Regulation of late-acting operons by three transcription factors and a CRISPR-Cas component during *Myxococcus xanthus* development

Shreya Saha and Lee Kroos*

*Department of Biochemistry and Molecular Biology, Michigan State University, East Lansing, MI 48824, USA.

Running title: Complex regulation of sporulation genes during *M. xanthus* development

*For correspondence. E-mail kroos@msu.edu; Tel. (+1) 517 355 9726; Fax (+1) 517 353 9334.
Abstract

Starvation induces *Myxococcus xanthus* multicellular development. Rod-shaped cells move, forming mounds. Within mounds, rods differentiate into round, stress-resistant spores. C-signaling is proposed to activate FruA, which binds DNA cooperatively with MrpC to increase transcription of many genes. We report that the regulation of late-acting operons involved in spore metabolism (*fadIJ*) and coat biogenesis (*exoA-I, exoL-P, nfsA-H*) is more complex. These operons appear to be negatively regulated by FruA prior to its activation, then positively regulated by C-signal-activated FruA, based on transcript levels in mutants. Although MrpC is required to produce FruA, loss of MrpC affected transcript levels differentially. Transcript measurements also indicated that transcription factor Nla6 is a positive regulator of late-acting operons, whereas the Devl component of a CRISPR-Cas system is a negative regulator. FruA bound to all four promoter regions *in vitro*, but each promoter was unique in terms of whether or not MrpC and/or Nla6 bound, and in terms of cooperative binding. Whereas FruA switches from negative to positive regulation of all four operons temporally, MrpC and Nla6 appear to exert operon-specific temporal regulation. We propose that complex, differential regulation of late-acting operons ensures that spore resistance and surface characteristics meet environmental demands.
Starving Myxococcus xanthus bacteria coordinate their movements to build multicellular mounds on a solid surface. Within the mounds rod-shaped cells transform into round spores that can survive starvation and other harsh conditions. Thousands of genes must be properly regulated in response to many signals to complete this developmental process. We investigated the regulation of genes whose products act near the end of the process to change metabolism of the spore and protect its contents by depositing a polysaccharide coat on its surface. We discovered that the regulation of these genes is unusually complex. It involves three transcription factors that respond to different signals and bind to the promoter regions in different combinations. This allows each promoter to be regulated uniquely. Combinatorial regulation of this complexity is common in higher eukaryotes, but not in bacteria. In addition, all the promoters are controlled by a CRISPR-Cas system. These systems typically defend bacteria against viral infection. Our results suggest that M. xanthus evolved complex eukaryotic-like combinatorial control of transcription and a link to a CRISPR-Cas system to thwart viral intrusion while making spores suited suited to withstand starvation and environmental insults.
Introduction

The Gram-negative soil bacterium *Myxococcus xanthus* provides an attractive model system to study signal-induced gene regulation and bacterial community behavior (1). Upon nutrient depletion, cells move on solid surfaces and form mounds, within which some of the rod-shaped cells differentiate into round, stress-resistant spores. During this multicellular developmental process of fruiting body formation, a majority of the population undergoes lysis, while some cells remain outside of fruiting bodies as peripheral rods (2, 3).

A signal-responsive gene regulatory network governs the developmental process of *M. xanthus* (4). Starvation triggers production of the intracellular secondary messenger molecules (p)ppGpp (5, 6) and c-di-GMP (7), which lead to production of the extracellular A- and C-signals (5, 8-10) and exopolysaccharide (7), respectively. The short-range C-signal appears to be a proteolytic fragment of the CsgA protein (11-13) and/or diacylglycerols produced by cardiolipin phospholipase activity of full-length CsgA (14), but the mechanisms of signal transduction remain to be elucidated. C-signaling appears to posttranslationally activate the transcription factor FruA (15, 16), but the mechanism is unknown. FruA is similar to response regulators of two-component signal-transduction systems (17). Response regulators are typically activated by phosphorylation by a histidine kinase (15). However, for several reasons, phosphorylation is unlikely to be the mechanism by which FruA is activated in response to C-signaling (16, 18).

Transcription of the *fruA* gene is regulated by a cascade of starvation-responsive transcription factors that includes MrpC (4, 19), which binds upstream of the *fruA* promoter and stimulates transcription (20, 21). In addition to transcriptional control of the *mrpC* gene by starvation (22), the level of MrpC protein is regulated by proteolysis, slowing the pace of...
development under the normal conditions of starvation (23, 24) and halting development if sufficient nutrients are added back before rods become committed to spore formation (25, 26).

*In vitro*, MrpC and FruA bind cooperatively to the promoter regions of many developmentally-regulated genes (27). Mutational analyses of five such promoter regions suggest that cooperative binding to a site just upstream of the promoter stimulates transcription *in vivo*, whereas binding to nearby sites can increase or decrease transcription (18, 28-31). The different arrangements of binding sites with different affinities for MrpC and FruA may explain the observed differences between genes in terms of their spatiotemporal patterns of expression and their extent of dependence on C-signaling for expression (4, 32, 33). The levels of MrpC, FruA, and CsgA normally rise in starving rods engaged in mound building (3), but the addition of sufficient nutrients triggers rapid proteolysis of MrpC and prevents the normal increase in transcripts for proteins involved in sporulation (25, 26). Combinatorial control of gene expression by MrpC and FruA appears to ensure that only starving rods (in which MrpC and FruA are synthesized and stably accumulate) aligned in mounds (which enhances short-range C-signaling between rods, presumably activating FruA within them) commit to spore formation (4, 18, 25, 33, 34).

Among the genes combinatorially controlled by MrpC and FruA (28), early studies showed that mutations in the *dev* operon can cause severe defects in sporulation (35-38). The *dev* operon includes eight genes comprising a CRISPR-Cas system that may protect developing *M. xanthus* against phage infection (39). The first gene in the *dev* operon, *devI*, codes for a 40-residue protein that strongly inhibits sporulation when overproduced (40), delays sporulation by about 6 h when produced at the normal level (16, 41), and exerts weak positive...
autoregulation on dev transcript accumulation (16). In contrast, in-frame deletions in three
genes of the dev operon (devTRS) increase accumulation of the dev transcript 10-fold during
development (16, 40, 41), indicating that DevTRS proteins exert strong negative autoregulation.
The negative autoregulation by DevTRS proteins is crucial in order to prevent DevI
overproduction from inhibiting sporulation (40, 41).

The products of several other operons act late during the developmental process, impacting
the metabolism of nascent spores and the biogenesis of their coat polysaccharide, but the
regulation of these operons is not fully understood. The putative fadIJ operon is normally
induced twofold in sporulating cells, but not in csgA, fruA, or mrpC mutants (42). Recently
published data support that fadIJ are co-transcribed as an operon (43). FadIJ are enzymes in a
fatty acid β-oxidation pathway that impacts spore structure and resistance properties (42). The
nfsA-H operon is up-regulated in spores formed during the starvation-induced developmental
process and in spores induced artificially by glycerol addition (44). The starvation-induced up-
regulation fails in a csgA mutant, is diminished in a devRS mutant, and, unusually, is greater in a
fruA mutant, suggesting negative regulation by FruA (44). The NfsA-H proteins form a complex
involved in biogenesis of the coat polysaccharide to generate compact and rigid stress-resistant
spores (45-47). The exoA-I operon (45) is normally induced late in development, but not in csgA
or devRS mutants (48). Transcription depends on binding of FruA (49) and Nla6 (50) to the
exoA-I promoter region. Interestingly, Nla6 is part of a cascade of enhancer-binding proteins
that responds to starvation early in development (19) and leads to production of MrpC and
FruA (4). Nla6 also binds to the promoter region of the predicted MXAN_3259–MXAN_3263
operon and appears to directly regulate transcription (50). Recently published data support
that the five genes are co-transcribed as an operon (43), which was renamed the exoL-P operon based on functional studies (51). The ExoA-I and ExoL-P proteins are involved in the synthesis, export, and modification of the spore coat polysaccharide (45, 46, 51).

Here, we report systematic investigation of the late-acting fadIJ, nfsA-H, exoA-I, and exoL-P operons with respect to the phenotypes of mutants and the regulation of gene expression. We found that exo mutants exhibit stronger defects in sporulation than an nfs mutant, whereas an fad mutant exhibited very little difference from a “wild-type” (WT) strain under our conditions.

Cooperative binding of MrpC and FruA appears to stimulate transcription of fadIJ, but regulation of fadIJ as well as nfsA-H, exoA-I, and exoL-P involves additional complexities, with FruA acting negatively prior to its activation and then C-signal-activated FruA acting positively later in development. Binding of MrpC appears to stimulate transcription of nfsA-H and exoL-P, albeit with some notable differences, and MrpC appears to indirectly decrease the exoA transcript level. Nla6 is a positive regulator of the late-acting operons early in development and binds cooperatively with FruA to the nfsA-H, exoL-P, and exoA-I promoter regions, and also binds cooperatively with MrpC to the nfsA-H promoter region. The DevI CRISPR-Cas component is a negative regulator of the late-acting operons. We conclude that three transcription factors and a CRISPR-Cas component regulate the late-acting operons and we propose that complex, differential regulation of genes involved in spore metabolism and coat biogenesis produces spores suited to withstand environmental insults.

Results

Mutations in exo genes impair sporulation more strongly than deletion of the nfs operon
In previous work, we established methods to systematically analyze *M. xanthus* development under submerged culture conditions (16). Submerged culture involves growing cells in nutrient medium followed by sedimentation, resuspension in starvation buffer, and incubation in a plastic container. Cells attach to the container bottom and develop under a layer of buffer.

Here, we examined the developmental time course of *exo*, *nfs*, and *fad* mutants microscopically and by harvesting samples for cellular and molecular analyses.

We compared submerged culture development of mutants with that of WT laboratory strain DK1622 (52) from which the mutant strains were derived. Specifically, we examined the effects of an insertion in *fadI* (42), a deletion of the entire *nfsA-H* operon (44), an insertion in *exoC* (45, 48), and an insertion in *exol* (50, 51). As expected (16), the WT strain formed mounds by 18 h poststarvation (PS) and the mounds began to darken by 30 h (Fig. 1). Darkening typically correlates with spore formation. The *fadI* mutant was indistinguishable from the WT strain. The *nfsA-H* mutant was delayed by about 3 h in mound formation and appeared to be delayed and reduced in mound darkening. The *exoC* and *exol* mutants formed normal-looking mounds by 18 h, but subsequent mound darkening was reduced compared to the WT strain, suggesting sporulation defects.

