Lysinibacillus varians sp. nov., an endospore-forming bacterium with a filament-to-rods cell cycle

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Six Gram-stain-positive, motile, filamentous and/or rod-shaped, spherical spore-forming bacteria (strains GY32T, L31, F01, F03, F06 and F07) showing polybrominated diphenyl ether transformation were investigated to determine their taxonomic status. After spore germination, these organisms could grow more than one hundred microns long as intact single cells and then divide into rod cells and form endospores in 33 h. The cell-wall peptidoglycan of these strains was type A43, the predominant menaquinone was MK-7 and the major fatty acids were iso-C16:0, iso-C15:0 and C16:1ω7C. Diphosphatidylglycerol, phosphatidylglycerol and phosphatidylethanolamine were detected in the polar lipid profile. Analysis of the 16S rRNA gene sequences indicated that these strains should be placed in the genus Lysinibacillus and they were most closely related to Lysinibacillus sphaericus DSM 28T (99% 16S rRNA gene sequence similarity). The gyrB sequence similarity and DNA–DNA relatedness between strain GY32T and L. sphaericus JCM 2502T were 81% and 52%, respectively. The G+C content of the genomic DNA of strain GY32T was 43.2 mol%. In addition, strain GY32T showed differences in nitrate reduction, starch and gelatin hydrolysis, carbon resource utilization and cell morphology. The phylogenetic distance from its closest relative measured by DNA–DNA relatedness and DNA G+C content, and its phenotypic properties demonstrated that strain GY32T represents a novel species of the genus Lysinibacillus, for which the name Lysinibacillus varians sp. nov. is proposed. The type strain is GY32T (=NBRC 109424T =CGMCC 1.12213T =CCTCC M 2011307T).

The genus Lysinibacillus is typically characterized by rod-shaped cells with an A43 cell-wall peptidoglycan type. The dominant respiratory lipoquinone system is MK-7, and major polar lipids are diphosphatidylglycerol, phosphatidylglycerol and ninyhdrin-positive phosphoglycolipid. At the time of writing, the genus contains the following species with validly published names: Lysinibacillus boronitolerans, L. fusiformis, L. sphaericus (Ahmed et al., 2007a), L. parviciboniciens (Miwa et al., 2009), L. xylanilyticus (Lee et al., 2010), L. macroides (Coorevits et al., 2012), L. mangiferahumi (Yang et al., 2012), L. sinduriensis, L. masiliensis, L. odyssyei (Jung et al., 2012), L. tabacioli (Duan et al., 2013) and L. chungkukjangi (Kim et al., 2013). During the selective enrichment experiments of deca-brominated diphenyl ether (BDE-209) biotransformation bacteria from electronic-waste-contaminated river sediment, six Lysinibacillus strains were obtained. They showed a filament-to-rods cell cycle in either enrichment medium or LB medium. The cell cycle of these novel isolates is different from those of other known species of the genus Lysinibacillus. The phenotypic and genetic properties were investigated to understand the interesting cell morphology and the phylogenetic characteristics of these isolates.

For screening of BDE-209 biodegrading bacteria, sediment samples were collected from an electronic-waste-recycling site in Guiyu, Guangdong, China. Sediment samples (20 g) were added to 80 ml sterilized H2O, sediment granule and microbes were separated by gradient centrifugation at 2000 g and 12 000 g. To enrich the BDE-209 biotransformation bacteria, the microbes were inoculated into a medium containing 20 mM formate, 20 mM lactate,
40 mM succinate, 5.7 mM Na₂HPO₄, 3.3 mM KH₂PO₄, 18 mM NH₄Cl, 0.1 % (w/v) yeast extract and a trace element solution and vitamins (Wolin et al., 1963). BDE-209 (1 μM) dissolved in dichloromethane was added to the culture bottle and evaporated in darkness before adding the medium above. Enrichment cultures were transferred into fresh media every month and the concentration of bromine ions in the systems before and after incubation were measured by ion chromatography (ICS-1500; Dionex). Six months later, the enriched populations were serially diluted and plated onto Luria–Bertani (LB) agar plates for single-colony isolation. Colonies displaying different morphologies were selected and tested for BDE-209 debroration ability after three purifications. Six strains (L31, GY32T, F01, F03, F06 and F07), producing more than 55.5 μg bromine ion l⁻¹ after incubation for 1 month in the medium containing 1 μM BDE-209, were selected and maintained on LB slopes at 4 °C or stored at −20 °C in LB supplemented with 30 % (v/v) glycerol for further studies. These strains displayed colonies that were yellowish, circular with jagged margins, opaque on LB plates and had glossy surfaces at 30 °C after 24 h of incubation.

