Selective Medium for Pseudomonas aeruginosa That Uses 1,10-Phenanthroline as the Selective Agent

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The MIC of 1,10-phenanthroline for 35 Pseudomonas aeruginosa strains was 128 to 160 µg/ml, whereas 32 µg or less per ml inhibited all other microorganisms tested. On the basis of these results, a selective agar for P. aeruginosa, which contained 15 g of Trypticase soy broth (BBL Microbiology Systems), 15 g of agar, and 0.1 g of phenanthroline per liter, was formulated. Forty-four P. aeruginosa strains yielded a mean efficiency of plating on this medium of 79% of the counts obtained on Trypticase soy agar, which was significantly higher than that obtained with Pseudomonas isolation agar or Pseudosel agar. Pseudomonas cepacia, Pseudomonas fluorescens, Pseudomonas putida, Pseudomonas stutzeri, representatives of 13 other genera (including gram-negative rods, gram-positive rods, and cocci), and a yeast were not recovered within 48 h at 35°C when approximately 107 CFU were plated on this medium. Only small colonies from one strain each of P. fluorescens and P. putida could be seen at 3 and 7 days, respectively, and they had an efficiency of plating of only <0.001%. When 107 CFU of either of these strains was plated with 102 CFU of P. aeruginosa, it did not interfere with the quantitative recovery of P. aeruginosa.

Pseudomonas aeruginosa is a nonfermentative gram-negative bacterium recoverable from most natural sources, especially wet environments. It is often found in preserved fluids, emulsions, and creams, largely because of its high level of resistance to many antimicrobial agents. Moreover, P. aeruginosa is also the most frequently isolated nonfermentative bacterium in clinical microbiology laboratories and now accounts for 10% of nosocomial infections.

It would be desirable to have a medium that is highly selective but not inhibitory for P. aeruginosa and that is also inexpensive and easy to use. Currently available media such as Pseudosel and pseudomonas isolation agar (PIA) inhibit for some strains of P. aeruginosa and do not inhibit some other species of bacteria. We have found that 1,10-phenanthroline (Sigma Chemical Co., St. Louis, Mo.), a common reagent that is inexpensive and easy to obtain, has selectivity for P. aeruginosa. The selective specificity of a Trypticase soy agar (TSA)-based medium containing phenanthroline was evaluated for 44 strains of P. aeruginosa and a variety of other species by spreading 0.1-ml samples of cell suspensions, adjusted in saline to the desired cell concentration, onto agar media with rotary turntables and glass spreaders. Results with the phenanthroline medium were compared with those obtained with TSA (a reference nonselective control medium), Pseudosel (a cetrimide-based formulation widely used to isolate P. aeruginosa), and PIA.

Representatives of 26 species of bacteria obtained from our culture collection were used to determine the MICs of phenanthroline (Table 1) and the recovery of the different species on selective media. Of the 44 strains of P. aeruginosa used for efficiency of plating (EOP) studies, five were from the American Type Culture Collection, Rockville, Md., and 22 were recent clinical isolates. The remaining 17 environmental isolates were from a variety of sources ranging from wastewaters to contaminated pharmaceuticals.

PIA was obtained from Difco Laboratories, Detroit, Mich. Pseudosel agar and Trypticase soy broth (TSB) were obtained from BBL Microbiology Systems, Cockeysville, Md. Agar was obtained from the U. S. Biochemical Corp., Cleveland, Ohio. Modified TSA (mTSA) was made with 15 g of TSB plus 15 g of agar per liter.

A mixture of mTSA and phenanthroline (mTSA+P) was prepared by adding 15 g of TSB, 15 g of agar, and 0.1 g of phenanthroline per liter to 1 liter of deionized water and heating in a microwave oven until the agar was melted. No additional heating was necessary, since the heat necessary to melt the agar kills any organisms that would not be inhibited by the phenanthroline. Other media were prepared according to the directions of the manufacturer. Twenty-five milliliters was dispensed per plate. The addition of phenanthroline to mTSA resulted in a light pink medium.

