JOURNAL OF BACTERIOLOGY, Jan. 2010, p. 360–364 Vol. 192, No. 1
0021-9193/10/$12.00 doi:10.1128/JB.01019-09

Identification of Enhancer Binding Proteins Important for *Myxococcus xanthus* Development[▽]

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Received 31 July 2009/Accepted 27 October 2009

Enhancer binding proteins (EBPs) control the temporal expression of fruiting body development-associated genes in *Myxococcus xanthus*. Eleven previously uncharacterized EBP genes were inactivated. Six EBP gene mutations produced minor but reproducible defects in fruiting body development. One EBP gene mutation that affected A-motility produced strong developmental defects.

When the intracellular starvation signal (p)ppGpp accumulates (10, 22, 23, 28), the deltaproteobacterium *Myxococcus xanthus* forms a biofilm containing a mat of peripheral rod cells and multicellular structures called fruiting bodies (29). Cells that aggregate into fruiting bodies differentiate into dormant and stress-resistant spores, while the peripheral rods outside these structures fail to sporulate (25). Fruiting body development is accompanied by large-scale changes in gene expression, and enhancer binding proteins (EBPs) form a regulatory cascade that controls the sequential expression of many developmental genes (N. B. Caberoy, K. M. Giglio, G. Suen, and A. G. Garza, submitted for publication). EBPs are transcriptional activators that work in conjunction with σ^{54}-RNA polymerase; EBPs help σ^{54}-RNA polymerase form a transcription-competent open promoter complex (33). To date, 17 EBPs that perform a variety of developmental functions have been linked to the formation of mature fruiting bodies (2, 6–9, 13, 14, 17, 30, 32).

Eleven *M. xanthus* genes that code for EBPs have yet to be characterized. Here, we examined whether these uncharacterized EBP genes are important for fruiting body development. Insertions in the chromosomal copies of the EBP genes in wild-type strain DK1622 were created and confirmed as previously described (2). (Tables 1 and 2 show the bacterial strains, plasmids, and primers used in this study.) Subsequently, EBP mutant cells and wild-type cells were placed on 1.5% agar plates containing TPM starvation buffer (10 mM Tris-HCl [pH 8.0], 1 mM KH_2PO_4, and 8 mM $MgSO_4$) to monitor the progress of fruiting body development and to determine sporulation efficiencies. Six of the EBP mutants exhibited relatively weak developmental defects (Table 3 and Fig. 1). The MXAN0172, MXAN5879, and MXAN7143 mutants had wild-type sporulation efficiencies, but they exhibited fruiting body formation defects. In particular, fruiting body formation in the MXAN0172 and MXAN5879 mutants was delayed, and the MXAN7143 mutant failed to produce fruiting bodies with characteristic shapes. Fruiting body formation