A light microscope (M165C; Leica), transmission electron microscope (H-7650; Hitachi) and scanning electron microscope (S-3000N; Hitachi) were employed for cell morphological observations. The Invitrogen LIVE/DEAD Baclight Bacterial Viability kit L7007 was used for nucleic acid staining according to the manufacturer’s protocols (Zeiss) and fluorescence microscopy (DMRA2; Leica). Cell length was measured with a Leica Qwin image processing and analysis application. All six strains were Gram-stain-positive, motile and spore-forming. Interestingly, both filamentous (10.0–466.1 μm, length × diameter) and long rod-shaped (5.0–10.0 × 0.8–1.3 μm, length × diameter) cells were observed in the field of vision (Fig. 1). The filamentous cell was not observed in Lysinibacillus sphaericus JCM 2502T (Movie S1, available in the online Supplementary Material). Strain GY32T was used as a representative to observe cell morphology characteristics in detail.

Time-lapse microscopy was used to monitor spore germination, single cell elongation and division, cell growth and spore formation. For sporulation, 1-week cultures were heat treated at 105 °C for an hour and stored at −4 °C. The purity of the spores was evaluated by phase-contrast microscopy and all preparations were free of intact living vegetative cells. Spore germination experiments were performed according to the method described by Hamoen & Errington (2003), modified as follows. Spores were heat shocked for 30 min at 30 °C and quickly cooled on ice. They were subsequently diluted to 1/2500 and inoculated into semi-solid LB medium. The inoculated medium was added to a cell culture dish (35 × 12 mm, 15 mm diameter, glass bottom; NEST Biotechnology) before medium gelling. After pre-incubation at 30 °C for 2 h, the culture was photographed under a laser scanning microscope, by which images were taken every 20 s for time-lapse experiments. All photomicrographs were saved with a scale rule. Cell length was measured with Leica Qwin and calculated as net size by scaleplate. Time lapse between consecutive images and cell length measurements were combined for the construction of a length–time diagram. These results showed that after a spore was pre-incubated at 30 °C for 2 h, the lifecycle from a spore germination to next-generation spore-forming, which required approximately 33 h, could be divided into seven major steps: (i) a spore expands and germinates in the first 4.5 h; (ii) the bud emerges from the spore and becomes thicker and longer in the next 2.0 h and then the rod-shaped bud releases from the cortex and crust of the spore; (iii) the rod cell elongates to form a filament in the next 1.5 h (Movie S2); (iv) the filamentous cell reproduces with a high growth rate and motile rod cells are observed after a further 3.0 h; (v) in another 5 h, filamentous cells and rod cells co-exist in the medium – some filaments which could not divide immediately undergo autolysis; (vi) rod cells become dominant in the following 12 h (Movie S3); and (vii) new endospores subsequently form after a further 5 h (Fig. 2). These results allow us to form a conceptual model for the lifecycle of strain GY32T (Fig. 3).

The physiological and biochemical characteristics of all isolates (GY32T, L31, F01, F03, F06 and F07) were determined along with those of L. sphaericus JCM 2502T. Growth at various temperatures (10–50 °C with 5 °C intervals), NaCl concentrations (0, 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, 6.0 and 7.0 %, w/v) and pH values (pH 4.0–10.0) was measured in the LB liquid medium. Optimal growth was
observed at 35 °C and pH 7.0 in the LB medium without NaCl. Use of carbon sources was determined by using the Biolog GP2 characterization system according to the manufacturer’s protocol. Oxidase and catalase activity, nitrate reduction and hydrolysis of starch and gelatin, were assessed as previously described (Dong & Cai, 2001). Except for the difference of delayed positive oxidase activity of strains L31 and F01, all novel isolates gave identical results in these tests (Tables S1 and S2). Such characteristics were compared in strain GY32T and in other species of the genus Lysinibacillus (Table 1); the results of the nitrate reduction test and hydrolysis of gelatin and starch revealed that strain GY32T was different from its relatives.

