

DNA Replication during Aggregation Phase Is Essential for *Myxococcus xanthus* Development

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Previous studies have demonstrated that fruiting body-derived *Myxococcus xanthus* myxospores contain two fully replicated copies of its genome, implying developmental control of chromosome replication and septation. In this study, we employ DNA replication inhibitors to determine if chromosome replication is essential to development and the exact time frame in which chromosome replication occurs within the developmental cycle. Our results show that DNA replication during the aggregation phase is essential for developmental progression, implying the existence of a checkpoint that monitors chromosome integrity at the end of the aggregation phase.

Myxococcus xanthus is a social gram-negative soil bacterium that when challenged with nutrient limitation enters a complex, multicellular developmental process that culminates in the formation of metabolically quiescent myxospores encased in a macroscopic fruiting body. Development is initiated by starvation for nitrogen, carbon, or phosphate and begins with a fairly homogenous distribution of cells. As development progresses, cells begin to aggregate and form dynamic aggregation centers. Eventually, these aggregation centers stabilize and recruit additional cells to become mounds, which darken into mature dome-shaped fruiting bodies containing environmentally resistant myxospores (Fig. 1).

As expected from such a highly complex process, *M. xanthus* cells have evolved a complex regulatory network which controls motility, behavior, and temporal gene expression. Of particular interest is the coordination of chromosome replication and the cell cycle in relation to development. We have recently reported that development in *M. xanthus* specifically produces myxospores, which each have two fully replicated copies of its single 9.1-Mbp chromosome (23, 24). From these observations, it is apparent that the possession of two chromosomes is the favored state for chromosome copy number in the myxospore. However, is this state necessary or essential for the process of development? This question is relevant because unlike sporulation in other developmental prokaryotes, such as *Bacillus subtilis*, which requires a cell division event to produce a single spore (13), each *M. xanthus* cell has the capacity to differentiate into a myxospore without the necessity of a cell division event (4).

In this study, we sought to use known chemical inhibitors of DNA replication to observe their effects on the developmental process in *M. xanthus*. The goal of this study was not only to determine if concurrent DNA replication is essential for progression through the developmental program, but to determine

the timing of this event in relation to the *M. xanthus* developmental program.

MATERIALS AND METHODS

Bacterial growth and media. *M. xanthus* was grown at 33°C in CTTYE liquid medium or on CTTYE containing 1.5% agar (1). Nalidixic acid, hydroxyurea, or novobiocin was added as described below. Nalidixic acid and novobiocin were used, each at a concentration of 20 µg/ml (10) (and 40 µg/ml for genetic selections), while hydroxyurea was used at a concentration of 20 mM (20).

Development and sporulation. Development was performed either with a submerged liquid culture buffer system (12) or on TPM agar plates (10 mM Tris [pH 7.6], 8 mM MgSO₄, and 1 mM KH₂PO₄ containing 1.5% agar), as described below. Cells were allowed to develop in a humidity chamber at 33°C. Nalidixic acid was added when indicated at a concentration of 20 µg/ml. Quantification of heat and sonication-resistant spore production was done as previously described (11).

Isolation of spontaneous nalidixic acid-resistant mutants. Wild-type *M. xanthus* DK1622 was plated on CTTYE agar plates containing 40-µg/ml nalidixic acid; this higher concentration of nalidixic acid was essential for reducing the background when selecting for nalidixic acid-resistant mutants. Nalidixic acid-resistant candidates were then tested for growth in both CTTYE liquid medium and CTTYE agar containing 20 µg of nalidixic acid/ml. Purified candidates were then subjected to DNA sequencing of the *gyrA* locus (University of California—Davis DNA Sequencing Facility) to identify the lesion. Only those mutants that contained a lesion at the *gyrA* locus were used for this study. Mx8-mediated transduction was used to transfer the nalidixic acid resistance marker to a genetically clean DK1622 background as previously described (14).

