Combinatorial Regulation of *fmgD* by MrpC2 and FruA during *Myxococcus xanthus* Development[▽]

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Upon starvation, a dense population of rod-shaped *Myxococcus xanthus* bacteria coordinate their movements to construct mounds in which some of the cells differentiate to spherical spores. During this process of fruiting body formation, short-range C-signaling between cells regulates their movements and the expression of genes important for sporulation. C-signaling activates FruA, a transcription factor that binds cooperatively with another transcription factor, MrpC2, upstream of the *fmgA* and *fmgBC* promoters, activating transcription. We have found that a third C-signal-dependent gene, herein named *fmgD*, is subject to combinatorial control by FruA and MrpC2. The two proteins appear to bind cooperatively upstream of the *fmgD* promoter and activate transcription. FruA binds proximal to the *fmgD* promoter, as in the *fmgBC* promoter region, whereas MrpC2 binds proximal to the *fmgA* promoter. A novel feature of the *fmgD* promoter region is the presence of a second MrpC2 binding site partially overlapping the promoter and therefore likely to mediate repression. The downstream MrpC2 site appears to overlap the FruA site, so the two transcription factors may compete for binding, which in both cases appears to be cooperative with MrpC2 at the upstream site. We propose that binding of MrpC2 to the downstream site represses *fmgD* transcription until C-signaling causes the concentration of active FruA to increase sufficiently to outcompete the downstream MrpC2 for cooperative binding with the upstream MrpC2. This would explain why *fmgD* transcription begins later during development and is more dependent on C-signaling than transcription of *fmgA* and *fmgBC*.

Myxococcus xanthus is a Gram-negative soil bacterium that undergoes multicellular development when starved, providing an attractive model to study signaling and gene regulatory mechanisms (49). Cells glide in swarms over solid surfaces, seeking prey bacteria on which to feed (3). When nutrients become limited, cells alter their movements, including the frequency with which they reverse their direction of gliding (15). Approximately 10^5 cells coordinate their movements to construct a mound-shaped fruiting body. A majority of the cells undergo programmed cell death during the developmental process, but some cells in the fruiting body differentiate into dormant, stress-resistant, spherical spores (30). The mature fruiting body is a dense mound of spores capable of germinating in response to nutrients and producing a swarm of cells ready to feed, grow, and divide.

Three signals governing *M. xanthus* fruiting body development are fairly well understood. Starvation triggers a stringent response that leads to the production of intracellular (p)ppGpp and induction of early developmental genes (11, 41). The second signal, A-signal, is a mixture of amino acids and peptides produced extracellularly by the activity of secreted proteases (24, 34). A-signaling appears to allow cells to measure their density and, if sufficient, proceed into development by beginning to form mounds and expressing A-signal-dependent genes (23, 25). The third signal is also extracellular, but unlike the A-signal, which is diffusible, the C-signal is a short-range signal involving CsgA (40), a protein associated with the

outer membrane, where it is cleaved by a secreted protease to a 17-kDa form that appears to act as the signal (18, 27, 35), although a receptor remains to be identified. C-signaling is necessary for cells to build large, stable mounds, apparently because cells become aligned in the outer domain of a nascent fruiting body as they move in circles (16, 37) and efficient C-signaling between aligned cells (19) causes some to form spores, which are swept to the interior of the fruiting body by continued movement of cells in the outer domain (37). Many studies support a model in which an increasing level of C-signaling governs mound formation followed by sporulation within mounds to produce mature fruiting bodies (reviewed in references 13, 39, and 42). C-signaling is also necessary for normal developmental gene expression after the early mound stage (20). Genes that are normally expressed shortly after early mound formation exhibited reduced expression in a *csgA* mutant incapable of C-signaling, and genes that are normally expressed later failed to be expressed. For these two classes of genes, their differential timing of expression and level of dependence on C-signaling is likely related but is not understood.

Recently, an understanding of the regulation of genes in the early class that depends only in part on C-signaling has been achieved. Two transcription factors bind cooperatively upstream of the *fmgA* and *fmgBC* promoters and activate transcription (28, 29). The *fmg* designation stands for "FruA- and MrpC2-regulated gene." FruA is similar to response regulators of two-component signal transduction systems and has been proposed to be phosphorylated in response to C-signal (6, 33), but a cognate histidine kinase has not been identified, and some evidence suggests that FruA might function without being phosphorylated (28). MrpC is similar to the cyclic AMP receptor protein (CRP) family (43), and it is inhibited during

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growth by phosphorylation (31, 32). Compared with the unphosphorylated form, the phosphorylated form of MrpC binds weakly to DNA. Starvation inhibits phosphorylation of MrpC, and some of the unphosphorylated form may be cleaved to MrpC2, lacking the 25 N-terminal residues (46). MrpC2 binds better than MrpC to sites upstream of the *mrpC* and *fruA* promoters, activating transcription so that the concentrations of MrpC, MrpC2, and FruA increase in starving cells (32, 46). MrpC also binds to the *mazF* promoter region and activates expression of MazF, a toxic endoribonuclease that causes programmed cell death during development (30). Paradoxically, MrpC acts as an antitoxin by binding to MazF and inhibiting its activity. Presumably, this allows some cells in the population to escape programmed cell death and eventually form spores inside fruiting bodies. Binding of MrpC2 to MazF and to the *mazF* promoter region remains to be tested, as does binding of MrpC to the *fmgA* and *fmgBC* promoter regions. MrpC2 binds cooperatively with FruA upstream of the *fmgA* and *fmgBC* promoters but in different arrangements; MrpC2 binds proximal to the *fmgA* promoter, and FruA binds proximal to the *fmgBC* promoter (28, 29). Cooperative binding by the two transcription factors has been proposed to integrate starvation signaling and cell death through MrpC and MrpC2 with positional information via C-signaling through FruA, determining spatiotemporal gene expression and cell fate (28).

