JOURNAL OF BACTERIOLOGY, Dec. 2000, p. 6622–6629 Vol. 182, No. 23
0021-9193/00/$04.00+0

Control of *asgE* Expression during Growth and Development of *Myxococcus xanthus*

ANTHONY G. GARZA,† BARUCH Z. HARRIS,‡ BRANDON M. GREENBERG,§ AND MITCHELL SINGER*

Section of Microbiology, University of California, Davis, Davis, California 95616

Received 8 May 2000/Accepted 21 September 2000

One of the earliest events in the *Myxococcus xanthus* developmental cycle is production of an extracellular cell density signal called A-signal (or A-factor). Previously, we showed that cells carrying an insertion in the *asgE* gene fail to produce normal levels of this cell-cell signal. In this study we found that expression of *asgE* is growth phase regulated and developmentally regulated. Several lines of evidence indicate that *asgE* is cotranscribed with an upstream gene during development. Using primer extension analyses, we identified two 5′ ends for this developmental transcript. The DNA sequence upstream of one 5′ end has similarity to the promoter regions of several genes that are A-signal dependent, whereas sequences located upstream of the second 5′ end show similarity to promoter elements identified for genes that are C-signal dependent. Consistent with this result is our finding that mutants failing to produce A-signal or C-signal are defective for developmental expression of *asgE*. In contrast to developing cells, the large majority of the *asgE* transcript found in vegetative cells appears to be monocistronic. This finding suggests that *asgE* uses different promoters for expression during vegetative growth and development. Growth phase regulation of *asgE* is abolished in a *relA* mutant, indicating that this vegetative promoter is induced by starvation. The data presented here, in combination with our previous results, indicate that the level of AsgE in vegetative cells is sufficient for this protein to carry out its function during development.

When *Myxococcus xanthus* is deprived of nutrients, approximately 100,000 rod-shaped cells initiate a complex social interaction that culminates in construction of a multicellular structure called a fruiting body (5, 15, 32). After cells aggregate into fruiting bodies, individual rod-shaped cells within these structures begin to differentiate into spherical spores that are resistant to certain types of environmental stress. Thus, the *M. xanthus* developmental cycle occurs in an ordered series of steps that include starvation, construction of a macroscopic fruiting body, and differentiation of rod-shaped cells into spherical spores.

Constructing multicellular structures requires cells to coordinate their activities. Previous analyses of conditional developmental mutants suggest that *M. xanthus* coordinates fruiting body development by producing cell-cell signals (4, 10, 25). Kuspa et al. (22) and Kroos and Kaiser (19) showed that two developmental signals, A-signal and C-signal, are required for expression of particular groups of developmentally regulated *lacZ* reporter gene fusions, indicating that these cell-cell signals may guide the developmental process by directing changes in gene expression. The fact that full expression of nearly all developmentally regulated *lacZ* reporter gene fusions requires an intact A-signaling system, whereas an intact C-signaling system is required only for expression of *lacZ* fusions activated after 6 h of development, suggests that A-signal is required earlier in development than C-signal.

Extracellular A-signal consists of a mixture of amino acids and peptides, which are heat stable, and at least two extracellular proteases, which are heat labile (23, 27). Based on these findings, it was proposed that A-signal is a mixture of amino acids and peptides generated by proteolysis (23, 27). Work done by Kuspa et al. (24) suggests that the concentration of A-signal produced by developing cells may serve as an indicator of cell density; A-signal is produced in proportion to the number of cells. A-signal may, therefore, allow *M. xanthus* cells to determine whether a sufficient number of cells is present to initiate fruiting body development.

Genetic analysis of the original collection of A-signal-defective mutants led to the discovery of three genes (*asgA*, *asgB*, and *asgC*) involved in the production of A-signal (21, 25, 29). Further studies demonstrated that the level of A-signal produced by these *asg* mutants is between 5.0 and 20.0% of that produced by wild-type cells, resulting in defects in aggregation, sporulation, and expression of developmentally regulated genes (3, 20, 21, 27, 29). Based on DNA sequence analysis of *asgA*, *asgB*, and *asgC*, it was proposed that the products of these genes are components of a signal transduction pathway regulating expression of genes directly involved in production of A-signal (3, 28, 29).

Recent studies of *M. xanthus* developmental mutants have led to the discovery of two new *asg* alleles, *asgD* and *asgE* (2, 9). Mutants carrying an *asgD* mutation appear to be unable to recognize starvation properly; these mutants fail to develop unless rapid starvation is induced. Cells carrying an insertion in the *asgE* gene generate a reduced level of A-signal. The level of A-signal produced by *asgE* cells, however, is higher than that produced by *asgA* or *asgB* cells. Thus, the developmental defects of an *asgE* mutant are less severe than those of an *asgA* or *asgB* mutant. Further analysis of *asgE* cells showed that they are almost completely lacking heat-labile A-signal activity.

* Corresponding author. Mailing address: Section of Microbiology, One Shields Ave., University of California, Davis, Davis, CA 95616. Phone: (530) 752-9005. Fax: (530) 752-9014. E-mail: mhsinger@ucdavis.edu.

† Present address: Departments of Biochemistry and Developmental Biology, Stanford University, Stanford, CA 94305.

‡ Present address: Department of Biochemistry and Biophysics, University of California, San Francisco, San Francisco, CA 94143.

§ Department of Microbiology and Molecular Genetics, The University of Texas Medical School, Houston, TX 77225.

