Evaluation of [^3H]Thymidine Uptake Method for Studying Growth of Spiroplasmas under Various Conditions

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[^3H]thymidine uptake and colony counts are quantitative and inexpensive methods for studying *Spiroplasma* growth. Using these techniques, we demonstrated subtle effects on the growth of suckling mouse cataract agent of medium alterations, inoculum size, and freezing of cultures. In addition, suckling mouse cataract agent multiplied more actively under aerobic than under anaerobic conditions. These techniques have wide application for the study of *Spiroplasma* growth and will be useful for the development of a defined medium.

Suckling mouse cataract agent (SMCA) is a member of the unique class *Mollicutes* (14) which may have significant medical importance (1-5; F. O. Bastian and R. A. Jennings, Infect. Dis. Newsl. 5:1-3, 1986). Knowledge about the biological growth characteristics of these *Spiroplasma* species is scanty (6), because imprecise, expensive methodology has been required for assaying spiroplasma viability (10). In the present study, by using [^3H]thymidine uptake, the growth of SMCA was measured under different culture conditions. *Spiroplasma* colony counts on Sp4 agar were also performed, and results from the two methods were correlated.

MATERIALS AND METHODS

***Spiroplasma* inoculum.** The experiments were carried out with SMCA, a strain of *Spiroplasma mirum* (provided by J. G. Tully, National Institutes of Health Mycoplasma Laboratory, Frederick, Md.). Primary inocula were prepared from frozen late-log-phase cultures shown to contain 5×10^5 spiroplasmas per ml as determined by counting CFU on Sp4 agar plates or from growing, early-stationary-phase cultures in Sp4 broth consisting of 1.5×10^9 CFU/ml. These cultures were diluted in Sp4 broth to achieve the desired inoculum density.

Media tested. Experiments were carried out in Sp4 broth developed for cultivating spiroplasma growth (13, 15). Agar consisted of Sp4 broth solidified with 1.5% agar (Difco Laboratories, Detroit, Mich.). Broth and agar cultures were maintained under aerobic or anaerobic conditions at an optimum temperature of 30°C (7). Anaerobic conditions were achieved with anaerobic gas packs, palladium catalysts, and anaerobic jars (BBL Microbiology Systems, Cockeysville, Md.).

The growth of SMCA was compared in Sp4 and Sp4 with HEPES buffer (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; 25 mM) as well as a chemically defined medium for spiroplasmas, H1, obtained from K. J. Hackett (8). H1 medium contained low concentrations of inorganic salt and high concentrations of amino acids, nucleic acid precursors, and cofactors as well as the component sphingomyelin (8).

Measurement of [^3H]thymidine incorporation. The growth of *Spiroplasma* species as measured by [^3H]thymidine incorporation into DNA was carried out at 24-h intervals. The SMCA were inoculated into a series of tubes, each containing 1 ml of medium. Three tubes were pulsed with 1 μCi of

[^3H]thymidine (specific activity, 50 Ci/mmol; Dupont, NEN Research Products, Boston, Mass.), 8 h before termination of the uptake. The assay was performed by first adding an equal volume of cold 10% trichloroacetic acid. The precipitate was centrifuged and washed twice with cold 5% trichloroacetic acid. The washed trichloroacetic acid precipitate was dissolved in 1 ml of 0.5 M NaOH and neutralized with HCl. The preparations were transferred into plastic scintillation vials and counted on a scintillation counter with 15 ml of Scintiverse II (Fisher Scientific Co., Pittsburgh, Pa.). Three tubes of Sp4 broth without *Spiroplasma* species, which served as negative controls, were similarly incubated, pulsed with [^3H]thymidine, precipitated, washed, and analyzed as described above. Each determination was performed in triplicate, and the mean was used for comparison with other data.

Determination of colony counts. Quantitation of SMCA was done by enumeration of *Spiroplasma* colonies on Sp4 agar dispensed into plastic petri plates (Becton Dickinson Labware, Oxnard, Calif.). The inoculum for the Sp4 agar was prepared by transferring 10 μl of the culture to 1 ml of fresh Sp4 broth. Subsequent 100-fold serial dilutions were carried out by transferring 10 μl of each dilution to 1 ml of fresh broth. Diluted sample (10 μl) was spread onto the Sp4 agar plates, giving final dilutions of 10^{-4} , 10^{-6} , or 10^{-8} as required. The inoculated plates were incubated at 30°C for 14 days before being counted.

