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Nla18, a Key Regulatory Protein Required for Normal Growth and Development of *Myxococcus xanthus*†

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NtrC-like activators regulate the transcription of a wide variety of adaptive genes in bacteria. Previously, we demonstrated that a mutation in the *ntrC*-like activator gene *nla18* causes defects in fruiting body development in *Myxococcus xanthus*. In this report, we describe the effect that *nla18* inactivation has on gene expression patterns during development and vegetative growth. Gene expression in *nla18* mutant cells is altered in the early stages of fruiting body development. Furthermore, *nla18* mutant cells are defective for two of the earliest events in development, production of the intracellular starvation signal ppGpp and production of A-signal. Taken together, these results indicate that the developmental program in *nla18* mutant cells goes awry very early. Inactivation of *nla18* also causes a dramatic decrease in the vegetative growth rate of *M. xanthus* cells. DNA microarray analysis revealed that the vegetative expression patterns of more than 700 genes are altered in *nla18* mutant cells. Genes coding for putative membrane and membrane-associated proteins are among the largest classes of genes whose expression is altered by *nla18* inactivation. This result is supported by our findings that the profiles of membrane proteins isolated from vegetative *nla18* mutant and wild-type cells are noticeably different. In addition to genes that code for putative membrane proteins, *nla18* inactivation affects the expression of many genes that are likely to be important for protein synthesis and gene regulation. Our data are consistent with a model in which Nla18 controls vegetative growth and development by activating the expression of genes involved in gene regulation, translation, and membrane structure.

In nature, biofilms formed by the soil bacterium *Myxococcus xanthus* feed on prey bacteria to obtain amino acids, which are used as a source of carbon, nitrogen, and energy (1, 7). Upon starvation for amino acids, *M. xanthus* initiates a complex developmental program that allows large groups of cells to migrate to aggregation centers and begin building multicellular fruiting bodies. Once a fruiting body is molded into its final shape, individual rod-shaped cells within this structure differentiate into dormant, spherically shaped spores that are resistant to many forms of environmental stress (8, 62).

When deprived of amino acids, *M. xanthus* cells accumulate (p)ppGpp (46, 47, 63), a molecule that serves as an intracellular starvation signal in bacteria (3, 4). After the intracellular pool of (p)ppGpp rises and *M. xanthus* cells initiate development, a series of cell-cell signals help coordinate large-scale changes in gene expression (6, 19, 38, 39, 43). Of these cell-cell developmental signals, the two that have been studied the most extensively are A-signal and C-signal. A-signal is a diffusible cell density signal that is required in the earliest stages of *M. xanthus* development, prior to the onset of aggregation (39, 40, 41, 57). In contrast, C-signal is a contact-stimulated signal that is required for the aggregation and sporulation phases of development to proceed normally (33, 34, 35, 38, 44).

Recent findings indicate that *M. xanthus* uses σ^{54}-like promoters to drive the expression of many developmentally regulated genes (11, 12, 13, 16, 17, 31, 59, 69). Work by Keseler and Kaiser (32) demonstrated that *rpoN*, which encodes σ^{54}, is essential for vegetative growth in *M. xanthus*. These results indicate that σ^{54}-like promoters are important for gene expression during vegetative growth and development. Transcriptional activation of σ^{54}-dependent promoters requires an NtrC-like activator, a DNA binding protein that helps σ^{54}-RNA polymerase form a transcriptionally active, open promoter complex (49, 70). Fifteen NtrC-like activators that are required for fruiting body development to proceed normally have been uncovered in the past 10 years (2, 15, 18, 20, 26, 27, 36, 67, 69).

More recently, Caberoy et al. (2) demonstrated that an insertion in the *ntrC*-like activator gene *nla18* (MXAN_3692; see http://cmr.tigr.org/tigr-scripts/CMR/GenomePage.cgi?org search=my&org=gmx) causes defects in aggregation and sporulation. In this paper, we show that the *nla18* mutation reduces or abolishes the expression of genes that are activated throughout the course of fruiting body development. In addition, cells carrying the *nla18* insertion are defective for ppGpp accumulation and A-signal production, indicating that Nla18 is required in the earliest stages of fruiting body development. We also found that in nutrient broth the doubling time of the *nla18* mutant is about two- to threefold longer than that of wild-type cells, indicating that the *nla18* mutant has a vegeta-

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† Supplemental material for this article may be found at http://jb.asm.org/.

TABLE 1. Bacterial strains, plasmids, and primers used in this study

Strain, plasmid, or primer	Relevant characteristic(s) or sequence	Source, reference, or amplicon size
Strains		
AG318	DK1622 pNBC18::*nla18*	2
AG339	DK101 pNBC18::*nla18*	This study
AG343	DK1622 pNBC31::*nla18*	2
AG390	pNBC31::*nla18 sdeK*::Ω4408 Tn*5lacZ*	This study
AG391	pNBC31::*nla18 spi*::Ω4521 Tn*5lacZ*	This study
AG392	pNBC31::*nla18 devR*::Ω4414 Tn*5lacZ*	This study
AG393	pNBC31::*nla18* Ω4403 Tn*5lacZ*	This study
AG395	pNBC31::*nla18* Ω4435 Tn*5lacZ*	This study
AG407	pNBC31::*nla18 exo*::Ω7536 Tn*5lacZ*	This study
AG1001	AG343 Mx8*attB*::pFOR18	This study
DK101	*pilQ1* (wild-type development)	24
DK476	*pilQ1 asgA476*	19
DK1622	Wild-type development	29
DK4323	*pilQ1 asgA476 spi*::Ω4521 Tn*5lacZ*	39
DK4300	*sdeK*::Ω4408 Tn*5lacZ*	37
DK4368	Ω4403 Tn*5lacZ*	37
DK4521	*spi*::Ω4521 Tn*5lacZ*	37
DK5204	Ω4435 Tn*5lacZ*	37
DK5208	*csgA*::Tn*5*-132 ΩLS205	38
DK5508	*devR*::Ω4414 Tn*5lacZ*	37
DK7536	*exo*::Ω7536 Tn*5lacZ*	45
DK11063	*fruA*::Ω7540 Tn*5lacZ*	64
MS1000	DK101Δ*relA1*	This study
Plasmids		
pBGS18	Kan^r	66
pCR2.1-TOPO	Kan^r	Invitrogen
pSWU19	Kan^r *attP*	S. S. Wu and D. Kaiser
pSWU22	Tet^r	S. S. Wu and D. Kaiser
pBJ114	Kan^r, *galK* vector	28
pMS302	4.8 PstI fragment containing *relA* in pBGS18	23
pMS302R	4.8 PstI fragment containing *relA* (opposite orientation) in pBGS18	This study
pMS330	pMS302 with 1.3 kb of *relA* deleted	This study
pMS331	pBJ114 carrying the 3.5-kb PstI fragment from pMS330	This study
pNBC18	477-bp internal fragment of *nla18* in pCR2.1-TOPO	2
pNBC31	477-bp internal fragment of *nla18* in pSWU22	2
pFOR18	Kan^r, pSWU19 containing 1.8 kb of *nla18* on a BamHI-HindIII fragment	This study
Primers		
*relA*fwd	5′-TCATCGCCTTTCGCATCATCGC-3′	129 bp
*relA*rev	5′-ACATGTTGGGCTTCGGAATCGC-3′	

tive growth defect. By DNA microarray analysis, we show that the *nla18* insertion alters the normal expression patterns of vegetative genes, including a large number of genes whose products are likely to be involved in gene regulation and protein synthesis, and genes that encode membrane and membrane-associated proteins. Taken together, our findings indicate that Nla18 is an important regulator of both vegetative and developmental gene expression.

MATERIALS AND METHODS

Bacterial strains. The strains used in this study are shown in Table 1. DNA sequence analysis of the *nla18* locus revealed that the *mx887* gene is located immediately downstream of *nla18*, suggesting that *nla18* and *mx887* may be part of the same operon. Two lines of evidence indicate that the *nla18* insertion does not impact *mx887* transcription. (i) A plasmid (pFOR18) carrying a wild-type copy of *nla18* and the upstream *nla18* promoter element rescues the defects of *nla18* mutant strain AG343 when it is integrated into the Mx*8* phage attachment site (*attB*) in the chromosome. (ii) Reverse transcription-PCR and DNA microarray analysis confirm that *mx887* is expressed in the *nla18* mutant.

