Paenibacillus koreensis sp. nov., a new species that produces an iturin-like antifungal compound

Young Ryun Chung,1 Chung Hwan Kim,3 Inhwan Hwang2 and Jongsik Chun4

A bacterial strain, YC300T, that produces an iturin-like antifungal antibiotic was isolated from compost and identified as member of the genus Paenibacillus. Gram reaction of the strain was variable depending upon growth stages and culture media. Three different types of colonies were developed on tryptic soy agar. The organism was facultatively anaerobic and grew at 50 °C. The DNA G+C content was 54 mol% and anteiso-C15:0 was the major fatty acid. A 0.9 kb fragment was produced by PCR amplification of strain YC300T DNA using primers PAEN515F and 1377R. Levels of 16S rDNA similarity between strain YC300T and other Paenibacillus species were between 89.8 and 94.8%. Phylogenetically, strain YC300T formed a significant monophyletic clade with Paenibacillus validus. It is clear from polyphasic evidence that the isolate should be classified as Paenibacillus koreensis sp. nov., the type strain of which is YC300T ( = KCTC 23935, KCCM 409033).

Keywords: Paenibacillus koreensis, iturin-like antifungal

INTRODUCTION

The genus Paenibacillus was created to harbour a phylogenetically coherent group of aerobic or facultatively anaerobic endospore-forming bacilli on the basis of 16S rRNA analysis (Ash et al., 1993; Shida et al., 1997a). The genus currently contains 24 species and is phenotypically related to other genera belonging to the family Bacillaceae. A highly specific primer, PAEN515F, was recently designed for the detection of 16S rRNA gene of the genus Paenibacillus and used to produce a 0.8 kb PCR amplicon in preparations from the 18 paenibacilli by amplifications in conjunction with primer 1377R (Shida et al., 1997a). The method proved to be useful for identifying and differentiating Paenibacillus species from other members of the family Bacillaceae. More recently, Paenibacillus campinensis, an alkaliphilic species, Paenibacillus dendritiformis, forming a distinctive growth pattern, and Paenibacillus lentimorbus and Paenibacillus popilliae, which cause milky disease in insects, have been introduced as new members of the genus (Pettersson et al., 1999; Tcherpakov et al., 1999; Yoon et al., 1998).

Some members of the genus, including Paenibacillus polymyxa and Paenibacillus thiaminolyticus, are known to produce antibacterial compounds such as polymyxin, octopytin and baciphelacin (Slepecky & Hemphill, 1991). However, no antifungal compound has been reported to be produced by Paenibacillus species so far, although iturins, cyclic peptide antifungals, are produced by several isolates of Bacillus subtilis (Lancini & Lorenzetti, 1993). In addition to the production of antibiotics, several species of Paenibacillus are also known to excrete a wide variety of enzymes that degrade natural biopolymers, such as chondroitin, curdlan and chitin (Claus & Berkeley, 1986; Kanzawa et al., 1995; Nakamura, 1987). A chitinolytic bacterial strain, designated YC300T, was isolated from a compost sample collected from Chinju, Republic of Korea, in the process of screening biological control agents for Rhizoctonia solani, a plant-pathogenic fungus. The strain also showed strong antifungal activity against Fusarium oxysporum, Colletotrichum lagenarium, Sclerotinia sclerotiorum and Botrytis cinerea (Chung, 1997). The taxonomic status of strain YC300T was investigated using a combination of phenotypic, chemical and molecular

Abbreviation: TSA, tryptic soy agar.

The GenBank accession number for the 16S rDNA sequence of strain YC300T is AF130254.

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systematic methods. On the basis of polyphasic evidence, we propose that strain YC300\(^{T}\) be classified in the genus Paenibacillus as Paenibacillus koreensis sp. nov.