To quantify changes at the cellular level, we harvested samples from submerged culture and either treated the samples with glutaraldehyde to fix cells or left the samples untreated (16).

Using the untreated samples, we quantified “sonication-resistant spores” microscopically and “mature spores” that were heat- and sonication-resistant and capable of germination and colony formation on nutrient agar. We used the fixed samples to quantify “sonication-sensitive cells” microscopically (i.e., the total number of cells observed in the fixed sample minus the...
number of sonication-resistant spores observed in the corresponding untreated sample). The vast majority of sonication-sensitive cells were rod-shaped, but we also observed a small percentage of “transitioning cells” (TCs) intermediate in morphology between rods and spores (33) in some of the fixed samples.

As expected (16), we first observed sonication-resistant spores at 27 h PS in samples of the WT strain, and the spores rose from ~0.2% (as a percentage of the number of rod-shaped cells present at $T_0$, the time starvation initiated development) to ~2% at 48 h (Fig. 2). The $fadI$ mutant formed more spores than the WT strain. Consistent with the observed mound darkening defects (Fig. 1), the $nfsA-H$ mutant formed less spores than the WT strain, and the $exoC$ and $exol$ mutants did not form spores (at a detection limit of 0.05% of the $T_0$ cell number) (Fig. 2 and Table S1). Neither did the $exoC$ and $exol$ mutants form mature spores at 72 h (at a detection limit of 0.00001% of the $T_0$ cell number), while the $nfsA-H$ mutant made ~15% as many mature spores as the WT strain, and the $fadI$ mutant made a similar number of mature spores as the WT strain (Table S1).

The WT strain exhibited a decline of sonication-sensitive cells during development similar to that reported previously (16), with only ~30% of the $T_0$ cell number remaining at 18 h PS and only ~6% remaining at 48 h (Fig. S1A). It was also reported previously that the decrease in cell number correlates with a decrease in the total protein concentration of developing cultures, which was suggested to reflect lysis of the majority of cells early during development under submerged culture conditions (25). The mutants showed similar decreases in cell number as the WT strain (Fig. S1A). As noted above, we observed a small percentage of TCs in some of the fixed samples. For the WT strain, ~3% of the $T_0$ cell number were TCs at 24 h (Fig. S1B), when
sonication-sensitive cells were \( \sim 20\% \) of the \( T_0 \) cell number (Fig. S1A). Thus, TCs comprised 15% of the total population at 24 h, before sonication-resistant spores were observed (Fig. 2), in agreement with \textit{in situ} observations using confocal microscopy (33). The percentage of TCs decreased at later times (Fig. S1B), presumably as TCs became sonication-resistant spores (Fig. 2). The \textit{fadI} mutant showed about half as many TCs as the WT strain at most time points (Fig. S1B), perhaps due to the \textit{fadI} mutant forming more sonication-resistant spores (Fig. 2). The \textit{exo} and \textit{nfs} mutants exhibited less TCs at 24 h (\( \sim 0.4-0.7\% \) of the \( T_0 \) cell number), but in each case the percentage rose to at least 1% later (Fig. S1B), suggesting that these mutants begin to change shape, but are impaired in their ability to make spores.

We conclude that the \textit{exoC} and \textit{exoL} mutants exhibited stronger defects in sporulation than the \textit{nfsA-H} mutant, whereas the \textit{fadI} mutant exhibited very little difference from the WT strain under our conditions.

\textbf{Transcript levels from the late-acting operons are very low in the absence of C-signaling}

We used methods established previously (16) to measure transcript levels from the late-acting operons of the WT strain and a \textit{csgA} insertion mutant at 18-30 h PS, the period leading up to and including the time that many cells commit to spore formation and sonication-resistant spores begin to be observed (25) (Fig. 2). As expected for the WT strain (26), transcript levels rose during development (Fig. 3). As noted previously (41), the \textit{exoA} transcript level varied greatly between biological replicates (Fig. 3A), which we do not understand. The \textit{exoL} transcript level likewise varied greatly (Fig. 3B). The \textit{nfsA} (Fig. 3C) and \textit{fadI} (Fig. 3D) transcript
levels varied less and on average increased only ~fourfold, much less than the exoA (~24-fold) and exol (~70-fold) transcript levels.

For the csgA mutant, which is unable to produce C-signal, the transcript levels did not increase during development (Fig. 3, asterisks indicate $p < 0.05$ in Student’s two-tailed $t$-tests comparing the mutant to the WT strain at each time point). Asterisks are absent above the exoA and exol transcript levels of the csgA mutant because the statistical test yielded $p > 0.05$ due to the large variation between biological replicates of the WT strain. Nevertheless, the exoA and exol transcript levels of the csgA mutant were low for all replicates at each time point. We conclude that transcript levels from the late-acting operons are very low in the absence of C-signaling. In agreement, reporter activity from fusions to exoC (48), nfsA (44), and fadl (42) was very low for csgA mutants relative to WT strains during development.

The low transcript levels could be due to decreased synthesis and/or increased degradation in the csgA mutant compared with the WT strain. To measure the transcript degradation rates, we added rifampicin to inhibit transcription at 30 h PS and determined the transcript levels at intervals thereafter. The transcript degradation rates did not differ significantly between the csgA mutant and the WT strain (Fig. 4), suggesting that decreased synthesis primarily accounts for the low transcript levels in the absence of C-signaling.

Considerable evidence supports a model in which C-signaling activates FruA posttranslationally in order to increase transcription of genes during M. xanthus development (15, 16). We showed previously that the FruA level of a csgA mutant is about twofold lower than that of the WT strain at 18-30 h PS (16). Boosting the FruA level in the csgA mutant to the WT level using a vanillate-inducible promoter ($P_{van}$) fused to fruA did not increase the transcript
levels of five genes or operons (*dev*, *fmgA*, *fmgBC*, *fmgD*, and *fmgE*) known to be under combinatorial control of FruA and MrpC. Neither did boosting the level of FruA D59E (with a phosphomimetic substitution in its receiver domain) using a $P_{van-fruA\ D59E}$ fusion.

We tested whether boosting the level of FruA or its D59E variant in the *csgA* mutant increases the transcript levels of the late-acting operons. The transcript levels remained low in all cases (Fig. S2), suggesting that neither a low level of FruA nor a lack of D59 phosphorylation causes the transcript levels to remain low in the *csgA* mutant. Rather, these results are consistent with evidence suggesting that C-signaling regulates FruA activity by a mechanism other than phosphorylation of its receiver domain (16, 18).

Prior to FruA activation by C-signaling, unactivated FruA negatively regulates the late-acting operons. If C-signaling activates FruA and the activated FruA increases transcription of the late-acting operons, then transcript levels are expected to be low in a *fruA* insertion mutant, as observed for the *dev* operon (16). Instead, the *exoA*, *exoL*, and *nfsA* transcript levels of the *fruA* mutant were greater than those of the WT strain at 18 h PS (Fig. 3), suggesting that FruA is a negative regulator of the late-acting operons early in development. The *exoA* and *exoL* transcript levels of the *fruA* mutant also exceeded those of the WT strain at 21 and 24 h. In contrast, the *fadI* transcript level of the *fruA* mutant was less than that of the WT strain (Fig. 3D), consistent with a model in which C-signaling activates FruA and the activated FruA increases *fadI* transcription. Activated FruA may also increase *nfsA* transcription at 27 and 30 h, since the *nfsA* transcript level of the *fruA* mutant was less than that of the WT strain at those times (Fig. 3C).
average exoA transcript level of the fruA mutant was less than that of the WT strain at 30 h, but this difference was due to one biological replicate of the WT strain with a much greater transcript level than the other three replicates (Fig. 3A), so the evidence that activated FruA increases exoA transcription at 30 h is weak. The average exol transcript level of the fruA mutant exceeded that of the WT strain at 27 and 30 h (Fig. 3B), providing no evidence that activated FruA is necessary to increase exol transcription.

Comparison of the fruA and csgA mutants suggests that FruA which has not been activated by C-signaling (i.e., unactivated FruA) negatively regulates all four of the late-acting operons early in development. The average transcript levels of the fruA mutant were greater than those of the csgA mutant (except for the very low fadl transcript levels in both mutants at 27 and 30 h PS) (Fig. 3). We also infer that negative regulation by unactivated FruA early in development of the WT strain accounts for its relatively low exoA (Fig. 3A) and exol (Fig. 3B) transcript levels at 18-24 h, and its low nfsA transcript level at 18 h (Fig. 3C), as compared with the fruA mutant. The fadl transcript level of the WT strain was comparable to that of the fruA mutant at 18 h (Fig. 3D), suggesting that positive regulation by activated FruA counteracts negative regulation by unactivated FruA already by 18 h in this case.

Taken together, the effects of mutations in fruA and csgA on transcript levels suggest that regulation of the late-acting operons during the period leading up to and including commitment to spore formation depends on negative regulation by unactivated FruA, which in at least some cases switches to positive regulation by C-signal-activated FruA.

The exoA, exol, and nfsA transcript levels of mrpC and fruA mutants differ
MrpC appears to directly stimulate transcription from the fruA promoter (20). In agreement, FruA was not detected in an mrpC in-frame deletion mutant at 18-30 h PS (16). Hence, the mrpC mutant lacks both MrpC and FruA. To compare the loss of both transcription factors with the loss of only FruA in terms of regulating the late-acting operons, we measured the transcript levels of the mrpC mutant in parallel with those of the fruA mutant and the WT strain.

Strikingly, the exoA, exoL, and nfsA transcript levels of the mrpC and fruA mutants differed, and in each case the pattern of effects was unique. The average exoA transcript level of the mrpC mutant was greater than that of both the WT strain and the fruA mutant at all times (Fig. 2). Asterisks above error bars indicate $p < 0.05$ in Student’s two-tailed $t$-tests comparing the mutant to the WT strain at the same time point and asterisks above brackets likewise compare the mutants. The comparison of mutants suggests that MrpC negatively regulates the exoA transcript level independently of FruA (i.e., loss of both transcription factors increased the exoA transcript level more than loss of only FruA). The exoL transcript level of the mrpC mutant exceeded that of the WT strain at 18 and 24 h PS, and on average exceeded that of the fruA mutant at 18 h, but on average was less than that of the fruA mutant at 24 and 30 h (Fig. 5B).