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Source or reference
E. coli strain		
DH5α	*supE44 ΔlacU169 ϕ80ΔlacZ*M15 *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	26
M. xanthus strains		
DK101	*pilQ1* (wild-type development)	13
DK476	*pilQ1 asgA4476*	10
DK480	*pliQ1 asgB480*	10
DK527	*pilQ1 relA*	12
DK767	*pilQ1 asgC767*	10
DK5216	*pilQ1 csgA*::*Tn5*132 (ΩLS205)	33
MS2020	*pilQ1 orf2*::pBMG3 (Ω5002)	9
MS2021	*pilQ1 asgE*::pREG-JP2B (Ω5003)	9
MS2036	*pilQ1 relA asgE*::pREG-JP2B (Ω5003)	This study
MS2037	*pilQ1 asgB480 asgE*::pREG-JP2B (Ω5003)	This study
MS2038	*pilQ1 asgA4476 asgE*::pREG-JP2B (Ω5003)	This study
MS2039	*pilQ1 asgC767 asgE*::pREG-JP2B (Ω5003)	This study
MS2040	*pilQ1 orf2*::pBMG4 (Ω5005)	This study
MS2041	*pilQ1 csgA*::*Tn5*132 (ΩLS205) *asgE*::pREG-JP2B (Ω5003)	This study
Plasmids		
pBGS18	Kan^r	34
pBluescript II SK	Amp^r	Stratagene
pREG1727	Amp^r Kan^r	6
pBMG3	Kan^r, pBGS18 containing 0.5 kb of *orf2* on a *Xba*I-*Bam*HI fragment	9
pBMG4	Amp^r Kan^r, pREG1727 containing 1.0 kb of *orf2* and 1.5 kb of upstream DNA on an *Xho*I-*Bam*HI fragment	This study
pELF3	Kan^r, pBGS18 containing *orf2*, *asgE*, and 0.4 kb of downstream DNA on a *Bam*HI-*Pst*I fragment	9
pREG-JP2B	Amp^r Kan^r, pREG1727 containing 1.0 kb of *asgE* on a *Bam*HI-*Hin*dIII fragment	9

Since we are interested in understanding how the genes required for production of A-signal are regulated, we examined developmental expression of the *asgE* gene in wild-type cells and in mutants that lack critical components of the *M. xanthus* developmental cycle. To further understand the mechanism of *asgE* regulation during development, the structure of the *asgE* operon was analyzed and putative transcriptional start sites were identified. Because we found that *asgE* is growth phase regulated, we examined the mechanism of *asgE* expression in vegetative cells and compared our results to those observed for cells placed under developmental conditions.

MATERIALS AND METHODS

Bacterial strains and plasmids. A complete list of strains and plasmids used in this study is given in Table 1. Construction of the *asgE*::*lacZ* fusion, Ω5003, was described previously (9). The *orf2*::*lacZ* fusion, Ω5005, was created by integrating pBMG4 (Table 1) into the *orf2* chromosomal locus as described below. Homologous recombinants were distinguished from site-specific recombinants by Southern blot analysis (30).

Plasmid transfer to *M. xanthus*. Plasmids containing DNA fragments from the *asgE* locus were electroporated into *M. xanthus* cells using the technique of Plamann et al. (28). Following electroporation, cells were placed into flasks containing 1.5 ml of CTT (see below) broth and incubated at 32°C for 8 h with vigorous agitation. Aliquots (500 μl) of these cultures were added to 5 ml of CTT soft agar and poured onto CTT plates containing kanamycin. Chromosomal DNA was isolated from Kan^r colonies (30) and used for Southern blot analysis (30) to identify transformants that contain a single copy of the appropriate plasmid integrated by homologous recombination into the *asgE* locus. Kan^r transformants carrying a single plasmid insertion were used in expression studies as described below.

Media for growth and development. *M. xanthus* strains were grown at 32°C in CTT broth containing 1% Casitone (Difco Laboratories), 10.0 mM Tris-HCl (pH 8.0), 1 mM KH_2PO_4, and 8 mM $MgSO_4$ or on plates containing CTT broth and 1.5% Difco Bacto-Agar. CTT broth and CTT plates were supplemented with 40 μg of kanamycin sulfate (Sigma)/ml or 12.5 μg of oxytetracycline (Sigma)/ml as needed. CTT soft agar is CTT broth containing 0.7% Difco Bacto-Agar. *Escherichia coli* strain DH5α was grown at 37°C in Luria broth (LB) containing 1% tryptone (Difco), 0.5% yeast extract (Difco), and 0.5% NaCl or on plates containing LB and 1.5% Difco Bacto-Agar. LB and LB plates were supplemented with 50 μg of ampicillin (Sigma)/ml or 40 μg of kanamycin sulfate (Sigma)/ml as needed. Fruiting body development was carried out at 32°C on plates containing TPM buffer (10.0 mM Tris-HCl [pH 8.0], 1 mM KH_2PO_4, and 8 mM $MgSO_4$) and 1.5% Difco Bacto-Agar.

Expression studies. The promoterless *lacZ* expression vector pREG1727 carries the Mx*8 attP* site, allowing for site-specific integration at the chromosomal Mx*8* phage attachment site *attB* (6). When we cloned fragments of the *asgE* locus into pREG1727 and electroporated these plasmids into DK101 cells, we found that a substantial portion of the Kan^r colonies (5.0 to 50.0%, depending on the fragment) contained a plasmid integration at the chromosomal *asgE* locus, rather than an integration at the Mx*8 attB*. Hence, many of the pREG1727 derivatives were integrating into the chromosomal *asgE* locus by homologous recombination. Taking advantage of this frequency of homologous recombination, we were able to create a series of *lacZ* reporter fusions in the vicinity of the *asgE* locus for expression studies.

For these studies, vegetatively growing cells and developmental cells were harvested and quick frozen in liquid nitrogen as described previously (8). β-Galactosidase assays were performed on quick-frozen cell extracts using the technique of Kaplan et al. (16). β-Galactosidase-specific activity is defined as nanomoles of *o*-nitrophenol (ONP) produced $minute^{-1}$ milligram of $protein^{-1}$.

RNA was isolated from quick-frozen cell extracts by the hot phenol method (30). Total cellular RNA was used for slot blot hybridization analysis as described previously (8, 16). The probe used for these experiments is a 1.0-kb *Sac*I-*Pvu*II fragment from the 5′ end of the *asgE* gene. Total RNA was isolated from cells after 12 h of development on TPM starvation agar or from cells grown to a density of 10^9 cells per ml in CTT nutrient broth.

Primer extension analyses. Primer extension analyses were carried out as described by Garza et al. (8) using RNA isolated from vegetatively growing cells (10^9 cells per ml) or from cells after 12 h of development on TPM agar. The primers used for this analysis, *ade-9* and *asgE*-veg1, are complementary to sequences in the 5′ end of *orf2* and *asgE*, respectively. DNA was sequenced by the dideoxynucleotide chain termination method (31) using a Sequi-Therm Cycle Sequencing Kit (Epicentre Technologies, Madison, Wis.) and custom-designed oligonucleotide primers. Primers were synthesized by Operon Technologies, Inc. (Alameda, Calif.).

