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Isolation of Actinomycetes Producing Extracellular Adenosin Deaminase

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세포외 Adenosine Deaminase를 생산하는 방선균의 분리

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ABSTRACT: Two strains of actinomycetes producing extracellular adenosine deaminase, strain J-845S and strain J-326TK, were isolated from soil. Strain J-845S was gram-positive and non-acid-fast. This strain formed whitish, rod-shaped, smooth and non-motile spores on the aerial mycelium, and the spore chain was spiral. The hyphae of the mycelium branched abundantly. Cell wall chemotypes of the strain were of type I containing LL-diaminopimelic acids, and of phospholipid type II, and then strain J-845S was designated as *Streptomyces* sp.. Strain J-326TK was gram-positive and non-acid-fast. The hyphae of primary and aerial mycelium fragmented into irregular rod or coccus-like elements. The aerial mycelium either did not branch or sparsely branched. Cell wall composition was of type I and phospholipid type I. Thus, strain J-326TK was identified as *Nocardioides* sp.

KEW WORDS □ **Extracellular adenosine deaminase, *Streptomyces* sp., *Nocardioides*, sp.**

Adenosine deaminase (adenosine aminohydrolase, EC 3.5.4.4) plays an important physiological role in the purine metabolism. This enzyme catalyzes the deamination of adenosine and deoxyadenosine to inosine and deoxyinosine, respectively. In man, an absence of the enzyme activity is associated with one form of severe combined immunodeficiency disease (Giblett *et al.*, 1972). The enzyme has been identified and characterized from a variety of sources including amphibians (Ma and Fisher, 1968a), birds (Hoagland and Fisher, 1967; Ma and Fisher, 1968b), and mammals (Akedo *et al.*, 1972; Brady and O'Connel, 1962; Ma and Fisher, 1966; Murphy *et al.*, 1969). However, there are a few reports on the enzyme from microbes (Bauer *et al.*, 1973; Gabellieri *et al.*, 1986; Pickard, 1975; Sakai and Jun, 1978), moreover, little information has been reported about the enzyme of actinomycetes. The studies on the microbial extracellular adenosine deaminase have not been published except those of *Aspregillus oryzae* (Minato *et al.*, 1965; 1966).

Actinomycetes have had a long history of usefulness to mankind. In nature, they degrade waste materials and play an integral part in the recycling of materials in nature. Of greatest importance of actinomycetes has been production of antibiotics and antitumor agents of use in medicine and agriculture. A large number of actinomycete enzymes are of current or potential interest in areas of biotechnology such as enzyme processes, clinical chemistry and medical theraphy (Demain, 1988).

The authors have been interested in purine metabolism of actinomycetes (Jun *et al.*, 1987a; 1987b). Therefore, to attempt a systematic study of extracellular adenosine deaminase in bacteria, as a first step, we isolated two strains of actinomycetes producing extracellular adenosine deaminase from soil. In this paper, we describe the taxonomic characteristics and identification of the isolates.

MATERIALS AND METHODS

Chemicals

Adenosine and inosine were purchased from Sigma Chemical Co., St. Louis MO (U.S.A.). All other chemicals were reagent grade.

Soil

The soil samples for the screening of actinomycetes were collected from the areas of Pusan, Kyungsang-Do, Chulla-Do and Chungcheung-Do. After the soil samples were dried under natural condition, they were used for the experiments.

Screening of actinomycetes

For the screening of actinomycetes, 0.5g of soil was suspended in 5 m*l* of sterilized water and a loopful of suspension was streaked on the agar medium consisting of 1.0% soluble starch, 0.05% K_2HPO_4 and 0.05% NH_4Cl. The plates were incubated at 30 °C for 5 to 7 days. The colonies of actinomycetes were selected and maintained on the agar slants composed of 1.0% glucose, 0.2% peptone, 0.15% meat extract and 0.15% yeast extract (pH 7.5, before sterilization).

