Patulibacter minatonensis gen. nov., sp. nov., a novel actinobacterium isolated using an agar medium supplemented with superoxide dismutase, and proposal of *Patulibacteraceae* fam. nov.

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A novel Gram-positive bacterial strain, designated KV-614^T, was isolated from a soil sample using an agar medium supplemented with superoxide dismutase. Based on 16S rRNA gene sequence analysis, it was found that the strain represented a novel deep-rooting lineage within the class *Actinobacteria* and clustered with yet-uncultivated bacteria from terrestrial environments and some unidentified strains isolated by unique methods. The most closely related established genus was *Conexibacter* (92.4 % sequence similarity to *Conexibacter woesei* DSM 14684^T). Cells of strain KV-614^T were rod-shaped and motile with long flagella. The strain was catalase-positive, oxidase-negative and grew under aerobic conditions. The cell-wall peptidoglycan contained *meso*-diaminopimelic acid as the diagnostic diamino acid and alanine and glutamic acid. The peptidoglycan acyl type was acetyl. The only detected isoprenoid quinone was demethylmenaquinone with seven isoprene units (DMK-7). Mycolic acids were not detected. The predominant cellular fatty acid was ω 9c-octadecenoic acid (C18 : 1 ω 9c). Minor components were 12-methyl tetradecanoic acid (anteiso-C15 : 0) and 14-methyl hexadecanoic acid (anteiso-C17 : 0). The DNA G + C content was 72 mol%. On the basis of phenotypic and genotypic characteristics, it is proposed that strain KV-614^T represents a new genus and a novel species, *Patulibacter minatonensis* gen. nov., sp. nov., in the class *Actinobacteria*. The type strain is KV-614^T (= NRRL B-24346^T = JCM 12834^T = NBRC 100761^T). The creation of the family *Patulibacteraceae* fam. nov. is proposed to encompass the genus *Patulibacter* gen. nov.

INTRODUCTION

The number of micro-organisms that have been successfully identified and cultured *in vitro* represents only a small portion of the total existing in nature; it has been estimated that the number of known bacteria is less than 10 % of the total number of bacterial species in the world (Whitman *et al.*, 1998; Schleifer, 2004). For many unknown micro-organisms, the appropriate cultivation conditions have not yet been found. In the course of searching for factors which promote bacterial colony growth, we found that using an isolation agar medium supplemented with superoxide dismutases (SODs) increased the number of colonies isolated from a

soil sample, a response that was further enhanced by the addition of catalase (Takahashi *et al.*, 2003). In this paper, we report the taxonomic characterization and classification of strain KV-614^T isolated from a soil sample using agar medium supplemented with SOD.

METHODS

Isolation and cultivation conditions. Strain KV-614^T was isolated from a soil sample collected at Minato-Ku, Tokyo, Japan, using an agar medium supplemented with SOD following the method of Takahashi *et al.* (2003). Glucose/peptone/meat extract (GPM) agar medium, consisting of 1 % D-glucose (Wako Pure Chem. Ind.), 0.5 % peptone (Kyokuto Seiyaku Co.), 0.5 % meat extract (Kyokuto Seiyaku Co.), 0.3 % NaCl and 1.2 % agar (Wako Pure Chem. Ind.) supplemented with 30 U ml⁻¹ *Escherichia coli* SOD (Sigma), was used for strain isolation. The strain was cultured on 1/5-strength nutrient agar (1/5 NA; Difco), ISP 3 medium (Shirling & Gottlieb, 1966), heart infusion agar, R2A agar and Todd–Hewitt agar (Difco) and yeast extract/glucose agar [YD agar; containing 1.0 % yeast

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Abbreviations: DAP, diaminopimelic acid; SOD, superoxide dismutase.

The GenBank/EMBL/DDBJ accession number for the partial 16S rRNA gene sequence of strain KV-614^T is AB193261.

extract (Difco), 1.0% glucose and 1.2% agar] for 7 days at 27 °C. Trypticase soy broth (BBL) was used for liquid culture. The strain was stored at 5 °C under lyophilization.

Morphology. The morphological characteristics of the strain were observed using a transmission electron microscope (JEM-1200EXII; JEOL) after incubation for 3 days at 27 °C in trypticase soy broth. Negative staining of cells was performed with 1% uranyl acetate. Gram-staining was performed using a Gram's stain reagent kit (Nacalai Tesque).

