Aquipuribacter nitratireducens sp. nov., isolated from a soil sample of a mud volcano

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A novel Gram-stain-positive, coccoid, non-motile bacterium, designated strain AMV4^T, was isolated from a soil sample collected from a mud volcano located in the Andaman Islands, India. The colony was pale orange. Strain AMV4^T was positive for oxidase, aesculinase, lysine decarboxylase and ornithine decarboxylase activities and negative for amylase, catalase, cellulase, protease, urease and lipase activities. 16S rRNA gene sequence analysis indicated that strain AMV4^T was a member of the order *Actinomycetales* and was closely related to *Aquipuribacter hungaricus* with a sequence similarity of 97.13 % (pairwise alignment). Phylogenetic analyses showed that strain AMV4^T clustered with *Aquipuribacter hungaricus* and was distantly related to the other genera of the family *Intrasporangiaceae*. DNA–DNA hybridization between strains AMV4^T and *Aquipuribacter hungaricus* IV-75^T showed a relatedness of 28 %. The predominant cellular fatty acids were iso-$C_{15:0}$ (6.9 %), anteiso-$C_{15:0}$ (25.3 %), $C_{16:0}$ (12.9 %), anteiso-$C_{16:0}$ (5.6 %), $C_{18:1}\omega9c$ (19.8 %) and $C_{18:3}\omega6,9,12c$ (9.1 %). The diagnostic diamino acid in the cell-wall peptidoglycan of strain AMV4^T was *meso*-diaminopimelic acid. Strain AMV4^T contained MK-10(H_4) as the predominant respiratory quinone. The polar lipids consisted of phosphatidylglycerol, one unidentified glycolipid, two unidentified phospholipids and five unidentified lipids. The DNA G+C content of strain AMV4^T was 74.3 mol%. Based on data from this taxonomic study using a polyphasic approach, it is proposed that strain AMV4^T represents a novel species of the genus *Aquipuribacter*, with the suggested name *Aquipuribacter nitratireducens* sp. nov. The type strain is AMV4^T (=CCUG 58430^T=DSM 22863^T=NBRC 107137^T).

The genus *Aquipuribacter* was established with a single species *Aquipuribacter hungaricus* by Tóth *et al.* (2012) as a member of the family *Intrasporangiaceae*. *A. hungaricus* is Gram-stain-positive, exhibits a rod-coccus cycle, is non-endospore forming and grows aerobically. Cells are non-motile and form pale orange, smooth, convex and circular colonies. Cells have *meso*-diaminopimelic acid (*m*-Dpm) as the cell wall component, MK-10(H_4) as the predominant quinone, phosphatidylglycerol as the major polar lipid, anteiso-$C_{15:0}$, $C_{18:1}\omega9c$ and $C_{16:0}$ as major cellular fatty acids with a DNA G+C content of 75 mol%. A strain of the species *A. hungaricus* has been isolated from the ultrapure water system of a Hungarian power plant (Tóth *et al.* 2012). In the present study, we focused on the characterization and classification, using a polyphasic approach, of strain AMV4^T, which was isolated from a mud volcano (Vandamme *et al.*, 1996) and propose the affiliation of strain AMV4^T to the genus *Aquipuribacter* as a distinct species.

Strain AMV4^T was isolated from a soil sample collected from a mud volcano on Baratang Island, Middle Andamans, India on 20 February 2009. The sample that yielded strain AMV4^T had a pH of 7.5. For isolation of bacteria, 100 mg of the soil sample suspended in 1 % saline water was plated on ZoBell marine agar (MA) plates (ZoBell, 1941) and incubated at 37 °C for 15 days. Out of the two pale-orange colonies that shared 100 % 16S rRNA gene sequence similarity, one was selected and characterized. Sub-cultivation of the isolate was carried out on marine agar medium at 37 °C. Stock cultures of the isolate in marine broth with 10 % (v/v) glycerol were preserved at −80 °C.

Abbreviations: FAMEs, fatty acid methyl esters; *m*-Dpm, *meso*-diaminopimelic acid

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain AMV4^T is FN397670.

