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# Purification and Characterization of SP21, a Development-Specific Protein of the Myxobacterium *Stigmatella aurantiaca*

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***Stigmatella aurantiaca* is a gram-negative bacterium with a complex life cycle, including cellular aggregation resulting in the formation of a characteristic three-dimensional structure, the so-called fruiting body. During fruiting and upon chemical induction of sporulation, a major development-specific protein, SP21, is synthesized. SP21 was purified to homogeneity from the membranous fraction of chemically induced spores. Expression of SP21 was studied with an antiserum raised against the purified protein.**

*Stigmatella aurantiaca* is a gram-negative, rod-shaped myxobacterium that grows on decaying organic matter in soil. The myxobacteria possess a biphasic cell cycle, during which they form a fruiting body. During vegetative growth the cells glide in swarms upon insoluble organic substrates which they degrade by secreted lytic enzymes. When nutrients are depleted, the cells migrate into aggregation centers from which the fruiting bodies arise (for a review, see reference 8). The fruiting body of *S. aurantiaca* consists of a branched stalk supporting the sporangioles, which in turn contain several thousand myxospores.

Spore formation in *S. aurantiaca* is not strictly coupled to fruiting and can be directly induced in liquid culture by a number of chemicals, of which the most potent are indole and some of its derivatives (2). Chemically induced sporulation is much more rapid than fruiting-body formation (3).

Features observed during eukaryotic multicellular morphogenesis, such as the processing of positional information and cell communication by close contact or diffusible molecules, are predicted to play an important role in fruiting of the myxobacteria (7). As morphogenic processes depend on the highly coordinated synthesis of development-specific proteins, the genes involved in fruiting have to be tightly regulated depending on the progress of development and on the location of the individual cell within the evolving fruiting body. To study this process, markers for the progress of development are needed. Proteins specifically expressed during development may serve as such markers.

We report here the detection of SP21, a marker protein for development, and its purification from chemically induced spores. Further, we demonstrate the pattern of SP21 expression in the course of sporulation and fruiting-body formation.

**Comparison of vegetative cells and chemically induced spores.** *S. aurantiaca* DW4 (7) was grown in 1% (wt/vol) Casitone (Difco)–0.15% (wt/vol) $MgSO_4$–0.1-mg/ml streptomycin sulfate in a 10-liter fermentor at 32°C with maximal aeration and a constant pH of 7.2. For analytical purposes, DW4 was cultured in shake flasks in 1% Tryptone–0.1% $MgSO_4$–0.1-mg/ml streptomycin sulfate. Spore formation was induced by addition of indole to a final concentration of 0.5 mM to a late-log-phase culture (ca. $8 \times 10^8$ cells per ml in a fermenter, $2 \times 10^8$ to $3 \times 10^8$ cells per ml in shake flasks). About 2 h after the addition of indole the cells formed shortened rods, and they started to become refractile after about 4 h. In the course of the indole treatment, 60 to 80% of the initial cells converted into sonication-resistant myxospores. Nonsporulated cells lysed after ca. 14 h. A 10-liter-fermentor culture yielded ca. 40 g (wet weight) containing ca. $4 \times 10^{12}$ spores. Spores were harvested by centrifugation 14 h after induction and washed twice in 10 mM Tris (pH 7.5)–2.5% NaCl. Spores were separated from vegetative cells by sonication on ice (in 1/100 culture volume of 10 mM Tris [pH 7.5]–2.5% NaCl for 6 min with 0.33-s pulses and 0.66-s time intervals with a Branson Sonifier with a 0.5-in. [1.27-cm] tip at 80 W) and twice-repeated centrifugation through a step gradient of 30% sucrose–2.5% NaCl and 40% sucrose–2.5% NaCl for 15 min at 4,000 × *g*. The spores sedimented while the debris floated at the interphase.

Gene products synthesized during development were detected by comparing the polypeptide patterns of vegetative cells and purified indole-induced spores. Lysates of cells were separated by centrifugation at 186,000 × *g* and analyzed in 15% (wt/vol) sodium dodecyl sulfate (SDS)-polyacrylamide gels (5) stained with Coomassie brilliant blue R-250 (Fig. 1). At an $M_r$ of 21,000 a polypeptide band which was predominant in spore lysates and apparently was absent from lysates of vegetative cells was detected. The corresponding protein, SP21, is synthesized in a development-specific manner. The residual SP21 appearing in the soluble fraction of spores sediments during prolonged centrifugation (see Fig. 2).

