

## Growth Curves of Anaerobic Bacteria in Solid Media

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Simple pour plate and spectrophotometric techniques for the evaluation of growth curves of several anaerobic bacteria on solid media are described. Three basic patterns of anaerobic growth were observed. The curves obtained were very reproducible when studied on separate occasions. The curves obtained by spectrophotometric measurement were comparable to those obtained by the pour plate method, especially when a large bacterial inoculum was used. Limitations in the interpretation of the results are discussed. The methods and principles reported could provide the basis for the determination of bacterial growth on solid media using other organisms and different experimental conditions.

Several bacterial characteristics are profoundly affected by the growth phase of the organism being studied, including structure, size, biochemical composition, antigenic determinants, susceptibility to serum bactericidal activity, etc. (4, 5, 9, 10, 12). Recently, we studied serum bactericidal activity against *Bacteroides fragilis* and for these studies the organism was grown on the surface of agar plates. However, only limited information is available about growth of bacteria proliferating on agar surfaces. To study the relationship of serum-killing and bacterial growth phases, we developed a method to determine the growth curve of anaerobic bacteria on solid media. This report describes the method and the growth curve characteristics of several anaerobic bacteria.

### MATERIALS AND METHODS

**Bacteria.** Eleven isolates representing seven species of five genera were studied, including *Bacteroides fragilis* subspecies *thetaiotaomicron* (isolated from stool), *B. fragilis* subspecies *fragilis*, *B. melaninogenicus* subspecies *intermedius*, *B. melaninogenicus* subspecies *melaninogenicus*, *Clostridium perfringens*, *Fusobacterium nucleatum*, *F. mortiferum*, *Peptococcus asaccharolyticus*, and *Peptostreptococcus intermedius*. A single fecal isolate was used and the remainder of the organisms were isolated from significant human infections. Frozen stock cultures were grown up in chopped meat glucose (Scott Laboratories, Fiskeville, R.I.), and then subcultured anaerobically at 37 C for 24 h on Brucella blood agar plates containing 10 µg of vitamin K<sub>1</sub> per ml (BAK) in a GasPak jar. Bacteria were harvested and washed in water containing 0.1% gelatin (water-gel). All organisms were shown in supplementary experiments to survive well in water-gel. The suspension was adjusted to a McFarland standard no. 3 and then

diluted 1:10 and 1:10,000 to provide inocula for the assays. The large inoculum contained approximately 10<sup>8</sup> bacteria/ml and the small inoculum contained approximately 10<sup>6</sup> bacteria/ml. The actual bacterial concentration of the inoculum was measured by making serial dilutions and pour plate counts.

A 0.1-ml portion of the inoculum was spread evenly on eight sets of duplicate or triplicate BAK plates. Each of the eight sets of plates was placed into a separate GasPak jar. The jars were incubated at 37 C for designated periods of time (usually 3, 6, 12, 18, 24, 30, 48, and 72 h). After each time interval 10.0 ml of water-gel was pipetted on to the BAK plates. The colonies were loosened with a platinum loop and were uniformly suspended by thorough, systematic swirling of the plates.

Completeness of bacterial growth removal was determined by washing BAK plates twice with 10.0 ml of water-gel and comparing pour plate counts after the first and second wash. The first wash harvested a mean of 86% and a minimum of 62% of the total organisms recovered by two washings. Greater than 90% of the bacteria were removed with the first wash on more than two-thirds of the compared plates. Subsequently, bacteria were harvested with a single wash of the BAK plates.

The bacterial concentration of the harvested 10.0-ml suspension was measured either as the number of bacteria per milliliter or as optical density (OD). In both methods the suspension was thoroughly shaken in tubes prior to taking portions for measurement. In the first method, the suspension was serially diluted and pour plates were made with brain heart infusion agar containing 5 µg of hemin per ml in petri dishes (10 by 100 mm). After 48 to 72 h of anaerobic incubation in GasPak jars, colonies were counted and the bacterial concentration of the harvested suspension was calculated. The results from replicate plates at each time interval were averaged and plotted on semilogarithmic coordinates.

