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ORIGINAL PAPER

# *Microvirga arsenatis* sp. nov., an arsenate reduction bacterium isolated from Tibet hot spring sediments

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**Abstract** Two novel Gram-stain negative, moderately thermophilic, aerobic, rod-shaped strains, designated 3D203$^T$ and 3D207, were isolated from hot spring sediment samples collected from Tibet, western China. Phylogenetic analyses based on 16S rRNA gene sequence similarities showed that two isolates belonged to the genus *Microvirga* and were most closely related to *Microvirga makkahensis* SV1470$^T$ (98.5% and 98.4%, respectively) and two strains had 99.8% similarity to each other. The average nucleotide identity (ANI) based on whole genome sequences of two strains and *M. makkahensis* SV1470$^T$ was 80.8% and 80.78%, respectively. Optimum growth was observed at 45 °C, pH 7.0 and 0.5% NaCl. They both could tolerate to high concentration arsenic. Ubiquinone 10 (Q10) was their predominant quinone. The differences of strains 3D203$^T$ and 3D207 were phosphatidyl dimethyl ethanolamine, phosphatidyl-N-methylethanolamine, phosphatidylglycerol, unidentified glycolipids and unidentified lipids. The major fatty acids (> 5%) were identified $C_{18:1}\omega7c$ and/or $C_{18:1}\omega6c$, $C_{18:0}$ and $C_{16:0}$. The genomic DNA G + C contents of strain 3D203$^T$ and 3D207 based on whole genome sequences were 64.8% and 64.7%, respectively. Phenotypic, chemotaxonomic, phylogenetic and genomic analyses suggested that two strains represent a novel species of the genus *Microvirga*, for which the name *Microvirga arsenatis* sp. nov. is proposed. The type strain is 3D203$^T$ (= CGMCC 1.17691$^T$ = KCTC 72653$^T$).

**Keywords** Hot spring · *Microvirga* · Arsenical · Taxonomy · ANI

Ze-Tao Liu and Wen-Dong Xian equally contributed to this work.

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## Introduction

The genus *Microvirga*, which belongs to the family *Methylobacteriaceae* of the order *Rhizobiales* in the α-*Proteobacteria*, was first described by Kanso and Patel (2003) with *Microvirga subterranean* as type species, and more species belonged to this genus were described subsequently. At the time of writing, there

are 17 valid species in the genus *Microvirga* listed in the LPSN (https://lpsn.dsmz.de/genus/microvirga). Among of them were isolated from various environments, such as sandy arid soil (Veyisoglu et al. 2016) or desert soil (Amin et al. 2016), root-nodule (Safronova et al. 2017; Ardley et al. 2012; Radl et al. 2014), stool sample (Caputo et al. 2016), thermal aquifer (Kanso and Patel 2003), air (Weon et al. 2010) and hot spring (Weon et al. 2010). Members of this genus are Gram-stain negative, aerobic and rod-shaped. Q10 is the predominant quinone and the major fatty acids are $C_{18:1}\omega7c$ and/or $C_{18:1}\omega6c$. The genomic DNA G + C contents are 61.1–65.1%. In the genus *Microvirga*, *M. indica* is capable of oxidise arsenite and possesses the *aioA* gene (Tapase et al. 2017). During the investigation of microbial diversity of hot springs in western of China, strains 3D203$^T$ and 3D207 were isolated from sediment samples. Besides, their taxonomic status were investigated using a polyphasic taxonomy approach.

