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Identification of Aquatic *Burkholderia* (*Pseudomonas*) *cepacia* by Hybridization with Species-Specific rRNA Gene Probes

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***Burkholderia* (*Pseudomonas*) *cepacia* is a common environmental bacterium which can be pathogenic for plants and humans. In this study, four strategies were used to identify aquatic isolates: API test strips, hybridization with species-specific DNA probes for the 16S and 23S rRNA genes, fatty acid methyl ester (FAME) profiles, and growth on selective medium (TB-T agar [C. Hagedorn, W. D. Gould, T. R. Bardinelli, and D. R. Gustarson, Appl. Environ. Microbiol. 53:2265–2268, 1987]). Only 59% of the isolates identified as *B. cepacia* with the API test strips were confirmed as *B. cepacia* by using fatty acid profiles. The 23S rRNA probe generated a few false-positive results but dramatically underestimated the number of *B. cepacia* isolates (i.e., 40% of the colonies that did not hybridize to the probe were *B. cepacia*, as determined by FAME). The 16S rRNA probe generated more false-positive results than the 23S rRNA probe but was effective in identifying the majority of the *B. cepacia* isolates. The selective medium was only partially successful in recovering *B. cepacia*. Use of the *B. cepacia*-specific 16S rRNA probe was the most efficient and accurate way of identifying *B. cepacia*.**

One of the most fundamental aspects of ecology is the identification and enumeration of species in nature. Microbial ecologists have not been able to determine the species composition of natural bacterial assemblages because most bacteria in nature cannot be cultured, and it is not known if these viable, nonculturable bacteria are of the same species as those that can be cultivated (4). In addition, many species of bacteria which can be cultivated frequently cannot be easily identified by conventional approaches (7). Much of this limitation is attributable to low usage of environmental bacteria in the development of identification schemes. Presently, there is intense interest in the development of methods for rapidly and efficiently identifying and quantifying the bacterial species present in environmental samples by molecular techniques.

The purpose of this study was to compare methods of identification of aquatic *Burkholderia* (*Pseudomonas*) *cepacia* isolates and to provide suggestions for future identification schemes. Four methods for identification of *B. cepacia*, one of the most commonly cultivated bacteria from the study site (Upper Three Runs Creek, Aiken, S.C. [9]), were compared. In addition to its abundance in soils and freshwater environments (3, 8, 9), *B. cepacia* is a plant pathogen (5), plant symbiont (6), and pathogen of cystic fibrosis patients (1). First, the physiological responses of unidentified environmental strains were determined via testing with API rapid test strips for nonfermenting, gram-negative bacteria (BioMerieux Vitek Inc., Hazelwood, Mo.). Second, 16S (2) and 23S (11) rRNA species-specific probes were hybridized with DNA from the isolates. Third, fatty acid methyl ester (FAME) profiles were determined and compared with those of known species by Microbial ID, Inc. (Newark, Del.). Fourth, growth on a *B. cepacia*-selective medium, TB-T agar (3), was determined.

Sediment samples were collected over the course of a year (1991 to 1992) from two sites (Tinker Creek and Box Landing) on Upper Three Runs Creek on the U.S. Department of Energy's Savannah River Site near Aiken, S.C. In the laboratory, *Pseudomonas* isolation agar plates (Difco), containing cycloheximide (20 mg ml^{-1}) to inhibit fungal growth, were inoculated with sediment samples suspended in streamwater. After incubation for 6 days, approximately 700 bacterial colonies were streaked for isolation, and pure cultures were used to inoculate API strips. The 106 isolates identified as *B. cepacia* by the API strips were frozen at −70°C for further study. These isolates were also sent to Microbial ID, Inc., for fatty acid analysis, where gas-liquid chromotography was performed on fatty acid methyl esters from the bacterial cultures (13).

The growth of isolates on a medium selective for *B. cepacia* was determined by inoculation of TB-T agar (3) with the putative *B. cepacia* isolates. This medium contains trypan blue and tetracycline (20 mg/liter), which inhibit the growth of many species of bacteria, glucose as the carbon source, and L-asparagine as the nitrogen source.

