Corynebacterium halotolerans sp. nov., isolated from saline soil in the west of China

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A halotolerant, non-spore-forming actinobacterium was isolated from a soil sample from the west of China. The strain, designated YIM 70093^T (=CCTCC AA 001024^T=DSM 44683^T), comprised Gram-positive, non-motile, diphtheroid and irregular rods. It grew in 0–25 % KCl (KCl could be substituted by NaCl or $MgCl_2.6H_2O$), with optimum growth at 10 % KCl, and its optimal pH and cultivation temperature were 7·2 and 28 °C, respectively. On the basis of its morphological, physiological and phylogenetic characteristics, strain YIM 70093^T should be classified in the genus *Corynebacterium*. However, it is sufficiently different from hitherto described *Corynebacterium* species to be considered as a novel species, for which the name *Corynebacterium halotolerans* sp. nov. is proposed.

The genus *Corynebacterium* was created by Lehmann & Neumann (1896) and represents a large group of Gram-positive, asporogenous, rod-shaped bacteria with a high DNA G+C content (Collins & Cummins, 1986; Liebl, 1992). In recent years, many novel *Corynebacterium* species have been described, the majority of which were isolated from clinical samples or animals. Some non-clinical species of the genus *Corynebacterium* originated from soil or plant materials. Here, we report the taxonomic characteristics of a novel *Corynebacterium* species that originated from saline soil in the west of China.

Strain YIM 70093^T was isolated from a saline soil sample that was collected in Xinjiang Province, China. Modified glycerol/asparagine agar (ISP 5), which contained [(l distilled water)$^{-1}$] 1·0 g L-asparagine, 10 g glycerol, 5 g yeast extract, 5 g KNO_3, 1·0 g K_2HPO_4, 150 g KCl and 1 ml trace element solution, was used for enrichment and isolation. The strain was cultivated aerobically at 28 °C for 2–3 days. Cells for biochemical and molecular systematic analyses were grown in shaken flasks (about 150 r.p.m.) of modified ISP 5 medium broth at 28 °C for 1 week. Stock cultures were maintained at 4 °C, using modified ISP 5 agar slants that contained 10 % KCl, and as glycerol suspensions (20 %, v/v) at −20 °C.

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The GenBank/EMBL/DDBJ accession number for the 16S rDNA sequence of strain YIM 70093^T is AY226509.

Strain YIM 70093^T was grown on modified ISP 5 medium and, for contrast, in other classical media without salt, such as trypticase/soy agar medium and Mueller–Hinton agar medium, for observation of cell and colony morphology. Motility of cells was studied on LB swarming agar (0·3 %, w/v). Temperature and pH ranges for growth were determined by using modified ISP 5 medium. For pH endurance experiments, modified ISP 5 medium that contained 10 % KCl and the following buffer solutions were used: pH 6·0, NaOH/KH_2PO_4; pH 7·0, NaOH/KH_2PO_4; pH 8·0, NaOH/KH_2PO_4; pH 9·0, borax/boric acid; pH 10·0, borax/NaOH; pH 11·0, Na_2HPO_4/NaOH; pH 12·0, KCl/NaOH; pH 13·0, KCl/NaOH. Tolerance to NaCl, $MgCl_2.6H_2O$ and KCl was tested at concentrations between 0 and 30 % (w/v), in combination with ISP 5 medium. Procedures and media that were used for determination of physiological and biochemical features were as described by Shirling & Gottlieb (1966). Metabolic properties of the strains studied were determined by using API Coryne and API ID 32 E test kits (bioMérieux), according to the manufacturer's instructions.

Amino acid and sugar analyses of whole-cell hydrolysates followed procedures described by Staneck & Roberts (1974). Polar lipids were extracted, examined by two-dimensional TLC and identified by using published procedures (Minnikin *et al.*, 1984). Menaquinones were isolated by using the methods of Minnikin *et al.* (1984) and separated by HPLC (Kroppenstedt *et al.*, 1981; Kroppenstedt, 1982). Fatty acid methyl esters and mycolic acid trimethylsilyl

esters were prepared and analysed as described previously (Klatte *et al.*, 1994), using the standard Microbial Identification system (MIDI Inc.) for automated GC analyses (Sasser, 1990).

Genomic DNA was isolated and purified by the method of Marmur (1961). The DNA G+C content of strain YIM 70093^T was measured by the thermal denaturation method (Marmur & Doty, 1962).

16S rRNA genes of the isolates were amplified by PCR using conserved primers close to the 3′ and 5′ ends of the gene, as described previously (Cui *et al.*, 2001). Multiple alignments with sequences of a broad selection of actinobacteria and calculations of sequence similarity levels were carried out by using CLUSTAL X (Thompson *et al.*, 1997). A phylogenetic tree was reconstructed from K_{nuc} values (Kimura, 1980, 1983) by using the neighbour-joining method of Saitou & Nei (1987). The topology of the phylogenetic tree was evaluated by the bootstrap resampling method of Felsenstein (1985) with 1000 replicates.

