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A Selective Medium for Enumeration and Recovery of *Pseudomonas cepacia* Biotypes from Soil

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TB-T medium provides a high degree of selectivity for and detection of *Pseudomonas cepacia* biotypes upon initial plating from soil. TB-T medium consists of a basal medium with glucose as the sole carbon source and asparagine as the sole nitrogen source. The selectivity of TB-T medium is based on the combination of trypan blue (TB) and tetracycline (T) (pH 5.5). On TB-T medium, 216 of 300 isolates (72%) from five different soil types were identified as *P. cepacia*. The remaining 28% were facultative organisms that could be separated readily from *P. cepacia* by anaerobic glucose fermentation and by their inability to grow at 41°C. Molds were controlled on low soil dilutions by adding crystal violet, nystatin, or both. Elimination of either ingredient or elevation of the pH to 7.5 resulted in a pronounced loss of selectivity. The efficiency of recovery varied considerably among *P. cepacia* strains but was high enough for some strains (76 to 86%) to permit quantitative studies. TB-T medium combines a defined formulation with high selectivity and allows recovery of *P. cepacia* biotypes from low soil dilutions (10^1 to 10^3).

Pseudomonas cepacia Burkholder 1950 is of current interest because a number of biotypes have been implicated in human disease (2, 13; L. L. Mackenzie and P. H. Gilligan, Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, C34, p. 317). However, this species has demonstrated wide natural diversity; for example, it can apparently incite disease in onions (8), cohabitate in alder nodules (10), repress soilborne plant pathogens (5, 12), and survive within pharmaceutical chemicals and clinical specimens (14; M. B. Ficke and B. T. Decicco, Abstr. Annu. Meet. Am. Soc. Microbiol. 1983, Q50, p. 268). This organism demonstrates considerable physiological versatility, including pectinase activity (6), tropolone synthesis (11), biodegradation of pesticides (4, 8), and broad resistance to antibiotics (16).

The objective of this study was to develop a more selective medium to facilitate isolation of *P. cepacia* from soil, where it is a common inhabitant (1, 6). To date, selective formulations for *P. cepacia* isolation are used mostly for clinical or water quality determinations and are nondefined (contain either blood or plate count agar-peptone); selectivity is gained by the incorporation of combinations of antibiotics (17; M. M. Muszynski, D. A. Pickett, and D. F. Welch, Program Abstr. 24th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 179, 1982; P. H. Gilligan and L. M. Bradshaw, 24th ICAAC, abstr. no. 178, 1984). These media are generally ineffective for isolations from soil, where, given a rich substrate, there is always a variety of microbes present that can grow in the presence of most antibiotics. Also, these clinical media contain no ingredients for controlling fungi. Burbage and Sasser (3) developed a *P. cepacia* isolation medium that includes azelaic acid and tryptamine as the sole sources of carbon and nitrogen, respectively, plus chlorothalonil (Bravo) (Diamond Shamrock Corp., Cleveland, Ohio) as an antifungal agent. Though this medium is useful, it permits only the growth of organisms capable of utilizing these specific compounds, which could limit the diversity of *P. cepacia* biotypes that are recovered. A secondary goal of this study was to compare the medium of Burbage and Sasser (3) with a new formulation.

Total gram-negative bacteria were enumerated on a 10% tryptic soy agar (TSA) medium containing tryptic soy agar (4 g/liter; Difco Laboratories, Detroit, Mich.), agar (15 g/liter), and crystal violet (0.005 g/liter) to reduce the growth of gram-positive bacteria (7). The isolation medium (TB-T) for *P. cepacia* contained the following components (per liter): 20 g of agar, 2 g of glucose, 1 g of L-asparagine, 1 g of $NaHCO_3$, 500 mg of KH_2PO_4, 100 mg of $MgSO_4 \cdot 7H_2O$, 50 mg of trypan blue (TB), and 20 mg of tetracycline (T). The pH was adjusted to 5.5 with 10% phosphoric acid (4 ml/liter), and the filter-sterilized tetracycline was added to the autoclaved medium. For lower soil dilutions in which fungi were numerous, crystal violet (5 mg/liter) and filter-sterilized nystatin (50 mg/liter) were added. The dyes were obtained from Fisher Scientific Co. (Pittsburgh, Pa.), the antibiotics were obtained from Sigma Chemical Co. (St. Louis, Mo.), and all other chemicals used were of reagent grade.

