	This study
pTH10-1	Ap^r (pUC19); 120-bp *Sma*I-*Eco*47III fragment from pTH4-4	This study
pGEM-7Zf	Ap^r *lacα*	Promega
pTH1-3	Ap^r Km^r (pGEM-7Zf); 13.3-kb *Xho*I fragment from DK4514	This study
pTH5-1	Ap^r (pGEM-7Zf); 0.7-kb *Eco*RI-*Bam*HI fragment from pTH4-6	This study
pTH5-2	Ap^r (pGEM-7Zf); 1.3-kb *Nru*I-*Bam*HI fragment from pTH4-4	This study
pGEME47Sp4514	Ap^r (pGEM-7Zf); 242-bp *Eco47*III-*Sph*I fragment from pTH4-4	This study
pTH9-3	Ap^r (pGEM-7Zf); 4.3-kb *Hind*III-*Bam*HI fragment from pTH7-2	This study
pBR322	Ap^r Tc^r	Bolivar et al., 1977
pBRE47Sp4514	Tc^r (pBR322); 250-bp *Nsi*I-*Aat*II fragment from pGEME47Sp4514	This study
pTH11-1	Tc^r (pBR322); 323-bp fragment of internal *orf2* sequence generated by PCR of pTH4-4	This study
pET21a	Ap^r	Novagen
pTH191	Ap^r (pET21a); complete *orf1* sequence generated by PCR from pTH4-4 using primers LK190 and LK191	This study
pTH192	Ap^r (pET21a); complete *orf1* sequence followed by a stop codon generated by PCR from pTH4-4 using primers LK190 and LK192	This study
pET16b	Ap^r	Novagen
pTH193	Ap^r (pET16b); complete *orf1* sequence generated by PCR from pTH4-4 using primers LK190 and LK193	This study

pREG1727	Ap^r Km^r P1-*inc attP 'lacZ*	Fisseha et al.,1996
pTH1-6	Ap^r Km^r (pREG1727); 4.3-kb *Xho*I-*Bam*HI fragment from pTH1-3	This study
pTH4-1	Ap^r Km^r (pREG1727); 2.2-kb *Sal*I-*Bam*HI fragment from pTH1-3	This study
pTH4-2	Ap^r Km^r (pREG1727); 2.1-kb *Xho*I-*Sal*I fragment from pTH1-3	This study
pTH4-3	Ap^r Km^r (pREG1727); 1.6-kb *Sma*I-*Bam*HI fragment from pTH1-3	This study
pTH5-3	Ap^r Km^r (pREG1727); 0.7-kb *Xho*I-*Bam*HI fragment from pTH5-1	This study
pTH5-4	Ap^r Km^r (pREG1727); 1.3-kb *Xho*I-*Bam*HI fragment from pTH5-2	This study
pTH5-5	Ap^r Km^r (pREG1727); 1.3-kb *Hind*III-*Bam*HI fragment from pTH4-7	This study
pTH10-3	Ap^r Km^r (pREG1727); 120-bp *Hind*III-*Bam*HI fragment from pTH10-1	This study
pREG429	Ap^r Km^r P1-*inc*	Gill et al.,1988
pTH9-1	Ap^r Km^r (pREG429); 1.6-kb *Eco*RI-*Bam*HI fragment from pTH4-4	This study
pTH9-2	Ap^r Km^r (pREG429); 2.2-kb *Acc*I-*Bam*HI fragment from pTH7-1	This study
pTH9-4	Ap^r Km^r (pREG429); 4.3-kb *Eco*RI-*Bam*HI fragment from pTH9-3	This study
pREG1175	Ap^r Km^r P1-*inc*	Gill and Bornemann, 1988
p1175SmSl4514	Ap^r Km^r (pREG1175); 2.7-kb *Xho*I-*Sma*I fragment from pTH7-2	Greg Velicer

[a] The vector is indicated in parentheses.

were digested with the same restriction enzymes used to produce the fragments, except as indicated below.

To construct pTH5-2, the 0.9-kb *Nru*I-*Bam*HI fragment from pTH4-4 was gel-purified and ligated into pGEM-7Zf that had been digested with *Sma*I and *Bam*HI.

Plasmid pTH9-2 was constructed as follows. The 2.2-kb *Acc*I-*Bam*HI fragment from pTH7-1 was gel-purified and ligated into pREG429, which had been digested with *Cla*I and *Bam*HI.

To subclone the 120-bp *Sma*I-*Eco*47III fragment from the region upstream of Tn*5* *lac* Ω4514 into pREG1727, the fragment was gel-purified after digestion of pTH4-4 and ligated into *Sma*I-digested pUC19. A plasmid with the fragment in the correct orientation for further cloning into pREG1727 (i.e., the *Sma*I end of the fragment close to the *Hind*III site in pUC19) was identified and named pTH10-1. A *Hind*III-*Bam*HI fragment of pTH10-1 that contains the 120-bp *Sma*I-*Eco*47III fragment of Ω4514 was obtained and cloned into *Hind*III-*Bam*HI-linerized pREG1727, resulting in pTH10-3, in which the 120-bp *Sma*I-*Eco*47III fragment was positioned before the promoterless *lacZ*.

Plasmid pGEME47Sp4514 was constructed by digestion of pTH4-4 with *Eco*47III and *Sph*I, gel-purification of the resulting 242-bp fragment and ligation into pGEM-7Zf, which had been linerized with *Sma*I and *Sph*I. This plasmid was digested with *Nsi*I and *Aat*II to obtain a 250-bp fragment, which was then ligated into *Aat*II-*Pst*I-digested pBR322 to construct pBRE47Sp4514.

Plasmid pTH11-1 was constructed by ligating part of the *orf2* sequence into pBR322. The partial *orf2* sequence was generated by PCR using pTH4-4 as a template. The upstream primer was 5'-AAACTGCAGTCCGGATGGCGCGTCG-3', which is

shown with *Pst*I site underlined. This primer anneals to the sequence between positions 814 and 829 (Figure 2.4), and the downstream primer was 5'-AAGAATTCGGGATGAAGGGCAGCC-3', which has an underlined *Eco*RI site and anneals to the sequence between positions 1137 and 1122 (Figure 2.4). The amplified fragment was digested with *Pst*I and *Eco*RI, gel-purified, and ligated to *Pst*I-*Eco*RI-digested pBR322 to construct pTH11-1. The insert was sequenced to ensure that no error occurred during the PCR.

Plasmids pTH191, pTH192 and pTH193 were constructed as follows. The insert for each plasmid was generated by PCR with the same upstream primer, 5'-AGAAGGGACATATGACGAACACCGGAGGA-3', which has an *Nde*I site (underlined) and the first 18 bases of *orf1* sequence. The downstream primer (LK191) for generating the insert of pTH191 was 5'-CTCCTCGAGTGCGTCCTCCGAATCCGT-3', which has a *Xho*I site (underlined) and the last 18 bases of *orf1* sequence without a stop codon. The downstream primer (LK192) for generating the insert of pTH192 was 5'-CTCCTCGAGTCATGCGTCCTCCGAATC-3', which has the stop codon TGA adjacent to the *Xho*I site. The downstream primer (LK193) for generating the insert of pTH193 was like LK192, except it has a *Bam*HI site instead of the *Xho*I site. The amplified fragments were digested with appropriate restriction enzymes, gel-purified and ligated into *Nde*I-*Xho*I-digested pET21a to construct pTH191 and pTH192, or into *Nde*I-*Bam*HI-digested pET16b to construct pTH193. Plasmids pET21a and pET16b are designed for high level protein expression in *E. coli* under the control of T7 RNA polymerase. The *orf1* DNA was upstream of an in-frame sequence encoding a 6xHis tag followed by a stop codon in pTH191, while in pTH192, the *orf1* DNA was followed immediately by a

stop codon. In pTH193, the *orf1* DNA was downstream of an in-frame sequence encoding a 6xHis tag. The inserts and junctions were sequenced to ensure that no error occurred during the PCR.

To construct p1175SmSl4514, the 2.7-kb *Xho*I-*Sma*I fragment from pTH4-4 was gel-purified and ligated into pREG1175 that had been digested with *Sal*I and *Sma*I.

DNA sequencing. Plasmid pTH4-4 was used as the template in sequencing reactions performed by the method of Sanger *et al.* (Sanger et al., 1977) with a Sequenase kit (United States Biochemical). Ambiguities arising from premature termination were resolved using the protocol of Fawcett and Bartlett (Fawcett and Bartlett, 1990). Briefly, 1µl of a reaction mixture containing terminal deoxynucleotide transferase (1 µM each deoxynucleoside triphosphate [pH 7.0], 2 U of terminal deoxynucleotide transferase per µl, 1x Sequenase reaction buffer) was added to each of the termination reactions (16 µl) and incubated at 37°C for 30 min. The reaction was terminated by adding 4 µl of stop buffer (United States Biochemical). 7-Deaza-dGTP reaction mixtures were used to resolve regions of compression. DNA and protein sequence analyses were done with the University of Wisconsin Genetics Computer Group software package. The Michigan State University Macromolecular Structure Facility synthesized oligonucleotide primers as needed to sequence both strands of the 1.9-kb Ω4514 upstream DNA.

Construction of *M. xanthus* strains. Strains containing pREG1727 derivatives integrated at Mx8 *attB* were constructed by P1 specialized transduction from the *rec*+ *E. coli* strain JM83 (Gill et al., 1988; Fisseha et al., 1996), or by electroporation, of the wild-type *M. xanthus* strain DK1622 or the *csgA* mutant strain DK5208. Except where noted otherwise, at least three derivatives, each containing a single copy of integrated plasmid,

were identified by Southern blot analysis (Sambrook et al., 1989; Fisseha et al., 1996), and β-galactosidase production was measured under developmental conditions as described previously (Kroos et al., 1986).

Strain MTH8-1 was constructed by transducing bacteriophage P1::Tn*5* *lac* (Tc^r) into DK4514 with selection for oxytetracycline resistance. Screening for kanamycin-sensitive transductants identified MTH8-1 in which the Km^r gene was replaced by the Tc^r gene, as verified by Southern blot analysis (Brandner and Kroos, 1998). MTH9-1, MTH9-2 and MTH9-4 are Km^r Tc^r strains resulting from P1 specialized transduction of pTH9-1, pTH9-2 and pTH9-4 from *E. coli* JM83 into *M. xanthus* MTH8-1, respectively.

MGV01 and MTH11-1 were constructed by electroporating pBR322 derivatives pBRE47Sp4514 and pTH11-1 into DK1622 and MTH10-3, respectively. Three transformants containing a single copy of either plasmid integrated by homologous recombination at *orf1* or *orf2* were identified by Southern blot analysis and PCR (data not shown). Likewise, MGV03 was constructed by electroporating p1175SmSl4514 into DK1622.

Polymerase chain reaction. Diagnostic PCR was used to identify *M. xanthus* integrants as follows. Cells were grown in CTT medium with appropriate antibiotics, collected by centrifugation, and resuspended in 500 μl of TE with 100 μg/ml RNAse A. The cell suspension was incubated first at room temperature for 15 min and then at 85°C for 15 min, followed by extraction with one volume of phenol, then two extractions with one volume of chloroform. The aqueous supernatant after microcentrifugation was used for the PCR reaction.

RNA analysis. RNA was prepared as described previously (Fisseha et al., 1996) from *M. xanthus* DK1622. An S1 nuclease protection assay was preformed with a probe from pTH7-2 digested with *Nru*I. The 2.6-kb fragment was gel-purified, phosphatase-treated, and 5' end-labeled with [γ-^{32}P] ATP and T4 polynucleotide kinase. RNA (100 μg) was precipitated with the labeled probe. The pellet was resuspended in 30 μl of hybridization buffer (80% formamide, 20 μM pipes [pH 6.4], 0.4 μM NaCl), and incubated for 10 min at 85°C followed by 3 hr at 52°C. S1 nuclease buffer (0.03 M NaOAc [pH4.6], 0.05 M NaCl, 1 mM $ZnSO_4$, 5% glycerol) and 500 u of S1 nuclease (Boeringer Mannheim) were then added, giving a final volume of 300 μl. After 30 min at 37°C, the reactions were extracted with 300 μl phenol-chloroform, precipitated with ethanol, and resuspended in formamide loading buffer (80% formamide, 10 mM EDTA, 0.01% xylene cyanol, 0.01% bromophenol blue). The protected products were resolved on a 5% polyacrylamide-8M urea gel and visualized by autoradiography.

Primer extension analysis was performed as described previously (Sambrook et al., 1989), using the oligonucleotide 5'-GATCTCCTGCATCGACGTGCCCTC-3', which corresponds to a sequence located about 140 bp downstream of the mRNA 5′ end mapped by S1 nuclease protection. The primer was end-labeled with T4 polynucleotide kinase and [γ-^{32}P] ATP and purified with a QIAquick Nucleotide Removal Kit (Qiagen). The reaction products were analyzed on a 5% polyacrylamide –8 M urea sequencing gel next to dideoxy sequencing reactions that utilized the same primer.

***In vitro* transcription.** Reactions were performed with partially purified *M. xanthus* σ^A RNAP or reconstituted σ^A RNAP holoenzyme as described previously (Biran and Kroos, 1997).

Western blot analysis. *E. coli* BL21 (λDE3) containing pTH192 was grown in LB medium containing ampicillin and harvested by centrifugation 3 h after 0.4 mM IPTG induction. The cell pellet was resuspended in 1x sample buffer (0.125 M Tris-HCl pH 6.8, 5% β-mercaptoethanol, 2% SDS, 10% glycerol, 0.1% bromophenol blue) and boiled for 5 min. DK1622 and *orf1* mutant (MGV01) cells were collected at the indicated times, resuspended in sample buffer, and boiled for 5 min. Equal amounts of *M. xanthus* protein, as determined by measuring total protein in sonic lysates of the same samples by the Bradford method (Bradford, 1976), were subjected to Western blot analysis. Proteins were separated by 10% or 12% SDS-PAGE and electrotransferred to a nitrocellulose or poly(vinylidene difluoride) membrane. The membrane was incubated in TBST (20 mM Tris-HCl pH 7.5, 0.5 M NaCl, 0.1% Tween 20) containing 2% nonfat dry milk for 4 h at room temperature with shaking in order to block nonspecific interaction between the primary antibodies and the membrane. The membrane was then probed for 4 h with shaking at room temperature with either a polyclonal antiserum to *B. subtilis* σ^{43} (a gift from B. –Y. Chang and R. Dori) or affinity-purified anti-ORF1-6xHis antibody diluted 1:500 in TBST/ 2% nonfat dry milk. Immunodetection using goat anti-rabbit-IgG horseradish peroxidase (Bio-Rad) and chemiluminescence (ECL; Amersham Pharmacia Biotech) was performed according to the manufacturer's instructions.

Production of ORF1-6xHis. pTH191 was transformed into *E. coli* strain BL21(λDE3) (Novagen), which contains the gene for T7 RNA polymerase under the control of an IPTG-inducible *lacUV5* promoter. Cells containing pTH191 were inoculated into LB medium containing ampicillin and grown at 37°C until the OD_{600} reached about 0.6, then the culture was induced with 0.4 mM IPTG. Cells were collected

3 hours after the induction by centrifugation (5,000xg, 5 min) and the cell pellet was stored at -70°C. Some experiments suggested that the ORF1-6xHis might be located in cytoplasmic inclusion bodies (data not shown). To minimize the proportion of ORF1-6xHis incorporated into inclusion bodies, cells were grown at 30°C and induced with IPTG as described above.

A whole-cell extract of the IPTG-induced cells was prepared as follows. The cell pellet was resuspended in 10 ml of 5 mM imidazole, 0.5 M NaCl, 20 mM Tris-Cl pH 7.9, 1 mM PMSF. Cells were lysed by sonication (Sonicator W-225; microtip maximum power setting at 6; Heat System-Ultrasonics, Inc.) while the mixture was kept cold on ice. The lysate was centrifuged at 39,000xg for 20 min at 4°C.

The ORF1-6xHis protein in the supernatant was isolated under nondenaturing conditions using a Ni-column as follows. The well-packed Ni-column was washed with 5 volumes of 1x column binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-Cl pH 7.9) and allowed to drain until the buffer reached the top of the column bed. After the whole-cell extract was loaded onto the column, it was washed with 10 volumes of 1x column binding buffer, followed by 6 volumes of 1x wash buffer (60 mM imidazole, 0.5 M NaCl, 20 mM Tris-Cl pH 7.9). Proteins were then eluted with 6 volumes of 1 M imidazole, 0.5 M NaCl, 20 mM Tris-Cl pH 7.9. The eluate was dialyzed against 2 L of gel-shift binding buffer (12% glycerol, 20 mM HEPES [pH 7.9], 50 mM KCl, 1 mM EDTA, 1 mM DTT, 5 mM $MgCl_2$) for 16 hr at 4°C. The protein solution (around 30 ml) was then concentrated 20-fold by centrifugation in a Millipore 15 filter device for 3 h at 4°C using the manufacturer's specifications and stored at 4°C.

Mobility shift experiments. The *Sma*I-*Eco*47III fragment containing DNA from –55 bp to +65 bp relative to the transcriptional start site was used as probe in the mobility shift experiments. This fragment from pTH4-4 digested with *Sma*I and *Eco*47III was gel-purified, phosphatase-treated, and 5' end-labeled with [γ-^{32}P] ATP and T4 polynucleotide kinase. The labeled DNA was purified with a QIAquick Nucleotide Removal Kit (Qiagen). Various amounts of affinity-purified ORF1-6xHis protein or crude extracts made from cells overexpressing ORF1-6xHis, ORF1 or 6xHis-ORF1 were incubated with 4.5ng (57 fmol) probe and 1 μg poly(dI·dC) at 30°C for 15 min to allow DNA binding. The 20 ul binding reactions contained 12% glycerol, 20 mM HEPES (pH 7.9), 50 mM KCl, 1 mM EDTA, 1mM DTT, 5 mM $MgCl_2$. Samples were then subjected to electrophoresis on a 5% or 8% polyacrylamide gel in low-ionic strength buffer at 4°C (Chodosh, 1988). After electrophoresis, the gels were dried and exposed to film.

Preparation and purification of antibodies. Purified ORF1-6xHis protein (500 μg) was mixed with Freund's complete adjuvant (BRL) and injected subcutaneously into a rabbit. Four weeks later, a booster injection (300 μg of ORF1-6xHis mixed with Freund's incomplete adjuvant) was performed. The rabbit was bled 1 week after the boost and the serum was prepared as described previously (Harlow and Lane, 1988).

Affinity purification of the antibodies was performed as follows. Purified ORF1-6xHis protein (100 μg) was electrophoresed on a 12% polyacrylamide-SDS gel and the protein was electrotransferred to a poly (vinylidene difluoride) (PVDF) membrane (Millipore). The region of the membrane with bound ORF1-6xHis was excised and incubated with TBST (20 mM Tris-HCl pH 7.5, 0.5 M NaCl, 0.05% Tween 20) containing 5% nonfat dry milk for 2 h to block non-specific binding of the antibodies.

Anti-ORF1-6xHis antiserum (2 ml) was incubated with the membrane overnight at 4°C with agitation. The membrane was then washed twice (5 min each) with TBST and twice with TBS (20 mM Tris-HCl pH 7.5, 0.5 mM NaCl). To elute antibodies bound to the membrane, 100 mM glycine pH 2.5 (1 ml) was added and incubated for 10 second with shaking. The solution was collected and immediately neutralized to pH 7.0 by adding 1 M Tris-Cl pH 8.0 (approximately 100 µl). The elution steps were repeated twice and the neutralized solutions were combined and stored at 4°C.

Sporulation efficiency. Cells were plated for development on TPM agar (1.5%) and harvested after 3 days at 32°C, then the samples were subjected to heat and sonication treatment to kill rod-shaped cells, and dilutions were plated to quantitate the number of spores able to germinate and form colonies, as described previously (Kroos and Kaiser, 1987).