TABLE 1. Bacterial strains and plasmids used in this study

Bacterial strain or plasmid	Relevant characteristic(s)	Reference or source
Strains		
AG1101	DK1622 pKG01::MXAN0172	This study
AG1102	DK1622 pKG02::MXAN0603	This study
AG1103	DK1622 pKG03::MXAN0907	This study
AG1104	DK1622 pKG04::MXAN1189	This study
AG1105	DK1622 pKG05::MXAN1565	This study
AG1106	DK1622 pKG06::MXAN3555	This study
AG1107	DK1622 pKG07::MXAN4196	This study
AG1108	DK1622 pKG08::MXAN4261	This study
AG1109	DK1622 pKG09::MXAN4977	This study
AG1110	DK1622 pKG10::MXAN5879	This study
AG1111	DK1622 pKG11::MXAN7143	This study
AG1112	DK1253 pKG05::MXAN4196	This study
AG1113	DK1218 pKG05::MXAN4196	This study
DK1218	A-motility defect	12
DK1253	S-motility defect	12
DK1622	Wild-type motility and development	15
DK2161	A-motility and S-motility defects	12
Plasmids		
pCR2.1-TOPO	Kan^r	Invitrogen
pKG01	Kan^r pCR2.1-TOPO containing a 455-bp MXAN0172 fragment	This study
pKG02	Kan^r pCR2.1-TOPO containing a 501-bp MXAN0603 fragment	This study
pKG03	Kan^r pCR2.1-TOPO containing a 548-bp MXAN0907 fragment	This study
pKG04	Kan^r pCR2.1-TOPO containing a 350-bp MXAN1189 fragment	This study
pKG05	Kan^r pCR2.1-TOPO containing a 404-bp MXAN1565 fragment	This study
pKG06	Kan^r pCR2.1-TOPO containing a 477-bp MXAN3555 fragment	This study
pKG07	Kan^r pCR2.1-TOPO containing a 502-bp MXAN4196 fragment	This study
pKG08	Kan^r pCR2.1-TOPO containing a 541-bp MXAN4261 fragment	This study
pKG09	Kan^r pCR2.1-TOPO containing a 485-bp MXAN4977 fragment	This study
pKG10	Kan^r pCR2.1-TOPO containing a 502-bp MXAN5897 fragment	This study
pKG11	Kan^r pCR2.1-TOPO containing a 350-bp MXAN7143 fragment	This study

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▽ Published ahead of print on 6 November 2009.

TABLE 2. Primers used in this study

Primer	Locus tag or gene	Sequence	Amplicon size (bp)
3558 up	MXAN0172	5′-CGCTGCATTCGATGACTGCTC-3′	
3558 down	MXAN0172	5′-GCGAGCGAAGAAGGAGACGAA-3′	455
1181a	MXAN0603	5′-CGTCATCGTCACCGGCGAGTCC-3′	
1181b	MXAN0603	5′-GTGAGCTGCCGGACGAAGTGCC-3′	501
mx2756-fwd	MXAN0907	5′-AGCGAGCTGCCCGTGCTGGTGTGC-3′	
mx2756-rev	MXAN0907	5′-GCGGACAGCTCCATCTCCTCACGG-3′	548
1156a	MXAN1565	5′-CCTTCGTCACGCTCAACTGCGC-3′	
1156b	MXAN1565	5′-GAGGAAGGCGCACAACTGCGGC-3′	404
980a	MXAN1189	5′-GGCTCGTCGCCGTCAACTGCG-3′	
980b	MXAN1189	5′-CTGGAGAGGCATCACGTTGAGG-3′	350
1930 up	MXAN3555	5′-GGAGCTCATCGCCACCGCGCT-3′	
1930 down	MXAN3555	5′-TGGCGTGCTTGGCCACGAAGT-3′	477
3656 up	MXAN4196	5′-GCAGGCCACGGTGCTGCTGGT-3′	
3656 down	MXAN4196	5′-GCGCAGCAGCAGCTCCGACAA-3′	502
939a	MXAN4261	5′-CGATGCGGAACCTCTACGAGC-3′	
939b	MXAN4261	5′-GTGAAGTGCTCCACCAACAAGG-3′	541
mx4346-fwd	MXAN4977	5′-CTGGCGAGAATGGGACGGGGAAGG-3′	
mx4346-rev	MXAN4977	5′-CACAGGTGGGCGCACTGATTGAGG-3′	485
6911 up	MXAN5879	5′-CATCGCCGCCTCATCCATGAC-3′	
6911 down	MXAN5879	5′-GTCCGGGGACAGGCCGGATAC-3′	502
1254a	MXAN7143	5′-GGTGCGGCGGCTCATCGAGCG-3′	
1254b	MXAN7143	5′-AGCCCACCGGATGCAGCTCGC-3′	350

in the MXAN0603 and MXAN4261 mutants was both delayed and incomplete, and their sporulation efficiencies were reduced about 1.5- to 1.8-fold compared to that of wild-type cells. Finally, the MXAN0907 mutant produced normal-looking fruiting bodies (data not shown), but its sporulation efficiency was reduced about 2.2-fold compared to that of wild-type cells.

