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Gene Expression during Development of *Myxococcus xanthus*: Pattern of Protein Synthesis

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The pattern of protein synthesis during development of *Myxococcus xanthus* was investigated. This gram-negative bacterium has a complex life cycle which involves a temporal sequence of cellular aggregation, mound formation, and myxosporulation. At various stages of development, cells were pulse-labeled with a ^{14}C-labeled amino acid mixture. Synthesis of soluble and membrane proteins was then analyzed by SDS-polyacrylamide gel electrophoresis. Of the 30 major soluble proteins, at least 25% showed significant changes in their rates of production during development. Several significant changes were also found in the membrane proteins as analyzed by two-dimensional polyacrylamide gel electrophoresis. The major proteins synthesized during development were classified into four different types: *accumulation proteins*, *peak proteins*, *late proteins*, and *constant proteins*. The synthesis of protein S, an accumulation protein, increases dramatically during development to a maximum of 15% of total soluble protein synthesis. When methionine was added to the culture medium, cells did not form fruiting bodies. Under these conditions, almost all of the protein changes observed in the early and middle periods of development still occurred. However, the production of *late proteins* (e.g., protein U) was not observed, suggesting that methionine blocks a late stage of development. During glycerol induction, many of the changes in protein synthesis which normally occur during development were not observed (e.g., protein S did not accumulate). These results indicate that gene expression in *M. xanthus* is complex and subject to tight regulation.

INTRODUCTION

Myxococcus xanthus is a gram negative bacterium 3 to 10 μm in length and about 0.5 μm in diameter. These bacteria commonly grow in soils on decaying organic material, on the bark of living trees, or by preying upon other microorganisms. The cells move by gliding over solid surfaces, usually in large groups. A striking property of this organism is its ability to undergo cellular differentiation. When nutrients are depleted from a solid culture medium, cells aggregate to form mounds. The rod-shaped vegetative cells then convert to round or ovoid spores, which are resting cells resistant to many environmental factors (Sudo and Dworkin, 1973). Mounds of mature myxospores are referred to as fruiting bodies. The developmental cycle of the myxobacteria shows several remarkable similarities to that of the eukaryotic cellular slime molds such as *Dictyostelium discoideum*. The complex life cycles of the myxobacteria make them attractive model systems for the study of gene regulation and cell-cell interactions.

There are several distinct advantages to the study of development in *M. xanthus*. The organisms are simple prokaryotes and are relatively easy to grow in large numbers. Development can be induced by simply placing the bacteria on a starvation agar medium. *M. xanthus* contains only one chromosome of $8.4 \pm 1.2 \times 10^9$ daltons (Zusman *et al.*, 1978). Recently several generalized transducing phages which enable the precise genetic analysis of *M. xanthus* have been isolated (Geisselsoder *et al.*,

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1978; Campos *et al.*, 1978; Martin *et al.*, 1978). Furthermore, the mode of chromosome replication of *M. xanthus* during vegetative growth has been established (Zusman and Rosenberg, 1970; Zusman *et al.*, 1978).

Although several morphological studies of fruiting body formation have been performed with *M. xanthus*, only fragmentary information on the biochemical changes which occur during development exists. Myxospores contain a spore coat, which is not present in vegetative cells (White, 1975). Also a development-specific hemagglutinin appears at the time of cellular aggregation (Cumsky and Zusman, 1977). We therefore investigated the pattern of protein synthesis during aggregation and fruiting body formation of *M. xanthus*. The results indicate a precise program of gene expression and will aid in illuminating the molecular mechanism involved in development.

MATERIALS AND METHODS

Cells and growth conditions. M. xanthus strain FB (DZF1) was used. Vegetative cultures of FB were grown at 30°C with vigorous aeration in CYE medium [1% Casitone (Difco), 0.5% yeast extract (Difco), 4 m*M* $MgSO_4$, adjusted to pH 7.3–7.5]. Development was induced on clone fruiting (CF) agar containing 10 m*M* Tris-HCl (pH 7.6), 1 m*M* K Na_2PO_4, 8 m*M* $MgSO_4$, 0.02% $(NH_4)_2SO_4$, 0.015% Casitone, 0.2% sodium citrate, 0.1% sodium pyruvate, and 1.2% Difco purified agar (Hagen *et al.*, 1978). Each CF plate contained 15 ml of CF agar. Plates were routinely preincubated for 1 day at 30°C and 4 days at room temperature before use.