Incorporation of radiolabeled nucleotide. Incorporation of ³H-labeled thymidine and ³H-labeled uridine was performed as follows. Cultures were grown in CTT (1) medium until mid-exponential phase (100 to 120 Klett units) and then split into two, with 20-µg/ml nalidixic acid added to the experimental culture. Samples were taken, pulsed for 3 min with either 2-µCi/ml of ³H-labeled thymidine ([6-³H]thymidine, 20 to 30 Ci/mmol; Amersham TRK61) or uridine ([5,6-³H]uridine, 47 Ci/mmol; Amersham TRK410), and precipitated with ice-cold 5% trichloroacetic acid. Precipitated counts were collected on a glass filter, washed three times with 3% trichloroacetic acid, and counted with a Beckman Coulter LS 6500 scintillation counter. For developmental incorporation of label, cells were allowed to develop in the submerged culture system described above for the times indicated in the text; then radiolabel was directly added to the surrounding buffer for the 3-min pulse. Cells were then harvested, and precipitated counts were quantified as described above.

RESULTS

DNA synthesis inhibitors block development at the early aggregation stage. The inhibitor of DNA synthesis nalidixic acid was chosen to address the question of whether replication

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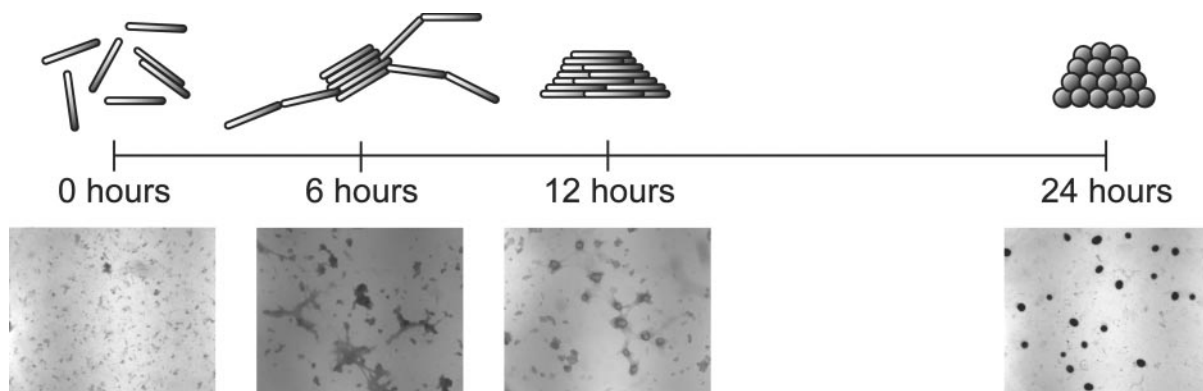


FIG. 1. Morphology of the *M. xanthus* developmental process. Shown are a pictorial representation of the major stages of development and a corresponding light micrograph of the morphology of wild-type *M. xanthus* DK1622 at each stage. Aggregation of the vegetative cells is apparent at 6 h and continues until 12 h when distinct, but still loose, mounds are formed. Fruiting bodies are dark, compact structures that begin to be apparent after 24 to 48 h.

was required for initiation of development. Nalidixic acid works by reversible inhibition of DNA gyrase subunits (5, 6, 22). Although nalidixic acid has been shown to affect RNA synthesis in *Escherichia coli* at higher concentrations (3, 21), previous studies with *M. xanthus* have established that 20 µg of nalidixic acid/ml specifically inhibits DNA synthesis without affecting protein synthesis or culture turbidity (10).

Nalidixic acid was added to wild-type *M. xanthus* DK1622 cells at the onset of development, using the submerged culture developmental system (12). As shown in Fig. 2, in the presence of inhibitor, development proceeded normally up to 6 h, cor-

responding to the early aggregation stage of development (Fig. 2A and B). Though cells began to aggregate by 6 h, similarly to what is observed in normal development, cells treated with nalidixic acid were unable to proceed past the aggregation stage and were unable to form spore-filled fruiting bodies by 48 h (Fig. 2F). Cells treated with nalidixic acid appeared to be totally incapable of forming mounds or developing into fruiting bodies, and studies done with two other inhibitors of DNA synthesis, hydroxyurea and novobiocin, showed similar results (data not shown). These observations demonstrate that inhibition of DNA synthesis severely blocks development, implying that DNA replication is essential in the normal developmental process of *M. xanthus*.

