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

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Four strains (3001T, 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 3001T, 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 3001T, 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 3001T (=IAM 14711T = ATCC BAA-476T).

The ‘Betaproteobacteria’ includes the families Comamonadaceae, Alcaligenaceae and Neisseriaceae, the genera Burkholderia, Ralstonia and Rhodococcus, ammonia-oxidizing bacteria and other species. The Comamonadaceae originally contained Variovorax (Willems et al., 1991b), the purple non-sulfur bacteria, including Rubrivivax and Rhodotherax (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 3001T, 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, 0–0.25 % yeast extract, 0–0.25 % peptone, 0–0.25 % K2HPO4, 0–0.7 % KH2PO4 and 0–0.3 % MgSO4. About 30 strains were found that formed clear zones on the colloidal chitosan medium. From these, four strains (3001T, 2, 12 and 13) that produced good clear zones were chosen for further study.

Strain 3001T has been studied extensively. The chitosanase produced by strain 3001T 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).
For these studies, strain 3001T was tentatively named ‘Matsuebacter chitosanotabidus’ 3001, but here we propose the name Mitsuria chitosanitabida gen. nov., sp. nov.

The cell morphology of strain 3001T 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 μm in width and 2.0 and 4.0 μm 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 3001T showed aerobic respiration with oxygen as the final electron acceptor. Catalase and oxidase were produced. Strain 3001T 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 3001T in Table 1. The physiological properties of strains 3001T, 12 and 13 were similar but quite different from those of strain 2.

To characterize the strains further, the 16S rRNA genes of strains 3001T, 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′-GGTTACCTTGTTACGACTT-3′ (positions 1509 to 1491). The amplified 15 kb fragments from the 16S rRNA genes of these strains were directly cloned into the pT7 Blue

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

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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<sup>T</sup> (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<sup>T</sup>, 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<sup>T</sup>, respectively. The 16S rRNA gene sequences of strains 3001<sup>T</sup>, 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), *Aquadibacterium citratophilum* (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 *Rhodoferax* (Hiraishi, 1994; Hiraishi et al., 1991; Hochkoeppler et al., 1995) and *Rhodocyclus* (Hiraishi et al., 1991; Willems et al., 1991a). Strains 3001<sup>T</sup>, 12 and 13 can be easily differentiated from related phototrophic species of *Rhodoferax*, *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 <i>a</i> in cultures of strains 3001<sup>T</sup>, 12 and 13 but the 870 nm peak was detected in cultured cells of *Rst. depolymerans*. Strains 3001<sup>T</sup>, 12 and 13 were distinguishable from *Rst. depolymerans* (Suyama et al., 1998, 1999) mainly by the absence of bacteriochlorophyll <i>a</i> 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<sup>T</sup>, 12 and 13, as found in *Aquadibacterium* (Kalmbach et al., 1999). As strains 3001<sup>T</sup>, 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<sup>T</sup> as a probe. *Comamonas testosteroni* ATCC 11996<sup>T</sup>, *I. dechloratans* CCUG 30898<sup>T</sup>, *Rvi. gelatinosus* ATCC 17011<sup>T</sup> and *V. paradoxus* IAM 12373<sup>T</sup> 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<sup>T</sup> based on 16S rRNA gene sequence. The 16S rRNA gene sequence of strain 3001<sup>T</sup> 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 3001T, 2, 12, 13, C. testosteroni, L. dechloratans, Rvi. gelatinosus and V. paradoxus, respectively. This result indicates that strains 3001T, 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 3001T. Hybridized DNAs were digested by a standard amount of SI nuclease. Non-digested DNA was sedimented by trichloroacetic acid and the remaining radioactivity was counted. DNA–DNA hybridization with strain 3001T 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, L. 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 3001T, 12 and 13 are very closely related and are different from strain 2, C. testosteroni, L. 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 was found for the 16S rRNA gene 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 3001T, 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 3001T, 12 and 13 are the same species.

The major fatty acids of strains 3001T, 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 3001T were palmitic acid (16:0), palmitoleic acid (16:1), 3-OH 10:0 and 3-OH 14:0. The DNA G+C content of the type strain 3001T is 69·2 mol% as determined by HPLC. The species is phylogenetically related to members of the ‘Betaproteobacteria’. The type species is Mitsuaria chitosanitabida.

The chitosan-degrading activity of Rvi. gelatinosus, L. 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 3001T, 12 and 13 showed them to be markedly different from closely related genera such as Rubrivivax, Rhodofexax, Sphaerotilus, Variovorax, Ideonella, Roseateles and Aquabacterium. For these reasons, we propose that strains 3001T, 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, the 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’.

**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-ketolactate, 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-glucoamine, maltose and glycerol are assimilated, but L-arabinose, D-fructose, D-sorbitol, D-raffinose, D-xyllose, 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 3-0H 10:0 and 3-0H 14:0. The G + C content of the DNA of the type strain is 69.2 mol% (as determined by HPLC).

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

Acknowledgements

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References


