Micropruina glycogenica gen. nov., sp. nov., a new Gram-positive glycogen-accumulating bacterium isolated from activated sludge

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A new Gram-positive non-spore-forming bacterium, strain Lg2[T], was isolated from an activated sludge reactor showing enhanced biological phosphorus removal activity. The new isolate was a slowly growing organism and was capable of accumulating large amounts of intracellular glycogen from substrate taken up. Both oxidase and catalase were produced. The new isolate contained *meso*-diaminopimelic acid (DAP) in the cell wall. Complex fatty acid patterns with iso-$C_{14:0}$, anteiso-$C_{15:0}$, $C_{16:0}$, iso-$C_{16:0}$ and four other minor saturated or unsaturated straight-chain fatty acids were detected. The isolate contained a high genomic G+C content (70·5 mol%). Phylogenetic analysis based on the 16S rRNA gene sequence placed the isolate in the high G+C Gram-positive group with *Microlunatus phosphovorus* and *Friedmanniella antarctica* as the closest relatives (sequence similarities are 93 and 92%, respectively). These three organisms shared common features in morphology, but strain Lg2[T] could be differentiated from the other species by its peptidoglycan type (*meso*-DAP), fatty acid composition, carbon source utilization profile and G+C content. On the basis of these findings, it is proposed that a new genus and species, *Micropruina glycogenica*, should be created for the new isolate; the type strain is strain Lg2[T] (= JCM 10248[T]).

Keywords: *Micropruina glycogenica* gen. nov., sp. nov., high G+C content group, Gram-positive bacteria, glycogen accumulation

INTRODUCTION

Activated sludge processes employ concentrated mixed microbial biomass (2–4 g l^{-1}) to eliminate organic materials from industrial wastewater and sewage before disposal. Recently, application of these processes has been further expanded to remove inorganic nutrients from wastewater. One good example is the enhanced biological phosphorus removal (EBPR) process. EBPR is established by the dominant growth of polyphosphate-accumulating bacteria in the activated sludge process utilizing anaerobic conditions with substrate addition and subsequent aerobic conditions. However, the EBPR process is difficult to control, and

sometimes fails in phosphorus removal performance. For stable operation, a better understanding of the micro-organisms in the activated sludge of the EBPR process is required. However, little is known about the microbiology of the EBPR process, and one of the reasons is the inability to isolate representative micro-organisms.

Acinetobacter spp. are the most common isolates from this process (Fuhs & Chen, 1975; Deinema *et al.*, 1980; Cloete *et al.*, 1985; Groenestijn *et al.*, 1989; Streichen *et al.*, 1990; Lötter & Pitman, 1992; Beacham *et al.*, 1992), but the community analyses using molecular techniques do not support their predominance in the EBPR process (Cloete & Steyn, 1987; Wagner *et al.*, 1993, 1994). Other known bacterial isolates include *Pseudomonas* sp. (Suresh *et al.*, 1985), *Arthrobacter globiformis* (Shoda *et al.*, 1980) and *Lampropedia* spp. (Stante *et al.*, 1997). Nevertheless, these isolates could not store substrate (i.e. cellular

glycogen and polyhydroxyalkanoate) and accumulate polyphosphate; although these abilities are observed in the EBPR process (Ohtake *et al.*, 1985; Groenestijn *et al.*, 1989). At present, only one bacterium, *Microlunatus phosphovorus*, isolated from the EBPR process has been demonstrated to accumulate intracellular polyphosphate and store substrate as a carbon reserve (Nakamura *et al.*, 1991, 1995a, b). Many researchers have suggested that, in contrast to the pure cultures used in the aforementioned studies, the microbial community of the EBPR process is composed of diverse micro-organisms at different phylogenetic positions (Wagner *et al.*, 1994; Bond *et al.*, 1995). To solve this microbial ecology puzzle, we have focused on isolating and identifying representative bacteria from EBPR processes.