Effect of adding nalidixic acid at specific times in development. The previous set of experiments demonstrated that inhibition of DNA replication blocks development at the aggregation stage. The next question to be addressed was at what point does development become independent of chromosome replication? Taking advantage of the submerged culture system, we could address this question by adding inhibitor at specific times into the developmental process to observe whether the developmental process becomes blocked or continues. Determining when or if a bypass occurs allows us to infer the point at which DNA replication is no longer coupled to developmental progression.

As demonstrated in Fig. 3, when *M. xanthus* cells are developing in submerged culture, the developmental process becomes independent of DNA replication by 12 h. This corresponds morphologically to the transition between the aggregation phase and early mound formation (Fig. 1). This was apparent when observing two snapshots of development, at 12 h and at 48 h. At 12 h into development, both samples with nalidixic acid added previously at 0 h (Fig. 3E) and at 6 h (Fig. 3F) appeared almost identical to the sample where nalidixic acid had just been added at 12 h (Fig. 3G) and similar to the no-addition case (Fig. 3H). In all cases at 12 h into development, cells were arranged into streams leading toward aggregation centers. However, at later times, such as 48 h into development, the samples with nalidixic acid added at either 0 h (Fig. 3I) or 6 h (Fig. 3J) had not formed the dense, darkened fruiting body structures that are normally associated with development at this time (Fig. 2E). Darkened

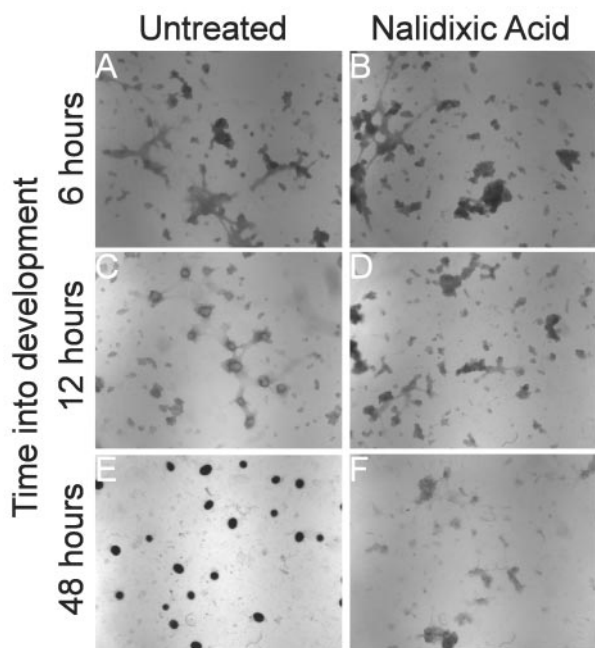
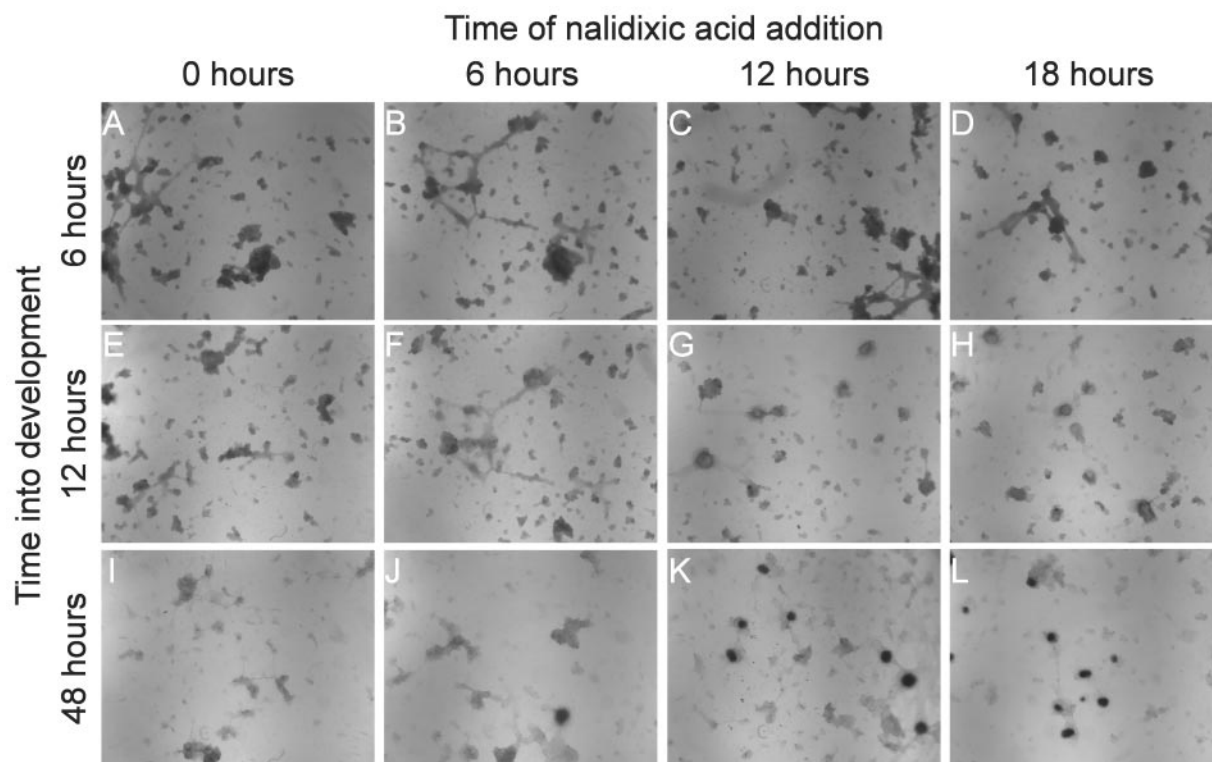