We scanned the sequences of the EBP gene loci (5) and our findings suggest that three (MXAN0172, MXAN0907, and MXAN7143) out of the six insertions that yielded relatively weak developmental phenotypes have the potential to be polar. The genes located immediately downstream of MXAN 0172, MXAN0907, and MXAN7143 are MXAN0171, MXAN 0906, and MXAN7142, respectively. Using quantitative PCR analysis (26), we found no obvious signs that the three insertions in question are polar; we detected wild-type levels of MXAN0171, MXAN0906, and MXAN7142 expression in the MXAN0172, MXAN0907, and MXAN7143 mutants, respectively (data not shown).

TABLE 3. Developmental phenotypes of wild-type and EBP gene mutant strains[a]

Strain (genotype)	Fruiting body formation[b]	Fruiting body spores (% of wild type)[c]
DK1622 (wild type)	+	100.0 ± 19.0
AG1101 (MXAN0172)	+/−	68.5 ± 8.5
AG1102 (MXAN0603)	+/−	54.3 ± 6.4[d]
AG1103 (MXAN0907)	+	46.3 ± 10.0[d]
AG1104 (MXAN1565)	+	100.1 ± 9.5
AG1105 (MXAN3077)	+	111.0 ± 11.1
AG1106 (MXAN3555)	+	85.9 ± 13.5
AG1107 (MXAN4196)	−	<0.01[d]
AG1108 (MXAN4261)	+/−	65.4 ± 3.8[d]
AG1109 (MXAN4977)	+	102.1 ± 2.3
AG1110 (MXAN5879)	+/−	72.2 ± 11.9
AG1111 (MXAN7143)	+/−	116.0 ± 5.6

[a] Cells were placed on TPM agar and allowed to develop for 5 days. Development was monitored visually using phase-contrast microscopy.

[b] Symbols: +, produced normal-looking fruiting bodies; −, failed to produce normal-looking fruiting bodies; +/−, produced normal-looking fruiting bodies but aggregation was delayed.

[c] Spore assays were performed three times for each strain. The mean values ± standard deviations for the spore assays are shown as percentages of DK1622 (wild type). The number of spores produced by wild-type cells ranged from 1.12×10^7 to 1.90×10^7. Values were determined by transferring sonication- and heat-resistant spores to CTTYE agar plates, incubating the plates for 5 days, and counting the number of colonies that arose from the spores.

[d] Variances compared to wild type were found to be significant using a two-tailed *t* test ($\alpha = 0.05$).

One EBP mutant, MXAN4196, showed strong defects in fruiting body formation and sporulation (Table 3 and Fig. 1). This mutant failed to form normal-looking fruiting bodies, even when it was given 5 days to develop. Furthermore, the MXAN4196 mutant produced no viable spores. On the basis of the *M. xanthus* genome sequence (5), MXAN4196 is the last gene in an operon that contains two genes. This finding indicates that the insertion in MXAN4196 is unlikely to have polar effects. Because the MXAN4196 mutant has strong defects in fruiting body development, we chose to analyze it further.

M. xanthus cells use gliding motility to aggregate into multicellular fruiting bodies, and many EBPs that are important for fruiting body development have been linked to gliding motility (19). To determine whether the MXAN4196 mutant has a gliding motility defect, we used swarm expansion assays (16). MXAN4196 mutant cells and wild-type cells were placed on CTTYE (1.0% Casitone, 0.5% yeast extract, 10 mM Tris-HCl [pH 8.0], 1 mM KH_2PO_4, and 8 mM $MgSO_4$) plates containing 0.4% or 1.5% agar, and colony diameters were determined after 3 days of incubation at 32°C. The mean diameters of MXAN4196 mutant colonies on 0.4% and 1.5% agar plates were 64.1% (±5.6% [standard deviation]) and 44.9% (±5.3%) of wild-type colonies, respectively. These results indicate that the MXAN4196 mutant has a gliding motility defect.

