Microbacterium natoriense sp. nov., a novel D-aminoacylase-producing bacterium isolated from soil in Natori, Japan

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A rod-shaped, Gram-positive bacterium, strain TNJL143-2^T, having *N*-acyl-D-amino acid amidohydrolase (D-aminoacylase) activity, was isolated from a soil sample from Natori, Japan. It was a non-spore-forming, strictly aerobic bacterium without motility, showing a temperature optimum for growth of 30 °C and a pH optimum for growth of 5–7. The 16S rRNA gene sequence of the strain showed the highest similarities to members of the genus *Microbacterium*, in particular, *Microbacterium aerolatum*, *Microbacterium foliorum* and *Microbacterium phyllosphaerae*. The chemotaxonomic characteristics, including the compositions of cellular menaquinones, cellular fatty acids and cell-wall amino acids, were consistent with those described for the genus *Microbacterium*. The G + C content of the genomic DNA was determined as 69.1 mol%. DNA–DNA hybridization studies using type strains of *M. aerolatum*, *M. foliorum* and *M. phyllosphaerae* showed only low levels of relatedness (11–12%). On the basis of these phenotypic and genotypic results, a novel species, *Microbacterium natoriense* sp. nov., is proposed, with TNJL143-2^T (=JCM 12611^T=ATCC BAA-1032^T) as the type strain.

Members of the genus *Microbacterium*, in which two genera formerly designated *Microbacterium* and *Aureobacterium* have been unified (Takeuchi & Hatano, 1998a, b), are widespread in nature, and some species of this genus are of significance in biotechnology, e.g. the amino acid industry (Nakayama & Soda, 1998). During the course of our studies to screen micro-organisms that were able to grow on *N*-acetyl-D-phenylalanine as the sole nitrogen and carbon source, we found that a yellow-pigmented bacterium, strain TNJL143-2^T, isolated from soil at Natori, Miyagi, Japan, produced a novel *N*-acyl-D-amino acid amidohydrolase (D-aminoacylase). As a result, extensive physiological, chemotaxonomic and phylogenetic analyses were carried out. On the basis of these results, we propose that strain TNJL143-2^T represents a novel species of the genus *Microbacterium*, which we have named *Microbacterium natoriense* sp. nov.

Strain TNJL143-2^T was isolated from soil, using an agar

medium (at pH 7.0) containing 0.1% (w/v) *N*-acetyl-D-phenylalanine, 0.01% (w/v) yeast extract, 0.5% (w/v) NaCl, 0.1% (w/v) KH₂PO₄, 0.0005% (w/v) MgSO₄·7H₂O and 1.5% (w/v) agar at 30 °C. The type strains *Microbacterium aerolatum* JCM 12137^T (=DSM 14217^T) (Zlamala *et al.*, 2002), *Microbacterium foliorum* JCM 11569^T (=DSM 12966^T) (Behrendt *et al.*, 2001) and *Microbacterium phyllosphaerae* JCM 11571^T (=DSM 13468^T) (Behrendt *et al.*, 2001) were obtained from the Japan Collection of Micro-organisms, Wako, Saitama, Japan. Cells of strains TNJL143-2^T, JCM 12137^T, JCM 11569^T and JCM 1571^T were stored at –80 °C in Luria–Bertani (LB) broth at pH 6.8 (Sambrook *et al.*, 1989) supplemented with 15% (w/v) glycerol.

General laboratory cultivation was performed using LB broth (5 ml) at 30 °C. Bacterial growth was monitored for up to 7 days after inoculation by measuring the turbidity, at 600 nm, of cultures in 25 ml test tubes, which were capped with air-permeable silicone plugs and incubated in a water-bath incubator. For measurement of turbidity, an uninoculated control was used as a blank. Cell morphology was observed by light microscopy after 24 h cell growth. Sporulation was observed by phase-contrast microscopy after the cells had reached stationary phase. Gram staining

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain TNJL143-2^T is AY566291.

The cellular fatty acid compositions of strain TNJL143-2^T and related type strains are available in a supplementary table in IJSEM Online.

was carried out using exponentially growing cells, according to Hucker's modification (Cowan & Steel, 1965), with reagents (Favour G) produced by Nissui. Flagellation was examined using Leifson's method (Cowan & Steel, 1965). The temperature range for growth of the organism was examined at 4 °C and at 10–50 °C in 5 °C steps. To determine the pH range for growth, the organisms were grown in LB medium supplemented with 0.1 % (w/v) potassium phosphate at 30 °C, as described above, except that the pH of the medium was adjusted to different values with 1.0 M H₂SO₄ or 1.0 M NaOH. Anaerobic growth was tested by incubation at 30 °C in 10 ml rubber-sealed screw-cap tubes containing LB medium (9 ml) that was covered with liquid paraffin.

