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Rapid Determination by Light Scattering of Growth Parameters of *Mycoplasma laidlawii* in Liquid Media

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An impediment to progress in the study of the course of growth, the effects of medium components, antibiotics, etc., of *Mycoplasma* has been the cumbersome methods of growth measurement currently in use. Heretofore, it required the plating of numerous samples during growth, at least in triplicate, after appropriate dilution, followed by a delay of 2 to 3 days before the colonies developed so that counts could be made. We applied the technique of light scattering to measure the growth of *Mycoplasma laidlawii* in liquid culture continuously in a manner analogous to the use of absorbancy for bacteria. Scattered light measurements precisely paralleled data obtained by the tedious method of plate counts and were available immediately during the development of the culture. The lower limit of sensitivity with the system described is 10^5 *Mycoplasma* per ml. The presence of serum in the medium lowers sensitivity somewhat. However, concentrations of serum up to 10% are easily tolerated. Higher serum content may require calibration curves. Thus the technique may be used with many pathogens, etc., that require serum to develop. One can easily and rapidly measure differences in growth rates as well as final cell yields during the course of growth, rather than 3 days later, after colonies have developed.

At present there is no rapid method to monitor the phases of growth of a culture of *Mycoplasma* during the growth of the culture. The most accurate and most widely used technique for the determination of a growth curve or the growth rate constant of *Mycoplasma* is to plot a graph of plate counts of samples taken during the development of the culture (2, 5, 10). The computations must be made after the colonies have developed to the point at which they can be counted under a dissecting microscope.

One solution which obviates the 2- to 5-day delay is the use of the principle of light scattering. The amount of light scattered by a suspension of particles or microbes is a function of the particle or cell number. The scattered light technique is most sensitive at low cell concentrations, precisely the region of the growth curve of *Mycoplasma* not easily measurable rapidly by turbidimetric techniques.

We have introduced the technique of light scattering to reduce the time and eliminate many of the manipulations previously required to determine the usual growth parameters of *Mycoplasma laidlawii*. This method allows one to continuously monitor the development of a *Mycoplasma* culture during the growth of the culture in the aqueous medium.

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MATERIALS AND METHODS

Organism, isolation and purification. *Mycoplasma laidlawii*, an isolate from sewage, obtained from H. Neimark (State University of New York, College of Medicine, Brooklyn, New York), was used in these studies.

Growth media. Medium I (Difco; PPLO Broth) consisted of: 0.5% Trypticase Soy Broth (BBL), 0.5% peptone (Difco), 0.2% Yeast Extract (BBL), and 0.5% NaCl. Medium II (7) consisted of: 2.0% Trypticase Soy Broth (BBL), 0.5% tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (Sigma), and 0.5% NaCl. Medium was adjusted to *p*H 8.0 with NaOH. Sterile bovine serum to a concentration of 1 to 10% and PPLO Serum Fraction (Difco) were added as specified below. For plate counts, medium I supplemented with 10% bovine serum was solidified with 1.5% agar. Plates were incubated at room temperature for 3 days to check on sterility as well as to remove excess surface moisture. Dilutions were made in medium I. After inoculation with *M. laidlawii*, the plates were sealed with tape to prevent dessication and incubated for 2 to 3 days at 37 C before counting

under 10 × magnification with a dissection microscope.

Fluorescence and light scattering measurements. A 36-hr culture of *M. laidlawii* containing 10^9 CFU (colony-forming units) per ml was harvested, and the cells were washed once in medium I, twice in buffer consisting of 0.15 M NaCl, 0.05 M Tris·hydrochloride, and 0.01 M β-mercaptoethanol adjusted to *p*H 7.4, and finally resuspended in this buffer at a concentration of 10^9 organisms per ml. The suspension was examined in the Aminco-Bowman spectrophotofluorimeter for fluorescence and light scattering. The initial recording was obtained with an excitation wavelength of 300 nm; the emission wavelength was scanned from 300 to 700 nm. The excitation wavelength was then successively increased by 20 nm until 700 nm was reached, a total of some 20 scans.

