Development-specific σ -factor essential for late-stage differentiation of *Myxococcus xanthus*

David Apelian and Sumiko Inouye

Department of Biochemistry, Robert Wood Johnson Medical School-UMDNJ at Rutgers, Piscataway, New Jersey 08854 USA

The gene for a developmentally expressed σ -factor, *sigB*, has been isolated from *Myxococcus xanthus* by use of the *sigA* gene (formerly *rpoD*) of the vegetative σ -factor as a probe. The sequence of *sigB* has been determined, and an open reading frame of 193 amino acid residues ($M_r = 21,551$) was identified. The amino-terminal region of SigB contains 69 residues, of which 35 are identical (50% identity) to the region of SigA required for core RNA polymerase binding and initiation of RNA polymerization. SigB also possesses many features commonly found in other prokaryotic σ -factors. Analysis of an *M. xanthus* strain carrying a *sigB-lacZ* fusion gene revealed that *sigB* is expressed from a middle to late stage of differentiation corresponding to the period from the onset of sporulation to late development. A *sigB* deletion mutant displayed normal mound formation and sporulation; however, production of the *ops* gene product in myxospores of the Δ *sigB* strain was shown to be blocked. Myxospores from the *sigB* deletion strain also exhibited severe defects in stability and viability during late development. Our data indicate that *sigB* encodes a σ -factor essential for the maturation of myxospores at a late stage of *M. xanthus* differentiation. Our results also suggest that differentiation of *M. xanthus* is regulated by development-specific σ -factors.

[Key Words: Myxobacteria; sporulation; fruiting body formation]

Received February 12, 1990; revised version accepted May 22, 1990.

Myxococcus xanthus is a gram-negative, soil-dwelling bacterium that moves by gliding on solid surfaces (for reviews, see Rosenberg 1984; Kaiser 1986; Shimkets 1987). *M. xanthus* grows vegetatively as a predator using the cooperative motility of many cells to surround and digest prey microorganisms. During nutritional starvation, cooperative motility is employed in the formation of multicellular resting structures called fruiting bodies. A portion of the rod-shaped cells within the fruiting bodies differentiates into spherical myxospores. Myxospores are resistant to heat, UV irradiation, dessication, and sonication. Myxospores are also metabolically dormant, enabling them to withstand prolonged periods of nutrient deprivation. This elaborate developmental strategy ensures that a highly localized population of spores can respond to favorable nutritional conditions as a multicellular, predatory colony.

The developmental cycle of *M. xanthus* has been shown to be executed by the appropriately timed expression of development-specific genes (Inouye et al. 1979; Kroos et al. 1986). Developmental regulation by gene selectivity is energetically efficient and well suited for response to nutritional starvation. Two well-characterized genes of *M. xanthus*, *ops* and *tps*, are developmentally regulated. These genes share 90% homology and are separated by 1.4 kb in the same orientation on the *M. xanthus* chromosome (Inouye et al. 1983a,b). Despite

their extensive similarity, the *ops* and *tps* genes are differentially expressed during development (Downard et al. 1984; Downard and Zusman 1985; Teintze et al. 1985). A more general analysis of gene regulation has been achieved by use of the random insertion of a *lacZ* gene into the *M. xanthus* chromosome (Kroos et al. 1986). This was accomplished by use of a Tn5-*lac* fusion construction capable of inserting in the proper orientation downstream of *M. xanthus* promoters (Kroos and Kaiser 1984). In this manner, 29 distinct *M. xanthus* strains were isolated that exhibited characteristic β -galactosidase expression at various stages of development. The aforementioned studies provide a body of evidence that indicates that *M. xanthus* development is governed by the orchestrated expression of stage-specific gene sets.

The temporal and spatial regulation of gene expression in the eubacteria has been shown to be governed largely by heterogeneous RNA polymerase σ -factors (Doi and Wang 1986). The σ -subunit aids the RNA polymerase enzyme in promoter recognition and transcription initiation (Helmann and Chamberlin 1988; Ishihama 1988). Studies on endospore formation in *Bacillus subtilis* demonstrate the means by which changes in the composition of the σ population of a cell cause corresponding changes in the specificity of gene expression (Losick et al. 1986). Stage-specific σ -factors in *B. subtilis* have been shown to temporally regulate gene expression in a cas-

cade-like fashion during endospore formation (Losick and Pero 1981). It has been demonstrated in *B. subtilis* that σ^{29} -directed gene expression is morphologically coupled to the formation of the sporulation septum (Stragier et al. 1988). This coupling is an effective means by which *B. subtilis* ensures the proper temporal regulation of its developmental σ cascade. Even more remarkably, a compartment-specific σ -factor is exclusively produced in the terminally differentiating mother cell compartment of *B. subtilis* during development by a chromosomal recombination event (Stragier et al. 1989). This σ -factor σ^K governs the expression of endospore coat proteins that are deposited from the mother cell compartment to the endospore surface (Donovan et al. 1987).

The major vegetative σ gene of *M. xanthus*, named *sigA* (formerly *rpoD*), has recently been cloned and sequenced (Inouye 1990). The deduced amino acid sequence of *sigA* displays extensive similarity to the major vegetative σ -factors of *Escherichia coli* and *B. subtilis*. In the present study the highly conserved carboxy-terminal coding region of the *M. xanthus sigA* gene has been used as a probe, and four additional *M. xanthus* genomic fragments have been cloned. We determined the DNA sequence of one of these fragments and found that it contains an open reading frame of 193 codons, designated *sigB*. The deduced amino acid sequence of the *sigB* product shows extensive similarity to the carboxy-terminal region of the *M. xanthus sigA* product and possesses many features commonly found in σ -factors of various origins. A deletion mutant of *sigB* has been constructed that exhibits severe defects in the stability and viability of myxospores during late development. Expression of the *ops* product in myxospores of the $\Delta sigB$ strain was also shown to be blocked. In contrast, the *sigB* deletion does not affect vegetative growth, fruiting mound formation, and early sporulation. Studies of a *lacZ* fusion of the *sigB* gene demonstrate that the *sigB* gene is expressed almost exclusively within myxospores from a middle to late stage of development. The stage-specific action of *sigB* and the hybridization of several genomic fragments to a *sigA* probe suggest that the differentiation of *M. xanthus* is regulated by a cascade of developmental σ -factors.

