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Localization of the Stress Protein SP21 in Indole-Induced Spores, Fruiting Bodies, and Heat-Shocked Cells of Stigmatella aurantiaca

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The localization and distribution of the stress protein SP21 in indole-induced vegetative cells, fruiting bodies, and heat shocked cells of Stigmatella aurantiaca were determined by immunoelectron microscopy. SP21 was found at the cell periphery in heat-shocked cells and either at the cell periphery or within the cytoplasm in indole-induced cells, often concentrated in clusters. In fruiting-body-derived spores, SP21 was located mainly at the cell wall, preferentially at the outer periphery. Furthermore, SP21 antigen was associated with cellular remnants within the stalk and within the peripheral horizon next to the fruiting body.

Stigmatella aurantiaca is a gram-negative, rod-shaped mycobacterium that grows on decaying organic matter in soil. The mycobacteria 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 partially degrade by means of secreted lytic enzymes. Cells that live in a dense swarm accumulate a high local concentration of enzymes which provide the cells with a higher concentration of soluble metabolites and efficiently prevent these metabolites from escaping (2). When nutrients are depleted, the cells migrate into aggregation centers, from which the fruiting bodies arise. The fruiting body of S. aurantiaca consists of a branched stalk supporting the sporangioles, which in turn contain several thousands of myxospores. During fruiting-body formation, large amounts of slime are produced and there is a concomitant massive cell lysis (22). Within the sporangiole, cells convert into myxospores as resting cells with increased resistance to environmental stresses, such as heat, UV light, and desiccation.

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 mycobacteria (17).

Spore formation of S. aurantiaca is not strictly coupled to fruiting-body formation 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 (3).

During development, a protein with an Mr of 21,000 (SP21) which was found in fruiting bodies and in indole-induced spores but was not detected in vegetative cells growing under standard conditions is synthesized (5). Stress conditions such as heat shock or limitation of oxygen also induce the synthesis of SP21. Sequence homology of the polypeptide sequence deduced from the corresponding gene (hsp4) identifies SP21 as a member of the family of low-molecular-weight heat shock proteins (LMW-HSP). The predicted polypeptide is more similar to some plant LMW-HSP (53% identity) than it is to any of the known bacterial LMW-HSP (4).

As SP21 was sedimented in the membrane fraction of cell lysates, we wanted to know if the protein is associated with the membranes or if it is part of a high-molecular-weight complex. Immunoelectron microscopy was used to locate SP21 within heat-shocked cells and indole-induced spores as well as in the different compartments of the S. aurantiaca fruiting body, i.e., the sporangioles and the stalk.

MATERIALS AND METHODS

Bacterial growth. S. aurantiaca DW4 was grown (13) and induced to differentiate as described earlier by Heidelbach et al. (4, 5).

Immunoelectron microscopy. Cells were harvested by centrifugation (10 min, 5,500 × g, 4°C), washed in ice-cold 10 mM HEPES (N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid)-1 mM CaCl2, pH 7.0, and suspended in the same buffer containing 0.5% (wt/vol) formaldehyde and 0.1% (vol/vol) glutaraldehyde.

Cellswereimmobilizedin2%(wt/vol)agar,bufferedwith50mMphosphate-bufferedsaline(PBS)–20mMglycine,pH7.0.Maturefruitingbodieswerefixedinsituonasheetofagar,bufferedwith50mMPBS, pH 7.0. Suitable pieces were excited, transferred to small glass petri dishes, and fixed in 0.5% (wt/vol) formaldehyde-0.1% (vol/vol) glutaraldehyde in PBS, pH 7.0, at 4°C for 3 h. Alddehydes were neutralized by washing the pieces three times for 10 min each in 50 mM PBS–20 mM glycine, pH 7.0. Induced spores and fruiting bodies were embedded in Lowicryl K4M resin and further processed for immunoelectron microscopy under labelling conditions identical to those described by Lünsdorf et al. (9), with 12-nm-diameter protein A-gold conjugates. Preimmune serum and protein A-gold conjugate controls were carried out in the same way for all types of specimens.

