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DNA-Based Diagnostic Approaches for Identification of *Burkholderia cepacia* Complex, *Burkholderia vietnamiensis*, *Burkholderia multivorans*, *Burkholderia stabilis*, and *Burkholderia cepacia* Genomovars I and III

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Bacteria of the *Burkholderia cepacia* complex consist of five discrete genomic species, including genomovars I and III and three new species: *Burkholderia multivorans* (formerly genomovar II), *Burkholderia stabilis* (formerly genomovar IV), and *Burkholderia vietnamiensis* (formerly genomovar V). Strains of all five genomovars are capable of causing opportunistic human infection, and microbiological identification of these closely related species is difficult. The 16S rRNA gene (16S rDNA) and *recA* gene of these bacteria were examined in order to develop rapid tests for genomovar identification. Restriction fragment length polymorphism (RFLP) analysis of PCR-amplified 16S rDNA revealed sequence polymorphisms capable of identifying *B. multivorans* and *B. vietnamiensis* but insufficient to discriminate strains of *B. cepacia* genomovars I and III and *B. stabilis*. RFLP analysis of PCR-amplified *recA* demonstrated sufficient nucleotide sequence variation to enable separation of strains of all five *B. cepacia* complex genomovars. Complete *recA* nucleotide sequences were obtained for 20 strains representative of the diversity of the *B. cepacia* complex. Construction of a *recA* phylogenetic tree identified six distinct clusters (*recA* groups): *B. multivorans*, *B. vietnamiensis*, *B. stabilis*, genomovar I, and the subdivision of genomovar III isolates into two *recA* groups, III-A and III-B. Alignment of *recA* sequences enabled the design of PCR primers for the specific detection of each of the six latter *recA* groups. The *recA* gene was found on the largest chromosome within the genome of *B. cepacia* complex strains and, in contrast to the findings of a previous study, only a single copy of the gene was present. In conclusion, analysis of the *recA* gene of the *B. cepacia* complex provides a rapid and robust nucleotide sequence-based approach to identify and classify this taxonomically complex group of opportunistic pathogens.

The *Burkholderia cepacia* complex is a very diverse group of bacteria (28). They are important opportunistic human pathogens that cause devastating infections in patients with cystic fibrosis (CF) (8, 13) and in other vulnerable individuals (25). The ability of *B. cepacia* to cause disease is not limited to the human host, as these bacteria are also important plant pathogens (7). In addition, *B. cepacia* complex bacteria may have commercially useful properties and have been used in agriculture as biocontrol agents and in the bioremediation of toxic agents (9, 14). Current taxonomic classification divides isolates previously classified as *B. cepacia* into five genomovars or discrete genomic species, all of which may be isolated from clinical infection (28). Strains of genomovar II have been proposed as the new species *Burkholderia multivorans* (28), and strains of genomovar V were found to be members of the proposed species *Burkholderia vietnamiensis* (6, 28). Strains within genomovar IV have recently been proposed as the new species *Burkholderia stabilis* (29). The remaining genomovars, I and III, await species designation if differential phenotypic tests can be found (28). Determination of the genomovar status of *B. cepacia* complex strains was based on a polyphasic taxonomic approach which utilized phenotypic tests such as whole-cell protein profile analysis and genotypic tests such as DNA-DNA hybridization (27, 28). A single test for identification of genomovar status is currently not available. Even conventional phenotypic identification of *B. cepacia* and its differentiation from closely related species are often not straightforward (3, 10, 24, 30). Incorrect diagnosis of infection, especially in patients with CF, may have serious clinical ramifications (13). A simple means of *B. cepacia* complex species identification will enhance our understanding of the pathogenesis and epidemiology of these opportunistic human pathogens.

Molecular diagnostic probes based on PCR provide a rapid and frequently highly discriminatory means of microbial identification (2, 15, 24, 30). In order to develop rapid tests to

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determine the genomovar status of *B. cepacia* complex isolates, we examined nucleotide sequence polymorphism in two genes, the 16S rRNA gene (16S rDNA) and *recA* gene. Although widely used for bacterial systematics, recent results demonstrate that 16S rDNA is limited in its ability to differentiate the *B. cepacia* complex (15, 23). Nucleotide sequence variation within this gene is not sufficient to enable all current genomovars within the complex to be easily identified (2, 15, 23). The *B. cepacia* complex *recA* (RecA is a protein essential for repair and recombination of DNA [5, 11]) was chosen as an alternative template gene for the following reasons. First, analysis of *recA* has proven very useful in previous studies of molecular systematics among closely related bacteria (5, 11). Second, although only a limited number of *B. cepacia* genes have been characterized, two *recA* sequences, each from a different strain, were available in the nucleotide sequence databases as a basis to initiate analysis (19, 31). Finally, in *B. cepacia*, *recA* has been shown to be diploid, with a single copy of the gene residing on each of the two large chromosomes present in *B. cepacia* strain ATCC 25416 (21). If both copies of *recA* are identical, they may form a more robust platform upon which to base diagnostic PCR probes than the multicopy rRNA genes, which are dispersed across the multiple replicons constituting the genome of *B. cepacia* and are potentially susceptible to genomic rearrangement (4, 12, 21). Diagnostic tests for the determination of *B. cepacia* complex genomovar status, developed using systematic genetic approaches, are described.

MATERIALS AND METHODS

Bacterial strains. *B. cepacia* complex strains were cultured and biochemically identified as described previously (10, 16, 28). Bacteria were routinely grown on blood agar, Trypticase soy agar, or Luria-Bertani broth (LB; 23) at 35°C for 24 to 48 h until confluent growth was obtained (10, 16). Molecular identification approaches were developed using the 35 isolates listed in Table 1, of which 29 isolates were derived from a published panel of strains representative of each genomovar (18). The sources of the six additional strains initially screened in this study are also provided in Table 1. The latter 35 isolates and 68 further *B. cepacia* complex isolates examined (103 total) were recovered from various sources including patients with CF, patients with non-CF infection, and the natural environment. Strains were selected to be representative of the genetic diversity of *B. cepacia* complex from previous studies (10, 16–18, 28). Additional reference strains were obtained from the American Type Culture Collection (ATCC, Manassas, Va.). Related bacterial species commonly recovered from patients with CF and misidentified as *B. cepacia* were derived from previous studies (10, 16).

