SOME CHARACTERISTICS OF AERIAL AND SUBMERGED SPORES OF *KITASATOSPORIA SETALBA*

YÔKÔ TAKAHASHI, TOMOKO KUWANA, YUZURU IWAI AND SATOSHI ÔMURA

*Kitasato Institute and School of Pharmaceutical Sciences, Kitasato University, Minato-ku, Tokyo 108, Japan*

(Received April 18, 1984)

*Kitasatosporia setalba* KM-6054 forms two kinds of spores: aerial spores on an agar medium and submerged spores in a liquid medium. In this study, both kinds of spores were resistant to sonication, but sensitive to lysozyme digestion and moist heat. LL-2,6-Diaminopimelic acid (A₃pm), alanine, glutamic acid and glycine were detected as the main constituents in the cell walls of the aerial and submerged spores. The respective molar ratios were similar; 1.0: 1.6: 0.5: 1.0 and 1.0: 1.5: 0.7: 0.8. On the other hand, the vegetative mycelium on agar media and the filamentous mycelium in submerged cultures were sensitive to sonication and differed from the two kinds of spores in both amino acid composition and A₃pm type.

The morphological and cultural characteristics of strain KM-6054, a setamycin producing organism (1), resemble those of strains of the genus *Streptomyces*. However, since the cell wall of the strain is a new type containing LL- and meso-2,6-diaminopimelic acid (A₃pm), glycine and galactose, we proposed a new genus *Kitasatosporia* and named the species *Kitasatosporia setalba* (2).

In previous papers (3, 4), we reported that the morphology of *K. setalba* related to the A₃pm type. When the strain was cultivated on an agar medium, aerial spores contained LL-A₃pm but were formed on vegetative mycelia which contained meso-A₃pm (3). Similarly, when the strain was cultivated in a liquid medium, the submerged spores contained LL-A₃pm but were produced by filamentous mycelia which contained meso-A₃pm (4). Thus the aerial and submerged spores of *K. setalba* are peculiar among spores of other actinomycete strains in that their A₃pm type is different from that of the mother mycelia.

In this paper, we report some other characteristics of the aerial and submerged spores of *K. setalba*. Both of the spores were found to be resistant to sonication,

---

1 To whom all correspondence should be addressed.
but sensitive to lysozyme digestion and moist heat. The amino acid composition of the cell walls was similar in the two kinds of spores but was different from that of the vegetative and filamentous mycelia.

MATERIALS AND METHODS

Actinomycete strains. Kitasatosporia setalba KM-6054 (ATCC 33774, IFO 14216), the type strain of K. setalba and the following nine reference strains were used: Streptomyces albus KA-1023, S. griseus KA-1198, Nocardia autotrophica KA-1050, S. alborubidus KA-1018, Nocardioides albus KA-603 (ATCC 27980), Nocardiopsis dassonvillei KA-602 (ATCC 23218), Pseudonocardia azurea AM-3696, P. thermophila KA-552 (KCC 0032), Actinomadura citrea KA-613 (ATCC 27887).

Preparation of aerial spores and vegetative mycelia. Aerial mycelia well grown on inorganic salts-starch agar at 27°C for two weeks were scraped off with sterile glass beads (4.0–6.0 mm) and suspended in 20 mM N-tris (hydroxymethyl)methyl-2-aminoethanesulfonic acid buffer (TES buffer, pH 7.3). The suspension of aerial mycelia was filtered through glass filter No. 3 to separate the aerial spores, the purity of which was confirmed by microscopic observation. After the agar surface was washed thoroughly with water, the agar was melted in a boiling water bath and filtered with cotton gauze. The residue was washed with hot water and air-dried at room temperature to give vegetative mycelia.

