Title

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

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NOTE

Mitsuaria chitosanitabida gen. nov., sp. nov., an aerobic, chitosanase producing member of the β-subclass of *Proteobacteria*

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Running title: *Mitsuaria chitosanitabida* gen. nov., sp. nov.

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Abstract

Four strains (3001^T (T=type strain), #2, #12 and #13), which were isolated as chitosanase producing bacteria from the soils of Matsue city (Japan), were studied phenotypically, genotypically and phylogenetically. Based on the sequence analysis of their 16S rRNA genes, the G+C contents (67.4-69.2%), quinone type (UQ-8), major fatty acid compositions (3-0H 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 β-subclass of the *Proteobacteria*. *Roseateles depolymerans, Rubrivivax gelatinosus* and *Ideonella dechloratans* are their closest neighbor (93-95% 16s rRNA gene sequence similarity). Meanwhile, the 16S rRNA sequence and other characteristics suggested that #2 belonged to *Flavobacterium*. DNA-DNA hybridization experiments supported that strains 3001^T, #12 and #13 are within the same species (72-78% hybridization level) and only distantly related to *I. dechloratans* and *R. gelatinosus*. We propose that the unknown bacteria 3001^T, #12 and #13 to be classified as *Mitsuaria chitosanitabida* gen. nov., sp. nov. The type strain of *Mitsuaria chitosanitabida* is strain 3001^T (=IAM 14711; ATCC BAA-476).

Keywords: chitosan, chitosanase, *Proteobacteria*, β–subclass, *Mitsuaria chitosanitabida*

Abbreviations:

UQ, Ubiquinone; MK, Menaquinone

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

The β-subclass of the *Proteobacteria* includes the following families the *Comamonadaceae*, the *Alcaligenaceae*, the *Neisseriaceae*, and the *Burkholderia* group, the *Ralstonia* group, the *Rhodocyclus* group, ammonia-oxidizing bacteria and other species. The *Comamonadaceae* originally contains *Variovorax* (Willems *et al.*, 1991A), the purple nonsulfur bacteria including *Rubrivivax* and *Rhodoferax* (Hiraishi, 1994; Willems *et al.*, 1991B) and the other genera such as *Comamonas* and *Acidovorax* formerly described in *Pseudomonas* (Willems *et al.*, 1991A, 1992). The genus *Aquabacterium* (Kalmbach *et al.*, 1999), the bacteriochlorophyll *a*-containing bacteria *Roseateles* (Suyama *et al.*, 1998, 1999), and sheathed bacteria including the genus *Leptothrix* and *Sphaerotilus* (Siering & Ghiorse, 1996) are closely related to the above genera. Besides those well characterized species, there still remain many unknown species which are thought to belong to the β-subclass of the *Proteobacteria*.

The present study was undertaken to determine the taxonomic position of certain novel bacteria which were originally isolated as chitosanase-producing bacteria and classified in the β-subclass of the *Proteobacteria*. We present evidence that the newly isolated strains 3001^T, #12 and #13, should be classified into a new genus, *Mitsuaria chitosanitabida* gen. nov., sp. nov.

We screened the soils of Matsue city in 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% K_2HPO_4, 0.07% KH_2PO_4 and 0.03% $MgSO_4$. We picked up about 30 strains that formed clear zones on the colloidal chitosan medium. Among them, four strains (3001^T, #2, #12 and #13) producing good clear zones were chosen for further study.

Among these, strain 3001^T was extensively studied. The chitosanase produced by strain 3001^T has been purified and characterized and its corresponding gene was cloned (Park *et al.*, 1999). Further analyses on this chitosanase have been conducted (Shimono *et al.*, 2002a; Shimono *et al.*, 2002b). Strain 3001^T was tentatively named *Matsuebacter chitosanotabidus* 3001 in those studies, but here we propose to name it *Mitsuaria chitosanitabida* 3001 ^{T}.

The cell morphology of the strain 3001^T was observed by a phase contrast microscopy and an electron scanning microscopy. Some cells are straight and elongated, and some are rod shaped. The cell size varied between 0.4 and 0.7 μm in width and 2.0 and 4.0 μm in length. Cells were actively motile with a single polar flagellum. Flagellation was examined with a model JEOL 1210 transmission electron microscope (JEOL, Akishima, Japan) after negative staining with 1% (wt/vol) phosphotungstenic acid (Fig. 1). The motile strain was a rod shape but not an elongated shape. No sheath was detected by an electron microscopy. Endospores were not produced. The Gram reaction was negative. No fluorescent pigments were produced. The strain 3001^T has an aerobic respiration type of metabolism with oxygen as the final electron acceptor and produced catalase and oxidase. The strain 3001^T could not grow either photosynthetically in the light nor in anaerobic-dark fermentation conditions. Additional physiological properties of strains #2, # 12 and #13 were also examined and are compared with neighboring strains in Table 1 along with those of strain 3001^T. The physiological properties of strains 3001^T, #12 and #13 were similar, but quite different from #2.

