Paenibacillus endophyticus sp. nov., isolated from nodules of Cicer arietinum

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A bacterial strain, designated PECAE04T, was isolated from root nodules of Cicer arietinum in Spain. Phylogenetic analysis based on 16S rRNA gene sequence placed the isolate into the genus Paenibacillus with its closest relative being Paenibacillus castaneae Ch-32T with 98.4 % 16S rRNA gene sequence similarity followed by Paenibacillus glycanilyticus DS-1T, Paenibacillus prosopidis PW21T, Paenibacillus xinjiangensis B538T and Paenibacillus catalpae D75T with similarities ranging from 97.9 to 96.8 %. DNA–DNA hybridization measurements showed values lower than 20 % between the strain PECAE04T and any of these species. The isolate was a Gram-stain-positive, motile, sporulating rod. Catalase and oxidase activities were positive. Aesculin was hydrolysed but casein and gelatin were not. Acetoin production, H2S production, nitrate reduction and urease and caseinase production were negative. Growth was supported by many carbohydrates and organic acids as carbon sources. MK-7 was the predominant menaquinone and anteiso-C15 : 0, iso-C16 : 0 and C16 : 0 were the major fatty acids. Major polar lipids were diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol, a glycolipid, three phospholipids and an unidentified lipid. Meso-diaminopimelic acid was not detected in the peptidoglycan. The DNA G+C content was 52.9 mol%. Phylogenetic, chemotaxonomic and phenotypic analyses showed that strain PECAE04T should be considered to be a representative of a novel species of the genus Paenibacillus, for which the name Paenibacillus endophyticus sp. nov. is proposed. The type strain is PECAE04T (=LMG 27297T=CECT 8234T).

Cicer arietinum is a species widely distributed in the world, constituting one of the most cultivated legumes in Spain where it is commonly nodulated by Mesorhizobium ciceri and Mesorhizobium mediterraneum (Rivas et al., 2006). However, together with rhizobia, other endophytes are commonly isolated from legume nodules, and specifically in those of C. arietinum, the presence of Gram-negative bacteria from the genus Ochrobactrum (Imran et al., 2010) and from the former genus Agrobacterium (Saïdi et al., 2011) have been reported to date.

In the present study, we characterized a novel endophytic strain belonging to the genus Paenibacillus, strain PECAE04T, isolated from root nodules of C. arietinum in Spain. The genus Paenibacillus comprises Gram-positive or Gram-variable endospore-forming rods with oval endospores formed in swollen sporangia. The major fatty acid is anteiso-C15 : 0 and meso-diaminopimelic is commonly present in the peptidoglycan (Priest, 2009). This genus is ubiquitous in nature and some species have been found in rhizosphere, phyllosphere and inner tissues of plants, including legume nodules. In this way, the novel strain isolated in this study is phylogenetically related to Paenibacillus castaneae, isolated from phyllosphere of Castanea sativa (Valverde et al., 2008), to Paenibacillus
obtained was compared with those from the GenBank Online). Oval sub-terminal endospores were formed in means of peritrichous flagella (Fig. S1A, available in IJSEM). Strain PECAE04$^T$ was isolated from a nodule of *Prosopis farcta* (Valverde et al., 2008) and to *Paenibacillus catalpae*, isolated from rhizosphere of *Catalpa speciosa* (Zhang et al., 2013).

