Intracellular pH regulation by *Mycobacterium smegmatis* and *Mycobacterium bovis* BCG

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Mycobacteria are likely to encounter acidic pH in the environments they inhabit; however intracellular pH homeostasis has not been investigated in these bacteria. In this study, *Mycobacterium smegmatis* and *Mycobacterium bovis* [Bacille Calmette–Guérin (BCG)] were used as examples of fast- and slow-growing mycobacteria, respectively, to study biochemical and physiological responses to acidic pH. *M. smegmatis* and *M. bovis* BCG were able to grow at pH values of 4.5 and 5.0, respectively, suggesting the ability to regulate internal pH. Both species of mycobacteria maintained their internal pH between pH 6.1 and 7.2 when exposed to decreasing external pH and the maximum Δ pH observed was approximately 2.1 to 2.3 units for both bacteria. The Δ pH of *M. smegmatis* at external pH 5.0 was dissipated by protonophores (e.g. carbonyl cyanide *m*-chlorophenylhydrazone), ionophores (e.g. monensin and nigericin) and *N,N*-dicyclohexylcarbodiimide (DCCD), an inhibitor of the proton-translocating F_1F_0 -ATPase. These results demonstrate that permeability of the cytoplasmic membrane to protons and proton extrusion by the F_1F_0 -ATPase plays a key role in maintaining internal pH near neutral. Correlations between measured internal pH and cell viability indicated that the lethal internal pH for both strains of mycobacteria was less than pH 6.0. Compounds that decreased internal pH caused a rapid decrease in cell survival at acidic pH, but not at neutral pH. These data indicate that both strains of mycobacteria exhibit intracellular pH homeostasis and this was crucial for the survival of these bacteria at acidic pH values.

Keywords: pH regulation, mycobacteria, membrane potential, proton ATPase

INTRODUCTION

Mycobacterium tuberculosis, the principal agent of tuberculosis (TB) in humans causes approximately three million deaths per year and it is estimated that up to one third of the world's population has been exposed to TB. Increased susceptibility of HIV-infected individuals and the emergence of multidrug-resistant strains make TB the leading cause of disease by an infectious agent (Bloom & Murray, 1992). Effective new TB control and prevention strategies will require additional knowledge of the growth mechanisms of *M. tuberculosis* and its interaction with the host.

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DCCD, *N,N*-dicyclohexylcarbodiimide; Δ p, protonmotive force; Δ pH, transmembrane proton gradient expressed as pH units; $\Delta\Psi$, membrane potential; TPP⁺, methyl triphenylphosphonium iodide; $Z\Delta$ pH, transmembrane proton gradient expressed in mV.

Mycobacteria are likely to encounter both acidic and mildly acidic pH in the environments they inhabit (Sturgill-Koszycki *et al.*, 1994; Oh & Straubinger, 1996; Iivanainen *et al.*, 1999). For example, acidic conditions (pH 3.5–4.3) often prevail in soil and aquatic habitats where saprophytic mycobacteria are found (Iivanainen *et al.*, 1999). In the host environment, *M. tuberculosis* has been shown to reside in the phagocytic vacuole of host macrophages where the intraphagosomal pH has been shown to be mildly acidic (pH 6.1–6.5) (Sturgill-Koszycki *et al.*, 1994; Oh & Straubinger, 1996). Reports in the 1960s demonstrated that *M. tuberculosis* H37Rv had a narrow pH range for growth between pH 6.2 and 7.3 with marked attenuation observed at pH 5.0 and 8.4 (Chapman & Bernard, 1962). Other species of mycobacteria, for example *Mycobacterium fortuitum*, *Mycobacterium marinum*, *Mycobacterium scrofulaceum*, *Mycobacterium avium* and *Mycobacterium chelonae*, grew at pH 6.0 in an

