Prolonged Stationary-Phase Growth of T-Strain Mycoplasmas in Broth Culture

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Growth of T-strain mycoplasmas in standard broth cultures has been characterized by rapid growth to peak titers of $10^4$ to $10^5$ color change units/ml at 20 to 24 hr, followed by a sharp decline in the viability over the next few hours. In a vacuum-flow (V-F) system utilizing negative pressure equal to 10 cm of water and air flow on the surface of broth, T strains were seen to enter the stationary phase of growth after 24 hr, and viability titers after 72 hr were $\geq 10^4$ color change units/ml. Maintenance of organisms in the stationary phase required both vacuum and air flow, a medium depth of 1 mm, and 1% urea in the broth. Concentration of ammonia in broth cultures under V-F remained below 200 $\mu$g/ml during 72 hr of observation. Ammonia levels in standard broth cultures exceeded 200 $\mu$g/ml after 24 hr, coincident with the decline in viability. Air passed over the medium surface in the V-F system contained large amounts of ammonia; the amount increased during each succeeding 24-hr period of observation.

T-strain mycoplasmas, common inhabitants of the human genital tract, have been suspected as a cause of non-gonococcal urethritis (NGU) in males since their original description by Shepard (7). Features of their behavior in culture have included formation of tiny colonies on agar (thus the designation "T strains") and growth in broth followed by a rapid demise over a total period of 24 to 30 hr (2). Maximum titers of broth cultures have ranged from $10^4$ to $10^5$ colony-forming units (CFU)/ml (3). In 1967, Ford and MacDonald (3) and Shepard and Lunceford (8) demonstrated the requirement of urea for growth, the possession of urease activity, and the production of ammonia (NH$_3$) by the organisms. Ford and MacDonald (3) suggested that NH$_3$ buildup was instrumental in the rapid death of the organisms, but they were unable to rid broth cultures of this metabolite. Manchee and Taylor-Robinson (5) in 1969 were able to enhance the size of T-strain colonies by the addition of N-2-hydroxyethylpiperezine-N'-2'-ethanesulfonic acid (HEPES) buffer to agar cultures. In broth cultures, HEPES buffer had only a transient effect, increasing the viability of the organisms only a few hours.

This paper describes a new method of growing T-strain mycoplasmas in broth. By combining a small amount of negative pressure and a constant air flow on the surface of the medium, we have prolonged viability of T-strain mycoplasmas for 72 hr. Growth curves of the organisms in this system resemble growth curves of bacteria and other mycoplasmas. Continued production of NH$_3$ over the 72 hr suggested continued metabolic activity of the organisms, but titers did not regularly exceed $10^2$ color change units (ccu)/ml.

MATERIALS AND METHODS

T-strain mycoplasmas. Mycoplasmas were identified as T strains by their ability to grow in broth, at pH 6, with production of an alkaline pH shift in the absence of turbidity, and by the inhibition of their growth on agar by erythromycin but not lincomycin (1). Strain SF2250 was isolated from the throat of an adult with a common cold, and strains U-3 and U-7 were obtained from the urine of university students with NGU. SF2250 was employed both uncloned and three times cloned, and U-3 and U-7 were cloned three times.

Viability titers of broth cultures were determined by serial 10-fold dilutions in broth tubes which were observed for an alkaline pH shift. Titers were expressed as color change units per milliliter.

Media. Broth medium employed has been described previously (4). In addition to PPLO broth base, yeast extract, penicillin, and phenol red, it contained 10% unheated horse serum (Scott Laboratories, Chapel Hill, N.C.) and 1% urea. Agar medium was similar but contained agar base instead of broth base and 0.05 M HEPES buffer; the 1% urea was not added.

Standard method of broth culture. Three milliliters of broth containing approximately $10^5$ ccu/ml of T-strain mycoplasmas were placed in a 3-oz screwcap prescription bottle. The bottle was incubated...
with the flat side down providing a medium layer of approximately 1 mm in depth. The temperature of incubation was 35°C.

**V-F apparatus.** Two 18-gauge needles, rubber tubing, a Pasteur pipette, glass tubing (5-mm outside diameter), a 3-oz prescription bottle, black rubber stoppers, and a 250-ml Erlenmeyer flask were used to construct the culture apparatus (Fig. 1). Vacuum was provided by water suction with a filter pump aspirator. Room air was pulled through a cotton plug in the Pasteur pipette and out of the end of the glass tubing located 10 cm under the surface of the water in the flask. Air was introduced at the rate of 5 to 10 bubbles per sec. Air then flowed through the connecting tubing and the 18-gauge needle in the rubber stopper of the prescription bottle, passed over the surface of the culture, and was drawn out through a second needle connected to two trap bottles (not shown) and then to the water suction. No air entered the system except through the Pasteur pipette; in this way, negative pressure equal to 10 cm of water and a constant air flow were applied to the surface of the culture.

