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A New Selective Medium for Isolating *Pseudomonas* spp. from Water

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A new medium, pseudomonas selective isolation agar, was developed to isolate *Pseudomonas* spp. from water. It consists of 350 μg of nitrofurantoin per ml and 2 μg of crystal violet per ml in a nutrient agar base. It is more selective for *Pseudomonas* spp. than are available commercial media. Its ingredients are inexpensive and readily available, and it is easy to prepare.

Pseudomonas selective media are available from Oxoid, Ltd., London, England, and Difco Laboratories, Detroit, Mich. Cephaloridine, Fucidin, and cetrimide are the selective agents in the Oxoid medium. Irgasan is the selective agent in the Difco medium. Hart and Kite (4) compared cetrimide agar (BP formula), Pseudosel (cetrimide; BBL Microbiology Systems, Cockeysville, Md.), Dettol (chloroxylenol; Reckitt and Colman Pharmaceutical Division) in nutrient agar, and nalidixic acid-cetrimide agar to isolate small numbers of *Pseudomonas* spp. They concluded that none of the media was suitable. Thom et al. (7) used 50 μg of nitrofurantoin per ml in an agar medium and compared its performance with that of cetrimide agar in selecting *P. aeruginosa*. They found that the nitrofurantoin media were as sensitive as cetrimide media, easier to prepare and use, and constant in performance. Grant and Holt (3) developed a medium (GH medium) to select *Pseudomonas* spp. from natural habitats with nitrofurantoin (10 μg/ml) and nalidixic acid as primary selective agents. They found that, compared with Pseudosel, Pseudomonas Agar F, Pseudomonas Agar P, and ampicillin-cycloheximide-chloramphenicol agars, their medium had superior selective properties, a short incubation time, and minimal inhibition of *Pseudomonas* spp.

It is important, especially in the pharmaceutical industry, to test for *Pseudomonas* spp. in deionized water. However, the lack of a suitable selective medium led us to develop a medium that would be simple, inexpensive, and effective in isolating *Pseudomonas* spp. from mixed bacterial populations in water. We evaluated nitrofurantoin as the selective agent because most pseudomonads are resistant to it at concentrations that inhibit most other gram-negative bacteria (5, 6). However, there is no agreement in the literature as to the optimum concentration of nitrofurantoin needed to select *Pseudomonas* spp. Therefore, we determined the optimum concentration of nitrofurantoin by testing concentrations from 10 to 500 μg/ml in the laboratory. The concentration of crystal violet selected, 2 μg/ml, was based on what others have used in agar media to inhibit gram-positive bacteria (1, 2). We compared the performance of our medium with that of Oxoid, Difco, and GH media. Optimum performance was recovery of most pseudomonads and inhibition of most nonpseudomonads after enrichment culture.

Pseudomonas agar base with C-F-C supplement SR103 (PAB-CFC; Oxoid) and pseudomonas isolation agar (PIA; Difco) were prepared as directed by the manufacturers. GH medium was prepared as directed except that cycloheximide, an antifungal agent, was omitted. We considered cycloheximide to be unnecessary for the performance of the medium in our application. Pseudomonas selective isolation agar (PSIA) was prepared as follows. A stock solution of 5% (wt/vol) nitrofurantoin, (Sigma Chemical Co., St. Louis, Mo.) was prepared in *N*,*N*-dimethylformamide (Fisher Scientific Co., Fair Lawn, N.J.). A stock solution of 0.1% (wt/vol) crystal violet (Sigma) was prepared in deionized water. The stock solutions were stored at room temperature, and the nitrofurantoin solution was protected from exposure to light. The stock solutions were not filter sterilized because they were so highly concentrated that sterile filtration was considered unnecessary. We prepared 1 l of PSIA by adding 2 ml of crystal violet stock solution to about 990 ml of soybean casein digest agar (SCD; BBL) and then autoclaving and cooling the SCD to ~50°C and adding 7 ml of nitrofurantoin stock solution. All media were dispensed in plastic petri dishes (100 by 15 mm) and either used immediately or stored in plastic bags in a refrigerator for up to 1 month.

