Microbacterium tumbae sp. nov., an actinobacterium isolated from the stone chamber of ancient tumulus

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Abstract

Eight strains characterised as Gram-stain-positive, non-spore-forming and non-motile rods were isolated from samples collected from stone chambers of the Takamatsuzuka and Kitora tumuli in Asuka village, Nara Prefecture, Japan. Among them, one strain, T7528-3-6b, was shown to form a novel lineage within the genus Microbacterium. The most closely phylogenetically related species to T7528-3-6b was Microbacterium panaciterrae, with 97.8 % sequence similarity. The major isoprenoid quinones of T7528-3-6b were MK-12, MK-13 and MK-11. The predominant cellular fatty acids for this isolate were anteiso-C₁₅ : 0, anteiso-C₁₇ : 0, iso-C₁₆ : 0 and iso-C₁₅ : 0. The diagnostic diamino acid of the peptidoglycan of this isolate was ornithine. Major polar lipids of the isolate were phosphatidylglycerol, diphosphatidylglycerol and an unknown glycolipid. The G+C content of the genomic DNA of this isolate was 70.1 mol%. On the basis of the results of physiological, biochemical and chemotaxonomic tests and molecular phylogenetic analysis, T7528-3-6b is considered to represent a novel species of the genus Microbacterium, for which the name M. tumbae sp. nov. has been proposed. The type strain is T7528-3-6b (=JCM 28836=NCIMB 15039). The results of comparisons of both phenotypic and genotypic (16S rRNA gene sequence) characteristics indicated that the remaining seven isolates were very closely related to Microbacterium shaanxiense. Although the sequence similarity between the two was 99.2 %, further detailed multifaceted comparisons are needed to determine their accurate taxonomic assignment.

The Takamatsuzuka Tumulus (TT) and Kitora Tumulus (KT), both circular burial mounds located in Asuka village, Nara Prefecture, Japan, are thought to have been built around the late seventh to early eighth century. When discovered, both tumuli contained 1300-year-old polychrome mural paintings on the thin plaster of the stone walls inside their stone chambers. These murals are well known to the Japanese. However, they have been damaged by severe biodeterioration and various disturbances since their respective discoveries in 1972 and 1983 [1]. Since 2004, we have been conducting microbiological surveys of the TT and KT stone chamber interiors to elucidate the cause of this biodeterioration of the mural paintings and plaster walls [2]. From the results of our taxonomic/systematic studies of TT and KT microbial isolates, the major colonisers and biodeteriogens of both tumuli have been described as members of nine novel bacterial species [3–6]. During these surveys, we also isolated eight strains of members of the genus Microbacterium from samples collected from both tumuli (Table S1, available in the online Supplementary Material). Some members of the class Actinobacteria have been thought to play an important role in the biodeterioration and bioprecipitation of minerals in caves, for example, at the Altamira Cave in Spain [7]. It was considered that CaCO₃ deposits or calcite crystals in this cave were products of the activity of members of the class Actinobacteria as biodeteriogens [8].

The murals inside the TT and KT stone chambers had been directly painted onto the thin layers of the plaster, which contained CaCO₃ as a major component [1, 9]. Therefore, damage to the plaster might have occurred via activities of microorganisms, including members of the class Actinobacteria that colonised the plaster surface.

At the time of writing, a total of 97 species of the genus Microbacterium have been recognised (LPSN, www."


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Abbreviations: TT, Takamatsuzuka Tumulus; KT, Kitora Tumulus.

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Two supplementary tables and five supplementary figures are available with the online Supplementary Material.
bacterio.net/), with *Microbacterium lacticum* being the type species [10]. This genus is thought to be widespread as it has been isolated from various samples, such as foods, fresh water, seawater, soil, sediment, activated sludge, animals and plants [11]. In this article, we described the taxonomic characterisation of the isolates from the stone chambers of the TT and KT and propose one novel species in the genus *Microbacterium* (family Microbacteriaceae, order Actinomycetales, class Actinobacteria), with the type strain T7528-3-6b\textsuperscript{T}. In addition, a brief note has been provided on the recently proposed novel species *Microbacterium shaanxiense*, which has been isolated from the nodule surface of soybean in China [12], in which seven of our isolates were accommodated.