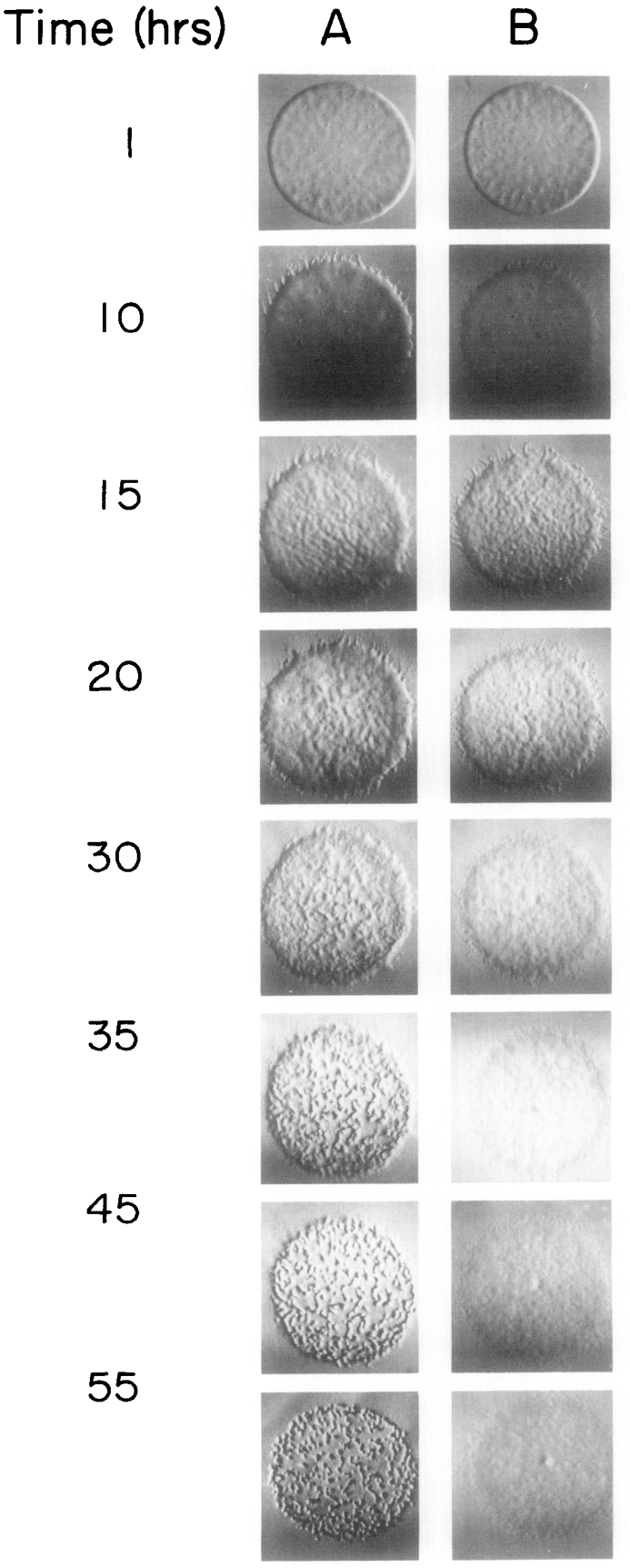
Fruiting body formation. An exponentially growing culture (200 Klett units, about 10^9 cells/ml) was centrifuged at 4000 *g* for 10 min at 4°C. The cell pellet was resuspended in 1/15 vol of 10 m*M* Tris-HCl buffer, pH 7.6, containing 8 m*M* $MgSO_4$. The suspension was kept in an ice bath. Aliquots (10 μl) of cell suspension were spotted on CF agar, 35 spots per plate. After spotting, the plates were allowed to dry at room temperature for about 30 min and then incubated at 30°C in the dark. The same spotting procedure was used for CF agar containing 200 μg/ml of L-methionine. In some experiments methionine did not inhibit fruiting body formation. Carefully controlled experiments showed that cell density and spot size are factors. We do not understand the variability of this inhibition.

At intervals, photographs of spots were taken through a dissecting microscope using a Polaroid camera.

Glycerol induction. Glycerol, final concentration 4%, was added to an exponentially growing culture (150 Klett units). After 6 hr of incubation at 30°C (with vigorous aeration), extracts were prepared and analyzed by SDS-polyacrylamide gel electrophoresis as described below.

Pulse-labeling experiments. Cells spotted on CF agar were pulse-labeled for 4 hr at 30°C by placing 100 μl (10 μCi) of a neutralized ^{14}C-labeled amino acid mixture (NEC-445; New England Nuclear) underneath the agar. Cells were labeled at the following time points: 1, 5, 10, 15, 20, 25, 30, 35, 45, 55, and 75 hr for the experiments without methionine and 1, 5, 10, 20, 35, 45, and 55 hr for the experiments with methionine. At the end of the incubation, cells were dispersed with a glass spreader (this step is important for the quantitative transfer of cells from a plate to a centrifuge

FIG. 1. Sequence of morphological changes observed on CF agar and CF agar plus methionine. Concentrated exponentially growing cells of *M. xanthus* were spotted on CF agar as described in Materials and Methods. Each spot contained about 1.5×10^8 cells, and the diameter of the spot at the 1-hr point was about 4 mm. At timed intervals the spots were photographed through a dissecting microscope. (A) Changes observed on CF agar; (B) changes observed on CF agar plus methionine (200 μg/ml).


Time (hrs)
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tube). Then 5 ml of 10 m*M* sodium phosphate buffer, pH 7.1 (P-buffer) was added. Cells were suspended with a Pasteur pipet and transferred to a 15-ml centrifuge tube. The plate was washed two more times with 2.5 ml of P-buffer. All of the washes were combined, and the suspension was centrifuged at 8000*g* for 10 min at 4°C. The pellet was stored at −20°C until all samples were collected.