**Purification of SP21.** Indole-induced spores (15 g [wet weight]) were harvested after 14 h and washed but not purified further. The spores were sonicated on ice for 30 min with 0.33-s pulses and 0.66-s time intervals at 80 W with a Branson Sonifier (0.5-in. [1.27-cm] tip) in the presence of 65 g of glass beads (diameter, 0.1 mm) in a final volume of 70 ml of buffer A (10 mM Tris [pH 7.5], 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride [PMSF]). Undisrupted spores and the glass beads were recycled twice and sonicated under the conditions described above. Pooled lysates were adjusted to 200 ml with buffer A and clarified by centrifugation at 25,000 × *g* for 15 min at 4°C. Fractions containing SP21 were identified by SDS-polyacrylamide gel electrophoresis (PAGE) in comparison with lysate of vegetative cells, as no activity or biological function of SP21 is known. The protein

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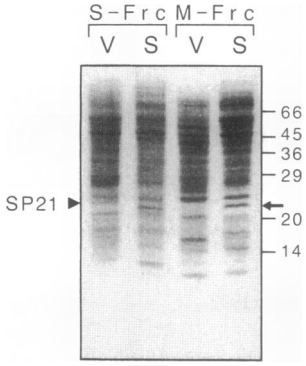


FIG. 1. Comparison of spores and vegetative cells of *S. aurantiaca*. Shown is a Coomassie-stained SDS gel of crude lysates from vegetative cells (V) and indole-induced spores (S) separated by centrifugation. S-Frc, supernatants; M-Frc, sediments. Numbers on the right show molecular weights (in thousands); the arrows indicate the SP21 band. The volume of fractions applied was normalized.

contents of fractions were determined by a modified Lowry protein assay (1). SP21 was enriched in the membrane fraction, which was separated from soluble cytoplasmatic proteins by centrifugation of the clear supernatant (900 mg of protein) for 10 h at 186,000 × *g* at 4°C (Fig. 2). From the sediment, SP21 was extracted quantitatively into 200 ml of buffer B (100 mM Tris, 2% Zwittergent SB14 [Serva], 0.5 mM PMSF [pH 7.7]). The insoluble material was sedimented at 186,000 × *g* at 10°C for 4 h.

All subsequent steps were performed at room temperature. The supernatant containing 270 mg of protein was filtered through a 0.45-μm-pore-size filter and applied at 150 ml/h onto a 160-ml Q-Sepharose FF column (Pharmacia) equilibrated with buffer B. The column was washed with 100 ml of buffer B and then with 100 ml of buffer C (100 mM Tris, 0.1% Zwittergent SB14, 0.5 mM PMSF [pH 7.7]) and developed with a linear gradient (0 to 200 mM NaCl; 500 ml of buffer C). Chromatography was monitored at $A_{280}$ in a

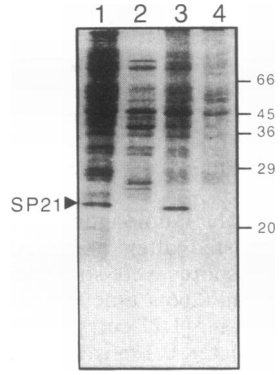


FIG. 2. Extraction of SP21 from the membrane fraction of *S. aurantiaca* spores. Shown is a Coomassie-stained SDS gel of samples from the extraction procedure. The crude lysate (lane 1) was centrifuged, yielding the soluble proteins (lane 2) and a pellet which was extracted with a zwitterionic detergent. Lane 3, detergent extract; lane 4, cell debris. Numbers on the right show molecular weights (in thousands); the arrowhead on the left indicates the SP21 band. The volume of fractions applied was normalized.

