Cohnella phaseoli sp. nov., isolated from root nodules of *Phaseolus coccineus* in Spain, and emended description of the genus *Cohnella*

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A bacterial strain designated GSPC1^T was isolated from root nodules of *Phaseolus coccineus* in Segovia (Spain). The 16S rRNA gene sequence of this strain showed 95.9 and 94.7 % sequence similarity, respectively, with those of the type strains of *Cohnella hongkongensis* and *Cohnella thermotolerans*. Strain GSPC1^T presented phenotypic, chemotaxonomic and molecular differences with respect to *Cohnella* species which indicated that it belonged to a different species. The isolate was a Gram-positive, aerobic, sporulated rod, motile by means of peritrichous flagella. The strain was catalase-positive and showed weak oxidase activity. It grew in the presence of 2 % NaCl. MK-7 was the predominant menaquinone. anteiso-C_{15:0}, iso-C_{15:0}, iso-C_{16:0} and C_{16:0} were the major fatty acids. Major polar lipids were diphosphatidylglycerol, phosphatidylglycerol and phosphatidylethanolamine. The G + C content was 60.3 mol%. The results of this study suggest that isolate GSPC1^T should be classified within a novel *Cohnella* species, for which the name *Cohnella phaseoli* sp. nov. is proposed, with strain GSPC1^T (=LMG 24086^T =DSM 19269^T) as the type strain.

The scarlet runner or runner bean (*Phaseolus coccineus* L.) is probably the third most economically important *Phaseolus* species and the second most important in the human diet. It is a climbing perennial vegetable often grown as an annual crop for dry seeds or immature pod production but in recent years it has also been grown as an ornamental plant in Europe, due to its gaudy inflorescences. There is general agreement that *P. coccineus* establishes symbiotic associations with rhizobial bacteria to form specific structures, root nodules, in which N₂ fixation takes place (Souza *et al.*, 1994). However, it is known that not only rhizobial bacteria may be found within root nodules (Trujillo *et al.*, 2006a, b). In the present study, we report on a novel non-nodulating strain from the genus *Cohnella*, GSPC1^T, isolated from root nodules of *P. coccineus* growing in Granja de San Ildefonso (Segovia, Spain). Several species of *Rhizobium* are able to nodulate *P. coccineus* in the same soil (García-Fraile *et al.*, 2006).

The genus *Cohnella* was proposed by Kämpfer *et al.* (2006) and at present includes three species, *Cohnella thermotolerans*, *C. hongkongensis* and *C. laeviribosi*, and forms a

homogeneous group within the family *Paenibacillaceae*. To our knowledge, this is the first report of the association of a bacterium of the genus *Cohnella* with a leguminous plant.

The root nodules used for the isolation of strain GSPC1^T were washed several times with sterile distilled water and then surface sterilized in HgCl₂ (2.5 % w/v) for 2 min. The nodules were rinsed five times with sterile distilled water and then crushed using a sterile glass rod. The homogenized nodule tissue was inoculated on modified yeast extract-mannitol agar (YMA; Vincent, 1970) (l⁻¹: 10 g mannitol, 1 g yeast extract, 0.2 g K₂HPO₄, 0.2 g MgSO₄ · 7H₂O, 0.5 g NaCl, 20 g agar) and the plates were incubated at 28 °C for 4 days. 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. To test the symbiotic characteristics of the new bacterial isolate, *P. coccineus* and *Phaseolus vulgaris* plants were inoculated as described by Velázquez *et al.* (2005), using *Rhizobium tropici* CIAT 899^T as a control. The strain isolated in this study was unable to produce nodules in *P. coccineus* or *P. vulgaris*.

Strain GSPC1^T was grown in nutrient agar for 48 h at 22 °C to check for motility by phase-contrast microscopy with the hanging-drop method. Gram staining was carried out by using the procedure described by Doetsch (1981).

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain GSPC1^T is EU014872.

Electron micrographs and results of 2D TLC of polar lipids of strain GSPC1^T are available as supplementary material with the online version of this paper.