Growth curve determination. Three ml of a culture was inoculated into 1 liter of test medium. The contents of the flask were mixed thoroughly and a 0.5-ml sample was withdrawn for the zero-time CFU assay and light scattering determination. The flask was incubated at 37 C on a rotary shaker set at 90 oscillations per min. Samples were examined at regular intervals.

Viable count determinations. The technique used for determining viable counts was essentially that described by Butler and Knight (2). Samples (0.5 ml) were serially diluted in 4.5 ml of medium I, and 0.01 ml of each dilution was spotted in sextuplicate onto dry agar plates. The plates were incubated at 37 C for 2 to 3 days. Plates containing 50 to 200 colonies per spot were counted.

Growth curve determinations by light scattering. Since maximal light scattering was noted at 460 nm, the extent of light scattering for succeeding experiments was measured at 460 nm. The fluorimeter was blanked on the 0.03 full scale with uninoculated medium. Samples from the inoculated flask and the uninoculated flask were removed for scattered light measurements and for plate-count determinations.

RESULTS

Figure 1 shows a typical growth curve of *M. laidlawii* growing in medium I supplemented with 2% serum, measured as viable cell count (CFU). Included in this figure is a concomitant measurement of the absorbancy of the culture. According to the viable cell count data (CFU), a lag of some 17 hr preceded the exponential growth phase, which continued for 10 hr before the culture entered the stationary phase. The generation time in the log phase was 80 min; $K = 0.524$. Absorbancy at 450 or 600 nm increased to a limited extent during most of the development of the culture and became readily apparent only late in the development of the culture at a cell concentration approximately 10^9 *Mycoplasma* per ml, which in this case was slightly less than two generation times before the onset of the stationary phase. An increase of approximately 0.08 absorbance units (A_{450}) heralds the

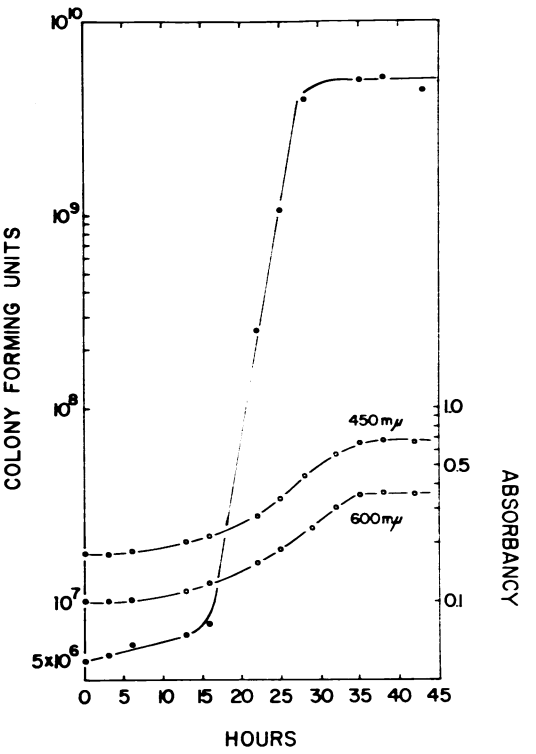

FIG. 1. *Typical growth curve of M. laidlawii growing in PPLO broth; CFU data,* ●.

passage of eight population doublings; in the log phase, an increase in A_{450} of 0.13 units corresponded to an increase in CFU from 10^7 to 3×10^9/ml.

Even after the culture entered the stationary phase according to the CFU data, the absorbancy readings continued to increase. Thus, not only is the sensitivity of this spectrophotometric method poor because of the relatively slight increase in absorbancy during the log phase of growth, but the resultant curve did not accurately reflect the late stages of growth.

Consequently, the usefulness of the light scattering technique for *Mycoplasma* and the wavelength which gives maximal light scattering were examined. Figure 2 shows a series of traces in which an exponentially growing 24-hr culture of *M. laidlawii* was "excited" at a given wavelength of light and scanned for "emission" continuously at wavelengths from 300 to 700 nm. The maximum intensity of emission for any given wavelength occurred at 460 nm. The fact that maximum transmittancy obtains when the emission-excitation wavelength corresponds to the same wavelength suggests that we are observing light scattering.

