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Myxococcus xanthus Protein C Is a Major Spore Surface Protein

WILLIAM R. McCLEARY,† BRENT ESMON,‡ AND DAVID R. ZUSMAN*

Department of Molecular and Cell Biology, University of California, Berkeley, California 94720

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Fruiting body formation in *Myxococcus xanthus* involves the aggregation of cells to form mounds and the differentiation of rod-shaped cells into spherical myxospores. The surface of the myxospore is composed of several sodium dodecyl sulfate (SDS)-soluble proteins, the best characterized of which is protein S (M_r, 19,000). We have identified a new major spore surface protein called protein C (M_r, 30,000). Protein C is not present in extracts of vegetative cells but appears in extracts of developing cells by 6 h. Protein C, like protein S, is produced during starvation in liquid medium but is not made during glycerol-induced sporulation. Its synthesis is blocked in certain developmental mutants but not others. When examined by SDS-polyacrylamide gel electrophoresis, two forms of protein C are observed. Protein C is quantitatively released from spores by treatment with 0.1 N NaOH or by boiling in 1% SDS. It is slowly washed from the spore surface in water but is stabilized by the presence of magnesium. Protein C binds to the surface of spores depleted of protein C and protein S. Protein C is a useful new marker for development in *M. xanthus* because it is developmentally regulated, spore associated, abundant, and easily purified.

Myxococcus xanthus is a gram-negative, rod-shaped bacterium that has a complex life cycle which includes fruiting body formation (6, 23, 26). When cells are placed under nutrient-limiting conditions on a solid surface, a developmental program is triggered which results in fruiting body morphogenesis and sporulation. Myxospores are metabolically quiescent and environmentally resistant cells that germinate when nutrient conditions become favorable. The time course of development (formation of mature fruiting bodies with maximum number of spores) is about 4 days under our standard laboratory conditions; however, spores continue to mature for several days thereafter (20).

Molecular markers of development are clearly useful for the study of gene expression during the developmental program. To date, however, only a few gene products have been described and characterized in any detail. (i) Myxobacterial hemagglutinin (MBHA) is a lectin (M_r, 28,000) that appears at the time of cellular aggregation (9 to 30 h in strain DZF1) (2). MBHA was found to be localized at the cell surface and the periplasmic space of aggregating cells (19). The gene for MBHA (called *mbhA*) was cloned, and its nucleotide sequence was determined (21, 22). Mutants deficient in the production of MBHA are unable to aggregate properly when development is initiated at low cell density and at low Mg^{2+} concentrations (22). (ii) Protein S is a spore surface protein (M_r, 19,000) whose synthesis begins by 5 to 10 h after the initiation of development and reaches a peak at about 50 h (4). During differentiation, the protein is deposited outside the cell (11). When the gene for protein S (called *tps*) was cloned and sequenced, it was discovered that there were actually two highly homologous genes adjacent to each other on the chromosome which encoded protein S (Tps) and protein S-1 (Ops) (12, 13). (iii) The *ops* gene product is about 80% homologous to Tps but has a different pattern of gene expression and localization. Synthesis of Ops begins about 24 h after the initiation of development, and Ops accumulates inside the spores rather than on the spore surface (4, 7, 24). In this report, we identify another abundant, developmentally regulated protein of *M. xanthus*, called protein C, which is localized on the spore surface along with protein S.

Protein C is an abundant spore surface protein. When spores of *M. xanthus* are purified on sucrose gradients and then boiled in sodium dodecyl sulfate (SDS), the spores maintain their integrity, although they are no longer viable (11). Examination of the solubilized proteins by SDS-polyacrylamide gel electrophoresis (PAGE) revealed three bands with apparent molecular weights of 16,000 (protein U), 23,000 (protein S) (the molecular weight deduced from DNA sequence analysis is 19,000), and 30,000, a protein we now refer to as protein C. The protein C band was observed (but not described) in an earlier report as a faint band above protein S (3, 11). However, in many subsequent experiments we have found that protein C is actually very abundant on the spore surface (Fig. 1). In our hands, protein U is always faint and often, as is the case in Fig. 1, not visible on the gels.

