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# Characterization of the Regulatory Region of a Cell Interaction-Dependent Gene in *Myxococcus xanthus*

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**Ω4403 is the site of a Tn*5 lac* insertion in the *Myxococcus xanthus* genome that fuses *lacZ* expression to a developmentally regulated promoter. Cell-cell interactions that occur during development, including C-signaling, are required for expression of Tn*5 lac* Ω4403. We have cloned DNA upstream of the Ω4403 insertion site, localized the promoter, and identified a potential open reading frame. From the deduced amino acid sequence, the gene disrupted by Tn*5 lac* Ω4403 appears to encode a serine protease that is dispensable for development. The gene begins to be expressed between 6 and 12 h after starvation initiates development, as determined by measuring mRNA or β-galactosidase accumulation in cells containing Tn*5 lac* Ω4403. The putative transcriptional start site was mapped, and sequences centered near −10 and −35 bp relative to this site show some similarity to the corresponding regions of promoters transcribed by *Escherichia coli* $\sigma^{70}$ RNA polymerase. However, deletions showed that an essential promoter element lies between −80 and −72 bp, suggesting the possible involvement of an upstream activator protein. DNA downstream of −80 is sufficient for C-signal-dependent activation of this promoter. The promoter is not fully expressed when fusions are integrated at the Mx8 phage attachment site in the chromosome. Titration of a limiting factor by two copies of the regulatory region (one at the attachment site and one at the native site) can, in part, explain the reduced expression. We speculate that the remaining difference may be due to an effect of chromosomal position. These results provide a basis for studies aimed at identifying regulators of C-signal-dependent gene expression.**

*Myxococcus xanthus* is a gram-negative soil bacterium that undergoes multicellular development (17). When starved at high cell density on a solid surface, cells move into aggregation centers, where they construct a fruiting body composed of approximately $10^5$ cells. Within the fruiting body, cells differentiate into dormant, ovoid spores.

In addition to morphological changes, *M. xanthus* development involves a highly ordered program of gene expression controlled by cell-cell interactions (35). At least five cell-cell signals, known as the A-, B-, C-, D- and E-signals, are required for normal development. Cells with a mutation in a gene required for the production of any of these signals are arrested in development at a specific stage; however, development is restored when these mutants codevelop with either wild-type cells or mutant cells from a different signaling group (15, 25, 48).

To study the role of cell-cell interactions in controlling *M. xanthus* gene expression, Tn*5 lac* has been used to identify developmentally regulated genes. Tn*5 lac* is a transposon containing a promoterless *lacZ* gene inserted near one end (44). Transposition of Tn*5 lac* into the *M. xanthus* chromosome can generate a transcriptional fusion between *lacZ* and an *M. xanthus* promoter. Among 2,374 Tn*5 lac* insertions, Kroos et al. (46) found 29 in distinct transcription units which increased activity during development. Dependence of developmental gene expression on cell-cell interactions was examined by monitoring β-galactosidase expression of the *lacZ* fusions in cell interaction mutants. A- and B-signaling were found to be required for normal gene expression beginning 1 to 2 h into development (21, 45, 47). D-signaling was first required at about 4 h (9), and C-signaling was needed for normal expression of nearly all genes that begin to be expressed after 6 h into development (45, 54). It has recently been shown that E-signaling is required early in development, as demonstrated by the lack of expression of an early developmental gene (*tps*) in an E-signaling mutant (15).

We are focusing on the regulation of developmental gene expression by C-signaling. The *csg* group of mutants, which are defective in C-signaling, are genetically simple and well characterized. All of the *csg* mutations map to a single gene, *csgA*, whose product appears to mediate the cell-cell interaction (26, 40, 70, 71). CsgA is associated with the cell surface membrane and/or extracellular matrix (41, 73). Upon solubilization and partial purification, CsgA, at concentrations of approximately 1 to 2 nM, restores normal development to *csgA* mutant cells (40). Recent results suggest that CsgA may function as an enzyme which generates an unknown signal molecule that is perceived by other cells (50). C-signaling is also of interest because it appears to couple morphogenesis of the fruiting body with expression of late genes and differentiation of cells into spores. Cell movement is required for transmission of the C-signal (38, 39, 43). During development, cells move into alignment and become densely packed in the outer domain of a nascent fruiting body (64). Sager and Kaiser (63) have proposed that end-to-end and/or side-to-side contacts between cells in the outer domain permit efficient C-signaling, triggering sporulation. Consistent with this model, patches of spore precursors and expression of several C-signal-dependent *lacZ* fusions are first observed in the outer domain (63). Also, efficient sporulation and activation of a later-expressed C-signal-dependent gene require a higher level of CsgA than does aggregation and activation of an earlier-expressed C-signal-dependent gene (42, 52). In addition, C-signaling is required for rippling (65, 72). Ripples form early in development when cells organize themselves into ridges that move as traveling waves. A rising level of CsgA has been proposed to entrain the

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developmental sequence of rippling, then aggregation, and finally sporulation (52).

To begin to understand how C-signaling regulates gene expression during *M. xanthus* development, we are characterizing the DNA regulatory regions of C-signal-dependent genes. Here, we report the characterization of the regulatory region of a transcriptional unit identified by insertion of Tn*5 lac* at site Ω4403 in the *M. xanthus* chromosome (46). Expression from Tn*5 lac* Ω4403 begins approximately 8 to 15 h into development and is absolutely dependent on C-signaling (45, 47). We constructed deletions to localize the sequences required for promoter activity, determined the nucleotide sequence of the promoter region, and mapped the transcriptional start site. Promoter activity was found to require sequences farther upstream of the transcriptional start site than is usually required for RNA polymerase binding, suggesting the involvement of an additional regulatory protein(s). Analysis of the minimal promoter region in *csgA* mutant cells showed that it does require C-signaling for activity. These studies therefore lay the foundation for the identification of regulatory proteins required for C-signal-dependent expression of genes.

## MATERIALS AND METHODS

**Bacterial strains and plasmids.** Strains and plasmids used in this work are listed in Table 1.

**Growth and development.** *Escherichia coli* cells were grown at 37°C in LB medium containing 50 μg of ampicillin or 25 μg of kanamycin per ml when necessary. *M. xanthus* was grown at 32 to 34°C in CTT medium (30) in liquid cultures or on agar plates as described previously (46). Forty micrograms of kanamycin or 12.5 μg of oxytetracycline per ml was used when required for selective growth. Fruiting body development was performed on TPM (10 mM Tris-HCl [pH 8.0], 1 mM $KH_2PO_4$, 8 mM $MgSO_4$ [final pH 7.6]) agar (1.5%) plates as described previously (46).

**Molecular cloning and construction of plasmids.** Recombinant DNA work was performed by using standard techniques (66). Plasmid DNA was prepared from *E. coli* DH5α or JM83.

To clone the DNA upstream of Tn*5 lac* Ω4403, chromosomal DNA was prepared (49) from *M. xanthus* DK4368 and digested with *Xho*I, the fragments were ligated to *Xho*I-digested pGEM7Zf, and the mixture was transformed into *E. coli* DH5α, selecting for both ampicillin resistance ($Amp^r$) of the vector and kanamycin resistance ($Km^r$) of the desired insert. One transformant with a plasmid bearing an insert of the expected size was characterized further. Restriction fragments of *M. xanthus* DNA from this plasmid, pMES003, were gel purified and ligated into vectors as indicated in Table 1. Vectors were digested with the same restriction enzymes used to produce the fragments, except as indicated below.

To test *M. xanthus* DNA fragments for promoter activity, we constructed pREG1666 and its derivative pREG1727. pREG1666 was derived from pREG1175 (20) as follows. A 3-kbp *Sma*I fragment from phage Mx8 (76) was ligated into *Sma*I-digested pUC18 (80), then excised as an *Eco*RI-*Bam*HI fragment, and ligated into *Eco*RI-*Bam*HI-digested pREG1175. The resulting plasmid was digested with *Bam*HI, and a 711-bp *Sau*3AI fragment from pRS577 (75) containing four copies of the T1 terminator of the *E. coli rrnB* operon was inserted. Only the *Sau*3AI end downstream of the terminators regenerated a *Bam*HI site upon insertion into the *Bam*HI-digested vector. A plasmid in which the unique remaining *Bam*HI site was proximal to *lacZ* was then digested with *Bam*HI, and a 49-bp *Bgl*II-*Bam*HI fragment containing multiple restriction sites was inserted. pREG1666 has the polylinker in the orientation that leaves the unique remaining *Bam*HI site proximal to *lacZ*. The polylinker fragment was generated by deleting the *Bgl*II site in the polylinker of pSP72 (Promega), digesting the resulting plasmid with *Xho*I, filling in the ends with the Klenow fragment of DNA polymerase I, ligating the plasmid onto *Bgl*II linkers (5′-GAA GATCTTC-3′), and digesting it with *Bgl*II and *Bam*HI. pREG1666 was used initially to test several *M. xanthus* fragments for promoter activity upon integration at the Mx8 phage attachment site (Mx8 *att*). To identify *M. xanthus* strains containing a single copy of the plasmid integrated at Mx8 *att*, the 3-kbp *Sma*I fragment from pREG1666 containing Mx8 *attP* was labeled and used to probe Southern blots of *Eco*RI-*Xho*I-digested chromosomal DNA as described below. In the desired strains, fragments 2 and 10 kbp in length hybridized the probe, and a 4-kbp fragment corresponding to unrecombined *attP* was absent. Occasionally, a fragment slightly smaller than the expected 2-kbp fragment was detected. We examined the structure of pREG1666 and found the terminator-containing fragment to be slightly larger than expected. We do not know the source of the additional DNA. pREG1727 is identical to pREG1666 except that the terminator-containing fragment is of the expected size. Therefore, pREG1727 was used subsequently to test *M. xanthus* fragments for promoter activity; however, this did not solve the problem of occasional integrants (about 15%) displaying a fragment slightly smaller than 2 kbp upon Southern blotting. We suspect that recombination within the terminator-containing fragment accounts for this observation. Integrants showing a fragment smaller than 2 kbp were not used. In addition, we compared developmental *lacZ* expression from pREG1666, pREG1727, and derivatives of these two plasmids containing a 0.6-kbp insert of the Ω4403 upstream region and detected no significant difference between the two vectors with *M. xanthus* strains bearing a single copy of each plasmid integrated at Mx8 *att*.

pMF3.4 was constructed by digesting pMF01 with *Sph*I, which cuts once in the 1.0-kb *M. xanthus* insert and once in the multiple cloning site of pUC19 near the end of the insert more distal from the Ω4403 insertion site, releasing a fragment of about 400 bp. The 3.4-kb vector-containing fragment was gel purified and ligated, generating pMF3.4.

