

Species Abundance and Diversity of *Burkholderia cepacia* Complex in the Environment

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Despite considerable interest in studying *Burkholderia cepacia* complex in the environment, we still do not have efficient methods to detect, isolate, and screen large numbers of *B. cepacia* isolates. To better describe the ecology and diversity of *B. cepacia* complex, a colony hybridization assay was developed to detect specifically all species of the complex based on polymorphism of the variable V3 region of the 16S rRNA sequence. The sensitivity of the assay was dramatically enhanced by using a probe consisting of three repeats of a *B. cepacia* complex-specific probe, each separated by a phosphoramidite spacer. In addition, a duplex PCR targeting *B. cepacia* complex-specific *recA* and 16S rRNA sequences was developed to enable a fast and reliable diagnostic assay for members of the complex. When applied to maize rhizosphere samples, colony hybridization results were in good agreement with those of most-probable-number duplex PCR, both indicating a >100-fold fluctuation of abundance between individual plants. Using restriction analysis of *recA* for a total of 285 confirmed isolates of the *B. cepacia* complex, up to seven *B. cepacia* complex species were identified; however, their diversity and abundance were not evenly distributed among individual plants, and several allelic variants were commonly found from the same rhizosphere sample. These results indicate that not only complex communities of *B. cepacia* complex species and closely related strains of the same species may coexist at high population levels but also species composition and abundance may dramatically vary between individual plants.

In the genus *Burkholderia* of the β subdivision of the proteobacteria, the *Burkholderia cepacia* complex consists of at least nine closely related *Burkholderia* species, namely, *B. cepacia* (formerly genomovar I), *B. multivorans* (II), *B. cenocepacia* (III), *B. stabilis* (IV), *B. vietnamiensis* (V), *B. dolosa* (VI), *B. ambifaria* (VII), *B. anthina* (VIII), and *B. pyrrocinia* (IX) (10, 11, 22, 49–53). They share moderate levels of DNA-DNA hybridization (30 to 50%) but high sequence similarity at the level of 16S rRNA (98 to 99%) and *recA* (94 to 95%) genes. Moreover, they all possess an unusually complex and plastic genome that consists of two to four replicons (chromosomes), which harbor several insertion sequences that may increase the frequency of genetic mutations and recombinations (7, 30). This may favor their adaptation to different environments, and it is not surprising that strains of the complex are found in habitats as diverse as soil, plant, and animal surfaces, rhizosphere, and water (9, 42).

This remarkable versatility is further exemplified by the wide array of properties they possess. Although first described as plant pathogens causing sour skin of onion (6), strains of the *B. cepacia* complex have also been reported to be effective biological control agents against soilborne (5, 25), foliar (28), and postharvest (27) plant pathogens, to promote plant growth (16, 48), and to decontaminate polluted soil or groundwater (29, 39). Despite the useful properties those organisms may offer to bioremediation or as an alternative to pesticides in agriculture, commercial application has been hampered by the risk *B. cepacia* complex may pose to human health. Indeed, in the last

decade, strains of the complex have been found associated with nosocomial infection of immunocompromised patients (26) and chronic or rapid decline in pulmonary function in patients with cystic fibrosis (31).

To date, no clear distinction has been established between clinical and environmental isolates on the basis of phenotypic or genotypic criteria (1, 3, 34), and all *B. cepacia* complex species have been isolated from sputum cultures of cystic fibrosis patients (9). Moreover, the emergence of new strains each year in cystic fibrosis patients suggests the ongoing acquisition of strains from the environment (8, 32, 34).

Although *B. cepacia* complex strains deposited in culture collections have been sampled from various habitats, information concerning major reservoirs of *B. cepacia* complex in the environment are still lacking. Indeed, there are few studies concerning the ecology of the *B. cepacia* complex in the environment, and they deal with a few strains isolated sporadically or were done when the description of the complex was limited to one species or a few species (9, 42). Recently, several studies have examined the rhizospheres of trees (43) and crop plants (1, 3, 14, 19) as potential reservoirs for *B. cepacia* complex. Although most of the latter studies dealt with 50 to 100 isolates, a more thorough description of *B. cepacia* complex populations in the environment is needed, despite the laborious work of isolation and screening that this entails. The main issue, however, is not *B. cepacia* complex identification because molecular identification of *B. cepacia* complex species is made possible using specific primers targeting *recA* (37) and 16S rRNA (2, 33) sequences. Furthermore, *recA* restriction analysis has been shown to be a convenient tool to distinguish each of the species in the complex (37). The greater difficulty is in the way those isolates are arbitrarily selected on dilution plates

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prior to identification. Indeed, Miller et al. (41) reported the presence of *B. cepacia* complex in more than 82% of 91 urban soils as detected by culture-independent methods, while *B. cepacia* complex colonies could be isolated in only 15% of all soils investigated. This underlines the need for better screening techniques to recover *B. cepacia* complex even when present in a sample at low population levels.

The low isolate yield may be attributed to the fact that semiselective media, e.g., trypan blue tetracycline medium (TB-T) (24) or *Pseudomonas cepacia* azelaic acid tryptamine medium (PCAT) (4), were developed when the taxonomic description of the *B. cepacia* complex was not yet fully established. Although unavoidable biases exist when recovering *B. cepacia* complex on selective or semiselective media (46), it is the only way to obtain isolates in pure cultures for further phenotypic and genotypic characterizations and to better describe their ecology and diversity.

The objectives of this study were to develop and assess the efficiencies of molecular assays to determine the population density and diversity of culturable organisms of the *B. cepacia* complex from environmental samples. The methods include colony hybridization (CH) and duplex PCR combined with most-probable-number PCR (MPN-PCR). The methods were first developed and tested using a representative collection of reference *B. cepacia* complex and non-*B. cepacia* complex species and further validated and compared with each other by thoroughly characterizing *B. cepacia* complex populations from a heterogeneous habitat, i.e., the maize rhizosphere.

