Mutants of *Mycobacterium smegmatis* unable to grow at acidic pH in the presence of the protonophore carbonyl cyanide *m*-chlorophenylhydrazone

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*Mycobacterium smegmatis* is able to grow and survive at acidic pH, and exhibits intracellular pH homeostasis under these conditions. In this study, the authors have identified low proton permeability of the cytoplasmic membrane, and high cytoplasmic buffering capacity, as determinants of intrinsic acid resistance of *M. smegmatis*. To identify genes encoding proteins involved in protecting cells from acid stress, a screening method was developed using the electrogenic protonophore carbonyl cyanide *m*-chlorophenylhydrazone (CCCP). CCCP was used to suppress intrinsic acid resistance of *M. smegmatis*. The screen involved exposing cells to pH 5-0 in the presence of CCCP, and survivors were rescued at various time intervals on solid medium at pH 7-5. Cells capable of responding to intracellular acidification (due to CCCP-induced proton equilibration) will survive longer under these conditions than acid-sensitive cells. From a total pool of 5000 transposon (Tn611) insertion mutants screened, eight acid-sensitive *M. smegmatis* mutants were isolated. These acid-sensitive mutants were unable to grow at pH 5-0 in the presence of 1–5 μM CCCP, a concentration not lethal to the wild-type strain mc²¹⁵⁵. The DNA flanking the site of Tn611 was identified using marker rescue in *Escherichia coli*, and DNA sequencing to identify the disrupted locus. Acid-sensitive mutants of *M. smegmatis* were disrupted in genes involved in phosphonate/phosphite assimilation, methionine biosynthesis, the PPE multigene family, xenobiotic-response regulation and lipid biosynthesis. Several of the acid-sensitive mutants were also defective in stationary-phase survival, suggesting that overlapping stress protection systems exist in *M. smegmatis*.

**INTRODUCTION**

Mycobacteria are likely to encounter both acidic and mildly acidic pH in the environments they inhabit (Iivanainen et al., 1999; Oh & Straubinger, 1996; Sturgill-Koszycki et al., 1994). 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, *Mycobacterium tuberculosis* resides in the phagocytic vacuole of host macrophages where the intraphagosomal pH is mildly acidic (pH 6-1–6-5) (Oh & Straubinger, 1996; Sturgill-Koszycki et al., 1994). Furthermore, a strong association between acidic pH and the core of granulomatous lesions has been suggested (Dannenberg, 1993; Saviola et al., 2003). In contrast to other intracellular pathogens, mycobacteria do not exhibit a classical acid-tolerant response (ATR) (O’Brien et al., 1996), and therefore the mechanisms they use to combat acid stress are of fundamental interest.

*Mycobacterium smegmatis*, *Mycobacterium bovis* Bacille Calmette–Guérin (BCG) and *M. tuberculosis* exhibit intracellular pH homeostasis (Rao et al., 2001; Zhang et al., 1999). The lethal internal pH for *M. smegmatis* and *M. bovis* BCG is <6-0, and once the internal pH decreases below this value, both species show a rapid decline in cell viability (Rao et al., 2001). The permeability of the cytoplasmic membrane to protons, and proton extrusion by the proton-translocating F₁F₀-ATPase, may play key roles in maintaining the internal pH near neutral (Rao et al., 2001). Piddington et al. (2000) have demonstrated that Mg²⁺ is required in the adaptation of *M. tuberculosis* to mildly acidic growth conditions, but the role of Mg²⁺ in this
process remains unknown. In *Mycobacterium paratuberculosis*, acid resistance can also be influenced by culture conditions. For example, cells grown in fatty-acid (i.e. oleic acid)-containing medium survive better at acidic pH than cells grown in glycerol-containing medium (Sung & Collins, 2003).

