The Measurement of the Growth of Mycoplasma in Liquid Media

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SUMMARY: The growth of *Mycoplasma laidlawii* strain A in liquid media was estimated by colony count, turbidity, and by measurement of dry weight, total-N and deoxyribonucleic acid (DNA) of washed deposits from centrifuged cultures. Samples from growing cultures were also examined with the phase-contrast microscope. The growth curves of cultures obtained by the colony count method resembled a bacterial growth curve. The other methods became useful only when nearly maximum growth had occurred because of the very small yield of organism per unit volume of culture. The growth of organisms in two different media was compared; the growth curves obtained by the colony count were nearly identical but values for total-N and DNA were different.

The colony count method can be very satisfactory for estimating the numbers of viable particles of *Mycoplasma* (Laidlaw & Elford, 1936; Smith, 1956; Butler & Knight, 1956, 1960*a*), but it does not give a measure of the mass of organism present at the time of sampling, nor can it be accepted without evidence that the number of viable particles per unit volume is directly proportional to the mass of organism. The work reported below was directed to compare several methods for measuring the growth of *Mycoplasma* in liquid media, so that the nutritional value of different media might be assessed.

METHODS

Organism. *Mycoplasma laidlawii* strain A (PG 8).

Culture medium. The medium generally used was that of Edward (1947) as modified by Edward & Fitzgerald (1951, 1952) and with the addition of dipotassium phosphate; this medium will be referred to as Edward medium. For convenience of reference the composition of this medium and the method of compounding it are recorded here in detail, because the source and quality of some of its components may be rather critical. The medium was made up from its various components as follows:

(1) 70 vol. of ox heart infusion (1 kg. minced defatted heart/2000 ml. water) at pH 8, containing 1 % (w/v) peptone (Evans Medical Supplies Ltd., London) and 0.5 % (w/v) NaCl. This infusion was solidified when necessary with 2 % (w/v) agar-agar (Japanese shredded; British Drug Houses, Ltd., Poole). Sufficient infusion was prepared at one time for about 3 months work.

(2) 20 vol. of horse serum (Normal no. 2; Burroughs Wellcome, Beckenham, Kent.

(3) 5 vol. of 10 % (w/v) yeast extract (Oxoid; Oxo Ltd., London) solution (pH 8); sterilized by passage through a Seitz filter.

(4) 5 vol. sterile distilled water.

(5) 1 vol. of 0.2 % (w/v) solution of deoxyribonucleic acid (Na salt from thymus; British Drug Houses Ltd.); sterilized by autoclaving for 20 min. at 121°.

(6) 2 vol. sterile m -potassium phosphate (K_2HPO_4) solution.

(7) 1 vol. sterile 1.25 % (w/v) thallium acetate solution.

(8) 1 vol. penicillin G (Na salt; Glaxo Laboratories Ltd., Greenford, Middlesex) solution containing 10,000 units/ml. Penicillin was incorporated in the liquid medium only. Drops of penicillin solution were spread on the surface of solid medium before inoculation.

Liquid Edward medium was prepared by mixing solutions 1 to 8, adjusting to pH 8 with N-NaOH . For solid medium solutions 2 to 7 were added to molten heart-infusion agar at *c.* 50°; 12.5 ml. volumes of the complete medium were pipetted into 4 in. diam. Petri dishes. Sufficient quantities of complete liquid and solid media were prepared for 3–4 weeks work and were stored at 4°. Petri dishes of solid medium were stored in closed tins.

A modification of Edward medium (2PSY medium) was also used; for this the heart infusion and the deoxyribonucleic acid were omitted and the concentration of peptone doubled.

Conditions of cultivations. Ten ml. liquid Edward medium in a L-shaped tube (van Heyningen & Gladstone, 1953) were inoculated with 0.1 ml. stock culture and rocked gently in a water bath at 37° for 24 hr. Organisms were harvested and washed according to the method of Butler & Knight (1960*a*). For experiments 10 ml. liquid medium in a L-shaped tube were inoculated with 0.1 ml. 10^{-3} dilution of washed suspension and rocked gently in a water bath at 37° for 3 days.

Measurement of growth. Growth was assessed by: (i) the colony count method of Miles & Misra (1938) as adapted for *Mycoplasma* (Butler & Knight, 1960*a*); (ii) the turbidity of cultures as measured by an EEL nephelometer (Evans Electroselenium Ltd., Essex); (iii) the dry weight of the washed centrifuged deposit from 80 ml. samples of culture; (iv) the measurement of the washed centrifuged deposit from 5 ml. samples of culture by (a) total-N (micro-Kjeldhal method; Ma & Zuazaga, 1942, with nesslerization instead of titration of the distillate) and (b) deoxyribonucleic acid (DNA) by the method of McIntyre & Sproull (1957) but taking ten times their volumes of reagents.

