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Guanosine Pentaphosphate and Guanosine Tetraphosphate Accumulation and Induction of *Myxococcus xanthus* Fruiting Body Development

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Development of multicellular fruiting bodies of *Myxococcus xanthus* can be induced by limitation of any of a number of different classes of amino acids. Investigated were amino acids that wild-type strains of *M. xanthus* are unable to synthesize (isoleucine, leucine, and valine), can synthesize at a low rate (phenylalanine), or can normally synthesize at an adequate rate (tryptophan and serine). In general, gradual rather than abrupt starvation for an essential amino acid was required for the induction of fruiting. Perhaps gradual starvation in general minimizes antagonism between amino acids present in the medium, as was documented for valine starvation. The previously reported induction of fruiting by a high concentration of threonine was shown to be specifically reversed by lysine. Threonine addition may starve cells for lysine by feedback inhibition of aspartokinase activity. Starvation for carbon-energy sources or inorganic phosphate also induced fruiting. As in other bacteria, amino acid starvation of *M. xanthus* leads to increases in cellular guanosine polyphosphate, usually consisting of large increases in the amount of guanosine pentaphosphate with smaller increases in the level of guanosine tetraphosphate. Guanosine polyphosphate accumulation is thus shown to be correlated with nutritional conditions that induce fruiting, and therefore may serve as an intracellular signal to trigger cells to end vegetative growth and initiate fruiting body development.

The preceding paper (11) presented experiments showing that guanosine tetraphosphate (Gp_4) accumulates in cultures of *Myxococcus xanthus* that are developing fruiting bodies in response to starvation for carbon and nitrogen. Since Gp_4 accumulates before morphological changes are evident, and since Gp_4 and guanosine pentaphosphate (Gp_5) are powerful general regulators of macromolecular synthesis in enteric bacteria, this accumulation may constitute the mechanism by which *M. xanthus* recognizes starvation and switches from growth to fruiting body formation. It is the aim of this paper to begin to test that hypothesis.

Escherichia coli and other bacteria accumulate guanosine polyphosphates (Gp_4 and Gp_5) in response to starvation for any amino acid (3). In contrast, the other two known general regulatory systems are activated by different starvation conditions: cyclic AMP accumulates in response to poor carbon sources (17), and glutamine synthetase deadenylylation is activated by nitrogen source limitation (20). Previous studies of *M. xanthus* have shown that amino acids are important in the control of fruiting. For example, in the first investigation of the relation between nutrition and fruiting body formation, Dworkin (5) found that on a medium containing high concentrations of 17 amino acids, fruiting was induced in prestarved vegetative cells uniquely by the simultaneous elimination of phenylalanine and tryptophan. Rosenberg et al. (18) later found that addition of amino acids derived from aspartate to an undefined low-nutrient medium can stimulate or inhibit fruiting, with the influence of an amino acid on fruiting inversely correlated with its effect on aspartokinase activity. These data were usually interpreted to mean that certain "key" amino acids regulated fruiting. Nevertheless, Hemphill and Zahler (9), using a medium containing 15 amino acids, found increased fruiting activity as a result of limitation for any of 10 of these amino acids, including each of 7 amino acids required or strongly stimulatory for growth. In these early studies amino acids served as carbon-energy sources as well as substrates for protein synthesis. Therefore, it was not possible to distinguish whether starvation for an amino acid led to fruiting because of the decrease in protein synthetic capacity or because it constituted loss of a carbon-energy source. Furthermore, as is shown here, the pres-

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ence of many nonessential amino acids in the media used probably led to antagonisms among amino acids that affected the patterns of fruiting induction observed.

If guanosine polyphosphate accumulation is the signal that induces myxobacteria to form fruiting bodies, and if guanosine polyphosphate is controlled in myxobacteria as it is in other bacteria, then fruiting and guanosine polyphosphate accumulation should result from starvation for any amino acid. The experiments described in this paper test these predictions.

MATERIALS AND METHODS

Strains. The wild-type strain used in these studies is DK501 (11). DK405 is a spontaneous mutant of DK101 that is resistant to 15 μg of rifampin per ml and was isolated by A. P. Bretscher. Tryptophan-requiring auxotrophs of DK405 (including DK149) were isolated after ethyl methane sulfonate mutagenesis by direct screening by T. Masuda.

Media. CTT broth consists of 1% Casitone, 10 mM Tris, and 8 mM $MgSO_4$, adjusted to pH 7.6 with HCl, and supports exponential growth of DK501 with a generation time of about 3.5 h at 33°C. CTT agar is CTT broth solidified with 1.5% agar (Difco). TM8 buffer consists of 10 mM Tris and 8 mM $MgSO_4$, adjusted to pH 7.6 with HCl. TPM buffer is TM8 buffer containing an additional 1 mM KPO_4. A1 medium, developed by A. P. Bretscher (1), consists of (per ml) 5 mg of sodium pyruvate, 5 mg of potassium aspartate, 100 μg of isoleucine, 50 μg of leucine, 100 μg of valine, 10 μg of methionine, 100 μg of phenylalanine, 100 μg of asparagine, 1 μg of vitamin B_{12}, 500 μg of ammonium sulfate, and 125 μg of spermidine-3HCl, plus 8 mM $MgSO_4$, 1 mM K_2HPO_4, 10 μM $FeCl_3$, 10 μM $CaCl_2$, and 10 mM Tris-hydrochloride, adjusted to pH 7.6. Low-phosphate A1 medium contains 0.2 mM rather than 1 mM inorganic phosphate. A1 medium supports growth with a generation time of 25 to 30 h in liquid suspension at 30°C. Solid A1 or A1 modified by deletion of normal constituents was made up at twice the normal concentration and was solidified after addition of supplements by further addition of concentrated agarose to 0.8% final concentration. Agarose is used to replace commercial agar, which contains about 34 μg of amino acids per ml (1). Plates were generally dried overnight at ambient temperature before cells were spotted onto them.

