**Natrinema versiforme** sp. nov., an extremely halophilic archaeon from Aibi salt lake, Xinjiang, China

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INTRODUCTION

Halobacteria (the family Halobacteraeaceae; Grant & Larsen, 1989) are a diverse group of extremely halophilic archaea that require at least 1·5 M NaCl for growth. In the last few years, the numbers of halobacterial genera and species have increased rapidly. Several new genera, e.g. Halogeometricum (Montalvo-Rodriguez et al., 1998) and Natronorubrum (Xu et al., 1999), have been created to accommodate newly isolated strains, and the others have been proposed by re-evaluation of misidentified or insufficiently described strains, e.g. *Natrinema* (McGenity et al., 1998) and Haloterrigena (Ventosa et al., 1999).

Abbreviations: PG, phosphatidylglycerol; PGP-Me, phosphatidylglycerol-phosphate-methyl ester; PGS, phosphatidylglycerol sulfate; S₃-DGD-1, 2,6-HSO₃-α-l-α-GlcC-α-l-α-GlcC; TMAO, trimethylamine N-oxide.

The DDBJ accession number for the 16S rDNA sequence of *Natrinema versiforme* XF10⁷ is AB023426.

A novel extremely halophilic archaeon, strain XF10⁷, was isolated from a salt lake in China. This organism was neutrophilic, non-motile and pleomorphic, and was rod, coccus or irregularly shaped. It required at least 1·5 M NaCl for growth and grew in a wide range of MgCl₂ concentrations (0·005–0·5 M). Lipid extract of whole cells contained two glycolipids with the same chromatographic properties as two unidentified glycolipids found in the two described *Natrinema* species, *Natrinema pellirubrum* and *Natrinema pallidum*. Phylogenetic analysis based on 16S rDNA sequence comparison revealed that strain XF10⁷ clustered with the two described *Natrinema* species and several other strains (strains T5.7, GSL-11 and Haloterrigena turkenica JCM 9743) with more than 98·1% sequence similarities, suggesting that strain XF10⁷ belongs to the genus *Natrinema*. Comparative analysis of phenotypic properties and DNA–DNA hybridization between strain XF10⁷ and the *Natrinema* species supported the conclusion that strain XF10⁷ is a novel species within the genus *Natrinema*. The name *Natrinema versiforme* sp. nov. is proposed for this strain. The type strain is XF10⁷ (= JCM 10478ᵀ = AS 1.2365ᵀ = ANMR 0149ᵀ).

Keywords: *Natrinema versiforme* sp. nov., halobacteria, archaea

Hypersaline environments are commonly found in China. In addition to many coastal saltlars, a number of salt lakes, soda lakes and salt-rich deserts are located in the west to northwest part of China, i.e. from Inner Mongolia, Qinghai Province, to Xinjiang and Tibet Autonomous Regions. From these saline environments, a number of halobacteria have been isolated, including strains A5 and B2, which have been shown to be related to the genus *Halococcus* based on polar lipid composition and 16S rDNA sequence (Zhou et al., 1994; Xu et al., 1995), strain HAM-2, which has been shown to be a close relative of *Natrolba magadii* on the basis of 16S rDNA sequence (Tian et al., 1997; Xu et al., 1999), two species of the genus *Natronorubrum* (*Natronorubrum bangense* and *Natronorubrum tibetense*) (Xu et al., 1999), and some incompletely characterized strains (Wang et al., 1984; Zhou et al., 1990). Isolation of novel strains of the genera *Halococcus*, *Natrolba* and *Natrinema* (H. Xin and others, unpublished work) has also exemplified the wide diversity of halobacteria in the hypersaline habitats in China.
In this paper, a novel halobacterial isolate, XF10T, which was isolated from a salt lake in the northwest part of China, is described.

