The Journal of Microbiology (2012) Vol. 50, No. 3, pp. 419–425
DOI 10.1007/s12275-012-1556-0

Evaluation of the Cell Growth of Mycobacteria Using *Mycobacterium smegmatis* mc^2 155 as a Representative Species

Jorge A. Gonzalez-y-Merchand[1]*, Ruben Zaragoza-Contreras[1], Rosalina Guadarrama-Medina[1], Addy C. Helguera-Repetto[1], Sandra Rivera-Gutierrez[1], Jorge F. Cerna-Cortes[1], Leopoldo Santos-Argumedo[2], and Robert A. Cox[3]

[1]*Departamento de Microbiologia, Escuela Nacional de Ciencias Biologicas, IPN, Prolong. Carpio y Plan de Ayala s/n, Col. Casco de Santo Tomas, Mexico, D.F. 11340, Mexico*
[2]*Departamento de Biomedicina Molecular, CINVESTAV, Zacatenco, Mexico, D.F., Mexico*
[3]*Division of Mycobacterial Research, National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, UK*

(Received November 7, 2011 / Accepted March 5, 2012)

The study of the *in vitro* cell growth of mycobacteria still remains a fastidious, difficult, and time-consuming procedure. In addition, assessing mycobacterial growth in the laboratory is often complicated by cell aggregation and slow growth-rate. We now report that the use of a stainless steel spring in the culture led to an absence of large cell clumps, to a decrease of dead cells in the exponential phase and to growth of a more homogeneous population of large cells. We also report that flow cytometry is a rapid, simple and reliable approach to monitor mycobacterial cell growth and viability. Here, we monitored *Mycobacterium smegmatis* cellular growth by optical density, dry cell mass, and colony forming units; in addition, viability, cell size and granularity profiles were analyzed by flow cytometry, and cell morphology by electron microscopy. Cultures monitored by flow cytometry may lead to a better understanding of the physiology of mycobacteria. Moreover, this methodology may aid in characterizing the cell growth of other fastidious species of microorganisms.

Keywords: mycobacterial growth, mycobacterial physiology, *Mycobacterium smegmatis*, flow cytometry

Introduction

Mycobacteria are rod-shaped, aerobic bacteria, non-motile, Gram-positive, and acid-fast, with genomes that have a high G+C content (Holt *et al.*, 1994). Within this genus, *Mycobacterium tuberculosis* is a leading cause of infectious disease (WHO, 2011); this microorganism is notable for its slow growth both *in vivo* and *in vitro*, with generation times of 16 h or longer (Wayne, 1994). Traditionally, mycobacteria are often classified as either slow or fast growers according to the time taken for colonies to appear on a solid medium (Holt *et al.*, 1994). Knowledge of mycobacterial physiology has been limited by the slow growth of these major pathogens and by other technical problems such as the presence of a high percentage of dead or dying cells in exponential phase and also by cell aggregation (Ratledge, 1982; Wheeler and Ratledge, 1994). Two theoretical approaches have been used in order to understand the growth of mycobacteria; first, a framework was established based on quantitative relationships between specific growth rates (μ) and cell macromolecular compositions of *M. tuberculosis*, *M. bovis* BCG, *Streptomyces coelicolor*, and *Escherichia coli* B/r (Cox, 2004). It was suggested from this analysis that the slow growth of *M. tuberculosis* reflects cell metabolism and not the possession of a single rRNA operon (*rrn*) per genome. From the second analysis, it was demonstrated that microarrays of specific genes involved in DNA and cell division can be applied to bacterial cultures of known growth rates; i.e., the number of copies of FtsZ per cell is proportional to the number of ribosomes per cell (Cox, 2007). Therefore, new methodologies should be developed that allow rapid and reliable monitoring of mycobacterial physiology and cell growth. *M. smegmatis* is of interest because it is regarded as a typical fast grower; under favorable conditions it has a generation time of about 3 h (Sander *et al.*, 1996; Gonzalez-y-Merchand *et al.*, 1998; Sander *et al.*, 2002). Although *M. smegmatis* might be an opportunistic pathogen (Newton and Weiss, 1994; Skiest and Levi, 1998; Wallace *et al.*, 1998), it can be safely handled in the laboratory. In this study, the growth of *M. smegmatis* mc^2 155 in batch cultures was followed as a function of time by measuring cell mass, the number of colony forming units (CFU), cell viability [flow cytometry (FCM)], cell morphology (electron microscopy) and profiles of granularity and cell size (FCM). Two conditions of cell culture were examined: cells were grown in the presence of 0.1% (v/v) Tween 80 in shaking cultures either with or without the addition of a stainless steel spring, to aid both aeration and dispersion of the mycobacteria. This report assesses the growth of *M. smegmatis* mc^2 155 by means of established methods and also by FCM. This procedure allows the simultaneous measurements of several physical and chemical properties of bacterial cells in a matter of seconds, providing an indication of the heterogeneity of a population of microorganisms within minutes (Álvarez-Barrientos *et al.*, 2000). FCM has been used to detect the