Genomovar status. The genomovar status of *B. cepacia* complex strains used to develop the molecular identification strategies was determined by whole-cell protein profile analysis and a polyphasic approach as described previously (27, 28).

PCR analysis. PCR was performed essentially as described previously (16, 17) however, PCR reagents were purchased from Qiagen Inc. Canada, and tests were performed in the presence of Qiagen Q solution, which enhances amplification of DNA templates which are rich in G+C content (Qiagen Inc. [http://www.qiagen.com]). Template DNA was prepared from fresh overnight cultures as previously described (17), with modification of the lysis buffer to include pronase at 0.5 mg/ml. DNA was quantitated by visualization on agarose gels, and approximately 20 ng was incorporated into 25-μl reactions which contained 1 U *Taq* DNA polymerase, 250 μM (each) deoxynucleoside triphosphate, 1.5 mM $MgCl_2$, and 1× PCR buffer. Approximately 20 pmol of each appropriate oligonucleotide primer (Table 2) was added to each reaction, and amplification was carried out using a Gene Cycler (Bio-Rad, Mississauga, Ontario, Canada) for 30 cycles of 30 s at 94°C, 45 s at the appropriate annealing temperature (Table 2), and 60 s at 72°C; a final extension of 10 min at 72°C was applied to all thermal cycles. The sequences and specificity of all PCR primers used in this study are provided in Table 2.

For restriction fragment length polymorphism (RFLP) analysis, 5 to 10 μl of PCR product was combined with the appropriate restriction enzyme buffer and endonuclease as outlined by the manufacturer (New England BioLabs Inc., Mississauga, Ontario, Canada) and incubated at 37°C for 1 to 3 h. PCR and RFLP products were analyzed by agarose gel electrophoresis, with agarose concentrations adjusted between 1% and 2.5%, appropriate for the size range of DNA being analyzed, and using 0.5× Tris-borate-EDTA buffer (24). Molecular size markers of the appropriate size range were included on all gels (100-bp DNA ladder or 1-kb DNA ladder [Life Technologies GIBCO BRL Products, Burlington, Ontario, Canada]: 50-bp ladder [Pharmacia, Uppsala, Sweden]). RFLP patterns were analyzed as described previously (16, 17).

Typing of *B. cepacia* strains by random amplified polymorphic DNA (RAPD) PCR analysis was performed exactly as described previously (16). Detection by PCR of the *B. cepacia* epidemic strain marker (BCESM) and cable pilin subunit gene (*cblA*) was carried out as described previously (17).

Nucleotide sequence analysis. Sequencing reactions were prepared using ABI PRISM DyeDeoxy Terminator cycle sequencing kits with AmpliTaq FS DNA polymerase according to the manufacturer's instructions and analyzed using either an ABI PRISM model 373 Stretch or a model 377 DNA sequencer (Perkin-Elmer Applied Biosystems, Foster City, Calif.). Raw sequences from both strands of the PCR products were then aligned, and a consensus sequence was derived using DNASTAR software (DNASTAR Inc., Madison, Wis.). Sequence identity was confirmed by analysis using the basic local alignment sequence tool (BLAST) (1) at the National Center for Biotechnology Information (NCBI, Bethesda, Md.).

Phylogenetic analysis. Evolutionary relationships between *recA* genes were determined using the Data Analysis in Molecular Biology software (DAMBE; http://web.hku.hk/~xxia/software/software.htm). After *recA* sequence determination, multiple sequence alignments were performed using CLUSTAL W (26). Phylogenetic trees were drawn from the resulting alignments using the genetic distance-based neighbor-joining algorithms of DAMBE. Paralinear and Jukes-Cantor-based algorithms were evaluated and found to demonstrate identical phylogenies for the strains examined (data not shown). Trees constructed using Jukes-Cantor distance matrices are presented in this report. Sequence input order was randomized, and 100 data sets were examined by bootstrapping resampling statistics for each analysis.

PFGE and Southern hybridization. Macrorestriction and pulsed-field gel electrophoresis (PFGE) genomic fingerprinting of *B. cepacia* strains were performed as described previously (18). Multiple replicons which constitute the *B. cepacia* genome were separated as previously described (4, 21). Southern blot transfer of the DNA to nylon membranes was allowed to proceed for 48 h and probed with a digoxigenin-labeled *recA* PCR probe as previously described (17).

Nucleotide sequence accession numbers. The complete *recA* nucleotide sequences were determined for the 20 *B. cepacia* complex strains listed in Table 1. Sequences were submitted to GenBank as an aligned set, and each was assigned an accession number as shown in Table 1.

RESULTS

Analysis of 16S rRNA genes of the *B. cepacia* complex and closely related species. Amplified-rDNA restriction analysis (ARDRA; 23, 27) was performed to examine sequence polymorphism of the 16S rRNA gene in strains representative of the *B. cepacia* complex. The primer pair used for ARDRA analysis of the 16S rDNA in this study is novel (UNI2 and UNI5 [Table 2]) and has proven effective for amplification of the gene from all bacterial species tested to date (S. K. Byrne, unpublished data). To evaluate the efficacy of both the 16S rRNA gene (and *recA* [see below]) for systematic differentiation of the *B. cepacia* complex, a panel of 35 (Table 1) strains representative of all five genomovars of the *B. cepacia* complex (18) was initially examined. The 16S rDNA 1-kb amplicon was successfully amplified with primers UNI2 and UNI5 from all 35 strains. The identity of the amplified DNA was confirmed to encode 16S rDNA by direct nucleotide sequence analysis of PCR products (data not shown). To detect nucleotide sequence variation within the amplified gene, several restriction endonucleases expected to cleave frequently within bacterial rDNA were screened for their ability to reveal genomovar-specific RFLPs (data not shown). The enzyme *Dde*I was found to produce the most discriminatory RFLPs for the *B. cepacia* complex (Fig. 1A). Three 16S rDNA ARDRA patterns were found among the 35 strains (Table 1): type 1, *B. vietnamiensis*; type 2, *B. cepacia* genomovars I and III and *B. stabilis*; and type 3, *B. multivorans* (Fig. 1A). Polymorphism detected by ARDRA analysis of the 16S rDNA was consistent with the taxonomic classification of the new species *B. multivorans* and *B. vietnamiensis* (28) but was not sufficient to separate *B. cepacia* genomovars I and III (28) and the new species *B. stabilis* (29).