In this case, comparison of the mutants suggests that MrpC negatively regulates the transcript level independently of FruA early in development, but that MrpC can positively regulate the transcript level independently of FruA later in development. The nfsA and fadl transcript levels of the WT strain increased at 30 h, but not those of the mrpC and fruA mutants (Fig. 5C and 5D), nor of the csgA mutant (Fig. 3C and 3D), suggesting that C-signal-activated FruA positively regulates the nfsA and fadl transcript levels late in development. However, earlier in development at 18 h, the nfsA transcript level of the fruA mutant was greater than that of the...
WT strain and the \textit{mrpC} mutant (Fig. 5C), suggesting that MrpC positively regulates the
transcript level in the absence of FruA. In contrast, the \textit{fadl} transcript level of both mutants
was less than that of the WT strain at 18 h (Fig. 5D), suggesting that both transcription factors
are required to positively regulate the transcript level. We conclude that each of the late-acting
operons is uniquely regulated by MrpC and FruA.

To determine whether the absence of MrpC and FruA in the \textit{mrpC} mutant affects the
transcript degradation rates, we added rifampicin to inhibit transcription at 18 h PS and
determined the transcript levels at intervals thereafter. We chose 18 h for this analysis since
the \textit{exoA} (Fig. 5A) and \textit{exol} (Fig. 5C) transcript levels were elevated in the \textit{mrpC} mutant relative
to the WT strain at that time. The transcript degradation rates did not differ significantly
between the \textit{mrpC} mutant and the WT strain (Fig. S3), suggesting that increased synthesis
(rather than decreased degradation) primarily accounts for the elevated \textit{exoA} and \textit{exol}
transcript levels in the absence of MrpC and FruA.

\textbf{FruA can positively regulate \textit{exoA}, \textit{exol}, and \textit{nfsA} in the absence of MrpC}

To examine the effects of FruA in the absence of MrpC, we used the \textit{P}_{van}\textit{fruA} fusion mentioned
previously (16) (Fig. S2) to produce FruA in the \textit{mrpC} mutant. The inducer (vanillate) was added
during growth and at 0 h PS. At 6 h, the FruA level of the \textit{mrpC P}_{van}\textit{fruA} mutant was about
threecfold greater than that of the WT strain, but their FruA level was similar at 12 and 18 h (Fig.
S4A). Interestingly, the \textit{mrpC P}_{van}\textit{fruA} mutant formed immature mounds by 12 h (Fig. S5),
although subsequent mound darkening was reduced compared to the WT strain (Fig. S6) and
spores were not detected (Table S1). Nevertheless, mound formation suggests that C-signaling

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is activating FruA to some extent, since a complete block, as in \textit{mrpC}, \textit{fruA}, and \textit{csgA} mutants, results in no mound formation (16) (Fig. S5).

To determine the effects of FruA on regulation of the late-acting operons in the absence of MrpC, we measured the transcript levels of the \textit{mrpC} \textit{P}_{van-fruA} mutant in parallel with those of the \textit{mrpC} and \textit{fruA} mutants and the WT strain early in development. The \textit{exoA} (Fig. 6A) and \textit{exoL} (Fig. 6B) transcript levels of the \textit{mrpC} \textit{P}_{van-fruA} mutant exceeded those of the WT strain at 6-18 h PS, and on average exceeded those of the \textit{mrpC} and \textit{fruA} mutants, except the \textit{exoL} transcript level of the \textit{mrpC} mutant was similar to that of the \textit{mrpC} \textit{P}_{van-fruA} mutant at 18 h. We conclude that FruA can positively regulate the \textit{exoA} and \textit{exoL} transcript levels in the absence of MrpC. This regulation may involve C-signal-activated FruA since the \textit{mrpC} \textit{P}_{van-fruA} mutant formed immature mounds (Fig. S5 and S6), whereas \textit{mrpC}, \textit{fruA}, and \textit{csgA} mutants fail to form mounds (16) (Fig. S5). The \textit{nfsA} transcript level of the \textit{mrpC} \textit{P}_{van-fruA} strain exceeded that of the WT strain at 6 h and the \textit{mrpC} mutant at 6-12 h (Fig. 6C), also indicative of positive regulation by FruA (perhaps activated FruA) in the absence of MrpC. On the other hand, the \textit{fadL} transcript level of the \textit{mrpC} \textit{P}_{van-fruA} strain was less than that of the WT strain (Fig. 6D), as observed for the \textit{mrpC}, \textit{fruA}, and \textit{csgA} mutants (Fig. 3D, 5D, and 6D), supporting that positive regulation requires both MrpC and activated FruA.

\textbf{Nla6 positively regulates the transcript levels of the late-acting operons}

The Nla6 transcription factor appears to be a direct regulator of the \textit{exoA-I} and \textit{exoL-P} operons since a protein with the \textit{E. coli} maltose-binding protein (MBP) fused to the Nla6 DNA-binding domain (DBD) bound to promoter region DNA fragments (50). A comparison of the \textit{exoA} and
exoL transcript levels of a WT strain and an nla6 insertion mutant suggested that Nla6 positively
regulates both during the first 8 h PS, and negatively regulates both at 24 h (50). We
constructed a new nla6 insertion mutant and compared it with the one characterized
previously (50, 53). The new mutant is tetracycline-resistant (Tc') and the one described
previously is kanamycin-resistant (Km'). Both mutants formed immature mounds by 12 h, but
failed to progress to more mature mounds with distinct, round edges by 18 h (Fig. S5). Later
during development, the Km' nla6 mutant mounds matured somewhat at 24-30 h, but failed to
darken by 36-48 h (Fig. S6). The Tc' nla6 mutant mounds did not mature until 36 h and also
failed to darken by 48 h. Spores were not detected for either mutant (Table S1).

The two nla6 mutants were indistinguishable in terms of the molecular markers we tested.
Since our results showed that FruA and MrpC impact transcript levels of the late-acting operons
at 18 h PS (Fig. 3 and 5) and in some cases earlier during development (Fig. 6), we measured the
levels of FruA, MrpC, and transcripts from the late-acting operons for the nla6 mutants in
parallel with the WT strain early in development. The FruA and MrpC protein levels of the nla6
mutants were similar to those of the WT strain at 6-18 h (Fig. S4). The exoA, nfsA, and fadI
transcript levels of the WT strain increased at 18 h, but those of the nla6 mutants failed to
increase (Fig. 7), suggesting that Nla6 positively regulates the exoA-I, nfsA-H, and fadIJ operons.
The exoL transcript level of the WT strain did not increase at 18 h, but on average that of the
nla6 mutants was less, suggesting that Nla6 also positively regulates the exoL-P operon.
Because the exoA, exoL, and nfsA transcript levels of the mrpC and/or fruA mutants exceeded
those of the WT strain early in development (Fig. 3, 5, and 6), we tried to construct mrpC nla6
and fruA nla6 double mutants, but our efforts were unsuccessful, so we were unable to
determine whether positive regulation by Nla6 could account for the elevated transcript levels in the \textit{mrpC} and \textit{fruA} mutants relative to the WT strain (see Discussion).

\textbf{Binding of the Nla6 DNA-binding domain, MrpC, and FruA differs at the promoter region of each of the late-acting operons}

As a possible indication of direct regulation by Nla6, MrpC, and FruA, we tested for binding to a DNA fragment upstream of the predicted start codon of the first gene in each of the late-acting operons. Figure S7 shows features of each promoter region fragment based on previous work. FruA and MrpC were shown to bind cooperatively to the \textit{dev} promoter region fragment (28). The FruA DBD was shown to bind to at least three sites in the \textit{exoA-I} promoter region fragment (49) and MBP-Nla6 DBD was also shown to bind, presumably via interaction with DNA sequences similar to a half-site consensus sequence (50). MBP-Nla6 DBD was also shown to bind to the \textit{exoL-P} promoter region fragment (50). ChIP-seq analysis suggested that MrpC is bound at a site upstream of \textit{nfsA-H} at 18 h PS (27). Here, we used electrophoretic mobility shift assays (EMSAs) to test for binding of purified MBP-Nla6 DBD, MrpC, and FruA separately, and in combination because MrpC and FruA were shown previously to bind cooperatively to the \textit{dev}, \textit{fmgA}, \textit{fmgBC}, \textit{fmgD}, and \textit{fmgE} promoter regions (18, 28-31).

As expected (28), MrpC and FruA bound individually to the \textit{dev} promoter region fragment and the combination of MrpC and FruA exhibited greater binding (Fig. 8), suggestive of cooperative binding. MrpC and FruA DBD also appeared to bind cooperatively (Fig. S8), indicating the N-terminal domain of FruA was not required. Binding of MBP-Nla6 DBD was not
detected at 4 μM (Fig. 8), consistent with previous reports (19, 50), but binding was detected at 16 μM and increased at higher concentrations (Fig. S8).

The fadIJ promoter region fragment yielded results that were in some ways similar to those for the dev fragment. MrpC and FruA bound individually to the fadIJ fragment, and the combination of MrpC and FruA appeared to bind cooperatively, but with much less cooperative binding than to the dev fragment (Fig. 8). The order of MrpC and FruA addition did not affect cooperative binding to either fragment (Fig. 8 and S8). However, MrpC and FruA DBD did not bind cooperatively to the fadIJ fragment, indicating a requirement for the FruA N-terminal domain, in contrast to the dev fragment (Fig. S8). On the other hand, for both fragments, MBP-Nla6 DBD binding was not detected at 4 μM and at that concentration did not affect binding of MrpC or FruA (Fig. 8), but MBP-Nla6 DBD binding was detected at 16 μM and increased at higher concentrations (Fig. S8). The apparent cooperative binding of MrpC and FruA to the fadIJ fragment, together with our measurements of the fadl transcript level, support a similar mechanism of developmental regulation of the fadIJ operon as proposed for the dev operon (16, 28), the fmg genes (18, 29-31), and likely many other genes (27), in which C-signaling activates FruA to bind DNA cooperatively with MrpC and increase transcription.

