Brevundimonas naejangsanensis sp. nov., a proteolytic bacterium isolated from soil, and reclassification of *Mycoplana bullata* into the genus *Brevundimonas* as *Brevundimonas bullata* comb. nov.

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A Gram-negative, motile and rod-shaped bacterial strain, BIO-TAS2-2^T, of the class *Alphaproteobacteria*, was isolated from a soil in Korea and studied using a polyphasic taxonomic approach. Strain BIO-TAS2-2^T grew optimally at pH 7.5–8.5 and 30 °C and in the presence of 0–1.0 % (w/v) NaCl. A neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showed that strain BIO-TAS2-2^T fell within the clade comprising species of the genus *Brevundimonas*, forming a coherent cluster with *Brevundimonas terrae* KSL-145^T and *Brevundimonas diminuta* LMG 2089^T. It exhibited 16S rRNA gene sequence similarity values of 96.0–98.7 % to members of the genus *Brevundimonas* and *Mycoplana bullata* IAM 13153^T. Strain BIO-TAS2-2^T contained Q-10 as the predominant ubiquinone and cyclo-C_{18:1}ω7c and C_{16:0} as the major fatty acids. The DNA G + C content was 67.0 mol%. Strain BIO-TAS2-2^T exhibited DNA–DNA relatedness levels of 12–19 % with the type strains of phylogenetically related *Brevundimonas* species and *M. bullata*. The novel strain could be differentiated from *Brevundimonas* species and *M. bullata* by differences in phenotypic characteristics. On the basis of phenotypic, phylogenetic and genetic data, strain BIO-TAS2-2^T is considered to represent a novel species of the genus *Brevundimonas*, for which the name *Brevundimonas naejangsanensis* sp. nov. is proposed. The type strain is BIO-TAS2-2^T (=KCTC 22631^T=CCUG 57609^T). In this study, it is also proposed that *Mycoplana bullata* be transferred to the genus *Brevundimonas* as *Brevundimonas bullata* comb. nov. (type strain TK0051^T=ATCC 4278^T=DSM 7126^T=JCM 20846^T=LMG 17157^T).

The genus *Brevundimonas* was proposed by Segers *et al.* (1994) and, at the time of writing, comprises 14 species with validly published names: *B. diminuta* and *B. vesicularis* (Segers *et al.*, 1994), *B. alba*, *B. aurantiaca*, *B. bacteroides*, *B. intermedia*, *B. subvibrioides* and *B. variabilis* (Abraham *et al.*, 1999), *B. nasdae* (Li *et al.*, 2004), *B. mediterranea* (Fritz *et al.*, 2005), *B. kwangchunensis* (Yoon *et al.*, 2006a), *B. terrae* (Yoon *et al.*, 2006b), *B. aveniformis* (Ryu *et al.*, 2007) and *B. lenta* (Yoon *et al.*, 2007). During the screening of

proteolytic micro-organisms from soil collected from Naejang Mountain in Korea, many novel bacterial strains have been isolated and characterized taxonomically. One of these isolates, designated BIO-TAS2-2^T, which had been found to be phylogenetically closely related to the genus *Brevundimonas*, is characterized in this study. The aim of the present work was to determine the exact taxonomic position of strain BIO-TAS2-2^T by using a polyphasic approach.

A soil sample collected from Naejang Mountain in Jeongeup, Korea, was used for the isolation of bacterial strains. Strain BIO-TAS2-2^T was isolated by means of the standard dilution plating technique on Luria–Bertani (LB) agar containing 3 % (w/v) skim milk at 30 °C. The type strains of two species of the genus *Brevundimonas* and

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain BIO-TAS2-2^T is FJ544245.

A supplementary table detailing the Biolog assimilation data for strain BIO-TAS2-2^T and related strains and an additional phylogenetic tree based on maximum-likelihood analysis of the 16S rRNA gene sequences are available with the online version of this paper.

Mycoplana bullata were used as reference strains for DNA–DNA hybridization. *B. diminuta* LMG 2089^T was obtained from the Laboratorium voor Microbiologie Universiteit Gent (LMG; Gent, Belgium) and *M. bullata* DSM 7126^T was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ; Braunschweig, Germany). *B. terrae* KSL-145^T was obtained from the study of Yoon *et al.* (2006b).