Strain MS1000 carries a 1,311-bp in-frame deletion in the *relA* gene. To construct the *relA* deletion mutant, we used pMS302, a plasmid that contains the *relA* gene and flanking DNA on a PstI fragment. Plasmid pMS302 was digested with SacI, and the large DNA fragment was gel purified and then self-ligated to generate plasmid pMS330. Plasmid pMS330 carries a 1,311-bp internal deletion of *relA* due to the removal of adjacent 87-bp and 1,224-bp SacI fragments. The PstI fragment carrying the internal deletion of *relA* was isolated from plasmid pMS330 and ligated into the PstI site of *galK* vector pBJ114, yielding plasmid pMS331. Plasmid pMS331 was introduced into DK101 cells by electroporation as described by Plamann et al. (58). Kan^r transformants with a single copy of pMS331 integrated into the *relA* locus were identified by Southern blot analysis (60) and used to isolate Gal^r Kan^s strains as previously described (11, 68). Gal^r Kan^s strains carrying the *relA* deletion were identified by Southern blot analysis (60). The presence of the *relA* deletion was confirmed by screening for (p)ppGpp accumulation in response to nutrient downshift. Strains AG390, AG391 AG392, AG393, AG395, and AG407 were constructed by introducing plasmid pNBC31 into strains DK4300, DK4521, DK5508, DK4368, DK5204, and DK7536, respectively.

Media used for growth and development. *M. xanthus* strains were grown at 28°C or 32°C in CTT broth (1.0% Casitone [Difco], 10 mM Tris-HCl [pH 8.0], 1 mM KH_2PO_4, 8 mM $MgSO_4$), on CTTYE broth (CTT containing 0.5% yeast extract [Difco]), or on solid support plates containing CTTYE broth and 1.5% Difco Bacto Agar. CTTYE broth and plates were supplemented with 40 μg of kanamycin sulfate/ml or 10 μg of oxytetracycline/ml as needed. Fruiting body development was carried out at 32°C on plates containing TPM buffer (10 mM

Tris-HCl [pH 8.0], 1 mM KH_2PO_4, 8 mM $MgSO_4$) and 1.5% Difco Bacto Agar. A-factor assays were performed with microtiter plates containing MC7 starvation buffer (10 mM MOPS, 1 mM $CaCl_2$, final pH 7.0). CTT soft agar contains CTT broth and 0.7% Difco Bacto Agar.

Escherichia coli strains were grown at 37°C in Luria broth (LB) containing 1.0% tryptone (Difco), 0.5% yeast extract (Difco), and 0.5% NaCl or in plates containing LB and 1.5% Difco Bacto Agar. LB and LB plates were supplemented with 40 μg of kanamycin sulfate/ml or 10 μg of oxytetracycline/ml as needed.

***M. xanthus* development.** *M. xanthus* strains were inoculated into flasks containing CTTYE broth, and the cultures were incubated at 28°C or 32°C with vigorous swirling. After the cultures reached a density of 5×10^8 cells/ml, the cells were pelleted, the supernatants were removed, and the cells were resuspended in TPM buffer to a density of 5×10^9 cells/ml. Aliquots (20 μl) of the cell suspensions were spotted onto TPM agar plates and incubated at 32°C. *M. xanthus* cells were harvested at various times during development on TPM agar and used for RNA slot blot hybridization studies, quantitative PCR (QPCR), β-galactosidase assays, or Western blot analysis as described below.

β-Galactosidase assays. Cells were harvested at different times during development on TPM and quick-frozen in liquid nitrogen as described previously (11). β-Galactosidase assays were performed on quick-frozen cell extracts by the technique of Kaplan et al. (30). β-Galactosidase specific activity is defined as nanomoles of *o*-nitrophenol produced per minute per milligram of protein.

A-factor assays. DK101, DK476, and AG339 cells were inoculated into flasks containing CTTYE broth, and the cultures were incubated at 32°C with vigorous swirling. After the cultures reached a density of 5×10^8 cells/ml, the cells were pelleted, washed with MC7 buffer, and resuspended in MC7 buffer to a density of 2.5×10^{10} cells/ml. The cell suspensions were placed in flasks and shaken at 32°C. After 3 h, the cells were pelleted and the conditioned MC7 buffer was removed. A-factor assays were performed with *M. xanthus* test strain DK4323 and aliquots of conditioned MC7 buffer as described previously (23, 57).

Western blot assays. Approximately 10^9 *M. xanthus* cells/ml were harvested from TPM agar plates, placed in sodium dodecyl sulfate (SDS) lysis buffer, and boiled for 10 min. Protein samples were separated by electrophoresis through a 12% polyacrylamide gel and transferred to an Immobilon P membrane (Millipore) with a semidry blotting apparatus. The blots were probed with anti-FruA antibody, followed by incubation with peroxidase-conjugated goat anti-rabbit immunoglobulin G (Boehringer Mannheim). The blots were developed with the Renaissance Chemiluminescence Reagent (NEN Life Science Products) and Amersham autoradiography Hyperfilm-MP.

Analysis of nucleotide pools. Nucleotides were isolated and separated by thin-layer chromatography as described previously (46, 63). ^{32}P-labeled ppGpp, GTP, and ATP were visualized with a STORM phosphorimaging scanner and quantified by Image Quant software (Molecular Dynamics).

QPCR and RNA slot blot hybridization analysis. Total cellular RNA was isolated from quick-frozen cells by the hot phenol method (60) and used to generate cDNA as described by Lancero et al. (42). One-microliter aliquots of the cDNA synthesis reaction mixtures were used for the subsequent PCR amplifications. PCR mixtures contained gene-specific forward and reverse primers (250 nM, final concentration) and the DyNamo HS SYBR Green qPCR Master Mix (Finnzymes). The primers used for QPCR are listed in Table 1. QPCR was performed with the Opticon 2 system from MJ Research. The rate of accumulation of PCR-generated DNA was measured by continuous monitoring of SYBR Green I (Molecular Probes) fluorescence. To confirm that RNA samples were not contaminated with residual genomic DNA, control cDNA synthesis reaction mixtures that lacked reverse transcriptase were prepared and then analyzed by QPCR as described above for the test samples. Gene expression was quantified by the absolute method of quantification (user bulletin 2, Applied Biosystems), and the expression levels were normalized to levels in wild-type cells at time zero (vegetative growth). Standard curves for each QPCR primer pair were made at 10^1, 10^2, 10^3, 10^5, 10^8, 10^{10}, and 10^{11} plasmid copies/μl. Standard curves for *relA* primers were made with pMS302R. Slot blot hybridizations were performed on total cellular RNA as described by Kaplan et al. (30). PCR-generated fragments of the *sdeK*, *relA*, and *nla18* genes were used as probes for slot blot hybridization experiments. The specificity of these probes was confirmed by using yeast mRNA, which yielded no detectable signal.

DNA microarrays. PCR generated DNA microarrays containing probes to the 7,235 *M. xanthus* open reading frames identified on the M1genome (26; R. D. Welch, personal communication) were spotted onto poly-L-lysine-coated glass slides by the Stanford Functional Genomics Facility (Stanford, CA). Production of *M. xanthus* DNA microarrays was done in conjunction with The Myxococcus Microarray Consortium, and construction was based on a pilot array previously described (26). Processing of the DNA arrays, cDNA synthesis, microarray hybridization, and posthybridization processing were performed as described by Jakobsen et al. (26), with the following modifications. Five independent biological replica pairs of wild-type and *nla18* mutant strains were used for this analysis, and each independent wild-type–*nla18* mutant pair was handled and processed identically. Briefly, each pair of wild-type and *nla18* mutant strains was grown at 28°C to a density of 5×10^8 cells/ml, the cells were pelleted by centrifugation, the supernatants were removed, and the cell pellets were quick-frozen in liquid nitrogen. Total cellular RNA was isolated from quick-frozen cells by the hot phenol method (60). Thirty micrograms of total RNA from matched cultures was used to synthesize cDNA with 10 μg of pdN6 primers (Amersham Pharmacia) in the presence of 40 μg/μl RNase inhibitor (Promega). Reverse transcriptase reaction times were modified as follows: 10 min at 37°C, then 42°C for 100 min, followed by a 10-min incubation at 50°C. RNA was hydrolyzed and neutralized as described by Jakobsen et al. (26) and purified with Micron 30 filters (Amicon), and cDNA was eluted and dried with a SpeedVac concentrator (Savant). The dried cDNA was resuspended in 9 μl of 0.1 M sodium bicarbonate (pH 9.0) and incubated for 5 min at 37°C. The cDNA was labeled with Cy3 (DK1622) or Cy5 (AG318) (Amersham Pharmacia) by addition of 2 μl of dye dissolved in 10 μl of dimethyl sulfoxide and incubated for 1 h in the dark. The labeled cDNA was purified with a QIA-quick PCR kit (QIAGEN) as described by the manufacturer and concentrated on a Micron 30 spin filter (Amicon). Labeled cDNA was then dried with a SpeedVac concentrator (Savant) and resuspended in 45 μl of hybridization buffer. Hybridization and posthybridization processing of the slides were performed as described previously (26).