Physiology. Substrate utilization patterns were studied by using a GP2 microplate from the Biolog identification system. Enzyme activities were examined by using API ZYM test strips (bioMérieux). The API 20 NE test system (bioMérieux) was used for the investigation of additional physiological characteristics. Cells were grown on 1/5 NA for these tests. For determination of the temperature range for growth, bacteria were cultured on 1/5 NA medium. NaCl tolerance was examined using YD agar medium. The pH growth range was also determined using YD agar that was adjusted to pH values ranging between 5 and 9 with HCl or NaOH. Antibiotic susceptibility was determined by placing antibiotic discs (KB Disk; Eiken) on 1/5 NA plates seeded with suspensions of strain KV-614^T.

Chemotaxonomic characterization. Purified cell-wall preparations were obtained using the method of Schleifer & Kandler (1972). The amino acid composition of cell-wall hydrolysates (Becker *et al.*, 1965) was identified by TLC (Hasegawa *et al.*, 1983). The *N*-acyl type of muramic acid was determined using the colorimetric method of Uchida & Aida (1977). Isoprenoid quinone was extracted as described by Collins *et al.* (1977). The sample was analysed by HPLC with 802-SC chromatography (JASCO) using a CAPCELL PAK C18 column (Shiseido) (Tamaoka *et al.*, 1983) and was further analysed by MS and NMR. The detection of mycolic acids was performed by TLC (Tomiya, 1982). Methyl esters of cellular fatty acids were prepared by direct transmethylation with methanolic hydrochloride using cells grown on trypticase soy agar at 27 °C for 4 days and analysed on a GLC system (HP 6890; Hewlett Packard). Identification and quantification of the fatty acid methyl esters, as well as the numerical analysis of the fatty acid profiles, were performed according to the instructions for the Microbial Identification System (MIDI).

DNA G+C content. Chromosomal DNA was prepared following the procedure of Marmur (1961) and the DNA G+C content was determined by reverse-phase HPLC according to Tamaoka & Komagata (1984).

Phylogenetic analysis. DNA was isolated following the method of Marmur (1961). The 16S rRNA gene was amplified using primers described by Takahashi *et al.* (2002). Amplifications were performed in a TaKaRa thermal cycler (Takara) with an initial incubation of 1 min at 94 °C followed by 30 cycles of 1 min at 94 °C, 1 min at 50 °C and 1.5 min at 72 °C, followed by a 2 min final extension at 72 °C. The PCR products were purified using a QIAquick gel extraction kit (Qiagen) and were sequenced directly on a DNA sequencer (ABI PRISM 3100; Applied Biosystems) using PRISM ready reaction dye primer cycle sequencing kits (Applied Biosystems), according to the manufacturer's instructions. The 16S rRNA gene sequence was manually aligned with the corresponding sequences of representative strains and clones retrieved from the DDBJ database. CLUSTAL W (Thompson *et al.*, 1994) was used to estimate evolutionary distances (the K_{nuc} value of Kimura, 1980) and similarity values were used to construct the phylogenetic tree by the neighbour-joining method (Saitou & Nei, 1987). The topology of the tree was evaluated by performing a bootstrap analysis (Felsenstein, 1985) using 1000 resamplings. The phylogenetic tree produced by the maximum-likelihood method was generated using PAUP* version 4.0b8 (Swofford, 2001).

RESULTS AND DISCUSSION

Cultural and morphological characteristics

Strain KV-614^T forms flat and nearly transparent colonies with a whitish colour on 1/5 NA, ISP 3 medium and R2A agar and with a pale-yellow colour on heart infusion agar and Todd-Hewitt agar. Bacterial cells are Gram-positive and rod-shaped (1.2–1.5 × 0.6–0.7 µm). Cells are motile due to the presence of long flagella (Fig. 1).

Phylogenetic analysis

The almost-complete 16S rRNA gene sequence (1528 nt) was determined for strain KV-614^T. Preliminary sequence comparisons with 16S rRNA gene sequences deposited in GenBank indicated that strain KV-614^T belonged to the class *Actinobacteria*.

A phylogenetic tree constructed using the 16S rRNA gene sequences of strain and those of cloned and cultured relatives retrieved from GenBank is shown in Fig. 2. Based on its 16S rRNA gene sequence, strain KV-614^T belongs to a phylogenetic cluster consisting of uncultured bacteria from terrestrial environments and unidentified bacterial strains. The closest sequence was that of bacterium Ellin 5025 (93.2% similarity; Joseph *et al.*, 2003).

