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Burkholderia rhizoxinica sp. nov. and *Burkholderia endofungorum* sp. nov., bacterial endosymbionts of the plant-pathogenic fungus *Rhizopus microsporus*

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Several strains of the fungus *Rhizopus microsporus* harbour endosymbiotic bacteria for the production of the causal agent of rice seedling blight, rhizoxin, and the toxic cyclopeptide rhizonin. *R. microsporus* and isolated endobacteria were selected for freeze–fracture electron microscopy, which allowed visualization of bacterial cells within the fungal cytosol by their two parallel-running envelope membranes and by the fine structure of the lipopolysaccharide layer of the outer membrane. Two representatives of bacterial endosymbionts were chosen for phylogenetic analyses on the basis of full 16S rRNA gene sequences, which revealed that the novel fungal endosymbionts formed a monophyletic group within the genus *Burkholderia*. Inter-sequence similarities ranged from 98.94 to 100 %, and sequence similarities to members of the *Burkholderia pseudomallei* group, the closest neighbours, were 96.74–97.38 %. In addition, the bacterial strains were distinguished from their phylogenetic neighbours by their fatty acid profiles and other biochemical characteristics. The phylogenetic studies based on 16S rRNA gene sequence data, together with conclusive DNA–DNA reassociation experiments, strongly support the proposal that these strains represent two novel species within the genus *Burkholderia*, for which the names *Burkholderia rhizoxinica* sp. nov. (type strain, HKI 454^T=DSM 19002^T=CIP 109453^T) and *Burkholderia endofungorum* sp. nov. (type strain, HKI 456^T=DSM 19003^T=CIP 109454^T) are proposed.

The genus *Burkholderia* comprises a group of metabolically versatile bacteria (Yabuuchi *et al.*, 1992) that occupy remarkably diverse ecological niches (Coenye & Vandamme, 2003), such as terrestrial and aquatic environments (Zhang *et al.*, 2000; Bramer *et al.*, 2001; Goris *et al.*, 2004; Yang *et al.*, 2006), the plant rhizosphere (Viallard *et al.*, 1998; Caballero-Mellado *et al.*, 2004), animals and humans (Coenye & Vandamme, 2003). Several members of the genus *Burkholderia* live in symbiosis, e.g. with vascular plants (Van Oevelen *et al.*, 2002, 2004), arbuscular mycorrhizal (AM) fungi (Bianciotto *et al.*, 2003) and basidiomycetes (Lim *et al.*, 2003). Recently, we discovered a unique symbiosis between bacteria belonging to the genus *Burkholderia* and the saprotrophic fungus *Rhizopus microsporus*. We noted that these bacteria live within the fungal cells and that they are the true producers of rhizoxin, the causative agent of rice seedling blight (Partida-Martinez & Hertweck, 2005, 2007; Scherlach *et al.*, 2006). In a subsequent study, we found that the endofungal bacteria are also responsible for the production of the 'mycotoxin' rhizonin (Partida-Martinez *et al.*, 2007a). This intriguing phytopathogenic alliance against rice seedlings represents an unprecedented example in which a fungus hosts a bacterial population for the production of a virulence factor (Partida-Martinez & Hertweck, 2005). Since the first observation of 'bacterium-like organelles' in the cytoplasm of AM fungi in the early 1980s, only very few reports about the occurrence of endofungal bacteria have been published (Bonfante, 2003; Bianciotto *et al.*, 2003; Boer *et al.*, 2005; Lumini *et al.*, 2006). However, in all cases, the symbionts resisted cultivation in cell-free medium and could only be investigated by using molecular methods (Van Oevelen *et al.*, 2002). In contrast, bacterial endosymbionts of *R. microsporus* strains could be isolated,

Abbreviations: AM, arbuscular mycorrhizal; MP, maximum-parsimony; NJ, neighbour-joining; PP, posterior probability.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains HKI 454^T and HKI 456^T are AJ938142 and AM420302, respectively.

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grown in pure culture and reintroduced into a cured fungal host strain (Partida-Martinez & Hertweck, 2005; Scherlach *et al.*, 2006; Partida-Martinez *et al.*, 2007a, b).

In the present study, we provide further support for the occurrence of endofungal bacteria in fungal strains of the genus *Rhizopus* by freeze–fracture electron microscopy, and determine the taxonomic position of two bacterial endosymbionts by 16S rRNA gene-based phylogenetic reconstruction, DNA–DNA hybridization and further biochemical data.

