Nutrient-regulated Proteolysis of MrpC Halts Expression of Genes Important for Commitment to Sporulation during *Myxococcus xanthus* Development

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ABSTRACT

Starved *Myxococcus xanthus* cells glide to aggregation centers and form fruiting bodies in which rod-shaped cells differentiate into ovoid spores. Commitment to development was investigated by adding nutrients at specific times after starvation and determining whether development halted or proceeded. At 24 h post-starvation, some rod-shaped cells were committed to subsequent shape change and to becoming sonication-resistant spores, but nutrients caused partial disaggregation of fruiting bodies. By 30 h post-starvation, 10-fold more cells were committed to becoming sonication-resistant spores, and compact fruiting bodies persisted after nutrient addition. During the critical period of commitment around 24-30 h post-starvation, the transcription factors MrpC and FruA cooperatively regulate genes important for sporulation. FruA responds to short-range C-signaling, which increases as cells form fruiting bodies. MrpC was found to be highly sensitive to nutrient-regulated proteolysis both before and during the critical period of commitment to sporulation. The rapid turnover of MrpC upon nutrient addition to developing cells halted expression of the *dev* operon, which is important for sporulation. Regulated proteolysis of MrpC appeared to involve ATP-independent metalloprotease activity and may provide a mechanism for monitoring whether starvation persists and halting commitment to sporulation if nutrients reappear.
INTRODUCTION

Myxococcus xanthus is a bacterium that undergoes multicellular development and provides an excellent model to study mechanisms of signaling, gene regulation, and cell fate determination (1). *M. xanthus* cells move in packs that feed on available prey bacteria and organic matter (2). When starved, the population undergoes development, including aggregation of cells into mounds that become fruiting bodies as some of the rod-shaped cells differentiate into dormant, ovoid spores capable of withstanding harsh environmental conditions (3). However, not all cells in the population become spores. Some cells persist outside of fruiting bodies as peripheral rods (4, 5), while the majority of the population undergoes cellular lysis (6-8). Hence, *M. xanthus* cells adopt one of three distinct fates during the developmental process. At some point post-starvation, a cell may “commit” to a particular fate irreversibly, adopting that fate even if nutrients reappear.

Early studies of developmental commitment to cellular lysis and spore formation were carried out with *M. xanthus* before work on the signaling and gene regulatory network began. Commitment to cellular lysis was shown to occur in 25% of the population by 36 h after being placed on starvation agar (8). Commitment to formation of optically refractile spores was also explored (9). Many of the rod-shaped cells harvested after 24-32 h on starvation agar appeared to be in an intermediate state, failing to form spores if placed directly into nutrient medium, but forming spores if incubated in magnesium phosphate buffer for 6 h prior to incubation in nutrient medium. By 36 h post-starvation, about 10% of the rod-shaped cells formed spores if placed directly into nutrient medium. These early studies established that commitment to cellular lysis and spore formation occur, but insight into the molecular mechanisms that might be involved
required a better understanding of the signaling and gene regulatory network that controls development.

A complex network of signals and gene expression regulates *M. xanthus* development (10) (Fig. S1). Expression of early developmental genes is triggered by the intracellular production of (p)ppGpp in response to starvation (11, 12). This is followed by production of the A-signal, which is a mixture of amino acids and peptides released by activity of extracellular proteases (13, 14). A-signaling has been proposed to play a quorum-sensing role that at a high enough cell density stimulates expression of certain genes and causes cells to begin building mounds (15, 16). Like A-signal, production of extracellular C-signal depends on starvation-linked (p)ppGpp accumulation inside cells and on Asg proteins, but C-signal is produced more slowly (17, 18), accumulates on the surface of cells (19, 20), and its effects are manifest slightly later as cells align during aggregation into mounds (21-24). C-signaling stimulates expression of many genes beginning at about 6 h post-starvation (24) and is required for cells to form compact mounds that mature into fruiting bodies as spores form inside (25). Abundant contacts between cells in nascent fruiting bodies have been proposed to facilitate C-signaling and a high level of C-signaling is necessary for expression of genes required for sporulation (26-30). Therefore, C-signaling may be involved in committing cells to spore formation. It may also be involved in commitment to cellular lysis since mutants unable to produce C-signal are defective for developmental lysis (31-33).

Recently, several C-signal-dependent genes have been shown to be under the combinatorial control of two transcription factors, MrpC and FruA, which bind cooperatively in promoter regions (34-37). Different arrangements of binding sites with different affinities influence the timing of promoter activity and its dependence on C-signaling. It was not surprising to find that...
FruA directly regulates C-signal-dependent genes since FruA was known to be similar to DNA-binding response regulators of two-component signal transduction systems and mutations in *fruA* interfered with the response to C-signaling (38-40). On the other hand, MrpC had not been implicated as a direct regulator of C-signal-dependent genes, though it was known to be similar to transcription factors of the cyclic AMP receptor protein family (41) and an N-terminally-truncated form of MrpC appeared to directly activate *fruA* transcription (42).

The discovery that MrpC directly regulates C-signal-dependent genes led to the hypothesis that the abundance of MrpC is responsive to nutrient conditions as development proceeds (35), because several mechanisms link MrpC activity to starvation (Fig. S1). This hypothesis predicts that nutrient addition to developing cells would diminish MrpC activity. Only if cells were both starving (to allow sufficient MrpC activity) and efficiently C-signaling within a nascent fruiting body (to allow sufficient FruA activity) would C-signal-dependent genes be expressed, committing the cell to form a spore. Consistent with possible involvement of MrpC, C-signal, and FruA in cell fate determination, all three proteins were found to be more abundant in aggregating cells than in non-aggregating cells of a starving population (33). Also, mutants that accumulate MrpC and FruA, or C-signal, earlier than normal during development, exhibit premature aggregation and sporulation (27, 43).

Here, we report that many *M. xanthus* cells commit to shape change and sonication resistance between 24 and 30 h post-starvation. During this critical period, MrpC is present and FruA accumulates, but if nutrients are added before or during this period, MrpC declines rapidly. The decline is more rapid than observed when chloramphenicol is added to inhibit protein synthesis, indicating that MrpC undergoes nutrient-regulated proteolysis (Fig. S1). The *mrpC* transcript declines less rapidly than MrpC protein, but transcriptional control also appears to be involved in
the response to nutrients. Importantly, the decline of MrpC halts expression of direct target genes important for sporulation. Experiments with cell extracts implicate ATP-independent metallloprotease activity in nutrient-regulated turnover of MrpC, which appears to provide a mechanism for sensing whether nutrients reappear before the critical period around 24-30 h post-starvation. If starvation persists, the MrpC level remains high, and together with a high level of activated FruA due to C-signaling between cells in close proximity in the nascent fruiting body, the two transcription factors coordinately regulate genes important for commitment to the spore fate.