Posthybridized DNA microarrays were scanned with a GenePix 4000A microarray scanner and read by GenePix Pro 3.0 (Axon Inc.). The GenePix array list (gal) file MyxoGALv2.gal, corresponding to the *M. xanthus* DNA microarrays, was constructed by GalFileMakerv1.2 (DeRisi Lab website; http://derisilab.ucsf.edu). Spots were flagged and removed from analyses based on stringent criteria for shape, signal intensity, and background by GenePix Pro 3.0 (Axon Inc.). Analyses were performed on all unflagged spots. All array analyses, including hierarchical clustering and statistical analysis, were performed by Cluster (Eisen Software; http://rana.lbl.gov/EisenSoftware.htm), Java TreeView software (Alok Saldanha, 2001; http://sourceforge.net/projects/jtreeview), and Significance Analysis of Microarrays (V. G. Tusher, R. Tibshirani and G. Chu, http://www-stat.stanford.edu/~tibs/SAM).

Isolation of membrane fractions. Wild-type cells were grown in CTTYE, and *nla18* mutant cells were grown in CTTYE containing 40 μg of kanamycin sulfate/ml to a density of 5×10^8 cells/ml. Following centrifugation, the pelleted cells were resuspended in CTTYE and the cell suspension was quick-frozen in liquid nitrogen. The crude cell envelope fraction used for sucrose density gradient centrifugation was prepared by an adaptation of the protocols previously developed by Nikaido (51) and by Orndorff and Dworkin (55). Briefly, after thawing of the frozen samples in the presence of 1 mM phenylmethylsulfonyl fluoride (PMSF), the cells were pelleted and resuspended in 8 ml of 10 mM HEPES (pH 7.4)/1 mM PMSF. The cell suspension was then lysed by passage through a French press (SLM Aminco) three times at 14,000 lb/in^2. Intact cells were removed from the cell lysates by collecting the supernatant fractions after several rounds of centrifugation at $5{,}000 \times g$ for 10 min. To pellet the bacterial envelope, the supernatants were centrifuged at $180{,}000 \times g$ for 1 h at 4°C and the resulting pellet was solubilized overnight with 1 ml of resuspension buffer (10 mM HEPES, 1 mM EDTA, 1 mM PMSF). The membrane fraction was then collected by sucrose gradient centrifugation as described by Osborn et al. (56) with the following steps: 46%, 49%, 52%, 55%, and 58% (wt/wt). Following centrifugation for 18 h in an SW41 rotor (Beckman), a single band was observed at the junction between the 46% and 49% steps. This band was removed, diluted with resuspension buffer, and centrifuged at $180{,}000 \times g$ for 1 h. Pellets were solubilized at 4°C overnight in 50 μl of resuspension buffer.

For quick whole-membrane isolations, the total membrane fractions were prepared by the small-scale cell envelope preparation procedure of Morona and Reeves (50). With *M. xanthus*, addition of lysozyme is not necessary for lysing cells but is important for separation of the membrane from the cell wall, thus avoiding smearing problems on SDS-polyacrylamide gels.

The protein composition of the membrane fractions was analyzed on 7.5% SDS-polyacrylamide gels with a 37.5:1 acrylamide/bisacrylamide ratio. Precision Plus Protein All Blue standards (Bio-Rad) or prestained SDS-polyacrylamide gel electrophoresis (PAGE) broad-range standards (Bio-Rad) were used, and the same total amount of protein was loaded into each lane. Gels were stained with Coomassie brilliant blue R-250 (Kodak) to visualize proteins. Peptide mass mapping by matrix-assisted laser desorption ionization–time of flight mass spectrometry was performed by the Molecular Structure Facility at the University of California, Davis, as described by Shevchenko et al. (61) and analyzed with an ABI 4700 Proteomics Analyzer mass spectrophotometer (Applied Biosystems).

