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Phenotypic Methods for Determining Genomovar Status of the *Burkholderia cepacia* Complex

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Recent taxonomic advances have demonstrated that *Burkholderia cepacia* is a cluster of at least seven closely related genomic species (or genomovars) collectively referred to as the *B. cepacia* complex, all of which may cause infections among cystic fibrosis patients and other vulnerable individuals. Thus, it is important for clinical microbiologists to be able to differentiate genomovars. Prior to this study, 361 *B. cepacia* complex isolates and 51 isolates easily confused with *B. cepacia* complex previously had been identified using a polyphasic approach, and in this study, a comparison of phenotypic and biochemical tests was carried out. It was determined that *Burkholderia multivorans* and *Burkholderia stabilis* could reliably be separated from other members of the *B. cepacia* complex by phenotypic methods. A combination of phenotypic and molecular tests such as *recA* PCR and 16S rRNA RFLP are recommended for differentiation among the genomovars of the *B. cepacia* complex. A biochemical reaction scheme for the identification of *B. gladioli*, Pandoraea species, and *Ralstonia pickettii* and the differentiation of these species from the *B. cepacia* complex is also presented.

Burkholderia cepacia has been recognized as a major pathogen in patients with cystic fibrosis (CF) since the late 1970s (15). Recent taxonomic advances have demonstrated that *B. cepacia* is actually a cluster of at least seven closely related genomic species (or genomovars) now called the *B. cepacia* complex (8, 8a, 11, 31, 33). Genomovars II, IV, and V are now formally named *Burkholderia multivorans, Burkholderia stabilis*, and *Burkholderia vietnamiensis*, respectively (with the species designation *B. cepacia* being reserved for genomovar I) (11, 31, 33); genomovar III has not been formally named, pending the availability of differential diagnostic tests. Genomovar VI has recently been described and is closely related to *B. multivorans* (8). Genomovar VII has also been described, and the name *Burkholderia ambifaria* (used herein) has been proposed (8a). These two newly proposed genomovars, *Burkholderia fungorum*, and *Pandoraea* species were initially described when *B. cepacia*-like organisms were submitted for whole-cell protein and amplified fragment length polymorphism (AFLP) analyses and were shown to be different from the presently recognized five genomovars (5, 25). Although all the genomovars thus far described can cause infections in patients with CF (21, 31), the prevalence of each genomovar has not been fully determined and may vary among different patient populations. Examination of large collections of isolates demonstrates that genomovar III is the predominant species isolated from patients with CF and that *B. multivorans* may frequently be recovered (22, 31). Genomovars I and VI, *B. vietnamiensis*, and *B. ambifaria* appear to be less commonly found in CF patients (8, 8a, 19, 31).

Commercial bacterial identification systems are not able to differentiate among the genomovars nor accurately confirm the identification of *B. cepacia* complex isolates while differentiating them from closely related species such as *Burkholderia gladioli, Ralstonia pickettii*, and the newly described *B. fungorum* and *Pandoraea* species (6, 7). Due to the marked differences in apparent pathogenicity and prevalence among the genomovars, a simple phenotypic scheme for classification is needed.

In this study, 412 isolates were selected from a larger collection that contains strains that had previously been thoroughly characterized by a polyphasic identification procedure including some or all of the following previously described methods: whole-cell protein electrophoresis (25), DNA-DNA hybridization (30), fatty acid analysis (30), AFLP (5), restriction fragment length polymorphism (RFLP) of the 16S rRNA PCR product (22), genomovar-specific *recA* PCR (22), species-specific PCR for *B. gladioli* (35), and random amplified polymorphic DNA (RAPD) fingerprinting (18, 19). From this information, 361 isolates from the *B. cepacia* complex and 51 isolates from phenotypically similar species were selected; phenotypic data were evaluated in correlation with genomovar or species, so that comparisons among the more classical and routine biochemical tests employed in clinical laboratories could be made. This report describes a battery of phenotypic tests which can differentiate *B. cepacia* complex organisms from other related species and can distinguish among several of the genomovars. Recommendations are given for combinations of phenotypic and genetic methods to aid in characterization of the *B. cepacia* complex.

