Reclassification of *Pseudomonas* sp. PB-6250^T as *Lysobacter firmicutimachus* sp. nov.

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Strain PB-6250^T, isolated from soil in Japan, was first identified in 1992. In contrast to its original taxonomic classification, its 16S rRNA gene sequence showed the highest similarity (99.2 %) to the sequence of *Lysobacter enzymogenes* DSM 2043^T, with *Lysobacter antibioticus* DSM 2044^T being the next most closely related species (98.7 %) with a validly published name. Chemotaxonomic data (fatty acid profile, quinone and polar lipid composition) and the G+C content of strain PB-6250^T were compared with those of the closely related type strains *L. enzymogenes* LMG 8762^T, *L. antibioticus* LMG 8760^T, *L. capsici* DSM 19286^T and *L. gummosus* LMG 8763^T; this supported the affiliation of strain PB-6250^T to the genus *Lysobacter*. Phylogenetic analyses, DNA–DNA-hybridization data, biochemical and physiological characteristics strongly supported the genotypic and phenotypic differentiation of strain PB-6250^T from species of *Lysobacter* with validly published names. Strain PB-6250^T, therefore represents a novel species, for which the name *Lysobacter firmicutimachus* sp. nov. is proposed. The type strain is PB-6250^T (=LMG 28994^T=DSM 102073^T).

Strain PB-6250^T (=FERM BP-2938) was isolated from soil collected at Okinawa-Honto, Okinawa Prefecture, Japan; it has been recognized primarily due to its production of a suite of depsilipopeptide antibiotics, termed plusbacins A_1–A_4 and B_1–B_4 (Shoji *et al.*, 1992a, b). Based on phenotypic and morphologic investigations, it was recognized to be most closely related to *Pseudomonas paucimobilis* and consequently assigned to the genus *Pseudomonas* at the time of its discovery. As part of our ongoing efforts to study *Pseudomonas* lipopeptide antibiotics, we have recently genome-sequenced the producer strain and analyzed it for the presence of the plusbacin biosynthetic gene cluster. Furthermore, we investigated the secondary metabolite profile of strain PB-6250^T. Due to these chemical and genetic investigations, doubts were raised about the correct taxonomic classification of the strain. Therefore, we re-examined the taxonomic position of strain PB-6250^T using a polyphasic approach.

Strain PB-6250^T was obtained from the International Patent Organism Depositary (IPOD) of the National Institute of Technology and Evaluation (NITE), Japan, and grown on tryptic soy agar (TSA, BBL 11768), at 28 °C under aerobic conditions for further analytical procedures.

The genome of strain PB-6250^T was sequenced by a combination of Illumina and PacBio technology (details of the genome sequence will be published elsewhere). The 16S rRNA gene of PB-6250^T was extracted from its *de novo* genome sequence, employing the web-based bioinformatics tool, RNAmmer 1.2 (Lagesen *et al.*, 2007), and the resulting consensus sequence (1488 nucleotides) was compared to the GenBank database using the BLAST program located at the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov) (Altschul *et al.*, 1997; Benson *et al.*, 2004). This analysis identified strains of the genus *Lysobacter* as the closest relatives, and suggested that strain PB-6250^T was a member of this genus. The 16S rRNA gene sequence of strain PB-6250^T was, therefore, compared to 16S rRNA gene sequences of type strains of species of the

Abbreviations: DPG, diphosphatidylglycerol; FAME, Fatty acid methyl esters; PE, phosphatidylethanolamine; PG, Phosphatidylglycerol; PMME, Phosphatidylmonomethylethanolamine.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of *Lysobacter firmicutimachus*^{T} is KU593484.

