Burkholderia latens sp. nov., *Burkholderia diffusa* sp. nov., *Burkholderia arboris* sp. nov., *Burkholderia seminalis* sp. nov. and *Burkholderia metallica* sp. nov., novel species within the *Burkholderia cepacia* complex

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The taxonomic position of five *recA* gene clusters of *Burkholderia cepacia* complex (Bcc) isolates was determined using a polyphasic taxonomic approach. The levels of 16S rRNA and *recA* gene sequence similarity, multilocus sequence typing (MLST) data and the intermediate DNA–DNA binding values demonstrated that these five clusters represented five novel species within the Bcc. Biochemical identification of these species is difficult, as is the case for most Bcc species. However, identification of these novel species can be accomplished through *recA* gene sequence analysis, MLST and BOX-PCR profiling and by *recA* RFLP analysis. For diagnostic laboratories, *recA* gene sequence analysis offers the best combination of accuracy and simplicity. Based on these results, we propose five novel Bcc species, *Burkholderia latens* sp. nov. (type strain FIRENZE 3^T = LMG 24064^T = CCUG 54555^T), *Burkholderia diffusa* sp. nov. (type strain AU1075^T = LMG 24065^T = CCUG 54558^T), *Burkholderia arboris* sp. nov. (type strain ES0263A^T = LMG 24066^T = CCUG 54561^T), *Burkholderia seminalis* sp. nov. (type strain AU0475^T = LMG 24067^T = CCUG 54564^T) and *Burkholderia metallica* sp. nov. (type strain AU0553^T = LMG 24068^T = CCUG 54567^T). In the present study, we also demonstrate that *Burkholderia ubonensis* should be considered a member of the Bcc.

Abbreviations: Bcc, *Burkholderia cepacia* complex; MLST, multilocus sequence typing; RFLP, restriction fragment length polymorphism.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains LMG 24064^T, LMG 24065^T, LMG 24066^T, LMG 24067^T and LMG 24068^T are AM747628–AM747632, and those of the *recA* sequences of strains LMG 24275, LMG 24066^T, R-24202, LMG 19587, LMG 24271, LMG 24265, R-22599, R-23401, LMG 24067^T, LMG 24065^T, LMG 24270, LMG 19239 and LMG 24064^T are AM748094–AM748105 and AM922300.

Results of DNA–DNA hybridization experiments, *HaeIII*/*MnII* *recA* gene RFLP patterns and a dendrogram derived from UPGMA linkage of correlation coefficients between BOX-PCR fingerprints are available as supplementary material with the online version of this paper.

INTRODUCTION

The *Burkholderia cepacia* complex (Bcc) is a diverse group of bacteria (Coenye *et al.*, 2001c; Vandamme *et al.*, 1997) which are ubiquitous in nature (Coenye & Vandamme, 2003). Some Bcc strains are recognized for their beneficial properties as plant-growth promoters, bioremediators and biocontrol strains (Parke & Gurian-Sherman, 2001), whereas others are notorious as pathogens causing infections in plants and immunocompromised individuals such as cystic fibrosis patients (Coenye & Vandamme, 2003; Mahenthalingam *et al.*, 2005).

The Bcc currently comprises nine species, which share a high degree of 16S rRNA gene (98–100%) and *recA*

(94–95 %) sequence similarity and moderate levels of DNA–DNA hybridization (30–50 %) (Coenye *et al.*, 2001c); they are *B. cepacia* (Vandamme *et al.*, 1997), *B. multivorans* (Vandamme *et al.*, 1997), *B. cenocepacia* (Vandamme *et al.*, 1997, 2003), *B. stabilis* (Vandamme *et al.*, 1997, 2000), *B. vietnamiensis* (Gillis *et al.*, 1995; Vandamme *et al.*, 1997), *B. dolosa* (Vermis *et al.*, 2004), *B. ambifaria* (Coenye *et al.*, 2001b), *B. anthina* (Vandamme *et al.*, 2002) and *B. pyrrocinia* (Vandamme *et al.*, 2002). Identification of these Bcc species is a complicated challenge, requiring a combination of multiple molecular diagnostic procedures, and is mostly restricted to reference centres (Coenye *et al.*, 2001c). Studies have revealed that the 16S rRNA gene, which is widely used for bacterial systematics, is limited in its ability to differentiate the Bcc species. In contrast, analysis of *recA* sequence variation correlated well with species status (Mahenthiralingam *et al.*, 2000b, 2002). Analysis of species-specific polymorphisms in the *recA* gene has now been superseded by a single, robust multilocus sequence typing (MLST) scheme for the Bcc that provides both species and strain identification (Baldwin *et al.*, 2005).