Materials. Sources for media components were as described (1, 11). β-Chloro-L-alanine, DL-serine hydroxamate, and DL-α,ε-diaminopimelic acid were from Sigma Chemical Co. L-Tyrosinol-HCl was from K+K Laboratories. Purines, nucleosides, and nucleotides were from Calbiochem.

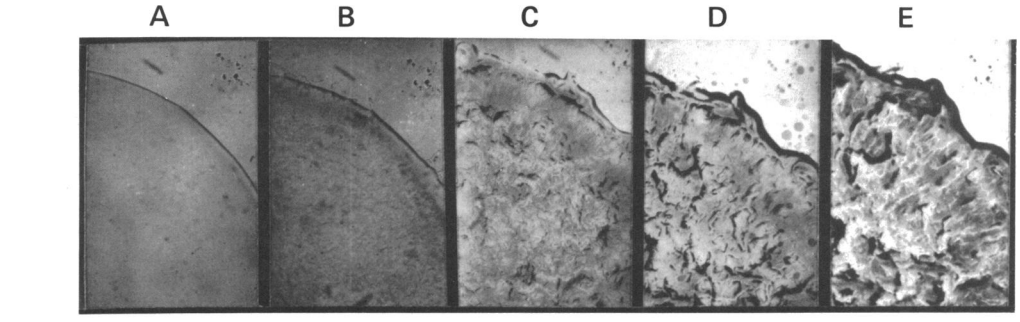
Fruiting test. Cultures of cells growing exponentially in A1 medium or CTT broth at a concentration of 2×10^8 to 3×10^8 cells per ml were centrifuged for 5 to 7 min at 6,500 × *g* and washed twice in equal volumes of ice-cold TM8 buffer before being suspended at a concentration of 2×10^9 to 5×10^9 cells per ml in cold TM8. Droplets of 10 μl of the concentrated cell suspensions were placed onto appropriate media and dried at room temperature (usually 1 to 2 h). After 4 days of incubation at 33°C, the spots were scored for growth and fruiting aggregation by using a Zeiss dissecting microscope and photographed under bright-field illumination using a Wild M7S dissecting microscope equipped with a PM-10 camera system onto Kodak Panatomic-X 35 mm film. Each photographic frame shows a field measuring 3.6 by 2.4 mm on the original fruiting spot. To score for the presence of myxospores, parts of each spot were picked up and suspended in 0.1% sodium dodecyl sulfate, and the suspensions were examined with a Zeiss phase-contrast microscope (40× objective). The detergent aids scoring by disrupting vegetative cells, without affecting the appearance of myxospores. Unless otherwise indicated, fruiting behavior was comparable for cells grown in A1 medium and CTT broth before spotting.

$^{32}PO_4$ labeling of cells in A1 medium. DK501 exponential cultures were labeled by at least 12 h of growth at 30°C in low-phosphate A1 medium containing 50 to 100 μCi of $^{32}PO_4$ per ml. To change media, cells were centrifuged for 5 min at 6,500 × *g*, washed twice in cold TM8 buffer, and then suspended in the new medium with $^{32}PO_4$ present at the same specific activity as before centrifugation. Extraction of nucleotides and two-dimensional chromatography were as described in the accompanying paper, except that solvent system 2 was used exclusively (11).

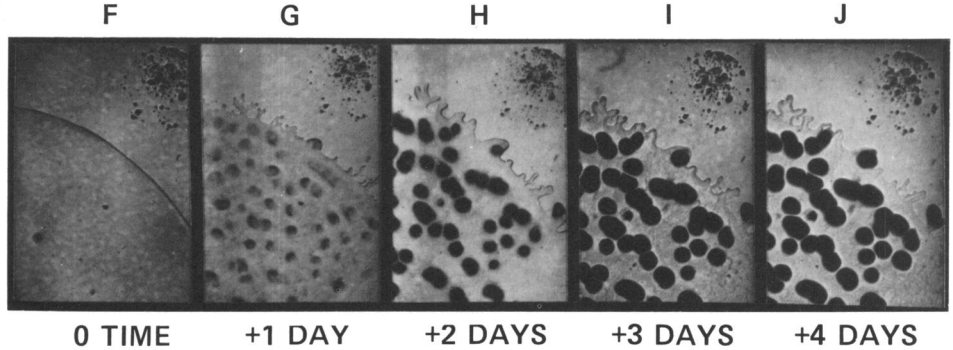
RESULTS

Vegetative growth and fruiting body formation on minimal medium. In the experiments presented in this paper, the nutritional control of fruiting body formation has been investigated using a minimal synthetic medium (A1 medium) which permits distinction between required amino acids and carbon and energy sources (1). A1 medium contains low levels of the three amino acids (isoleucine, leucine, and valine) that myxococci are unable to synthesize, along with a fourth amino acid (phenylalanine) which can be made only at a low rate that severely limits growth. In addition to these four amino acids, A1 medium contains pyruvate and aspartate as carbon-energy sources, vitamin B_{12}, methionine, asparagine, and a mixture of inorganic salts.

Figure 1A to E shows the progress of vegetative growth of *M. xanthus* on A1 medium. Drops of concentrated cells had been spotted onto the medium, and each photograph in Fig. 1A to E shows a portion of that spot, including its edge. Immediately after drying, the cell mass was thin and its boundary was barely detectable (Fig. 1A). With time the culture grew (Fig. 1B to E), the boundary became more pronounced, and the spot itself became darker due to the increased number of cells present. After 4 days (Fig. 1E), the surface of the spot of cells was not smooth, but instead showed irregularities, indicating some lack of uniformity in the distribution of



	0 TIME	+1 DAY	+2 DAYS	+3 DAYS	+4 DAYS
AGG	−	−	−	−	−
SPO	−	−	−	−	−



	0 TIME	+1 DAY	+2 DAYS	+3 DAYS	+4 DAYS
AGG	−	±	+	+	+
SPO	−	−	+	+	+

FIG. 1. *Time course of vegetative growth or fruiting body formation. DK501 cells were spotted onto A1 medium, which supports vegetative growth (A to E), or A1 medium lacking phenylalanine, which leads to fruiting body formation (F to J). Directly after spotting ("0 time") and at 1-day intervals, spots were photographed and examined for spores. The carbon grains in the upper right-hand corner of each frame serve as reference points. The cells shown in A to E were grown in CTT broth before spotting, whereas those in F to J were grown in A1 medium. In this and following figures of spots of cells, each frame shows a field 3.6 by 2.4 mm. Aggregation (AGG) was judged to have occurred (scored as "+") if mounding was evident to the extent observed in (H). Spots were scored as showing spores (SPO) if large clusters estimated to contain greater than 100 spores were seen.*

cells, a phenomenon characteristic of motile strains.

