APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Jan. 1985, p. 28–32
0099-2240/85/010028-05$02.00/0

Vol. 49, No. 1

New Selective Media for Enumeration and Recovery of Fluorescent Pseudomonads from Various Habitats

W. D. GOULD,* C. HAGEDORN, T. R. BARDINELLI, AND R. M. ZABLOTOWICZ

Crop Science Laboratory, Syracuse Research Laboratory, Allied Corp., Solvay, New York 13209

Received 13 August 1984/Accepted 10 October 1984

New media (S1 and S2) were formulated that provide a high degree of selectivity and detection of fluorescent pseudomonads on initial plating. The selectivity of the S-type media was based on a detergent, sodium lauroyl sarcosine, and an antibiotic, trimethoprim. A total of five soils from different geographical locations and one sewage sludge sample were examined. On S1 medium, isolates from two soils with low fluorescent pseudomonad populations exhibited a high frequency of arginine dihydrolase (78%) and oxidase-positive (95%) phenotypes, but no fermentative isolates were recovered. Medium S2 was more defined and selective than S1, but lower numbers of fluorescent pseudomonads were recovered on S2. In soils in which fluorescent pseudomonads represent a small proportion of the total population, S1 medium consistently recovered high percentages of fluorescent phenotypes (82.5%).

The isolation and enumeration of bacteria belonging to the genus *Pseudomonas* from various habitats are often necessary due to their importance in a diverse range of microbiological phenomena. The fluorescent pseudomonads are of particular interest because a number of species are plant pathogens (14), and at least one (*P. aeruginosa*) is a human pathogen (5). The fluorescent pseudomonads have also been considered as biological control agents against various root diseases (8, 9, 21) and in the degradation of oil spills (13). The fluorescent pseudomonads have been frequently reported as contaminants in certain meat and dairy products (1, 15), in the biodegradation of pesticides and chemical wastes (20), and in the industrial fermentation of organic acids (19) and have been suggested as indicators of water quality (22).

Antibiotic-based and detergent-based selective media have been developed for the isolation of fluorescent pseudomonads. Because these microorganisms generally possess high intrinsic resistance to antibiotics, Sands and co-workers (16–18) developed selective media containing antibiotics to eliminate other bacteria. The D4 medium of Kado and Heskett (10) contains sodium dodecyl sulfate to eliminate nonpseudomonads by altering cell surface components. Katoh and Itoh (11) developed four media selective for several species of fluorescent pseudomonads based on desoxycholate and defined carbon sources as the selective agents. The medium described by Burr and Katz (3) for isolation of *P. syringae* contains both detergents and antibiotics. These media have disadvantages. The Burr and Katz (3) and the Katoh and Itoh (11) media are selective for only one species or biotype of pseudomonad, and the Sands and Rovira medium is only semiselective in that resistance to the recommended antibiotics is not unique to the pseudomonads. D4 medium is the most widely used for the isolation of fluorescent pseudomonads. There are several difficulties with D4: many other gram-negative bacteria grow; fluorescence is not observed; and sodium dodecyl sulfate is a known plasmid-curing agent. The currently accepted diagnostic medium for the detection of fluorescence is King B medium (12). However, King B medium is unsuitable for the isolation of fluorescent pseudomonads because it is relatively nonselective (16).

The objective of this study was to develop a medium that is more selective than D4 for the isolation of fluorescent pseudomonads and that would promote the expression of observable fluorescence upon initial isolation.