An efficient working algorithm for the identification of Bcc strains starts with a *recA*-based Bcc-specific PCR test (Mahenthiralingam *et al.*, 2000b), followed by restriction fragment length polymorphism (RFLP) analysis of the amplicon using restriction enzymes *Hae*III and *Mn*II, which reveal sequence variation within the complex. Currently, more than 70 Bcc *Hae*III RFLP types have been found (P. Vandamme, unpublished data). Several species include multiple RFLP types (Coenye *et al.*, 2001c; Mahenthiralingam *et al.*, 2000b), and overlap of certain RFLP types is observed in different species (Mahenthiralingam *et al.*, 2000a; McDowell *et al.*, 2001; Vandamme *et al.*, 2002). In particular, isolates showing RFLP types H, J and W should be further investigated using the second restriction enzyme, *Mn*II, for unambiguous species identification. At present, the large majority of these *Hae*III RFLP types can be assigned to one of the established nine Bcc species. To avoid identification problems due to inaccurate or overlapping RFLP types, strains with novel RFLP types should be subjected to *recA* gene nucleotide sequence analysis to enable phylogenetic prediction of species status (Mahenthiralingam *et al.*, 2002). This working strategy has revealed several new groups of isolates that could not be assigned to one of the established species. The aim of the present study was to determine the taxonomic status of five such clusters using a polyphasic approach. We also present data that demonstrate that *Burkholderia ubonensis* (Yabuuchi *et al.*, 2000) should be considered a member of the Bcc based on its genetic relatedness with the other Bcc species.

METHODS

Bacterial strains and growth conditions. The strains studied represented five *recA* lineages that are referred to as clusters 1 to 5

(Table 1). Clusters 1 and 5 contained six and five isolates, respectively, which were all recovered from sputum samples of cystic fibrosis patients. Clusters 2, 3 and 4 comprised clinical and environmental isolates and contained 14, 17 and 21 isolates, respectively. All strains were routinely cultured on trypticase soy agar (TSA) and incubated at 28 °C unless indicated otherwise. Pure cultures were maintained on Microbank vials at –80 °C.

DNA preparation. For PCR and RFLP experiments, DNA was prepared by alkaline lysis as described before (Storms *et al.*, 2004). For DNA–DNA hybridization experiments and the determination of the DNA base composition, DNA was prepared as described by Pitcher *et al.* (1989) or by Marmur (1961). The latter procedure was followed when the former did not yield sufficient pure DNA.

Bcc-specific *recA* gene PCR. The Bcc *recA* gene (1040 bp) was amplified using primers BCR1 and BCR2, as described previously (Mahenthiralingam *et al.*, 2000b).

***recA* RFLP and *recA* gene sequence analysis.** Amplified *recA* fragments of all isolates were subjected to *Hae*III-based RFLP analysis (Mahenthiralingam *et al.*, 2000b). When the *recA* RFLP pattern was type H, J or W, *Mn*II RFLP analysis was also performed. Electrophoretic separation of the restriction fragments was performed by PAGE, as described previously (Vanlaere *et al.*, 2005). Restriction patterns were analysed using the Bionumerics 4.5 software package (Applied Maths) and compared to those of Bcc reference strains. *recA* sequence analysis was performed as described previously (Mahenthiralingam *et al.*, 2000b). If not available, nucleotide sequences of the *recA* gene from representative strains were determined and deposited in the EMBL public database. All other sequences were available from public databases. Multiple alignment was performed by using the CLUSTAL_X program (Thompson *et al.*, 1997). The aligned sequences were analysed phylogenetically using the Bionumerics 4.5 software. Distances were calculated using the Jukes & Cantor algorithm. Phylogenetic trees based on the neighbour-joining method were constructed with bootstrap values of 1000 replications.

16S rRNA gene sequence analysis. 16S rRNA gene sequence analysis was performed as described previously (Coenye *et al.*, 2001a). 16S rRNA gene sequences were determined and deposited in the EMBL public database.

Determination of the DNA base composition. DNA was degraded enzymically into nucleosides as described previously (Mesbah & Whitman, 1989). The nucleoside mixture obtained was then separated using a Waters Breeze HPLC system and XBridge Shield RP18 column thermostatted at 37 °C. The solvent was 0.02 M NH₄H₂PO₄ (pH 4.0) with 1.5 % (v/v) acetonitrile. Non-methylated lambda phage (Sigma) and *Escherichia coli* LMG 2093 DNA were used as calibration reference and control, respectively.

DNA–DNA hybridization experiments. Hybridization reactions were performed with photobiotin-labelled probes in microplate wells as described before (Ezaki *et al.*, 1989; Goris *et al.*, 1998), using an HTS7000 Bio Assay Reader (Perkin-Elmer) for the fluorescence measurements. The hybridization temperature was 50 °C.

BOX-PCR fingerprinting. BOX-PCR profiles were obtained as described previously (Coenye *et al.*, 2002). The BOX profiles were analysed using the Molecular Analyst-Fingerprinting (MA-F) software. A dendrogram was derived from the UPGMA linkage of correlation coefficients between the BOX-PCR fingerprint patterns from Bcc isolates. Data for reference strains were available from previous studies (Chen *et al.*, 2001; Coenye *et al.*, 2002, 2004; LiPuma *et al.*, 2002) or were unpublished (J. J. LiPuma).