To construct pMF100, the insert fragment was generated by digesting pMF01 with *Hae*II and making these ends blunt by digestion with mung bean nuclease (66). After phenol-chloroform extraction and ethanol precipitation, the DNA was digested with *Bam*HI and fractionated on an agarose gel. The supposed 523-bp fragment was purified and directionally cloned into pREG1727 that had been digested with *Hin*dIII, subjected to end filling using the Klenow fragment of DNA polymerase I (66), digested with *Bam*HI, and gel purified. The *Hin*dIII-*Hae*II junction in pMF100 was sequenced by first subcloning the insert as a *Xho*I-*Bam*HI fragment into *Xho*I-*Bam*HI-digested pGEM7Zf, generating pMF0100. The M13 pUC forward sequencing primer (United States Biochemical) was used to sequence this junction by the Sanger method (67) as described below. The sequence showed that mung bean nuclease had overdigested the *Hae*II end by 2 bp. Thus, pMF100 contains a 521-bp insert rather than the 523-bp insert predicted by the location of the *Hae*II restriction site.

pMF200 was constructed by directional cloning of the 452-bp *Rsa*I-*Bam*HI fragment from pMF01 into pREG1727 that had a filled-in *Hin*dIII blunt end and a *Bam*HI cohesive end (prepared as described above for pMF100).

pMF072, -062, and -052 contain PCR-generated fragments of Ω4403 upstream DNA. The regions between −72, −62, and −52 bp (relative to the putative Ω4403 transcriptional start site) and the *Bam*HI site at the left end of Tn*5 lac* were amplified by using pMF01 as the template. For this purpose, the downstream primer was the M13 pUC forward sequencing primer (United States Biochemical) that binds pUC19 downstream of the *Bam*HI site, and the upstream primers were 34-mers that bind between 454 and 429, 444 and 419, 434 and 409 bp upstream of the Ω4403 insertion site. The 5′ ends of the upstream primers had a 9-base sequence containing a *Xho*I site that does not bind to the template. The amplified fragments were digested with *Bam*HI and *Xho*I, gel purified, and ligated into *Xho*I-*Bam*HI-digested pGEM7Zf, resulting in pMF072, -062, and -052. The sequences of inserts in these plasmids were determined and found to be identical to the wild-type sequence. pMF301 also contains a PCR-generated fragment of Ω4403 upstream DNA. In this case, the upstream primer binds between 424 and 405 bp upstream of the Ω4403 insertion site, and the downstream primer was the same as described above. The amplified fragment was digested with *Xho*I and *Bam*HI, gel purified, and ligated into *Xho*I-*Bam*HI-digested pREG1727, generating pMF301. To sequence the insert fragment, the *Xho*I-*Bam*HI fragment from pMF301 was first cloned into *Xho*I-*Bam*HI-digested pGEM7Zf, generating pMF0301, and sequencing was performed on this plasmid.

To clone the 17-kb *Eco*RI-*Bam*HI fragment upstream of Ω4403, pSH1 (Table 1) was transduced into DK1622 by using bacteriophage P1 (22), with selection for $Km^r$. One of the $Km^r$ transductants, MSH1, had pSH1 integrated at the native site (designated Ω*4403* in Table 1) by homologous recombination, as verified by Southern blot analysis (data not shown). Chromosomal DNA from MSH1 was digested with *Eco*RI, which cuts *M. xanthus* DNA 17 kb upstream of the Ω4403 insertion site and cuts downstream at the distal end of the integrated vector. Religation at low DNA concentration to favor recircularization, followed by transformation into *E. coli* DH5α with selection for $Km^r$ and $Ap^r$, resulted in the cloning of pLK1. To test the 17-kb Ω4403 upstream segment in pLK1 for promoter activity, the plasmid was digested with *Eco*RI, the ends were filled in with the Klenow fragment of DNA polymerase I, and the plasmid was digested with *Bam*HI. The 17-kb fragment was gel purified and ligated to pREG1727 that had a filled-in *Hin*dIII blunt end and a *Bam*HI cohesive end (prepared as described above for pMF100), resulting in pMF71-1.

**DNA sequencing.** DNA fragments to be sequenced were cloned into either pUC19 or pGEM7Zf and sequenced on both strands by using synthetic oligonucleotide primers. Double-stranded sequencing was performed by the Sanger method (67), using a Sequenase kit (United States Biochemical). Ambiguities arising from premature termination were resolved by using the protocol of Fawcett and Bartlett (18). Briefly, 1 μl of a reaction mixture containing terminal deoxynucleotide transferase (1 μM [each] deoxynucleoside triphosphate [pH 7.0], 2 U of terminal deoxynucleotide transferase per μl, 1× Sequenase reaction buffer) was added to each of the termination reactions (total of 7 μl) and incubated at 37°C for 30 min. The reaction was terminated by using 4 μl of stop buffer (United States Biochemical). 7-Deaza-dGTP reaction mixes (United States Biochemical) were used to resolve regions of compression. DNA and protein sequence analyses were done with the University of Wisconsin Genetics Computer Group software package.

TABLE 1. Bacterial strains and plasmids used

| Strain(s) or plasmid(s) | Relevant characteristic(s) | Source or reference |
|---|---|---|
| *E. coli* | | |
| DH5α | ϕ80 Δ*lacZ* ΔM15 Δ*lacU169 recA1 endA1 hsdR17 supE44 thi-1 gyrA relA1* | 27 |
| JM83 | *ara* Δ*lac-pro strA thi* ϕd*lacZ* ΔM15 | 56 |
| *M. xanthus* | | |
| DK1622 | Wild type | 34 |
| DK4368 | Tn*5 lac* ($Km^r$) Ω4403 | 46 |
| DK4499 | Tn*5 lac* ($Km^r$) Ω4499 | 46 |
| JW103, -104, -107 | *attB*::pREG1666 | Jamie White |
| MMF1727-7, -9, -11 | *attB*::pREG1727 | This work |
| MES005, -008, -012 | *attB*::pMES004 | This work |
| MMF71-6, -15, -22 | *attB*::pMF71-1 | This work |
| MES034, -036, -047 | *attB*::pMES116 | This work |
| MES053, -064, -077 | *attB*::pMES115 | This work |
| MMF31-3, -7, -9 | *attB*::pMF31 | This work |
| MMF81-1, -8, -32 | *attB*::pMF81 | This work |
| MMF100-6, -17, -24 | *attB*::pMF100 | This work |
| MMF72-2, -10, -32 | *attB*::pMF72 | This work |
| MMF62-4, -34 | *attB*::pMF62 | This work |
| MMF52-35, -44, -45 | *attB*::pMF52 | This work |
| MMF301-3, -4, -5 | *attB*::pMF301 | This work |
| MMF200-5, -6, -7 | *attB*::pMF200 | This work |
| MES014, -016, -017 | Ω*4403*::pMES108 | This work |
| MSH1 | Ω*4403*::pSH1 | Stacie Hill |
| JPB07 | Tn*5 lac* ($Tc^r$) Ω4403 | Janine Brandner |
| MLK1-3, -4, -5 | Ω*4403*::pLK1 Tn*5 lac* ($Tc^r$) Ω4403 | This work |
| DK5208 | *csgA*::Tn*5-132* ($Tc^r$) Ω205 | 70 |
| MES119 | *csgA*::Tn*5-132* ($Tc^r$) Ω205 *attB*::pREG1666 | This work |
| MMF100C-6, -17, -24 | *csgA*::Tn*5-132* ($Tc^r$) Ω205 *attB*::pMF100 | This work |
| MMF200C-4, -7, -11 | *csgA*::Tn*5-132* ($Tc^r$) Ω205 *attB*::pMF200 | This work |
| Plasmids | | |
| pUC19 | $Ap^r$ *lac*α | 80 |
| pGEM7Zf | $Ap^r$ *lac*α | Promega |
| pREG1666/pREG1727 | $Ap^r$ $Km^r$ P1-*inc attP* | This work |
| pREG1175 | $Ap^r$ $Km^r$ P1-*inc* | 20 |
| pREG429 | $Ap^r$ $Km^r$ P1-*inc* | 22 |
| pMES003 | $Ap^r$ $Km^r$ (pGEM7Zf), 17-kb *Xho*I fragment from DK4368 | This work |
| pMES004 | $Ap^r$ $Km^r$ P1-*inc attP* (pREG1666), 8.5-kb *Xho*I-*Bam*HI fragment from pMES003 | This work |
| pMES110 | $Ap^r$ (pGEM7Zf), 4.0-kb *Cla*I-*Bam*HI fragment from pMES003 | This work |
| pMES112 | $Ap^r$ (pUC19), 2.0-kb *Pst*I-*Bam*HI fragment from pMES003 | This work |
| pMES115 | $Ap^r$ $Km^r$ P1-*inc attP* (pREG1666), 2.0-kb *Hin*dIII-*Bam*HI fragment from pMES112 | This work |
| pMES116 | $Ap^r$ $Km^r$ P1-*inc attP* (pREG1666), 4.0-kb *Xho*I-*Bam*HI fragment from pMES110 | This work |
| pMF01 | $Ap^r$ (pUC19), 1.0-kb *Sal*I-*Bam*HI fragment from pMES112 | This work |
| pMF31 | $Ap^r$ $Km^r$ P1-*inc attP* (pREG1666), 1.0-kb *Hin*dIII-*Bam*HI fragment from pMF01 | This work |
| pMF3.4 | $Ap^r$ (pUC19), 674-bp *Sph*I-*Bam*HI fragment from pMF01 | This work |
| pMF81 | $Ap^r$ $Km^r$ P1-*inc attP* (pREG1727), 680-bp *Hin*dIII-*Bam*HI fragment from pMF3.4 | This work |
| pMF100 | $Ap^r$ $Km^r$ P1-*inc attP* (pREG1727), 521-bp *Hae*II-*Bam*HI fragment from pMF01 | This work |
| pMF0100 | $Ap^r$ (pGEM7Zf), 537-bp *Xho*I-*Bam*HI fragment from pMF100 | This work |
| pMF072 | $Ap^r$ (pGEM7Zf), 513-bp *Xho*I-*Bam*HI fragment generated by PCR of pMF01 | This work |
| pMF72 | $Ap^r$ $Km^r$ P1-*inc attP* (pREG1727), 513-bp *Xho*I-*Bam*HI fragment from pMF072 | This work |
| pMF062 | $Ap^r$ (pGEM7Zf), 503-bp *Xho*I-*Bam*HI fragment generated by PCR of pMF01 | This work |
| pMF62 | $Ap^r$ $Km^r$ P1-*inc attP* (pREG1727), 503-bp *Xho*I-*Bam*HI fragment from pMF062 | This work |
| pMF052 | $Ap^r$ (pGEM7ZF), 493-bp *Xho*I-*Bam*HI fragment generated by PCR of pMF01 | This work |
| pMF52 | $Ap^r$ $Km^r$ P1-*inc attP* (pREG1727), 493-bp *Xho*I-*Bam*HI fragment from pMF052 | This work |
| pMF301 | $Ap^r$ $Km^r$ P1-*inc attP* (pREG1727), 483-bp *Xho*I-*Bam*HI fragment generated by PCR of pMF01 | This work |
| pMF0301 | $Ap^r$ (pGEM7Zf), 483-bp *Xho*I-*Bam*HI fragment from pMF301 | This work |
| pMF200 | $Ap^r$ $Km^r$ P1-*inc attP* (pREG1727), 452-bp *Rsa*I-*Bam*HI fragment from pMF01 | This work |
| pMES108 | $Ap^r$ $Km^r$ P1-*inc* (pREG1175), 8.5-kb *Xho*I-*Bam*HI fragment from pMES003 | This work |
| pSH1 | $Ap^r$ $Km^r$ P1-*inc* (pREG429), 4.0-kb *Cla*I-*Bam*HI fragment from pMES110 | Stacie Hill |
| pLK1 | $Ap^r$ $Km^r$ P1-*inc* (pREG429), 17-kb *Eco*RI-*Bam*HI fragment from MSH1 | This work |
| pMF71-1 | $Ap^r$ $Km^r$ P1-*inc attP* (pREG1666), 17-kb *Bam*HI-*Eco*RI fragment from pLK1 | This work |

**Construction of *M. xanthus* strains.** Strains containing pREG1666 or pREG1727, or derivatives of these plasmids, integrated at Mx8 *att* (designated *attB* in Table 1) were constructed by P1 specialized transduction from the $rec^+$ *E. coli* strain JM83 into either the wild-type *M. xanthus* strain DK1622 or the *csgA* mutant strain DK5208 as described previously (22). For each plasmid, three transductants (Table 1), each containing a single copy of the plasmid integrated at Mx8 *att*, were identified by Southern blot analysis (data not shown). Similarly, pMES108 was transduced into DK1622, and three transductants containing a single copy of the plasmid integrated at the native site (designated Ω*4403* in Table 1) by homologous recombination were identified by Southern blotting (data not shown).

