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Novel Method for Rapid Measurement of Growth of Mycobacteria in Detergent-Free Media

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We describe a novel, rapid, and inexpensive method for the measurement of growth of *Mycobacterium tuberculosis*, *Mycobacterium bovis*, and *Mycobacterium smegmatis* in the presence or absence of detergent. The method, which employs hot NaOH treatment of mycobacterial cells to release total cellular protein, compares favorably with other methods for monitoring mycobacterial growth but is particularly useful for heavily clumped cultures grown in defined minimal medium.

Quantitation of mycobacterial biomass is notoriously difficult as the hydrophobic cells aggregate into clumps of various sizes. The addition of detergents such as Tween 80 to the growth medium only partially alleviates the problem of clumping. The use of detergents may also cause experimental design problems and artifacts. Since hydrolyzed Tween 80 can be used as a carbon source by *Mycobacterium tuberculosis* (15), it may be an undesired nutrient in the medium. It is also toxic to *M. tuberculosis* in the absence of bovine serum albumin (BSA) (15), making it difficult to grow well-dispersed cultures in defined, BSA-free media. Furthermore, detergents cause a reduction in the virulence of *M. tuberculosis* (3) and, at high concentration, affect the composition of the cell envelope (6, 10).

Despite the problem of cell aggregation, CFU counts are routinely used for the enumeration of mycobacteria (15). This method is time consuming and underestimates the number of cells in the culture (the use of mild sonication does not break up all clumps of bacilli [11]). Optical density (OD) measurements have limited value and work best for well-dispersed cultures containing detergent. The extraction and measurement of ATP has been used for the reliable measurement of mycobacterial cultures (11) but requires expensive reagents and specialized equipment. Other quantitative methods include micro-Kjeldahl nitrogen determination (7, 16) and labelling cells with radioisotopes (14), but both are laborious and time consuming. Although the BACTEC system is widely used in clinical laboratories for the radiometric quantitation of mycobacterial growth (4), it is inflexible in that it is limited to a single, prepacked growth medium (Middlebrook 7H12 broth). It is also expensive. We wished to monitor the growth of mycobacteria in a BSA-free, Tween-free defined medium and describe here the development of a rapid and reliable protein extraction method for measuring mycobacterial growth in these heavily clumped cultures.

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Cultures of *M. tuberculosis* H37Rv, *Mycobacterium bovis* BCG (Tokyo), and *Mycobacterium smegmatis* mc^{2}155 (13) were grown with agitation at 37°C in 400 ml of Middlebrook 7H9 broth (Difco Laboratories, Detroit, Mich.), containing sterile OADC (0.5% BSA, 0.2% glucose, 0.006% oleic acid, 140 mM NaCl) or ADC (0.5% BSA, 0.2% glucose, 3 μg of catalase per ml) supplement as indicated in 1-liter screw-cap bottles. Tween 80 (0.05%) was used where indicated. All cells used as inocula were washed in saline. All experiments with *M. tuberculosis* were performed in a Biosafety Level III facility for the safe handling of pathogenic tubercle bacilli. Cultures of *M. bovis* BCG mc^{2}798 (*leuD2*::Tn*5366*; a kanamycin-resistant, leucine auxotroph) (9) were grown with agitation in detergent-free Sauton's medium (1), pH 7.0, containing kanamycin (20 μg/ml) and various concentrations of L-leucine. The heavily clumped cultures of the auxotroph settled rapidly when the mixtures were allowed to stand and therefore had to be stirred continuously while sampling was conducted with large-bore pipettes. For all growth curves, duplicate samples were taken at intervals and used for protein, OD, or ATP measurements.