MATERIALS AND METHODS

Bacterial cultures and storage. All reference *B. cepacia* complex and non-*B. cepacia* complex strains were selected from the collection of the *Burkholderia cepacia* Research Laboratory and Repository (at the University of Michigan, Ann Arbor). Twenty-four strains representing the nine *B. cepacia* complex species were used to validate the molecular techniques. In the following list, a strain name followed by a T and/or P superscript indicates the strain is a species type strain and/or a strain included in previously published experimental strain panels (12, 36), respectively. Their designations in the American Type Culture Collection (ATCC) and the Laboratorium voor Microbiologie (LMG) (Ghent University, Ghent, Belgium) are provided when available. The reference *B. cepacia* complex and non-*B. cepacia* complex strains were *B. cepacia* genomovar I PC783^{PT} (ATCC 25416, LMG 1222), HI2140^P, and HI2284; *B. multivorans* HI229^P (ATCC 17616, LMG 17588), HI2132^P, and HI2240^P; *B. cenocepacia* J2315^{TP} (ATCC BAA-245, LMG 16656), HI3240^P, and HI3248^P; *B. stabilis* HI2210^{TP} (ATCC BAA-67, LMG 14294) and AU0244; *B. vietnamiensis* PC259^P (LMG 18835, CEP40, PC259), AU1344, and HI2278^P; *B. dolosa* AU0645^{TP} (LMG 18943, R-5670), AU0158, and HI2238; *B. ambifaria* AMMD^{TP} (ATCC BAA-244, LMG 19182), AU0212 (LMG 19466), and HI2474^P; *B. anthina* AU1293^P (LMG 21821) and HI2725^P; and *B. pyrrocinia* BC011^P (LMG 21823) and HI2710^P. Negative controls consisted of six strains that were not *B. cepacia* complex strains but were closely related to them, i.e., *Burkholderia gladioli* AU3923, AU5002, AU5085, and HI2870; *Burkholderia xenovorans* LB400^T (LMG 21463) (23); and *Ralstonia pickettii* PKO1 (21). Reference strains and rhizosphere isolates were routinely grown at 29°C on plate count agar (Difco Laboratories, Detroit, Mich.) and stored at -80°C in Luria-Bertani broth medium (Difco) containing 30% glycerol.

Duplex PCR. *B. cepacia* complex-specific primers targeting 16S rRNA and *recA* (Table 1) were synthesized by Integrated DNA Technologies (Coralville, Iowa). Using Primer3 software (44), a new forward primer (i.e., Burkf) was designed to amplify specifically 16S rRNA sequences of *B. cepacia* complex based on a CLUSTAL W (47) sequence alignment of *B. cepacia* complex and closely related genera and species.

Each DNA preparation consisted of 1- μ l loopful of a colony grown overnight that was lysed in 4 μ l of lysis solution (50 mM KCl, 0.1% Tween 20, 10 mM Tris-HCl [pH 8.3]) at 99°C for 10 min. Sixteen microliters of PCR cocktail was subsequently added to the cell lysate. The PCR cocktail consisted of 1 \times PCR

TABLE 1. *B. cepacia* complex-specific primers and probes

Assay	Target	Primer or probe ^a	Positions ^b	Sequence (5' → 3') ^c	Length (no. of bases)	Reference
PCR	16S rRNA gene	Burkf (f)	133–153	GGCGAAGCCGGATTAATACC	21	This study
		CeMuVi-16-2 (r)	452–435	CCGRCTGTATTAGAGCCA	18	2
	<i>recA</i>	BCR1 (f)	2–20	TGACCCGGAGAAAGCAA	19	37
		BCR2 (r)	1044–1024	CTCTTCTCCATCGCCTC	21	37
CH	16S rRNA gene	V3-30	460–431	GTCATCCCCGGGTGTTAGAGCCAAGGA	30	This study
		V3x2	NA	GTCATCCCCGGGTGTTAGAGCCAAGGA/SpC3/GTCATCCCCGGGTGTTAGAGCCAAGGA	60	This study
		V3x3	NA	GTCATCCCCGGGTGTTAGAGCCAAGGA/SpC3/GTCATCCCCGGGTGTTAGAGCCAAGGA/SpC3/GTCATCCCCGGGTGTTAGAGCCAAGGA	90	This study

^a (f), forward; (r), reverse.

^b For 16S rRNA gene-based assays, positions in the sequence of *B. multivorans* LMG 13010^T (GenBank accession number Y18703) are shown. For *recA*-based assays, positions in the sequence of *B. multivorans* ATCC 17616 (U70431) are shown. The first coordinate corresponds to the 5' end of the oligonucleotide sequence. NA, not applicable.

^c Wobble base, R = A or G; /SpC3/, hydrophilic C3 phosphoramidite spacer.

TABLE 2. Population density, species abundance, and diversity of *B. cepacia* complex strains from maize rhizosphere samples

Sample or parameter	Population level [\log_{10} (CFU/g)] ^a		No. of isolates of <i>B. cepacia</i> complex species ^b :										Total no. of isolates	S ^c		
	Total	<i>B. cepacia</i> complex	I		II	III	IV	VI	VII						IX	
			A	B					A	B	C	D				E
Plant 1	5.90 (0.07)	5.41			2				19	3		6	2		30	2
Plant 2	6.48 (0.14)	5.81	20	1					3		3	1		2	30	3
Plant 3	6.57 (0.08)	6.51	1	35	3	5	1		2	1	1	1	1	1	51	6
Plant 4	7.02 (0.08)	6.72							13	6	6	3			28	1
Plant 5	5.86 (0.10)	5.83							5		46	5			56	1
Plant 6	5.15 (0.04)	4.58				2		7	8		1				18	3
Plant 7	4.76 (0.06)	4.61	1					19	27		3	20			70	3
Total abundance and richness			22	36	3	9	1	26	75	10	60	36	2	3	285	7
Mean (SD)	5.96 (0.80)	5.64 (0.84)													40 (18.7)	2.7 (1.7)
CV ^d (%)	14	15													47	63

^a Total population levels were determined by plating on TB-T plates, and *B. cepacia* complex abundance was determined by CH, followed by confirmatory duplex PCR. Standard deviations of three replicate measurements per plant are indicated in parentheses.

^b The *B. cepacia* complex species found were *B. cepacia* (formerly genomovar I), *B. multivorans* (II), *B. cenocepacia* (III), *B. stabilis* (IV), *B. dolosa* (VI), *B. ambifaria* (VII), and *B. pyrrocinia* (IX). This classification is based on the results of *recA* RFLP analysis and was confirmed by sequencing. Different *recA* alleles are indicated by letters (A, B, C, and D) that correspond to the sequences of representative isolates KBC-1 (VII-E), KBC-2 (I-A), KBC-3 (I-B), KBC-4 (III), KBC-5 (VII-B), KBC-6 (VII-C), and KBC-7 (VII-D). The results of a phylogenetic analysis of those sequences are shown in Fig. 4. Amplicons yielding patterns that were similar to those of reference strains were not sequenced.

^c Observed species richness (S) was calculated by considering *B. cepacia* complex species and not the occurrence of multiple *recA* patterns within a species.

