APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Apr. 1988, p. 910–916
0099-2240/88/040910-07$02.00/0

Vol. 54, No. 4

# A Model for Analyzing Growth Kinetics of a Slowly Growing *Mycobacterium* sp.

RANDALL S. LAMBRECHT,[1]* JACQUES F. CARRIERE,[2] AND MICHAEL T. COLLINS[1]

*Department of Pathobiological Sciences, School of Veterinary Medicine,[1] and Department of Statistics, College of Letters and Sciences,[2] University of Wisconsin, Madison, Wisconsin 53706*

Received 19 October 1987/Accepted 29 December 1987

**This report describes a simple method for quantifying viable mycobacteria and for determining generation time. We used statistical models and computer analysis of growth curves generated for the slowly growing mycobacterium *Mycobacterium paratuberculosis* under controlled conditions to derive a mathematical formula relating the dependent variable, growth, to the independent variables, $\log_{10}$ number of organisms in the inoculum (inoculum size) and incubation time. Growth was measured by a radiometric method which detects $^{14}CO_2$ release during metabolism of a $^{14}C$-labeled substrate. The radiometric method allowed for early detection of growth and detected as few as three viable bacteria. The coefficient of variation between culture vials inoculated with the same number of *M. paratuberculosis* was 0.083. Radiometric measurements were highly correlated to spectrophotometric and plate count methods for measuring growth ($r$ = 0.962 and 0.992, respectively). The proportion of the total variability explained by the model in a goodness of fit test was 0.9994. Application of the model to broth cultures provided accurate estimates of the number of *M. paratuberculosis* (standard error = 0.21, $\log_{10}$ scale) and the growth rate (coefficient of variation, 0.03). Generation time was observed to be dependent upon the number of organisms in the inoculum. The model accurately described all phases of growth of *M. paratuberculosis* and can likely be applied to other slowly growing microorganisms.**

Mycobacteria are grouped into two categories on the basis of their rate of growth. The fast-growing *Mycobacterium* spp., however, demonstrate longer generation times than do most other bacteria. In addition to their relatively slow growth, the tendency of mycobacteria to clump, forming aggregates in tissues and broth media, makes it difficult to quantify or study mycobacterial growth by methods commonly used for other bacteria (23). Turbidimetric measurements and enumeration of mycobacteria by microscopy or plate count methods are time-consuming and subject to significant error (13, 19). Microscopy counts do not differentiate between viable cells and those which are dead or dying. Plate count methods require at least 3 weeks of incubation and may underestimate the number of mycobacteria. Turbidimetry is not very sensitive; it fails to detect *M. paratuberculosis* at concentrations of $<10^{5.5}$/ml (25) and is often affected by pigmented organisms or their products or by light-absorbing components of the medium.

In 1969, Deland and Wagner (11) developed a technique for automated detection of microbial metabolism which measures the conversion of a $^{14}C$-labeled substrate to $^{14}CO_2$. The extremely sensitive and quantitative capability of radiometry allows for faster detection of mycobacteria and subsequent evaluation of their growth. In 1977, this technology was adapted for the detection and susceptibility testing of *M. tuberculosis* (8, 21). Mycobacteria grown in sealed vials containing [$^{14}C$]palmitate produce respired $^{14}CO_2$, which can be detected by an automated instrument, the BACTEC 460 ionization detector (Johnston Laboratories, Cockeysville, Md.). The use of radiometry for detecting mycobacterial growth has made significant contributions to the clinical microbiology laboratory, which is predominantly concerned with early detection, identification, and susceptibility testing (9, 10, 14, 17, 18, 24). The value of radiometry as a tool for studying the growth of mycobacteria in the research laboratory, however, has not been fully appreciated.

*M. paratuberculosis* is the etiologic agent of paratuberculosis, or Johne's disease, a chronic enteritis primarily affecting ruminants (6). Recently, *M. paratuberculosis* was isolated from several patients with Crohn's disease (7, 20). Strains of *M. paratuberculosis* often take 6 to 10 weeks to appear as colonies on Middlebrook medium or Herrold's egg yolk medium. For this reason, we proposed to take advantage of the speed and sensitivity of radiometry as a technique to study the growth of *M. paratuberculosis*. The statistical model that we have developed can be used to relate growth response, as a function of incubation time, to inoculum size and may prove to be a useful tool for studies that require quantitation of slowly growing mycobacteria.

