Cohnella laeviribosi sp. nov., isolated from a volcanic pond

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A novel thermophilic and endospore-forming Gram-positive bacterium capable of assimilating and isomerizing L-ribose was isolated from a volcanic area in Likupang, Indonesia. The isolate, RI-39^T, was able to grow at high temperatures (37–60 °C); optimum growth was observed at pH 6.5 and 45 °C. The strain contained MK-7 (87%) as the main respiratory quinone and had a DNA G + C content of 51 mol%. The major cellular fatty acids of the isolate were iso-C_{16:0} and anteiso-C_{15:0} and the predominant polar lipids were diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine and lysyl-phosphatidylglycerol. Phylogenetic analysis based on 16S rRNA gene sequences revealed that the isolate represents an evolutionary lineage that is distinct from those of other *Cohnella* species. Based on morphological, physiological and chemotaxonomic characteristics and 16S rRNA gene sequence comparisons, it is proposed that strain RI-39^T represents a novel species, *Cohnella laeviribosi* sp. nov. The type strain is RI-39^T (=KCTC 3987^T =KCCM 10653P^T =CCUG 52217^T).

Recently, non-naturally produced sugars have attracted much attention as synthetic intermediates for pharmaceutical drugs and agricultural chemicals (Chu *et al.*, 1998). In the case of ribose, the D-isomer is abundantly distributed throughout the living world, as a component not only of DNA, but also of various vitamins and coenzymes (Sugisawa *et al.*, 2005). However, the L-isomer of ribose is a pentose that is not found in nature and no known organism can assimilate or degrade this rare aldo-pentose (Ahmed *et al.*, 1999; Muniruzzaman *et al.*, 1996; Granstrom *et al.*, 2004). L-Ribose is used for the synthesis of L-nucleosides, which have more potent biological activities and lower toxicities than their corresponding D-nucleosides. For example, several modified L-nucleosides have been developed as antiviral agents that are more effective and less toxic than their D-counterparts; examples include (–)-(2′R5′S)-1-(2-hydroxymethyl oxathiolan-5-yl)-cytosine, L-thymidine, L-3′-thiacytidine, L-5-fluoro-3′-thiacytidine, L-2′,3′-dideoxycytidine, L-5-fluoro-2′,3′-dideoxycytidine and

L-2′-fluoro-5-methylarabinofuranosyl uracil. L-Nucleosides are phosphorylated by cellular kinases, but they interact selectively with viral polymerases and only rarely with cellular polymerases (Shi *et al.*, 2002; Hunsucker *et al.*, 2005; Ma *et al.*, 1996). There are some methods for the chemical synthesis of L-carbohydrates (Takahashi *et al.*, 2002; Cho *et al.*, 2005; Yun *et al.*, 2005). However, chemical synthesis methods are very lengthy, with many reaction steps, and have other drawbacks, such as low overall yields and the difficulty of large-scale synthesis. In addition, these chemical methods produce unnecessary by-products. Therefore, the biochemical production of L-ribose from L-ribulose using micro-organisms and their enzymes has been attempted.

L-Ribose isomerase from *Acinetobacter* sp. strain DL-28 has been used for the reversible isomerization of L-ribose to L-ribulose (Shimonishi & Izumori, 1996). However, the enzyme is not stable over 30 °C, which limits the overall process productivity significantly. A thermophilic L-ribose isomerase would offer many biotechnological advantages. Enzymic reactions at high temperatures allow higher substrate concentrations, lower viscosity, little risk of microbial contamination, higher resistance to chemical denaturants and often a higher reaction rate (Brown *et al.*, 1993).

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain RI-39^T is DQ459874.

An extended phylogenetic tree showing the position of isolate RI-39^T compared with related taxa based on 16S rRNA gene sequences is available as supplementary material with the online version of this paper.