Here, we report that MrpC2 and FruA combinatorially regulate a gene in the late class that depends completely on C-signaling. The gene was identified by an insertion of the transposon Tn*5 lac* at the Ω4403 locus, because it created a transcriptional fusion to *lacZ*, and the *M. xanthus* strain exhibited developmentally regulated β-galactosidase activity (22). Developmental *lacZ* expression was abolished in a *csgA* mutant incapable of C-signaling (20). The gene at the Ω4403 locus (MXAN1501) (9) is predicted to code for a subtilisin-type serine protease, but the Tn*5 lac* insertion in the coding region caused no discernible growth or developmental defect (8, 22). DNA downstream of position −80 (relative to the transcriptional start site) was shown to be sufficient for C-signal-dependent activation of the promoter (8), and mutational analysis identified three positive *cis*-regulatory sequences upstream of the promoter (47). Two of the sequences, a 5-bp element (consensus sequence GAACA) at positions −63 to −59 and a C box (consensus sequence CAYYCCY, in which Y means C or T) at positions −52 to −46, are also present upstream of the *fmgA* and *fmgBC* promoters in regions important for promoter activity that are bound by MrpC2 and FruA (7, 28, 29, 50–52). Therefore, we tested whether MrpC2 and FruA bind upstream of the MXAN1501 promoter. The two proteins appear to bind cooperatively to DNA with FruA proximal to the promoter, as in the *fmgBC* promoter region, and this combination of proteins activates transcription, based on the effects of mutations upstream of the promoter that impair both DNA binding *in vitro* and promoter activity *in vivo*. Hence, we name the MXAN1501 gene *fmgD* (for FruA- and MrpC2-regulated gene D). Importantly, the *fmgD* promoter region contains a second MrpC2 binding site that partially overlaps the promoter and the FruA binding site. We propose that cooperative binding of MrpC2 to the two sites accounts for the differential timing and dependence on C-signaling of *fmgD* compared with those of *fmgA* and *fmgBC*.

MATERIALS AND METHODS

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

Growth and development. *M. xanthus* strains were grown at 32°C in CTT (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]) medium (12) or on CTT agar (1.5%) plates. When required, 40 μg kanamycin sulfate per ml was added. Fruiting body development was performed on TPM (10 mM Tris-HCl [pH 8.0], 1 mM KH_2PO_4-K_2HPO_4, 8 mM $MgSO_4$ [final pH 7.6]) agar (1.5%) plates as described previously (22).

Site-directed mutagenesis and construction of plasmids. The QuikChange site-directed mutagenesis kit (Stratagene) was used to create mutations in pMF0100 using primer pairs LK714 and LK715 or LK716 and LK717 (Table 2). Sequencing of the *M. xanthus* DNA portion of the plasmid was performed to ensure the presence of only the desired mutation. The BamHI-XhoI fragment bearing the mutant *fmgD* promoter region was gel purified and subcloned into BamHI-XhoI-digested pREG1727. *Escherichia coli* DH5α served as the host for plasmid construction and propagation.

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 (17). 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/ml, in order to avoid rare transformants with unusual developmental *lacZ* expression (47). Three transformants were chosen for further analysis, and β-galactosidase activity was measured as described previously (22).

ChIP. *M. xanthus* strains DK1622 and DK5285 were used for ChIP as described previously (28, 52), except Dynabeads protein G (Invitrogen) (100 μl/ml of cell extract) was used instead of protein A Sepharose beads for preclearing and immunoprecipitation. The primers used for PCR of the *fmgD* promoter region were LK1359 and LK1376, and the primers used for PCR of the *rpoC* coding region were LK1861 and LK1862 (Table 2).

Preparation of His_{10}-MrpC2 and FruA-His_6. Recombinant proteins were expressed in *E. coli* and purified as described previously (28, 32).

Preparation of DNA fragments. DNA fragments from the *fmgD* promoter region were generated by PCR using wild-type or mutant plasmid (Table 1) as the template and the oligonucleotide primers listed in Table 2. For electrophoretic mobility shift assays (EMSAs), ^{32}P-labeled DNA was synthesized by PCR after labeling the primers with [γ-^{32}P]ATP using T4 polynucleotide kinase (New England BioLabs), and the DNA fragment was purified after 15% PAGE (38). Alternatively, complementary primers were labeled with ^{32}P as just described, mixed, boiled for 10 min, and placed at room temperature for 3 h, and then the double-stranded DNA fragment was purified as just described.

EMSAs. EMSAs were performed as described previously (52), except that binding reaction mixtures were incubated at 25°C for 15 min.

RESULTS

Expression of *fmgD* depends on *fruA*. To determine whether FruA is involved in regulation of *fmgD*, expression from an *fmgD-lacZ* transcriptional fusion was measured during development of wild-type and *fruA* mutant *M. xanthus* strains. The *fmgD-lacZ* fusion was integrated at the Mx8 phage attachment site in the *M. xanthus* chromosome via site-specific recombination as described previously (8). Expression from the *fmgD* promoter was abolished in the *fruA* mutant (Fig. 1), indicating that FruA directly or indirectly regulates transcription of *fmgD*.

MrpC and FruA associate with the *fmgD* promoter region *in vivo*. To determine whether MrpC (and/or MrpC2) and FruA are associated with the *fmgD* promoter region *in vivo*, we performed ChIP assays with polyclonal antibodies against MrpC that also recognize MrpC2 and with polyclonal antibodies against FruA. Wild-type *M. xanthus* cells were collected after 18 h of development and subjected to ChIP with affinity-purified immunoglobulin G (IgG) antibodies against MrpC (or, as a control, with total IgG from nonimmunized rabbits) or with antiserum against FruA (or, as a control, preimmune antiserum). DNA recovered after ChIP was analyzed by PCR with primers