METHODS AND MATERIALS

***B. cepacia* complex isolates.** Isolates were collected from various international laboratories as described previously (12). From this collection, 412 isolates were selected for this study as follows: 297 isolates from CF patients, 65 isolates from non-CF clinical specimens, and 50 isolates from environmental sources. Three hundred sixty-one isolates were members of the *B. cepacia* complex, and 51

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organisms belonged to phenotypically similar species that can be confused with *B. cepacia* complex. Isolates were selected to represent a variety of geographical and epidemiological groups. Between 8 and 12 clonal isolates (from different geographic locations) from each of the common strain types identified by RAPD analysis of isolates from Canadian CF patients (ST001, ST002, and ST004) were chosen (19). The remaining genomovar III isolates were from other RAPD groups (19). For genomovar I, *B. multivorans, B. vietnamiensis,* and *B. ambifaria*, most strains selected belonged to a unique RAPD group. *B. stabilis* isolates were also chosen for geographic diversity but were mainly clonal (RAPD strain type BS016) due to the genetic stability of this species (33). Genomovar VI isolates, although from different geographic areas, were also highly clonal and consisted mainly of RAPD strain type ST010.

Phenotypic identification of *B. cepacia* complex and other organisms. Isolates were identified as described previously (12). In brief, purity, morphology, and hemolysis were observed and oxidase activity (Pathotec cytochrome oxidase; Remel, Lenexa, Kans.) was tested after growth on Columbia agar with 5% sheep blood (PML Microbiologicals, Richmond, British Columbia, Canada). Oxidase reactions were considered fast if a positive reaction occurred within 10 s and slow if a positive reaction occurred between 10 and 30 s; isolates that were negative after 30 s were subjected to repeated testing using a 1% aqueous solution of tetramethylparaphenylenediamine dihydrochloride to confirm the negative oxidase test result. From agar-grown bacteria, a heavy emulsion in saline was made, and a drop was placed in either Hugh and Leifson oxidation-fermentation sugars (14) or a modification of ammonium phosphate sugars (9, 13). The agar concentration was modified from 14 g/liter to 2 g/liter resulting in a sloppy agar tube instead of a slant. Bacteria were incubated in the following sugars for up to 7 days at 35°C: glucose, maltose, lactose, xylose, sucrose, and adonitol. Moellers lysine, ornithine, and a negative control were also heavily inoculated and incubated at 35°C for 2 days (with a modification to a 2-ml volume with a 0.5-ml oil overlay). The API 20 NE strip (Biomerieux Vitek Inc., Hazelwood, Mo.) was set up according to manufacturer's instructions except that the strip was incubated at 35°C and observed at 24 and 48 h. Isolates exhibiting negative *p*-nitrophenyl-β-D-glucoside (PNPG) reactions from the API 20 NE strip were tested again using the *o*-nitrophenyl-β-D-galactopyranoside (ONPG) method as described previously (9). Growth on MacConkey agar without crystal violet (Difco Laboratories, Detroit, Mich.) and on *B. cepacia* selective agar (BCSA) (12) at 35°C was observed at 24 and 48 h. Pigment production and growth on tryptic soy agar at 35 and 42°C was observed at 24 and 48 h.

Preparation of bacterial DNA. For AFLP fingerprinting, DNA was prepared as described previously (5). For RAPD analysis, *B. cepacia* epidemic strain marker (BCESM) detection by dot blot hybridization (20), *recA* gene PCR (22), and *B. gladioli* species-specific (SS) PCR (35), genomic DNA was extracted from the strains after mechanical disruption as described previously (18). In addition, for strains whose genomovar status was known but that produced false negatives by *recA* gene PCR (see below), DNA was also extracted using Instagene matrix (Bio-Rad Laboratories, Hercules, Calif.) per the manufacturer's instructions.