Strain JPB07 was constructed by transducing bacteriophage P1::Tn*5 lac* ($Tc^r$)

into DK4368 with selection for oxytetracycline resistance ($Tc^r$). Screening for kanamycin-sensitive transductants identified JPB07 in which the $Km^r$ gene was replaced by the $Tc^r$ gene, as verified by Southern blot analysis (6). The maximum level of developmental *lacZ* expression from JPB07 (and similar, independent isolates) is consistently about twofold lower than that from DK4368 (6); the reason for this difference is unknown. MLK1-3, -4, and -5 are $Km^r$ $Tc^r$ strains resulting from P1 specialized transduction (22) of pLK1 from *E. coli* JM83 into *M. xanthus* JPB07.

**Southern blot analysis.** *M. xanthus* chromosomal DNA was isolated by the method of Avery and Kaiser (4). Three to 5 μg of DNA was digested with the appropriate restriction enzyme(s) and fractionated on a 0.5% agarose gel. DNA was transferred as described previously (12) except that nylon membranes were used. Probe synthesis and hybridization of the membrane were carried out with a Genius kit (Boehringer Mannheim) according to the manufacturer's specifications.

**RNA isolation.** Total RNA was purified from DK4368 (which contains the Tn*5* *lac* Ω4403 fusion transcript) or DK4499 (which contains the wild-type Ω4403 transcript) by using the protocol of Igo and Losick (31), with the following modifications. Cells were harvested from exponentially growing cultures (50 ml of $5 \times 10^8$ cells per ml) or from an equivalent number of cells at 24 h of development on TPM agar plates, washed with ice-cold TPM, and resuspended in 2 ml of LETS buffer (100 mM LiCl, 10 mM EDTA, 10 mM Tris [pH 7.8] with LiCl, 1% sodium dodecyl sulfate). After isolation (31), the nucleic acid was resuspended in 200 μl of diethylpyrocarbonate (0.1% [vol/vol])-treated water and treated with RNase-free DNase (Boehringer Mannheim) to eliminate DNA. DNase was removed by extraction with phenol-chloroform and precipitation with ethanol. RNA was resuspended in diethylpyrocarbonate-treated water and used for further analysis.

**S1 nuclease protection experiments.** Low-resolution mapping of the 5′ end of the Ω4403-associated transcript was performed as described previously (66). Plasmid pMES112 was digested with *Bam*HI, phosphatase treated, 5′ end labeled with [γ-$^{32}$P]ATP and T4 polynucleotide kinase, and used as a probe. 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 of the nucleic acids at 85°C for 10 min, the samples were incubated at 53°C for 16 h. Unhybridized, single-stranded DNA and RNA were digested for 1 h at 37°C with S1 nuclease (25 to 250 U; Boehringer Mannheim) in a final volume of 200 μl containing 1× S1 nuclease mapping buffer (66). The reaction was stopped by the addition of 40 μl of stop solution and was extracted with phenol-chloroform (150 μl). The samples were precipitated with ethanol, resuspended in formamide loading buffer, and loaded onto a 5% polyacrylamide–8 M urea gel. The protected products were separated by electrophoresis at 25 mA and visualized by autoradiography.

Quantitative S1 nuclease mapping experiments were carried out as just described except that yeast tRNA was added to some samples to maintain a constant total amount of input RNA (50 μg). Signals were quantified with a Visage Digital Imager and fell within the linear response range of the assay as determined with different amounts (0 to 100 μg) of RNA from DK4368 cells harvested at 18 h of development.

**Primer extension analysis.** Primer extension reactions were performed as described previously (66), using 20 μg of RNA. The oligonucleotide used for the primer extension analysis (5′-CGCCCCCATCAGCAACATCATGCCC ACGGA-3′) corresponds to a sequence approximately 100 bp downstream of the putative transcriptional start site mapped by S1 nuclease protection. The primer was end labeled with T4 polynucleotide kinase and [γ-$^{32}$P]ATP and purified as described previously (3). Primer extension products were electrophoresed at 60 W on a sequencing gel (6% polyacrylamide, 5 M urea) in parallel with sequencing reactions performed with the same end-labeled primer.

**Nucleotide sequence accession number.** The DNA sequence of the Ω4403 upstream region as reported here has been deposited in the GenBank database with accession number U41374.

## RESULTS

**Cloning DNA upstream of Ω4403 and testing it for promoter activity.** To clone the putative promoter region upstream of the developmentally regulated Tn*5* *lac* insertion Ω4403, we took advantage of a *Xho*I restriction site approximately 8 kb upstream of the site of insertion Ω4403 (46) and a *Xho*I site in Tn*5* *lac* about 9 kb from the left end (Fig. 1). Since the *Xho*I site in Tn*5* *lac* is downstream of the *aphII* gene (encoding aminoglycoside phosphotransferase, which confers $Km^r$), *Xho*I digestion of chromosomal DNA from *M. xanthus* containing Tn*5* *lac* Ω4403 should yield a 17-kb fragment able to confer $Km^r$ when cloned into *E. coli*. We cloned the 17-kb fragment as described in Materials and Methods. Restriction mapping of the resulting plasmid, pMES003, showed the patterns expected on the basis of restriction sites in DNA upstream of Ω4403 that had been mapped by Southern blotting (46) and on the basis of known restriction sites in Tn*5* *lac* and the vector (data not shown). Figure 1 shows a restriction map of DNA upstream of Ω4403 generated on the basis of these results.

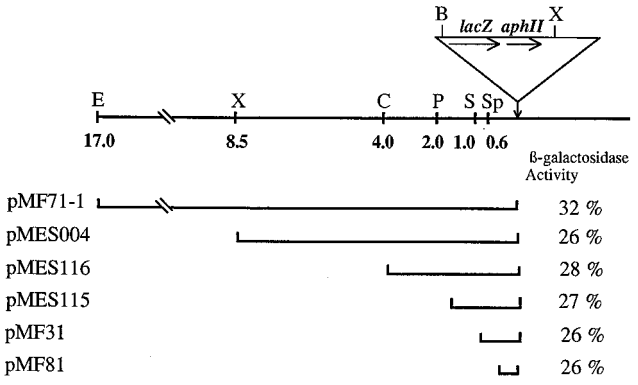


FIG. 1. Physical map of the Ω4403 insertion region and summary of deletions tested for promoter activity. The top part shows restriction sites in Tn*5* *lac* and the adjacent *M. xanthus* chromosome that were used in cloning experiments. Distances of restriction sites from the Tn*5* *lac* Ω4403 insertion are given in kilobases. B, *Bam*HI; C, *Cla*I; E, *Eco*RI; P, *Pst*I; S, *Sal*I; Sp, *Sph*I; X, *Xho*I. The bottom part shows different segments of Ω4403 upstream DNA that were fused to *lacZ* to permit testing for promoter activity as described in Materials and Methods. The maximum β-galactosidase specific activities during a 48-h developmental time course of wild-type *M. xanthus* strain DK1622 derivatives containing a single copy of each plasmid at Mx8 *att* are given as percentages of the maximum activity observed for strain DK4368 containing Tn*5* *lac* Ω4403. In each case, the maximum activity of three independent transductants was measured as described previously (46) and the average is given.

To test the DNA upstream of Ω4403 for promoter activity, the *Xho*I-*Bam*HI restriction fragment from pMES003, which includes 8.5 kb of *M. xanthus* DNA and about 50 bp of the left end of Tn*5* *lac* (Fig. 1), was subcloned into *Xho*I-*Bam*HI-digested pREG1666 (Fig. 2) to construct pMES004. Because the multiple cloning site in pREG1666 is upstream of the same *lacZ*-containing segment found in Tn*5* *lac*, pMES004 contains Ω4403 upstream DNA fused to *lacZ* in exactly the same way as in the chromosome of *M. xanthus* DK4368, which has Tn*5* *lac*

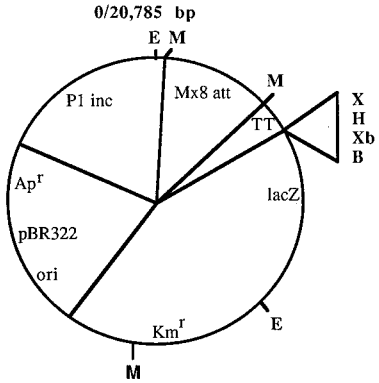


FIG. 2. Map of the plasmids used to test for promoter activity. A single map is shown to represent pREG1666 and pREG1727 (see Materials and Methods for a description of the difference between these two closely related plasmids). Unique cloning sites include *Bam*HI (B), *Hin*dIII (H), *Xba*I (Xb), and *Xho*I (X). Additional restriction sites indicated are *Eco*RI (E) and *Sma*I (M). These plasmids contain Mx8 *att*, *E. coli* transcriptional terminators (TT), the promoterless *lacZ* gene, the *aphII* gene (conferring $Km^r$) with its own promoter, part of pBR322 including the origin of replication (ori) and the *bla* gene (conferring $Ap^r$), and a P1 incompatibility fragment (P1 inc).

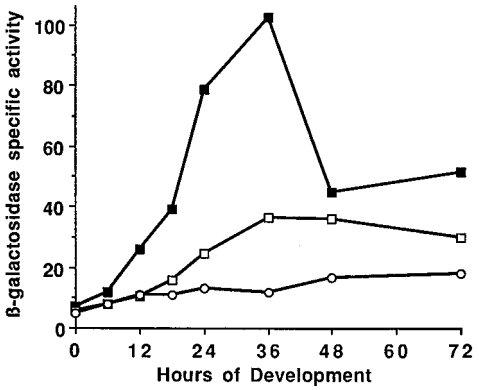


FIG. 3. Expression of developmental *lacZ* under the control of the Ω4403 promoter. Developmental *lacZ* expression was determined as described previously (46) for *M. xanthus* DK4368 containing Tn*5 lac* Ω4403 (■) and for three independently isolated transductants containing a single copy of the 8.5-kb Ω4403 upstream DNA fused to *lacZ* and integrated at Mx8 *att* (strains MES005, -008, and -012 in Table 1) (□) or the vector with no insert DNA at Mx8 *att* (JW103, -104, and -107) (○). The average β-galactosidase activity from three determinations for DK4368 or from one determination for each of three independent transductants is plotted. The units of β-galactosidase specific activity are nanomoles of *o*-nitrophenyl phosphate per minute per milligram of protein.

inserted at site Ω4403 (46). When pMES004 was transduced from *E. coli* JM83 into the wild-type *M. xanthus* strain DK1622 by using bacteriophage P1 specialized transduction, it integrated into the chromosome efficiently at Mx8 *att*. Transductants containing a single copy of pMES004 integrated at Mx8 *att* were identified by Southern blot hybridization (data not shown; see Materials and Methods). Several of these transductants were assayed for β-galactosidase activity during development. In parallel, developmental *lacZ* expression of *M. xanthus* DK4368 containing Tn*5 lac* Ω4403 was measured. Also, as a negative control, expression of transductants containing a single copy of pREG1666 (with no *M. xanthus* DNA insert) integrated at Mx8 *att* was examined. Figure 3 shows that transductants containing pMES004 integrated at Mx8 *att* expressed *lacZ* with timing similar to that for the strain containing Tn*5 lac* Ω4403 but reached only about 25% of the maximum level (after subtraction of the background expression observed for the negative control). These results indicate that the 8.5-kb Ω4403 upstream DNA has a promoter that is able to drive development-specific expression of a *lacZ* reporter gene.