Although the 16S rDNA ARDRA method was limited with regard to its differentiation of the *B. cepacia* complex, it is a

TABLE 1. *B. cepacia* complex strains used to develop molecular diagnostic approaches

Genomovar and strain[a]	16S rDNA *Dde*I RFLP[b]	Result of *recA* RFLP by digestion with:		*recA* GenBank accession no.	*recA* phylogenetic and PCR identification group
		*Hae*III[c]	*Mnl*I[d]		
B. cepacia genomovar I					
ATCC 25416^T	2	D	d	AF143786	
J1050[e]	2	D	d		I
ATCC 17759	2	E	e	AF143788	I
CEP509	2	E	e	AF143787	I
LMG 17997	2	E	e		I
B. multivorans (formerly genomovar II)					
C5393	3	F	a	AF143776	*B. multivorans*
LMG 13010^T	3	F	a		*B. multivorans*
CF-A1-1	3	F	a		*B. multivorans*
C1576	3	C	a	AF143774	*B. multivorans*
HI-2308[e]	3	C	a	AF143777	*B. multivorans*
JTC	3	F	a	AF143778	*B. multivorans*
C1962	3	F	a		*B. multivorans*
ATCC 17616	3	F	a	AF143775	*B. multivorans*
249-2	3	F	a		*B. multivorans*
B. cepacia genomovar III					
J2315	2	G	f		III-A
BC7	2	G	f		III-A
K56-2	2	G	f	AF143779	III-A
C5424	2	G	f	AF143781	III-A
C6433	2	G	f	AF143780	III-A
C4455[e]	2	G	f	AF143782	III-A
C1394	2	H	g	AF143783	III-B
ATCC 17765	2	H	g		III-B
CEP511	2	I	h	AF143785	III-B
PC184	2	J	i	AF143784	III-B
B. stabilis (formerly genomovar IV)					
LMG 07000[e]	2	J	b	AF143789	*B. stabilis*
LMG 14291[e]	2	J	b	AF143790	*B. stabilis*
LMG 14294	2	J	b		*B. stabilis*
C7322	2	J	b		*B. stabilis*
LMG 14086	2	J	b		*B. stabilis*
LMG 18888	2	J	b		*B. stabilis*
B. vietnamiensis (formerly genomovar V)					
PC259	1	A	c	AF143791	*B. vietnamiensis*
LMG 16232	1	A	c		*B. vietnamiensis*
FC441	1	A	c		*B. vietnamiensis*
LMG 10929^T	1	B	c	AF143793	*B. vietnamiensis*
C2822[e]	1	B	c	AF143792	*B. vietnamiensis*

[a] All strains except those indicated in footnote *b* below were derived from the *B. cepacia* complex strain panel where source and other data relevant to each are described (18).
[b] Data are numerical 16S rRNA RFLP types as indicated in Fig. 1.
[c] Letters correspond to alphabetical RFLP types as shown in Fig. 2A.
[d] Letters correspond to alphabetical RFLP types as shown in Fig. 2B.
[e] Strains not derived from the *B. cepacia* complex panel (18): J1050, isolated from non-CF patient within the U.K. and kindly provided by J. R. W. Govan; HI-2308, recovered from neonatal infection and kindly provided by J. J. LiPuma; C4455, recovered from a patient with CF (16); LMG 07000, recovered from a patient with septicaemia (20); LMG 14291, recovered from a patient with CF (20); and C2822, recovered from a patient with CF (16).

versatile means of rapid identification and discrimination for other bacterial species which may be recovered from CF patient sputum (24). *Burkholderia gladioli*, *Ralstonia picketti*, *Chryseobacterium meningosepticum*, *Stenotrophomonas maltophilia*, and *Commomonas acidovorans* are examples of organisms which in clinical settings have been incorrectly identified as members of the *B. cepacia* complex (3, 10). ARDRA analysis of the 16s rDNA with *Dde*I of the latter species is shown in Fig. 1B. Each of these microbial species was easily distinguished from bacteria of the *B. cepacia* complex by ARDRA analysis (Fig. 1).

PCR amplification of *B. cepacia* complex *recA* gene. Two *recA* sequences, each from a different strain of *B. cepacia*, were available within GenBank. The genomovar status of *B. cepacia* strain JN25, from which one *recA* sequence (GenBank accession no. D90120) was derived, was not known; it was an isolate of clinical origin characterized in Japan in 1990 (19). The second *recA* sequence (accession no. U70431) was derived from the *B. cepacia* reference strain ATCC 17616 (31); this strain has been classified as the new species *B. multivorans* (28). Each sequence was aligned using BLAST (1). PCR primers BCR1 and BCR2 (Table 2) were designed from homolo-