For this reason, we searched for a thermostable L-ribose isomerase (Cho *et al.*, 2007). In this study, a novel Gram-positive endospore-forming bacterium capable of catalysing the interconversion of L-ribulose and L-ribose was isolated. Soil from Likupang, a volcanic area in Indonesia, was screened for thermophilic micro-organisms that showed L-ribose isomerase activity at high temperatures. Several strains were isolated on minimal salt medium containing L-ribose as sole carbon source at 45–55 °C and strain RI-39^T was selected for further study. Based on phenotypic, chemotaxonomic and phylogenetic characteristics, the isolate represents a novel *Cohnella* species.

Cultivation of strain RI-39^T was carried out on trypticase soy agar (TSA; Difco) adjusted to pH 6.5 for 20 h at 45 °C. The cell morphology of RI-39^T was examined by light microscopy and motility was observed with an optical microscope using the hanging-drop technique (Skerman, 1967). To examine flagellum type, cells from the exponential phase of growth were negatively stained with 1 % (w/v) phosphotungstic acid and, after air-drying, the grids were examined using a model CM-20 transmission electron microscope (Philips). Spores fixed with glutaraldehyde/osmium were sectioned, stained and observed with a transmission electron microscope. Anaerobic growth was recorded in an anaerobic chamber (CO₂/H₂/N₂, 7:7:86; Forma Scientific) on TSA for up to 1 week. Growth at different temperatures was observed in trypticase soy broth at 10, 20, 30, 37, 40, 43, 45, 48, 50, 52, 55, 60, 70 and 80 °C. Growth was assessed by monitoring the optical density at 600 nm of bacteria in 50 ml trypticase soy broth adjusted to pH 4.0–10.0 using 100 mM citric acid/200 mM Na₂HPO₄ buffer at pH 4.0–5.0, 100 mM Na₂HPO₄/NaH₂PO₄ buffer at pH 6.0–8.0 or 100 mM glycine/NaOH buffer at pH 9.0–10.0, based on a previously described method (Yumoto *et al.*, 1998). The ability of the isolate to grow in different NaCl concentrations (0, 0.2, 0.5, 1, 2, 5, 10, 15 and 20 %, w/v) was tested with nutrient broth (Difco) as the basal medium at pH 6.5 and 45 °C. Physiological and biochemical characterizations were performed using the API 20NE and API 20E strips and 50CH strips combined with API 50CHB/E medium (bioMérieux), in accordance with the manufacturer's directions. Catalase activity was determined by production of oxygen bubbles in 3 % (v/v) aqueous hydrogen peroxide solution. Oxidase activity was determined by oxidation of 1 % (w/v) tetramethyl-*p*-phenylenediamine (Merck). Hydrolysis of gelatin, casein, starch and aesculin and production of urease were examined according to the methods of Cowan & Steel (1965) and Kurup & Fink (1975).

Strain RI-39^T was aerobic, non-motile, rod-shaped and Gram-positive. It formed spherical subterminal spores. Colonies were circular, flat, smooth, opaque and white. Growth occurred at 30–60 °C and pH 5.5–8.0; optimal growth occurred at 45 °C and pH 6.5. Strain RI-39^T did not grow under anaerobic conditions on TSA. Optimal growth occurred in the presence of 0.2–0.5 % (w/v) NaCl.

The strain did not grow in the presence of >1 % (w/v) NaCl or in 0.001 % (w/v) lysozyme. In our studies, RI-39^T showed no evidence of oxidase, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase or tryptophan deaminase activity and was unable to hydrolyse gelatin or produce indole. On the other hand, the isolate showed catalase activity and could hydrolyse aesculin. The isolate tested positive for β -galactosidase and β -glucosidase activities, but negative for urease and protease activities. Furthermore, RI-39^T produced acid from adonitol, D-arabinose, L-arabinose, fructose, L-fucose, galactoside, glucose, glycerol, D-lyxose, mannose, maltose, melibiose, rhamnose, D-ribose, starch, sucrose, D-xylose and methyl β -D-xyloside. Acid was not produced from arbutin, dulcitol, D-fucose, gentiobiose, gluconate, 2-ketogluconate, 5-ketogluconate, *N*-acetylglucosamine, methyl α -D-mannoside, erythritol, inositol, inulin, mannitol, melezitose, salicin, sorbose, sorbitol, D-tagatose or L-xylose. RI-39^T utilized arabinose, glucose, maltose, mannitol and mannose, but not phenylacetate, citrate, gluconate, *N*-acetylglucosamine or malate. Phenotypic properties of strain RI-39^T and other *Cohnella* species are shown in Table 1.

