Burkholderia phytofirmans sp. nov., a novel plant-associated bacterium with plant-beneficial properties

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A Gram-negative, non-sporulating, rod-shaped, motile bacterium, with a single polar flagellum, designated strain PsJN^T, was isolated from surface-sterilized onion roots. This isolate proved to be a highly effective plant-beneficial bacterium, and was able to establish rhizosphere and endophytic populations associated with various plants. Seven related strains were recovered from Dutch soils. Based on 16S rRNA gene sequence data, strain PsJN^T and the Dutch strains were identified as representing a member of the genus *Burkholderia*, as they were closely related to *Burkholderia fungorum* (98·7 %) and *Burkholderia phenazinium* (98·5 %). Analysis of whole-cell protein profiles and DNA–DNA hybridization experiments confirmed that all eight strains belonged to a single species. Strain PsJN^T had a DNA G+C content of 61·0 mol%. Only low levels of DNA–DNA hybridization to closely related species were found. Qualitative and quantitative differences in fatty acid composition between strain PsJN^T and closely related species were identified. The predominant fatty acids in strain PsJN^T were 16 : 0, 18 : 1ω7*c* and summed feature 3 (comprising 16 : 1ω7*c* and/or iso-15 : 0 2-OH). Isolate PsJN^T showed high 1-aminocyclopropane-1-carboxylate deaminase activity and is therefore able to lower the ethylene level in a developing or stressed plant. Production of the quorum-sensing signal

Abbreviations: ACC, 1-aminocyclopropane-1-carboxylate; GFP, green fluorescent protein; NAHL, *N*-acyl-homoserine lactone.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of *B. phytofirmans* strains PsJN^T, G44-5 and G6-5 are AY497470, AY836218 and AY836219.

A neighbour-joining tree showing the position of *B. phytofirmans* sp. nov. within the genus *Burkholderia*, a dendrogram derived from the protein patterns of the strains studied and cross-sections showing chickpea roots with strain PsJN^T tagged with GFP are available as supplementary figures in IJSEM Online.

compound 3-hydroxy-C8-homoserine lactone was detected. Based on the results of this polyphasic taxonomic study, strain PsJN^T and the seven Dutch isolates are considered to represent a single, novel species, for which the name *Burkholderia phytofirmans* sp. nov. is proposed. The type strain is strain PsJN^T (=LMG 22146^T=CCUG 49060^T).

Strain PsJN^T (Frommel *et al.*, 1991) was originally isolated as a contaminant from *Glomus vesiculiferum*-infected onion roots and was subsequently shown to be a highly effective plant-beneficial bacterium (reviewed by Nowak, 1998; Nowak & Shulaev, 2003). Strain PsJN^T is able to establish rhizosphere and endophytic populations associated with various plants, including potato, tomato and grapevines, where it stimulates plant growth (Frommel *et al.*, 1991; Nowak *et al.*, 1995; Pillay & Nowak, 1997; Bensalim *et al.*, 1998; Ait Barka *et al.*, 2000, 2002; Compant *et al.*, 2005) and induces developmental changes leading to better water management (Nowak *et al.*, 1995; Lazarovits & Nowak, 1997). Plants inoculated with strain PsJN^T have been reported to produce much larger root systems, with enhanced secondary roots and more root hairs, more and larger leaf hairs, sturdier stems, and greater lignin deposits around the vascular system (Nowak, 1998). Furthermore, plants inoculated with strain PsJN^T were found to contain larger amounts of phenolics and chlorophyll (Nowak *et al.*, 1997), as well as increased levels of cytokinins (Lazarovits & Nowak, 1997) and enhanced activity of phenylalanine ammonia lyase (Nowak *et al.*, 1997). Strain PsJN^T is also able to enhance resistance to low levels of potato pathogens (Nowak *et al.*, 1995) and tomato pathogens (Sharma & Nowak, 1998) as well as to reduce *in vitro* infection of grapevine by *Botrytis cinerea* (Ait Barka *et al.*, 2000, 2002). Recently, during a field experiment performed in The Netherlands, seven strains with high 16S rRNA gene sequence similarity to strain PsJN^T were isolated (RG31-12, RG47-8, RG47-15, G44-5, G6-5, RG44-4, RG6-12), mainly from the bulk and rhizosphere soil of maize and grass plants growing in an old grassland field (>50 years) (J. F. Salles and others, unpublished results). Additionally, *in vitro* dual-culture assays performed with the Dutch strains revealed that some were antagonistic to the potato pathogen *Rhizoctonia solani* AG-3 (Salles *et al.*, 2005).