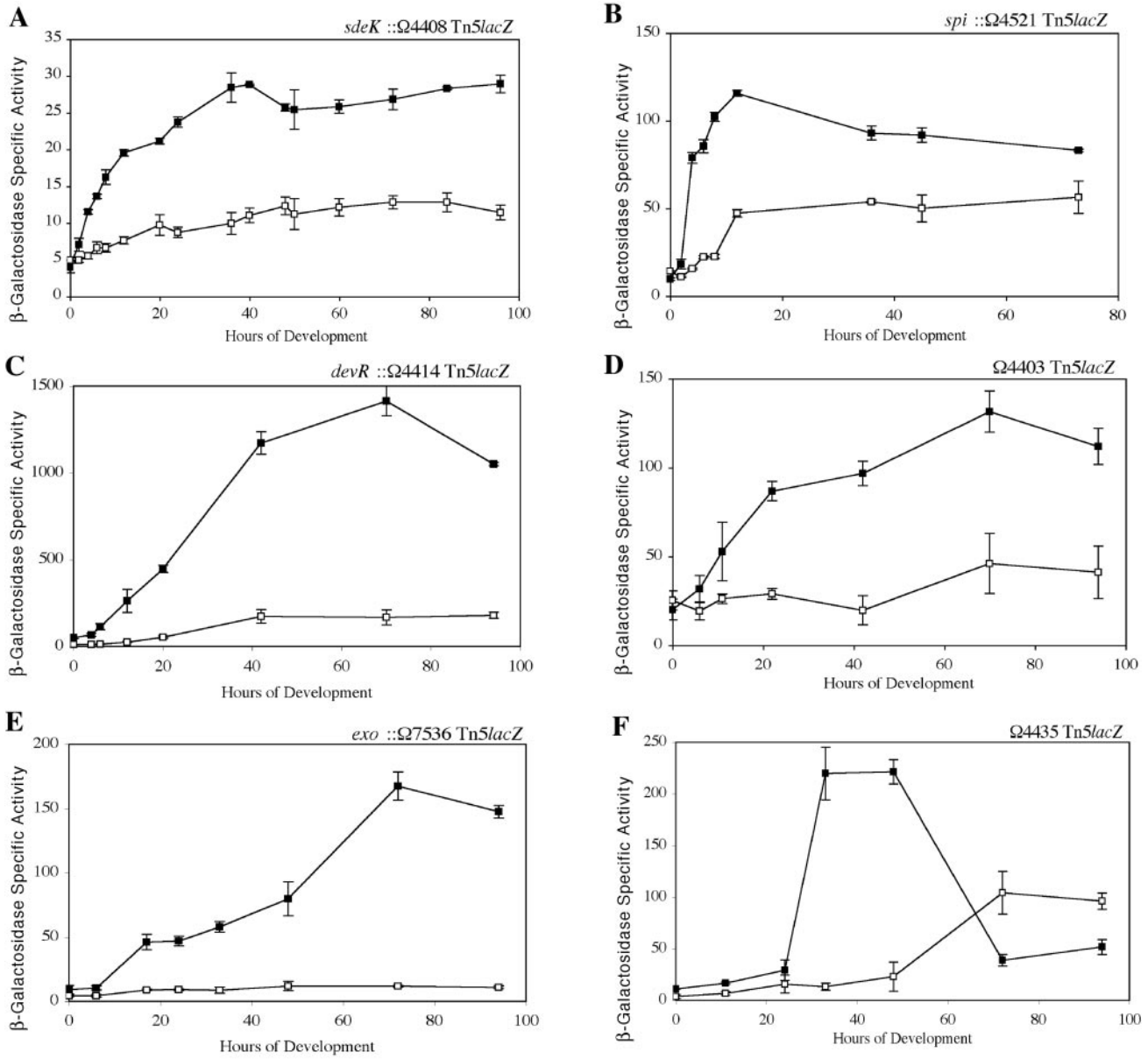


FIG. 1. Developmental expression in wild-type cells and *nla18* mutant cells. Expression of Tn*5lacZ* reporter gene fusions was monitored at various times of development on TPM agar. Mean β-galactosidase specific activities were determined from three independent experiments. Error bars are the standard deviations of the means. Black squares show β-galactosidase specific activities for strains carrying the wild-type *nla18*$^+$ alleles, whereas empty squares show β-galactosidase specific activities for strains carrying the *nla18* mutant alleles. The specific fusions monitored were *sdeK*::Ω4408 Tn*5lacZ* (A), *spi*::Ω4521 Tn*5lacZ* (B), *devR*::Ω4414 Tn*5lacZ* (C), Ω4403 Tn*5lacZ* (D), *exo*::Ω7536 Tn*5lacZ* (E), and Ω4435 Tn*5lacZ* (F).

Measured monoisotopic masses of tryptic peptides were used as inputs to search the *M. xanthus* protein database (The Institute for Genomic Research [TIGR]-Monsanto; G. Suen and R. D. Welch, personal communication) with the Mascot search engine and a probability-based scoring algorithm (http://www.matrixscience.com).

Nucleotide sequence accession numbers. All of the DNA microarray results in this study have been submitted to Gene Expression Omnibus (GEO) at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/projects/geo/). Accession numbers are provided in Fig. 5.

RESULTS

Domain structure of Nla18. The putative domain structure of Nla18 (MXAN3692) based on the completed *M. xanthus* genome sequence (TIGR website; http://www.tigr.org) was recently described by Jelsbak et al. (27). The C-terminal domain of Nla18 contains a helix-turn-helix motif, which is characteristic of many DNA binding proteins. The central region of the protein contains the highly conserved σ^{54} activator domain. This domain is required for ATP binding and hydrolysis, which helps σ^{54}-bound RNA polymerase become transcriptionally active. Since these domains are hallmarks of NtrC-like activators, Nla18 is likely to be a bona fide NtrC-like activator protein. Based on a partial sequence of the *M. xanthus* genome, Caberoy et al. (2) reported that the N-terminal signal recog-

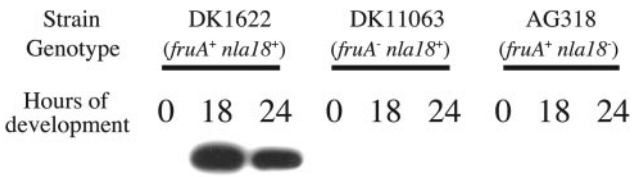


FIG. 2. FruA protein levels in wild-type cells and *nla18* mutant cells developing on TPM agar. Whole-cell lysates were prepared from strains DK1622 (*nla18*$^+$ *fruA*$^+$), DK11063 (*nla18*$^+$ *fruA*), and AG318 (*nla18 fruA*$^+$) at the indicated times of development. Protein samples were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with anti-FruA antibody as described in Materials and Methods. The same total amount of protein was loaded into each lane. The experiments were repeated three times, and a representative experiment is shown.

nition domain of Nla18 is about 30 to 40 amino acids (GenBank accession number AY337505). However, the completed *M. xanthus* genome sequence suggests that the N-terminal signal input domain of the protein is about 210 to 220 amino acids. This region of the Nla18 protein appears to contain a forkhead-associated domain (27), which is a phosphothreonine-specific recognition domain. This finding suggests that the N-terminal signal input domain of Nla18 may interact with a serine/threonine protein kinase, signal transduction proteins that are abundant in *M. xanthus*.

Developmental gene expression. Caberoy et al. (2) found that the *nla18* mutant is defective for two important landmark events in fruiting body development, aggregation and sporulation. To determine whether the *nla18* mutant is defective for the changes in gene expression that accompany these morphological events, a panel of developmentally regulated *lacZ* reporter fusions were introduced into *nla18* mutant cells. The expression profiles of these *lacZ* fusions in wild-type and *nla18* mutant cells developing on TPM starvation agar are shown in Fig. 1. In wild-type cells, expression of *spi*::Tn*5lacZ* and of *sdeK*::Tn*5lacZ* was induced prior to the onset of aggregation (Fig. 1A and B), expression of *dev*::Tn*5lacZ* and of Ω4403 Tn*5lacZ* was induced during aggregation (Fig. 1C and D), and expression of *exo*::Tn*5lacZ* and of Ω4435 Tn*5lacZ* was induced as sporulation commences (Fig. 1E and F). In *nla18* mutant cells, however, peak expression of the two early reporters *spi*::Tn*5lacZ* and *sdeK*::Tn*5lacZ* was only 42% to 44% of the peak expression in wild-type cells. The peak expression of the remaining four reporters in *nla18* mutant cells ranged from about 10% to 35% of the peak expression observed in wild-type cells. These findings indicate that inactivation of *nla18* affects gene expression throughout *M. xanthus* fruiting body development. They also suggest that Nla18 is required in the early stages of fruiting body development, prior to the start of aggregation.

TABLE 2. A-factor activity in wild-type cells and mutant cells

Strain	A-factor activity (U/ml)[a]
DK101 (*nla18*$^+$ *asgA*$^+$)	32.2 ± 4.2
AG339 (*nla18 asgA*$^+$)	12.0 ± 0.6
DK476 (*nla18*$^+$ *asgA*)	4.4 ± 0.2

[a] The values shown are means derived from three independent experiments. Standard deviations are shown next to the means.

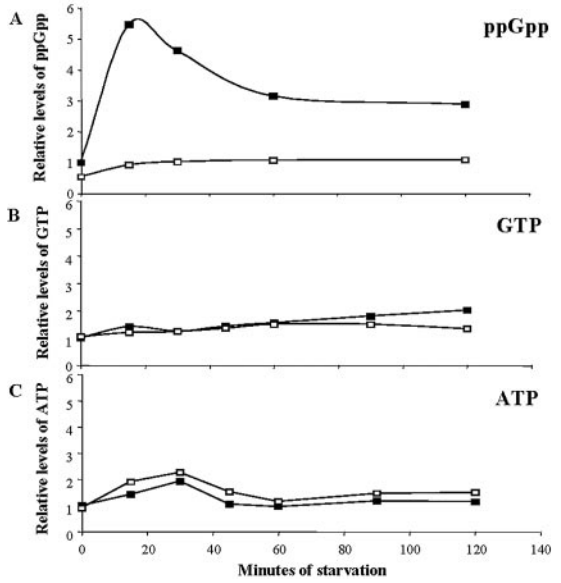


FIG. 3. Relative levels of nucleotides in wild-type cells and *nla18* mutant cells. Nucleotides were isolated from DK101 (*nla18*$^+$ *relA*$^+$) and AG339 (*nla18 relA*$^+$) cells at different times following starvation and analyzed as described in Materials and Methods. Signal intensities were normalized to that of vegetative wild-type cells at 0 min (vegetative growth). These assays were repeated four times, and a representative sample set is shown. Black squares show the relative levels of ppGpp, GTP, and ATP in DK101 cells, and empty squares show the relative levels of ppGpp, GTP, and ATP in AG339 cells. The levels of ppGpp were similar when the *nla18* insertion was placed in a DK101 or DK1622 strain background (data not shown). Strain MS1000 served as the negative control for the ppGpp assays (data not shown).

