

# Dormant forms of *Mycobacterium smegmatis* with distinct morphology

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Cultivation of *Mycobacterium smegmatis* cells in a nitrogen-limited minimal medium (SR-1) followed by prolonged storage at room temperature without shaking resulted in the gradual accumulation of morphologically distinct ovoid forms characterized by (i) low metabolic activity; (ii) elevated resistance to antibiotics and to heat treatment; and (iii) inability to produce colonies on standard agar plates (non-platable cells). Detailed microscopic examination confirmed that ovoid cells possessed an intact cell envelope, specific fine structure and large electron-transparent bodies in the cytoplasm. Cell staining with Nile red and analysis of the lipid content by TLC revealed the presence of significant amounts of apolar lipids in these bodies. The ovoid forms could be stored for significant periods (up to 5 months) and resuscitated afterwards in a modified Sauton's medium. Importantly, resuscitation of ovoid cells was accompanied by their transformation into the typical rod-shaped cells. We suggest that the observed ovoid cells represent dormant forms, resembling morphologically distinct cells of *Mycobacterium tuberculosis* previously isolated from tuberculosis patients and infected animals.

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## INTRODUCTION

*Mycobacterium tuberculosis* (MTB), the causative agent of tuberculosis (TB), is responsible for more deaths than any other infectious agent. This organism is estimated to infect about one-third of the world population (Bloom & Murray, 1992). Nevertheless, 90 % of these 2 billion people remain clinically healthy and never develop overt TB because of quiescent persistence of the tubercle bacilli that they harbour. Such latent TB infections are difficult to diagnose and treat; moreover in adults they can reactivate at any time and are often accompanied by severe destruction of lung tissue (Wallis & Ellner, 1994). Persisting organisms evidently are in a state of dormancy, defined before as 'a reversible state of low metabolic activity, in which cells can persist for extended periods without division' (Kaprelyants *et al.*, 1993). In recent years, significant progress has been achieved in characterization of various aspects of mycobacterial infection and adaptation to different environmental conditions (Young *et al.*, 2005; Boshoff & Barry, 2005). However, our knowledge on persisting mycobacteria associated with latent TB is still very limited, mainly due to presumably very low numbers of such organisms and the lack of clear indication of their

precise localization in living tissues of the host (Neyrolles *et al.*, 2006).

Only a few studies have demonstrated isolation of supposedly dormant MTB cells. Their existence was originally proposed to account for the presence of acid-fast, but transiently 'non-culturable' bacteria in closed pulmonary lesions (Medlar *et al.*, 1952) and further reinforced by interesting findings in the Cornell model of latent TB (McCune *et al.*, 1966). Later, Khomenko and colleagues found filterable or mini-forms of MTB that remained within the tissues of guinea pigs upon the completion of anti-TB treatment. After several months of therapy, viable MTB could no longer be detected by standard plating procedures, although microscopic examination of organ homogenates filtered through 0.2–0.7 µm filters revealed the presence of electron-dense forms with a rounded shape and an average diameter of 0.25 µm. Direct administration of these forms to guinea pigs induced the development of TB, and after several passages, MTB could be isolated by standard culture methods. The authors suggested that these filterable forms might represent persistent organisms, which were able to convert to actively growing cells under the appropriate conditions (Khomenko & Golyshevskaya, 1984). In the last two decades, significant efforts have been invested to develop several *in vitro* models of mycobacterial dormancy (reviewed by Young *et al.*, 2005 and Zhang,

Abbreviations: MTB, *Mycobacterium tuberculosis*; PI, propidium iodide; TB, tuberculosis.

2004). In particular, dormant, 'non-culturable' MTB cells were obtained *in vitro* in prolonged stationary phase (Sun & Zhang, 1999; Shleeva *et al.*, 2002). These tubercle bacilli were resuscitated using early-stationary-phase culture supernatant of the same strain (Sun & Zhang, 1999), and in a later study, by addition of phospholipids or 8 kDa secreted protein found in the supernatant (Zhang *et al.*, 2001). Also, the importance of a family of proteinaceous resuscitation-promoting factors (Rpf) secreted by MTB for resuscitation of 'non-culturable' tubercle bacilli cells was established (Kana *et al.*, 2008). With great anticipation of important findings resulting from these and other studies, it should be admitted that any single model has failed to reproduce the complexity of the *in vivo* environment and to provide conditions for generation of morphologically distinct persisting cells similar to the forms described by Khomenko's group. In the present study, we attempted to fill this obvious gap in mycobacterial physiology and find requirements essential for production of such forms *in vitro*.