The 106 putative *B. cepacia* isolates were subjected to colony blotting with species-specific probes directed against 16S and 23S rDNA targets (5′CCTCTGTTCCGACCA3′ and 5′CCCA TCGCATCTAACAAT3′, respectively) (2, 11). The positive control used in blots was the *B. cepacia* type strain (ATCC 24561), and the negative controls were *Pseudomonas putida* (12633 and 1064) and *B. solanacearum* (AW). The 23S rRNA oligonucleotide probe was labeled with digoxigenin (Boehringer Mannheim, Indianapolis, Ind.). Colonies attached to Hybond N^+ nylon (Amersham) were lysed with 0.5 M NaOH, neutralized in 1 M Tris-HCl (pH 7.5), and immersed in 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate [pH 7.2]) and then in 95% ethanol (12). Hybridizations were performed with 5× SSC–0.5% blocking reagent–0.1% sodium *N*-lauroyl sarcosine–0.02% sodium dodecyl sulfate (SDS) at 47°C for 12 to 18 h. The filters were washed three times with 0.1× SSC–0.1% SDS for 20 min each at 47°C. Hybridization results were visualized by using Lumi-Phos (Boehringer Mannheim).

Hybridization with the 16S probe was performed as described by Braun-Howland et al. (2). Briefly, blots were prehybridized for 1 h in 6× SSPE (1× SSPE is 180 mM NaCl, 10 mM $NaPO_4$, 1 mM disodium EDTA)–0.1% SDS–1× Den-

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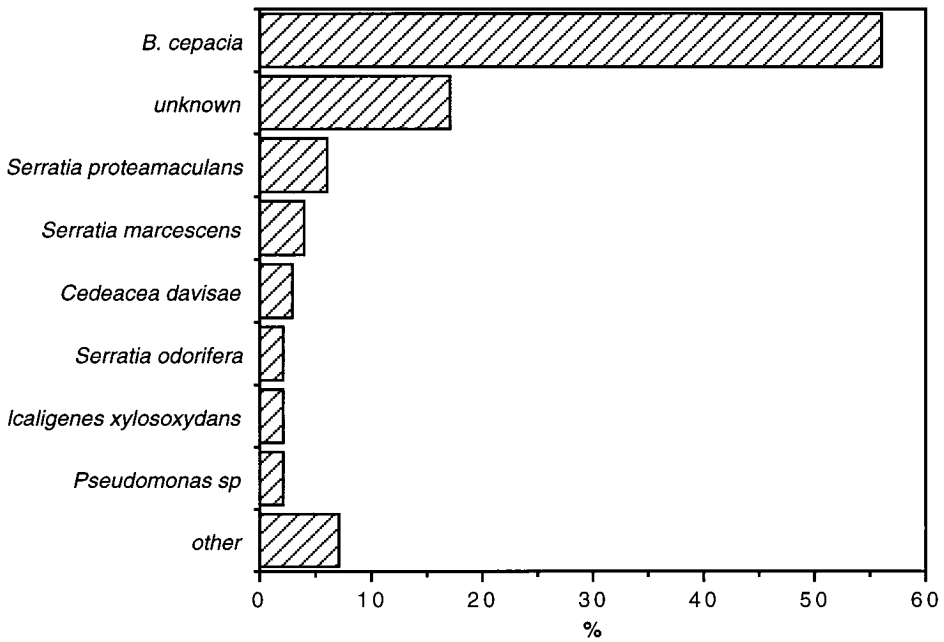

FIG. 1. Results of FAME profile analysis of 106 putative *B. cepacia* isolates. FAME identifications were considered valid only if there was strong separation from other species and the profile matched that of control strains in the Microbial ID, Inc., database. Values are percentages of the 106 putative *B. cepacia* isolates.

hardt's solution (0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin), and hybridization was carried out overnight with a ^{32}P-labeled probe. Blots were washed in three changes of 6× SSPE–0.1% SDS for 1 h.

The 106 isolates examined in this study were selected from a large collection of stream bacterial isolates because they all gave responses on the API test strips that were identical to those of the *B. cepacia* type strain (ATCC 24561). However, only 59% of the isolates identified as *B. cepacia* by the API test results were confirmed to be *B. cepacia* by using FAME profiles (Fig. 1). More than 15% of the isolates could not be identified to species level from FAME analysis, and the remaining isolates were identified as various other species.