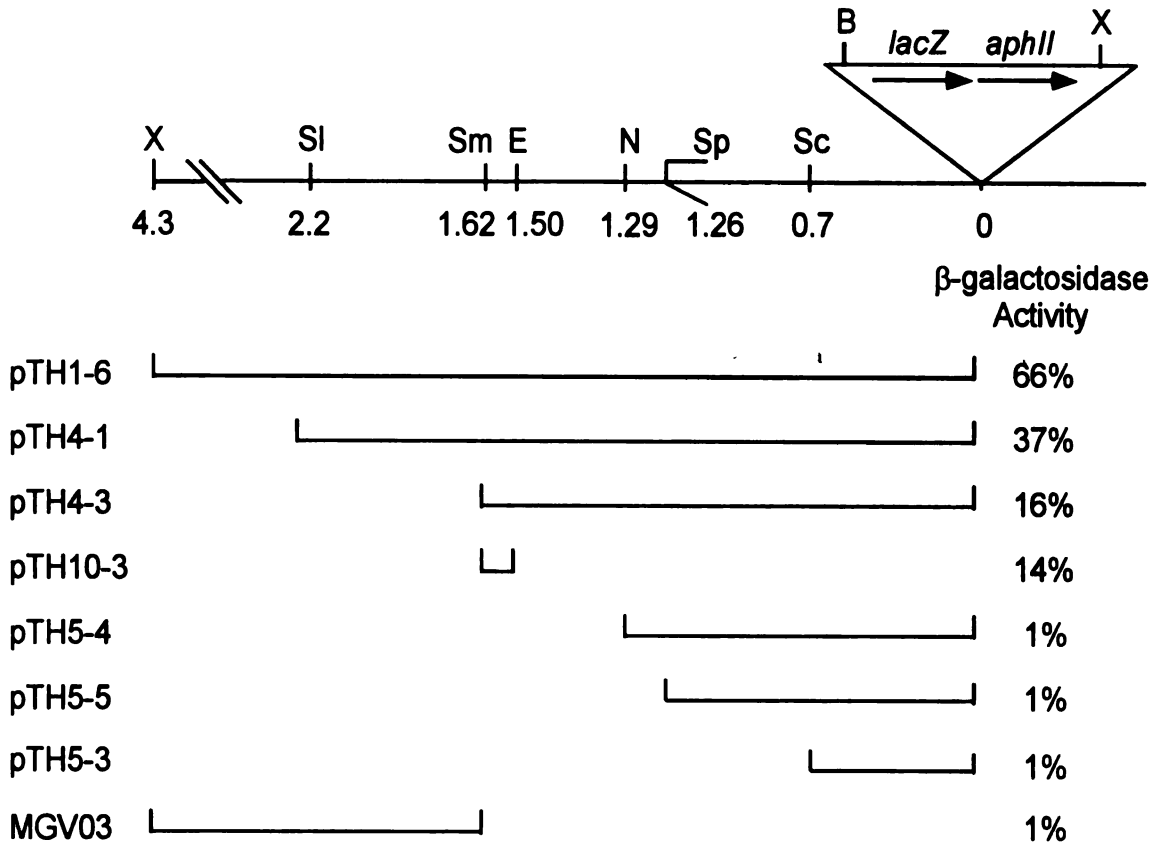
Results

Localization of the developmental promoter controlling Ω4514 expression.

To clone the putative promoter located upstream of the developmentally regulated Tn*5* *lac* insertion Ω4514, I took advantage of a *Xho*I restriction site approximately 4.3 kb upstream of the Ω4514 insertion in *M. xanthus* DK4514 (Kroos et al., 1986) and a *Xho*I site in Tn*5* *lac* approximately 9 kb from the left end (Figure 2.1). Chromosomal DNA from DK4514 was digested with *Xho*I, cloned into pGEM-7Zf, and transformed into *E. coli* cells. Since the 13.3 kb *Xho*I fragment described above includes the *aphII* gene of Tn*5* *lac*, which encodes aminoglycoside phosphotransferase and confers Km^r, *E. coli* cells that contain plasmids with the desired *Xho*I fragment of *M. xanthus* chromosomal DNA will survive under Km selection. Several restriction sites upstream of the Ω4514 insertion in DK4514 have been mapped by Kroos et al. (Kroos et al., 1986). Restriction mapping of the resulting plasmid, pTH1-3, showed the patterns expected on the basis of restriction sites in DNA upstream of Ω4514 and on the basis of known restriction maps of pGEM-7Zf and Tn*5* *lac*. Figure 2.1 shows a restriction map of DNA upstream of Ω4514 generated on the basis of these results.

To test Ω4514 upstream DNA for promoter activity, the *Xho*I-*Bam*HI restriction fragment from pTH1-3, which contains 4.3 kb of *M. xanthus* DNA and approximately 60 bp of the left end of Tn*5* *lac* (Figure 2.1), was subcloned into *Xho*I-*Bam*HI-digested pREG1727 to construct pTH1-6. Since the *Bam*HI site of pREG1727 is located immediately upstream of the same *lacZ*-containing fragment found in Tn*5* *lac* (Fisseha et al., 1996), pTH1-6 contains Ω4514 upstream DNA fused to a promoterless *lacZ* gene in

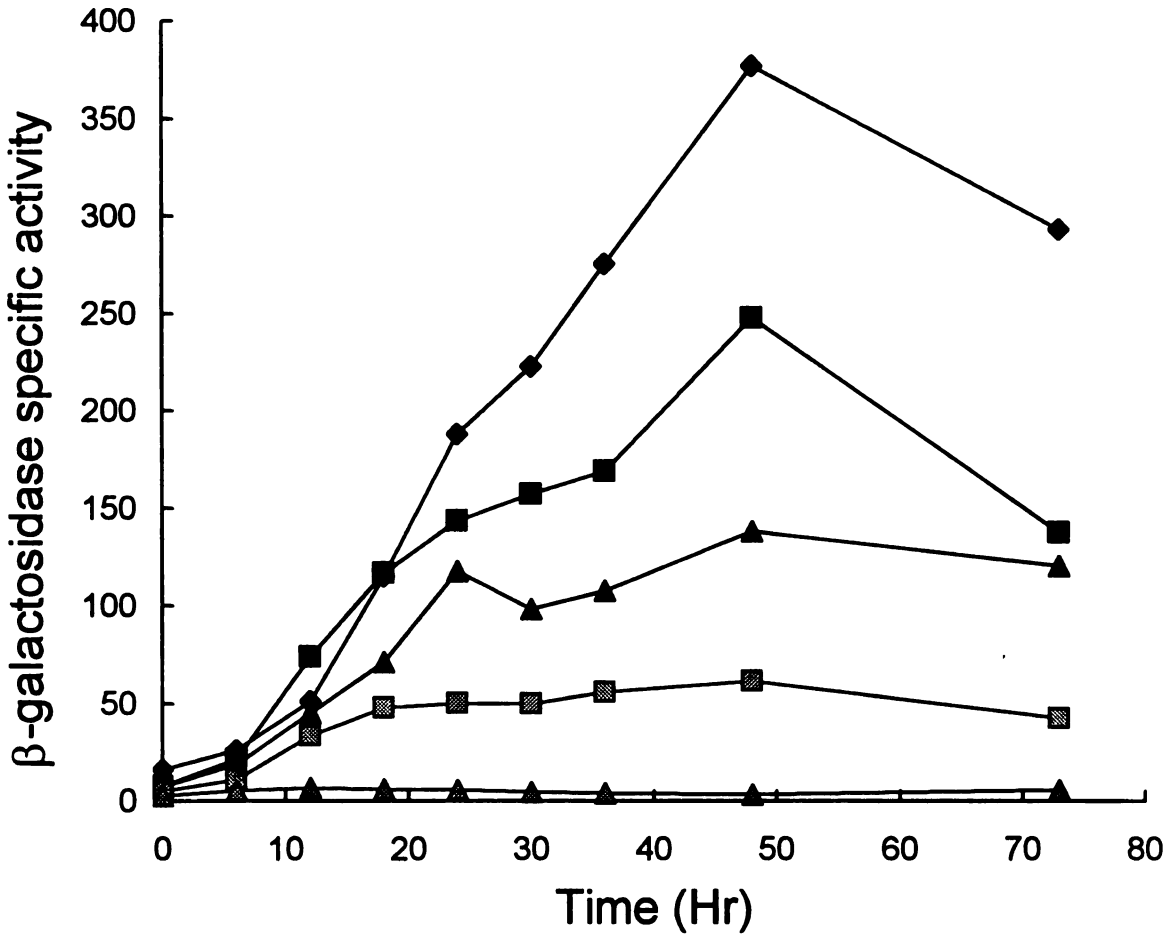
Figure 2.1. Physical map of the Ω4514 insertion region and summary of deletions tested for promoter activity. The upper schematic depicts the restriction sites in Tn*5 lac* and the upstream *M. xanthus* chromosome that were used in this study. Distances of restriction sites from the Tn*5 lac* Ω4514 insertion are given in kilobases. B, *Bam*HI; E, *Eco47*III; N, *Nru*I; Sc, *Sac*I; Sl, *Sal*I; Sm, *Sma*I; Sp, *Sph*I; X, *Xho*I. The lower diagram depicts the segments of Ω4514 upstream DNA fused to the promoterless *lacZ* gene in pREG1727 or in pREG1175 in the case of strain MGV03, to test for promoter activity. The plasmid or strain designation is indicated on the left (Table 1). Derivatives of wild-type *M. xanthus* DK1622 containing a single copy of each pREG1727 derivative integrated at Mx8 *attB* or a single copy of the pREG1175 derivative integrated by homologous recombination were measured for ß-galactosidase. The maximal ß-galactosidase specific activities during a 72-hr developmental time course are given as percentages of the maximum activity observed for Tn*5 lac* Ω4514-containing strain DK4514. In each case, the activity of at least three independent transductants was measured at least once as described previously (Kroos et al., 1986) and the average is given.


B
lacZ
aphII
X
X
Sl
Sm
E
N
Sp
Sc
4.3
2.2
1.62
1.50
1.29
1.26
0.7
0
β-galactosidase Activity
pTH1-6
66%
pTH4-1
37%
pTH4-3
16%
pTH10-3
14%
pTH5-4
1%
pTH5-5
1%
pTH5-3
1%
MGV03
1%

the same manner as Ω4514-containing *M. xanthus* DK4514. pTH1-6 was transduced from *E. coli* JM83 into the wild-type *M. xanthus* strain DK1622 using bacteriophage P1 specialized transduction (Fisseha et al., 1996). Due to the presence of the *attP* segment from myxophage Mx8, pTH1-6 integrated efficiently into the *M. xanthus* chromosome at *attB* (Stellwag et al., 1985; Stephens and Kaiser, 1987). Transductants containing a single copy of pTH1-6 integrated at Mx8 *attB* were identified by Southern blot hybridization (data not shown). Several of these transductants were assayed for β-galactosidase activity during development and showed, on average, 66% of the maximal level observed in DK4514 (Figures 2.1 and 2.2). The results indicate that the 4.3 kb Ω4514 upstream DNA segment contains a promoter(s) that is able to direct development-specific expression.

To further localize the developmental promoter(s) within this region, additional deletions of the Ω4514 upstream DNA were generated and tested for promoter activity as described above. The results showed that the 2.2-kb and 1.62-kb Ω4514 upstream DNA segments directed *lacZ* expression with a similar timing as the Ω4514-containing strain, but reached about 37% and 16% of the maximal level, respectively (Figures 2.1 and 2.2), however, transductants containing the 1.29-kb, 1.26-kb, or 0.7-kb Ω4514 upstream DNA segments produced no developmental β-galactosidase above the background (Figure 2.1). These results indicate that a development-specific promoter driving the expression of Tn*5* *lac* Ω4514 lies between the *Sma*I and *Nru*I sites, and that DNA farther upstream also contributes significantly to promoter activity. To test for an additional promoter upstream of the *Sma*I site, the 2.7-kb *Xho*I-*Sma*I fragment was inserted into pREG1175 (Gill and Bornemann, 1988), resulting in p1175SmSl4514, in which the *Xho*I-*Sma*I fragment is fused to the same promoterless *lacZ*-containing fragment found in Tn*5* *lac*. Since

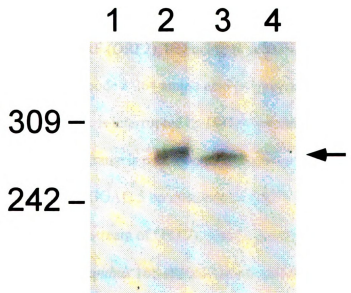
Figure 2.2. Developmental expression of *lacZ* under the control of the Ω4514 promoter. Developmental ß-galactosidase specific activity was measured as described previously (Kroos et al., 1986) for Tn*5* *lac*-containing strain DK4514 (♦) and for at least three independent isolated transductants of DK1622 containing a single copy of the 4.3-kb (■), 2.2-kb (▲), or 1.62-kb (□) Ω4514 upstream DNA fused to promoterless *lacZ* within pREG1727 and integrated at Mx8 *attB*. The ß-galactosidase specific activity of DK1622 containing the pREG1727 vector alone with no insert integrated at Mx8 *attB* (Δ) is also shown. The average ß-galactosidase specific activity from three determinations for DK4514 and from at least one determination for each of three independent transductants is plotted. ß-galactosidase specific activity is expressed in nanomoles of *o*-nitrophenyl phosphate per minute per milligram of protein.


400
350
300
250
200
150
100
50
0
β-galactosidase specific activity
0
10
20
30
40
50
60
70
80
Time (Hr)

pREG1175 does not contain the *attP* segment of Mx8, when p1175SmSl4514 was transduced into wild-type DK1622, homologous recombination between the plasmid *M. xanthus* DNA segment and the *M. xanthus* chromosome resulted in MGV03, in which the *lacZ* reporter is positioned immediately downstream of the *Sma*I site in the chromosome. No developmental promoter activity was observed in three such independent transductants (Figure 2.1). We conclude that there is no additional promoter upstream of the *Sma*I site that can function in the absence of downstream DNA to drive Tn*5 lac* Ω4514 expression.

Localization of an mRNA 5′ end upstream of Ω4514. To test whether an mRNA 5′ end maps upstream of the Ω4514 insertion, an S1 nuclease protection assay was performed with a probe labeled at the *Nru*I site located 1.29-kb upstream of Ω4514 (Figure 2.1) and including about 2.6-kb of *M. xanthus* DNA farther upstream. The probe was hybridized to RNA from *M. xanthus* DK1622 and subjected to S1 nuclease digestion. The probe protected a development-specific RNA species as shown in Figure 2.3. RNA from developing cells harvested at 12 or 18 h after starvation protected a fragment of about 270 bases in length. No protection was observed with RNA from growing cells and very weak protection was observed with RNA from 24-h developing cells. These results indicate that a development-specific mRNA is transcribed from the region upstream of the Ω4514 Tn*5 lac* insertion and the 5′ end of this mRNA species is located approximately 1.56 kb upstream of the insertion site. Together with the deletion analysis (Figures 2.1 and 2.2), the results suggest that a promoter lies 1.56 kb upstream of Ω4514 and the mRNA 5′ end reflects the transcriptional start site.

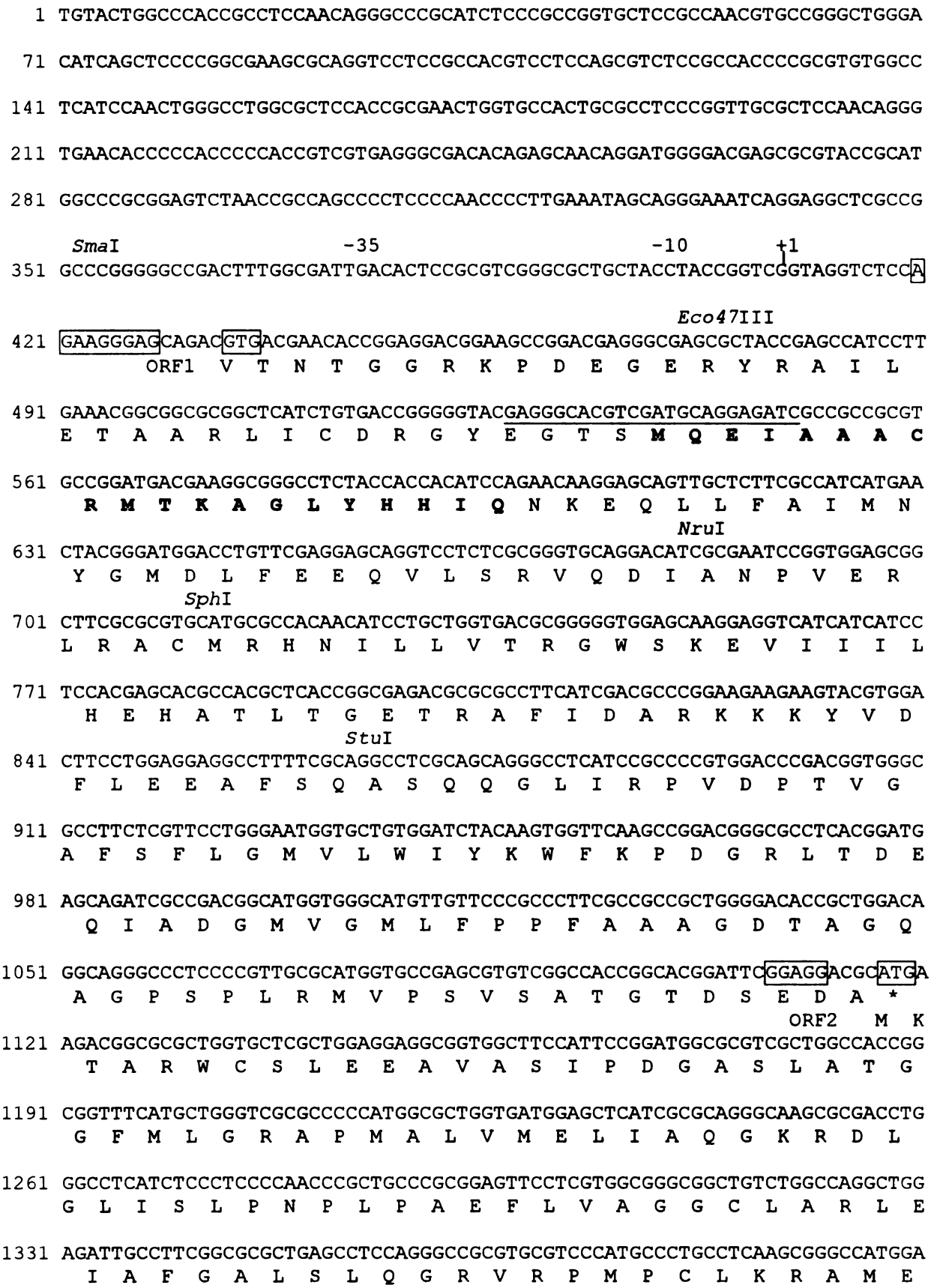
Figure 2.3. Localization of an mRNA 5′ end within the Ω4514 upstream region. Low-resolution S1 nuclease mapping of the Ω4514-associated transcript was performed by using RNA prepared from DK1622 cells growing vegetatively (lane 1) or cells that had undergone 12, 18, and 24 hr of development in lanes 2 to 4, respectively. The numbers on the left indicate the length (in bases) of end-labeled *Msp*I-digested pBR322 marker fragments. The arrowhead denotes the protected fragment.


