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C SIGNAL-DEPENDENT, DEVELOPMENTAL GENE EXPRESSION IN MYXOCOCCUS XANTHUS

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Monica Ellen Semancik

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Lee Kroos

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C SIGNAL-DEPENDENT, DEVELOPMENTAL GENE EXPRESSION IN *MYXOCOCCUS XANTHUS*

by

Monica Ellen Semancik

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ABSTRACT

C SIGNAL-DEPENDENT, DEVELOPMENTAL GENE EXPRESSION IN *MYXOCOCCUS XANTHUS*

by

Monica Ellen Semancik

Four classes of cell-cell interactions (A, B, C, and D) are absolutely required for starvation-induced morphogenesis and developmental gene expression in *Myxococcus xanthus*. To investigate the mechanisms which couple intercellular signaling and gene activation, DNA adjacent to previously identified C signal-dependent Tn*5lac* fusions was cloned and tested for the ability to direct developmental gene expression. A 2 kbp region adjacent to Tn*5lac* Ω4403 promotes developmental β-galactosidase expression and at least partially mediates the C signal dependence. DNA between 2 and 8.5 kbp of the Ω4403 fusion also appears to positively affect the level of expression. Accumulation of the Ω4403-associated transcript is developmentally regulated suggesting that control of transcription initiation may be one mechanism for modulating gene expression. To facilitate the identification of factors involved in C-dependent transcription, core RNA polymerase was purified from vegetative *M. xanthus*. Core RNA polymerase transcriptional activity is stimulated by both *E. coli* σ^{70} and a 50 kDa *M. xanthus* protein.

To my parents, John and Martha,

whose love, patience, and unending encouragement

are the cornerstones of my success

and to Mark

who has taught me that nothing is impossible

(except skiing through a revolving door)!

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DEPC	diethylpyrocarbonate
DTT	dithiothreitol
EDTA	(ethelyenedinitrilo)tetraacetic acid
IgG	immunoglobulin G
KIU	Kallikrein-inhibitor units
KU	Klett units
ONP	o-nitrophenol
ONPG	2-nitrophenyl-b-D-galactopyranoside
PMSF	phenylmethylsulfonyl fluoride
PVDF	polyvinylidene difluoride
SDS	sodium dodecyl sulfate
TAE	Tris-acetate-EDTA buffer
TBS	Tris buffered saline
TCA	trichloroacetic acid
Tris-HCl	2-amino-2-(hydroxymethyl)-1,3-propandiol-hydrochloride
UAS	upstream activation site

INTRODUCTION

Myxococcus xanthus is a Gram-negative prokaryote which offers a unique opportunity to study phenomena most often associated with higher multicellular organisms, particularly cell-cell interactions. Commonly found in the soil, groups of *Myxococcus* cells coordinately prey on other bacteria or feed on decomposing organic material by secreting a multitude of enzymes that degrade protein, lipid, peptidoglycan, and polysaccharide. In the laboratory, *M. xanthus* can be grown in liquid cultures or on solid agar containing hydrolyzed casein and a few salts (1). Organized groups of *Myxococcus* tend to move together in "swarms" within which cell-cell contact is maintained, although independent cells are capable of a slow, gliding movement. Perhaps most interesting to developmental biologists are the morphogenesis and social interactions which occur in response to nutrient deprivation (2).

Under conditions of amino acid starvation, approximately 10^5 individual cells participate in forming a three-dimensional fruiting body in which myxospores mature. Along with nutrient limiting conditions, a high initial cell density and a solid surface are required for development (3,4). Development proceeds through a specific sequence of events (5). Within 5 to 7 hours after the onset of starvation, cells begin to accumulate in asymmetric ridges.

Shortly thereafter as cells continue to pile on top of one another, stable, circular mounds held together by an extracellular matrix form at specific aggregation centers. As the fruiting bodies mature, cells within the structures begin to differentiate from long, rod-shaped cells into ovoid myxospores. Several coat proteins are added to the exterior of the outer membrane and provide the spore with resistance to a variety of environmental stresses including heat, radiation, detergent, and dessication (6). Only about 20% of the cells actually complete the sporulation process (3).

The differentiation of myxobacteria is unique among the sporulation processes of other bacteria in that it absolutely requires cell-cell interactions. Four classes of conditional, non-sporulating mutants (A, B, C, D) have been isolated which arrest development at particular stages. The ability to sporulate can be recovered by mixing a mutant strain from one class with either wild-type cells or mutant cells from another class, suggesting these mutants are defective in different cell-cell interactions (7). The rescue does not require the exchange of genetic material or direct contact between cells since sporulation can be recovered by the addition of wild-type cells separated from the mutants by membrane filters (8). In some cases, the addition of media "conditioned" by wild-type cells (9) or supplementation with a putative signal molecule (10) also transiently restores the ability to sporulate. Together

these results support the hypothesis that extracellular signals essential for proper development (designated Asg for A signal, Bsg, Csg, and Dsg) are exchanged via cell-cell interactions.

Additionally, correct developmental gene expression requires all of these signals. A transposon probe containing a promoterless *trp-lac* fusion, Tn5*lac*, has previously been constructed (11) and used to identify developmentally regulated genes in *M. xanthus* (12). When Tn5*lac* inserts within a transcription unit in the correct orientation, transcription of the *lacZ* gene is dependent on the native promoter and regulatory regions. Among 2374 Tn5*lac* insertion strains obtained, thirty-six strains increase developmental β-galactosidase expression at specific times (from 0-25 hours) after starvation initiates development. Surprisingly, only eight of the 2374 insertion-containing strains resulted in a defect in the aggregation or sporulation pathway suggesting that most developmentally regulated genes are not essential for development (12, 13). The expression from Tn5*lac* insertions is dependent on cell-cell interactions since many fail to show the proper levels of β-galactosidase activity in *asg, bsg, csg,* or *dsg* mutant backgrounds. (9, 14, 15). Again, the addition of wild-type cells to the mutant Tn5*lac*-containing strains restores normal patterns of gene expression.

What are the components of the signal transduction machinery and how is intercellular communication coupled to

the changes in gene expression underlying morphogenesis and differentiation? These key questions are being investigated in a number of eukaryotic, model development systems including *Drosophila melanogaster* neurogenesis (16), chick limb bud formation (17), and fruiting body formation in *Dictyostelium discoideum* (18). However, the relatively small genome size (approximately twice the size of the *E. coli* genome, 19), the availability of technology for genetic analyses (20-23), as well as the general properties of prokaryotic cells (relatively short generation times and high density populations) make *M. xanthus* particularly inviting for studying the complicated processes of development.

One approach to understanding the role of cell-cell interactions in *M. xanthus* development is the identification and purification of the signal molecules themselves. Progress has been made in mapping loci involved in the signaling pathways as well as in purifying factors that can restore proper morphogenesis to developmental mutants.

Relatively little is known about two of the signal molecules. Strains containing defective Bsg signaling systems form loosely-associated ridges that never progress to the mound stage. Sporulation is blocked and the expression of all developmental markers is reduced or completely abolished suggesting that B signal is essential early in the developmental pathway (14). One of the *bsg* loci has been cloned (*bsgA*, 24); antibodies raised against a

bsgA-lacZ fusion detect comparable levels of the *bsgA* product in both vegetative and developing cells until the time of fruiting body formation. The function of the *bsgA* product is currently unknown. *dsg* signal mutants are severely delayed in the timing of aggregation and sporulation; however, fruiting body morphogenesis is complete after 3 days and spores form by 7 days. Although early gene expression (within 4 hours after starvation) in *dsg* mutants is essentially equivalent to wild-type levels, later developmental gene expression is reduced (15). One *dsg* locus has been identified (15) and its product shown to be essential for cell viability (26).

Asg mutants are blocked early in development (8); they fail to form tight aggregates, do not sporulate, and exhibit altered patterns of expression for genes activated within 1 to 2 hours after the onset of sporulation (9). Mutations in one of three different loci (*asgA, asgB, asgC*) appear to be responsible for the developmental defects (27). The addition of media "conditioned" by wild-type cells rescues both gene expression and morphogenesis at the time when *asg* mutants originally arrest their development (9). Media "conditioned" by *asgA, asgB,* and *asgC* mutants contains only 5% the wild-type level of A factor which suggests that these mutants are defective in their ability to produce, release, or modify sufficient amounts of A factor. Further purification of A-factor is currently underway.

Characterization of another signal molecule, C signal, has advanced rapidly in the past five years. In contrast to the tight aggregates formed by developing wild-type cells (6 to 8 hours after starvation), only loose ridges and mounds are assembled in *csg* mutant strains and their formation is delayed until about 18 hours after nutrient depletion. Eventually, cells in the loose aggregates disperse and never complete the sporulation process (14). Genes normally activated between 6 to 30 hours into development also exhibit either reduced or abolished expression in *csg* mutant strains. All *csg* mutations map to one locus (*csgA*, 21, 28).

Recently, a 17 kDa polypeptide capable of restoring normal aggregation and sporulation to *csgA* mutants has been purified from wild-type *M. xanthus* (10) and has subsequently been shown to be the product of the *csgA* gene (10, 28). It appears that this molecule (called C-factor) possesses several thresholds of activity (29). At low concentrations, C-factor is incapable of rescuing development or gene expression; however, at intermediate concentrations, aggregation is restored as well as early C-dependent gene expression. At higher concentrations (1 to 2 nM), both early and late developmental gene expression are activated and sporulation occurs. C-factor also positively affects its own expression. Both cell motility and proper spatial orientation during development are necessary for transmission of C factor (30, 31). Additionally, C-factor appears to be tightly associated with membranes suggesting

it interacts via receptors at the cell surface (10). Identification of the receptor(s) or other targets of C signal will provide a more complete picture of the initial steps in this developmental signal transduction pathway.

Another approach to understanding the connection between signaling and gene activation requires examining the molecular mechanisms directly involved in modulating signal-dependent gene expression. By analogy to other prokaryotic systems, regulation of transcription initiation is likely to be one mechanism coordinating the complex patterns of gene activation observed during *M. xanthus* development. In *Bacillus subtilis*, the sequential expression of σ factors and other DNA binding proteins which modify, enhance, or inhibit RNA polymerase activity plays a critical role in organizing the program of endospore development (32, 33). Similarly, the tight spatial and temporal regulation of flagellum biosynthesis in *Caulobacter crescentus* is controlled by a cascade of trans-acting factors (34, 35). Characterization of the regulatory and promoter sequences of several signal-dependent genes should provide clues about the requirements for trans-acting factors during *Myxococcus* developmental gene expression. Purification of these factors followed by *in vitro* biochemical and *in vivo* mutational and genetic analyses will reveal their roles in mediating cell-cell signalling.

Investigations into the mechanisms of C signal-dependent gene expression have been the focus of this thesis. Since the C signaling system is the best characterized, knowledge about the nature of the C signal itself as well as the components it directly interacts with may suggest specific mechanisms that link cell-cell interactions and gene activation. Also, the availability of the signal components will facilitate the testing of possible models. Chapter 1 describes attempts to clone and characterize the promoter and regulatory regions adjacent to two previously described C-dependent Tn*5lac* fusions (12, 14). The upstream regions were tested for the ability to direct developmental gene expression in the presence and absence of C signal. Additionally, the approximate start site of one of the insertion-associated transcripts was located and its levels throughout development were measured. Chapter 2 discusses experiments aimed at characterizing *M. xanthus* core and holoenzyme RNA polymerase. *In vitro* reconstitution of C-dependent transcription with core polymerase and other purified developmental factors will firmly establish the requirements for differentially-regulated gene expression. Vegetative core polymerase was partially purified and tested for the ability to be stimulated by *Escherichia coli* σ^{70} and *M. xanthus* proteins. Holoenzyme transcriptional activity was assayed on templates from several bacteria. The experiments documented in this thesis provide a starting point for understanding the

components involved in producing developmental, C-dependent gene expression.

CHAPTER 1

CHARACTERIZATION OF C-DEPENDENT REGULATORY REGIONS IN *MYXOCOCCUS XANTHUS*

Underlying the exquisite morphological development of *M. xanthus* is a strictly regulated program of gene expression (12, 14). Cell-cell communication is essential for allowing the complete differentiation of vegetative, rod-shaped cells into mature myxospores since mutants defective in producing any one of the four signals (A, B, C, or D) fail to complete the developmental process (7). Previous studies using a promoterless Tn5*lac* transposon have identified at least 29 different transcriptional units that are activated at specific times throughout development (12). By moving many of the developmental fusions into signal-deficient mutants, the dependence of each of the insertions on the four signaling interactions has been examined (14). Fusions that require the exchange of a particular signal for their expression have reduced (partial requirement for the signal) or abolished (absolute requirement for the signal) β-galactosidase activity in the mutant strain. Using information about both the timing of expression and signal dependence of the Tn5*lac* insertions, a developmental map was constructed (Figure 1, [14]). The site of the Tn5*lac* insertion is designated by Ω followed by a number. An interesting observation apparent in this diagram is that

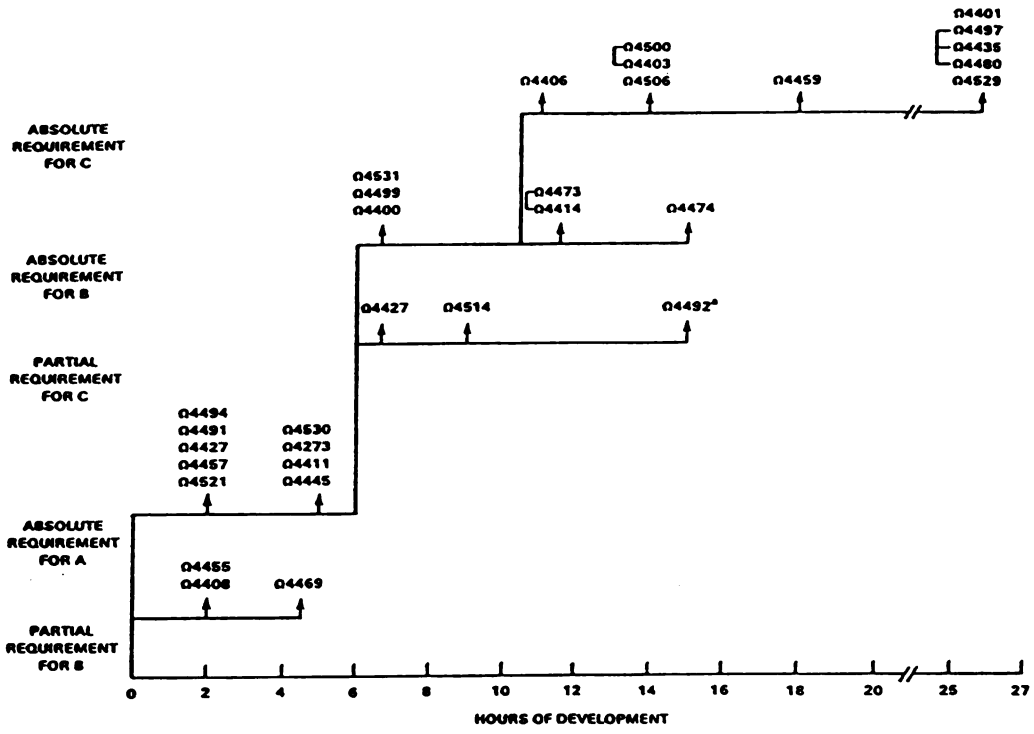


Figure 1. **Dependence of gene expression on the A, B, and C signals.** Several developmentally-regulated Tn5*lac* insertions were transduced into signal-deficient backgrounds. This diagram summarizes the effects of the signal mutations on β-galactosidase expression. The Tn5*lac* insertions are placed along the x-axis according to the time at which they normally begin to be expressed in wild-type cells; they are placed along the y-axis according to their dependence on each of the signals. A partial requirement indicates that β-galactosidase expression is reduced in the signal mutant while an absolute requirement indicates abolished expression in the mutant. Tn5*lac* insertions with similar signal dependence patterns are placed on the same horizontal line. Reprinted with permission of the author (14).

several fusions that are activated at approximately the same time after nutrient depletion have different signal requirements (compare Ω4492, Ω4474, and Ω4403 at 14 hours after the start of development). By comparing classes of insertions with respect to their regulatory regions and protein components necessary for differential expression, particularly those activated at these signal-dependence "transition points", insight into the molecular events coordinating cell-cell interactions and developmental gene activation will be gained.

Initial studies have focused on cloning C-dependent promoter regions. In particular, this chapter examines the regulatory regions of Ω4403 and Ω4414. These insertions are strongly regulated; that is, vegetative levels of β-galactosidase expression are increased 10- to 50-fold during development. The significant change between basal and developmental activity offers a fairly large window within which background and true signal can be discriminated. Additionally, both of these fusions are activated at or near the time when C signal becomes absolutely required for continued development and production of β-galactosidase, suggesting a more direct link between the initial signaling event and increased gene expression. DNA adjacent to each of these insertions was cloned and analyzed for the ability to direct developmental β-galactosidase expression. Regions that exhibited promoter activity were subcloned to further define their functional boundaries. An approximate start

site for the Ω4403-associated transcript was located and levels of this transcript were quantified throughout development. Finally, the cloned regulatory regions were examined for their roles in mediating the C signal dependence of gene expression.

MATERIALS AND METHODS

Bacterial strains and plasmids

The strains and plasmids used in this study are listed in Table 1. DK1622 (36) is the wild-type strain. The coliphage P1 *clr-100 cam* is a chloramphenicol resistant, temperature-sensitive, inducible variant of P1 (37).

Growth conditions

Myxococcus vegetative cells were grown at 30°C with vigorous shaking in CTT liquid (1% Casitone [Difco Laboratories], 10 mM Tris-HCl [pH 8.0], 1 mM KPO_4 [pH 7.6], 8 mM $MgSO_4$; final pH 7.6) or on CTT agar (CTT liquid with 1.5% Bacto-agar [Difco Laboratories]). Growth media was supplemented with kanamycin sulfate (40 μg/ml) or oxytetracycline (12.5 μg/ml [Sigma Chemical Co.]), when appropriate. Developmental *M. xanthus* was spotted on TPM starvation agar (10 mM Tris-HCl, 1 mM KPO_4 [pH 7.6], 8 mM $MgSO_4$, 1.5% Bacto-agar) as previously described (12). *E. coli* cells were grown at 37°C in LB liquid (1% tryptone, 0.5% yeast extract [Difco Laboratories], 0.5% NaCl) or on LB agar (LB liquid with 1.2% Bacto-agar) containing ampicillin (50 μg/ml) or kanamycin sulfate (25 μg/ml) when appropriate.

Cloning strategy and plasmid construction

Standard cloning procedures were used as described by Maniatis et al. (45). All fragments used in cloning were

Table 1. Bacterial strains and plasmids.