Preparation of submerged spores and filamentous mycelia. A loopful of aerial mycelia of K. setalba was transferred to 100 ml of Y-D medium (pH 7.0 before sterilization) consisting of 1% yeast extract (Difco) and 1% glucose in a 500-ml Sakaguchi flask and incubated on a reciprocal shaker at 27°C. The 3-day culture (stationary phase) was filtered through a Toyo Roshi No. 1 filter. Submerged spores were obtained by centrifugation (2,940 x g, 20 min) of the filtrate. The submerged spores thus obtained under sterile conditions were inoculated into 100 ml of Y-D medium in a 500-ml Sakaguchi flask and incubated at 27°C for 8 hr on a reciprocal shaker. The culture was centrifuged at 1,200 x g for 10 min to give filamentous mycelia.

Viability measurement. After the spore suspension was diluted with sterile TES buffer (pH 7.3), 0.1 ml of the diluted suspension was spread onto the surface of modified Waksman’s agar (pH 7.0 before sterilization) consisting of 1% glucose, 0.5% peptone, 0.5% meat extract, 0.3% NaCl and 1% agar. The plates were incubated at 27°C for a week, and then the colonies were counted.

Sensitivity to sonication, lysozyme digestion and moist heat. Sonic tests were carried out by subjecting 1.0 ml of spore suspension to 10 or 60 min of sonication (50% pulse, 170 W, sonicator model W-225 R of Heat System-Ultrasonic Inc.). For lysozyme-sensitivity tests 1.0 ml of spore suspension was incubated with 20 or 200 µg/ml of lysozyme (egg white lysozyme, Sigma) at 37°C for 60 min. Heat-sensi-
tivity tests were carried out by incubating 1.0 ml of spore suspension for 20 min at 27°, 40°, 60° or 80°. After the heating, the viable cells were counted.

Preparation of cell walls. The cell walls were prepared according to the method of YAMAGUCHI (5) with modifications as follows. Vegetative and filamentous mycelia were disrupted with a sonicator at 150 W, 10–20 min, and aerial and submerged spores at 170 W, 2–3 hr in the presence of glass beads (0.1–0.2 mmØ, Willy A. Bachoten). After that treatment, the suspension was centrifuged three times at 2,940 × g each for 20 min to remove glass beads and unbroken cells. The supernatant fluid was then centrifuged at 8,000 × g for 30 min to give a crude cell wall preparation, which was washed with 1 M NaCl and then with ethanol. Next, the preparation was saponified in 2% KOH in ethanol with stirring in the presence of glass beads (4.0–6.0 mmØ) on a shaker at 27° for 2 days, and then washed successively with ethanol, water and 0.05 M tris-(hydroxymethyl)aminomethane-HCl buffer (Tris buffer, pH 8.0). The crude cell walls were suspended in a fresh solution of trypsin (0.3 mg/ml, Sigma) in Tris buffer (pH 8.0) and digested in the presence of glass beads (4.0–6.0 mmØ) on a shaker at 37° for 2 hr. The suspension was centrifuged, washed successively with Tris buffer (pH 8.0), water, ethanol and acetone to yield a purified preparation of cell wall.

Amino acid analysis. A cell wall preparation was hydrolyzed in 6 N HCl at 100° for 18 hr. The hydrolyzate was filtered with cotton gauze. The filtrate was concentrated in vacuo to dryness to give the hydrolyzate used for the quantitative amino acid analysis in an amino acid analyzer (model 200A, JEOL).

Scanning electron micrography. A scanning electron microscope (model 5-430, Hitachi) was used to observe K. setalba grown on solid and in liquid media.

After the strain was incubated at 27° for 2 weeks on inorganic salts-starch agar, an agar piece containing aerial and vegetative mycelia was cut out, put onto an aluminium film, fixed with 1% OsO₄ and coated with Pt-Pd. Submerged spores and filamentous mycelia grown in Y-D medium with shaking were centrifuged, washed and spread onto a dialysis membrane (Seamless Cellulose Tubing, Visking Co.), and they were stained by the method of WATANABE et al. (6).

