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A NOVEL MECHANISM OF C-SIGNAL-DEPENDENT GENE REGULATION DURING MYXOCOCCUS XANTHUS DEVELOPMENT

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A NOVEL MECHANISM OF C-SIGNAL-DEPENDENT GENE REGULATION DURING *MYXOCOCCUS XANTHUS* DEVELOPMENT

By

SHEENU MITTAL

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ABSTRACT

A NOVEL MECHANISM OF C-SIGNAL-DEPENDENT GENE REGULATION DURING *MYXOCOCCUS XANTHUS* DEVELOPMENT

By

Sheenu Mittal

The Gram-negative soil bacterium, *Myxococcus xanthus*, undergoes a multicellular developmental process upon nutrient limitation. Intercellular signals expressed at different times during the developmental process coordinate cell movements and regulate spatiotemporal gene expression. C-signal, a short-range signal that may require cell-cell contact, is essential for cells to move into aggregates and differentiate into spores within the mound-shaped fruiting bodies.

To understand gene regulation in response to C-signaling, the control of two C-signal-dependent transcription units, whose products play a role in aggregation and sporulation, *fmgA* and *fmgBC*, was studied. MrpC2 was discovered as a direct activator of *fmgA* and *fmgBC* expression. MrpC2, an N-terminally truncated form of the protein, MrpC, is a development-specific transcription factor. MrpC functions as an antitoxin to mediate programmed cell death of about 80% of the cells during development. Gel-shift assays showed that MrpC2 binds to sequences important for *fmgA* and *fmgBC* expression. Chromatin immunoprecipitation assays demonstrated association of MrpC and/or MrpC2 with the *fmgA* and *fmgBC* promoter regions *in vivo*.

Previously, FruA, a transcription factor believed to be phosphorylated in response to C-signaling, was shown to be essential for *fmgA* expression. MrpC2 binds to a sequence

adjacent to the binding site of FruA in the *fmgA* promoter region. FruA was found to be essential for association of MrpC and/ or MrpC2 with the *fmgA* promoter region *in vivo*. DNase I footprinting revealed cooperative binding of FruA and MrpC2 to *fmgA* promoter region DNA, and the combination of proteins enhanced formation of shifted complexes with *fmgA* DNA, unless the DNA had a mutation in the FruA- or MrpC2-binding site. This is a novel mechanism of gene regulation since cooperative binding of a response regulator (FruA) and another transcription factor (MrpC2) has not been observed previously.

Expression of the *fmgBC* operon was found to be regulated by a similar mechanism as *fmgA*. *fmgB-lacZ* expression depended absolutely on FruA. Association of FruA with the *fmgBC* promoter region was observed *in vivo* and *in vitro*. FruA bound to a key *cis*-regulatory sequence in the *fmgBC* promoter region *in vitro*, although it bound downstream of MrpC2, as opposed to binding upstream of MrpC2 in the *fmgA* promoter region. Nevertheless, similar to *fmgA* gene, FruA was required for *in vivo* occupancy of the *fmgBC* promoter region by MrpC and/or MrpC2. Also, FruA and MrpC2 appeared to bind cooperatively to *fmgBC* promoter region DNA *in vitro*.

The results with *fmgA* and *fmgBC* (and preliminary results with two other C-signal-dependent genes) suggest that combinatorial regulation by FruA and MrpC2 is a conserved mechanism during *M. xanthus* development. This mechanism is proposed to monitor C-signaling via FruA phosphorylation, and persistent starvation via the MrpC2 level, resulting in sporulation of some cells and programmed cell death of others.

To my parents, Rajendra and Rekha Mittal, and my husband, Shireesh Srivastava, without them I could not have accomplished this.

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1. ATP – adenosine triphosphate
2. bp – base pair
3. cGMP – cyclic guanosine monophosphate
4. CTT – casitone-tris media
5. DNA – deoxyribonucleic acid
6. dNTP – deoxynucleotide triphosphate
7. GTP – guanosine triphosphate
8. His – histidine
9. h – hour
10. IgG – immunoglobulin G
11. kb – kilobase
12. kDa – kilodalton
13. Km – kanamycin
14. M – molar
15. Mb – megabase
16. mM – millimolar
17. min – minute
18. ml – millilitre
19. μg – microgram
20. μl – microlitre
21. μM – micromolar
22. NAD – nicotinamide adenine dinucleotide

23. ng – nanogram

24. nM – nanomolar

25. NTP – nucleotide triphosphate

26. PAGE – polyacrylamide gel electrophoresis

27. PCR – polymerase chain reaction

28. (p)ppGpp – guanosine (penta) tetraphosphate

29. RNA – ribonucleic acid

30. SDS – sodium dodecyl sulfate

31. TPM – tris-phosphate-magnesium media

Chapter 1: Introduction

INTRODUCTION

The myxobacteria were discovered in the nineteenth century and are Gram-negative bacteria that display a social lifestyle. Based on 16S ribosomal RNA sequence data, the myxobacteria belong to the delta-subgroup of the Proteobacteria (Shimkets and Woese 1992). They feed on organic matter in the soil, as well as on other bacteria and protozoa. When feeding, they travel like packs of wolves, secreting enzymes to degrade and hydrolyze food sources (Rosenberg et al. 1977). Among the myxobacteria, *Myxococcus xanthus* has been the best-studied. The sequence of *M. xanthus* strain DK1622 revealed a single circular chromosome of 9.14 Mb with 7,388 putative coding sequences (Goldman et al. 2006). Gene duplication has been proposed to contribute to the large size of the *M. xanthus* genome. *M. xanthus* is a proficient producer of secondary metabolites including antimicrobials, polyketides (PKs), nonribosomally-made peptides (NRPs), and carotenoids (Goldman et al. 2006). Around 9% of the *M. xanthus* genome encodes enzymes involved in secondary metabolism.

Motility

M. xanthus is a rod-shaped, top-soil dwelling bacterium that has been isolated from many sites around the world. It lacks flagella, but moves by gliding on solid surfaces. *M. xanthus* employs two distinct motility systems for gliding; the A (adventurous) and S (social) systems (Hodgkin and Kaiser 1979a, Hodgkin and Kaiser 1979b). Lone cell movements have been attributed to A-motility and group movements to S-motility. There are currently two models to explain the A-motility mechanism. These models differ from one another in terms of the location of the gliding motor in a cell. According to the first

model, there are small nozzle-like structures present at both poles of the cell and slime secretion from one end provides the required force to push cells forward (Wolgemuth et al. 2002). These nozzle-like structures have been proposed to be analogous to the nozzles that mediate gliding motility via slime secretion in cyanobacteria (Hoiczyk and Baumeister 1998). According to the second model, the gliding motor is not localized at the poles, but instead it is distributed along the cell body (Sun et al. 1999, Mignot et al. 2007, Sliusarenko et al. 2007). According to this model, membrane-anchored adhesion complexes exert force on the cytoskeleton to move the cell forward by pushing it against the substratum (Mignot et al. 2007). S-motility requires type IV pili, lipopolysaccharide O-antigen, extracellular polysaccharide (EPS) or fibrils and FrzS (Li et al. 2003, Mignot et al. 2005). S-motility results from retraction of the polar pili, pulling the cell forward (Li et al. 2003). This retraction mechanism in *M. xanthus* is similar to that seen in *Neisseria gonorrhoeae* and *Pseudomonas aeruginosa* (Wolfgang et al. 1998, Skerker and Berg 2001).

The frequency of cellular reversals during motility is controlled by genes of the Frz (Frizzy) chemosensory pathway in *M. xanthus* (Blackhart and Zusman 1985). The Frz pathway is similar to the Che (Chemotaxis) system found in enteric bacteria like *E. coli*. The Frz pathway regulates cell reversals, which is essential for directed cell movements during *M. xanthus* development. Elasticotaxis is another sensory mechanism found in *M. xanthus*. Elasticotaxis has been described as an effect that results in an asymmetrically elongated swarm on a compressed agar surface (Stanier). A-motility has been shown to play a role in elasticotaxis (Fontes and Kaiser 1999).

Signal transduction

M. xanthus harbors a vast number of proteins similar to those that typically form bacterial two-component systems (TCSs) including 137 His protein kinases (HPKs) and 118 response regulators (RRs) (Goldman et al. 2006). Two-component His/Asp systems are the most common bacterial signal transduction systems. They contain a His kinase which senses a signal via its N-terminal input domain, resulting in autophosphorylation of a His residue in the C-terminal transmitter domain. The phosphoryl group from the His residue is then transferred to a conserved Asp residue in the receiver domain of the RR. This results in the activation of the C-terminal effector domain of the RR. Most RRs contain a DNA-binding motif in the effector domain and when activated via phosphorylation, bind to DNA to activate gene expression. *M. xanthus* employs His-Asp signal transduction systems in a variety of processes including motility, development, pili biogenesis, heat shock, and osmotic tolerance (Whitworth 2008). The genome sequence also revealed an abundance of 102 eukaryotic-like protein Ser/Thr kinases (PSTKs) in *M. xanthus* (Goldman et al. 2006). The first bacterial PSTK was identified in *M. xanthus* (Munoz-Dorado et al. 1991) and PSTKs were subsequently discovered in many other bacteria (Av-Gay and Everett 2000, Wang et al. 2002, Petrickova and Petricek 2003). *M. xanthus* utilizes both TCSs and PSTKs to couple gene expression to continuously changing environmental cues.

Sigma factors

Additional mechanisms for coupling external stimuli with coherent cellular responses are provided by 38 putative extra-cytoplasmic function (ECF) sigma factors present in the *M.*

xanthus genome (Goldman et al. 2006). ECF sigmas belong to the σ^{70} family and typically respond to external stimuli to affect expression of genes whose products are involved in functions of the cell envelope and periplasmic space (Helmann 2002). Of the 38 putative ECF sigmas, only three are well-characterized. CarQ regulates light-induced synthesis of carotenoids (Gorham et al. 1996). RpoE1 plays a role in motility during vegetative growth and development (Ward et al. 1998). EcfA regulates expression of an early developmental gene (Kroos and Inouye 2008). Our group has created insertion mutants of each of the remaining 35 putative ECF sigma genes, and the mutants are being characterized for defects in motility, development, and secondary metabolite production, in collaboration with other groups to gain insight into the role of ECF sigma factors (D. Srinivasan, S. Mittal, P. Luethy and L. Kroos, unpublished data).

In addition to the ECF sigmas, the σ^{70} family contains nine additional putative sigma factors, of which seven have been characterized, while the remaining two exhibit limited similarity to known sigma factors (Goldman et al. 2006, Kroos and Inouye 2008). The primary sigma factor, SigA, is essential for growth (Inouye 1990). SigB is a development-specific sigma factor that is essential for maturation of spores (Apelian and Inouye 1990). *sigC* is expressed early in development and affects fruiting body formation (Apelian and Inouye 1993). SigD is similar to the stationary-phase sigma factor, σ^{S}, of *E. coli*. *M. xanthus* sigD is present during vegetative growth and early in development. A *sigD* mutation results in growth defects, compromised stress responses, and abnormal development (Ueki and Inouye 1998). A *sigD* mutant is impaired in the synthesis of the

intercellular A- and C-signals, but responds to starvation by inducing the synthesis of the stringent response molecule, (p)ppGpp (Viswanathan et al. 2006b). SigE is present during vegetative growth and development, and is similar to heat shock sigma factors in other bacteria, but it does not appear to have a role in the heat shock response in *M. xanthus* (Ueki and Inouye 2001). SigF plays a role in S-motility and development (Ueki et al. 2005). SigG is similar to the *E. coli* FliA protein, which directs flagellar gene expression. An *M. xanthus sigG* mutant displays no obvious defects in growth or development (Kroos and Inouye 2008). *M. xanthus* also has one σ^{54} (Goldman et al. 2006), which is essential for growth, unlike in most bacteria that have been studied (Keseler and Kaiser 1997).

Enhancer-binding proteins

In bacteria, transcription at σ^{54} promoters involves activators that bind to enhancer-like sequences upstream of the core promoter, and by DNA looping interact with σ^{54} RNA polymerase (RNAP) to assist in open complex formation (Buck et al. 2000). These activators have a conserved central ATPase domain and a C-terminal DNA-binding domain. *M. xanthus* is predicted to have 52 genes that code for activators, based on the presence of the conserved ATPase domain (Goldman et al. 2006). These transcriptional activators have been designated σ^{54}-activators (Gorski and Kaiser 1998), NtrC-like activators (Caberoy et al. 2003), or enhancer-binding proteins (EBPs) (Jakobsen et al. 2004). In *M. xanthus*, most EBPs have an N-terminal sensory domain, suggesting a role in signal transduction. Gene knock-out and expression studies have demonstrated that

certain EBPs play a role in aggregation and sporulation (Kroos and Inouye 2008). Some EBPs like Nla18 affect vegetative growth as well as development (Diodati et al. 2006).

Fruiting body formation

Nutrient depletion triggers the multicellular developmental process of *M. xanthus*. Deprivation of amino acids, carbon source, or phosphate initiates development (Manoil and Kaiser 1980b, Shimkets 1987, Dworkin 1996). Starvation conditions induce a rise in the intracellular concentration of the stringent response molecule (p)ppGpp (Manoil and Kaiser 1980a, Manoil and Kaiser 1980b Singer and Kaiser 1995). (p)ppGpp is essential for the initiation of the developmental process (Singer and Kaiser 1995). (p)ppGpp levels are affected by several genes and are subject to complex regulation.

When starved at a high density, *M. xanthus* cells glide to form aggregation foci. Prior to and during the formation of aggregation centers, cells exhibit rippling behavior (Shimkets and Kaiser 1982). During rippling, cells accumulate in parallel ridges that appear to travel as waves under time-lapse microscopy (Welch and Kaiser 2001). As starvation continues, aggregation foci fuse and grow larger, resulting in the formation of mound-shaped structures over a period of 24 h (Kroos 2007). There are approximately 100,000 cells in a nascent fruiting body, and around 10-15% of the cells differentiate into heat and desiccation-resistant spherical spores, while other cells undergo programmed cell death (PCD) mediated by a toxin (Wireman and Dworkin 1977, Nariya and Inouye 2008). Cells that remain outside of the fruiting bodies are called peripheral rods (O'Connor and

Zusman 1991). Peripheral rods are distinct from vegetative cells and they neither lyse nor sporulate. Their role in the developmental process remains to be understood.

Intercellular signaling during development

The process of development is controlled by temporal and spatial gene expression that is regulated by a network of intercellular signals and transcription factors. The involvement of intercellular signals in fruiting body development was discovered by the isolation of nonautonomous mutants that exhibit developmental defects (Hagen et al. 1978). The sporulation defects displayed by these mutants could be rescued by mixing with wild-type cells and this was defined as extracellular complementation (Hagen et al. 1978). The nonautonomous mutants were classified into five groups based on extracellular complementation experiments: *asg* (A-signal), *bsg* (B-signal), *csg* (C-signal), *dsg* (D-signal) and *esg* (E-signal) mutants (Hagen et al. 1978, Downard et al. 1993). In these experiments, codevelopment of mutants from two different groups resulted in the rescue of developmental defects, while codevelopment of mutants from the same group did not show rescue (Hagen et al. 1978, Downard et al. 1993). The signals are required at different times into the developmental process, with the A- and B-signals acting early in development at around 2 h, the D- and E-signals acting at 3-5 h, and the C-signal acting after 6 h (Kaiser 2004). The A- and C-signals have been studied in the most detail biochemically, while B-, D-, and E-signal molecules remain to be identified.

A-signaling

The A-signal is a mixture of extracellular proteases, peptides, and amino acids that is involved in sensing cell density early during the developmental process (Kuspa et al. 1992a, Kuspa et al. 1992b). Mutants that are defective in A-signal production have a defect in any one of the five *asg* genes (Kuspa and Kaiser 1989, Cho and Zusman 1999, Garza et al. 2000b). *asgA* encodes a hybrid protein containing an N-terminal receiver domain (typically found in RRs) and a C-terminal histidine kinase domain (Plamann et al. 1995). *agsA* is expressed during vegetative growth and during development. AsgA has autokinase activity and has been proposed to be involved in a phosphorelay process (Plamann et al. 1995). *asgB* codes for a putative DNA-binding protein that is essential for growth and development (Plamann et al. 1994). AsgB has a C-terminal helix-turn-helix domain that is similar to region 4 of the major sigma factors in *M. xanthus*, *E. coli*, and *B. subtilis* (Plamann et al. 1994). *asgC* encodes the major sigma factor of *M. xanthus*, sigA (Davis et al. 1995). *asgD* codes for a two-component signal transduction hybrid protein (Cho and Zusman 1999) similar to that encoded by *asgA*. *asgE* encodes a protein with two putative membrane-spanning domains and shares homology with aminohydrolases (Garza et al. 2000a).

The A-signal is comprised of heat-labile and heat-stable components (Kuspa et al. 1992a, Plamann et al. 1992). The heat-labile component was shown to have trypsin-like protease activity. Inactivation of this activity by heat treatment resulted in 40 to 60% reduced activity of an early developmental gene at the Ω4521 locus (Plamann et al. 1992). It was proposed that the heat-labile component of A-signal consists of secreted proteases

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required for degradation of proteins and peptides early in development. The heat-stable component was shown to primarily consist of amino acids; in particular, tyrosine, proline, phenylalanine, tryptophan, leucine, and isoleucine (Kuspa et al. 1992a). These and subsequent studies suggested that at the onset of starvation, cells release a mixture of proteases that act on outer membrane proteins to generate peptides and amino acids, and as starvation continues, increased levels of extracellular proteases, peptides, and amino acids result in induction of early developmental gene expression [reviewed in (Shimkets 1999, Kaiser 2004)].

The Ω4521 promoter was shown to be A-signal-dependent (Kuspa et al. 1986). This promoter is similar to σ^{54}-regulated promoters (Keseler and Kaiser 1995) and appears to be regulated by SasS and SasR, which are similar to HPKs and RRs respectively (Yang and Kaplan 1997).

B-signaling

A single locus, *bsgA*, is associated with all B-signaling mutants (Gill and Bornemann 1988, Gill et al. 1988, Tojo et al. 1993b). The *bsgA* gene codes for an ATP-dependent intracellular protease which is very similar to Lon protease of *E. coli* (Gill et al. 1993, Tojo et al. 1993b, Tojo et al. 1993a). *M. xanthus* has two Lon proteases; LonV is essential for growth, and BsgA is essential for development (Tojo et al. 1993b). The mechanism of extracellular complementation is not known Because the BsgA protein is cytoplasmic.

Interestingly, two suppressor mutations have been identified that bypass BsgA protease function during development. One mutation has been mapped to the *spdR* gene that codes for an EBP (Hager et al. 2001) and another mutation has been mapped to the *bcsA* locus that codes for a protein similar to flavin-containg monooxygenases (Cusick et al. 2002). It appears that *spdR* and *bcsA* might not function in the same pathway. *spdR* mutations also bypass the requirement for A-signaling but not that for C-signaling (Hager et al. 2001), while mutations in *bcsA* suppress defects of C-signaling mutants but not of A-signaling mutants (Cusick et al. 2002).

Recent work has shed some light on the possible function of the BsgA protease during development. It has been proposed to be involved in the cleavage of the transcriptional activator MrpC to an N-terminally truncated form, MrpC2, early in development (Nariya and Inouye 2006). MrpC2 appears to be a direct activator of *fruA*, a gene essential for fruiting body development (Ogawa et al. 1996, Ellehauge et al. 1998, Ueki and Inouye 2003). MrpC2 is undetectable in a *bsgA* mutant and *fruA* expression is also drastically reduced (Nariya and Inouye 2006). However, it remains to be tested whether MrpC is a direct substrate of the BsgA protease. Conceivably, MrpC2 and/or FruA activate transcription of one or more genes involved in production of the unknown B-signal. Alternatively, BsgA may cleave a protein other than MrpC that is involved in B-signaling.

D-signaling

Similar to B-signaling, little is known about D-signaling, but a single locus, *dsgA*, has been implicated by genetic studies. *dsgA* codes for a putative translation initiation factor 3, IF3 (Cheng and Kaiser 1989b, Cheng and Kaiser 1989a, Cheng et al. 1994, Kalman et al. 1994). IF3 helps ribosomes in selection of initiation codons. *M. xanthus dsgA* complemented an *E. coli infC* (the gene for IF3) mutant (Cheng et al. 1994). DsgA is essential for *M. xanthus* growth and development (Cheng and Kaiser 1989b, Cheng and Kaiser 1989a). There was limited rescue of sporulation of *dsgA* mutants by wild-type cells upon co-development (Cheng and Kaiser 1989a), and the mechanism of extracellular complementation is unknown.

E-signaling

Mutations leading to defects in E-signaling have been mapped to two genes that code for the E1α and E1β subunits of branched-chain keto acid dehydrogenase (BCKDH) (Downard et al. 1993, Toal et al. 1995). *esg* mutants had aggregation and sporulation defects (Toal et al. 1995). BKCDH is involved in conversion of branched-chain amino acids like leucine, isoleucine, and valine to isovaleryl-CoA, 2-methylbutyryl-CoA, and isobutyryl-CoA, respectively, which are required for the synthesis of iso-fatty acids and secondary metabolites (Ring et al. 2006). *esg* mutants have reduced iso-fatty acids and their developmental defects can be rescued by addition of isovalerate, suggesting that an iso-fatty acid or a corresponding lipid could be required during development (Toal et al. 1995). Kearns et al. showed the likely involvement of a phosphatidylethanolamine (PE) containing a straight-chain fatty acid (16:1ω5c) in E-signaling (Kearns et al. 2001),

however, a recent study suggests that this PE is not required for *M. xanthus* development (Bode et al. 2006).

Interestingly, the loss of BKCDH activity in *esg* mutants does not result in complete loss of iso-fatty acid production due to the presence of an alternative unique pathway in myxobacteria (Mahmud et al. 2002). A double mutant for both pathways is severely hampered in iso-fatty acid production (Bode et al. 2006). Developmental defects in this double mutant have been proposed to be due to the loss of unusual iso-branched ether lipids found in myxospores and therefore suggested to be novel biochemical markers of development (Ring et al. 2006). Whether extracellular complementation of *esg* mutants reflects developmental signaling or metabolite cross-feeding remains an open question, but the study of *esg* mutants has revealed an important role of iso-fatty acid biosynthesis in *M. xanthus* development.

C-signaling

C-signaling is mediated by the product of the *csgA* gene (Shimkets et al. 1983) and begins about 6 h after starvation (Kroos and Kaiser 1987). Mutants defective in C-signal synthesis are unable to ripple, aggregate, or sporulate (Shimkets et al. 1983). Developmental defects of *csgA* mutants can be rescued by either codevelopment with wild-type cells or by exogenous addition of purified C-signal (Kim and Kaiser 1990a, Kim and Kaiser 1991). Li et al. manipulated *csgA* expression *in vivo* to demonstrate that low levels of *csgA* expression induce rippling, intermediate levels induce aggregation, and high levels induce sporulation (Li et al. 1992). Kruse et al. showed that

overexpression of *csgA* *in vivo* resulted in early aggregation, gene expression and sporulation (Kruse et al. 2001). These studies suggest that C-signaling coordinates the rippling, aggregation and sporulation behavior of cells.

Initially, the C-signal was identified as a 17 kDa (p17) protein and purified by detergent extraction and biochemical fractionation of starving *M. xanthus* cells (Kim and Kaiser 1990d). The purified C-signal was able to restore development of *csgA* mutant cells (Kim and Kaiser 1990d). However, later findings suggested that the *csgA* gene product could possibly function as a short-chain alcohol dehydrogenase (SCAD) to produce the C-signal. The *csgA* gene encodes a 25 kDa (p25) protein that shares homology with SCADs and contains two conserved motifs, an N-terminal NAD^+-binding pocket and a C-terminal catalytic domain, found in SCADs (Lee et al. 1995). *csgA* alleles carrying substitutions in either the putative NAD^+-binding pocket or catalytic domain were unable to complement *csgA* mutant cells (Lee et al. 1995). Likewise, exogenous addition of p25, purified after expression in *E. coli*, could rescue a *csgA* mutant, but there was no rescue with p25 having substitutions in either NAD^+-binding pocket or catalytic domain (Lee et al. 1995). Also, overproduction of the SocA protein, which shares homology with SCADs, restores development of *csgA* mutant cells (Lee and Shimkets 1994).

Recently, it was shown that p25 and p17 are two forms of the CsgA protein. p25 is proteolytically cleaved to p17 by a serine protease in the starving cells and p17 lacks the putative NAD^+-binding pocket (Lobedanz and Sogaard-Andersen 2003). p25 is present

during vegetative growth, while p17 is present only during development (Kruse et al. 2001). Both p25 and p17 are cell-surface associated proteins (Lobedanz and Sogaard-Andersen 2003). p17 has been proposed to be perceived by an unidentified receptor on the adjacent cell (Figure 1.1).

csgA expression is regulated in response to nutrient depletion. Li et al. showed that 930 bp upstream from the transcriptional start site (TSS) are required for full *csgA* expression in the presence of limited nutrients, while in the absence of nutrients 400 bp upstream of the TSS is sufficient for development and full *csgA* expression (Li et al. 1992). The products of the *act* operon have been shown to affect the level and timing of *csgA* expression (Gronewold and Kaiser 2001), but exactly how the Act proteins sense and respond to starvation is unknown.

C-signal transduction requires cell alignment and possibly end-to-end contact between cells, and acts over a short range, communicating positional information (Kim and Kaiser 1990c, Kim and Kaiser 1990b, Kim and Kaiser 1990a, Sager and Kaiser 1994). As mentioned above, distinct levels of C-signaling are required for rippling, aggregation, and sporulation. The expression of various genes during development also requires different levels of C-signaling (Kim and Kaiser 1991).

The C-signal Transduction Pathway

FruA plays an important role in the C-signal transduction pathway (Figure 1.1) and is believed to be regulated post-translationally by C-signaling (Ogawa et al. 1996,

Ellehauge et al. 1998). FruA regulates aggregation by activating Frz proteins (directly or indirectly) and FruA regulates sporulation by controlling expression of at least 50 genes (Horiuchi et al. 2002).

