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Methylation of Macromolecules during Development in *Myxococcus xanthus*

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Covalent modification of macromolecules can serve to alter their biological activities and is therefore frequently involved in regulation. I examined methylation of proteins and carbohydrates during development and vegetative growth in the procaryote *Myxococcus xanthus*. Striking differences in the patterns of protein methylation occurred when cell development was induced by nutrient deprivation on solid media and when cells were starved in liquid. In addition, a methylated, protease-resistant macromolecule which contained carbohydrate and which may have been an unusual type of lipopolysaccharide was observed on sodium dodecyl sulfate-polyacrylamide gels. A comparison of methylation patterns in various media and an analysis of the time course of methylation indicated that changes in methylation were part of the developmental pathway which includes aggregation. Induction of development in liquid by glycerol produced no changes in methylation.

Those seeking to understand biochemical regulation during development sometimes find it advantageous to study a model system. *Myxococcus xanthus* is a procaryote which, when induced by starvation or other environmental factors, will undergo a developmental program in which millions of cells aggregate, form fruiting bodies, and differentiate into spores. These features make *M. xanthus* an attractive model for the study of development, and several reviews are available which describe the excellent work done in various areas of myxobacterial development, such as genetics, regulation of gene expression, motility, cell-to-cell interaction, and changes in morphology (17). All of these investigations clearly demonstrate profound changes in the behavior and metabolism of developing *M. xanthus*. However, there are few reports of cases in which such changes have been attributed to a specific biochemical reaction. In this paper, I report that methylation of polypeptides and of an unusual carbohydrate occurred in *M. xanthus* and that during the early stages of development, several changes in methylation took place. Methylation is an easily monitored event; therefore, an examination of the role of the methylated macromolecules and of the factors which influence methylation should provide valuable insight into the biochemistry of bacterial development.

MATERIALS AND METHODS

Bacterium and growth conditions. Yellow variants of *M. xanthus* DZF1 were grown at 32°C with vigorous aeration in Casitone (Difco Laboratories, Detroit, Mich.)-yeast extract (CYE) medium (2) or LM medium (10). Clone fruiting (CF) medium is a limited medium which promotes fruiting (9). TM buffer is 10 mM Tris hydrochloride (pH 7.6)–8 mM $MgSO_4$; morpholinepropanesulfonic acid (MOPS) buffer is 10 mM MOPS (pH 6.8)–1 mM $CaCl_2$.

Methylation on solid medium. Small plates containing 5 ml of CF or CYE agar were prepared and allowed to dry at least 24 h. Logarithmically growing cells were concentrated to approximately 10^{10} cells per ml by centrifugation and suspension in TM buffer. For continuous labeling, 37 μl of L-[*methyl*-^{3}H]*S*-adenosylmethionine (1.0 mCi/ml, 70 Ci/mmol; New England Nuclear Corp., Boston, Mass.) was dried under N_2 and suspended in 0.1 ml of the concentrated cells. This mixture was applied to the plates in spots of 20 μl, the spots were allowed to dry, and the plates were incubated at 32°C for 6 h. For pulse-labeling, cells were labeled on filters by the method of Smith and Dworkin (21). Labeled cells were removed from filters or agar by scraping, washed in TM buffer, suspended in sodium dodecyl sulfate (SDS) buffer, and boiled briefly. Parallel unlabeled cultures were examined under a dissecting microscope at various times for aggregation and fruiting body formation.

Methylation in liquid cultures. A 3-ml sample of logarithmically growing cells was harvested from CYE or LM medium, washed twice, and suspended in the appropriate labeling medium. After a 30-min preincubation at 32°C with shaking, label was added (85 μCi of L-[*methyl*-^{3}H]methionine at 80 Ci/mmol or 90 μCi of L-[*methyl*-^{3}H]*S*-adenosylmethionine at 70 Ci/mmol), and incubation was continued for 90 min. Labeled cells were harvested, suspended in 400 μl of SDS buffer, and boiled.

Glycerol induction. Cells were grown and labeled in liquid CYE medium as described above except that the labeling medium contained 0.5 M glycerol. Samples were removed, harvested, and suspended in SDS buffer at the times indicated. Parallel control cultures were examined by microscope for the presence of spherical spores among the rod-shaped vegetative cells.

Enzymatic digestion. DNase I or RNase I (0.2 mg/ml final concentration) or pronase (1 mg/ml) was added to denatured samples in SDS buffer, and samples were incubated overnight at 37°C.