The flagellation type was determined by electron microscopy after 48 h incubation of strain GSPC1^T in nutrient agar at 22 °C. The cells were gently suspended in sterile water and then stained with 0.2% uranyl acetate and examined at 80 kV with a Zeiss EM 209 transmission electron microscope. Cells of strain GSPC1^T were Gram-positive, rod-shaped (2.5 × 0.7 µm) and motile by means of peritrichous flagella (see Supplementary Fig. S1 available in IJSEM Online); cells of *C. thermotolerans*, *C. hongkongensis* and *C. laeviribosi* are not flagellated (Table 1).

For sequencing of the 16S rRNA gene, DNA extraction was carried out as described previously (Rivas *et al.*, 2001). Amplification of the gene and sequencing were performed according to methods described previously (Rivas *et al.*, 2002). An almost-complete 16S rRNA gene sequence was obtained and compared with those deposited in the GenBank using the BLASTN program (Altschul *et al.*, 1990). Sequences were aligned using CLUSTAL_X software (Thompson *et al.*, 1997) and distances were calculated according to Kimura's two-parameter method (Kimura, 1980). Phylogenetic trees were inferred using neighbour-joining (Saitou & Nei, 1987) and parsimony analysis (Felsenstein, 1983). Bootstrap analysis was based on 1000 resamplings. The MEGA2 package (Kumar *et al.*, 2001) was used for all analyses.

Comparison of the nearly complete 16S rRNA gene sequence of GSPC1^T (1429 nt) with sequences held in GenBank indicated that strain GSPC1^T was phylogeneti-

cally related to members of the genus *Cohnella* within the phylum *Firmicutes*. Fig. 1 shows the relationship of strain GSPC1^T within the genus *Cohnella* and its nearest phylogenetic relatives based on the neighbour-joining method. Similar results were obtained using the maximum-parsimony method (not shown). The sequence similarity of GSPC1^T to the type strains of *C. hongkongensis*, *C. thermotolerans* and *C. laeviribosi* was respectively 95.9, 94.7 and 94.2%; these relationships were supported by high bootstrap values. These levels of 16S rRNA gene sequence similarity are generally considered sufficient to define a novel species since, in general, organisms that share less than 97.0% 16S rRNA gene sequence similarity do not show more than 70% DNA–DNA reassociation (Rosselló-Mora & Amann, 2001; Stackebrandt & Goebel, 1994; Stackebrandt *et al.*, 2002); DNA–DNA hybridization was therefore not performed.

DNA for the determination of G + C content was prepared according to Chun & Goodfellow (1995); the G + C content of strain GSPC1^T was determined by using the thermal denaturation method (Mandel & Marmur, 1968) as 60.3 mol%.

Chemotaxonomic analyses were performed by the Identification Service of the DSMZ. Isolate GSPC1^T was cultivated in tryptic soy broth (Becton Dickinson) for 24 h at 28 °C in a rotary shaker (90 r.p.m.). Biomass was harvested, washed in distilled water and freeze-dried. Menaquinones and polar lipids were analysed as described by Tindall (1990) and cellular fatty acids were analysed according to the instructions of the Microbial Identification System (MIDI; Microbial ID). Unsaturated menaquinone with seven isoprene units (MK-7) was the predominant isoprenoid quinone (88%) found in strain GSPC1^T. MK-6 was also detected (12%). This quinone profile is characteristic of species within the family *Paenibacillaceae* and supports the affiliation of strain GSPC1^T to the genus *Cohnella* (Kämpfer *et al.*, 2006). The cellular fatty acid profile of GSPC1^T was characterized by 15 different fatty acids and consisted predominantly of iso- and anteiso-branched fatty acids. The major fatty acids detected in strain GSPC1^T were anteiso-C_{15:0} (41.5%), iso-C_{15:0} (14.3%), iso-C_{16:0} (14.1%) and C_{16:0} (8.9%). The fatty acid profile of GSPC1^T differed in the proportions of some fatty acids with respect to the type strains of related *Cohnella* species (Table 2). Strain GSPC1^T shared a similar polar lipid profile with *C. thermotolerans* CCUG 47242^T, *C. hongkongensis* HKU3^T and *C. laeviribosi* RI-39^T, made up predominantly of diphosphatidylglycerol, phosphatidylglycerol and phosphatidylethanolamine. Four unknown phospholipids (PL1–PL4) and two unknown aminophospholipids (PN1, PN2) were also detected (Supplementary Fig. S2). Two unknown glycolipids (GL1 and GL2) were detected in very small amounts. The presence of these two glycolipids and aminophospholipid PN1 and the absence of lysylphosphatidylglycerol clearly distinguish strain GSPC1^T from the remaining species of *Cohnella* (Kämpfer *et al.*, 2006; Cho *et al.*, 2007).

