JOURNAL OF CLINICAL MICROBIOLOGY, Mar. 1997, p. 614–619 Vol. 35, No. 3
0095-1137/97/$04.00+0
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Identification of *Burkholderia cepacia* Isolates from Patients with Cystic Fibrosis and Use of a Simple New Selective Medium

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Received 31 July 1996/Returned for modification 8 October 1996/Accepted 5 December 1996

We evaluated 819 isolates referred to us as "*Burkholderia cepacia*" from cystic fibrosis (CF) clinics and research laboratories from five countries; 28 (3.4%) were not *B. cepacia*. A further 12 (1.5%) organisms appeared to be other *Burkholderia* species, but identification could not be confirmed by conventional means. The most prevalently misidentified organisms were *Stenotrophomonas maltophilia*, *Alcaligenes xylosoxidans*, and *Comamonas acidovorans*. Many of these organisms grew on oxidation-fermentation polymyxin-bacitracin-lactose (OFPBL) and *Pseudomonas cepacia* agars, selective media currently used for *B. cepacia* isolation. We developed a new medium, *B. cepacia* selective agar (BCSA), which is more enriched for the growth of *B. cepacia* yet which is more selective against other organisms than currently available selective agars. A total of 190 of 191 (99.5%) isolates of *B. cepacia* from patients with CF grew on BCSA without vancomycin, whereas 100% grew on OFPBL agar and 179 (94.2%) grew on *P. cepacia* agar. Of 189 other gram-negative and gram-positive organisms tested, 10 (5.3%) grew on BCSA without vancomycin. The addition of vancomycin to BCSA lowered the false positivity rate to 3.7% without further inhibition of *B. cepacia*. The false positivity rates for OFPBL and *P. cepacia* agars were 19.6 and 13.8%, respectively. Isolates of *B. cepacia* from CF patients grew most quickly on BCSA, with 201 of 205 (98.0%) being readily visible within 24 h, whereas 182 (88.8%) grew on OFPBL agar and 162 (79.0%) grew on *P. cepacia* agar within 24 h. We propose that the use of BCSA will allow investigators to overcome many of the difficulties associated with the identification of *B. cepacia* and should be considered for use as a primary isolation agar for specimens from patients with CF.

Recovery of *Burkholderia cepacia* from a patient with cystic fibrosis (CF) has serious repercussions on the patient's management and may be associated with a poor prognosis (5, 9, 14). Therefore, it is important that the initial isolation of the organism and its subsequent identification as *B. cepacia* be carried out as accurately and efficiently as possible (1, 8, 12, 17).

Our laboratory has become an international repository for typing of *B. cepacia*. Before proceeding with molecular typing studies, we subjected all isolates referred to us as "*B. cepacia*" to a thorough biochemical identification process to confirm the identification. Because several organisms misidentified as *B. cepacia* grew on commercially available agars intended to be selective for *B. cepacia*, we believed a medium that was more inhibitory yet that was enriched to support the growth of *B. cepacia* within 24 to 48 h was required. In this paper we describe a new *B. cepacia* selective agar (BCSA) and the results of our identification procedures, which show that biochemical patterns may vary with the source of the isolate.

(Preliminary findings were presented at the Ninth North American Cystic Fibrosis Conference [9a].)

MATERIALS AND METHODS

***B. cepacia* isolates.** Isolates of *B. cepacia* were kindly provided from CF clinics and from clinical and research laboratories in Canada, the United States, the United Kingdom, France, and Australia, as follows: University of Alberta Hospital (Edmonton, Alberta, Canada); Alberta Children's Hospital (Calgary, Alberta, Canada); Janeway Child Health Centre (St. John's, Newfoundland, Canada); University of Laval Hospital Centre (Ste. Foy, Quebec, Canada); Centre Hospitalier Rouyn-Noranda (Rouyn-Noranda, Quebec, Canada); Izaak Walton Killam Children's Hospital (Halifax, Nova Scotia, Canada); McMaster University Medical Centre (Hamilton, Ontario, Canada); Laurentian Hospital (Sudbury, Ontario, Canada); Children's Hospital of Eastern Ontario and the Laboratory Centre for Disease Control (Ottawa, Ontario, Canada); Hospital for Sick Children and Wellesley Hospital (Toronto, Ontario, Canada); British Columbia's Children's Hospital, St. Paul's Hospital, Vancouver Hospital and Health Sciences Centre, University of British Columbia site (Vancouver, British Columbia, Canada); Victoria General Hospital and the Royal Jubilee Hospital (Victoria, British Columbia, Canada); Children's Hospital and Medical Center (Seattle, Wash.); Medical College of Pennsylvania (Philadelphia, Pa.); Genentech Inc. (South San Francisco, Calif.); University of Minnesota Health Center (Minneapolis, Minn.); Hôpital Robert Debré (Paris, France); and Prince of Wales Hospital (Randwick, New South Wales, Australia). John Govan, University of Edinburgh (Edinburgh, Scotland), contributed many isolates from various centers in the United Kingdom and the United States.