A total of six samples collected from the TT and two samples collected from the KT were used for bacterial isolation (Table S1). Two of the TT samples were collected using a cotton swab from mouldy spots on the surface of the murals, whereas the remaining four samples were collected during dismantling of the stone chamber. The KT samples were collected after the relocation of the mural paintings from the interior of the stone chamber. Bacterial isolation was performed by a method previously described [5]. The common bacteria were isolated using nutrient agar (Code: CM3; Oxoid) under aerobic conditions at 30 °C for 3 days. After purification of the bacterial colonies, 16S rRNA gene sequence determination was performed for the representative strains of each isolate group. For preservation, cells of the isolates were grown on CM3 plates at 30 °C for 2 days, suspended in sterilised distilled water supplemented with 20 % (v/v) glycerol and stored at −80 °C. A total of eight isolates were used (Table S1).

Type strains of species of the genus *Microbacterium* closely related to our eight isolates, namely, *Microbacterium profundi* DSM 14840\textsuperscript{T}, *Microbacterium murale* DSM 22178\textsuperscript{T} and *Microbacterium panaciterrae* JCM 17839\textsuperscript{T}, were used as references. These reference strains were cultured in accordance with the recommended methods of the respective suppliers.

The almost complete 16S rRNA gene sequences of all eight isolates (1483–1485 bp) were determined using previously described methods [3]. The results of sequence comparisons based on the 16S rRNA gene sequences in DDBJ (www.ddbj.nig.ac.jp/) by BLAST [13] indicated that the eight isolates represented members of the genus *Microbacterium*. These isolates were separated into two groups based on the similarity in the 16S rRNA gene sequences. The sequence of one strain, T7528-3-6b\textsuperscript{T} (one group), showed high sequence similarity to those of *Microbacterium kyungjeense* THGC26\textsuperscript{T} (98.0 % similarity), *Microbacterium arabinogalactanolyticum* DSM 8611\textsuperscript{T} (97.9 %), *M. panaciterrae* DCY37\textsuperscript{T} (97.8 %), *Microbacterium ginsengiterrae* DCY37\textsuperscript{T} (97.8 %) and *Microbacterium azadirachtae* AI-S262\textsuperscript{T} (97.7 %). On the other hand, the sequences of T6220-7-3b and the other six isolates (another group) were completely identical and were most closely related to those of *M. shaanxiense* CCNWS60\textsuperscript{T} (99.2 %), *M. profundi* Shh49\textsuperscript{T} (98.7 %) and *M. murale* DSM 22178\textsuperscript{T} (98.7 %). The sequence similarity between the two groups of isolates was 97.9 %. Phylogenetic trees were reconstructed using MEGA version 6.0 [14], and three algorithms, neighbour-joining (NJ) [15], maximum likelihood (ML) [14, 16, 17] and maximum-parsimony (MP) [18, 19] were used. Cluster formation reliability was assessed by bootstrapping [20]. All three algorithms we used gave similar tree topologies and bootstrap robustness values (Figs 1 and S1). The respective strains T6220-7-3b (and six other strains) and T7528-3-6b\textsuperscript{T} were assigned together in a cluster of the genus *Microbacterium* in the phylogenetic trees depicted in Figs 1 and S1. One isolate, T7528-3-6b\textsuperscript{T}, was placed in a novel lineage in the genus *Microbacterium* and formed a cluster distantly with *M. panaciterrae* DCY56\textsuperscript{T}. On the other hand, the 16S rRNA gene sequence-based NJ (Fig. 1) and MP (Fig. S1) trees showed that the seven other isolates formed a monophyletic cluster with *M. shaanxiense* CCNWS60\textsuperscript{T} incidentally, the statistical confidences, determined by bootstrap analysis, in the NJ and MP trees were 100 and 84 %, respectively. The phylogenetic positions of the isolates among all known species of the genus *Microbacterium* are shown in Fig. S2. Together with the low 16S rRNA gene sequence similarities of isolate T7528-3-6b\textsuperscript{T} with the respective type strains of the closely related species of the genus *Microbacterium*, these results indicate that isolate T7528-3-6b\textsuperscript{T} represents a novel species of the genus *Microbacterium*.

