Caldisericum exile gen. nov., sp. nov., an anaerobic, thermophilic, filamentous bacterium of a novel bacterial phylum, *Caldiserica* phyl. nov., originally called the candidate phylum OP5, and description of *Caldiseriaceae* fam. nov., *Caldisericales* ord. nov. and *Caldisericia* classis nov.

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An anaerobic, thermophilic, thiosulfate-reducing bacterium, strain AZM16c01^T, isolated from a hot spring in Japan [Mori, K., Sunamura, M., Yanagawa, K., Ishibashi, J., Miyoshi, Y., Iino, T., Suzuki, K. & Urabe, T. (2008). *Appl Environ Microbiol* **74**, 6223–6229] was characterized in detail. The 16S rRNA gene sequence analysis had revealed that strain AZM16c01^T was the first cultivated representative of the candidate phylum OP5. The cells were multicellular filaments with a single polar flagellum. The strain contained iso-C_{17:0} as the major fatty acid and menaquinone-8(H₈), menaquinone-8(H₉) and menaquinone-8(H₁₀) as the respiratory quinones. The G + C content of the genomic DNA of strain AZM16c01^T was 34.6 mol%. Optimum growth was obtained at 65 °C, pH 6.5 and in the absence of NaCl, with a doubling time of 10.6 h. On the basis of the results of phylogenetic analysis based on the 16S rRNA gene sequence and the characterization of the strain in this study, we propose the name *Caldisericum exile* gen. nov., sp. nov. for strain AZM16c01^T (=NBRC 104410^T=DSM 21853^T). In addition, we propose the new phylum name *Caldiserica* phyl. nov. for the candidate phylum OP5 represented by *C. exile* gen. nov., sp. nov., and *Caldiseriaceae* fam. nov., *Caldisericales* ord. nov. and *Caldisericia* classis nov.

A novel anaerobic, thermophilic, thiosulfate-reducing bacterium, strain AZM16c01^T, was isolated from a hot spring in Otari, Nagano Prefecture, Japan (Mori *et al.*, 2008). Phylogenetic analysis based on the 16S rRNA gene sequence revealed that strain AZM16c01^T was not closely related to any of the recognized phyla and was reproducibly placed in a candidate phylum called OP5. The candidate phylum OP5 was represented originally by environmental clone sequences retrieved from the Obsidian Pool in Yellowstone National Park (Hugenholtz *et al.*, 1998a) and indicated an independent phylum-level lineage in the domain *Bacteria* (Hugenholtz *et al.*, 1998a, b; Mori *et al.*, 2008; Rappé & Giovannoni, 2003). Since then, environmental clone

sequences belonging to this phylum have been detected in various environments (Dojka *et al.*, 1998; Humayoun *et al.*, 2003; Inagaki *et al.*, 2006; Kaksonen *et al.*, 2004; Ley *et al.*, 2006; Skirnisdottir *et al.*, 2000; Teske *et al.*, 2002). Strain AZM16c01^T was the first cultivable isolate accommodated in OP5. In the present study, we have characterized strain AZM16c01^T in detail and propose the name *Caldisericum exile* gen. nov., sp. nov. for this strain. In addition, the higher taxa of the genus *Caldisericum* are proposed in this paper.

Cells of strain AZM16c01^T were multicellular filaments (0.3 µm in width and 1.6–8.3 µm in length) with a single polar flagellum and formed complicated aggregates (Fig. 1 a–c). Observation of negative-staining with a transmission electron microscope (model H-7600; Hitachi) revealed that cells of strain AZM16c01^T possessed an envelope outside of the cell membrane (Fig. 1c). Although this envelope was sometimes observed in ultrathin sections, it was composed

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain AZM16c01^T is AB428365.

Graphs showing the effects of temperature, pH and NaCl concentration on the growth of strain AZM16c01^T are available as supplementary material with the online version of this paper.