Phenotypic identification of *B. cepacia*. Pure cultures were stored at −70°C in Mueller-Hinton broth (Difco Laboratories, Detroit, Mich.) with 8% dimethyl sulfoxide. Frozen isolates were passaged twice on Columbia agar containing 5% sheep blood (PML Microbiologicals, Richmond, British Columbia, Canada) before testing. The primary identification system used was the API Rapid NFT system (BioMerieux Vitek Inc., Hazelwood, Mo.) with glucose, maltose, lactose, mannitol, xylose, and sucrose oxidation-fermentation (OF) sugars (10), and Moeller lysine decarboxylase (Difco). Resistance to polymyxin (600 U/ml) and gentamicin (10 μg/ml) was also determined. All incubations were performed at 32°C in ambient air. OF sugars were incubated for up to 7 days, and for the other tests incubation was for up to 3 days. Identification of *B. cepacia* was confirmed when API Rapid NFT system results to the genus level were supported by key positive biochemical reactions (8). These positive reactions were (i) oxidation of glucose and xylose and at least one positive reaction among oxidation of maltose, oxidation of lactose, oxidation of sucrose, and/or oxidation of lysine decarboxylase; (ii) weakly positive oxidase reaction, defined as a faint purple color occurring at between 20 and 60 s with the Pathotec cytochrome oxidase strip (Remel, Lenexa, Kans.) (a strong oxidase reaction was considered a deep purple color developing within 10 s); (iii) *p*-nitrophenyl-β-D-galactopyranoside (PNPG) positivity and no nitrogen gas production from the reduction of nitrate (both read from the API Rapid NFT strip); and (iv) resistance to polymyxin and gentamicin.

The Vitek Gram-negative identification panel (Biomerieux Vitek) and/or the RapID NF Plus system (Innovative Diagnostic Systems, Norcross, Ga.) were used for selected isolates which did not meet the criteria given above and were set up according to the manufacturers' instructions. Additional tests used to

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confirm the identities of other genera and species were Gram staining, tests for growth at 42°C, tests for the production of arginine dihydrolase, DNase and urease, nitrate reduction tests, tests for motility in swarm agar (L broth with 0.3% agar), flagella staining, and the API 20E system test (4, 6, 8, 18).

For comparisons of the biochemical reactions, 361 *B. cepacia* isolates were selected from the collection. Organisms were chosen so that multiple strains from a single patient were not included unless there were differences in two or more biochemical reactions between the strains. In cases in which some CF centers had sent clonally related isolates from different patients (13), a representative 10 to 15% of strains from that center showing some phenotypic heterogeneity were selected for the study.

Genotypic identification of *Burkholderia* species. Isolates which failed to be satisfactorily identified by the phenotypic methods described above were blindly analyzed by PCR amplification of chromosomal DNA by using species-specific and genus-specific oligonucleotide primers. In brief, chromosomal DNA was recovered from bacterial cells as described previously (16). Amplification of DNA was performed in 50-μl reaction mixtures containing 10 mM Tris HCl (pH 9.0), 50 mM KCl, 2.0 mM $MgCl_2$, 200 μM (each) deoxynucleoside triphosphate, 1% Triton X-100, 50 ng of chromosomal DNA, 1 to 2 U of *Taq* polymerase (Fisher Scientific, Pittsburgh, Pa.), and 300 mM (each) primer. PCR was performed in a PTC-100 (MJ Research) thermal cycler with an initial denaturation of 3 min at 95°C, followed by 30 cycles of 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C. After a final extension of 4 min at 72°C, the samples were cooled to 4°C. After amplification, 10 μl of each reaction mixture was electrophoresed in a 2% agarose gel. PCR products were visualized and photographed after ethidium bromide staining.

Initially, these isolates were analyzed by PCR with two pairs of oligonucleotide primers. The first pair targets species-specific 16S and 23S rRNA gene sequences and amplifies the internal transcribed spacer region of *B. cepacia* rRNA operons (unpublished data). The second pair targets 16S rRNA sequences specific for all members of the genus *Burkholderia*. Any isolate not amplified by using the first pair of species-specific primers was analyzed by using a second pair of species-specific primers that targets 16S rRNA gene sequences, as described previously (2). Any isolate amplified with either pair of species-specific primers was identified as *B. cepacia*. Any isolate amplified by the genus-specific primer pair but not by either species-specific pair was designated as belonging to a *Burkholderia* species other than *B. cepacia.*

Sixteen laboratory isolates and American Type Culture Collection (ATCC) strains ATCC 10856, ATCC 17759, ATCC 25416, ATCC 25608, ATCC 27515, and ATCC 35130 of *B. cepacia*, one laboratory strain and strains ATCC 49129 and ATCC 27511 of *Burkholderia pickettii*, and two laboratory strains and strain ATCC 10248 of *Burkholderia gladioli* were used as controls for the PCR method.