To determine whether a higher level of promoter activity could be observed by cloning additional upstream DNA, we cloned 17 kb of DNA upstream of Ω4403 (see Materials and Methods) and tested it for promoter activity as described above. Like the 8.5-kb segment, the 17-kb segment directed only about 25% of the maximum level of developmental *lacZ* expression seen with *M. xanthus* DK4368 containing Tn*5 lac* Ω4403 (Fig. 1). This result suggests that the lower activity of fusions integrated at Mx8 *att* than of the fusion created by insertion of Tn*5 lac* at site Ω4403 is unlikely to be due to insufficient upstream DNA.

To determine the approximate location of the promoter in the 8.5-kb Ω4403 upstream DNA, smaller fragments were tested for promoter activity after fusion to *lacZ* and integration at Mx8 *att* as described above. Figure 1 shows that promoter activity localized to 0.6 kb of DNA immediately upstream of Ω4403. No significant difference was observed in the timing or level of developmental *lacZ* expression for any of the fragments that showed promoter activity.

**Accumulation of the Tn*5 lac* Ω4403 fusion mRNA during development.** Localization of promoter activity within 0.6 kb upstream of Ω4403 indicated that an RNA 5′ end should map within this region. A probe labeled at the *Bam*HI site near the left end of Tn*5 lac* (Fig. 1), including about 2 kb of *M. xanthus* DNA upstream of Ω4403, was hybridized to RNA from *M. xanthus* DK4368, which contains Tn*5 lac* Ω4403 (46), and subjected to S1 nuclease protection analysis. RNA from 24-h developing cells, but not from growing cells, protected a fragment about 430 bases in length. We used this assay to quantitate the relative amount of fusion mRNA from Tn*5 lac* Ω4403 in strain DK4368 during development. Figure 4 shows that the fusion mRNA was first detected at 12 h of development and reached a maximum level at 24 h. β-Galactosidase activity was measured in samples collected in the same experiment. Figure 4 also shows that accumulation of β-galactosidase activity lagged slightly behind accumulation of fusion mRNA. We conclude that Tn*5 lac* Ω4403 identifies a transcriptional unit with a putative start site approximately 430 bp upstream of the *Bam*HI site in Tn*5 lac*. The increase in the level of fusion mRNA during development appears to account for the observed developmentally regulated β-galactosidase activity of strain DK4368. The increase in fusion mRNA probably results from activation of a developmentally regulated promoter, although we cannot exclude the possibility that the fusion mRNA is stabilized in a developmentally regulated fashion.

We were unable to detect any discrete species of fusion mRNA from developing DK4368 cells by Northern (RNA) blot analysis (data not shown). Apparently, the large fusion mRNA is susceptible to degradation. The ability to map the 5′ end of the fusion mRNA suggests that the S1 nuclease assay is more sensitive and/or may involve hybridization of probe to partial breakdown products of the fusion mRNA. Using *M. xanthus* cells that do not contain Tn*5 lac* Ω4403 and using

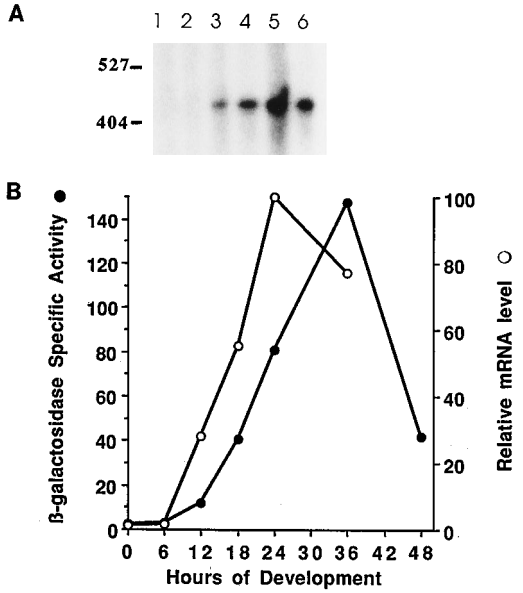


FIG. 4. Levels of fusion mRNA and β-galactosidase specific activity from Tn*5 lac* Ω4403 during development. (A) Quantitative S1 nuclease protection analysis of developmental RNA from *M. xanthus* DK4368, performed as described in Materials and Methods with 50 μg of RNA harvested from cells at 0, 6, 12, 18, 24, and 36 h of development in lanes 1 to 6, respectively. The numbers on the left indicate lengths (in bases) of end-labeled *Msp*I-digested pBR322 marker fragments. (B) Quantitation of the S1 nuclease-protected products in panel A (○) and β-galactosidase specific activity of samples harvested in the same experiment (●).

```
SphI
GCATGCCCACCTGGTGAACGCAGCACGAGAGATGGGCATCAACGACATCCTGTCACGCCC

CGGGAAACGTCCACTGGAAGCAGTCTTGTACGAATGGGGAACCACCATTCGTCCTTGGTA
                          HaeII-80     -72       -62
GGTCATGGCCAGACATCGCGCCTTGAAGCGCCATGGCATGTTCAATCACGGACCGCCGTC
-52       -42                  -11       +1
TCATCCCTCCGGGTTGATTCATGAATAAGCCGTTTTTGATGTACACCCGTTTTACCCATC

ACAACCATTTTCCTCTGGGTAAAACATGAATCGACACACACTGAAGCTGTTCGTCTCCGT

GGGCATGATGTTGCTGATGGGGGCGTGCGCATCGCGCAGGTGCCCTGCAAGGACCGCACG

GCCAGCAGGCCCACGATGGGCCAGAAGCAGACCGGCAAGTTCATCACTGTCCGAAAGAAG
               M  G  Q  K  Q  T  G  K  F  I  T  V  R  K  K

ATTCCGGGTGAATACATTGTCGTCCTGAAGTCGCCCGCACAAAGCCTGGAACAGGTCGAG
 I  P  G  E  Y  I  V  V  L  K  S  P  A  Q  S  L  E  Q  V  E

GTCCAGCAAGCCACGACGAGCCTCATCACGGCTTACGGTGGCACCGCATTCGCGATGTAT
 V  Q  Q  A  T  T  S  L  I  T  A  Y  G  G  T  A  F  A  M  Y

GAGAATGCGTTGCGTGGTTTCGCGGCCAAGATGACGGAAGCCCAGGCGCGGGCCATGGCG
 E  N  A  L  R  G  F  A  A  K  M  T  E  A  Q  A  R  A  M  A

AACGACCCCGCAGCTG
 N  D  P  A  A
```

FIG. 5. Nucleotide sequence upstream of Tn*5 lac* Ω4403. The transcriptional start site is indicated by +1, and the primer used for primer extension analysis is underlined. The putative translational start codon and a potential ribosome binding site are boxed, and the deduced amino acid sequence of the Ω4403 partial ORF is shown below the nucleotide sequence. Restriction sites used to generate some deletions in the Ω4403 promoter region are shown. The numbers above the nucleotide sequence indicate the 5′ ends of deletions that were tested for promoter activity.

DNA immediately upstream of Ω4403 as the labeled probe, we detected a single transcript of approximately 1.5 kb by Northern blotting of RNA isolated from 24-h developing cells but not from growing cells (data not shown). Since the native Ω4403 mRNA has the same 5′ end as the Tn*5 lac* Ω4403 fusion mRNA (see below), the 1.5-kb size of the native transcript suggests that transcription terminates about 1.1 kb downstream of the Ω4403 insertion site.

**DNA sequence of the Ω4403 upstream region.** Figure 5 shows the nucleotide sequence of 616 bp of *M. xanthus* DNA immediately upstream of the Ω4403 insertion site. An open reading frame (ORF) beginning with ATG at position 376 is preceded 6 nucleotides upstream by the sequence 5′-AGC AGG-3′, which is complementary (except for one mismatch) to a sequence near the 3′ end of *M. xanthus* 16S rRNA (58), suggesting that it could serve as a ribosome binding site. The ORF exhibits a codon preference typical of *M. xanthus* genes (26), including a strong bias toward usage of guanine or cytosine at the third codon position (69). The ORF remains uninterrupted for at least 80 amino acids, extending to the Ω4403 insertion site at the end of the sequenced region. The deduced amino acid sequence of the ORF shows 38% amino acid identity and 73% amino acid similarity over a 63-amino-acid stretch to aqualysin I (Fig. 6), a secreted protease of *Thermus aquaticus* (78). Both the ORF and aqualysin I exhibit sequences highly similar to the sequence YIVGFK, which is conserved in subtilisin-type proteases and influences processing of the precursor protein to the active protease (33, 51, 74). On the basis of these observations, we speculate that Tn*5 lac* Ω4403 disrupts the first (and perhaps only) gene in a developmentally regulated transcriptional unit and that this gene encodes a subtilisin-type serine protease. The function of the putative protease remains unclear because *M. xanthus* cells bearing Tn*5 lac* Ω4403 show no discernible growth or developmental defect (46) (see below).

```
                                YIVGFK
Ω4403          1  MGQKQTGKFITVRKKIPGEYIVVLK- SPAQSLEQVEVQQATTSLI
                  |||  |||| : | :  : | ||  |  ::       ::|
Aqualysin I   36  WPKEAPVYGLDDPEAIPGRYIVVFKKGKGQSLLQGGI TT LQARLA

Ω4403         45  TAYGGTAFAMYENALRGFAAKMTEAQARAMANDPAA
                  :: |  ::  :  | : : || : || | |: | :  :  |:  :: |
Aqualysin I   81  PQ-GVVVTQA YTGALQGFAAEMAPQALEAFRQSPDVE
```

FIG. 6. Alignment of amino acid sequences of the Ω4403 partial ORF and the N-terminal region of aqualysin I. Identical amino acids are indicated by vertical lines, and similar amino acids are indicated by two dots. A conserved amino acid sequence in subtilisin-type proteases (33, 74) is shown in boldface letters above the corresponding regions.

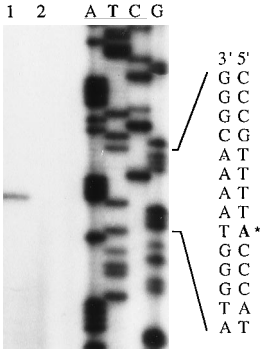


FIG. 7. Primer extension analysis of Ω4403 mRNA. RNA was isolated from *M. xanthus* DK4499 (which makes the native Ω4403 mRNA) and subjected to primer extension analysis as described in Materials and Methods. The same end-labeled primer was also used for sequencing of Ω4403 upstream DNA. A portion of the DNA sequence is indicated at the right. The asterisk marks the putative transcriptional start site that was observed with RNA isolated from 24-h developing cells (lane 1) but not with RNA from vegetatively growing cells (lane 2).

**Precise mapping of the Ω4403 mRNA 5′ end.** Knowing the approximate location of the 5′ end of the Tn*5 lac* Ω4403 fusion mRNA (Fig. 4) and the sequence of Ω4403 upstream DNA (Fig. 5), we designed a primer to precisely map the 5′ end of Ω4403 mRNA by using primer extension analysis. The position of the primer is shown in Fig. 5. Figure 7 shows that the 5′ end of the native Ω4403 mRNA mapped to an adenine nucleotide 382 bp upstream of the Ω4403 insertion site. The 5′ end of the Tn*5 lac* Ω4403 fusion mRNA also mapped to the same position (data not shown). This is about 430 bp upstream of the *Bam*HI site near the left end of Tn*5 lac* (Fig. 1), in good agreement with the S1 nuclease protection result (Fig. 4). The position of the putative transcriptional start site is shown in Fig. 5. Inspection of the DNA sequence of the putative promoter region revealed no striking similarity to known *M. xanthus* promoters, although hexanucleotide sequences in the −35 and −10 regions bear some resemblance to the corresponding regions of *E. coli* promoters transcribed by $\sigma^{70}$ RNA polymerase (55).

