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Combinatorial Regulation of the *dev* Operon by MrpC2 and FruA during *Myxococcus xanthus* Development

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Proper expression of the *dev* operon is important for normal development of *Myxococcus xanthus*. When starved, these bacteria coordinate their gliding movements to build mounds that become fruiting bodies as some cells differentiate into spores. Mutations in the *devTRS* genes impair sporulation. Expression of the operon occurs within nascent fruiting bodies and depends in part on C signaling. Here, we report that expression of the *dev* operon, like that of several other C-signal-dependent genes, is subject to combinatorial control by the transcription factors MrpC2 and FruA. A DNA fragment upstream of the *dev* promoter was bound by a protein in an extract containing MrpC2, protecting the region spanning positions −77 to −54. Mutations in this region impaired binding of purified MrpC2 and abolished developmental expression of reporter fusions. The association of MrpC2 and/or its longer form, MrpC, with the *dev* promoter region depended on FruA *in vivo*, based on chromatin immunoprecipitation analysis, and purified FruA appeared to bind cooperatively with MrpC2 to DNA just upstream of the *dev* promoter *in vitro*. We conclude that cooperative binding of the two proteins to this promoter-proximal site is crucial for *dev* expression. 5′ deletion analysis implied a second upstream positive regulatory site, which corresponded to a site of weak cooperative binding of MrpC2 and FruA and boosted *dev* expression 24 h into development. This site is unique among the C-signal-dependent genes studied so far. Deletion of this site in the *M. xanthus* chromosome did not impair sporulation under laboratory conditions.

Myxococcus xanthus is a Gram-negative bacterium that undergoes multicellular development (1). Upon starvation on a solid surface, cells coordinate their movements to build mounds that contain thousands of cells. Within these nascent fruiting bodies, some cells differentiate from rods to ovoid spores. Other cells lyse during the developmental process or remain outside fruiting bodies as peripheral rods. The spores remain viable during prolonged starvation and resist environmental insults. Under favorable conditions, spores germinate, producing rod-shaped cells capable of growth and division.

The developmental process of *M. xanthus* provides an attractive model to study signaling and gene regulatory mechanisms (1). Here, we focus on regulation of the *dev* operon in response to extracellular C signaling. The *dev* locus was identified by two transposon insertions that created reporter fusions induced during development (2, 3). Expression from the fusions was reduced in a *csgA* mutant incapable of C signaling (4). The transposon insertions in the *dev* locus prevented darkening of nascent fruiting bodies and reduced sporulation >100-fold (3, 5). The sporulation defect can be accounted for by the observation that a *dev* mutant fails to express the Ω7536 locus (6), the site of the *exo* operon, whose products are necessary for spore formation (7). How the products of the *dev* operon regulate the expression of the *exo* operon is unknown. The *dev* operon includes eight genes plus at least two repeats of the downstream CRISPR (clustered regularly interspaced short palindromic repeats) (8). The eight genes include a short upstream gene, three genes implicated in development (*devTRS*) (3, 8, 9), and four *cas* (CRISPR-associated) genes that typically form small interfering RNAs to inhibit the expression of plasmid and bacteriophage genes (10).

Expression of the *dev* operon and other C-signal-dependent genes is localized to nascent fruiting bodies (11). Numerous end-to-end contacts between cells in the outer domain of a nascent fruiting body have been proposed to facilitate C signaling, triggering the expression of the *dev* operon and then the *exo* operon and leading to spore formation (6, 11–14). Efficient C signaling requires that cells move into alignment (15–17), and sporulation demands a higher level of C signaling than does nascent fruiting body formation (18–20). Hence, regulation of the *dev* operon in response to C signaling appears to be a key step that couples the movement of cells into nascent fruiting bodies with differentiation into spores.

The mechanism of C signaling is partly understood. It involves CsgA (21), a 25-kDa protein (p25) that appears to associate with the inner membrane during cell growth (22) but with the outer membrane during development (23). Considerable evidence supports a model in which p25 is cleaved to a 17-kDa form (p17) that appears to be the C signal (19, 23–25). Starvation initiates a RelA-dependent proteolytic cascade in which $FtsH^D$-dependent degradation of PopD (where $FtsH^D$ represents FtsH important for development) releases PopC for secretion (26) and PopC-dependent cleavage of p25 forms p17 at the cell surface (27). However, a p17 receptor has not been identified. The similarity of CsgA to short-chain alcohol dehydrogenases and evidence that $NAD(P)^+$ bind-

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ing is essential for activity suggested that CsgA is an enzyme that generates a C signal (28). A related protein, SocA, which, when overexpressed, can substitute for CsgA (29), oxidized lipid substrates, but the products were unstable and failed to rescue the development of a *csgA* mutant (30). If CsgA is an enzyme, its substrate and product remain to be identified. Interestingly, the outer membrane porin Oar is required for C signaling and has been proposed to be the channel for the export of the C signal (31).

Cellular responses to C signaling involve FruA, which is similar to the response regulators of two-component signal transduction systems (32, 33). The putative histidine protein kinase that would phosphorylate FruA in response to C signaling has not been identified. Some evidence suggests that FruA might function as a pseudoresponse regulator without being phosphorylated (34). In any case, FruA has a C-terminal DNA-binding domain that was shown previously to bind to promoter regions of FruA-dependent genes *in vitro* (35–37). Mapping of the *dev* operon promoter revealed an upstream sequence centered at position −91 with similarity to a site recognized by the FruA DNA-binding domain (8). It was shown that the region between positions −101 and −75 upstream of the *dev* promoter can be bound by the FruA DNA-binding domain *in vitro* and that mutations in this region impair *dev* expression *in vivo* (38). Hence, FruA appears to activate *dev* transcription by binding to an upstream *cis*-regulatory element.

The expression of several C-signal-dependent genes has been shown to be under the combinatorial control of FruA and MrpC2 (34, 39–41). MrpC2 is an N-terminally truncated form of MrpC (42), a protein similar to transcription factors in the cyclic AMP receptor protein (CRP) family (43). A protein serine/threonine kinase cascade negatively regulates *mrpC* expression during growth, apparently by phosphorylating MrpC (44), which reduces its DNA-binding activity (45) and perhaps its ability to positively autoregulate (43). MrpC2 may be produced by utilization of an alternative translation start codon, or it may be derived from MrpC by proteolytic cleavage. MrpC2 appears to activate the transcription of *fruA* (42). MrpC2 appeared to have higher DNA-binding activity than MrpC (45), but the two forms of the protein had similar DNA-binding activity for the *fruA* promoter region in a recent study (62). Since MrpC2 cannot be phosphorylated, it might play an important role in escaping negative regulation by the protein kinase cascade during development (45).

MrpC2 binds cooperatively with FruA to the promoter regions of several C-signal-dependent genes *in vitro* (34, 39–41). Mutations in the cooperative binding sites affect promoter activity *in vivo*. In each case, binding of the two proteins cooperatively to a site located immediately upstream of the promoter appears to activate transcription. However, insertion mutations in these genes cause, at most, mild defects in fruiting body formation (34, 39–41). In contrast, insertions in the *dev* operon impair sporulation dramatically (3, 5). To determine whether a key developmental operon is regulated by a mechanism similar to that of other C-signal-dependent genes, we undertook the investigation reported here. We show that MrpC2 and FruA appear to bind cooperatively just upstream of the *dev* promoter. Mutations in the MrpC2-binding site abolished promoter activity, as did mutations in the FruA-binding site described previously (38). In addition, we identify a second *cis*-regulatory element located further upstream of the *dev* promoter, where MrpC2 and FruA appear to bind with weak cooperativity and enhance the expression of the *dev* operon after 24 h of development.