A new bacterium capable of accumulating intracellular glycogen was isolated from an EBPR process. The organism, designated Lg2^T, was morphologically similar to *Microlunatus phosphovorus*, but exhibited no polyphosphate-accumulation ability. In this paper, we describe the phenotypic and phylogenetic characteristics of the new isolate, and propose to create a new genus and species name, *Micropruina glycogenica*, for the isolate.

METHODS

Bacterial strain and culture conditions. Strain Lg2^T was previously isolated from an anaerobic–aerobic sequential batch reactor exhibiting EBPR activity (Liu *et al.*, 1997). A previously described NM-1 agar medium (Nakamura *et al.*, 1995b) contained (l^{-1}) 0·5 g glucose, 0·5 g peptone, 0·5 g monosodium glutamate, 0·5 g yeast extract, 0·44 g KH$_2$PO$_4$, 0·1 g (NH$_4$)$_2$SO$_4$, 0·1 g MgSO$_4$.7H$_2$O and 15 g agar (final pH was 7·0). Liquid cultures were established by using the same medium without agar, and the cultures were incubated at 30 °C with a gentle shaking. For estimation of the optimal temperature and pH range for growth, the isolate was aerobically cultured under the following conditions: 5, 9, 16, 20, 23, 26, 30, 33, 35, 37 and 40 °C, pH 4, 5, 6, 7, 8, 9 and 10. The growth rate of the isolate was determined by measuring the OD$_{600}$ with a spectrophotometer (Beckman DU 640).

Morphological characteristics. Gram and Neisser staining were performed as described by Magee *et al.* (1975) and Jenkins *et al.* (1993), respectively. Morphology was examined by phase-contrast microscopy and transmission electron microscopy. For transmission electron microscopy, a centrifuged cell pellet was fixed with 5% (v/v) glutaraldehyde and 1% (v/v) osmium tetroxide. Ultrathin sections of the sample embedded in epoxy resin (Kushida, 1980) were prepared with a Reichert ultramicrotome. Samples were stained with uranyl acetate and lead citrate, and examined using a Hitachi H-7000 transmission electron microscope.

Physiological and biochemical characterization. Oxidase activity was determined by monitoring the oxidation of tetramethyl-*p*-phenylenediamine on a filter paper (Smibert & Krieg, 1981). Catalase activity was determined by adding cells to a 3% hydrogen peroxide solution (Smibert & Krieg, 1981). For the determination of nitrate reduction, strain Lg2^T was grown in a medium containing 1·0 g NaNO$_3$ l^{-1} under anaerobic conditions. The anaerobic conditions were established by replacing the gas phase in a butyl rubber stopper sealed test tube with N$_2$. Denitrification was determined by detecting the production of nitrogen gas during anaerobic growth on nitrate. The organic substrate utilization activities under aerobic conditions were determined using the BIOLOG system.

Quinone, fatty acid and cell wall analyses. Quinones were extracted from freeze-dried cells with chloroform/methanol (2:1, v/v) and n-hexane. The extract was purified by Sep-Pak Plus (Waters) and analysed by reverse-phase HPLC for identification (Tamaoka *et al.*, 1983). Whole-cell fatty acids were converted to methyl esters by treatment with anhydrous methanolic HCl. Methyl esters were extracted with n-hexane (Komagata & Suzuki, 1987), and analysed by using a gas chromatograph equipped with a flame-ionization detector (model HP5890II; Hewlett Packard). GC-MS analysis was performed with a JEOL SX102A mass selective detector directly connected to a gas chromatograph. The presence of diaminopimelic acid (DAP) isomers in the cell wall peptidoglycan was determined by TLC (Merck; no. 5716) after hydrolysis with 6 M HCl at 100 °C for 18 h (Komagata & Suzuki, 1987).