MATERIALS AND METHODS

Media. Total heterotrophic aerobic bacteria were enumerated on 10% tryptic soy agar (TSA; Difco Laboratories, Detroit, Mich.) (7), and gram-negative bacteria were enumerated on 10% TSA containing 5 g of crystal violet per ml to eliminate gram-positive bacteria (4). The isolation medium for detection of fluorescence (S1 medium) contained the following reagents (per liter): 18 g of agar, 10 g of sucrose, 10 ml of glycerol, 5.0 g of Casamino Acids (Difco), 1.0 g of $NaHCO_3$, 1.0 g of $MgSO_4 \cdot 7H_2O$, 2.3 g of K_2HPO_4, 1.2 g of sodium lauroyl sarcosine (SLS), and 20 mg of trimethoprim (2) (5-[(3,4,5-trimethoxyphenyl)methyl]-2,4-pyrimidinediamine). Trimethoprim was obtained from Sigma Chemical Co., St. Louis, Mo., and all of the other chemicals used were of reagent grade. S2 medium contained 4.0 g of asparagine per liter in place of Casamino Acids. Trimethoprim was added after the medium had been autoclaved and cooled. The final pH of the media was 7.4 to 7.6, and no adjustment was necessary. Three media identical to S1, except as noted below, were used to characterize one of the soil samples; trimethoprim was omitted from S3, SLS was omitted from S4, and SLS and trimethoprim were both omitted from S5.

Fluorescence and plating efficiency. The plating efficiency of 10 American Type Culture Collection (ATCC; Bethesda, Md.) strains on the S1 and S2 media were compared with D4 (10) and 10% TSA. A 24-h culture of each strain in nutrient broth was serially diluted in 0.1% peptone (7) and plated on each of the media. Counts on 10% TSA were arbitrarily set at 100% recovery. Each of the ATCC cultures was also streaked on King B and the three selective media (S1, S2, and D4), and growth, fluorescence, and pigment production were evaluated after 24 h of incubation at 23°C.

Isolation of pseudomonads from various habitats. Soils from five locations and one sewage sludge sample were used for the isolation and enumeration of pseudomonads. The

* Corresponding author.

TABLE 1. Bacteria enumerated on one selective and three partially selective media from a Naff silt loam and physiological properties of organisms isolated on the media

Medium	Selective agent	CFU/g of soil	% Positive[a]						
			Fluorescent	Producing N_2	Urease	ADH	Oxidase	Anaerobic glucose	Gram negative
S1	Trimethoprim + SLS	9.0×10^2	100	0	40	90	100	0	100
S3	SLS	6.4×10^3	50	10	25	55	100	0	100
S4	Trimethoprim	1.6×10^5	10	0	75	15	95	0	10
S5	None	8.9×10^5	0	0	15	0	90	0	5

[a] Twenty isolates from each medium were evaluated.

TABLE 2. Growth, fluorescence, and pigment production by ATCC *Pseudomonas* cultures on four selective media after 24 h at 23°C

Organism	Growth				Diffusible pigment				Fluorescence			
	S1	S2	KB[a]	D4	S1	S2	KB	D4	S1	S2	KB	D4
P. fluorescens ATCC 17816	+	+	+	+	+	+	+	−	+	+	+	−
P. fluorescens ATCC 11250	+	+	+	+	+	+	+	−	+	−	+	−
P. fluorescens ATCC 17397	+	+	+	+	+	+	+	−	+	+	+	−
P. fluorescens ATCC 17559	+	+	+	+	+	+	+	−	+	+	+	−
P. fluorescens ATCC 17518	+	+	+	+	+	+	+	−	+	+	+	−
P. putida ATCC 17522	+	+	+	+	+	+	+	−	+	+	+	−
P. putida ATCC 12633	+	+	+	+	+	+	+	−	+	+	+	−
P. maltophilia ATCC 13270	+	−	+	−	−	−	−	−	−	−	−	−[b]
P. cichorii ATCC 10857	+	+	+	+	+	+	+	−	+	+	+	+
P. aeruginosa ATCC 19429	+	+	+	+	+	+	+	−	+	+	+	−

[a] KB, King B.
[b] Nonfluorescent pseudomonad.

soils were: 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). The sewage sludge sample was taken from the waste recycle line of the Metro Wastewater Treatment Plant, Syracuse, N.Y. The samples were serially diluted in 0.1% peptone and plated on S1, S2, D4, 10% TSA, and 10% TSA containing 5 g of crystal violet per ml. The Washington soil was also plated on S3, S4, and S5 media. Colonies were enumerated after 3 days of incubation at 23°C. A total of 20 colonies were picked from two representative plates of each of the three selective media and streaked on 10% TSA.

