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Identification of Adenosine Deaminase Inhibitor-producing Bacterium Isolated from Soil

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An adenosine deaminase inhibitor-producing bacterium was isolated from soil. An isolate exhibiting high adenosine deaminase inhibitory activity, was designated J-89, and classified as a strain of *Bacillus subtilis* on the basis of its morphological, phenotypic characteristics, the menaquinone content and cellular fatty acid composition. To confirm the taxonomic position of the strain we need more information such as DNA-DNA homology and other chemotaxonomic characteristics. In this paper we provisionally named strain J-89 as *Bacillus* sp. J-89 pending further chemotaxonomic study and analysis of adenosine deaminase inhibitor.

Adenosine deaminase (ADA), present in virtually all mammalian cells and a key enzyme in purine metabolism, catalyzes the irreversible hydrolysis of adenosine and 2'-deoxyadenosine to inosine and 2'-deoxyinosine, respectively. ADA plays a key role in a variety of biological processes (21) and is particularly crucial in the development of the lymphoid system (16). Lack of the enzyme is associated with severe combined immunodeficiency disease(SCID) (16), which makes ADA a paradigm for structure-function studies of a genetic disease. The first clinical trial of gene therapy was carried out on a patient with SCID caused by a defect in the ADA gene.

ADA inhibitors have helped enormously in understanding the mechanism and action of adenosine metabolites and their analogs. ADA inhibitors have also enabled us to understand the regulatory processes associated with immunodeficiencies caused by a lack of ADA, and to further understand the maturation of the immune response (8).

ADA is also a well-studied model for catalytic function through binding numerous inhibitors (6, 17, 22), including ground-state and very potent transition-state analogs. Potent inhibitors of ADA have evolved from isolated natural products (8), including coformycin and 2'-deoxycoformycin (1). Coformycin produced by *Streptomyces kaniharaensis* and 2'-deoxycoformycin produced by *Aspergillus nidulans* were purified by Umezawa's laboratory and by Parke-Davis investigators, respectively (18, 23). Several ADA inhibitors, which are known as purine analogues, have been studied in various microorganisms such as *Actinomycetes* and fungi. However, no ADA inhibitor from bacteria has been reported.

In this study, an ADA inhibitor producing bacterium was isolated from soil and its taxonomic position was studied.

MATERIALS AND METHODS

Isolation of the ADA Inhibitor-producing Bacterial Strain

The soil samples obtained from an area of Pusan were suspended in sterilized water and inoculated onto isolation medium composed of soluble starch 10 g/l, KH_2PO_4 0.5 g/l, NH_4Cl 0.5 g/l and agar 16 g/l. The isolated microorganisms were reselected on ADA inhibitor production medium composed of the following in 1,000 ml of distilled water; glucose 10 g; yeast extract 2 g; meat extract 2 g; polypeptone 2 g; KH_2PO_4 0.5 g and $MgCl_2 \cdot 6H_2O$ 0.1 g adjusted to pH 7.3 before sterilization. A colony from the isolation medium was inoculated into a test tube (2.4×21 cm) containing 10 ml of the inhibitor production medium. Fermentation was carried out at 30°C for 24 h on a reciprocal shaker. Culture supernatant was collected by centrifugation at 10,000 rpm for 20 min, and tested for inhibitory activity against ADA. The isolated strain which showed the highest inhibitory activity was selected and used for further studies. Stock culture of the strains was maintained on nutrient agar (Difco Laboratories, Detroit, MI) slants and transferred at one month intervals.

Bacillus subtilis type strain IAM 12118^T (T=type

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strain) was used for a reference strain. The strains with an IAM number were provided by the Institute of Molecular and Cellular Biosciences, the University of Tokyo (Tokyo, Japan).

Preparation of ADA Enzyme

Calf intestinal ADA (type VIII; Sigma Chemical Co.) was dissolved in 3 ml of potassium phosphate buffer (pH 7.0). Aliquots (50 µl) were properly diluted and used to determine the amount of inhibitor.

Spectrophotometric Assay of ADA and Inhibitor

ADA activity based on the differential absorptivity of the substrate adenosine and the product inosine was measured by Kalckar's method (13). One unit of enzyme activity was defined as the amount of enzyme required to form 1 µmol of inosine/h per ml under these conditions. The inhibition of ADA by the inhibitor was determined using the same assay system as for the enzyme. One unit of inhibitory activity was defined as that inhibiting 50% of the enzyme activity in the standard assay system.

