APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Oct. 1984, p. 743–746
0099-2240/84/100743-04$02.00/0

Vol. 48, No. 4

Selective Medium for *Pseudomonas cepacia* Containing 9-Chloro-9-(4-Diethylaminophenyl)-10-Phenylacridan and Polymyxin B Sulfate

BO JACK WU* AND STEPHEN T. THOMPSON†

Norwich Eaton Pharmaceuticals, Inc., Norwich, New York 13815

Received 13 January 1984/Accepted 19 July 1984

Contamination of solutions and lotions with *Pseudomonas cepacia* is a growing concern among health professionals. The identification of *P. cepacia* usually requires a long series of biochemical tests. In an effort to develop a more direct method, we evaluated plate count agar containing 9-chloro-9-(4-diethylaminophenyl)-10-phenylacridan and polymyxin B sulfate at respective concentrations of 1 and 75 μg/ml as a medium for selectively isolating *P. cepacia*. The medium inhibited the growth of all gram-negative bacilli and gram-positive cocci tested except *P. cepacia* and *Serratia marcescens*. These two microorganisms could easily be differentiated by their colony morphology and their reactions in the oxidase test. When nonsterilized water samples were inoculated with *P. cepacia* and spread or streaked on the selective medium, all *P. cepacia* organisms were recovered. These results demonstrate the usefulness of 9-chloro-9-(4-diethylaminophenyl)-10-phenylacridan and polymyxin B sulfate in the detection of *P. cepacia*. We believe that this selective medium could be useful in isolating *P. cepacia* from mixed bacterial flora that might be present in environmental water and water-related samples, such as solutions and lotions.

Pseudomonas cepacia is a glucose-nonfermenting, gram-negative bacillus with a wide geographic distribution which has been shown to be present in natural and tap water, in soil and hospital environments, and on medical devices (1, 5, 6, 8–11). This organism has also been isolated from disinfectants (2), saline solution, distilled water, and products containing antimicrobial compounds (4). According to Food and Drug Administration scientists, *P. cepacia* has been implicated in the contamination of povidone-iodine solutions used to disinfect skin before blood collection (10). As a result, this organism is a growing concern among health officials and in hospitals, as well as in cosmetic and pharmaceutical industries. The identification of *P. cepacia* is tedious, usually requiring a long series of biochemical tests. The objective of this study was to devise a medium which can selectively isolate *P. cepacia*, even when present in small numbers, from materials containing a wide variety of microorganisms.

MATERIALS AND METHODS

Organisms. *P. cepacia* strains B4943A, D7072, E8980, F1046, and F2111 were obtained from Robert Weaver, Centers for Disease Control, Atlanta, Ga. Strains B89b, B464-78, and B493-78 were from Mehdi Shayegani, New York State Department of Health, Albany, N.Y. Strain CH was from Diane Harrington, Chenango Memorial Hospital, Norwich, N.Y. *P. cepacia* ATCC 25416 was used as a reference organism. The other 84 bacterial strains (see Table 1) were obtained from the collection of Norwich Eaton Pharmaceuticals, Inc., Norwich, N.Y.

All organisms except *P. cepacia* were maintained on CTA medium (BBL Microbiology Systems, Cockeysville, Md.). *P. cepacia* strains were maintained on motility medium (BBL Microbiology Systems). All organisms were subcultured daily in brain heart infusion broth (BHI; Difco Laboratories, Detroit, Mich.) and incubated at 30°C. A minimum of three transfers were made before the cultures were used.

Chemicals. 9-Chloro-9-(4-diethylaminophenyl)-10-phenylacridan (C-390) was synthesized and provided by Norwich Eaton Pharmaceuticals, Inc. Polymyxin B sulfate (PBS), USP micronized, was obtained from Pfizer Inc., Groton, Conn.