Phenol extraction. The method for labeling on solid medium was adapted for large-scale preparations by applying 45 spots (30 μl each) of cells plus radioactive *S*-adenosylmethionine to small plates of CF agar. After incubation at 32°C for 24 h, cells were scraped from the agar, suspended in 10 ml of TM buffer, and disrupted by sonication. Pancreatic RNase I was added, and the extract was incubated overnight at 32°C. After centrifuging at 6,000 × *g* for 10 min to remove debris, the supernatant was centrifuged at 85,000 × *g* for 2 h in a type 50 rotor. This pellet was suspended in 10 ml of H_2O, warmed to 65°C, and extracted with an equal volume of 89% phenol (also prewarmed to 65°C) by a modification of the procedure of Westphal and Jann (23). After 15 min at 65°C, the phenol mixture was cooled in an ice bath and centrifuged to separate the phases, and the aqueous layer was removed.

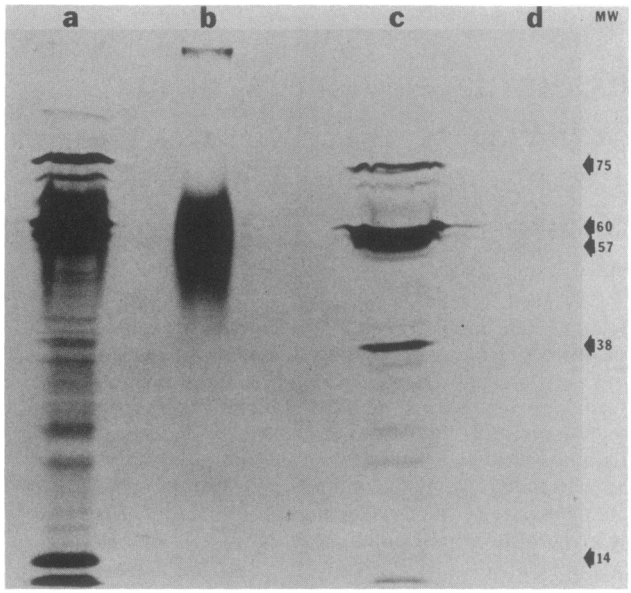

FIG. 1. Methylation of macromolecules during vegetative growth and development in *M. xanthus*. Cells were labeled with L-[*methyl*-^{3}H]*S*-adenosylmethionine on solid media and analyzed by autoradiography of 12% polyacrylamide gels containing SDS. Lanes: a and b, CF medium; c and d, CYE. Samples in lanes b and d were treated with protease before electrophoresis. Molecular weights (MW) were calculated relative to standards.

The phenol layer was warmed and reextracted with water. The two aqueous phases were combined and dialyzed extensively against water.

Periodate treatment. After dialysis, the aqueous phase from the phenol extraction was dried under nitrogen, suspended in 1 ml of 50 mM sodium acetate (pH 4.5) containing 25 mM sodium periodate (omitted from control tubes), and left at 4°C for 22 h. The samples were quenched with 250 mM (final concentration) ethylene glycol, dialyzed extensively against water, dried, and suspended in SDS buffer for gel electrophoresis.

Polyacrylamide gel electrophoresis. SDS-polyacrylamide gel electrophoresis (12% polyacrylamide, 0.32% bis-acrylamide) was as described previously (16). Gels were analyzed by fluorography with En^{3}Hance (New England Nuclear). Proteins were stained with Coomassie brilliant blue, and carbohydrate was stained with Schiff's reagent as described previously (8) or by reaction with silver (22).

RESULTS

Developing cells of *M. xanthus* differed dramatically from vegetative cells when their patterns of methylation were compared (Fig. 1). Vegetative growth on CYE medium (lane c) caused methylation of polypeptides of approximately 75,000, 60,000, 57,000, and 38,000 daltons (Da). On the other hand, incubation on CF medium (lane a), which caused the cells to fruit, reduced the methylation of the 38,000-Da protein and caused the appearance of a new methylated band at 14,000 Da. Treatment of these samples with protease before electrophoresis exposed an additional striking difference in methylation. Cells grown on CYE showed no protease-resistant material (lane d). In contrast, protease-resistant material appeared in the CF sample (lane b) as a diffuse band of 40,000 to 60,000 Da.