One supplementary figure is available with the online Supplementary Material.

genus *Lysobacter* with validly published names, which were retrieved from the EMBL and NCBI databases. Phylogenetic trees were reconstructed with the software package BioNumerics 7.5 (Applied Maths) and MEGA 6.0 (Tamura *et al.*, 2013) using the neighbour-joining, maximum-likelihood and maximum-parsimony methods (Felsenstein, 1981; Saitou & Nei, 1987). All trees generally showed the same topology (Fig. 1). The phylogenetic position of strain PB-6250^T is in the genus *Lysobacter* with *Lysobacter enzymogenes* LMG 8762^T as the closest relative and this branch is supported by a high bootstrap value in all three treeing methods (Fig. 1). Using the BioNumerics 7.5 software package (Applied Maths), *Lysobacter enzymogenes* DSM 2043^T=LMG 8762^T (99.2 %), *Lysobacter antibioticus* DSM 2044^T=LMG 8760^T (98.7 %), *Lysobacter capsici* YC5194^T=DSM 19286^T (98.6 %) and *Lysobacter gummosus* KCTC 12132^T=LMG 8763^T (98.6 %) were found to be the closest relatives (values based on pairwise similarities). Although no precise correlation exists between 16S rRNA sequence divergence values and species delineation, divergence values of 1.3 % or more are generally recognized as significant (Stackebrandt & Ebers, 2006; Stackebrandt & Goebel, 1994).

Genomic DNA was isolated from strain PB-6250^T for determination of the DNA base composition by the method of Wilson (1987) with minor modifications (Cleenwerck *et al.*, 2002). The DNA was enzymatically degraded into nucleosides that were then separated using HPLC as described by Mesbah *et al.* (1989). The DNA G+C content (68.7 mol%) obtained was similar to the value reported in the literature (69.4 mol%; Shoji *et al.* 1992a) and is within the range reported for *Lysobacter* spp. (Christensen & Cook, 1978; Lee *et al.*, 2006; Wei *et al.*, 2012).

To confirm the novel species status of strain PB-6250^T, DNA–DNA hybridization experiments were carried out with DNA of this strain and of *L. enzymogenes* LMG 8762^T and *L. antibioticus* LMG 8760^T, the nearest phylogenetic neighbours. The DNA used for DNA–DNA-hybridizations was isolated using the same DNA isolation protocol as applied for the DNA G +C content analysis. DNA-DNA hybridizations were conducted at 50 °C in the presence of 50 % formamide, according to a modification (Cleenwerck *et al.*, 2002; Goris *et al.*, 1998) of the microplate method described by Ezaki *et al.* (1989). Reciprocal reactions were performed in quadruplicate and strain PB-6250^T exhibited 37±1 % and 30±2 % DNA–DNA relatedness to *L. antibioticus* LMG 8760^T and *L. enzymogenes* LMG 8762^T, respectively. As these values are well below the generally accepted limit for species delineation (70 %; Wayne *et al.*, 1987), these data confirm the novel species status of strain PB-6250^T.

Furthermore, chemotaxonomic studies were undertaken, in order to support classification of strain PB-6250^T into the genus *Lysobacter*. Respiratory quinone and polar lipid analyses were carried out by the Identification Service of DSMZ, Braunschweig, Germany. Cells were grown in R2A medium [0.5 g bacto-yeast extract, 0.5 g proteose peptone, 0.5 g bacto-casamino acids, 0.5 g bacto-dextrose, 0.5 g soluble starch, 0.3 g sodium-pyruvate, 0.3 g KH_2PO_4, 50 mg $MgSO_4.7H_2O$ (all l^{-1}) of demineralized water], washed with TE-buffer and freeze-dried.

For determination of the cellular fatty acid methyl esters (FAME) composition, cells were grown for 24 h at 28 °C under aerobic conditions on TSA medium (BBL 11768).