To investigate its morphological, physiological and biochemical characteristics, strain BIO-TAS2-2^T was routinely cultivated on trypticase soy agar (TSA; Difco) at 30 °C. Cell morphology was examined by light microscopy (E600; Nikon) using cells from exponentially growing cultures. The Gram reaction was determined using the bioMérieux Gram Stain kit according to the manufacturer's instructions. Growth at various temperatures (4, 10, 20, 25, 28, 30, 35, 40, 45, 50 and 55 °C) was measured on TSA. The pH range for growth was determined in nutrient broth (Difco) adjusted, prior to sterilization, to various pH values (pH 4.5–10.5 at intervals of 0.5 pH units) by the addition of HCl or Na₂CO₃. Growth at various NaCl concentrations (0, 0.5, 1.0, 2.0, 3.0, 4.0 and 5.0 %; w/v) was investigated using trypticase soy broth prepared according to the formula of the Difco medium except that NaCl was excluded. Growth under anaerobic conditions was determined after incubation in an anaerobic chamber on TSA and on TSA supplemented with potassium nitrate (0.1 %; w/v), both of which had been prepared anaerobically under a nitrogen atmosphere. Catalase and oxidase activities and hydrolysis of gelatin, hypoxanthine, starch, Tweens 20, 40, 60 and 80, tyrosine, urea and xanthine were determined as described by Cowan & Steel (1965). Hydrolysis of aesculin and nitrate reduction were examined as described previously (Lányi, 1987).

Susceptibility to antibiotics was tested by placing antibiotic-impregnated discs on TSA plates that were seeded with suspensions of the test strain. The concentrations of antibiotics used were as follows; polymyxin B (100 U), streptomycin (50 µg), penicillin G (20 U), chloramphenicol (100 µg), ampicillin (10 µg), cephalothin (30 µg), gentamicin (30 µg), novobiocin (5 µg), tetracycline (30 µg), kanamycin (30 µg), lincomycin (15 µg), oleandomycin (15 µg), neomycin (30 µg) and carbenicillin (100 µg). The assimilation of various substrates was determined by using the Biolog GN2 MicroPlate assay as recommended by the manufacturers. Enzyme activities and other physiological and biochemical properties were tested by using the API ZYM and API 20E systems (bioMérieux).

Cell biomass of strain BIO-TAS2-2^T for DNA extraction and for isoprenoid quinone analysis was obtained from cultures grown in trypticase soy broth (Difco) at 30 °C. Chromosomal DNA was isolated and purified according to the method described by Yoon *et al.* (1996), with the exception that RNase T1 was used in combination with RNase A to minimize contamination with RNA. The 16S rRNA gene was amplified by PCR using two universal

primers as described previously (Yoon *et al.*, 1998). Sequencing of the amplified 16S rRNA gene and phylogenetic analysis were performed as described by Yoon *et al.* (2003). Isoprenoid quinones were extracted according to the method of Komagata & Suzuki (1987) and analysed using reversed-phase HPLC and a YMC ODS-A (250 × 4.6 mm) column. For cellular fatty acid analysis, cell mass of strain BIO-TAS2-2^T was harvested from TSA plates after incubation for 2 days at 30 °C. The fatty acids were extracted and fatty acid methyl esters were prepared according to the standard protocol of the MIDI/Hewlett Packard Microbial Identification System (Sasser, 1990). The DNA G + C content was determined by the method of Tamaoka & Komagata (1984) with the modification that DNA was hydrolysed and the resultant nucleotides were analysed by reversed-phase HPLC. DNA–DNA hybridization was performed fluorometrically by the method of Ezaki *et al.* (1989) using photobiotin-labelled DNA probes and microdilution wells. Hybridization was performed with five replications for each sample. The highest and lowest values obtained in each sample were excluded and the means of the remaining three values were quoted as DNA–DNA relatedness values.

The morphological, cultural, physiological and biochemical characteristics of strain BIO-TAS2-2^T are given in the species description (see below) or in Table 1. The almost complete 16S rRNA gene sequence of strain BIO-TAS2-2^T determined in this study comprised 1418 nucleotides, representing approximately 96 % of the *Escherichia coli* 16S rRNA gene sequence. The phylogenetic analyses based on 16S rRNA gene sequences revealed that strain BIO-TAS2-2^T fell within the clade comprising species of the genus *Brevundimonas* (Fig. 1). In the phylogenetic tree based on the neighbour-joining algorithm, strain BIO-TAS2-2^T joined the cluster comprising *B. terrae* and *B. diminuta* with a bootstrap resampling value of 77.7 % (Fig. 1). This relationship was also maintained in trees constructed using the maximum-parsimony (Fig. 1) and the maximum-likelihood algorithms (see Supplementary Fig. S1 in IJSEM Online). Strain BIO-TAS2-2^T exhibited 16S rRNA gene sequence similarity values of 98.0, 98.7, 96.8, 96.6 and 97.5 % to the type strains of *B. terrae*, *B. diminuta*, *B. lenta*, *B. subvibrioides* and *M. bullata*, respectively, and 96.0–98.0 % to other *Brevundimonas* species used in the phylogenetic analysis.