## Material and methods

### Isolation and culture conditions

During the investigation of microbial diversity of hot springs in western of China, sediment samples were collected from geothermal fields of Tibetan Plateau (E87.14°, N29.17°). Sampling was done using a sterile spoon and the sample collected into a sterile sampling bag. They were then transported back to the laboratory under ambient condition and stored at 4 °C. Isolation of two strains was done using the standard dilution plate method on Reasoner's 2A agar (Reasoner and Geldreich 1985). The colonies of strains 3D203$^T$ and 3D207 were obtained after incubation for 1 week at 28 °C. Selected colonies were then purified on T5 (glucose 1 g, lotus root starch 1 g, yeast extract 2 g, tyrptone 0.5 g, $CaCO_3$ 1 g, agar 1.2%, water 1 L, trace element 1 mL ($FeSO_4$ 0.2%, $MnCl_2$ 0.1%, $ZnSO_4$ 0.1%)) agar. Besides, the purified colonied were stored as glycerol suspensions with 20% w/v concentration at − 80 °C. The experimental control strain *Microvirga makkahensis* SV1470$^T$ was provided by the Korean Collection for Type Cultures (KCTC). All the strains were maintained routinely on T5 medium for 5 days in 28 °C incubator. Biomass of strains 3D203$^T$ and 3D207 and the experimental control strain for chemotaxonomic and molecular investigations were harvested from cultures grown on T5 medium (28 °C, 5 days).

### Phenotypic characterization

Growth tests were performed on Luria–Bertani Broth, Potato Dextrose Agar, Yeast Malt Agar, Reasoner's 2A agar, Tryptic Soy Agar and T5 at 37 °C for 3 days. Gram-stain reaction was tested by using the standard Gram reaction and was confirmed by using the KOH test (Cerny 1978). Cell morphology was observed by using a transmission electron microscope (JEM1400-FLASH) with strains grown on T5 agar for 3 days at 45 °C. Growth temperatures from 4 to 60 °C were determined on T5 medium (without addition $CaCO_3$) for 2 weeks. Tolerance to different NaCl concentrations (0–4%, at intervals of 1%, w/v) and pH (pH 4–10, at intervals of 1 unit) were tested in T5 medium without addition $CaCO_3$ for 2 weeks. Tolerance to arsenate and arsenite was tested on T5 medium with non-supplemented $CaCO_3$ for two weeks. The concentration range of arsenite is 1–20 mM, and arsenate is 5–200 mM, respectively. Catalase and oxidase activity, urease, $H_2S$ production and hydrolyses of starch and Tweens 20, 40, 60, 80 were determined as described by Tindall et al. (2007). Carbon-source utilisation tests were performed according to the methods of Shirling and Gottlieb (1966) and Locci (1989). Nitrogen-source utilization tests were analysed as described by Williams et al. (1983). Other phenotypic characteristics were tested using API 20NE, API ZYM and API 50CHB/E kits (bioMérieux) according to the manufactures' instructions. Antibiotic susceptibility test was performed by the agar-diffusion method on T5 agar medium (37 °C, 5 days).

### Chemotaxonomy

The fatty acids were extracted and performed by gas chromatography (Agilent Technologies 7890A GC System) according to the standard protocol of the Microbial Identification System (Sherlock Version 6.1; MIDI database: TSBA6) (Sasser 1990), with the two strains and related type strain grown on T5 at 37 °C for 3 days. Respiratory quinones were extracted (Collins et al. 1977) and analysed using HPLC (Kroppenstedt 1982). The polar lipids were prepared

as described by Minnikin et al. (1979), and identified by two-dimensional TLC (Collins and Jones 1980).

### Molecular characterisation

Genomic DNAs extraction and the amplification of 16S rRNA genes were performed as described by Li et al. (2007). The obtained sequences were submitted to the Ezbiocloud server for similarity analysis (Yoon et al. 2017). Multiple alignments with sequences of the most closely related taxa were carried out by using CLUSTAL_X programs (Thompson et al. 1997). Phylogenetic analyses were performed by using three tree-making algorithms: neighbor-joining (Naruya and Nei 1987), maximum-likelihood (Joseph 1981) and maximum-parsimony (Walter 1971). The trees constructed by using the MEGA version 7.0 (Sudhir et al. 2016). Kimura's two parameter model was used to calculate evolutionary distance matrices of the phylogenetic trees (Motoo 1980). Bootstrap analysis was performed with 1000 replications (Joseph 1985). The whole genomes of two strains and closely related type strain were sequenced and annotated by Novogene Biotech (Beijing, China) using Illumina Miseq platform. The average nucleotide identity (ANI) based on the whole genome sequence was calculated by using the ANI calculator (www.ezbiocloud.net/tools/ani).