unrestricted manner indicating that *M. tuberculosis* was unique among the mycobacteria in its extreme sensitivity to acid (Chapman & Bernard, 1962). Portaels & Pattyn (1982) reported that *Mycobacterium smegmatis* was capable of growth over a wide pH range with optimum growth observed between pH 5.0 and 7.4, and partial growth at pH 4.6. A more recent study by O'Brien *et al.* (1996) has demonstrated that exposure of *M. smegmatis* to sublethal, adaptive acidic pH (e.g. pH 5.0) conferred a significant level of protection against subsequent exposure to lethal pH (e.g. pH 3.0) compared to unadapted cells grown at pH 7.6, but no mechanism for this acid-tolerant response was provided. In contrast to other micro-organisms that exhibit an acid-tolerant response or acid habituation, the magnitude of protection was only a two- to threefold increase in cell viability for *M. smegmatis* compared to adapted cells of *Salmonella typhimurium* which are 100 to 1000 times more resistant to strong acid challenge (e.g. pH 3.3) compared to unadapted cells (Foster & Hall, 1990).

Due to the paucity of basic information that exists on how mycobacteria cope with acidic pH and pH in general, we have studied the effect of external pH on intracellular pH homeostasis in *M. smegmatis* and *Mycobacterium bovis* BCG. The results reported in this communication demonstrate that both species of mycobacteria adopt intracellular pH homeostasis and this was essential for survival at acidic pH.

METHODS

Chemicals and radiochemicals. CCCP (carbonyl cyanide *m*-chlorophenylhydrazone), DCCD (*N,N'*-dicyclohexylcarbodiimide), monensin, valinomycin and nigericin were obtained from Sigma. [¹⁴C]salicylic acid (56 mCi mmol⁻¹) was obtained from ICN Biomedicals. The following radiochemicals were obtained from NEN: [³H]methyltriphenylphosphonium iodide (TPP⁺) (30–60 Ci mmol⁻¹), [⁷⁻¹⁴C]-benzoic acid (10–25 mCi mmol⁻¹), [1,2-³H]taurine (5–30 Ci mmol⁻¹) and [³H]water (25 mCi g⁻¹).

Growth and maintenance. Cultures of *M. smegmatis* mc²155 (Snapper *et al.*, 1990) and *M. bovis* BCG (Pasteur 1173P2) were used in this study. For liquid culture, cells were grown with gentle agitation at 37 °C in supplemented Middlebrook 7H9 broth (Difco Laboratories) containing sterile Middlebrook ADC enrichment (Becton Dickinson) and 0.05 % Tween 80 (Sigma). For solid medium, supplemented Middlebrook 7H11 (1.5 % agar) with OADC (Becton Dickinson) and glycerol (0.5 %, v/v) was used. All cells used as inocula were washed in saline (0.85 % NaCl). To acidify supplemented and non-supplemented 7H9 medium, the pH was adjusted with 2 M HCl. Culture optical density was measured with a Beckman DU-64 spectrophotometer at 600 nm (OD₆₀₀) using culture samples diluted with saline to bring the OD₆₀₀ to below 0.7 when measured in cells of 1 cm light path length.

Protonmotive force (Δp) measurements. Mid-exponential phase cultures were harvested by centrifugation (8000 g, 15 min, 10 °C) and washed in 100 mM sodium citrate/phosphate buffer (pH 7.0). Cells were resuspended to a final OD₆₀₀ of 1.0 in a volume of 2 ml (glass tubes). Where the external pH was varied (pH 4.0 to 7.0), initial experiments were carried out

using non-supplemented 7H9 medium, but there was no detectable difference in ΔpH values between using this medium and one of the following buffers; 100 mM sodium citrate/phosphate buffer, 100 mM potassium- and sodium-phosphate buffer at the external pH being studied. Citrate/phosphate buffer has been used extensively for measuring internal pH and acid survival in other bacteria (Baronofsky *et al.*, 1984; Terracciano & Kashket, 1986; McGowan *et al.*, 1998) and this was the buffer routinely used in this study. Cells were energized with 20 mM glucose for 15 min followed by the addition of [³H]TPP⁺ (5 μM final concentration), [⁷⁻¹⁴C]benzoate (11 μM, pK_a 4.2) or [¹⁴C]salicylic acid (10 μM, pK_a 3.0) at pH values below 5.0. [1,2-³H]taurine (50 μM) and ³H₂O (25 mM) were used to determine intracellular volume. Taurine has been shown to be non-metabolizable by *M. tuberculosis* (Zhang *et al.*, 1999) and this was confirmed for the mycobacteria used in this study. After incubation for 10 min at 37 °C, the cultures were centrifuged through 0.35 ml silicon oil (BDH Laboratory Supplies) in 1.5 ml microcentrifuge tubes (13000 g, 5 min, 22 °C) and 20 μl samples of supernatant were removed. The tubes and contents were frozen (–20 °C), and cell pellets removed with dog nail clippers. Supernatant and cell pellets were dissolved in scintillation fluid and counted. The silicon oil mix used in this study was a 40 % mixture of phthalic acid bis(2-ethylhexyl ester) and 60 % silicone oil (40 % part mixture of DC200/200 silicone oil and 60 % DC 550). Silicone oils were left overnight at room temperature to equilibrate.