Strain or plasmid	Description	Source
E. coli		
DH5α	*supE*44 Δ*lacU*169 (ø80 *lacZ* ΔM15) *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1	(38)
JM83	*ara* Δ*lac-pro strA thi* ø*dlacZ* ΔM15	(39)
MC1061	*hsdR mcrB araD*139 Δ(*araABC-leu*)7679 Δ*lacX*74 *galU galK rpsL thiL*	(40)
plasmids		
pLJS60	Ap^r	(41)
pREG1175	Km^r Ap^r	(25)
pREG1666	Km^r Ap^r	(42)
pMES001	Km^r Ap^r, 10.9 kbp *SalI* fragment of DK5279 ligated into pUC19	This study
pMES002	Km^r Ap^r, 10.9 kbp *SalI* fragment of pMES001 ligated into pLJS60	This study
pMES003	Km^r Ap^r, 16.7 kbp *XhoI* fragment of DK4368 ligated into pGEM7Zf	This study
pMES004	Km^r Ap^r, 8.5 kbp *XhoI-BamHI* fragment of pMES003 ligated into pREG1666	This study
pMES108	Km^r Ap^r, 8.5 kbp *XhoI-BamHI* fragment of pMES003 ligated into pREG1175	This study
pMES110	Km^r Ap^r, 4 kbp *ClaI-BamHI* fragment of pMES003 ligated into pGEM7Zf	This study
pMES111	Km^r Ap^r, 4.5 kbp *XhoI-ClaI* fragment of pMES003 ligated into pGEM7Zf	This study
pMES112	Km^r Ap^r, 2 kbp *PstI-BamHI* fragment of pMES003 ligated into pGEM7Zf	This study
pMES114	Km^r Ap^r, 4.5 kbp *XhoI-BamHI* fragment of pMES111 ligated into pREG1666	This study
pMES115	Km^r Ap^r, 2 kbp *HindIII-BamHI* fragment of pMES112 ligated into pREG1666	This study

(Table 1 cont.)

Strain or plasmid	Description	Source
pMES116	Km^r Ap^r, 4 kbp *XhoI-BamHI* fragment of pMES110 ligated into pREG1666	This study
M. xanthus		
DK1622	wild type	(36)
DK4368	Km^r, Tn*5*lac Ω4403	(12)
DK5208	Tc^r, *csgA*	(43)
DK5270	Km^r Tc^r, Tn*5lac* Ω4403, *csgA*	(14)
DK5279	Km^r, Tn*5lac* Ω4414	(12)
DZF1	wild type	(85)
JW103	P1(pREG1666) x DK1622[a] to Km^r	J. White unpublished
LK6 LK7	P1(pMES002) x DK1622 to Km^r	L. Kroos unpublished
MES005 MES008 MES012	P1(pMES004) x DK1622 to Km^r	This study
MES014 MES016 MES017 MES022 MES024	P1(pMES108) x DK1622 to Km^r	This study
MES034 MES036 MES039 MES040 MES047	P1(pMES114) x DK1622 to Km^r	This study
MES053 MES064 MES065 MES068 MES077	P1(pMES115) x DK1622 to Km^r	This study

(Table 1 cont.)

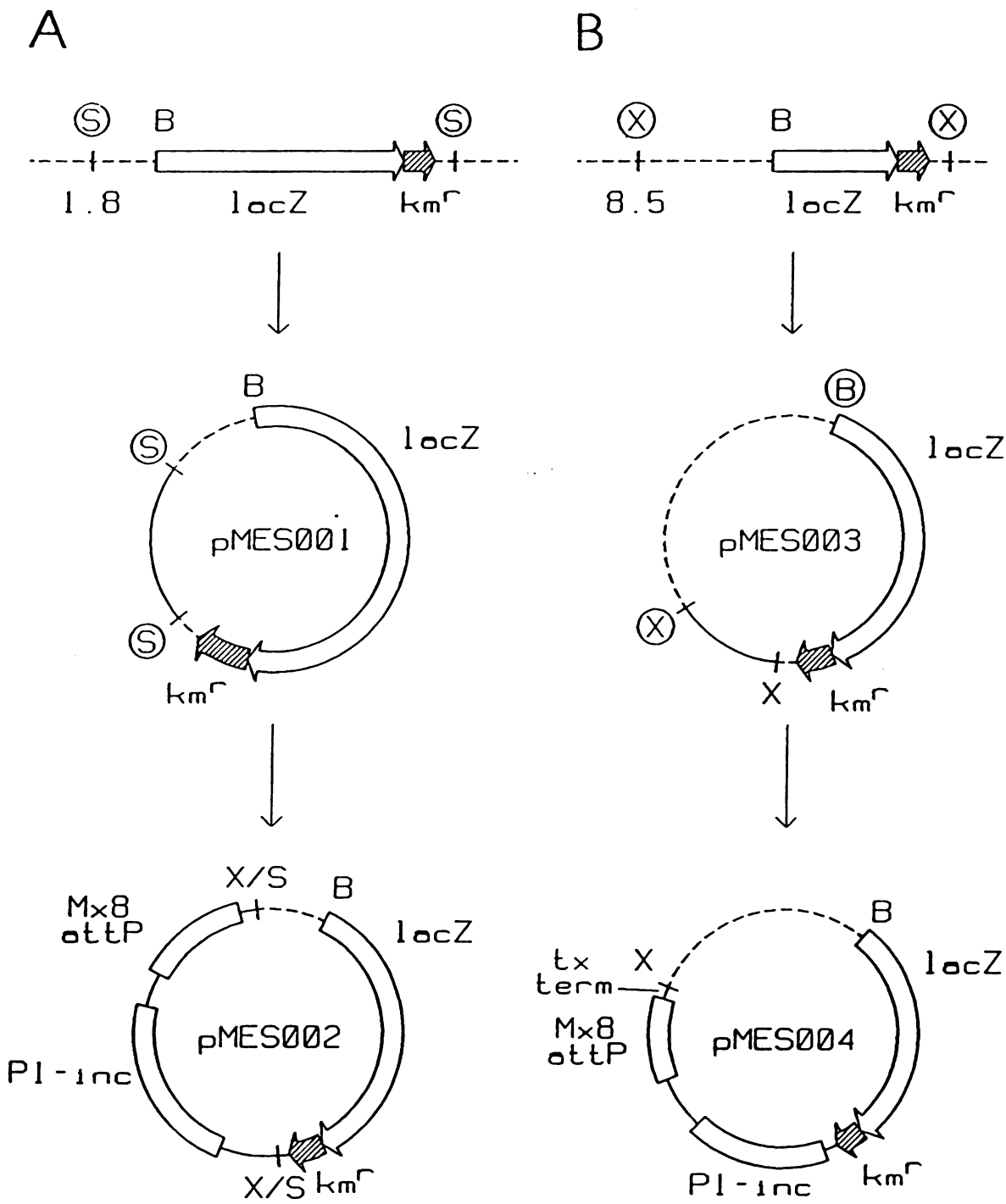
Strain or plasmid	Description			Source
MES079 MES083 MES094 MES097 MES100	P1(pMES116) x DK1622	to	Km^r	This study
MES107 MES117 MES119	P1(pREG1666) x DK5208	to	Km^r Tc^r	This study
MES123 MES124	P1(pMES004) x DK5208	to	Km^r Tc^r	This study
MES137 MES141 MES149 MES155	P1(pMES115) x DK5208	to	Km^r Tc^r	This study

[a] Constructions are described in an abbreviated form. For MES107, a P1 *clr-100 cam* phage stock was propagated on an *E. coli* strain carrying pREG1666 and was used to infect DK5208. Transductants were selected for both Km^r and Tc^r.

gel-purified from low melting point agarose (Sea-Plaque, FMC) or were directly ligated with vector in the remelted gel. The structure of all constructs was verified by restriction mapping. Restriction enzymes were obtained from Boehringer Mannheim Biochemicals, Betheseda Research Laboratories, New England Biolabs, or Promega Biological Research Products). DNA adjacent to developmentally regulated Tn5*lac* insertions was cloned using previously described restrictions maps of the upstream DNA (12). Chromosomal DNA from DK5279 (Ω4414) was digested with *SalI*, ligated into *SalI*-digested pUC19 and used to transform *E. coli* DH5α (Figure 2A). Chromosomal DNA from DK4368 (Ω4403) was digested with *XhoI*, ligated into *XhoI*-digested pGEM7Zf (Promega) and also used to transform *E. coli* DH5α (Figure 2B). Both *SalI* and *XhoI* cut immediately downstream of the kanamycin resistance gene in Tn5*lac* and upstream of the insertion site; hence, desired clones confer kanamycin resistance to the *E. coli* host. Plasmid pMES001 contains a 10.9 kbp *SalI* fragment of DK5279 DNA while plasmid pMES003 contains a 16.7 kbp *XhoI* fragment of DK4368 DNA.

The upstream regions were subcloned into appropriate shuttle vectors for transfer to *Myxococcus*. The vectors pLJS60 (41) and pREG1666 (42) are pBR322-derived plasmids that have several features useful for analyzing DNA adjacent to Tn5*lac* insertions. Both plasmids contain a P1 incompatibility fragment which allows their packaging into P1 specialized transducing particles for efficient

Figure 2. **Cloning strategy for isolating DNA adjacent to developmentally-regulated Tn5*lac* fusions.** (A) Chromosomal DNA from the Ω4414 containing-strain was digested with *SalI* and cloned into *SalI*-digested pUC19 to generate pMES001. The 10.9 kbp *SalI* fragment containing Ω4414 upstream DNA fused to *lacZ* as well as the kanamycin resistance gene was transferred to *XhoI*-digested shuttle vector (pLJS60) to construct pMES002. (B) Chromosomal DNA from the Ω4403-containing strain was digested with *XhoI* and cloned into *XhoI*-digested pGEM7Zf to generate pMES003. The 8.5 kbp *XhoI-BamHI* fragment containing the Ω4403 upstream DNA was fused to *lacZ* in *XhoI-BamHI*-digested shuttle vector (pREG1666) to construct pMES004. Restriction sites are indicated by abbreviations: *XhoI* (X), *BamHI* (B), and *SalI* (S); numbers under the restriction sites indicate the distance in kbp to the *BamHI* site near the 5' end of Tn5*lac*. The Mx8 phage attachment site (Mx8 *attP*), P1-incompatibility fragment (P1-inc), and rho-independent terminators (tx term) are indicated on the shuttle vectors.


A
B
S
B
S
1.8
lacZ
km
B
lacZ
S
pMES001
S
km
X/S
B
Mx8
attP
lacZ
pMES002
PI-inc
X/S
km
B
X
B
8.5
lacZ
km
B
lacZ
pMES003
X
X
km
B
tx
term
X
lacZ
Mx8
attP
pMES004
PI-inc
km

transduction into *M. xanthus* (21). These vectors also have the mxyophage Mx8 attachment site (*attP,* 46) which permits their integration at a specific chromosomal location (Figure 3B). Additionally, pREG1666 includes the kanamycin resistance gene, a promoterless *lacZ* gene (identical to that in Tn5*lac*) downstream from several unique restriction sites, and rho-independent transcription terminators upstream of the multiple cloning site. Plasmid pMES001 was digested with *SalI* and a 10.9 kbp gel-purified fragment was ligated into *XhoI*-digested pLJS60 to form pMES002. Likewise, an 8.5 kbp gel-purified *XhoI-BamHI* fragment from pMES003 was ligated into *XhoI-BamHI*-digested pREG1666 to generate pMES004.

A more detailed restriction map of the Ω4403 upstream region was generated (Figure 6). Plasmid pMES114 was constructed by subcloning a 4.5 kbp *XhoI-ClaI* fragment of pMES003 into *XhoI-ClaI*-digested pGEM7Zf to generate pMES111 and then transferring a similar-sized *XhoI-BamHI* fragment of pMES111 into *XhoI-BamHI*-digested pREG1666. Plasmid pMES115 was generated by subcloning a 2 kbp *PstI-BamHI* fragment of pMES003 into *PstI-BamHI*-digested pUC19 to create pMES112 and then moving a similar-sized *HindIII-BamHI* fragment from pMES112 into *HindIII-BamHI*-digested pREG1666. Plasmid pMES116 was obtained by ligating a 4 kbp *ClaI-BamHI* fragment of pMES003 into *ClaI-BamHI* digested pGEM7Zf to generate pMES110 and then subcloning a similar-sized *XhoI-BamHI* fragment from pMES110 into *XhoI-BamHI*-digested pREG1666.

Plasmid pMES108 was constructed by joining the 8.5 kbp *XhoI-BamHI* fragment of pMES003 with *XhoI-BamHI* digested pREG1175 (25), a plasmid similar to pREG1666 but lacking the transcription terminators and Mx8 *attP* site thus forcing the plasmid to integrate into the chromosome by homologous recombination (Figure 3A).

Transfer of plasmid DNA from *E. coli* to *M. xanthus*

Specialized P1 transducing particles were constructed as previously described (21, 24, 47) Coliphage P1 *clr 100 cam* was grown on the *rec*$^+$ *E. coli* JM83 or MC1061 strains containing either pLJS60, pREG1175, or pREG1666 derivatives. Phage were preadsorbed for 20 minutes with a multiplicity of infection of 0.1 or 1. The cells were diluted 20-fold into LB liquid, shaken at 32°C for 1 hour, and kanamycin (25 μg/ml) and chloramphenicol (12.5 μg/ml) were subsequently added. Growth continued overnight at 32°C.

The overnight lysogens were diluted 20-fold into LB liquid containing 20 mM $MgCl_2$, 5 mM $CaCl_2$, 25 μg/ml kanamycin, and 12.5 μg/ml chloramphenicol and grown at 32°C with shaking to approximately 2.5 x 10^8 cells/ml. After the addition of 0.2% glucose, lysis was induced by shifting the cultures to 42°C for 30 minutes followed by further incubation at 37°C for 30 minutes. Chloroform was added to complete the lysis and cell debris was pelleted by centrifugation in an SS34 rotor (10,000 rpm, 10 minutes).

Exponentially growing DK1622 cells (5 x 10^8) were mixed at room temperature with 10 µl or 100 µl of the P1-lysate. After 15 minutes, the cells were added to CTT soft agar (CTT liquid with 0.7% Bacto-agar) and plated onto CTT agar containing 40 µg/ml kanamycin. Plates were incubated at 30°C for 3-5 days.

Southern analysis

The chromosomal structures of all transductants obtained in this study were verified by Southern blot analysis (48).

β-galactosidase assays

The timing and level of β-galactosidase expression during development were determined as described in Kroos and Kaiser (12). Cells were disrupted by sonicating for three 10-second intervals with cooling on ice in between. After pelleting cell debris by centrifugation in an Eppendorf microfuge (14,000 rpm, 1 minute), an aliquot of each sample (100 µl) was added to 0.4 ml of Z buffer containing 1 mg/ml ONPG. Reactions were incubated at 37°C for 1 to 3 hours and stopped by the addition of 1 M Na_2CO_3 (0.5 ml). The absorbance at 415 nm of each assay (350 µl) was measured against a blank prepared and incubated identically except lacking ONPG using a Bio-Tek Instruments MicroPlate Autoreader.

Protein concentrations were obtained using a Bradford assay (50) according to the manufacturer's specifications (Bio-Rad Chemical Company). Bovine IgG was utilized as a protein standard. Specific activity was calculated as described in Kroos and Kaiser (12).

RNA isolation

Vegetative *M. xanthus* cells were grown in 50 ml of CTT liquid to a density of 7.5 x 10^8 cells/ml (150 KU) and harvested by centrifugation in an SS-34 rotor (10,000 rpm, 10 minutes). Developmental cells were obtained by growing *Myxococcus* in 1 liter of CTT liquid to 4 x 10^8 cells/ml (80 KU), pelleting the cells by spinning in an SS34 rotor (10,000 rpm, 10 minutes), resuspending them in TPM liquid to 5 x 10^9 cells/ml and plating 2-ml aliquots of the cell suspension onto TPM agar (dried overnight at 37°C and incubated an additional 3 hours at 37°C with the lids slightly ajar). An initial 12-ml sample was saved and stored at -70°C. Additional samples were collected at 6- or 12-hour intervals by scraping the cells from 6 plates, resuspending them in 12 ml of TPM liquid, and storing them at -70°C until all samples were obtained.

Total RNA was prepared as described by Igo and Losick (51) and was resuspended in DEPC-treated water. Samples were digested with DNAse (RNAse-free, Boehringer Mannheim Biochemicals) for 1 hour at 37°C to remove contaminating DNA. The concentration and yield were determined by

measuring the absorbance at 260 nm; one absorbance unit at 260 nm is equivalent to 40 μg/ml RNA. Samples were stored at -20°C.

S1-nuclease protection experiments

Low-resolution mapping of the 5' end of the Ω4403-associated transcript was performed as described in Burton et al. (52). Hybridization buffer, S1-nuclease mapping buffer, stop solution, and formamide loading buffer were prepared as described by Maniatis et al (45). The mapping strategy was designed to determine whether the start site for transcription was located between the *SalI* and *BamHI* sites (0 to 1 kbp) or the *PstI* and *SalI* sites (1 to 2 kbp) upstream of the Ω4403 insertion (Figure 9A). Plasmid pMES112, a pUC19 derivative containing the 2 kbp *PstI-BamHI* segment of DNA adjacent to the Ω4403 fusion, was digested with *BamHI* or *SalI*, 5' end-labelled with ^{32}P-γATP and used as probe. A third probe was generated by digesting a portion of the *BamHI*-end-labelled plasmid with *SalI*. Developmental or vegetative RNA (20 to 50 μg) was precipitated with probe (0.5 μg) and the pellet was resuspended in 10 μl of hybridization buffer. After denaturing the nucleic acids at 85°C for 10 minutes, the samples were incubated at 53°C for 16 hours. Unhybridized, single-stranded DNA and RNA was digested with S1-nuclease (Boehringer Mannheim Biochemicals, 25 to 250 units in 100 μl S1-nuclease mapping buffer) for 1 hour at 37°C. The

reaction was stopped by the addition of 40 µl of stop solution and was extracted with phenol-$CHCl_3$ (150 µl). The samples were precipitated with EtOH, resuspended in formamide loading buffer, and the entire sample was loaded onto a 5% polyacrylamide-8M urea gel. The protected products were separated by electrophoresis at 25 mA and visualized by autoradiography.

For quantitative S1-mapping experiments, the procedure of Chamberlin and Gilman (53) was used. Yeast tRNA was added to some samples to maintain a constant total amount of input RNA (50 µg). *BamHI*-digested pMES112 was 5' end-labelled and used as probe (0.5 µg). Hybridization, S1-nuclease digestion, and polyacrylamide electrophoresis conditions were identical to those described above. The protected products were quantified using a Kodak Bio-Image densitometer.