1
2
3
4
309 –
242 –

DNA sequence of the Ω4514 upstream region. The nucleotide sequence of both strands of the 1.9-kb DNA fragment immediately upstream of the Ω4514 insertion site was determined (Figure 2.4). Two complete open reading frames (ORFs) and one partial ORF interrupted by Ω4514 were inferred from the sequence downstream of the Ω4514 transcriptional start site. The two ORFs exhibit codon preference typical of *M. xanthus* genes, including a strong bias towards usage of guanine or cytosine at the third codon position (Shimkets, 1993). The first ORF (ORF1), beginning with GTG at position 434 and ending with a TGA stop codon at position 1118, is predicted to encode a 228 amino acid polypeptide. The second ORF (ORF2), beginning at position 1117 and ending at position 1909, is predicted to encode a 264 amino acid polypeptide. A putative third ORF, beginning at position 1908, is interrupted by Ω4514 insertion after the first two amino acids. Each open reading frame is preceded by a sequence that might serve as a ribosomal binding site (AGAAGGGAG 5 bp upstream of ORF1, GGAGG 4 bp upstream of ORF2 and GGAGGG 4 bp upstream of ORF3), since it is complementary to the sequence near the 3′ end of *M. xanthus* 16S rRNA (Oyaizu and Woese, 1985). The positions of the start points of ORF2 and ORF3 suggest that these three open reading frames might be translationally coupled.

The deduced amino acid sequence of ORF1 was analyzed by the Motif program (Wisconsin Genetics Computer Group sequence analysis software) and shown to contain a helix-turn-helix motif near the N-terminus (Figure 2.4), suggesting that the product of ORF1 is a DNA-binding protein (Brennan and Matthews, 1989; Harrison and Aggarwal, 1990). Analysis of the deduced amino acid sequence by the BLAST program supported this observation by revealing that the ORF1 product shared significant similarity to

Figure 2.4. Nucleotide sequence of 1.9-kb of DNA upstream of Tn*5* *lac* Ω4514. The transcriptional start site is indicated by +1, and the -35 and -10 promoter regions are marked. The complementary sequence of the primer used for the primer extension analysis is underlined. The putative translational start codons and potential ribosome binding sites are boxed, and the deduced amino acid sequences of the Ω4514 ORFs are shown below the nucleotide sequence. The putative helix-turn-helix motif is shown in bold letters. Restriction sites used to generate some deletions in the Ω4514 upstream region are also indicated.


1 TGTACTGGCCCACCGCCTCCAACAGGGCCCGCATCTCCCGCCGGTGCTCCGCCAACGTGCCGGGCTGGGA
71 CATCAGCTCCCCGGCGAAGCGCAGGTCCTCCGCCACGTCCTCCAGCGTCTCCGCCACCCCGCGTGTGGCC
141 TCATCCAACTGGGCCTGGCGCTCCACCGCGAACTGGTGCCACTGCGCCTCCCGGTTGCGCTCCAACAGGG
211 TGAACACCCCCACCCCCACCGTCGTGAGGGCGACACAGAGCAACAGGATGGGGACGAGCGCGTACCGCAT
281 GGCCCGCGGAGTCTAACCGCCAGCCCCTCCCCAACCCCTTGAAATAGCAGGGAAATCAGGAGGCTCGCCG
SmaI
-35
-10
+1
351 GCCCGGGGGCCGACTTTGGCGATTGACACTCCGCGTCGGGCGCTGCTACCTACCGGTCGGTAGGTCTCCA
Eco47III
421 GAAGGGAGCAGACGTGACGAACACCGGAGGACGGAAGCCGGACGAGGGCGAGCGCTACCGAGCCATCCTT
ORF1 V T N T G G R K P D E G E R Y R A I L
491 GAAACGGCGGCGCGGCTCATCTGTGACCGGGGGTACGAGGGCACGTCGATGCAGGAGATCGCCGCCGCGT
E T A A R L I C D R G Y E G T S M Q E I A A A C
561 GCCGGATGACGAAGGCGGGCCTCTACCACCACATCCAGAACAAGGAGCAGTTGCTCTTCGCCATCATGAA
R M T K A G L Y H H I Q N K E Q L L F A I M N
NruI
631 CTACGGGATGGACCTGTTCGAGGAGCAGGTCCTCTCGCGGGTGCAGGACATCGCGAATCCGGTGGAGCGG
Y G M D L F E E Q V L S R V Q D I A N P V E R
SphI
701 CTTCGCGCGTGCATGCGCCACAACATCCTGCTGGTGACGCGGGGGTGGAGCAAGGAGGTCATCATCATCC
L R A C M R H N I L L V T R G W S K E V I I I L
771 TCCACGAGCACGCCACGCTCACCGGCGAGACGCGCGCCTTCATCGACGCCCGGAAGAAGAAGTACGTGGA
H E H A T L T G E T R A F I D A R K K K Y V D
StuI
841 CTTCCTGGAGGAGGCCTTTTCGCAGGCCTCGCAGCAGGGCCTCATCCGCCCCGTGGACCCGACGGTGGGC
F L E E A F S Q A S Q Q G L I R P V D P T V G
911 GCCTTCTCGTTCCTGGGAATGGTGCTGTGGATCTACAAGTGGTTCAAGCCGGACGGGCGCCTCACGGATG
A F S F L G M V L W I Y K W F K P D G R L T D E
981 AGCAGATCGCCGACGGCATGGTGGGCATGTTGTTCCCGCCCTTCGCCGCCGCTGGGGACACCGCTGGACA
Q I A D G M V G M L F P P F A A A G D T A G Q
1051 GGCAGGGCCCTCCCCGTTGCGCATGGTGCCGAGCGTGTCGGCCACCGGCACGGATTCGGAGGACGCATGA
A G P S P L R M V P S V S A T G T D S E D A *
ORF2 M K
1121 AGACGGCGCGCTGGTGCTCGCTGGAGGAGGCGGTGGCTTCCATTCCGGATGGCGCGTCGCTGGCCACCGG
T A R W C S L E E A V A S I P D G A S L A T G
1191 CGGTTTCATGCTGGGTCGCGCCCCCATGGCGCTGGTGATGGAGCTCATCGCGCAGGGCAAGCGCGACCTG
G F M L G R A P M A L V M E L I A Q G K R D L
1261 GGCCTCATCTCCCTCCCCAACCCGCTGCCCGCGGAGTTCCTCGTGGCGGGCGGCTGTCTGGCCAGGCTGG
G L I S L P N P L P A E F L V A G G C L A R L E
1331 AGATTGCCTTCGGCGCGCTGAGCCTCCAGGGCCGCGTGCGTCCCATGCCCTGCCTCAAGCGGGCCATGGA
I A F G A L S L Q G R V R P M P C L K R A M E

```
1401 GCAAGGCACCCTCGCCTGGCGCGAACATGATGGCTACCGCGTCGTCCAGCGGCTGCGCGCCGCGTCCATG
      Q  G  T  L  A  W  R  E  H  D  G  Y  R  V  V  Q  R  L  R  A  A  S  M

1471 GGGCTGCCCTTCATCCCCGCGCCGGACGCGGACGTGTCCGGGCTGGCACGGACGGAGCCGCCTCCCACGG
      G  L  P  F  I  P  A  P  D  A  D  V  S  G  L  A  R  T  E  P  P  P  T  V

1541 TGGAGGACCCCTTCACCGGCCTGCGCGTGGCGGTGGAGCCTGCCTTCTATCCGGACGTGGCGTTGCTCCA
       E  D  P  F  T  G  L  R  V  A  V  E  P  A  F  Y  P  D  V  A  L  L  H

1611 CGCGCGCGCCGCGGACGAGCGCGGCAACCTCTACATGGAAGACCCGACCACGGACCTGCTGGTGGCGGGC
      A  R  A  A  D  E  R  G  N  L  Y  M  E  D  P  T  T  D  L  L  V  A  G

1681 GCGGCGAAGCGGGTGATTGCCACGGTGGAGGAGCGGGTGGCGAAGCTGCCTCGCGCCACCCTGCCCGGCT
     A  A  K  R  V  I  A  T  V  E  E  R  V  A  K  L  P  R  A  T  L  P  G  F

1751 TCCAGGTGGACCGCATCGTCCTGGCTCCCGGCGGCGCCCTGCCCAGCTGCGCCGGACTCTACCCGCACGA
       Q  V  D  R  I  V  L  A  P  G  G  A  L  P  S  C  A  G  L  Y  P  H  D

1821 CGACGAAATGCTGGCCCGCTACCTGTCGCTGGCGGAGACGGGCCGTGAAGCGGAGTTCCTGGAAACGTTG
      D  E  M  L  A  R  Y  L  S  L  A  E  T  G  R  E  A  E  F  L  E  T  L

1891 CTGACGCGGAGGGCGGCATGAGCG
     L  T  R  R  A  A  *
               ORF3  M  S
```

several transcriptional regulatory proteins. Among these is a putative transcription factor of the TetR family identified in the *Deinococcus radiodurans* genome sequencing project, to which ORF1 exhibits 31% identity and 46% similarity over a 182 amino acid stretch (White et al., 1999). ORF1 also shows significant similarity to other TetR family members. TetR is a repressor that regulates tetracycline resistance of many Gram-negative bacteria (Hillen and Berens, 1994). In the absence of tetracycline, TetR binds to operator sites, preventing transcription of *tetA*, the tetracycline resistance gene, and transcription of *tetR* itself. When tetracycline is present, TetR binds to tetracycline and releases the operators, so *tetA* and *tetR* are transcribed. These analyses suggest that the protein product of the first open reading frame in the developmentally regulated Ω4514 transcriptional unit might function as a transcriptional regulator.

A BLAST search with ORF2 revealed significant similarity to several glutaconate CoA transferases. The deduced amino acid sequence of ORF2 shows 29% identity and 44% similarity over its entire length to glutaconate CoA-transferase subunit A of *Acidaminococcus fermentans* (Mack etal, 1994; Jacob etal, 1997). Glutaconate CoA transferase is involved in glutamate fermentation. These results suggest that the Ω4514 operon might encode components of an alternative metabolic pathway induced during *M. xanthus* development.

Precise localization of the Ω4514 mRNA 5′ end. The DNA sequence of the Ω4514 upstream region and the S1 nuclease protection assay results facilitated the design of a primer for precise mapping of the location of the Ω4514 mRNA 5′ end by primer extension analysis. The position of the primer is shown in Figure 2.4; however, the actual primer contains a sequence complementary to that underlined. When primer extension

analysis was performed using RNA from DK1622 cells that had undergone 24 h of development, a single primer extension product was identified (Figure 2.5). This localizes the Ω4514 mRNA 5′ end to a guanine nucleotide at position 409 in the sequence (Figures 2.4 and 2.5). The mRNA 5′ end is 272 bp upstream of the *Nru*I site that was labeled in the S1 nuclease protection experiment (Figure 2.3), so the results of the two methods are in good agreement. No primer extension product was generated when mRNA prepared from growing *M. xanthus* cells was subjected to primer extension analysis (Figure 2.5).

Further deletion analysis of the Ω4514 upstream region. The 1.62-kb DNA segment upstream of Tn*5 lac* Ω4514 extends to a *Sma*I site located at 54 bp upstream of the putative transcriptional start site (Figures 2.1 and 2.4) and exhibits developmental promoter activity (Figures 2.1 and 2.2). A 3′ deletion was constructed to further define the sequence required for developmental promoter activity. An *Eco*47III site (Figures 2.1 and 2.4) allowed construction of a 3′ deletion to 65 bp downstream of the putative transcriptional start site. The *Sma*I-*Eco*47III fragment was inserted into pREG1727 and *M. xanthus* strains with a single copy of the plasmid integrated at the chromosomal *attB* site were tested for developmental promoter activity. Figure 2.6 shows that β-galactosidase activity reached a similar maximal level as for strains containing the 1.62-kb Ω4514 upstream segment (with its 5′ end also at -54 but its 3′ end downstream of *orf2*) fused to *lacZ*, although there was a reproducible difference in *lacZ* expression at 6 h of development (see discussion). These results demonstrate that a developmental promoter exists between –54 bp and +65 bp relative to the location in the DNA of the Ω4514 mRNA 5′ end.

Figure 2.5. Primer extension analysis of Ω4514 mRNA. RNA was isolated from wild-type cells grown vegetatively (lane V) or cells that had undergone 24 h of development (lane D). The same primer was used to sequence Ω4514 upstream DNA. A portion of the DNA sequence is indicated at the left. The arrow indicates the extension product that was observed with RNA isolated from 24 h developing cells but not with RNA from vegetatively growing cells. The asterisk marks the putative transcriptional start site as determined by the position of the primer extension product.

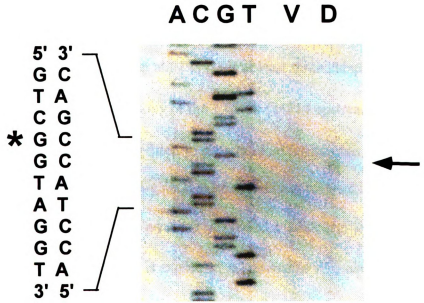
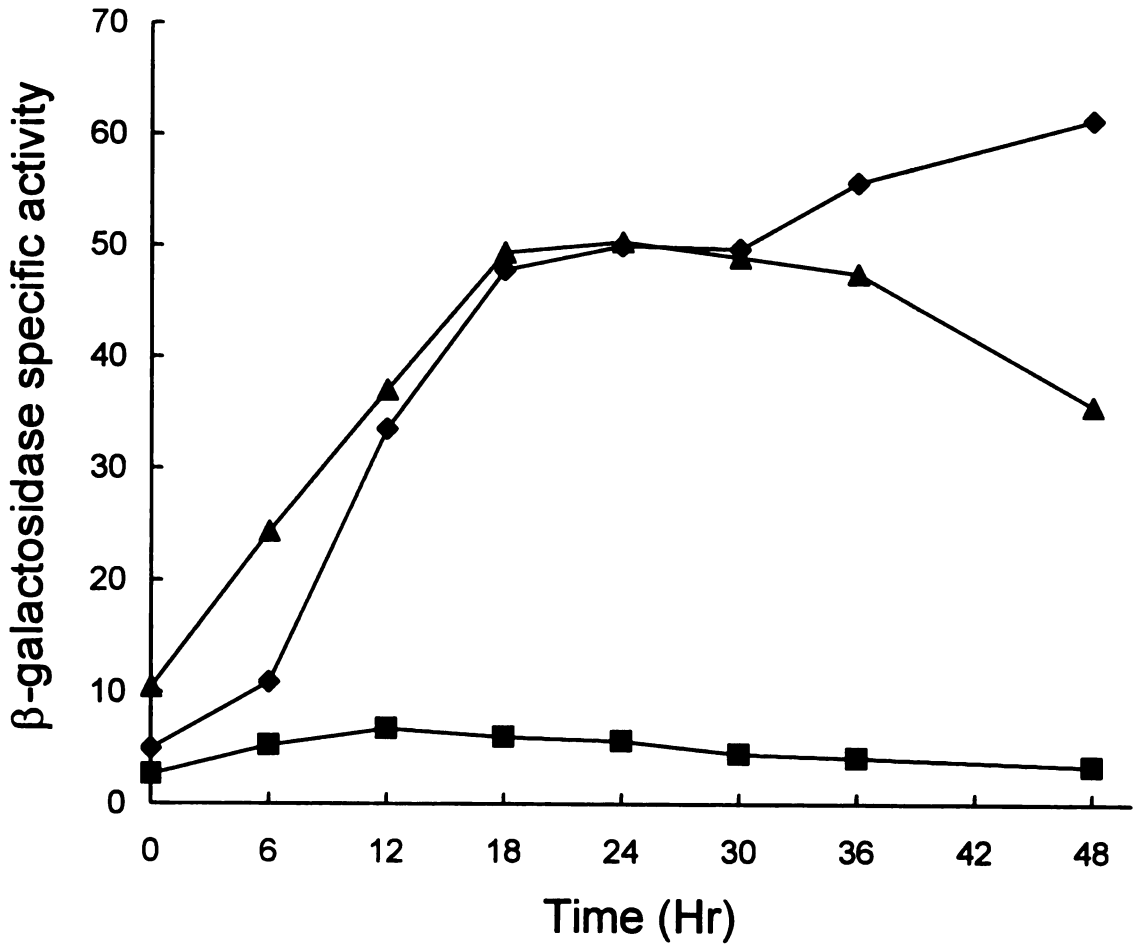

A C G T V D
5' 3'
G C
T A
C G
* G C
G C
T A
A T
G C
G C
T A
3' 5'

Figure 2.6. Developmental expression of *lacZ* from a small segment containing the Ω4514 promoter. Developmental ß-galactosidase specific activity was measured as described previously (Kroos et al., 1986) for at least three independent transductants of DK1622 containing a single copy of the 1.62-kb Ω4514 upstream segment (♦) or the *Sma*I-*Eco*47III fragment (▲) fused to promoterless *lacZ* within pREG1727 and integrated at Mx8 *attB*. The ß-galactosidase specific activity of DK1622 containing the pREG1727 vector alone with no insert integrated at Mx8 *attB* (■) is also shown. ß-galactosidase specific activity is expressed in nanomoles of *o*-nitrophenyl phosphate per minute per milligram of protein.


β-galactosidase specific activity
70
60
50
40
30
20
10
0
0
6
12
18
24
30
36
42
48
Time (Hr)

Ω4514 can be transcribed by RNA polymerase isolated from growing *M. xanthus* cells. Inspection of the DNA sequence upstream of the apparent Ω4514 transcriptional start site revealed a perfect match in the –35 region to the *E. coli* σ^{70} consensus (TTGACA), and a 2 out of 6 match to the –10 region consensus (TATAAT) separated by 18 base pairs (Figure 2.4). To test whether RNA polymerase isolated from growing *M. xanthus* cells can transcribe from the Ω4514 promoter, *in vitro* transcription experiments were performed. Two different DNA segments containing the Ω4514 promoter were used and run-off transcription products of the expected sizes were observed (Figure 2.7A). *M. xanthus* core RNA polymerase alone did not transcribe from the Ω4514 promoter (Figure 2.7B lane 1). Only when the core RNA polymerase was mixed with σ^A, the major vegetative σ factor of *M. xanthus*, was transcription observed (Figure 2.7B lane 2). Primer extension analysis of RNA produced *in vitro* using reconstituted σ^A RNA polymerase mapped the 5′ end to the same position (data not shown) as Ω4514 mRNA produced *in vivo* (Figure 2.5). Together, these results indicate that Ω4514 can be transcribed by the major vegetative RNA polymerase of *M. xanthus*, suggesting that σ^A RNA polymerase may be responsible for Ω4514 transcription *in vivo*.

Since Ω4514 is transcribed primarily, if not exclusively, during development (Figures 2.2, 2.3, 2.5, and 2.6), we next ask whether σ^A is present during development. Antiserum against *B. subtilis* σ^{43} was used to detect σ^A in growing and developing *M. xanthus* cells (Figure 2.8) using Western blot analysis as described previously (Biran and Kroos, 1997). The level of σ^A remained fairly constant until 12 h into development, then

Figure 2.7. *In vitro* transcription from the Ω4514 promoter. DNA fragments containing the Ω4514 promoter were transcribed *in vitro* with *M. xanthus* σ^A RNA polymerase and the reactions were subjected to 5% polyacrylamide-8 M urea gel electrophoresis. (A) Two different linear DNA templates containing the Ω4514 promoter region (*Sma*I-*Stu*I and *Sma*I-*Sph*I fragments in lanes 1 and 2, respectively) were transcribed with partially purified σ^A RNAP (Biran and Kroos, 1997). Run-off transcription products of 458 (lane 1) and 303 bases (lane 2) are expected based on the Ω4514 mRNA 5′ end mapped in Figure 5. Arrows mark the expected run-off transcription products. The upper band in each lane is the end-to-end transcript of the template. The numbers on the left indicate lengths (in bases) of end-labeled *Msp*I-digested pBR322 marker fragments. (B) Transcription from the *Sma*I-*Sph*I Ω4514 upstream fragment by *M. xanthus* core RNA polymerase alone (lane 1) or core RNA polymerase plus σ^A (lane 2). The arrow marks the expected 303 base run-off transcription product.

