Tetrasphaera remsis sp. nov., isolated from the Regenerative Enclosed Life Support Module Simulator (REMS) air system

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Two Gram-positive, coccoid, non-spore-forming bacteria (strains 3-M5-R-4^T and 3-M5-R-7), cells of which formed diploid, tetrad and cluster arrangements, were isolated from air of the Regenerative Enclosed Life Support Module Simulator system. On the basis of 16S rRNA gene sequence similarity, these strains were shown to belong to the family *Intrasporangiaceae* and were related to members of the genus *Tetrasphaera*, with similarities to the seven known species of the genus *Tetrasphaera* of 96.71–97.76 %. The fatty acid profile supported affiliation of these novel isolates to the genus *Tetrasphaera*, although larger amounts of octadecanoic acid (C_{18:0}) and *cis*-9-octadecenoic acid (C_{18:1}) were observed in the isolates, thus enabling them to be differentiated from other *Tetrasphaera* species. In addition, DNA–DNA hybridization studies indicated that these strains belonged to a novel species that could be readily distinguished from its nearest neighbour, *Tetrasphaera japonica* DSM 13192^T, which had less than 20 % DNA–DNA relatedness. Physiological and biochemical tests showed few phenotypic differences, but genotypic analysis enabled these gelatin-liquefying strains to be differentiated from the seven *Tetrasphaera* species. The strains described in this study therefore represent a novel species, for which the name *Tetrasphaera remsis* sp. nov. is proposed; the type strain is 3-M5-R-4^T (=ATCC BAA-1496^T =CIP 109413^T).

The genus *Tetrasphaera* currently comprises seven species, *Tetrasphaera australiensis*, *T. elongata*, *T. duodecadis*, *T. japonica*, *T. jenkinsii*, *T. vanveenii* and *T. veronensis*

Abbreviations: DAP, diaminopimelic acid; EM, electron microscope; ISS, International Space Station; REMS, Regenerative Enclosed Life Support Module Simulator.

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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains 3-M5-R-4^T and 3-M5-R-7 are DQ447774 and EF028236, respectively.

A figure showing TLC analysis of whole-cell hydrolysates is available as supplementary material with the online version of this paper.

(Hanada *et al.*, 2002; Ishikawa & Yokota, 2006; Maszenan *et al.*, 2000; McKenzie *et al.*, 2006). Most of these species were isolated from wastewater sludge. However, two phenotypically and genotypically distinct strains of bacteria related to the genus *Tetrasphaera* were isolated recently from air samples collected from a class 100K clean room.

The Regenerative Enclosed Life Support Module Simulator (REMS) system was constructed to simulate conditions aboard the International Space Station (ISS) and to create an environment for testing reclamation technologies for future ISS implementation. Crew activity was simulated using volunteers who exercised, performed prescribed hygienic activities (e.g. face washing and tooth brushing with carefully measured quantities of water) and prepared

pre-packaged meals (Carter *et al.*, 2005). The REMS facility as a whole was maintained as a class 100K clean room (cumulative distribution of particles $>0.5\ \mu\text{m}$ per one cubic foot of air) during its operational phase through stringent maintenance protocols that included controlled air circulation and filtration. The REMS system provided an extreme, unique, encapsulated environment for humans and their associated microbes. The microbial diversity of the REMS atmosphere was characterized recently using state-of-the-art molecular methods to establish criteria necessary for providing an environment that promotes the health, safety and productivity of the ISS crew-members (Moissl *et al.*, 2007). While cataloguing cultivable microbes, two novel strains, 3-M5-R-4^T and 3-M5-R-7, with unique morphology and taxonomic characteristics were isolated and characterized.