Recent reports have shed light on the molecular responses of mycobacteria to acidic pH (Fisher et al., 2002; Saviola et al., 2003). Using microarray analysis, Fisher et al. (2002) reported that 81 genes were differentially expressed >1.5-fold in *M. tuberculosis* when exposed to pH 5.5 versus 6.9, and many of these genes were involved in fatty acid metabolism. Genes that were highly induced showed homology with non-ribosomal peptide synthases/polyketide synthases (Fisher et al., 2002). To identify promoters induced at low pH (pH 4.5) in *M. tuberculosis*, Saviola et al. (2003) developed a recombinase-based in vivo expression technology. Two promoters, belonging to genes encoding a putative lipase/esterase (*lipF, Rv3487c*) and a PE-PGRS gene (*Rv0834c*), were reproducibly upregulated at acidic pH (Saviola et al., 2003).

The aim of this study was to identify genetic factors that contribute to acid resistance in *M. smegmatis*. We report here that *M. smegmatis* exhibits intrinsic resistance to acid stress, which is mediated by low proton permeability of the cytoplasmic membrane, and high cytoplasmic buffering capacity. To suppress this intrinsic resistance, the electrogenic protonophore CCCP was used to equilibrate internal pH with external pH in order to place the cells under greater acid stress. Mutants that were either unable to counteract the proton equilibrating effect of CCCP on intracellular pH, or were sensitive to CCCP at acidic pH, were isolated, and the results of this investigation are presented here.

**METHODS**

**Chemicals and radiochemicals.** All metabolic inhibitors were obtained from Sigma-Aldrich. The following radiochemicals were obtained from NEN Life Science Products: [7,14C]-benzoic acid (10–25 mCi mmol⁻¹); 370–925 MBq mmol⁻¹), [1,2-3H]taurine (5–30 Ci mmol⁻¹); 0.185–1.111 GBq mmol⁻¹) and [3H]water (25 mCi g⁻¹; 925 MBq g⁻¹). The fluorescent pH probe 8-hydroxy-1,3,6-pyrenetrisulfonic acid (pyranine) was from Sigma-Aldrich.

**Growth and maintenance.** For liquid culture of *M. smegmatis* mc²155 (Snapper et al., 1990), 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-Aldrich). 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. *Bacillus subtilis* was grown in Luria broth at 37 °C with shaking (150 r.p.m.). Optical density was measured with a Beckman DU-64 spectrophotometer at 600 nm (OD₆₀₀) using culture samples diluted with saline to bring OD₆₀₀ to below 0.7 when measured in cuvettes of 1 cm light path length. Protein concentration was determined by the method of Markwell et al. (1978). Bovine serum albumin was used as a standard.

**Membrane proton permeability and cytoplasmic buffering determinations.** Proton permeability was determined in membrane vesicles by monitoring the change in external pH with the fluorescent pH probe pyranine (pKₐ 7.3, excitation and emission wavelengths of 450 and 508 nm respectively), as described by van de Vossenberg et al. (1995). The preparation of membrane vesicles of *M. smegmatis* and *B. subtilis* was based on previously described protocols (Cook et al., 2003; Rao et al., 2001). These vesicles were prepared with a high buffering capacity on the inside (50 mM MOPS, pH 7.0, 75 mM KCl and 25 mM choline chloride), and dispersed into a solution (2 mg protein ml⁻¹) of low buffering capacity (0.5 mM MOPS, pH 7.0, 75 mM KCl and 75 mM sucrose), containing 10 μM pyranine. In order to overcome the generation of an opposing (reversed) membrane potential as a result of electrogenic proton uptake, valinomycin (1 μM) was included in the assay buffer. The external pH was lowered by an acid pulse (HCl, approx. 100 nmol H⁺), and measured by the fluorescence of externally added pyranine. The initial drop in the external pH (i.e. fluorescence) was followed by an increase in pH (partial recovery of fluorescence) due to the slow movement of protons into the lumen of the membrane vesicle loaded with a higher buffering capacity. Typical traces at 37 °C are shown for *M. smegmatis* (Fig. 1a) and *B. subtilis* (Fig. 1b). This slow increase in pyranine fluorescence after the proton pulse was fitted to a first-order kinetic rate equation to yield the rate constant k for H⁺ influx based on the calculations of Elferink et al. (1994) and van de Vossenberg et al. (1995). In order to calibrate the fluorescence signal, experiments were performed in
which rapid H⁺ equilibration was achieved using the ionophore nigericin (5 μM), which mediates an electroneutral exchange between potassium and protons (Fig. 1a). In combination with valinomycin, nigericin causes a complete dissipation of K⁺ and H⁺ gradients across the cytoplasmic membrane in *M. smegmatis* (Rao et al., 2001) and *B. subtilis* (van de Vossenberg et al., 1995). Fluorescence measurements were performed using a Perkin-Elmer LS-50B fluorimeter with a thermostated flow cell.

