JOURNAL OF BACTERIOLOGY, July 1973, p. 29-34
Vol. 115, No. 1

Aspartokinase Activity and the Developmental Cycle of *Myxococcus xanthus*

EUGENE ROSENBERG, DAVID FILER, DINA ZAFRITI, AND S. H. KINDLER
Department of Microbiology, Tel Aviv University, Tel Aviv, Israel

Received for publication 13 February 1973

The relationship between aspartokinase activity and fruiting body formation in *Myxococcus xanthus* was investigated. Two required amino acids, methionine and isoleucine, which stimulated the enzyme in vitro also inhibited fruiting body formation when added to 0.1% Casitone agar. Threonine, a potent feedback inhibitor of the aspartokinase, completely reversed the effects of methionine and isoleucine both on enzyme activity and fruiting body formation. A mutant, *M. xanthus* FB-S, which had the unusual property of forming fruiting bodies on 1.0% Casitone agar, also exhibited an altered regulation of aspartokinase activity. Spermidine, which is a strong stimulator of the enzyme in vitro, interfered with the developmental cycle of both *M. xanthus* FB and FS-S. During glycerol induction of myxospores the level of aspartokinase dropped more than 75% during the first hour. These data indicate a strong correlation between aspartokinase activity and the induction of the developmental cycle in *M. xanthus*. It is suggested that the decrease in aspartokinase activity results in diaminopimelic acid starvation, blockage of cell wall growth, and subsequent induction of the developmental cycle.

Myxobacteria of the genus *Myxococcus* characteristically go through a developmental cycle (8) consisting of both cellular morphogenesis (myxospore formation) and colonial morphogenesis (fruiting body formation). An early observation (15) and one that has been confirmed frequently (e.g., reference 7) is that relatively low concentrations of peptone (0.1-0.5%) support vegetative growth and subsequent fruiting body formation on solid medium, whereas high concentrations of peptone (1-3%) encourage vegetative growth but inhibit completely the development of fruiting bodies and myxospores. The cellular morphogenesis became amenable to laboratory investigation with the discovery by Dworkin and Gibson (9) that addition of 0.5 M glycerol to exponentially growing cells induced rapid and synchronous myxospore formation. Although the glycerol induction technique proved invaluable for the study of various biochemical (2, 3, 17, 18, 20, 21, 23) and morphological (1, 10, 16, 19) changes accompanying myxospore formation, the technique provided little insight into the underlying induction mechanism.

Recently, induction of myxospore formation in a defined medium by methionine starvation or by addition of 0.1 M threonine plus 0.1 M isoleucine was reported (22). Spermidine (2 and 5 mM) inhibited induction of myxospores brought about by either methionine starvation or high isoleucine-threonine concentrations. This led to the hypothesis that spermidine is an intracellular inhibitor of myxospore formation; when spermidine formation is blocked by methionine, starvation morphogenesis is induced. Two problems arose in attempting to provide further evidence for this hypothesis. (i) Even high concentrations of methionine could not reverse the effects of isoleucine-threonine, suggesting that isoleucine-threonine was working by a mechanism other than by inducing methionine starvation. (ii) If the hypothesis was correct, then it should have been possible to isolate spermidine-requiring mutants of *M. xanthus* by (positive) selection for myxospores in the absence of the polyamine. All attempts to obtain such mutants failed.

An alternative explanation for myxospore induction came from an investigation of the regulation of aspartokinase (ATP:L-aspartate 4-phosphotransferase, EC 2.7.2.4) in *M. xanthus* (11). The aspartokinase activity of *M. xanthus* is repressed and feedback-inhibited by

L-lysine and L-threonine and "feedback stimulated" by L-isoleucine and L-methionine. Thus, these amino acids could exert their influence on the induction process by interacting with the aspartokinase. Evidence is presented in this paper that alteration of aspartokinase activity either by nutritional or mutational means influences the induction of the developmental cycle of *M. xanthus* FB, possibly by regulating the biosynthesis of diaminopimelic acid (DAP).

(Part of this report was taken from a dissertation submitted by David Filer to Tel Aviv University in partial fulfillment of the requirement of the M.Sc. degree.)