A neighbour-joining phylogenetic tree constructed from the 16S rRNA gene sequence of strain KV-614^T and those of other recognized species is shown in Fig. 3. Strain KV-614^T represents a novel lineage within the order *Rubrobacterales* of the

Fig. 1. Transmission electron micrograph of negatively stained cells of strain KV-614^T. The bacterial cells exhibit long flagella. Bar, 1 µm.

Fig. 2. Neighbour-joining phylogenetic tree based on 16S RNA gene sequences of strain KV-614^T and its closest clonal and cultured relatives. Clones prefixed 'TM' were from a peat bog (Rheims *et al.*, 1996, 1999) and strains prefixed 'Ellin' were from Australian soils (Sait *et al.*, 2002; Joseph *et al.*, 2003). The sequences of clones YNPFFP1 and 1959-7 were available from public databases. Numbers at nodes are bootstrap values based on 1000 resamplings; only values higher than 500 are shown. The tree was rooted with *Bacillus subtilis*. Bar, 20 inferred nucleotide substitutions per 1000 nucleotides.

class *Actinobacteria* (Stackebrandt *et al.*, 1997), adjacent to the genera *Conexibacter* (Monciardini *et al.*, 2003) and *Solirubrobacter* (Singleton *et al.*, 2003). The 16S rRNA gene

sequence similarity of strain KV-614^T to *Conexibacter woesei* DSM 14684^T is 92.4 % and it shows 89.7 % similarity to that of *Solirubrobacter pauli* B33D1^T. The tree constructed by the

Fig. 3. Phylogenetic tree showing the position of strain KV-614^T based on 16S rRNA gene sequences. Numbers at nodes indicate the level of bootstrap support based on neighbour-joining analysis of 1000 resampled datasets. Only values higher than 700 are shown. The tree was rooted with *Escherichia coli*. Bar, 10 inferred nucleotide substitutions per 100 nucleotides.

maximum-likelihood method supported this result (data not shown).

Chemotaxonomic characteristics

To characterize strain KV-614^T further, bacterial cell chemical constituents were analysed. Cell-wall peptidoglycan contained *meso*-diaminopimelic acid (DAP) as the diagnostic diamino acid and alanine and glutamic acid. The peptidoglycan appeared to be of the A1 γ type (based on *meso*-DAP, direct cross-linkage). The peptidoglycan was of the acetyl type. The isoprenoid quinone was demethylmenaquinone with seven isoprene units (DMK-7), which was detected as the only component by HPLC. DMK-7 has also been found in *Enterococcus faecalis* (Hiraishi, 1988), *Pasteurella* and *Haemophilus* (Kroppenstedt & Mannheim, 1989); however, this unique isoprenoid quinone has been not reported previously for established genera of the class *Actinobacteria*. Mycolic acids were not detected. The predominant cellular fatty acid components were ω 9*c*-octadecenoic acid (oleic acid, C18:1 ω 9*c*, 63.2 %), 12-methyl tetradecanoic acid (anteiso-C15:0, 9.8 %), 14-methyl hexadecanoic acid (anteiso-C17:0, 7.7 %), hexadecanoic acid (C16:0, 4.5 %) and octadecanoic acid (C18:0, 4.3 %). There was no match to any entry in the TDBA50 MIS library, further supporting the classification of strain KV-614^T as a member of a novel genus and species. The DNA G + C content of strain KV-614^T was 72 mol%.

Physiological characteristics

Strain KV-614^T was aerobic, catalase-positive, oxidase-negative and able to reduce nitrate to nitrite. It did not grow on media containing ≥ 2 % (w/v) NaCl. The pH range for growth was 6–8. The temperature range for growth was 16–28 °C and the optimum temperature for growth was 24–27 °C.

Taxonomic conclusions

On the basis of phylogenetic analysis, strain KV-614^T belongs to the order *Rubrobacterales* of the subclass *Rubrobacteridae* (Stackebrandt *et al.*, 1997). An A residue at position 906 (*E. coli* sequence; Brosius *et al.*, 1978) and a U residue at position 955, characteristics of members of the subclass *Rubrobacteridae*, were confirmed to be present in the 16S rRNA gene sequence of strain KV-614^T. The characteristic pattern of 16S rRNA gene sequence signature nucleotides of the subclass *Rubrobacteridae* (Stackebrandt, 2004), 127–234 (G–C), 291–309 (U–A), 955–1225 (U–A), 1115–1185 (C–G) and 1410–1490 (A–U), was also present.