**Further deletion analysis of the Ω4403 promoter.** We tested smaller fragments of Ω4403 upstream DNA for promoter activity in vivo to determine whether a promoter exists at the location predicted by the 5′ end mapping and, if so, to determine the extent of sequences required for promoter activity. Figure 5 shows the positions of 5′ deletions that were generated by digestion with restriction enzymes or by PCR. The 3′ ends of all fragments were the same, being produced by *Bam*HI digestion at the site near the left end of Tn*5 lac* (Fig. 1). Each fragment was inserted in the proper orientation into the vector shown in Fig. 2, and *M. xanthus* strains with a single copy of the plasmid at Mx8 *att* were tested for developmental *lacZ* expression. A segment of 80 bp of DNA upstream of the putative transcriptional start site was sufficient to promote *lacZ*

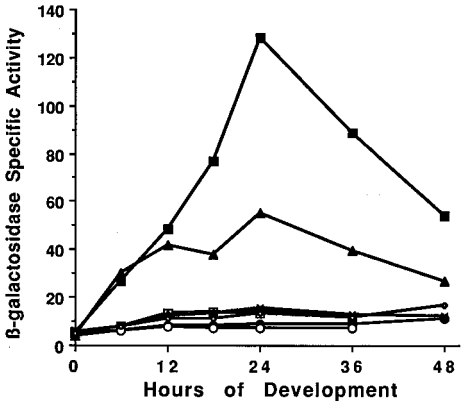


FIG. 8. Deletion analysis of the Ω4403 promoter. Developmental *lacZ* expression was determined as described previously (46) for two or three independently isolated transductants containing a single copy of Ω4403 upstream DNA fused to *lacZ* and integrated at Mx8 *att* (see Table 1 for strain designations). This 5′ deletion series included 80 (▲), 72 (□), 62 (△), 52 (×), 42 (●), and 11 (○) bp of DNA upstream of the putative transcriptional start site. Vector with no insert DNA (◆) was also included. Each point is the average β-galactosidase activity for transductants of the same type. β-Galactosidase activity in DK4368 cells containing Tn*5 lac* Ω4403 is also plotted (■). The units of enzyme activity are given in the legend to Fig. 3.

expression with developmental kinetics and amount similar to the pattern observed for larger upstream segments (Fig. 8). Developmental *lacZ* expression was abruptly abolished upon deletion to −72 bp or farther downstream (Fig. 8). These results show that a promoter does exist in the predicted region, that 80 bp of DNA upstream of the transcriptional start site is sufficient for developmentally regulated expression of *lacZ* comparable to that observed with much larger upstream segments, and that DNA between −80 and −72 bp is critical for promoter activity.

**Possible explanations for the low level of expression from fusions integrated at Mx8 *att*.** Segments of Ω4403 upstream DNA fused to *lacZ* and integrated at Mx8 *att* promote only about 25% of the maximum level of developmental *lacZ* expression seen with *M. xanthus* DK4368 (which contains Tn*5 lac* Ω4403) regardless of whether the segment ends 80 bp upstream of the Ω4403 transcriptional start site (Fig. 8) or much farther (up to 17 kb) upstream (Fig. 1 and 3) and despite the identical nature of the fusion junctions. It seemed unlikely that the difference was due to a promoter or promoter element (e.g., an enhancer) located farther upstream. A promoter element could be located downstream of the Ω4403 insertion site; however, this explanation seemed unlikely for two reasons. First, the downstream element would have to function over a distance of at least 12 kb (the size of Tn*5 lac*) in order to account for the higher level of expression seen with the strain containing Tn*5 lac* Ω4403. Second, the distance between the Ω4403 insertion site and the Mx8 *att* site is estimated to be 5 to 25 kb (68); therefore, the hypothetical downstream element would have to be incapable of functioning over this distance in order to account for the low level of expression from fusions integrated at *att*. These considerations led us to test other possible explanations.

One simple explanation for the low level of expression from fusions integrated at Mx8 *att* is DNA instability. If the plasmid integrated at Mx8 *att* were lost or rearranged at a high frequency during development, it could explain the 75% reduction in *lacZ* expression compared with cells containing Tn*5 lac* Ω4403. We isolated DNA from *M. xanthus* MMF31-7, which contains 1 kb of Ω4403 upstream DNA fused to *lacZ* and integrated at Mx8 *att*. DNA from cells grown in the presence of kanamycin (to maintain selection for the integrated plasmid) or from cells developing for 24 h in the absence of antibiotic was digested with *Xho*I and *Eco*RI and then subjected to Southern blot hybridization using a probe that detects fragments of 10 and 2 kb if the plasmid is integrated at Mx8 *att* (see Materials and Methods). Equal amounts of DNA from growing or developing cells resulted in hybridization signals of similar intensities at 10 and 2 kb, and fragments of other sizes were not detected (data not shown). These results show that a plasmid with Ω4403 upstream DNA integrated at Mx8 *att* is neither lost nor rearranged in a substantial proportion of developing cells. Also, after 3 days of development, all 500 heat- and sonication-resistant MMF31-7 spores that we tested produced colonies of $Km^r$ cells.

Another simple explanation for the low level of expression from fusions integrated at Mx8 *att* is that the integrated DNA hinders development. We examined fruiting body formation microscopically and measured the number of heat- and sonication-resistant spores produced (45) from three strains: the wild-type strain DK1622, strain DK4368 containing Tn*5 lac* Ω4403, and strain MMF31-7 containing 1 kb of Ω4403 upstream DNA fused to *lacZ* and integrated at Mx8 *att*. No significant differences were observed among the three strains, indicating that a plasmid with Ω4403 upstream DNA integrated at Mx8 *att* does not inhibit development and supporting the idea (46) that Tn*5 lac* Ω4403 does not cause a developmental defect.

A third possible explanation for the low level of expression from fusions integrated at Mx8 *att* compared with expression from Tn*5 lac* Ω4403 is that the transposon disrupts a gene whose product negatively regulates its own transcription. According to this model, expression from fusions integrated at Mx8 *att* is negatively regulated, directly or indirectly, by the protein produced by the intact gene at site Ω4403. Thöny-Meyer and Kaiser (79) observed negative autoregulation of *devR-lacZ* by a tandem copy of the intact *devRS* locus. We subcloned 8.5 kb of Ω4403 upstream DNA into a plasmid (pREG1175) (20) much like the one shown in Fig. 2 but lacking the Mx8 *attP* portion. Rather than integrating at Mx8 *att* upon transduction into the wild-type *M. xanthus* strain DK1622, the plasmid recombined into the chromosome via a single crossover at the homologous site, resulting in the structure depicted at the top of Fig. 9A; this structure was verified by Southern blot analysis (data not shown). Strains with this structure have all normal upstream sequences fused to *lacZ*, followed by 8.5 kb of Ω4403 upstream DNA fused to an intact copy of the Ω4403 gene. These strains expressed *lacZ* at about 70% of the maximum level observed for strain DK4368 containing Tn*5 lac* Ω4403 (Fig. 9A). To determine whether the intact copy of the Ω4403 gene in tandem caused the 30% reduction in developmental *lacZ* expression, we constructed strains with the structure depicted at the top of Fig. 9B. In this case, a plasmid (pLK1) with 17 kb of Ω4403 upstream DNA but devoid of Mx8 *attP* and *lacZ* was transduced into *M. xanthus* JPB07 containing Tn*5 lac* Ω4403-$Tc^r$ (in which the $Km^r$ gene was replaced with a $Tc^r$ gene to permit selection for the incoming plasmid). Transductants expressed developmental *lacZ* at about 70% of the maximum level observed for strain JPB07 containing Tn*5 lac* Ω4403-$Tc^r$ (Fig. 9B), despite the lack of an intact Ω4403 gene. Hence, the Ω4403 gene product does not appear to be autoregulatory. The results shown in Fig. 9 for two different types of strains with *lacZ* fusions integrated by homologous recombination at the native Ω4403 chromosomal site suggest that having two copies of Ω4403 upstream DNA

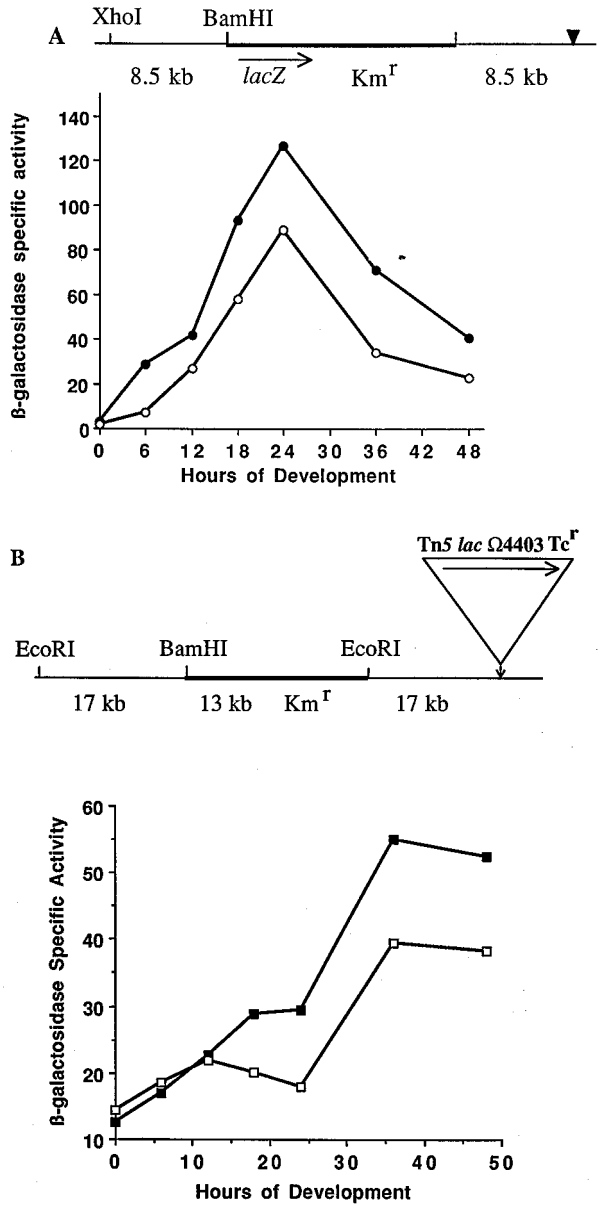


FIG. 9. Developmental *lacZ* expression from fusions at the native Ω4403 position in the chromosome. The arrangement of *M. xanthus* DNA (thin line) and plasmid vector DNA (thick line) is shown at the top in each panel. In panel A, the triangle indicates the site of the Ω4403 insertion as a reference point (Tn*5 lac* Ω4403 is not present). The graph shows the average β-galactosidase activity of three independently isolated transductants (strains MES014, -016, and -017) with this structure (○) and the activity of strain DK4368 containing Tn*5 lac* Ω4403-Km$^r$ (●). In panel B, the triangle shows the position of Tn*5 lac* Ω4403-Tc$^r$. The graph shows the average β-galactosidase activity of three strains (MLK1-3, -4, and -5) with this structure (□) and the activity of strain JPB07 containing Tn*5 lac* Ω4403-Tc$^r$ (■). The units of enzyme activity are given in the legend to Fig. 3.