^d CV, coefficient of variation (standard deviation divided by the mean).

buffer (50 mM KCl, 10 mM Tris-HCl [pH 9.0], 0.1% Triton X-100, 1.5 mM MgCl₂; Promega Corp., Madison, Wis.), bovine serum albumin (0.5 g liter⁻¹; Sigma, St. Louis, Mo.), 5% dimethyl sulfoxide (Sigma), 200 μ M concentrations of the four deoxynucleoside triphosphates (Promega), a 0.50 μ M concentration of each primer targeting 16S rRNA (i.e., Burkf and CeMuVi-16-2), a 0.40 μ M concentration of each primer targeting *recA* (i.e., BCR1 and BCR2), and 1.5 U of *Taq* DNA polymerase (Promega). After an initial denaturation step (3 min at 94°C), 30 PCR cycles (1 cycle consisting of 30 s at 94°C, 30 s at 55°C, and 1 min at 72°C) and a final extension step (10 min at 72°C) were used. PCR products were electrophoresed on 1.5% agarose gels in 1 \times Tris-acetate-EDTA (TAE) and stained with ethidium bromide by standard protocols (45). A 100-bp ladder (Invitrogen Corp., Carlsbad, Calif.) was used as molecular size markers.

***recA* RFLP.** PCR cocktail composition was as described above, except that only primers BCR1 and BCR2 (0.40 μ M each) were used. After PCR amplification, 5 μ l of amplified product was used for restriction analysis with 2 U of either HaeIII or MnlI (New England Biolabs, Inc., Beverly, Mass.) in a total volume of 15 μ l. After 3-h digestion at 37°C, restriction fragments were separated by electrophoresis on 2.5% agarose gels by standard procedures (45). A 100-bp ladder and a 50-bp ladder (Invitrogen) were used as molecular size markers. Restriction analysis was generally done once and was repeated if restriction patterns could not be assigned to any of the reference patterns, i.e., those obtained by *recA* restriction fragment length polymorphism (RFLP) with reference *B. cepacia* complex strains of the nine *B. cepacia* complex species.

16S rRNA and *recA* sequencing. To determine 16S rRNA sequences, PCR products were purified with the Qiaquick PCR purification kit (QIAGEN, Inc., Valencia, Calif.), and both strands were sequenced using universal eubacterial primers. Alternatively, when specific PCR products could not be obtained, PCR amplicons were first cloned with TOPO TA cloning kit for sequencing (Invitrogen) and then sequenced using *Taq* dye terminator chemistry and an ABI cycle sequencer (Genomic Technology Support Facility, Michigan State University). Both strands of *recA* sequences were sequenced using PCR primers BCR1, BCR2, BCR3, and BCR4 (37).

Phylogenetic reconstruction. Sequences determined in this study were used to search for similarities among known sequences using the basic local alignment tool (BLAST) at the National Center for Biotechnology Information (Bethesda, Md.), and SEQUENCE MATCH of the Ribosomal Database Project. Multiple sequences yielding the highest similarity scores were aligned using CLUSTAL W, and sites presenting gaps or unknown bases were excluded from analysis.

Evolutionary distances were calculated according to the two-parameter Kimura model, and phylogenies were reconstructed on the basis of the neighbor-joining method of the Phylogeny Inference Package (PHYLP) (17). Input order

was randomized 5 to 10 times in all phylogenetic reconstructions. Nodal support of the inferred trees was assessed by 1,000 bootstrap replicates. DNAPars in the PHYLIP package was also used to perform ordinary parsimony analyses using exhaustive search, and nodal support was assessed with 100 bootstrap replicates to confirm the phylogenetic positioning of the clades.

Isolation from rhizospheric soil. Maize rhizosphere samples were obtained in mid-July, 2003, from the Michigan State University, W. K. Kellogg Biological Station Long-Term Ecological Research (KBS) site (Hickory Maizeers, Mich.) (<http://lter.kbs.msu.edu>). The maize field was managed under maize monoculture (one annual crop with no cover crop), with conventional tillage, and without application of fertilizers, herbicides, or insecticides. Entire root systems were sampled at 5, 10, 20, 120, 150, and 160 m from the first sample, and they were assigned sample number 2, 3, 4, 5, 6, and 7, respectively (Table 2). For each sample, 1 g of root system with closely adhering soil (rhizospheric soil) was transferred to a sterile tube containing 10 ml of 0.85% NaCl solution, and the tube was shaken at 350 rpm at room temperature for 30 min (Fig. 1A). The bacterial suspension was diluted 10-fold in 0.85% NaCl, and 100 μ l of each dilution was plated in triplicate on *B. cepacia* complex semiselective medium trypan blue tetracycline medium (TB-T) (24), which contains (per liter) 2 g of glucose, 1 g of L-asparagine, 1 g of NaHCO₃, 0.5 g of KH₂PO₄, 0.1 g of MgSO₄ · 7H₂O, 20 g of agar, 5 mg of crystal violet, and 50 mg of trypan blue. The pH was adjusted to 5.5, and 20 mg of tetracycline and 100 mg of cycloheximide were added after autoclaving. TB-T plates were incubated at 29°C for 4 days prior to counting the number of CFU. TB-T was chosen over other *B. cepacia* complex semiselective media, because all known *B. cepacia* complex species can grow on it, it yields more *B. cepacia* complex isolates than other media (41; our unpublished results), and it was formulated for environmental samples (24).

DNA-DNA and colony hybridization. A CLUSTAL W alignment of rRNA sequences of *B. cepacia* complex species and closely related organisms was inspected manually to identify putative *B. cepacia* complex-specific probes. Candidate probes were evaluated in silico using the Probe Match tool of the Ribosomal Database Project (38). A universal probe (for members of the domain *Bacteria*) served as a positive control for DNA fixation on the membranes. It consisted of a 510-bp fragment (produced with primers RHG-F and RHG-R) (33) that hybridized with all bacterial DNA spotted on the membrane. This probe was washed off the nylon membrane prior to hybridization with *B. cepacia* complex-specific probes.

For dot blot hybridization, full-length PCR-amplified 16S rRNA was spotted onto Hybond-N⁺ membranes (Amersham, Piscataway, N.J.), and fixed by UV cross-linking (Stratalinker; Stratagene, La Jolla, Calif.). For CH, colonies were grown at 29°C either overnight on PCAT or for 4 days on TB-T dilution plates.