Based on various biochemical and physiological tests, strain PsJN^T was originally classified as representing a non-fluorescent *Pseudomonas* sp. (Frommel *et al.*, 1991). However, subsequent studies revealed that it represents a member of the genus *Burkholderia*. Phylogenetically, the genus *Burkholderia* belongs to the *β-Proteobacteria* and currently comprises more than 30 species (Coenye & Vandamme, 2003). There are several *Burkholderia* species known to interact with plants. *Burkholderia cepacia*, the type species of the genus, was initially described as the causative agent of onion soft rot (Burkholder, 1950), but many strains belonging to the *B. cepacia* complex are also able to promote plant health (Parke & Gurian-Sherman, 2001). Similarly, other *Burkholderia* species have been reported to exhibit plant-growth-promoting or biocontrol effects (El Banna & Winkelmann, 1998; Tran Van *et al.*, 2000; Estrada de los Santos *et al.*, 2001).

Sequencing of the 16S rRNA gene was performed as described by Reiter *et al.* (2002) using the primers 8f (5′-AGAGTTTGATCCTGGCTCAG-3′), 1520r (5′-AAGGAGGTGATCCAGCCGCA-3′) and 926r (5-CCGTCAATTCCTTT(AG)AGTTT-3′). Sequence assembly was performed by using the program SEQUENCHER 4.0 (Gene Codes Corporation). Phylogenetic analysis was performed with the software package ARB (Strunk *et al.*, 2000). Multiple alignment was performed with the MULTALIN tool (Corpet, 1988) and a neighbour-joining tree based on 1298 nt was constructed using the TREECON software (Van de Peer & De Wachter, 1994) (Supplementary Fig. A in IJSEM Online). The levels of similarity between strain PsJN^T and the Dutch strains were 98·8 % (strain G44-5) and 99·2 % (strain G6-5). According to 16S rRNA gene sequence analysis, strain PsJN^T was closely related to *Burkholderia fungorum* LMG 16225^T and *Burkholderia caledonica* LMG 19076^T (both 98·6 %). 16S rRNA gene sequence similarity to *Burkholderia phenazinium* LMG 2247^T and to *Burkholderia terricola* LMG 20594^T was 98 %. Similarity values of 97·8, 97·5 and 97·4 % were found to the 16S rRNA gene sequences of *Burkholderia graminis* C4D1M^T, *Burkholderia xenovorans* LMG 21463^T and *Burkholderia phymatum* LMG 21445^T, respectively. Similarity levels to other *Burkholderia* species were below 97 %, and similarity levels to other genera belonging to the *β-Proteobacteria* were below 95·8 %.

Whole-cell protein profiles of strain PsJN^T and the seven Dutch strains were determined by SDS-PAGE (Coenye *et al.*, 2001a) and compared with over 6000 profiles in a database comprising all recognized *Burkholderia*, *Ralstonia* and *Pandoraea* species (Coenye *et al.*, 2001b, 2002). Computer-assisted numerical analysis and visual comparison of the whole-cell protein profiles of strain PsJN^T and the seven Dutch strains revealed that they were identical to each other and were clearly different from those of the recognized *Burkholderia*, *Ralstonia* and *Pandoraea* reference species (Supplementary Fig. B). Determination of the cellular fatty acid profile was determined by GLC, using the Sherlock Microbial Identification System (MIDI Inc.; database TSBA40) according to a standard protocol (Paisley, 1996). Briefly, the procedure involved saponification of whole cells in methanolic NaOH, esterification of fatty acids in acidic methanol and extraction of fatty acid methyl esters with methyl-tert-butyl ether/hexane. Strain PsJN^T showed qualitative and quantitative differences in its fatty acid composition compared with closely related species. Predominant fatty acids were 16 : 0, 18 : 1ω7*c* and summed features 2 and 3 (Table 1). A higher 18 : 1ω7*c*