**MATERIALS AND METHODS**

**Bacterial strains, plasmids, and primers.** Strains, plasmids, and primers used in this study are listed in Table S1. To generate pRR010 that was used in the RNA analysis, a 110-bp internal fragment of *mrpC* was amplified using primers mrpC qPCR F and mrpC qPCR R with chromosomal DNA from *M. xanthus* strain DK1622 as template. The fragment was cloned into pCR2.1-TOPO (Invitrogen) as described by the manufacturer. The presence of the correct fragment was verified by DNA sequencing.

**Growth and development.** *Escherichia coli* containing plasmids were grown at 37°C in Luria-Bertani (LB) medium supplemented with 50 μg/ml of kanamycin sulfate as required. *M. xanthus* strains were grown at 32°C in CTTYE liquid medium (1% Casitone, 0.2% yeast extract, 10 mM Tris-HCl [pH 8.0], 1 mM KH2PO4-K2HPO4, 8 mM MgSO4, [final pH 7.6]) with shaking or on CTT agar plates (1.5% agar, 1% Casitone, 10 mM Tris-HCl [pH 8.0], 1 mM KH2PO4, K2HPO4, 8 mM MgSO4, [final pH 7.6]) supplemented with 40 μg/ml of kanamycin sulfate as required. Fruiting body development was performed in submerged culture with MC7 (10 mM
morpholinepropanesulfonic acid [MOPS, pH 7.0], 1 mM CaCl₂) as the starvation buffer (44).

Briefly, log-phase cultures of *M. xanthus* growing in CTTYE liquid medium were collected by centrifugation and resuspended in MC7 at a concentration of 1000 Klett units. Either 247 μl of the cell suspension plus 1.65 ml MC7 was added to a 3.5 cm diameter well of a 6-well plastic plate or in the case of samples for RNA preparation, 1.5 ml of cell suspension plus 10.5 ml MC7 was added to an 8.5 cm diameter plastic Petri plate. Upon incubation at 32°C, cells adhere to the bottom of the plate. At the indicated times, the culture supernatant was replaced with fresh MC7 starvation buffer or CTTYE nutrient medium supplemented with inhibitors as required. For collection of samples, cells were scraped from the plate bottom using a sterile 1-ml plastic pipette tip and the entire contents were aspirated into a 15-ml centrifuge tube. Samples were mixed thoroughly by vortexing for 15 seconds followed by pipetting the mixture up and down 15 times, and this was done a total of 3 times. For observation of cellular shape change, the tubes were then left undisturbed for 5-10 min to allow cell aggregates to settle to the bottom of the tube. The aggregates were then removed for microscopic observation. For measurement of sonication resistance, 400 μl of the thoroughly-mixed sample was transferred to a microcentrifuge tube, sonicated using a Model 450 Sonifier (Branson) at output setting 2 for 10 s intervals 3 times with cooling on ice in between, and ovoid spores were counted microscopically using a Neubauer counting chamber. To quantify mature spores that are heat- and sonication-resistant and capable of germination, 400 μl of the thoroughly-mixed sample was heated at 50°C for 60 min before sonication as described above, then the sample was serially diluted 10-fold in MC7 buffer and 100 μl samples were spread onto CTT soft (1.0%) agar plates. Colonies were counted after incubation at 32°C for 5-7 days.
Microscopy. Low-resolution images of fruiting bodies were obtained with a Leica Wild M8 microscope equipped with an Olympus E-620 digital camera. High-resolution images of presumed nascent fruiting bodies to visualize cellular shape change were obtained with an Olympus BX51 microscope using a DIC filter and a 100X oil-immersion objective, and equipped with an Olympus DP30BW digital camera.

Total protein concentration and immunoblot analysis. Thoroughly-mixed developmental samples were prepared as described above and stored at -20°C in 400 μl aliquots. One aliquot was used to determine the total protein concentration by sonicating the sample as described above, centrifuging it at 10,000 x g for 1 min to sediment cell debris, and using the supernatant in a Bradford (45) assay (Bio-Rad Laboratories). A second aliquot was mixed with an equal volume of 2X sample buffer (0.125 M Tris-HCl [pH 6.8], 20% glycerol, 4% SDS, 0.2% Bromophenol blue, 0.2 M DTT) and boiled for 5 min. Samples (equal amounts of total protein; typically 1 μg) were subjected to SDS-PAGE and immunoblot as described (46). For detection of MrpC and MrpC2, anti-MrpC antibodies were used at a 1:10,000 dilution. Anti-FruA antibodies were used at a 1:2,000 dilution. Protein-antibody complexes were detected using HRP-conjugated goat anti-rabbit immunoglobulin G (Bio-Rad) at 1:5,000 dilution and chemiluminescence (Western Lightning; Perkin-Elmer) according to the manufacturers’ instructions. Signals were detected using a LAS 3000 imager (Fujifilm) with exposure times short enough to ensure the signals were not saturated. Signal intensities were quantified using Multi Gauge (Fujifilm) software. Protein half-lives were calculated assuming a first order kinetic degradation reaction as described (47). Signal intensities, after subtracting background, were normalized to the relevant intensity at t = 0, and the natural log of the resulting values were plotted versus min after nutrient, chloramphenicol, or protease inhibitor/sonication treatment.
The slope of a linear fit of the data was used to calculate the protein half-life. For MrpC, only the upper band corresponding to the full-length protein was quantified and used to calculate the half-life since lower bands could be MrpC2 and/or other proteolytic fragments of MrpC.

**RNA extraction and analysis.** Immediately prior to harvesting the culture, its supernatant was replaced with a mixture of 0.5 ml RNase stop solution (5% phenol pH < 7 in ethanol) (48) and 4.5 ml of MC7 buffer. Cells were scraped from the bottom of the plate as described above and the entire contents were aspirated into a 15-ml centrifuge tube, which was subjected to flash freezing in a dry ice-ethanol bath and stored at -80°C. After thawing and centrifugation at 8,700 x g for 10 min at 4°C, RNA was extracted using the hot-phenol method and digested with DNase I (Roche) as described (43). Total RNA (1 μg) was used to synthesize cDNA in a 20 μl reaction with Superscript III reverse transcriptase (Lifetech) and random primers (Promega) according to the manufacturers’ instructions. qRT-PCR was done in 20 μl reactions using SYBR green PCR master mix (Applied Biosystems), 2 μl of 10-fold diluted cDNA product, and primers (20 pmole each) in a Model 7300 Real Time PCR System (Applied Biosystems). qRT-PCR reactions were done in triplicate for each biological replicate. Control reactions were done with reverse transcriptase omitted at the cDNA synthesis step prior to the qPCR reaction. A standard curve for each set of qPCR reactions was generated using 10-fold serial dilutions of plasmid pRR010 or DK1622 chromosomal DNA, and gene expression was quantified using the relative standard curve method (user bulletin 2, Applied Biosystems). 16S rRNA was used as the internal standard. The primers used to quantify mrpC, dev, and 16S rRNA transcripts are listed in Table S1.