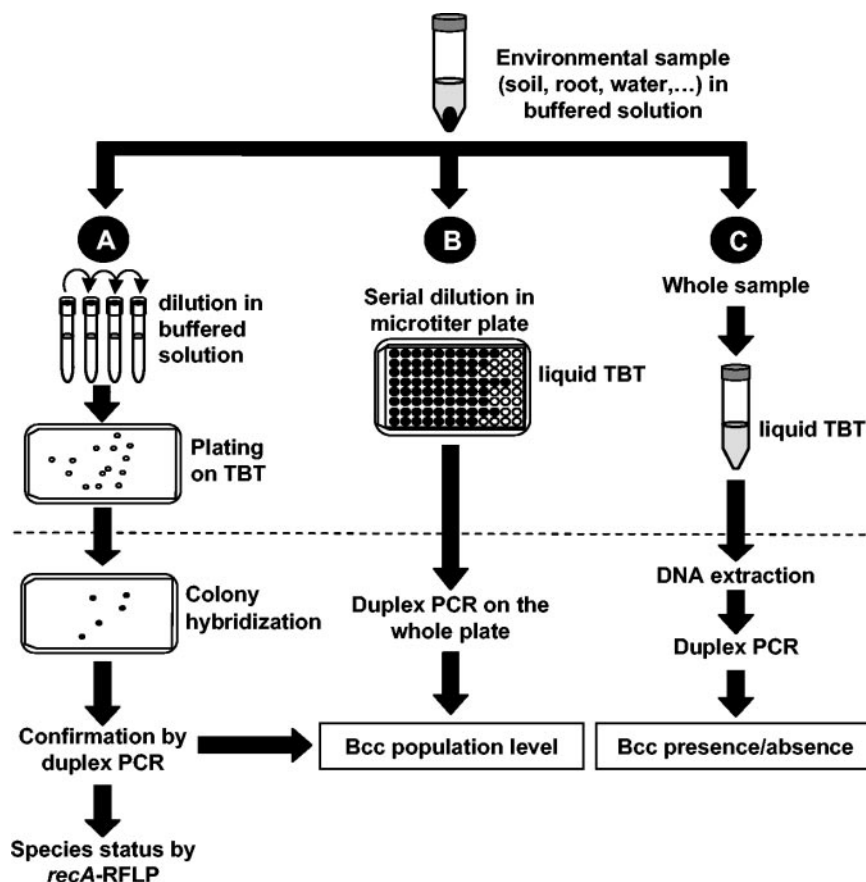


FIG. 1. Determination of the presence of *B. cepacia* complex (Bcc) and *B. cepacia* complex population densities from environmental samples. (A) After the sample is shaken in buffered solution, it is serially diluted and plated on semiselective TB-T medium (TBT). Colonies are transferred to nylon membranes, and their DNA is hybridized with the probe V3x3 to localize *B. cepacia* complex candidates on dilution plates. The candidates are confirmed as *B. cepacia* complex members by performing duplex PCR targeting 16S rRNA gene and *recA* *B. cepacia* complex-specific sequences. (B) In parallel, the sample is diluted serially in liquid TB-T medium, and after 4 days of growth, an aliquot of each well is subjected to duplex PCR to detect whether *B. cepacia* complex cells are present at a given dilution. (C) When population levels are below the detection limit for strategies A and B [around 2 to 3 log (CFU/g)], the presence of *B. cepacia* complex in a sample can be assessed by enriching the sample for 4 days in liquid TB-T medium, extracting DNA from total cells, and testing by duplex *B. cepacia* complex-specific PCR. The dotted line represents the 4-day incubation period at 29°C for all methods.

Prior to applying colonies to membranes, it was generally necessary to incubate the colonies overnight at 4°C to increase colony adhesiveness. For TB-T dilution plates, larger plates (Omnitray; Nalge Nunc International, Rochester, N.Y.) were used instead of round petri dishes to maximize distance between colonies. DNA from colonies adhering to the membranes was denatured and neutralized following the manufacturer's recommendations (Amersham) and fixed by UV cross-linking.

Oligoprobes were synthesized by Integrated DNA Technologies. They were diluted to a concentration of 1.25 μ M and labeled according to the instructions for the Alkphos direct labeling kit (Amersham) for 30 min for fragment probes and for at least 2 h for oligoprobes. Prehybridization, hybridization, and post-hybridization stringency washes were done at 40°C using the manufacturer's recommended buffers and protocols (Amersham). After signal generation in the presence of CDP-Star chemiluminescent substrate (Amersham), light emission was detected by autoradiography using film sensitive to blue light (Hyperfilm ECL; Amersham).

Prior to hybridizing the same membranes with a new probe, probes were removed by dipping membranes into boiling 0.5% (wt/vol) sodium dodecyl sulfate and allowing them to cool down to room temperature with gentle agitation. The absence of signal was checked by autoradiography before further hybridization with other probes.

DE-PCR and MPN-PCR. A dilution endpoint PCR (DE-PCR) was performed by the method of McSpadden Gardener et al. (40) with the following modifications. Ten microliters of the initial rhizospheric wash suspension was diluted

10-fold six times in a 96-well microtiter plate with liquid TB-T as the diluent (Fig. 1B). Four replicate samples were prepared, which enabled the simultaneous analysis of four rhizospheric samples in each 96-well plate. Plates were incubated at 29°C with gentle shaking (150 rpm) for 4 days. Five microliters from each well was transferred to 95 μ l of lysis buffer using a 12-channel pipetman, and cell lysis was performed as described above. Two microliters of the DNA lysate was used in 10- μ l screening duplex PCR with the PCR cocktail composition and cycling conditions described above. Population levels were estimated on the basis of the last dilution in which a PCR product could be observed after electrophoresis on standard agarose gels. Based on the same PCR results, estimates of *B. cepacia* complex population levels were also calculated using the MPN method and its corresponding 95% confidence intervals (20). DE-PCR and MPN-PCR differ only in the way PCR results are used to calculate final population levels.

Enrichment and detection at low population levels. Samples (1 g) were shaken in liquid TB-T medium 4 days at 29°C (Fig. 1C). Total DNA was extracted and purified using the UltraClean soil DNA isolation kit (Mo Bio Laboratories, Inc., Carlsbad, Calif.). Duplex PCR was performed as described above using 4 μ l of undiluted or 10-fold-diluted soil extracts for a total volume of 20 μ l.

