Cohnella rhizosphaerae sp. nov., isolated from the rhizosphere environment of *Zea mays*

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A Gram-staining-positive, aerobic, non-endospore forming organism, isolated as a seed endophyte (colonizing the internal healthy tissue of plant seed) of sweet corn (*Zea mays*), strain CSE-5610^T, was studied for its taxonomic allocation. On the basis of 16S rRNA gene sequence comparisons, strain CSE-5610^T was grouped into the genus *Cohnella*, most closely related to *Cohnella ginsengisoli* GR21-5^T (98.1 %) and '*Cohnella plantaginis*' YN-83 (97.5 %). The 16S rRNA gene sequence similarity to other members of the genus *Cohnella* was <96.6 %. DNA–DNA hybridization of strain CSE-5610^T with *C. ginsengisoli* DSM 18997^T and '*C. plantaginis*' DSM 25424 was 58 % (reciprocal 24 %) and 30 % (reciprocal 27 %), respectively. The fatty acid profile from whole cell hydrolysates supported the allocation of the strain to the genus *Cohnella*; iso- and anteiso-branched fatty acids were found as major compounds. *meso*-Diaminopimelic acid was identified as the cell-wall diamino acid. The quinone system consisted predominantly of menaquinone MK-7. The polar lipid profile was composed of diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine, two aminophospholipids, a phospholipid and minor amounts of two polar lipids. In the polyamine pattern, spermidine was the major polyamine. The G+C content of the genomic DNA was 60 mol%. In addition, the results of physiological and biochemical tests also allowed phenotypic differentiation of strain CSE-5610^T from the two closely related strains. Hence, CSE-5610^T represents a novel species of the genus *Cohnella*, for which we propose the name *Cohnella rhizosphaerae* sp. nov., with CSE-5610^T (=LMG 28080^T=CIP 110695^T) as the type strain.

The genus *Cohnella* was described as a homogeneous group within the family *Paenibacillaceae* (Kämpfer *et al.*, 2006). Members of the genus *Cohnella* can be differentiated from members of the genus *Paenibacillus* on the basis of 16S rRNA gene sequence analysis, polar lipid patterns and fatty acid compositions. MK-7 is detected as the major menaquinone. Major fatty acids are iso-$C_{16:0}$, anteiso-$C_{15:0}$ and $C_{16:0}$ and the predominant polar lipids are diphosphatidylglycerol, phosphatidylglycerol and phosphatidylethanolamine (Kämpfer *et al.*, 2006). At present, the genus contains 21 species with validly published names, *Cohnella hongkongensis* and *C. thermotolerans* (Kämpfer *et al.*, 2006), *C. laeviribosi* (Cho *et al.*, 2007), *C. phaseoli* (García-Fraile *et al.*, 2008), *C. damuensis* (Luo *et al.*, 2010), *C. fontinalis* (Shiratori *et al.*, 2010), *C. ginsengisoli* and *C. yongneupensis* (Kim *et al.*, 2010), *C. luojiensis* (Cai *et al.*, 2010), *C. thailandensis* (Khianngam *et al.*, 2010a), *C. terrae* and *C. xylanilytica* (Khianngam *et al.*, 2010b), *C. panacarvi* (Yoon *et al.*, 2007), *C. cellulosilytica* (Khianngam *et al.*, 2012), *C. soli* and *C. suwonensis* (Kim *et al.*, 2012), *C. boryungensis* (Yoon & Jung, 2012), *C. arctica* (Jiang *et al.*, 2012), *C. formosensis* (Hameed *et al.*, 2013), *C. lupini* (Flores-Félix *et al.*, 2014) and *C. ferri* (Mayilraj *et al.*, 2011), as well as '*Cohnella plantaginis*' (Wang *et al.*, 2012), the name of which has not yet been validly published. Most of the type strains were isolated from different soils (Yoon *et al.*, 2007; Cai *et al.*, 2010; Kim *et al.*, 2010; Kim *et al.*, 2012; Jiang *et al.*, 2012; Yoon & Jung 2012; Khianngam *et al.*, 2010a, b); some were isolated from the rhizosphere (Hameed *et al.*, 2013) or endophytic compartments, root nodules, of plants (Flores-Félix *et al.*, 2014; García-Fraile *et al.*, 2008).