Table 1. *B. cepacia* complex isolates studied

Culture collections: ATCC, American Type Culture Collection, Manassas, VA, USA; CCUG, Culture Collection, University of Göteborg, Department of Clinical Bacteriology, Göteborg, Sweden; DMST, Department of Medical Sciences Thailand, Nonthaburi, Thailand; LMG, BCCM/LMG Bacteria Collection, Laboratorium voor Microbiologie, Universiteit Gent, Gent, Belgium; NCTC, National Collection of Type Cultures, Health Protection Agency, London, UK; R, Research Collection, Laboratorium voor Microbiologie, Universiteit Gent. Other strain numbers are personal designations of the depositor. CF, Cystic fibrosis; ND, not determined.

Strain	Other strain designation(s)	RFLP PAGE type		Origin*	Source
		<i>HaeIII</i>	<i>MnII</i>		
<i>B. ubonensis</i>					
LMG 20358 ^T	EY3383 ^T , NCTC 13147 ^T , CCUG 48852 ^T	W	a	NCTC	Soil (Thailand)
LMG 24263	DMST 6406	W	a	S. Dejsirilert	Nosocomial infection (Thailand)
Cluster 1 (<i>Burkholderia latens</i> sp. nov.)					
LMG 24064 ^T	CCUG 54555 ^T , FIRENZE 3 ^T	W	b	S. Campana	CF patient (Italy)
R-5631	FIRENZE 4	W	b	S. Campana	CF patient (Italy)
LMG 24264	CCUG 54556, E26	W	b	J. R. W. Govan	CF patient (UK)
LMG 24265	CCUG 54557, ERL449	W	b	J. R. W. Govan	CF patient (UK)
R-11768	E27	W	b	J. R. W. Govan	CF patient (UK)
R-17385	ERL450	W	b	J. R. W. Govan	CF patient (UK)
Cluster 2 (<i>Burkholderia diffusa</i> sp. nov.)					
LMG 24065 ^T	CCUG 54558 ^T , AU1075 ^T	AP	c	Own isolate	CF patient (USA)
LMG 24266	CEP0472	H	c	Own isolate	CF patient (Canada)
R-9913	CEP0495	H	c	Own isolate	Non-CF, blood (Canada)
R-9915	CEP0568	AP	c	Own isolate	CF patient (Canada)
R-9916	CEP0607	H	c	Own isolate	CF patient (Canada)
R-18861	CEP1228	H	c	Own isolate	CF patient (Canada)
R-36771	AU8889	ND	ND	Own isolate	CF patient (USA)
R-36772	HI3672	ND	ND	Own isolate	Environment (USA)
R-2607	ATCC 29352, CCUG 54885	H	c	ATCC	Soil (USA)
R-23401	A 9954/02	H	c	F. Daxboeck	CF patient (Austria)
R-23408	A 7881/02	H	c	F. Daxboeck	CF patient (Austria)
LMG 24268	CCUG 54560, 15330612/04	H	c	N. O. Hermansøn	CF patient (Norway)
LMG 24267	CCUG 54559, Bv12	H	c	M. Magelhães	Water, haemodialysis unit (Brazil)
R-16537	Bv9	H	c	M. Magelhães	Human blood (Brazil)
Cluster 3 (<i>Burkholderia arboris</i> sp. nov.)					
LMG 24066 ^T	CCUG 54561 ^T , ES0263A ^T	H	d	Own isolate	Arboretum soil (USA)
R-8833	AU1125	J	c	Own isolate	CF patient (USA)
R-16920	cry/079/01	J	c	Own isolate	Industrial contaminant (Belgium)
R-20536	14 a/c/G	J	c	Own isolate	River water (Belgium)
R-14554	5-33	J	c	E. Bingen	CF patient (France)
R-11808	VERONA 21	J	c	S. Campana	CF patient (Italy)
LMG 24269	CCUG 54563, W45F, J2861	J	c	J. R. W. Govan	Rhizosphere soil (UK)
R-132	J1757	J	c	J. R. W. Govan	CF patient (USA)
R-13059	J2544	J	c	J. R. W. Govan	Botanic garden soil (UK)
R-13064	J2539	J	c	J. R. W. Govan	Botanic garden soil (UK)
R-18413	ERL580	J	c	J. R. W. Govan	CF patient (UK)
R-25659	B04-C00423	J	c	M. Hogardt	CF patient (Germany)
LMG 14939	A40-O	J	c	S. Lauwers	CF patient (Belgium)
R-18163	IST 404	H	d	I. Sa'-Correia	CF patient (Portugal)
R-18164	IST 407	H	d	I. Sa'-Correia	CF patient (Portugal)
LMG 24270	CCUG 54562, 9-06	J	c	C. Ségonds	CF patient (France)
R-22599	10-35	J	c	C. Ségonds	Non-CF patient (France)

Table 1. cont.