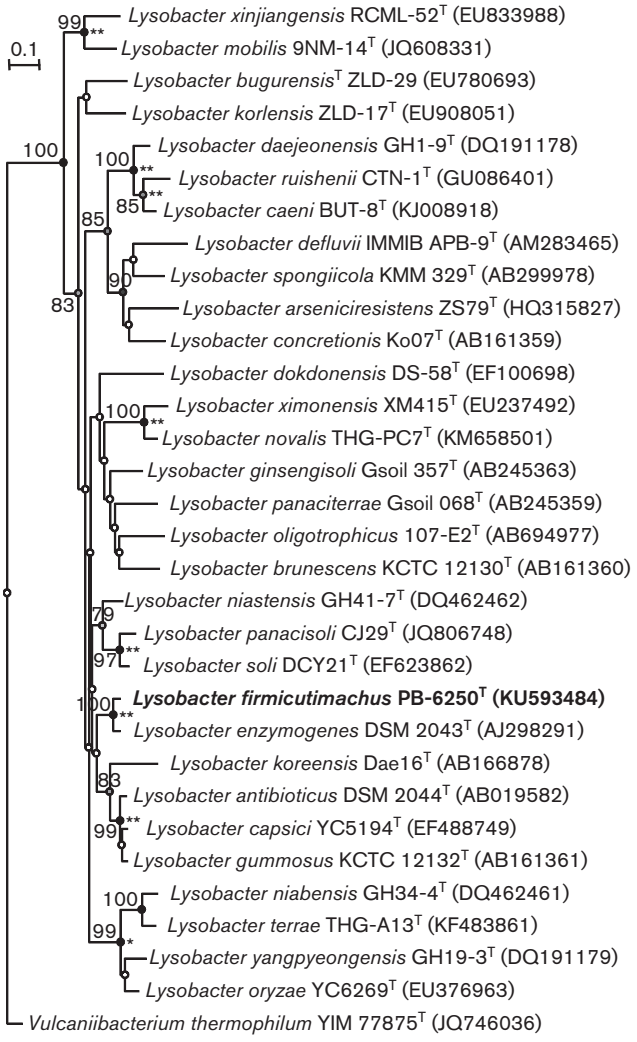

Fig. 1. Neighbour-joining tree, based on 16S rRNA gene sequence analysis, showing the position of *Lysobacter firmicutimachus* sp. nov. strain PB-6250^T and members of the genus *Lysobacter*. The GenBank accession numbers are shown in parentheses. Numbers at nodes indicate bootstrap percentages based on 1000 resampled datasets (filled circles indicate that for the corresponding node a bootstrap value of more than 75 % was found). Nodes marked with one asterisk were supported by one of the other applied treeing methods (maximum-likelihood or maximum-parsimony); two asterisks indicate that nodes were supported in trees calculated with both the other treeing methods. Bar, 1 nucleotide substitution per 10 nucleotides. *Vulcaniibacterium thermophilum* YIM 77875^T was used as an outgroup.

Inoculation and harvesting of the cells, and the extraction and analysis were performed according to the recommendations of the commercial identification system MIDI (Microbial Identification System). The whole-cell fatty acid composition was determined by using an Agilent Technologies 6890N gas chromatograph (Santa Clara) and the peaks of the profile were identified using the TSBA50 identification library version 5.0 (MIDI). *L. enzymogenes* LMG 8762^T, *L. antibioticus* LMG 8760^T, *L. gummosus* LMG 8763^T and *L. capsici* DSM 19286^T were included in the FAME analysis as reference strains.

Strain PB-6250^T contained ubiquinone Q-8 as the sole lipoquinone. In the polar lipid analysis phosphatidylglycerol (PG), phosphatidylethanolamine (PE), phosphatidylmonomethylethanolamine (PMME) diphosphatidylglycerol (DPG) were detected as major components, with DPG as the most predominant polar lipid. In addition, moderate amounts of an unknown aminophospholipid, together with trace amounts of four unknown phospholipids and of one single polar lipid were detected (Fig. S1, available in the online Supplementary Material). The major polar lipids of closely related strains were the same (Park *et al.*, 2008), but the unknown amino-group-containing phospholipids differed from species to species.