Bacterial endosymbionts of *R. microsporus*

Bacterial endosymbionts of *R. microsporus* ATCC 62417 were initially visualized in the cytosol by staining fungal preparations from a growing fungal culture with a mixture of dyes (SYTO 9 and propidium iodide). The samples were treated with a fluorescence enhancer and analysed by using a confocal laser microscope, showing that mycelium of *R. microsporus* harboured a high number of living endobacteria (Partida-Martinez & Hertweck, 2005). Endosymbionts of *R. microsporus* CBS 112285, a toxinogenic strain isolated from ground nuts, were visualized by transmission electron microscopy (Partida-Martinez *et al.*, 2007a). We succeeded in isolating bacterial symbionts from mycelium of both fungal strains (Table 1). The mycelium was broken by mechanical stress (pipetting) and sedimented by centrifugation for 30 min at 13 200 r.p.m. (16 100 ***g***). A loop of the supernatant was plated on nutrient agar I (Serva) plates and incubated at 30 °C until bacterial growth was observed. Bacterial colonies were then cultured in Bacto tryptic soy broth (TSB; Difco) and preserved at −80 °C as a 1 : 1 mixture of culture broth and glycerol.

Fungus *R. microsporus* van Tieghem ATCC 62417 and its recovered isolated endobacteria were selected for freeze–fracture electron microscopy. Samples were cultivated as described above and washed twice with saline solution or fresh medium. For cryoprotection, glycerol was added to all cell suspensions to a final concentration of 20 % (v/v). Aliquots of these suspensions were enclosed between 0.1 mm thick copper profiles (BAL-TEC) as used for the sandwich double-replica technique. The sandwiches were frozen rapidly by plunging them into a 1 : 1 mixture of liquid ethane : propane cooled by liquid nitrogen. Freeze–fracturing was performed in a BAF400T (BAL-TEC) freeze–fracture device at −150 °C using a double replica stage. For replication, in a first step, 2 nm platinum was evaporated under an angle of 35° and, in a second step, about 30 nm carbon under 90°. Evaporation was done by electron-gun evaporators and controlled by thin-layer quartz crystal monitoring. Replicas were floated onto the surface of a small amount of water, cleaned in sodium hypochlorite (bacteria) and hot sulphuric acid [70 % (v/v), hyphae], washed three times with double-distilled water and mounted to mesh 400 electron microscopical copper grids. Replicas were investigated in an EM 900 electron microscope (Zeiss). Bacterial cells can be identified clearly within the fungal cytosol by their two parallel-running envelope membranes, in particular by the fine structure of the lipopolysaccharide layer of the outer membrane (Fig. 1b, d).

Table 1. Fungal strains and their symbionts

Taxon	Collection no.	Origin	Bacterial endosymbiont
Rhizopus microsporus van Tieghem var. *chinensis*	ATCC 62417	Rice seedlings, Japan	*Burkholderia rhizoxinica* HKI 454^T
Rhizopus microsporus Tieghem	CBS 112285	Ground nuts, Mozambique	*Burkholderia endofungorum* HKI 456^T

Phylogenetic analysis

To establish the taxonomic position of the bacterial symbionts, both metagenomic DNA from rhizoxin-producing fungal species and genomic DNA from the recovered endobacterial strains were isolated and amplified by using 16S universal primers, as described previously (Partida-Martinez & Hertweck, 2005). Database searches based on their full-length 16S rRNA gene sequences (1525 and 1527 bp) revealed that the isolated endosymbiotic bacteria are related closely to organisms belonging to the genus *Burkholderia*, and that each fungus contains only one bacterial strain.

