

Burkholderia insecticola sp. nov., a gut symbiotic bacterium of the bean bug *Riptortus pedestris*

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Abstract

A Gram-negative, aerobic, rod-shaped, non-spore-forming, motile bacterium, designated strain RPE64^T, was isolated from the gut symbiotic organ of the bean bug *Riptortus pedestris*, collected in Tsukuba, Japan, in 2007. 16S rRNA gene sequencing showed that this strain belongs to the *Burkholderia glathei* clade, exhibiting the highest sequence similarity to *Burkholderia peredens* LMG 29314^T (100%), *Burkholderia turbans* LMG 29316^T (99.52%) and *Burkholderia pterochthonis* LMG 29326^T (99.04%). Phylogenomic analyses based on 107 single-copy core genes and Genome BLAST Distance Phylogeny confirmed *B. peredens* LMG 29314^T, *B. pterochthonis* LMG 29326^T and several uncultivated, endophytic *Burkholderia* species as its nearest phylogenetic neighbours. Digital DNA–DNA hybridization experiments unambiguously demonstrated that strain RPE64^T represents a novel species in this lineage. The G+C content of its genome was 63.2 mol%. The isoprenoid quinone was ubiquinone 8 and the predominant fatty acid components were C_{16:0}, C_{18:1ω7c} and C_{17:0} cyclo. The absence of nitrate reduction and the capacity to grow at pH 8 clearly differentiated strain RPE64^T from related *Burkholderia* species. Based on these genotypic and phenotypic characteristics, strain RPE64^T is classified as representing a novel species of the genus *Burkholderia*, for which the name *Burkholderia insecticola* sp. nov. is proposed. The type strain is RPE64^T (=NCIMB 15023^T =JCM 31142^T).

The bean bug *Riptortus pedestris* (Insecta: Heteroptera) is known as a notorious pest of leguminous crops in East Asia. It develops a number of sac-like tissues, called crypts, at the posterior region of the midgut, the lumen of which is densely colonized by a symbiotic bacterium [1, 2]. In addition to the insect gut, the symbiont lives in soil environments where the insect inhabits, and is acquired orally from the ambient soil to colonize the gut symbiotic organ [3]. This bacterial symbiont positively enhances host growth and fecundity [3, 4], indicating its beneficial nature to the host insect.

Previous studies provided evidence that this symbiont belongs phylogenetically to the *Burkholderia glathei* clade (BGC) of the genus *Burkholderia* [1, 2, 5]. The genus *Burkholderia* is phylogenetically diverse and BGC species have formally been reclassified into the novel genus *Caballeronia* on the basis of their phylogenetic position and the presence of five conserved sequence indels [6]. As argued earlier [7],

the coherence of the genus *Caballeronia* is inconsistent in different phylogenetic and phylogenomic analyses [7–9], and these bacteria have genomes that consist of 6.2–10.1 million nucleotides [5]. Taken together, we do not consider the presence of conserved sequence indels in five genes that are not essential for growth on either rich or minimal media [10] to be a sufficient biological basis for revising the taxonomy of dozens of species. In the present study we provide a complete taxonomic description of the *R. pedestris* symbiont and formally propose to classify it as representing a novel *Burkholderia* species, pending broader biological support for species that now also have validly published names within the genus *Caballeronia*.

In 2007, an adult insect of *R. pedestris* was collected from a pea field in Tsukuba, Japan. The insect was killed, superficially sterilized in 70% ethanol, and the symbiotic organ (midgut crypts) was carefully dissected out. The symbiotic organ was homogenized in sterilized water, and the crypt

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Abbreviations: BGC, *Burkholderia glathei* clade; dDDH, digital DNA–DNA hybridization.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA and complete genome sequences of strain RPE64^T are AB558208 and AP013058–AP013062, respectively. The accession number for the whole-genome sequence of *B. novacaledonica* LMG 28615^T is OGTP01000000. Two supplementary tables are available with the online version of this article.

contents were spread onto a yeast glucose (YG) agar plate (0.5 % yeast extract, 0.4 % glucose, 0.1 % NaCl and 1.5 % agar, pH 7.0) [11]. After incubation at 26 °C for 3 days, uniform, whitish colonies developed, from which strain RPE64^T was purified and stored as a glycerol suspension (27 %, v/v) at –80 °C.

