JOURNAL OF BACTERIOLOGY, Mar. 1968, p. 1018-1023
Vol. 95, No. 3

Nutritional Induction and Suppression of Fruiting in *Myxococcus xanthus* FBa

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Received for publication 5 January 1968

A defined agar medium (A agar) containing 15 amino acids in concentrations between 0.5 and 2 mM was developed for studying the fruiting cycle of *Myxococcus xanthus* FBa. Cells grew only vegetatively in this medium unless the initial concentration of one of nine required or stimulatory amino acids was lowered about 50-fold. In the latter circumstance, fruiting bodies developed after several days of vegetative growth. The conclusion was that fruiting occurred when any amino acid required for normal growth became limiting in the environment. High concentrations (10 mM) of phenylalanine, tryptophan, or methionine prevented fruiting without affecting growth. Mutants requiring arginine, thymidine, or adenine could not be induced to fruit by limiting their unique requirement although they responded to the same deprivations which brought about fruiting of the wild type. A histidine auxotroph formed fruiting bodies when histidine was lowered to growth-limiting concentrations, provided that the medium was supplemented with purines. A uracil auxotroph was isolated that, perhaps secondarily, had lost some of the mechanisms which control the formation of fruiting bodies; if uracil was present, it formed fruits even when no amino acid was limiting. No concentration of uracil was sufficient to prevent fruiting. Fruiting bodies were formed when mixtures of the uracil auxotroph and wild-type cells were inoculated on A agar plus uracil, even when 75% of the cells were wild type. Microcysts of both strains were present in the fruiting bodies.

It has long been known that the induction of the fruiting cycle in myxobacteria is intimately related to nutrition. [For a review of this and other aspects of myxobacterial physiology, see Dworkin (3).] Many investigators who have worked with these organisms have noted that, whereas myxobacters often grow luxuriantly on a variety of laboratory media, the formation of fruiting bodies is usually restricted to cells growing on relatively poor nutrient media (3, 9, 12).

Interpretations of this observation fall mainly into two classes. One is that fruiting occurs in response to the depletion of nutrients below the level necessary to sustain vegetative growth. Alternatively, fruiting may be specifically suppressed by high concentrations of one or more substances present in the environment.

With the development by Dworkin (1, 2) of defined media for the growth of *Myxococcus xanthus* FB, it was possible to examine this problem. Dworkin reported that, when cells grown in rich medium were prestarved in buffer and then plated on his defined medium, fruiting took place, but only when phenylalanine and tryptophan were omitted or reduced in concentration. This result has been interpreted to mean that aromatic amino acids act overtly to suppress fruiting. Evidence implying the importance of these amino acids has been obtained in other strains of *Myxococcus* (7) and in *Chondromyces* (8).

The present study reports the development of a defined medium which supports both growth and fruiting of *M. xanthus* FBa. This medium has been used to study which elements of nutrition influence fruiting in the wild-type organism and to determine the effects of various metabolites on fruiting in several auxotrophs. We have found that depletion of some nutrients induces fruiting and that the presence of high concentrations of some nutrients suppresses fruiting.

MATERIALS AND METHODS

Organism. Most of the strains have been described in the previous paper (6). Arginine and histidine

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auxotrophs were isolated by the following variations of the methods described there for auxotroph isolation. C broth was replaced by LM. Penicillin treatment was carried out in LM lacking either arginine or histidine, rather than in LM. Two cycles of penicillin selection were needed for the isolation of the histidine auxotroph, and three cycles for the isolation of the arginine auxotroph.

Media. Liquid cultures for inoculation were grown in C Broth (6). Soft agar contains 7.5 g of Difco agar per liter. A agar contains 2.0 mM asparagine, proline, and histidine; 1.0 mM glycine, lysine, serine, valine, and alanine; 0.5 mM leucine, isoleucine, arginine, methionine, tryptophan, threonine, and phenylalanine; 1.0 mM $MgSO_4$; 2.5 mM potassium phosphate buffer, *p*H 7.6; and 15 g of agar per liter. Unless otherwise specified, L-amino acids and Difco agar were used.

Solutions containing amino acids were made up in 5× concentration, filter-sterilized, added to molten agar at 60 C, and poured into plastic petri plates. Solutions of free purine and pyrimidine bases were autoclaved; those of nucleotides were filter-sterilized.

Inoculation. Most inoculations (spot tests) were made by diluting cells grown in liquid medium into buffer to give about 2×10^7 cells/ml, centrifuging and resuspending twice, and inoculating about 0.01 ml on the surface of each plate.