Identification Methods

One- to two-day old culture on nutrient agar incubated at 30°C was used as the inoculum unless otherwise specified. Methods and procedures described in the Manual of Methods for General Bacteriology (7) and in the Manual for the Identification of Medical Bacteria (5) were usually followed. Gram staining was carried out by the Hucker-Conn modification (11). Cell morphology and motility were observed under a phase-contrast microscope, and spores were stained using malachite green (5). The flagellation and the morphology of vegetative cells and spores were pictured by transmission electron microscope. Anaerobic growth was determined according to Gordon *et al.* (9). Hydrolysis of gelatin was observed in stab culture (Bacto gelatin, 120 g; distilled water, 1,000 ml; pH 7.0). Stab cultures were cooled in a refrigerator at 4°C for 1 h and then liquefaction was investigated. Urease activity was determined on Christensen medium (2) after incubation for 1 week. This test was done for 4 weeks. Catalase activity was detected by the production of bubbles in a 3% hydrogen peroxide solution. Decarboxylase base Moeller (Difco 0890) was used for the examination of arginine dihydrolase and of lysine decarboxylase. Utilization of carbon compounds was tested by using basal media of Iizuka and Komagata (12) at 30°C for 2 weeks with shaking.

Analyses of Quinones and Fatty Acids

Chemotaxonomic analyses of chemical macromolecules, e.g., isoprenoid quinones and cellular fatty acids provide valuable information for classification and identification. Cells were grown in nutrient broth at 30°C, and harvested at the early stationary growth phase. Quinone systems were determined according to the methods of Collins *et al.* (4) and Shin *et al.* (19). For the analysis of fatty acids, harvested cells were freeze-dried. Whole-cell fatty acids were extracted, purified according to a standard protocol (10, 20), and then analyzed by gas-liquid chromatography (Shimadzu GC-14A, Japan) (15).

RESULTS AND DISCUSSION

One of the isolates, J-89 was found to produce an maximum level of ADA inhibitory activity of interest and was used for the taxonomic studies.

Production of ADA Inhibitor of Isolated Strain J-89

The growth curve and time course of ADA inhibitor production are shown in Fig. 1. The cells were grown at 30°C in ADA production medium (pH 7.3) and the ADA production was monitored at 2 h intervals. ADA inhibitory activity appeared when bacterial growth was in the exponential growth phase, and its production increased rapidly after 6 h of growth. The ADA inhibitor production increased in proportion to the growth of the cell, and reached its maximum after 18 h of cultivation.

Phenotypic Characteristics

To identify the taxonomic position of strain J-89 morphological and physiological studies on this strain were performed. This strain was Gram-positive and a motile rod of cell. It formed an oval-shaped, central endospore (Fig. 2A). The cell wall of the vegetative cell was not distended during spore formation and the endospore was enclosed by a loose outer coat of exosporium. The vegetative cell was less than 1.0 µm in width (Fig. 2B) and possessed several peritrichous flagella (Fig. 3).

According to the physiological studies of this strain (Table 1), it could not ferment sugars under anaerobic conditions. Gelatin was liquefied. Urease activity was not observed. The activities of catalase and arginine dihydrolase were found to be present, but not those of lysine decarboxylase. Acid was produced from L-arabinose, D-glucose, D-mannitol and D-xylose. Thus, this strain may

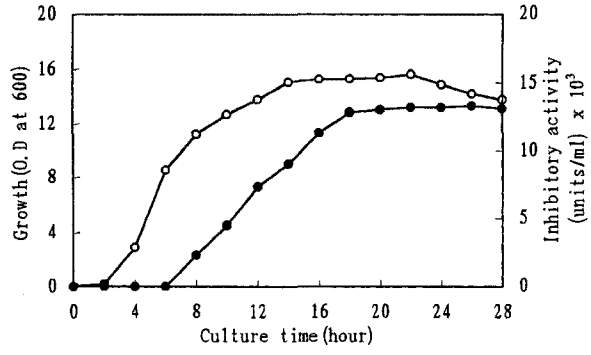

Fig. 1. Time course of growth and ADA inhibitor production of isolated strain J-89.
Cell growth (O.D. 600 nm), —○—; ADA inhibitory activity (units/ml), —●—.