Samples of the growing cultures were also examined with the phase-contrast microscope.

RESULTS

Comparison of different methods of measuring growth in Edward medium

The growth curve of *Mycoplasma laidlawii* strain A grown in Edward medium obtained by the colony method count resembled a bacterial growth curve (Fig. 1). The doubling time during the logarithmic phase was about 1.3 hr.

Whereas it was possible to obtain a record of growth from the start of an experiment by the colony count method, all the other techniques became practically useful only when near maximal growth had occurred, because of the small mass of organism/unit volume culture (Fig. 1). Even then the values for

dry weight, total-N and DNA were very small, e.g. the dry weight value for a 48 hr. culture was *c.* 0.2 mg./ml.

The values obtained on washed centrifuged deposits have not been corrected for a small deposit which occurs on incubating uninoculated Edward and 2PSY media and which might also occur in cultures.

Fig. 1. The growth of *Mycoplasma laidlawii* strain A in Edward medium as measured by different methods: colony count of viable particles in culture, —○—; turbidity of culture, —×—; washed centrifuged deposit from culture: dry weight, —□—; total-N, —▼—; DNA, —△—.

At the end of the stationary phase as measured by colony count the values for total-N and DNA decreased, but the dry weight and turbidity continued to increase. This increase coincided with increases in the amount of miscellaneous crystalline material as seen under the phase-contrast microscope.

The culture became slightly turbid (granular) to the naked eye at a colony count of 10^6 to 10^7 viable particles/ml., i.e. after incubation for 18–20 hr. As seen with a phase-contrast microscope this turbidity corresponded with the appearance of granular structures (roughly spherical, $2\text{--}20\mu$ diameter); many smaller and less compact groups of granules and single granules (*c.* $0.2 \times 0.4\mu$) were also seen. About 10^4 large granules/ml. were counted in a haemocytometer chamber after staining with methylene blue. These granules were difficult to break up, e.g. by crushing under a coverslip. They disappeared at *c.* 24 hr.

when the colony count was 1 to 2×10^8 viable particles/ml. and the cultures became uniformly turbid to the naked eye. Microscopical examination of samples taken after incubation for 86 hr. and later showed increasing amounts of miscellaneous crystalline and granular particles.

Comparison of growth in two different media

The colony count of samples of *Mycoplasma laidlawii* strain A grown in 2PSY medium were similar to that for growth in Edward medium, but from cultures in 2PSY medium the values for total-N and DNA were c. 20 and 37 % lower, respectively, than from Edward medium (Fig. 2). These differences as between the two media were not accounted for by the deposits which occurred in uninoculated media. No microscopical differences were observed between the cultures in the two different media and the turbidity values were very similar.

Fig. 2. A comparison of the growth of *Mycoplasma laidlawii* strain A in Edward medium (solid lines) and 2PSY medium (dotted lines) as measured by different methods: colony count of viable particles in culture, ○ ○; in washed centrifuged deposit from culture: total-N, ▼ ▼; DNA, △ △.

DISCUSSION

Even the most profuse growth of *Mycoplasma laidlawii* strain A in the best available medium only gave small yields of organism, of the order of 0.2 mg./ml.; many bacteria, for example, give at least ten times this yield. This restricts

the use of methods for assessing growth of *Mycoplasma* which depend on measurements made on washed centrifuged deposits from samples of culture of reasonable size (e.g. 5–10 ml.).

The difference between the organic content of corresponding deposits from cultures grown in Edward medium and 2PSY medium, while the colony count was the same, deserves some comment. While the discrepancy might at least be partly affected by deposition of material as occurred in uninoculated media, this could not account for all of it. There must have been different masses of organism produced in the two media. Further evidence in favour of this was the observation that the values for total-N and DNA in washed centrifuged deposits from cultures in the two media decreased after the stationary phase, corresponding with decreases in colony count. The lower total-N and DNA values in 2PSY medium might be due to a smaller size of the viable particles which are perhaps really micro-colonies. If the 'viable particles' are the micro-colonies then they appear to be relatively stable under our conditions, e.g. they did not break up on handling, otherwise the colony count method would not have been found so reliable (Butler & Knight, 1960*a*). It would appear that reliance should not be placed on the results of using only one method for assessing the 'growth' of *Mycoplasma* when comparing the nutrient values of different media.

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