Conventional electron microscopy. Mature fruiting bodies were fixed in situ for 7 days on the agar surface in a saturated glutaraldehyde atmosphere in sealed petri dishes. They were covered with low-melting-point agarose buffered with Veronal-sodium acetate buffer (12), supplemented with 5% (wt/vol) sucrose, pH 7.0, and washed once for 10 min at room temperature. Osmium fixation was done with 1% (wt/vol) OsO4 in Veronal-sodium acetate buffer, pH 7.0, overnight at 4°C. The fruiting bodies were further processed according to the general protocol for epoxy resins (15, 18).

Electron microscopy of ultrathin sections. Ultrathin sections of 120 nm thickness for electron microscopy were made with a Reichert Ultratome 3 ultramicrotome (Leica, Bensheim, Germany) and mounted on carbon-coated Formvar grids. Samples were observed with a CEM 902 microscope (Zeiss, Oberkochen, Germany) at a magnification range from ×5,300 to ×31,600, an acceleration voltage of 80 kV, and an objective aperture of 30 μm.

RESULTS

Localization of SP21 in S. aurantiaca cells after anoxia, indole treatment, and heat shock. The LMW-HSP of S. au-
number of 247 gold conjugates per square micrometer (mean ± SD) was found to be significantly increased in comparison with that in well-aerated cells (see Fig. 4b; Table 1). Interestingly, SP21 antigen concentration was increased in the vicinity of the cytoplasmic membrane (Fig. 2a, double arrow). Apparently, either SP21 is attached to a matrix of the cell surface which protrudes from the cells into the surrounding space or SP21 is focally condensed on the cell surface.

At 42°C the cells also produced SP21 within 1 h. SP21 was preferentially located near the outer membrane (Fig. 2b). In contrast to cells at 37°C, which remained viable, cells at 42°C turned into spheroplasts and died. This was preceded by tremendous changes in the cytoplasmic ultrastructure: electron-dense aggregates were formed (Fig. 2b, asterisks), mainly located at the cytoplasmic membrane. The spheroplasts (Fig. 2b, single arrowhead) formed coned protrusions before the occurrence of cell wall disruption, followed by cell lysis (Fig. 2b, double arrowhead).

Localization of SP21 antigen within the fruiting body. According to the arrangement and morphology of the cells, the fruiting body of *S. aurantiaca* can be divided into at least three compartments: the sporangiole, the stalk, and a layer of cells at the base of the fruiting body and the surrounding area (basal layer). In each of these compartments, *hsp4* expression followed a distinct pattern.

In the sporangiole, vegetative cells are converting into myxospores. The sporangiole represents a compartment which is characterized by a unique ultrastructural appearance. Conventional embedding results in high ultrastructural but low immunocytochemical quality (Fig. 3a). The results of this embedding method have to be compared with those obtained by using Lowicryl embedding, which results in high immunocytochemical but low ultrastructural quality (Fig. 3b). The cell wall of the myxospore (Fig. 3a, inset) is composed of four horizons: (i) the cytoplasmic membrane, about 6.3 nm thick; (ii) the darkly stained murein sacculus, about 4.2 nm thick; (iii) the outer membrane, about 8.3 nm thick; and (iv) the spore coat, ranging from 12.5 to 20.8 nm thick. The overall thickness of the spore cell wall ranged from 54 to 67 nm. The myxospores were densely packed and were in tight contact. In the areas of dense packing, the spores had an irregular shape. The spore coat layers did not fuse. In the contact region, the spore coats of both cells were considerably thickened and showed a stronger contrast (Fig. 3a, arrows).

