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Transport and Localization of Protein S, a Spore Coat Protein, During Fruiting Body Formation by *Myxococcus xanthus*

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Protein S, the most abundant soluble protein synthesized by *Myxococcus xanthus* FB during early fruiting body formation, accumulates in the soluble fraction of developing cells, reaching a peak at about 24 h; at late stages of fruiting body formation, protein S is found on the surface of spores (M. Inouye et al. Proc. Natl. Acad. Sci. U.S.A. **76**:209–213, 1979). In this study, the transport and localization of protein S were investigated. Cells were fractionated to give osmotic shock, membrane, cytoplasmic, and spore fractions. The various fractions were then analyzed for protein S. Protein S was first detected in the cytoplasmic fraction at about 3 to 6 h of development. However, transport of protein S through the cytoplasmic membrane was not observed until 15 to 18 h of development. Thus, protein S is unusual among translocated proteins in that it accumulates as a soluble cytoplasmic protein before translocation. Biosynthesis of protein S ceased after 48 h; by 72 h, protein S was only found on the surface of spores. Pulse-chase experiments were performed to determine the transport kinetics of protein S. The results showed that in 24-h developing cells, the transport of protein S across the cytoplasmic membrane was rapid, occurring in less than 2 min. However, transport across the outer membrane was slow, requiring 10 to 15 min. Pulses of 15 s with [^{35}S]methionine failed to reveal any short-lived precursor form in immunoprecipitated material separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Isoelectric focusing also failed to detect any precursor form of protein S. Thus, protein S appears to be translocated in the absence of a cleaved signal peptide.

Myxococcus xanthus is a gram-negative, rod-shaped bacterium (12, 27). Under conditions of starvation on a solid surface, a developmental program is triggered which results in cellular aggregation and sporulation (17). Aggregation involves the movement of cells toward specific aggregation centers where about 10^5 to 10^6 cells form raised mounds. The sporulation program controls the conversion of individual rod-shaped cells to ovoid, environmentally resistant resting cells called myxospores. Whole cells are converted to myxospores in contrast to endospore formation in *Bacillus* species. For *M. xanthus*, mounds of myxospores are termed fruiting bodies.

Previously, Inouye et al. (9) described a spore coat protein called protein S. Protein S is a very abundant developmental protein comprising up to 15% of the total protein synthesis at the time of maximum synthesis. It is induced early in development. It is initially found as a soluble protein, although late in development it is insoluble; at these times it is located on the outermost layer of spores. This deposition of protein S appears to be a self-assembly process mediated by Ca^{2+} or Mg^{2+} (9).

In this study, we investigated the transport and localization of protein S during fruiting body formation by *M. xanthus*. We found that protein S initially accumulated as a soluble protein in the cytoplasm, beginning at 3 to 6 h. Later in development (15 to 18 h), protein S was transported out of the cytoplasm since it was then located in the periplasm as well as the cytoplasm. Synthesis of protein S ended after 48 h; by 72 h, protein S was only found on myxospores. Protein S transport across the cytoplasmic and outer membranes occurred without the cleavage of a signal peptide. Thus, protein S appears to be an unusual secreted protein, the study of which may better elucidate the process of both secretion and development.

MATERIALS AND METHODS

Cells and growth conditions. *M. xanthus* FB (DZF1, derived from DK101 [7]) was used. Vegetative cultures were grown in Casitone-yeast extract broth and

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maintained on Casitone-yeast extract agar plates (5). Development was induced on clone fruiting (CF) agar (7). CF agar plates were routinely dried at 35°C for 3 to 4 days before use.

Fruiting body formation. Vegetative cultures (200 to 300 Klett units with a no. 66 filter; ca. 10^9 cells per ml) were harvested by centrifugation (8,000 × *g* for 8 min at 4°C). The cells were suspended in 10 mM Tris-hydrochloride buffer (pH 7.6) plus 8 mM $MgSO_4$ (TM buffer) to 3,000 Klett units. Cells were immediately spotted onto CF plates with a multisample inoculator (86 spots per plate; 5 μl per spot). Plates were incubated at 30°C. Fruiting bodies were harvested from plates by gently disrupting the aggregates with a glass spreader, adding 0.5 to 1.0 ml of TM buffer to each plate, scraping again, then pipetting the cell suspension into a centrifuge tube. Harvested fruiting cells were used immediately for experiments.