Genomovar-specific PCR for the *recA* gene. PCR using selected *recA* primers was performed essentially as described previously (22), but with differences in the preparation of template DNA and the PCR reagents used. The DNA used was extracted for RAPD analysis as described above instead of by the extraction method described previously (22), which incorporated additional extract digestion with pronase. Amplification was repeated for some false-negative strains with an increased concentration of DNA and/or with Instagene matrix-extracted DNA. Tests were performed with the six *recA* subgroup genomovar-specific primers described previously (one primer pair each for genomovars I, III-A, and III-B, *B. multivorans, B. stabilis,* and *B. vietnamiensis*) (22). A seventh primer pair, forward primer BCRGC1 (5′-GTCGGGTAAAACCACGCTG-3′) and reverse primer BCRGC2 (5′-TCCGCAGCCGCACCTTCA- 3′), was used for genomovar VII; this primer pair produced a product of 810 bp for genomovar VII strains under appropriate PCR conditions (8a). Primers for genomovar VI have not been developed yet. Reactions mixtures (25 μl) contained 20 mM Tris-Cl, 50 mM KCl, 0.1% Triton X-100, 0.01% gelatin, 1.5 mM $MgCl_2$, 1 U of *Taq* polymerase, 250 μl of each deoxynucleoside triphosphate (Amersham Pharmacia Biotech Inc.), 40 ng of DNA, and 40 pmol of oligonucleotide and were overlaid with 25 μl of mineral oil. PCR was performed in a Perkin-Elmer model TC-1 thermal cycler with a 1-min denaturation at 94°C, annealing for 1 min at 60 to 64°C (depending on the PCR test being performed [22]), and extension for 2 min at 72°C; this cycle was repeated 30 times. After a final extension of 10 min at 72°C, samples were cooled to 8°C. After amplification, 8 μl of each reaction mixture was electrophoresed in a 1.5% agarose gel. PCR products were photographed after ethidium bromide staining.

Speciation using the 16S rRNA gene. Restriction fragment length polymorphism (RFLP) analysis of the 16S rRNA gene PCR product using the enzyme *Dde*I was performed as described previously (22), with the exception that the DNA extract used was the same as that used for RAPD and *recA* gene PCR analyses described above.

BCESM. Southern dot blot analysis of the BCESM was performed as previously described (20).

Genotypic identification of *B. gladioli*. Genomic DNA was extracted as described previously (18). A PCR with the primer pair LP1-LP4, directed towards a species-specific region of the 23S rRNA gene, was performed (35). The above-described thermal cycler protocol and PCR mixture and an annealing temperature of 60°C were used.

RESULTS

A total of 412 organisms were chosen from a larger, well characterized collection to represent a wide variety of geographic areas and to reduce skewing of results by major epidemiologically related clones. Three hundred sixty-one isolates were identified as members of the *B. cepacia* complex, and 51 organisms were identified as members of phenotypically closely related species that can be confused with *B. cepacia* complex (most of the isolates had been isolated from the lungs of CF patients) (Table 1). Initial analyses using a polyphasic approach identified each organism by species and genomovar. The isolates were then evaluated by biochemical and phenotypic tests.

***B. cepacia recA* PCR.** The *recA* PCRs for each genomovar are very specific (22), an observation that was confirmed in the current study. No cross-reactivity between the seven present members of the *B. cepacia* complex and closely related organisms was seen, except for between genomovar VI and *B. multivorans*, which are phenotypically very similar. Identification based on the present panel of *recA* primers could not discriminate isolates of genomovar VI; therefore, the 16S rRNA RFLP pattern (see below) was used to confirm the identification of isolates as being of this new genomovar. For 43 *B. cepacia* complex isolates, protein electrophoresis was unable to differentiate among presently recognized genomovars to provide a *B. cepacia* complex subclassification. PCR analysis of the *recA* gene was used to provide a working genomovar classification for these problematic isolates; 36 of 43 isolates were identified as being of genomovar III, and 7 of 43 isolates were identified as being of genomovar I.

