NOTE

***Kineosphaera limosa* gen. nov., sp. nov., a novel Gram-positive polyhydroxyalkanoate-accumulating coccus isolated from activated sludge**Wen-Tso Liu,¹ Satoshi Hanada,² Terrence L. Marsh,³ Yoichi Kamagata² and Kazunori Nakamura²¹ Department of Civil Engineering, National University of Singapore, Singapore 117576² Research Institute of Biological Resources, National Institute of Advanced Industrial Science and Technology, Central 6, 1-1 Higashi, Tsukuba 305-8566, Japan³ Center for Microbial Ecology, Michigan State University, East Lansing, MI, USAAuthor for correspondence: Wen-Tso Liu. Tel: +65 8741315. Fax: +65 7791635.
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A high-G+C Gram-positive, motile, non-spore-forming coccus capable of accumulating significant amounts of polyhydroxyalkanoate (PHA) was isolated from an inefficient biological phosphorus removal activated sludge reactor. The cell wall of strain Lpha5^T was characterized by the presence of meso-diaminopimelic acid, menaquinone MK-8(H₄) and a complex fatty-acid pattern consisting of C_{16:0} and at least five other major straight-chain saturated and unsaturated fatty acids. Strain Lpha5^T also had a high G+C content (71.3 mol%). The nearest phylogenetic relative of strain Lpha5^T, based on 16S rDNA sequence analysis, was the high-G+C Gram-positive bacterium *Dermatophilus congolensis* (similarity value of 94%), of the family *Dermatophilaceae*, class *Actinobacteria*. As strain Lpha5^T was distinct from *D. congolensis* in its morphological, phenotypical (i.e. its PHA-accumulating ability and fatty-acid profile) and genetic traits (phylogeny and G+C content), it is proposed that strain Lpha5^T be designated as the type species of a novel genus within the *Dermatophilaceae*. The name *Kineosphaera limosa* is proposed for strain Lpha5^T (= JCM 11399^T = DSM 14548^T).

Keywords: *Kineosphaera* gen. nov., *Kineosphaera limosa* sp. nov., high-G + C group, Gram-positive bacteria, polyhydroxyalkanoate accumulation

Polyhydroxyalkanoates (PHAs) are intracellular granules that are found within a number of bacterial species. These granules are found predominantly in microbes associated with anaerobic and aerobic activated sludge processes (Liu *et al.*, 1997). Accumulation of PHA granules occurs most often during the anaerobic phase when carbon sources such as short-chained acids are introduced in excess into the sludge environment. The physiology of PHA accumulation and the metabolism of organisms involved in anaerobic–aerobic activated sludge processes is thought to be distinct from that observed for *Pseudomonas* spp. and *Ralstonia* spp. (Anderson & Dawes, 1990). PHA-accumulating *Acinetobacter* spp. are found in anaerobic–aerobic activated sludge processes, but pure cultures of these organisms show a different pattern of

PHA accumulation than *in situ* populations (Mino *et al.*, 1998). Molecular examination of the microbial populations of anaerobic–aerobic activated sludge systems indicates that *Acinetobacter* spp. are not the most dominant microbes in these environments (Wagner *et al.*, 1994). A number of other Gram-negative and Gram-positive bacteria, besides *Acinetobacter* spp., have been isolated from anaerobic–aerobic processes (Mino *et al.*, 1998; Seviour *et al.*, 2000), but none have been found to accumulate PHA under anaerobic conditions. This suggests that novel PHA-accumulating organisms are present in activated sludge.

We have previously reported on the ability of Gram-positive, high-G + C bacteria isolated from an inefficient biological phosphorus removal activated sludge reactor to accumulate up to 35% (w/w) poly(3-hydroxybutyrate-co-3-hydroxyvalerate) under anaerobic or aerobic batch culture conditions (Liu *et al.*, 2000). The strains examined in that study were

Abbreviations: DAP, diaminopimelic acid; PHA, polyhydroxyalkanoate.