Selective medium preparation. Plate count agar (PCA; Difco Laboratories) was rehydrated in accordance with the manufacturer's specifications. Aqueous stock solutions containing 0.1% C-390 or 7.5% PBS were prepared. A portion (1 ml) of each stock solution was added to 1 liter of rehydrated PCA to yield concentrations of 1 μg of C-390 and 75 μg of PBS per ml of selective medium. The medium was autoclaved at 121°C for 15 min; the final pH of the medium was 6.95 ± 0.05. Approximately 20 ml of the selective medium was poured into each sterilized, disposable, plastic petri plate (100 by 15 mm) and allowed to solidify in a laminar flow hood. The agar plates were stored in plastic bags at room temperature until used.

Water sample collection. Well water was collected from the overflow outlet of a cooling tower. The cooling tower water was pooled from two wells located in the manufacturing plant area. After the water was softened and treated with phosphate, it was pumped to the cooling tower and then conducted to the air-conditioning system to absorb heat from the environment. This warm water was then circulated back to the cooling tower.

Air-conditioning condensate was collected from an air-conditioning unit (model 39EP12; Carrier Air Conditioning Co., Div. of Carrier Corp., Syracuse, N.Y.).

Tap water was collected from a laboratory sink faucet in 250-ml sterile, screw-capped plastic bottles which contained 0.2 ml of 10% sodium thiosulfate (2 mg/250 ml) for neutralizing the residual chlorine.

Distilled water was collected from a 50-gal. (ca. 190-liter) storage tank in which a UV light had been installed to prevent microbial contamination. Water was distilled with a model A-1015 Sybron Barnstead electrically heated still. Both the storage tank and the still were manufactured by the Barnstead Div. of Sybron Corp., Boston, Mass.

Determination of the MBC. A total of 10 *P. cepacia* strains were tested. C-390 was added to sterile BHI (200 μg/ml) to

* Corresponding author.
† Present address: Sterling Drug, Inc., McPherson, KS 67460.

make a stock solution. Twofold serial dilutions of the stock solution were made with BHI. An overnight BHI culture was diluted to 10^{-3} with sterile saline. The test was conducted by adding 0.5 ml of diluted culture to glass tubes containing 10 ml of C-390 medium. The tubes were incubated at 30°C for 24 h. After incubation, a loopful (0.01 ml) of broth from each of the assay tubes was streaked onto the surface of PCA. The plates were incubated at 30°C for 48 h. The MBC was defined as the lowest concentration of C-390 at which no bacterial growth was observed when 0.01 ml of the test medium was subcultured onto PCA containing no C-390.

Determination of inhibition by the selective medium. An overnight BHI culture of each bacterial strain was diluted to 10^{-1} with sterile saline. A loopful (0.01 ml) of the diluted culture containing ca. 10^5 CFU was streaked onto the selective medium. The plates were incubated at 30°C and examined at 24, 48, and 72 h. After each incubation period, the presence or absence of growth was noted.

Determination of growth of *P. cepacia* on the selective medium. An overnight BHI culture of each *P. cepacia* strain was diluted to 10^{-6} with sterile saline. A 0.1-ml volume from each dilution containing ca. 30 to 100 CFU was spread onto the selective medium with a bent glass rod. The plates were incubated at 30°C and examined at 16, 24, and 48 h. The presence or absence of colonies was noted.

Recovery efficiency of the selective medium. An overnight BHI culture of each *P. cepacia* strain was diluted to 10^{-6} with sterile saline. A 0.1-ml volume from each dilution containing ca. 30 to 100 CFU was spread onto the selective medium with a bent glass rod. By the same procedure and with the same organism dilutions each strain of the test organism was spread onto PCA. The plates for both recovery processes were incubated at 30°C for 48 h. After incubation, the colonies were counted with the aid of a colony counter (model 92-11-2 Colony Counter with Instally; Wilson Diagnostics, Inc., Glenwood, Ill.).

Microbial concentrations in water samples. Each water sample, with the exception of the distilled water sample, was serially diluted 10-fold with its own type of water, which had been filter sterilized. A 0.1-ml volume from each dilution was spread with a bent glass rod onto PCA as well as onto the selective medium in duplicate. All plates were incubated at 30°C for 48 h. After incubation, the plates were examined for growth, and the colonies were counted. Microbial counts were obtained from an average of the duplicate plates which showed colony counts between 30 and 300.