Two representative isolates from the two different groups, T7528-3-6b\textsuperscript{T} and T6220-7-3b, were selected for subsequent experiments. These isolates were cultivated at 30 °C using soybean–casein digest agar [SCD agar; 15 g pancreatic digest of casein (BBL), 5 g papaic digest of soybean meal (BBL), 5 g sodium chloride, 15 g agar and 1 l distilled water; pH 7.3]. Anaerobic cultivation was conducted by absorbing oxygen using an Anaero Pack gas system (Anaero Pack disposable, Mitsubishi Gas Chemical), and the isolates were inoculated on SCD plates for 7 days at 30 °C. The methods for observing cell morphology and characterising conventional phenotypic features were in accordance with the procedures described by Barrow and Feltham [21] and Tindal et al. [22]. API 20NE, API 20E, API 50CHB and API ZYM kits (bioMérieux) were used for assessing biochemical features, acid production from carbon sources and enzyme activities, in accordance with the manufacturer’s instructions, except that the incubation time in API ZYM tests was extended to 24 h, which gave reliable results. In addition, assimilation of the carbon source with the M70 medium [23] was examined for the selected carbon sources. Growth at a range of temperatures (10, 15, 20, 25, 30, 37 and 40 °C) was assessed on SCD agar. Growth at various salt concentrations (0–10 % w/v NaCl at 1 % increments as well as 12, 15 and 20 % w/v NaCl) was determined in SCD broth, which was made without NaCl from the normal formula as a basal medium (normal formula: 17 g pancreatic digest of casein, 3 g papaic digest of soybean meal, 5 g sodium chloride, 2.5 g dipotassium phosphate, 2.5 g glucose and 1 l
distilled water; pH 7.3), supplemented with an appropriate amount of NaCl. Growth for the pH range (values tested pH 5.5–10.5 in 0.5 increments) was tested using SCD broth with Good’s buffer [24].

The two isolates were Gram-stain-positive, catalase-positive and oxidase-negative, non-spore forming and non-motile. The cells of both isolates were rods with no flagellum. The isolates possessed a respiratory-type metabolism, growing aerobically and being incapable of growing under anaerobic conditions. Colonies of T7528-3-6b T grew at temperatures between 10 and 37°C, with an optimum range of 25–30°C; in the presence of 0–8% (w/v) NaCl, with an optimum range of 0–1% and at pH 5.5–9.0, with an optimum pH of 7.5–8.5. On the other hand, T6220-7-3b grew at temperatures between 4 and 37°C, with an optimum range of 25–30°C; in the presence of 0–12% (w/v) NaCl, with an optimum range of 0–3%, and at pH 5.0–10.0, with an optimum pH of 6.5–7.0. The other

**Fig. 1.** Neighbour-joining tree based on the nearly complete 16S rRNA gene sequences (1322 nt) of the isolates and type strains of their related species. Bootstrap values (percentages of 1000 replications) of >50% are shown at the respective selected branches. Filled circles indicate that the corresponding nodes were recovered in a tree generated with the maximum-likelihood method with bootstrap values of >70%. *Cellulomonas flavigena* was used as an outgroup. Bar, one change per 100 nucleotide positions.
The isoprenoid quinone system of the genus Microbacterium was determined. The predominant cellular fatty acids for T7528-3-6b were anteiso-C₁₅:0 (46.5 %), anteiso-C₁₇:0 (21.1 %), iso-C₁₆:0 (18.3 %) and iso-C₁₅:0 (10.1 %). Major fatty acids of T6220-7-3b were anteiso-C₁₅:0 (43.3 %), iso-C₁₆:0 (27.1 %), iso-C₁₅:0 (12.0 %) and anteiso-C₁₇:0 (11.9 %) (Table 2). These dominant fatty acids of the isolates were similar to those of other known species of the genus Microbacterium [11]. For polar lipid analysis, the cells of the two isolates were grown in SCD broth with shaking at 30 °C for 48 h. Polar lipids of the two isolates were extracted from freeze-dried cells by a modified version of the method by Bligh and Dyer [26], as described by Minnink et al. [27]. Extracted lipids were then separated by two-dimensional HPTLC on silica gel 60 plates (Merck) using chloroform/methanol/water (65:25:4 by volume) in the first dimension and chloroform/acetone/diethyl ether/methanol/water (80:18:12.5:5 by volume) in the second dimension. Total lipid components were detected by spraying with 10 % (w/v)
molybdophosphoric acid in isopropanol and incubating at 180 °C for 20 min. Specific lipids were detected using spray reagents (α-naphthol-sulfuric acid, ninhydrin, Dittmer–Lester and periodate–Schiff), in accordance with the methods described by Tindall et al. [22]. Major polar lipids of both isolates were phosphatidylglycerol, diphosphatidylglycerol and an unknown glycolipid (Fig. S3). The preparation of cell wall peptidoglycan and analysis of amino acids in the cell wall were performed in accordance with the work by Nakai et al. [28]. Amino acids in the cell wall hydrolysate were determined to be phenylisothiocyanate (Wako) derivatives by ultra-performance liquid chromatography (Acquity Ultra-Performance LC System, Waters). The cell wall of T7528-3-6bT contained 3-hydroxyglutamic acid (denoted as Hyg), glutamic acid (Glu), glycine (Gly), alanine (Ala) and ornithine (Orn). These results are similar to those for the most closely related species M. panaciterrae [29]. The cell wall of isolate T6220-7-3bT contained Hyg, Glu, Gly, homoserine (Hse), Ala and Orn, and these coincided well with the cell walls of the closely related species, M. murale [30] and this study) and M. profundii (this study). However, the peptidoglycan type of T6220-7-3bT was different from that of M. shaanxiense [12] (Table 1). In the original paper, the peptidoglycan of M. profundii was reported to contain Ala, Gly, Orn and two unidentified amino acids [31]. Muramic acid acetylation was analysed in accordance with the work by Uchida et al. [32]. The muramic acid type of peptidoglycan of both isolates was the glycolyl type.