RESULTS

To begin to characterize the C-dependent regulatory regions of *M. xanthus*, DNA adjacent to previously identified Tn5*lac* insertions (12) was isolated and subsequently tested for the ability to direct developmental gene expression. Chromosomal DNA from *Myxococcus* strains containing Tn5*lac* insertions was digested with a restriction enzyme that cut both downstream of the kanamycin resistance gene in Tn5*lac* and somewhere upstream of the point of insertion (Figure 2). The generated fragments were cloned into pGEM7Zf (a pUC19-based plasmid) and after transformation into *E. coli*, clones containing both *Myxococcus* DNA and portions of Tn5*lac* were selected on the basis of their kanamycin resistance. Putative regulatory regions still fused to the *lacZ* gene were subcloned into a shuttle vector (pLJS60). Alternatively, the regulatory regions alone were fused to a promoterless *lacZ* gene (identical to the *lacZ* fragment in Tn5*lac*) contained on a similar shuttle vector (pREG1666).

P1-specialized transducing particles were used to efficiently transfer the constructs containing putative regulatory regions into wild-type *Myxococcus*. Whereas P1 DNA is rapidly degraded in *Myxococcus*, the pLJS60- and pREG1666-derivatives are stably maintained after integration into the bacterial chromosome (21). Because the putative

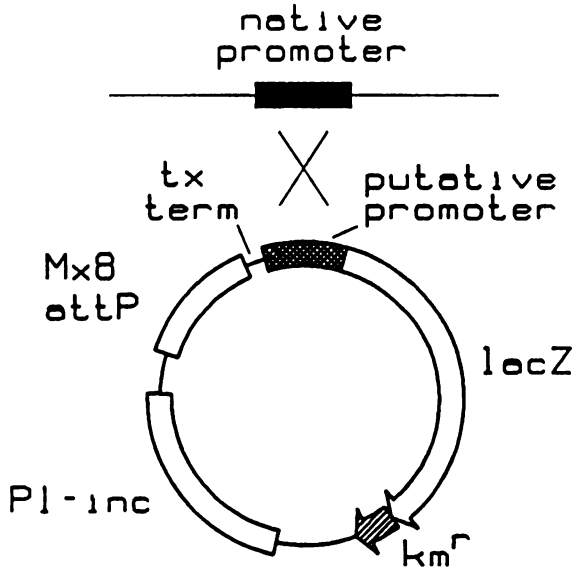
regulatory regions are homologous to native sequences in the *Myxococcus* chromosome, recombination between the two regions is possible (Figure 3A). Since a homologous recombination event fuses the *lacZ* marker gene to all of the native regulatory sequences, integrants of this type are not useful in assessing the functionality of the region of interest. However, because the vectors contain the myxophage Mx8 *attP* site (analagous to the *attP* site of coliphage lambda), 99% of the integration events occur by a site-specific mechanism (Figure 3B, 41). In a site-specific integrant, the putative regulatory regions remain fused to the *lacZ* gene and are located at a separate position from the native promoter. The structures of all transductants generated in this study were verified by Southern analysis (data not shown).

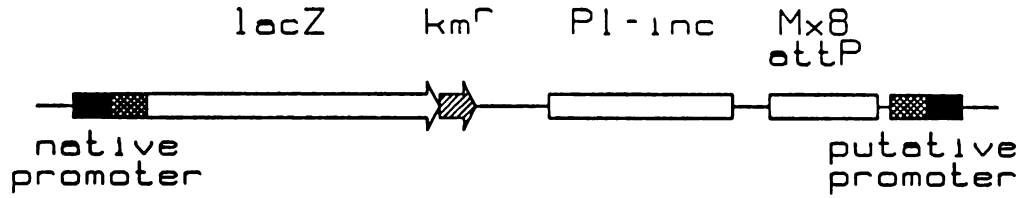
A 1.8 kbp Ω4414 upstream region is insufficient to direct proper developmental gene expression.

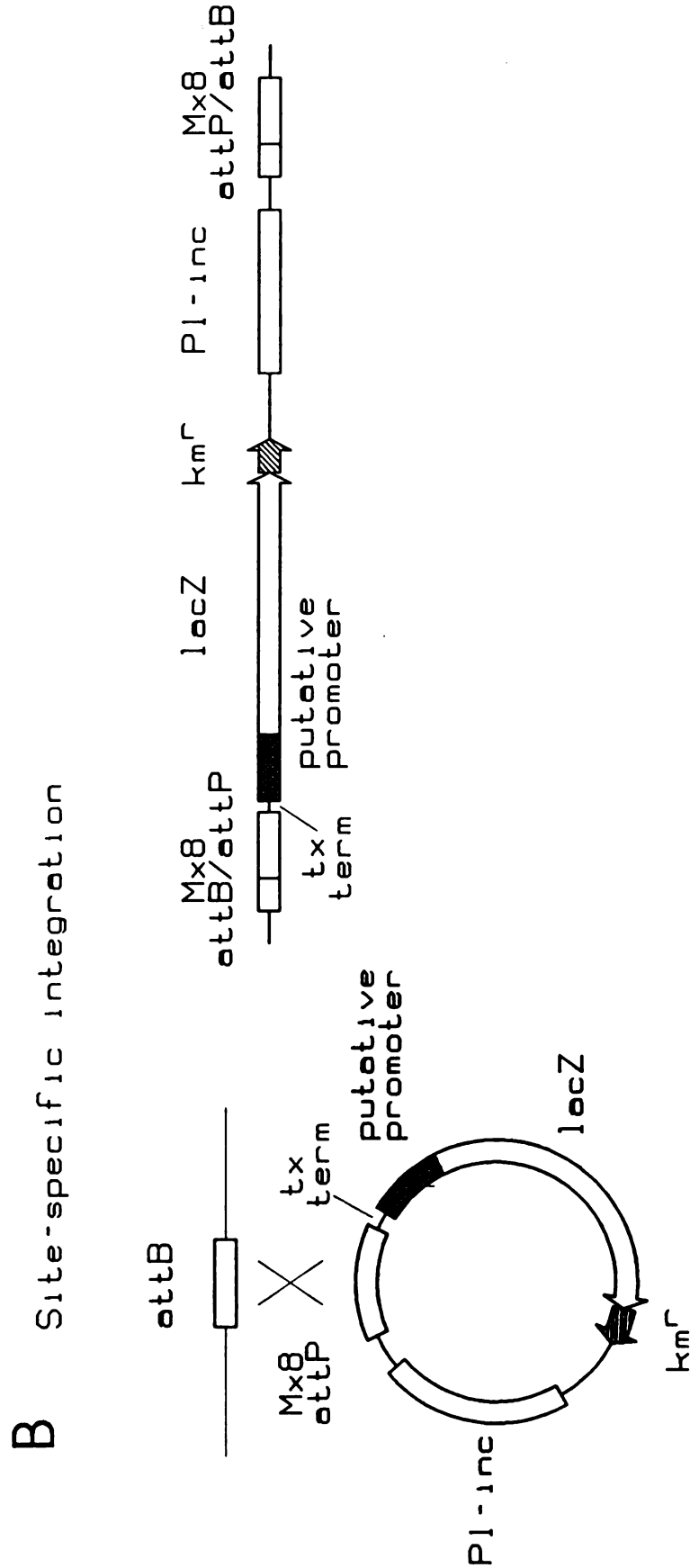
Using the cloning strategy described above, 1.8 kbp of DNA upstream of the Ω4414 insertion was fused to a promoterless *lacZ* gene (pMES002) and placed back into wild-type *Myxococcus*. Figure 4 shows the development-specific β-galactosidase expression of several strains. β-galactosidase activity dramatically increases throughout development in the strain containing the original Ω4414 fusion (DK5279). In contrast, the wild-type strain (DK1622) lacks development-specific β-galatosidase expression. The average specific activity during development of two independently-

Figure 3. **Chromosomal structures resulting from homologous and site-specific integration events.** (A) A homologous recombination event fuses *lacZ* to the native promoter region while the putative promoter is located downstream of the marker gene. (B) A site specific recombination event integrates the putative promoter-*lacZ* fusion at the Mx8 *attB* site (a chromosomal location separate from the native promoter).

A Homologous integration






B
Site-specific integration
attB
Mx8 attP
tx term
putative promoter
lacZ
kmr
Pl-inc
Mx8 attB/attP
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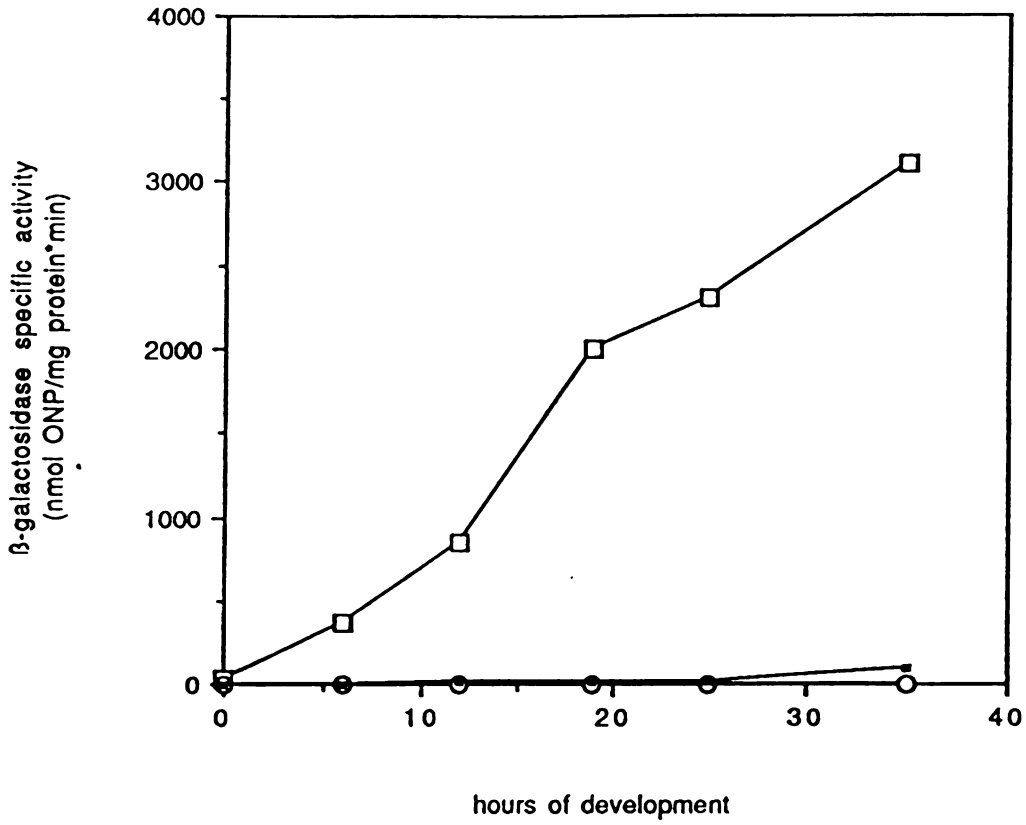


Figure 4. **Developmental β-galactosidase expression from *lacZ* fused to the putative regulatory region upstream of Ω4414.** A promoterless *lacZ* gene was fused to 1.8 kbp of DNA located immediately upstream from the Ω4414 insertion (pMES002) and introduced into wild type *M. xanthus* at the Mx8 phage attachment site. β-galactosidase expression was measured in cells harvested at various times during their development on TPM starvation agar as described in the Materials and Methods. The specific activity of the wild type strain (DK1622; ○—○), the original Ω4414 fusion strain (DK5279; □—□), and the average specific activity of two independently-isolated transductants containing a single copy of pMES002 integrated at the Mx8 *attB* site (■—■). Error bars showing one standard deviation are too small to be seen.

isolated transductants containing a single copy of pMES002 integrated at the Mx8 *attB* site clearly resembles the pattern seen in DK1622; only a basal level of expression is observed. This result indicates that 1.8 kbp of DNA upstream of Ω4414 is insufficient to direct developmental gene expression and implies that the promoter and possibly important regulatory regions lie further upstream.

Developmental gene expression can be directed by DNA within 2 kbp of the Ω4403 insertion site.

An 8.5 kbp segment of DNA adjacent to the Ω4403 insertion was cloned (pMES004) and reintroduced into wild-type *M. xanthus*. As illustrated in Figure 5, β-galactosidase activity starts to increase between 6 and 12 hours after the onset of starvation in the original Ω4403 fusion strain (DK4368). Similarly, the average β-galactosidase activity of 3 independently-isolated transductants containing a single copy of pMES004 integrated at the Mx8 *attB* site increases during the same time interval, although the maximum level of expression for these transductants is only 25% of that observed for DK4368. These results suggest that at least some of the regulatory regions required for developmental gene expression are located within 8.5 kbp of the Ω4403 insertion point.

In order to more closely define the regions within the 8.5 kbp of upstream DNA responsible for directing β-galactosidase expression, portions of this segment were

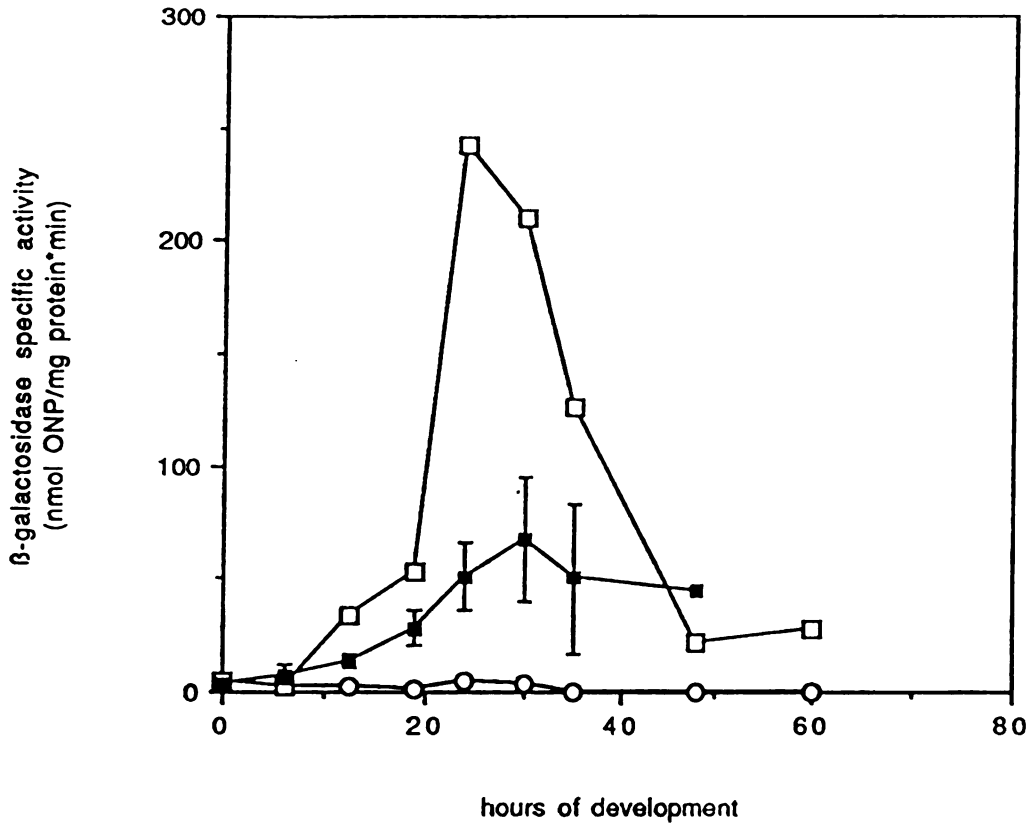


Figure 5. **Developmental β-galactosidase expression from *lacZ* fused to the putative regulatory region upstream of Ω4403.** A promoterless *lacZ* gene was fused to 8.5 kbp of DNA located immediately upstream from the Ω4403 insertion (pMES004) and introduced into wild type *M. xanthus* at the Mx8 phage attachment site. β-galactosidase expression was measured in cells harvested at various times during their development on TPM starvation agar as described in the Materials and Methods. The specific activity of the wild type strain (DK1622; ○—○), the original Ω4403 fusion strain (DK4368; □—□), and the average specific activity of three independently-isolated transductants containing a single copy of pMES004 integrated at the Mx8 *attB* site (■—■). Error bars show one standard deviation.

fused to *lacZ* (Figure 6) and integrated into wild-type *M. xanthus* at the *attB* site. Strains containing 2, 4, or 8.5 kbp of DNA immediately upstream of the Ω4403 fusion or 4.5 kbp of DNA normally separated by 4 kbp of sequence from the insertion were examined for developmentally regulated β-galactosidase expression; the results are presented in Figure 7. Only 2 kbp of DNA adjacent to Ω4403 is required to direct developmental β-galactosidase expression. This 2 kbp segment appears to be responsible for 60% of the β-galactosidase activity driven by the 8.5 kbp region; fusion of an additional 2 kbp of upstream DNA to *lacZ* increases the level of expression to 75% the level observed with the 8.5 kbp segment. Transcription from promoters near the *attB* site is not responsible for the β-galactosidase activity as strains containing the vector alone (pREG1666) produce only a low basal level of activity (54, also see Figure 11B). This same basal level of activity is observed in strains containing 4.5 kbp of DNA normally separated from the Ω4403 insertion by intervening sequences (Figure 7) demonstrating that the developmental gene expression is dependent specifically on the 2 kbp region immediately adjacent to Ω4403. This data suggests that the start site for transcription lies within the same 2 kbp of DNA. Additionally, the region between 2 and 8.5 kbp upstream of the Ω4403 insertion point has a role in regulating the level of expression.