Separation of the soluble and membrane fractions. P-buffer (1 ml) was added to the cell pellet and the suspension was sonicated to break the cells. Unbroken cells were collected by centrifugation (6000*g* for 10 min at 4°C) and resonicated in 1 ml of P-buffer. The pellet after the second sonication was resuspended in 1 ml of P-buffer, and the number of spores was measured with the use of a Petroff-Hauser counting chamber. The supernatants were combined and centrifuged at 100,000*g* for 30 min at 4°C. The resulting pellet is the membrane fraction, and the supernatant is the soluble fraction (Inouye and Pardee, 1970). The membrane fraction was washed once with 5 ml of P-buffer, and the final pellet was dissolved in 60 μl of solubilizing solution (0.08 *M* Tris-HCl, pH 6.8, containing 2% SDS and 10% glycerol). Trichloroacetic acid (TCA; 0.2 ml, 100%) was added to the soluble fraction. The resulting precipitate was collected by centrifugation (12,000*g* for 10 min at 4°C) and washed with 4 ml of ethanol and then 4 ml of ether to remove the TCA. The final pellet was dissolved in 100 μl of solubilizing solution.

Polyacrylamide gel electrophoresis. SDS gel electrophoresis was performed with 17.5% acrylamide according to the method of Anderson *et al.* (1973). (It should be noted that *Myxococcus* is "smooth" and has a complete lipopolysaccharide; in addition, it produces copious amounts of slime. These substances interfere with the banding patterns of crude cytoplasmic and membrane fractions, limiting the resolution of the gels.) Two-dimensional gel electrophoresis was carried out according to the method of Ames and Nikaido (1976). The samples were solubilized in SDS. The first dimension, isoelectric focusing, was performed with urea using ampholytes in the range of pH 3–10; the second dimension, SDS electrophoresis, was performed using 17.5% acrylamide.

After electrophoresis, the gels were stained with Coomassie blue, dried, and stored with X-ray film for autoradiography. Individual gel bands were cut out with scissors and extracted overnight with 0.1 ml of Protosol (New England Nuclear). After 3 ml of 0.4% Omnifluor (New England Nuclear) in toluene was added to the extract, radioactivity was measured in a liquid scintillation counter.

RESULTS

Time Course of Fruiting Body Formation

Standard conditions were established for the synchronous induction of fruiting bodies. Exponentially growing cells were spotted on CF agar, incubated at 30°C, and examined under a dissecting microscope at timed intervals. Figure 1A shows the normal sequence of development under these conditions. Figure 1B shows the sequence observed when cells were spotted on CF agar containing 200 μg/ml of methionine, which is known to inhibit fruiting body formation (Rosenberg *et al.*, 1973; Campos and Zusman, 1975). During the first 20 hr of incubation, spot morphologies were similar in the presence or absence of methionine. One can observe wrinkled patterns on the spot surfaces in both cases. This suggests that the cells aggregate normally during the first 20 hr of development even in the presence of methionine. However, after 30 hr of incubation, cells started to form mounds in the absence of methionine, while cells failed to form mounds in the presence of methionine. In the absence of methionine, the formation of mounds became more and more pronounced, and at 50 hr, fruiting body formation appears to be completed. In the presence of methionine, the initial aggregates disperse with continued