Strain PECAE04$^T$ was isolated from a nodule of *C. arietinum* growing in Salamanca, Spain during a study of rhizobia and nodular endophytes present in different legumes. Root nodules were sterilized by washing several times with sterile distilled water and then surface-sterilizing in 2.5 % (w/v) HgCl$_2$ for 2 min. The nodules were rinsed five times with sterile distilled water and then crushed using a sterile pestle. The homogenized nodule tissue was inoculated on modified yeast extract mannitol agar (Vincent, 1970) (10 g l$^{-1}$ mannitol, 1 g l$^{-1}$ yeast extract, 0.2 g l$^{-1}$ K$_2$HPO$_4$, 0.2 g l$^{-1}$ MgSO$_4$.7H$_2$O, 0.5 g l$^{-1}$ NaCl, 20 g l$^{-1}$ agar) and the plates were incubated at 28 °C for 4 days. In parallel, some of the disinfected entire nodules were incubated in the same medium in order to ensure their complete external disinfection, and no growth was observed around these nodules. The cultures used in further phenotypic and molecular studies were purified from a single colony after 2 days incubation at 28 °C on YMA. The colonies were white, mucoid, translucent and convex on this medium. The strain isolated in this study was unable to produce nodules in *C. arietinum*.

Following isolation, strain PECAE04$^T$ was grown in TSA medium (Difco, Becton Dickinson, BBL). On this medium colonies of strain PECAE04$^T$ were white–cream, round, smooth and convex with approximate diameters of 1–3 mm.

Strain PECAE04$^T$ was grown in nutrient agar (NA) for 48 h at 22 °C to check for motility by phase-contrast microscopy using the hanging drop method. Gram staining was carried out by the procedure described by Doetsch (1981) after 24 h incubation at 28 °C. The flagellation type was determined by electron microscopy after 48 h incubation in NA at 22 °C. For electron microscopy analysis, cells were gently suspended in sterile water, stained with 0.2 % uranyl acetate and examined at 80 kV with a Zeiss EM 209 transmission electron microscope. Strain PECAE04$^T$ was Gram-stain-positive and motile by means of peritrichous flagella (Fig. S1A, available in IJSEM Online). Oval sub-terminal endospores were formed in swollen sporangia (Fig. S1B).

Amplification and sequencing of the 16S rRNA gene were performed according to Rivas et al. (2007). The sequence obtained was compared with those from the GenBank database using the BLASTN (Altschul et al., 1990) and EzTaxon-e server (Kim et al., 2012) programs. Sequences were aligned using CLUSTAL X software (Thompson et al., 1997) and distances were calculated according to Kimura's two-parameter model (Kimura, 1980). The phylogenetic trees were inferred using the neighbour-joining (NJ) and maximum-likelihood (ML) models (Saitou & Nei, 1987, Rogers & Swofford, 1998) including species of the genus *Paenibacillus* with more than 95 % similarity with respect to strain PECAE04$^T$. The MEGA5 software package (Tamura et al., 2011) was used for all analyses. Comparison of the 16S rRNA gene sequence of strain PECAE04$^T$ (1482 nt) against those of type strains held in the EzTaxon-e database indicated that the novel strain belonged to the genus *Paenibacillus*. The closest related species was *Paenibacillus castaneae* Ch-32$^T$ with 98.4 % 16S rRNA gene sequence similarity followed by *Paenibacillus glycanilyticus* DS-1$^T$, *Paenibacillus prosopidis* PW21$^T$, *Paenibacillus xinjiangensis* B538$^T$ and *Paenibacillus catalpae* D75$^T$ with similarities ranging from 97.9 to 96.8 %. Both NJ and ML analyses showed that strain PECAE04$^T$ grouped with *P. castaneae* Ch-32$^T$ within a wider cluster containing the remaining closely related species of the genus *Paenibacillus* (Fig. 1).