CF is a very limited medium which both depletes cells of nutrients and induces them to aggregate, form fruiting bodies, and sporulate. Thus, two different but related conditions may have influenced the patterns of methylation shown in Fig. 1. However, to some extent, these conditions could be differentiated because normal fruiting requires a solid surface. To determine whether, like fruiting, the alterations in methylation required solid media, I compared methylation in liquid media with the patterns observed on solid media. Liquid CYE and LM were chosen as rich media; they supported vegetative growth of *M. xanthus* with doubling times of 4 and 22 h, respectively. CF liquid medium allowed very little growth (doubling time 75 h, whereas LM medium lacking methionine, TM buffer, and MOPS buffer permitted no growth whatever. Incubation in these different media affected the patterns of methylation as demonstrated in Fig. 2. The three cultures tested in MOPS buffer (lane b), CF medium (lane c), and TM buffer (lane d) produced a methylated polypeptide of 14,000 Da, such as that seen on solid CF medium. However, none of the three cultures showed significant labeling of the 75,000- or the 60,000-Da proteins, which were apparent in Fig. 1 and which occurred in lane a. Cells labeled in CF medium (lane c) and in LM medium lacking methionine (lane a) produced a methylated band at 57,000 Da. In other respects, the liquid cultures differed from each other. For example, cells labeled in TM buffer produced a 30,000-Da band which was labeled much less strongly (if at all) in the other cultures. LM medium lacking methionine (lane a) was the most complete medium shown in Fig. 2, although the omission of methionine prevented

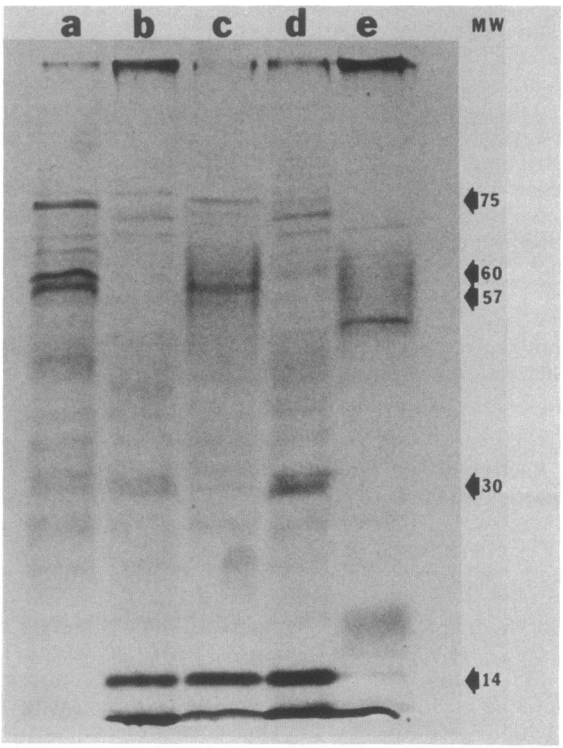

FIG. 2. Methylation patterns in liquid media and buffers. Lanes: a, LM medium lacking methionine; b, MOPS buffer; c, CF medium; d, TM buffer; e, LM medium lacking methionine. Lanes a through d were labeled with L-[*methyl*-^{3}H]*S*-adenosylmethionine; lane e was labeled with L-[*methyl*-^{3}H]methionine in the presence of chloramphenicol. MW, Molecular weight standards.

growth and (if this were a solid medium) could have induced fruiting. The methylation observed in LM medium lacking methionine was very similar to that observed in LM medium plus methionine (data not shown) and in liquid CYE medium (see Fig. 4, lane a). An example of methylation by methionine is shown in Fig. 2, lane e, and the results were surprisingly different from those shown in lane a, although the media were identical (LM lacking methionine).

In liquid culture, starvation was not sufficient to cause methylation of the protease-resistant material (Fig. 3), which was methylated by cells incubated in CF medium (lane c) but not by cells in TM buffer (lane b) or in MOPS buffer (lane d). Comparison of lanes a and e revealed a further anomaly in the behavior of *S*-adenosylmethionine and of methionine as methyl donors, since methionine methylated the protease-resistant material, whereas *S*-adenosylmethionine, under apparently identical conditions, did not.

Glycerol, when added to *M. xanthus* growing in rich medium, induced a type of sporulation which resembled that found on solid media, yet which differed in several respects. Methylation of polypeptides and protease-resistant material remained unaffected by glycerol induction (Fig. 4), although the cells changed from the rod-shaped morphology typical of vegetative growth to the spherical shape typical of spores.

