Continuous opacity monitoring of the growth of bacteria under strict anaerobic conditions

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SUMMARY A newly designed instrument is described which generates continuous records of the opacities of six bacterial cultures growing under strict anaerobic conditions. Additions (for example, of antibacterial agents) or withdrawal of culture (for example, for viable counting) can be made at any time without breach of anaerobiosis. Use of the instrument is illustrated by growth curves obtained from small inocula of two strict anaerobes, *Bacteroides asaccharolyticus* and *Peptostreptococcus anaerobius* and by the effects on the growth curve of *Bacteroides fragilis* of adding various concentrations of metronidazole at different times.

Continuous opacity measurement of the growth of aerobic bacteria, particularly when supplemented by morphological studies, has provided considerable insight into the nature of bacterial responses to antimicrobial agents. In our own studies, continuous measurements of the opacity of growing bacteria have been generated by twelve-channel devices^{1,2} based on the very simple system devised by Watson *et al*³ in which light from a 12 V festoon bulb supplied by a stabilised power source is focused by the tube containing the growing culture onto a silicon photocell. Simple controls make it possible to adjust the output on a multichannel 10 mV dotting recorder to read zero when the tube contains uninoculated broth and full scale when it contains fully grown culture. Magnets act on Teflon-coated magnetic bars within the tubes to stir the cultures which are housed in an insulated box maintained at $37 \pm 1^\circ\text{C}$ by a thermostatically controlled fan heater.

By suitable adjustment of the cultural conditions, these and other commercially available opacity reading devices can be used to record the growth of the less demanding anaerobic bacteria, but the methods have several limitations. The cultures (in prereduced medium) must be rendered airtight by covering with liquid paraffin or Plasticine^{4,5} and this restricts the ease with which additions and withdrawals for morphological studies or viable counts can be made. Large inocula are required⁶ and even then consistent growth of all test organisms does not occur.⁵ Better results may be obtained by using media containing

potent reducing agents, but commercial thiol broth inactivates penicillins and gentamicin⁷ and penicillins have been held to be unstable in the presence of sodium thioglycolate.⁸

We describe a device which generates continuous opacity records of bacteria growing in conditions corresponding with those of the conventional anaerobic jar and in which antibiotics can be added or samples removed without disturbing the anaerobic conditions.

Material and methods

Ability of the instrument to support the growth of strict anaerobes was tested with *Bacteroides fragilis* (NCTC 9334), *Bacteroides asaccharolyticus* (NCTC 9337) and a clinical isolate of *Peptostreptococcus anaerobius* kindly supplied by Dr AT Willis. Brain-heart infusion broth, Oxoid (BHI) supplemented with 5 mg yeast extract, 5 μg haemin and 1 μg menadione/ml (BHIS) was used throughout. Susceptibility of *B. fragilis* in this system was tested by making appropriate additions of metronidazole dissolved in sterile distilled water.

INSTRUMENT

Fig. 1 shows a diagrammatic cross section through the instrument. A circular aluminium block, seen in Fig. 2, was drilled *vertically* to accommodate a single central light source (A), *radially* to allow light from the central source to fall on six photocells mounted on the outside of the block (a cover is seen at B) and *vertically* (C) through the light channels to accom-

Fig. 1 Cross section through the main instrument block and cover; slightly simplified; not to scale.

A: Aluminium body of block, B: Water circulation channel, C: water-tight top plate sealing water-channel, D: wells with culture-tubes containing magnetic stirrers, E: Festoon bulb, F: upper bulb connection to body of block, G: lower bulb connection through air-tight insulator, H: light path, J: stout glass disc, K: O ring, L: washer clamping glass onto O ring producing air-tight seal, M: photocell, N: photocell cover, O: channel for electrical connections, P: Perspex wall of cover, Q: O ring air-tight seals between Perspex cylinder and block below and cover above, R: stainless steel cover plate, S: covers for culture tubes, T: needles for additions to and withdrawals from cultures, U: Luer fitting taps, V: Schrader valve W: supporting pillars, X: Base-plate, Y: clamping ring, Z: clamping screws.

Fig. 2 Main block of the instrument showing (A) cap of central light source (B) cover of photocell (C) hole for insertion of culture tube (D) plate covering circulating channel for temperature-controlled water (E) O rings providing air-tight seal for cover.

modate six 70 × 20 mm flat-bottomed screw-capped tubes at points which caused the fluid-filled tubes to focus the light onto the photocells. A deep channel was milled close to the edge of the upper surface of the block in an almost complete circle and covered by a plate (D) to produce a watertight channel. Tubes leading to the ends of the channel were connected to a thermostatically controlled

Fig. 3 View of underside of main block showing (A) magnets driven by (B) gear train.

water circulator (Grant FH/5) which maintained the block and the contained cultures at $37 \pm 0.5^\circ\text{C}$. Scaling arrangements as used in the original Watson device³ were applied to each channel. As seen in Fig. 3, beneath each tube rotating magnets (A) driven by a nylon-gear chain (B) activated Teflon-coated magnetic bars in the tubes.

To render the system gas-tight, the wells for the culture tubes were drilled to within 3 mm of the base leaving sufficient metal to withstand evacuation of the system but allow the external magnets to stir the cultures. One connection to the central light source was made to the body of the block and the other brought out below through an airtight seal. In the ports for the photocells, circles of stout glass were pressed onto O rings by metal washers to render the light channels airtight. The photocells were mounted against the glass. A shelf (Fig. 2E) was formed around the upper surface of the block carrying O rings to provide an airtight seal for the cover.