The GenBank accession number for the 16S rRNA gene sequence of strain Lpha5^T is AF109792.

reported to represent novel isolates. Here we describe the characteristics of one of the strains isolated from the inefficient biological phosphorus removal activated sludge reactor.

Strain Lpha5^T was cultured on GM1 medium, as previously described (Liu *et al.*, 1997, 2000). The temperature growth range (from 5 to 40 °C) and pH growth range (in pH-adjusted GM1 medium between pH 4 and 10) of the strain were determined by measuring the optical density of cultures at 600 nm. Gram-staining and Neisser-staining procedures were done as described by Magee *et al.* (1975) and the polyhydroxybutyrate-staining procedure was done as described by Jenkins *et al.* (1986). The cell morphology of strain Lpha5^T was examined under phase-contrast microscopy (using an Axioscope II light microscope; Zeiss), and by scanning and transmission electron microscopy (Shintani *et al.*, 2000). Oxidase, catalase and nitrate reduction activities of strain Lpha5^T were determined according to protocols described previously (Smibert & Krieg, 1981; Shintani *et al.*, 2000). The organic substrate utilization activities of Lpha5^T under aerobic conditions were determined using the BIOLOG system (BIOLOG). For the determination of PHA accumulation, strain Lpha5^T was cultured in a 1 l flask containing 200 ml of GM1 medium for 8 days under aerobic conditions at 30 °C. Cells were harvested at the end of cultivation, rinsed with 0.85% (w/v) NaCl and freeze-dried. The PHA composition of the lyophilized cells was determined by GC after their digestion with strong sulfuric acid, methylation by treatment with methanol and chloroform extraction, as described previously (Brandl *et al.*, 1988). Determinations of the quinones, diaminopimelic acid (DAP) isomers and whole-cell fatty acids of strain Lpha5^T were carried out according to protocols described previously (Tamaoka & Komagata, 1984; Hanada *et al.*, 2001). Polar lipids were extracted and purified by the method of Minnikin *et al.* (1979) and analysed by using two-dimensional TLC. The determination of the G+C content of the DNA of strain Lpha5^T, amplification of its 16S rRNA gene, and sequencing and subsequent analysis of the 16S rDNA data were performed as described previously (Shintani *et al.*, 2000).

Strain Lpha5^T grew slowly on GM1 agar and required 1–2 weeks incubation for colonies to develop. Colonies of strain Lpha5^T were light-yellow, irregular and umbonate (2–3 µm in diameter) with undulated margins. The optimum growth temperature of strain Lpha5^T was 30 °C (temperature growth range between 10 and 35 °C) and its optimum growth pH was 7.0 (pH growth range between pH 6.0 and 10.2). Growth was not inhibited by the presence of up to 3.0% (w/v) NaCl in the growth medium. When grown in liquid GM1 medium (pH 7.0) at 30 °C under aerobic conditions, the doubling time for strain Lpha5^T was 1.7 days.

Strain Lpha5^T stained Gram-positive. Upon microscopic examination, the strain appeared as motile, non-

Fig. 1. Scanning electron micrographs of strain Lpha5^T (A and B) showing the coccus-shaped morphology of the cells. Transmission electron micrograph of strain Lpha5^T (C) showing the presence of intracellular PHA granules. Bar, 1 µm in all cases.

spore-forming cocci (1–2 µm) that occurred singly, in pairs or in clusters (Fig. 1A). Cells of strain Lpha5^T had a rough surface on their outer cell walls (Fig. 1B) and contained intracellular granules (Fig. 1C). The cells appeared to divide by binary fission, and septum formation was frequently observed in the middle of a dividing cell (Fig. 1C). Phosphate accumulation was negative, as demonstrated by Neisser-staining; this observation was supported by an undetectable level of cellular polyphosphate. Accumulation of PHA (approx. 13% cell dry weight), with 3-hydroxybutyrate and 3-hydroxyvalerate as the major components, was detected.

Strain Lpha5^T was an obligately aerobic heterotroph. It was oxidase-negative and catalase-positive, and could not utilize nitrate as an electron acceptor. Strain

Fig. 2. Phylogenetic position of strain *Lpha5^T* within the suborder *Micrococccineae*. The sequence of *Bacillus subtilis* was used as an outgroup to root the tree. Bootstrap probabilities, based on 100 replications, are indicated at the branch points. Bar, two nucleotide substitutions per 100 nucleotides.