During the characterization of organisms isolated from REMS air, strains 3-M5-R-4^T and 3-M5-R-7 were recovered from low nutrient media under mesophilic conditions, appearing as beige-coloured colonies. All further cultivation was carried out on R2A medium (Difco) at 25 °C for 7 days. Isolates were capable of growth at 15–45 °C, but did not grow under psychrophilic conditions. Results of physiological characterization using API 20E and API 20NE kits (bioMérieux) are given in the species description. Oxidation of various carbon sources was tested by using Biolog GP2 MicroPlates. The plates were used according to the manufacturer's instructions except that R2A liquid medium was used instead of Biolog Universal Growth medium. The inoculated plates were incubated for 72 h and the results were read with a MicroPlate Reader using Microlog 4.2 computer software. Table 1 shows the differential characteristics of the newly described strains and *T. japonica* DSM 13192^T, a closely related strain (16S rRNA gene sequence similarity of ~98 % with the isolates). The newly described strains were oxidase-negative and able to liquefy gelatin and so could be readily distinguished from their nearest neighbour. Several sugars (e.g. sucrose and trehalose) and organic compounds were utilized by the novel strains, but not by *T. japonica*. Also, *T. japonica* grew between 15 and 37 °C, but failed to grow at 45 °C.

Morphology of cells grown for 3 days at 25 °C on R2A or in liquid medium was observed under an Olympus light microscope at $\times 1000$ magnification. For cryo-electron tomography, cells grown for 3 days in liquid medium were plunge-frozen together with 10 nm gold fiducial markers across a glow-discharged, copper/rhodium electron microscope (EM) grid with R 2/2 carbon-on-film Quantifoil (Quantifoil Micro Tools). A 4 μl drop of cell culture mixed with fiducial gold markers was applied to the EM grid at 100 % humidity, automatically blotted and plunged into liquid ethane using a Vitrobot (FEI). The prepared grids were stored under liquid nitrogen. Images were collected using the FEI Polara, a 300 kV field emission gun transmission EM equipped with an energy filter (slit width 20 eV) and a Gatan Ultrascan CCD camera. Single projections were collected from -63° to $+63^\circ$ in 1 degree

increment steps using the predictive UCSF_Tomo software package (Zheng *et al.*, 2004). The dataset was recorded at 12 μm underfocus and a total electron dose of 160 $\text{e}^- \text{\AA}^{-2}$. The tomogram was reconstructed by weighted back-projection using the IMOD software package (Mastronarde, 1997).

All cells showed a Gram-positive wall. As observed in *T. japonica* (Maszenan *et al.*, 2000), the cell wall of the novel strains consisted of three visible layers: an inner electron-dense layer, which was closely associated with the cytoplasmic membrane, an electron-lucent layer and, on the outside, another electron-dense layer (Fig. 1). As observed in *T. vanveenii* (McKenzie *et al.*, 2006), there were two layers of capsular material in 3M5-R-4^T, an electron-lucent layer that surrounded each cell and an electron-dense layer that surrounded the entire cluster of cells (Fig. 1). A thin strand of material from this outer capsule continued into the centre of the septum separating the cells. In contrast to the type strains of *T. jenkinsii*, *T. vanveenii* and *T. veronensis*, the novel isolates always grew as cocci in diploid, tetrad and cluster arrangements on all the media examined and filamentous growth was not observed.

The G+C content of genomic DNA was determined as described previously (Saha *et al.*, 2005). The G+C content of strain 3MSR-4^T was 69.2 mol% (T_m 81.5 °C). Both strains examined in this study produced polyphosphate (Murray *et al.*, 1994), as observed in other *Tetrasphaera* species. The diastereoisomer of diaminopimelic acid (DAP) in cell-wall hydrolysates of strains 3M5-R-4^T and 3M5-R-7 was determined (Staneck & Roberts, 1974). Briefly, 20 mg freeze-dried cells was hydrolysed with 200 μl 6 M HCl in a 100 °C water-bath for 18 h. Following hydrolysis, the cell mixture was filtered through an activated charcoal column and dried completely in a concentrator (Turbo-Vap; Zymarck) and then dissolved in 200 μl sterile double-distilled water. A total of 5 μl hydrolysate was applied on a TLC plate (Merck 5577) along with standard DAP and developed in a TLC tank containing a methanol/water/10 M HCl/pyridine (80:17.5:2.5:10, by vol.) mixture for 3 h. The TLC plate was dried under a fume hood and sprayed with a 0.2 % ninhydrin solution. Plates were finally heated to 100–105 °C. After about 5 min, hydrolysate DAP was visualized as a grey-green colour when compared against the standard DAP. Analysis of the cell-wall hydrolysates of strains 3M5-R-4^T and 3M5-R-7 revealed the presence of *meso*-DAP as its diagnostic cell-wall dibasic amino acid, which was also present in the *Tetrasphaera* type strains analysed in this study (see Supplementary Fig. S1 available in IJSEM Online).

