Characteristics of spores formed by surface and submerged cultures of *Streptomyces albidoflavus* SMF301

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Streptomyces albidoflavus SMF301 produced abundant spores in submerged cultures (submerged spores) as well as on solid media (aerial spores). The content of carbon, hydrogen, nitrogen, and phosphorus in submerged and aerial spores was similar; however, the contents of metal ions (K^+ , Na^+ , Ca^{2+} and Mg^{2+}) were very different. Glutamic acid, alanine, and glycine, all known to be cell-wall components, were the major amino acids in both types of spores. However, cysteine was more abundant in submerged spores than in aerial spores. The major fatty acid in aerial spores was n-C₁₈ (61.74%), whereas in submerged spores it was ai-C₁₆ (33.68%). The contents of ai-C₁₄, and ai-C₁₇ in submerged spores were also very much higher than in aerial spores. Unsaturated fatty acids were found in both kinds of spores but not in mycelium; they were particularly abundant in submerged spores. The composition of menaquinones in the two kinds of spores also varied. The resistance of aerial spores to lysozyme digestion, mild acid treatment, heating and desiccation was higher than that of submerged spores, but the submerged spores were more resistant to sonication.

Introduction

Streptomyces spp. are important as producers of useful metabolites. They are also of interest because of their unique morphological complexity: they form a substrate mycelium, as well as an aerial mycelium that differentiates into arthrospores. Aerial growth and spore formation probably reflect nutrient limitation, reduced growth rate and the accumulation of growth inhibitors or extracellular pheromones. Studies on the mechanisms involved in cell differentiation have been carried out mostly on solid cultures (Chater, 1984, 1989, 1991; Chater *et al.*, 1988). However, spore formation in submerged cultures would give some advantages in the elucidation of relationships between environmental change and spore formation, and in quantitative analysis of the processes. Some species of *Streptomyces* can sporulate in submerged culture, and this has led to some physiological analyses (Kendrick & Ensign, 1983; Ensign, 1988; Koepsel & Ensign, 1984; Huber *et al.*, 1987; Ochi, 1987; Daza *et al.*, 1989; Glazebrook *et al.*, 1990).

We found, in soil, a strain of *Streptomyces* (SMF301) that produced abundant spores both in submerged

cultures and on solid cultures (Shin & Lee, 1986; Jeong *et al.*, 1988; Rho *et al.*, 1988, 1989). This strain was classified as *Streptomyces albidoflavus* by numerical analysis of taxonomic characters (Williams *et al.*, 1983*a, b*) using TAXON, an identification probability matrix (Rho *et al.*, 1992). We report here the cellular composition of the spores and their resistance to extreme conditions.

Methods

Micro-organism and media. The micro-organism used was *Streptomyces albidoflavus* SMF301. Rich medium consisted of (w/v): 1% glucose, 0.2% peptone, 0.1% yeast extract, 0.1% beef extract, and 1.8% agar for solid cultures. Chemically defined medium contained (w/v): 2% glucose, 0.1% NH₄Cl or 0.5% Na-caseinate, 0.13% KH₂PO₄, 0.09% Na₂HPO₄, 0.06% MgSO₄·7H₂O, 0.0001% trace elements (FeSO₄·7H₂O, MnCl₂·4H₂O, CaCl₂·2H₂O, and ZnSO₄·7H₂O). The initial pH of the media was adjusted to 7.0 before steam sterilization; the phosphate and magnesium salts were sterilized separately and added aseptically.

Strain maintenance and culture conditions. Strains were maintained by transferring to slopes of rich agar medium each month and storing at 4 °C. The spores developed on rich agar medium were suspended in rich liquid medium supplemented with glycerol (10%, v/v) and stored at –70 °C (Wellington & Williams, 1978). The frozen spore suspensions (about 10⁹ spores ml⁻¹) were thawed at ambient temperature for use as inocula.

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Surface culture was carried out in Petri plates on rich or chemically defined medium at 28 °C. Submerged cultures in 50 ml chemically defined medium in 100 ml baffled flasks were inoculated with 1 ml of spore suspension and incubated for 5 d at 28 °C on a rotary shaker (150 r.p.m.). In flask cultures, the pH was maintained at 7.1 by addition of 48 mM-MOPS buffer. The seed culture was used to inoculate 2 litres of chemically defined medium in a jar fermentor (3 litre; Korea Fermentor Co.). The culture temperature was maintained at 28 °C, and the pH was controlled at 7.0 by automatic addition of 1 M-HCl or 1 M-NaOH. Aeration and agitation were controlled at 1.0 v/v min⁻¹ and 200 r.p.m., respectively.