MATERIALS AND METHODS

Bacterial strains, plasmids, and primers. Strains and plasmids used in this study are listed in Table 1. To construct plasmids containing different segments of *dev* DNA, pPV1004 was used as the template for PCR with upstream primers containing a HindIII site and downstream primer LK1344 containing a BamHI site (see Table 2 for primers). Each PCR product was cloned by using pCR2.1-TOPO and *Escherichia coli* strain TOP10 as described by the manufacturer. To create mutations, pPV695 served as the template, using the QuikChange site-directed mutagenesis kit (Stratagene) and pairs of primers. Cloned PCR products and genes subjected to site-directed mutagenesis were verified by DNA sequencing. To construct pREG1727 derivatives, each derivative of pCR2.1-TOPO or pPV695 was digested with HindIII and BamHI, and the DNA insert was gel purified and cloned into HindIII-BamHI-digested pREG1727 by using *E. coli* strain DH5α and standard methods (46). The plasmid used to delete the distal upstream site of MrpC2 and FruA binding was constructed by using overlap extension PCR (47) to synthesize a DNA fragment of ~1 kbp spanning the region but lacking the site. In the first step, primers SS1 and SS2 and primers SS3 and SS4 were used in separate PCRs with pPV1515 as the template. In the second step, the two PCR products served as the template with primers SS1RII and SS4RII. The resulting ~1-kbp fragment was digested with EcoRI and BamHI, and the DNA insert was gel purified and cloned into EcoRI-BamHI-digested pBJ113 to create pSS3. The insert was verified by DNA sequencing.

Growth and development. *E. coli* DH5α strains containing plasmids were grown at 37°C in Luria-Bertani (LB) medium containing 50 μg/ml of either ampicillin or kanamycin sulfate. *M. xanthus* strains were grown at 32°C in CTT medium (1% Casitone, 10 mM Tris-HCl [pH 8.0], 1 mM KH_2PO_4-K_2HPO_4, 8 mM $MgSO_4$ [final pH 7.6]) (48) or on CTT agar (1.5%) plates. When required, 40 μg of kanamycin sulfate per ml was added. Fruiting body development was performed on TPM agar (1.5%) plates (10 mM Tris-HCl [pH 8.0], 1 mM KH_2PO_4-K_2HPO_4, 8 mM $MgSO_4$ [final pH 7.6]), as described previously (2), except for experiments in which spores were measured, in which case a submerged culture (49) was used, as described previously (50). Measurement of sonication-resistant spores, and of mature spores that were heat and sonication resistant and capable of germination, was performed as described previously (50).

ChIP. *M. xanthus* strains DK1622 and DK5285 were used for chromatin immunoprecipitation (ChIP) as described previously (34, 37, 41). The primers used for PCR of the *dev* promoter region were LK1298 and LK1331, and those used for PCR of the *rpoC* coding region were LK1861 and LK1862.

Preparation of proteins. DNA-binding proteins partially purified (ammonium sulfate [AS] fraction) from 12-h-developing *M. xanthus* DZF1 cells, as described previously (42), were a gift from Sumiko Inouye. Recombinant His_{10}-MrpC2 (34) and FruA-His_6 (45) were expressed in *E. coli* BL21(DE3) and purified as described previously.

EMSAs and footprinting. For electrophoretic mobility shift assays (EMSAs), ^{32}P-labeled DNA fragments from the *dev* promoter region were generated by PCR using the wild-type or mutant plasmid as the template and primers labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase (New England BioLabs). The DNA fragment was purified after 15% PAGE (46). EMSAs were performed as described previously (37), except that binding reaction mixtures were incubated at 25°C for 15 min. For footprinting, a ^{32}P-labeled DNA probe was synthesized by PCR after labeling primer LK1331 as described above, and probes were purified after 5% PAGE (46). Footprint analysis of gel slices using 1,10-phenanthroline-copper ion was performed as described previously (51).

TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Description[a]	Source or reference
Strains		
E. coli		
BL21(DE3)	F$^-$ *ompT hsdS*$_B$(r_B^- m_B^-) *gal dcm* with DE3, a λ prophage carrying the T7 RNA polymerase gene	Novagen
DH5α	λ$^-$ ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 recA1 endA1 hsdR17*(r_K^- m_K^-) *supE44 thi-1 gyrA relA1*	59
SMhisMrpC2	BL21(DE3) containing pET16b/His$_{10}$-MrpC2	34
SMFruAhis	BL21(DE3) containing pET11km/FruA-His$_6$	34
TOP10	λ$^-$ F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) ϕ80 *lacZ*ΔM15 Δ*lacX74 recA1 araD139* Δ(*ara-leu*)*7697 galU galK rpsL* (Str^r) *endA1 nupG*	Invitrogen
M. xanthus		
DK1622	Wild type	60
DK5285	*fruA*::Tn*5 lac* Ω4491	5
DK11209	Δ*devS*	8
MAC1	Δ*devS attB*::pAC01 (pREG1727 with a 696-bp HindIII-BamHI fragment from pTB1)	This study
MAC2A	Δ*devS attB*::pAC02A (pREG1727 with a 696-bp HindIII-BamHI fragment from pTB2A)	This study
MAC2B	Δ*devS attB*::pAC02B (pREG1727 with a 696-bp HindIII-BamHI fragment from pTB2B)	This study
MAC5	Δ*devS attB*::pAC05 (pREG1727 with a 696-bp HindIII-BamHI fragment from pTB5)	This study
MLK299	Δ*devS attB*::pLK0299 (pREG1727 with a 300-bp HindIII-BamHI fragment from pAC2)	This study
MLK324	Δ*devS attB*::pLK0324 (pREG1727 with a 325-bp HindIII-BamHI fragment from pAC1)	This study
MPV35N	Δ*devS attB*::pPV035N (pREG1727 with a 1,515-bp HindIII-BamHI fragment from pPV35N)	8
MPV184	Δ*devS attB*::pPV0184 (pREG1727 with a 185-bp HindIII-BamHI fragment from pPV184)	8
MPV391	Δ*devS attB*::pPV0391 (pREG1727 with a 392-bp HindIII-BamHI fragment from pPV391)	This study
MPV605	Δ*devS attB*::pPV0605 (pREG1727 with a 606-bp HindIII-BamHI fragment from pPV695)	This study
MPV695	Δ*devS attB*::pPV0695 (pREG1727 with a 696-bp HindIII-BamHI fragment from pPV695)	38
MPV1004	Δ*devS attB*::pPV01004 (pREG1727 with a 1,005-bp HindIII-BamHI fragment from pPV1004)	8
MSS1	A distal upstream site of MrpC2 and FruA binding at positions −254 to −228 was deleted using pSS3	This study
MTB3	Δ*devS attB*::pTB03 (pREG1727 with a 696-bp HindIII-BamHI fragment from pTB3)	This study
MTB4	Δ*devS attB*::pTB04 (pREG1727 with a 696-bp HindIII-BamHI fragment from pTB4)	This study
MTB6	Δ*devS attB*::pTB06 (pREG1727 with a 696-bp HindIII-BamHI fragment from pTB6)	This study
Plasmids		
pAC1	pCR2.1-TOPO with *dev* DNA spanning positions −254 to +71 generated by PCR using LK2489 and LK1344	This study
pAC2	pCR2.1-TOPO with *dev* DNA spanning positions −229 to +71 generated by PCR using LK2497 and LK1344	This study
pBJ113	Ap^r Km^r *galK*	11
pCR2.1-TOPO	Ap^r Km^r *lacZ*α	Invitrogen
pET16b/His$_{10}$-MrpC2	pET16b with a gene encoding His$_{10}$-MrpC2 under the control of a T7 RNA polymerase promoter	45
pET11km/FruA-His$_6$	pET11km with a gene encoding FruA-His$_6$ under the control of a T7 RNA polymerase promoter	S. Inouye
pPV35N	pPV1515 with a TTGACG-to-GGTCAT mutation spanning positions −38 to −33	8
pPV184	pCR2.1-TOPO with *dev* DNA spanning positions −114 to +71	8
pPV391	pCR2.1-TOPO with *dev* DNA spanning positions −321 to +71 generated by PCR using Cover 7 and LK1344	This study
pPV605	pCR2.1-TOPO with *dev* DNA spanning positions −535 to +71 generated by PCR using Cover 6 and LK1344	This study
pPV695	pCR2.1-TOPO with *dev* DNA spanning positions −114 to +581	38
pPV1004	pCR2.1-TOPO with *dev* DNA spanning positions −934 to +71 generated by PCR using Cover 4 and LK1344	8
pPV1515	pCR2.1-TOPO with *dev* DNA spanning positions −934 to +581	8
pREG1727	Ap^r Km^r P1-*inc attP′ lacZ*	61
pSS3	pBJ113 with a ~1-kbp region of *M. xanthus* DNA spanning a deletion of positions −254 to −228 upstream of the *dev* transcriptional start site	This study
pTB1	pPV695 with M1 mutation using LK2381 and LK2382	This study
pTB2A	pPV695 with M2A mutation using LK2491 and LK2492	This study
pTB2B	pPV695 with M2B mutation using LK2493 and LK2494	This study
pTB3	pPV695 with M3 mutation using LK2385 and LK2386	This study
pTB4	pPV695 with M3 mutation using LK2387 and LK2388	This study
pTB5	pPV695 with M3 mutation using LK2389 and LK2390	This study
pTB6	pPV695 with M3 mutation using LK2391 and LK2392	This study