## MATERIALS AND METHODS

**Organism.** All experiments were performed with the type strain of *M. paratuberculosis*, ATCC 19698.

**Preparation of *M. paratuberculosis* single cell suspensions.** Two seedlots, A and B, were prepared on separate occasions by cultivating *M. paratuberculosis* ATCC 19698 in Middlebrook 7H9 broth supplemented with 10% OADC enrichment (Difco Laboratories, Detroit, Mich.), 0.1% Tween 80 (Sigma Chemical Co., St. Louis, Mo.), and 1 μg of mycobactin-J per ml (Allied Laboratories, Ames, Iowa). After approximately 3 weeks of growth, the suspension was pelleted by centrifugation at 10,000 × *g* for 20 min in a high-speed centrifuge (model J2-21M; Beckman Instruments, Palo Alto, Calif.), washed six times in phosphate-buffered saline, and inoculated into 100 ml of fresh medium. The culture was then incubated for 3 additional weeks, after which time the suspensions were transferred to 25-ml glass centrifuge tubes and centrifuged, and the pellets were washed three times

* Corresponding author.

with fresh 7H9 broth. After the final wash, the pellet was suspended in 5 ml of 7H9 broth and the cells were dispersed (15) with a motor-driven overhead stirrer and glass-Teflon (Du Pont) homogenizer (Wheaton Instruments, Millville, N.J.). The homogenates were pooled and passed through an 8-μm-pore-size filter (Millipore Corp., Bedford, Mass.). An acid-fast stain of the filtrates revealed primarily single cells. Aliquots (1 ml each) of the filtrates were placed into freezer vials (Nunc, Copenhagen, Denmark) and stored frozen at −70°C. The number of bacteria in each seedlot was determined by counting the number of CFU from serial 10-fold dilutions on Middlebrook 7H9 agar supplemented with OADC, 2 μg of mycobactin-J per ml, and Tween 80. Seedlot A was shown to contain $1.45 \times 10^6$ organisms per ml (95% confidence interval, $1.17 \times 10^6$ to $1.74 \times 10^6$). Seedlot B was shown to contain $1.15 \times 10^8$ organisms per ml (95% confidence interval, $1.02 \times 10^8$ to $1.28 \times 10^8$).

**Radiometric growth assay.** Tenfold dilutions of seedlot B were prepared in Middlebrook 7H9 broth. Eight 0.3-ml portions of the dilution were inoculated into eight vials by using a Tridak Stepper repetitive pipette (Vangard International, Neptune, N.J.). Each vial contained 4 ml of Middlebrook 7H12B medium (1 μCi of [$^{14}$C]palmitate per ml) supplemented with 2 μg of mycobactin-J per ml. The inocula contained from $10^{0.5}$ to $10^{7.5}$ bacteria per vial. All vials were swirled gently and incubated at 37°C under ambient atmosphere without shaking. Growth was measured every 48 h for 14 weeks by using the BACTEC 460. Four of the eight vials were randomly dedicated to establishing growth curves, whereas the other four vials were used in the sensitivity study outlined below. Results were reported in growth units of $^{14}CO_2$ released (1 growth unit = 0.25 nCi of $^{14}$C) and were evaluated as raw units per 48 h and as the cumulative sum of growth units at each reading time. Background readings of uninoculated vials were <10 units. Control vials, which were analyzed at the same time as the test vials, included: (i) 7H12B medium without mycobactin-J, inoculated with *M. paratuberculosis*; (ii) uninoculated 7H12B medium that contained individual growth supplements; and (iii) a standard performance control (Johnston Laboratories), which, when prepared according to the manufacturer's directions, releases a specific amount of $^{14}CO_2$. Acceptable limits for the negative and no-growth controls were interpreted as <30 growth units. The standard positive control was considered to be satisfactory when 45 to 65 units of activity was detected, as recommended by the manufacturer.

**Sensitivity of the radiometric method.** Sensitivity of the radiometric assay was defined as the least number of organisms inoculated into vials which eventually gave rise to growth above background after 90 days. The least number of *M. paratuberculosis* inoculated into vials by the dilution scheme described above was approximately three (inoculum size, $10^{0.5}$). The number of *M. paratuberculosis* in a vial when cumulative growth reached a minimum positive value of 30 units was also considered an indication of the level of sensitivity. When the mean of cumulative growth measurements from quadruplicate vials reached a minimum threshold of 30 units, the vials were vigorously vortexed and samples were removed, serially diluted, and plated to determine the number of CFU. Additionally, new radiometric vials were inoculated in triplicate with 0.1-ml portions of culture from the minimum-threshold-positive vials, and growth was measured every 48 h. The number of *M. paratuberculosis* in the inoculum was then calculated by using the statistical model described below and was compared with the number of CFU observed by the plate count method.

**Correlation of the radiometric method to turbidimetric and plate count methods.** Owing to the different levels of sensitivity inherent in the turbidimetric and radiometric techniques, a concentration of *M. paratuberculosis* which could be measured by both methods had to be chosen carefully. Accordingly, $10^{5.5}$ organisms were inoculated into 28 vials each containing 4 ml of Middlebrook 7H12B medium supplemented with 2 μg of mycobactin-J per ml and were incubated at 37°C for 24 days. Growth in all vials was measured with the BACTEC 460 every 48 h. On every fourth day, four vials were randomly chosen, samples were removed and serially diluted, and plate counts were performed. The remaining vials were frozen at −20°C for later spectrophotometric measurements (Beckman model DU-50). The correlation between radiometric, spectrophotometric, and plate count measurements of *M. paratuberculosis* growth was tested by using Spearman's correlation coefficient.

