

# Replication of *Mycoplasma pneumoniae* in Broth Culture

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## ABSTRACT

LOW, IOLANDA E. (Harvard Medical School, Boston, Mass.), AND MONROE D. EATON. Replication of *Mycoplasma pneumoniae* in broth culture. *J. Bacteriol.* **89**:725-728. 1965.—Reproducible growth curves of *Mycoplasma pneumoniae* can be obtained with the use of shaking cultures, which allows earlier and higher yields of the organism than stationary or roller cultures. Decreasing oxygen tension clearly decreases the growth of *M. pneumoniae* to barely detectable levels, with or without glucose or pyruvate as substrates. The use of phenol red as a pH indicator in the medium is helpful in judging the replication of a culture so that log-phase organisms can be harvested in large amounts.

A major difficulty in working with many *Mycoplasma* strains (pleuropneumonia-like organisms, PPLO) is their irregular, slow growth, with such low yields as to impede research on their antigenic composition, biochemical nature, and physiological mechanisms. This has been especially true with *M. pneumoniae* (Eaton agent), an important human pathogen.

In an earlier preliminary report (Low and Eaton, 1964), we described the use of aeration in the growth of *M. pneumoniae*, which resulted in reproducible growth curves with earlier, higher yields than previously reported (Chanock et al., 1962; Clyde, 1964).

The present communication details and expands the results obtained on the growth of *M. pneumoniae* in broth cultures.

## MATERIALS AND METHODS

**Medium.** The medium used was that of Chanock, Hayflick, and Barile (1962), consisting of seven parts Difco PPLO broth, two parts unheated horse serum, and one part 25% fresh yeast extract, plus thallium acetate, at a final concentration of 1:2,000, and 1% dextrose, unless otherwise noted. Phenol red (0.002%) was added as an indicator to detect pH changes. Agar plates for colony counts were similarly prepared, except for the use of Difco PPLO agar instead of broth.

**Strains.** The FH strain of *M. pneumoniae*, kindly provided by L. Hayflick, was used as the 67th passage on artificial medium, and the Mac strain, isolated from the 79th chick embryo lung passage in our laboratory, was used as the 12th and 13th passage on PPLO medium.

All materials were stored at  $-70^{\circ}\text{C}$ .

**Cultivation and viable counts.** The broth cultures

were incubated at  $36^{\circ}\text{C}$  on a shaker table at a rate of 90 oscillations per minute. Initially, cultures of 40 ml of broth in 250-ml flasks were used in determining the growth curves. However, the volume employed was found not to be a critical factor, since amounts of from 2 to 250 ml have been used, allowing a ratio of 1:4 or 1:5 of fluid volume to air space in the vessels for adequate aeration.

The number of viable organisms was determined by colony counts of 10-fold dilutions of broth samples, spread on PPLO agar plates, and stained with a 1:100 dilution of Dienes stain. In our hands, this proved more accurate than the hemolytic "plaque" counts with guinea pig (Somerson et al., 1963) or sheep (Clyde, 1963) red blood cells in an overlay.

## RESULTS

**Effects of conditions of incubation and omission of dextrose.** Shaking cultures gave an overall 80% success on subculture from agar plates, in contrast to stationary cultures that gave 40% positive, and anaerobic conditions that have, up to this time, been unsuccessful. Fresh isolates from human cases have not yet been tested.

Once the organisms were adapted to artificial medium, shaking cultures, as compared with stationary conditions, gave increased yields of viable PPLO. The yield was, on the average, 2 logs higher, and the time for maximal titers to appear was shortened by 2 to 4 days (Fig. 1).

Since stationary cultures showed only slight turbidity, as would be expected from the lower titers, but of a granular character, the question of dispersion had to be considered. Under conditions (such as roller cultures or direct breakage of clumps) assuring dispersion without increasing

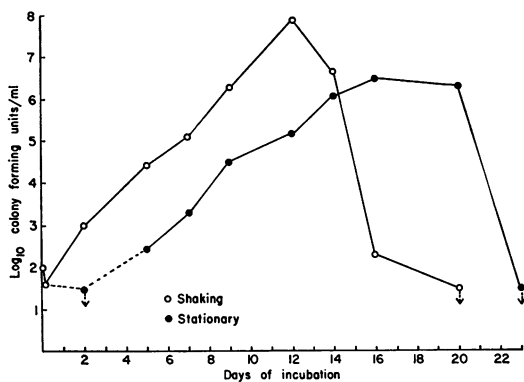


FIG. 1. Growth curves of *Mycoplasma pneumoniae* (Mac strain, passage 13). Comparison of shaking and stationary cultures.