Strain	Other strain designation(s)	RFLP PAGE type		Origin*	Source
		<i>Hae</i> III	<i>Mn</i> II		
Cluster 4 (<i>Burkholderia seminalis</i> sp. nov.)					
LMG 24067 ^T	CCUG 54564 ^T , AU0475 ^T	H	e	Own isolate	CF patient (USA)
R-24198	AU2283	H	e	Own isolate	CF patient (USA)
R-24202	HI2490	H	e	Own isolate	Environment (USA)
R-36768	AU0086	ND	ND	Own isolate	CF patient (USA)
R-36769	AU2877	ND	ND	Own isolate	CF patient (USA)
R-14422	MILANO 23	H	e	S. Campana	CF patient (Italy)
LMG 24272	CCUG 54565, 1288	H	e	S. Dejsirilert	Nosocomial infection (Thailand)
R-26472	CCUG 50452	H	e	CCUG	CF patient (Hungary)
LMG 24273	CCUG 54566, SAB	H	e	M. Govindarajan	Suger cane (India)
R-18169	IST 4114	H	e	I. Sa'-Correia	CF patient (Portugal)
R-18173	IST 4118	H	e	I. Sa'-Correia	CF patient (Portugal)
R-18178	IST 4123	H	e	I. Sa'-Correia	CF patient (Portugal)
LMG 19587	BPJ3262	H	e	T. Mew	Rice seed surface (Philippines)
LMG 24271	BPJ4997	H	e	T. Mew	Rice seed surface (Philippines)
R-2817	BPJ829	H	e	T. Mew	Rice seed surface (Philippines)
R-5597	BPJ1247	H	e	T. Mew	Rice seed surface (Philippines)
R-9239	BPJ4917	H	e	T. Mew	Rice seed surface (Philippines)
R-9243	BPJ4989	H	e	T. Mew	Rice seed surface (Philippines)
R-22005	BC14	H	e	C. Winstanley	CF patient (Brazil)
R-22006	BC35	H	e	C. Winstanley	CF patient (Brazil)
R-27260	V07 5780/05	H	e	R. Zbinden	CF patient (Switzerland)
Cluster 5 (<i>Burkholderia metallica</i> sp. nov.)					
LMG 24068 ^T	CCUG 54567 ^T , AU0553 ^T	S	ND	Own isolate	CF patient (USA)
LMG 24275	CCUG 54569, AU4810	S	ND	Own isolate	CF patient (USA)
R-2712	C7522	S	ND	Own isolate	CF patient (Canada)
R-36770	AU7680	ND	ND	Own isolate	CF patient (USA)
LMG 24274	CCUG 54568, MILANO 4	S	ND	S. Campana	CF patient (Italy)

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MLST analysis. MLST analysis was performed as described by Baldwin *et al.* (2005). A phylogenetic tree of concatenated sequences (2773 bp) [including fragments of seven genes: *atpD* (443 bp), *gltB* (400 bp), *gyrB* (454 bp), *recA* (393 bp), *lepA* (397 bp), *phaC* (385 bp) and *trpB* (301 bp)] of each isolate was constructed based on the neighbour-joining method using MEGA software package version 3 (Baldwin *et al.*, 2005; Kumar *et al.*, 2004). The significance of branching within the trees was evaluated by bootstrap analysis of 1000 computer-generated trees. This publication made use of the Bcc MLST website (<http://pubmlst.org/bcc/>) developed by Keith Jolley and sited at the University of Oxford (Jolley *et al.*, 2004).

Biochemical characterization. A biochemical characterization was performed as described previously (Henry *et al.*, 1997, 2001).

RESULTS AND DISCUSSION

B. ubonensis is member of the Bcc

As mentioned above, Bcc species typically share a high degree of 16S rRNA gene (98–100 %) and *recA* (94–95 %)

sequence similarity and moderate levels of DNA–DNA hybridization (30–50 %) (Coenye *et al.*, 2001c). *B. ubonensis* shares at least 98 % of its 16S rRNA gene sequence and 94 % of its *recA* sequence with the other members of the Bcc. The *recA* gene was amplified using the Bcc-specific *recA* PCR (Mahenthiralingam *et al.*, 2000b). DNA–DNA hybridization values obtained between *B. ubonensis* LMG 20358^T and the other established members of the Bcc ranged from 40 to 56 % (Supplementary Table S1, available in IJSEM Online), which indicate a close relationship with Bcc species, since values between strains of Bcc species and other *Burkholderia* species are typically below 30 % (Coenye *et al.*, 2001c). By MLST analysis, *B. ubonensis* shares at least 93 % similarity with other Bcc species. These results demonstrate that *B. ubonensis* constitutes a tenth species within the Bcc and corroborate the initial phylogenetic positioning of its *recA* sequence within the complex (Payne *et al.*, 2005). Although *B. ubonensis* is very similar on the molecular level, it differs biochemically in two major tests; *B. ubonensis* is positive for arginine dihydrolase and negative for β -galactosidase activity (Table 2). At present, only a single *B. ubonensis* strain, LMG 20358^T, has been reported. In the course of our biodiversity analyses of Bcc and similar bacteria, we identified a second strain, LMG 24263, as *B. ubonensis*. The latter strain was isolated from a nosocomial infection in Thailand (S. Dejsirilert, personal communication).