Selection of the organisms producing extracellular adenosine deaminase

The isolated actinomycetes were transferred to 5 m*l* of the media composed of 3.0% glucose, 0.2% meat extract, 0.2% polypeptone, 0.05% KH_2PO_4 and 0.01% $MgCl_2 \cdot 6H_2O$ (pH 7.3). The cells in a test tube (23 × 200 mm) were incubated at 30 °C with reciprocation for 24 to 48 hrs. After incubation, the culture broth was centrifugated at 10,000 rpm for 10 min, and the resultant supernatant was used as the crude enzyme preparation.

The degradation of adenosine by the crude enzyme solution of the isolated strains was investigated using paper chromatographic analysis. The reaction mixture contained 5 mM adenosine, 50 mM potassium phosphate buffer (pH 7.0) and the crude enzyme solution in a total volume of 1 m*l*. It was incubated at 37 °C for 30 min. After incubation, 50 μl of the reacted solution was spotted on Whatman No. 1 filter paper. A spot of each 50 μl of 5 mM adenosine and inosine was put on the paper as reference standards. The paper was developed in the distilled water which was adjusted to pH 10 with 1N-NH_4OH. The formation of inosine was detected under UV lamp (Mineralight lamp, U.S.A.). The strains producing extracellular adenosine deaminase were purified again on a agar plate and used for this study.

Taxonomical studies

Morphological characteristics of the isolated strains were observed by means of optical and scanning electron microscope. Cultural and physiological characteristics were tested based on the methods of Shirling and Gottlieb (1964) and Pridham and Gottlieb (1948). The inoculation of the organisms was done by the technique of Pridham and Lyons (1980). In the study of cell wall composition, diamino acids of the cell wall were determined by the methods of Becker *et al.* (1964), the sugars were determined using the methods of Lechevalier and Lechevalier (1970). Phospholipids from whole cells were extracted by the techniques of Bligh and Dyer (1959), and analyzed by thin layer chromatography (Kates, 1972; Lechevalier and Lechevalier, 1980). The isolated strains were identified according to 'Bergey's Manual of Determinative Bacteriology' (Buchanan *et al.*, 1974), 'Bergey's Manual of Systematic Bacteriology' Vol. 2 (Sneath *et al.*, 1986) and Vol. 4 (Williams *et al.*, 1989), and the paper of Shirling and Gottlieb (1968).

RESULTS AND DISCUSSION

The selection of the microorganisms

About 1,500 strains of actinomycetes were isolated from soil and 5 strains of them were found to produce extracellular adenosine deaminase. Among these 5 strains, strain J-845S and strain J-326TK produced a large amount of extracellular adenosine deaminase and they had apparent differences in their morphological and cultural characteristics. And they were selected for the further experiments of this study.

Taxonomical characterization of strain J-845S

Morphological characteristics

The hyphae of the primary mycelium grew on the agar surface and penetrated the agar and branched abundantly. The aerial mycelium at maturity formed chains of 30 to 50 spores. The spore was rod-shaped, 0.3 to 0.4 um in diameter and 1.0 to 1.3 μm in length, and the chains of spores formed spirals (Fig. 1, A). The surface of spores was smooth (Fig. 1, B).

Cultural characteristics

The organism grew on the standard media used for the cultivation of bacteria including actinomycetes (Table 1). Colonies appeared 1 or 2 days after incubation at 30 °C and developed quickly. The whitish aerial mycelium formed usually good on the tested media, however, it did not form on nutrient agar and peptone-yeast extract-iron agar. Substrate mycelium was yellowish white, gray, or brown in some media.

Biochemical characteristics

The organism was gram-positive and non-acid-fast. The hydrolysates of cell wall contained LL-diaminopimelic acid, and no readily detectable arabinose,