flow-through cell. NaCl concentration was determined by measuring the conductivity of the eluate. SP21 eluted with a peak at 130 mM NaCl in a total volume of 100 ml (Fig. 3). SP21-containing fractions were pooled, and proteins (15 mg) were precipitated with 10% trichloroacetic acid. The pellet was washed twice with ethanol-ether (1:1 [vol/vol]), dried, and suspended in 3.5 ml of 1× Laemmli sample buffer (5). Part of the protein (3 mg) was loaded onto a preparative SDS gel and electrophoresed as described above. The $M_r$-21,000 polypeptide band was excised and crushed. Proteins were electroeluted in a BioTrap (Schleicher & Schuell), precipitated with 2.5 volumes of acetone at −20°C, harvested, dried, and suspended in 400 μl of 10 mM Tris–0.1% (wt/vol) SDS. From the excised gel slice, 390 μg of pure SP21 was recovered. In SDS-PAGE, no contaminating bands were detected in the preparation (Fig. 4).

The purity of SP21 isolated from the $M_r$-21,000 band was shown by gas-phase Edman degradation. The sequence of 25 N-terminal amino acids was unambiguously legible, demonstrating the purity of the SP21 preparation. Furthermore, the amino acid sequence will be necessary to identify the gene coding for SP21. The sequence of the 25 N-terminal amino acids is ADLSVRRGTGSTPQRTREWDPFQQM. The EMBL/GenBank data base was searched by using the TFasta program (6), but no significant homology to a known sequence was found.

**Immunological detection of SP21.** An antiserum was prepared in a rabbit (primed and given boosters three times by subcutaneous injection of 50 μg of SP21 in 500 μl of RAS adjuvant [Sebak]). The antibody titer was monitored by enzyme-linked immunosorbent assay and Western blot (immunoblot).

The formation of fruiting bodies was induced as described previously (9). Fruiting bodies obtained from ca. $2 \times 10^9$ cells were suspended in 20 μl of $H_2O$ and incubated at 100°C for 2 min. The sample was boiled for a further 2 min after the addition of 20 μl of 2× Laemmli sample buffer. Debris was spun down, and the supernatant was subjected to SDS-PAGE and subsequently electroblotted to a sheet of Immobilon polyvinylidene difluoride membrane (Millipore) with a semidry blotting apparatus as described elsewhere (4). Western blot analysis of lysates from fully differentiated fruiting bodies showed the presence of SP21 in fruiting bodies (Fig. 4).

There are at least two possibilities for the development-specific origin of SP21: (i) the protein is expressed specifically in the course of development, or (ii) the protein is a derivative of a vegetative precursor protein which is processed in a spore-specific manner. These alternatives can be distinguished by Western blot analysis. The polyclonal antiserum to denatured SP21 is expected to cross-react with any precursor protein in vegetative cells. As the antiserum does not detect any protein in vegetative cells (Fig. 4), SP21 is not generated by processing a vegetative precursor protein. Therefore, the expression of SP21 (and not processing of a hypothetical precursor) is the development-specific process. Thus, SP21 is a valid marker for the development of *S. aurantiaca*.

Synthesis of SP21 during chemically induced sporulation was monitored by Western blot analysis. Samples from a sporulating culture were taken at the times indicated and processed as described above (Fig. 5). Expression of the protein was detectable 1 to 2 h after induction and persisted throughout sporulation; between 12 and 24 h after induction, the accumulation of the protein ceased, most probably because sporulation itself had terminated. No substantial