Fig. 1. (a) Phase-contrast and (b–h) transmission electron micrographs of strain AZM16c01^T. Negatively stained cells are shown in (b) and (c). Ultrathin sections of cells are shown in d–h. (d) The cellular membrane and outer envelope are indicated by open and solid arrows, respectively. For the observation of ultrathin sections, cells were prepared by using rapid freezing and the freeze-substitution method with acetone containing 2% (w/v) osmium tetroxide. After these procedures, cells were embedded in Spurr's resin (Spurr, 1969), stained with 0.5% (w/v) KMnO₄ and lead citrate (Reynolds, 1963), and observed using a transmission electron microscope (model H-7600; Hitachi) operating at 100 kV.

of an electron-lucent material and often could not be observed (Fig. 1d–h). The cell-wall structure of strain AZM16c01^T was shown to be Gram-negative, by using both conventional Gram-staining and Gram test with 3% (w/v) KOH (Powers, 1995). Stalk structures (Fig. 1e and g) and intracellular vacuole-like structures (Fig. 1e and h) were often observed. Motility and spore formation were not observed. Strain AZM16c01^T was negative for catalase and oxidase activities.

Cellular fatty acids, respiratory quinones and the genomic DNA G+C content were analysed using methods described previously by Mori & Suzuki (2008). Strain AZM16c01^T possessed iso-C_{17:0} (49% of the total fatty acids) as the predominant fatty acid; iso-C_{15:0} (23%), iso-C_{17:0} 3-OH (16%), C_{16:0} (8%) and anteiso-C_{17:0} (4%) were also present. Strain AZM16c01^T possessed the respiratory quinones menaquinone-8(H₆), menaquinone-8(H₈) and menaquinone-8(H₁₀) (39, 40 and 21% of the total isoprenoid quinones, respectively). The genomic DNA G+C content of strain AZM16c01^T was 34.6 mol%.

For a detailed study of the cell envelope, we attempted to extract the cell wall to analyse its peptidoglycan content by using the method employed for Gram-positive bacteria (Schleifer & Kandler, 1972; Yamaguchi, 1965); however, our attempt was unsuccessful. To investigate whether the component of the cell wall was peptidoglycan or protein S-layer, we observed negative-stained cells using an electron

microscope after enzyme treatments with lysozyme [Nacalai Tesque; 2 mg l⁻¹ in 10 mM Tris buffer (pH 8) containing 1 M urea], proteinase K [Nacalai Tesque; 2 mg l⁻¹ in 10 mM Tris buffer (pH 8) containing 1 mM Na₂EDTA and 1 M urea], proteinase mix (Pronase, Roche; 2 mg l⁻¹ in 10 mM Tris buffer containing 1 mM Na₂EDTA and 1 M urea) or trypsin (Nacalai Tesque; 2 mg l⁻¹ in a solution comprising 1 mM Na₂EDTA, 72 mM NaCl, 2.6 mM KCl, 0.2 mM NaH₂PO₄·2H₂O and 1 M urea, adjusted to pH 8 with 8% NaHCO₃) at 37 °C for overnight. Compared with untreated cells in 10 mM Tris buffer containing 1 M urea, the cells treated with lysozyme, proteinase K and proteinase mix were shrunk in the envelope, but these digestions could not remove the envelope (data not shown). Because of these results, we could not come to a conclusion on the outer envelope structure. However, the detection of 3-hydroxy fatty acid in the cellular fatty acid analysis suggested that strain AZM16c01^T possessed an outer membrane identical to that of Gram-negative bacteria.