Table 1. Differential phenotypic characteristics of strain GSPC1^T and type strains of phylogenetically related species

Strains: 1, GSPC1^T (*C. phaseoli* sp. nov.); 2, *C. thermotolerans* CCUG 47242^T (data from Kämpfer *et al.*, 2006); 3, *C. hongkongensis* HKU3^T (Kämpfer *et al.*, 2006; Teng *et al.*, 2003); 4, *C. laeviribosi* RI-39^T (Cho *et al.*, 2007). +, Positive; –, negative; w, weak; ND, no data available.

Characteristic	1	2	3	4
Flagella	+	–	–	–
Growth at 55 °C	–	+	+	+
Oxidase	w	+	+	–
<i>o</i> -Nitrophenyl-β-D-galactosidase	–	–	+	+
Growth in presence of 2% NaCl	+	ND	ND	–
Assimilation of carbon sources				
Gluconate	–	+	+	–
L-Rhamnose	–	w	+	+
Galactose	–	+	+	+
Sucrose	+	–	–	+
Trehalose	+	–	–	+
D-Xylose	+	–	–	+
Adonitol	–	–	–	+
Mannitol	–	–	–	+
Sorbitol	–	–	–	+
Salicin	+	–	–	ND*
N-Acetylglucosamine	+	–	+	ND*

*Acid production was negative.

Fig. 1. Comparative sequence analysis of 16S rRNA genes from strain GSPC1^T and representative species from related genera using the neighbour-joining method. *Lactobacillus delbrueckii* subsp. *lactis* DSM 20072^T was used as an outgroup. The significance of each branch is indicated by a bootstrap value calculated for 1000 subsets. Bar, 2 substitutions per 100 nucleotide positions.

Phenotypic characterization was performed according to the standard methods described by Claus & Berkeley (1986) and Logan & Berkeley (1984) and by using the API 50CH (with CHB/E medium), API 20NE and API 20E systems (bioMérieux) according to the manufacturer's instructions. Acid production from D-glucose, D-xylose, mannitol and L-arabinose, gas production from glucose, acetoin production, the ability to grow in the presence of 2 and 7 % NaCl, nitrate reduction, anaerobic growth and phenylalanine deaminase and caseinase activities were analysed as described by Claus & Berkeley (1986). Catalase activity was determined by observing bubble formation in a culture after the addition of 3 % hydrogen peroxide. Oxidase activity was determined by oxidation of 1 % tetramethyl *p*-phenylenediamine. Growth at temperatures ranging from 4 to 50 °C was determined in YED medium (0.5 % yeast extract, 0.7 % glucose and 2 % agar). Growth at pH 5.7 and 6.8 was tested as described by Claus & Berkeley (1986), growth at pH 7–8 was tested in YED medium containing 100 mM Na₂HPO₄/NaH₂PO₄ and growth at pH 9 and 10 was tested in the same medium containing 100 mM NaHCO₃/Na₂CO₃. Differentiating characteristics of strain GSPC1^T and other *Cohnella* species are shown in Table 1. The other characteristics determined are given in the species description.

On the basis of phylogenetic, chemotaxonomic and phenotypic data, we propose that isolate GSPC1^T belongs

to a novel species of genus *Cohnella*, *Cohnella phaseoli* sp. nov.

Description of *Cohnella phaseoli* sp. nov.

Cohnella phaseoli (pha.se.o'li. N.L. masc. n. *Phaseolus* botanical genus name; N.L. gen. n. *phaseoli* of *Phaseolus*, referring to the isolation source of the type strain, nodules of *Phaseolus coccineus*).