The results obtained from chemotaxonomic analyses, showing the properties that are shared by species of the genus *Brevundimonas*, were in agreement with the result of phylogenetic analysis, i.e. that strain BIO-TAS2-2^T is a member of the genus *Brevundimonas*. The predominant isoprenoid quinone detected in strain BIO-TAS2-2^T was ubiquinone-10 (Q-10), as previously found for other *Brevundimonas* species (Segers *et al.*, 1994; Abraham *et al.*, 1999). The cellular fatty acid profile of strain BIO-TAS2-2^T is shown in Table 2, together with those of *B. diminuta* LMG 2089^T, *B. terrae* KSL-145^T and *M. bullata* DSM 7126^T, which were also analysed in this study. The

Table 1. Differential phenotypic characteristics of strain BIO-TAS2-2^T, phylogenetically related species of the genus *Brevundimonas* and *M. bullata*

Taxa: 1, strain BIO-TAS2-2^T; 2, *B. terrae* (data from Yoon *et al.*, 2006b); 3, *B. diminuta* (Palleroni, 1984; Segers *et al.*, 1994; Li *et al.*, 2004; Yoon *et al.*, 2006a); 4, *B. lenta* (Yoon *et al.*, 2007); 5, *B. subvibrioides* (Poindexter, 1964; Yoon *et al.*, 2006a); 6, *M. bullata* (Urakami *et al.*, 1990; Yoon *et al.*, 2007). +, Positive; –, negative; w, weakly positive; ND, not determined; NG, no growth. Data in parentheses are for the type strain.

Characteristic	1	2	3	4	5	6
Oxidase	+	+	+*	+	ND	+ or w
Catalase	+	+	+	+	ND	w or –
Urease	–	–	–*	–	ND	NG
Arginine dihydrolase	–	–	–*	–	ND	ND
α-Glucosidase	–	–	–	+	ND	ND
Nitrate reduction	–	–	–	–	–	w or –
Hydrolysis of:						
Aesculin	–	–	–	+	ND	(–)
Gelatin	–	–	–*	+	ND	(–)
Starch	–	–	–	–	+	(–)
Tween 80	+	+	–	+	ND	(–)
Utilization of:†						
Acetic acid	+	–	(+)	–	(–)	(–)
Alaninamide	+	+	(+)	–	(+)	(+)
D-Alanine	+	+	(+)	–	(–)	(+)
L-Alanine	+	+	(+)	–	(–)	(+)
L-Alanyl glycine	+	+	(+)	–	(–)	(+)
L-Asparagine	+	+	(+)	–	(–)	(+)
L-Aspartic acid	+	+	(+)	–	(–)	(+)
Cellobiose	–	–	(–)	–	(+)	(–)
Dextrin	–	–	(–)	+	(+)	(+)
Glucose 1-phosphate	–	–	(–)	–	(–)	(+)
Glucose 6-phosphate	–	–	(–)	–	(–)	(+)
D-Glucose	–	–	(–)	–	(+)	(+)
Glycyl L-aspartic acid	+	+	(+)	–	(–)	(+)
Glycyl L-glutamic acid	+	+	(+)	–	(+)	(+)
L-Histidine	+	w	(–)	–	(–)	(–)
Hydroxy L-proline	+	+	(+)	–	(–)	(–)
β-Hydroxybutyric acid	+	+	(+)	–	(–)	(+)
α-Ketobutyric acid	–	–	(–)	–	(–)	(+)
α-Ketoglutaric acid	+	–	(–)	+	(–)	(–)
α-Ketovaleric acid	+	–	(+)	–	(–)	(–)
L-Leucine	+	+	(–)	–	(–)	(–)
Methylpyruvate	+	+	(+)	–	(–)	(+)
Monomethyl succinate	+	–	(–)	–	(–)	(–)
L-Proline	+	+	(+)	+	(–)	(+)
Propionic acid	+	+	(–)	–	(–)	(–)
L-Serine	+	+	(+)	–	(–)	(+)
Succinic acid	+	–	(–)	–	(–)	(–)
L-Threonine	+	+	(–)	–	(–)	(+)
Turanose	–	–	(–)	–	(–)	(w)
Tween 40	+	+	(+)	–	(–)	(–)
Tween 80	+	+	(–)	–	(–)	(–)
Presence of cyclo C _{19:0} ω8c	+	–	(+)	–	–	(–)
DNA G + C content (mol%)	67.0	61.8	66–68	68.7	67‡	66–68

*Data from Palleroni (1984) and Segers *et al.* (1994); different results for the type strains were obtained from Li *et al.* (2004).

