Humibacillus xanthopallidus gen. nov., sp. nov.

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Strain KV-663^T was isolated from a soil sample collected from a paddy field in Japan by using GPM agar plates. Strain YM21-029 was obtained from a lake sediment sample by using HSV agar. The two novel strains were Gram-positive, catalase-positive, rod-shaped bacteria with LL-diaminopimelic acid as the diagnostic diamino acid of the cell-wall peptidoglycan. The major menaquinone was MK-8(H₄). Mycolic acids were not detected. The G + C content of the DNA was 69–70 mol%. 16S rRNA gene sequence analysis revealed that these strains represent a novel lineage within the family *Intrasporangiaceae*, order *Actinomycetales*, and are related to members of the genera *Intrasporangium* and *Terracoccus*. Based on morphological, biochemical and chemotaxonomic properties, together with phylogenetic analysis based on 16S rRNA gene sequences, the two new strains are considered to represent a novel species of a new genus, for which the name *Humibacillus xanthopallidus* gen. nov., sp. nov. is proposed. The type strain of the type species, *Humibacillus xanthopallidus*, is KV-663^T (=NRRL B-24471^T=NBRC 101803^T).

The family *Intrasporangiaceae* Rainey *et al.* 1997 was proposed by Stackebrandt *et al.* (1997) and the description of the family was later emended by Stackebrandt & Schumann (2000). At the time of writing, the family comprised 13 genera, and could be divided into three groups based on the occurrence of LL-diaminopimelic acid (A₂pm), meso-A₂pm or L-ornithine as the diagnostic diamino acid in the cell-wall peptidoglycan. Two novel strains within the family are described herein. Strain KV-663^T was isolated from the same soil sample from which strains of the recently described genus *Oryzihumus* (Kageyama *et al.*, 2005) were obtained. In contrast, strain YM21-029 was isolated from a sediment sample collected in Japan by using HSV agar plates. The components of the HSV medium are detailed in Supplementary Table S1 (see IJSEM Online). Humic acid was purchased from Acros Organics Inc. and was purified according to the protocol of the International Humic Substances Society (<http://www.ihss.gatech.edu/soilhafa.html>). Strains KV-663^T and YM21-029 were isolated from different sources and by using two different methods. However, morphological, physiological and biochemical characteristics, 16S rRNA

gene sequence analysis and DNA–DNA hybridization experiments revealed that the two strains represented a single novel species of a new genus.

Strain KV-663^T was isolated from a soil sample collected from a paddy field in Saitama Prefecture, Japan. A 2 g soil sample was suspended in 18 ml sterile water and mixed. Soil particles were allowed to sediment, the liquid phase was then diluted 10⁵-fold and 100 µl of the liquid was spread onto the surfaces of GPM agar plates (per litre: 10 g glucose, 5 g peptone, 5 g meat extract, 3 g NaCl, 12 g agar; pH 7.0) (Takahashi *et al.*, 2003). The plates were cultured at 27 °C. Strain YM21-029 was isolated from a sediment sample collected from Lake Jusanko, Aomori Prefecture, Japan (depth, 20 cm; GPS location, 41° 59' 48.8" N 140° 21' 33.2" E), in September 2005. The samples (0.5–1 cm³) were homogenized and suspended in 5 ml sterile seawater. Strain YM21-029 was isolated on HSV medium, by using a 50 µl sample of the suspension, and the plate was cultured at 25 °C for 30 days. Biomass for biochemical and chemotaxonomic studies was prepared by culturing the strains in trypticase soy broth (TSB; Difco) at 27 °C for 3 or 4 days.

Morphological observation via a scanning electron microscope (JSM-5600; JEOL) was performed on cultures grown on GPM agar medium at 27 °C for 2 days. The ability of each strain to grow on a range of sole carbon sources at 1 % (w/v) was determined by using carbon utilization media (Pridham & Gottlieb, 1948). Tolerance of NaCl and pH

Abbreviation: A₂pm, LL-diaminopimelic acid.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains KV-663^T and YM21-029 are AB282888 and AB286022, respectively.

A table detailing the components of medium HSV is available with the online version of this paper.

and temperature ranges for growth were determined on one-fifth-strength nutrient agar. The two isolates were characterized biochemically by using the API ZYM system in accordance with the manufacturer's instructions (bioMérieux). Assimilation of sugars by strain KV-663^T was determined by using the API 50CH system (bioMérieux).