Strain AMV4^T was characterized simultaneously with *A. hungaricus* IV-75^T, which was obtained from Dr E. M. Tóth (Tóth *et al.*, 2012). Cell morphology and motility were observed by using phase-contrast microscopy (Eclipse, 80i; Nikon, Japan). Morphology was also observed using transmission electron microscopy [TEM (H7600; Hitachi)]. Specimens for TEM were negatively stained with phosphotungstic acid. Motility was also assessed on tryptic soy agar (TSA) containing (l^{-1}) a pancreatic digest of casein (17 g), a papaic digest of soyabean meat (3 g), NaCl (20 g), dipotassium hydrogen phosphate (2.5 g), glucose (2.5 g) and agar (0.4 g) and on a motility Indole Lysine agar slant (HIMEDIA). Growth at 4, 10, 20, 30, 37, 40, 45 and 50 °C was ascertained using marine broth (MB) and salt tolerance [0, 1, 2, 3, 4, 5, 6, 8 and 10 % (w/v) NaCl] was determined using nutrient broth (NB) containing (l^{-1}) peptone (5 g) and beef extract (3 g). Growth of strain AMV4^T at pH 5, 6, 7, 7.5, 8, 8.5, 9, 9.5, 10, 11 and 12 was assessed on MB buffered with citric acid/NaOH (for pH 5 and 6), $NaHPO_4$-Na_2HPO_4 (for pH 7 and 8), glycine/NaOH (for pH 9 and 10) or Tris/HCl or NaOH (for pH 11 and 12). Biochemical characteristics, such as activity of oxidase, lysine decarboxylase, ornithine decarboxylase, nitrate reduction, hydrolysis of aesculin, gelatin, ONPG, starch, Tweens 20, 40, 60 and 80, carbon source assimilation, H_2S production and the sensitivity to 19 different antibiotics using the disc diffusion method with commercially available discs (HIMEDIA) were determined by previously described methods (Lányí, 1987; Smibert & Krieg, 1994). Anaerobic growth was assessed: (i) in screw-capped tubes containing ZoBell marine broth with 0.05 % sodium ascorbate filled up to the top of the tube without an air gap and, (ii) by streaking the cultures on marine anaerobic agar (HIMEDIA) slants and flushing with N_2 to expel all the air. Biochemical characteristics were assessed using the Hi25 Enterobacteriaceae identification kit and the HiCarbohydrate kit parts A, B and C (HIMEDIA) according to the manufacturer's protocol. Biochemical and enzymic characterization of the strains was also performed using Vitek 2 GN (bioMérieux) according to the manufacturer's protocol, except that sterile 2.0 % (w/v) NaCl was used to prepare the inoculum.

Standardization of the physiological age of strains AMV4^T and *A. hungaricus* IV-75^T was carried out based on the protocol (http://www.microbialid.com/PDF/TechNote_101.pdf) given by the Sherlock Microbial Identification System (MIDI). For cellular fatty acid analysis, strains AMV4^T and *A. hungaricus* IV-75^T were grown on MA plates at 37 °C for 7 and 4 days respectively. Cellular fatty acid methyl esters (FAMEs) were obtained from cells by saponification, methylation and extraction following the protocol of MIDI. Cellular FAMEs were separated by GC (Agilent 6890) and analysed using the Sherlock Microbial Identification System (MIDI-6890 with database TSBA6) according to the protocol described by the Sherlock Microbial Identification System. The cell-wall diamino acid of strain AMV4^T was extracted from whole-cell hydrolysates and analysed as described by Hasegawa *et al.* (1983). Polar lipids were extracted following the method of Bligh & Dyer (1959) and analysed by two-dimensional TLC followed by spraying with appropriate detection reagents (Komagata & Suzuki, 1987). Menaquinones were extracted as described by Collins *et al.* (1977) and analysed by HPLC (Groth *et al.*, 1997). Menaquinones and polar lipids were analysed using freeze-dried cells. The polar lipids were identified as phospho-, amino- or glyco-lipids by spraying with molybdenum blue, ninhydrin, and α-naphthol reagents, respectively. The DNA of strain AMV4^T was isolated according to the procedure of Marmur (1961) and the G+C content was determined from melting point (T_m) curves (Sly *et al.*, 1986) obtained by using a Lambda 2 UV-Vis spectrophotometer (Perkin Elmer) equipped with the Templab 2.0 software package (Perkin Elmer). *Escherichia coli* DH5-α DNA was used as the standard in determining the DNA G+C content.