Aggregation is an aspect of development on solid media which is absent during starvation in liquid and during glycerol induction. Under the conditions used in these labeling experiments, aggregation began at 4 to 6 h after plating, rippling began at 8 to 10 h, and raised mounds became visible by 24 h. The time course of methylation of the protease-resistant material corresponded with the time course of aggregation. Cells which were developing on filters on CF plates were pulse-labeled, and the results for selected times are shown graphically in Fig. 5. Methylation of the protease-resistant material increased during the early stages of aggregation and peaked at the onset of rippling. Methylation of the 14,000-Da polypeptide followed a similar time course. At later times, recovery of samples became increasingly difficult as the cells became more and more resistant to extraction by SDS; the extent to which this affected the decline in methylation observed in Fig. 5 and 6 is not known. Methyl groups which were incorporated early in development remained relatively stable as determined by pulse-chase experiments (shown in Fig. 6).

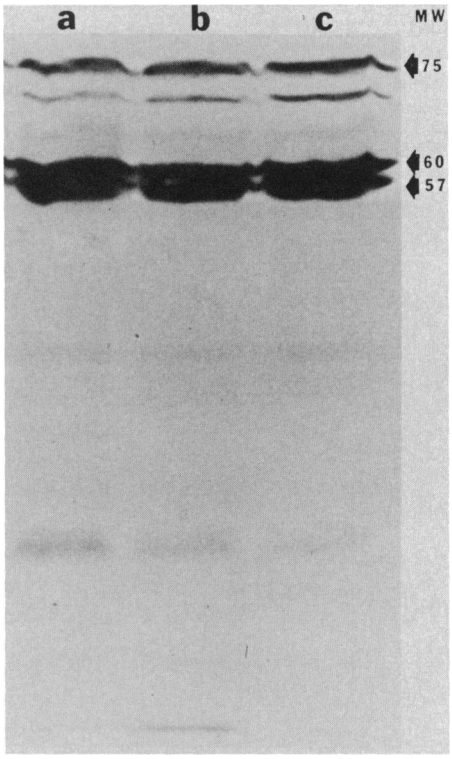

FIG. 4. Methylation during glycerol induction in *M. xanthus*. Cells were labeled in liquid CYE after glycerol induction. Lanes: a, uninduced control; b, 30 min; c, 90 min. MW, Molecular weight standards.

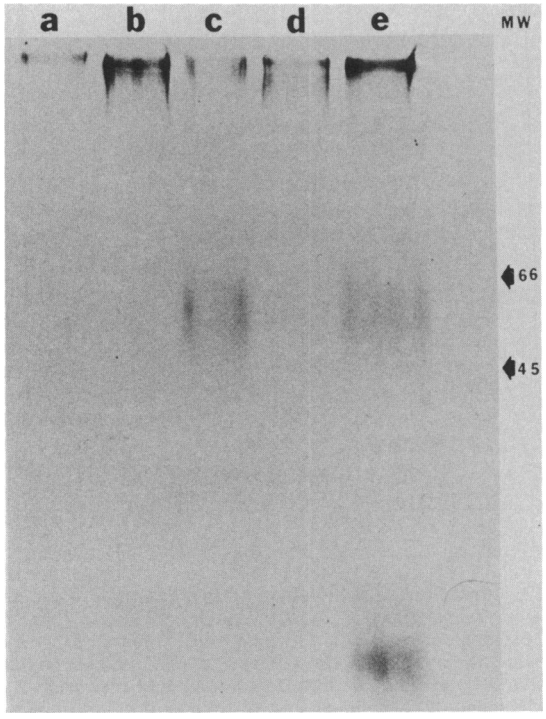

FIG. 3. Protease-resistant methylation in liquid media. Samples were identical to those described in the legend to Fig. 2, except that they were treated with protease before electrophoresis.

Characteristics of the protease-resistant material. Digestion with RNase or DNase had no effect on the electrophoretic mobility of the protease-resistant material. However, treatment with periodate completely eliminated the band from the autoradiographs (data not shown).