Whole-cell hydrolysates were analysed for A₂pm isomers by using TLC (Hasegawa *et al.*, 1983). Purified cell walls were obtained according to the method of Kawamoto *et al.* (1981). A 1 mg sample of purified cell wall was hydrolysed at 100 °C with 1 ml 6 M HCl for 16 h. The residue was dissolved in 100 µl water and this solution was used for amino acid analysis. The amino acid composition was determined by HPLC by using the Pico Tag method (Waters). Samples were derivatized with phenylisothiocyanate and UV (254 nm) detection was used. The presence of mycolic acids was assessed by using the TLC method of Tomiyasu (1982). Polar lipid profiles were determined by TLC (Minnikin *et al.*, 1977). Whole-cell sugars were identified by the method described by Becker *et al.* (1965). Menaquinones were extracted and purified according to Collins *et al.* (1977) and then analysed by HPLC (802-SC; Jasco) by using a chromatograph equipped with a Capcell Pak C18 column (Shiseido) (Tamaoka *et al.*, 1983). Methyl esters of cellular fatty acids were prepared and analysed by GLC (HP6890; Hewlett Packard).

DNA base composition and levels of DNA–DNA relatedness were determined. DNA was isolated as described by Saito & Miura (1963), with some modifications. DNA base composition was estimated by HPLC (Tamaoka & Komagata, 1984). Levels of DNA–DNA relatedness were determined by using the method of Ezaki *et al.* (1989).

DNA was prepared by sonication (Yu *et al.*, 2002) or by using the InstaGene matrix (Bio-Rad). The 16S rRNA gene was amplified by PCR and sequenced with an automatic sequence analyser (ABI Prism 3130 or 3730; PE Applied Biosystems) by using a dye terminator cycle sequencing kit (PE Applied Biosystems).

Species related to the novel isolates were identified by performing sequence database searches with the BLAST program (Altschul *et al.*, 1990). Sequence data of related species were retrieved from GenBank. Nucleotide substitution rates (K_{nuc} values) were calculated (Kimura & Ohta, 1972) and phylogenetic trees were constructed by using the neighbour-joining method (Saitou & Nei, 1987). Statistical significance of the tree topology was evaluated by bootstrap analysis of sequence data by using CLUSTAL W software (Thompson *et al.*, 1994). Sequence similarity values were determined by visual comparison and manual calculation.

Nearly-complete 16S rRNA gene sequences were determined for the two isolated strains. A database search demonstrated that these strains belonged to the suborder *Micrococcineae* of the family *Intrasporangiaceae*. It was clear from the phylogenetic tree (Fig. 1) that the two strains formed a monophyletic clade that was associated with *Intrasporangium calvum* and *Terracoccus luteus*. Strains KV-663^T and YM21-029 shared a 16S rRNA gene sequence similarity of 99.2%; levels of sequence similarity between strain KV-663^T and the type strains of *Intrasporangium calvum*, *Terracoccus luteus* and *Arsenicicoccus bolidensis* were 97.4, 97.8 and 93.8 %, respectively.

The DNA base composition of the novel isolates was 69–70 mol% G + C based on HPLC of nucleosides. The cell-wall peptidoglycan of strain KV-663^T contained glutamic acid, glycine, alanine and LL-A₂pm at a molar ratio of 1:1.7:1.3:0.8. The polar lipid of strain KV-663^T was phosphatidylethanolamine. Whole-cell sugars of strain KV-663^T were galactose, glucose and rhamnose. The two novel isolates had peptidoglycan of type A3γ (Schleifer & Kandler, 1972). The predominant menaquinone was MK-8(H₄) (about 90 %). Mycolic acids were not detected. The cellular fatty acids of strains KV-663^T and YM21-029 were iso-C_{14:0} (4.4 and 6.0 %, respectively), iso-C_{15:0} (39.7 and 34.9 %), anteiso-C_{15:0} (8.1 and 11.1 %), iso-C_{16:0} (8.0 and 8.8 %), iso-C_{17:0} (4.4 and 4.1 %), anteiso-C_{14:0} (4.2 and 5.8 %), C_{17:1ω8c} (7.5 and 5.7 %), C_{17:0} (4.0 and 5.7 %) and C_{18:1ω9c} (4.3 and 3.7 %). The predominant cellular fatty acid component was iso-C_{15:0}. Strain

Fig. 1. Phylogenetic tree showing the position of strains KV-663^T and YM21-029 derived from 16S rRNA gene sequences and constructed according to the neighbour-joining method and K_{nuc} values. Numbers at the branch points are bootstrap values (1000 resamplings). The tree was unrooted and *Janibacter limosus* DSM 11140^T was used as an outgroup. Bar, 0.002 K_{nuc} .

KV-663^T was able to assimilate glycerol, ribose, glucose, fructose, mannose, mannitol, maltose, sucrose and trehalose, but not 40 other compounds in the API 50CH system.