## METHODS

**Strain and growth conditions.** *Mycobacterium smegmatis* strain mc<sup>2</sup>155 was routinely maintained on solid medium plates, containing NB (Nutrient Broth, HiMedia Laboratories, India) and 1.5% agar; only fresh 3–5-day-old colonies were used for production of starter cultures. Cells were grown in standard Sauton's medium or in SR-1 medium of the following composition: glucose, 20 g; MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.5 g; NaCl, 0.5 g; KNO<sub>3</sub>, 1 g; K<sub>2</sub>HPO<sub>4</sub>, 0.5 g; CaCO<sub>3</sub>, 3 g; H<sub>2</sub>O, 1 l; pH 7.0 (Krasilnikov, 1950). In some experiments, SR-1 medium was modified by reducing the concentration of KNO<sub>3</sub> to 0.2 g l<sup>-1</sup>. For c.f.u. estimations, solid NB medium with 1.5% agar was used. Modified Sauton's medium (KH<sub>2</sub>PO<sub>4</sub>, 0.25 g; MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.25 g; L-asparagine, 2 g; glycerol, 6 ml; ferric ammonium citrate, 0.025 g; sodium citrate, 1 g; 1% ZnSO<sub>4</sub>, 0.05 ml; H<sub>2</sub>O, 1 l; pH 7.0) was used for resuscitation of non-platable cells.

**Formation of dormant *M. smegmatis* cells.** Starter cultures for all experiments were produced by inoculation of one colony of *M. smegmatis* into a 75 ml flask containing 25 ml Sauton's medium, followed by incubation at 37 °C with agitation (200 r.p.m.) for 35 h. This culture served as the inoculum (200 µl) for the second passage under similar conditions. One millilitre of the second-passage culture was transferred to 100 ml nitrogen-limited SR-1 medium in 500 ml flasks, followed by incubation at 30 °C for 72 h in a shaker (200 r.p.m.). Then the culture was kept at ambient temperature under stationary conditions (without agitation) for a further 2.5–5 months.

**Resuscitation of non-platable cells.** Resuscitation of the cultures containing non-platable cells was performed in plastic 48-well microplates (Corning), using the most probable number (MPN) assay as described before (Downing *et al.*, 2005). Briefly, cells (10 ml of the culture) were centrifuged for 20 min, 3000 g to remove spent medium; the pellets were resuspended and homogenized using a thin syringe needle in 10 ml modified Sauton's medium. The cell suspension was decimally diluted by inoculation of 0.1 ml of cell suspensions into 0.9 ml of the same medium.

**Fractionation of morphologically distinct cells of *M. smegmatis*.** Two millilitres of the culture was carefully applied onto the top of a 1.1–2.1 M sucrose gradient (total volume, 8 ml) in 12 ml glass centrifuge tubes. After centrifugation of the tubes for 60 min at

3000 g, the bottom fractions (120 µl) were collected and used for c.f.u. estimation and MPN assay.

**Incorporation of radioactive tracers.** Ten millilitres of 2.5-month-old cell culture, grown in nitrogen-limited SR-1 medium, was centrifuged for 20 min, 3000 g. The supernatant was discarded, and the pellet was resuspended in 15 ml modified Sauton's medium for resuscitation. Every 4 h, samples from the resuscitating culture were placed into Eppendorf tubes with 1 µl L-[<sup>3</sup>H]asparagine and [5,6-<sup>3</sup>H]uracil (each 10 µCi ml<sup>-1</sup>; 370 kBq ml<sup>-1</sup>) and incubated at 37 °C with agitation for 4 h. A 0.2 ml sample of the culture was placed into a 15 ml Falcon tube with 3 ml 10% ice-cold CCl<sub>3</sub>COOH and incubated at 0 °C for 15 min. The mixture was then passed through a Whatman glass microfibre filter followed by washing with 3 ml 10% CCl<sub>3</sub>COOH and 4 ml 96% ethanol. Filters were placed into 10 ml scintillation mixture, and impulse counts were determined using an LS analyser (Beckman Instruments).

**Flow cytometry.** The FACSCalibur system (Becton Dickinson) was used to measure red fluorescence (650 nm) of microbial cells after homogenization with a thin syringe needle. This parameter was acquired as pulse height signals for 20 000 events at a rate of 800–1100 events per second. Fluorescence was measured before and after treatment of cell suspensions with Nile red (4 µg ml<sup>-1</sup>). Data analysis was done and graphics were acquired using the WinMDI 2.8 software package.

**Light and fluorescence microscopy.** Cell suspensions were examined under an Eclipse E4000 microscope (Nikon, Japan) in the phase-contrast and epifluorescence modes after staining with propidium iodide (PI, 3 µM) to detect injured/dead cells, with ethidium bromide (5 µM) to identify DNA-containing cells, or with Nile red (4 µg ml<sup>-1</sup>) to detect lipid inclusions; excitation was at 510 nm, and emission was at >560 nm.

**Electron microscopy.** Harvested cells were prefixed with 1.5% (w/v) glutaraldehyde in 0.05 M cacodylate buffer (pH 7.2) at 4 °C for 1 h. After washing three times with this buffer, the material was post-fixed with 1% OsO<sub>4</sub> in 0.05 M cacodylate buffer at 20 °C for 3 h. After dehydration, the material was embedded in Epon 812 epoxy resin and cut into thin sections on an LKB III ultratome. The sections were mounted on copper Formvar-coated grids and contrasted with a 3% solution of uranyl acetate in 70% ethanol for 30 min and then stained with lead citrate at 20 °C for 4–5 min. Thin sections were examined in a JEM-100B electron microscope (JEOL) operating under standard conditions at 60 kV.