Table 1 shows the phenotypic characteristics of strain KV-614^T and members of two phylogenetically related genera, *Conexibacter* and *Solirubrobacter*. Like strain KV-614^T, *C. woesei* has *meso*-DAP as the diagnostic diamino acid in the cell-wall peptidoglycan and also produces long flagella, but it clearly differs in the major menaquinone MK-7(H₄) and predominant fatty acids. *S. pauli* is distinguished from strain KV-614^T in that it has non-motile cells and a different fatty acid content. Data on isoprenoid quinones and cell-wall peptidoglycan amino acids are not yet available for this genus. On the basis of phylogenetic analysis and phenotypic characteristics, we propose that strain KV-614^T represents a novel genus and species, *Patulibacter minatonensis* gen. nov., sp. nov. (type strain KV-614^T = NRRL B-24346^T = JCM 12834^T = NBRC 100761^T).

At present, the order *Rubrobacterales* (Stackebrandt *et al.*, 1997) contains four families, *Rubrobacteraceae*, *Conexibacteraceae*, *Solirubrobacteraceae* and *Thermoleophilaceae* (Stackebrandt, 2004). Based on the distinct phylogenetic position of *Patulibacter minatonensis* gen. nov., sp. nov. within the order *Rubrobacterales* and the differences observed in the pattern of 16S rRNA gene sequence signature nucleotides, a new family, *Patulibacteraceae* fam. nov., is proposed.

Table 1. Differential characteristics of strain KV-614^T and members of related genera

Strains: 1, KV-614^T; 2, *Conexibacter woesei* ID 131577^T (data from Monciardini *et al.*, 2003); 3, *Solirubrobacter pauli* B33D1^T (Singleton *et al.*, 2003). ND, Not determined; –, negative; +, positive.

Characteristic	1	2	3
Length of rods (μm)	1.2–1.5	0.9–1.2	1.4
Motility	Motile, long flagella	Motile, long flagella	Non-motile
Optimum growth temperature (°C)	24–27	28–37	28–30
pH range	6–8	7–7.5	6–7.7
Oxidase	–	+	–
Major fatty acids (>8 %)	C18:1 ω 9 <i>c</i> (63 %); a-C15:0 (10 %); a-C17:0 (8 %)	C18:1 ω 9 <i>c</i> (41 %); i-C16:0 (16 %); C17:1 ω 9 <i>c</i> (14 %); C16:0 (13 %)	i-C16:0 (54 %); C18:1 ω 9 <i>c</i> (36 %)
Diagnostic amino acid in peptidoglycan	<i>meso</i> -DAP	<i>meso</i> -DAP	ND
Isoprenoid quinone	DMK-7	MK-7(H ₄)	ND

Description of *Patulibacter* gen. nov.

Patulibacter (Pat.ul.i.bac'ter. L. adj. *patulus* spreading; N.L. masc. n. *bacter* from Gr. n. *baktron* a rod; N.L. masc. n. *Patulibacter* bacterium with spreading growth).

Cells are Gram-positive, non-endospore-forming rods. Bacterial respiration is aerobic. Cell-wall peptidoglycan contains *meso*-DAP as the diagnostic diamino acid and alanine and glutamic acid. The peptidoglycan is of the acetyl type. The fatty acid profile is dominated by C18:1 ω 9c. Mycolic acids are absent. The predominant isoprenoid quinone is DMK-7. The DNA G+C content is 72 mol%. The type species is *Patulibacter minatonensis* sp. nov.

Description of *Patulibacter minatonensis* sp. nov.

Patulibacter minatonensis (mi.na.to.nen'sis. N.L. masc. adj. *minatonensis* pertaining to Minato-ku, the ward of Tokyo, Japan, where the type strain was isolated).