For DNA base composition analysis, DNA was prepared according to Chun & Goodfellow (1995). The DNA G+C content was determined using the thermal denaturation method (Mandel & Marmur, 1968). The DNA G+C content of strain PECAE04$^T$ was 52.9 mol%. This value is within the range defined for members of the genus *Paenibacillus* (Shida et al., 1997) and it was similar to those of some closely related species such as *P. glycanilyticus*, *P. prosopidis* and *P. catalpae* (Dasman et al., 2002; Valverde et al., 2010; Zhang et al., 2013), and slightly higher than that of *P. castaneae* with 46.0 mol% (Valverde et al., 2008), *P. xinjiangensis* with 47.0 mol% (Lim et al., 2006) and *Paenibacillus polymyxa*, the type species of genus *Paenibacillus*, with 43–46 mol% (Priest, 2009). DNA–DNA hybridization was performed by using the method of Ezaki et al. (1989), following the recommendations of Willems et al. (2001). DNA–DNA relatedness values between strain PECAE04$^T$ and its closest relatives *P. castaneae* Ch-32$^T$, *P. xinjiangensis* DSM 16970$^T$, *P. catalpae* DSM 24714$^T$, *P. glycanilyticus* DSM 17608$^T$ and *P. prosopidis* PW21$^T$ were 19 ± 3 %, 7.7 ± 1 %, 7.5 ± 1 %, 16.2 ± 3 % and 18.2 ± 5 %, respectively, confirming that the novel isolate represents a novel species of the genus *Paenibacillus* according to the current species concept (Wayne et al., 1987).

Cellular fatty acids were analysed by using the Sherlock Microbial Identification System 6.1 (MIDI; Microbial ID) and the library RTSBA6 according to the technical instructions provided by this system (Sasser, 1990). *P. castaneae* Ch-32$^T$, *P. glycanilyticus* DSM 17608$^T$, *P. prosopidis* PW21$^T$, *P. xinjiangensis* DSM 16970$^T$, *P. catalpae* DSM 24714$^T$ and *P. polymyxa* CECT 155$^T$ were included as reference strains. Strains were grown on TSA plates (Difco, Becton Dickinson, BBL) at 28 °C for 48 h. Other chemotaxonomic analyses were carried out by the Identification Service of the DSMZ (Braunschweig, Germany) for which strain PECAE04$^T$ was cultivated in TSA (Difco, Becton Dickinson, BBL) at 28 °C for 48 h. Respiratory quinones and polar lipids were analysed as described by Tindall (1990). For peptidoglycan analysis, whole cells of strain PECAE04$^T$ were hydrolysed with HCl at 100 °C for 15 h. The hydrolysates were subjected to thin-layer chromatography on cellulose plates using the solvent system of Rhuland et al. (1955). Menaquinone 7 (MK-7) from *strain PECAE04T*.
was the major respiratory quinone (91%) and small amounts (9%) of menaquinone 9 (MK-9) were also detected in strain PECAE04\textsuperscript{T}. MK-7 is the major menaquinone in species of the genus Paenibacillus (Shida et al., 1997) including those closely related to strain PECAE04\textsuperscript{T} (Lim et al., 2006; Valverde et al., 2008; Valverde et al., 2010; Zhang et al., 2013). MK-9 was neither detected in the closest related species, P. castaneae (Valverde et al., 2008) nor in P. prosopidis (Valverde et al., 2010). Nevertheless since in most species of the genus Paenibacillus only the major menaquinone has been reported and only low levels of MK-9 were found in strain PECAE04\textsuperscript{T}, it is difficult to establish the significance of this result. The presence of meso-diaminopimelic acid (DAP) in the peptidoglycan is diagnostic for members of the genus Paenibacillus (Shida et al., 1997), which typically present peptidoglycan type A1\textsubscript{c} (Schumann, 2011). Nevertheless in some paenibacilli the amount of peptidoglycan in the cell wall is small and DAP stays below the detection limit as occurred in strain PECAE04\textsuperscript{T} and other related species of genus Paenibacillus, such as P. prosopidis (Valverde et al., 2010). The fatty acid profile of strain PECAE04\textsuperscript{T} consisted of anteiso-C\textsubscript{15:0} (58.1%); C\textsubscript{16:0} (12.8%); iso-C\textsubscript{16:0} (10.7%); anteiso-C\textsubscript{17:0} (5.6%); iso-C\textsubscript{15:0} (2.3%); C\textsubscript{15:0} (1.7%); iso-C\textsubscript{14:0} (1.6%); iso-C\textsubscript{17:0} (1.1%); C\textsubscript{14:0} (1.6%); C\textsubscript{12:0} (1.1%). The fatty acid profile of strain PECAE04\textsuperscript{T} mainly differed with respect to those of the type strains of the closest related species and P. polymyxa CECT 155\textsuperscript{T} in the amounts of C\textsubscript{16:0} and iso-C\textsubscript{16:0} (Table S1). Strain PECAE04\textsuperscript{T} displayed a lipid profile (Fig. S2) consisting of diphosphatidylglycerol (DPG), phosphatidylglycerol (PG), phosphatidylethanolamine (PE), an unidentified glycolipid (GL1), three unidentified phospholipids (PL1–PL3) and an unidentified lipid (L1). This polar lipid profile was similar to that of the type species of the genus Paenibacillus, P. polymyxa, with diphosphatidylglycerol (DPG), phosphatidylethanolamine (PE) and phosphatidylglycerol (PG) as major polar lipids and an unidentified phospholipid (PL) detected in low amounts (Kämpfer et al., 2006). It was also similar to that of the recently described P. catalpae (Zhang et al., 2013), which clusters in the same phylogenetic group as strain PECAE04\textsuperscript{T}.

