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Development of a *recA* Gene-Based Identification Approach for the Entire *Burkholderia* Genus

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Burkholderia **is an important bacterial genus containing species of ecological, biotechnological, and pathogenic interest. With their taxonomy undergoing constant revision and the phenotypic similarity of several species, correct identification of *Burkholderia* is difficult. A genetic scheme based on the *recA* gene has greatly enhanced the identification of *Burkholderia cepacia* complex species. However, the PCR developed for the latter approach was limited by its specificity for the complex. By alignment of existing and novel *Burkholderia recA* sequences, we designed new PCR primers and evaluated their specificity by testing a representative panel of *Burkholderia* strains. PCR followed by restriction fragment length polymorphism analysis of an 869-bp portion of the *Burkholderia recA* gene was not sufficiently discriminatory. Nucleotide sequencing followed by phylogenetic analysis of this *recA* fragment differentiated both putative and known *Burkholderia* species and all members of the *B. cepacia* complex. In addition, it enabled the design of a *Burkholderia* genus-specific *recA* PCR that produced a 385-bp amplicon, the sequence of which was also able to discriminate all species examined. Phylogenetic analysis of 188 novel *recA* genes enabled clarification of the taxonomic position of several important *Burkholderia* strains and revealed the presence of four novel *B. cepacia* complex *recA* lineages. Although the *recA* phylogeny could not be used as a means to differentiate *B. cepacia* complex strains recovered from clinical infection versus the natural environment, it did facilitate the identification of clonal strain types of *B. cepacia*, *B. stabilis*, and *B. ambifaria* capable of residing in both niches.**

Burkholderia is a genus with complex taxonomy that currently contains 34 validly described species (5), nine of which are a closely related group, known as the *Burkholderia cepacia* complex (6). *Burkholderia* species are widely distributed in the natural environment, and although the majority appear to live either freely or as symbionts or commensals with a variety of higher organisms, several species also cause disease (5). Plant-pathogenic species include *Burkholderia glumae* and *Burkholderia plantari*, which are important rice pathogens. The genus also includes mammalian primary pathogens such as *Burkholderia pseudomallei*, the cause of meliodosis in humans, and *Burkholderia mallei*, which causes glanders in horses; both species have attracted recent interest as potential bioterrorism agents (19).

Many other *Burkholderia* species are capable of causing opportunistic infections in humans and animals; for example, the *B. cepacia* complex (6) can cause serious infections in persons with cystic fibrosis (20) and other vulnerable individuals (31), as well as disease in plants (8) and animals (3). In contrast to these detrimental pathogenic properties, several *Burkholderia* species have considerable commercial and ecological importance. They have been used in agriculture as biopesticides and plant growth promoters (27), and in the bioremediation of major pollutants such as trichloroethylene (30) and polychlorinated biphenyls (26).

The taxonomy and identification of the genus *Burkholderia* are complex, with new species being described rapidly (5, 6). Closely related species such as the *B. cepacia* complex are difficult to identify using conventional biochemical and phenotypic tests, and species belonging to other betaproteobacterial genera (including *Pandoraea* and *Ralstonia*) may be misidentified as *Burkholderia* species (6). A polyphasic taxonomic approach (39) utilizing multiple diagnostic tests is often required to identify *Burkholderia* species accurately.

Although 16S rRNA gene sequence analysis forms an integral part of taxonomical analysis for many bacterial genera (39), its utility in the genus *Burkholderia* is more limited, especially within the *B. cepacia* complex, where it cannot be used as a means to accurately distinguish all species (16, 21). The *recA* gene has been widely applied in bacterial systematics (13) and has proven very useful for the identification of *B. cepacia* complex species, with phylogenetic analysis of sequence variation within the gene enabling discrimination of all nine current species within the *B. cepacia* complex (21). However, the PCR primers designed for the original *recA*-based approach, BCR1 and BCR2, are specific only to members of the *B. cepacia* complex and fail to amplify this gene from other *Burkholderia* species (21). While this can be used as a positive means to confirm an isolate's position within the complex, it limits the application of the approach to identify other *Burkholderia* species in diverse natural habitats.

Given the ecological, biotechnological, and pathogenic im-

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portance of these bacteria, there is a clear need for a molecular diagnostic scheme capable of discrimination across all *Burkholderia* spp. This paper describes the use of genome sequence data from several *Burkholderia* species genome sequencing projects, in combination with an extensive collection of *recA* sequences from *B. cepacia* complex bacteria (21), to develop and evaluate a scheme for identification of all *Burkholderia* spp. based on the *recA* gene. New PCR primers were designed to amplify 87% of the *recA* gene, and novel *recA* sequence data were obtained from known and unknown species. The large size of this *recA* PCR product enabled the development of a PCR-restriction fragment length polymorphism method. Once *recA* sequence information for each *Burkholderia* species had been obtained, PCR primers specific for the genus were designed and tested. Finally, to further examine the phylogenetic relationships between strains of clinical and environmental origin, the *recA* genes from a large collection of *B. cepacia* complex strains were sequenced and compared.

MATERIALS AND METHODS

Bacterial strains, identification, and culture. Molecular identification approaches were developed and evaluated on the collection of strains listed in Table 1. The panel was selected to be representative of the current species diversity of *Burkholderia* and contained 28 species from the genus, with the exception of the primary pathogens *B. pseudomallei* and *B. mallei*. These *Burkholderia* species and all *B. cepacia* complex strains were obtained from the Belgian Co-ordinated Collections of Microorganisms (BCCM), LMG Bacteria collection, the Cardiff University collection (over 800 isolates) (2, 21), and U.S. *B. cepacia* Complex Research Laboratory and Repository (18) and included representatives of the published strain panels (7, 22). Nine isolates representing putative novel *Burkholderia* species, a selection of non-*Burkholderia* control species for PCR, and four isolates of the closely related genus *Pandoraea* were also included in the test strain panel (Table 1). The *Burkholderia ubonensis* type strain, which appears to be a new species member of the *B. cepacia* complex (42), was also included in the study. The ecologically and biotechnologically relevant *B. cepacia* complex strains examined are described in Table 2. All *Burkholderia* species were cultured and identified as described previously (6, 21).

Chromosomal DNA extraction. DNA was prepared for PCR amplification from overnight cultures as described previously (21). Rapid DNA extraction by boiling in the presence of a chelating resin was also carried out as follows. Bacteria from 1 ml of a liquid culture were harvested by centrifugation and resuspended in 100 μl of a sterile solution containing 5% Chelex 100 (Sigma Aldrich, Gillingham, United Kingdom). The suspension was boiled for 5 min and immediately placed on ice for a further 5 min, and then this procedure was repeated. The final supernatant was recovered after centrifugation and stored at −20°C. Before use DNA was diluted in sterile water, and approximately 20 ng of template DNA was incorporated into each PCR.

PCR analysis. PCR was performed as described previously (21) using QIAGEN reagents (QIAGEN Ltd., Crawley, United Kingdom). Each 25-μl PCR contained the following: 1 U *Taq* polymerase, 250 μM of each deoxynucleoside triphosphate, 1x PCR buffer (including 1.5 mM $MgCl_2$), 10 pmol of each appropriate oligonucleotide primer, and 10 to 50 ng of template DNA.

