Diaminopimelic Acid Profiles of Alkalophilic and Alkaline-resistant Strains of Actinomycetes

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Twenty alkalophilic actinomycetes with optimum growth pH of 9.0 to 9.5 were isolated. Although these new isolates morphologically closely resembled conventional streptomycetes containing LL-diaminopimelic acid, nine out of 20 isolates contained *meso*-diaminopimelic acid.

Among 420 International *Streptomyces* Project (ISP) *Streptomyces* strains tested for their maximum growth pH, only 6 strains grew at pH 11.5. Of these six strains, three strains, *S. caeruleus* ISP 5103, *A. alborubidus* ISP 5465 and *S. autotrophicus* ISP 5011 contained *meso*-diaminopimelic acid.

INTRODUCTION

In the classification of *Actinomycetales*, cell wall composition has become widely accepted as an aid to the differentiation of the genera, in addition to more traditional taxonomic characters (Lechevalier & Lechevalier, 1970).

Recently, we have isolated alkalophilic actinomycetes from soil as a possible source of new antibiotics. In the course of taxonomic studies of these isolates, it was found that about half of them contained *meso*-diaminopimelic acid, even though they were morphologically indistinguishable from conventional *Streptomyces* containing LL-diaminopimelic acid in the cell wall.

Here some characteristics of the newly isolated alkalophilic actinomycetes, as well as those of alkaline-resistant International *Streptomyces* Project (ISP) *Streptomyces* strains are described, with emphasis on the cell wall diaminopimelic acid configuration.

METHODS

Isolation of alkalophilic actinomycetes from soil. Medium for the isolation of alkalophilic actinomycetes contained (g l⁻¹): polypeptone (Wakojunyaku, Co., Tokyo, Japan), 10; meat extract (Kyokuto Co., Tokyo, Japan), 10; yeast extract (Difco), 3; glucose, 3; Na₂CO₃ (anhydrous), 10; agar (Koso Chemical Co., Tokyo, Japan), 15; pH 10.0 or 11.0 (after autoclaving). Na₂CO₃ or NaOH were separately sterilized and used for adjusting the pH. Alkalophilic actinomycetes were isolated from the agar medium by plating water suspensions of soil samples from Chiba and Ibaraki prefectures (Japan), and incubating at 27 °C for one to two weeks.

After primary isolation, growth on media incubated at 27 °C for one week at pH 6.0 and 11.5 was compared. Isolates unable to grow at pH 6.0, but able to grow at pH 11.5 or above were used in the subsequent experiments.

Determination of alkaline-resistance of ISP Streptomyces strains. A total of 420 ISP strains of *Streptomyces* (Shirling & Gottlieb, 1972), maintained in this laboratory or kindly given by Dr A. Seino (Kaken Chemical Co., Tokyo, Japan), were used. Growth at pH 6.0 and 11.5 was checked as above.

Cell wall analysis. For cell wall analysis, cultures were grown in shake flasks containing (g l^{-1}): peptone (Difco), 10; meat extract, 10; glycerol, 10; NaCl, 8; pH 10.0 before autoclaving. After incubation in a reciprocal shaker ($200 \text{ rev. min}^{-1}$) at 27°C for 78 to 96 h, the cultures were collected by centrifugation, and thoroughly washed with distilled water. The washed mycelia were treated as described by Cummins & Harris (1958).

The purified cell wall preparation was used for amino acid analysis using an automatic amino acid analyser (Hitachi Seisakujo Co., Japan). For sugar and diaminopimelic acid determination, the washed whole cell preparation was hydrolysed (Becker *et al.*, 1965) and chromatographed on microcrystalline cellulose TLC plates (Avicel SF cellulose, Asahi Kasei Co., Japan). Solvents used were pyridine/ethyl acetate/acetic acid/water (36:36:7:21, by vol.) for sugars, and methanol/pyridine/HCl (10 M)/water (80:10:25:17.5, by vol.) for diaminopimelic acid. After 7 to 8 h development, the dried chromatograms were sprayed with aniline phthalate and ninhydrin, respectively.

Determination of optimum pH for growth. The range of pH allowing optimum growth was determined by the incorporation of [^3H]adenine into actinomycete cells. The reaction mixture contained 2 ml of the following medium (g l^{-1}): L-asparagine, 1; glycerol, 10; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.01; K_2HPO_4 , 0.1; 2 ml Good's buffer (Good *et al.*, 1966) at intervals of 1 pH unit in the range pH 6.0 to 11.0, and 0.2 ml [^3H]adenine ($2 \mu\text{Ci ml}^{-1}$; 74 kBq). The reaction was initiated by adding 0.5 ml spore suspension from the organisms grown on oatmeal agar slants (ISP medium 3) adjusted to pH 10.0. The radioactivity uptake by actinomycetes after 5 h incubation at 27°C , was counted by a Packard Tri-Carb Liquid Scintillation Spectrometer.

