Mitsuaria chitosanitabida gen. nov., sp. nov., an aerobic, chitosanase-producing member of the 'Betaproteobacteria'

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Four strains (3001^T, 2, 12 and 13), which were isolated as chitosanase-producing bacteria from soil from Matsue city (Japan), were studied phenotypically, genotypically and phylogenetically. Based on sequence analysis of 16S rRNA genes, DNA G + C content (67.4–69.2 mol%), quinone type (UQ-8), major fatty acid composition (3-OH 10 : 0, 3-OH 14 : 0) and other phylogenetic studies, strains 3001^T, 12 and 13 were found to occupy a separate position in the 'Betaproteobacteria'. *Roseateles depolymerans*, *Rubrivivax gelatinosus* and *Ideonella dechloratans* were their closest neighbours (93–95 % 16S rRNA gene sequence similarity). The 16S rRNA gene sequence and other characteristics suggested that strain 2 belonged to the genus *Flavobacterium*. DNA–DNA hybridization experiments supported the conclusion that strains 3001^T, 12 and 13 were of the same species (72–78 % DNA hybridization) and only distantly related to *I. dechloratans* and *R. gelatinosus*. It is proposed that strains 3001^T, 12 and 13 represent a novel genus and species for which the name *Mitsuaria chitosanitabida* gen. nov., sp. nov. is proposed. The type strain of *Mitsuaria chitosanitabida* is 3001^T (= IAM 14711^T = ATCC BAA-476^T).

The 'Betaproteobacteria' includes the families *Comamonadaceae*, *Alcaligenaceae* and *Neisseriaceae*, the genera *Burkholderia*, *Ralstonia* and *Rhodocyclus*, ammonia-oxidizing bacteria and other species. The *Comamonadaceae* originally contained *Variovorax* (Willems *et al.*, 1991b), the purple non-sulfur bacteria, including *Rubrivivax* and *Rhodoferrax* (Hiraishi, 1994; Willems *et al.*, 1991a), and other genera such as *Comamonas* and *Acidovorax*, formerly described as *Pseudomonas* (Willems *et al.*, 1991b, 1992). The genus *Aquabacterium* (Kalmbach *et al.*, 1999), the bacteriochlorophyll *a*-containing bacteria *Roseateles* (Suyama *et al.*, 1998, 1999) and sheathed bacteria, including *Leptothrix* and *Sphaerotilus* (Siering & Ghiorse, 1996), are closely related to the above genera. Besides these well-characterized species, it is thought that there are many more as yet unknown species that belong to the 'Betaproteobacteria'.

The present study was undertaken to determine the taxonomic position of certain novel strains that were originally isolated as chitosanase-producing bacteria and classified in the 'Betaproteobacteria'. We present evidence that the newly isolated strains 3001^T, 12 and 13 should be classified as a new genus and species, *Mitsuaria chitosanitabida* gen. nov., sp. nov.

We screened soils from Matsue city, Japan, for bacteria producing chitosanase, an enzyme which degrades glucosamine polymers and forms clear zones on a chitosan-containing minimal medium. This defined medium consisted of 1 % colloidal chitosan, 0.025 % yeast extract, 0.025 % peptone, 0.025 % K₂HPO₄, 0.07 % KH₂PO₄ and 0.03 % MgSO₄. About 30 strains were found that formed clear zones on the colloidal chitosan medium. From these, four strains (3001^T, 2, 12 and 13) that produced good clear zones were chosen for further study.

Strain 3001^T has been studied extensively. The chitosanase produced by strain 3001^T has already been purified and characterized and the corresponding gene has been cloned (Park *et al.*, 1999). Further analyses on this chitosanase have been conducted (Shimono *et al.*, 2002a, b; Yun *et al.*, 2005).

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Abbreviations: MK, menaquinone; UQ, ubiquinone.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains 3001^T, 2, 12 and 13 are AB006851, AY856841, AB024307 and AB024306, respectively.

Fig. 1. Cell morphology of strain 3001^T observed by transmission electron microscopy after negative staining. Bar, 1 μ m.

For these studies, strain 3001^T was tentatively named '*Matsuebacter chitosanotabidus*' 3001, but here we propose the name *Mitsuaria chitosanitabida* gen. nov., sp. nov.

The cell morphology of strain 3001^T was observed by phase-contrast and transmission electron microscopy. Some cells were straight and elongated and some were rod-shaped. Cells varied between 0.7 and 1.0 μ m in width and 2.0 and

4.0 μ m in length. Cells were actively motile with a single polar flagellum. Flagellation was examined by transmission electron microscopy (JEOL 1210; JEOL) after negative staining with 1% (w/v) phosphotungstic acid (Fig. 1). The motile strain was rod-shaped. No sheath was detected by electron microscopy. Endospores were not produced. The Gram reaction was negative. No fluorescent pigments were produced. Strain 3001^T showed aerobic respiration with oxygen as the final electron acceptor. Catalase and oxidase were produced. Strain 3001^T could not grow photosynthetically under light conditions or under anaerobic dark fermentation conditions. Additional physiological properties of strains 2, 12 and 13 were examined and are compared with neighbouring strains and with strain 3001^T in Table 1. The physiological properties of strains 3001^T, 12 and 13 were similar but quite different from those of strain 2.