Figure 1 shows a Coomassie blue-stained SDS-polyacrylamide gel of myxospores that were harvested from CF agar plates and boiled in 1× Laemmli loading buffer (17). Protein C is almost as abundant on the spore surface as protein S in our wild-type strain DZF1 (Fig. 1, lane 1). Since protein C is also found on the spore surface in strain DZF3367 (which contains Tn*5* mutations in both *tps* and *ops*) (3), its accumulation on the spore surface is not dependent on the presence of the *tps* or *ops* gene product. The data in Fig. 1 also suggest that spores from strain DZF3367 would be a good source for the purification of protein C. We therefore prepared spores from that strain by spotting exponentially growing cells (about 5×10^8/ml) concentrated 3,000-fold onto 100 CF agar plates as described previously (11). The plates were incubated for 96 h at 28°C. The fruiting bodies were scraped from the plates with a glass rod, and the spores and peripheral cells were washed in ice-cold TM buffer (10 mM Tris-HCl [pH 7.6], 8 mM $MgSO_4$) and collected by centrifugation at 5,000 × *g* for 10 min at 4°C. The cell suspension was sonicated for 2.5 min. The broken cell suspension and spores were layered onto sucrose step gradients (7.5, 15, 30, and 60% sucrose in TM buffer) and centrifuged for 6 min at 2,000 rpm in a Sorvall HS-4 swinging-bucket rotor. The spores

* Corresponding author.
† Present address: Department of Molecular Biology, Princeton University, Princeton, NJ 08544.
‡ Present address: Infra-gene Inc., Benecia, CA 94510.

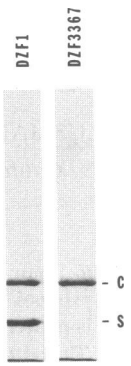

FIG. 1. SDS-PAGE of spores from *M. xanthus*. Strains were spotted onto CF agar plates and incubated at 28°C for 4 days. Spores were harvested and purified as described in the text. DZF1 is wild type; DZF3367 contains Tn*5* insertions in both *ops* and *tps* (3).

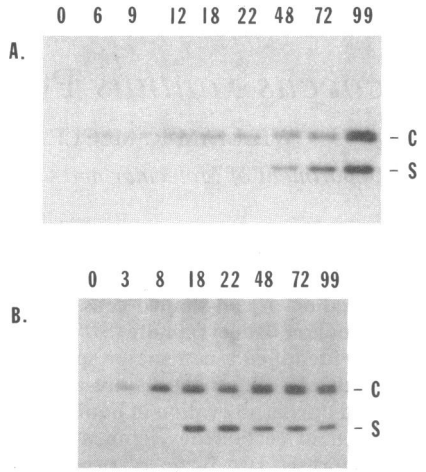

FIG. 2. (A) Developmental induction of protein C and protein S. Vegetatively growing cells were plated on CF agar to induce development. Plates were incubated at 34°C. Cells were harvested at the indicated hours after the initiation of development and processed as described in the text. (B) Time course of expression of protein C and protein S during incubation of *M. xanthus* in liquid CF shaker culture. Vegetatively growing cells were washed in cold TM buffer and resuspended in liquid CF medium as described by Downard et al. (4). Samples were collected by centrifugation at the indicated times (hours) and processed as described for panel A.

banded at the 30 to 60% sucrose interface. The top of the gradient was removed, and the spores were pelleted (8,000 rpm for 20 min in a Sorvall SS34 rotor), washed, and resuspended in TM buffer. The spores were sonicated again and repurified in a second sucrose gradient as described above. The purified spores were boiled for 2 min in Laemmli sample buffer and subjected to SDS-PAGE on a 10% preparative slab gel. The protein C band was located by staining the gel with 4 M sodium acetate (10), cut from the gel, and then electroeluted from the acrylamide. We estimate that we recovered 200 μg of protein from the gel. Antiserum to protein C was prepared by injecting two rabbits intradermally with an emulsion of 100 μg of purified protein and Freund complete adjuvant.