**Lipid extraction and analysis.** Cells from stationary-phase cultures (Sauton's medium, 72 h growth) and 3-month stored cultures (N-limited SR-1 medium) of *M. smegmatis* were sedimented by centrifugation (4000 g, 15 min), washed three times in PBS and freeze-dried. Apolar lipids were extracted from 50 mg freeze-dried biomass and analysed by TLC using the solvent system petroleum ether/acetone (96:4, v/v) as described by Dobson *et al.* (1985). Chromatograms were scanned and images were analysed using the TotalLab 2.01 software package.

**Antibiotic sensitivity and heat treatment.** Four-millilitre aliquots from stationary-phase (48 h) cultures in Sauton's medium and from cultures stored for 2.5 months in N-limited SR-1 medium were transferred into 15 ml tubes and incubated with different concentrations (25, 50, 75 µg ml<sup>-1</sup>) of hygromycin or doxycycline at 37 °C for 24 h with agitation or heated at 60–80 °C for 10 min. The number of c.f.u. was determined as described above. PI-positive and negative cells were counted under an epifluorescence microscope using a Helber's chamber.

## RESULTS

### Formation of ovoid forms of *M. smegmatis*

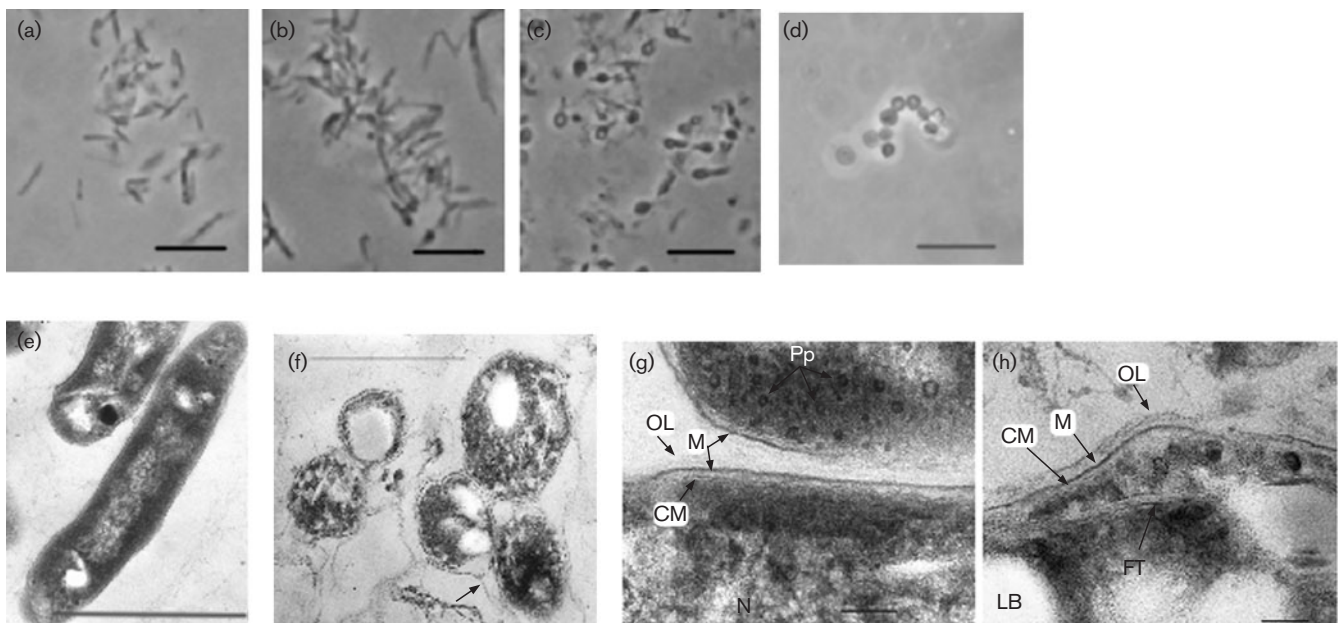
Our previous studies on dormant forms of *M. smegmatis* showed the importance of suboptimal growth conditions for transition of cells to the non-culturable state (Shleeva *et al.*, 2004); therefore, in the present study, we selected the simple, defined SR-1 medium, originally formulated for isolation and cultivation of actinobacteria (Krasilnikov, 1950). This medium supported the growth of *M. smegmatis*, even though it was less efficient than in Sauton's medium. Thus, after 3 days of cultivation, *M. smegmatis* cultures reached  $10^{10}$  c.f.u. ml<sup>-1</sup> in Sauton's medium,  $10^9$  c.f.u. ml<sup>-1</sup> in SR-1, and only  $10^7$  c.f.u. ml<sup>-1</sup> in nitrogen-limited SR-1 medium. *M. smegmatis* cells were grown in nitrogen-limited SR-1 medium for 3 days, and stored without agitation on a bench at room temperature for a further 5 months.