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

Construction of *M. xanthus* strains and determination of *lacZ* expression during development. Strains containing a plasmid integrated at the Mx8 phage attachment site, *attB*, were constructed by electroporation (52). Transformants were selected on CTT agar plates containing kanamycin sulfate and screened on TPM agar plates containing 40 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside per ml, in order to avoid rare transformants with unusual developmental *lacZ* expression (53). Three transformants were typically chosen for further analysis, and β-galactosidase activity was measured as described previously (2). Strains containing a deletion of the distal upstream site of cooperative MrpC2 and FruA binding were constructed by electroporating pSS3 into *M. xanthus*, selecting a kanamycin-resistant transformant, growing the transformant

TABLE 2 Primers used in this study

Primer	Sequence[a]	Description[b] or reference
Cover 6	CCCAAGCTTAAGGAGAGACCTTCTTGTGGCG	Positions −535 forward with HindIII site
Cover 7	CCCAAGCTTCGCCGTTGGCTCGGATGCGGAC	Positions −321 forward with HindIII site
LK1298	CGAGGACCAGCGCTCGTC	Position −19 reverse
LK1331	CCAAGCTTGCTCACGTTGCAGACGGGG	Position −114 forward with HindIII site
LK1344	GGCGGATCCACCTCGTACTTCGACTTCCG	Position +71 reverse with BamHI site
LK1861	CCTTGAGCGCGATGGAGATA	56
LK1862	CTCGGCGGCCTCATCGAC	56
LK2381	GGGGCAATACAG*TTGGAC*AAGTGAGACGATTG	M1 mutation
LK2382	CAATCGTCTCACTT*GTCCAA*CTGTATTGCCCC	M1 mutation
LK2385	GGTTCAAAGTGA*TCATC*TTGCATGCATCAG	M3 mutation
LK2386	CTGATGCATGCAA*GATGA*TCACTTTGAACC	M3 mutation
LK2387	CAAAGTGAGACGA*GGTACGT*CATCAGCGAACG	M4 mutation
LK2388	CGTTCGCTGATG*ACGTACC*TCGTCTCACTTTG	M4 mutation
LK2389	GACGATTGCATG*ACGACTA*GAACGTTGACGAG	M5 mutation
LK2390	CTCGTCAACGTTC*TAGTCGT*CATGCAATCGTC	M5 mutation
LK2391	GCATGCATCAGC*TCCAT*TTGACGAGCGCTG	M6 mutation
LK2392	CAGCGCTCGTCAA*ATGGA*GCTGATGCATGC	M6 mutation
LK2475	GTCTGCAACGTGAGCGCG	Position −100 reverse
LK2476	CACGACGGCGCCGTTGG	Position −329 forward
LK2481	CGTGCTGCCTGTTCCTGG	Position −202 forward
LK2482	GCCGCTGGCCCAGGAAC	Position −176 reverse
LK2489	CAAGCTTCGCAACGAGCTGCGCACG	Position −254 forward with HindIII site
LK2491	CAATACAGGGTTCA*CCTT*GAGACGATTGC	M2A mutation
LK2492	GCAATCGTCTCA*AGGT*GAACCCTGTATTG	M2A mutation
LK2493	CAATACAGGGTTCA*CCTGTC*GACGATTGCATGCATCAGCG	M2B mutation
LK2494	CGCTGATGCATGCAATCGTC*GACAGGT*GAACCCTGTATTG	M2B mutation
LK2497	CAAGCTTCACATCTCATCCTCTATGCC	Position −229 forward with HindIII site
SS1	GCGAATTCGCGCCAACGCCTCGCAGC	Position +292 reverse with EcoRI site
SS2	**GCGCGCG**CATCTCATCCTCTATGCC	Position −227 forward with overlap
SS3	**TGAGATG**CGCGCGCCGGAGTTGCTC	Position −255 reverse with overlap
SS4	GCGGATCCGGACTGGACGCCTCACTTC	Position −762 forward with BamHI site
SS1RII	GCGAATTCCACAGCAACAAGGACTTCCAAG	Position +272 reverse with EcoRI site
SS4RII	ATAGGATCCGTTGATTGACCTGCGGGGCAAG	Position −639 forward with BamHI site
ΔSite 1F	CACTTCCGCGCCGTGCTCTAC	Position −371 forward
ΔSite 1R	GAACCCTGTATTGCCCCAAAGG	Position −70 reverse

[a] Restriction sites are underlined. Mutations are italicized. Overlaps are in boldface type.
[b] The position is relative to the start site of *dev* transcription, and the orientation (forward or reverse) is relative to the direction of *dev* transcription.

without selection, and then selecting galactose-resistant clones as described previously (54). Three clones in which the wild-type allele had been replaced with the deletion by homologous recombination were identified by colony PCR with primers ΔSite 1F and ΔSite 1R and verified by sequencing of the PCR product. The three clones were collectively designated MSS1.

RESULTS

Binding of a protein in a fraction from developing *M. xanthus* cells to the *dev* promoter region. A preparation containing DNA-binding proteins that was partially purified from 12-h-developing *M. xanthus* cells was shown previously to contain MrpC2 (34, 42). The preparation, called the AS fraction, was incubated with a ^{32}P-labeled DNA probe spanning positions −114 to −19 of the *dev* promoter region, and EMSAs revealed a single shifted complex (Fig. 1A). This finding suggested that a protein in the AS fraction binds to the *dev* promoter region. To characterize the position of binding, the protein-DNA complex and the unbound DNA probe (as a control) were excised from the gel and subjected to footprinting by treatment with 1,10-phenanthroline-copper followed by electrophoresis on a DNA sequencing gel. The complex showed protection from positions −77 to −54 (Fig. 1B). Interestingly, this region is adjacent to a site spanning positions −101 to −75 that is bound by FruA (38). Since FruA and MrpC2 bind cooperatively to adjacent sites in the promoter regions of several *M. xanthus* genes (34, 39–41), we hypothesized that MrpC2 in the AS fraction was binding to the region spanning positions −77 to −54.