The cells of both isolates were grown in the SCD broth at 30 °C for 5 days for the analysis of whole-cell sugars. The whole-cell sugars were analysed in accordance with the method described by Staneck and Roberts [33], except that the solvent 1-butanol/pyridine/water (5 : 3 : 2, by volume) [34] was used for TLC development. The whole-cell sugars for T7528-3-6bT were ribose, glucose and galactose (Fig. S4), whereas those for T6220-7-3bT were rhamnose, ribose and galactose as major components and glucose as a minor component. Additionally, an unknown hexose was detected above the rhamnose spot on the TLC plate as a major component (Fig. S4).

For matrix-assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI–TOF/MS) bacterial profiling, the cells of all eight isolates (Table S1) and three reference strains we used were grown on SCD agar plates at 25 °C for 48 h. The ethanol–formic acid sample preparation method was used for analysis using the VITEK MS Plus System (bioMérieux). Data obtained were analysed using the VITEK MS database (VITEK MS V2.0 Knowledge Base) with default settings. A dendrogram was reconstructed based on the data obtained on the isolates and their related reference strains and some data for species of the genus Microbacterium in the VITEK MS database using the SARAMIS Premium program (bioMérieux), as shown in Fig. S5. T7528-3-6bT was positioned separately from other species, including the phylogenetically most closely related species M. panaciterrae JCM 17839T. On the other hand, the other seven isolates, including isolate T6220-7-3bT, showed identical 16S rRNA gene sequences and formed a cluster with greater than 70 % similarity based on the MS data. Therefore, these seven isolates were thought to represent the same species of the genus Microbacterium.

DNA extraction, purification and determination of the G+C content of genomic DNA of the isolates were performed in accordance with previously described methods [3] with two lytic enzymes, achromopeptidase (Wako Pure Chemical Industries) and lysozyme (Wako Chemical), for cell wall lysis [35]. The G+C contents of the DNA of T7528-3-6bT and T6220-7-3bT were 70.1 and 67.4 mol%, respectively.

Table 1 summarises the characteristics that differentiate the novel species proposed herein based on one isolate, T7528-3-6bT, and its closest phylogenetic neighbour, M. panaciterrae [29]. T7528-3-6bT can be distinguished from M. panaciterrae on the basis of its quinone composition, whole-cell sugars and major cellular fatty acids, along with the phylogenetic positions. Additionally, the MALDI-TOF/MS profile indicated that these two strains, T7528-3-6bT and M. panaciterrae JCM 17839T, represented different species within the genus Microbacterium.

In conclusion, the results derived from our polyphasic systematic approach for T7528-3-6bT indicate that it represents a novel species of the genus Microbacterium, for which we propose the name M. tumbae sp. nov.

