

Mutational Analysis of the *Myxococcus xanthus* Ω 4406 Promoter Region Reveals an Upstream Negative Regulatory Element That Mediates C-Signal Dependence

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C signaling plays a key role in coordinating cell movement and differentiation during the multicellular developmental process of *Myxococcus xanthus*. C signaling regulates expression of genes induced after about 6 h into development, when cells are forming mounds. One gene whose expression depends absolutely on C signaling was identified by insertion of a transposable element at site Ω 4406 which generated a transcriptional fusion between *lacZ* and an upstream promoter. We have investigated regulation of the Ω 4406 promoter. A 5' deletion revealed a negative regulatory element located between bp –533 and –100 relative to the transcriptional start site. In the absence of this element, the promoter was still developmentally regulated but about fourfold more active. Also, the truncated promoter region retained normal dependence on two developmental regulators, FruA and DevS, but lost its dependence on the C-signaling protein CsgA. We infer that C signaling partially overcomes the negative effect of the upstream element on activity of the Ω 4406 promoter. Deletion of downstream DNA between bp 50 and 140 caused a threefold loss in expression, suggesting that a positive regulatory element lies in this region. Additional positive and negative regulatory elements are present in the region from bp –69 to –49, based on the effects of multiple-base-pair mutations. Within this region, a 5-bp element and a C-box-like sequence resemble sequences found in other developmentally regulated *M. xanthus* promoter regions, but the effects of single-base-pair changes in these sequences suggest that each functions uniquely. We conclude that regulation of the Ω 4406 promoter involves multiple positive and negative regulatory elements located upstream and downstream of the region typically bound by RNA polymerase.

Myxococcus xanthus is a gram-negative, soil-dwelling bacterium that undergoes a multicellular developmental process involving signaling between cells and changes in gene expression (4). When these bacteria are starved at a high cell density on a solid surface, the rod-shaped cells move in a synchronized fashion, forming aggregation centers, where cells pile on top of one another. Within the resulting mounds, cells differentiate into dormant, spherical spores that are resistant to heat and desiccation. The spore-filled mounds are called fruiting bodies and are typically about 0.1 mm in height and width, containing on the order of 10^5 spores.

Five extracellular signals called A, B, C, D, and E have been inferred to control *M. xanthus* development, based on genetic and cell mixing experiments (3, 10), but only the A and C signals have been identified. A signaling is important at the start of development and involves the release of proteases, peptides, and amino acids that are believed to permit quorum sensing (25–27, 35). At a sufficient cell density, the population embarks on development. C signaling is required later, beginning at about 6 h into development, as judged from expression of developmentally regulated gene fusions to a reporter (23). C signaling has been hypothesized to occur through end-to-end contact between cells (36) and involve interaction of a proteolytic cleavage product of CsgA from the donor cell (31) with a

receptor on the surface of the recipient cell (reviewed in reference 18). C signaling controls cell movements, with a low level sufficing for rippling (cells accumulate in parallel ridges that appear to travel as waves in time-lapse microscopy) and a higher level necessary for aggregation (reviewed in references 17 and 41). Alignment of cells in fruiting bodies may permit an even higher level of C signaling which triggers sporulation (21, 28).

In addition to coordinating cell movement and differentiation, C signaling also coordinates gene expression during *M. xanthus* development. This regulation involves both temporal and spatial control. Genes that depend on C signaling for expression are activated at different times after 6 h into development, and some show partial expression in a *csgA* mutant, whereas others show no expression (23, 29). Expression of all such genes examined thus far could be rescued by codeveloping the *csgA* mutant with wild-type cells (to supply C signal) (1, 6, 7, 23, 32) or by adding a 17-kDa fragment of CsgA (22). A higher level of the CsgA fragment was needed to rescue expression of a gene normally induced at the onset of sporulation than for a gene induced earlier, indicating different thresholds for response (21). Possibly related to this, C-signal-dependent genes have been shown to be expressed specifically in cells within fruiting bodies and not in the peripheral rods that remain outside of fruiting bodies (16, 37).

How are genes regulated by C signaling during *M. xanthus* development? One component of the C-signal transduction pathway is FruA, a protein similar to response regulators of two-component systems (34). This protein has a critical aspartate residue, suggesting it may be phosphorylated by an unidenti-

