NOTE

***Tetrasphaera elongata* sp. nov., a polyphosphate-accumulating bacterium isolated from activated sludge**Satoshi Hanada,¹ Wen-Tso Liu,² Tomoyoshi Shintani,³ Yoichi Kamagata¹ and Kazunori Nakamura¹¹ Research Institute of Biological Resources, National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba Central 6, 1-1-1 Higashi, Tsukuba 305-8566, Japan² Department of Civil Engineering, National University of Singapore, Singapore 119260³ Industrial Research Center of Ehime Prefecture, Matsuyama 791-1101, Japan

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A new meso-diaminopimelic acid-containing, Gram-positive bacterium was isolated from an activated sludge reactor showing enhanced biological phosphorus removal activity. The isolate was an asporogenous oval to rod-shaped bacterium, but occasionally formed clumps. The Neisser staining was positive, suggesting intracellular polyphosphate granules. The isolate was an aerobic chemoheterotroph which was capable of utilizing various sugars, sugar alcohols and organic acids. It contained anteiso-C15:0, iso-C15:0, iso-C14:0 and C16:0 as the major cellular fatty acids, and menaquinone-8(H₄) as the major quinone. The G+C content of the genomic DNA was 69.6 mol%. Analysis of the 16S rDNA sequence revealed that the isolate is a new member of the family Intrasporangiaceae. The closest relatives were *Tetrasphaera* species. On the basis of the phenotypic and phylogenetic distinctiveness of the isolate, it was concluded that the organism represents a new species in the genus *Tetrasphaera*, for which the name *Tetrasphaera elongata* sp. nov. is proposed. The type strain is strain Lp2^T (= JCM 11141^T = DSM 14184^T).

Keywords: *Tetrasphaera elongata* sp. nov., Intrasporangiaceae, polyphosphate accumulation, activated sludge, meso-diaminopimelic acid

The family *Intrasporangiaceae* was proposed as a family in the suborder *Micrococcineae* by Stackebrandt *et al.* (1997), on the basis of 16S rDNA sequence analysis, and recently this family has been slightly emended (Stackebrandt & Schumann, 2000). The family *Intrasporangiaceae* is currently composed of the following genera: *Intrasporangium*, *Terrabacter*, *Terracoccus*, *Janibacter* and *Tetrasphaera*. Although the family *Intrasporangiaceae* is composed of morphologically and physiologically heterogeneous members, the family was divided into two groups on the basis of the type of diamino acid in the cell-wall structure: the genera *Intrasporangium*, *Terrabacter* and *Terracoccus* contain LL-diaminopimelic acid (A₂pm); and meso-A₂pm is the diagnostic diamino acid in the genera *Janibacter* and *Tetrasphaera*. Most members in the meso-A₂pm-containing group were isolated from activated sludge in sewage-treatment systems (the only

exception is *Janibacter brevis*, which was isolated from groundwater; Imamura *et al.*, 2000). Of the members, the genus *Tetrasphaera*, consisting of two species, *Tetrasphaera australiensis* and *Tetrasphaera japonica* (Maszenan *et al.*, 2000), is a coccus that is Neisser-stain-positive, indicating that the organisms accumulate polyphosphate in the cells. Thus, *Tetrasphaera* species are considered to be important micro-organisms for use in conjunction with biological phosphorus removal from wastewater. Blackall *et al.* (2000) reported that a Neisser-positive, filamentous organism – ‘*Candidatus Nostocoida limicola*’ – that inhabits activated sludge was closely related to *Tetrasphaera* species. These findings suggest that certain members of the family *Intrasporangiaceae* may be involved in the phosphorus removal in activated sludge systems.

Previously, a new Gram-positive bacterium that contained meso-A₂pm, and which showed distinctive phosphate-removal activity, had been isolated from an anaerobic-aerobic sequential batch reactor (Liu, 1995; Liu *et al.*, 1997, 2000). The isolate, designated strain

Abbreviation: A₂pm, diaminopimelic acid.

The GenBank/EMBL/DBJ accession number for the 16S rRNA gene sequence of strain Lp2^T is AB030911.

Fig. 1. Phase-contrast photomicrograph of strain Lp2^T cells grown aerobically at 25 °C in MN-1 medium (left) and R medium (right). Bar, 5 µm.

Lp2^T, like *Tetrasphaera* species, exhibited positive Neisser-staining, implying a role in the biological phosphorus-removal process. In this report, we describe the phenotypic traits and taxonomic position of this strain.