MrpC and FruA associate with the *dev* promoter region *in vivo*. To test whether MrpC and FruA associate with the *dev* promoter region in developing *M. xanthus* cells, we performed ChIP assays with polyclonal antibodies to MrpC, which also recognize MrpC2 (45), and polyclonal antibodies to FruA. Cells that had been developing for 18 h, when both transcription factors are known to be expressed, were subjected to ChIP with affinity-purified anti-MrpC immunoglobulin G (IgG) (or IgG from a non-immunized rabbit as a control) or anti-FruA serum (or preimmune serum from the same rabbit as a control). The ChIP DNA was analyzed by PCR with primers designed to amplify the *dev* promoter region (positions −119 to −19) or, as a control, the *rpoC* coding region (positions +1780 to +1905) (37). PCR analysis showed enrichment of the *dev* promoter region by ChIP with anti-MrpC compared to control IgG (Fig. 2A, lanes 5 and 6) and

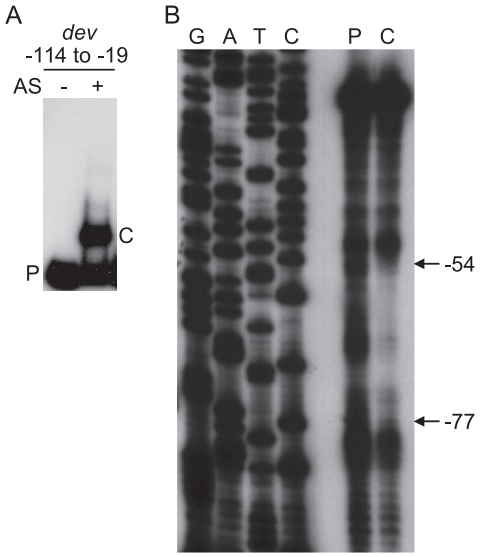

FIG 1 Binding of a protein in a fraction from developing *M. xanthus* cells to the *dev* promoter region. (A) EMSAs. A ^{32}P-labeled DNA probe (2 nM) spanning positions −114 to −19 of the *dev* promoter region was incubated alone or with proteins in the AS fraction (0.7 μg/μl) and subjected to EMSAs. The unbound probe (P) and the shifted complex (C) produced by the AS fraction are indicated. (B) Footprinting. The unbound probe (P) and the shifted complex (C) from a gel like that shown in panel A were subjected to footprint analysis with 1,10-phenanthroline-copper. Lanes G, A, T, and C show sequence ladders generated by primer LK1331.

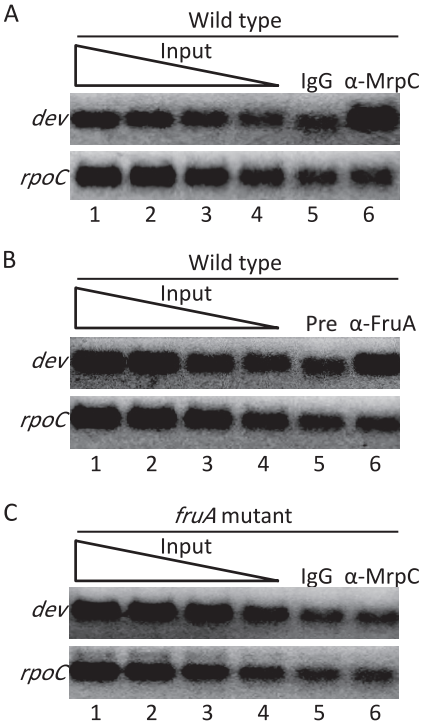

FIG 2 Association of MrpC and FruA with the *dev* promoter region in developing *M. xanthus* cells. Eighteen hours into development, cells were subjected to ChIP analysis. DNA was amplified with primers for the *dev* promoter region spanning positions −114 to −19 or for the *rpoC* coding region spanning positions +1780 to +1905 as a control. A 2-fold dilution series of input DNA (lanes 1 to 4 in each panel) was used as a template in parallel PCRs to show that the PCR conditions allow detection of differences in DNA concentrations for each primer set. (A) Wild-type strain DK1622 with affinity-purified IgG antibodies against MrpC (α-MrpC) or, as a control, with total IgG (IgG) from nonimmunized rabbits. (B) Wild-type strain DK1622 with antiserum against FruA (α-FruA) or, as a control, preimmune antiserum (Pre). (C) *fruA* mutant strain DK5285 with antibodies as described above for panel A.

with anti-FruA compared to control preimmune serum (Fig. 2B, lanes 5 and 6). Similar results were observed in two additional experiments. As expected, PCR analysis with primers designed to amplify the *rpoC* coding region showed no enrichment by ChIP with anti-MrpC or anti-FruA compared to the controls. We conclude that MrpC and/or MrpC2 and FruA associate with the *dev* promoter 18 h into development.

Association of MrpC and/or MrpC2 with the *dev* promoter region requires FruA. It was shown previously that the association of MrpC and/or MrpC2 with the *fmgA* (34), *fmgBC* (40), and *fmgE* (41) promoter regions depends on FruA. This appears to be due to cooperative binding of the two proteins just upstream of the promoters, based on *in vitro* DNA-binding studies. To test whether the association of MrpC and/or MrpC2 with the *dev* promoter region requires FruA, we performed ChIP-PCR analysis of a *fruA* mutant at 18 h of development. PCR analysis showed no enrichment of the *dev* promoter region by ChIP with anti-MrpC compared to control IgG (Fig. 2C, lanes 5 and 6). Similar results were observed in two additional experiments. Likewise, there was no enrichment of the *rpoC* coding region compared to the control, as expected. Taken together, the results of our ChIP-PCR analysis show that the association of MrpC and/or MrpC2 with the *dev* promoter region 18 h into development requires FruA. These results suggest that FruA binds cooperatively with MrpC and/or MrpC2 to the *dev* promoter region.

MrpC2 and FruA bind to the *dev* promoter region *in vitro*. Purified His_{10}-MrpC2 and FruA-His_6 were tested for binding to a ^{32}P-labeled DNA fragment spanning positions −114 to −19 of the *dev* promoter region in EMSAs. His_{10}-MrpC2 produced two shifted complexes, one more abundant and faster migrating than the other (Fig. 3, lane 2). The slower-migrating complex presumably has more than one His_{10}-MrpC2 bound. FruA-His_6 produced a single shifted complex (Fig. 3, lane 3). Together, the two proteins produced a single shifted complex that migrated more slowly and was more abundant (lane 4) than the complex produced by FruA-His_6 alone or the faster-migrating complex produced by His_{10}-MrpC2 alone. This pattern was shown previously to be indicative of cooperative binding (34).

To test the hypothesis mentioned above, that MrpC2 binds to the region spanning positions −77 to −54 of the *dev* promoter region, we made several mutations between positions −74 and −39 (Fig. 3) and measured the effects on the binding of His_{10}-MrpC2 and FruA-His_6 in EMSAs. The mutations were multiple-base-pair transversions that dramatically altered the sequence. Mutations M1 and M3 have changes at the ends of a sequence ($CGTCN_8AAC$, on the opposite strand of the one shown in bold italic type in Fig. 3) very similar to a consensus sequence for MrpC binding ($TGTYN_8RAC$). The consensus sequence was identified by bioinformatic analysis of sequences bound by MrpC, as determined by chromatin immunoprecipitation followed by DNA sequencing (ChIP-seq) of *M. xanthus* that had formed nascent fruiting bodies (62). Mutations M2A and M2B have changes in the middle (i.e., the nonspecific N_8 part) of the sequence matching the consensus. Mutation M1 eliminated detectable binding of His_{10}-