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Source or reference
Strains		
<i>E. coli</i> DH5 α	ϕ 80 <i>lacZ</i> Δ M15 Δ <i>lacU169</i> <i>recA1</i> <i>endA1</i> <i>hsdR17</i> <i>supE44</i> <i>thi-1</i> <i>gyrA</i> <i>relA1</i>	11
<i>M. xanthus</i>		
DK1622	Wild type	19
LS203	<i>csgA653</i>	40
<i>fruA</i> ::Tc Ω 5	<i>fruA</i> ::Tc ^r	34
DK11209	Δ <i>devS</i>	A. Garza and D. Kaiser
MPV1727-8	<i>attB</i> ::pREG1727	46
MKV4	<i>attB</i> ::pKV4 (pREG1727 with 1.0-kb XhoI-BamHI fragment from pPV4406-1.0) ^a	This study
MKV6	<i>attB</i> ::pKV6 (pREG1727 with 150-bp XhoI-BamHI fragment from pKV3)	This study
MKV11	<i>attB</i> ::pKV11 (pREG1727 with 0.6-kb XhoI-BamHI fragment from pKV9)	This study
MKV20	<i>attB</i> ::pKV20 (pREG1727 with 420-bp XhoI-BamHI fragment from pKV12)	This study
MPV251	<i>attB</i> ::pPV0251 (pREG1727 with 351-bp XhoI-BamHI fragment from pPV251)	This study
MPV182	<i>attB</i> ::pPV0182 (pREG1727 with 282-bp XhoI-BamHI fragment from pPV182)	This study
MKV31	<i>attB</i> ::pKV31 (pREG1727 with 420-bp XhoI-BamHI fragment from pKV28)	This study
MKV32	<i>attB</i> ::pKV32 (pREG1727 with 420-bp XhoI-BamHI fragment from pKV29)	This study
MKV33	<i>attB</i> ::pKV33 (pREG1727 with 420-bp XhoI-BamHI fragment from pKV30)	This study
MKV37	<i>attB</i> ::pKV37 (pREG1727 with 240-bp XhoI-BamHI fragment from pKV34)	This study
MKV39	<i>attB</i> ::pKV39 (pREG1727 with 420-bp XhoI-BamHI fragment from pKV36)	This study
MKV55	<i>attB</i> ::pKV55 (pREG1727 with 420-bp XhoI-BamHI fragment from pKV46)	This study
MKV56	<i>attB</i> ::pKV56 (pREG1727 with 420-bp XhoI-BamHI fragment from pKV47)	This study
MKV57	<i>attB</i> ::pKV57 (pREG1727 with 420-bp XhoI-BamHI fragment from pKV48)	This study
MKV58	<i>attB</i> ::pKV58 (pREG1727 with 420-bp XhoI-BamHI fragment from pKV49)	This study
MKV59	<i>attB</i> ::pKV59 (pREG1727 with 420-bp XhoI-BamHI fragment from pKV50)	This study
MKV60	<i>attB</i> ::pKV60 (pREG1727 with 420-bp XhoI-BamHI fragment from pKV51)	This study
MKV61	<i>attB</i> ::pKV61 (pREG1727 with 420-bp XhoI-BamHI fragment from pKV52)	This study
MKV63	<i>attB</i> ::pKV63 (pREG1727 with 420-bp XhoI-BamHI fragment from pKV53)	This study
MKV65	<i>attB</i> ::pKV65 (pREG1727 with 420-bp XhoI-BamHI fragment from pKV62)	This study
MKV67	<i>attB</i> ::pKV67 (pREG1727 with 420-bp XhoI-BamHI fragment from pKV64)	This study
MKV4 Δ CP	<i>csgA653 attB</i> ::pKV4	This study
MKV11 Δ CP	<i>csgA653 attB</i> ::pKV11	This study
MKV4 Δ F	<i>fruA</i> ::Tc ^r <i>attB</i> ::pKV4	This study
MKV11 Δ F	<i>fruA</i> ::Tc ^r <i>attB</i> ::pKV11	This study
MKV4 Δ devS	Δ <i>devS attB</i> ::pKV4	This study
MKV11 Δ devS	Δ <i>devS attB</i> ::pKV11	This study
Plasmids		
pCR2.1-TOPO	Ap ^r Km ^r ; <i>lacZ</i> α	Invitrogen
pREG1727	Ap ^r Km ^r ; P1- <i>inc attP lacZ</i>	7
pPV4406-1.0	pCR2.1-TOPO with 1.0-kb Ω 4406 DNA segment from bp -533 to 485 generated by PCR	32
pKV3	pCR2.1-TOPO with Ω 4406 DNA segment from bp -100 to 50 generated by PCR	This study
pKV9	pCR2.1-TOPO with 0.6-kb Ω 4406 DNA segment from bp -100 to 485 generated by PCR	This study
pKV12	pCR2.1-TOPO with Ω 4406 DNA segment from bp -100 to 320 generated by PCR	This study
pPV251	pCR2.1-TOPO with Ω 4406 DNA segment from bp -100 to 251 generated by PCR	This study
pPV182	pCR2.1-TOPO with Ω 4406 DNA segment from bp -100 to 182 generated by PCR	This study
pKV28	pKV12 with CATCGTG to ACGATGT mutation from bp -55 to -49	This study
pKV29	pKV12 with GGACA to TTCAC mutation from bp -69 to -65	This study
pKV30	pKV12 with GAACC to TCCAA mutation from bp -64 to -60	This study
pKV34	pCR2.1-TOPO with Ω 4406 DNA segment from bp -100 to 140 generated by PCR	This study
pKV36	pKV12 with GGCGTTTCA to TTATGGGAC mutation from bp -90 to -82	This study
pKV46	pKV12 with C to A mutation at bp -55	This study
pKV47	pKV12 with A to C mutation at bp -54	This study
pKV48	pKV12 with T to G mutation at bp -53	This study
pKV49	pKV12 with C to A mutation at bp -52	This study
pKV50	pKV12 with G to T mutation at bp -51	This study
pKV51	pKV12 with T to G mutation at bp -50	This study
pKV52	pKV12 with G to T mutation at bp -49	This study
pKV53	pKV12 with GTC to TGA mutation from -59 to -57 bp	This study
pKV62	pKV12 with T to G mutation at bp -56	This study
pKV64	pKV12 with C to A mutation at bp -61	This study

^a Where possible, the plasmid description is given in parentheses after the strain description.

fied histidine protein kinase in response to C-signaling (5). Recently, the C-terminal domain of FruA has been shown to bind specifically to the promoter region of *fdgA*, a gene whose expression depends partially on C signaling (45). Hence, FruA (or its phosphorylated form) may directly activate *fdgA* transcription. Other potential targets of FruA, or regulators that function downstream of FruA in the C-signal transduction pathway, have been identified by random insertion of the transposon Tn5 *lac* into the *M. xanthus* chromosome (24). Insertions that generated a transcriptional fusion between a developmentally regulated *M. xanthus* promoter and the *Escherichia coli lacZ* gene have been tested for dependence of expression on *csgA* (23, 29). The promoter regions upstream of two insertions that exhibited partial dependence on *csgA* (Ω 4400 and Ω 4499) and two that exhibited absolute dependence on *csgA* (Ω 4403 and Ω 4406) have been identified (1, 6, 7, 32). Three of these promoter regions have been subjected to mutational analysis to identify important regulatory elements (46–48).

Here, we report mutational analysis of the fourth promoter region, which drives expression of the Ω 4406 locus. Unlike the three other promoters, the Ω 4406 promoter is regulated by an upstream DNA element that acts negatively. This element is necessary for expression from the promoter to be C-signal dependent. Our results provide the first example of an upstream negative element in an *M. xanthus* promoter region and the first example of C-signal dependence mediated by negative regulation. We also obtained evidence that a downstream regulatory element boosts Ω 4406 expression and that the Ω 4406 promoter region contains positive elements at bp -64 to -49 which resemble those in other developmentally regulated promoters but appear to function uniquely in each case.

MATERIALS AND METHODS

Bacterial strains and plasmids. Strains and plasmids used in the present study are listed in Table 1.

Growth and development. *Escherichia coli* DH5 α strains containing plasmids were grown at 37°C in Luria-Bertani medium (38) containing 50 μ g of ampicillin per ml. *M. xanthus* strains were grown at 32°C in CTT broth or agar (1.5%) plates (1% Casitone, 10 mM Tris-HCl [pH 8.0], 1 mM KH₂PO₄, 8 mM MgSO₄ [final pH = 7.6]) (13). When necessary, 40 μ g of kanamycin (Km) per ml and 12.5 μ g of oxytetracycline (Tc) per ml were used for selection. Fruiting body development was performed on TPM agar (1.5%) plates (10 mM Tris-HCl [pH 8.0], 1 mM KH₂PO₄-K₂HPO₄, 8 mM MgSO₄ [final pH = 7.6]) as described previously (24).