Conditions for labeling *M. xanthus* cells with [^{35}S]methionine. Developmental cells, harvested as described above, were centrifuged (10,000 × *g* for 5 min at 4°C) and suspended in CF broth at 3,000 Klett units. The cell suspension was incubated with shaking at 30°C. After a preincubation period of 10 to 20 min, the cells were labeled with L-[^{35}S]methionine (New England Nuclear Corp., Boston, Mass.; initial specific activity, ~1,200 Ci/mmol). The amount of radioisotope and the time of the pulses are described below for each experiment. [^{35}S]Methionine was chased with the addition of at least a 10^6-fold excess of unlabeled L-methionine. Pulse-chase experiments were terminated by the addition of cold trichloroacetic acid (10%).

Cell fractionation procedures. The procedures described by Nelson et al. (18) were used to obtain the wash, osmotic shock, cytoplasmic, and membrane fractions of *M. xanthus*. The spore fraction was obtained as the pellet from the low-speed centrifugation (3,000 × *g* for 3 min at 4°C) of osmotically shocked cells which had been sonicated.

Electrophoretic methods. Polyacrylamide gel electrophoresis was carried out on 1-mm-thick, 10% polyacrylamide slab gels with the buffer system of Laemmli (13). Samples for gel electrophoresis were usually concentrated by precipitation in 10% trichloroacetic acid. Polyacrylamide gels were usually fixed in a solution of 25% isopropanol and 10% acetic acid for 1 h before drying and autoradiography.

Analytical isoelectric focusing was carried out by the procedures of Ames and Nikaido (1). Gels were run for 16 h at 350 V and 1.5 h at 500 V for a total of 6,000 V · h.

Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose and solid-phase radioimmunoassay (Western blots). Proteins were electrophoretically transferred from polyacrylamide gels to nitrocellulose sheets (Millipore Corp., Bedford, Mass.; type HA; pore size, 0.45 μm) by using a modification of the procedures of Towbin et al. (23) and an electrophoretic transfer chamber designed in this laboratory (currently available from Bio-Rad Laboratories, Richmond, Calif.). After the protein transfer, the nitrocellulose sheet was soaked in a solution containing 10 mM sodium phosphate and 0.9% NaCl (PBS) plus 1% gelatin for 1 h at 35°C to saturate any nonspecific protein-binding sites on the nitrocellulose. The nitrocellulose was rinsed with PBS four times over a 1-h period at 35°C. Then, the nitrocellulose was incubated at 20°C in 100 ml of a 1:200 dilution of rabbit anti-protein S serum in PBS. The nitrocellulose was washed with PBS four times over a 1-h period at 35°C and placed in a sealed plastic bag. The bound antibody was detected by adding about 10^6 cpm of a ^{125}I-labeled protein A solution from *Staphylococcus aureus* in PBS buffer and by incubating at 35°C for 1 h. The blot was then washed extensively with PBS, dried, and subjected to autoradiography.

Immunoprecipitation of protein S. Protein S from both whole cells and cell fractions, prepared as described above, was immunoprecipitated by the protocol of Ito et al. (10).