Phenotypic analysis of strain RPE64^T and of '*Burkholderia novacaledonica*' LMG 28615^T was performed as described previously [12] to enable a comprehensive phenotypic comparison of all validly named BGC species [5, 13, 14]. Cell morphology, motility, flagellation pattern and spore formation were observed under phase-contrast microscopy (DMI-4000; Leica) and transmission electron microscopy (H-7600; Hitachi). In addition, strain RPE64^T was further characterized by testing anaerobic cultivation on YG agar at 27 °C using the Anaero Pack system (Mitsubishi Gas Chemical) and by assessing growth in YG liquid cultures at 27 °C in the presence of 0–4.0 % (w/v) NaCl (at intervals of 0.5 % units) and at different temperatures (4, 10, 15, 20, 25, 30, 35, 37 and 40 °C). Finally, physiological and biochemical characteristics were investigated by using Biolog GN MicroPlates (Biolog) according to the manufacturer's instructions.

Cells of strain RPE64^T were Gram-negative, aerobic, non-spore-forming rods (0.9 µm wide × 2.4–3.2 µm long), which were motile with two or three polar flagella (Fig. 1). The diameter of the colonies was 1–2 mm after 2 days of incubation on YG agar plates at 27 °C. Colonies were circular, white-creamy, and moist with an entire margin. Growth of strain RPE64^T was observed at 15–35 °C (optimum 30 °C), at pH 6–8 and with 0–2.5 % (w/v) NaCl (optimum 1.5 %). The lack of nitrate reduction and the capacity to grow at pH 8 clearly differentiated strain RPE64^T from related *Burkholderia* species. Table S1 (available in the online version of this article) provides a comprehensive overview of the differential biochemical characteristics of BGC species. The results from the Biolog GN MicroPlate tests are shown in Table S2.

The complete genome of strain RPE64^T has been sequenced in a previous study [15] and is available in the GenBank/EMBL/DDBJ nucleotide sequence databases under accession numbers AP013058–AP013062. The G+C content of strain RPE64^T calculated from its complete genome sequence was 63.2 mol%, a value similar to that of its nearest neighbouring species [5]. The full-length (1522 bp) 16S rRNA gene sequence of strain RPE64^T was retrieved from its genome sequence [15] and showed highest similarity [16] to those of *Burkholderia peredens* LMG 29314^T (100 %), *Burkholderia turbans* LMG 29316^T (99.52 %) and *Burkholderia pterochthonis* LMG 29326^T (99.04 %).

A phylogenomic analysis of 107 single-copy core genes, which mainly encode ribosomal and general housekeeping proteins and which are found in a majority of bacteria [17], using bcgTree [18] demonstrated that the nearest phylogenomic neighbours of strain RPE64^T were *B. peredens*, *B.*