Figure 1F to J shows the behavior of cells in a spot on A1 minimal medium lacking phenylalanine, a nutritional condition leading to fruiting body formation. Immediately after it was applied (Fig. 1F), the spot appeared the same as that placed under vegetative growth conditions (Fig. 1A). However, after 1 day of incubation (Fig. 1G), there was aggregation into small mounds, many of which matured into fruiting bodies, evident as the approximately 40 dark, round structures within the spot in Fig. 1J.

To aid in interpreting experiments involving the formation of fruiting bodies, each spot was scored for its extent of aggregation. Aggregation was judged to have occurred (scored as "+") if mounding was evident to an extent comparable to that observed in Fig. 1H. Since the scoring of fruiting aggregation required a judgment of morphology, photographs of the spots scored have been included to show the data directly.

Mature myxococcal fruiting bodies consist predominantly of myxospores (10). Since the presence of spores cannot be distinguished at the low magnification shown in Fig. 1, spots tested for fruiting were also routinely examined at higher magnification. There, spores are evident as bright, ovoid cells, whereas vegetative cells have the morphology of long rods and

appear dark relative to spores (10). A spot was scored as showing spores only if large clusters estimated to contain greater than 100 spores were observed, whether or not vegetative cells were also present.

Figure 1F to J shows that aggregation of cells into small mounds precedes sporulation. For example, after 1 day of incubation immature aggregates but no spores were present (Fig. 1G). As aggregates matured into fruiting bodies, they became larger and more opaque (compare Fig. 1G, H, and I).

Starvation for essential amino acids: leucine, isoleucine, and valine. Figure 1F to J shows that starvation for phenylalanine, which is not essential but is growth-limiting in A1 medium, leads to fruiting body formation. What is the effect of starvation for the essential amino acids leucine, isoleucine, and valine? Figure 2B shows that when isoleucine was present at a low, limiting concentration (2 or 5 μg/ml), fruiting bodies did form. The normal level of isoleucine in A1 medium is 100 μg/ml. Although fruiting did not usually occur when isoleucine was entirely eliminated (Fig. 2A), occasionally it did. Similar behavior was observed in response to leucine limitation, where fruiting occurred on 2 μg of leucine per ml, but not on 0 or 50 μg of leucine per ml.

By contrast, limiting the levels of valine in A1 medium did not induce fruiting, even though small increments in valine concentration at levels below 5 μg/ml were examined. To test the possibility that other amino acids in the medium might prevent induction by valine limitation, their levels were varied at limiting valine concentrations (Fig. 3). Indeed, valine limitation did induce fruiting if the leucine and isoleucine concentrations of A1 medium were reduced 2.5-fold and the nonessential amino acid methionine was eliminated altogether (Fig. 3A–C). Fruiting bodies were made when low amounts of valine were present (Fig. 3B), but not when valine was eliminated entirely (Fig. 3A). The normal level of valine (i.e., the concentration in A1 medium, 100 μg/ml) abolished fruiting (Fig. 3C). The addition of methionine when valine was limited prevented fruiting entirely (Fig. 3D). The presence of normal levels of isoleucine and leucine decreased the total number of fruiting bodies, and those that did form were of irregular shape (Fig. 3E), although they did contain spores. Thus, valine limitation can induce fruiting body formation, but this induction is antagonized by methionine and, to a lesser extent, by leucine and isoleucine.

Starvation for nonessential amino acids. As we pointed out in the introduction, if fruiting is induced by guanosine polyphosphate, then starvation for any amino acid, including nonessential ones, should induce fruiting. The effect on fruiting body development of limiting nonessential amino acids was examined using auxotrophic mutants of *M. xanthus* and amino acid analog treatment of prototrophic strains. In testing a set of tryptophan auxotrophs for fruiting

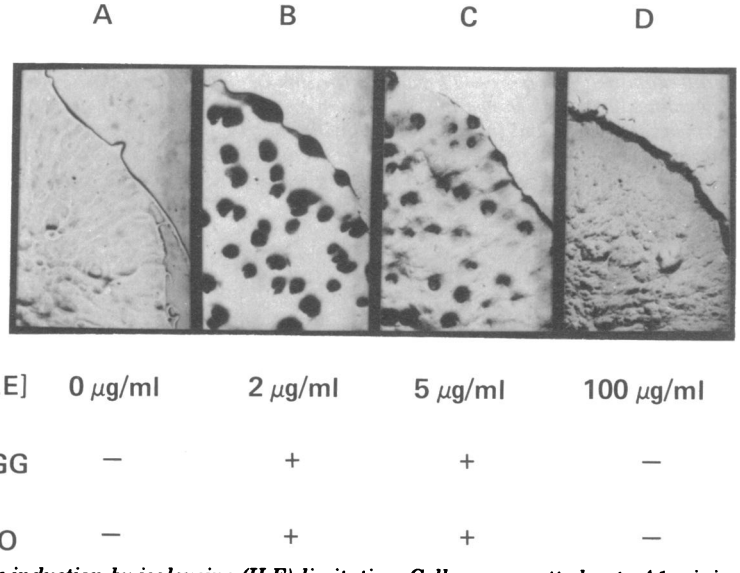


FIG. 2. *Fruiting induction by isoleucine (ILE) limitation. Cells were spotted onto A1 minimal medium (A) lacking isoleucine, or containing (B) 2, (C) 5, or (D) 100 μg of isoleucine per ml. DK501 cells had been grown in CTT broth before spotting.*