Biochemical examinations (nitrate reduction, pyrazinamidase, pyrrolidonyl arylamidase, alkaline phosphatase, β -glucuronidase, β -galactosidase, α -glucosidase, *N*-acetyl- β -glucosaminidase, β -glucosidase, urease and gelatinase) were examined using API Coryne test strips (bioMérieux). Cells were resuspended in API GP suspension medium (bioMérieux) at a cell density corresponding to tube no. 6 of the MacFarland series of standard opacities (Cowan & Steel, 1965). The cell suspensions (150 μ l) were added to API Coryne test-strip wells as recommended by the manufacturer, then incubated at 30 °C. Acid production from carbon sources was examined under aerobic conditions by using API 50 CH test strips (bioMérieux). Cells were resuspended in API 50 CHB/CHE suspension medium (bioMérieux) at a cell density corresponding to tube no. 2 of the MacFarland series. The cell suspensions (150 μ l) were added to API 50 CH test-strip wells as recommended by the manufacturer, then incubated at 30 °C. Acidification was observed daily for 7 consecutive days; if the medium turned yellow, the cells were regarded as positive with respect to acid production. Strain TNJL143-2^T was tested (as described previously; Barrow & Feltham, 1993) for catalase activity, oxidase activity and the hydrolysis of casein, and was subjected to an oxidation/fermentation test. The cellular menaquinones, cellular fatty acids and cell-wall diamino acids of TNJL143-2^T were analysed as described previously (Komagata & Suzuki, 1987).

Isolation and purification of chromosomal DNA and estimation of DNA base composition (by HPLC) were performed according to Tamaoka & Komagata (1984). The 16S rRNA gene was amplified by a PCR (Weisburg *et al.*, 1991) with the universal bacterial primers 10F (5'-AGT-TTGSTCCTGGCTC-3') and 1500R (5'-GGCTACCTTG-TTACGA-3'). PCR products were purified on Microspin S-400 HR columns (Amersham Biosciences) and sequenced using a CEQ2000XL DNA system (Beckman Coulter). Previously published 16S rRNA gene sequences were obtained from the GenBank/DBJ/EMBL databases. Multiple alignment of the sequences, calculation of nucleotide substitution rates (K_{nuc} values; Kimura, 1980), construction of a neighbour-joining phylogenetic tree

(Saitou & Nei, 1987) and bootstrap analysis with 1000 replicates for the evaluation of phylogenetic tree topology (Felsenstein, 1985) were carried out using the CLUSTAL X (Thompson *et al.*, 1997) and MEGA (Kumar *et al.*, 1993) software programs. DNA reassociation values were determined as described by Ezaki *et al.* (1989). In a typical experiment, probes for DNA hybridization were prepared from cells of *M. aerolatum* JCM 12137^T, *M. foliorum* JCM 11569^T and *M. phyllosphaerae* JCM 11571^T. Probe DNAs were biotinylated with photobiotin and hybridized with single-stranded unlabelled chromosomal DNA fragments of strain TNJL143-2^T. Hybridized DNA fragments were visualized using alkaline-phosphatase-based fluorometric methods (Ezaki *et al.*, 1989). Means from three independent determinations of DNA–DNA reassociation values were determined.

Cells of strain TNJL143-2^T were rod-shaped (0.5–0.6 $\times$ 1.5 μ m) and stained Gram-positive. Neither spore formation nor motility was observed and anaerobic growth did not occur. TNJL143-2^T cells tested positive in a catalase test but gave a negative result in an oxidase test (Collins & Kieddie, 1986). Colonies on LB agar were slightly yellow, shiny, moist, circular, slightly convex and 1 mm in diameter after 30 h growth at 30 °C.