RESULTS

Morphological aspects of the spores and mycelia of Kitasatosporia setalba grown on agar and liquid media

Figure 1 shows a scanning electron micrograph of an aerial spore and vegetative mycelium of K. setalba. Good sporulation of the aerial mycelium (Fig. 1a) and no fragmentation of the vegetative mycelium (Fig. 1b) were observed. The morphology of K. setalba resembles that of strains of the genus Streptomyces.

Figure 2 shows a scanning electron micrograph of K. setalba grown in a submerged culture. The strain developed filamentous mycelia and submerged spores, which were morphologically distinguishable from each other.
Table 1 shows a comparison of the spores of *K. setalba* with related actinomycete strains in sensitivity to sonication and lysozyme digestion. Nine actinomycete strains of six known genera that produce aerial spores were used as references. The vegetative and filamentous mycelia of *K. setalba* were easily broken by sonication for 10 min, whereas both spores of the strain were resistant to sonication even for 60 min, as were the spores of the reference strains. The two kinds of spores were sensitive to lysozyme digestion and so were the aerial spores of the actinomycetes except *Streptomyces albus*, *S. griseus* and *Actinomadura citrea*.

The heat sensitivity of *Streptomyces albus* and *S. griseus*, which contain LL-A_{2}pm in the cell walls, were compared with that of the *K. setalba* spores. As shown in Table 2, the aerial and submerged spores of *K. setalba* were more sensitive to moist heat at 40° than the aerial spore of two streptomycete strains.

### Amino acid composition in cell walls of spores and mycelia

The amino acid composition of the cell walls of the four kinds of *K. setalba* cells, aerial and submerged spores and vegetative and filamentous mycelia, was analyzed (Table 3). Four kinds of amino acids, A_{2}pm, Ala, Glu and Gly, were detected as main components in the hydrolyzates of the cell walls. The molar

---

*Fig. 1.* Scanning electron micrograph of aerial spores (a) and vegetative mycelia (b) of *Kitasatospora setalba* grown on inorganic salts-starch agar. The bar marker represents 1 µm.
Spores of *Kitasatosporia setalba*

Fig. 2. Scanning electron micrograph of a submerged culture of *Kitasatosporia setalba* grown in Y-D medium. The bar marker represents 1 μm. A, submerged spore; B, filamentous mycelium.

Table 1. Comparison of spores of *K. setalba* and the related actinomycete strains regarding sensitivity to ultrasonication and lysozyme digestion.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Spore</th>
<th>Ultrasonication (min)</th>
<th>Lysozyme (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>10</td>
<td>60</td>
</tr>
<tr>
<td><em>Kitasatosporia setalba</em> KM-6054</td>
<td>AS</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>SS</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td><em>Streptomyces albus</em> KA-1023</td>
<td>AS</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td><em>S. griseus</em> KA-1198</td>
<td>AS</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td><em>Nocardia autotrophica</em> KA-1050</td>
<td>AS</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td><em>S. alborubidus</em> KA-1018</td>
<td>AS</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td><em>Nocardioides albus</em> KA-603 (ATCC 27980)</td>
<td>AS</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td><em>Nocardiosis dassonvillei</em> KA-602 (ATCC 23218)</td>
<td>AS</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td><em>Pseudonocardia azuroa</em> AM-3696</td>
<td>AS</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td><em>P. thermophila</em> KA-552 (KCC 0032)</td>
<td>AS</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td><em>Actinomadura citrea</em> KA-613 (ATCC 27887)</td>
<td>AS</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

Values indicate survival ratio (%).

AS, aerial spore; SS, submerged spore.
The amino acid composition of vegetative and filamentous mycelia was also essentially the same. It was found that the Gly content in the two mycelia was lower than that of the two kinds of spores.

**DISCUSSION**

*K. setalba* forms the two kinds of spores, aerial spores on solid medium and submerged spores in liquid medium. It is of particular interest that both kinds of spores contain L-L-A2pm but are produced by vegetative and filamentous mycelia, containing meso-A2pm. The aerial and submerged spores are similar in their sensitivity to sonication, lysozyme digestion and moist heat as well as the amino acid composition of their cell walls (Tables 1 and 2).