Construction of plasmids. The 1.0-kb XhoI-BamHI fragment from pPV4406-1.0, which contains DNA from bp -533 to 485 relative to the Ω 4406 transcriptional start site, was subcloned into XhoI-BamHI-digested pREG1727 to construct pKV4. This plasmid served as the template for PCR amplification of Ω 4406 DNA segments using upstream primers containing a XhoI site and downstream primers containing a BamHI site. Each PCR product was cloned using pCR2.1-TOPO as described by the manufacturer. Each DNA insert was sequenced at the Michigan State University Genomics Technology Support Facility to ensure the correct sequence was obtained. Each pCR2.1-TOPO derivative was digested with XhoI and BamHI, and the DNA insert was purified by agarose gel extraction and subcloned into pREG1727 that had been digested with XhoI and BamHI.

A QuikChange site-directed mutagenesis kit (Stratagene) was used to create mutations in the Ω 4406 promoter region that were A \leftrightarrow C or G \leftrightarrow T single-base-pair or multiple-base-pair transversions. The plasmid pKV12 containing Ω 4406 DNA from bp -100 to 320 served as the DNA template for the mutagenesis with various combinations of primers. Candidate mutant plasmids were sequenced at the Michigan State University Genomics Technology Support Facility to identify plasmids with only the desired mutations. Each mutant plasmid was digested with XhoI and BamHI, and the mutant DNA insert was purified by agarose gel extraction and subcloned into pREG1727 that had been digested with XhoI and BamHI.

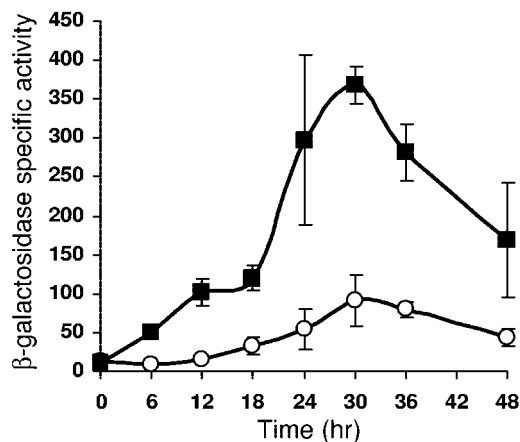


FIG. 1. An upstream DNA element negatively regulates Ω 4406 expression. Developmental expression from the 1.0-kb (bp -533 to 485) (○) or 0.6-kb (bp -100 to 485) (■) DNA segment containing the Ω 4406 promoter fused to *lacZ* and integrated at the Mx8 phage attachment site in the chromosome of *M. xanthus* wild-type DK1622, was measured for three independently isolated transformants (see Materials and Methods). The graph shows the average β -galactosidase specific activity expressed in nanomoles of *o*-nitrophenyl phosphate per minute per milligram of protein, and error bars show 1 standard deviation of the data.

Construction of *M. xanthus* strains and determination of *lacZ* expression during development. Strains containing pREG1727 derivatives integrated at the Mx8 phage attachment site (designated *attB* in Table 1) were constructed by electroporation of *M. xanthus* (20), and transformants were selected on CTT-Km plates. Based on previous experience in our laboratory (1, 6, 7, 12, 32), the majority of transformants have a single copy of the plasmid integrated at *attB*. To eliminate colonies with unusual developmental *lacZ* expression, we screened at least 10 transformants on TPM agar plates containing 40 μ g of 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside per ml. Any colonies with unusual expression of *lacZ* were discarded and, of the remaining candidates, three independent isolates of each mutant construct were chosen for development. In all cases, the three transformants gave similar results when developmental β -galactosidase activity was measured as described previously (24).

RESULTS

Deletion analysis reveals an upstream negative regulatory element in the Ω 4406 promoter region. An \sim 1.0-kb DNA segment spanning from bp -533 to 485 relative to the start site of Ω 4406 transcription was shown previously to drive developmental expression of a *lacZ* reporter when the fusion was integrated at the Mx8 phage attachment site in the *M. xanthus* chromosome (32). The maximum level of expression (about 200 U) was similar to that observed for Tn5 *lac* Ω 4406-Tc^r (32). The fusion at the phage attachment site had been created in a plasmid (pPV12K) that encodes Tc^r. For the present study, we fused the 1.0-kb segment to *lacZ* in a plasmid (pREG1727) that encodes Km^r. After integration at the Mx8 phage attachment site, this fusion exhibited developmental *lacZ* expression, reaching a maximum of 90 U at 30 h (Fig. 1 and Table 2). This pattern is similar to that reported previously for Tn5 *lac* Ω 4406-Km^r (24, 32). Although we do not understand the reason for the higher expression of fusions associated with Tc^r than Km^r, this effect was observed previously with a much larger (3.2-kb) segment spanning the Ω 4406 promoter region (32).

To localize the DNA elements important for Ω 4406 transcription, we performed deletion analysis, beginning with a 5'

TABLE 2. Summary of activities of mutant Ω 4406 promoters

Promoter assayed	Avg maximum β -galactosidase specific activity during development ^a \pm SD	% Control activity measured in the same experiment ^b \pm SD
Vector (no insert)	16 \pm 5	
bp -533 to 485	90 \pm 32	100 \pm 36
Deletions		
bp -100 to 485	368 \pm 24	476 \pm 7
bp -100 to 320	272 \pm 35	346 \pm 13
bp -100 to 251	182 \pm 12	205 \pm 7
bp -100 to 182	220 \pm 50	244 \pm 23
bp -100 to 140	199 \pm 30	247 \pm 15
bp -100 to 50	57 \pm 6	55 \pm 11
Mutations in the C box-like sequence		
CATCGTG -55 to -49 ACGATGT	31 \pm 10	11 \pm 32
C -55 A	161 \pm 3	59 \pm 2
A -54 C	797 \pm 199	293 \pm 25
T -53 G	24 \pm 9	9 \pm 38
C -52 A	176 \pm 34	65 \pm 19
G -51 T	33 \pm 4	12 \pm 12
T -50 G	98 \pm 22	36 \pm 22
G -49 T	568 \pm 93	209 \pm 16
Mutations farther upstream		
T -56 G	350 \pm 6	129 \pm 2
GTC -59 to -57 TGA	160 \pm 25	59 \pm 16
GAACC -64 to -60 TCCAA	67 \pm 22	25 \pm 32
C -61 A	95 \pm 31	35 \pm 33
GGACA -69 to -65 TTCAC	835 \pm 175	307 \pm 21
GGCGTTTCA -90 to -82 TTATGGGAC	421 \pm 23	155 \pm 5

^a The maximum β -galactosidase specific activity in nanomoles of *o*-nitrophenyl phosphate per minute per milligram of protein (average \pm 1 standard deviation) is listed for three independently isolated *M. xanthus* transformants (one determination each), except in the case of the vector control (one transformant, seven determinations) and the deletion with Ω 4406 DNA from bp -100 to 320 (three transformants, two determinations each). Samples were assayed at 0, 6, 12, 18, 24, 30, 36, and 48 h during development.