TABLE 2. Species-specific and nucleotide-sequencing PCR primers used in study

Specificity and primer name	Sequence (5′ to 3′)[a]	Position	PCR annealing temperature (°C)	Product size
Universal 16S rDNA primer				
UNI2	GACTCCTACGGGAGGCAGCAG	336–356[b]	60	1,020 bp[c]
UNI5[a]	CTGATCCGCGATTACTAGCGATTC	1337–1360[b]		
B. cepacia complex *recA*				
BCR1	TGACCGCCGAGAAGAGCAA	2–20[d]	58	1,043 bp[d]
BCR2[a]	CTCTTCTTCGTCCATCGCCTC	1044–1024[d]		
recA sequencing primers				
BCR3	GTCGCAGGCGCTGCGCAA	513–530[d]	58	532 bp,[d] 3′ half of *B. cepacia* complex *recA* gene in combination with primer BCR2
BCR4[a]	GCGCAGCGCCTGCGACAT	528–511[d]	58	527 bp,[d] 5′ half of *B. cepacia* complex *recA* gene in combination with primer BCR1
B. cepacia genomovar I				
BCRG11	CAGGTCGTCTCCACGGGT	112–129[e]	62	492 bp[e]
BCRG12[a]	CACGCCGATCTTCATACGA	603–585[e]		
B. multivorans (*Bm*)				
BCRBM1	CGGCGTCAACGTGCCGGAT	321–339[d]	62	714 bp[d]
BCRBM2[a]	TCCATCGCCTCGGCTTCGT	1034–1016[d]		
B. cepacia genomovar III-A				
BCRG3A1	GCTCGACGTTCAATATGCC	294–309[f]	62	378 bp[f]
BCRG3A2[a]	TCGAGACGCACCGACGAG	671–654[f]		
B. cepacia genomovar III, RG-B				
BCRG3B1	GCTGCAAGTCATCGCTGAA	228–246[g]	60	781 bp[g]
BCRG3B2[a]	TACGCCATCGGGCATGCT	1008–991[g]		
B. cepacia genomovar IV, RG-4				
BCRG41	ACCGGCGAGCAGGCGCTT	361–378[h]	64	647 bp[h]
BCRG42[a]	ACGCCATCGGGCATGGCA	100–990[h]		
B. vietnamiensis, RG-BV				
BCRBV1	GGGCGACGGCGACGTGAA	84–101[i]	62	378 bp[i]
BCRBV2[a]	TCGGCCTTCGGCACCAGT	461–444[i]		

[a] Base positions in primer sequence which were mismatched in other genomovars are underlined.
[b] Position in relation to *Escherichia coli* 16S rRNA gene (GenBank accession number J01859).
[c] Size in relation to *B. cepacia* 16S rRNA gene (GenBank accession number X87275).
[d] Size and position in relation to *B. multivorans* ATCC 17616 *recA* sequence U70431.
[e] Size and position in relation to *B. cepacia* genomovar I strain ATCC 17759 *recA* (Table 1).
[f] Size and position in relation to *B. cepacia* genomovar III-A strain K56-2 *recA* (Table 1).
[g] Size and position in relation to *B. cepacia* genomovar III-B strain C1394 *recA* (Table 1).
[h] Size and position in relation to *B. stabilis* strain LMG14291 *recA* (Table 1).
[i] Size and position in relation to *B. vietnamiensis* strain C2822 *recA* (Table 1).

gous sequences at the 5′ and 3′ ends of the *recA* open reading frame. These primers amplified a single 1-kb amplicon from all 35 strains representative of the *B. cepacia* complex (Table 1). The identity of the 1-kb fragment was subsequently confirmed to be *recA* by direct nucleotide sequence analysis of PCR products (see below). A further 68 isolates, each with biochemical- (10) and genomovar-specific (28) properties characteristic of the *B. cepacia* complex, also tested positive with these *recA* primers. PCR with primers BCR1 and BCR2 failed to amplify PCR products from the following species: *B. gladioli*, *R. pickettii*, *C. meningosepticum*, *S. maltophilia*, *C. acidovorans*, *Escherichia coli*, and *Pseudomonas aeruginosa* (data not shown).

RFLP analysis of the *B. cepacia* complex *recA* gene. RFLP analysis with *Alu*III, *Bsa*WI, *Mnl*I, and *Hae*III was investigated. RFLP types generated by digestion with *Hae*III were the most discriminatory among the four restriction enzymes (data not shown). Ten distinct *Hae*III RFLP patterns were found among the 35-isolate *B. cepacia* complex panel initially examined, and each pattern was assigned an alphabetical code (Fig. 2; Table 1). *Hae*III RFLP analysis was capable of discriminating among all five genomovars (Fig. 2A) except for RFLP pattern J, which was shared by the genomovar III strain PC184 and all of the *B. stabilis* strains examined. To distinguish genomovar III and *B. stabilis* strains with this RFLP type, analysis with an additional enzyme (such as *Mnl*I [Fig. 2B]) was required.

RFLP analysis with *Mnl*I revealed eight patterns among the 35 *B. cepacia* complex isolates (Table 1). Single-signature RFLP types were produced for the *B. multivorans*, *B. vietnamiensis*, and *B. stabilis* strains examined (Fig. 2B; Table 1). The *B. stabilis* and *B. multivorans Mnl*I RFLP types (patterns a and b, respectively) were only slightly different, with the second largest RFLP fragment being consistently smaller in size among