Colonies of the isolate on NM-1 medium (Nakamura *et al.*, 1995) were beige-coloured and convex with a glossy surface. The cells grown in liquid NM-1 medium were non-spore-forming oval to short rods, 0.7–1.0 µm in diameter and 1.0–1.8 µm long (Fig. 1, left). The cells had no flagella and showed no motility. The isolate could also grow in liquid R medium (Martin *et al.*, 1997), which contains concentrations of carbon sources that are approximately 15-fold higher than those in NM-1 medium. The cells grown in R medium clearly elongated to approximately 4 µm in length (Fig. 1, right). Unlike cultures of the known species of the genus *Tetrasphaera*, cultures of this isolate contained L-shaped clumps and irregular clusters. Electron microscopy revealed several septa within elongated cells grown in R medium, and showed that the cells possessed the typical cell-wall structure of a Gram-positive bacterium (Fig. 2).

Strain Lp2^T is an obligate aerobic chemoheterotrophic bacterium. Although the isolate was able to reduce nitrate to nitrite under anaerobic conditions, the nitrate respiration could not support growth under the conditions used. The isolate showed no fermentative growth. The test for organic substrate-oxidation activities (the BIOLOG system; BIOLOG) revealed that the cells aerobically oxidize various sugars and sugar alcohols, i.e. cellobiose, glucose, lactose, maltose, mannose, melibiose, sucrose, trehalose, xylose, dulcitol, inositol and mannitol. It also oxidized acetate, pyruvate, alanine and arginine, and hydrolysed starch and glycogen. The isolate, however, did not oxidize the following compounds: arabinose, galactose, ethanol, glycerol, methanol, propanol, malate, propionate, succinate, asparagine, glutamate, glutamine and his-

Fig. 2. Transmission electron micrograph of strain Lp2^T cells, showing storage materials and septa in the cells. The ultra-thin sections of cells were prepared and observed with a Hitachi H-7000 transmission electron microscope as described previously (Shintani *et al.*, 2000). Cells were grown in R medium at 25 °C under aerobic conditions. Bar, 0.5 µm.

Table 1. Cellular fatty acid composition (%) of strain Lp2^T and related species that belong to the family *Intrasporangiaceae*

Data, except that for strain Lp2^T, were taken from the following references: Maszenan *et al.* (2000); Martin *et al.* (1997). Abbreviations: i, iso; ai, anteiso.

Fatty acid	Strain Lp2 ^T	<i>Tetrasphaera australiensis</i>	<i>Tetrasphaera japonica</i>	<i>Janibacter limosus</i>
i-C14:0	7.9	2.6	–	0.5
i-C15:0	19.9	12.1	6.4	1.7
ai-C15:0	30.6	7.6	3.0	0.3
i-C16:0	4.2	27.1	25.8	17.5
i-C16:1	–	9.7	3.2	–
C16:0	7.9	5.9	–	3.9
C16:1	–	8.4	5.6	2.4
ai-C17:0	1.5	14.1	20.5	–
C17:0	3.2	–	1.3	20.5
C17:1	1.1	–	–	29.7
ai-C17:1	–	4.7	6.9	0.2
C18:1	–	3.7	2.2	8.1

tidine. The catalase test was positive, but oxidase and indole were not produced. Optimal growth of the isolate in NM-1 medium occurred at 25 °C and at pH 7.0. The temperature range for growth was 20–35 °C. Growth did not occur at 16 or 37 °C. The isolate grew between pH 6 and 9, and did not grow below pH 5 or above pH 10. Fatty acid methyl ester analysis with a GC/MS system (Hanada *et al.*, 2002) revealed that the main cellular fatty acids in strain Lp2^T are anteiso-C15:0, iso-C15:0, iso-C14:0 and C16:0 (Table 1). The cellular fatty acids in the isolate were predominantly saturated fatty acids. The isolate contained phospho-

Fig. 3. Phylogenetic position of strain Lp2^T within the family Intrasporangiaceae in the Micrococcineae. CLUSTAL W program version 1.6 (Thompson *et al.*, 1994) was used to align the 16S rDNA sequences. The tree was constructed on the basis of the evolutionary distances calculated by using the neighbour-joining method (Saitou & Nei, 1987) with the Kimura two-parameter model (Kimura, 1980). The neighbour-joining analysis was performed with a MEGA program (Kumar *et al.*, 1994). All gaps in the alignment were excluded; 1359 bases were compared. The bar represents one nucleotide substitution per 100 nucleotides in the 16S rDNA sequences. The sequences of *Friedmanniella antarctica* and *Microlunatus phosphovorius* (belonging to the family Propionibacteriaceae) were used to root the tree. Bootstrap probabilities (Felsenstein, 1985) are indicated at the branch points. Bold type indicates Neisser-positive bacteria in the family Intrasporangiaceae. The accession numbers are shown in parentheses.