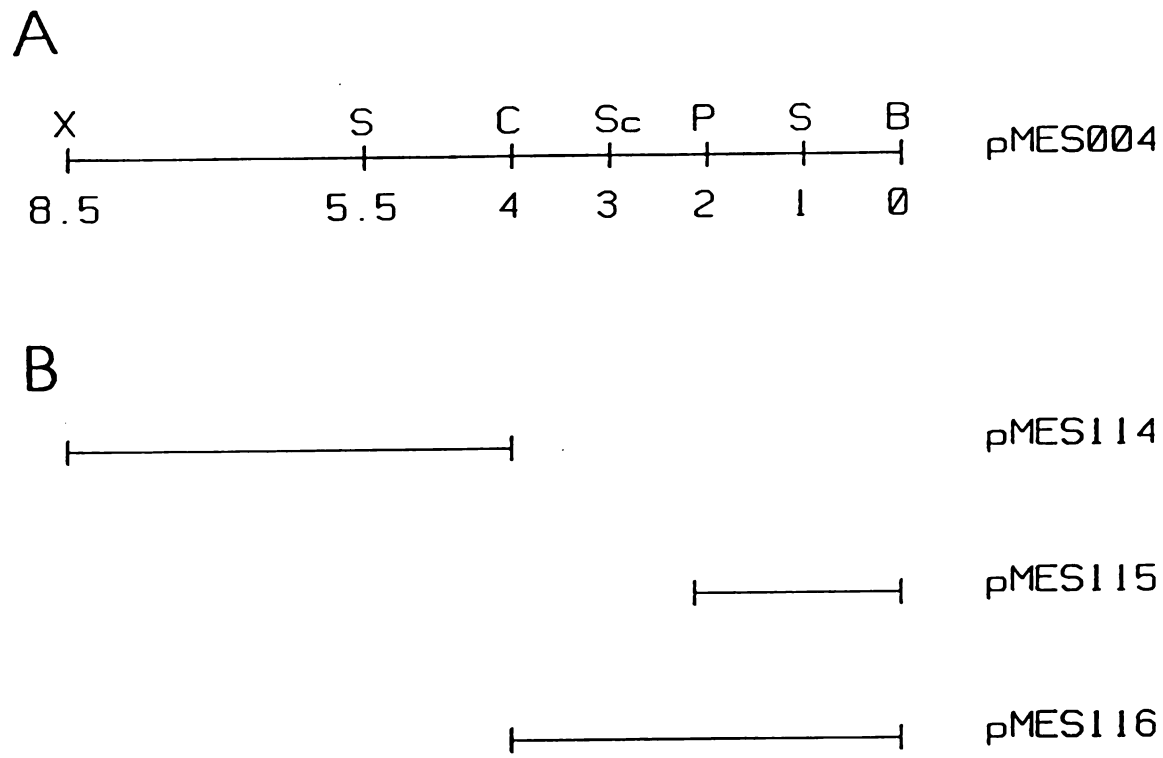


Figure 6. Strategy for subcloning regulatory regions adjacent to Ω4403. (A) Additional restriction sites were located within the 8.5 kbp *XhoI-BamHI* segment adjacent to Ω4403 by digesting pMES003 with *XhoI* (X), *SalI* (S), *ClaI* (C), *SacI* (Sc), *PstI* (P), and *BamHI* (B), separating the products on a 0.5% TAE agarose gel, and visualizing the fragments with ethidium bromide. The approximate distance in kbp from the *BamHI* site is given by the number under each restriction site. (B) A 4.5 kbp *XhoI-ClaI* fragment, a 2 kbp *PstI-BamHI* fragment and a 4.0 kbp *ClaI-BamHI* fragment were subcloned into pREG1666 generating pMES114, pMES115, and pMES116 respectively.

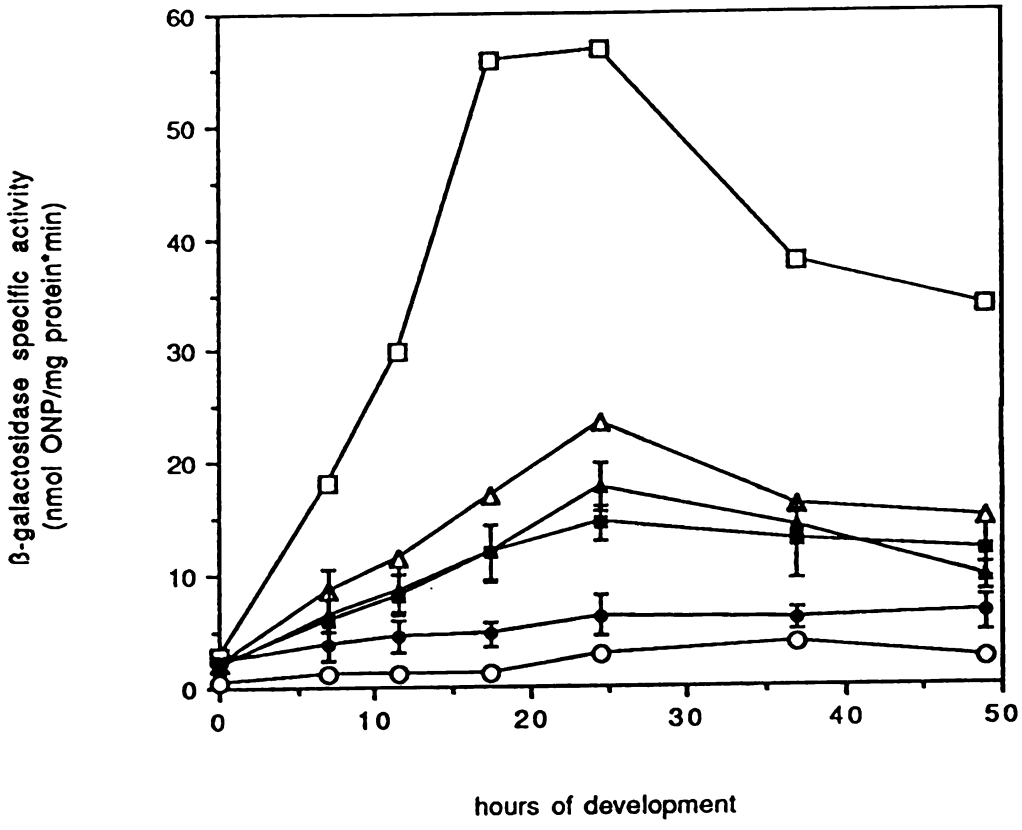


Figure 7. **Developmental β-galactosidase expression from *lacZ* fused to portions of the 8.5 kbp regulatory region upstream of Ω4403.** Plasmids pMES114, pMES115, pMES116, and pMES004 were introduced into wildtype *M. xanthus* at the Mx8 phage attachment site. β-galactosidase activity was measured throughout development on TPM starvation agar as described in the Materials and Methods. The specific activity of the wild type strain (DK1622; ○—○), the original Ω4403 fusion strain (DK4368;□—□), a transductant containing a single copy of pMES004 integrated at the Mx8 *attB* site (MES008; △—△) and the average specific activities of five independently-isolated transductants containing a single copy of pMES114 (●—●), pMES115 (■—■), or pMES116 (▲—▲) integrated at the Mx8 *attB* site. Error bars show one standard deviation.

Despite the fact that 8.5 kbp of DNA upstream from the Ω4403 fusion was sufficient to direct developmentally regulated β-galactosidase expression, the activity was only 25% of the maximum level produced in the original insertion-containing strain. A similar observation was noted by Li and Shimkets (41). In their experiments, 1.3 kbp of DNA adjacent to the C-dependent Ω4435 insertion was capable of directing β-galactosidase expression, but only to 50% of the maximum level seen in the original Ω4435 strain. One explanation for these observations is that additional sequences further upstream are required for proper levels of expression. However, in the case of Ω4435, inclusion of an additional 10 kbp of upstream DNA had no effect on the expression levels. A second hypothesis is that the presence of two copies of an important regulatory sequence (one at the *attB* site and one at the native promoter) causes competition for a necessary factor present in limiting amounts. To test this "titration model", a control plasmid was constructed by placing the 8.5 kbp of Ω4403 upstream DNA into pREG1175 (25), a plasmid similar to pREG1666 but lacking the Mx8 *attP* segment (pMES108). This plasmid can only integrate into the *Myxococcus* chromosome by a homologous recombination event (Figure 3A) and places the *lacZ* marker under the control of the native regulatory regions. If the entire promoter is contained within the 8.5 kbp fragment, expression in homologous integrants would also be expected to be reduced relative to the original insertion

strain since two copies of the regulatory region are present and compete for the postulated limiting factor. Figure 8 demonstrates that homologous integrants containing one copy of pMES108 exhibit β-galactosidase activity that is about 70% of the level seen in DK4368 (the original fusion strain). Therefore, although an additional copy of the regulatory region appears to have a small effect on gene expression, the presence of two copies of the 8.5 kbp Ω4403 upstream region may not be solely responsible for the markedly reduced level of expression originally observed (Figure 6).

Ω4403 is located approximately 380 bp downstream from the start of the transcription unit into which it has inserted.

Low resolution, S1-nuclease mapping experiments were utilized to define the 5' end of the transcription unit to which Ω4403 was fused. A plasmid containing the 2 kbp region shown to be sufficient for directing developmental β-galactosidase expression (pMES112) was digested with *BamHI* or *SalI* and labelled at the 5' ends; a portion of the *BamHI* end-labelled probe was subsequently digested with *SalI* (Figure 9A and 9B, lanes 1-3. The *BamHI* site is located approximately 50 bp into the 5' end of Tn5*lac* while the *SalI* site lies 1 kbp upstream of the *BamHI* site, Figure 6). These three probes were hybridized separately to developmental RNA from *Myxococcus* (24 hours of development, designated T_{24}). The hybrids were treated with S1-nuclease,

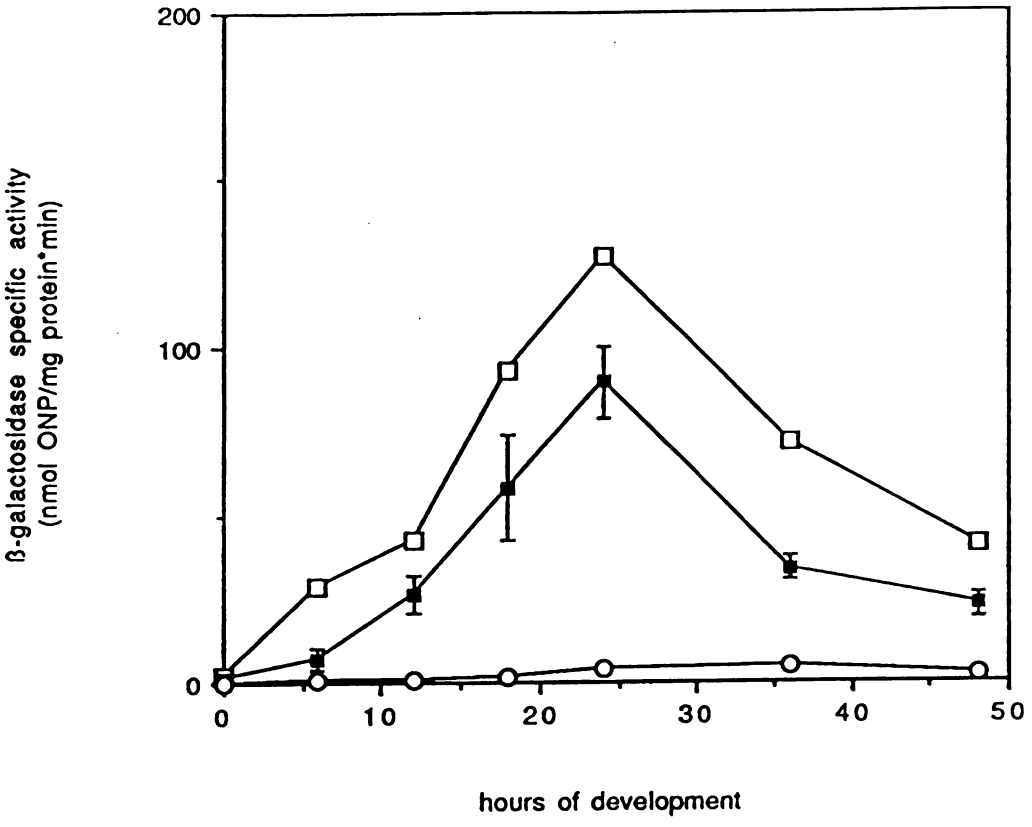
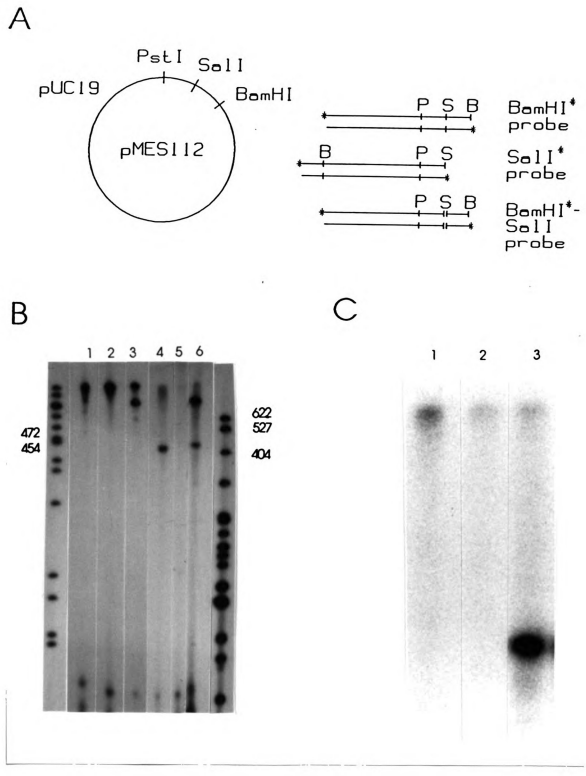


Figure 8. **Developmental β-galactosidase expression in homologous integrants.** A promoterless *lacZ* gene was fused to 8.5 kbp of DNA located immediately upstream from the Ω4403 insertion (pMES108) and introduced into wild type *M. xanthus* at the native chromosmal site. β-galactosidase activity was measured in cells harvested at various times during their development on TPM starvation agar as described in the Materials and Methods. The specific activity of the wild type strain (DK1622; ○—○), the original Ω4403 fusion strain (DK4368; □—□), and the average specific activity of five independently-isolated transductants containing a single copy of pMES108 integrated at the native promoter site (■—■). Error bars show one standard deviation.

Figure 9. **Low resolution, S1-nuclease mapping of developmental transcripts from DK4368.** Developmental (T_{24}) or vegetative (T_0) RNA (50 μg) was hybridized for 16 hours at 53° C to 5' end-labelled probe (0.5 μg) as described in the Materials and Methods. Each reaction was digested with 25 u (B) or 250 u (C) S1-nuclease. The protected products were separated on a 5% polyacrylamide-urea gel and visualized by autoradiography. Molecular size markers are indicated by horizontal bars in the margin. (A) Plasmid pMES112 5' end-labelled at *BamHI*, *SalI*, or labelled at *BamHI* and recut with *SalI* was used as probe. (B) *BamHI**-probe (lane 1), *SalI**-probe (lane 2), *BamHI**-*SalI*-probe (lane 3), and S1-nuclease protected products of developmental RNA hybridized to *BamHI**-probe (lane 4), *SalI**-probe (lane 5), or *BamHI**-*SalI*-probe (lane 6). (C) S1-nuclease protected products of *BamHI**-probe hybridized to yeast tRNA (50 μg, lane 1), vegetative RNA (lane 2) or developmental RNA (lane 3).


A
PstI
SalI
pUC19
BamHI
pMES112
P S B
BamHI*
probe
B P S
SalI*
probe
P S B
BamHI*-
SalI
probe
B
1 2 3 4 5 6
472
454
622
527
404
C
1 2 3

and the protected products were separated by gel electrophoresis. As shown in Figure 9, the same protected-fragment (about 430 bases) is observed for both the *BamHI**-probe and *BamHI**-*SalI*-probe (panel B, lanes 4 and 6); none of the SalI*-probe is protected by developmental RNA (panel B, lane 5). Furthermore, the 430 base, protected-fragment is not observed in experiments in which vegetative RNA (T_0) was hybridized to the *BamHI**-probe (Figure 9C, lane 2). These observations are consistent with the idea that the 5' end of the Ω4403-associated transcript is located approximately 380 bases upstream of the insertion point and also suggests that the transcript levels are developmentally regulated.

Regulation of Ω4403-associated gene expression occurs at the level of mRNA accumulation.

Initial experiments indicated that the transcript fused to Ω4403 was produced 24 hours after starvation but not during vegetative growth (Figure 9C). To determine whether mRNA levels were responsible for the developmental pattern of β-galactosidase activity, a quantitative S1-nuclease experiment was performed (53). Increasing amounts of developmental RNA (T_{18}) were hybridized to equal amounts of probe (pMES112 digested with *BamHI* and labelled at the 5' end, Figure 9A); the amount of probe used was in excess (data not shown). After treatment with S1-nuclease, the protected fragments were separated by gel electrophoresis,

visualized by autoradiography, and quantified using a Kodak Bio-Image densitometer. Figure 10A demonstrates that the amount of protected probe is a linear function of the amount of input RNA (at least to 100 μg of T_{18} RNA) and indicates the reproducibility of individual S1-nuclease protection experiments (correlation coefficient=0.975).

Identical amounts of RNA prepared from cells harvested throughout development were hybridized to a constant amount of probe and subjected to the same S1-nuclease treatment described above. The amount of transcript present at various times during development (relative to T_{24} RNA) is shown in Figure 9B. For each time point, the amount of protected probe is within the linear range established in Figure 10A. The developmental increase in β-galactosidase activity correlates well with the increase in transcript abundance supporting the idea that regulation of the Ω4403 fusion occurs at the level of mRNA accumulation. Whether this accumulation involves differences in transcription initiation or mRNA stability is not known.

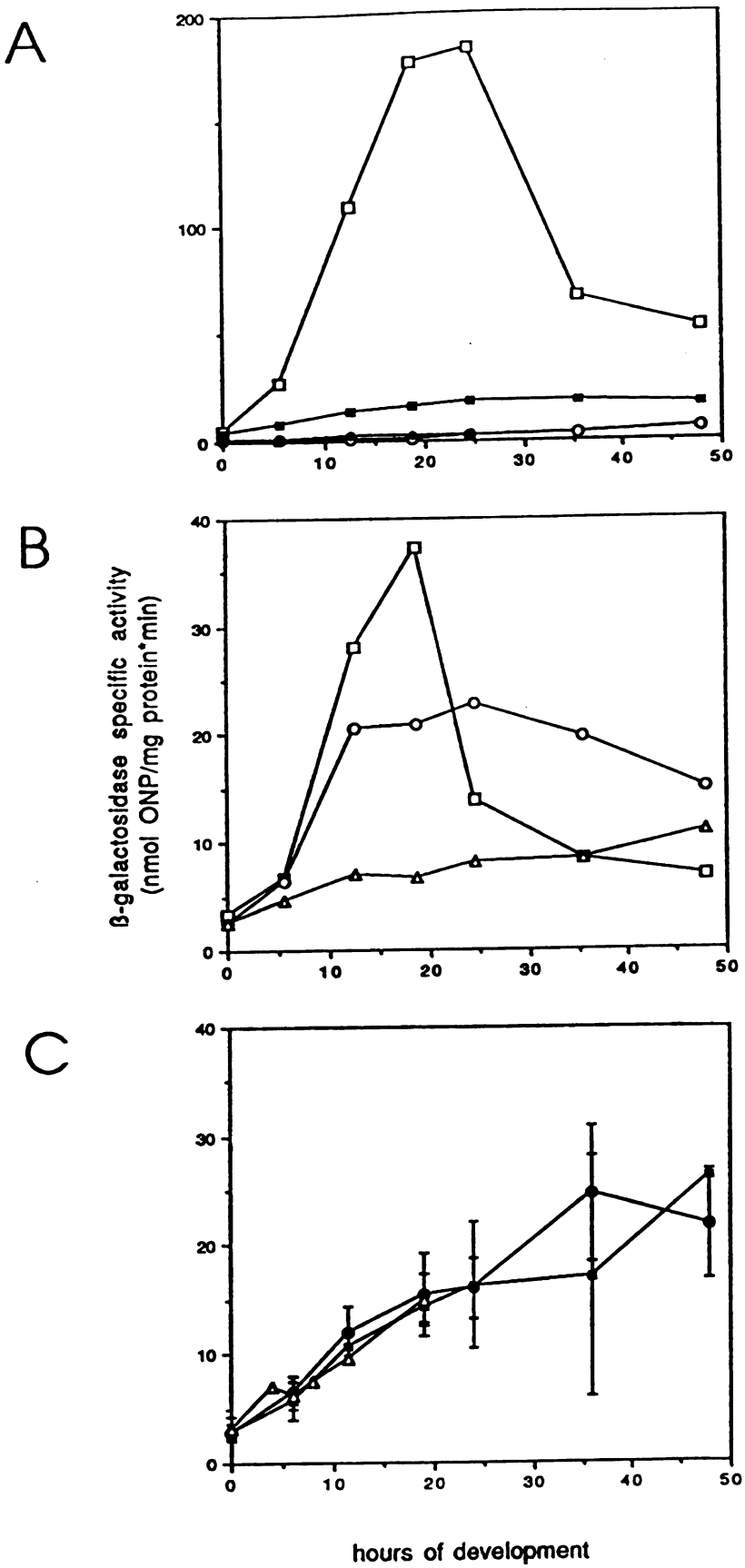
C-dependent activation of gene expression occurs at least partially through DNA within 2 kbp of the Ω4403 fusion.