MBP-Nla6 DBD at 2 μM bound to the exoA-I promoter region fragment (Fig. 8), as expected (50). FruA also bound (Fig. 8), as expected based on FruA DBD binding observed previously (49). Strikingly, MBP-Nla6 DBD and FruA appeared to bind cooperatively and the order of addition made no difference (Fig. 8 and S8). FruA DBD exhibited very little cooperative binding with MBP-Nla6 DBD (Fig. S8), suggesting the N-terminal domain of FruA plays an important role. Although MrpC appeared to negatively regulate the exoA transcript level independently of FruA.
(Fig. 5A), binding of MrpC was not detected at 4 µM (Fig. 8) or at concentrations ranging up to 20 µM (Fig. 8S), and MrpC did not affect MBP-Nla6 DBD or FruA binding (Fig. 8). Taken together, our results suggest that MrpC regulates exoA indirectly both independently of FruA (Fig. 5A) and because MrpC is required for synthesis of FruA (16, 49), which together with Nla6 appears to directly regulate exoA-I.

We discovered that FruA binds to the exol-P promoter region fragment (Fig. 8). Binding of MBP-Nla6 DBD was not detected at 4 µM in the experiment shown in Figure 8, but was detected at 4 µM in two other experiments (Fig. S8). Interestingly, MBP-Nla6 DBD and FruA appeared to bind cooperatively and the order of addition made no difference, similar to our results for the exoA-I promoter region fragment (Fig. 8 and S8). Both fragments also yielded similar results in other experiments; FruA DBD exhibited very little cooperative binding with MBP-Nla6 DBD (Fig. S8) and MrpC did not affect MBP-Nla6 DBD or FruA binding (Fig. 8).

However, the two fragments differed in terms of MrpC binding, which was not detected for the exoA-I promoter region fragment at MrpC concentrations ranging up to 20 µM (Fig. 8S). In contrast, for the exol-P promoter region fragment, although binding of MrpC was not detected at 4 µM in the experiment shown in Figure 8D, binding was detected at 4 µM and increased at higher concentrations in another experiment (Fig. S8). Therefore, MrpC, as well as FruA and Nla6, appear to directly regulate exol-P, differentiating its regulation from that of exoA-I (see Discussion).

We found that MBP-Nla6 DBD, MrpC, and FruA could each bind individually to the nfsA-H promoter region fragment (Fig. 8). In combination with MBP-Nla6 DBD at 4 µM, individual binding of MrpC was not detected and the intensity of the shifted complex increased at the
lagging edge (Fig. 8), suggestive of cooperative binding. In agreement, MBP-Nla6 DBD at 2 μM appeared to bind cooperatively with MrpC, and the order of addition made little or no difference (Fig. S8). We note that the large size of MBP-Nla6 DBD and the DNA fragment likely explain the small difference in migration of the putative MBP-Nla6 DBD/MrpC/DNA complex compared to the MBP-Nla6 DBD/DNA complex. Like MrpC, FruA appeared to bind cooperatively with MBP-Nla6 DBD (Fig. 8 and S8). In the mixtures with MBP-Nla6 DBD, individual binding of FruA was detected, suggestive of less cooperativity than in the case of MrpC. FruA DBD did not appear to bind cooperatively MBP-Nla6 DBD, and FruA did not appear to bind cooperatively with MrpC (Fig. S8). We conclude that MBP-Nla6 DBD, MrpC, and FruA appear to directly regulate \( nfas-A-H \) and we note that the apparent cooperative binding of MBP-Nla6 DBD and MrpC to the \( nfas-A-H \) promoter region fragment distinguishes it from the \( exoA-I \) and \( exol-P \) fragments we tested.

DevI negatively regulates the transcript levels of the late-acting operons

Reporter activity from fusions to \( exoc \) (48) and \( nfasA \) (44) was reduced in a devRS insertion mutant compared with a WT strain during development. The \( exoA \) transcript level was also reduced in a devS in-frame deletion mutant compared with a WT strain during development (41). To further investigate the effects of mutations in components of the dev CRISPR-Cas system on regulation of the late-acting operons, we measured transcript levels in devl and devS in-frame deletion mutants. Sporulation occurs about 6 h earlier than normal in the devl mutant (16, 41), suggesting that Devl delays sporulation of the WT strain. Sporulation is severely
impaired in the devS mutant (16, 39) due to loss of negative autoregulation of dev transcription
and resulting overproduction of Devl, which strongly inhibits sporulation (40).

We found that the exoA, exol, nfsA, and fadl transcript levels remain low in the devS
mutant at 18-30 h PS (Fig. 9). We infer that Devl overproduction in the devS mutant inhibits
accumulation of transcripts from the late-acting operons. Although Student’s two-tailed t-tests
rarely yielded $p < 0.05$ comparing transcript levels in the devS mutant with the WT strain at the
corresponding time PS, this was due to the large variation between biological replicates of the
WT strain. We emphasize that transcript levels were low in all biological replicates of the devS
mutant at all times PS.

The average transcript level was greater in the devl mutant than the WT strain, except the
nfsA transcript levels were equal at 30 h PS (Fig. 9). These results are consistent with the notion
that Devl inhibits accumulation of transcripts from the late-acting operons. Here again,
Student’s two-tailed t-tests rarely yielded $p < 0.05$, but in this case there was large variation
between biological replicates of both the devl mutant and the WT strain. Even so, taken
together, the measurements of transcript levels in the devl and devS mutants support that Devl
is a negative regulator of the late-acting operons.

Discussion

Our results reveal complex regulation of genes involved in spore metabolism and spore coat
biogenesis during M. xanthus development (Fig. 10). Prior to its activation by C-signaling, FruA
appears to negatively regulate transcription of the late-acting fadlJ, nfsA-H, exoA-I, and exol-P
operons (red line with barred end), then C-signal-activated FruA (designated FruA*) appears to
positively regulate transcription of these operons during spore formation (green arrow).

However, each operon is unique in terms of the strength of negative and positive regulation by FruA. Moreover, MrpC differentially regulates each operon, exerting negative and/or positive effects independently of FruA/FruA* or cooperatively. The negative effect of MrpC on exoA-I appears to be indirect since binding to the promoter region \textit{in vitro} was not detected (dashed red line with barred end), but the effects of MrpC on the other three operons may be direct since MrpC binds to their promoter regions (red line with barred end to \textit{exoL-P} early, green arrow to \textit{nfsA-H} early, and green arrows to \textit{exoL-P} and \textit{fadIJ} late). FruA binds to all four promoter regions. Unusually for bacterial genes, a third transcription factor, Nla6, also binds to the promoter regions. Interestingly, Nla6 bound cooperatively with FruA and with MrpC to the \textit{nfsA-H} promoter region, but Nla6 bound cooperatively only with FruA to the \textit{exoA-I} and \textit{exoL-P} promoter regions. Nla6 appears to positively regulate all four operons early in development (green arrows). The positive effect of Nla6 on \textit{fadIJ} may be indirect (dashed green arrow) since binding to the promoter region required a high concentration of Nla6. DevI negatively regulates transcript levels from all four operons (dashed red line with barred end), providing new insight into the regulation of \textit{M. xanthus} development by a CRISPR-Cas system. We conclude that three transcription factors and a CRISPR-Cas component regulate the late-acting operons and we propose this ensures that spore resistance and surface characteristics meet environmental demands.

\textit{New insights into the functions of the late-acting operons}
Our results show that mutations in exoC, nfsA-H, and exoL do not prevent the initial cellular shape change associated with sporulation (Fig. S1B), but do impact the formation of sonication-resistant spores beginning at 27 h PS (Fig. 2). Previously, these mutants were examined for starvation-induced spore formation at 120 h (45, 50, 51). Our findings indicate a much earlier role of the ExoA-I, NfsA-H, and ExoL-P proteins during starvation-induced sporulation than established previously. Glutaraldehyde fixation of cells followed by brief sonication allowed us to visualize and enumerate cells that appeared to be transitioning from rods to spores in samples of the starved mutants (Fig. S1B). These TCs likely resemble cells of exo and nfs mutants that fail to complete morphogenesis upon chemical induction of sporulation in liquid culture (45, 46, 51).

The WT strain may exhibit a higher percentage of TCs than the fadI mutant (Fig. S1B) because the mutant makes more sonication-resistant spores (Fig. 2). The fadI insertion mutant presumably has a reduced rate of fatty acid β-oxidation, as appeared to be the case for a fadIJ (MXAN5372-MXAN5371) deletion mutant (42), so perhaps altered metabolism enhanced formation of sonication-resistant spores by the fadI mutant at 27-48 h (Fig. 2), albeit not mature spores at 72 h (Table S1).

The nfsA-H mutant made about twofold less sonication-resistant spores than the WT strain, whereas the exoC and exoL mutants made less than the detection limit (Fig. 2). For these three mutants, the lower percentage of TCs as compared with the WT strain at 24 and 27 h (Fig. S1B) may reflect reduced ability to initiate and/or maintain the cellular shape change associated with sporulation. Upon chemical induction of sporulation, exoC, nfsA-H, and exoL mutants appeared to initiate the transition from rods to spores, but then revert into rods or form branched rods,
ovoid-shaped cells, or large spheres (45, 46, 51). Given the evidence that Exo and Nfs proteins function in spore coat polysaccharide export (45, 46, 51, 54) and assembly (45-47), respectively, our results suggest that defective spore coat biogenesis of the exoC, nfsA-H, and exoL mutants reduces their ability to maintain cellular shape change as early as 24 h (Fig. S1B) and blocks or reduces their ability to form sonication-resistant spores by 27 h (Fig. 2) and mature spores by 72 h (Table S1) (Fig. 10).

The milder sporulation defect of the nfsA-H mutant compared with the exoC and exoL mutants (Fig. 2, Table S1) is consistent with previous reports (45, 50, 51). The exoC mutant fails to export spore coat polysaccharide, whereas the nfsA-H mutant appears to export it, but fails to assemble it properly, producing an amorphous, unstructured spore coat that nevertheless appears to provide some heat- and sonication-resistance (45). The Nfs machinery shortens the polysaccharide chains, decreases their surface abundance, and with the help of the AglQRS motor assembles the compact, rigid, fully stress-resistant coat of mature spores (46, 47).