in response to tryptophan limitation on minimal medium, it was found that the parent strain of these auxotrophs (DK405, a rifampin-resistant mutant), fruited on complete A1 minimal medium (Fig. 4A). To test the effect of tryptophan limitation on the auxotrophs therefore first required that nutritional conditions be adjusted to allow growth without fruiting of the parent strain. One such condition was found to be addition of 500 μg each of proline and glycine per ml to A1 minimal medium (Fig. 4B). These amino acids may function as supplementary carbon-energy sources (10). With this adjustment, three independently isolated tryptophan auxotrophs were induced to fruit when tryptophan was limiting for growth (shown for one strain in Fig. 4D). An increased tryptophan concentration prevented fruiting (Fig. 4E), and, as was observed for limitation of essential amino acids, fruiting did not occur when tryptophan was completely eliminated (Fig. 4C).

Serine hydroxamate, a serine analog that com-

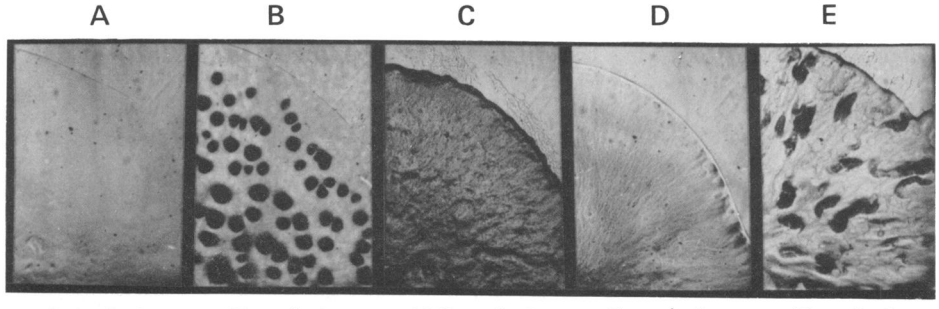


FIG. 3. *Fruiting induction by valine limitation. Cells were spotted onto A1 medium lacking methionine and containing reduced amounts of isoleucine and leucine (40 μg of isoleucine and 20 μg of leucine per ml) with (A) 0 μg of valine (VAL) per ml, (B) 5 μg of valine per ml, (C) 100 μg of valine per ml, (D) 5 μg of valine and 10 μg of methionine per ml, and (E) 5 μg of valine, 100 μg of isoleucine, and 50 μg of leucine per ml. Cells were grown in CTT broth before spotting; cells grown in A1 medium gave similar results except that looser aggregates formed that did not contain mature myxospores.*

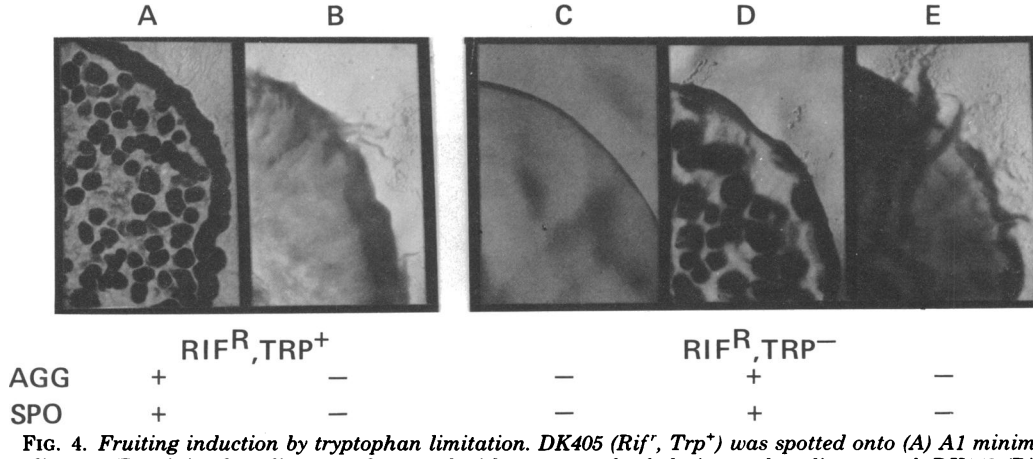


FIG. 4. *Fruiting induction by tryptophan limitation. DK405 (Rif^r, Trp$^+$) was spotted onto (A) A1 minimal medium or (B) minimal medium supplemented with 500 μg each of glycine and proline per ml. DK149 (Rif^r, Trp$^-$) was spotted onto minimal medium containing 500 μg each of glycine and proline per ml, further supplemented with (C) 0, (D) 10, or (E) 100 μg of tryptophan per ml. Cells were grown in CTT broth before spotting; growth in minimal medium before spotting was not examined.*

petitively inhibits seryl-tRNA synthetase in *Escherichia coli* (19), induced fruiting in *M. xanthus* (Fig. 5B). Induction was reversed by addition of serine to the serine hydroxamate-treated culture (Fig. 5C), showing that it was starvation for serine or seryl-tRNA that led to fruiting. Higher concentrations of serine hydroxamate were found to inhibit aggregation, though spores were still detected.

The amino acid analogs tyrosinol and β-chloro-L-alanine also induced fruiting on A1 medium (C. Manoil, Ph.D. thesis, Stanford University, Stanford, Calif., 1978). The induction of fruiting by tyrosinol was reversed by phenylalanine but not tyrosine, implying that it acts through phenylalanine (perhaps along with tyrosine) starvation.

Is the induction of fruiting restricted to starvation for amino acids? Figure 6A shows that starvation of cells for the carbon-energy sources pyruvate and aspartate led to fruiting in the form of long, ridge-like fruiting bodies that contained spores. Elimination of pyruvate alone also led to mature fruiting bodies (data not shown). This result is expected since pyruvate contributes three times more to cell carbon than does aspartate (1). In addition, Fig. 6B shows that starvation for inorganic phosphate led to fruiting body formation.