Results

Cloning and sequencing of *sigB*

The gene encoding the *M. xanthus* major vegetative σ -factor, *sigA* has recently been cloned and sequenced (Inouye 1990). The highly conserved carboxy-terminal region of this gene (*Pst*I–*Xho*I) was used to probe various restriction digests of *M. xanthus* chromosomal DNA (Fig. 1). By optimizing conditions of hybridization stringency, distinct secondary bands were observed in addition to the prominent *sigA* band in each restriction digest. The *Xho*I digest of the chromosomal DNA yielded four distinct bands that hybridized with the *sigA* probe at 5.5, 6.3, 7.4, and 8.4 kb, in addition to the 2.7-kb *sigA* fragment. In the present work, we attempted to charac-

Figure 1. Southern blot analysis of the chromosomal DNA from *M. xanthus* DZF1 with nick-translated *M. xanthus sigA* as a probe. The DNA was digested with *Eco*RI (lane 1), *Bam*HI (lane 2), *Pst*I (lane 3), *Sal*I (lane 4), *Xho*I (lane 5), and *Sma*I (lane 6). In each lane, 3 μ g of the DNA digest was applied, and blot hybridization analysis was carried out, as described in Materials and methods. Numbers at left represent molecular weights (in kb).

terize the 7.4-kb fragment. For this purpose, this fragment was cloned into the unique *Xho*I site of pUX (Dhundale et al. 1988), and the clone was identified by the *sigA* probe. The clone designated pUXsigB was digested further with various endonucleases, and the restriction map of the 7.4-kb *Xho*I fragment is shown in Figure 2. The 3-kb *Sma*I fragment located within the 7.4-kb fragment was found to hybridize with the *sigA* probe. The 3-kb *Sma*I fragment was subcloned into the unique *Sma*I site of pUC9 (Vieira and Messing 1982). Subsequently, a DNA sequence of 918 bp within the *Sma*I fragment was determined, as shown in Figure 3. In this DNA sequence, an open reading frame (ORF) of 193 amino acid residues, designated *sigB*, can be found, as shown in Figure 3. As will be discussed later (see Fig. 7), the amino-terminal sequence of 69 amino acid residues in SigB contains extensive similarity to the region of SigA and several other prokaryotic σ -factors. This region is known to be required for interaction of the σ -factor with the core RNA polymerase and for the initiation of RNA polymerization by the holoenzyme.

sigB ORF characterization

The ORF of *sigB* was established on the basis of the complete identity of SigB with SigA in amino acid res-

Figure 2. Restriction map of the 7.4-kb DNA fragment encompassing the *M. xanthus sigB* gene and a definitive map of the *sigB* gene derived from the sequence data. Arrows represent the *sigB* ORF and its orientation. (X) *Xho*I; (S) *Sal*I; (Sm) *Sma*I; (B) *Bam*HI; (P) *Pst*I; (R) *Rsa*I; (T) *Taq*I; (M) *Mbo*II; (N) *Nco*I.

idues 25–38 of SigB. Two stop codons are located in-frame at bases 868–870 and 883–885. The designation of the initiation codon at bases 289–291 is supported by the codon usage upstream and downstream of this start site by use of the predicted reading frame. G and C bases constitute only 46.4% of the third codon position within the 288 bases upstream of the predicted start codon and only 58.8% of the third codon position within the 48 bases downstream of the first predicted stop codon. In contrast, G and C bases comprise 92.8% of the third codon position in the predicted ORF. *M. xanthus* chromosomal DNA possesses a GC content of 70% (Johnson and Ordal 1968) and would be expected to select for G and C bases at the third codon position in the ORF. SigA demonstrates 94% GC content at its third codon positions (Inouye 1990). A purine-rich Shine–Dalgarno-like sequence (GGCGG) is located 10 bases upstream of the initiation codon, further supporting the designated start codon. The deduced amino acid sequence of SigB has a molecular weight of 21,551 and an estimated pI of 8.25. High levels of both acidic (13.5%) and basic (15.5%) residues are present in SigB, which is a characteristic feature of σ -factors (Helmann and Chamberlin 1988). A detailed analysis of the similarity of SigB to other characterized σ -factors will be discussed later.

sigB–*lacZ* fusion analysis

To determine the circumstances under which SigB is expressed, we constructed a fusion of the *sigB* gene with the *lacZ* gene of *E. coli* and inserted it into the chromosome of *M. xanthus*. A *Bam*HI site that cuts in-frame within the 3' end of *sigB* (after codon 181; see Fig. 3) was joined to a *Bam*HI site that cuts in-frame within the 5' end of *lacZ*. This fusion construction put the *lacZ* gene under the transcriptional regulation of the *sigB* promoter and potential upstream regulatory regions. This *sigB*–*lacZ* fusion was inserted into the *M. xanthus* chromosome by use of specialized P1 transduction (Shimkets et al. 1983). Transduction by this method results in the tandem arrangement of the *sigB*–*lacZ* gene, followed by the wild-type *sigB* gene. It should be noted that the wild-type *sigB* gene existing in the fusion strain, desig-

nated B/Z, is fully functional during development. When the B/Z strain was plated on CF (clone-fruited; Hagen et al. 1978) agar containing X-gal and incubated at 30°C, it developed normally like the wild-type strain. After ~20 hr of incubation, blue color induction was observed in the B/Z strain. This corresponds to the early mound formation stage of development. The blue color increased in intensity with prolonged incubation. No color induction was observed in the wild-type strain under these conditions.