Physiological tests. 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.), Gram stain, and streaking on two media (King B and S1) to observe fluorescence and diffusible pigment production. Because a number of the isolates were unable to grow at 37°C, the Oxi-ferm tubes were incubated for 72 h at 23°C rather than for 48 h at 37°C. Isolates that were identified as fluorescent biotypes by the Oxi-ferm computer coding system and also produced fluorescent pigments on King B were classified as fluorescent pseudomonads. Because the isolates obtained from the activated sludge sample on D4 medium were primarily oxidase negative, they were also characterized by the Enterotube II series of tests (Roche Diagnostics).

TABLE 3. Plating efficiency of three selective media with 10 ATCC *Pseudomonas* cultures

Organism	Plating efficiency (%)[a]		
	S1	S2	D4
P. fluorescens ATCC 17816	79	<0.1	85
P. fluorescens ATCC 11250	66	14	80
P. fluorescens ATCC 17397	93	<0.1	95
P. fluorescens ATCC 17559	91	87	80
P. fluorescens ATCC 17518	82	36	54
P. putida ATCC 17552	97	46	88
P. putida ATCC 12633	87	78	86
P. maltophilia ATCC 13270	11	<0.1	<0.1
P. cichorii ATCC 10857	52	1	<0.1
P. aeruginosa ATCC 19429	87	61	97

[a] Plating efficiency is the recovery of bacteria compared with 10% TSA, which is arbitrarily set at 100%.

RESULTS

During initial experiments in which the concentration of SLS was varied, it was found that 1.2 g of SLS per liter was required to prevent the growth of gram-positive bacteria (data not shown). The elimination of trimethoprim from the medium formulation (S3) reduced the recovery of fluorescent pseudomonads to 50%, and elimination of SLS (S4) reduced the recovery of fluorescent pseudomonads to 10% of the isolates (Table 1). Only 5% of the isolates were gram negative, and no fluorescent pseudomonads were detected among these when both trimethoprim and SLS were eliminated (S5).

All of the ATCC cultures classified as fluorescent pseudomonads grew, produced diffusible pigments, and fluoresced on S1 medium, and all but one culture fluoresced on S2 medium (Table 2). Fluorescence and pigment production were more intense on S1 medium than King B. On D4, none of the ATCC cultures produced diffusible pigments, and only weak fluorescence of the colonies could be detected. *P. maltophilia* (ATCC 13270) did not grow on D4 or S2 media (Table 2).

TABLE 4. Bacterial populations from five soils on various media

Media	CFU/g of soil				
	Marietta sandy loam (Mississippi)	Dothan sandy loam (South Carolina)	Herkimer silt loam (New York)	Bowie fine sandy loam (Texas)	Naff silt loam (Washington)
10% TSA	4.3×10^7	8.6×10^6	5.6×10^7	3.2×10^8	7.4×10^6
10% TSA + crystal violet (5 μg/ml)	6.4×10^6	3.5×10^6	2.8×10^6	1.1×10^7	7.4×10^4
S1	4.6×10^5	9.8×10^4	2.3×10^6	2.5×10^6	9.0×10^2
S2	3.5×10^5	1.3×10^5	1.2×10^6	4.6×10^5	5.5×10^2
D4	3.9×10^5	9.5×10^4	1.7×10^6	3.3×10^6	8.1×10^3

The plating efficiency of S1 medium was higher than that of S2 medium for all of the ATCC strains and was also higher than the efficiency of D4 for 6 of the 10 ATCC strains (Table 3). Three fluorescent ATCC isolates and one nonfluorescent isolate had very low plating efficiencies on S2 medium compared with one fluorescent and one nonfluorescent culture which had low plating efficiencies on D4 medium. Only *P. maltophilia* exhibited low plating efficiencies on S1 medium (Table 3).

The total bacterial numbers from the five soils as determined on 10% TSA were in the following order: Texas > New York = Mississippi > Washington = South Carolina (Table 4). The total numbers of gram-negative bacteria as determined on 10% TSA amended with crystal violet were, except for the South Carolina soil, an order of magnitude lower than the total bacterial numbers. For each soil, bacterial counts were all within 1 log unit on each of the three selective media (S1, S2, and D4). Of the isolates obtained from the Mississippi and Washington soils on S1 and S2 media, a higher percentage were arginine dihydrolase (ADH) positive and oxidase positive and produced fluorescence compared with those isolated from D4 (Table 5). The phenotypes isolated from the three other soils were in equivalent proportions on all of the selective media. Both fluorescence and pigment production on S1 medium were consistently observed on initial isolation from all soils (Fig. 1A and B). Anaerobic glucose-positive bacteria were isolated on D4, but none were detected on the two S media (Table 5).