Lpha5^T could utilize dextrin, D-arabitol, fructose, α -D-glucose, maltose, 3-O-methylglucose, psicose, trehalose, turanose, xylitol, adenosine, Tween 40, Tween 80, N-acetyl-D-mannosamine, D-mannose, methyl β -D-glucoside, palatinose, salicin, D-sorbitol, sucrose, D-trehalose, γ -hydroxybutyric acid, methylpyruvate, propionic acid, succinamic acid, succinic acid and glycerol.

Strain *Lpha5^T* contained menaquinone MK-8(H_4) and meso-DAP as its dominant respiratory quinone and DAP, respectively. The fatty-acid profile of strain *Lpha5^T* contained $C_{16:0}$ (35%), $C_{17:1}$ (19%), $C_{18:1}$ (17%), $C_{17:0}$ (15%), $C_{16:1}$ (8%), $C_{15:0}$ (5%) and $C_{14:0}$ (1%) as its major components. The G+C content of the genomic DNA of the strain was 71.3 mol%. The polar-lipid profile of strain *Lpha5^T* comprised phosphatidylglycerol, phosphatidylinositol, diphosphatidylglycerol, phosphatidylethanolamine and phosphatidylcholine.

Phylogenetic analysis of approximately 1438 bp of the 16S rRNA gene of *Lpha5^T* [equivalent to > 95% of the *Escherichia coli* 16S rRNA gene sequence (Brosius *et al.*, 1981)] indicated that the strain was closely related to *Dermatophilus congolensis* (94% sequence similarity), which belongs to the family *Dermatophilaceae* within the suborder *Micrococccineae*. Strain *Lpha5^T* was also closely related to other families belonging to the suborder *Micrococccineae* (Fig. 2). The branch point between strain *Lpha5^T* and its closest relative, *Dermatophilus congolensis*, was supported by a high bootstrap value (92%). The presence of all of the signature nucleotides of the family *Dermatophilaceae* and the suborder *Micrococccineae* (Stackebrandt *et al.*, 1997) within the 16S rDNA sequence of strain *Lpha5^T* provided further support for this relationship.

Currently, the family *Dermatophilaceae*, belonging to the suborder *Micrococccineae*, contains four genera

that are differentiated primarily on the basis of their cell morphology and motility, and their DNA G+C contents, phylogeny and cell-envelope profiles [i.e. peptidoglycan type, menaquinone(s) content, fatty acids and polar lipids] (Table 1). Strain *Lpha5^T* can be clearly differentiated from the genera *Dermacoccus*, *Kytococcus*, *Dermatophilus* and *Demetria* on the basis of its peptidoglycan type, menaquinone content, fatty-acid profile, polar-lipid profile and G+C content. Furthermore, strain *Lpha5^T* is a motile coccus, whereas *Dermatophilus* spp. form mycelia and *Demetria* spp. are non-motile, coccoid cells. In addition, strain *Lpha5^T* is unique amongst the genera belonging to the family *Dermatophilaceae* and other known high-G+C strains isolated from activated sludge in its ability to accumulate intracellular PHAs (Liu *et al.*, 2000). Based on the results presented in this study, we propose to assign strain *Lpha5^T* as the type species of a new genus within the family *Dermatophilaceae*, *Kineosphaera limosa* gen. nov., sp. nov.

Description of *Kineosphaera* gen. nov.

Kineosphaera (Ki.ne.o.sphae'ra. Gr. n. *kinesis* motion; M.L. fem. n. *sphaera* sphere; N.L. fem. n. *Kineosphaera* a motile sphere).