An initial lack of sensitivity with the genomovar-specific *recA* PCR could be attributed to the reagent and setup differing from the published method (22). In this study, the reagents and methods originally applied during the development stage of the *recA*-based genomovar identification method (22) were used. False-negative results were generally overcome by repeated testing, suggesting that while the sensitivity of PCR assays varies from user to user, the specificity of this identification approach is reliable when optimized and used in conjunction with the appropriate controls.

RFLP analysis of the 16S rRNA gene. Analysis of *B. cepacia* complex demonstrated the presence of three distinct RFLP patterns when the 16S rRNA amplification product was digested with the enzyme *Dde*I (22): pattern 1 (including *B. vietnamiensis*), pattern 2 (including genomovars I and III and *B. stabilis*), and pattern 3 (including *B. multivorans*) (Fig. 1). In this study, we have expanded these observations to include genomovar VI, *B. ambifaria, Pandoraea* species, and *B. fungorum* (Fig. 1). Seven genomovar VI strains were subjected to *Dde*I digest of the 16s rRNA PCR product and yielded the

TABLE 1. Characteristics of *B. cepacia* complex and phenotypically similar organisms[a]

Test or specimen source	% of strains positive for test specimen or source									
	Genomovar I	Genomovar II	Genomovar III	Genomovar IV	Genomovar V	Genomovar VI	Genomovar VII	*B. gladioli*	*Pandoraea* species	*R. pickettii*
Tests										
Oxidation[b] of:										
Glucose	100	100	95 (96)	100	100	100	100	100	11 (89)	100
Maltose	39 (70)	98 (99)	78 (86)	93	97 (100)	100	100	0	0	92
Lactose	61 (91)	100	79 (88)	93	97 (100)	100	100	0	0	92
Xylose	87 (100)	98 (99)	88 (92)	44 (78)	75 (86)	100	100	96	0	83 (92)
Sucrose	87 (91)	0	88 (91)	0	94 (97)	0	94	0	0	0
Adonitol	70 (78)	91 (92)	79 (87)	78 (96)	0	100	100	93 (96)	0	0
Lysine decarboxylase	100	53	99	100	100	0	100	0	0	0
Ornithine decarboxylase	30	0	71	100	0	0	0	0	0	0
Growth at 42°C	43	100	84	0	100	100	22	4	89	83
PNPG[c] or ONPG	100	98	99	0	100	100	100	100	0	0
Oxidase[d]	100	100	100	100	100	100	100	0	67	100
Nitrate reduction[c]	4	94	31	4	47	100	67	33	11	17
Gelatin liquefaction[c]	74	2	55	93	0	0	94	70	0	33
Esculin hydrolysis[c]	56	2	33	0	0	0	56	11	0	0
Growth on MacConkey agar	83	96	84	93	83	100	100	96	100	50
Pigment brown	4	2	14	0	0	0	6	33	0	0
Pigment yellow	78	2	3	0	0	0	0	44	0	0
Alpha hemolysis	9	1	9	0	36	0	83	22	0	0
Beta hemolysis	9	0	3	0	36	0	83	22	0	0
BCSA	100	100	100	100	100	100	100	18	100	100
API 20 NE[e]	96	94	86	4	83	100	100	70	0	0
BCESM	0	0	63	0	3	0	0	0	0	0
Specimen sources										
CF	26	88	76	56	56	100	22	82	100	75
Non-CF clinical	30	6	20	33	19	0	0	11	0	25
Environmental	44	6	4	11	25	0	78	7	0	0

[a] The number of strains positively identified as being of a particular genomovar or species are as follows: for genomovar I, 23; for genomovar II (*B. multivorans*), 109; for genomovar III, 139; for genomovar IV (*B. stabilis*), 27; for genomovar V (*B. vietnamiensis*), 36; for genomovar VI, 9; for genomovar VII (*B. ambifaria*), 18; for *B. gladioli*, 27; for *Pandoraea* species, 9; and for *R. pickettii*, 12.