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
Strains		
E. coli		
DH5α	λ^- ϕ80d*lacZΔM15* Δ(*lacZYA-argF*)*U169 recA1 endA1 hsdR17*($r_K{}^-$ $m_K{}^-$) *supE44 thi-1 gyrA relA1*	10
BL21(DE3)	F^- *ompT hsdS*$_B$($r_B{}^-$ $m_B{}^-$) *gal dcm* with DE3, a λ prophage carrying the T7 RNA polymerase gene	Novagen
SMhisMrpC2	BL21(DE3) containing pET16b/His$_{10}$-MrpC2	28
SMFruAhis	BL21(DE3) containing pET11km/FruA-His$_6$	28
M. xanthus		
DK1622	Wild type	14
DK5285	*fruA*::Tn5 *lac* Ω4491	21
MPL1-6, -7, -8	*sglA1 attB*::pMF100	This work
MPL2-2, -7, -9	*sglA1 fruA*::TnV Ω786 *attB*::pMF100	This work
MPV4037-1, -5, -8	*attB*::pPV04037	This work
MPV4441-1, -2, -3	*attB*::pPV04441	This work
Plasmids		
pET16b/His$_{10}$-MrpC2	pET16b with a gene encoding His$_{10}$-MrpC2 under the control of a T7 RNA polymerase promoter	32
pET11km/FruA-His$_6$	pET11km with a gene encoding FruA-His$_6$ under the control of a T7 RNA polymerase promoter	S. Inouye
pREG1727	Ap^r Km^r P1-*inc attP ′lacZ*	8
pMF100	pREG1727 with *fmgD* DNA from positions -80 to $+382$	8
pGEM7Zf	Ap^r *lac*α	Promega
pMF0100	pGEM7Zf with *fmgD* DNA from positions -80 to $+382$	8
pPV74	pMF0100 with a G-to-T mutation at position -74	47
pPV4037	pMF0100 with a TTGA-to-GGTC mutation at positions -40 to -37	This work
pPV04037	pREG1727 with a 537-bp XhoI-BamHI fragment from pPV4037	This work
pPV4441	pMF0100 with a CGGG-to-ATTT mutation at positions -44 to -41	This work
pPV04441	pREG1727 with a 537-bp XhoI-BamHI fragment from pPV4441	This work

designed to amplify the *fmgD* promoter region or, as a control, the *rpoC* coding region. The PCR analysis showed that the *fmgD* promoter region, but not the *rpoC* coding region, was reproducibly enriched by ChIP with the anti-MrpC antibodies relative to the IgG control (Fig. 2A, lanes 5 and 6) and with the anti-FruA antibodies relative to the preimmune control (Fig. 2B, lanes 5 and 6). These results indicate that MrpC and/or MrpC2 and FruA are present in the vicinity of the *fmgD* promoter at 18 h into development, when expression of *fmgD-lacZ* was observed (Fig. 1).

Association of MrpC and/or MrpC2 with the *fmgA* and *fmgBC* promoter regions was dependent on FruA, presumably due to cooperative binding of the two proteins just upstream of the promoters (28, 29). To determine whether association of MrpC and/or MrpC2 with the *fmgD* promoter region depends on FruA, ChIP assays were performed on a *fruA* mutant at 18 h into development. Interestingly, the *fmgD* promoter region was reproducibly enriched by ChIP with the anti-MrpC antibodies relative to the IgG control (Fig. 2C, lanes 5 and 6). We conclude that MrpC and/or MrpC2 do not require FruA to associate with the *fmgD* promoter region, in contrast to the *fmgA* and *fmgBC* promoter regions.

MrpC2 and FruA bind cooperatively to the *fmgD* promoter region. Previous studies showed that MrpC2 and FruA bind cooperatively just upstream of the *fmgA* and *fmgBC* promoter regions (28, 29). To determine whether this is the case for the *fmgD* promoter region, EMSAs were performed with a DNA

fragment spanning from positions -80 to -16 and purified His$_{10}$-MrpC2 and FruA-His$_6$. His$_{10}$-MrpC2 produced two shifted species (Fig. 3, lane 2), suggesting that the fragment includes two binding sites (see below). We reasoned that the more abundant, lower species was a mixture of His$_{10}$-MrpC2 bound singly to one site, and the upper species was two molecules of His$_{10}$-MrpC2 bound to the DNA fragment (i.e., both sites occupied). FruA-His$_6$ produced a single shifted complex (Fig. 3, lane 3), indicative of a single binding site. The combination of proteins produced two shifted species (Fig. 3, lane 4). The total amount of shifted species was greater than expected for additive binding, a pattern shown previously to be indicative of cooperative binding (28). The upper species is presumably a mixture of DNA fragments with two sites occupied (one His$_{10}$-MrpC2 and one FruA-His$_6$, or two His$_{10}$-MrpC2), and the lower species is presumably a mixture of DNA fragments with one site occupied. Much more of the lower species is formed by the combination of His$_{10}$-MrpC2 and FruA-His$_6$ than by either protein alone, which might reflect initial cooperative binding of the two proteins followed by dissociation of FruA-His$_6$ (whose binding is weaker), as discussed previously for *fmgA* and *fmgBC* (28, 29). Occasionally, binding of both proteins may be lost during the electrophoresis, giving rise to the observed smear between the lower species and the unbound probe DNA fragment.

MrpC2 binds to sequences important for *fmgD* promoter activity. Mutational analysis of the *fmgD* promoter region pre-