The cytoplasmic buffering capacity of whole cells (*B. subtilis* and *M. smegmatis* grown at pH 7-5) was determined by the acid pulse method as described by Rius & Loren (1998). The total buffering capacity (*Bt*) and the external buffering capacity (*Bε*) were calculated as described by Maloney (1979). The internal cytoplasmic buffering capacity (*Bi*) was the H⁺ difference between *Bt* and *Bε*. To rule out the potential involvement of the proton-pumping respiratory chain and the H⁺-conducting F₁F₀-ATPase in contributing to proton efflux, *Bi* was determined in the presence of the inhibitors rotenone and azide, and the metabolic inhibitor, *N,N*-dicyclohexylcarbodiimide (DCCD).

All inhibitors were effective at blocking respiration and the F₁F₀-ATP synthase of *M. smegmatis* and *B. subtilis*. Each inhibitor (100 μM) was added to the cell suspension 1 h prior to the acid pulse.

**Intracellular pH measurements.** Mid-exponential phase cultures were harvested by centrifugation (8000 g, 15 min, 4 °C), and washed in 100 mM sodium citrate/phosphate buffer (pH 7-0 or pH 5-0). Cells were resuspended to a final OD₆₀₀ of 1.0 in a volume of 2 ml (glass tubes). The transmembrane pH gradient (ΔpH) was measured as previously described (Rao et al., 2001). The ΔpH was determined from the distribution of [¹⁴C]benzoic acid using the Henderson–Hasselbalch equation (Riebling et al., 1975). Non-specific [¹⁴C]benzoate binding was estimated from cells which had been treated with valinomycin and nigericin (10 μM each) for 25 min.

**Isolation of *M. smegmatis* mutants sensitive to acidic pH.** A mutant library was generated by transposon mutagenesis using Tn611 (pCG79), as described by Guilhot et al. (1994). Southern blotting of DNA from random colonies was used to confirm replicative transposition of IS6100 to different sites in the *M. smegmatis* genome. For Southern hybridization experiments, chromosomal DNA was isolated from Tn611 mutants as described by Martin et al. (1990), and digested with PstI. Digested DNA was separated by agarose gel electrophoresis, and probed with pCG79.

In total, 5000 Tn611 insertional mutants, obtained from seven independently generated libraries, were screened for acid-sensitive mutants. Two screening strategies were employed. The first of these approaches involved growing Tn611 mutants at pH 7-5 until colonies appeared on agar, followed by replica plating onto agar at pH 5-0 (buffered with 100 mM potassium citrate/phosphate buffer) and pH 7-5 (buffered with 100 mM potassium citrate/phosphate buffer). The second strategy was based on an approach used by Foster & Bearson (1994) in which a protonophore is added to acidified medium to provide greater acid stress (via proton equilibration), and survivors are rescued at various time intervals. We have previously demonstrated that CCCP is effective at lowering the intracellular pH of *M. smegmatis* to a lethal level (i.e. <pH 6-0) (Rao et al., 2001). Mutants were grown on agar at pH 7-5, and individual colonies were transferred to microtitre dishes (200 μl), and grown to an OD₆₀₀ of approximately 1.0 in supplemented 7H9 medium at pH 7-5. Cells (25 μl) were then transferred to supplemented 7H9 medium at pH 5-0 (total volume 200 μl), and grown to late exponential phase (OD₆₀₀ approximately 1.0), with the intent that any genes required for acid resistance would be induced at this pH. These pH 5-0-grown cells were then transferred (20 μl into 180 μl) into 7H9 medium (pH 5-0) containing 200 μM CCCP. At various time intervals (0–4 h), cells (10 μl) were removed by pelleting, and spotted (10 μl) onto supplemented 7H11 medium at pH 7-5, and mutants that could not be rescued (i.e. no colonies or pinpoint colonies) were considered putative acid-sensitive mutants.