*For correspondence. E-mail: jgonzal1212@yahoo.com.mx; Tel.: +00-5255-57296300, ext 62495.; Fax: +00-5255-57296000, ext 62495.

presence of live pathogenic mycobacteria such as *M. tuberculosis* in sputum (Burdz *et al.*, 2003), to measure the induction of Tumor Necrosis Factor (TNF) in macrophages infected with *M. tuberculosis* (Engele *et al.*, 2002) and to assess the viability of *M. tuberculosis* grown in a chemostat (James *et al.*, 2000). To the best of our knowledge, this report might be the first to show the reliability, speed and scope of FCM for measuring properties of mycobacterial cells during the course of batch cultures. Other methods are more expensive, elaborate and time-consuming; for example, measurements of CFU, dry cell mass (DCM) or electron microscopy (EM).

Materials and Methods

Bacterial strain and growth conditions

The strain investigated was *M. smegmatis* mc^2 155. Cells were cultured in Kohn-Harris succinate (1% w/v) Tween 80 (0.1% v/v) medium (K-HTM) (Gonzalez-y-Merchanol *et al.*, 1998). Cells were grown at 37°C while shaken at 200 rpm. In some cultures a sterile stainless steel spring 10 cm long and 1.3 cm in diameter (Alliance Spring Co., UK) was present in order to reduce cell aggregates and maximize homogeneity. The spring has a vigorous movement that increases the agitation of the growth medium.

A single colony of *M. smegmatis* mc^2 155 was grown in 5 ml of K-HTM for two days. A dilution (1:200) of this culture in fresh K-HTM was incubated for approximately 20 h until an OD_{640} of 0.3 ($[7\pm0.4]\times10^7$ CFU/ml) was reached. This second culture was used to inoculate the experimental samples. An inoculum (0.4 ml) from the same culture was added to each of 100 ml of K-HTM contained in a 250 ml conical flask with or without a stainless steel spring. The cultures were grown for 72 h and samples were removed from the cultures at intervals of 4 h, while the temperature was kept at 37°C. More than seven independent cultures were examined and the results obtained were found to be reproducible. Succinate as the sole source of carbon leads to the release of ammonia (MacFaddin, 1980) which increases the pH of the medium. Initially, the pH was 6.3± 0.05, which increased to 6.7±0.05 after 16 h and to 7.65±0.05 after 36 h.

Growth measurements

M. smegmatis growth was monitored by optical density (OD_{640}), dry cell mass, colony forming units per milliliter (CFU/ml) and by flow cytometry (FCM). Dry cell mass was measured by taking a 5 ml sample of the culture which was then passed through a 0.22 µm pore size nitrocellulose membrane (Millipore, USA); mycobacterial cells retained by the membrane were washed twice with 5 ml of deionized water and the membranes loaded with cells were dried in an oven at 90°C for 24 h then weighed with a OHAUS VOYAGER digital balance with an accuracy of ± 0.1 mg. Colony forming units per ml of culture (CFU/ml) were determined by plating appropriate dilutions. Ten-fold dilutions (from 10^{-1} to 10^{-7}) were made in sterile microtubes, previously packed with 300 µl of glass beads (150–212 µm in diameter, Sigma, USA) and 900 µl of a 0.05% (w/v) solution of Tween 80. Each dilution was briefly vortexed in order to reduce cell aggregation. Treatment with glass beads led to higher values of CFU/ml with smaller standard deviations.

An aliquot of 100 µl was plated from the appropriate dilution (10^{-1} and 10^{-2} for the samples taken from a mycobacterial culture of 0 h and 4 h of growth; 10^{-3} to 10^{-5} for the samples from a culture of 8 h to 20 h; and 10^{-5} to 10^{-7} for the samples from a culture of 24 h to 72 h), on Middlebrook 7H11 agar plus albumin/dextrose/catalase (ADC, Difco, USA). The plates were incubated at 37°C for five days before quantifying the CFU/ml. Colonies first became visible after three days of plating.