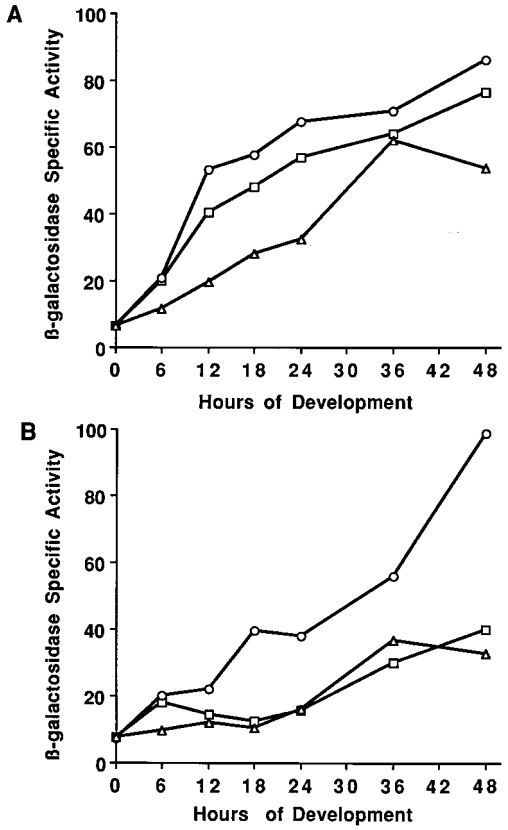


FIG. 10. Extracellular complementation of developmental *lacZ* expression from the Ω4403 promoter in *csgA* cells. (A) Developmental *lacZ* expression was measured as described previously (46) for three independently isolated transductants containing a single copy of Ω4403 upstream DNA extending to −80 bp (MMF100C-6, -17, and -24) (○) or −11 bp (MMF200C-4, -7, and -11) (△) fused to *lacZ* and integrated at Mx8 *att* in a *csgA* mutant background. Points represent averages. The β-galactosidase activity of a similar strain (MES119) with no insert of Ω4403 upstream DNA (□) is also plotted. (B) The strains used for panel A were codeveloped with an equal number of wild-type DK1622 cells (which do not express β-galactosidase), and the β-galactosidase specific activity was determined as described previously (45). The units of enzyme activity are given in the legend to Fig. 3.

titrates a limiting factor for Ω4403 expression. Because strains with fusions integrated at Mx8 *att* contain two copies of Ω4403 upstream DNA, a titration effect can partially account for the lower level of expression observed from these strains than from the strain containing Tn*5 lac* Ω4403. Even so, expression from fusions integrated by homologous recombination at the native site was about 70% of the maximum level observed for strains containing the corresponding Tn*5 lac* insertion (Fig. 9), whereas expression from fusions integrated at Mx8 *att* was only about 25% (Fig. 1, 3, and 8). The remaining difference may be due to an effect of chromosomal position on expression of the fusions (see below).

**Dependence of expression on C-signaling.** Intercellular C-signaling is mediated by the product of *csgA* (26, 40, 70, 71) and is required for the expression of many *M. xanthus* genes that begin to be expressed after 6 h into development (45, 54). Introduction of a *csgA* mutation into cells containing Tn*5 lac* Ω4403 abolished developmental *lacZ* expression (45). To determine whether the minimal Ω4403 promoter region defined by our deletion analysis also exhibits dependence on C-signaling, we measured expression of fusions integrated at Mx8 *att* in *csgA* mutant cells. Surprisingly, we found that *csgA* cells with the vector alone (i.e., containing no insert of *M. xanthus* DNA; Fig. 2) integrated at Mx8 *att* expressed *lacZ* at a considerable level and in a developmentally regulated fashion (Fig. 10A). This result suggests that a developmentally regulated promoter lies upstream of *lacZ* in the vector or in adjoining *M. xanthus* DNA (after integration at Mx8 *att*). Expression from this promoter appears to be inhibited by C-signaling, since expression

was observed in *csgA* mutant cells (Fig. 10A) but not in wild-type cells (Fig. 3). *csgA* cells containing the vector with different amounts of Ω4403 upstream DNA fused to *lacZ* and integrated at Mx8 *att* exhibit a pattern of developmental *lacZ* expression similar to that with the vector alone. Figure 10A shows the results for Ω4403 DNA extending to −80 or −11 bp, and similar results were observed for DNA extending to −616 or about −8,000 bp (data not shown). In wild-type cells, the deletion to −80 bp retained Ω4403 promoter activity and the deletion to −11 bp abolished activity (Fig. 8). In *csgA* mutant cells, the two deletions exhibit expression similar to that of the vector alone (Fig. 10A). These results suggest that the Ω4403 promoter is inactive in *csgA* mutant cells.

To determine whether C-signaling could restore Ω4403 promoter activity in *csgA* mutant cells, we used wild-type cells as C-signal donors. *csgA* cells with a fusion integrated at Mx8 *att* were mixed with an equal number of wild-type strain DK1622 cells and allowed to codevelop. The specific activity of β-galactosidase in the mixture was determined at different times of development. Figure 10B shows that expression from cells with Ω4403 DNA extending to −80 bp was considerably higher than expression from cells with Ω4403 DNA extending to −11 bp, which was comparable to the vector-alone control. C-signaling appears to stimulate the Ω4403 promoter that is present in cells with the deletion to −80 bp and absent from cells with the deletion to −11 bp. *csgA* mutant cells containing the deletion to −11 bp or the vector alone expressed *lacZ* at a lower level when mixed with wild-type cells (Fig. 10B) than when unmixed (Fig. 10A), consistent with the idea that C-signaling inhibits expression of an upstream promoter that lies in the vector or in adjoining *M. xanthus* DNA (after integration at Mx8 *att*). If expression of this promoter is inhibited by C-signaling, it seems possible that expression of other promoters (possibly including the Ω4403 promoter) is inhibited (partially) when the promoters are located at Mx8 *att* in wild-type cells (see the Discussion).

## DISCUSSION

The analysis of the Ω4403 promoter reported here is part of a larger effort to understand gene regulation by intercellular C-signaling during *M. xanthus* development. Ω4403 is one of seven Tn*5 lac* insertions in distinct transcriptional units that have been shown to depend absolutely on C-signaling for expression (45). Expression of nine additional Tn*5 lac* insertions in different genes exhibits a partial dependence upon C-signaling (45, 54). Two fairly well characterized genes also appear to fall into the latter class: *mbhA*, which encodes a hemagglutinin (11, 48, 61), and *csgA*, which encodes the mediator of C-signaling (42, 52). By characterizing the regulatory regions of several genes in each class, we hope to uncover common features of C-signal-dependent gene regulation. These features may suggest approaches to identify regulatory proteins involved in C-signal-mediated gene expression.

To facilitate characterization of promoters identified by Tn*5 lac* insertions, we constructed a plasmid designed specifically for this purpose (Fig. 2). A multiple cloning site present in the plasmid upstream of the same promoterless *lacZ* segment found in Tn*5 lac* (44) permits construction of fusions identical to those created by a Tn*5 lac* insertion. This allows direct comparison between the promoter activity of a cloned segment and expression from the native promoter (with all normal upstream DNA present) as detected by the chromosomal Tn*5 lac* insertion. Upstream (relative to *lacZ*) of the multiple cloning site is a series of factor-independent transcriptional terminators (75) designed to prevent transcription from upstream vector or chromosomal (after plasmid integration) sequences from reading through the cloned segment and expressing *lacZ*. Other features of the plasmid include a bacteriophage P1 incompatibility segment that mediates cointegrate formation for efficient transfer of plasmid from *E. coli* to *M. xanthus* via specialized P1 transduction (22) and a segment from myxophage Mx8 that promotes site-specific recombination at the phage attachment site (76, 77). Upon transduction of the plasmid into *M. xanthus*, we estimate that >85% of the $Km^r$ transductants contain a single copy of the plasmid integrated at Mx8 *att*, which is easily determined by Southern blotting. Hence, the plasmid makes it simple to test a DNA segment for promoter activity under well-defined conditions of copy number and chromosomal position.

The plasmid just described allowed us to test a series of deletions and localize Ω4403 promoter activity to within 0.6 kb upstream of the insertion site (Fig. 1). S1 nuclease analysis of RNA from developing cells identified a single 5′ end in this region, providing evidence for a single promoter with a start site located about 380 bp upstream of Tn*5 lac* Ω4403 (Fig. 4). The promoter appeared to be inactive in growing cells and 6-h developing cells, since no signal was observed upon S1 nuclease analysis of RNA from these cells and *lacZ* expression from Tn*5 lac* Ω4403 remained at a low background level. The promoter was clearly active by 12 h into development, as evidenced by accumulation of fusion mRNA (detected by the S1 nuclease assay) and β-galactosidase activity from Tn*5 lac* Ω4403. The simplest explanation of these results is that the Ω4403 promoter is developmentally regulated at the level of transcription initiation. We cannot exclude the possibilities that the Ω4403 promoter is active in growing cells and/or early developing cells and that the fusion mRNA is rapidly degraded. According to this model, the rise in β-galactosidase activity from Tn*5 lac* Ω4403 in 12-h developing cells would result from stabilization of the fusion mRNA. It seems unlikely a priori that a short (382-base) segment of *M. xanthus* RNA at the 5′ end could stabilize the long (at least several kilobases) fusion mRNA in a developmentally regulated fashion. Indeed, we were unable to detect any discrete species of fusion mRNA in developing cells by Northern blot analysis, suggesting that it is unstable. The native Ω4403 mRNA may be more stable, since a 1.5-kb species was detected by Northern blot analysis of RNA from developing cells lacking Tn*5 lac* Ω4403. Several *M. xanthus* genes that appear to be developmentally regulated at the level of transcription initiation produce transcripts with unusually long half-lives (16, 57, 62).

The DNA sequence of the Ω4403 upstream region revealed an ORF that has striking similarity to the gene encoding aqualysin I, a secreted protease of *T. aquaticus* (78). The ORF could extend farther upstream, beyond the predicted translational start at position 376 (Fig. 5). Potential (GTG) start codons are located at positions 325 and 340, but neither of these codons exhibits a satisfactory ribosome binding site, and the longer ORFs do not exhibit the codon preference and third codon position GC bias typical of *M. xanthus* genes. If translation starts at the ATG at position 376, the ORF should encode 80 amino acids and be interrupted downstream by the Tn*5 lac* Ω4403 insertion. A 63-amino-acid stretch within the ORF shows high similarity to aqualysin I (Fig. 6). Our sequence ends a few amino acids after this stretch. It will be interesting to clone DNA downstream of Tn*5 lac* Ω4403 and determine whether the similarity to aqualysin I can be extended. The 1.5-kb Ω4403 mRNA could encode a polypeptide similar in size to the aqualysin I precursor (51 kDa) (78), if it were monocistronic. Activation of the aqualysin I precursor involves self-processing of N- and C-terminal pro sequences (78). The

Ω4403 ORF is similar to the N-terminal pro sequence of aqualysin I, and both contain sequences highly similar to the sequence YIVGFK, which is conserved in subtilisin-type proteases (33, 74). Amino acid substitutions in the YIVVFK sequence of aqualysin I enhanced processing of the precursor protein to the active protease, but deletion of this six-amino-acid sequence abolished accumulation of precursor and active enzyme (51). Amino acids 61 to 80 of the Ω4403 ORF (Fig. 6) exhibit a pattern of hydrophobic and charged residues similar to that of part of motif N2, another highly conserved feature of the propeptide region of subtilisin family members, including aqualysin I (74). Unlike the aqualysin I gene, the Ω4403 ORF does not appear to encode an N-terminal signal sequence, which is found in many proteins that are translocated across the inner membrane of gram-negative bacteria (60). Perhaps the Ω4403 gene product is not secreted. Alternatively, the protein might exit the cytoplasm by a mechanism that does not require a signal peptide, as appears to be the case for some extracellular and periplasmic developmental proteins of *M. xanthus* (23).