Techniques which are commonly used to purify lipopolysaccharides (23) were useful in the isolation of this methylated material from *M. xanthus*. During the purification, methylated material was assayed by autoradiography of SDS-polyacrylamide gels. After disruption of cells by sonication, the material sedimented with the membrane fraction, remained in the aqueous phase during extraction with warm phenol, and banded at a density of 1.38 g/ml in isopycnic CsCl gradients. Recovery during this isolation was monitored by the phenol-sulfuric acid assay of Dubois et al. (4), and a preparation of 10^{11} cells generally yielded 1 to 2 mg of material.

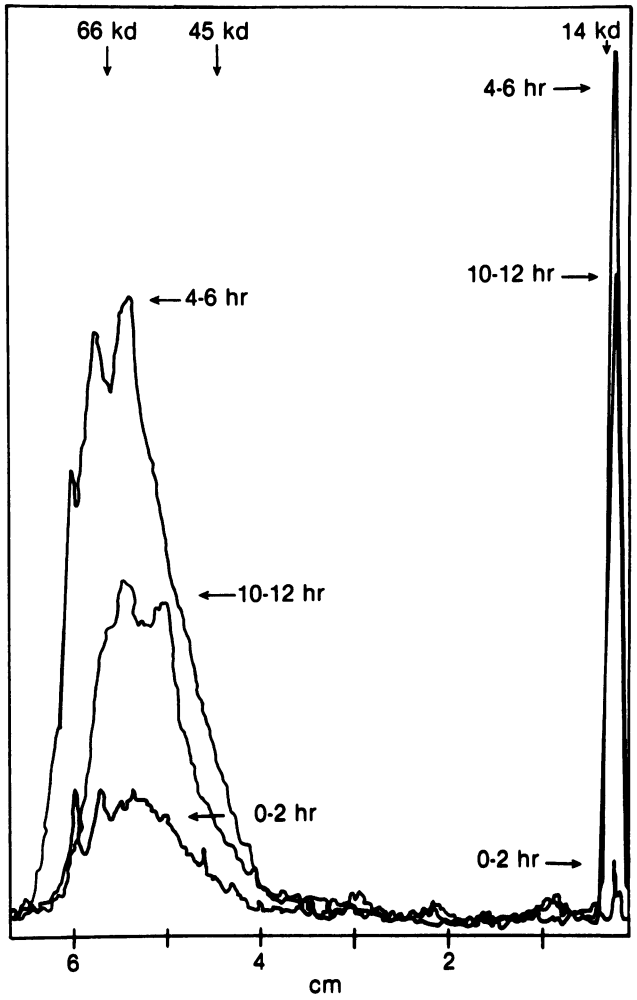

FIG. 5. Time course of methylation during development in *M. xanthus*. Cells were labeled for 2 h at the times indicated after plating on solid CF medium. Autoradiographs were scanned, and film density was plotted against distance (cm) from the dye front. The positions of the following molecular weight standards were measured on the Coomassie-stained gels and are shown by arrows on the density scan: bovine serum albumin, 66,000 (66 kd); ovalbumin, 45,000 (45 kd); lysozyme, 14,000 (14 kd).

DISCUSSION

Methylation of macromolecules is part of the developmental cycle in *M. xanthus*. Two macromolecules, a 14,000-Da polypeptide and a protease-resistant macromolecule, showed significant increases in methylation when developing cells were analyzed by gel electrophoresis. However, although methylation of the macromolecules was readily apparent, their function and their role in the developmental program of *M. xanthus* were not.

Fruiting is a complex process in *M. xanthus*, and much of the biochemistry of fruiting is poorly understood. The fruiting program begins when cells are depleted of nutrients (5, 10) and continues through formation of fruiting bodies containing myxospores. A solid surface is required for fruiting body formation, and starvation in liquid does not lead to fruiting body formation. The many events involved make it convenient to subdivide fruiting body formation into readily identifiable phases, such as aggregation, rippling, and the formation of mounds (15, 19). In which phase of development does methylation occur? The following criteria may be applied in attempting to assign roles to the methylation events I observed. If methylation were related only to the earliest phase, starvation, it might occur whether cells were starved in liquid or solid cultures. This was the case for the 14,000-Da polypeptide, which became heavily methylated whenever nutrients were severely limited.

On the other hand, methylation related to a later phase, such as aggregation, might occur only when cells were analyzed under conditions in which aggregation took place.