Unactivated FruA negatively regulates transcription of the late-acting operons early in development

Our measurements of transcript levels in a fruA mutant indicate that FruA negatively regulates the late-acting operons. The fruA mutation elevated the average exoA, exoL, and nfsA transcript levels 17-, 57-, and 4-fold, respectively, relative to the WT strain at 18 h PS (Fig. 3). The fruA mutation did not elevate the average fadl transcript level compared to the WT strain, but the fadl transcript level was 8-fold greater in the fruA mutant than in the csgA mutant (p = 0.002 in a Student’s two-tailed t-test). Since FruA* appears to be present by 18 h in the WT
strain (16), positive regulation by FruA* likely counteracts negative regulation by unactivated FruA. However, in the csgA mutant, only unactivated FruA is present, resulting in a very low fadl transcript level, as well as very low transcript levels from the other late-acting operons (Fig. 3). Neither mrpC nor csgA mutations altered the degradation rates of transcripts from the late-acting operons (Fig. 4 and S3). Therefore, since unactivated FruA is absent from the mrpC mutant and present in the csgA mutant (16), we conclude that unactivated FruA regulates the synthesis rather than the degradation of transcripts from the late-acting operons at 18 h, which we consider early in development since mounds (Fig. 1) but not spores have formed (Fig. 2).

This negative regulation of transcription by unactivated FruA is summarized in Figure 10 by a red line with barred end that extends from FruA toward a gray box including all four late-acting operons.

Our results agree qualitatively with two prior reports of negative regulation by FruA early in development. Expression of a reporter fused to the nfsA-H promoter region was elevated 2-fold in a fruA mutant compared with a WT strain at 12-18 h PS (44). We observed that the average nfsA transcript level was consistently elevated at least 4-fold in a fruA mutant compared with a WT strain at 18 h (Fig. 3C, 5C, and 6C), but elevated in only one of three replicates at 12 h (Fig. 6C). The difference in the strength of regulation between the studies may be due to differences in the strains and/or the methods. The difference between replicates at 12 h in our study reflects experimental variation in the timing of induction of nfsA-H transcription.

While our work was in progress, transcriptomic analysis of a fruA mutant compared with a WT strain identified > 1000 genes that were negatively regulated by FruA at one or more time
points during the first 24 h of development (55). The transcript levels of all genes in the late-acting operons were elevated in a fruA mutant relative to a WT strain at 12 h (Table S2). In our study, the transcript levels were not elevated in the fruA mutant relative to the WT strain at 12 h (Fig. 6), except the one replicate for nfsA mentioned above (Fig. 6C). The strength of regulation also differed between the studies (compare Fig. 3 and Table S2). Interestingly, the fold increase in transcript levels varied greatly for different genes in the exoA-I and exoL-P operons (Table S2), perhaps reflecting differential regulation. We measured transcript levels of the first gene in each operon, but even focusing on the results for the first gene in each operon reveals large differences between the studies in the strength of regulation (i.e., 25-, 11-, and 15-fold differences for exoA, exoL, and nfsA, respectively, between the 12-h values in Table S2 and the 18-h values mentioned above for Fig. 3). In support of our conclusion that regulation of fadIJ switches from negative by unactivated FruA to positive by FruA*, fadIJ transcript levels were elevated 2-fold in a fruA mutant compared with a WT at 12 h, but elevated 4-fold in the WT strain compared with the fruA mutant at 24 h (Table S2). Importantly, we note that exoA-I, exoL-P, and nfsA-H transcript levels decline sharply in the fruA mutant relative to the WT strain between 12 and 24 h, which may be a signature for late-acting genes in the data of McLoon et al. (55).

MrpC and Nla6 contribute to differential regulation of the late-acting operons

The switch from negative regulation of fadIJ transcription by unactivated FruA to positive regulation by FruA* requires MrpC (Fig. 10) since the fadI transcript level did not increase in the mrpC mutant (Fig. 5D and 6D) or the mrpC mutant engineered to express FruA from a vanillate-
inducible promoter (Fig. 6D). MrpC and FruA bound individually and with weak cooperativity to
the fadIJ promoter region in vitro (Fig. 8 and S8). Cooperative binding of MrpC and FruA in vitro
has been correlated with positive regulation in vivo for dev (28) and fmg (16, 18, 29-31) genes,
leading to a model in which MrpC and FruA* bind cooperatively upstream of the promoter and
increase transcription. Positive regulation of fadIJ transcription appears to fit this model since
expression from a fadl reporter fusion was twofold lower in mrpC, fruA, and csgA mutants than
in a WT strain at 18 and 24 h PS (42), and matches to the consensus sequences for MrpC and
FruA binding are present upstream of the promoter (Fig. S9). In particular, the matches to the
consensus sequences for MrpC and FruA binding centered at -96 and -69.5, respectively, are
well-positioned to explain cooperative binding of MrpC and FruA* that could increase
transcription. Bullock et al. did not observe elevated expression in a fruA mutant relative to the
other strains (i.e., evidence for negative regulation by unactivated FruA), perhaps due to
differences in the strains and/or the methods. The negative regulation of fadIJ by unactivated
FruA that both we and McLoon et al. observed (as explained above) distinguishes the regulation
of fadIJ transcription from that of dev (Fig. 10) and fmg genes since neither reporter fusion
expression nor transcript levels of dev (15, 16, 55, 56) and fmg (29, 30, 55, 57, 58) genes are
elevated in fruA mutants (Table S3). Perhaps unactivated FruA binds to the putative site
centered at -69.5 and/or other putative FruA-binding sites upstream of the fadIJ promoter (Fig.
S9), preventing a low level of transcription that can occur in the absence of FruA (Fig. 3D, 5D,
and 6D) (55) (Table S2) but not in the absence of MrpC (Fig. 5D and 6D).

Regulation of the nfsA-H operon by MrpC was unique among the late-acting operons since
the nfsA transcript level was much greater in the fruA mutant than in the WT strain or the mrpC
mutant at 18 h PS (Fig. 5C and 6C). Since the MrpC level in the fruA mutant is similar to that in
the WT strain (16) and both MrpC and FruA bind independently (without cooperativity) to the
nfsA-H promoter region (Fig. 8 and S8), we infer that MrpC can directly increase nfsA-H
transcription but that unactivated FruA directly opposes an increase early in development (Fig.
10). Later (e.g., at 27 and 30 h), FruA* appears to be required to increase nfsA-H transcription
(Fig. 3C and can do so without MrpC (Fig. 6C, note the y-axis scale is different than in Fig. 3C)
(Fig. 10). The best match to the consensus sequence for MrpC binding is centered at -29 in the
nfsA-H promoter region (Fig. S9), consistent with the location of an MrpC-binding site identified
by ChIP-seq analysis (27) (Fig. S7). The best match to the consensus sequence for FruA binding
is centered at -82.5. Binding of MrpC and FruA to sites centered at -29 and -82.5, respectively,
may explain the lack of cooperative binding in vitro (Fig. 8 and S8), but how unactivated FruA
bound to the upstream site would oppose an increase in transcription caused by MrpC bound
to the downstream site early in development is unclear (Fig. 10). We note that upstream of the
putative FruA-binding site centered at -82.5 there are several overlapping sequences that
match the FruA and MrpC consensus sequences except at one or two positions, respectively
(Fig. S9). If those putative MrpC-binding sites increase transcription directly or indirectly (e.g.,
by aiding recruitment of MrpC to the site centered at -29), then binding of unactivated FruA to
overlapping sites could oppose an increase in transcription early in development (Fig. 10).
Alternatively, unactivated FruA bound to any of three putative upstream sites may oppose an
increase in transcription mediated by Nla6. MBP-Nla6 DBD bound cooperatively with FruA to
the nfsA-H promoter region (Fig. 8 and S8), and a sequence matching the Nla6 half-site
consensus sequence (50) except at one position is centered at -100.5 (Fig. S9) and may be
involved in the cooperative binding. Testing these hypotheses and elucidating how FruA* increases transcription later in development will require much more work. MBP-Nla6 DBD also bound cooperatively with MrpC to the nfsA-H promoter region (Fig. 8 and S8), and a putative Nla6 half-site centered at -42.5 appears to be well-positioned for cooperative binding, but it is unclear whether this affects transcription.

Regulation of the exoA-I and exoL-P operons by MrpC was different from that of the nfsA-H and fadJ operons since the average exoA and exoL transcript levels were greater in the mrpC mutant than in the WT strain or the fruA mutant at 18 h PS (Fig. 5AB and 6AB). These results suggest that MrpC contributes to negative regulation of the exoA-I and exoL-P operons both independently of FruA and because MrpC is required for synthesis of unactivated FruA (16, 49), which negatively regulates the operons early in development (Fig. 10).

Negative regulation of exoA-I by MrpC independently of FruA appears to be indirect (dashed red line with barred end in Fig. 10) since binding of MrpC to the promoter region in vitro was not detected (Fig. S8). Negative regulation of exoA-I by unactivated FruA early in development may involve overlapping FruA- and Nla6-binding half-sites (Fig. S7 and S9). The FruA-binding site depicted as centered at -75.5 in Figure S7 based on DNase I footprinting (49), encompasses two matches to the FruA consensus binding sequence, and the match centered at -83 overlaps a putative Nla6-binding half-site centered at -88.5 (Fig. S9). A putative FruA-binding site centered at -42 overlaps two putative Nla6-binding half-sites. FruA and MBP-Nla6 DBD bound cooperatively to the exoA-I promoter region (Fig. 8 and S8). We propose that unactivated FruA interferes with transcriptional activation by Nla6 early in development. The very low exoA transcript level in a csgA mutant (Fig. 3A) despite a similar transcript degradation rate as the
WT strain (Fig. 4AE), and our finding that FruA expression increases the transcript level in the absence of MrpC (Fig. 6A), suggest that FruA* increases exoA-I transcription later in development (Fig. 10). In contrast, Nla6 negatively regulates the exoA transcript level twofold at 24 h PS (50) (Fig. 10). Perhaps the concentration of Nla6 decreases during development and/or C-signal-dependent formation of FruA* increases its binding to DNA or changes its cooperative binding with Nla6, allowing FruA* to activate exoA-I transcription.