Determination of cell viability by flow cytometry

For the viability staining of *M. smegmatis*, the Live/Dead BacLight Viability kit (Molecular Probes Inc., USA) was used. This kit contains a green fluorochrome SYTO 9 as a marker for viable cells, and propidium iodide (PI) as a marker for non-viable (membrane-compromised) cells. The fluorochromes were used according to the manufacturer's instructions; briefly, an equimolar mix of both fluorochromes was combined with 10^6 to 10^8 *M. smegmatis* cells/ml and left at room temperature for 25 min. This sample was analyzed by FCM as described below. The total number of bacteria was determined by the Bacteria Counting kit (Molecular Probes Inc.). This kit contains the fluorochrome SYTO BC and a microsphere standard of 0.6 µm; both components were combined with an appropriate *M. smegmatis* culture dilution according to the manufacturer's instructions. The total number of *M. smegmatis* cells/ml was calculated using the formula provided by the manufacturer. Fisher's exact test was used in order to statistically analyze the viability results.

Flow cytometry (FCM) analysis

FCM analyses were performed on a FACS Calibur Flow Cytometer (Becton Dickinson, USA) equipped with a 15 mW, 488 nm, air-cooled argon ion laser and a cell sorting catching tube. Cell samples were diluted to have approximately 10^6–10^8 cells/ml and delivered at a low flow rate (12 µl/min). Forward Scattered light (FSC), Side Scattered light (SSC) and the two fluorescent signals (green and red) were measured. A band pass filter of 530 nm (515–545 nm) was used to collect the green fluorescence (FL1) and a band pass filter of 585 nm (564–606 nm) was used to collect the yellow-orange fluorescence (FL2). FSC was collected with a diode detector. SSC and the fluorescence signals were measured by photomultiplier tubes. Because of the small cell size, all signals were collected by using logarithmic amplifications of intensities and on each occasion at least ten thousand events were analyzed. A combination of FSC and SSC was used to discriminate bacteria from background, and a combination of FL1 and FL2 was used to discriminate between viable and non viable cells. Data were analyzed with the CellQuest program (version 3.1f; Becton Dickinson, USA).

Transmission electron microscopy

Cells were harvested by centrifugation at 16,000×g, washed in sterile phosphate buffered saline (PBS), and fixed in

Table 1. Properties of *M. smegmatis* mc^2 155 grown in batch cultures

Parameter property[a]	Cultures with spring	Cultures without spring
Exponential phase		
μ (h^{-1})[b]	0.200±0.004	0.196±0.009
t_D (h)[b]	3.48±0.262	3.53±0.107
μ (h^{-1})[c]	0.244±0.006	0.299±0.010
t_D (h)[c]	2.90±0.07	2.32±0.111
CFU/OD_{640}	$(2.10\pm0.05)\times10^8$	$(2.45\pm0.70)\times10^8$
dry cell mass/CFU (pg/CFU)	3.95±1.05	3.54±0.98
Stationary phase		
CFU/OD_{640}	$(6.3\pm0.7)\times10^8$	$(1.91\pm0.46)\times10^9$
dry cell mass/CFU (pg/CFU)	0.631±0.092	0.508±0.088

[a] Values represent the mean of at least four independent cultures±one standard deviation. t_D, generation time. μ, specific growth rates. [b] Calculated from data for OD_{640}. Values represent the mean. [c] Calculated from data for CFU/ml (see Fig. 1B).

2.5% glutaraldehyde/0.1 M sodium phosphate (v/v) for 1 h. Cells were recovered and resuspended in 1% osmium tetroxide (w/v) for 1 h. Cells were then dehydrated through treatment with a series of ethanol solutions (30%, 40%, 50%, 60%, 70%, 80%, and 90%; each treatment was performed twice for 10 min each time). A final dehydration step was done twice with 100% ethanol for 15 min. Samples were embedded in an EPON 812 resin (EMS, USA). This resin was polymerized at 60°C for 24 h. Resin blocks were cut in a LEICA Ultracut UCT ultratome to 70 nm thickness, placed on copper, 200 mesh grids (EMS) and stained with 30% uranyl acetate (w/v) / 70% methanol (v/v) and counterstained with Reynold's lead citrate buffer. Samples were examined using a Jeol JEM-1010 Transmission Electron Microscope with an acceleration voltage of 60 kV.