In 2010, a Gram-staining-positive organism was isolated as a seed endophyte (colonizing the internal healthy tissue

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain CSE-5610^T is KF840792.

of plant seed) of sweet corn (*Zea mays*). The bacterium was subcultivated on tryptone soy agar (TSA; Oxoid) at 28 °C for 24 h. Gram-staining was performed as described previously (Gerhardt *et al.*, 1994). Cell morphological traits were observed under a Zeiss light microscope at a magnification of ×1000, using cells that had been grown for 3 days at 28 °C on TSA (Oxoid).

EzTaxon-e search was applied for a first phylogenetic identification (Kim *et al.*, 2012). Thereafter, detailed phylogenetic analysis was performed in ARB release 5.2 (Ludwig *et al.*, 2004) in the All-Species Living Tree Project (LTP; Yarza *et al.*, 2008) database release s111 (February 2013). Sequences that needed to be implemented in the LTP database were aligned with SINA (version 1.2.9) according to the SILVA seed alignment (http://www.arb-silva.de; Pruesse *et al.*, 2012) and implemented into the LTPs database. The alignment was checked manually based on secondary structure information. Pairwise sequence similarities were calculated in ARB without the use of an evolutionary substitution model. Phylogenetic trees were calculated with the maximum-likelihood method using RAxML version 7.04 (Stamatakis, 2006) with GTR-GAMMA and rapid bootstrap analysis and PhyML (without bootstrap analysis), the neighbour-joining method (ARB neighbour-joining) with the Jukes–Cantor correction (Jukes & Cantor, 1969) and the maximum-parsimony method using DNAPARS version 3.6 (Felsenstein, 2005). Phylogenetic trees were calculated with 100 resamplings (bootstrap analysis; Felsenstein, 1985) and based on 16S rRNA gene sequences between sequence positions 98 and 1423 (according to *Escherichia coli* numbering; Brosius *et al.*, 1978). The phylogenetic trees were checked for consistency, and nodes that were conserved in other trees were marked by asterisks in the RAxML tree (Fig. 1). The obtained 16S rRNA gene sequence of strain CSE-5610^T was a continuous stretch of 1478 unambiguous