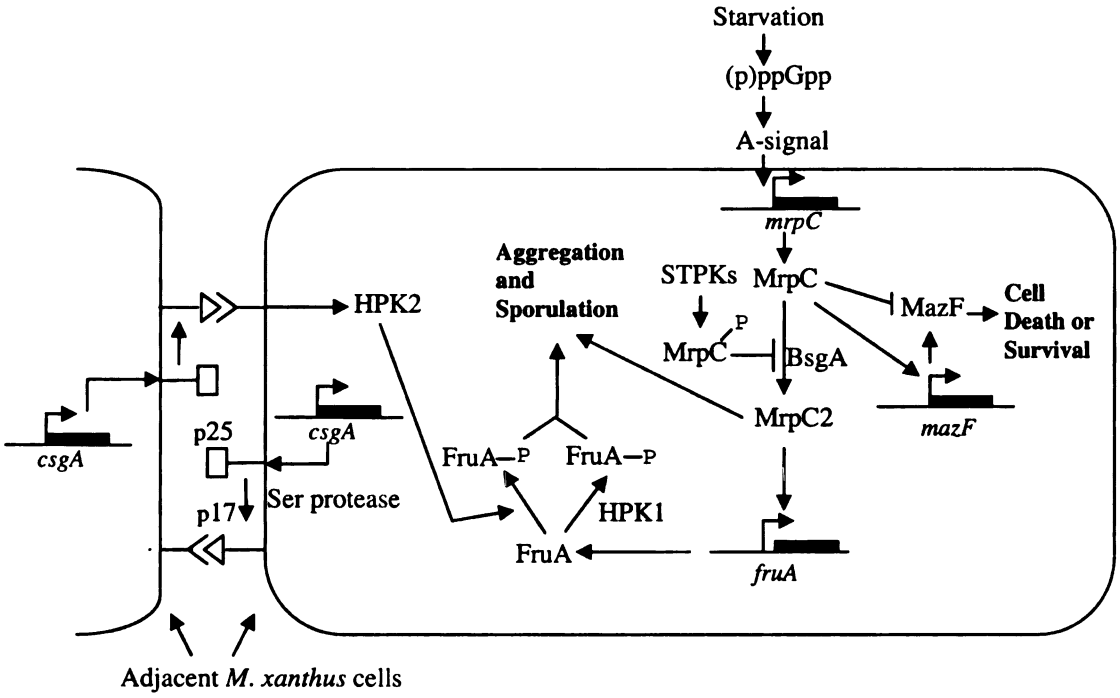

Figure 1.1. Regulatory network during *M. xanthus* development. Positive and negative effects (direct or indirect) are depicted by arrows and lines with barred ends, respectively. The C-signal (p17) is perceived by an unknown receptor on the surface of an adjacent cell and results in activation of an unknown kinase, HPK2. FruA has been proposed to be phosphorylated to a low level by an unknown HPK1, independent of C-signaling, and later phosphorylated to a higher level by HPK2 in response to C-signaling. HPK, histidine protein kinase; STPKs, serine/threonine protein kinases; MrpC-P, phosphorylated MrpC; FruA-P, phosphorylated FruA. See text for detailed description.

fruA transcription is induced around 6 h after the onset of starvation and depends on A- and E-signaling but not C-signaling (Ogawa et al. 1996, Ellehauge et al. 1998). FruA is

similar to response regulators of the FixJ subfamily of two-component His-Asp signal transduction systems. The D59 residue in the N-terminal part has been proposed to be the site of phosphorylation and genetic evidence shows that it is indispensable for the function of FruA (Ellehauge et al. 1998). *M. xanthus* cells carrying D59A or D59N or D59Q mutant alleles were unable to complement aggregation and sporulation defects of a *fruA* mutant (Ellehauge et al. 1998). However, a D59E substitution designed to mimic the phosphorylated state of the protein was able to restore development of a *fruA* mutant (Ellehauge et al. 1998). A histidine kinase(s) that can phosphorylate FruA remains to be identified. The C-terminal domain of FruA consists of a helix-turn-helix DNA-binding motif (Ellehauge et al. 1998). The C-terminal DNA-binding domain of FruA (FruA-DBD) has been shown to bind to promoter regions of several developmental genes, including both C-signal-dependent and C-signal-independent genes. It has been proposed that early during development, FruA is phosphorylated to a small extent to activate transcription of C-signal-independent genes like *dofA* and *tps*, while later in development, as C-signaling increases, more FruA is phosphorylated to activate C-signal-dependent genes like *fdgA*, *fmgA* and *dev* (Ueki and Inouye 2005b, Ueki and Inouye 2005a, Yoder-Himes and Kroos 2006, Viswanathan et al. 2007b).

fruA expression is developmentally-regulated (Figure 1.1) and appears to be under the direct control of the development-specific transcription factor MrpC2 (Ogawa et al. 1996, Ellehauge et al. 1998, Ueki and Inouye 2003). MrpC2 is an N-terminally truncated form of MrpC (Nariya and Inouye 2006). The *mrpC* locus was identified by transposon insertion mutagenesis as essential for aggregation and sporulation (Sun and Shi 2001b).

Upstream of *mrpC*, there is a two-gene operon, *mrpAB*, that code for an HPK and a RR. It has been shown that MrpB is required for *mrpC* transcription and that MrpC autoregulates its own transcription (Sun and Shi 2001b). MrpC is similar to cyclic-AMP receptor family transcriptional activators. MrpC is present during vegetative growth, whereas MrpC2 is detectable only during development beginning at around 6 h (Nariya and Inouye 2006). BsgA, an ATP-dependent protease has been proposed to cleave off the 25 N-terminal residues of MrpC to produce MrpC2 (Figure 1.1). A serine-threonine protein kinase (STPK) cascade controls the activity of MrpC and the generation of MrpC2. Pkn8, an integral membrane STPK, phosphorylates a cytoplasmic STPK, Pkn14, which then phosphorylates MrpC (Nariya and Inouye 2005). Phosphorylation inhibits processing of MrpC to MrpC2. MrpC2 cannot be phosphorylated, suggesting that Thr21 and/or Thr22 are the likely sites of MrpC phosphorylation. Phosphorylation reduces MrpC DNA-binding activity, and MrpC2 binds to the *mrpC* and *fruA* promoter regions with higher affinity than does MrpC (Nariya and Inouye 2006). The inhibitory effects of the Pkn8-Pkn14 cascade are relieved by an unknown mechanism early during development, causing a rise in the levels of MrpC and eventually MrpC2.

MrpC plays an important role in cell fate decisions during development (Figure 1.1). MrpC was shown to act as an anti-toxin that interacts directly with the toxin, MazF, an mRNA interferase, which mediates PCD during development (Nariya and Inouye 2008). MrpC also binds to the *mazF* promoter region and appears to activate transcription. Phosphorylation of MrpC affects its antitoxin properties as it has been shown that nonphosphorylated MrpC has higher affinity for MazF compared to the phosphorylated

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form of MrpC (Nariya and Inouye 2008). During growth, MrpC is phosphorylated by the STPK cascade, but MazF is kept in check Because phosphorylated MrpC presumably activates *mazF* transcription poorly. During development, MrpC and/or MrpC2 presumably activate *mazF* gene expression, leading to PCD of some cells in the population. MrpC and/or MrpC2 presumably inhibit MazF in other cells, preventing PCD and activating genes required for aggregation and sporulation. Binding of MrpC2 to the *mazF* promoter region and MazF has not been tested, but it is important to do so Because processing of MrpC to MrpC2 could be an important determinant of whether a cell undergoes PCD or forms a spore.

C-signal-dependent gene expression

Developmentally-regulated promoters in *M. xanthus* have been identified by transposition of a Tn*5 lac* into *M. xanthus* chromosome, generating transcriptional fusions of the promoter-less *E. coli lacZ* gene to genomic regulatory regions (Kroos and Kaiser 1984). Each insertion locus was assigned a four digit number. These fusions proved extremely useful for studying developmental gene expression in response to intercellular signals. Of the 2,374 insertions, 29 fusions were found to be developmentally-regulated (Kroos et al. 1986). In one study, 18 fusions were found to be A-signal-dependent (Kuspa et al. 1986). In another study, 26 insertions were found to be B-signal-dependent and 15 were C-signal-dependent (Kroos and Kaiser 1987). The Kroos lab has cloned DNA upstream of several C-signal-dependent Tn*5 lac* insertions, mapped transcriptional start sites, performed deletion analysis to identify promoter regions and conducted extensive mutational analysis to identify *cis*-regulatory sequences (Fisseha et al. 1996, Brandner

and Kroos 1998, Fisseha et al. 1999, Viswanathan and Kroos 2003, Yoder and Kroos 2004a, Yoder and Kroos 2004b, Loconto et al. 2005, Viswanathan et al. 2006a). Sequence comparison and mutational analysis identified two conserved *cis*-acting sequences, the C box (consensus CAYYCCY; Y is C or T) and the 5-bp element (consensus GAACA) (Fisseha et al. 1999, Viswanathan et al. 2006a). These sequences are present in all C-signal-dependent promoter regions studied so far and are crucial for promoter activity (Fisseha et al. 1996, Brandner and Kroos 1998, Fisseha et al. 1999, Viswanathan and Kroos 2003, Yoder and Kroos 2004a, Yoder and Kroos 2004b, Loconto et al. 2005, Viswanathan et al. 2006a). These studies have provided a foundation for understanding the regulation of C-signal-dependent gene expression.

Mutational analysis of the Ω4403 promoter region revealed that a 10-bp element, in addition to the C box and the 5-bp element, is critical for expression (Viswanathan and Kroos 2003). The Ω4403 fusion is absolutely dependent on C-signaling Because expression is completely abolished in a *csgA* mutant background but is restored upon codevelopment with wild-type cells, which supply C-signal (Kroos and Kaiser 1987).

The Ω4400 promoter region has a 5-bp element and a C box at exactly the same position as present in the Ω4403 promoter region (Brandner and Kroos 1998, Yoder and Kroos 2004a). However, single base-pair mutations in these sequences have different effects on promoter activity (Viswanathan and Kroos 2003, Yoder and Kroos 2004a). A region from -101 to +155 is sufficient for Ω4400 promoter activity (Yoder and Kroos 2004a). FruA is indispensable for expression from the Ω4400 promoter region (Yoder-Himes and Kroos

2006). FruA-DBD binds to a region between -86 and -77, which contains a region (-86 to -81) that is responsible for partial dependence on C-signaling (Yoder-Himes and Kroos 2006). σ^A RNA polymerase, the major form of vegetative RNA polymerase, was unable to produce transcripts from the Ω4400 promoter (Brandner and Kroos 1998). Null mutations in *sigB* and *sigC* genes did not affect expression from the Ω4400 promoter (Brandner and Kroos 1998). However, mutations in *sigD* and *sigE* abolished and reduced Ω4400 promoter activity, respectively, suggesting a direct or indirect role of SigD and SigE in Ω4400 transcription (Yoder and Kroos 2004a).

There are two C boxes and two 5-bp elements present in the Ω4499 promoter region, which are crucial for the Ω4499 promoter activity (Yoder and Kroos 2004b). Single base-pair mutations in one of the 5-bp elements and one of the C boxes had different effects on promoter activity than mutations at corresponding positions in the Ω4400 and Ω4403 promoter regions, suggesting a difference in the interaction with *trans*-acting factors. A region from -100 to +50 contains the regulatory sequences required for Ω4499 promoter activity (Yoder and Kroos 2004b). Expression from a Ω4499 fusion, like that from a Ω4400 fusion, is partially dependent on C-signaling (Kroos and Kaiser 1987). Also, like the Ω4400 promoter, σ^A RNA polymerase was unable to produce transcripts from the Ω4499 promoter (Fisseha et al. 1999). There was no effect of *sigB* or *sigC* null mutations on expression from the Ω4499 promoter (Brandner and Kroos 1998). A null mutation in *sigD* and *sigE* reduced expression from the Ω4499 promoter to about 30% of the level observed in wild-type cells (Yoder and Kroos 2004b). This suggests that *sigD* and *sigE* directly or indirectly affect Ω4499 promoter activity.

Expression of *lacZ* from the Ω4406 Tn*5* *lac* fusion depends absolutely on C-signaling (Kroos and Kaiser 1987, Loconto et al. 2005). An upstream negative regulatory element mediates C-signal dependence of the Ω4406 promoter (Viswanathan et al. 2006a). Interestingly, DNA 50 to 140 bp downstream of the TSS contains a positive regulatory element. Similar to other C-signal-dependent promoter regions, the 5-bp element and the C box are important for Ω4406 promoter activity (Viswanathan et al. 2006a).

The *dev* operon, whose products are essential for sporulation, is partially dependent on C-signaling (Kroos et al. 1986, Kroos and Kaiser 1987, Kroos et al. 1990). The regulatory region controlling *dev* expression spans more than 1 kb and includes positive and negative regulatory elements surrounding the promoter (Viswanathan et al. 2007a). The *dev* promoter region has the 5-bp element and C box-like sequences, but mutational analysis has not been performed on these sequences. *dev* expression is under combinatorial control by FruA and LadA (Viswanathan et al. 2007b). FruA binds to an upstream positive regulatory region centered at -91, while LadA binds around 350 bp downstream of the *dev* promoter (Viswanathan et al. 2007b).

A 5-bp element and a C box are present in the *fruA* promoter region, although *fruA* transcription is C-signaling-independent (Ellehauge et al. 1998, Srinivasan and Kroos 2004). Mutational analysis has shown that these sequences are important for *fruA* expression (Srinivasan and Kroos 2004).

To understand differential gene expression in response to C-signaling at the molecular level, my research focused on elucidating the regulation of two developmentally-regulated C-signal-dependent transcription units, *fmgA* (FruA- and MrpC2-regulated gene A) and *fmgBC* (FruA- and MrpC2-regulated gene B and C). *fmgA* and *fmgBC* genes were identified by insertion of Tn*5 lac* into the *M. xanthus* genome at loci Ω4400 and Ω4499, respectively (Kroos et al. 1986).

Chapter 2 describes the identification of MrpC2 as a direct activator of *fmgA* expression. It was found that *fmgA* expression is under combinatorial control of the previously identified activator, FruA, and MrpC2. FruA is essential for MrpC and/or MrpC2 association with the *fmgA* promoter region *in vivo*. This is a novel mechanism of gene regulation in which a response regulator, FruA, recruits an independent transcription factor, MrpC2. Biochemical studies showed that FruA and MrpC2 bind cooperatively to the *fmgA* promoter region. We propose that cooperative binding subjects *fmgA* promoter activity to positional information via C-signaling and phosphorylation of FruA, and to starvation signaling via MrpC2 during development. This chapter will be submitted to Proceedings of National Academy of Sciences in August 2008.

In Chapter 3, regulation of *fmgBC* operon expression is described. MrpC2 and FruA were identified as direct activators of *fmgBC* expression since both proteins bind to important *cis*-regulatory sequences in the *fmgBC* promoter region. Similar to *fmgA*, cooperative binding of FruA and MrpC2 was observed at the *fmgBC* promoter region. This chapter will be submitted to the Journal of Bacteriology in September 2008.

Chapter 4 is conclusions and future directions, which contains a summary of the research and possible future directions.

Chapter 2: Cooperative binding of a response regulator and a bifunctional transcription factor/antitoxin controls gene expression and cell fate during *Myxococcus xanthus* development

The work in this chapter will be submitted to Proceedings of National Academy of Sciences in August 2008.

ABSTRACT

Myxococcus xanthus is a bacterium that undergoes multicellular development requiring coordinate regulation of multiple signaling pathways. One pathway governs aggregation and sporulation of some cells in a starving population and requires C-signaling, while another pathway causes programmed cell death and requires the MazF toxin. In response to starvation, the levels of the antitoxin MrpC and its related proteolytic fragment MrpC2, are increased, inhibiting the cell death pathway via direct interaction of MrpC with MazF. Herein, we demonstrate that MrpC2 plays a direct role in the transcriptional response to C-signaling. We show that MrpC2 binds to sequences upstream of the C-signal-dependent *fmgA* promoter. These sequences are present in other C-signal-dependent promoter regions, indicating a general role for MrpC2 in developmental gene regulation. Recruitment of MrpC2 to promoters is enhanced by FruA, a protein that responds to C-signaling and is similar to response regulators of two-component signal transduction systems. DNA binding studies showed that this involves a novel mechanism for a response regulator, in which FruA and MrpC2 bind cooperatively to adjacent sites upstream of the *fmgA* promoter. This novel mechanism of combinatorial control allows coordination of morphogenetic C-signaling via FruA with starvation signaling and cell death via MrpC2 and MrpC, determining spatiotemporal gene expression and cell fate.

INTRODUCTION

Understanding how cells integrate many different signals to regulate genes and determine cell fates during multicellular development is a fundamental question. *Myxococcus xanthus* provides an attractive model to address this question because starvation initiates a relatively simple developmental process (Whitworth 2008). Thousands of rod-shaped cells coordinate their movements to build fruiting bodies in which cells differentiate into dormant, spherical spores (Figure 2.1). However, not all cells form spores. Alternative fates are programmed cell death (PCD) (Nariya and Inouye 2008) or persistence outside of fruiting bodies as peripheral rods (O'Connor and Zusman 1991).

Signals act at different times during the *M. xanthus* developmental process to control gene expression, coordinate cell movements, and determine cell fates (Kroos 2007). Starvation triggers the stringent response, which involves production of the second messenger (p)ppGpp (Figure 2.1). This intracellular signal leads to activation of early developmental genes (like *csgA*) (Harris et al. 1998, Crawford and Shimkets 2000) and secretion of protease activity that generates a mixture of peptides and amino acids known as A-signal (Kuspa et al. 1992a, Plamann et al. 1992). This extracellular signal is believed to allow quorum sensing (Kuspa et al. 1992b). At a sufficient concentration, expression of genes like those in the *mrp* operon is induced (Figure 2.1) (Sun and Shi 2001a). Later, when cells begin to aggregate, C-signaling takes over. The C-signal appears to be a proteolytic cleavage product of CsgA, which is associated with the cell surface (Kim and Kaiser 1990a, Lobedanz and Sogaard-Andersen 2003), but a receptor has not been identified. C-signaling requires cell alignment (Kim and Kaiser 1990b) and

possibly end-to-end contact (Sager and Kaiser 1994), so it is paracrine or short-range signaling, which is common in eukaryotes but rare among bacteria (Bassler and Losick 2006). The short-range nature of C-signaling and its effects on cell movement and gene expression can explain its critical role in coordinating aggregation with sporulation (Kaiser 2003, Sogaard-Andersen et al. 2003). Cell alignment within a nascent fruiting body has been proposed to allow a high level of C-signaling and activation of genes required for sporulation (Sager and Kaiser 1993). Consistent with this model, elevated CsgA accelerates aggregation and sporulation, but the fruiting bodies are smaller than normal, and reduced CsgA causes the opposite effects (Li et al. 1992, Gronewold and Kaiser 2001, Kruse et al. 2001). Also, the expression of genes important for sporulation is temporally and spatially restricted to nascent fruiting bodies (Sager and Kaiser 1993, Julien et al. 2000).

Here, we focused on gene regulation in response to C-signaling during *M. xanthus* development, a process in which FruA plays a key role (Figure 2.1). FruA is similar to response regulators of two-component signal transduction systems (Ogawa et al. 1996). FruA's N-terminal domain is believed to be phosphorylated by one or more histidine protein kinases (HPKs) in response to C-signal and perhaps other signals (Ellehauge et al. 1998, Jelsbak et al. 2005), but the cognate HPK(s) remains to be identified. Phosphorylation presumably enhances DNA binding by the C-terminal domain of FruA, which resembles that of the NarL/FixJ subfamily of response regulators (West and Stock 2001). The FruA DNA-binding domain (FruA-DBD) has been shown to bind to sites in the promoter regions of developmentally-regulated genes, including one whose

expression does not depend on C-signaling (*dofA*; Figure 2.1) (Ueki and Inouye 2005a) and others whose expression is C-signal-dependent (e.g., *fmgA*; Figure 2.1) (Ueki and Inouye 2005b, Yoder-Himes and Kroos 2006). FruA positively regulates expression of these genes, but in the case of *fmgA*, mutational analysis of the promoter region implied that an additional transcriptional activator is required (Yoder and Kroos 2004a).

The *fmgA* gene (named herein) was identified by insertion of transposon Tn*5 lac* into the *M. xanthus* genome at locus Ω4400 (Kroos et al. 1986). Expression of *lacZ* from Tn*5 lac* Ω4400 was shown to be developmentally-regulated and partially dependent on *csgA* (Kroos et al. 1986, Kroos and Kaiser 1987). *M. xanthus* DNA upstream of Tn*5 lac* Ω4400 was cloned, a putative transcriptional start site was mapped, and the region from -101 to +155 was shown to encompass the *fmgA* promoter (Brandner and Kroos 1998). Expression from this promoter region, as measured by a transcriptional fusion to *lacZ* integrated ectopically into the *M. xanthus* chromosome, was reduced in a *csgA* mutant (as was expression from Tn*5 lac* Ω4400), but expression was restored upon co-development of the *csgA* mutant with wild type cells, which supply C-signal. This indicated that *fmgA* promoter activity is partially dependent on C-signaling. Mutational analysis identified *cis*-regulatory sequences at -86 to -77 and -63 to -46 upstream of the *fmgA* promoter (Figure 2.2) (Yoder and Kroos 2004a), and subsequent analysis showed that FruA-DBD binds to the sequence between -86 and -77 (Yoder-Himes and Kroos 2006). The sequence between -63 and -46 contains two elements found in other C-signal-dependent promoter regions, a 5-bp element (consensus GAACA) and a C box (consensus CAYYCCY; Y means C or T) (Fisseha et al. 1999, Viswanathan and Kroos 2003, Yoder and Kroos

2004a, Loconto et al. 2005, Viswanathan et al. 2006a, Viswanathan et al. 2007a). Mutations in this region nearly abolish *fmgA* promoter activity (Figure 2.2) (Yoder and Kroos 2004a) but do not affect binding of FruA-DBD (Yoder-Himes and Kroos 2006). Taken together, these studies suggested that a transcriptional activator binds to the sequence between -63 and -46 upstream of the *fmgA* promoter, and perhaps to similar sequences in other C-signal-dependent promoter regions.

Here, we report identification of the activator as MrpC2 and we name the gene at the Ω4400 locus *fmgA* (FruA- and MrpC2-regulated gene A). MrpC2 lacks the N-terminal 25 residues of MrpC and appears to be generated by proteolytic activity of a developmentally-regulated protease (LonD; Figure 2.1) (Nariya and Inouye 2006). MrpC is similar to transcription factors in the cyclic AMP receptor protein (CRP) family (Sun and Shi 2001b). Recently, MrpC was shown to interact with the toxin MazF, which mediates PCD during development (Nariya and Inouye 2008). Phosphorylation of MrpC by a cascade of serine/threonine protein kinases (STPKs) appears to inhibit its proteolytic processing to MrpC2 during growth (Nariya and Inouye 2005, Nariya and Inouye 2006). Early during development, due to relief from the inhibitory effects of the STPK cascade, MrpC and MrpC2 levels are increased. MrpC2 has a higher affinity than MrpC for binding sites in the *mrpC* and *fruA* promoter regions, so MrpC2 presumably boosts transcription from these promoters. In addition to identifying MrpC2 as an activator of *fmgA* transcription, we show that FruA is required for association of MrpC and/or MrpC2 with the *fmgA* promoter region *in vivo*, and that FruA and MrpC2 bind cooperatively to *fmgA* promoter region DNA *in vitro*. Cooperative binding of a response regulator with

another transcription factor represents a novel mechanism of gene regulation. Preliminary results indicate that this mechanism is shared by other C-signal-dependent genes (see Discussion). We propose that cooperative binding facilitates integration of positional information (via short-range C-signaling through FruA) with nutritional status and other signals (through transcriptional and post-transcriptional control of MrpC), governing cell fate decisions, analogous to combinatorial control during development of multicellular eukaryotes.

MATERIALS AND METHODS

Bacterial strains and plasmids

Strains and plasmids used in this study are listed in Table 2.1.

Growth and development

E. coli BL21(DE3) containing plasmids were grown at 37^{o}C in Luria-Bertani medium (Sambrook et al. 1989) containing 200 μg ampicillin per ml. Growth and development of *M. xanthus* was as described (Viswanathan and Kroos 2003).

Preparation of *fmgA* DNA fragments

DNA fragments spanning the *fmgA* promoter region from -101 to +25 were generated by PCR using wild-type or mutant plasmid as the template and the primers 5′-CTTAAGCTTTGCACTGCGACGCGAGTC-3′ (for -101) and 5′-GCGGATCCCGGTCCTTCGCGTCGCCG-3′ (for +25). For EMSAs, ^{32}P-labeled DNA was synthesized by PCR after labeling the primers with [γ-^{32}P]ATP using T4 polynucleotide kinase, and the labeled DNA was purified and the concentration was estimated as described (Yoder-Himes and Kroos 2006). For DNase I footprinting, only one primer was labeled and a different upstream primer, 5′-AGGCTTTCGATGCACTGCG-3′ (for -139), was used to allow resolution of digestion products of interest. The -139 to +25 DNA fragment produced a pattern of shifted complexes in EMSAs with His_{10}-MrpC2 and/or FruA-His_6 that was indistinguishable from the -101 to +25 DNA fragment (data not shown). The modified (+5 bp) -76 to -41

fragment was generated by annealing 5'-CACAATGCGGTGGGGAGCGAACAGTCCCACATCCCTGGCGG-3' (the non-*fmgA* sequence is underlined) with its complement; after each oligonucleotide had been labeled as above, they were mixed, boiled for 10 min, placed at room temperature for 3 h, then the double-stranded DNA fragment was purified as above.

EMSAs and DNase I footprinting

EMSAs were performed as described (Yoder-Himes and Kroos 2006) except the binding reaction mixtures were incubated at 25°C for 15 min. For footprinting, 0.2 U of DNase I (Promega) was added to the binding reaction mixture (20 μl) for 2 min at 25°C. The binding mixture was as described previously (Yoder-Himes and Kroos 2006), except that it included 5mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.025 μg/μl double-stranded poly(dI-dC), and no glycerol. Reactions were stopped by adding 100 μl of solution containing 300 mM sodium acetate, 20 mM EDTA, 0.2% SDS, 0.02 μg/μl proteinase K, and 100 μg/ml yeast tRNA, and incubating at 52°C for 15 min. After extraction with 100 μl of phenol (twice), DNA was precipitated with ethanol. The DNA was resuspended in formamide loading buffer (Sambrook et al. 1989), boiled for 3 min, subjected to electrophoresis on an 8% polyacrylamide gel containing 8 M urea, and visualized by autoradiography. Sequencing ladders were generated using the SequiTherm EXCEL™ II DNA Sequencing Kit protocol (Epicentre Biotechnologies).