MATERIALS AND METHODS

Bacterial strains and culture conditions. The following strains of *M. xanthus* have been employed in this study: FB, a dispersed growing mutant (6) which was obtained from M. Dworkin; FBmp, which was derived from FB (22) and which grows well in defined medium M1 but does not form fruiting bodies either on 0.1 CT agar or *E. coli* agar (8); and FB-S, spontaneous mutant of FB which formed fruiting bodies on 1% CT agar.

Synthetic medium M1 was as described previously (24) except that tryptophan was omitted. In some cases, M1 media lacking lysine or threonine, or both, were employed. Casitone media, 1 CT and 0.1 CT, contained 1% and 0.1% Casitone (Difco), respectively. Solid media were prepared by addition of 1.5% agar (Difco). All media were supplemented with 1.0 mg $MgSO_4$ per ml. In addition, the 0.1 CT liquid medium was supplemented with 0.01 M phosphate buffer, pH 7.4. Organisms were grown at 32 C with vigorous gyratory shaking. For myxospore induction in liquid media (2), 10 M glycerol was added directly to exponentially growing cultures to yield a final concentration of 0.5 M glycerol. The growth rates of *M. xanthus* FB on 1 CT and 0.1 CT media are identical to those reported for *M. xanthus* FBmp (11).

Fruiting body formation. To check for fruiting body formation, 0.1-ml samples of a culture of exponentially growing *M. xanthus* FB or *M. xanthus* FB-S in 1 CT medium (10^8 cells/ml) were applied as small droplets to a variety of solid media. The plates were incubated at 32 C. Where fruiting body formation was evident (e.g., *M. xanthus* FB on 0.1 CT agar), the process was complete in 3 to 4 days. In all cases, the resulting fruiting bodies or vegetative colony were examined further for the presence of myxospores with a phase microscope. Unless stated otherwise fruiting bodies contained only myxospores, and vegetative colonies only vegetative cells.

Chemicals. Amino acids used in growth media were L-isomers purchased from Nutritional Biochemicals Corp., Cleveland, Ohio. Amino acids used in the enzyme assay were products of Calbiochem Corp., Los Angeles, Calif. Spermidine was purchased from Serva Feinbiochemica, Heidelberg, Germany. Diaminopimelic acid was purchased from Calbiochem Corp.

Paper chromatography. To determine the level of contamination of methionine in the commercial preparations of leucine employed in these studies, the following solvent systems were utilized for descending paper chromatography on Whatman no. 1 paper at room temperature: (i) *n*-butanol-acetic acid-water (4:1:4, vol/vol/vol) and (ii) butyric acid-*n*-butanol-water (2:2:1, vol/vol/vol). After developing and air-drying, chromatograms were searched for amino acids by being dipped in a 0.2% solution of ninhydrin in acetone, which contained 2% of pyridine, added just prior to use, followed by heating at 105 C.

Aspartokinase assay. The aspartokinase activity was assayed by measuring the formation of L-aspartic-β-hydroxamate by the procedure described in the accompanying paper (11). The standard reaction mixture contained 5 mM $MgSO_4$, 10 mM adenosine 5'-triphosphate (ATP), 20 mM 2-mercaptoethanol, 800 mM $NH_2OH \cdot HCl$, 30 mM L-aspartic acid, 100 mM tris(hydroxymethylaminomethane-hydrochloride (pH 7.5), and enzyme. Specific activity is defined as nanomoles of aspartyl hydroxamate formed per minute per milligram of protein. The 30 to 50% $(NH_4)_2SO_4$ fraction (11) was utilized for all of the experiments reported here.

