Burkholderia cordobensis sp. nov., from agricultural soils

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Two Gram-negative, rod-shaped bacteria were isolated from agricultural soils in Córdoba province in central Argentina. Their 16S rRNA gene sequences demonstrated that they belong to the genus *Burkholderia*, with *Burkholderia zhejiangensis* as most closely related formally named species; this relationship was confirmed through comparative *gyrB* sequence analysis. Whole-cell fatty acid analysis supported their assignment to the genus *Burkholderia*. *Burkholderia* sp. strain YI23, for which a whole-genome sequence is available, represents the same taxon, as demonstrated by its highly similar 16S rRNA (100 % similarity) and *gyrB* (99.1–99.7 %) gene sequences. The results of DNA–DNA hybridization experiments and physiological and biochemical characterization further substantiated the genotypic and phenotypic distinctiveness of the Argentinian soil isolates, for which the name *Burkholderia cordobensis* sp. nov. is proposed, with strain MMP81^T (=LMG 27620^T=CCUG 64368^T) as the type strain.

Members of the genus *Burkholderia* are betaproteobacteria and are aerobic, chemo-organotrophic and motile (except for *Burkholderia mallei*), Gram-negative rods, that display a wide physiological versatility, allowing them to inhabit extremely different ecological niches (Coenye & Vandamme, 2003; Compant *et al.*, 2008). Several species of the genus *Burkholderia* are human or animal pathogens; these include members of the *Burkholderia cepacia* complex, a group of closely related species commonly isolated from respiratory specimens of cystic fibrosis patients (Vandamme & Dawyndt, 2011), and species belonging to the *Burkholderia pseudomallei* group, which includes *B. pseudomallei*, the aetiological agent of melioidosis in humans, and *B. mallei*, the causative agent of glanders in animals (Galyov *et al.*, 2010). However, strains of *Burkholderia*, including *B. cepacia* complex bacteria (Parke & Gurian-Sherman, 2001), may also have beneficial characteristics that include biological nitrogen fixation, phytohormone synthesis, plant defence induction and xenobiotic activity (Suárez-Moreno *et al.*, 2012; Vial *et al.*, 2011). Also, a growing number of species of the genus *Burkholderia* lives associated with other organisms: these include endophytes from plant tissues including root nodules, and inhabitants of insect guts or fungal mycelium and spores (Gyaneshwar *et al.*, 2011; Kikuchi *et al.*, 2005; Levy *et al.*, 2003; Mahenthiralingam *et al.*, 2008; Verstraete *et al.*, 2013).

Strains of *Burkholderia* occur commonly in pristine, agricultural or polluted soils, in which their population density can reach levels of 10^3–10^5 c.f.u. (g soil)$^{-1}$ (Pallud *et al.*, 2001; Salles *et al.*, 2006b), but antagonistic species of the genus *Burkholderia* can be lost when soils are used continuously under arable management schemes (Salles *et al.*, 2006a). As part of the BIOSPAS Consortium (http://www.biospas.org/en), we analysed the composition of populations of *Burkholderia* across Argentinian agricultural soils that were subjected to different agricultural management regimes. A thorough description of site characteristics and treatment definitions was published elsewhere (Figuerola *et al.*, 2012). From these samples, 1 g soil was suspended in

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA and *gyrB* gene sequences of strains LMG 27620^T and LMG 27621 are HG324048 and HG324055 (LMG 27620^T) and HG324049 and HG324056 (LMG 27621), respectively. The accession number for the *gyrB* gene sequence of *B. grimmiae* LMG 27580^T is HG324054.

A supplementary figure is available with the online version of this paper.