To characterize the strains further, the 16S rRNA genes of strains 3001^T, 2, 12 and 13 were amplified by PCR using synthetic oligonucleotides. The primers used were; 5'-AGAGTTTGATCCTGCTCAG-3' (positions 8 to 26, *Escherichia coli* numbering system, Brosius *et al.*, 1978) and 5'-GGTACCTTGTTACGACTT-3' (positions 1509 to 1491). The amplified 1.5 kb fragments from the 16S rRNA genes of these strains were directly cloned into the pT7 Blue

Table 1. Comparison of physiological properties of isolated and related strains

Strains: 1, strain 2; 2, strain 12; 3, strain 13; 4, strain 3001^T; 5, *Ideonella dechloratans*; 6, *Leptothrix discophora*; 7, *Roseateles depolymerans*; 8, *Aquabacterium citratiphilum*; 9, *Rubrivivax gelatinosus*. Chitosan degradability and quinone type were tested in this study. Other results are taken from Kalmbach *et al.* (1999), Malmqvist *et al.* (1994), Spring *et al.* (1996), Suyama *et al.* (1999) and Willems *et al.* (1991a). NT, Not tested; NA, not available.

Characteristic	1	2	3	4	5	6	7	8	9
Flagella	NT	1, Polar	1, Polar	1, Polar	Several polar	1, Polar	Several polar	1, Polar	1, Polar
Sheath formation	—	—	—	—	—	+	—	—	—
Polyalkanoate inclusion body	—	—	—	—	—	NA	NA	+	NA
pH for growth	5–8	5–9	5–9	5–9	5–9	NA	6–8	6.5–9.5	5–9
Maximum temperature for growth (°C)	37	34	34	34	42	NA	45	34	37
Enzyme activity:									
Catalase	+	+	+	+	+	—	—	—	+
Oxidase	+	+	+	+	+	+	+	NA	+
Urease	+	—	—	—	+	NA	—	+ / —	—
Production of:									
Dihydroxyacetone	+	—	—	—	—	NA	—	NA	—
Indole	+	—	—	—	—	NA	—	NA	—
2-Ketogluconate	+	—	—	—	NA	NA	NA	NA	NA
Pigment:									
Bacteriochlorophyll <i>a</i>	—	—	—	—	—	+	—	NA	+
Fluorescent (King's A and B)	+	—	—	—	NA	NA	NA	NA	NA
Nitrate reduction	—	+	+	+	+	—	—	+	—
Hydrolysis of Tweens 20, 40, 80	+	+	+	+	+	NA	+	+	+
Chitosan degradation	+	+	+	+	—	—	—	NA	—
Quinone type	MK-6	UQ-8	UQ-8	UQ-8	UQ-8	UQ-8	UQ-8	NA	UQ-8, MK-8
DNA G+C content (mol%)	35.6	67.4	69.1	69.2	68.1	67.8–71.1	66.2	66	70.0–72.5
16S rRNA gene similarity to 3001 ^T (%)	72.0	98.9	98.7	100	93–94	94–95	94–95	93–94	94–95

T-vector (Novagen). Sequencing was performed by the dideoxy-nucleotide chain-termination method (Sanger *et al.*, 1977) using an ABI Prism 377 DNA sequencer (Perkin-Elmer). The 16S rRNA gene sequences of the fragments from strain 3001^T (1490 bp), strain 2 (1476 bp), strain 12 (1489 bp) and strain 13 (1489 bp) were deposited in GenBank/EMBL/DDBJ. A homology search analysis of the 16S rRNA gene sequences was conducted using the Ribosomal Database Project (RDP) software (Maidak *et al.*, 1999). Phylogenetic trees were constructed from the 16S rRNA gene sequences by using the CLUSTAL W program and the neighbour-joining method (Saitou & Nei, 1987). The phylogenetic tree suggested the presence of an independent group consisting of strains 3001^T, 12 and 13 (Fig. 2). The 16S rRNA genes of strains 12 and 13 displayed sequence similarities of 98.9 and 98.7% with strain 3001^T, respectively. The 16S rRNA gene sequences of strains 3001^T, 12 and 13 were most closely related to those of *Rubrivivax gelatinosus* (94–95% similarity) (Willems *et al.*, 1991a), *Leptothrix discophora* (94–95%) (Emerson & Ghiorse, 1993; Spring *et al.*, 1996), *Roseateles depolymerans* (94–95%) (Suyama *et al.*, 1998, 1999), *Aquabacterium citratiphilum* (93–94%) (Kalmbach *et al.*, 1999) and *Ideonella dechloratans* (93–94%) (Malmqvist *et al.*, 1994) (Fig. 2). The genus *Rubrivivax* has been proposed for certain strains of phototrophic bacteria that are phenotypically similar to strains of *Rhodospirillum rubrum* (Hiraishi, 1994; Hiraishi *et al.*, 1991; Hochkoeppler *et al.*, 1995) and *Rhodocyclus* (Hiraishi *et al.*,