To further characterize these strains, the 16S rRNA genes of strain 3001^T, #2, #12 and #13 were amplified by the polymerase chain reaction (PCR) using synthetic oligonucleotides. The primers used were; 5'-AGAGTTTGATCCTGCTCAG-3' (positions 8 to 26; *E. coli* numbering system)

and 5'-GGTTACCTTGTTACGAC TT-3' (positions 1509 to 1491) (Brosius *et al.*, 1978). The amplified 1.5-kb fragments from the 16S rRNA genes of these strains were directly cloned into the pT7 Blue T-vector (Novagen). Sequencing was done by the dideoxy- nucleotide chain termination method (Sanger *et al.*, 1977) using an ABI PrismTM 377 DNA sequencer (Perkin-Elmer). The 16S rRNA sequences of the fragments from strain 3001^T (1,490 bp), strain #2 (1,476 bp), strain #12 (1,489 bp) and strain #13 (1,489 bp) have been deposited in DDBJ/GenBank/EMBL. A homology search analysis of the 16S rRNA sequences was done using the program of the Ribosomal Database Project (RDP) (Maidak *et al.*, 1999). To construct the phylogenetic trees using the 16S rRNA sequences, the CLUSTAL W program and the neighbor-joining method (Saitou & Nei, 1987) were used. The constructed tree suggested an independent group consisting of strains #3001^T, #12 and #13 (Fig. 2). The 16S rRNA of strains #12 and #13 displayed sequence similarities of 98.9% and 98.7% with strain 3001^T, respectively. The 16S rRNAs of strains 3001^T, #12 and #13 were most closely related to those of the genera *Rubrivivax gelatinosus* (94-95% identity) (Willems *et al.*, 1991B), *Leptotrix discophora* (94-95% identity) (Emerson & Ghiorse,1993; Spring *et al.*, 1996), *Roseateles depolymerans* (94-95% identity) (Suyama *et al.*, 1998, 1999), *Aquabacterium citratiphilum* (93-94% identity) (Kalmbach *et al.*, 1999), and *Ideonella dechloratans* (93-94% identity) (Malmqvist *et al.,* 1994) (Fig. 2). The genus *Rubrivivax* was proposed for certain strains of phototrophic bacteria that were 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.*, 1991B). Strains 3001^T, #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 a specific absorption peak at 870nm of bacteriochlorophyll *a* from

culture cells of strain 3001 ^{T}, #12 and #13 under the condition we detected the peak at 870nm from culture cells of *R. depolymerans*. Strains 3001^{T}, #12 and #13 are distinguishable from *R. depolymerans* (Suyama *et al.*, 1998, 1999) mainly by their absence of bacteriochlorophyll *a* and by some differences of physiological properties such as flagellation, possession of catalase and carbon source utilization. We did not see large polyalkanoate inclusion bodies in strains 3001^{T}, #12 and #13 as observed in *Aquabacterium* (Kalmbach *et al.*, 1999). As strains 3001^{T}, #12 and #13 lacked sheath production, they can be easily distinguished from genera such as *Leptothrix* and *Sphaerotilus* which display sheath production (Rogers & Anderson, 1976; Siering & Ghiorse,1996).

DNA-DNA dot hybridization against chromosomal DNAs from various strains was performed using the chromosomal DNA of strains 3001^{T} as a probe. *Comamonas testosteroni* ATCC11996^{T}, *I. dechloratans* CCUG30898^{T}, *R. gelatinosus* ATCC17011 ^{T}, and *V. paradoxus* IAM12373 ^{T}, were used as reference strains. The 12.5 mg purified chromosomal DNAs were cross-linked to the nitrocellulose membrane by UV light, pre-hybridized at 42^{o}C for 1h and then incubated at 42^{o}C for 14h in a hybridization solution containing the 100 ng DNA probe. Spots were detected by the ECL system (Amersham). The relative intensity of the hybridization was estimated by densitometry to be 100%, 3.8 %, 78.8%, 96.2%, 7.7%, 5.8%, 19.2% and 7.7% for strain 3001^{T}, #2, #12, #13, *C. testosteroni, I. dechloratans*, *R. gelatinosus* and *V. paradoxus,* respectively. This result indicates 3001^{T}, #2, and #13 are within the same genera, but different from other tested strains.