DNA-affinity chromatography

An *fmgA* DNA fragment (-101 to +25) was synthesized by PCR with a 5'-biotin label at -101, bound to streptavidin beads, and DNA-affinity chromatography was performed with the AS fraction as described (Viswanathan et al. 2007b).

Antibody supershift assays

Binding reactions were performed as described above and then antibodies were added and the mixture was incubated at 4^{o}C for 30 min, followed by EMSAs as described (Yoder-Himes and Kroos 2006).

Preparation of MrpC2 and FruA

His_{10}-MrpC2 (Nariya and Inouye 2006) and FruA-DBD-His_8 (Yoder-Himes and Kroos 2006) were purified as described previously from *E. coli* strains SMhisMrpC2 and EDYFruA, respectively. FruA-His_6 was purified from *E. coli* SMFruAhis as described (S. Inouye, personal communication).

ChIP

M. xanthus strains MDY4400.DZF1 and MDY4400.FA were used for ChIP as described (Yoder-Himes and Kroos 2006) with the following modifications: anti-MrpC antibodies (500 ng) (Nariya and Inouye 2006) or control IgG (500 ng) (Santa Cruz Biotechnology) were used for immunoprecipitation, twofold serial dilutions were made of the input DNA samples, and the primers used for PCR of the *fmgA* promoter region were the one for +25

described above and one upstream (yielding a product of about 180 bp) in the vector used for ectopic integration (5′-CTGCCAGGAATTGGGGATC-3′).

TABLE 2.1. Bacterial strains and plasmids used in this study

Bacterial strain or plasmid	**Description**	**Reference or Source**
E. coli strains		
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	This work
SMFruAhis	BL21(DE3) containing pET11km/FruA-His$_6$	This work
EDYFruA	BL21(DE3) containing pET11a/FDBD-H$_8$	(Yoder-Himes and Kroos 2006)
M. xanthus strains		
DK1622	Wild type	(Kaiser 1979)
DK4292	Tn*5 lac* Ω4400	(Kroos et al. 1986)
MDY4400.DZF1	*sglA1 attB*::pJB40030	(Yoder-Himes and Kroos 2006)
MDY4400.FA	*sglA1 fruA*::TnV Ω786 *attB*::pJB40030	(Yoder-Himes and Kroos 2006)
Plasmids		
pET11a/FDBD-H$_8$	pET11a with a gene encoding FruA-DBD-His$_8$ under control of a T7 RNA polymerase promoter	(Ueki and Inouye 2005b)
pET16b/His$_{10}$-MrpC2	pET16b with a gene encoding His$_{10}$-MrpC2 under control of a T7 RNA polymerase promoter	(Nariya and Inouye 2006)
pET11km/FruA-His$_6$	pET11km with a gene encoding FruA-His$_6$ under control of a T7 RNA polymerase promoter	S. Inouye
pJB4001	pGEM7Zf with the 1.1 kb *EcoRI-BamHI* fragment from pJB4400	(Brandner and Kroos 1998)
pJB40029	pGEM7Zf with *fmgA* DNA from -101 to +155	(Yoder and Kroos 2004a)
pDY69	pJB40029 with GTC-to-TGA mutation at –86 to –84	(Yoder and Kroos 2004a)
pDY79	pJB40029 with GGGGGTG-to-TTTTTGT mutation at –83 to –77	(Yoder and Kroos 2004a)
pDY67	pJB40029 with TG-to-GT mutation at –76 to –75	(Yoder and Kroos 2004a)

Table 2.1 (cont'd)

pDY37	pJB40029 with CGGTG-to-ATTGT mutation at –74 to –70	(Yoder and Kroos 2004a)
pDY65	pJB40029 with GGGAGC-to-TTTCTA mutation at –69 to –64	(Yoder and Kroos 2004a)
pDY35	pJB40029 with GAAC-to-TCCA mutation at –63 to –60	(Yoder and Kroos 2004a)
pDY61	pJB40029 with A-to-C mutation at –59	(Yoder and Kroos 2004a)
pDY59	pJB40029 with GTCCC-to-TGAAA mutation at –58 to –54	(Yoder and Kroos 2004a)
pDY57	pJB40029 with A-to-C mutation at –53	(Yoder and Kroos 2004a)
pGV4400.1	pJB40029 with CATCCCT-to-ACGAAAG mutation at –52 to –46	(Yoder and Kroos 2004a)

RESULTS

An insertion in *fmgA* delays aggregation

Tn*5 lac* Ω4400 in *M. xanthus* DK4292 is inserted in codon 138 of an open reading frame predicted previously to code for a protein that binds ATP or GTP (Brandner and Kroos 1998). The genomic sequence of *M. xanthus* revealed a long open reading frame (MXAN2884) preceded by a gene in the opposite orientation and followed closely by five genes in the same orientation that may form an operon (Goldman et al. 2006). The predicted start codon of MXAN2884 (Goldman et al. 2006) is likely incorrect, as it is not preceded by a good potential ribosome-binding site and it is located at -79 relative to the mRNA 5' end mapped previously (Brandner and Kroos 1998), which mutational analysis confirmed as the *fmgA* transcriptional start site (Yoder and Kroos 2004a). On the other hand, Brandner and Kroos (Brandner and Kroos 1998) identified a potential ATG start codon beginning at +66 that is preceded 5 bp upstream by the sequence AGGGAGG, which is a good potential ribosome-binding site since it is complementary (except for one mismatch) to a sequence near the 3' end of *M. xanthus* 16S rRNA (Oyaizu and Woese 1985). The ATG beginning at +66 is likely the correct start codon of MXAN2884, which we name FmgA herein. A BLAST search with the predicted FmgA sequence revealed two putative domains; an N-terminal ATPase domain and a C-terminal tetratricopeptide repeat domain. The N-terminal domain was most similar to proteins found in other myxobacteria (a putative STPK of *Sorangium cellulosum* [e^{-15}] and a putative adenylyl or guanylyl cyclase of *Stigmatella aurantiaca* [e^{-10}] that also had a C-terminal tetratricopeptide repeat domain).

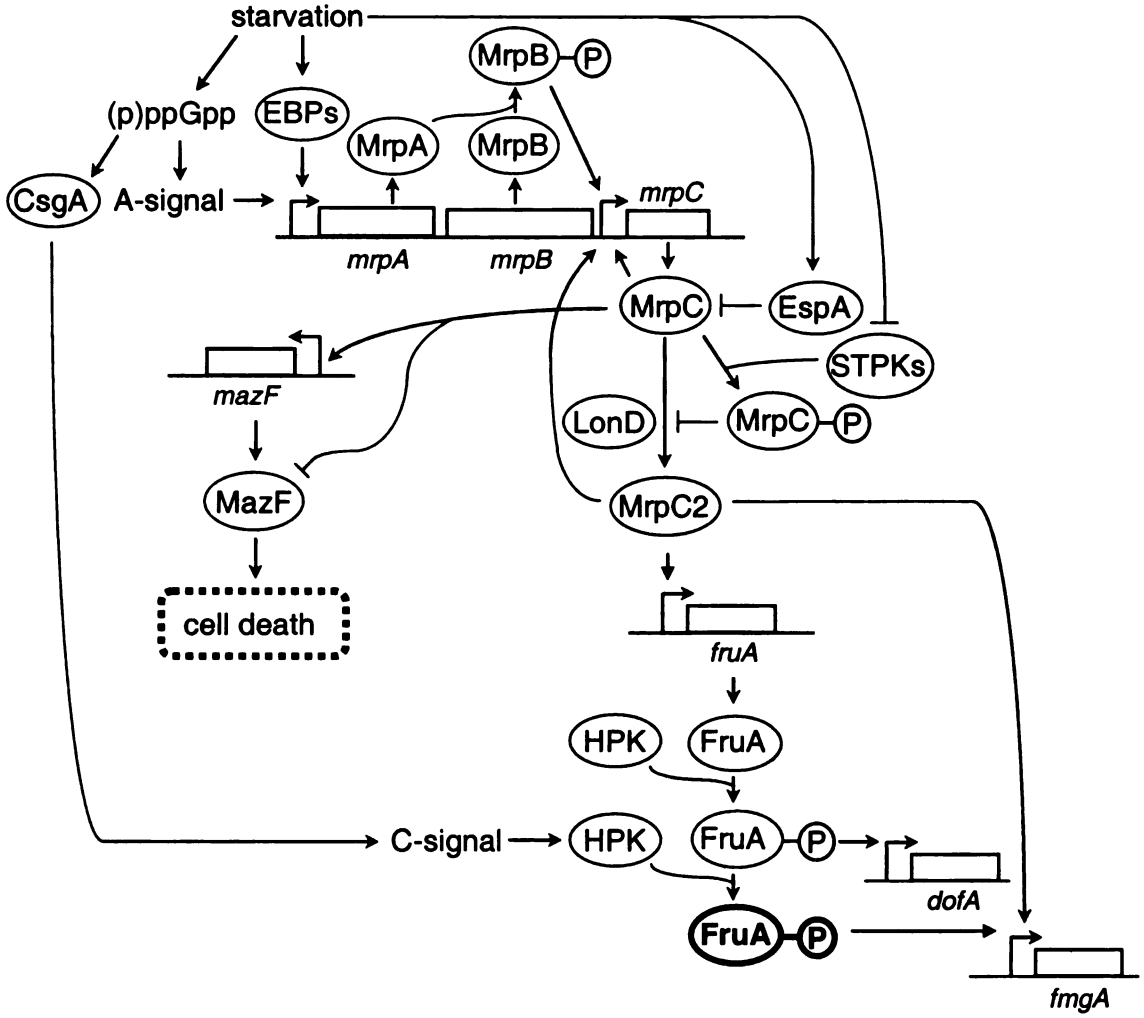


Figure 2.1. Model of the *M. xanthus* regulatory network. Effects depicted in the network may be direct or indirect. The *mrpA* and *mrpB* genes are co-transcribed from a promoter that depends on a cascade of enhancer-binding proteins (EBPs) (N. Caberoy & A.G. Garza, personal comm.), which activate transcription by σ^{54} RNA polymerase. MrpA is a putative histidine protein kinase (HPK) thought to phosphorylate MrpB, a putative EBP hypothesized to activate *mrpC* transcription (Sun and Shi 2001b). Phosphorylation of MrpC depends on a cascade of serine/threonine protein kinases (STPKs) (Nariya and Inouye 2005). Other effects are described in the text.

M. xanthus DK4292 bearing Tn*5 lac* Ω4400 inserted in *fmgA* exhibited delayed aggregation by about 6-h compared to wild-type DK1622 but both strains formed a similar number of heat- and sonication-resistant spores that were able to germinate and form a colony (data not shown). The effect on aggregation could be due to loss of FmgA and/or due to loss of one or more downstream gene products (due to a polar effect of the Tn*5 lac* insertion) that might also be transcribed from the *fmgA* promoter.

MrpC2 binds to a *cis*-regulatory sequence in the *fmgA* promoter region

As described above, mutational analysis identified a *cis*-regulatory sequence located at -63 to -46 upstream of the *fmgA* promoter (Figure 2.2) (Yoder and Kroos 2004a). This sequence contains two elements found in other C-signal-dependent promoter regions, a 5-bp element and a C box, suggesting that these elements might be bound by a transcriptional activator of *fmgA* that also regulates other C-signal-dependent genes. To identify the putative activator, DNA-binding proteins were partially purified as described previously (Ueki and Inouye 2003) from *M. xanthus* that had undergone 12 h of development, a time when *fmgA* is expressed (Kroos et al. 1986). Proteins in the AS fraction were incubated with a ^{32}P-labeled DNA fragment (-101 to +25) spanning the *fmgA* promoter region and electrophoretic mobility shift assays (EMSAs) revealed a single shifted complex (Figure 2.2). EMSAs with DNA probes having a mutation between -63 and -46 eliminated or greatly reduced formation of the shifted complex, with the exception of the single base pair change at -53, which also had a smaller effect on promoter activity *in vivo* (Figure 2.2) (Yoder and Kroos 2004a). The shifted complex

appeared to be formed by a protein in the AS fraction that binds specifically to sequences between -63 and -46 upstream of the *fmgA* promoter.

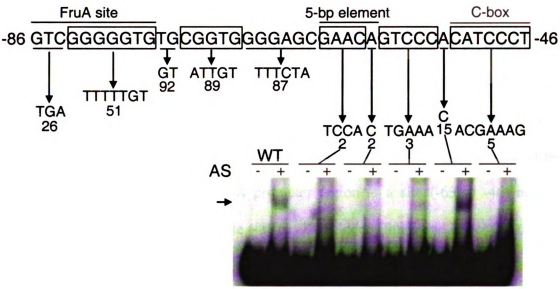


Figure 2.2. Effects of mutations on *fmgA* promoter activity *in vivo* and on DNA binding *in vitro*. The top part summarizes mutational effects on developmental *fmgA-lacZ* expression (Yoder and Kroos 2004a). The wild-type *fmgA* upstream sequence is alternately underlined or boxed to indicate mutations. Mutant sequences are shown below. The number beneath each mutant sequence indicates the percentage of wild-type promoter activity. The bottom part shows EMSAs performed with ^{32}P-labeled *fmgA* DNA (6 nM) spanning from -101 to +25 and proteins in the AS fraction (0.7 μg/μl). The arrow indicates the shifted complex produced by incubating the wild-type (WT) DNA fragment with the AS fraction, and other lanes show the effects of mutations.

To purify the putative activator protein from the AS fraction, DNA-affinity chromatography was performed with the *fmgA* promoter region (-101 to +25). The major protein species after purification had an apparent molecular weight of about 30 kDa (Figure 2.3A). The affinity-purified protein (APP) generated a shifted complex

indistinguishable from that observed with the AS fraction (Figure 2.3B). Also, like the AS fraction (Figure 2.2), APP failed to generate a shifted complex with mutant (-63 to -60) *fmgA* promoter region DNA (Figure 2.3B). Therefore, APP was subjected to mass spectrometry analysis after protease digestion. Peptide sequences matching MrpC were the only significant matches to *M. xanthus* proteins predicted from the genome sequence. MrpC is similar to CRP-family transcription factors and was shown previously to be essential for development (Sun and Shi 2001b). A form of MrpC lacking the N-terminal 25 residues, called MrpC2, was identified previously in an AS fraction based on binding to the *fruA* promoter region (Ueki and Inouye 2003). Our results suggested that MrpC2 in the AS fraction binds to the *fmgA* promoter region at a site (-63 to -46) near the previously identified FruA binding site (-86 to -77) (Yoder-Himes and Kroos 2006).

To test the idea that MrpC2 in the AS fraction was responsible for the shifted complex (Figure 2.2), antibodies against MrpC were added after the complex had been allowed to form. EMSAs revealed the formation of supershifted complexes and loss of the original shifted complex (Figure 2.4), supporting the idea that MrpC2 in the AS fraction binds to *fmgA* promoter region DNA, producing the original shifted complex.

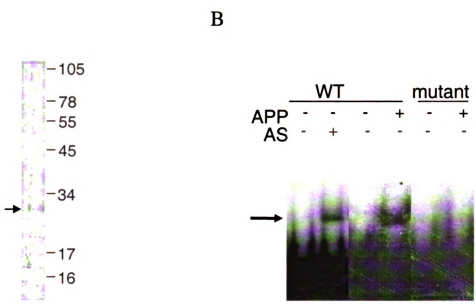


Figure 2.3. DNA-affinity purification of protein that binds to the *fmgA* promoter region. (A) SDS-PAGE of protein purified from the AS fraction using *fmgA* DNA (-101 to +25). The arrow indicates the major species in the affinity-purified protein (APP) after staining with silver. Numbers indicate the migration positions of molecular weight (kDa) standards. (B) EMSAs with ^{32}P-labeled *fmgA* DNA (6 nM) spanning from -101 to +25 and proteins in the AS fraction or the APP. The arrow indicates the shifted complex produced with the wild-type (WT) DNA fragment. APP failed to form the shifted complex with a DNA fragment bearing the GAAC to TCCA mutation at -63 to -60 (mutant).

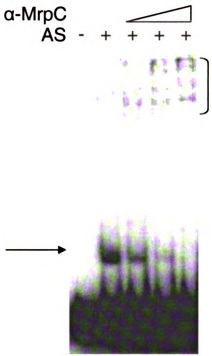


Figure 2.4. Anti-MrpC antibodies supershift the complex formed by *fmgA* promoter region DNA and the AS fraction. ^{32}P-labeled *fmgA* DNA (6 nM) spanning from -101 to +25 was incubated with the AS fraction followed by addition of 0.03 μM, 0.06 μM, or 0.1 μM anti-MrpC antibodies. The shifted complex is indicated by the arrow and supershifted complexes are indicated by the bracket.

To confirm that MrpC2 binds to the *fmgA* promoter region, N-terminally His-tagged MrpC2 (His_{10}-MrpC2) was expressed in *E. coli* and purified. His_{10}-MrpC2 exhibited a similar pattern of binding to wild-type and mutant *fmgA* promoter region DNA (-101 to +25) as seen with the AS fraction (Figure 2.5). The complex produced by His_{10}-MrpC2 migrates more slowly than the complex produced by the AS fraction, presumably due to the 10 His residues plus 8 additional residues present in the His_{10}-MrpC2 fusion protein.

Mutations between -63 and -46 eliminated or reduced MrpC2 binding, with the exception of a single base pair change at -53. These results, taken together with the effects of mutations in this region on *fmgA* promoter activity (Figure 2.2) (Yoder and Kroos 2004a), imply that MrpC2 binding to this region activates *fmgA* transcription. Since the region includes a 5-bp element and a C box, which are found in a similar arrangement, separated by 5-8 base pairs, upstream of other C-signal-dependent promoters (Fisseha et al. 1999, Viswanathan and Kroos 2003, Yoder and Kroos 2004b, Loconto et al. 2005, Viswanathan et al. 2006a, Viswanathan et al. 2007a), another implication is that MrpC2 might directly activate other C-signal-dependent genes (see Discussion). Interestingly, mutations upstream of -63 appeared to enhance (-74 to -70) or reduce (-76 to -75) MrpC2 binding, whereas two mutations between -86 and -77 that impair binding of FruA-DBD (Yoder-Himes and Kroos 2006) did not affect MrpC2 binding (Figure 2.5). We conclude that FruA and MrpC2 bind to adjacent, important *cis*-regulatory sequences upstream of the *fmgA* promoter.

The MrpC2 binding site in the *fmgA* promoter region is the third type of MrpC2 binding site to be described. It includes a 5-bp element and a C box, but other sequences between -76 and -46 also affected binding (Figures 2.2 and 2.5), so MrpC2 interacts with a long segment of DNA at this type of site. Two other types of MrpC2 binding sites have been described previously (consensus sequences GTGTC-N_8-GACAC and G/ATTTC/GAA/G) (Nariya and Inouye 2006).

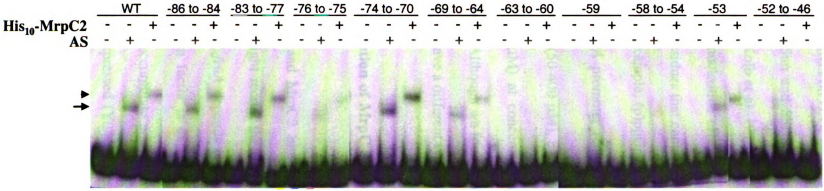


Figure 2.5. Comparison of purified His_{10}-MrpC2 and the AS fraction for binding to the *fmgA* promoter region. EMSAs with ^{32}P-labeled *fmgA* DNA (1.2 nM) spanning from -101 to +25, wild-type (WT) or mutant as indicated (see Fig. 2 for mutations), and His_{10}-MrpC2 (1 μM) or the AS fraction (0.7 μg/μl). The arrowhead and arrow indicate the shifted complexes produced by His_{10}-MrpC2 and the AS fraction, respectively. The image is a composite from three experiments (and intervening lanes were deleted from each image). In each experiment, the WT *fmgA* DNA served as a control and the signal intensity of the shifted complexes was comparable to that shown.

We tested nucleotides for an effect on DNA binding by MrpC2 since it is similar to CRP-family transcription factors (Sun and Shi 2001b). Cyclic AMP (cAMP) allosterically controls CRP DNA binding (Kolb et al. 1993), but the family also includes well-studied proteins like FNR with non-nucleotide effectors of DNA binding (Kiley and Beinert 1998). MrpC2 lacks certain residues that contact cAMP, so it was suggested that it might bind another nucleotide; for example, (p)ppGpp (Ueki and Inouye 2003). We measured His_{10}-MrpC2 binding to *fmgA* promoter region DNA (-101 to +25) by EMSAs and observed no effect of ppGpp (50-400 μM), cAMP (4-8 μM), cGMP (20-40 nM), NTPs (400 μM), or dNTPs (200 μM) at concentrations designed to reflect physiological conditions (data not shown). Although we found no evidence for a nucleotide effector of DNA binding, MrpC2 might have a different type of effector.

FruA is required for association of MrpC and/or MrpC2 with the *fmgA* promoter region *in vivo*

The proximity of the FruA and MrpC2 binding sites in the *fmgA* promoter region suggested that one protein might recruit the other or that the two proteins might bind cooperatively. Expression of *fruA* depends on MrpC2 (Figure 2.1) (Ueki and Inouye 2003), so neither transcription factor is expected to accumulate in an *mrpC* mutant. However, MrpC and MrpC2 accumulate normally in a *fruA* mutant (Nariya and Inouye 2006), yet *fmgA* fails to be expressed (Yoder-Himes and Kroos 2006). Why are MrpC and MrpC2 insufficient to activate *fmgA* transcription? We hypothesized that they fail to bind to the *fmgA* promoter region in the absence of FruA. To test this hypothesis, chromatin immunoprecipitation (ChIP) with polyclonal antibodies against MrpC (which

also recognize MrpC2) (Nariya and Inouye 2006) was used to measure the association of MrpC and/or MrpC2 with the *fmgA* promoter region (-101 to +155) integrated ectopically into the chromosome of wild-type or *fruA* mutant cells that had undergone development. DNA recovered from ChIP was subjected to PCR with primers designed to amplify the ectopic copy of the *fmgA* promoter region. The PCR analysis revealed that the *fmgA* promoter region was enriched by ChIP with antibodies against MrpC relative to control antibodies for wild type, but not for the *fruA* mutant (Figure 2.6, top panel). Neither strain showed enrichment of *rpoC* coding region DNA (as a negative control) (Figure 2.6, bottom panel). We conclude that FruA is required for association of MrpC and/or MrpC2 with the *fmgA* promoter region *in vivo*.

Cooperative binding of MrpC2 and FruA to the *fmgA* promoter region

The requirement for FruA for association of MrpC and/or MrpC2 with the *fmgA* promoter region *in vivo* is consistent with recruitment or cooperative binding. To distinguish between these models and to test the notion that FruA directly affects binding of MrpC2 to the *fmgA* promoter region, recombinant His-tagged FruA (FruA-His_6) was mixed with His_{10}-MrpC2 and *fmgA* promoter region DNA (-101 to +25) for analysis of DNA binding by EMSAs. FruA-His_6 bound to the *fmgA* promoter region weakly compared with His_{10}-MrpC2, but there was a strong enhancement of shifted complex formation when both proteins were incubated with *fmgA* DNA (Figure 2.7A). In the presence of both proteins, two complexes were observed. The abundant lower complex (LC) co-migrated with the complexes formed by either protein alone, suggesting that the LC is a mixture of

complexes composed of DNA bound by His_{10}-MrpC2 or FruA-His_6. The upper complex (UC) was suggestive of a complex of two protein molecules bound to DNA.

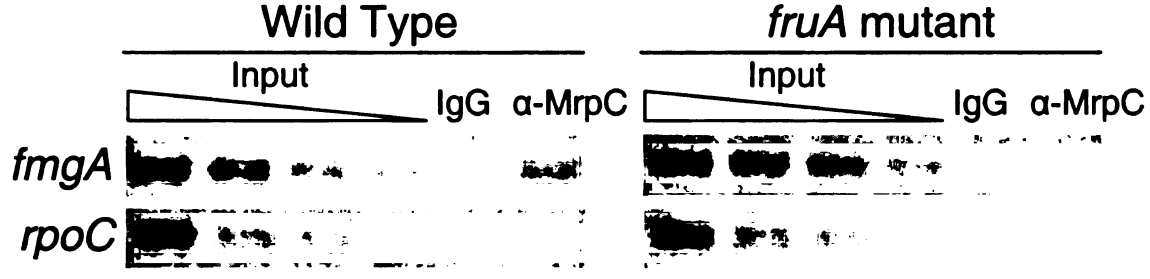


Figure 2.6. FruA is required for association of MrpC and/or MrpC2 with the *fmgA* promoter region *in vivo*. ChIP analysis of *M. xanthus* with the *fmgA* promoter region (-101 to +155) integrated ectopically in otherwise wild-type or *fruA* mutant backgrounds. After 18 h of development, cells were treated with formaldehyde, lysed, and crosslinked chromatin was immunoprecipitated with anti-MrpC antibodies or IgG as a control. DNA was amplified with appropriate primers for the *fmgA* promoter region at the ectopic chromosomal site or for the *rpoC* coding region as a negative control. A twofold dilution series of input DNA purified from 0.25%, 0.125%, 0.0625% or 0.03125% 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.

Interestingly, the C-terminal DNA-binding domain of FruA was insufficient to enhance shifted complex formation in combination with MrpC2. The complexes formed by the combination of proteins were indistinguishable from those produced when FruA-DBD-His_8 or His_{10}-MrpC2 alone was incubated with *fmgA* promoter region DNA (Figure 2.7B). We conclude that the N-terminal domain of FruA contains an important determinant for enhancement of shifted complex formation in combination with MrpC2 and the *fmgA* promoter region.