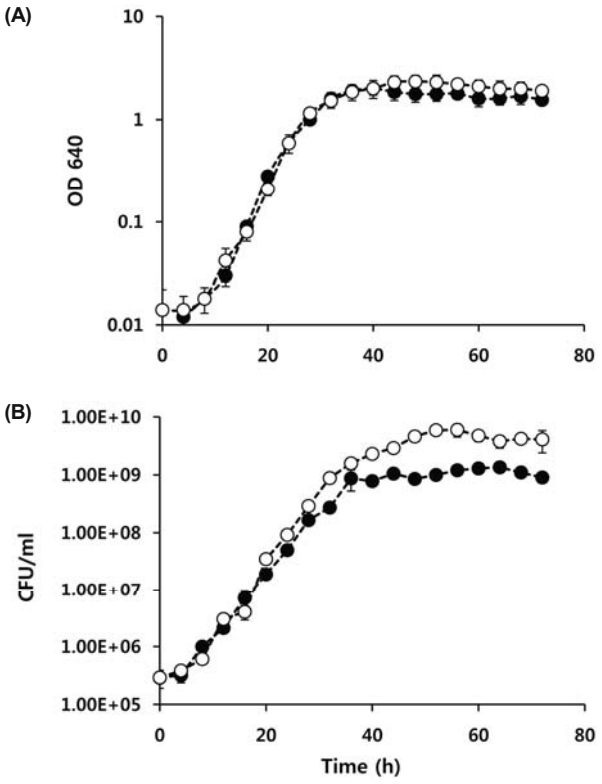

Fig. 1. The growth of *M. smegmatis* mc^2 155 at 37°C in cultures agitated with and without a spring. (A) Measurements of cell growth by OD_{640}. The plot represents five independent experiments. The bar denotes ± 1 standard deviation. (B) Measurements of CFU/ml of culture. The plot represents four independent experiments. The bar denotes±1 standard deviation. Open circles denote cultures grown without a spring. Filled circles denote cultures grown with a spring.

Results

Growth curves (0–72 h) for cells grown with and without the aid of a spring are presented in Fig. 1. The dependence of OD_{640} on incubation time is shown in Fig. 1A and the corresponding measurements for CFU are shown in Fig. 1B. A lag phase of at least 4 h was observed even though the inoculum was obtained from exponentially growing cultures and the fresh medium was pre-heated to 37°C. Exponential phase extended over a period of at least 12 h from 12 h–24 h after inoculation. Entry into stationary phase was observed after 28 h as judged by OD_{640} measurements [see Fig. 1A]. The increase of CFU/ml in stationary phase when OD_{640} increased very little or even decreased, suggests that although the overall biomass/ml remained largely unchanged, the number of cells/ml increased as the mass/cell diminished (see Table 1). Properties of *M. smegmatis* mc^2 155 grown in batch cultures with and without the aid of a spring are summarized in Table 1. The specific growth rates (μ) in exponential phase were found to be very similar for both conditions of culture, as judged by OD_{640} measurements. However, the profiles of CFU versus time yielded different values of specific growth rate (μ) depending on whether or not a spring was used.

The specific growth rate measured by OD_{640} reflects the rate at which cell mass is replicated; cell mass is dependent on both the number and sizes (mass) of the cells. The use of a spring had little or no effect on the specific growth rate measured OD_{640} (see Table 1) and therefore no effect on the rate of synthesis of cell mass. In contrast, the specific growth rate measured by CFUs depends only on the number of cells (the CFUs). Ideally, this method should reflect the generation time of the cells under scrutiny. It was found (see Table 1) that the use of a spring increased the generation time by about 25%.

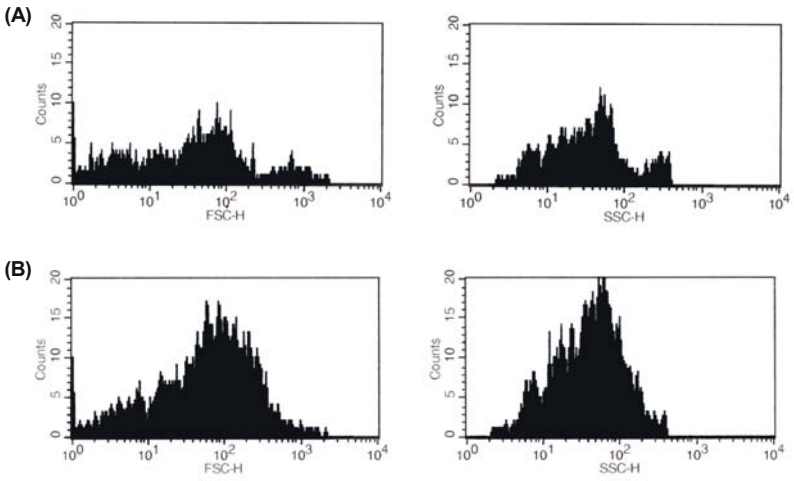

Fig. 2. Profiles of granularity and size of cells grown with and without a spring. Cell samples were taken after 16 h incubation at 37°C. The ordinate (cell counts) denotes single events. The profiles of events versus forward scatter (FSC-H) reflect the size of cells. The profiles of counts versus side scatter (SSC-H) reflect the granularity of cells, (A), cells grown without a spring; (B), cells grown with a spring. At least ten thousand events were analyzed in each sample.