Fatty acid methyl esters of 24 h cultures were extracted and prepared by the method described by Xu et al. (2005). Extracts were analysed using a Hewlett Packard model HP6890 GC equipped with a flame-ionization detector (HP CHEMSTATION version A 5.01) and an Ultra-2 column (0.2 mm internal diameter; 25 m long). The major fatty acids of strain GY32T were iso-C16:0, iso-C15:0 and C16:1ω7c. Strain GY32T had larger relative amounts of iso-C16:0 and iso-C14:0 than other species of the genus Lysinibacillus.

For peptidoglycan analysis, strain GY32T and L. sphaericus JCM 2502T were grown in shaking flasks containing liquid

Fig. 2. Spore germination and filament formation observed by laser scanning microscopy. (a) Spore swelling; (b) spore germination and outgrowth; (c) vegetative cell release from cortex and crust of the spore; (d) cell elongation; (e) cell division; (f) endospore formation. Bars, 2 μm (a) and 5 μm (b–f).

Fig. 3. A conceptual model for the lifecycle of strain GY32T in LB culture at 30 °C. After spore germination, a rod cell outgrows and elongates into a filament. Immediately, the filament asymmetrically divides into long rod-shaped cells. Subsequently, rod cells become predominant and spores are formed in the medium. The lifecycle from spore germination to next-generation spore formation requires approximately 33 h. The length of rod cells are 5.0–10.0 μm and filaments are 10.0–466.1 μm. The diameter of cells maintained at 0.8–1.3 μm.
LB on a rotary shaker for 24 h at 30 °C. Quantitative analysis of cell-wall peptidoglycan showed that strain GY32T contained lysine and aspartic acid, representing the type A4α (Schleifer & Kandler, 1972). This result is a key marker used to discriminate the genus Lysinibacillus or the genus Kurthia from other members of Bacillus group 2 (Rheims et al., 1999; Lee et al., 2010).

Respiratory quinones were extracted and purified from 500 mg dried cells and were separated by HPLC and individually identified by MS (Nishijima et al., 1997). Polar lipids were extracted and purified from 200 mg dried cells using the protocol described by Minnikin et al. (1984) and were identified by two-dimensional TLC (Merck). The predominant menaquinone of strain GY32T was MK-7 (95.7 %) and the major polar lipids were diphosphatidylglycerol, phosphatidylglycerol and phosphatidylethanolamine. Ninhydrin-positive phosphoglycolipid was not observed in strain GY32T as in all other species of the genus Lysinibacillus.

The 16S rRNA gene was amplified by universal primers 27f and 1492r (Dunbar et al., 2001). The gyrB gene was amplified using primers UP-1 and UP-2r (Yamamoto & Harayama, 1995). The 16S rRNA gene sequence of strain GY32T, consisting of 1456 bp, was used to search the GenBank database. Sequence searches showed that strain GY32T was most closely related to L. sphaericus DSM 28T (99 % 16S rRNA gene sequence similarity) (Fig. 4). The gyrB sequence similarity between strain GY32T (GenBank accession no. JN860069) and its most closely related strain L. sphaericus JCM 2502T was 81 % (Fig. 5).

DNA G+C content of strain GY32T was determined by HPLC using a procedure previously described (Ahmed et al., 2007b). The DNA–DNA renaturation rate was determined using a Lambda 35 UV/VIS spectrometer equipped with a PTP-1 Peltier temperature programmer (PerkinElmer) and the experiment was carried out by the service of the Institute of Microbiology, Chinese Academy of Sciences. The DNA G+C content of strain GY32T was 43.2 mol% compared with 38.4 mol% for L. sphaericus JCM 2502T. The mean DNA–DNA hybridization (DDH) value between strain GY32T and L. sphaericus JCM 2502T was 52 ± 0.1 % (SD). A value of 70 % DDH was proposed by Wayne et al. (1987) as a recommended standard for delineating species. According to the equation described by

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<td>3.0–5.0 × 1.2–1.3</td>
<td>3.0–5.0 × 1.0–1.3</td>
<td>3.0–5.0 × 0.9–1.1</td>
<td>3.0–5.0 × 0.8–1.5</td>
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<td>pH range for growth</td>
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![Fig. 4. Phylogenetic tree of the genus Lysinibacillus based on 16S RNA gene sequences. The tree was reconstructed by using the neighbour-joining method. GenBank accession numbers are given in parentheses; the sequence determined in this study is in bold type. Bar, 0.01 substitutions per nucleotide position.](http://ijs.sgmjournals.org)
Goris et al. (2007), the 52% DDH corresponds to 89% average nucleotide identity of common genes and 46% conserved DNA between a pair of strains.