In the spectrophotometric method, portions of the

suspension harvested from the plates were pipetted into semimicro cuvettes and the OD of the suspension was measured at 620 nm with a Gilford spectrophotometer. The replicate values were averaged and the OD was plotted against time on linear coordinates.

## RESULTS

Growth curves with identifiable lag, accelerated, and stationary phases were obtained by both the pour plate and OD methods in nine of the 11 isolates which were studied. In some cases, the declining phase was also observed during the 72 h of the assay. The assays were highly reproducible; three separate studies of one strain of *B. fragilis* resulted in essentially identical curves. Growth curves were not obtained on two isolates (*P. asaccharolyticus* and *B. melaninogenicus* subspecies *intermedius*) because of marked bacterial clumping.

**Effect of inoculum size.** Figure 1 compares pour plate growth curves of *B. fragilis* using both large and small inocula. The time periods of the lag, accelerated, and stationary growth phases, were identical for the two inocula. In both cases the stationary phase occurred at about  $3 \times 10^{10}$  organisms/plate, reflecting undefined factors of the spent medium limiting the maximum bacterial population. The ranges of triplicate plates growth at each time interval is indicated in the graph. Clearly, the precision of replicate values was excellent compared to log changes in bacterial numbers.

The growth-phase intervals identified by OD measurements did depend upon inoculum size. Figure 2 shows the OD growth curves of *C. perfringens* using large ( $10^7$  organisms) and small ( $10^4$  organisms) inocula. The growth curve resulting from the small inoculum was shifted to the right, i.e., the growth-phase intervals were observed 6 to 12 h later than when using the large inoculum. This same comparison of small and large inocula OD growth curves was observed in nearly all of the other isolates. The OD curves resulting from large inocula usually correlated best with curves derived from pour plates.

### Comparisons of pour plate and OD results.

Comparison of OD and pour plate growth curves of *B. fragilis* is shown in Fig. 3. Identical small inocula were used to determine both curves. Even though OD was not sensitive enough to record the initial phases of growth, the accelerated and stationary phases were clearly identified by this method and corresponded well to the growth phases indicated by the pour plate curves. This correlation was confirmed with the other anaerobic bacteria which were studied by both methods.

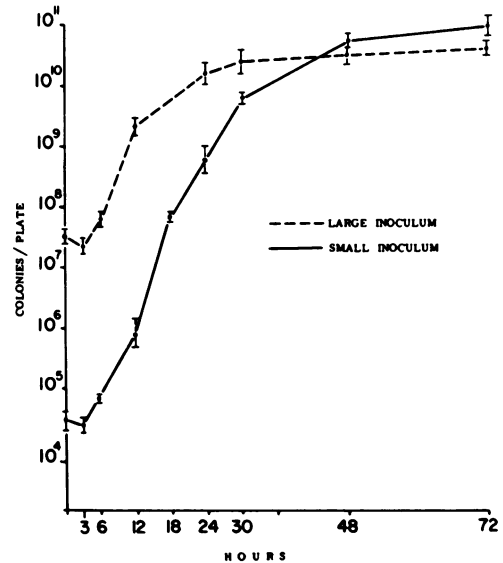


FIG. 1. Pour plate growth curves for *B. fragilis* using small and large inocula.

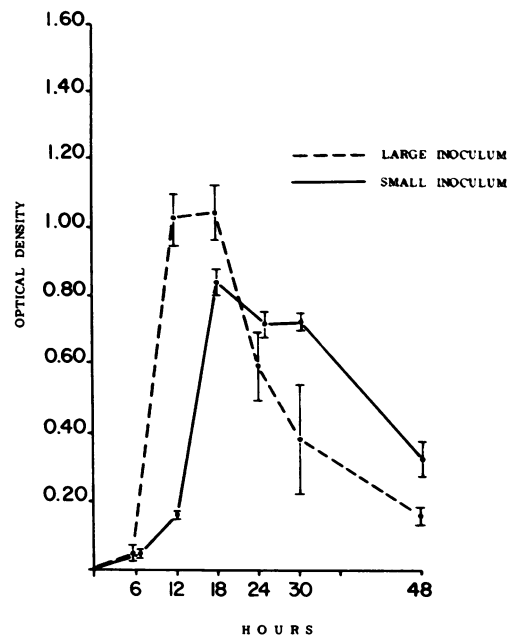


FIG. 2. Optical density growth curves for *C. perfringens*, using small and large inocula.