Protein C synthesis is developmentally regulated. The antiserum to purified protein C was used to determine the time course of protein C synthesis during development. In this experiment (Fig. 2A), cells were spread onto CF agar plates as described previously (25) and incubated at 34°C. At various times of development, cells were harvested and rapidly frozen for subsequent processing. The cells were disrupted with zirconium beads in a Minibeadbeater (Biospec Products, Bartlesville, Okla.) for seven 60-s cycles. The extracts were kept on ice between cycles. Cell disruption was estimated to be greater than 98% by microscopic examination using phase-contrast optics. Equivalent amounts of protein, as measured by the method of Bradford (1), were separated on 10% SDS-polyacrylamide gels and blotted onto nitrocellulose membranes, and protein C was detected immunologically with ^{125}I-protein A. To detect protein S on the same blot, the nitrocellulose filter was erased of protein C antibodies and the bound ^{125}I-protein A by a wash in 0.1 M glycine-HCl (pH 2.2)–0.02 M magnesium acetate–0.05 M KCl for 90 min (18). The filter was then reprobed with antiserum to protein S. Figure 2A shows a typical experiment consisting of superimposed autoradiograms. It is apparent that protein C is much more abundant in mature 99-h spores than in vegetative cells. Protein C is detected by 6 h after the initiation of development, and its accumulation continues throughout development. Protein S is first detected in this experiment between 12 and 18 h of development, with a continual accumulation observed through 99 h. From these data, it appears that protein C is produced before protein S. However, because of differences in antibody titer and avidity, it was not possible to compare absolute amounts of either protein by the relative intensities of their respective bands.

Induction of protein C in CF liquid medium. When *M. xanthus* cells are placed in liquid starvation media, certain developmental proteins are expressed but spores are not formed. It has been previously shown that Tps is produced during nutritional downshift experiments (4). It is interesting to note, however, that Ops is not expressed under these conditions. To investigate whether protein C was expressed in liquid media subsequent to nutritional downshift, exponentially growing cells were washed in TM buffer and resuspended in CF broth at 3×10^8 cells per ml. Samples were removed at various times, centrifuged, and rapidly frozen. After all samples were collected, cells were disrupted with zirconium beads in a Minibeadbeater and prepared for SDS-PAGE as described above. The induction of protein C was rapid, with significant increases in the amount of protein C observed by 3 h after the nutritional downshift (Fig. 2B). The amount of protein C increased through 48 h and then remained constant through 99 h. We first detected protein S by 8 h; its amount increased until 18 h and subsequently declined through 99 h.

Glycerol spores do not contain protein C. When *M. xanthus* cells are introduced into a rich medium containing high levels (0.5 M) of glycerol, they rapidly and synchronously differentiate into sonication-resistant glycerol spores (5). The profiles of many biochemical markers demonstrate that glycerol-induced spores are clearly different from fruiting body spores (20a). Ops is expressed in glycerol-induced spores, while Tps is not expressed (4). Protein C, like Tps, was not detected in glycerol-induced spores (data not shown).

Protein C regulation in developmental mutants of *M. xanthus*. The expression of protein C was also investigated in four classes of conditional developmental mutants: *asg*, *bsg*, *csg*, and *dsg*. These classes are unable to complete devel-

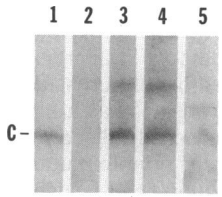

FIG. 3. Western blot analysis of the expression of protein C in conditional developmental mutants. Cells were harvested after 48 h of incubation at 34°C and prepared for SDS-PAGE as described in the text. Lanes: 1, DZF1; 2, DK5057 (*asg*); 3, DK5209 (*bsg*); 4, DK2630 (*csg*); 5, DK3261 (*dsg*).