For 16S rRNA gene sequencing, DNA was prepared using a microbial DNA isolation kit (Mo Bio Laboratories) and sequenced as described previously (Lane, 1991). The resultant, almost complete sequence, of the 16S rRNA gene (1492 nt) was subjected to BLAST sequence similarity searches (Altschul *et al.*, 1990) and the EzTaxon-e server (Kim *et al.*, 2012) was used to identify the nearest taxa. 16S rRNA gene sequences of the type strains of *A. hungaricus* and type species of the genera within the family *Intrasporangiaceae* were downloaded from the NCBI database

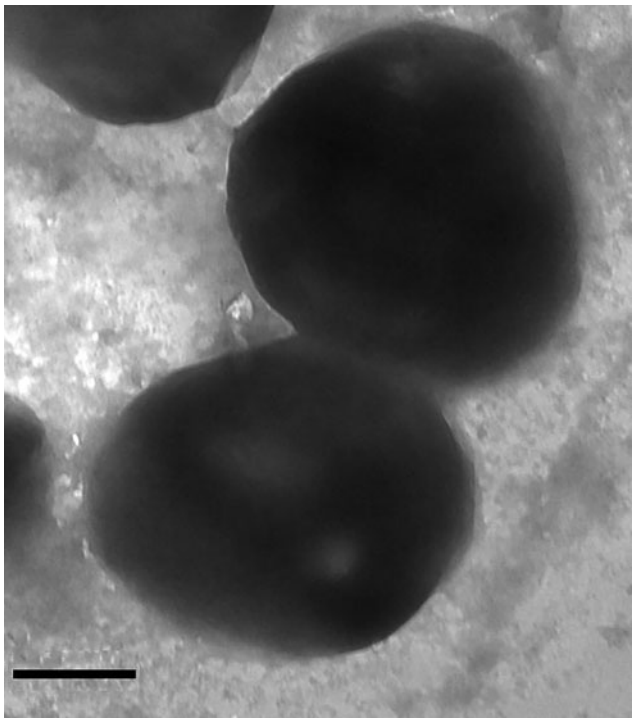

Fig. 1. Electron micrograph of negatively stained cells of strain AMV4^T. Bar, 0.5 μm.

(*http://www.ncbi.nlm.nih.gov*) and aligned using the CLUSTAL W program within the MEGA5 package (Tamura *et al.*, 2011). Phylogenetic trees were reconstructed using the neighbour-joining and maximum-likelihood methods using the MEGA5 package (Tamura *et al.*, 2011) and the resultant tree topologies were evaluated based on 1000 bootstrap resamplings. DNA–DNA hybridization was performed by the membrane filter method, as described previously (Shivaji *et al.*, 1992; Tourova & Antonov, 1988).

Cells of strain AMV4^T were Gram-stain-positive, non-motile, coccoid shaped, 1.5–2.0 μm in diameter and they multiplied by binary fission (Fig. 1). Colonies, after 7 days, were circular, 0.2–0.6 mm in diameter, smooth, shiny, pale orange, translucent and raised with entire margins on marine agar. The strain was positive for catalase, lysine decarboxylase and ornithine decarboxylase activities and negative for oxidase and phenylalanine deaminase activities and indole production. Strain AMV4^T reduced nitrate and

Table 1. Distinguishing phenotypic characteristics of strain AMV4^T and *A. hungaricus* IV-75^T