tidylethanolamine, phosphatidylglycerol, phosphatidylinositol diphosphatidylglycerol and an unknown aminophospholipid as the polar lipids (Komagata & Suzuki, 1987). The major respiratory quinone (Tamaoka *et al.*, 1983) in strain Lp2^T was menaquinone-8(H₄). The G + C content (Komagata & Mikami, 1991) of the total DNA of the isolate was 69.6 mol %.

The phylogenetic tree (Fig. 3), based on the neighbour-joining method (Saitou & Nei, 1987) and with the Kimura two-parameter model as a distance corrector (Kimura, 1980), showed that the isolate belongs to the family Intrasporangiaceae in the high-G + C group of the Gram-positive bacteria, and that it forms a cluster with the genera *Tetrasphaera*, *Janibacter* and '*Candidatus Nostocoida limicola*'. The sequence similarities between strain Lp2^T and the relatives were as follows: *T. australiensis*, 96.9 %; *T. japonica*, 96.7 %; *Janibacter limosus*, 96.2 %; *J. brevis*, 95.8 %; *Janibacter terrae*, 95.8 %; '*Candidatus Nostocoida limicola*', 97.1 %. The tree indicated that the isolate is clearly affiliated with the genus *Tetrasphaera*. However, the 16S rDNA sequence similarities between the isolate and known *Tetrasphaera* species were less than 97 %. These low values suggested that strain Lp2^T should be classified as a new species in this genus.

Table 2 shows comparative phenotypes for the isolate and *Tetrasphaera* and *Janibacter* species. Like its relatives, strain Lp2^T contained meso-A₂pm in the cell-wall structure, contained menaquinone-8(H₄) as the major quinone, and had a high G + C content. In addition, several common features were found between the isolate and *Tetrasphaera* species, e.g. both cells produced indole and were positively Neisser-stained (Maszenan *et al.*, 2000). These characteristics are not found in *Janibacter* species (Martin *et al.*, 1997; Imamura *et al.*, 2000; Yoon *et al.*, 2000). The cellular fatty acid profile of the isolate resembles that of *Tetrasphaera* species more than that of *Janibacter*

species (Table 1). *Janibacter* species contain considerable amounts of C17:1, but *Tetrasphaera* species and the isolate scarcely contain any of this unsaturated fatty acid. These phenotypic traits supported the view that the isolate belongs to the genus *Tetrasphaera* rather than the genus *Janibacter*. However, there were obvious dissimilarities between the isolate and *Tetrasphaera* species, as illustrated by the following phenotypic features. (1) The cells formed clumps in R medium. The formation of elongated linear or L-shaped clumps was characteristic of strain Lp2^T and was rarely observed in the known species of the genus *Tetrasphaera* (Maszenan *et al.*, 2000). The isolate did not form tetrads typical of *Tetrasphaera* species under any conditions tested in this study. (2) The major cellular fatty acids in Lp2^T were anteiso-C15:0, iso-C15:0, iso-C14:0 and C16:0. There were few unsaturated fatty acids in the isolate, whereas the known *Tetrasphaera* species contained a significant amount of unsaturated fatty acids (e.g. C16:1, C17:1 and C18:1). (3) The isolate was able to reduce nitrate and produce oxidase, while *Tetrasphaera* species could not. On the basis of the genotypic, chemotaxonomic and morphological data, we propose strain Lp2^T as a new species in the genus *Tetrasphaera*, namely *Tetrasphaera elongata* sp. nov.