Analysis of growth and spore formation. Mycelium and spores in submerged cultures were harvested by centrifugation at 15000 g for 10 min, and then washed twice with physiological saline solution and once with distilled water. The washed cells were dried at 100 °C for 12 h. Growth is expressed as dry cell weight. The procedure for distinguishing spores from mycelium was based on results obtained during the present studies; the number of spores formed in submerged cultures (spore forming units) was counted as follows: culture broth (5 ml) was sonicated for 5 min at 100 W using a sonic dismembrator (Model 300, Fisher). The sonicated cell suspension (0.5 ml) was mixed gently with 0.1 M-HCl (4.5 ml); after 5 min, it was diluted adequately in physiological saline solution and spread on rich agar medium. Colonies were counted after 4 d at 28 °C.

Characterization of spores from surface and submerged cultures. Morphological characteristics were observed with a phase-contrast microscope (Nikon Labophot) and a scanning electron microscope (Stereoscan 260 SEM). Vegetative mycelium growing rapidly in submerged culture was harvested by centrifugation at 3000 g for 5 min. Aerial spores and submerged spores were separated from mycelium by passing suspensions through glass wool (Hopwood *et al.*, 1985).

The C, H, and N contents of spores and mycelia were analysed with an elemental analyser (model 2400CHN, Perkin-Elmer) and the P content was estimated spectrophotometrically after reaction with ammonium molybdate and 1-amino-2-naphthol-4-sulphonic acid by the method of Herbert *et al.* (1971). Trace elements were measured with an atomic absorption analyser (Model AA-880 Mark II, Jarrel-Ash). Total carbohydrate was determined by reaction with phenol in sulphuric acid (Herbert *et al.*, 1971). Trehalose was measured by HPLC using a differential refractometer for detection as reported by McBride & Ensign (1987a). Crude protein content was calculated by assuming that crude protein = total N × 6.25. In the same samples, the Biuret method (Gornall *et al.*, 1949) was used to measure protein content; the latter measurement gave the protein content without interference from nucleic acids and most other cellular macromolecules containing nitrogen (Herbert *et al.*, 1971). The amino acid profiles were determined with an amino acid analyser (Model 510, Waters) as described by Bidlingmeyer *et al.* (1984) and Gaitonde (1967). Fatty acids were analysed by gas chromatography [Model MSD (5791A), Hewlett Packard] as described by Minnikin *et al.* (1978).

The resistance of vegetative mycelia, aerial spores, and submerged spores to various treatments was measured by known procedures as follows: sonication (Koepsel & Ensign, 1984); lysozyme (Glazebrook *et al.*, 1990); mild acid (McBride & Ensign, 1987a, b); heat (Daza *et al.*, 1989). In the desiccation treatment, spore suspensions (about 10⁹ spores) were passed through a membrane filter disc (0.2 µm, Millipore) and the spore filter cakes were dried for 2 weeks over P₂O₅; the moisture content in the desiccator was maintained at less than 0.01 % humidity, measured by a moisture meter (Karl Fisher).

Chemicals, reagents, and reproducibility. Lysozyme, NTG, amino acids and fatty acids were from commercial suppliers. Each experiment was repeated three times; mean values are given.

Results

Growth characteristics and spore formation in submerged culture

Mycelium and spores of *S. albidoflavus* SMF301 grown on chemically defined agar medium were observed with a scanning electron microscope (Fig. 1). The spore surface was smooth and lacked ornamentation. Morphological changes (Fig. 2) and spores formed in a submerged culture (Fig. 3) were also examined. The inoculum spores germinated and then formed a vegetative mycelium. The vegetative mycelium was extensively branched but not fragmented, and verticils were not observed. However, from the middle of the exponential growth phase the vegetative mycelium developed chains of spores.

Sporulation in a submerged culture of SMF 301 began in the middle of the growth phase; at that time the amounts of residual glucose and ammonium ions were relatively high (Fig. 4). Thus, carbon or nitrogen limitation did not seem to be responsible. The spore chains were segmented to give free submerged spores in the stationary phase of the culture. The submerged spores were slightly larger than aerial spores, but were otherwise similar.

Cellular components of the spores and mycelia

The contents of C, H, N, P, K, Na, Ca, and Mg in mycelia and spores are shown in Table 1. The contents of C, and H were comparable, but the content of N in the mycelium was higher than in spores, indicating a higher content of protein. Moreover, spores contained more P, Ca, and Mg. Conversely, the content of K and Na in the

Fig. 1. Scanning electron photomicrograph of *S. albidoflavus* SMF301 grown on rich agar medium for 7 d at 28 °C. Bar, 5 µm.

Fig. 2. Phase contrast photomicrographs of *S. albidoflavus* SMF301 from a submerged culture in chemically defined medium. Cells were harvested from the culture at (a), 0 h; (b), 24 h; (c), 48 h; (d), 60 h; (e), 72 h; (f), 96 h. Bar, 10 µm.