Other inoculations were made by cloning the cells in 3-ml soft agar overlays. Incubations were at 30 C in a saturated incubator.

Counting fruiting clones. In each experiment involving cloned cells, about 200 clones were examined after 10, 12, and 15 days of incubation for the presence of fruiting bodies. A clone was said to be fruiting if it contained at least one aggregation of cells with microcysts, even if the sharp outline typical of normal fruiting bodies was not visible. Some fruiting bodies (particularly on control A agar plates) disappeared between the 10th and 15th days of incubation. The percentage of fruiting clones on the day with the highest percentage was used throughout this work. The result of this procedure is to exaggerate the percentage of fruiting clones when the percentage is small.

Assays of fruiting bodies. Fruiting bodies were scraped from agar plates with an inoculating loop, suspended in 0.01 M potassium phosphate buffer (*p*H 7.6), and blended in a Lourdes blender for 60 sec. To assay for the presence of microcysts, samples were heated in a water bath at 60 C for 5 min. In assays of mixed fruiting bodies containing both wild-type and auxotrophic cells, comparisons were made of the number of viable cells of each strain before and after the heat test by plating on appropriate selective media.

RESULTS

A agar is a modification of LM medium (6), which in turn is a modification of the Dworkin defined medium (1). It contains 15 amino acids ranging in concentrations from 0.5 to 2.0 mM. With a few exceptions, these concentrations are considerably lower than those in LM and in the Dworkin medium. While this medium supported excellent growth on agar, A medium (unlike the other two defined media) did not support good turbid growth in liquid unless the concentrations of its components were increased three- or four-fold.

When prepared with 1.5% agar, A agar, like LM and the Dworkin medium, supported only vegetative growth of cells spotted on the surface. When cells were cloned on A agar, about 5% of the colonies had one or more fruiting bodies.

A agar could be modified to support both growth and fruiting by lowering the concentration of isoleucine from 0.5 to 0.01 mM. On this medium the cells grew vegetatively for 5 or 6 days (10 to 14 days for cloned cells) and then fruited. The results compared to A agar were most striking in cloning experiments; the percentage of fruiting clones increased from 5% on A agar to 98% on A agar with 0.01 mM isoleucine.

However, as shown in Table 1, isoleucine was only one of several amino acids that controlled or influenced fruiting. This table compares the results with cloned cells and spotted inocula on A agar with various amino acids lowered in concentration, usually to 0.01 mM. In some cases (e.g., valine) 0.01 mM was too low a concentration, and considerable fluctuation in cloning efficiency occurred. This was accompanied by large fluctuations from experiment to experiment in the percentage of clones that fruited. In such instances, better results both in cloning efficiency and in fruiting were obtained at 0.03 mM concentrations.

These results indicate that fruiting was induced when the concentration of any amino acid required for normal growth became limiting. The degree to which any given amino acid influenced fruiting was roughly proportional to its indispensability to the cell; i.e., required amino acids exerted a stronger influence than stimulatory amino acids, whereas indifferent amino acids had no influence.

To expand these results, we utilized auxotrophs of *M. xanthus* FBa to study fruiting control when the unique requirement of the mutant was limited. Attempts were first made to control fruiting in two amino acid auxotrophs requiring, respectively, arginine and histidine. We did not succeed by limiting arginine in the former strain, although this mutant did not fruit well even under conditions where FBa did; it may have a second genetic defect related to fruiting. The histidine auxotroph, on the other hand, fruited normally under conditions where the wild type fruits, and we hoped that it might also be induced to fruit by limiting histidine.

Table 2 demonstrates the results we obtained when we tried to induce fruiting in the auxotroph

TABLE 1. *Fruiting by Myxococcus xanthus FBa when the concentrations of single amino acids in A agar were decreased*