Periodic examinations of these cultures by phase-contrast microscopy revealed accumulation of atypical cells with significantly altered morphology. After 14 days of incubation, 10–20% of the cells in the population were rods with easily distinguishable 'bulges' at the cell ends. During further incubation, cells became racket-like, and after 1.5 months, 50–70% of the cell population was represented by almost round (ovoid) cells with central highly refractive

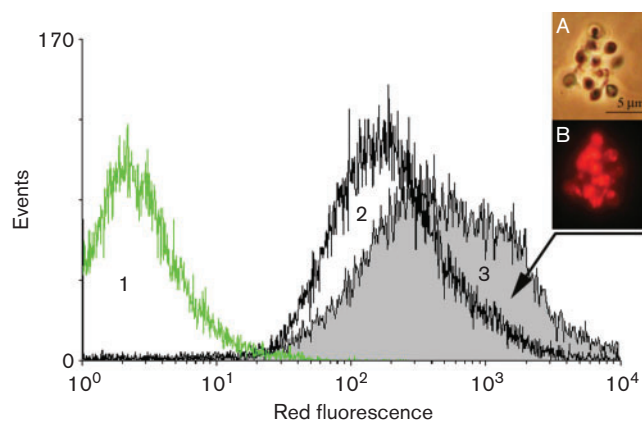
regions (Figs 1 and 2). The percentage of ovoid cells did not change significantly up to 5 months of further incubation. The length to width ratio of these cells was 1.0–1.4 (average diameter 0.8–1.4  $\mu\text{m}$ ), in contrast to 5–7 for stationary-phase cells. The ovoid cells were stainable with Ziehl–Neelsen reagent similarly to stationary-phase, rod-shaped cells. The formation of these ovoid cells was absolutely dependent on the conditions of cell cultivation: replacement of nitrogen-limited SR-1 medium by either standard Sauton's medium, its modified versions with different limitations, or normal, unlimited SR-1 medium did not support the accumulation of ovoid cells in prolonged stationary phase. Under those conditions, moribund or dead (according to PI staining) cells appeared with time. The amount of ovoid cells in populations also depended on the age of inoculum used; the maximum yield was obtained with 24 h inoculum. The storage of cultures without agitation was also a prerequisite for the formation of ovoid cells in sufficient amounts.

### Electron microscopy studies

Electron microscopy of thin sections revealed differences between stationary-phase (Fig. 1e, g) and long-stored *M. smegmatis* cells (Fig. 1f, h). In stationary-phase cells, grown in Sauton's medium, the cell wall exhibited an electron-dense layer of murein and an outer loosened layer of low



**Fig. 1.** Light microscopy (a–d) and thin-section transmission electron microscopy (e–h) of stationary-phase and ovoid *M. smegmatis* cells. (a, b, c) Cells grown in nitrogen-limited SR-1 medium and stored for 2, 3, 4 weeks respectively or for 5 months (f, h) as described in Methods; (d) pure fraction of ovoid cells obtained after centrifugation in a sucrose gradient. (e, g) Stationary-phase cells (3 days) grown in standard Sauton's medium, under low and high magnification; (f, h) 5-month-old cells under low and high magnification. OL, outer layer of the cell wall; M, murein layer; CM, cytoplasmic membrane; Pp, electron-dense granules; N, nucleoid; FT, threads with fibrillar structure; LB, electron-transparent lipid bodies. Bars: (a, b, c, d), 6  $\mu\text{m}$ ; (e, f), 2.0  $\mu\text{m}$ ; (g, h), 0.1  $\mu\text{m}$ .



**Fig. 2.** Flow cytometric analysis of *M. smegmatis* cells. Cells grown in modified SR-1 medium followed by storage for 2.5 months and stationary-phase (52 h) cells grown in Sauton's medium were stained with Nile red ( $4 \mu\text{g ml}^{-1}$ ) and analysed by flow cytometry as described in Methods. The experiment was repeated six times; a typical result is shown. 1, Unstained cells; 2, stationary-phase cells; 3, 2.5 month stored cells. Inset: phase-contrast microscopy (A) and fluorescence microscopy with Nile red staining (B) of ovoid cells.

electron density (Fig. 1g). The cytoplasm of these cells was electron-dense, homogeneous, finely textured and contained small electron-transparent bodies and abundant electron-dense inclusions similar to polyphosphate granules. The nucleoid with net-like structured electron-dense threads was easily distinguishable in the cytoplasm of stationary-phase cells (Fig. 1g). Similar ultrastructural features were found for *M. smegmatis* in cultures grown to the stationary phase in the nitrogen-limited SR-1 medium (not shown here).

In 5-month-old nitrogen-limited cultures, we observed intact ovoid cells (Fig. 1f, h), which were morphologically distinct from stationary-phase cells. The cell wall contained an additional layer with altered sublayers and an outermost electron-dense layer (Fig. 1h). Some ovoid cells contained an electron-transparent tail-like structure surrounded by the outer layer of cell wall (indicated with an arrow in Fig. 1f). The cytoplasm in ovoid cells was inhomogeneous and coarsely granulated and contained large vesicular and polygonal electron-transparent bodies and small electron-dense granules. In some cells, these bodies occupied most of the cytoplasm. Intracytoplasmic fibrillar threads of unknown composition were also observed along the cell's longitudinal axis (Fig. 1h). The nucleoid was poorly visible in ovoid cells.