Phylogenetic analysis was performed using the ARB software package (Ludwig *et al.*, 2004). Distances and clustering with the neighbour-joining and maximum-parsimony methods were determined by using bootstrap values based on 1000 replications. The 16S rRNA gene sequences of

Table 1. Differential characteristics of strains 3M5-R-4^T and 3M5-R-7 and *T. japonica* DSM 13192^T

Characteristics shown in bold can be used to distinguish the novel strains, 3M5-R-4^T and 3M5-R-7, from *T. japonica* DSM 13192^T. All three strains were cocci, occurring singly and in diploid, tetrad and cluster arrangements (cells 0.5–1.0 µm diameter). They all grew at pH 6.0–8.0; optimum growth was observed at pH 7.0 and 25 °C. All were positive for catalase and negative for nitrate reduction and production of urease, indole and H₂S. All strains were positive for oxidation of D-galactose, maltose, D-ribose, turanose and L-malic acid and negative for oxidation of α-cyclodextrin, glycogen, inulin, mannan, N-acetyl-D-glucosamine, N-acetyl-β-D-mannosamine, amygdalin, D-arabitol, D-gluconic acid, myo-inositol, α-D-lactose, lactulose, mannitol, D-melibiose, methyl α-D-galactoside, methyl β-D-glucoside, methyl α-D-mannoside, D-psicose, D-raffinose, L-rhamnose, sedoheptulosan, stachyose, D-tagatose, α-hydroxybutyric acid, β-hydroxybutyric acid, p-hydroxyphenylacetic acid, α-ketoglutaric acid, α-ketovaleric acid, lactamide, D-lactic acid methyl ester, L-lactic acid, D-malic acid, pyruvic acid methyl ester, succinic acid monomethyl ester, propionic acid, succinamic acid, succinic acid, N-acetyl-L-glutamic acid, L-alaninamide, L-alanine, L-alanyl glycine, L-asparagine, glycyl L-glutamic acid, L-pyrogutamic acid, L-serine, putrescine, 2,3-butanediol, 2'-deoxyadenosine, inosine, thymidine, uridine, AMP, TMP, UMP, D-fructose 6-phosphate, α-D-glucose 1-phosphate, D-glucose 6-phosphate and DL-α-glycerol phosphate. ND, Not determined.

Characteristic	Strain 3M5-R-4 ^T	Strain 3M5-R-7	<i>T. japonica</i> DSM 13192 ^T
Growth temperature range (°C)	15–45	15–45	15–37
Oxidase	–	–	+
Gelatin hydrolysis	+	+	–
Oxidation of substrates using Biolog GP2 MicroPlates			
β-Cyclodextrin	–	–	+
Dextrin	+	+	–
Tween 40	–	+	–
Tween 80	+	–	–
L-Arabinose	–	+	+
Arbutin	+	–	–
D-Cellobiose	+	+	–
D-Fructose	+	+	–
L-Fucose	–	–	+
D-Galacturonic acid	–	+	–
Gentiobiose	–	+	–
α-D-Glucose	+	+	–
Maltotriose	+	+	–
Mannose	–	+	–
D-Melezitose	+	+	–
Methyl β-D-galactoside	+	–	–
3-Methyl D-glucose	+	+	–
Methyl α-D-glucoside	+	–	–
Palatinose	+	+	–
Salicin	+	+	–
D-Sorbitol	–	+	–
Sucrose	+	+	–
Trehalose	+	+	–