BCSA test protocol. The testing of the efficacy of BCSA was conducted in two phases, as described below. After the analyses of the results obtained in phase 1, it was determined that BCSA without vancomycin was not inhibitory enough against certain gram-positive organisms. BCSA was modified by the addition of vancomycin, and in phase 2, those organisms that had grown on BCSA in phase 1 were retested on BCSA-vancomycin.

BCSA phase 1. The following ingredients were combined to make BCSA (per liter of distilled water): 5.0 g of sodium chloride, 10.0 g of sucrose, and 10.0 g of lactose (ICN Biomedicals, Inc., Aurora, Ohio), 0.08 g of phenol red (BDH Chemicals, Poole, England), 0.002 g of crystal violet (Sigma Chemical Co., St. Louis, Mo.), 10.0 g of trypticase peptone, 1.5 g of yeast extract, and 14.0 g of agar (Becton Dickinson & Co., Cockeysville, Md.). The phenol red and crystal violet were prepared as 10× aqueous solutions, and 10 ml of each was added per liter. The pH of the medium was 7.0 ± 0.1. After autoclaving for 20 min at 15 lb/in.^{2}, 600,000 U of polymyxin (Sigma) and 10 mg of gentamicin (Calbiochem Corp., La Jolla, Calif.) were added.

BCSA phase 2. Vancomycin (2.5 mg/liter; Eli Lilly Canada Inc., Scarborough, Ontario, Canada) was added, after the autoclaving step, to the formulation described above for BCSA phase 1.

Phase 1 study. A total of 281 *B. cepacia* isolates were selected from the repository for testing: 191 from respiratory secretions from CF patients, 44 from non-CF patients, and 46 from environmental sources. *B. cepacia* was chosen so that similar strains from one patient were not included. Organisms that had been misidentified as *B. cepacia* were also assessed. Other isolates were included to represent organisms that may be found in the sputa of CF patients: *Staphylococcus aureus* and most nonfermenting gram-negative bacilli other than *B. cepacia* were recovered from CF patients attending the clinics in Vancouver, and most members of the family *Enterobacteriaceae* and other gram-positive organisms were recovered from non-CF patients attending the Vancouver Hospital and Health Sciences Centre, University of British Columbia site. The following organisms were tested: 8 *Burkholderia gladioli*, 27 *Pseudomonas aeruginosa*, 12 *Pseudomonas fluorescens*, 17 *Stenotrophomonas maltophilia*, 10 *Alcaligenes xylosoxidans*, 2 *Agrobacterium radiobacter*, 6 *Comamonas acidovorans*, 5 *Flavobacterium meningosepticum*, 8 *Serratia marcescens*, 13 *Escherichia coli*, 11 *Enterobacter* spp., 13 *Klebsiella pneumoniae*, 10 *S. aureus*, 10 *Staphylococcus epidermidis*, 11 *Enterococcus faecalis*, and 9 *Candida* spp.

Organisms were grown on blood agar for 24 to 48 h. A saline inoculum with a turbidity equivalent to that of a 0.5 McFarland standard was prepared, and agar plates were inoculated by using a Steers replicator, resulting in approximately 5×10^5 CFU/spot (15). Plates were incubated at 32°C in ambient air and were examined for growth at approximately 24, 48, and 72 h. Growth on BCSA, OF polymyxin-bacitracin-lactose (OFPBL) agar (19) prepared in house, and *P. cepacia* agar (purchased from PML Microbiologicals) (7) was compared to growth on BCSA base, OFPBL agar base, and Mueller Hinton agar (Difco) without antibiotics, respectively. Organisms with fewer than 10 colonies per spot or pinpoint colonies after 72 h of incubation were considered inhibited or negative. The control Mueller-Hinton agar plate was also used to determine pigment production.

Quality control strains *B. cepacia* ATCC 25416 and *P. aeruginosa* ATCC 27853 were included on every plate, as were two of the following: *S. aureus* ATCC 25923, *E. faecalis* ATCC 29212, *B. gladioli* ATCC 10248, or a resistant yeast isolate (*Candida parapsilosis*) that had originally been misidentified as *B. cepacia* by a referring laboratory.

At the conclusion of the phase 1 study, BCSA was sent to three clinical microbiology laboratories in the Vancouver regional district for inclusion in their routine replicator susceptibility test procedures. These participants were Burnaby General Hospital, St. Paul's Hospital, and BC-Biomedical Laboratories. Any gram-negative organism and some gram-positive organisms that grew on BCSA were returned to us for further evaluation, along with information regarding the number and species of organisms tested.