^b Controls were included in each experiment. For the deletions, the controls were Ω 4406 DNA from bp -533 to 485 and the vector with no insert. The maximum for each deletion is expressed as a percentage of the maximum observed for bp -533 to 485 in the same experiment, after subtraction from both values of the maximum observed for vector alone in that experiment. Likewise, the maximum for each mutation is expressed as a percentage of the maximum observed for bp -100 to 320 in the same experiment, after subtraction from both values of the maximum observed for vector alone in that experiment. The average percentage \pm 1 standard deviation is listed.

deletion. A DNA segment spanning from bp -100 to 485 was fused to *lacZ* in the *Km*^r-encoding plasmid (pREG1727) and integrated at the Mx8 phage attachment site. Developmental *lacZ* expression reached a higher maximum (368 U, Table 2) than for the 1.0-kb segment (Fig. 1). We conclude that DNA between bp -533 and -100 inhibits expression of a downstream *lacZ* reporter by at least fourfold.

The upstream negative element mediates C-signal dependence of the Ω 4406 promoter. Previously, expression from the 1.0-kb segment spanning the Ω 4406 promoter region was shown to be abolished in a *csgA* mutant incapable of C signaling (32). Developmental *lacZ* expression was restored upon

codevelopment of the fusion-bearing *csgA* mutant with an equal number of wild-type cells, which supply C signal (32). These experiments used the fusion created in the Tc^r-encoding plasmid (pPV12K). We repeated these experiments with the fusion of the 1.0-kb segment to *lacZ* in the *Km*^r-encoding plasmid (pREG1727). Expression was greatly reduced in the *csgA* mutant background (Fig. 2A). Upon codevelopment with wild type, induction of *lacZ* expression was partially restored, suggesting that transcription from the Ω 4406 promoter within the 1.0-kb segment depends on extracellular C signaling.

We next tested the segment spanning from bp -100 to 485, fused to *lacZ* in the *Km*^r-encoding plasmid (pREG1727) and integrated at the Mx8 attachment site of the *csgA* mutant. Developmental expression in the *csgA* mutant was indistinguishable from that in wild type, except at 48 h (Fig. 2B). In the absence of the negative element located between bp -533 and -100, expression from the Ω 4406 promoter appeared to be *csgA* independent and therefore independent of C signaling.

Expression from the Ω 4406 promoter depends on *fruA* and *devS* even in the absence of the upstream negative element. FruA mediates both motility and gene regulatory responses to

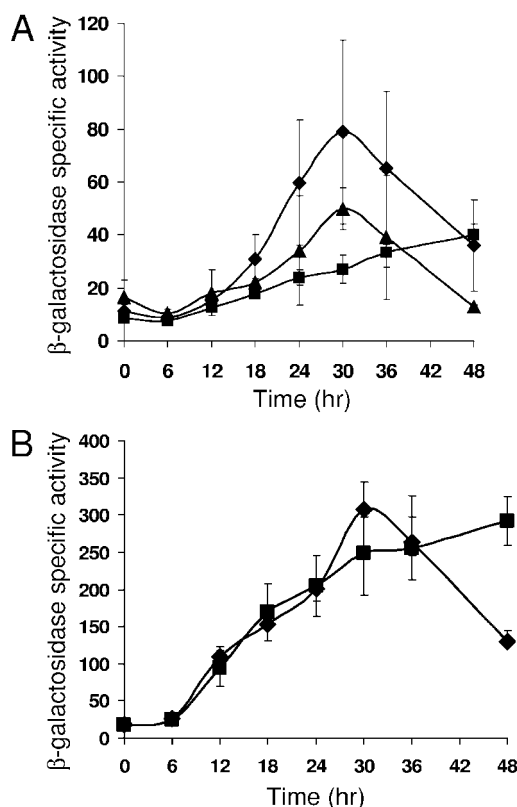


FIG. 2. C-signal dependence of Ω 4406 expression requires the upstream negative element. (A) Developmental expression from the 1.0-kb DNA segment containing the Ω 4406 promoter fused to *lacZ* and integrated at the Mx8 phage attachment site in the chromosome of *M. xanthus* wild-type DK1622 (\blacklozenge) or *csgA* mutant LS203 in the absence (\blacksquare) or presence (\blacktriangle) of an equal number of wild-type DK1622 cells (lacking *lacZ* but capable of C signaling). (B) Developmental expression from the 0.6-kb (bp -100 to 485) segment in wild-type DK1622 (\blacklozenge) or *csgA* mutant LS203 (\blacksquare). The meaning of points and error bars is the same as described in the Fig. 1 legend.

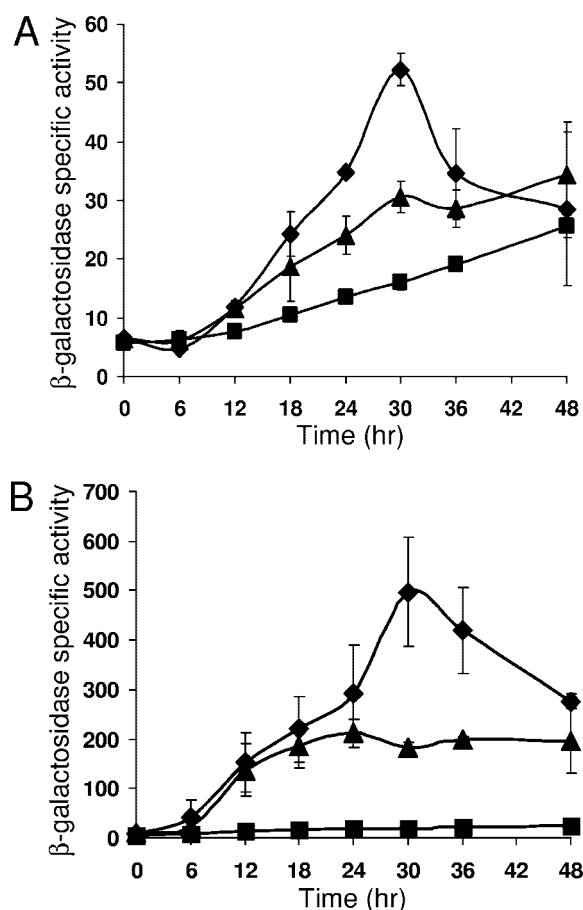


FIG. 3. Dependence of Ω 4406 expression on *fruA* and *devS*. Developmental expression from the 1.0-kb (A) or 0.6-kb (B) DNA segment containing the Ω 4406 promoter fused to *lacZ* and integrated at the Mx8 phage attachment site in the chromosome of *M. xanthus* wild-type DK1622 (◆), *fruA* mutant *fruA*::Tc Ω 5 (■), or *devS* mutant DK11209 (▲) was measured for three independently isolated transformants (see Materials and Methods). The meaning of points and error bars is the same as described in the Fig. 1 legend. The error bars are too small to be seen for the *fruA* mutant in both panels.