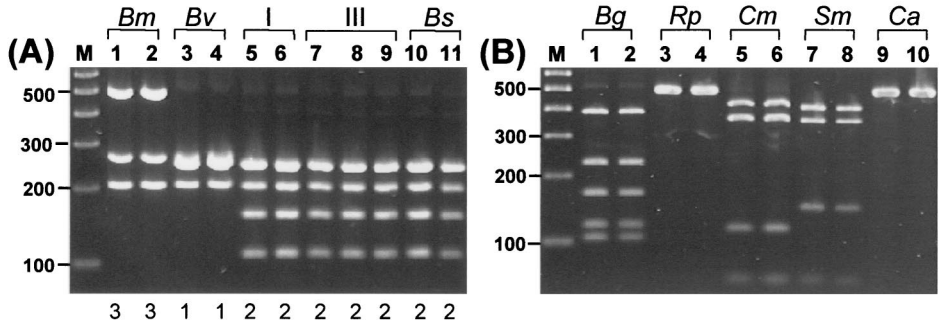

FIG. 1. RFLP analysis of the 16S rRNA gene. (A) Analysis of the 16S rRNA gene of strains from the *B. cepacia* complex. Lanes: 1, LMG 13010^T; 2, C1576; 3, LMG 10929^T; 4, LMG 16232; 5, ATCC 25416; 6, ATCC 17759; 7, C5424; 8, C1394; 9, CEP511; 10, LMG 07000; and 11, LMG 14294. The genomovar status of each strain is indicated above the lane numbers, and the numerical 16S rRNA RFLP type is shown below each lane. Molecular size markers are shown in lane M (100-bp ladder). (B) Analysis of 16S rRNA genes of other bacterial species which may be misidentified as *B. cepacia* or grow on BCSA (10). Reference strains from Henry et al. (10): Lanes: 1, *B. gladioli* (*Bg*) CEP82; 2, *B. gladioli* CEP89; 3, *R. picketti* (*Rp*) ATCC 27511; 4, *R. picketti* ATCC 49129; 5, *C. meningosepticum* (*Cm*) FC113; 6, *C. meningosepticum* FC224; 7, *S. maltophilia* (*Sm*) CEP272; 8, *S. maltophilia* C4525; 9, *C. acidovorans* (*Ca*) FC77; 10, *C. acidovorans* CEP145. In panel A, the numerical 16S rRNA RFLP type of each isolate is shown below each lane.

the *B. stabilis* strains examined (Fig. 2B). The same number of distinct patterns as observed with *Hae*III were also obtained by *Mnl*I digestion for the remaining genomovar I and III strains. Genomovar III strain PC184 produced a distinct *Mnl*I *recA* RFLP type (pattern i [Fig. 2; Table 1]) which was easily distinguished from the *Mnl*I RFLP type of *B. stabilis* (pattern b [Fig. 2; Table 1]). The remaining 68 *B. cepacia* complex isolates examined were all analyzed using *Hae*III for RFLP analysis of their amplified *recA* genes (Table 3).

Nucleotide sequence analysis of *recA*. To confirm the sequence variation detected by RFLP analysis of the amplified *recA* gene and facilitate the design of PCR primers specific to each *B. cepacia* genomovar, complete nucleotide sequence analysis of the gene was performed for 20 strains representative of each *recA* RFLP type (Table 1). The complete *recA* gene was sequenced in two 500-bp segments using the combinations of PCR primers BCR1, BCR2, BCR3, and BCR4 described in Table 2. The sequence of each segment was combined to produce the full-length *recA*. Sequence analysis of the *recA* amplicon of *B. multivorans* ATCC 17616 was performed as a control for the sequencing strategy; the sequence determined in this study was identical to the published sequence for this strain (31). BLAST analysis (1) of each nucleotide sequence confirmed that each encodes RecA with homology to the published *B. cepacia* sequences (19, 31). Computational analysis of the *Hae*III restriction sites within each nucleotide sequence matched those *Hae*III sites determined by RFLP analysis of the complete *recA* amplicon for each strain. Sequence-based restriction mapping of *recA* from genomovar III strain PC184 and the *B. stabilis* strains demonstrated the presence of closely overlapping *Hae*III sites. However, seven cleavage sites were present in PC184 and six in the *B. stabilis recA*, and each of these *recA* sequences was quite different (see phylogenetic analysis below). The minor differences in the *Hae*III fragment sizes were not distinguished under the electrophoresis conditions used for RFLP analysis (Fig. 2A).

Phylogenetic analysis of *recA*. Construction of a *recA* phylogenetic tree of the *B. cepacia* complex was carried out as described in Materials and Methods. The *Bordetella pertussis recA* gene (accession no. X53457) was used to root the tree because previous molecular systematic analysis of bacterial *recA* genes had demonstrated that it was closely related to *B. cepacia recA* and had placed both bacteria within the β-subgroup of the *Proteobacteria* (5, 11). The *P. aeruginosa* (accession no. X05691), *Methylobacillus flagellatum* (accession no. M35525), and *Xanthomonas campestris* (accession no. U49086) *recA* sequences were also included in the phylogenetic analysis as indicators of the ability of the analysis to differentiate among unrelated species. The resulting nucleotide sequence-based phylogenetic tree is shown in Fig. 3. All five previously determined genomovars (28) formed distinct arms within the tree, consistent with their proposal as new species (28, 29) or un-

TABLE 3. Evaluation of genomovar-specific primers on a collection of genetically diverse *B. cepacia* complex strains

PCR primer set specificity	No. of isolates testing positive with each primer set	*recA Hae*III RFLP type within positive isolates	No. of isolates within each RFLP type	No. of genetically heterogeneous strains within each RFLP type	16s rDNA RFLP type (*Dde*I)
Genomovar I	8	D	5	3	2
		E	3	3	2
B. multivorans	20	C	3	3	3
		F	17	16	3
Genomovar III-A	16	G	16	8	2
Genomovar III-B	24	H	4	3	2
		I	10	8	2
		J	10	7	2
B. stabilis	22	J	22	4	2
B. vietnamiensis	13	A	6	5	1
		B	7	6	1
Total	103	10	103	66	