Amino acid	Concn (mM)	Status	Percentage of clones fruiting		Results with spot tests
			Range[a]	Mean	
A control			0–13	5	Heavy growth
Phenylalanine	0.01	Required	76–98	91	Fruiting
	0.03			98	
Isoleucine	0.01	Required	92–99	98	Fruiting
Leucine	0.01	Required	55–92	78	Fruiting
	0.03			88	
Methionine	0.01	Required	68–92	78	Fruiting
Valine	0.01	Required	35–96	64	Fruiting
	0.03			73	
Tryptophan[b]	0.01	Stimulatory	28–70	48	Some unstable fruits
Glycine	0.01	Stimulatory	52–57	55	Fruiting
Proline	0.01	Stimulatory	2–15	9	Fruiting
Threonine	0.01	Stimulatory	32–96	69	Unstable fruits
Asparagine[c]	0.03	Stimulatory		28	
Alanine[d]	0.1	Stimulatory	1–15	8	Heavy growth
Lysine	0.01	Stimulatory	1–5	3	Occasional fruits
Histidine	0.01	Indifferent	11–6	13	Heavy growth
Serine	0.01	Indifferent	0–9	5	Heavy growth
Arginine	0.01	Indifferent	0–6	3	Heavy growth

[a] Range of percentages when four or more tests were made at the indicated concentration.
[b] Fruits on tryptophan are unstable and collapse before maturing.
[c] Cloning efficiency is close to zero at 0.01 mM asparagine.
[d] Cloning efficiency is close to zero at 0.01 and 0.03 mM alanine.

TABLE 2. *Control of fruiting in a histidine auxotroph of Myxococcus xanthus FBa by limiting histidine in A agar*

Histidine concentration (mM)	Results	
	Without purine mixture	With purine mixture[a]
0–0.005	Little or no growth	Little or no growth
0.005–0.01	Moderate growth	Moderate growth
0.01–0.03	Good growth, many aggregations, a few fruits	Fruits
0.03–above	Heavy growth	Heavy growth

[a] Mixture contained 0.5 mM 5'-adenosine monophosphate, 0.5 mM adenosine, and 0.1 mM guanosine.

by limiting histidine. At limiting concentrations of histidine, the mutant formed aggregations of cells but did not develop mature fruiting bodies. However, if the medium were supplemented with 0.5 mM adenosine or with a mixture of purine derivatives, mature fruiting bodies with heat-resistant microcysts were formed.

Attempts to control fruiting in the adenine and thymidine auxotrophs by limiting their particular requirements were not successful.

The uracil auxotroph gave a unique pattern of fruiting on A medium, as shown in Table 3. At concentrations of 0.03 mM uracil and higher, the mutant formed fruiting bodies under conditions where the wild type grew only vegetatively. The structure of the fruits varied from normal to abnormally shaped mounds, but all contained heat-resistant microcysts.

The cause of fruiting in the uracil auxotroph was apparently not the limitation of environmental uracil, because no concentration of uracil was too high to allow fruiting. It is possible that the determinant of fruiting in this strain is a second mutation at another locus, or the uracil may actually be limiting because of uptake limitation.

Mixing experiments with two fruiting strains were performed by taking advantage of the fact that the uracil auxotroph fruited under conditions where the wild type did not. Washed cells of the two strains were mixed in various proportions and then spotted onto A agar plates containing 0.5 mM uracil. The mixtures were analyzed by cloning cells on A agar and on A agar with uracil. Fruits were analyzed by scraping them off the

plates, blending, and then assaying before and after heating to differentiate vegetative cells from microcysts.

Table 4 presents the results of one such experiment. Large numbers of wild-type cells formed microcysts when mixed with the uracil auxotroph. Indeed, even in mixtures where the great majority of the cells were wild type and where the structure of the fruiting bodies had degenerated to mere masses of cysts, there were about 100,000 times as many wild-type microcysts as occurred in the control.

We believe that these results indicate that: (i) the wild type did not excrete an inhibitor which prevented microcyst formation by the mutant, and thus, by implication, fruiting in rich medium is not prevented by excretions or metabolic products of the cells; and (ii) the uracil auxotroph, in the process of fruiting, produced substances which induced many wild-type cells to become microcysts (and perhaps also to enter into the process of aggregation), even though the wild-type cells were not nutritionally prepared to do so.

TABLE 3. *Fruiting of a uracil-requiring mutant of Myxococcus xanthus FBa on A agar with added uracil*

Concn of phenylalanine (mM)	Concn of uracil (mM)	Results	
		Wild-type	Ura⁻ strain
0.01	0.1	Fruiting	Fruiting
0.5	0	Heavy growth	No growth
0.5	0.0001	Heavy growth	No growth
0.5	0.0005	Heavy growth	Poor growth
0.5	0.001	Heavy growth	Moderate growth
0.5	0.003	Heavy growth	Good growth, occasional fruiting
0.5	0.01	Heavy growth	Good growth, occasional fruiting
0.5	0.03	Heavy growth	Abnormal fruiting
0.5	1.0	Heavy growth	Abnormal fruiting

Suppression of fruiting by particular amino acids. We were interested in the apparent discrepancy between our results, which suggest that fruiting is induced by depletion of nutrients, and those of other investigators suggesting that fruiting is overtly suppressed by the presence of high concentrations of specific amino acids. We felt that the most likely explanation was that both observations are correct and that fruiting, while normally induced by the disappearance of nutrients, can be suppressed under conditions where it would otherwise occur if certain amino acids are present at very high levels.

