Isolation of Fluorescent Pseudomonads with a Selective Medium

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Received for publication 7 April 1970

Incorporation of novobiocin, penicillin, and cycloheximide into a standard medium for fluorescence selects for fluorescent pathogenic and free-living pseudomonads.

Although there have been extensive studies of the physiology and pathology of fluorescent pseudomonads, less research has been undertaken on the ecology of these bacteria in natural habitats, namely, soil, plants, and animals. A factor which has limited such research has been the difficulty of detecting fluorescent pseudomonads where they form a minor component of a mixed microflora. We report the use of a medium which is selective for fluorescent pseudomonads encountered in the fields of medicine and soil microbiology and most of those that incite plant diseases.

In a previous study of the taxonomy of phytopathogenic pseudomonads, Sands, Schroth, and Hildebrand (4) assayed the nutritional requirements and antibiotic sensitivity of representative strains of the biotypes of Pseudomonas aeruginosa, P. fluorescens, P. putida, and over 30 species of phytopathogenic pseudomonads of the P. syringae and P. aeruginosa types. They found few nutritional characteristics which were common to all phytopathogenic fluorescent pseudomonads. The universal carbon sources such as glucose, fumarate, and glutarate held little promise as selective factors in a medium. However, resistance to certain broad-spectrum antibiotics is a common feature of most of the fluorescent pseudomonads, and this has provided the basis of selectivity in the medium. We found that penicillin (75 units/ml), novobiocin (45 μg/ml), and cycloheximide (75 μg/ml) limited the growth of nearly all fungi and bacteria except fluorescent pseudomonads. The addition of these antibiotics to the standard diagnostic medium for fluorescence of pseudomonads resulted in a highly selective medium, useful for detecting very low numbers of fluorescent pseudomonads. In this note we describe the medium, abbreviated to NPC (novobiocin, penicillin, cycloheximide), and its relative selectivity for fluorescent pseudomonads.

The basal medium is medium B of King, Ward, and Raney (2) which contains Difco Proteose, Peptone no. 3, 20 g; Oxoid Ionagar No. 1, 12 g; glycerol, 8 ml; K₂SO₄, 1.5 g; MgSO₄·7H₂O, 1.5 g; distilled water, 940 ml; pH was adjusted to 7.2 with 0.1 N NaOH before autoclaving for 15 min at 121 C. The types of peptone and agar should be adhered to for maximum fluorescence. Also, care should be taken to keep the medium iron free as iron represses pigment production and quenches fluorescence.

Penicillin G (75,000 units), novobiocin (Upjohn, Albamycin; 45 mg), and cycloheximide (Upjohn, Acti-Dione; 75 mg) were mixed together in 3 ml of 95% ethanol, then diluted with 50 ml of sterile distilled water, and added to 940 ml of the melted basal medium at 45 C. The plates can be dried overnight before use and then refrigerated in plastic bags until needed, but it should be noted that the antibiotic activity decreases over several weeks.

To demonstrate the selective value of this medium for detecting fluorescent pseudomonads when greatly outnumbered by soil bacteria, we used a red brown earth, DR 2-23 (3) from Parafield, South Australia. This soil, with exceptionally low numbers of fluorescent pseudomonads, was sieved free from most organic matter and used for plate-count experiments. Ten grams of soil was vigorously shaken for 15 min in 100 ml of sterile distilled water, and then 0.1 ml was spread onto the NPC medium with a glass rod. All dilutions were done with soil extract (1%, w/v; centrifuged and autoclaved) since, as with many other bacteria, viable counts of P. aeruginosa Corvino declined rapidly in distilled water but not in soil extract. For comparison of selectivity, the relatively nonselective YPS medium (1) was used for pour plates of soil dilutions. Counts on YPS were made after 10 days.