A higher percentage of isolates obtained from a sewage sludge sample were fluorescent on S1 and S2 media (50 and 75%, respectively) compared with isolates on D4 medium (20%) (Table 6). Greater numbers of bacteria were isolated from sewage sludge on D4 medium (10^6 CFU/ml) than were isolated on S1 and S2 media (10^5 CFU/ml) (Table 6). A high percentage of D4 isolates were anaerobic glucose positive (65%) and oxidase negative (70%) as compared with 0% anaerobic glucose-positive and 100% oxidase-positive isolates obtained from both of the S media. Approximately 60% of D4 isolates were members of the family *Enterobacteriaceae*, and 35% were presumptive *Shigella* spp. Other identified organisms recovered from the sewage sludge sample on D4 included *Citrobacter* spp., *Klebsiella* spp., *Acinetobacter* spp., and *Escherichia coli*. No fungi were observed on S1, S2, and D4 media even at low dilutions (10^{-1}) from the sludge and soil platings.

DISCUSSION

SLS was chosen as one of the selective agents because it is a weaker detergent than sodium dodecyl sulfate and would potentially provide higher recovery and be less likely to

TABLE 5. Physiological characteristics of bacteria isolated from five soils on three selective media

Soil (location)	Medium	% Positive[a]					
		Fluorescent	Producing N_2	Urease	ADH	Oxidase	Anaerobic glucose
Marietta sandy loam (Mississippi)	S1	65	10	0	55	90	0
	S2	90	0	5	80	100	0
	D4	30	0	45	35	55	45
Dothan sandy loam (South Carolina)	S1	100	0	100	100	100	0
	S2	100	0	100	90	100	0
	D4	90	0	100	75	85	10
Herkimer silt loam (New York)	S1	100	35	20	95	100	0
	S2	85	65	15	85	100	0
	D4	95	35	5	90	95	0
Bowie fine sandy loam (Texas)	S1	100	0	40	90	100	0
	S2	100	0	60	95	100	0
	D4	75	0	20	95	100	0
Naff silt loam (Washington)	S1	100	0	40	90	100	0
	S2	100	5	60	100	100	0
	D4	20	0	10	20	90	10

[a] Twenty isolates from each medium were evaluated.

interfere with fluorescence. SLS prevented the growth of gram-positive organisms (Table 1), but eliminating trimethoprim allowed the growth of nonfluorescent pseudomonads. Trimethoprim appeared to be most active against facultative gram-negative bacteria, but it was ineffective against many gram-positive bacteria (Table 1). When both trimethoprim and SLS were omitted from the medium (S5), selectivity was lost (Table 1). The combination of the two selective agents provided an apparent synergistic effect on selectivity and was essential for the selectivity of the S media.

The greater numbers of oxidase- and ADH-positive bacteria obtained on S1 medium as compared with D4 (Tables 5 and 6) were consistent with the greater numbers of fluorescent phenotypes isolated on S1. All of the fluorescent pseudomonads are oxidase positive, and many of them are ADH positive (6). The S media were not compared with the Sands and Rovira medium because initial studies showed that D4 was superior to the Sands and Rovira medium (unpublished data). Sands and co-workers reported that in some soils the fluorescent pseudomonads constituted a very low percentage (6%) of the isolates obtained on their medium (18).