**Development of a statistical model.** Cumulative growth responses were obtained from radiometric growth analysis of *M. paratuberculosis* prepared from dilutions of seedlot A. From these data we sought a statistical model which accurately described the relationship of the dependent variable, growth (defined as units of $^{14}CO_2$ released), as a function of the independent variables, $\log_{10}$ number of organisms in the inoculum (inoculum size) and incubation time. The model was then validated with dilutions of *M. paratuberculosis* prepared from seedlot B.

The S shape of the growth curves suggested a model similar to a logistic cumulative distribution function which takes the form:

$$Y = \frac{Y_m}{1 + Be^{k_{(x,\,t)}}} \tag{1}$$

where $Y$ is the cumulative growth response in units of $^{14}CO_2$ released, $Y_m$ is a fixed value bounded by the maximum cumulative growth response possible owing to substrate limitation, and $k_{(x,\,t)}$ is a linear function of the $\log_{10}$ number of *M. paratuberculosis* in the inoculum ($x$) and of incubation time ($t$). Seeking the best model for describing the relationship between the cumulative growth response and the inoculum size and incubation time, we applied a stepwise multiple regression approach which permitted the choice of the best estimate of $k$ with the least number of parameters (12).

**Growth rate and generation time.** The relationship of cumulative growth units, $Y$, to raw growth units, $g$, can be expressed by the differential equation:

$$\frac{dy}{dt} = g_{(x,\,t)} \tag{2}$$

Raw growth curves for *M. paratuberculosis* were generated from actual growth experiments and from the statistical model, which incorporated the same inoculum sizes to calculate growth response over time. Growth rates were determined by plotting $\ln g_{(x,\,t)}$ over time and by graphically identifying the linear segment representing exponential growth. The slope of this segment describes the maximum constant rate of $^{14}CO_2$ release. Thus, $g_{(x,\,t)}$ is proportional to the rate of growth of *M. paratuberculosis* and can be expressed as:

$$\frac{dg}{dt} = \mu g \tag{3}$$

where $\mu$ is the maximum growth rate constant. This implies that the linear portion of the growth curve can be approximated by:

$$g_{(x,\,t)} = g_{(x)}e^{\mu t} \tag{4}$$

The natural logarithm of this equation yields the equation:

$$\ln g_{(x,\,t)} = \ln g_{(x)} + \mu t \tag{5}$$

Growth rate was subsequently found to be a function of $x$ which satisfied the form:

$$\mu_x = \mu_o e^{ax} \tag{6}$$

Substitution of this expression into equation (5) yields:

$$\ln g_{(x,\,t)} = \ln g_{(x)} + t\mu_o e^{ax} \tag{7}$$

By using regression analysis, we estimated $\mu_o$ to be 0.105 and $a$ to be 0.292 (correlation coefficient, 0.991).

Generation time, the time necessary for the number of organisms to double, was calculated by solving for $\Delta$:

$$\Delta = \frac{\ln 2}{\mu_x} = \frac{0.69315}{\mu_x} \tag{8}$$

## RESULTS

**Radiometric growth determination.** Growth curves for each inoculum size were expressed in raw and cumulative growth units. Each growth unit represents 0.25 nCi of $^{14}CO_2$ released as a result of metabolism of [$^{14}$C]palmitate. Cumulative or total growth is the cumulative $^{14}CO_2$ activity released from the beginning of incubation (Fig. 1A). The differential $^{14}CO_2$ production is the amount of $^{14}CO_2$ produced between measurements and is plotted as raw growth units over time (Fig. 1B). No growth curves are shown for inoculum sizes greater than $10^{5.5}$ because the rapid release of high levels of $^{14}CO_2$ read off scale even when more-frequent measurements were taken. The apex of each curve in Fig. 1B is represented by the point of inflection of the respective curve in Fig. 1A. The decrease in $^{14}CO_2$ production resulted from depletion of the [$^{14}$C]palmitate in the medium, which was overcome by adding more substrate to the vials.

Precision of the radiometric method was determined by calculating the coefficient of determination of raw growth unit measurements from four vials inoculated with the same number of organisms and sampled every 48 h during the exponential growth phase. This was done for each of the five inoculum sizes. The coefficients were then averaged ($n$ = 205), and the reproducibility of measurements between vials was found to be ±8.3%, which more likely represents error associated with pipetting rather than instrument measurements. The inoculum was prepared as a single-cell suspension to minimize the error associated with using a nonhomogeneous mixture of cells since *M. paratuberculosis* tends to clump. Variability of the precision pipette for inoculating the vials was stated by the manufacturer to be ±2.5%.