**Protein degradation assays.** For the experiments shown in Figures 7, S5, and S7, we modified a procedure described previously (17). At 18 or 24 h post-starvation in submerged
culture, the supernatant was replaced with fresh starvation buffer or nutrient medium as described above for 15 min at 32°C, then the supernatant was replaced with phosphate-buffered saline and samples were collected immediately and mixed thoroughly as described above.

Samples (400 µl) were transferred to microcentrifuge tubes and protease inhibitors were added as follows: Complete PIC (Roche) at the concentration recommended by the manufacturer, EDTA (5 mM), Pefabloc (0.4 mM), aprotinin (1.5 µM), leupeptin (20 µM), E-64 (28 µM), or pepstatin (40 µM). Samples were sonicated as described above. Alternatively, Apyrase (2 µM) was added after sonication. After 0, 15, 45, and 105 min at 25°C, 40 µl of sample was mixed with 20 µl of 3X sample buffer (containing PIC) and heated at 96°C for 5 min. Samples were stored at -20°C.

Equal volumes of the samples were subjected to SDS-PAGE and immunoblot analysis.

For the experiment shown in Figure S6, samples were prepared at 24 h post-starvation as described above with sonication in the absence of protease inhibitor or in the presence of EDTA (5 mM). To one set of extracts prepared in the absence of protease inhibitor, Apyrase (2 µM) was added and the sample was placed at 4°C for 10 min. Recombinant His10-MrpC (60 ng in 5 ul) or His10-MrpC2 (60 ng in 5 ul), expressed in E. coli and purified as described (49, 50), was added to 5 µl of a 2-fold dilution series of extract in phosphate-buffered saline and the 10 µl reactions were incubated at 25°C for 10 min, mixed with 5 µl of 3X sample buffer, heated, stored, and subjected to SDS-PAGE (1 µl per well) and immunoblot analysis as described above.

**RESULTS**

**Commitment to fruiting body formation and cellular shape change.** To investigate commitment to morphological changes during *M. xanthus* development, we used submerged culture in which growing cells are resuspended in starvation buffer, they attach to the bottom of
the culture vessel, and they undergo development (44). If left undisturbed, aggregation centers resolved into small mounds by 18 h (Fig. 1A). Darkening of some nascent fruiting bodies was observed by 36 h post-starvation, with more growing darker by 42 h. A similar developmental pattern was observed if culture supernatant was replaced with fresh starvation buffer at different times (Fig. 1B). In this and subsequent figures, 18S-24, for example, means the culture supernatant was replaced with fresh starvation buffer at 18 h and the culture was photographed at 24 h. The area between developing fruiting bodies became clearer over time, presumably due to movement of cells into fruiting bodies and cellular lysis. The total protein concentration of developing cultures showed a substantial decrease as time progressed (Fig. S2A), with a six-fold decrease by 30 h and a 50-fold decrease by 66 h. This suggests that developing cells contain less protein or they lyse, since only a small proportion of the input cells form spores (that would not release protein upon sonication) inside fruiting bodies.

Strikingly, if the culture supernatant was replaced with nutrient medium at 18 or 24 h post-starvation, aggregates failed to progress to compact, darkened fruiting bodies and instead became less compact over time (Fig. 1C, compare 24N-30 and 24N-36). Also, the areas between fruiting bodies failed to clear normally. On the other hand, nutrient addition at 30 or 36 h post-starvation did not stop the formation of compact, darkened fruiting bodies (Fig. 1C), indicating that a starving culture commits to fruiting body formation by 30 h. Nutrient addition at all time points tested (18, 24, 30) did not prevent the eventual reduction of total protein concentration, although it did not decrease as rapidly (Fig. S2B). These results suggest that some cells in the population commit to lowering their protein concentration or undergoing lysis before 18 h, but nutrient addition delays these events. Delayed cellular lysis and movement of cells out of fruiting bodies
may account for the failure of areas between nascent fruiting bodies to clear when nutrients were added at 18 or 24 h.

Commitment to cellular shape change was examined by microscopic observation after gentle dispersion. Developing cells were scraped from the substratum along with the culture supernatant and samples were subjected to mild dispersion by vortexing and pipetting. This treatment was designed to preserve cells in the process of converting from rods into spores, but perhaps not yet resistant to sonication. Indeed, thickened rods and ovoid spores were first observed at 24 h post-starvation within or at the edge of large cell aggregates (presumably nascent fruiting bodies) (Fig. 2A). By 30 h a larger percentage of cells had changed shape and by 36 h the majority of cells in aggregates had morphed into spores. Darkening of cell aggregates was observed at 36 h, consistent with the darkening of nascent fruiting bodies at 36 h seen under lower magnification (Fig. 1A). Replacing the culture supernatant with fresh starvation buffer did not affect normal progression of cellular shape change (Fig. 2B and 2D). In contrast, replacing the culture supernatant with nutrient medium at 18 h prevented cellular shape change (Fig. 2C and data not shown for later times after nutrient addition). However, addition of nutrients at 24 h did not stop a large proportion of the cells in some aggregates from changing shape, although a delay was observed, with few cells having changed shape by 30 h (Fig. 2E, 24N-30), but many spores by 36 h (Fig. 2E, 24N-36, left half). Therefore, at least some cells in a developing culture commit to cellular shape by 24 h. Other aggregates lacked spores, but contained some rods and what appeared to be cell debris, as if some cells had undergone lysis (Fig. 2E, 24N-36, right half). This is consistent with observations noted above at lower magnification.
Taken together, we conclude that in submerged culture under our conditions a few cells are changing from rods to spores by 24 h post-starvation and many more cells are committed by this time to subsequently change their shape by 36 h even if nutrients are added. Other cells appear to be committed by 18 h to subsequently lower their protein concentration or undergo lysis. If starvation persists during the period between 24 and 30 h, many cells in nascent fruiting bodies morph into spores (or are committed to do so by 36 h). Since spores are non-motile, nascent fruiting bodies persist and darken even if nutrients are added at 30 h. A computational simulation suggested that formation of non-motile spores stabilizes nascent fruiting bodies (51).