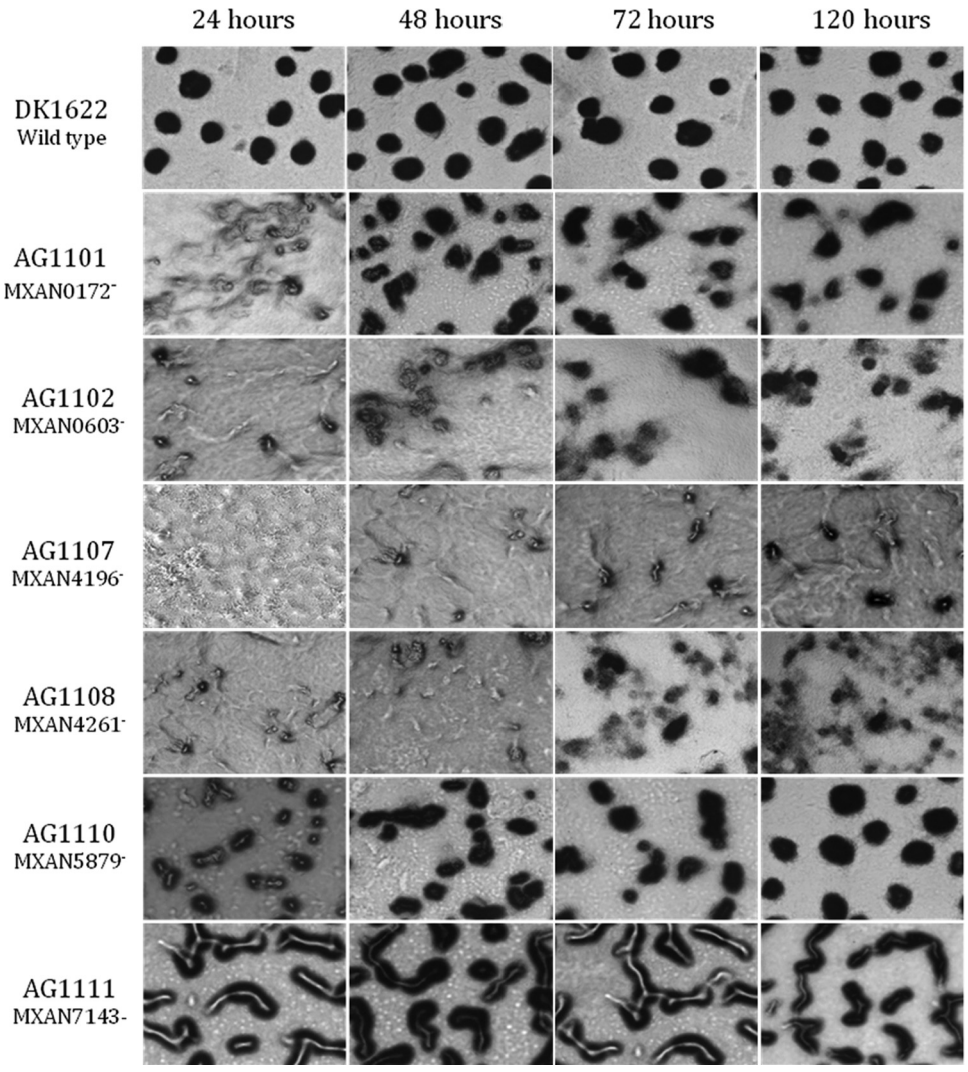

FIG. 1. Development of EBP gene mutants on TPM agar plates. Wild-type and mutant cells were placed on TPM starvation agar, and the progress of fruiting body development was monitored for 5 days using phase-contrast microscopy. Photographs were taken at 24, 48, 72, and 120 h poststarvation using a total magnification of ×40.