RESULTS

Localization of protein S during development. Previous work has shown that at early times in development, protein S is found in the soluble fraction of cells and at later times in the insoluble spore fraction (9). To study the transport of protein S to the cell surface, we first examined the localization of protein S at different stages of development. Cells were harvested from CF plates at various times during fruiting body formation and treated to obtain the cytoplasmic, periplasmic (shock), membrane, and spore (low-speed pellet) fractions as described above. Proteins from these fractions were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and transferred to nitrocellulose filter sheets (Western blots). The blots were radioimmunologically stained for protein S by treating them with antiprotein S serum followed by ^{125}I-labeled protein A (from *S. aureus*). Figure 1 shows an autoradiogram of one such experiment. The location of protein S in the cytoplasmic (panel A), periplasmic (panel B), and spore (panel C) fractions are compared at specific times of development (mature fruiting bodies are observed between 48 and 72 h of plating under our standard fruiting conditions). At early times of development (6 to 11 h), protein S was located almost exclusively in the cytoplasmic fraction, with little or no protein S detectable in the other fractions. The cytoplasmic fraction also contained a minor band of higher molecular weight which cross-reacted with the antiserum. We do not think that this protein is a precursor of protein S since it was not labeled in pulse experiments described below. At 24 h (15 to 18 h in other experiments), protein S was also detected in the periplasmic and spore fractions, although the cytoplasmic fractions clearly contained most of the protein S. From 36 to 48 h, most of the protein S was located in the spore fraction. This fraction also showed a second minor band of higher molecular weight which cross-reacted with the antiserum. This protein was only detected by the Western blot technique and was not seen by the pulse-labeling experiments described below. By

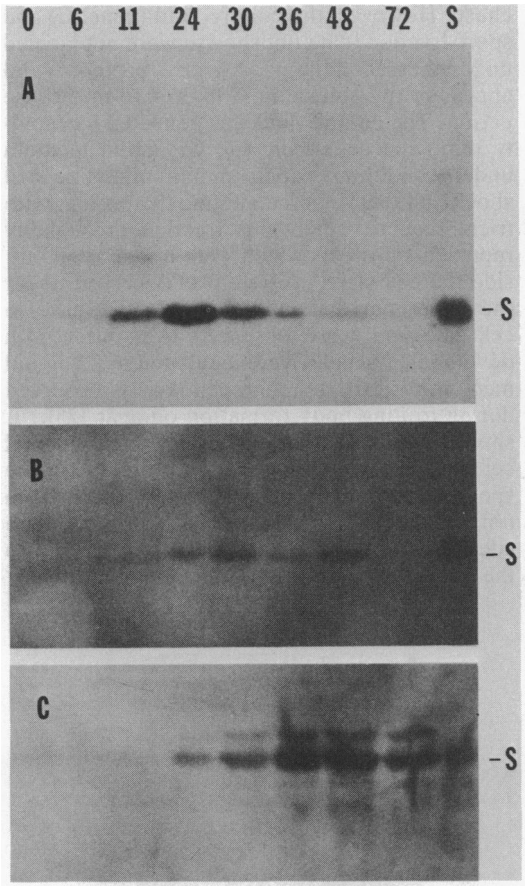

FIG. 1. Localization of protein S in developing *M. xanthus* cells. Developing cells (0, 6, 11, 24, 30, 36, 48, and 72 h) were harvested from CF agar plates and fractionated to yield cytoplasmic (A), periplasmic (B), and spore (C) fractions. Lane S contains protein S purified from mature spores. Proteins from these fractions were separated by SDS-polyacrylamide gel electrophoresis and then electrophoretically transferred to nitrocellulose sheets. The sheets were probed with antiprotein S serum, washed, and stained with ^{125}I-labeled protein A as described in the text. The autoradiograms were exposed for 16 h at −70°C.

72 h, protein S was detected only in the spore fraction. It should be noted that although fruiting body formation is complete at 72 h, many cells never sporulate so that cultures harvested at this time contain both spores and rod-shaped cells. At no time could protein S be detected in the membrane fraction (data not shown). These results suggest that protein S is initially synthesized as a soluble cytoplasmic protein which accumulates for several hours before translocation and assembly onto the spore surface.