Commitment to sonication resistance and formation of mature spores. Mature spores are resistant to heat and sonication, and they are capable of germinating in the presence of nutrients and growing to produce a colony. We reasoned that cells might change shape and become sonication-resistant before they become fully mature spores. To quantify spores that were resistant to sonication but not necessarily fully mature, we sonicated submerged culture samples to disperse spores so they could be counted microscopically. Sonication-resistant spores were present by 30 h post-starvation and increased about 10-fold by 36 h (Fig. 3A, no replacement), consistent with our observations of cellular shape change reported above. The number of sonication-resistant spores rose more gradually thereafter. Replacing the culture supernatant with fresh starvation buffer did not affect development of sonication resistance (Fig. 3A, starvation). In striking agreement with our observations of cellular shape change reported above, replacing the culture supernatant with nutrient medium at 18 h but not at 24 h prevented cells from becoming sonication-resistant (Fig. 3A, nutrient). Therefore, many cells that are not yet sonication-resistant at 24 h are nevertheless committed to become sonication-resistant spores by 30 h (Fig. 3A, 24N-30). Interestingly, addition of nutrients at 24 h blocked the large increase
in the number of sonication-resistant spores between 30 and 36 h (24N-36). Addition of
nutrients at 30 h did not block this increase, indicating that a large number of cells that are not
yet sonication resistant at 30 h are committed to become so by 36 h (30N-36). The number of
sonication-resistant spores decreased slightly after prolonged exposure to nutrients (30N-54 and
36N-54), perhaps due to a small amount of spore germination.

To measure the number of mature spores, samples were subjected to heat treatment and
sonication followed by plating on nutrient agar to allow spore germination and colony formation.
Mature spores were observed starting at 36 h post-starvation, and the number gradually went up
more than 1000-fold by 96 h (Fig. 3B, no replacement). Formation of mature spores lags behind
development of sonication resistance (Fig. 3A, no replacement). Addition of nutrients at 18 h
post-starvation prevented the development of mature spores by 36 h (Fig. 3B, 18N-36).
However, addition of nutrients at 24 h did not stop the formation of mature spores when
analyzed at 48 h (24N-48), although the number of mature spores formed was much lower than
the number at 48 h in the undisturbed culture (48) or after replacement of the culture supernatant
with fresh starvation buffer (24S-48). Thus, at least some cells in a starving population are
committed by 24 h to becoming mature spores. Similarly, nutrient addition at 30 h did not stop
the formation of a small number of mature spores by 54 h (30N-54). Nutrient addition at 36 h
allowed more cells to form mature spores by 42 h (36N-42), resulting in only about 10-fold less
than in the undisturbed culture (42). Waiting longer after nutrient addition at 36 h resulted in
slight decreases in the number of mature spores (36N-48 and 36N-54), perhaps because a small
number of spores begin to germinate in the presence of nutrients. However, spore germination
cannot account for the relatively small numbers of mature spores in the 24N-48, 30N-54, and
36N-54 samples (Fig. 3B, nutrient), since germination would render cells sensitive to sonication, and the number of sonication-resistant spores remained high in those samples (Fig. 3A, nutrient). We conclude that commitment to formation of sonication-resistant spores, like commitment to cellular shape change, occurs primarily during a critical period at 24-30 h post-starvation. In contrast, only a small number of cells commit to forming a mature spore during this period, and even at 36 h post-starvation, when a large number of sonication-resistant spores have formed (Fig. 3A, no replacement or starvation), very few of these spores are committed to maturation (Fig. 3B, nutrient). The number of mature spores increases sharply during the period between 36 and 48 h post-starvation (Fig. 3B, no replacement or starvation); we were unable to define a shorter period of commitment to spore maturation. Therefore, we focused on molecular events before and during the critical period between 24 and 30 h post-starvation when cells commit to subsequent shape change and sonication resistance.

MrpC and FruA are required for commitment to development and persist through the period of commitment to shape change and sonication resistance. To begin testing possible involvement of MrpC and FruA in commitment to development, we examined mutants under our conditions of submerged culture. Neither the mrpC mutant nor the fruA mutant formed compact nascent fruiting bodies (Fig. S3A), as expected based on previous reports of their developmental phenotypes on starvation agar plates (41, 52). Neither mutant initiated detectable cellular shape change (Fig. S3B). Hence, the absence of MrpC or FruA prevented detectable commitment to development. Also, mature spores failed to form (data not shown), as reported previously (38, 41, 52). Next, we examined MrpC and FruA levels in wild-type cells developing under our submerged culture conditions. The levels of these proteins in different “wild-type” backgrounds have been examined previously under various conditions of development (38, 39, 43, 50), but it
was important to measure the levels in our wild-type strain under our conditions of development for comparison with the results described above. Immunoblots showed that MrpC and its smaller form, MrpC2, were not detected at 0 h, when cells were sedimented from nutrient medium and resuspended in starvation buffer. Both forms were detected at 12 h and increased by 15 h, with MrpC persisting through the period of commitment to shape change and sonication resistance (i.e., 24-30 h post-starvation), at least until 42 h, and MrpC2 decreasing reproducibly between 30 and 36 h (Fig. 4A). FruA was barely detectable at 12 h and its accumulation lagged behind that of MrpC by about 6 h (Fig. 4B), as expected since transcription of fruA depends on MrpC (42). The FruA level rose steadily throughout the period of commitment. Thus, both MrpC and FruA accumulate prior to the time we observed commitment to cellular shape change (24 h) (Fig. 2) and persist at least until 42 h (Fig. 4), the period during which cells in the developing population commit to forming compact nascent fruiting bodies (Fig. 1) and sonication-resistant and mature spores (Fig. 3).

**MrpC declines rapidly upon nutrient addition to developing cells.** What roles do MrpC and FruA play in commitment to development? The discovery that the two proteins cooperatively regulate developmental genes suggested that positional information through C-signaling and FruA is integrated with nutrient sensing by regulating the abundance of MrpC (35). Therefore, we hypothesized that the role of MrpC in commitment is to have its abundance regulated in response to nutrients. This hypothesis predicts that MrpC would decline rapidly upon nutrient addition to developing cells. In agreement with our hypothesis, immunoblots showed a rapid decline in MrpC upon nutrient addition at 6, 12, 18, 24, or 30 h post-starvation (Fig. 5). Nutrient addition caused the MrpC level to decrease faster than addition of chloramphenicol (Cm), an inhibitor of protein synthesis, indicating that nutrient addition triggers
MrpC turnover rather than simply halting MrpC synthesis. The signals were quantified and used to calculate the half-life of MrpC (Fig. 5, right). Reproducibly, the MrpC half-life was shorter after nutrient addition than after Cm addition at all times tested (compare t_{1/2} for N with S+Cm). Moreover, simultaneous addition of nutrient and Cm at 24 h post-starvation caused MrpC to decline at least as rapidly as addition of nutrient alone (Fig. 5D, compare N+Cm with N). Therefore, protein synthesis is not required for nutrients to activate one or more pre-existing proteases to rapidly degrade MrpC.