Myxospores, which had been processed according to the high-quality immunocytochemical procedure, showed few and poor ultrastructural details within their cell walls (Fig. 3b). The membrane, the murein sacculus, and the spore coat were indistinguishable, and thus the cytoplasm appeared to be surrounded by an electron-translucent zone. The cells were outlined by a diffuse darkish layer (Fig. 3b, double arrowheads). However, the apparent distance between two adjacent protoplasts in an area of tight contact (Fig. 3, arrows) differed considerably depending on the embedding procedure used. Conventionally embedded myxospores had a protoplast-protoplast distance of 83 to 104 nm, and for Lowicryl-embedded spores the distance was 125 to 168 nm. The length of the myxospores ranged from 1.68 to 2.36 μm, the width ranged from 540 nm to 670 nm, and the length-to-width ratio was about 3. This implies either a shrinkage of the spore protoplast or a dilation of the spore cell wall during Lowicryl

### Table 1. Area densities of the SP21 antigen

<table>
<thead>
<tr>
<th>Physiological state</th>
<th>Protein A-gold conjugates/μm² (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vegetative cell (physiological control)</td>
<td>0.0 ± 0.8</td>
</tr>
<tr>
<td>Beginning of anoxic stress</td>
<td>4.6 ± 1.7</td>
</tr>
<tr>
<td>6 h of indole induction</td>
<td>3.4 ± 0.8</td>
</tr>
<tr>
<td>Heat-shocked cells (37°C)</td>
<td>13.6 ± 9.5</td>
</tr>
<tr>
<td>Heat-shocked cells (42°C)</td>
<td>13.6 ± 0.9</td>
</tr>
<tr>
<td>Sporangiole spores</td>
<td>0.0 ± 0.0</td>
</tr>
</tbody>
</table>

*Note: SP21, a stress protein of *S. aurantiaca*, is formed during anoxia, in the course of indole-induced sporulation and fruiting-body formation (4, 5). SP21 was localized by immunoelectron microscopy with an SP21-specific antiserum and 5- or 12-nm-diameter protein A-gold conjugates.

Localizing SP21 distribution in a vegetative mid-logarithmically growing cell, which had recently been exposed to anoxic stress, is shown in Fig. 1a. SP21 was present in small amounts, but its concentration was significantly increased in comparison with that in well-aerated cells (see Fig. 4b; Table 1). Interestingly, SP21 was restricted mainly to the cell wall, where a mean number of 247 gold conjugates per μm² (Table 1) were found. The level of SP21 expression and the concentration of the antigen increased at the early stage of indole-induced sporulation at 6 h (Fig. 1b; Table 1). The number of gold conjugates per μm², the width ranged from 540 nm to 670 nm, and the length-to-width ratio was about 3. This implies either a shrinkage of the spore protoplast or a dilation of the spore cell wall during Lowicryl embedding.
FIG. 1. Immunocytochemical detection of SP21 antigen in *S. aurantiaca*. (a) Vegetative cells stressed by anoxia for only about 5 min. Gold targets are distributed mainly at the outer membrane. (b) Antigen distribution within a cell after 6 h of indole induction. The SP21 antigen is homogeneously distributed at the cell wall periphery. Circled areas mark clusters which indicate a focal antigen accumulation. (c) Antigen distribution within a cell after 26 h of indole induction. The antigen is dispersed within the cytoplasm. Focal accumulations are indicated by circles. Electron-translucent inclusions, possibly polyhydroxybutyrate, are indicated by asterisks. PP, polyphosphate granules; Chr, bacterial chromosome; om, outer membrane. Bars, 500 nm (lower bar is valid for panels b and c).
embedding. By both embedding methods, the bacterial chromosome was displayed.

An intracellular organelle was detected by both embedding methods. It is a circular electron-transparent structure (104 to 125 nm in diameter), the border of which is studded with about 18 electron-dense particles, 21.3 ± 1.0 nm in diameter. This organelle is characteristic for the sporangiole-derived myxospores and has not been found in chemically induced spores. In fruiting bodies, SP21 was most strongly produced in the myxospores, where it was preferentially localized in the electron-transparent zone surrounding the cytoplasm (Fig. 3b). Some SP21 was located within the cytoplasm, mainly at the border with the electron-transparent margin. Occasionally, small clusters were formed (Fig. 3b, circles). Compared with stressed cells or chemically induced spores, the cells of the sporangioles had an immunocytochemical label of reduced intensity.