***recA* RFLP analysis and *recA* gene sequencing**

Among the isolates studied, five different *Hae*III *recA* RFLP patterns were found, designated W (cluster 1 strains), AP (cluster 2 strains), H (cluster 2, 3 and 4 strains), J (cluster 3 strains) and S (cluster 5 strains). Patterns H and J have also been observed for *B. cenocepacia* strains (Mahenthiralingam *et al.*, 2000b), pattern J for *B. stabilis* (Mahenthiralingam *et al.*, 2000b) and pattern W for *B. ubonensis* (Supplementary Fig. S1). Therefore, these isolates were further examined by *Mn*II *recA* RFLP. Cluster 1 and cluster 4 isolates were each characterized by a unique *Mn*II restriction profile. Cluster 2 and some of the cluster 3 isolates shared the same distinct *Mn*II restriction profile; the remaining cluster 3 isolates again had a unique *Mn*II restriction profile. For all isolates, the *Hae*III and *Mn*II *recA* RFLP types are listed in Table 1. Computer-generated gel images illustrating the different *Hae*III and *Mn*II restriction profiles among the strains studied are shown in Supplementary Fig. S1.

Strains representing each RFLP type were chosen for *recA* sequence analysis. All sequences obtained were more than 94 % similar to those of other members of the Bcc. As mentioned above, phylogenetic analysis of these sequences revealed five *recA* clusters, referred to as clusters 1 to 5. A *recA* sequence-based phylogenetic tree, which illustrates the five *recA* lineages supported by high bootstrap values (>98 %), is shown in Fig. 1.

16S rRNA gene sequencing and phylogenetic analysis

The 16S rRNA gene sequence of one representative strain per cluster was determined. Comparison of these 16S rRNA gene sequences with those of other Bcc strains revealed similarity levels above 98 % (data not shown). These values were similar to those obtained between strains of other Bcc species. Similarity levels of less than 97.5 % were calculated towards representatives of other *Burkholderia* species (data not shown).

DNA–DNA hybridization and determination of the DNA base composition

DNA–DNA hybridization experiments were performed between representative strains of the five clusters and established members of the Bcc including *B. ubonensis*. For each cluster, two strains were analysed. For clusters 2 and 3, strains with different *Hae*III *recA* restriction profiles were selected. An overview of the DNA–DNA hybridization values and G + C contents is given in Supplementary Table S1. The values obtained among isolates within each cluster were high (>80 %), including clusters 2 and 3. Moderate to high DNA–DNA hybridization values, in the range of 39 to 67 %, were obtained between strains representing the five clusters and established species of the Bcc.

All strains investigated had G + C contents between 66 and 68 mol%. These DNA–DNA hybridization values and G + C contents are typical for Bcc species (Coenye *et al.*, 2001c) and demonstrate that these five *recA* lineages can be considered to represent five novel species within the Bcc.

BOX-PCR fingerprinting

Strain typing methods such as repetitive element PCR using BOX A1R primers are used for typing Bcc strains (Coenye *et al.*, 2002). Nevertheless, numerical analysis of BOX-PCR fingerprints often yields species-specific clusters of strains (Goris *et al.*, 2002; Rademaker *et al.*, 2000). Representative strains of the five clusters and members of the established Bcc species were examined by BOX-PCR fingerprinting. Supplementary Fig. S2 shows the result of a numerical analysis of the BOX profiles. Strains of four clusters grouped together, in congruence with results obtained by other methods in the present study, confirming their genetic homogeneity. Cluster 3 isolates, however, were divided in two clades, revealing more substantial diversity.

MLST analysis

Recently, Baldwin *et al.* (2005) reported a single MLST scheme that allowed differentiation of all established Bcc species. Clusters 1 to 5, including four, eight, fifteen, seven and one representative strains, respectively, were analysed by MLST. Phylogenetic analysis of concatenated sequences demonstrated that strains of the five clusters occupied

distinct positions in the MLST tree supported by 100% bootstrap values, except for cluster 3, which showed a bootstrap value of 84% (Fig. 2). In clusters 1 to 5, one, eight, eleven, five and one different sequence types (STs), respectively, have been found. None of these clusters contained alleles that were found in STs of other Bcc species. For clusters 2, 3 and 4, which show at least five STs, an index of association (Ia) value was calculated to examine whether or not those populations were clonal (Baldwin *et al.*, 2005; Haubold & Hudson, 2000). Cluster 2 had an Ia of 0.3683 ($P < 0.05$) and cluster 3 had a Ia of 0.2933 ($P < 0.05$), whereas cluster 4 had a Ia of 0.0185 ($P > 0.05$). As the Ia of cluster 4 was not significantly greater than zero, it implies that cluster 4 is a freely recombining population, whereas the other two clusters have Ia values significantly greater than zero and are therefore more clonal populations, though further investigations by MLST are necessary to obtain larger numbers of STs in order to study their population biology. Nevertheless, the present study demonstrates the usefulness of MLST for strain typing and species identification in bacterial taxonomy. Comparison of our results with data from other studies showed that strains belonging to clusters 1 to 3 have been reported in previous studies under the designations Bcc 1, 2 and 3, respectively (Baldwin *et al.*, 2005).