We note that although nutrient-regulated proteolysis of MrpC occurs at 24 h post-starvation (Fig. 5D), it does not stop a large proportion of the cells in some aggregates from subsequently changing shape by 36 h (Fig. 2E, 24N-36, left panel), presumably because MrpC-dependent genes required for cellular shape change had already been expressed. Likewise, nutrient-regulated MrpC proteolysis occurs at 30 h (Fig. 5E), but does not prevent formation of compact nascent fruiting bodies that subsequently darken by 42 h (Fig. 1C, 30N-42) and does not prevent a large increase in the number of sonication-resistant cells by 36 h (Fig. 3A, 30N-36). Presumably, MrpC-dependent genes required for these morphological and physical changes are expressed between 24 and 30 h. It appears that MrpC must persist after 30 h in order for genes to be expressed that are important for mature spores to form in large numbers after 36 h (Fig. 3B). In this context, it is worth noting that very little of the smaller form, MrpC2, was present after 30 h (Fig. 4A), suggesting that MrpC2 is not required for spore maturation. We also note that very little MrpC2 was present at any of the times tested in our nutrient addition experiments (Fig. 5, unfilled arrowheads). In these experiments, the culture supernatant was replaced with fresh starvation buffer prior to collecting samples, whereas the culture was undisturbed prior to collecting samples for the experiment shown in Figure 4. Although replacement with fresh
starvation buffer greatly diminished MrpC, there was no apparent effect on fruiting body formation (Fig. 1B), cellular shape change (Fig. 2B and 2D), or formation of sonication-resistant or mature spores (Fig. 3, starvation). MrpC2 has been reported to bind sites in the mrpC and fruA promoter regions with higher affinity than MrpC (50), but individual roles of the two proteins during development have not been dissected.

The level of FruA also decreased after nutrient and/or Cm addition (Fig. S4), but the effect of nutrient addition was not as profound as for MrpC (Fig. 5). When nutrients were added at 18 h post-starvation, MrpC was nearly undetectable after 1 h (Fig. 5C), but FruA was still visible (Fig. S4A). Nutrient addition at 24 h post-starvation caused a more rapid and complete loss of MrpC (Fig. 5D) than FruA (Fig. S4B). At both times, nutrient addition caused a more rapid loss of MrpC than Cm addition (Fig. 5C and 5D, compare N with S+Cm and the corresponding t1/2 values), but this was not the case for FruA (Fig. S4). The half-life of FruA was similar after nutrient or Cm addition at 18 h, and it was longer after nutrient addition than after Cm addition at 24 h. Therefore, we cannot be certain whether FruA is subject to nutrient-regulated proteolysis.

Since transcription of fruA depends on MrpC (42), synthesis of FruA is expected to decline rapidly upon nutrient addition, although probably not as rapidly as upon Cm addition. We conclude from the data in Figure S4 that FruA might be subject to nutrient-regulated proteolysis, in addition to its expected decline as fruA transcription ceases due to the declining MrpC level. Since the decline of MrpC appeared to be the primary response of developing cells to nutrient addition, we focused on this response and its consequences in subsequent experiments.

 mrpC transcript increases, then decreases, upon nutrient addition to developing cells.

To explore further the response of developing cells to nutrient addition prior to commitment of most cells to sonication resistance, we measured mrpC transcript levels after nutrient addition to
24-h developing cells (Fig. 6A). Nutrient addition resulted in more than a 3-fold increase in the
mrpC transcript level after 15 min (N+15), followed by a decline to the original level after 30
min (N+30), and a further decline to about half the original level by 120 min (N+120). As a
control, the culture supernatant was replaced with fresh starvation buffer rather than nutrient
medium. Surprisingly, the mrpC transcript level increased about 2-fold after 15 min (S+15) and
remained high after 30 min (S+30), as if an inhibitor of mrpC transcript accumulation had been
removed, followed by a decline to the original level after 60 min (S+60) or 120 min (S+120). In
contrast, if the fresh starvation buffer contained rifampicin, which blocks transcription, the mrpC
transcript level declined about 10-fold after 15 min (S+Rif+15) and was barely detectable after
30 min (S+Rif+30). The result with rifampicin indicates that the mrpC transcript is unstable in
24-h developing cells. Nutrient addition, like addition of fresh starvation buffer, may remove an
inhibitor of mrpC transcript accumulation, resulting in the observed increase after 15 min.
However, the mrpC transcript level remains high 30 min after the addition of fresh starvation
buffer, but declines to the original level 30 min after nutrient addition, indicating that nutrients
inhibit mrpC transcript accumulation between 15 and 30 min after their addition. The inhibition,
though, returns the mrpC transcript to its original level, which does not explain the observed
decrease in MrpC protein level by 30 min after nutrient addition to 24-h developing cells (Fig.
5D), consistent with our interpretation above that nutrients activate one or more proteases that
rapidly degrade MrpC. The rapid decline of MrpC may account for the decline of the mrpC
transcript, since MrpC has been reported to positively autoregulate its own expression, likely at
the transcriptional level (41). Since the primary effect of nutrients prior to commitment of most
cells to sonication resistance appeared to be rapid degradation of MrpC, we focused on gaining
further insight into this process.
Nutrient addition halts expression of MrpC target genes important for sporulation. To test whether nutrient-regulated proteolysis of MrpC affects transcription of its target genes that code for proteins important for commitment to sporulation, we measured transcript levels from the dev operon after nutrient addition to 24-h developing cells (Fig. 6B), when most cells have not yet committed to sonication resistance (Fig. 3A). MrpC and FruA cooperatively bind in the promoter region of the dev operon and appear to directly activate transcription (53) (A. Campbell, P. Viswanathan, B. Son, T. Barrett, and L. Kroos, unpublished data). Products of the dev operon have been shown to be important for sporulation (54-56) and to positively regulate expression of genes that play a major role in maintenance of cellular shape change (57-59). Under our submerged culture conditions, null mutants of devT or devR failed to change cell shape or become sonication resistant by 72 h post-starvation (data not shown). We measured dev transcript levels using qPCR with primers that would amplify from within devT near its 3’ end to within devR near its 5’ end (Fig. 6B). At 24 h post-starvation, nutrient addition resulted in a steady decline in dev transcripts, which decreased about 3-fold by 120 min (N+120) to a level significantly below the starting value (P = 0.02, Student’s t-test). In the control, where the culture supernatant was replaced with fresh starvation buffer, the dev transcript level remained similar to the starting value. In contrast, if the fresh starvation buffer contained rifampicin, the dev transcripts were barely detectable after 15 min (S+Rif+15), indicating that the transcript is unstable in 24-h developing cells. We conclude that nutrient-regulated proteolysis of MrpC halts expression of the dev operon whose products are important for commitment to sporulation. The decline in dev transcripts is less rapid after nutrient addition than after rifampicin addition, suggesting that transcription of dev continues for a short time after nutrient addition until MrpC and FruA levels are too low to support further expression.
Nutrient-regulated proteolysis of MrpC may involve ATP-independent metalloprotease activity. We investigated the possibility that BsgA (also called LonD) is involved in rapid turnover of MrpC after nutrient addition to developing cells. BsgA is an ATP-dependent serine protease shown previously to be required for development \((60, 61)\). Since a \(bsgA\) mutant fails to produce MrpC2 during development, it was suggested that BsgA may be responsible for proteolytic conversion of MrpC to MrpC2 \((50)\) (Fig. S1). Under our conditions of submerged culture development, a \(bsgA\) mutant shows a severe developmental defect similar to \(mrpC\) or \(fruA\) mutants, failing to form fruiting bodies or initiate cellular shape change (Fig. S3A and S3B). Immunoblots revealed that addition of nutrients to the \(bsgA\) mutant at 18 h post-starvation resulted in a rapid decline of MrpC, more rapid than the addition of Cm (Fig. S3C), as observed for wild type (Fig. 5C). The half-life of MrpC was shorter after nutrient addition than after Cm addition (Fig. S3C, right), so BsgA is not required for nutrient-regulated proteolysis of MrpC, but the half-lives were longer in the \(bsgA\) mutant than in wild type (Fig. 5C, right), suggesting that BsgA directly or indirectly impacts MrpC proteolysis. As reported previously for a different \(bsgA\) mutant \((50)\), we did not detect conversion of MrpC to MrpC2 in the \(bsgA\) mutant we used (Fig. S3C).