TABLE 1. Comparison of growth of *M. paratuberculosis* observed in Middlebrook 7H12B medium with that determined by using the statistical model

| Observed growth | | | Growth calculated from model | |
|---|---|---|---|---|
| $Log_{10}$ inoculum size | Time to detection[a] (days) | Generation time (days) | $Log_{10}$ inoculum size | Generation time (days) |
| 5.5 | 4 | 1.3 | 5.4 | 1.3 (1.2, 1.4)[b] |
| 4.5 | 7 | 1.7 | 4.4 | 1.8 (1.6, 1.9) |
| 3.5 | 11 | 2.4 | 3.5 | 2.4 (2.3, 2.6) |
| 2.5 | 17 | 3.2 | 2.4 | 3.4 (3.3, 3.5) |
| 1.5 | 25 | 4.4 | 1.3 | 4.0 (2.3, 5.5) |

[a] Detectable growth defined as ≥30 cumulative growth units.
[b] Values in parentheses are the 95% confidence intervals.

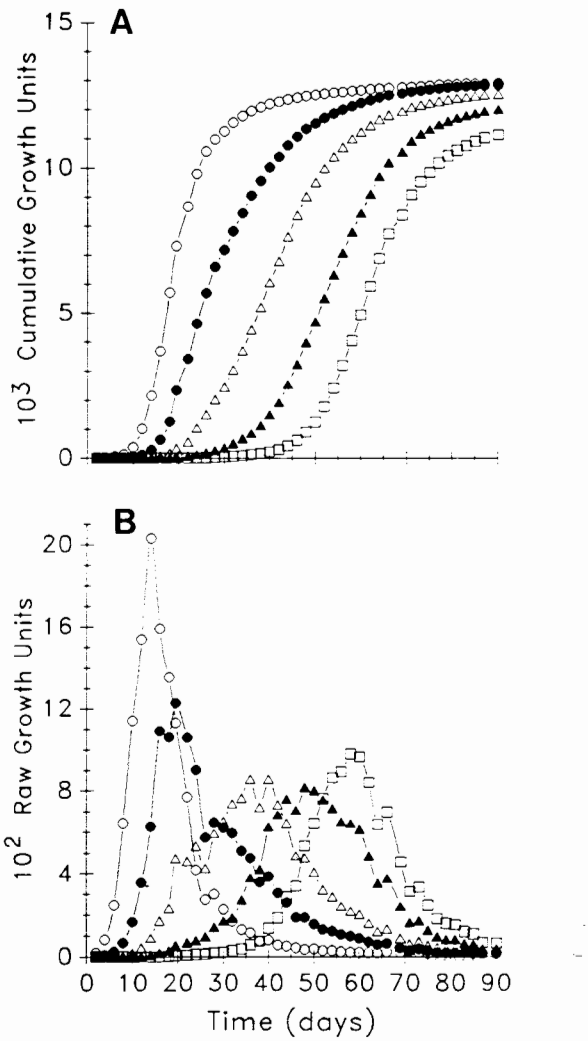


FIG. 1. Growth kinetics of *M. paratuberculosis* in Middlebrook 7H12B medium. Growth was measured as described in Material and Methods. One growth unit equals 0.25 nCi of $^{14}CO_2$. (A) Cumulative growth curves. (B) Raw growth curves. Inoculum sizes are: $10^{5.5}$ (○), $10^{4.5}$ (●), $10^{3.5}$ (△), $10^{2.5}$ (▲), and $10^{1.5}$ (□).

The time required for detection of growth was inversely proportional to the number of organisms in the inoculum (Table 1). Except when $<10^{1.5}$ organisms were present, the time necessary for detecting growth was significantly less with the radiometric assay than the 20 days required to observe colonies by microscopy (×10) and the 29 days to observe them with the naked eye on Middlebrook 7H9 agar medium. It is worth noting that the time necessary for the laboratory-adapted *M. paratuberculosis* ATCC 19698 to form colonies on artificial medium may not reflect the growth rate of isolates recovered directly from infected material. Positive growth, or growth above background, in the radiometric assay was defined as cumulative growth measurements ≥30 units. Cumulative growth was a convenient

means of expressing growth without loss of sensitivity. The sensitivity of the radiometric assay permitted detection of as few as three viable organisms. The mean logarithmic concentration of *M. paratuberculosis* in Middlebrook 7H12B medium at the minimum positive threshold of 30 units was 4.3 ± 0.16 (standard deviation) CFU/ml, as determined by the plate count method. The concentration of *M. paratuberculosis* was also determined by using the model. New radiometric vials were inoculated in triplicate with culture from the minimum-positive-threshold bottles, and resultant growth responses were used to calculate inoculum size. The mean logarithmic number of *M. paratuberculosis* per ml in the original culture vials was 4.48 ± 0.25, as determined by using the model, and was not significantly different from the number of CFU per milliliter obtained by the plate count method.