For phylogenetic analyses, 16S rRNA gene sequences of 36 type strains of species belonging to the genus *Burkholderia* were retrieved from GenBank. We also included three representatives of the closely related genus *Pandoraea* and four members of the genus *Ralstonia*, which were used as outgroups. Sequences were aligned by using CLUSTAL W (Thompson *et al.*, 1997). The manually optimized alignment included 1422 sites, 264 of which were variable and 213 of which were parsimony-informative. Phylogenetic analyses were carried out in a Bayesian, maximum-parsimony and distance-matrix framework (Schmitt & Lumbsch, 2004). Bayesian tree sampling was performed by using the MRBAYES 3.1.2 program (Huelsenbeck & Ronquist, 2001; Huelsenbeck *et al.*, 2001). We employed the general time-reversible model of nucleotide evolution, including estimation of invariant sites and assuming a discrete gamma distribution (GTR + I + Γ). Two analyses, including 5×10^6 generations each, were run in parallel. Eight chains were run simultaneously. Trees were sampled every 1000 generations for a total of 2×5000 trees. The first 1×10^6 generations (i.e. the first 1000 trees of each analysis) were deleted as the 'burn-in' of the chain. We plotted the log-likelihood scores of sample points against generation time by using TRACER 1.0 (http://evolve.zoo.ox.ac.uk/software.html?id=tracer) to ensure that stationarity was

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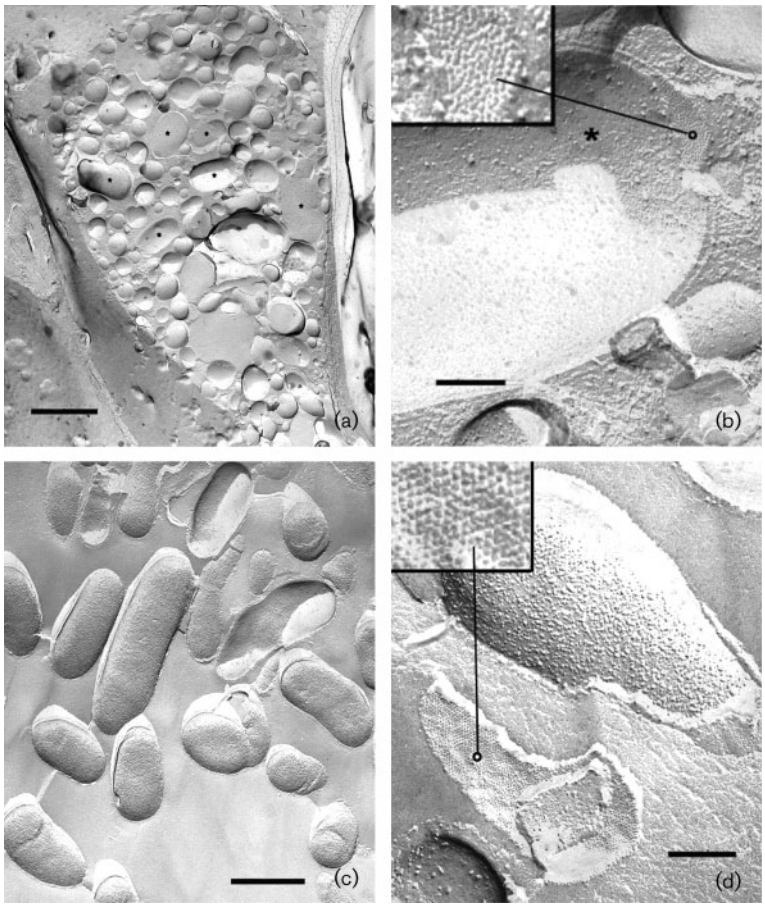

Fig. 1. Freeze–fracture electron images. (a) Fungus *R. microsporus* van Tieghem ATCC 62417 with intrahyphal bacteria (small stars). (b) Enlarged view of (a), showing the two parallel-running envelopes of a bacterium, the cytoplasmic membrane (star) and the lipopolysaccharide (LPS) layer of the outer membrane (o), with its special fine structure (inset) typical of Gram-negative bacteria. (c) Bacterial cells isolated from *R. microsporus* van Tieghem ATCC 62417 and cultivated in pure culture. (d) Cultivated bacteria at high magnification, showing the LPS layer of the outer membrane (o, inset) in comparison to (b). Micrographs were mounted with direction of platinum evaporation from bottom to top. Bars, 2 μm (a); 1 μm (c); 200 nm (b, d).