FruA is a response regulator that plays a critical role in the C-signal transduction pathway, the cell-cell signaling system that regulates changes in gene expression during aggregation and sporulation (10, 54, 64). Since gene expression during

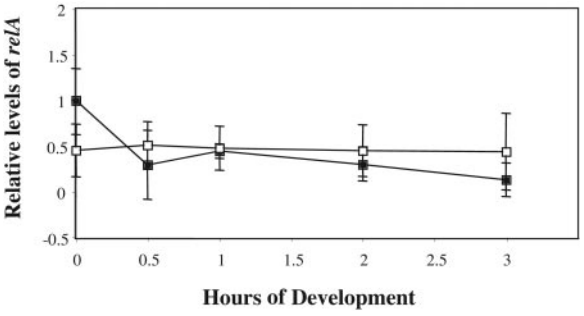
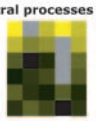
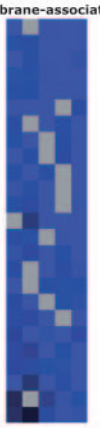


FIG. 4. Expression of *relA* in wild-type cells and *nla18* mutant cells. QPCR was used to examine developmental expression of *relA* in the wild-type and *nla18* mutant backgrounds as described in Materials and Methods. Expression of the *relA* gene was determined by the absolute method of quantification, and the expression levels were normalized to levels in wild-type cells at time zero (vegetative growth). Expression of *relA* in wild-type strain DK1622 (black squares) and *nla18* mutant strain AG318 (empty squares) was determined with primers *relA*fwd and *relA*rev (Table 1). The values shown are means derived from four replicates. Error bars are standard deviations of the means.

UPREGULATED

Mx number*		Experiment**								
	1 2 3 4 5	1	2	3	4	5	Ave	Std dev	Fold	Annotation***
Membrane/Membrane-associated proteins										
Mx_5132		14.96	15.40	8.37	37.81	14.23	18.15	11.35	18.15	lipoprotein, putative; PurE
Mx_6480		3.92	2.42	3.72	1.74	1.76	2.71	1.05	2.71	chemoreceptor Mcp2, putative
Mx_6697		2.57	N/A	1.69	2.10	1.44	1.95	0.49	1.95	ABC transporter, permease protein, putative
Mx_640		1.83	2.08	1.52	1.69	1.66	1.76	0.21	1.76	Choline transporter/probable transmembrane protein
Regulators										
Mx_6096		5.73	2.96	5.99	24.75	4.50	8.79	9.01	8.79	Ser/Thr protein phosphatase family protein
Mx_4965		3.36	3.17	7.13	4.62	1.36	3.93	2.13	3.93	sigma-54 dependent transcriptional regulator, Nla34
Mx_2379		2.20	N/A	2.30	3.20	1.24	2.23	0.80	2.23	transcription termination/antitermination factor NusG
Translation/Ribosomal proteins										
Mx_423		31.86	N/A	12.83	10.27	9.05	16.00	10.69	16.00	translation elongation factor G
Mx_423		21.06	8.81	14.60	28.92	6.19	15.92	9.25	15.92	translation elongation factor G
Mx_3228		2.97	3.31	8.85	6.03	3.72	4.97	2.48	4.97	ribosomal protein L13
Mx_3047		5.54	2.98	5.56	4.35	3.20	4.33	1.23	4.33	ribosomal protein L20
Mx_1544		4.58	4.46	4.73	3.91	2.50	4.04	0.91	4.04	translation elongation factor Tu
Mx_3048		4.10	1.98	6.28	4.25	3.53	4.03	1.55	4.03	ribosomal protein L35, putative
Mx_5413		2.45	3.41	3.61	2.40	2.52	2.88	0.58	2.88	translation elongation factor Tu
Mx_1544		3.20	2.59	3.91	1.84	1.37	2.58	1.02	2.58	translation elongation factor Tu
Mx_5413		3.00	2.69	2.90	1.94	1.07	2.32	0.81	2.32	translation elongation factor Tu
Mx_3229		2.37	N/A	2.16	2.71	1.47	2.18	0.52	2.18	30S ribosomal protein S9
Mx_1543		2.09	1.86	2.46	1.59	N/A	2.00	0.37	2.00	30S ribosomal protein S10
Metabolism/Central processes										
Mx_4095		12.04	N/A	6.98	27.31	6.65	13.25	9.70	13.25	HNH endonuclease
Mx_4095		5.68	3.09	4.57	N/A	5.76	4.78	1.25	4.78	HNH endonuclease
Mx_2850		2.26	1.25	1.90	N/A	2.82	2.06	0.66	2.06	RNA polymerase beta subunit
Mx_6850		1.51	1.61	2.33	N/A	2.47	1.98	0.49	1.98	RtcB-like protein
Mx_369		2.37	1.56	1.80	2.33	1.73	1.96	0.37	1.96	putative serine protease, HtrA/DegQ/DegS family
Mx_1241		1.68	2.24	2.24	1.30	1.85	1.86	0.40	1.86	glutathione-disulfide reductase

DOWNREGULATED

Mx number*		Experiment**								
	1 2 3 4 5	1	2	3	4	5	Ave	Std dev	Fold	Annotation***
Membrane/ Membrane-associated proteins										
Mx_42		0.16	N/A	0.19	0.03	0.13	0.13	0.07	7.79	cytochrome p450
Mx_4484		0.22	0.25	0.13	0.07	0.01	0.14	0.10	7.37	S-layer-like protein
Mx_5263		0.11	0.24	0.11	0.17	0.22	0.17	0.06	5.95	conserved hypothetical/hypothetical membrane protein
Mx_6566		0.22	0.21	0.21	0.14	0.08	0.17	0.06	5.85	oxidoreductase, short chain family
» Mx_4187		0.25	0.22	0.14	0.16	0.09	0.17	0.06	5.77	**Oar**, OmpA-related protein precursor
Mx_4506		0.18	0.10	0.10	N/A	0.32	0.17	0.10	5.75	oxidoreductase, short chain putative
Mx_68		0.29	N/A	0.10	0.26	0.06	0.18	0.11	5.64	paral putative outer membrane receptor
Mx_6677		0.23	0.01	N/A	0.28	0.19	0.18	0.12	5.61	development-specific protein s
Mx_5503		0.13	0.24	N/A	0.32	0.03	0.18	0.13	5.58	membrane protein, putative
Mx_1782		0.25	0.02	0.31	N/A	0.14	0.18	0.13	5.57	lipoprotein, putative
Mx_1521		0.19	0.24	0.19	N/A	0.12	0.18	0.05	5.42	membrane protein, putative
Mx_1704		0.23	0.32	0.06	N/A	0.19	0.20	0.11	4.94	isoquinoline 1-oxidoreductase, alpha subunit
Mx_5708		N/A	0.55	0.12	0.06	0.11	0.21	0.23	4.78	permease, putative domain protein
Mx_7218		0.28	0.20	N/A	0.19	0.18	0.21	0.05	4.72	lipoprotein, putative
Mx_3060		0.19	0.32	0.16	0.25	0.17	0.22	0.06	4.60	lipoprotein, putative
Mx_1547		0.31	N/A	0.07	0.30	0.20	0.22	0.11	4.59	integral membrane protein, putative
Mx_1368.3		0.11	N/A	0.30	0.22	0.26	0.22	0.08	4.49	ABC transporter, ATP-binding protein, putative
Mx_1246		0.30	0.16	N/A	0.24	0.22	0.23	0.06	4.35	outer membrane protein, OMP85 family
Mx_3608		0.29	0.29	0.17	N/A	0.16	0.23	0.07	4.35	preprotein translocase, YaiC subunit
Mx_5355		0.42	0.04	0.24	0.11	0.35	0.23	0.16	4.34	oxidoreductase, short chain family
Mx_2747		0.32	0.43	0.12	0.25	0.07	0.24	0.15	4.19	phosphonates transport system (permease)
Mx_6912		0.37	0.27	0.26	0.22	0.09	0.24	0.10	4.13	Rhomboid-like, integral membrane protein
Mx_7234		0.08	0.58	0.16	0.29	0.15	0.25	0.20	3.97	oxidoreductase, FAD-binding
» Mx_4332		0.78	N/A	0.60	0.19	0.24	0.45	0.28	2.21	**membrane protein, putative**
Mx_4186		0.72	0.91	0.36	0.18	0.21	0.48	0.32	2.10	MlpA, putative lipoprotein precursor
Regulators										
Mx_2776		0.11	N/A	0.20	0.06	0.12	0.12	0.06	8.16	two-component, response regulator
Mx_6693.3		0.19	0.23	0.01	N/A	0.16	0.15	0.09	6.81	two-component, response regulator
Mx_1523		0.31	N/A	0.07	0.09	0.22	0.17	0.12	5.76	serine/threonine kinase Pkn14
Mx_4299		0.32	0.23	0.18	0.15	0.09	0.19	0.08	5.15	transcriptional regulator, HthA
Mx_3772		0.26	0.27	0.11	N/A	0.13	0.20	0.08	5.10	two-component, sensor histidine kinase
Mx_890		0.24	N/A	0.07	0.40	0.27	0.24	0.14	4.10	serine/threonine kinase, PskB5
Mx_5136		0.27	0.46	0.14	0.24	0.13	0.25	0.13	4.05	transcriptional regulator, MarR family
Translation/Ribosomal proteins										
Mx_2851		0.14	0.04	0.15	0.05	0.29	0.13	0.10	7.42	50S ribosomal protein L7/L12
Mx_3743		0.08	0.16	0.27	N/A	0.15	0.16	0.07	6.09	ribosomal large subunit pseudouridine synthase D, RluD
Mx_2707		0.03	0.29	0.41	0.17	0.24	0.23	0.14	4.38	leucyl-tRNA synthetase
Mx_4393		0.12	0.11	0.36	0.17	0.43	0.24	0.15	4.17	translation elongation factor EF-P
Metabolism/Central processes										
Mx_5928		0.16	0.12	0.10	N/A	0.05	0.11	0.04	9.38	phosphoribosylaminoimidazole synthetase, PurM
Mx_4268		0.09	0.24	0.16	0.13	0.17	0.16	0.05	6.40	putative epoxide hydrolase, EphA
Mx_6495		0.24	N/A	0.11	0.10	0.20	0.16	0.07	6.23	putative monooxygenase
Mx_3492		0.06	N/A	0.12	0.25	0.21	0.16	0.09	6.22	putative methyltransferase
Mx_2366		0.21	0.17	0.21	0.14	0.08	0.16	0.05	6.22	methylguanine-DNA methyltransferase
Mx_6548		0.01	0.17	0.18	0.26	0.23	0.17	0.10	5.75	SAM-dependent methyltransferase
Mx_1269		0.22	0.35	0.12	N/A	0.02	0.18	0.14	5.60	phosphopeptide-binding protein, putative
Mx_7005		0.05	0.12	0.07	0.53	N/A	0.19	0.23	5.16	DNA polymerase I
Mx_5583		0.21	N/A	0.17	0.27	0.17	0.20	0.05	4.89	calcium-binding protein-like
Mx_3058		0.26	0.09	0.41	0.22	0.06	0.21	0.14	4.78	glutathione peroxidase family protein
Mx_4431		0.30	0.01	0.10	0.23	0.41	0.21	0.16	4.74	spoVG-like
Mx_5667		0.46	0.31	0.10	0.09	0.25	0.24	0.15	4.13	polynucleotide adenylyltransferase
Mx_5788		0.08	0.62	0.29	0.09	0.14	0.24	0.23	4.10	phosphoserine phosphatase, SerB
Mx_1215		0.21	0.27	0.23	0.31	0.25	0.25	0.04	3.94	hypothetical RTX exoprotein-like protein
Mx_3086		0.41	0.37	0.15	N/A	0.11	0.26	0.15	3.85	homoserine kinase, putative
Mx_574		0.23	0.38	0.19	0.31	0.23	0.27	0.08	3.71	aminotransferase, class I, putative
Mx_2194		0.28	0.21	0.29	0.32	N/A	0.28	0.05	3.62	non-ribosomal peptide synthetase
Other										
» Mx_2915		0.34	0.44	0.14	N/A	0.27	0.30	0.13	3.32	**hypothetical protein**