To study the fate of newly synthesized protein S, cells were harvested from CF plates at various times of fruiting body formation and suspended in CF broth. The cells were then pulse-labeled with L-[^{35}S]methionine for 5 min and chased with unlabeled L-methionine for 15 min. Cellular fractions were prepared, and protein S was immunoprecipitated and analyzed by SDS-polyacrylamide gel electrophoresis. This experimental approach was used because it is very difficult to briefly pulse-label *M. xanthus* fruiting clones on agar plates or even nitrocellulose filters. Although the disrupted aggregates do not proceed to fruiting body formation in suspension culture, they do continue to express protein S for at least 2 h (D. R. Nelson and D. R. Zusman, Proc. Natl. Acad. Sci. U.S.A., in press). Autoradiograms from this experiment are shown in Fig. 2. The location of newly synthesized protein S in the cytoplasmic (panel A), periplasmic (panel B), and spore (panel C) fractions are compared at specific times of development. Labeled protein S was found almost exclusively in the cytoplasm from 6 to 18 h. At 24 h, labeled protein S was still primarily located in the cytoplasm but could also be found in the shockable fraction. At 48 h, labeled protein S was barely detectable in the cytoplasm but was clearly present in the shockable fraction and in the spore fraction. At 72 h, no protein S synthesis was detected. These results show that at early times of fruiting body formation (6 to 18 h) newly synthesized protein S was not transported out of the cytoplasm in the 15-min chase period al-

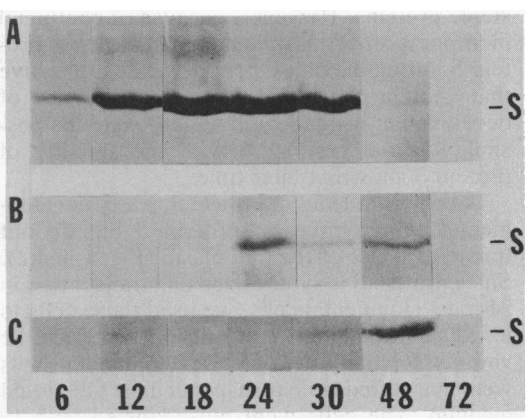

FIG. 2. Localization of [^{35}S]methionine-labeled protein S in developing *M. xanthus* cells. Developing *M. xanthus* cells (6, 12, 18, 24, 30, 48, 72 h) were harvested from CF agar plates and suspended in CF broth at 30°C. Fractions of 0.5 ml were labeled with L-[^{35}S]methionine (10 μCi) for 5 min, chased with cold L-methionine for 15 min, and immediately fractionated into cytoplasmic (A), periplasmic (B), and spore (C) fractions. Protein S was immunoprecipitated and run on an SDS-polyacrylamide gel as described in the text. The autoradiogram was exposed for 9 days at −70°C.

lowed in this experiment, although this chase period was adequate to allow transport to the spore fraction at 48 h. These results with suspension cultures agree with those of Fig. 1 (with cells harvested directly from plates) in showing that protein S remains compartmentalized in the cytoplasmic fraction at early times in development and is translocated only at later times.

An interesting question raised by these experiments is whether protein S labeled at early times of development could be recovered in the spore fraction at later times. In other words, is the protein S that is synthesized early in development exported at a later time or is it degraded and only that protein that is synthesized later in development exported? In an attempt to resolve this question, cells were inoculated onto nitrocellulose filters placed on CF agar and incubated under conditions in which fruiting bodies form. At 8 h, the cells were pulse-labeled with [^{35}S]methionine for 30 min; the cells were then chased for 20 or 60 h after transfer to fresh plates containing unlabeled methionine. The fruiting bodies were harvested, and the spore fraction was analyzed for protein S. The results (data not shown) indicated that the protein S isolated from mature spores was very heavily labeled. The amount of label in the protein S fraction was in fact much higher than that observed immediately after the labeling period. The results are consistent with the fact that protein S is very stable. Because of extensive protein turnover, however, there is a net increase in the amount of labeled protein S at the expense of other, less stable proteins. However, because of continued incorporation of [^{35}S]methionine into new protein S during the chase period, we cannot prove that protein S synthesized at early times of development is not degraded, although this possibility is not consistent with the stability of protein S shown at later times.