Negative regulation of exoL-P by MrpC independently of FruA may involve overlap of an MrpC-binding site with an Nla6-binding half-site. The best match to the consensus sequence for MrpC binding is centered at -99 and partially overlaps a putative Nla6-binding half-site centered at -89.5 (Fig. S7 and S9). Both MrpC and MBP-Nla6 DBD bound weakly to the exoL-P promoter region and the two proteins did not bind cooperatively (Fig. 8 and S8). In this case, MrpC may compete with Nla6 for binding and thus interfere with transcriptional activation by Nla6 early in development (Fig. 10). In contrast, negative regulation by unactivated FruA may involve cooperative binding with Nla6, as proposed for exol-A and possibly nfsA-H transcription, since FruA and MBP-Nla6 DBD bound cooperatively to the exoL-P promoter region (Fig. 8 and S8). Putative Nla6-binding half-sites centered at -115.5 and -46.5 are adjacent to putative FruA-binding sites centered at -130 and -62, respectively (Fig. S7 and S9), which may allow cooperative binding that interferes with transcriptional activation by Nla6 early in development (Fig. 10). For similar reasons as mentioned above for the exoA transcript, our results for the exoL transcript (Fig. 3B, 4BE, and 6B) suggest that FruA* increases exoL-P transcription later in development, and previous work showed that Nla6 negatively regulates the exoL transcript level twofold at 24 h PS (50) (Fig. 10). Therefore, similar mechanisms may allow FruA* to
activate exoL-P and exoA-I transcription. A striking difference between regulation of exoL-P and exoA-I late in development is that MrpC positively regulates the exoL-P transcript level independently of FruA (Fig. 5AB and 10). Consistent with MrpC acting independently of FruA, the two proteins did not appear to bind cooperatively to the exoL-P promoter region (Fig. 8), perhaps because the distance between putative binding sites is too great (Fig. S9).

To summarize, null mutations in mrpC and fruA uniquely affect the transcript levels from each of the late-acting operons. The observed differential regulation may be explained in part by the positions and affinities of binding sites for MrpC, FruA, and Nla6 in each promoter region. Cooperative binding also appears to be involved, exerting positive or negative effects.

The C-signal-dependent switch from negative regulation by unactivated FruA early in development to positive regulation by FruA* later also plays an important role. This process, as well as potential changes in the levels and activities of MrpC and Nla6, will require further investigation in order to better understand the differential regulation of each late-acting operon.

DevI is a negative regulator of the late-acting operons

Our results confirm and extend the role of Devl as a negative regulator of late-acting operons during development. Previous studies implicated DevRS proteins in positive regulation of exoA-I (48) and nfsA-H (44). Subsequently, DevTRS proteins were shown to negatively autoregulate the dev operon, which codes for Devl, a 40-residue protein that strongly inhibits sporulation when overproduced (40) and delays sporulation by about 6 h when produced at the normal level (16, 41). As expected, the exoA transcript level was very low in a devS null mutant (41).
We confirmed that result and also showed that the exoL, nfsA, and fadl transcript levels are very low in the devS mutant at 18-30 h PS (Fig. 9). We also found that transcript levels from the late-acting operons were slightly greater on average in a devI null mutant than in the WT strain, supporting our conclusion that DevI is a negative regulator of the late-acting operons late in development (Fig. 10). However, the mechanism of negative regulation by DevI is unknown.

The devS mutant failed to undergo normal cellular shape change as determined by light microscopic observation (41), presumably because DevI overproduction inhibits cellular shape change (Fig. 10). This could be a cumulative effect of failure to express the late-acting operons, given that mutations in the individual operons did not prevent cellular shape change (Fig. S1B).

Alternatively, one or more other genes subject to negative regulation by DevI overproduction may account for the failure of the devS mutant to undergo normal cellular shape change. The method devised in this work to quantify TCs after glutaraldehyde fixation and brief sonication, and the recently reported in situ confocal microscopy approaches (33), would provide more sensitive measures of cellular shape change by the devS mutant than the light microscopy method (41).

Combinatorial control of genes involved in spore formation

Eukaryotic-like signaling and gene regulation has been known in M. xanthus for decades (59, 60). More recently, combinatorial control, a common theme in eukaryotic gene regulatory networks (61), emerged from studies of M. xanthus gene regulation (4). Combinatorial control allows signal integration by utilizing two or more signal-responsive transcription factors to directly regulate the same gene. During M. xanthus development, the cascade of enhancer-
binding proteins (EBPs) that includes Nla6 has several instances of combinatorial control by two  
EBPs (4, 19, 62). MrpC and FruA appear to combinatorially control the dev operon (16, 28), the  
fmg genes (18, 29-31), and likely many other genes (27) by binding cooperatively to the  
promoter regions. MrpC and FruA also bind cooperatively to the promoter region of the fadII  
operon (Fig. 8 and S8) involved in spore fatty acid metabolism (42). Combinatorial control by  
cooperative binding of MrpC and FruA may ensure that only starving rods aligned in mounds  
commit to spore formation (4, 18, 25, 33, 34).  
Importantly, combinatorial control of the nfsA-H, exoA-I, and exoL-P operons involved in  
spore coat biogenesis does not appear to involve cooperative binding of MrpC and FruA (Fig. 8  
and S8). Rather, MBP-Nla6 DBD bound cooperatively with FruA to all three promoter regions  
and with MrpC only to the nfsA-H promoter region. Based on our current understanding of the  
signaling and gene regulatory network, Nla6 indirectly responds to starvation and cell density-  
dependent A-signaling early in development (4, 19). It is unclear whether Nla6 responds to  
other signals later in development. A high cell density near the radial center of early nascent  
fruiting bodies correlates with a high spore proportion later (33), so perhaps A-signaling plays a  
role in early spatial patterning of subsequent sporulation via Nla6-mediated positive regulation  
(Fig. 10). Alternatively or in addition, negative regulation by Nla6 of exoA-I and exoL-P later in  
development may prevent premature synthesis, export, and modification of the spore coat  
polysaccharide. Combinatorial control of exoL-P also appears to involve MrpC binding  
separately (i.e., without cooperativity) (Fig. 8) and regulating negatively early in development  
and positively later (Fig. 10).
We propose that combinatorial control of the late-acting operons by signal-responsive transcription factors allows proper temporal and spatial expression of genes involved in spore metabolism and coat biogenesis in order to produce spores that are densely packed in fruiting bodies and suited to withstand environmental insults. Several transcription factors likewise positively and negatively fine-tune the expression of hundreds of genes during *Bacillus subtilis* endospore formation (63-65), ensuring that the resulting spores are endowed with resistance and surface properties tailored for their environment (66-68). In *M. xanthus*, the Dev proteins of a CRISPR-Cas system provide yet another layer of regulation of the late-acting operons (Fig. 9 and 10), and one signal to which this system responds may be bacteriophage infection (39).

In summary, our investigation uncovered complex regulation of genes involved in spore metabolism and spore coat biogenesis by three transcription factors and a CRISPR-Cas component during *M. xanthus* development. Further elucidating the signals to which Nla6, MrpC, and FruA respond, understanding how these transcription factors interact at promoter regions with many potential binding sites (Fig. S9), and elucidating how DevI inhibits the cellular shape change associated with sporulation, are important goals for the future.

**Material and Methods**

**Bacterial strains, plasmids and primers**

The strains, plasmids and primers used in this study are listed in Table S4.

*M. xanthus* strain MSS2 with P*van−fruA* integrated ectopically was constructed by electroporating (69) pSS10 into strain DK1622, selecting transformants on CTT agar (1% Casitone, 10 mM Tris-HCl [pH 8.0], 1 mM KH<sub>2</sub>PO<sub>4</sub>-K<sub>2</sub>HPO<sub>4</sub>, 8 mM MgSO<sub>4</sub> [final pH 7.6] solidified
with 1.5% agar) containing 15 µg/mL tetracycline (70), and verification by colony PCR using primers pMR3691 MCS G-F and pMR3691 MCS G-R.

*M. xanthus* strain MSS10 with a plasmid insertion mutation in *nla6* was constructed by electroporating pSS11 into strain DK1622, selecting transformants on CTT agar containing tetracycline (15 µg/mL), and verification by colony PCR using primers PMR3487 Rev, Nla6 Fwd4, and Nla6 Fwd5. To construct pSS11, primers Nla6 Fwd and Nla6 Rev were used to generate a PCR product using chromosomal DNA from *M. xanthus* strain DK1622 as a template. The product was combined with DNA amplified from pMR3487 using primers PMR3487G Fwd and PMR3487G Rev primers, and a Gibson assembly reaction was used to enzymatically join the overlapping DNA fragments (71). The reaction mixture was transformed into *E. coli* strain DH5α, transformants were selected at 37°C on Luria-Bertani (LB) (72) 1.5% agar containing 15 µg/mL tetracycline, and the cloned DNA sequence was verified using primers 3487 seq Fwd1, 3487 seq Fwd2, 3487 seq Fwd3, 3487 seq Fwd4, and 3487 seq Fwd5.

To provide a DNA template for amplification of the *nfsA-H* promoter region, pSS14 was constructed as follows. Primers Nfs -290G and Nfs +83G were used to generate a PCR product using chromosomal DNA from *M. xanthus* strain DK1622 as a template. The product was combined with DNA amplified from pMR3487, the overlapping DNA fragments were joined using a Gibson assembly reaction, the reaction mixture was transformed into *E. coli* strain DH5α, transformants were selected, and the cloned DNA sequence was verified as described above for pSS11.

To express MBP-Nla6 DBD for purification, primers Nla DBD For and Nla DBD Rev were used to generate a PCR product using *nla6* DNA as a template. The PCR product was digest with *Sbf1*
and ligated to XmnI-SbfI-digested pMAL-c5x (NEB), resulting in pMAL-c5x/MBP-ΔCNla6 DBD, which lacked a CG bp at the beginning of the Nla6 DBD-encoding segment. The missing CG bp was added by performing site-directed mutagenesis using the QuikChange strategy (Stratagene) with primers MBP-Nla6 add C fwd and MBP-Nla6 add C rev, resulting in pMAL-c5x/MBP-Nla6 DBD, and the DNA sequence was verified using primer mbp fwd colpcr.