†Data from Biolog GN2 MicroPlate assay for reference strains from Yoon *et al.* (2006a, b, 2007).

‡Determined for one isolate from Poindexter (1989).

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the positions of strain BIO-TAS2-2^T and some other related taxa. Bootstrap values (expressed as percentages of 1000 replications) of >50% are shown at branching points. Filled circles indicate that the corresponding nodes were also recovered in trees generated with both the maximum-likelihood and maximum-parsimony algorithms. Open circles indicate that the corresponding nodes were recovered in the tree generated with the maximum-parsimony algorithm. *Rhodospirillum rubrum* ATCC 11170^T (GenBank accession no. D30778) was used as an outgroup. Bar, 0.01 substitutions per nucleotide position.

major fatty acids (>10% total fatty acids) were C_{18:1ω7c} and C_{16:0}. The fatty acid profiles of these species were similar, although there were differences in the proportions of some fatty acids between the taxa (Table 2). The DNA G + C content of strain BIO-TAS2-2^T was 67.0 mol%.

Strain BIO-TAS2-2^T exhibited mean DNA–DNA relatedness levels of 12, 19 and 17% to *B. diminuta* LMG 2089^T, *B. terrae* KSL-145^T and *M. bullata* DSM 7126^T, respectively, indicating that it is genetically different from these three reference strains (Wayne *et al.*, 1987). Strain BIO-TAS2-2^T is distinguishable from recognized species of the genus *Brevundimonas* and *M. bullata* on the basis of differences in several phenotypic characteristics (Table 1 and Supplementary Table S1 in IJSEM Online). Therefore, this phylogenetic and genetic distinctiveness, together with the differential phenotypic properties, suggests that strain BIO-TAS2-2^T represents a novel species of the genus *Brevundimonas*, for which the name *Brevundimonas naejangsensis* sp. nov. is proposed.

On the basis of the phylogenetic and chemotaxonomic data determined in this study, it is considered appropriate for *M. bullata* to be reclassified as a member of the genus *Brevundimonas*. The phylogenetic analyses based on 16S rRNA gene sequences showed that *M. bullata* is phylogenetically more closely related to members of the genus *Brevundimonas* than to *Mycoplana dimorpha* IAM 13154^T, the type species of the genus, and *Mycoplana ramosa* IAM 13949^T (Fig. 1; Yoon *et al.*, 2006b). The results obtained from chemotaxonomic analyses revealed the properties that are shared by species of the genus *Brevundimonas*. *M. bullata* has previously been described as having Q-10 as the predominant ubiquinone (Urakami *et al.*, 1990). The fatty acid profile of *M. bullata* DSM 7126^T is similar to those of *Brevundimonas* species in that C_{18:1ω7c} and C_{16:0} are the

major fatty acids (Table 2; Urakami *et al.*, 1990; Yoon *et al.*, 2006b). Accordingly, it is proposed that *Mycoplana bullata* is transferred into the genus *Brevundimonas* as *Brevundimonas bullata* comb. nov.

Description of *Brevundimonas naejangsensis* sp. nov.

Brevundimonas naejangsensis (nae.jang.san.en'sis. N.L. fem. adj. *naejangsensis* pertaining to Naejangsan, the Korean name of Naejang Mountain, from where the type strain was isolated).

Cells are Gram-negative and rod-shaped (0.3–0.5 × 0.7–4.0 μm). Motile by means of a single polar flagellum. Colonies on TSA are circular, slightly convex, smooth, glistening, greyish yellow and 1.0–2.0 mm in diameter after incubation for 2 days at 30 °C. Growth occurs at 4 and 50 °C, but not at 55 °C. Optimal pH for growth is between 7.5 and 8.5; growth occurs at pH 5.5 and pH 10.0, but not at pH 5.0 or pH 10.5. Growth occurs in the presence of 0–4.0% (w/v) NaCl, with an optimum in the presence of 0–1.0% (w/v) NaCl. Growth occurs under anaerobic conditions. Casein, L-tyrosine and Tweens 20, 40 and 60 are hydrolysed, but hypoxanthine and xanthine are not. H₂S is not produced. Susceptible to chloramphenicol, gentamicin, kanamycin, neomycin, novobiocin, streptomycin and tetracycline, but not to ampicillin, carbenicillin, cephalothin, lincomycin, oleandomycin, penicillin G or polymyxin B. In assays with the API ZYM system, alkaline phosphatase, esterase (C4), leucine arylamidase, valine arylamidase, trypsin, acid phosphatase and naphthol-AS-BI-phosphohydrolase are present, but esterase lipase (C8), lipase (C14), cystine arylamidase, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, β-glucosi-