achieved after the first 1×10^6 generations by checking whether the log-likelihood values of the sample points reached a stable equilibrium value (Huelsenbeck & Ronquist, 2001). Of the remaining 2×4000 trees, a majority-rule consensus tree including branch lengths was calculated by using the 'sumt' option in MRBAYES. Branch support is calculated as posterior probability (PP), indicating the number of times a branch is present in the tree topology in relation to its presence in the total number of sampled trees. PPs $\geqslant$95 % are regarded as significant. In addition, maximum-parsimony (MP) and neighbour-joining (NJ) analyses were performed by using the program PAUP* (Swofford, 2003). For the MP analysis, 200 random-addition replicates were run. We obtained 70 most-parsimonious trees, of which we calculated the strict consensus (897 steps, consensus index=0.41, reliability index=0.72). Neighbour-joining trees were constructed by fitting the LogDet model to the data (minimum evolution), which corrects for nucleotide-substitution inequalities along branches. Branch support for MP and NJ trees was obtained by performing 2000 simple-addition bootstrap replicates. Phylogenetic trees were visualized by using the program TreeView (Page, 1996). Tree topologies obtained with different reconstruction methods were very similar and showed no supported conflicts. Therefore, only the Bayesian phylogenetic estimate is presented (Fig. 2).

Phylogenetic analyses show that the novel fungal endosymbionts form a monophyletic group within the highly supported genus *Burkholderia* (Fig. 2). Inter-sequence similarity is 99.22 %. As most phylogenetic relationships within the genus *Burkholderia* are not supported, it is difficult to determine the closest relative of the novel endosymbiont species by using the current dataset. BLAST searches against GenBank indicate that *Burkholderia mallei*, *Burkholderia pseudomallei*, *Burkholderia vietnamiensis* and *Burkholderia thailandensis* (Brett *et al.*, 1998) are probably most similar to the novel species in 16S rRNA gene sequence. Sequence similarities to these sequences are 96.74–97.38 %. Members of the *Burkholderia cepacia* complex, e.g. *B. cepacia*, *Burkholderia multivorans*, *Burkholderia pyrrocinia*, *Burkholderia stabilis* and *B. vietnamensis* (Coenye *et al.*, 2001a, b, c, d), and *Burkholderia caryophylli*, a parasitic endophyte infecting vascular plants (Burkholder, 1942), also appear to be related closely to the novel species.

Several members of the genus *Burkholderia* live in symbiosis or are associated with other organisms, e.g. with plants (Viallard *et al.*, 1998; Van Oevelen *et al.*, 2002, 2004; Caballero-Mellado *et al.*, 2004; Reis *et al.*, 2004; Sessitsch *et al.*, 2005), AM fungi (Bianciotto *et al.*, 2003) and basidiomycota (Coenye *et al.*, 2001b; Lim *et al.*, 2003). The phylogenetic placement of the novel bacterial species,

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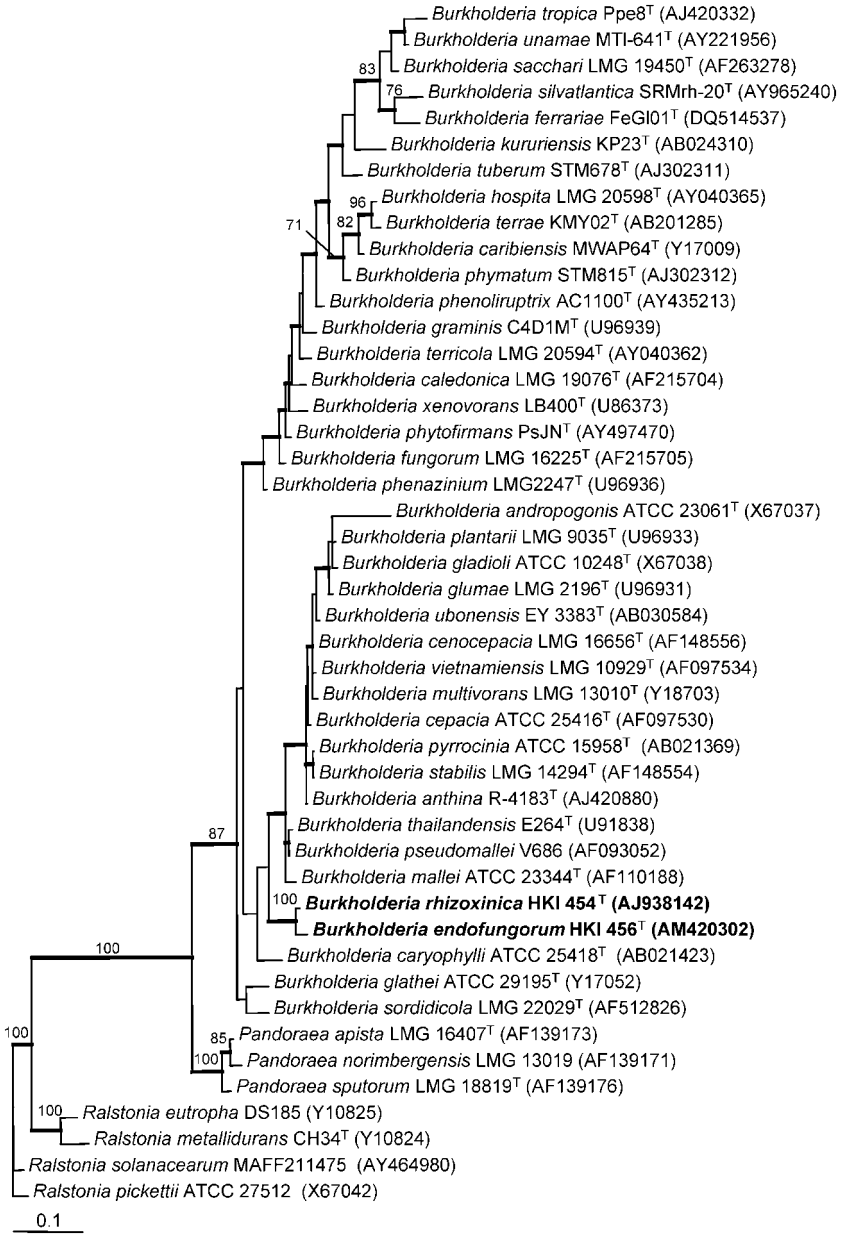