The Ω4403 fusion was one of 16 Tn*5lac* insertions whose β-galactosidase expression absolutely depended on a functional C-signaling system (14). This dependence is illustrated in Figure 11A. β-galactosidase expression in a *csgA* mutant strain containing Ω4403 (DK5270) is reduced to

Figure 10. **Quantitative S1-nuclease protection of RNA from DK4368 throughout development.** (A) Upper panel: Increasing amounts of developmental RNA (T_{18}) were hybridized to probe (0.5 μg *BamHI*-probe) at 53° C for 16 hours. Carrier tRNA was included with some samples to bring the total amount of RNA to 50 μg. Each sample was digested with S1-nuclease (250 units); the protected fragments were separated on a 5% polyacrylamide-urea gel and were visualized by autoradiography. Size markers are indicated in the margin. (Lanes 1-5) 0, 12.5, 25, 50, and 100 μg T_{18} RNA. Lower panel: The integrated intensity of each protected band (obtained by two-dimensional densitometry on a Kodak Bio-Image densitometer) was plotted against the total amount of input RNA (correlation coefficient=0.975). (B) Upper panel: RNA (50 μg) prepared from cells harvested throughout development was hybridized to *BamHI**-probe (0.5 μg) at 53° C for 16 hours. Each sample was digested with S1-nuclease (250 units); the protected products were run on a 5% polyacrylamide-urea gel and visualized by autoradiography. (Lanes 1-6) T_0, T_6, T_{12}, T_{18}, T_{24}, T_{36}. Lower panel: The amount of protected product was quantitated using a Kodak Bio-Image densitometer. The integrated intensity at each time point was compared to the curve in panel A (lower) to determine whether it was in the linear range of the assay. The amount of protected probe (relative to T_{24}; ○—○) and β-galactosidase specific activity (●—●) for each time point were plotted against hours of development.

A
1 2 3 4 5
527
404
integrated intensity
Input RNA (µg)
B
1 2 3 4 5 6
relative mRNA levels
β-galactosidase specific activity
(nmol ONP/mg protein*min)
hours of development

Figure 11. **Developmental β-galactosidase expression from *lacZ* fused to the Ω4403 regulatory region in *csgA* mutant strains.** Plasmids pREG1666, pMES115, and pMES004 were introduced into the *csgA* mutant strain DK5208 and β-galactosidase activity was measured in cells harvested throughout their development on TPM starvation agar as described in the Materials and Methods. (A) The specific activity of the wild type strain (DK1622; ○—○), the original Ω4403 fusion strain (DK4368; □—□), the *csgA* mutant strain (DK5208; ●—●), and the *csgA* Ω4403 fusion strain (DK5270; ■—■). (B) The specific activity of individual transductants containing a single copy of pREG1666 (JW103; △—△), pMES115 (MES053; ○—○), or pMES004 (MES008; □—□) integrated at the *attB* site. (C) The average specific activities of independently-isolated transductants containing a single copy of pREG1666 (five transductants, △—△), pMES115 (five transductants, ●—●), or pMES004 (two transductants, ■—■) integrated at the Mx8 *attB* site in a *csgA* background. Error bars show one standard deviation.


A
B
C
ß-galactosidase specific activity
(nmol ONP/mg protein*min)
hours of development
0
10
20
30
40
50
100
200

10% of the level seen in the original insertion strain (DK4368). In order to investigate whether the C-dependent activation of gene expression occurs through the 8.5 kbp Ω4403 upstream region, *lacZ* fused to 0, 2, or 8.5 kbp of DNA adjacent to Ω4403 was integrated into a *csgA* strain (DK5208) at the Mx8 *attB* site and developmental β-galactosidase activity was measured. The 2 kbp Ω4403 upstream region was previously demonstrated to be sufficient for directing developmental gene expression (Figure 7 and Figure 11B); additional DNA within 8.5 kbp of the Ω4403 fusion appeared to be involved in increasing the level of expression. Figure 11C shows that developmental β-galactosidase activity in *csgA* strains containing either 2 kbp or 8.5 kbp of Ω4403 regulatory DNA fused to *lacZ* is reduced to the basal level observed in a *csgA* control strain containing the vector alone (pREG1666). These results suggest that the mechanism of C-dependent gene activation is at least partially mediated through DNA within 2 kbp of the Ω4403 fusion point. The data further suggests that the region between 2 and 8.5 kbp upstream of the insertion may also participate in C-dependent gene activation. (Alternatively, the mechanism by which the 2 to 8.5 kbp region increases gene expression may actually be C-independent with an initial requirement for C-dependent activation through only the 2 kbp of DNA immediately adjacent to Ω4403). The basal β-galactosidase levels in *csgA* or wild-type strains containing the vector alone were

expected to be equivalent; however, the specific activity observed in the *csgA* strain is slightly higher than that of the wild-type strain. Among other explanations, overall protein or mRNA stability may be greater in *csgA* strains. Because the control strains show this discrepancy, the direct comparison of a particular regulatory region-*lacZ* fusion in the *csgA* mutant and wild-type strain is not possible.

DISCUSSION

Four signaling systems are absolutely required for *M. xanthus* differentiation and gene expression (3, 9, 14). Knowledge about the C-signal system, particularly the signal molecule itself (C-factor) is rapidly increasing (10, 28, 29, 30). To begin to define cis-acting elements involved in C-dependent gene activation DNA adjacent to developmentally-regulated Tn5*lac* fusions was cloned and tested for promoter activity. Although DNA within 1.8 kbp of the Ω4414 insertion is unable to direct developmental β-galactosidase expression (Figure 4), a 2 kbp region immediately adjacent to the Ω4403 insertion is sufficient to promote β-galactosidase activity (Figure 7). Interestingly, the level of activity increases when an additional 2 or 6.5 kbp of Ω4403 upstream DNA is fused to the *lacZ* marker gene. This observation suggests that several different segments of upstream DNA are involved in activating Ω4403-associated gene expression. Similar activating sequences have been identified in the developmentally regulated genes *mbhA* (55), *ops*, and *tps* (57, 58). A cis-acting region of DNA located between 89 and 276 nucleotides upstream of the *mbhA* transcription start site is required for the accumulation of *mbhA* transcripts during development and DNA further than 2 kbp from the transcription start site is also believed to

play a role in gene expression (55). Expression from *lacZ* fused to DNA upstream of the *ops* gene increases substantially when a region between -131 to -208 bp is included (56). This same *ops* segment functions as an upstream activating site (UAS) for transcription of the *tps* gene (located about 2 kbp downstream of the UAS, 57) Together, this evidence indicates that DNA separate from the transcription start site plays an essential role in regulating *M. xanthus* developmental gene expression.

The specific mechanisms coupling intercellular signaling to gene activation during *M. xanthus* development are currently unknown; however, by comparison to other prokaryotic and eukaryotic organisms which undergo similar developmental cycles, regulation of transcription initiation is likely to be involved. The start site of the Ω4403-associated transcript was located about 380 bases upstream of the insertion point (Figure 9). Using quantitative S1-nuclease experiments, the levels of transcript were demonstrated to be developmentally regulated (Figure 10B). These results provide preliminary evidence that modulation of gene expression occurs at least partly through the regulation of transcription initiation or mRNA stability. In the case of the Ω4403-associated transcription unit, the mechanism of gene activation is clearly dependent on a functional C-signaling system as β-galactosidase expression from the original Tn*5lac* insertion is essentially abolished in a *csgA* mutant (Figure 11A; [14]). This C-dependent

activation is mediated to at least some extent through the 2 kbp Ω4403 upstream region (Figure 11B and 11C). Perhaps in response to C-signaling, trans-acting factors are produced or already existing factors are modified which interact with this region to stimulate transcription. The exchange of C signal could also trigger the degradation of a repressor normally blocking the transcription of the Ω4403-associated gene. Expression of another C-dependent gene, *ops*, requires the product of the *sigB* locus (a putative developmental σ factor, 58) and a DNA binding activity that preferentially associates with the *ops* UAS was identified in developmental *M. xanthus* crude extracts (57). It will be very interesting to biochemically identify the trans-acting factors that are involved in C-dependent gene activation. Further characterization of the Ω4403-associated regulatory regions including a more precise mapping of the start site, sequence analysis of the promoter, and localization of the upstream activation sites within the 8.5 kbp adjacent to Ω4403 will facilitate the identification of individual components of the transcriptional machinery.

The maximum level of β-galactosidase expression observed in transductants containing up to 8.5 kbp of Ω4403 upstream DNA fused to the *lacZ* marker was only about 20% to 30% of the level produced in the original fusion strain (Figures 5 and 7). The lower activity level was not due to the titration of a limiting factor through competition between binding sites at the native and putative promoters

since strains containing both *lacZ* fused to the native promoter as well as a copy of the putative promoter approximately 20 kbp downstream produce β-galactosidase levels comparable to the original Ω4403 fusion strain (Figure 8). A possible explanation for the decreased β-galactosidase expression is that additional regulatory sequences located further than 8.5 kbp from the fusion site may be required for maximal gene expression. Since the transcription start site is located about 380 bases from the 5' end of the insertion, these putative, additional regulatory regions would have to be strong activating sequences (as they would be responsible for 60% to 70% of the β-galactosidase activity observed in the original Ω4403 fusion strain) and would have to function over a substantial distance (greater than 8 kbp). Although eukaryotic enhancer elements are often located within a few hundred base pairs of the transcription start site, long-range activation sequences which function at distances greater than several kilobases upstream or downstream of the transcription start site have been identified in several systems (59, 60, 61). The *M. xanthus ops* UAS also affects the expression of the *tps* gene located 2 kbp downstream (57) suggesting that long-range activating sequences might be involved in *Myxococcus* developmental gene expression. The possibility also exists that regions downstream of the start site may affect gene activation. C-dependent gene expression may be novel in its requirements for DNA extremely distant from the

transcription start site. Confirmation of these hypotheses requires cloning additional Ω4403 upstream or downstream regions and testing them for transcription-enhancing activity.

Several alternative explanations for the lower β-galactosidase activities can be postulated including a positional effect on gene expression as well as autorepression by the Ω4403-associated gene product on its own transcription. Besides being fused to the entire complement of native promoter sequences, the original Tn5*lac* insertion is at a completely different chromosomal location with respect to the Mx8 *attB* site. Variations in the levels of gene expression which are solely dependent on chromosomal position have been documented in both eukaryotic and prokaryotic systems including *M. xanthus* (56, 62, 63). In the current studies, the *attB* site may be a particularly poor location for C-dependent gene expression. The position-effect model can be directly tested by integrating the regulatory region-*lacZ* fusions at a different chromosomal location, such as near the original locus. Position effects would be eliminated and β-galactosidase levels should approach those observed in the original fusion strain.

The reduced levels of β-galactosidase expression may, however, reflect the true regulation of the Ω4403-associated transcript. If the original Ω4403 Tn5*lac* inserted within a gene whose product negatively affects its own expression, β-galactosidase activity would be

artificially elevated in the insertion strain due to the absence of the autorepressor product. Transductants containing the Ω4403 upstream region fused to *lacZ* and integrated at the *attB* site would still produce the repressor molecule from the native locus and thus be subjected to autoinhibition. The autorepression model can be investigated by introducing the regulatory region-lacZ fusions into a modified insertion strain possessing a Tn5-Tc^r replacement of Tn5*lac*-Km^r. If this model is correct, the developmental β-galactosidase levels should be comparable to the original Tn5*lac* Ω4403-containing strain. Alternatively, a comparison of the levels of the Ω4403-associated transcript in the wild-type strain (DK1622) and the Tn5*lac* fusion strain (DK4368) could be performed; the autorepressor model predicts that the transcript levels would be 3- to 5-fold higher in DK4368 than in DK1622. The results of these proposed experiments should yield valuable information that will allow a more definitive explanation for the decreased β-galactosidase expression. At the same time, this information should provide insight into increasing the window of sensitivity for detecting changes in the level of β-galactosidase activity which is necessary for a more detailed analysis of the cloned regulatory regions.

CHAPTER 2

CHARACTERIZATION OF VEGETATIVE RNA POLYMERASE FROM *MYXOCOCCUS XANTHUS*

One mechanism utilized by a variety of bacteria to coordinate proper sequential gene activation with environmental conditions is the modification of the promoter specificity of RNA polymerase (32). Promoter recognition is mediated by a sigma (σ) subunit which binds to both the RNA polymerase core (α_2, β, β') and to the template's promoter. Nitrogen regulation and the heat shock response in *E. coli* are regulated by sigma factors (σ^{54} and σ^{32} respectively; 64, 65) with promoter specificities quite different from the major *E. coli* σ factor (σ^{70}). In *B. subtilis*, the endospore developmental program induced by nutrient depletion is tightly coupled to the production or activation of alternative σ factors (32). Similarly, heterogeneous RNA polymerase molecules discovered in *Streptomyces coelicolor* may be involved in differential gene expression during mycelial and hyphal development (66, 67).

During differentiation, *M. xanthus* exhibits dramatic changes in both protein synthesis and patterns of gene expression (12, 68) suggesting the need for mechanisms to effect these tightly regulated changes. A comparison of four well-characterized promoters from *M. xanthus* genes which are utilized during growth (*vegA*, [69]) or development

(*ops* and *tps* [70, 71], *mbhA* [55]) reveals differences indicative of polymerase heterogeneity. Three of the promoters (*vegA*, *ops*, and *tps*) share weak homology with the -10 and/or -35 sequences of the *E. coli* σ^{70} consensus sequence. The promoter of the remaining gene (*mbhA*) is clearly distinct as it resembles an *E. coli* σ^{54} consensus sequence (72). The variations indicate that modification of the promoter-recognition specificity of RNA polymerase must occur to allow utilization of these distinct promoters.

Indeed, the gene for a development-specific σ factor was recently cloned (*sigB*, 58). This new σ factor is expressed during middle to late development and is required for proper maturation of myxospores. Deletions of the *sigB* gene do not affect the production of protein S (*tps* gene product). However, the *sigB* deletions abolish the production of protein S1 (*ops* gene product), suggesting that the *sigB*-encoded factor directly interacts with the *ops* promoter or indirectly affects its expression by interrupting the program of developmental gene expression. Evidence, therefore, is accumulating to support the theory that differentiation in *M. xanthus* is coordinated by the regulated expression and utilization of alternative σ factors.

Intercellular signaling is absolutely required for developmental gene expression in *M. xanthus* and modulation of transcription initiation may be at least one mechanism coupling the two. An *in vitro* system for reconstituting

signal-dependent transcription will ultimately be required to test this hypothesis. Chapter 1 of this study described initial characterization of C signal-dependent promoter and regulatory regions in *M. xanthus;* these promoters could serve as templates for *in vitro* reconstitution experiments. In addition to a repertoire of well characterized C-dependent templates, several proteins are required. Included among these proteins are core RNA polymerase, developmental σ factors, and additional regulatory proteins that might act at upstream (or downstream) sites and be necessary for efficient transcription.

In preparation for the eventual establishment of an *M. xanthus in vitro* transcription system, core RNA polymerase from *M. xanthus* was purified for reconstitution with developmental factors. The availability of vegetative promoters from several bacteria prompted initial purification attempts with vegetative *M. xanthus* as a model system. Using a modified Burgess-Jendrisak procedure (73, 74), both core and holoenzyme were obtained. A protein was isolated from holoenzyme fractions that stimulated both *M. xanthus* and *E. coli* core polymerase transcribing activity. Additionally, the transcriptional activity of holoenzyme on a variety of vegetative and developmental templates was examined.

MATERIALS AND METHODS

Growth of strains

Vegetative *Myxococcos xanthus* DK1622 cells were grown in six liters of CYE (1% Casitone [Difco Laboratories], 0.5% yeast extract, 0.1% $MgSO_4$) with shaking (250 rpm) at 30°C to the mid-exponential phase of growth (7.5 x 10^8 cells/ml). Cells were harvested by centrifugation in a GS-3 rotor (7500 rpm, 10 minutes), quickly frozen in a dry ice-ethanol bath, and stored at -70°C. Approximately 80 grams of cell (wet weight) were obtained.

Buffers

Buffers and other solutions were prepared as described previously (73, 74). Grinding buffer contained 0.05 M Tris-HCl (pH 7.9), 10% (v/v) glycerol, 1 mM EDTA, 0.2 mM DTT, 1 mM 2-mercaptoethanol, 130 μg/ml lysozyme, 0.23 M NaCl, 1 mM PMSF, and Trasylol (10^6 KIU/L, Boehringer Mannheim Biochemicals). The buffer used throughout most of the isolation was TGED (0.1 M Tris-HCl [pH 7.9], 10% glycerol, 1 mM EDTA, 0.2 mM DTT) or TGEDM (TGED buffer with 10 mM $MgCl_2$) containing sodium chloride as mentioned. Ammonium sulfate dilution buffer consisted of 40 mM Tris-HCl (pH 7.9), 1 mM EDTA, 0.2 mM DTT, and 65% saturated ammonium sulfate. Samples were dialyzed into storage buffer (0.1 M Tris-HCl [pH 7.9], 50% glycerol, 0.1 M NaCl, 1 mM EDTA, 0.3

mM DTT, and 10 mM $MgCl_2$) and kept at -20° C as indicated.