Statistical analyses. *B. cepacia* complex population levels were determined at least twice, and representative results of one experiment are presented. Restriction analysis to determine *B. cepacia* complex species was done for one of those experiments. The numbers of CFU expressed per gram of fresh roots were \log_{10} transformed (35) prior to statistical analysis. Analysis of variance, regression analysis followed by Bonferroni's two-tailed significance test, and nonparametric

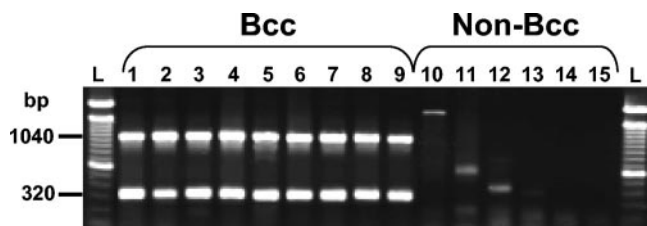


FIG. 2. *B. cepacia* complex-specific duplex PCR. The *B. cepacia* complex-specific PCR yielded fragment sizes of 1,040 and 320 bp, corresponding to 16S rRNA and *recA* amplicons, respectively. Representative strains of the nine *B. cepacia* complex (Bcc) species are shown in lanes 1 to 9. Lanes 1 to 9, *B. cepacia* PC783, *B. multivorans* HI229, *B. cenocepacia* J2315, *B. stabilis* HI2210, *B. vietnamiensis* PC259, *B. dolosa* AU0645, *B. ambifaria* AMMD, *B. anthina* AU1293, and *B. pyrrocinia* BC011, respectively. Lanes 10 to 13, *B. gladioli* strains AU3923, AU5002, AU5085, and HI2870, respectively; lane 14, *B. xenovorans* LB400; lane 15, *R. pickettii* PKO1. L, 100-bp ladder.

tests were done using SYSTAT (version 8.0; SPSS, Inc., Chicago, Ill.), and a *P* value of <0.05 was considered statistically significant.

Nucleotide sequence accession numbers. 16S rRNA and *recA* sequences have been deposited in the GenBank and EMBL databases under accession numbers AY769902 to AY769913 for 16S rRNA sequences and AY769895 to AY769901 for *recA* sequences.

RESULTS AND DISCUSSION

Duplex PCR. In order to determine quickly and reliably whether an isolate belongs to the *B. cepacia* complex, a duplex PCR assay was designed to specifically differentiate *B. cepacia* complex from closely related bacteria. Existing primers and a new forward primer targeting 16S rRNA sequences (Table 1) were combined to enable the simultaneous amplification of *recA* (1,040 bp) and 16S rRNA (320 bp) in only *B. cepacia* complex species (Fig. 2). All reference *B. cepacia* complex strains used in this study produced the double band, whereas closely related genera and species did not. The Burkf primer was designed to provide more specificity to the 16S rRNA gene-based PCR assay than the combination of eubacterial primer Eub-16-1 with the *B. cepacia* complex-specific CeMuVi-16-2 (2, 41). Initially, the two primer pairs were mixed at equal concentrations, but this sometimes produced a weaker signal for the 16S rRNA gene band than for the *recA* band. Comparable band intensities were obtained by using 25% more of the 16S rRNA gene primers than of the *recA* primers. As a result, a reliable and efficient method to identify all *B. cepacia* complex species was obtained, eliminating closely related isolates that could produce weak, unspecific fragments (e.g., Fig. 2, lanes 10 to 12). This was particularly valuable for high-throughput PCR screening and rapid identification of environmental isolates displaying the characteristic double band (data not shown).

Specificity and sensitivity of *B. cepacia* complex-specific probes. We identified the variable V3 region of the 16S rRNA sequence (positions 430 to 457 in *B. multivorans* LMG 13010^T; accession number Y18703) as the best candidate to differentiate *B. cepacia* complex species from closely related species and genera, such as *B. gladioli*, *Ralstonia* spp., *Chryseobacterium meningosepticum*, *Stenotrophomonas maltophilia*, *Comamonas acidovorans*, and *Pseudomonas* spp. These bacteria are often found in mixed cultures with *B. cepacia* complex isolates from either environmental or clinical samples, often

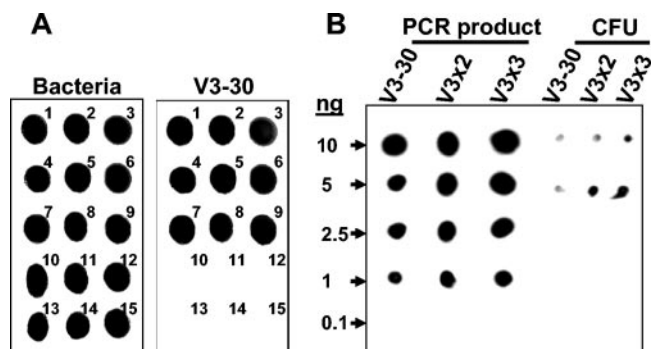


FIG. 3. Specificity and sensitivity of the CH assay. (A) The specificity of the V3-30 probe was tested against all reference strains. 16S rRNA was amplified by PCR and fixed to the membranes as described in Materials and Methods. DNA fixation on the membranes was controlled by using a universal probe (for members of the domain *Bacteria*) that was subsequently washed off the membrane before hybridization with different *B. cepacia* complex-specific probes. Representative strains of each of the nine *B. cepacia* complex species are numbered 1 to 9, with the numbers 1 to 9 corresponding to *B. cepacia* PC783, *B. multivorans* HI229, *B. cenocepacia* J2315, *B. stabilis* HI2210, *B. vietnamiensis* PC259, *B. dolosa* AU0645, *B. ambifaria* AMMD, *B. anthina* AU1293, and *B. pyrrocinia* BC011, respectively. The numbers 10 to 13 indicate *B. gladioli* strains AU3923, AU5002, AU5085, and HI2870, respectively. The numbers 14 and 15 indicate *B. xenovorans* LB400 and *Ralstonia pickettii* PKO1, respectively. (B) Comparison of the sensitivities of probes V3-30 and its corresponding dimer (V3x2) and trimer (V3x3 [Table 1]) for different amounts of PCR-amplified 16S rRNA of *B. cepacia* LMG 1222 strain (left) and for a minimal number of colonies (CFU) deposited on the membrane with a toothpick (right).

have similar growth requirements as *B. cepacia* complex species, and thus, may often be misidentified as *B. cepacia* complex species (9). In the *B. cepacia* complex, sequence polymorphism in the V3 region consisted of two or three mismatches distantly located from each other, whereas more than seven mismatches (i.e., with *B. gladioli*) were found for non-*B. cepacia* complex species. Generally, about 10 mismatches were obtained in the genus *Burkholderia*, with the exception of *Burkholderia andropogonis*, which displayed 15 mismatches. Other closely related genera listed above displayed either 15 to 20 mismatches or complete deletions (e.g., *Chryseobacterium meningosepticum* with gaps at seven sites [data not shown]).