**Conserved growth measurements.** Thirty-two radiometric vials were supplemented with mycobactin-J and inoculated in the same way with 0.1 ml of seedlot A. Each vial was randomly allocated to one of seven groups. Vials in groups 1 through 5 were read only once, on day 8, 12, 16, 20, or 24 of the incubation period. Those in group 6 were read every 2 days, and those in group 7 were read every 4 days. Readings from the four vials in each group were averaged. For groups 6 and 7, the averages were cumulative. The total $^{14}CO_2$ content detected in vials that were repeatedly sampled throughout incubation was not significantly different from that in vials sampled once at the end of the incubation period (Table 2). Thus, growth was conserved, and a single reading could be used to evaluate growth over the entire incubation period.

**Correlation of the radiometric assay with spectrophotometric and plate count methods.** Three methods of measuring growth were compared to determine whether measurements based on metabolism of [$^{14}$C]palmitate correlated with conventional methods for measuring growth. Because of the relative insensitivity of turbidimetric measurements, an initial concentration of *M. paratuberculosis* above the lower limit of detection of a spectrophotometer had to be used. As long as the substrate was not significantly depleted, total evolution of $^{14}CO_2$ was proportional to the total number of *M. paratuberculosis* (Fig. 2). The correlation coefficients of cumulative growth units to CFU and to optical density units at 540 nm were determined to be 0.996 and 0.962, respectively.

**Statistical model of *M. paratuberculosis* growth.** A statistical model for characterizing the growth kinetics of *M. paratuberculosis* was developed from growth measurement data from seedlot A. The model was then tested on seedlot B. By using multiple linear regression, the following model best described the data:

$$\hat{Y}_{(x,\ t)} = \frac{Y_m}{1 + B(C_0C_1^x)^t\ (D_0D_1^x)^{t^2}\ (E_0E_1^x)^{t^3}} \qquad (9)$$

TABLE 2. Comparison of total growth from a single measurement and from multiple measurements at 2- and 4-day intervals

| Frequency of measurements | Cumulative growth units (Mean ± 2 SD)[a] after incubation for (days): | | | | |
|---|---|---|---|---|---|
| | 8 | 12 | 16 | 20 | 24 |
| One-time reading | 8 ± 0 | 21 ± 4 | 116 ± 14 | 263 ± 2 | 564 ± 26 |
| Every 2 days | 5 ± 2 | 22 ± 0 | 91 ± 14 | 255 ± 20 | 523 ± 64 |
| Every 4 days | 6 ± 4 | 22 ± 4 | 85 ± 14 | 239 ± 18 | 493 ± 50 |

[a] Mean and standard deviation (SD) calculated from triplicate vials.

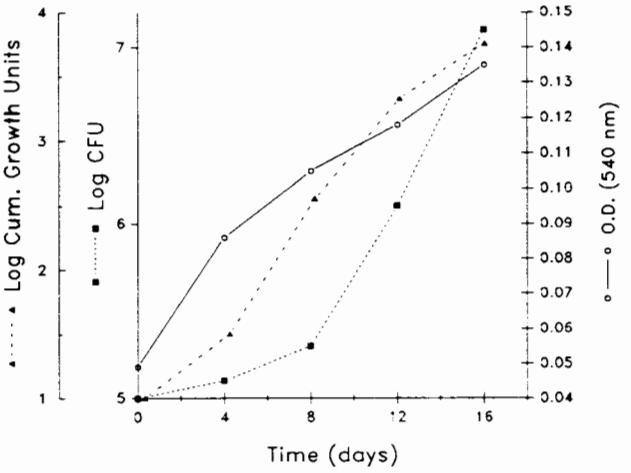


FIG. 2. Comparison of three methods for measuring growth of *M. paratuberculosis* in Middlebrook 7H12B medium. Mean determinations: ▲, $n$ = 4, average coefficient of determination (CD) = 0.20; ■, $n$ = 3, CD = 0.15; ○, $n$ = 8, CD = 0.15.