RNA polymerase purification

RNA polymerase was purified from *Myxococcus* using procedures described by Jendrisak and Burgess (74) and Rudd and Zusman (73). The entire purification was performed rapidly and in the cold (10°C). More specifically, 80 grams of vegetative cells were resuspended in 240 ml of grinding buffer by blending at low speed for 2 minutes in a Waring blender. After letting the solution sit for 20 minutes in an ice-water slurry, sodium deoxycholate (4%) was added to a final concentration of 0.05%. The mixture was blended for 30 seconds at low speed and again set in the ice-water slurry for 20 minutes. The viscous solution was sheared at high speed for 60 seconds, diluted with 320 ml TGEDM buffer containing 0.2 M NaCl, and blended for 30 additional seconds at low speed. Cell debris was pelleted by centrifugation in a GSA rotor (8000 rpm, 45 minutes). The supernatant was collected and a portion retained as the "low speed supernatant".

Protein was precipitated by slowly adding 10% Polymin P (polyethyleneimine [Sigma Chemical Co.] prepared as detailed in [75]) to a final concentration of 0.25%. The slurry was stirred for 5 minutes, spun in a GSA rotor (6000 rpm, 15 minutes), and the liquid phase removed. The Polymin P pellet was resuspended in 320 ml TGEDM buffer containing 0.35 M NaCl (using a homogenizer) and was washed for 5

minutes with gentle stirring. After centrifugation (6000 rpm, 15 minutes), the supernatant was discarded and RNA polymerase was eluted from the Polymin P by resuspending and stirring the pellet in TGEDM containing 1.0 M NaCl as outlined above. The Polymin P was removed from the suspension by spinning (8000 rpm, 30 minutes), and the yellow supernatant was saved.

A fractional ammonium sulfate precipitation was performed to remove additional contaminating proteins. Powdered ammonium sulfate was slowly added to 40% saturation (over a period of 20 minutes) with stirring for another 20 minutes. The precipitated proteins were collected by centrifugation in a GSA rotor (8000 rpm, 45 minutes) and discarded. RNA polymerase was recovered from the liquid phase by increasing the ammonium sulfate to 65% saturation, stirring for 20 minutes, diluting the mixture with 65% saturated-ammonium sulfate dilution buffer, and spinning at 8000 rpm for 45 minutes. The dark, yellow pellet was dissolved in 87 ml TGED buffer and an aliquot saved as the "ammonium sulfate enzyme".

The ammonium sulfate enzyme was applied to a 30-ml double-stranded DNA-cellulose affinity column (7mg/ml calf-thymus DNA [Sigma Chemical Company], equilibrated with TGED containing 0.15 M NaCl) at a rate of 43 ml/hour. RNA polymerase was eluted with a 0.15 M to 1.3 M NaCl gradient in 172 ml TGED buffer at the same flow rate. Two-ml fractions were collected and assayed for transcribing

activity. Fractions with polymerase activity were dialyzed against 3 liters of storage buffer for 4 hours (one change after 1 hour) and stored at -20°C.

In vitro transcription assays

Polymerase activity was initially located using a nonspecific transcription reaction. The standard assay conditions were similar to those used in the procedure of Rudd and Zusman (73) except 4 μg poly d(AT) was utilized as template, 0.1 mM each ATP and UTP were included as the unlabeled ribonucleotides, 2 μCi ^{3}H-UTP (New England Nuclear) was the labeled ribonucleotide, and KCl was omitted. Ten microliters of the samples to be tested were added to each reaction and the subsequent incubation, precipitation, and wash steps were followed exactly as described (73).

RNA polymerase-containing fractions were analyzed for their ability to produce run-off transcripts on linearized plasmids possessing one of several bacterial promoters. Reaction conditions were those of Kroos et. al. (76). For reconstitution experiments, 2 μl of core RNA polymerase were mixed with 8 μl of gel-purified protein (77) and incubated on ice for 10 minutes. Ten microliters of the fractions to be tested, or reconstituted enzyme, were included in each assay. *E. coli* σ^{70}-RNA polymerase was a gift from A. Revzin; *E. coli* core RNA polymerase was a gift from C. Gross; *B. subtilis* σ^{43}-RNA polymerase was purified by L. Kroos.

SDS-polyacrylamide gels

Core polymerase subunits (α, β, β') and total protein were separated using SDS-polyacrylamide gel electrophoresis. Aliquots from fractions were mixed with one-third volume sample buffer (0.375 M Tris-HCl [pH 6.8], 6% SDS, 15% 2-mercaptoethanol, 30% glycerol, 0.3% bromophenol blue) and boiled 2 minutes. The samples were loaded onto a 10% discontinuous SDS-polyacrylamide gel and were separated by electrophoresis at a constant voltage (100-200 V) for several hours. Proteins were visualized by staining in a Coomassie blue R250 solution (50% Methanol, 7.5% acetic acid, 0.1% Coomassie blue R250) for 15-30 minutes, followed by rapid destaining (10% EtOH, 7.5% acetic acid) with four 15 minute rinses. Alternatively, a more sensitive staining was performed using a Bio-Rad silver stain kit according to the manufacturer's specifications.

When proteins were to be gel-purified, total protein was precipitated with one volume of 20% TCA, washed with one volume 5% TCA, and dried 5 minutes at room temperature. The pellets were resuspended in 1x sample buffer, boiled 2 minutes, and loaded onto a preparative gel. The desired bands were sliced out of the Coomassie-stained gel and recovered by the procedure of Hager and Burgess (78) for use in reconstitution experiments as mentioned.

Western blot analysis

Proteins separated on SDS-polyacrylamide gels (as described above) were transferred to PVDF membranes using

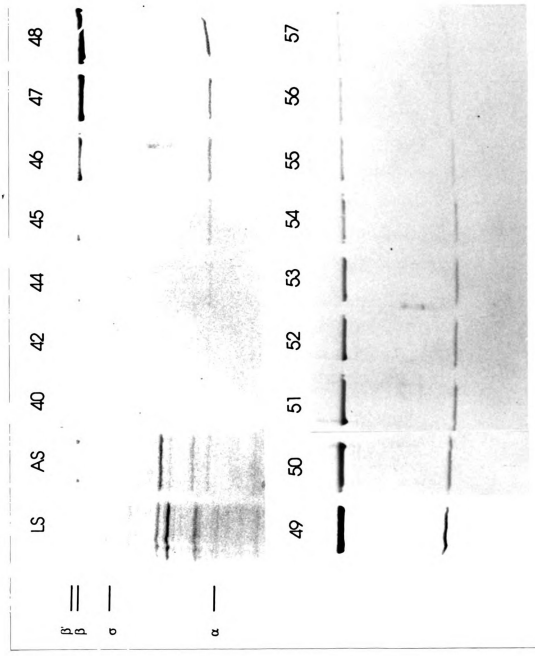
the procedure of Matsudaira (78). The blots were incubated in TBS blocking buffer (20 mM Tris-HCl, [pH 7.9], 500 mM NaCl, and 2% nonfat dry milk) for 2 hours at room temperature to prevent nonspecific binding of the antibodies. The membranes were shaken overnight at room temperature in a 1:600 dilution of polyclonal antiserum raised against B. subtilis σ^{43} (gift of R. Doi) in antibody buffer (TBS containing 2% nonfat dry milk and 0.05% Tween 20). Immunodetection using a secondary goat anti-rabbit antibody conjugated to alkaline phosphatase was performed according to the manufacturer's specifications (Bio-Rad).

RESULTS

Purification of vegetative RNA polymerase by DNA-cellulose chromatography.

Using a modified version of a polymerase purification scheme described by Jendrisak and Burgess (73) and Rudd and Zusman (74), RNA polymerase was purified from vegetative *M. xanthus*. After lysing the cells and pelleting debris by low speed centrifugation, a supernatant is obtained that contains non-specific transcribing activity on a poly d(AT) template (300-850 cpm under the conditions in Figure 2). This low speed supernatant is incapable of producing run-off transcripts from the *B. subtilis veg* promoter which has the *E. coli* σ^{70} consensus at its -35 and -10 regions (79). Furthermore, polymerase subunits (α, β, β', σ) are also not easily distinguishable from the many other contaminating proteins on Coomassie blue-stained SDS-polyacrylamide gels (Figure 1, LS). Following additional purification steps involving Polymin P precipitation and ammonium-sulfate fractionation, core polymerase subunits are visible on Coomassie blue-stained SDS-polyacrylamide gels (Figure 1, AS) and non-specific transcribing activity increases 2- to 3-fold (900-1200 cpm under the conditions in Figure 2). Additionally, the ammonium-sulfate enzyme is capable of utilizing the *Bacillus veg* promoter to produce run-off

Figure 1. **SDS-polyacrylamide gel analysis of proteins in various fractions of the purification.** Proteins in the low speed supernatant (5 μg), ammonium sulfate enzyme (3.7 μg), and DNA-cellulose fractions (25 μl) adjacent to or containing the poly d(AT) peak (Figure 2) were separated on 10% SDS-polyacrylamide gels and visualized by Coomassie blue staining. The numbers above each lane indicate the fraction; low speed supernatant (LS), ammonium sulfate enzyme (AS). *E. coli* polymerase subunits (α, β, β', σ) are indicated as size markers in the left margin.


LS
AS
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42
44
45
46
47
48
49
50
51
52
53
54
55
56
57
58
60
β'
β
σ
α

transcripts of the appropriate size. However, the background of both shorter and longer transcription products is quite high (data not shown).

The ammonium-sulfate enzyme was chromatographed on a double-stranded DNA-cellulose column as described in the Materials and Methods. A narrow peak (Figure 2, fractions 45-51) followed by a small tail (Figure 2, fractions 52-56) of poly d(AT) activity elutes within a 0.45 M to 0.52 M NaCl gradient. Fractions across the gradient, particularly those near or containing the peak of poly d(AT) activity, were examined for their protein composition by SDS-polyacrylamide gel electrophoresis (Figure 1) and were also tested for the production of run-off transcripts on the *B. subtilis veg* promoter (Figure 3). Core polymerase is detectable in fractions 40-60 with the highest levels appearing in fractions 48-51 (Figure 1). These latter fractions are also coincident with the peak of non-specific transcribing activity (Figure 2). In contrast, fractions containing the "tail" of poly d(AT) activity (Figure 2, fractions 52-56) have 2- to 10-fold less core polymerase subunits visible on SDS-polyacrylamide gels. Interestingly, the levels of core polymerase subunits do not always correlate with the ability to produce specific transcripts from the *veg* promoter. Fractions eluting early in the poly d(AT) peak (Figure 2, fractions 46 and 47) that have amounts of α, β, and β' comparable to those in the "tail" fractions (Fractions 54 and 52, respectively) produce at least 10-fold

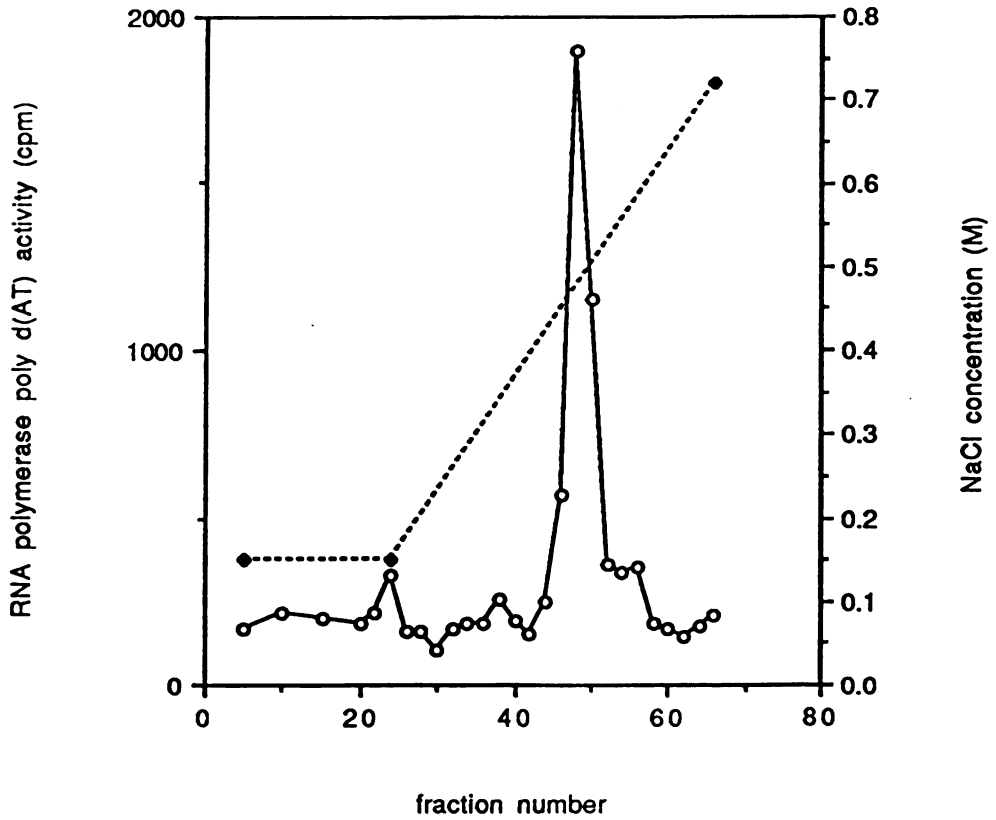
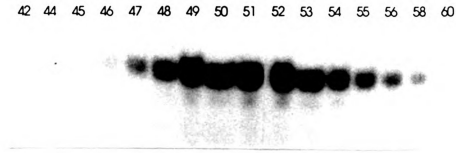
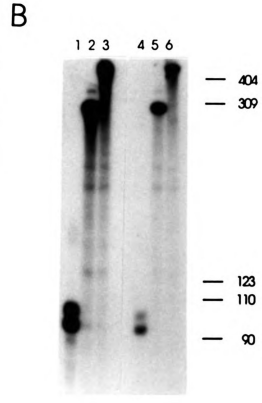


Figure 2. DNA-cellulose chromatography. The ammonium sulfate enzyme was dissolved in TGED buffer (87 ml) and applied to a 30-ml double-stranded, calf-thymus DNA-cellulose column as described in the Materials and Methods. RNA polymerase was eluted with a 0.15 M to 1.3 M linear NaCl gradient (●--●). Fractions (2 ml) were collected and assayed for incorporation of ^{3}H-UTP into RNA with poly d(AT) as template (○--○). (A void volume of one-third the column volume was assumed).

Figure 3. **Run-off transcription by vegetative RNA polymerase from *M. xanthus* and *B. subtilis*.** (A) DNA-cellulose fractions (10 μl) across the poly d(AT) activity peak (Figure 2) were tested for the production of run-off transcripts as described in the Materials and Methods. *BamHI*-digested pMS530 containing the *B. subtilis veg* promoter was utilized as template. Only the region of the gel containing the run-off transcripts is shown. Numbers above each lane indicate the fraction being tested. (B) Run-off transcripts from the *B. subtilis veg* promoter were produced by *B. subtilis* (lanes 1-3) and *M. xanthus* (lanes 4-6; fraction 54 from Figures 1 and 2) DNA-cellulose-purified RNA polymerase. Template (pMS530) was digested with either *BamHI* (lanes 1 and 4), *EcoRV* (lanes 2 and 5), or *HindIII* (lanes 3 and 6). Size markers are indicated in the margin.


A
42 44 45 46 47 48 49 50 51 52 53 54 55 56 58 60


B
1 2 3 4 5 6
404
309
123
110
90

lower levels of specific transcripts. These results suggest that fractions eluting early in the salt gradient, which contain both fairly high levels of α, β, and β' subunits and poly d(AT) activity but low specific transcribing activity (Fractions 46-48), are σ-depleted, core RNA polymerase. Fractions eluting later in the gradient which have higher levels of specific transcribing activity despite lower amounts of α, β, and β' and poly d(AT) activity (Fractions 52-56), are σ-enriched, holoenzyme-containing samples.

To ensure that transcription was initiating at the promoter and proceeding to the correct end of the template, a plasmid containing the *Bacillus veg* promoter was digested with several restriction enzymes and was utilized as a template in transcription reactions. DNA-cellulose-purified *B. subtilis* and *M. xanthus* RNA polymerase (fraction 54) produce identical transcripts of the expected sizes on the *Bacillus veg* promoter (Figure 3B) indicating that transcription is in the correct direction. (Two bands are sometimes seen when *BamHI*-digested *veg* template is used in transcription reactions with either *Bacillus* or *Myxococcus* RNA polymerase; the identity of the second band is unknown. The lower band could be a degradation product of the larger transcript or different termination or initiation sites could be used to generate the two products.)

M. xanthus core polymerase activity can be stimulated by *E. coli* σ^{70}.

Several fractions from the DNA-cellulose column (fractions 46-48; Figures 1, 2, and 3) which showed a reasonable abundance of polymerase subunits α, β, and β' were capable of nonspecific transcription. Because these fractions produced fewer run-off transcripts from the *Bacillus veg* promoter, they were postulated to contain core RNA polymerase. To test this hypothesis, *E. coli* σ^{70} purified from an SDS-polyacrylamide gel and renatured by the Hager-Burgess procedure (77) was used with the putative *M. xanthus* core RNA polymerase in transcription reconstitution experiments. Figure 4 clearly shows that the transcriptional activity of putative *M. xanthus* core polymerase on the *Bacillus veg* promoter (Fraction 47, lane 1) is increased 2- to 3-fold by σ^{70} from *E. coli* (lane 2). The amount of stimulation is identical to that observed for *E. coli* core polymerase (lane 4) upon supplementation with gel-purified σ^{70} (lane 5). Additional *M. xanthus* fractions (46 and 48) are similarly stimulated by *E. coli* σ^{70} (data not shown). These observations indicate that fractions 46-48 obtained by DNA-cellulose chromatography contain σ-depleted, *M. xanthus* core RNA polymerase which can function with a heterologous sigma factor to produce specific transcripts.

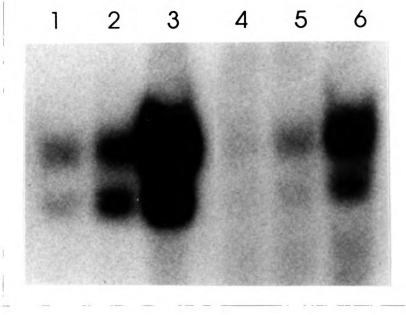


Figure 4. **Stimulation of *E. coli* and *M. xanthus* core RNA polymerase transcriptional activity.** Run-off transcripts from the *B. subtilis veg* promoter were produced using reconstituted *E. coli* and *M. xanthus* polymerase. Core RNA polymerase (2 μl of *E. coli* core or *M. xanthus* fraction 47 from Figure 1) and either gel-purified *E. coli* σ^{70} (8 μl), gel purified *M. xanthus* 50 kDa protein (8 μl), or guanidine dilution buffer (8 μl, as a control) were mixed and incubated on ice for 10 minutes prior to the addition of template. Transcription reactions were performed as described in the Materials and Methods. The products were separated on a 5% polyacrylamide-urea gel and visualized by autoradiography. *M. xanthus* core RNA polymerase (fraction 47) + control buffer (lane 1), *E. coli* σ^{70} (lane 2), or *M. xanthus* 50 kDa protein (lane 3); *E. coli* core RNA polymerase (fraction 47) + control buffer (lane 4), *E. coli* σ^{70} (lane 5), or *M. xanthus* 50 kDa protein (lane 6). Only the region of the gel containing the run-off transcripts is shown.