[b] Oxidation test results were recorded after 3 days of incubation (data in parentheses were recorded after 7 days of incubation).

[c] Results presented are from API 20 NE strip test.

[d] In tests using the Pathotech cytochrome oxidase strip, the slow, weak reaction occurred in 10 to 30 s and the fast, strong reaction occurred in less than 10 s. Only *R. pickettii* displayed a fast, strong reaction.

[e] For *B. cepacia* complex strains, results represent very good or excellent identification (≥99.0%), although API database admits that any given strain may be *B. gladioli*.

same RFLP pattern as *B. vietnamiensis* (pattern 1), and *B. ambifaria* strains shared the same polymorphisms as genomovars I and III and *B. stabilis* (pattern 2). *Pandoraea* species and *B. fungorum* each had unique banding patterns that were easily differentiated from those of *B. cepacia* complex bacteria (Fig. 1). 16s rRNA PCR products from *R. pickettii* strains failed to be digested with *Dde*I (data not shown), so the lack of distinct bands allowed it to be distinguished from the *B. cepacia* complex.

***B. gladioli* 23S rRNA SS PCR.** Twelve of 27 *B. gladioli* isolates were identified by protein electrophoresis. The remaining 15 *B. gladioli* isolates had previously been identified by biochemical methods, and identification was confirmed by 23S rRNA SS PCR (35), which showed 100% sensitivity for all 27 isolates included in the collection. To confirm specificity of the PCR for *B. gladioli*, 30 organisms from the *B. cepacia* complex, *Ralstonia* species, and *Pandoraea* species were tested and yielded negative results (data not shown).

BCESM. Genomovar III strains comprised the majority of isolates examined from the *B. cepacia* complex (Table 1), since this is the major species isolated from patients with CF. The BCESM, although almost exclusively found among genomovar III isolates, was present in fewer than two-thirds of the genomovar III isolates.

Phenotypic analysis. During the course of this study the medium base for oxidation-fermentation studies was changed from the classical Hugh and Leifson base (14) to the ammonium phosphate base described by Cowan and Steel (9) and modified by Holmes et al. (13). It was observed that acidification frequently occurred more quickly in the $(NH_4)_2PO_4$ sugars than in the Hugh and Leifson sugars, and no alkalinity that could mask weakly acidic sugars, as can sometimes occur with Hugh and Leifson sugars, was observed (D. A. Henry, unpublished data). The API 20 NE strip contains only a glucose fermentation test, not oxidative acidification tests.

The distinctive, slow oxidase reactions for the *B. cepacia* complex and some *Pandoraea* species were observed from the Pathotec cytochrome oxidase strips. A slow reaction occurred between 10 and 30 s and was usually weaker in color development than that of the *Pseudomonas aeruginosa* positive control, which provided a quick and strong reaction within 10 s. *R. pickettii* demonstrated a strong, fast oxidase reaction, while *B. gladioli* was negative. This difference in slow and fast reactions may not be as noticeable with other oxidase test methods.

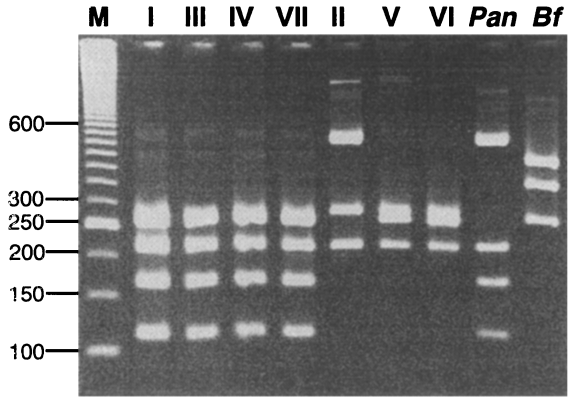

FIG. 1. PCR-RFLP analysis of 16S rRNA products of *B. cepacia* complex bacteria and related organisms. Lanes: M, molecular size markers (the sizes [in base pairs] of appropriate bands are indicated on the left); I, II, III, IV, V, VI, and VII, RFLP profiles for genomovars I, II, III, IV, V, VI, and VII, respectively, of the *B. cepacia* complex; Pan, RFLP profile for *Pandoreae* spp.; *Bf*, RFLP profile for *fungorum*.

