

The *Myxococcus xanthus* Nla4 Protein Is Important for Expression of Stringent Response-Associated Genes, ppGpp Accumulation, and Fruiting Body Development^{∇†}

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Changes in gene expression are important for the landmark morphological events that occur during *Myxococcus xanthus* fruiting body development. Enhancer binding proteins (EBPs), which are transcriptional activators, play prominent roles in the coordinated expression of developmental genes. A mutation in the EBP gene *nla4* affects the timing of fruiting body formation, the morphology of mature fruiting bodies, and the efficiency of sporulation. In this study, we showed that the *nla4* mutant accumulates relatively low levels of the stringent nucleotide ppGpp. We also found that the *nla4* mutant is defective for early developmental events and for vegetative growth, phenotypes that are consistent with a deficiency in ppGpp accumulation. Further studies revealed that *nla4* cells produce relatively low levels of GTP, a precursor of RelA-dependent synthesis of (p)ppGpp. In addition, the normal expression patterns of all stringent response-associated genes tested, including the *M. xanthus* ppGpp synthetase gene *relA*, are altered in *nla4* mutant cells. These findings indicate that Nla4 is part of regulatory pathway that is important for mounting a stringent response and for initiating fruiting body development.

In soil, vegetative swarms of *Myxococcus xanthus* feed on prey bacteria to obtain amino acids, which are used as a source of carbon, nitrogen, and energy (2, 10). When deprived of amino acids, large groups of *M. xanthus* cells migrate to aggregation centers and begin building multicellular fruiting bodies. Once a fruiting body is formed, rod-shaped cells within this structure differentiate into spherical, stress-resistant spores (for reviews, see references 11 and 62).

Amino acid starvation in *M. xanthus* cells leads to accumulation of the intracellular starvation signal (p)ppGpp and induction of an adaptive response known as the stringent response (52, 53, 63). In *Escherichia coli*, the ribosome-associated RelA protein catalyzes production of (p)ppGpp molecules that are capable of altering the promoter specificity of RNA polymerase (for reviews, see references 4 and 5). A mutation in the *M. xanthus relA* gene blocks (p)ppGpp synthesis, and cells carrying this mutation fail to initiate early developmental events (24). Furthermore, Singer and Kaiser (63)

showed that ectopic expression of the *E. coli relA* gene causes *M. xanthus* cells to initiate early developmental gene expression in the absence of starvation. These findings indicate that (p)ppGpp accumulation is necessary and sufficient to initiate the *M. xanthus* developmental cycle.

Recent studies suggest that SocE inhibits (p)ppGpp production when nutrients are plentiful and that CsgA is required for maintaining relatively high levels of (p)ppGpp under starvation conditions (6, 7). Thus, it appears that there are both positive and negative input signals that modulate RelA-mediated synthesis of (p)ppGpp in *M. xanthus*.

After (p)ppGpp levels rise and the developmental process begins, *M. xanthus* cells produce a series of cell-cell developmental signals (9, 22, 40, 45, 49). Of these cell-cell developmental signals, the two that have been studied the most extensively are A-signal and C-signal. A-signal is produced early in development, and it serves as an indicator of cell density (46, 47), allowing *M. xanthus* to determine whether a sufficient number of cells are present to build a multicellular fruiting body. In contrast, C-signal is a contact-stimulated cell-cell signal that guides the aggregation and sporulation stages of *M. xanthus* development (30, 36–38, 50, 51). It is believed that *M. xanthus* uses these (p)ppGpp-dependent cell-cell signals to coordinate the large-scale changes in gene expression that occur during fruiting body development.

Many early developmental genes have σ^{54} -like promoters (14, 15, 19, 20, 34, 41, 59). Transcription at σ^{54} promoters requires σ^{54} -RNA polymerase and an enhancer binding protein (EBP). EBPs are often components in signal transduction pathways, and upon activation, EBPs help σ^{54} -RNA polymerase form a transcription-competent open promoter complex (for reviews, see references 54, 65, and 73). In the last 10 years,

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17 EBPs that are required for fruiting body development to proceed normally have been uncovered (3, 18, 21, 23, 28, 29, 32, 39, 66, 71), indicating that the σ^{54} system plays an important role in expression of *M. xanthus* developmental genes. In addition to its role in activating developmental genes, the σ^{54} system regulates expression of *M. xanthus* genes that are important for growth (8, 18, 35).

Recently, Caberoy et al. (3) showed that a mutation in the EBP gene *nla4* affects fruiting body formation and sporulation. Here we show that an *nla4* mutant accumulates relatively low levels of the stringent nucleotide ppGpp. We also show that the *nla4* mutant is defective for early developmental events and for vegetative growth, phenotypes that are consistent with a deficiency in ppGpp accumulation. Further studies revealed that *nla4* cells produce relatively low levels of GTP, a (p)ppGpp precursor. In addition, we found that expression of *relA* and of other stringent response-associated genes is altered in *nla4* cells. These results indicate that Nla4 is part of the regulatory pathway that is important for mounting a stringent response.

MATERIALS AND METHODS

Bacterial strains and plasmids. The strains and plasmids used in this study are shown in Table 1. *M. xanthus* strain DK1622 (31) is wild type for growth, fruiting body development, sporulation, and motility. AG304 is a derivative of DK1622 (3) that carries the pNBC4 plasmid (which confers resistance to kanamycin) insertion in the EBP gene, *nla4*. To examine developmental gene expression in the presence of an *nla4* mutation, plasmid pNBC29 (which confers resistance to oxytetracycline) was introduced into *lacZ* transcriptional reporter strains DK4300 (*sdeK::* Ω 4408 Tn5*lacZ*) and DK4521 (*spi::* Ω 4521 Tn5*lacZ*), creating strains AG375 and AG376, respectively. DK101, which carries the *sglA1* (*pilQ*) mutation that allows dispersed growth in liquid media, forms fruiting bodies on agar surfaces but not in submerged cultures (27, 43, 69). Strain AG337 is a derivative of DK101 that carries the pNBC4 plasmid insertion in the *nla4* gene.