To investigate β -galactosidase expression in the B/Z strain more closely, the specific activity was measured at time intervals during development, as described by Kroos et al. (1986). *M. xanthus* developing populations contained both rod-shaped cells and spores (O'Connor and Zusman 1988). By use of an improved method of developmental harvest, as described by O'Connor and Zusman (1988), it was observed that the rod-shaped cells are maintained in the developing populations of B/Z until 200 hr of development (Fig. 4B). The wild-type strain showed a pattern of developmental cell type similar to that of B/Z (data not shown). β -Galactosidase activity was assayed throughout the development of the B/Z strain in both the nonspore and spore fractions (as described in Materials and methods). As shown in Figure 4A, β -galactosidase was expressed predominantly in the spore fraction from 30 hr to 150 hr of development. Very low β -galactosidase activity (~2% of that found in the

GCA	CGC	CAA	CCC	CTC	GCA	GAG	ATT	GGG	AAT	GCC	CGT	TCG	GGG	CAG	GTT	GGC	ATT	TCT	GTT	60
GCT	CAA	GTT	CCC	TTC	GTC	ACG	TCG	TCA	GAC	GAA	ATC	CAT	CCA	TCC	CGG	CCT	CCC	GTG	AGG	120
CCC	ACC	GGC	GGA	GAA	ATC	ATT	CAT	GCA	GCC	ATC	CAC	CGA	GCA	GTC	GTC	CAA	CTC	CGG	CTC	180
CCT	GGC	GAT	GTA	GCT	CTC	GGA	GAT	CAA	CCA	CTA	CTC	CCT	CAA	GGT	GGA	GGA	GGA	GCA		240
GGA	GCT	CGC	GGC	CGG	CTT	CCT	CAA	GGG	CGA	CCT	GGC	GGC	CGG	GCA	CCG	ATG	GTG	AGC	CCC	300
																Met	Val	Thr	Ala	
AAC	CTG	CGC	TTC	GTG	GTG	AAG	GTC	GCC	TAT	GAG	TAC	CGC	TCA	TAC	GGC	ATC	AAG	ATG	TGG	360
Asn	Leu	Arg	Phe	Val	Val	Lys	Val	Ala	Tyr	Glu	Tyr	Arg	Ser	Tyr	Gly	Ile	Lys	Met	Ser	
GAC	CTC	ATC	CAG	GAG	GGG	AAC	ATC	GCC	CTG	ATG	AAG	GCC	GTG	CAG	AAG	TTC	GAT	CCG	GAC	420
Asp	Leu	Ile	Gln	Glu	Gly	Asn	Ile	Gly	Leu	Met	Lys	Ala	Val	Gln	Lys	Phe	Asp	Pro	Asp	
AAG	GGC	ATC	CGC	CTC	ATC	TCC	TAC	GGG	GTG	TGG	TGG	ATT	CGC	CGG	TAC	ATC	CAG	AAC	TAC	480
Lys	Gly	Ile	Arg	Leu	Ile	Ser	Tyr	Ala	Val	Trp	Trp	Ile	Arg	Ala	Tyr	Ile	Gln	Asn	Tyr	
ATC	CTC	AAG	AGC	TGG	TGG	CTC	GTG	AAG	CTG	GGG	ACC	ACC	CAG	CGC	CAC	GGG	AAG	CTG	TTC	540
Ile	Leu	Lys	Ser	Trp	Ser	Leu	Val	Lys	Leu	Gly	Thr	Thr	Gln	Ala	His	Gly	Lys	Leu	Phe	
TTC	AGT	CTG	GGC	CGC	ACG	CGC	CGC	GAG	CTG	GAG	AAG	TTT	GGC	AGC	GGT	GAC	GGC	GGG	GTG	600
Phe	Ser	Leu	Ala	Arg	Thr	Arg	Arg	Glu	Leu	Glu	Lys	Phe	Gly	Ser	Gly	Asp	Ala	Ala	Val	
AAC	GTG	GAT	GAC	ATC	GCC	CGC	CGG	CTC	GAC	GTG	AAG	CGG	GGC	GAG	GTG	CGG	GAG	ATG	GAG	660
Asn	Val	Asp	Asp	Ile	Ala	Arg	Arg	Leu	His	Val	Lys	Pro	Gly	Glu	Val	Arg	Glu	Met	Glu	
CAG	CGC	ATG	GGC	GGC	GAC	CTG	TGG	CTG	GAC	GGC	CCC	ATG	GCC	GAG	GAC	GGC	GGC	AAG		720
Gln	Arg	Met	Gly	Gly	Arg	Asp	Leu	Ser	Leu	Asp	Ala	Pro	Met	Gly	Glu	Asp	Gly	Gly	Asn	
AGC	CAC	GTG	GAC	TTC	GTG	GTG	AGC	GGC	GGC	GGC	CGG	CAG	GAC	GAC	GAG	TTC	CGG	GAC	AAG	780
Ser	His	Val	Asp	Phe	Val	Val	Ser	Ala	Ala	Ala	Pro	Gln	Asp	Asp	Glu	Phe	Ala	Asp	Lys	
GAG	GAG	GGC	GGC	CTC	ATC	AAC	GCC	CGC	GTG	CGT	GGC	GGC	CTG	ATG	CGC	CTG*	GAT	CGG	CGT	840
Glu	Glu	Ala	Gly	Leu	Ile	Asn	Ala	Arg	Val	Arg	Ala	Ala	Leu	Met	Arg	Leu	Asp	Pro	Arg	
GAG	CCT	TCA	TCA	TGG	AGC	AAC	GCG	TCA	TGA	ACG	AGC	GCC	CCA	TGA	CGC	TCA	AGG	AAC	TGG	900
Glu	Pro	Ser	Ser	Ser	Ser	Asn	Ala	Ser	OPA											
GTG	AGC	ACT	TGG	GCT	TCT															918

Figure 3. Nucleotide sequence of the *sigB* region and its deduced amino acid sequence. The asterisk (*) identifies the location of the *Bam*HI cleavage site. Numbers at right enumerate the nucleotide bases.