Glycogen accumulation measurement. Strain Lg2^T was cultured in a 500 ml flask containing 200 ml NM-1 medium up to 8 d under aerobic conditions at 30 °C. At a 1 d interval, cells were harvested, rinsed with 0·85% NaCl (w/v), and dried overnight. Before the determination of glycogen content, the dried cells (100 mg) were washed with ethanol and hydrolysed using 4 M HCl for 6 h at 100 °C (Elson & Morgan, 1993; Lott & Turner, 1975). The glycogen content of cells was measured with a Glucose CII test kit (Wako Pure Chemical Industries) according to the manufacturer's protocol.

DNA base composition. Total DNA of strain Lg2^T was extracted according to the method described previously (Kamagata & Mikami, 1991). The extracted total DNA was digested with P1 nuclease using a Yamasa GC kit (Yamasa Shoyu). The G+C content of the total DNA was measured by HPLC (Shimadzu model LC-6A system) equipped with a CLC-ODS column (6 by 150 mm; Shimadzu).

16S rRNA gene sequence and phylogenetic analysis. The 16S rRNA gene (rDNA) fragment of strain Lg2^T was amplified by PCR (Hiraishi, 1992; Hiraishi *et al.*, 1994) and sequenced directly with a dRhodamine dye terminator cycle sequencing kit (Applied Biosystems) and a model ABI 377 DNA sequencer. The 16S rDNA sequence obtained and reference sequences were aligned by using the CLUSTAL W program, version 1.5 (Thompson *et al.*, 1994). Phylogenetic trees were constructed from the evolutionary distance matrix calculated by the neighbour-joining method (Saitou & Nei, 1987) with Kimura's two-parameter correction. The neighbour-joining analysis was performed with the MEGA program (Kumar *et al.*, 1993). All gaps in the alignment were excluded; 970 bases were compared.

Nucleotide sequence accession numbers. The accession numbers of the reference sequences used in the phylogenetic analysis are as follows: *Aeromicrobium erythreum*, AF005021; *Bacillus subtilis*, X60646; *Corynebacterium xerosis*, X81914; *Friedmanniella antarctica*, Z78206; *Luteococcus japonicus*, D21245; *Microlunatus phosphovorus*, D26169; *Nocardioides albus*, X53211; *Nocardioides jensenii*, Z78210; *Nocardioides plantarum*, Z78211; *Nocardioides simplex*, Z78212; *Propionibacterium freudenreichii*, X53217; and *Propionibacterium thoenii*, X53220. The sequence of *Propioniferax innocua* was directly taken from the report by Pitcher & Collins (1991).

RESULTS

Isolation and growth conditions

Strain Lg2^T was previously isolated from a sequential batch reactor for EBPR (Liu *et al.*, 1997), and was extensively characterized in this study. When grown on the NM-1 agar medium plate, the strain required 2–3 weeks to develop a white, punctate colony. Growth was very slow in the liquid medium under aerobic conditions. The doubling time was approximately 12·6 h at 30 °C. The temperature range for growth was 20–35 °C, and the optimal growth temperature was 30 °C. Growth was not observed below 16 °C or above 37 °C. The pH range for growth was between 6 and 8 with the optimum at pH 7·0. The strain could grow in the presence of NaCl up to 3·0 % (w/v).

Morphological characteristics

Cells of strain Lg2^T were Gram-positive, non-motile cocci, occurring in pairs, packets or clusters. The size of each cell varied from 0·5 to 2·2 μm in diameter (Fig. 1). No spore formation was observed. Electron microscopic observations of ultrathin sections (Fig. 2) showed that the individual cells of the strain were approximately 1 μm wide. Cells possessed the typical cell wall structure of a Gram-positive bacterium. A septum was often observed in the middle of each growing cell. Capsules were present around the cells. No intracytoplasmic membrane was observed.