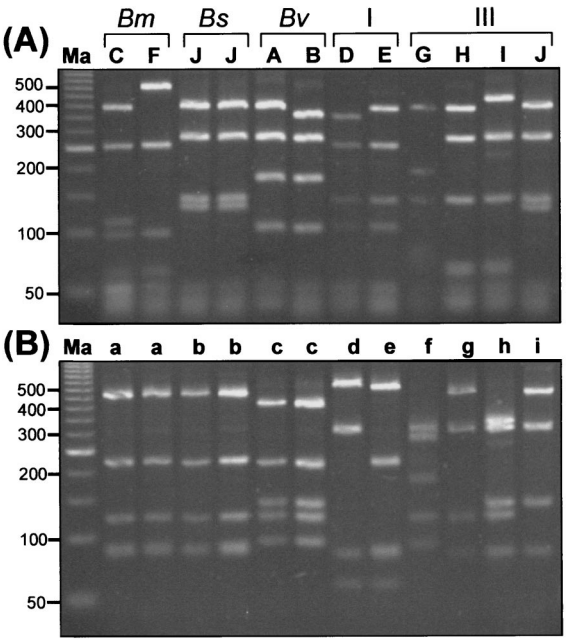

FIG. 2. RFLP analysis of the *B. cepacia* complex *recA* amplified by PCR. (A) The alphabetical RFLP type is shown above each lane. Lanes: (left to right) C, C1576; F, ATCC 17616; J, LMG 14086; J, LMG 14294; A, FC441; B, LMG 10929^T; D, ATCC 25416; E, ATCC 17759; G, K56-2; H, C1394; I, CEP511; and J, PC184. The genomovar status of each strain is indicated above the relevant lane. Molecular size standards (50-bp ladder) are in lane Ma. B. *Mnl*I RFLP analysis of the *recA* gene. The designated *Mnl* RFLP type is shown above each lane. Molecular size standards (50-bp ladder) are in lane Ma.

named genomic species awaiting formal binomial classification (28). In total, six distinct phylogenetic groups were present within the *recA* tree and were given the following designations as *recA* groups (Fig. 3): I, *B. cepacia* genomovar I; III-A and III-B, *B. cepacia* genomovar III *recA* clusters A and B, respectively; *Bs*, *B. stabilis*; *Bm*, *B. multivorans*; and *Bv*, *B. vietnamiensis*. These *B. cepacia* complex phylogenies were also distinct from the unrelated bacterial species chosen as out groups for the tree (Fig. 3). Phylogenetic trees based on the protein translation of each *recA* nucleotide sequence also demonstrated the same clustering of genomovars and new species (data not shown).

The separation of *B. multivorans*, *B. vietnamiensis*, and *B. stabilis* strains within the *recA* tree (Fig. 3) was consistent with their designation as new species (28, 29). The published sequence of strain JN25 (21) aligned closely with the *B. vietnamiensis recA* sequences. Separation of *B. cepacia* complex genomovars I and III was also apparent in the *recA* phylogenetic tree and consistent with their designation as distinct genomovars (28), except for the subdivision of isolates which had previously been identified as genomovar III by protein profile analysis (27, 28). RecA group III-A (Fig. 3) included epidemic CF strains from the cable pilus-encoding lineage (18, 22) and Vancouver outbreaks (16, 18). RecA group III-B (Fig. 3) contained epidemic CF strains from outbreaks in Cleveland, Ohio, in Manchester, England, and in Australia (18). *B. cepacia* genomovar III strain PC184, which shared the same *recA Hae*III RFLP as *B. stabilis* (Fig. 2), did not align with the latter (Fig. 3), clearly demonstrating the presence of genomovar III-specific sequence variation in the complete *recA* gene.

Genomovar-specific PCR. Using the nucleotide sequence alignment of the 20 novel *recA* genes determined above and the two within the databases (19, 31), specific PCR primers were designed to detect the six *recA*-derived clusters of the *B. cepacia* complex identified by phylogenetic analysis (see Fig. 3). Primer pairs for the identification of *recA* groups I, III-A, III-B, *B. multivorans*, *B. stabilis*, and *B. vietnamiensis* are listed in Table 2. Each primer pair was designed to match all sequences determined within each phylogenetic subgroup (see Fig. 3) and be mismatched at the 3′ base (and as many other bases as possible) with all other *B. cepacia* complex *recA* sequences (Table 2). Each primer set was tested on all 103 *B. cepacia* complex isolates. Amplification products of the correct size (Table 2) were obtained from strains of the appropriate *recA* group with each specific primer pair (data not shown). No products of the predicted size were seen in strains outside the target group for each primer pair (data not shown). The identity of each *recA* group-specific PCR product was confirmed to be the appropriate portion of the *recA* gene by direct sequence analysis of the PCR product for each of the following strains: *B. vietnamiensis* PC259, *B. multivorans* ATCC 17616, *B. stabilis* LMG 7000, *B. cepacia* ATCC 25416 (I), *B. cepacia* C5424 (III-A), and *B. cepacia* C1394 (III-B) (data not shown). The *recA*-specific PCR, *Hae*III *recA* RFLP analysis, and 16S ARDRA results obtained from all 103 *B. cepacia* complex isolates are summarized in Table 3.

The majority of 103 strains examined were genetically heterogeneous (66 out of 103) and possessed unique RAPD or PFGE fingerprints (Table 3). Each *recA* primer set was 100% specific for isolates which possessed *recA Hae*III RFLP types (Table 1) characteristic of the respective new species, conventional genomovar, or *recA* phylogenetic subgroup for which they were designed (Table 3). RFLP types and specific PCR results remained stable for sequential or multiple isolates of a given genetic strain type. ARDRA profiles obtained with the 16S rDNA primers also correlated as expected with the *recA*-specific primer results (Table 3).

Genomic location of *recA*. Given the multichromosomal structure of the *B. cepacia* complex genome (4, 12, 21), it was important to determine the copy number and genomic location of *recA*. Previous studies on *B. cepacia* genomovar I strain ATCC 25416 (Table 1) demonstrated the presence of two copies of the gene, one on each large chromosome (21). Electrophoretic separation of linear replicons from *B. multivorans* genomovars I, III-A, and III-B and *B. stabilis* strains is shown in Fig. 4A. Each genomovar possessed a multireplicon structure, with between two and four large replicons, ranging from approximately 0.6 to 3.8 Mb in size, being detected (data not shown for *B. vietnamiensis*). For each *B. cepacia* complex genomovar tested, only the largest chromosome produced a positive signal when probed with *recA* by Southern hybridization (Fig. 4B). Further analysis of the genomic location of the *recA* gene using RFLP approaches was also consistent with the presence of only one copy of the *recA* gene within the *B. cepacia* complex genome (data not shown).