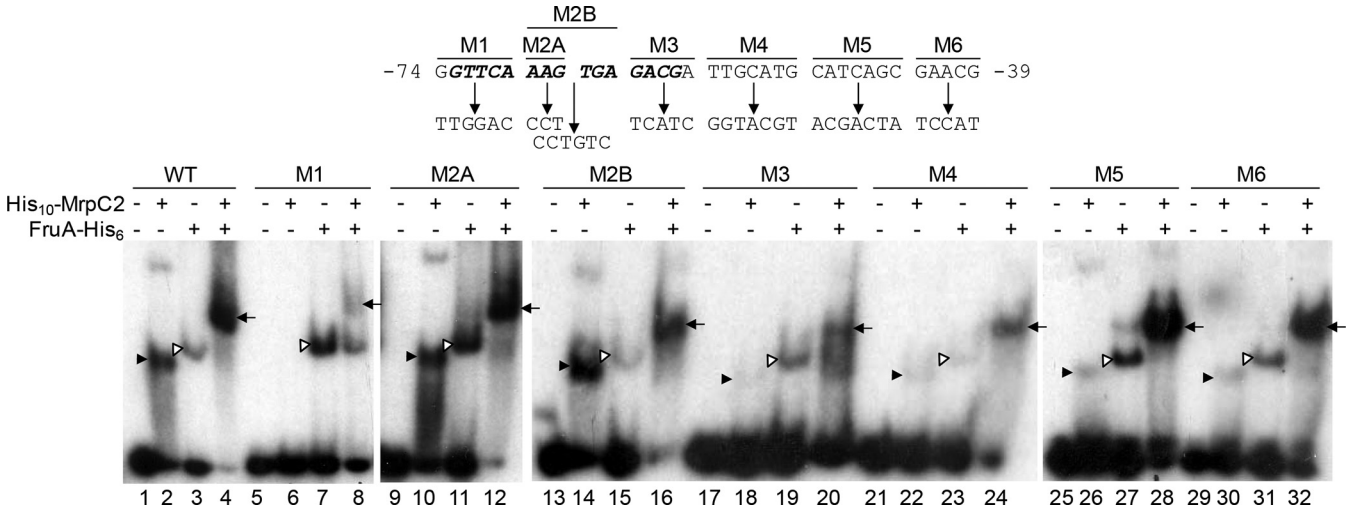

FIG 3 Binding of MrpC2 and FruA to the *dev* promoter region. (A) Mutations made in the *dev* promoter region between positions −74 and −39. The name of each multiple-base-pair transversion is shown at the top. Boldface italic type indicates the complement of the sequence $CGTCN_8AAC$, which best matches the MrpC2 consensus binding sequence $TGTYN_8RAC$ (62). (B) EMSAs with ^{32}P-labeled *dev* DNA (2 nM) spanning positions −114 to −19 and His_{10}-MrpC2 (0.13 μM), FruA-His_6 (3 μM), or both His_{10}-MrpC2 (0.13 μM) and FruA-His_6 (3 μM), as indicated. The probe DNA had the wild-type (WT) sequence or the indicated mutation. Filled arrowheads point to shifted complexes produced by His_{10}-MrpC2 alone, open arrowheads point to complexes produced by FruA-His_6 alone, and arrows point to complexes indicative of cooperative binding of the two proteins.

MrpC2 (Fig. 3, lane 6), and mutations M3 (lane 18), M4 (lane 22), M5 (lane 26), and M6 (lane 30) greatly diminished binding. These results demonstrate that His_{10}-MrpC2 binds to the sequence between positions −74 and −39 in the *dev* promoter region. Mutations M2A (Fig. 3, lane 10) and M2B (lane 14) did not impair His_{10}-MrpC2 binding, nor did mutations M2A (lane 12) and M2B (lane 16) impair the formation of the slower-migrating complex indicative of cooperative binding of His_{10}-MrpC2 and FruA-His_6. In contrast, mutations M1 (Fig. 3, lane 8) and M3 (lane 20) reduced the formation of the slower-migrating complex. The ability of mutation M1 to form a small amount of this complex suggests that His_{10}-MrpC2 can bind in the presence of FruA-His_6, even though His_{10}-MrpC2 alone did not bind detectably (Fig. 3, lane 6). Interestingly, mutations M4 (Fig. 3, lane 24), M5 (lane 28), and M6 (lane 32) formed considerable amounts of the slower-migrating complex, leaving little of the probe unbound and very little of the probe bound by just one of the proteins, as if cooperative binding occurred but not to the extent observed for the wild-type DNA sequence (Fig. 3, lane 4). Altogether, our results indicate that FruA binds cooperatively with MrpC and/or MrpC2 to sequences located immediately upstream of the *dev* promoter, with MrpC and/or MrpC2 being proximal to the promoter and MrpC2 very likely accounting for the binding of a protein in the AS fraction to the *dev* promoter region (Fig. 1).

Mutations in the MrpC2-binding site abolish *dev* expression. To test the effects of the mutations described above on *dev* expression *in vivo*, we constructed *M. xanthus* strains with mutant promoter regions transcriptionally fused to the *E. coli lacZ* reporter gene and measured β-galactosidase specific activity during development. As shown previously (38), the wild-type *dev* promoter region spanning positions −114 to +581 fused to *lacZ* and integrated ectopically in a *devS*-null mutant (to relieve negative autoregulation) resulted in increasing expression by 12 h into development that reached ~1,000 units by 48 h (Fig. 4). Likewise, the corresponding promoter region with the M2B mutation, which

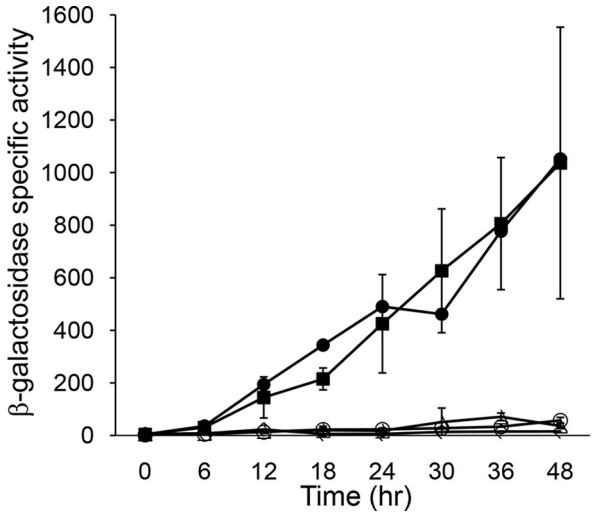

FIG 4 Effects of mutations in the MrpC2-binding site on developmental *dev-lacZ* expression. β-Galactosidase specific activity during development was measured for *lacZ* fused to *dev* spanning positions −114 to +581. Fragments were inserted into pREG1727 to create *lacZ* fusions, and the resulting plasmids were transformed into *M. xanthus* Δ*devS* mutant strain DK11209. Expression from transformants bearing the wild-type promoter (●) or the M2B (■), M5 (△), or M3 (○) mutant promoter region was measured, as was that from the M1, M4, and M6 mutant promoter regions, which is not shown since it was indistinguishable from the negative control. As a negative control, expression from a fragment spanning positions −934 to +581 with a mutation in the −35 region of the *dev* promoter that abolishes activity (◇) was likewise measured. The units of activity are nanomoles of *o*-nitrophenyl phosphate per minute per milligram of protein. For the mutant promoter regions, points show average values for three independent transformants, and error bars represent 1 standard deviation from the mean. Expression from the wild-type promoter and the negative control was measured once and was consistent with results reported previously (8, 38).