Detailed phenotypic and biochemical reactions from isolates of each genomovar and species are shown in Table 1. Nitrate, gelatin, esculin, and PNPG results were obtained from the API 20 NE strip. The API 20 NE strip was reasonably reliable for identifying all members of the *B. cepacia* complex, except for *B. stabilis*, as *B. cepacia*. Discrimination could be improved if the APE 20 NE database were to incorporate negative PNPG results and no growth at 42°C as diagnostic criteria for *B. stabilis*. The uniformity of the last four digits, representing the assimilation tests, was a first indication that further testing of this organism was warranted. A total of 79% of *B. cepacia* complex isolates (excluding *B. stabilis*) had API 20 NE numerical profiles of XXX7577 (which indicated that all carbon sources on the strip, except for maltose, were assimilated), with the Xs referring to variations in nitrate, gelatin, or esculin reactions. A total of 63% of *B. stabilis* isolates had the API 20 NE profile number 0056577. Since the strain collection had only three isolates of *B. fungorum*, it was not included in Table 1; *B. fungorum* was identified as *B. cepacia* by the API 20 NE strip.

The genus *Pandoraea* consists of five species, of which four were available for this study. The small numbers tested here were included to demonstrate the lack of saccharolytic activity and should not be used to draw definitive phenotypic conclusions (see the article by Coenye et al [6] for a discussion of this newly described genus). The API 20 NE database does not contain the *Pandoraea* genus but gave the following results for the nine *Pandoraea* isolates: five had low discrimination (less than 80% reliability) between *Alcaligenes denitrificans, Alcaligenes faecalis*, CDC IV C2 (proposed name, *Ralstonia paucula* [32]), and *Acinetobacter* species; two had an unacceptable identification; one had a good identification as CDC IV C2; and one had low discrimination between *R. pickettii* and *Pseudomonas putida*.

For the 12 *R. pickettii* isolates, the API 20 NE results were as follows: five had low discrimination between *R. pickettii* and either *P. putida, Ochrobacter anthropii*, or *Pseudomonas fluorescens*; three had a good identification as *P. fluorescens*; three had a good identification as *R. pickettii*; and one had low discrimination between *P. fluorescens* and *B. cepacia*. The oxidation sugars and oxidase reaction were useful to distinguish *B. gladioli, R. pickettii,* and *Pandoraea* species from the *B. cepacia* complex and from each other.

Combined phenotypic and molecular analyses. *B. multivorans, B. stabilis, B. vietnamiensis*, and genomovar VI were distinguished from the other members of the *B. cepacia* complex by tests such as sucrose and adonitol acidification, ONPG utilization, and growth at 42°C (Table 1). Genomovar VI isolates, which frequently had a pungent rotten potato odor, were distinguished from *B. multivorans* by odor and by 16S rRNA gene RFLP pattern (Fig. 1). *B. vietnamiensis* and genomovar VI, which shared the same 16S rRNA gene RFLP pattern (Fig. 1), could be readily discriminated biochemically; *B. vietnamiensis* was negative for adonitol oxidation and positive for sucrose oxidation, while genomovar VI had the reverse results (Table 1). Genomovars I and III, *B. stabilis*, and *B. ambifaria* possessed the same 16S rRNA gene RFLP pattern (Fig. 1) and shared many biochemical traits but could be separated from each other by *recA* PCR analysis. Of these four genomovars, only *B. stabilis* was consistently phenotypically distinct, being unable to grow at 42°C and ONPG negative. For genomovars I and III and *B. ambifaria*, several phenotypic properties used in conjunction with genetic testing for the BCESM (20) provided the means to distinguish these species. Genomovar III isolates were frequently positive for the BCESM; genomovar I strains frequently produced a yellow pigment; and *B. ambifaria* strains were frequently beta hemolytic (Table 1). However, nonpigmented, nonhemolytic, BCESM-negative strains of genomovars I and III and *B. ambifaria* could not be phenotypically differentiated (Table 1).