RESULTS

Expression of *asgE* during growth and development. To determine whether *asgE* is developmentally regulated, we cloned a 1.0-kb internal fragment of this gene into the promoterless *lacZ* expression vector pREG1727 (6). When this plasmid (pREG-JP2B) is integrated into the chromosome of wild-type

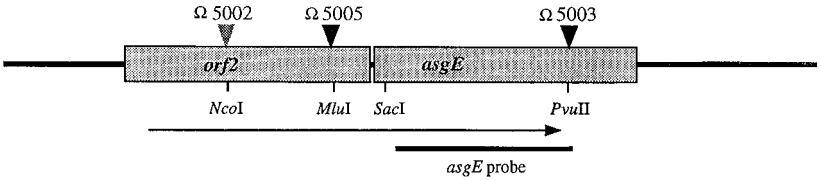

FIG. 1. Physical map of the *asgE* locus. Boxes show the locations of the indicated open reading frames, and the arrows show their predicted direction of transcription. Black triangles mark the locations of *lacZ* reporter gene fusions Ω5003 and Ω5005; the gray triangle shows the location of the *orf2* insertion in strain MS2020. The broadened black line represents the 1-kb fragment of the *asgE* gene that was used as a probe for RNA slot blot analysis.

DK101 cells by homologous recombination, a transcriptional fusion between *lacZ* and the *asgE* gene is created. The location of this reporter gene fusion (Ω5003) is shown on the physical map of the *asgE* locus (Fig. 1), and the pattern of *asgE*::*lacZ* expression in cells developing on TPM agar is shown in Fig. 2A. β-Galactosidase-specific activity in cells carrying the *asgE*::*lacZ* fusion began to increase relatively early in the developmental process (2 to 4 h) and continued to increase until about 24 h of development on TPM agar. Between 0 and 24 h, the levels of β-galactosidase in cells carrying *asgE*::*lacZ* increased approximately threefold, indicating that *asgE* is developmentally regulated.

Cells carrying the *asgE*::*lacZ* fusion produce approximately 300 to 350 U of β-galactosidase-specific activity prior to the onset of development, which suggests that *asgE* is expressed during vegetative growth. To investigate this further, we monitored β-galactosidase levels in cells carrying the *asgE*::*lacZ* reporter while they were growing in CTT nutrient broth. The data in Fig. 2B indicate that expression of *asgE* is induced around mid- to late exponential growth phase in nutrient broth and that expression continues to increase until cells begin to enter stationary phase. Between exponential growth and stationary phases, *asgE* expression increases approximately 2.5-fold. Taken with our previous findings, these results indicate that *asgE* is both growth phase regulated and developmentally regulated.

Organization of the *asgE* operon. Previously, Garza et al. (9) demonstrated that *asgE* is located immediately downstream of an open reading frame designated *orf2* (Fig. 1). The results of genetic studies and DNA sequence analysis suggest that *asgE* and *orf2* may be cotranscribed during development. To examine whether *asgE* and *orf2* are part of the same operon, we generated the Ω5005 transcriptional fusion between *orf2* and *lacZ*. Subsequently, we monitored the levels of β-galactosidase produced by cells carrying the *orf2* reporter fusion at various times during development on TPM starvation agar (Fig. 3A). Consistent with the idea that *orf2* and *asgE* are under the transcriptional control of the same developmental promoter, the pattern of β-galactosidase production from cells carrying the *orf2*::*lacZ* fusion was virtually identical to the pattern observed for cells carrying the *asgE*::*lacZ* fusion. Furthermore, the mean fold increase in β-galactosidase-specific activity during development was similar for both strains: 2.7 ± 0.1-fold for *orf2*::*lacZ* cells and 2.8 ± 0.2-fold for *asgE*::*lacZ* cells.

To confirm that *asgE* and *orf2* are cotranscribed during development, we used RNA slot blots to show that an insertion in *orf2* has a polar effect on transcription of the downstream *asgE* gene. For these experiments, 12-h developmental RNA was isolated from wild-type DK101 cells and from isogenic cells carrying the Ω5002 insertion in *orf2* (MS2020). RNA slot blots were probed with a 1.0-kb fragment of the *asgE* gene, and the relative levels of *asgE* mRNA were quantified. The results shown in Fig. 4 demonstrate that the level of *asgE* mRNA in MS2020 cells after 12 h of development is approximately 8.0% of that in wild-type cells, supporting the idea that *asgE* and *orf2* are under the control of the same developmental promoter.

To examine whether *asgE* and *orf2* are cotranscribed in vegetative cells, we monitored expression of the *orf2*::*lacZ* reporter gene fusion in MS2040 cells while they were growing in CTT nutrient broth (Fig. 3B). In contrast to the 2.5-fold in-

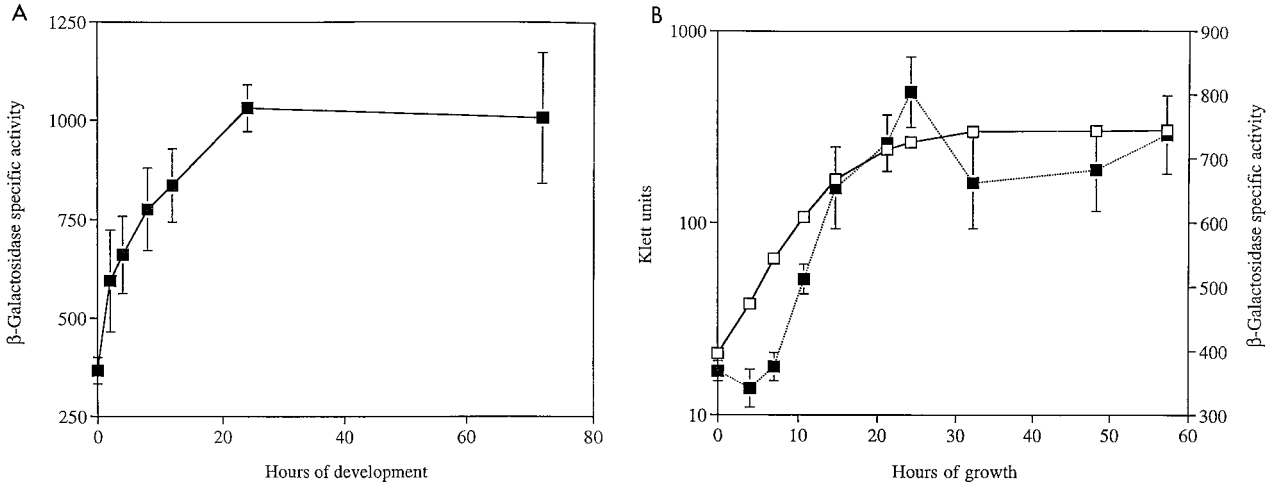

FIG. 2. Patterns of *asgE* expression during growth and development. Expression was monitored using the *asgE*::*lacZ* reporter gene fusion Ω5003 in strain MS2021. Mean β-galactosidase-specific activities (defined as nanomoles of ONP produced minute^{-1} milligram of protein^{-1}) were determined from three independent experiments. Error bars represent the standard deviations of the mean. Black squares represent β-galactosidase-specific activity, while empty squares represent cell density. Expression of *asgE* during development on TPM starvation agar (A) and growth in CTT nutrient broth (B) is shown.