PCR of *B. cepacia* complex *recA* genes was carried out using primers BCR1 and BCR2 as previously described (21). New primers for specific amplification and sequencing of *Burkholderia* species *recA* were designed: BUR1, GATC GA(AG)AAGCAGTTCGGCAA, and BUR2, TTGTCCTTGCCCTG(AG)C CGAT, amplifying an 869-bp fragment; and BUR3, GA(AG) AAG CAG TTC GGC AA, and BUR4, GAG TCG ATG ACG ATC AT, amplifying a 385-bp fragment. OligoCheck (1; http://www.cf.ac.uk/biosi/research/biosoft) was used to assist in primer design and analyze primers BUR1 and BUR2 by rating the likelihood of publicly available bacterial *recA* sequences being amplified. Thermal cycling was carried out in a Flexigene thermal cycler (Techgene Ltd., Cambridge, United Kingdom) for 30 cycles of 30 s at 94°C, annealing for 30 s at 60°C, and extension at 72°C for 45 s, with a final 5-min extension at 72°C. Approximately 2 μl of each PCR product was visualized by agarose gel electrophoresis as described previously (21).

Restriction fragment length polymorphism analysis. Restriction fragment length polymorphism (RFLP) fingerprinting of the *recA* amplicons of *B. cepacia* complex bacteria were performed as previously described (21). RFLP analysis of *Burkholderia* species and other strains (Table 1) was performed on 5 μl of PCR product in a mixture containing the appropriate restriction enzyme buffer and restriction endonuclease as outlined by the manufacturer (Promega, Southampton, United Kingdom) and incubated at 37°C for 4 h. Analysis of the BUR1 and BUR2 *recA* amplicons was performed with HaeIII. RFLP patterns obtained from *recA* were recorded and compared using computer software (GeneSnap, GeneTools, and GeneDirectory, Syngene, Cambridge, United Kingdom). RFLP pattern similarity was calculated as a Dice coefficient at 1% tolerance and clustered using the unweighted pair-group method average. RFLP patterns with a similarity index of 0.75 or higher were clustered as a single group. Macrorestriction and pulsed-field gel electrophoresis (PFGE) analysis was performed as described previously (38); PFGE fingerprints were also compared and analyzed using computer software as described above.

Nucleotide sequence analysis. All *recA* PCR products were sequenced directly using the appropriate primers as described previously (21) as well as those developed in this study (BUR1 and BUR2). Sequencing reactions were prepared using Applied Biosystems Big Dye Terminator ready reaction mix version 3.1 in accordance with the manufacturer's instructions and analyzed using Applied Biosystems ABI-Prism 3100 genetic analyzer capillary electrophoresis system running Applied Biosystems Performance Optimized Polymer 6 (POP-6). Raw sequences from both strands of the PCR products were aligned, and a consensus sequence was derived using the CAP contig assembly program within the BioEdit software (10). Analysis also involved the use of Basic Local Alignment Search Tool (BLAST; www.ncbi.nlm.nih.gov) to establish the correct gene identity.

Phylogenetic analysis. Multiple nucleotide sequence alignments spanning 760 nucleotides of the *recA* gene were constructed using CLUSTAL W (34). Phylogenetic and molecular evolutionary analyses were conducted using genetic-distance-based neighbor-joining algorithms within MEGA version 2.1 (http://www.megasoftware.net/). Gaps and missing data were completely deleted in MEGA before trees were constructed using the Jukes and Cantor matrix model with random sequence input order and 1,000 data sets examined by bootstrapping. All trees were rooted with the *Pseudomonas aeruginosa* PAO1 *recA* gene.

Nucleotide sequence accession numbers and aligned sequence sets. Novel *recA* nucleotide sequences were determined for 28 *Burkholderia* strains, four *Pandoraea* strains, and the *B. ubonensis* type strain (GenBank accession numbers are listed in Table 1). Sequences were also determined for 106 *B. cepacia* complex strains representative of the diversity seen in the *recA* gene RFLP, and these have been submitted under accession numbers AF143782, AF143797, AF143800, AF456003 to AF456015, AF456017, AF456018, AF456020, AF456023, AF456024, AF456026 to AF456028, AF456035, AF456036, AF456038, AF456039 to AF456050, AF456052, AF456054, AF456056, AF456057, AF456062 to AF456064, AF456066, AF456067, AF456069 to AF456083, AF456085 to AF456124, and AY753187. Phylogenetic analysis was performed on the latter novel sequences, 52 previously published *recA* genes (4, 21, 35, 36, 41, 42), and four sequences obtained from the following genome sequence projects: *B. cenocepacia* strain J2315 (NC_004503; www.sanger.ac.uk/Projects/B_cenocepacia/), *B. vietnamiensis* strain G4 (NZ_AAEH00000000; http://genome.jgi-psf.org/draft_microbes/bur08/bur08.home.html), *B. cepacia* strain ATCC 17660 (strain 383; http://genome.jgi-psf.org/draft_microbes/bur94/bur94.home.html), and the *Burkholderia* Sargasso Sea Metagenome (40) strain SAR-1 (NS_000028; www.ncbi.nlm.nih.gov/genomes/static/es.html). Aligned sequence sets of all the *recA* sequences used in this study are available from ftp://cepacia.bios.cf.ac.uk

RESULTS

Design of PCR probes and amplification of *Burkholderia* species *recA* gene. Data for the design of a *Burkholderia* genus-specific *recA* PCR were obtained from the alignment of 10 published *recA* genes spanning the *B. cepacia* complex (21) and genomic regions spanning the *recA* genes from the *B. xenovorans* LB400^T, *B. cenocepacia* J2315, and *B. pseudomallei* K96243 genome sequence projects. Few primer sites capable of amplification across the genus were identified in silico either within or just outside the *recA* coding sequence. Sites that facilitated amplification of a large (869-bp) internal fragment suitable for both RFLP and sequence analysis were selected and used to design primers BUR1 (21-mer; spanning bases 72 to 92 relative to the *B. cenocepacia* J2315 genome *recA* gene