Phase 2 study. The organisms that in phase 1 had grown on BCSA (both in house and from outside laboratories), plus additional *B. cepacia* isolates received after phase 1 was completed, were tested. BCSA-vancomycin was compared to BCSA without vancomycin, OFPBL agar, and P. cepacia agar by using the same replicator methodology used in phase 1. Growth on MacConkey agar (Que-Bact; Quelab Laboratories, Montreal, Quebec, Canada) with and without crystal violet was also assessed. Replicator plates were set up in duplicate, incubated at 32 and 37°C, and examined for growth at approximately 24, 48, and 72 h.

RESULTS

Among the 819 organisms in the collection, 28 (3.4%) were misidentified by the referring laboratory according to our phenotypic identification with commercial panels and by traditional biochemical tests. These were 1 *Enterococcus* species, 1 *C. parapsilosis* isolate, 3 members of the family *Enterobacteriaceae* (2 *Enterobacter agglomerans* isolates and 1 *S. marcescens* isolate), and 23 nonfermenting gram-negative bacilli. Of these organisms, 46 and 38% grew on OFPBL agar and *P. cepacia* agar, respectively. Gram stains would have ruled out the misidentification of *Enterococcus* sp. and the yeast. A fermentative reaction for glucose and a negative oxidase reaction for the *Enterobacteriaceae* would have indicated the necessity of an alternate identification pathway for these organisms. Although both isolates of *E. agglomerans* yielded dry wrinkled colonies, similar to the morphologies of some *B. cepacia* isolates, neither grew on BCSA, OFPBL agar, or *P. cepacia* agar. The *S. marcescens* isolate grew on OFPBL agar, but not on BCSA or *P. cepacia* agar.

The following nonfermenting organisms were misidentified: five *A. xylosoxidans*, one *A. radiobacter*, three *B. gladioli*, five *C. acidovorans*, two *F. meningosepticum*, one *P. aeruginosa*, one *P. fluorescens*, and five *S. maltophilia* isolates. All except *B. gladioli* and *S. maltophilia* were strongly oxidase positive (two of the latter were weakly oxidase positive); all except *S. maltophilia* were lysine decarboxylase negative; all except *A. radiobacter*, *P. fluorescens*, and one *S. maltophilia* did not oxidize sucrose. Three of the *S. maltophilia* isolates grew on *P. cepacia* agar (two as bright pink colonies), two grew on OFPBL agar, and one grew on BCSA. All except the *B. gladioli* isolates were correctly identified by the API Rapid NFT strip. The *B. gladioli* isolates were all oxidase negative and gentamicin susceptible, which were the initial clues indicating that further workup was required; tests for the oxidation of maltose, lactose, and sucrose and production of lysine decarboxylase were all negative.

A further 12 (1.5%) of 819 isolates from nine patients appeared to be other *Burkholderia* species or closely related organisms, and one isolate per patient received further evaluation. For one patient, three strains with identical biochemical and DNA patterns were isolated 14 months apart, and another isolate represented one of two similar strains received from a

different CF center. The nine isolates all oxidized glucose; were all negative for sucrose oxidation, lysine decarboxylation, arginine dihydrolation, and nitrate reduction; and were not clearly identified as a specific species by the biochemical test protocol. As well, all except one isolate (isolate C4964) oxidized xylose and had strongly positive oxidase reactions. All except two isolates (isolates FC124 and C3370) grew on BCSA, OFPBL agar, and *P. cepacia* agar. Only one isolate (isolate FC124) was from a non-CF patient (bronchoscopy specimen from a patient with chronic granulomatous disease); two reference laboratories could not confirm the identification but felt that it was most like *B. cepacia*, and the Vitek GNI panel plus PCR with genus-specific and species-specific primers indicated that it was *B. cepacia*. Our hesitation in agreeing with this identification rested with the fact that it was strongly oxidase positive and was susceptible to penicillin, gentamicin, and ticarcillin and therefore did not grow on *P. cepacia* agar or BCSA.

A subgroup of five isolates (including the isolates representing three strains from one patient and two strains from one clinic) were most likely *Burkholderia pickettii*, but because the isolates in this group were either nonmotile or had small zones in motility agar, we were unable to confirm the species identification on the basis of the number of flagella (*B. cepacia* normally has more than one polar flagella, while *B. pickettii* has a single polar flagellum [8]). PCR with genus-specific and species-specific primers indicated that the isolates were *Burkholderia* species but not *B. cepacia*. The API Rapid NFT system identification of *P. fluorescens* for four isolates from this group was ruled out by negative reactions for arginine and by growth at 42°C (the fifth organism was identified as *B. pickettii*). The organisms were not pigmented, did not have a discernible odor, and were saccharolytic, ruling out the RapID NF Plus system identification of *Flavobacterium odoratum*. The Vitek GNI panel identified two of this group as *B. pickettii*, one as *Eikenella corrodens*, and one as *Pseudomonas stutzeri* and one was unidentified. They all oxidized maltose and OF of lactose and grew on BCSA, OFPBL agar, and *P. cepacia* agar. A sixth isolate had the same biochemical reactions as those for the organisms in this subgroup except that it was unidentified by the Vitek GNI panel and was identified as *Pseudomonas putida* by the API Rapid NFT system, and PCR analysis indicated that it was *B. cepacia.*

Isolate C3770, being negative for oxidation of maltose, lactose, and sucrose and negative for the decarboxylation of lysine, may have been *B. gladioli*, except that the strong oxidase reaction and the PCR identification of "not *Burkholderia* species" contradicted this identification. The API Rapid NFT system identified it as *B. cepacia*, but the Vitek GNI panel and RapID NF Plus system could not identify it. It was susceptible to gentamicin and therefore did not grow on BCSA.