Media used for growth and development. *M. xanthus* strains were grown at 28 or at 32°C in CTT broth (1.0% Casitone, 10 mM Tris-HCl [pH 8.0], 1 mM KH_2PO_4 , 8 mM MgSO_4), in CTTYE broth (CTT containing 0.5% yeast extract), or on plates containing CTTYE broth and 1.5% agar. CTT broth, CTTYE broth, and CTTYE agar plates were supplemented with 40 μg of kanamycin sulfate/ml or 10 μg of oxytetracycline/ml as needed. CTT soft agar contained CTT broth and 0.7% agar.

Fruiting body development was carried out at 32°C on plates containing TPM buffer (10 mM Tris-HCl [pH 8.0], 1 KH_2PO_4 , 8 mM MgSO_4) and 1.5% agar. A-factor assays were performed in microtiter plates containing MC7 starvation buffer (10 mM morpholinepropanesulfonic acid [MOPS], 1 mM CaCl_2 ; final pH, 7.0).

E. coli strains were grown at 37°C in LB broth containing 1.0% tryptone, 0.5% yeast extract, and 0.5% NaCl or on plates containing LB broth and 1.5% agar. LB broth and LB agar 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 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 β -galactosidase assays or Western blot analysis as described below.

For real-time quantitative PCR (QPCR), cells were grown as described above, the cells were pelleted, the supernatants were removed, and the cells were resuspended in MC7 buffer to a density of 2×10^{10} cells/ml. The suspensions of concentrated cells were placed in a petri dish containing 28 ml of MC7 buffer and incubated at 32°C. Cells were harvested at various times during development and processed as described below.

β -Galactosidase assays. Cells were harvested at different times during development on TPM agar plates. The cells were resuspended in 400 μl of TPM buffer, quick-frozen in liquid nitrogen as described previously (14), and stored at -80°C . β -Galactosidase assays were performed as described by Kaplan et al.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Reference or source
<i>M. xanthus</i> strains		
AG304	DK1622 pNBC4:: <i>nla4</i>	3
AG306	DK1622 pNBC6:: <i>nla6</i>	3
AG337	DK101 pNBC4:: <i>nla4</i>	This study
AG375	pNBC29:: <i>nla4 sdeK::</i> Ω 4408 Tn5 <i>lacZ</i>	This study
AG376	pNBC29:: <i>nla4 spi::</i> Ω 4521 Tn5 <i>lacZ</i>	This study
DK101	<i>pilQ1</i> (wild-type growth and development)	27
DK476	<i>pilQ1 asgA476</i>	22
DK1622	Wild-type growth and development	31
DK4300	<i>sdeK::</i> Ω 4408 Tn5 <i>lacZ</i>	41
DK4323	<i>pilQ1 asgA476 spi::</i> Ω 4521 Tn5 <i>lacZ</i>	45
DK4521	<i>spi::</i> Ω 4521 Tn5 <i>lacZ</i>	41
DK5208	<i>csgA::</i> Tn5-132 Ω LS205	40
DK11063	<i>fruA::</i> Ω 7540 Tn5 <i>lacZ</i>	64
MS1000	DK101 Δ <i>relA1</i>	8
Plasmids		
pCR 2.1-TOPO	Kan ^r	Invitrogen
pSWU22	Tet ^r	S. S. Wu and D. Kaiser
pNBC4	624-bp internal fragment of <i>nla4</i> in pCR2.1-TOPO	3
pNBC29	624-bp internal fragment of <i>nla4</i> in pSWU22	3

(33). β -Galactosidase specific activities were measured in wild-type and *nla4* mutant cells containing developmentally regulated *lacZ* reporter gene fusions. β -Galactosidase specific activity was expressed in nanomoles of *o*-nitrophenol produced per minute per milligram of protein.

A-factor assays. MC7 buffer conditioned by wild-type strain DK101 (*nla4*⁺ *asgA*⁺), AG337 (*nla4 asgA*⁺), or DK476 (*nla4*⁺ *asgA*) cells served as the source of A-factor. A-factor was isolated as described by Diodati et al. (8). DK4323, a test strain that is defective for A-factor production and that carries the A-factor-dependent *spi::* Ω 4521 Tn5*lacZ* reporter fusion, was prepared as described previously (24, 57). Test cell aliquots (25 μl ; 1.25×10^8 cells) were added to the wells of 24-well microtiter plates containing 400 μl of conditioned MC7 buffer. The microtiter plates were incubated at 32°C, and the cells were harvested at various time intervals and assayed for β -galactosidase activity as described above. One unit of A-factor activity is defined as the amount required to stimulate test cells to produce 1 U of β -galactosidase activity.

Western blots. Approximately 1×10^9 *M. xanthus* cells were harvested from TPM agar and placed in a lysis buffer (Tris-sodium dodecyl sulfate [pH 7.2])-protease inhibitor cocktail (Sigma). The cell suspensions were vortexed for 30 s, an equal volume of loading buffer (60) was added to each suspension, and the cell mixtures were boiled for 10 min. Samples containing equal amounts of protein were separated by electrophoresis through a 12% polyacrylamide gel and transferred to an Immobilon P membrane (Millipore) using a semidry blotting apparatus (Bio-Rad). The blots were probed with anti-FruA antibody or anti-CsgA antibody, followed by incubation with peroxidase-conjugated goat anti-rabbit immunoglobulin G (Boehringer Mannheim). Blots were developed using the Renaissance chemiluminescence reagent (NEN Life Science Products) and Amersham autoradiography Hyperfilm-MP.

Analysis of nucleotide pools. ^{32}P -labeled nucleotides were isolated and separated by thin-layer chromatography as described previously (52, 63). Labeled nucleotides were visualized using a STORM phosphorimaging scanner, and the relative nucleotide levels were determined using Image Quant software (Molecular Dynamics). Strains DK1622 and AG304 yielded results similar to those obtained for DK101 and AG337, respectively.

