Microcalorimetric Detection of Growth of Mycoplasmatales

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SUMMARY

A static ampoule microcalorimeter was used to study the growth of mycoplasmas, achleoplasmas and ureaplasmas. Growth as indicated by thermograms was compared with the results of conventional methods, namely, terminal dilution counts, plate counts, turbidimetric measurements, glucose consumption and pH changes. Removal of oxygen had little effect on mycoplasma growth. The microcalorimetric method is potentially useful for identifying and enumerating the members of the Mycoplasmatales.

INTRODUCTION

All biological processes produce heat, which can be detected quantitatively by calorimetry, and so it is possible to record microbial growth without disturbing the culture system.

The low specificity of calorimetry sometimes limits its use as a general analytical tool, particularly in chemistry. When complex biological systems are studied, however, it is unlikely that observations made with a single specific method are adequate for the characterization of the whole process. Thus, as non-specific methods are more likely to be useful than more specific ones, calorimetry has considerable potential for investigating general processes in bacteria and yeasts. Microcalorimetric methods have recently been improved and their use as general analytical tools in microbiology is now being pursued (for review, see Spink & Wadsö, 1975).

We have applied the microcalorimetric method to members of the order Mycoplasmatales, which is divided into two families, Achleplasmataceae and Mycoplasmataceae. The former includes one genus, *Achleplasma*, and the latter, two, *Mycoplasma* and *Ureaplasma* (Edward, 1974). All species of *Achleplasma* and most species of *Mycoplasma* ferment carbohydrates such as glucose. The species of *Mycoplasma* that do not ferment carbohydrates break down arginine by the dihydrolase pathway as a major energy-yielding mechanism, but this may not be the only energy source which they can use (Woodsen, McCarty & Shepard, 1965). In contrast to achleoplasmas, mycoplasmas have an obligate need for sterols. Ureaplasmas are unique within the order in being able to hydrolyse urea (Taylor-Robinson *et al.*, 1966).

Our results showed that microcalorimetry could be used to detect growth and to identify and count the microbial population. The use of the method for optimization of growth media and for antibiotic sensitivity tests was also considered.

METHODS

Calorimeter. A twin microcalorimeter of the heat conduction type was used. The instrument and its operation have been described previously (Wadsö, 1974; Spink & Wadsö, 1975). Samples were enclosed in Teflon-coated stainless-steel ampoules, volume 7.5 ml. The temperature of the calorimeter was maintained at 37.00 °C. The voltage signal was amplified with a Keithley 150 B Microvolt Ammeter (10, 30 or 100 μ V range; Keithley Instruments, Cleveland, Ohio, U.S.A.) and was recorded by a Servogor RE51 recorder (100 mV range; Goerz Electro, Vienna, Austria). The baseline stability for the instrument was 1.5 μ W over 24 h.

Organisms. *Mycoplasma fermentans* M274 and *M. hominis* M18 had been isolated from clinical specimens sent to the Department of Bacteriology, University Hospital, Lund. The strains of *Acholeplasma laidlawii* A and *A. granularum* were obtained from Dr D. Taylor-Robinson, CRC, Harrow, Middlesex. The strain of *Ureaplasma urealyticum*, serotype I (Black, 1974), was obtained from Dr M. Shepard, Camp Lejeune, North Carolina, U.S.A.

Growth media. Mycoplasmas and acholeplasmas were grown in PPLO broth (Difco) with 20 % (v/v) horse serum, 10 % (v/v) of a 25 % (w/v) extract of dried yeast, 0.05 % (w/v) thallium acetate, penicillin G (1.5×10^6 i.u. l⁻¹), and glucose (5 g l⁻¹). The glucose was replaced by L-arginine (7.16 g l⁻¹) when culturing *M. hominis*. Solid medium, for plate counts, contained PPLO agar instead of PPLO broth. *Ureaplasma urealyticum* was grown in 3 % (w/v) trypticase soy broth (BBL) with 20 % (v/v) horse serum, 0.04 % (w/v) urea and penicillin G (1.5×10^6 i.u. l⁻¹). In some experiments urea was omitted. Solid medium for the growth of ureaplasmas contained 1.13 % (w/v) Ionagar no. 2 (Oxoid) in trypticase soy broth medium.