**Growth and development of *M. xanthus***

Strains of *M. xanthus* were grown at 32°C in CTYTE liquid medium (CTT medium with 0.2% yeast extract) with shaking at 350 rpm. CTT agar was used for growth on solid medium and was supplemented with 40 µg/mL of kanamycin sulfate or 15 µg/mL of tetracycline as required. Fruiting body development under submerged culture conditions was performed using MC7 (10 mM morpholinepropanesulfonic acid [MOPS; pH 7.0], 1 mM CaCl₂) as the starvation buffer as described previously (25). Briefly, cells in the mid-exponential phase of growth were collected by centrifugation, the supernatant was removed, and the cells were resuspended in MC7 buffer at a density of approximately 1,000 Klett units. A 96-µL sample (designated T₀) was removed and 4 µL of glutaraldehyde was added from a 50% stock solution to achieve a 2% final concentration. The sample was stored at 4°C for at least 24 h in order to fix the cells for later quantification of total cells as described below. For each developmental sample, 1.5 mL of the 1,000-Klett-unit cell suspension plus 10.5 mL of MC7 buffer was added to an 8.5-cm-diameter plastic petri plate. Upon incubation at 32°C, cells adhere to the bottom of the plate and undergo development. At the indicated times, developing populations were photographed using a Leica Wild M8 microscope equipped with an Olympus E-620 digital camera.
Sample collection

At the indicated times PS, the MC7 buffer overlay was replaced with 5 mL of fresh MC7 buffer or 5 mL of MC7 buffer containing 50 μg/mL of rifampicin to inhibit transcription for measurement of transcript degradation rates. Developing cells were scraped from the bottom of the plates, the entire contents were collected in a 15-mL centrifuge tube, and samples were mixed thoroughly as described previously (16). A 96-μL sample was removed, glutaraldehyde was added, and the sample was stored as described above for the T₀ sample, fixing the cells for quantification of total cells as described below. A 400 µL sample was removed and stored at -20°C for measurement of sonication-resistant spores as described below. For the experiments shown in Figures 7 and S4, a 100 μl sample was removed and added to an equal volume of 2× sample buffer (0.125 M Tris-HCl [pH 6.8], 20% glycerol, 4% sodium dodecyl sulfate [SDS], 0.2% bromophenol blue, 0.2 M dithiothreitol), boiled for 5 min, and stored at -20°C for immunoblot analysis. Immediately after collecting the samples just described, the rest of the 1,000-Klett-unit cell suspension was mixed with 0.5 mL of RNase stop solution (5% phenol [pH < 7] in ethanol), followed by rapid cooling in liquid nitrogen until almost frozen, centrifugation at 8,700 × g for 10 min at 4°C, removal of the supernatant, freezing of the cell pellet in liquid nitrogen, and storage at -80°C until RNA extraction.

Quantification of total cells, transitioning cells, and sonication-resistant spores

The total number of cells, including rods, elliptical TCs, and round spores, was determined using the glutaraldehyde-fixed samples collected as described above. Each sample was thawed and
mixed by vortexing and pipetting, diluted with MC7 buffer, sonicated for 10 s, and then all cells were counted microscopically as described previously (16), except noting the number of cells that were elliptical or round. The number of sonication-resistant spores in the 400-µL samples collected as described above was quantified as described previously (25). These samples were not glutaraldehyde-fixed and were sonicated for 10 s intervals three times with cooling on ice in between, leaving only round spores (i.e., neither rods nor elliptical TCs were observed). The total cell number minus the number of sonication-resistant spores was designated the number of sonication-sensitive cells (consisting primarily of rods and a small percentage of TCs) and was expressed as a percentage of the total cell number in the corresponding T₀ sample (consisting only of rods). The number of cells that were elliptical or round in the glutaraldehyde-fixed sample minus the number of sonication-resistant spores in the corresponding sample that was not glutaraldehyde-fixed, was designated the number of TCs and was also expressed as a percentage of the total cell number in the corresponding T₀ sample.

**RNA extraction and analysis**

RNA was extracted using the hot-phenol method, followed by digestion with DNase I (Roche) as described previously (23). The RNA (1 µg) was then subjected to cDNA synthesis using Superscript III reverse transcriptase (InVitrogen) and random primers (Promega), as instructed by the manufacturers. In parallel, RNA (1 µg) was subjected to cDNA synthesis reaction conditions without Superscript III reverse transcriptase, as a control. One µl of cDNA at the appropriate dilution (as determined empirically) and 20 pmol of each primer were subjected to qPCR in a 25 µl reaction using 2× reaction buffer as described previously (16). qPCR was done...
in quadruplicate for each cDNA using a LightCycler® 480 System (Roche). In parallel, a standard
curve was generated for each pair of qPCR primers using genomic DNA (gDNA) from *M. xanthus*
WT strain DK1622 and gene expression was quantified using the relative standard curve
method (user bulletin 2; Applied Biosystems). 16S rRNA was used as the internal standard for
each sample. The primers used for RT-qPCR analysis of 16S rRNA (73) and the late-acting
operons (26) have been described previously.

Relative transcript levels for mutants are the average of three biological replicates after
each replicate was normalized to the transcript level observed for one replicate of the WT
strain at the earliest time point (i.e., either 6 or 18 h PS) in the same experiment (16).

Transcript levels for the WT strain at other times PS were likewise normalized to that observed
for the WT strain at the earliest time point in the same experiment. For the WT strain at the
earliest time point, the transcript levels of at least three biological replicates from different
experiments were normalized to their average, which was set as 1.

To measure transcript degradation rates, samples were collected immediately after addition
of rifampicin (designated t₀) and 8 and 16 min later for RNA extraction and analysis as described
above. For each biological replicate the transcript levels after 8 and 16 min were normalized to
the transcript level at t₀, which was set as 1, and the natural log of the resulting values was
plotted versus minutes after rifampicin treatment (16). The slope of a linear fit of the data was
used to compute the mRNA half-life.

**Expression and purification of proteins**
To express His-tagged proteins, *E. coli* strain BL21(DE3) was transformed with pET11a/FruA-DBD-H8, pET11km/FruA-H6, or pPH158 (for H6-MrpC) and transformants were selected on LB 1.5% agar containing 50 µg/mL ampicillin or kanamycin sulfate. In each case, a single colony was used to inoculate 10 mL of LB supplemented with antibiotic, followed by overnight incubation at 37°C with shaking. Five mL of overnight culture was used to inoculate 500 mL of the same medium, followed by continued shaking at 37°C until the culture reached 60-80 Klett units. IPTG (1 mM final concentration) was added to induce synthesis of the recombinant protein. After 2 h, cells were harvested as reported previously (57) and stored at -80°C.

To purify His-tagged proteins, a modification of a protocol described previously (74) was used. Each cell pellet was resuspended in 35 mL of lysis buffer (50 mM Na-phosphate buffer pH 8.0, 500 mM NaCl, 14 mM β-mercaptoethanol, protease inhibitors [1 Roche Mini EDTA-free tablet/10 mL]). Cells were disrupted by sonication (4 times for 1 min with intermittent cooling on ice). After centrifugation at 18,000 × g for 10 min at 4°C, the supernatant was mixed with 5 mL of lysis buffer containing 10% w/v Triton X-100, 1 mL of 1 M imidazole (pH 8.0), enough lysis buffer for 50 mL total, and finally 0.5 mL of Ni-NTA agarose (Qiagen) that had been washed 3 times with lysis buffer containing 20 mM imidazole. The mixture was rotated for 1 h at 4°C. The Ni-NTA agarose was collected by centrifugation at 700 × g for 3 min at 4°C and washed 4 times with 50 mL of lysis buffer containing 20 mM imidazole, 0.5% w/v Triton X-100, and 25% w/v glycerol. The Ni-NTA agarose was rotated for 30 min at 4°C with 10 mL of elution buffer (50 mM Na-phosphate buffer pH 8.0, 500 mM NaCl, 1.4 mM β-mercaptoethanol, protease inhibitors [1 Roche Mini EDTA-free tablet], 250 mM imidazole, 0.5% w/v Triton X-100, 25% w/v glycerol). Eluates were dialyzed twice at 4°C against 1 L of storage buffer (10 mM Tris-HCl pH
874 8.0, 100 mM NaCl, 1 mM β-mercaptoethanol, 10% w/v glycerol) and aliquots were stored at -
875 80°C. The protein concentration was determined using a Bradford (75) assay kit (Bio-Rad).
876 To express MBP-Nla6 DBD, *E. coli* strain BL21(DE3) was transformed with pMAL-c5x/MBP-
877 Nla6 DBD and transformants were selected on LB 1.5% agar containing 100 µg/mL ampicillin.
878 Five colonies were used to inoculate 80 mL of LB supplemented with antibiotic and 0.2%
879 dextrose, followed by incubation at 37°C with shaking at 350 rpm until the culture reached 60
880 Klett units. IPTG (0.3 mM final concentration) was added to induce synthesis of the
881 recombinant protein. After 2 h, cells were harvested, the cell pellet was resuspended in 10 mL
882 of column buffer (20 mM Tris-HCl pH 7.5, 200 mM NaCl, 1 mM EDTA, protease inhibitors [1
883 Roche Mini EDTA-free tablet], 10 mM β-mercaptoethanol), and the cell suspension was stored
884 at -20°C.
885 MBP-Nla6 DBD was purified according to instructions in the pMAL™ Protein Fusion &
886 Purification System manual (NEB). Briefly, the cell pellet was thawed and cells were disrupted
887 by sonication (8 times for 15 s with intermittent cooling on ice). After centrifugation at 18,000
888 × g for 20 min at 4°C, the supernatant was loaded onto a 1 mL amylose gravity-flow column,
889 which had been prewashed with 5 mL of column buffer. The loaded column was washed with
890 12 mL of column buffer, then eluted with 2.5 mL of column buffer supplemented with 10 mM
891 maltose, collecting fractions of 4 drops each (approximately 200 µL). Fractions were subjected
892 to SDS-PAGE with Coomassie Blue staining. Fractions 4-9, with maximal purified MBP-Nla6
893 DBD, were pooled, dialyzed as described above, and aliquots were stored at -80°C. The protein
894 concentration was determined by measuring the absorbance at 280 nm and using the
895 calculated extinction coefficient of MBP-Nla6 DBD (1.32 [mg/mL]⁻¹cm⁻¹).
Electrophoretic mobility shift assays

$^{32}$P-labelled DNA fragments were generated by PCR using primers labelled with $\gamma^{32}$P-ATP using T4 polynucleotide kinase (NEB) according to the manufacturer’s instructions. The templates for PCR were either plasmid DNA or gDNA from *M. xanthus* WT strain DK1622. The primers and templates were as follows: primers LK1298 and LK1331 with template pPV391 for *dev*, primers *exoA-I*-171 fwd and *exoA-I*-1 rev with template gDNA for *exoA-I*, primers *fadIJ*-214 fwd and *fadIJ*-5 rev with template gDNA for *fadIJ*, primers *exol-P*-217 fwd and *exol-P* +56 rev with template gDNA for *exol-P*, and primers *nfsA-H*-166 fwd and *nfsA-H* +35 with template pSS14 for *nfsA-H*. The labeled DNA fragments were purified by electrophoresis on 15% polyacrylamide gels, followed by visualization using autoradiography, excision, and overnight elution by soaking in TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA) as described previously (57).