The usefulness of light scattering for cell enu-

meration was examined. *M. laidlawii* was inoculated into PPLO Broth to an initial concentration of 10^4 CFU/ml. Samples were withdrawn periodically and plated by the procedure we have detailed. Duplicate samples were examined in the Aminco-Bowman spectrophotofluorimeter at 460 nm for excitation and emission, i.e., by the use of the spectrophotofluorimeter to determine light scattered 90°. Uninoculated medium served as the blank on the 0.03 full scale.

The data are shown in Fig. 3. The upper curve (open circles) is the growth curve as measured by viable count. The lower curve (solid circles) was derived from scattered light measurements. The curves parallel each other; it is obvious that the light scattering technique can be used to monitor growth of *M. laidlawii* during most of the development of the culture. The generation time computed from the light scattered data was 90 min; $K = 0.465$. Although the plate counts showed that exponential growth began at about the 13th hr after inoculation, we did not detect any evidence of growth by light scattering until about the 25th hr, that is, until the population reached a concentration of 10^5 CFU/ml. Thus, the lower limit of sensitivity of the instrument fell into the 10^5 particles/ml range. The upper limit of sensitivity on the 0.03 scale full range is about 10^6 particles/ml, but of course this problem is resolved by reading dilutions of the growth suspension. Light scattering permits continuous monitoring of the culture. The 1,000-fold change in the range of 10^5 to 10^8 CFU/ml was easily detected.

Figure 4 shows a similar growth curve in which the medium was prefiltered through a microporous membrane, 0.3-μm diameter pore size. The culture entered the exponential phase at about the 6th hr after inoculation according to the CFU data. Again, light scattering was found to be insensitive at cell concentrations below 10^5/

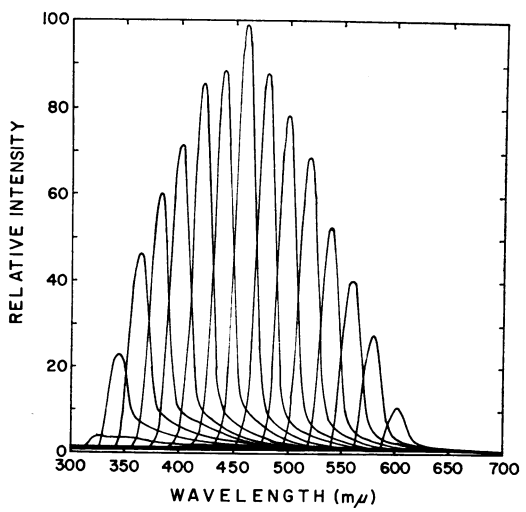

FIG. 2. *Relative intensity of light scattered through a suspension of M. laidlawii measured at an angle of 90°. The initial recording was obtained with an excitation wavelength of 300 nm; the emission wavelength was scanned from 300 to 700 nm. The excitation wavelength was then successively increased by 20 nm until 700 nm was reached.*

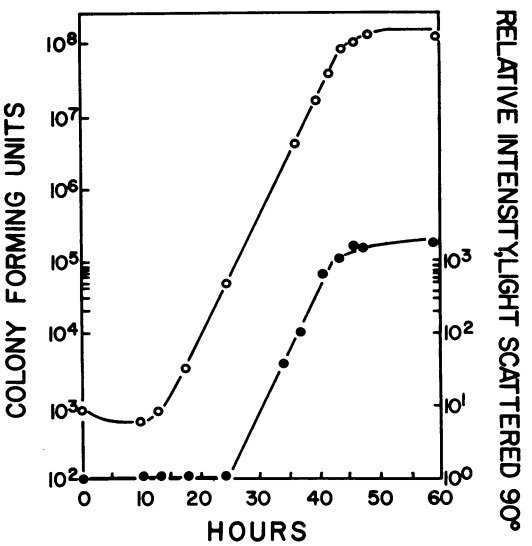

FIG. 3. *Growth curve of M. laidlawii determined by plate counts and scattered light measurements. CFU, ○; scattered light data, ●.*