1991; Willems *et al.*, 1991a). Strains 3001^T, 12 and 13 can be easily differentiated from related phototrophic species of *Rhodospirillum rubrum*, *Rubrivivax* and *Rhodocyclus* mainly on the basis of their failure to grow photosynthetically. We did not detect the specific absorption peak at 870 nm for bacteriochlorophyll *a* in cultures of strains 3001^T, 12 and 13 but the 870 nm peak was detected in cultured cells of *Rst. depolymerans*. Strains 3001^T, 12 and 13 were distinguishable from *Rst. depolymerans* (Suyama *et al.*, 1998, 1999) mainly by the absence of bacteriochlorophyll *a* and by some differences in physiological properties such as flagellation, possession of catalase and carbon source utilization. We did not observe large polyalkanoate inclusion bodies in strains 3001^T, 12 and 13, as found in *Aquabacterium* (Kalmbach *et al.*, 1999). As strains 3001^T, 12 and 13 lacked sheath production, they were easily distinguished from genera such as *Leptothrix* and *Sphaerotilus* (Rogers & Anderson, 1976; Siering & Ghiorse, 1996).

DNA–DNA dot hybridization against chromosomal DNAs from various strains was performed using chromosomal DNA of strain 3001^T as a probe. *Comamonas testosteroni* ATCC 11996^T, *I. dechloratans* CCUG 30898^T, *Rvi. gelatinosus* ATCC 17011^T and *V. paradoxus* IAM 12373^T were used as reference strains. Purified chromosomal DNAs (12.5 µg) were cross-linked to a nitrocellulose membrane by UV light, pre-hybridized at 42 °C for 1 h and then incubated at 42 °C for 14 h in a hybridization solution containing 100 ng DNA

Fig. 2. Phylogenetic position of strain 3001^T based on 16S rRNA gene sequence. The 16S rRNA gene sequence of strain 3001^T was determined and compared with the complete 16S rRNA gene sequences of other related genera. Sequences were aligned by using CLUSTAL W and the tree was constructed by the neighbour-joining method (Saitou & Nei, 1987). The numbers on the branches are confidence limits (expressed as percentages) estimated from the bootstrap analysis performed with 1000 replicates. Bar, 1 nucleotide substitution per 100 nucleotides.

probe. Spots were detected by the ECL system (Amersham). The relative intensity of hybridization was estimated by densitometry to be 100, 3.8, 78.8, 96.2, 7.7, 5.8, 19.2 and 7.7 % for strains 3001^T, 2, 12, 13, *C. testosteroni*, *I. dechloratans*, *Rvi. gelatinosus* and *V. paradoxus*, respectively. This result indicates that strains 3001^T, 12 and 13 are within the same genus, but different from the other tested strains.

To confirm this result, solution DNA–DNA hybridization was performed as described previously (Tanasupawat *et al.*, 1992). In brief, chromosomal DNAs from various strains were prepared and hybridized in solution with ³²P-labelled chromosomal DNA from strain 3001^T. Hybridized DNAs were digested by a standard amount of S1 nuclease. Non-digested DNA was sedimented by trichloroacetic acid and the remaining radioactivity was counted. DNA–DNA hybridization with strain 3001^T was calculated to be 23.3, 71.9, 78.1, 44.7, 43.7, 39.0 and 41.0 % for strains 2, 12, 13, *C. testosteroni*, *I. dechloratans*, *Rvi. gelatinosus* and *V. paradoxus*, respectively. These values were the means of three independent experiments. DNA–DNA hybridization values of over 70 % suggest that two strains belong to the same species and values lower than 50 % suggest that the strains belong to different genera. These results indicate that strains 3001^T, 12 and 13 are very closely related and are different from strain 2, *C. testosteroni*, *I. dechloratans*, *Rvi. gelatinosus* and *V. paradoxus*. Strain 2 was also isolated as a chitosanolytic bacterium using the same screening test, but seemed to belong to the genus *Flavobacterium* based on its 16S rRNA gene sequence. For strain 2, the highest sequence similarity found for the 16S rRNA gene was with that of *Flavobacterium indologenes* (98 %).