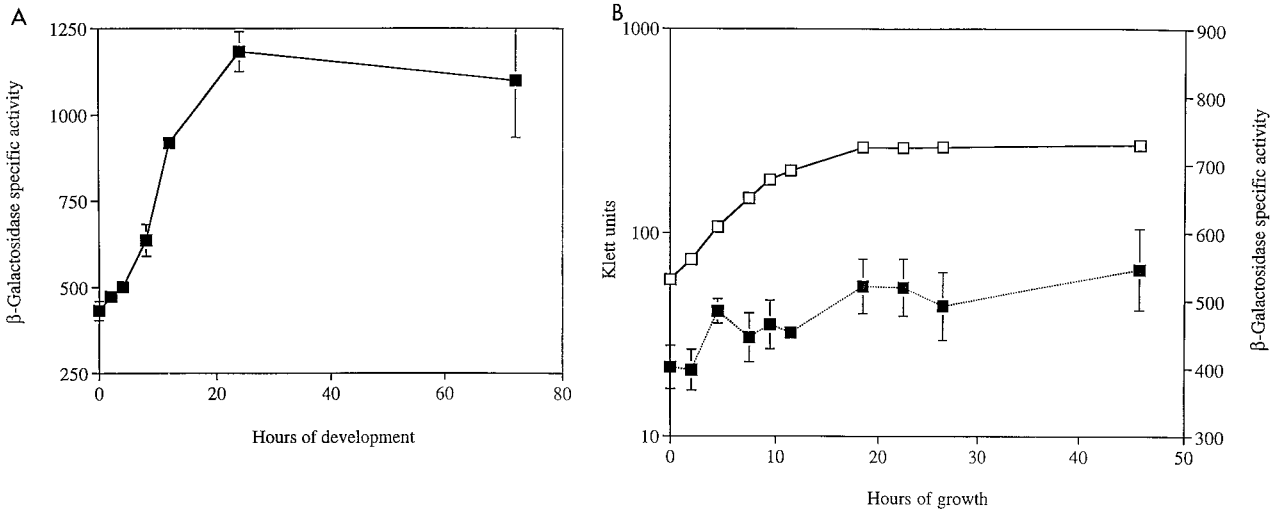

FIG. 3. Patterns of *orf2* expression during growth and development. Expression was monitored using the *orf2*::*lacZ* reporter gene fusion Ω5005 in strain MS2040. Mean β-galactosidase-specific activities (defined as nanomoles of ONP produced minute^{-1} milligram of protein^{-1}) were determined from three independent experiments. Error bars represent the standard deviations of the mean. Black squares represent β-galactosidase-specific activity, while empty squares represent cell density. Expression of *orf2* during development on TPM starvation agar (A) and growth in CTT nutrient broth (B) is shown.

duction observed for the *asgE*::*lacZ* fusion, the levels of β-galactosidase in cells carrying a *lacZ* reporter gene fusion to *orf2* remain relatively unchanged during growth phase, suggesting that *orf2* and *asgE* may be under the control of different vegetative promoters. To confirm this proposal, we used RNA slot blots to show that an insertion in *orf2* fails to abolish the transcription of *asgE* via a polar effect. For these experiments, we used a 1-kb fragment of the *asgE* gene as the probe and RNA isolated from cells grown in CTT broth. The data presented in Fig. 4 show that the level of *asgE* mRNA is approximately 70.0% of the wild-type level in the *orf2* insertion mutant. Taken with the *lacZ* expression studies, these data indicate that *asgE* has its own promoter for driving transcription during vegetative growth. However, *asgE* mRNA levels are reduced by 30.0% in the *orf2* insertion mutant, indicating that at least some *asgE* expression during vegetative growth is coming from a promoter located upstream of *orf2*.

Developmental expression of *asgE* in signaling mutants. As described above, *asgE* and *orf2* appear to be cotranscribed during development, and expression is induced at about 2 to 4 h poststarvation, indicating that the *asgE* operon (*orf2* and *asgE*) is induced relatively early in the *M. xanthus* developmental process. To further our understanding of how the *asgE* operon is regulated during development, we introduced the promoterless *asgE*::*lacZ* fusion plasmid into the chromosome of different developmental mutants and monitored the patterns of expression on TPM starvation agar.

Expression of the *asgE*::*lacZ* fusion was first examined in a strain carrying a mutation in the *relA* gene. An intact copy of *relA* is required for synthesis of the intracellular starvation signal (p)ppGpp, and accumulation of this signaling molecule is required for the earliest events in development, including production of A-signal (12). Consistent with the finding that *asgE* is part of the A-signal-generating pathway (9), we found that developmental expression of *asgE*::*lacZ* is abolished in cells carrying the *relA* mutation (Fig. 5A).

Previous work suggests that the products of *asgA*, *asgB*, and *asgC* genes may serve as regulatory factors that modulate the expression of genes required for A-signal production (3, 28, 29). Because *asgE* is required for production of heat-labile A-signal, we wanted to determine whether the developmental expression of *asgE* requires functional copies of these three *asg* genes. Accordingly, we introduced the *asgE*::*lacZ* fusion into strains carrying a mutation in *asgA*, *asgB*, or *asgC*. As shown in Fig. 5B, the level of β-galactosidase produced by cells carrying an *asgA*, *asgB*, or *asgC* mutation is lower than in cells carrying the wild-type counterpart. The effect, however, that each *asg* mutation has on the expression of *asgE*::*lacZ* appears to be somewhat different; peak expression (24 h poststarvation) of *asgE*::*lacZ* is about 40.0% of the wild-type level in *asgB* cells, 30.0% in *asgA* cells, and 20.0% in *asgC* cells. These findings indicate that full expression of *asgE* during development is dependent on the *asgA*, *asgB*, and *asgC* gene products.