TABLE 1. Bacterial strains used for *recA* PCR primer development and evaluation

Species	Strain	*recA* GenBank accession no.[a]	BUR1 and BUR2 PCR	BUR1 and BUR2 RFLP type	BUR3 and BUR4 PCR
Pandoraea species					
Pandoraea pulmonicola	#40	AY619660	Positive	67	Negative
Pandoraea apista	Patient W	AY619657	Positive	04	Negative
Pandoraea sputorum	AU0012	AY619659	Positive	16	Negative
Pandoraea pnomenusa	Keulen	AY619658	Positive	15	Negative
Known *Burkholderia* species					
Burkholderia kururiensis	LMG 19447^T	AY619654	Positive	80	Positive
Burkholderia caledonica	LMG 19076^T	AY619669	Positive	78	Positive
Burkholderia phenazinium	LMG 2247^T	AY619668	Positive	—	Positive
Burkholderia plantarii	LMG 9035^T	AY619655	Positive	08	Positive
Burkholderia glathei	LMG 14190^T	AY619666	Positive	10	Positive
Burkholderia glumae	LMG 2196^T	AY619675	Positive	89	Positive
Burkholderia gladioli	LMG 2216^T	AY619665	Positive	70	Positive
Burkholderia fungorum	LMG 16225^T	AY619664	Positive	12	Positive
Burkholderia caryophylli	LMG 2155^T	AY619663	Positive	53	Positive
Burkholderia graminis	LMG 18924^T	AY619653	Positive	77	Positive
Burkholderia caribensis	LMG 18531^T	AY619662	Positive	18	Positive
Burkholderia thailandensis	LMG 20219^T	AY619656	Positive	83	Positive
Burkholderia sacchari	LMG 19450^T	AY619661	Positive	71	Positive
Burkholderia terricola	LMG 20594^T	AY619672	Positive	85	Positive
Burkholderia tuberum	LMG 21444^T	AY619674	Positive	86	Positive
Burkholderia phymatum	LMG 21445^T	AY619667	Positive	87	Positive
Burkholderia xenovorans	LB400^T	AAAJ00000000	Positive	01	Positive
Burkholderia andropogonis	LMG 2129^T	—	Negative	—	Negative
Burkholderia hospita	LMG 20598^T	—	Positive[b]	—	Positive
Indeterminate *Burkholderia* species					
	R-20943	AY619679	Positive	71	Positive
	R-8349	AY619681	Positive	84	Positive
	R-15273	AY619677	Positive	81	Positive
	R-701	AY619680	Positive	90	Positive
	R-15821	AY619678	Positive	01	Negative
	R-13392	AY619676	Positive	77	Positive
	LMG 21262	AY619673	Positive	02	Positive
	LMG 19510	AY619670	Positive	82	Positive
	LMG 20580	AY619671	Positive	84	Positive
B. cepacia complex species					
Burkholderia vietnamiensis	LMG 10929^T	AF143793	Positive	66	Positive
Burkholderia multivorans	C1576	AF143774	Positive	73	Positive
Burkholderia multivorans	LMG 13010^T	—	Positive	—	Positive
Burkholderia cepacia	ATCC 25416^T	AF143786	Positive	88	Positive
Burkholderia cenocepacia	J2315/LMG16656^T	www.sanger.ac.uk	Positive	20	Positive
Burkholderia stabilis	LMG 14294^T	AF456031	Positive	63	Positive
Burkholderia pyrrocinia	LMG 14191^T	AF143794 BPP	Positive	75	Positive
Burkholderia ambifaria	LMG 19182^T	AF323985	Positive	48	Positive
Burkholderia dolosa	LMG 18943^T	AF323971	Positive	58	Positive
Burkholderia anthina	LMG 20980^T	AF456059	Positive	57	Positive
Burkholderia cenocepacia	CFLG	AF456021	Positive	25	Positive
Burkholderia ubonensis	LMG 20358^T	AY780511	—	—	—
PCR control species					
Bordetella parapertussis	LMG 14449^T	—	Positive	—	Negative
Rhizobium vitis	LMG 8750^T	—	Negative	—	Negative
Xanthomonas sacchari	LMG 471^T	—	Positive	—	Negative
Ralstonia metallidurans	LMG 1195^T	—	Negative	—	Negative
Ralstonia gilardii	LMG 5886^T	—	Positive	—	Negative
Ralstonia eutropha	JMP134/LMG1197	www.jgi.doe.gov	Positive	—	Negative
Neisseria elongata	LMG 5124^T	—	Negative	—	Negative
Mycobacterium smegmatis	MC^{2}155	—	Negative	—	Negative
Pseudomonas aeruginosa	C3719	—	Negative	—	Negative
Pseudomonas aeruginosa	PAO1	NC_002156	Negative	—	Negative

[a] —, *recA* sequence or RFLP not available or not determined.
[b] PCR amplification with BUR1 and BUR2 resulted in one product of the correct size, 869 bp, and another of approximately 400 bp which could not be resolved by optimization.

TABLE 2. Characteristics of selected *B. cepacia* complex strains

Species (*recA* phylogenetic cluster)	Strain name (other strain designations)	Source and relevant information	Reference(s)
B. cepacia (genomovar I type strain cluster)	ATCC 49709	Grass seed biological control strain	11
B. cepacia (genomovar I group K)	SAR-1	*Burkholderia* species metagenomic strain from Sargasso Sea	40
B. cepacia (genomovar I group K)	383 (ATCC 17660; LMG 6991)	Forest soil, Trinidad; genome draft available (http://genome.jgi-psf.org/draft_microbes/bur94/bur94.home.html)	32
B. cepacia (genomovar I type strain cluster)	ATCC 25416^T (LMG 1222)	Onion rot; *B. cepacia* type strain	32, 37
B. cepacia (genomovar I type strain cluster)	J1050	Clinical infection, USA	28
B. cepacia (genomovar I type strain cluster)	ATCC 17759 (LMG 2161)	Forest soil, Trinidad	32
B. cepacia (genomovar I type strain cluster)	LMG 14087	Wound swab, UK	37
B. cenocepacia (III-B)	M36	Corn rhizosphere, USA; registered biopesticide withdrawn from commercial use; type Wisconsin strain; encodes *B. cenocepacia* pathogenicity island	27
B. cenocepacia (III-B)	BC-1	Corn rhizosphere, USA; biological control strain; encodes *B. cenocepacia* pathogenicity island	24
B. cenocepacia (III-B)	BC-2	Corn rhizosphere, USA; biological control strain; encodes *B. cenocepacia* pathogenicity island	24
B. stabilis[a]	HI-2482	Veterinary shampoo contaminant, USA	This study
B. stabilis[a]	LMG 14294	Cystic fibrosis patient, Belgium	38
B. vietnamiensis[a]	G4 (ATCC 53617; R-1808)	Waste water, USA; capable of trichloroethylene degradation; derivative strain ENV435 effective in commercial field test on a contaminated aquifer; genome draft available (http://genome.jgi-psf.org/draft_microbes/bur08/bur08.home.html)	29, 33
B. ambifaria[a]	M54 (R-5142)	Corn rhizosphere, USA; registered biopesticide in commercial use; type Wisconsin strain with antifungal properties	27
B. ambifaria[a]	J82 (R-5140)	Corn rhizosphere, USA; registered biopesticide in commercial use; type Wisconsin strain with antinematodal properties	27
B. ambifaria[a]	BC-F	Corn rhizosphere, USA; U.S. Department of Agriculture biological control strain; production of antifungal agents	43
B. ambifaria[a]	AMMD^T (LMG 19182^T)	Pea rhizosphere, USA; biological control strain and species type strain	4, 27
B. ambifaria[a]	AU212 (LMG 19466)	Cystic fibrosis strain isolated from a patient in Wisconsin, USA	4
B. pyrrocinia[a]	BC11	Soil; antifungal-producing biological control strain	12
B. pyrrocinia[a]	ATCC 39277	Corn field soil; production of antifungal agents	25

[a] Species group and *recA* cluster are identical.

[all subsequent primer positions are given relative to this sequence]) and BUR2 (20-mer; bases 819 to 938).