We propose that these observations can be reconciled in the following way, remembering that the use of a spring had no effect on the rate of cell mass synthesis. We suppose that the spring affects the distribution of cell mass and not the total amount by stimulating the synthesis of a fewer number of larger cells. Although the larger cells contribute more to OD_{640} than smaller cells, the fewer number of larger cells keeps the cell mass per ml the same as for cultures grown without a spring. Because the spring has no effect on the rate of synthesis of cell mass, the larger cells will require a longer generation time, as was shown by the CFU-based measurements. Furthermore, the ratio of the generation times should, in this case, provide an estimate of the ratio of the cell sizes.

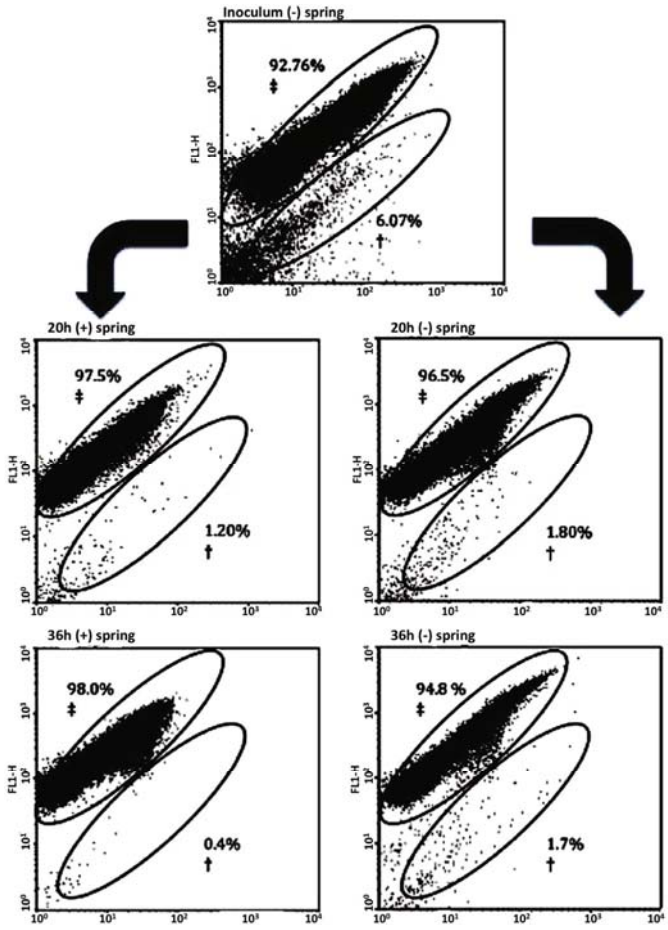

Fig. 3. Viability of cells grown with and without spring. In each case the inoculum was a sample (OD_{640}=0.3) taken from a culture grown without a spring. These samples were not diluted for fluorescence measurements. All other samples were diluted one hundred fold. The times (h) after inoculation at which the samples were taken are shown. Abscissa (FL2-H) indicates the measurement of red fluorescence while ordinate depicts (FL1-H) measurement of green fluorescence. Viable cells take up green stain more strongly than red stain. Dead or dying cells take up red more strongly than green stain. Note that few dead or dying cells were found after cultures were grown with a spring.

‡Viable cells; † dead or dying cells.

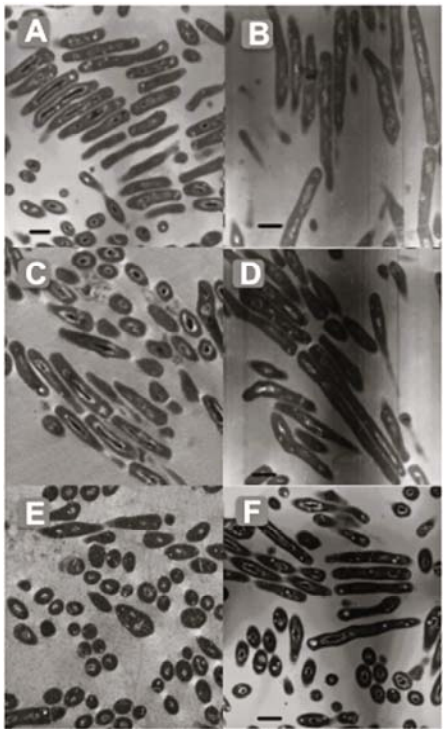

Fig. 4. Electron micrographs of samples of *M. smegmatis* mc^2 155. Samples (A), (B), and (C), respectively, were obtained from a culture grown without a spring for 12 h (exponential phase), 24 h (exponential phase), and 36 h (stationary phase) after inoculation. Samples (D), (E), and (F), respectively, were obtained from a culture grown with a spring for 12 h, 24 h, and 36 h after inoculation. Magnification was ×10,000; in each case the bar represents 0.5 μm. For the growth curve see Fig. 5.

