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Identification of an Alkaliphilic Actinomycetes Producing PrpSc-degrading Enzyme

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Summary

A novel alkaliphilic actinomycetes strain TOA-1 producing PrpSc-degrading keratinase was isolated from the surface of bathroom tile joints. The strain produced substrates and aerial mycelis. Growth occurred at a pH range of 7.0 - 12.0, with an optimal pH at 10.0. TOA-1 contained *meso* -diaminopimelic acid, no diagnostic sugars, iso-, anteiso-, 10-methyl-branched fatty acids with 16-18 carbon atoms. Various organic substrates were used for growth, and various alkaline enzymes were produced. All these characteristics and analysis of 16S rDNA indicated that the strain belonged to the genus *Nocardiopsis*, but genetically differed from the other *Nocardiopsis* species.

Key words: *Nocardiopsis* sp., alkaliphilic, prion, keratinase

Introduction

Prion diseases are characterized by the conversion of the normal cellular form of the prion protein (PrpC) into an insoluble, protease-resistant abnormal form (PrpSc) [15, 22]. There have been some reports on PrpSc-degrading enzymes, and it has been shown that the keratinase from *Bacillus licheniformis* [8] and the subtilisin-enzyme Properase [9] needed additional chemical and physical treatments to degrade PrpSc. Only the alkaline protease E77 from *Streptomyces* sp. has been reported to be able to degrade PrpSc without further treatment [2, 3].

In a previous paper, we reported the isolation of a keratinase (NAPase) from the alkaliphilic actinomycetes strain TOA-1 from the surface of bathroom tile joints [11, 12]. This enzyme can efficiently degrade PrpSc, as does E77 [13]. In the present study, a novel alkaliphilic actinomycetes

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strain TOA-1 was identified using a polyphasic approach, and is believed to be a novel species of the genus *Nocardiopsis*.

Materials and Methods

Strain and culture conditions

For morphological studies, strain TOA-1 was grown on AWA medium containing 10 g/l glucose, 5 g/l peptone, 1 g/l K_2HPO_4, 0.5 g/l $MgSO_4 \cdot 7H_2O$, 15 g/l agar, and 10 g/l Na_2CO_3. Sodium carbonate was separately sterilized, and added to the medium. For physiological studies and chemotaxonomic analyses, strain TOA-1 was grown on AW medium consisting of AWA but without agar. For chemotaxonomic analysis, cells were collected by filtration, washed twice with distilled water, and freeze dried.

Morphology

Strain TOA-1 was cultivated on AWA medium, and was microscopically observed for sporophores, spore chains, and spore surfaces using light and scanning electron microscopy (HITACHI, S-4700). Culture characteristics were studied on ISP media [18].

Physiology

All physiological tests were performed at 30°C, and at pH 9.5-10.0 unless otherwise specified. Production of melanoid pigments was tested on ISP 7 plates [18]. Carbon source utilization was examined using ISP 9 as basal medium [18], supplemented with a final concentration of 1% of the tested carbon source. Degradations of casein, gelatin, starch, and slim milk were detected in modified Bennett's agar medium (MBA) [23]. Effects of temperature, and pH on growth and tolerance to salt were determined using MBA as basal medium. To investigate the ability of strain TOA-1 to produce extracellular alkaline enzymes, plate assays were carried out using alkaline media with various substrates [14].

Chemotaxonomy

Amino acid and sugar analyses of whole cell hydrolysates were performed as described by Hasegawa *et al.* [1] and Staneck and Roberts [20]. Fatty acid methyl esters were prepared from 50 mg wet cells. Mixtures of fatty acid methyl esters were analyzed by capillary GC, using a Hewlett Packard gas chromatograph (model 5898) run using the MIDI system [17].

16S rDNA analysis

Genomic DNA of TOA-1 was extracted by the methods of Murray and Thompson [4], and 16S rDNA was amplified by PCR using the following oligonucleotide primers: 5'-AGAGTTTGATC CTTGGCTCAG-3' and 5'-GGTTACCTTGTTAAGACTT-3' [10]. PCR was performed using a DNA thermal cycler (Biometra) according to the following amplification profile: 98°C (5 min) followed by 30 cycles at 95°C (1 min), 52°C (40 s), and 72°C (2 min). *Taq* DNA polymerase (0.5 U, Promega) and 100 ng genomic template DNA were used with 50 pmol of each primer per 50 μl reaction volume. PCR products of 16S rDNA were directly sequenced by the dideoxy-chain termination method, as described by Sanger *et al.* [16] using a Thermo Sequence kit (Amersham Biosciences) and a DNA sequencer 4000L (Li-Cor). Sequence homologies were analyzed using BLASTP. Phylogenetic tree analysis was performed using the Clustal X software [21]. The tree was

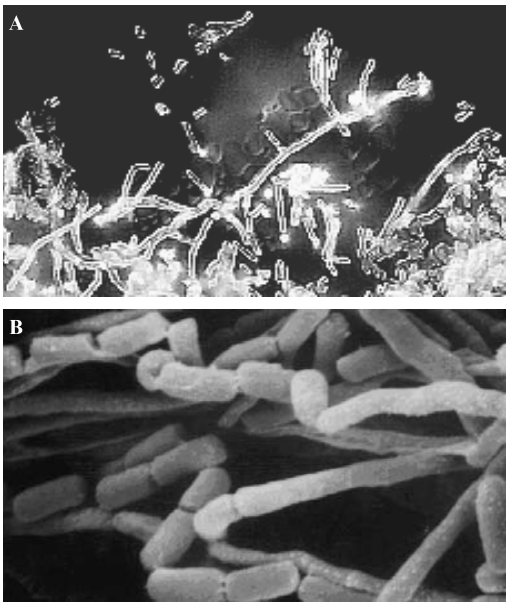

Figure 1. Micrographs of a sporulating aerial mycelium of strain TOA-1. (A) Light micrograph (×1,000). (B) Scanning electron micrograph (×10,000).

rooted using *Bacillus subtilis* as outgroup.