Mutants defective for either A-motility (A^- S^+ cells) or S-motility (A^+ S^- cells) swarm at a reduced rate, while mutants that are defective for both types of motility (A^- S^- cells) have a nonswarming phenotype and smooth colony edges (12). To determine whether the MXAN4196 insertion causes a defect in the A- or S-motility system, it was introduced into A^- S^+ (DK1218) and A^+ S^- (DK1253) mutant strains, and the colony edges of the double mutants were examined using phase-contrast microscopy (Fig. 2). When the MXAN4196 insertion was introduced into the DK1218 (A^- S^+) recipient, the colony edge was similar to that of wild-type cells carrying the same insertion. When the insertion was introduced into the DK1253 (A^+ S^-) background, we detected a smooth colony edge that was similar to that of the nonswarming A^- S^- double mutant DK2161. These findings indicate that the MXAN4196 insertion causes a defect in A-motility.

Since sporulation takes place inside fruiting bodies and the MXAN4196 insertion disrupts A-motility and fruiting body formation, we examined whether this insertion has a direct effect on sporulation by performing glycerol spore assays (21). When glycerol is added to a nutrient broth culture, rod-shaped vegetative cells undergo a rapid and synchronous conversion into spores, bypassing many of the early events that are required for production of fruiting body spores by directly activating at least part of the sporulation program (4). Interestingly, the MXAN4196 mutant produced no viable glycerol spores in our assays (data not shown). Our interpretation of this result is that MXAN4196 plays a direct and important role in the *M. xanthus* sporulation process.

In this study, we identified six EBP mutants that have relatively minor defects in fruiting body development and one EBP mutant (MXAN4196) that has strong developmental defects. The MXAN4196 mutant fails to produce normal-looking fruiting bodies, and it fails to produce viable spores during development. Our data indicate that

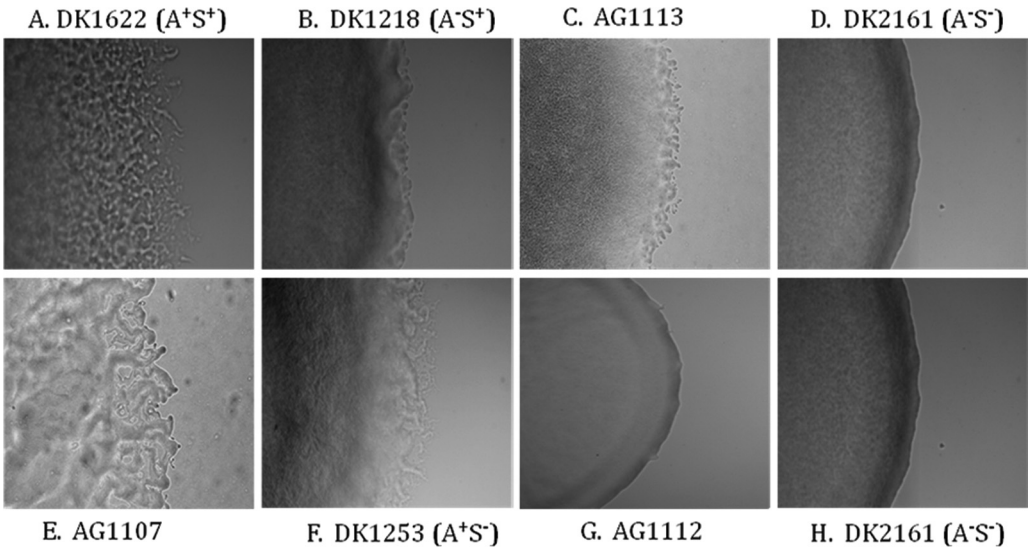

FIG. 2. Colony edge morphologies produced by the MXAN4196 insertion. Colony edge morphologies produced by A^+ S^+ strain DK1622 (A), A^- S^+ strain DK1218 (B), A^+ S^- strain DK1253 (F), and A^- S^- strain DK2161 (D and H) are shown. The MXAN4196 insertion was introduced into strain DK1622 to generate strain AG1107 (E), into strain DK1218 to generate strain AG1113 (C) and into strain DK1253 to generate strain AG1112 (G). Colony edges were observed after 3 days of growth on CTTYE agar using phase-contrast microscopy (40× magnification).