To identify the type of protease that might be involved in rapid turnover of MrpC after nutrient addition to developing cells, various protease inhibitors were tested for the ability to slow the degradation of MrpC in cell extracts. Immunoblots showed that MrpC was degraded rapidly in sonic extracts of developing cells prepared at 18 h (Fig. 7) or 24 h (Fig. S5) post-starvation, covering time points both before and at the beginning of the critical period of commitment to sporulation. Loss of MrpC appeared to be more rapid when the culture supernatant was replaced with fresh starvation buffer for 15 min prior to sonication [designated
S(-) with minus indicating absence of protease inhibitors] than when it was replaced with nutrient medium [designated N(-)]. We expected the opposite to be the case since nutrient addition caused MrpC to decline rapidly in developing cells (Fig. 5); however, we loaded equal volumes rather than equal amounts of total protein in the experiments shown in Figures 7 and S5, and there could be a larger burst of MrpC synthesis shortly after nutrient addition than after starvation buffer addition. In any case, the half-life of MrpC could not be determined because it was too unstable in sonic extracts without protease inhibitor. The addition of a protease inhibitor cocktail (PIC) during sonication greatly stabilized full-length MrpC in samples to which nutrients had been added before sonication (N+PIC), and to a lesser degree in samples to which fresh starvation buffer had been added (S+PIC) (Fig. 7 and S5). The greater stabilization of MrpC in extracts of developing cells to which nutrients had been added suggests that protease activity is altered in these cells. Perhaps nutrients activate a protease that is effectively inhibited by the cocktail, and this directly or indirectly protects MrpC from attack by other proteases. Since the PIC was a mixture of protease inhibitors at proprietary concentrations, we tested individual protease inhibitors at standard concentrations. Only the metalloprotease inhibitor EDTA stabilized MrpC, albeit typically not quite as well as the PIC, as judged from the half-lives (Fig. 7 and S5). Inhibitors of other types of proteases did not stabilize MrpC. Like PIC, EDTA stabilized full-length MrpC in samples to which nutrients had been added before sonication (N+EDTA) to a greater degree than in samples to which fresh starvation buffer had been added (S+EDTA). Apyrase, an enzyme that hydrolyzes ATP, was also tested, to see if proteolysis of MrpC depends on ATP. Addition of apyrase immediately after sonication did not stabilize MrpC (Fig. 7 and S5). Neither did pre-incubation of sonic extracts with apyrase inhibit their ability to produce a discrete breakdown product of purified recombinant His_{10}-MrpC or
His10-MrpC2, although addition of EDTA during sonication did inhibit breakdown product formation (Fig. S6). Taken together, our results suggest that nutrient-regulated proteolysis of MrpC may involve ATP-independent metalloprotease activity.

Since the FruA level also decreased after nutrient addition (Fig. S4), we examined FruA degradation in cell extracts. As with MrpC, the addition of PIC or EDTA during sonication stabilized FruA in samples at 18 or 24 h post-starvation, and addition of apyrase immediately after sonication did not stabilize FruA (Fig. S7). These results suggest that proteolysis of FruA in developing cells may also involve ATP-independent metalloprotease activity.

**DISCUSSION**

Our examination of the consequences of adding nutrients to developing *M. xanthus* revealed progressive commitment to differentiation and established a foundation for investigating the molecular basis of commitment to particular cell fates. Between 18 and 24 h post-starvation, mounds become well-defined, a few cells begin to change shape, and some cells commit to subsequent shape change and sonication resistance. Nevertheless, nutrient addition at 24 h post-starvation still profoundly changes the developmental outcome, resulting in 10-fold less sonication-resistant spores (as measured 6 or 12 h later) than if starvation persists until 30 h. Hence, the period around 24-30 h post-starvation, when nascent fruiting bodies become well-defined and cells are close-packed in mounds, is a critical period for commitment to cellular shape change and sonication resistance. During this period, short-range C-signaling is proposed to reach a maximum and stimulate expression of genes required for sporulation (26-30). The transcription factor FruA was known to be involved in the response to C-signaling (38-40), yet each of several C-signal-dependent genes or operons studied was found to be under coordinate...
control by MrpC (34-37). Here, we have shown that MrpC is rapidly degraded upon nutrient addition to developing cells before and during the critical period of commitment, halting expression of direct target genes in the dev operon that are important for sporulation (Fig. S1). Therefore, we propose that nutrient-regulated proteolysis of MrpC provides a mechanism for monitoring whether starvation persists and halting commitment to sporulation if nutrients reappear before the critical period around 24-30 h post-starvation.

**Advantages of delayed, step-wise commitment to development.** Sporulation is a costly decision both in terms of lost time for reproduction and in terms of resources spent to produce a cell capable of surviving a long period without nutrients in a harsh environment. *Bacillus subtilis* and related endospore formers delay commitment to sporulation by secreting toxins that enable cells to cannibalize siblings (62, 63). *M. xanthus* may likewise feed off of lysing siblings, although whether this involves autocide production (64-66), programmed cell death (6, 33), or both, is unclear. Our results agree with previous work showing that *M. xanthus* does not commit to sporulation until it has been starving for about 24 h (9). The obvious advantage of delaying commitment to sporulation is that cells could resume growth if nutrients reappear.