Figure 4. (A) β -Galactosidase-specific activity [in nanomoles of ONP/(per milligram $\times$ minutes)] in the spore fraction of developing *M. xanthus*. (B) The relative population of rods and spores in developing *M. xanthus*, as determined by the method of O'Connor and Zusman (1988).

spore fraction) was observed in the nonspore fraction just prior to and during spore formation (data not shown). It is important to note that the designation of nonspore fractions is based solely on their sensitivity to sonication in the absence of glass beads. Under these conditions, the wild-type strain demonstrated no β -galactosidase activity in either spore or nonspore fractions.

Effects of *sigB* deletion on development

To examine further the role of *sigB* in the development of *M. xanthus*, a *sigB* deletion strain ($\Delta sigB$) was constructed. A 1.5-kb DNA fragment between the *SalI* and *BamHI* sites was replaced by the Tn5 kanamycin resistance gene, as described in Materials and methods.

Vegetative growth of the $\Delta sigB$ strain was completely normal, and fruiting mound formation and early sporulation appeared normal on CF agar plates or in submerged culture (Kuner and Kaiser 1982). The $\Delta sigB$ strain was also capable of sporulation by glycerol induction (Dworkin and Gibson 1964) and by liquid starvation in MCM buffer (Rosenbluh and Rosenberg 1989). However, during prolonged development (from 200 to 500 hr) on CF agar plates, the fruiting mounds of $\Delta sigB$ became increasingly translucent when viewed under a dissecting microscope, whereas wild-type fruiting mounds remained dark and opaque under these conditions (Fig. 5).

The morphological changes that we observed in the

fruiting mounds of $\Delta sigB$ prompted us to analyze the appearance of spores during prolonged development on CF agar plates. Developmental harvests were sonicated both to disrupt nonspores and to disperse the spores for quantitation by phase-contrast microscopy with a Petroff-Hausser chamber. We have defined refractile spores as those spores that were round and phase-contrast refractile. Table 1 shows characteristic data from such an experiment. The wild-type strain maintained a significantly higher level of refractile spores during late development. In contrast, $\Delta sigB$ exhibited an increasing number of nonrefractile spores throughout late development, which accounted for $\sim 90\%$ of the $\Delta sigB$ spore population at 500 hr (data not shown). The wild-type fruiting bodies contained these nonrefractile spores at a level of only 1% of the total spore population throughout development. After 250 hr of development, >10 times the number of wild-type refractile spores were present than $\Delta sigB$ refractile spores. It should be noted that the appearance of nonrefractile spores in $\Delta sigB$ corresponded well in timing with the increased translucence of $\Delta sigB$ fruiting mounds.

The viability of the wild-type and $\Delta sigB$ spores was also monitored throughout development. Spores were harvested from CF agar, sonicated, quantitated using a Petroff-Hausser chamber, and plated on CYE agar at 30°C . Colonies were counted after 7 days. The viability of each harvest was calculated as the percentage of refractile spores that were capable of germination (Table 1). There was a striking reduction of viability in the $\Delta sigB$ refractile spores starting at ~ 250 hr of development. After 316 hr of development, there was an $\sim 10^3$ -fold difference in the viability of wild-type spores compared to $\Delta sigB$ spores. The plating efficiency of the wild-type spores was between 40% and 60% during late development.

Effects of *sigB* on the expression of *ops* and *tps*

The expression of the *ops* and *tps* genes in the $\Delta sigB$ strain was investigated by Western blot analysis. The *tps*

Figure 5. Fruiting mound morphology of late developing *M. xanthus*. Columns A and B show wild-type and $\Delta sigB$ fruiting mounds, respectively. Rows 1 and 2 show 140- and 240-hr fruiting mounds, respectively. Photographs were taken, using an incandescent light source and a dissecting microscope.

Table 1. Yield and viability of myxospores of wild type and Δ SIGB

	Time (hr)	34	50	120	167	213	246	285 ^a	316 ^a	339 ^a	367 ^a
I. Spores ($\times 10^8$)	wild type	1.7	5.6	7.7	10.8	8.9	8.0	ND	ND	ND	ND
	Δ SIGB	0.2	5.5	8.0	6.7	0.8	0.7	ND	ND	ND	ND
	Δ SIGB wild type	0.12	0.98	1.04	0.62	0.09	0.09	ND	ND	ND	ND
II. Viability (%)	wild type	ND	1.0	8.4	10.4	30.4	25.0	16.6	59.2	45.6	46.0
	Δ SIGB	ND	1.2	7.4	13.4	33.8	15.0	6.8	0.4	0.02	0.4
	Δ SIGB wild type	ND	1.20	0.84	1.29	1.11	0.60	0.41	$<10^{-2}$	$<10^{-3}$	$<10^{-2}$

I. Spore counts represent the round, phase contrast refractile spores harvested from one CF plate (0.42 ml of cells plated at a Klett value of 4000).

II. Viability (%) represents the percentage of round, phase contrast refractile spores that are capable of germination on CYE agar. Time (hr) indicates the duration of incubation of CF agar prior to spore counting and viability determination.