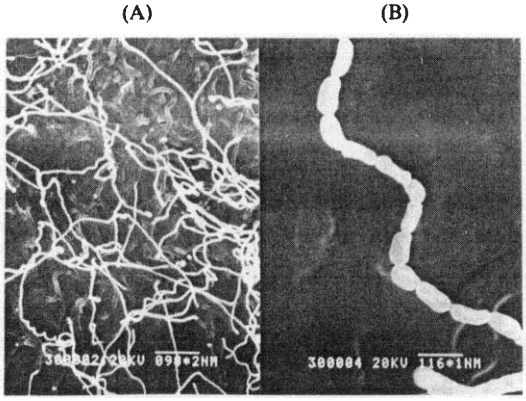

Fig. 1. *Electron microphotography of aerial mycelium (A) and spore chain (B) of strain J-845S on oatmeal agar after 4 days culture at 28°C.*

galactose, xylose and madurose, and so cell wall composition was of type I according to the classification of Lechevalier and Lechevalier (1970). Phosphatidylethanolamine of diagnostic phospholipid was present in the cell wall but phosphatidylcholine was lacking, and it belonged to phospholipid type II (Lechevalier *et al.*, 1981).

Physiological characteristics

The optimum temperature for growth was 25 °C to 30 °C; growth was also observed at 11 °C and 38 °C. Melanoid pigments were produced on peptone-yeast extract-iron agar. Other physiological properties were listed on Table 2. L-Arabinose, D-glucose and D-galactose were well utilized by the strain as carbon sources. However, no growth of the culture was observed on rhamnose, sucrose and inositol (Table 3).

Identification

In comparison with the description of 'Bergey's Manual of Determinative Bacteriology' (Buchanan *et al.*, 1974) and 'Bergey's Manual of Systematic Bacteriology' Vol. 4 (Williams *et al.*, 1989) and the paper of Shirling and Gottlieb (1968), the various properties of strain J-845S were similar to those of *Streptomyces bobili.* However, strain J-845S was found to be different from *S. bobili* in their ability of carbon utilization. Unlike *S. bobili,* strain J-845S utilized salicine but could not utilize rhamnose, inositol and sucrose. From these results, strain J-845S was identified to be a *Streptomyces* sp. and named *Streptomyces* sp. J-845S.

Taxonomical characterization of strain J-326TK

Morphological characteristics

The hyphae of the primary mycelium grew on the agar medium and fragmented into irregular rod or coccus-like elements (Fig. 2, A). The hyphae of the aerial mycelium, 0.4 um to 0.6 um in diameter, either did not branched or sprsely branched (Fig. 2, B), and

Table 1. *Cultural characteristics of strain J-845S*

Medium	Growth	Aerial mycelium	Reverse side	Soluble pigment
Sucrose-nitrate agar (Waksman meidum No. 1)	poor	white	white	none
Glucose-asparagine agar (waksman medium No. 2)	abundant	pale pinkish white	white to pale yellow	none
Glycerol-asparagine agar (ISP No. 5)	abundant	white, cottony	light yellow	none
Tyrosine agar (ISP No. 7)	abundant raised wrinkled	white	dark brownish gray	none
Inorganic salts-starch agar (ISP No. 4)	moderate	grayish white	yellowish gray	none
Nutrient agar	moderate	none	light brownish gray	none
Yeast ext.-malt-ext. agar (ISP No. 2)	moderate	white(partially)	yellowish brown	none
Oatmeal agar (ISP No. 3)	abundant	pinkish white	light yellow	none
Peptone-yeast ext.-iron agar (ISP No. 6)	moderate	none	dark brownish gray	purplish black

fragmented into irregular rod or spore-like elements with smooth surfaces (Fig. 2, C), thus resembling the structure of the aerial mycelium of nocardiae. The sheath which was a characteristic for streptomyceae was absent.

Cultural characteristics

The colonies of the strain was hard and whitish to faint yellowish-brown, and had wrinkled and bright surfaces. The formation of substrate mycelium was usually good, while the aerial mycelium was poor. A very thin, chalky aerial mycelium appeared a few days after the colonies became visible on some media. Soluble pigments were produced on peptone-yeast extract-iron agar, tyrosine agar and skim milk agar (Table 4).

Biochemical characteristics

Strain J-326TK was gram-positive and non-acid-fast. Diagnostic amino acid of the cell wall was LL-diaminopimelic acid. Arabinose, galactose, xylose and madurose were not detected. These results were in accord with cell wall type I (Lechevalier and Lechevalier, 1970). Both phosphatidyl-ethanolamine and phosphatidylcholine were absent in the cell wall, and it belonged to phospholipid type I (Lechevalier *et al.*, 1981).