The intracellular volume [3.45 ± 0.59 μl (mg protein)⁻¹] was estimated from the difference between the partitioning of ³H₂O and [¹⁴C]taurine. The electrical potential across the cell membrane (membrane potential; ΔΨ) was calculated from the uptake of [³H]TPP⁺ according to the Nernst relationship. Non-specific TPP⁺ binding was estimated from cells which had been treated with valinomycin and nigericin (10 μM each) for 25 min. These inhibitors have been used previously with *M. smegmatis* (De Rossi *et al.*, 1998; Choudhuri *et al.*, 1999). The ΔpH was determined from the distribution of [¹⁴C]-benzoate or [¹⁴C]salicylic acid using the Henderson–Hasselbalch equation (Reibeling *et al.*, 1975) and ZΔpH was calculated as 62 mV × ΔpH.

Determination of lethal internal pH for *M. smegmatis* and *M. bovis* BCG. The determination of lethal internal pH for *M. smegmatis* and *M. bovis* BCG was as reported by Foster & Hall (1991). Mycobacteria were grown to the exponential phase (OD₆₀₀ 0.5–1.0) in supplemented 7H9 broths (pH 7.0) and harvested by centrifugation (8000 g, 15 min, 10 °C). Cells were resuspended in non-supplemented 7H9 medium and 20 mM glucose at pH 5.0 or 5.5. Preliminary experiments indicated that 20 min exposure to 500 μM CCCP prior to the measurement of intracellular pH was required to reduce the internal pH from 6.7 to 5.5, but CCCP did not eliminate ΔpH completely. An exposure time of 4 h to 250 μM CCCP at pH 5.0 was required to cause a 68 % decline in viability of *M. smegmatis*; this was the time chosen for cell-survival experiments. To reduce the level of CCCP associated with the cells prior to plating, cell suspensions were harvested by centrifugation (13000 g, 5 min, 22 °C) and washed twice in 1.5 ml sterile 0.9 % NaCl. Cell viability (survival) was determined as the number of bacteria remaining as a percentage of the starting count. All samples were diluted in sterile saline (pH 7.0) and three 100 μl volumes of each dilution (10⁻⁴–10⁻⁶) were spread plated on 7H11 agar plates in duplicate. Cell viability, as measured by c.f.u., was determined after 2–3 d for *M. smegmatis* and 2–3 weeks for *M. bovis* BCG, or after colonies were visible at 37 °C. The minimum detection limit was 100 c.f.u. ml⁻¹. The results are expressed as log percentage

survival and represent the mean values of two independent experiments.

Measurement of oxygen consumption by washed cell suspensions, and other analyses. For oxygen consumption measurements, cells were harvested from exponentially growing cultures ($OD_{600} \sim 0.5$) by centrifugation (8000 g, 15 min, 10 °C), washed in 100 mM sodium citrate/phosphate buffer (pH 7.0) and resuspended in the same buffer to give protein concentrations of 5–10 mg protein ml⁻¹. Respiration rates were measured in a Rank Bros Clark-type oxygen electrode at 37 °C as described by Carneiro de Melo *et al.* (1996). Glucose (20 mM) was added as an oxidizable carbon source. The oxygen electrode was calibrated with air-saturated sodium citrate/phosphate buffer (220 nmol dissolved O₂ ml⁻¹ at 37 °C). Protein from NaOH-hydrolysed cells (0.2 M NaOH, 100 °C, 20 min) and cell membranes was assayed by the method of Markwell *et al.* (1978).