10 ml saline solution (0.85 % w/v NaCl), shaken at 240 r.p.m. for 30 min, vortexed for 1 min, sonicated in a water bath for 30 s and centrifuged for 2 min at 500 r.p.m. An aliquot (200 µl) of the soil suspension was plated onto modified semi-selective medium PCAT (but using citrulline instead of tryptamine as the main nitrogen source; PCAT refers to *Pseudomonas cepacia*, azelaic acid and tryptamine) containing (l^{-1}) 0.1 g $MgSO_4$, 0.2 g citrulline, 2 g azelaic acid, 4 g K_2HPO_4, 4 g KH_2PO_4, 0.02 g yeast extract and 15 g agar. The pH was adjusted to 5.7. The medium was supplemented with cycloheximide (0.2 g l^{-1}) and crystal violet (0.002 g l^{-1}) to inhibit growth of fungi and Gram-positive bacteria, respectively (Burbage *et al.*, 1982). After 5 days of incubation at 28 °C, colonies were selected randomly, subcultured on LB medium (l^{-1}: 10 g tryptone, 5 g yeast extract, 10 g NaCl, 15 g agar) to check purity and maintained at −80 °C as a suspension with 20 % (v/v) glycerol. Two isolates from soils from Monte Buey, in Córdoba province (32° 58′ 14″ S 62° 27′ 06″ W), MMP81^T (=LMG 27620^T, isolated in 2010) and MAN52 (=LMG 27621, isolated in 2011), were characterized further in the present study.

When analysing our data, we found highly similar 16S rRNA gene and *gyrB* sequences in the whole-genome sequence of strain YI23, which was isolated from a golf course soil in South Korea and which showed the ability to degrade the pesticide fenitrothion (GenBank accession numbers CP003087–CP003092; Lim *et al.*, 2012).

For PCR, genomic DNA was prepared using the method described by Niemann *et al.* (1997). Randomly amplified polymorphic DNA patterns were generated using the primers 5′-TGCGCGCGGG and 5′-AGCGGGCCAA, as described previously (Williams *et al.*, 1990), and demonstrated that isolates LMG 27620^T and LMG 27621 represent genetically distinct strains (Fig. S1, available in the online Supplementary Material).

Nearly complete sequences of the 16S rRNA genes of strains LMG 27620^T and LMG 27621 were obtained as described previously (Peeters *et al.*, 2013). The MOTHUR software package (Schloss *et al.*, 2009) was used to align the 16S rRNA gene sequences of strains LMG 27620^T, LMG 27621 and YI23 (1484 bp) with those of type strains of phylogenetically related species of the genus *Burkholderia* (1388–1525 bp) against the SILVA reference database (http://www.arb-silva.de). Phylogenetic analysis was conducted in MEGA5 (Tamura *et al.*, 2011) (Fig. 1). Uncorrected pairwise distances were calculated using MEGA5, and all ambiguous positions were removed for each sequence pair. Strains LMG 27620^T, LMG 27621 and YI23 had identical 16S rRNA gene sequences, which were highly similar to those of *Burkholderia zhejiangensis* OP-1^T (99.7 % similarity) and *Burkholderia grimmiae* R27^T (99.3 %) (Fig. 1).

Partial sequences of the *gyrB* genes of strains LMG 27620^T and LMG 27621 and their nearest neighbours as determined by 16S rRNA gene sequence proximity were obtained using the method described by Spilker *et al.* (2009) as modified by