In one experiment, vacuum without airflow was provided by drawing air from the water bottle through a T tube and then directly to the trap bottles. The egress needle was removed from the stopper of the prescription bottle, and a single line from the T tube was connected to the needle piercing the stopper. In this way, the prescription bottle containing the culture was connected to 10 cm of water negative pressure, but there was no movement of air in the bottle. In another experiment, airflow without vacuum was provided by reducing the depth of water in the Erlenmeyer flask so that the tip of the glass tubing was only 0.5 cm below the surface.

**Ammonia production.** The amount of NH₃ present in the culture was determined by the method described by Manchee and Taylor-Robinson (5), with the single modification that Na₂CO₃ instead of K₂CO₃ was used for alakalization of the sample to be analyzed. The NH₃ being removed from the culture in the V-F system was quantitated by using a modification of the V-F arrangement shown in Fig. 1. Air being drawn into the system was bubbled through 10% H₂SO₄ and then through water before entering the Pasteur pipette. Airflow through the system was the same except that the air coming out of the prescription bottle was bubbled through 20 ml of 2% boric acid in a milk dilution bottle. After passage through the boric acid, air was led to the trap bottles and thus to the water suction. In this way, the NH₃ coming out of the culture was trapped in the 20 ml of boric acid. The boric acid bottle was changed after each 24-hr period, and the contents were titrated to a red end point with 0.001 N HCl to measure the amount of base which had been trapped.

**RESULTS**

**Growth curves.** Duplicate 3-ml amounts of broth inoculated with strain SF2250 were incubated under both standard and V-F conditions. Viability titrations were done at selected intervals, and the titers from the two cultures under each set of conditions were averaged (Fig. 2). The growth curves were similar from zero through 24 hr of incubation; peak titers of 10⁶ ccc/ml were reached at or near 20 hr. After 26 hr, the viability of organisms under standard conditions declined rapidly, and, by 45 hr, a concentration of 10 ccc/ml remained. In contrast, organisms incubated under V-F entered the stationary phase of growth after 24 hr. Gradual decline in viability occurred over the succeeding 48 hr, but a titer of greater than 10⁴ ccc/ml remained at 72 hr. In subsequent experiments, titers at various times of incubation were obtained by using the same pool of strain SF2250 under V-F and standard conditions. The range of titers obtained in five experiments under V-F and two under standard conditions did not overlap after 36 hr (Fig. 3).

Two other strains (U-3 and U-7) were incubated under V-F conditions to test their ability to enter the stationary phase. Both organisms entered the stationary phase of growth as determined by curves drawn from titrations done at 24, 48, and 72 hr. Viability titers at 72 hr were 10⁵ ccc/ml with both strains.

Duplicate 3-ml amounts of broth inoculated with strain SF2250 were incubated under vacuum without airflow and titers were obtained at 24 and 48 hr. At 48 hr, the viability titer in the duplicate samples was 10 ccc/ml. The effect of airflow without vacuum was tested in the same way. At 48 hr, the titers in duplicate samples were 10 ccc/ml. Neither negative pressure nor airflow...
alone provided suitable conditions for the organisms to enter the stationary phase of growth.

The depth of the medium was found to be critical for maintaining T-strain mycoplasmas in the stationary phase under V-F conditions. Three milliliters of medium in a 3-oz prescription bottle provided a depth of approximately 1 mm, whereas the depth was approximately 2 mm with 5 ml of medium. The organism remained in the stationary phase of growth with the 1-mm depth, but, with a depth of 2 mm, the viability titer at 48 hr had fallen to 10 ccu/ml. The volume of the culture could be increased if accompanied by a proportional increase in the surface area of the container so that medium depth was approximately 1 mm. Thus, organisms remained in the stationary phase of growth in 15 ml of broth in a 12-oz prescription bottle.

Rocking the bottle containing broth cultures under V-F conditions on an aliquot mixer (LabTek Instruments Co., Westmont, Ill.) at 16 tilt cycles per min had no effect on growth curves.

Ammonia production and urea utilization. Viability titers and concentrations of NH₃ were determined at 24, 48, and 72 hr for cultures of strain SF2250 incubated under V-F conditions in two experiments and under standard conditions in one experiment (Fig. 4). The concentration of NH₃ in the broth rose to 150 to 200 μg/ml in both systems at 24 hr, concomitant with a rise in the viability titer to 10⁶ to 10⁷ ccu/ml. By 48 hr, the NH₃ level in the cultures under standard conditions had risen to approximately 350 μg/ml and the viability titer had dropped.