Glycogen-accumulating activity

Strain Lg2^T was able to take up and convert glucose into cellular carbohydrate (i.e. glycogen) under either aerobic or anaerobic conditions (Liu, 1995). Fig. 3 indicates the cellular glycogen profile of strain Lg2^T during cultivation in NM-1 medium under aerobic conditions. The cellular glycogen content increased
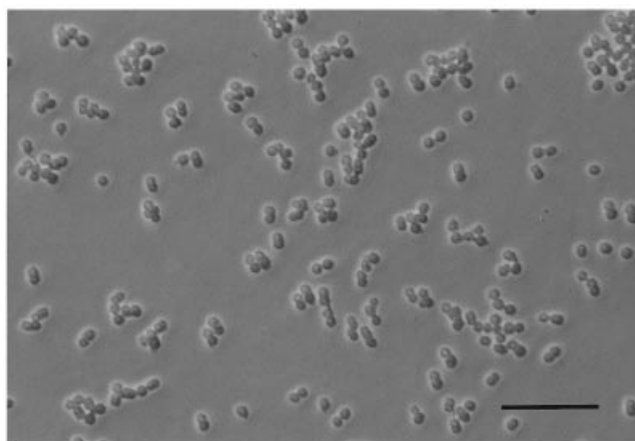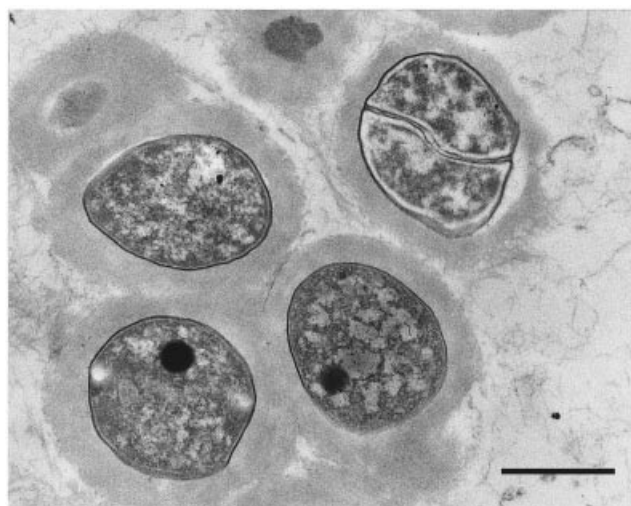
Fig. 2. Transmission electron micrograph of strain Lg2^T cells showing capsules outside the cells. Cells were grown in NM-1 medium at 30 °C under aerobic conditions. Bar, 1 μm.

significantly in the exponential phase, and gradually decreased in the stationary phase. The highest cellular glycogen content that we have ever detected was 8·4 % based on dry cell weight.
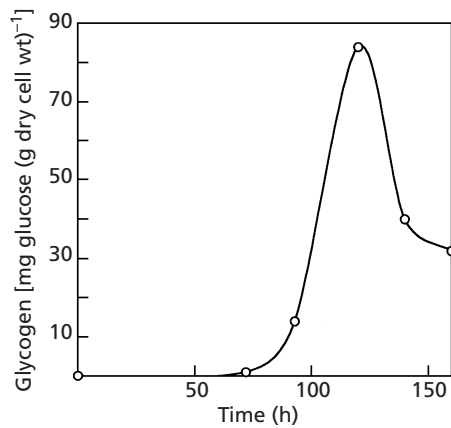
Physiological and biochemical characteristics

Strain Lg2^T could utilize arabinose, cellobiose, galactose, glucose, lactose, maltose, mannose, melibiose, saccharose, xylose, inositol, mannitol, acetate, propionate, pyruvate, succinate, alanine, arginine and histidine as carbon sources (all tests were performed at 30 °C under aerobic conditions). The isolate could also hydrolyse starch and glycogen. However, the isolate did not utilize trehalose, dulcitol, ethanol, glycerol, methanol, propanol, malate, asparagine, glutamate and glutamine as growth substrates.

Table 1 summarizes the major biochemical characteristics of strain Lg2^T. Catalase and oxidase activities of strain Lg2^T were found to be positive. Strain Lg2^T could utilize nitrate as an electron acceptor, but could not further reduce nitrite to nitrogen gas under anaerobic conditions. The isolate was judged to exhibit no orthophosphate uptake activity or accumulation of intracellular polyphosphate, because no cells were stained positive with Neisser staining.