Real-time QPCR. Total cellular RNA was isolated from 2×10^{10} quick-frozen cells using the hot phenol method (60) and used to generate cDNA as described by Lancero et al. (48). Briefly, cDNA was generated from 1.5 to 2.0 μg of an RNA sample using 1 μl of Superscript III reverse transcriptase (Invitrogen) and 250 ng of random hexamers (Amersham Biosciences). The subsequent 16- μl PCR mixtures contained 0.5- μl aliquots of the cDNA synthesis reaction mixtures, gene-specific forward and reverse primers (1 μM), and 7.5 μl of iQ SYBR green Supermix (Bio-Rad). Primers were designed to yield approximately 100-bp PCR products. The primers used for QPCR are listed in Table S1 in the supplemental material. QPCR was performed using the iCycler iQ system from Bio-Rad. The

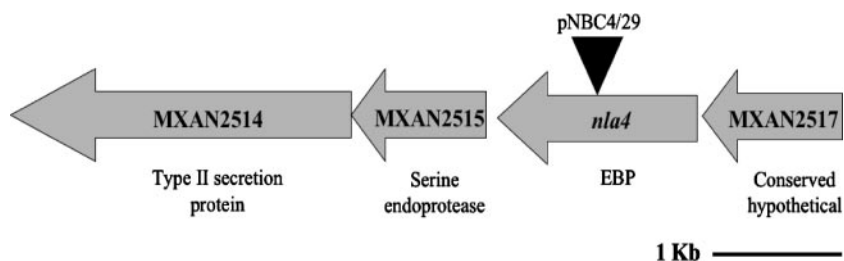


FIG. 1. Schematic diagram of the *nla4* locus. The arrows show the locations of the indicated genes and the predicted directions of gene transcription. The designations of genes in the *nla4* locus and the potential functions of the proteins that they encode were obtained from reference 17. Approximately 160 bp separates *nla4* and the downstream gene, MXAN_2515. The inverted triangle indicates the position of the pNBC4 and pNBC29 plasmid insertions in the *nla4* gene.

rate of PCR-generated DNA accumulation 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 reactions without reverse transcriptase were performed, and the synthesis reaction mixtures were analyzed using QPCR as described above for the test samples. The relative levels of *relA*, *socE*, and MXAN_1364 (Mx_1594) expression in wild-type cells and *nla4* mutant cells were obtained using the relative standard curve method (user bulletin no. 2, Applied Biosystems) or as described previously (48). Standard curves were generated using 16S rRNA primers and various concentrations of wild-type cDNA (10^{-7} to 10 ng). The expression level of *relA*, *socE*, or MXAN_1364 in each test sample was normalized to that of 16S rRNA and compared to the expression level in vegetatively growing wild-type cells (zero time).

RESULTS

Expression of MXAN_2515 (Mx_838) and MXAN_2514 (Mx_837) in *nla4* mutant cells. Previously, we showed that an insertion in *nla4* causes aggregation and sporulation defects (3). The DNA sequence of the *nla4* locus (17) places *nla4* in close proximity to the downstream MXAN_2515 gene (Fig. 1). MXAN_2515 and *nla4* are separated by approximately 160 bp, suggesting that these two genes are not part of the same operon and that the insertion in *nla4* is unlikely to have a polar effect on MXAN_2515 transcription. However, since an insertion in MXAN_2515 yielded developmental phenotypes similar to that of the *nla4* insertion (3), we wanted to confirm that the *nla4* insertion does not affect MXAN_2515 transcription. To do this, we monitored MXAN_2515 expression in *nla4* cells and wild-type cells during vegetative growth and development using QPCR analysis. We found that the levels of MXAN_2515 expression in wild-type cells and *nla4* mutant cells were similar (data not shown). Furthermore, we found that expression of MXAN_2514, the gene immediately downstream of MXAN_2515, was similar in wild-type cells and *nla4* cells (data not shown). These data indicate that the developmental defects of the *nla4* mutant are due to inactivation of the *nla4* gene.

Expression of early developmental genes. As described by Caberoy et al. (3), aggregation of the *nla4* mutant is delayed and incomplete, and this strain shows a dramatic drop in sporulation efficiency relative to wild-type cells. This strong aggregation defect suggests that the developmental process in the *nla4* mutant goes awry early. To determine whether the *nla4* mutation affects early developmental gene expression, we used Tn5lacZ transcriptional fusions to *sdeK* and *spi*, genes that are activated shortly after development is induced by ppGpp accumulation (41, 63). Expression of *sdeK*::Tn5lacZ

and *spi*::Tn5lacZ in wild-type and *nla4* mutant cells was monitored using β -galactosidase assays as described previously (33). The peak expression of *sdeK*::Tn5lacZ in *nla4* mutant cells was about 42% of the peak expression in wild-type cells (Fig. 2A), whereas the peak expression of *spi*::Tn5lacZ in *nla4* cells was about 26% of the peak expression in wild-type cells (Fig. 2B). These findings indicate that the *nla4* mutation alters gene expression early in development. Furthermore, these data show that the *nla4* mutation affects expression of the key early developmental gene *sdeK*; *sdeK* codes for a histidine kinase that is important for the aggregation and sporulation phases of development (14, 42, 58). Another early developmental gene that is known to be important for aggregation and sporulation is *fruA*. FruA is a response regulator that plays an important role in the C-signaling pathway (12, 55, 64). To determine whether the *nla4* mutation affects the levels of FruA in developing cells, wild-type cells and *nla4* 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. In contrast to wild-type cells, no FruA was detected in *nla4* cells at either the 18- or 24-h developmental time point (Fig. 3).