Prior to laboratory testing, the OligoCheck software was used to examine the performance of primers in silico against all available *recA* sequences. A panel of nine non-*Burkholderia* control strains, each containing fewer than five putative mismatches to the PCR primers, were selected from the OligoCheck output for testing (Table 1). BUR1 and BUR2 produced an 869-bp amplicon from all *Burkholderia* species (Fig. 1A) except *B. andropogonis* (Table 1). Four of the control species, *Bordetella parapertussis*, *Xanthomonas sacchari*, *Ralstonia gilardii*, and *Ralstonia eutropha*, also produced the same-sized amplicon; the remaining control strains were negative (Table 1). Because of its broad specificity for *Burkholderia* species, the PCR employing BUR1 and BUR2 was subsequently used as a means to test a *recA*-based RFLP approach and to obtain further nucleotide sequence information for specific primer design.

RFLP analysis of *Burkholderia* species *recA* gene. BUR1 and BUR2 PCR amplification and digestion with HaeIII (21) were applied to analyze the *Burkholderia* genus. RFLP analysis with other enzymes was not performed since previous work had already shown HaeIII to be the most discriminatory enzyme for restriction of *B. cepacia* complex *recA* genes (21). A total of

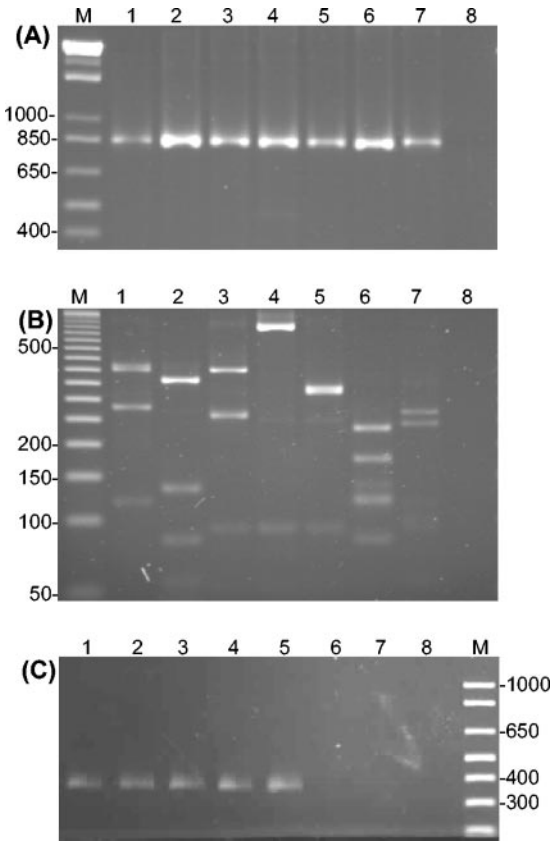

FIG. 1. *Burkholderia recA* gene PCR analysis. Panel A shows PCR products obtained with primers BUR1 and BUR2 from *Burkholderia* and control species as follows in each lane: 1, *B. stabilis* LMG14294; 2, *B. caryophylli* LMG2155^T; 3, *B. fungorum* LMG16225^T; 4, *B. graminis* LMG18924^T; 5, *B. plantarii* LMG9035^T; 6, *Bordetella parapertussis* LMG14449; 7, *Ralstonia eutropha* JMP134; 8, negative control. Panel B shows the RFLP analysis of the BUR1 and BUR2 *recA* amplicon performed by digestion of PCR products with the enzyme HaeIII. Samples in each lane are as follows: 1, *B. stabilis* LMG14294; 2, *B. caryophylli* LMG2155^T; 3, *B. fungorum* LMG16225^T; 4, *B. graminis* LMG18924^T; 5, *B. plantarii* LMG9035^T; 6, *Bordetella parapertussis* LMG14449; 7, *Ralstonia eutropha* JMP134; 8, negative control. Panel C shows the specific amplification of the *Burkholderia recA* gene with primers BUR3 and BUR4 with the following species run in each lane as follows: 1, *B. stabilis* LMG14294; 2, *B. caryophylli* LMG2155^T; 3, *B. fungorum* LMG16225^T; 4, *B. graminis* LMG18924^T; 5, *B. plantarii* LMG9035^T; 6, *Bordetella parapertussis* LMG14449; 7, *Ralstonia eutropha* JMP134; 8, H_2O negative control. Molecular size markers are shown in lane M for all panels, and the sizes of relevant bands are indicated in bp.

TABLE 3. *Burkholderia* and *Pandoraea* isolates found to possess matching *recA* RFLP types

Species	Strain name	HaeIII RFLP type
B. xenovorans	LB400^T	01
"*Burkholderia*" sp. nov.	R-15821	01
P. pulmonicola	BCC0150	15
P. pnomenusa	BCC0580	15
B. caribiensis	LMG 18531	18
B. cepacia	BCC0679	18
B. cepacia complex unknown	AU0553	38
B. cepacia complex unknown	BCC0095	38
B. pyrrocinia	LMG 21823	38
B. vietnamiensis	CLO	45
B. ubonensis	R-11767	45
B. ambifaria	MVP/C1 64	46
B. cenocepacia	MDII 367	46
B. cenocepacia	MVP/C1 73	62
B. cenocepacia	BELF 2	62
B. stabilis	LMG 14294	63
B. cenocepacia	PC184	63
B. gladioli	LMG 2216	70
B. gladioli	BCC0238	70
B. sacchari	LMG 19450	71
"*Burkholderia*" sp. nov.	R-20943	71
B. graminis	LMG 18924	77
"*Burkholderia*" sp. nov.	R-13392	77
"*Burkholderia*" sp. nov.	R-8349	84
"*Burkholderia*" sp. nov.	LMG 20580	84

103 isolates representative of both *Burkholderia* species (Table 1) and RFLP pattern diversity with the *B. cepacia* complex (21; E. Mahenthiralingam, unpublished data) were examined; 81 unique and 13 shared RFLP patterns were identified. Examples of unique RFLP types from *B. cepacia* complex members, *Burkholderia* species, and two non-*Burkholderia* species bacteria are shown in Fig. 1B. Shared RFLP types are described in Table 3.

Five known species and several strains representing putative novel species possessed overlapping RFLP patterns (Table 3). Therefore, although the majority of strains and species analyzed (86%) possessed unique patterns, the discriminatory power of the *recA*-RFLP analysis was limited. However, it could serve as a useful primary screen in a *Burkholderia* species identification scheme in an analogous fashion to its use for determining species within the *B. cepacia* complex (21, 42). Further resolution was achieved with nucleotide sequence analysis of *Burkholderia recA*.

Development of *Burkholderia*-specific *recA* PCR. To confirm the sequence variations detected by RFLP analysis of the amplified *recA* fragment and facilitate the design of *Burkholderia*-specific PCR primers, the 869-bp BUR1 and BUR2 *recA* amplicons of 16 *Burkholderia* strains, nine indeterminate *Burkholderia* species strains, the *B. ubonensis* type strain, and four *Pandoraea* strains (Table 1) were sequenced. These novel *recA* sequences were analyzed by alignment with published *B. cepacia* complex sequences and genomic *recA* sequences.

