Proposal to accommodate *Burkholderia cepacia* genomovar VI as *Burkholderia dolosa* sp. nov.

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Phenotypic and genotypic studies revealed new tools for differentiating *Burkholderia cepacia* genomovar VI from *Burkholderia multivorans* and other *B. cepacia*-complex species. Hence, the name *Burkholderia dolosa* sp. nov. is proposed, with LMG 18943^T (=CCUG 47727^T) as the type strain. *B. dolosa* can be differentiated from other *B. cepacia*-complex bacteria by its inability to assimilate tryptamine, azelaic acid and salicin and by its failure to grow on the *B. cepacia*-selective medium PCAT. Both 16S rDNA and *recA* RFLP analysis revealed unique *B. dolosa* restriction patterns. In addition, new 16S rDNA- and *recA*-based PCR assays allowed its specific identification.

The *Burkholderia cepacia* complex harbours notorious lung pathogens for patients with cystic fibrosis and comprises nine genomovars: *B. cepacia* (genomovar I), *Burkholderia multivorans* (genomovar II), *Burkholderia cenocepacia* (genomovar III), *Burkholderia stabilis* (genomovar IV), *Burkholderia vietnamiensis* (genomovar V), *B. cepacia* genomovar VI, *Burkholderia ambifaria* (genomovar VII), *Burkholderia anthina* (genomovar VIII) and *Burkholderia pyrocinia* (genomovar IX) (Coenye *et al.*, 2001a, b; Vandamme *et al.*, 1997, 2002, 2003). In the present study, we describe biochemical tests, a 16S rDNA-based specific PCR test and several PCR-RFLP approaches for differentiating *B. cepacia* genomovar VI from other *B. cepacia*-complex bacteria, and propose to classify this bacterium formally as *Burkholderia dolosa* sp. nov.

Details of 18 *B. dolosa* isolates collected in the course of a long-term study on the biodiversity of *B. cepacia*-complex bacteria are available as supplementary material in IJSEM Online. The isolates were obtained from specimens from cystic fibrosis patients from the USA, Canada, France and the UK and from environmental samples. Isolates were grown aerobically on trypticase soy agar at 30 °C.

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The GenBank/EMBL/DDBJ accession numbers for the *recA* genes of strains LMG 21820 and LMG 21443 are AY324807 and AY324808.

Details of the 18 isolates of *B. dolosa* sp. nov. included in this study are available as supplementary material in IJSEM Online.

DNA was prepared as described by Pitcher *et al.* (1989). Amplification of the *recA* gene and subsequent digestion of the amplicon with *Hae*III and *Mn*II were performed as described by Mahenthiralingam *et al.* (2000). Three *Hae*III restriction patterns could be distinguished. Most isolates (including all isolates described by Coenye *et al.*, 2001a) were characterized by a previously described RFLP type designated Q (Vandamme *et al.*, 2002). Two other RFLP types, one represented only by isolate LMG 21443 and the other represented by isolates LMG 21820 and R-17373, were observed (data not shown). *Mn*II restriction patterns of type Q isolates and of isolates LMG 21820 and R-17373 were identical, but distinct from that of isolate LMG 21443 (data not shown).

DNA–DNA hybridization experiments were performed with photobiotin-labelled probes in microplate wells as described by Ezaki *et al.* (1989), using an HTS7000 Bio Assay reader (Perkin Elmer) for the fluorescence measurements. The hybridization temperature was 50 °C. DNA–DNA binding values between isolates representing the three *Hae*III *recA* RFLP types (LMG 18941, LMG 21443 and LMG 21820) were above 87 %. Combined with the previously reported high values (> 97 %) obtained between type Q isolates LMG 18941, LMG 18942 and LMG 18943^T (Coenye *et al.*, 2001a), these high DNA–DNA hybridization values indicate that *B. dolosa* forms a homogeneous genospecies.

Analysis of the *recA* gene sequences of isolates LMG 21820

and LMG 21443 was performed as described by Mahenthiralingam *et al.* (2000). The sequences were aligned and compared with *recA* gene sequences from *B. dolosa* and other *B. cepacia*-complex isolates analysed in previous studies (Coenye *et al.*, 2001b; LiPuma *et al.*, 2001; Mahenthiralingam *et al.*, 2000; Vandamme *et al.*, 2000; Vermis *et al.*, 2002a). The *recA* sequence of *Burkholderia* sp. LB400 was obtained from the LB400 genome sequencing project (http://genome.jgi-psf.org/draft_microbes/burfu/burfu.home.html) and was used as the outgroup. A phylogenetic tree based on the neighbour-joining method (Saitou & Nei, 1987) was constructed by using the KODON (Applied Maths) software package. All *B. dolosa* isolates formed a single *recA* lineage (Fig. 1).

Amplification of 16S rDNA was performed using the primers described by Ségonds *et al.* (1999). Subsequent digestion of the amplicon using the restriction enzymes *AluI*, *CfoI* and *DdeI* was performed as described by Mahenthiralingam *et al.* (2000). The *B. dolosa* isolates generated two restriction-profile combinations that were not observed for other bacteria. Thirteen cystic fibrosis isolates, all from the USA, revealed a *CfoI* restriction profile distinct from that of isolates R-8868, R-15304, LMG 21443, LMG 21820 and R-17373. These two sets of restriction profiles correspond to the *AluI*–*CfoI*–*DdeI* restriction patterns ACC and ADC, respectively (Vermis *et al.*, 2002b).