A small proportion of genomovar III strains were unable to acidify any sugars, most likely due to some auxotrophic requirement (1), and were described as biovar IIIc according to the scheme described by Vandamme et al. (31). Almost all of these strains were of the ET12 lineage (ST002), as determined by RAPD analysis. These auxotrophic, ST002 strains were ONPG positive and lysine positive, which distinguished them from *Pandoraea* spp.

Phylogenetic analysis of *recA* demonstrated two distinct lineages (requiring two pairs of *recA* primers for identification), III-A and III-B, among strains assigned to genomovar III (22). Biochemical differences between the two *recA* subgroups of genomovar III were not generally apparent, except for the ability to reduce nitrate, which was never observed for subgroup III-B but was variable among III-A strains. DNA-DNA hybridization between representatives of *recA* groups III-A and III-B clearly indicated that they belonged to the same genomic species (T. Coenye and P. Vandamme, unpublished data).

DISCUSSION

Several studies have indicated problems with misidentification of *Burkholderia* species using phenotypic methods (12, 16, 23, 29, 34). Identification of *Burkholderia* species, *Ralstonia* sp. strain CDC IV C2, or *Alcaligenes* spp. by commercial methods such as the API 20 NE, Remel, RapID NF Plus, Microscan, Crystal, Sherlock, and Vitek tests lacks accuracy (16, 29, 34), and results must be confirmed by other methods. In a recent study, McMenamin et al. (23) found that only one-third of laboratories used biochemical tests to supplement commercial

test systems for the identification of *B. cepacia*. Shelly et al. (29) reported positive predictive values between 71 and 98% for these systems when they were used as the primary identification method, and they reported that there was at least a 20% probability of a strain identified by these tests as non-*B. cepacia* actually being a member of the *B. cepacia* complex (29). Commercial systems should be supplemented with additional biochemical tests, but phenotypic methods alone should not be relied upon for definitive identification.

We recommend that isolates identified by commercial test methods as being suggestive of *B. cepacia* complex or related suspect species be screened by the following phenotypic tests: growth on BCSA, lysine and ornithine decarboxylase, sucrose and adonitol oxidation, oxidase activity, hemolysis, pigment production, and growth at 42°C. With this information, appropriate molecular tests can be chosen for subclassification within the *B. cepacia* complex. If *recA* PCR fails to identify the genomovar, then RFLP of the 16S rRNA PCR product can be performed. Alternatively, if facilities are available, laboratories may adopt a completely molecular approach based on amplification of the *recA* gene, which is specific for most groups within the *B. cepacia* complex, followed by RFLP analysis of the product and specific PCR testing to provide a final identification (22). Different combinations of approaches to identification can be chosen depending on availability of methods.

Incomplete or incorrect identification of an organism as *B. cepacia* can lead to inappropriate segregation or cohorting of CF patients. Infection control is a significant problem associated with bacteria of the *B. cepacia* complex, and there have been extensive studies of its molecular epidemiology (3, 10, 19, 27). Many of the strains examined in these studies were initially identified based on their phenotypic properties. In retrospect, it can now be seen that some of these organisms are actually different species. A study by Ryley et al (26) found no evidence of nosocomial cross-infection among Danish CF patients; the isolates they examined were examined in the present study and were identified as *B. multivorans* (D. A. Henry, unpublished data). Contrasting studies (24, 37) demonstrated patient-to-patient spread of *B. cepacia* complex strains that were subsequently identified as *B. multivorans* (21, 31). Nosocomial acquisition and spread of *B. gladioli* infection, based on biochemical identification, had also been described (38); it was subsequently found that these strains were members of *B. cepacia* complex genomovar III (F. E. Clode, L. A. Metherell, and T. L. Pitt, Letter, Am. J. Crit. Care Med. **160:**374–375, 1999).