RESULTS

Methionine requirement for the growth of M. xanthus FBmp. It was reported previously (22) that *M. xanthus* FBmp could grow in the absence of methionine if supplied with 2.0 mg of leucine per ml. Since methionine starvation and microcyst formation took place when the culture contained 0.5 mg of leucine per ml, it was postulated that leucine somehow regulates the methionine biosynthetic pathway. An alternative and less interesting explanation for the observation was suggested by A. L. Demain based on his experience that commercial grades of "methionine-free" leucine often contain appreciable quantities of methionine (5). By paper chromatography it was determined that the batch of L-leucine previously employed (Nutritional Biochemical Corp., chemically pure and "methionine free," 5833) contained 0.5 to 1.25% methionine. Thus, the 2 mg of leucine per ml employed in the medium contained 10 to 25 μg of methionine per ml, which is sufficient for maximal growth. Furthermore, neither *M. xanthus* FB nor *M. xanthus* FBmp will grow in the absence of added methionine even when supplemented with 2 mg of leucine per ml (Calbiochem. Corp., less than 0.01% methionine contamination).

Aspartokinase activity of M. xanthus FB. A comparison of the aspartokinase activities of *M. xanthus* FB when grown on 1 CT and 0.1 CT media is shown in Table 1; the doubling times were 4.4 and 11.5 h, respectively. The weak activity derived from cells grown on 1 CT

TABLE 1. *Aspartokinase activity of M. xanthus FB*

Growth medium[a]	Sp act[b] in the presence of 1 mM:				
	No addition	Lys	Thr	Ileu	Met
1.0 CT	8.6	7.4	3.1	15.8	11.8
0.1 CT	23.3	10.0	9.0	39.0	29.0

[a] The composition of 1.0 CT and 0.1 CT media are described in Materials and Methods.

[b] Specific activities were determined on the 30–50% $(NH_4)_2SO_4$ fractions and were measured as nanomoles of aspartyl hydroxamate formed per minute per milligram of protein.

medium was feedback-inhibited by threonine and lysine and stimulated by isoleucine and methionine. Growth on 0.1 CT medium resulted in about a threefold increase of the activity. This activity was inhibited approximately 60% by 1 mM lysine or threonine and stimulated 25% and 67% by 1 mM methionine and isoleucine, respectively. These data do not differ significantly from those obtained from the nonfruiting strain FBmp (11).

Fruiting body formation of M. xanthus FB on supplemented 0.1 CT agar. To test the hypothesis that variation in aspartokinase activity influences the developmental cycle of *M. xanthus*, cells were spotted on solid media supplemented with effectors of aspartokinase (Table 2, Fig. 1). The two amino acids, methionine and isoleucine, which stimulated the enzyme in vitro also inhibited fruiting body formation when added to 0.1 CT agar. The minimum concentrations required to completely block fruiting body formation were 2 and 6 mM for methionine and isoleucine, respectively. Threonine, a potent feedback inhibitor of the aspartokinase, completely reversed the effects of both methionine and isoleucine. The results of lysine supplementation were less clear; at low concentration (1–3 mM) there were no discernible effects, whereas at concentrations greater than 4 mM, lysine blocked fruiting body formation. Thus, with the possible exception of lysine, which will be discussed later, there is a good inverse correlation between the affect of these amino acids on aspartokinase activity in vitro and fruiting body formation in vivo.

Characterization of M. xanthus FB-S. A spontaneous mutant of *M. xanthus* FB, which had the unusual property of forming fruiting bodies on 1 CT agar, was isolated for further investigation. The strong tendency of this mutant, referred to as *M. xanthus* FB-S, to form fruiting bodies on solid media is shown in Table 3. Fruiting body formation took place even when the medium was supplemented with relatively high concentrations of methionine or isoleucine. The growth of *M. xanthus* FB-S in liquid 1 CT medium also differed significantly from that of the parent culture. The cells clump and form rings on the glass surface even with vigorous shaking, making accurate determination of growth rates impossible. Furthermore, shortly after the culture reaches stationary phase, the cells begin to lyse. Thus, frequent transfers are necessary to maintain *M. xanthus* FB-S in liquid culture.