On the basis of previously published 16S rDNA sequences (Coenye *et al.*, 2001a), a *B. dolosa*-specific PCR assay was developed. The assay was performed in a final volume of 25 µl composed of 50 mM KCl, 20 mM Tris/HCl, 1.5 mM MgCl₂, 1 U *Taq* polymerase (Invitrogen), 0.4 µM each primer (G6F1, 5'-GATTAATACCGCATACGATCCAC-3'; G6B2, 5'-GGATTTCACATCGGTCTTAGGG-3'), 200 µM

each dNTP and 2 µl lysed whole bacteria as template. Amplification was carried out using a PTC-100 programmable thermal cycler (BIOzym). After initial denaturation for 3 min at 95 °C, 30 amplification cycles were completed, each consisting of 1 min at 94 °C, 1 min at 62 °C and 2 min at 72 °C, followed by a final extension for 6 min at 72 °C. A total of 160 isolates were tested: all 18 *B. dolosa* isolates from the present study and an additional 20 *B. dolosa* isolates from US patients with cystic fibrosis, nine *B. cepacia* (genomovar I) isolates, 10 *B. multivorans* isolates, 18 *B. cenocepacia* isolates, seven *B. stabilis* isolates, 10 *B. vietnamiensis* isolates, nine *B. ambifaria* isolates, 19 *B. anthina* isolates, nine *B. pyrrocinia* isolates, four *Burkholderia gladioli* isolates, 13 *Pandorea* isolates, five *Ralstonia* isolates, three *Achromobacter* isolates and six *Pseudomonas* isolates. All *B. dolosa* isolates, except one, generated an amplicon of 448 bp. There were no false-positive reactions, resulting in sensitivity and specificity of 97 and 100 %, respectively.

A *B. dolosa*-specific *recA* primer was also designed. Published *recA* genes from all current genomovars were aligned and the *B. dolosa*-diagnostic primer G6N (positions 136–120 in the *recA* gene of LMG 18943^T; 5'-CGAGC-GAGCCGGTCTCGAT-3') was designed. The primer contained three bases specific to *B. dolosa recA* and absent at those positions in all other *B. cepacia*-complex *recA* genes: the 3' base (T; A in coding sequence) and the two further mismatched bases shown underlined in the primer sequence given above. In combination with *recA* primer BCR1 as the forward primer (5'-TGACCGCCGAGAAGAGCAA-3'; Mahenthiralingam *et al.*, 2000) and primer G6N as the reverse primer, a PCR under standard conditions (Mahenthiralingam *et al.*, 2000) with an annealing temperature of 67 °C produced a 135 bp amplicon from *B. dolosa* strains R-2879, LMG 18942, LMG 18943^T, R-6140 and R-8869. The *B. dolosa recA* PCR failed to produce any amplification products when tested against controls representing genomovars I–VII (data not shown). The *B. dolosa recA* diagnostic PCR was not tested against *B. anthina* or *B. pyrrocinia* strains. However, analysis of *recA* sequence from these species demonstrated that the mismatches described above were present, and indicated that the diagnostic PCR would not amplify *recA* from these species.

The carbon-source-assimilation patterns and growth ability on *B. cepacia*-selective media [BCSA (Henry *et al.*, 1997), PCAT (Burbage & Sasser, 1982)] and *B. cepacia*-selective agar (Mast Diagnostics) of 142 *B. cepacia*-complex isolates, representing all genomovars, were evaluated in a separate study (Vermis *et al.*, 2003). All *B. dolosa* isolates could be grown on BCSA and MAST selective agar, but not on PCAT agar; in contrast, more than 85 % of the remaining *B. cepacia*-complex isolates did. *B. dolosa* isolates did not assimilate azelaic acid or tryptamine, the two carbon sources present in PCAT. In addition, none of the *B. dolosa* isolates could utilize salicin, but they were all able

Fig. 1. *recA*-based phylogenetic tree showing the position of *B. dolosa* sp. nov. among other *B. cepacia*-complex bacteria. The scale bar represents 10 % sequence dissimilarity. The *recA* sequence of *Burkholderia* sp. LB400 was obtained from the LB400 genome sequencing project (http://genome.jgi-psf.org/draft_microbes/burfu/burfu.home.html).

to grow on L-arabinose. In comparison, all other *B. cepacia*-complex genomovars were able to utilize azelaic acid. Growth on tryptamine, salicin and L-arabinose was strain dependent for *B. vietnamiensis* (growth was observed for 1 of 15 isolates), *B. multivorans* (5 of 19 isolates) and *B. stabilis* (4 of 14 isolates).

Description of *Burkholderia dolosa* sp. nov.

Burkholderia dolosa (do.lo'sa. L. fem. adj. *dolosa* deceitful, unwilling, referring to the absence of growth on a *B. cepacia*-selective medium).

Gram-negative, small (1.5–2.5 µm long), motile, rod-shaped cells. Biochemical characteristics are listed by Coenye *et al.* (2001a). In addition, isolates fail to grow on the *B. cepacia*-selective medium PCAT and are unable to utilize azelaic acid, tryptamine or salicin. Major fatty acid components are 16:0 (about 26%), 17:0 cyclo (about 16%), 19:0 cyclo ω8c (about 15%), 18:1 (about 10%), summed feature 3 (comprising 14:0 3-OH, 16:1 iso, 12:0 alde, an unidentified fatty acid with equivalent chain length of 10.928 or a combination of these fatty acids) (about 9%) and 16:0 3-OH (about 6%). The G+C content of the DNA is 66.9–67.7 mol%. Compared with other *B. cepacia*-complex bacteria, strains generate unique 16S rDNA RFLP and *recA* RFLP patterns and can be identified using a specific 16S rDNA-based PCR assay. Isolates have been obtained from the environment and from sputum of patients with cystic fibrosis.

The type strain is LMG 18943^T (=CCUG 47727^T). The type strain and other *B. dolosa* reference strains are available from the BCCM/LMG and CCUG culture collections.

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