Chemotaxonomic characteristics

The GC of methylated cellular fatty acid analysis indicates that strain Lg2^T contained anteiso-C$_{15:0}$ (37·1 %), iso-C$_{14:0}$ (14·1 %), C$_{16:0}$ (12·9 %) and iso-C$_{16:0}$ (10·8 %). In addition, substantial amounts of C$_{15:0}$ (5·9 %), C$_{18:0}$ (4·6 %), C$_{14:0}$ (3·7 %) and iso-C$_{15:0}$ (2·2 %) were also detected. Analysis of the DAP isomer by TLC revealed that *meso*-DAP was

Fig. 1. Phase-contrast photomicrograph of strain Lg2^T cells aerobically grown in NM-1 medium at 30 °C. Bar, 10 μm.

Fig. 3. Profile of glycogen uptake by strain Lg2^T under aerobic conditions. Cells were grown in NM-1 medium. Glycogen was extracted from dry cells. After extraction and hydrolysis, the amount of glucose was determined by enzyme assay.

contained in the cell wall peptidoglycan of the isolate. The dominant respiratory quinone of strain Lg2^T was menaquinone-9(H$_4$). The G+C content of the genomic DNA of strain Lg2^T was 70·5 mol%.

Phylogenetic analysis based on the 16S rRNA gene sequence

Almost the full length sequence (1438 bp) of the 16S rRNA gene of strain Lg2^T was determined. A phylogenetic tree (Fig. 4) was constructed by the neighbour-joining method (Kimura, 1980; Saitou & Nei, 1987). The sequence of *B. subtilis*, which belongs to the low G+C Gram-positive bacteria, was used to root the tree. Strain Lg2^T formed a cluster with the genera *Microlunatus*, *Friedmanniella*, *Nocardioides* and *Aeromicrobium* in the high G+C group of Gram-positive bacteria. The isolate was closely related to *Microlunatus phosphovorus* (93% sequence similarity) and *F. antarctica* (92%).

DISCUSSION

In the past few decades, the microbiological aspect of EBPR or activated sludge processes has been extensively studied to improve the process operation (Bond *et al.*, 1995; Cloete *et al.*, 1985; Cloete & Steyn, 1987; Nakamura *et al.*, 1991; Streichen *et al.*, 1990; Suresh *et al.*, 1985; Wagner *et al.*, 1993, 1994; Yoshimi *et al.*, 1996). One approach to understanding the biochemical mechanism was the isolation of representative bacteria enriched in the process. In our previous studies, we successfully isolated two bacterial strains with unique chemotaxonomic and metabolic characteristics: these included a polyphosphate-accumulating bacterium, *Microlunatus phosphovorus* (Nakamura *et al.*, 1995b), and a polysaccharide-accumulating bacterium, *Microsphaera multipartita* (Yoshimi *et al.*, 1996). Here, we have extended our study to a Gram-positive, non-spore-forming, and non-motile coccus, strain Lg2^T, which was isolated from a reactor with good biological phosphorus removal activity (Liu *et al.*, 1997). Interestingly, this strain was not able to accumulate intracellular polyphosphate or take up

Table 1. Phenotypic characteristics of strain Lg2^T and related species in the high G+C content Gram-positive group

Data were taken from the following references: *Microlunatus phosphovorus*, Nakamura *et al.* (1995b); *F. antarctica*, Schumann *et al.* (1997). +, Positive; −, negative.