Of the 13 recognized genera within the family *Intrasporangiaceae*, *Arsenicicoccus* (Collins *et al.*, 2004), *Intrasporangium* (Kalakoutskii *et al.*, 1967), *Terrabacter* (Collins *et al.*, 1989) and *Terracoccus* (Prauser *et al.*, 1997) have LL-A₂pm in their cell walls. Differential characteristics between strains KV-663^T and YM21-029 and LL-A₂pm-containing members of the family *Intrasporangiaceae* are detailed in Table 1. The major cellular fatty acids of strains KV-663^T and YM21-029 were a complex mixture of straight-chain saturated, monounsaturated, and iso- and anteiso-methyl-branched acids. Members of the genus *Arsenicicoccus* show the same major cellular fatty acid profile, but differ with respect to cell morphology and the number of glycine residues in the peptidoglycan. Strain KV-663^T has two glycine residues within the peptidoglycan. In contrast, *Arsenicicoccus* has a single glycine residue within the murein interpeptide bridge, whereas the genera *Intrasporangium*, *Terrabacter* and *Terracoccus* have three glycine residues within the murein interpeptide bridge (Collins *et al.*, 2004). Given these observations, we suggest that strains KV-663^T and YM21-029 belong to a new genus in the family *Intrasporangiaceae*.

In order to confirm that these two strains belong to the same species, DNA–DNA hybridization experiments were performed (duplicate procedures). Levels of relatedness between strains KV-663^T and YM21-029 were 77–78 %, indicating that they belong to the same species.

The two novel strains were also examined for a range of phenotypic and chemotaxonomic characteristics. Physiological characteristics are provided in the species description below.

Based on their distinct phylogenetic position within the family *Intrasporangiaceae*, together with characteristic cell morphology, fatty acid composition and cell-wall murein type, strains KV-663^T and YM21-029 are considered to represent a novel species of a new genus, for which the name *Humibacillus xanthopallidus* gen. nov., sp. nov. is proposed.

Description of *Humibacillus* gen. nov.

Humibacillus (Hu.mi.ba.cil'lus. L. fem. n. *humus* soil; L. masc. n. *bacillus* rod; N.L. masc. n. *Humibacillus* rod isolated from soil).

Gram-positive, catalase-positive, aerobic, irregular rods. The peptidoglycan is of A3 γ type, containing LL-A₂pm, glycine, alanine and glutamic acid. Mycolic acids are absent. The major menaquinone is MK-8(H₄). The major cellular fatty acids are a complex mixture of straight-chain saturated, monounsaturated, and iso- and anteiso-methyl-branched components. The DNA G + C content is 69–70 mol%. Phylogenetically, the genus is a member of the family *Intrasporangiaceae*, suborder *Micrococcineae*. With regard to signature nucleotides defined for the family *Intrasporangiaceae*, at pair positions 1134 to 1140 strain KV-663^T has an A–U rather than a G–C pair. The type species is *Humibacillus xanthopallidus*.

Description of *Humibacillus xanthopallidus* sp. nov.

Humibacillus xanthopallidus (xan.tho.pal'li.dus. Gr. adj. *xanthos* yellow; L. adj. *pallidus* pale; N.L. masc. adj. *xanthopallidus* pale yellow).

Cells have diameters of 0.4–1.1 $\times$ 1.1–1.9 μ m (Fig. 2). Colonies are pale yellow. Growth occurs between pH 5 and 11 (optimal is pH 7), and between 7 and 32 °C (optimal temperatures are 27 and 32 °C for strains KV-663^T and YM21-029, respectively). In one-fifth-strength nutrient agar medium, growth occurs at up to 3 % (w/v) NaCl. Assimilates D-galactose, D-glucose, D-mannitol, D-mannose, raffinose, L-rhamnose, sucrose and trehalose, but not L-arabinose, D-fructose, maltose or D-xylose. Alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α -galactosidase, β -galactosidase, α -glucosidase and β -glucosidase are detected with the API ZYM enzyme assay; negative for N-acetyl- β -glucosaminidase and α -fucosidase. Weakly positive for lipase (C14). The DNA G + C content is 69–70 mol%.

Table 1. Differential characteristics between strains KV-663^T, YM21-029 and LL-diaminopimelic-acid-containing members of the family *Intrasporangiaceae*

Taxa: 1, strains KV-663^T and YM21-029; 2, *Arsenicicoccus* (data from Collins *et al.*, 2004); 3, *Intrasporangium* (Schumann *et al.*, 1997); 4, *Terrabacter* (Martin *et al.*, 1997); 5, *Terracoccus* (Prauser *et al.*, 1997). All taxa have LL-A₂pm as the cell-wall diamino acid.

Characteristic	1	2	3	4	5
Cell morphology	Irregular rods	Cocci	Hyphae	Cocci to rods	Cocci
DNA G + C content (mol%)	69–70	72	68	70–73	73
Major menaquinone	MK-8(H ₄)	MK-8(H ₄)	MK-8	MK-8(H ₄)	MK-8(H ₄)
Fatty acid type*	SAIU	SAIU	SAI	SAI	SAI

*A, anteiso methyl branched; I, iso methyl branched; S, straight-chain saturated; U, monounsaturated.

Fig. 2. Scanning electron micrograph of cells from a 2-day-old culture of strain KV-663^T grown on GPM agar medium at 27 °C. Bar, 2 µm.

The type strain, KV-663^T (=NRRL B-24471^T=NBRC 101803^T), was isolated from a soil sample collected from a paddy field. YM21-029, isolated from lake sediment, is a second strain of the species.

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