

Paenibacillus cucumis sp. nov., isolated from a cucumber plant

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A Gram-positive-staining, aerobic, endospore-forming bacterial strain, isolated from the stem of a cucumber plant, was studied in detail for its taxonomic position. Based on 16S rRNA gene sequence similarity comparisons, strain AP-115^T was grouped into the genus *Paenibacillus*, most closely related to *Paenibacillus amylolyticus* (98.8%), *Paenibacillus tundrae* and *Paenibacillus barcinonensis* (both 98.4%). The 16S rRNA gene sequence similarity to other species of the genus *Paenibacillus* was ≤98.4%. Chemotaxonomic characterization supported allocation of the strain to the genus *Paenibacillus*. The quinone system contained exclusively menaquinone MK-7, and in the polar lipid profile diphosphatidylglycerol, phosphatidylglycerol, phosphatidylethanolamine and phosphatidylserine were predominating. The major component in the polyamine pattern was spermidine, and the diagnostic diamino acid of the peptidoglycan was meso-diaminopimelic acid. The major fatty acids were iso- and anteiso-branched fatty acids. The results of physiological and biochemical tests allowed phenotypic differentiation of strain AP-115^T from closely related species. Thus, AP-115^T represents a novel species of the genus *Paenibacillus*, for which the name *Paenibacillus cucumis* sp. nov. is proposed, with AP-115^T (=LMG 29222^T=CCM 8653^T) as the type strain.

The genus *Paenibacillus* was initially proposed by Ash *et al.* (1993) to accommodate members of '16S rRNA group 3' bacilli. The type species of this genus was finally decided to be *Paenibacillus polymyxa* (Tindall, 2000; Judicial Commission, 2005). The genus is still growing, now comprising more than 100 species isolated from various sources.

Novel species of the genus *Paenibacillus* have frequently been isolated as endophytes and from other plant-associated environments. Endospore-forming bacilli, including members of the genus *Paenibacillus*, are of particular interest for their capacity to promote plant growth and form endospores. The production of spores provides resistance to desiccation, an attribute that is particularly attractive to agri-business. Stable spores in a dry formulation means formulated products have the potential for longer shelf-life. The ability to promote plant growth is of particular interest to the biotechnology industry for the purpose of formulating commercial products for agricultural applications. Bacterial strains belonging to the type species of the genus, *Paenibacillus polymyxa*, have been shown to have the

capacity to promote plant growth and/or provide biological disease control utilizing a variety of possible mechanisms, including biofilm formation, niche exclusion (Haggag & Timmusk, 2008), enzymic or antibiotic activity (He *et al.*, 2009), and production of phytohormones such as indole-3-acetic acid (Holl *et al.*, 1988) and cytokinin (Timmusk *et al.*, 1999). Strain AP-115^T was initially isolated in our effort to find beneficial endospore-forming bacilli for potential use in sustainable agricultural applications. AP-115^T was isolated in 1998 from a healthy cucumber plant. Subcultivation was performed on tryptone soy agar (TSA; Oxoid) at 25 °C for 24 h. The cell morphology and motility was observed under a Zeiss light microscope at a magnification of ×1000, using cells that had been grown for 3 days at 25 °C on TSA (Oxoid). Gram staining was performed by the modified Hucker method according to the protocol of Gerhardt *et al.* (1994). The KOH test was carried out according to the method of Moaledj (1986). Physiological characterization was done according to the methods described by Kämpfer *et al.* (1991) and Kämpfer (1990). In addition, presence of urease was tested on urea agar (Merck) supplemented with 2% urea according to the manufacturer's instructions (Christensen, 1946). Indole

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain AP-115^T is KU201962.

and sulphide production was tested in SIM agar according to the instructions of the manufacturer (Merck).