TB-T medium was developed by testing several thousand combinations of antibiotics, dyes, and various carbon and nitrogen sources. A multipoint inoculator (Cathra Replicator System, St. Paul, Minn.) was used to transfer 30 cultures simultaneously to each plate, and the cultures consisted of a wide range of common soil bacteria, as well as *P. cepacia* isolates. This procedure identified trypan blue and tetracycline as agents that were selective for only the *P. cepacia* cultures. The same procedures were used to determine the optimal pH of the formulation and the choice of antifungal agents.

Soil samples from five locations were used for the isolation and enumeration of *P. cepacia* biotypes. The soil types were as follows: a Marietta sandy loam (pH 6.6, from Mississippi), a Herkimer silt loam (pH 6.9, from New York), a Dothan sandy loam (pH 4.7, from South Carolina), a Bowie fine sandy loam (pH 5.8, from Texas), and a Naff silt loam (pH 5.2, from Washington). Soil samples (100 g each) were serially diluted in 0.1% peptone and plated on TB-T medium

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TABLE 1. Physiological characteristics of *P. cepacia* isolates recovered with five formulations of selection medium[a]

Supplement to basal medium[b] (pH)	% of isolates testing positive for:									
	Fluorescence	Yellow pigment	Arginine dihydrolase	Oxidase	Anaerobic utilization of glucose	Growth at 41°C	Utilization of:			Identification as *P. cepacia*
							L-Arginine	P-B-H[c]	Citrate	
None (5.5)	35	40	63	59	35	25	65	34	87	11
T (5.5)	13	10	27	56	19	14	47	19	97	25
TB (5.5)	36	30	58	42	27	23	67	43	93	39
TB + T (5.5)[d]	0	38	0	82	18	67	72	85	100	74
TB + T (7.5)	38	38	48	50	35	33	51	41	3	35

[a] Ninety isolates per medium per soil type were examined. Isolates were taken from soil samples from Washington and New York.
[b] Basal medium is the defined medium with no selective agents. T, Tetracycline; TB, trypan blue.
[c] P-B-H, Poly-β-hydroxybutyrate.
[d] Formulation is the complete medium used to isolate *P. cepacia*.

and 10% TSA. The soil samples from Washington and New York were also plated on various formulations of TB-T medium lacking specific ingredients, as well as on the medium described by Burbage and Sasser (3). Colonies were enumerated after plates were incubated for 4 days at 23°C. A total of 30 colonies were picked from each of three representative plates of the different media (90 isolates per medium per soil type) and were streaked on 10% TSA (without crystal violet).

Each of the isolates from the selective media was characterized by the Oxi-ferm series of tests (Roche Diagnostics, Div. Hoffmann-La Roche Inc., Nutley, N.J.) and was tested for the Gram stain reaction, oxidase reaction (Marion Scientific, Div. Marion Laboratories, Inc., Kansas City, Mo.), growth at 41°C, and utilization of poly-β-hydroxybutyrate and L-arginine. The Enterotube II series of tests (Roche Diagnostics) was also used to differentiate between *P. cepacia* and other organisms that grew on the TB-T medium. Key tests for the confirmation of *P. cepacia* were a positive oxidase reaction, utilization of L-arginine and citrate, accumulation of poly-β-hydroxybutyrate, a negative arginine dihydrolase reaction, no fluorescence, growth at 41°C, and utilization of *m*-hydroxybenzoate (3, 9, 15, 17).

The plating efficiencies of six culture collection strains on TB-T medium were compared with those on 10% TSA. A 36-h culture of each strain in nutrient broth was serially diluted in 0.1% peptone and plated on each of the media. The counts of bacteria recovered on 10% TSA were arbitrarily set at 100% recovery. Each of the cultures was also examined with appropriate physiological tests to ensure its identity as *P. cepacia*.