Since many of the nutritional conditions leading to fruiting permit some increase in total cell mass, an experiment was performed to determine whether fruiting is possible when there is no exogenous source of nutrient. Thoroughly washed cells were spotted onto agarose plates containing in addition only buffer. Unlike commercial agar, agarose is free of amino acids and utilizable sugars (1). Although cells grown in rich CTT broth before spotting formed only immature aggregates without spores (Fig. 7B), those grown in A1 minimal medium formed fruiting bodies under these conditions (Fig. 7A). These fruiting bodies were smaller, having less than 1/10 the volume of those formed in response to gradual starvation in amino acid-deficient A1 medium (compare Fig. 1J).

Guanosine polyphosphate accumulation during amino acid starvation. To examine the hypothesis that amino acid starvation induces fruiting via the accumulation of guanosine polyphosphate, the effects of amino acid starvation on the levels of Gp_4 and Gp_5 were measured. The basal level of Gp_4 in cells grown in A1 minimal medium was three- to fourfold higher than that in the richer Casitone broth, whereas Gp_5 levels were low in both media (Table 1). The high level of Gp_4 in cells grown in minimal medium correlates with their lower growth rate:

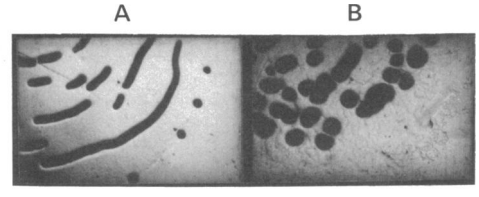


FIG. 6. *Fruiting induction by carbon-energy source and phosphate starvation. Cells were spotted onto A1 medium lacking (A) pyruvate and aspartate, or (B) inorganic phosphate. Photographs were taken after 2 days of incubation. (A) Cells were grown in A1 medium before spotting, although CTT-grown cells gave a similar fruiting response. (B) Cells were grown in CTT broth; if grown in A1 minimal medium, aggregates formed which did not contain myxospores.*

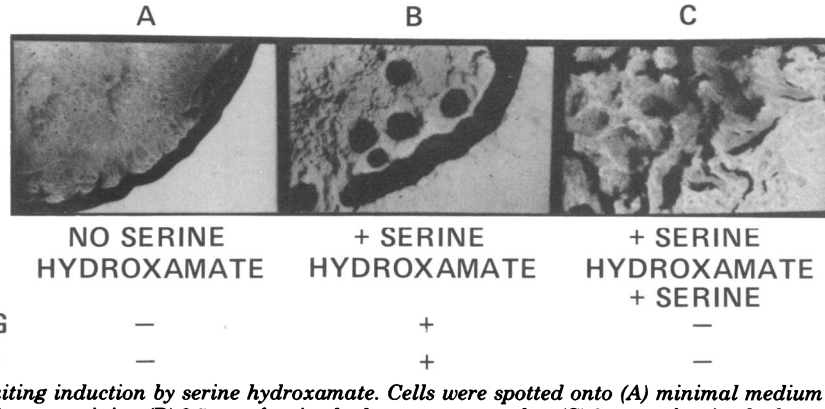


FIG. 5. *Fruiting induction by serine hydroxamate. Cells were spotted onto (A) minimal medium alone, or minimal medium containing (B) 2.5 mg of serine hydroxamate per ml or (C) 2.5 mg of serine hydroxamate and 1 mg of serine per ml. Cells were grown in CTT broth before spotting.*

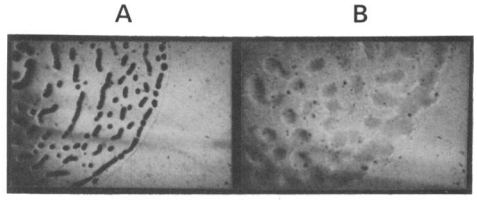


FIG. 7. *Fruiting response to complete starvation. DK501 was spotted onto plates containing TPM buffer solidified with agarose. Before spotting, cells were grown in (A) A1 minimal medium or (B) CTT broth.*

TABLE 1. *Representative guanosine polyphosphate levels under various nutritional conditions*

Condition	Gp_5[a]	Gp_4[a]
0.5% Casitone		
Exponential growth	1×10^{-14}	4×10^{-14}
Stationary phase	1×10^{-14}	8×10^{-14}
A1 minimal medium		
Exponential growth	2×10^{-14}	15×10^{-14}
Stationary phase	2×10^{-14}	30×10^{-14}
Carbon nitrogen source starvation	2×10^{-14}	80×10^{-14}
Amino acid starvation		
Severe (valine elimination)	20×10^{-14}	20×10^{-14}
Gradual (serine hydroxamate addition)	6×10^{-14}	20×10^{-14}
Glycerol-induced sporulation (2 h)	15×10^{-14}	25×10^{-14}

[a] Expressed in moles of nucleotide per 5×10^6 cells. Values are collected from data for DK501 presented in this paper, the accompanying paper (11), and other experiments not shown.

the generation time in A1 is 25 to 30 h as opposed to 5 h in 0.5% Casitone.

Guanosine polyphosphate levels were measured during starvation for essential amino acids as described in Materials and Methods. Figure 8 shows that the major response to starvation for valine or isoleucine was a greater than 10-fold increase in the level of Gp_5 (Fig. 8A). An initial drop in Gp_4 levels when cells were resuspended in complete medium (Fig. 8B) can be accounted for by the fact that Gp_4 levels had increased during the growth of the inoculum in A1 as exemplified by the increase in Gp_4 between exponential- and stationary-phase cultures shown in Table 1. Therefore, the steady-state Gp_4 levels after resuspension of cells in complete media or media lacking amino acid offer the most valid comparisons. Thus, in addition to the large increase in Gp_5, there is also a small but definite increase in Gp_4. Gp_4 and Gp_5 accumulated during leucine starvation in a fashion similar to that shown for valine and isoleucine.