**Table 2.** Comparison of the heat sensitivity of *K. setalba* spores with those of the genus *Streptomyces* strains.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Spore</th>
<th>27°C</th>
<th>40°C</th>
<th>60°C</th>
<th>80°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kitasatosporia setalba KM-6054</td>
<td>AS</td>
<td>100</td>
<td>15.6</td>
<td>0.3</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>SS</td>
<td>100</td>
<td>10.3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Streptomyces albus KA-1023</td>
<td>AS</td>
<td>100</td>
<td>100</td>
<td>16.8</td>
<td>0.3</td>
</tr>
<tr>
<td><em>S. griseus</em> KA-1198</td>
<td>AS</td>
<td>100</td>
<td>100</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Values indicate survival ratio (%).

AS, aerial spore; SS, submerged spore.

**Table 3.** Comparison of the amino acid composition (molar ratio) in the cell walls of four kinds of cells of *K. setalba*.

<table>
<thead>
<tr>
<th></th>
<th>A2pm (type)</th>
<th>Ala</th>
<th>Glu</th>
<th>Gly</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aerial spore</td>
<td>1.0 (L-L)</td>
<td>1.6</td>
<td>0.5</td>
<td>1.0</td>
</tr>
<tr>
<td>Submerged spore</td>
<td>1.0 (L-L)</td>
<td>1.5</td>
<td>0.7</td>
<td>0.8</td>
</tr>
<tr>
<td>Vegetative mycelium</td>
<td>1.0 (meso)</td>
<td>1.6</td>
<td>0.7</td>
<td>0.2</td>
</tr>
<tr>
<td>Filamentous mycelium</td>
<td>1.0 (meso*)</td>
<td>1.6</td>
<td>0.7</td>
<td>0.4</td>
</tr>
</tbody>
</table>

* A small amount of L-L-A2pm was also detected.

ratios in the spores were similar with minor difference in Glu and Gly contents. The amino acid composition of vegetative and filamentous mycelia was also essentially the same. It was found that the Gly content in the two mycelia was lower than that of the two kinds of spores.
Spores of *Kitasatospora setalba*

Composition of vegetative and filamentous mycelia was also similar. In both, the Gly content was lower than in the spores. These results suggest that the structures of the peptidoglycans of two kinds of spores are similar to each other, but are different from those of the two kinds of mycelia.

Nakamura et al. (8) reported the amino acid composition of the cell walls of mycelia in submerged cultures of nine species of the genus *Streptomyces*. The Apm, Ala and Gly contents of the two kinds of *K. setalba* spores are similar to *Streptomyces*, except for the Glu content. Thus far the amino acid composition of the cell walls of the separated cells of actinomycete strains has not been analyzed. To our knowledge, our studies are the first investigation of the amino acid composition of the cell walls of the four kinds of cells that are produced by a single strain and are morphologically distinguishable from each other.

Compared with the studies of aerial spores, only a few descriptions of spores produced in submerged culture of actinomycete strains have been reported (9). Previously we called the spores in a submerged culture of *K. setalba* "spore-like cells." Since the properties of the spores described in this paper indicate that they are true spores, we now call them "submerged spores," the name proposed by Carvajal (10).

Recently we isolated two new strains of the genus *Kitasatospora*, strains KA-338 and AM-9660, which are different from *K. setalba* but also produce submerged spores. We plan to investigate the mechanism of formation of the submerged spores of three *Kitasatospora* strains.

The authors wish to thank Kyowa Hakko Co. Ltd. for analysis of amino acids, and Dr. A. Seino of The Institute of Physical and Chemical Research for the generous gift of *Pseudonocardia thermophila*. We thank Dr. Y. Tanaka of The Kitasato Institute and Dr. H. Tanaka of Kitasato University for their suggestions.

REFERENCES