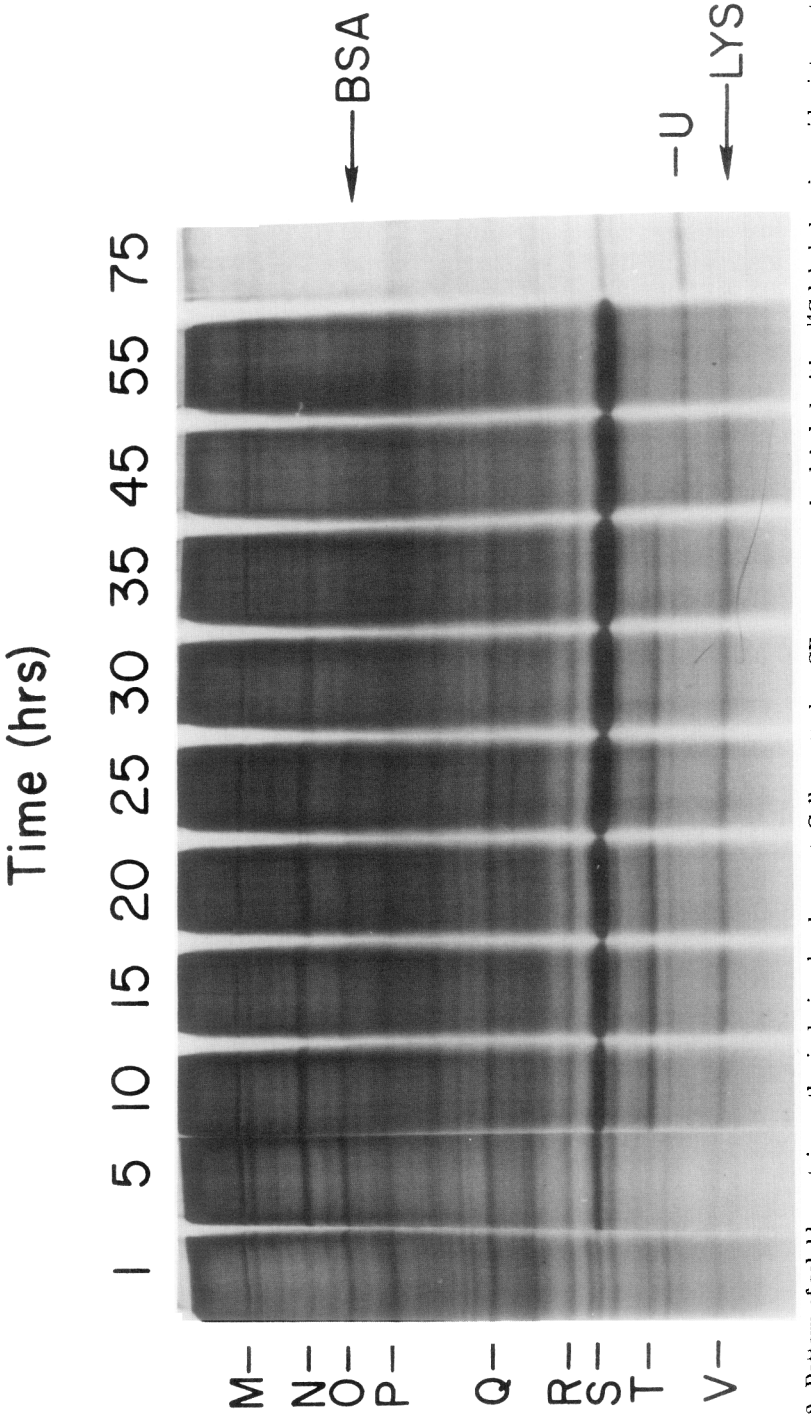


FIG. 2. Pattern of soluble protein synthesis during development. Cells spotted on CF agar were pulse-labeled with a ^{14}C-labeled amino acid mixture at various time intervals. The soluble fractions were then analyzed by SDS–polyacrylamide gel electrophoresis. The gel was dried and prepared for autoradiography (10 days' exposure). The arrows indicate the positions of molecular weight standards: BSA, bovine serum albumin; LYS, hen egg white lysozyme.

incubation. Spore formation was first observed at 35 hr; the number of spores reached a maximum level at about 60 hr (data not shown). In contrast, no spore formation occurred in the presence of methionine.

Pattern of Soluble Protein Synthesis during Development

Cells spotted on CF agar were pulse-labeled at various intervals with a ^{14}C-labeled amino acid mixture. It should be noted that the label period used, 4 hr, was determined to be the most practical labeling time for sufficient incorporation in this system. This label period is actually a small fraction of the entire development cycle. However, because of the length of the pulse, we can only measure the rate of synthesis of "stable" proteins during this interval. Cells were then harvested and sonicated. Soluble and membrane fractions were separated by centrifugation and the samples analyzed by SDS-polyacrylamide gel electrophoresis. The gels were stained to determine the protein content and then subjected to autoradiography to determine the rates of synthesis of proteins. Control experiments indicated that extensive *in vitro* proteolysis was not a significant problem under the experimental conditions (see Materials and Methods). Radioactive membrane fractions of *Escherichia coli*, which are known to be protease sensitive (Inouye and Yee, 1972), were mixed with unlabeled *Myxococcus* extracts and analyzed by gel electrophoresis and autoradiography. The pattern obtained was not detectably modified by proteolysis.

Figure 2 shows a sample autoradiogram of the soluble polypeptides labeled at various time points in the absence of methionine. Equivalent amounts of each sample were applied to the gel, except for 1-hr (2×) and 5-hr (1.3×) samples; these contained increased amounts in order to compensate for the lower incorporation of radioactivity at these two time points. This reduced level of incorporation at these early stages of development was probably due to the amino acid content of CF agar. At later times, when the amino acids in the medium have probably been depleted, the rate of incorporation of radioactivity into the soluble fraction was relatively constant. Except for proteins S and U, little radioactivity was incorporated into soluble proteins at 75 hr, when fruiting body formation is completed.

Figure 2 shows that at least 30 major

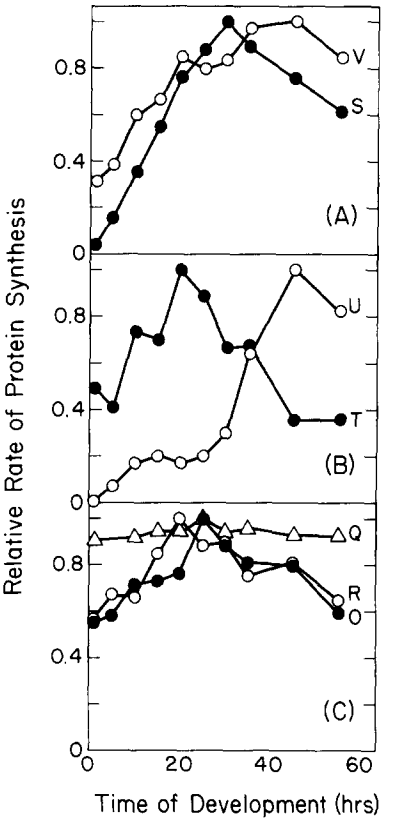


FIG. 3. Changes in rates of soluble protein synthesis during development. Bands M to V were cut out from the gel in Fig. 2, and their radioactivities were measured. The results are expressed as relative rates by normalizing the highest rates observed for each protein to 1. The radioactivities (cpm) of these highest rates were 750, 738, 469, 406, 387, 247, 2352, 225, 148, and 199 for proteins M, N, O, P, Q, R, S, T, U, and V, respectively. (A) ●——●, Protein S; ○——○, protein V. (B) ●——●, Protein T; ○——○, protein U. (C) ●——●, Protein O; ○——○, protein R; △——△, protein Q.

