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Burkholderia novacaledonica sp. nov. and *B. ultramafica* sp. nov. isolated from roots of *Costularia* spp. pioneer plants of ultramafic soils in New Caledonia

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ABSTRACT

The taxonomic status of eleven rhizospheric bacterial strains belonging to the genus *Burkholderia* and isolated from roots of *Costularia* (*Cyperaceae*), tropical herbaceous pioneer plants growing on ultramafic soils in New Caledonia, was investigated using a polyphasic taxonomic approach. The genetic analyses (16S rRNA genes, *gyrB*, *recA*, *nreB* and *cnr*) confirmed that all strains are *Burkholderia* and cluster into two separated groups. The DNA hybridization results showed low relatedness values to the closest relatives *Burkholderia* species. The phenotypic analyses confirmed that the two groups of strains could be differentiated from each other and from other known *Burkholderia* species. This polyphasic study revealed that these two groups of strains represent each a novel species of *Burkholderia*, for which the names *Burkholderia novacaledonica* sp. nov. (type strain STM10272^T = LMG28615^T = CIP110887^T) and *B. ultramafica* sp. nov. (type strain STM10279^T = LMG28614^T = CIP110886^T) are proposed, respectively. These strains of *Burkholderia* presented specific ecological traits such as the tolerance to the extreme edaphic constraints of ultramafic soils: they grew at pH between 4 and 8 and tolerate the strong unbalanced Ca/Mg ratio (1/19) and the high concentrations of heavy metals i.e. Co, Cr, Mn and Ni. Noteworthy *B. ultramafica* tolerated nickel until 10 mM and *B. novacaledonica* up to 5 mM. The presence of the nickel (*nreB*) and cobalt/nickel (*cnr*) resistance determinants encoding for protein involved in metal tolerance was found in all strains of both groups. Moreover, most of the strains were able to produce plant growth promoting molecules (ACC, IAA, NH_3 and siderophores). Such ecological traits suggest that these new species of *Burkholderia* might be environmentally adaptable plant-associated bacteria and beneficial to plants.

Bacteria belonging to the genus *Burkholderia*, a Betaproteobacteria group, are a multi-faceted group with large genetic and metabolic diversity and very versatile lifestyles [10,42]. Members of the genus *Burkholderia* are characterized as Gram-negative, aerobic, non-spore-forming, non-fermentative, straight or curved rod-shaped, catalase-positive bacteria and most species are motile by using a single polar flagellum or a tuft of polar flagella. *Burkholderia* are characterized by the presence of hydroxyl fatty acids of 14, 16 and 18 carbon atoms, the most characteristic of these is the $C_{16:0,3\text{-OH}}$ [49]. They show a DNA G+C content between 59.0 and 69.5 mol% [40]. Over the past two decades, research on *Burkholderia* species has been steadily expanding, as members of the genus *Burkholderia* are very abundant, occupying diverse ecological niches including soil and hospital environments [9]. At the time of the writing, the genus *Burkholderia* comprises more than 60 species, which are distributed in diverse habitats from human and animals to plants and soils [15]. Some species are regarded as beneficial to plants as they possess traits such as ability to promote plant growth, kill pest organisms, fix nitrogen or degrade man-made

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pollutants whereas others are important pathogens towards plants, animals and humans [8]. *Burkholderia* are also known as common inhabitants of the phytosphere and the internal tissues of plants in both natural and managed ecosystems. In addition, this genus is considered as the most diverse and environmentally adaptable plant-associated species including strains that are beneficial to plants and potentially exploited in biotechnological processes [10].

In the recent years, *Burkholderia* sp. have been isolated from the rhizosphere of plants growing on extreme ultramafic soils [18,37]. Ultramafic soils (also known as serpentinic soils) are produced by weathering and pedogenesis of ultramafic rocks that are characterized by high levels of heavy metals as Co, Cr, Mn and Ni, but contain low levels of available essential nutrients for plant growth such as N, P, K, and present a strong unbalanced Ca/Mg ratio (1/20) [4,22]. *Burkholderia* isolated from plants able to grow on such extreme soils, showed both tolerance to the edaphic conditions and the capacities to promote plant growth [1,18]. In this study, we report the full description and characterization of two new species of *Burkholderi*a spp. that have been recently isolated from root of pioneers plants belonging to the genus *Costularia* (*Cyperaceae*) growing on ultramafic soils in New Caledonia [18].

The locations of the study sites where the bacterial isolates were collected are shown in a map of New Caledonia (Fig. 1). The 3 sites are located in ultramafic ligno-herbaceous maquis ecosystems of which climate, geology, geomorphology and vegetation structure have been previously described by Jaffré [24] and Perrier et al. [35,36]. Sites location, geographical description and soil characteristics are summarized in Table 1. Full geochemical soil characteristics are reported in Supplemental Table 1. Plants were identified according to morphological features using the Nouméa Herbarium (New Caledonia, Nou): *Costularia* species were *C. arundinacea*, *C. nervosa*, and *C. pubescens*. A full botanical description of these sedges has been recently reported in Wulff et al. [50]. *Costularia arundinacea* was collected from sites 1, 2 and 3 while *C. nervosa* and *C. pubescens* were collected only from site 3. Full description of plant sampling and bacterial strain isolation, were previously reported in Gonin et al. [18]. The eleven strains denomination and their relationship with the site and the host plant are reported in Table 2.