(ND) Not determined.

^aPartial harvests were collected for these time points, and the relative % of viable spores was determined.

gene is expressed prior to sporulation, and its product (protein S) is found predominantly on the surface of spores. In contrast, the *ops* gene product (protein S1) is expressed only after sporulation has occurred, and its product is found only within myxospores. Total protein was harvested from both nonspore and spore fractions at several time points during development (see Materials and methods). The protein fractions were subjected to SDS-PAGE separation and transferred to a PVDF membrane. Protein S and protein S1 were identified by use of anti-S antibodies. Protein S appeared normally in both the nonspore fractions (data not shown) and in the spore fraction of the Δ sigB strain (Fig. 6). However, protein S1 was absent in the spore fraction of the Δ sigB strain (Fig. 6, lane 4), indicating that *sigB* is essential for the expression of *ops* but not *tps*. The results of the *sigB-lacZ* fusion indicates that *sigB* plays an important role in the maturation of myxospores at a late stage of development.

Discussion

SigB sequence homology

The sequence of *M. xanthus* SigB not only shows significant similarity to *M. xanthus* SigA but also exhibits many other features that are conserved in other characterized prokaryotic σ -factors (Fig. 7). It has been shown that secondary σ -factors do not share the extensive sequence homology demonstrated among the major vegetative σ -factors (Helmann and Chamberlin 1988). However, structural and functional features of secondary σ -factors are well conserved, including three functionally distinct regions found in most prokaryotic σ -factors; regions 2–4 (Helmann and Chamberlin 1988). Region 2 is the most highly conserved of these regions, and evidence indicates that it is responsible for core polymerase binding and initiation at the -10 region of target promoters. Region 2 is divided into four subregions (2.1, 2.2, 2.3, and 2.4), as is shown in Figure 7. Region 2.1 is proposed to be a DNA-melting region that aids in the formation of the open promoter complex at the -10 region

Figure 6. Western blot analysis of spore fraction proteins of wild-type and Δ sigB strain *M. xanthus*. The protein samples (10 μ g/lane) were solubilized in loading buffer and analyzed as described in Materials and methods. (Lane 1) Protein from 48-hr wild-type spores; (lane 2) protein from 48-hr Δ sigB spores; (lane 3) protein from 97-hr wild-type spores; (lane 4) protein from 97-hr Δ sigB spores. Protein bands were detected by the immunoblot procedure, with *M. xanthus* anti-S as the first antibody. Arrows labeled S and S1 identify protein S and protein S1 positions, respectively. Arrows at left indicate positions of molecular weight (from top to bottom: bovine serum albumin, ovalbumin, carbonic anhydrase, and lysozyme).

of the promoter. SigB shares 47% identity with SigA in region 2.1 (7 residues out of 15). SigB also contains many aromatic and basic residues in region 2.1, as are found in region 2.1 of other σ -factors (Helmann and Chamberlin 1988). The aromatic residues are believed to intercalate between the exposed DNA bases on the nontranscribed single strand. The basic residues are believed to neutralize the negatively charged backbone phosphate groups of the nontranscribed single strand. Region 2.2 of SigB shows complete identity with region 2.2 of SigA. This region is proposed to form a core polymerase-binding surface based primarily on its universal conservation in all characterized σ -factors. Region 2.3 of SigB shows 41% identity with that of SigA (7 residues out of 17). Region 2.3 is also a proposed DNA-melting region,

Figure 7. Amino acid sequence alignment of region 2 of *M. xanthus* SigB, *M. xanthus* SigA, *E. coli* σ^{32} , *B. subtilis* σ^{29} , and *B. subtilis* SigA. The subregions of region 2 are denoted by the brackets above the aligned sequences. Amino acid sequences are compared with matching residues, assigned as follows: (●) Residues at this position are identical to *M. xanthus* SigB in each of the four other sequences; (○) these residues are identical to *M. xanthus* SigB at three of the four other sequences.

and this region of SigB also contains many basic and aromatic residues. Evidence indicates that region 2.4 recognizes the -10 promoter region of target genes. It is believed that region 2.4 forms an α -helix with a hydrophobic brace on one side, which aligns the opposing helix face in the proper orientation for specific promoter binding. Region 2.4 of SigB contains well-conserved isoleucine residues spaced 4 residues apart, which could serve as the hydrophobic brace. As would be expected, there is significant variability of the residues between the conserved isoleucines of SigA and SigB. This variability is indicative of functional differences between σ -factors with distinct promoter specificities.

In Figure 7, region 2 of SigB is aligned with the vegetative σ -factors of *M. xanthus* and *B. subtilis*. Also included in this alignment is a sporulation σ -factor of *B. subtilis*, σ^{29} . The *E. coli* heat shock σ -factor, σ^{32} (Grossman et al. 1984) has also been included in this alignment. Each of these σ -factors shows a significant level of identity to region 2 of *M. xanthus* SigB (Mx SigA, 50%; Bs SigA, 50%; Bs σ^{29} , 42%; Ec σ^{32} , 59%). It is of special interest to note that the heat shock σ -factor of *E. coli*, in contrast to the other σ -factors mentioned, shows significant similarity to *M. xanthus* SigB even in regions 3 and 4. Overall, σ^{32} shows 38% identity with SigB. σ^{32} also shows 62% similarity to SigB when including conservative replacements. Both SigB and σ^{32} are expressed in response to stress (starvation and heat shock, respectively), and the significant level of similarity between the two may be indicative of their analogous roles in gene regulation. Also of particular interest is the similarity of region 2.4 of σ^{32} to region 2.4 of SigB. Of the 10 variable residues, 4 are identical, and 3 of the remaining variable residues are conservatively replaced. This indicates that SigB and σ^{32} recognize similar promoter sequences at the -10 region and may possess similar target genes. This observation suggests further that heat-shock response in *Escherichia coli* and late-stage differentiation of *M. xanthus* may be ancestrally related genetic programs.