Cells are strictly aerobic, non-spore-forming, Gram-positive, motile cocci (1–2 μ m in diameter), which grow in pairs and packets. Catalase-negative and oxidase-positive. Cells do not use nitrite as an electron acceptor. The cell wall contains meso-DAP as its characteristic diamino acid. The major menaquinone is MK-8(H_4). The G+C content of the DNA is 71 mol%. The predominant cellular fatty acid is $C_{16:0}$, followed by $C_{17:1}$, $C_{18:1}$, $C_{17:0}$ and $C_{16:1}$. The genus is a member of the family *Dermatophilaceae*, suborder *Micrococccineae* of the class *Actinobacteria* (high-G+C group), and contains nucleotide signatures of its family

Table 1. Morphological and chemotaxonomic features of *Kineosphaera* gen. nov. (strain Lpha5^T) and members of the family *Dermatophilaceae*

Characteristics	Genus*				
	<i>Kineosphaera</i>	<i>Dermatophilus</i> †	<i>Dermacoccus</i> ‡	<i>Kytococcus</i> ‡	<i>Demetria</i> †
Cell morphology	Cocci, single, in pairs and in clusters	Mycelium, spore-forming	Coccoid	Coccoid	Coccoid, non-sporulating
Motility	+	+	—	—	—
Peptidoglycan type	meso-DAP	meso-DAP	Lys-MCA, DCA	Lys-DCA	Lys-MCA, DCA
Major menaquinone(s)	MK-8(H ₄)	MK-8(H ₄)	MK-8(H ₂)	MK-8, MK-9, MK-10	MK-8(H ₄)
Fatty acids	U, S	S, U	S, A, I	(S), A, I	S, U, A, I
Polar lipids	PI, PG, DPG, PE, PC	DPG, PG, PI	DPG, PG, PI	DPG, PG, PI	PI, PG, DPG, PE, PL
G + C content (mol %)	71.3	57–59	66–71	68–69	66

* +, Positive; —, negative; MCA, monocarboxylic amino acid; DCA, dicarboxylic amino acid; S, straight-chain saturated; U, unsaturated; A, anteiso-methyl branched; I, iso-methyl branched; PI, phosphatidylinositol; PG, phosphatidylglycerol; DPG, diphosphatidylglycerol; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PL, unknown phospholipid(s).

† Data from Groth *et al.* (1997).

‡ Data from Stackebrandt *et al.* (1995).

and suborder, with the exception of an A at position 1251 of the 16S rRNA gene sequence instead of a G. The type and only species of the genus is *Kineosphaera limosa*.

Description of *Kineosphaera limosa* sp. nov.

Kineosphaera limosa (li.mo'sa. L. adj. *limosa* muddy, pertaining to sludge, the natural habitat of the species).

Strain Lpha5^T is Neisser-stain negative and polyhydroxybutyrate-stain positive. Colonies are light-yellow, irregular and umbonate (2–3 µm in diameter) with undulating margins when the strain is grown on GM1 medium. The optimum growth temperature is 30 °C (temperature growth range between 10 and 35 °C), and the optimum growth pH is 7.0 (pH growth range is between pH 6.0 and 10.2). Grows in the presence of up to 3.0% (w/v) NaCl. Strain Lpha5^T utilizes dextrin, D-arabitol, fructose, α-D-glucose, maltose, 3-O-methylglucose, psicose, trehalose, turanose, xylitol, adenosine, Tween 40, Tween 80, N-acetyl-D-mannosamine, D-mannose, methyl β-D-glucoside, palatinose, salicin, D-sorbitol, sucrose, D-trehalose, γ-hydroxybutyric acid, methylpyruvate, propionic acid, succinamic acid, succinic acid and glycerol. Cells accumulate PHA. The major cellular fatty acids of strain Lpha5^T are C_{16:0} (35%), C_{17:1} (19%), C_{18:1} (17%), C_{17:0} (15%), C_{16:1} (8%), C_{15:0} (5%) and C_{14:0} (1%). Phospholipids are phosphatidylglycerol, phosphatidylinositol, diphosphatidylglycerol, phosphatidylethanolamine and phosphatidylcholine. The G + C content of the genomic DNA is 71.3 mol %. The type strain of *Kineosphaera limosa* is Lpha5^T (= JCM 11399^T = DSM 14548^T).

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