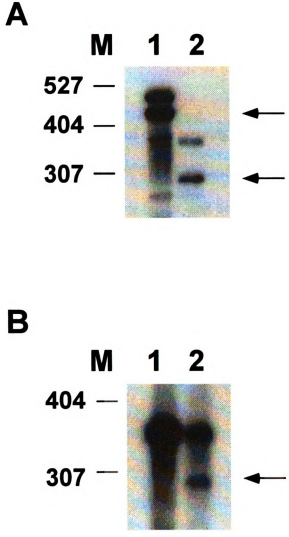
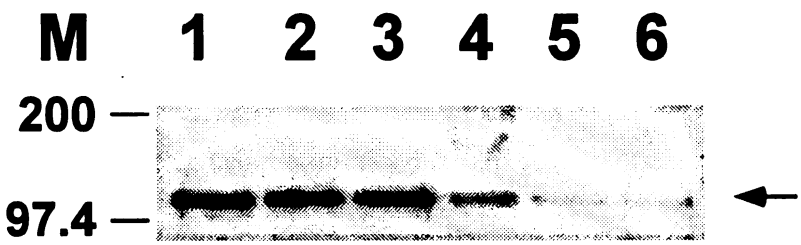

A
M
1
2
527
404
307
B
M
1
2
404
307

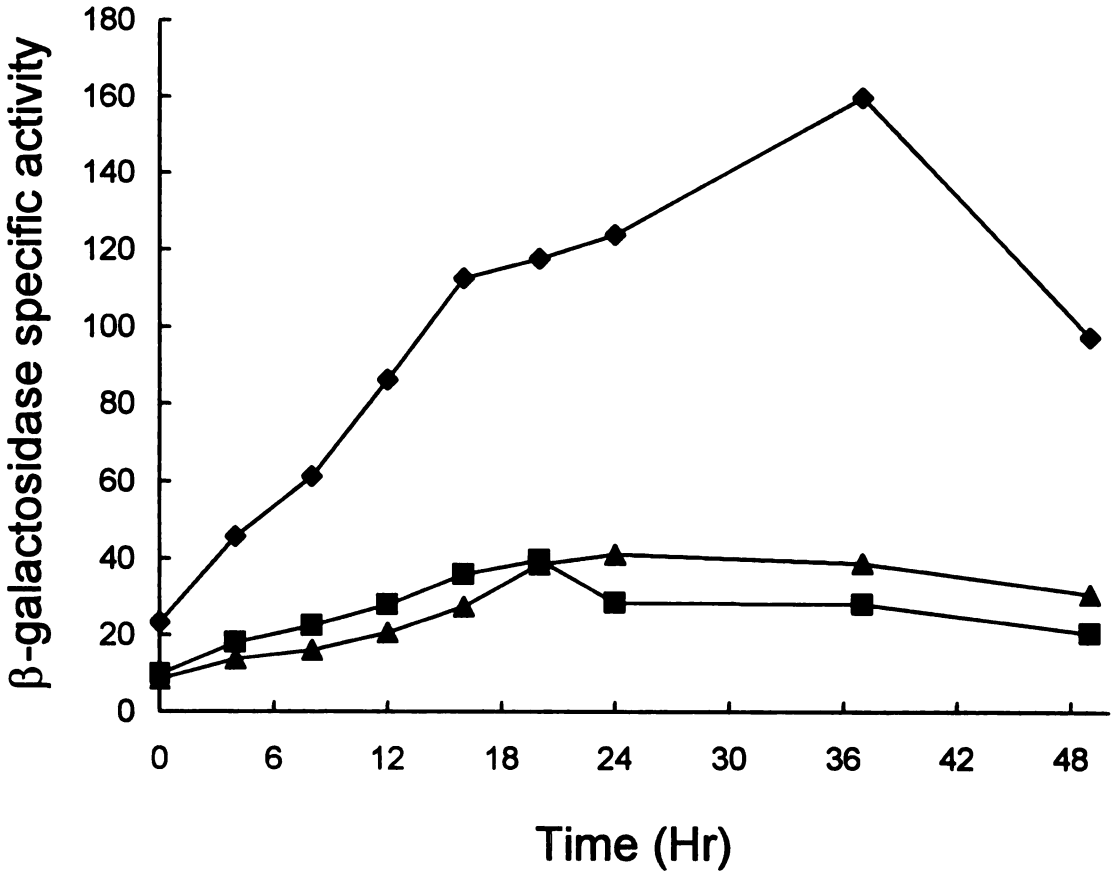
Figure 2.8. Western blot analysis of σ^A in growing and developing *M. xanthus* cells. DK1622 cells were collected at 0, 9, 12, 15, 18 and 24 h after the onset of development (lanes 1 to 6), resuspended in sample buffer, and boiled for 5 min. Equal amounts of *M. xanthus* protein, as judged by measuring total protein in sonic lysates of the same samples, were separated on 10% SDS-PAGE and electrotransferred to a nitrocellulose filter, which was incubated with a polyclonal antiserum to *B. subtilis* σ^{43} and visualized with goat anti-rabbit IgG-horseradish peroxidase. The σ^A is indicated by an arrow and protein standards are marked in kDa.


M
1
2
3
4
5
6
200
97.4

decreased markedly by 15 h. Thus, σ^A is present early in development when the level of Ω4514 mRNA rises (Figure 2.3).

Effect of an insertional mutation in ORF1 on Ω4514 expression. Since the Ω4514 promoter can be transcribed by σ^A RNA polymerase (Figure 2.7) and since σ^A is present in growing cells (Figure 2.8), we reasoned that Ω4514 transcription must be negatively regulated during growth, because Ω4514 mRNA was not detected in growing cells (Figures 2.3 and 2.5) and *lacZ* expression from Tn*5 lac* Ω4514 was at a low background level (Figures 2.2 and 2.6). The first ORF of the Ω4514 operon is predicted to encode a protein with a DNA-binding motif and sequence similarity to an auto-repressor. Therefore, we hypothesized that the ORF1 product functions as a repressor which prevents Ω4514 from being transcribed during growth. To test this hypothesis, an insertional disruption mutation of *orf1* was constructed. An *Eco47*III-*Sph*I restriction fragment (Figure 2.1) from position 473 to 713 (Figure 2.4), which contains the putative helix-turn-helix motif, was subcloned into an integrational vector and transformed into wild-type DK1622. Transformants containing the plasmid integrated by homologous recombination were identified by Southern blot hybridization (data not shown). The effect of this *orf1* disruption mutation on Ω4514 expression was tested by transforming the mutant (MGV01) with the plasmid (pTH10-3) containing the Ω4514 promoter region from –54 bp to +65 bp fused to *lacZ*. Transformants containing a single copy of pTH10-3 integrated at Mx8 *attB* were identified by Southern blot hybridization (data not shown). At least three of these transformants (MGV02) were assayed for β-galactosidase activity during growth and development along with MTH10-3, the strain with a wild-type *orf1* gene and a single copy of pTH10-3 at *attB*. Figure 2.9 shows that significantly higher

Figure 2.9. Effect of *orf1* and *orf2* mutations on developmental *lacZ* expression under the control of the Ω4514 promoter. Developmental ß-galactosidase specific activity from pTH10-3 integrated at *attB* was measured as described previously (Kroos et al., 1986) for at least three independent isolates of *M. xanthus* containing wild-type *orf1* and *orf2* (▲), the *orf1* mutation (♦), or the *orf2* mutation (■). ß-galactosidase specific activity is expressed in nanomoles of *o*-nitrophenyl phosphate per minute per milligram of protein.

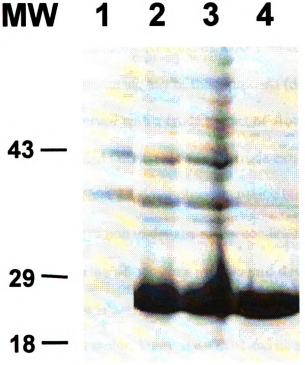

β-galactosidase specific activity
180
160
140
120
100
80
60
40
20
0
0
6
12
18
24
30
36
42
48
Time (Hr)

lacZ expression was observed from the Ω4514 promoter in the *orf1* mutant than in the wild-type strain during growth and development.

To determine whether the higher *lacZ* expression observed in the *orf1* mutant was due to disruption of *orf1*, or to a polar effect on expression of *orf2*, I constructed an *orf2* insertional disruption mutant using a similar approach. A DNA segment containing part of *orf2* from position 1164 to 1487 (Figure 2.4) was amplified by the PCR, verified by DNA sequencing, and cloned into pBR322 to construct pTH11-1. This plasmid was transformed into MTH10-3, the strain containing the Ω4514 promoter fused to *lacZ* at the *attB* site in the chromosome. Transformants (MTH11-1) in which pTH11-1 had integrated by homologous recombination, disrupting *orf2*, were identified by diagnostic PCR (data not shown). As shown in Figure 2.9, the *orf2* mutation did not affect *lacZ* expression from the Ω4514 promoter. We conclude that disruption of *orf1* increases Ω4514 expression during growth and development. This conclusion is consistent with the idea that ORF1 protein represses Ω4514 transcription directly, but we cannot rule out the possibility that ORF1 affects expression of *lacZ* fused to the Ω4514 promoter indirectly.

Production of ORF1 in *E. coli* and testing for binding to the Ω4514 promoter. To test whether ORF1 protein binds to the Ω4514 promoter region, I engineered *E. coli* to overproduce ORF1 or ORF1 chimeras tagged with 6xHis at the N- or C-terminus; using an IPTG-inducible T7 RNA polymerase expression system. As shown in Figure 2.10, a polypeptide of approximately the expected size (26 kDa) for ORF1-6xHis (tagged at the C-terminus) was enriched in cells after IPTG induction as compared to the uninduced cells. *E. coli* overproduced ORF1 or 6xHis-ORF1 (tagged at the N-terminus) upon IPTG induction (data not shown) about as well as ORF1-6xHis was overproduced

Figure 2.10. Production of ORF1-6xHis in *E. coli* and affinity purification. Whole-cell extracts (5 μl) of BL21(λDE3) containing pTH191 designed to produce ORF1-6xHis before (lane 1) and 2 or 3 h after IPTG induction (lanes 2 and 3). Eluted fraction (10 μl) from Ni-affinity column (lane 4).

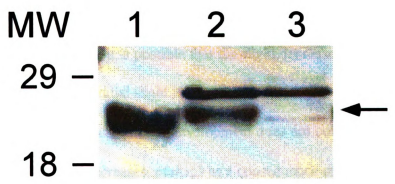

MW
1
2
3
4
43
29
18

(Figure 2.10, lanes 2 and 3). The ORF1-6xHis protein was purified under nondenaturing conditions with the aid of a Ni-affinity column (Figure 2.10, lane 4).

To test whether affinity-purified ORF1-6xHis binds to the Ω4514 promoter, mobility shift experiments were performed using a fragment, which spanned from –54 to +65 bp, because expression from this segment was elevated in the *orf1* mutant (Figure 2.9). Binding reactions incubated under standard conditions (Chodosh, 1988) or with several modifications, such as changing the monovalent and divalent salt concentrations, showed no specific shifted complex (data not shown). Likewise, no specific shifted complex was observed with gel-purified ORF1-6xHis, ORF1, or 6xHis-ORF1, or with crude extracts from *E. coli* overproducing any of these proteins (data not shown). Also no specific shifted complex was observed with a crude extract of growing *M. xanthus* cells or with a mixture of affinity-purified ORF1-6xHis and a crude extract of growing *M. xanthus* cells (data not shown). Thus, I did not observe specific binding of ORF1 products to the Ω4514 promoter region under any of the conditions I tested.

ORF1 protein levels during *M. xanthus* growth and development. To measure the ORF1 level in *M. xanthus*, the affinity-purified ORF1-6xHis fusion protein was used to raise polyclonal antibodies in a rabbit. These anti-ORF1-6xHis antibodies detected as little as 0.3 ng of native ORF1 overproduced in *E. coli* when diluted 1:5000 and used in Western blot analysis (data not shown). However, the antiserum (as well as the preimmune serum) cross-reacted with many proteins in Western blot analysis of proteins from growing *M. xanthus* cells (data not shown). Therefore, the anti-ORF1-6xHis antibodies were affinity-purified as described in Materials and Methods. Figure 2.11 shows that the affinity-purified antibodies detected a polypeptide in the extract of wild-

Figure 2.11. Detection of ORF1 protein in extracts of growing *M. xanthus*. Western blot analysis of *E. coli* BL21 (λDE3) overproducing ORF1 (lane 1), wild-type *M. xanthus* DK1622 (lane 2), and the *M. xanthus orf1* mutant was performed using affinity-purified anti-ORF1-6xHis antibodies as described in Materials and Methods. The arrow indicates ORF1 protein and the positions of protein markers are indicated in kDa.

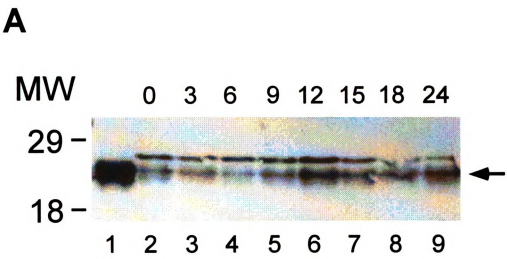

MW
1
2
3
29
18

type *M. xanthus* that was not present in the *orf1* mutant extract. This polypeptide migrated at a similar position as ORF1 made in *E. coli.*

To determine whether the ORF1 protein level changes during *M. xanthus* development, Tn*5 lac* Ω4514-containing strain DK4514 was induced to develop and samples were subjected to Western blot analysis and assayed for β-galactosidase specific activity. Figure 2.12A shows that the ORF1 protein level decreased slightly at 3-6 h then rose later in development to a level higher than in growing cells. Figure 2.12B shows that β-galactosidase activity increased slightly at 3-6 h, remained about the same at 9-12 h, then rose later in development. Since ORF1 negatively regulates Ω4514 expression directly or indirectly (Figure 2.9), it is possible that the slight decrease in the ORF1 level early in development allows the initial slight increase in Ω4514 expression. However, the major increase in Ω4514 expression later in development does not correlate with a loss of ORF1. Rather, the ORF1 level increases, perhaps as a consequence of increased Ω4514 promoter activity. The continued presence of ORF1 during development is consistent with the observation that the *orf1* mutant overexpresses Ω4514 during development (Figure 2.9), suggesting that ORF1 continues to negatively autoregulate Ω4514 expression during development. Hence, the major increase in Ω4514 expression later in development appears to involve a mechanism other than loss or inactivation of ORF1.

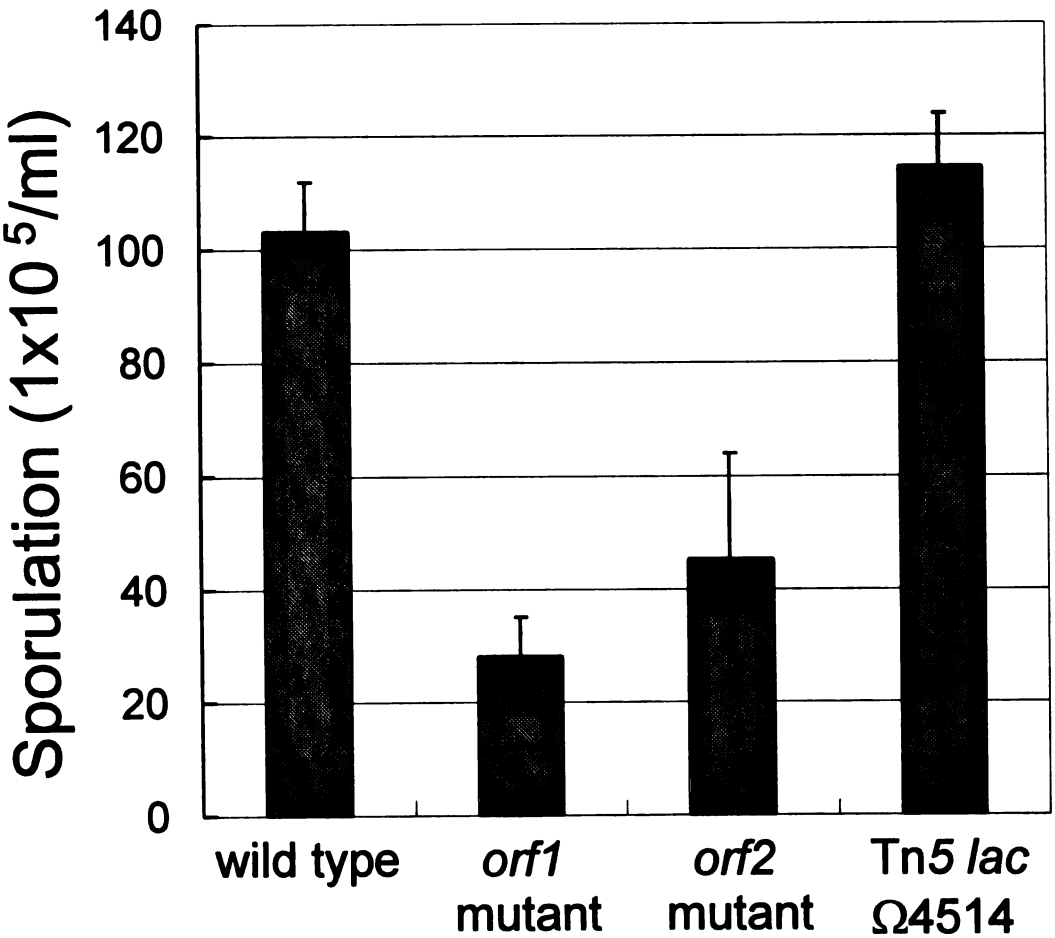
Sporulation efficiency of Ω4514 mutants. Tn*5 lac* Ω4514 appears to be inserted at the beginning of the third open reading frame of an operon. A previous study showed that Tn*5 lac* Ω4514 did not cause a developmental defect, as judged qualitatively by the normal appearance of fruiting bodies (Kroos et al., 1986). To be more quantitative, and to test whether the *orf1* or *orf2* insertional mutants exhibit development defects, the

Figure 2.12. Level of ORF1 protein and β-galactosidase specific activity during development of DK4514. (A) Western blot analysis of proteins in extracts of *M. xanthus* DK4514 collected during growth (lane 2) and at 3, 6, 9, 12, 15, 18, 24 h after the onset of development (lanes 3 to 9, respectively) using affinity-purified anti-ORF1-6xHis antibodies as described in Materials and Methods. ORF1 protein overproduced in *E. coli* was detected in lane 1. Equal amounts of *M. xanthus* protein, as judged by measuring total protein in sonic lysates of the same samples, were subjected to SDS-PAGE and Western blot analysis. The arrow indicates ORF1 protein and the positions of protein markers are indicated in kDa. (B) β-galactosidase specific activity of DK4514 harvested in the same experiment.


A
MW
0 3 6 9 12 15 18 24
29
18
1 2 3 4 5 6 7 8 9
B
β-galactosidase specific activity
140
120
100
80
60
40
20
0
0 3 6 9 12 15 18 21 24
Time (Hr)

sporulation efficiency was measured. The Tn*5* *lac* Ω4514-containing strain DK4514 had a similar sporulation efficiency as wild-type DK1622 (Figure 2.13), indicating that interruption of *orf3* did not affect sporulation. The *orf1* and *orf2* mutants appeared to aggregate like wild type, but exhibited about fourfold and twofold reduced sporulation efficiency, respectively (Figure 2.13), indicating that the *orf1* and *orf2* products play a role in development.

Figure 2.13. Sporulation of Ω4514 mutants. Sporulation tests were performed as described in Materials and Methods. The chart shows the average spore count of three independent isolates of each strain and one standard deviation of the data.