Cells of strain YIM 70093^T were aerobic, Gram-positive, non-motile, non-spore-forming, diphtheroid and irregular rods. Colonies on modified ISP 5 medium were moderately yellow, circular, entire, somewhat convex, opaque and approximately 0·5 mm in diameter after 24 h at 28 °C, while those on trypticase/soy agar medium and Mueller–Hinton agar medium differed from the former only in their diameter. Strain YIM 70093^T grew in modified ISP 5 medium with 0–25 % KCl, NaCl or $MgCl_2.6H_2O$. The isolate was catalase-positive and oxidase-negative. Urease, tyrosinase and Tween esterase activities were negative; nitrate reduction was positive, whilst nitrite reduction was negative. The carbon utilization range was wide, as the isolate could utilize most carbon sources that were tested.

The enzymic profile, obtained after 3 days incubation with API ZYM strips, was as follows: lipase and β-glucuronidase activities were positive and ornithine decarboxylase, arginine dihydrolase, lysine decarboxylase, α- and β-galactosidase, *N*-acetyl-β-glucosaminidase and β-glucosidase activities were negative.

The optimum pH, cultivation temperature and NaCl, KCl and $MgCl_2.6H_2O$ concentrations for growth were 7·2, 28 °C and 10 %, respectively.

Cell walls of strain YIM 70093^T contained *meso*-diaminopimelic acid. Whole-cell hydrolysates contained mainly galactose and arabinose. Menaquinones were MK-8(H_2) (35·5 %) and MK-9(H_2) (64·5 %). Polar lipid extract contained diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol, glycolipid and phosphatidylinositol mannosides. Predominant cellular fatty acids were $C_{14:0}$ (7·3 %), *cis*-9-$C_{16:1}$ (9·8 %), $C_{16:0}$ (42·1 %), *cis*-9-$C_{18:1}$ (28·9 %), $C_{18:0}$ (4·5 %) and 10-methyl $C_{18:0}$ (7·4 %). Short-chain mycolic acids (C_{32}–C_{36}) were present; predominant mycolic acids were $C_{32:0}$ (36·0 %), $C_{34:0}$ (20·8 %), $C_{34:1}$ (25·1 %), $C_{36:0}$ (3·6 %), $C_{36:1}$ (8·4 %) and $C_{36:2}$ (5·1 %). The DNA G+C content of strain YIM 70093^T was 63 mol%.

To determine the phylogenetic position of the unknown bacterium, the 16S rRNA gene was amplified by PCR. An almost-complete 16S rDNA sequence (1492 bp) was obtained and subjected to comparative analysis. Members of the genus *Corynebacterium* were its closest phylogenetic neighbours (Fig. 1). Binary similarity values ranged between 93·5 % (*Corynebacterium callunae* CCUG 28793^T) and 95·8 % (*Corynebacterium xerosis* DSM 20743^T); no sequence similarity of >97 % was obtained with any member of the genus *Corynebacterium*.

The genus *Corynebacterium* embraces a very diverse range of organisms; over 50 different species are currently assigned to the genus. It is, however, recognized that the genus is not monophyletic and actually comprises several distinct rDNA lineages. It is evident from the present 16S rDNA study that strain YIM 70093^T forms a distinct subclade with *C. xerosis* DSM 20743^T and *Corynebacterium freneyi* CIP 106767^T within the phylogenetic tree. High sequence divergence values (>4·2 %) with other members of this genus clearly indicate that the isolate represents a novel

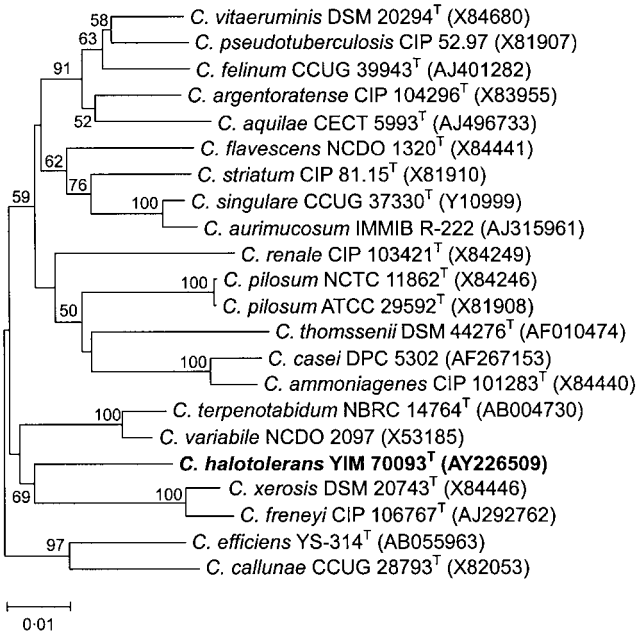

Fig. 1. Phylogenetic dendrogram obtained by distance matrix analysis of 16S rDNA sequences, showing the position of strain YIM 70093^T among its phylogenetic neighbours. Numbers on branch nodes are bootstrap values (1000 resamplings). The sequence of *Rhodococcus equi* DSM 20307^T (GenBank no. X80614) was used as root. Bar, 1 % sequence divergence.