It has been shown that *csgA* mutants, which are defective for production of C-signal, fail to fully express genes that are induced after 6 h of development (19). Since developmental expression of *asgE* begins around this time, we wanted to know whether developmental expression of the *asgE* operon requires

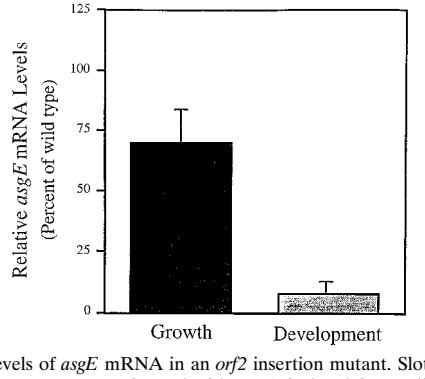

FIG. 4. Levels of *asgE* mRNA in an *orf2* insertion mutant. Slot blot hybridization experiments were performed with RNA isolated from wild-type strain DK101 and from isogenic strain MS2020, which carries the Ω5002 insertion in *orf2*. RNA was isolated from cells after 12 h of development on TPM agar and from cells that were grown to a density of 10^9 cells per ml in nutrient broth. The probe for these experiments was a 1-kb *Sac*I-*Pvu*II fragment of the *asgE* gene. For each experiment, the level of *asgE* mRNA in the MS2020 mutant was normalized to the level in wild-type DK101 cells. The mean values shown were derived from three independent experiments. Error bars represent the standard deviations of the mean.

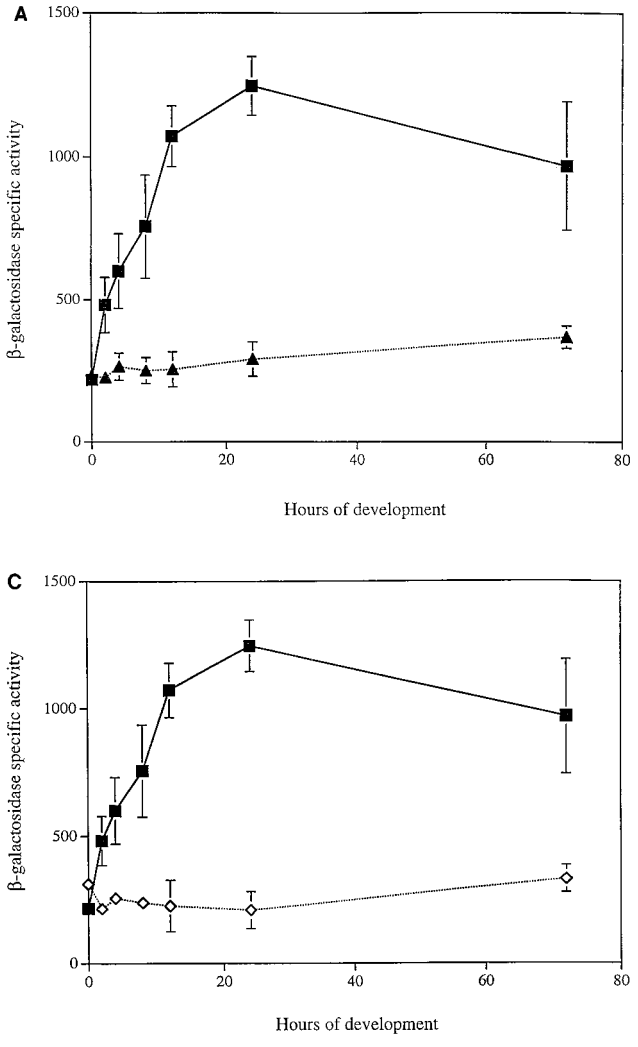

FIG. 5. Developmental expression of *asgE* in signaling mutants. The *asgE*::*lacZ* reporter gene fusion Ω5003 was introduced into mutants as described in Materials and Methods, and β-galactosidase-specific activity (defined as nanomoles of ONP produced minute^{-1} milligram of protein^{-1}) was monitored at various times during development on TPM agar. Mean β-galactosidase-specific activities were determined from three independent experiments. Error bars represent the standard deviations of the mean. β-Galactosidase-specific activities for strain MS2021 (black squares) are compared to those of *relA* strain MS2036 (black triangles) (A); *asgA* strain MS2036 (black circles), *asgB* strain MS2037 (empty circles), and *asgC* strain MS2039 (empty triangles) (B); and *csgA* strain MS2041 (empty diamonds) (C).

C-signaling. Therefore, we introduced the *asgE*::*lacZ* fusion plasmid into a strain that carries a *csgA* mutation and assayed for β-galactosidase expression during development on TPM starvation agar. The results shown in Fig. 5C indicate that developmental induction of the *asgE*::*lacZ* fusion is abolished in the *csgA* mutant.

Vegetative expression of *asgE* in a *relA* mutant. The pattern of growth phase regulation that we observed for *asgE* is strikingly similar to that of *sdeK*, a (p)ppGpp-dependent gene required for development in *M. xanthus* (8, 12). Because of this similarity, we wanted to know whether the growth phase regulation of *asgE* is (p)ppGpp dependent. Hence, we examined expression of the *asgE*::*lacZ* reporter fusion in a *relA* mutant during growth in CTT nutrient broth (Fig. 6). Consistent with the proposal that vegetative induction of *asgE* is (p)ppGpp dependent, we found that growth phase regulation of *asgE* is abolished in the *relA* mutant; no increase in β-galactosidase-specific activity is observed when cells enter mid- to late exponential growth phase in CTT. In contrast, the *asgA*, *asgB*, *asgC*, and *csgA* mutations, which block developmental expression of *asgE*, have no observable effect on the growth phase regulation of *asgE* (data not shown).