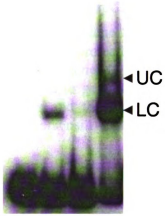
To characterize the enhanced DNA binding observed in the presence of His_{10}-MrpC2 and FruA-His_6 (Figure 2.7A), DNase I footprinting of complexes in solution was performed. Protection was observed with His_{10}-MrpC2 alone in the region spanning the 5-bp element and the C box (Figure 2.8A), where MrpC2 binding was previously mapped by EMSAs (Figure 2.5). There were prominent hypersensitive sites in this region (Figure 2.8A), suggesting that His_{10}-MrpC2 bends the DNA upon binding. The protection and hypersensitivity in this region increased when FruA-His_6 was present in combination with His_{10}-MrpC2, but was not observed with FruA-His_6 alone, suggesting that His_{10}-MrpC2 binding was increased in the presence of FruA-His_6. DNase I footprinting of the other strand with FruA-His_6 alone revealed a hypersensitive site near -86 (Figure 2.8B), a region that was previously shown to bind FruA-DBD-His_8 (Yoder-Himes and Kroos 2006). The intensity of this hypersensitive site increased when His_{10}-MrpC2 was present in combination with FruA-His_6, but hypersensitivity was not observed with His_{10}-MrpC2 alone, suggesting that FruA-His_6 binding was increased in the presence of His_{10}-MrpC2. As observed with the other strand (Figure 2.8A), there were protected and hypersensitive sites near the C box with His_{10}-MrpC2 alone and these signals were increased in the presence of both proteins (Figure 2.8B). Hypersensitive sites were observed near -77 and -70 when both proteins were present but not with either protein alone (Figure 2.8B), suggesting simultaneous binding of MrpC2 and FruA to the same DNA molecule. The

DNase I footprinting results demonstrate cooperative binding of FruA and MrpC2 to the *fmgA* promoter region, providing plausible explanations for the observed dependence of MrpC and/or MrpC2 on FruA for association with the *fmgA* promoter region *in vivo* (Figure 2.6) and for the observed enhancement of shifted complex formation *in vitro* (Figure 2.7A).

Figure 2.7. Enhancement of shifted complex formation. (A) The combination of FruA-His_6 and His_{10}-MrpC2 enhances complex formation. EMSAs with ^{32}P-labeled *fmgA* DNA (1.2 nM) spanning from -101 to +25 with 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. Arrowheads indicate upper complex (UC) and lower complex (LC). (B) The combination of FruA-DBD-His_8 and His_{10}-MrpC2 does not enhance complex formation. EMSAs with ^{32}P-labeled *fmgA* DNA (1.2 nM) spanning from -101 to +25 with no protein, His_{10}-MrpC2 (1 μM), FruA-DBD-His_8 (14 μM) or both His_{10}-MrpC2 (1 μM) and FruA-DBD-His_8 (14 μM) as indicated. The arrowhead and arrow indicate the complexes produced by His_{10}-MrpC2 and FruA-DBD-His_8, respectively.

A

FruA-His_6 - - + +
His_{10}-MrpC2 - + - +



B

FruA-DBD-His_8 - - + +
His_{10}-MrpC2 - + - +

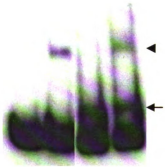
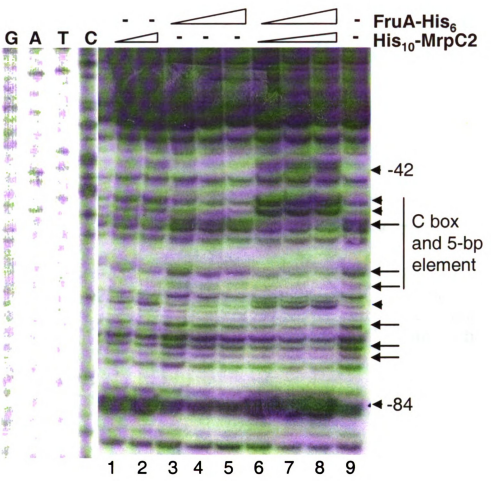
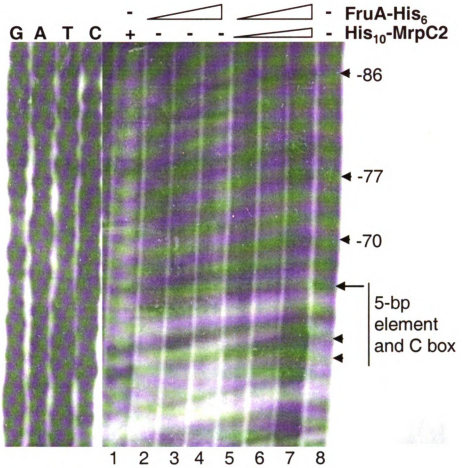


Figure 2.8. DNase I footprinting shows cooperative binding of MrpC2 and FruA to the *fmgA* promoter region. (A) *fmgA* promoter region DNA (-139 to +25) was 5′-labeled at -139, incubated with 1 or 1.5 μM His_{10}-MrpC2 (lanes 1-2), or with 1.5, 3 or 4.5 μM FruA-His_6 (lanes 3-5), or with 0.5, 1 or 1.5 μM His_{10}-MrpC2 in combination with 1.5, 3 and 4.5 μM FruA-His_6 (lanes 6-8), or with no protein (lane 9), and subjected to DNase I footprinting. (B) *fmgA* promoter region DNA (-101 to +25) was 5′-labeled at +25, incubated with 1.5 μM His_{10}-MrpC2 (lane 1), or with 1.5, 3 or 4.5 μM FruA-His_6 (lanes 2-4), or with 0.5, 1 or 1.5 μM His_{10}-MrpC2 in combination with 1.5, 3 or 4.5 μM FruA-His_6 (lanes 5-7), or with no protein (lane 8), and subjected to DNase I footprinting. In both panels, arrows indicate sites protected from DNase I digestion and arrowheads indicate hypersensitive sites. Also, lanes G, A, T and C show sequence ladders generated by the same labeled primer used to generate the probe for DNase I footprinting. Lanes between the sequence ladders and the footprints were deleted from the images. (C) Summary of protected and hypersensitive site.

A


G A T C
- - - -
FruA-His6
His10-MrpC2
-42
C box
and 5-bp
element
-84
1 2 3 4 5 6 7 8 9

B


G A T C
- + - - - - -
FruA-His6
His10-MrpC2
-86
-77
-70
5-bp element and C box
1 2 3 4 5 6 7 8

C


-86 GTCGGGGGTGTGCGGTGGGGAGCGAACAGTCCCACATCCCTGGCG -42
CAGCCCCCACACGCCACCCCTCGCTTGTCAGGGTGTAGGGACCGC

To determine whether the binding sites for both His_{10}-MrpC2 and FruA-His_6 in the *fmgA* promoter region are important for enhanced formation of shifted complexes, EMSAs were performed with mutant DNA fragments expected to impair binding of one or the other protein. Mutations in the region from -86 to -77 greatly reduce binding of FruA-DBD-His_8 (Yoder-Himes and Kroos 2006), and reduced the enhancement of shifted complex formation by the combination of FruA-His_6 and His_{10}-MrpC2 (Figure 2.9).

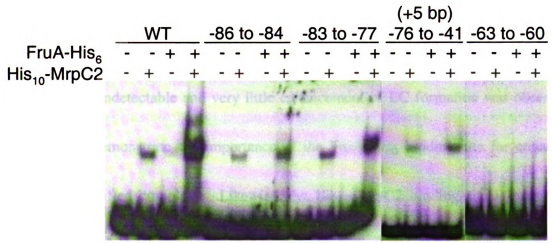


Figure 2.9. Enhancement of shifted complex formation depends on binding sites for both FruA and MrpC2. EMSAs with ^{32}P-labeled *fmgA* DNA (1.2 nM) spanning from -101 to +25, wild-type (WT) or mutant as indicated (see Fig. 2 for mutations), and His_{10}-MrpC2 (1 μM) and/or FruA-His_6 (3 μM) as indicated. The modified (+5 bp) -76 to -41 DNA fragment has non-*fmgA* sequence (CACAA) at its upstream end.

UC was undetectable and LC was greatly diminished with DNA fragments bearing mutations at -86 to -84 or -83 to -77. The small amount of enhancement of LC formation

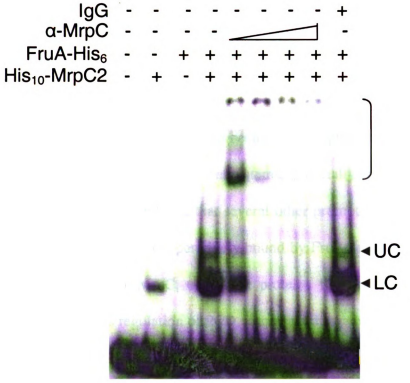
could be due to a small amount of FruA-His_6 binding that is undetectable when FruA-His_6 alone is incubated with DNA. To test this possibility, we attempted to eliminate the FruA-His_6 binding site without impairing His_{10}-MrpC2. A DNA fragment from -76 to -41 was insufficient for His_{10}-MrpC2 binding (data not shown), indicating that the site required for His_{10}-MrpC2 binding may partially overlap the site required for FruA-His_6 binding. However, adding 5 bp of non-*fmgA* sequence (CACAA) to the upstream end allowed His_{10}-MrpC2 binding (Figure 2.9). No FruA-His_6 binding was detected with this modified (+5 bp) -76 to -41 DNA fragment. In the presence of His_{10}-MrpC2 and FruA-His_6, UC was undetectable and very little enhancement of LC formation was observed. These results demonstrate the importance of the FruA-His_6 binding site for enhanced formation of shifted complexes. Likewise, the His_{10}-MrpC2 binding site is extremely important since a DNA fragment containing a mutation at -63 to -60, which eliminates detectable His_{10}-MrpC2 binding, also abolished detectable enhancement of shifted complex formation (Figure 2.9).

Supershift assays provided further evidence that both His_{10}-MrpC2 and FruA-His_6 are responsible for enhanced formation of shifted complexes with *fmgA* promoter region DNA. After incubating His_{10}-MrpC2 and FruA-His_6 with *fmgA* DNA to allow formation of complexes, purified antibodies against MrpC or FruA were added and incubation was

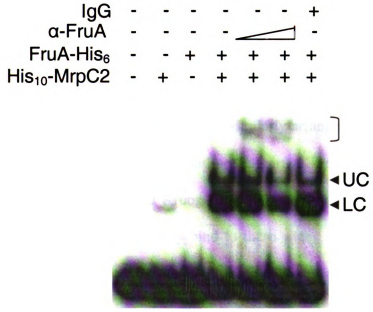
continued, followed by EMSAs. An increasing amount of anti-MrpC antibodies resulted in progressive loss of UC and LC, and appearance of supershifted complexes (Figure 2.10A). A greater amount of anti-MrpC antibodies did not result in more of the supershifted complexes. Rather, there appeared to be more unbound DNA fragment, as if the antibodies interfered with the equilibrium between bound and unbound His_{10}-MrpC2. Similar results were obtained with FruA antibodies, although inhibition of UC and LC formation was incomplete (Figure 2.10B). These results support the interpretation that enhancement of shifted complex formation involves binding of both His_{10}-MrpC2 and FruA-His_6 to adjacent (possibly overlapping) sites upstream of the *fmgA* promoter, and together with our footprinting and ChIP results support a model in which FruA and MrpC2 bind cooperatively to regulate *fmgA* transcription during *M. xanthus* development.

Figure 2.10. Antibodies against MrpC2 or FruA cause appearance of supershifted complexes and loss of UC and LC. ^{32}P-labeled *fmgA* promoter region DNA (1.2 nM) spanning from -101 to +25 was incubated with His_{10}-MrpC2 (1 μM) and/or FruA-His_6 (3 μM) as indicated followed by addition of antibodies. (A) Supershift assay with an increasing amount of anti-MrpC antibodies (0.2, 0.3, 0.5 or 0.6 μM) as indicated. IgG (0.7 μM) served as a negative control. (B) Supershift assay with an increasing amount of anti-FruA antibodies (7 or 8 μM) as indicated. IgG (8 μM) served as a negative control. In both panels, arrowheads indicate the UC and LC, and a bracket indicates the supershifted complexes.

A


IgG - - - - - - - +
α-MrpC - - - - - -
FruA-His6 - - + + + + + + +
His10-MrpC2 - + - + + + + + +
UC
LC

B


IgG - - - - - - +
α-FruA - - - - -
FruA-His6 - - + + + + +
His10-MrpC2 - + - + + + +
UC
LC

DISCUSSION

We have discovered that a crucial *cis*-regulatory element in the *fmgA* promoter region is bound by MrpC2, but that recruitment of MrpC2 to its binding site is enhanced by FruA. Our DNA binding studies revealed cooperative binding of FruA and MrpC2 to adjacent (possibly overlapping) sites upstream of the *fmgA* promoter. This represents a novel mechanism of gene regulation since recruitment of another transcription factor (MrpC2) by a response regulator (FruA) has not been observed previously, despite the prevalence of two-component signal transduction systems, especially in bacteria. Our preliminary results, described below, indicate that several other promoter regions that depend on C-signaling for activation are cooperatively bound by FruA and MrpC2. Since MrpC2 is a proteolytic fragment of MrpC, and these proteins function not only as transcription factors, but also in the regulation of PCD, it appears that cooperative binding of FruA and MrpC2 facilitates the coordination of multiple signaling pathways to ensure proper control of gene expression and cell fate during *M. xanthus* development.

Cooperative binding of MrpC2 and FruA appears to be a conserved mechanism of gene regulation in response to C-signaling during *M. xanthus* development. The *cis*-regulatory element to which MrpC2 binds in the *fmgA* promoter region includes a 5-bp element and a C box. These two sequences are similarly arranged immediately upstream of other C-signal-dependent promoters and are important for promoter activity (Viswanathan and Kroos 2003, Yoder and Kroos 2004b, Loconto et al. 2005, Viswanathan et al. 2006a), suggesting that MrpC2 may bind to these sites. Indeed, in the promoter region of the operon identified by Tn*5* *lac* Ω4499, MrpC2 binds near a 5-bp element and it appears to

bind cooperatively with FruA (S. M. and L. K., unpublished data). In the promoter region of the *dev* operon, whose products are important for sporulation (Thony-Meyer and Kaiser 1993, Boysen et al. 2002, Viswanathan et al. 2007b), MrpC2 binds to a region that includes a 5-bp element followed 3 or 7 bp downstream by two C-box-like sequences, and it appears to bind cooperatively with FruA (S. M., P. Viswanathan, and L. K., unpublished data). In the promoter region of the gene identified by Tn*5* *lac* Ω4403, MrpC2 binds to a region that includes two 5-bp elements in inverted orientation, and it appears to bind cooperatively with FruA (J. Lee, S. M., and L. K., unpublished data). Our preliminary studies, taken together with the evidence presented here for *fmgA*, indicate that cooperative binding of MrpC2 and FruA is a conserved mechanism of C-signal-dependent gene regulation.

Cooperative binding of MrpC2 and FruA to promoter regions of C-signal-dependent genes represents a novel mechanism of gene regulation. Typically, DNA-binding response regulators are phosphorylated by an HPK and this modification enhances DNA binding (West and Stock 2001). The bound response regulator recruits RNA polymerase to the promoter or facilitates another step during transcription initiation. Phosphorylation of FruA's N-terminal regulatory domain may relieve an inhibitory effect on its C-terminal DNA-binding domain, since FruA-DBD-His_8 appears to bind with higher affinity than (presumably unphosphorylated) full-length FruA-His_6 to the *fmgA* promoter region (Fig. 6) and other promoter regions (Viswanathan et al. 2007b) (S. M. and L. K., unpublished data). We found that FruA is required for association of MrpC and/or MrpC2 with the *fmgA* promoter region *in vivo* (Figure 2.6). This presumably explains why *fmgA*

expression is abolished in a *fruA* mutant (Yoder-Himes and Kroos 2006). FruA and/or MrpC2 probably interact with RNA polymerase at the *fmgA* promoter. The two proteins occupy a location typical for Class I activators, which function by contacting the C-terminal domain of the α subunits of RNA polymerase (Barnard et al. 2004).

The detailed mechanism of cooperative binding of MrpC2 and FruA to the *fmgA* promoter region remains to be explored. The binding sites of the two proteins may partially overlap since a 7-bp mutation at -83 to -77 impairs FruA-DBD-His_8 binding (Yoder-Himes and Kroos 2006) and since DNA upstream of -76 is required for His_{10}-MrpC2 binding (data not shown). The two proteins may interact with opposite faces of the DNA in a region of overlap, analogous to certain homeodomain proteins, which bind DNA cooperatively (Tullius 1995, Passner et al. 1999). As for some homeoprotein-DNA complexes, cooperativity might depend not only on protein-protein interactions but on bending of the DNA by one or both proteins. Binding of either MrpC2 or FruA alone to the *fmgA* promoter region produced DNase I hypersensitivity indicative of DNA bending, and the combination of proteins increased the intensity and number of hypersensitive sites (Figure 2.8). While these results demonstrated cooperative binding, we did not observe much protection from DNase I digestion, suggestive of limited occupancy. Likewise, EMSAs clearly showed that the combination of proteins enhances formation of shifted complexes (Figure 2.7A) and that this depends on sequences important for binding of each protein (Figure 2.9), consistent with cooperative binding, yet the predominant shifted complex, LC, co-migrated with complexes produced by either protein alone. This suggests limited co-occupancy by MrpC2 and FruA, although we

cannot rule out the possibility that LC is a mixture of binary (one protein bound to DNA) and ternary (both proteins bound to DNA) complexes since the ternary complex might migrate unexpectedly due to DNA bending. The enhancement of shifted complex formation by MrpC2 and FruA requires the N-terminal regulatory domain of FruA (Figure 2.7B), which is believed to be phosphorylated by one or more HPKs in *M. xanthus* (Ellehauge et al. 1998). Treatment of FruA-His_6 (purified from *E. coli* and therefore conceivably phosphorylated to a small extent) with phosphatase from bacteriophage λ did not diminish its ability to enhance formation of shifted complexes (data not shown). It seems likely that the N-terminal domain of FruA interacts directly with MrpC2, but further studies will be needed to distinguish this model from the possibility that FruA's N-terminal domain alters the DNA structure in a way that facilitates MrpC2 binding. No enhancement of shifted complex formation was observed when the FruA and MrpC2 binding sites were on separate DNA fragments (only one of which was ^{32}P-labeled in each of two separate experiments) (data not shown). Although the detailed mechanism of cooperative binding is unknown, it is worth exploring because response regulators like FruA and CRP-family transcription factors like MrpC are abundant in bacterial signaling and gene regulatory networks, and cooperative binding between such proteins could provide a general mechanism to achieve tight combinatorial control of target genes.

The regulation of *fmgA* by the combination of MrpC2 and FruA constitutes a coherent feed-forward loop. Such loops, in which one transcription factor (MrpC2) positively regulates another (FruA), and the two factors both positively regulate target genes, are

common in regulatory networks (Milo et al. 2002, Shen-Orr et al. 2002, Mangan and Alon 2003). They allow filtering out of noise in input stimuli, rapid response to step-like stimuli in one direction (e.g., off to on), delayed response to steps in the opposite direction (e.g., on to off), and delay of target gene expression until both transcription factors reach a sufficient concentration (Mangan et al. 2003). These characteristics could have obvious benefits for regulation of *fmgA* and other genes in response to starvation, C-signaling, and other signals via MrpC2 and FruA.

Combinatorial regulation of gene expression is common in bacteria and eukaryotes because it allows multiple signals to control individual genes (Barnard et al. 2004, Stathopoulos and Levine 2005). Cooperative interactions between activators is one of several mechanisms for achieving combinatorial control in bacteria; however, not many examples (and none involving a response regulator) have been reported previously, perhaps due to evolutionary constraints it places on the activators (Barnard et al. 2004). For transcription factors like MrpC and FruA that are devoted to a developmental program, such evolutionary constraints may be tolerable. On the other hand, both MrpC and FruA probably interact with multiple protein partners, in addition to interacting with DNA, and possibly with each other and RNA polymerase.

It has been proposed that distinct HPKs phosphorylate FruA in response to an early (unidentified) signal and later in response to C-signal (Ueki and Inouye 2003) (Figure 2.1). This model can explain how different FruA-dependent genes exhibit different levels of dependence on C-signaling, if in the absence of C-signaling the level of

phosphorylated FruA supports full (e.g., *dofA*), partial (e.g., *fmgA*), or no expression due to differential affinity for binding sites in promoter regions. It will be interesting to test whether C-signal-independent genes like *dofA* are not only directly regulated by FruA (Ueki and Inouye 2005a) but also by cooperative binding of MrpC2.

MrpC is phosphorylated by an STPK cascade, presumably in response to an unknown signal during growth, inhibiting accumulation of MrpC and MrpC2 (Nariya and Inouye 2005). Starvation conditions may remove the signal (Figure 2.1), allowing MrpC and MrpC2 to accumulate. Recently, it was shown that the EspA signal transduction pathway influences the MrpC and MrpC2 concentrations (Higgs et al. 2008), presumably providing another link to starvation (Figure 2.1), although the exact signal to which EspA responds is unknown. Also, MrpC was shown to interact with the toxin MazF, inhibiting PCD (Nariya and Inouye 2008) (Figure 2.1). On the other hand, MrpC appears to directly activate *mazF* transcription. Whether MrpC2 differs from MrpC in either of these activities is important to test. The concentrations of MrpC and its phosphorylated or cleaved forms, and their interactions with MazF, and at different promoters, may determine the fate of cells in a developing population of *M. xanthus*.

Nariya and Inouye (Nariya and Inouye 2008) suggested that the position of an individual cell in the cell cycle at the time of nutrient depletion might determine its fate. Alternatively, by analogy with many cell fate decisions in bacteria (Dubnau and Losick 2006, Smits et al. 2006) and eukaryotes (Ferrell 2002), we suggest that the MrpC positive autoregulatory loop could be a source of bistability in the developing cell population.

This model predicts heterogeneity in the MrpC concentration in cells undergoing development. The concentrations of MrpC and MrpC2 also influence the pace of aggregation and the morphology of fruiting bodies. Mutants defective in the STPK cascade (that leads to phosphorylation of MrpC) or EspA exhibit accelerated aggregation (Nariya and Inouye 2005, Higgs et al. 2008). These mutants make fairly normal spore numbers. Whether MazF-mediated PCD is aberrant in these mutants and whether this contributes to the disorganized appearance of fruiting bodies and loss of coordination between aggregation and sporulation (i.e., spore formation outside of fruiting bodies) are intriguing questions.

Commitment to form a spore has been hypothesized to involve induction of genes at the Ω7536 locus, which in turn depends on induction of the *dev* operon (Kroos 2007). Since *dev* appears to be regulated by cooperative binding of MrpC2 and FruA (S. M., P. Viswanathan, and L. K., unpublished data), we propose that commitment to sporulation is governed by these key transcription factors. MrpC is a major hub in the regulatory network, linked extensively to starvation (Figure 2.1). Its direct involvement in commitment to sporulation might couple persistent starvation to the decision to form a spore. FruA is likewise a major hub in the regulatory network. Phosphorylation of FruA in response to short-range C-signaling might contribute positional information (i.e., cell alignment in the nascent fruiting body) to the decision to sporulate, ensuring that spores form within fruiting bodies (Kaiser 2003, Sogaard-Andersen et al. 2003). Other potential inputs via FruA include phosphorylation in response to another signal(s) (Jelsbak et al. 2005) and regulation at the transcriptional level. In addition to direct regulation by

MrpC2 (Ueki and Inouye 2003), at least four other inputs directly or indirectly control *fruA* transcription; SasR (responsive to A-signal) (Guo et al. 2000), HthA (Nielsen et al. 2004), CarD (Penalver-Mellado et al. 2006), and DevT (feeding back positively from the *dev* operon) (Boysen et al. 2002). Commitment to sporulation may also be governed by a third activator of *dev* transcription, LadA (Viswanathan et al. 2007b). This LysR-type transcription factor likely responds to a signal and, unusually, it acts positively from a site downstream of the *dev* promoter, perhaps by counteracting negative regulatory elements. Combinatorial regulation of *dev* by at least three signal-responsive transcription factors and a regulatory region spanning more than 1 kb resembles regulation of developmental genes in multicellular eukaryotes (Stathopoulos and Levine 2005, Viswanathan et al. 2007a, Viswanathan et al. 2007b).

Integration of environmental, cell-to-cell, and intracellular signals by transcription factors to control gene expression and cell fate is crucial for multicellular organisms, especially during development. We have discovered that during *M. xanthus* development the signal integration potential of MrpC is combined with that of FruA. Cooperative binding, and a coherent feed-forward loop design, create a powerful regulatory circuit to achieve correct temporal and spatial expression of target genes. Examples of spatiotemporal differentiation of bacteria in architecturally complex biofilms continue to emerge (Vlamakis et al. 2008), so it would be surprising if the design principles discovered in *M. xanthus* are not utilized in other bacterial communities, as well as in multicellular eukaryotes.

Chapter 3: Combinatorial regulation by a novel arrangement of FruA and MrpC2 transcription factors during *Myxococcus xanthus* development

The work in this chapter will be submitted to the Journal of Bacteriology in September 2008.

ABSTRACT

Myxococcus xanthus is a Gram-negative soil bacterium that undergoes multicellular development upon nutrient limitation. Intercellular signals control cell movements and regulate gene expression during the developmental process. C-signal is a short-range signal essential for aggregation and sporulation. C-signaling regulates the *fmgA* gene by a novel mechanism involving cooperative binding of the response regulator FruA and the transcription factor/antitoxin MrpC2. Here, we demonstrate that regulation of the C-signal-dependent *fmgBC* operon is under similar combinatorial control by FruA and MrpC2, but the arrangement of binding sites is different than in the *fmgA* promoter region. MrpC2 was shown to bind to a crucial *cis*-regulatory sequence in the *fmgBC* promoter region. FruA was required for MrpC and/or MrpC2 to associate with the *fmgBC* promoter region *in vivo*, and expression of an *fmgB-lacZ* fusion was abolished in a *fruA* mutant. Recombinant FruA was shown to bind to an essential regulatory sequence located slightly downstream of the MrpC2-binding site in the *fmgBC* promoter region. Full-length FruA, but not its C-terminal DNA-binding domain, enhanced the formation of complexes with *fmgBC* promoter region DNA, when combined with MrpC2. This effect was abolished with *fmgBC* DNA fragments having a mutation in either the MrpC2- or FruA-binding site, indicating that both proteins must bind to DNA in order to enhance complex formation. These results are similar to those observed for *fmgA*, where FruA and MrpC2 bind cooperatively upstream of the promoter, except in the *fmgA* promoter region the FruA-binding site is located slightly upstream of the MrpC2-binding site. Cooperative binding of FruA and MrpC2 appears to be a conserved mechanism of gene

regulation that allows a flexible arrangement of binding sites, and coordinates multiple signaling pathways.