To allow conditions inside the chamber to be seen, culture to be withdrawn and additions to be made, the cover was constructed of a stout Perspex cylinder, cut away at the top to form a shelf carrying an O ring onto which a stainless steel plate was pressed by a metal ring which clamped the cover into position (Fig. 4). This plate carried six stainless steel taps with Luer fittings in positions corresponding with the culture tubes and two Schrader valves for evacuation and admission of gas. Built into the plate below each luer fitting were six caps which served to cover the tubes when the instrument was in operation. The whole cover unit was sterilised by autoclaving. Fig. 5 shows the complete instrument consisting of the anaerobic chamber (A); the stirrer drive (B); the housing (C) for the motor and a fan arranged to cool the motor and culture block; the panel (D) for setting the readings on the six channels for uninoculated broth and fully grown culture and

Fig. 4 Cover of the instrument showing Luer fitting taps in positions corresponding with culture tubes, Schrader valves and circular cover-clamp.

Fig. 5 The complete instrument showing (A) the main block with cover in position (B) the magnetic stirrer assembly (C) the motor and fan housing (D) the scaling controls for each channel (E) six-channel dotting recorder.

the six-channel dotting recorder (E).

SETTING UP

Sterile tubes with loosened caps containing 10 ml BHIS broth were prereduced in a conventional anaerobic jar containing 10% CO₂, 10% H₂ and 80% N₂. Each tube contained a sterile 16 × 4 mm Teflon-coated magnetic stirrer paddle. For use, the jar was opened and the tubes inoculated immediately *in situ* with minimum disturbance.

The caps were closed and the tubes transferred to the opacity measuring device. Conventional catalyst envelopes and a Gaspak disposable anaerobic indicator (BBL) were placed in the chamber. The caps on the culture tubes were removed and the cover immediately clamped into position. The chamber was evacuated, drawing the vacuum not below 700 mm Hg. Greater evacuation caused degassing of the tubes with loss of contents, contamination of

the chamber and possible interference with the light path. After a pressure gauge reading had established that the vacuum was being held, the anaerobic gas mixture was admitted through the second Schrader valve from a football bladder. Evacuation and gas replacement were repeated three times. The stirring mechanism was activated only when the indicator strip was fully decolourised, thereby minimising possible introduction of air into the broth cultures while the system was being made anaerobic.

In some experiments, antibacterial agents under test were added at the time of inoculation; in others they were added when the opacity trace showed that growth had reached the desired level. Syringes were charged with the appropriate quantities and concentrations of antibiotics so that the syringe tips were completely filled. The syringes were then tightly pressed into the Luer fittings of the taps to form an airtight seal. As the whole assembly below the syringe was strictly anaerobic, opening the tap allowed the addition of antibiotic without the introduction of air.

Results and discussion

To fulfil its purpose the device had to operate under strict anaerobic conditions and permit the addition or withdrawal of material without disturbing anaerobiosis. Fig. 6 shows growth curves of *Bacteroides asaccharolyticus* and *Peptostreptococcus*

Fig. 6 Growth curves of *Bacteroides asaccharolyticus* (upper trace) from inocula of (A) 1 (B) 2 (C) 5 (D) 20 drops of overnight culture; and *Peptostreptococcus anaerobius* (lower trace) from inocula of (A) 1 (B) 2 (C) 5 (D) 10 (E) 20 drops of overnight culture.

Fig. 7 Effect of stated concentrations of metronidazole (mg/l) on the growth of *Bacteroides fragilis* when added (upper trace) at the time of inoculation (lower trace) at arrow (C). Arrow at (A) marks the point of inoculation and at (B) activation of stirring.

anaerobius in tubes containing 10 ml BHIS broth inoculated with overnight cultures grown in the same medium in a conventional anaerobic jar. The effect of increasing the inoculum size in reducing the lag time is clearly shown, but growth was regularly obtained from a single drop of overnight culture ($\sim 1.0 \times 10^6$ organisms/ml). After overnight incubation, cultures contained: *Bacteroides asaccharolyticus*: $8-9 \times 10^8$ /ml; *Peptostreptococcus anaerobius*: $7-8 \times 10^8$ /ml. Differences in the form of the growth curves of the two organisms are also apparent and even more striking differences have been found in preliminary experiments with other organisms, notably *Fusobacterium* spp, which show poor growth of an extraordinary polyauxic form probably indicative of inadequacy of the medium used.

Figure 7 shows the effect of adding various concentrations of metronidazole to a culture of *Bacteroides fragilis* at the time of inoculation or when growth had reached 30% of the opacity of a fully grown culture ($\sim 1.0 \times 10^8$ organisms/ml). The results clearly show the lytic effect of high concentrations of drug and a marked inoculum effect. After overnight incubation, control cultures contained $\sim 1.0 \times 10^9$ organisms/ml while those exposed at 30% opacity to 8 mg metronidazole/l contained $5-6 \times 10^4$ organisms/ml and those exposed to 32 or more mg/l, $<10^2$ organisms/ml.

Byrne and Cater⁹ have given an outline description of an interesting, but considerably more com-

plex, microcomputer-based 11-channel instrument which uses rotating prism distribution of laser illumination in a manner evidently similar to that of the instrument described by Marchand *et al.*¹⁰ The instrument is gas-tight but its performance in generating bacterial growth curves has not yet been reported.

We believe, therefore, that this is the first description of an instrument shown to generate continuous growth curves of strict anaerobic bacteria by opacity measurement which also allows the addition and withdrawal of material without breach of anaerobiosis, thereby making it possible to conduct concurrent studies such as viable counts, antibiotic induced morphological changes and antibiotic degradation.

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