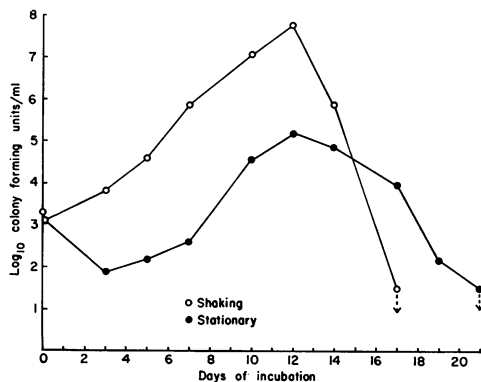


FIG. 2. Growth curves of *Mycoplasma pneumoniae* (Mac 13). Comparison of shaking and stationary cultures without added dextrose.

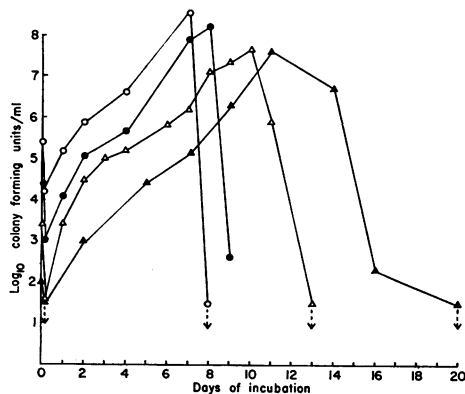


FIG. 3. Growth curves of *Mycoplasma pneumoniae* (Mac 12) at various inoculum concentrations. Inoculum in CFU/ml. Symbols:  $\circ = 10^{5.5}$ ,  $\bullet = 10^{4.4}$ ,  $\triangle = 10^{3.4}$ , and  $\blacktriangle = 10^{2.0}$ .

aeration to any extent, growth curves, in fact, did not differ from stationary cultures, thus excluding clumping as an important factor.

Omission of the added dextrose had little effect on the growth curve of shaking cultures, but caused a considerable lag in the growth curve of the stationary cultures, with maximal titers only 2 logs higher than the original inoculum (Fig. 2).

*Effect of inoculum size.* Figure 3 shows the growth curves of the Mac strain (12th passage) with various concentrations of the inoculum. With a large inoculum of  $10^{5.5}$  colony-forming units (CFU) per milliliter, titers of over 8 logs could be reached regularly by 7 days. With smaller inocula, the appearance of maximal titers was proportionally delayed.

The rapid loss of viability, once maximal titers were attained, was due to the accumulation of acid from dextrose. The rate of this loss was, in turn, proportional to the mycoplasma input and titers obtained.

*Effect of continuing adaptation to artificial medium.* Since the FH strain, which we had received as a well-adapted strain, showed, at a comparable high input, maximal titers by 4 days, we felt that this was a reflection of longer adaptation to artificial medium rather than a strain difference. This proved to be the case (Fig. 4).

The log phase of growth for both the Mac and the FH strains was shortened to 4 days after 67 passages; after 125 passages, the FH strain reached maximal titers at 3 days, whereas the Mac strain did not change significantly. Thus, adaptation has meant a decrease from 12 to 5 hr as the average doubling time of the organism.

*Changes in pH and loss of viability.* The pH

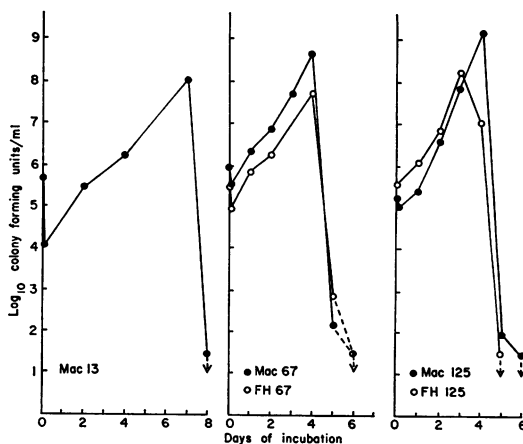


FIG. 4. Growth curve of adapted *Mycoplasma pneumoniae* strains.