The majority of the 106 isolates grew on TB-T agar, and many strains which did not have *B. cepacia* FAME profiles were able to grow on this selective medium (Table 1). Also, 22% of the isolates identified as *B. cepacia* by FAME profiles failed to grow on TB-T agar.

The use of species-specific rRNA probes was quicker and less expensive than identification based on FAME profiles. Of the isolates with FAME profiles indicative of *B. cepacia*, 40% failed to hybridize with the 23S rRNA *B. cepacia*-specific probe. Of the isolates which had FAME profiles that did not resemble that of *B. cepacia*, 7% hybridized with the 23S rRNA *B. cepacia*-specific probe. The 23S probe generated a few false-positive results but dramatically underestimated the number of *B. cepacia* isolates. In contrast, only 3% of FAME-identified *B. cepacia* isolates failed to hybridize with the 16S probe, while 14% of non-*B. cepacia* isolates (from the FAME identification) hybridized with the probe. Although there were more false-positive results with the 16S probe than with the 23S probe, the 16S probe allowed identification of the vast majority of the *B. cepacia* isolates.

There were differences between sites in the relative success of the various identification approaches (Table 1). At the downstream site, Box Landing, 69% of the isolates identified as *B. cepacia* by the API test strips were confirmed to be *B. cepacia* by FAME profiles, while at the upstream site, Tinker Creek, 44% of the putative *B. cepacia* isolates were found to be *B. cepacia*. More *B. cepacia* isolates from Tinker Creek than from Box Landing were able to grow on TB-T medium. The majority of *B. cepacia* isolates from both sites hybridized with the 16S probe, while 50 and 62% of isolates from Tinker Creek and Box Landing, respectively, hybridized with the 23S probe.

The majority of the *B. cepacia* isolates were in *B. cepacia* subgroup B, based on the FAME profiles (Table 2) (10). The success of the 16S and 23S probes in identifying *B. cepacia* varied with subgroup. Most of the subgroup A isolates hybridized to both probes (88% for both the 16S and 23S probes). Subgroup B isolates hybridized with the 16S probe 98% of the time, but hybridization results with the 23S probe were frequently negative (45% hybridization).

TABLE 1. Responses of *B. cepacia* identified by FAME profiles

Site	No. of isolates identified by FAME analysis	% of isolates showing:		
		Growth on TB-T medium	Hybridization	
			23S probe	16S probe
Tinker Creek	20	90	50	95
Box Landing	42	79	62	98

TABLE 2. Distribution of *B. cepacia* isolates identified by FAME profiles between *B. cepacia* subgroups A and B

Site	Subgroup	No. of isolates (FAME analysis)	No. of isolates hybridizing with:	
			23S probe	16S probe
Tinker Creek	A	3	3	3
	B	17	7	16
Box Landing	A	5	4	4
	B	37	23	37

B. cepacia is a common and widely distributed species with tremendous intraspecies diversity (8, 9). The diversity of environmental isolates in terms of both variations among strains and the number of different species which may be represented has made identification of environmental isolates difficult. API test strips alone were not adequate for identification of *B. cepacia*, as the identifications were inaccurate 41% of the time. The selective medium was helpful in screening *B. cepacia* isolates, but more than 20% of the *B. cepacia* isolates did not grow on this medium. Because of the unavailability of selective media for the many diverse species present in most environments, this approach will not be helpful in most studies.

FAME profiles allowed identification of 85% of the stream isolates. The utility of this approach may be limited when hundreds of isolates need to be identified because of the expense of FAME profile generation.

Taxon-specific gene probes allowed quick and relatively inexpensive identification of the *B. cepacia* environmental isolates. Although both the 16S and 23S probes used in this study were developed for identification of environmental bacteria (2, 11), the 16S probe, which was tested with riverine bacteria in the original publication (2), was more effective in identifying the stream bacteria collected during this study.

In general, the effectiveness of taxon-specific gene probes as a tool for identifying the great multitude of environmental bacteria is limited by the availability of suitable probes. Newly developed probes need to be tested against type strains and dozens of natural isolates to determine the strain variation present in the species. Although the probe may hybridize with the type strain of a species, other members of the species may show variation in the nucleotide sequence in the target region of the probe.

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