**METHODS**

**Bacterial strain.** Strain YC300\(^{T}\) was isolated from a compost sample prepared with rice husk and fishery industrial wastes collected from Chinju, Republic of Korea, using the standard dilution agar plating method. Aliquots of serially diluted soil suspension were inoculated onto minimal salt agar medium containing 0.5 g MgSO\(_4\), 7H\(_2\)O, 0.7 g K\(_2\)HPO\(_4\), 0.3 g KH\(_2\)PO\(_4\), 0.01 g Fe\(_2\)SO\(_4\), H\(_2\)O, 0.001 g ZnSO\(_4\) and 0.001 g MnCl\(_2\) per litre distilled water supplemented with 0.2% (w/v) colloidal chitin prepared from crab shell chitin (Sigma) as sole carbon and nitrogen source (Chung et al., 1989). Bacterial colonies were picked up 3 d following incubation at 28 °C and their inhibitory activities against Rhizoctonia solani and Fusarium oxysporum were determined by paired bioassay on potato dextrose agar (Difco) and 0.1 x tryptic soy agar (TSA; Difco) plates. An isolate, designated YC300\(^{T}\), showing substantial inhibition of mycelial growth of fungi was selected and studied further. The strain was maintained on TSA at 4 °C.

**Morphological and physiological characteristics.** The cells were grown at 28 °C using a fermenter (5 l; Korea Fermentation; aeration: 31 min\(^{-1}\), 300 r.p.m.) containing soybean meal media (SM; 2 g yeast extract, 1 g beef extract, 20 g soluble starch, 25 g soybean meal, 5 g dextrose, 0.5 g K\(_2\)HPO\(_4\), 2 g NaCl, 0.5 g MgSO\(_4\), 7H\(_2\)O, 6 g CaCO\(_3\) per litre distilled water) and sampled after 4, 12, 24 and 48 h. The morphological properties were examined by light microscopy after Gram staining. Vegetative cells and spores were fixed with glutaraldehyde/osmium and after sectioning and staining the specimens were observed by using a transmission electron microscope (Hitachi model H-600). The methods described by Smibert & Krieg (1981) were used for the following physiological tests: oxidase, catalase, indole production, Voges-Proskauer reaction, nitrate reduction, motility and hydrolysis of casein, starch, chitin and chitosan. Carbonhydrate utilization and other tests described below were performed with GNI and BAC cards according to the instructions of the VITEK system (AMS 60; bioMérieux). Growth in the presence of 7% (w/v) NaCl and 0.001% lysozyme, and the temperature range for growth were determined using 0.1 x TSA as basal medium.

**Chemotaxonomy.** The biomass for cellular fatty acid analysis was prepared from a 1-d-old culture grown on a TSA plate at 28 °C. Fatty acid methyl esters were prepared using the method described in the manual of the MIDI Microbial Identification System (Hewlett Packard). The resultant esters were separated using a gas chromatograph (model 5890; Hewlett Packard) fitted with a phenylmethyl silicone fused silica capillary column (25 m x 0.2 mm; Hewlett Packard).

**DNA base composition.** The G + C content of the DNA was determined from the midpoint value of the thermal denaturation profile using a spectrophotometer (Ultraspex 2000; Pharmacia Biotech) equipped with programmable peltier temperature control unit according to the equation of Marmur & Doty (1962) as modified by De Lay (1970).

**16S rDNA sequencing.** 16S rDNA was enzymically amplified using two oligonucleotide primers as described previously (Chung et al., 1999). The sequences of the two primers are 5'-TATGGATCATTCTACGGAGAGTTGATCC-3' and 5'-TATGGATCCACCTTCCGGTACGGTACCC-3' (BamHI sites are indicated by underlining). The temperatures for denaturation, annealing and extension were 94, 55 and 72 °C, respectively. The PCR products were digested with BamHI and cloned into linearized pBluescript II SK(+ ) vector. Overlapping subclones were generated by exonuclease III digestion of the insert DNA. The nucleotide sequences of these clones were sequenced using an automatic sequencer (ABI), according to the manufacturer’s protocol.