INTRODUCTION

Myxococcus xanthus is a rod-shaped bacterium that glides on solid surfaces, forming a single-species biofilm that provides an attractive model to study how signaling couples gene expression to environmental and cellular cues (Whitworth 2008). *M. xanthus* cells in the biofilm grow and divide when nutrients are available, but upon starvation, a multicellular developmental process ensues, during which cells move into aggregates and form mound-shaped structures called fruiting bodies. Approximately 10^5 cells participate in forming a fruiting body, in which a portion of the cells differentiate into dormant, stress-resistant, spherical spores. Other cells undergo programmed cell death (Nariya and Inouye 2008) or autolysis caused by siblings in the developing biofilm (Wireman and Dworkin 1977, O'Connor and Zusman 1988), and some cells remain outside of fruiting bodies as peripheral rods (O'Connor and Zusman 1991). These fates are met by different proportions of cells in the biofilm, depending on genetic and environmental factors (O'Connor and Zusman 1988, Berleman and Kirby 2007). The spores in a fruiting body can germinate and resume growth and division when nutrients become available.

Signals act at different times during the developmental process to coordinate cell behavior and determine cell fate. Nutrient limitation causes a stringent response that results in production of (p)ppGpp and the induction of early developmental genes (Harris et al. 1998). A mixture of amino acids and peptides, known as A-signal, is generated by secreted proteases and is believed to allow quorum sensing (Kuspa et al. 1992b). A-signal-dependent genes are expressed and cells alter their pattern of movement so that aggregates begin to form. Subsequent gene expression, and the maturation of aggregates

into spore-filled fruiting bodies, depends on C-signaling, which is mediated by the product of the *csgA* gene (Shimkets et al. 1983). CsgA is associated with the outer membrane of the cell, where it is processed by a secreted protease to a 17 kDa form that appears to act as a short-range signal (Kim and Kaiser 1990d, Lobedanz and Sogaard-Andersen 2003). C-signal transduction requires cell-alignment (Kim and Kaiser 1990b) and possibly end-to-end contact between cells (Sager and Kaiser 1994), so it communicates positional information. Cells become aligned as aggregates transform into nascent fruiting bodies and the resulting high level of C-signaling has been proposed to trigger expression of genes required for sporulation (Sager and Kaiser 1993). Indeed, the expression of C-signal-dependent genes that are important for sporulation is restricted to nascent fruiting bodies (Sager and Kaiser 1993, Julien et al. 2000), and many studies support a model in which an increasing level of C-signaling controls gene expression to coordinate aggregation and sporulation during development (Kim and Kaiser 1991, Li et al. 1992, Gronewold and Kaiser 2001, Kruse et al. 2001).

How does C-signaling regulate expression of target genes? FruA plays a key role in the C-signal transduction pathway (Ogawa et al. 1996, Ellehauge et al. 1998). It is similar to response regulators of two-component signal transduction systems and is believed to be phosphorylated in its N-terminal regulatory domain in response to C-signal and perhaps other signals (Ellehauge et al. 1998, Jelsbak et al. 2005), but the cognate histidine protein kinase(s) has not been identified. Presumably, phosphorylation enhances DNA binding by the C-terminal domain of FruA, which is similar to that of the NarL/FixJ subfamily of response regulators (West and Stock 2001). The C-terminal domain of FruA has been

shown to bind to sites in the promoter regions of developmentally-regulated genes that fail to be expressed in *fruA* mutant cells, suggesting that FruA is a transcriptional activator (Ueki and Inouye 2005a, Ueki and Inouye 2005b, Yoder-Himes and Kroos 2006). Recently, FruA was shown to bind cooperatively with MrpC2 to the promoter region of the C-signal-dependent *fmgA* (FruA- and MrpC2-regulated gene A) gene (Mittal and Kroos 2008), revealing a novel mechanism of combinatorial control, as cooperative binding of a response regulator (FruA) and a distinct transcription factor (MrpC2) had not been observed previously.

MrpC2 is a smaller form of MrpC (Ueki and Inouye 2003), which is similar to the cyclic AMP receptor protein (CRP) family of transcriptional regulators (Sun and Shi 2001b). MrpC is expressed during vegetative growth and is phosphorylated by a cytoplasmic serine/threonine protein kinase (STPK) called Pkn14 (Nariya and Inouye 2005, Nariya and Inouye 2006). Pkn14 is in turn phosphorylated by a membrane STPK called Pkn8. Phosphorylation of MrpC by the Pkn8/Pkn14 cascade results in weaker binding of MrpC to DNA and also appears to inhibit proteolytic cleavage of MrpC to MrpC2 (Nariya and Inouye 2006), which lacks the 25 N-terminal residues of MrpC (Ueki and Inouye 2003). The STPK cascade is counteracted by an unknown mechanism early in development, allowing MrpC and MrpC2 concentrations to rise. MrpC2 binds to DNA with higher affinity than MrpC (Nariya and Inouye 2006), and appears to play a key role as a transcriptional activator during development. Recently, MrpC was shown to function as an antitoxin by interacting directly with the toxin MazF, an mRNA interferase that mediates programmed cell death during development (Nariya and Inouye 2008). MrpC

also binds to the *mazF* promoter region and activates expression. Binding of MrpC2 to the *mazF* promoter region and MazF has not been tested. The dual functions of MrpC, and possibly MrpC2, as an antitoxin and a transcription factor make it an important determinant of cell fate. The finding that MrpC2 and FruA bind cooperatively to crucial *cis*-regulatory sequences upstream of the *fmgA* promoter suggests that starvation and other signals that regulate MrpC2 are integrated with positional information via short-range C-signaling that leads to phosphorylation of FruA (Mittal and Kroos 2008). This novel mechanism of combinatorial control was predicted to be conserved because similar *cis*-regulatory sequences have been found upstream of other developmentally-regulated *M. xanthus* promoters (Fisseha et al. 1999, Viswanathan and Kroos 2003, Yoder and Kroos 2004b, Srinivasan and Kroos 2004, Loconto et al. 2005, Viswanathan et al. 2006a, Viswanathan et al. 2007a).

The promoter region of a putative operon (named herein *fmgBC* for FruA- and MrpC2-regulated genes B and C) at the Ω4499 locus in the *M. xanthus* chromosome has *cis*-regulatory sequences similar to those bound by MrpC2 in the *fmgA* promoter region. The *fmgBC* operon was identified by an insertion of the transposon Tn*5 lac* into *fmgC* (Kroos et al. 1986). FmgB and FmgC are similar to reductase and oxidase components, respectively, of bacterial cytochrome P-450 systems, which typically are involved in catabolism or anabolism of unusual compounds (Fisseha et al. 1999). *M. xanthus* DNA upstream of *fmgBC* was cloned, a putative transcriptional start site was mapped, and the region from -100 to +50 was shown to encompass the promoter (Fisseha et al. 1999, Yoder and Kroos 2004b). Expression from the *fmgBC* promoter was reduced in a *csgA*

mutant but was restored upon co-development of the *csgA* mutant with wild-type cells, which supply C-signal, demonstrating that promoter activity is partially dependent on C-signaling (Kroos and Kaiser 1987, Fisseha et al. 1999). Mutational analysis identified critical *cis*-regulatory sequences at -71 to -45 upstream of the promoter (Yoder and Kroos 2004b). This region contains two C boxes (consensus CAYYCCY; Y means C or T) and two 5-bp elements (consensus GAACA) (Figure 3.1), which are sequence motifs found in the promoter regions of several developmentally-regulated genes (Fisseha et al. 1999, Viswanathan and Kroos 2003, Yoder and Kroos 2004b, Srinivasan and Kroos 2004, Loconto et al. 2005, Viswanathan et al. 2006a, Viswanathan et al. 2007a). In the *fmgA* promoter region, between -63 and -46, a 5-bp element is located 6 bp upstream of a C box, and this region is bound by MrpC2, while FruA binds cooperatively to a site located slightly upstream (Mittal and Kroos 2008).

Here, we report that MrpC2 and FruA bind to sequences between -71 and -45 upstream of the *fmgBC* promoter, but the arrangement of binding sites is the reverse of that found in the *fmgA* promoter region. Nevertheless, the association of MrpC and/or MrpC2 with the *fmgBC* promoter region *in vivo* required FruA. Furthermore, there appeared to be cooperative binding of MrpC2 and FruA to *fmgBC* promoter region DNA *in vitro*. Our results demonstrate combinatorial control by MrpC2 and FruA at a second promoter, and reveal surprising flexibility in the arrangement of the binding sites.

MATERIALS AND METHODS

Bacterial strains and plasmids. Strains and plasmids used in this study are listed in Table 3.1.

Growth and development. *Escherichia coli* BL21(DE3) containing plasmids were grown at 37°C in Luria-Bertani (LB) medium (Sambrook et al. 1989) containing 200 μg ampicillin per ml. *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 (Hodgkin and Kaiser 1977) or on CTT agar (1.5%) plates. When required, 40 μg kanamycin sulfate per ml was added. Fruiting body development was performed on TPM agar (1.5%) plates (10 mM Tris-HCl [pH 8.0], 1 mM KH_2PO_4-K_2HPO_4, 8 mM $MgSO_4$, [final pH 7.6] as described previously (Kroos et al. 1986).

Construction of *M. xanthus* strains and determination of *lacZ* expression during development. Strains containing pREG1727 or its derivatives integrated at the Mx8 phage attachment site, *attB*, were constructed by electroporation (Kashefi and Hartzell 1995) of *M. xanthus*, and transformants were selected on CTT agar plates containing kanamycin sulfate. Transformants were screened on TPM agar plates containing 40 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside per ml, in order to avoid rare transformants with unusual developmental *lacZ* expression (Viswanathan and Kroos 2003). Three transformants were chosen for further analysis and β-galactosidase activity was measured as described previously (Kroos et al. 1986).

Preparation of DNA fragments. DNA fragments spanning the *fmgBC* promoter region from -104 to -29 were generated by PCR using wild-type or mutant plasmid (Table 3.1) as the template and the oligonucleotide primers 5′-CGCGAGGAGATTGCGTTCATAC-3′ (for -104) and 5′- GAGGAATGGGCCGGAAGTTC-3′ (for -29). For 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 (Sambrook et al. 1989).

EMSAs. EMSAs were performed as described previously (Yoder-Himes and Kroos 2006), except that binding reaction mixtures were incubated at 25°C for 15 min.

DNA-affinity chromatography. An *fmgBC* DNA fragment (-104 to -29) was synthesized by PCR with a 5′-biotin label at -104, bound to streptavidin beads, and DNA-affinity chromatography was performed with the AS fraction as described previously (Viswanathan et al 2007b).

Preparation of His_{10}-MrpC2, FruA-His_6 and FruA-DBD-His_8. Recombinant proteins were expressed in *E. coli* and purified as described previously (Yoder-Himes and Kroos 2006, Nariya and Inouye 2006, Mittal and Kroos 2008).

ChIP. *M. xanthus* strains MDY1727.DZF1, MSM4499.DZF1 and MSM4499.FA were used for ChIP as described previously (Yoder-Himes and Kroos 2006). The primers used

for PCR of the *fmgBC* promoter region integrated ectopically were 5′-CTGCCAGGAATTGGGGATC-3′ (upstream primer in the vector) and 5′-CGGATCCAGCGGGTGAGGTCGACGACG-3′ (downstream primer with its 5′ end at +50 of *fmgBC*). The primers used for PCR of the vector alone integrated ectopically were the same upstream primer as above and 5′- CGGGCCATCCGCCAGTGG-3′ (downstream primer in the vector). The primers used for PCR of the *rpoC* coding region were described previously (Yoder-Himes and Kroos 2006).

TABLE 3.1 Strains and plasmids used in this study.

Strain or plasmid	**Description**	**Reference or Source**
E. coli		
BL21(DE3)	F⁻ *ompT hsdS*$_B$(r_B^- m_B^-) *gal dcm* with DE3, a λ prophage carrying the T7 RNA polymerase gene	Novagen
SMhisMrpC2	BL21(DE3) containing pET16b/His$_{10}$-MrpC2	(Mittal and Kroos 2008)
SMFruAhis	BL21(DE3) containing pET11km/FruA-His$_6$	(Mittal and Kroos 2008)
EDYFruA	BL21(DE3) containing pET11a/FDBD-H$_8$	(Yoder-Himes and Kroos 2006)
M. xanthus		
DK1622	Wild type	(Kaiser 1979)
DK4499	Tn*5 lac* Ω4499	(Kroos et al. 1986)
MDY1727.DZF1	*sglA1 attB*::pREG1727	(Yoder-Himes and Kroos 2006)
MSM1727.FA	*sglA1 fruA*::TnV Ω786 *attB*::pREG1727	This work
MSM4499.DZF1	*sglA1 attB*::pDY51	This work
MSM4499.FA	*sglA1 fruA*::TnV Ω786 *attB*::pDY51	This work
Plasmids		
pET11a/FDBD-H$_8$	pET11a with a gene encoding FruA-DBD-His$_8$ under control of a T7 RNA polymerase promoter	(Ueki and Inouye 2005a)
pET16b/His$_{10}$-MrpC2	pET16b with a gene encoding His$_{10}$-MrpC2 under control of a T7 RNA polymerase promoter	(Nariya and Inouye 2006)
pET11km/FruA-His$_6$	pET11km with a gene encoding FruA-His$_6$ under control of a T7 RNA polymerase promoter	S. Inouye
pREG1727	Ap^r Km^r P1-*inc attP 'lacZ*	(Fisseha et al. 1996)
pDY51	pGEM7Zf with *fmgBC* DNA from -100 to +50 generated by PCR using pDY100 as template	(Yoder and Kroos 2004b)
pDY100	pGEM7Zf with *fmgBC* DNA from -218 to +50	(Yoder and Kroos 2004b)
pDY133	pDY100 with GCCGC-to-TAATA mutation at –81 to –77	(Yoder and Kroos 2004b)
pDY129	pDY100 with GGAC-to-TTCA mutation at –71 to –68	(Yoder and Kroos 2004b)
pDY127	pDY100 with ACCA-to-CAAC mutation at –67 to –64	(Yoder and Kroos 2004b)

Table 3.1 (cont'd)

pDY125	pDY100 with CCGG-to-AATT mutation at –63 to –60	(Yoder and Kroos 2004b)
pDY49	pDY100 with TCATTC-to-GACGGA mutation at –59 to –54	(Yoder and Kroos 2004b)
pDY121	pDY100 with CCTTC-to-AAGGA mutation at –53 to –49	(Yoder and Kroos 2004b)
pDY47	pDY100 with GAAC-to-TCCA mutation at –48 to -45	(Yoder and Kroos 2004b)
pDY117	pDY100 with C-to-A mutation at -37	(Yoder and Kroos 2004b)
pDY45	pDY100 with CATTCCT-to-ACGGAAG mutation at –36 to 30	(Yoder and Kroos 2004b)

RESULTS

An insertion in *fmgC* reduces spore formation. *M. xanthus* strain DK4499 contains Tn*5 lac* Ω4499 inserted in *fmgC*, which was predicted previously to encode an oxidase of a cytochrome P-450 system (Fisseha et al. 1999). *fmgC* corresponds to MXAN4127 in the annotation of the genomic sequence (Goldman et al. 2006). Only 59 bp upstream of *fmgC* is *fmgB* (MXAN4126), which was predicted previously to code for a reductase likely to function in the same P-450 system as FmgC, although the substrate and products of the system are unknown (Fisseha et al. 1999). The short distance between *fmgB* and *fmgC*, and the finding that their products are likely components of a P-450 system, suggested that the two genes might be co-transcribed. In agreement, 5'-deletion analysis and mapping of an mRNA 5' end, located a promoter upstream of *fmgB* capable of driving expression of *lacZ* during development similar to that observed for DK4499 containing Tn*5 lac* Ω4499 (Fisseha et al. 1999). The gene upstream of *fmgB* is in the opposite orientation (Goldman et al. 2006). The gene downstream of *fmgC* is in the same orientation, but is separated from the end of *fmgC* by an intergenic region of at least 243 bp and is predicted to encode a transposase, so it is unlikely to be co-transcribed with the putative *fmgBC* operon.

M. xanthus DK4499 bearing Tn*5 lac* Ω4499 aggregated normally under conditions that induce development, but the number of heat- and sonication-resistant spores that were able to germinate and form a colony was 6-fold lower than observed for wild-type DK1622. The reduced sporulation of DK4499 is likely due to loss of FmgC, although we cannot rule out an effect of the Tn*5 lac* insertion on expression of *fmgB* (e.g., due to

altered mRNA stability) or a gene downstream of *fmgC* (i.e., if transcription from the *fmgBC* promoter normally reads through a downstream gene). Nevertheless, our results suggest that transcription from the *fmgBC* promoter is important for sporulation.

MrpC2 binds to a key *cis*-regulatory sequence in the *fmgBC* promoter region. Mutational analysis of the *fmgBC* promoter region was performed previously (Yoder and Kroos 2004b) and showed that sequences upstream of the promoter are important for its activity (Figure 3.1). These regulatory sequences include two 5-bp elements and two C boxes, which are found in the promoter regions of several developmentally-regulated genes (Fisseha et al. 1999, Viswanathan and Kroos 2003, Yoder and Kroos 2004b, Srinivasan and Kroos 2004, Loconto et al. 2005, Viswanathan et al. 2006a, Viswanathan et al. 2007a). To identify putative transcription factors, we performed electrophoretic mobility shift assays (EMSAs) with a DNA fragment from the *fmgBC* promoter region and partially-purified DNA-binding proteins (AS fraction) from *M. xanthus* cells that had undergone 12 h of development, since *fmgBC* is expressed at this time (Kroos et al. 1986). A single shifted complex was observed with a DNA fragment spanning from -104 to -29, but no complex was observed when the DNA fragment contained a mutation in the sequence from -67 to -64 (Figure 3.1). Since this mutation was shown previously to eliminate *fmgBC* promoter activity *in vivo* (Yoder and Kroos 2004b), these results showed that a protein in the AS fraction binds to a crucial *cis*-regulatory sequence upstream of the *fmgBC* promoter.

To purify the putative activator protein from the AS fraction, DNA-affinity chromatography was performed with the *fmgBC* DNA fragment (-104 to -29). The major species after purification was approximately 30 kDa in size (Figure 3.2A). The affinity-purified protein (APP) generated a shifted complex of similar mobility as observed with the AS fraction when the *fmgBC* DNA fragment with the wild-type sequence was used in EMSAs, and no complex was observed with the APP and the mutant (-67 to -64) *fmgBC* promoter region (Figure 3.2B). It appeared that APP contained the putative activator protein from the AS fraction.

To identify the putative activator protein, the APP was subjected to mass spectrometry analysis after protease digestion. The peptide sequences primarily matched MrpC, a protein that is about 30 kDa in size, consistent with the size of the major species in the APP (Figure 3.2A). MrpC is similar to CRP-family transcription factors and is an essential for *M. xanthus* development (Sun and Shi 2001b). MrpC2, a shortened form of MrpC that lacks the 25 N-terminal residues, is produced during development and was identified in an AS fraction previously by DNA-affinity chromatography with the *fruA* promoter region (Ueki and Inouye 2003). We infer that MrpC2 in the AS fraction and in the APP is responsible for the shifted complex we observed with *fmgBC* promoter region DNA.

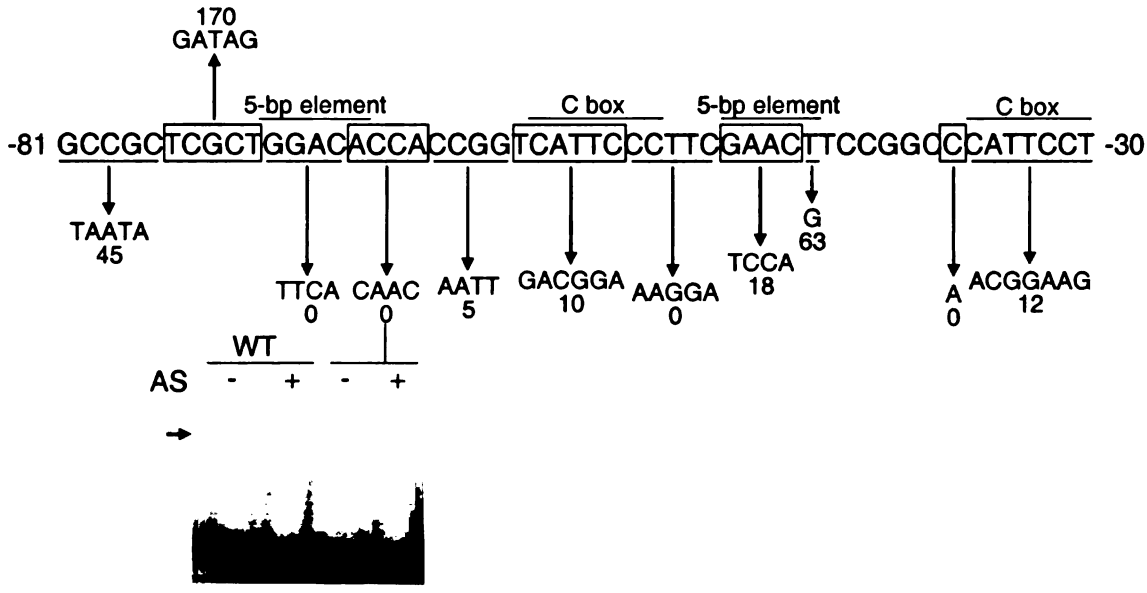


Figure 3.1. Effects of mutations on *fmgBC* promoter activity *in vivo* and on DNA binding *in vitro*. The top part shows a summary of mutational effects on developmental *fmgB-lacZ* expression (Yoder and Kroos 2004). The wild-type *fmgBC* upstream sequence is alternately boxed or underlined to indicate changed sequences, which are shown below the downward arrows. The number beneath each mutant sequence indicates the maximum β-galactosidase activity during development, expressed as a percentage of the maximum activity observed for the wild-type promoter. The bottom part shows EMSAs performed with ^{32}P-labeled *fmgBC* DNA (12 nM) spanning from -104 to -29 and proteins in the AS fraction (0.7 μg/μl). The arrow indicates the shifted complex produced by incubating the wild-type (WT) DNA fragment with the AS fraction. No complex was observed with a DNA fragment bearing the indicated mutation at -67 to -64.

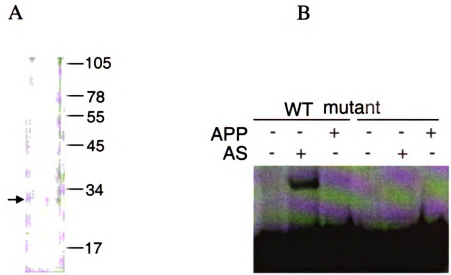


Figure 3.2. DNA-affinity purification of protein that binds to the *fmgBC* promoter region. (A) SDS-PAGE of protein purified from the AS fraction using *fmgBC* DNA (-104 to -29). The arrow indicates the major species in the affinity-purified protein (APP) after staining with silver. Numbers indicate the migration positions of molecular weight (kDa) standards. (B) EMSAs with ^{32}P-labeled *fmgBC* DNA (12 nM) spanning from -104 to -29 and proteins in the AS fraction or the APP. Arrowheads indicate the shifted complexes produced with the wild-type (WT) DNA fragment. No complex was observed with a DNA fragment bearing the ACCA to CAAC mutation at -67 to -64 (mutant).

To confirm that MrpC2 binds to the *fmgBC* promoter region fragment, N-terminally His-tagged MrpC2 (His_{10}-MrpC2) was expressed in *E. coli* and purified. His_{10}-MrpC2 displayed a similar pattern of binding to wild-type and mutant *fmgBC* DNA fragments as the AS fraction (Figure 3.3). The slower migration of the complex produced by His_{10}-MrpC2, as compared with the complex produced by the AS fraction, is presumably due to the 10 His residues plus 8 additional residues present in the His_{10}-MrpC2 fusion protein. The mutation from -67 to -64 that resulted in loss of shifted complex formation with the

AS fraction (Figure 3.1) and the APP (Figure 3.2B), also caused loss of shifted complex formation with His_{10}-MrpC2 (Figure 3.3). This mutation includes one base pair of a 5-bp element (Figure 3.1); however, an adjacent mutation at -71 to -68, which changes the remaining four base pairs of the 5-bp element, did not impair formation of shifted complexes with the AS fraction or with His_{10}-MrpC2 (Figure 3.3). Likewise, none of the other mutations between -63 and -30 impaired complex formation. The mutation from -81 to -77 resulted in diminished formation of the complex that we believe contains MrpC2, by the AS fraction, and the appearance of a novel shifted complex. The novel complex appears to be due to an unknown protein in the AS fraction that is capable of binding to this mutant *fmgBC* DNA fragment, since purified His_{10}-MrpC2 did not show this effect. Indeed, His_{10}-MrpC2appeared to bind normally to this mutant *fmgBC* DNA fragment, suggesting that formation of the novel complex by the AS fraction might account for its diminished ability to form the complex that we believe contains MrpC2. We conclude that MrpC2 binds to an important *cis*-regulatory sequence at -67 to -64 in the *fmgBC* promoter region.