Mapping the 5′ ends of *asgE* transcripts. To identify the 5′ end(s) of the *asgE* developmental transcript, primer extension analysis was performed with 12-h developmental RNA and a primer that is complementary to the region immediately downstream of the 5′ end of the *orf2* gene. The results given in Fig. 7A show that two bands corresponding to two 5′ ends ($TSS1_{dev}$ and $TSS2_{dev}$) were identified using primer extension. One of the 5′ ends maps to a guanine nucleotide 23 bp upstream of the putative start for the Orf2 protein coding sequence. The second 5′ end maps to a cytosine nucleotide 44 bp upstream of the Orf2 start codon. No bands were identified by primer extension when we used developmental RNA and primers complementary to the 5′ end of the *asgE* gene (data not shown), further

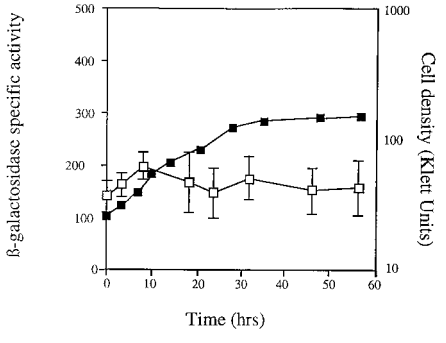

FIG. 6. Effect of a *relA* mutation on vegetative expression of *asgE*. Expression was monitored using the *asgE*::*lacZ* reporter gene fusion Ω5003 in strain MS2036. Mean β-galactosidase-specific activities were determined from three independent experiments. Error bars represent the standard deviations of the mean. Empty squares represent β-galactosidase-specific activity (defined as nanomoles of ONP produced minute^{-1} milligram of protein^{-1}), while black squares represent cell density.

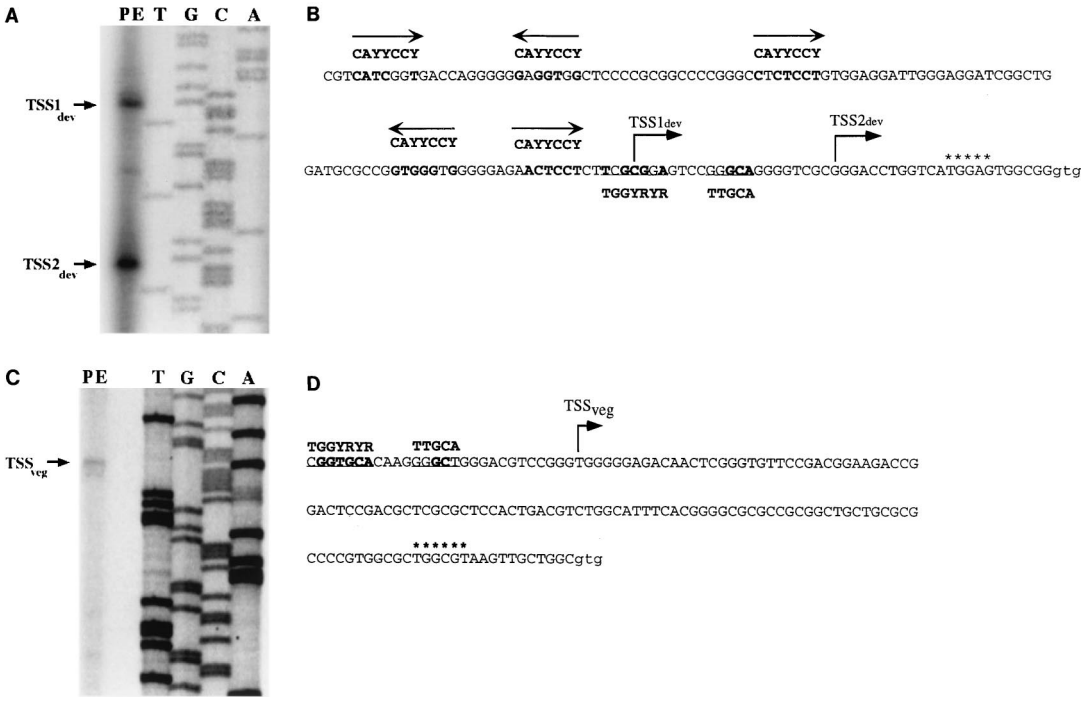

FIG. 7. (A) Mapping the 5′ ends of the *asgE* developmental transcript by primer extension analysis. A, C, T, and G show the DNA sequencing ladders. PE, primer extension using total RNA prepared from DK101 cells developing on TPM agar for 12 h and primer *ade9* (Materials and Methods). (B) DNA sequence of the region upstream of *orf2*. The putative GTG start codon for the ORF2 coding sequence is shown in lowercase letters, and nucleotides that form a potential ribosome binding site are marked with asterisks. The bent arrows above the guanine nucleotide at position −23 and the cytosine nucleotide at position −44 represent the two 5′ ends identified by primer extension. Regions of similarity to the consensus sequence for the σ^{54} family of promoters (35) (depicted below the sequence) and to the C box consensus sequence (7) (5′-CAYYCCY-3′, depicted above the sequence) are in boldface. Arrows above the C box sequences indicate the directionality of the C box with respect to the DNA strand shown. (C) Mapping of the 5′ end of the *asgE* vegetative transcript by primer extension analysis. A, C, T, and G show the DNA sequencing ladders. PE, primer extension using total RNA from DK101 cells grown in CTT to a density of 10^9 cells per ml and primer *asgE*-veg1. (D) DNA sequence of the region upstream of *asgE*. Symbols and descriptions are the same as for panel B, except that the σ^{54} consensus sequence is shown above the DNA sequence.

supporting the idea that developmental expression of *asgE* is driven solely by a promoter(s) located upstream of *orf2*.

When we examined the region preceding $TSS2_{dev}$, we found sequences with similarity to the σ^{54} family of promoters (35) and Fig. 7B). This family of promoters has two conserved regions centered 12 and 24 bp upstream of the transcriptional start site. We found the strongest overall similarity in the −24 region, with five of seven nucleotides identical to the σ^{54} consensus sequence. The −24 region for $TSS2_{dev}$ has a CG dinucleotide instead of the highly conserved GG dinucleotide. Keseler and Kaiser (17) found a similar result when they analyzed the σ^{54}-type promoter that directs transcription of the *4521* gene during development. In the −12 region of $TSS2_{dev}$, three of five matches to the σ^{54} consensus were found, including the highly conserved GC dinucleotide.

Two sets of sequences positioned around −14 and −65 bp upstream of $TSS1_{dev}$ show similarity to the CAYYCCY heptanucleotide (C box) found in the promoter regions of several C-signal-dependent genes (1, 6, 7) (Fig. 7B). At both the −14 and −65 positions, six of seven nucleotides matched those found in the C box. When we examined the DNA strand opposite the one shown in Fig. 7B, we found two additional regions centered around −23 bp (six of seven matches) and −89 bp (five of seven matches) upstream of $TSS1_{dev}$ that show similarity to the C box sequences.