opment unless they are in proximity to wild-type cells (9). This phenotype has been interpreted to mean that these classes are defective in either the production or release of extracellular signals. The effects of mutations in each of these classes of genes on the expression of many developmental markers have been examined, and it appears that each of these groups blocks development at different stages (15, 16). Normal expression of Tps is dependent on the *asg* and *bsg* genes, whereas *ops* gene expression depends on the *asg*, *bsg*, and *csg* genes (8, 15, 16). Accumulation of protein C, as determined by Western immunoblot analysis, was also influenced by mutations in two of these groups of genes (Fig. 3). Strains carrying mutations in each of the synergizable groups were grown in CYE broth (2), and equivalent numbers of cells were spread onto developmental plates. Cells were scraped from one plate after 48 h of development, resuspended in 0.5 ml of Laemmli loading buffer, and boiled for 5 min; equivalent volumes of extract were then separated on 12.5% SDS-polyacrylamide gels. After transfer onto Immobilon-P membranes (Millipore Corp., Bedford, Mass.) with a semidry electroblotter (Integrated Separation Systems, Hyde Park, Mass.), protein C was detected as described in the legend to Fig. 2. Protein C was not produced in the *asg* mutant, DK5057 (Fig. 3, lane 2). The accumulation of protein C was also significantly reduced and delayed in the *dsg* strain, DK3261 (lane 5), in comparison with the wild-type levels produced by strain DZF1 (lane 1). Although *dsg* mutants are greatly impaired in development, they eventually do form a reduced number of fruiting bodies. The production of protein C in strain DK3261 seems to parallel this general pattern. However, DK5209 (lane 3) and DK2630 (lane 4) (*bsg* and *csg* mutants, respectively) showed normal levels of protein C production. From these results, it appears that the expression of protein C, like that of protein S, is controlled by intercellular signaling.

Analysis of protein C by SDS-PAGE. It should be noted that protein C often migrates as a doublet on low-percentage (8 to 10%) SDS-polyacrylamide gels. After staining of the gels with Coomassie blue, the intensity of the upper band is about 20 to 40% of that of the lower band. When extracts were prepared from 4-day-developmental cells and separated by two-dimensional PAGE (Fig. 4A), blotted onto a nitrocellulose membrane, and detected with antiserum to protein C and ^{125}I-protein A, two spots were also observed (Fig. 4B). Since the two spots migrated to similar positions after isoelectric focusing in the first dimension and SDS-polyacrylamide slab gel electrophoresis in the second dimension, we suspect that the two bands of the doublet are related to each other. This view was confirmed by one-dimensional protein mapping. Similar banding patterns for each band of the doublet were observed when each protein C band was excised with a razor blade and incubated with either V8 protease, papain, or subtilisin (data not shown). The nature of the difference between the two bands is not known.