During initial experimentation, it was found that the use of L-asparagine as the sole nitrogen source restricted the growth of many common soil organisms. Altering the concentration of trypan blue resulted in a level (50 mg/liter) that prevented the growth of most gram-positive bacteria (data not shown). The inclusion of tetracycline at 20 mg/liter reduced the growth of many gram-negative bacteria, and the combination of trypan blue and tetracycline further reduced the growth of gram-negative bacteria (Table 1). Reducing the pH of the medium to 5.5 also allowed the growth of a higher proportion of *P. cepacia* biotypes. Soil fungi were capable of growth on all formulations, although they were somewhat controlled by the trypan blue. Incorporation of nystatin at 50 mg/liter controlled fungi, whereas the addition of 5 mg of crystal violet per liter (with nystatin) repressed fungal growth even on the plates with the lowest dilutions. All of the formulations of medium, except TB-T medium, permitted the growth of substantial populations of fluorescent pseudomonads (0% on TB-T), whereas *P. cepacia* isolates averaged 74% growth on TB-T medium and only 11 to 39% growth on the other formulations. On TB-T medium, the *P. cepacia* colonies appeared to be white, yellow, or blue pigmented, and the proportion of pigmented colonies differed among soils.

Approximately 65% of the isolates from two soil samples recovered by using the medium of Burbage and Sasser (3) were bacteria other than *P. cepacia*, and 50% were fluorescent (Table 2). With their medium, only 34% of the isolates were confirmed as *P. cepacia*, compared with 74% confirmed as *P. cepacia* with TB-T medium. Although the Burbage and Sasser formulation was selective to the point that a significant proportion of isolates were *P. cepacia*, the majority of isolates were still non-*P. cepacia* types, and additional biochemical tests were necessary to separate them. Molds did not represent a serious problem; some fluorescent pseudomonads formed very large, gummy colonies that spread over the plate and made isolations difficult.

For each soil, bacterial counts with the selective medium (TB-T) were from 1 to 3 orders of magnitude lower than the total counts of gram-negative colonies, and the selectivity for *P. cepacia* was proportionately higher than that with TSA (Table 3). It was necessary to examine many colonies to find a smaller number of *P. cepacia* isolates on the less selective 10% TSA-crystal violet media (10% average *P. cepacia* colonies), whereas the TB-T formulation yielded an

TABLE 2. Physiological characteristics of *P. cepacia* isolates recovered from two soil types by using two selective media[a]

Test or parameter	Percent of isolates testing positive with:	
	Medium of Burbage and Sasser	TB-T[b]
Oxidase	64	88
Arginine dihydrolase	63	0
Fluorescence	50	0
Yellow pigment[c]	55	39
Anaerobic glucose utilization	0	2
Growth at 41°C	20	79
Percent bacteria accumulating P-B-H[d]	34	84
L-Arginine	57	75
Citrate	100	85

[a] Soil samples were from Washington and New York; 90 isolates were recovered by using each medium per soil type. With the medium of Burbage and Sasser (3), 34% of the isolates were confirmed as *P. cepacia*, whereas 74% of the isolates were confirmed as *P. cepacia* with TB-T medium.
[b] TB-T medium was supplemented with crystal violet (5 mg/liter) and nystatin (50 mg/liter).
[c] Yellow pigment was nondifferentiated.
[d] P-B-H, Poly-β-hydroxybutyrate.

TABLE 3. Physiological characteristics of bacteria isolated from five soil types with two media

Soil type (location)	Medium[a]	CFU/g of soil	% of isolates testing positive for:									
			Fluorescence	Yellow pigment	Arginine dihydrolase	Oxidase	Anaerobic utilization of glucose	Growth at 41°C	Utilization of:			Identification as *P. cepacia*
									L-Arginine	P-B-H[b]	Citrate	
Marietta sandy loam (Miss.)	10% TSA + CV	6.4×10^6	23	26	37	50	57	20	60	40	73	3
	TB-T	7.4×10^3	0	47	0	60	40	87	93	93	100	56
Dothan sandy loam (S.C.)	10% TSA + CV	3.5×10^5	37	13	23	43	43	0	80	29	76	8
	TB-T	9.3×10^2	0	56	0	77	13	72	82	67	100	72
Herkimer silt loam (N.Y.)	10% TSA + CV	3.3×10^6	62	20	53	37	23	22	61	40	83	15
	TB-T	9.2×10^3	0	55	0	88	6	56	78	83	100	77
Bowie fine sandy loam (Tex.)	10% TSA + CV	7.4×10^5	33	17	60	51	13	23	74	36	60	12
	TB-T	2.8×10^4	0	57	0	93	3	79	83	81	70	84
Naff silt loam (Wash.)	10% TSA + CV	6.1×10^6	67	24	21	41	15	27	70	36	88	9
	TB-T	2.2×10^4	0	21	0	79	1	81	67	87	100	73