A-factor production. Early developmental genes, such as *spi* and *fruA*, are activated in response to A-factor (or A-signal) production. Given that the *nla4* mutant fails to express normal levels of *spi* and *FruA*, we examined whether *nla4* cells are defective for production of A-factor. The A-factor levels in the *nla4* mutant were compared to those in wild-type cells and the *asgA* mutant, a strain that produces almost no A-factor (44). The peak levels of A-factor produced by *nla4* mutant cells were about 14% of the peak levels produced by wild-type cells. The peak values, determined by measuring A-factor activity at various times during 24 h of development in submerged cultures, were 48.0 ± 5.9 U/ml for DK101 (*asgA*⁺ *nla4*⁺), 6.6 ± 0.6 U/ml for AG337 (*asgA*⁺ *nla4*), and 4.0 ± 0.6 U/ml for DK476 (*asgA* *nla4*⁺) (means \pm standard deviations from three independent experiments). However, it did not appear that A-factor production was abolished in the *nla4* mutant since the peak levels in this strain were 1.7-fold more than those in the *asgA* mutant. We concluded that the *nla4* mutant has a strong defect in A-factor production, which is consistent with the defects in A-signal-dependent gene expression (Fig. 2B and 3). These data support the idea that Nla4 is important for events that occur very early in development, prior to the onset of aggregation and sporulation.

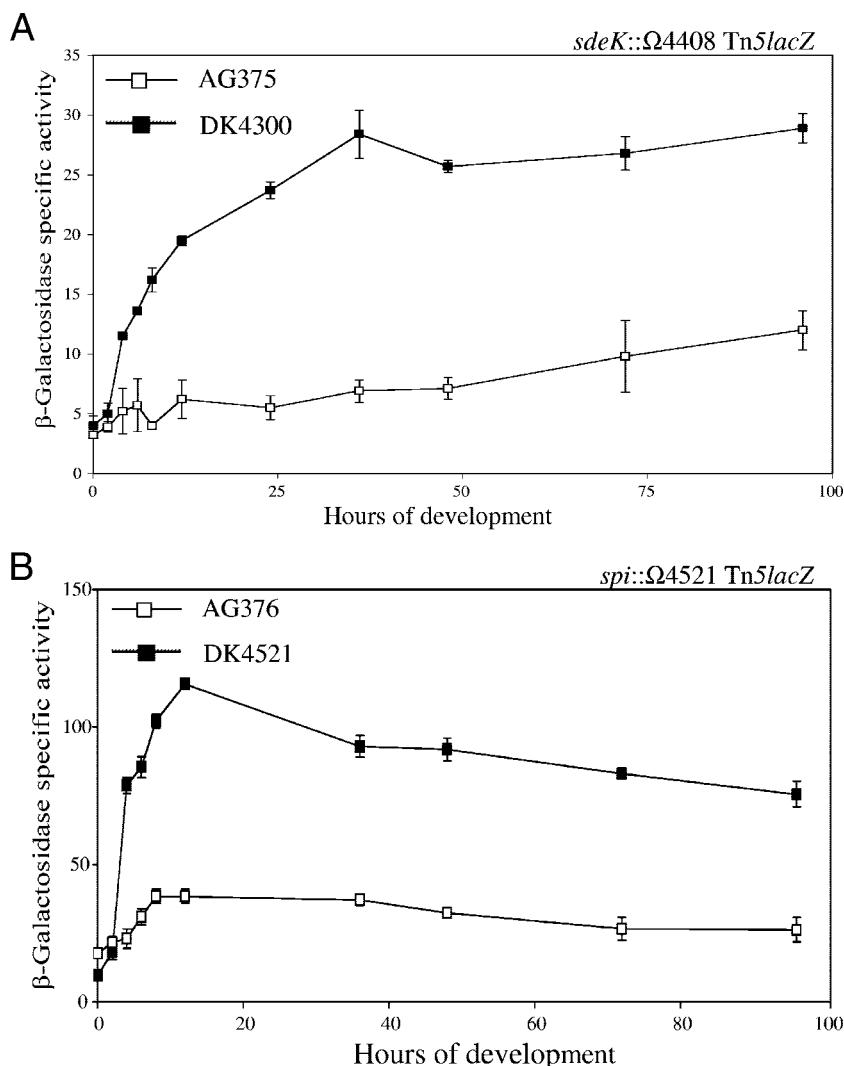


FIG. 2. Developmental expression of *sdeK* and *nla4* mutant cells. Expression of *sdeK* and *spi* was monitored at various times during development on TPM agar using the *sdeK::Ω4408 Tn5lacZ* and *spi::Ω4521 Tn5lacZ* reporter gene fusions, respectively. Mean β -galactosidase specific activities were determined from three replicates. The error bars indicate standard deviations of the means. (A) β -Galactosidase specific activity for strain DK4300 (*nla4*⁺ *sdeK::Ω4408 Tn5lacZ*) (■) and strain AG375 (*nla4* *sdeK::Ω4408 Tn5lacZ*) (□). (B) β -Galactosidase specific activities for strain DK4521 (*nla4*⁺ *spi::Ω4521 Tn5lacZ*) (■) and strain AG376 (*nla4* *spi::Ω4521 Tn5lacZ*) (□).

ppGpp and GTP accumulation. Accumulation of the intracellular starvation signal (p)ppGpp triggers A-signal production and early developmental gene expression (24, 63). Given that the *nla4* mutation affects these (p)ppGpp-dependent

events, we hypothesized that the *nla4* mutant may be defective for (p)ppGpp accumulation. Therefore, we examined ppGpp levels in the *nla4* mutant and wild-type cells. For these assays, wild-type cells and *nla4* cells were grown in nutrient broth and subjected to a nutrient downshift, and the relative amounts of ppGpp were measured as previously described (8, 52, 63). The results of these studies are shown in Fig. 4A. In wild-type cells, the level of ppGpp increased about sixfold after 15 min of starvation, which was followed by a decrease to a new steady-state level (after 60 min) that was about fourfold higher than the vegetative growth level (zero time). During vegetative growth, the level of ppGpp in *nla4* mutant cells was about 54% of the wild-type level. The peak ppGpp level in *nla4* mutant cells was detected at 60 min poststarvation, compared to 15 min poststarvation in wild-type cells. In addition, the peak poststarvation level of ppGpp in *nla4* cells was about 38% of that found in wild-type cells, and it did not increase significantly when the time of starvation was extended (data not

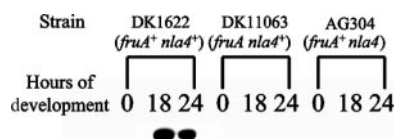


FIG. 3. FruA protein levels in wild-type cells and *nla4* mutant cells developing on TPM agar. Whole-cell lysates were prepared from strains DK1622 (*fruA*⁺ *nla4*⁺), DK11063 (*fruA* *nla4*⁺), and AG304 (*fruA*⁺ *nla4*) at the indicated times during development. Samples containing 30 μ g of total protein were resolved using sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and probed with anti-FruA antibody. The same total amount of protein was loaded into each lane. The experiment was repeated three times, and the results of a representative experiment are shown.