aggregation and sporulation appears to be severely impaired in *nla18* mutant cells, we examined whether they express FruA. Isogenic wild-type and *nla18* mutant cells were harvested after 18 h (when FruA levels peak) and 24 h of development on TPM agar, the cells were lysed, and whole-cell extracts were probed with anti-FruA antibody. The FruA expression profiles shown in Fig. 2 revealed that *nla18* mutant cells were producing little or no FruA after 18 and 24 h of development. Similar results were observed when *nla18* mutant cells were given additional time to develop (data not shown), suggesting that FruA production in the *nla18* mutant is abolished.

A-factor production. Genes on the early developmental pathway such as *spi* and *fruA* are activated in response to A-factor (or A-signal) production. The inability of the *nla18* mutant to express normal levels of these genes suggested that this mutant is defective for production of A-factor. To test this hypothesis, A-factor assays were performed as previously described (23, 57) with conditioned medium from wild-type DK101 (*nla18*$^+$ *asgA*$^+$), AG339 (*nla18 asgA*$^+$), or DK476 (*nla18*$^+$ *asgA*) cells as the source of A-factor. The ability of conditioned medium to rescue β-galactosidase production in an *asg* mutant (DK4323) carrying the A-factor-dependent *spi*::Ω4521 Tn*5lacZ* transcriptional fusion was used to determine A-factor activity; 1 U of β-galactosidase specific activity is equal to 1 U of A-factor activity. The results of the A-factor assays are shown in Table 2. The levels of A-factor produced by *nla18* mutant cells were about 37% of those in wild-type cells. However, *nla18* mutant cells generated 2.7-fold more A-factor than *asgA* cells, which are known to be defective for production of A-factor. Thus, it seems that A-factor production in *nla18* mutant cells is impaired, which is consistent with the defects in early developmental gene expression (Fig. 1 and 2).

ppGpp accumulation. Inactivation of *nla18* affects early developmental gene expression and A-signal production, events that are dependent on accumulation of the intracellular starvation signal (p)ppGpp (23, 63). Therefore, we hypothesized that the *nla18* mutant may also be defective for (p)ppGpp accumulation. To test this hypothesis, we assayed the relative levels of ppGpp in *nla18* mutant cells during vegetative growth and starvation. In the experiment shown in Fig. 3, wild-type DK101 cells and *nla18* mutant cells were grown in nutrient broth and subjected to a nutrient downshift, and the relative amounts of ppGpp were measured (Fig. 3A). In wild-type cells, the levels of ppGpp increased about sixfold after 15 min of starvation, followed by a decrease to new steady-state levels that were about threefold higher than the vegetative growth levels. The levels of ppGpp in *nla18* mutant cells were about 50% of the wild-type levels during vegetative growth. Furthermore, the peak poststarvation levels of ppGpp in *nla18* mutant cells were about 18% of the wild-type peak, indicating that *nla18* mutant cells failed to fully initiate a starvation response. No additional rise in ppGpp levels was observed in *nla18* mutant cells when we extended the period of starvation (data not shown). Presumably, the relatively low levels of ppGpp in *nla18* mutant cells affect downstream developmental gene expression and cell-cell signal production.

RelA converts GTP into (p)ppGpp with ATP as the pyrophosphate donor (3, 63). Hence, GTP and ATP are essential precursors for the synthesis of the (p)ppGpp starvation signal. To determine whether *nla18* mutant cells are defective in the production of these ppGpp precursors, we quantified the relative levels of GTP and ATP in *nla18* mutant and wild-type cells. As shown in Fig. 3B and C, GTP and ATP levels in *nla18* mutant cells were similar to those found in wild-type cells, indicating that *nla18* mutant cells produce the nucleotides required for RelA-dependent synthesis of ppGpp.

Expression of genes implicated in ppGpp accumulation. One testable model of Nla18 function is that Nla18 modulates ppGpp levels by regulating expression of the *relA* gene. To test this hypothesis, we monitored *relA* mRNA levels in wild-type cells and *nla18* mutant cells during vegetative growth and development by QPCR (Fig. 4). The QPCR studies revealed that wild-type cells and *nla18* mutant cells expressed similar levels of *relA* mRNA. The results were confirmed by RNA slot blot hybridization studies (data not shown). Based on the results of these expression studies, we conclude that *relA* is not under transcriptional control of Nla18, implying that Nla18 modulates ppGpp levels by an alternative mechanism (see Discussion).

