

## NOTES

# Cultivation of Mycoplasmas on Cellulose Ester Substrates

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The ability of mycoplasmas to grow on cellulose ester substrates was evaluated. *Mycoplasma pneumoniae*, *M. hominis*, *M. arthritis*, *M. gallisepticum*, and *Acholeplasma laidlawii* grew on Millipore (mixed cellulose ester) filters and Sepraphore III (cellulose polyacetate) membranes.

No single medium or method is optimal for the cultivation of mycoplasmas. They have been grown in broth (3), and on agar (3), glass (4), and plastic (5). The latter two methods are especially valuable because they can be used to prepare organisms relatively free of contaminating proteins from the complex media. Standard bacterial forms do not attach well to such hard surfaces but can grow on cellulose-base filters. Various L-forms also have this property, and it has been reported to exist for mycoplasmas as well (2). The purpose of this study was to verify this observation with the use of several substrates and mycoplasma species and to develop techniques which could facilitate the application of this method.

*Mycoplasma pneumoniae* strain FH (obtained from J. Tulley, NIH, Bethesda, Md.) was used, along with species purchased from the American Type Culture Collection: ATCC no. 14192 (*Acholeplasma laidlawii*), ATCC no. 19610 (*M. gallisepticum*), ATCC no. 19611 (*M. arthritis*), and ATCC no. 14027 (*M. hominis*). These were grown in standard Hayflick broth containing 20% horse serum (3). Solid medium (15 ml per 60- by 15-mm petri dish) contained an additional 0.75% Ionagar no. 2S (Colab Laboratories, Greenwood, Ill.). Chlorazol black E (1) plates contained 0.1% of the dye (Matheson, Coleman, and Bell, Cincinnati, Ohio) in addition to agar.

Millipore filters (Millipore Corp., Bedford, Mass.) of mixed cellulose esters, or Sepraphore III electrophoresis strips (Gelman Instrument Co., Ann Arbor, Mich.) of cellulose polyacetate were autoclaved and placed on the agar surface.

A sample (0.1 ml) of a log-phase culture was spread evenly over the filter (47-mm-diameter disk or 25-mm square) with a sterile pipette. Plates were incubated for 7 to 10 days in 5% CO<sub>2</sub>-95% air at 37 C. Filters from chlorazol black plates were then air-dried without further processing. Filters from standard agar plates were air-dried, stained in 2% Dienes stain for 10 s, rinsed in phosphate-buffered saline (PBS, pH 7) for 30 s, and air-dried.

*A. laidlawii* grew well, regardless of whether the inoculum yielded confluent growth or isolated colonies (Fig. 1). Filters with less than 200 colony-forming units (CFU) had colonies 1 to 2 mm in diameter, whereas confluent growth was composed of pin-point colonies similar to those seen when organisms are plated directly on agar. Filter disks placed on chlorazol black plates yielded similar results. However, colonies were colored dark black, whereas the dried filters were light gray. This method, in which the dye is incorporated in the agar and is later concentrated in the colonies during growth, permits easy observation without fixation and staining and may be valuable in other applications.

The ability to grow on Millipore filters was not unique to *Acholeplasma*, since *M. pneumoniae*, *M. gallisepticum*, *M. hominis*, and *M. arthritis* grew in a similar fashion (Fig. 2). Differences in morphology were apparently due to differences in cell density. Sepraphore III cellulose polyacetate membranes, as well as Millipore filters of various mean pore sizes (0.10, 0.22, 0.30, and 0.65  $\mu$ m), all supported growth.

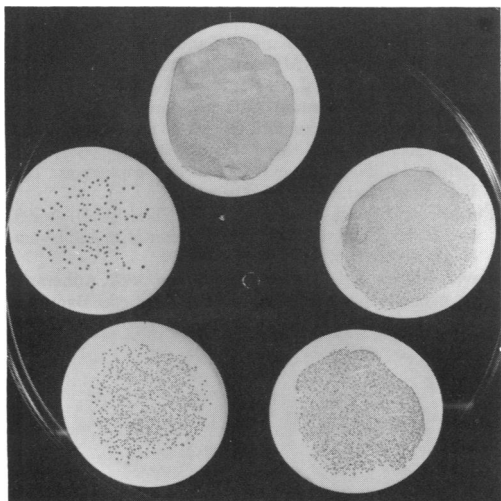


FIG. 1. Millipore filters (0.45- $\mu$ m pores) with various dilutions of *Acholeplasma laidlawii*. Filters were incubated on Hayflick agar and stained with Dienes stain. Dilutions, beginning at the top and reading clockwise, were  $10^{-1}$ ,  $10^{-2}$ ,  $10^{-3}$ ,  $10^{-4}$ , and  $10^{-5}$  of a log-phase culture.