The new bacterium was isolated from activated sludge in an anaerobic-aerobic sequential batch reactor exhibiting biological phosphorus-removal activity (Liu, 1995; Liu *et al.*, 1997). Neisser staining indicated that polyphosphate granules were stored in the cells. Thus, strain Lp2^T appears to be involved in the phosphorous-removal process. Its phylogenetic relatives, *Tetrasphaera* species, are also Neisser-positive bacteria showing polyphosphate accumulation (Maszenan *et al.*, 2000). Blackall *et al.* (2000) isolated a positively Neisser-stained, filamentous bacterium – '*Candidatus Nostocoida limicola*' – from activated

Table 2. Phenotypic characteristics of *meso*-A₂pm-containing members of the *Intrasporangiaceae* and strain Lp2^T

Abbreviations: +, positive; –, negative; ND, not determined; DPG, diphosphatidylglycerol; PG, phosphatidylglycerol; PI, phosphatidylinositol; PE, phosphatidylethanolamine; PL, unidentified glycolipid(s); APL, unidentified amino phospholipid; MK, menaquinone.

Phenotypic characteristic	Strain Lp2 ^T	<i>Tetrasphaera australiensis</i> *	<i>Tetrasphaera japonica</i> *	<i>Janibacter limosus</i> †
Cell morphology	Oval to short rods or irregular clumps	Cocci	Cocci	Rods or cocci
Habitat	Activated sludge	Sewage-treatment plant	Sewage-treatment plant	Activated sludge
Optimum growth temperature (°C)	25	25	25	28
pH range for growth	6.0–9.0	6.0–8.0	6.0–8.0	ND
Catalase	+	+	±	+
Oxidase	–	+	+	–
Nitrate reduction	+	–	–	+
Production of indole	–	–	–	+
Polar lipids	DPG, PE, PG, PI, APL	DPG, PG, PI, PL	DPG, PG, PI, PL, APL	DPG, PG, PI
Major quinone	MK-8(H ₄)	MK-8(H ₄)	MK-8(H ₄)	MK-8(H ₄)
G + C content (mol %)	69.6	70	71	70

* Data taken from Maszenan *et al.* (2000).

† Data taken from Martin *et al.* (1997).

sludge by using micro-manipulation, and found that the organism was closely related to the *meso*-A₂pm-containing members of the *Intrasporangiaceae* on the basis of 16S rDNA sequence analysis (its phylogenetic position is indicated in Fig. 3). The ability of these organisms and their population in the community to remove phosphate remain to be clarified, but it is likely that the Neisser-positive group comprising *Tetrasphaera* species, '*Candidatus* Nostocoida limicola' and our new isolate contribute to the biological phosphorous removal in activated sludge processes.

Description of *Tetrasphaera elongata* sp. nov.

Tetrasphaera elongata (e.lon'ga.ta. L. adj. *elongatus* elongated, pertaining to the formation of elongated clumps).

Cells are Gram-positive, non-motile, oval to short rods, 0.7–1.0 µm in diameter and 1.0–1.8 µm long, and occasionally form elongated linear clumps up to 4 µm long. Spore formation is not observed. Neisser staining is positive. Intracellular polyphosphate granules are observed. Colonies are beige-coloured and convex with a glossy surface. Aerobic chemoheterotroph. Cells reduce nitrate to nitrite under anaerobic conditions, but do not reduce nitrite to nitrogen. No anaerobic growth occurs. Cells oxidize acetate, alanine, arginine, cellobiose, glucose, lactose, maltose, mannose, melibiose, sucrose, trehalose, xylose, dulcitol, inositol, mannitol and pyruvate under aerobic conditions. Cells do not oxidize the following compounds: arabinose, galactose, ethanol, glycerol, methanol, propanol, malate, propionate, succinate, aspara-

gine, glutamate, glutamine and histidine. Starch and glycogen are hydrolysed. Catalase is positive. Oxidase and indole are not produced. Growth occurs at 20–35 °C and at pH 6–9. The optimal growth temperature and pH are 25 °C and 7.0, respectively. The major cellular fatty acids are anteiso-C15:0, iso-C15:0, iso-C14:0 and C16:0. The cell-wall peptidoglycan contains *meso*-diaminopimelic acid. The major quinone is menaquinone-8(H₄). On the basis of a phylogenetic comparison of 16S rDNA sequences, the bacterium belongs to the family *Intrasporangiaceae* of the high-G + C, Gram-positive bacteria. The closely related species are *Tetrasphaera australiensis* (the sequence similarity is 96.9%), *Tetrasphaera japonica* (96.7%), *Janibacter limosus* (96.2%) *Janibacter brevis* (95.8%), *Janibacter terrae* (95.8%) and '*Candidatus* Nostocoida limicola' (97.1%). The G + C content of the genomic DNA is 69.6 mol %. The type strain is strain Lp2^T (= JCM 11141^T = DSM 14184^T).

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