## Results and discussion

### Phenotypic characteristics

Strain 3D203$^T$ was able to grow on Yeast Malt Agar, Reasoner's 2A agar and T5, but not on Luria–Bertani Broth, Tryptic Soy Agar and Potato Dextrose Agar. Colonies on T5 were non-pigmented with cells 1.5–3.1 μm long and 0.8–1.1 μm wide (Fig. S3A and Fig. S4A). It was able to grow at 28–55 °C (optimum, 37–45 °C) and pH 4–8 (optimum, 7) and in the presence of 0–1% (w/v) NaCl (optimum, 0.5%). While strain 3D207 was able to grow on Yeast Malt Agar, Tryptic Soy Agar, Reasoner's 2A agar and T5, but not on Luria–Bertani Broth and Potato Dextrose Agar. Colonies on T5 were pink pigmented with cells 2.4–2.5 μm long and 0.9–1.1 μm wide (Fig. S3B and Fig. S4B). It was able to grow at 28–50 °C (optimum, 37–45 °C) and pH 5–8 (optimum, 7) and in the presence of 0–2% (w/v) NaCl (optimum, 0.5%). Cells of strains 3D203$^T$ and 3D207 were observed to be Gram-stain negative, aerobic and rod-shaped. They both could tolerate high concentration arsenite and arsenate with 1–5 mM and 5–100 mM, respectively. However, the parallel reference type strain SV1470$^T$ not, which suggests members of this group may play distinct roles in different ecosystem, and mainly because of the unique evolution under isolated hot habitats. Arsenate metabolite genes among newly isolated strains and strain *M. makkahensis* SV1470$^T$ were further compared (Table S2), the results showed the similar component of related genes, which suggests the two strains might possess arsenic transformation as reported by Tapase et al. (2017). They showed negative for oxidase catalase, urease, $H_2S$, Tweens 20, 40, 60, 80 and starch. Other phenotypic characteristics are detailed in the species description and Table 1.

### Molecular characteristics

The 16S rRNA gene sequences of strains 3D203$^T$ and 3D207 were obtained with the length of 1494 bp (accession no: MN879271) and 1484 bp (accession no: MN879275), respectively. They shared the highest similarity to *M. makkahensis* SV1470$^T$ (98.5% and 98.4%) and two strains had 99.8% similarity to each other. Phylogenetic analyses based on the 16S rRNA gene showed that they belonged to the genus *Microvirga* and was most closely related to *M. makkahensis* SV1470$^T$ (Fig. 1, Figs. S1 and S2). The draft genome sequences of strains 3D203$^T$, 3D207 and *M. makkahensis* SV1470$^T$ were 5,053,196 bp, 5,053,091 bp and 5,580,646 bp in length with 73 contigs, 81 contigs and 94 contigs, respectively. Their DNA G + C contents were 64.8%, 64.7% and 63.1%, respectively (Table 2). Genomic analysis showed that their ANI values were 80.8% and 80.78% after comparing strains 3D203$^T$ and 3D207 with *M. makkahensis* SV1470$^T$. The two strains were determined to belong to same species based on high value (99.9%) of ANI between them. Considering the recommended threshold value for species discrimination (ANI $< 95\%$), it is clear that the two strains represent a novel species of the genus *Microvirga*.