Fatty acids were extracted and analysed according to the instructions of the Microbial Identification System (MIDI; Microbial ID). The isoprenoid quinones were extracted with chloroform/methanol (2:1, v/v) and purified by TLC on Merck Kieselgel 60 F₂₅₄ plates (20 × 20 cm, 0.5 mm thickness) using petroleum ether/diethyl ether (9:1, v/v) as the solvent. The identity of the quinones was determined by reversed-phase HPLC analysis (Shin *et al.*, 1996). The DNA G+C content of strain RI-39^T was determined by reversed-phase HPLC using the method of Tamaoka & Komagata (1984). Extraction and analysis of polar lipids by two-dimensional TLC was performed according to Komagata & Suzuki (1987). The polar lipid profile of strain RI-39^T was compared with that of *Cohnella thermotolerans* KCTC 13053^T. Lipids were extracted from dried cells using methanol/chloroform (2:1, v/v) and the total polar lipid fraction was collected by evaporation. Polar lipids were dissolved in chloroform/methanol (2:1, v/v) and stored in chloroform/methanol at 4 °C. Total polar lipids were subjected to TLC on 20 × 20 cm, 0.5 mm silica gel 60 glass plates (Merck). Solvent systems used for development were chloroform/methanol/water (65:25:4, v/v) for the first direction and chloroform/acetic acid/methanol/water (80:18:12:5, v/v) for the second direction. Spots were detected by spraying with 50 % H₂SO₄.

The major fatty acids in the isolate were iso-C_{16:0} (40.5 %) and anteiso-C_{15:0} (22.0 %) (Table 2). The strain had MK-7 (87 %) as the major isoprenoid quinone and MK-6 (13 %) as a minor isoprenoid quinone. The DNA G+C content was 51 mol%. The predominant polar lipids of the isolate were diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine and lysyl-phosphatidylglycerol (Fig. 1); these polar lipids are characteristic of members of the genus *Cohnella* (Kämpfer *et al.*, 2006).

Table 1. Differential characteristics of *Cohnella laeviribosi* sp. nov. RI-39^T and the other members of the genus *Cohnella*

Strains: 1, *C. laeviribosi* RI-39^T; 2, *C. thermotolerans* CCUG 47242^T; 3, *C. hongkongensis* HKU3^T. +, Positive; w, weakly positive; –, negative; ND, no data available. All three strains had MK-7 as major menaquinone and diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine and lysyl-phosphatidylglycerol as the major polar lipids and were negative for anaerobic growth and acid production from erythritol, dulcitol, inositol, *N*-acetylglucosamine and salicin.