**METHODS**

**Isolation procedure, strains and growth conditions.** Strain XF10T was isolated from clay collected from the shallow near-edge floor of Aibi salt lake in Xinjiang Autonomous Region, China. After enrichment of the sample in Sehgal and Gibbons medium (Sehgal & Gibbons, 1960) at 37 °C with shaking for 1–2 weeks, a pure culture was obtained by plating serial dilutions of enrichment cultures and repeated transfers of separate colonies on agar plates of the same medium. The purity of the strain was checked by colony morphology and randomly amplified polymorphic DNA patterns of total DNAs derived from different colonies on the agar plate. The growth medium used for the following studies contained (l–l): 5 g Casamino acids (Difco); 5 g yeast extract (Difco); 1 g sodium glutamate; 3 g trisodium citrate dihydrate; 30 g MgCl₂, 6H₂O; 5 g KH₂PO₄; 36 mg FeCl₃, 6H₂O; 0.36 mg MnCl₂, 4H₂O; and 220–250 g NaCl (pH 7.0). Agar slants and plates were prepared by adding 20 g agar l⁻¹. The strain was maintained on agar slants for short periods (several months) or stored in growth medium with glycerol (final 20%, v/v) at −80 °C for long-term preservation. *Haloterrigena turkenica* JCM 9743 (for genus and species designation, see Discussion; deposited in JCM by M. Kamekura in 1995), *Haloterrigena turkenica* JCM 9101T (=VKM B-1734T; transferred from VKM to JCM in 1993), *Natrinema pallidum* JCM 8980T (=NCIMB 777T; transferred from NCIMB to JCM in 1993) and *Natrinema pillirubrum* JCM 10476T (=NCIMB 786T; transferred from NCIMB to JCM in 1999) were used as reference strains and cultivated in the above growth medium with 20 g MgSO₄·7H₂O l⁻¹ and 2 g KCl l⁻¹ (instead of MgCl₂, 6H₂O and K₂SO₄), and 200 g NaCl l⁻¹. If not specified, strains were cultivated at 37 °C with shaking at 180 r.p.m. in 500 ml Erlenmeyer flasks containing 100 ml medium. Inoculated agar plates were wrapped in plastic bags and incubated at 37 °C.

**Morphology and growth characteristics of strains.** Cells were observed under a phase-contrast light microscope (Optiphot-2; Nikon). Gram staining was performed with acetic-acid-fixed cells as described by Dussault (1955). Cell lysis was observed by diluting dense cell suspensions with the diluted medium or distilled water. Growth ranges and optima of NaCl and MgCl₂ levels were determined using growth medium containing various concentrations of NaCl (1–2–5 M) and MgCl₂ (0.005–0.5 M), respectively. Various buffer systems were employed in the determination of growth pH (50 mM of each): MES (pH 5.5–6.5), PIPES (pH 6.5–7.5), HEPES (pH 7.0–8.0), Tricine (pH 7.5–9.0) and CHES (pH 9.0–10.0). Growth temperature was determined in the range 4–60 °C by using a temperature gradient incubator (model TN-3; ADVANTEC). Growth rate was determined by measuring culture turbidity at 660 nm.

**Nutrition.** Anaerobic growth in the presence of arginine, DMSO, trimethylamine N-oxide (TMAO) and nitrate (5 g l⁻¹ of each) was determined in rubber-stoppered tubes completely filled with the growth medium in the dark for 1–3 weeks and compared to growth on medium without the test compounds. Reduction of nitrate was detected by using the sulfanilic acid and p-naphthylamine reagent (Smibert & Krieg, 1981). Formation of gas from nitrate was detected by using Durham tubes under anaerobic conditions.

To estimate the utilization of various carbohydrates as carbon and energy sources, the basal medium [0.1 g yeast extract (Difco), 0.5 g NH₄Cl l⁻¹, 0.05 g K₂HPO₄ l⁻¹, at pH 7.0 with 50 mM HEPES] was supplemented with 10 g test carbohydrate l⁻¹. Production of acids from carbohydrates was tested in the basal medium supplemented with 0.5 g test substrate l⁻¹ without buffer. The cultures were incubated at 37 °C without shaking for 2 weeks. Growth was determined visually and pH was measured with a pH electrode.

**Biochemical tests.** Tests for catalase and oxidase activities, formation of indole, and hydrolysis of starch, gelatin, casein and Tween 80 were performed according to the standard or modified procedures of Oren et al. (1997). Formation of sulfide was determined by incubating cells in the growth medium supplemented with elemental sulfur (6.4 g l⁻¹) or sodium thiosulfate (Na₂S₂O₃·5H₂O, 5.0 g l⁻¹) without shaking, and detecting sulfide with a strip of paper impregnated with 10% (w/v) lead acetate solution.