Isolate C4964 was particularly difficult in that tests with the reference OF sugars failed; it was only weakly acidic in a test for oxidation of glucose after 4 days of incubation and was alkaline for the other OF sugars tested. This organism was either oxidase negative or occasionally weakly oxidase positive; if the oxidase reaction was read as positive, the API Rapid NFT strip reading would have been *Alcaligenes faecalis*, and if it was negative, the reading would have been *Acinetobacter anitratus*. The Vitek GNI panel result of presumptive *Acinetobacter lwoffi* indicated an organism that produces few positive biochemical reactions. It possessed polar flagella, which ruled out both genera, because *Alcaligenes* spp. have peritrichous flagella and *Acinetobacter* spp. are nonmotile (18). Isolate C4964 was most likely *B. cepacia*, based on the oxidase reaction, antibiotic resistance pattern, PCR results, RapID NF Plus strip, and flagellar staining (we have seen other *B. cepacia* isolates from different centers with similar alkaline results). It grew on all three isolation media.

TABLE 1. Biochemical reactions of *B. cepacia* isolates separated by specimen source after 3 and 7 days of incubation at 32°C

Biochemical reaction	% Positive after 3 days (7 days)			
	CF (n = 266)	Non-CF (n = 43)	Env., hospital (n = 12)[a]	Env., true (n = 40)[b]
Oxidation of:				
Glucose	97 (98)	100	100	100
Maltose	63 (83)	95 (98)	100	55 (80)
Lactose	77 (91)	100	100	68 (90)
Mannitol	66 (85)	88 (100)	92 (100)	70 (98)
Xylose	87 (97)	63 (93)	67 (75)	73 (88)
Sucrose	81 (82)	72 (72)	83 (83)	58 (63)
Lysine decarboxylase	90	93	75	80
Oxidase (slow)	97	100	100	100
PNPG	94	81	67	93
Pigment, brown	35	0	17	0
Pigment, yellow	1	5	0	60

[a] Isolates from hospital environments.
[b] Isolates from environmental sources other than hospitals, such as plant or soil.

ATCC and known strains of *Burkholderia* species were tested as controls by the PCR method. The results were as follows. Three *B. pickettii* grew on BCSA (not tested on OFPBL agar or *P. cepacia* agar) and were determined to be *Burkholderia* species, but not *B. cepacia*, by PCR. Three *B. gladioli* isolates grew on OFPBL agar, but not on BCSA or *P. cepacia* agar, and were identified as *Burkholderia* species, but not *B. cepacia*, by PCR. Five ATCC strains and nine laboratory isolates of *B. cepacia* grew on BCSA, OFPBL agar, and *P. cepacia* agar and were identified as *B. cepacia* by PCR analysis. ATCC 35130 and seven *B. cepacia* isolates grew on the three media but were determined to be *Burkholderia* species, but not *B. cepacia*, by PCR.

Table 1 presents the percentage of positive biochemical reactions for the *B. cepacia* isolates, with the isolates separated by specimen source. Because the tests with the sugars were incubated for 7 days, the percentage of positive reactions after 3 days of incubation, followed by the total reactions (7 days), are presented. Sucrose and glucose oxidation generally occurred within the first 3 days, whereas the other OF sugars tested often required extended incubations. The true environmental strains (from plant and soil sources) were less likely to utilize sucrose (62.5%) and lysine (80.0%) compared to the isolates from patients with CF, with results of 82 and 90%, respectively. The rate of positivity for oxidation of maltose and lactose by isolates from patients with CF were 83.1 and 91.0%, respectively, after 7 days of incubation. Of 23 (8.6%) *B. cepacia* isolates from CF patients that were negative for oxidation of both maltose and lactose, only two (strains C5241 and C6122) were also negative for both oxidation of sucrose and decarboxylation of lysine. Both of these isolates were from patients who had been colonized with *B. cepacia* for a number of years, with minor changes in the strains' biochemical reactions noted over time. Random amplified polymorphic DNA (RAPD) PCR fingerprints were stable (data not shown) (13), so it was presumed that these two strains were still *B. cepacia*. Ten (43.5%) of the strains negative for the oxidation of maltose and lactose came from various Ontario clinics; all of these were of the RAPD group predominant in Ontario, and seven were brown pigmented. This brown water-soluble pigment, thought to be mel-