Strains: 1, AMV4^T; 2, *Aquipuribacter hungaricus* IV-75^T. All data are from the present study. Both taxa grew optimally at 2 % NaCl (w/v) and produced pale orange colonies, were positive for catalase, glutamyl arylamidase, β-glucosidase activities and for the hydrolysis of aesculin. Both strains were negative for oxidase, phenylalanine deaminase, L-pyrrolydonyl-arylamidase, γ-glutamyl-transferase, β-alanine arylamidase, L-proline arylamidase, tyrosine arylamidase, glycine arylamidase, β-glucoronidase, β-xylosidase and glu-gly-arg-arylamidase activities,as well as for L-lactate and succinate alkalinization, fermentation of glucose, H_2S production, indole production and the Voges–Proskauer reaction. Neither strain could hydrolyse casein, cellulose, Tweens 20, 40, 60 and 80, or urea. Both strains utilized glucosamine, ribose and serine, but not adonitol, L-arabitol, cellobiose, D-glucose, maltose, D-mannitol, D-mannose, isomaltulose, D-sorbitol, sucrose, D-tagatose, trehalose, sodium citrate, malonate, L-histidine, 4-coumarate, L-malate, L-lactate, D-arabinose, fructose, galactose, glycerol, lactose, melibiose, melezitose, methyl α-D-mannoside, raffinose, rhamnose, salicin, sodium gluconate, sorbose, xylitol, xylose, dulcitol, erythritol, inositol, inulin or methyl α-D-glucoside. Both taxa were resistant to kanamycin, nitrofurantoin and nalidixic acid, and sensitive to erythromycin, tetracycline and vancomycin. (+), Weakly positive; +, positive; −, negative; R, resistant; S, sensitive; M, moderately resistant.

Characteristic	1	2
Salinity range for growth (%)	13	0–3
Temperature for growth (°C)		
Range	37–45	20–37
Optimum	37	20–28
pH range for growth	7–8	6–9
Biochemical characteristics		
Lysine decarboxylase	+	−
Ornithine decarboxylase	+	−
Ala-Phe-Pro-arylamidase	(+)	−
μ-Galactosidase	−	+
β-*N*-Acetylglucosaminidase	−	+
β-*N*-Acetylgalactosaminidase	−	+
Lipase	−	+
α-Glucosidase	−	+
α-Galactosidase	−	+
Phosphatase	−	+
Methyl red test	+	−
Nitrate reduction to nitrite	+	−
Hydrolysis of starch	−	+
Utilization of:		
5-Keto-D-gluconate	−	+
L-Arabinose	−	+
Antibiotic susceptibility:		
Norfloxacin	R	M
Penicillin G	R	S
Streptomycin	R	S
Tobramycin	R	M
DNA G+C content (mol%)	74.3	75*
Habitat	Soil sample from a mud volcano	Ultra-pure water from a power plant*

*Data from Tóth *et al.*, (2012).

hydrolysed aesculin, but not casein, cellulose, starch, Tweens 20, 40, 60 and 80, or urea. Strain AMV4^T exhibited growth between 37–45 °C, at pH 7–8 and tolerated 3 % (w/v) NaCl. Studies of growth at temperatures below 37 °C and above 45 °C, below pH 7 and above pH 8 and below 1 % (w/v) NaCl and above 3 % (w/v) NaCl were repeated three times (to confirm the results) and no growth was observed even after several weeks of incubation. Strain AMV4^T exhibited optimum growth at 37 °C, at pH 7.5 and in the presence of 2 % (w/v) NaCl. Other phenotypic characteristics of strain AMV4^T are listed in the species description and in Table 1.

The cellular fatty acid composition of strain AMV4^T showed branched, saturated and unsaturated fatty acids, with a high abundance of iso-$C_{15:0}$, anteiso-$C_{15:0}$, $C_{16:0}$, anteiso-$C_{16:0}$, $C_{18:1}\omega9c$ and $C_{18:3}\omega6,9,12c$ (Table 2). When compared with *A. hungaricus* IV-75^T, strain AMV4^T showed a very similar composition with respect to the major fatty acids (Table 2). The diagnostic diamino acid in the cell-wall peptidoglycan of strain AMV4^T was *m*-Dpm. The predominant respiratory quinone of strain AMV4^T was MK-10(H_4). The polar lipids consisted of phosphatidylglycerol, one unidentified glycolipid, two unidentified phospholipids and five unidentified lipids (Fig. S1, available in the online Supplementary Material). Strain AMV4^T showed similar chemotaxonomic characteristics (diamino acid, quinone, major polar lipids and fatty acids) to *A. hungaricus* IV-75^T (Tóth *et al.*, 2012). The DNA G+C content of strain AMV4^T was 74.3 mol%.