C signaling (5, 34, 42). Since expression from the 1.0-kb segment containing the Ω 4406 promoter was strongly dependent on C signaling, but expression from the \sim 0.6-kb segment (i.e., bp -100 to 485) lacking the upstream negative element was C-signal independent (Fig. 2), we tested the effect of a *fruA* mutation on expression from these segments by integrating the fusion-containing, Km^r plasmids at the Mx8 attachment site of *M. xanthus* bearing the *fruA*::Tc Ω 5 mutation, which confers Tc^r (34). Expression from both segments was greatly reduced in the *fruA* mutant (Fig. 3). These results demonstrate that FruA positively regulates Ω 4406 expression and that it does not do so solely by counteracting the negative effect of the upstream element. Our results do not rule out the possibility that in response to C signaling, FruA both counteracts the negative upstream element and causes transcriptional activation (directly or indirectly) through a DNA element(s) located downstream of bp -100 in the Ω 4406 promoter region.

The *dev* locus functions after *fruA* on a pathway leading to sporulation (5). Previously, expression of Tn5 *lac* Ω 7536-Km^r

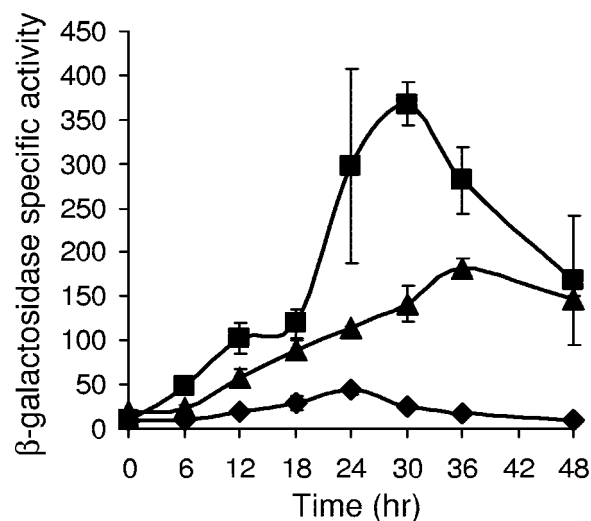


FIG. 4. Effect of 3' deletions on Ω 4406 expression. Developmental expression from DNA segments spanning from bp -100 to 485 (■) (i.e., the 0.6-kb segment; the data shown in Fig. 1 is also shown here), bp -100 to 251 (▲), or bp -100 to 50 (◆) relative to the Ω 4406 transcriptional start site, fused to *lacZ* and integrated at the Mx8 phage attachment site in the chromosome of *M. xanthus* wild-type DK1622, was measured for three independently isolated transformants (see Materials and Methods). The meaning of points and error bars is the same as described in the Fig. 1 legend. The error bars are too small to be seen for the bp -100 to 50 segment.

was shown to be abolished in *csGA* and *devRS* mutants (30). Like the *devRS* mutation, an in-frame deletion in *devS* causes a severe sporulation defect (9). This *devS* mutation reduced expression from both the 1.0- and 0.6-kb segments containing the Ω 4406 promoter, by \sim 2-fold (Fig. 3). We conclude that expression of Ω 4406 depends less strongly on *devS* than on *fruA*, and neither of these effects relies on the upstream negative element.

A downstream element may boost expression from the Ω 4406 promoter. To further characterize the DNA elements important for Ω 4406 expression, we performed 3' deletion analysis. We chose to construct 3' deletions with a common 5' end at bp -100 (i.e., lacking the upstream negative element described above) since the 0.6-kb segment spanning from bp -100 to 485 drove a high level of developmental *lacZ* expression (Fig. 1). Fusions with 3' ends at bp 320 (data not shown) or bp 251 (Fig. 4) exhibited similar timing of expression during development as the fusion at bp 485 , but the maximum level of expression progressively decreased (Table 2). Fusions with 3' ends at bp 182 or 140 showed similar expression as the fusion at bp 251 (Table 2). A fusion with a 3' end at bp 50 exhibited much less developmental *lacZ* expression (Fig. 4), reaching less than 30% the maximum level observed for the fusion at bp 140 (Table 2). These results indicate that DNA between bp 50 and 140 boosts expression of a downstream reporter. The effect could be on transcription initiation from the Ω 4406 promoter (e.g., due to a transcriptional activator protein that binds between bp 50 and 140 bp) or on a postinitiation event (e.g., transcription termination, mRNA stability, or translation). Another possibility, which we think is unlikely, is that the fusion at bp 50 results in reduced expression of the downstream *lacZ*

reporter, due to a polar effect. Each fusion with a different 3' end was designed to create an in-frame fusion between an open reading frame predicted to start at bp 37 and *trpA*, which precedes *lacZ* in pREG1727 (32). If translation instead begins between bp 50 and 140, so that only the fusion at bp 50 fails to create an in-frame fusion with *trpA*, it might cause a polar effect on expression of *lacZ*. However, it is unlikely that translation begins between bp 50 and 140, because the only potential translation start codon in this region, an ATG that begins at bp 97, is not preceded by a sequence likely to serve as a ribosomal binding site. On the other hand, the ATG that begins at bp 37 is preceded 5 bp upstream by the sequence AGGAG, which likely serves as a ribosomal binding site (32). Therefore, it is likely that DNA between bp 50 and 140 not only boosts expression of the downstream *lacZ* reporter in our fusions, but also boosts expression of the *M. xanthus* gene downstream of the Ω 4406 promoter in its native context.

A C-box-like sequence centered at bp -52 is important for Ω 4406 promoter activity. Inspection of the promoter regions of several developmentally regulated *M. xanthus* genes revealed sequences that conform to the consensus CAYYCCY (Y means C or T), which was called the C box (6). Mutational analyses of C boxes centered near bp -50 have demonstrated the importance of this DNA element for promoter activity (43, 46-48). In the Ω 4406 promoter region, the sequence CATCGTG centered at bp -52 matches the C box consensus at only the first four positions. To test whether this C-box-like sequence is important for Ω 4406 promoter activity, we changed the sequence to ACGATGT in the context of DNA from bp -100 to 320. This mutant promoter region was fused to *lacZ* in a plasmid (pREG1727) and integrated at the Mx8 phage attachment site in the *M. xanthus* chromosome as described above. Developmental *lacZ* expression was nearly abolished (Table 2), demonstrating the importance of the C-box-like sequence for Ω 4406 promoter activity.