The aspartokinase activity of *M. xanthus* FB-S is summarized in Table 4. The enzyme derived from the mutant grown on 1 CT medium exhibited a two- to threefold higher specific activity than the parent strain grown under identical conditions. An even greater difference between the *M. xanthus* FB and FB-S enzyme preparations after growth on 1 CT was the extent of methionine and isoleucine stimulation. The mutant enzyme was stimulated only 13% and 12% by isoleucine and methionine, respectively, whereas the FB enzyme was stimulated 84% and 37% by these amino acids (Table 1). Growth of the mutant on 0.1 CT caused a four- to fivefold decrease of enzyme formation. The low activity present under these conditions was, however, strongly stimulated by isoleucine and methionine. These data demonstrate the strikingly different control patterns for the biosynthesis of aspartokinase in the two strains. Whether these differences are due di-

TABLE 2. *Nutritional control of fruiting body formation in M. xanthus FB*

Agar composition[a]	Result after 4 days[b]
1.0 CT	VEG
0.1 CT	FB
0.1 CT + Met (1–3 mM)	VEG
0.1 CT + Ileu (6 mM)	VEG
0.1 CT + Thr (1–5 mM)	FB
0.1 CT + Lys (1–3 mM)	FB
0.1 CT + Lys (5–10 mM)	VEG
0.1 CT + Met (2 mM) + Thre (1 mM)	FB
0.1 CT + Ileu (6 mM) + Thre (1 mM)	FB
0.1 CT + Met (2 mM) + Lys (2 mM)	VEG
0.1 CT + Ileu (6 mM) + Lys (2 mM)	VEG

[a] The composition of 1.0 CT and 0.1 CT agar are described in Materials and Methods.

[b] Approximately 0.01 ml of a suspension of 10^8 vegetative cells per ml were spotted; incubation was at 32 C. Abbreviations: VEG, vegetative colony; FB, fruiting bodies.