M. xanthus holoenzyme can utilize different bacterial promoters.

DNA-cellulose fractions that generated run-off transcripts on the *Bacillus veg* promoter were tested for their ability to utilize other bacterial promoters. A comparison of the -35 and -10 sequences of the promoters used in this study to the *E. coli* σ^{70} consensus is shown in Table 1. *M. xanthus* holoenzyme (fraction 52) produces run-off transcripts of the appropriate size from the *E. coli lac*UV5 promoter (80) as efficiently as it does from the *B. subtilis veg* promoter (data not shown). Both the *lac*UV5 and *veg* promoters have -35 and -10 sequences that are (within one base pair) identical to the *E. coli* consensus sequences. In contrast, the *M. xanthus vegA* promoter (69) which has very low sequence similarity to the *E. coli* consensus sequence, is unable to direct transcription using *M. xanthus* polymerase fractions (fractions 40-60 from Figure 2, data not shown). DNA-cellulose-purified *B. subtilis* σ^{43}-polymerase and *E. coli* holoenzyme are also incapable of producing transcripts from this *vegA* promoter.

DNA-cellulose fractions across the gradient were tested for the production of transcripts from an *M. xanthus*, A signal-dependent, developmentally-regulated promoter. A plasmid containing 300 bp of DNA upstream of the A-dependent Ω4521 insertion (which has been shown to be sufficient for directing developmental gene expression, 81) was utilized as template. No specific transcripts are produced by vegetative polymerase (fractions 42-59 from Figure 1 were

Table 1. Comparison of several bacterial promoters.

	-35		-10
E. coli σ^{70} consensus	TTGACA		TATAAT
B. subtilis veg	TTGACA		TACAAT
E. coli lacUV5	TTTACA		TATAAT
M. xanthus vegA	TAGACA		AAGGGT
M. xanthus tps	TTGCAT		AATGCT
M. xanthus ops	TTGCTC		TCTGCT
M. xanthus mbhA	TTGGCA	N_5	TCTGCT
E. coli σ^{54} consensus	CTGGCA	N_5	TTTGCA

Table 1. Comparison of several bacterial promoters. A comparison of the -10 and -35 sequences of the bacterial promoters utilized in this study to the *E. coli* σ^{70}-consensus sequence is shown. The three, well-characterized developmental promoters from *M. xanthus* and the *E. coli* σ^{54}-consensus sequence are also listed.

tested, data not shown).

A 50 kDa protein present in *M. xanthus* holoenzyme containing fractions is capable of stimulating transcription.

Previous studies have suggested that *Myxococcus* vegetative RNA polymerase is associated with a σ factor(s) having a molecular weight of 70 to 80 kDa. Rudd and Zusman (73) demonstrated that two proteins (σI and σII) present in vegetative *M. xanthus* migrated slightly higher than *E. coli* σ^{70} on SDS-polyacrylamide gels and consistently copurified with RNA polymerase activity. Additionally, the gene for a putative vegetative σ factor was cloned from *Myxococcus* based on homology to the *E. coli rpoD* gene (82), and sequence analysis subsequently predicted two possible protein products of 73 kDa and 80 kDa molecular weights. Analysis of the subunit composition of holoenzyme-containing fractions from this study by SDS-polyacrylamide gel electrophoresis and Coomassie blue-staining fails to reveal an obvious σ factor in the 70 to 80 kDa range (Figure 1, fractions 50-55). A more sensitive, silver stain was used to examine core and holoenzyme fractions (Figure 5). Although several bands in the 65-95 kDa range are faintly visible in holoenzyme fractions (fractions 50-55), none of the proteins is as abundant as would have been predicted from the high levels of specific transcribing activity or the amounts of α, β, and β' present. Furthermore, all of these proteins appear to be present in core fractions as well.

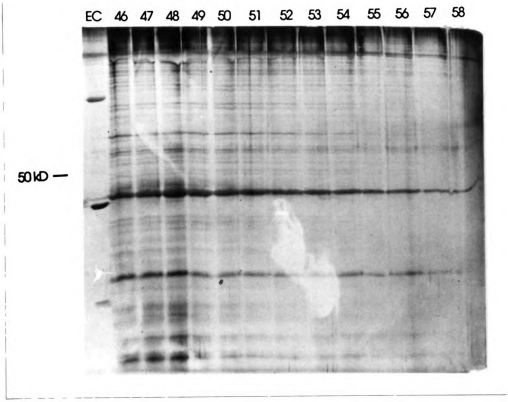


Figure 5. **Comparison of proteins present in fractions containing *M. xanthus* core and holoenzyme.** Proteins in DNA-cellulose fractions across the peak of specific transcribing activity (20 µl, Figure 3) were separated on a 10% polyacrylamide gel and visualized by silver staining. The numbers above each lane indicate the fraction; one microgram of *E. coli* RNA polymerase was used as a marker. The putative 50 kDa transcription-stimulating protein is indicated in the margin.

To facilitate the identification of a σ factor, antibodies raised against *Bacillus* σ^{43} (a vegetative σ factor that recognizes promoters with *E. coli* σ^{70} consensus sequences) were used in Western blot analysis of DNA-cellulose-purified fractions. The anti-σ^{43} antibodies recognize many proteins in both *Bacillus* vegetative extracts (Figure 6B, lane 1) and in *E. coli* holoenzyme (Figure 6B, lane 2) and react quite strongly with *Bacillus* σ^{43} and *E. coli* σ^{70}. Similarly, most of the *M. xanthus* proteins visible on a Coomassie blue-stained blot (Figure 6A, lanes 3 and 4) are also recognized by the polyclonal antiserum (Figure 6B, lanes 3 and 4). However, two proteins present only in the holoenzyme fraction (fraction 50) which are undetectable on the Coomassie-stained blot (Figure 6A, lane 4) are recognized by the anti-σ^{43} antibodies (Figure 6B, lane 4; indicated in the margin). These two proteins migrate with molecular weights of approximately 50 kDa and 70 kDa and are not detectable in the core fraction (fraction 47, Figure 6B, lane 3) suggesting they may be candidates for a σ factor.

A preparative SDS-polyacrylamide gel was used to separate proteins in holoenzyme-containing fractions (Figure 7A). The entire samples from fractions 52 and 53 were precipitated and loaded into one lane of the gel as described in the figure legend. Several slices in the 70-90 kDa range as well as one in the 50 kDa range were cut from the gel. The proteins within these slices were

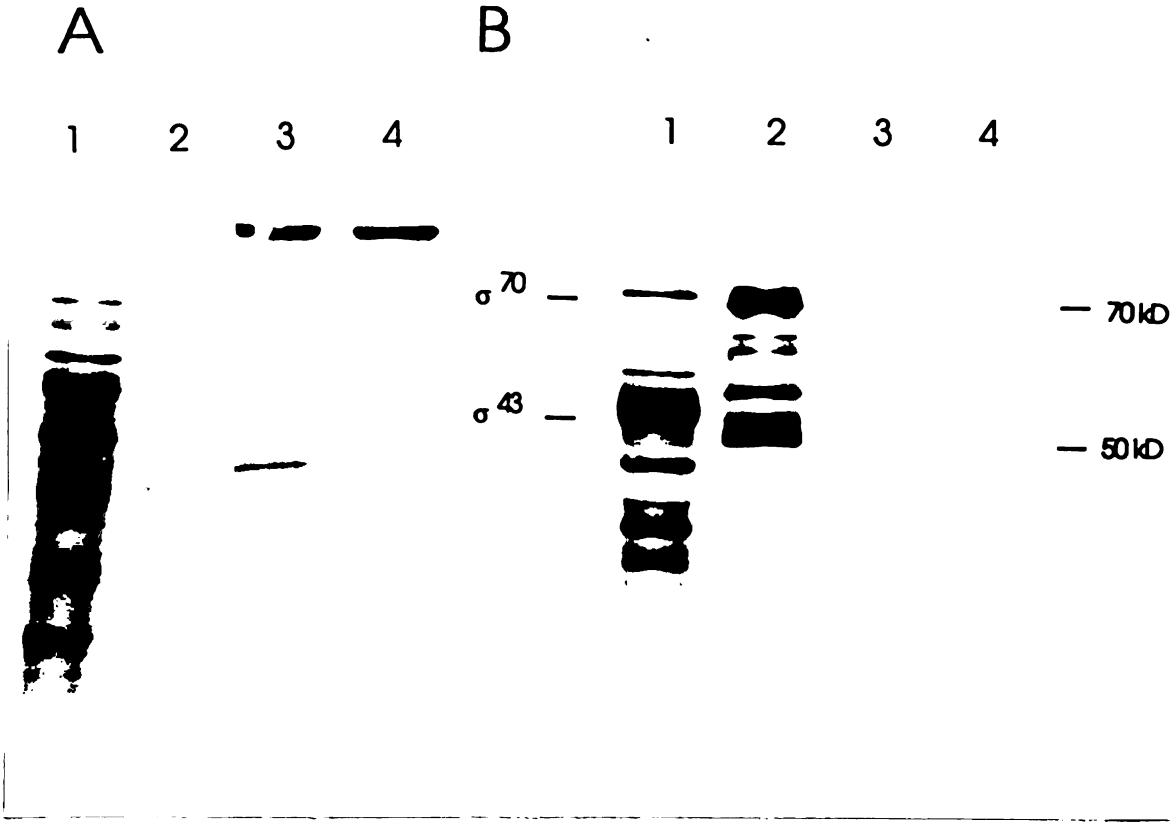


Figure 6. Western blot analysis of core- and holoenzyme-containing fractions. DNA-cellulose fractions containing high (fraction 50, Figure 3) or low (fraction 47) specific transcribing activity were TCA precipitated, separated on 10% SDS-polyacrylamide gels, and blotted onto PVDF membranes (in duplicate). The membranes were stained with Coomassie blue (A) or probed with *B. subtilis* polyclonal, anti-σ^{43} antiserum (B) as described in the text. Vegetative *B. subtilis* whole-cell extract (5 µg, lane 1), *E. coli* RNA polymerase holoenzyme (1.2 µg, lane 2), *M. xanthus* DNA-cellulose fraction 47 (65 µl, lane 3) and fraction 50 (65 µl, lane 4). *B. subtilis* σ^{43}, *E. coli* σ^{70}, and two proteins detectable only in *M. xanthus* holoenzyme-containing fractions are indicated in the margin.

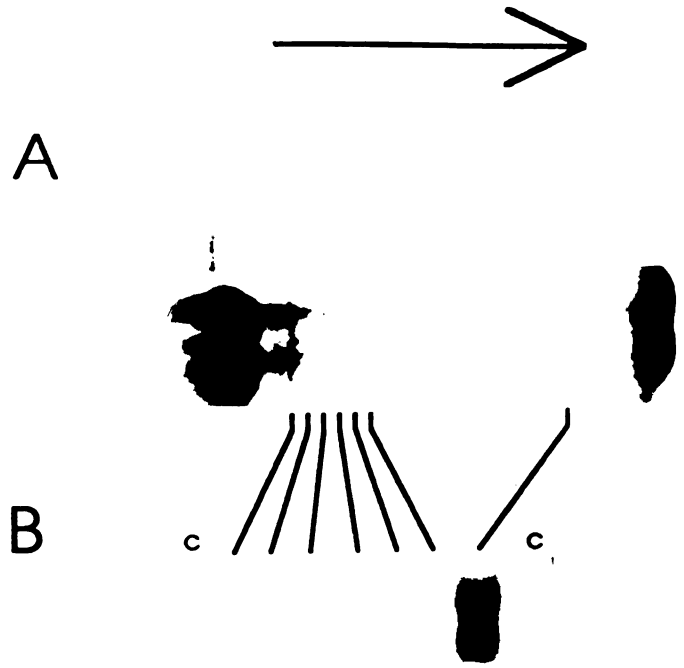


Figure 7. Isolation of an *M. xanthus* transcription stimulating protein. (A) A holoenzyme-containing fraction (1 ml of pooled DNA-cellulose fractions 52 and 53, Figure 2) was TCA-precipitated, the proteins were separated on a 14-cm 10% SDS-polyacrylamide gel and were visualized by Coomassie blue staining (lower lane). *E. coli* holoenzyme (1 μg) was used as a marker (upper lane). The direction of migration through the gel is indicated by an arrow. *M. xanthus* proteins were subsequently purified from the indicated gel slices (1 mm) and used in reconstitution experiments. (B) Run-off transcripts from the *B. subtilis veg* promoter were produced with reconstituted *M. xanthus* polymerase. *M. xanthus* core polymerase (2 μl of fraction 47, Figure 1) and either guanidine dilution buffer (8 μl as a control, lane C) or gel-purified protein (8 μl from the corresponding gel slices in panel [A]) were mixed and incubated on ice for 10 minutes prior to the addition of template (pMS530 digested with *BamHI*). Transcription reactions were performed as described in the Materials and Methods. The products were separated on a 5% polyacrylamide-urea gel and were visualized by autoradiography. Only the region of the gel containing the run-off transcript is shown.

eluted, denatured, and renatured using the Hager-Burgess procedure (Figure 7A). When proteins from gel slices in the 70-90 kDa range are mixed with *M. xanthus* core polymerase and are tested for the production of run-off transcripts from the *B. subtilis veg* promoter, no stimulation of transcription is observed (Figure 7B). These results indicate that either the concentration of proteins eluted from the gel slices in the 70-90 kDa range is too low to stimulate transcription or that none of the 70-90 kDa proteins is the missing σ factor(s).

In contrast, a protein which was eluted from the 50 kDa gel slice has a strong transcription-stimulating activity (Figure 7B). This 50 kDa protein is capable of stimulating both *M. xanthus* and *E. coli* core polymerase about 20-fold (Figure 4, lanes 3 and 6) on the *Bacillus veg* promoter, but is unable to stimulate *B. subtilis* core (data not shown). Additionally, upon re-examination of a silver-stained SDS-polyacrylamide gel of proteins across the elution gradient (Figure 5), a protein clearly present in holoenzyme-containing fractions (fraction 50-55) that migrates at approximately 50 kDa is less abundant or absent in early core fractions (Figure 5, fractions 46 and 47 indicated in the margin). Whether this protein is the 50 kDa protein that stimulates transcription or merely migrates to the same position is unknown. Overall, these results suggest that a 50 kDa *M. xanthus* protein may be a vegetative σ factor.

DISCUSSION

Regulation of transcription initiation by the use of alternative σ factors has been postulated as a mechanism involved in developmental gene expression during *M. xanthus* differentiation. Initial purification of RNA polymerase from vegetative *M. xanthus* was attempted to obtain core RNA polymerase for use in future reconstitution experiments aimed at identifying developmental transcription factors and to gain an appreciation for the difficulties that might be encountered in the isolation of developmental RNA polymerase. RNA polymerase was purified from vegetative *M. xanthus* using a relatively simple procedure (73, 74) including double-stranded DNA-cellulose chromatography. Roughly 1.8 mg of total polymerase (core and holoenzyme) was obtained from 80 g of cells. This yield was lower than that reported by Rudd and Zusman (73 at the same point in their purification). Much of this difference can be attributed to the overall lower levels of protein consistently observed in the low speed supernatant fraction. The low yield may increase the difficulty of purifying developmental polymerase since large amounts of starting material are not easy to obtain. By washing the cells prior to lysis to remove extracellular proteases, achieving better cell lysis (perhaps by including a sonication step), and decreasing the

initial centrifugation speed used to prepare the low speed supernatant, yields may be increased.

Polymerase subunits α, β, and β' are clearly the most abundant proteins by SDS-polyacrylamide gel analysis after the DNA-cellulose column. No easily identifiable σ-factor is present (Figure 1), suggesting that most of the polymerase obtained is core RNA polymerase. However, all fractions are contaminated with some σ as at least low levels of run-off transcripts from the *Bacillus veg* promoter are produced even by "core" fractions (Figure 3, fractions 46-48). The contaminating proteins (including σ) can possibly be removed by chromatography on a phosphocellulose (83) or Bio-Rex 70 column (74); at low salt concentration, core RNA polymerase binds to these columns while σ may be released into the flow-through. Alternatively, core RNA polymerase can be separated from holoenzyme by single-stranded DNA cellulose chromatography using a salt step-elution (74, 85) in combination with Bio-Rex chromatography.

The transcriptional activity of *Myxococcus* core polymerase on the *veg* promoter can be stimulated by supplementation with either gel-purified σ^{70} or a gel-purified 50 kDa *M. xanthus* protein (Figure 4). Reconstitution of developmental transcription may not be as simple. DNA located up to 2 kbp from the transcription start site of several development-specific genes (*ops* and *tps* [56, 57], *mbhA* [58]) appears to be involved in the regulation of their expression. Furthermore, gel mobility

retardation experiments have demonstrated the presence of developmental DNA binding proteins in crude cell extracts of *M. xanthus* which recognize an *ops* upstream activation sequence (57). These observations suggest that additional developmental activators and repressors may complicate transcription reconstitution experiments. Nonetheless, vegetative core RNA polymerase provides a convenient starting point upon which to build the additional transcription machinery.

The gene for the major vegetative σ factor in *M. xanthus* (*sigA*) has been cloned based on its homology to the *rpoD* gene of *E. coli* (82). In amino acid sequence comparisons, the carboxy-terminal domain of the *Myxococcus* σ-factor shares 78% similarity with *E. coli* σ^{70}. In particular, region 2 which is involved in core binding and recognition of the -10 sequence shows only 3 conservative changes between these two σ factors. As predicted by the structural analysis, vegetative RNA polymerase purified from *M. xanthus* recognizes promoters with the *E. coli* σ^{70} consensus sequence (Figure 5). On the other hand, neither the *vegA* promoter from *Myxococcus* (69) which conforms only weakly to the same consensus sequence (specifically at the -10 region) nor the A-dependent, Ω4521-associated devlopmental promoter (81) is able to serve as template. These observations can potentially be explained by several hypotheses. The amount of σ-saturated enzyme obtained at this point in the purification may not have been sufficient

to stimulate transcription from these promoters. Alternatively, a minor vegetative σ factor, a developmental σ factor (in the case of the Ω4521-associated promoter), or additional activators may be needed. Finally, a repressor that copurifies with the polymerase may inhibit its activity on these promoters.