If the Ω4403 gene does encode a developmentally regulated subtilisin-type protease, as we have speculated, this protease does not seem to be essential for aggregation or sporulation. The insertion of Tn*5 lac* at codon 81 of the putative protease gene would probably abolish function, yet the *M. xanthus* strain containing Tn*5 lac* Ω4403 aggregates normally and produces a normal number of heat- and sonication-resistant spores (measured after 3 days of development). Of course, the Ω4403 gene product may have subtle effects on aggregation and/or spore viability that escaped detection. It is also possible that the Ω4403 gene product is functionally redundant with another *M. xanthus* protein(s).

Our principal interest in the Ω4403 gene is its mechanism of regulation. Inspection of the DNA sequence immediately upstream of the putative Ω4403 transcriptional start site revealed hexanucleotide sequences in the −35 and −10 regions with some similarity to sequences found in these regions of *E. coli* (55) and *Bacillus subtilis* (29) promoters transcribed by the major vegetative RNA polymerase containing $\sigma^{70}$ and $\sigma^{43}$, respectively. The best match in the −35 region is the sequence 5′-TTGATT-3′ centered at −37.5 (Fig. 5), with only two mismatches to the 5′-TTGACA-3′ consensus. In the −10 region, the sequences 5′-TGTACA-3′ and 5′-TACACA-3′ centered at −11.5 and −9.5, respectively, each contain three matches to the 5′-TATAAT-3′ consensus. However, the 20- to 22-bp spacing between the −35 and −10 sequences is more than the optimal spacing. *B. subtilis* $\sigma^{43}$ RNA polymerase tolerates spacings of 22 and 21 bp between the −35 and −10 regions of the *spoIIG* and *spoIIE* promoters, respectively, but transcription requires an activator protein, Spo0A-phosphate (5, 81). A similar mechanism might regulate the Ω4403 promoter. *M. xanthus* contains a gene, *sigA*, predicted to encode the major sigma factor, and SigA is very similar to both *B. subtilis* $\sigma^{43}$ and *E. coli* $\sigma^{70}$, including the domains that interact with the −35 and −10 promoter regions (32). Alternatively, the Ω4403 promoter might be recognized by a development-specific form of RNA polymerase. Two developmentally regulated sigma factors, SigB and SigC, have been found in *M. xanthus*, and more are likely to exist (1, 2). Neither SigB nor SigC appears to be required for Ω4403 transcription because null mutations in the *sigB* and *sigC* genes do not prevent developmental expression of Tn*5 lac* Ω4403-Tc$^r$ (6).

Deletion analysis showed that DNA between −80 and −72 bp is critical for Ω4403 promoter activity. Although RNA polymerase does interact with DNA upstream of the −35 region of some promoters (8), more typically a required sequence element upstream of the −35 region is indicative of a binding site for a transcriptional activator protein (10). Transcriptional activators often bind to palindromic DNA sequences (59). The region critical for Ω4403 promoter activity has two copies of the palindromic sequence 5′-CATG-3′ separated by 1 bp (Fig. 5); however, the −80 bp deletion that retains promoter activity (Fig. 8) replaces the CA of the upstream palindrome with GC from the vector. Nucleotide substitutions will be required to establish the importance of the palindromic sequence, or the −35 and −10 regions, for Ω4403 promoter activity. Mutations that reduce promoter activity could provide a means to identify regulatory proteins by isolating suppressor mutants with restored promoter activity. We are currently pursuing biochemical approaches to identify proteins that bind to the Ω4403 regulatory region.

The Ω4403 promoter is not unusual among developmentally regulated *M. xanthus* genes in requiring an upstream element. All such genes examined so far exhibit this requirement (14, 24). In some cases, multiple elements appear to act over distances of several kilobases from the transcriptional start site (37). In the case of Ω4403, however, our results show that 80 bp of DNA upstream of the transcriptional start site (Fig. 8) and 17 kb of upstream DNA direct the same level of developmentally regulated gene expression, providing no evidence for additional upstream promoter elements.

It seems unlikely that a promoter element lies more than 17 kb upstream of Ω4403 or can still function even when located downstream of the 12 kb Tn*5 lac* element, yet cells containing Tn*5 lac* Ω4403 express three- to fourfold more β-galactosidase activity during development than any strains that we tested with fusions integrated at Mx8 *att*. We ruled out several possible explanations for this difference. The fusion-containing plasmid integrated at the phage attachment site did not appear to be unstable, nor was it a hindrance to the normal developmental process. Tn*5 lac* Ω4403 did not disrupt a gene whose product negatively autoregulates, as was observed for Tn*5 lac* Ω4414 (79). Cells with one copy of the Ω4403 regulatory region fused to *lacZ* and another copy in tandem at the native site expressed *lacZ* at about 70% of the maximum level observed for cells containing Tn*5 lac* Ω4403 (Fig. 9). This finding suggests that titration of a limiting positive-acting factor by a second copy of the Ω4403 regulatory region in cells with fusions integrated at Mx8 *att* can, in part, account for their reduced expression compared with cells containing Tn*5 lac* Ω4403 (with only one copy of the regulatory region).

An effect of chromosomal position may also hinder expression from some fusions located at Mx8 *att*. Our results show that a putative promoter in the vicinity of the phage attachment site is down-regulated by C-signaling (Fig. 10). Perhaps this effect is also observed for the Ω4403 promoter and certain other promoters upon integration at Mx8 *att*. In particular, it seems to be difficult to observe full expression at Mx8 *att* of late developmental genes that depend absolutely on C-signaling. Li and Shimkets (53) observed about 50% as much developmental β-galactosidase activity in cells with 1.3 or 11 kb of Ω4435 upstream DNA fused to *lacZ* and integrated at Mx8 *att* as in cells containing Tn*5 lac* Ω4435. Similarly, 1.3 or 10 kb of Ω4459 upstream DNA inserted in the plasmid that we described here (Fig. 2) and integrated at Mx8 *att* drives only about 10 to 20% as much developmental *lacZ* expression as seen with cells containing Tn*5 lac* Ω4459 (7). Also, Thöny-Meyer and Kaiser (79) reported poorer expression of *devRS* at Mx8 *att* than at the native site. These authors invoked differential condensation of the chromosome during development as a possible explanation of the positional effects. Given that Tn*5 lac* Ω4403 is within 5 to 25 kb of Mx8 *att* (68), the differential condensation would

have to be highly localized. Perhaps this involves a silencing mechanism similar to that used in yeast mating-type switching (13). Some genes that depend less strongly on C-signaling for expression as compared with Ω4403 escape the inhibition. For example, cells with Ω4499 (19, 45) or Ω4514 (28, 45) upstream DNA inserted in the plasmid shown in Fig. 2 and integrated at Mx8 *att* show a level of developmental *lacZ* expression comparable to that of cells containing the corresponding Tn*5* *lac* insertions. Similarly, the Ω4521 promoter, which depends on A-signaling for expression (47), but not on C-signaling (45), escapes inhibition when integrated at Mx8 *att* (36). Taking all of these observations together, we speculate that C-signaling leads to an altered chromosomal state of DNA integrated at Mx8 *att* and DNA nearby, partially inhibiting expression of genes that depend strongly on C-signaling. The Ω4403 promoter was sufficiently active upon integration at Mx8 *att* to permit deletion analysis (Fig. 1 and 8). For promoters less active at Mx8 *att*, integration of plasmids by homologous recombination to produce structures like that depicted in Fig. 9B might be the preferred approach (24).

Unexpectedly, our plasmid vector alone, with no insert of *M. xanthus* DNA, expressed *lacZ* upon integration at Mx8 *att* in *csgA* mutant cells (Fig. 10A). A promoter active in developing *csgA* mutant cells must lie upstream of *lacZ* in the vector, or in adjoining *M. xanthus* DNA after integration at Mx8 *att*. We did not map this promoter; however, it is likely to be located upstream of the multiple cloning site present in the vector. The same sequences downstream of the multiple cloning site in the plasmid are also present in Tn*5* *lac* (44), and many Tn*5* *lac* insertions express only a low background level of β-galactosidase when present in *csgA* mutant cells (45). The multiple cloning site itself cannot be the origin of the transcription because it is almost completely deleted in some of the plasmids that contain Ω4403 upstream DNA inserts and show a pattern of *lacZ* expression similar to that of the vector alone. Neither the transcriptional terminators engineered into the vector nor inserts of Ω4403 upstream DNA up to 8.5 kb in size seemed to impede the transcription, suggesting that the RNA polymerase is in an antitermination mode. Interestingly, this transcription is negatively regulated by C-signaling. $csgA^+$ cells express only a low background level of β-galactosidase when the vector is present at Mx8 *att* (Fig. 3). Moreover, mixing $csgA^+$ cells with *csgA* mutant cells containing the vector at Mx8 *att* inhibits *lacZ* expression from the *csgA* cells (Fig. 10B).

The ability of $csgA^+$ cells to donate C-signal and substantially reduce *lacZ* expression from *csgA* mutant cells containing the vector alone integrated at Mx8 *att* allowed us to demonstrate that the minimal Ω4403 promoter, as defined by deletions, is responsive to C-signaling (Fig. 10B). We conclude that elements needed for C-signal-dependent expression of Ω4403 lie between −80 and +382 bp. Our deletion analysis showed that an element critical for Ω4403 promoter activity lies between −80 and −72 bp. A simple model is that this DNA element interacts with a transcriptional activator protein whose synthesis or activity is controlled directly or indirectly by C-signaling. The challenge now is to identify the regulatory protein(s).