Increases in guanosine polyphosphate were also observed when the amino acid limitation was less severe, although the extent of accumulation was lower. Elimination of phenylalanine, which cells can biosynthesize at a low rate, from A1 medium, resulted in a small but detectable elevation of the level of Gp_5 (Figure 9). The Gp_5 level may have increased only slightly when phenylalanine was removed from the medium because the endogenous rate of phenylalanine synthesis may suffice to prevent abrupt starvation during the 12 h over which these measurements were made. This view is consistent with the observation that when the limitation of phenylalanine (and tyrosine) was intensified by the addition of the amino acid analog tyrosinol, a higher level of Gp_5 was observed (Fig. 9), one that is close to that seen after elimination of amino acids that cells cannot biosynthesize at all (Fig. 8). Thus severe phenylalanine limitation (induced by tyrosinol) is analogous to starvation for valine, leucine, or isoleucine, which *Myxococcus* is completely unable to synthesize.

When cells growing exponentially in A1 liquid medium were treated with a concentration of serine hydroxamate that induced fruiting on plates (3 mg/ml), there were approximate two-fold increases in relative Gp_5 levels over minimal medium alone, with smaller increases in the level of Gp_4 (see Table 1).

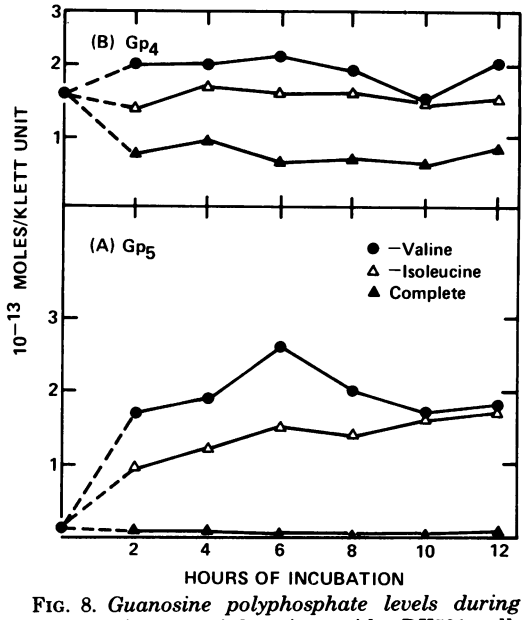


FIG. 8. *Guanosine polyphosphate levels during starvation for essential amino acids. DK501 cells previously grown in low-phosphate A1 medium were suspended either in complete low-phosphate A1 medium (▲) or in medium lacking valine (●) or isoleucine (△). The zero-hour values are derived from the original minimal medium culture samples just before centrifugation.*

Induction of fruiting by addition of threonine. Rosenberg et al. (18) made the interesting discovery that *M. xanthus* would fruit when high levels of threonine were added to an undefined, low-nutrient medium, an observation which at first sight seems difficult to reconcile with the hypothesis that fruiting is initiated by guanosine polyphosphate accumulation. Figure 10B confirms and extends the observation of Rosenberg et al. by showing that high levels (2 mg/ml) of threonine induced fruiting on defined minimal medium. However, we find in addition that threonine-induced fruiting can be reversed by the addition of low levels (100 μg/ml) of lysine (Fig. 10C), but not by any of 15 other amino acids tested individually, suggesting that threonine addition induces fruiting through starvation for lysine. *meso*-Diaminopimelic acid, a precursor of lysine in *M. xanthus*, also reversed threonine-induced fruiting, but only when present at a high concentration (1 mg/ml). The need for a high concentration may be due to an inability of *M. xanthus* cells to transport diaminopimelate efficiently. Even in the presence of lysine (Fig. 10C) there was some residual aggregation activity. However, the residual aggregation was eliminated by addition of low levels of glycine (Fig. 10D), although glycine alone did not reverse threonine-induced fruiting. The mechanism of the glycine effect has not been further investigated.

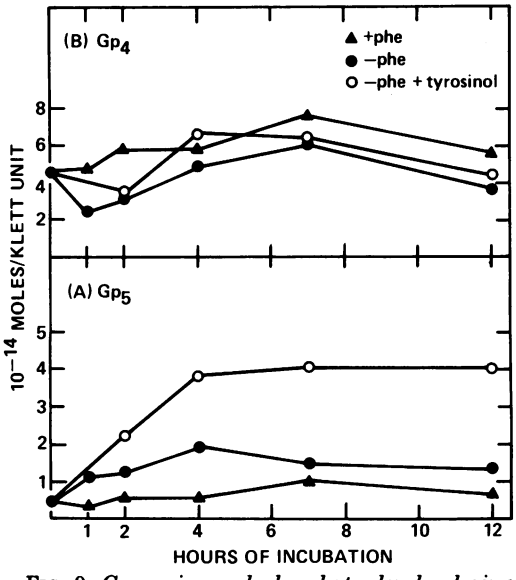


FIG. 9. *Guanosine polyphosphate levels during phenylalanine limitation. DK501 cells were suspended in complete low-phosphate A1 medium (▲), medium lacking phenylalanine (●), or medium lacking phenylalanine and containing 100 μg of L-tyrosinol per ml (○).*

When threonine at 4 mg/ml was added to *M. xanthus* minimal medium cultures, there were significant increases in both Gp_5 and Gp_4 without appreciable changes in GTP (Fig. 11). Moreover, when low levels of lysine or lysine and glycine were added along with threonine the accumulation of Gp_5 was nearly completely eliminated, although some Gp_4 still accumulated. If the decrease in GTP level evident in the culture containing threonine and lysine is used to normalize the guanosine polyphosphate levels, a decrease in the Gp_5 level from that seen in the presence of threonine alone remains evident, but the Gp_4