The A4x cell-wall peptidoglycan type and the 16S rRNA gene sequence analysis clearly placed strain GY32\textsuperscript{T} in the genus Lysinibacillus. However, strain GY32\textsuperscript{T} differs from all other previously described species of the genus Lysinibacillus in several aspects, such as DNA G+C content, gyrB sequence similarity, biochemical and physiological features, cell cycle and DNA–DNA relatedness. Based on these results, strains GY32\textsuperscript{T}, L31, F01, F03, F06 and F07 were assigned to a novel species of the genus Lysinibacillus, for which the name Lysinibacillus varians sp. nov. is proposed.

**Description of Lysinibacillus varians sp. nov.**

*Lysinibacillus varians* (va’r.i.ans. L. part. adj. *variare* varying, referring to the change of cell shape).

Cells are motile, Gram-stain-positive, filamentous (10.0–466.1 μm x 0.8–1.3 μm, length x diameter), and/or rod-shaped (5.0–10.0 μm x 0.8–1.3 μm, length x diameter), spore-forming with cell length changes during the growth cycle. Colonies are yellowish, circular with jagged margins, varying, referring to the change of cell shape). A4x (Lys–Asp) and menaquinone MK-7 is the predominant quinone. Phosphatidylethanolamine, diphosphatidylglycerol are the major polar lipids. The major fatty acids are iso-C\textsubscript{16:0}, iso-C\textsubscript{15:0} and C\textsubscript{16:1}ω7c. Oxidase, catalase and nitrate reduction tests are positive, whereas gelatin and starch hydrolysis and Voges–Proskauer tests are negative. The following compounds can be utilized as sole sources of carbon: mannann, L-arabinose, D-ribose, D-tagatose, D-xyllose, acetic acid, d-hydroxybutyric acid, D-ketovaleric acid, L-lactic acid, L-malic acid, L-asparagine, L-glutamic acid, D-serine, 2′-deoxyadenosine, propionic acid, thymidine and uridine. The following compounds are not utilized as sole sources of carbon: α-cyclodextrin, α-cyclodextrin, dextrin, glycogen, nulin, Tween 40, Tween 80, N-acetyl-D-galactosamine, N-acetyl-D-glucosamine, amygdalin, D-arabitol, arbutin, D-cellobiose, D-fructose, L-fucose, D-galactose, D-galacturonic acid, gentiobiose, D-gluconic acid, α-D-glucose, m-inositol, α-D-lactose, lactulose, maltose, maltotriose, D-mannitol, D-mannose, D-melezitose, D-melibiose, α-Methyl-D-Galactoside, β-methyl-D-galactoside, 3-methyl-D-glucose, α-methyl-D-glucoside, β-methyl-D-glucoside, α-methyl-D-mannoside, palatinose, D-psicose, D-raffinose, L-rhamnose, salicin, sedoheptulose, D-sorbitol, stachyose, sucrose, D-trehalose, turanose, xylitol, β-hydroxy butyric acid, γ-hydroxy butyric acid, p-hydroxy-phenylacetic acid, α-ketoglutaric acid, lactamic acid, D-lactic acid methyl ester, D-malic acid, pyruvic acid methyl ester, succinic acid mono-methyl ester, pyruvic acid acid, succinimonic acid, succinic acid, N-acetyl-l-lactamid glutamic acid, L-alanine, L-alanine, L-α-aminoglucine, glycyll-L-glutamic acid, l-pyroglutamic acid, putrescine, 2,3-butanediol, glycerol, adenosine, inosine, adenosine-5′-monophosphate, thymidine-5′-monophosphate, uridine-5′-monophosphate, d-fructose-6-phosphate, α-D-glucose-1-phosphate, D-glucose-6-phosphate, D-L-α-2-glucoronic acid.

The type strain is GY32\textsuperscript{T} (=NBRC 109424\textsuperscript{T}=CGMCC 1.12212\textsuperscript{T}=CCTCC M 2011307\textsuperscript{T}), isolated from an electronic-waste-contaminated river sediment from Guiyu, Guangdong, China. The DNA G+C content of the type strain is 43.2 mol%.

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**References**

**Lysinibacillus varians sp. nov.**