Examination of the plates was carried out by dividing the base of the plate into grids with a felt-tip pen. The colonies were counted by using a Zeiss dissecting microscope equipped with dark-field illumination at a magnification of approximately $\times 200$. The mean of several counts was used to calculate the number of CFU per milliliter. Colonies on Sp4 agar resembled those previously described for *Spiroplasma* species (14) and possessed well-defined umbilicated or "fried egg" configurations.

RESULTS

Thymidine uptake. The [^3H]thymidine uptake analyses of SMCA after inoculation of late-log-phase cultures into Sp4 broth demonstrated a classic growth curve pattern. Spiroplasma growth peaked at 4 days for inocula from an actively growing stationary-phase culture, as opposed to 6 days for inocula prepared directly from a frozen culture (Fig. 1). Thereafter, thymidine counts were gradually reduced. Colony counts revealed similar growth patterns with a peak count of 1.5×10^9 CFU/ml and a 6-h generation time during

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FIG. 1. Comparison of SMCA growth under different conditions as determined by $[^3\text{H}]$ thymidine uptake. Spiroplasma growth was compared by using inoculation of actively growing stationary-phase ($\circ$) and frozen ($\bullet$) cultures in Sp4 broth. Frozen cultures were inoculated into Sp4 medium with HEPES buffer ($\blacktriangle$) and a newly defined medium (H1) ($\triangle$). Each data point represents the mean of three determinations.

log-phase growth (Fig. 2). Peak counts were demonstrated at the same time as by thymidine uptake. The inoculum prepared from the freezer preparation contained 5×10^4 CFU/ml, and that prepared from actively growing stationary-phase cultures contained 1.5×10^6 CFU/ml.

Effect of medium alteration. Frozen cultures inoculated into Sp4 broth containing HEPES buffer produced a longer lag phase in the SMCA growth curve with a peak at 7 days compared with growth in Sp4 alone (6-day peak) (Fig. 1). SMCA grew more sluggishly in the newly chemically defined H1 medium, which has HEPES as a buffer system. In this medium, frozen SMCA cultures showed a prolonged lag period with a slowly developing log phase, reaching an ill-defined peak at 10 days and a secondary peak at 16 days after inoculation (Fig. 1).

Effect of inoculum size. In this experiment, the effect of inoculum size on the *Spiroplasma* growth curve was tested by using three different inocula (1×10^7 , 1.8×10^5 , and $1 \times$

FIG. 2. Colony counts (CFU/ml) of SMCA from either actively growing ($\circ$) or frozen ($\bullet$) culture in Sp4 broth.

FIG. 3. $[^3\text{H}]$ thymidine uptake of different SMCA inocula (1×10^7 [$\blacktriangle$], 1.8×10^5 [$\circ$], and 1×10^4 [$\triangle$] CFU/ml) in Sp4 broth.

10^4 CFU/ml) from a stationary-phase culture in Sp4. These *Spiroplasma* inocula, when placed into fresh Sp4 medium, showed growth peaks at 3, 5, and 6 days, respectively, as measured by thymidine uptake and colony counts (Fig. 3 and 4). Overlapping death-phase curves, occurring at the same time after inoculation, were demonstrated by both methods for all inocula, although cultures from each inoculum density entered log phase at different times.

Gaseous environment. SMCA derived from aerobic actively growing Sp4 broth cultures grew on Sp4 agar plates maintained for 14 days under aerobic conditions with the formation of typical umbilicated colonies, which were optimally visualized under dark-field microscopy.