TABLE 2. Primers used in this study

Primer	Sequence	Description[a] or reference
LK714	CACGGACCGCCGTCTCATCCCTCATTTTTGATTCATGAA TAAGCCG	−67 forward with a CGGG-to-ATTT mutation at −44 to −41
LK715	CGGCTTATTCATGAATCAAAAATGAGGGATGAGACGGC GGTCCGTG	−22 reverse, complement of LK714
LK716	GCCGTCTCATCCCTCCGGGGGTCTTCATGAATAAGCCG	−59 forward with a TTGA-to-GGTC mutation at −40 to −37
LK717	CGGCTTATTCATGAAGACCCCCGGAGGGATGAGACGGC	−22 reverse, complement of LK716
LK1359	CAAAAACGGCTTATTCATGAATC	−16 reverse
LK1376	CGCCATGGCATGTTCAATCA	−85 forward
LK1407	CTCGAGCAGCTGAAGCTGG	MCS of pPV74 plus −80 to −78 forward
LK1861	CCTTGAGCGCGATGGAGATA	52
LK1862	CTCGGCGGCCTCATCGAC	52
LK2104	TGGCATGTTCAATCACGGACCGCCGTCT	−80 forward
LK2105	AGACGGCGGTCCGTGATTGAACATGCCA	−53 reverse, complement of LK2104
LK2106	TCACGGACCGCCGTCTCATCCCTCCGGG	−68 forward
LK2107	CCCGGAGGGATGAGACGGCGGTCCGTGA	−41 reverse, complement of LK2106
LK2108	CGTCTCATCCCTCCGGGTTGATTCATGA	−57 forward
LK2109	TCATGAATCAACCCGGAGGGATGAGACG	−30 reverse, complement of LK2108
LK2304	TGGCAGTGGAAATCACGGACCGCCGTCT	−80 forward with a TGTTC-to-GTGGA mutation at −75 to −71
LK2305	AGACGGCGGTCCGTGATTTCCACTGCCA	−53 reverse, complement of LK2304
LK2306	TGGCATGTTCAATCACGTCAATCCGTCT	−80 forward with a GACCG-to-TCAAT mutation at −63 to −59
LK2307	AGACGGATTGACGTGATTGAACATGCCA	−53 reverse, complement of LK2306
LK2308	TGGCAGTGGAAATCACGTCAATCCGTCT	−80 forward with a TGTTC-to-GTGGA mutation at −75 to −71 and a GACCG-to-TCAAT mutation at −63 to −59
LK2309	AGACGGATTGACGTGATTTCCACTGCCA	−53 reverse, complement of LK2308
LK2446	CATCCCTCCGGGTTGATTCATGAATAAGCCGTTTTTG	−52 forward
LK2447	CAAAAACGGCTTATTCATGAATCAACCCGGAGGGATG	−16 reverse, complement of LK2446
LK2453	CAAAAACGGCTTATTCATGAAGA	−16 reverse for PCR with pPV4037 as template

[a] The number is relative to the start site of *fmgD* transcription, and the orientation (forward or reverse) is relative to the direction of *fmgD* transcription.
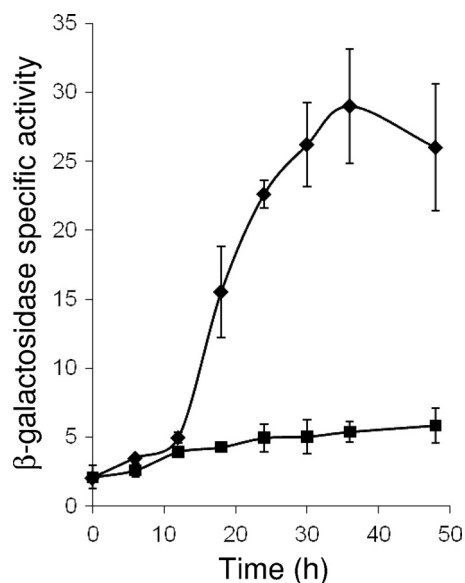

FIG. 1. Expression of *fmgD-lacZ* during development. β-galactosidase-specific activity during development was measured for *lacZ* fused to *fmgD* (positions −80 to +382) in the wild type (◆) or in a *fruA* mutant (■). The units of activity are nanomoles of *o*-nitrophenyl phosphate per minute per milligram of protein. Points show the averages of results from three transformants, and error bars depict one standard deviation of the data.

viously identified three positive *cis*-regulatory sequences, a 10-bp element at positions −79 to −70, a 5-bp element at positions −63 to −59, and a C box at positions −52 to −46 (Fig. 4A) (47). We note that within the 10-bp element, there is a sequence matching the 5-bp element consensus sequence (GAACA), on the other strand at positions −71 to −75. Sequences similar to the 5-bp element consensus sequence and the C box (consensus sequence CAYYCCY, in which Y means C or T) are present in the promoter regions of other C-signal-dependent genes (7, 50, 51) and have been shown to be important for the binding of MrpC2 and FruA in the *fmgA* and *fmgBC* promoter regions (28, 29, 52). To localize MrpC2 and FruA binding in the *fmgD* promoter region, three oligonucleotide pairs were used as probes in EMSAs. Each oligonucleotide pair forms a 28-bp duplex that was tested for binding of His$_{10}$-MrpC2, FruA-His$_6$, and the combination of proteins. His$_{10}$-MrpC2 produced a shifted complex only with DNA from positions −80 to −53 (Fig. 4A, lane 2), whereas FruA-His$_6$ produced a shifted complex only with DNA from positions −57 to −30 (Fig. 4A, lane 11). No enhancement of complex formation was observed with the combination of proteins, apparently because none of the 28-bp duplexes allows binding of both proteins. We conclude that MrpC2 binds upstream of FruA in the *fmgD* promoter region.

To determine whether MrpC2 recognizes the inverted 5-bp elements at positions −63 to −59 and at −71 to −75, we measured binding of His$_{10}$-MrpC2 to three mutant versions of the DNA spanning from positions −80 to −53. A 5-bp change
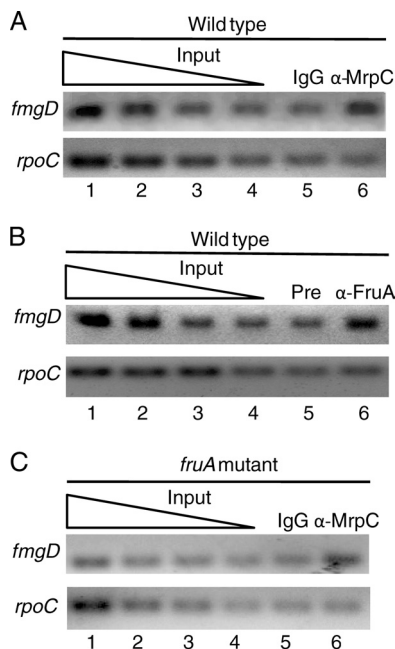
FIG. 2. Association of MrpC and/or MrpC2 and FruA with the *fmgD* promoter region during development. ChIP analysis of *M. xanthus* at 18 h into development. Cells were treated with formaldehyde and lysed. Cross-linked chromatin was immunoprecipitated with antibodies. DNA was amplified with primers for the *fmgD* promoter region (positions −85 to −16 relative to the start site of transcription) or for the *rpoC* coding region (positions +1780 to +1905 relative to the predicted translation start) as a control. A 2-fold dilution series of input DNA purified from 0.025, 0.0125, 0.00625, or 0.003125% of the total cellular extract prior to immunoprecipitation was used as a template in parallel PCRs to show that the PCR conditions were in the linear range of amplification for each primer set. (A) Wild-type strain DK1622 with affinity-purified IgG antibodies against MrpC (α-MrpC), or, as a control, 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 antibodies, as described for panel A.