MXAN416 is an A-motility mutant. Although the mechanism of *M. xanthus* A-motility is not well understood, two models have been proposed: one model suggests that A-motility is powered by slime extrusion from the cell poles (31), and the other model suggests that A-motility is powered by motors associated with focal adhesion complexes (24). The A-motility system is known to require a complex network of more than 30 genes (reviewed in reference 11). Mutations in most A-motility genes have little or no effect on the formation of spore-filled fruiting bodies. Mutations that do produce developmental phenotypes seem to primarily affect sporulation. At this point, it is unclear whether the A-motility defect of the MXAN4196 mutant contributes to its developmental phenotype. However, we can state that the MXAN4196 mutant has a particularly strong developmental defect for an A-motility mutant. The MXAN4196 mutant also has a strong defect in glycerol-induced sporulation. This is a rather unique phenotype for an A-motility mutant, but we are aware of one other A-motility mutant that has such a defect, the EBP gene mutant *nla24* (2, 20). Gliding motility is not required for glycerol-induced sporulation, suggesting that the MXAN4196 protein plays a critical role in sporulation that is distinct from its role in A-motility. Since EBPs regulate transcription at σ^{54} promoters, we looked for σ^{54} promoter signature sequences upstream of operons containing A-motility genes and operons containing sporulation-specific genes. As shown in Table 4, we found five A-motility gene operons and three sporulation gene operons that have putative σ^{54} promoters. This finding suggests that MXAN4196 might play a direct role in the regulation of both A-motility genes and sporulation genes. The goal of future work will be to determine whether any of these operons are under direct transcriptional control of this EBP.

TABLE 4. Operons containing genes with putative σ^{54} promoters

Gene type	First gene in operon	Relevant gene	No. of genes in operon	σ^{54} promoter sequence[a]	Reference
Genes known to be required for A motility	MXAN2991	*aglZ*	1	**TGGCA**AC-N4-C**TGCT**	34
	MXAN3502	*agmI*	2	**TGG**GG**CG**-N4-**TTGC**C	35
	MXAN4799	*agmC*	2	**TGACA**GA-N4-**TTTCA**	35
	MXAN5818	*agmR*	2	**TGGCAC**A-N4-G**TGCT**	35
	MXAN5820	*agmM*	1	**TGGC**C**C**T-N4-C**TGCT**	35
Genes known to be required for sporulation	MXAN2269	*mspA*	1	**TGGC**CTA-N4-G**TGCT**	3
	MXAN3225	*exo*	5	**TGGCAC**A-N4-C**TGCT**	21
	MXAN5432	*tps*	2	**TGG**GG**C**A-N4-**TTGCT**	18

[a] The putative promoter regions of operons containing A-motility and sporulation genes were analyzed using the *M. xanthus* genome sequence (5) and PromScan (http://molbiol-tools.ca/promscan/), a bioinformatics tool that was specifically developed to identify σ^{54}-RNA polymerase binding sites in the sequences of bacterial DNA. To be designated a σ^{54} promoter, there had to be a potential binding site for σ^{54}-RNA polymerase and a potential EBP binding site, which is a tandem repeat of at least 7 bp (27). On the basis of tests done with known promoter sequences and intragenic sequences, we estimated that our analysis had a false-positive rate of about 4% and a false-negative rate of about 23%. The −12 and −24 regions of the putative σ^{54} promoters are shown. Bold, underlined nucleotides are those that match nucleotides in the σ^{54} consensus sequence, which is TGGCACG-N4-TTGC(T/A) (1).

This work was supported by National Science Foundation grant 0717653 to A. Garza.

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