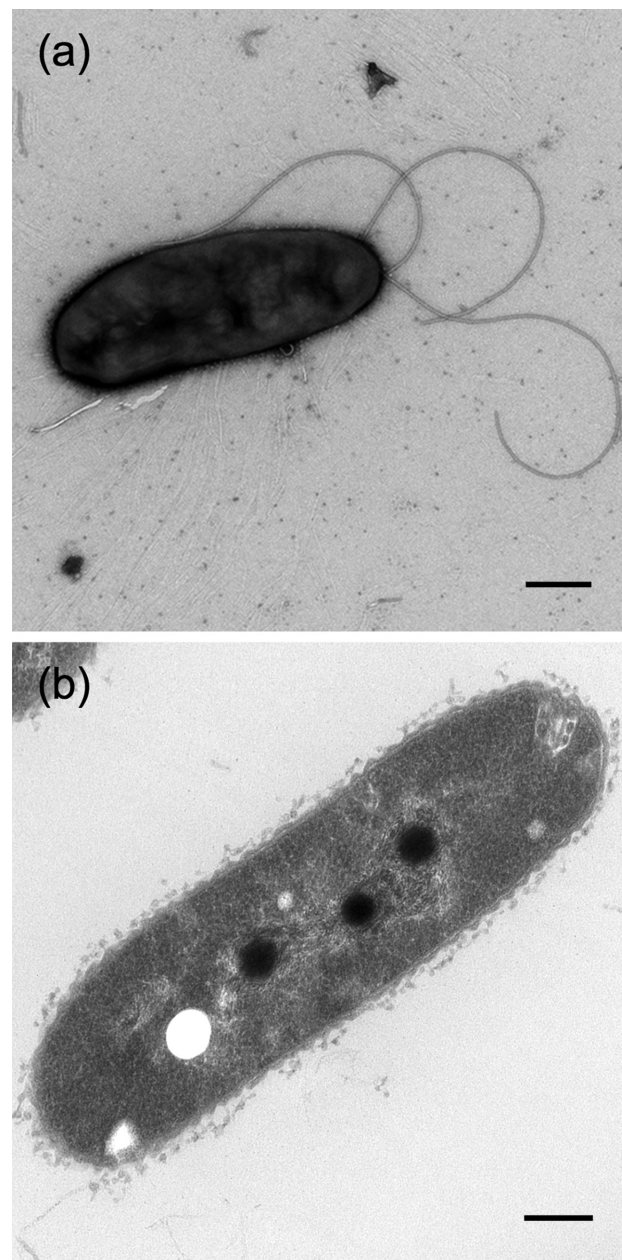


Fig. 1. Electron micrographs of cells of strain RPE64^T. (a) Transmission electron micrograph of a cell visualized by negative staining (1 %, w/v, uranyl acetate for 30 s), showing three polar flagella. Bar, 0.5 µm. (b) Ultra-thin section image of a cell taken by transmission electron microscopy. Bright and black granules within the cell are probably polyhydroxyalkanoate and polyphosphate, respectively. Bar, 0.2 µm.

pterochthonis and uncultivated, leaf nodule endophytes from *Psychotria kirkii* [19] and *Pavetta schumanniana* [20] (Fig. 2). This result corroborates and extends results from two earlier phylogenomic analyses of the BGC genomes [5]. The Genome BLAST Distance Phylogeny method was used to calculate pairwise digital DNA–DNA hybridization (dddH) values and their confidence intervals (GGDC 2.1; <http://ggdc.dsmz.de>)