DISCUSSION

Systematic analysis of two conserved genes, 16S rDNA and *recA*, in strains of the *B. cepacia* complex demonstrated that each may have a use in the identification and classification of this important group of opportunistic pathogens. Although widely used for bacterial identification, analysis of the 16S rDNA of the *B. cepacia* complex by an ARDRA approach demonstrated that this gene was useful for discrimination of the two new species, *B. vietnamiensis* and *B. multivorans*, but insufficient to separate genomovars I and III and *B. stabilis*. Because of these discriminatory limitations, a novel PCR-RFLP analysis was developed based on the *B. cepacia recA*

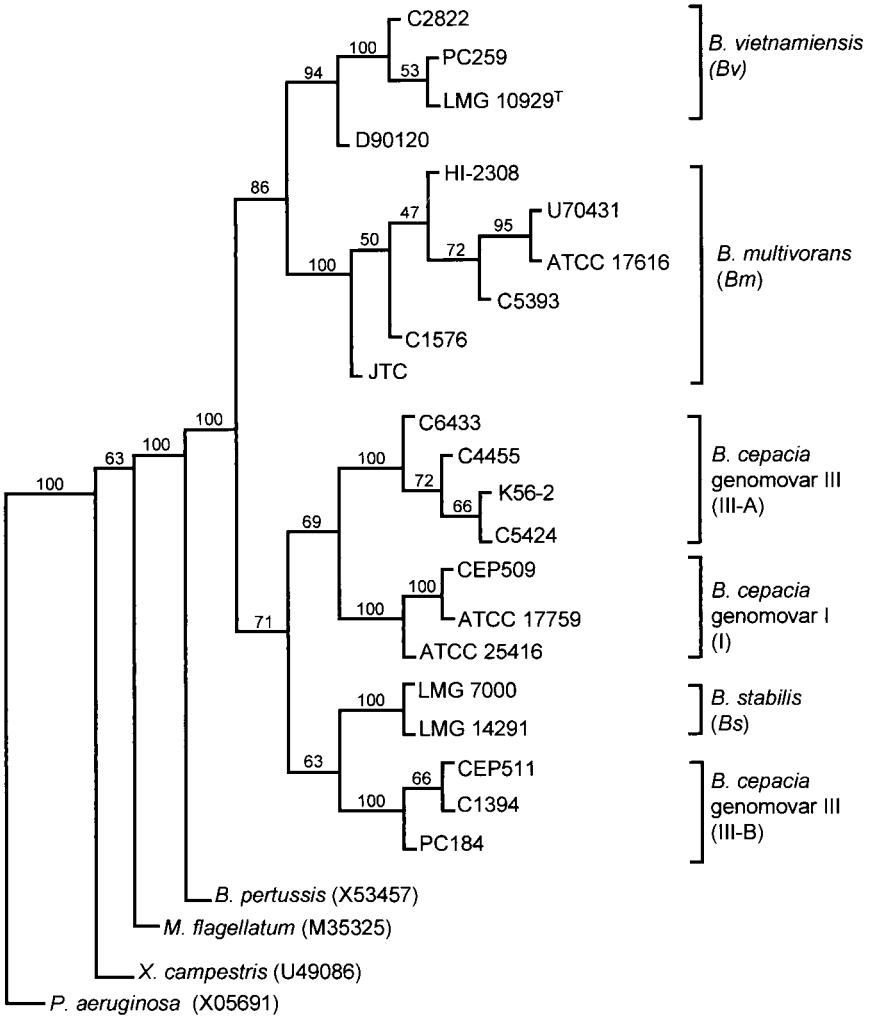

FIG. 3. Phylogenetic tree of the *B. cepacia* complex based on the complete *recA* gene sequence. Multiple sequence alignment was performed on these genes and the published *recA* sequences from *B. pertussis*, *M. flagellatum*, *X. campestris*, and *P. aeruginosa*. The phylogenetic tree was rooted with the *B. pertussis recA* gene. Bootstrapping resampling statistics were applied to the tree (100 data sets), and bootstrap values are shown on each horizontal limb of the tree. The genomovar status of each *B. cepacia* complex strain is indicated on the right of the figure (the corresponding *recA* phylogenetic group is shown in brackets).

gene. Nucleotide sequence polymorphism was identified in *recA* which was sufficient to identify all five current *B. cepacia* complex genomovars defined by a polyphasic approach (28). This study validates the use of nonribosomal housekeeping genes in the development of alternative molecular diagnostic strategies, especially among taxonomically complex species such as *B. cepacia*. A complete nucleotide sequence-based identification approach based on this conserved, stable, and single-copy gene was developed.

Four approaches based on the *B. cepacia* complex *recA* gene may be used to identify the genomovar status of clinical isolates and, depending on the resources of the diagnostic laboratory, they may be applied as individual tests or multiple complementary analyses. First, amplification of *recA* with primers BCR1 and BCR2 can be used as an initial means of placing an isolate within the *B. cepacia* complex since cross-reaction of these primers with other species commonly found in CF sputum was not detected. In the future, direct testing and application of these *B. cepacia* complex-specific primers to CF sputum, which contains multiple bacterial species, may also be possible. Second, after successful amplification of *recA*, RFLP with *Hae*III and *Mnl*I may be used to place the isolate within a specific genomovar or *recA* group (Table 1). Third, the design of *recA* group-specific primers capable of identifying all of the genomovars within the current *B. cepacia* complex (28, 29) provides a means of genomovar identification in a single test. With further development such *recA*-based primer sets may be employed in a single multiplex PCR for rapid genomovar detection. Finally, nucleotide sequence determination of *recA* provides a powerful means of both identification and classification of these poorly defined bacteria. Overall, the availability of several complementary tests based on a single diagnostic gene provides a robust approach to *B. cepacia* complex identification.