where $Y_{(x,\ t)}$ is cumulative growth units as a function of inoculum size ($x$) and incubation time ($t$), $t > 0$, and $x > 0$. Total growth is bounded above by $Y_m$ and below by $Y_m/(1 + B)$, where $Y_m$ = 12,950. The letters B through E are regression coefficient constants determined to have the following values: $B$ = 10,340, $C_0$ = 1.2217, $C_1$ = 0.84345, $D_0$ = 0.98959, $D_1$ = 1.004644, $E_0$ = 1.00008339, and $E_1$ = 0.99996559. The formula is extremely accurate about the median values of $Y$ and $t$ and remains satisfactory for extreme values. The proportion of the total variability explained by this model was found to be 0.9994. Natural background at time zero was derived from the equation $Y_m/(1 + B)$ and was found to be 9.9 growth units. Estimated cumulative growth was calculated from the formula by solving for $Y$ by using the same values for $x$ and $t$ as were used in the actual experiment. Cumulative growth curves calculated from the model equation (Fig. 3A) were similar to those calculated from the data actually observed (Fig. 1A) and represent the integral form of raw growth units plotted over time (Fig. 3B). The correlation coefficient of observed $Y$ values to calculated $Y$ values was found to be 0.998 over all observations (Fig. 4).

Inversion of equation 9 relates the inoculum size, $x$, as a function of growth response, $Y$, observed after time, $t$, and permits calculation of the number of *M. paratuberculosis* in an inoculum from a single growth measurement. That is,

$$\hat{x} = \frac{\ln\ [Y_m/(Y - 1)] - \ln\ (B\ C_0^t\ D_0^{t^2}\ E_0^{t^3})}{\ln\ (C_1^t\ D_1^{t^2} E_1^{t^3})} \qquad (10)$$

The mean of the calculated inoculum size ($\hat{x}$) from all observations of $Y_{(x,\ t)}$ by using the model compared well to the actual inoculum size used to generate each growth curve (Table 1). The correlation coefficient of actual inoculum size to calculated inoculum size for all measurements was 0.971. Standard error of $\hat{x}$ was determined to be 0.21. Prediction of $x$ in the early lag or late stationary growth phase was less reliable than during exponential growth.

**Growth rate and generation time.** Growth rates were not constant but increased with increasing inoculum size. Accordingly, generation times were inversely proportional to the inoculum size (Table 1). The relationship between inoculum size and growth rate could be expressed mathemati-

cally by the equation $\mu_x = \mu_o e^{ax}$, where $\mu_o = 0.105$ and $a = 0.292$. Generation time can thus be predicted when the inoculum size is known or when it can be estimated from the model. Alteration of the conditions for a given inoculum size and comparison of generation times would allow for the evaluation of the effect of various factors on growth. Generation times as determined from the actual growth curves and from the model were not different (Table 1). Although little information is available regarding generation time for *M. paratuberculosis*, those values calculated are biologically plausible in terms of what is known about other mycobacteria. The generation time of 1.33 days for $10^{5.5}$ *M. paratuberculosis* is in good agreement with the mean generation time of 1.38 days derived by using plate counts to quantitate growth over the same time period.

## DISCUSSION

There are many methods for measuring bacterial growth. Defining growth and its significance is important in deciding which method to choose. Growth can be defined as the orderly increase in all components of a cell (16). Although comprehensive, this definition is not practical in the laboratory. Cell multiplication is probably the most applied definition of bacterial growth. Regardless of the method of measuring growth used, the marker trait used to monitor growth must increase at a specific rate proportional to the increase in the amount of biomass. Likewise, if the ratio of one trait to another is to remain constant, the same proportionality constant must apply. Practically, this proportionality allows the production of a metabolite, such as $CO_2$, to be used as an index of growth (22).

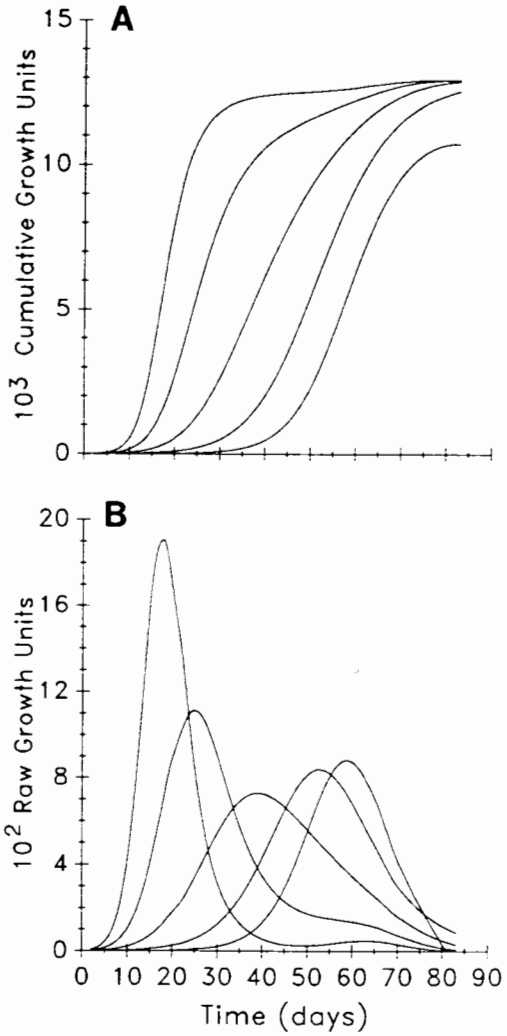


FIG. 3. Predicted growth curves of *M. paratuberculosis* by using the statistical model. Inoculum sizes correspond to those used in the actual growth experiment and are (from the left): $10^{5.5}$, $10^{4.5}$, $10^{3.5}$, $10^{2.5}$, and $10^{1.5}$. (A) Calculated cumulative growth curves. (B) Derivative form of cumulative growth curves.