**Growth curve patterns.** Three general growth curve patterns resulted from studying nine isolates of six anaerobic species and these patterns are represented in Fig. 4. Small bacterial inocula and OD measurements were employed in each case. The observed patterns were related to the species, but not to the genera, of

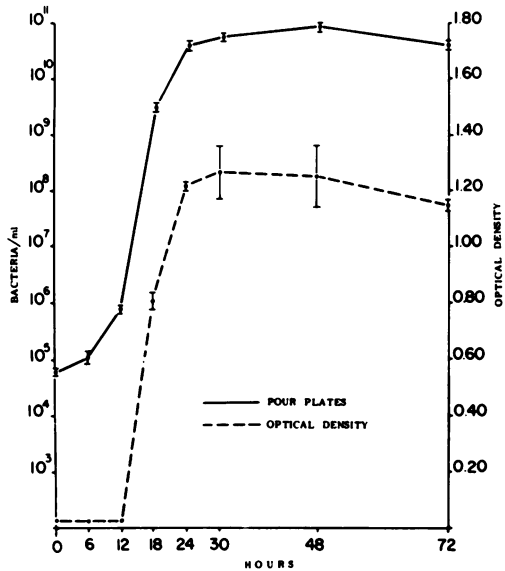


FIG. 3. Comparison of OD and pour plate growth curves for *B. fragilis*.

the isolates. Four isolates of *B. fragilis* grew in essentially the same pattern.

One growth pattern (Fig. 4A) was seen with *C. perfringens* and *P. intermedius*. These curves were characterized by: (i) a relatively rapid onset of growth, (ii) the termination of accelerated growth by 24 to 30 h, (iii) the development of a lower maximal turbidity in the stationary phase (corresponding to fewer maximal numbers of organisms per plate) than with the other isolates, and (iv) in the case of *C. perfringens* the rapid development of bacteriolysis after 30 h of growth.

The second pattern of growth (Fig. 4B) was seen with *B. fragilis* and *F. mortiferum*. These curves were characterized by: (i) a somewhat slower onset of growth, (ii) the termination of accelerated growth by 24 to 30 h, (iii) a stationary phase from 30 to 48 h, and (iv) a declining phase after 48 h.

The third pattern of growth (Fig. 4C) was seen with *B. melaninogenicus* and *F. nucleatum*. These curves were characterized by a very long lag phase, significant growth not occurring before 24 h of incubation, and the persistence of accelerated growth to 48 h.

## DISCUSSION

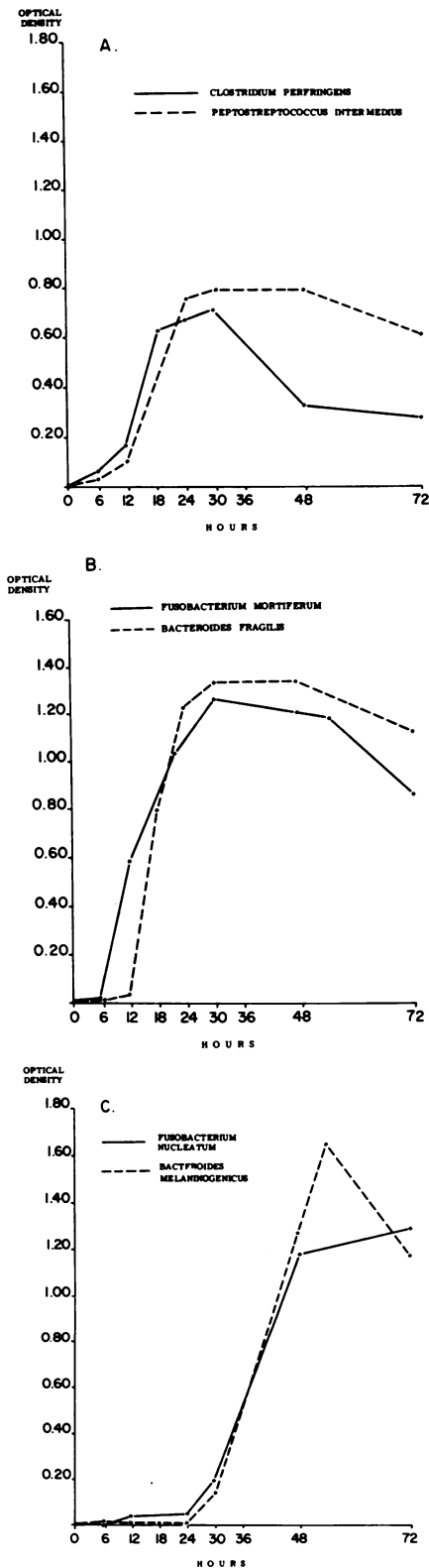
Growth curves in liquid media have been determined by pour plate counts, turbidimetry, measurement of dry weight, cell packing by centrifugation, analyses for cellular components or extracellular end products, and electronic and microscopy determination of cell numbers