FIG. 2. Fruiting body morphology with addition of DNA synthesis inhibitors at the onset of development. Nalidixic acid was added to the surrounding buffer medium of developing *M. xanthus* wild-type DK1622 cells at the onset of development (0 h). Shown are light micrographs taken at 6, 12, and 48 h and corresponding to aggregation (A and B), mound formation (C and D), and fruiting body (E and F) stages of development, respectively.



Spore production

Time of addition	Total spore production	% sporulation compared to untreated
untreated	$1.98 \times 10^6 \pm 8.03 \times 10^5$	100%
0 hours	$1.58 \times 10^5 \pm 1.12 \times 10^5$	7.96%
6 hours	$1.27 \times 10^6 \pm 4.59 \times 10^5$	64.4%
12 hours	$2.16 \times 10^6 \pm 5.02 \times 10^5$	109%
18 hours	$2.04 \times 10^6 \pm 6.02 \times 10^5$	103%
24 hours	$2.19 \times 10^6 \pm 8.78 \times 10^5$	111%

FIG. 3. Fruiting body morphology with addition of nalidixic acid at specific times after the initiation of development. Shown are light micrographs of developmental morphology of wild-type DK1622 taken at 6, 12, and 48 h into development with 20- μ g/ml nalidixic acid added to the surrounding buffer medium at specific times (0, 6, 12, and 18 h). Viable spore production is shown in a table below. The total numbers of viable spores produced at the end of development were determined for each specific time of addition, were compared to untreated samples, and are shown as a percentage.

fruiting bodies were clearly seen in the samples with inhibitor added at 12 h (Fig. 3K) or 18 h (Fig. 3L), and these fruiting bodies appeared visually to be identical those formed in the absence of nalidixic acid (Fig. 2E).

To determine if sporulation is altered by the addition of nalidixic acid, spore production was assayed. Myxospore production appeared to reach wild-type levels if nalidixic acid was added at 12 h or beyond, but when nalidixic acid was added any time previous to 12 h, a substantial reduction in spore production was observed (Fig. 3), correlating well with the visually observed morphological markers of development. It appears that DNA synthesis inhibitors only affect development when added before or during the aggregation phase. This implies that during this phase, cells actively replicate their DNA and that

the process of DNA replication is coupled to progression through development. These results also support our earlier observations: that development in the presence of DNA replication inhibitors appears morphologically normal during the aggregation stage and then can no longer proceed to the mound formation stage.