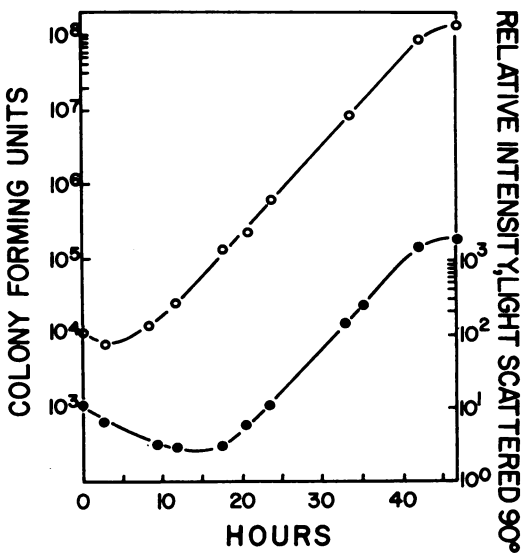

FIG. 4. *Effect of prefiltering the culture medium on the growth of M. laidlawii. CFU, ○; scattered light data, ●.*

ml. Filtration did not increase the sensitivity of the method. However, filtration reduced the growth rate almost 50%. The generation time increased to 150 min; $K = 0.279$.

The effect of serum on the sensitivity of the method was examined. Sensitivity of the technique decreased with increasing serum content until, at a concentration of 10% serum, the sensitivity was reduced by a factor of 10. However, even when sensitivity is reduced to this extent, reliable and useful growth curves are obtained. Thus, the method is probably applicable for many pathogens and other strains of *Mycoplasma* requiring serum for growth. We are currently adapting this method for use with other strains of *Mycoplasma*, e.g., pathogens that require serum for growth.

DISCUSSION

Several procedures have been investigated to obtain growth curves. Smith (8) suggested that the sensitivity of the turbidimetric method could be increased by a preliminary concentration of cells by centrifugation. However, as many as 5×10^5 viable cells per ml may remain in the supernatant fluid after centrifugation at forces up to $40,000 \times g$. As many as 10^8 cells per ml may remain in the supernatant fluid after centrifugation at 10,000 rev/min. Measurement of mean colony diameter on plates under standardized conditions is useful for some species (9). The method of most probable numbers has been applied (3) but can be used only with species capable of developing in liquid media from a few cells. The measurement of acid production has been used to assay growth of fermentative species (2). Growth may be assayed quantitatively by measurement of dry weight (2, 8), deoxyribonucleic acid and protein nitrogen (2, 6). However, in addition to the cautions noted by Smith (10), i.e., cells must be washed free of medium, although diffusion of cell contents must be minimized, the loss of cells during centrifugation may yield inconsistent data. More recently, Anderson, Pollock, and Brower (1) have used the electron microscope for cell counts and for the study of the size distribution of *M. laidlawii*. The methods enumerated above require 2 to 3 days before colonies develop or are impractical for use on a routine basis. At present, viable cell count is generally accepted as the most accurate method for *Mycoplasma* enumeration.

We have applied the technique of light scattering to measure growth in a manner analogous to the use of absorbancy for bacterial cultures. Growth curves of *M. laidlawii* based on light scattering data precisely parallel viable cell count data. After calibration, curves relating CFU to degree of light scattering are plotted; all that is required to determine cell numbers and growth rate or generation times during the course of growth are a few simple readings on a fluorimeter.

With the instrumentation we have employed, the lower limit of sensitivity of the technique is 10^5 *Mycoplasma*/ml. Dilutions of the culture as cell numbers increase beyond 10^6 per ml extend the upper limit indefinitely.

The method should be applicable to species requiring serum. Serum lowers the sensitivity of the method somewhat because of its inherent light-scattering properties. Medium filtration with filters of pore size diameter less than 0.3 μm may obviate this difficulty. We are currently exploring several alternative solutions to this problem.

The relatively high cost of the Aminco-Bowman spectrophotofluorimeter led us to examine less expensive instruments which would still give reasonable sensitivity. The Coleman Jr. Universal Spectrophotometer was not satisfactory because most of the scale was utilized solely to blank out the optically dense growth medium used for *Mycoplasma*. The Turner 110 fluorimeter may be of value if square cuvettes and the corresponding holder are employed. It was necessary to reduce the slit width to its narrowest position, however, and this in turn decreased the lower limit of sensitivity 10-fold.

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