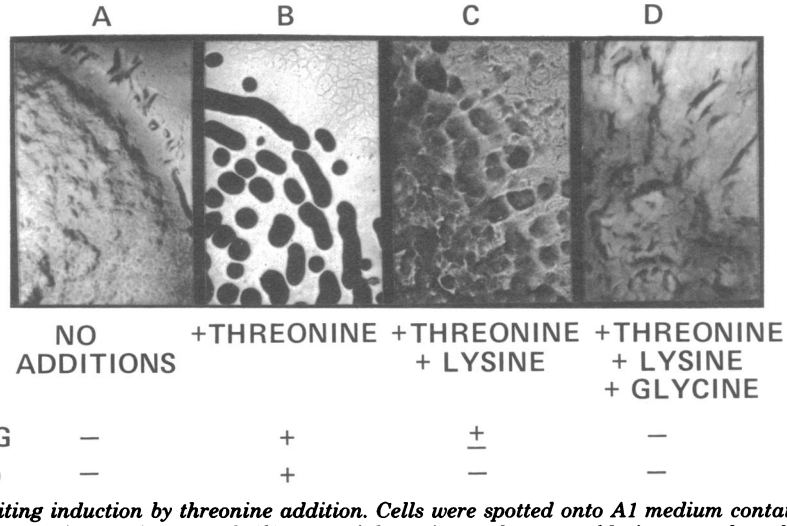


FIG. 10. *Fruiting induction by threonine addition. Cells were spotted onto A1 medium containing (A) no additions, (B) 2 mg of threonine per ml, (C) 2 mg of threonine and 100 μg of lysine per ml, and (D) 2 mg of threonine, 100 μg of lysine, and 100 μg of glycine per ml. Cells were grown in minimal medium before spotting.*

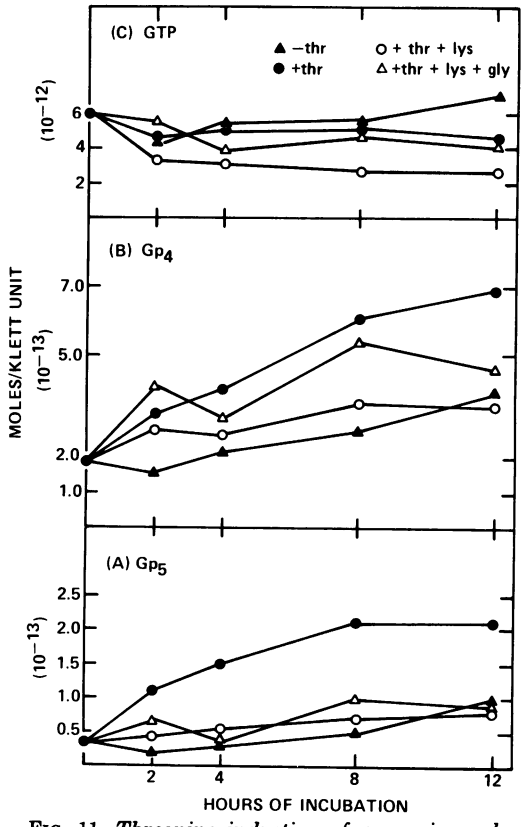


FIG. 11. *Threonine induction of guanosine polyphosphate accumulation. To portions of a DK501 culture were added threonine (4 mg/ml final concentration)* (●); *threonine (4 mg/ml) and lysine (100 μg/ml)* (○); *threonine (4 mg/ml), lysine (100 μg/ml), and glycine (100 μg/ml)* (△); *or an equivalent volume of water* (▲).

level is approximately the same with and without lysine. Thus high levels of threonine appear to starve cells for lysine, and possibly for another substance that can be replaced by glycine.

DISCUSSION

Table 2 summarizes the conditions here shown to lead *M. xanthus* to form fruiting bodies. Fruiting is induced by limitation of any of a number of different amino acids, including those that are essential for growth (isoleucine, leucine, and valine), as well as the nonessential amino acids tryptophan and serine. Elimination from the minimal medium of phenylalanine, which is not essential but is growth-limiting, also leads to fruiting. The fact that fruiting is induced by limitation of any of the six amino acids that have been systematically examined implies that fruiting is a general response to amino acid limitation, rather than a special response limited to starvation for certain "key" amino acids. This suggestion is further supported by the finding that induction by addition of threonine or, as described elsewhere (12), of purines can be accounted for by depletion of certain amino acids.

However, fruiting is not usually induced if an essential amino acid is completely eliminated from A1 medium. Instead, reliable induction of fruiting requires the presence of low, though still growth-limiting, levels of the amino acid. Examination of induction by valine limitation sheds light on this requirement. Valine limitation leads to optimal fruiting only if methionine is eliminated from the medium and isoleucine and leucine are reduced in concentration, as if these amino acids antagonize fruiting when valine is limited. Similar antagonisms may prevent fruiting when other amino acids are totally eliminated from the medium. Consequently, low levels of the limiting amino acids must be present to overcome the antagonisms. This view is supported by the finding that cells can fruit when deprived of all nutrients (Fig. 7A), a condition in which there can be no antagonisms from amino acids in the medium.

Antagonisms between amino acids can account for Dworkin's observation (5) that fruiting was induced only in response to the joint elimination of phenylalanine and tryptophan from a complex defined medium (Manoil, thesis). Antagonisms between amino acids can also account for the findings that methionine and isoleucine

TABLE 2. *Nutrient limitations that cause fruiting*

Compound limited	Condition leading to optional fruiting[a]
Essential amino acids	
Leucine	Low levels of leucine
Isoleucine	Low levels of isoleucine
Valine	Low levels of valine with reduced leucine, isoleucine, and methionine
Nonessential amino acids	
Phenylalanine (and tyrosine)	Phenylalanine elimination or tyrosinol treatment
Tryptophan	Low levels of tryptophan with auxotroph
Serine	Serine hydroxamate addition
Lysine	Threonine addition
Other compounds	
Carbon-energy sources	Elimination of pyruvate and aspartate
Inorganic phosphate	Elimination of inorganic phosphate

[a] All treatments refer to changes in A1 medium except tryptophan limitation, which refers to A1 medium supplemented with 500 μg each of glycine and proline per ml.

inhibit fruiting on undefined, low-nutrient media (2, 17).