Several probes ranging from 30 to 150 bases long, spanning the V3 region of the 16S rRNA, were tested for specificity using PCR-amplified DNA from *B. cepacia* complex and closely related organisms. Non-*B. cepacia* complex strains were chosen on the basis of the high similarities of their rRNA gene sequences, since they would be more difficult to differentiate from the *B. cepacia* complex, and consisted of *B. gladioli*, *B. xenovorans*, and *Ralstonia* spp.

Only probe V3-30 specifically detected all *B. cepacia* complex species (Fig. 3A), while longer probes lacked specificity (data not shown). However, the hybridization assay using probe V3-30 was not sensitive enough to satisfactorily detect small colonies growing on dilution plates (Fig. 3B), for which a weak signal was often difficult to distinguish from the low level of background hybridization signal.

Longer probes were designed by linking together several copies of the V3-30 probe using one or two phosphoramidite spacers (Table 1), because signal intensity was directly related

to probe length due to probe labeling with alkaline phosphatase, but probes of increased sequence length were found to lack specificity. The sensitivities of dimer (V3x2) and trimer (V3x3) probes (Table 1) were substantially higher than that of V3-30, especially when working with colonies (Fig. 3B), without losing the specificity of the monomer (data not shown). Generally, the limit of detection of the CH assay using V3x3 ranged from 0.1 to 1 ng, and the limit of detection of the assay using V3-30 or V3x2 was >1 ng (Fig. 3B; our unpublished data).

The difference in the sensitivities of the V3-30 and V3x3 probes was further confirmed by a preliminary experiment in which 1,100 isolates from a maize rhizosphere sample were randomly selected from TB-T dilution plates, transferred to PCAT plates, and submitted to CH with either V3-30 or V3x3. Among the 700 isolates that were identified as *B. cepacia* complex strains by using V3-30, only 78% were confirmed by duplex PCR. In comparison, 99% of the 548 colonies identified as *B. cepacia* complex with V3x3 were duplex PCR positive. This can be explained by the weaker hybridization signals that are generated with V3-30 compared with those of V3x3; hence, when using V3-30, more doubtful colonies had to be picked on the plates to be screened by duplex PCR. In conclusion, probe V3x3 was the best probe in terms of both specificity and sensitivity and was used in subsequent studies on *B. cepacia* complex population densities in maize rhizospheres.

Population levels in the maize rhizosphere. Seven individual rhizosphere samples were used to determine the *B. cepacia* complex population densities, species abundance, and diversity in the same field. The number of total bacteria growing on TB-T plates was high, with an average of 6.01 ± 0.83 log (CFU/g of root) (mean \pm standard deviation) and with noticeable differences between maize plants (e.g., plants 4 and 7 [Table 2]). CH revealed that *B. cepacia* complex population levels were generally found within 0.5 to 1 log unit below the total number of bacteria growing on TB-T plates, with an average of 5.64 ± 0.84 log (CFU/g of root), very close to the estimates previously found in Italy on maize with 6.20 ± 0.20 log (CFU/g of root) (15). As for total counts on TB-T, *B. cepacia* complex colonization was found to vary by several orders of magnitude between individual rhizospheres (e.g., plants 4 and 6, with a 140-fold difference). Experimental repeatability that consisted of sampling the same root system at different times ranged from 0.5 to 1.5 log units, depending on the rhizosphere sample considered (data not shown). The percentage of *B. cepacia* complex isolates recovered from TB-T plates ranged from 17 to 81, which led to the isolation of about 40 ± 18 isolates per rhizosphere for a total of 285 isolates (Table 2).

Restriction analysis of *recA* amplicons with two restriction enzymes (HaeIII and MnlI [37]) for all *B. cepacia* complex strains identified by CH was used to determine species abundance and richness. Samples giving patterns that were not identical to those of reference strains were sequenced further to ascertain the correct species (Table 2 and Fig. 4).

Sequencing of the *recA* and 16S rRNA gene amplicons was performed for seven randomly selected isolates belonging to the main *recA* types and allelic variants to confirm that they were *B. cepacia* complex isolates and determine their phylogenetic positions. The 16S rRNA gene-based phylogeny indicated that all selected isolates were indeed *B. cepacia* complex isolates (Fig. 4A). They clustered with *B. ambifaria* (KBC-1, -5,

-6, and -7), *B. cepacia* (formerly genomovar I) (KBC-2 and -3) or close to but separately from *B. anthina* (genomovar VIII) (KBC-4) (Fig. 4A).

The phylogenetic positions of the isolates in the *B. cepacia* complex were further confirmed by the analysis of *recA* sequences (Fig. 4B). Noticeably, while all selected isolates clustered similarly when *recA* or 16S rRNA gene sequences were considered, KBC-4 was found between *B. cenocepacia* III-B and III-C clusters in the *recA*-based phylogeny, with significant nodal support (Fig. 4B) for this topology. The existence of low bootstrap values between *B. cepacia* complex species (e.g., between clusters III, V, VI, and VII [Fig. 4B]) may suggest that nucleotide substitutions at this locus could display some phylogenetic saturation (our unpublished data).

As a whole, one to seven *B. cepacia* complex species were recovered on each plant rhizosphere with *B. ambifaria* (formerly genomovar VII) found in all samples (i.e., 64% of all isolates), followed by *B. cepacia* (genomovar I) with 58 isolates (i.e., 20% of the total number) found on three plants (Table 2). The other species consisted of *B. multivorans* (genomovar II) with three isolates found on one plant, *B. cenocepacia* (genomovar III) with nine isolates found on three plants, *B. stabilis* (genomovar IV) with a unique isolate, *B. dolosa* (genomovar VI) found on two plants for a total of 26 isolates, and *B. pyrocinia* (genomovar IX) found on two plants for a total of three isolates. To our knowledge, this is the first report of *B. stabilis* and *B. dolosa* isolated from the environment. Noteworthy, *B. multivorans* and *B. cenocepacia*, although rarely evidenced in our study, are the most frequent species isolated in patients with cystic fibrosis. Indeed, those two species were found in up to 38 and 50%, respectively, of patients with cystic fibrosis in the United States, accounting for mortality rates of up to 15 and 45%, respectively, in cystic fibrosis patients in Canada (42).

Interestingly, in the dominant species, substantial allelic polymorphism was evidenced for *recA* with two and four alleles for *B. cepacia* (genomovar I) and *B. ambifaria*, respectively. This allelic polymorphism was also observed for populations from the same sample, e.g., two *recA* variants found in genomovar I and three to four *recA* variants of *B. ambifaria* on plants 2 and 3 (Table 2).