To determine susceptibility to lethal acid pH, *M. smegmatis* and Tn611 mutants were grown to exponential phase (OD₆₀₀: 0-5) in supplemented 7H9 broth (10 ml, pH 7-0), and harvested by centrifugation (8000 g, 15 min, 4 °C). Cells were then resuspended in acid saline (5 ml, OD₆₀₀: 1-0, pH 2-7), and incubated at 37 °C with gentle shaking. Cell viability (survival) was determined at various time intervals: samples (100 μl) were spread onto sterile saline (pH 7-0), and three 100 μl volumes of each dilution (10⁻¹⁻¹⁰⁻⁵) were spread- plated on 7H11 agar in duplicate. Cell viability, as measured by colony forming units (c.f.u.), was determined after 2–3 days. The minimum detection limit was 100 c.f.u. ml⁻¹. The results are expressed as percentage survival, and represent the mean values of two independent experiments.

**Identification of Tn611-interrupted genes using marker rescue in *E. coli*.** Transposon-disrupted genes were identified by marker rescue, as described by Patterson et al. (2000). DNA was sequenced directly using the ABI PRISM BigDye terminator cycle sequencing ready reaction kit (PE Applied Biosystems). Sequence reaction mixtures were electrophoresed by using a model ABI 377 automated DNA sequencer (PE Applied Biosystems). Other DNA manipulations were carried out according to standard molecular biology protocols (Sambrook et al., 1989).

**RESULTS AND DISCUSSION**

*M. smegmatis* exhibits intrinsic resistance to acid stress through low membrane proton permeability and high cytoplasmic buffering capacity

To elucidate the genetic determinants that allow *M. smegmatis* to grow and survive at acidic pH, Tn611-interrupted mutants were screened to identify mutants that could grow at pH 7-5, but not at pH 5-0. From a pool of 5000 Tn611 mutants screened, no acid-sensitive mutants were obtained. Based on these results, we hypothesized that *M. smegmatis* exhibited a high degree of intrinsic resistance to acidic pH. To determine the extent of this intrinsic resistance, the proton permeability of the cytoplasmic membrane, and the cytoplasmic buffering capacity of *M. smegmatis*, were determined. For these experiments, the neutrophilic *B. subtilis* was included for comparison. These parameters have been well documented in *B. subtilis* (Krukwich et al., 1985; Rius & Loren, 1998; van de Vossenberg et al., 1995, 1999).

When the proton permeability of membrane vesicles of *M. smegmatis* was measured, the cytoplasmic membrane showed very high resistance to proton passage at the optimum growth temperature (37 °C) when compared with similarly prepared membrane vesicles of *B. subtilis* (compare Fig. 2a and b). At 37 °C, the proton permeability (rate constant of H⁺ influx) of *M. smegmatis* was threefold lower (i.e. 0.015; Fig. 2a) than that of *B. subtilis* (i.e. 0.05; Fig. 2b). Both strains exhibited increasing proton permeability with increasing temperature, as reported in other bacterial genera by van de Vossenberg et al. (1995).
The cytoplasmic buffering capacity of whole cells grown at neutral pH values was determined (Fig. 3). As the external pH of the buffer was lowered from 7 to 4, and once the external pH reached <6.0, the B\textsubscript{i} of *M. smegmatis* increased significantly [approx. 2300 nmol H\textsuperscript{+} (pH unit\textsuperscript{-1} (mg protein\textsuperscript{-1})] (Fig. 3a). A second maximum was observed when the external pH was raised above 7.6, but these B\textsubscript{i} values were lower than those observed at acidic pH values.