As indicated in a previous report (Mori *et al.*, 2008), strain AZM16c01^T could grow on yeast extract but not on any other substrate in the presence of sulfur compounds (thiosulfate, sulfite and elemental sulfur) as electron acceptors. Optimum conditions for growth with 10 mM thiosulfate and 10 g Bacto yeast extract l⁻¹ (Difco) were determined by assessing the time-course of optical density

with a spectrophotometer (model U-2800; Hitachi) and the decrease in thiosulfate concentration by using HPLC (Mori & Suzuki, 2008). The results are shown in Supplementary Fig. S1 (available in IJSEM Online). Strain AZM16c01^T could grow between 55 and 70 °C, the optimum temperature being 65 °C. The pH range for growth was 5.5–7.5 and the optimum pH was 6.5. Strain AZM16c01^T was very sensitive to the NaCl concentration. Optimum growth was observed when no NaCl was added to the medium. Although growth was observed in 0.5 and 1 % (w/v) NaCl, the cell concentration increased only scarcely and the amount of thiosulfate reduction was very slight. Strain AZM16c01^T had a doubling time of 10.6 h under the optimum growth conditions (65 °C, pH 6.5 and in 0 % NaCl).

Phylogenetic analysis based on the 16S rRNA gene sequence (Mori *et al.*, 2008) indicated that strain AZM16c01^T belonged to the OP5 lineage (Fig. 2). This lineage was independent at the phylum level from other bacteria, and the branching point of the OP5 lineage was strongly supported with 93 and 97 % bootstrap values by using the neighbour-joining and maximum-likelihood methods, respectively. The neighbour-joining tree of the OP5 phylum is shown in Fig. 3. This phylum consisted

mainly of clone sequences obtained from anaerobic environments, and the sequence divergence for the phylum was 27.2 %.

The aim of this study was to characterize in detail strain AZM16c01^T, isolated from the Otari hot spring. The phylogenetic analysis based on the 16S rRNA gene sequence revealed that strain AZM16c01^T belonged in the OP5 lineage described by Hugenholtz *et al.* (1998a) and Rappé & Giovannoni (2003). In addition, strain AZM16c01^T was not closely related to any of the known bacteria in recognized phyla, with less than 85 % sequence similarity (Mori *et al.*, 2008), and was sufficiently distinct to represent a new bacterial phylum (Hugenholtz *et al.*, 1998a). The low DNA G + C content of strain AZM16c01^T and the presence of the highly saturated menaquinone-8 in strain AZM16c01^T were not common in members of the domain *Bacteria* (Collins, 1994). Also, the fatty acid profile was typical of thermophiles and resembled particularly that of the phylum '*Deinococcus-Thermus*' (Garrrity & Holt, 2001). Considering the morphological observations, although the occurrence of 3-hydroxy fatty acid strongly suggested the presence of an outer membrane containing lipopolysaccharide as observed for Gram-negative bacteria, it would be interesting to analyse the cell envelope of this

Fig. 2. Phylogenetic relationship based on 16S rRNA gene sequences of strain AZM16c01^T and major recognized bacterial phyla, inferred from the ARB program (Ludwig *et al.*, 2004). Solid wedges indicate phyla with cultivated representatives and open wedges indicate phyla currently represented only by environmental clone sequences. Bar, 0.1 substitutions per compared nucleotide site.

Fig. 3. Neighbour-joining tree based on 16S rRNA gene sequences of strain AZM16c01^T and environmental clones belonging to the OP5 phylum. The probability scores at branch points obtained using four analysis methods (neighbour-joining, maximum-likelihood, maximum-parsimony and Bayesian) are indicated by solid circles (above 95% by all methods) and open circles (>90% bootstrap probability supported by two methods). GenBank/EMBL/DBJ accession numbers are shown in parentheses. Bar, 0.02 substitutions per compared nucleotide site.

organism in detail in a further study. In conclusion, the phylogenetic and physiological findings indicated that strain AZM16c01^T represents a new genus and novel species within the candidate phylum OP5. Thus, we propose the name *Caldisericum exile* gen. nov., sp. nov. for this strain. In addition, corresponding to the candidate phylum OP5, we propose the phylum name *Caldiserica* phyl. nov., after *C. exile*, the first and sole representative of the phylum.

Description of *Caldisericum* gen. nov.

Caldisericum (Cal.di.se'ri.cum. L. adj. *calidus* hot; L. neut. n. *sericum* silk; N.L. neut. n. *Caldisericum* a silk existing in a hot environment).