A 1429 nt stretch of the 16S rRNA gene of strain TNJL143-2^T was sequenced and compared with available 16S rRNA gene sequences to construct a phylogenetic tree (Fig. 1). It showed the highest similarities to the 16S rRNA gene sequences of *M. phyllosphaerae* DSM 13468^T (98.7 %), *M. foliorum* DSM 12966^T (98.6 %), *Microbacterium keratanoliticum* DSM 8606^T (98.1 %) (Yokota *et al.*, 1993; Takeuchi & Hatano, 1998a) and *M. aerolatum* DSM 14217^T (97.6 %). Phylogenetic analysis showed that TNJL143-2^T fell within the radiation of the genus *Microbacterium* and was most closely related to *M. aerolatum* DSM 14217^T. The G + C contents of the genomic DNAs of strain TNJL143-2^T, *M. aerolatum* JCM 12137^T, *M. foliorum* JCM 11569^T and *M. phyllosphaerae* JCM 11571^T were determined (by HPLC) as 69.1, 68.1, 68.5 and 68.7 mol%, respectively. DNA–DNA reassociation studies on strain TNJL143-2^T were performed using *M. aerolatum* JCM 12137^T, *M. foliorum* JCM 11569^T and *M. phyllosphaerae* JCM 11571^T. The DNA–DNA reassociation values for strain TNJL143-2^T with these type strains were 12, 11 and 12 %, respectively. In comparison, the DNA–DNA reassociation value between *M. foliorum* and *M. phyllosphaerae*, determined by the method described by Ezaki *et al.* (1989), was 11.5 %, which is even lower than the value (41.0 %) previously obtained by the spectrophotometric method (Behrendt *et al.*, 2001; Martin *et al.*, 1997). The values for strain TNJL143-2^T with respect to type strains are significantly lower than the threshold value (70 %) recommended for the delineation of a novel species (Wayne *et al.*, 1987). Thus, strain TNJL143-2^T can be distinguished from representatives of all previously described species of *Microbacterium*.

The cellular fatty acid composition of strain TNJL143-2^T, in

Fig. 1. Phylogenetic tree derived from 16S rRNA gene sequence data for TNJL143-2^T and other species of the genus *Microbacterium*. GenBank/EMBL/DBJ accession numbers are shown in parentheses. The branching pattern was generated by the neighbour-joining method. Numbers indicate bootstrap percentages greater than 80 %. Bar, 0.01 % nucleotide substitutions per site. *C.*, *Curtobacterium*.

comparison with those reported for *M. aerolatum* DSM 14217^T, *M. foliorum* DSM 12966^T and *M. phyllosphaerae* DSM 13468^T, is available in a supplementary table in IJSEM Online. C_{15:0} anteiso was the most abundant cellular fatty acid (45.0 mol%) in strain TNJL143-2^T, as is the case for related species. Strain TNJL143-2^T contained a significant amount (17.0 mol%) of C_{15:0} iso, which is present in only minor amounts (2–4 mol%) in cells of related species. The cell-wall diamino acid of TNJL143-2^T was ornithine. The quinone system of strain TNJL143-2^T consisted of MK-9, MK-10, MK-11 and MK-12. In comparison, *M. aerolatum* DSM 14217^T contains MK-11, MK-12 and MK-13 (Zlamala *et al.*, 2002), whereas *M. foliorum* DSM 12966^T and *M. phyllosphaerae* DSM 13468^T contain MK-10, MK-11 and MK-12.

Strain TNJL143-2^T was able to grow in LB medium at pH 5–9, with a broad optimum at around pH 5–7 (at 30 °C). Optimum growth was observed at 30 °C in LB medium; no growth occurred below 4 °C or above 45 °C. The organism was able to grow in the presence of 6 % (w/v) NaCl in LB medium; however, no growth was observed at NaCl concentrations at or above 7 % (w/v). Strain TNJL143-2^T gave a negative result in an oxidation/fermentation test. Several other biochemical and physiological characteristics of TNJL143-2^T were also compared with those of *M. aerolatum* JCM 12137^T, *M. foliorum* JCM 11569^T and *M. phyllosphaerae* JCM 11571^T (Tables 1 and 2). The results obtained with these three related species in the present study were generally in good agreement with those

reported in the literature; exceptions are noted in the footnotes of Tables 1 and 2. TNJL143-2^T was positive for catalase, pyrazinamidase, alkaline phosphatase, *N*-acetyl-β-glucosaminidase and gelatinase, but was negative for nitrate reduction (Table 1). Acid production under aerobic conditions was observed with *N*-acetylglucosamine, inulin and glycogen, but not with L-rhamnose or D-mannitol

Table 1. Biochemical characteristics that differentiate *M. natoriense* sp. nov. TNJL143-2^T from related type strains

Characteristics shown were examined using an API Coryne kit. Strains: 1, TNJL143-2^T; 2, *M. aerolatum* JCM 12137^T; 3, *M. foliorum* JCM 11569^T; 4, *M. phyllosphaerae* JCM 11571^T. Symbols: +, positive; w+, weakly positive; –, negative. All strains were positive for β-galactosidase, α-glucosidase and β-glucosidase activities [according to Zlamala *et al.* (2002), *M. aerolatum* is negative for β-galactosidase activity]. All were negative for casein hydrolysis, β-glucuronidase, pyrrolidonyl arylamidase and urease.