Calorimetric measurements. A sterile calorimetric ampoule was charged with 5 ml of a broth culture (test ampoule) and another was charged with sterile growth medium (reference ampoule). Unless otherwise stated, the gas phase (2.5 ml) in the ampoules was air. At the start of the experiment, the media in the ampoules were in equilibrium with the atmosphere. In a few experiments, nitrogen (purity 99.95 %) was bubbled through the culture medium for 10 min at a rate of 1 l min⁻¹ in a nitrogen tent, and the ampoules, which had been so treated, were charged under the same atmospheric conditions. Ampoules were held at 37 °C for 5 min before they were introduced into the calorimeter. They were allowed to rest for 3 min and 7 min in the two heat exchange positions and were then inserted into the ampoule holder in the thermopile zone.

When the experimental conditions had been established, each experiment was done twice.

Titration of organisms and turbidimetric measurements. Initially, microbial counts were done on broth medium cultures in calorimeter ampoules, but identical results were obtained with plastic-capped test-tubes and these were used subsequently. The ampoules used for microbial counts were charged simultaneously with those used for calorimetric measurement and they were incubated in the calorimetric thermostat. Usually counts were determined as the number of colour changing units (c.c.u.) by the microtitration method (Taylor-Robinson *et al.*, 1966). Tenfold dilutions were made in medium containing phenol red (20 μ g ml⁻¹) as a pH indicator, and cultures were incubated at 37 °C until colour changes ceased. One colour changing unit was taken as the highest dilution that caused the medium to change colour.

In some experiments samples from serial dilutions were transferred directly to solid medium. The plates were incubated at 37 °C in an atmosphere of 10 % CO₂ in nitrogen. Colonies were counted with a stereomicroscope and results were expressed as colony-forming units (c.f.u.) per ml culture medium.

Fig. 1. (a) Typical thermogram of *Mycoplasma fermentans*; (b) its integrated form; and (c) the logarithmic representation. ○, Cell count (c.c.u. ml⁻¹).

Turbidity was measured at 540 nm.

Other determinations. Glucose concentration was normally determined by the GOD-PERID method (Boehringer, Mannheim, Germany), but in a few experiments a Beckman Glucose Analyser (Beckman Instruments, Geneva, Switzerland) was used. Lactic acid was determined enzymically using lactic dehydrogenase (Sigma), and pH was measured with a no. 26 Radiometer pH meter (Radiometer, Copenhagen, Denmark).

Antibiotic susceptibility tests. In some calorimetric experiments, *M. hominis* M18 was cultured in the presence of various concentrations of doxycycline (Pfizer, New York, U.S.A.).

RESULTS

Mycoplasmas

Mycoplasma fermentans. The calorimetric curve, the 'thermogram', for the growth of *M. fermentans* (Fig. 1) has a simple bell-shaped form. The integrated form of the thermogram, however, shows no clear resemblance to that of the microbial count. The log *Q* curve (heat quantity versus time) shows a linear phase which deviates when the pH of the medium falls below 7. The decrease in glucose concentration from about 5 to 2.5 g l⁻¹ approximately paralleled the drop in pH value.

In experiments on the effect of oxygen tension, the growth medium in one ampoule was equilibrated with nitrogen and that in a second ampoule was equilibrated with air. The 'anaerobic' and 'aerobic' ampoules were placed in the heat measuring position alternately, the ampoule not in the calorimeter being placed in a metal tube in the calorimeter thermostat to maintain its temperature. Measurements of the cell count and the turbidity were also made. Results were the same for both aerobic and anaerobic conditions, as were the results of glucose determinations and pH measurements.

In several of the growth experiments, lactate concentrations were measured, but no changes were found.

Mycoplasma hominis. Growth experiments with *M. hominis* showed that the length of the lag phase depended approximately on the size of the inoculum. The lag phase was followed by a short phase of rapid heat evolution. The thermograms showed marked differences in the decline phase. A typical thermogram is shown in Fig. 2, together with the variation in cell count, expressed in c.f.u. ml⁻¹. The c.c.u. method gave higher counts. The pH increased slightly during growth.

Fig. 2

Fig. 3

Fig. 2. Typical thermogram of *Mycoplasma hominis*. ●, Cell count (c.f.u. ml⁻¹).

Fig. 3. Thermograms of *Mycoplasma hominis* grown in the presence of doxycycline, (a) 0.25 μg ml⁻¹, (b) 0.10 μg ml⁻¹; or (c) in its absence. Readings were taken sequentially; the continuous lines indicate times when each sample was being measured.

Fig. 4. Typical thermograms of (a) *Acholeplasma laidlawii* A, (b) *Acholeplasma granularum* and (c) *Ureaplasma urealyticum*.