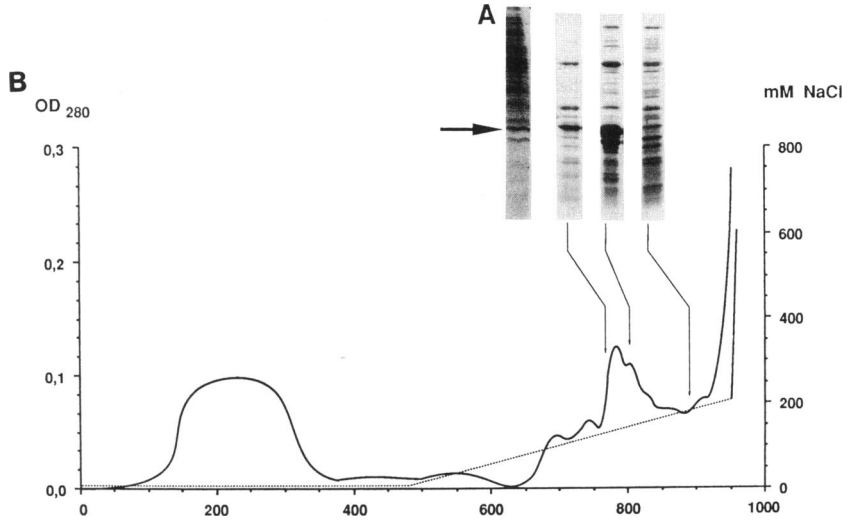


FIG. 3. Anion-exchange chromatography of SP21. (A) Detergent extract (200 ml) containing 270 mg of protein was applied to a 160-ml Q-Sepharose column, washed, and eluted with a linear NaCl gradient (0 to 200 mM). Protein patterns of selected fractions are shown. The arrow indicates the SP21 band. (B) On the abscissa the volume of the eluent (in milliliters) is depicted. SP21 eluted between 770 and 870 ml at a NaCl concentration around 150 mM. The 280-nm optical density ($OD_{280}$) profile does not reflect the protein content because pigments were present in the extract. ——, $OD_{280}$; · · · ·, NaCl concentration (millimolar).

decrease of band intensity was observed in Western blots up to 72 h after induction (data not shown), suggesting that SP21 is not considerably degraded during late sporulation or processed in a manner leading to an altered mobility in SDS-PAGE. Alternatively, the lack of further accumulation could also be due to the reaching of a steady state between synthesis and degradation. But we regard this as rather unlikely because the spores of the myxobacteria are known to be physiologically silent (8) and therefore, major turnover of proteins is not expected. Minor degradation of SP21 resulting in a number of weak bands below an $M_r$ of 21,000 detected in a Western blot may be due to proteolysis during sporulation or preparation of the samples. At approximately 2 h after induction, a weak $M_r$-22,000 band appeared. At this time, the relationship of this band to SP21 is unknown.

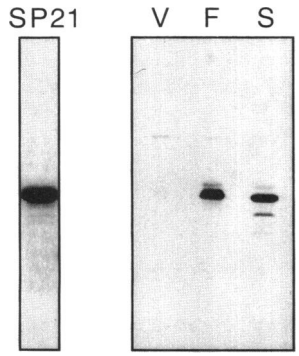


FIG. 4. Presence of SP21 in different types of *S. aurantiaca* cells. Left, Coomassie-stained SDS gel of pure SP21; right, Western blot analysis of vegetative cells (V), fruiting bodies (F), and indole-induced spores (S) of *S. aurantiaca*. Crude lysates (protein contents normalized) were separated by SDS-PAGE, transferred to a polyvinylidene difluoride membrane, and analyzed with a polyvalent antiserum against SP21 (dilution, 1:3,000).

**Properties of SP21.** SP21 is a major constituent of chemically induced spores, constituting 0.1 to 0.2% of total cell proteins as judged by Coomassie staining. Its apparent molecular weight is 21,000. A very rough estimate of SP21 yield suggests that the protein is present at a concentration of at least 100,000 molecules per cell.

The ability to sediment SP21 indicates that the protein is associated with the cell membranes or is part of a larger complex. Solubilization of the protein by detergent would then be due to the disruption of the complex either by disturbing hydrophobic interactions of its components or by solubilizing the lipids and proteins attached to them. Some intrinsic capacity of SP21 to form aggregates was observed in SDS-PAGE of the purified protein. When the sample was not boiled prior to electrophoresis, a polypeptide band with an $M_r$ of 43,000 was detected by Coomassie staining and by the antiserum (data not shown).

The small size of SP21, its relative abundance, and its membrane association or ability to form aggregates make it likely that SP21 is a component of the spore coat. Furthermore, the development-specific expression of SP21 makes the protein a valuable marker for the study of development of *S. aurantiaca*.

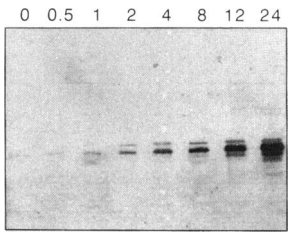


FIG. 5. Synthesis of SP21 during indole-induced sporulation. Exponentially growing *S. aurantiaca* cells were induced by 0.5 mM indole. Samples were withdrawn at the times indicated (hours) and prepared for Western blot analysis. Samples applied were normalized for their protein content.

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