To further 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 the [^{32}P]-labeled chromosomal DNA of strain 3001^{T}. Hybridized DNAs were digested by a certain amount of S1 nuclease. Non-digested DNA was

sedimented by trichloro acetic acid and the remaining radio activities were counted. As the result, the levels of DNA hybridizations with strain 3001^T were 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*, *R. gelatinosus* and *V. paradoxus,* respectively. These numbers are the average of three independent experiments. In this method, the number up to 70% hybridization level suggests that two strains belong in the same species, and values lower than 50% identifiy the other strain as belonging in different taxa. These results indicate that strain 3001^T, #12 and #13 are very closely related strains and different from #2, *C. testosteroni, I. dechloratans*, *R. gelatinosus* and *V. paradoxus.* Strain #2 was also isolated as a chitosanolytic bacterium using the same screening test, but seems to belong to the *Flavobacterium* based on its 16S rRNA sequence. The best sequence similarity for the 16S rRNA of strain #2 was with that of *Flavobacterium indologenes* (98%).

The G+C contents of the isolated strains were determined by the method described previously (Mesbah *et al.*, 1989). The G+C contents of strains 3001^T, #2, #12 and #13 were 69.2, 35.6, 67.4 and 69.1%, respectively. These results support also that strain 3001^T, #12, #13 are the close species.

The major fatty acids of strains 3001^T, #2, #12 and #13 were examined as described previously (Takeuchi *et al.*, 1995) and found to be quite similar, except for those of strain #2. The major components of 3001^T were palmitic acid (16:0), palmitoleic acid (16:1), 3-OH 10:0 and 3-OH 14:0. A similar distribution in fatty acid composition was observed in *R. gelatinosus* (Hiraishi *et al.,* 1991). *L. discophora* contains 3-hydroxydodecanoic 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 were determined by the

method using HPLC as described previously (Okada *et al.*, 1997). Strains 3001^T, #12 and #13 all contained UQ-8 as the major ubiquinone, while strain #2 contained MK-6. Because no menaquinone was detected in strains 3001^T, #12 and #13, they are clearly differentiated from *R. 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 acids compositions (3-OH 10:0) and biological features of these strains are quite different from strains 3001^T, #12 and #13.

We tested the chitosan degrading activity of *R. gelatinosus, I. dechloratans, L. discophora*, *S. natans, V. paradoxus* and *R. depolymerans,* but could not find any chitosan-degrading activity.

A comparison of the physiological properties, phylogenetic relationships, G+C contents, quinone species, whole-cell fatty acid profiles and DNA hybridization levels of strains 3001^T, #12 and #13 showed them to be markedly different from closely related genera such as *Rubrivivax*, *Rhodoferax*, *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 β-subclass of the *Proteobacteria*.

Description of *Mistuaria* gen. nov. Mistuaria (L. fem. suff. -aria, suffix meaning 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 0.4 to 0.7 μm wide and 2.0 to 4.0 μm long. Cells are motile by means of a single polar flagellum. Endospores are not formed. Gram negative. Obligatory aerobic. Oxidase and catalase positive.

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 DNA of the type

strain is 69.2 mol% (as determined by HPLC). The species is phylogenetically related to members of the β -subclass of the *Proteobacteria*. The type species is *Mitsuaria chitosanitabida*.

Description of *Mitsuaria chitosanitabida* sp. nov. *Mitsuaria chitosanitabida* (N. L. n. chitosanum, chitosan; L. adj. tabida, dissolving, decaying, consuming, putrefying; N.L. fem. adj. chitosanitabida, dissolving chitosan, a deacetylated derivative of chitin, which is a polysaccharide contained in *Crustacea*). Cells are rod shaped (0.4 to 0.7 μm by 2.0 to 4.0 μm), Gram negative and motile with a single polar flagellum. Colonies are circular with entire margins and are light brown in color. Phototrophic growth is negative. Good growth occurs on nutrient agar at 20 to 30℃ between pH 5.0 and 9.0. Cells are obligatory aerobic and oxidase and catalase positive. Nitrate is reduced to nitrite. H_2S is not produced. Production of urease, indole, 3-ketolactose, dihydroxyacetone and 2-keto-gluconate are negative. The Voges-Proskauer and methyl red tests are negative. Tween 40, 60 and 80 are hydrolyzed. Fluorescent pigment is not produced on King's medium A and 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-hexadecan are not. 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 is strain 3001^T (IAM 14711; ATCC BAA-476).