Fig. 3. Scanning electron photomicrograph of spores of *S. albidoflavus* SMF301. The spores and mycelia were collected from a submerged culture in chemically defined medium at 7 d. Mycelia and spores were coated with gold in an argon gas chamber. Bar, 5 µm.

mycelium was much higher than in aerial and submerged spores.

The contents of total carbohydrate, trehalose, and

Fig. 4. Changes in concentration of glucose (●), NH_4^+ (△), biomass (□), and spore forming units (■) in a submerged culture of *S. albidoflavus* SMF301. Cultures were grown at 28 °C in chemically defined medium containing Na-caseinate as the sole nitrogen source.

Table 1. Contents of C, H, N, P, K, Na, Ca and Mg in mycelium, aerial spores and submerged spores of *S. albidoflavus* SMF301

Cultures were grown at 30 °C for 5 d in rich liquid medium for submerged spores and vegetative mycelium, and on rich agar medium for aerial spores

	Percentage content in:		
	Mycelium	Aerial spores	Submerged spores
C	43.18	43.43	43.01
H	6.75	6.83	6.91
N	10.24	8.10	8.49
P	1.25	2.47	2.71
K	2.13	1.39	0.69
Na	1.31	1.41	0.87
Ca	0.04	0.06	0.07
Mg	0.11	0.13	0.14

protein in mycelia and spores are shown in Table 2. Aerial spores contained more carbohydrate than submerged spores, and their content of trehalose was higher; trehalose was not detected in the vegetative mycelium. The ratio of Biuret protein to crude protein was higher in the mycelium than in submerged and aerial spores, indicating that the spore forms contained more non-protein nitrogen compounds such as nucleic acids and cell water mucopeptides. The molar percentage amino acid compositions in vegetative mycelia, aerial spores and submerged spores are shown in Table 3. Except for the cysteine content, the amino acid compositions of the

Table 2. Total carbohydrate, trehalose, crude protein and purified protein in mycelium, aerial spores and submerged spores of *S. albidoflavus* SMF301

Culture conditions were the same as described in Table 1.

	Percentage content in:		
	Mycelium	Aerial spores	Submerged spores
Total carbohydrate*	16.02	17.49	13.82
Trehalose*	ND	8.70	6.90
Crude protein*†	64.00	50.63	53.06
Pure protein*‡	61.30	44.50	33.70

ND, Not detected.

* Lyophilized samples were used for assay.

† Based on a protein nitrogen index of 6.25.

‡ Analysed by the Biuret method.

Table 3. Molar percentage composition of amino acids in mycelium aerial spores and submerged spores of *S. albidoflavus* SMF301

Culture conditions were the same as described in Table 1.

Amino acid	Percentage content in:		
	Mycelium	Aerial spores	Submerged spores
Aspartic acid	7.56	6.68	4.76
Asparagine	0.80	0.69	0.50
Glutamic acid	16.55	18.97	18.25
Glutamine	1.79	2.10	1.98
Serine	5.78	6.09	6.25
Glycine	11.27	14.08	14.09
Histidine	1.79	1.50	1.28
Arginine	5.57	4.09	4.06
Threonine	5.39	4.70	3.94
Alanine	14.94	16.07	19.57
Proline	5.57	4.18	4.17
Tyrosine	0.70	0.40	0.39
Valine	6.48	6.57	6.54
Methionine	0.80	0.50	0.59
Isoleucine	3.08	2.89	2.78
Leucine	5.67	4.99	4.56
Phenylalanine	2.49	2.39	2.78
Lysine	3.29	2.79	2.48
Cysteine	0.25	0.15	0.80
Tryptophan	0.23	0.17	0.23
Total	100.00	100.00	100.00

spore samples were nearly identical. Glutamic acid, alanine, and glycine were the major amino acids in all morphological forms.

The major fatty acid in aerial spores was n-C₁₈ (61.74%) and that in submerged spores was ai-C₁₆ (33.66%) (Table 4). The content of unsaturated fatty acids, such as n-C_{15:1}, n-C_{16:1}, i-C_{17:1}, n-C_{17:1} and n-C_{18:2} in submerged spores was much higher than in aerial

Table 4. Fatty acid composition (%) in mycelium, aerial spores and submerged spores of *S. albidoflavus* SMF301

Culture conditions were the same as described in Table 1.