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## REFERENCES

1. **Apelian, D., and S. Inouye.** 1990. Development-specific σ-factor essential for late-stage differentiation of *Myxococcus xanthus*. Genes Dev. **4:**1396–1403.
2. **Apelian, D., and S. Inouye.** 1993. A new putative sigma factor of *Myxococcus xanthus*. J. Bacteriol. **175:**3335–3342.
3. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.).** 1989. Current protocols in molecular biology. John Wiley & Sons, New York.
4. **Avery, L., and D. Kaiser.** 1983. *In situ* transposon replacement and isolation of a spontaneous tandem genetic duplication. Mol. Gen. Genet. **191:**99–109.
5. **Baldus, J., B. Green, P. Youngman, and C. P. Moran.** 1994. Phosphorylation of *Bacillus subtilis* transcription factor Spo0A stimulates transcription from the *spoIIG* promoter by enhancing binding to weak OA boxes. J. Bacteriol. **176:**296–306.
6. **Brandner, J., and L. Kroos.** Unpublished data.
7. **Brandner, J., J. White, and L. Kroos.** Unpublished data.
8. **Busby, S., and R. H. Ebright.** 1994. Promoter structure, promoter recognition, and transcription activation in prokaryotes. Cell **79:**743–746.
9. **Cheng, Y., and D. Kaiser.** 1989. *dsg*, a gene required for cell-cell interaction early in *Myxococcus* development. J. Bacteriol. **171:**3719–3726.
10. **Collado-Vides, J., B. Magasanik, and J. D. Gralla.** 1991. Control site location and transcriptional regulation in *Escherichia coli*. Microbiol. Rev. **55:** 371–394.
11. **Cumsky, M. G., and D. R. Zusman.** 1979. Myxobacterial hemagglutinin: a development-specific lectin of *Myxococcus xanthus*. Proc. Natl. Acad. Sci. USA **76:**5505–5509.
12. **Davis, R., D. Botstein, and J. Roth.** 1980. Advanced bacterial genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
13. **Dillin, A., and J. Rine.** 1995. On the origin of a silencer. Trends Biochem. Sci. **20:**231–235.
14. **Downard, J., and L. Kroos.** 1993. Transcriptional regulation of developmental gene expression in *Myxococcus xanthus*, p. 183–199. *In* M. Dworkin and D. Kaiser (ed.), Myxobacteria II. American Society for Microbiology, Washington, D.C.
15. **Downard, J., S. V. Ramaswamy, and K. Kil.** 1993. Identification of *esg*, a genetic locus involved in cell-cell signaling during *Myxococcus xanthus* development. J. Bacteriol. **175:**7762–7770.
16. **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.
17. **Dworkin, M., and D. Kaiser (ed.).** 1993. Myxobacteria II. American Society for Microbiology, Washington, D.C.
18. **Fawcett, T., and S. Bartlett.** 1990. An effective method for eliminating "artifact banding" when sequencing double-stranded DNA templates. BioTechniques **9:**46–48.
19. **Fisseha, M., and L. Kroos.** Unpublished data.
20. **Gill, R. E., and M. C. Bornemann.** 1988. Identification and characterization of the *Myxococcus xanthus bsgA* gene product. J. Bacteriol. **170:**5289–5297.
21. **Gill, R. E., and M. G. Cull.** 1986. Control of developmental gene expression by cell-to-cell interactions in *Myxococcus xanthus*. J. Bacteriol. **168:**341–347.
22. **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.
23. **Guespin-Michel, J., B. Letouvet-Pawlak, and F. Petit.** 1993. Protein secretion in myxobacteria, p. 235–255. *In* M. Dworkin and D. Kaiser (ed.), Myxobacteria II. American Society for Microbiology, Washington, D.C.
24. **Gulati, P., D. Xu, and H. Kaplan.** 1995. Identification of the minimum regulatory region of a *Myxococcus xanthus* A-signal-dependent developmental gene. J. Bacteriol. **177:**4645–4651.
25. **Hagen, D. C., A. P. Bretscher, and D. Kaiser.** 1978. Synergism between morphogenetic mutants of *Myxococcus xanthus*. Dev. Biol. **64:**284–296.
26. **Hagen, T. J., and L. J. Shimkets.** 1990. Nucleotide sequence and transcriptional products of the *csg* locus of *Myxococcus xanthus*. J. Bacteriol. **172:**15–23.
27. **Hanahan, D.** 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166:**557.
28. **Hao, T., and L. Kroos.** Unpublished data.
29. **Helmann, J.** 1995. Compilation and analysis of *Bacillus subtilis* $\sigma^A$-dependent promoter sequences: evidence for extended contact between RNA polymerase and upstream promoter DNA. Nucleic Acids Res. **23:**2351–2360.
30. **Hodgkin, J., and D. Kaiser.** 1977. Cell-to-cell stimulation of motility in nonmotile mutants of *M. xanthus*. Proc. Natl. Acad. Sci. USA **74:**2938–2942.
31. **Igo, M. M., and R. Losick.** 1986. Regulation of a promoter region that is utilized by minor forms of RNA polymerase holoenzyme in *Bacillus subtilis*. J. Mol. Biol. **191:**615–624.
32. **Inouye, S.** 1990. Cloning and DNA sequence of the gene coding for the major sigma factor from *Myxococcus xanthus*. J. Bacteriol. **172:**80–85.
33. **Jacobs, M., M. Eliasson, M. Uhlen, and J. I. Flock.** 1985. Cloning, sequencing and expression of subtilisin Carlsberg from *Bacillus licheniformis*. Nucleic Acids Res. **13:**8913–8926.
34. **Kaiser, D.** 1979. Social gliding is correlated with the presence of pili in *Myxococcus xanthus*. Proc. Natl. Acad. Sci. USA **76:**5952–5956.

35. **Kaiser, D., and L. Kroos.** 1993. Intercellular signaling, p. 257–284. *In* M. Dworkin and D. Kaiser (ed.), Myxobacteria II. American Society for Microbiology, Washington, D.C.
36. **Keseler, I., and D. Kaiser.** 1995. An early A-signal-dependent gene in *Myxococcus xanthus* has a $\sigma^{54}$-like promoter. J. Bacteriol. **177:**4638–4644.
37. **Kil, K.-S., G. Brown, and J. Downard.** 1990. A segment of *Myxococcus xanthus ops* DNA functions as an upstream activation site for *tsp* gene transcription. J. Bacteriol. **172:**3081–3088.
38. **Kim, S. K., and D. Kaiser.** 1990. Cell alignment required in differentiation of *Myxococcus xanthus*. Science **249:**926–928.
39. **Kim, S. K., 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 Dev. **4:**896–905.
40. **Kim, S. K., and D. Kaiser.** 1990. C-factor: a cell-cell signaling protein required for fruiting body morphogenesis of M. xanthus. Cell **61:**19–26.
41. **Kim, S. K., and D. Kaiser.** 1990. Purification and properties of *Myxococcus xanthus* C-factor, an intercellular signaling protein. Proc. Natl. Acad. Sci. USA **87:**3635–3639.
42. **Kim, S. K., and D. Kaiser.** 1991. C-factor has distinct aggregation and sporulation thresholds during *Myxococcus* development. J. Bacteriol. **173:** 1722–1728.
43. **Kroos, L., P. Hartzell, K. Stephens, and D. Kaiser.** 1988. A link between cell movement and gene expression argues that motility is required for cell-cell signaling during fruiting body development. Genes Dev. **2:**1677–1685.
44. **Kroos, L., and D. Kaiser.** 1984. Construction of Tn*5 lac*, a transposon that fuses *lacZ* expression to exogenous promoters, and its introduction into *Myxococcus xanthus*. Proc. Natl. Acad. Sci. USA **81:**5816–5820.
45. **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.
46. **Kroos, L., A. Kuspa, and D. Kaiser.** 1986. A global analysis of developmentally regulated genes in *Myxococcus xanthus*. Dev. Biol. **117:**252–266.
47. **Kuspa, A., L. Kroos, and D. Kaiser.** 1986. Intercellular signaling is required for developmental gene expression in *Myxococcus xanthus*. Dev. Biol. **117:** 267–276.
48. **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.
49. **Laue, B. E., and R. Gill.** 1994. Use of a phase variation-specific promoter of *Myxococcus xanthus* in a strategy for isolating a phase-locked mutant. J. Bacteriol. **176:**5341–5349.
50. **Lee, B.-U., K. Lee, J. Mendez, and L. Shimkets.** 1995. A tactile sensory system of *Myxococcus xanthus* involves an extracellular NAD(P)$^+$-containing protein. Genes Dev. **9:**2964–2973.
51. **Lee, Y.-C., Y. Miyata, I. Terada, T. Ohta, and H. Matsuzawa.** 1991. Involvement of $NH_2$-terminal pro-sequence in the production of active aqualysin I (a thermophilic serine protease) in *Escherichia coli*. Agric. Biol. Chem. **55:**3027–3032.
52. **Li, S.-F., B. Lee, and L. J. Shimkets.** 1992. *csgA* expression entrains *Myxococcus xanthus* development. Genes Dev. **6:**401–410.
53. **Li, S.-F., and L. J. Shimkets.** 1988. Site-specific integration and expression of a developmental promoter in *Myxococcus xanthus*. J. Bacteriol. **170:**5552–5556.
54. **Li, S.-F., and L. J. Shimkets.** 1993. Effect of *dsp* mutations on the cell-to-cell transmission of CsgA in *Myxococcus xanthus*. J. Bacteriol. **175:**3648–3652.
55. **Lisser, S., and H. Margalit.** 1993. Compilation of *E. coli* mRNA promoter sequences. Nucleic Acids Res. **21:**1507–1516.
56. **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, p. 43–48. National Institutes of Health, Bethesda, Md.
57. **Nelson, D., and D. Zusman.** 1983. Evidence for long-lived mRNA during fruiting body formation in *Myxococcus xanthus*. Proc. Natl. Acad. Sci. USA **80:**1467–1471.
58. **Oyaizu, H., and C. Woese.** 1985. Phylogenetic relationships among the sulfate respiring bacteria, myxobacteria and purple bacteria. Syst. Appl. Microbiol. **6:**257–263.
59. **Pabo, C. O., and R. T. Sauer.** 1992. Transcription factors: structural families and principles of DNA recognition. Annu. Rev. Biochem. **61:**1053–1095.
60. **Pugsley, A.** 1993. The complete general secretory pathway in gram-negative bacteria. Microbiol. Rev. **57:**50–108.
61. **Romeo, J. M., and D. R. Zusman.** 1991. Transcription of the myxobacterial hemagglutinin gene is mediated by a $\sigma^{54}$-like promoter and a *cis*-acting upstream regulatory region of DNA. J. Bacteriol. **173:**2969–2976.
62. **Romeo, J. M., and D. R. Zusman.** 1992. Determinants of an unusually stable mRNA in the bacterium *Myxococcus xanthus*. Mol. Microbiol. **6:**2975–2988.
63. **Sager, B., and D. Kaiser.** 1993. Spatial restriction of cellular differentiation. Genes Dev. **7:**1645–1653.
64. **Sager, B., and D. Kaiser.** 1993. Two cell-density domains within the *Myxococcus xanthus* fruiting body. Proc. Natl. Acad. Sci. USA **90:**3690–3694.
65. **Sager, B., and D. Kaiser.** 1994. Intercellular C-signaling and the traveling waves of *Myxococcus*. Genes Dev. **8:**2793–2804.
66. **Sambrook, J., E. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
67. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
68. **Shimkets, L. J.** Personal communication.
69. **Shimkets, L. J.** 1990. Social and developmental biology of the myxobacteria. Microbiol. Rev. **54:**473–501.
70. **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.
71. **Shimkets, L. J., R. E. Gill, and D. Kaiser.** 1983. Developmental cell interactions in *Myxococcus xanthus* and the *spoC* locus. Proc. Natl. Acad. Sci. USA **80:**1406–1410.
72. **Shimkets, L. J., and D. Kaiser.** 1982. Induction of coordinated movement of *Myxococcus xanthus* cells. J. Bacteriol. **152:**451–461.
73. **Shimkets, L. J., and H. Rafiee.** 1990. CsgA, an extracellular protein essential for *Myxococcus xanthus* development. J. Bacteriol. **172:**5299–5306.
74. **Shinde, U., and M. Inouye.** 1994. The structural and functional organization of intramolecular chaperones: the N-terminal propeptides which mediate protein folding. J. Biochem. **115:**629–636.
75. **Simons, R., F. Houman, and N. Kleckner.** 1987. Improved single and multicopy *lac*-based cloning vectors for protein and operon fusions. Gene **53:** 85–96.
76. **Stellwag, E., J. M. Fink, and J. Zissler.** 1985. Physical characterization of the genome of the *Myxococcus xanthus* bacteriophage MX-8. Mol. Gen. Genet. **199:**123–132.
77. **Stephens, K., and D. Kaiser.** 1987. Genetics of gliding motility in *Myxococcus xanthus*: molecular cloning of the *mgl* locus. Mol. Gen. Genet. **207:**256–266.
78. **Terada, I., S. Kwon, Y. Miyata, H. Matsuzawa, and T. Ohta.** 1990. Unique precursor structure of an extracellular protease, aqualysin I, with $NH_2$- and COOH-terminal pro-sequences and its processing in *Escherichia coli*. J. Biol. Chem. **265:**6576–6581.
79. **Thöny-Meyer, L., and D. Kaiser.** 1993. *devRS*, an autoregulated and essential genetic locus for fruiting body development in *Myxococcus xanthus*. J. Bacteriol. **175:**7450–7462.
80. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences M13 mp18 and pUC19 vectors. Gene **33:**103–119.
81. **York, K., T. Kenney, S. Satola, C. P. Moran, H. Poth, and P. Youngman.** 1992. Spo0A controls the $\sigma^{A}$-dependent activation of *Bacillus subtilis* sporulation-specific transcription unit *spoIIE*. J. Bacteriol. **174:**2648–2658.