**Table 1** Differential characteristics of strains 3D203$^T$, 3D207 and closely related members of the genus *Microvirga*

| Characteristics | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| Isolation source | Hot spring | Hot spring | Sandy arid soil | Root nodule | Air | Soil |
| Oxidase | − | − | + | ND | + | + |
| Catalase | − | − | + | + | − | + |
| *Carbon source* | | | | | | |
| Sorbitol | − | − | + | + | − | + |
| Glucose | − | − | + | + | + | + |
| Mannitol | − | − | + | − | − | + |
| Sucrose | − | − | + | − | − | ND |
| Maltose | + | + | − | − | − | + |
| Trehalose | − | − | + | + | ND | + |
| Sodium acetate | − | − | + | ND | ND | ND |
| *Nitrogen source* | | | | | | |
| Lysine | − | − | + | ND | ND | − |
| Glutamic acid | − | − | + | ND | ND | ND |
| Histidine | − | − | + | ND | ND | ND |
| Aspartic acid | − | − | + | ND | ND | ND |
| *API 20NE* | | | | | | |
| Nitrate reduction | + | + | − | + | − | + |
| Assimilation D-glucose | − | − | + | + | ND | + |
| Assimilation D-mannose | − | − | + | + | ND | ND |
| Assimilation Trisodium citrate | − | − | + | ND | ND | ND |
| *API ZYM* | | | | | | |
| Alkaline phosphatase | + | − | − | ND | + | − |
| Cystine arylamidase | − | − | + | ND | − | ND |
| α-chymotrypsin | − | − | + | ND | − | ND |
| *API 50CH* | | | | | | |
| D-fructose | − | + | − | + | ND | + |
| DNA G + C contents (%) | 64.8 | 64.7 | 63.1 | 61.8 | 62.2 | 64.3 |

Strains: 1. 3D203$^T$; 2. 3D207; 3. *M. makkahensis* SV1470$^T$ (Veyisoglu et al. 2016); 4. *M. ossetica* V5/3M$^T$ (Safronova et al. 2017); 5. *M. aerophila* 5420S-12 $^T$ (Weon et al. 2010); 6. *M. guangxiensis* 25B$^T$ (Zhang et al. 2009). All data were obtained in this study under the same conditions unless otherwise specified

+ Positive; − negative; *ND* not determined

## Chemotaxonomical characteristics

Q-10 was found to be the major respiratory quinone of two strains. The polar lipids of strain 3D203$^T$ included phosphatidylcholine, diphosphatidylglycerol, phosphatidyl dimethyl ethanolamine, phosphatidyl ethanolamine, three unidentified glycolipids and three unidentified lipids, while strain 3D207 consisted of phosphatidylcholine, diphosphatidylglycerol, phosphatidyl ethanolamine, phosphatidyl-N-methylethanolamine, phosphatidylglycerol and two unidentified lipids (Fig. S5). The major fatty acids (> 5%) of strains 3D203$^T$ and 3D207 were Summed Feature 8 ($C_{18:1}\omega7c$ and/or $C_{18:1}\omega6c$) (72.9% and 67.8%), $C_{18:0}$ (8.8% and 12.8%) and $C_{16:0}$ (9.5% and 10.5%) (Table S1). There were significant differences between two strains and their closely related type strains in their major fatty acid contents. The fatty acid analysis clearly showed that two strains represent a novel species.

Based on the phenotypic, phylogenetic, chemotaxonomic analyses, two strains should be affiliated to the