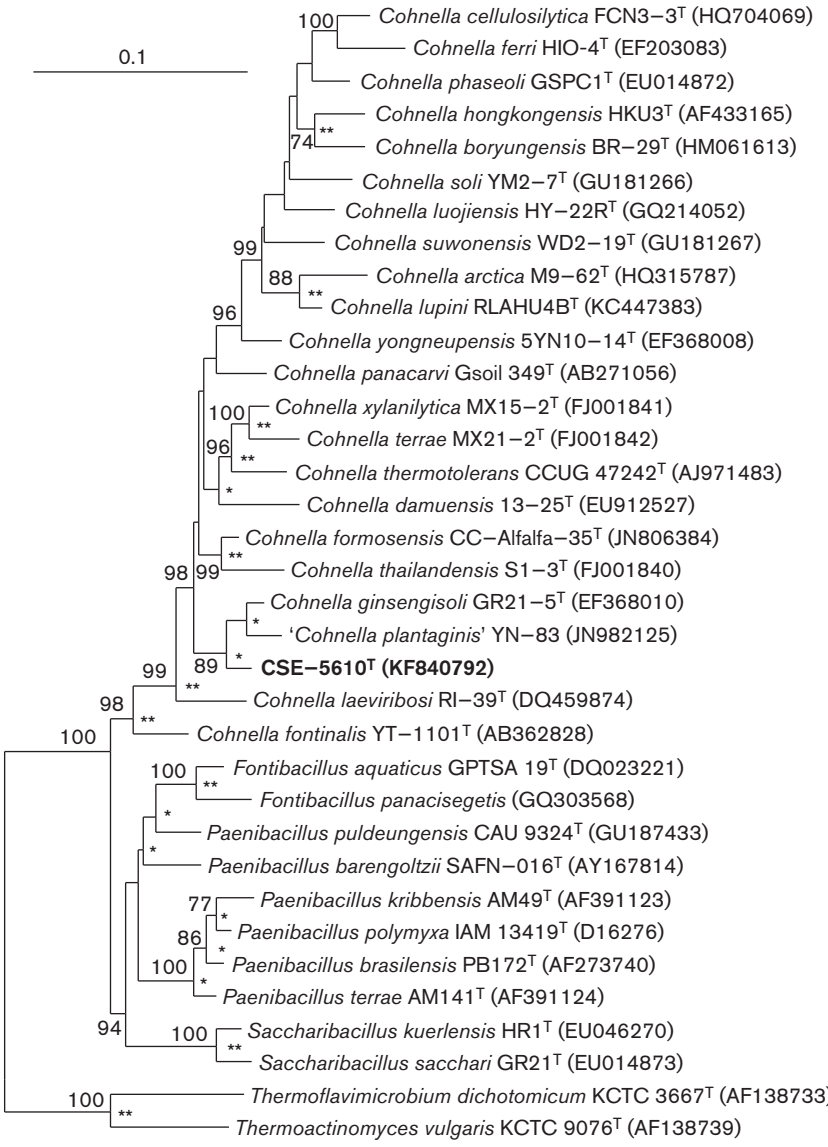

Fig. 1. Maximum-likelihood tree showing the phylogenetic relationship of strain CSE-5610^T to species of the genus *Cohnella* and members of closely related genera of the *Paenibacillaceae.* The tree was generated in ARB using RAxML (GTR-GAMMA, rapid bootstrap analysis) and based on 16S rRNA gene sequences between positions 98 and 1423 (*E. coli* numbering; Brosius *et al.*, 1978). Numbers at branch nodes refer to bootstrap values >70 (100 replicates). Nodes marked with one asterisk were supported by one of the other applied treeing methods (neighbour-joining or maximum-parsimony); two asterisks indicate nodes supported in trees calculated with both other applied treeing methods. *Thermoflavimicrobium dichotomicum* KCTC 3667^T and *Thermoactinomyces vulgaris* KCTC 9076^T were used as an outgroup. Bar, 0.10 substitutions per nucleotide position.

nucleotides spanning *E. coli* sequence positions 20–1492 (Brosius *et al.*, 1978). EzTaxon-e analysis showed that CSE-5610^T shared the highest 16S rRNA gene sequence similarity with type strains of species of the genus *Cohnella*. Pairwise analysis in ARB showed that strain CSE-5610^T shared 92.8–98.1 % 16S rRNA gene sequence similarity with type strains of species of the genus *Cohnella*, with the highest sequence similarity to the type strain of *C. ginsengisoli* (98.1 %) followed by the proposed type strain of '*C. plantaginis*' (97.5 %). 16S rRNA gene sequence similarities to all other type strains of species of the genus were below 97 %. Phylogenetic trees clearly indicated that strain CSE-5610^T is placed within the monophyletic cluster of the genus *Cohnella* and forms a distinct subcluster (high bootstrap support) with the type strains of *C. ginsengisoli* and '*C. plantaginis*' (Fig. 1).

For more detailed genotypic analysis genomic DNA was extracted by the method of Pitcher *et al.* (1989). DNA–DNA hybridization was performed between strain CSE-5610^T and *C. ginsengisoli* DSM 18997^T and '*C. plantaginis*' DSM 25424, as described by Ziemke *et al.* (1998). DNA–DNA hybridization for strain CSE-5610^T and *C. ginsengisoli* DSM 18997^T was 58 % (reciprocal 24 %) and for strain CSE-5610^T and '*C. plantaginis*' DSM 25424 was 30 % (reciprocal 27 %). The genomic DNA G+C content of strain CSE-5610^T was determined as described previously (Glaeser *et al.*, 2013) based on the DNA melting temperature methods established by Gonzales & Saiz-Jimenez (2002). Strain CSE-5610^T had a genomic G+C content of 60 mol%. This was in the same range as the G+C content obtained for the type strain of the type species of the genus *Cohnella, C. thermotolerans* (60.9 mol%; Kämpfer *et al.*, 2006) and the closely related *Cohnella* species *C. ginsengisoli* (61.3 mol%; Kim *et al.*, 2010).