Table 1. Fatty acid composition of *Burkholderia phytofirmans* sp. nov. PsJN^T and related *Burkholderia* species

Species/strains: 1, *B. phytofirmans* sp. nov. PsJN^T; 2, *B. fungorum* (9 strains studied); 3, *B. caledonica* (7); 4, *B. phenazinium* (2); 5, *B. graminis* (4); 6, *B. caribensis* (2). Values are mean percentages of total fatty acids (±SD as appropriate). Summed feature 2 comprises 14:0 3-OH, iso-16:1 I, an unidentified fatty acid with an equivalent chain-length value of 10·928 and/or 12:0 ALDE. Summed feature 3 comprises 16:1ω7*c* and/or iso-15:0 2-OH. Fatty acids for which the mean amount in all taxa was less than 1% are not given. tr, Trace amount (<1%). Data for reference species are from Coenye *et al.* (2001a).

Fatty acid	1	2	3	4	5	6
14:0	3·6	4·6±0·1	4·7±0·2	5·1±0·5	4·6±0·0	4·2±0·0
16:0	13·8	14·7±0·9	13·6±1·6	15·7±2·7	14·4±4·7	19·1±0·6
cyclo 17:0	2·1	5·1±1·6	8·4±1·5	8·4±0·5	6·9±1·8	10·0±0·2
16:1 2-OH	2·3	3·5±0·7	2·7±0·4	3·0±1·8	5·3±2·2	1·7±0·1
16:0 2-OH	2·1	3·6±0·5	2·4±0·4	2·0±1·0	3·7±1·6	2·2±0·3
16:0 3-OH	4·1	5·6±0·5	6·0±0·4	4·2±2·0	7·0±0·3	4·6±0·4
18:1ω7*c*	44·3	35·6±2·1	34·2±1·7	30·1±0·1	26·5±0·9	33·8±0·4
cyclo 19:0ω8*c*	1·8	2·5±0·7	3·7±0·7	6·3±0·1	3·3±0·9	5·3±0·1
18:1 2-OH	1·4	1·7±0·2	1·1±0·3	1·6±1·0	1·8±0·3	tr
Summed feature 2	5·6	8·1±1·1	7·4±0·9	7·3±0·7	9·8±1·7	6·1±0·6
Summed feature 3	17·6	13·6±1·9	14·5±1·8	12·6±0·1	15·7±3·0	11·2±0·2

content was found in strain PsJN^T compared with other *Burkolderia* species, whereas strain PsJN^T produced lower levels of 14:0, cyclo 17:0 and cyclo 19:0ω8*c* fatty acids. In addition to these differences, strain PsJN^T showed different levels of 16:0 3-OH, and summed features 2 and 3 compared with *B. fungorum* and *B. caledonica* (Table 1).

Strain PsJN^T was originally recovered on King's B medium (King *et al.*, 1954) incubated at 30 °C (method outlined in Nowak & Shulaev, 2003), and forms beige-coloured colonies on 1:10 strength trypticase soy agar (Becton–Dickinson) at 30 °C but not at 37 °C. Classical phenotypic tests were performed with strain PsJN^T and all Dutch strains as described by Vandamme *et al.* (1993) and Coenye *et al.* (2001a). API ZYM, API20 NE, ONPG and PNPG tests were performed according to the recommendations of the manufacturer (bioMérieux). Results of the phenotypic analyses are shown in Table 2 and in the species description. Strain PsJN^T and the seven Dutch strains could be distinguished from closely related *Burkholderia* species such as *B. fungorum* and *B. caledonica* on the basis of additional biochemical properties, including growth in 10% lactose, no growth at 37 °C, no nitrate reduction and oxidase activity (Table 2).

DNA–DNA hybridization experiments were performed as described by Coenye *et al.* (2001a). Based on 16S rRNA gene sequence data, protein profiles and biochemical data, *B. fungorum*, *B. terricola*, *Burkholderia caribensis* and *B. xenovorans* were selected as reference strains for DNA–DNA hybridization experiments. Strain PsJN^T and strain R23375 (one of the Dutch isolates) showed a high DNA–DNA hybridization value (90%). In contrast, strain PsJN^T showed relatively low DNA–DNA hybridization values towards *B. fungorum* LMG 16225^T (27%), *B. terricola* LMG 20594^T (21%), *B. caribensis* LMG 18531^T (17%), *B. graminis* LMG 18924^T (11%), *B. caledonica* LMG 19076^T (36%) and *B. xenovorans* LMG 21463^T (9%).