The correlation between antigen expression and the different physiological states of S. aurantiaca is summarized in Table 1. The mean label density of protein A-gold conjugates per square micrometer was determined for the cell wall, cytoplasm, and the periphery of the cells. The complex which consists of protein A-gold, antibody, and SP21 spans about 12 to 18 nm (8). As the protein A-gold antibody conjugate flips around the antigen, the score range in which the gold signal can be detected is 24 to 36 nm. Therefore, a differential localization of the antigen in the outer membrane, the periplasm, or the cytoplasmic membrane is not possible. Thus, in a 30-nm score range all gold conjugates were indicated as cell wall targets.

Apart from very few intact cells, the stalk consisted mostly of spheroplasts and electron-translucent cell ghosts which had a much larger diameter than did vegetative cells or spores (Fig. 4a, asterisk). SP21 was present in the stalk, mainly attached to the residual outer membrane and cellular debris.

**DISCUSSION**

The LMW-HSP of the α-crystallin family possess a very conserved consensus sequence which is supposed to mediate the formation of high-molecular-weight complexes (11). During its purification, SP21 was sedimented in the membrane fraction (5). The absence of any membrane-spanning region in the amino acid sequence (4) tempted us to study the localization of SP21 by immunogold electron microscopy. In the course of these investigations, morphological information about the organism in different developmental states has been obtained.

Indole-induced spores and spores from fruiting bodies differ in shape (Fig. 1b and 3a). We explain this discrepancy as follows. The cell wall of myxobacteria is supposed to consist of peptidoglycan patches, which are interconnected by rather long nonpeptidoglycan bridges (21). During the transition from the vegetative state to the spore, there is extensive restructuring of the cell wall, in the course of which cells become prone to lysis (16). We suggest that in this process, the cell wall attains increased plasticity. In densely populated areas, the prespores snuggle up to each other and take on a bent, polyhedral shape. Spore maturation leads to a solidification of the spore cell wall, thereby fixing the acquired shape of the spores. In other studies, by Voelz and Reichenbach (20), cross-sections of sporangial myxospores had exclusively circular or elliptical contours. This may be a strain-specific feature or may be due to a different stage of fruiting-body maturation or simply variant experimental designs. Furthermore, electron-translucent inclusions surrounded by globular particles have been detected in spores from fruiting bodies (20) and in glycerol-induced myxospores of S. aurantiaca (14). They also resemble those found in Cystobacter fuscus and Melittangium lichenicol, in these organisms termed TGP particles (10).

When the specimen was fixed with osmium tetroxide and post-contrasted with uranium acetate and lead citrate, the particles surrounding the vesicles showed intense contrast and were of similar size (diameter, 21.3 ± 1.0 nm). These structures are probably not ribosomes, because they appear to be larger than the dark spots in the cytoplasm which we assume to be ribosomes. The diameter of prokaryotic ribosomes is smaller (16 to 18 nm for Escherichia coli and Myxococcus fulvus in osmium tetroxide-fixed ultrathin sections [data not shown]). Though one may speculate on the physiological significance of these electron-translucent inclusions studded with dense particles, it is interesting that they could not be detected in indole- or heat shock-induced myxospores of S. aurantiaca though they were present in glycerol-induced spores, as shown by Reichenbach et al. (14). Possibly the reason for these discrepancies may be different incubation periods during spore induction for the two experiments.