To test this, we prepared a series of plates containing A agar plus 0.5 mM uracil. To each plate we added enough of one of the 15 amino acids of A agar to increase its concentration to 10 mM, which was 5 to 20 times higher than normal. The uracil auxotroph was then spot-tested on each of these media. Fruiting occurred normally when alanine, asparagine, leucine, serine, or threonine was present at 10 mM concentration, or when no amino acids were present. Growth was slowed by 10 mM histidine, glycine, or valine, but fruiting was not prevented. Growth was also slowed by 10 mM lysine, and no fruiting bodies were formed. Arginine prevented growth completely at this concentration.

The other five amino acids of A agar, when raised to 10 mM concentration, had no apparent effect upon growth of the uracil auxotroph, but they interfered with fruiting body formation. Fruits produced in the presence of elevated proline were abnormal in appearance. High isoleucine concentration caused a delay of several days in the appearance of fruiting bodies. Most strikingly, methionine, tryptophan, and phenylalanine completely prevented the formation of fruiting bodies when present at 10 mM concentration.

TABLE 4. *Composition of mixed fruits of wild-type and ura⁻ cells on A agar containing 0.5 mM uracil*

Wild-type cells				Per centage of cells heat resistant	Description
In inoculum		In fruiting bodies (day 9)			
Calculated	Measured	Total cells	Microcysts		
%	%	%	%		
100	—	—	—	0.0006	Heavy vegetative growth
0 (ura⁻ control)	—	—	—	102	Fruiting
25	26	64	22	59	Abnormal fruits
50	67	67	22	40	Abnormal fruits
75	85	45	11	41	Masses of microcysts

Agar effects. The work reported in this paper was done by using Difco agar as a solidifying agent. The choice of this agar was based on a number of factors which reflect on the fruiting cycle.

Many of our original studies were done with Difco Purified Agar. This agar contains reduced concentrations of contaminating amino acids and makes experiments with very low concentrations of amino acids more meaningful. In most respects, results on one agar were comparable to those on the other, but there were exceptions. Neither tryptophan nor threonine could be used to control fruiting on Purified Agar, and proline and glycine only occasionally gave successful control.

Studies with cloned cells were difficult on Purified Agar. Cloning efficiency was low and variable; clones in soft agar overlays rarely fruited. When the cloning was done by spreading the cells on the surface of the agar, the clones fruited under the same conditions as did spot tests on this medium. However, quantification was difficult as the clones tended to coalesce because of the gliding motility of the myxobacters.

The reason for the differences between the two agars is unknown although we have some evidence that the surface properties of the two are different.

Fruiting in the presence of analogues. Leadbetter (7) and McCurdy (8) have reported that some strains of myxobacteria can be induced to fruit on rich media if phenylalanine analogues are incorporated into the medium.

We have investigated this with *M. xanthus* FBa by using a slightly modified A medium. In these studies, we used A medium with 0.1 mM phenylalanine and Difco Purified Agar as the solidifying agent. The lowering of the phenylalanine level was necessary because some phenylalanine analogues are not very soluble, and it was impossible to attain high ratios of analogue to phenylalanine when the level of the latter was high. Purified Agar was used because fruiting occasionally occurred at this lowered phenylalanine level when Difco agar was used.

We found that DL-*p*-fluorophenylalanine at 1.0 mM concentration would permit fruiting on A medium with 0.1 mM phenylalanine, whereas cultures on plates not containing the analogue grew only vegetatively. The *o*- and *meta*-fluoro analogues did not permit formation of normal fruits under these conditions, but they did greatly inhibit growth and often caused the formation of microcysts without the usual preformation of fruiting bodies.

DISCUSSION

The results of these experiments suggest that nutrition influences fruiting in several ways, and that the original observation that rich media enhance vegetative growth but suppress fruiting is really a composite of effects. At least three amino acids (methionine, phenylalanine, and tryptophan), when present at high concentrations, were able to suppress fruiting under conditions where it would otherwise have occurred, without noticeably affecting vegetative growth. Their combined effect would seem ample explanation for the failure of *M. xanthus* FBa to fruit on such media as LM, Dworkin's medium, and 2% Casitone (C agar).