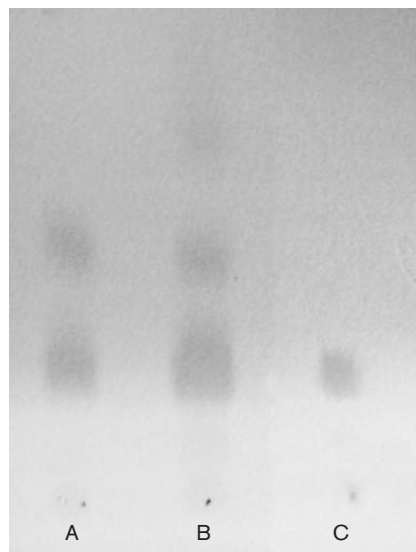
### Fluorescence microscopy and flow cytometry

Ovoid cells had an intact permeability barrier, as judged from PI staining, after up to 5 months storage, in contrast to rod-shaped cells, which also occurred (<9%) in cultures

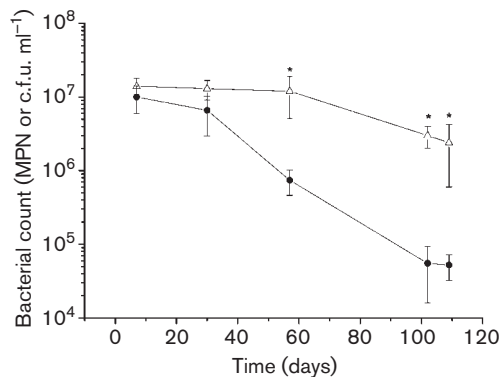
of the same age. To elucidate the nature of the refractive central regions of ovoid cells, we stained the cells with Nile red, widely used for identification of intracellular lipid inclusions (Garton *et al.*, 2002). The inset in Fig. 2 shows that the granules in the majority of ovoid cells indeed showed strong red fluorescence after staining with Nile red. Accordingly, flow cytometry demonstrated a significant fraction of cells ( $45 \pm 17\%$ ) with much higher fluorescence in a population of ovoid cells than among stationary-phase cells grown on standard Sauton's medium (Fig. 2). An elevated content of triacylglycerides in ovoid cells was confirmed by TLC analysis of isolated apolar lipids (Fig. 3): the triacylglyceride content per 1 mg dried cell weight was  $0.05 \pm 0.006 \mu\text{g}$  for stationary-phase cells and  $0.08 \pm 0.007 \mu\text{g}$  for ovoid cells.

### Culturability of starved *M. smegmatis* cells

The number of culturable (platable) cells (c.f.u.) gradually decreased with time of storage in the culture grown in nitrogen-limited SR-1 medium; however, the numbers of viable cells estimated by MPN assay were significantly higher than c.f.u. numbers. This difference was more pronounced in older cultures, suggesting the accumulation of cells with a 'non-culturable' (non-platable) phenotype,



**Fig. 3.** TLC of *M. smegmatis* lipids. Stationary-phase cells (48 h or 72 h incubation) and ovoid cells were obtained as described in the legend to Fig. 2. Apolar lipids were extracted as described in Methods and dissolved in 0.2 ml dichloromethane. One-microlitre samples of lipids isolated from stationary-phase cells (A) or from ovoid cells (B) were applied on TLC plates. Trioleinoyl glycerol ( $2.7 \mu\text{g}$ ) was used as the standard (C). TLC plates were developed in petroleum ether/acetone (96:4, v/v), dried and stained with iodine vapour. The experiment was repeated four times; a typical result is shown.



**Fig. 4.** *M. smegmatis* cells grown in nitrogen-limited SR-1 medium show a partial loss of culturability during storage. Cells were grown and stored, and determinations of MPN ( $\Delta$ ) and c.f.u. ( $\bullet$ ) were done over time, as described in Methods. Each point represents the mean of eight independent experiments; error bars represent SD. Asterisks indicate a significant difference ( $P < 0.01$ ) between c.f.u. and MPN according to Student's *t*-test.

defined by the loss of ability to produce colonies on standard solid media (Fig. 4).

Separation of morphologically distinct rod-shaped and ovoid cells was achieved by centrifugation in a density gradient of sucrose. We found that rod-shaped and most ovoid cells had different buoyant densities, corresponding to 1.6 M and 2.1 M sucrose, respectively. Unlike fully platable, 1.5-month-old stationary-phase cells, grown in Sauton's medium, some ovoid forms were able to pass through 2.1 M sucrose. This difference allowed us to obtain a homogeneous fraction of ovoid cells, which contained no rod-shaped cells according to microscopy (Fig. 1d). In addition, zero c.f.u. or MPN found in the 2.1 M sucrose fraction after centrifugation of 1.5-month-old stationary rod-shaped cells confirmed purity of the ovoid fraction obtained. Some cells in the 'ovoid' population were able to produce colonies on agar plates; however, the majority of them were only able to grow in liquid medium as estimated by MPN assay. For comparison, in a homogeneous population the total number of cells was  $2.7 \times 10^5 \text{ ml}^{-1}$ , c.f.u.  $8 \times 10^2 \text{ ml}^{-1}$  and cells grown in MPN assay  $0.55 \times 10^5 \text{ ml}^{-1}$ . Thus, more than 99% of the cells in the pure ovoid cell fraction were non-platable but could be resuscitated in a liquid medium.