Transport kinetics of protein S. Early developmental cells synthesize protein S but do not transport it out of the cytoplasm (Fig. 1 and 2). Since protein S is synthesized and transported in 24-h developmental cells, we used these cells to investigate the kinetics of transport through the various cellular fractions. Developmental cells were harvested and resuspended in CF liquid medium. The cells were pulse-labeled with L-[^{35}S]methionine for 2 min and chased with unlabeled L-methionine for up to 30 min. The cells were then fractionated to yield cytoplasmic, periplasmic, and spore fractions. Protein S was immunoprecipitated with antiprotein S serum and then analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography (Fig. 3). Both the cytoplasmic (panel A) and periplasmic (panel B) fractions contained labeled protein S even in the absence of any chase. However, the culture fluid (panel C) and spore fraction (data not shown) were not labeled under these conditions. Labeled protein S did appear in the culture fluid after a 10-min chase period. The culture fluid was collected by rapidly removing cells from the CF liquid medium under conditions which should minimize cell shock and then by precipitating the protein with trichloroacetic acid. This fraction presumably represents proteins which were transported outside the cell since no lysis was observed under the experimental conditions. (No decrease in cell numbers were observed even after 24-h developmental cells were incubated in CF liquid medium for 1 h, although cell lysis is observed during fruiting body formation on agar [25].) It should be noted that the 24-h developmental cells did not show any labeled protein S in the spore fraction even after a 30-min chase (data not shown). This chase period was more than adequate for the translocation of protein S into the spore fraction of 48-h developmental cells.

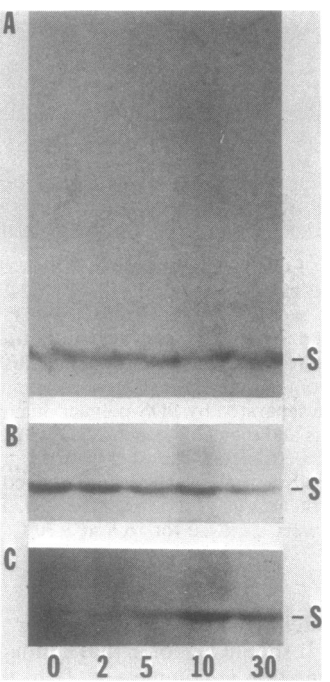

FIG. 3. Transport of protein S in 24-h developing *M. xanthus* cells. Developing cells (24 h) were harvested from CF agar plates and suspended in CF broth at 30°C. The cells (3 ml) were labeled with L-[^{35}S]methionine (25 μCi) for 2 min and chased with 100 μl of 0.2 M unlabeled L-methionine. Samples (0.5 ml) were taken at 0, 2, 5, 10, and 30 min and immediately fractionated to yield cytoplasmic (A), periplasmic (B), and culture fluid (C) fractions. Protein S was immunoprecipitated and run on 10% SDS-polyacrylamide gels. Autoradiograms were exposed for 9 days at −70°C.

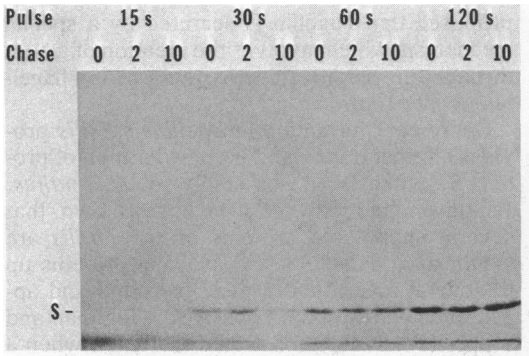

FIG. 4. Immunoprecipitation of protein S after a rapid pulse-chase of [^{35}S]methionine. Developing *M. xanthus* cells (24 h) were harvested from CF plates and suspended in CF broth at 30°C. Cells (1.5 ml) were pulsed with L-[^{35}S]methionine (20 μCi) for 15, 30, 60, or 120 s, and 0.5-ml samples were taken after being chased with 100 μl of 0.2 M methionine for 0, 2, and 10 min. The samples were immunoprecipitated and run on SDS-polyacrylamide gels. The autoradiogram was exposed for 10 days at −70°C.