For phylogenetic analysis, the 16S rRNA gene of strain AP-115^T was PCR-amplified and sequenced with universal 16S rRNA gene sequence targeting primers 27F and 1492R (Lane, 1991). The final, corrected 16S rRNA gene sequence of strain AP-115^T had a size of 1480 nt and spanned gene termini 8 to 1474 (numbering according to Brosius *et al.*, 1978). Phylogenetic identification was performed with EzTaxon (Kim *et al.*, 2012) by BLAST analysis in the EzTaxon type strain 16S rRNA gene sequence database. Strain AP-115^T shared highest 16S rRNA gene sequence similarity with the type strains of *Paenibacillus amylolyticus* (98.8%), *Paenibacillus tundrae* and *Paenibacillus barcinonensis* (both 98.4%). Sequence similarities to all other species of the genus *Paenibacillus* were below 98.4%. The phylogenetic relationship of strain AP-115^T was analysed in more detail

in ARB release 5.2 (Ludwig *et al.*, 2004) using the 'All-Species Living Tree' Project (LTP; Yarza *et al.*, 2008) database release LTPs123 (September 2015). SINA (v1.2.9) was used to align sequences not included in the LTP database according to the SILVA seed alignment (<http://www.arb-silva.de>; Pruesse *et al.*, 2007) before the sequences were implemented in the LTP database. Maximum-likelihood trees were calculated with RAXML v7.04 (Stamatakis, 2006), GTR-GAMMA and rapid bootstrap analysis, neighbour-joining trees with the Jukes–Cantor correction (Jukes & Cantor, 1969), and maximum parsimony trees with DNAPARS v 3.6 (Felsenstein, 2005). All trees were calculated with 100 resamplings (bootstrap analysis; Felsenstein, 1985) and 16S rRNA gene sequences between gene termini 86 and 1402 (*Escherichia coli* numbering; Brosius *et al.*, 1978). First, trees were calculated including all species of the genus *Paenibacillus*. Further trees were then calculated, including only type strains which