### Resuscitation of starved cells of *M. smegmatis*

Consistent with their dormant phenotype, ovoid forms did not accumulate radioactive precursors of nucleic acid or protein synthesis. However, a substantial increase in uptake of radioactive precursors was observed during resuscitation of such cells in fresh modified Sauton's medium between 0 and 25 h. It preceded the restoration of cell culturability (Fig. 5a), reflecting the importance of activation of cellular

metabolism for initiation of cell division. After 25 h of incubation, the number of c.f.u. rose, accompanied by a significant increase in the incorporation of radioactive precursors ( $>50\,000 \text{ c.p.m.}$ ). The effective 'generation time' calculated from the increase in c.f.u. between 24 and 32 h was about 1 h, whereas the actual generation time for *M. smegmatis* grown in the same medium was  $4 \pm 0.4 \text{ h}$ . This difference evidently reflects true resuscitation (restoration of culturability) of cells, whilst the regrowth of initially culturable and resuscitated cells could also contribute to the observed c.f.u. increase. Since maximum restoration of the c.f.u. number after resuscitation could not exceed the MPN ( $2 \times 10^7 \text{ ml}^{-1}$ ) for the same culture, it is possible to estimate the restoration period as being no longer than 4 h. However, the first 20 h of incubation (between 4 and 24 h) should also be regarded as an initial resuscitation step.

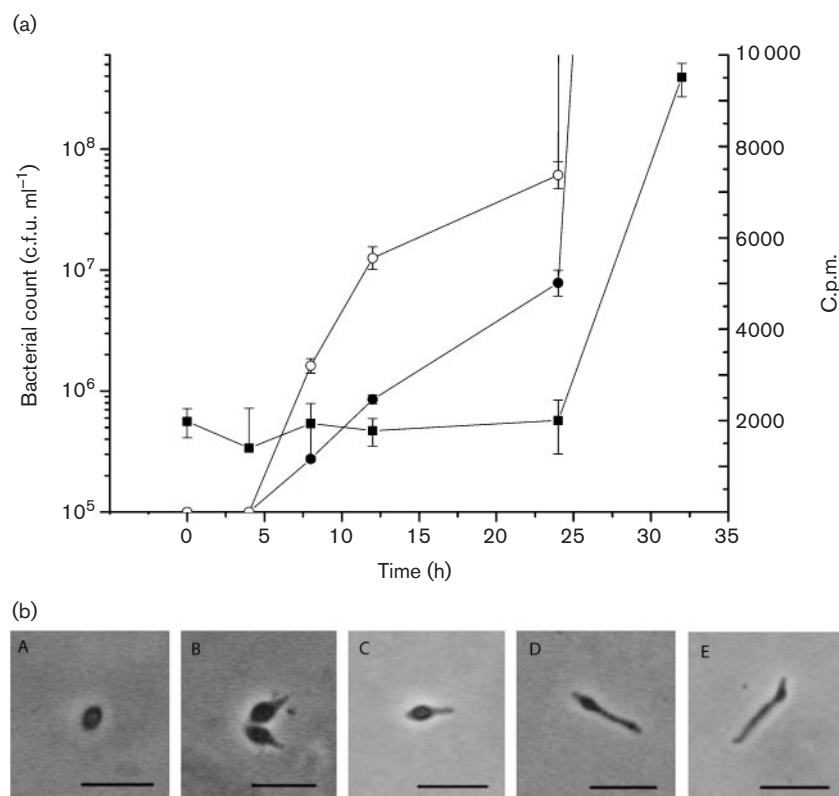
Microscopic examinations confirmed the conversion of ovoid cells to normal rod-shaped cells during resuscitation (Fig. 5b). Ovoid cells in the pure fraction were also capable of resuscitation and conversion to rod-shaped cells. Analysis of the 16S rRNA sequences of these cells after resuscitation revealed 100% identity to the initial wild-type of *M. smegmatis* and to cells in the original nitrogen-starved culture.

### Sensitivity to stresses

Dormant forms are known for their remarkable resistance to different stresses. Therefore, we studied the resistance of vegetative and ovoid cells to elevated temperature and antibiotics. Indeed, as compared with 48-h-old stationary-phase cells, the ovoid cells in 2.5-month-old nitrogen-limited cultures were more resistant to high temperature in the range 60–80 °C (Fig. 6a, b) and to killing by hygromycin and doxycycline as judged by PI staining (Fig. 6c). It is worth mentioning that the rod-shaped cells in these cultures underwent degradation after exposure to 65–70 °C, in contrast to ovoid cells.