The immunocytochemical data for hspA-expressing S. aurantiaca cells are consistent with those reported previously (4, 5). The synthesis of SP21 is associated with the reaction to unfavorable environmental conditions such as heat shock, oxygen limitation, and nutrient depletion. In liquid culture, each cell is responding individually to stress, e.g., heat shock. During fruiting, the situation is different. The cells respond to starvation stress not as individuals but as a swarm. The differential expression of hspA according to time and position supports this assumption. (i) SP21 appears late in morphogenesis. By this time, the fruiting body is already differentiated into stalk and sporangioles. The sensing of nutrient depletion, the coordinated movement of cells, and cell lysis are already accomplished. It is unlikely that during maturation of the fruiting body the spores (or prespores) would suffer stress or respond to stress in the same manner as heat-shocked cells do. (ii) The cells within the basal layer have most probably encountered starvation stress, because they have been the last to arrive in the grazed area. If they had reacted individually and not according to the developmental program, they would have been expected to show a stress response and express hspA. The fact that they did not lets us assume that hspA is regulated differentially during fruiting-body morphogenesis. We therefore suggest that during fruiting-body formation, SP21 synthesis is not an independent process but is strictly embedded into the system of morphogenetic regulation. The different time courses of SP21 appearance during stress response and fruiting-body formation argue for distinct signal transduction pathways leading to SP21 synthesis.

For several of the eukaryotic LMW-HSP, a chaperone-like activity has been demonstrated in vitro (6, 7, 11, 14). This activity was located in the C-terminal part of these α-crystallin-like proteins (1, 19), which is most conserved across the species. As SP21 also has this region, it too may be needed to protect structures of the myxospores. SP21 may also have enzymatic activities or serve as a component of the cell signalling apparatus.

SP21 is transported across the bacterial membranes. After heat shock, nearly all SP21 was found outside the cells, whereas considerable amounts were found in the cytoplasm of spores. Presently it is unclear if SP21 serves its function inside and outside the cell or if its function involves crossing of the bacterial membranes, or if we observed an intermediate state in our study and all SP21 is eventually exported.

The swarm is vital for myxobacteria. Its maintenance is dependent on cell-cell communication which at least partially
FIG. 2. Immunocytochemical detection of SP21 antigen after physical stress of vegetative cells. (a) Heat-shocked cell at 37°C. SP21 antigen is evenly distributed along the outer membrane; clusters of antigen can be observed both within the cytoplasm, near to the cytoplasmic membrane (arrows), or extracellularly (double arrow). (b) Heat-shocked cell at 42°C. A vegetative cell (V), showing electron-opaque deposits (asterisks) near the cytoplasmic membrane at the cell pole, can be seen. These deposits can be equally recognized in closed and lysed spheroplasts (S). Arrowheads point to spheroplast sites, about to perforate for lysis. Antigen is distributed along the outer membrane with only few clusters. Bars, 500 nm.
FIG. 3. Ultrastructural comparison of conventionally prepared (a) and immunocytochemically labelled (b) myxospores within an *S. aurantiaca* sporangiole. (a) Osmium-fixed cells, poststained with uranyl acetate and lead citrate. The cytoplasm is homogeneously packed, with typical inclusions (asterisks) interspersed. Oligolaminar membrane bodies (MB) are indicated; the outer surface of the myxospore is covered by the spore coat (SC). Arrows point to intercellular tight-contact areas. (Inset) Ultrastructure of the fourfold layered cell wall. (b) Immunocytochemical localization of SP21 antigen. The antigen is distributed mainly along the cytoplasmic borderline and the outer spore coat (double arrowheads); intracellular clusters (circles) are shown near the cytoplasmic borderline, while electron-transparent inclusions (asterisks) appear alone or as a cluster. Chr, bacterial chromosome; om, outer membrane; m, murein sacculus; cm, cytoplasmic membrane. Bars, 500 (lower bar is valid for panels a and b) and 50 nm (inset).
FIG. 4. (a) Localization of SP21 antigen in the stalk. (b) SP21 expression and localization in logarithmically growing cells. The antigen is found in low numbers at the cell wall, as indicated by arrowheads marking individual protein A-gold 5-nm conjugates. (c) Label specificity control of the SP21 preimmune serum with immunoglobulin G concentrations and label conditions identical to those for the SP21 antiserum. Individual protein A-gold 12-nm conjugates are circled; the low level of gold conjugates at the sporangiolar spores underlines the high specificity of the SP21 antiserum (for direct comparison, see Fig. 3b). Bars, 1 μm (all panels).
involves structures (fimbriae and pili, etc.) on the surface of the cells. A function of SP21 may be to stabilize these structures under several stress conditions.

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