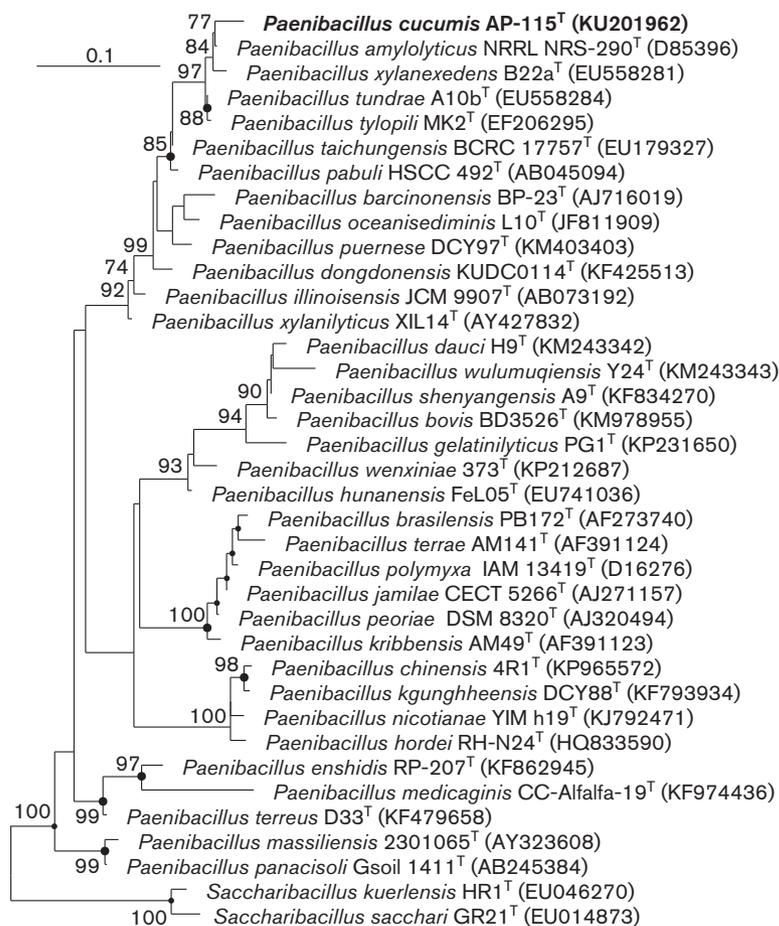


Fig. 1. Maximum-likelihood tree showing the phylogenetic position of strain AP-115^T among closely related species of the genus *Paenibacillus*. The tree was generated in ARB using RAXML (GTR-GAMMA, Rapid Bootstrap analysis) and based on 16S rRNA gene sequences between positions 86 and 1427 according to *E. coli* numbering (Brosius *et al.*, 1978). GenBank/EMBL/DBJ accession numbers are given in parentheses. Numbers at branch nodes refer to bootstrap values >70% (100 replicates). Filled circles mark nodes that were also present in respective maximum-parsimony and neighbour-joining trees. Larger circles indicate nodes that were also supported by high bootstrap values in the other trees. *Saccharibacillus kuerlensis* and *Saccharibacillus sacchari* were used as outgroups. Bar, 0.10 substitutions per site.

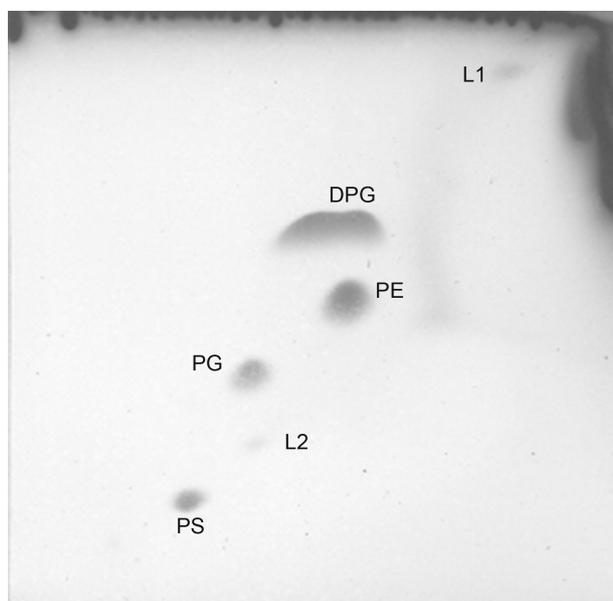


Fig. 2. Polar lipid profile of strain AP-115^T separated by two-dimensional TLC and detected with molybdatophosphoric acid. DPG, diphosphatidylglycerol; PG, phosphatidylglycerol; PE, phosphatidylethanolamine, PS, phosphatidylserine; L1, L2, unidentified polar lipids not reacting with the spray reagents specific for free amino groups, phosphate or sugar moieties.

clustered close to AP-115^T. The novel strain formed, independent of the treeing method applied, a cluster with the type strains of *P. amylolyticus*, *Paenibacillus xylanexedens*, *P. tundrae* and ‘*Paenibacillus tylopili*’ (Fig. 1). The cluster was supported by high bootstrap values.

The genomic DNA G+C content of AP-115^T was determined by the DNA melting temperature method established by Gonzales & Saiz-Jimenez (2002) as described previously (Glaeser *et al.*, 2013). The genomic DNA G+C content determined for strain AP-115^T was 51.4 mol%.

DNA–DNA hybridization was applied according to the method of Ziemke *et al.* (1998) using genomic DNA extracted by the method of Pitcher *et al.* (1989). Hybridization of genomic DNA of strain AP-115^T and *P. amylolyticus* LMG 11153^T, *P. tundrae* DSM 21291^T and *P. barcinonensis* DSM 15478^T resulted in values of 27% (reciprocal 17%), 58% (reciprocal 56%) and 41% (reciprocal 58%), respectively.