## DISCUSSION

We have previously described the formation of 'non-culturable' (non-platable) but resuscitable cells of *M. smegmatis* (Shleeva *et al.*, 2004) and MTB (Shleeva *et al.*, 2002) after prolonged cultivation in stationary phase. Whilst these cells displayed specific features attributed to dormancy, they could persist in this state for only a limited period. Despite the reduction in size, 'non-culturable' cells of both these mycobacterial species did not undergo significant morphological alterations and maintained a rod shape (Shleeva *et al.*, 2004, 2002). In Wayne's model of non-replicating persistence, MTB cells, grown under microaerophilic conditions, developed a thickened outer cell wall layer. However, the authors did not find any spore-like or ovoid forms in such populations (Cunningham & Spreadbury, 1998). In the present study, we developed a



**Fig. 5.** Resuscitation of ovoid *M. smegmatis* cells in modified Sauton's medium. A population of ovoid cells, obtained as described in Methods, was washed and inoculated into fresh modified Sauton's medium with further agitation at 37 °C. (b) C.f.u. (■) and c.p.m. (●, [5,6-<sup>3</sup>H]uracil; ○, L-[<sup>3</sup>H]asparagine) were monitored every 4 h (for more details see Methods). Cell concentration (c.f.u.) after inoculation was  $6 \times 10^5$  ml<sup>-1</sup>. This experiment was repeated three times, and the results of a typical experiment are shown. Each point of c.f.u. and c.p.m. determination represents the mean of five and three replicates, respectively; error bars represent SD. (b) Photographs were taken at the time of resuscitation and the percentage of ovoid cells was counted. Cell concentration (c.f.u.) after inoculation was  $3.6 \times 10^6$  ml<sup>-1</sup>. A, 0 h; B, 5 h; C, 16 h; D, 20 h; E, 24 h. The percentage of ovoid cells remained at  $57 \pm 9\%$  until 20 h of resuscitation; it was  $44 \pm 10\%$  at 20 h and,  $21 \pm 6\%$  at 24 h. No ovoid cells were detected after 25 h of resuscitation.

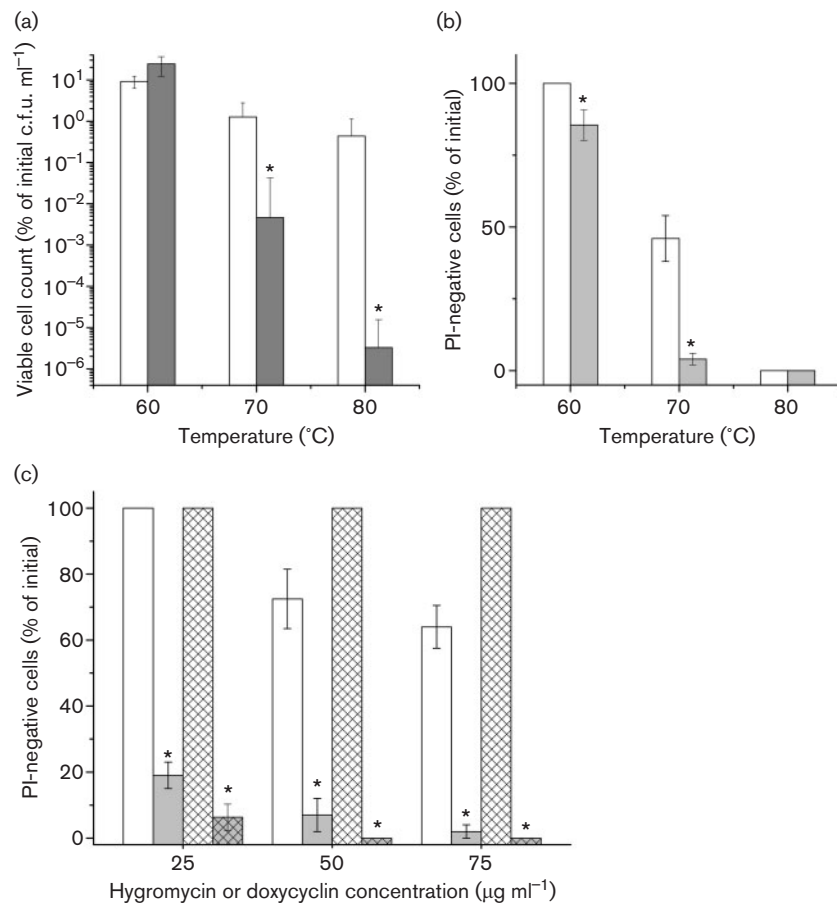
procedure for formation of morphologically distinct 'cyst-like' cells that were able to persist in the dormant state for prolonged periods. These spherical cells were positively stained by Ziehl–Neelsen reagent and had an intact cell wall with additional envelope structures (identified by electron microscopy); therefore they should not be considered as L-forms, well-documented for mycobacteria isolated from the *in vivo* environment (Zhang, 2004).