The properties of exponentially growing cultures revealed by FCM were found to support the notion that a population of larger cells was obtained when growth was aided by the use of a spring (see Fig. 2B, lower left). The profile of counts (events) versus forward (0°) scattered light (FSC-H) provides a measure of the distribution of cell sizes within a cultured population of mycobacteria. The profile of side (90°) scattered light (SSC-H) measures the internal and surface complexity of cells. The profiles of FSC-H and SSC-H observed for cultures grown with a spring indicates that the cell population was more homogeneous and the cells were larger than the cell population obtained without the use of a spring.

Dead (or badly damaged) cells were found to be present in exponential phase when cells were grown without a spring and to be almost absent from the exponential phase when grown with a spring, as shown by FCM (Fig. 3). As few as 0.4% dead cells were found to be present in bacterial cultures agitated with a spring, compared with 1.7% found in cultures without a spring ($p<0.05$).

Examination of cell morphology by electron microscopy (Fig. 4) revealed that *M. smegmatis* mc^2 155 grew as slender rods of variable length and constant diameter. Exponential phase mycobacteria cultured with the aid of a spring showed dimensions of 0.3–0.4 μm×4.4 μm (upper limit). Cells of similar dimensions were observed for *M. smegmatis* grown in Middlebrook 7H9 broth (Dr. S. A. Sattar, The Centre for Research on Experimental Microbiology, The University of Ottawa; unpublished results) and viewed by scanning electron microscopy; namely 0.3–0.4 μm×4.3 μm. In contrast, the larger cells observed without a spring were 0.3–0.4 μm×2.7 μm (38.6 rate of decrease in total length).

Discussion

The tendency of *M. smegmatis* cells to clump in minimal medium was described previously (Smeulders *et al.*, 1999), where it was found 'that the clumps become larger as exponential phase progresses, and they become more compact after entry into stationary phase'. Examination, by phase contrast microscopy, of culture samples grown with 0.1% Tween 80 but without a spring revealed large clumps of cells and few single cells, in accord with the observations of Smeulders *et al.* (1999). In contrast, some clumps of a few cells and many single cells were found in cultures grown with 0.1% Tween 80 and the spring (data not shown). Besides, the measurements of μ cited in Table 1 are consistent with the observations that cells grown with the aid of a spring were larger than those grown without it.

In an ideal case, the dry cell mass/CFU is equal to the dry cell mass/cell. The dry cell mass for an *E. coli* cell ranges from 0.204 pg to 0.918 pg according to the specific growth rate (Bremer and Dennis, 1996). The dry cell mass of *M. smegmatis* (μ=0.244/h) would be expected to lie within this range. However, the observed values (see Table 1) of 3.95±1.05 pg/CFU and 3.54±0.98 pg/CFU for exponential phase are higher than those expected for single cells. Values of 2.8–6.4 pg/CFU were reported for *M. tuberculosis* grown exponentially in a chemostat (James *et al.*, 2000). High values of dry cell mass/CFU indicative of either large aggregates of cells or dead cells, or both, were reported (Beste *et al.*, 2005) for *M. bovis* BCG Pasteur grown in a chemostat; namely, 69 pg/CFU (μ=0.03/h) and 13.5 p/CFU (μ=0.01/h). In contrast, the values of 0.631±0.092 pg/CFU and 0.508±0.088 pg/CFU obtained for stationary phase cells of *M. smegmatis* are closer to values expected for single cells.

Except during lag phase, we observed a high correlation between CFU and viability measured by FCM when cultures

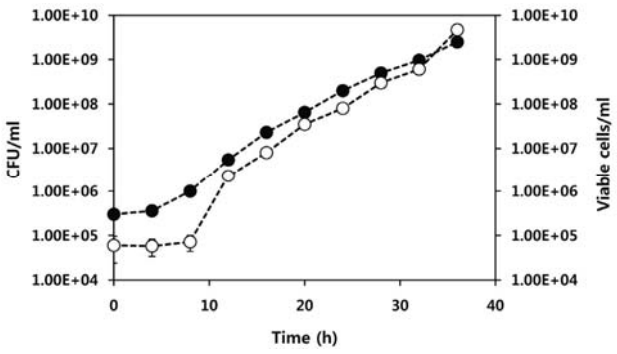

Fig. 5. Correlation between the number of viable cells/ml (FCM) and the number of CFU/ml of *M. smegmatis* cultures grown with a spring. Open circles represent viable cells/ml and filled circles represent CFU/ml.

were grown with a spring (Fig. 5). Hence, we found it both simpler and quicker to measure cell viability by FCM rather than by CFU. The differences noticed in lag phase appear to reflect the differences in the procedures followed. The 5 days of incubation in a rich medium, which is needed for measurements of CFU, may allow time for damaged cells present in the inoculum to recover and form colonies: such cells are not included in measurements of viable cells.

