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Use of Dialyzing Culture Technique for High Yield of *Mycoplasma*

MARY E. POLLOCK[1]

Department of Microbiology, University of Minnesota, Minneapolis, Minnesota

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ABSTRACT

POLLOCK, MARY E. (University of Minnesota, Minneapolis). Use of dialyzing culture technique for high yield of *Mycoplasma*. J. Bacteriol. **90**:1682–1685. 1965.—The saprophytic pleuropneumonia-like organism, *Mycoplasma laidlawii*, type A, was cultivated within a dialyzing membrane suspended in a reservoir of uninoculated medium. Generation time was not decreased by this method, but the period of active growth was prolonged, and the final yield was 10-fold that attained by the usual cultural methods. A defined mixture, which alone would not support growth of *Mycoplasma*, could replace the complex soy peptone-yeast extract medium in the inner membrane vessel of the dialyzing culture flask with no decrease in growth rate or yield.

It has been demonstrated (Gerhardt and Gallup, 1963) that concentrated cultures of microorganisms can be obtained by use of a dialyzing membrane through which a continuing supply of diffusable nutrients is made available from a large reservoir of suitable medium. The many applications of dialyzing culture systems have been reviewed by these investigators.

Present methods for cultivation of *Mycoplasma* (pleuropneumonia-like organisms) do not yield suspensions of sufficient density to allow analytical or morphological examination without resort to harsh physical procedures for concentration. Because of the small size of these organisms, high centrifugal force is necessary for quantitative recovery from broth culture. When an organism lacking an intact cell wall is subjected to such treatment, one might predict the occurrence of a high incidence of cell distortion, rupture, and leakage of cytoplasmic constituents. Because the extent of distortion or cell damage may be expected to vary with age of culture, growth sequence studies of washed suspensions must be evaluated with caution. If methods can be devised to increase yield of *Mycoplasma*, then some types of investigations, in particular, studies of morphology, may be carried out without centrifugation.

In addition to the problem of low yield, the necessity for complex media also limits the techniques which can be applied to these organisms. The requirement of most species for serum or comparable undefined supplement often makes the cultivation of large populations economically prohibitive, especially when the presence of extraneous macromolecular components limits the usefulness of some analytical techniques.

With these problems in mind, a simple dialyzing culture flask was devised which combined the advantages of economy and accessibility to the culture vessel. This report describes the technique and results of pilot growth studies with the saprophytic *M. laidlawii* type A.

MATERIALS AND METHODS

Culture. M. laidlawii type A was obtained from the American Type Culture Collection (ATCC 14089).

Media. The basal medium employed for both routine cultivation and experimental purposes (SP-YE; Pollock, Treadwell, and Kenny, 1963) contained, w/v: 2% soy peptone (Sheffield Chemical, Norwich, N.Y.; USP grade), 1% yeast extract (Difco), and 0.5% sodium chloride in distilled water. The *p*H was adjusted to 7.8 before autoclaving. When solid medium was desired, Difco Special Agar (Noble) was incorporated in 0.85% concentration. The defined media employed were L-15 Tissue Culture Medium and Arginine Assay Medium (Difco); these were sterilized by filtration through an 03 Selas after dilution to single strength and *p*H adjustment (7.8).

Dialyzing culture flask. The component parts of the dialyzing flask are shown in Fig. 1. When complex medium was employed, the assembled unit was autoclaved ready for use. For studies with defined mixtures sterilized by filtration, distilled water was used in place of medium during sterilization, and was replaced aseptically

[1] Present address: Scripps Clinic and Research Foundation, La Jolla, Calif.