Table 2. Cellular fatty acid contents (%) of strain BIO-TAS2-2^T, the type strains of two *Brevundimonas* species and *M. bullata*

Strains: 1, BIO-TAS2-2^T; 2, *B. diminuta* LMG 2089^T; 3, *B. terrae* KSL-145^T; 4, *M. bullata* DSM 7126^T. All data are from this study. –, Not detected. Fatty acids that represented <0.5% in all strains were omitted.

Fatty acid	1	2	3	4
Straight-chain fatty acid				
C _{14:0}	0.9	0.6	3.3	1.0
C _{15:0}	0.7	6.1	9.1	5.3
C _{16:0}	25.3	15.5	24.7	21.2
C _{17:0}	0.7	9.7	2.4	1.9
Unsaturated fatty acid				
C _{15:1} ω8c	–	1.7	1.0	0.9
C _{17:1} ω6c	0.35	4.5	1.5	2.1
C _{17:1} ω8c	0.7	7.8	3.2	3.9
C _{18:1} ω5c	1.0	1.2	0.9	1.3
C _{18:1} ω7c	53.8	42.2	41.1	52.7
C _{20:2} ω6,9c	0.1	0.7	–	–
Hydroxy fatty acid				
C _{12:1} 3-OH	0.2	0.2	2.1	0.8
C _{12:0} 3-OH	2.2	1.7	–	1.6
11-methyl C _{18:1} ω7c	0.1	–	5.6	1.5
Cyclo C _{19:0} ω8c	9.5	5.4	–	0.2
Summed features*				
1	–	0.8	0.3	0.2
3	3.8	1.2	3.5	4.9

*Summed features represent groups of two or three fatty acids which could not be separated by GLC with the MIDI system. Summed feature 1 contained iso-C_{15:1} and/or C_{13:0} 3-OH. Summed feature 3 contained C_{16:1}ω7c and/or iso-C_{15:0} 2-OH.

dase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase are absent. L-Glutamic acid is assimilated, but the following substrates are not assimilated: α-cyclodextrin, glycogen, *N*-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine, adonitol, L-arabinose, D-arabitol, *i*-erythritol, D-fructose, L-fucose, D-galactose, gentiobiose, *myo*-inositol, D-lactose, lactulose, maltose, D-mannitol, D-mannose, melibiose, methyl β-D-glucoside, D-psicose, raffinose, L-rhamnose, D-sorbitol, sucrose, trehalose, xylitol, *cis*-aconitic acid, citric acid, formic acid, D-galactonic acid lactone, D-galacturonic acid, D-gluconic acid, α-hydroxybutyric acid, D-glucosaminic acid, D-glucuronic acid, γ-hydroxybutyric acid, *p*-hydroxyphenyl acetic acid, itaconic acid, DL-lactic acid, malonic acid, quinic acid, D-saccharic acid, sebacic acid, bromosuccinic acid, succinamic acid, glucuronamide, L-ornithine, L-phenylalanine, L-pyroglutamic acid, D-serine, DL-carnitine, γ-aminobutyric acid, urocanic acid, inosine, uridine, thymidine, phenylethylamine, putrescine, 2-aminoethanol, 2,3-butanediol, glycerol and DL-α-glycerol phosphate. The predominant ubiquinone is

Q-10. The major fatty acids (>10% of total fatty acids) are C_{18:1}ω7c and C_{16:0}. Other phenotypic characteristics are given in Table 1.

The type strain, BIO-TAS2-2^T (=KCTC 22631^T=CCUG 57609^T), was isolated from a soil from Naejang Mountain, Korea. The DNA G+C content of the type strain is 67.0 mol% (determined by HPLC).

Description of *Brevundimonas bullata* comb. nov.

Brevundimonas bullata (bull.a.ta. L. fem. adj. *bullata* wearing a bulla, with a knob).

Basonym: *Mycoplana bullata* Gray and Thornton 1928 (Approved Lists 1980) emend. Urakami *et al.* 1990.

The description is the same as that given by Urakami *et al.* (1990). The type strain is TK0051^T (=ATCC 4278^T=DSM 7126^T=JCM 20846^T=LMG 17157^T).

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