Note

The strain name *Matsuebacter chitosanotabidus* appeared in the references (Park. *et al.*, 1999; Shimono *et al.*, 2002a; Shimono *et al.*, 2002b) is here after

called *Mitsuaria chitosanitabida*.

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FIGURE LEGENDS

FIG. 1. Cell morphology of strain 3001^T. Cell was observed by the scanning electron micrography after negatively staining the cells (magnification of 20,000).

FIG. 2. Phylogenetic position of strain 3001^T based on the sequence of the 16S rRNA gene. The sequence of the 16S rRNA gene from 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 Neighbor-Joining Method (Saitou & Nei, 1987). Horizontal lines indicate the evolutionary distance, while vertical lines are meaningless. The numbers on the branches are confidence limits (expressed as percentages) estimated from the bootstrap analysis performed with 1,000 replicates. The bar represents one nucleotide substitution per 100 nucleotides in 16S rRNA sequences.

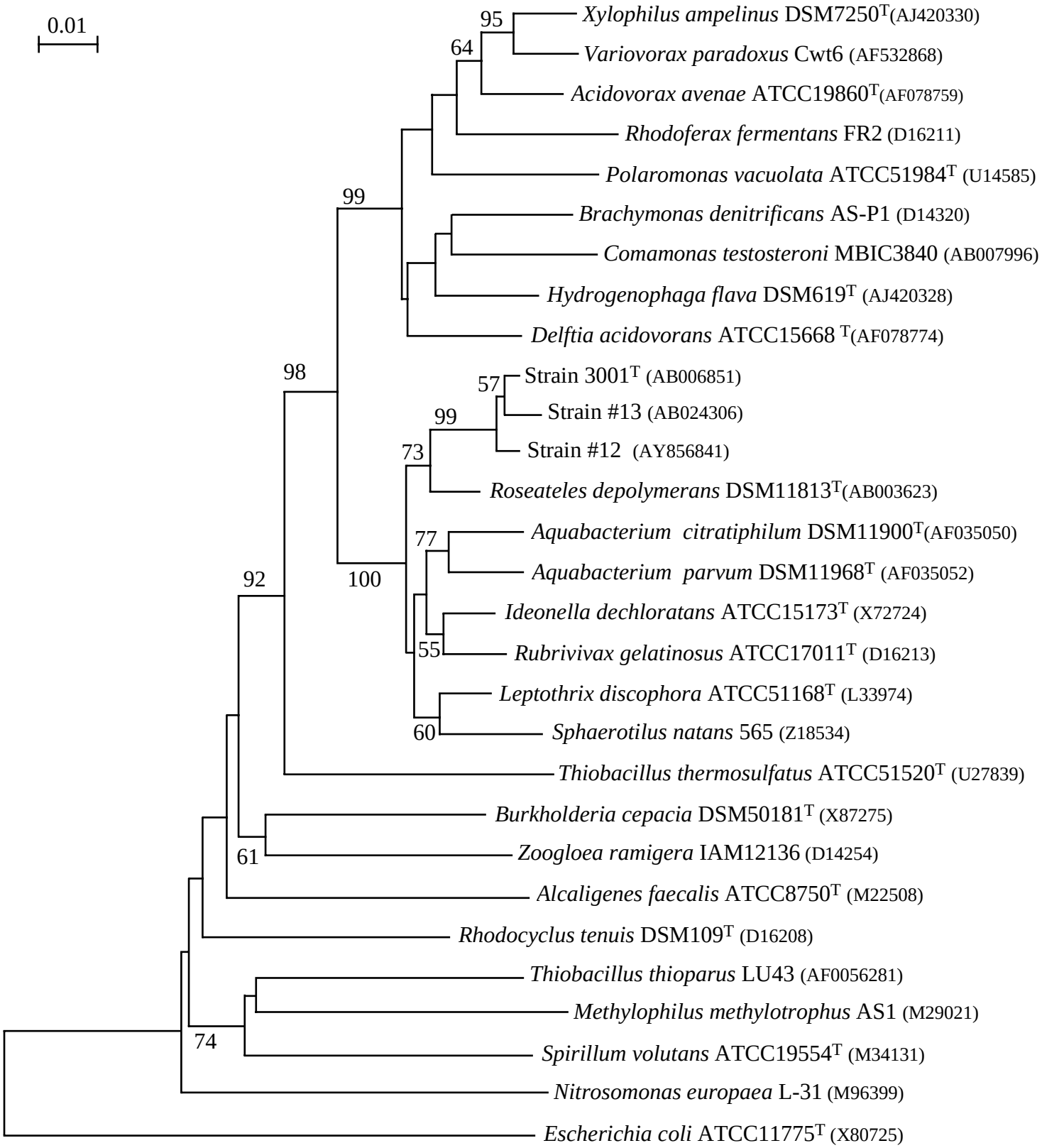
0.01
95
64
99
98
57
99
73
77
92
100
55
60
61
74
Xylophilus ampelinus DSM7250^T(AJ420330)
Variovorax paradoxus Cwt6 (AF532868)
Acidovorax avenae ATCC19860^T(AF078759)
Rhodoferax fermentans FR2 (D16211)
Polaromonas vacuolata ATCC51984^T (U14585)
Brachymonas denitrificans AS-P1 (D14320)
Comamonas testosteroni MBIC3840 (AB007996)
Hydrogenophaga flava DSM619^T (AJ420328)
Delftia acidovorans ATCC15668 ^{T}(AF078774)
Strain 3001^T (AB006851)
Strain #13 (AB024306)
Strain #12 (AY856841)
Roseateles depolymerans DSM11813^T(AB003623)
Aquabacterium citratiphilum DSM11900^T(AF035050)
Aquabacterium parvum DSM11968^T (AF035052)
Ideonella dechloratans ATCC15173^T (X72724)
Rubrivivax gelatinosus ATCC17011^T (D16213)
Leptothrix discophora ATCC51168^T (L33974)
Sphaerotilus natans 565 (Z18534)
Thiobacillus thermosulfatus ATCC51520^T (U27839)
Burkholderia cepacia DSM50181^T (X87275)
Zoogloea ramigera IAM12136 (D14254)
Alcaligenes faecalis ATCC8750^T (M22508)
Rhodocyclus tenuis DSM109^T (D16208)
Thiobacillus thioparus LU43 (AF0056281)
Methylophilus methylotrophus AS1 (M29021)
Spirillum volutans ATCC19554^T (M34131)
Nitrosomonas europaea L-31 (M96399)
Escherichia coli ATCC11775^T (X80725)