**Sensitivity to antimicrobial agents.** Sensitivity to antimicrobial agents was determined in the growth medium containing each antimicrobial agents at 50 mg l⁻¹ for at least 2 weeks. Antimicrobial agents used were ampicillin, anisomycin, bacitracin, chloramphenicol, erythromycin, neomycin, novobiocin, penicillin G and rifampicin.

**Lipid analyses.** Total lipids were extracted by the modified method of Kamekura (1993) and separated by TLC on Merck Kieselgel 60-HPTLC. For one-dimensional development, the solvent was chloroform/methanol/acetic acid/water (85:22.5:10:4, v/v/v). For two-dimensional development, the solvents were chloroform/methanol/water (65:25:4, v/v/v) followed by chloroform/methanol/acetic acid/water (50:12:15:4, v/v/v). Phospholipids were detected with the Zinzadze reagent of Dittmer & Lester (1964). Glycolipids were detected by spraying the plate with 0.5% 1-naphthol in methanol/water (1:1) and then with sulfuric acid/ethanol (1:1) followed by heating at 120 °C for 5–10 min; all the other polar lipids were detected by further heating of the plate at about 250 °C for several minutes (Ihara et al., 1997).

**Sequencing of 16S rDNA.** Total DNAs were extracted by the method of Cline et al. (1989). The 16S rRNA genes were amplified by PCR with the following forward and reverse primers: 5'-TCCGCTTTGATCCTGCGC (positions 8–24 according to *Escherichia coli* numbering) and 5'-GGAGGT-GATCCAGCCG (positions 1540–1525). The amplified
16S rDNAs were cloned into pT7Blue T-vector (Novagen) and sequenced using the SequiTherm Long-Read Cycle Sequencing kit (Epicentre Technologies) on the ALF red DNA sequencer (Pharmacia Biotech). The sequence obtained was aligned with the other reported halobacterial 16S rDNA sequences by using the CLUSTAL W 1.7 program (Thompson et al., 1994). The phylogenetic tree was reconstructed by the neighbour-joining method (Saitou & Nei, 1987) and was evaluated by bootstrap sampling (Felsenstein, 1985).

**G+C content and DNA–DNA hybridization.** G+C content was determined by the HPLC method of Tamaoka (1994). DNA–DNA hybridization was assessed by the fluorometric method of Ezaki et al. (1989).

**RESULTS**

**Morphology and growth characteristics**

Cells of strain XF10<sup>T</sup> growing exponentially under optimal condition were non-motile and pleomorphic, with rods, coccii or irregular shapes (Fig. 1). The pleomorphism was confirmed in media containing various concentrations of NaCl (2.6–5.2 M) or MgCl<sub>2</sub> (0.005–0.5 M). Cell lysis occurred in the diluted medium containing less than 1.0 M NaCl or in distilled water. There were no gas vesicles formed inside cells. Cells stained Gram-negative. Colonies formed on agar plates were light-red, opaque, small and circular, 0.5–1.0 mm in diameter, and elevated. The strain grew at 1.5 M to saturation of NaCl and grew optimally at 3.4–4.3 M (in the presence of 0.15 M MgCl<sub>2</sub>). The strain grew in a wide range of MgCl<sub>2</sub> concentrations from 0.005 to 0.5 M and grew optimally around 0.15 M. It was neutrophilic (pH 6.0–8.0) with an optimum pH at 6.5–7.0. Growth occurred in the temperature range 20–53 °C with an optimum at 37–46 °C. Under the optimal growth conditions (3.4–4.3 M NaCl, 0.15 M MgCl<sub>2</sub>, at 37 °C and pH 7.0), the doubling time was 11 h.