Fig. 2. Phylogenetic position of the genus *Burkholderia* and related genera based on 16S rRNA gene sequences. The newly described endofungal species are indicated in bold. GenBank accession numbers are given in parentheses. This is a 50 % majority-rule consensus tree based on 8000 trees of a Bayesian Markov chain Monte Carlo analysis. Bold branches indicate posterior probabilities >94 %. Numbers above branches are maximum-parsimony bootstrap-support values >70 %, based on 2000 pseudoreplicates. Bar, 0.1 genetic distance.

which form endosymbioses with zygomycota, gives further evidence of the broad ecological amplitude of the genus *Burkholderia*.

DNA–DNA relatedness

The results obtained from phylogenetic studies were supported strongly by DNA–DNA hybridization experiments performed by the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany. Type strains of *Burkholderia rhizoxinica* (HKI 454) and *B. thailandensis* exhibited a low DNA–DNA reassociation level (19.2 %). Analysis of the closely related strains HKI 454^T and HKI 456^T revealed a reassociation value of 43.2 %. Both values are well below 70 %. This result demonstrates that not only is HKI 454^T sufficiently distant from related *Burkholderia* species to be recognized as an independent taxospecies, but also that HKI 454^T and HKI 456^T represent distinct species.

Other taxonomic characteristics

Morphological studies, determination of growth parameters and cellular fatty acid analyses of the bacterial isolates and the type strain of *B. thailandensis*, DSM 13276^T (Brett *et al.*, 1998), were performed in TSB or on Bacto tryptic soy agar (TSA; Difco), nutrient agar I and Luria–Bertani (LB) agar at an incubation temperature of 30 °C. Cell morphology and cell dimensions were examined by phase-contrast microscopy using a Zeiss Axioscope 2

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microscope equipped with image-analysing software (Axio Vision 2.05) and by freeze–fracture electron microscopy as described above.