The long, thin rod shape of *M. smegmatis* provides the cell with a high surface area-to-volume ratio which is a favorable property for a bacterium with a cell envelope that has a low surface density of porin channels (Stephan *et al.*, 2005).

There was a high concordance among different parameters used (CFU, DCM, EM, and FCM), for example, the particularly large size of *M. smegmatis* cells growing with the aid of the spring at log phase, is shown in Table 1 (DCM) and Figs. 2B and 4E. The lengthy and laborious procedures of most approaches mentioned above, except FCM, emphasize the potential benefits of using FCM to study mycobacterial viability and physiological parameters throughout a growth curve. Furthermore, FCM is not only a rapid (a few min) and reproducible approach, but is also economical since just two samples provide us with data concerning cell size, percentages of live and dead cells, and cellular complexity for a given population of bacteria.

Mycobacterial cells have a tendency to clump during cell culture. Tween 80 is often added to the growth medium to reduce aggregation but clumps of up to twenty or so cells persist (James *et al.*, 2000). In our experience stirrer bars offer no benefit over shaking in reducing aggregation. We have shown that the use of a spring reduces aggregation below the level observed by the use of Tween 80 alone. The spring visibly increases the agitation of the growth medium because of its rapid reciprocating motion. Two methods suitable for FCM studies of *M. tuberculosis* have been described. One procedure (James *et al.*, 2000) requires that the flow cytometer be housed under level 3 containment conditions. The second method (Moore *et al.*, 1999) uses fixation with 1% (w/v) paraformaldehyde to inactive the pathogen and thereby allow the samples to be removed from containment. In conclusion, we have shown that FCM constitutes a powerful tool for revealing growth profiles of mycobacterial cell populations, providing several advantages over other methods due to its rapid measuring, high sensitivity and reproducible results. These procedures may aid us to characterize mycobacterial and other fastidious bacterial cultures in a fast and simple fashion.

Acknowledgements

We thank Simon A. Cox for his help in the preparation of this manuscript, Iris Estrada-Garcia for helpful review of the manuscript, and Oliver Ornelas-Lopez and Esther Ramirez-Espindola from the Microscopy Unit of ENCB, IPN, for their help with the electron microscopy analysis. This work is supported as part of the 7th Framework Program, European Commission, contract no. HEALTH-F3-2008-200999. It was also partially supported by grants SIP20110750, SIP-20110106 and SIP 20110448 from IPN, Mexico. J.A.G-y-M, J.F.C-C, S.R-G and A.C.H-R are fellows of COFAA and EDI, IPN, Mexico.

References

Álvarez-Barrientos, A., Arroyo, J., Cantón, R., Nombela, C., and Sánchez-Pérez, M. 2000. Applications of flow cytometry to clinical microbiology. *Clin. Microbiol. Rev.* **13**, 167–195.

Beste, D.J., Peters, J., Hooper, T., Avignone-Rossa, C., Bushell, M.E., and McFadden, J. 2005. Compiling a molecular inventory for *Mycobacterium bovis* BCG at two growth rates: evidence for growth rate-mediated regulation of ribosome biosynthesis and lipid metabolism. *J. Bacteriol.* **187**, 1677–1684.

Bremer, H. and Dennis, P.P. 1996. Modulation of chemical composition and other parameters of the cell growth rate, pp. 1553–1568. *In* Neidhardt, F.C., Curtis, R., Ingraham, J.L., Lin, E.C.C., Low, K.B., Magasanik, B., Reznikoff, W.S., Riley, M., Schaechter, M., and Umbarger, H.E. (eds.), *Escherichia coli* and *Salmonella*: cellular and molecular biology. 2nd ed. American Society for Microbiology, Washington, D.C., USA.

Burdz, T.V.N., Wolfe, J., and Kabani, A. 2003. Evaluation of sputum decontamination methods for *Mycobacterium tuberculosis* using viable colony counts and flow cytometry. *Diagn. Microbiol. Infect. Dis.* **47**, 503–509.