TABLE 2. Comparison study (phase 1) of selective media

Organism	No. of isolates	% Growth after 48 h of incubation at 32°C		
		OFPBL agar	*P. cepacia* agar	BCSA
B. cepacia (all)	281	100	95.4	99.6
B. cepacia (CF patients only)	191	100	94.2	99.5
B. cepacia (non-CF patients)	44	100	97.7	100
B. cepacia (environmental)	46	100	97.8	100
All non-*B. cepacia*	189	19.6	13.8	5.3
B. gladioli	8	100	12.5	25.0
Pseudomonas spp.[a]	53	0.0	0.0	0.0
F. meningosepticum	5	100	100	60.0
A. xylosoxidans	10	30.0	20.0	0.0
C. acidovorans	6	16.7	83.3	0.0
S. maltophilia	16	12.5	43.8	6.3
Other NFB[b]	6	0.0	0.0	0.0
E. coli	13	0.0	0.0	0.0
Enterobacter spp.	11	9.1	9.1	0.0
Klebsiella spp.	13	0.0	0.0	0.0
S. marcescens	8	100	25.0	12.5
E. faecalis	11	0.0	0.0	27.3
Staphylococcus spp.[c]	20	0.0	0.0	0.0
Candida spp.	9	100	33.3	0.0

[a] Including 27 *P. aeruginosa*, 12 *P. fluorescens*, 7 *P. putida*, 4 *P. stutzeri*, and 3 *P. mendocina* isolates.
[b] NFB, nonfermenting gram-negative bacilli (four *Acinetobacter* spp. and two *A. radiobacter* isolates).
[c] Ten isolates each of *S. aureus* and coagulase-negative *Staphylococcus* spp.

anin (9), was also present in some isolates from patients with CF in various clinics in British Columbia, eastern Canada, and the United Kingdom and in two hospital environmental samples, but it was not found in clinical isolates from non-CF patients or true environmental isolates (from plant or soil sources). The presence of a yellow pigment has classically been an identifiable feature of *B. cepacia*, but it was most often found in strains from plant or soil sources, confirming other reports that clinical strains are usually not pigmented (6, 8). The two CF patient strains with a yellow pigment were much paler yellow than was found in the isolates from the true environmental samples.

Nineteen (7.1%) of 266 isolates from CF patients, 9 of 52 (17.3%) environmental isolates, and 2 of 43 (4.7%) clinical isolates from non-CF patients were negative for both lysine decarboxylase and oxidation of sucrose. Except for isolates C5241 and C6122, all isolates were positive for oxidation of lactose, and only one (an environmental isolate) was negative for oxidation of maltose. The environmental isolates that were lysine decarboxylase negative and negative for oxidation of sucrose were all from England, and 55% of the isolates from patients with CF were from patients in British Columbia. For those organisms that were oxidase or PNPG negative, positive results for the OF sugars or lysine decarboxylase confirmed the identification of *B. cepacia*. The strains from patients with CF usually yielded much smaller colonies and grew more slowly than clinical strains from non-CF patients or environmental strains.

Phase 1 evaluation of BCSA. Growth of *B. cepacia* was not affected by polymyxin at 600,000 U/liter (300,000 U/liter is present in OFPBL agar and *P. cepacia* agar), but many other nonfermenting gram-negative bacilli, coagulase-negative staphylococci, and yeast were inhibited by the increased concentration (data not shown). The results for the quality control organisms verified the performance of all media and the reproducibility of the test procedures. A total of 280 of 281 (99.6%) *B. cepacia* isolates grew on BCSA, whereas 268 (95.4%) grew on *P. cepacia* agar and 100% grew on OFPBL agar (Table 2). One isolate from a patient with CF was inhibited on BCSA; it grew as pinpoint colonies after 72 h of incubation and grew poorly on *P. cepacia* agar. This isolate was one of 15 related strains (identical RAPD PCR patterns) from a geographically isolated center; the other 14 strains grew well on BCSA, but because related strains were not included in this part of the study, these organisms were not tested on OFPBL agar or *P. cepacia* agar.

The overall false-positive growth rate for OFPBL agar was 19.6%, that for *P. cepacia* agar was 13.8%, and that for BCSA was 5.3%. *E. faecalis* was the only organism that grew on BCSA but not on OFPBL agar or *P. cepacia* agar. *B. gladioli* was not inhibited on OFPBL agar; the isolates that did grow on *P. cepacia* agar or BCSA grew poorly. Fifteen of the 16 *S. maltophilia* isolates tested were from CF patients; one isolate grew on all three selective media.

The results obtained from other laboratories that used BCSA in their routine replicator runs confirmed the ability of BCSA to inhibit most gram-negative organisms. All identifications were performed by the contributing centers; when possible, the identities of the organisms growing on BCSA were confirmed by our laboratory. Six of 283 (2.1%) of the isolates of the family *Enterobacteriaceae* tested grew on BCSA; these were 1 of 18 *Enterobacter cloacae* isolates, 1 of 4 *Yersinia* spp., 1 of 7 *Morganella morganii* isolates, and 3 of 3 *Providencia* spp. Of 67 *Enterococcus* spp. tested, 18 (26.9%) grew on BCSA; these were all *E. faecalis* (only one was from a respiratory source).