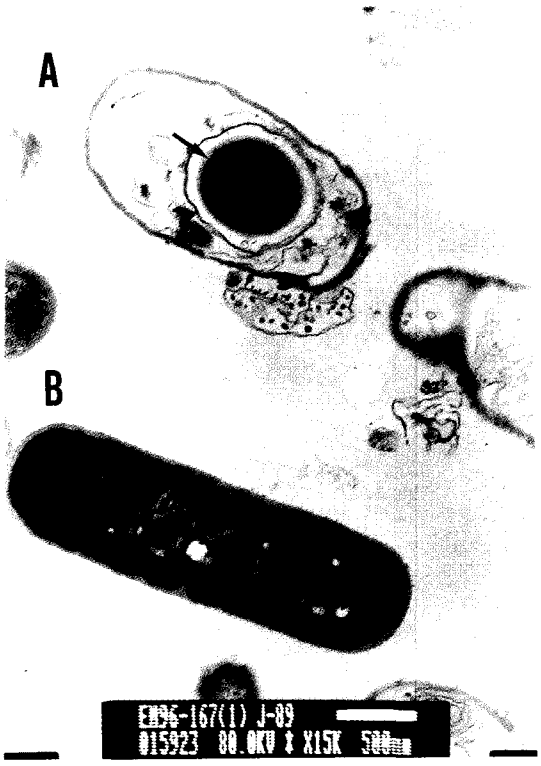

Fig. 2. Transmission electron micrograph (15,000×) of the isolated strain J-89.
A, endospore; B, vegetative cell.

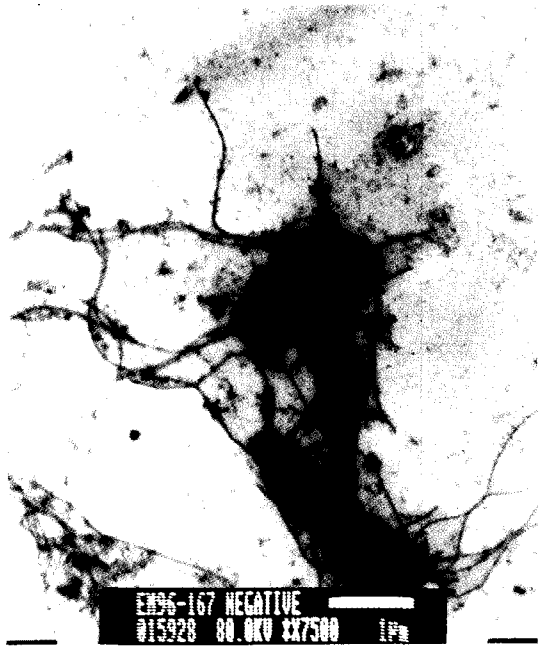

Fig. 3. Electron micrograph of the isolated strain J-89 showing peritrichous flagellation (7,500 ×).

correspond to *Bacillus subtilis* in phenotypic characteristics except for the activity of arginine dihydrolase which is present in *B. subtilis*.

Chemotaxonomic Characteristics

For more information about strain J-89, the chemotaxonomic characteristics of the strain were investigated. Strain J-89 contained menaquinone (MK) as the sole respiratory quinone, with MK-7 representing 99% of the total menaquinone content. The presence of MK-7 is one of the most/more common traits of genus *Bacillus* (3) and supports the results of the phenotypic studies. As cellular fatty acid composition of strain J-89, shown in Table 2, is very similar to characteristic profiles of genus *Bacillus*, having four major fatty acids, C15 : 0 iso, C15 : 0 anteiso, C17 : 0 iso, and C17 : 0 anteiso (14) (Fig. 4). Kampfer (14) proposed the characterization of *Bacillus* species on the basis of the ratio of the quantitatively predominant fatty acids C15 : 0 iso and C15 : 0 anteiso. *B. subtilis* could be characterized by a ratio of C15 : 0 iso/C15 : 0 anteiso ranging from 0.4 to 1.7. The ratio of C15 : 0

Table 1. Phenotypic characteristics and quinone system of J-89 strain.