A single *Burkholderia* genus-specific adenosine was identified at base 445 in the *recA* gene and used to design the downstream primer BUR4 (17-mer; bases 445 to 461). The upstream primer, BUR3 (17-mer; bases 76 to 92), was designed to prime off the same base as BUR1 but was shorter to facilitate potential future use in a nested PCR priming off a primary amplicon resulting from BUR1 and BUR2 PCR. BUR3 and BUR4 produced a 385-bp amplicon for all *Burkholderia* species examined (examples shown in Fig. 1C) except *Burkholderia andropogonis* and novel *Burkholderia* sp. strain R-15821. The *recA* sequence of the last strain was subsequently shown to be more closely related to that of *Bordetella* (see below; Table 1 and Fig. 2). BUR3 and BUR4 PCR did not produce amplicons with non- closely *Burkholderia*-related species, including those that had originally amplified with BUR1 and BUR2 (Table 1).

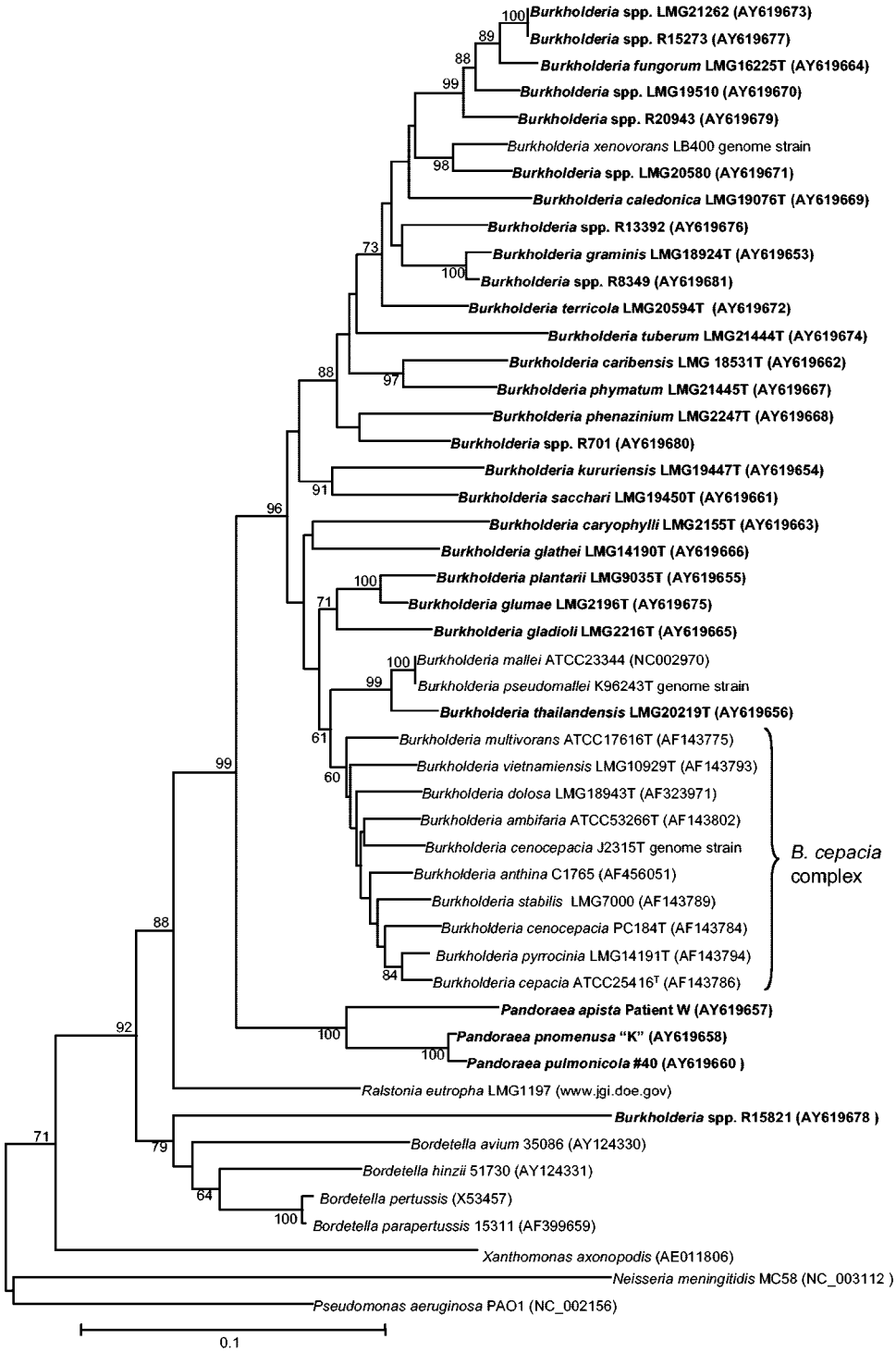

FIG. 2. Phylogenetic analysis of the *Burkholderia* genus using novel *Burkholderia recA* sequences. A phylogenetic tree comparing representatives from each species is shown; 27 novel *Burkholderia recA* genes and four novel *Pandoraea recA* genes are highlighted. Bootstrap values over 70% and genetic distance scale are indicated. The species name and strain number are shown, with the accession number in brackets. The tree was rooted with the *P. aeruginosa* PAO1 *recA* gene taken from the complete genome sequence as a representative member of a species outside the betaproteobacteria group. The following *recA* sequences were also included in the phylogenetic analysis as indicators of the ability of the analysis to differentiate among genera closely related to *Burkholderia*: *Ralstonia eutropha* JMP135, *Bordetella avium* AY124330, *Bordetella hinzii* AY124331, *Bordetella parapertussis* AF399659, *Bordetella pertussis* X53457, *Xanthomonas axonopodis* AE011806, and *Neisseria meningitidis* NC_0031112. Sequences from the species comprising the *B. cepacia* complex were included.

Phylogenetic analysis of *Burkholderia* species *recA* gene. A phylogenetic tree constructed with novel *recA* sequences from *Burkholderia* species and *Pandoraea* species (Table 1) is shown in Fig. 2 to illustrate the diversity of the *Burkholderia* genus. The *B. cepacia* complex formed a distinct phylogenetic cluster. *Burkholderia thailandensis*, *B. pseudomallei*, and *B. mallei* (the *B. pseudomallei* group) formed a cluster, which, although distinct, was more closely related to the *B. cepacia* complex than other *Burkholderia* species. The *Pandoraea* genus also formed a distinct cluster consistent with their recent separation from the *Burkholderia* genus. All the indeterminate *Burkholderia* species (Table 1) strains clustered within the *Burkholderia* genus cluster except strain R-15821, which was more closely related to *Bordetella* species.

To test if the same cluster assignments were made using sequence obtained from the smaller BUR3 and BUR4 amplicon, the sequences were trimmed to the 300 bases within the 385-bp amplicon produced by these primers and subjected to an identical analysis. The trimmed sequences produced a tree with the same topology and clusters that had been observed with the 800-base sequences derived from the 865-bp BUR1 and BUR2 amplicon (data not shown).