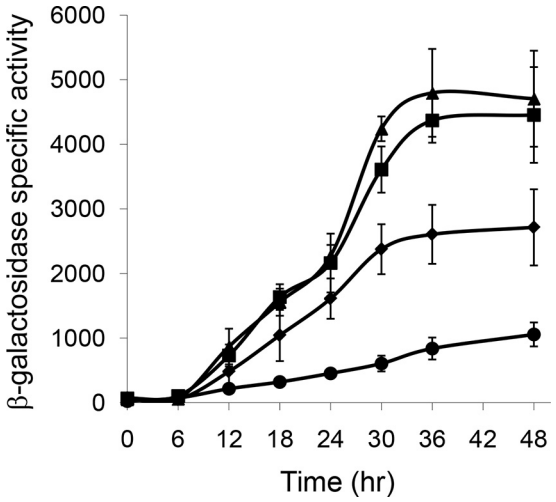

FIG 5 Effects of 5′ deletions on developmental *dev-lacZ* expression. Fragments spanning positions −934 (◆), −535 (■), −321 (▲), or −114 (●) to +71 were inserted into pREG1727, the resulting plasmids were transformed into *M. xanthus* Δ*devS* mutant strain DK11209, and β-galactosidase specific activity during development was determined for three independent transformants. The units of activity are nanomoles of *o*-nitrophenyl phosphate per minute per milligram of protein. Points show average values, and error bars represent 1 standard deviation from the mean.

did not impair His_{10}-MrpC2 binding *in vitro* (Fig. 3, lane 14), resulted in similar developmental *lacZ* expression (Fig. 4). In contrast, the M1, M3, M4, M5, and M6 mutations that impaired His_{10}-MrpC2 binding *in vitro* (Fig. 3) abolished *dev* expression *in vivo* (Fig. 4). Although these mutations appeared to allow cooperative binding of His_{10}-MrpC2 and FruA-His_6 *in vitro* to some extent (Fig. 3), the binding in developing cells was apparently insufficient to activate transcription from the *dev* promoter (Fig. 4). As a negative control, a mutation in the −35 region of the *dev* promoter abolished *dev* expression (Fig. 4), as expected (8). We conclude that high-affinity, cooperative binding of MrpC and/or MrpC2 with FruA to a site immediately upstream of the *dev* promoter is crucial to activate transcription of the *dev* operon during development.

Localization of a second positive *cis*-regulatory element. A series of 5′ deletions with upstream ends located at position −934, −535, or −114 and the same downstream end at position +834 fused to *lacZ* showed a graded loss of *dev* promoter activity in previous work (8). These results implied that additional positive *cis*-regulatory elements lie upstream of position −114. However, other results suggested that upstream and downstream regulatory elements functionally interact (8). To minimize such interactions and to further localize upstream regulatory elements, a series of 5′ deletions with upstream ends at position −934, −535, −321, or −114 and the same downstream end at position +71 fused to *lacZ* was constructed. These *dev-lacZ* fusions were integrated ectopically into *M. xanthus* with a *devS*-null mutation, and developmental *lacZ* expression was measured. Surprisingly, the fusions with upstream ends at position −535 or −321 exhibited higher expression levels during development than the fusion with its upstream end at position −934 (Fig. 5). This suggests that a negative regulatory element lies between positions −934 and −535. The expression level from the fusion with its upstream end at position −114 was lower than the expression level from the fusions with upstream ends at position −934 or −535 (Fig. 5), as observed previously for fusions with their downstream end at position +834 (8). The much higher expression level from the fusion with its upstream end at position −321 than from the fusion with its upstream end at position −114 (Fig. 5) suggests that a strong positive *cis*-regulatory element lies between positions −321 and −114. We further characterized this regulatory element, as described below.

MrpC2 and FruA bind to a second site upstream of the *dev* promoter. Since MrpC and/or MrpC2 binds cooperatively with FruA immediately upstream of the *dev* promoter to activate transcription, as described above, and since these transcription factors bind in the vicinity of other promoters in various arrangements to regulate their transcription (34, 39–41), we tested whether His_{10}-MrpC2 and FruA-His_6 bind to two overlapping DNA probes spanning positions −329 to −176 and positions −202 to −100 upstream of the *dev* promoter, which covers the region corresponding to the second positive *cis*-regulatory element mentioned above plus a few base pairs on each end in case a binding site is close to an end. His_{10}-MrpC2 produced a single shifted complex with the probe spanning positions −329 to −176 (Fig. 6, lane 2) but not with the probe spanning positions −202 to −100 (Fig. 6, compare lanes 5 and 6) (note that this probe was contaminated with a species migrating more slowly, but there was no difference when His_{10}-MrpC2 was added). FruA-His_6 did not bind detectably to either probe (Fig. 6, lanes 3 and 7); however, in combination with His_{10}-MrpC2, FruA-His_6 appeared to enhance the formation of a shifted complex with the probe spanning positions −329 to −176, although little if any of the complex migrated more slowly than the complex produced by His_{10}-MrpC2 alone (Fig. 6, compare lanes 2 and 4). Because these results suggested that there might be weak cooperative binding of the two proteins to the probe spanning positions −329 to −176, we repeated the experiment with slightly more concentrated probe and slightly less FruA-His_6. Under these conditions, FruA-His_6 alone produced a shifted complex that was barely detectable (Fig. 6, lane 11), and in combination with His_{10}-MrpC2, the two proteins produced two shifted complexes (Fig. 6, lane 12). The amount of the two shifted complexes was slightly larger than expected from the binding of the two proteins individually, indicative of weak cooperative binding. A probe spanning positions −254 to −176 exhibited a similar pattern of binding (Fig. 6, lanes 13 to 16). In contrast, a probe spanning residues −229 to −176 showed no detectable binding by FruA-His_6 and no indication of cooperative binding, although it was bound by His_{10}-MrpC2 (Fig. 6, lanes 17 to 22). Because we were unable to prepare this probe at a high concentration, we cannot be certain whether FruA-His_6 alone is able to bind, but it did not enhance complex formation in combination with His_{10}-MrpC2, as we had observed with the probe spanning positions −329 to −176 at a lower concentration (Fig. 6, compare lanes 4 and 22). Therefore, we conclude that DNA between positions −254 and −229 in the *dev* promoter region is necessary for the weak cooperative binding of FruA and MrpC2 to a site located between positions −254 and −176, with MrpC2 binding being proximal to the promoter.

The second site of MrpC2 and FruA binding is important for *dev* expression. To test whether DNA between positions −254 and −229 contributes to *dev* transcription *in vivo*, 5′ deletions with upstream ends at position −254 or −229 and the same downstream end at position +71 fused to *lacZ* were constructed

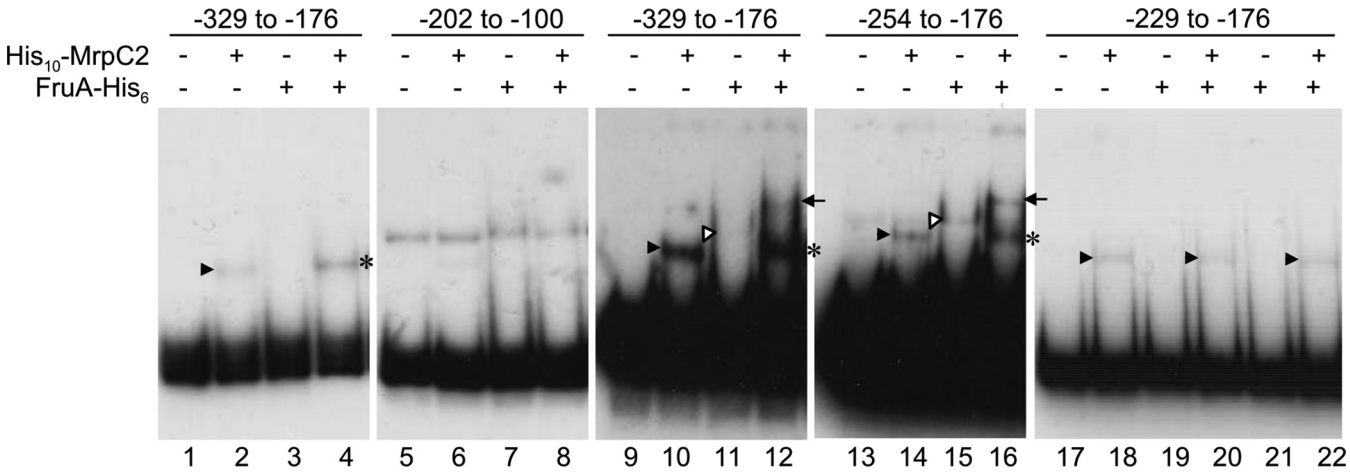

FIG 6 Binding of MrpC2 and FruA to a second site in the *dev* promoter region. Shown are results of EMSAs with ^{32}P-labeled *dev* DNA spanning the indicated positions with His_{10}-MrpC2 (1 μM) and/or FruA-His_6, as indicated. The probe DNA concentration was 2 nM (lanes 1 to 8 and 17 to 22) or 6 nM (lanes 9 to 12). The FruA-His_6 concentration was 3 μM (lanes 3, 4, 7, 8, and 19 to 22) or 1.5 μM (lanes 11, 12, 15, and 16). Filled arrowheads point to shifted complexes produced by His_{10}-MrpC2 alone, open arrowheads point to complexes produced by FruA-His_6 alone, arrows point to complexes indicative of cooperative binding of the two proteins, and asterisks indicate FruA-His_6-dependent enhancement of complexes that comigrate with those produced by His_{10}-MrpC2 alone.