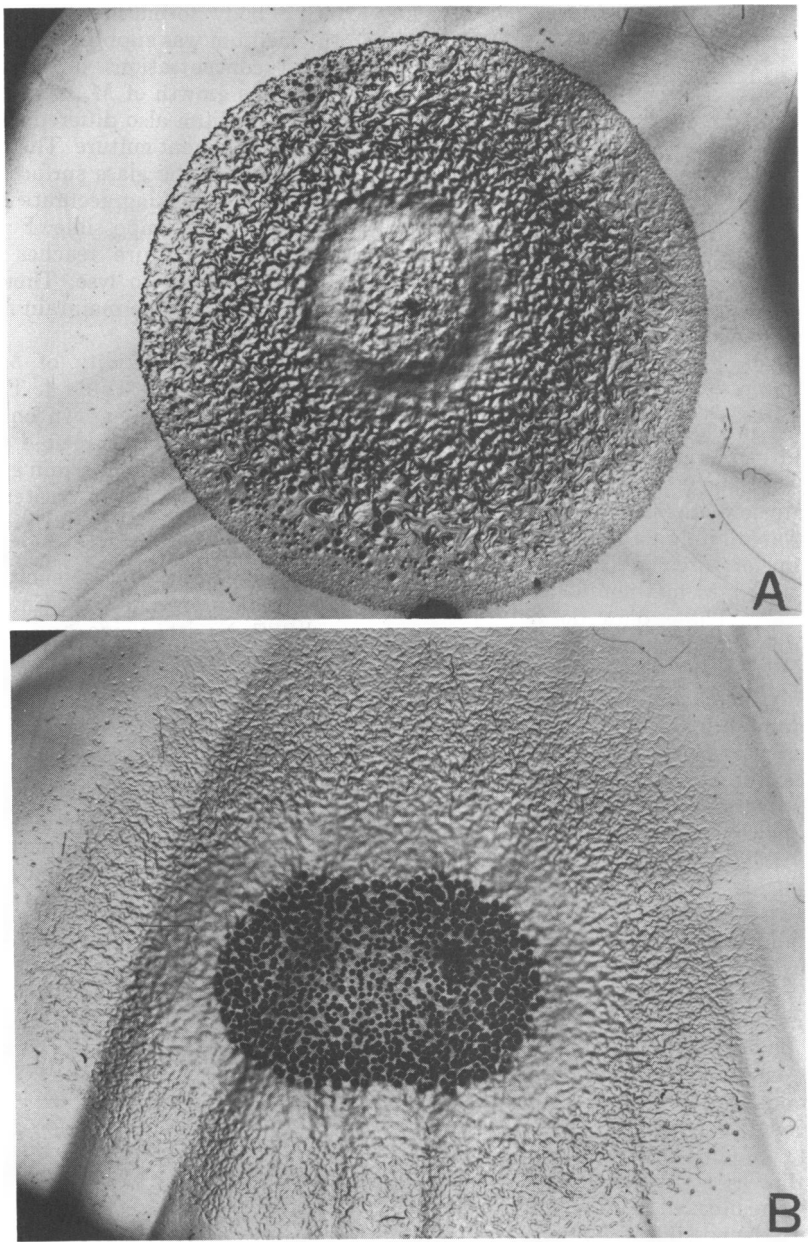

FIG. 1. *Nutritional control over fruiting body formation in Myxococcus xanthus FB. Samples (0.1 ml) of an exponentially growing culture (10^8 cells per ml) were applied to 0.1 CT agar plates supplemented with either 2 mM methionine (A) or 2 mM methionine plus 1 mM threonine (B). The plates were incubated at 32 C for 4 days.*

rectly to a mutation in a regulator gene for aspartokinase or are the indirect consequence of mutation at a different site can not be established by these experiments.

Effect of spermidine on fruiting body formation and aspartokinase activity. Since previously it had been reported that spermidine blocked induction of myxospores in liquid medium (22) and also was a potent stimulator of the aspartokinase in vitro (11), it seemed worthwhile to examine the effect of this polyamine upon fruiting body formation. Spermidine had a profound effect upon the developmental cycle of both *M. xanthus* FB and *M. xanthus* FB-S.

TABLE 3. *Fruiting body formation of M. xanthus FB-S*

Agar composition	Results after 4 days[a]
1.0 CT	FB
0.1 CT	Poor growth (some FB)
1.0 CT + Met (1–7 mM)	FB
1.0 CT + Ileu (1–10 mM)	FB

[a] The experiment was performed as described in Table 2. Abbreviation: FB, fruiting bodies.

When the parent strain, FB, was spotted on 0.1 CT agar containing spermidine (2–4 mM), aggregation and fruiting body formation took place, but microscope examination of the cells inside the fruiting bodies revealed about 50% vegetative cells and 50% abnormal myxospores (heavily granulated ovoids). Even after prolonged periods of incubation, normal spherical refractile myxospores were never observed. Spermidine (2–5 mM) completely blocked the developmental cycle of *M. xanthus* FB-S on 1 CT agar. Instead of aggregating and forming fruiting bodies, the cells developed into normal colonies containing only vegetative cells.

Aspartokinase activity during glycerol induced myxospore formation. The rapid and synchronous glycerol induction technique (9) provides an excellent system for examining enzymatic changes during the early stages of morphogenesis (e.g., reference 3). Accordingly, enzyme levels of aspartokinase were examined during the 2-h period after addition of inducer (Table 5). In 1.0 CT medium the specific activity decreased more than 75% during the first hour; the residual activity exhibited the characteristic stimulation by methionine and isoleucine and inhibition by threonine and lysine. The little activity that remained at 2 h could not be stimulated by addition of isoleucine or methionine.

DISCUSSION

The results of this investigation indicate a strong correlation between aspartokinase activity and the induction of the developmental cycle in *M. xanthus*. (i) Amino acids which modify the activity of the enzyme in vitro affect fruiting body formation in a reciprocal manner in vivo. The only exception to this conclusion is lysine which feedback-inhibits and represses the enzyme, but does not apparently stimulate fruiting body formation. It should be pointed out, however, that data from the accompanying paper (11) indicate that lysine is a much poorer inhibitor of aspartokinase than threonine at low concentration. (ii) A mutant, *M. xanthus* FB-S, with altered control over fruiting body formation also contained altered regulation of aspartokinase activity. (iii) Spermidine, which is a potent stimulator of the enzyme in vitro, interfered with the developmental cycle of both *M. xanthus* FB and FB-S. (iv) The enzyme activity decreased during glycerol induction of myxospores. This decrease of aspartokinase in *M. xanthus* is in sharp contrast to the rapid rise in specific activities of isocitrate lyase and malate synthase during the initial 2 h after initiation of myxospore formation (3).