Binding reactions (10 µL) were performed as reported previously (57), except the reaction mixtures were incubated at 37°C for 10 min prior to loading on 8% polyacrylamide gels. To determine whether the order of protein addition was important, one protein was added to the reaction mixture during an initial 10 min incubation at 37°C, followed by addition of a second protein and another 10 min incubation at 37°C, prior to loading on 8% polyacrylamide gels. Gels were dried and exposed to X-ray film for autoradiography as described (57).

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Conception or design of the study: LK, SS
Acquisition of the data: SS, LK
Analysis or interpretation of the data: SS, LK
Writing of the manuscript: LK, SS

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Supporting information captions

Table S1  Cell and spore numbers

Table S2  Fold change in transcript levels in a fruA mutant relative to a WT strain for genes in late-acting operons based on McLoon et al. 2021

Table S3  Fold decrease in transcript levels in a fruA mutant relative to a WT strain for dev and fmg genes based on McLoon et al. 2021
Table S4 Strains, plasmids and primers

Figure S1. Sonication-sensitive cells during M. xanthus development.

Figure S2. Transcript levels during M. xanthus development.

Figure S3. Transcript stability.

Figure S4. Protein levels during M. xanthus development.

Figure S5. Early development of M. xanthus strains.

Figure S6. Late development of M. xanthus strains.

Figure S7. Features of promoter region DNA fragments used in DNA-binding assays.

Figure S8. Binding of the Nla6 DBD, MrpC, FruA, and the FruA DBD to the promoter regions of the late-acting operons.

Figure S9. DNA sequences in the promoter regions of late-acting operons match consensus sequences for binding of MrpC, FruA, and Nla6.
Figure 1. Development of *M. xanthus* strains. The WT strain and its indicated mutant derivatives were subjected to starvation under submerged culture condition and microscopic images were obtained at the indicated times PS. The WT strain and all the mutants except *nfsA-H* formed compact mounds by 18 h (an arrow points to one in each panel). The *nfsA-H* mutant formed compact mounds by 21 h. Mounds of the WT strain began to darken by 30 h. Mounds of the *nfsA-H, exoC*, and *exoL* mutants darkened by 30 h but remained slightly less dark than mounds of the WT strain and the *fadl* mutant by 48 h. Bar, 100 μm. Similar results were observed in at least three biological replicates.
Figure 2. Sonication-resistant spores during *M. xanthus* development. The WT strain and its indicated mutant derivatives were subjected to starvation under submerged culture conditions. Samples were collected at the indicated times PS for quantification of sonication-resistant spores. Values are expressed as a percentage of the number of rod-shaped cells present at the time starvation initiated development (*T₀*) (Table S1). Bars show the average of three biological replicates and error bars indicate one standard deviation.
Figure 3. Transcript levels during *M. xanthus* development. The WT strain and its indicated mutant derivatives were subjected to starvation under submerged culture conditions. Samples were collected at the indicated times PS for measurement of the *exoA* (A), *exoL* (B), *nfsA* (C), and *fadI* (D) transcript levels by RT-qPCR. Graphs show the data points and the average of at least three biological replicates, relative to the WT strain at 18 h PS, and error bars indicate one standard deviation. Asterisks indicate a difference (*p* < 0.05 in Student’s two-tailed *t*-tests) from the WT strain at the corresponding time PS.
Figure 4. Transcript stability. The WT strain and the csgA mutant were subjected to starvation under submerged culture conditions for 30 h. The overlay was replaced with fresh starvation buffer containing rifampicin (50 μg/mL) and samples were collected immediately (t₀) and at the indicated times (tₓ) for measurement of the exoA (A), exoL (B), nfsA (C), and fadI (D) transcript levels by RT-qPCR. Transcript levels at tₓ were normalized to that at t₀ for each of three biological replicates and used to determine the transcript half-life for each replicate. The graph shows the average ln(tₓ/t₀) and one standard deviation for the three biological replicates of the WT strain (black dashed line) and the csgA mutant (gray solid line). The average half-life and one standard deviation, as well as the p value from a Student’s two-tailed t-test, are reported in (E).
Figure 5. Transcript levels in mrpC and fruA mutants. The WT strain and its indicated mutant derivatives were subjected to starvation under submerged culture conditions. Samples were collected at the indicated times PS for measurement of exoA (A), exoL (B), nfsA (C), and fadl (D) transcript levels by RT-qPCR. Graphs show the data points and the average of at least three biological replicates, relative to the WT strain at 18 h PS, and error bars indicate one standard deviation. Asterisks above error bars indicate a difference ($p < 0.05$ in Student’s two-tailed $t$-tests) from the WT strain at the corresponding time PS and asterisks above brackets indicate a difference between the mutants.
Figure 6. Transcript levels early during *M. xanthus* development. The WT strain and its indicated mutant derivatives were subjected to starvation under submerged culture conditions. Samples were collected at the indicated times PS for measurement of exoA (A), exoL (B), nfsA (C), and fadI (D) transcript levels by RT-qPCR. Graphs show the data points and the average of at least three biological replicates, relative to the WT strain at 6 h PS, and error bars indicate one standard deviation. Asterisks above error bars indicate a difference (\(p < 0.05\) in Student’s two-tailed \(t\)-tests) from the WT strain at the corresponding time PS and asterisks above brackets indicate a difference between the mutants.
Figure 7. Transcript levels in nla6 mutants early during M. xanthus development. The WT strain and its nla6 mutant derivatives were subjected to starvation under submerged culture conditions. Samples were collected at the indicated times PS for measurement of exoA (A), exoL (B), nfsA (C), and fdl (D) transcript levels by RT-qPCR. Graphs show the data points and the average of at least three biological replicates, relative to the WT strain at 6 h PS, and error bars indicate one standard deviation. Asterisks indicate a difference ($p < 0.05$ in Student’s two-tailed t-tests) from the WT strain at the corresponding time PS and the asterisk above the bracket indicates a difference between the mutants.
Figure 8. Binding of the Nla6 DBD, MrpC, and FruA to the promoter regions of the late-acting operons. EMSAs were performed with $^{32}$P-labeled DNA fragments (2 nM) of the *dev* (-114 to -19), *exoA-I* (-171 to -1), *fadIJ* (-214 to -5), *exol-P* (-217 to +56), and *nfsA-H* (-166 to +35) promoter regions, and MBP-Nla6 DBD, His$_6$-MrpC, and FruA-His$_6$ at the $\mu$M concentrations indicated by numbers above each autoradiographic image. A white number on a black background indicates a protein that was added to the DNA-binding reaction 10 min later than the other protein. Shifted complexes produced by MBP-Nla6 DBD alone (gray arrowheads), His$_6$-MrpC alone (black arrowheads), FruA-His$_6$ alone (white arrowheads), or binding of two proteins (arrows) are indicated. Lanes are numbered below images.
Figure 9. Transcript levels in *devI* and *devS* mutants during *M. xanthus* development. The WT strain and its *devI* and *devS* mutant derivatives were subjected to starvation under submerged culture conditions. Samples were collected at the indicated times PS for measurement of *exoA* (A), *exol* (B), *nfsA* (C), and *fadI* (D) transcript levels by RT-qPCR. Graphs show the data points and the average of at least three biological replicates, relative to the WT strain at 18 h PS, and error bars indicate one standard deviation. Asterisks indicate a significant difference (*p* < 0.05 in Student’s two-tailed t-tests) from the WT strain at the corresponding time PS.
Figure 10. Model for regulation of late-acting operons during *M. xanthus* development. In response to starvation, phosphorylated Nla6 (Nla6-P) positively regulates all four late-acting operons directly (solid green arrows) or indirectly (dashed green arrow) early in development (upper gray area reflecting transcript measurements from 6-24 h PS). Nla6-P also positively regulates MrpC via Nla28-P (19) and MrpB-P (76). MrpC negatively regulates *exoA*-I indirectly (dashed red line with barred end) and *exoL*-P directly (solid red line with barred end), and positively regulates the *nfsA*-H operon directly, early in development. Nutrients (25, 26) and Esp signaling (23, 24) can negatively regulate MrpC via proteolysis, and MrpC negatively autoregulates by competing with MrpB-P for binding to the mrpC promoter region (22). MrpC positively regulates C-signaling (77) and *fruA* transcription (20), leading to production of unactivated FruA, which negatively regulates the late-acting operons early in development. C-signaling activates FruA to FruA* by an unknown mechanism (15, 16). FruA* positively regulates mound formation (17), which enhances C-signaling (34, 78, 79), forming a positive feedback loop. FruA* also positively regulates the dev operon (28, 56) and the late-acting operons late in development (lower gray area reflecting transcript measurements at 27 and 30 h). Dev TRS proteins negatively autoregulate, decreasing DevI (40, 41), which negatively regulates the late-acting operons and cellular shape change. MrpC positively regulates *dev*, *exoL*-P, and *fadIJ*, while Nla6-P negatively regulates *exoA*-I and *exoL*-P late in development. Under our submerged culture conditions, mounds form by 18 h (Fig. 1 and S5) and fruiting bodies with many spores form by 36 h (Fig. 2).