Methionine Inhibits Developmental Aggregation of *Myxococcus xanthus* by Blocking the Biosynthesis of S-Adenosyl methionine

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Previous studies showed that high concentrations of methionine (>1 mM) inhibited aggregation and fruiting body formation in *Myxococcus xanthus* (E. Rosenberg, D. Filer, D. Zafriti, and S. H. Kindler, J. Bacteriol. 115: 29–34, 1973, and J. M. Campos and D. R. Zusman, Proc. Natl. Acad. Sci. USA 72:518–522, 1975). However, the mechanism for the inhibition was unclear. In this study, we found that high levels of methionine inhibited the biosynthesis of S-adenosylmethionine (SAM) and that reduced intracellular levels of SAM are correlated with defective chemotactic movements and reduced developmental gene expression. In addition, we found that methionine analogs and high concentrations of amino acids which are known to affect SAM synthesis in other bacteria, such as threonine, lysine, and isoleucine, also caused reduced cellular levels of SAM and blocked fruiting body formation in *M. xanthus*. These results indicate that SAM is required for development of *M. xanthus* and the inhibitory effect of methionine on development results, at least in part, from its blocking of the biosynthesis of SAM.

Myxococcus xanthus is a gram-negative soil bacterium which, when stressed by starvation on a solid surface at a high cell density, undergoes a developmental program in which cells aggregate, form fruiting bodies, and differentiate into spores. Some early studies noted that addition of methionine to starvation medium inhibited fruiting body formation (3, 17). Furthermore, it was shown that the biosynthesis of some developmental proteins was blocked in the presence of methionine (9). The mechanism of the inhibitory effect by methionine and several other amino acids on development was not clear, although some studies suggested that the activity of aspartokinase, which is regulated by methionine, may play a role in this inhibition (6, 17).

In this study, we reexamined the effect of the addition of high levels of methionine on development of *M. xanthus*. We were able to reproduce the previously published inhibitory effects of methionine on fruiting body formation. Furthermore, we found that cells treated with high concentrations of methionine for extended periods of time (>20 h) showed less-frequent cell reversals and unmethylated FrzCD (a methyl-accepting chemotaxis protein). These results suggested an absence of S-adenosylmethionine (SAM) as a methyl donor for FrzCD during development. High-pressure liquid chromatographic (HPLC) analysis showed that the intracellular levels of SAM were indeed dramatically reduced in cells treated with methionine. In addition, we found that methionine analogs and other amino acids (Thr, Lys, and Ile) which were reported to affect SAM biosynthesis (4, 8, 21) also caused reduced levels of SAM and blocked fruiting body formation in *M. xanthus*. These results suggest that SAM is required for development of *M. xanthus* and that the inhibitory effect of methionine on development may result from blocking the biosynthesis of SAM.

Myxobacterial strains and culture conditions. *M. xanthus* DK1622 is wild type for motility and fruiting body formation (10). Strains DK4491, DK4300, DK4469, DK4414, DK4293, and DK5204 are DK1622 derivatives containing *lacZ* fusions which are developmentally regulated and expressed at different times (11). *M. xanthus* DZF1 shows wild-type fruiting body formation and does not form cell clumps (2); however, it contains a motility defect (*sglA* [leaky]). *M. xanthus* cells were grown in a medium consisting of 10 g of Casitone per liter, 5 g of yeast extract per liter, and 8 mM MgSO₄ in 10 mM MOPS (morpholinepropanesulfonic acid) buffer, pH 7.6 (CYE) (2) at 32°C on a rotary shaker at 225 rpm. MOPS medium contains 10 mM MOPS (pH 7.6) and 8 mM MgSO₄.

Assays. For fruiting, 20 µl of cells at 1,000 Klett units (~1 × 10⁸ cells) was concentrated by centrifugation and placed on MOPS medium plates (1.5% agar) containing various concentrations of L-amino acids (unless otherwise specified) and incubated at 32°C for 2 to 3 days. For sporulation, the plates were incubated for 7 days. Spores were examined by microscopy. They are refractile spherical cells which are resistant to 1% sodium dodecyl sulfate (SDS) and limited exposure to high temperature (55°C for 1 h). For growth inhibition studies, various concentrations of amino acids were added to CYE medium to test their effect on cell growth. For studies on the effect of added amino acids on cell viability, DZF1 cells from MOPS medium plates containing the amino acid addition were collected after 20 h, resuspended to the same optical density, and plated on CYE plates after dilution. Strain DZF1 was used for these experiments since it is easy to disperse, which is essential for accurate colony counts. Cell motility was assayed by time-lapse video microscopy as described by Shi and Zusman (20). The assays for chemotaxis and methylation of FrzCD were performed by the methods described previously (18, 19). β-Galactosidase activities of developmentally regulated Tn5 *lac* fusions were assayed by the procedures described by Kroos et al. (11).