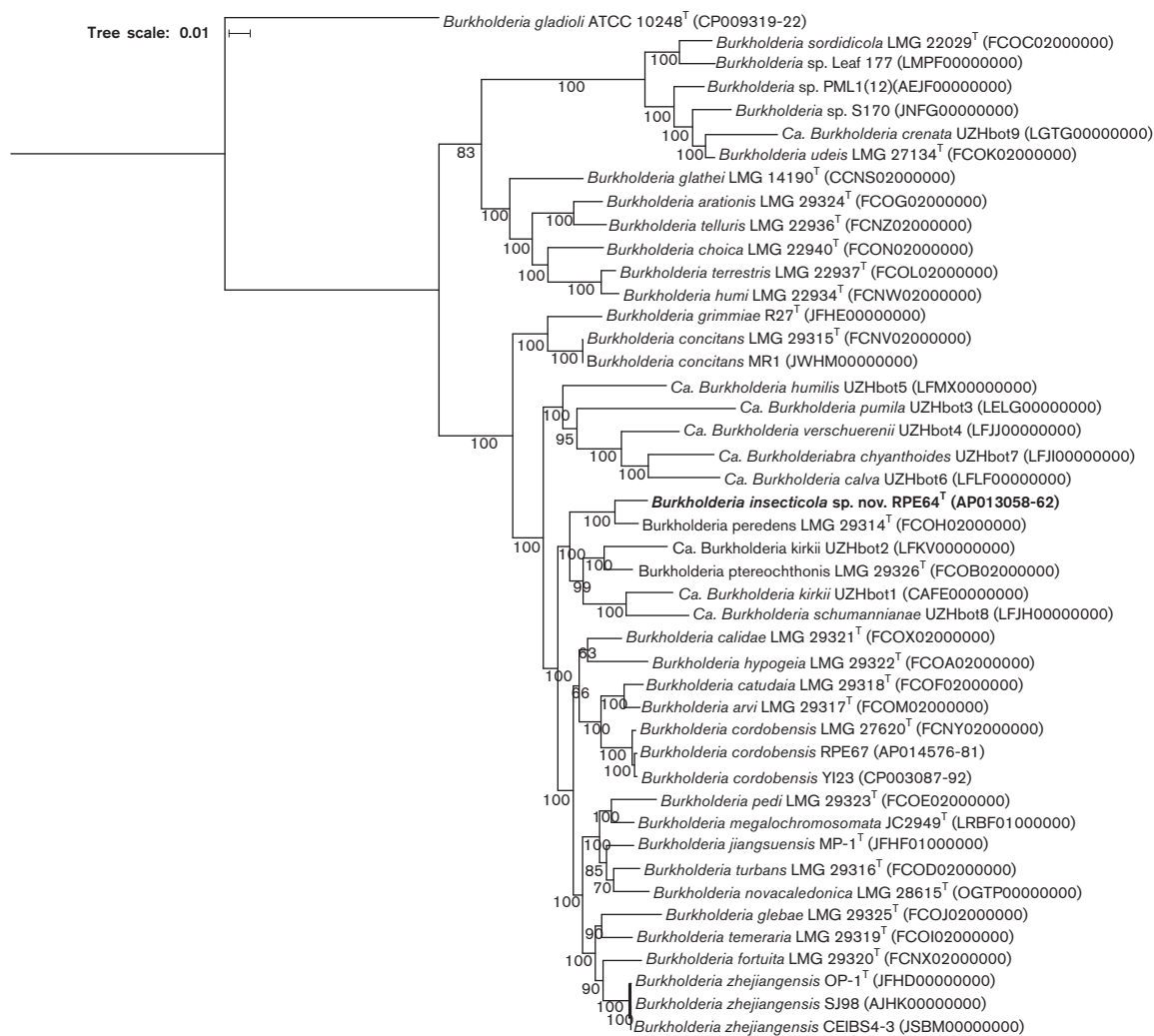


Fig. 2. Phylogenetic tree based on 107 single-copy core genes. bcgTree was used to extract the nucleotide sequence of 107 single-copy core genes and to reconstruct their phylogeny by partitioned maximum-likelihood analysis. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches. *Burkholderia gladioli* ATCC 10248^T was used as the outgroup. Bar, 0.01 changes per nucleotide position.

[21]. Values of 44.8 and 33.8% dDDH towards *B. peredens* LMG 29314^T and *B. pterochthonis* LMG 29326^T, respectively, confirmed that strain RPE64^T represents a distinct BGC species (data were taken from reference [5]).

We finally determined the cellular fatty acid and respiratory quinone components of strain RPE64^T. Whole-cell fatty acid methyl esters were extracted according to the MIDI protocol (http://www.microbialid.com/PDF/Tech-Note_101.pdf). All characteristics such as temperature, cultivation medium and physiological age (overlap area of the second and third quadrant from a quadrant streak for cells at the late logarithmic phase of growth) were as in the MIDI protocol. The profiles were generated using an Agilent Technologies 6890N gas chromatograph, identified and clustered using the Microbial Identification System software and MIDI TSBA database version 5.0.