Organisms could be recovered by mechanically scraping the colonies from the surface into PBS. Starting with an inoculum of  $9 \times 10^8$  CFU, the average yield ( $n = 15$ ) was  $3 \times 10^8$  CFU/47-mm filter. A relatively high protein content (5.0 mg/disk) and dry wt (5.7 mg/disk) indicated that media or filter fragments contaminated the cells. A rinsing step, using a standard vacuum apparatus (Millipore Corp.) would be advisable. Sonic disruption for 15 or 30 s did not significantly increase cell recovery from scraped filters.

The manner in which the mycoplasmas grew on cellulose ester substrates was investigated by using scanning electron microscopy. Samples of isolated colonies of *M. pneumoniae* were fixed by using 2.5% glutaraldehyde in PBS for 2 h at 4 C. They were washed for 15 min in PBS, rinsed twice in distilled water, and air-dried.

Examination of critical-point dried specimens by using a Jeolco U3 microscope indicated that the colonies were round, flat, and raised slightly (Fig. 3). Organisms could apparently "weave" themselves into the interstices of the filter so that the colony gave the filter a slight increase in mechanical rigidity. The colony in Fig. 3 extends over the fracture plane. The filter cleaved around the colony instead of through it. (The porous area to the lower right of the pancake-shaped colony of *M. pneumoniae* is the exposed cellulose ester matrix from within the filter.)

The benefits of using this method for the cultivation of mycoplasmas on cellulose ester substrates may be similar to those of glass-surface culture methods. Masses of cells can be washed and collected without recourse to high-speed centrifugation of protein-rich media. In addition, the methodology described here should prove useful in studying the morphology and development of mycoplasma colonies. This

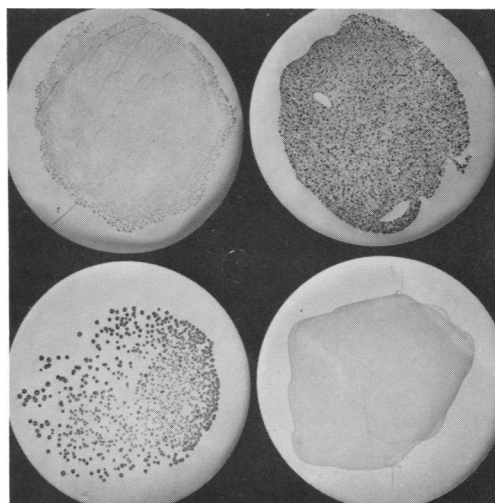


FIG. 2. Millipore filters (0.45- $\mu$ m pores) with colonies of *M. hominis* (upper left), *M. pneumoniae* (upper right), *M. gallisepticum* (lower left), and *M. arthritis* (lower right).

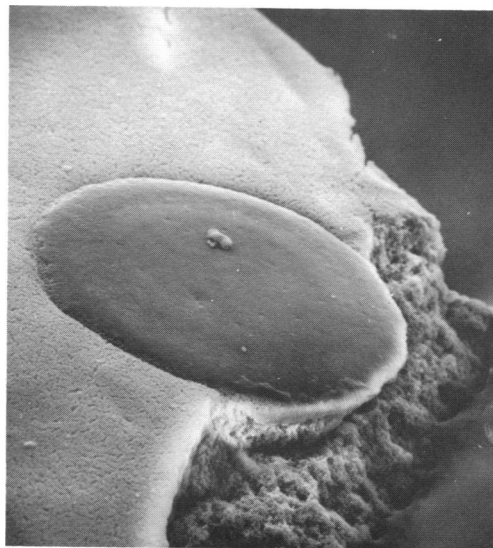


FIG. 3. Scanning electron micrograph of an *M. pneumoniae* colony on the cleavage plane of a Millipore filter (0.45- $\mu$ m pores). Approximately  $\times 200$ .

technique also has potential application as a replica plating process to be used in mutation studies, and as a means of transferring cells to fresh media for induction or labeling experiments.

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