Despite their sequence similarity, C boxes appear to function differently in the promoter regions thus far tested, since single-base-pair transversion mutations in each C box produced a different pattern of effects on promoter activity (43, 46-48). We subjected the C-box-like sequence centered at bp -52 in the Ω 4406 promoter region to this type of analysis. These mutations, as well as others described below, were analyzed in the context of DNA from bp -100 to 320. Certain single-base-pair changes in the C-box-like sequence had a dramatic effect on Ω 4406 promoter activity. Developmental expression from the two mutant promoter regions with the highest and lowest activity is compared to that from the wild-type promoter region in Fig. 5A. The timing of expression from mutant promoter regions was similar to that from wild type (Fig. 5A and data not shown). The maximum level of expression during development for each mutant promoter region is compared to that for the wild-type promoter region (bp -100 to 320) in Table 2. The pattern of effects on promoter activity for mutations in the C-box-like sequence centered at -52 bp in the Ω 4406 promoter region is compared to the effects of mutations in C boxes of other promoter regions in Fig. 5B. The pattern is unique for the C-box-like sequence in the Ω 4406 promoter region, even considering just the first two positions, at which in each case the C at position 1 was changed to an A and, separately, the A at position 2 was changed to a C. Like the C boxes in other promoter regions, the C-box-like se-

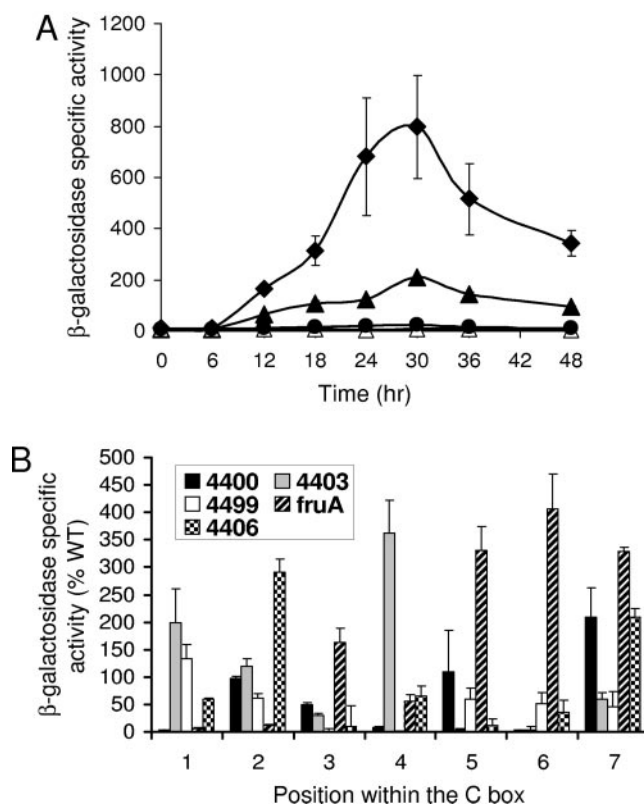


FIG. 5. Single-base-pair changes in the C-box-like sequence centered at bp -52 have dramatic effects on Ω 4406 expression, and the pattern of effects is different than for C boxes mutated previously. (A) Developmental expression from the wild-type DNA segment spanning from bp -100 to 320 (\blacktriangle) or the same segment with a T-to-G change at bp -53 (\bullet) or an A-to-C change at bp -54 (\blacklozenge), fused to *lacZ* and integrated at the Mx8 phage attachment site in the chromosome of *M. xanthus* wild-type DK1622, was measured for three independently isolated transformants (see Materials and Methods). The vector with no insert was included as a control (\triangle). The meaning of points and error bars is the same as described in the Fig. 1 legend. The error bars are too small to be seen except in the case of the base pair segment from -100 to 320 with the A-to-C change at -54 bp. (B) Comparison of the effects of single-base-pair transversion mutations in the C-box-like sequence centered at bp -52 in the Ω 4406 promoter region and in four different C boxes. The x-axis represents the position in the C box (or C-box-like sequence) corresponding to the consensus sequence CAYYCCY with the A being position 2, etc. The bars represent the average maximum developmental *lacZ* activity expressed as a percentage of the wild-type promoter activity for the C boxes centered at bp -49 in the Ω 4400 (47) and Ω 4403 (46) promoter regions, at bp -33 in the Ω 4499 promoter region (48), at bp -51 in the *fruA* promoter region (43), or for the C-box-like sequence centered at bp -52 in the Ω 4406 promoter region (Table 2). Error bars show one standard deviation of the data.

quence in the Ω 4406 promoter region appears to function in a unique fashion (e.g., due to binding of a different transcription factor in each case).

Mutations upstream of the C-box-like sequence reveal differences and similarities compared to other developmental promoter regions. C boxes are preceded 5 to 8 bp upstream by sequences similar to the consensus GAACA in the promoter regions of several developmentally regulated *M. xanthus* genes (46). These sequences, called 5-bp elements, have been shown to be important for promoter activity (43, 46-48). The se-

quence of DNA between the 5-bp element and the C box also affects promoter activity, leading to speculation that the 5-bp element, the C box, and the sequence in between together constitute a recognition site for a transcription factor, which may be different for each promoter examined thus far (48). To test the importance of these sequences in the Ω 4406 promoter region, we made four mutations and measured the activity of the mutant promoter regions as described above (Table 2). The timing of expression from mutant promoter regions was similar to that from wild type (data not shown). Changing T-to-G at position -56 , immediately upstream of the C box-like sequence, caused a slight rise in the maximum level of developmental *lacZ* expression, to 130% of the maximum level observed for the wild-type promoter region (bp -100 to 320) in the same experiment (summarized in Fig. 6A). This is a unique result compared to three other C-signal-dependent promoter regions (Ω 4400, Ω 4403, and Ω 4499), where transversion mutations at the position preceding a C box greatly reduced promoter activity (46–48). Changing GTC to TGA at base pair positions -59 to -57 slightly decreased promoter activity (Fig. 6A). In contrast, multiple-base-pair changes in sequences between 5-bp elements and C boxes abolished promoter activity (Ω 4400 and Ω 4499) or had no effect (Ω 4403) (46–48). Changing the 5-bp element, GAACC, to TCCAA at base-pair positions -64 to -60 reduced activity \sim 4-fold, and changing just the perfectly conserved C at position -61 bp to A reduced activity about threefold (Fig. 6A). As in other promoter regions (Ω 4400, Ω 4403, Ω 4499, and *fruA*) (43, 46–48), the 5-bp element is very important for Ω 4406 promoter activity. However, a threefold reduction in activity has not been observed previously for a C-to-A change at position 4 within a 5-bp element; the corresponding change in the Ω 4403 (46), Ω 4400 (47), and *fruA* (43) promoter regions resulted in 180, 1, and 270% of wild-type activities, respectively. We conclude that the Ω 4406 promoter region has an architecture similar to that of other developmental promoter regions that have been examined in detail (Ω 4400, Ω 4403, Ω 4499, and *fruA*) (43, 46–48), which includes a C-box-like sequence preceded 4 bp upstream by a 5-bp element. The different effects of many mutations in this region compared to the corresponding region of the other promoters is consistent with the idea (48) that each constitutes a recognition site for one or more transcription factors that differ between the promoters.