Fatty acid	Percentage content in:		
	Mycelium	Aerial spores	Submerged spores
i-C ₁₄	2.85	0.39	0
ai-C ₁₄	0	0	2.33
n-C ₁₄	0	0.58	2.40
i-C ₁₅	12.52	1.01	3.61
ai-C ₁₅	28.82	1.74	9.34
n-C ₁₅	0.19	1.43	1.43
Unsaturated n-C _{15:1}	0	0.24	1.39
i-C ₁₆	11.34	0	0
ai-C ₁₆	0	1.84	33.69
Unsaturated n-C _{16:1}	0	0	2.30
n-C ₁₆	16.10	5.86	0
Unsaturated i-C _{17:1}	0	0	1.39
Unsaturated n-C _{17:1}	0	0.11	1.54
i-C ₁₇	11.78	0.39	0
ai-C ₁₇	16.59	1.15	5.37
n-C ₁₇	0	0.27	0
Cyclopropane-C ₁₅	0	0.88	0
Unsaturated n-C _{18:2}	0	0	13.84
2-Hexyl cyclopropane-C ₁₈	0	0	4.78
n-C ₁₈	0	61.74	9.21
n-C ₁₉	0	7.09	0
ai-C ₂₀	0	0.18	0
n-C ₂₀	0	0.87	7.39
ai-C ₂₂	0	15.44	0
Total	100	100	100

spores but the unsaturated fatty acids were not present in the vegetative mycelium. Furthermore, the content of anteiso-branched fatty acids in submerged spores was particularly different from that in aerial spores.

The composition of menaquinones in the mycelium showed the typical pattern of the genus *Streptomyces*, with only MK-9(H₆) and MK-9(H₈) present. However, the major and minor menaquinones in the aerial spores were MK-9(H₄) and MK-9(H₆), respectively, and in the submerged spores were MK-9(H₆) and MK-9(H₄), respectively.

Survival characteristics of spores and mycelia

Aerial and submerged spores were more resistant to mild acid, lysozyme, desiccation and heat than the vegetative mycelia (Fig. 5*a-d*). However, significant differences between submerged spores and aerial spores appeared in lysozyme sensitivity, aerial spores being more resistant to lysozyme than submerged spores (Fig. 5*b*). However, submerged spores were more resistant to sonication than aerial spores (Fig. 5*e*). Since the submerged spores were clearly distinguished from vegetative mycelia by soni-

Fig. 5. Resistance of vegetative mycelium (●), aerial spores (△), and submerged spores (■) of *S. albidoflavus* SMF301 to treatment with (a) acid, (b) lysozyme, (c) desiccation, (d) heat and (e) sonication.

cation, we selected sonication treatment to differentiate quantitatively between submerged spores and the vegetative mycelium of *S. albidoflavus* SMF301.

Discussion

The content of Ca and Mg were higher in submerged spores. This finding is in agreement with a report that the content of Ca in spores of *Streptomyces* spp. was high, and that Ca^{2+} ions were incorporated into spores during spore formation and released at spore germination (Salas *et al.*, 1983). The content of trehalose in aerial spores was

higher than in the submerged spores. It has been reported that trehalose is the major carbohydrate in *Streptomyces* spores (Ensign, 1978; Brana *et al.*, 1986), and also that the resistance of actinomycete spores to heat and desiccation is linked closely to their content of trehalose (Kendrick & Ensign, 1983; Dworkin, 1985; McBride & Ensign, 1987a, b).

The profiles of amino acids in spores were very similar; glutamic acid, alanine and glycine were the major amino acids. LL-diamino pimelic acid, alanine, glutamic acid, and glycine were found in the hydrolysate of cell walls of *S. albidoflavus* SMF301 (Rho *et al.*, 1992). The higher contents of glutamic acid, alanine, and glycine in spores

compared to those in vegetative mycelia suggest that the spore cell wall was thicker than that of the vegetative mycelium. The cysteine contents of submerged spores and aerial spores were different, which might contribute to the different resistances to various treatments (Fig. 5a-e).

The profiles of fatty acids in aerial and submerged spores were very different. The unsaturated fatty acid content of submerged spores was remarkably high; it is noteworthy that unsaturated fatty acids have not been detected previously in *Streptomyces* (Locci, 1989). Furthermore, the ai-C₁₄, ai-C₁₅, ai-C₁₆, ai-C₁₇ contents were also very much higher in submerged spores, although ai-C₂₀ and ai-C₂₂ were not detected.

Although MK-9(H₆) and MK-9(H₈) represent the typical pattern of menaquinones in the genus of *Streptomyces* (Lechevalier & Lechevalier, 1980), we found that the major and minor menaquinones in aerial spores were MK-9(H₄) and MK-9(H₆), respectively, while in submerged spores, they were MK-9(H₆) and MK-9(H₈), respectively. Thus the menaquinone profiles change during cell morphogenesis in *S. albidoflavus* SMF301.

The higher survival of spores subjected to harsh treatments might be due to their thicker cell wall. The higher contents of P, Ca, and Mg in aerial and submerged spores compared to vegetative mycelia might also contribute to their resistance to such treatments. The resistance of submerged spores to mild acid, desiccation and heat was similar to that of aerial spores, but the higher resistance of the submerged spores to sonication might result from the higher contents of unsaturated fatty acids and anteiso-branched fatty acids, which are thought to greatly increase membrane fluidity (Tung *et al.*, 1991).

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