The major cellular fatty acids (>5 %) detected (percentages of the total cellular fatty acids) in strain PB-6250^T were iso-$C_{11:0}$ (5.1 %), iso-$C_{11:0}$ 3-OH (9.5 %), iso-$C_{15:0}$ (42.5 %), iso-$C_{17:0}$ (5.9 %) and iso-$C_{17:1}\omega9c$ (21.5 %). Similar cellular fatty acid profiles were obtained for the reference strains (Table 1).

All these chemotaxonomic traits clearly distinguish PB-6250^T from members of the genus *Pseudomonas sensu stricto*, which show a quinone system containing ubiquinone Q-9 (Palleroni, 1984, 1993, 2003 ; Yokota*et al.*, 1992), while members of the genus *Lysobacter* typically contain Q-8 as the major respiratory quinone (Wang *et al.*, 2009). Regarding the lipid content, typical characteristics of the genus *Lysobacter* are the predominance of iso-branched fatty acids, and PG, PE and DPG as the major polar lipids (Park *et al.*, 2008; Romanenko *et al.*, 2008; Wang *et al.*, 2011; Zhang *et al.*, 2011).

Further phenotypic characterization was carried out for strain PB-6250^T and *L. enzymogenes* LMG 8762^T, *L. antibioticus* LMG 8760^T, *L. gummosus* LMG 8763^T and *L. capsici* DSM 19286^T with API20NE and APIZYM strips, according to the manufacturer's instructions (BioMérieux). Cells for the inoculation of the tests were grown aerobically for 24 h at 28 °C on TSA medium.

In addition, extensive growth tests were performed. The growth of strain PB-6250^T and the four type strains of species of the genus *Lysobacter* mentioned above on Nutrient Agar (NA, Oxoid CM3), Marine Agar (MA, Difco 0979), R2A agar (Difco 1826–17), Luria Broth (LB) medium (Difco 244620), MacConkey agar (Oxoid CM0007) was investigated. Cells were inoculated on the agar plates and growth was detected after 4 days of aerobic incubation at 28 °C. Under anaerobic conditions, growth on Nutrient Agar after 4 days at 28 °C was examined.

Table 1. Cellular fatty acid profiles of *Lysobacter firmicutimachus* sp. nov. strain PB-6250^T and related type strains of the genus *Lysobacter*

Strains: 1, PB-6250^T; 2, *L. enzymogenes* LMG 8762^T; 3, *L. antibioticus* LMG 8760^T; 4, *L. gummosus* LMG 8763^T; 5, *L. capsici* DSM 19286^T. All data were obtained in this study using the same methodology (cells were grown on TSA medium for 24 h at 28 °C and analysis was performed according to the MIDI system). Data are expressed as percentages of total fatty acids present. ECL, Equivalent chain length; –, not detected; TR, trace amounts (fatty acids representing less than 0.5 % of the total fatty acids present).

Fatty acid	1	2	3	4	5
$C_{10:0}$	–	TR	–	–	0.8
$C_{10:0}$ 3-OH	–	0.8	–	–	1.9
iso-$C_{11:0}$	5.1	7.4	5.3	7.9	10.6
iso-$C_{11:0}$ 3-OH	9.5	18.2	11.0	15.9	26.4
$C_{12:0}$	TR	TR	–	–	1.1
iso-$C_{13:0}$	TR	TR	–	–	–
$C_{14:0}$	–	0.7	TR	–	1.2
iso-$C_{14:0}$	TR	–	–	–	–
iso-$C_{15:0}$	42.5	32.3	42.4	40.0	24.2
iso-$C_{15:0}$ 3-OH	TR	–	–	–	–
iso-$C_{15:1}$ AT 5	3.3	–	2.7	1.1	0.9
anteiso-$C_{15:0}$	TR	TR	0.6	–	–
$C_{16:0}$	0.7	3.7	1.8	2.0	3.1
$C_{16:1}\omega7c$ alcohol	1.0	TR	TR	–	–
$C_{16:1}\omega11c$	TR	–	1.6	–	2.1
iso-$C_{16:0}$	1.8	TR	1.3	0.7	–
iso-$C_{17:0}$	5.9	3.1	5.2	9.0	1.6
iso-$C_{17:1}\omega9c$	21.5	7.9	15.7	14.7	4.4
$C_{18:1}\omega7c$	TR	0.9	0.9	0.6	0.7
Unknown (ECL 11.799)	2.7	5.1	3.2	4.6	7.6
Summed feature 3*	2.7	17.7	7.5	3.5	13.4