Protein was extracted from the mycobacterial cultures by using a modification of the method of Makkar et al. (8). Duplicate 1-ml culture samples were centrifuged in a benchtop centrifuge (13,800 × *g*) for 5 min, and the tubes were rotated through 180° and centrifuged again for 5 min to produce compact cell pellets. *M. tuberculosis* cultures were centrifuged in a sealed rotor in a benchtop centrifuge (LABOFUGE 400R; Heraeus Instruments). The pellets were washed with 1 ml of phosphate-buffered saline (PBS), pH 7.0 (without resuspending the cells), and were centrifuged as described above. At this point the pellets were frozen at −20°C for later analysis or resuspended in 0.1 ml of 1 M NaOH and the sealed tubes were placed in boiling water for 10 min. The samples were neutralized by adding 0.02 ml of 5 M HCl, and the volumes were adjusted to 1 ml by adding 0.88 ml of PBS, pH 7.0. Samples were centrifuged for 30 min, and 0.8 ml of each supernatant was removed for protein determination. For each sample, the absorbance was measured at 230 and 260 nm, and the protein concentration (μg/ml) was determined from the equation [Protein] = $(183 \times A_{230}) - (75.8 \times A_{260})$ (5). The assay is linear over the range of 6 to 225 μg of protein/ml (5), and extracts from heavily turbid cultures were diluted in PBS to ensure that measurements remained within the linear range. The following modifications did not significantly enhance protein extraction from *M. tuberculosis* H37Rv cells: heating for a longer period in boiling water (20 or 30 min), adding 3% sodium dodecyl

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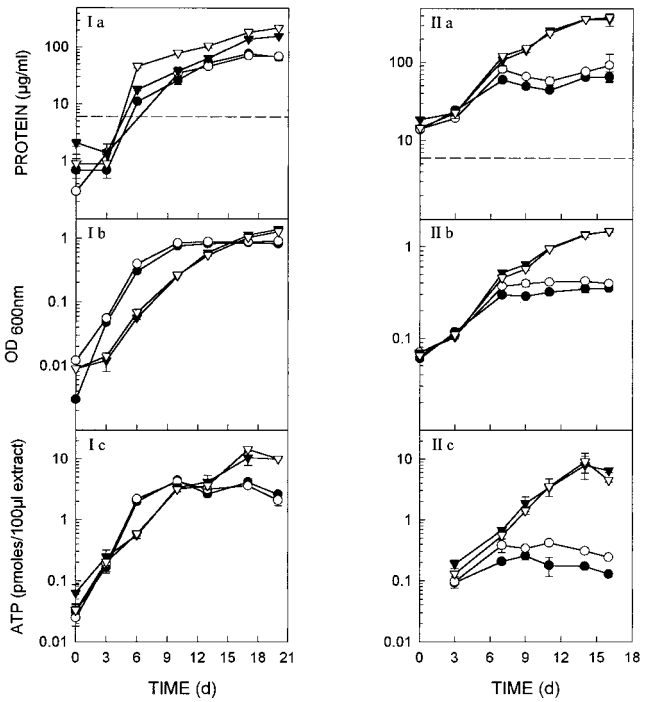

FIG. 1. Growth curves of *M. tuberculosis* H37Rv (I) and *M. bovis* BCG Tokyo (II) cultures in Middlebrook 7H9 broth. Duplicate cultures were grown with (●, ○) and without (▼, ▽) 0.05% Tween 80 and supplemented with ADC (*M. tuberculosis* H37Rv) or OADC (*M. bovis* BCG Tokyo). Curves were plotted from total protein yield (a), OD (b), and total ATP (c) data. The dashed horizontal lines on the protein growth curves indicate the lower limit of sensitivity of the protein assay (6 μg of protein/ml). Each data point represents the average of two measurements. Error bars show the standard deviations of the means.

sulfate to the 1 M NaOH, and reversing the order of NaOH and HCl addition (i.e., extracting the protein in 1 M HCl and neutralizing with NaOH) (data not shown).

Protein extraction from 5 day-old, log-phase *M. bovis* BCG (Tokyo) cells, grown in Middlebrook 7H9 broth with OADC supplement and then labelled for 48 h with 36.5 μCi [^{35}S]methionine/ml (323.1 Ci/mmol), showed that the hot NaOH extraction led to a good recovery of label in the protein extracts from cells grown in the presence (79.3% ± 12.3%) or absence (87.7% ± 6.2%) of Tween 80. The recovery of the label was calculated by determining the counts per minute (cpm) in the final protein extract as a percentage of the total cpm in each sample. The total cpm of the sample was determined after the boiling step but before the final centrifugation step.