The G+C content of the DNA was determined as described by Coenye *et al.* (2001a). Strains PsJN^T and R23375 had G+C contents of 61·0 and 62·1 mol%, respectively, matching well with values for closely related species.

In order to show the endophytic colonization potential of strain PsJN^T, a green fluorescent protein (GFP)-tagged derivative was applied for visualization of the strain on chickpea (*Cicer arietinum* L.) plants. Plants were inoculated with cells of the GFP-tagged PsJN^T strain [10^7 c.f.u. (g vermiculite)$^{-1}$] as described by Ait Barka *et al.* (2000). Plants were harvested at different times after sowing. Aggregations of GFP-expressing bacterial cells were visualized by using an Olympus fluorescence stereomicroscope (model BH2) equipped with a double set of fluorescence filters. The filter sets consisted of a 450–490 nm band-pass excitation filter and a 520 nm suppressor filter. Microscopic analyses were performed on live intact plant tissue. Just 1 day after incubation, the rhizoplane was densely colonized by strain PsJN^T. More GFP-expressing cells were found on lateral roots than on the main root, and root cracks resulting from lateral root emergence were densely colonized. Four days after inoculation, PsJN^T cells were found in association with root epidermal cells, parenchyma cells (Supplementary Fig. C) and xylem vessels (Supplementary Fig. D). After 6 days, PsJN^T also colonized stems and leaves endophytically. In addition to chickpea endophytic colonization, the potential of strain PsJN^T was also shown with potato (Frommel *et al.*, 1991), tomato (Pillay & Nowak, 1997) and grapevines (Ait Barka *et al.*, 2002).

Activity of the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase was determined according to Penrose &

Table 2. Phenotypic characteristics of *B. phytofirmans* sp. nov. in comparison with related *Burkholderia* species

Species: 1, *B. phytofirmans* sp. nov. (8 strains tested); 2, *B. fungorum* (9); 3, *B. caledonica* (7); 4, *B. phenazinium* (2); 5, *B. graminis* (4); 6, *B. caribensis* (2). Characteristics of type strains are scored as positive (+) or negative (−); V(+), strain dependent, type strain positive; V(−), strain dependent, type strain negative. Data for reference species are taken from Coenye *et al.* (2001a). The following features are positive for all type strains: growth at 30 °C, hydrolysis of Tween 80, assimilation of L-arabinose, *N*-acetylglucosamine, D-glucose, D-mannose, D-mannitol, D-gluconate, phenylacetate and L-malate and activity for acid and alkaline phosphatase, C_8-esterase lipase and leucine arylamidase. The following features are negative for all type strains: haemolysis, growth at 42 °C, production of fluorescent pigment, growth in the presence of 4·5 % NaCl, production of acid or H_2S in triple-sugar-iron agar, assimilation of maltose, aesculin hydrolysis and activity for DNase, urease, gelatinase, tryptophan deaminase, α-glucosidase, α-galactosidase, α-fucosidase, α-mannosidase, β-glucuronidase, trypsin, α-chymotrypsin, arginine dehydrolase, ornithine decarboxylase and *N*-acetylglucosaminidase.

Characteristic	1	2	3	4	5	6
Oxidase activity	+	V(+)	−	V(+)	V(−)	−
Growth at 37 °C	−	+	V(−)	V(+)	+	+
Growth in OF medium with:						
D-Glucose	+	+	+	V(−)	V(+)	+
Maltose	V(−)	V(−)	V(+)	V(−)	−	−
Adonitol	−	V(−)	V(+)	−	V(+)	V(+)
D-Fructose	+	V(+)	+	+	+	V(+)
D-Xylose	+	V(+)	+	V(−)	V(+)	−
Growth on cetrimide	V(+)	V(+)	−	−	−	−
Nitrate reduction	−	+	−	−	+	−
Growth in the presence of:						
0·5 % NaCl	+	+	V(+)	−	V(+)	−
1·5 % NaCl	V(+)	V(+)	V(−)	−	V(+)	−
3·0 % NaCl	V(+)	−	−	−	−	−
4·5 % NaCl	V(−)	−	−	−	−	−
10 % Lactose	+	−	V(+)	−	−	−
Activity of:						
C_4-esterase	+	V(+)	V(−)	+	V(+)	V(−)
C_{14}-lipase	−	V(−)	−	−	−	−
β-Galactosidase	−	−	−	−	−	+
Acetamide deamidase	V(−)	−	−	−	−	−