SAM analysis was performed by HPLC according to a method described by Payne and Ames (16), with the following

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TABLE 1. Effect of methionine on development of *Myxococcus xanthus*^a

Developmental process	Experimental condition	
	No methionine	0.5 mM methionine
Aggregation on MOPS plates	Yes	Blocked
Sporulation on MOPS plates	Yes	Blocked
Cell growth on MOPS plates	No	No
Cell viability after 20 h on MOPS plates ^b	92%	87%
Cell motility after 20 h on MOPS plates	Motile	Motile
Time between two cell reversals ^c (after 48 h on MOPS plates)	6–10 min	>20 min
Modification of FrzCD after 48 h on MOPS plates	Mainly methylated	Mainly demethylated

^a See the section headed "Assays" in the text for experimental procedures.

^b Cell viabilities were calculated in comparison with cells directly put onto CYE medium without any treatment. The actual colony numbers of cells in control group, cells on MOPS plates, and cells on MOPS-methionine plates were 2.35×10^8 , 2.16×10^8 , and 2.04×10^8 cells per ml, respectively.

^c Cellular reversal frequencies listed were determined by monitoring >15 single cells by time-lapse video microscopy.

modification: 1 ml of 2×10^{10} cells were placed on MOPS medium plates containing various amino acids and incubated for 24 h. Cells were collected from the plates and resuspended in 5 ml of MOPS medium. All cell solutions were adjusted to the same density using a Klett-meter and filtered through Whatman 3-mm filters. The small-molecule fraction of the cells was extracted from the cells with formic acid. Immediately after filtration, 10 ml of cold 1.0 M formic acid (pH 2) was added on the filter and allowed to stand at 4°C for 30 min. The formic acid was collected and centrifuged to remove cells and debris, and the supernatant was lyophilized. The residue after lyophilization (whole-cell extract) was redissolved in 100 μ l of water. The extracts were then deproteinized and filtered through a Millipore filter (pore size, 0.2 μ m). A Hewlett Packard HPLC system (Model HP1090) was used in this study with a Spectroflow 757 UV absorbance detector (ABI Analytical Co.). An Ultrasphere ODS/reverse-phase C-18 column was used to analyze the extract contents. The chromatographic conditions were as follows: buffer A, 0.25 M ammonium acetate, pH 6.0; solution B, methanol; 20-min gradient curve no. 7, 0 to 50% B, 1.5 ml/min. Similar amounts of filtered cell extracts were loaded onto the HPLC column. The SAM peak was identified at 7.1 min on the basis of retention time and UV spectrum of a pure SAM standard purchased from Sigma Chemical Co.

Methionine blocks development of *M. xanthus*. Strains DK1622 and DZF1 aggregate and form fruiting bodies containing spores on MOPS medium. However, as reported previously (3, 17), when methionine (>0.5 mM) was added to the MOPS medium, the developmental process was blocked (Table 1). Instead of forming fruiting bodies, cells on MOPS medium containing methionine exhibited the "rough" phenotype (14), and no obvious aggregates were observed. With further incubation (up to 10 days), no spores were observed (Table 1). It is interesting that sometimes the methionine-treated DZF1 cells formed frizzy filaments, a characteristic phenotype caused by cell movement with infrequent cell reversals (1).

There are a number of ways for chemicals to block fruiting body formation on MOPS medium. For example: (i) the chemical could be a nutrient which feeds the cell and blocks the starvation signal; (ii) the chemical could have toxic effects on cells; (iii) the chemical could block cell motility or interfere

MOPS MET
0 2 48 0 2 48 (h)

FIG. 1. Effect of methionine on the modification of FrzCD. Cells were collected from plates containing either MOPS or MOPS plus 5 mM methionine at the time points indicated and studied for methylation or demethylation of FrzCD by the procedure described in the text in the section headed "Assays." The arrow indicates the position of demethylated FrzCD.

with chemotactic movements; or (iv) the chemical could block developmental gene expression.

We found that methionine was not a nutrient which could restore cell growth on minimal plates (Table 1). Conversely, we found that methionine (up to 20 mM) did not have an inhibitory effect on cell growth when added to the rich growth medium CYE (data not shown). Cells placed on MOPS medium containing 0.5 mM methionine for up to 20 h had cell viability similar to that of a control group cultured on MOPS medium alone (Table 1). These results show that methionine does not have a direct killing effect on *M. xanthus* cells. However, with further incubation, cells on MOPS medium formed spores, which made them resistant to starvation conditions; in contrast, cells on MOPS medium containing methionine began to die after 2 days and were all dead after 4 to 5 days (there were no survivors of 10^8 cells).