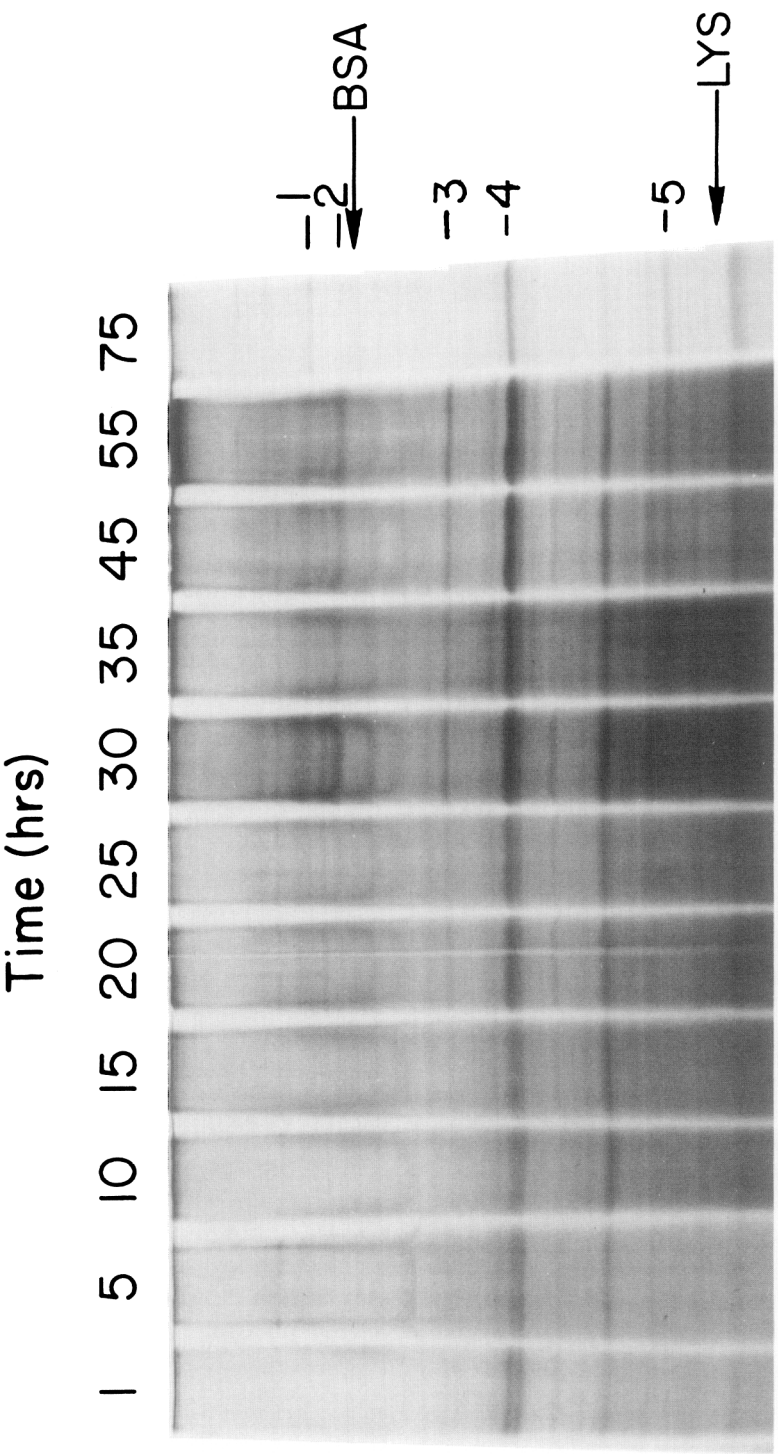


FIG. 4. Pattern of membrane protein synthesis during development. The membrane fraction from the experiment described in Fig. 2 was analyzed as described in that figure except that the autoradiograms were exposed for 15 days. The arrows indicate the positions of molecular weight standards: (BSA, bovine serum albumin; LYS, hen egg white lysozyme.

soluble polypeptides are synthesized during development. Synthesis of many of these major proteins continues during the entire period of aggregation and fruiting body formation. However, a few polypeptides show significant changes in their rates of synthesis. The most striking change is observed in protein S, which showed progressively increased rates of synthesis during development. On the other hand, protein T disappeared after 45 hr, while protein U appeared only after 45 hr.

In order to quantitate these changes in the rates of protein synthesis during development, various bands were cut from the gel and solubilized; radioactivity was measured in a scintillation counter. The results, summarized in Fig. 3, were normalized to facilitate direct comparisons. These results suggest several distinct patterns of synthesis during development: *accumulation proteins*—proteins which show accelerated and continuous synthesis throughout development (e.g., proteins S, and V). In the case of protein S, the change is rather striking. Its production increased from 1% of the total soluble protein synthesis at 1 hr to as high as 15% at 30 hr, and this high level of production was maintained until the end of development (Figs. 2 and 3A). The amount of protein S at 55 hr, as estimated from the Coomassie-blue staining band, is about 10% of the total soluble protein. *Peak proteins*—proteins which show a peak in synthesis midway in development. For example, protein T production reached a peak at 20 hr of development (Fig. 3B); proteins M, N, and P (Fig. 2; not shown in Fig. 3) and proteins O and R (Fig. 3C) are also classified as peak proteins. *Late proteins*—proteins which are only synthesized at a very late period of development. For example, protein U started to appear at 35 hr, and its production reached the highest level at 45 hr (Fig. 2 and 3B). *Constant proteins*—proteins which show constant rates of synthesis throughout development (for example, protein Q in Fig. 3C).