Glick (2003). ACC deaminase, commonly found in plant-growth-promoting rhizobacteria (e.g. Shah *et al.*, 1998), cleaves the plant ethylene ACC, thereby lowering the ethylene level in a developing or stressed plant. Strain PsJN^T had high ACC deaminase activity; it was able to cleave 308 nmol α-ketobutyrate (mg protein)$^{-1}$ min^{-1}. It has been reported that ⩾20 nmol α-ketobutyrate (mg protein)$^{-1}$ min^{-1} is sufficient to show plant-growth-promoting effects (Penrose & Glick, 2003).

Many bacteria have evolved mechanisms to allow gene expression only when cell density is appropriate. This phenomenon is known as quorum sensing (for reviews see, for example, Whitehead *et al.*, 2001; Fuqua & Greenberg, 2002), which, in Gram-negative bacteria, is mediated by *N*-acyl-homoserine lactones (NAHLs). These low-molecular-mass compounds diffuse in and out of bacterial cells and control important biological functions such as pathogenicity or plant-growth-promoting functions (Pierson *et al.*, 1998). The production of NAHLs is common among *B. cepacia* strains (Gotschlich *et al.*, 2001) and was found to control the synthesis of protease and siderophores (Lutter *et al.*, 2001; Lewenza *et al.*, 1999). NAHL production by strain PsJN^T was determined here by TLC as described by Shaw *et al.* (1997). PsJN^T extracts and pure NAHL were visualized with the biosensors *Chromobacterium violaceum* CV026 (McClean *et al.*, 1997) and *Agrobacterium tumefaciens* NTLR4 (Cha *et al.*, 1998). Results indicated the production of 3-hydroxy-C8-homoserine lactone in strain PsJN^T.

Description of *Burkholderia phytofirmans* sp. nov.

Burkholderia phytofirmans (phy.to.fir′mans. Gr. n. *phyton* plant; L. part. adj. *firmans* strengthening; N.L. part. adj. *phytofirmans* plant-strengthening).

Cells are Gram-negative, non-sporulating, straight rods, 0·5–0·8 μm wide and 0·8–1·8 μm long, and are motile by a single polar flagellum (Frommel *et al.*, 1991). Growth is observed at 30 °C but not at 37 °C. Nitrate and nitrite are not reduced. Able to assimilate D-fructose, D-xylose and D-glucose but not adonitol. Growth occurs in the presence of 0·5 % NaCl and 10 % lactose. Additional characteristics are listed in Table 2. The following fatty acids are present in significant amounts (above 1 %): 14 : 0, 16 : 0, cyclo 17 : 0, 16 : 1 2-OH, 16 : 0 2-OH, 16 : 0 3-OH, 18 : 1ω7*c*, 18 : 0, cyclo 19 : 0ω8*c*, 18 : 1 2-OH, summed feature 2 (comprising 14 : 0 3-OH, iso-16 : 1 I, an unidentified fatty acid with an equivalent chain-length value of 10·928 and/or 12 : 0 ALDE) and summed feature 3 (comprising 16 : 1ω7*c* and/or iso-15 : 0 2-OH). The G+C content is 61·0–62·1 mol%.

The type strain, PsJN^T (=LMG 22146^T=CCUG 49060^T), was isolated from surface-sterilized onion roots at the Nova Scotia Agricultural College, Truro, NS, Canada (Nowak *et al.*, 1997). Other strains (LMG 22849–LMG 22855) were isolated from the rhizosphere of maize and grasses and from the bulk soil of a Dutch field. The properties of the type strain are the same as described above for the species. In addition, the type strain will grow on cetrimide and in the presence of 1·5 and 3·0 % NaCl, will not grow in OF medium with maltose or in the presence of 4·5 % NaCl and does not show acetamide deamidase activity. The G+C content of the type strain is 61·0 mol%.

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