Characteristic	Lg2^T	*Microlunatus phosphovorus*	*Friedmanniella antarctica*
Cell shape	Cocci (single, pair or packet)	Cocci (single or pair)	Cocci
Cell size (diameter)	0·5–2·2 μm	0·8–2·0 μm	1·8–2·5 μm
Gram staining	+	+	+
Motility	−	−	−
Spore formation	−	−	−
Colour of colony	White	Cream	Orange
Polyphosphate granules	−	+	−
Oxidase	+	+	−
Catalase	+	+	+
Nitrate reduction	+	+	−
Denitrification	−	−	−
Acid production from glucose	+	+	−
DNA G+C content	70·5 mol%	67·9 mol%	73·0 mol%
Major quinone	Menaquinone-9(H$_4$)	Menaquinone-9(H$_4$)	Menaquinone-9(H$_4$)
Cellular fatty acids	iso-C$_{14:0}$, anteiso-C$_{15:0}$, C$_{16:0}$	anteiso-C$_{15:0}$, iso-C$_{15:0}$, C$_{16:0}$	anteiso-C$_{15:0}$, iso-C$_{15:0}$
Peptidoglycan	*meso*-DAP	LL-DAP	LL-DAP
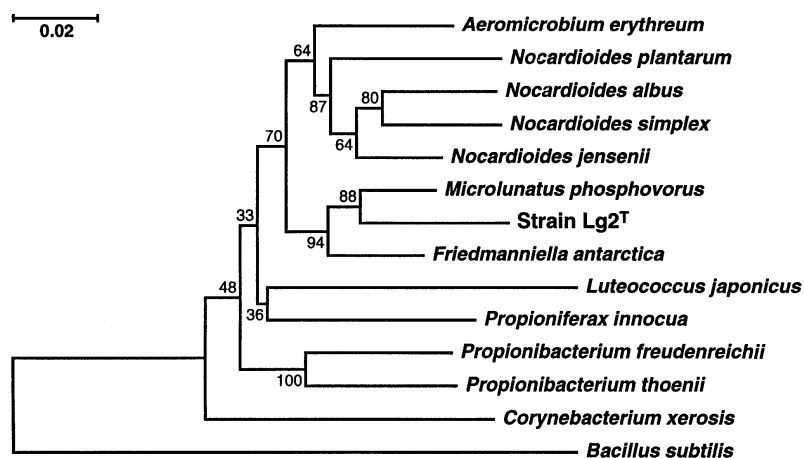
Fig. 4. Phylogenetic tree showing the relationship between strain Lg2^T and related species in the high G+C content group of Gram-positive bacteria. The tree was constructed from the evolutionary distance matrix by the neighbour-joining method (Saitou & Nei, 1987). Bar, 2 nucleotide substitutions per 100 nucleotides in 16S rDNA sequences. The sequence of *B. subtilis* (belonging to the low G+C content Gram-positive bacteria) was used to root the tree. Bootstrap probabilities (Felsenstein, 1985) are indicated at the branch points.

orthophosphate under either anaerobic or aerobic conditions. Instead, like *Microsphaera multipartita*, it could accumulate a large amount of carbon substrates as cellular glycogen.

A phylogenetic analysis based on the 16S rRNA gene sequence revealed that strain Lg2^T is associated with the high G+C content group of the Gram-positive bacteria. It was closely related to *Microlunatus phosphovorus* (Nakamura *et al.*, 1995a) and *F. antarctica* (Schumann *et al.*, 1997). The phenotypic characteristics summarized in Table 1 further indicate that strain Lg2^T, *Microlunatus phosphovorus* and *F. antarctica* share several common features. They are all non-spore-forming, non-motile cocci, contain anteiso-$C_{15:0}$ as the major fatty acid component, contain menaquinone-9(H$_4$) as the major quinone, are catalase-positive, and do not possess denitrification capability. However, the similarities of the 16S rRNA gene sequence between the isolate and these two authentic species were 92–93 %, which are low enough to place the isolate in a new and distinct taxon.

Strain Lg2^T was also distinguishable from both *Microlunatus phosphovorus* and *F. antarctica* in the following phenotypic aspects: (1) the isolate contains *meso*-DAP in the cell wall peptidoglycan, while the two relatives have a different peptidoglycan type (LL-DAP); (2) iso-$C_{14:0}$ is one of the major fatty acid components in the isolate (14·1 % of total fatty acids), while it is detected in the other relatives as a trace amount (<4 %); (3) strain Lg2^T has a genomic G+C content of 70·6 mol%, and this value is different from those of *Microlunatus phosphovorus* (67·9 %) and *F. antarctica* (73 %); (4) the isolate produces no pigment and its colonies are white, while colonies of the other two are cream or orange.