Gram staining was performed according to Hucker's modification (Cowan & Steel, 1965) with reagents produced by bioMérieux. Cytochrome *c* oxidase activity was studied by monitoring the oxidation of *N*,*N*,*N*′,*N*′-tetramethylphenylenediamine dihydrochloride on DrySlide oxidase (Difco). Catalase activity was determined as described by Smibert & Krieg (1994). For testing tolerance to NaCl, the cultures were grown on TSA and in TSB at 30 °C. Growth parameters of strains HKI 454^T and HKI 456^T were investigated with 300 ml aerated submerged cultures using 500 ml vessels of the multifermenter system Biostat Q (B. Braun Biotech International). Trypticase soy broth (BBL; 30 g l^{-1}) supplemented with glycerol (10 g l^{-1}) was used for cultivation. The pH value and dissolved oxygen tension were measured by respective sensors. pH of cultures was adjusted and controlled by the pH-control system of the fermenter using 0.5 M NaOH and 0.5 M HCl. For determining the pH range for growth, the pH value of the culture medium was first adjusted to the pH of the inoculum. After inoculation, the pH of the respective culture was adjusted within 4 h to the desired end value. In total, 10 different pH values ranging from 3.5 to 9.5 were tested. Viability of cells during the cultivation process was examined by plating samples on agar medium at different intervals. Growth was examined by measuring OD_{600}. Concentration of glycerol was determined by HPLC. Oxygen requirement was studied by incubation of inoculated agar plates (nutrient agar I) in GENbag microaer and GENbag anaer incubation systems (bioMérieux). For analysis of cellular fatty acids, the strains were cultivated for 48 h at 30 °C in shake flasks containing TSB. Biomass was separated from the culture broth by centrifugation. Due to the poor growth of the strains under study, biomass had to be collected from several flasks inoculated with the same culture. Fatty acid profiles were determined by using the MIDI system (Agilent) according to the instructions of the manufacturer. For testing enzymic activities by using the API ZYM system (bioMérieux), the strains under investigation were cultivated for 24 or 48 h on TSA plates. To determine the utilization of various carbon sources, the commercial test systems API 20NE, API 50CH/BE (bioMérieux) and GN2 MicroPlates (Biolog) were used. Susceptibility to antibiotics was examined by placing antibiotic discs (Difco) on nutrient agar I plates seeded with the test strains grown for 24–48 h at 30 °C in submerged cultures.

Although the endosymbiotic bacteria could be cultivated in both liquid and solid media, many of the required tests to characterize them fully could not be carried out. Growth of all isolates was poor on agar plates and typically occurred as a very thin lawn. Single colonies, if they appeared, grew very slowly. Subcultivation was generally erratic and only successful when the agar plates were incubated under high humidity. On dry agar plates, no growth occurred. Due to the poor growth of the strains under investigation, biomass had to be collected from several flasks inoculated with the same culture. Despite the facts that different cultures of the isolates were used as inocula for the test systems API 20NE, API 50CH/BE (bioMérieux) and GN2 MicroPlates (Biolog) and that repeated tests were performed, in all cases a very high number of negative results was obtained. Only a few variable results indicated that the isolates were able to hydrolyse aesculin, gelatin and Tween 80 and to utilize glycerol, L-glutamic acid and succinamic acid. Confident results were obtained for the cellular fatty acid composition (Table 2), enzymic activity using the API ZYM system and susceptibility to some antibiotics (Table 3). All major results obtained are summarized in the species description.

In conclusion, we have demonstrated that *R. microsporus* is associated with bacterial endosymbionts that can be distinguished clearly from their phylogenetic neighbours by their fatty acid profiles and other biochemical characteristics. Apparently, the endosymbionts are highly adapted to their hosts. For this reason, growth of these bacteria is erratic in pure culture and does not allow a polyphasic taxonomic study. Nevertheless, our phylogenetic studies based on 16S rRNA gene sequence data, together with the phenotypic characteristics and DNA–DNA hybridization, strongly support the proposal that the strains represent two novel species within the genus *Burkholderia*, for which the names *Burkholderia rhizoxinica* sp. nov. and *Burkholderia endofungorum* sp. nov. are proposed.

Description of *Burkholderia rhizoxinica* sp. nov.

Burkholderia rhizoxinica (rhi.zo.xi′ni.ca. N.L. n. *rhizoxinum* rhizoxin; L. f. suff. *-ica* suffix used with various meanings; N.L. fem. adj. *rhizoxinica* referring to the ability of this organism to produce the antimitotic agent rhizoxin).

Irregularly cultured, Gram-negative, motile, short or coccoid rods that occur singly, in pairs or irregular clusters. Cell size varies from 1.2 to 2.0 μm in length and from 0.6 to 1.2 μm in width. Cells are positive for oxidase and catalase. Single colonies are tiny, circular, flat, glistening and of cream colour (about 1 mm in diameter). Growth is poor on agar plates and typically occurs as a very thin lawn. Production of diffusible pigments is not observed. NaCl is tolerated well up to a concentration of 2 % (w/v). Growth is negative or weak at a concentration of 3 % (w/v) NaCl and is inhibited completely at 5 % (w/v) NaCl. The type strain is able to grow at temperatures between 16 and 45 °C and at a pH range of 5.0–7.4. Optimum pH is 6.0–6.5. Cells are not viable at pH values below 4.5 or above 7.5. In cultures without pH control, growth stops at pH 7.5. Glucose is not metabolized, but glycerol is utilized. Able to grow under aerobic and microaerophilic conditions, but not in an anaerobic atmosphere containing CO_2.