FIG. 3. Binding of MrpC2 and FruA to the *fmgD* promoter region. EMSAs with ^{32}P-labeled *fmgD* DNA (2 nM) spanning from positions −80 to −16 and no protein, His$_{10}$-MrpC2 (1 μM), FruA-His$_6$ (3 μM), or both His$_{10}$-MrpC2 (1 μM) and FruA-His$_6$ (3 μM) as indicated, were electrophoresed on an 8% polyacrylamide gel. The two arrowheads indicate the shifted species produced by the combination of proteins.

important for cooperative binding of FruA and for *fmgD* promoter activity.

Localization of a second MrpC2 binding site and of the FruA binding site. His$_{10}$-MrpC2 produced two shifted species with the DNA segment from positions −80 to −16 (Fig. 3, lane 2), but the 28-bp duplex from positions −80 to −53 produced only a single shifted complex, and neither of the other 28-bp duplexes (−68 to −41 and −57 to −30) produced a shifted complex (Fig. 4A), suggesting that DNA between positions −30 and −16 might be important for binding of a second molecule of MrpC2. To test this idea and to further localize the site of FruA binding, additional DNA segments were used as probes in EMSAs. A segment from positions −80 to −30 produced a single shifted complex with His$_{10}$-MrpC2 (Fig. 5A, lane 10), consistent with the idea that binding of a second MrpC2 molecule requires DNA between positions −30 and −16 (Fig. 5A, lane 2). FruA-His$_6$ also produced a single shifted complex with DNA from positions −80 to −30 (Fig. 5A, lane 11), as expected since FruA bound the 28-bp duplex from positions −57 to −30 (Fig. 4A, lane 11). The combination of His$_{10}$-MrpC2 and FruA-His$_6$ exhibited enhanced binding (relative to either protein alone) to DNA from positions −80 to −30 (Fig. 5A, lane 12), similar to that observed with the DNA fragment from positions −80 to −16 (Fig. 5A, lane 4). These results provide additional evidence that MrpC2 at the upstream site can bind cooperatively with FruA and indicate that binding of MrpC2 to a second site, which we refer to as the "downstream" site below, requires DNA between positions −30 and −16.

Binding of MrpC2 to the downstream site does not promote cooperative binding of FruA. A fragment from positions −68 to −16, lacking the upstream MrpC2 site, produced single, faint, shifted complexes with His$_{10}$-MrpC2 or FruA-His$_6$ but no enhancement of binding by the combination of proteins (Fig. 5A and B, lanes 6 to 8). The pattern is much like that observed for the DNA fragment from positions −80 to −16 bearing the 1-bp change at position −74 (Fig. 4C, lanes 6 to 8). We infer that in both cases, MrpC2 bound to the downstream site and did not bind cooperatively with FruA. We note that FruA-His$_6$ alone bound the fragment from positions −80 to −16 more strongly than the fragment from positions −68 to

in either 5-bp element, or simultaneous 5-bp changes in both elements, eliminated binding (Fig. 4A, lanes 14, 16, and 18). These results indicate that MrpC2 binds to the inverted 5-bp elements, presumably as a dimer (31, 32). We refer to this as the "upstream" MrpC2 binding site.

The mutation in the 5-bp element at positions −63 to −59 (GACCG to TCAAT) that eliminated binding of His$_{10}$-MrpC2 *in vitro* (Fig. 4A, lane 16) was shown previously to abolish *fmgD* promoter activity *in vivo* (47). The effect on *fmgD* promoter activity of the 5-bp change at positions −71 to −75 has not been measured; however, a 1-bp change at positions −74 (G to T) abolished promoter activity (47). Therefore, binding of His$_{10}$-MrpC2 and FruA-His$_6$ to a DNA fragment (−80 to −16) bearing the 1-bp change was measured in EMSAs. Binding of His$_{10}$-MrpC2 was greatly reduced (Fig. 4B, lane 6), although a single, faint, shifted complex was detected in a long exposure (Fig. 4C, lane 6). Binding of FruA-His$_6$ was unchanged (Fig. 4B, lane 7). The combination of proteins exhibited little or no enhancement of binding (Fig. 4B, lane 8). Taken together, the data suggest that binding of MrpC2 to the upstream site is
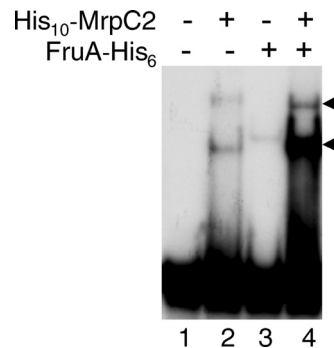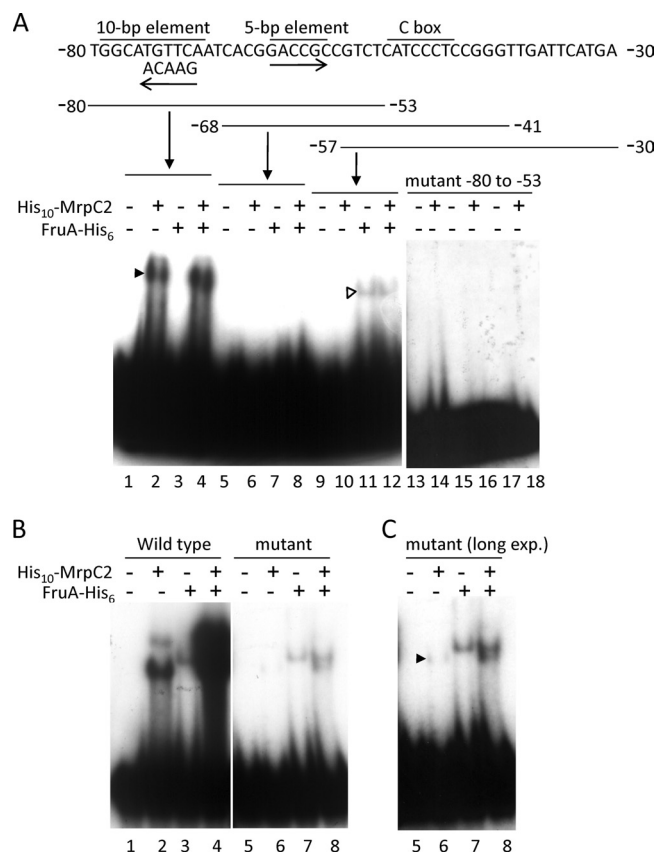
FIG. 4. MrpC2 binds to sequences important for *fmgD* promoter activity. (A) Sequences important for *fmgD* promoter activity and binding of MrpC2 and FruA to different probes. The top part shows the sequence of the *fmgD* promoter region with three positive *cis* regulatory elements labeled. A short segment of the other strand and divergent arrows highlight the inverted 5-bp elements. The location and ends of three oligonucleotide pairs used as probes (1 nM) are depicted with downward arrows pointing to the corresponding EMSAs (lanes 1 to 12). No protein, His_{10}-MrpC2 (1 μM), FruA-His_6 (3 μM), or both His_{10}-MrpC2 (1 μM) and FruA-His_6 (3 μM) were added as indicated, and reactions were electrophoresed on an 8% polyacrylamide gel. The black arrowhead points to a shifted complex produced by His_{10}-MrpC2, and the white arrowhead points to a shifted complex produced by FruA-His_6. Lanes 13 to 18 show EMSAs with mutant −80 to −53 oligonucleotide pairs in which TGTTC at positions −75 to −71 was changed to GTGGA (lanes 13 and 14), GACCG at positions −63 to −59 was changed to TCAAT (lanes 15 and 16), or both changes were made (lanes 17 and 18). The light gray spots in lanes 16 to 18 are artifacts of X-ray film processing, not shifted complexes. (B) Effect of a 1-bp change at position −74 on binding of MrpC2 and FruA. EMSAs were performed as described above except with wild-type or mutant *fmgD* DNA (2 nM) spanning from positions −80 to −16. Exposure to film was longer in this experiment than in the experiment shown in Fig. 3, so the two shifted species in lane 4 are unresolved. Intervening lanes were deleted from the image. (C) A longer exposure of the experiment described for panel B allowed detection of a faint, shifted complex in lane 6 (arrowhead).