*Summed features are groups of two or three fatty acids that cannot be separated by GC with the Microbial Identification System (MIDI); summed feature 3 comprises iso-$C_{15:0}$ 2-OH and/or $C_{16:1}\omega7c$.

All five strains studied grew well on NA, R2A, LB and MacConkey agar under aerobic conditions. Weak growth on NA under anaerobic conditions was observed for *L. capsici* DSM 19286^T, but not for strain PB-6250^T, *L. enzymogenes* LMG 8762^T, *L. antibioticus* LMG 8760^T and *L. gummosus* LMG 8763^T. Growth on MA was observed for strain PB-6250^{T}and *L. enzymogenes* LMG 8762^T, weak growth was observed for *L. antibioticus* LMG 8760^T, but no growth for *L. gummosus* LMG 8763^T and *L. capsici* DSM 19286^T.

The temperature range for the growth of strain PB-6250^T was determined on R2A agar and cultures were incubated at different temperatures 4, 10, 15, 18, 25, 28, 30, 35, 37 and 42 °C. The pH range for growth was determined using different pH values (pH 4.0–10.0, at intervals of 0.5 pH unit) in R2A broth for 4 days at 28 °C. The following buffers were used to set pH values: acetic acid for pH 4.0–4.5, acetate buffer for pH 5.0–6.5 and phosphate buffer for pH 7.0–

10.0. The pH of R2A broth was confirmed after autoclaving. Salt tolerance was tested with 0 to 5 % (w/v) NaCl (at 0.5 % intervals) in R2A broth after 4 days at 28 °C. Growth was estimated by monitoring the optical density at 600 nm.

Growth of PB-6250^T occurred at temperatures of 15–37 °C, at pH 5.5–9.0 and in the presence of 0–0.5 % (w/v) NaCl. Optimum growth occured at 25–28 °C and pH 5.5 and in the absence of NaCl. The biochemical properties of PB 6250^T and of the reference strains were very similar. This demonstrates the affiliation of PB-6250^T to the genus *Lysobacter*, but also demonstrated that PB-6250^T could be differentiated from its closest phylogenetic relatives only by a small set of biochemical properties (Table 2). PB-6250^T could be differentiated from *L. enzymogenes* LMG 8762^T by its inability to assimilate citrate and its lack of α-glucosidase activity, both properties of *L. enzymogenes* LMG 8762^T. Furthermore, PB-6250^T could be differentiated from *L. antibioticus* LMG 8760^T by its positive esterase (C4) enzymatic activity and a lack of β-glucosidase activity.

In summary, phylogenetic, genetic, chemotaxonomic and physiological data all support the reclassification of *Pseudomonas* sp. strain PB-6250^T (=FERM BP-2938=LMG 28994^T=DSM 102073^T) as a novel species of the genus *Lysobacter*. Based on its antibiotic properties, the name *Lysobacter firmicutimachus* sp. nov. is proposed, with PB-6250^T as the type strain.

Description of *Lysobacter firmicutimachus* sp. nov.

Lysobacter firmicutimachus sp. nov. (fir.mi.cu.ti.máchus. N. L. n. (nominative in apposition) *firmicutimachus* from Gr. *machos*, 'fighting' and Firmicutes phylum of bacteria that currently include all low G+C Gram-positive bacteria), referring to the antibacterial activity of the strain, particularly combatting Gram-positive bacteria due to the production of antibiotics of the plusbacin class.