Phylogenetic distinctiveness is supported by the fatty acid profile, which is characterized by major amounts of

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Table 2. Cellular fatty acid profiles of novel *Burkholderia* strains

Strains: 1, *B. thailandensis* DSM 13276^T; 2, *B. rhizoxinica* HKI 454^T; 3, *B. endofungorum* HKI 456^T.

Fatty acid	1	2	3
Saturated			
10 : 0	0.05	–	–
12 : 0	0.05	0.14	0.13
14 : 0	3.71	3.29	3.94
15 : 0	0.43	0.11	0.14
16 : 0	28.14	14.86	17.48
17 : 0	0.64	0.11	0.13
18 : 0	0.92	1.62	1.35
12 : 0 3-OH	–	–	–
15 : 0 3-OH	0.08	–	–
16 : 0 2-OH	2.42	1.92	1.47
16 : 0 3-OH	5.10	4.75	4.95
18 : 0 2-OH	–	–	0.11
Unsaturated			
13 : 1AT12–13	–	–	–
14 : 1ω5*c*	–	–	0.09
16 : 1ω5*c*	0.30	0.13	0.29
18 : 1ω5*c*	0.49	0.21	0.23
17 : 1ω7*c*	–	–	–
18 : 1ω7*c*	0.91	37.27	21.94
18 : 3ω6*c*	–	–	–
20 : 2ω6,9*c*	0.35	0.29	0.36
16 : 1 2-OH	0.61	2.47	3.61
18 : 1 2-OH	1.63	2.77	2.51
Branched			
i-15 : 0	–	–	–
i-16 : 0	–	0.12	0.92
i-19 : 0	0.31	0.26	0.33
ai-11 : 0	0.04	–	–
ai-15 : 0	–	–	–
ai-19 : 0	0.24	–	–
10-Methyl			
17 : 0	–	–	–
11-Methyl			
18 : 1ω7*c*	0.44	0.37	0.39
Cyclo			
17 : 0	19.36	6.45	12.06
19 : 0ω8*c*	28.46	11.09	16.42
Others			
Feature 1*	–	–	–
Feature 2*	4.61	3.08	2.61
Feature 3*	0.52	8.71	8.48
Unknown 14.502	0.17	–	–
Unknown 18.814	–	–	–
16 : 0 *N* alcohol	–	–	–
ai 17 : 1ω9*c*	–	–	0.06

*Unknown fatty acids; these compounds do not have a listed name in the Peak Library File of the MIDI system and therefore were not identified.

hexadecanoic acid (C16 : 0), cyclo-heptadecanoic acid (C17 : 0 cyclo), ω-7-*cis*-octadecenoic acid (C18 : 1ω7*c*) and cyclo-ω-8-nanodecanoic acid (C19 : 0ω8*c*). Positive reactions are obtained for esterase (C4), esterase lipase (C8), leucine arylamidase, L-mannosidase and naphthol-AS-BI-phosphohydrolase. Alkaline phosphatase, cystine arylamidase, acid phosphatase and *N*-acetyl-β-glucosamidase are absent.

Table 3. Enzymic activity obtained by using the API ZYM system

Strains: 1, *B. thailandensis* DSM 13276^T; 2, *B. rhizoxinica* HKI 454^T; 3, *B. endofungorum* HKI 456^T. Characteristics: –, no enzymic reaction; +, positive enzymic reaction; w, weak enzymic reaction observed. Antibiotic susceptibility by novel endofungal bacteria (concentration per disc): –, no inhibition of growth; +, inhibition of growth; (+), slight inhibition of growth; NT, not tested.