Biomass subjected to analyses of the diagnostic diamino acid of the peptidoglycan, polyamines, the quinone system and polar lipids was grown in PYE broth (0.3% peptone from casein, 0.3% yeast extract, pH 7.2) at 28 °C. Quinones and polar lipids were extracted and analysed by applying the integrated procedure reported by Tindall (1990a, b) and Altenburger *et al.* (1996). Polyamines were extracted and analysed from cells harvested at the late exponential

growth phase as reported by Busse & Auling (1988) and Altenburger *et al.* (1997). HPLC analysis was carried out using the equipment described by Stolz *et al.* (2007). The diagnostic diamino acid of the peptidoglycan was analysed according to the method of Schumann (2011).

The fatty acids were extracted and analysed as described by Kämpfer & Kroppenstedt (1996). Strains were grown under identical conditions (TSA after 72 h of incubation at 28 °C), and the cells for extractions were taken from colonies of the same size. Fatty acids were identified with Sherlock version 2.11, TSBA40 Rev. 4.1 (MIDI).

The quinone system consisted exclusively of MK-7, which supported the affiliation of strain AP-115^T to the genus *Paenibacillus*. The polar lipid profile of strain AP-115^T contained the major lipids diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol and phosphatidylserine and trace amounts of two lipids only detectable after total lipid staining (Fig. 2). The polyamine pattern contained only the major compound spermidine [15.2 µmol (g dry weight)⁻¹] and traces of putrescine, cadaverine and spermine [each <0.1 µmol (g dry weight)⁻¹]. *meso*-Diaminopimelic acid was identified as the diagnostic diamino acid of the peptidoglycan.

The fatty acids comprised mainly iso- and anteiso-branched fatty acids and were very similar to those of the most closely related species of the genus *Paenibacillus*. The detailed fatty acid profile obtained is shown in Table 1.

The results of the physiological characterization, performed using methods described previously (Kämpfer, 1990;

Table 1. Cellular fatty acid profiles of strain AP-115^T and the most closely related species of the genus *Paenibacillus*

Strains: 1, AP-115^T; 2, *P. amylolyticus* LMG 11153^T; 3, *P. tundrae* DSM 21291^T, 4, *P. barcinonensis* DSM 15478^T. All data from this study. All strains were cultured on TSA at 28 °C for 72 h. Values are percentages of total fatty acids. Only fatty acids showing relative amounts >1% are given.

| Fatty acid | 1 | 2 | 3 | 4 |
|-------------------------------|------|------|------|------|
| Saturated | | | | |
| C _{14:0} | 5.3 | 2.9 | | 3.3 |
| C _{15:0} | | | | 3.4 |
| C _{16:0} | 10.5 | 9.5 | 2.5 | 5.0 |
| Branched | | | | |
| iso-C _{14:0} | 7.2 | 2.0 | 7.1 | 16.3 |
| iso-C _{15:0} | 5.4 | 10.2 | 4.9 | 6.4 |
| anteiso-C _{15:0} | 43.8 | 51.5 | 62.3 | 38.6 |
| iso-C _{16:0} | 12.1 | 9.9 | 11.1 | 16.5 |
| iso-C _{17:0} | 1.4 | 4.5 | | |
| anteiso-C _{17:0} | 3.5 | 9.5 | 5.0 | |
| Unsaturated | | | | |
| C _{16:1} ω7c alcohol | 1.3 | | 3.5 | 2.5 |
| C _{16:1} ω11c | 6.4 | | 3.5 | 7.9 |

Table 2. Differential phenotypic characteristics of strain AP-115^T and the most closely related species of the genus *Paenibacillus*

Strains: 1, AP-115^T; 2, *P. amylolyticus* LMG 11153^T; 3, *P. tundrae* DSM 21291^T; 4, *P. barcinonensis* DSM 15478^T. All data from this study. +, Positive reaction; –, negative reaction. All species are positive for the production of catalase and negative for H₂S production.