Characteristic	1	2	3
Gram reaction	+	+	–
Catalase	+	ND	w
Oxidase	–	+	+
Temperature range (°C)	37–52	20–55	37–65
Fermentation/oxidation/assimilation of:			
Glycerol	+	ND	–
D-Arabinose	+	ND	–
L-Arabinose	+	w	–
D-Ribose	+	+	–
D-Xylose	+	–	–
L-Xylose	–	ND	–
Adonitol	+	–	–
Methyl β-D-xyloside	+	ND	–
Galactoside	+	ND	–
D-Glucose	+	+	–
Fructose	+	+	–
D-Mannose	+	+	–
Sorbitol	–	ND	–
Rhamnose	+	+	–
Mannitol	+	–	–
Sorbitol	+	–	–
Methyl α-D-mannoside	–	ND	–
Methyl α-D-glucoside	+	ND	–
Amygdalin	+	ND	–
Arbutin	–	+	–
D-Cellobiose	+	+	–
Maltose	+	+	–
Lactose	+	–	–
Melibiose	+	+	–
Sucrose	+	–	–
Trehalose	+	–	–
Inulin	–	ND	–
Melezitose	–	ND	–
Raffinose	+	–	–
Starch	+	ND	ND
Glycogen	+	ND	–
Xylitol	+	ND	–
Gentiobiose	–	ND	–
Turannose	+	ND	–
D-Lyxose	+	ND	–
D-Tagatose	–	ND	–
D-Fucose	–	ND	–
L-Fucose	+	ND	–
D-Arabitol	+	–	–
L-Arabitol	+	ND	–
Gluconate	–	+	–
2-Ketogluconate	–	ND	–

Characteristic	1	2	3
5-Ketogluconate	–	ND	–
DNA G + C content (mol%)	51	59	47.6

Chromosomal DNA was extracted and purified by a modification of the method of Marmur (1961). Two universal primers [9F and 1492R; described by Stackebrandt & Liesack (1993)] were used for PCR amplification of the 16S rRNA gene of strain RI-39^T. The amplified PCR product was purified using the QIAquick PCR purification kit (Qiagen). The purified 16S rRNA gene was sequenced using an ABI Prism BigDye Terminator cycle sequencing ready reaction kit (Applied Biosystems) and automatic DNA sequencer (model 377; Applied Biosystems). The majority of the 16S rRNA sequence (1435 bp) for isolate RI-39^T was determined and it was aligned with representative sequences from members of the genus *Cohnella* and related genera using CLUSTAL W software (Thompson *et al.*, 1994). Phylogenetic trees were constructed using three different methods, the neighbour-joining, maximum-likelihood and maximum-parsimony algorithms available in the PHYLIP software, version 3.6 (Felsenstein, 2002). Evolutionary distance matrices were calculated using the model of Jukes & Cantor (1969). The PHYLIP software package (Felsenstein, 1993) was used for analysis. Topology of the phylogenetic tree was evaluated

Table 2. Fatty acid content (%) of *C. laeviribosi* sp. nov. RI-39^T and the other members of the genus *Cohnella*

Strains: 1, *C. laeviribosi* RI-39^T; 2, *C. thermotolerans* CCUG 47242^T; 3, *C. hongkongensis* HKU3^T. Data for *C. thermotolerans* CCUG 47242^T and *C. hongkongensis* HKU3^T are from Kämpfer *et al.* (2006). tr, Trace (<1%); ND, not detected.

Fatty acid	1	2	3
Saturated fatty acids			
C _{14:0}	1.9	1.0	5.0
C _{15:0}	1.3	1.4	8.0
C _{16:0}	9.2	6.6	25.3
C _{17:0}	tr	ND	1.2
Unsaturated fatty acids			
C _{14:1ω5c}	tr	ND	ND
C _{16:1ω11c}	tr	ND	tr
C _{17:1ω6c}	tr	1.0	ND
Branched fatty acids			
iso-C _{13:0}	tr	ND	tr
anteiso-C _{13:0}	tr	ND	ND
iso-C _{14:0}	3.9	2.1	2.3
iso-C _{15:0}	11.7	3.2	8.1
anteiso-C _{15:0}	22.0	28.4	31.2
iso-C _{16:0}	40.5	45.5	11.9
iso-C _{17:0}	2.8	ND	ND
anteiso-C _{17:0}	5.78	6.7	2.6

Fig. 1. Two-dimensional TLC showing polar lipid profiles of *C. thermotolerans* KCTC 13053^T (a) and *C. laeviribosi* sp. nov. RI-39^T (b). DPG, Diphosphatidylglycerol; LPG, lysyl-phosphatidylglycerol; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PL, unknown polar lipid; PN, unknown aminolipid.

using bootstrap analysis (Felsenstein, 1985) of the neighbour-joining method based on 1000 replications.