When the cytoplasmic buffering capacity of *B. subtilis* was measured, the B\textsubscript{i} values also increased as the external pH decreased, with maximum B\textsubscript{i} values [approx. 2500 nmol H\textsuperscript{+} (pH unit\textsuperscript{-1} (mg protein\textsuperscript{-1})] observed at external pH values around 5.0 (Fig. 3b). At pH values above 7.2, the B\textsubscript{i} values increased to the maximum value at external pH 8.4 (Fig. 3b).

*M. smegmatis* exhibited low proton permeability of the cytoplasmic membrane compared to *B. subtilis*, and a B\textsubscript{i} that was comparable to *B. subtilis*. Both these bacteria have high B\textsubscript{i} values [e.g. at external pH 5–5, B\textsubscript{i} > 1500 nmol H\textsuperscript{+} (pH unit\textsuperscript{-1} (mg protein\textsuperscript{-1})] when compared to other neutrophilic bacteria [e.g. *E. coli* at external pH 5–0, B\textsubscript{i} approx. 200 nmol H\textsuperscript{+} (pH unit\textsuperscript{-1} (mg protein\textsuperscript{-1})] (Kruwijk et al., 1985). On the basis of these results, *M. smegmatis* exhibited a high intrinsic resistance to acid stress, and this finding may also be consistent with the lack of a significant ATR or acid habituation observed in *M. smegmatis*; the magnitude of acid protection is only two- to threefold for acid-adapted cells of *M. smegmatis* (O’Brien et al., 1996), compared to the ATR response of *Salmonella typhimurium*, where acid-adapted cells are 100–1000 times more resistant to strong acid challenge (pH 3–3) (Foster & Hall, 1990, 1991).

**Isolation of *M. smegmatis* mutants sensitive to acid pH in the presence of the protonophore CCCP**

Previously, we have demonstrated that the electrogenic protonophore CCCP can be used to equilibrate internal pH with external pH in *M. smegmatis* and *M. bovis* BCG (Rao et al., 2001). Using this technique, we demonstrated that the lethal internal pH (i.e. the internal pH at which cells rapidly lose viability) was less than pH 6.0, and also showed that protonophores could be used, as in the approach of Foster & Bearson (1994), to cause intracellular acid stress to cells at external pH values that are ordinarily not harmful to the cell (Rao et al., 2001). For the screening method used here, the rationale was that any Tn611 mutant disrupted in a gene important for resistance to acid stress should show a greater decrease in cell viability.
than those mutants that can respond to such intracellular acid stress. Experiments were carried out with wild-type *M. smegmatis* to determine the concentration of CCCP, and duration of time at pH 5-0, in which CCCP could cause intracellular acid stress, but was not completely lethal to wild-type cells. We found that the most reproducible results were obtained by resuspending cells in buffer containing 200 μM CCCP for 2-4 h, followed by rescue onto solid medium at pH 7-5. Using this protocol, we isolated eight Tn611 mutants out of 5000 tested that were unable to be rescued after exposure to acid pH in the presence of CCCP (Table 1). All mutants shared the property that their growth was sensitive to low concentrations of CCCP (1·5–5·0 μM), which were not inhibitory to the wild-type strain mc²155 (MIC 10 μM) at pH 5-0 (Table 1). Concentrations of CCCP that were inhibitory to the growth of mutants at pH 5-0 had no effect on the growth of cells at pH 7-0, indicating that either the effect of CCCP was due to equilibration of internal pH with external pH (i.e. intracellular acidification), or the mutants were sensitive to CCCP at acidic pH. Another uncoupler, DNP, was also tested for its ability to inhibit the growth of mutants at acidic pH. The MIC of the wild-type strain mc²155 for DNP was 200 μM, and the MICs of mutants ranged from 120 to 160 μM (data not shown). The elevated MICs for DNP compared to CCCP are consistent with the observations of Prasada Reddy et al. (1975), who showed that DNP is not a very effective uncoupler of oxidative phosphorylation in *M. smegmatis* when compared to CCCP. At pH 7-0, only two mutants (A2 and M7) displayed a significantly increased doubling time when compared to the wild-type strain. At pH 5-0, the doubling times of mutants M7, M6, A2, A4 and A6 were significantly increased, yet only one (A6) of these mutants was defective in intracellular pH homeostasis at pH 5-0 (Table 1). However, it should be noted that previous work has shown that while the internal pH appears to be optimal (i.e. >pH 6·5) in *M. smegmatis* cells resuspended at external pH 4-0, no growth is observed below pH 4·5 (Rao et al., 2001). Survival at lethal acidic pH (i.e. pH 2·7) was impaired for the majority of the mutants (except M6) compared to the wild-type strain (Table 1). Four mutants (A2, A4, A6 and M13) showed a stationary-phase survival defect (percentage survival approximately ≤0·01 % after 45 days in 7H9 medium, compared to wild-type survival of 10 %) (data not shown).