Fig. 2

Table1. Comparison of physiological properties of isolated strains and neighboring strains

	No.2	No.12	No.13	3001^T	*I.d.*	*L.d*	*R. d.*	*A. c.*	*R.g*
Flagellation	NT	1,polar	1,polar	1,polar	Several polar	1,polar	Several polar	1, polar	1,polar
Formation of sheaths	-	-	-	-	-	+	-	-	-
Nitrate reduction	-	+	+	+	+	-	-	+	-
Polyalkanoate inclusion body	-	-	-	-	-	NA	NA	+	NA
Oxidase	+	+	+	+	+	+	+	NA	+
Indole production	+	-	-	-	-	NA	-	NA	-
Catalase	+	+	+	+	+	-	-	-	+
Hydrolysis of Tween20,40,80	+	+	+	+	+	NA	+	+	+
Pigments: Bchl a	-	-	-	-	-	+	-	NA	+
Urease	+	-	-	-	+	NA	-	+/-	-
Fluorescent Pigment (KingsA & B)	+	-	-	-	NA	NA	NA	NA	NA
2-keto-gluconate production	+	-	-	-	NA	NA	NA	NA	NA
Dihydroxyacetone production	+	-	-	-	-	NA	-	NA	-
pH for growth	5-8	5-9	5-9	5-9	5-9	NA	6-8	6.5-9.5	5-9
Highest temperature for growth	37ºC	34ºC	34ºC	34ºC	42ºC	NA	45ºC	34ºC	37ºC
Chitosan	+	+	+	+	-	-	-	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 with 3001 (%)	72.0	98.9	98.7	100	93-94	94-95	94-95	93-94	94-95

I.d. Ideonella dechloratans; *L. d. Leptothrix discophora*; *R. d. Roseateles depolymerans*; *A.c. Aquabacterium citratiphilum*;*R. g. Rubrivivax gelatinosus*

Chitosan degradability and quinone type were tested in our hands. Other results were taken from the literatures (Kalmbach *et al.* (1999); Spring *et al.* (1996); Suyama *et al.* (1999); Malmqvist *et al.* (1994); Willems et al. (1991b)). * N.A, Not Available