Sporulation (1x10 5/ml)
140
120
100
80
60
40
20
0
wild type
orf1 mutant
orf2 mutant
Tn5 lac Ω4514

Discussion

We have cloned the DNA upstream of Tn*5 lac* Ω4514 and identified a promoter that is induced during *M. xanthus* development. Promoter activity was lost in a graded fashion in a 5′ deletion series, suggesting that multiple upstream elements contribute to activity, but a deletion to –54 bp still permitted a low level of developmental induction. Interestingly, the promoter shares sequence similarity with promoters of genes that are transcribed during growth, and σ^A RNA polymerase (RNAP), the major form of RNAP in growing *M. xanthus*, initiated transcription from this promoter *in vitro.* Our results show that the Ω4514 promoter is negatively regulated by ORF1, the product of a downstream gene. This negative autoregulation keeps Ω4514 expression low during growth and early in development. Full induction of the Ω4514 operon during development involves positive regulation acting through DNA elements both near the promoter and far upstream.

Our deletion analysis of the Ω4514 promoter region indicates that multiple DNA elements spanning at least 650 bp upstream of the transcriptional start site contribute to developmental promoter activity (Figures 2.1 and 2.2). Deletion of DNA between *Xho*I and *Sal*I sites located approximately 2.7 kb and 650 bp, respectively, upstream of the start site, reduced developmental *lacZ* expression by about 30% (Figures 2.1 and 2.2). Therefore, DNA more than 650 bp upstream of the start site is necessary for full Ω4514 promoter activity. Likewise, deletion of DNA between the *Sal*I site located approximately 650 bp upstream of the start site, and the *Sma*I site located at -54 bp, reduced developmental *lacZ* expression an additional 20%, indicating the importance of

this region (Figures 2.1 and 2.2). The graded loss of promoter activity for these deletions was not specific to placement at the Mx8 *attB* site. Similar results were observed when plasmids were integrated by homologous recombination upstream of Tn*5* *lac* Ω4514 to create the corresponding 5′ deletions fused to *lacZ* at the native chromosomal location (data not shown). The Ω4514 promoter is not unique among developmentally regulated *M. xanthus* genes in requiring multiple upstream elements (Downard and Kroos, 1993). The *tps*, *csgA*, and Ω4499 promoters also exhibit large upstream regulatory regions (Kil et al., 1990; Li et al., 1992; Fisseha et al., 1999). In the case of *tps*, multiple elements appear to act over distances of several kb from the transcriptional start site (Kil et al., 1990). In the cases of *csgA* and Ω4499, upstream regions spanning more than 500 bp were required for full expression, and in both cases expression was lost in a graded fashion as 5' deletion endpoints approached the transcriptional start site (Li et al., 1992; Fisseha et al., 1999). It was proposed that multiple transcription factors bind to these upstream regions and regulate expression in response to different developmental cues. Similarly, expression of the Ω4514 promoter may involve several transcription factors that respond to developmental cues by binding to upstream DNA elements and activating transcription.

One cue that the Ω4514 promoter does not respond to is C signaling. Intercellular C signaling is mediated by the product of *csgA* (Shimkets et al., 1983; Shimkets and Asher, 1988) and is required for the expression of many *M. xanthus* genes that begin to be expressed after 6 h into development (Kroos and Kaiser, 1987). Introduction of a *csgA* mutation into cells containing Tn*5* *lac* Ω4514 delayed developmental *lacZ* expression slightly in a previous study (Kroos and Kaiser, 1987). However, when we repeated this

experiment, we found no significant difference between wild-type and a *csgA* mutant when we compared developmental expression from Tn*5 lac* Ω4514. Also, expression of 5′ deletions (i.e., pTH1-6, pTH4-1, and pTH4-3; see Figure 2.1 and Table 2.1) was tested at Mx8 *attB* in a *csgA* mutant and was indistinguishable from that in wild-type cells (data not shown). Moreover, when extracellular C signal was provided (by co-development with wild-type cells) to the *csgA* mutants harboring the 5′ deletions, no increase in expression was observed (data not shown), in contrast to the results observed for C signal-dependent promoters (Fisseha et al., 1996; Brandner and Kroos, 1998; Fisseha et al., 1999). Promoters that depend on C signaling for expression exhibit one or more sequences (near –50 bp) matching the consensus CAYYCCY (Y means pyrimidine), which has been called the C box (Fisseha et al., 1999). It has been speculated that C box sequences are cis-acting regulatory elements important for the expression of C signal-dependent genes. The Ω4514 promoter does not have a sequence matching the C box consensus near –50 bp. However, there are five sequences in Figure 2.4 that match the C box consensus. Two of these are in the upstream region, centered at –191 (position 218 in Figure 2.4) and –185 (position 224). These may be in the coding region of an upstream gene, because an ORF that exhibits codon preference typical of *M. xanthus* genes stops at –113 (position 296) and extends upstream to the end of the region that has been sequenced. The C-terminal polypeptide predicted by this partial ORF does not show significant similarity to any protein in the databases. The other three sequences that match the C box consensus are located in ORF1 (centered at position 744 and 928 on the transcribed strand) or ORF2 (centered at position 1485 on the nontranscribed strand), well downstream of the Ω4514 promoter. A sequence matching the C box consensus is

expected to occur on average every 1200 bp in a high GC (70%) organism like *M. xanthus*, so the occurrence of C box sequences is higher than expected in the Ω4514 region; but it remains to be tested whether any of these sequences are positioned properly to influence Ω4514 expression.

The Ω4514 promoter is the first example of a developmental *M. xanthus* promoter that appears to be recognized by σ^A RNAP. Figure 2.14 compares the –35 and –10 regions of the Ω4514 promoter with the corresponding regions of two promoters shown previously to be transcribed by σ^A RNAP *in vitro* (Biran and Kroos, 1997), and with the consensus –35 and –10 regions of promoters recognized by *E. coli* σ^{70} and *B. subtilis* σ^{43} RNA polymerases. The Ω4514 promoter has a perfect match in the –35 region to the consensus. It resembles the *vegA* and *aphII* promoters in that the –10 region is more GC-rich than the consensus sequence. It was proposed previously that *M. xanthus* σ^A interacts better with a more GC-rich –10 sequence than the primary sigma factors of *E. coli* and *B. subtillis* (Biran and Kroos, 1997).

We showed that σ^A persists at a high level at least until 12 h into development (Figure 2.8), when the Ω4514 mRNA level is also high (Figure 2.3). By 24 h into development, σ^A and Ω4514 mRNA were barely detectable, yet β-galactosidase activity from Tn*5 lac* Ω4514 increased until 48 h of development (Figure 2.2). The continued rise in *lacZ* expression after 18 h of development required upstream DNA beyond the *Sma*I site located at –54 (Figures 2.2 and 2.6). We considered the possibility that another promoter located upstream of the *Sma*I site was expressed later in development, despite detecting a single apparent mRNA 5′ end (Figure 2.3). However, integration of a plasmid (p1175SmSl4514, Table 2.1) into the chromosome (strain MGV03 in Table 2.1), placing

Figure 2.14. Comparison of promoter sequences. The –10 and –35 promoter regions and the distance between them are shown for Ω4514, for the *M. xanthus vegA* (Komano et al., 1987) and *aphII* (Rothstein and Reznikoff, 1981) genes, which are transcribed by σ^A RNAP *in vitro* (Biran and Kroos, 1997), and for genes transcribed by *E. coli* σ^{70} (Lisser and Margalit, 1993) and *B. subtilis* σ^{43} (Helmann, 1995) RNA polymerases. Matches to the σ^{70}/σ^{43} consensus are indicated by boldface letters.

	-35		-10
Ω4514	**TTGACA**	18	**TA**CCTA
vegA	**TAGACA**	17	**TA**AGGG
aphII	**TTGCCA**	17	**TA**AGG**T**
σ^{70}/σ^{43} consensus	**TTGACA**	17	**TATAAT**

lacZ immediately downstream of the *Sma*I site, showed no developmental β-galactosidase activity (Figure 2.1). Since there appears to be no additional promoter upstream, we speculate that high-level expression of the Ω4514–*lacZ* fusion mRNA at 12-18 h of development allows this message to persist later in development, giving rise to continued β-galactosidase production. Another possibility is that Tn*5 lac* Ω4514 may disrupt or exert a polar effect on a gene whose product negatively regulates Ω4514 expression. The level of Ω4514 mRNA was measured in wild-type cells in which the postulated negative regulation would be intact, decreasing the mRNA level after 12-h into development. In cells containing Tn*5 lac* Ω4514, this negative autoregulatory loop would be broken. Therefore, β-galactosidase activity would continue to increase later in development. Both models predict that the level of Ω4514-*lacZ* fusion mRNA would remain higher than was observed for the level of Ω4514 mRNA late in development.

The product of the first ORF downstream of the Ω4514 promoter negatively regulates expression of the Ω4514 operon during both growth and development (Figure 2.9). Although ORF1 has a helix-turn-helix DNA-binding motif and is most similar in sequence to a family of repressors, we were unable to demonstrate specific binding of ORF1 to the Ω4514 promoter region. It is possible that ORF1 regulates another gene(s) whose product(s) directly represses Ω4514 transcription (Figure 2.15). Alternatively, ORF1 may be a direct repressor of Ω4514 transcription, but may require an additional factor that was not present under the conditions we tested. One such factor could be post-translational modification of ORF1. However, we observed no significant difference in migration on SDS-PAGE of ORF1 produced in *E. coli* compared with ORF1 produced during *M. xanthus* growth and development (Figures 2.11 and 2 12), providing no

evidence for post-translational modification of ORF1. Perhaps ORF1 requires binding of a small molecule in order to bind specifically to the Ω4514 promoter, analogous to the case of tryptophan and the *trp* repressor (Joachimiak et al., 1983).

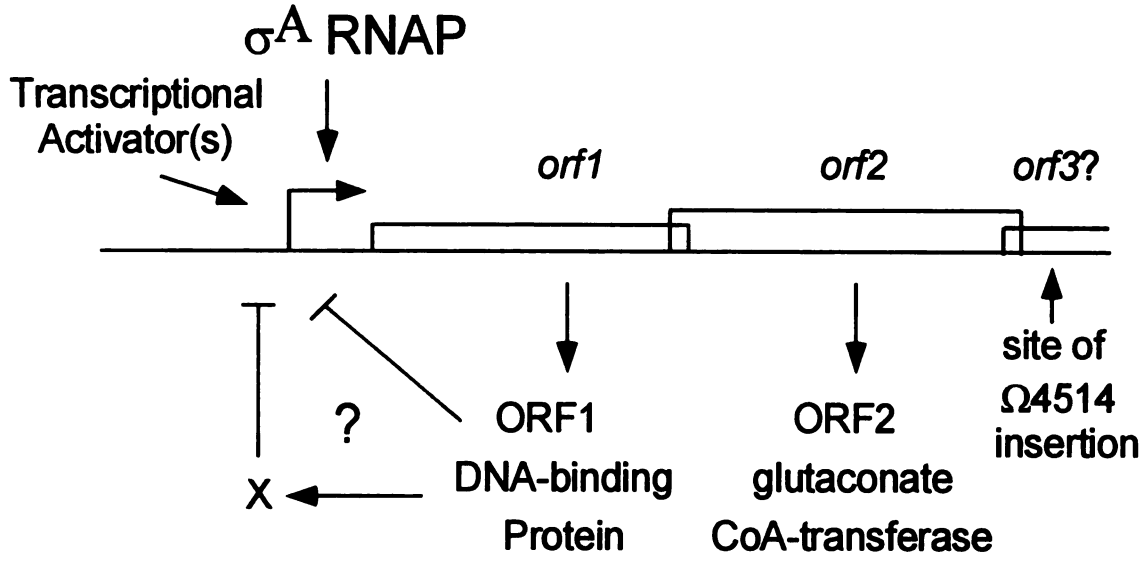
Developmental induction of Ω4514 expression can occur in the absence of ORF1 and in the absence of upstream DNA beyond –54 (Figure 2.9). This suggests that σ^A RNAP transcribes from the Ω4514 promoter more efficiently during development than during growth, because the level of σ^A remains about the same during growth and early in development (Figure 2.8). If many σ^A-dependent genes are repressed as cells enter development, perhaps σ^A RNAP becomes available to transcribe the Ω4514 promoter. Another possibility is that a transcriptional activator is induced upon starvation and binds between –54 and +65 in the Ω4514 promoter region.

Insight into the regulation of Ω4514 expression can be gained from comparison of the level and timing of developmental *lacZ* expression observed for different promoter constructs. β-galactosidase activity from the promoter region between –54 and +65 reached 160 U in the absence of ORF1 (Figure 2.9). However, β-galactosidase activity reached nearly 400 U from the Ω4514 promoter with additional upstream DNA, even when ORF1 was present (Figure 2.2). Clearly, upstream DNA elements enhance Ω4514 promoter activity. It will be interesting to test the activity of the Ω4514 promoter with upstream elements in the absence of ORF1. We might observe a higher maximum level of expression, as suggested by the results shown for the Ω4514 promoter from –54 to +65 in the presence or absence of *orf1* at the native chromosomal site (Figure 2.9). We might also observe a change in the timing of *lacZ* expression. Figure 2.9 shows that *lacZ* expression increased significantly by 4 h of development for strains with *lacZ* fused at

+65. This is most obvious for the strain completely lacking ORF1 due to a disruption at the native site (Figure 2.9, diamonds). The other strains examined in this experiment had *orf1* intact at the native site, and therefore exhibited a lower level of expression due to ORF1 negative regulation in *trans* (since the *lacZ* fusion is located at the Mx8 *attB* site), but like the strain completely lacking *orf1*, these strains showed no obvious lag in *lacZ* expression early in development (Figure 2.9). One of these strains was also used in the experiment shown in Figure 2.6 (triangles), and it is clear that expression increased by 6h of development. In contrast, *lacZ* expression appeared to be delayed, increasing dramatically only after 6h of development, for strains with *lacZ* fused downstream of *orf2* (Figure 2.2 and Figure 2.6, diamonds). In these strains *orf1* is expressed from the Ω4514 promoter at Mx8 *attB* as well as from the native site, so ORF1 may be responsible for the delay in expression, by directly or indirectly inhibiting Ω4514 promoter activity (Figure 2.15). We cannot exclude the possibility that DNA between +65 and the site of Tn*5 lac* Ω4514 insertion (downstream of *orf2*) causes the delay in developmental expression, due to regulation at the transcriptional or translational level.

If ORF1 is responsible for delaying Ω4514 expression early in development, how is this negative regulation overcome? There was a small decrease in the level of ORF1 at 3-6 h of development that correlated with a small increase in expression from Tn*5 lac* Ω4514, but the major rise in *lacZ* expression did not correlate with a loss of ORF1 (Figure 2.12). The finding that *lacZ* expression from the Ω4514 promoter continues to rise during the first 36 h of development in *orf1* mutant cells, but not in cells expressing *orf1* (Figure 2.9), suggests that ORF1 continues to negatively regulate Ω4514 expression during development. We speculate that positive regulation by one or more transcriptional

Figure 2.15. A model describing regulation of the Ω4514 operon. During growth and development, ORF1 negatively regulates transcription by σ^A RNAP either by binding directly to the promoter region or by regulating another gene(s) whose product(s) (x) represses transcription. Developmental induction may initially involve a decrease in ORF1-mediated negative regulation early in development but our data suggest that ORF1 continues to negatively autoregulate Ω4514 expression during development and we propose that a transcriptional activator(s) is primarily responsible for developmental induction of Ω4514.


σA RNAP
Transcriptional
Activator(s)
orf1
orf2
orf3?
site of
Ω4514
insertion
?
X
ORF1
DNA-binding
Protein
ORF2
glutaconate
CoA-transferase

activators overcomes ORF1-mediated negative regulation and causes the major rise in Ω4514 transcription during development (Figure 2.15).

Disruption of *orf1* caused a mild sporulation defect (Figure 2.13). This may be due to a polar effect on expression of *orf2* since disruption of *orf2* also caused a mild sporulation defect (Figure 2.13). ORF2 shows similarity to glutaconate CoA-transferase subunit A of *Acidaminococcus fermentans* and related proteins in other organisms. These enzymes catalyze transfer of the CoA moiety from acetyl-CoA to 2-hydroxyglutarate and related compounds like glutamate in the glutamate fermentation pathway (Mack et al, 1994). Our results suggest that the Ω4514 operon might encode components of an alternative metabolic pathway that is induced during *M. xanthus* development and plays a role in sporulation. Sequence analysis suggested the possible existence of a third ORF overlapping the ORF2 stop codon and interrupted after just two amino acids by Tn*5 lac* Ω4514. In *Acidaminococcus fermentans*, subunit B of glutaconate CoA-transferase is encoded in the same operon downstream of subunit A. By analogy, ORF3 might encode subunit B of glutaconate CoA-transferase. However, DK4514, in which *orf3* is disrupted by Tn5 lac Ω4514, did not show a sporulation defect (Figure 2.13). It is unlikely that a functional ORF3 protein is made in DK4514, because the Tn*5 lac* insertion disconnects *orf3* from its normal transcription and translation start signals. Although many questions remain about the function and regulation of the Ω4514 operon, it is the first example of a developmentally regulated *M. xanthus* operon that is transcribed by the major vegetative RNAP, and it is subject to complex control, including negative autoregulation by the product of the first gene in the operon and positive regulation by upstream DNA elements.

Chapter III

Identification of regulatory proteins involved in the expression of a C-signal-dependent gene

Abstract

Ω4403 is a Tn*5 lac* insertion in the *Myxococcus xanthus* chromosome that fuses *lacZ* expression to a developmental promoter, which is expressed between 6 and 12 h after starvation. Cell-cell interactions, including C-signaling, are required for the developmental expression of Tn*5 lac* Ω4403. It has been shown previously that DNA downstream of –80 bp relative to the Ω4403 transcriptional start site is sufficient for C-signal-dependent developmental expression of *lacZ*, comparable to that observed with much larger upstream segments. However, the developmental *lacZ* expression was abolished upon deletion to –72 bp, suggesting the possible involvement of an upstream activator protein that interacts with DNA between –80 and –72. To search for this protein, I first cloned DNA from –103 to +36, which has less sequence downstream of the transcriptional start site than the previously tested constructs, and showed that it had similar promoter activity. Therefore, this fragment was used to search for a regulatory protein(s) that interacts with the Ω4403 promoter region. I identified a development-specific complex using an electrophoretic mobility shift assay. It appears that a protein binds between –41 and –11, and the formation of the shifted complex does not depend on C-signaling, or on A-, D-, or E-signaling. Shifted complexes were also formed with the C-signal-dependent Ω4400 promoter and with three promoters that are not C-signal-dependent (Ω4514, *vegA* and *aphII*). Taken together, the data suggest that the protein forming the shifted complex with the Ω4403 promoter fragment is unlikely to mediate C-signaling-dependent expression of Ω4403.

Introduction

Cell-cell interactions play a critical role in multicellular development and cellular differentiation. *Myxococcus xanthus* is a gram-negative, rod-shaped soil bacterium that undergoes multicellular development when nutrients are depleted (Dworkin and Kaiser, 1993). When cells are starved at a high density on a solid surface, they move in a coordinated fashion into aggregation centers and form mound-shaped multicellular structures known as fruiting bodies, inside of which most of the cells lyse and some cells differentiated into ovoid spores. When nutrients are available, these dormant, heat- and sonication-resistant spores germinate.

This multicellular process requires cell motility and communication between cells (Shimkets, 1999). At least five extracellular signaling interactions have been identified and are known as the A-, B-, C-, D-, and E-signaling interactions (Hagen et al., 1978; LaRossa et al., 1983; Downard et al., 1993). Mutants unable to produce any one of these signals are arrested in development, but the defects are rescued upon codevelopment with wild-type cells or mutants of a different signaling group. Cells defective in different signaling interactions arrest development at different stages. Mutants defective in A-, B-, D-, or E-signaling block development before 5 h, whereas C-signaling affects genes that are expressed after 6 h into development (Downard et al., 1993; Kaiser and Kroos, 1993; Downard and Toal, 1995).