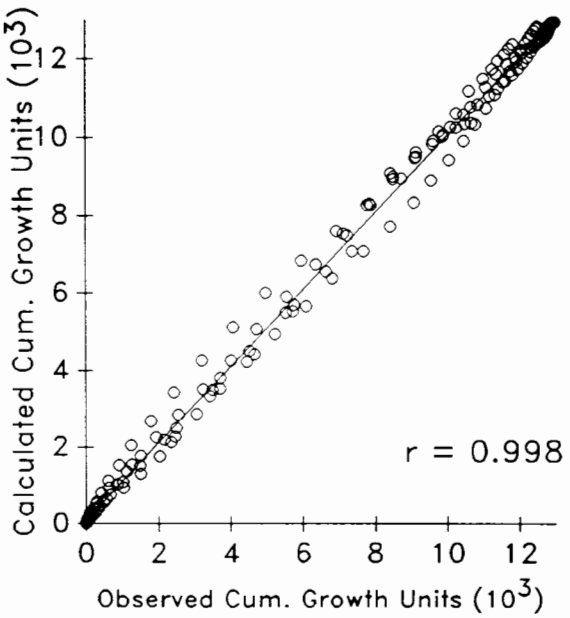


FIG. 4. Comparison of observed cumulative growth units to calculated cumulative growth curves by using the statistical model. For comparison, the same inoculum sizes and incubation times were used in the model as were used in the actual growth experiment. Pearson's correlation coefficient ($r$) was calculated.

In measuring growth of an organism such as *M. paratuberculosis*, the slow growth rate coupled with the aggregation of bacterial cells makes it difficult to apply many methods of quantitation. Strain ATCC 19698 formed visible colonies within 3 to 5 weeks, but primary clinical isolates often take 6 to 10 weeks to form colonies on artificial media. This strain was used here because it is the type strain of *M. paratuberculosis*. Turbidimetry was not sensitive enough to detect $<10^{5.5}$ *M. paratuberculosis* per ml and, like plate counting, assumes a homogeneous population of bacteria. Changes in radiometric measurements coincided with changes in viable cell numbers and correlated highly with plate count and turbidimetric measurements. Sensitivity was sufficient to permit detection of as few as three organisms and allowed for precise measurements during the early phase of exponential growth. Reproducibility, which included all errors associated with the technique, was shown to have a precision of ±8.3%. Radiometric growth detection is less likely to be influenced by bacterial clumping than are plate count methods. Interference from turbid specimens or from components in the medium is also not a problem in the radiometric assay. In addition, radiometric measurements provide quantitative data which can be linked to on-line computer analysis.

Radiometric systems for measuring growth of bacteria are not novel; several approaches have been described. A

two-compartment scintillation vial was evaluated with rapidly growing bacteria (3, 4) and consisted of an inner vial containing the bacterial culture and an outer vial containing the scintillator detector. Radioactivity evolved from bacterial metabolism was then measured in a scintillation counter. This method suffered from low counting efficiency but has been significantly improved by modifying the detector (2). Caution must be exercised with this system so as not to allow culture medium to come in contact with the fluor detector. For these reasons, this system does not lend itself well to long-term growth measurements. In contrast, the radiometric approach described in this paper requires an ionization chamber to measure evolved $^{14}CO_2$ and has been applied to even the most difficult-to-grow *Mycobacterium* spp. with good results (5).

Our results indicate that the statistical model can accurately predict the number of *M. paratuberculosis* in an inoculum from a single growth measurement under controlled conditions. Likewise, the rate of growth and the generation time can be accurately determined. A culture can be analyzed as often as required without loss of information since the method of sampling does not perturb growth. There is no need to monitor growth repeatedly or for long periods. A single growth measurement can be used to obtain growth parameters as long as the measurement is not taken at either extreme phase of the growth curve, i.e., early lag or late decline. It should be noted that differences in single-point estimates can be interpreted in several ways since they may reflect differences in lag periods, specific growth rates, population densities, or inhibitory products; only when certain of these variables are controlled can a particular parameter be evaluated. Although we have quantified *M. paratuberculosis* only in broth cultures, it may be possible to quantify bacteria in tissues or other specimens.