### Identification of Tn611-interrupted genes in acid-sensitive mutants

Southern hybridization analysis of mutant DNA digested with *Pst*I, and probed with pCG79, showed bands identical to the plasmid (viz. 2, 1·2 and 0·9 kb), and extra bands corresponding to the duplication of IS6100 (Fig. 4) (Guilhot et al., 1994). Two variable bands of high molecular mass were observed for each mutant, representing distinct junctions (Tn611 insertions) within the chromosome. To identify the Tn611-disrupted genes, we used marker rescue in *E. coli*. All mutants had insertions in distinct genetic loci, and were disrupted in genes that had gene homologues in *M. tuberculosis* (Cole et al., 1998) (Table 2). Three of the genes were homologous to *metB* (M4), *lipF* (M13) and *PPE11* (M9) of *M. tuberculosis*. Four of the genes were conserved hypothetical proteins, and further analysis via BLAST search and a conserved domain search revealed that A2 and A4 were similar to a phosphonate/phosphite ABC transporter in *Rhodopseudomonas palustris*, and a transcriptional GntR regulator protein from *Streptomyces coelicolor*, respectively (Table 2). Analysis of M7 revealed Tn611 insertion in a putative regulatory protein with similarity

### Table 1. Characterization of acid-sensitive mutants of *M. smegmatis* mc²155

<table>
<thead>
<tr>
<th>Mutant or wild-type</th>
<th>MIC for CCCP (μM)*</th>
<th>Doubling time (μ)</th>
<th>Internal pH</th>
<th>Survival (%)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>pH&lt;sub&gt;ext&lt;/sub&gt; 7·0</td>
<td>pH&lt;sub&gt;ext&lt;/sub&gt; 5·0</td>
<td>at pH&lt;sub&gt;ext&lt;/sub&gt; 5·0†</td>
</tr>
<tr>
<td>mc²155</td>
<td>10</td>
<td>2·3</td>
<td>5·1</td>
<td>6·7</td>
</tr>
<tr>
<td>M7</td>
<td>1·5</td>
<td>4·3</td>
<td>12·8</td>
<td>6·4</td>
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<tr>
<td>A4</td>
<td>5·0</td>
<td>2·9</td>
<td>7·7</td>
<td>6·3</td>
</tr>
<tr>
<td>M6</td>
<td>5·0</td>
<td>2·8</td>
<td>10·3</td>
<td>6·5</td>
</tr>
<tr>
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<td>3·6</td>
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<td>6·6</td>
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<tr>
<td>A2</td>
<td>1·5</td>
<td>6·9</td>
<td>9·9</td>
<td>7·1</td>
</tr>
<tr>
<td>A6</td>
<td>3·0</td>
<td>2·2</td>
<td>10·3</td>
<td>5·5</td>
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</table>

*The MIC for CCCP was determined by standard macrobroth dilution, and is reported as the lowest concentration of CCCP that resulted in complete inhibition of growth.
†Internal pH was measured by [¹⁴C]benzoic acid (pK<sub>a</sub> 4·2, pH 5·0) equilibration in glucose-energized cells. The internal pH values are the means of two independent experiments, and the SE was less than 15 %.
‡Percentage cell survival was determined after 4 h exposure to acid saline at pH 2·7, and the values represent the means ± SE of two independent experiments.