(7, 12). However, growth curves for bacteria proliferating on solid media have usually involved comparison of colony diameters (3), a relatively inexact measurement. This report describes a simple technique for the determination of growth rates on solid agar surfaces, involving the delivery of a known quantity of diluent to the petri dishes and the systematic harvesting of bacterial growth. The resultant bacterial suspension was measured both by pour plate determination of colony-forming units and by spectrophotometric assessment of OD. The precision of measurement of growth on replicate plates was very good and the curves were reproducible when studied on several occasions.

Spectrophotometric determination of bacterial growth involves several problems, including the inability to differentiate dead from living organisms, variable light scattering by living and dead organisms, discrepant measurements with low and high bacterial concentrations and with aggregating organisms, and interference with by-products of growth (7, 8). Furthermore, light transmission and scattering by microorganisms change between the logarithmic and stationary phases of growth (8). In our studies, major limiting factors also included bacterial aggregation and hemolysis in the blood agar plates. When aggregation was prominent, (isolates of *B. melaninogenicus* subspecies *intermedius* and *P. assacharolyticus*) growth curves could not be obtained by either the OD or pour plate methods. Several isolates hemolyzed the blood agar during growth and the pigment was recordable; however, the pigment accounted for an insignificant OD (usually less than 0.05 U) compared to turbidity changes secondary to cell multiplication. Curves obtained by the OD method were also reproducible when repeated on several occasions. The growth curves corresponded well to those obtained by the pour plate method, and the growth-phase intervals usually (but not always) corresponded best when the higher inoculum was used. The prime advantages of the OD method were that it was technically easier and required much less time.

Correlation between OD and actual bacterial counts was performed only for *B. fragilis*. Such correlations are possible only when standard curves are constructed for each species and for each set of experimental conditions. We have also observed that such standard curves probably are required for each subspecies.

Limited information has been available regarding growth-curve characteristics of anaerobic bacteria. Previous studies involved growth



in closed or stirred fermentor liquid media systems (2, 11). In this report the growth-curve characteristics on a solid media were described for several anaerobes which could tolerate exposure to air for prolonged periods of time (6). If more fastidious anaerobes were to be studied, the methodology described should be readily adaptable to application within the anaerobic glove box (1). If facultative organisms were to be studied, the same methods could be employed without establishing anaerobiosis. Finally, for clearer definition of growth-phase intervals, the number of time periods studied could be increased accordingly.

The curves reported here reflect the growth characteristics of heterogeneous populations of several organisms, suspended in the reported diluents, grown on BAK medium at 37 C after anaerobiosis was established by the GasPak reaction. Changing any of these or other variables could well lead to changes in the growth curves. To define the growth phases of organisms to be studied, the curves must be determined for each set of experimental conditions. The principles, general characteristics of the curves, and the methodology described in this report could be employed to determine the growth curves of other organisms on different solid media.

#### ACKNOWLEDGMENT

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FIG. 4. Growth curves for several anaerobic species. The three general growth patterns involved a rapid (A), intermediate (B), and late (C) onset of growth.

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