Spore-forming rods, 0.7 µm wide and 2.5 µm long. Gram-positive. Motile by means of peritrichous flagella. Round or ovoid spores are formed in slightly swollen sporangia and they are in a central or subterminal position within cells. Colonies on YED are circular, flat, white-cream, opaque and usually 1–3 mm in diameter after 48 h growth at 28 °C. The major quinone is MK-7. The main fatty acids are anteiso-C_{15:0} and iso-C_{16:0}. Growth occurs from 10 to 45 °C (optimal growth at 28 °C) and pH 6–8 (optimal pH 7). Aerobic. Weakly positive for oxidase and positive for catalase. Acetoin is produced. The type strain grows in the presence of 2 % NaCl. Nitrate is not reduced to nitrite. β-Galactosidase is produced, but arginine dihydrolase, indole, lysine decarboxylase, ornithine decarboxylase, urease, tryptophan deaminase, phenylalanine deaminase and hydrogen sulfide are not. Milk coagulation is positive. Gelatinase production is weak. Aesculin is hydrolysed. D-Glucose, L-arabinose, maltose, mannose, N-acetylglucosamine, malate

Table 2. Cellular fatty acid composition of strain GSPC1^T and type strains of phylogenetically related species

Strains: 1, *C. phaseoli* GSPC1^T; 2, *C. thermotolerans* CCUG 47242^T (data from Kämpfer *et al.*, 2006); 3, *C. hongkongensis* HKU3^T (Kämpfer *et al.*, 2006) 4, *C. laeviribosi* RI-39^T (Cho *et al.*, 2007). ND, Not detected/not reported; tr, trace.

Fatty acid	1	2	3	4
Saturated acids				
C _{14:0}	1.8	1	5	1.9
C _{15:0}	5.3	1.4	8	1.3
C _{16:0}	8.9	6.6	25.3	9.2
C _{17:0}	0.5	ND	1.2	tr
iso-C _{12:0}	0.3	ND	ND	ND
iso-C _{13:0}	0.8	ND	ND	tr
iso-C _{14:0}	2.6	2.1	2.3	3.9
iso-C _{15:0}	14.3	3.2	8.1	11.7
iso-C _{16:0}	14.1	45.5	11.9	40.5
iso-C _{17:0}	3.1	ND	ND	2.8
anteiso-C _{13:0}	1.4	ND	0.8	tr
anteiso-C _{15:0}	44.5	28.4	31.2	22.0
anteiso-C _{17:0}	2.3	6.7	2.6	5.8
Unsaturated acids				
iso-C _{17:1}	ND	1.1	1.9	ND
C _{14:1} ω5c	ND	ND	ND	tr
C _{16:1} ω7c	1.9	ND	ND	ND
C _{16:1} ω11c	1.2	ND	0.9	tr
C _{17:1} ω6c	ND	1	ND	tr
C _{18:1} ω7c	ND	4	ND	ND

and gluconate are assimilated in the API 20NE system. Acid is produced from D-glucose, D- and L-arabinose, D-ribose, D-xylose, methyl β-D-xyloside, fructose, mannose, methyl α-D-glucoside, N-acetylglucosamine, amygdalin, arbutin, salicin, cellobiose, maltose, lactose, melibiose, sucrose, trehalose, raffinose, starch, glycogen, gentiobiose, turanose and L-fucose in the API 50CH system. Assimilation of galactose, L-xylose, L-sorbose, L-rhamnose, sucrose, melibiose, erythritol, adonitol, dulcitol, inositol, mannitol, sorbitol, methyl α-D-mannoside, inulin, melezitose, xylitol, D-lyxose, D-tagatose, D-fucose, D- and L-arabitol, gluconate, 2-ketogluconate, 5-ketogluconate, caprate, propionate, adipate, citrate and phenylacetate is negative. The DNA G+C content of the type strain is 60.3 mol%.

The type strain, GSPC1^T (=LMG 24086^T =DSM 19269^T), was isolated from root nodules of *Phaseolus coccineus* in Segovia (Spain).

Emended description of the genus *Cohnella* Kämpfer *et al.* 2006

The description is as given by Kämpfer *et al.* (2006) with the following amendments. Cells are non-motile or motile. Most species are thermotolerant. Predominant polar lipids are diphosphatidylglycerol, phosphatidylglycerol and phosphatidylethanolamine. In addition, lysylphosphatidylglycerol and several unknown phospholipids, unknown

aminophospholipids and unknown glycolipids might be present.

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