Our results suggest a temporal progression of *M. xanthus* in terms of commitment to differentiation, with some cells committing to shape change and sonication resistance during the critical period around 24-30 h, followed by gradual commitment to formation of mature spores. The advantage of gradual step-wise commitment to development may be to provide adaptability under changing environmental conditions. Single-cell analysis of gene expression and modeling of different stress conditions suggests that *B. subtilis* cells remain responsive to environmental fluctuations as they proceed toward commitment to endospore formation (67). Methods have not yet been developed to track gene expression in individual cells during *M. xanthus* fruiting body
formation. However, in a recent study, aggregating cells were separated from non-aggregating
cells and MrpC, C-signal, and FruA were found to be more abundant in the aggregating cells
(33). Therefore, high levels of the three proteins are correlated with the subpopulation in which
cells commit to sporulation.

**Molecular basis of commitment to particular cell fates.** In a wide variety of systems,
commitment to particular cell fates is governed by key signals and gene regulators with
characteristic features (68-70). For example, many are part of a positive feedback loop that
results in bistable expression with some cells in a population producing a much higher level of
the key molecule than other cells. MrpC, C-signal, and FruA are in positive feedback loops (Fig.
S1). These positive feedback loops likely result in bistable expression of MrpC, C-signal, and
FruA, accounting for their higher abundance in aggregating cells than in non-aggregating cells
(33).

We propose that MrpC, C-signal, and FruA are key regulators of commitment to sporulation.
C-signal accumulates gradually during development and distinct thresholds have been observed
for aggregation, sporulation, and gene expression (27-29). FruA mediates the response to C-
signal (38) and binds cooperatively with MrpC to promoter regions of C-signal-dependent genes
(34-37), including the dev operon (56) (A. Campbell, P. Viswanathan, B. Son, T. Barrett, and L.
Kroos, unpublished data) (Fig. S1). Since MrpC appears to activate transcription of fruA (42),
and MrpC and FruA together appear to activate dev transcription, the expression of the dev
operon appears to be controlled by a coherent feed-forward loop (71) expected to delay full
expression until FruA* (i.e., FruA activated in response to C-signaling by an unknown
mechanism) reaches a sufficient concentration. Products of the dev operon are important for
sporulation (53-55), including cellular shape change and development of sonication resistance
(this study), and they positively regulate the *exo* operon, whose products are necessary to maintain spore differentiation (57, 59). Also, transcription of the *exo* operon appears to be directly activated by FruA* (72). So, together MrpC, C-signal, and FruA appear to activate targets including the *dev* and *exo* operons whose products are important for committing cells in a nascent fruiting body to the spore fate. Commitment to sporulation also involves blocking growth and division so that differentiation proceeds even if nutrients become available. In *B. subtilis*, the SpoIIP and SpoIIQ proteins play key roles in commitment to endospore formation (73), but how they block growth and division is unknown. In *M. xanthus*, proteins that block growth and division, as well as proteins directly involved in peptidoglycan rearrangement that presumably accompanies cell shape change, remain to be identified.

**Regulation of MrpC as a mechanism to sense nutrients and escape commitment.** As a key component of a feed-forward loop that appears to govern *dev* transcription, MrpC is well-positioned to halt commitment to sporulation. Our results show that MrpC is rapidly degraded upon nutrient addition to developing cells before and during the critical period of commitment. Further, nutrient addition to developing cells at 24 h post-starvation resulted in a steady decline in *dev* transcripts to a low level within 2 h. We conclude that nutrient-regulated proteolysis of MrpC halts expression of direct target genes important for sporulation, and we propose that this provides a rapid mechanism to escape commitment if nutrients reappear.

Regulated proteolysis in bacteria is used to control adaptive, cell cycle, and developmental events by degrading or activating regulatory proteins, or by producing signals (74). During *M. xanthus* development, extracellular proteases produce A-signal (13, 14) and a proteolytic cascade produces C-signal (17, 75). The protease BsgA has been implicated in production of the unidentified B-signal (60) and in conversion of MrpC to MrpC2 (50) (Fig. S1). Our results show
that replacing the developing culture supernatant with fresh starvation buffer markedly diminishes MrpC2 accumulation, but has no detectable effect on development, suggesting that MrpC2 need not accumulate continuously for development to occur. A bsgA mutant failed to accumulate MrpC2, as expected (50), and the level of MrpC was reduced compared with wild type, but addition of nutrients resulted in a rapid decline of MrpC (Fig. S3C), indicating that BsgA is not responsible for nutrient-regulated proteolysis of MrpC. Instead, our experiments involving addition of protease inhibitors to sonic extracts of developing cells implicate ATP-independent metalloprotease activity. Analysis of the *M. xanthus* genome (76) reveals at least 29 genes predicted to code for metalloproteases that are likely to be ATP-independent and cytosolic. These putative metalloproteases are candidates for involvement in nutrient-regulated proteolysis of MrpC. The Esp signaling system negatively regulates MrpC stability (Fig. S1) to delay *M. xanthus* development (43) and has been proposed to do so by activating a protease or protease targeting factor to stimulate MrpC turnover (47). However, the signal(s) sensed by the Esp system is unknown.

Transcriptional regulation of *mrpC* plays a secondary role in the response of developing cells to nutrient addition. Our results show that the *mrpC* transcript level increases transiently after nutrient addition to developing *M. xanthus* at 24 h post-starvation, returns to the original level, and then decreases (Fig. 6A). The decrease in transcript level occurs well after the decrease in MrpC protein level (Fig. 5D). Indeed, the loss of MrpC is expected to cause the *mrpC* transcript level to decrease since MrpC has been reported to positively autoregulate and to positively regulate the *mrpAB* operon, whose products positively regulate *mrpC*, forming a positive feedback loop (Fig. S1). Hence, nutrient-regulated proteolysis destroys MrpC rapidly and loss of
positive autoregulation and feedback likely accounts for the decline of mrpC transcript observed later.  

**Conclusion.** *M. xanthus* delays commitment to sporulation until a critical period around 24-30 h post-starvation. Nutrient addition before or during this period causes a rapid decline of MrpC. The primary response is proteolysis of MrpC that may involve ATP-independent metalloprotease activity, followed by declines of mrpC and dev transcripts, and FruA protein. This response may allow cells to escape commitment to sporulation and resume growth if nutrients reappear in a changing environment. More work is needed to precisely define the signal(s), signal transduction pathway(s), and protease(s) involved in the response, and to identify genes acting after the dev operon (and perhaps in parallel with it) that commit a cell to the spore fate if starvation persists.  