Methionine (at concentrations tested) had almost no effect on cellular motility (Table 1). Using time-lapse video microscopy, we examined the gliding motility of cells placed on the MOPS medium containing methionine and found that cells were fully motile for at least 20 h.

Methionine caused defective chemotaxis after prolonged incubations. Our recent studies showed that chemotactic movements play a very important role in cellular aggregation (19). We tested the effect of methionine on chemotaxis in *M. xanthus*. The chemotaxis assays showed that methionine was neither an attractant nor a repellent (data not shown). Previously it was shown that cells treated with attractants (for up to 1 h) contained methylated FrzCD while cells treated with repellents (for up to 1 h) contained unmethylated FrzCD (13, 18). We tested the effect of methionine on methylation/demethylation of FrzCD and found that it did not have any clear effect on the modification of FrzCD when incubated with cells for a short period of time (up to 2 h) (Fig. 1). Therefore, it is unlikely that the inhibitory effect on fruiting body formation was due to chemoattraction or chemorepulsion. However, we

TABLE 2. Intracellular levels of SAM under various conditions^a

Conditions	SAM peak
MOPS.....	272.8
MOPS + 5 mM methionine.....	<5
MOPS + 0.1 mM selenomethionine.....	<5
MOPS + 10 mM lysine.....	52.3
MOPS + 5 mM phenylalanine.....	156.7
MOPS + 20 mM serine.....	246.5

^a See the section headed "Assays" in the text for detailed experimental procedures. Each cell group was treated under various conditions for 20 h, and total area of peaks for each sample was around 1,800 mAU · sec. The SAM peak was located at 7.1 min. The numbers listed are in mAU · sec.

TABLE 3. Effect of amino acid additions on development of *M. xanthus*

Amino acid(s)	MIC (mM)	Motility	Fruiting	Sporulation ^a
MOPS alone		+	+	+
Met	0.5	+	—	—
Selenomethionine	0.01	+	—	—
Ethionine	0.1	+	—	—
Lys	5	+	—	+/-
Thr	10	+	—	+/-
Ile, Leu, Val	10	+	—	+/-
Phe, Trp, Tyr	5	+	—	+/-
Other amino acids	20	+	+	+

^a + indicates that >90% of cells became spores after 10 days; — indicates that <1% of cells became spores after 10 days; +/- indicates <30% of cells became spores after 10 days.

found that with prolonged incubation (i.e., 48 h), cells treated with methionine showed greatly reduced cell reversal frequencies (>20 min per reversal), while untreated cells had a reversal frequency of 6 to 10 min (Table 1). In addition, we found that the methylation pattern of FrzCD was altered in cells after prolonged methionine treatment: cells harvested from methionine plates (after 48 h) contained mainly the unmethylated form of FrzCD, while untreated cells at this time are enriched for methylated FrzCD (Fig. 1).

It has been shown that in enteric bacteria, excess methionine in the medium turns off the *met* regulon which includes *metK* (SAM synthetase), reducing the level of SAM synthesis (21); SAM starvation causes the demethylation of methyl-accepting chemotaxis proteins (MCPs) and causes reduced tumbling (22). Therefore, it seemed likely that in *M. xanthus* in the presence of methionine, methylation of FrzCD (a MCP analog) was inhibited and cellular reversal frequency reduced; both of these may be caused by reduced cellular levels of SAM.

Methionine and its analogs block the biosynthesis of SAM and development in *M. xanthus*. To test the hypothesis that methionine and its analogs block SAM biosynthesis and development in *M. xanthus*, we examined the intracellular levels of SAM by HPLC (see the section headed "Assays" above for detailed procedures). We found that SAM levels during development were about the same as they were during vegetative growth. However, cells exposed to high levels of methionine contained no detectable SAM after 20 h (Table 2). These results are consistent with the pattern of demethylation of FrzCD and strongly suggest that SAM plays an important role in developmental chemotaxis.