Table 2. *Physiological properties of strain J-845S*

Test	Results
Temperature range of growth	11-38 °C
	(Opt. temp. 25-30 °C)
Liquefaction of gelatin	positive
Hydrolysis of starch	positive
Coagulation of milk	negative
Peptonization of milk	positive
Melanin formation	positive
	(ISP No. 6 medium)
Nitrate reduction	positive
Catalase	positive

Table 3. *Utilization of carbon sources by strain J-845S*

Carbon source	Utilization
L-Arabinose	+
D-Xylose	+
D-Glucose	+
L-Rhamnose	–
D-Galactose	+
Sucrose	–
D-Mannitol	–
Raffinose	+
Inositol	–
Salicin	+
D-Fructose	+

+, positive utilization; –, negative utilization.

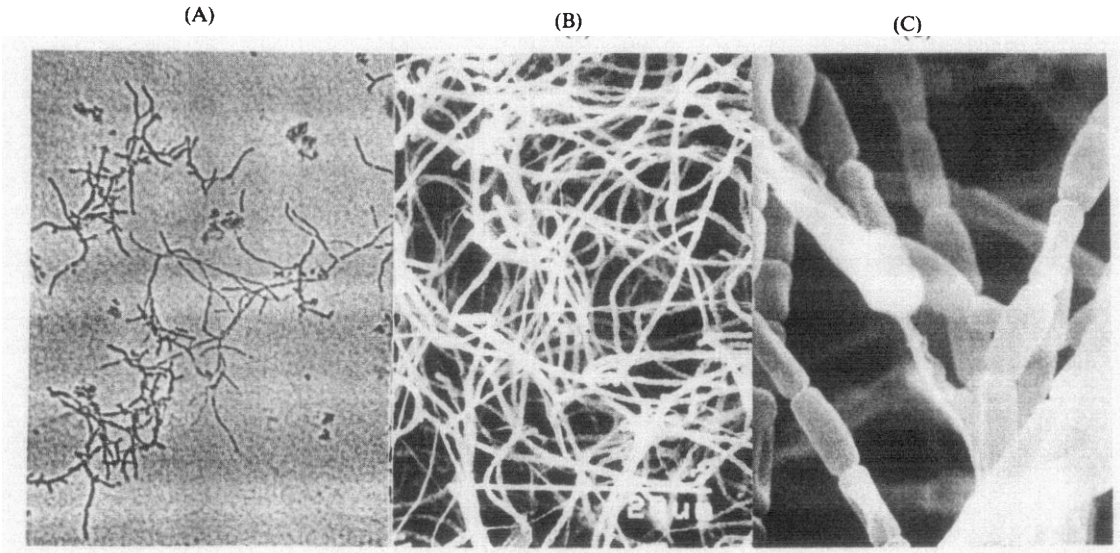

Fig. 2. *Microphotography of substrate mycelium of strain J-326TK on glucose-aspergine agar after 7 days culture at 37°C (A). Electron microphotography of aerial mycelium (B) and sporelike elements (C) of the strain on inorganic salts-starch agar after 14 days culture at 30°C. Mycelial fragmentations were observed.*

Table 4. *Cultural characteristics of strain J-326TK*

Medium	Growth	Aerial mycelium	Substrate mycelium	Soluble pigment
Sucrose-nitrate agar (Waksman medium No. 1)	poor	none	white	none
Glucose-asparagine agar (Waksman medium No. 2)	moderate	whitish yellow	whitish yellow	none
Glycerol-asparagine agar (ISP No. 5)	moderate	white	yellowish white	none
Tyrosine agar (ISP No. 7)	abundant	none	brown	dark brown
Inorganic salts-starch agar (ISP No. 4)	abundant	white	whitish yellow	none
Nutrient agar	moderate	none	whitish yellow	none
Yeast ext.-malt ext. agar (ISP No. 2)	abundant	none	brown	none
Oatmeal agar (ISP No. 3)	poor	white	whitish yellow	none
Peptone-yeast ext.-iron agar (ISP No. 6)	moderate	none	gray	dark brown
Skim milk agar	moderate	none	yellow	yellow
Starch agar	abundant	none	yellow	none

Physiological characteristics

The organism was aerobic and mesophilic. The optimum temperature for growth was about 30°C. Starch was hydrolyzed. Melanoid pigments were formed on some media. H_2S was produced (Table 5). Mannose, arabinose, cellobiose, galactose, melobiose, fructose, glucose and dextrin were utilized for growth, but rhamnose, sucrose and mannitol were not (Table 6).