Growth was also evident on Sp4 agar plates inoculated directly from aerobic Sp4 broth cultures and maintained for 14 days under anaerobic conditions. These colonies did not develop the complete umbilicated appearance seen under aerobic conditions and were difficult to visualize, even under dark-field microscopy. There was no significant $[^3\text{H}]$ thymidine uptake in broth cultures maintained under anaerobic conditions for up to 16 days of incubation. Samples from

FIG. 4. Log_{10} CFU/ml of three SMCA inocula (1×10^7 [$\blacktriangle$], 1.8×10^5 [$\circ$], and 1×10^4 [$\triangle$] CFU/ml) grown in Sp4 broth.

the anaerobic broth cultures were taken at 1-day intervals and assayed by [^3H]thymidine uptake and colony counts on aerobically incubated Sp4 agar plates. There was a waxing and waning of *Spiroplasma* growth, but numbers remained below that of the inoculum (1.8×10^6). The *Spiroplasma* colonies developed an umbilicated configuration but were smaller than the usual aerobic forms.

DISCUSSION

These studies evaluated new methodology for use in the examination of the biologic growth characteristics of spiroplasmas. Previous viability studies of these microorganisms have used an elaborate and uncertain titration method (8, 11). The reason given for abandoning classic colony counting for evaluation of *Spiroplasma* growth has been the small indiscernible colonies produced by spiroplasmas (10). We found that the small *Spiroplasma* colonies could be easily seen by examining agar plates under dark-field microscopy using adequate magnification. Undoubtedly, the CFU assay is still the most accurate and sensitive method for the quantitative evaluation of spiroplasma viability. The companion method, [^3H]thymidine uptake studies, has been previously attempted with spiroplasmas in our laboratory and by others but gave spurious results when traditional filtration methodology was used (12). Our modification of this analytical method for the examination of SMCA growth under a variety of conditions produced reliable, reproducible, and quantitative results which correlated with the corresponding colony counts. The thymidine uptake study used small amounts of media, produced immediate results, and aided our choosing the proper dilutions for inoculations of the agar plates for parallel plate counts.

In this study we examined the growth characteristics of SMCA. We found that the *Spiroplasma* colonies, when inoculated at approximately 10^6 CFU/ml, reached a growth peak at 4 days with a 6-h doubling time during log phase. Variations in inocula altered the length of time cultures remained in lag phase, but the return of thymidine counts to base line occurred the same number of days after inoculation in each case. Excess amounts of thymidine did not alter the shape or peak of the *Spiroplasma* growth curve relative to base line. It was apparent that evaluation of viable counts in the *Spiroplasma* inocula was critical in order to compare different growth curves. Some differences could be explained by variations in inoculum size, such as the faster transition to log phase by actively growing cultures versus frozen cultures. Frozen-culture inocula produced growth curves comparable in shape to growth curves of spiroplasmas derived from active cultures. Figures 1 and 3 demonstrate that inoculum size alone does not account for this observation, since death phases overlapped in time when inoculum size was the only variable.

Besides the obvious use of this methodology in comparing the growth characteristics of different spiroplasmas, the thymidine incorporation assay in combination with CFU determinations has important applications for studying growth conditions for spiroplasmas. In these experiments, the simple addition of HEPES buffer to the Sp4 broth gave a 1-day delay in the initiation of log-phase growth with a slower deterioration of the viability compared with controls. SMCA showed a stepwise growth pattern in the new chemically defined H1 medium, with the log-phase growth beginning near 14 days. SMCA achieved 1 log₁₀ less growth, as determined by thymidine counts, at the peak of the curve in this defined medium compared with Sp4. These experiments

show the powerful application of this new methodology for further development of a defined medium for spiroplasmas by allowing the detection of small differences in growth rates with changing nutrients.

When we tested SMCA growth under strict anaerobic conditions using our methodology, we discovered that SMCA did not prefer anaerobic growth as previously reported (7). Although SMCA grew on Sp4 agar in an anaerobic atmosphere, the spiroplasmas showed evidence of stress, with poorly formed colonies compared with colonies in parallel studies under aerobic conditions. Furthermore, SMCA showed waves of attempted growth activity in anaerobically incubated broth, as documented by colony assay and thymidine uptake, without the development of the growth pattern seen under aerobic conditions. The persistence of *Spiroplasma* viability with periodic attempts to grow under anaerobic conditions may represent anaerobic dormancy (9).

These precise and quantitative results of SMCA growth characteristics, obtained by using a modified thymidine uptake method in tandem with traditional colony counts and dark-field microscopy, suggest that these simple and inexpensive tools should replace current dilution methods for studying growth patterns of spiroplasmas.

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