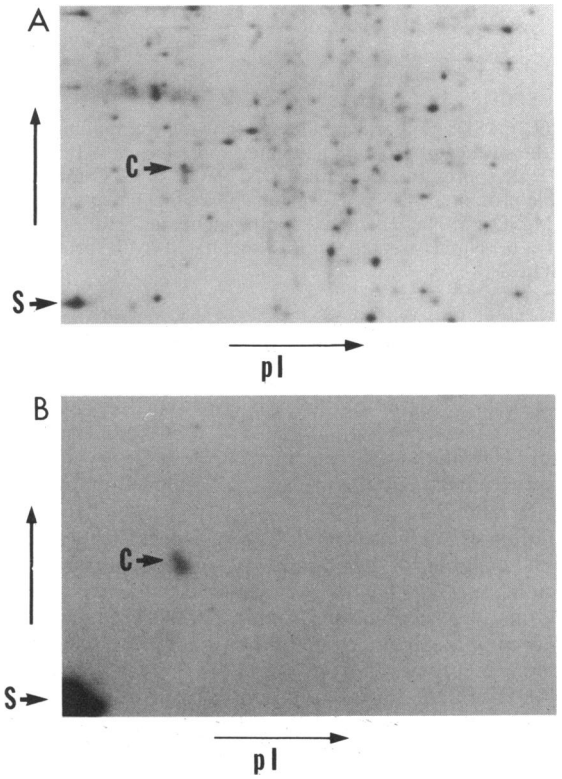

FIG. 4. Two-dimensional PAGE analysis of spore proteins from *M. xanthus*. Samples of DZF1 spores were disrupted with the Minibeadbeater and treated with SDS–β-ME and DNase-RNase solutions, provided by Protein Databases, Inc. (Huntington Station, N.Y.). The gels were run and silver stained at Protein Databases. Nonequilibrium isoelectric focusing gels were run in the first dimension, and 12.5% SDS-containing Laemmli gels were run in the second dimension. (A) Photograph of a section of the silver-stained gel. Protein C and protein S (labeled with arrows) were identified on the two-dimensional gel by Western blot analysis with antibodies to both proteins (B).

Extraction of protein C from spores. Protein S is released from spores by chelating Ca^{2+} with ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) or EDTA or by adding 1 M NaCl. Protein S and protein C were quantitatively released from the spore surface by treatment with 0.1 N NaOH or by boiling in 1% SDS (Fig. 5, lane 1).

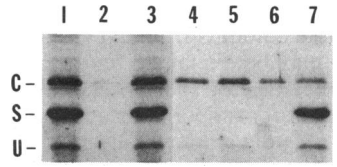

FIG. 5. Coomassie blue-stained SDS-polyacrylamide gel showing the loss of protein C from the spore surface when magnesium is not present. Spores from DZF1 were prepared in TM (10 mM Tris-HCl [pH 7.6], 8 mM $MgSO_4$) buffer and washed in either TM or water. Lanes: 1, DZF1 spores boiled in 1% SDS; 2, TM wash fraction; 3, spores after TM wash; 4, first H_2O wash; 5, second H_2O wash; 6, third H_2O wash; 7, spores after three H_2O washes.

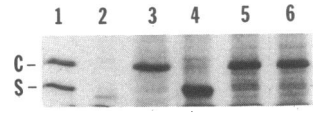

FIG. 6. SDS-polyacrylamide gel showing the binding of protein C to the surface of protein C-depleted spores. Lanes: 1, spores from DZF1; 2, spores treated with 0.1 N NaOH at 22°C for 60 min; 3, soluble protein C; 4, NaOH-treated spores incubated with protein S and $MgSO_4$; 5, NaOH-treated spores incubated with protein C and $CaCl_2$; 6, NaOH-treated spores incubated with protein C and $MgSO_4$.

EGTA, EDTA, NaCl, and pH treatments from 3 to 10 were ineffective in quantitatively releasing protein C from the spore surface (data not shown). However, repeated washes in distilled water (Fig. 5, lanes 4 to 6) did release some protein C from the spore surface. Protein C was stabilized on the spore surface by the presence of magnesium in the buffer (Fig. 5, lane 2).