The description is based on Shoji *et al.* (1992a) and the present study. Cells are aerobic, Gram-stain-negative, non-sporulating rods (0.9×2.0–4.0 μm) with rounded ends, which occur singly or in pairs. They are either non-motile or weakly motile by means of one or several polar flagella. Catalase- and oxidase-positive. Grows on Nutrient Agar, R2A agar, LB medium, TSA agar, Marine Agar and MacConkey agar under aerobic conditions. Shows no growth on Nutrient Agar under anaerobic conditions. Colonies on these agar media are round and smooth, convex and cream-yellow. The temperature range for growth is 15–37 °C, but the optimum temperature range is 25–28 °C; no growth is observed at 42 °C. The pH range for growth is 5.5–9.0 with an optimum at pH 5.5. Can only grow at NaCl concentrations of ≤0.5 % (w/v). Gelatin and aesculin are hydrolyzed. Does not reduce nitrate. Positive for the following enzyme activities: alkaline and acid phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, trypsin, naphthol-AS-BI-phosphohydrolase and *N*-

Table 2. Differential characteristics of *Lysobacter firmicutimachus* sp. nov. strain PB-6250^T and related type strains

Strains: 1, PB-6250^T; 2, *L. enzymogenes* LMG8762^T; 3, *L. antibioticus* LMG 8760^T; 4, *L. gummosus* LMG8763^T; 5, *L. capsici* DSM 19286^T. +, Positive; (+), weakly positive; −, negative.

Characteristic	1	2	3	4	5
Assimilation of:					
Citrate	−	+	−	(+)	−
Enzyme activities					
Esterase (C4)	+	(+)	−	(+)	(+)
Valine arylamidase	−	(+)	−	−	−
Cystine arylamidase	−	(+)	−	−	−
α-Chymotrypsin	−	+	−	−	(+)
α-Galactosidase	−	−	−	−	+
β-Galactosidase	−	−	(+)	−	(+)
α-Glucosidase	−	+	−	−	+
β-Glucosidase	−	−	+	+	+
N-Acetyl-β-glucosaminidase	(+)	+	−	+	(+)
DNA G+C content (mol%)*	68.7	69.0[a]	69.2[a]	65.7[a]	65.4[b]
Cell size (μm)*	0.9×2.0–4.0	0.5×38[a]	0.4×4–40[a]	0.4×2.0[a]	0.3–0.5×2.0–20[b]
Colony colour*	Cream-yellow	cream-brown[a]	Pinkish[a]	Pale yellowish-gray[a]	Cream white/yellow[b]
NaCl tolerance (%)*	0–0.5	0–1[b]	0–2[a]	0–2[a,b]	0–2[b]
Temperature range for growth (°C)*	15–37	10–35[a]	10–40[a]	10–40[a]	15–37[b]

*Data taken from other studies as indicated:
a, Data taken from Christensen & Cook (1978).
b, Data taken from Park *et al.* (2008).

acetyl-β-glucosaminidase. The strain gives negative results in the methyl red and Voges-Proskauer tests, as well as in the lipase (C14), valine- and cystine arylamidase, α- and β-galactosidase, β-glucuronidase, α- and β-glucosidase, α-mannosidase and α-fucosidase assays. Glucose, mannose, *N*-acetyl-glucosamine, maltose and malate are utilized as carbon and energy sources. Predominantly contains the polar lipids phosphatidylglycerol, phosphatidylethanolamine, phosphatidylmonomethylethanolamine and diphosphatidylglycerol. The only respiratory quinone is ubiquinone Q-8 (100 %) and the major cellular fatty acids are iso-$C_{11:0}$, iso-$C_{11:0}$ 3-OH, iso-$C_{15:0}$, iso-$C_{17:0}$ and iso-$C_{17:1}\omega$9c.

The type strain, PB-6250^T (=LMG 28994^T=DSM 102073^T), was isolated from a soil sample collected in Okinawa-Honto, Okinawa Prefecture, Japan. The DNA G+C content of the type strain is 68.7 mol%.

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