Characteristic	Strain 3M5-R-4 ^T	Strain 3M5-R-7	<i>T. japonica</i> DSM 13192 ^T
Xylitol	–	–	+
D-Xylose	+	+	–
Acetic acid	+	+	–
γ-Hydroxybutyric acid	–	+	–
Pyruvic acid	+	+	–
D-Alanine	–	+	–
L-Glutamic acid	–	+	–
Glycerol	+	+	–
Adenosine	+	+	–
Uridine	–	+	+
D-Fructose 6-phosphate	–	+	–
DNA G + C content (mol%)	69.2	ND	70

strains 3-M5-R-4^T and 3-M5-R-7 were continuous stretches of 1250 bp and were compared with those of known *Tetrasphaera* species (Blackall *et al.*, 2000; Hanada *et al.*, 2002; Maszenan *et al.*, 2000). The parsimony-analysis-based phylogenetic tree is illustrated in Fig. 2. Sequences from strains 3M5-R-4^T and 3M5-R-7 were >99.9% similar to each other and formed a well-defined and separate cluster supported by high bootstrap values (~99%). Sequence similarity calculations indicated that the closest relative of these strains was *T. japonica* (98%). Lower sequence similarities (~97%) were found with all species of the genus *Tetrasphaera* with validly published names.

DNA–DNA hybridization was performed using microplate hybridization methods (Ezaki *et al.*, 1989). Isolates 3-M5-R-4^T and 3-M5-R-7 were closely related to each other (71 and 84% DNA relatedness values with each other) suggesting that they are members of the same species, but exhibited relatively low levels of hybridization with

Fig. 1. Electron microscope image of cells of *Tetrasphaera remsis* sp. nov. 3M5-R-4^T. The image is a 19 nm-thick section of the tomogram. Bar, 100 nm. Cells contain vesicles with electron-dense material. Diploid cells and a single cell are shown, but the cells can also form tetrads (not shown).

their nearest neighbour, *T. japonica* ACM 5116^T (less than 16%). These findings strongly indicate that the two isolates represent a novel species of the genus *Tetrasphaera*.

Cellular fatty acid methyl esters were analysed with the Microbial ID system according to the manufacturer's instructions; results are given in Table 2. The fatty acid profiles of strains 3-M5-R-4^T and 3-M5-R-7 revealed a high percentage of branched-chain fatty acids (iso-C_{16:0}), as reported in the majority of other *Tetrasphaera* species (Hanada *et al.*, 2002). The presence of both stearic and oleic acids distinguished 3-M5-R-4^T and 3-M5-R-7 from other *Tetrasphaera* species. The two strains contained larger amounts of octadecanoic acid (C_{18:0}) and *cis*-9-octadecenoic acid (C_{18:1}) than the other *Tetrasphaera* species (approx. 12 and 22% greater, respectively). Although C_{18:0} has been reported in *Sanguibacter* species (Fernandez-Garayzabal *et al.*, 1995), C_{18:1} was not found. In addition, C_{16:0} was not found in strains 3-M5-R-4^T and 3-M5-R-7 in the elevated quantities (33–53%) observed in *Sanguibacter* species.

Physiological and biochemical tests showed few phenotypic differences between the novel species described herein and other *Tetrasphaera* species. However, detection of elevated levels of certain fatty acids (C_{18:0} and C_{18:1}) as well as genotypic analyses allowed these gelatin-liquefying strains to be differentiated from the seven *Tetrasphaera* species. It is therefore proposed that strains 3-M5-R-4^T and 3-M5-R-7 represent a novel species in the genus *Tetrasphaera*, for which the name *Tetrasphaera remsis* sp. nov. is suggested.

Description of *Tetrasphaera remsis* sp. nov.

Tetrasphaera remsis (rem'sis. N.L. gen. n. *remsis* off/from REMS, acronym for Regenerative Enclosed Life Support Module Simulator).