Fifefold serial dilutions of 2 mg of phenanthroline per ml in a volume of 0.2 ml of 15 g of TSB per liter in microdilution plate wells were used to determine the activity of phenanthroline against various bacterial species. The base medium contained 15 g of TSB per liter instead of the manufacturer-recommended 30 g per liter, because all of the tested strains grew well at this concentration and the inhibitory effects of alkaline by-products of amino acid metabolism on pseudomonads were minimized. Each dilution was inoculated with 106 CFU of the test organism per ml by using a 1% inoculum of a suspension adjusted to a turbidity that yielded about 106 CFU/ml. This equivalent to an optical density at 540 nm of 0.2 for gram-negative bacteria, 1.0 for Candida spp., and 0.1 for all others. Suspensions were prepared in 0.85% NaCl from overnight mTSA cultures (30°C). Duplicate 0.1-ml samples were plated onto test media directly or after dilution in saline. All plates were incubated for 48 h at 35 to 37°C before colonies were counted and average yields (per plate) were calculated. All plates were reincubated and rechecked daily through day 7.

Results of MIC tests with 49 strains of bacteria representing 11 species showed that phenanthroline at 32 µg or less per ml inhibited the growth of all microorganisms tested except P. aeruginosa (Table 1). Thirty-five strains of P. aeruginosa were tested, and the MIC for each was 160 ppm (160 µg/ml). To better assess the MIC of phenanthroline for

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**TABLE 1.** MICs of phenanthroline for selected bacteria

<table>
<thead>
<tr>
<th>Species</th>
<th>No. of strains</th>
<th>MIC* (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enterobacter spp.</td>
<td>5</td>
<td>32</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>1</td>
<td>6.4</td>
</tr>
<tr>
<td>Klebsiella pneumoniae</td>
<td>1</td>
<td>6.4</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>35</td>
<td>160</td>
</tr>
<tr>
<td>Pseudomonas cepacia</td>
<td>1</td>
<td>32</td>
</tr>
<tr>
<td>Pseudomonas fluorescens</td>
<td>1</td>
<td>32</td>
</tr>
<tr>
<td>Pseudomonas maltophilia</td>
<td>1</td>
<td>6.4</td>
</tr>
<tr>
<td>Pseudomonas putida</td>
<td>1</td>
<td>32</td>
</tr>
<tr>
<td>Proteus spp.</td>
<td>1</td>
<td>6.4</td>
</tr>
<tr>
<td>Serratia marcescens</td>
<td>1</td>
<td>32</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>1</td>
<td>6.4</td>
</tr>
</tbody>
</table>

* Determined by fivefold serial dilutions of 2 µg of phenanthroline per ml in 15 g of TSB per liter. Each dilution was inoculated with 10^6 CFU of the test organism per ml. Within the limits of the dilutions used, there was no variation in MICs for strains of each species.

**P. aeruginosa** in the range between 32 and 160 ppm, six strains (two each from environmental, clinical, and American Type Culture Collection sources) were tested by using phenanthroline concentrations of 32, 64, 128, and the previously employed 160 ppm. MICs for five of the six strains were 128 ppm, while the sixth, one of the American Type Culture Collection strains, grew in 128 ppm but not in 160 ppm phenanthroline.

The mean EOP of **P. aeruginosa** strains from different sources on mTSA+P is shown in Fig. 1. When 44 strains, including strains from the American Type Culture Collection as well as clinical and environmental sources, were tested, mTSA+P yielded a mean EOP of 79% compared with their EOPs on nonselective mTSA. Pseudosel had an EOP of 29%; PIA had an EOP of 59%. Mean EOPs were consistently high (about 80%) on mTSA+P regardless of source. Mean EOPs of strains from different sources on Pseudosel were low (24 to 30%) while EOPs on PIA were variable; clinical strains were high (79%) and environmental strains were low (39%). Thirty-two of 44 strains yielded higher average counts on mTSA+P than on PIA, and all but two strains yielded higher average counts on mTSA+P than on Pseudosel. Some strains of **P. aeruginosa** produced large colonies on mTSA+P after 24 h, and almost all did so after 48 h. This compared favorably with growth on Pseudosel; however, PIA produced larger colonies faster.