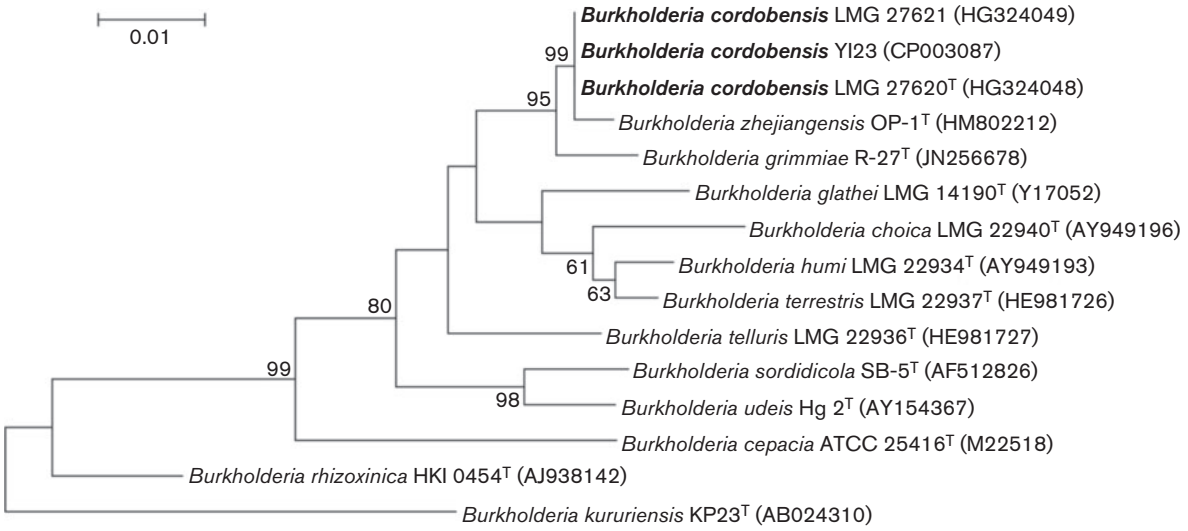

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences of strains LMG 27620^T, LMG 27621 and YI23 and phylogenetically related members of the genus *Burkholderia*. The tree was reconstructed using the maximum-likelihood method based on the Hasegawa–Kishino–Yano model (Hasegawa *et al.*, 1985). The tree with the highest log-likelihood (−3205.5147) is shown. The percentage of trees in which the associated taxa clustered together (from 1000 bootstrap replicates) is shown next to branches if greater than 50 %. A discrete gamma distribution was used to model evolutionary rate differences among sites [5 categories (+*G*, parameter=0.0500)] and allowed for some sites to be evolutionarily invariable ([+*I*], 10.0430 % sites). All positions containing gaps and missing data were eliminated. There were a total of 1335 positions in the final dataset. The 16S rRNA gene sequence of *Burkholderia kururiensis* KP23^T was used as an outgroup. Bar, 0.01 substitutions per site.

De Meyer *et al.* (2013). Sequence assembly was performed using BioNumerics version 5.10 (Applied Maths). Sequences (589–704 bp) were aligned based on amino acid sequences using MUSCLE (Edgar, 2004) in MEGA5 (Tamura *et al.*, 2011). Phylogenetic analysis of nucleotide sequences was conducted in MEGA5 (Fig. 2) and uncorrected pairwise distances were calculated using MEGA5. Strains LMG 27620^T, LMG 27621 and YI23 formed a homogeneous cluster (sequences were >99 % similar), which was supported by a bootstrap value of 75 % (Fig. 2) and grouped with *B. zhejiangensis* LMG 27258^T (95.3–95.9 %) and *B. grimmiae* LMG 27580^T (90.8–91.6 %) as nearest neighbours.

For DNA–DNA hybridization and the determination of the DNA G+C content, high-molecular-mass DNA was prepared as described by Pitcher *et al.* (1989). DNA–DNA hybridizations were performed using a microplate method and biotinylated probe DNA (Ezaki *et al.*, 1989). The hybridization temperature was 45±1 °C. Reciprocal reactions (A×B and B×A) were performed and the variation was within the limits of this method (Goris *et al.*, 1998). DNA–DNA hybridization experiments were performed between strain LMG 27620^T and *B. zhejiangensis* LMG 27258^T, its nearest named phylogenetic neighbour (Figs 1 and 2). DNA–DNA hybridization between the two strains was 44 %. The DNA G+C content was determined by HPLC according to the method of Mesbah & Whitman (1989) using a Waters Breeze HPLC system and XBridge Shield RP18 column thermostabilized at 37 °C. The solvent was 0.02 M $NH_4H_2PO_4$ (pH 4.0) with 1.5 % (v/v) acetonitrile. Non-methylated lambda phage (Sigma) and *Escherichia coli* DNA were used as calibration reference and control, respectively. The DNA G+C content of strain LMG 27620^T was 63.6 mol%, which is within the range reported for members of the genus *Burkholderia* (59.0–69.9 mol%; Gillis *et al.*, 1995; Yabuuchi *et al.*, 1992) and which corresponds well to the DNA G+C content of 63.5 mol% reported for the whole-genome sequence of strain YI23 (Lim *et al.*, 2012).