The primary structure of the 16S rRNA gene sequence of the strain was compared with those of closely related reference strains. The phylogenetic tree indicated that strain RI-39^T belonged to the genus *Cohnella* in the neighbour-joining analysis (Fig. 2; an extended version of this tree is available as Supplementary Fig. S1 in IJSEM Online). The topologies of phylogenetic trees generated

Fig. 2. Phylogenetic tree constructed using neighbour-joining analysis showing the position of isolate RI-39^T compared with other species from the genus *Cohnella* and related taxa based on 16S rRNA gene sequences. Bootstrap values are noted at branch points. Bar, 0.1 accumulated changes per nucleotide.

using the maximum-likelihood and maximum-parsimony algorithms were similar to that of the tree constructed by neighbour-joining analysis (data not shown). Isolate RI-39^T was closely related to *C. thermotolerans* KCTC 13053^T; these two strains shared 93.5 % 16S rRNA gene sequence similarity, which indicates that strain RI-39^T represents a novel species (Rosselló-Mora & Amann, 2001; Stackebrandt *et al.*, 2002). The phylogenetic definition of a species generally includes 'strains with approximately 70 % or greater DNA–DNA relatedness' (Wayne *et al.*, 1987), based on DNA–DNA reassociation experiments. According to currently available data, DNA from organisms with sequence identities of less than 97.0 % will not reassociate to more than 60 %, no matter which hybridization method is applied (Stackebrandt & Goebel, 1994).

On the basis of the above results, a novel species, *Cohnella laeviribosi* sp. nov., is proposed to accommodate strain RI-39^T.

Description of *Cohnella laeviribosi* sp. nov.

Cohnella laeviribosi [lae.vi.ri'bo.si. L. adj. *laevus* left, on the left side; N.L. n. *ribosum* ribose; N.L. gen. n. *laeviribosi* referring to L-ribose (isomerase), because the type strain exhibits L-ribose isomerization ability].

Aerobic, non-motile and Gram-positive. Cells are rods, about 0.5–0.7 µm wide and 2.0–7.0 µm long. In old cultures, cells become shorter rods or spherical elements. Colonies are circular, flat, smooth, opaque and white. No growth is seen in the presence of >1 % (w/v) NaCl, with 0.001 % (w/v) lysozyme or under anaerobic conditions on TSA. Grows at 30–60 °C and pH 5.5–8.0, with optimal

growth at 45 °C and pH 6.5. Optimal growth occurs in the presence of 0.2–0.5% (w/v) NaCl. Positive for aesculin hydrolysis, β -galactosidase, β -glucosidase, catalase and acid production from adonitol, D-arabinose, L-arabinose, fructose, L-fucose, galactoside, glucose, glycerol, D-lyxose, mannose, maltose, melibiose, rhamnose, D-ribose, starch, sucrose, D-xylose and methyl β -D-xyloside. Does not produce acid from arbutin, dulcitol, D-fucose, gentiobiose, gluconate, 2-ketogluconate, 5-ketogluconate, N-acetylglucosamine, phenylacetate, citrate, malate, methyl α -D-mannoside, erythritol, inositol, inulin, mannitol, melezitose, salicin, sorbose, sorbitol, D-tagatose or L-xylose. Negative for oxidase, lysine decarboxylase, ornithine decarboxylase, urease and tryptophan deaminase activities, indole production, arginine hydrolysis and gelatin hydrolysis. The major isoprenoid quinone is MK-7. The major fatty acids are iso-C_{16:0} and anteiso-C_{15:0}. Predominant polar lipids are diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine and lysyl-phosphatidylglycerol.

The type strain is RI-39^T (=KCTC 3987^T =KCCM 10653P^T =CCUG 52217^T), isolated from Likupang, a volcanic area in Indonesia. The DNA G+C content of the type strain is 51 mol%.

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