In the phylogenetic analyses based on 16S rRNA gene sequences, strain AMV4^T appeared most closely related to *A. hungaricus* (97 % sequence similarity). Phylogenetic trees were reconstructed using the maximum-likelihood (Fig. 2) and neighbour-joining (Fig. S2) methods. The trees revealed a clear affiliation of the novel isolate with species of the genus *Aquipuribacter*; it clustered with the type strain of *A. hungaricus* and appeared distinct from the clades representing the other genera of the family *Intrasporangiaceae*; DNA–DNA hybridization between strains AMV4^T and *A. hungaricus* IV-75^T showed a relatedness value of 28 %. This value is much lower than 70 %, so it confirms that strain AMV4^T is not a member of the same species as *A. hungaricus* IV-75^T (Wayne *et al.*, 1987). We found that, when compared with suborders described within the order *Actinomycetales*, strain AMV4^T and *A. hungaricus* had many unique 16S rRNA gene signature nucleotides, which were reflected particularly in fourteen different positions: in 73 : 97 (A-G), 74 : 96 (A-G), 129 : 232 (C-G), 444 : 490 (U-A), 445 : 489 (C-G), 446 : 488 (C-G), 610-611 (GU), 629-631 (AUC), 665 : 741 (A-G), 787-788 (CU), 841-843 (AUC), 1006 : 1023 (A-C), 1133-1135 (GGG) and 1261-1263 (AAA).

The characteristics that differentiate strain AMV4^T from *A. hungaricus* IV-75^T are given in Table 1. The data obtained from the phenotypic characterization support strain AMV4^T occupying a separate position in the 16S rRNA gene sequence-based phylogenetic tree for the class *Actinobacteria*. Based on the results described above, it is concluded that strain AMV4^T represents a novel species of the genus *Aquipuribacter*, for which the name *Aquipuribacter nitratireducens* sp. nov. is proposed.

Table 2. Fatty acid compositions differentiating strain AMV4^T from *A. hungaricus* IV-75^T

Strains: 1, AMV4^T; 2, *A. hungaricus* IV-75^T. Data for first three taxa is from the present study. Strain AMV4^T and *A. hungaricus* IV-75^T were grown on MA plates at 37 °C for 7 and 4 days, respectively. Results are presented as percentages of the total fatty acids. Fatty acids amounting to ⩾5 % of the total fatty acids are in bold. Data in parentheses are from Tóth *et al.* (2012) for *A. hungaricus*. Values <1 % for all strains are not shown. ND, Not detected.

Fatty acid	1	2
$C_{14:0}$	1.1	1.6 (1.4)
iso-$C_{14:0}$	0.8	2.0 (2.2)
$C_{15:0}$	1.3	0.9 (0.7)
iso-$C_{15:0}$	**6.9**	**8.3 (5.3)**
anteiso-$C_{15:0}$	**25.3**	**19.8 (29.8)**
$C_{16:0}$	**12.9**	**15.5 (9.2)**
iso-$C_{16:0}$	3.1	3.8 (3.8)
anteiso-$C_{16:0}$	**5.6**	2.9 (ND)
$C_{16:0}$ N alcohol	ND	0.2 (1.2)
$C_{16:1}\omega7c$	ND	0.8 (1.4)
$C_{17:0}$	2.7	3.9 (3.9)
iso-$C_{17:0}$	1.9	3.5 (3.0)
anteiso-$C_{17:0}$	4.6	3.6 (4.6)
$C_{18:0}$	3.1	4.3 **(7.3)**
$C_{18:1}\omega9c$	**19.8**	**17.5 (17.5)**
$C_{18:3}\omega6,9,12c$	**9.1**	**7.8 (7.8)**

Description of *Aquipuribacter nitratireducens* sp. nov.