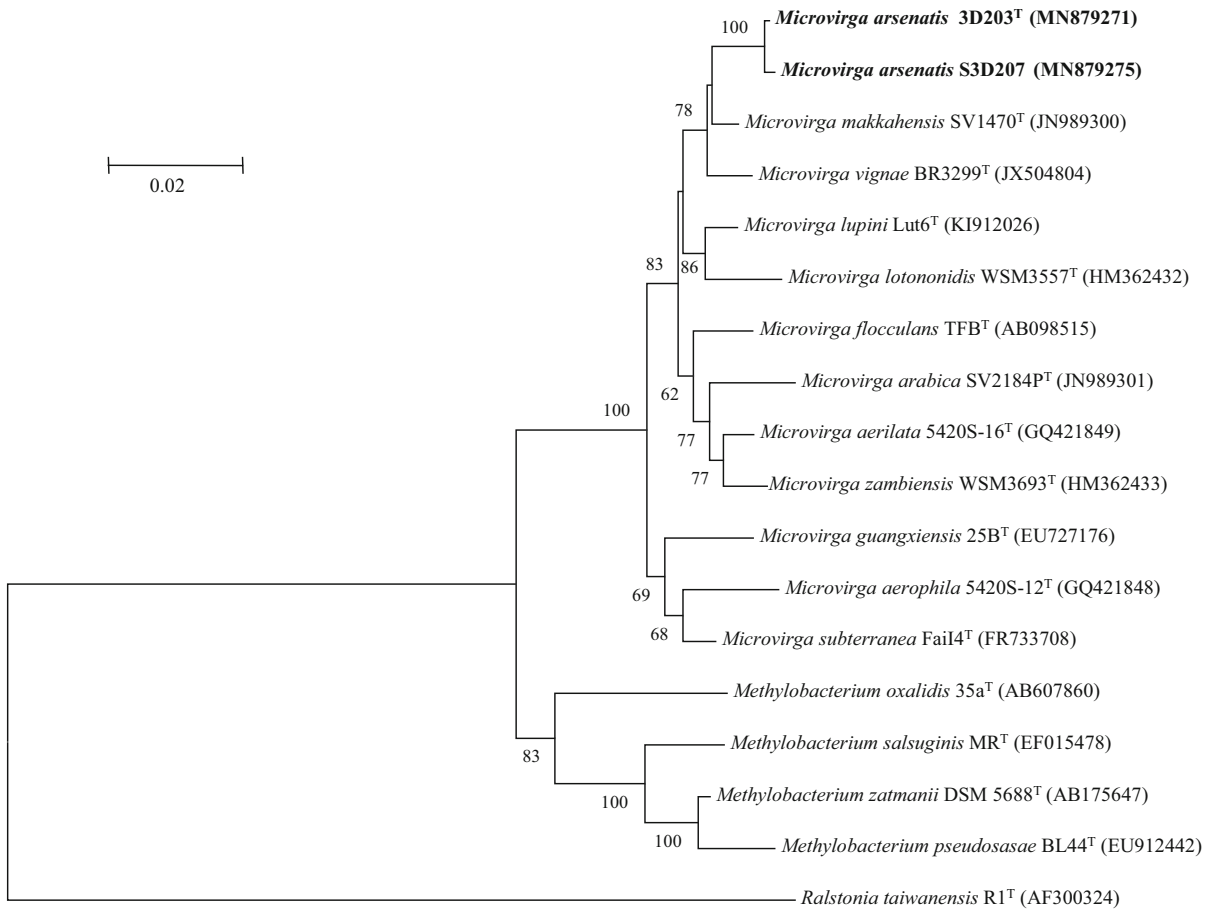


**Fig. 1** Neighbour-joining phylogenetic tree showing the relationship between strains 3D203$^T$, 3D207 and its closest relatives. Asterisks indicate branches that were also recovered using the maximum-parsimony and maximum-likelihood methods. Bootstrap values (expressed as percentages of 1000 replications) of above 50% are shown at the branch points. *Ralstonia taiwanensis* R1$^T$ was used as an outgroup. Bar, 0.01 substitutions per nucleotide position

genus *Microvirga*. However, two strains can be distinguished from the type strain *M. makkahensis* SV1470$^T$ by differences in several properties, such as utilisation of carbon and nitrogen source, catalase, oxidase, as well as the proportions of fatty acids and polar lipid composition. Moreover, the low ANI values, it is clear that strains 3D203$^T$ and 3D207 represent a novel species of the genus *Microvirga*, for which the name *Microvirga arsenical* sp. nov. is proposed.