Morphological characterization. The morphology of spore chains and spore surface was observed with a Hitachi HU-12 transmission electron microscope, and an Hitachi S-450 scanning electron microscope.

RESULTS AND DISCUSSION

When newly isolated alkalophilic actinomycetes were compared with reference strains such as *Streptomyces lavendulae* ISP 5069, *S. aureofaciens* ISP 5127, *S. antibioticus* ISP 5234, *S. albus* ISP 5313, *S. hygroscopicus* ISP 5578, *S. erythraeus* ISP 5517 and *Streptoverticillium netropsis* ISP 5259, clear differences in the optimum growth pH were observed (Fig. 1). The characteristic features of the isolates were good growth in alkaline media, with optimum activity at pH 9.0 to 9.5, and little or none at pH 6.0 to 7.0. The growth optimum of most *Streptomyces* type cultures tested was around pH 7.0.

When 420 ISP *Streptomyces* strains were tested for growth at pH 6.0 and 11.5, all strains (except for *S. caeruleus* ISP 5103) were able to grow on the medium adjusted to pH 6.0, whereas only six strains, *S. caeruleus* ISP 5103, *Actinomyces* (*Streptomyces*) *alborubidus* ISP 5465, *S. canescens* ISP 5001, *S. autotrophicus* ISP 5011, *S. cavourensis* ISP 5300, and *S. hydrogenans* ISP 5586, grew at pH 11.5. Cell wall analysis of these six ISP streptomycetes revealed that three strains, *S. caeruleus* ISP 5103, *Actinomyces alborubidus* ISP 5465 and *S. autotrophicus* ISP 5011, had *meso*-diaminopimelic acid. Therefore it was decided to study the diaminopimelic acid configuration of the alkalophilic and alkaline-resistant isolates.

Analysis of 20 newly isolated alkalophilic actinomycetes revealed that nine strains had *meso*-diaminopimelic acid. The strains containing *LL*-diaminopimelic acid had glycine, which is one of the characteristic amino acids of *Streptomyces*, but no glycine was detected in those containing *meso*-diaminopimelic acid. No characteristic sugars such as madurose, galactose and arabinose were observed among alkalophilic isolates of actinomycetes containing either *LL*- or *meso*-diaminopimelic acid.

All isolates developed abundant aerial mycelium, frequently some shade of blue, purple or yellow in colour.

Nineteen out of 20 strains belonged to the section *Rectiflexibiles* (Shirling & Gottlieb, 1966), with smooth spore surface, and the remaining one to the section *Retinaculiaperti*, also with smooth spore surface.

Taber (1959, 1960) first reported 24 strains of 'alkalophilic actinomycetes' as acid-sensitive strains, not growing below pH 6.1 to 6.8. However, no further detailed taxonomic studies on the strains were reported. To our knowledge, only two species, *S. caeruleus* and *S. alcalophilus*, both antibiotic producers, have been shown to be alkalophilic. According to the original description, *S. alcalophilus* (Yoshida *et al.*, 1979) contained *LL*-diaminopimelic acid,

Fig. 1. Incorporation of [³H]adenine into three alkalophilic streptomycete strains and three ISP strains of *Streptomyces*. The type of diaminopimelic acid (DAP) in the cell walls of the alkalophilic isolates is indicated.

and our experiment revealed that *S. caeruleus* contained *meso*-diaminopimelic acid in the cell wall.

Although several genera such as *Streptomyces*, *Nocardioides*, *Streptoverticillium*, *Elytrosporangium*, *Microellobosporia*, *Chainia* and *Sporichthya* (Prauser, 1976*a, b*) have been reported to have cell wall type I (LL-diaminopimelic acid) and to bear aerial mycelium, the alkalophilic isolates with LL-diaminopimelic acid resemble *Streptomyces* morphologically. On the other hand, the isolates with cell wall type III (*meso*-diaminopimelic acid and no glycine) showed some similarity to *Nocardioopsis* (Meyer, 1976).

Studies of the pH requirements of the alkalophilic isolates and the six ISP *Streptomyces* strains indicated that they fell into one of two groups. Firstly, there were those which grew well in the range from pH 8.0 to 11.5 with optimum growth around 9.0 to 9.5; these could be designated 'alkalophilic actinomycetes'. Secondly, there were strains which grew well around pH 7.0 and still grew at pH 11.5 with an optimum growth pH around pH 7.0; these could be designated 'alkaline-resistant actinomycetes'. According to this distinction, the new isolates were alkalophilic, while the five ISP strains were alkaline resistant, with the exception of *S. caeruleus* ISP 5103, which grew in the range from pH 6.0 to 11.5.

Maximum incorporation of adenine across the membrane of alkalophilic actinomycetes occurred between pH 9.0 and 9.5. This suggested that their membrane may exhibit alkalophilic properties when adenine is used as a substrate.

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