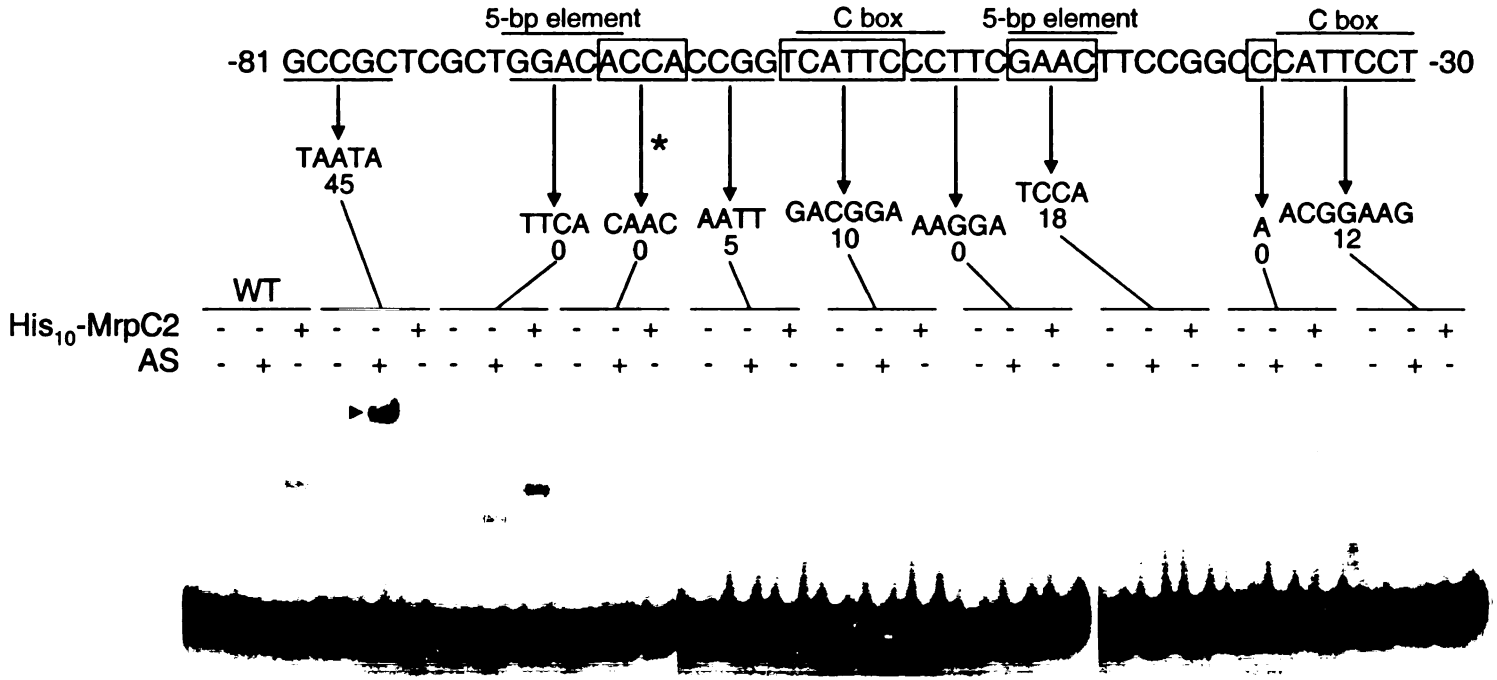


Figure 3.3. Comparison of purified His_{10}-MrpC2 and the AS fraction for binding to the *fmgBC* promoter region. EMSAs with ^{32}P-labeled *fmgBC* DNA (2 nM) spanning from -104 to -29, wild-type (WT) or mutant as indicated, and His_{10}-MrpC2 (1 μM) or the AS fraction (0.7 μg/μl). An asterisk indicates the -67 to -64 mutation that impairs shifted complex formation. The -81 to -77 mutation causes a novel shifted complex to form with the AS fraction (arrowhead). The image is a composite from three experiments, and in each experiment the WT *fmgBC* DNA served as a control and the signal intensity of the shifted complexes was comparable to that shown.

Since MrpC2 is similar to CRP-family transcription factors, and cyclic nucleotides affect DNA binding by some family members (Kolb et al. 1993), we examined His_{10}-MrpC2 binding to the *fmgBC* promoter region (-104 to -29) in the presence of different nucleotides. At concentrations designed to reflect physiological conditions, no effect of cAMP (4-8 μM), cGMP (20-40 nM), ppGpp (50-400 μM), NTPs (400 μM) or dNTPs (200 μM) was observed (data not shown).

MrpC and/or MrpC2 associates with the *fmgBC* promoter region *in vivo* and this depends on FruA. Chromatin immunoprecipitation (ChIP) assays were performed with polyclonal antibodies against MrpC, which also recognize MrpC2 (Nariya and Inouye 2006), to determine whether MrpC and/or MrpC2 associate with the *fmgBC* promoter region during development. *M. xanthus* cells with the *fmgBC* promoter region (-100 to +50) integrated ectopically at a phage attachment site via site-specific recombination, were collected after 12 or 18 h of development, and subjected to ChIP with antibodies against MrpC or, as a control, IgG. DNA recovered after ChIP was analyzed by PCR with primers designed to amplify the ectopic copy of the *fmgBC* promoter region. The PCR analysis showed that the *fmgBC* promoter region was enriched by ChIP with the anti-MrpC antibodies relative to the IgG control at 12 and 18 h into development (Figure 3.4). PCR analysis with primers designed to amplify the *rpoC* coding region showed no enrichment of this region by ChIP with anti-MrpC antibodies relative to control antibodies at 18 h, as reported previously (Yoder-Himes and Kroos 2006), but at 12 h we unexpectedly yet reproducibly observed enrichment of the *rpoC* coding region by ChIP with anti-MrpC antibodies relative to control antibodies. These results indicate that MrpC

and/or MrpC2 is present in the vicinity of the *rpoC* coding region at 12 h into development, but not at 18 h, and that MrpC and/or MrpC2 is associated with the *fmgBC* promoter region at both times.

Recently, regulation of the *fmgA* gene was shown to be under combinatorial control by MrpC2 and FruA (Mittal and Kroos 2008). Since expression of *fmgA* occurs with similar timing during development as *fmgBC* (Kroos et al. 1986) and expression of both genes depends partially on C-signaling (Kroos and Kaiser 1987, Fisseha et al. 1999, Brandner and Kroos 1998), to which FruA responds (Ellehauge et al. 1998), we hypothesized that *fmgBC* is also under direct control by FruA. In the case of *fmgA*, association of MrpC and/or MrpC2 with the promoter region *in vivo*, as measured by ChIP-PCR analysis, was dependent on FruA (Mittal and Kroos 2008). We carried out a similar analysis for *fmgBC* by performing ChIP-PCR analysis of a *fruA* mutant with the *fmgBC* promoter region (-100 to +50) integrated ectopically as above. In contrast to the wild-type strain, no enrichment of the *fmgBC* promoter region was observed with anti-MrpC antibodies relative to control antibodies at 12 h or 18 h into development (Figure 3.4). Likewise, no enrichment of the *rpoC* coding region was observed with anti-MrpC antibodies relative to control antibodies. We conclude that FruA is necessary for the association of MrpC and/or MrpC2 with the *fmgBC* promoter region during development, and for the association of MrpC and/or MrpC2 with the *rpoC* coding region at 12 h into development.

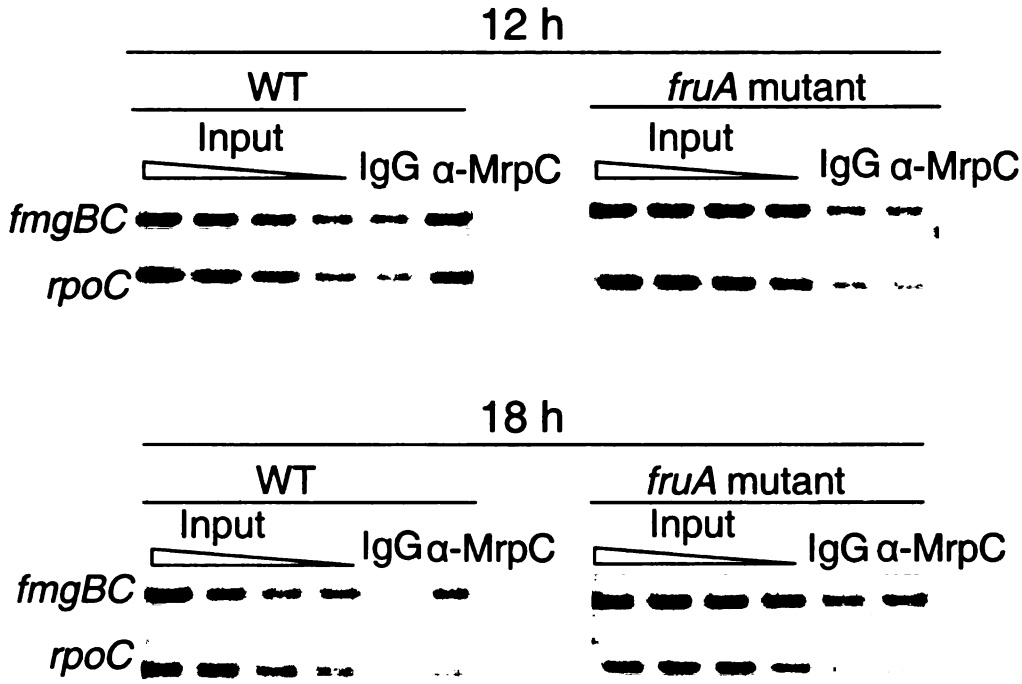


Figure 3.4. Association of MrpC and/or MrpC2 with the *fmgBC* promoter region during development of wild-type and *fruA* mutant cells. ChIP analysis of *M. xanthus* with the *fmgBC* promoter region (-100 to +50) integrated ectopically in otherwise wild-type (WT) or *fruA* mutant backgrounds. At 12 and 18 h into development, cells were treated with formaldehyde, lysed, and crosslinked chromatin was immunoprecipitated with anti-MrpC antibodies or IgG as a control. DNA was amplified with appropriate primers for the *fmgBC* promoter region at the ectopic chromosomal site, or with appropriate primers for the *rpoC* coding region as a control. A twofold dilution series of input DNA purified from 0.25%, 0.125%, 0.0625% or 0.03125% 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.

FruA associates with the *fmgBC* promoter region *in vivo* and governs expression. If FruA plays a direct role in recruitment of MrpC and/or MrpC2 to the *fmgBC* promoter region, as observed previously for *fmgA* (Mittal and Kroos 2008), it should be possible to detect FruA at the *fmgBC* promoter region by ChIP with antibodies against FruA. To test this expectation, ChIP was performed on the wild-type strain with the *fmgBC* promoter

region (-100 to +50) integrated ectopically. At 12 h into development, enrichment of the *fmgBC* promoter region was observed with anti-FruA antibodies as compared to control pre-immune serum (Figure 3.5). No enrichment was observed for a strain with vector lacking the *fmgBC* promoter region integrated ectopically. We conclude that FruA associates with the *fmgBC* promoter region *in vivo*, consistent with the notion that it directly recruits MrpC and/or MrpC2.

If FruA plays a key role in regulation of *fmgBC*, expression of *fmgBC* is predicted to be impaired in a *fruA* mutant, as observed previously for *fmgA* (Yoder-Himes and Kroos 2006). To test this prediction, *fruA* mutant and wild-type *M. xanthus* cells were transformed with a plasmid containing the -100 to +50 region of the *fmgBC* promoter transcriptionally fused to the *E. coli lacZ* gene. The plasmid integrates into the *M. xanthus* genome ectopically via site-specific recombination at a phage attachment site. As negative controls, strains bearing the vector with promoterless *lacZ* were also constructed. β-galactosidase specific activity was measured in cell extracts at different times during development. The activity of each negative control strain was subtracted from that of the corresponding promoter-containing strain. The *fruA* mutation abolished developmental *lacZ* expression from the *fmgBC* promoter region (Figure 3.6). This demonstrates that FruA governs *fmgBC* expression, and together with our other data, strongly suggests that FruA binds to the *fmgBC* promoter region and recruits MrpC and/or MrpC2, activating transcription.

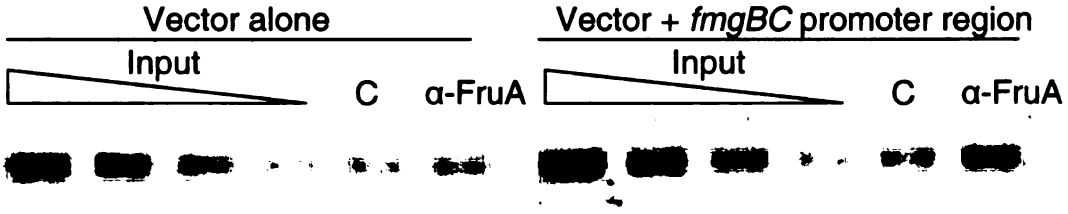


Figure 3.5. Association of FruA with the *fmgBC* promoter region *in vivo*. ChIP analysis of *M. xanthus* with the vector alone or with the *fmgBC* promoter region (-100 to +50) integrated ectopically. At 12 h into development, cells were treated with formaldehyde, lysed, and crosslinked chromatin was immunoprecipitated with anti-FruA antibodies or pre-immune serum as a control (C). A twofold dilution series of input DNA purified from 0.25%, 0.125%, 0.0625% or 0.03125% 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.

The FruA DNA-binding domain binds to a key *cis*-regulatory sequence in the *fmgBC* promoter region. To determine whether FruA binds to the *fmgBC* promoter region, the C-terminally His-tagged FruA DNA-binding domain (FruA-DBD-His_8) was overexpressed in *E. coli*, purified, and used in EMSAs. FruA-DBD-His_8 generated a single shifted complex with a DNA fragment spanning from -104 to -29 of the *fmgBC* promoter region (Figure 3.7). EMSAs with mutant probes localized the binding to -53 to -49, since a mutation in this region abolished FruA-DBD-His_8 binding. This region was shown previously to be critical for *fmgBC* promoter activity (Yoder and Kroos 2004b). It includes part of a C box and lies immediately upstream of a 5-bp element.

Enhanced complex formation in the presence of FruA-His_6 and His_{10}-MrpC2. The combination of FruA and MrpC2 led to enhanced formation of shifted complexes with *fmgA* promoter region DNA, due to cooperative binding of the two proteins to adjacent

(possibly overlapping) sites (Mittal and Kroos 2008). Both sites were required for the enhancement of shifted complex formation, as was full-length FruA (i.e., FruA-DBD-His_8 was insufficient), suggesting that the N-terminal regulatory domain of FruA, which is believed to be phosphorylated in response to C-signal (Ellehauge et al. 1998), might directly interact with MrpC2 (Mittal and Kroos 2008). To test whether similar effects could be observed with the *fmgBC* promoter DNA, FruA-His_6 was overexpressed in *E. coli* and purified. As observed previously with *fmgA* promoter region DNA (Mittal and Kroos 2008), the *fmgBC* promoter region (-104 to -29) was bound weakly by FruA-His_6 in EMSAs, but the combination of FruA-His_6 and His_{10}-MrpC2 resulted in formation of an abundant lower complex (LC) and a faint upper complex (UC) (Figure 3.8A). Migration of the LC was similar to that of complexes formed by either protein alone, suggesting that the LC is composed of DNA bound by His_{10}-MrpC2 or FruA-His_6. The slower migration of the UC was suggestive of DNA bound by both proteins simultaneously.

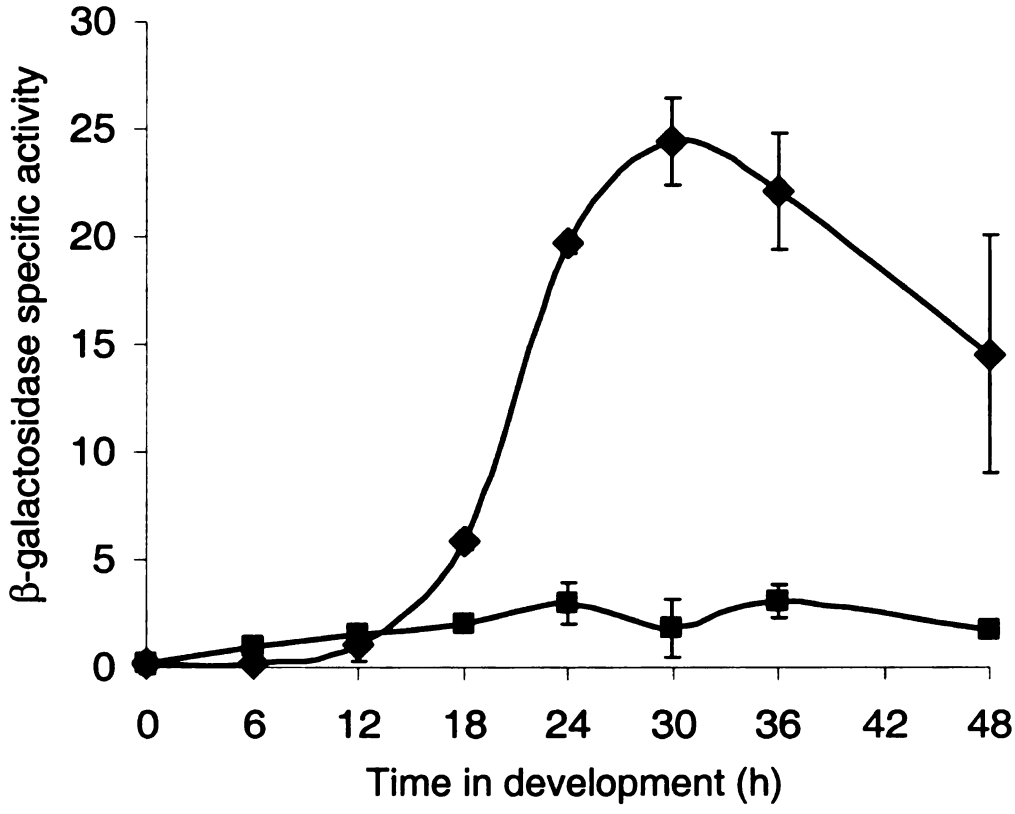


Figure 3.6. Developmental expression from *fmgB-lacZ*. The *fmgBC* promoter region from -100 to +50 was fused to *lacZ*, and β-galactosidase specific activity was measured during development of *M. xanthus* wild-type (diamonds) and *fruA* mutant cells (squares). In each background, activity from the vector with no promoter was measured as a negative control. Points show the average of three transformants, after subtracting the average of three transformants with the promoterless vector. The units of activity are nanomoles of *o*-nitrophenyl phosphate per minute per milligram of protein. Error bars depict 1 standard deviation of the data.

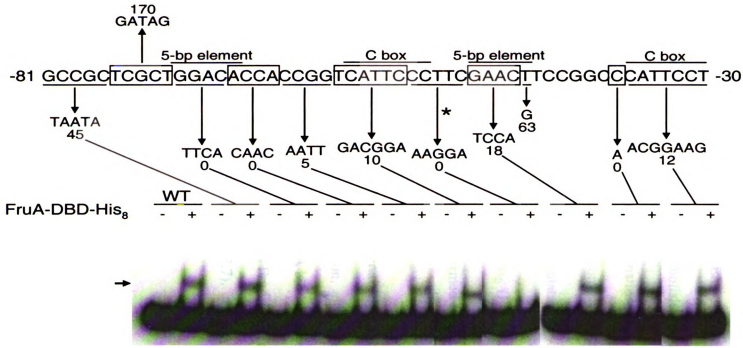


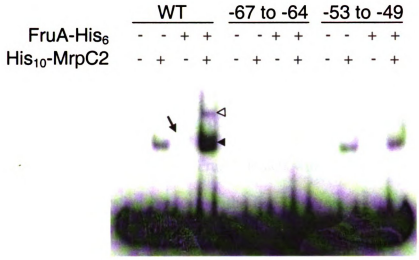
Figure 3.7. Effects of mutations on binding of FruA-DBD-His$_8$ to *fmgBC* promoter region DNA. EMSAs with ^{32}P-labeled *fmgBC* DNA (2 nM) spanning from -104 to -29, wild-type (WT) or mutant as indicated, and FruA-DBD-His$_8$ (14 μM). A horizontal arrow indicates the shifted complex produced with WT DNA. An asterisk indicates the -53 to -49 mutation that impairs shifted complex formation. The image is a composite from three experiments, and intervening lanes were removed from one of the images. In each experiment, the WT *fmgBC* DNA served as a control and the signal intensity of the shifted complex was comparable to that shown.

To determine whether binding of both proteins to DNA is required for the observed enhancement of complex formation, EMSAs were performed with mutant DNA fragments. A mutation at -67 to -64 that abolished His_{10}-MrpC2 binding (Figure 3.3) also abolished enhancement of complex formation by the combination of proteins; the UC was undetectable and the faint LC was comparable in intensity to that formed by FruA-His_6 alone (Figure 3.8A). Similarly, a mutation at -53 to -49 that abolished binding of FruA-DBD-His_8 (Figure 3.7) or FruA-His_6 (Figure 3.8A) resulted in no detectable UC, and LC of comparable intensity as that formed by His_{10}-MrpC2 alone (Figure 3.8A).

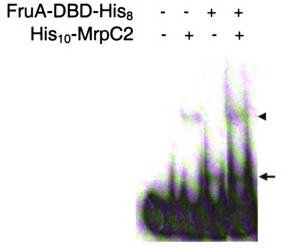
MrpC2 and FruA appear to bind cooperatively to the *fmgBC* promoter region, as seen previously for the *fmgA* promoter region, although the arrangement of binding sites relative to the promoter is different. FruA binds upstream of MrpC2 in the *fmgA* promoter region (Mittal and Kroos 2008), whereas FruA binds downstream of MrpC2 in the *fmgBC* promoter region (Figure 3.3 and Figure 3.7).

Figure 3.8. EMSAs with MrpC2 and full-length FruA or just the DNA-binding domain of FruA. (A) Shifted complex formation with His_{10}-MrpC2 and full-length FruA-His_6, and the effect of mutations. EMSAs with ^{32}P-labeled *fmgBC* DNA (2 nM) spanning from -104 to -29, wild-type (WT) or mutant 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. A slanted arrow indicates the faint shifted complex produced by FruA-His_6 alone. The unfilled and filled arrowheads indicate the upper and lower complexes, respectively, produced by the combination of proteins. (B) Shifted complex formation with His_{10}-MrpC2 and FruA-DBD-His_8. EMSAs with ^{32}P-labeled *fmgBC* DNA (2 nM) spanning from -104 to -29 and no protein, His_{10}-MrpC2 (1 μM), FruA-DBD-His_8 (14 μM), or both His_{10}-MrpC2 (1 μM) and FruA-DBD-His_8 (14 μM) as indicated. The arrowhead indicates the complex produced by His_{10}-MrpC2 and the arrow indicates the complex produced by FruA-DBD-His_8. Intervening lanes were removed from the image.

A


WT
-67 to -64
-53 to -49
FruA-His6 - - + + - - + + - - + +
His10-MrpC2 - + - + - + - + - + - +

B


FruA-DBD-His8 - - + +
His10-MrpC2 - + - +

Despite the different arrangement of binding sites, we found that the *fmgA* and *fmgBC* promoter regions share the characteristic that the FruA DNA-binding domain itself is insufficient to enhance complex formation in combination with His_{10}-MrpC2 (Figure 3.8B). The complexes formed by the combination of proteins were similar to the complexes formed by His_{10}-MrpC2 or FruA-DBD-His_8 alone. We propose that the N-terminal regulatory domain of FruA interacts with MrpC2 at the *fmgBC* promoter region, mediating cooperative binding of the two transcription factors, and subjecting *fmgBC* expression to combinatorial control similar to that observed for *fmgA*.

DISCUSSION

Our results demonstrate that MrpC2 and FruA bind to key *cis*-regulatory sequences upstream of the *fmgBC* promoter, placing it under similar combinatorial control as observed previously for *fmgA* (Mittal and Kroos 2008). Surprisingly, the arrangement of binding sites for MrpC2 and FruA is different in the two promoter regions. FruA binds downstream of MrpC2 in the *fmgBC* promoter region (Figure 3.3 and Figure 3.7), whereas FruA binds upstream of MrpC2 in the *fmgA* promoter region (Mittal and Kroos 2008). In both cases, FruA is required for promoter activity and for recruitment of MrpC and/or MrpC2 to the promoter region *in vivo*. *In vitro*, FruA and MrpC2 appear to bind cooperatively to both promoter regions, and this depends on the N-terminal regulatory domain of the FruA response regulator. Preliminary results, described below, indicate that cooperative binding by FruA and MrpC2 is a common mechanism of gene regulation during *M. xanthus* development. This mechanism is proposed to allow integration of positional information via short-range C-signaling through FruA, with starvation signaling and cell death via MrpC and MrpC2, to control spatiotemporal gene expression and determine cell fate.

Combinatorial control of C-signal-dependent genes involving cooperative binding of FruA and MrpC2 appears to be a common mechanism of gene regulation during *M. xanthus* development. In addition to *fmgA* and *fmgBC*, the promoter region of the *dev* operon appears to utilize this mechanism. MrpC2 binds to a region that includes a 5-bp element and two C box-like sequences, and appears to bind cooperatively with FruA (S. M., P. Viswanathan, and L. K., unpublished data). Expression of the *dev* operon is

confined to fruiting bodies (Sager and Kaiser 1993, Julien et al. 2000) and has been proposed to be a crucial step in commitment of cells to differentiate into spores (Kroos 2007, Mittal and Kroos 2008). The gene identified by Tn*5 lac* Ω4403 encodes a putative serine protease whose role in development is unknown, but whose expression depends absolutely on C-signaling (Kroos and Kaiser 1987, Fisseha et al. 1996). The promoter region contains two 5-bp elements in inverted orientation that are bound by MrpC2, and FruA appears to bind cooperatively (J. Lee, S. M., and L. K., unpublished data). Therefore, at least four promoter regions appear to be bound cooperatively by MrpC2 and FruA, since the combination of proteins greatly enhances formation of shifted complexes in EMSAs, and this was shown to correlate with cooperative binding at the *fmgA* promoter region by DNase I footprinting (Mittal and Kroos 2008). Moreover, enhancement of shifted complex formation was shown to require the binding sites for both MrpC2 and FruA at both the *fmgA* (Mittal and Kroos 2008) and *fmgBC* (Figure 3.8A) promoter regions.