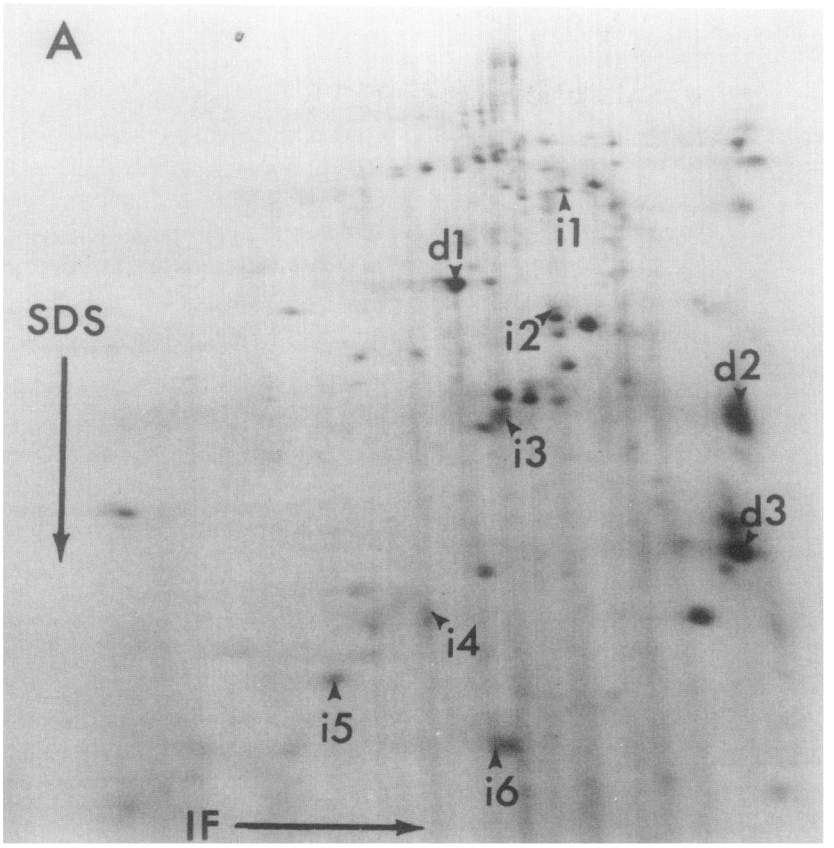
Pattern of Membrane Protein Synthesis during Development

Figure 4 shows a sample autoradiogram of the membrane proteins labeled at various time points during development. About 20 bands can be seen. Many of them (for example, bands 1 to 5) appear to show increased rates of synthesis towards the end of development. We therefore examined the membrane proteins further by two-dimensional polyacrylamide gel electrophoresis. Gel patterns for 10 and 45 hr of development are shown in Fig. 5. Three of the major proteins at 10 hr (d1, d2, and d3) exhibited decreased rates of synthesis at 45 hr. In contrast, many proteins (e.g., i1 to i6) exhibited increased synthesis during development. In these gel patterns, spot i3 corresponds to band 4 in Fig. 4. The synthesis of this protein (apparent molecular weight, about 34,000) increases more dramatically during fruiting body formation than the synthesis of any other membrane protein.

Effect of Methionine on Protein Synthesis during Development

Because methionine inhibits fruiting body formation (see Fig. 1B), pulse-label experiments were also carried out with cells spotted on CF agar containing methionine (200 μg/ml). Figure 6 shows an autoradiogram of the soluble polypeptides labeled at various time points. A comparison with the results obtained in the absence of methionine (the control experiment; Fig. 2) shows several interesting differences: (a) The accumulation proteins, proteins S and