Characteristic	1	2	3
Phosphatase alkaline	+	–	–
Esterase (C4)	+	+	+
Esterase lipase (C8)	+	+	+
Lipase (C14)	+	–	–
Leucine arylamidase	+	+	+
Valine arylamidase	+	–	+
Cystine arylamidase	+	–	–
Trypsin	–	–	–
α-Chymotrypsin	–	–	–
Phosphatase acid	+	–	–
Naphthol-AS-BI-phosphohydrolase	+	+	+
α-Galactosidase	–	–	–
β-Galactosidase	–	–	–
β-Glucuronidase	–	–	–
α-Glucosidase	–	–	–
β-Glucosidase	–	–	–
N-Acetyl-β-glucosamidase	+	–	–
α-Mannosidase	–	+	–
α-Fucosidase	–	–	–
Antibiotic sensitivity			
Ampicillin, 10 µg	–	–	–
Chloramphenicol, 30 µg	+	+	+
Ciprofloxacin, 5 µg	+	+	+
Imipenem, 10 µg	+	+	+
Kanamycin, 30 µg	–	+	+
Lincomycin, 2 µg	–	–	–
Meticillin, 5 µg	–	–	–
Nalidixic acid, 30 µg	+	+	+
Norfloxacin, 10 µg	+	+	+
Novobiocin, 5 µg	+	–	–
Ofloxacin, 10 µg	+	NT	NT
Oxytetracycline, 30 µg	+	+	+
Penicillin G, 10 IU	–	–	–
Polymyxin B, 300 IU	–	+	–
Rifampicin, 30 µg	+	NT	NT
Rifampicin, 5 µg	–	+	+
Streptomycin, 10 µg	–	+	+
Sulfonamide, 200 µg	–	–	–
Tetracycline, 30 µg	+	+	+
Vancomycin, 30 µg	–	–	–

Susceptible to chloramphenicol, ciprofloxacin, imipenem, kanamycin, nalidixic acid, norfloxacin, oxytetracycline,

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rifampicin, streptomycin and tetracycline. Resistant to ampicillin, lincomycin, meticillin, novobiocin, penicillin G, sulfonamide and vancomycin.

The type strain is HKI 454^T (=DSM 19002^T=CIP 109453^T). Isolated from the fungus *Rhizopus microsporus* van Tieghem var. *chinensis*.

Description of *Burkholderia endofungorum* sp. nov.

Burkholderia endofungorum (en.do.fun.go′rum. N.L. pref. *endo-* from Gr. *endon* within; L. gen. pl. n. *fungorum* of fungi; N.L. gen. n. *endofungorum* referring to the endosymbiotic nature of this organism with fungi).

Irregularly cultured, Gram-negative, motile, short or coccoid rods that occur singly, in pairs or irregular clusters. Cell size varies from 1.2 to 2.0 μm in length and from 0.6 to 1.2 μm in width. Cells are positive for oxidase and catalase. Single colonies are tiny, circular, flat, glistening and of cream colour (about 1 mm in diameter). Growth is poor on agar plates and typically occurs as a very thin lawn. Production of diffusible pigments is not observed. NaCl is tolerated well up to a concentration of 2 % (w/v). Growth is negative or weak at a concentration of 3 % (w/v) NaCl and is inhibited completely at 5 % (w/v) NaCl. The type strain is able to grow at temperatures between 16 and 45 °C and at a pH range of 5.0–7.4. Optimum pH is 6.0–6.5. Cells are not viable at pH values below 4.5 or above 7.5. In cultures without pH control, growth stops at pH 7.5. Glucose is not metabolized, but glycerol is utilized. Able to grow under aerobic and microaerophilic conditions, but not in an anaerobic atmosphere containing CO_2.

Phylogenetic distinctiveness is supported by the fatty acid profile, which is characterized by major amounts of hexadecanoic acid (C16 : 0), cyclo-heptadecanoic acid (C17 : 0 cyclo), ω-7-*cis*-octadecenoic acid (C18 : 1ω7*c*) and cyclo-ω-8-nanodecanoic acid (C19 : 0ω8*c*). Positive reactions are obtained for esterase (C4), esterase lipase (C8), leucine arylamidase and naphthol-AS-BI-phosphohydrolase. Alkaline phosphatase, cystine arylamidase, acid phosphatase and *N*-acetyl-β-glucosamidase are absent.

Susceptible to chloramphenicol, ciprofloxacin, imipenem, kanamycin, nalidixic acid, norfloxacin, oxytetracycline, rifampicin, streptomycin and tetracycline. Resistant to ampicillin, lincomycin, meticillin, novobiocin, penicillin G, sulfonamide and vancomycin.

The type strain is HKI 456^T (=DSM 19003^T=CIP 109454^T). Isolated from the fungus *Rhizopus microsporus* Tieghem.

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