Enumeration of *P. cepacia* in water samples on spread plates. A 100-ml sample of nonsterilized and nondiluted well water, air-conditioning condensate, tap water, or distilled water was inoculated with a 1-ml volume of *P. cepacia* culture saline dilution. The final concentration of *P. cepacia* in the water sample was ca. 3.0×10^2 to 1.0×10^3 CFU/ml of water. A 0.1-ml volume of the inoculated sample was spread onto PCA in duplicate. An identical procedure was performed with the selective medium. All PCA and selective medium spread plates were incubated at 30°C for 48 h. After incubation, the colonies on the plates were enumerated with the aid of a colony counter (Wilson Diagnostics, Inc.). The microbial count obtained was the average of the duplicate plates.

Identification of *P. cepacia* in water samples on streaked plates. A 0.01-ml volume of the inoculated sample (see above) was streaked onto PCA and onto the selective medium. The plates were incubated at 30°C for 48 h. After incubation, the growth on the streaked plates was observed.

Confirmation of *P. cepacia* on the selective medium. A Minitek Nonfermenter Set (BBL Microbiology Systems) was used to confirm that the colonies on the selective medium were those of the inoculated strain.

RESULTS

Determination of the MBC. Results of the determination of the MBC showed that *P. cepacia* was highly resistant to C-390. The MBC for eight of the reference strains was 100 μg/ml. The remaining reference strains, B89b and ATCC 25416, were killed at a concentration of 50 μg/ml.

Inhibition by the selective medium. A total of 94 bacterial strains were tested on the selective medium. All *P. aeruginosa* and other gram-negative bacilli except *P. cepacia* and *Serratia marcescens* were inhibited after incubation at 30°C for 24 h, and no gram-positive bacilli were detected on the selective medium (Table 1).

Growth of *P. cepacia* on the selective medium. Table 2 summarizes the effect of the selective agents C-390 and PBS on the 10 *P. cepacia* reference strains. At 30°C after 16 h incubation, colonies of all strains were tiny but identifiable. Most of the colonies grew larger after 24 h of incubation, and all colonies were fully developed after 48 h.

Recovery efficiency of the selective medium. The recovery of *P. cepacia* on PCA and on the selective medium is summarized in Table 3. The recovery efficiency of the selective medium was compared with that of PCA by means of a *t* test. Statistical significance (*P*) is shown in the last column of Table 3. The recovery efficiency of the selective medium is also expressed in terms of percent recovery, which was obtained by comparing the mean colony count on PCA with the mean colony count on the selective medium.

Microbial concentrations in water samples. The results of spread plate counting on PCA with 0.1 ml of an appropriate dilution of the water showed that the well water and air-conditioning condensate were heavily contaminated; the counts were 7.2×10^3 and 2.4×10^3 CFU/ml, respectively. The microbial count for the tap water was ca. 70 CFU/ml, ranging from 48 to 92 CFU/ml. The distilled water was practically sterile. When 0.1 ml of the water samples was spread in parallel on the selective medium, no microorganisms were detected.

Enumeration of *P. cepacia* in water samples on spread plates. The results of colony counting of the inoculated water samples are summarized in Table 4. The counts shown

TABLE 1. Inhibitory effect of the selective medium

Organism	No. of strains tested	Growth[a] after indicated incubation time (h)		
		24	48	72
Alcaligenes spp.	1	−	−	−
Escherichia coli	5	−	−	−
Klebsiella pneumoniae	3	−	−	−
Proteus mirabilis	1	−	−	−
Pseudomonas alcaligenes	1	−	−	−
P. aeruginosa	53	−	−	−
P. cepacia	10	+	+	+
P. diminuta	1	−	−	−
P. fluorescens	4	−	−	−
P. maltophilia	4	−	−	−
P. putida	1	−	−	−
P. stutzeri	2	−	−	−
Serratia marcescens	4	+	+	+
Staphylococcus aureus	3	−	−	−
Streptococcus faecalis	1	−	−	−

[a] +, Growth; −, no growth.