FIG. 5. Two-dimensional electrophoresis of membrane proteins pulse-labeled at 10 and 45 hr of development. Membrane fractions prepared from the experiment described in Fig. 4 were subjected to two-dimensional gel electrophoresis. Autoradiograms (45 days' exposure) from 10-hr samples (A) and 45-hr samples (B) are presented. Arrows with the letter "d" indicate spots that showed decreased intensities during development; arrows with the letter "i" indicate spots that showed increased intensities during development.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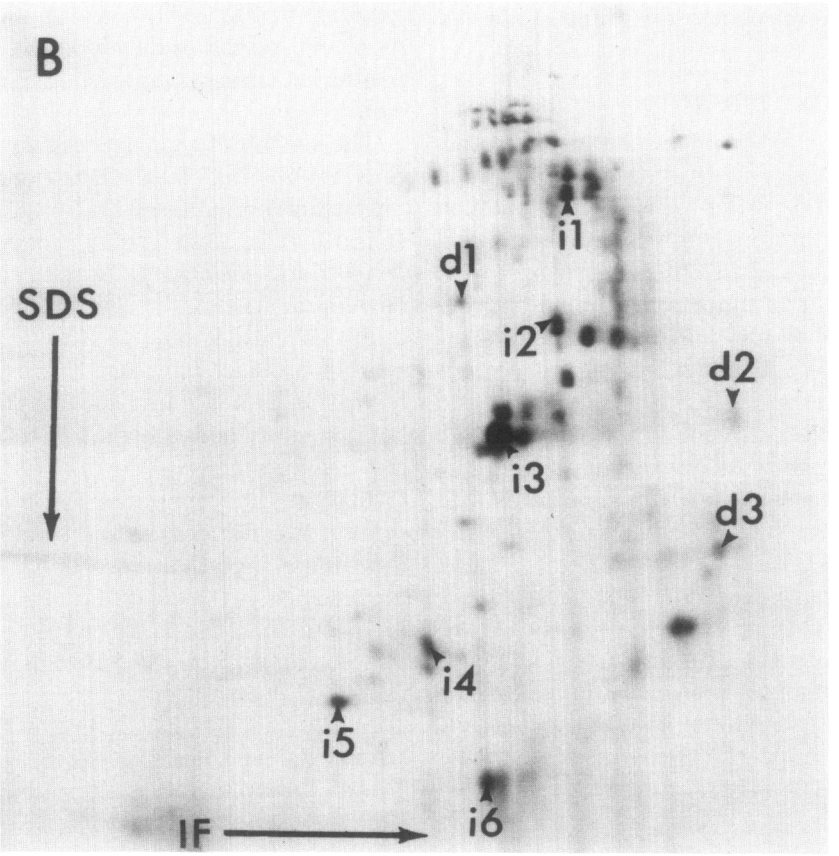
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V, are induced at early times. However, their production at late periods (45 and 55 hr) is markedly decreased in comparison with the control experiment (Fig. 2). (b) Protein T, a peak protein, appears to be induced a little later than the control experiment, but more importantly, its production was not shut off at late times. This is especially evident at 55 hr. (c) The late protein, protein U, was not produced at all in the presence of methionine. (d) The constant proteins also show several changes. For example, the production of proteins P and Q appears to decrease during the late period. On the other hand, the production of protein R seems to increase during the late period.

No significant changes in the synthesis of membrane proteins were observed in the presence of methionine. There was no induction of protein 4, the major membrane protein in the developmental cells, or of proteins 3 and 5 (data not shown). There was also little induction of proteins 1 and 2 in the presence of methionine (data not shown).

Preliminary Characterization of Protein S

Protein S is the most abundant protein synthesized during fruiting body formation (see Fig. 2). In order to gain information on the role of protein S in the developing cell, we determined if this protein is synthesized during glycerol induction. The addition of glycerol to liquid cultures of *M. xanthus* induces the conversion of vegetative rods to spherical spore-like cells (Dworkin and Gibson, 1964). No induction of protein S synthesis occurred during glycerol induction (data not shown). This indicates that protein S may not be essential for glycerol spore formation.

Preliminary experiments indicate that in fruiting bodies protein S is specifically associated with the exterior surface of spores but is probably not essential for spore viability and resistance to heat and sonication. Protein S appears to exist as a monomer when extracted from cells, and its apparent molecular weight as determined by SDS gel electrophoresis is about 23,000. Recently protein S has been purified to near-homogeneity and its properties have been studied in greater detail (manuscript in preparation).

DISCUSSION

In this paper, we describe the pattern of synthesis of the major proteins produced during fruiting body formation in *M. xanthus*. The most striking finding in this study is that many proteins do show significant changes in their amounts and patterns of synthesis. These temporal changes in gene expression can now be related to the morphological stages of development and can be used as biochemical markers for stage-specific events and for the characterization of developmental mutants. Proteins S, T, and U from the soluble fraction, and protein 4 from the membrane fraction are good candidates for markers in studying control mechanisms of gene expression during development. Since they are abundant at a particular period of development, the purification of these proteins should be easy as well.

The control of gene expression of protein S is interesting. Vegetative cells contain only trace amounts of protein S (i.e., less than 1% of the total soluble protein; unpublished data). Synthesis is induced early in development, and the rate of synthesis accelerates until it reaches 15% of the total soluble protein synthesis. Since protein S formation is not found during glycerol induction, it is possible that it functions in cell–cell interactions during fruiting body formation. The regulation of this burst in protein S synthesis during development is now under investigation. We have already purified protein S to near-homogeneity, and its biochemical properties have been investigated (Inouye, Inouye, and Zusman, manuscript in preparation).

Another interesting finding is the correlation between the disappearance of protein T and the appearance of protein U. The