Biochemical characterization

In general, species of the Bcc are phenotypically nearly indistinguishable, making their biochemical differentiation difficult, even with an extended panel of biochemical tests (Henry *et al.*, 2001). Biochemical characteristics were determined for clusters 1 to 5, including six, six, thirteen, thirteen and three strains, respectively. A comparison of the biochemical characteristics of the five novel species with those of established members of the Bcc is shown in Table 2. Additional characteristics are given in the species descriptions below.

In conclusion, the aim of the present study was to determine the taxonomic position of five *recA* gene clusters of Bcc isolates using a polyphasic taxonomic approach. The 16S rRNA and *recA* gene similarity levels, the MLST data and the intermediate DNA–DNA binding values demonstrate that these five clusters represent five novel species within the Bcc. Biochemical identification of these species is difficult, as is the case for most Bcc species (Henry *et al.*, 2001; LiPuma *et al.*, 2007; Vandamme *et al.*, 2007). However, identification of these novel species can be accomplished through *recA* gene sequence analysis, MLST and BOX-PCR profiling and by *recA* RFLP analysis. For diagnostic laboratories, *recA* gene sequence analysis offers the best combination of accuracy and simplicity. Based on these results, we propose to classify clusters 1 to 5 as *Burkholderia latens* sp. nov., *Burkholderia diffusa* sp. nov., *Burkholderia arboris* sp. nov., *Burkholderia seminalis* sp. nov. and *Burkholderia metallica* sp. nov., respectively.

Description of *Burkholderia latens* sp. nov.

Burkholderia latens (la'tens. L. fem. part. pres. *latens* concealed, hidden, because the taxonomic status of these strains remained unclear for a long time).

Cells are Gram-negative, aerobic, non-sporulating rods. Colonies are moist. The isolates can show a mucoid and a non-mucoid morphotype. Known strains grow on MacConkey agar. Strains grow on *B. cepacia*-selective agar (BCSA), forming acids. Growth is observed at 30, 37 and 42 °C; some isolates produce a diffusing, melanin-like, brown pigment at 37 and 42 °C on Luria–Bertani agar. Pigmented strains or haemolysis have not been detected. Known strains assimilate glucose, L-arabinose, D-mannose, D-mannitol, *N*-acetylglucosamine, D-gluconate, caprate, adipate, L-malate, citrate and phenylacetate, but not maltose. Acidification of glucose, maltose, lactose, xylose, sucrose and adonitol is observed. Nitrate reduction is absent. Oxidase, β -galactosidase and lysine decarboxylase activities are present, but not ornithine decarboxylase, tryptophanase, arginine dihydrolase, aesculin hydrolase or urease. Gelatin liquefaction is strain-dependent. The G+C content is 67 mol%. Strains have been isolated from sputum samples of cystic fibrosis patients.

The type strain is FIRENZE 3^T (=LMG 24064^T =CCUG 54555^T) and was recovered from sputum of a cystic fibrosis patient in Italy in 2000. Phenotypic and biochemical characteristics are as described above for the species.

Description of *Burkholderia diffusa* sp. nov.

Burkholderia diffusa (dif.fu'sa. L. fem. adj. *diffusa* widespread).

Cells are Gram-negative, aerobic, non-sporulating rods. Colonies are moist or smooth. Known strains grow on MacConkey agar. Strains can grow on BCSA, turning the medium either alkaline or acidic. Growth is observed at 30 and 37 °C, but is strain-dependent at 42 °C. So far, pigmented strains have not been detected and haemolysis has not been observed. Known strains assimilate glucose, L-arabinose, D-mannose, D-mannitol, *N*-acetylglucosamine, D-gluconate, caprate, adipate, L-malate, citrate and phenylacetate, but not maltose. Acidification of glucose, maltose, lactose, xylose and sucrose is observed, but acidification of adonitol is strain-dependent. Nitrate is reduced. Oxidase, β -galactosidase and lysine decarboxylase activities are present, but not ornithine decarboxylase, tryptophanase, arginine dihydrolase, aesculin hydrolase or urease. Gelatin liquefaction is strain-dependent. The G+C content is 67 mol%. Strains have been isolated from sputum samples of cystic fibrosis patients, blood samples from non-cystic fibrosis patients and from soil and water.

The type strain is AU1075^T (=LMG 24065^T =CCUG 54558^T) and was recovered from sputum of a cystic fibrosis patient in the USA in 1999. Phenotypic and biochemical characteristics are as described above for the species.