Aquipuribacter nitratireducens (ni.tra.ti.re.du′cens. N.L. n. *nitras -atis* nitrate; L. part. adj. *reducens* leading back, bringing back and in chemistry, converting to a different oxidation state; N.L. part. adj. *nitratireducens* reducing nitrate).

The main characteristics are the same as those previously given for the genus. In addition, cells are non-motile, coccoid, 1.5–2.0 μm in diameter, occur singly and multiply by binary fission. Colonies on MA are circular, 0.2–0.6 mm in diameter, smooth, pale-orange, translucent and raised with entire margins. Grows at 37 to 45 °C with an optimum growth temperature of 37 °C and tolerates 1–3 % NaCl (w/v) with optimum growth in the presence of 2 % NaCl (w/v). Grows at pH 7–8, with optimum growth at pH 7.5. Catalase, lysine decarboxylase and ornithine decarboxylase activities are present, but β-galactosidase, oxidase and phenylalanine deaminase activities are absent. In the VITEK GN ID card, positive for glutamyl arylamidase and

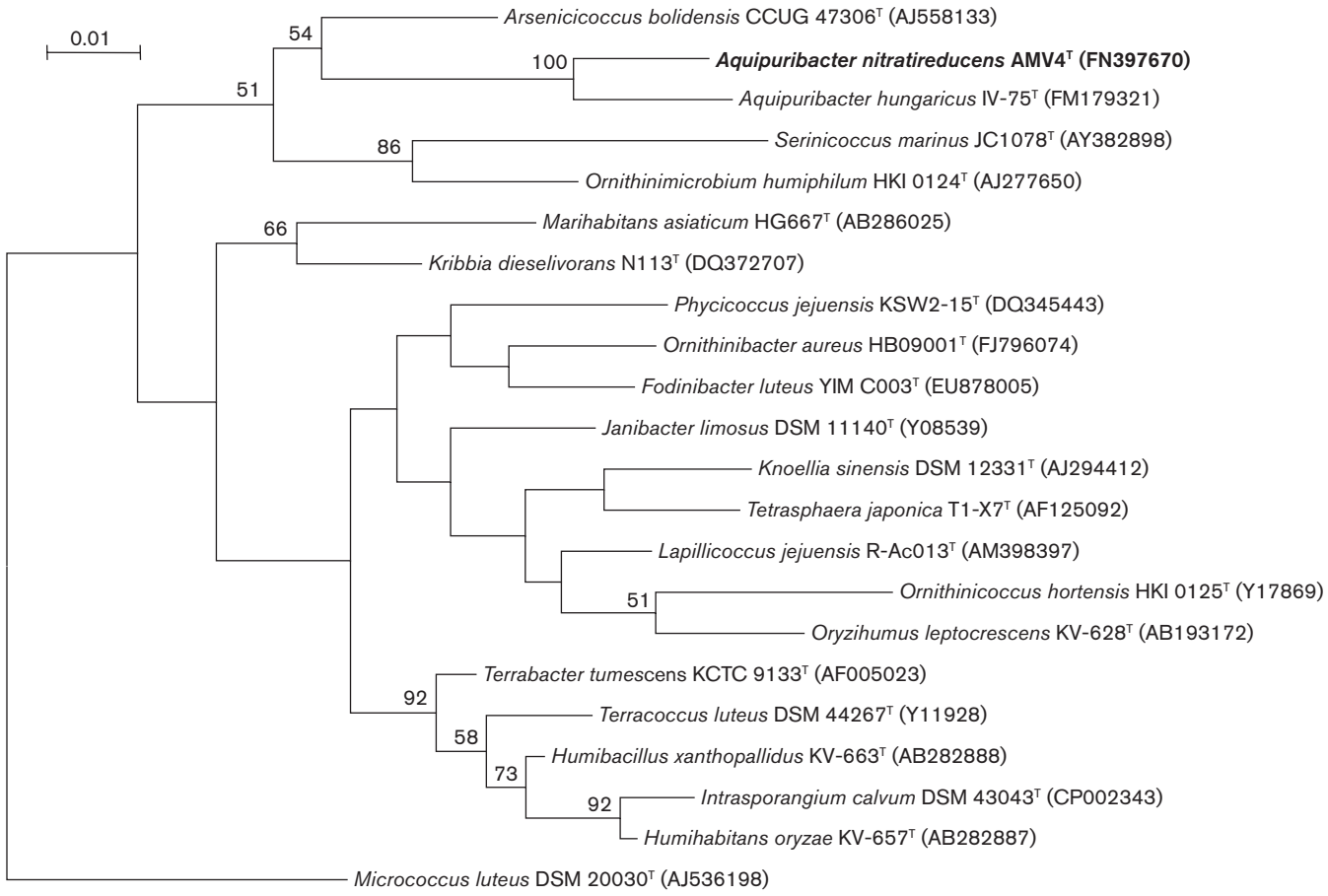

Fig. 2. Maximum-likelihood phylogenetic tree, based on 16S rRNA gene sequences, showing the relationship between strain AMV4^T and representatives of the species belonging to the family *Intrasporangiaceae*. Numbers at the nodes are bootstrap values >50 %. *Micrococcus luteus* DSM 20030^T was used as an out group. Bar, 0.01 substitutions per nucleotide position.

β-glucosidase activities, weakly positive for Ala-Phe-Pro-arylamidase activity, but negative for β-*N*-acetylglucosaminidase, lipase, α-glucosidase, β-*N*-acetylgalactosaminidase, α-galactosidase, phosphatase, L-pyrrolydonyl-arylamidase, γ-glutamyl-transferase, β-alanine arylamidase, L-proline arylamidase, tyrosine arylamidase, glycine arylamidase, β-glucoronidase, β-xylosidase and glu-gly-arg-arylamidase activities, L-lactate and succinate alkalinization and the fermentation of glucose. The methyl red reaction is positive and the Voges–Proskauer reaction is negative. Nitrate is reduced and H_2S and indole are not