In contrast to most mathematical models, which describe bacterial growth through the stationary phase, our model is based upon cumulative growth through the decline phase (see the Appendix for further discussion). During exponential growth of *M. paratuberculosis*, total $^{14}CO_2$ production increased in proportion to the total cell population. All phases of the growth curve were accurately described by plotting the logarithm of raw growth units over time. The exponential growth phase was then expressed as the linear portion of the curve, and generation time was calculated from the slope. For *M. paratuberculosis*, the generation time was found to be dependent on the number of organisms in the inoculum; thus, generation time can be determined quite easily if the inoculum size is known. Alternatively, it can be estimated from a single growth measurement by using the model. It is interesting that the growth rate was influenced by inoculum size, and one can only speculate that clumping or development of microcolonies with large inocula may accelerate growth.

Growth expressed in terms of generation time is a convenient and meaningful parameter. By using a constant inoculum size for test and control vials, the effects of changes in medium composition or of altered culture conditions on bacterial generation times can be evaluated. Growth inhibitors or enhancers can also be compared by using standard tests that compare the difference between two generation times.

The statistical model should be adaptable to other strains of *M. paratuberculosis* and to other species of mycobacteria. The upper and lower asymptotes of the cumulative growth curves are predetermined by the limited amount of $^{14}C$ in each vial and the relatively constant low-level background activity. The only other variable in the model, which is likely to differ among strains, is the rate of growth and death of the population of bacteria. This characteristic is responsible for defining the point of inflection of the S shape of the growth curve and can be determined by calculating new values for the parameters (regression coefficients) in the linear model, $k_{(x,\ t)}$. This is done simply by generating growth curves with inocula of different sizes and by analyzing the resulting data by using the model described. The application of this statistical model to estimation of numbers and generation times of *M. paratuberculosis* has already facilitated our understanding of the biology of this organism. We caution the reader that our parametric model is not unique and that other, more-complex models may be more appropriate, but the model presented here provides an excellent approximation of the observed data.

## APPENDIX

**Development of a statistical model.** The simplest model that can describe the microbial growth process is based upon first-order chemical reaction kinetics and assumes that no increasingly effective retardation is operative:

$$\frac{dN}{dt} = \mu N \tag{A1}$$

This model states that the change in growth of the microbe population ($dN/dt$) is directly related to the value for microbe population multiplied by a growth rate constant ($\mu$). If the size of the microbe population approaches a maximum limit ($N_m$), the specific growth rate approaches zero (1). This situation can be explained by using the logistics model:

$$\frac{dN}{dt} = \mu N\left(\frac{N_m - N}{N_m}\right) \tag{A2}$$

which is the differential equation of the integral function:

$$N = \frac{N_m}{1 + Be^{-\mu t}} \tag{A3}$$

This function plots as a symmetrical S-shaped curve and describes the growth of a variety of living organisms through stationary phase. In contrast, the model presented in this paper is also a logistics model which plots as an S-shaped curve but is unique in that it is based upon cumulative growth measurements and describes all events associated with growth of *M. paratuberculosis* through the decline phase. This model was found to take the form:

$$Y = \frac{Y_m}{1 + Be^{k(x,\ t)}} \tag{A4}$$

where $Y$ is cumulative growth units and $k$ is a function of the number of organisms ($x$) and incubation time ($t$) with the property $e^{k(x,\ 0)} = 1$. This means that background can be expressed as:

$$Y_{(x,\ 0)} = \frac{Y_m}{1 + B} \tag{A5}$$

Transformation of equation A5 results in the following linear equation:

$$\ln B + k_{(x,\,t)} = \ln\left(\frac{Y_m}{Y} - 1\right) \tag{A6}$$

Next, we considered the following class of linear models for $k_{(x,\,t)}$:

$$k_{(x,\,t)} = \sum_{i=0}^{3} \sum_{j=1}^{4} s_{ij} x^i t^j \tag{A7}$$

A stepwise multiple regression approach was used to choose the best model with the least number of parameters. The least-squares method was then used to estimate parameters in equation A6. With the usual normal assumptions, each parameter in the model was tested for significance by using Student's $t$ test. The model does not have too many parameters, as each parameter contributes to a significant improvement in the fit. These estimated parameters were then used in equation A4 to analyze the model by the nonlinear least-squares method, and new parameters which fit the data better were derived.

As an indicator of the goodness of fit, the proportion of the total variability explained by the model was defined as:

$$R^2 = \frac{\Sigma(\hat{Y} - Y)^2}{Y^2} \tag{A8}$$

where $Y$ is the observed value, $\hat{Y} = Y_m/(1 + e^k)$, and

$$k = \sum_i \sum_j s_{ij} x^i t^j.$$

## ACKNOWLEDGMENTS

This work was supported in part by a grant from the U.S. Department of Agriculture (84-CRSR-2-2447).

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