The DNA sequencing was performed by Macrogen (Seoul, Korea). For this purpose, all purified isolates were cultivated separately in fresh Yeast Mannitol (YM) liquid medium [47] for 3 days at 30 °C until a dense bacterial culture was obtained. For each isolate, 1 mL of each dense culture was added to 60% sterile glycerol and sent to Macrogen (Seoul, Korea) for *recA*, *gyrB*, *nreB* and *cnrT*, amplifications and sequencing according to their procedures. Specific primers used for the sequencing were sent to Macrogen and are listed in Supplemental Table 2a. The nucleotide sequences obtained were corrected and analyzed using the sequence analysis software ChromasPro (Technelysium Pty, Helensvale, Queensland, Australia). For each bacterial isolate tested, the corrected sequences were deposited into the DNA Data Bank of Japan (DDBJ) database. Sequences were also compared with sequences already deposited in the Genbank database using BlastN analysis [2]. The phylogeny analyses were performed by using the online software "Phylogeny.fr" (http://phylogeny.lirmm.fr/phylo_cgi/index.cgi) as described by Dereeper et al. [12,13]. This software aligns DNA sequences using the Muscle algorithm [14], achieves the phylogeny using the phylogenetic algorithm phylML [3,21] and the tree rendering software TreeDyn [7].

The analyze of the 16S rRNA gene sequences and the phylogeny of the eleven strains (see Table 2 for accession numbers) clearly indicate that the strains isolated from the rhizosphere of *Costularia* plants sampled in New Caledonia (i) all belong to the genus *Burkholderia* and (ii) constitute two separate but homogenous groups (Fig. 2). The first group (G1) is constituted of the strains STM10272, STM10274, STM10275, STM10276, STM10277 and STM10278 among which the chosen type strain is STM10272^T. As shown in Fig. 2 presenting the phylogenetic relationship based upon the 16S rRNA gene sequence analysis within the closest *Burkholderia* species, the group of strains G1 clusters with *Burkholderia zhejiangensis* OP1^T [30] and *B. grimmiae* R27^T [45] of which the 16S rRNA gene sequence of the type strain shows 97.0% of similarity to each of them. The second group (G2) is constituted of the strains STM10279, STM10280, STM10281, STM10282 and STM10283 among which the chosen type strain is STM10279^T. As shown in Fig. 2, the group of strains G2 clusters with *Burkholderia ginsengisoli* KMY03^T [25], *B. terricola* R8118^T [19] and *B. xenovorans* LB400^T [20]. The 16S rRNA gene sequence of the type strain shows 98.0% of similarity to *B. ginsengisoli* and *B. terricola* and 97.0% of similarity to *B. xenovorans*.

In addition, the phylogenetic relationship based upon analysis of the *gyrB* sequences (cf. accession number in Table 2) within the closest *Burkholderia* species showed that the group of strains G1 clusters also with these most highly related type strains indicated above as shown in Supplemental Fig. 1: the *gyrB* gene segment of the type strain STM10272^T showed 95.0% and 92.0% similarities with the sequences of *Burkholderia zhejiangensis* OP1^T and *B. grimmiae* R27^T, respectively. The phylogenetic relationship based upon the analysis of *gyrB* sequences of the group of strains G2 (accession number in Table 2) within the closest *Burkholderia* species showed that sequences cluster also with the most highly related type strains indicated above (Supplemental Fig. 1): the *gyrB* gene segment of the type strain STM10279^T showed 87.0% and 85% similarities with the sequences of *Burkholderia terricola* and *B. xenovorans*, respectively.

Similarly, the phylogenetic relationship based upon the analysis of the *recA* [34] sequences (cf. accession number in Table 2) within the closest *Burkholderia* species showed that the group of strains G1 clusters also with these most highly related type strains indicated above (Supplemental Fig. 2). The *recA* gene segment of the type strain STM10272^T showed 95.0% and 91.0% similarities with the sequences of *Burkholderia zhejiangensis* OP1^T [30] and *B. grimmiae* R27^T [45], respectively (Supplemental Fig. 2). The phylogenetic relationship based upon the analysis of the *recA* sequences (accession number in Table 2) within the closest *Burkholderia* species showed that the group of strains G2 clusters also with most of highly related type strains indicated above (Supplemental Fig. 2). The *recA* gene segment of the type strain STM10272^T showed 95.0% similarities with the sequences of *Burkholderia xenovorans* and *B. terricola*, respectively (Supplemental Fig. 2).

The spectroscopic DNA–DNA hybridization was carried out as described by De Ley et al. [11] under consideration of the modifications described by Huss et al. [23]. Using a model Cary 100 Bio UV/VIS-spectrophotometer equipped with a Peltier-thermostated 6 × 6 multicell changer and a temperature controller with in situ temperature probe (Varian, Germany). The whole genome DNA–DNA hybridization experiments were performed between strains STM10272^T and the type strains of the nearest phylogenetic neighbours: *Burkholderia glathei* [46], *B. grimmiae and B. zhejiangensis*. The percentage of DNA–DNA similarity reached 48.4% between strain STM10272^T and *B. zhejiangensis* DSM 28073^T, 34.0% between strain STM10272^T and *B. grimmiae* DSM 25160^T and 22.9% between strain STM10272^T and *B. glathei* DSM 50014^T. The whole genome DNA–DNA hybridization experiments were performed between strains STM10279^T and the type strains of the nearest phylogenetic neighbours: *Burkholderia ginsengisoli*, *B. terricola and B. xenovorans*. The percentage of DNA–DNA similarity reached 39.9% between the strain STM10279^T and *B. terricola* DSM17221^T, 23.0% between the strain STM10279^T and *B. ginsengisoli* DSM21638^T, and only 13.7% between the strain STM10279^T and *B. xenovorans* DSM17367^T.