A second sequence similar to the 5-bp element consensus sequence was noted in the Ω 4406 promoter region previously (32). This sequence, GGACA, is immediately upstream of the 5-bp element described above. Changing this sequence to TTCAC at base pair positions -69 to -65 increased promoter activity threefold (Table 2 and Fig. 6A). The timing of expression from the mutant promoter region was similar to that from wild type (data not shown). Clearly, the effect of mutating this sequence is different than for 5-bp elements located 5 to 8 bp upstream of a C box (43, 46–48) or for the 5-bp element located 4 bp upstream of the C-box-like sequence in the Ω 4406 promoter region (Fig. 6A). It is worth noting that the GGACA sequence at base pair positions -69 to -65 is near the 3' end of a 39-bp segment with an imperfect inverted repeat (-101 TCATTCTGTGCGGCGTTTCAGGGAAACCGCACGGAC AGA -63) recognized previously (32). Also within this repeat is the sequence GGCGTTTCA, centered at bp -86 (32),

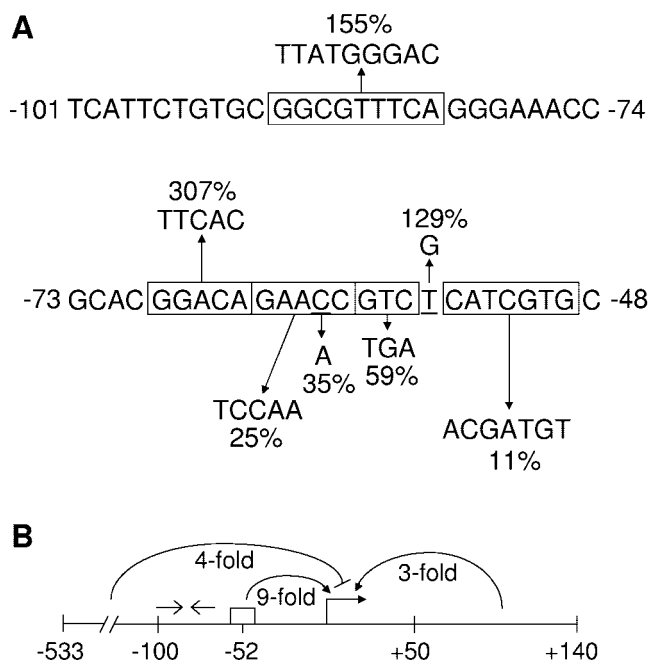


FIG. 6. Summary of the Ω 4406 promoter region. (A) Effects of mutations upstream of the Ω 4406 promoter. The indicated changes were made in the context of DNA spanning from bp -100 to 320 bp relative to the Ω 4406 transcriptional start site. Upward and downward arrows indicate that developmental *lacZ* expression was increased or decreased, respectively, and numbers indicate the maximum β -galactosidase specific activity observed for the mutant promoter region, expressed as a percentage of wild-type promoter activity measured in the same experiment (Table 2). (B) Summary of deletion analysis and the effect of a multiple-base-pair mutation in the C-box-like sequence. Deletions show that DNA between bp -533 and -100 exerts a four-fold negative effect on Ω 4406 promoter activity and suggest that DNA between bp 50 and 140 exerts a threefold positive effect. A multiple-base-pair change in the C-box-like sequence centered at bp -52 (rectangle) indicates it exerts a ninefold positive effect. Open arrows indicate the position of an imperfect inverted repeat sequence that appears to be involved in negative regulation.

which resembles an essential 10-bp element, GGCATGTTCA, centered at bp -74.5 in the Ω 4403 promoter region (46). Changing this sequence to TTATGGGAC at base pair positions -90 to -82 increased promoter activity \sim 1.6-fold (Table 2 and Fig. 6A) without altering the timing of expression (data not shown). Clearly, the sequence does not function like the 10-bp element in the Ω 4403 promoter region. Rather, since both mutations in the large inverted repeat resulted in increased promoter activity, we speculate that it might be a site for binding of a dimeric repressor. This putative negative regulatory site would be in addition to the negative element located between bp -533 and -100 (Fig. 6B).

DISCUSSION

We have performed mutational analysis of the Ω 4406 promoter region. Our most novel findings are that developmental activity of this promoter is governed by an upstream negative DNA element and that this element mediates the promoter's dependence on C signaling. We also obtained evidence that DNA downstream of the transcriptional start site boosts ex-

pression of the gene. Despite these unusual regulatory features, we found that the Ω 4406 promoter region resembles that of other developmentally regulated *M. xanthus* genes in its general architecture. It has a C-box-like sequence and a 5-bp element, both of which are important for promoter activity. However, our detailed mutational analysis revealed unique features of the Ω 4406 promoter region in comparison to other *M. xanthus* promoter regions studied thus far. We conclude that regulation of Ω 4406 expression is complex and involves multiple positive and negative *cis*-acting DNA elements located upstream and downstream of the region typically bound by RNA polymerase (Fig. 6B).

Our results indicate that DNA between bp -533 and -100 negatively regulates Ω 4406 promoter activity by about fourfold. Important goals of further research are to localize this *cis*-acting element and determine whether it is bound by a repressor protein. Upstream repressor binding sites typically function in cooperation with repressor bound farther downstream, forming a DNA loop (reviewed in reference 39). We speculate that the large inverted repeat between bp -101 and -63 might be such a downstream repressor binding site, since two different multiple-base-pair mutations in the repeat each caused Ω 4406 promoter activity to increase. We searched for a sequence similar to the large inverted repeat, located between bp -533 and -100 . Interestingly, the sequence GCGTTTGAT TGCAACCGC at bp -317 to -300 matches the center of the large inverted repeat at 14 of 18 positions.

In the absence of the negative element located between bp -533 and -100 , expression from the Ω 4406 promoter was still developmentally regulated, but it did not depend on C signaling. The finding that loss of the negative element did not result in constitutive expression during growth indicates the existence of additional mechanisms of developmental control, and our analysis uncovered multiple positive regulatory DNA elements, which will be discussed below. The finding that loss of DNA upstream of bp -100 rendered expression from the Ω 4406 promoter independent of C signaling is striking. It implies that the role of C signaling at this promoter is to overcome the negative effect of the upstream element. In fact, our results suggest the C signaling only partially overcomes this negative regulation, since developmental expression reached a fourfold-higher level in the absence of the upstream negative element. The only other *M. xanthus* promoter for which a regulatory DNA element mediating C-signal-dependent expression has been defined is the Ω 4400 promoter. In this case, DNA from bp -86 to -81 exerts a two- to fourfold positive effect on expression and is at least partially responsible for C-signal dependence (47).