Granted the inherent difficulty in ascribing cause and effect relationships from correlations, the following simple hypothesis might, nevertheless, explain the data: decrease in aspartokinase activity results in DAP starvation, blockage of vegetative cell wall synthesis, and subsequent induction of the developmental cycle. It should be emphasized that DAP biosynthesis is required for cell growth but that there is no net increase in DAP during myxospore formation (21).

The "DAP hypothesis" provides an explanation (at an enzymatic level) for myxospore induction in liquid medium by methionine

TABLE 4. *Aspartokinase activity of Myxococcus xanthus FB-S*

Growth medium[a]	Sp act[b] in the presence of 1 mM:				
	No addition	Lys	Thr	Ileu	Met
1.0 CT	19.7	12.7	5.7	22.3	22.0
0.1 CT	4.3	2.0	2.5	14.3	8.7

[a] The composition of 1.0 CT and 0.1 CT media are described in Materials and Methods.

[b] Specific activities were determined on the 30–50% $(NH_4)_2SO_4$ fraction and were measured as nanomoles of aspartyl hydroxamate formed per minute per milligram of protein.

TABLE 5. *Aspartokinase activity of M. xanthus FBmp during glycerol-induced myxospore formation*

Time after glycerol induction (h)[a]	Sp act[b] in the presence of 1 mM:				
	No addition	Lys	Thr	Ileu	Met
0	8.6	7.4	3.1	15.8	11.8
1	1.9	0.8	0.0	4.6	11.2
2	1.8	0.0	1.1	1.4	1.8

[a] The cells were grown in 1.0 CT and induced to form myxospores by addition of 0.5 M glycerol as described in Materials and Methods.

[b] Specific activities were determined on the 30–50% $(NH_4)_2SO_4$ fraction and were measured as nanomoles of aspartyl hydroxamate formed per minute per milligram of protein.

starvation, addition of high isoleucine-threonine, or glycerol, and the inhibitory effect of spermidine. In addition to a direct effect upon the enzyme, methionine starvation and high concentrations of isoleucine plus threonine cause a lowering of the spermidine pool (22), thereby decreasing the most efficient stimulator of the aspartokinase. This hypothesis might also explain how high concentrations of peptone generally permit vegetative growth but prevent fruiting body formation. At high concentrations of amino acids, aspartokinase is required only for the formation of DAP; at low concentrations, the cells must also utilize aspartokinase for the synthesis of lysine or threonine, or both (as evidenced by the derepression of the enzyme in 0.1 CT medium). As long as the intracellular concentration of the two required amino acids, methionine and isoleucine, remain high, the derepressed and stimulated enzymatic activity is adequate for vegetative growth; however, when the methionine and isoleucine levels also fall, then insufficient aspartylphosphate is formed, leading to DAP starvation and fruiting body formation. Such a regulatory system could serve as at least one indicator of substrate availability in a natural environment for a protein degrading organism that has the option of converting into a relatively stable form under adverse conditions.

The DAP hypothesis does not provide a direct explanation for the reported (i) inhibition of fruiting body formation by phenylalanine and tryptophan (7) on defined media and (ii) induction of the developmental cycle on a dilute defined medium which was further modified by starvation for any required or stimulatory amino acid (12, 13). Certainly much more needs to be known about general amino acid metabolism and metabolic interlocks (14) in *M. xanthus* for these data to be explained. One relevant point is that the aspartokinase activity of *M. xanthus* is strongly inhibited by adenosine diphosphate in vitro; thus any nutritional alteration which would bring about a change in the adenine nucleotide levels might lead to DAP starvation either by decreasing the energy charge (4) or reversing the aspartokinase reaction simply by mass action.