The microorganisms used were 85 *Pseudomonas* strains representing about 15 species (Table 1), 63 strains of gram-negative bacteria other than *Pseudomonas* spp., and 5

TABLE 1. Growth of *Pseudomonas* spp. on selective media after 48 to 72 h of incubation at 32°C during laboratory trials

Species[a]	No. of strains tested	No. of strains failing to grow on each selective test medium			
		PSIA	PIA	PAB-CFC	GH
P. aeruginosa	29	0	0	0	0
P. cepacia	21	0	1	0	0
P. fluorescens-P. putida group	15	0	1	0	0
P. maltophilia	5	1	3	1	2
P. stutzeri	2	0	1	1	0
P. pickettii	2	0	0	0	0
P. diminuta	3	0	3	3	0
P. acidovorans	1	1	1	0	0
P. testosteroni	1	1	0	1	0
P. alcaligenes	2	2	2	1	1
P. solanacearum	1	0	1	0	0
Other *Pseudomonas* spp.	3	0	3	2	1

[a] Identified by the API 20E or Rapid NFT biochemical identification system (Analytab), conventional macro methods, and analysis of fatty acids by gas chromatograph (Hewlett-Packard HP5898A; Microbial Identification Systems). Sources: American Type Culture Collection, Rockville, Md.; Presque Isle, Cultures, Presque Isle, Pa.; Lorenzo Forbes, Sacred Heart Hospital, Chester, Pa.; Larry Borbon, Riverside Hospital, Wilmington, Del.; Myron Sasser, University of Delaware, Newark; Jan Bonalsky, CIBA-GEIGY Corp., Summit, N.J.; and laboratory isolates.

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TABLE 2. Growth of nonpseudomonads[a] on selective media after 48 to 72 h of incubation at 32°C during laboratory trials

Organism (no. of strains tested)	No. of strains that grew on each medium[b]			
	PSIA	PIA	PAB-CFC	GH
Serratia marcescens (7)	5	7	7	3
Proteus mirabilis (4)	0	0	1	4
Citrobacter freundii (1)	0	0	1	0
Hafnia alvei (1)	0	0	1	0
Enterobacter cloacae (8)	0	0	8	0
Bordetella bronchiseptica (2)	2	0	2	2
Xanthomonas campestris (1)	1	1	1	0
Xanthomonas dieffenbachiae (1)	0	1	1	0
Xanthomonas pruni (1)	0	0	0	1
Aeromonas hydrophila (1)	0	1	1	0
Flavobacterium meningosepticum (2)	2	1	0	2
Flavobacterium multivorans (1)	0	0	0	1
Alcaligenes sp. (2)	1	1	2	2
Acinetobacter sp. (2)	0	0	2	1
Alteromonas putrefaciens (2)	1	2	2	1

[a] All nonpseudomonads not listed grew on none of the media. See the text for a complete list of nonpseudomonads used in the study.
[b] Growth ranged from very poor to excellent.

strains of gram-positive bacteria. Nonpseudomonads were the following: *Bacillus subtilis* (ATCC 9372), *B. cereus*, *Staphylococcus aureus* (ATCC 6538), *S. epidermidis*, *Streptococcus* sp., *Klebsiella pneumoniae* (eight strains), *Escherichia coli* (five strains), *Salmonella enteritidis*, *S. flexneri*, *S. typhimurium* (ATCC 14028), *Serratia marcescens* (seven strains), *S. liquefaciens*, *Enterobacter cloacae* (eight strains), *E. agglomerans* (two strains), *E. sakazakii*, *Citrobacter freundii*, *Hafnia alvei*, *Proteus mirabilis* (four strains), *Kluyvera* sp., *Bordetella bronchiseptica* (two strains), *Alcaligenes faecalis* (two strains), *Acinetobacter* sp. (two strains) *Alteromonas putrefaciens*, (two strains) *Flavobacterium meningosepticum*, (two strains) *F. multivorans*, *Moraxella lacunata*, *Xanthomonas campestris* pv. *campestris*, *X. campestris* pv. *pelargonii*, *X. campestris* pv. *pruni*, *X. campestris* pv. *vesicatoris* (two strains), and *X. campestris* pv. *dieffenbachiae*, *Aeromonas hydrophila*, and *Agrobacterium* sp. (two strains). The organisms were stored at −80°C in defibrinated 5% sheep blood (Scott Laboratories, Inc., Richmond, Calif.).

In laboratory trials, organisms were cultured in SCD broth at ~30°C for 72 h. A loopful of the broth culture was streaked onto the surface of each selective medium and an SCD control. The inoculated media were incubated at ~30°C and examined for growth after 24, 48, and 72 h. The results were recorded as growth or no growth, although subjective judgments were made regarding how well the organisms grew on each medium.