Preparation of bacterial cell membranes and ATPase assays. Cell membranes of *M. smegmatis* were prepared as previously described (Basu *et al.*, 1992). Membrane-bound ATPase activity was determined in triplicate by the colourimetric assay of inorganic phosphate liberated from ATP hydrolysis as described by Kobayashi & Anraku (1972). The incubation time and concentration of membrane protein was adjusted so that the assay was linear with time and less than 50% of the ATP hydrolysed. Non-enzymic degradation of ATP under these conditions was less than 10% of the total phosphate. ATPase activity was expressed as μmol inorganic phosphate liberated min⁻¹ (mg protein)⁻¹ at 37 °C.

RESULTS

The effect of extracellular pH on the growth of *M. smegmatis* and *M. bovis* BCG

M. smegmatis grew rapidly in 7H9 broth at pH 7.0 and the maximum specific growth rate observed was approximately 0.28 h⁻¹ (Fig. 1). When the initial pH of

Fig. 1. The effect of extracellular pH on specific growth rate (h⁻¹) of *M. smegmatis* (■) and *M. bovis* BCG (□) grown in supplemented 7H9 broth at the pH indicated. The specific growth rate (h⁻¹) was determined in the mid-exponential phase of growth.

7H9 broth was adjusted to pH values in the range 4.5 to 7.0, the growth rate decreased with declining extracellular pH to approximately 0.09 h⁻¹ at pH 4.5 (Fig. 1). A comparative analysis with *M. bovis* BCG under identical growth conditions revealed a similar pattern of pH sensitivity (Fig. 1). In these experiments, the final pH was between 0.2 and 0.5 units higher than the initial pH value.

Regulation of internal pH by *M. smegmatis* and *M. bovis* BCG

Oxygen consumption measurements were performed to determine the energy status of washed cell suspensions for internal pH measurements. Non-energized cell suspensions of *M. smegmatis* at pH 7.0 consumed oxygen at a rate of 86 nmol min⁻¹ (mg protein)⁻¹ and the addition of glucose increased the rate to 165 nmol min⁻¹ (mg protein)⁻¹. At pH 5.0, the rate of oxygen consumption by non-energized cells was 99 nmol min⁻¹ (mg protein)⁻¹ and this was increased to 126 nmol min⁻¹ (mg protein)⁻¹ by glucose addition. Non-energized cell suspensions of *M. bovis* BCG at pH 7.0 consumed oxygen at a rate of 32 nmol min⁻¹ (mg protein)⁻¹ and the addition of glucose increased the rate to 42 nmol min⁻¹ (mg protein)⁻¹. At pH 5.5, the rate of oxygen consumption by non-energized cells was 21 nmol min⁻¹ (mg protein)⁻¹ and this was increased to 24 nmol min⁻¹ (mg protein)⁻¹ by glucose addition.

The use of radioactive probes to measure the total Δp requires corrections for non-specific binding of probes such as TPP⁺. Since the cell wall of mycobacteria is unlike that of conventional eubacteria, we first tested the effect of ionophores and protonophores on the growth of these bacteria. The following inhibitors at the concentrations listed completely arrested growth of exponentially growing cells of *M. smegmatis* at pH 7.0: CCCP, 100 μM ; DCCD, 200 μM ; monensin, 100 μM ; nigericin/valinomycin, 10 μM each. At pH 5.0, the following concentrations were required to inhibit growth of *M. smegmatis*: CCCP, 50 μM ; DCCD, 300 μM ; monensin, 10 μM ; nigericin/valinomycin, 10 μM each. These inhibitors were also effective against *M. bovis* BCG at similar concentrations (data not shown). These results show that the cell wall of *M. smegmatis* and *M. bovis* BCG did not pose a barrier to these compounds.