**Phylogenetic analysis.** The resultant 16S rDNA sequence of strain YC300\(^{T}\) was manually aligned with representative sequences of paenibacilli and related taxa obtained from the Ribosomal Database Project (Maidak et al., 1997) and GenBank databases, using known 16S rRNA secondary structure information. Phylogenetic trees were inferred by using three treeing algorithms, namely the Fitch–Margoliash (Fitch & Margoliash, 1967), maximum-parsimony (Fitch, 1972) and neighbour-joining (Saitou & Nei, 1987) methods. Evolutionary distance matrices for the neighbour-joining and Fitch–Margoliash methods were calculated according to the model of Jukes & Cantor (1969). The trees were rooted using Alicyclobacillus acidocaldarius (accession no. X60742) and Brevibacillus laterosporus (D16271) as outgroups. The PHYLIP package (Felsenstein, 1993) was used for all analyses. The resultant unrooted tree topology was evaluated in bootstrap analyses (Felsenstein, 1985) of the neighbour-joining method based on 1000 resamplings.

**Paenibacillus specific PCR.** The Paenibacillus-specific primer PAEN515F (5’-GCTGGAGAGTGACGGTACCTGA-3’) and universal primer 1377R (5’-GGCATGCTGATCCCGGATTACTAGC-3’) were applied to isolate YC300\(^{T}\) following the method of Shida et al. (1996, 1997a). The universal forward and reverse primers, 27FC and 1377R, were also used for PCR amplification.

**RESULTS**

**Morphological and physiological characteristics.** Cells grown vegetatively were Gram-positive and rod-shaped, measuring 5-0–9 x 2-3–4.5 µm. Ellipsoidial endospores were produced in swollen sporangia in a fermenter at 28 °C (Fig. 1). During the early and

**Fig. 1.** Electron micrograph of vegetative cell (a), endospore in swollen sporangia (b) and endospore (c) of strain YC300\(^{T}\). Bar, 1 µm.
cells were motile by peritrichous flagella. Strain YC300<sup>T</sup> formed three types of colonies on 0.1% TSA: (i) circular, flat, smooth and opaque; (ii) circular, convex, glistening and translucent; and (iii) circular or irregular, wrinkled hollow and opaque (Fig. 2). The first type was mainly developed. The strain was facultatively anaerobic and grew at 10–50 °C with an optimum growth temperature of 38–40 °C. This strain grew in the presence of 0.001% lysozyme, but not in the presence of 7% NaCl. Oxidase, catalase, arginine dihydrolase and ornithine decarboxylase activities were observed, but not urease or lysine decarboxylase activities. Casein, chitin, chitosan, ascuscin and starch were hydrolysed. Sodium acetate and malonate were utilized, but acetamide and citrate were not. Nitrate was reduced and tests for indole production and Voges–Proskauer reaction were negative. Reactions for the oxidation and fermentation of carbohydrates as sole carbon sources are shown in Table 1. Strain YC300<sup>T</sup> inhibited growth of several plant and human fungal pathogens, such as Rhizoctonia solani, Fusarium oxysporum, Botrytis cinerea, Sclerotinia sclerotiorum, Colletotrichum lagenarium, Magnaporthe grisea, Botryosphaeria dothidea, Candida albicans and Trichophyton mentagrophyte, by producing iturin-like anti-fungal compounds. The active substance is composed of seven amino acids (Glu, Tyr, Ser, Pro, Asp<sub>1</sub>, Asp<sub>2</sub>, Lys) and one β-amino acid, typical components of the iturins (S. S. Moon, personal communication).

### Table 1. Acid production from carbohydrates as sole carbon source by strain YC300<sup>T</sup>

+,-, positive or negative for carbohydrate fermentation; (+), positive for carbohydrate oxidation.