Region 3, extending from Glu-93 to Glu-160, is known to be largely negatively charged in many σ -factors. Region 3 of SigB contains a total of 16 negatively charged residues (Asp + Glu; 24%) in contrast to 7 positively charged residues (Arg + Lys; 10.5%). The rest of SigB contains only 8 acidic residues of 126 (Asp + Glu; 6.5%). The function of this region is not clearly understood, but some evidence indicates that it may be required for the structural integrity of the σ -factor (Helmann and Chamberlin 1988).

Region 4.2 is believed to form a helix–turn–helix (HTH) structure that is proposed to bind the -35 region of target promoters. Although little similarity is present between SigA and SigB in this region, the structural features appear to be conserved in SigB. Chou–Fasman analysis (Chou and Fasman 1974) of SigB predicts two closely spaced helices located between Asp-159 and Ala-167, and Ala-172 and Leu-181. The intervening 4 residues (Gly-Leu-Ile-Asn) conform reasonably well to the consensus sequence for HTH turns (Gly-Val-Ile-Ser).

Possible functions of sigB

Analysis of *sigB* expression with the B/Z strain demonstrated that *sigB* is predominantly expressed in the spore fraction; however, very low-level β -galactosidase activity appeared in nonspore fractions just prior to sporulation. There was also a low level of β -galactosidase activity in the nonspore fractions at a late developmental stage. This nonspore-derived activity may actually originate from sonication-sensitive prespores early in development (O'Connor and Zusman 1988) and spores that might have become sensitive to sonication late in development.

The phenotypic effects of *sigB* deletion on the late development of *M. xanthus* suggest that *sigB* may be required for the complete maturation of spores. The deletion mutant showed a dramatic reduction in the number of refractile spores, the concurrent appearance of large numbers of nonrefractile blackened spores, translucent fruiting mounds, and almost complete loss of viability in the spores of late development. In contrast, the wild-type strain demonstrated none of these anomalies during late development. These results suggest that *sigB* may be required for the expression of genes that are essential for a state of dormancy in maturing spores. Spore dormancy would be required for the long-term survival of myxospores under conditions of prolonged starvation and desiccation. Because *M. xanthus* is a soil-dwelling organism, it would be expected to experience extremes of temperature, drought, and seasonally varying nutrient supply. The defects observed in the late-stage differentiation of the $\Delta sigB$ strain may be due to an unrestrained metabolism of the myxospores in the presence of nutrient depletion.

Our data suggest that *sigB* is not involved directly in the germination process, because normal levels of viability are observed in the $\Delta sigB$ strain until ~ 225 hr of development. If *sigB* were directly involved in germination it would be expected that the viability of $\Delta sigB$

spores would also be deficient at the early time points during development.

The *sigB*-dependent program of gene expression is apparently separate from the earlier developmental programs of mound formation and sporulation, because the deletion mutant is indistinguishable from the wild-type strain during these stages. The *sigB* expression is coincident with sporulation and may be morphologically coupled to sporulation as a temporally significant event. We have shown that the Δ *sigB* strain does not produce the spore-specific *ops* gene product, indicating that *ops* may be a direct target gene of *sigB*. This result supports the observation that the *sigB-lacZ* gene is predominantly expressed in the spore fraction from a middle to late stage of development. It should also be noted that the deletion of *sigB* may disrupt a possible σ cascade of *M. xanthus* development. Thus, the inability to produce other σ -factors expressed later in the developmental program may contribute to the wide range of deficiencies observed in the late development of the Δ *sigB* strain.

Identification of the target genes of *sigB* will greatly facilitate the further investigation of the mechanisms governing the maturation of myxospores during late development. *ops* appears to be a target gene of SigB. Another σ -factor gene (designated *sigC*) has been identified in the 8.4-kb *XhoI* fragment of *M. xanthus* chromosomal DNA. Further characterization of the *sigC* gene is presently in progress. Characterization of the other *M. xanthus* chromosomal fragments that hybridize with the *sigA* probe is also in progress, which will provide important information concerning a σ cascade of *M. xanthus* development.

Materials and methods

Materials

Restriction enzymes were purchased from either Bethesda Research Laboratories (BRL) or New England Biolabs. T4 DNA ligase was from BRL, and DNA polymerase I Klenow fragment was from Boehringer-Mannheim. Sequenase enzyme was from U.S. Biochemical. [α - 32 P]dCTP was from Amersham. PVDF was purchased from Millipore. Nitrocellulose was purchased from Schleicher & Schuell.

Cells and growth conditions

M. xanthus FB (DZF1) was used in this study and grown in CYE medium (Campos et al. 1984). *E. coli* strain JM83 (Yanisch-Perron et al. 1985) was used for cloning and grown in Luria broth.

Plasmids and phages

pUX (Dhundale et al. 1988) was used to clone *XhoI* genomic fragments from DZF1. pUC9 (Vieira and Messing 1982) was used for subsequent subcloning and sequencing experiments. Phage P1 was used for transduction of DNA from *E. coli* to *M. xanthus*, as originally described (Shimkets et al. 1983).

DNA manipulations and sequencing

Chromosomal DNA was prepared as described previously (Yee and Inouye 1981). Plasmid preparation was as originally described (Birnbom and Dolly 1979). Blot hybridization was performed according to the method of Southern (1975) under the

following conditions: 40% formamide, 5 $\times$ SSPE [1 $\times$ SSPE: 180 mM NaCl, 10 mM NaH₂PO₄ (pH 7.4), 1 mM EDTA (pH 7.4)], 0.5% SDS, 5 $\times$ Denhardt's solution, and 5 $\times$ 10⁵ cpm of 32 P-labeled probe/ml at 37°C. Posthybridization washes with 2 $\times$ SSC were carried out at room temperature.