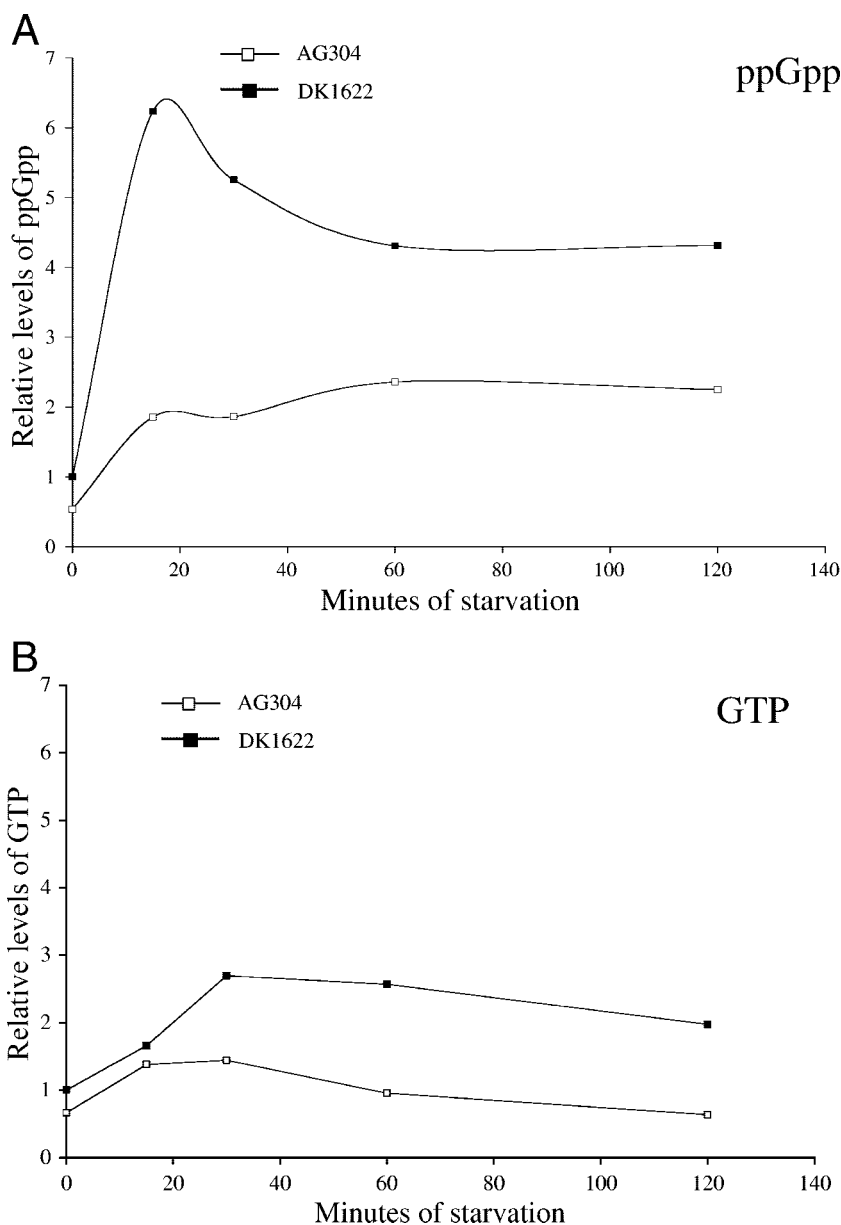


FIG. 4. Relative levels of ppGpp and GTP in wild-type and *nla4* mutant cells. Nucleotides were isolated from DK1622 (*nla4*⁺ *relA*⁺) and AG304 (*nla4* *relA*⁺) cells at different times following starvation and analyzed as described in Materials and Methods. The signal intensities were normalized to that of wild-type cells at zero time (vegetative growth). (A) Relative levels of ppGpp. (B) Relative levels of GTP. The assays were repeated four times, and the data for a representative sample are shown. The levels of ppGpp and GTP were similar when the *nla4* insertion was placed in a DK1622 or DK101 strain background (data not shown). Strain MS1000 served as the negative control for the nucleotide assays (data not shown).

shown). These results show that the level of ppGpp in *nla4* cells is relatively low during vegetative growth and starvation. The ppGpp accumulation defect in *nla4* cells is likely to be a major contributing factor in the downstream defects in early developmental gene expression and A-signal production.

Since GTP is an essential precursor for RelA-dependent synthesis of (p)ppGpp (4, 63), we tested whether the *nla4* mutant produces wild-type levels of GTP. The relative levels of GTP in *nla4* mutant and wild-type cells are shown in Fig. 4B. The level of GTP in vegetatively growing *nla4* cells was about 67% of that in wild-type cells. Under starvation conditions, the peak level of GTP in *nla4* cells was about 53% of the peak

wild-type level. These results indicate that *nla4* mutant cells are defective for production of GTP.