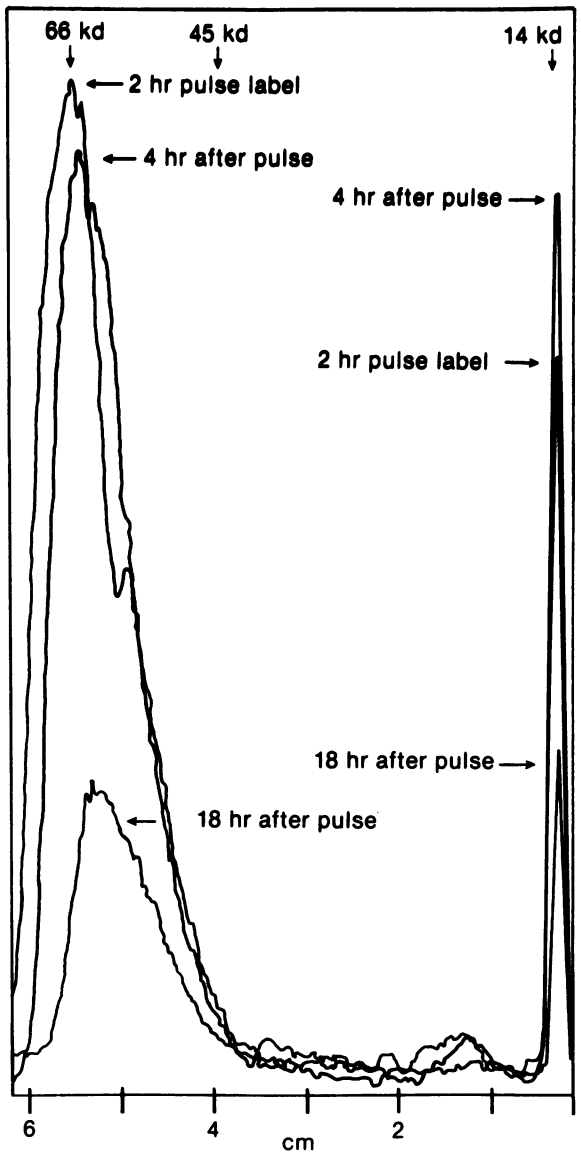

FIG. 6. Analysis of methylation during and after a 2-h pulse-label. Cells were plated on top of Nuclepore filters (Nuclepore Corp., Pleasanton, Calif.) on CF agar and incubated for 4 h. The filters were then removed, transferred to CF medium containing L-[*methyl*-^{3}H]*S*-adenosylmethionine for 2 h, and returned to unlabeled CF medium. The times shown refer to the durations of the incubation on unlabeled medium after the 2-h pulse. Autoradiographs were scanned beginning at the dye front. Molecular weight standards are the same as in the legend to Fig. 5.

Methylation of the protease-resistant material did not occur when cells were starved in liquid buffer, but methylation did occur on several types of limited solid media; thus, the protease-resistant material met this criterion. Protease-resistant material was methylated to a very slight extent in liquid CF medium, but data from other laboratories indicate that several metabolic changes characteristic of developing cells are also observed in liquid CF medium (3). Thus, the conclusion that methylation of the protease-resistant material is normally part of the poststarvation phases of development is consistent with the observed labeling of protease-resistant material.

Methionine is frequently used as a methyl donor in in vivo labeling experiments because many types of cells are impermeable to *S*-adenosylmethionine. Two factors make methionine undesirable for use in these experiments: labeling is feeble in rich medium, and protein synthesis must be blocked. *M. xanthus*, however, takes up *S*-adenosylmethionine (16), and it was therefore possible to compare methylation during vegetative growth and development. The methylation pattern obtained from methionine-labeled cells was not identical to the pattern obtained from *S*-adenosylmethionine-labeled cells, because, for example, methionine labeled protease-resistant material (although rather poorly) under conditions in which *S*-adenosylmethionine did not. Characterization of material that was labeled by both donors is underway, and no differences in composition have been found (S. M. Panasenko, manuscript in preparation). The cause of this difference in methylation patterns remains unclear.

Changes in methylation are not part of the glycerol-induced sporulation pathway. Glycerol not only eliminates the nutritional basis for sporulation, it allows sporulation to occur in liquid culture, bypassing aggregation and the production of fruiting bodies. The spores induced by glycerol share many properties with fruiting body spores, such as resistance to heat and to sonication (6). Methylation is, therefore, not likely to be required for acquisition of these properties, which argues against a role for methylation in, for example, formation of the spore coat. On the other hand, glycerol spores differ from fruiting body spores in several respects, including the composition of the coat (11, 13), so such a role for the products of developmentally induced methylation cannot be entirely ruled out.