The 3-kb *SmaI* subclone of the 7.4-kb *XhoI* genomic clone was digested separately by *RsaI* and *SalI*. These *RsaI* and *SalI* fragments were shotgun-cloned into the *SmaI* site of pUC9 for sequencing. The DNA sequence of *sigB* was determined in both orientations by use of the chain-termination method (Sanger 1977) directly on the denatured double-stranded *SalI* and *RsaI* shotgun clones. Synthetic oligonucleotides were used as sequencing primers to clarify gaps and ambiguities from the shotgun sequencing data. 7-Deazaguanosine triphosphate (Mizusawa et al. 1986) was used to minimize spontaneous chain terminations that can result from the high GC content of myxobacterial DNA.

Construction of *sigB* strain

The fragment between the *SalI* and *BamHI* sites of the 7.4-kb genomic clone of *sigB* (see Fig. 2) was replaced with a 2.5-kb fragment containing the Tn5 kanamycin resistance gene. This construct was introduced into the *M. xanthus* chromosome by use of specialized P1 transduction (Shimkets et al. 1983). Double crossover recombination of this construct into the chromosome resulted in the deletion of the first 181 codons of the *sigB* gene, including $\sim$ 1 kb of upstream DNA. Deletion of the *sigB* gene was verified by Southern hybridization (Southern 1975) of Δ *sigB* chromosomal digests with a radioactively labeled *sigA* probe (*PstI-XhoI* fragment, containing the highly conserved carboxy-terminal coding region). Kanamycin-resistant candidates that were incapable of hybridizing with the *sigA* probe at the 7.4-kb position were designated as Δ *sigB* (data not shown).

Developmental conditions

Glycerol sporulation was performed as described previously (Dworkin and Gibson 1964). Liquid starvation in MCM was carried out as described previously (Rosenbluh and Rosenberg 1989). Submerged fruiting was performed as originally described (Kuner and Kaiser 1982). Fruiting body formation on CF agar was carried out as described previously (Hagen et al. 1978). β -Galactosidase assays were performed on developmental *M. xanthus*, as described previously (Kroos et al. 1986).

Developmental spores and nonspores were harvested from CF plates by use of a straight-edged spatula to scrape the agar surface. Harvests were suspended in TM buffer (10 mM Tris, 8 mM MgSO₄). All subsequent manipulations of developmental harvests were conducted in TM buffer. Developmental spores and nonspores were disrupted by use of a heat-systems sonicator with a cup horn. Developmental nonspores were disrupted by sonication at 40 watts for 2 min in an ice bath. The remaining spore fraction was washed in TM buffer and disrupted by sonication in the presence of 100- μ m glass beads (Sigma) at 30 Watts for 4 min as described previously (Teintze et al. 1985). Developmental cell types were determined by the method of O'Conner and Zusman (1988).

Western blot analysis

Spore and nonspore fractions were suspended in loading buffer [80 mM Tris-HCl (pH 6.8), 2% SDS, 0.1 M β -mercaptoethanol, 10% glycerol, 0.12% bromophenol blue] and incubated in a boiling water bath for 5 min. Samples were applied to a 17.5% polyacrylamide-SDS gel. After electrophoresis, protein bands were electrotransferred onto PVDF membranes by use of a Sar-toblot semidry apparatus as described by the manufacturer (Sar-

torious, Gottingen, FRG). The immunodetection of protein S and protein S1 was accomplished by use of *M. xanthus* polyclonal anti-S antiserum and the alkaline phosphatase-conjugated goat anti-rabbit antibody, as described by the manufacturer (Promega).

Acknowledgments

We thank Dr. Masayori Inouye for helpful discussions throughout this research and in the preparation of this manuscript. This work was supported by U.S. Public Health Service grant GM-26843 from the National Institutes of Health.

The publication costs of this article were defrayed in part by payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 USC section 1734 solely to indicate this fact.