[a] TB-T medium was supplemented with crystal violet (5 mg/liter) and nystatin (50 mg/liter). CV, Crystal violet.
[b] P-B-H, Poly-β-hydroxybutyrate.

average of 72% *P. cepacia* isolates identified (56 to 84%) from five soil samples. The non-*P. cepacia* colonies that appeared on the medium were predominantly white or yellow pigmented, were all facultatively anaerobic, and were keyed to the genus *Serratia*. Even though the TB-T formulation is not 100% selective, these contaminants can quickly be distinguished by performing an anaerobic glucose test, which is included in most of the commercial rapid identification tests (e.g., Enterotube II). Wu and Thompson (17) also reported finding *Serratia* spp. as a common contaminant on their selective formulation, but they identified their *Serratia* isolates as *S. marcescens*. The oxidase test is variable for *Serratia* spp. and, although there are other ways to distinguish between *Serratia* spp. and *P. cepacia*, the facultative anaerobe test is probably the easiest to perform.

The efficiency of recovery of culture collection *P. cepacia* strains varied from 1 to 86% (Table 4). The two cultures that were recovered at the highest levels (PC 23 and PC 742) were both relatively new isolates that had been stored at 4°C for only 2 weeks prior to use. These isolates had not been isolated on TB-T medium. All of the *P. cepacia* cultures grew rapidly in 10% TSA and appeared to be typical in all other characteristics. The recovery efficiencies for isolates PC 23 and PC 742 appeared to be high enough for quantitative studies and, by selecting for spontaneous antibiotic resistance in these strains, the medium (plus the antibiotic) could be made more specific for an individual strain, which would be necessary for soil studies in which it is desirable to prevent indigenous *P. cepacia* strains from growing.

TABLE 4. Plating efficiency of culture collection *P. cepacia* strains on TB-T medium[a]

Strain[b]	Count of bacteria recovered from:		% *P. cepacia* isolates recovered
	10% TSA	TB-T	
PC 23	1.9×10^9	1.6×10^9	86.0
PC 742	1.6×10^9	1.2×10^9	76.0
PC 64-22	4.9×10^8	1.2×10^8	24.0
CCPPBP47	3.1×10^9	4.0×10^7	1.0
ATCC 10856	2.0×10^9	3.7×10^8	18.0
ATCC 17759	1.4×10^9	2.5×10^8	18.0

[a] Plating efficiency is the percent *P. cepacia* isolates recovered from TB-T medium compared with that recovered from 10% TSA. The counts of bacteria recovered from TSA were arbitrarily set at 100%.
[b] The ATCC strains were from the American Type Culture Collection, Rockville, Md. PC 742 was from C. R. Howell, National Cotton Pathology Laboratory, College Station, Tex., and PC 23, PC 64-22, and CCPPBP47 were from J. Gardner, University of Florida Agricultural Experiment Station, Lake Alfred, Fla.

There were difficulties in classifying some of the soil isolates on TB-T medium as belonging to *P. cepacia*. The Oxi-ferm scheme identifies *P. cepacia* isolates as being oxidase negative, whereas *Bergey's Manual* (15) describes them as being oxidase positive. Many of the TB-T medium isolates did not fit the *P. cepacia* description in the Oxi-ferm scheme but did follow the *Bergey's Manual* description (15). Some isolates were weakly oxidase positive, and it is highly probable that some of the *P. cepacia* isolates are actually closely related *Pseudomonas* species. However, this same situation would be encountered with all of the currently described selective formulations of media.

The TB-T medium exhibits high selectivity for *P. cepacia*, and no fluorescent biotypes will grow on it. The medium has a simple, defined formulation and could be refined or perhaps made more selective through the use of different carbon and nitrogen sources. It is likely that TB-T medium selects only for certain biotypes of *P. cepacia* and that altering the carbon or nitrogen sources or both in TB-T medium will change the biotypes that are recovered. Even though approximately 25 to 30% of the isolates were not *P. cepacia*, these could be distinguished by one biochemical test (anaerobic utilization of glucose). Thus, TB-T medium provides for recovery of *P. cepacia* at low soil dilutions (10^1 to 10^3), with a minimum of contamination, noncluttered plates, and discrete nonspreading colonies even at the lowest dilutions.

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