Mutants have been isolated which are defective in aggregation or fruiting but which form glycerol-induced spores normally, and parallel pathways of development have been proposed to account for the phenotypes of these mutants (15). Thus, such mutations are thought to affect only those phases of fruiting which are required for development on a solid surface. Similarly, methylation of the 14,000-Da polypeptide and the protease-resistant material may be part of the aggregation pathway which does not come into play during sporulation in glycerol. These results do not point to a specific function for methylation. However, again, they suggest that the relevant phase for methylation of the 14,000-Da protein is starvation, whereas the relevant phase for methylation of the protease-resistant material is aggregation. Currently, testing of the various aggregation, fruiting, and sporulation mutants for methylation is in progress, and this work may allow us to assign methylation to a specific physiological or morphological event.

The time course of methylation further indicated a correlation between methylation and aggregation. Poor synchrony among developing cells of *M. xanthus* makes it difficult to pinpoint events within more than a 2-h range. Thus, the timing of aggregation, rippling, etc., varied somewhat from day to day. However, in repeated experiments, peak methylation of both the polypeptide and the protease-resistant material occurred within 2 h of the onset of rippling.

Changes in methylation during development had been reported previously (12). However, those authors used methionine as the methyl donor, and the labeling was done in starvation buffer. Thus, it would not have been possible to differentiate between starvation and development. Furthermore, even vegetative cells were starved during the labeling. This probably accounts for the observed methylation of the lower-molecular-weight polypeptide and of the protease-resistant material during vegetative growth as well as during glycerol induction (12). An additional difference in procedure (a higher-percentage acrylamide gel) enabled Komano et al. (12) to resolve the low-molecular-weight polypeptides into three components, whereas I observed only a single band near the dye front. When I used higher-percentage gels, I also observed the three lower-molecular-weight bands; however, the gels shown in this paper are at a lower acrylamide concentration to optimize visualization of the protease-resistant material.

What is the methylated protease-resistant material? The sensitivity to periodate oxidation indicated that the material contained carbohydrate, yet the resistance of the protease-resistant material to degradative enzymes ruled out nucleic acid or glycoprotein. The migration of bacterial lipopolysaccharides in SOS gels has been reported previously (18), and the behavior of the methylated material from *M. xanthus* is very similar. The methylated compound from *M. xanthus* pelleted with the membrane fraction during differential centrifugation, entered the aqueous phase during extraction with warm phenol, and had a buoyant density of 1.38 g/ml in CsCl. These also are characteristics of bacterial lipopolysaccharides. One may suggest, therefore, that *M. xanthus* produced a lipopolysaccharide which was methylated during development. An analysis of the carbohydrate content, sequence, and lipid content of this unusual methylation product is in progress, and confirmation of this hypothesis must await the results of these experiments.

What is the significance of methylation during development? Methylation of proteins, lipids, and carbohydrates has been an area of active investigation for some time, and several interesting functions for such methylation products have been proposed. For example, protein methylation is involved in signal processing during bacterial chemotaxis (14, 20), and lipid methylation is required for transmembrane signal transmission in eucaryotes (1). Signaling is certainly important in development in *M. xanthus*, and methylation may be a mechanism by which signaling occurs. Protein methylation may also serve as a mechanism for regulation of enzymatic activity, and, by analogy, carbohydrate methylation might alter specific structure-dependent interactions. In this regard, it is especially intriguing to note the effects of modification of carbohydrate content in systems such as the cell adhesion molecules characterized by Edelman and coworkers (7). The biological stability of the methyl groups on both the 14,000-Da protein and the carbohydrate was apparent from the pulse-chase labeling experiments. Methylation during chemotaxis has been found to be quite transient; thus, an analogous type of signaling in *M. xanthus* seems unlikely. However, these results are consistent with a long-term regulatory function for methylation.

Although it is yet too early to propose a function for the methylation of either polypeptides or carbohydrates in *M. xanthus*, the correlation of these events with development is

in itself of great significance. Methylation is an easily monitored event which lends itself to analysis at the biochemical level. Heretofore, few biochemical "handles" have been available in *M. xanthus*, a system which is unusually well characterized with respect to the genetics and physiology of development. Methylation therefore offers the opportunity to study the regulation of methylating and demethylating activities and to link this regulation with the developmental cycle.

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