LITERATURE CITED

1. Bacon, K., and F. A. Eiserling. 1968. A unique structure of microcysts of *Myxococcus xanthus*. J. Ultrastruct. Res. **21**:378–382.
2. Bacon, K., and E. Rosenberg. 1967. Ribonucleic acid synthesis during morphogenesis in *Myxococcus xanthus*. J. Bacteriol. **94**:1883–1889.
3. Bland, J., W.-K. Yeh, D. White, and A. Hendricks. 1971. Increase in glyoxylate shunt enzymes during cellular morphogenesis in *Myxococcus xanthus*. Can. J. Microbiol. **17**:209–211.
4. Chapman, A. G., L. Fall, and D. E. Atkinson. 1971. Adenylate energy charge in *Escherichia coli* during growth and starvation. J. Bacteriol. **108**:1072–1086.
5. Demain, A. L. 1965. Contamination of commercial L-leucine preparations with methionine and cystine. J. Bacteriol. **89**:1162.
6. Dworkin, M. 1962. Nutritional requirements for vegetative growth of *Myxococcus xanthus*. J. Bacteriol. **84**:250–257.
7. Dworkin, M. 1963. Nutrional regulation of morphogenesis in *Myxococcus xanthus*. J. Bacteriol. **86**:67–72.
8. Dworkin, M. 1966. Biology of myxobacteria. Annu. Rev. Microbiol. **20**:75–106.
9. Dworkin, M., and S. Gibson. 1964. A system for studying microbial morphogenesis: rapid formation of microcysts in *Myxococcus xanthus*. Science **146**:243–244.
10. Dworkin, M., and W. Sadler. 1966. Induction of cellular morphogenesis in *Myxococcus xanthus*. I. General description. J. Bacteriol. **91**:1516–1519.
11. Filer, D., E. Rosenberg, and S. H. Kindler. 1973. Aspartokinase of *Myxococcus xanthus*: "feedback stimulation" by required amino acids. J. Bacteriol. **115**:23–28.
12. Hemphill, H. E., and S. A. Zahler. 1968. Nutrition of *Myxococcus xanthus* FBa and some of its auxotrophic mutants. J. Bacteriol. **95**:1011–1017.
13. Hemphill, H. E., and S. A. Zahler. 1968. Nutritional induction and suppression of fruiting in *Myxococcus xanthus* FBa. J. Bacteriol. **95**:1018–1023.
14. Jensen, R. A. 1969. Metabolic interlock: regulatory interactions exerted between biochemical pathways. J. Biol. Chem. **244**:2816–1823.
15. Quehl, A. 1906. Untersuchungen über die Myxobakterien. Zentralbl. Bakteriol. Parasitenk. Infektionskr. Abt. 2. **16**:9–34.
16. Ramsey, W. S., and M. Dworkin. 1968. Microcyst germination in *Myxococcus xanthus*. J. Bacteriol. **95**:2249–2257.
17. Rosenberg, E., M. Katarski, and P. Gottlieb. 1967. Deoxyribonucleic acid synthesis during exponential growth and microcyst formation in *Myxococcus xanthus*. J. Bacteriol. **93**:1402–1408.
18. Sadler, W., and M. Dworkin. 1966. Induction of cellular morphogenesis in *Myxococcus xanthus*. II. Macromolecular synthesis and mechanism of an inducer action. J. Bacteriol. **91**:1520–1525.
19. Voelz, H., and M. Dworkin. 1962. Fine structure of *Myxococcus xanthus* during morphogenesis. J. Bacteriol. **84**:943–952.
20. Watson, B. F., and M. Dworkin. 1968. Comparative intermediary metabolism of vegetative cells and microcysts of *Myxococcus xanthus*. J. Bacteriol. **96**:1465–1473.
21. White, D., M. Dworkin, and D. J. Tipper. 1968. Peptidoglycan of *Myxococcus xanthus*: structure and relation to morphogenesis. J. Bacteriol. **95**:2186–2197.
22. Witkin, S. S., and E. Rosenberg. 1970. Induction of morphogenesis by methionine starvation in *Myxococcus xanthus*: polyamine control. J. Bacteriol. **103**:641–649.
23. Zusman, D., and E. Rosenberg. 1968. Deoxyribonucleic acid synthesis during microcyst germination in *Myxococcus xanthus*. J. Bacteriol. **96**:981–986.
24. Zusman, D., and E. Rosenberg. 1970. DNA cycle of *Myxococcus xanthus*. J. Mol. Biol. **49**:609–619.