For the detection of the diagnostic diamino acid of the cell wall, biomass was used that had been grown at 28 °C in 3.3 × PYE broth (1.0 g peptone from casein, 1.0 g yeast extract, pH 7.2). Detection was carried out as described by Schleifer (1985) and revealed the presence of *meso*-diaminopimelic acid. Polyamines, quinones and polar lipids were also extracted from biomass that had been grown in 3.3 × PYE broth. Biomass subjected to polyamine analysis was harvested at the late exponential growth phase, as recommended by Busse & Auling (1988), whereas quinones and polar lipids were extracted from cells harvested at the stationary growth phase. Extraction of polyamines was carried out as described by Busse & Auling (1988), applying HPLC conditions reported by Busse *et al.* (1997). For extraction and analyses of quinones and polar lipids, the integrated procedure reported by Tindall (1990a, b) and Altenburger *et al.* (1996) was applied. The HPLC equipment used was reported by Stolz *et al.* (2007). The polyamine pattern consisted of (per g dry weight) 32.0 μmol spermidine, 2.7 μmol spermine, 0.5 μmol putrescine and 0.1 μmol cadaverine. The quinone system contained menaquinones MK-7 (99.8 %) and MK-8 (0.2 %). Diphosphatidylglycerol was identified as the major polar lipid; in addition, large proportions of phosphatidylethanolamine, phosphatidylglycerol, two unidentified aminophospholipids and an unidentified phospholipid and small amounts of two unidentified lipids only detectable after total lipid staining were detected (Fig. 2). Lysyl-phosphatidylglycerol was absent. Like the presence of *meso*-diaminopimelic acid as the diagnostic diamino acid of the peptidoglycan and the major quinone MK-7, this polar lipid profile conforms well to the description of the genus (Kämpfer *et al.*, 2006; García-Fraile *et al.*, 2008; Khianngam *et al.*, 2010a). To the best of our knowledge, strain CSE-5610^T is the first member of the genus *Cohnella* to be analysed for its polyamine content, but the fact that its polyamine profile resembles those of other endospore-formers (Hamana *et al.*, 1989) suggests that this diagnostic feature is of minor importance for this group of bacteria.

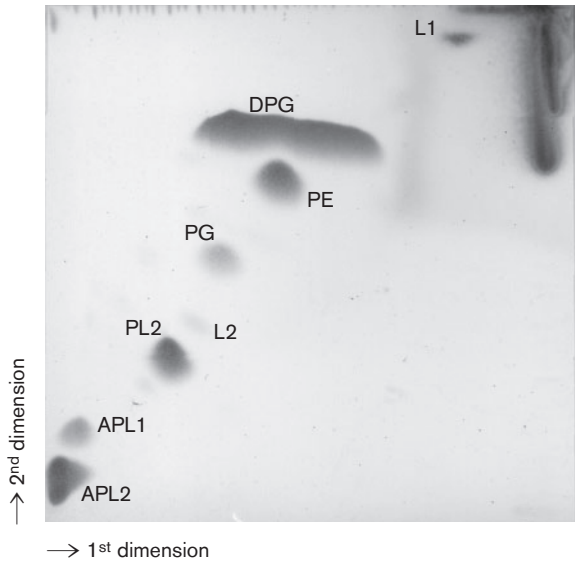

Fig. 2. Polar lipid profile of strain CSE-5610^T after staining with molybdatophosphoric acid. DPG, Diphosphatidylglycerol; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PL2, unidentified phospholipid; APL1, 2, unidentified aminophospholipids; L1, 2, unidentified lipids only detectable with molybdatophosphoric acid.

Fatty acid analysis was done as described by Kämpfer & Kroppenstedt (1996) using an HP-6890 gas chromatograph, Sherlock MIDI software version 2.11 and TSBA peak-naming table version 4.1. Strains were cultivated on R2A agar at 28 °C for 48 h prior to extraction. The fatty acid profile comprised mainly iso- and anteiso-branched fatty acids, and the fatty acid profile was similar to those of the most closely related species of the genus *Cohnella*. The detailed fatty acid profile obtained from cells grown on R2A agar after 48 h of incubation at 28 °C is shown in Table 1.