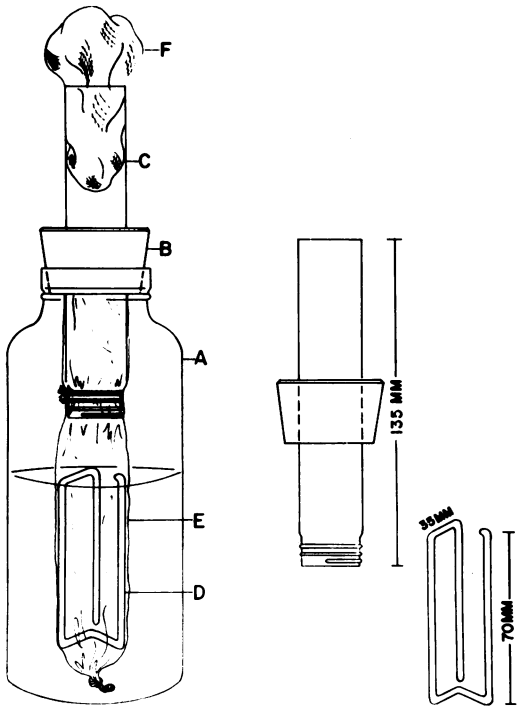

FIG. 1. *Dialyzing flask for cultivation of Mycoplasma laidlawii: A, 500-ml flint glass bottle (A. H. Thomas, 2207); B, no. 8 rubber stopper bored to accommodate C; C, 25 × 150-mm screw-cap tube (Kimax) cut 15 mm from the bottom; D, one-piece glass frame of 4-mm solid glass rod; E, dialyzer tubing (regenerated cellulose, 1.75 inches flat width), hydrated and rinsed, doubly knotted, and secured about the constricted end of the access tube; F, gauze-covered cotton plug.*

with suitable volumes of the desired sterile media. The ratio of reservoir medium volume to that within the membrane was about 10; 250- to 300-ml volumes were most conveniently employed in the reservoir. To assure equalization of pressure on either side of the membrane during autoclaving, an 18-gauge hypodermic needle plugged with cotton was inserted between the rubber stopper and the neck of the vessel.

Incubation. Cultures were used for inoculation after 5 to 8 hr of incubation at 37 C. The number of colony-forming units (CFU) could be predicted from previous growth curves, and the suspension was appropriately diluted with broth; this usually required a final dilution of about 2×10^{-5} to give 10^4 colonies per milliliter.

To facilitate diffusion between the two compartments, the inoculated vessel was agitated by use of a reciprocal shaking water bath (100 oscillations per minute). A magnet in the reservoir, activated by a magnetic stirrer, could also be used, but did not always give as consistent results. Incubation was at 37 C.

The problem of escape of *Mycoplasma* from the inner vessel into the reservoir was encountered only if agitation was too vigorous or the dialyzing membrane was not sufficiently long to envelop the glass access tube to the base of the rubber stopper.

Colony counts. Hanks' solution (Hanks and Wallace, 1949), lacking calcium and magnesium salts and supplemented with 2% human serum, was used for dilution. Fractions (0.01 ml) of serially diluted culture were deposited on SP-YE agar plates which had been allowed to stand at room temperature for 18 hr after preparation. After 48 hr of incubation at 37 C, colonies were enumerated on at least three replicate spots on which between 30 and 300 colonies were present. Colonies were viewed at 20× magnification with a stereoscopic microscope, and accurate counts were obtained with the aid of an electronic colony counter. The number of CFU per milliliter of culture was calculated according to the formula: number colonies counted/number spots counted × dilution × 100 = number of CFU.

RESULTS

Growth in complex medium. Figure 2 shows growth curves of *M. laidlawii* when complex medium was employed in the dialyzing flask. When the medium was inoculated uniformly throughout the vessel, growth both within the membrane and in the reservoir was characteristic of that observed in any standard culture flask. Growth continued to a higher level (approximately 10-fold) when the medium within the membrane only was inoculated.