The susceptibility of *M. hominis* to doxycycline was measured calorimetrically (Fig. 3) and by parallel viable counts. Calorimetric measurements were carried out interchangeably as described above. There was a marked difference between the thermograms, although this was not reflected in the viable counts. Changes in pH similar to those in normal cultures were observed. With higher concentrations of the antibiotic (0.5 and 1.0 μg ml⁻¹), the thermograms approached a steady state and no viable organisms could be detected after about 20 h. The constant heat production, about 3.5 μW ml⁻¹, continued until the experiments were terminated after 45 h.

Acholeplasmas

Acholeplasma laidlawii A. In all experiments, the thermograms had characteristic profiles (Fig. 4a).

Acholeplasma granularum. In two experiments in which ampoules were inoculated with 10⁶ organisms ml⁻¹ (c.c.u.), the thermograms were highly structured with two main peaks.

There was good qualitative agreement between the finer details of the two thermograms, one of which is shown in Fig. 4(b).

Ureaplasmas

Ureaplasma urealyticum. Narrow, nearly symmetrical bell-shaped curves were obtained in two experiments; one of them is shown in Fig. 4(c). There was a large increase in pH from 6.2 to 8.0; but the viable count (c.c.u. ml⁻¹) remained constant at 10⁶ organisms ml⁻¹ during the time when the pH increased and the major heat evolution occurred. When the medium did not contain urea (other than that present in the horse serum), only very small heat effects were observed.

DISCUSSION

Our results show that the heat effects usually produced in cultures of members of the *Mycoplasmatales* are well in excess of what is required for reliable microcalorimetric measurement and that there seem to be no particular difficulties involved in using the calorimetric technique. The precision of the calorimetric measurements was about 1 % when the heat effect was $\geq 10 \mu\text{W}$. The reproducibility of cell counts (c.c.u. and c.f.u.) was at least one order of magnitude less. The thermograms obtained with *M. fermentans* suggest that these organisms are less sensitive to variations in oxygen pressure than are bacteria (see Binford, Binford & Adler, 1973; Monk & Wadsö, 1975).

The number of mycoplasma organisms in a broth culture is usually determined as colour changing units, since this is more convenient than counting numbers of colonies. The colour changing unit method usually gives a higher count than the latter, and we found this in our experiments. The organisms have a tendency to aggregate which may partly explain the differences noted.

Detection of changes in turbidity is an important method for measuring bacterial growth, but it is unsuitable for mycoplasmas; the changes are small and are easily obscured by the presence of insoluble particles from the medium, which also make dry weight measurements difficult. In addition the reliability of turbidity measurements may be affected by the filaments which these organisms form, and which vary in length during the growth cycle, and by the tendency of the organisms to adhere to glass surfaces.

We believe that because of the unreliability of present methods of determining mycoplasma growth, the calorimetric method should be considered as a feasible alternative. Calorimetric measurements are much more precise and the growth is recorded instantly and continuously.

When curves showing variation in viable counts are compared with the corresponding thermograms, correlation is usually poor. This may be partly due to the unreliable numbers obtained by cell counting, but there is no reason why the heat effect values should be strictly proportional to the number of living organisms because calorimetric values measure the heat evolved by the total metabolic activity of the culture. It is likely, therefore, that the conversion factors between 'cell counts' and heat effect values will differ not only for different species, but also for different growth phases. The heat effect produced per organism during the lag phase may well be different from the amount produced during the exponential phase of growth.

The smallest concentration of growing organisms of the order *Mycoplasmatales* that can be detected with our ampoule calorimetric method is 10⁶ organisms ml⁻¹. The corresponding number for fast growing bacteria is about 10⁴ organisms ml⁻¹, as found in flow calorimetric studies (Beezer *et al.*, 1974).

Boling, Blanchard & Russel (1973) have suggested that the characteristic thermograms obtained from different bacteria may be used for identification purposes. Certainly in view

of the shapes of the thermograms that we obtained with achleoplasmas, mycoplasmas and ureaplasmas, it may be possible to use a calorimetric technique to differentiate between organisms belonging to these three genera. There may also be differences between species within these genera. However, defined growth media should always be used when calorimetric methods are used for identification, which is an obstacle in microcalorimetric studies of Mycoplasmatales.

The differences in heat effects obtained when *M. hominis* was cultured in the presence of sublethal doses of doxycycline (Fig. 3) suggest that calorimetry may be valuable in studying the antibiotic susceptibilities of organisms belonging to the order Mycoplasmatales.

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