| Characteristic | 1 | 2 | 3 | 4 |
|--------------------------|---|---|---|---|
| Acid produced from: | | | | |
| Glucose | – | + | + | – |
| Lactose | – | + | + | – |
| Raffinose | – | + | + | – |
| Assimilation of: | | | | |
| N-Acetyl-D-galactosamine | – | + | – | – |
| Cellobiose | + | – | + | + |
| D-Fructose | + | – | + | + |
| L-Rhamnose | + | – | – | – |
| myo-Inositol | – | + | – | – |
| Pyruvate | + | – | – | – |
| Hydrolysis of: | | | | |
| pNP-β-D-glucuronide | + | + | – | + |
| L-Alanine-pNA | + | – | + | + |

pNP, *para*-nitrophenyl; pNA, *para*-nitroanilide.

Kämpfer *et al.*, 1991), are given in Table 2 and in the species description.

Based on the summary of genotypic, phenotypic and chemotaxonomic results, we describe a novel species of the genus *Paenibacillus*, for which the name *Paenibacillus cucumis* is proposed. The minimal standards, as provided by Logan *et al.* (2009), were considered.

Description of *Paenibacillus cucumis* sp. nov.

Paenibacillus cucumis (cu.cu'mis. L. gen. n. *cucumis* of cucumber).

Cells (with rounded ends) stain Gram-positive. No chains or filaments can be observed after growth on TSA at 28 °C for 48 h. Cells are 2.0–3.0 µm in length and 0.8–1.0 µm in width) and show no motility. Oval endospores are formed in a central position. No other cell inclusions can be detected. Colonies grown on TSA after 48 h of incubation are circular, convex and beige with a shiny appearance and an average diameter of 2 to 3 mm. Weak growth on MacConkey agar. Optimum temperature for growth is 28–30 °C; growth occurs between 10 and 45 °C but not at 4 °C or 50 °C. Optimal pH for growth is pH 7–8; growth occurs between pH 5.5 and 9.5. Grows in the presence of 1–5 % NaCl (but not 6 % or above) tested in TS broth. Tests for catalase and oxidase activities are positive. Tests for production of indole and sulphide, gelatinase, β-galactosidase,

arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase and tryptophan deaminase, citrate utilization, and gelatin, starch and casein hydrolysis are negative. No acid formation from the sugars or sugar-related compounds D-glucose, lactose, sucrose, D-mannitol, dulcitol, salicin, D-adonitol, D-sorbitol, L-arabinose, raffinose, L-rhamnose, maltose, D-xylose, trehalose, cellobiose, erythritol, melibiose, D-arabitol or D-mannose. Urease production is weakly positive. Several sugars or sugar-related 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, gluconate, maltose, L-rhamnose, sucrose, salicin, trehalose, D-xylose, myo-inositol, D-maltitol, D-mannitol, D-mannose, D-sorbitol, malate and pyruvate. D-Adonitol, melibiose, ribose, putrescine, acetate, propionate, *cis*-aconitate, *trans*-aconitate, adipate, 4-aminobutyrate, azelate, citrate, itaconate, 2-oxoglutarate and mesaconate are not utilized as sole carbon sources. Hydrolysis of *para*-nitrophenyl-β-D-glucuronide and L-alanine-*para*-nitroanilide is positive. The predominant polyamine is spermidine. The quinone system contains mainly menaquinone MK-7. The polar lipid profile consists of the major lipids diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylglycerol and phosphatidylserine and trace amounts of two unidentified lipids. *meso*-Diaminopimelic acid is the characteristic diamino acid of the peptidoglycan, and spermidine is the predominant compound in the polyamine pattern. Major fatty acids are anteiso-C_{15:0}, iso-C_{16:0} and C_{16:0}. In addition, iso-C_{14:0}, C_{16:1ω11c}, iso-C_{15:0}, C_{14:0}, anteiso-C_{17:0} and iso-C_{17:0} are detected.

The type strain AP-115^T (=LMG 29222^T=CCM 8653^T) was isolated in Auburn, AL, USA. The isolate came from a field-grown cucumber plant.

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