Phylogenetic analysis of *recA* from a range of bacteria has demonstrated that the gene may be very useful for the separation of closely related species and may define evolutionary trees that are consistent with those observed for rRNA genes (5, 11). Its application herein confirms these observations and illustrates the benefits of examining protein-encoding DNA when systematic analysis of ribonucleotide-encoding sequences fails to yield discriminatory classifications among closely re-

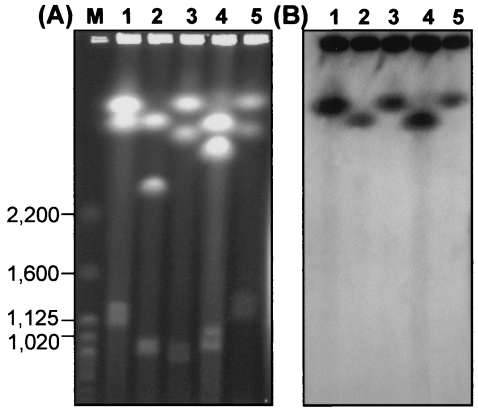

FIG. 4. Genomic location of the *recA* gene in strains of the *B. cepacia* complex. (A) Lanes: M, molecular size standards (*S. cerevisiae* chromosomes; relevant size bands are indicated in kilobases); 1, strain ATCC 25416 (genomovar I); 2, *B. multivorans* strain 17616; 3, strain C5424 (*recA* group III-A); 4, strain C1394 (*recA* group III-B); and 5, *B. stabilis* strain LMG 14291. (B) Southern hybridization of separated chromosomes from panel A with a *recA* gene probe from *B. cepacia* strain K56-2 (genomovar III-A). A positive hybridization signal was obtained from the largest chromosome in each strain examined.

lated species. Analysis of a single genomic locus, *recA*, in this study was consistent with the polyphasic taxonomic approach (27) originally used to define the *B. cepacia* complex genomovars, with the exception of the phylogenetic subdivision of organisms classified as genomovar III by DNA-DNA hybridization experiments (28). The taxonomic significance of the subdivision of genomovar III-classified strains into *recA* groups III-A and III-B awaits further research. In addition *B. cepacia* complex strains possessing novel *recA* RFLPs, not reported in this study, have recently been identified (E. Mahenthiralingam and P. Vandamme, unpublished data); preliminary polyphasic analysis (27) of these strains indicates that they are members of novel genomic species within the complex (Mahenthiralingam and Vandamme, unpublished data). The ability to use *recA* analysis as a means to assist taxonomic classification of the *B. cepacia* complex was demonstrated in the recent proposal of *B. stabilis* (formerly genomovar IV) as a new species (29) and is also corroborated by the data presented in this study.

Analysis of *recA* also revealed new insights into the epidemiology and pathogenesis of the *B. cepacia* complex. Concerns about commercial use of these bacteria (9, 14) are confirmed by the *recA* phylogenetic analysis. Strains isolated from clinical infection and those recovered from the natural environment cluster within the *B. multivorans*, *B. vietnamiensis*, and *B. cepacia* genomovar I arms of the evolutionary tree (Fig. 3). In addition, among the 103 isolates screened, there were clinical and environmental strains of *B. cepacia* genomovar III-B which shared the same RFLP type (Table 3) and hence would cluster phylogenetically if nucleotide sequence analysis were subsequently performed. Only within *recA* group III-A and *B. stabilis* were no environmental isolates found in this study. The results of *recA* analysis corroborate the taxonomic findings of Vandamme et al. (28) which demonstrate that all genomovars within the *B. cepacia* complex can cause human opportunistic infection. Phylogenetic distinctions between environmental and clinical strains do not appear to exist, and all strains within the complex appear to possess conserved traits which enable them to cause infection in vulnerable individuals.

Determination of the phylogeny of the *B. cepacia* complex enabled the association of known epidemiological features with each *recA* subgroup to be examined. The cable pilus gene was primarily associated with genomovar III strains of the ET12 lineage (22), such as K56-2 and C5424, which clustered in *recA* group III-A (Table 1; Fig. 3). However, one unique strain recovered from an individual CF patient at a center in Australia, which possessed DNA homologous to *cblA*, was identified as *recA Hae*III RFLP type E and *B. cepacia* genomovar I by specific PCR (Table 3). Therefore, possession of *cblA* still appears to be a rare feature for the *B. cepacia* complex (17), but it is not solely possessed by genomovar III strains of the ET12 lineage (22). The prevalence of the BCESM within the *B. cepacia* complex *recA* phylogeny also appears more widespread that originally observed (17). The marker was associated with strains that had spread among patients with CF when it was originally described (17); however, unlike *cblA*, no specific virulence trait has been associated with the BCESM locus. From the results of this study, DNA homologous to the BCESM appears to be primarily associated with epidemic CF strains belonging to *recA* groups III-A and III-B (all classified as genomovar III) (18). However, BCESM was also found in *B. vietnamiensis* strain ATCC 29424, but none of the other *B. vietnamiensis* strains examined (Table 3), suggesting that it may occasionally occur outside of the highly transmissible genomovar III strains (16–18). The *recA* probes described herein provide an additional means of identifying strains with an epidemiological precedent (16–18) for patient-to-patient spread among individuals with CF. The *recA* group III-A- and III-B-specific primers detect strains which have caused several outbreaks (16–18) and may ultimately prove to be better diagnostic probes than the not-yet fully-defined BCESM DNA (17). Early diagnosis of infection by *B. cepacia recA* PCR may in future facilitate rapid-enactment infection control measures and aggressive therapy to improve the poor outcome associated with *B. cepacia* infection in patients with CF (13).

In conclusion, nucleotide sequence analysis of *recA* is a rapid and reproducible means of identifying all of the current genomovars and new species within the *B. cepacia* complex. Rapid identification of bacteria recovered from patients with CF and from other vulnerable individuals is vital if we are to further understand the clinical risks posed by each genomovar and new species within the *B. cepacia* complex.

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