Characteristic	1	2	3	4
Catalase	+	w+	+	+
Nitrate reduction	–	+	–	–
Pyrazinamidase	+	–	+	–
Alkaline phosphatase	+	–	+	+
<i>N</i> -Acetyl-β-glucosaminidase	+	+	–	+
Gelatinase	+	–	+	–*

*Positive according to Behrendt *et al.* (2001).

Table 2. Acid production from carbohydrates by *M. natoriense* sp. nov. TNJL143-2^T and related type strains

Strains: 1, TNJL143-2^T; 2, *M. aerolatum* JCM 12137^T; 3, *M. foliorum* JCM 11569^T; 4, *M. phyllosphaerae* JCM 11571^T. Symbols: +, positive; w+, weakly positive; −, negative. Acid production was examined by using an API 50 CH kit under aerobic conditions. All strains were negative for acid production from D-erythritol, L-xylose, D-adonitol, methyl β-D-xylopyranoside, L-sorbose, dulcitol, inositol, D-sorbitol, xylitol, D-tagatose, D-fucose, D-arabitol, L-arabitol, potassium gluconate, potassium 2-ketogluconate and potassium 5-ketogluconate. All strains were positive for acid production from L-arabinose, D-xylose, D-galactose, D-glucose, D-fructose, D-mannose, methyl α-D-glucopyranoside, amygdalin, arbutin, aesculin, salicin, D-cellobiose, D-maltose, sucrose, trehalose, D-melezitose and D-turanose.

Carbohydrate	1	2	3	4
Glycerol	+	−	+	+
D-Arabinose	−	+	−	+ ^{a*}
D-Ribose	−	+ ^b	w+	+
L-Rhamnose	−	+	w+	+
D-Mannitol	−	+	+	+
N-Acetyl-D-glucosamine	+	−	−	−
D-Lactose	−	−	−	w+
D-Melibiose	+	−	−	+
Inulin	+	−	−	−
D-Raffinose	+	−	w+	+
Starch	+	+	−	−
Glycogen	+	−	−	−
Gentiobiose	−	+	+ ^a	+

*Results that contradict published data are indicated by: *a*, negative according to Behrendt *et al.* (2001); *b*, negative according to Zlamala *et al.* (2002).

(Table 2). These characteristics distinguish strain TNJL143-2^T from the phylogenetically related species of *Microbacterium*.

Description of *Microbacterium natoriense* sp. nov.

Microbacterium natoriense (na.to.ri.en'se. N.L. neut. adj. *natoriense* of Natori, Miyagi, Japan, where the type strain was isolated).

Rod-shaped, Gram-positive, non-spore-forming, strictly aerobic organism. The rods measure 0.5–0.6 × 1.5 µm. No motility is observed. The temperature range for growth is 10–40 °C (optimum 30 °C). The pH range for growth is 5–9 (optimum pH 5–7). The organism grows in the presence of 6% (w/v) NaCl in LB medium. Positive results are obtained for catalase, pyrazinamidase, alkaline phosphatase, β-galactosidase, α-glucosidase, N-acetyl-β-glucosaminidase, β-glucosidase and gelatinase, but test results for oxidation/fermentation, oxidase, casein hydrolysis, nitrate reduction, pyrrolidonyl arylamidase,

β-glucuronidase and urease are negative. Test results for acid production from the following carbohydrates are positive: glycerol, L-arabinose, D-xylose, D-galactose, D-glucose, D-fructose, D-mannose, methyl α-D-glucopyranoside, N-acetyl-D-glucosamine, amygdalin, arbutin, aesculin, salicin, D-cellobiose, D-maltose, D-melibiose, sucrose, trehalose, inulin, D-melezitose, D-raffinose, starch, glycogen and D-turanose. Acid production is not observed from the following: erythritol, D-arabinose, D-ribose, L-xylose, D-adonitol, methyl β-D-xylopyranoside, L-sorbose, L-rhamnose, dulcitol, inositol, D-mannitol, D-sorbitol, lactose, xylitol, gentiobiose, D-tagatose, D-fucose, D-arabitol, L-arabitol, potassium gluconate, potassium 2-ketogluconate or potassium 5-ketogluconate. The cell-wall diamino acid is ornithine. The major fatty acids are C_{15:0} anteiso (45.0 mol%), C_{15:0} iso (17.0 mol%) and C_{17:0} anteiso (16.9 mol%). The major menaquinones are MK-9, MK-10, MK-11 and MK-12. The G + C content of the genomic DNA of the type strain is 69.1 mol%.

The type strain, strain TNJL143-2^T (=JCM 12611^T =ATCC BAA-1032^T), was isolated from a soil sample obtained at Natori, Miyagi, Japan.

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