and integrated ectopically into *M. xanthus* with a *devS*-null mutation, and developmental *lacZ* expression was measured. The expression level from the fusion with its upstream end at position −254 was only slightly lower than the expression level from the fusion with its upstream end at position −321, which was measured as a control (Fig. 7). Interestingly, the expression level from the fusion with its upstream end at position −229 increased similarly during the first 24 h of development, then stopped increasing until 36 h, and finally rose slightly by 48 h, reaching about half the level observed for the fusion with its upstream end at position −254. As a control, expression from the fusion with its upstream end at position −114 was measured in parallel. The expression level increased less rapidly during the first 24 h but continued to rise later in development, reaching a level by 48 h slightly below that observed for the fusion with its upstream end at position −229. Taken together, these results demonstrate that the region between positions −254 and −229 of the *dev* promoter region, which is necessary for weak cooperative binding of MrpC2 and FruA *in vitro* (Fig. 6), is important for the normal pattern of *dev* expression *in vivo*, particularly after 24 h of development (Fig. 7). Additional *cis*-regulatory elements between positions −114 and −229 appear to boost *dev* expression during the first 24 h of development, and elements between positions −254 and −321 appear to boost expression later during development.

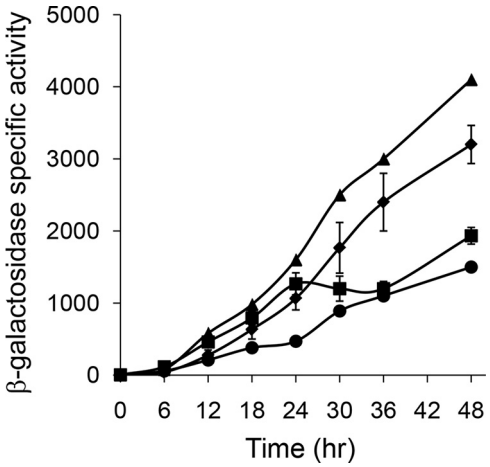

FIG 7 Effects of 5′ deletions to position −254 or −229 on developmental *dev-lacZ* expression. Fragments spanning positions −321 (▲), −254 (◆), −229 (■), or −114 (●) to +71 were inserted into pREG1727, the resulting plasmids were transformed into *M. xanthus* Δ*devS* mutant strain DK11209, and β-galactosidase specific activity during development was determined. The units of activity are nanomoles of *o*-nitrophenyl phosphate per minute per milligram of protein. For the 5′ deletions to positions −254 and −229, points show average values for three independent transformants, and error bars represent 1 standard deviation from the mean. Expression from the 5′ deletions to positions −321 and −114 was measured once and was consistent with the results shown in Fig. 5.

Deletion of the distal upstream site of MrpC2 and FruA binding does not impair spore formation. The period between 24 and 30 h poststarvation is crucial for commitment to sporulation during *M. xanthus* development (50). Since the distal upstream site of MrpC2 and FruA binding, located at least in part between positions −254 and −229, boosts *dev* expression at 24 to 36 h of development (Fig. 7), we hypothesized that this *cis*-regulatory element is important for commitment to sporulation. To test this hypothesis, we deleted DNA between positions −254 and −228 in the *M. xanthus* chromosome, which also creates a small in-frame deletion in the upstream gene MXAN_7267. We derived three such strains from wild-type strain DK1622 and measured sonication-resistant spore formation at 24, 30, 36, 48, and 72 h poststarvation, with strain DK1622 as a control. We observed no significant difference between the deletion and control strains, and the results were very similar to those described previously for strain DK1622 (50). There was also no significant difference in the number of mature spores (i.e., heat- and sonication-resistant spores capable of germination) at 72 h poststarvation. We conclude that the distal upstream site of MrpC2 and FruA binding is not required for sporulation. Together with our results shown in Fig. 7, this suggests that a boost in *dev* expression at 24 to 36 h is not

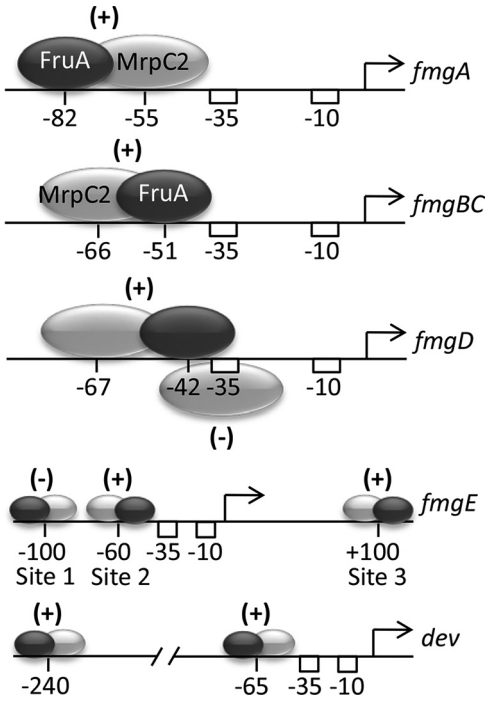

FIG 8 Arrangements of MrpC2- and FruA-binding sites in promoter regions of C-signal-dependent genes. The approximate positions of His_{10}-MrpC2 (light gray ovals) and FruA-His_6 (dark gray ovals) binding *in vitro* are shown, based on footprinting and the effects of mutations in DNA probes in EMSAs for *fmgA* (34, 37) and *dev* (38) (Fig. 1 and 3) or just on the effects of mutations in DNA probes in EMSAs for *fmgBC*, *fmgD*, and *fmgE* (39–41). (+) and (−) indicate positive and negative effects on promoter activity, respectively, as inferred from expression of *lacZ* fusions in promoter regions with mutations in transcription factor-binding sites (38, 53, 56–58) (Fig. 4). A larger region is shown for the *fmgE* and *dev* promoter regions.

important for commitment to sporulation, at least under laboratory conditions.

DISCUSSION

Our results show that the regulation of a key developmental operon is under combinatorial control of MrpC2 and FruA, which appear to bind cooperatively to two sites upstream of the *dev* promoter and contribute to positive regulation. Below, we discuss the implications of our findings related to this novel arrangement of MrpC2- and FruA-binding sites.

Cooperative binding of MrpC2 and FruA immediately upstream of the *dev* promoter is crucial for expression. A common theme among several C-signal-dependent genes studied previously is that MrpC2 and FruA bind cooperatively just upstream of the promoter (34, 39–41) (Fig. 8). At this location, either protein may bind proximal to the promoter. Immediately upstream of the *dev* promoter, MrpC2 and FruA bind cooperatively, with MrpC2 being proximal to the promoter, as in the *fmgA* promoter region. Cooperative binding to the *dev* promoter region is supported by our *in vivo* and *in vitro* data. We found that the association of MrpC and/or MrpC2 with the *dev* promoter region *in vivo* requires FruA by performing ChIP-PCR analysis of a *fruA* mutant (Fig. 2C). We did not perform a similar analysis of an *mrpC* mutant since *fruA* is not transcribed in such a mutant (42). Our evidence for the cooperative binding of MrpC2 and FruA *in vitro* is based on the migration and abundance of shifted complexes produced in EMSAs (Fig. 3, lanes 2 to 4). This pattern of shifted complexes was initially observed for the *fmgA* promoter region, and cooperative binding was demonstrated by using DNase I footprinting (34). A similar pattern of shifted complexes in EMSAs provided evidence for cooperative binding just upstream of the *fmgBC*, *fmgD*, and *fmgE* promoters (39–41). In these cases, FruA binds proximal to the promoter (Fig. 8).