Phenotypic analysis of strains LMG 27620^T and LMG 27621 and *B. zhejiangensis* LMG 27258^T was performed on tryptone soya agar (TSA) at 28 °C unless indicated otherwise (LMG medium 14; http://bccm.belspo.be/db/media_search_form.php). Cell morphology and motility were observed by phase-contrast microscopy. Oxidase activity was detected by immersion of cells in 1 % *N*,*N*,*N*′,*N*′-tetramethyl *p*-phenylenediamine solution and catalase activity was determined by bubble formation after flooding a colony with 10 % H_2O_2. Lipase activity was determined according to the method described by Sierra (1957). Growth on MacConkey medium was observed after 48 h of incubation at 28 °C. Starch hydrolysis was observed after 48 h of incubation at 28 °C in LMG 14 medium amended with 2 % starch. DNase activity was observed after 48 h of incubation at 28 °C on BD Difco DNase test agar, according to the method of Jeffries *et al.* (1957). Casein hydrolysis was observed after 48 h of incubation at 28 °C on TSA plates amended with 1.3 % skimmed milk, through the observation of clear haloes around colonies. Other biochemical tests were performed by inoculating API 20NE and API ZYM strips (bioMérieux) according to the manufacturer's instructions and incubating for 48 h at 28 °C or for 4 h at 28 °C, respectively. Growth was tested at 28 °C in nutrient broth (NB; BD Difco) at pH 4–9 using appropriate biological buffers (acetate, citrate/

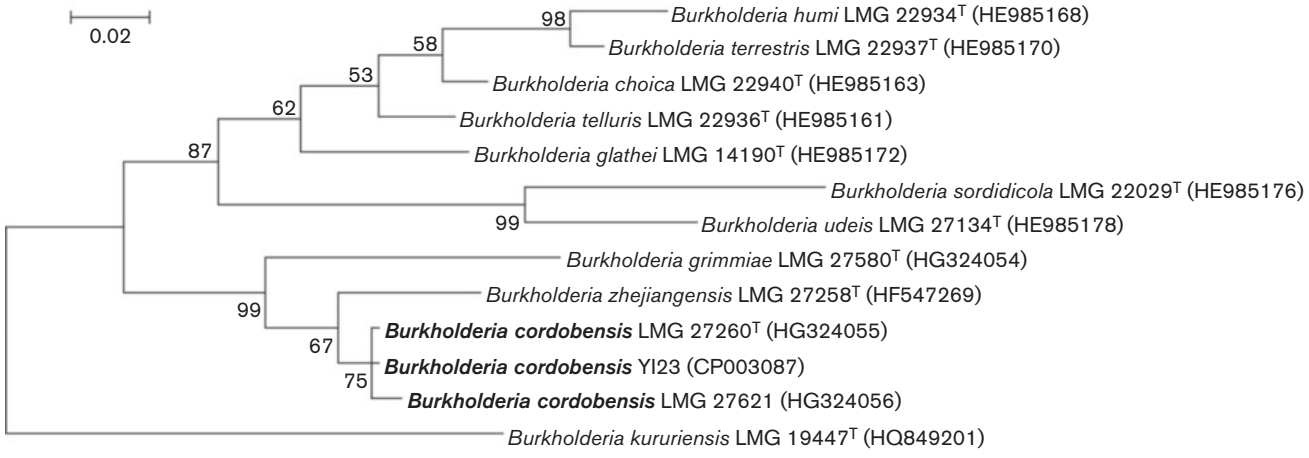

Fig. 2. Phylogenetic tree based on *gyrB* gene sequences of strains LMG 27620^T, LMG 27621 and YI23 and phylogenetically related members of the genus *Burkholderia*. The tree was reconstructed using the maximum-likelihood method based on Tamura's three-parameter model (Tamura, 1992). The tree with the highest log-likelihood (−2409.0868) is shown. The percentage of trees in which the associated taxa clustered together (from 1000 bootstrap replicates) is shown next to branches. A discrete gamma distribution was used to model evolutionary rate differences among sites [5 categories (+*G*, parameter=0.2933)]. All positions containing gaps and missing data were eliminated. There were a total of 570 positions in the final dataset. The *gyrB* sequence of *Burkholderia kururiensis* LMG 19447^T was used as an outgroup. Bar, 0.02 substitutions per site.