**Fig. 2.** TLC of polar lipids extracted from strain XF10<sup>T</sup> and some halobacteria by using the 1-naphthol/sulfuric acid-ethanol stain. (a) Two-dimensional TLC of polar lipids from strain XF10<sup>T</sup>. The plate was heated to 120 °C to show the glycolipids and then to about 250 °C to show all the polar lipid components. First dimension, from left to right; second dimension, from bottom to top. (b) One-dimensional TLC (from bottom to top). The plate was heated to 120 °C to show the glycolipids. Lanes: 1, strain XF10<sup>T</sup>; 2, Natrinema pellirubrum JCM 10476<sup>T</sup>; 3, Haloterrigena turkmenica JCM 9101<sup>T</sup>; 4, Haloterrigena turkmenica JCM 9743; 5, Natrinema pallidum JCM 8980<sup>T</sup>. GL, unidentified glycolipid; other abbreviations are defined in the text. The designation pairs (1-1 and 1-2; 2-1 and 2-2) refer to the two adjacent glycolipids in each set which may contain the same sugar residues but different diether core lipids (C<sub>20</sub>C<sub>20</sub> or C<sub>20</sub>C<sub>25</sub>).
Two-dimensional TLC revealed that strain XF10\textsuperscript{T} possessed the glycerol diether analogues of phosphatidylglycerol (PG), phosphatidylglycerophosphomethyl ester (PGP-Me), phosphatidylglycerol sulfate (PGS) and some glycolipids (Fig. 2a). The core lipids were C\textsubscript{20}C\textsubscript{20} and C\textsubscript{20}C\textsubscript{25} diethers, as shown from the two PG or PGP spots. Strain XF10\textsuperscript{T} showed two glycolipid spots, tentatively designated GL 1-1 and 1-2, which might correspond to molecules containing C\textsubscript{20}C\textsubscript{20} and C\textsubscript{20}C\textsubscript{25} diether moieties. These two glyco-

lipid spots were also found in varying amounts in the two described Natrinema species, Natrinema pellirubrum JCM 10476\textsuperscript{T} and Natrinema pallidum JCM 8980\textsuperscript{T}, as well as Haloterrigena turkmenica JCM 9743 (Fig. 2b, lanes 2, 5 and 4, respectively). Like Natrinema pellirubrum JCM 10476\textsuperscript{T}, strain XF10\textsuperscript{T} did not contain two glycolipids (tentatively designated GL 2-1 and 2-2) found in Natrinema pallidum JCM 8980\textsuperscript{T} and Haloterrigena turkmenica JCM 9743 (Fig. 2b, lanes 5 and 4), whereas it lacked two glycolipids found in Natrinema pellirubrum JCM 10476\textsuperscript{T} which appeared below GL 1-1 and 1-2 (Fig. 2b, lane 2). On the other hand, the type strain of Haloterrigena turkmenica, JCM 9101\textsuperscript{T}, contained S\textsubscript{2}-DGD-1[2,6-HSO\textsubscript{3}-Manp-α(1→2)-GlcP-α(1→1)-sn-glycerolidiether] as a major glycolipid (Fig. 2b, lane 3), which was not detected in strain XF10\textsuperscript{T}.

Sequencing of 16S rRNA gene, alignment and reconstruction of phylogenetic tree

Sequences of several clones containing PCR-amplified 16S rDNA were determined. There were few heterogeneities among the sequences. The sequence determined was 1438 bp. Phylogenetic analysis of this 16S rDNA sequence with those of relevant halobacteria available from the database was conducted by comparing 1367 bases of each sequence, excluding gaps and uncertain bases. On the phylogenetic tree, strain XF10\textsuperscript{T}, the two described Natrinema species, strains T5.7, GSL-11 (formerly L-11: Kamekura & Dyall-Smith, 1995) and Haloterrigena turkmenica JCM 9743 formed a tight cluster (Fig. 3). This cluster was supported by a high bootstrap value (100\%). Strain
Table 1. Levels of DNA–DNA relatedness between strain XF10T and related strains