<table>
<thead>
<tr>
<th>Carbohydrate</th>
<th>Reaction</th>
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<tr>
<td>N-Acetyl-D-glucosamine</td>
<td>+</td>
</tr>
<tr>
<td>Amygdalin</td>
<td>-</td>
</tr>
<tr>
<td>Amylopectin</td>
<td>+</td>
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<tr>
<td>L-Arabinose</td>
<td>+</td>
</tr>
<tr>
<td>Adonitol</td>
<td>-</td>
</tr>
<tr>
<td>Arabinol</td>
<td>+</td>
</tr>
<tr>
<td>D-Galactose</td>
<td>+ (+)</td>
</tr>
<tr>
<td>D-Glucose</td>
<td>+ (+)</td>
</tr>
<tr>
<td>Inositol</td>
<td>+</td>
</tr>
<tr>
<td>Inulin</td>
<td>-</td>
</tr>
<tr>
<td>Lactose</td>
<td>+ (+)</td>
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<tr>
<td>Maltose</td>
<td>+ (+)</td>
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<tr>
<td>Mannitol</td>
<td>+ (+)</td>
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<tr>
<td>Palatinose</td>
<td>+</td>
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<tr>
<td>D-Raffinose</td>
<td>+</td>
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<tr>
<td>L-Rhamnose</td>
<td>-</td>
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<tr>
<td>D-Ribose</td>
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<tr>
<td>Salcin</td>
<td>-</td>
</tr>
<tr>
<td>Sorbitol</td>
<td>+</td>
</tr>
<tr>
<td>Sucrose</td>
<td>+</td>
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<tr>
<td>Tagatose</td>
<td>-</td>
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<tr>
<td>D-Trehalose</td>
<td>+</td>
</tr>
<tr>
<td>D-Xylose</td>
<td>- (+)</td>
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</table>

exponential growth stages under well aerated conditions, cells were Gram-positive, but with the onset of spore formation, the cells became Gram-negative. The

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**Fig. 2.** Various colony morphologies of strain YC300<sup>T</sup>. The organism was grown on TSA at 28 °C for 10 d.
analyses based on 16S rDNA sequence data that strain YC300\textsuperscript{T} belongs to the genus \textit{Paenibacillus}. Shida et al. (1997a) reported that a 0.8 kb fragment was produced from paenibacilli using the same primers under the reaction conditions used in the present study. Careful examination of 16S rDNA sequences of all \textit{Paenibacillus} type strains clearly indicated that the primer pair set should yield a fragment in the size range 895 (\textit{Paenibacillus apiarius}) to 907 bp (\textit{P. dendritiformis}). Therefore, the size of the \textit{Paenibacillus}-specific PCR amplicon should be 0.9 instead of 0.8 kb. The genetic uniqueness of our isolate among the paenibacilli was evident, as none of the validly described species showed 95\% or greater 16S rDNA sequence similarity, a level of similarity far less than the borderline for defining bacterial genomic species, i.e. 97\%, proposed by Stackebrandt & Goebel (1994).