Expression of genes implicated in ppGpp accumulation. One way in which Nla4 might influence ppGpp levels is by directly or indirectly regulating expression of stringent response-associated genes, such as *relA*, which encodes the sole *M. xanthus* ppGpp synthetase (17, 24, 63). To determine whether the *nla4* mutation affects expression of *relA*, we monitored *relA* mRNA levels in wild-type and *nla4* cells during vegetative growth and development using QPCR. We found that the level of *relA* mRNA in *nla4* cells was about 16% of the level in the wild type during vegetative growth and that the

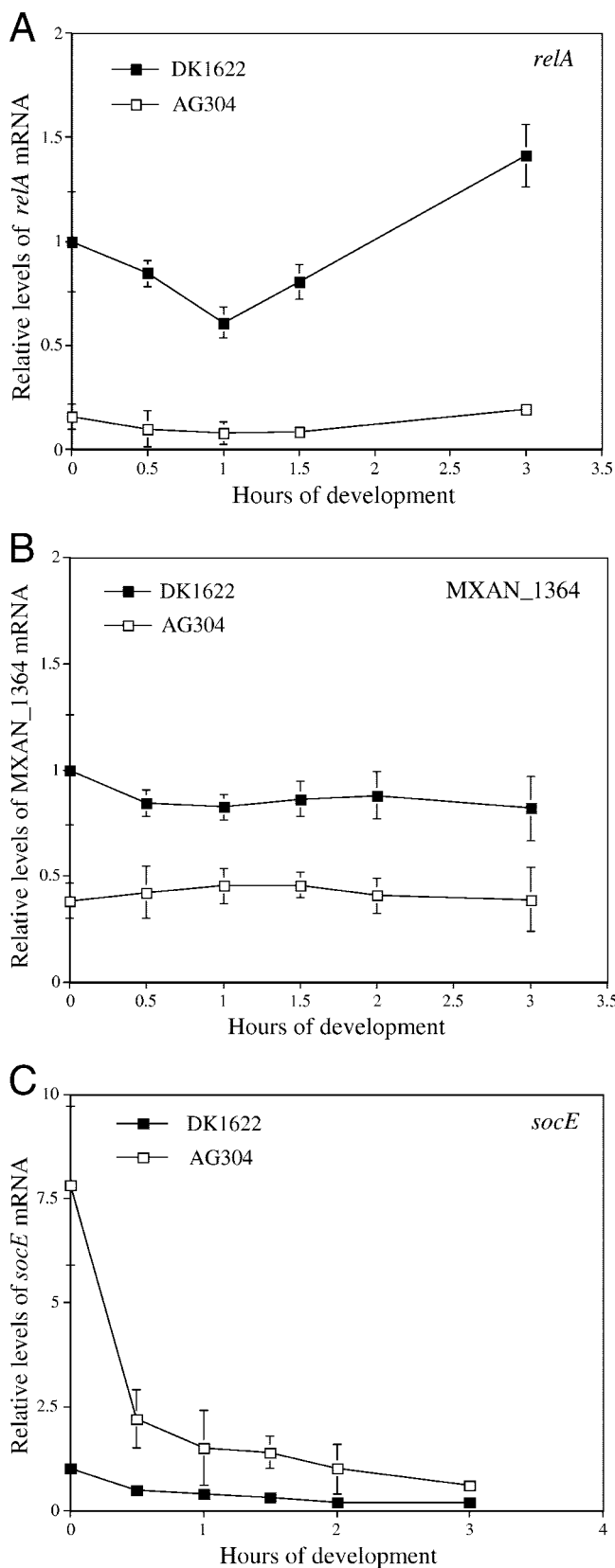


FIG. 5. Expression of *relA*, MXAN_1364, and *socE* in wild-type and *nla4* mutant cells. QPCR was used to examine developmental expression of *relA* (A), MXAN_1364 (B), and *socE* (C) in wild-type strain DK1622 (■) and *nla4* mutant strain AG304 (□). The expression

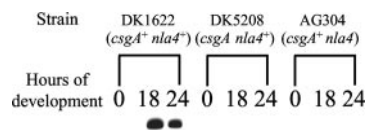


FIG. 6. CsgA protein levels in wild-type cells and *nla4* mutant cells developing on TPM agar. Whole-cell lysates were prepared from strains DK1622 (*csgA*⁺ *nla4*⁺), DK5208 (*csgA* *nla4*⁺), and AG304 (*csgA*⁺ *nla4*⁻) at the indicated times during development. Samples containing 30 μg of total protein were resolved using sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and probed with anti-CsgA antibody. The same total amount of protein was loaded into each lane. The experiment was repeated three times, and the results of a representative experiment are shown.

peak level in *nla4* cells during development was about 12% of the wild-type peak level (Fig. 5A). Since RelA is essential for synthesis of (p)ppGpp (24, 63), these results suggest that a major reason for the ppGpp accumulation defect in *nla4* cells is that *relA* expression is severely impaired.

CsgA is required for maintaining relatively high levels of (p)ppGpp during fruiting body development, and developmental expression of the *csgA* gene is known to be dependent on a wild-type copy of *relA* and, presumably, the induction of a normal stringent response (7). Given that *relA* expression is reduced about six- to eightfold in the *nla4* mutant, we speculated that developmental expression of *csgA* might be impaired or abolished in the *nla4* mutant. Therefore, we examined the levels of CsgA in wild-type and *nla4* mutant cells developing on TPM starvation agar. Cells were harvested after 18 h (when peak CsgA levels are observed [36]) and 24 h of development and were lysed, and whole-cell extracts were probed with anti-CsgA antibody. As shown in Fig. 6, no CsgA was detected in *nla4* cells at 18 or 24 h of development. Furthermore, no CsgA was detected when *nla4* mutant cells were given additional time to develop (data not shown).

There are two additional *M. xanthus* genes that have been implicated in the stringent response, *socE* and MXAN_1364 (Mx_1594). The *socE* gene codes for a negative regulator of (p)ppGpp accumulation (6, 7). Hence, *socE* expression is relatively high during vegetative growth and relatively low during development. The product of the MXAN_1364 gene has similarity to the N-terminal hydrolase domain of *E. coli* SpoT (8; K. A. O'Connor and D. R. Zusman, personal communication), a protein that modulates (p)ppGpp levels in response to certain starvation cues (16). Based on this finding, it has been proposed that MXAN_1364 codes for a protein involved in the stringent response (8). To determine whether the *nla4* mutation affects expression of MXAN_1364 and/or *socE*, QPCR was used to examine MXAN_1364 and *socE* mRNA levels in *nla4* and wild-type cells during vegetative growth and development (Fig. 5). The level of MXAN_1364 mRNA in *nla4* cells during vegetative growth was about 38% of the wild-type level,

of the *relA*, MXAN_1364, and *socE* genes was normalized to that of 16S rRNA. The indicated *relA*, MXAN_1364, and *socE* expression levels are relative to the levels found in vegetatively growing wild-type cells (zero time). The values are means derived from three replicates. The error bars indicate standard deviations of the means.