Characteristics	J-89	*B. subtilus* IAM 12118^T
Gram staining	+	+
Cell shape	rod	rod
Motility	+	+
Endospore formation	+	+
Anaerobic growth	−	−
Hydrolysis of: gelatin	+	+
urea	−	−
Catalase	+	+
Arginine dihydrolase	+	−
Lysine decarboxylase	−	−
Utilization of: L-Arabinose	+	+
D-Glucose	+	+
D-Mannitol	+	+
D-Xylose	+	+
Quinone system	MK-7	MK-7

Table 2. Cellular fatty acid composition of J-89 strain.

Compound[a]	Retention time	Percentage[b]
13;0 anteiso (a)	6.297	4.1
14;0 iso (b)	7.908	t[c]
15;0 iso (c)	9.794	19.2
15;0 anteiso (d)	9.949	33.2
16;0 iso (e)	11.712	3.1
16;0 (f)	12.406	3.2
17;0 iso (g)	13.648	21.1
17;0 anteiso (h)	13.802	15.1
18;0 (i)	16.199	1.0

[a]Cellular fatty acid components are shown as peaks (a~i) in Fig. 3. [b]Data were expressed as percentage of total peak area. [c]Trace (less than 1% of total peak area).

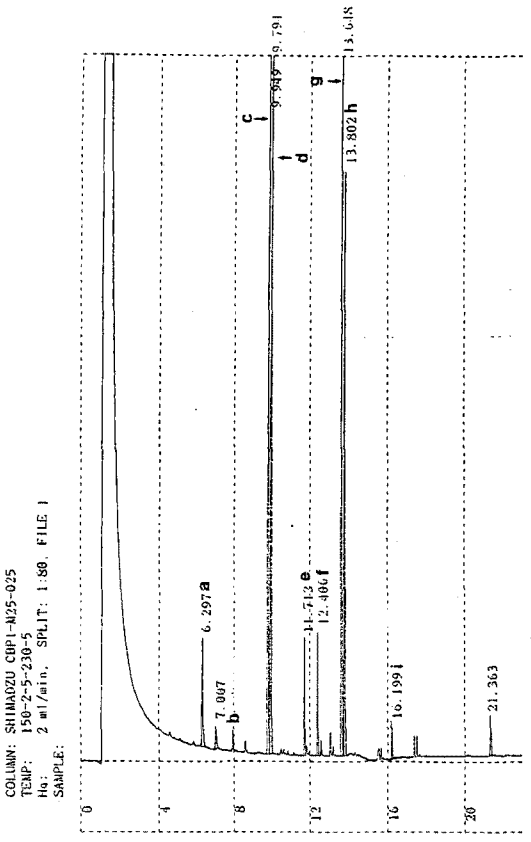

Fig. 4. Gas-liquid chromatogram of cellular fatty acids of the isolated strain J-89.

iso/C15 : 0 anteiso for J-89 showed 0.57.

Therefore, besides the phenotypic characteristics, the menaquinone content and cellular fatty acid composition of strain J-89 were consistent with those of *Bacillus subtilis*. We classified this strain as *Bacillus subtilis* and named it as *Bacillus* sp. J-89. To confirm the taxonomic position of this strain we need more information such as the G+C content, comparison of DNA-DNA homology with the *Bacillus subtilis* type strain and 16S rRNA sequences of *Bacillus* sp. J-89.

At present, we are trying to purify ADA inhibitor produced by *Bacillus* sp. J-89 and analyze its structure. We suspect that this inhibitor might have a novel structure which is different from other known inhibitors.