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<th>Strain</th>
<th>Relatedness (%) with biotinylated DNA from:</th>
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<td>Strain XF10T</td>
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<tr>
<td>Strain XF10T</td>
<td>100</td>
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<tr>
<td>Natrinema pellirubrum</td>
<td>26</td>
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<tr>
<td>Natrinema pallidum</td>
<td>29</td>
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<td>Haloterrigena turkmenica JCM 9743</td>
<td>22</td>
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<td>Haloterrigena turkmenica JCM 9101T</td>
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XF10T had 98.5% and 98.8% sequence similarities to the two described Natrinema species, Natrinema pellirubrum NCIMB 786T and Natrinema pallidum NCIMB 777T (also Natrinema pallidum NCIMB 784), respectively, 99.4% to strain T5.7, and 98.1–98.4% to the Haloterrigena turkmenica JCM 9743 strain GSL-11 group. The closest relatives to this cluster were strain SR1.5 and Haloterrigena turkmenica VKM B-1734T (= JCM 9101T), which had 97.1–98.0% and 96.2–97.2% sequence similarities, respectively, to the cluster members (97.3% and 96.6%, respectively, to strain XF10T). Sequence similarities between strain XF10T and the rest of halophilic archaea compared were 90.5–95.7%.

G + C content and DNA–DNA hybridization

The G + C content of total DNA of strain XF10T was 64.2 mol%, which is similar to that of Natrinema pellirubrum JCM 10476T, Natrinema pallidum JCM 8980T and Haloterrigena turkmenica JCM 9743 (62.9, 63.9 and 64.4 mol%, respectively). The DNA–DNA hybridization results are shown in Table 1. Low levels of DNA–DNA hybridization were observed between strain XF10T and the two described Natrinema species (17–29%), and Haloterrigena turkmenica JCM 9743 (11–22%), as well as Haloterrigena turkmenica JCM 9101T (5–6%). The DNA–DNA hybridization values between Natrinema pellirubrum JCM 10476T and Natrinema pallidum JCM 8980T were almost the same as that reported by Ross & Grant (1985) (50%).

DISCUSSION

Strain XF10T was isolated from a neutral salt lake in the northwest part of China. It was isolated along with many other halobacterial strains (and some eubacteria) after enrichment in complex medium containing 20% NaCl.

On the phylogenetic tree based on 16S rDNA sequences, strain XF10T clustered with the two described Natrinema species as well as strains T5.7, GSL-11 and Haloterrigena turkmenica JCM 9743 (≥ 98.1% sequence similarities). This cluster is still phylogenetically coherent when compared with several halobacterial genera such as Halorubrum (≥ 92.8% sequence similarity), Natrialba (≥ 93.3%) and Natronorubrum (95.2%) (McGenity & Grant, 1995; Kamekura et al., 1997; Xu et al., 1999). Therefore, all members of this cluster, including strain XF10T, should belong to the genus Natrinema, which was proposed validly in 1998 (McGenity et al., 1998). Although Haloterrigena turkmenica JCM 9743 should be renamed, this strain is still in taxonomic confusion as discussed below. The presence of the two unidentified glycolipid spots shared by the two described Natrinema species would support assignment of strain XF10T to the genus Natrinema. Strain XF10T was also similar to the two described Natrinema species in possessing C20-C26 core lipids (McGenity et al., 1998; Kamekura & Dyall-Smith, 1995). Although strain XF10T (and the Natrinema species) showed slightly high 16S rDNA sequence similarity to Haloterrigena turkmenica VKM B-1734T (96.6%), the latter had a significant amount of S1-DGD-1 as a characteristic glycolipid (Ventosa et al., 1999), which suggested that strain XF10T should not belong to the genus Haloterrigena.