Because FruA mediates responses to C signaling during development (5, 34, 42), it was of interest to determine whether FruA relieves negative regulation of the Ω 4406 promoter. We found that FruA is required positively for Ω 4406 expression, even when the upstream negative element is absent (Fig. 3). This prevented us from determining whether FruA counteracts the negative effect of the upstream element. In the absence of the upstream element, Ω 4406 regulation is like that of a few other *M. xanthus* genes, including *tps* and *dofA*, whose expression depends on FruA but not on C signaling (14, 15, 23, 34).

Expression of the *dev* locus depends on *fruA* (5), and expres-

sion of the Ω 7536 locus depends on *dev* (30). This placed the Ω 7536 locus downstream of *dev* on a pathway that leads to sporulation, since all of these genes are required for sporulation (30, 34, 44). Although the disruption caused by Tn5 *lac* Ω 4406-Km^r did not cause a sporulation defect (24), we showed that Ω 4406 expression depends partially on *dev*, being reduced about twofold in a *devS* mutant (Fig. 3). The effect was independent of the upstream negative element. It appears that *dev* function is not exclusively devoted to regulation of genes that are required for sporulation.

DNA between bp 50 and 140 boosted expression from a downstream *lacZ* reporter more than threefold when the upstream end of the Ω 4406 promoter region was at bp -100 . A similar observation was made previously using 3' deletions and *lacZ* fusions to analyze the *M. xanthus crtI* promoter region (33). *CrtI* is involved in light-induced carotenoid biogenesis and *crtI* is induced in response to blue light (8). Martinez-Argudo et al. (33) found that a downstream regulatory element located between bp 30 and 130 is required for light-induced expression from the *crtI* promoter. This element has enhancer-like properties since it remained active when placed upstream of the *crtI* promoter. Although enhancers are common in promoter regions recognized by σ^{54} RNA polymerase, they are unusual in promoter regions recognized by holoenzymes bearing a σ in the σ^{70} family (reviewed in reference 2). The Ω 4406 promoter does not resemble *M. xanthus* promoters believed to be transcribed by σ^{54} RNA polymerase (32, 48). It will be interesting to determine whether Ω 4406 downstream DNA contains an enhancer-like regulatory element. We searched Ω 4406 downstream DNA between bp 1 and 140 for inverted repeats but found nothing strongly suggestive of a binding site for a dimeric transcription factor or a transcription terminator that might be indicative of an antitermination regulatory mechanism.

The Ω 4406 promoter region has blocks of sequence that are strikingly similar to sequences in the promoter regions of other developmentally regulated *M. xanthus* genes (32). These alignments helped us identify targets for mutagenesis in the Ω 4406 promoter region, such as the C-box-like sequence centered at bp -52 . Our mutational analysis showed that single-base-pair changes within this sequence can dramatically decrease or increase promoter activity. Likewise, single-base-pair changes in C boxes have decreased or increased activity of other promoters, but in each case the pattern of effects has been different (32, 43, 46–48). In keeping with this trend, the pattern of effects of single-base-pair changes in the C-box-like sequence centered at -52 bp in the Ω 4406 promoter region is also unique (Fig. 5B). On the other hand, a multiple-base-pair change in the Ω 4406 C-box-like sequence (Table 2 and Fig. 6) or in C boxes in other promoter regions (32, 43, 46–48) has in each case strongly decreased promoter activity. This suggests that these sequences are positive regulatory elements, and we have speculated that each is bound by a different transcriptional activator protein (43, 48), although these proteins may be evolutionarily related since they recognize similar sequences. According to this model, single-base-pair changes in C boxes (or the Ω 4406 C-box-like sequence) that increase promoter activity do so by making the sequence a better binding site for the putative C-box-binding protein and therefore might be useful for biochemical approaches aimed at identifying these proteins.

The putative C-box-binding proteins may in fact recognize

not only the C box but also the 5-bp element located 5 to 8 bp upstream of the C box (43, 48). The 5-bp element also appears to be a positive regulatory element, since multiple-base-pair changes have decreased promoter activity in every case examined thus far (43, 46–48). Four base pairs upstream of the C-box-like sequence in the Ω 4406 promoter region is the sequence GAACC, which matches the 5-bp element consensus sequence, GAACA, in four of five positions. A multiple-base-pair change in this sequence reduced promoter activity four-fold. Hence, the Ω 4406 promoter region has a similar architecture (i.e., a 5-bp element located 4 to 8 bp upstream of a C-box or C-box-like sequence) as the Ω 4400, Ω 4403, Ω 4499, and *fruA* promoter regions (43, 46–48). DNA between the 5-bp element and the C box has also been shown to be important for activity of the Ω 4400, Ω 4403, and Ω 4499 promoters (46–48); however, two mutations in the corresponding region had only modest effects on Ω 4406 promoter activity. Also, changing C to A at position 4 of the 5-bp element had a different effect on Ω 4406 promoter activity than on the activity of the Ω 4400, Ω 4403, or *fruA* promoters (43, 46, 47). These differences strengthen the impression that the 5-bp element, the C-box (or C-box-like) sequence, and the DNA in between function differently in each developmentally regulated promoter examined thus far.

Further differences between the Ω 4406 promoter region and the promoter regions of other developmentally regulated *M. xanthus* genes that have been subjected to detailed mutational analysis are evident from the results of two mutations we made upstream of the 5-bp element. Most strikingly, changing GGACA at base pair positions –69 to –65 to TTCAC dramatically increased promoter activity. Despite its similarity to the 5-bp element consensus sequence, the GGACA sequence does not appear to function like 5-bp elements that are located 4 to 8 bp upstream of C-box or C-box-like sequences, since multiple-base-pair changes in these 5-bp elements greatly reduced promoter activity (43, 46–48). Neither does the GGC GTTTC sequence centered at –86 bp in the Ω 4406 promoter region appear to function like the 10-bp element, GGC ATGTTCA, which is centered at bp –74.5 in the Ω 4403 promoter region (46). Rather, our mutational analysis suggests that these sequences might be part of a repressor binding site, and we have speculated above that repressor bound to the large inverted repeat between bp –101 and –63 might interact with repressor bound farther upstream to form a repression loop.

In conclusion, our analysis of the Ω 4406 promoter region revealed an upstream negative DNA element that mediates C-signal dependence, a likely downstream positive regulatory element, and upstream 5-bp and C-box-like elements. The latter act positively and may do so in conjunction, as observed for other developmentally regulated *M. xanthus* promoters, but somehow functioning uniquely in each promoter region, based on detailed mutational analyses. In the future, we hope to identify activator and repressor proteins that bind to the positive and negative regulatory DNA elements we have uncovered in the Ω 4406 promoter region.

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