To identify the 5′ end(s) of the *asgE* vegetative transcript, primer extension analysis was performed with RNA isolated from vegetative cells and a primer that is complementary to the region immediately downstream of the 5′ end of the *asgE* gene. The results given in Fig. 7C show that one band corresponding to one 5′ end (TSS_{veg}) was identified 128 bp upstream of *asgE* by using primer extension. As shown in Fig. 7D, sequences upstream of TSS_{veg} show similarity to the σ^{54} family of promoters (35). The strongest overall similarity is in the −24 region, with six of seven nucleotides identical to the σ^{54} consensus sequence, including the highly conserved GG dinucleotide. The similarity is less conserved around the −12 region, with only two of five matches to the consensus. However, the highly conserved GC dinucleotide is present.

DISCUSSION

When confronted with nutrient limitation, *M. xanthus* cells must decide whether or not to initiate development and begin to build a multicellular fruiting body. Because A-signal is produced in proportion to cell numbers (24), nutrient-deprived cells can sample the concentration of this extracellular signal and determine whether the population is sufficient to complete development. Consequently, A-signal helps *M. xanthus* make this critical decision at the onset of starvation, and mutants that fail to produce normal levels of this signal are defective for most of the important events associated with development.

Many of the steps that lead to A-signal production are unclear, although two forms of A-signal have been identified. One form of A-signal consists of a mixture of amino acids and peptides, which are heat stable, and the other form contains at least two extracellular proteases, which are heat labile (23, 27). It has been previously demonstrated that disruption of the *asgE* gene causes cells to be almost completely devoid of the extracellular protease activity associated with heat-labile A-sig-

nal (9). Hence, *asgE* mutants are defective for a variety of A-signal-dependent events, including aggregation, sporulation, and developmental gene expression.

In the work presented here, we examined the regulation of *asgE* to help uncover the events that lead to production of heat-labile A-signal. We found that the regulation of *asgE* is complex; expression of *asgE* is both growth phase regulated and developmentally regulated. During development, expression of *asgE* begins to increase relatively early (2 to 4 h), around the time that cells are beginning to aggregate into mounds. Peak expression of *asgE* occurs at about 24 h of development, and input from the A-signaling and C-signaling systems is required for this peak level of induction. During vegetative growth, expression is induced when cells reach mid- to late exponential phase, and expression continues to increase until cells begin to enter stationary phase.

In a previous study, the DNA sequence of the *asgE* locus led researchers to speculate that *asgE* may be part of a two-gene operon, which includes the upstream gene *orf2* (9). In this study, we have examined the structure of the *asgE* operon during growth and development. During growth, the dominant promoter (P_{veg}) controlling *asgE* expression appears to be located immediately upstream of the *asgE* gene itself, rather than upstream of *orf2*. We base this conclusion on several lines of evidence. First, *asgE* appears to be growth phase regulated, while *orf2* is not. Second, an insertion in *orf2* reduces transcription of *asgE* mRNA by only 30.0%. Third, using RNA from growing cells, we identified a putative transcriptional start site about 130 bp upstream of the *asgE* gene, within the protein coding sequence for Orf2. Like the *sdeK* promoter, which is a starvation-induced promoter that we examined previously (8), the P_{veg} promoter is dependent on production of the intracellular starvation signal (p)ppGpp; expression of *asgE* during growth is reduced by fourfold in a *relA* mutant. Moreover, the results of primer extension analyses suggest that starvation induction of both *sdeK* and *asgE* may be driven by a σ^{54}-like promoter element.

Developmental expression of *asgE* is also dependent on the (p)ppGpp starvation signal. It appears, however, that control of *asgE* expression is being shifted to a promoter(s) located upstream of *orf2*. Hence, we propose that *asgE* and *orf2* are coexpressed from the same developmental promoter(s) (P_{dev}). This proposal is based on three pieces of data. First, an insertion in *orf2* abolishes transcription of *asgE* developmental mRNA. Second, the patterns of *asgE* and *orf2* expression during development are virtually identical. Finally, primer extension experiments with developmental RNA yielded two potential transcriptional start sites located upstream of *orf2*, while no transcriptional start sites were identified immediately upstream of *asgE* (data not shown).

The DNA sequences upstream of this first transcriptional start site have similarity to the σ^{54} family of promoters, including the σ^{54}-like promoters that drive developmental expression of several A-signal-dependent genes (17, 35). Upstream of the second start site, we found similarity to sequences located upstream of the C-signal-dependent genes Ω4400, Ω4403, and Ω4499 (1, 6, 7). These DNA sequence similarities are consistent with the finding that full induction of *asgE* during development requires wild-type copies of *asgABC*, as well as *csgA*.

Although *asgE* expression increases during development, we believe that the levels of AsgE in vegetative cells are sufficient for this protein to carry out its function during development. We base this conclusion on the observation that *csgA* cells, which express *asgE* during vegetative growth but fail to induce *asgE* expression during development, can fully rescue the developmental defect of an *asgE* mutant when the two strains are codeveloped (9). Therefore, *csgA* cells appear to be able to provide the *asgE* mutant with sufficient levels of A-signal to rescue its developmental defects. The finding that *asgE* expression during growth is relatively high compared with that of other developmentally regulated genes is also consistent with the idea that sufficient AsgE is present before development begins (18, 19, 20, 22). Thus, the threefold increase in expression of *asgE* during development may serve to specifically adjust the levels of the AsgE protein that are already present during growth.

ACKNOWLEDGMENTS

We thank members of the Singer lab for helpful discussions and for critical reading of the manuscript. This work was supported (in part) by a National Institutes of Health postdoctoral fellowship (GM19080) to A.G.G. and by a National Institutes of Health grant (GM54592) to M.S.