After observing that 27% of the enterococci tested grew on BCSA, cultures of throat swabs from five staff members were tested on BCSA and blood agar. This additional testing revealed scant growth of viridans group streptococci from some samples, resulting in the modification of the BCSA medium by the addition of 2.5 mg of vancomycin per liter. Repeat cultures of throat swabs from the same individuals showed no growth on BCSA-vancomycin, while viridans group streptococci were still present on the blood agar control plates.

Phase 2 evaluation of BCSA. The addition of vancomycin to BCSA inhibited the enterococci but had no effect on the growth of the gram-negative organisms. Incubation at 32 and 37°C demonstrated no differences between temperatures for OFPBL agar BCSA, or BCSA-vancomycin; therefore, only the results for incubations performed at 37°C are presented (Table 3). *P. cepacia* agar performed better at 37°C (92.7% with growth after 48 h) than at 32°C (87.8%) for *B. cepacia* isolates from CF patients. *B. cepacia* isolates grew more frequently on MacConkey agar with and without crystal violet when they were incubated at 37°C (51.7 and 91.1%, respectively) than at 32°C (38.3 and 83.6%, respectively). The formulation of MacConkey agar-crystal violet had an inhibitory effect on *B. cepacia*; only 48.3% of *B. cepacia* isolates from CF patients grew on MacConkey agar with crystal violet, whereas 87.8% grew on MacConkey agar alone.

The quality and rapidity of growth of *B. cepacia* at 37°C were also compared. Growth was considered "good" if it was greater than 60% compared to that on the control plate and "poor" if there were more than 10 colonies larger than pinpoint size but less than 60% growth compared to that on the control plate. For isolates from patients with CF grown on BCSA-vancomycin, good growth was demonstrated more quickly (98.0% by 24 h, increasing to 99.5% by 48 h) than the time to good growth for isolates grown on OFPBL agar (88.8 and 99.0%, respectively) and *P. cepacia* agar (79.0 and 86.8%, respectively). Maximal good growth occurred by 48 h on BCSA-vancomycin and

TABLE 3. Comparison study of (phase 2) selective media

Organism	No. of isolates	% Growth after 48 h of incubation at 37°C[a]				
		BCSA + Vnc	OFPBL agar	*P. cepacia* agar	Mac + CV	Mac − CV
B. cepacia (all)	292	99.7	100	93.8	51.7	91.1
B. cepacia (CF patients)	205	99.5	100	92.7	48.3	87.8
B. cepacia (non-CF patients)	44	100	100	92.7	54.5	100
B. cepacia (environmental)	43	100	100	95.3	65.1	97.7
Enterococcus sp.	14	0	0	0	64.3	100

[a] BCSA + Vnc, BCSA-vancomycin; Mac + CV, MacConkey agar with crystal violet; Mac − CV, MacConkey agar without crystal violet.

OFPBL agar; for *P. cepacia* agar, increasing the incubation time to 72 h improved the good growth rate by 2.0% (with an additional 3.9% of isolates still considered to exhibit poor growth). All *B. cepacia* isolates from other sources displayed good growth on BCSA-vancomycin and OFPBL agar by 48 h compared to that on *P. cepacia* agar, on which good growth rates of 97.7% were achieved for isolates in clinical specimens from non-CF patients and 93.0% for environmental isolates by 48 h.

DISCUSSION

Due to the impact on patient treatment, the initial identification of *B. cepacia* must be carefully performed. Some of the identification errors that we encountered could have occurred because the organisms may have grown on *P. cepacia* agar or OFPBL agar, or both. *S. maltophilia* is particularly problematic since, like *B. cepacia*, it can utilize sucrose and lysine and can occasionally be weakly or slowly oxidase positive (1, 6). DNase results are usually positive within 24 h for *S. maltophilia* but can be delayed, and it has been recommended that incubation of DNase plates be extended to 72 h (1). *B. cepacia* is DNase negative.

We found several isolates from various centers that appeared to be closely related to *Burkholderia* species but whose identities could not be confirmed by our methods or with the commercial systems used in this study. We conjecture that *B. cepacia* and related species may undergo selective pressures to survive in the lungs of CF patients, resulting in the loss of typical phenotypic markers. What is needed is a definitive genotypic test that can easily be used by clinical laboratories. Recognized reference centers specializing in the identification of this group of organisms should be established. The PCR assays used here appear to be accurate to the genus level and capable of identifying most isolates defined as *B. cepacia* by phenotypic parameters; work is progressing to improve the accuracy of PCR assays for organism identification to the species level.