The DNA G+C content of the strains was determined by the method of Mesbah & Whitman (1989). The DNA G+C content for strains 3001^T, 2, 12 and 13 was 69.2, 35.6, 67.4 and 69.1 mol%, respectively. These results are consistent with the suggestion that strains 3001^T, 12 and 13 are the same species.

The major fatty acids of strains 3001^T, 2, 12 and 13 were examined according to the method of Takeuchi *et al.* (1995) and found to be quite similar, except for those of strain 2. The major components for strain 3001^T were palmitic acid (16:0), palmitoleic acid (16:1), 3-OH 10:0 and 3-OH 14:0. A similar distribution of fatty acid composition was observed for *Rvi. gelatinosus* (Hiraishi *et al.*, 1991). *L. discophora* contains 3-hydroxydecanoic acid (3-OH 10:0) as the major 3-OH fatty acid (Stead, 1992). Strain 2 had a totally different fatty acid composition and contained iso 15:0 non-polar fatty acids, iso 15:0 and iso 17:0 3-hydroxy acids.

The quinone type present in the isolated strains was determined by HPLC (Okada *et al.*, 1997). Strains 3001^T, 12 and 13 all contained UQ-8 as the major ubiquinone, while strain 2 contained MK-6. As no menaquinone was detected in strains 3001^T, 12 and 13, they were clearly differentiated from *Rvi. gelatinosus*, which contains both UQ-8 and MK-8

(Hiraishi *et al.*, 1991). UQ-8 is also found in species related to *C. testosteroni* and *V. paradoxus*, but the fatty acid composition (3-OH 10:0) and biological features of these strains are quite different from those of strains 3001^T, 12 and 13.

The chitosan-degrading activity of *Rvi. gelatinosus*, *I. dechloratans*, *L. discophora*, *Sphaerotilus natans*, *V. paradoxus* and *Rst. depolymerans* was tested and no activity could be detected.

A comparison of the physiological properties, phylogenetic relationships, DNA G+C content, quinone species, whole-cell fatty acid profiles and DNA–DNA hybridization of strains 3001^T, 12 and 13 showed them to be markedly different from closely related genera such as *Rubrivivax*, *Rhodoferrax*, *Sphaerotilus*, *Variovorax*, *Ideonella*, *Roseateles* and *Aquabacterium*. For these reasons, we propose that strains 3001^T, 12 and 13 be given the name *Mitsuaria chitosanitabida* and that they be placed in a new genus within the 'Betaproteobacteria'.

Description of *Mitsuaria* gen. nov.

Mitsuaria (Mit'su.ar.i.a. L. fem. suff. -aria belonging to; N.L. fem. n. *Mitsuaria* belonging to Matsue City, an inhabitant of Matsue City, the source of the soil samples from which the organism was isolated).

Cells are 0.7–1.0 µm wide and 2.0–4.0 µm long. Cells are motile by means of a single polar flagellum. Endospores are not formed. Gram-negative. Obligately aerobic. Oxidase and catalase-positive. The major respiratory quinone is UQ-8. The major cellular hydroxy fatty acids are 3-OH 10:0 and 3-OH 14:0. The DNA G+C content of the type strain is 69.2 mol% (as determined by HPLC). The species is phylogenetically related to members of the 'Betaproteobacteria'. The type species is *Mitsuaria chitosanitabida*.

Description of *Mitsuaria chitosanitabida* sp. nov.

Mitsuaria chitosanitabida (chi'to.san.it.ab'i.da. N.L. n. *chitosanum* chitosan; L. adj. *tabida* dissolving, decaying, consuming, putrefying; N.L. fem. adj. *chitosanitabida* dissolving chitosan, a polysaccharide found in Crustacea, which is a deacetylated derivative of chitin).

Displays the following properties in addition to those given in the genus description. Colonies are circular with entire margins and are light brown in colour. Phototrophic growth is negative. Good growth occurs on nutrient agar at 20–30 °C between pH 5.0 and 9.0. Nitrate is reduced to nitrite. H₂S is not produced. No production of urease, indole, 3-ketolactose, dihydroxyacetone and 2-ketogluconate. Voges–Proskauer and methyl red tests are negative. Tweens 40, 60 and 80 are hydrolysed. Fluorescent pigment is not produced on King's media A or B. D-Glucose, D-glucosamine, maltose and glycerol are assimilated, but L-arabinose, D-fructose, D-sorbitol, D-raffinose, D-xylose, D-galactose, sucrose,

D-mannose, N-acetyl-D-glucosamine, lactose and n-hexadecane are not assimilated. The major respiratory quinone is ubiquinone 8. The major cellular hydroxy fatty acids are 3-OH 10:0 and 3-OH 14:0. The G + C content of the DNA of the type strain is 69.2 mol% (as determined by HPLC).

The type strain, 3001^T (= IAM 14711^T = ATCC BAA-476^T), was isolated from soil from Matsue City, Japan.

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