The NH₃ concentration increased very little after 48 hr in the standard setup. In contrast, the NH₃ concentration in cultures under V-F conditions remained below 200 μg/ml throughout the 72-hr period of observation, whereas the viability titer remained above 10⁶ ccu/ml.

In the same experiments, the amounts of NH₃ recovered from the air passed over the V-F culture surface in the first, second, and third 24-hr periods were 123, 621, and 742 μg/ml of inoculated broth, respectively. The total amount of

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**Fig. 3. Range of values in growth curves of strain SF2250.**

**Fig. 4. Viability of strain SF2250 and concentration of NH₃ in broth under vacuum-flow and standard conditions.**

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NH₃ produced per milliliter of inoculated broth in each 24-hr period is shown in Fig. 5. Values shown for NH₃ production in V-F system include the amount drawn off in the air plus the net change in NH₃ in the broth over the 24-hr period. The amount produced under V-F far exceeded that in the standard system, and this excess increased in each successive 24-hr period.

**Urea concentration of broth.** Three-point growth curves (24, 48, and 72 hr) of strain SF2250 were obtained under V-F and standard conditions. On cultures containing 2%, 0.25% or 0.05% urea in the starting broth, the values in replicate cultures were averaged (Fig. 6). Under V-F, the growth curves with 1 and 2% urea in the broth were similar, whereas with 0.25 and 0.05% urea the titers at 48 and 72 hr were lower. The curves for cultures with 0.25 and 0.05% urea under standard conditions followed the curve for organisms grown in 1% broth under standard conditions shown in Fig. 2.

**Growth on agar.** Colonies of strain SF2250 grown on agar plates incubated in a sealed jar under V-F conditions were tiny, had no lateral growth, and were much inferior in appearance to colonies produced on HEPES agar in an atmosphere of 20% CO₂ and 80% N₂.

**DISCUSSION**

T-strain mycoplasmas in broth culture regularly went into the stationary phase of growth when volatile substances, probably NH₃ produced by the metabolic breakdown of urea, were removed with the V-F system described. Assuming that the accumulation of NH₃ is primarily responsible for the decline in viability, it appeared that there was a critical concentration of NH₃ (ca. 200 μg/ml) which produced this effect. Several conditions were necessary to maintain NH₃ concentrations at or below 200 μg/ml: a medium depth of approximately 1 mm, negative pressure of 10 cm of water, and a continuous flow of air on the medium surface. Aerobic conditions were satisfactory for broth cultures but not for agar cultures, in accord with previous work (2).

In addition to an environment low in volatile substances, T-strain mycoplasmas required sufficient urea for continued metabolism to remain in the stationary phase of growth. However, the exact function of the urea metabolism remains unclear. Urea is required for growth (8), but in excess it is metabolized with the production of NH₃ without an additional increase in the number of viable organisms (3). Shepard and Lunceford (8) noted inhibition of growth of T-strains by urea concentrations of 1 to 2% in standard broth cultures. Urea utilization was maximal at a urea concentration of 0.06%, and viability of the organisms was increased since excessive accumulation of NH₃ was limited. In the V-F system, however, excess urea did not lead to death of the organisms since the NH₃ produced was being removed. The continued availability of urea after 24 hr in broth containing an excess of urea at the outset then allowed maintenance of the organisms in the stationary phase, whereas relatively little urea remained in the broths with low initial concentrations.

On the basis of these results, it appears that the T-strain mycoplasmas are well adapted to their natural habitat, the urinary tract. In this location
urea is continuously available in the urine at an average concentration of 1% (9), whereas the metabolites produced, including NH₃, are removed several times daily by urine flow.

T-strain mycoplasmas have seemed to differ from “classical” mycoplasmas in that colonies on agar were tiny and growth curves in broth were sharply truncated. Recently, Manchee and Taylor-Robinson (5) demonstrated the ability of T strains to produce typical “fried egg” colonies on agar buffered with HEPES. They suggested that the tiny colony size was not an essential feature of these organisms. In addition, T-strain growth curves in broth similar to those of other mycoplasmas were demonstrated in the current study when volatile substances were removed. However, T-strain mycoplasmas have still not been shown to grow to concentrations of 10⁸ ccu/ml in broth, whereas titers of this magnitude have regularly been reached with many strains of classical mycoplasmas.

The original stimulus for the current studies was a desire to produce potent T-strain antigens for hyperimmunization of animals. The effect of NH₃ on the antigenicity of these organisms is not known. The acidity produced by M. pneumoniae in broth cultures has been reported to have a deleterious effect on the antigenicity of that organism (6). This raises the possibility that NH₃ may have a similar effect on the antigenicity of T-strain mycoplasmas, and this will be explored with antigens produced under V-F conditions.

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LITERATURE CITED