Na_2HPO_4, phosphate buffer and Tris/HCl). Growth on LMG 14 medium was tested at 4, 15, 28, 30, 37, 40 and 45 °C. The results of phenotypic and biochemical tests are given in the species description and in Table 1. They allow clear differentiation of the taxon represented by strains LMG 27620^T and LMG 27621 and its nearest phylogenetic neighbour, *B. zhejiangensis*: the former exhibits oxidase activity but not arginine dihydrolase or urease activity, reduces nitrate, hydrolyses Tween 80 and does not assimilate caprate; opposite test results were obtained for the latter. In addition, on the basis of phenotypic data published by Tian *et al.* (2013) using the same test gallery, strains LMG 27620^T and LMG 27621 can be differentiated from *B. grimmiae* R27^T: the former exhibits no arginine dihydrolase or urease activity and assimilates phenylacetate. In addition, it does not hydrolyse starch and grows on MacConkey agar; opposite test results were obtained for *B. grimmiae* R27^T. Differential characteristics towards other species of the genus *Burkholderia* in this same lineage are presented in Table 1.

Whole-cell fatty acid methyl esters were extracted according to the MIDI protocol (http://www.microbialid.com/PDF/TechNote_101.pdf). All characteristics such as temperature, medium and physiological age (overlap area of the second and third quadrant from a quadrant streak for late-exponential-phase growing cells) were as in the MIDI protocol. The profiles were generated using an Agilent Technologies 6890N gas chromatograph and identified and clustered using the Microbial Identification System software and MIDI TSBA database version 5.0. The most abundant fatty acids of strains LMG 27620^T and LMG 27621 were $C_{18:1}\omega7c$, summed feature 3 (probably $C_{16:1}\omega7c$) and $C_{16:0}$. Moderate to small amounts of summed feature 2 (probably $C_{14:0}$ 3-OH), $C_{17:0}$ cyclo, $C_{16:0}$ 3-OH, $C_{14:0}$, $C_{19:0}$ cyclo $\omega8c$, $C_{16:1}$ 2-OH and $C_{16:0}$ 2-OH were also detected. The presence of $C_{16:0}$ 3-OH supports the placement of these strains in the genus *Burkholderia* (Yabuuchi *et al.*, 1992), but the overall profile of the novel taxon is very similar to those of its nearest neighbours (Table 2).

The phenotypic, chemotaxonomic and genotypic data from the present study demonstrate that strains LMG 27620^T, LMG 27621 and YI23 represent a novel species of the genus *Burkholderia* that can be distinguished from its nearest phylogenetic neighbours both phenotypically as well as genotypically. We therefore propose to classify these bacteria

Table 1. Phenotypic characteristics that distinguish *B. cordobensis* sp. nov. from its nearest phylogenetic neighbours

Strains: 1. *B. cordobensis* sp. nov. LMG 26720^T and LMG 27621; 2, *B. zhejiangensis* LMG 27258^T, LMG 26180 and LMG 26181; 3, *B. grimmiae* R27^T; 4, *B. choica* LMG 22940^T; 5, *B. glathei* LMG 14190^T; 6, *B. humi* LMG 22934^T;7, *B. sordidicola* LMG 22029^T; 8, *B. telluris* LMG 22936^T; 9, *B. terrestris* LMG 22937^T; 10, *B. udeis* LMG 27134^T. Results for *B. cordobensis* sp. nov. and its nearest neighbour *B. zhejiangensis* are from the present study; test results of the type strains are given first, followed by the remaining strains in the order given above. Data for *B. grimmiae* R27^T were extracted from Tian *et al.* (2013); data for the remaining strains were taken from Vandamme *et al.* (2013). +, Present; −, absent; w, weak reaction; v, variable; ND, not determined.