In addition to the above-mentioned proposal, the phenotypic and genotypic (molecular) characteristics of the seven isolates were found to be almost identical to those of M. shaanxiense, which was recently proposed by Peng et al. [12] based on an isolate from the nodule surface of a soybean (Glycine max) cultivar, Zhonghuang 13, from a field in Yangling, Shaanxi Province, China. The major phenotypic characteristics are shown in Tables 1 and 2. The phylogenetic placement of the isolates based on 16S rRNA gene sequence divergence is shown in Figs 1 and S1. Strain data on the respective isolates assignable to M. shaanxiense are provided in Tables 1 and S2. Some characteristics such as enzyme activities, acid production from carbon sources, cellular fatty acid composition, cell wall peptidoglycan and whole-cell sugars differed from those of isolate T6220-7-3bT. The seven isolates and M. shaanxiense were positioned within the same cluster based on the results of phylogenetic analysis (Figs 1 and S1). Therefore, it seemed that the seven isolates represented M. shaanxiense. However, further detailed comparisons are needed to unravel the identity of the seven TT and KT isolates that are currently assigned to M. shaanxiense.

**DESCRIPTION OF MICROBACTERIUM TUMBAE SP. NOV.**

Microbacterium tumbae [tum’bae. L. gen. fem. n. tumbae of tomb, referring to the isolation source of the type strain].
Cells are Gram-stain-positive rods measuring 0.6–0.7×1.0–1.2 µm and are non-motile. Strictly aerobic, catalase-positive, and oxidase-negative. Grows well on R2A agar, nutrient agar and SCD agar, but not on MacConkey agar. Growth occurs at pH 5.5–9.0, with the optimum pH being 7.5–8.5, and at 10–37 °C, with the optimum temperature being 25–30 °C. NaCl tolerance is up to 8%, with an optimum range of 0–1%. Nitrate is not reduced to nitrite. Not able to ferment glucose. Positive for the hydrolysis of DNA and Tween 80 (weakly). Negative for the hydrolysis of starch, casein, lecithin, tyrosine and Tween 20 and gelatin liquefaction. In API 20NE tests, positive for asaccharolysis and 2-nitrophenyl-β-D-galactopyranoside (PNPG) test; assimilation of D-glucose, L-arabinose, D-mannose, D-mannitol, maltose and malic acid. Negative for production of indole, activities of urease and arginine dihydrolase; assimilation of N-acetylglucosamine, potassium gluconate, capric acid, adipic acid, trisodium citrate and phenylacetic acid. In API 20E tests, negative for H₂S production, and activities of L-lysine decarboxylase, ornithine decarboxylase and tryptophan deaminase, and at 10°C, 15°C and 20°C. Acetate, L-glucose, L-xylose, turanose, xyitol (weakly), gentiobiose (weakly), turanose, D-lyxose and L-fucose (weakly) but not from glycerol, erythritol, D-arabinose, L-xylene, L-adonitol, methyl-β-D-xlyopyranoside, L-sorbitose, L-rhamnose, dulcitol, inositol, D-sorbitol, methyl-α-D-mannopyranoside, methyl-α-D-glucopyranoside, N-acetylgalactosamine, lactose, melibiose, inulin, melezitose, raffinose, starch, glycollen, D-tagatose, D-fucose, D-arabitol, L-arabinose, potassium gluconate, potassium 2-ketogluconate and potassium 5-ketogluconate. In sole carbon source utilization, examined on M70 medium, succinate is utilised, whereas celllobiose, propionate, fumarate, citrate, L-arginine and L-alanine are not utilised. Acetate, L-aspartate, malate and pyruvate are weakly utilised. The predominant menaquinones are MK-12, MK-13 and MK-11. The predominant cellular fatty acids are anteiso-C₁₅:₀, anteiso-C₁₇:₀, iso-C₁₅:₀ and iso-C₁₅:₀. The polar lipids are phosphatidylglycerol, diphosphatidylglycerol and an unknown glycolipid. The cell wall peptidoglycans contain 3-hydroxyglutamic acid, glutamic acid, glycine, alanine and ornithine. The muramic acid type of the peptidoglycan is the glycyl type. The whole-cell sugars are ribose, glucose and galactose.

The type strain T7528-36bT (=JCM 28836T=NCIMB 15039T) was isolated from a sample collected (May 28, 2007) between east wall 1 and ceiling stone 2 during the relocation of the Takamatsuzuka Tumulus stone chamber in Asuka village, Nara Prefecture, Japan. The DNA G+C content of the type strain is 70.1 mol%.