Table 2. Biochemical characteristics useful for the differentiation of established Bcc species and novel Bcc clusters

Species: 1, *B. latens* sp. nov. (cluster 1; 6 strains); 2, *B. diffusa* sp. nov. (cluster 2; 6); 3, *B. arboris* sp. nov. (cluster 3; 13); 4, *B. seminalis* sp. nov. (cluster 4; 13); 5, *B. metallica* sp. nov. (cluster 5; 3); 6, *B. cepacia* (22 strains); 7, *B. multivorans* (108); 8, *B. cenocepacia* (137); 9, *B. stabilis* (27); 10, *B. vietnamiensis* (35); 11, *B. dolosa* (12); 12, *B. ambifaria* (17); 13, *B. anthina* (18); 14, *B. pyrrocinia* (5); 15, *B. ubonensis* (2). All strains grow on BCSA, assimilate D-glucose, D-gluconate, L-malate, D-mannose and citrate, acidify D-glucose and exhibit oxidase activity. L-Tryptophanase and urease activity is not observed for all strains. For Bcc species: +, >90% of all isolates positive; v, 10–90% positive; –, <10% of strains positive. For the five novel Bcc clusters, the number of positive reactions is indicated for strain-dependent reactions. The reaction of the type strain is given in parentheses for variable reactions. β , β -Haemolysis; w, weak. Results for *B. cepacia*, *B. multivorans*, *B. cenocepacia*, *B. stabilis*, *B. vietnamiensis*, *B. dolosa* and *B. ambifaria* are taken from Henry *et al.* (2001). Data for three additional strains of *B. dolosa* and for *B. anthina*, *B. pyrrocinia* and *B. ubonensis* are unpublished data from D. Henry and D. Speert.

Test	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Growth on MacConkey agar	+	+	+	+	+	v (+)	+	v (+)	+	v (+)	+	+	+	+	+
Growth at 42 °C	+	5 (+)	2 (–)	12 (–)	+	v (–)	+	v (+)	–	+	+	v (+)	v (–)	v (w)	v (–)
Pigment*	–	–	2 py (–)	8 y (+)	y (+)	v y (+)	–	v b (+)	–	–	–	v (–)	–	v y (–)	–
Haemolysis (sheep blood)	–	–	6 β (+)	–	–	–	–	–	–	v β (–)	–	v β (+)	–	v β (–)	–
Assimilation of:															
L-Arabinose	+	+	12 (+)	+	+	+	+	+	–	+	+	+	+	+	+
D-Mannitol	+	+	12 (+)	+	+	+	+	+	+	+	+	+	+	v (+)	+
N-Acetylglucosamine	+	+	12 (+)	+	+	+	+	+	+	+	+	+	+	v (+)	+
Maltose	–	–	3 (+)	–	–	–	–	–	–	v (+)	–	–	v (–)	v (+)	+
Caprate	+	+	12 (+)	+	+	+	+	+	+	+	+	+	+	+	+
Adipate	+	+	+	+	+	+	+	+	+	v (+)	+	+	+	+	+
Phenylacetate	+	+	12 (+)	+	+	+	+	+	+	+	+	+	+	+	+
Acidification of:															
Maltose	+	+	+	+	+	v (–)	+	v (–)	+	+	+	+	+	+	+
Lactose	+	+	+	+	+	+	+	v (+)	+	+	+	+	+	+	+
D-Xylose	+	+	w	+	+	+	+	+	v (–)	v (+)	+	+	+	+	+
Sucrose	+	+	12 (+)	+	+	+	–	+	–	+	–	+	v (–)	+	+
Adonitol	+	3 (+)	+	+	+	v (+)	+	v (+)	+	–	+	+	v (+)	+	–
Nitrate reduction	–	+	8 (+)	–	–	–	+	v (–)	–	v (+)	+	v (+)	v (+)	v (+)	v (+)
Activity of:															
Lysine decarboxylase	+	+	11 (+)	11 (+)	+	+	v (–)	+	+	+	–	+	v (+)	+	–
Ornithine decarboxylase	–	–	12 (+)	11 (+)	–	v (–)	–	v (+)	+	–	–	–	–	+	–
Aesculin hydrolase	–	–	1 (+)	10 (+)	+	v (+)	– (+)	v (–)	–	–	–	v (–)	–	–	–
Arginine dihydrolase	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+
Gelatinase	5 (+)	5 (+)	12 (+)	+	+	v (–)	–	v (–)	+	–	–	+	–	+	+
β -Galactosidase	+	+	+	+	+	+	+	+	–	+	+	+	v (+)	+	–

*b, Brown; py, pale yellow; y, yellow.

Fig. 1. Phylogenetic tree derived from analysis of *recA* gene sequences of Bcc bacteria and five novel Bcc clusters. Different *B. cenocepacia* *recA* lineages are designated IIIA, IIIB, IIIC and IIID (Vandamme *et al.*, 2003). The tree was constructed using the neighbour-joining method based on the Jukes & Cantor parameter. Accession numbers are given in parentheses. Bootstrap values (>70%) are shown for 1000 replicates. *B. xenovorans* LB400^T was used as the outgroup. Bar, 1% sequence dissimilarity.

Description of *Burkholderia arboris* sp. nov.

Burkholderia arboris (ar.bo'ris. L. gen. n. *arboris* of the forest, referring to the source of isolation of the type strain, the Morris Arboretum in Philadelphia).