species. Although there is no precise correlation between 16S rDNA divergence values and species delineation, it is generally recognized that organisms that display sequence divergence values of $\geqslant 3\,\%$ do not belong to the same species (Stackebrandt & Goebel, 1994). The sequence divergence of $>3\,\%$ that was observed between the unknown actinobacterium and *Corynebacterium* species with validly published names is therefore consistent with separate species status. Support for the distinctiveness of the unknown actinobacterium also comes from phenotypic evidence, when compared to the species *C. xerosis* (DSM 20743^T) and *C. freneyi* (CIP 106767^T) of the genus *Corynebacterium* (Table 1). The isolate originated from saline soil and has high tolerance to KCl, NaCl and $MgCl_2.6H_2O$ (0–25 %, w/v) in ISP 5 medium. β-Glucuronidase activity of strain YIM 70093^T is positive and α-glucosidase activity is negative, whereas the two most closely related species show the opposite characteristics (Table 1). In addition, the unknown bacterium has different types and amounts of fatty and mycolic acids from these two species (data not shown). Therefore, based on phenotypic and molecular genetic results, it is evident that the unknown *Corynebacterium* strain described above should be classified as a member of a novel species of the genus *Corynebacterium*, for which we propose the name *Corynebacterium halotolerans* sp. nov.

Table 1. Characteristics that differentiate *Corynebacterium halotolerans* sp. nov. from its two closest phylogenetic relatives

Species: 1, *C. halotolerans* YIM 70093^T; 2, *C. freneyi* CIP 106767^T; 3, *C. xerosis* DSM 20743^T. Characteristics are scored as: +, positive; −, negative; W, weak; V, variable; ND, not determined. All strains have the same results for the following characteristics: acid production from glucose, but not from xylose, mannitol or lactose. Activities for β-galactosidase, β-glucosidase and *N*-acetyl-β-glucosaminidase are negative.

Characteristic	1	2*	3*
Nitrate reduction	+	V	V
Hydrolysis of:			
Gelatin	+	−	−
Urea	+	−	−
Starch	−	ND	+
Acid production from:			
Maltose	−	+	+
Sucrose	−	+	+
Ribose	−	W	+
5-Ketogluconate	−	ND	+
Activity for:			
β-Glucuronidase	+	−	−
α-Glucosidase	−	+	+

*Data were taken from Funke *et al.* (1996) and Renaud *et al.* (2001).

Description of *Corynebacterium halotolerans* sp. nov.

Corynebacterium halotolerans (ha.lo.to′le.rans. Gr. n. *halos* salt; L. part. adj. *tolerans* tolerating; N.L. pres. part. *halotolerans* referring to the ability to tolerate high salt concentrations).

Cells are aerobic, Gram-positive, non-motile, non-spore-forming, diphtheroid and irregular rods. Colonies on modified ISP 5 medium, trypticase/soy agar medium and Mueller–Hinton agar medium are moderately yellow, circular, entire, somewhat convex, opaque and 0·5–1·5 mm in diameter after 24 h at 28 °C. Optimum growth temperature is 28 °C. Optimum growth concentration of KCl, NaCl and $MgCl_2.6H_2O$ is 10 %. Positive for nitrate reduction, but negative for milk peptonization and coagulation, gelatin liquefaction, growth in cellulose, production of H_2S and melanin, starch hydrolysis and urease production. Activities for lipase and β-glucuronidase are positive. Ornithine decarboxylase, arginine dihydrolase, lysine decarboxylase, α- and β-galactosidase, *N*-acetyl-β-glucosaminidase and β-glucosidase activities are negative. The following substrates are utilized: glucose, galactose, sucrose, arabinose, mannose, mannitol, maltose, starch, xylose, ribose, salicin and dextrin. Cellobiose, fructose, amygdalin and lactose are not utilized. Acid production occurs only from glucose. Cell wall contains *meso*-diaminopimelic acid. Whole-cell hydrolysates contain mainly galactose and arabinose. Menaquinones are MK-8(H_2) (35·5 %) and MK-9(H_2) (64·5 %); phospholipids are diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol, glycolipid and phosphatidylinositol mannosides. Major cellular fatty acids are $C_{14:0}$ (7·3 %), *cis*-9-$C_{16:1}$ (9·8 %), $C_{16:0}$ (42·1 %), *cis*-9-$C_{18:1}$ (28·9 %), $C_{18:0}$ (4·5 %) and 10-methyl $C_{18:0}$ (7·4 %). Predominant mycolic acids are $C_{32:0}$ (36·0 %), $C_{34:0}$ (20·8 %), $C_{34:1}$ (25·1 %), $C_{36:0}$ (3·6 %), $C_{36:1}$ (8·4 %) and $C_{36:2}$ (5·1 %). DNA G+C content is 63 mol%.

The type strain is YIM 70093^T (=CCTCC AA 001024^T=DSM 44683^T). Isolated from saline soil collected in Xinjiang Province, west China.

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