Our studies have focused on the regulation of developmental gene expression by C-signaling. The *csg* group of mutants, which are defective in C-signaling, are genetically simple and well-characterized. All *csg* mutations map to a single locus in the

chromosome, *csgA*, and the cells are defective in rippling, aggregation, fruiting body formation and sporulation (Hagen et al., 1978; Shimkets and Asher, 1988). Almost all genes expressed later than 6 h into development exhibit reduced expression or no expression in a *csgA* mutant background (Kroos and Kaiser, 1987). The *csgA* gene appears to encode a 25-kDa protein of the short-chain alcohol dehydrogenase family and the CsgA protein is associated with the cell surface and/or the extracellular matrix (Shimkets and Rafiee, 1990; Lee et al., 1995). Efficient C-signal transmission requires cell movement (Kroos et al., 1988; Kim and Kaiser, 1990b). During development, cells move into alignment and become densely packed in the outer domain of a nascent fruiting body (Sager and Kaiser, 1993). It has been proposed that end-to-end contact brought about by cell movement mediates efficient C-signaling (Sager and Kaiser, 1994). C-signaling is self-reinforcing. CsgA affects the gliding movements of starved individual cells in a way that is thought to promote aggregation, thus bringing about cell alignment that permits more efficient C-signaling (Sogaard-Anderson and Kaiser, 1996; Jelsbak and Sogaard-Anderson, 1999). Also, CsgA addition to starved cells stimulates expression of *csgA*, creating a positive feedback loop (Kim and Kaiser, 1991). A lower level of C-signaling is sufficient for aggregation and expression of genes partially dependent on C-signaling, whereas a higher level of C-signaling is required for sporulation and expression of genes absolutely dependent on C-signaling (Kim and Kaiser, 1991; Li et al., 1992). Thus, CsgA appears to act as a developmental timer that triggers the normal developmental sequence of aggregation, fruiting body formation and finally sporulation as the CsgA concentration increases during development.

Sixteen C-signal-dependent genes have been identified by using Tn*5 lac*, a transposon that contains a promoterless *lacZ* (Kroos and Kaiser, 1984). Transposition of Tn*5 lac* into the *M. xanthus* chromosome can generate a transcriptional fusion between *lacZ* and an upstream *M. xanthus* promoter (Kroos et al., 1986). Tn*5 lac* insertions in C-signal-dependent genes were identified by comparing *lacZ* expression in wild-type cells and *csgA* mutants (Kroos and Kaiser, 1987).

The expression of Tn*5 lac* Ω4403 is absolutely dependent on C-signaling (Kroos and Kaiser, 1987). The Ω4403 promoter has been localized and mapped (Fisseha et al., 1996). It was shown that DNA downstream of –80 is sufficient to direct C-signal-dependent expression, whereas the expression is completely abolished when 8 bp more is deleted from the 5′ end. Since DNA from –80 to –72 is upstream of the typical RNAP binding site, we speculate that a regulatory protein(s) binds to this region and mediates C-signal-dependent expression. To search for this protein and any other proteins that interact with the Ω4403 upstream region, I first cloned a small Ω4403 promoter-containing DNA segment (–103 to +36) and showed that this segment has similar promoter activity as DNA segments with more downstream sequence. The small DNA segment was used as a probe to search for DNA-binding proteins. A development-specific, DNA-binding activity was identified. However, it does not require the –80 to –72 sequence to bind, nor does binding require C-signaling. Moreover, the DNA-binding activity did not appear to be specific for C-signal-dependent promoters, so it is unlikely to be responsible for C-signal-dependent gene expression.

Materials and Methods

Bacterial strains and plasmids. Strains and plasmids used in this work are listed in Table 3.1.

Growth and development. *Escherichia coli* cells were grown at 37°C in LB medium (Sambrook et al., 1989) containing 50 μg ampicillin or 25 μg kanamycin per ml, as necessary. *M. xanthus* was grown at 32°C in CTT medium (Hodgkin and Kaiser, 1977) in liquid culture or on agar (1.5%) plates with 40 μg kanamycin or 12.5 μg oxytetracycline per ml when required (Kroos et al., 1986). Fruiting body development was performed on TPM (10 mM Tris-HCl, pH 8.0, 1 mM KH_2PO_4, 8 mM $MgSO_4$, final pH 7.6) agar (1.5%) plates as described previously (Kroos et al., 1986).

Molecular cloning. Recombinant DNA work was performed using standard techniques (Sambrook et al., 1989). Plasmid DNA was prepared from *E. coli* DH5α or JM83.

To construct a pREG1727 derivative with a small DNA segment containing the Tn*5 lac* Ω4403 promoter, a DNA fragment from –103 to +36 bp relative to the Ω4403 transcriptional start site was generated by PCR using pMF3.4 as a template. The upstream primer was 5'-CTCTCTAGAAGACATCGCGCCTTGAA-3', which has an underlined *Xba*I site and anneals to the sequence between positions –103 and –87. The downstream primer was 5'-CTCGGATCCTTCATGTTTTACCCAGA-3', which has an underlined *Bam*HI site and anneals to the sequence between position +20 and +36. The amplified fragment was digested with *Bam*HI and *Xba*I, gel-purified and ligated to *Bam*HI/*Xba*I-digested pREG1727 to construct pTH6-1. The insert was sequenced to ensure that no errors occurred during the PCR.

Table 3.1. Bacterial Strains and Plasmids

Strain or plasmid	Characteristics	Source
E. coli		
DH5α	*supE*44 Δ*lacU*169 ϕ80 Δ*lacZ* ΔM15 *hsdR*17 *recA*1 *endA*1 *gyrA thi*-1*relA*1	Hanahan, 1983
JM83	*ara* Δ*lac-pro strA thi* ϕd*lacZ* ΔM15	Messing, 1979
M. xanthus		
DK1622	wild type	Kaiser, 1979
MMF1727	*attB*::pREG1727	Fisseha et al., 1996
MTH6-1	*attB*::pTH6-1	This study
MMF100	*attB*::pMF100	Fisseha et al., 1996
DK4746	Tn*5* Ω4678 *asgB480*	Dale Kaiser
DK5270	Tn*5 lac* (Km^r) Ω4403 *csgA*::Tn*5-132* (Tc^r) Ω205	Kroos and Kaiser, 1987
DK3261	Tn*5* Ω1867 *dsg439*	Cheng and Kaiser, 1989b
JD300	*esg*::Tn*5* (Ω258)	J. Downard
Plasmid		
pUC19	Ap^r *lacα*	Yanisch-Perron et al., 1985
pMF3.4	Ap^r (pUC19)[a], 674-bp *Sph*I-*Bam*HI fragment upstream of Ω4403 Tn*5 lac*	Fisseha et al., 1996
pMF100	Ap^r Km^r (pREG1727), 521-bp *Hae*II-BamHI fragment upstream of Ω4403 Tn*5 lac*	Fisseha et al., 1996

pREG1727	Ap^r Km^r P1-*inc attP 'lacZ*	Fisseha et al., 1996
pTH6-1	Ap^r Km^r (pREG1727), DNA from -103 to +36 bp of Ω4403 transcription start site generated by PCR using pMF3.4	This study
pTH10-1	Ap^r (pUC19); 120-bp *Sma*I-*Eco*47III fragment from pTH4-4	Chapter 2

[a] The vector is indicated in parentheses.

Construction of *M. xanthus* strains. Strain MTH6-1 was constructed by P1 specialized transduction of pTH6-1 from the *rec*$^+$ *E. coli* strain JM83 into wild-type DK1622 (Gill et al., 1988; Fisseha et al., 1996). Because of the presense of *attP* on the plasmid, pTH6-1 integrated into the *M. xanthus* chromosome at Mx8 *attB*. At least three derivatives, each containing a single copy of integrated plasmid, were identified by Southern blot analysis (Sambrook et al., 1989; Fisseha et al., 1996), and β-galactosidase production was measured under developmental conditions as described previously (Kroos et al., 1986).

Crude extract preparation for gel mobility shift assay. Wild-type DK1622 cells or signaling mutant cells were grown in CTT liquid medium with appropriate selection until the density reached 100 Klett units. Cells were then sedimented at 4300 x g for 10 min. The cell pellet was resuspended to 2000 Klett units using TPM buffer and 0.5 ml aliquots were spread on TPM plates and incubated at 32°C. Vegetative and developmental *M. xanthus* cells collected at the indicated time points were harvested and stored at −70°C. Extracts were prepared by the method of Gross and Burgess (Gross et al., 1976) with some modifications. Thawed cell pellets (approximately 0.4 ml) were suspended in 0.25 ml of solution A (10 mM Tris-HCl [pH 8.0], 25% sucrose, 100 mM NaCl, 1 mM phenylmethylsulphonyl floride [PMSF]), followed by addition of a mixture of protease inhibitors (around 5 μl total) of 1 μg aprotinin, 0.5 μg leupeptin and 0.7 μg pepstatin A. The mixture was incubated on ice for 15 min. This was followed by addition of 0.06 ml of solution B (300 mM Tris-HCl [pH 8.0], 100 mM EDTA, 4 mg/ml lysozyme, 1 mM PMSF) and incubation on ice for 10 min. Finally, 0.31 ml of solution C (1 M NaCl, 20 mM EDTA, 0.08% deoxycholate, 1 mM PMSF) was added and the

mixture was incubated at 10°C for 10 min. The resulting lysates were centrifugated for 40 min at 30,000 x *g*. The protein concentration of the supernatants was quantified by the Bradford method (Bradford, 1976). The extracts were used immediately for mobility shift assays or quick-frozen in a 95% ethanol/dry ice bath after addition of glycerol to 50% final concentration.

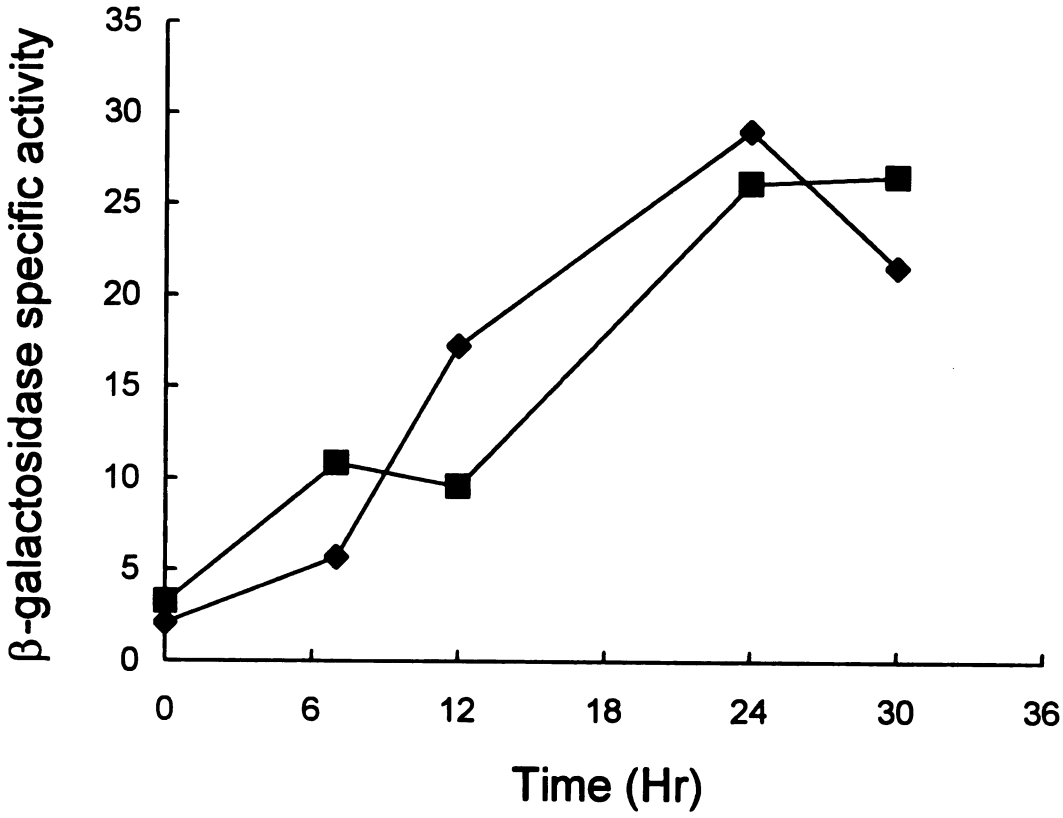
Mobility shift experiments. PCR products containing Ω4403 upstream DNAs were 5' end-labeled with [γ-^{32}P] ATP and T4 polynucleotide kinase. A restriction fragment from –105 to +53 relative to the Ω4403 transcriptional start site, obtained from pMF3.4 digested with *Msc*I and *Xmn*I, was gel-purified, phosphatase-treated, and 5' end-labeled with [γ-^{32}P] ATP and T4 polynucleotide kinase. The labeled DNAs were purified with a QIAquick Nucleotide Removal Kit (Qiagen). Ten μg of crude extract was incubated with 10,000 cpm of probe (typically 0.75 ng) and 1 μg of poly(dI·dC) as nonspecific competitor at 30°C for 15 min to allow DNA binding. The 20 μl binding reaction contained 12% glycerol, 12 mM HEPES (pH 7.9), 200 mM NaCl, 4 mM Tris-HCl (pH 7.9), 0.6 mM EDTA, and 5 mM $MgCl_2$. The samples were then subjected to electrophoresis on 5% native polyacrylamide gels at 4°C in low ionic strength buffer (Chodosh, 1988). After electrophoresis, the gels were dried for autoradiography.

Results

Further deletion analysis of the Ω4403 promoter region. Ω4403 is a Tn*5 lac* fusion that is expressed only during development in a C-signaling dependent manner. A previous study showed that DNA between –80 and +382 bp relative to the transcriptional start site was sufficient for C-signal-dependent promoter activity, but a deletion that removed 8 bp from the upstream end of this fragment abolished promoter activity (Fisseha et al., 1996). To better define the downstream boundary of DNA needed for promoter activity, DNA between –103 and +36 bp was amplified by PCR and cloned into pREG1727 upstream of a promoterless *lacZ* gene, resulting in pTH6-1. pTH6-1 was integrated into the *M. xanthus* chromosome at the myxophage Mx8 *attB*. Transductants containing a single copy of pTH6-1 integrated at *attB* were identified by Southern blot hybridization (data not shown). Several of these transductants were assayed for β-galactosidase activity during development and showed similar promoter activity as compared to MMF100, a strain containing Ω4403 DNA between –80 and +382 bp fused to *lacZ* at the *attB* site (Figure 3.1).

Detection of a development-specific shifted complex with the Ω4403 promoter region. To search for a protein(s) that interacts with the Ω4403 promoter region, a PCR product containing DNA between –103 and +36 bp or a restriction fragment containing DNA between -105 and +53 bp was utilized in electrophoretic mobility shift assays. Crude extracts from vegetatively growing or developing wild-type *M. xanthus* DK1622 were prepared and used in mobility shift assays as described in Materials and Methods. A shifted complex was detected with extract made from cells harvested 12 h into development, in the absence or the presence of the nonspecific

Figure 3.1. Developmental expression of *lacZ* from a DNA segment containing the Ω4403 promoter. Developmental ß-galactosidase specific activity was measured as described previously (Kroos et al., 1986) for at least three independent transductants of DK1622 containing a single copy of the Ω4403 upstream segment from –80 to +382 bp (♦) or the DNA segment from –103 to +36 bp relative to the Ω4403 transcriptional start site (■). ß-galactosidase specific activity is expressed in nanomoles of *o*-nitrophenyl phosphate per minute per milligram of protein.


β-galactosidase specific activity
35
30
25
20
15
10
5
0
0
6
12
18
24
30
36
Time (Hr)

competitor poly(dI·dC), whereas no shifted complex was detected with vegetative cell extract (Figure 3.2).

Competition assays with unlabeled fragments. To test whether the formation of the shifted complex is specific to the probe DNA, competition experiments including an excess amount of the same unlabeled DNA in the binding reaction were performed in the presence of the nonspecific competitor poly(dI·dC). The intensity of the shifted band started to decrease with a 10x molar excess of the unlabeled DNA and became very faint with a 100x molar excess of the unlabeled DNA (Figure 3.3). These results show that the DNA-binding activity is somewhat specific for the Ω4403 promoter containing fragment.

Developmental timing of formation of the shifted complex. To determine the level of the DNA-binding activity during development, crude extracts made from DK1622 cells harvested at 4, 8, 12, 20 or 24 h into development, as well as during vegetative growth, were tested. A shifted complex was observed at 4 h into development, reached its strongest intensity at 12 h, and disappeared after 24 h into development (Figure 3.4).

Dependence of the DNA-binding activity on extracellular signals. The expression of Ω4403 is absolutely dependent on C-signaling (Kroos and Kaiser, 1987). To test whether the DNA-binding activity exhibits dependency on C-signaling, crude extracts made from vegetative and developmental *csgA* mutant cells were tested. A similar shifted complex was observed with developmental *csgA* mutant cells (Figure 3.5A), suggesting that neither the production nor the DNA-binding activity of the protein in the shifted complex is dependent on C-signaling. No shifted complex was seen with vegetative cell extracts.

Figure 3.2. Gel mobility shift analysis of the Ω4403 promoter region. A PCR product spanning −103 to +36 bp was used as probe with extracts made from vegetatively growing (T0) or 12-h developing (T12) wild-type DK1622 *M. xanthus* cells in a mobility shift experiment as described in Materials and Methods. Poly(dI·dC) was added to binding reactions, unless indicated otherwise. The arrow indicates the position of a shifted complex with the developmental extract.

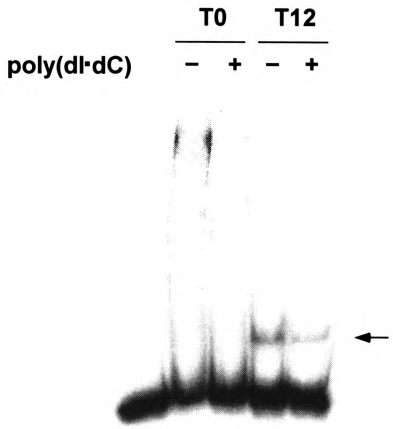

T0
T12
poly(dI·dC)
–
+
–
+

Figure 3.3. Competition with unlabeled DNA. The binding reaction was carried out with 0.75 ng (7 fmol) ^{32}P-labeled Ω4403 DNA fragment (-105 to +53 bp) alone (lane 1) or with the addition of extract (10 μg protein) made from 12 h-developing DK1622 cells and 1μg poly(dI·dC) (lanes 2-5). The same Ω4403 DNA fragment, except unlabelled, was also included in the binding reaction in 10-fold (lane3), 50-fold (lane 4) or 100-fold (lane 5) molar excess over the labeled probe. The shifted complex is marked by the arrow.

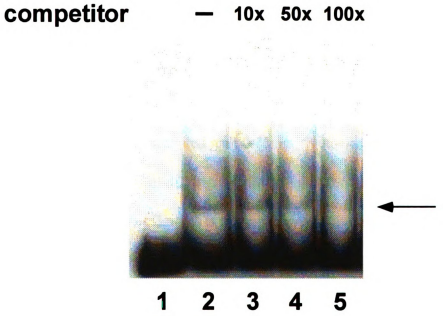
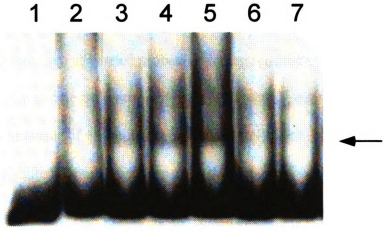

competitor
—
10x
50x
100x
1
2
3
4
5

Figure 3.4. Developmental timing of formation of the shifted complex. Extracts (10 μg protein) made from DK1622 cells collected during growth (lane 2) or at 4, 8, 12, 20 or 24 h after starvation (lanes 3 to 7) were incubated with 0.75 ng ^{32}P-labeled Ω4403 DNA probe (-105 to +53 bp) and 1μg poly(dI·dC) at 30°C for 15 min. The mixtures were then subjected to gel mobility shift analysis. Lane 1 is the probe alone and the arrow indicates the shifted complex.