The observations of the latter studies and the urgent need for a detailed understanding of the clinical risks posed by each genomovar stress the necessity for accurate methods for differentiating among genomovars of the *B. cepacia* complex. Thorough initial biochemical analysis as described herein should be an integral part of this identification process.

The BCESM is a specific but not a sensitive marker for genomovar III strains, although in previous studies it has served as an excellent marker for those *B. cepacia* genomovar III strains which have spread from patient to patient (20). A study looking at distribution of putative transmissibility factors among the *B. cepacia* complex genomovars initially separated on the basis of phenotypic test results showed that 2 of 9 genomovar I or IV isolates and 6 of 49 *B. multivorans* isolates were positive for the BCESM (4). In our collection, where all phenotypic identification was confirmed by molecular methods, we have found only one non-genomovar III strain, *B. vietnamiensis* ATCC 29424, that was BCESM positive by Southern hybridization but failed to amplify the BCESM when examined by PCR (E. Mahenthiralingam, unpublished data).

Difficulties associated with phenotypic overlap, especially among genomovars I and III and *B. ambifaria* and between genomovar VI and *B. multivorans*, may now be overcome by several molecular assays. Methods based on targeting either the 16S or 23S rRNA genes by PCR or RFLP cannot discriminate among genomovars I and III, *B. stabilis*, and *B. ambifaria* (2, 17, 28; our unpublished data) unless multiple testing with different primers or restriction enzymes and analysis with an algorithm are used (36). In contrast, diagnostic tests based on the *recA* gene are very specific and discriminate between genomovars I and III, *B. stabilis*, and *B. ambifaria*. For genomovar VI and *B. multivorans*, there is cross-reaction with *recA* primers originally designed to be specific for *B. multivorans*. However, these two species may be distinguished by RFLP analysis of both the amplified *recA* gene (E. Mahenthiralingam, unpublished data) and the 16S rRNA gene (Fig. 1). In addition, sufficient nucleotide sequence variation is present within the *recA* gene of genomovar VI to enable the design of specific primers (E. Mahenthiralingan, unpublished data). In the past, when the *recA* primers failed to amplify a product from a suspected *B. cepacia* complex isolate, the organism under examination has actually been a new genomovar or an as-yet-undescribed species such as *Pandoraea* spp. or *B. fungorum* (6, 7).

In conclusion, while phenotypic characteristics may not be sufficient for differentiating all genomovars within the *B. cepacia* complex, we have clearly demonstrated that isolates from this group may be discriminated readily from closely related species such as *B. gladioli, Pandoraea* spp., and *R. pickettii*. Among members of *B. cepacia* complex, *B. vietnamiensis, B. multivorans*, and *B. stabilis* remain the biochemically most distinct genomovars, consistent with their naming as new species. However, it must be noted that phenotypic similarity between *B. multivorans* and genomovar VI is high, except for the characteristic earthy, rotten potato odor associated with the latter genomovar. Genomovars I and III and *B. ambifaria* remain very difficult to differentiate by biochemical testing.

We recommend that all organisms suspected of being of the *B. cepacia* complex or *B. gladioli* and any unusual assacharolytic gram-negative bacilli isolated from patients with CF be subjected to a combination of biochemical tests and recently described molecular diagnostic assays for genomovar identification. If facilities are unavailable for specialized testing, new isolates should be sent to referral laboratories capable of state-of-the-art identification of the *B. cepacia* complex. However, thorough biochemical analysis, as described herein, may be used as a primary screen for the genomovar or species status or to confirm the identification of clinical isolates previously identified by molecular methods. A greater understanding of the health risks associated with infection by each genomovar can only be obtained if isolates are accurately identified.

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