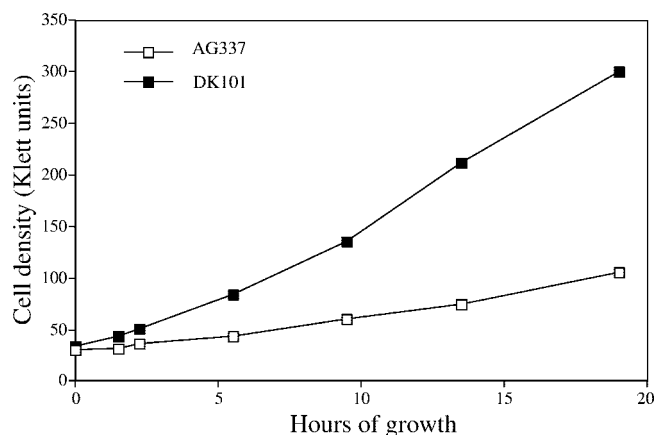


FIG. 7. Growth of wild-type and *nla4* mutant cells. Cells were grown at 28°C in CTTYE broth. Representative growth curves produced by wild-type cells and by *nla4* mutant cells are shown. In this experiment, the doubling time of wild-type cells was 5.1 h and the doubling time of *nla4* mutant cells was 10.3 h. Based on data from several growth experiments, the average doubling time of wild-type cells is 5.0 ± 0.2 h and the average doubling time of *nla4* mutant cells is 10.7 ± 0.6 h.

and the peak level in *nla4* cells during development was about 52% of the wild-type peak level (Fig. 5B). As reported previously (7), *socE* mRNA levels were relatively high when wild-type cells were growing vegetatively and they declined when wild-type cells initiated development (Fig. 5C). A similar trend in *socE* expression was observed in *nla4* cells (Fig. 5C). However, the *socE* mRNA levels in vegetatively growing *nla4* cells were about eightfold higher than those found in vegetatively growing wild-type cells. Thus, the *nla4* mutation affects expression of all stringent response-related genes that we tested in this study. The implications of these findings are addressed in the Discussion.

Vegetative growth. Low vegetative growth rates have been reported for *relA* and *nla18* mutants, both of which accumulate very low levels of ppGpp (8, 24). When grown in CTT broth at 32°C, which are standard laboratory conditions, *nla4* cells had a generation time of 12 to 16 h, whereas wild-type cells had a generation time of about 5 h. The vegetative growth defect of *nla4* mutant cells was less severe when the cells were grown at 28°C and the CTT broth was supplemented with yeast extract (CTTYE broth). Under these conditions, *nla4* mutant cells had a generation time of about 11 h, while wild-type cells had a generation time of about 5 h (Fig. 7). Thus, like other *M. xanthus* mutants that fail to mount a normal stringent response, the *nla4* mutant is defective for growth.

The *nla4* mutant (AG304) used in the vegetative growth studies was generated by pNBC4 plasmid insertion in the *nla4* gene (Table 1). Plasmid pNBC4 imparted kanamycin resistance to the *nla4* mutant, and we found that to maintain the *nla4* mutant phenotype, this strain had to be grown in nutrient broth containing kanamycin; *nla4* cells rapidly lost kanamycin resistance and their vegetative growth defect when they were placed in nutrient broth without kanamycin. To confirm that the slow-growth phenotype of the *nla4* mutant was not due to the presence of kanamycin in CTTYE broth, AG306 cells containing the pNBC6 plasmid insertion that conferred kana-

mycin resistance were grown in CTTYE broth supplemented with kanamycin and were shown to have typical wild-type doubling times, about 5 h (data not shown). Therefore, the slow-growth phenotype of *nla4* mutant cells resulted from inactivation of the *nla4* gene rather than the presence of the antibiotic.

DISCUSSION

Fifty-two genes encoding EBPs were identified in the *M. xanthus* genome sequence (17), and most of these transcriptional activators have been characterized by mutational analyses (3, 18, 21, 23, 28, 29, 32, 39, 66, 71). Among the characterized EBP mutants, *nla4* and *nla18* mutants are the only mutants that have severe defects in vegetative growth and fruiting body development (3, 8). This phenotype suggests that the Nla4 and Nla18 regulatory pathways play unique roles in the transition from vegetative growth to development.

In this report, we focused on the effects that *nla4* inactivation has on *M. xanthus* fruiting body development. Previous morphology studies suggested that fruiting body development goes awry relatively early in *nla4* cells (3), and here we found that the *nla4* mutation affects expression of early developmental genes. Two of these early developmental genes, *sdeK* and *fruA*, encode proteins that are important for downstream morphological events and for downstream changes in gene expression (12, 14, 42, 55, 58). In addition, we found that production of A-signal, a cell density signal that is required for expression of the vast majority of developmental genes that have been tested (45–47, 57), is almost completely abolished in *nla4* cells. Given that the *nla4* mutation has a strong effect on important early developmental events, perhaps it is not too surprising that *nla4* cells fail to fully express genes that are induced during aggregation and sporulation (data not shown) and to aggregate and sporulate normally.

Since the *M. xanthus* early developmental pathway is activated by the accumulation of the intracellular starvation signal (p)ppGpp, the defects in early developmental gene expression and A-signal production are likely to be linked to the strong negative effect that the *nla4* insertion has on ppGpp accumulation. We propose that the relatively low levels of ppGpp in *nla4* cells are primarily due to (i) the six- to eightfold reduction in expression of the (p)ppGpp synthetase gene, *relA*, and (ii) the relatively low levels of GTP, which, among other things, serves as a (p)ppGpp precursor (4). In addition to the stringent response, low GTP levels might affect other cellular processes that are important for fruiting body development.