1 2 3 4 5 6 7

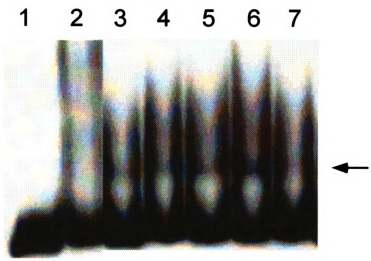
The dependence of DNA-binding activity on three other extracellular signaling interactions (A-, D- and E-signals) was also tested using extracts prepared from signaling-defective mutant cells that had undergone development. The DNA-binding activity was present in all three mutant extracts (Figure 3.5B). Together, these results indicate that the DNA-binding activity is not dependent on the A-, C-, D-, or E-signaling interactions.

Localization of DNA sequence in the Ω4403 promoter region required for binding. To localize the DNA sequence that was recognized by the DNA-binding activity, a series of 5′ deletions were generated by PCR. The deletions all have the same 3′ end (at +36) and were used as labeled probes in binding reactions. As shown in Figure 3.6, a shifted complex was detected with DNA fragments with 5′ ends at -80, -72, or -41 bp in the absence or presence of 1μg poly(dI·dC). When DNA with a 5′ end at -31 bp was used as probe, a shifted complex with much weaker intensity was observed without poly(dI·dC) and disappeared when 1 μg poly(dI·dC) was included in the reaction. This result suggests that DNA between –41 and –31 contributes to formation of the shifted complex.

These deletion segments, as well as DNAs from –105 to –11 and from -11 to +53 bp, were also used as competitors in experiments with DNA between -105 and +53 as probe. The deletions with 5′ ends at -80, -72 or -41 bp (all having the same 3′ end at +36) and the DNA from –105 to –11 competed away the formation of the shifted complex as well as full length fragment (-105 to +53), whereas DNA from -11 to +53 bp failed to compete (data not shown). Taken together, the results suggest that a protein binds to Ω4403 promoter DNA between -41 and -11 bp relative to the transcriptional start site.

Figure 3.5. Dependence of the DNA-binding activity on extracellular signals. (A) Extracts (10 μg protein) from *csgA* cells collected during growth (lane 2) or at 4, 8,12, 20 or 24 h after starvation were incubated with 0.75 ng ^{32}P-labeled Ω4403 DNA probe (-105 to +53 bp) and 1μg poly(dI·dC) at 30°C for 15 min. The mixtures were then subjected to electrophoresis on a 5% native polyacrylamide gel. (B) Extracts (10 μg protein) made from wild-type cells (lane 2) or *asg* (lane 3), *dsg* (lane 4), or *esg* (lane 5) cells harvested at 12 h into development were incubated with probe as described for panel A. No extract was added to probe in lane 1 of both panels. Shifted complexes are indicated by the arrows.

A



B

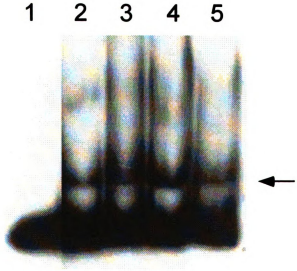
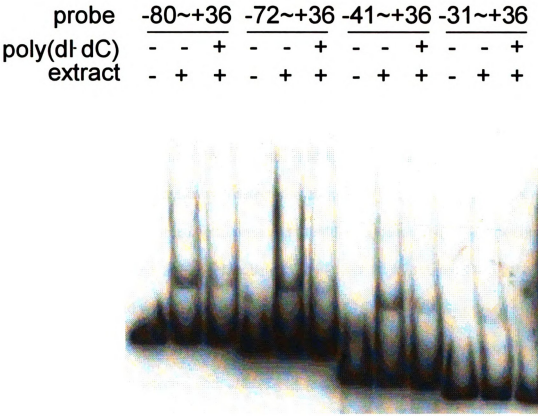


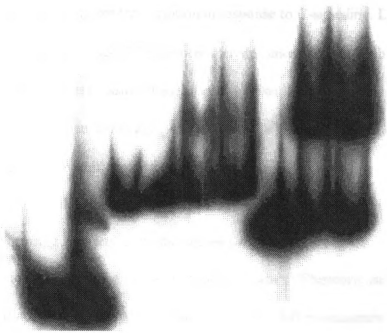
Figure 3.6. Gel mobility shift assays of 5′ deletion fragments containing the Ω4403 promoter. Ω4403 DNA (40 fmol) from –80 to +36, -72 to +36, -41 to +36, or –31 to +36 was incubated with extract (10 μg protein) from wild-type DK1622 cells at 12 h into development in the presence or absence of 1μg poly(dI·dC) at 30°C for 15 min. The mixtures were then subjected to electrophoresis on a 5% native polyacrylamide gel.


probe
-80~+36
-72~+36
-41~+36
-31~+36
poly(dI-dC)
- - + - - + - - + - - +
extract
- + + - + + - + + - + +

Binding to other promoters. Do the extracts from developing cells possess DNA-binding activity for other promoters, or is the Ω4403 promoter unique in this respect? To answer this question, two other *M. xanthus* developmental promoter-containing DNAs were tested. Ω4514 DNA spanning from -54 to +65 bp (chapter 2) was shifted in a similar fashion as Ω4403 promoter DNA, both in terms of the amount of shifted complex produced and its migration (data not shown). Likewise, Ω4400 DNA spanning –101 to +101 bp (Brandner and Kroos, 1998) was shifted similarly (data not shown). Hence, the extract shifts C-signal-dependent (Ω4403 and Ω4400) and C-signal-independent (Ω4514) developmental promoters. I also tested two *M. xanthus* vegetative promoter DNAs; *vegA*, spanning from –320 to +100 bp and *aphII*, spanning from –250 to +70 bp. A similar shifted complex was detected with *vegA* DNA (Figure 3.7). To ask whether the same protein is binding to *vegA* and Ω4403 promoter DNAs, a competition experiment was performed. A 50-fold molar excess of *vegA* DNA competed away the shifted complex formed with Ω4403 promoter DNA (data not shown); suggesting that same protein binds to both promoters. A more abundant and perhaps slightly slower-migrating complex was detected with *aphII* DNA (Figure 3.7). No shifted complex was detected when part of the coding sequence of Ω4514 DNA (from +5 to +273 bp relative to the transcriptional start site) was tested (data not shown). This shows that not every DNA segment can be shifted by proteins in developing cell extracts and suggests that complex formation may require promoter DNA.

Figure 3.7. Gel shift experiments with different promoters. Ω4403 DNA (–103 to +36 bp), *vegA* DNA (–320 to +100 bp) or *aphII* DNA (–250 to +70 bp) was incubated with extract (10 μg protein) of DK1622 cells 12 h into development in the presence or absence of 1μg poly(dI·dC) at 30°C for 15 min. The mixtures were then subjected to eletrophoresis on a 5% native polyacrylamide gel.

probe	Ω4403		*vegA*				*aphII*		
poly(dI·dC)	-	+	-	-	-	+	-	-	+
extract	-	+	-	-	+	+	-	+	+



Discussion

A previous study showed that elements needed for C-signal-dependent expression of Ω4403 lie between –80 and +382 bp relative to the transcriptional start site (Fiseha et al., 1996). In this study, I showed that Ω4403 DNA between –103 and +36 bp has similar developmental promoter activity as the –80 to +382 bp segment (Figure 3.1). Together, the results localize the Ω4403 promoter between –80 and +36 bp.

The principal aim of this study was to identify a regulatory protein that binds to the Ω4403 upstream region and activates transcription in response to C-signaling. DNA between –80 and –72 bp is critical for Ω4403 promoter activity, since deletion of this sequence abolishes developmental expression (Fisseha et al., 1996). Initially, a synthetic, double-stranded DNA with sequence identical to –92 to –64 bp of the Ω4403 promoter was used as a probe in gel mobility shift experiments to search for a DNA-binding protein. No specific shifted complex was obtained using the conditions described in Materials and Methods, or using a variety of modifications (data not shown). We reasoned that the synthetic DNA might be too short to allow binding. Therefore, larger Ω4403 promoter DNA fragments were used as probes in mobility shift experiments. Using DNA between –103 and +36, a development-specific shifted complex was observed (Figure 3.2). A competition experiment with unlabelled Ω4403 promoter DNA demonstrated that binding was somewhat specific for the Ω4403 promoter (Figure 3.3). A 10-fold molar excess of specific competitor was sufficient to reduce formation of the shifted complex in the presence of more than 1300-fold molar excess of nonspecific competitor. Because the crude extracts used in my experiments contain many DNA-

binding proteins, some of which may bind to the Ω4403 promoter region with some specificity, it may not be surprising that a 100-fold molar excess of specific competitor was needed to virtually eliminate formation of the shifted complex (Figure 3.3).

The ability of extracts prepared from cells at different times during development to produce the shifted complex (Figure 3.4) correlated roughly with the timing of Ω4403 expression during development (Figure 3.1). The DNA-binding activity was present by 4 h, the earliest time tested, and β-galactosidase activity from Tn*5 lac* Ω4403 rose by 7 h into development. DNA-binding activity reached a maximum at 12 h, whereas β-galactosidase activity continued to rise until 24 h, but the latter could be due to persistence of the fusion mRNA, its continued translation, and accumulation of β-galactosidase.

The development-specific DNA-binding activity was present in extracts prepared from mutants defective in extracellular signaling (Figure 3.5) despite the fact that developmental expression of Tn*5 lac* Ω4403 depends on signaling (Kroos and Kaiser, 1987). This could mean that the protein responsible for the DNA-binding activity is not involved in activation of Ω4403 transcription. However, it remained possible that the protein's ability to activate transcription was controlled not at the level of production or DNA-binding, but rather by a modification (e.g., phosphorylation), so I continued to characterize the DNA-binding activity.The DNA-binding activity appeared to recognize DNA between –41 and –11 bp relative to the Ω4403 transcriptional start site, based on mobility shift experiments with smaller DNA fragments (Figure 3.6) and competition experiments (data not shown). This region of the Ω4403 promoter is expected to be recognized by RNA polymerase, but it is unlikely that RNA polymerase is responsible for

the DNA-binding activity in the extracts because partially purified σ^A RNA polymerase (Biran and Kroos, 1997) bound to promoter-containing DNA produced a shifted complex that migrated much more slowly through the gel (data not shown). There are many examples of transcription factors that bind to promoters within the region recognized by RNA polymerase (Ishihama, 1993). It remains possible that the DNA-binding activity in the extracts is such a transcription factor. However, we were particularly interested in identifying a protein that binds specifically to DNA between –80 and –72 bp because deletion analysis had demonstrated that this region was essential for Ω4403 promoter activity *in vivo*. To search for such a DNA-binding activity in the extracts, the conditions of the binding reaction were varied with respect to the monovalent (NaCl or KCl) or divalent ($MgCl_2$) cation concentrations, the pH, the amount of nonspecific competitor [poly(dI·dC)], and the amount of crude extract. The only shifted complex detected migrated at the position shown in Figures 3.2-3.6, and the conditions of the binding reaction reported in Materials and Methods were those found to be optimal for formation of this shifted complex.

If there exists a transcription factor that binds specifically to DNA between –80 and –72 bp of the Ω4403 promoter, there are several reasons why this protein might have escaped detection. First, it may not be concentrated enough in the crude extract to allow detectable binding. Proteins from crude extracts were concentrated and partially purified using a heparin-agarose column with 1 M NaCl elution, followed by a DNA-cellulose column with 0.15 M to 1 M NaCl gradient elution. In the dialyzed eluate from the heparin-agarose column, DNA-binding activity producing a shifted complex at the position shown in Figures 3.2-3.6 was concentrated 10-fold, but no other shifted

complexes were observed (data not shown). Likewise, no other shifted complexes were observed with dialyzed DNA-cellulose column fractions (data not shown). These experiments do not rule out the possibility that a protein binding between –80 and –72 bp is present and produces a shifted complex that comigrates with the complex produced by a protein that binds between -41 and -11 bp. Another reason why a protein binding between –80 and –72 bp might have escaped detection is that this protein was susceptible to proteolysis, despite the presence of several protease inhibitors. A third possibility is that this protein needs another molecule in order to interact with the promoter, and this molecule is not present or active in the crude extract.

Our goal was to understand the molecular mechanism of C-signal-dependent activation of Ω4403 transcription. As noted above, the DNA-binding activity that recognizes DNA between -41 and -11 (Figure 3.6 and data not shown) is present in a mutant defective in C-signaling (Figure 3.5A), which fails to express Ω4403 (Kroos and Kaiser, 1987). Nevertheless, C-signaling might modify the DNA-binding protein so that it activates transcription, so it remained possible that this DNA-binding protein is important for C-signal-dependent activation of gene expression. This possibility was weakened by the finding that extracts produce similar shifted complexes with promoters that do not depend on C-signaling for expression (Figure 3.7). Moreover, when DNA-cellulose column fractions containing partially purified DNA-binding proteins were tested in mobility shift assays with vegetative promoters (*vegA* and *aphII*), similar shifted complexes were observed as with the Ω4403 promoter (data not shown). These results strongly suggest that the DNA-binding activity we have detected in developing-cell

extracts is not specific for C-signal-dependent promoters. Therefore, we decided not to pursue purification and further characterization of the DNA-binding activity.

It remains possible that this DNA-binding activity represents a transcription factor for both developmental and vegetative genes. A shifted complex was not observed with a DNA fragment that does not contain a promoter (data not shown). Further studies would be needed to determine whether the DNA-binding activity is indeed specific for promoter DNA and whether it binds to a similar region in different promoters.

Summary and Perspectives

The multicellular developmental process of *M. xanthus* is governed by extracellular signaling interactions. My research focused on characterizing two developmental genes. The aim of these studies was to investigate the regulatory mechanisms that mediate signaling interactions and developmental gene expression.

Tn*5 lac* Ω4514, a developmentally induced *lacZ* fusion originally thought to be partially dependent on C-signaling (Kroos and Kaiser, 1987), was shown to be C-signal-independent (chapter 2 Discussion). The regulation of the Ω4514 operon was shown to be complex and unique for *M. xanthus*, being negatively autoregulated by one of its own gene products, ORF1, and positively regulated by one or more putative activators that bind to upstream DNA elements. I was unable to demonstrate binding of ORF1 protein to the smallest Ω4514 promoter DNA segment that exhibits negative autoregulation by ORF1, and possible explanations have been discussed in chapter 2. Another possible explanation is that the binding site for ORF1 is too close to one end of the fragment used in the gel shift experiments, so a larger fragment could be tested.

Since multiple upstream regulatory elements are needed for full Ω4514 expression, as demonstrated by deletion analysis, finer deletions to localize the DNA elements that participate in regulation could be carried out. If a small DNA segment is shown to be critical, biochemical approaches could be used to identify regulatory proteins that interact with this DNA, as was attempted for Ω4403 (chapter 3). Any proteins so identified would be candidates for future studies aimed at comparing their expression and activity in wild-type cells and signaling defective mutants.

Disrupting the negative autoregulation of the Ω4514 operon by creating an *orf1* disruption mutant caused expression from the smallest Ω4514 promoter segment tested to

increase dramatically both during growth and development. It would be interesting to test a promoter segment containing the upstream regulatory elements for expression in the absence of ORF1. The results presented in chapter 2 suggest that such an experiment might show even more dramatic effect of ORF1 on the timing and level of Ω4514 expression.

The fact that Ω4514 mRNA peaked at 12-h into development whereas β-galactosidase activity from Tn*5* *lac* Ω4514 increased until 48-h into development is intriguing (chapter 2 Results). No additional promoter was identified upstream by S1 nuclease protection assays, primer extension, or by an *in vivo* experiment in which *lacZ* was integrated just upstream (i.e., at –55) of the Ω4514 promoter characterized here. One possible explanation is that Tn*5* *lac* Ω4514 disrupts or exerts a polar effect on a gene whose product negatively regulates Ω4514 expression (chapter 2 Discussion). To address this hypothesis, the level of Ω4514 mRNA in cells containing Tn*5* *lac* Ω4514 could be checked during development.

The DNA region between –80 and –72 is crucial for C-signal-dependent expression of Ω4403 (Fisseha et al., 1996). No protein binding specifically to this sequence was identified in my experiments (chapter 3). The regulatory protein that interacts with this region may exist at a very low level so that it is hard to observe the protein-DNA interaction even when the extract is partially purified and concentrated by column chromatography. Several other approaches could be tried to identify the putative regulatory protein, including a DNA affinity column using the sequence between –80 and –72, using this sequence as a probe in Southwesterns, or as the target for binding in a

yeast one-hybrid screen, or by doing a classical genetic screen for mutants that fail to express Tn*5* *lac* Ω4403.

Bibliography

Bibliography

Apelian, D. and S. Inouye. 1990. Development-specific σ-factor essential for late-stage differentiation of *Myxococcus xanthus*. Genes Dev. **4**: 1396-1403.

Apelian, D. and S. Inouye. 1993. A new putative sigma factor of *Myxococcus xanthus*. J. Bacteriol. **175**: 3335-3342.

Baker, M. E. 1994. *Myxococcus xanthus* C-factor, a morphogenetic paracrine signal, is similar to *Escherichia coli* 3-oxoacyl-[acyl-carrier-protein] reductase and human 17ß-hydroxysteroid dehydrogenase. Biochem. J. **301**: 311-312.

Biran, D. and L. Kroos. 1997. *In vitro* transcription of *Myxococcus xanthus* genes with RNA polymerase containing σ^A, the major sigma factor in growing cells. Mol. Microbiol. **25**: 463-472.

Bolivar, F., R. L. Rodriguez, P. J. Greene, M. C. Betlach, H. L. Heyneker and H. W. Boyer. 1977. Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. Gene **2**: 95-113.

Bradford, M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**: 248-254.

Brandner, J. P. and L. Kroos. 1998. Identification of the Ω4400 regulatory region, a developmental promoter of *Myxococcus xanthus*. J. Bacteriol. **180(8)**: 1995-2004.

Brennan, R. G. and B. Matthews. 1989. The helix-turn-helix DNA binding motif. J. Bio. Chem. **264**: 1903-1906.

Cashel, M., D. R. Gentry, V. J. Hemandez and D. Vinella. 1996. The stringent response. In *Escherichia coli and Salmonella*. F. Neidhardt. Washington, DC, American Society of Microbiology: 1458-1496.

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

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

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

Cho, K. and D. R. Zusman. 1999. AsgD, a new two-component regulator required for A-signaling and nutrient sensing during early development of *Myxococcus xanthus*. Mol. Microbiol. **34**(2): 268-281.

Chodosh, L. A. 1988. Mobility shift DNA-binding assay using gel electrophoresis. In *Current protocols in molecular biology*. F. M. Ausubel, R. Brent, R. E. Kingstonet al, John Wiley & Sons, Inc.: 12.2.1-12.2.10.

Crawford, E. W. J. and L. J. Shimkets. 2000. The stringent response in *Myxococcus xanthus* is regulated by SocE and the CsgA C-signaling protein. Genes Dev. **14**(483-492).

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

Downard, J. and L. Kroos. 1993. Transcriptional regulation of developmental gene expression in *Myxococcus xanthus*. In *Myxobacteria II*. M. Dworkin and D. Kaiser. Washington, DC, American Society for Microbiology: 183-199.

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

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

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

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

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

Dworkin, M. and D. Kaiser. 1993. Myxobacteria II. Washington, DC, American Society for Microbiology.

Ellehauge, E., M. Norregaard-Madsen and L. Sogaard-Andersen. 1998. The FruA signal transduction protein provides a checkpoint for the temporal co-ordination of intercellular signals in *Myxococcus xanthus* development. Mol. Microbiol. **30**: 807-817.

Fawcett, T. and S. Bartlett. 1990. An effective method for eliminating "artifact banding" when sequencing double-stranded DNA templates. Biotechniques **9**: 46-48.

Fisseha, M., D. Biran and L. Kroos. 1999. Identification of the Ω4499 regulatory region controlling developmental expression of a *Myxococcus xanthus* cytochrome P-450 system. J. Bacteriol. **181**(17): 5467-5475.