Characteristic	1	2	3	4	5	6	7	8	9	10
Growth at:										
37 °C	+−	+++	+	w	+	−	−	w	−	−
40 °C	−−	+++	+	−	−	−	−	−	−	−
pH 8	ww	+++	+	−	−	+	w	−	−	+
Hydrolysis of Tween 60	++	+++	ND	+	−	−	−	+	−	−
Nitrate reduction (API 20NE)	w+	+++	+	−	−	−	+	+	−	+
β-Galactosidase (API 20NE)	−−	−−−	−	−	−	−	+	−	−	w
Assimilation of (API 20NE):										
Arabinose	+−	+++	+	w	w	−	+	+	−	+
Mannose	++	+++	+	−	+	+	+	+	+	+
Mannitol	++	−−−	+	w	w	+	+	+	+	−
N-Acetylglucosamine	++	+++	+	w	+	+	+	+	+	+
Gluconate	++	+++	+	w	+	+	+	+	+	+
Caprate	−−	+++	−	−	+	w	−	+	w	−
Malate	++	+++	+	w	+	+	v	+	+	+
Citrate	−−	−−−	−	−	+	+	−	+	+	+
Phenylacetate	++	++w	−	−	+	+	−	+	+	−
Enzyme activity (API ZYM)										
C_4 lipase	−−	−−−	+	w	+	+	+	+	+	+
C_8 lipase	−−	−−−	+	+	w	+	+	w	w	+
Valine arylamidase	−−	−−−	−	w	w	−	−	−	w	+
Cystine arylamidase	−−	−−−	−	+	−	−	−	−	−	−
β-Galactosidase	−−	−−−	−	−	−	−	+	−	−	+

Table 2. Mean fatty acid compositions of *B. cordobensis* sp. nov. and of its nearest phylogenetic neighbours

Species/strains: 1, *B. cordobensis* sp. nov. LMG 27620^T and LMG 27621; 2, *B. zhejiangensis* LMG 27258^T, LMG 26180 and LMG 26181; 3, *B. grimmiae* LMG 27258^T; 4, *B. choica*; 5, *B. glathei*; 6, *B. humi*; 7, *B. sordidicola*; 8, *B. telluris*; 9, *B. terrestris*; 10, *B. udeis*. Data for *B. cordobensis* sp. nov. and *B. grimmiae* LMG 27258^T are from the present study. The remaining data were extracted from Vandamme *et al.* (2013) and were generated under the same cultivation and analysis conditions. Values are mean ± SD percentages of total fatty acids. Those fatty acids for which the mean amount for all taxa was <1 % are not included; therefore, the percentages may not add up to 100 %. TR, Trace amount (<1 %); ND, not detected.