Obligately anaerobic and chemoheterotrophic growth. Gram-negative. Filamentous morphology with an outer envelope. Thermophilic and non-halophilic. Spores are not formed. Cells are not motile. Growth occurs with anaerobic respiration using sulfur compounds. Respiratory isoprenoid quinones are menaquinone-8(H₆), menaquinone-8(H₈) and menaquinone-8(H₁₀). The DNA G+C content of the type species is 34.6 mol%. Type species is *Caldisericum exile*.

Description of *Caldisericum exile* sp. nov.

Caldisericum exile (e.xi'le. L. neut. adj. *exile* slender).

Exhibits the following properties in addition to those given in the genus description. Cells are multicellular filaments, 0.3 µm wide and 1.6–8.3 µm long, with single polar flagella. Cells possess an outer envelope outside the cell membrane. Catalase- and oxidase-negative. Obligately

chemoheterotrophic and anaerobic growth is observed with the reduction of sulfur compounds (thiosulfate, sulfite and elemental sulfur). Sulfate, fumarate, nitrate, nitrite and oxygen are not used as electron acceptors. Fermentation is not observed. Yeast extract is necessary for growth; the following substrates do not support growth: H₂/CO₂, H₂/CO₂ + acetate, acetate, butyrate, formate, fumarate, glutamate, lactate, malate, propionate, pyruvate, succinate, L-arginine, L-cysteine, arabinose, fructose, galactose, glucose, inositol, mannose, raffinose, sucrose, xylose, CH₄, methanol, ethanol, 2-propanol, starch, Casamino acids (Difco), or polypeptone (Nihon Seiyaku). Temperature range for growth is 55–70 °C, with optimum growth at 65 °C. pH range for growth is 5.5–7.5, with optimum growth at pH 6.5. Optimum growth occurs in the absence of NaCl; growth is inhibited above 1% (w/v) NaCl. Major cellular fatty acid is iso-C_{17:0}; iso-C_{15:0}, iso-C_{17:0} 3-OH, C_{16:0} and anteiso-C_{17:0} are also present. The DNA G+C content of the type strain is 34.6 mol%.

The type strain, AZM16c01^T (=NBRC 104410^T=DSM 21853^T), was isolated from a hot spring, Otari, Nagano Prefecture, Japan.

Description of *Caldiseriaceae* fam. nov.

Caldiseriaceae (Cal.di.se'ri.ca.ce'ae. N.L. neut. n. *Caldisericum* type genus of the family; suff. *-aceae* ending to denote a family; N.L. fem. pl. n. *Caldiseriaceae* the family of the genus *Caldisericum*).

The description is the same as for the genus *Caldisericum*.

Type genus is *Caldisericum* gen. nov.

Description of *Caldisericales* ord. nov.

Caldisericales (Cal.di.se'ri.cal'es. N.L. neut. n. *Caldisericum* type genus of the order; suff. *-ales* ending to denote an order; N.L. fem. pl. n. *Caldisericales* the order of the genus *Caldisericum*).

The description is the same as for the genus *Caldisericum*.

Type genus is *Caldisericum* gen. nov.

Description of *Caldisericia* classis nov.

Caldisericia (Cal.di.se'ri.ci'a. N.L. neut. n. *Caldisericum* type genus of the type order of the class; suff. *-ia*, ending to denote a class; N.L. neut. pl. n. *Caldisericia* the class of the order *Caldisericales*).

The description is the same as for the genus *Caldisericum*.

Type order is *Caldisericales* ord. nov.

Description of *Caldiserica* phyl. nov.

Caldiserica (Cal.di.se'ri.ca. N.L. neut. n. *Caldisericum* type genus of the type order of the phylum; N.L. fem. pl. n. *Caldiserica* phylum of the genus *Caldisericum*).

The phylum *Caldiserica* is defined on a phylogenetic analysis based on 16S rRNA gene sequences of a sole isolated strain and uncultured representatives from various environments. Gram-negative bacteria with an outer envelope.

Type order is *Caldisericales* ord. nov.

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