Identification

The hyphae of the aerial mycelium of strain J-326TK either did not branch or sparsely branched, thus resembling the structure of the mycelium of nocardiae, while the developed sporelike elements were similar to streptomycetes. However, the spores were not produced in or on special parts of the mycelium as in the rule in streptomycetes. In streptomycetes, the cell wall composition was type I and phospholipid type II. On the other hand, cell wall chemotypes of nocardiae were of type IV and phospholipid type II. Unlike streptomycetes and nocardiae, the strain was cell wall type I and phospholipid

Table 5. *Physiological characteristics of strain J-326TK*

Test	Result
Decarboxylase test	positive
Indol formation	negative
Catalase	positive
Methyl red test	negative
Voges-Proskauer test	negative
Gelatin liquefaction test	negative
Urease test	negative
Oxidase test	negative
Hydrolysis of starch	positive
Melanin formation	positive(ISP No. 6, No. 7, trypton-yeast ext. broth)
Nitrate reduction	positive
Litmus milk	negative
Casein hydrolysis	negative
H_2S formation	positive
NaCl tolerance	not inhibited 5% NaCl

Table 6. *Utilization of carbon sources by strain J-326TK.*

Carbon source	Utilization	Carbon source	Utilization
L-Rhamnose	−	Melobiose	+
Sucrose	−	Mannitol	−
D-Mannose	+	D-Fructose	+
Inuline	−	Sorbose	−
Arabinose	+	Glucose	+
Trehalose	±	Inositol	−
Raffinose	±	Saliacin	±
Cellubiose	+	Maltose	±
Xylose	±	Dextrin	+
Galactose	+		

+, positive utilization; −, negative utilization; ±, doubtful utilization.

type I. From these cell wall chemotypes and morphological characteristics, strain J-326TK was found to place in the genus *Nocardioides*. Since the genus *Nocardioides* was first proposed by Prauser (1976), a few species containing *Nocardioides albus* and *N. luteus* have been described (Sneath *et al.*, 1986). The study organism was identical with *N. albus* and *N. luteus* in morphological characteristics and cell wall chemotypes. However, it was found to be different from them in its carbon utility and ability of melanoid pigments formation. In contrast to *N. albus* and *N. luteus*, strain J-326TK produced melanoid pigments and did not use rhamnose, sucrose and mannitol. Thus we suggested the name *Nocardioides* sp. J-326TK for the isolate.

적 요

토양으로부터 세포외 adenosine deaminase를 생산하는 두종의 방선균 J-845S주와 J-326TK 주를 분리하여 각각 *Streptomyces* sp. J-845S와 *Nocardioides* sp. J-326TK로 동정하였다. *Streptomyces* sp. J-845S는 그람염색 양성, 비항산성균으로 기균사의 흰색계열의 간상형의 비운동성의 포자를 형성하였으며, 포자의 표면은 평활하였고 나선상의 포자연쇄를 형성하였다. 균사체의 분지는 양호하였다. 세포벽 구성성분을 분석한 결과 LL-diaminopimelic acid를 함유하는 cell wall type I이었으며, phospholipid type II였다. *Nocardioides* sp. J-326TK는 그람염색반응 양성, 항산성염색반응 음성이었으며, 균사체는 불규칙한 간상 또는 구상의 절편으로 분절되었다. 기균사의 분지는 그다지 인정되지 않았으며, 포자형태의 긴 elements의 표면은 평활하였다. 세포벽 구성성분을 검토한 결과 cell wall type I과 phospholipid type I으로 나타났다.

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