Cells are Gram-positive, non-motile cocci (0.5–1.0 µm diameter), occurring in diploid, tetrad and cluster arrangements. Spore formation is not observed. Colonies are beige-coloured and convex with a glossy surface. Aerobic chemoheterotroph. Does not reduce nitrate to

Table 2. Cellular fatty acid compositions (%) of the novel strains and type strains of *Tetrasphaera* species

Strains: 1, 3M5-R-4^T; 2, 3M5-R-7 (data from this study); 3, *T. vanveenii* DSM 17518^T; 4, *T. jenkinsii* DSM 17519^T; 5, *T. veronensis* DSM 17520^T (data in columns 3–5 from McKenzie *et al.*, 2006); 6, *T. japonica* DSM 13192^T (data from Maszenan *et al.*, 2000); 7, *T. australiensis* ACM 5117^T (Hanada *et al.*, 2002); 8, *T. elongata* DSM 14184^T (Hanada *et al.*, 2002); 9, *T. duodecadis* IAM 14868^T (Ishikawa & Yokota, 2006). —, Not detected or below 1%. Values over 10% are in bold. Abbreviations: i, iso; ai, anteiso.

Fatty acid	1	2	3	4	5	6	7	8	9
i-C _{14:0}	0.8	0.9	2.5	2.2	5.1	—	2.6	7.9	1.9
C _{14:0}	1.0	1.0	1.4	3.2	1.7	—	—	—	—
i-C _{15:0}	9.0	9.4	18.7	17.2	8.8	6.4	12.1	19.9	15.8
ai-C _{15:0}	—	—	2.4	2.8	1.3	3.0	7.6	30.6	—
C _{15:0}	—	—	6.8	3.1	3.2	1.1	4.0	—	2.0
C _{15:1}	—	—	3.3	1.0	1.9	—	—	—	—
i-C _{16:0}	13.7	14.6	30.3	21.0	49.9	25.8	27.1	4.2	17.8
C _{16:0}	5.8	5.9	2.8	8.1	3.6	—	5.9	7.9	2.7
i-C _{16:1}	—	—	7.8	2.3	10.1	3.2	9.7	—	—
C _{16:1}	3.3	3.1	3.1	—	5.9	5.6	8.4	—	—
i-C _{17:0}	6.1	6.2	1.1	4.4	—	2.2	2.0	—	3.9
ai-C _{17:0}	1.8	1.8	2.5	5.8	6.0	20.5	14.1	1.5	21.8
C _{17:0}	6.7	6.5	2.8	4.9	1.1	1.3	—	3.2	6.1
i-C _{18:0}	2.4	2.3	—	1.9	—	5.3	—	—	—
i-C _{17:1}	0.8	1.1	2.1	1.2	—	2.3	—	—	—
C _{17:1}	5.4	5.8	7.1	—	—	—	—	1.1	—
ai-C _{17:1}	—	—	1.5	1.1	1.5	6.9	4.7	—	—
i-C _{18:1}	—	—	—	—	—	1.3	—	—	5.1
C _{18:0}	12.9	12.2	—	1.6	—	—	—	—	11.8
C _{18:1}	23.4	22.4	—	—	—	2.2	3.7	—	—

nitrite. Does not grow anaerobically or ferment glucose. Liquefies gelatin and hydrolyses aesculin. Produces catalase and β-galactosidase. Utilizes D-glucose, D-mannose, D-mannitol, maltose, N-acetylglucosamine and malate. Does not assimilate the following compounds: L-arabinose, gluconate, capric acid, adipic acid, phenylacetic acid and citrate. Oxidase, urease, arginine dihydrolase, lysine and ornithine decarboxylases and indole are not produced.

Fig. 2. Phylogenetic position of strains of *Tetrasphaera remsis* sp. nov. with members of *Tetrasphaera* and related genera. The tree was constructed on the basis of evolutionary distances calculated using the maximum-parsimony method and 1250 bases of the 16S rRNA gene sequence were compared. Bar, 10 substitutions per 100 nucleotide positions. Significant bootstrap probabilities (>90%, 1000 replications) are indicated at branch points. GenBank accession numbers are shown.

Growth occurs at 15–45 °C and pH 6–9; optimal growth occurs at 25 °C and pH 7. The major cellular fatty acids are iso-C_{15:0}, iso-C_{16:0}, C_{18:0} and C_{18:1}.

The type strain is strain 3-M5-R-4^T (=ATCC BAA-1496^T =CIP 109413^T).

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