REFERENCES

1. **Brandner, J. P., and L. Kroos.** 1998. Identification of the Ω4400 regulatory region, a developmental promoter of *Myxococcus xanthus*. J. Bacteriol. **180:** 1995–2004.
2. **Cho, K., and D. R. Zusman.** 1999. AsgD, a new two-component regulator required for A-signalling and nutrient sensing during early development of *Myxococcus xanthus*. Mol. Microbiol. **34:**268–281.
3. **Davis, J. M., J. Mayor, and L. Plamann.** 1995. A missense mutation in *rpoD* results in an A-signalling defect in *Myxococcus xanthus*. Mol. Microbiol. **18:**943–952.
4. **Downard, J., S. V. Ramaswamy, and K.-S. Kil.** 1993. Identification of *esg*, a genetic locus involved in cell-cell signaling during *Myxococcus xanthus* development. J. Bacteriol. **175:**7762–7770.
5. **Dworkin, M.** 1996. Recent advances in the social and developmental biology of myxobacteria. Microbiol. Rev. **60:**70–102.
6. **Fisseha, M., M. Gloudemans, R. E. Gill, and L. Kroos.** 1996. Characterization of the regulatory region of a cell interaction-dependent gene in *Myxococcus xanthus*. J. Bacteriol. **178:**2539–2550.
7. **Fisseha, M., D. Biran, and L. Kroos.** 1999. Identification of the Ω4499 regulatory region controlling developmental expression of a *Myxococcus xanthus* cytochrome P-450 system. J. Bacteriol. **181:**5467–5475.
8. **Garza, A. G., J. S. Pollack, B. Z. Harris, A. Lee, I. M. Keseler, E. F. Licking, and M. Singer.** 1998. SdeK is required for early fruiting body development in *Myxococcus xanthus*. J. Bacteriol. **180:**4628–4637.
9. **Garza, A. G., B. Z. Harris, J. S. Pollack, and M. Singer.** 2000. The *asgE* locus is required for cell-cell signaling during *Myxococcus xanthus* development. Mol. Microbiol. **35:**812–824.
10. **Hagen, D. C., A. P. Bretscher, and D. Kaiser.** 1978. Synergism between morphogenetic mutants of *Myxococcus xanthus*. Dev. Biol. **64:**284–296.
11. **Hanahan, D.** 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166:**557–580.
12. **Harris, B. Z., D. Kaiser, and M. Singer.** 1998. The guanosine nucleotide (p)ppGpp initiates development and A-factor production in *Myxococcus xanthus*. Genes Dev. **12:**1022–1035.
13. **Hodgkin, J., and D. Kaiser.** 1977. Cell-to-cell stimulation of movement in nonmotile mutants of *Myxococcus*. Proc. Natl. Acad. Sci. USA **74:**2938–2942.
14. **Kaiser, D.** 1979. Social gliding is correlated with the presence of pili in *Myxococcus xanthus*. Proc. Natl. Acad. Sci. USA **76:**5952–5956.
15. **Kaiser, D., and R. Losick.** 1993. How and why bacteria talk to each other. Cell **73:**873–885.
16. **Kaplan, H. B., A. Kuspa, and D. Kaiser.** 1991. Suppressors that permit A-signal-independent developmental gene expression in *Myxococcus xanthus*. J. Bacteriol. **173:**1460–1470.
17. **Keseler, I. M., and D. Kaiser.** 1995. An early A-signal-dependent gene in *Myxococcus xanthus* has a σ^{54}-like promoter. J. Bacteriol. **177:**4638–4644.
18. **Kroos, L., and D. Kaiser.** 1984. Construction of Tn*5lac*, a transposon that fuses *lacZ* expression to exogenous promoters, and its introduction into *Myxococcus xanthus*. Proc. Natl. Acad. Sci. USA **81:**5816–5820.
19. **Kroos, L., and D. Kaiser.** 1987. Expression of many developmentally regulated genes in *Myxococcus* depends on a sequence of cell interactions. Genes Dev. **1:**840–854.
20. **Kroos, L., A. Kuspa, and D. Kaiser.** 1986. A global analysis of developmentally regulated genes in *Myxococcus xanthus*. Dev. Biol. **117:**252–266.
21. **Kuspa, A., and D. Kaiser.** 1989. Genes required for developmental signalling in *Myxococcus xanthus*: three *asg* loci. J. Bacteriol. **171:**2762–2772.
22. **Kuspa, A., L. Kroos, and D. Kaiser.** 1986. Intercellular signaling is required for developmental gene expression in *Myxococcus xanthus*. Dev. Biol. **117:** 267–276.
23. **Kuspa, A., L. Plamann, and D. Kaiser.** 1992. Identification of heat-stable

A-factor from *Myxococcus xanthus*. J. Bacteriol. **174:**3319–3326.
24. **Kuspa, A., L. Plamann, and D. Kaiser.** 1992. A-signaling and the cell density requirement for *Myxococcus xanthus* development. J. Bacteriol. **174:**7360–7369.
25. **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.
26. **Messing, J., B. Gronenborn, B. Muller-Hill, and P. Hopschneider.** 1977. Filamentous coliphage M13 as a cloning vehicle: insertion of a *Hin*dII fragment of the *lac* regulatory region in M13 replicative form *in vitro*. Proc. Natl. Acad. Sci. USA **74:**3642–3646.
27. **Plamann, L., A. Kuspa, and D. Kaiser.** 1992. Proteins that rescue A-signal-defective mutants of *Myxococcus xanthus*. J. Bacteriol. **174:**3311–3318.
28. **Plamann, L., J. M. Davis, B. Cantwell, and J. Mayor.** 1994. Evidence that *asgB* encodes a DNA-binding protein essential for growth and development of *Myxococcus xanthus*. J. Bacteriol. **176:**2013–2020.
29. **Plamann, L., Y. Li, B. Cantwell, and J. Mayor.** 1995. The *Myxococcus xanthus asgA* gene encodes a novel signal transduction protein required for multicellular development. J. Bacteriol. **177:**2014–2020.
30. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
31. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
32. **Shimkets, L. J.** 1990. Social and developmental biology of the myxobacteria. Microbiol. Rev. **54:**473–501.
33. **Shimkets, L. J., and S. J. Asher.** 1988. Use of recombination techniques to examine the structure of the *csg* locus of *Myxococcus xanthus*. Mol. Gen. Genet. **211:**63–71.
34. **Spratt, B. G., P. J. Hedge, S. T. Heesen, A. Edelman, and J. K. Broome-Smith.** 1986. Kanamycin-resistant vectors that are analogs of plasmids pUC8, pUC9, pEMBL8 and pEMBL9. Gene **41:**337–342.
35. **Thöny, B., and H. Hennecke.** 1989. The −24/−12 promoter comes of age. FEMS Microbiol. Rev. **63:**341–358.