−16 (Fig. 5A, compare lanes 3 and 7), indicating that DNA upstream of position −68 enhances FruA binding.

A fragment from positions −52 to −16 showed weak binding by His_{10}-MrpC2 (Fig. 5C, lane 2), presumably to the downstream site, and showed no binding by FruA-His_6 (Fig. 5C, lane 3). Since FruA bound the 28-bp duplex from positions
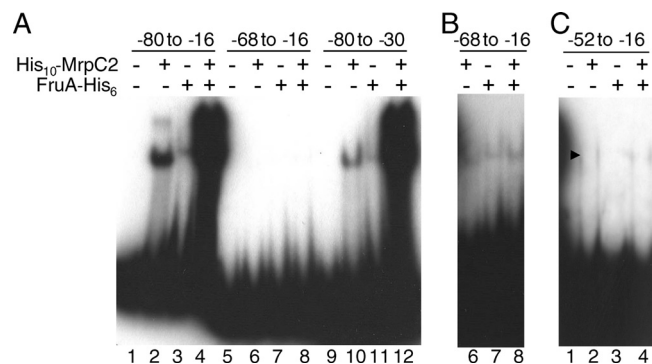

FIG. 5. Localization of a second MrpC2 binding site and of the FruA binding site. (A) EMSAs with ^{32}P-labeled DNA fragments (2 nM) as indicated and no protein, His_{10}-MrpC2 (1 μM), FruA-His_6 (3 μM), or both His_{10}-MrpC2 (1 μM) and FruA-His_6 (3 μM) as indicated, were electrophoresed on an 8% polyacrylamide gel. (B) A longer exposure of the experiment described for panel A allowed detection of faint, shifted complexes with DNA from positions −68 to −16 (lanes 6 to 8). (C) EMSAs were performed as described for panel A with DNA from positions −52 to −16. The arrowhead points to a faint, shifted complex produced by His_{10}-MrpC2.

−57 to −30 (Fig. 4A, lane 11), DNA between positions −57 and −52 is important for FruA binding.

FruA acts positively and the downstream MrpC2 binding site likely acts negatively to regulate *fmgD* promoter activity. Within the region bound by FruA (−57 to −30) is a C box sequence at positions −52 to −46 (Fig. 4A) that was shown previously to function as a positive regulatory element for *fmgD* promoter activity (47). We noticed an imperfect match to the C box consensus sequence at positions −38 to −44 on the other DNA strand (Fig. 6A) and hypothesized that FruA binds to these inverted repeats as a dimer, since FruA-His_6 shifted the 28-bp duplex from positions −57 to −30 to about the same position as a presumed dimer of His_{10}-MrpC2 (32) shifted the 28-bp duplex from positions −80 to −53 (Fig. 4A). Monomers of the two transcription factors are predicted to be similar in size. The sequence from positions −44 to −37 had not been subjected to mutational analysis. Therefore, we tested the effects of two 4-bp changes in this region on *fmgD* promoter activity. Strikingly, a 4-bp change from CGGG to ATTT at positions −44 to −41 abolished *fmgD-lacZ* expression, and a 4-bp change from TTGA to GGTC at positions −40 to −37 increased expression about 4-fold (Fig. 6B). These results suggested adjacent or partially overlapping activator and repressor binding sites. We reasoned that binding of MrpC2 to the downstream site might repress transcription, and binding of MrpC2 to the upstream site cooperatively with FruA might activate transcription (Fig. 6C).