TABLE 2. Growth of *P. cepacia* on the selective medium

P. cepacia strain	Colonies observed[a] after indicated incubation time (h)		
	16	24	48
ATCC 25416	++	+++	+++
B89b	++	+++	+++
B464-78	++	+++	+++
B493-78	+	++	+++
B4943A	++	+++	+++
CH	++	+++	+++
D7072	++	+++	+++
E8980	++	+++	+++
F1046	+	++	+++
F2111	++	+++	+++

[a] +, Pinpoint (<0.5 mm in diameter) and small (0.5 to 1.0 mm in diameter) colonies were observed; ++, small and large (1.1 to 2.0 mm in diameter) colonies were observed; +++, large colonies were observed.

represent the averages of the values obtained on four plates in two experiments.

Identification of *P. cepacia* in water samples on streaked plates. As the well water and air-conditioning condensate were heavily contaminated, a mixed culture of these two samples was observed on the PCA plates, but a pure culture was observed on the selective medium plates. Pure cultures of the tap and distilled water samples were detected on PCA and on the selective medium.

Results of *P. cepacia* confirmation. On the spread and streaked PCA plates, mixed cultures of the well water, air-conditioning condensate, and tap water samples were found. A pure culture of the distilled water sample was found. However, on the spread and streaked selective medium plates, pure cultures of all water samples were detected. As it was not economically feasible to identify all colonies on the spread and streaked plates, representative colonies were chosen at random only from the spread and streaked selective medium plates and identified with the Minitek Nonfermenter Set. The colonies chosen were all identified as *P. cepacia*.

DISCUSSION

The antimicrobial chemical C-390 was found by Marold et al. to be a selective agent for the isolation of *P. aeruginosa* (8). Davis et al. further demonstrated that *P. aeruginosa* was the only organism that grew in BHI containing 15 μg of C-390 per ml (3). PBS, known to be highly active against gram-negative bacilli, on the other hand, inactivates the growth of *P. aeruginosa* but not *P. cepacia*. Washington found that 93% of *P. aeruginosa* strains were inhibited by PBS at a concentration of 5 μg or less per ml (12). Manniello et al. reported that the viability of *P. cepacia* spheroplasts and whole cells was unchanged after treatment with PBS at 500 μg/ml for 60 min (7). Because of the complementary nature of the spectra of C-390 and PBS, their combination was thought likely to yield a medium selective for *P. cepacia*. In addition, these antimicrobial agents are both water soluble and stable under autoclaving and do not interfere with the oxidase test reaction.

In preliminary studies, C-390 and PBS were combined in PCA at concentrations of 1 to 3 and 10 to 100 μg/ml, respectively. We found that low concentrations of C-390 and PBS allowed *P. aeruginosa* to grow and that high concentrations of C-390 and PBS delayed the growth of *P. cepacia*. Consequently, the combination of 1 μg of C-390 and 75 μg of PBS per ml in PCA was chosen as the selective medium for the study reported here.

Upon examining the inhibitory effect of the selective medium, we noted that in addition to *P. cepacia*, *S. marcescens* also grew. As these two microorganisms have distinct colony morphologies, *P. cepacia* colonies being cream colored, small, and raised, with even edges, and *S. marcescens* colonies being pink, large, and convex, with undulate edges, they can be readily differentiated visually. They may also be identified by the oxidase test reaction, as *P. cepacia* is oxidase positive and *S. marcescens* is oxidase negative.

Fourteen tests were performed for each *P. cepacia* strain to compare the recovery of the test organisms on PCA and on the selective medium. The sizes of the inocula ranged from 21 to 115 CFU/0.1 ml on a plate. The recovery of organisms on PCA was higher than that on the selective medium. This indicates that the combination of C-390 and PBS has an inhibitory effect on the test organisms. For the 10 *P. cepacia* strains tested, the recovery on PCA was significantly different from that on the selective medium for 6 strains ($P = 0.00$ to 0.26), whereas the difference in recovery for the other 4 strains was not significant ($P = 0.4$ to 0.6). More experiments with different *P. cepacia* strains should be done before a conclusion is drawn.