Our studies showed that some of the isolates from patients with CF were negative or delayed in their oxidation of various sugars, while it had been reported previously that greater than 98% of *B. cepacia* isolates were positive for the oxidation of glucose, maltose, lactose, mannitol, and xylose (8). *B. cepacia* isolates whose biochemical reactions were exceptions to the expected biochemical reactions of *B. cepacia* isolates formed patterns common to a patient, clinic, or geographic area; therefore, keeping laboratory records of the biochemical reaction patterns of a patient's or clinic's strains may simplify identification of future isolates and aid in epidemiological studies. Positive reactions for the oxidation of maltose and lactose are used to differentiate *B. cepacia* from *B. gladioli*, which has occasionally been isolated from CF patients (3). Few commercially available systems include *B. gladioli* in their organism identification databases, and commercially available systems frequently misidentify *B. gladioli* as *B. cepacia* (11). It is important to be aware of the limitations of the various commercially available identification panels, since weaknesses in their abilities to identify *B. cepacia* isolates and to separate *B. cepacia* from other *Burkholderia* species have been demonstrated. Kiska et al. (11) evaluated several commercially available systems for their ability to identify organisms from secretions from CF patients. They found that the RapID NF Plus system misidentified 8 of 58 *B. cepacia* isolates as *Alcaligenes faecalis*/*Alcaligenes oderans*, and that the API Rapid NFT system and the Vitek GNI panel misidentified 5 and 7 *B. cepacia* isolates, respectively, as other organisms. Both the RapID NF Plus and the API Rapid NFT systems misidentified five of nine *B. gladioli* isolates and two of six *B. pickettii* isolates as *B. cepacia*, the Vitek GNI panel misidentified one of nine *B. gladioli* isolates and one of six *B. pickettii* isolates as *B. cepacia*, and the Remel system misidentified two of nine *B. gladioli* isolates and two of six *B. pickettii* isolates as *B. cepacia* (11).

Growth of *B. cepacia* on MacConkey agar with and without crystal violet was studied, since many laboratories use MacConkey agar for primary isolation. The MacConkey agar with 0.001 g of crystal violet per liter contained 1.5 g of an undefined bile salts mixture per liter, while MacConkey agar without crystal violet contained 5.0 g of bile salts per liter. BCSA and BCS-vancomycin contained crystal violet at 0.002 g/liter, which had no effect on the growth of *B. cepacia* or any other gram-negative organism tested, as demonstrated by good growth on the BCSA base control plate (without antibiotics). It is possible that the combination of bile salts and crystal violet was inhibitory to some isolates of *B. cepacia*. Fifty percent of *B. cepacia* isolates from CF patients failed to grow on MacConkey agar with crystal violet, which emphasized the importance of including an agar with enrichments for *B. cepacia* in the primary isolation protocol for respiratory specimens from patients with CF.

In-house replicator trials demonstrated that BCSA was more inhibitory than OFPBL agar or *P. cepacia* agar for organisms other than *B. cepacia* and had a quicker recovery rate for *B. cepacia*. To further test the efficacy of BCSA, we are comparing BCSA, OFPBL agar, and *P. cepacia* agar in the routine examination of sputa from patients with CF in two clinical laboratories servicing large CF clinics. Preliminary data indicate that BCSA may be superior to OFPBL agar and *P. cepacia* agar in its ability to support the rapid growth of *B. cepacia* and to inhibit the growth of other organisms (unpublished data).

We recommend that OF sucrose and lysine decarboxylase plus a decarboxylase control be combined with a commercial nonfermenting, gram-negative identification panel to confirm the identification of *B. cepacia* by the panel. If the panel fails

to identify the organism as *B. cepacia* or if both lysine and sucrose are negative, any initial isolate that is possibly *B. cepacia* or some other *Burkholderia* species should be sent to a recognized reference laboratory for confirmation of its identity. Screening identification methods can be used for subsequent isolates (1). Very few oxidase-positive, nonfermenting, gram-negative bacilli other than *B. cepacia* utilize both sucrose and lysine; thus, positive results for these tests (combined with growth on BCSA, a negative result for DNase, and a weakly positive oxidase reaction) can be useful as a screen for repeat isolates from the same patient if the patient's previous isolates were positive for lysine decarboxylase and OF sucrose.

ACKNOWLEDGMENTS

This study was supported by the Canadian Cystic Fibrosis Foundation.

We thank Janet Zemkov of St. Paul's Hospital for assistance and identification of some of the misidentified organisms with the Vitek GNI panel and Beth Harper, Alison Clarke, and Joyce Wilson and their respective staffs of the Microbiology Laboratories at Burnaby General Hospital, St. Paul's Hospital, and BC-Biomedical Laboratories for participation in field trials. We thank the clinic directors and microbiologists at Canadian CF treatment centers and Edouard Bingen, Jane Burns, Patricia Ferrieri, John Govan, Margaret Roy, Terrence Stull, and Peter Taylor for providing *B. cepacia* isolates.

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