References

- Birboim, H.C. and J. Dolly. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res.* **7**: 1513–1523.
- Campos, J.M., J. Geisselsoder, and D.R. Zusman. 1984. Isolation of bacteriophage Mx4, a generalized transducing phage for *Myxococcus xanthus*. *J. Mol. Biol.* **119**: 167–178.
- Chou, P.Y. and G.D. Fasman. 1974. Prediction of protein conformation. *Biochemistry* **13**: 222–244.
- Dhundale, A., T. Furuichi, M. Inouye, and S. Inouye. 1988. Mutations that affect production of branched RNA-linked msDNA in *Myxococcus xanthus*. *J. Bacteriol.* **170**: 5620–5624.
- Doi, R.H. and L.F. Wang. 1986. Multiple prokaryotic ribonucleic acid polymerase σ -factors. *Microbiol. Rev.* **50**: 227–243.
- Donovan, W., L. Zheng, K. Sandman, and R. Losick. 1987. Genes encoding spore coat polypeptides from *Bacillus subtilis*. *J. Mol. Biol.* **196**: 1–10.
- Downard, J.S. and D.R. Zusman. 1985. Differential expression of protein S genes during *Myxococcus xanthus* development. *J. Bacteriol.* **161**: 1146–1155.
- Downard, J. S., D. Kupfer, and D. R. Zusman. 1984. Gene expression during development of *Myxococcus xanthus*: analysis of the genes for protein S. *J. Mol. Biol.* **175**: 469–492.
- Dworkin, M. and S.M. Gibson. 1964. A system for studying microbial morphogenesis: Rapid formation of microcysts in *Myxococcus xanthus*. *Science* **146**: 243–244.
- Grossman, H.D., J.W. Erikson, and C.A. Gross. 1984. The *htpR* gene product of *E. coli* is a σ -factor for heat shock promoters. *Cell* **38**: 383–390.
- Hagen, D.C., A.P. Bretscher, and D. Kaiser. 1978. Synergism between morphogenetic mutants of *Myxococcus xanthus*. *Dev. Biol.* **64**: 284–296.
- Helmann, J.D. and M.J. Chamberlin. 1988. Structure and function of bacterial σ -factors. *Annu. Rev. Biochem.* **57**: 839–872.
- Inouye, S. 1990. Cloning and DNA sequence of the gene coding for the major σ -factor of *Myxococcus xanthus*. *J. Bacteriol.* **172**: 80–85.
- Inouye, S., T. Franceschini, and M. Inouye. 1983a. Structural similarities between the development-specific protein S from a Gram-negative bacterium, *Myxococcus xanthus* and calmodulin. *Proc. Natl. Acad. Sci.* **80**: 6829–6833.
- Inouye, S., Y. Ike, and M. Inouye. 1983b. Tandem repeat of the genes for protein S, a development-specific protein of *Myxococcus xanthus*. *J. Biol. Chem.* **258**: 38–40.
- 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.
- Ishihama, A. 1988. Promoter selectivity of prokaryotic RNA polymerases. *Trends Genet.* **4**: 282–286.
- Johnson, J.L. and E.J. Ordal. 1968. Deoxyribonucleic acid homology in bacterial taxonomy: effect of incubation temperature on reaction specificity. *J. Bacteriol.* **95**: 893–900.
- Kaiser, D. 1986. Control of multicellular development: *Dictyostelium* and *Myxococcus*. *Annu. Rev. Genet.* **20**: 539–566.
- 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.* **81**: 5816–5820.
- Kroos, L., A. Kuspa, and D. Kaiser. 1986. A global analysis of developmentally regulated genes in *Myxococcus xanthus*. *Dev. Biol.* **117**: 252–266.
- Kuner, J.M. and D. Kaiser. 1982. Fruiting body morphogenesis in submerged cultures of *Myxococcus xanthus*. *J. Bacteriol.* **151**: 458–461.
- Losick, R. and J. Pero. 1981. Cascades of σ -factors. *Cell* **25**: 582–584.
- Losick, R., P. Youngman, and P.J. Piggot. 1986. Genetics of endospore formation in *Bacillus subtilis*. *Annu. Rev. Genet.* **20**: 625–669.
- Mizusawa, S., S. Nishimura, and F. Seela. 1986. Improvement of the dideoxy chain termination method of DNA sequencing by use of deoxy-7-deazaguanosine tri-phosphate in place of dGTP. *Nucleic Acids Res.* **14**: 1319–1324.
- O'Conner, K.A. and D.R. Zusman. 1988. Reexamination of the role of autolysis in the development of *Myxococcus xanthus*. *J. Bacteriol.* **170**: 4103–4112.
- Rosenberg, E. 1984. *Myxobacteria: Development and cell interactions*. Springer-Verlag, New York.
- Rosenbluh, A. and E. Rosenberg. 1989. Sporulation of *Myxococcus xanthus* in liquid shake flask cultures. *J. Bacteriol.* **171**: 4521–4524.
- Sanger, F., S. Nicklen, and A.R. Coulson. 1977. DNA sequencing with chain terminating inhibitors. *Proc. Natl. Acad. Sci.* **74**: 5463–5467.
- Shimkets, J.L. 1987. Control of morphogenesis in myxobacteria. *Crit. Rev. Microbiol.* **14**: 195–227.
- Shimkets, L.J., R.E. Gill, and D. Kaiser. 1983. Developmental cell interactions in *Myxococcus xanthus* and the *spoC* locus. *Proc. Natl. Acad. Sci.* **80**: 1406–1410.
- Southern, E.M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* **98**: 503–517.
- Stragier, P., C. Bonamy, and C. Karmazyn-Campelli. 1988. Processing of a sporulation σ -factor in *Bacillus subtilis*: How morphological structure could control gene expression. *Cell* **52**: 697–704.
- Stragier, P., B. Kunkel, L. Kroos, and R. Losick. 1989. Chromosomal rearrangement generating a composite gene for a developmental transcription factor. *Science* **243**: 507–512.
- Teintze, M., R. Thomas, T. Furuichi, M. Inouye, and S. Inouye. 1985. Two homologous genes coding for spore specific proteins are expressed at different times during development of *Myxococcus xanthus*. *J. Bacteriol.* **163**: 121–125.
- Vieira, J. and J. Messing. 1982. The pUC plasmids, an M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. *Gene* **19**: 259–268.
- Yanisch-Perron, C., J. Vieira, and J. Messing. 1985. Improved M13 phage cloning vectors and host strains: Nucleotide sequences of the M13mp18 and pUC19 cloning vectors. *Gene* **33**: 103–119.
- Yee, T. and M. Inouye. 1981. Reexamination of the genomic size of myxobacteria, including the use of a new method for genome size analysis. *J. Bacteriol.* **145**: 1257–1265.

Development-specific sigma-factor essential for late-stage differentiation of *Myxococcus xanthus*.

D Apelian and S Inouye

Genes Dev. 1990, 4:

Access the most recent version at doi:[10.1101/gad.4.8.1396](https://doi.org/10.1101/gad.4.8.1396)

References

This article cites 37 articles, 17 of which can be accessed free at:
<http://genesdev.cshlp.org/content/4/8/1396.full.html#ref-list-1>

License

Email Alerting Service

Receive free email alerts when new articles cite this article - sign up in the box at the top right corner of the article or [click here](#).