Strain CSE-5610^T grew well on nutrient agar, TSA and R2A agar (all Oxoid), Columbia agar supplemented with 5 % sheep blood and 3.3 × PYE agar at 28 °C after 48 h; no haemolysis was observed on blood agar. No growth was

Table 1. Fatty acid content of strain CSE-5610^T in comparison with those of closely related strains

Strains: 1, CSE-5610^T; 2, '*C. plantaginis*' DSM 25424; 3, *C. ginsengisoli* DSM 18997^T; 4, *C. thermotolerans* CCUG 47242^T (data from Kämpfer *et al.*, 2006). Data are from this study unless indicated. Data in parentheses from Wang *et al.* (2012). Values are percentages of total fatty acids.

Fatty acid	1	2	3	4
Saturated				
$C_{12:0}$	3.3	5.7 (−)	3.6 (−)	−
$C_{14:0}$	5.6	− (1.6)	7.3 (1.6)	1.0
$C_{15:0}$	−	−	−	1.4
$C_{16:0}$	25.4	14.7 (7.7)	36.5 (9.6)	6.6
Branched				
iso-$C_{14:0}$	−	− (4.1)	− (6.6)	2.1
iso-$C_{15:0}$	10.2	11.7 (11.3)	6.6 (13.9)	3.2
iso-$C_{16:0}$	12.2	15.2 (18.6)	10.6 (21.6)	45.5
iso-$C_{17:0}$	−	− (1.2)	− (1.7)	−
anteiso-$C_{15:0}$	36.5	43.8 (44.3)	30.2 (37.2)	28.4
anteiso-$C_{17:0}$	6.7	8.9 (3.1)	5.3 (2.8)	6.7
Unsaturated				
$C_{18:1}\omega 6c$	−	−	−	4
$C_{18:1}\omega 9c$	−	− (1.4)	− (2.8)	−

observed on MacConkey agar. Growth was tested on nutrient agar at 4, 10, 15, 20, 25, 28, 30, 36, 45, 50 and 55 °C. The strain grew well at 15–45 °C; no growth was observed at 10 °C or below or at 50 °C or above. When a suspension of cells in 3.3 × PYE broth was incubated for 15 min at 80 °C and afterwards incubated overnight at 28 °C, growth was demonstrated unambiguously by strongly increased turbidity. These data demonstrate either that cells of strain CSE-5610^T tolerate high temperatures or that growth resulted from germinated endospores. Light microscopic analysis of strain CSE-5610^T at × 1000 magnification did not show endospore formation after growth on TSA at 28 °C for 48 days. The O/F test for glucose was negative. In the SIM test, no production of H_2S or indole was observed, but motility was indicated by diffuse turbidity around the inoculation channel. Salinity-dependent growth was tested in tryptone soy broth (TSB; Oxoid) by the addition of 1, 2 and 3 % (w/v) NaCl. Strain CSE-5610^T grew without NaCl and in the presence of 1 % NaCl. The pH range for growth (tested in TSB adjusted to pH 4.5–12.5) was pH 5.5–9.5. No growth was observed at pH 4.5 or 10.5. The results of physiological characterization, performed using methods described previously (Kämpfer, 1990; Kämpfer *et al.*, 1991), are given in Table 2 and in the species description. After 72 h of incubation at 25 °C, strain CSE-5610^T was not able to produce acids from any tested sugars or sugar-related compounds, but was able to utilize several of them (weakly) as sole sources of carbon. A distinct physiological and biochemical profile allowed differentiation of the strain from the type strains of the two most closely related species.

Phylogenetic data based on the 16S rRNA gene sequence suggest that strain CSE-5610^T belongs in the genus *Cohnella*, and the quinone system as well as the fatty acid profile are in accordance with this assignment; hence, strain CSE-5610^T can be assigned to the genus *Cohnella*. DNA–DNA hybridization analysis to the type strains of the closest related species of the genus *Cohnella* clearly showed that strain CSE-5610^T represents a novel species, for which we propose the name *Cohnella rhizosphaerae* sp. nov.