The chemotaxonomic data, i.e. G + C content of DNA (54 mol\%) and the anteiso-15:0 fatty acid as the major cellular fatty acid (51:1\%), also fall within the ranges exhibited by \textit{Paenibacillus} species (Shida et al., 1997a, b; Yoon et al., 1998). In a comparison of phenotypic characteristics of strain YC300\textsuperscript{T} with those of phylogenetically closely related \textit{Paenibacillus} species, \textit{P. validus}, \textit{P. chondroitinus} and \textit{P. alginitolyticus}, strain YC300\textsuperscript{T} exhibited several differential properties. Strain YC300\textsuperscript{T} grew anaerobically and had oxidase activity in opposition to those related species. Xylose was not fermented by the isolate. The Gram reaction of YC300\textsuperscript{T} was variable, depending on the growth stage and culture medium, a phenomenon often observed in \textit{Bacillus} species (Claus & Berkeley, 1986). The appearance of colonies was variable depending on culture media and three different colony types were developed on TSA. Each colony produced these various colony types in many repeated experiments. We could not find the culture conditions necessary to induce colonial variation of strain YC300\textsuperscript{T}. Such variations in colony morphology can also be observed in many \textit{Bacillus} species (Claus & Berkeley, 1986). Like other \textit{Paenibacillus} species, including \textit{Paenibacillus alvei}, \textit{Paenibacillus macerans}, \textit{Paenibacillus polymyxa} and \textit{P. thiaminolyticus}, strain YC300\textsuperscript{T} has the ability to degrade many natural biopolymers, such as chitin (Nielson & Sorensen, 1997; Shida et al., 1997a). The isolate has been used as the biological control agent of soil-borne pathogens because of its ability to degrade chitin, a major component of the fungal cell wall (Singh et al., 1999). YC300\textsuperscript{T} produces iturin-like antifungal agents, the structures of which were elucidated by a series of chromatographic and spectroscopic analyses. Some \textit{Bacillus} and \textit{Paenibacillus} species are known to produce antifungal and antibacterial substances, such as bacillomycin and polymyxin (Slepecky & Hemphill, 1991). However, no production of an antifungal by \textit{Paenibacillus} species has been reported to date. To our knowledge, this is the first report of the production of an iturin-like antifungal by \textit{Paenibacillus} species.

**DISCUSSION**

It is evident from the results of \textit{Paenibacillus}-specific PCR amplification and comprehensive phylogenetic

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**Fig. 3.** A rooted neighbour-joining tree based on 1396 unambiguously aligned nucleotide positions, including positions 45–70, 98–1133 and 1141–1431 (\textit{E. coli} numbering; Brosius et al., 1981). The numbers at the nodes exhibit the levels of bootstrap support based on neighbour-joining analyses of 1000 resampled data sets. The scale bar represents 0.1 nt substitutions per position.
strain YC300T be assigned in the genus *Paenibacillus* as *Paenibacillus koreensis* sp. nov.

**Description of *Paenibacillus koreensis* sp. nov.**

*Paenibacillus koreensis* (ko.re.en’sis, M.L. masc. adj. *koreensis* indicating Korea, the geographical origin of isolation).

Cells are rod-shaped, measuring 0.5–0.9 x 2.3–4.5 μm and motile by peritrichous flagella. Ellipsoidal spores are formed in swollen sporangia. Three types of colonies are formed when grown on 0.1 x TSA: circular, flat, smooth and opaque; circular, convex, glistening and translucent; and circular or irregular, wrinkled hollow and opaque. Facultatively anaerobic, growing at 10–50 °C with an optimum growth temperature of 38–40 °C. Growth occurs in the presence of 0.001 % lysosome, but not in the presence of 7 % NaCl. Positive for oxidase, catalase, arginine dihydrolase, ornithine decarboxylase and nitrate reduction. Negative for urease, lysine decarboxylase, indole production and Voges–Proskauer reaction. Casein, chitin, chitosan, ascorbin and starch are hydrolysed. Utilizes sodium acetate and malonate as sole carbon sources, oxidatively, and fermentatively from arabinose, arabinose, glucose, lactose, maltose, mannitol, palatinose, raffinose, sorbitol and sucrose. Carbohydrates not utilized by the species are shown in Table 1. The major fatty acid is anteiso-15:0. The G+C content is 54 mol%. A 16S rDNA fragment (0.54 km) is amplified by PCR with primers PAEN515F and PAEN1499R. Inhibits the growth of fungi by producing an iturin-like antifungal compound. The type strain is YC300T, isolated from commercial bed soils originating from Korea, the geographical origin of isolation. Strain YC300T has been deposited in the Korean Collection for Type Cultures and the Korean Culture Center of Microorganisms under the accession numbers KCTC 2393 and KCCM 40903T, respectively.

**ACKNOWLEDGEMENTS**

This work was supported by a grant No. KOSEF 96-0402-01-01-3 from the Korea Science and Engineering Foundation (KOSEF).

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