The fatty acid components which represented more than 1% of the total were: C_{14:0} (4.8%), summed feature 2 (probably C_{14:0} 3-OH; 7.2%), summed feature 3 (probably C_{16:1}ω7c; 9.6%), C_{16:0} (13.7%), cyclo-C_{17:0} (13.1%), C_{16:1} 2-OH (1.9%), C_{16:0} 2-OH (7.0%), C_{16:0} 3-OH (7.1%), C_{18:1}ω7c (28.9%), cyclo-C_{19:0}ω8c (4.3%) and C_{18:1} 2-OH (1.1%). The presence of C_{16:0} 3-OH supports the placement of this strain in the genus *Burkholderia* and the overall profile is very similar to those of its nearest neighbours [5].

Respiratory quinone components were determined using the LC10 HPLC system (Shimadzu) with a C18 reversed-phase HPLC column, Zorbax SB-C18 (Agilent Technologies), as described previously [22]. The isoprenoid quinone of strain RPE64^T was ubiquinone 8, which is consistent with that of all other *Burkholderia* species.

In conclusion, the present genomic, phenotypic and chemotaxonomic data demonstrate that strain RPE64^T represents a novel BGC species that can be distinguished from its nearest phylogenetic neighbours both phenotypically and genotypically. We therefore propose to classify this insect symbiont [2–4, 15, 23] as representing *Burkholderia insecticola* sp. nov., with RPE64^T (=NCIMB 15023^T=JCM 31142^T) as the type strain.

DESCRIPTION OF *BURKHOLDERIA INSECTICOLA* SP. NOV.

Burkholderia insecticola sp. nov. [in.sec.ti'co.la. L. part. *insectus* divided into sections; N.L. neut. n. *insectum* insect; L. suff. *-cola* (from L. n. *incola*) a dweller, inhabitant; N.L. fem. n. *insecticola* a dweller of insects].

Cells are Gram-negative, aerobic, non-spore-forming rods that are motile by two or three polar flagella, and about 0.9 µm wide and 2.4–3.2 µm long. Colonies on YG agar plates are circular with entire margins, white-creamy in colour and moist. Growth occurs between 15 and 35 °C (optimum 30 °C) and in the presence of 0–2.5 % (w/v) NaCl (optimum 1.5 %), in YG liquid cultures at 27 °C. Grows at pH 6.0–8.0 in liquid nutrient broth at 28 °C. Grows on MacConkey agar. Catalase activity is present but no oxidase activity. Grows on Tween 40 and 60, but not on Tween 20 or 80. Hydrolyses Tween 60 but not Tween 20, 40 or 80. No hydrolysis of starch or casein. No DNase activity. In API 20NE tests, negative for reduction of nitrate, fermentation of glucose, activity of tryptophanase, arginine dihydrolase, urease and PNPG β-galactosidase, hydrolysis of aesculin and liquefaction of gelatin. Assimilates glucose, arabinose, mannose, mannitol, *N*-acetylglucosamine, gluconate, caprate, malate and phenylacetate, but not maltose, adipate or citrate. In API ZYM tests, positive for activities of C₄ lipase, C₁₄ lipase and acid phosphatase, but negative for alkaline phosphatase, C₈ lipase, leucyl arylamidase, valine arylamidase, cystine arylamidase, trypsin, chymotrypsin, phosphoamidase, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase. Other biochemical characteristics are detailed in Table S1. The major fatty acid components are C_{16:0}, C_{18:1ω7c} and cyclo-C_{17:0}. The isoprenoid quinone is ubiquinone 8.

The type strain, RPE64^T (=NCIMB 15023^T=JCM 31142^T), was isolated from the midgut crypts of the bean bug, *R. pedestris*, collected in Tsukuba, Japan in 2007 [3, 11]. The DNA G+C content of the type strain is 63.2 mol%. The complete genome sequence of the type strain is 6.96 Mb, consisting of three chromosomes and two plasmids [15], which has been deposited in GenBank/EMBL/DDBJ under accession numbers AP013058–AP013062.

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Conflicts of interest

The authors declare that there are no conflicts of interest.

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