Phylogenetic diversity of the *B. cepacia* complex. Although the *recA* gene has proven to be a valuable tool in determining the taxonomy of the *B. cepacia* complex, species such as *B. cenocepacia* and *B. cepacia* are split into distinct phylogenetic clusters when analyzed in this way. Two *recA* lineages were originally observed in *B. cepacia* genomovars III, III-A, and III-B (21), and subsequently clusters III-C and III-D were reported when the formal name *B. cenocepacia* was proposed for this genomovar (36). Similarly, *B. cepacia* strains also divide into two lineages by phylogenetic polymorphism in the *recA* gene; one cluster includes the type strain (ATCC 25416^T) (21), while the second group was named group K based on the most common *recA* RFLP found within that cluster (42). Further novel *recA* phylogenetic groups have also been observed for other strains. However, the significance of these clusters was difficult to interpret within a phylogeny based solely on the *B. cepacia* complex (E.M., unpublished data), and so further analysis with the new data obtained in this study was carried out to resolve phylogenetic relationships on a broader scale.

A phylogenetic tree comprising a subset of 101 *B. cepacia* complex strain *recA* genes and including 28 novel *Burkholderia* and *Pandoraea* sequences (Table 1) is shown in Fig. 3. Seventeen phylogenetically distinct clusters were observed for *B. cepacia* complex species. As observed with the *Burkholderia* genus phylogeny (Fig. 2), the *B. pseudomallei* group clustered adjacent to the *B. cepacia* complex. *B. gladioli* and *B. plantarii*, and *B. glathei* and *B. carophylli* also formed distinct clusters. All the remaining *Burkholderia* species formed a diverse separate group.

Within the *B. cepacia* complex, all *B. stabilis*, *B. pyrrocinia*, *B. anthina*, *B. vietnamiensis*, *B. dolosa*, *B. ambifaria*, and *B. multivorans* strains and the *B. ubonensis* type strain formed single discrete phylogenetic clusters. As shown previously (21), *B. cepacia* strains were split into sublineages (Fig. 3), while *B. cenocepacia* III-A, III-B, III-C, and III-D were all distinct. Three *B. cepacia* clusters were observed, one containing the type strain, ATCC 25416^T, one composed of RFLP type K strains (42), and another composed of RFLP type AW strains that previously belonged to *B. cepacia* (9) (Fig. 3). However, it must be noted that *B. cepacia* RFLP group K cluster was the least robust group within the phylogenetic analysis (bootstrap value 30) (Fig. 3), suggesting that further species diversity may be present. Several strains which previously were difficult to assign (E.M., unpublished work), fell into distinct branches of the new tree. *B*. cepacia complex group 1 strains were closely related to *B. cenocepacia* III-A, while *B. cepacia* complex group 2 strains clustered between *B. cenocepacia* III-D and *B. anthina* (Fig. 3). Again all the clusters remained intact when the *recA* sequences were trimmed to 300 bp within the BUR3 and BUR4 amplicon (data not shown).

Phylogenetic positions of clinical and environmental strains. Of the 101 *B. cepacia* strains analyzed phylogenetically (Fig. 3), 41 were from the environment and 60 were from human sources. No clinical strains were present in *B. cepacia* type AW, *B. pyrrocinia*, and *B. cenocepacia* III-C clusters, and no environmental strains were present in the *B. cenocepacia* III-D cluster. All the remaining *B. cepacia* complex phylogenetic clusters contained strains from both sources, with the exception of *B. ubonensis*, which comprised the single type strain (Fig. 3).

The phylogenetic positions of several environmentally, biotechnologically and clinically relevant strains were also resolved (Table 2). Strains with documented biological control activities were found to be present in the *B. cepacia* type strain cluster, *B. cenocepacia* III-B, *B. ambifaria*, and *B. pyrrocinia* (Table 2). Interestingly, all three *B. cenocepacia* III-B biological control strains contained the *B. cenocepacia* pathogenicity island (2) (Table 2), including strain M36, which was originally registered for commercial use in the United States and has now been withdrawn (23). One other registered biopesticide strain, M54 (27), was assigned to the *B. ambifaria* cluster, where the majority of strains with biological control activity were found (Table 2). The *recA* genes drawn from *Burkholderia* genomic resources clustered as follows (Fig. 1; Table 2): strain G4, a well-known bioremediation strain, clustered with *B. vietnamiensis*, as expected; and strain 383, one of the isolates from the pioneering study of Stanier et al. (32), clustered within the group K lineage of *B. cepacia*, as did the metagenomic Sargasso Sea strain SAR-1 (40).

Genetic identity of *B. cepacia* complex strains. Several strains were found to possess the same *recA* RFLP type and identical *recA* sequences, yet were of distinct environmental or clinical origin. To resolve their clonality to the strain type level, macrorestriction and PFGE fingerprinting was performed, resulting in the definition of four strain types, each comprising a strain from an environmental and a clinical source (Fig. 4). Each strain pair possessed almost identical genomic fingerprints (all Dice coefficients of similarity >0.93 for each pair) clearly defining each pair as a distinct genetic strain type. Pairs were found within the following species (see Table 2 and Fig. 4): *B. cepacia* strains, ATCC 17759 (environmental, isolated before 1966), and LMG 14087 (clinical, isolated in 1988 from a wound swab in the United Kingdom); ATCC 25416^T (environmental, isolated in the 1940s) and J1050 (clinical); *B. ambifaria* strains AMMD^T (environmental) and AU0212 (clinical); and *B. stabilis* strains HI-2482 (environmental) and LMG 14294 (clinical, isolated from sputum from a patient with cystic fibrosis in Belgium in 1993).