Field trials were conducted. Deionized water samples were collected in sterile containers from sites throughout a pharmaceutical manufacturing plant. Water (50 ml) from each sampling site was combined with 50 ml of double-strength SCD broth and incubated at ~30°C for up to 72 h. A loopful of the broth culture was streaked onto the surface of each selective medium and an SCD control. We incubated the inoculated agar plates at ~30°C for 72 h and then left them at room temperature for an additional 96 h to recover as many pseudomonads as possible. Developing colonies

TABLE 3. Recovery of *Pseudomonas* spp.[a] from 41 deionized water samples on selective media during field trials

Recovery medium	Total no. of samples from which *Pseudomonas* spp. were isolated	Identification of *Pseudomonas* spp. recovered	No. of samples from which *Pseudomonas* spp. were recovered	No. of samples from which nonpseudomonad spp. were recovered	Identification of nonpseudomonad spp. recovered
PSIA	38	*P. stutzeri*	26	1	CDC group IIf[b]
		P. aeruginosa	10		
		P. paucimobilis	5		
		Other *Pseudomonas* spp.	5		
		Fluorescent *Pseudomonas* group	4		
		P. maltophila	2		
		P. pickettii	2		
		P. fluorescens	1		
PIA	14	*P. aeruginosa*	5		
		P. maltophila	2	1	*Serratia marcescens*
		P. stutzeri	2	2	*Aeromonas hydrophila*
		Fluorescent *Pseudomonas* group	2		
PAB-CFC	18	*P. aeruginosa*	8	2	CDC group IIf[b]
		P. stutzeri	5	2	CDC group IVC-s[c]
		P. maltophila	4		
		Other *Pseudomonas* spp.	2		
		Fluorescent *Pseudomonas* group	2		
		P. paucimobilis	1		
GH	33	*P. stutzeri*	22	2	CDC group IIf[b]
		P. aeruginosa	8	2	CDC group IVC-2[c]
		Other *Pseudomonas* spp.	4		
		Fluorescent *Pseudomonas* group	4		
		P. maltophila	1		
		P. paucimobilis	1		
		P. pickettii	1		

[a] Identified with the API 20E and Rapid NFT nonfermenter miniature identification systems and conventional macro methods.
[b] Nonsaccharolytic flavobacterium; CDC, Center for Disease Control, Atlanta, Ga.
[c] *Alcaligenes* sp.-like.

were carefully examined throughout the incubation period. Colonies showing different colony morphology were transferred to SCD plates and incubated at ~30°C. Soon after colonies appeared, they were identified with the API 20E and Rapid NFT Nonfermenters miniature identification systems (Analytab Products, Plainview, N.Y.). In addition, determinations of motility, including flagellar staining, cytochrome oxidase activity, growth on MacConkey agar, and glucose metabolism in oxidative-fermentative medium, were performed. During laboratory trials, all 29 strains of *P. aeruginosa* grew on the four selective media (Table 1). Of the remaining *Pseudomonas* species, representing 56 strains, more strains grew on PSIA and GH medium than on the commercially available media. No medium permitted the growth of all 85 *Pseudomonas* strains. However, 80 strains grew on PSIA versus 69 on PIA, 76 on PAB-CFC, and 81 on GH medium. None of the four media was completely effective in preventing the growth of all gram-negative nonpseudomonads (Table 2). However, PSIA and PIA were better in this respect than GH medium and PAB-CFC. Overall, 12 gram-negative bacteria grew on PSIA, while 14 grew on PIA, 31 grew on PAB-CFC, and 19 grew on GH medium. Gram-positive bacteria grew on none of the media.

The results of field trials comparing the effectiveness of each medium at isolating *Pseudomonas* spp. from 41 samples of deionized water are contained in Table 3. PSIA recovered *Pseudomonas* spp. from 93% of the water samples, with *P. stutzeri* the most common isolate. GH medium, PAB-CFC, and PIA recovered *Pseudomonas* spp. from 80, 44, and 34% of the water samples, respectively. PSIA recovered *P. aeruginosa* from more samples than did the other media. In addition to poor recovery of *Pseudomonas* spp., PAB-CFC allowed the growth of more nonpseudomonads than did the other media. Overall, field trial results demonstrated the superior performance of PSIA over the other media. Gram-positive bacteria were not recovered. In summary, PSIA performed somewhat better overall than the other media. It was more selective for *Pseudomonas* spp. than were the commercial media and as selective as GH medium. It was more effective at inhibiting gram-negative nonpseudomonads than was GH medium. Its performance in the field trials was superior to that of the other media, and finally its formulation, consisting of only two readily available ingredients in an SCD base, is inexpensive and easy to prepare. Qualitative evaluation of PSIA will continue as additional isolates of *Pseudomonas* spp. and other gram-negative bacteria are tested for growth on the medium.

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