Our results demonstrate that, even when the concentrations of methionine, phenylalanine, and tryptophan were below suppressive levels, fruiting did not occur in relatively rich media such as A agar, unless a required or stimulatory amino acid was lowered to trace levels. It seems that, if the concentration of nutrients is not limiting, vegetative growth continues until either the sheer mass of cells makes it physically impossible to form the fruiting bodies or the buildup of metabolic end-products prevents fruiting. Thus, the failure to fruit on rich media is a composite of the suppression by methionine, phenylalanine, and tryptophan and the lack of growth-limiting Conditions at a critical time.

It is not possible to compare our results directly with those of Dworkin (2), who studied the fruiting of washed, starved cells of strain FB (from which FBa is derived) plated on a medium containing more than three times as much nutrient as A agar. However, his medium contained approximately 6 mM phenylalanine and 5 mM tryptophan. These concentrations are probably high enough to inhibit fruiting completely; 10 mM concentrations certainly are. Therefore, it is not surprising that he was able to demonstrate good fruiting only when the concentrations of these two amino acids were lowered. Decreasing the concentration of leucine, for example, could not have induced fruiting while the phenylalanine and tryptophan concentrations were still high.

The actual induction of fruiting bodies seems to be a direct response to the disappearance of critical elements of nutrition. When an amino acid which the cells cannot synthesize in sufficient quantity for normal growth becomes limiting in the environment, the fruiting cycle is initiated, i.e., fruiting is a response to starvation. The biochemical mechanism by which fruiting is induced is not known, but, since many of the critical amino acids are not metabolically related in their synthesis

(e.g., phenylalanine and isoleucine), the point of action is probably in the areas of protein synthesis where amino acids interact—either in the formation of charged transfer ribonucleic acids (11) or in protein synthesis itself. The immediate signal to the cell may be the accumulation of one or more uncharged transfer ribonucleic acids, or general slow-down of protein synthesis. The action of analogues such as *p*-fluorophenylalanine can be reconciled with either mechanism.

The mixing experiments between wild type and the uracil auxotroph indicate, however, that once fruiting is initiated, its development is a coordinate effect no longer dependent solely on the nutritional state of the individual cells. Thus, cells which are not yet starved of a vital growth requirement are impressed into participating in microcyst formation, and perhaps in aggregations as well. Dworkin and Gibson (4) have shown that cells growing in a rich liquid medium can be induced to form microcysts when glycerol is added to the medium.

The mechanism by which this control is maintained over the developing fruit is not known, but evidence indicating a role of chemotaxis has been noted by several investigators (5, 10). The fact that some nonfruiting mutants of *M. xanthus* FB can act synergistically to form fruits (10) may indicate that there is considerable biochemical communication among the cells entering the fruit.

One is left to ponder what relationship the findings in the laboratory have to the induction of fruiting in nature. A agar has almost 2 mg/ml of amino acids, which is unrealistically high compared to some natural habitats. Conversely, some common habitats such as dung, on which myxobacteria both grow and fruit, have a bacterial mass far exceeding the total mass of nutrient available even in C agar.

There is some laboratory evidence that starvation is only a partial answer to the triggering of morphogenesis. For example, when A agar is made with phenylalanine at very low concentration (0.001 mM) and inoculated as described earlier with spot tests, the cells grow very slowly. At the end of a week, when control plates containing 0.01 mM phenylalanine are fruiting, the cells on lower phenylalanine show only a small amount of growth. The cells continue to grow very slowly until the mass of cells is sufficient to permit the formation of tiny fruits. It seems probable that limiting growth conditions occur throughout the 2 weeks or more of growth, but the cells continue to grow until some signal indicates that there are enough cells to form fruits.

It is also possible that other unfavorable changes in the environment besides depletion of nutrient may induce fruiting. We have noted that desiccation of the plates may radically alter the fruiting pattern, but we have found no way to quantitate these effects.

ACKNOWLEDGMENTS

This research was supported by National Science Foundation grant GB-638. The senior author was supported by a fellowship from the Woodrow Wilson National Fellowship Foundation and a predoctoral traineeship in the Genetics Training Program under grant T1 GM 1035 from the National Institute of General Medical Sciences. The excellent technical assistance of Janet Adams is gratefully acknowledged.

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