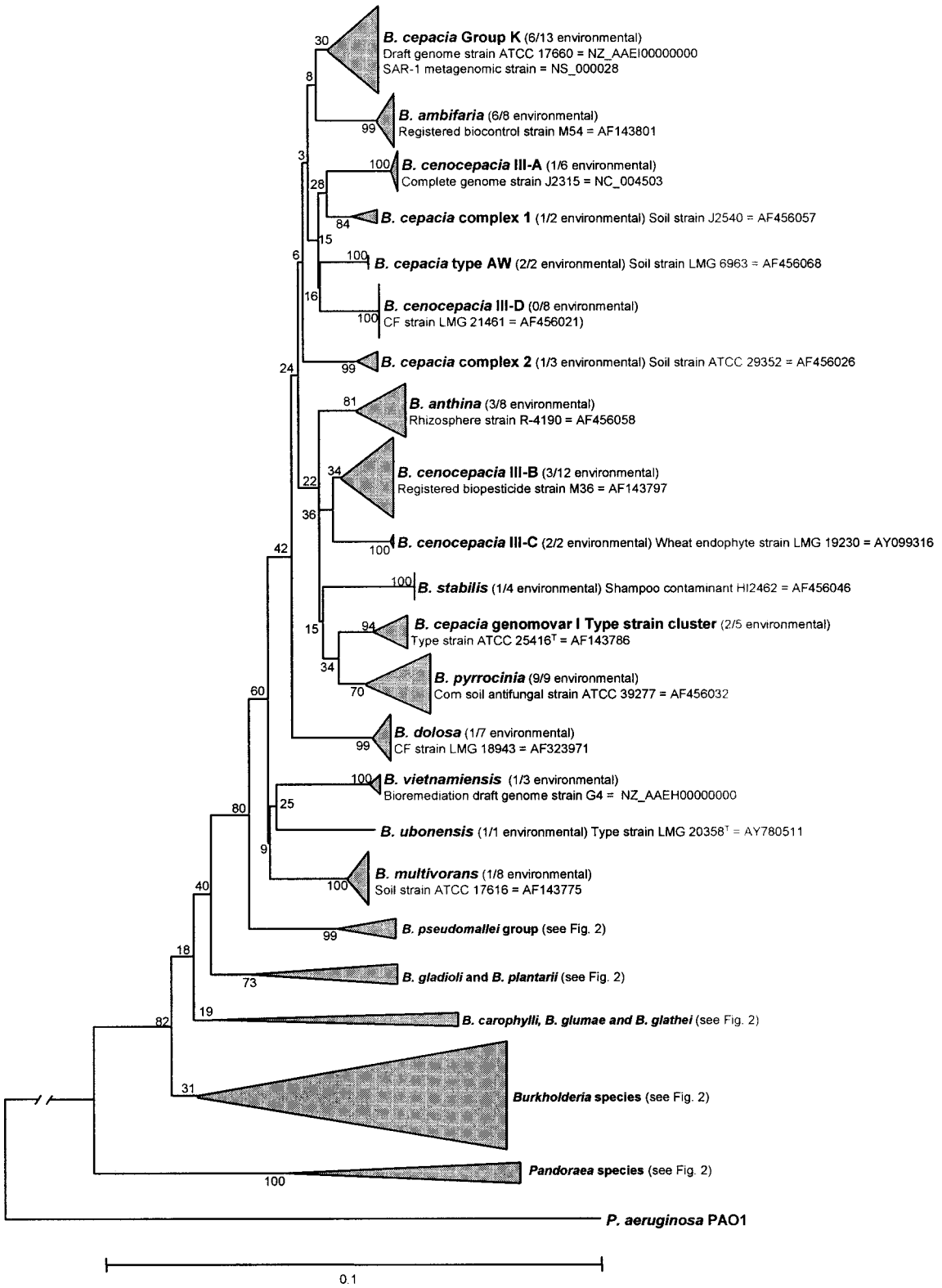

FIG. 3. Phylogenetic analysis of *B. cepacia* complex strains using novel *Burkholderia recA* sequences. A phylogenetic tree comparing 160 *B. cepacia* complex strains, 28 *Burkholderia*, and four *Pandoraea recA* sequences is shown as a composite of phylogenetically distinct sequence clusters. Bootstrap values and genetic distance scale are indicated. The species or group name for each cluster is shown in bold. Additional information for one or more reference or interesting strains within the group is also shown. The number of sequences deriving from environmental strains versus the total number of sequences in the group is shown in brackets.

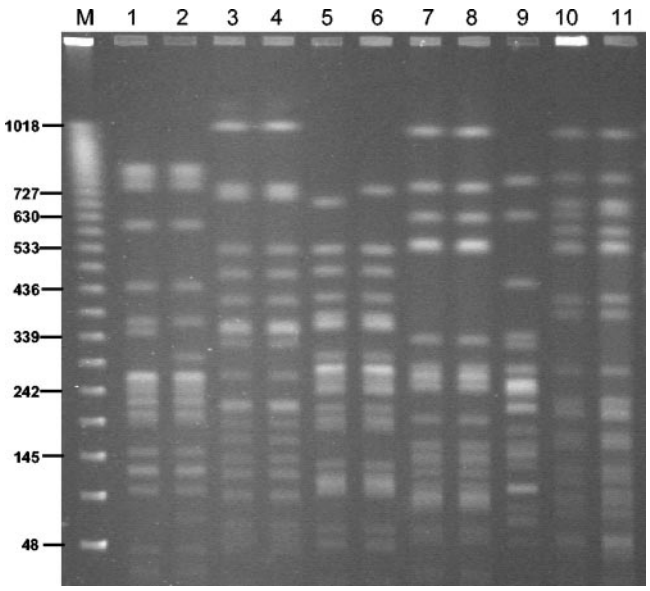

FIG. 4. PFGE fingerprinting of environmental and clinical *B. cepacia* complex strains. Macrorestriction analysis with SpeI of the following strains is shown in each lane as follows (given, respectively, for each strain pair): 1 and 2, *B. cepacia* strains ATCC 17759 and LMG 14087; 3 and 4, *B. cepacia* strains ATCC 25416^T and J1050; 5 and 6, *B. ambifaria* strains AMMD^T and AU0212; 7 and 8, *B. ambifaria* strains M54 and J82; 9, *B. cenocepacia* strain M36; 10 and 11, *B. stabilis* strains HI-2482 and LMG 14294. Molecular size markers were run in lane M, and the sizes of relevant fragments are indicated in kb.

Strain AMMD^T, a well-characterized biocontrol isolate (4, 27), was almost identical to strain AU0212 recovered from a cystic fibrosis patient, differing only in one macrorestriction fragment (Fig. 4). Interestingly, both strains had the same geographic origin, Wisconsin, even though their sources were distinct, soil and cystic fibrosis sputum. Genomic fingerprinting of the three *B. cepacia* complex biopesticide strains registered for commercial use (Table 2) (27) also revealed an interesting feature of these isolates in that they were all registered as type Wisconsin, a designation derived from their phenotypic features and source. However, it is clear that this designation was not related to their genotype or even species identity. Strain M36 was a member of *B. cenocepacia* III-B (Fig. 3; Table 3) and possessed a macrorestriction profile clearly distinct from strains M54 and J82 (Fig. 4). Moreover, the latter biocontrol isolates were in fact exactly the same *B. ambifaria* strain type (Fig. 4), even though each possess slightly different biopesticidal properties (Table 2) (27).

DISCUSSION

We have developed a genetic identification approach for the entire *Burkholderia* genus that can discriminate between members of the closely related *B. cepacia* complex. Sequence polymorphism within the *recA* gene has proven very useful in defining the taxonomy of the *B. cepacia* complex (6, 21), but the original approach could not be applied to *Burkholderia* species outside the complex. By designing new *Burkholderia* species *recA* PCRs, we have demonstrated that RFLP analysis of the gene can be used to differentiate the majority of *Burkholderia* strains. However, this pattern-matching technique was still limited in its ability to completely discriminate between all species and required comparison to known reference strains for preliminary grouping of strains. The *Burkholderia*-specific *recA* primers and an extensive set of reference *recA* sequences provided by this study have facilitated further definition of species diversity within both *Burkholderia* and the *B. cepacia* complex and were also used to examine the phylogenetic and genotypic relationships of strains from clinical versus environmental sources.

The BUR1 and BUR2 primers enabled amplification of an 869-bp *recA* fragment, but were not absolutely specific to *Burkholderia* and cross-reacted with control strains of the genera *Bordetella*, *Pandoraea*, *Ralstonia*, and *Xanthomonas*. However, nucleotide sequence deriving from analysis of novel *Burholderia* and *Pandoraea recA*s enabled the design of primers BUR3 and BUR4, which were found to be specific for the genus. Only *B. andropogonis* repeatedly failed to produce correct amplification products with both the BUR1 and BUR2, and BUR3 and BUR4 primers. Southern hybridization of *B. andropogonis* chromosomal DNA demonstrated that DNA homologous to the *recA* gene of *B. cenocepacia* J2315 was present, suggesting that the lack of amplification had not resulted from gene deletion in this isolate (data not shown).