To test the model depicted in Fig. 6C, binding of His_{10}-MrpC2 and FruA-His_6 to DNA fragments (−80 to −16) bearing the 4-bp changes was measured in EMSAs. In agreement with the model, the mutation at positions −44 to −41 eliminated binding of FruA-His_6 alone, and no enhancement of binding was observed in combination with His_{10}-MrpC2 (Fig. 6D). This result, together with the finding that *fmgD-lacZ* expression was abolished by the mutation at positions −44 to −41, provides evidence that FruA is a direct activator of *fmgD* transcription.
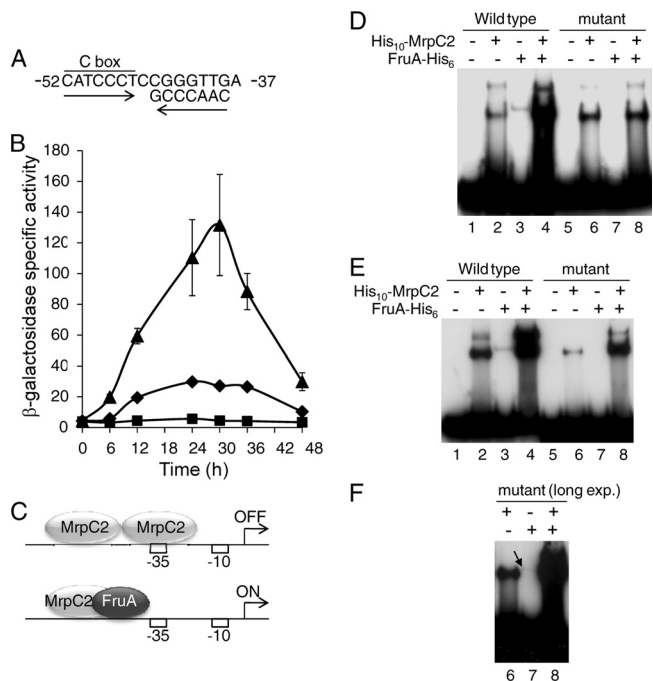
FIG. 6. FruA acts positively and the downstream MrpC2 binding site likely acts negatively to regulate *fmgD* promoter activity. (A) Sequences within the region bound by FruA. Perfect and imperfect matches to the C box consensus sequence (CAYYCCY [Y means T or C]) are indicated by arrows. (B) Effects of two mutations on *fmgD* promoter activity. β-Galactosidase-specific activity during development was measured for *lacZ* fused to *fmgD* (positions −80 to +382) with no mutation (◆), a 4-bp mutation at positions −44 to −41 (■), or a 4-bp mutation at positions −40 to −37 (▲). The units of activity are nanomoles of *o*-nitrophenyl phosphate per minute per milligram of protein. Points show the averages of results from three transformants, and error bars depict one standard deviation of the data for mutant promoter regions. The error bars are too small to be seen in the case of the mutation at positions −44 to −41. For the wild-type promoter region, one transformant was measured in this experiment. (C) Model for regulation of the *fmgD* promoter. Early in development, MrpC2 binds cooperatively to the upstream and downstream sites, preventing transcription (top). Later in development, C-signaling activates FruA, which binds cooperatively with MrpC2 bound to the upstream site, activating transcription (bottom). (D) Effect of a 4-bp change at positions −44 to −41 on binding of MrpC2 and FruA. EMSAs were performed as described in the Fig. 3 legend, with wild-type or mutant *fmgD* DNA (2 nM) spanning from positions −80 to −16. A shorter exposure of the result for wild-type DNA (lanes 1 to 4) is shown in Fig. 3. A longer exposure is shown here for comparison with mutant DNA (lanes 5 to 8). (E) Effect of a 4-bp change at positions −40 to −37. EMSAs were performed as described for panel D. (F) A longer exposure of the experiment described for panel E allowed detection of a faint, shifted complex in lane 7 (arrow).

The EMSA result for the mutation at positions −40 to −37 was more complex but also consistent with the model. Binding of His$_{10}$-MrpC2 alone was reduced, and only one shifted complex was observed (Fig. 6E, lane 6), even in a long exposure (Fig. 6F, lane 6), suggesting that the mutation eliminated His$_{10}$-MrpC2 binding to the downstream site. The mutation also weakened binding of FruA-His$_6$ alone; a very faint shifted complex was observed in a long exposure (Fig. 6F, lane 7). The effects of the mutation on binding of both proteins suggest that the downstream MrpC2 site partially overlaps the FruA site.

Importantly, the combination of proteins produced a pattern indicative of cooperative binding (Fig. 6E, lane 8). Despite weakened binding of FruA alone, it can still bind cooperatively with MrpC2 bound to the upstream site. We infer that the 4-fold increase in *fmgD-lacZ* expression brought about by the mutation at positions −40 to −37 is likely due to loss of MrpC2 binding to the downstream site, consistent with the notion that MrpC2 represses transcription when bound to this site (Fig. 6C). Because the mutation at positions −40 to −37 is adjacent to the promoter −35 region, we cannot exclude the possibility that the mutation enhances RNA polymerase binding that is productive for transcription.

DISCUSSION

The main finding of this work is that *fmgD*, like *fmgA* and *fmgBC*, is subject to combinatorial control by MrpC2 and FruA. The two transcription factors appear to bind cooperatively in all three promoter regions, but the arrangements of binding sites differ. FruA binds upstream of MrpC2 in the *fmgA* promoter region, whereas MrpC2 binds upstream of FruA in the *fmgBC* promoter region. In terms of cooperative binding that activates transcription, the *fmgD* promoter region resembles the *fmgBC* promoter region, with MrpC2 binding to an upstream site and FruA binding downstream, adjacent to the promoter. However, our results show that MrpC2 also binds to a downstream site that overlaps the *fmgD* promoter, likely repressing transcription when bound at this position. The downstream MrpC2 site appears to overlap the FruA site, so the two transcription factors may compete for binding, resulting in repression when MrpC2 is bound (Fig. 6C, top) and in activation when FruA is bound (Fig. 6C, bottom). This model would explain why *fmgD* transcription begins later during development and exhibits greater dependence on C-signaling than *fmgA* and *fmgBC* transcription, since C-signaling activates FruA, and a higher concentration of active FruA might be needed to outcompete MrpC2 for binding to its downstream site in the *fmgD* promoter region.