To further study the requirement of SAM for fruiting body formation, we tested several chemicals which are known to affect SAM biosynthesis and examined their effects on fruiting body formation. Selenomethionine is a methionine analog which has a strong and specific inhibition for SAM synthetase (8). We found that at very low concentrations (10 to 100 μ M), selenomethionine was able to block SAM biosynthesis and fruiting body formation (Tables 2 and 3). Similar results were obtained with another methionine analog, ethionine (Table 3). It has been shown that in the enteric bacteria, addition of excess lysine, threonine, or isoleucine can cause methionine starvation and, consequently, reduced SAM synthesis (4, 8, 21). Our data showed that when these amino acids were added to *M. xanthus* at high concentrations, they caused reduced cellular levels of SAM and blocked fruiting body formation (Tables 2 and 3). The above data established a consistent correlation between cellular levels of SAM and fruiting body formation. This suggests that SAM is very important for *M. xanthus* de-

velopment and that the inhibitory effect of methionine addition may be caused by a block in SAM biosynthesis. However, when we added SAM (up to 100 μ M) to MOPS medium containing methionine (1 mM), we were not able to rescue fruiting body formation (data not shown). A previous study showed that small amounts of SAM will enter *M. xanthus* cells (15); therefore, this result appears to be inconsistent with our hypothesis. However, it is possible that there is not enough SAM to enter cells to relieve starvation.

We tested all the other amino acids (neutralized to pH 7.0 and added to MOPS plates) to see their effects on fruiting body formation and SAM biosynthesis. Most amino acids (for example, serine) have no effect on fruiting body formation even at the highest concentration tested (up to 20 mM) (Table 3). However, phenylalanine, tyrosine, and tryptophan were also found to block fruiting body formation at concentrations higher than 5 mM (Table 3), as has been reported previously (5, 7). HPLC analysis showed that cells exposed to phenylalanine contained only slightly reduced SAM levels (Table 2); therefore, its inhibitory effect on fruiting body formation must be through a different mechanism. One such alternative pathway is A signaling (12).

Reduced developmental gene expression under the conditions in which SAM biosynthesis is blocked. Previously, it was found that many developmentally regulated polypeptides observed by SDS-polyacrylamide gel electrophoresis are not induced in the presence of methionine (9). Here, we studied the effect of methionine on developmental gene expression using a set of developmentally regulated Tn5::lac fusion strains made and described by the Kaiser laboratory (11). Table 4 shows that methionine had an inhibitory effect on developmental gene expression; in particular, it had a strong inhibitory effect on late gene expression. These results are in agreement with the SDS-polyacrylamide gel electrophoresis data previously described (9). It is of interest that selenomethionine, ethionine, lysine, and threonine had inhibitory effects similar to that of methionine; however, most other amino acids (for example, serine) had no effect at all (Table 4 and data not shown). These results indicate that the pathway for developmental gene expression requires SAM. It is also interesting that phenylalanine also inhibited developmental gene expression, even though it did not significantly affect SAM biosynthesis (data not shown).

In conclusion, in this paper we have reexplored the effect of methionine on development of *M. xanthus*. We were able to verify the inhibitory effects of high levels of methionine on

TABLE 4. Effect of methionine on developmental gene expression^a

Strain	Time of expression (h)	Developmental gene expression (%)				
		MOPS alone	MOPS + 1 mM Met	MOPS + 0.1 mM selenomet ^b	MOPS + 10 mM Lys	MOPS + 20 mM Ser
DK4411	0-1	100	94	97	92	93
DK4491	0-1	100	46	66	54	99
DK4300	0-1	100	51	56	41	94
DK4469	5	100	60	65	56	90
DK4414	10	100	20	11	35	101
DK4293	25	100	13	8	18	92
DK5204	25	100	5	2	12	105

^a Numbers shown are β -galactosidase activities of developmentally regulated Tn5 lac fusions assayed by the procedures described in Kroos et al. (11). The actual numbers for DK4411, DK4491, DK4300, DK4469, DK4414, DK4293, and DK5204 (under the MOPS-alone condition) were 1,423, 68, 87, 1,104, 1,673, 431, and 1,537 nmol of ONP/min/mg of protein, respectively.

^b Selenomet, selenomethionine.

cellular aggregation and fruiting body formation. We found that cells treated with high concentrations of methionine for extended time intervals (>20 h) exhibited less frequent cell reversals and contained unmethylated FrzCD. These findings are consistent with the absence of SAM in the cells under these conditions. HPLC analysis showed that the cellular levels of SAM were indeed extremely low in the cells treated with methionine. Methionine, its analogs, and several other amino acids in the aspartate family such as threonine, lysine, and isoleucine also caused reduced cellular levels of SAM and resulted in blocked fruiting body formation in *M. xanthus*. These results establish a strong correlation between cellular levels of SAM and development, indicating that SAM is important for development of *M. xanthus*; the inhibitory effects of methionine on development result, at least in part, from blocking the biosynthesis of SAM. It would be of interest to investigate further how SAM affects fruiting body formation at the molecular and biochemical levels.

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