The effect of extracellular pH on intracellular pH regulation was studied over the pH range 4.0 to 7.0 (Fig. 2). The $\Delta\Psi$ of *M. smegmatis* was approximately -178 mV at pH 7.0 and decreased with declining pH (Fig. 2a). As the $\Delta\Psi$ decreased, the total Δp remained greater than -180 mV and there was an increase in the ΔpH from -8 mV at pH 7.0 to -155 mV at pH 4.0 (Fig. 2a). These results indicated that *M. smegmatis* was interconverting $\Delta\Psi$ to ΔpH to maintain the Δp constant. The internal pH as a function of the external pH is shown in Fig. 2b. The maximum ΔpH (2.3 units) was observed at pH 4.5. Identical experiments were carried out with *M. bovis*

Fig. 2. The effect of extracellular pH on the individual components of the protonmotive force (a) and internal pH (b) of *M. smegmatis*. (a) $\Delta\Psi$ (■), $Z\Delta pH$ (□) and Δp (●). (b) Internal pH (▲) was calculated using the data in (a). Measurements of ΔpH and $\Delta\Psi$ and preparation of cell suspensions are described in Methods. The cells (glucose-energized) were resuspended in sodium citrate/phosphate buffer at the pH indicated. The ΔpH values are the means of three independent experiments and the standard error associated with these determinations is shown.

Fig. 3. The effect of extracellular pH on the individual components of the protonmotive force (a) and internal pH (b) of *M. bovis* BCG. (a) $\Delta\Psi$ (■), $Z\Delta pH$ (□) and Δp (●). (b) Internal pH (▲) was calculated using the data in (a). Measurements of ΔpH and $\Delta\Psi$ and preparation of cell suspensions are described in Methods. The cells (glucose-energized) were resuspended in sodium citrate/phosphate buffer at the pH indicated. The ΔpH values are the means of three independent experiments and the standard error associated with these determinations is shown.

BCG (Fig. 3). As the external pH declined, the $\Delta\Psi$ decreased to approximately -50 mV and the $Z\Delta pH$ increased to -130 mV, but the Δp did not remain constant (Fig. 3a). The internal pH as a function of the external pH is shown in Fig. 3b and the maximal ΔpH was 2.1 units observed at an external pH of 4.0.

Lethal pH for *M. smegmatis* and *M. bovis* BCG

Based on the experiments of Foster & Hall (1991), one can estimate the lethal internal pH by adding protonophores, compounds that move protons to equilibrate the internal pH with the external pH, to cells suspended in medium at pH 5.0, an external pH that is ordinarily not harmful to the cell. Correlations between measured internal pH and viability will indicate the internal pH at which viability declines. When increasing concen-

trations of CCCP (0–1 mM) were added to *M. smegmatis* cells (OD_{600} 1.0) resuspended in non-supplemented 7H9 medium containing 20 mM glucose at pH 5.0, the internal pH declined and at 1 mM CCCP the internal pH was 5.1 (Fig. 4a). The viability of *M. smegmatis* decreased with increasing CCCP concentration and at 500 μ M CCCP there was a 99 % reduction in cell viability (Fig. 4b). At an external pH of 7.0, the addition of 500 μ M CCCP caused only a 20 % reduction in cell viability (data not shown) indicating that the decline in cell viability caused by CCCP was a low-pH-induced lethality.

Identical experiments were performed with *M. bovis* BCG, but in these experiments the extracellular pH used was 5.5. The addition of increasing concentrations of CCCP (0–1 mM) to *M. bovis* BCG cells resuspended in non-supplemented 7H9 medium containing 20 mM

Fig. 4. Effect of increasing concentrations of CCCP on the internal pH and cell viability of *M. smegmatis*. (a) Glucose-energized cell suspensions were incubated at pH 5.0 in the presence of CCCP (0–1 mM) for 20 min and the internal pH ($\blacktriangle$) was measured as described in Methods. (b) CCCP (0–1 mM) was added to cell suspensions at pH 5.0 in non-supplemented 7H9 medium containing 20 mM glucose and cell viability (c.f.u. ml⁻¹) was measured 4 h after the addition of CCCP. The results are expressed as log percentage survival and represent the mean values of two independent experiments.

glucose at pH 5.5 caused the internal pH to decline, and at 1 mM CCCP the internal pH was 5.9 (data not shown). The viability of *M. bovis* BCG decreased with increasing CCCP concentration and at 1 mM CCCP there was a 90% reduction in cell viability (data not shown). At an external pH of 7.0, the addition of 1 mM CCCP caused only a 15–20% reduction in cell viability (data not shown).