Binding of purified protein C to the spore surface. Because Tps is capable of self-assembly on the surface of Tps-deficient spores (11), we investigated whether protein C could also be reconstituted onto spores depleted of protein C. To quantitatively remove Tps and protein C from the spore surface, spores from strain DZF1 were washed in 0.1 N NaOH at 22°C for 60 min, neutralized with 0.1 volume of 1 M HCl, and then washed three times in a large excess of 10 mM Tris-HCl (pH 7.6). Protein C was prepared by washing spores four times in 10 mM Tris-HCl (pH 7.6) at 22°C for 60 min. The wash fractions were pooled and concentrated four times in a Centricon 10 microconcentrator (Amicon, Danvers, Mass.). Protein S was prepared from protein C-depleted spores by a wash with 0.1 N NaOH and then neutralization with 1 M HCl. Spores that had been stripped of both proteins S and C by treatment with NaOH were incubated in the presence of excess protein C and either 10 mM $CaCl_2$ or 10 mM $MgSO_4$ or with Tps and $MgSO_4$ at 4°C for 18 h. Spores were then washed twice with vigorous vortexing in a 300-fold excess of TM buffer, boiled in Laemmli loading buffer, and subjected to SDS-PAGE. Protein C was capable of binding to the surface of NaOH-washed spores in the presence of $CaCl_2$ (Fig. 6, lane 5) or $MgSO_4$ (lane 6). Protein S also bound to the spore surface when it was incubated in the presence of $MgSO_4$ (lane 4). Thus, protein C, like protein S, is capable of binding to the surface of NaOH-washed spores. We do not yet know whether this binding represents a self-assembly process similar to that observed for protein S.

In this report we have identified a new, abundant spore surface protein of *M. xanthus*. Protein C has previously been observed in papers describing protein S but has not been characterized (3, 11). The significance of protein C on the spore surface has probably been overlooked because it was present in variable amounts from experiment to experiment. This variability may be explained by the absence of magnesium during some stage of spore preparation or storage. As might be predicted for a spore surface protein, the expression of protein C is developmentally regulated. Its patterns of expression are very similar to those observed for protein S (Tps); however, they are not identical. For example, protein C is expressed earlier in development than protein S. In addition, expression of protein C is not impaired in a *bsg* background, while expression of protein S is both delayed and markedly reduced. Another interesting finding is that protein C, like protein S, binds to the surface of spores.

We do not yet know the function of protein C or protein S. Mutants lacking both Tps and Ops are viable and do not display significant defects in aggregation, fruiting body formation, or heat and sonication resistance of spores (3, 7, 14). It is possible that protein C has a function similar to that of protein S and that an easily discernible phenotype was not observed in the *ops tps* double mutant because of functional redundancy with protein C. It is also possible that protein C is involved in other functions, such as spore cohesion, resistance to UV light, or germination. Further work is necessary to determine the function of these spore surface proteins. Nevertheless, protein C is extremely useful because it is an abundant and easily detected developmental marker.

We especially thank the Dale Kaiser laboratory for providing us with the *asg*, *bsg*, *csg*, and *dsg* strains of *M. xanthus*. We thank Kathleen O'Connor for the two-dimensional PAGE analysis. We also acknowledge Kathleen O'Connor, Mark McBride, Robin Weinberg, and Joe Romeo for helpful discussions during the course of this work.

