APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Feb. 1983, p. 701-702 Vol. 45, No. 2
0099-2240/83/020701-02$02.00/0
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Method for Rapid Detection of Cyanogenic Bacteria

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Received 4 August 1982/Accepted 18 October 1982

An agar plate method is described in which the production of hydrogen cyanide by as many as 50 microbial isolates per plate may be detected. Cyanide produced by the organisms reacts with copper(II) ethylacetoacetate and 4,4′-methylenebis-(*N*,*N*-dimethylaniline) in a paper disk suspended above the microbial colonies. Cell growth occurs in depressions in the agar surface, which allows separation of colonies and enhances sensitivity of hydrogen cyanide detection.

Hydrogen cyanide (HCN) is commonly found in surface waters, where its presence may often be attributed to biological sources (5, 6). Potential biological HCN sources include: (i) the runoff from soil supporting cyanogenic plants and (ii) cyanogenic microbes. Examples of soil and water microbes capable of producing HCN include bacteria (3), fungi (1, 10), and algae (9). The detection of HCN-producing microbes has been hampered by the cumbersome procedures involved. The potentially cyanogenic organism was grown on individual agar slants, with filter paper strips impregnated with a cyanide-sensitive chemical, usually alkaline picrate, suspended above the growing cells (7, 8). This procedure required considerable manipulation for each isolate, thus making the screening of a large number of strains difficult. An agar plate method, suggested by a procedure for visualizing cyanogenic regions of mushroom tissue (S. G. Saupe, Ph.D. thesis, University of Illinois, Urbana, 1981) was devised which allows the rapid screening of up to 50 isolates per plate. Figure 1 shows the components used in this technique and the manner in which they are assembled. The agar is poured in plates in two layers: 15 ml of 2% agar for the lower layer and 10 ml of 1.5% agar for the upper layer. The first layer is allowed to solidify before the second layer is poured. The nutrient composition of these plates is conducive to cyanogenesis; nutrient agar, 2% peptone agar, or a synthetic medium (2) may be employed.

To produce depressions of uniform dimensions, a sterile cork borer barrel (size no. 1), attached to a vacuum source with a tube, is touched lightly to the agar surface. This procedure withdraws only the upper layer of the agar and leaves a pit with a uniform diameter and a flat lower surface corresponding to the top of the 2% agar layer. Fifty pits could be easily placed in a standard agar plate (Fig. 1) by using a mutant grid template. The purpose of these depressions is to increase the surface area available to cell growth, and so increase the intensity of the HCN spot produced on the detection paper above the depression.

Next to the agar surface are three layers of sterile nylon mesh which prevent the HCN-sensitive paper from coming in contact with the agar or the cells. If the paper picks up moisture from the plate, a smeared reaction is seen. The HCN-sensitive paper is a circle of Whatman 3MM chromatography paper soaked in the HCN detection reagent of Feigl and Anger (4): copper(II) ethyl acetoacetate (5 mg) and 4,4′-methylenebis-(*N*,*N*-dimethylaniline) (5 mg) in chloroform (1 to 2 ml). Increasing the concentration of these compounds causes a weakened cyanide reaction. When dry, these pale aqua disks can be stored for several weeks before use. A wire mesh disk, placed above the HCN detection paper, functions as a weight to keep the paper in uniform proximity with the growing cells, thus producing an unambiguous response.

The protocol requires first the inoculation of the depressions by a suitable method from either a solid or liquid medium. Colonies are "patched" from a master plate by using sterile toothpicks. If particularly motile organisms are anticipated, the plates may be predried before inoculation. After assembly, the plates are incubated in an upright position at a temperature conducive to cyanogenesis. With *Pseudomonas aeruginosa*, the temperature was 35°C. HCN-positive strains could be seen in 3 to 18 h, depending on the amount of inoculum and cyanogenic nature of the isolate. A positive reaction showed a dark blue spot against the pale aqua background of the HCN detection disk (Fig. 2). The spot occurred directly above the pits containing the cyanogenic strains; thus a key to the relative position of the spot and pits must be maintained.

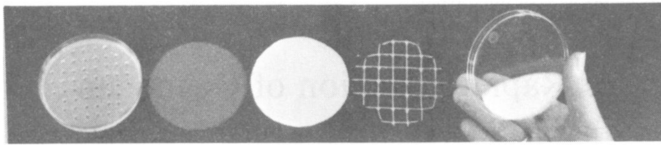

FIG. 1. Agar plate apparatus for the detection of cyanogenic microbes. The component parts are described in the text.

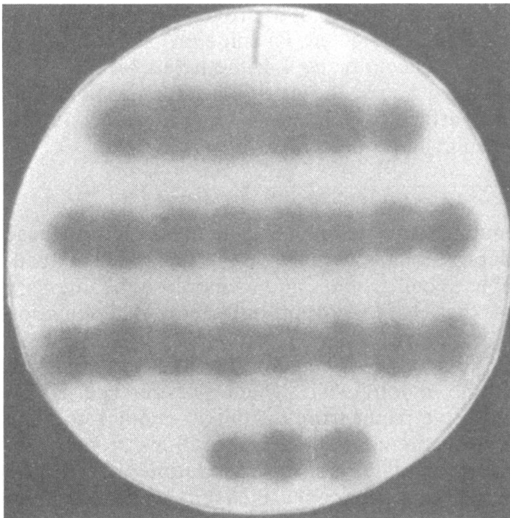

FIG. 2. Results of an HCN detection test. This was an 18-h incubation with *P. aeruginosa* growing on peptone-agar plates at 35°C. Only the depressions corresponding to the spots were inoculated.

Fourteen strains of *P. aeruginosa*, previously shown to be cyanogenic by the picrate strip method (2), were also shown to be cyanogenic by the present method. Several organisms, previously shown to be non-cyanogenic (*P. putida, Escherichia coli, Bacillus megaterium, B. subtilis, Candida utilis, Saccharomyces cerevisiae*) also gave negative results by the depression plate method. Finally, nine non-cyanogenic mutants, derived from the cyanogenic *P. aeruginosa* strain 9-D2, proved to be cyanide negative by the procedure described here.

This procedure may be used in a rapid screening of bacterial isolates from natural environments and could easily be adapted for use with other cyanogenic microbes, including fungi and algae. Other volatile products capable of forming a colored product with chemicals impregnated in a paper disk could potentially be detected by this general method.

This work was supported by National Science Foundation grant PCM 77-24144.

The valuable assistance of E. Hyde is gratefully acknowledged.

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