Results and Discussion

Morphological characteristics

The substrate mycelium was colorless. The spore contained a straight chain of 10 to 50 components, and its surface was smooth and white (Fig. 1). No soluble pigments were produced on any medium. These morphological characteristics were consistent with those described for *Nocardiopsis* species [5].

Physiological characteristics

Growth occurred at a pH range of 7.5 - 13, with optimal growth at pH 10.0. Temperature range for growth was 15 - 40°C; with optimal growth at 30°C. No melanoid pigments were produced. Arabinose, xylose, glucose, fructose, sucrose, raffinose, manittol, rhamnose, and inositol were used as good carbon sources. Strain TOA-1 could degrade casein, gelatin, starch, and skim milk, and formed large clear zones on plates containing starch, carboxymethyl cellulose, chitin, Tween 80, pectin, casein, and xylan, indicating that this strain produced at least 7 alkaline hydrolytic enzymes such as amylase, cellulase, chitinase, lipase, pectinase, protease, and xylanase.

Chemotaxonomic characteristics

Whole cell hydrolysates contained *meso*-diaminopimelic acid as the only diamino acid of the peptidoglycans. Diagnostic sugars such as arabinose, xylose, madurose [6], and rhamnose [7] were not detected. Whole cell fatty acid analyses of strain TOA-1 and the phylogenetically most closely related *N. dassonvillei* revealed that the major fatty acids were iso-C16:0, antiiso-17:0, and 10-

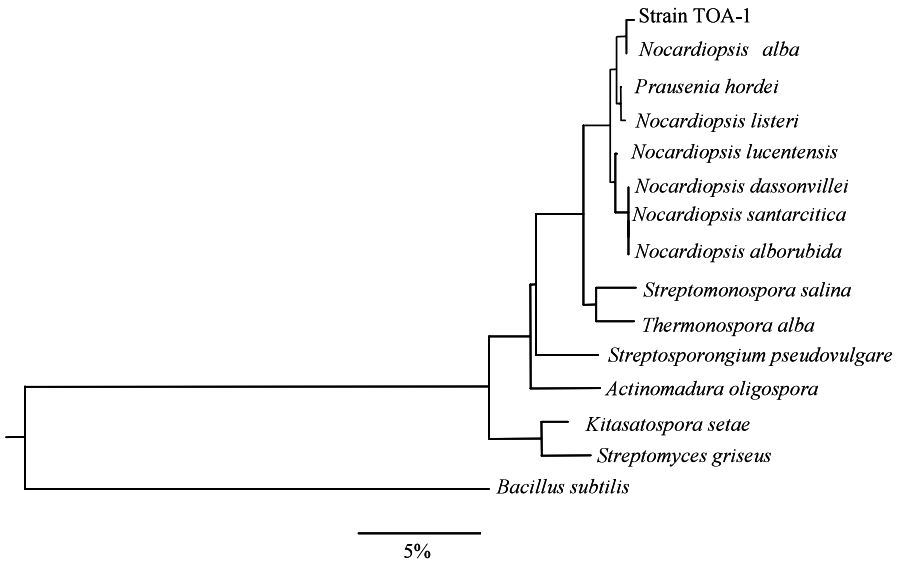

Figure 2. Phylogenetic tree depicting the relationship of TOA-1 to other related organisms based on 16S rDNA sequences. *Bacillus subtilis* was incorporated as outgroup.

methyl-C18:0. All these characteristics were typical of the genus *Nocardiopsis* [5].

16S rDNA analysis

The almost complete 16S rDNA sequence of strain TOA-1 which was 1515 bp, was determined. Preliminary comparison of the sequence against those in GenBank indicated that members of the genus *Nocardiopsis* were the closest phylogenetic neighbors. Pairwise similarity values of > 97% were also found for *N. alba* DSM43845^T (97.6%), *Prouseria hordei* (97.3%), *N. histeri* (97.2%), *P. antarcica* DSM43377^T (97.0%), and *N. lucentensis* (97.0%). These 16S rDNA sequence similarity values were less than the similarity values between closely related *Nocardiopsis* species such as *N. alba* and *N. listeri* (98.7%), *N. dassonvillei* and *N. santalcica* (98.4%). The phylogenic tree is shown Fig. 2. The closest phylogenetic neighbor of strain TOA-1 was *N. alba* DSM43845^T. These data indicated that strain TOA-1 probably belonged to a novel species. However, sequence similarity values of ≧ 97% were reported to be of limited usefulness in species differentiation, and DNA paring studies remain to be performed to confirm species affiliation [19]. Determinations of G+C content and DNA-DNA similarities are currently in progress.

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