Effect of protonophores, ionophores and DCCD on intracellular pH regulation in *M. smegmatis*

To gain a better understanding of how mycobacteria regulate their internal pH, experiments were performed with *M. smegmatis* and known inhibitors of key proteins (e.g. proton-translocating F_1F_0 -ATPase) and the generation of ionic gradients (e.g. H^+ , K^+ , Na^+) across the

Table 1. Effect of CCCP, DCCD, monensin and nigericin/valinomycin on the intracellular pH of *M. smegmatis*

Internal pH was measured by [¹⁴C]benzoic acid equilibration in cells oxidizing glucose. Glucose was added to achieve a final concentration of 20 mM. All inhibitors were added 15 min prior to the addition of [¹⁴C]benzoic acid. The internal pH values are the means of two independent experiments and the standard error is shown.

Treatment	Intracellular pH	
	External pH 5.0	External pH 7.0
Control (ethanol treated)	6.90 $\pm$ 0.11	7.38 $\pm$ 0.18
Valinomycin (10 μM)	6.74 $\pm$ 0.08	7.64 $\pm$ 0.15
Nigericin/valinomycin (10 μM each)	5.60 $\pm$ 0.12	7.28 $\pm$ 0.09
DCCD (1 mM)	5.70 $\pm$ 0.21	7.60 $\pm$ 0.19
Monensin (10 μM)	5.67 $\pm$ 0.17	7.30 $\pm$ 0.13
CCCP (500 μM)	5.54 $\pm$ 0.06	7.59 $\pm$ 0.11

cell membrane that have been implicated in pH homeostasis. The addition of either the protonophore CCCP or the ionophore nigericin (K^+/H^+ antiporter) in combination with valinomycin (K^+ uniporter), or monensin alone (Na^+/H^+ antiporter) to cells at pH 5.0 almost completely dissipated the ΔpH gradient (Table 1). Valinomycin alone had no effect on the ΔpH gradient. When DCCD (1 mM), an inhibitor of the F_1F_0 -ATPase, was added to cells at pH 5.0, the internal pH was 5.70 (Table 1). At neutral pH, under all conditions tested, the internal pH was alkaline with respect to the external pH (7.28–7.60) in the presence of the inhibitors used (Table 1).

Growth at low external pH has been shown to induce the synthesis of the F_1F_0 -ATPase in *Enterococcus faecalis* (Kobayashi *et al.*, 1984, 1986). To determine if a membrane-bound proton-translocating ATPase was being synthesized in *M. smegmatis* in response to low external pH, cells were grown at pH 7.0 and pH 5.0, and the amount of ATPase activity determined in purified cell membranes. The cell membranes from cells that were grown at pH 7.0 had $4.63 \pm 0.49 \mu\text{mol min}^{-1} (\text{mg protein})^{-1}$ ATPase activity. The ATPase activity in cell membranes of cells grown at pH 5.0 was $6.48 \pm 0.43 \mu\text{mol min}^{-1} (\text{mg protein})^{-1}$. The pH optimum of the ATPase was approximately 7.0 from both pH 5.0 and 7.0 grown cells.

The F_1F_0 -ATPase has been shown to be essential for the survival of *Escherichia coli* at external pH values below 4.0 (Bearson *et al.*, 1997). Further experiments were conducted to determine the effect of DCCD on the survival of *M. smegmatis* at both acidic and neutral pH. *M. smegmatis* cells were incubated in sodium citrate/phosphate buffer at pH 3.0, 5.0 and 7.0 in the presence

Fig. 5. Effect of DCCD on the survival of exponential-phase *M. smegmatis* at external pH 7.0 (●), 5.0 (▲) and 3.0 (■). *M. smegmatis* cells (initial inoculum $\sim 10^8$ c.f.u. ml $^{-1}$) were incubated for 0–2 h in sodium citrate/phosphate buffer at pH 3.0, 5.0 and 7.0 in the absence or presence of 1 mM DCCD. A single line (□) is shown for the controls (averaged survival values of pH 3.0, 5.0 and 7.0 in the absence of DCCD). The results are expressed as log percentage survival and represent the mean values of two independent experiments.