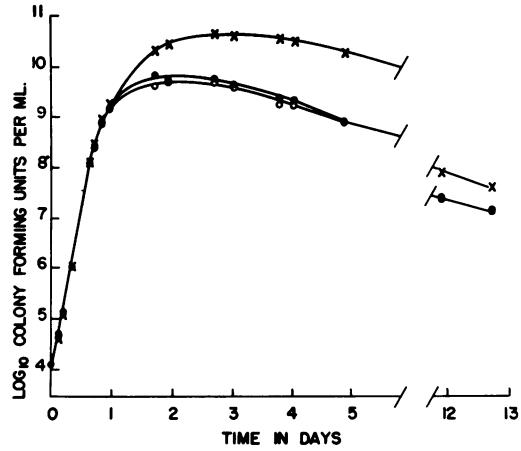

FIG. 2. *Growth of Mycoplasma laidlawii type A in complex medium in dialyzing culture flask. Symbols: × = inner vessel only inoculated; ○ = uniformly inoculated throughout, growth in inner vessel; ● = uniformly inoculated throughout, growth in reservoir.*

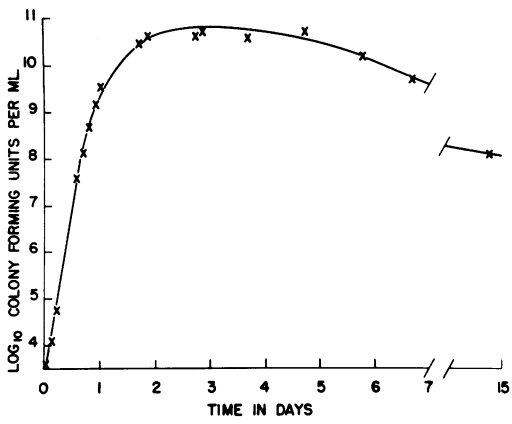

FIG. 3. *Growth of Mycoplasma laidlawii type A in dialyzing culture flask containing defined mixture L-15 suspended within complex medium reservoir; inner vessel only inoculated.*

Figure 3 demonstrates the course of growth when the defined medium L-15 was employed within the membrane suspended in a reservoir of complex SP-YE. Only the inner vessel was inoculated. The maximal population attained and the nature of the curve were almost identical to that of the same culture propagated in complex medium. Results obtained with use of Arginine Assay Medium were indistinguishable from those observed with L-15. Growth did not occur in either L-15 or Arginine Assay Medium alone when a conventional culture vessel was used.

Irrespective of the medium employed, the generation time during the logarithmic phase was approximately 1 hr. Generation time was not shortened by use of the dialyzing technique.

DISCUSSION

Studies of morphology of *Mycoplasma* without resort to methods for concentration which result in distortion have long been indicated, but were impractical because of the low density of culture suspensions and the requirement by most species for undefined supplements which interfered with preparative methods. In investigations of the saprophytic *M. laidlawii* A, the attainment of populations of 5 to 6 $\times$ 10^{10} CFU per milliliter in a dialyzing culture flask has made possible electron microscopic examination of cell morphology without need for centrifugation. Results of these studies will be reported in other communications (Anderson, Pollock, and Brower, 1965).

The generation time was about 1 hr whether or not the dialyzing technique was used, but the period of active growth was increased by the dialyzing flask method. Although yield per milliliter calculated on the basis of total medium volume was not higher than by conventional methods, the achievement of denser suspensions within a confined volume made it possible to work with smaller samples, an advantage even when centrifugation was necessary.

Defined mixtures have been used for cultivation of *M. laidlawii* by conventional techniques, but until recently none supported growth without an undefined supplement (Razin and Knight, 1960). Tourtellotte, Morowitz, and Kasimer (1964) successfully cultivated this species in a defined mixture to which were added purified peptides of known amino acid sequence. A study of the dialysate of the SP-YE medium here described is being undertaken to identify more specifically the peptide requirement and other low molecular weight components which may contribute to high-level growth in an initially defined mixture.

The usefulness of this technique for study of parasitic *Mycoplasma* is more limited, because of the requirement for undefined macromolecular components. However, preliminary studies indicate that higher yields of *M. hominis*, type 1, may be obtained when the undefined supplement is added only to the inner culture vessel. Studies are continuing to determine whether yield of other parasitic strains of *Mycoplasma* can be significantly increased under these conditions.

ACKNOWLEDGMENT

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