Cox, R.A. 2004. Quantitative relationships for specific growth rates and macromolecular compositions of *Mycobacterium tuberculosis*, *Streptomyces coelicolor* A3(2) and *Escherichia coli* B/r: an integrative theoretical approach. *Microbiology* **150**, 1413–1426.

Cox, R.A. 2007. A scheme for the analysis of microarray measurements based on a quantitative theoretical framework for bacterial cell growth: application to studies of *Mycobacterium tuberculosis*. *Microbiology* **153**, 3337–3349.

Engele, M., Stöbel, E., Castiglione, K., Schwerdtner, N., Wagner, M., Bölcskei, P., Röllinghoff, M., and Stenger, S. 2002. Induction of TNF in human alveolar macrophages as a potential evasion mechanism of virulent *Mycobacterium tuberculosis*. *J. Immunol.* **168**, 1328–1337.

Gonzalez-y-Merchand, J.A., Colston, M.J., and Cox, R.A. 1998. Roles of multiple promoters in transcription of ribosomal DNA: effects of growth conditions on precursor rRNA synthesis in mycobacteria. *J. Bacteriol.* **180**, 5756–5761.

Holt, J.G., Krieg, N.R., Sneath, P.H.A., Staley, J.T., and Williams, S.T. 1994. Bergey's Manual of Determinative Bacteriology, pp. 597–603. 9th ed, The Williams & Wilkins Co., Baltimore, Maryland, USA.

James, B.W., Williams, A., and Marsh, P.D. 2000. The physiology and pathogenicity of *Mycobacterium tuberculosis* grown under controlled conditions in a defined medium. *J. Appl. Microbiol.* **88**, 669–677.

MacFaddin, J.F. 1980. Biochemical tests for identification of medical bacteria, pp. 59–63. 2nd ed. The Williams & Wilkins Co., Baltimore, Maryland, USA.

Moore, A.V., Kirk, S.M., Callister, S.M., Mazurek, G.H., and Schell, R.F. 1999. Safe determination of susceptibility of *Mycobacterium tuberculosis* to antimycobacterial agents by flow cytometry. *J. Clin. Microbiol.* **37**, 479–483.

Newton, J.A.Jr. and Weiss, P.J. 1994. Aspiration pneumonia caused by *Mycobacterium smegmatis*. *Mayo Clin. Proc.* **69**, 296–298.

Ratledge, C. 1982. Nutrition, growth and metabolism, pp. 185-271. *In* Ratledge, C. and Stanford, J.L. (eds.), Biology of the Mycobacteria, Academic Press Inc Ltd. London, UK.

Sander, P., Belova, L., Kidan, Y.G., Mankin, A.S., and Böttger, E.C. 2002. Ribosomal and non-ribosomal resistance to oxazolidinones: species-specific idiosyncrasy of ribosomal alterations.

Mol. Microbiol. **46**, 1295–1304.

Sander, P., Prammananan, T., and Böttger, E.C. 1996. Introducing mutations into a chromosomal rRNA gene using a genetically modified eubacterial host with a single rRNA operon. *Mol. Microbiol.* **22**, 841–848.

Skiest, D.J. and Levi, M.E. 1998. Catheter-related bacteremia due to *Mycobacterium smegmatis*. *South Med. J.* **91**, 36–37.

Smeulders, M.J., Keer, J., Speight, R.A., and Williams, H.D. 1999. Adaptation of *Mycobacterium smegmatis* to stationary phase. *J. Bacteriol.* **181**, 270–283.

Stephan, J., Bender, J., Wolschendorf, F., Hoffmann, C., Roth, E., Mailänder, C., Engelhardt, H., and Niederweis, M. 2005. The growth rate of *Mycobacterium smegmatis* depends on sufficient porin-mediated influx of nutrients. *Mol. Microbiol.* **58**, 714–730.

Wallace, R.J.Jr., Nash, D.R., Tsukamura, M., Blacklock, Z.M., and Silcox, V.A. 1998. Human disease due to *Mycobacterium smegmatis*. *J. Infect. Dis.* **158**, 52–59.

Wayne, L.G. 1994. Cultivation of *Mycobacterium tuberculosis* for research purposes, pp. 73–83. *In* Bloom, B.R. (ed.), Tuberculosis: pathogenesis, protection and control. American Society for Microbiology, Washington, D.C., USA.

Wheeler, P.R. and Ratledge, C. 1994. Metabolism of *Mycobacterium tuberculosis*, pp. 353–380. *In* Bloom, B.R. (ed.). Tuberculosis: pathogenesis, protection and control, American Society for Microbiology, Washington, D.C., USA.

WHO (World Health Organization). 2011. Global tuberculosis control 2011. Available at: http://www.who.int/tb/publications/global_report/2011/gtbr11_full.pdf