Although the combination of MrpC2 and FruA produces a strikingly similar enhancement of shifted complex formation in EMSAs with *fmgA* or *fmgBC* promoter region DNA, the arrangement of the MrpC2 and FruA binding sites is different in the two promoter regions. In the *fmgA* promoter region, mutations from -86 to -77 impaired binding of FruA-DBD-His_8 (Yoder-Himes and Kroos 2006) and mutations from -76 to -46 affected binding of His_{10}-MrpC2 (Mittal and Kroos 2008). In addition, DNA upstream of -76 was found to be required for His_{10}-MrpC2 binding, suggesting that the MrpC2- and FruA-

binding sites might partially overlap, with the two proteins presumably interacting with opposite faces of the DNA in the region of overlap (Mittal and Kroos 2008). In contrast, FruA-DBD-His_8 and His_{10}-MrpC2 binding to the *fmgBC* promoter region was impaired only by mutations from -53 to -49 and -67 to -64, respectively (Figure 3.3 and Figure 3.7). Adjacent mutations did not impair binding of either protein, although these mutations had previously been shown to reduce promoter activity (Yoder and Kroos 2004b), suggesting that sequences important for binding *in vivo* might be missed under the *in vitro* conditions of the EMSAs. Alternatively, other transcription factors might bind to the adjacent sequences. In any case, FruA binds downstream of MrpC2 in the *fmgBC* promoter region, whereas FruA binds upstream of MrpC2 in the *fmgA* promoter region.

The different arrangement of FruA and MrpC2 binding sites in the *fmgA* and *fmgBC* promoter regions suggests a somewhat different mechanism of transcriptional activation from the two promoters. As noted previously, in the *fmgA* promoter region, the two proteins occupy a location typical for Class I activators (Mittal and Kroos 2008), which contact the C-terminal domain of the α subunits of RNA polymerase (Barnard et al. 2004). In the *fmgBC* promoter region, FruA and MrpC2 occupy a similar location, but their positions relative to the promoter are reversed, so presumably a different contact(s) with the C-terminal domain of the α subunits of RNA polymerase would be involved in activation of transcription. Two activators can contact the C-terminal domain of the α subunits of RNA polymerase at the same promoter, based on studies of both synthetic (Langdon and Hochschild 1999) and natural promoters (Beatty et al. 2003).

Despite the different arrangement of FruA and MrpC2 binding sites with respect to the *fmgA* and *fmgBC* promoters, the two proteins might interact with each other similarly at the two promoter regions. Our results show that the N-terminal regulatory domain of FruA is required for enhancement of shifted complex formation in combination with MrpC2 at both promoter regions (Mittal and Kroos 2008) (Figure 3.8B). This domain is believed to be phosphorylated by one or more histidine protein kinases in *M. xanthus* (Ellehauge et al. 1998, Ueki and Inouye 2003, Jelsbak et al. 2005); however, the histidine protein kinase(s) has not been identified, so the effect of FruA phosphorylation on its ability to enhance complex formation in combination with MrpC2 remains to be explored. The presumed lack of phosphorylation of FruA-His_6 purified from *E. coli* might account for its low DNA-binding affinity in EMSAs (Figure 3.8A). Phosphorylation enhances DNA binding of many response regulators (West and Stock 2001). Phosphorylation of FruA's N-terminal domain might relieve an inhibitory effect on its C-terminal DNA-binding domain, since FruA-DBD-His_8 appeared to bind to the *fmgBC* promoter region with higher affinity than full-length FruA-His_6 (Figure 3.8), and this has been observed for two promoter regions (Viswanathan et al. 2007b, Mittal and Kroos 2008). Low affinity of FruA-His_6 for DNA might also explain why, when it was combined with His_{10}-MrpC2, the predominant shifted complex co-migrated with complexes produced by either protein alone, for both *fmgBC* (Figure 3.8A) and *fmgA* promoter region DNA (Mittal and Kroos 2008). Perhaps the predominant shifted complex contains only His_{10}-MrpC2 bound to DNA, and FruA-His_6 has dissociated after initial cooperative binding.

Another observation consistent with the idea that FruA and MrpC2 might interact similarly with each other in the *fmgA* and *fmgBC* promoter regions is that sequences matching the consensus binding site for FruA are in the opposite orientation in the two promoters. The consensus sequence for binding of FruA-DBD-His_8 is GGGC/TA/G(N_{4-6})C/TGGG (Viswanathan et al. 2007b). The sequence GGGTG(N_5)TGGG from -81 to -68 in the *fmgA* promoter region matches the consensus perfectly, and some mutations in this sequence impair FruA-DBD-His_8 binding *in vitro* (Yoder-Himes and Kroos 2006). In the *fmgBC* promoter region, in the opposite orientation, the sequence GGGAA(N_4)CGGT from -52 to -64 matches the consensus except at two positions, and the mutation at -53 to -49 that impaired FruA-DBD-His_8 binding *in vitro* overlaps this sequence (Figure 3.7). MrpC is dimeric, and one type of site to which MrpC and MrpC2 bind is palindromic, with a consensus sequence of GTGTC(N_8)GACAC (Nariya and Inouye 2006). Presumably, a dimer of MrpC or MrpC2 bound to such a palindromic site could present the same surface to FruA bound upstream or downstream. In the *fmgA* promoter region, the sequence GAGCG(N_8)CACAT from -67 to -50 is the best match to the consensus between -76 and -46, where mutations affected His_{10}-MrpC2 binding (Mittal and Kroos 2008). In the *fmgBC* promoter region, the sequence ACGCC(N_8)GACAC from -83 to -66 matches half the consensus perfectly, and the mutation at -67 to -64 that impaired His_{10}-MrpC2 binding *in vitro* overlaps this sequence (Figure 3.3). We hypothesize that the N-terminal domain of FruA can interact directly with dimeric MrpC2 to permit cooperative

DNA binding, whether FruA binds upstream of MrpC2 (as at the *fmgA* promoter region) or whether FruA binds to a site in the opposite orientation downstream of MrpC2 (as at the *fmgBC* promoter region). This flexibility in the arrangement of FruA and MrpC2 at different promoters would presumably result in a different contact(s) with RNA polymerase and different levels of transcriptional activation.

The implications of discovering a novel mechanism of gene regulation involving cooperative binding of the FruA response regulator and the MrpC transcription factor/antitoxin have been discussed previously (Mittal and Kroos 2008). Briefly, since MrpC2 appears to activate *fruA* transcription (Ueki and Inouye 2003), combinatorial regulation of target genes by MrpC2 and FruA constitutes a coherent feed-forward loop, which is a motif found commonly in regulatory networks since it has beneficial characteristics (Milo et al. 2002, Mangan and Alon 2003). One characteristic is that expression of target genes is delayed until both transcription factors reach a sufficient concentration. Since phosphorylation of FruA is believed to occur in response to short-range C-signaling (Ellehauge et al. 1998), expression of target genes like *fmgBC* and the *dev* operon, which are important for sporulation, may be delayed until cell alignment in the nascent fruiting body causes a high level of C-signaling. The concentration of MrpC2 is influenced by proteolytic cleavage of MrpC, and accumulation of both these proteins is inhibited by the STPK cascade that leads to phosphorylation of MrpC during growth (Nariya and Inouye 2006). Starvation triggers accumulation of MrpC and MrpC2 by counteracting the STPK cascade (Nariya and Inouye 2006); however, the EspA signal transduction pathway appears to delay the accumulation of MrpC and MrpC2 during

development in response to an unknown signal (Higgs et al. 2008). Therefore, the concentrations of MrpC and MrpC2 appear to be linked to starvation and perhaps other signals via several pathways. Only if starvation persists and the other putative signals are received, would the MrpC2 concentration rise to a level that permits expression of target genes in combination with FruA, committing the cell to form a spore. In its role as an antitoxin, binding of MrpC to the MazF toxin would prevent programmed cell death in cells destined to form spores (Nariya and Inouye 2008). In cells destined to undergo programmed cell death, binding of MrpC to the *mazF* promoter region would activate transcription, leading to increased MazF. According to this model, MrpC is a key determinant of cell fate, and determining whether MrpC2 binds to MazF and/or the *mazF* promoter region is an important goal.

Chapter 4: Conclusions and Future Directions

CONCLUSIONS

The objective of this research was to understand C-signal-dependent gene regulation during *Myxococcus xanthus* development. To achieve this goal, two C-signal-dependent transcription units, *fmgA* and *fmgBC*, were investigated. A novel mechanism of gene regulation was discovered that involves cooperative binding of a response regulator, FruA, and a bifunctional transcription factor/antitoxin, MrpC2, to the *fmgA* and *fmgBC* promoter regions. A Tn*5* *lac* insertion in the *fmgA* gene resulted in an aggregation defect (a 6-h delay compared to wild type), and a Tn*5* *lac* insertion in the *fmgC* gene caused a sporulation defect (a 6-fold reduction compared to wild type), suggesting these genes are important for development. It had been shown previously that distinct levels of C-signaling are required for different developmental behaviors like rippling, aggregation, and sporulation (Kim and Kaiser 1991, Li et al 1992) and for expression of different genes after 6 h into development (Kroos and Kaiser 1987). To account for these observations, FruA has been proposed to be phosphorylated to different levels (low, medium and high) in response to different threshold levels of C-signaling, activating distinct set of genes that control rippling, aggregation, and sporulation (Sager and Kaiser 1994, Sogaard-Andersen et al. 1996, Ellehauge et al. 1998, Jelsbak and Sogaard-Andersen 2002). My discovery that MrpC2 is also a direct regulator of C-signal-dependent genes, and that MrpC2 binds cooperatively with FruA in different arrangements upstream of promoters, provides insight into the mechanism and complexity of gene regulation in response to C-signaling. MrpC2, in addition to activating C-signal-dependent genes, is derived from MrpC, which plays a central role in cell fate decisions by interacting with the toxin MazF and by activating *mazF*

transcription to regulate programmed cell death during development. Based on my results with *fmgA* and *fmgBC*, a model for regulation of C-signal-dependent genes is proposed that involves integration of positional signals by phosphorylation of FruA in response to short-range C-signaling, with nutritional and other signals via MrpC and MrpC2 levels.

FUTURE DIRECTIONS

The studies presented in this dissertation have revealed a novel mechanism of C-signal-dependent gene regulation during *M. xanthus* development. Greater insights may be achieved by performing some of the following studies.

Determinants of FruA and MrpC2 essential for cooperative binding

To further understand the mechanism of enhanced shifted complex formation in the presence of FruA and MrpC2 *in vitro*, and the absolute requirement for FruA for association of MrpC and/or MrpC2 with the *fmgA* and *fmgBC* promoter regions *in vivo*, it is reasonable to determine whether all, or only parts, of FruA and MrpC2 are necessary for these effects. Work presented in this dissertation has shown that the C-terminal DNA-binding domain of FruA is insufficient for enhancement of shifted complex formation with MrpC2, suggesting that the N-terminal regulatory domain of FruA is required either to interact with MrpC2 directly or to change the structure of DNA to stabilize MrpC2 binding. Deletions can be made in the N-terminal domain of FruA to determine the minimal region required for enhancement of shifted complex formation, and similar deletion analysis of MrpC2 could be performed. It would be interesting to see the developmental phenotypes of strains expressing these minimal regions in the corresponding *fruA* or *mrpC* mutant background. In the case of the minimal FruA region, recruitment of MrpC and /or MrpC2 to the *fmgA* and *fmgBC* promoter regions could be examined to see if recruitment *in vivo* correlates with enhancement of shifted complex formation *in vitro*.

One known determinant of FruA activity is residue D59, which genetic studies have shown can be changed to E (mimicking phosphorylation of D) but not A (which cannot be phosphorylated), suggesting that D59 is the site of phosphorylation (Ellehauge et al. 1998). It would be interesting to see the effect of the D59A or D59E substitution in FruA on enhancement of complex formation in combination with MrpC2 and *fmgA* or *fmgBC* promoter region DNA.

Identification of the RNAP holoenzyme responsible for *fmgA* and *fmgBC* transcription

A key step toward understanding the regulation of *fmgA* and *fmgBC* gene expression would be reconstitution of their transcription *in vitro*. It has been shown previously that σ^A RNAP was unable to produce *fmgA* and *fmgBC* transcripts *in vitro*. It was also shown that there is no effect of *sigB* and *sigC* mutations on *fmgA-lacZ* or *fmgB-lacZ* expression; however *sigD* and *sigE* mutations impaired expression, suggesting a direct or indirect role of σ^D and σ^E in transcription of these genes. It is possible that σ^A RNAP alone is unable to transcribe *fmgA* and *fmgBC* genes but in the presence of activators like MrpC2 and FruA, would produce transcripts. If it does not, then σ^D and σ^E RNAPs can be tested in the absence or presence of MrpC2 and FruA.

Determining the roles of MrpC2 and FruA in *fmgA* and *fmgBC* transcriptional regulation

Reconstitution of *fmgA* and *fmgBC* transcription *in vitro*, with activation by MrpC2 and FruA, would open the door to more detailed mechanistic studies. From the data presented in this dissertation, it is evident that MrpC2 and FruA bind to *cis*-regulatory sequences in the *fmgA* and *fmgBC* promoter regions that are critical for expression. In order to further understand the mechanism, it is important to investigate the role of MrpC2 and FruA in *fmgA* and *fmgBC* transcription. At the *fmgA* and *fmgBC* promoter regions, MrpC2 and FruA bind to a region typically occupied by bacterial Class I activators, which interact with the α-CTD of RNAP to assist binding of RNAP to the promoter. DNA-binding studies (EMSAs) can be performed to determine whether FruA and MrpC2 facilitate RNAP to bind to *fmgA* and *fmgBC* promoters. The binding of RNAP holoenzyme to *fmgA* and *fmgBC* promoter region DNA can be tested in the absence or in the presence of MrpC2, FruA, and a combination of both, MrpC2 and FruA.

MrpC2 is similar to CRP-family transcription factors (Sun and Shi 2001b), which function by a Class I activation mechanism. Hence, it is likely that MrpC2 activates *fmgA* and *fmgBC* transcription (and more broadly the transcription of other genes regulated by MrpC2) by making direct contact with the α-CTD of RNAP. Available data on the residues in CRP that contact the α-CTD of RNAP could be utilized to perform mutational analyses of the corresponding residues in MrpC2 to determine the activation region, utililizing activation of *fmgA* and *fmgBC* transcription *in vitro* as the functional assay. Likewise, knowledge about residues of response regulators that contact the α-CTD of

RNAP could inform mutational analysis of FruA. Alternatively or in addition, chemical crosslinking studies can be performed to identify residues in MrpC2 and FruA that might contact RNAP.

Identifying FruA and MrpC targets genome-wide and investigating combinatorial regulation by FruA and MrpC2 at other C-signal-dependent and C-signal-independent promoters

To identify binding sites of FruA and MrpC and/or MrpC2 genome-wide, ChIP-chip analysis could be performed. Alternatively, the available information on FruA- and MrpC and/or MrpC2-binding sites can be utilized to perform bioinformatic analyses (by utilizing position weight matrices and other algorithms) to identify regulatory sequences bound by FruA and MrpC2 (work in progress in the Kroos lab). In either case, experimental verification could include ChIP-PCR of candidate promoter regions and EMSAs with cloned DNA sequences and FruA and MrpC2.

Preliminary data suggests that other C-signal-dependent transcription units like *dev* (S. M., P. Viswanathan and L. K., unpublished data) and Ω4403 (J. Lee, S. M., and L. K., unpublished data) are subject to a regulatory mechanism similar to that observed for *fmgA* and *fmgBC*. FruA and MrpC2 appear to bind cooperatively to sequences containing a 5-bp element and a C box in the *dev* and Ω4403 promoter regions *in vitro*. Studies as conducted and proposed for *fmgA* and *fmgBC*, can be performed with the *dev* and Ω4403 promoter regions to gain insights into their regulation. Also, binding of MrpC2 and FruA can be tested at another C-signal-dependent promoter, Ω4406, which contains a 5-bp

element and a C box, and is predicted to be regulated similar to *fmgA* and *fmgBC*. It would be interesting to investigate whether regulation of C-signal-independent genes like *dofA*, to which FruA-DBD binds (Ueki and Inouye 2005a), is under combinatorial control by FruA and MrpC2.

The *fmgA* and *fmgBC* transcription units provide examples where compact promoter regions are regulated combinatorially by FruA and MrpC2. As noted above, preliminary data suggests that the *dev* operon, whose regulatory region spans more than 1 kb, is under similar regulation by FruA and MrpC2. In addition, *dev* expression is regulated by a LysR-type regulator (Viswanathan et al. 2007a), LadA, which presumably responds to a signal that remains to be discovered. LadA acts from a site located downstream of the promoter (at about +350), possibly by counteracting the effects of negative regulatory elements. Whether this represents a distinct input, or whether LadA interacts via DNA looping with MrpC2, FruA, and/or other transcription factors bound upstream of the promoter, is an interesting question. Other key developmental genes of *M. xanthus* also have large regulatory regions. For example, the *csgA* gene that encodes the protein involved in C-signaling has a regulatory region that spans around 930 bp. Presumably, multiple transcription factors (perhaps including MrpC2 and/or FruA) bind to this region in order to bring about proper spatiotemporal regulation in response to multiple signals. Further understanding of the mechanism of cooperative binding of the response regulator, FruA, and transcription factor MrpC2, and studies to explore how these and other transcription factors respond to signals, and interact at promoters, will provide insight into how *M. xanthus* cells integrate multiple signals in a developing biofilm to control

gene expression and cell fate. Response regulators like FruA and activators like MrpC are abundant in bacteria, so the kind of combinatorial gene regulation discovered here suggests that similar mechanisms will be found in bacterial biofilms that exist in a variety of physiological contexts (e.g., *Pseudomonas aeruginosa* infection of the lung in cystic fibrosis patients). Understanding such mechanisms could lead to novel ways to modulate bacterial signaling and gene regulation to improve human health.

REFERENCES

Apelian, D. and Inouye, S. 1990. Development-specific sigma-factor essential for late-stage differentiation of *Myxococcus xanthus*. *Genes Dev* **4**(8): 1396-1403.

Apelian, D. and Inouye, S. 1993. A new putative sigma factor of *Myxococcus xanthus*. *J Bacteriol* **175**: 3335-3342.

Av-Gay, Y. and Everett, M. 2000. The eukaryotic-like Ser/Thr protein kinases of *Mycobacterium tuberculosis*. *Trends Microbiol* **8**(5): 238-244.

Barnard, A., Wolfe, A., and Busby, S. 2004. Regulation at complex bacterial promoters: how bacteria use different promoter organizations to produce different regulatory outcomes. *Curr Opin Microbiol* **7**(2): 102-108.

Bassler, B.L. and Losick, R. 2006. Bacterially speaking. *Cell* **125**(2): 237-246.

Beatty, C.M., Browning, D.F., Busby, S.J., and Wolfe, A.J. 2003. Cyclic AMP receptor protein-dependent activation of the *Escherichia coli acsP2* promoter by a synergistic class III mechanism. *J Bacteriol* **185**(17): 5148-5157.

Berleman, J.E. and Kirby, J.R. 2007. Multicellular development in *Myxococcus xanthus* is stimulated by predator-prey interactions. *J Bacteriol* **189**(15): 5675-5682.

Blackhart, B.D. and Zusman, D.R. 1985. "Frizzy" genes of *Myxococcus xanthus* are involved in control of frequency of reversal of gliding motility. *Proc Natl Acad Sci USA* **82**: 8767-8770.

Bode, H.B., Ring, M.W., Kaiser, D., David, A.C., Kroppenstedt, R.M., and Schwar, G. 2006. Straight-chain fatty acids are dispensable in the myxobacterium *Myxococcus xanthus* for vegetative growth and fruiting body formation. *J Bacteriol* **188**(15): 5632-5634.

Boysen, A., Ellehauge, E., Julien, B., and Sogaard-Andersen, L. 2002. The DevT protein stimulates synthesis of FruA, a signal transduction protein required for fruiting body morphogenesis in *Myxococcus xanthus*. *J Bacteriol* **184**(6): 1540-1546.

Brandner, J.P. and Kroos, L. 1998. Identification of the Ω4400 regulatory region, a developmental promoter of *Myxococcus xanthus*. *J Bacteriol* **180**(8): 1995-2004.

Buck, M., Gallegos, M.T., Studholme, D.J., Guo, Y., and Gralla, J.D. 2000. The bacterial enhancer-dependent σ^{54} (σ N) transcription factor. *J Bacteriol* **182**(15): 4129-4136.

Caberoy, N.B., Welch, R.D., Jakobsen, J.S., Slater, S.C., and Garza, A.G. 2003. Global mutational analysis of NtrC-like activators in *Myxococcus xanthus*: identifying activator mutants defective for motility and fruiting body development. *J Bacteriol* **185**(20): 6083-6094.

Cheng, Y. and Kaiser, D. 1989a. *dsg*, a gene required for *Myxococcus* development, is necessary for cell viability. *J Bacteriol* **171**: 3727-3731.

Cheng, Y. and Kaiser, D. 1989b. *dsg*, a gene required for cell-cell interaction early in *Myxococcus* development. *J Bacteriol* **171**: 3719-3726.

Cheng, Y.L., Kalman, L.V., and Kaiser, D. 1994. The *dsg* gene of *Myxococcus xanthus* encodes a protein similar to translation initiation factor IF3. *J Bacteriol* **176**(5): 1427-1433.

Cho, K. and Zusman, D.R. 1999. AsgD, a new two-component regulator required for A-signalling and nutrient sensing during early development of *Myxococcus xanthus*. *Mol Microbiol* **34**(2): 268-281.

Crawford, E.W., Jr. and Shimkets, L.J. 2000. The *Myxococcus xanthus socE* and *csgA* genes are regulated by the stringent response. *Mol Microbiol* **37**(4): 788-799.

Cusick, J.K., Hager, E., and Gill, R.E. 2002. Characterization of *bcsA* mutations that bypass two distinct signaling requirements for *Myxococcus xanthus* development. *J Bacteriol* **184**(18): 5141-5150.

Davis, J.M., Mayor, J., and Plamann, L. 1995. A missense mutation in *rpoD* results in an A-signalling defect in *Myxococcus xanthus*. *Mol Microbiol* **18**(5): 943-952.

Diodati, M.E., Ossa, F., Caberoy, N.B., Jose, I.R., Hiraiwa, W., Igo, M.M., Singer, M., and Garza, A.G. 2006. Nla18, a key regulatory protein required for normal growth and development of *Myxococcus xanthus*. *J Bacteriol* **188**: 1733-1743.

Downard, J., Ramaswamy, S.V., and Kil, K.S. 1993. Identification of *esg*, a genetic locus involved in cell-cell signaling during *Myxococcus xanthus* development. *J Bacteriol* **175**(24): 7762-7770.

Dubnau, D. and Losick, R. 2006. Bistability in bacteria. *Mol Microbiol* **61**(3): 564-572.

Dworkin, M. 1996. Recent advances in the social and developmental biology of the myxobacteria. *Microbiol Rev* **60**: 70-102.

Ellehauge, E., Norregaard-Madsen, M., and Sogaard-Andersen, L. 1998. The FruA signal transduction protein provides a checkpoint for the temporal co-ordination of intercellular signals in *Myxococcus xanthus* development. *Mol Microbiol* **30**: 807-817.

Ferrell, J.E. 2002. Self-perpetuating states in signal transduction: positive feedback, double-negative feedback and bistability. *Curr Opin Cell Biol* **14**(2): 140-148.

Fisseha, M., Biran, D., and Kroos, L. 1999. Identification of the Ω4499 regulatory region controlling developmental expression of a *Myxococcus xanthus* cytochrome P-450 system. *J Bacteriol* **181**(17): 5467-5475.

Fisseha, M., Gloudemans, M., Gill, R.E., and Kroos, L. 1996. Characterization of the regulatory region of a cell interaction-dependent gene in *Myxococcus xanthus*. *J Bacteriol* **178**(9): 2539-2550.

Fontes, M. and Kaiser, D. 1999. *Myxococcus* cells respond to elastic forces in their substrate. *Proc Natl Acad Sci USA* **96**(14): 8052-8057.

Garza, A.G., Harris, B.Z., Greenberg, B.M., and Singer, M. 2000a. Control of *asgE* expression during growth and development of *Myxococcus xanthus*. *J Bacteriol* **182**(23): 6622-6629.

Garza, A.G., Harris, B.Z., Pollack, J.S., and Singer, M. 2000b. The *asgE* locus is required for cell-cell signalling during *Myxococcus xanthus* development. *Mol Microbiol* **35**(4): 812-824.

Gill, R.E. and Bornemann, M.C. 1988. Identification and characterization of the *Myxococcus xanthus bsgA* gene product. *J Bacteriol* **170**: 5289-5297.

Gill, R.E., Cull, M.G., and Fly, S. 1988. Genetic identification and cloning of a gene required for developmental cell interactions in *Myxococcus xanthus*. *J Bacteriol* **170**: 5279-5288.

Gill, R.E., Karlok, M., and Benton, D. 1993. *Myxococcus xanthus* encodes an ATP-dependent protease which is required for developmental gene transcription and intercellular signaling. *J Bacteriol* **175**: 4538-4544.

Goldman, B.S., Nierman, W.C., Kaiser, D., Slater, S.C., Durkin, A.S., Eisen, J., Ronning, C.M., Barbazuk, W.B., Blanchard, M., Field, C., Halling, C., Hinkle, G., Iartchuk, O., Kim, H.S., Mackenzie, C., Madupu, R., Miller, N., Shvartsbeyn, A., Sullivan, S.A., Vaudin, M., Wiegand, R., and Kaplan, H.B. 2006. Evolution of sensory complexity recorded in a myxobacterial genome. *Proc Natl Acad Sci USA* **103**(41): 15200-15205.

Gorham, H.C., McGowan, S.J., Robson, P.R., and Hodgson, D.A. 1996. Light-induced carotenogenesis in *Myxococcus xanthus*: light-dependent membrane sequestration of ECF sigma factor CarQ by anti-sigma factor CarR. *Mol Microbiol* **19**(1): 171-186.

Gorski, L. and Kaiser, D. 1998. Targeted mutagenesis of sigma54 activator proteins in *Myxococcus xanthus*. *J Bacteriol* **180**(22): 5896-5905.

Gronewold, T.M. and Kaiser, D. 2001. The *act* operon controls the level and time of C-signal production for *Myxococcus xanthus* development. *Mol Microbiol* **40**(3): 744-756.

Guo, D., Wu, Y., and Kaplan, H.B. 2000. Identification and characterization of genes required for early *Myxococcus xanthus* developmental gene expression. *J Bacteriol* **182**(16): 4564-4571.

Hagen, D.C., Bretscher, A.P., and Kaiser, D. 1978. Synergism between morphogenetic mutants of *Myxococcus xanthus*. *Dev Biol* **64**: 284-296.