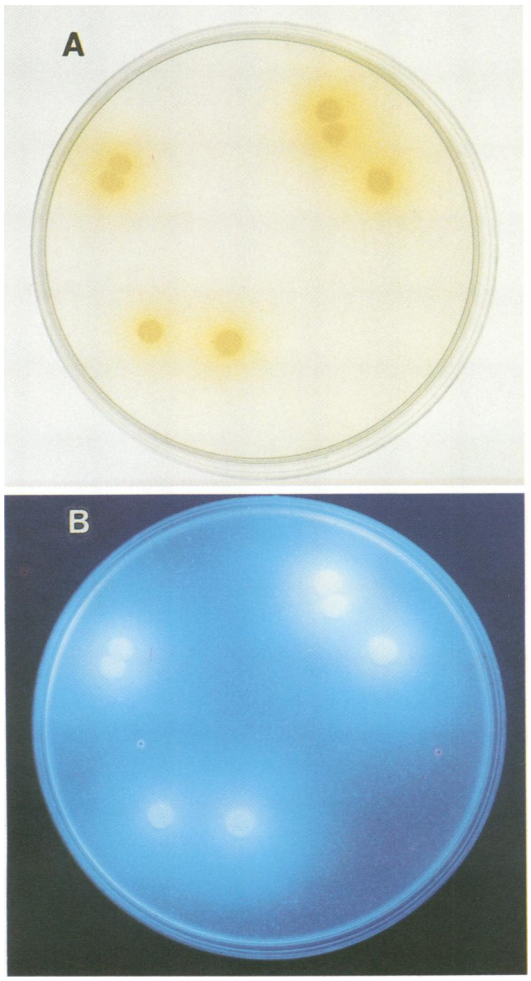

FIG. 1. Soil dilution on a plate of S1 medium after 3 days of incubation at 23°C. (A) Pigment production under 3400 K photographic lights, color-corrected with an 80B lens filter; (B) fluorescence when illuminated with UV light at 366 nm.

TABLE 6. Bacteria enumerated on three selective media from a sewage sludge sample and physiological properties of organisms isolated on the media

Medium	CFU/g of sludge	% Positive[a]					
		Fluorescent	Producing N_2	Urease	ADH	Oxidase	Anaerobic glucose
S1	5.2×10^5	50	35	5	80	100	0
S2	1.5×10^5	75	40	30	80	95	0
D4	2.0×10^6	20	0	35	65	30	65

[a] Twenty isolates from each medium were evaluated.

S1 medium has several advantages over other media used for the isolation of fluorescent pseudomonads. The medium consistently gives high selectivity and good recoveries of fluorescent pseudomonads with samples obtained from a variety of habitats. Fluorescence can also be observed on initial isolation on S1 medium, and subsequent streaking on King B medium to observe fluorescent pigment production is unnecessary.

S1 medium could be modified for a number of other applications. The medium could be used for the selective isolation of one species or biotype of fluorescent pseudomonad by the replacement of Casamino Acids with a defined nitrogen source. Sucrose and glycerol are essential to S1 medium because the combination of these compounds provides an osmotic stress that is one of the selective features of the medium. Because these compounds are carbon sources it would be inappropriate to use a defined carbon source to obtain selectivity. Although asparagine as the sole nitrogen source in S2 medium did not improve selectivity, there are other nitrogen sources that could potentially be used.

P. aeruginosa (ATCC 19429) produced a green pigment on S1 medium, but several other strains of *P. aeruginosa* did not produce the same colored pigment. A modified S1 medium could possibly be employed as the base for a diagnostic formulation to provide for the selection and identification of *P. aeruginosa* if green fluorescent pigments could be consistently observed.

ACKNOWLEDGMENT

We express appreciation to Donald R. Gustavson for the photographs and for assistance with the manuscript.

LITERATURE CITED

1. **Ayres, J. C.** 1960. The relationship of organisms of the genus *Pseudomonas* to the spoilage of meat, poultry, and eggs. J. Appl. Bacteriol. **23**:471–475.
2. **Burchall, J. J.** 1975. Trimethoprim and pyrimethamine, p. 304–320. *In* J. W. Corcoran and F. E. Hahn (ed.), Antibiotics, vol. 3. Springer-Verlag, New York.
3. **Burr, T. J., and B. Katz.** 1982. Evaluation of a selective medium for detecting *Pseudomonas syringae* pv *papulans* and *P. syringae* pv *syringae* in apple orchards. Phytopathology **72**:564–567.
4. **Conn, H. J.** 1957. Staining methods, p. 10–48. *In* Manual of microbiological methods. McGraw-Hill Book Co., New York.
5. **Curtin, J. D., R. G. Petersdorf, and I. L. Bennett, Jr.** 1961. *Pseudomonas* bacteremia: review of ninety-one cases. Ann. Intern. Med. **54**:1077–1107.
6. **Doudoroff, M., and N. J. Palleroni.** 1974. Genus *Pseudomonas* Migula 1894, 237, p. 217–242. *In* R. E. Buchanan and N. E. Gibbons (ed.), Bergey's manual of determinative bacteriology, 8th ed. The Williams & Wilkins Co., Baltimore.
7. **Hagedorn, C., and J. G. Holt.** 1975. Ecology of soil arthrobacters in Clarion-Webster toposequences of Iowa. Appl. Microbiol. **29**:211–218.