## Description of *Microvirga arsenatis* sp. nov

*Microvirga arsenatis* (ar.sen.a'tis. M.L. gen. n. *arsenatis*, of arsenate, referring to the ability of the organism to tolerate high concentration of arsenate).

Cells are Gram-stain negative, aerobic, and rod-shaped (0.8–1.1 × 1.5–3.1 μm). Growth occurs Reasoner's 2A agar, Yeast Malt Agar and T5, but not on Luria–Bertani Broth or Potato Dextrose Agar. Colonies are smooth, convex and circular on T5 medium at 37 °C for 3 days. Growth is observed at a range of 28–50 °C, pH 5–8 and 0–1% (w/v) NaCl. Nitrate reduction is positive, but oxidase, catalase, urease, $H_2S$ production, and hydrolyses of Tweens 20, 40, 60, 80 and starch are negative. Assimilates maltose, arabinose, xylose, lactose, sorbitol, ribose, cellobiose, glycerol as sole carbon source, and alanine, serine, tyrosine, threonine, arginine, glycine, phenylalanine, valine, ornithine, asparagine, cystine, tryptophan, proline as sole nitrogen source. Positive for esterase (C4), esterase lipase (C8), leucine arylamidase, trypsin, naphthol-AS-BI-phosphohydrolase. The

**Table 2** Comparison between the genomes of strains 3D203$^T$, 3D207 and the closest type strain *M. makkahensis* SV1470$^T$, *M. subterranea* DSM 14364 $^T$

| Attributes | 3D203$^T$ | 3D207 | *M. makkahensis* SV1470$^T$ | *M. subterranea* DSM 14364$^T$ |
|---|---|---|---|---|
| Genome size (bp) | 5,053,196 | 5,053,091 | 5,580,646 | 5,147,800 |
| G + C content | 64.8 | 64.7 | 63.1 | 65.1 |
| Status | Draft | Draft | Draft | Draft |
| Contigs | 73 | 81 | 94 | 46 |
| $N_{50}$ length (bp) (scaffolds) | 306,102 | 203,749 | 257,062 | 519,753 |
| tRNA Numbers | 53 | 53 | 51 | 59 |
| GenBank ID | JAAAXJ000000000 | JAAAXI000000000 | WURB00000000 | QQBB00000000 |
| Protein-coding genes count | 4943 | 4941 | 5331 | 4895 |
| Genes assigned to COGs | 3524 | 3516 | 2828 | 2607 |
| Coding density | 0.851 | 0.850 | 0.856 | 0.881 |
| Genes assigned to KEGG | 4333 | 4673 | 5625 | 4975 |
| rRNA gene counts | 3 | 3 | 3 | 3 |

major respiratory quinone is Q-10 and the major fatty acids are Summed Feature 8 ($C_{18:1}\omega7c$ and/or $C_{18:1}\omega6c$), $C_{18:0}$ and $C_{16:0}$. The major polar lipids contain phosphatidylcholine, diphosphatidylglycerol, phosphatidyl ethanolamine.

The type strain 3D203$^T$ (= CGMCC 1.17691 $^T$ = KCTC 72653 $^T$) was isolated from hot spring sediment in geothermal fields of Tibetan Plateau, western China. The genomic DNA G + C content is 64.8%. The GenBank accession numbers for 16S rRNA gene sequence and draft genome sequence of the strain 3D203$^T$ are MN879271 and JAAAXJ000000000.

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**Author contributions** LZT and WJL designed research and project outline. LZT, XWD, LMM, LL, MYZ and JJY performed isolation, deposition, and identification. LZT, FBZ, XM and WJL drafted the manuscript. All authors read and approved the final manuscript.

**Compliance with ethical standards**

**Conflict of interest** The authors declare that there is no conflict of interest.

**Ethical standard** This article does not contain any studies with human participants or animals performed by any of the authors.

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