Hager, E., Tse, H., and Gill, R.E. 2001. Identification and characterization of *spdR* mutations that bypass the BsgA protease-dependent regulation of developmental gene expression in *Myxococcus xanthus*. *Mol Microbiol* **39**(3): 765-780.

Harris, B.Z., Kaiser, D., and Singer, M. 1998. The guanosine nucleotide (p)ppGpp initiates development and A-factor production in *Myxococcus xanthus*. *Genes Dev* **12**(7): 1022-1035.

Helmann, J.D. 2002. The extracytoplasmic function (ECF) sigma factors. *Adv Microb Physiol* **46**: 47-110.

Higgs, P.I., Jagadeesan, S., Mann, P., and Zusman, D.R. 2008. EspA, an orphan hybrid histidine protein kinase, regulates the timing of expression of key developmental proteins of *Myxococcus xanthus*. *J Bacteriol*.

Hodgkin, J. and Kaiser, D. 1977. Cell-to-cell stimulation of movement in nonmotile mutants of *Myxococcus*. *Proc Natl Acad Sci U S A* **74**(7): 2938-2942.

Hodgkin, J. and Kaiser, D. 1979a. Genetics of gliding Motility in *Myxococcus xanthus* (Myuxobacterales): Genes Controlling Movement of Single Cells. *Mol Gen Genet* **171**: 167-176

Hodgkin, J. and Kaiser, D. 1979b. Genetics of Gliding Motility in *Myxococcus xanthus* (Myxobacterales): Two Gene Systems Control Movement. *Mol Gen Genet* **171**: 177-191.

Hoiczyk, E. and Baumeister, W. 1998. The junctional pore complex, a prokaryotic secretion organelle, is the molecular motor underlying gliding motility in cyanobacteria. *Curr Biol* **8**(21): 1161-1168.

Horiuchi, T., Taoka, M., Isobe, T., Komano, T., and Inouye, S. 2002. Role of *fruA* and *csgA* genes in gene expression during development of *Myxococcus xanthus*:

analysis by two-dimensional gel electrophoresis. *J Biol Chem* **277**(30): 26753-26760.

Inouye, S. 1990. Cloning and DNA sequence of the gene coding for the major sigma factor from *Myxococcus xanthus*. *J Bacteriol* **172**: 80-85.

Jakobsen, J.S., Jelsbak, L., Jelsbak, L., Welch, R.D., Cummings, C., Goldman, B., Stark, E., Slater, S., and Kaiser, D. 2004. Sigma54 enhancer binding proteins and *Myxococcus xanthus* fruiting body development. *J Bacteriol* **186**(13): 4361-4368.

Jelsbak, L., Givskov, M., and Kaiser, D. 2005. Enhancer-binding proteins with an FHA domain and the σ^{54} regulon in *Myxococcus xanthus* fruiting body development. *Proc Natl Acad Sci USA* **102**: 3010-3015.

Jelsbak, L. and Sogaard-Andersen, L. 2002. Pattern formation by a cell surface-associated morphogen in *Myxococcus xanthus*. *Proc Natl Acad Sci USA* **99**(4): 2032-2037.

Julien, B., Kaiser, A.D., and Garza, A. 2000. Spatial control of cell differentiation in *Myxococcus xanthus*. *Proc Natl Acad Sci USA* **97**(16): 9098-9103.

Kaiser, D. 1979. Social gliding is correlated with the presence of pili in *Myxococcus xanthus*. *Proc Natl Acad Sci USA* **76**: 5952-5956.

Kaiser, D. 2003. Coupling cell movement to multicellular development in myxobacteria. *Nature Rev Microbiol* **1**: 45-54.

Kaiser, D. 2004. Signaling in Myxobacteria. *Annu Rev Microbiol* **58**: 75-98.

Kalman, L.V., Cheng, Y.L., and Kaiser, D. 1994. The *Myxococcus xanthus dsg* gene product performs functions of translation initiation factor IF3 in vivo. *J Bacteriol* **176**(5): 1434-1442.

Kashefi, K. and Hartzell, P.L. 1995. Genetic suppression and phenotypic masking of a *Myxococcus xanthus frzF*⁻ defect. *Mol Microbiol* **15**(3): 483-494.

Kearns, D.B., Venot, A., Bonner, P.J., Stevens, B., Boons, G.J., and Shimkets, L.J. 2001. Identification of a developmental chemoattractant in *Myxococcus xanthus* through metabolic engineering. *Proc Natl Acad Sci U S A* **98**(24): 13990-13994.

Keseler, I.M. and Kaiser, D. 1995. An early A-signal-dependent gene in *Myxococcus xanthus* has a sigma 54-like promoter. *J Bacteriol* **177**(16): 4638-4644.

Keseler, I.M. and Kaiser, D. 1997. sigma54, a vital protein for *Myxococcus xanthus*. *Proc Natl Acad Sci U S A* **94**(5): 1979-1984.

Kiley, P.J. and Beinert, H. 1998. Oxygen sensing by the global regulator, FNR: the role of the iron-sulfur cluster. *FEMS Microbiol Rev* **22**(5): 341-352.

Kim, S.K. and Kaiser, D. 1990a. C-factor: a cell-cell signaling protein required for fruiting body morphogenesis of *M. xanthus*. *Cell* **61**: 19-26.

Kim, S.K. and Kaiser, D. 1990b. Cell alignment required in differentiation of *Myxococcus xanthus*. *Science* **249**: 926-928.

Kim, S.K. and Kaiser, D. 1990c. Cell motility is required for the transmission of C-factor, an intercellular signal that coordinates fruiting body morphogenesis of *Myxococcus xanthus*. *Genes Dev* **4**: 896-905.

Kim, S.K. and Kaiser, D. 1990d. Purification and properties of *Myxococcus xanthus* C-factor, an intercellular signaling protein. *Proc Natl Acad Sci USA* **87**: 3635-3639.

Kim, S.K. and Kaiser, D. 1991. C-factor has distinct aggregation and sporulation thresholds during *Myxococcus* development. *J Bacteriol* **173**: 1722-1728.

Kolb, A., Busby, S., Buc, I.I., Garges, S., and Adhya, S. 1993. Transcriptional regulation by cAMP and its receptor protein. *Annu Rev Biochem* **62**(1): 749-797.

Kroos, L. 2007. The *Bacillus* and *Myxococcus* developmental networks and their transcriptional regulators. *Ann Rev Genet* **41**: 13-39.

Kroos, L. and Inouye, S. 2008. Transcriptional regulatory mechanisms during *Myxococcus xanthus* development. in *Myxobacteria: Multicellularity and Differentiation* (ed. D.E. Whitworth), pp. 149-168. ASM Press, Washington, D.C.

Kroos, L. and Kaiser, D. 1984. Construction of Tn*5 lac*, a transposon that fuses *lacZ* expression to exogenous promoters, and its introduction into *Myxococcus xanthus*. *Proc Natl Acad Sci USA* **81**: 5816-5820.

Kroos, L. and Kaiser, D. 1987. Expression of many developmentally regulated genes in *Myxococcus* depends on a sequence of cell interactions. *Genes Dev* **1**: 840-854.

Kroos, L., Kuspa, A., and Kaiser, D. 1986. A global analysis of developmentally regulated genes in *Myxococcus xanthus*. *Dev Biol* **117**: 252-266.

Kroos, L., Kuspa, A., and Kaiser, D. 1990. Defects in fruiting body development caused by Tn*5 lac* insertions in *Myxococcus xanthus*. *J Bacteriol* **172**: 484-487.

Kruse, T., Lobedanz, S., Berthelsen, N.M., and Sogaard-Andersen, L. 2001. C-signal: a cell surface-associated morphogen that induces and co-ordinates multicellular fruiting body morphogenesis and sporulation in *Myxococcus xanthus*. *Mol Microbiol* **40**(1): 156-168.

Kuner, J.M. and Kaiser, D. 1982. Fruiting body morphogenesis in submerged cultures of *Myxococcus xanthus*. *J Bacteriol* **151**: 458-461.

Kuspa, A. and Kaiser, D. 1989. Genes required for developmental signalling in *Myxococcus xanthus*: three *asg* loci. *J Bacteriol* **171**(5): 2762-2772.

Kuspa, A., Kroos, L., and Kaiser, D. 1986. Intercellular signaling is required for developmental gene expression in *Myxococcus xanthus*. *Dev Biol* **117**: 267-276.

Kuspa, A., Plamann, L., and Kaiser, D. 1992a. Identification of heat-stable A-factor from *Myxococcus xanthus*. *J Bacteriol* **174**: 3319-3326.

Kuspa, A., Plamann, L., and Kaiser, D. 1992b. A-signalling and the cell density requirement for *Myxococcus xanthus* development. *J Bacteriol* **174**: 7360-7369.

Langdon, R.C. and Hochschild, A. 1999. A genetic method for dissecting the mechanism of transcriptional activator synergy by identical activators. *Proc Natl Acad Sci USA* **96**(22): 12673-12678.

Lee, B.U., Lee, K., Mendez, J., and Shimkets, L.J. 1995. A tactile sensory system of *Myxococcus xanthus* involves an extracellular NAD(P)(+)-containing protein. *Genes Dev* **9**(23): 2964-2973.

Lee, K. and Shimkets, L.J. 1994. Cloning and characterization of the *socA* locus which restores development to *Myxococcus xanthus* C-signaling mutants. *J Bacteriol* **176**(8): 2200-2209.

Li, S., Lee, B.U., and Shimkets, L.J. 1992. *csgA* expression entrains *Myxococcus xanthus* development. *Genes Dev* **6**(3): 401-410.

Li, Y., Sun, H., Ma, X., Lu, A., Lux, R., Zusman, D., and Shi, W. 2003. Extracellular polysaccharides mediate pilus retraction during social motility of *Myxococcus xanthus*. *Proc Natl Acad Sci USA* **100**(9): 5443-5448.

Lobedanz, S. and Sogaard-Andersen, L. 2003. Identification of the C-signal, a contact-dependent morphogen coordinating multiple developmental responses in *Myxococcus xanthus*. *Genes Dev* **17**(17): 2151-2161.

Loconto, J., Viswanathan, P., Nowak, S.J., Gloudemans, M., and Kroos, L. 2005. Identification of the Ω4406 regulatory region, a developmental promoter of *Myxococcus xanthus*, and a DNA segment responsible for chromosomal position-dependent inhibition of gene expression. *J Bacteriol* **187**(12): 4149-4162

Mahmud, T., Bode, H.B., Silakowski, B., Kroppenstedt, R.M., Xu, M., Nordhoff, S., Hofle, G., and Muller, R. 2002. A novel biosynthetic pathway providing

precursors for fatty acid biosynthesis and secondary metabolite formation in myxobacteria. *J Biol Chem* **277**(36): 32768-32774.

Mangan, S. and Alon, U. 2003. Structure and function of the feed-forward loop network motif. *Proc Natl Acad Sci USA* **100**(21): 11980-11985.

Mangan, S., Zaslaver, A., and Alon, U. 2003. The coherent feedforward loop serves as a sign-sensitive delay element in transcription networks. *J Mol Biol* **334**(2): 197-204.

Manoil, C. and Kaiser, D. 1980a. Accumulation of guanosine tetraphosphate and guanosine pentaphosphate in *Myxococcus xanthus* during starvation and myxospore formation. *J Bacteriol* **141**: 297-304.

Manoil, C. and Kaiser, D. 1980b. Guanosine pentaphosphate and guanosine tetraphosphate accumulation and induction of *Myxococcus xanthus* fruiting body development. *J Bacteriol* **141**(1): 305-315.

Mignot, T., Merlie, J.P., Jr., and Zusman, D.R. 2005. Regulated pole-to-pole oscillations of a bacterial gliding motility protein. *Science* **310**(5749): 855-857.

Mignot, T., Shaevitz, J.W., Hartzell, P.L., and Zusman, D.R. 2007. Evidence that focal adhesion complexes power bacterial gliding motility. *Science* **315**(5813): 853-856.

Milo, R., Shen-Orr, S., Itzkovitz, S., Kashtan, N., Chklovskii, D., and Alon, U. 2002. Network motifs: simple building blocks of complex networks. *Science* **298**(5594): 824-827.

Mittal, S. and Kroos, L., 2008. Cooperative binding of a response regulator and bifunctional transcription factor/antitoxin controls gene expression and cell fate during *Myxococcus xanthus* development. *PNAS* Submitted.

Munoz-Dorado, J., Inouye, S., and Inouye, M. 1991. A gene encoding a protein serine/threonine kinase is required for normal development of *M. xanthus*, a Gram-negative bacterium. *Cell* **67**: 995-1006.

Nariya, H. and Inouye, M. 2008. MazF, an mRNA interferase, mediates programmed cell death during multicellular *Myxococcus* development. *Cell* **132**(1): 55-66.

Nariya, H. and Inouye, S. 2005. Identification of a protein Ser/Thr kinase cascade that regulates essential transcriptional activators in *Myxococcus xanthus* development. *Mol Microbiol* **58**(2): 367-379.

Nariya, H. and Inouye, S. 2006. A protein Ser/Thr kinase cascade negatively regulates the DNA-binding activity of MrpC, a smaller form of which may be necessary for the *Myxococcus xanthus* development. *Mol Microbiol* **60**(5): 1205-1217.

Nielsen, M., Rasmussen, A.A., Ellehauge, E., Treuner-Lange, A., and Sogaard-Andersen, L. 2004. HthA, a putative DNA-binding protein, and HthB are important for fruiting body morphogenesis in *Myxococcus xanthus*. *Microbiology* **150**: 2171-2183.

O'Connor, K.A. and Zusman, D.R. 1988. Reexamination of the role of autolysis in the development of *Myxococcus xanthus*. *J Bacteriol* **170**(9): 4103-4112.

O'Connor, K.A. and Zusman, D.R. 1991. Development in *Myxococcus xanthus* involves differentiation into two cell types, peripheral rods and spores. *J Bacteriol* **173**(11): 3318-3333.

Ogawa, M., Fujitani, S., Mao, X., Inouye, S., and Komano, T. 1996. FruA, a putative transcription factor essential for the development of *Myxococcus xanthus*. *Mol Microbiol* **22**(4): 757-767.

Oyaizu, H. and Woese, C. 1985. Phylogenetic relationships among the sulfate respiring bacteria, myxobacteria and purple bacteria. *System Appl Microbiol* **6**: 257-263.

Passner, J.M., Ryoo, H.D., Shen, L., Mann, R.S., and Aggarwal, A.K. 1999. Structure of a DNA-bound Ultrabithorax-Extradenticle homeodomain complex. *Nature* **397**(6721): 714-719.

Penalver-Mellado, M., Garcia-Heras, F., Padmanabhan, S., Garcia-Moreno, D., Murillo, F.J., and Elias-Arnanz, M. 2006. Recruitment of a novel zinc-bound transcriptional factor by a bacterial HMGA-type protein is required for regulating multiple processes in *Myxococcus xanthus*. *Mol Microbiol* **61**(4): 910-926.

Petrickova, K. and Petricek, M. 2003. Eukaryotic-type protein kinases in Streptomyces coelicolor: variations on a common theme. *Microbiology* **149**(Pt 7): 1609-1621.

Plamann, L., Davis, J.M., Cantwell, B., and Mayor, J. 1994. Evidence that *asgB* encodes a DNA-binding protein essential for growth and development of *Myxococcus xanthus*. *J Bacteriol* **176**(7): 2013-2020.

Plamann, L., Kuspa, A., and Kaiser, D. 1992. Proteins that rescue A-signal-defective mutants of *Myxococcus xanthus*. *J Bacteriol* **174**: 3311-3318.

Plamann, L., Li, Y., Cantwell, B., and Mayor, J. 1995. The *Myxococcus xanthus asgA* gene encodes a novel signal transduction protein required for multicellular development. *J Bacteriol* **177**: 2014-2020.

Ring, M.W., Schwar, G., Thiel, V., Dickschat, J.S., Kroppenstedt, R.M., Schulz, S., and Bode, H.B. 2006. Novel iso-branched ether lipids as specific markers of developmental sporulation in the myxobacterium *Myxococcus xanthus*. *J Biol Chem* **281**(48): 36691-36700.

Rosenberg, E., Keller, K., and Dworkin, M. 1977. Cell density-dependent growth of *Myxococcus xanthus* on casein. *J Bacteriol* **129**: 770-777.

Sager, B. and Kaiser, D. 1993. Spatial restriction of cellular differentiation. *Genes Dev* **7**: 1645-1653.

Sager, B. and Kaiser, D. 1994. Intercellular C-signaling and the traveling waves of *Myxococcus*. *Genes Dev* **8**: 2793-2804.

Sambrook, J., Fritsch, E., and Maniatis, T. 1989. *Molecular Cloning: a laboratory manual, 2nd ed.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

Shen-Orr, S.S., Milo, R., Mangan, S., and Alon, U. 2002. Network motifs in the transcriptional regulation network of *Escherichia coli*. *Nat Genet* **31**(1): 64-68.

Shimkets, L. and Woese, C.R. 1992. A phylogenetic analysis of the myxobacteria: basis for their classification. *Proc Natl Acad Sci U S A* **89**(20): 9459-9463.

Shimkets, L.J. 1987. Control of morphogenesis in myxobacteria. *Crit Rev Microbiol* **14**(3): 195-227.

Shimkets, L.J. 1999. Intercellular signaling during fruiting-body development of *Myxococcus xanthus*. *Annu Rev Microbiol* **53**: 525-549.

Shimkets, L.J., Gill, R.E., and Kaiser, D. 1983. Developmental cell interactions in *Myxococcus xanthus* and the *spoC* locus. *Proc Natl Acad Sci USA* **80**: 1406-1410.

Shimkets, L.J. and Kaiser, D. 1982. Induction of coordinated movement of *Myxococcus xanthus* cells. *J Bacteriol* **152**: 451-461.

Singer, M. and Kaiser, D. 1995. Ectopic production of guanosine penta-and tetraphosphate can initiate early developmental gene expression in *Myxococcus xanthus*. *Genes and Dev* **9**: 1633-1644.

Skerker, J.M. and Berg, H.C. 2001. Direct observation of extension and retraction of type IV pili. *Proc Natl Acad Sci U S A* **98**(12): 6901-6904.

Sliusarenko, O., Zusman, D.R., and Oster, G. 2007. The motors powering A-motility in *Myxococcus xanthus* are distributed along the cell body. *J Bacteriol* **189**(21): 7920-7921.

Smits, W.K., Kuipers, O.P., and Veening, J.W. 2006. Phenotypic variation in bacteria: the role of feedback regulation. *Nat Rev Microbiol* **4**(4): 259-271.

Sogaard-Andersen, L., Overgaard, M., Lobedanz, S., Ellehauge, E., Jelsbak, L., and Rasmussen, A.A. 2003. Coupling gene expression and multicellular morphogenesis during fruiting body formation in *Myxococcus xanthus*. *Mol Microbiol* **48**(1): 1-8.

Sogaard-Andersen, L., Slack, F.J., Kimsey, H., and Kaiser, D. 1996. Intercellular C-signaling in *Myxococcus xanthus* involves a branched signal transduction pathway. *Genes Dev* **10**(6): 740-754.

Srinivasan, D. and Kroos, L. 2004. Mutational analysis of the *fruA* promoter region demonstrates that C-box and 5-base-pair elements are important for expression of an essential developmental gene of *Myxococcus xanthus*. *J Bacteriol* **186**(17): 5961-5967.

Stanier. *The Myxobacteria.*

Stathopoulos, A. and Levine, M. 2005. Genomic regulatory networks and animal development. *Dev Cell* **9**(4): 449-462.

Sun, H. and Shi, W. 2001a. Analyses of *mrp* genes during *Myxococcus xanthus* development. *J Bacteriol* **183**(23): 6733-6739.

Sun, H. and Shi, W. 2001b. Genetic studies of *mrp*, a locus essential for cellular aggregation and sporulation of *Myxococcus xanthus*. *J Bacteriol* **183**(16): 4786-4795.

Sun, H., Yang, Z., and Shi, W. 1999. Effect of cellular filamentation on adventurous and social gliding motility of *Myxococcus xanthus*. *Proc Natl Acad Sci U S A* **96**(26): 15178-15183.

Thony-Meyer, L. and Kaiser, D. 1993. *devRS*, an autoregulated and essential genetic locus for fruiting body development in *Myxococcus xanthus*. *J Bacteriol* **175**: 7450-7462.

Toal, D.R., Clifton, S.W., Roe, B.A., and Downard, J. 1995. The *esg* locus of *Myxococcus xanthus* encodes the E1 alpha and E1 beta subunits of a branched-chain keto acid dehydrogenase. *Mol Microbiol* **16**(2): 177-189.

Tojo, N., Inouye, S., and Komano, T. 1993a. Cloning and nucleotide sequence of the *Myxococcus xanthus lon* gene: indispensability of lon for vegetative growth. *J Bacteriol* **175**(8): 2271-2277.

Tojo, N., Inouye, S., and Komano, T. 1993b. The *lonD* gene is homologous to the *lon* gene encoding an ATP-dependent protease and is essential for the development of *Myxococcus xanthus*. *J Bacteriol* **175**: 4545-4549.

Tullius, T. 1995. Homeodomains: together again for the first time. *Structure* **3**(11): 1143-1145.

Ueki, T. and Inouye, S. 1998. A new sigma factor, SigD, essential for stationary phase is also required for multicellular differentiation in *Myxococcus xanthus*. *Genes Cells* **3**: 371-385.

Ueki, T. and Inouye, S. 2001. SigB, SigC, and SigE from *Myxococcus xanthus* homologous to sigma32 are not required for heat shock response but for multicellular differentiation. *J Mol Microbiol Biotechnol* **3**(2): 287-293.

Ueki, T. and Inouye, S. 2003. Identification of an activator protein required for the induction of *fruA*, a gene essential for fruiting body development in *Myxococcus xanthus*. *Proc Natl Acad Sci USA* **100**(15): 8782-8787.

Ueki, T. and Inouye, S. 2005a. Activation of a development-specific gene, *dofA*, by FruA, an essential transcription factor for development of *Myxococcus xanthus*. *J Bacteriol* **187**(24): 8504-8506.

Ueki, T. and Inouye, S. 2005b. Identification of a gene involved in polysaccharide export as a transcription target of FruA, an essential factor for *Myxococcus xanthus* development. *J Biol Chem* **280**(37): 32279-32284.

Viswanathan, K., Viswanathan, P., and Kroos, L. 2006a. Mutational analysis of the *Myxococcus xanthus* Ω4406 promoter region reveals an upstream negative regulatory element that mediates C-signal dependence. *J Bacteriol* **188**: 515-524.

Viswanathan, P. and Kroos, L. 2003. *cis* elements necessary for developmental expression of a *Myxococcus xanthus* gene that depends on C signaling. *J Bacteriol* **185**(4): 1405-1414.

Viswanathan, P., Murphy, K., Julien, B., Garza, A.G., and Kroos, L. 2007a. Regulation of dev, an operon that includes genes essential for *Myxococcus xanthus* development and CRISPR-associated genes and repeats. *J Bacteriol* **189**: 3738-3750.

Viswanathan, P., Singer, M., and Kroos, L. 2006b. Role of sigmaD in regulating genes and signals during *Myxococcus xanthus* development. *J Bacteriol* **188**(9): 3246-3256.

Viswanathan, P., Ueki, T., Inouye, S., and Kroos, L. 2007b. Combinatorial regulation of genes essential for *Myxococcus xanthus* development involves a response regulator and a LysR-type regulator. *Proc Natl Acad Sci USA* **104**: 7969-7974.

Vlamakis, H., Aguilar, C., Losick, R., and Kolter, R. 2008. Control of cell fate by the formation of an architecturally complex bacterial community. *Genes Dev* **22**(7): 945-953.

Wang, L., Sun, Y.P., Chen, W.L., Li, J.H., and Zhang, C.C. 2002. Genomic analysis of protein kinases, protein phosphatases and two-component regulatory systems of the cyanobacterium Anabaena sp. strain PCC 7120. *FEMS Microbiol Lett* **217**(2): 155-165.

Ward, M.J., Lew, H., Treuner-Lange, A., and Zusman, D.R. 1998. Regulation of motility behavior in *Myxococcus xanthus* may require an extracytoplasmic-function sigma factor. *J Bacteriol* **180**(21): 5668-5675.

Welch, R. and Kaiser, D. 2001. Cell behavior in traveling wave patterns of myxobacteria. *Proc Natl Acad Sci USA* **98**(26): 14907-14912

West, A.H. and Stock, A.M. 2001. Histidine kinases and response regulator proteins in two-component signaling systems. *Trends Biochem Sci* **26**(6): 369-376.

Whitworth, D.E., ed. 2008. *Myxobacteria: Multicellularity and Differentiation.* ASM Press, Washington, D.C.

Wireman, J.W. and Dworkin, M. 1977. Developmentally induced autolysis during fruiting body formation by *Myxococcus xanthus*. *J Bacteriology* **129**: 796-802.

Wolfgang, M., Lauer, P., Park, H.S., Brossay, L., Hebert, J., and Koomey, M. 1998. PilT mutations lead to simultaneous defects in competence for natural transformation and twitching motility in piliated *Neisseria gonorrhoeae*. *Mol Microbiol* **29**(1): 321-330.

Wolgemuth, C., Hoiczyk, E., Kaiser, D., and Oster, G. 2002. How myxobacteria glide. *Curr Biol* **12**(5): 369-377.

Yang, C. and Kaplan, H.B. 1997. *Myxococcus xanthus sasS* encodes a sensor histidine kinase required for early developmental gene expression. *J Bacteriol* **179**(24): 7759-7767.

Yoder-Himes, D. and Kroos, L. 2006. Regulation of the *Myxococcus xanthus* C-signal-dependent Ω4400 promoter by the essential developmental protein FruA. *J Bacteriol* **188**: 5167-5176.

Yoder, D. and Kroos, L. 2004a. Mutational analysis of the *Myxococcus xanthus* Ω4400 promoter region provides insight into developmental gene regulation by C signaling. *J Bacteriol* **186**: 661-671.

Yoder, D. and Kroos, L. 2004b. Mutational analysis of the *Myxococcus xanthus* Ω4499 promoter region reveals shared and unique properties in comparison with other C-signal-dependent promoters. *J Bacteriol* **186**: 3766-3776.