While *fmgD* transcription exhibits greater dependence on C-signaling than *fmgA* and *fmgBC* transcription, all three promoters depend absolutely on FruA (29, 52) (Fig. 1). This implies that FruA is active to some extent in the absence of C-signaling. In a *csgA* mutant incapable of C-signaling, developmental expression of *fmgA* and *fmgBC* is reduced 2-fold to 4-fold (5, 7, 20). Apparently, the concentration of active FruA is high enough to permit some cooperative binding with MrpC2, partially activating the *fmgA* and *fmgBC* promoters. In contrast, developmental expression of *fmgD* is abolished in a *csgA* mutant (20). We infer that the concentration of active FruA is insufficient to permit cooperative binding with MrpC2 bound to the upstream site in the *fmgD* promoter region, perhaps due to MrpC2 bound to the downstream site. A *csgA* mutant accumulates MrpC and MrpC2 normally (data not shown).

Consistent with the notion that the *fmgD* promoter region differs from the *fmgA* and *fmgBC* promoter regions, ChIP analysis revealed MrpC and/or MrpC2 associated with the *fmgD* promoter region even in a *fruA* mutant (Fig. 2C). In contrast, association of MrpC and/or MrpC2 with the *fmgA* and *fmgBC* promoter regions required FruA (28, 29). We infer

that MrpC and/or MrpC2 can occupy the *fmgD* promoter region in the absence of FruA due to the presence of a higher-affinity binding site (i.e., the upstream MrpC2 site) and/or due to the presence of two binding sites (to which MrpC2 binds cooperatively; see below).

Comparison of MrpC2 and FruA binding to different DNA fragments suggests that MrpC2 binding to the upstream site in the *fmgD* promoter region is cooperative with either a second molecule of MrpC2 binding downstream or with FruA binding downstream. A 1-bp change at position −74 in the upstream MrpC2 binding site nearly abolished binding of His$_{10}$-MrpC2 *in vitro* (Fig. 4B). The small amount of binding detected in a long exposure likely reflects weak binding of MrpC2 to the downstream site (Fig. 4C). In agreement with this interpretation, His$_{10}$-MrpC2 bound very weakly to DNA fragments lacking the upstream site (Fig. 5). FruA-His$_6$ bound to DNA with the 1-bp change at position −74, but in combination with His$_{10}$-MrpC2 there was little or no enhancement of binding (Fig. 4B), and a similar result was seen with a DNA fragment from positions −68 to −16 lacking the upstream MrpC2 site (Fig. 5B). Likewise, a DNA fragment lacking the FruA site due to a mutation at positions −44 to −41 showed little or no enhancement of binding by the combination of proteins (Fig. 6D), and DNA fragments lacking the downstream MrpC2 site due to truncation (Fig. 5A, lane 10) or due to a mutation at positions −40 to −37 (Fig. 6E, lane 6) exhibited considerably less binding by His$_{10}$-MrpC2 than a DNA fragment from positions 80 to −16 that contains both MrpC2 sites (Fig. 5A, lane 2, and 6E, lane 2). Taken together, the results suggest that MrpC2 binds cooperatively to upstream and downstream sites in the *fmgD* promoter region, and MrpC2 binds cooperatively to the upstream site, with FruA binding downstream (Fig. 6C). The combination of FruA and MrpC2 produced much more shifted species than either protein alone, indicative of strong cooperative binding.

The sites bound by MrpC2 and FruA in the *fmgD* promoter region contain sequences that match consensus binding sequences. The upstream MrpC2 site contains the sequence ATGTT(N$_8$)GACCG from positions −76 to −59, and the downstream MrpC2 site contains the sequence GGGTT(N$_8$)AATAA from positions −43 to −26, which match the GTGTC(N$_8$)GACAC consensus sequence (32) at six and five out of 10 positions, respectively. The FruA site contains the sequence TCTCA(N$_6$)CGGG from positions −55 to −41, which matches the consensus sequence for binding of the FruA DNA-binding domain, GGG(C/T)(A/G)(N$_{4–6}$)(C/T)GGG (48), at six out of nine positions.

Binding of both MrpC2 to its upstream site and FruA to its site appears to be required for *fmgD* promoter activity. Mutations at positions −63 to −59 or at position −74 that impair binding of MrpC2 to its upstream site (Fig. 4), as well as a mutation at positions −44 to −41 that impairs FruA binding (Fig. 6D), abolish developmental *fmgD-lacZ* expression (47) (Fig. 6B). Hence, *fmgD*, like *fmgA* and *fmgBC*, is subject to combinatorial control by MrpC2 and FruA. As noted previously (28), the *dev* operon also appears to utilize this mechanism (S. Mittal, P. Viswanathan, A. Campbell, and L. Kroos, unpublished data), and expression of this operon is crucial for sporulation (4, 45). Also as noted previously (28, 29), at promoters utilizing this mechanism, MrpC2 and FruA occupy a location typical for class I activators, which contact the C-terminal domain of the α subunits of RNA polymerase (1), and both MrpC2 and FruA might make such contacts at each promoter, based on studies of activator pairs at both synthetic (26, 44) and natural (2) promoters.

Our work with *fmgD* reveals complexity in combinatorial control by MrpC2 and FruA. Our previous work with *fmgA* and *fmgBC* revealed flexibility in the arrangement of MrpC2 and FruA binding sites (28, 29). The new feature uncovered here is the presence of a second MrpC2 binding site that we propose mediates repression of *fmgD* transcription until the concentration of active FruA increases enough to overcome the repression. According to our model, the added complexity delays *fmgD* expression relative to that of *fmgA* and *fmgBC* and makes *fmgD* expression more dependent on C-signaling (since C-signaling activates FruA). Since cell alignment in the outer domain of the nascent fruiting body is believed to promote C-signaling (reviewed in references 13, 39, and 42), our model predicts that *fmgD* expression would be localized to the fruiting body outer domain, as has been observed (36). We have speculated that the role of MrpC and MrpC2 in combinatorial control of *fmg* genes is to signal persistent starvation and prevent MazF-mediated programmed cell death of cells destined to form spores (28). Our work with *fmgD* suggests that MrpC2 also fine-tunes expression of particular *fmg* genes via more than one binding site in the promoter region.

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