Mutants in *gyrA* are resistant to the developmental block imposed by nalidixic acid. If nalidixic acid specifically inhibits DNA replication and this inhibition directly leads to the developmental block, then we would predict that a mutant that is specifically resistant to the DNA replication block would be able to develop normally. Six spontaneously occurring nalidixic acid-resistant mutants were isolated from wild-type DK1622 cells and then sequenced for mutations in a known target of

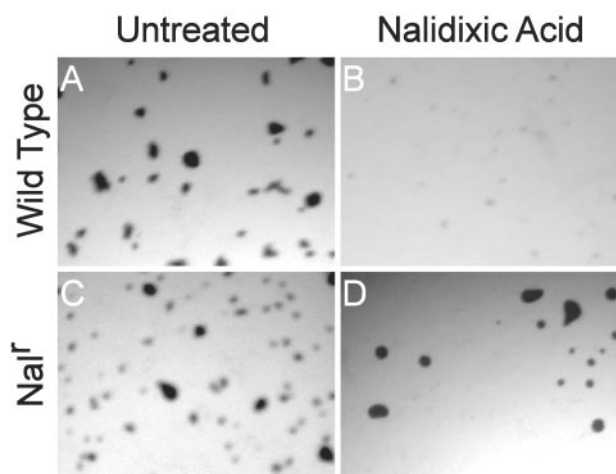


FIG. 6. Fruiting body morphology of nalidixic acid-resistant mutant MS1701 compared to the wild type. Shown are light micrographs of the developmental morphology of wild-type DK1622 and the mutant nalidixic acid-resistant strain MS1701 after 5 days of development when fruiting bodies have fully formed. The untreated samples have no inhibitor added to the surrounding medium, while the nalidixic acid-treated samples are exposed to inhibitor at a concentration of 20 $\mu\text{g}/\text{ml}$ from the onset of development.

in timing of the incorporation peak, it was clear that radiolabel incorporation was highest during the early stages of development, decreasing prior to 12 h into development with little or no incorporation of label into macromolecules after this time. The time period of this decrease occurred just prior to the morphological formation of mounds.

DISCUSSION

In this paper, we demonstrate that active DNA replication is necessary for the completion of the developmental pathway, fruiting body formation, and sporulation in the social soil bacterium *M. xanthus*. In these studies, we observed that the addition of three DNA replication inhibitors all caused developmental arrests corresponding to the time of aggregation (between 6 and 12 h postinduction under submerged culture conditions) and that addition of the same DNA replication inhibitors past the aggregation stage had no effect on further developmental progression. Our data are supported by radiolabeling studies, which demonstrated that incorporation of radiolabeled thymidine no longer occurs in DNA past the aggregation phase. These data implicate the aggregation phase as a key stage in developmental regulation of chromosome replication. Previously, it was shown that in *M. xanthus* undergoing glycerol spore formation, the addition of nalidixic acid does not halt sporulation but merely leads to DNA-incomplete spores (10). Our findings further support the hypothesis that chemical induction of sporulation and starvation-induced developmental formation of myxospores proceed by two very different mechanisms. It was previously demonstrated that glycerol spores appear to be less tightly controlled in terms of DNA replication (24) and that the two types of spores differ in terms of gene expression (18). Our results serve to further highlight the complexity of regulation in the multicellular pathway of *M. xanthus* sporulation.

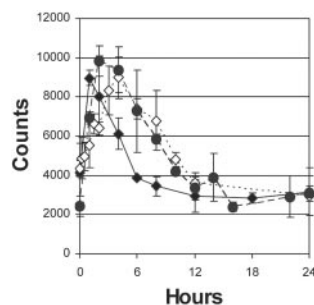


FIG. 7. Incorporation of radiolabeled thymidine during development. Shown are the results of three independent experiments on the incorporation of ^3H -labeled thymidine by wild-type DK1622 during a developmental time course from 0 to 24 h. No DNA replication inhibitors were used. The x axis represents hours after the onset of development, and the y axis represents absolute levels of radiolabel incorporation.