Other organisms could not be recovered on mTSA+P when inoculated to 10^7 CFU per plate. The following non-**Pseudomonas** species were tested: one strain each of *Achromobacter* spp., *Aceintobacter calcoaceticus*, *Bacillus subtilis*, *Candida albicans*, *Enterobacter aerogenes*, *Micrococcus luteus*, and *Staphylococcus epidermidis*; two strains each of *Alcaligenes faecalis*, *Alcaligenes odorans*, *Enterobacter cloacae*, *Serratia marcescens*, and *Staphylococcus aureus*, and seven strains of *Proteus* spp. Other **Pseudomonas** species, including four *Pseudomonas cepacia*, one *Pseudomonas facilis*, four *Pseudomonas maltophilia*, one *Pseudomonas pickettii*, one *Pseudomonas paucimobilis*, and three *Pseudomonas stutzeri* strains, could not be recovered on mTSA+P when inoculated to 10^7 CFU per plate. One of five *Pseudomonas fluorescens* strains and one of four *Pseudomonas putida* strains grew slowly and with a very low EOP. These strains were recovered at an EOP of about 0.001%, and were visible after 3 and 7 days, respectively. In mixed cultures of *P. aeruginosa* (200 CFU per plate) and the recoverable *P. fluorescens* or *P. putida* strain (10^7 CFU per plate), recovery of *P. aeruginosa* was not inhibited but recovery of *P. fluorescens* and *P. putida* was inhibited and delayed. Because of delayed appearance and small size, *P. fluorescens* and *P. putida* did not interfere with recovery of *P. aeruginosa* from mixtures of the two organisms. When incubation was at 30°C, recovery of *P. fluorescens* and *P. putida* increased to 0.1% for some strains, although colonies were still small and recovery time was still longer than for *P. aeruginosa*. PIA and Pseudosel do not completely inhibit the growth of microorganisms other than *P. aeruginosa*, particularly other pseudomonads (2-4).

Platings of lower numbers of organisms (10^1, 10^2, and 10^5 CFU per plate) did not improve recovery. This ruled out possible selective inhibition of growth on mTSA+P due to overcrowding when high numbers of cells are plated.

9-Chloro-9-(4-diethylaminophenyl)-10-phenylacridan (C-390) has been proposed as a primary selective agent in a *P. aeruginosa* selective medium (5) and as a selective agent with polymyxin sulfate for a *P. cepacia*-selective medium (8). The C-390-based *P. aeruginosa* selective medium was reported to inhibit the growth of all organisms tested except *P. aeruginosa* at 10^2 to 10^4 CFU per plate, although *P. cepacia* was not tested against this medium. Recovery of *P. aeruginosa* on C-390 medium is reported to compare favorably with recovery on nonselective media and to be better than recovery on PIA- or cetrimide-based agar (5). However, C-390 selective medium was not tested for selectivity against higher numbers of organisms, whereas mTSA+P is selective against large numbers and is effective in isolating *P. aeruginosa* from mixtures of organisms when *P. aeruginosa* is present in comparatively low numbers. Furthermore, when C-390 medium was used for recovery of *P. aeruginosa* from clinical specimens (1), three of 30 samples containing *K. pneumoniae* and three of 10 samples containing *Serratia* spp. showed growth of those species. To our knowledge, C-390 is not presently commercially available.

The selective agent used in this study, 1,10-phenanthroline, is inexpensive and commercially available. In addition, mTSA+P need not be autoclaved; boiling to melt the agar is all that is required.

The excellent selectivity without significant inhibition of *P. aeruginosa*, the ease of preparation, and the low cost of this medium should make it widely applicable for the isolation of *P. aeruginosa*.
LITERATURE CITED