If the abundance of each species is expressed in \log_{10} so as to identify significant difference in species abundance in a given sample, it becomes clear that although up to six *B. cepacia* complex species may coexist on a given maize rhizosphere (e.g., plant 3 [Table 2]), each plant is colonized by a dominant species and/or one variant type. For instance, plant 1 is dominated by *B. ambifaria* variant A, while other *recA* variants were three to six times less numerous (Table 2). The only case where abundance was similar between species in a given sample was for plant 6 with seven to nine isolates for both *B. dolosa* and *B. ambifaria*. Note that no direct relationship was found between the location of the sample and the total population density of *B. cepacia* complex strains, species distribution, and abundance ($P > 0.05$ by regression [Table 2]).

In summary, our results indicate that in each maize rhizosphere, a complex community of *B. cepacia* complex species and variants of the same species coexist at high population densities. Since *recA* restriction analysis is rather a coarse method to resolve intraspecific diversity, further studies using

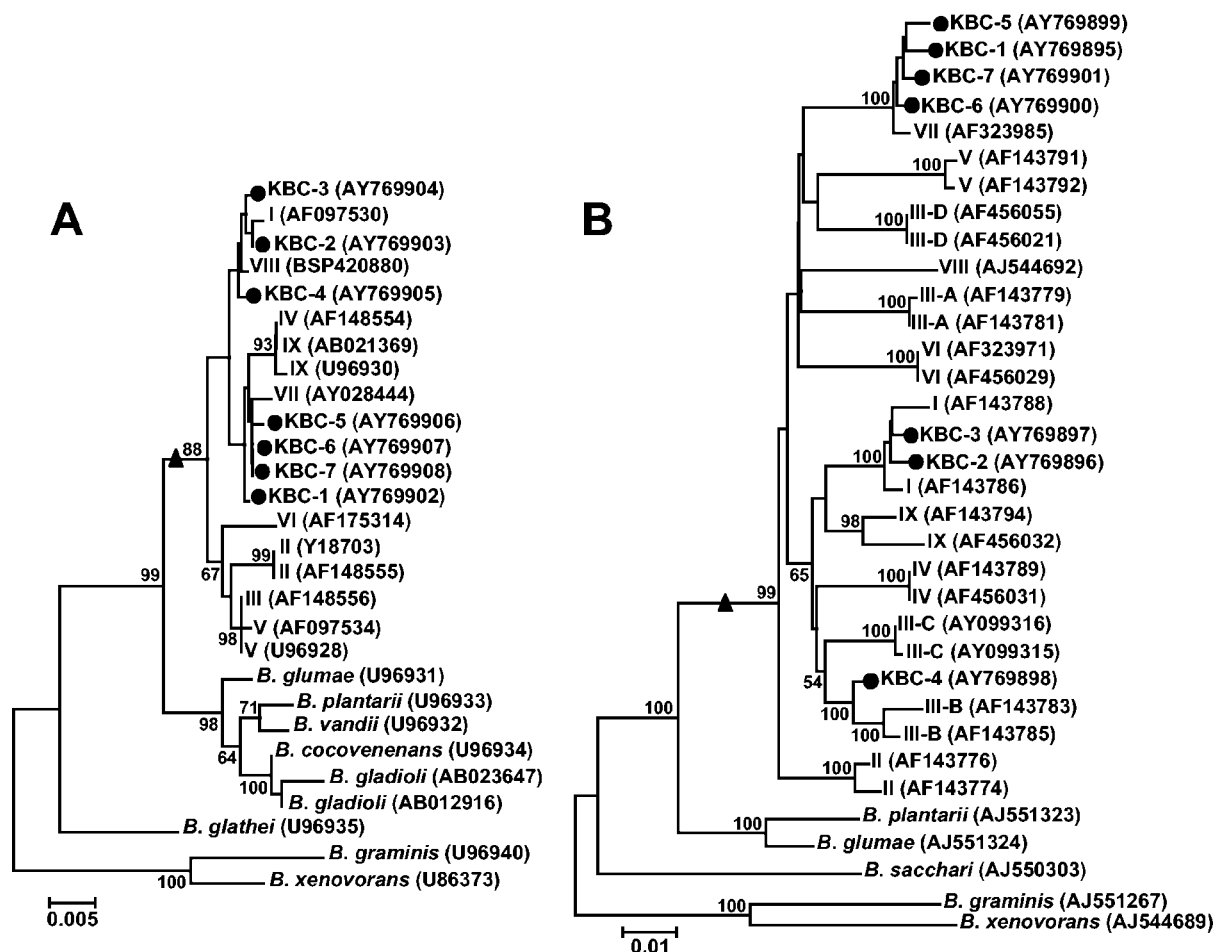


FIG. 4. Phylogenetic positions of representative isolates of the maize field based on their 16S rRNA gene sequences (A) and *recA* sequences (B). Phylogenetic trees were generated by the neighbor-joining method based on the two-parameter Kimura correction of evolutionary distances. For both phylogenies, nodal support was assessed by 1,000 bootstrap replicates. Support values lower than 50% are not indicated. The number of substitutions per site is indicated by the bar at the bottom left of each phylogeny. Sequences determined in this study are indicated by a solid black circle (i.e., KBC-1 to -7). For presentation purposes, roman numerals were used to designate *B. cepacia* complex species and correspond to *B. cepacia* (formerly genomovar I), *B. multivorans* (II), *B. cenocepacia* (III), *B. stabilis* (IV), *B. vietnamiensis* (V), *B. dolosa* (VI), *B. ambifaria* (VII), *B. anthina* (VIII), and *B. pyrrocinia* (IX). Different *recA* lineages in *B. cenocepacia* (genomovar III) (37, 48) are indicated by capital letters A, B, C, and D after a hyphen in the genomovar designation. GenBank accession numbers of the sequences are given in parentheses. For each phylogeny, the *B. cepacia* complex monophyletic clade is indicated by a solid black triangle.

DNA fingerprinting may prove very valuable to reveal the level of genetic differentiation of *B. cepacia* complex species on each individual plant.