Fatty acid	1	2	3	4	5	6	7	8	9	10
$C_{12:0}$	ND	ND	TR	ND	ND	ND	3.70	ND	ND	4.63 ± 0.07
$C_{14:0}$	4.16 ± 0.04	4.08 ± 0.13	4.02	3.54 ± 0.13	4.29 ± 0.13	3.71 ± 0.27	0.79	3.00 ± 0.01	3.80	TR
$C_{16:0}$	17.00 ± 0.80	16.24 ± 0.64	12.14	20.37 ± 4.40	19.11 ± 1.93	12.00 ± 0.76	18.29	13.31 ± 0.15	16.02	15.72 ± 0.96
$C_{16:0}$ 2-OH	2.49 ± 0.79	3.37 ± 0.82	4.41	3.26 ± 1.54	2.06 ± 0.86	2.43 ± 0.55	3.32	1.61 ± 0.14	2.29	2.05 ± 0.10
$C_{16:0}$ 3-OH	5.92 ± 0.04	5.91 ± 0.13	5.02	6.93 ± 2.32	6.13 ± 0.49	5.17 ± 0.38	4.90	5.05 ± 0.04	5.36	6.75 ± 0.91
$C_{16:1}$ 2-OH	TR	TR	4.37	ND	TR	1.71 ± 0.32	4.06	TR	1.29	2.02 ± 0.22
$C_{17:0}$ cyclo	5.31 ± 1.86	4.96 ± 0.65	TR	14.30 ± 4.67	13.05 ± 2.41	7.64 ± 2.05	13.94	4.54 ± 0.13	6.58	6.57 ± 0.99
$C_{18:1}\omega7c$	36.62 ± 1.2	36.88 ± 1.05	38.78	30.97 ± 1.41	31.10 ± 1.83	42.19 ± 1.77	27.61	43.66 ± 0.55	38.00	32.56 ± 1.27
$C_{19:0}$ cyclo $\omega8c$	1.82 ± 0.72	2.14 ± 0.37	ND	ND	2.64 ± 1.29	TR	7.01	TR	ND	3.61 ± 0.68
Summed features*										
2	7.21 ± 0.15	6.82 ± 0.35	5.32	7.80 ± 2.28	7.08 ± 0.53	6.24 ± 0.42	4.86	5.71 ± 0.08	6.18	7.53 ± 0.60
3	17.55 ± 2.11	17.43 ± 0.60	23.16	12.84 ± 4.21	12.79 ± 2.79	17.14 ± 1.55	11.53	20.55 ± 0.57	18.98	17.20 ± 1.46

*Summed feature 2 comprised iso-$C_{16:1}$ I and/or $C_{14:0}$3-OH; summed feature 3 comprised iso-$C_{15:0}$ 2-OH and/or $C_{16:1}\omega7c$.

in a novel species, for which the name *Burkholderia cordobensis* sp. nov. is proposed.

Description of *Burkholderia cordobensis* sp. nov.

Burkholderia cordobensis (cor.do.ben′sis. N.L. fem. adj. *cordobensis* pertaining to the Argentinian province of Córdoba, where the first strains were isolated).

Gram-negative, aerobic, motile, non-spore-forming rods, about 0.4–0.7 μm wide and 1.2–1.8 μm long. Colonies are round, with entire margins, a convex elevation, a white–creamy colour and a moist appearance, and are 1–2 mm in diameter after 48 h of growth on TSA at 28 °C. Grows on MacConkey agar. Growth occurs at 28–37 °C and at pH 6–8 in NB at 28 °C. Catalase and oxidase activities are present, but not arginine dihydrolase, urease, β-glucosidase, gelatinase or β-galactosidase. When tested by using API ZYM strips, activities of the following enzymes are present: alkaline and acid phospatases, leucyl arylamidase and phosphoamidase; activities are absent for C_4 lipase, C_8 lipase, C_{14} lipase, valine and cystine arylamidases, trypsin, chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α- mannosidase and α-fucosidase. Tweens 20, 40, 60 and 80 are degraded, but casein and starch are not hydrolysed. When tested by using API 20NE strips, nitrate is reduced and glucose, arabinose (variable; type strain positive), mannose, mannitol, *N*-acetylglucosamine, gluconate, malate and phenylacetate are assimilated, but not maltose, caprate, adipate or citrate. Negative for fermentation of glucose, activities of tryptophanase, arginine dihydrolase, urease and β-galactosidase (PNPG), hydrolysis of aesculin and gelatin liquefaction. The most abundant fatty acids are $C_{18:1}\omega7c$, summed feature 3 (probably $C_{16:1}\omega7c$) and $C_{16:0}$.

The type strain, MMP81^T (=LMG 27620^T=CCUG 64368^T), was isolated from agricultural soils in Córdoba province, Argentina. The DNA G+C content of the type strain is 63.6 mol%. The whole-genome sequence of strain YI23 is 8.89 Mb and consists of three chromosomes and three plasmids (Lim *et al.*, 2012).

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