Fisseha, M., M. Gloudemans, R. Gill and L. Kroos. 1996. Characterization of the regulatory region of a cell interaction-dependent gene in *Myxococcus xanthus*. J. Bacteriol. **178**: 2539-2550.

Garza, A. G., B. Z. Harris, J. S. Pollack and M. Singer. 2000. The *asgE* locus is required for cell-cell signaling during *Myxococcus xanthus* development. Mol. Microbiol. **35**: 812-824.

Garza, A. G., J. S. Pollack, B. Z. Harris, A. Lee, I. M. Keseler, E. F. Licking and M. Singer. 1998. SdeK is required for early fruiting body development in *Myxococcus xanthus*. J. Bacteriol. **180**(17): 4628-4637.

Gill, J. S. and M. Dworkin. 1986. Cell surface antigens during submerged development of *Myxococcus xanthus* examined with monoclonal antibodies. J. Bacteriol. **168**: 505-511.

Gill, J. S., B. Jarvis and M. Dworkin. 1987. Inhibition of development in *Myxococcus xanthus* by Monoclonal Antibody 1604. Proc. Natl. Acad. Sci. USA **84**: 4505-4508.

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

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

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

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

Gorham, H., S. McGowan, P. Robson and D. Hodgson. 1996. Light-induced carotenogenesis in *Myxococcus xanthus*: light-dependent membrane sequestration of ECF sigma factor CarQ by anti-sigma factor CarR. Mol. Microbiol. **19**: 171-186.

Gorski, L., T. Gronewold and D. Kaiser. 2000. A sigma(54) activator protein necessary for spore differentiation within the fruiting body of *Myxococcus xanthus*. J. Bacteriol. **182**(9): 2438-2444.

Gross, C., F. Engbaek, T. Flammang and R. Burgess. 1976. Raped micromethod for the purification of *Escherichia coli* ribonucleic acid polymerase and the preparation of bacterial extracts active in ribonucleic acid synthsis. J. Bacteriol. **128**(1): 382-389.

Guo, D., G. Bowden and H. B. Kaplan. 1995. Analysis of the *Myxococcus xanthus sasA* locus a negative regulator of an A-signal-dependent gene. Abstr. of the 22nd Intl. Conf. on the Biol. of the Myxobacteria : 19.

Guo, D., Y. Wu and H. B. Kaplan. 2000. Identification and characterization of genes required for early *Myxococcus xanthus* developmental gene expression. J. Bacteriol. **182**(16): 4564-4571.

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

Haldenwang, W. G. 1995. The sigma factors of *Bacillus subtilis*. Microbiol. Rev. **59**: 1-30.

Hanahan, D. 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166**: 557.

Harlow, E. and D. Lane. 1988. Antibodies. New York, NY. Cold Spring Harbor.

Harris, B. Z., D. Kaiser and M. Singer. 1998. The guanosine nucleotide (p)ppGpp initiates development and A-factor production in *Myxococcus xanthus*. Genes Dev. **12**: 1022-1035.

Harrison, S. C. and A. Aggarwal. 1990. DNA recognition by proteins with the helix-turn-helix motif. Annual review of biochemistry **59**: 933-969

Hartzell, P. and D. Kaiser. 1991a. Function of MglA, a 22-kilodalton protein essential for gliding in *Myxococcus xanthus*. J. Bacteriol. **173**: 7615-7624.

Hartzell, P. and D. Kaiser. 1991b. Upstream gene of the *mgl* operon controls the level of MglA protein in *Myxococcus xanthus*. J. Bacteriol. **173**: 7625-7635.

Helmann, J. 1995. Compilation and analysis of *Bacillus subtilis* σ^A-dependent promoter sequences: evidence for extended contact between RNA polymerase and upstream promoter DNA. Nucleic Acids Res. **23**: 2351-2360.

Hillen, W. and C. Berens. 1994. Mechanisms underlying expression of Tn10 encoded tetracycline resistance. Annu. Rev. Microbiol. **48**: 345-369.

Hodgkin, J. and D. Kaiser. 1977. Cell-to-cell stimulation of motility in nonmotile mutants of *M. xanthus*. Proc. Natl. Acad. Sci. USA **74**: 2938-2942.

Hodgkin, J. and D. Kaiser. 1979a. Genetics of gliding Motility in *Myxococcus xanthus* (Myuxobacterales): Genes Controlling Movement of Single Cells. Mol. Gen. Genet. **171**: 167-176.

Hodgkin, J. and D. Kaiser. 1979b. Genetics of Gliding Motility in *Myxococcus xanthus* (Myxobacterales): Two Gene Systems Control Movement. Mol. Gen. Genet. **171**: 177-191.

Hodgson, D. and F. J. Murillo. 1993. Genetics of regulation and pathway of synthesis of carotenoids. In *Myxobacteria II*. M. Dworkin and D. Kaiser. Washington, D.C., American Society for Microbiology: 157-182.

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

Inouye, M., S. Inouye and D. R. Zusman. 1979b. Gene expression during development of *Myxococcus xanthus*: pattern of protein synthesis. Dev. Biol. **68**: 579-591.

Inouye, S. 1990. Cloning and DNA sequence of the gene coding for the major sigma factor from *Myxococcus xanthus*. J. Bacteriol. **172**: 80-85.

Inouye, S., T. Franceschini and M. Inouye. 1983a. Structural similarities between the development-specific protein S from a Gram-negative bacterium, *Myxococcus xanthus*, and calmodulin. Proc. Natl. Acad. Sci. USA **80**: 6829-6833.

Inouye, S., Y. Ike and M. Inouye. 1983b. Tandem repeat of the genes for protein S, a development-specific protein of *Myxococcus xanthus*. J. Biol. Chem. **258**: 38-40.

Ishihama, A. 1993. Protein-protein communication within the transcription apparatus. J. Bacteriol. **175**: 2483-2489.

Jacob. U, M. Mack, T. Clausen, R. Huber, W. Buckel and A. Messerschmidt. 1997. Glutaconate CoA-transferase from Acidaminococcus fermentans: the crystal structure reveals homology with other CoA-transferase. Structure **5**: 415-426.

Jelsbak, L. and L. Sogaard-Anderson. 1999. The cell surface-associated intercellular C-signal induces behavioral changes in individual *Myxococcus xanthus* cells during fruiting body morphogenesis. Proc. Natl. Acad. Sci. USA **96**: 5031-5036.

Joachimiak, A., R. C. Kelley, R. P. Gunsalus, C. Yanofsky and P. Sigler. 1983. Purification and characterization of *trp* aporepressor. Proc. Natl. Acad. Sci. USA **80**: 668-672.

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

Kaiser, D. 1999. Cell fate and organogenesis in bacteria. Trends in genetics **15**(7): 273-277.

Kaiser, D. and C. Crosby. 1983. Cell movement and its coordination in swarms of *Myxococcus xanthus*. Cell Motil. **3**: 227-245.

Kaiser, D. and L. Kroos. 1993. Intercellular signaling. In *Myxobacteria II*. M. Dworkin and D. Kaiser. Washington, DC, American Society for Microbiology: 257-284.

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

Kalos, M. and J. Zissler. 1990. Transposon tagging of genes for cell-cell interactions in *Myxococcus xanthus*. Proc. Natl. Acad. Sci. USA **87**: 8316-8320.

Keseler, I. and D. Kaiser. 1995. An early A-signal-dependent gene in *Myxococcus xanthus* has a σ^{54}-like promoter. J. Bacteriol. **177**: 4638-4644.

Keseler, I. and D. Kaiser. 1997. σ^{54}, a vital protein for *Myxococcus xanthus*. Proc. Natl. Acad. Sci. USA **94**: 1979-1984.

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

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

Kim, S. K. and D. Kaiser. 1990b. Cell motility is required for the transmission of C-factor, an intercellular signal that coordinates fruiting body morphogenesis of *Myxococcus xanthus*. Genes Dev. **4**: 896-905.

Kim, S. K. and D. Kaiser. 1990c. C-factor: a cell-cell signaling protein required for fruiting body morphogenesis of *M. xanthus*. Cell **61**: 19-26.

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

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

Komano, T., T. Franceschini and S. Inouye. 1987. Identification of a vegetative promoter in *Myxococcus xanthus*: a protein that has homology to histones. J. Mol. Biol. **196**: 517-524.

Kroos, L. and S. Cutting. 1994. Intercellular and intercompartmental communication during *Bacillus subtilis* sporulation. In *Regulation of Bacterial Differentiation*. P. J. Piggot, J. C.P. Moran and P. Youngman. Washington, DC, Amer. Soc. for Microbiol.: 155-180.

Kroos, L., P. Hartzell, K. Stephens and D. Kaiser. 1988. A link between cell movement and gene expression argues that motility is required for cell-cell signaling during fruiting body development. Genes Dev. **2**: 1677-1685.

Kroos, L. and D. Kaiser. 1984. Construction of Tn*5 lac*, a transposon that fuses *lacZ* expression to exogenous promoters, and its introduction into *Myxococcus xanthus*. Proc. Natl. Acad. Sci. USA **81**: 5816-5820.

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

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

Kroos, L., A. Kuspa and D. Kaiser. 1990. Defects in fruiting body development caused by Tn*5 lac* insertions in *Myxococcus xanthus*. J. Bacteriol. **172**: 484-487.

Kuspa, A. and D. Kaiser. 1989. Genes required for developmental signaling in *Myxococcus xanthus*: three *asg* loci. J. Bacteriol. **171**: 2762-2772.

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

Kuspa, A. and W. F. Loomis. 1992. Tagging developmental genes in Dictyostelium by restriction enzyme mediated integration of plasmid DNA. Proc. Natl. acad. Sci. USA **89**: 8803-8807.

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

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

LaRossa, R., J. Kuner, D. Hagen, C. Manoil and D. Kaiser. 1983. Developmental cell interactions of *Myxococcus xanthus*: analysis of mutants. J. Bacteriol. **153**: 1394-1404.

Laue, B. E. and R. Gill. 1994. Use of a phase variation-specific promotor of *Myxococcus xanthus* in a strategy for isolating a phase-locked mutant. J. Bacteriol. **176**: 5341-5349.

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

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

Li, S.-F., B. Lee and L. J. Shimkets. 1992. *csgA* expression entrains *Myxococcus xanthus* development. Genes Dev. **6**: 401-410.

Li, S.-F. and L. J. Shimkets. 1993. Effect of *dsp* mutations on the cell-to-cell transmission of CsgA in *Myxococcus xanthus*. J. Bacteriol. **175**: 3648-3652.

Lisser, S. and H. Margalit. 1993. Compilation of *E. coli* mRNA promoter sequences. Nucleic Acids Res. **21**: 1507-1516.

Lonetto, M., K. Brown, K. Rudd and M. Buttner. 1994. Analysis of the *Streptomyces coelicolor sigE* gene reveals the existence of a subfamily of eubacterial polymerase σ factors involved in the regulation of extracytoplasmic functions. Proc. Natl. Acad. Sci. USA **91**: 7573-7577.

Mack, M., K. bendrat, O. Zelder, E. Eckel, D. Linder and W. Buckel. 1994. Location of the two genes encoding glutaconate coenzyme A-transferase at the beginning of the hydroxyglutarate operon in Acidaminococcus fermentans. Eur. J. Biochem. **226**: 41-51.

MacNeil, S. D., F. Calara and P. Hartzell. 1994. New clusters of genes required for gliding motility in *Myxococcus xanthus*. Mol. Microbiol. **14**: 61-71.

Manoil, C. and D. Kaiser. 1980. Accumulation of guanosine tetraphosphate and guanosine pentaphosphate in *Myxococcus xanthus* during starvation and myxospore formation. J. Bacteriol. **141**: 297-304.

McBride, M. J. 1993. FrzCD, a methyl-accepting taxis protein from *Myxococcus xanthus*, shows modulated methylation during fruiting body formation. J. Bacteriol. **175**: 4936-4940.

McBride, M. J., T. Kohler and D. R. Zusman. 1992. Methylation of FrzCD, a methyl-accepting taxis protein of *Myxococcus xanthus*, is correlated with factors affecting cell behavior. J. Bacteriol. **174**(13): 4246-4257.

Messing, J. 1979. A multipurpose cloning system based on the single-stranded DNA bacteriophage M13. Recombinant DNA bulletin, Publication no. 71-99, National Institutes of Health, Bethesda, Maryland, p. 43-48. .

Ogawa, M., S. Fujitani, X. Mao, S. Inouye and T. Komano. 1996. FruA, a putative transcription factor essential for the development of *Myxococcus xanthus*. Mol. Microbiol. **22**(4): 757-767.

Oyaizu, H. and C. Woese. 1985. Phylogenetic relationships among the sulfate respiring bacteria, myxobacteria and purple bacteria. System. Appl. Microbiol. **6**: 257-263.

Persson, B., M. Krook and H. Jornvall. 1991. Characteristics of short-chain alcohol dehdrogenases and related enzymes. Eur. J. Biochem. **200**: 537-543.

Plamann, L., J. Davis, B. Cantwell and J. Mayor. 1994. Evidence that *asgB* encodes a DNA-binding protein essential for growth and development of *Myxococcus xanthus*. J. Bacteriol **176**: 2013-2022.

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

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

Rhie, H.-G. and L. J. Shimkets. 1991. Low-temperature induction of *Myxococcus xanthus* developmental gene expression in wild-type and *csgA* suppressor cells. J. Bacteriol. **173**: 2206-2211.

Rodriguez, A. M. and A. M. Spormann. 1999. Genetic and molecular analysis of *cglB*, a gene essential for single-cell gliding in *Myxococcus xanthus*. J. Bacteriol. **181**(14): 4381-4390.

Romeo, J. M. and D. R. Zusman. 1987. Cloning of the gene for myxobacterial hemagglutinin and isolation and analysis of structural gene mutations. J. Bacteriol. **169**: 3801-3808.

Romeo, J. M. and D. R. Zusman. 1991. Transcription of the myxobacterial hemagglutinin gene is mediated by a σ^{54}-like promoter and a *cis*-acting upstream regulatory region of DNA. J. Bacteriol. **173**: 2969-2976.

Rosenberg, E., K. Keller and M. Dworkin. 1977. Cell density-dependent growth of Myxococcus xanthus on casein. J. Bacteriol. **129**: 770-777.

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

Rothstein, S. and W. Reznikoff. 1981. The functional differences in the inverted repeats of Tn*5* are caused by a single base pair nonhomology. Cell **23**: 191-199.

Russo-Marie, F., M. Roederer, B. Sager, L. A. Herzenberg and D. Kaiser. 1993. β-galactosidase activity in single differentiating bacterial cells. Proc. Natl. Acad. Sci. USA **90**: 8194-8198.

Sager, B. and D. Kaiser. 1993. Spatial restriction of cellular differentiation. Genes Dev. **7**: 1645-1653.

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

Sambrook, J., E. Fritsch and T. Maniatis. 1989. Molecular Cloning: a laboratory manual. Cold Spring Harbor, New York, Cold Spring Harbor Laboratory Press.

Sanger, F., S. Nicklen and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**: 5463-5467.

Shi, W., T. Kohler and D. R. Zusman. 1993. Chemotaxis plays a role in the social behaviour of *Myxococcus xanthus*. Mol. Microbiol. **9**(3): 601-611.

Shi, W. and D. R. Zusman. 1995. The *frz* signal transduction system controls multicellular behavior in *Myxococcus xanthus*. Two-component signal transduction. J. A. Hoch and T. J. Silhavy. Washington, D. C., American Society for Microbiology: 419-430.

Shimkets, L. 1993. The myxobacterial genome. Myxobacteria II. M. Dworkin and D. Kaiser. Washington, D.C., American Society for Microbiology: 85-107.

Shimkets, L. J. 1990. Social and developmental biology of the myxobacteria. Microbiol. Rev. **54**: 473-501.

Shimkets, L. J. 1999. Intercellular signaling during fruiting-body development of *Myxococcus xanthus*. Annu. Rew. Microbiol. **53**: 525-549.

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

Shimkets, L. J., R. E. Gill and D. Kaiser. 1983. Developmental cell interactions in *Myxococcus xanthus* and the *spoC* locus. Proc. Natl. Acad. Sci. USA **80**: 1406-1410.

Shimkets, L. J. and D. Kaiser. 1982. Induction of coordinated movement of *Myxococcus xanthus* cells. J. Bacteriol. **152**: 451-461.

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

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

Sogaard-Andersen, L., F. Slack, H. Kimsey and D. Kaiser. 1996. Intercellular C-signaling in *Myxococcus xanthus* involves a branched signal transduction pathway. Genes Dev. **10**: 740-754.

Sogaard-Anderson, L. and D. Kaiser. 1996. C-factor, a cell-surface-associated intercellular signaling protein, stimulates the cytoplasmic Frz signal transduction system in *Myxococcus xanthus*. Proc. Natl. Acad. Sci. U. S. A. (93): 2675-2679.

Stellwag, E., J. M. Fink and J. Zissler. 1985. Physical characterizatin of the genome of the *Myxococcus xanthus* bacteriophage MX-8. Mol. Gen. Genet. **199**: 123-132.

Stephens, K., P. Hartzell and D. Kaiser. 1988. Gliding motility in *Myxococcus xanthus* : *mgl* locus, RNA and predicted protein products. J. Bacteriol. **171**: 819-830

Stephens, K. and D. Kaiser. 1987. Genetics of gliding motility in *Myxococcus xanthus*: molecular cloning of the *mgl* locus. Mol. Gen. Genet. **207**: 256-266.

Teintze, M., R. Thomas, T. Furuichi, M. Inouye and S. Inouye. 1985. Two homologous genes coding for spore-specific proteins are expressed at different times during development of *Myxococcus xanthus*. J. Bacteriol. **163**: 121-125.

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

Ueki, T. and S. Inouye. 1998. A new sigma factor, SigD, essential for stationary phase is also required for multicellular differentiation in *Myxococcus xanthus*. Genes Cells **3**: 371-385.

Ward, M., H. Lew, A. Treuner-Lange and D. Zusman. 1998. Regulation of motility behavior in *Myxococcus xanthus* may require an extracytoplasmic-function sigma factor. J. Bacteriol. **180**: 5668-5675.

White, O., J. A. Eisen, J. F. Heidelberg, E. K. Hickey, J. D. Peterson, R. J. Dodson, D. H. Haft, M. L. Gwinn, W. C. Nelson, D. L. Richardson et al. 1999. Genome sequence of the radioresistant bacterium *Deinococcus radiodurans* R1. Science **286**: 1571-1577.

Wu, S. S. and D. Kaiser. 1995. Genetic and functional evidence that Type IV pili are required for social gliding motility in *Myxococcus xanthus*. Mol. Microbiol. **18**(3): 547-558.

Wu, S. S. and D. Kaiser. 1997a. The *Myxococcus xanthus pilT* locus is required for social gliding motility although pili are still produced. Mol. Microbiol. **23**(1): 109-121.

Wu, S. S. and D. Kaiser. 1997b. Regulation of expression of the *pilA* gene in *Myxococcus xanthus*. J. Bacteriol. **179**(24): 7748-7758.

Xu, D., C. Yang and H. Kaplan. 1998. *Myxococcus xanthus sasN* encodes a regulator that prevents developmental gene expression during growth. J. Bacteriol. **180**(23): 6215-6223.

Yang, C. and H. B. Kaplan. 1997. *Myxococcus xanthus sasS* encodes a sensor histidine kinase required for early developmental gene expression. J. Bacteriol. **179**(24): 7759-7767.

Yang, Z., Y. Geng, D. Xu, H. B. Kaplan and W. Shi. 1998. A new set of chemotaxis homologues is essential for *Myxococcus xanthus* social motility. Mol. Microbiol. **30**(5): 1123-1130.

Yanisch-Perron, C., J. Vieria and J. Messing. 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences M13 mp18 and pUC19 vectors. Gene **33**: 103-119.