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REFERENCES

1. Agarwel, R. P., T. Spector, and R. E. Parks, Jr. 1977. Tight-binding inhibitors. IV. Inhibition of adenosine deaminases by various inhibitors. *Biochem. Pharmacol.* **26**: 359-367.
2. Christensen, W. B. 1946. Urea decomposition as a means of differentiating *Proteus* and Paracolon cultures from each other. *J. Bacteriol.* **52**: 461-466.
3. Collins, M. D. and D. Jones. 1981. Distribution of isoprenoid quinone structural types in bacteria and their taxonomic implications. *Microbiol. Rev.* **45**: 316-354.
4. Collins, M. D., T. Pirouz, and M. Goodfellow. 1977. Distribution of menaquinones in actinomycetes and corynebacteria. *J. Gen. Microbiol.* **100**: 221-230.
5. Cowan, S. T. 1974. *Manual for the identification of medical bacteria*, 2nd ed. Cambrige University Press, Cambridge.
6. Frieden, C., L. C. Kurz, and H. R. Gilbert. 1980. Adenosine deaminase and adenylate deaminase: Comparative kinetic studies with transition state and ground state analogue inhibitors. *Biochemistry* **19**: 5303-5309.
7. Gerhardt, P., R. G. E. Murray, R. N. Costilow, E. W. Nester, W. A. Wood, N. R. Krieg, and G. B. Phillips. 1981. *Manual of methods for general bacteriology*. American Society for Microbiology, Washington, D. C.
8. Glazer, R. I. 1980. Adenosine deaminase inhibitor: Their role in chemotherapy and Immunosuppression. *Cancer Chemother. Pharmacol.* **4**: 227-235.
9. Gordon, R. E., W. C. Haynes, and C. H. N. Pang. 1973. *The genus Bacillus* (Agricultural handbook No. 427). Washington, D. C. United States Department of Agriculture.
10. Hiraishi, A., Y. K. Shin, J. Sugiyama, and K. Komagata. 1992. Isoprenoid quinones and fatty acids of *Zoogloea*. *Antonie van Leeuwenhoek* **61**: 231-236.
11. Hucker, G. J. and H. C. Conn. 1923. Method of gram staining. *Technol. Bull.* 93. New York State Agricultural Experiment Station, Ithaca.
12. Iizuka, H. and K. Komagata. 1963. Taxonomy of genus *Pseudomonas* with special reference to their modes of metabolism of carbon compounds. *J. Gen. Appl. Microbiol.* **9**: 83-95.
13. Kalckar, Br H. M. 1947. Differential spectrophotometry of purine compounds by means of specific enzymes. II. Determination of adenine compounds. *J. Biol. Chem.* **167**: 445-459.
14. Kampfer, P. 1994. Limits and possibilities of total fatty acid analysis for classification and identification of *Bacillus* species. *System. Appl. Microbiol.* **17**: 86-98.
15. Kawahara, K., I. Mizuta, W. Katabami, M. Koizumi, and S. Wakayama. 1994. Isolation of *Sphingomonas* strains from ears of rice and other plants of family *Gramineae*. *Biosci. Biotechnol. Biochem.* **58**: 600-601.
16. Kredich, N. M. and M. S. Hershfield. 1989. p. 1045-1075. *In* C. R. Scrivner (ed.), *The Metabolic Basis of Inherited Disease*, McGraw-Hill, New York.
17. Kurz, L. C. and C. Frieden. 1983. Adenosine deaminase:

Solvent isotope and pH effects on the binding of transition-state and ground-state analogue inhibitors. *Biochemistry* **22**: 382-389.

18. Nakamura, H., G. Koyama, Y. Iitake, M. Ohno, N. Yagisawa, S. Kondo, K. Maeda, and H. Umezawa. 1974. Structure of coformycin, an unusual nucleoside of microbial origin. *J. Am Chem. Soc.* **96**: 4327.
19. Shin, Y. K., J. S. Lee, C. O. Chun, H. J. Kim, and Y. H. Park. 1996. Isoprenoid quinone profiles of the *Leclercia adecarboxylata* KCTC 1036^T. *J. Microbiol. Biotechnol.* **6**: 68-69.
20. Suzuki, K. and K. Komagata. 1983. Taxonomic significance of cellular fatty acid composition in some corynebacteria. *Int. J. Syst. Bacteriol.* **33**: 188-200.
21. Wilson, D. K., F. B. Rudolph, and F. A. Quiocho. 1991. Atomic structure of adenosine deaminase complexed with a transition-state analog: understanding catalysis and immunodeficiency mutations. *Science* **252**: 1278-1284.
22. Wilson, D. K. and F. A. Quiocho. 1993. A pre-transition-state mimic of an enzyme: X-ray structure of adenosine deaminase with bound 1-deazaadenosine and zinc-activated water. *Biochemistry* **32**: 1689-1694.
23. Woo, P. W. K., H. W. Dion, S. M. Lange, L. F. Dahl, and L. J. Durham. 1974. A novel adenosine and ara-A deaminase inhibitor, (R)-3-(2-deoxy-β-D-*erythro*-pentofuranosyl) 3,6,7,8-tetrahydoimidazo[4,5-*d*]diazepin-8-ol. *J. Hetero Chem.* **11**: 641.

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