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REFERENCES

1. **Bradford, M. M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248–254.
2. **Cumsky, M., and D. R. Zusman.** 1979. Myxobacterial hemagglutinin: a development-specific lectin of *Myxococcus xanthus*. Proc. Natl. Acad. Sci. USA **76**:5505–5509.
3. **Downard, J. S., D. Kupfer, and D. R. Zusman.** 1984. Gene expression during development of *Myxococcus xanthus*: analysis of the genes for protein S. J. Mol. Biol. **175**:469–492.
4. **Downard, J. S., and D. R. Zusman.** 1985. Differential expression of protein S genes during *Myxococcus xanthus* development. J. Bacteriol. **161**:1146–1155.
5. **Dworkin, M., and S. M. Gibson.** 1964. A system for studying microbial morphogenesis: rapid formation of microcysts in *Myxococcus xanthus*. Science **146**:243–244.
6. **Dworkin, M., and D. Kaiser.** 1985. Cell interactions in myxobacterial growth and development. Science **230**:18–24.
7. **Furuichi, T., T. Komano, M. Inouye, and S. Inouye.** 1985. Functional complementation between the two homologous genes, *ops* and *tps*, during differentiation of *Myxococcus xanthus*. Mol. Gen. Genet. **199**:434–439.
8. **Gill, R. E., and M. G. Cull.** 1986. Control of developmental gene expression by cell-to-cell interactions in *Myxococcus xanthus*. J. Bacteriol. **168**:341–347.
9. **Hagen, D. C., A. P. Bretscher, and D. Kaiser.** 1978. Synergism between morphogenetic mutants of *Myxococcus xanthus*. Dev. Biol. **64**:284–296.
10. **Higgins, R. C., and M. E. Dahmus.** 1979. Rapid visualization of protein bands in preparative SDS polyacrylamide gels. Anal. Biochem. **93**:257–260.
11. **Inouye, M., S. Inouye, and D. R. Zusman.** 1979. Biosynthesis and self-assembly of protein S, a development-specific protein of *Myxococcus xanthus*. Proc. Natl. Acad. Sci. USA **76**:209–213.
12. **Inouye, S., T. Franceschini, and M. Inouye.** 1983. Structural similarities between the development-specific protein S from a Gram-negative bacterium, *Myxococcus xanthus*, and calmodulin. Proc. Natl. Acad. Sci. USA **80**:6829–6833.
13. **Inouye, S., Y. Ike, and M. Inouye.** 1983. Tandem repeat of the genes for protein S, a development-specific protein of *Myxococcus xanthus*. J. Biol. Chem. **258**:38–40.
14. **Komano, T., T. Furuichi, M. Teintze, M. Inouye, and S. Inouye.** 1984. Effects of deletion of the gene for the development-specific protein S on differentiation in *Myxococcus xanthus*. J. Bacteriol. **158**:1195–1197.

15. **Kroos, L., and D. Kaiser.** 1987. Expression of many developmentally regulated genes in *Myxococcus* depends on a sequence of cell interactions. Genes Dev. **1**:840–854.
16. **Kuspa, A., L. Kroos, and D. Kaiser.** 1986. Intercellular signaling is required for developmental gene expression in *Myxococcus xanthus*. Dev. Biol. **117**:267–276.
17. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
18. **Legocki, R. P., and D. P. S. Verma.** 1981. Multiple immunoreplica techniques: screening for specific proteins with a series of different antibodies using one polyacrylamide gel. Anal. Biochem. **111**:385–392.
19. **Nelson, D. R., M. Cumsky, and D. R. Zusman.** 1981. Localization of myxobacterial hemagglutinin in the periplasmic space and on the cell surface of *Myxococcus xanthus* during developmental aggregation. J. Biol. Chem. **256**:12589–12595.
20. **O'Connor, K. A., and D. R. Zusman.** 1989. Patterns of cellular interactions during fruiting body formation in *Myxococcus xanthus*. J. Bacteriol. **171**:6013–6024.
20a. **O'Connor, K. A., and D. R. Zusman.** Unpublished data.
21. **Romeo, J. M., B. Esmon, and D. R. Zusman.** 1986. Nucleotide sequence of the myxobacterial hemagglutinin gene contains four homologous domains. Proc. Natl. Acad. Sci. USA **83**:6332–6336.
22. **Romeo, J. M., and D. R. Zusman.** 1987. Cloning of the gene for myxobacterial hemagglutinin and isolation and analysis of structural gene mutations. J. Bacteriol. **169**:3801–3808.
23. **Rosenberg, E.** 1984. Myxobacteria: development and cell interactions. Springer-Verlag, New York.
24. **Teintze, M., R. Thomas, T. Furuichi, M. Inouye, and S. Inouye.** 1985. Two homologous genes coding for spore-specific proteins are expressed at different times during development of *Myxococcus xanthus*. J. Bacteriol. **163**:121–125.
25. **Weinberg, R. A., and D. R. Zusman.** 1990. Alkaline, acid, and neutral phosphatase activities are induced during development in *Myxococcus xanthus*. J. Bacteriol. **172**:2294–2302.
26. **Zusman, D. R.** 1984. Cell-cell interactions and development in *Myxococcus xanthus*. Q. Rev. Biol. **59**:119–138.