(Figure 2 shows labeled protein S in the spore fraction of 48-h cells after a 15-min chase.) This experiment shows that 24-h developmental cells are capable of transporting protein S through the cytoplasmic membrane and that this transport is very rapid (<2 min). These cells can also transport protein S through the outer membrane, although at a much slower rate (ca. 10 min). Assembly of protein S onto material in the spore fraction of 24-h cells (Fig. 1) was not observed in the 30-min chase period used in this experiment.

Protein S modification during transport. Most secreted proteins have been shown to contain cleavable amino-terminal sequences or signal peptides 11 to 30 amino acids long (10, 19). Soluble cytoplasmic protein S has the same apparent molecular weight as spore-associated protein S (Fig. 1 through 3). Thus, protein S does not appear to have a cleavable sequence necessary for secretion. However, a short-lived precursor may not have been detected in these experiments. To test this idea, a rapid pulse-chase experiment followed by immunoprecipitation of protein S, SDS-polyacrylamide gel electrophoresis, and autoradiography was performed with 24-h developmental cells. The results (Fig. 4) show that even pulses as short as 15 to 30 s could label protein S; the molecular weight of this protein remained unchanged during a subsequent 10-min chase period. Thus, we were unable to detect a short-lived precursor of protein S.

Figure 5 shows an autoradiogram of protein S that was analyzed by isoelectric focusing. Developmental cells (12 or 24 h) were pulse-labeled with L-[^{35}S]methionine for 2 min, and protein S was immunoprecipitated. No change in the isoelectric point of purified spore-associated protein S (lane A) and protein S labeled in 24-h developmental cells (lane B) or 12-h developmental cells (data not shown) was observed. Since protein S from 24-h developmental cells is a mixture of cytoplasmic and periplasmic forms (Fig. 3), if there were modifications in protein S which affect the net charge of the protein, a change in isoelectric point of the protein should have been observed. Thus, if protein S is modified during transport, the modifications do not involve significant changes in molecular weight or net charge. At this time, there is no evidence for a cleavable signal peptide associated with the translocation of protein S across the cytoplasmic membrane.

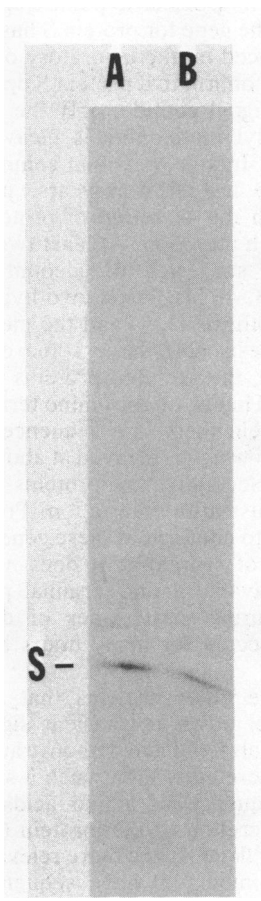

FIG. 5. Isoelectric focusing of protein S. Developing cells of *M. xanthus* (24 h) were harvested from CF agar plates and suspended in CF broth. The cells (1 ml) were labeled with L-[^{35}S]methionine (10 μCi) for 2 min, immunoprecipitated, and run on an isoelectric focusing gel (lane B). Lane A shows protein S purified from spores (9) and labeled with ^{125}I that was run on the same isoelectric focusing gel.

DISCUSSION

The data presented show that protein S, a spore coat protein produced during fruiting body formation by *M. xanthus*, is a cytoplasmic protein at early times in development. It accumulates in the cytoplasm as a pool for several hours and is not transported across the cytoplasmic membrane until about 18 h of development. At later times of development, it may be found in the cytoplasm, periplasm, and culture fluid and on the surface of spores. Once transport is initiated, protein S transverses the cytoplasmic membrane rapidly (<2 min) and, at a slower rate, the outer membrane (10 to 15 min). Additionally, there appear to be no transport-associated modifications to the protein during transport since changes in neither its molecular weight nor its isoelectric point can be detected. Recently, the gene for protein S has been cloned and sequenced by the laboratory of M. Inouye. Their data confirm that protein S does not have a cleavable signal peptide; only the amino-terminal *N*-formyl methionine is cleaved from the protein (M. Inouye, personal communication).