The phenotypic characterization included the characteristics recommended in the minimal standards for aerobic endospore-forming bacteria (Logan et al., 2009) and was performed according to the standard methods described by Claus & Berkeley (1986) and Logan & Berkeley (1984) and by using API 20NE, API20E and API50CH systems (bioMérieux) according to the manufacturer’s instructions. The results were read after 72 h incubation at 30 °C. Anaerobic growth was tested in fluid tetrathionate medium (Sigma). Acetoin production, the ability to grow in the presence of 2, 5 and 7% NaCl, nitrate reduction and phenylalanine deaminase, catalase, gelatinase, caseinase, amylase and oxidase activities were analysed as described.

**Fig. 1.** Neighbour-joining phylogenetic tree based on the nearly complete 16S rRNA gene sequence (1482 nt) of strain PECAE04\textsuperscript{T} and closely related species of the genus Paenibacillus. Lactobacillus delbrueckii subsp. lactis DSM 20072\textsuperscript{T} was used as an outgroup. The significance of each branch is indicated by a bootstrap value calculated for 1000 subsets; only values $\geq 50$% are shown. Filled circles indicate corresponding nodes were also obtained with the maximum-likelihood algorithm. Bar, 2 nt substitutions per 100 nt.
Collectively, these results showed that strain PECAE04<sup>T</sup> differs with the related species are shown in Table 1. Results are given in the species description and the production from D-glucose, D-xylose, D-mannitol and L-arabinose, and gas from glucose were analysed in liquid medium as described by Claus & Berkeley (1986). Growth was determined at temperatures of 4, 15, 25, 37, 40 and 45 °C in TSA medium (Difco, Becton Dickinson, BBL). Growth at pH 5.7 and 6.8 was tested as described by Claus & Berkeley (1986) in Sabouraud broth without chloramphenicol (Difco, Becton Dickinson, BBL) and nutrient broth (Difco, Becton Dickinson, BBL), respectively. Growth at pH 7–8 was tested in YMA medium containing 200 mM Na<sub>2</sub>HPO<sub>4</sub>/NaH<sub>2</sub>PO<sub>4</sub> and growth at pH 9 and 10 was tested in the same medium containing 200 mM NaHCO<sub>3</sub>/Na<sub>2</sub>CO<sub>3</sub>. <i>P. castaneae</i> Ch-32<sup>T</sup>, <i>P. glycanilyticus</i> DSM 17608<sup>T</sup>, <i>P. prosopidis</i> PW21<sup>T</sup> <i>P. xinjiangensis</i> DSM 16970<sup>T</sup>, <i>P. catalpae</i> DSM 24714<sup>T</sup> and <i>P. polymyxa</i> CECT 155<sup>T</sup> were included in the phenotypic study as reference strains. Results are given in the species description and the differences with the related species are shown in Table 1.