Table 2. Differential phenotypic characteristics between strain CSE-5610^T and closely related strains

Strains: 1, CSE-5610^T; 2, '*C. plantaginis*' DSM 25424; 3, *C. ginsengisoli* DSM 18997^T; 4, *C. thermotolerans* CCUG 47242^T (data from Kämpfer *et al.*, 2006). Data are from this study unless indicated. Data in parentheses from Wang *et al.* (2012). +, Positive; W, weakly positive; −, negative; ND, not determined.

Characteristic	1	2	3	4
Growth temperature (°C)	15–45	10–45 (10–45)	10–36 (10–40)	20–54
Growth pH	5.5–9.5	4.5–8.5 (5.0–8.0)	4.5–8.5 (6.0–9.0)	ND
Growth in the presence of 3 % NaCl	+	+ (+)	(−)	ND
Nitrate reduction	−	− (−)	(+)	ND
Assimilation of:				
Alanine	−	− (+)	−* (−)	−
Histidine	−	− (+)	−* (−)	−
Serine	−	− (+)	−* (−)	−
Sucrose	W	− (+)	−* (−)	−
L-Arabinose	W	− (−)	W* (+)	W
Salicin	−	− (−)	W* (+)	−
Melibiose	W	− (−)	W* (+)	+
D-Fructose	W	− (+)	W* (−)	+

*Data in congruence with those published by Kim *et al.* (2010).

Description of *Cohnella rhizosphaerae* sp. nov.

Cohnella rhizosphaerae (rhi.zo.sphae′rae. Gr. n. *rhiza* a root; L. n. *sphaera* ball, sphere; N.L. fem. n. *rhizosphaera* the rhizosphere; N.L. gen. n. *rhizosphaerae* of the rhizosphere).

Cells are Gram-staining-positive, non-motile, strictly aerobic rods (0.8–1.0 μm in diameter, 2.0–3.0 μm long). Endospore formation is not detected. Colonies grown on R2A agar are circular, convex and beige. Optimal temperature for growth is 28 °C; growth occurs at 15–45 °C but not at 10 or 50 °C. Optimal growth in TSB at pH 6.5; growth occurs at pH 5.5–9.5 and in TSB containing NaCl concentrations up to 1 % (w/v). Test for catalase is negative; oxidase activity is weakly positive. Tests for urease, gelatinase, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, tryptophan deaminase and citrate utilization are negative. Starch, casein and gelatin are hydrolysed. Indole production, H_2S formation, DNase activity and the Voges–Proskauer reaction are also negative. Acid formation from sugars is not observed with the following compounds: D-glucose, D-xylose, lactose, sucrose, D-mannitol, dulcitol, salicin, D-adonitol, *myo*-inositol, D-sorbitol, L-arabinose, raffinose, L-rhamnose, maltose, trehalose, cellobiose, erythritol, melibiose and D-arabitol. Only a few tested compounds are utilized as sole sources of carbon according to the method of Kämpfer *et al.* (1991): *N*-acetyl-D-glucosamine, L-arabinose, arbutin, cellobiose, D-fructose, D-glucose, D-galactose, maltose, L-rhamnose, ribose, sucrose, D-xylose, D-maltitol and D-mannitol. D-Adonitol, gluconate, *myo*-inositol, D-mannose, melibiose, salicin, trehalose, D-sorbitol, malate, pyruvate, putrescine, acetate, propionate, *cis*- and *trans*-aconitate, adipate, 4-aminobutyrate, azelate, citrate, itaconate, 2-oxoglutarate, alanine, histidine, serine and mesaconate are not utilized as sole carbon sources. *meso*-Diaminopimelic acid is the cell wall diamino acid. The quinone system contains predominantly menaquinone MK-7. In the polar lipid profile, diphosphatidylglycerol is predominant and large amounts of phosphatidylglycerol, phosphatidylethanolamine, two unidentified aminophospholipids and one phospholipid are also present. Lysyl-phosphatidylglycerol is absent. The polyamine pattern contains the major compound spermidine. Major fatty acids are iso-$C_{15:0}$, iso-$C_{16:0}$ and anteiso-$C_{15:0}$. The G+C content of the genomic DNA is 60 mol%.

The type strain, CSE-5610^T (=LMG 28080^T=CIP 110695^T), was isolated as a seed endophyte (colonizing the internal healthy tissue of plant seed) of sweet corn (*Zea mays*).

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