A very low metabolic activity as revealed by the absence of incorporation of radioactive precursors (Fig. 5a), and the resistance to elevated temperatures (Fig. 6a, b) and to antibiotics (Fig. 6c), displayed by ovoid cells further confirmed their similarity to constitutive dormant bacterial cells. Interestingly, ovoid forms were heavily loaded with large lipid inclusions, probably triacylglycerides, according to staining with Nile red and TLC analysis (Figs 2 and 3), as was found for non-replicating MTB cells recovered from sputum (Garton *et al.*, 2002) or for MTB cells persisting in adipose tissue (Neyrolles *et al.*, 2006). The observed

correlation between the number of MTB cells containing lipid bodies and the proportion of non-replicating (presumably dormant) cells in sputum samples highlighted the significance of triacylglyceride accumulation for non-replicating persistence *in vivo* (Garton *et al.*, 2008).

In contrast to MTB cells, metabolically active *M. smegmatis* cells are able to produce lipid-inclusion bodies, especially in stationary phase (Garton *et al.*, 2002). Indeed, flow cytometry revealed the ability of stationary-phase cells to be stained with Nile red. However, we observed a major difference in staining pattern between active cells and ovoid forms: the latter contained very large lipid bodies of characteristic form (Fig. 2). In fact, these characteristic lipid bodies together with ovoid morphology could be considered as specific and readily recognizable markers of dormant mycobacterial cells.

It is important that populations of ovoid forms of *M. smegmatis* contained a significant proportion of non-platable



**Fig. 6.** Sensitivity of *M. smegmatis* cells to heat or antibiotic treatment. Samples were taken from a 2.5-month-old culture containing ovoid cells (white columns) and from a 48 h culture grown in standard Sauton's medium (grey columns) and heated at 60–80 °C for 10 min (a, b) or treated with antibiotics (c). (a, b) C.f.u. (a) and PI-negative cells (b) as a percentage of the initial value were determined. Initial c.f.u. for stationary-phase cells and for 2.5-month-old stored cells were on average  $2 \times 10^9$  ml<sup>-1</sup> and  $3 \times 10^5$  ml<sup>-1</sup>, respectively. This experiment was repeated three times; representative results are shown. Each point represents the mean of five replicates for c.f.u. determination and three replicates for percentage of PI-negative cells; error bars represent SD. Asterisks indicate a significant difference ( $P < 0.01$ ) between stationary-phase culture and 2.5-month-old culture containing ovoid cells according to Student's *t*-test. (c) Percentage of PI-negative cells in populations was determined after 24 h of incubation with doxycycline (cross-hatched columns) or hygromycin (columns without hatching). This experiment was repeated five times; representative results are shown. Each point represents the mean of three replicates; error bars represent SD. Asterisks indicate a significant difference ( $P < 0.01$ ) between stationary-phase culture and 2.5-month-old culture containing ovoid cells according to Student's *t*-test.

cells (>99 % in 5-month-old cultures), which were able to resuscitate in liquid medium. 'Non-culturable' cells of MTB were found in macrophages (Biketov *et al.*, 2000), in the organs of mice with chronic (but not acute) type of infection (Dhillon *et al.*, 2004) and in Cornell's mouse model of latent TB (Hu *et al.*, 2000). Moreover, the coccoid MTB forms, isolated by Khomenko from infected animal tissues, were also non-platable and required several passages through animals to restore virulence and culturability (Khomenko & Golyshevskaya, 1984).

Earlier, we concluded that the development of a 'non-culturable' phenotype could be considered as a result of

adaptation of mycobacterial cells to cultivation under suboptimal conditions (Young *et al.*, 2005). This conclusion was confirmed in the present study, as the SR-1 medium used supported relatively poor growth of *M. smegmatis*. However, poor growth was an important but not sufficient requirement for the production of ovoid forms, since other non-optimal media did not trigger morphological transformations of *M. smegmatis* cells (Shleeva *et al.*, 2004). Evidently, a combination of different factors (medium composition, nitrogen starvation, agitation regime) should be applied to induce the successful transformation of growing active cells to dormant ovoid forms and switch on as yet unknown mechanisms,

resulting in dramatic reorganization of cell morphology and cell metabolism. Presumably, such a transformation is a versatile property of many micro-organisms, as cyst-like resting cells of other non-sporulating bacteria (*Micrococcus luteus*, *Arthrobacter globiformis*, *Pseudomonas aurantiaca*) were accumulated during cultivation under nutrient limitations (Suzina *et al.*, 2004). Whilst the molecular mechanisms underlying the formation of ovoid, dormant cells in this study remain unclear, we may hypothesize an important role of ppGpp in this process, as overexpression of the *relA* gene in *M. smegmatis* resulted in similar morphological transformation. Unfortunately, the authors did not characterize the physiological state of these cells (Ojha *et al.*, 2000).

In conclusion, we suggest that the ovoid forms of *M. smegmatis* found in this study could be considered as specialized dormant forms with a long lifetime (up to 5 months). Because of their similarity to morphologically distinct cells of MTB previously isolated from TB patients (Khomenko, 1987) and infected animals, a comprehensive study of these forms will be important for elucidation of the molecular mechanisms that underlie non-replicating persistence of mycobacteria. Finally, in some experiments, we observed the presence of a few MTB cells morphologically similar to the ovoid forms described in this study, after prolonged incubation in stationary phase (unpublished results). The task for the future is to develop a procedure for production of significant amounts of such MTB forms and to characterize them in detail.

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