The extraction of ATP was performed by using a modification of the boiling-buffer method of Prioli et al. (11). Duplicate 0.05-ml volumes of culture were mixed with 0.5 ml of preheated buffer (100 mM Tris-acetate–2 mM EDTA, pH 7.75) and placed in boiling water for 5 min. Extracts were allowed to cool, and the ATP was assayed with the ENLITEN luciferase and luciferin reagent (Promega Corporation, Madison, Wis.). A total of 0.1 ml of extract was added to 0.398 ml of monitoring reagent (0.348 ml of Tris-acetate-EDTA buffer plus 0.05 ml of luciferase and luciferin reagent) in 4-ml polystyrene cuvettes, and the light output (millivolts) was measured with a Bio-Orbit 1253 luminometer (Bio-Orbit, Turku, Finland). ATP standard (2.5 μl; 0.1 μM) was added, and the light output was measured. The ATP content was calculated from the two light output readings obtained for each sample. The assay is linear over the concentration range 10^{-11} to 10^{-6} M ATP (0.001 to 100 pmol of ATP/100 μl) (2).

The third method for monitoring mycobacterial growth was culture turbidity measurements. OD measurements were made at 600 nm. Heavily turbid cultures were diluted before measurement so that readings did not exceed 1.0.

Figure 1 shows a comparison of the protein, OD, and ATP methods for monitoring the growth of *M. tuberculosis* H37Rv and *M. bovis* BCG (Tokyo) cultures with and without Tween 80. For both organisms, the protein and ATP data show that the cell yields were higher for the Tween-free cultures than the Tween-containing cultures. This agrees with the observation reported by Sattler and Youmans (12). The protein method was also used successfully for monitoring the growth of *M. smegmatis* mc^{2}155 cultures (results not shown).

The detergent-free Sauton's medium cultures of the leucine auxotroph *M. bovis* BCG mc^{2}798 contained large clumps of cells which settled so rapidly on standing that OD measurements were not possible (results not shown). Despite the heavy clumping, the protein data points traced out smooth growth curves (Fig. 2), which show that the cell yield increased as the concentration of L-leucine in the medium increased.

It is clear from these results that the protein extraction method is limited by low sensitivity at low-cell densities, and it is therefore recommended that large inocula be used when growth is monitored by this method (compare Fig. 1 Ia and IIa). Where the measurement of growth in low-cell-density cultures is necessary, the ATP method is the more suitable. Although OD measurements for Tween-free Middlebrook cultures of both *M. tuberculosis* and *M. bovis* BCG were expected to be erratic, they nevertheless produced growth curves that are broadly similar to the corresponding curves obtained with the protein and ATP data. The application of the protein extraction method for monitoring the growth of severely clumped cultures was, however, clearly demonstrated with *M. bovis* BCG mc^{2}798 grown in Tween-free Sauton's medium, where OD measurements were not possible.

We have developed a simple, rapid, and inexpensive method for the measurement of mycobacterial growth in detergent-free cultures by using common laboratory equipment and reagents. The method compares favorably with OD and ATP measurements for the quantitation of well-dispersed cultures but is particularly useful for heavily clumped, detergent-free,

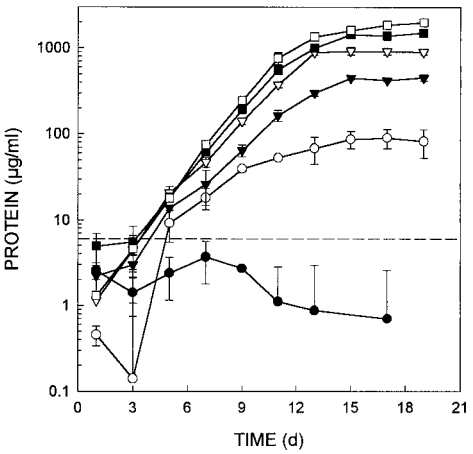

FIG. 2. Growth curves, monitored by total protein yield, of *M. bovis* BCG mc^{2}798 in detergent-free Sauton's medium containing 0 (●), 25 (○), 50 (▼), 125 (▽), 250 (■), and 500 (□) μg of L-leucine/ml. The dashed horizontal line indicates the lower limit of sensitivity of the protein assay (6 μg of protein/ml). Each data point represents the average of two measurements. Error bars show the standard deviations of the means.

defined-medium cultures in which accurate OD measurements and CFU counts are impossible. We propose that monitoring the growth of mycobacterial cultures by protein measurement is a suitable alternative to ATP measurement where the materials and apparatus for quantitation of ATP are not available.

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