In *M. xanthus*, several other genes have been implicated in ppGpp regulation, including *socE* (5) and mx_1594. The product of the mx_1594 gene has strong sequence similarity to the N-terminal hydrolase domain of *E. coli* SpoT (K. A. O'Connor and D. R. Zusman, personal communication; M. E. Diodati and M. Singer, unpublished data), a protein that modulates (p)ppGpp levels in response to starvation stimuli (14). To determine whether inactivation of *nla18* affects expression of *socE* or mx_1594, we monitored the levels of *socE* and mx_1594 mRNAs in wild-type cells and *nla18* mutant cells during vegetative growth and development by QPCR. Similar levels of both *socE* and mx_1594 mRNAs were detected in wild-type cells and *nla18* mutant cells (data not shown), indicating that *nla18* inactivation does not affect *socE* or mx_1594 expression.

Finally, for completeness, we performed the reciprocal of the experiment above, assaying for whether *nla18* expression is under the control of *relA*. By both QPCR and RNA slot blot hybridization analysis, we determined that *nla18* mRNA levels are not affected by a *relA* deletion (data not shown). These data

FIG. 5. Genes showing significant changes in expression. *, Annotation based on M1genome construction of microarrays (26; R. D. Welch, personal communication). Repeated Mx numbers represent duplicate features on the array; not all features are duplicated on each array. **, Five independent biological replicates were prepared as described in Materials and Methods. Averages, standard deviations, and *n*-fold values are reported. Data are presented graphically as heat maps (yellow is upregulated, blue is downregulated) and as corresponding non-log-transformed ratios of the *nla18* mutant to the wild type. A gray box in the head map corresponds to N/A in the table. N/A represents flagged spots on the array (see Materials and Methods). ***, *M. xanthus* sequence completed by TIGR/Monsanto (personal communication) and submitted to GEO (platform, GPL2848; series, GSE3323; samples, GSM78450, GSM78452, GSM78453, GSM78455, and GSM78456).

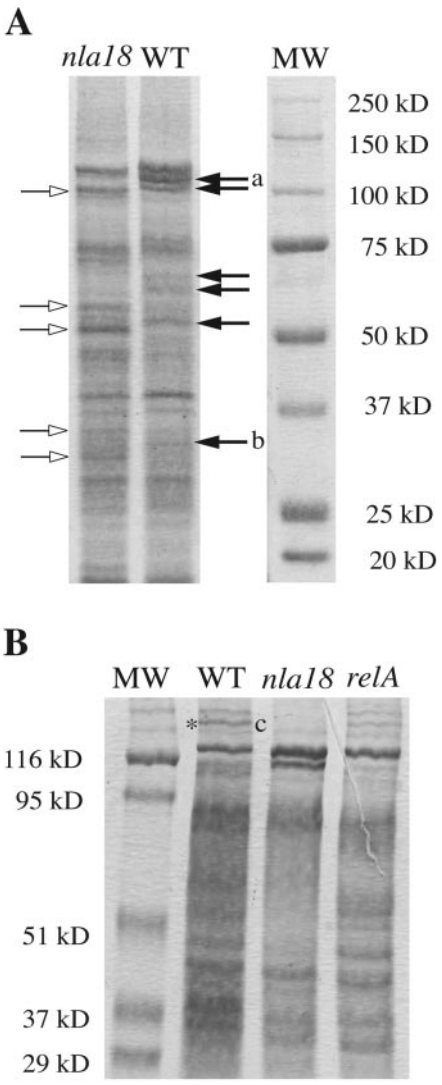


FIG. 6. Membrane protein profiles of wild-type (WT) and mutant cells. (A) Membranes of wild-type (DK1622) and *nla18* mutant (AG318) cells were isolated by sucrose density gradient centrifugation and run on a 7.5% SDS-PAGE gel. The same total amount of protein was loaded into each lane. Proteins were visualized with Coomassie brilliant blue R-250 stain, and Precision Plus Protein All Blue standards (Bio-Rad) were used as a reference (MW). The empty and black arrows represent protein bands whose expression appears to increase or decrease in the *nla18* mutant, respectively. Bands excised and identified by peptide mass mapping are labeled "a" and "b." The larger band, band a, is the Oar protein, and band b is Mx_2915, which is a hypothetical protein. The absence of these bands in the *nla18* mutant is consistent with our DNA microarray data (Fig. 5). (B) Membranes of isogenic wild-type (DK101), *nla18* mutant (AG339), and *relA* mutant (MS1000) cells were isolated via quick whole-membrane preparation as described in Materials and Methods and run on a 7.5% SDS-PAGE gel. The same total amount of protein was loaded into each lane. Proteins were visualized with Coomassie brilliant blue R-250 stain, and prestained SDS-PAGE broad-range standards (Bio-Rad) were used as a reference (MW). The band excised and identified by peptide mass mapping is flanked by an asterisk and the letter "c." This band, Mx_4332 (a putative membrane protein), is present in the *relA* mutant, albeit at a lower intensity than in the wild type. The absence of this band in the *nla18* mutant is consistent with our DNA microarray data (Fig. 5).

suggest that *nla18* is not downstream of *relA* on the *M. xanthus* developmental pathway.

Vegetative growth. We found that the phenotypes caused by *nla18* inactivation are not specific to the *M. xanthus* developmental process; an *nla18* mutation alters colony color, cell cohesion, and the vegetative growth rate of *M. xanthus* cells. The growth defect is dependent upon growth media and temperature. When they are grown in CTT broth at 32°C, which are standard laboratory conditions, *nla18* mutant cells have a generation time of approximately 14 to 16 h, whereas wild-type cells have a generation time of 5 h. This defect is less severe when *nla18* mutant cells are grown at 28°C and the CTT broth is supplemented with yeast extract (CTTYE broth). Under these conditions, *nla18* mutant cells have a generation time of 10.5 to 12 h, whereas wild-type cells have a generation time of 5 to 6 h. In addition, *nla18* mutant cells display a 60- to 72-h lag phase prior to exponential growth, while wild-type cells begin exponential growth after 4 to 5 h.

Gene expression during vegetative growth. Our preliminary phenotypic analysis of the *nla18* mutant revealed a vegetative growth rate reduction, implying that Nla18 plays an important role in regulating gene expression in vegetative cells. We used a global DNA microarray approach to examine vegetative gene expression patterns in *nla18* mutant cells, in an attempt to identify genes under Nla18 control. As described in Materials and Methods, wild-type and *nla18* mutant cells were grown to a density of 5×10^8 cells/ml (mid-exponential phase), total cellular RNA was harvested from these cells, and the RNA was used for DNA microarray studies. More than 700 genes showed altered patterns of expression in *nla18* mutant cells compared to wild-type cells, some of which are presented in Fig. 5. A complete listing of all of the significant down-regulated and up-regulated genes is provided in Tables S1 and S2, respectively, in the supplemental material, and a complete list of all of the genes on the array with data in at least four of the five experiments is presented in Table S3 in the supplemental material. Inactivation of *nla18* affected the expression of several genes whose products are likely to be required for protein synthesis. Perhaps misregulation of these genes in *nla18* mutant cells perturbs the *M. xanthus* translation machinery, affecting the ribosome-associated RelA protein's ability to monitor the translation state of cells and its ability to synthesize (p)ppGpp. Inactivation of *nla18* also affected the expression of genes that are likely to encode membrane and membrane-associated proteins, the largest class of *nla18*-dependent genes with known or predicted functions. Among these genes, expression of *oar*, which encodes an OmpA-related protein (48), is one of the most severely impacted. In addition, expression of *mlpA*, a putative lipoprotein gene in the *oar* operon (21), was down about 2.1-fold in the *nla18* mutant (Fig. 5). Other categories of genes whose expression was altered in *nla18* mutant cells include a number of putative regulatory genes and genes that are likely to encode metabolic enzymes.

Membrane protein profiles. Because the DNA microarrays indicate that expression of many genes encoding putative membrane and membrane-associated proteins is impaired in *nla18* mutant cells, we compared the membrane protein profile of *nla18* mutant cells to that of wild-type cells by two different membrane protein preparation methods (see Materials and Methods). Previous studies have demonstrated that the pres-

ence or absence of membrane proteins can vary based on the method of preparation (51, 55, 56). The data shown in Fig. 6A revealed that the membrane protein profiles of *nla18* mutant cells and wild-type cells were different. Many bands appeared to be underrepresented or missing in the *nla18* lanes. Several other protein bands are overrepresented in the *nla18* lanes, suggesting a complex role for the Nla18 activator in the regulation of these proteins.