Although the primary goal of this study was to purify core RNA polymerase from *M. xanthus*, the fact that an easily identifiable σ factor was not present in any fraction during the purification (even those with the highest levels of specific transcribing activity) was quite puzzling. Previous experiments have demonstrated the presence of two proteins (designated σI and σII) which copurify with *M. xanthus* vegetative polymerase activity (73). Additionally, antibodies raised against *Bacillus* σ^{43} have been shown to recognize 3 proteins (with apparent molecular weights of 86, 80 and 51 kDa) in *Myxococcus* whole-cell extracts (82). When anti-σ^{43} antibodies are used to probe *M. xanthus* core and holoenzyme-containing fractions, 2 proteins present only in holoenzyme fractions are detectable amidst the background (Figure 6). These proteins appear to migrate at positions corresponding to the lower 2 bands seen by Inouye (82) in her Western analysis. Proteins from both these regions were gel-purified and tested for transcription stimulating activity, but only the 50 kDa region contains a protein which acts with core RNA polymerase to transcribe the *Bacillus veg* promoter (Figure 7; although the larger 80 kDa

protein could possibly also have stimulated transcription if present at higher concentration). Furthermore, a protein migrating to the 50 kDa position of an SDS-polyacrylamide gel is slightly more abundant in holoenzyme fractions than in core fractions (Figure 5). Together, these observations suggest that a 50 kDa protein is the missing σ-factor.

Evidence seems to indicate that the 50 kDa protein is a degradation product of a larger σ factor. First, Rudd and Zusman (73) described protease activity as a "substantial problem" in their attempts to purify vegetative *M. xanthus* polymerase. Of the 2 closely-related proteins (σI and σII) coeluting with polymerase activity, σII was postulated to be a degradation product of σI. Second, bands migrating with the lower 2 of 3 proteins identified by Inouye using Western analysis (82) are detectable in holoenzyme-containing fractions (Figure 5). Efforts to repeat Inouye's results using whole cell extracts from both DK1622 and DZF1 (86) (the strain originally used by Rudd and Zusman [73] and Inouye [82]) have failed to detect the largest σ-band. Finally, *M. xanthus* holoenzyme appears to recognizes the *E. coli* σ^{70} consensus sequence and the 50 kDa protein present in holoenzyme fractions can stimulate *E. coli* core transcriptional activity. These results suggest that the 50 kDa protein and *E. coli* σ^{70} share structural and functional similarities in the regions necessary for core and promoter binding, which was predicted previously from sequence comparison of the major *Myxococcus* vegetative σ

factor (*sigA*) and *E. coli* σ^{70} (*rpoD*) genes (82). Taken together, these observations suggest that the 50 kDa protein and the product of the *sigA* gene are related. The 50 kDa protein might, however, be an entirely different vegetative σ-factor or a transcriptional activator. Multiple vegetative σ factors have been identified in *S. coelicolor* (66). Unequivocal identification of the 50 kDa protein awaits purification of quantitative amounts for N-terminal sequencing.

In conclusion, the major goal of the polymerase study was achieved; namely, to purify core RNA polymerase from *M. xanthus* for future reconstitution experiments with developmental factors. Identification of developmental σ factors and transcription regulators will likely require further minimization of protease activity, a maximization of polymerase yields, as well as the availability of a variety of well-characterized, developmentally-regulated promoters. Ultimately, by reconstructing the transcription of signal-dependent genes, the molecular events coupling changes in gene expression to intercellular communication will begin to be elucidated.

SUMMARY AND CONCLUSIONS

All organisms, regardless of their complexity, need mechanisms to sense and respond to an ever-changing environment. When challenged with nutrient limiting conditions, thousands of seemingly simple myxobacteria cooperatively participate in a developmental program that culminates in the production of environmentally-resistant spores. The multicellular behaviors and social interactions exhibited by *M. xanthus* during its differentiation can serve as models for higher developmental systems.

A complete understanding of the role of cell interactions in the *M. xanthus* developmental pathway requires knowledge about many components. At the start of the pathway lies the extracellular stimulus whose interactions with the sensory machinery commences differentiation. At the end, rests the morphogenic consequences of a tightly and temporally regulated program of gene expression: intricate fruiting bodies and coat-protected spores. In between, an unknown array of activators, repressors, and modifiers functions to couple the intercellular signalling events and developmental gene activation. The experiments described in this thesis have aimed at the eventual identification of these intracellular mediators.

One of at least four signaling events, the transmission of C-signal is required for the progression of development past loose aggregation and is also necessary for proper gene expression. DNA adjacent to C-dependent Tn5*lac* insertions was cloned to define regions that are important for regulated gene expression. DNA within 1.8 kbp of the Ω4414 insertion is incapable of promoting developmental β-galactosidase activity suggesting that additional regulatory regions further upstream may be required for gene expression. In contrast, DNA within 2 kbp of the Ω4403 insertion directs developmental β-galactosidase activity. Transcription from the Ω4403-associated promoter initiates approximately 380 bp from the insertion point and the levels of transcript are developmentally regulated. This same 2 kbp regulatory region at least partially mediates the C-signal dependence of gene expression. An additional regulatory region lies between 2 and 8.5 kbp upstream of the Tn5*lac* Ω4403 fusion and appears to positively affect the level of β-galactosidase activity. DNA even further than 8.5 kbp from the insertion site may participate in activating gene expression since *lacZ* fused to the 8.5 kbp Ω4403 upstream region produces only 25% of the maximum β-galactosidase activity observed in the original insertion-containing strain. Alternatively, the reduced expression may reflect a position effect (dependent on the chromosomal location of integration) or autorepression by the product of the Ω4403-associated gene.

Since the Ω4403-associated transcript is differentially expressed throughout development, changes in β-galactosidase activity are most likely due to changes in the level of transcript. Although the mechanism of this mRNA accumulation can not be definitively ascertained yet, by analogy to other systems, modulation of transcription initiation is likely to be involved. Core RNA polymerase was purified from vegetative *M. xanthus* for use in reconstitution experiments with other developmental factors. Core RNA polymerase, followed immediately by holoenzyme, elutes from a double-stranded DNA-cellulose column within a shallow salt gradient. The transcriptional activity of core polymerase on the *B. subtilis veg* promoter can be stimulated by both *E. coli* σ^{70} and an approximately 50 kDa *M. xanthus* protein purified from holoenzyme fractions. The identity of the 50 kDa protein is currently unknown; it may be a degradation product of two previously described σ-factors (73, 82), a new vegetative σ factor, or a potent transcriptional activator.

M. xanthus holoenzyme appears to recognize bacterial promoters with *E. coli* σ^{70} consensus sequences. Both the *B. subtilis veg* and *E. coli lac*UV5 promoters are efficiently transcribed *in vitro*; transcription from the *M. xanthus vegA* promoter or Ω4521-associated promoter has not been observed. These latter two promoters differ noticeably from the σ^{70} consensus sequence and transcription from them may require additional activators, alternative σ factors, or the

removal of a repressor.

By beginning near the end of the signal transduction pathway and working backwards through the machinery, the mechanisms coupling gene expression to intercellular signaling will be revealed. Information obtained by studying *M. xanthus* cell interactions should be generally applicable to other developmental systems.

LIST OF REFERENCES

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1. Reichenbach, H. (1984). Myxobacteria: A most peculiar group of social prokaryotes, pp 1-50. *In* E. Rosenberg (ed.), *Myxobacteria: Development and cell interactions*, Springer Verlag, New York.

2. Burchard, R. P. (1984). Gliding motility and taxes, pp 139-161. *In* E. Rosenberg (ed.), *Myxobacteria: Development and cell interactions*, Springer Verlag, New York.

3. Wireman, J. W. and M. Dworkin. (1975). Morphogenesis and developmental interactions in myxobacteria. Science **189**, 516-522.

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

5. Kaiser, D. (1984). Regulation of multicellular development in myxobacteria, pp 197-218. *In* R. Losick and L. Shapiro (eds.), *Microbial development*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

6. Sudo, S. Z., and M. Dworkin. (1969). Resistance of vegetative cells and microcysts of *Myxococcus xanthus*. J. Bacteriol. **98**, 883-887.

7. Hagen, D., A. Bretscher, and D. Kaiser. (1978). Synergism between morphogenetic mutants of *Myxococcus xanthus*. Dev. Biol. **64**, 284-296.

8. 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.

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

10. Kim, S., and D. Kaiser. (1990). C-factor: A cell-cell signaling protein required for fruiting body morphogenesis of *Myxococcus xanthus*. Cell **61**, 19-26.

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

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

13. Kroos, L., A. Kuspa, and D. Kaiser. (1990). Defects in fruiting body development caused by Tn5*lac* insertions in *Myxococcus xanthus*. J. Bacteriol. **172**, 484-487.

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

15. Cheng, Y., and D. Kaiser. (1989). *dsg*, a gene required for cell-cell interactions early in *Myxococcus* development. J. Bacteriol. **171**, 3719-3726.

16. Campos-Ortega, J. A. (1988). Cellular interactions during early neurogenesis of *Drosophila melanogaster*. Trends Neurosc. **11**, 400-404.

17. Thaller, C. and G. Eichele. (1987). Identification and spatial distribution of retinoids in the developing chick limb bud. Nature **327**, 625-628.

18. Devreotes, P. (1989). *Dictyostelium discoideum*: a model system for cell-cell interactions in development. Science **245**, 1054-1058.

19. Chen, H.-W., I. M. Keseler, and L. J. Shimkets. (1990). The genome size of *Myxococcus xanthus* determined by pulse-field gel electrophoresis. J. Bacteriol **172**, 4206-4213.

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

21. 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.

23. O'Connor, K. A., and D. R. Zusman. (1983). Coliphage P1 mediated transduction of cloned DNA from *Escherichia coli* to *Myxococcus xanthus*: use for complementation and recombinational analyses. J. Bacteriol. **155**, 317-329.

23. Martin, S., E. Sodergren, T. Masuda, and D. Kaiser. (1978). Systematic isolation of transducing phages for *Myxococcus xanthus*. Virology **88**, 44.

24. 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.

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

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

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

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

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

30. Kim, S., and D. Kaiser. (1990). Cell motility is required for the transmission of C-factor, an intercellular signal that coordinates fruiting body morphogenesis of *Myxococcus xanthus*. Genes @ Devel. **4**, 896-905.

31. Kim, S., and D. Kaiser. (1990). Cell alignment is required in differentiation of *Myxococcus xanthus*. Science **249**, 926-928.

32. Losick, R., and J. Pero. (1981). Cascades of sigma factors. Cell **25**, 582-584.

33. Losick, R., and L. Kroos (1989). Dependence pathways for the expression of genes involved in endospore formation in *B. subtilis*, pp 223-241. *In* I. Smith, R. A. Slepecky, and P. Setlow. (eds.), American Society for Microbiology, Washington D. C.

34. Newton, A., N. Ohta, G. Ramakrishnan, D. A. Mullin, and G. Raymond. (1989). Genetic switching in the flagellar gene hierarchy requires both positive and negative regulation of transcription. Proc. Natl. Acad. Sci. USA **86**, 6651-6665.

35. Xu, H., A. Dingwall, and L. Shapiro. (1989). Negative transcriptional regulation in the *Caulobacter* flagellar hierarchy. Proc. Natl. Acad. Sci. USA. **76**, 6656-6660.

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

37. Rosner, J. L. (1972). Formation, induction and curing of bacteriophage P1 lysogens. Virology **48**, 679-689.

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

39. Messing, J. (1979). A multipurpose cloning system based on the single-stranded DNA bacteriophage M13. Recombinant DNA bulletin, NIH Publication No 71-99; 2, No 2, 43-48.

40. Meissner, P. S., W. P. Sisk, and M. L. Berman. (1987). Bacteriophage λ cloning system for the construction of directional cDNA libraries. Proc. Natl. Acad. Sci. USA. **84**, 4171.

41. Li, S. and L. Shimkets. (1988). Site-specific integration and expression of a developmental promoter in *Myxococcus xanthus*. J. Bacteriol. **170**, 5552-5556.

42. Gill, R. E. Personal communication.

43. Kroos, L. Unpublished data. DK5208 was constructed by growing Mx8*c1p2* (23) on the *csgA* donor strain LS523 (*csgA-205* Tc^r [44]) and transducing DK1622 to tetracycline resistance.

44. Shimkets, L. J., and S. J. Asher. (1987). Use of recombination techniques to examine the structure of the *csg* locus of Myxococcus xanthus. Mol. Gen. Genet. **211**, 63-71.

45. Maniatis, T., E. F. Fritsch, and J. Sambrook. (1989). Molecular cloning; a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

46. Stellwag, E., J. M. Fink, and J. Zissler. (1985). Physical characterization of the genome of the *Myxococcus xanthus* bacteriophage Mx8. Mol. Gen. Genet. **199**, 123-132.

47. Sternberg, N., D. Tiemeier, and L. Enquist. (1977). *In vitro* packaging of a λ *Dam* vector containing *EcoR11* DNA fragments of *Escherichia coli* and phage P1. Gene **1**, 255-280.

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

49. Miller, J. (1972). Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

50. Bradford, M. (1976). A rapid and sensitive method for quantization of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **73**, 248-254.

51. Igo, M. M., and R. Losick. (1986). Regulation of a promoter that is utilized by minor forms of RNA polymerase holoenzyme in *Bacillus subtilis*. J. Mol. Biol. **191**, 615-624.

52. Burton, Z. F., C. A. Gross, K. K. Watanabe, and R. R. Burgess. (1983). The operon that encodes the sigma subunit of RNA polymerase also encodes ribosomal protein S21 and DNA primase in *E. coli* K12. Cell **32**, 335-349.

53. Gilman, M. Z. and M. J. Chamberlin. (1983). Developmental and genetic regulation of *Bacillus subtilis* genes transcribed by σ^{28}-RNA polymerase. Cell **35**, 285-293.

54. White, J. Unpublished data.

55. 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.

56. Downard, J., S.-H. Kim, and K.-S. Kil. (1988). Localization of the cis-acting regulatory DNA sequences of the *Myxococcus xanthus tps* and *ops* genes. J. Bacteriol. **170**, 4931-4938.

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

58. Apelian, D., and S. Inouye. (1990). Development-specific σ-factor essential for late-stage differentiation of *Myxococcus xanthus*. Gene @ Devel. **4**, 1396-1403.

59. Hama, C., S. Ali, and T. B. Kornberg. (1990). Region specific recombination and expression are directed by portions of the *Drosophila engrailed* promoter. Gene @ Devel. **4**, 1079-1093.

60. Banerjii, J., S. Rusconi, and W. Schaffner. (1981). Expression of a β-globin gene is enhanced by remote SV40 DNA sequences. Cell **27**, 299-308.

61. Moreau, P., B. Wasylyk, R. Everett, M. P. Gaub, and P. Chambon. (1981). The SV40 72 base pair repeat has a striking effect on gene expression both in SV40 and other chimeric recombinants. Nuc. Acids Res. **9**, 6047-6067.

62. Lewis, E. B. (1950). Advan. Genet. **3**, 73-115.

63. Feinstein, S. C., S. R. Ross, and K. R. Yamamoto. (1982). Chromosomal position effects determine transcriptional potential of integrated mammary tumor virus DNA. J. Mol. Bio. **156**, 549-565.

64. Hunt, T. P. and B. Magasanik. (1985) Transcription of *glnA* by purified *E. coli* components: core RNA polymerase and the products of *glnF, glnG*, and *glnL*. Proc. Natl. Acad. Sci. USA. **82**, 8453-8457.

65. Grossman, A. D., J. W. Erickson, and C. A. Gross. (1984) The *htpR* gene product of *E. coli* is a σ factor for heat shock promoters. Cell **38**, 383-390.

66. Buttner, M. J. (1988). RNA polymerase heterogeneity in *Streptomyces coelicolor* A3(2). Mol. Micro. **3**, 1653-1659.

67. Chater, K. F., C. J. Bruton, K. A. Plaskitt, M. J. Buttner, C. Mendez, and J. D. Helman. (1989). The developmental fate of *S. coelicolor* hyphae depends upon a gene product homologous with the motility σ factor of *B. subtilis*. Cell **59**, 133-143.

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

69. 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. Bio. **196**, 517-524.

70. Downard, J. S. (1987). Identification of the RNA products of the *ops* gene of *Myxococcus xanthus* and mapping of *ops* and *tps* RNAs. J. Bacteriol. **169**, 1522-1528.

71. 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. Bio. **175**, 469-492.

72. Beynon, J., M. Cannon, V. Buchanan-Wollaston, and F. Cannon. (1985). The *nif* promoters of *Klebsiella pneumoniae* have a characteristic primary structure. Cell **34**, 665-671.

73. Rudd, K., and D. Zusman. (1982). RNA polymerase of *Myxococcus xanthus*: purification and selective transcription *in vitro* with bacteriophage templates. J. Bacteriol. **151**, 89-105.

74. Burgess, F., and J. Jendrisak. (1975). A procedure for the rapid large-scale purification of *Escherichia coli* DNA-dependent RNA polymerase involving polymin P precipitation and DNA-cellulose chromatography. Biochemistry **14**, 4634-4638.

75. Jendrisak, J. J. and R. R. Burgess. (1975). A new method for the large scale purification of wheat germ DNA-dependent RNA polymerase II. Biochemistry **14**, 4639-4645.

76. Kroos, L., B. Kunkel, and R. Losick. (1989). Switch protein alters specificity of RNA polymerase containing a compartment-specific σ factor. Science **243**, 526-529.

77. Hager, D., and R. Burgess. (1980). Elution of proteins from sodium dodecyl sulfate-polyacrylamide gels, removal of sodium dodecyl sulfate, and renaturation of enzymatic activity: results with sigma subunit of RNA polymerase, wheat germ DNA, topoisomerase, and other enzymes. Anal. Biochem. **109**, 76-86.

78. Matsudaira, P. (1987). Sequence from picomole quantities of proteins electroblotted onto a polyvinylidene difluoride membrane. J. Biol. Chem. **262**, 10035-10038.

79. Moran, C., N. Lang, S. LeGrice, G. Lee, M. Stephens, A. Sonenshein, J. Pero, and R. Losick. (1982). Nucleotide sequences that signal the initiation of transcription and translation in *Bacillus subtilis*. Mol. Gen. Genet. **186**, 339-346.

80. Silverstone, A., R. Arditi, and B. Magasanik. (1970). Catabolite-insensitive repression of *lac* promoter mutants. Proc. Natl. Sci. Acad. USA. **66**, 773-779.

81. Kaplan, H. B. and D. Kaiser. Personal communication.

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

83. Burgess, R. R., A. A. Travers, J. J. Dunn, and E. K. F. Bautz. (1969). Factor stimulating transcription by RNA polymerase. Nature **221**, 43-46.

84. Lowe, P. A., D. A. Hager, R. R. Burgess. (1979). Purification and properties of the σ subunit of *Escherichia coli* DNA-dependent RNA polymerase. Biochemistry **18**, 1344-1352.

85. Morrison, C. E., and D. R. Zusman. (1979). *Myxococcus xanthus* mutants with temperature-sensitive, stage specific defects: evidence for independent pathways in development. J. Bacteriol. **140**, 1036-1042.