produced. Aesculin is hydrolysed, but casein, cellulose, starch, Tweens 20, 40, 60 and 80, and urea are not hydrolysed. Acid is produced from glucosamine and ribose after incubation for one week at the optimum temperature and pH. Utilizes glucosamine, ribose and serine, but not xylose, adonitol, rhamnose, cellobiose, melibiose, sucrose, raffinose, trehalose, glucose, lactose, maltose, fructose, galactose, L- or D-arabinose, mannose, inulin, sodium gluconate, glycerol, salicin, dulcitol, inositol, sorbitol, mannitol, adonitol, methyl α-D-glucoside, melezitose, methyl α-D-mannoside, xylitol, citrate, malonate, sorbose, glycine, alanine, valine, leucine, isoleucine, arginine, phenylalanine, tyrosine, tryptophan, aspartic acid, glutamic acid, threonine, histidine, cysteine, methionine and glutamine. Does not utilize L-arabitol, cellobiose, D-glucose, maltose, D-mannitol, D-mannose, isomaltulose, 5-keto-D-gluconate, D-sorbitol, D-tagatose, trehalose, L-histidine, 4-coumarate, L-malate and L-lactate (VITEK GN card system). Susceptible to chloramphenicol, ciprofloxacin, erythromycin, lomefloxacin, tetracycline and vancomycin and resistant to co-trimoxazole, nalidixic acid, cefotaxime, nitrofurantoin, norfloxacin, penicillin G, cefuroxime, cefoperazone, streptomycin, amikacin, cefazolin, kanamycin and tobramycin. The diagnostic diamino acid in the cell-wall peptidoglycan is *m*-Dpm; MK-10(H_4) is the predominant respiratory quinone; phosphatidylglycerol, one unidentified glycolipid, two unidentified phospholipids and five unidentified lipids are the polar lipids, and iso-$C_{15:0}$, anteiso-$C_{15:0}$, $C_{16:0}$, anteiso-$C_{16:0}$, $C_{18:1}\omega9c$ and $C_{18:3}\omega6,9,12c$ are the major cellular fatty acids.

The type strain, AMV4^T (=CCUG 58430^T=DSM 22863^T=NBRC 107137^T) was isolated from a soil sample collected

from a mud volcano located on Baratang Island in the Andaman Islands, India. The DNA G+C content of the type strain is 74.3 mol%.

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