We mentioned earlier that the well water and air-conditioning condensate were heavily contaminated. It was expected that at least some background contaminants associated with water, such as the gram-negative bacteria *Achromobacter*, *Acinetobacter*, *Flavobacterium*, and *Mor-*

TABLE 3. Recovery of *P. cepacia* on PCA and on the selective medium

P. cepacia strain	CFU of *P. cepacia* recovered (per 0.1 ml per plate)[a] on:				% Recovery	Statistical significance (P)[b]
	PCA		Selective medium			
	Range	Mean	Range	Mean		
ATCC 25416	28–96	46.7	26–92	41.6	89.1	0.45
B89b	32–111	56.7	21–98	46.2	81.5	0.26
B464-78	49–111	86.1	42–115	72.9	84.7	0.14
B493-78	39–75	53.8	20–59	36.5	67.8	0.00
B4943A	30–72	53.9	31–74	49.6	92.0	0.42
CH	26–48	40.1	26–52	32.4	80.8	0.09
D7072	30–74	48.7	30–79	44.0	90.3	0.40
E8980	35–71	52.2	22–72	41.9	80.3	0.06
F1046	46–103	72.0	42–83	64.1	89.0	0.16
F2111	23–60	39.1	26–48	36.8	94.1	0.60

[a] A total of 14 plates were used.
[b] Recoveries on PCA and on the selective medium were compared by means of a *t* test.

TABLE 4. Recovery of *P. cepacia* from nonsterilized water samples on PCA and on the selective medium

P. cepacia strain	CFU of *P. cepacia* recovered (per 0.1 ml per plate)[a] on the indicated medium from:							
	Well water		Air-conditioning condensate		Tap water		Distilled water	
	PCA	Selective medium	PCA	Selective medium	PCA	Selective medium	PCA	Selective medium
ATCC 25416	TNTC	96	TNTC	103	195	105	130	115
B89b	TNTC	91	TNTC	89	179	95	90	97
B464-78	TNTC	82	TNTC	68	176	74	86	72
B493-78	TNTC	66	TNTC	70	142	68	74	64
B4943A	TNTC	50	TNTC	44	143	48	46	58
CH	TNTC	64	TNTC	50	147	61	56	72
D7072	TNTC	70	TNTC	63	158	72	62	52
E8980	TNTC	48	TNTC	50	153	65	44	61
F1046	TNTC	81	TNTC	70	167	41	73	64
F2111	TNTC	51	TNTC	49	129	60	54	46

[a] The CFU per 0.1 ml obtained represent the average from four plates in two experiments. TNTC, Too numerous to count.

axella spp., would appear on the selective medium. However, all the water background contaminants that grew on PCA did not survive on the selective medium. The next logical step is to experiment with pure cultures of these gram-negative bacteria on the selective medium. As our requests to various sources for pure cultures of these gram-negative strains have not been fulfilled and as it was economically infeasible for us to obtain these organisms from the American Type Culture Collection, Rockville, Md., we did not pursue the experiment along this line.

We inoculated nondiluted and nonsterilized water samples, such as well water, air-conditioning condensate, tap water, and distilled water, with *P. cepacia* and plated samples on PCA or the selective medium. All *P. cepacia* strains were recovered from both spread plates (high inoculum, 30 to 100 CFU/0.1 ml) and streaked plates (low inoculum, 3 to 10 CFU/0.01 ml). Even though the efficiency of plating for *P. cepacia* on the selective medium has not been determined, the results obtained from the spread and streaked plates indicate that the growth of all bacteria present in the water samples except *P. cepacia* was inhibited by the selective agents.