Mutations that reduce or eliminate binding *in vitro* of MrpC2 or FruA to their sites near the *dev* promoter impair or abolish promoter activity *in vivo*. This was shown previously for the FruA DNA-binding domain and mutations in the region between positions −101 and −75 (38) and was shown here for MrpC2 and mutations in the region between positions −74 and −39 (Fig. 3 and 4). We conclude that cooperative binding of MrpC2 and FruA immediately upstream of the *dev* promoter is crucial for expression. Presumably, the transcription factors recruit RNA polymerase to the promoter and/or facilitate a subsequent step in transcription initiation.

Interestingly, mutations M1 and M3, which strongly impair MrpC2 binding and cooperative binding *in vitro*, have changes at the ends of a sequence very similar to a consensus sequence for MrpC binding, whereas mutations M2A and M2B, which did not impair MrpC2 or cooperative binding, have changes in the middle (i.e., the nonspecific N_8 part) of the sequence matching the consensus (Fig. 3). Mutations M4, M5, and M6 had less of an impact on MrpC2 and cooperative binding than did mutations M1 and M3 (Fig. 3), yet all five mutations abolished *dev* expression *in vivo* (Fig. 4). A plausible explanation for this finding is that the binding reaction conditions *in vitro* (i.e., purified proteins and DNA) do not fully reflect the conditions present *in vivo* (e.g., many potential binding sites competing for binding of the proteins).

Why is the expression of the *dev* operon and several other C-signal-dependent genes under the combinatorial control of MrpC2 and FruA? Since FruA responds to C signaling, which appears to increase as cells become aligned in nascent fruiting bodies, FruA may communicate positional information to the gene regulatory network. Since several mechanisms link MrpC and MrpC2 levels to starvation, it was proposed previously that the abundance of these proteins is responsive to nutrient conditions as development proceeds (34). In agreement with that proposal, it was found that MrpC and MrpC2 are highly sensitive to nutrient-regulated proteolysis both before and during a critical period of commitment to sporulation at ~24 to 30 h poststarvation (50). The addition of nutrients halted the expression of the *dev* operon. Hence, combinatorial regulation appears to ensure that the *dev* operon and other C-signal-dependent genes are fully expressed, committing cells to form spores, only if cells are both starving (to allow sufficient MrpC and MrpC2 activity) and aligned within a nascent fruiting body (to allow efficient C signaling and therefore sufficient FruA activity).

Binding of MrpC2 and FruA to a distal upstream site boosts *dev* expression during the critical period of commitment to sporulation. Our findings imply that the expression of the *dev* operon is subject to a novel regulatory strategy compared with other C-signal-dependent genes studied so far. We found that a second *cis*-regulatory element located further upstream of the *dev* promoter corresponds to a site of weak cooperative binding of MrpC2 and FruA and contributes positively to *dev* expression after 24 h of development. This is a more complex arrangement of cooperative

binding sites than those identified in the *fmgA* (34) and *fmgBC* (40) promoter regions, where a single cooperative binding site just upstream of the promoter was found (Fig. 8). Expression from these promoters depends in part on C signaling, as does the expression of the *dev* operon (4), but 5′ deletion analysis indicated additional complexity in the regulation of the *dev* promoter (8). Two positive *cis*-regulatory elements were inferred, one between positions −934 and −535 and the other between positions −535 and −114, both upstream of the promoter-proximal cooperative binding site for MrpC2 and FruA discussed above. This type of complexity has not been observed for the promoter regions of other C-signal-dependent genes examined so far, although two genes that depend completely on C signaling for expression appear to have more complex regulatory strategies than those of *fmgA* and *fmgBC*. The *fmgD* promoter region has two MrpC2-binding sites (39) (Fig. 8). The downstream MrpC2 site overlaps the FruA site and the promoter. Mutational analysis supports a model in which the two transcription factors compete for binding, resulting in repression when two MrpC2s bind cooperatively (early in development) and in activation when FruA binds cooperatively with upstream MrpC2 (late in development, as C signaling causes the concentration of active FruA to increase). The *fmgE* promoter region has three sites where MrpC2 and FruA bind cooperatively (41) (Fig. 8). Site 1, centered at about position −100, mediates negative regulation, whereas site 2, immediately upstream of the promoter, and site 3, at about position +100, mediate positive regulation. The relative binding affinities support a model in which site 3 recruits MrpC2 and FruA to the promoter region, site 1 competes with site 2 for binding of the two proteins, and binding to site 2 is required to activate the promoter, which occurs only as C signaling produces a high concentration of active FruA late in development. Regulation of the *dev* operon might also rely on differential affinities of binding of MrpC2 and FruA to cooperative binding sites. Binding to the site immediately upstream of the promoter was stronger *in vitro* than binding to the distal upstream site (Fig. 3 and 6). Perhaps the proximal upstream site can be occupied at relatively low MrpC2 and FruA concentrations *in vivo*, accounting for expression early in development that does not depend on C signaling to increase the concentration of active FruA. We propose that the occupancy of the distal upstream site requires a higher concentration of active FruA, produced in response to efficient C signaling as cells become aligned in nascent fruiting bodies and accounting for the boost in *dev* expression observed after 24 h of development. Since the boost in *dev* expression coincides with the critical period of commitment to sporulation mentioned above (50), we hypothesized that increased *dev* expression is important for sporulation. However, we found that deletion of the distal upstream region of MrpC2 and FruA binding did not impair spore formation under laboratory conditions. Perhaps under other conditions, the boost in *dev* expression brought about by the distal upstream site is important for sporulation.

In addition to the two upstream cooperative binding sites for MrpC2 and FruA characterized here, regulation of the *dev* operon also involves downstream elements. A positive regulatory element located at about position +350 is bound by LadA, a LysR-type transcription factor (38). Two other positive regulatory elements and one negative regulatory element are inferred from 3′ deletion analysis, and several results suggest that downstream regulatory elements interact functionally with upstream elements (8). DNA looping was proposed to explain the apparent long-range interactions. It seems likely that DNA looping also allows MrpC2 and FruA bound to the distal upstream site to activate transcription from the *dev* promoter, as looping often explains how transcription factors exert effects from promoter-distal sites (55). In any case, the identification of the distal upstream site was facilitated here by performance of a 5′ deletion analysis with *lacZ* fusions containing very little DNA downstream of the *dev* promoter, to eliminate potential long-range interactions between upstream and downstream regulatory elements. The effect of DNA between positions −934 and −535 was negative for fusions ending at position +71 (Fig. 5) but positive for fusions ending at position +834 (8), supporting the notion of long-range interactions in the latter case. Although some mysteries remain about regulation of the *dev* operon, our results show that combinatorial regulation by MrpC2 and FruA is an important aspect.

Combinatorial regulation by MrpC2 and FruA appears to be used widely during *M. xanthus* development. In addition to the *dev* promoter and the four *fmg* promoters mentioned above, ChIP-seq analysis of cells that had formed nascent fruiting bodies revealed 1,608 putative MrpC-binding sites, and when 15 of these were tested for cooperative binding of MrpC2 and FruA, there was evidence for cooperative binding in 13 cases (62). These include sites near the 5′ ends of genes important for development, such as genes that code for protein kinases or transcription factors, and genes involved in signal production, spore formation, and motility.

Combinatorial regulation by MrpC2 and FruA is also versatile. Depending on the arrangement and affinities of binding sites for MrpC2 and FruA, a variety of regulatory patterns can be achieved in terms of the strength and timing of expression. Our analysis of *dev* regulation provides the first example of a distal upstream cooperative binding site for MrpC2 and FruA that boosts expression late in development.

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