Previous work with *Vibrio cholerae* suggests that defects in ppGpp accumulation may lead to changes in membrane protein profiles (22). Therefore, it is possible that the ppGpp accumulation defect in *nla18* mutant cells is responsible for the altered membrane protein profile. To explore this idea, we compared the membrane profiles of isogenic wild-type, *nla18* mutant, and Δ*relA1* mutant strains (Fig. 6B). Under these conditions, the relative levels of most membrane proteins in Δ*relA1* mutant cells were similar to those in wild-type cells. In contrast, the membrane protein profile of *nla18* mutant cells was quite different from that of wild-type cells. Furthermore, band c (Mx_2195), which is differentially expressed in Δ*relA1* mutant cells, is absent in *nla18* mutant cells (Fig. 6B). These data imply that the altered membrane protein profile of *nla18* mutant cells is not simply due to a decrease in ppGpp levels.

To further examine the differences in membrane protein profiles and to confirm the results of the DNA microarray analysis, we attempted to identify three proteins missing in *nla18* mutant cells but present in both wild-type and *relA* cells. Three wild-type protein bands were excised and digested with trypsin, and the peptide fragments were subjected to matrix-assisted laser desorption ionization–time of flight mass spectrometry. Three proteins were identified: the OmpA-related protein Oar (Mx_4187), a hypothetical protein (Mx_2915), and the putative membrane protein Mx_4332 (Fig. 6A and B, bands a, b, and c, respectively). These results are consistent with the DNA microarray analysis showing that expression of the mx_4187 (Oar), mx_2915, and mx_4332 genes is reduced between 2.2-fold and 5.8-fold (Fig. 5), which supports the hypothesis that *nla18* mutant cells have a general defect in the expression of genes that code for membrane and membrane-associated proteins.

DISCUSSION

M. xanthus genomic analyses have identified 52 NtrC-like activators (27), proteins required for transcription from σ^{54}-dependent promoters (49, 70). Because RpoN (σ^{54}) has previously been shown to be essential in *M. xanthus*, it has been suggested that one or more of these NtrC-like activators are likely to be essential (31). While mutational analyses have uncovered 15 NtrC-like activators that are required for normal development, these studies failed to identify an *M. xanthus* activator protein that is absolutely required for vegetative growth (2, 15, 18, 20, 26, 27, 36, 67, 69). Interestingly, mutations in two (*nla4* and *nla18*) of these 15 activator genes cause severe vegetative phenotypes, as well as developmental defects. Based on these phenotypes, we propose that inactivation of both *nla4* and *nla18* may be lethal like null mutations in *rpoN*.

In this paper, we establish connections among the key NtrC-like activator Nla18, the starvation response, and vegetative gene expression. When starved for nutrients, *nla18* mutant cells accumulate about sixfold less ppGpp than their wild-type counterparts. This result suggests that inactivation of *nla18* affects the earliest stages of fruiting body development, when *M. xanthus* cells are assessing the status of available nutrients.

Our results demonstrate that *nla18* and *relA* strains have several phenotypes in common: they are severely impaired for (p)ppGpp accumulation, and they are defective for vegetative growth and development (23, 46, 47, 63). Like *relA* mutations (5, 23), mutations in *nla18* affect the normal function of *M. xanthus* cell-cell signaling systems. Cells carrying an inactivated copy of *nla18* fail to produce normal levels of A-signal, a cell density signal that is required early in fruiting body development (39, 40, 41, 57). In addition, *nla18* mutant cells produce little or no FruA, a response regulator that is essential for the C-signal response pathway (10, 64). Since the C-signaling system guides aggregation and sporulation, the lack of FruA in *nla18* mutant cells is likely to have dire consequences for the later stages of development. This idea is consistent with the finding that inactivation of *nla18* affects aggregation and sporulation (2).

The fact that the vegetative and developmental phenotypes that we observed for *nla18* mutant cells were similar to *relA* cells suggested to us that Nla18 may be directly or indirectly involved in the ppGpp starvation response. The *nla18* mutation could affect ppGpp levels by altering the expression patterns of genes coding for regulators of ppGpp synthesis/turnover such as *relA*, *socE*, mx_1594 (putative SpoT hydrolase domain-containing gene), and *csgA* (5, 23). When we examined the levels of *relA*, mx_1594, and *socE* mRNAs in *nla18* mutant and wild-type cells, we found no significant differences during vegetative growth or development. These results rule out the simple hypothesis that during vegetative growth, Nla18 is working through any of these known or predicted regulators of ppGpp accumulation. It is a formal possibility that during development, Nla18 is working through CsgA; there is sufficient signal for extracellular complementation (2); however, it is not known what level of CsgA is necessary to maintain a stringent response. All of our data, taken as a whole, suggest that the effect the *nla18* mutation has on the stringent response is probably quite complex and that indirect effects play a significant role (see below).

How might the *nla18* mutation affect ppGpp accumulation? The results of our DNA microarray analysis of vegetative gene expression patterns indicate that inactivation of *nla18* affects the expression of several genes whose products are likely to be required for protein synthesis (e.g., EF-Tu, EF-G, and RluD). In *nla18* mutant cells, the expression of some of these genes increases while the expression of others decreases relative to that in the wild type (Fig. 5; Tables S1 and S2 in the supplemental material), suggesting that *nla18* mutant cells fail to properly regulate genes that encode important components of the *M. xanthus* translation machinery. It is possible that the differential expression patterns observed for *nla18* mutant and wild-type cells are due to growth rate effects; the generation time of the *nla18* mutant is more than twice the generation time of a wild-type strain. However, the fact that *nla18* mutant cells overexpress some translation-associated genes and underexpress others argues that the altered expression patterns in *nla18* mutant cells are not simply due to growth rate effects.

Therefore, we propose that Nla18 either directly or indirectly modulates the expression of the *M. xanthus* translational machinery itself. In the absence of Nla18, the ribosome-associated RelA protein is unable to properly monitor the translation state of cells and adjust ppGpp levels accordingly. Examining the distribution of the various populations of ribosomes, polysomes, and their subunits in the *nla18* mutant cells and wild-type cells will allow us to directly test this hypothesis.

In addition to genes required for protein synthesis, *nla18* inactivation strongly affects the vegetative expression of a large set of genes for putative membrane and membrane-associated proteins. These results were corroborated by our analysis of the membrane protein profile of *nla18* mutant cells; the membrane protein profile of *nla18* mutant cells is quite different from that of wild-type cells. Work on the OmpA-related protein Oar supports our findings (21, 48). Strains carrying deletions in *oar* and *mlpA*, genes whose expression is affected by *nla18* inactivation, show a developmental delay similar to that of the *nla18* mutant (21, 48). Furthermore, the *oar mlpA* double mutant is defective for sporulation, although its sporulation defect is not as severe as that of the *nla18* mutant.

Because the membrane protein profile of *relA* cells is similar to that of wild-type cells, it seems unlikely that the altered membrane protein profile of *nla18* mutant cells is simply due to a decrease in ppGpp levels. However, it has been shown in *E. coli* that *relA* mutations can affect phospholipid and fatty acid production (9, 52, 53, 65). It is therefore possible that the *M. xanthus relA* mutant has a compromised membrane that was not detectable in our protein analysis. This caveat aside, our data strongly suggest that the *nla18* mutant's vegetative and developmental phenotypes are due to at least two defects: (i) decreased levels of ppGpp, a defect that may be caused by the misexpression of components of the translational machinery, and (ii) the altered expression patterns of genes that code for membrane and membrane-associated proteins.

These two defects may be linked; the misexpression of genes such as those encoding transporters could have significant effects on the *nla18* mutant cell's ability to acquire nutrients from the environment, causing unbalanced growth and defects in ppGpp synthesis. However, the fact that *nla18* mutant cells grow on minimal A1 medium indicates that these defects are not simply due to auxotrophy (2). There is precedence for the regulation of important membrane and/or membrane-associated proteins by the σ^{54} system. In *Borrelia* and *Pseudomonas*, it has been reported that several genes for membrane and membrane-associated proteins are directly controlled by RpoN (25, 71). Our next challenges are to determine which genes uncovered in these studies are directly regulated by Nla18 and to elucidate the signal transduction networks that modulate Nla18 activity.

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