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**FIGURE LEGENDS**

**FIG 1** Effect of nutrient addition on fruiting body formation. (A) Fruiting body formation by *M. xanthus* DK1622 under submerged culture conditions. Aggregates form compact fruiting bodies by 24 h (an arrow points to one), which darken by 36-42 h. Spaces between fruiting bodies become clearer over time. The culture supernatant was replaced with (B) fresh starvation buffer (designated “S”) or (C) nutrient medium (designated “N”) at 18, 24, 30, or 36 h and observed at the indicated time (e.g., 18S-24 means the culture supernatant was replaced with fresh starvation buffer at 18 h and the culture was photographed at 24 h). Bar = 100 μm. Similar results were observed in at least two biological replicates.

**FIG 2** Effect of nutrient addition on cellular shape change. (A) Cellular shape change in *M. xanthus* DK1622 under submerged culture conditions. Developing cultures were subjected to mild dispersion and examined microscopically. Photos show densely-packed cell aggregates...
presumed to be nascent fruiting bodies and insets show the edge of a cell aggregate. Arrows indicate thickened rods or ovoid spores. The culture supernatant was replaced with (B) fresh starvation buffer (designated “S”) or (C) nutrient medium (designated “N”) at 18 or 24 h and observed at the indicated time (e.g., 24N-36 means the culture supernatant was replaced with nutrient medium at 24 h and the culture was photographed at 36 h). The panel labeled 24N-36 shows a cell aggregate with many spores (left half) and a cell aggregate lacking spores but with rods and what appears to be cell debris (right half). Bar = 5 μm. Similar results were observed in at least two biological replicates.

**FIG 3** Effect of nutrient addition on development of (A) sonication resistance and (B) mature spores. *M. xanthus* DK1622 was subjected to starvation under submerged culture conditions for the time indicated by the first number. The culture supernatant was left undisturbed or replaced with fresh starvation buffer (designated “S” and indicated by the bracket labeled “starvation”) or nutrient medium (designated “N” and indicated by the bracket labeled “nutrient”), then the culture was harvested for measurement of sonication-resistant or mature spores at the time indicated by the second number (e.g., 24N-36 means the culture supernatant was replaced with nutrient medium at 24 h and the culture was harvested at 36 h). Values (log_{10}) are the average of at least three biological replicates and error bars represent 1 standard deviation from the mean.

**FIG 4** Immunoblot analysis of (A) MrpC and (B) FruA during development. *M. xanthus* DK1622 was starved under submerged culture conditions. Samples were collected at the indicated times and equal amounts of protein were analyzed by immunoblot using anti-MrpC or anti-FruA antibodies. Intervening lanes were removed between the 36 and 42 h samples in panel...
A. Representative immunoblots are shown. Similar results were observed in at least two biological replicates.

**FIG 5** Effect of nutrient addition on the MrpC level during development. *M. xanthus* DK1622 was starved under submerged culture conditions and after 6 (A), 12 (B), 18 (C), 24 (D), or 30 h (E) the culture supernatant was replaced with fresh starvation buffer (S), nutrient medium (N), fresh starvation buffer with 200 μg/ml chloramphenicol (S+Cm), or nutrient medium with 200 μg/ml chloramphenicol (N+Cm). At the indicated times (in min) after replacement, cells were harvested and equal amounts of total protein were analyzed by immunoblot using anti-MrpC antibodies. Filled and unfilled arrowheads indicate MrpC and MrpC2, respectively. Intervening lanes were removed from the image in panel A. Representative immunoblots are shown. The half-life in minutes (t₁/₂, min) of MrpC after the indicated treatment is shown to the right. A similar ratio of the half-life after nutrient treatment to that after chloramphenicol treatment was observed in at least two biological replicates.

**FIG 6** Effect of nutrient addition on the (A) *mrpC* transcript level and (*dev* transcript level) during development. *M. xanthus* DK1622 was starved under submerged culture conditions and after 24 h the culture supernatant was replaced with fresh starvation buffer (S), nutrient medium (N), fresh starvation buffer with 50 μg/ml rifampicin (S+Rif), or nutrient medium with 50 μg/ml rifampicin (N+Rif). At the indicated times (in min) after replacement (e.g., S+15 means the culture supernatant was replaced with fresh starvation buffer and the culture was harvested 15 min later), cultures were harvested and RNA was isolated and subjected to qRT-PCR analysis to determine the *mrpC* or *dev* transcript level. Values are the average of three technical replicates.
for at least three biological replicates and are reported relative to the untreated 24-h sample (labeled “none”). Error bars indicate one standard deviation from the mean.

FIG 7  Effects of nutrient addition and protease inhibitors on degradation of MrpC in extracts of developing cells. *M. xanthus* DK1622 was starved under submerged culture conditions and after 18 h the culture supernatant was replaced with fresh starvation buffer (S) or nutrient medium (N) for 15 min. Cultures were harvested and protease inhibitor(s) was added prior to sonication, or no protease inhibitor was added as a control (-). After sonication, samples were taken at the indicated times (in min). Apyrase was added immediately after sonication. Equal sample volumes were analyzed by immunoblot using anti-MrpC antibodies. Gaps in the image indicate samples analyzed on separate immunoblots. Representative immunoblots are shown. The half-life in minutes ($t_{1/2}$, min) of MrpC under the indicated condition is shown to the right. A similar ratio of the half-life in the presence of PIC to that with EDTA was observed in at least two biological replicates.
FIG 1
FIG 3

A

Replacement:

None

Starvation

Nutrient

Log SBoiication-Resistant
cells/ml

Hours After Starvation

B

Replacement:

None

Starvation

Nutrient

Log Colony-forming units/ml

Hours After Starvation
FIG 5

A

6 h

0 15 30 60 15 30 60 15 30 60 6 h

N = 12
S+Cm = 21

t₁/₂, min
A

S N S + C m

B

12 h

0 15 30 60 120 180 15 30 60 120 180 15 30 60 120 180

N = 17
S+Cm = 35

C

18 h

0 30 60 120 30 60 120 30 60 120 30 60 120

N = 15
S+Cm = 25

D

24 h

0 30 60 120 30 60 120 30 60 120 30 60 120

N = 22
S+Cm = 28
N+Cm = 18

E

30 h

0 30 60 120 30 60 120 30 60 120

N = 30
S+Cm = 50
FIG 6

A. Replacement: Starvation Nutrient Rifampicin

Relative mrpC Transcript Level

None S+15 S+30 S+60 S+120 N+15 N+30 N+60 N+120 S+Rif+15 N+Rif+30 N+Rif+30

Culture Supernatant Replacement and Min Incubated

B. Replacement: Starvation Nutrient Rifampicin

Relative dev Transcript Level

None S+30 S+60 S+120 N+30 N+60 N+120 S+Rif+15 N+Rif+30 N+Rif+30

Culture Supernatant Replacement and Min Incubated
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- S+PIC = 70
- S+EDTA = 50
- N+PIC = 110
- N+EDTA = 70