In terms of phenotypic properties, the difference between strain XF10T and the two described Natrinema species (McGenity et al., 1998) is clear despite the close phylogenetic relationships. Strain XF10T does not exhibit the typical rod shape of the two described Natrinema species and it differs from them in several biochemical and physiological properties as shown in Table 2. Moreover, strain XF10T shows some differences in polar lipid composition from both the two described Natrinema species. Low DNA–DNA relatedness between strain XF10T and the two described Natrinema species clearly indicated that strain XF10T is independent from them. Likewise, differences in polar lipid composition (Fig. 2b, lane 4) and low DNA–DNA relatedness between strain XF10T and Haloterrigena turkmenica JCM 9743 support the proposal that strain XF10T is also separate from Haloterrigena turkmenica JCM 9743. According to the properties described above, strain XF10T should be a new species of the genus Natrinema, and the name Natrinema versiforme sp. nov. is proposed. The type strain is strain XF10T, which has been deposited in the Japan Collection of Microorganisms, RIKEN (The Institute of Physical and Chemical Research), Saitama,
Recently, it was shown that *Haloterrigena turkmenica* JCM 9743 (formerly *Halobacterium trapanicum* JCM 9743), which had been thought to be the descendant of the original type strain of *Halobacterium trapanicum* (NRC 34021), was not identical to the original strain (Kamekura, 1998). Ventosa *et al.* (1999) regarded it as a strain of *Haloterrigena turkenmenica*, although this needs to be reconsidered. Our DNA–DNA hybridization data revealed that *Haloterrigena turkmenica* JCM 9743 was unrelated to *Haloterrigena turkenmenica* JCM 9743 (an equivalent strain of VKM B-1734) (Table 1). Moreover, the two strains differed in their polar lipid composition (Ventosa *et al.*, 1999; also shown in Fig. 2b, lanes 3 and 4), and the 16S rDNA sequence similarity between both the strains was too low (96.6%) for inclusion in the same species. On the basis of the very high DNA–DNA hybridization value (Table 1), it appears that *Haloterrigena turkenmenica* JCM 9743 and *Natrinema pallidum* JCM 8980 are the same species. However, a final conclusion should not be drawn until a thorough phenotypic comparison with *Natrinema pallidum* is accomplished.

Inclusion of strain XF10" as a new species in the genus *Natrinema* seems to require the amendment of the description of this genus, which is given below.

**Amended description of the genus *Natrinema* McGenity, Gemmell and Grant 1998**

Cells rod-shaped, 1–5 µm by 0.6–1.0 µm, or pleomorphic. Cells lyse at low NaCl concentration (<1.0 M). Colonies light orange-red or pale orange, 0.5–2.0 mm in diameter, smooth, circular, convex. Gram-negative. Chemo-organotrophic. Some species are strict aerobes, whereas others show anaerobic growth with nitrate. Nitrogen source: Casamino acids. Carbon sources: Casamino acids and certain sugars. Requires at least 1.5–1.7 M NaCl for growth; optimum 3.4–4.3 M NaCl. Optimum pH around 7.0. Possesses C<sub>20</sub>–C<sub>22</sub> and C<sub>20</sub>–C<sub>22</sub> diether core lipids (Ross *et al.*, 1985; Ross & Grant, 1985). Possesses several unidentified glycolipids (Ross *et al.*, 1985). Sensitive to anisomycin, bacitracin and novobiocin. Type species: *Natrinema pellirubrum*.

**Description of *Natrinema versiforme* sp. nov.**

*Natrinema versiforme* (ver.si.for.me. L. neut. adj. versiforme of various shapes; the various-shaped *Natrinema*).

Cells non-motile and pleomorphic. Lyse in dilute medium containing less than 1.0 M NaCl or in distilled water. Colonies light red, 0.5–1.0 mm in diameter, smooth, circular and elevated. Requires at least 1.5 M NaCl for growth; optimum 3.4–4.3 M (in the presence of 0.15 M MgCl<sub>2</sub>). Growth occurs at 0.005–0.5 M MgCl<sub>2</sub>; optimum around 0.15 M. Temperature range for growth 20–53 °C, optimum 37–46 °C. pH range for growth 6.0–8.0; optimum 6.5–7.0. Chemo-organotrophic. Anaerobic growth in the presence of nitrate. Carbon sources: Casamino acids and several sugars, such as fructose, glucose, glycerol (with strong acid formation), galactose, maltose, mannose, D-ribose, sucrose and D-xylene, but not lactose. Reduces nitrate and forms gas. Forms sulhide from sulfur or thiosulfate. Forms indole. Does not hydrolyse gelatin or casein. Starch hydrolysis doubtful and weak hydrolysis of Tween 80. Possesses C<sub>20</sub>–C<sub>22</sub> and C<sub>20</sub>–C<sub>22</sub> diether core lipids and some unidentified glycolipids. Possesses phospholipids: PG, PGP-<i>Me</i> and PGS. Sensitive to anisomycin, bacitracin, novobiocin and rifampicin. Insensitive to ampicillin, chloramphenicol, erythromycin, neomycin and penicillin G. Type strain: XF10" (=JCM 10478" = AS 1.2365" = ANMR 0149")

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