It seems likely that low levels of ppGpp and GTP contribute significantly to the vegetative growth defect of the *nla4* mutant. There are precedents for vegetative growth defects in *M. xanthus* mutants that fail to mount a normal stringent response; vegetative growth defects have been previously reported for the *relA* and *nla18* mutants, both of which have ppGpp accumulation defects (8, 24). Furthermore, GTP is known to be important for a variety of growth-related functions (13, 56, 61). The finding that Nla4 is important for vegetative growth is consistent with previous data showing that *rpoN*, which codes for σ^{54} , is an essential *M. xanthus* gene (35).

The *nla4* mutant's defect in expression of stringent response-associated genes is not limited to *relA*; the *nla4* mutation alters expression of *csa4* and *socE*, genes whose protein products are

known to be positive and negative regulators of (p)ppGpp accumulation, respectively (6, 7). It has been proposed that *M. xanthus* balances the levels of the CsgA and SocE proteins in order to modulate the stringent response. In cells that are maintaining vegetative growth, ppGpp levels are low. Hence, expression of the negative regulatory gene *socE* is relatively high and expression of the positive regulatory gene *csgA* is relatively low. The reverse is true for *csgA* and *socE* expression during fruiting body development, when ppGpp levels are high.

In cells carrying an *nla4* mutation, the trend in *socE* expression is similar to that found in wild-type cells; *socE* expression is high during vegetative growth and decreases during development. However, the level of *socE* expression in vegetatively growing *nla4* cells is about eightfold higher than the level of *socE* expression in their wild-type counterparts, indicating that the presence of the Nla4 protein has a negative effect on *socE* expression in vegetative cells. Since EBPs function as transcriptional activators, it is highly likely that this negative effect on *socE* transcription is indirect. The fact that the *nla4* mutation abolishes CsgA expression in developing cells suggests that Nla4 is a positive regulator of *csgA* transcription during fruiting body development. It seems likely that the absence of detectable CsgA in *nla4* cells is due at least in part to the six- to eightfold reduction in *relA* expression; developmental expression of the *csgA* gene is known to be *relA* dependent (7, 24). The idea that Nla4 regulates expression of *csgA* indirectly is consistent with evidence suggesting that *csgA* uses a σ^{70} -type promoter (50).

Expression of MXAN_1364, a gene that is thought to play a role in the stringent response (8; O'Connor and Zusman, personal communication), is reduced in *nla4* cells. The product of MXAN_1364 was tagged as a (p)ppGpp hydrolase based on DNA sequence analysis. This suggests that the role of the MXAN_1364 protein in the stringent response might be (p)ppGpp turnover, an idea that has yet to be examined experimentally. Studies in *E. coli* have shown that (p)ppGpp accumulation depends on the opposing synthetic and hydrolytic activities of the RelA and SpoT enzymes (16, 25, 26, 67, 68, 70, 72). Presumably, *M. xanthus* also accommodates the need for certain levels of (p)ppGpp by adjusting these activities accordingly. Since RelA and MXAN_1364 are predicted to have opposing activities, perhaps the relatively low levels of MXAN_1364 expression in *nla4* cells is an adjustment to the relatively low levels *relA* expression.

Is it possible that Nla4 directly regulates expression of *M. xanthus* stringent response genes? The *csgA* gene does not appear to use a σ^{54} -type promoter (50), indicating that *csgA* expression is indirectly regulated by Nla4. Given that EBPs are positive regulators of gene transcription and that Nla4 has a negative effect on *socE* expression, we believe that Nla4 is an indirect regulator of *socE* transcription as well.

Using total RNA isolated from vegetative cells, the primer extension studies of Harris et al. (24) uncovered a putative σ^{70} -like promoter for the *relA* gene. This σ^{70} -like promoter is located within the coding sequence of an upstream gene (MXAN_3203) that appears to be transcribed in the opposite orientation with respect to *relA*. When we scanned the MXAN_3203 gene for additional promoters using the PromScan bioinformatics tool (<http://molbiol-tools.ca/promscan/>)

	-24	-12
σ^{54} consensus	TGGYRYR N4	TTGCA/T
MXAN1364	AGGCACG N4	GTGCA
<i>relA</i>	<u>TGGTCT</u> N4	<u>CTGCT</u>

FIG. 8. Alignment of putative σ^{54} promoters found upstream of the *relA* and MXAN_1364 genes. Putative σ^{54} promoters for *relA* and MXAN_1364 were identified using the PromScan bioinformatics tool (<http://molbiol-tools.ca/promscan/>) and the *M. xanthus* genome sequence (17). Nucleotides in the putative *relA* and MXAN_1364 promoters that match those found in the σ^{54} promoter consensus sequence are in bold type and are underlined. Y = C or T; R = A or G.

(1), a putative σ^{54} -like promoter was found upstream of the σ^{70} -like promoter identified by Harris et al. (24). As shown in Fig. 8, this putative σ^{54} promoter has the highly conserved GG dinucleotide in the -24 region and the highly conserved GC dinucleotide in the -12 region. Furthermore, five of seven nucleotides in the -24 region and four of five nucleotides in the -12 region of this promoter are identical to nucleotides found in the σ^{54} consensus sequence. These findings suggest that *relA* might use two different promoters, a σ^{70} -like promoter and a σ^{54} -like promoter. These findings also imply that Nla4 might directly regulate *relA* transcription, an idea that warrants further investigation.

The promoter of MXAN_1364 has not been analyzed experimentally. However, when PromScan was used to examine the region upstream of MXAN_1364, a putative σ^{54} -type promoter was identified (Fig. 8). We found the highly conserved GG dinucleotide in the -24 region of the putative MXAN_1364 promoter and the highly conserved GC dinucleotide in the -12 region of this putative promoter. The putative MXAN_1364 promoter shows a great deal of similarity to the σ^{54} consensus sequence, with six of seven nucleotides identical in the -24 region and four of five nucleotides identical in the -12 region. These findings suggest that Nla4 might directly regulate transcription of MXAN_1364. Additional work is needed to test this proposal and to identify the components in the signal transduction network that modulates the activity of Nla4.

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