Phylogenetic analysis of eight of the nine strains representing putative novel *Burkholderia* species (Table 1) corroborated the results of the phenotypic data assigning them to the genus (Fig. 2). Only strain R-15281 clustered outside the genus and adjacent to members of *Bordetella*. Two of the novel *Burkholderia* species strains, R-15273 and LMG 21262, possessed identical *recA* genes, suggesting they were members of the same novel species group that was closely related to *B. fungorum* by phylogenetic analysis (Fig. 2). The remaining six *Burkholderia* species strains were all highly distinct according to the bootstrap values observed in the phylogenetic analysis, suggesting they were members of novel *Burkholderia* species. These findings were consistent with the analysis of their whole-cell protein profiles (P.V., unpublished data). Two clusters within the *B*. cepacia complex were only weakly grouped by the phylogenetic analysis, *B. cepacia* group K and *B. cenocepacia* group III-B (Fig. 3; bootstrap values of 30 and 34, respectively). All other *B. cepacia* complex species *recA* genes fell into clusters associated with bootstrap values of greater than 70, consistent with their recognition as distinct species. The considerable polymorphism observed within the *recA* genes of *B. cepacia* complex group K and *B. cenocepacia* III-B indicates that further species diversity may be present within these groups.

The topology of the *Burkholderia* genus *recA* phylogeny (Fig. 2) was congruent with the 16S rRNA gene-based phylogenetic tree presented by Coenye and Vandamme (5) with respect to the following: (i) all *Burkholderia* species were distinct from other genera; (ii) all taxonomically classified *Burkholderia* species formed separate arms within both trees; (iii) species within the *B. cepacia* complex formed a distinct cluster; and (iv) the *B. cepacia* complex cluster was most closely linked to a group containing *B. mallei*, *B. pseudomallei*, and *B. thailandensis*. Clusters which were divergent in phylogenetic analysis based on the *recA* gene and 16S rRNA gene gene (5) were as follows: (i) *B. plantari* and *B. glumae* formed a distinct cluster in both trees, however, this group also contained *B. gladioli* in the *recA*

analysis (Fig. 2); (ii) *B. fungorum*, *B. caledonica*, *B. graminis*, and *B. caryophylli*, which formed a distinct cluster in the 16S rRNA gene tree, were not closely associated according to *recA*; and (iii) the positions of all the remaining *Burkholderia* species examined were not conserved between the *recA* and 16S rRNA gene analyses. The major advantage *recA* phylogenetic analysis provides over the 16S rRNA gene for classification of *Burkholderia* is the greater degree of resolution among closely related species within the genus, such as the *B. cepacia* complex.

Although RFLP analysis of the *recA* gene did not discriminate between all species of *Burkholderia*, sequence analysis of the BUR1 and BUR2 amplicon was sufficient to separate all strains, including their differentiation from members of the *B. cepacia* complex (Fig. 2 and 3). In particular, the degree of resolution of the *recA* phylogenetic trees for members of the *B. cepacia* complex was much greater than observed with 16S rRNA gene analysis (6). However, the presence of discrete *recA* lineages within species such as *B. cenocepacia* and *B. cepacia* adds an additional level of complexity. A promising finding of the study was that analysis of just a 300-bp region of *recA* sequence within the BUR3 and BUR4 amplicon produced phylogenetic trees with the same topology and discrimination as the nearly full-length trees derived from analysis of the BUR1 and BUR2 PCR. These data suggest that the BUR3 and BUR4 PCR could provide a useful and rapid non-culture-based approach to explore the *Burkholderia* diversity in various environments.

Genomic resources for *Burkholderia* have increased substantially in the last few years, and we have shown that by phylogenetic analysis of whole-genome sequence derived *recA*s the classification of both cultured and uncultured genomic strains can be clarified. The genome strains *B. cepacia* ATCC 17760 (strain 383) (32) and *Burkholderia* Sargasso Sea strain SAR-1 reconstructed from metagenomic data (38) were both found to be members of *B. cepacia* group K. The *recA* gene of SAR-1 was identical to that of strain R-12710 (data not shown), cultivated from sheep with mastitis (3), and another marine isolate (obtained from the Sea of Japan) also belongs to *B. cepacia* group K (P.V., unpublished data). While the presence of *B. cepacia* complex members in river water has been reported (15), their isolation from marine environments is thought to be rare. The novel primers developed in this study can be applied to investigate this area of scientific interest.

Phylogenetic analysis of *Burkholderia recA* genes also demonstrated that, like other taxonomic criteria, it could not be used as a means to distinguish environmental from clinical strains. Strains from both sources were found throughout the extensive phylogenetic tree derived from this study (Fig. 3). Although no clinical strains were found in four clusters and no environmental strains found in one other *B. cepacia* complex cluster (Fig. 3), these clusters contained small numbers of strains. It is likely that as more *B. cepacia* complex strains are examined, so that the number of strains in these clusters is increased, environmental and clinical stains will be identified for each cluster.

Analysis of the *recA* gene of *B. cepacia* complex strains in this study resulted in the identification of several clonal pairs of strains each from distinct environmental and clinical sources (Fig. 4). Such pairs were found for *B. cepacia*, *B. stabilis*, and *B. ambifaria* and clearly showed that genotypically identical *B. cepacia* complex strains can be isolated from both human infections and natural or environmental sources. This work extends the work of LiPuma and colleagues (17), who demonstrated that an epidemic cystic fibrosis strain of *B. cenocepacia* was identical to a strain found in the soil. *B. ambifaria* strain AMMD^T is the first strain with known biopesticidal properties (4, 27) to have an essentially clonal relative, strain AU0212, cultured from a patient with cystic fibrosis (Fig. 4). In conclusion, it appears that all *B. cepacia* complex bacteria are capable of colonizing a wide range of habitats, and in the case of infectious niches, this trait appears more dependent on the vulnerability of the host than the taxonomic or phylogenetic classification of the strain.

B. cepacia complex strains with useful biotechnological properties were also found throughout the *recA* phylogeny. *B. cepacia*, *B. cenocepacia*, *B. ambifaria*, and *B. pyrrocinia* strains with biopesticidal or antifungal properties were identified (Table 2). The ability to obtain accurate species information using *recA* sequence data may prove vital in the characterization of future biotechnologically useful strains, especially since detailed understanding of the strain taxonomy forms one of the major risk assessment criteria for commercial registration of bacteria (27). In addition, our study has shown that classification of biopesticidal strains based on phenotypic criteria alone is not adequate. The two type Wisconsin strains of *B. ambifaria*, M54 and J82, which remain registered for commercial use were found to be genotypically identical (Fig. 4) even though they possess different biopesticidal properties (Table 2) (27). This kind of phenotypic variability within a single clone has also be observed in isolates from cystic fibrosis patient infections (14). Although the *B. cepacia* complex has been highlighted as a group of bacterial species which require risk assessment as biological control agents (27), the case for this evaluation is not limited to these organisms.

In summary, the *recA*-based approach developed in this study provides molecular tools for the identification of *Burkholderia* species that will help to enable researchers to keep pace with the ever-increasing ecological, pathogenic, and genomic interest in the genus.

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