The direct culturing of environmental water and water-related samples on a medium nonselective for the isolation of *P. cepacia* is tedious. On the basis of the selective properties of C-390 and PBS, we believe that this selective medium could be useful in isolating *P. cepacia* from a mixed bacterial flora that might be present in environmental water or water-related samples, such as solutions and lotions.

The mechanism of the resistance of *P. cepacia* to C-390 is unknown. Further biochemical, physiological, and morphological studies on the interaction between bacteria and the combination of C-390 and PBS should provide a better understanding of how this selective medium exerts its effects.

ACKNOWLEDGMENTS

We thank Robert Weaver of the Centers for Disease Control, Atlanta, Ga., Mehdi Shayegani of the New York State Department of Health, Albany, N.Y., and Diane Harrington of the Chenango Memorial Hospital, Norwich, N.Y., for cultures from their collections and Lorraine Marold for her professional consultation. We are also grateful to Samuel K. Wang for his statistical analysis and Mary R. Liddell for her help in the preparation of the manuscript. The technical assistance of Deannia Opabola and Christine Andrews is gratefully acknowledged.

LITERATURE CITED

1. **Carson, L. A., M. S. Favero, W. W. Bond, and N. J. Petersen.** 1973. Morphological, biochemical, and growth characteristics of *Pseudomonas cepacia* from distilled water. Appl. Microbiol. **25**:476–483.
2. **Close, J., and P. A. Nielsen.** 1976. Resistance of a strain of *Pseudomonas cepacia* to esters of *p*-hydroxybenzoic acid. Appl. Environ. Microbiol. **31**:718–722.
3. **Davis, J. R., C. E. Stager, and G. F. Araj.** 1983. 4-h Identification of *Pseudomonas aeruginosa* with 9-chloro-9-(4-diethylaminophenyl)-10-phenylacridan. J. Clin. Microbiol. **17**:1054–1056.
4. **Geftic, S. G., H. Heymann, and F. W. Adair.** 1979. Fourteen-year survival of *Pseudomonas cepacia* in a salts solution preserved with benzalkonium chloride. Appl. Environ. Microbiol. **37**:505–510.
5. **Gelbart, S. M., G. F. Reinhardt, and H. B. Greenlee.** 1976. *Pseudomonas cepacia* strains isolated from water reservoirs of unheated nebulizers. J. Clin. Microbiol. **3**:62–66.
6. **Hugh, R., and G. L. Gilardi.** 1980. *Pseudomonas*, p. 288–317. *In* E. H. Lennette, A. Balows, W. J. Hausler, Jr., and J. P. Truant (ed.), Manual of clinical microbiology, 3rd ed. American Society for Microbiology, Washington, D.C.
7. **Manniello, J. M., H. Heymann, and F. W. Adair.** 1978. Resistance of spheroplasts and whole cells of *Pseudomonas cepacia* to polymyxin B. Antimicrob. Agents. Chemother. **14**:500–503.
8. **Marold, L. M., R. Freedman, R. E. Chamberlain, and J. J. Miyashiro.** 1981. New selective agent for isolation of *Pseudomonas aeruginosa*. Appl. Environ. Microbiol. **41**:977–980.
9. **Martone, W. J., C. A. Osterman, K. A. Fisher, and R. P. Wenzel.** 1981. *Pseudomonas cepacia*: implications and control of epidemic nosocomial colonization. Rev. Infect. Dis. **3**:708–715.
10. **Quagliaro, D. A., L. Quinones, and J. F. Yager.** 1981. Microbiological analysis of povidone iodine products, p. 191–195. *In* Food and Drug Administration By-Lines no. 4. Food and Drug Administration, Washington, D.C.
11. **Richards, R. M. E., and J. M. Richards.** 1979. *Pseudomonas cepacia* resistance to antibacterials. J. Pharm. Sci. **68**:1436–1437.
12. **Washington, J. A., II.** 1969. Antimicrobial susceptibility of Enterobacteriaceae and nonfermenting gram-negative bacilli. Mayo Clin. Proc. **44**:811–824.