and absence of 1 mM DCCD, a concentration shown to lower the internal pH at external pH 5.0 (Table 1). In the absence of DCCD, *M. smegmatis* survived at each of the pH values tested (Fig. 5). The addition of 1 mM DCCD caused a 60% reduction in viable cell numbers of *M. smegmatis* at pH 7.0 and a 72% reduction at pH 5.0 (Fig. 5). At an external pH of 3.0, DCCD caused a 99% decrease in cell viability. These results demonstrate that ATPase activity is required for survival of *M. smegmatis* at all pH values tested, but appears to be more essential for survival at low pH values (e.g. pH 3.0).

DISCUSSION

In this study we have carried out a detailed investigation of how mycobacteria cope with low pH using two model organisms; the fast-growing *M. smegmatis* and the slow-growing *M. bovis* BCG. Our findings indicate that the growth of *M. smegmatis* is inhibited as the external pH decreases below 4.5, thus supporting previous studies (Chapman & Bernard, 1962; Portaels & Pattyn, 1982). *M. tuberculosis* H37Rv has been reported to have a narrow pH range for growth between 6.2 and 7.3 with marked attenuation observed at pH 5.0 and 8.4 (Chapman & Bernard, 1962). In this study, growth of *M. bovis* BCG was inhibited at pH values below 5.0. The results reported here demonstrated that both species of mycobacteria adopt intracellular pH homeostasis. *M. smegmatis* and *M. bovis* BCG maintained their internal pH in the range 6.1–7.2 when examined over the external pH range of 4.0–7.0. At an external pH of 5.0, the internal pH of these organisms was 6.6–6.7. Zhang *et al.* (1999) have reported that at an

external pH of 5.0, the internal pH of *M. tuberculosis* H37Ra was close to 7.0, supporting the work in this communication and suggesting that *M. tuberculosis* adopts pH homeostasis.

The maintenance of intracellular pH near neutrality when faced with decreasing external pH requires changes in the ΔpH that is a component of the $\Delta\mu$. One mechanism bacteria employ to modify their ΔpH while maintaining $\Delta\mu$ constant is to make compensatory changes in $\Delta\Psi$. This may be accomplished by the use of various cation transport systems (Booth, 1985). For example, *Enterococcus faecalis* and *E. coli* are able to interconvert ΔpH for $\Delta\Psi$ by electrogenic K^+ transport (Bakker & Mangerich, 1981; Booth, 1985). Results here indicated that *M. smegmatis* interconverted $\Delta\Psi$ for ΔpH to maintain the $\Delta\mu$ constant with declining external pH; the $\Delta\mu$ values were in good agreement with those published for other respiring neutrophiles (Kashket, 1985). In contrast to *M. smegmatis*, the $\Delta\mu$ for *M. bovis* BCG varied over the pH range studied. The differences in $\Delta\mu$ were largely reflected in the $\Delta\Psi$ values that varied significantly between the two species. *M. tuberculosis* has been shown to express a multidrug efflux pump, Mmr, that confers resistance to TPP^+ , ethidium bromide and erythromycin (De Rossi *et al.*, 1998). Importantly, *M. smegmatis* does not contain the *mmr* gene and accumulates $^3\text{H}\text{TPP}^+$ with no efflux observed (De Rossi *et al.*, 1998). *M. bovis* has been shown to contain the *mmr* gene (De Rossi *et al.*, 1998) and therefore we cannot rule out the operation of a TPP^+ efflux pump in *M. bovis* BCG; this may explain the low levels of TPP^+ accumulation (e.g. low $\Delta\Psi$) observed in this species and therefore the large fluctuations in the $\Delta\mu$ observed with external pH.