Interestingly, when radiolabel incorporation of [^3H]thymidine into developing *M. xanthus* cells is observed, there appears to be a controlled increase in DNA synthesis early in development. This is an unexpected result because in previous studies of glycerol spores, it was shown that no new rounds of DNA replication are initiated during sporulation (18). While this may be an artifact of experimental procedures, it may in fact represent the first developmental checkpoint for chromosome replication in *M. xanthus*. Since DNA replication would take approximately 5 h in *M. xanthus* (25), cells that are to become myxospores need to initiate chromosome replication very early into development to allow for completion. Cells at this point would need to evaluate their chromosome replication status, initiating DNA replication when appropriate. Recent studies in the Kaiser laboratory using *M. xanthus* DNA microarrays have suggested that *dnaA* expression is activated three- to fourfold during the early stages of development (8), which correlates with this result. We have also proposed previously that chromosome replication during development may be directly controlled by the stringent response (24) which also occurs within this time frame, at the onset of development (19).

A possible second checkpoint in development is after completion of chromosome replication at the end of aggregation phase. This is based on the observation that DNA replication is essential for development to proceed past the aggregation phase. When DNA replication is inhibited past the aggregation phase, we observed a specific block at this stage of development. It perhaps is no coincidence that a number of regulatory pathways are active during the aggregation stage of development, including the C-signal (11) and the SdeK (17) pathways and the activation of the *devTRS* operon (23). A checkpoint mechanism monitoring DNA replication state could easily feed into one or more of these previously described regulatory pathways, halting cells at aggregation if cells have not fully replicated their DNA or if there are errors in replication. It has been shown with *B. subtilis* that DnaA regulates *sda* expression, preventing sporulation from occurring before the completion of DNA replication (2). Although *M. xanthus* does not appear to possess an Sda homologue, these observations would suggest that *M. xanthus*, like *B. subtilis*, has

a regulatory circuit that prevents progression of development until DNA replication functions are fully completed.

Previously, we have reported that the two populations formed at the end of development, myxospores and peripheral rods (15), have differing chromosomal copy numbers. Myxospores appear to have two copies of the chromosome, while peripheral rod cells have only one copy (24). Given our current observation that there is a general increase in DNA replication during the aggregation phase of development, how are these two distinct states established? It would be interesting to propose a model where stringent response-induced activation of *dnaA* expression and initiation of chromosome replication are events that occur universally across the entire developing population. At the end of aggregation phase, the integrity of chromosome replication is checked. A majority of cells may have incomplete replication or errors due to lack of energy, and these cells will abort development, either simply arresting the developmental process or undergoing autolysis. The remaining cells all contain two copies of the chromosome but contain differing levels of C signaling because of spatial distribution within the aggregating mass of cells. It has been shown previously that a key difference between peripheral rods and cells within the fruiting body (presumably fated to become myxospores) is in the level of C signaling. Peripheral rods have low levels of C signaling and low expression of C-signal-dependent genes, while cells within the fruiting body have a much higher level of expression of these genes (9). So with low levels of C signaling, we propose that cells will be induced to septate a reducing chromosomal copy number to one to become peripheral rods; however, with high levels of C signaling, cells will inhibit septation, retaining their two-chromosome state, and proceed into the later stages of development.

From our initial experiments, we proposed that the developmental block in the presence of nalidixic acid was caused specifically by the effect on DNA replication. This hypothesis is supported by the fact that a nalidixic acid-resistant mutation in *gyrA* is not only capable of synthesizing DNA normally but also able to grow and develop in the presence of nalidixic acid, bypassing the block in development at the aggregation stage and strongly suggesting that DNA gyrase is the primary target of the inhibitor.

All previous characterizations of chromosome replication during sporulation in *M. xanthus* have been based on studies of glycerol spores (10, 18, 26). The data presented here show that in terms of chromosome replication in *M. xanthus* development, there is a significant difference between what occurs in glycerol sporulation and what happens in fruiting body-associated sporulation, leading us to alter the basic assumptions that are currently held about locus or if chromosome replication is related to development in this organism.

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