8. **Howell, C. R., and R. D. Stipanovic.** 1979. Control of *Rhizoctonia solanii* on cotton seedlings with *Pseudomonas fluorescens* and with an antibiotic produced by the bacterium. Phytopathology **69**:480–482.
9. **Howell, C. R., and R. D. Stipanovic.** 1980. Suppression of *Pythium ultimum*—induced damping-off of cotton seedlings by *Pseudomonas fluorescens* and its antibiotic, pyoleutorin. Phytopathology **70**:712–715.
10. **Kado, C. I., and M. G. Heskett.** 1970. Selective media for isolation of *Agrobacterium*, *Corynebacterium*, *Erwinia*, *Pseudomonas*, and *Xanthomonas*. Phytopathology **60**:969–976.
11. **Katoh, K., and K. Itoh.** 1983. New selective media for *Pseudomonas* strains producing fluorescent pigment. Soil Sci. Plant Nutr. **29**:525–532.
12. **King, E. O., M. K. Ward, and D. E. Raney.** 1954. Two simple media for the demonstration of pyocyanin and fluorescin. J. Lab. Clin. Med. **44**:301–307.
13. **McGill, W. B., M. J. Rowell, and D. W. S. Westlake.** 1981. Biochemistry, ecology, and microbiology of petroleum components in soil, p. 229–296. *In* E. A. Paul and J. N. Ladd (ed.), Soil biochemistry, vol. 5. Marcel Dekkar, Inc., New York.
14. **Misaghi, I., and R. G. Grogan.** 1969. Nutritional and biochemical comparisons of plant-pathogenic and saprophytic fluorescent pseudomonads. Phytopathology **59**:1436–1450.
15. **Nickerson, J. T., and A. J. Sinskey.** 1973. Microbiology of dairy products, p. 176–196. *In* J. T. Nickerson and A. J. Sinskey (ed.), Microbiology of foods and food processing. Elsevier/North-Holland Publishing Co., New York.
16. **Sands, D. C., and A. D. Rovira.** 1970. Isolation of fluorescent pseudomonads with a selective medium. Appl. Microbiol. **20**: 513–514.
17. **Sands, D. C., and A. D Rovira.** 1971. *Pseudomonas fluorescens* biotype G, the dominant fluorescent pseudomonad in south Australian soils and wheat rhizospheres. J. Appl. Bacteriol. **34**:261–275.
18. **Simon, A., A. D. Rovira, and D. C. Sands.** 1973. An improved selective medium for isolating fluorescent pseudomonads. J. Appl. Bacteriol. **36**:141–145.
19. **Underkofler, L. A.** 1954. Gluconic acid, p. 446–469. *In* L. A. Underkofler and R. J. Hickey (ed.), Industrial fermentations, vol. 1. Chemical Publishing Co., New York.
20. **Walker, N.** 1975. Microbial degradation of plant protection chemicals, p. 181–192. *In* N. Walker (ed.), Soil microbiology—a critical review. John Wiley & Sons, Inc., New York.
21. **Weller, D. M., and R. J. Cook.** 1983. Suppression of take-all of wheat by seed treatments with fluorescent pseudomonads. Phytopathology **73**:463–469.
22. **Wolf, H. W.** 1972.The coliform count as a measure of water quality, p. 333–345. *In* R. Mitchell (ed.), Water pollution microbiology. John Wiley & Sons, Inc., New York.