Studies with the protonophore CCCP, used to equilibrate internal pH with external pH, indicated that the lethal internal pH for mycobacteria was less than pH 6.0 and this was associated with a rapid decrease in cell survival and viability suggesting that in mycobacteria, acid death is related to internal pH rather than external pH. However, it should be pointed out that at the high concentrations of CCCP used, not only may H^+ be moved but also other ions (e.g. internal K^+) and therefore the effect on viability may not only be due to acidification of the cytoplasm. Foster & Hall (1991) showed that high concentrations of the protonophore dinitrophenol (200–400 μM) were required to collapse the ΔpH of *S. typhimurium*. At an external pH of 4.0, non-growing cells of both mycobacteria had a significant $\Delta\mu$ (greater than -150 mV) and the internal pH was greater than pH 6.0, but neither species of mycobacteria could grow at this external pH. The reason why these bacteria are unable to grow under conditions where the $\Delta\mu$ and internal pH appear to be favourable for growth is unknown. Perhaps the act of maintaining a high internal pH under conditions of low growth rate and ATP generation decreases the amount of energy available for growth. The mycobacteria may stop growing at acidic pH in order to direct energy towards maintenance of their internal pH, which is crucial for their survival.

Alternatively, there may be a pH-sensitive element (e.g. membrane-bound protein) that is essential for the growth of *M. smegmatis* and *M. bovis* BCG at acidic pH.

The basic mechanism(s) that mycobacteria use to cope with acidic pH is not known, but the Δ pH gradient of *M. smegmatis* at pH 5.0 was dissipated by the protonophore CCCP and the ionophores monensin and nigericin (Pressman, 1976), indicating that permeability of the cytoplasmic membrane to protons plays a key role in maintaining internal pH near neutral. Harold *et al.* (1970) demonstrated that the generation and maintenance of the pH gradient of *Ent. faecalis* was energy dependent, and could be prevented by incubation with the F_1F_0 -ATPase inhibitor DCCD. Furthermore, *Ent. faecalis* has been shown to increase the amount of F_1F_0 -ATPase twofold when the internal pH is lowered artificially by gramicidin D or growth at low pH (Kobayashi *et al.*, 1984, 1986). The ability of low pH to increase the amount and activity of proton ATPases has also been reported in other bacteria (Nannen & Hutkins, 1991; Miwa *et al.*, 1997; Amachi *et al.*, 1998; Kullen *et al.*, 1999). In *E. coli* and *Bacillus subtilis*, environmental pH does not influence expression of the *atp* operon (Santana *et al.*, 1994; Kasimoglu *et al.*, 1996). In this study, DCCD caused a reduction in the Δ pH of *M. smegmatis* and there was an increase in the level of membrane-bound ATPase activity in cells that were grown at pH 5.0, implying a potential role for this enzyme in intracellular pH homeostasis. Piddington *et al.* (2000) have demonstrated a role for Mg^{2+} in the adaptation of *M. tuberculosis* to mildly acidic growth conditions, but the role of Mg^{2+} was unknown. The authors hypothesized that Mg^{2+} may play a role in the maintenance of neutral pH, perhaps by influencing the Mg^{2+} -dependent proton translocating ATP synthase. Inspection of the *M. tuberculosis* genome sequence reveals homologues of ATP synthase genes (e.g. *atpBFEADHCG*) (Cole *et al.*, 1998).

Datta & Benjamin (1997) have demonstrated that DCCD at 1 mM inhibits the survival of *Listeria monocytogenes* at pH 3.0 but had no effect on the survival of *L. monocytogenes* at pH 7.3, indicating that the effect of DCCD was due to low-pH-induced lethality. Our results demonstrated that *M. smegmatis* exhibited a striking sensitivity towards DCCD at acidic pH, suggesting that the ATPase may be more essential for survival at acidic pH. *E. coli* mutants defective in the F_1F_0 -ATPase (e.g. *atp::Tn10* or Δ *atp*) are extremely acid sensitive, but only to external pH values below 4.0. To test the hypothesis that the ATPase is essential for survival of *M. smegmatis* at low pH values and rule out potential non-specific effects of DCCD, further work is required using *atp* mutants of *M. smegmatis*.

In conclusion, our data demonstrate that both species of mycobacteria studied here adopt intracellular pH homeostasis to maintain their internal pH near neutral at acidic pH. The permeability of the membrane to protons and the activity of the membrane-bound proton

F_1F_0 -ATPase play important roles in this process. Current studies are aimed at determining the molecular responses of mycobacteria to pH stress.

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