The mechanism of antagonism has not yet been studied in detail. However, an interesting possibility arises from studies of *E. coli* showing that misincorporation of one amino acid for another into protein is enhanced when amino acids are limiting (13, 15). Misincorporation during amino acid starvation in *M. xanthus* could lead to the synthesis of dysfunctional fruiting proteins (Manoil, thesis). Misincorporation is a strong possibility since all the essential amino acids for *M. xanthus* have a uracil residue as their second codon letter. Misincorporation could then occur through misreading at either the first or third base of codons for limiting amino acids, inserting instead another amino acid present at high level since it is a component of A1 medium.

If amino acid starvation leads to fruiting, how is that starvation sensed? The finding that fruiting is induced by serine hydroxamate (Fig. 5), a compound known to inhibit seryl-tRNA synthetase in *E. coli* (19), suggests that limiting aminoacylated tRNA rather than amino acid itself may initiate fruiting. Furthermore, the fact that limitation of a number of metabolically unrelated amino acids leads to fruiting itself suggests that the cell recognizes amino acid starvation at a step related to protein synthesis, where the different amino acids come together. A monitoring of the level of tRNA aminoacylation would be a simple way to accomplish this.

In *E. coli* and other bacteria, an excess of uncharged tRNA stimulates Gp_5 and Gp_4 synthesis, so that the level of these nucleotides increases in response to amino acid starvation (3). Guanosine polyphosphates also accumulate during amino acid starvation of *M. xanthus*, although in A1 medium the level of Gp_5 appears to be a much more sensitive indicator of amino acid starvation than is the level of Gp_4, in part because the level of Gp_4 is relatively high to begin with. The magnitude of Gp_5 accumulation is a function of the severity of the amino acid starvation. Thus, elimination of an amino acid that *M. xanthus* is incapable of synthesizing, such as valine or isoleucine, leads to maximal accumulation (Fig. 8), whereas limitation of an amino acid that the cell is capable of synthesizing, such as phenylalanine (by removal from the medium) or serine (by addition of serine hydroxamate), leads to smaller increases in levels of Gp_5 (e.g., see Fig. 9).

These studies illustrate the correlation of initiation of fruiting body formation with guanosine polyphosphate accumulation. As might be expected, a gradual amino acid starvation that permits some growth leads to the greatest mass of fruiting bodies and to small rather than large increases in guanosine polyphosphate over the high basal levels present in minimal medium. When cells are severely starved by deprivation of all exogenous nutrient, large guanosine polyphosphate increases are seen (11), and the total mass of fruiting bodies is small (Fig. 7A).

Induction of fruiting is not unique to amino acid starvation, since starvation for inorganic phosphate or the major carbon-energy sources also leads to fruiting. The finding by Hemphill and Zahler (9), that limitation of prototrophic *M. xanthus* for five amino acids nonessential for growth stimulates fruiting, may be due to the use of these as carbon and energy sources. Although not yet further investigated in *M. xanthus*, comparable starvations of *E. coli* are thought to lead to guanosine polyphosphate increase by mechanisms independent of that activated by amino acid starvation (8, 14). It is also not excluded that starvation for inorganic phosphate or for carbon and energy indirectly leads to fruiting through the intermediate of starvation for some amino acid(s).

A high concentration of threonine induces *M. xanthus* to fruit (Fig. 10B) and to elevate its guanosine polyphosphate (Fig. 11). Both effects are reversed by low levels of lysine, indicating that threonine acts by causing lysine limitation. The feedback relationships that exist between amino acids of the aspartate family suggest a mechanism for threonine induction (Fig. 12). There are two aspartokinase isozymes in *M. xanthus*: a major species, feedback-inhibited by threonine, and a minor one, feedback-inhibited by lysine (6). Threonine may cause lysine starvation by inhibiting the major aspartokinase isozyme, the remaining activity being insufficient to provide enough aspartyl phosphate for diaminopimelate and lysine biosynthesis.

Table 1 summarizes representative levels of Gp_4 and Gp_5 in *M. xanthus* cultures under dif-

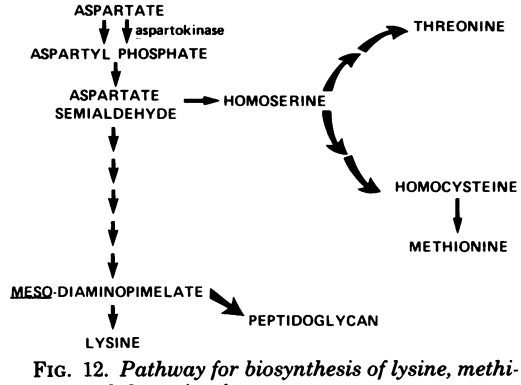


FIG. 12. *Pathway for biosynthesis of lysine, methionine, and threonine from aspartate.*

ferent conditions of nutrition and development drawn from this and the previous paper (11). Two patterns of nucleotide levels are evident: (i) Gp_4 rises without any major increase of Gp_5, as is seen during growth in 0.5% Casitone or A1 minimal medium and during carbon and nitrogen starvation, or (ii) Gp_4 and Gp_5 rise together, as during amino acid starvation or glycerol-induced sporulation. These patterns are similar to those observed in *E. coli* and other bacteria in analogous nutritional conditions (4) and may result from the activities of two different guanosine polyphosphate synthetic mechanisms (7, 14). The recent discovery of a guanosine polyphosphate synthetic mechanism which responds to carbon source starvation (14) extends the generality of the stringent response and helps to explain the stimulation of β-galactosidase synthesis by Gp_4 (16).

The experiments reported here show that without exception the induction of fruiting bodies is correlated with guanosine polyphosphate accumulation. By analogy to *E. coli* and other bacteria (4), it is likely that guanosine polyphosphate accumulation acts to inhibit functions of vegetative cell growth, such as ribosome and cell wall biosynthesis, that are arrested during fruiting. We suggest that the accumulation of guanosine polyphosphate also activates the early developmental steps of fruiting body formation, so that fruiting may be an evolutionary extension of the stringent response.

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