Previous studies that examined *B. cepacia* complex population levels in maize rhizospheres reported that *B. cenocepacia* and *B. ambifaria* were predominant, while *B. cepacia* and *B. pyrrocinia* were found less frequently (3, 13, 19). This was partly corroborated here because *B. ambifaria* and *B. cepacia* were the most dominant in our study, whereas *B. cenocepacia* and *B. pyrrocinia* were less frequently isolated (Table 2). Furthermore, species not yet encountered on maize, such as *B. multivorans* and *B. stabilis*, were also found at significant population densities at the KBS site (Table 2). Therefore, without higher capacity methods, such as proposed here, sampling bias may distort our understanding of the diversity and ecology of *B. cepacia* complex strains, which may underestimate species distribution and abundance, and hence could potentially miss the identification of true environmental reservoirs.

rRNA sequencing was also done for five randomly chosen isolates that were not identified as *B. cepacia* complex isolates by any of the methods used in this study but that were often present at high densities on TB-T dilution plates. These organisms belonged to the α , β , and γ subdivisions of the proteobacteria. In the α subdivision, one isolate (S8 [accession number AY769909]) showed 70 to 90% identity of its rRNA sequence with *Rhizobium* or *Agrobacterium*. In the β subdivision, isolates S9 (accession number AY769910) and S12 (AY769913) displayed 98% sequence similarity with *Burkholderia* spp. and *Variovorax* spp., respectively. Two isolates belonged to the γ subdivision (S10 [accession number AY769911] and S11 [AY769912]) and shared more than 97% identity of their 16S rRNA sequences with *Lysobacter* spp. and *Xanthomonas* spp., respectively. The diversity of organisms growing on semiselective media in company with *B. cepacia* complex strains further underlines the necessity of using an efficient and

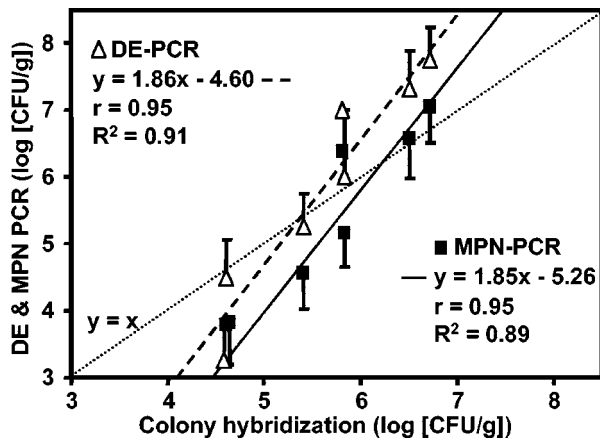


FIG. 5. Comparison of DE-PCR, MPN-PCR, and CH methods to estimate the densities of environmental *B. cepacia* complex populations isolated from seven independent maize rhizospheres in the same field. CH was done using probe V3x3 on TB-T dilution plates. All positive colonies were confirmed as *B. cepacia* complex by duplex PCR and sequencing for representative isolates (Fig. 4 and Table 2). *B. cepacia* complex population levels were estimated by DE-PCR and MPN-PCR as described in Materials and Methods. Error bars indicate standard deviations for DE-PCR and 95% confidence intervals for MPN-PCR (both based on four measurements per sample). Regression equations, Pearson correlation coefficients (r), and the coefficients of determination (R^2) are indicated for each PCR-based method. The theoretical line representing the same population estimate by PCR-based methods and CH is depicted by a dotted line ($y = x$).

specific screening strategy to recover *B. cepacia* complex organisms from environmental samples.

Determination of *B. cepacia* complex population levels by DE- and MPN-PCR. In parallel to the detection by CH, a liquid culture screening assay was developed to determine *B. cepacia* complex population levels (Fig. 1B). CH gave the best estimate, since all dilution plates are screened, and positive isolates are further confirmed as *B. cepacia* complex by duplex PCR, leading to an accurate counting of *B. cepacia* complex at each dilution level. Liquid culture PCR screening assays gave results that showed good agreement with the results of CH (Fig. 5). Both DE-PCR and MPN-PCR indicated a strong linear relationship ($r > 0.90$, $P < 0.001$, $R^2 = 0.90$) with CH estimates of population levels (Fig. 5). DE-PCR and CH gave the same results, ranging from 4.5 to 6.5 log (CFU/g), whereas above 6.5 log (CFU/g), DE-PCR overestimated the abundance of *B. cepacia* complex. For MPN-PCR, good estimates of population levels were obtained over the range 5.5 to 7 log (CFU/g) with an underestimation below 5.5 log (CFU/g). As a consequence, the three methods agreed with each other in the range 5 to 5.5 log (CFU/g) to 6.5 to 7 log (CFU/g). Below 4.5 to 5 log (CFU/g of sample), liquid culture PCR screening methods gave lower estimates of *B. cepacia* complex abundance than those of CH. These results are supported by previous studies reporting that MPN counts generally underestimate the actual number of bacteria in a sample (18). Therefore, a quick estimation of *B. cepacia* complex population abundance can be obtained using liquid culture screening methods in order to decide whether isolation should be performed by randomly picking isolates or by CH in the case of low *B. cepacia* complex population levels.

The actual detection limit of liquid culture PCR screening is 1,000 CFU/g, given that only 10 μ l of the initial 10 ml of sample is inoculated into the wells of the dilution plate (Fig. 1B). For CH, the detection limit is 10-fold lower (i.e., 100 CFU/g), since it is possible to detect one *B. cepacia* complex colony on the semiselective medium plate corresponding to the lowest dilution of the sample suspension. Because not finding *B. cepacia* complex by these methods cannot definitely rule out their presence at population levels below 100 CFU/g (i.e., what is not detected by CH), incubation of the sample in liquid TB-T medium for 4 days prior to plating on TB-T plates (Fig. 1C) can be used to enrich as few as 1 to 10 cells in the original sample to levels detectable by duplex PCR after DNA extraction of the whole sample (our unpublished results). If the sample gave positive results by PCR, screening of dilution plates by CH may then be successfully used to isolate *B. cepacia* complex. However, due to the enrichment step, a reliable estimate of the actual population level in the sample is no longer possible. This enrichment step can prove valuable in the case of bulk soil in which *B. cepacia* complex abundance may range from 10^2 to 10^4 CFU/g of soil (24) or even lower (our unpublished results).

In conclusion, a combination of the methods presented here offers efficiency and flexibility, depending on the specific aims of each study. For instance, enrichment and duplex PCR may be used to screen numerous samples in parallel for just the presence or absence of the *B. cepacia* complex, liquid culture PCR screening assay may estimate *B. cepacia* complex abundance, and CH provides the best means of isolating the target organisms in pure cultures. Another variation would be to perform CH on the bacterial suspension from each well of liquid culture plates (Fig. 1B). Indeed, by directly spotting liquid cultures from several plates on hybridization membranes, determining *B. cepacia* complex population densities in tens of samples in parallel will no longer be an obstacle. This will undoubtedly yield more data about the ecology and diversity of the *B. cepacia* complex in various habitats and environments.

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