Over the last several years, processes involved with the secretion of proteins have received much attention. At least two hypotheses have been suggested to account for protein secretion (3, 4, 24). These two hypotheses, the signal hypothesis (3, 4) and the membrane trigger hypothesis (24), have some common features. First, the secreted proteins have precursor forms. That is, on the amino terminal portion of the protein there is a sequence of 11 to 30 amino acids which is cleaved at about the time of secretion. Secondly, the proteins are secreted during or just after translation. Protein S does not appear to conform to these generally accepted models of secretion. It does not appear to have a cleavable amino-terminal precursor sequence. During early times of development, synthesis occurs for many hours before secretion.

There are other proteins that are secreted without the aid of a transient signal peptide. Palmiter et al. (20) have demonstrated that ovalbumin is secreted without such a sequence. An internal sequence of amino acids appears to promote secretion of this protein (14, 16). The secretion of flagellin is a more relevant example. Zieg and Simon (26) have sequenced the H2 gene of *Salmonella* species and have found no cleavable leader sequence, yet this protein is initially a soluble cytoplasmic protein which is secreted to form an insoluble, self-assembled structure. Additionally, no leader sequence for the H1 gene of *Salmonella* species or for the *hag* gene of *Escherichia coli* has been found (M. Simon, personal communication). It has been proposed that flagellin is secreted by a special mechanism which involves the creation of a pore through the cell envelope by the growing flagellum itself (22).

Spore coat formation in *Bacillus subtilis* provides another paradigm for consideration of protein S synthesis and assembly in *M. xanthus*. Jenkinson and Sawyer (11) have shown that several spore coat proteins of *B. subtilis* are synthesized as soluble cytoplasmic proteins up to several hours before their secretion and appearance on the spore coat. Also, Goldman and Tipper (6) have demonstrated that even when a spore coat protein has a precursor form, the precursor is readily recovered as a soluble cytoplasmic protein.

The mechanism of protein S secretion is not understood at this time. One striking feature of protein S transport is the rather sudden appearance of the ability of cells to transport protein S at a particular stage of development. This new transport ability appears at about 18 h of development. One possible hypothesis which might account for this transport ability is the insertion of a developmentally regulated transport protein (possibly a porin) in the cytoplasmic membrane which is specific for protein S. An alternate hypothesis is that major changes in the cell membrane occur between 18 and 24 h which allow protein S to transverse the cytoplasmic membrane. Our previous work shows that myxobacterial hemagglutinin is a periplasmic protein and that it is transported across the cytoplasmic membrane almost immediately after synthesis, about 6 to 9 h of development (18). In contrast to protein S, myxobacterial hemagglutinin does not accumulate in the cytoplasmic fraction. This suggests that whatever mechanism is responsible for the transport of protein S is probably specific for protein S.

What advantages are there for *M. xanthus* to employ a special mechanism for the translocation of protein S? Protein S is the most abundant protein synthesized by *M. xanthus* FB during fruiting body formation. Up to 15% of developmental protein synthesis may be devoted to the production of protein S (8). Cells begin synthesis of protein S soon after being plated on CF medium, a semistarvation agar, perhaps to get a head start in the production of this protein. However, we know that protein S is a spore coat protein and is not assembled onto spores until late in myxospore formation, starting at 24 to 30 h of development. Clearly, if protein S were secreted according to the currently accepted models for secretion, several things could go wrong. Protein S, which is synthesized early and before it is needed for spore coat formation, would be secreted as it is made and possibly lost to the developing cell. Cells could probably not

synthesize the massive amounts of protein S needed in the relatively short time just before spore formation (15). Alternatively, if protein S had a signal peptide, it might bind to the membrane (21), and if not secreted, it would block the proper functioning of the membrane, resulting in improper processing of proteins normally secreted by this mechanism (10), or it could cause cell death (2). Therefore, we think that there is considerable advantage to *M. xanthus* to regulate the transport of protein S so that cells can accumulate a large cytoplasmic pool while nutrients are still available and then transport the protein to the outer surface of the cell when it is needed for the final stages of spore formation.

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