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TABLE 1. Relative selectivity of three media plated with soil suspensions

| Medium*       | Colonies/gram of soil | Fluorescent colonies detected/gram of soil |
|---------------|-----------------------|------------------------------------------|
| YPS           | \(1.2 \times 10^7\)   | None                                     |
| Medium B      | \(2.8 \times 10^4\)   | \(<8 \times 10^1\)                        |
| NPC           | \(4.0 \times 10^6\)   | \(1.8 \times 10^2\)                      |

*YPS is the medium of Bunt and Rovira (1). Medium B is that of King, Ward, and Raney (2). NPC is the standard diagnostic medium for fluorescence of pseudomonads plus novobiocin (45 \(\mu\)g/ml), penicillin (75 units/ml), and cycloheximide (75 \(\mu\)g/ml).

TABLE 2. Recovery with NPC medium of P. aeruginosa Corvino* introduced into soil suspensions

| No. of fluorescent bacteria introduced/\(mL\) of soil suspension* | No. of fluorescent bacteria per cent recovered/\(mL\) of soil suspension |
|-------------------------------------------------------------------|-------------------------------------------------------------------------|
| None                                                              | None                                                                    |
| \(2.2 \times 10^7\)                                               | \(2.2 \times 10^7\) (100\%)*                                           |
| \(2.2 \times 10^6\)                                               | \(2.5 \times 10^4\) (114)                                              |
| \(2.2 \times 10^6\)                                               | \(2.2 \times 10^4\) (100)                                              |
| \(2.2 \times 10^4\)                                               | \(2.4 \times 10^4\) (109)                                              |

*NPC is the standard diagnostic medium for fluorescence of pseudomonads plus novobiocin (45 \(\mu\)g/ml), penicillin (75 units/ml), and cycloheximide (75 \(\mu\)g/ml).

After inoculation, the NPC plates were incubated at 28 to 30 C for 2 to 3 days before examination with ultraviolet light. Yellow-green or blue-white fluorescence in and around the colonies is diagnostic for fluorescent pseudomonads. Most fluorescent pseudomonads will be apparent after incubation for 3 days, with the exception of many plant pathogenic pseudomonads which exhibit a slower rate of growth and pigment production. The NPC medium is highly selective as can be seen from the results of direct plating of a soil suspension (Table 1). Medium B, without antibiotics, is somewhat selective but fewer fluorescent pseudomonads are detected with it than on NPC, probably because of inhibition by other organisms. In a study of fluorescent bacteria in 14 soils, from 10% to 100% of the bacterial colonies on NPC were fluorescent. All of several hundred fluorescent strains from the soil fit the description of Stanier, Palleroni, and Doudoroff (6) of fluorescent pseudomonads; i.e., they were oxidase-positive, arginine dihydrolase-positive, negative for glucose fermentation, and unable to grow on a nitrogen-free medium. The nonfluorescent strains that grew on NPC were also gram-negative, but all differed from the fluorescent strains in at least one test in addition to pigment production. Many were fermentative and oxidase-negative.

A high rate of recovery of P. aeruginosa was obtained when a suspension of soil was inoculated with decreasing numbers of these bacteria and counted with NPC medium (Table 2). The NPC counts of a cell suspension were as high as the medium B or the YPS counts. High recovery rates were also observed with two strains of P. fluorescens and two strains of P. putida.

We found no effect of NPC culture on the virulence of two strains of plant pathogenic pseudomonads, although Schwinghamer (5) found that penicillin- and novobiocin-resistant mutants of Rhizobium were no longer able to nodulate clover. Three out of 10 strains of fluorescent plant pathogens from Australia would not grow on NPC; however, prolonged maintenance of these cultures on laboratory media may have caused a loss of resistance to these antibiotics. We have not found antibiotic-sensitive fluorescent strains from nature, suggesting that resistance to these antibiotics may be necessary for the survival of fluorescent pseudomonads in nature.

All seven tested strains of P. aeruginosa of medical origin grew well on NPC, suggesting its value for hospital studies. We found the medium useful for studying the transmission of bacterial plant diseases by seed, insects, soil, and water.

This investigation was supported by a National Science Foundation postdoctoral fellowship no. 48047 to D. C. Sands.

We are indebted to A. Simon for technical assistance and to J. Harris, P. Brisbane, and A. Kerr for helpful suggestions and critical discussions.

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