Exhibits the following properties in addition to those given in the genus description. Forms flat and nearly transparent colonies with a whitish or pale yellow colour. Cells are 1.2–1.5 $\times$ 0.6–0.7 μ m in size, motile by long flagella, catalase-positive and oxidase-negative. Utilizes the following substrates: dextrin, inulin, mannan, *N*-acetyl-D-mannosamine, L-arabinose, D-arabitol, cellobiose, D-fructose, L-fucose, D-galactose, D-galacturonic acid, α -D-glucose, *myo*-inositol, maltose, maltotriose, D-mannitol, D-mannose, D-melezitose, methyl α -D-galactoside, methyl β -D-glucoside, methyl α -D-glucoside, methyl α -D-mannoside, D-psicose, L-rhamnose, salicin, sedoheptulosan, turanose, α -hydroxybutyric acid, lactamide, D-lactic acid methyl ester, L-lactic acid, L-serine, 2,3-butanediol, adenosine, inosine, uridine, adenosine 5'-monophosphate, fructose 6-phosphate, glucose 1-phosphate, glucose 6-phosphate, DL- α -glycerol phosphate, D-ribose, D-tagatose, D-xylose, acetic acid, propionic acid, D-alanine, putrescine, maltose, potassium gluconate, adipic acid and L-malic acid. Shows the following enzyme activities: alkaline phosphatase, elastase (C4), elastase lipase (C8), lipase (C4), leucine arylamidase, acid phosphatase and naphthol-AS-BI-phosphohydrolase. Negative result in Biolog substrate panels, API ZYM enzyme assay and API NE gallery tests for: α -cyclodextrin, β -cyclodextrin, glycogen, Tween 40, Tween 80, amygdalin, arbutin, gentiobiose, D-gluconic acid, α -D-lactose, lactulose, D-melibiose, methyl β -D-galactoside, 3-methyl glucose, palatinose, D-raffinose, D-sorbitol, stachyose, sucrose, D-trehalose, xylitol, β -hydroxybutyric acid, γ -hydroxybutyric acid, *p*-hydroxyphenylacetic acid, α -ketoglutaric acid, α -ketovaleric acid, D-malic acid, methylpyruvate, monomethyl succinate, pyruvic acid, succinamic acid, succinic acid, *N*-acetyl L-glutamic acid, alaninamide, L-alanine, L-alanylglycine, L-asparagine, L-glutamic acid, glycyl L-glutamic acid, L-pyrogutamic acid, glycerol, 2'-deoxyadenosine, thymidine, thymidine 5'-monophosphate, uridine 5'-monophosphate, *N*-acetyl-D-glucosamine, n-decanoic acid, citric acid, phenylacetate, valine arylamidase,

cystine arylamidase, trypsin, chymotrypsin, α -galactosidase, β -glucuronidase, α -glucosidase, β -glucosidase, *N*-acetyl- β -glucosaminidase, α -mannosidase, α -fucosidase, nitrate reduction, tryptophan degradation, glucose fermentation, gelatin hydrolysis, arginine dihydrolase, urease and β -galactosidase. Does not grow on media containing 2% (w/v) NaCl. The pH range for growth is 6–8. The temperature range for growth is 16–28 °C. The type strain is susceptible to tetracycline (30 μ g), polymyxin B (300 U), amikacin (30 μ g), chloramphenicol (30 μ g), erythromycin (15 μ g), ciprofloxacin (5 μ g), tobramycin (10 μ g), vancomycin (30 μ g), kanamycin (30 μ g), gentamicin (10 μ g), ampicillin (10 μ g) and imipenem (10 μ g). Cells are resistant to aztreonam (30 μ g), ceftazidime (30 μ g), nalidixic acid (1 μ g), norfloxacin (10 μ g) and oxacillin (1 μ g). The DNA G+C content is 72 mol%.

The type strain, KV-614^T (= NRRL B-24346^T = JCM 12834^T = NBRC 100761^T), was isolated from soil.

Description of *Patulibacteraceae* fam. nov.

Patulibacteraceae (Pat.ul.i.bact.era'ce.ae. N.L. masc. n. *Patulibacter* type genus of the family; -aceae ending to denote a family; N.L. fem. pl. n. *Patulibacteraceae* the *Patulibacter* family).

Cells are Gram-positive and non-endospore-forming. Cell-wall peptidoglycan is based upon *meso*-DAP. The main isoprenoid quinone is demethylmenaquinone. Mycolic acids are absent. The pattern of 16S rRNA gene sequence signature nucleotides of members of the family consists of 52–359 (C–G), 98 (A), 139 (G), 144–178 (C–G), 370–391 (C–G), 580–761 (U–A), 590–649 (U–A), 600–638 (U–G), 670–736 (A–U), 953–1228 (G–C), 954–1226 (G–C), 999–1041 (U–A), 657–749 (U–A), 681–709 (U–A), 941–1342 (A–U), 1051–1207 (G–C) and 1311–1326 (A–U). Phylogenetically, the family is a member of the class *Actinobacteria*, subclass *Rubrobacteridae*, order *Rubrobacterales*. The type genus is *Patulibacter*.

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