Collectively, these results showed that strain PECAE04<sup>T</sup> represents a novel species of the genus <i>Paenibacillus</i> distinguishable from the closest phylogenetically related species by the 16S rRNA gene sequence and DNA–DNA relatedness values as well as by chemotaxonomic features and phenotypic characteristics including enzyme production and assimilation of several carbohydrates and organic acids. The name <i>Paenibacillus endophyticus</i> sp. nov. is proposed for the novel species.

### Description of <i>Paenibacillus endophyticus</i> sp. nov.

<i>Paenibacillus endophyticus</i> (en.do phy’ti. cus. Gr. pref. endo- within; Gr. n. phyton plant; L. masc. suff. -icus suffix used with the sense of pertaining to; N.L. masc. adj. <i>endophyticus</i> within plant, because the type strain was first isolated from the inner of a plant nodule).

Cells are Gram-stain-positive rods (width 0.6–1.0 μm, length 2.5–2.9 μm) and motile by means of peritrichous flagella. Oval sub-terminal endospores are formed in swollen sporangia. Catalase- and oxidase-positive. Colonies on TSA are white–cream, round, smooth and convex with approximate diameters of 1–3 mm. Anaerobic growth is positive. Grows at pH 9 but not at pH 5.7; optimal pH is 7. Can grow in the presence of 2 % NaCl but not in 5 % NaCl and the optimal NaCl concentration for growth is 0.5 %. Grows at temperatures of 10–40 °C; optimal temperature for growth is 30 °C. Nitrate is not reduced to nitrite. Produces amylase and β-galactosidase but does not produce gelatinase, caseinase, indole, phenylalanine deaminase, urease, arginine dehydrodrolase, ornithine or lysine decarboxylase. Tween 80 was not

### Table 1. Differential characteristics of strain and phylogenetically related species of the genus <i>Paenibacillus</i>

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Data are from this study. +, Positive; −, negative; w, weakly positive; v, variable.

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hydrolysed. Acetoin and H$_2$S production were negative and aesculin hydrolysis was positive. In the API 20NE system, assimilation of glucose, l-arabinose, N-acetylglucosamine and maltose was positive and that of mannose, mannotetol, gluconate, caprate, adipate, malate, citrate and phenylacetylated was negative. Acid but not gas was produced from glucose. In API 50CH tests, acid production from l-arabinose, D-ribose, D-xyllose, galactose, glucose, fructose, methyl $\alpha$-D-glucoside, N-acetylglucosamine, amygdalin, cellulbiose, maltose, lactose, melibiose, sucrose, trehalose, inulin, melezitose, raffinose and starch was positive, but acid production from glycerol, erythritol, D-arabinose, L-xyllose, adonitol, methyl $\alpha$-D-xylloside, D-mannose, L-sorbitol, L-rhamnose, dulcitol, inositol, mannotetol, sorbitol, methyl $\alpha$-D-mannoside, glycogen, xylitol, gentiobiose, turanose, L-lyxose, tagatose, D-fucose, L-fucose, D-arabinitol and L-arabinose was negative. Acid production from salicin was weak. Hydrolysis of arbutin and aesculin was positive. Assimilation of gluconate and 2- and 5-ketogluconate was negative. The major quinone is MK-7 and meso-diaminopimelic acid was not detected in the peptidoglycan. The lipid profile consists of diphosphatidylglycerol (DPG), phosphatidylglycerol (PG), phosphatidylethanolamine (PE), an unidentified glycolipid (GL1), three unidentified phospholipids (PL1–PL3) and an unidentified lipid (L1). The main fatty acids are anteiso-C$_{15:0}$, iso-C$_{16:0}$ and C$_{16:0}$. The type strain PECAE04$^T$ (=LMG 27297$^T$=CECT 8234$^T$) was isolated from the nodules of *Cicer arietinum* in Spain. The DNA G+C content of strain PECAE04$^T$ is 52.9 mol%.

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**References**