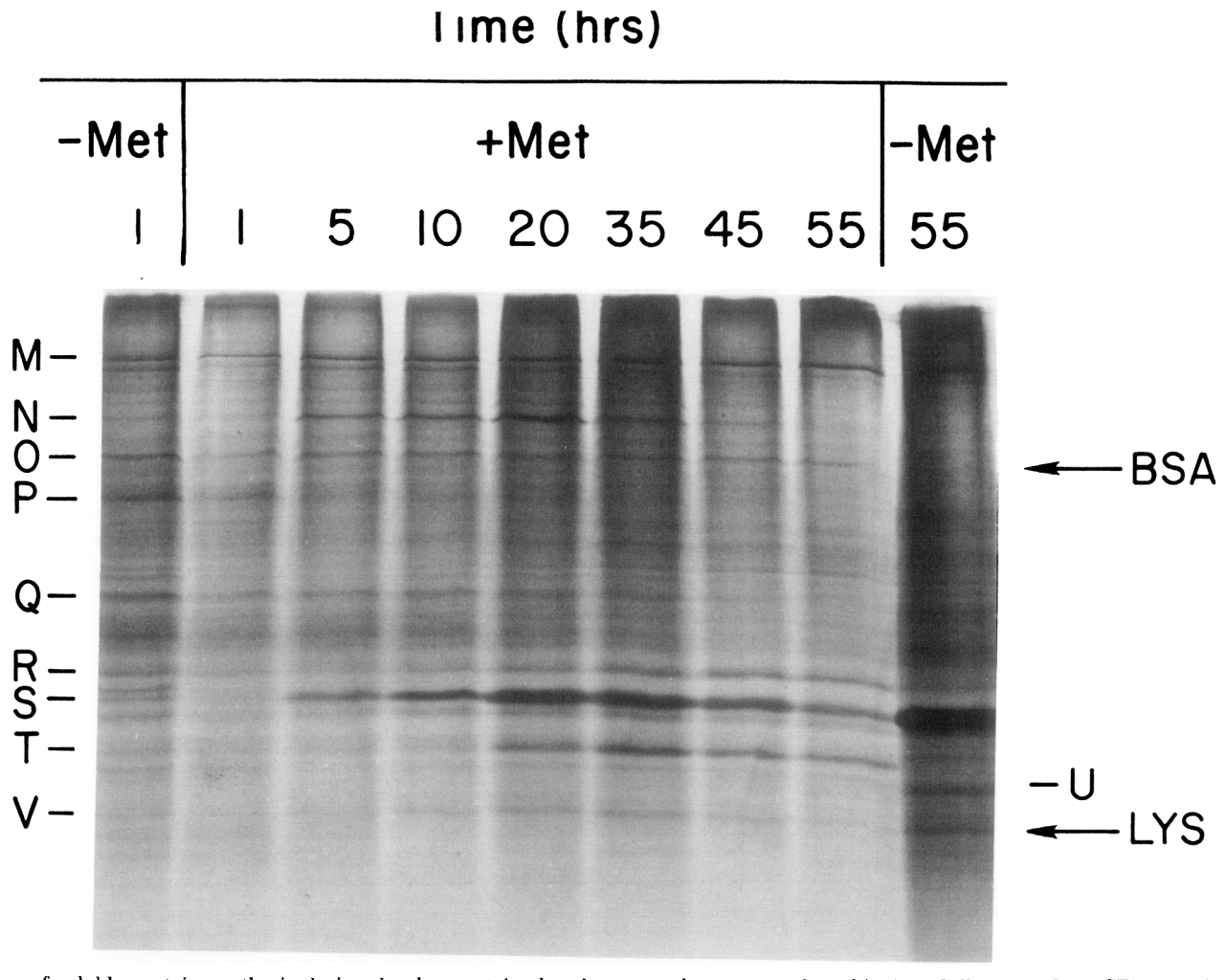

FIG. 6. Pattern of soluble protein synthesis during development in the absence and presence of methionine. Cells spotted on CF agar with or without methionine (200 μg/ml) were pulse-labeled with a ^{14}C-labeled amino acid mixture at various time points indicated in the figure. The wells at the left (1 hr) and right (55 hr) extremes contain samples labeled in the absence of methionine; all other wells contain samples labeled in the presence of methionine. The autoradiograms were exposed for 10 days. Arrows indicate the positions of molecular weight standards: BSA, bovine serum albumin; LYS, hen egg white lysozyme.

production of protein T rapidly dropped between 30 and 45 hr of development, while the production of protein U sharply increased at the same time (Fig. 3B). In the presence of methionine, the production of protein T was not shut off, and the production of protein U was not detected. We are now determining if a relationship exists between these two proteins. It should be pointed out that the appearance of protein U seems to coincide with the appearance of spores.

The role of methionine in inhibiting the developmental program was also investigated. While methionine does not inhibit early aggregation, it does inhibit the formation of mounds and the subsequent maturation of these structures into fruiting bodies. After continued incubation in the presence of methionine, even the initial aggregates disperse. (Methionine cannot be very toxic, since total protein synthesis is not greatly affected.) Surprisingly, under these conditions, almost all of the protein changes observed in the early and middle periods of development still occurred. However, the production of *late proteins* (e.g., protein U) was not observed. These results are difficult to interpret. One hypothesis is that methionine only blocks a late stage of development. However, this is difficult to determine, since development in *Myxococcus* may involve both dependent and independent pathways. If developmental proteins are controlled by independent pathways, then it is impossible to determine the inhibitory stage for methionine.

In the present paper, we have focused only on the major proteins synthesized during development. The synthesis of many minor proteins probably undergoes similar changes during development. However, examining such changes in minor proteins is not feasible using crude extracts. One way to study some of these minor proteins is to partially purify developmental extracts by using development specific antibodies or by purifying special subclasses of proteins. We are currently using this approach to study changes in minor proteins.

The pattern of protein synthesis during development indicates that gene expression is complex and subject to tight regulation. Thus, *M. xanthus* is an excellent model system for elucidating the molecular mechanisms which control gene expression during development.

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