Cells are Gram-negative, aerobic, non-sporulating rods. Colonies are moist. Known strains grow on MacConkey agar. Strains can grow on BCSA and turn the medium either alkaline or acidic. Growth is observed at 30 and 37 °C; only a few strains are able to grow at 42 °C. Strains R-13059 and R-20536 produce a purple pigment after several days. Six of thirteen strains known show β -haemolysis, a characteristic not commonly observed among Bcc species. Known strains assimilate glucose, D-mannose, D-gluconate, adipate, L-malate and citrate. The assimilation of L-arabinose, D-mannitol, N-acetylglucosa-

mine, maltose, caprate and phenylacetate is strain-dependent. Acidification of glucose, sucrose, maltose, lactose and adonitol is observed, but not of xylose. Nitrate reduction is strain-dependent. Oxidase and β -galactosidase activities are present, but not tryptophanase, arginine dihydrolase or urease activity. Gelatin liquefaction and activities of aesculin hydrolase and lysine and ornithine decarboxylases are strain-dependent. The G+C content is 67 mol%. Strains have been recovered from sputum of cystic fibrosis patients and from environmental sources.

The type strain is ES0263A^T (=LMG 24066^T =CCUG 54561^T) and was isolated in the Morris Arboretum in Philadelphia in 1999. Phenotypic and biochemical characteristics are as described above for the species.

Fig. 2. Phylogenetic tree of concatenated nucleotide sequences from seven loci (*atpD*, *gltD*, *gyrB*, *recA*, *lepA*, *phaC* and *trpB*) of Bcc bacteria and novel Bcc clusters using the neighbour-joining method (Jukes & Cantor). Different *B. cenocepacia* *recA* lineages are designated IIIA, IIIB, IIIC and IIID (Vandamme *et al.*, 2003). Bootstrap values are shown for 1000 replicates. *B. pseudomallei* ATCC 23343^T was used as the outgroup. Bar, 1% sequence dissimilarity.

Description of *Burkholderia seminalis* sp. nov.

Burkholderia seminalis (se.mi.na'lis. L. fem. adj. *seminalis* pertaining to the seed, referring to the rice seed surface, from which several strains were isolated).

Cells are Gram-negative, aerobic, non-sporulating rods. Colonies are moist. Known strains grow on MacConkey agar. All known strains grow on BCSCA, forming acids. Growth is observed at 30 and 37 °C; growth at 42 °C is strain-dependent. Most strains are yellow-pigmented. Haemolysis has not been observed. Known strains assimilate glucose, L-arabinose, D-mannose, D-mannitol, N-acetylglucosamine, D-gluconate, caprate, adipate, L-malate, citrate and phenylacetate, but not maltose. Acidification of glucose, sucrose, maltose, lactose, xylose (weakly positive) and adonitol is observed. Oxidase, β -galactosidase activities and gelatin liquefaction are present, but no tryptophanase, arginine dihydrolase or urease activity. Ornithine and lysine decarboxylase and aesculin hydrolase activities are strain-dependent. Nitrate reduction is absent. The G+C content is 67 mol%. Strains have been isolated from sputum of cystic fibrosis patients, nosocomial infections and environmental samples.

The type strain is AU0475^T (=LMG 24067^T =CCUG 54564^T) and was recovered from sputum of a cystic fibrosis

patient in the USA in 1998. Phenotypic and biochemical characteristics are as described above for the species.

Description of *Burkholderia metallica* sp. nov.

Burkholderia metallica (me.tal'li.ca. L. fem. adj. *metallica* metallic, referring to the metallic shine on the colony surface of the four known isolates).

Cells are Gram-negative, non-sporulating, aerobic rods. Colonies are moist, showing a metallic shine. Known strains grow on MacConkey agar. Known strains grow on BCSCA, forming acids. Growth is observed at 30, 37 and 42 °C. Strains are not haemolytic and most strains are yellow-pigmented. Known isolates assimilate glucose, L-arabinose, D-mannose, D-mannitol, N-acetylglucosamine, D-gluconate, caprate, adipate, L-malate, citrate and phenylacetate, but not maltose. Acidification of glucose, sucrose, maltose, lactose, xylose and adonitol is observed. Nitrate reduction is absent. Activity of oxidase, β -galactosidase, lysine decarboxylase, aesculin hydrolase and gelatin liquefaction is present, but not tryptophanase, arginine dihydrolase or urease activity. The G+C content is 67 mol%. Known strains have been isolated from sputum samples of cystic fibrosis patients.

The type strain is AU0553^T (=LMG 24068^T =CCUG 54567^T) and was recovered from sputum of a cystic fibrosis patient in the USA in 1998. Phenotypic and biochemical characteristics are as described above for the species.

The five type strains have been deposited in the Belgian Co-ordinated Collections of Microorganisms/Laboratorium Microbiologie Gent and the Culture Collection of the University of Göteborg, Sweden. Additional strains (designated with LMG and CCUG numbers) have also been deposited in these culture collections (Table 1).

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