changes revealed by the phenol red were a useful tool in judging the replication of a culture and in the harvesting of log-phase organisms. In this experimental system (under shaking), a pH drop from the initial pH of 7.8 to 7.4, accompanied by slight turbidity, usually indicated a colony-forming titer of 6.5 logs per milliliter, or greater. A pH of 7.0, or slightly below, with increasing turbidity, reflected maximal growth, but once the pH had dropped below 6.5 (sometimes as low as 5.5 to 5.0 overnight), there was rapid loss of viability without concomitant loss of turbidity. The rate of this loss depended on the size of inoculum.

The rapid loss of viability due to the accumulation of acid(s) could be delayed as long as 4 days by adjusting the pH to neutrality with 1.0 N NaOH, without change of medium. During this interval, there was only 1 to 1.5 log loss, as compared with controls resuspended in fresh medium.

*Effect of decreased oxygen tension.* Table 1 shows the effect of decreased oxygen tension on shaking cultures. Experiments with an oxygen electrode revealed 10 to 20% of atmospheric oxygen still present after gassing with nitrogen for 1 hr, yet considerable diminution of growth occurred, as seen in Table 1 (addition a). When nitrogen gassing was continued for 1.5 hr, with experimental conditions approaching, but not quite achieving, complete anaerobiosis, the data seen in Table 1 (addition b) were obtained. It is evident that practically no growth occurred with almost complete exclusion of oxygen.

A change in substrate from dextrose to 1% pyruvate (Table 1, addition c) delayed the appearance of maximal titers by about 3 days, but there was no drop of pH below 7.0. Anaerobic conditions, again, are inimical to *M. pneumoniae* replication in this system.

TABLE 1. *Effect of decreasing oxygen tension on Mycoplasma pneumoniae Mac 13 (shaker cultures)*

Addition to medium	N <sub>2</sub> gassing	Initial titer*		Maximal titer	Day reached
		0 min	10 min		
a. None	None 1 hr	2.5	<1.6	8.3	12
				5.4	10
b. 1% dextrose	None 1.5 hr	2.5	<1.6	8.2	12
				2.7	7
c. 1% pyruvate	None 1.5 hr	4.2	2.1	7.7	12
				No growth	

\* Titers in log colony-forming units per milliliter.

## DISCUSSION

Though shaking cultures have been used to obtain higher yields with many PPLO strains, no reports have as yet appeared describing the feasibility of aeration in the growth of *M. pneumoniae*. The immediate usefulness of consistently harvesting large quantities of *M. pneumoniae* at exactly the desired growth phase of the organism is evident. The fact that one can delay the loss of viability associated with high yields by neutralizing the acidity of the cultures gives one, also, considerable latitude in planning experiments. But aside from the convenience and practicality, the experiments present two puzzling problems.

Smith (1964) classified the *Mycoplasma* into two broad metabolic groups, fermentative types and oxidative types. The first puzzle is that *M. pneumoniae*, on one hand, is clearly a fermentative strain, but, on the other, seems to be oxygen-dependent. The only other strain that presents this dichotomy is *M. mycoides* (Rodwell and Rodwell, 1954a, b, c). Of interest, and, perhaps, of significance, is the fact that both *M. pneumoniae* and *M. mycoides* are pathogens.

The absence of glucose degradation under anaerobic conditions was explained by Rodwell and Rodwell (1954a, b, c) as being due to O<sub>2</sub> requirement for the reoxidation of reduced nicotinamide adenine dinucleotide formed by the oxidation of triose phosphate. However, *M. mycoides* could utilize pyruvate anaerobically via the dismutation system to lactic acid, acetic acid, and CO<sub>2</sub> (Rodwell and Rodwell, 1954b), whereas *M. pneumoniae* seems to lack this system.

The second puzzle is the previously mentioned gap between the unpublished statements of many preferential isolations under strict anaerobic cultivation (Mufson et al., 1963) and the dependence of our two laboratory-adapted strains on aerobic conditions. This merits further investigation, since "domestication" (Morton, 1960) may involve changes in metabolism, and perhaps play a role in diminution of pathogenicity.

## ACKNOWLEDGMENT

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