Furthermore, the substrate utilization profiles of strain Lg2^T and the two related species were different. *Microlunatus phosphovorus* can utilize trehalose, dulcitol, asparagine, glutamate and glutamine, while strain Lg2^T can not. On the other hand, strain Lg2^T can utilize lactose, glycogen, propionate and succinate,

while *Microlunatus phosphovorus* can not (Nakamura *et al.*, 1995b). In contrast with strain Lg2^T and *Microlunatus phosphovorus*, which can use a wide range of organic compounds as energy sources, *F. antarctica* has a very limited carbon utilization ability (Schumann *et al.*, 1997).

In addition, *F. antarctica* was rather psychrophilic (the optimum growth temperature is 22 °C), whereas the isolate is a mesophilic bacterium with the optimum at 30 °C. *Microlunatus phosphovorus* can accumulate cellular polyphosphate, and can take up and store several different sugars as polyglucose under both anaerobic and aerobic conditions (Nakamura *et al.*, 1995a, b). Strain Lg2^T can store substrates as intracellular glycogen but can not accumulate polyphosphate.

On the basis of the results of the phenotypic and genotypic comparisons, we propose that a new genus and species, *Micropruina glycogenica* gen. nov., sp. nov., should be established for strain Lg2^T described in this study.

Description of *Micropruina* gen. nov.

Micropruina (Mi.cro.prui′na. Gr. adj. *micros* small, fine; M. L. fem. n. *pruina* hoarfrost; M. L. fem. n. *Micropruina* fine hoarfrost).

Cells are Gram-positive, non-motile, cocci (diameter 0·5–2·2 μm), and usually occur in pairs, packets or clusters. Spore formation is not observed. Capsules are formed. Colonies and liquid cultures are white in colour. Neisser stain is negative. An aerobic chemoorganotroph. Catalase and oxidase are produced. The cell wall peptidoglycan contains *meso*-DAP. The major cellular fatty acids are iso-$C_{14:0}$, anteiso-$C_{15:0}$, $C_{16:0}$ and iso-$C_{16:0}$. The cells contain menaquinone-9(H$_4$) as the major quinone. The phylogenetic position is in the high G+C Gram-positive bacteria; the genera *Microlunatus* and *Friedmanniella* are closely related to this genus. The G+C content of the genomic DNA is 70·5 mol%. The type species is *Micropruina glycogenica*.

Description of *Micropruina glycogenica* sp. nov.

Micropruina glycogenica (gly.co.gen.i′ca. Gr. suff. -icus related to, belonging to; N. L. adj. *glycogenica* referring to the ability to accumulate glycogen).

The morphology and chemotaxonomic features of the species are the same as those described above for the genus.

The species can store substrates as intracellular glycogen. Growth occurs at 20–35 °C and at pH 6–8. The optimal growth temperature and pH are 30 °C and 7·0, respectively. A slow growing organism. The species tolerates up to 3·0 % NaCl in the medium. Cells reduce nitrate to nitrite under anaerobic conditions, but do not reduce nitrite to nitrogen. Cells grow on glucose, mannose, galactose, xylose, arabinose, sucrose, maltose, lactose, cellobiose and melibiose, but not trehalose. Starch and glycogen are utilized. Cells utilize sugar alcohols, including inositol and mannitol, but not dulcitol. Acetate, propionate, pyruvate and succinate are utilized, but not malate. On the basis of the phylogenetic comparison of 16S rRNA sequences, the bacterium belongs to the high $G+C$ Gram-positive bacteria. The closest relative is *Microlunatus phosphovorus* (93 % sequence similarity). The $G+C$ content of the genomic DNA is 70·5 mol %. The type strain is Lg2^T, which has been deposited in the Japan Collection of Microorganisms (RIKEN) as JCM 10248^T.

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