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to the xenobiotic-response element (XRE) family of transcriptional regulators (Table 2).

We are aware of only two studies on how mycobacteria respond to acidic pH at a genetic level (Fisher et al., 2002; Saviola et al., 2003). In the microarray analysis of *M. tuberculosis*, cells were resuspended at pH 5.5 (acidified 7H9 medium) at 37 °C for 15 min, 30 min and 18 h prior to RNA extraction (Fisher et al., 2002). Saviola et al. (2003) conducted a screen at pH 4.5 (3 h) in HCl-adjusted 7H9 medium. The most highly induced genes in the screen by Fisher et al. (2002) were those that showed homology with non-ribosomal peptide synthases/polyketide synthases, implicating a role for a bioactive product in the intracellular survival of mycobacteria at acidic pH (Fisher et al., 2002). A set of genes identified in our study [e.g. *lipF* (lipase or esterase) and a gene encoding a putative oxygenase], and in genetic screens by Fisher et al. (2002) and Saviola et al. (2003), are those involved in fatty acid metabolism or synthesis. Fisher et al. (2002) hypothesized that *M. tuberculosis* may sense low pH as a signal that the bacteria are entering the phagosome, and express genes involved in fatty acid metabolism needed for long-term survival under these conditions. The induction of *lipF* may potentially function to hydrolyse toxic fatty acids present in caseous necrotic debris during tuberculosis, or alternatively it may be responsible for modifying the external cell wall of mycobacteria (Saviola et al., 2003). In view of the fact that M13 was disrupted in a gene (i.e. *lipF*) that may be involved in altering the cell wall of mycobacteria, we determined the proton permeability of the cytoplasmic membrane of M13. No significant difference in the proton permeability of M13 compared to the wild-type strain mc²155 was observed, suggesting that *lipF* mutation did not compromise membrane proton permeability of *M. smegmatis* (data not shown).

One of the genes identified here (mutant A2) encoded a putative ABC transporter involved in phosphonate/phosphite uptake. The Tn611 insertion in A2 was localized to the gene encoding a periplasmic binding protein (designated *phnD*) that was the first gene in a putative three-gene operon (*phnDCE*) encoding the membrane-spanning and ATP-binding domains of an ABC transporter. A second mutant, A4, was identified, in which Tn611 had inserted into the gene adjacent to A2, but divergently transcribed from *phnDCE*. This gene (designated *phnF*) encodes a putative transcriptional regulator of the GntR family that potentially may regulate *phnDCE*

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**Table 2.** *M. tuberculosis* genes homologous to DNA sequences obtained from Tn611 insertions in acid-sensitive mutants of *M. smegmatis*

<table>
<thead>
<tr>
<th>Mutant</th>
<th>ORF/gene name</th>
<th>Gene product</th>
<th>Similarity with genes from other micro-organisms</th>
</tr>
</thead>
<tbody>
<tr>
<td>M7</td>
<td>Rv0474</td>
<td>Probable transcriptional regulatory protein</td>
<td>Putative DNA-binding protein (XRE family) of <em>Streptomyces coelicolor</em></td>
</tr>
<tr>
<td>M4</td>
<td>Rv1079/metB</td>
<td>Probable cystathionine γ-synthase</td>
<td></td>
</tr>
<tr>
<td>M9</td>
<td>Rv0453/PPE11</td>
<td>PPE family protein</td>
<td></td>
</tr>
<tr>
<td>A2</td>
<td>Rv1680</td>
<td>Hypothetical protein</td>
<td>Putative PhnD protein, phosphonate ABC transporter of <em>Rhodopseudomonas palustris</em></td>
</tr>
<tr>
<td>A4</td>
<td>Rv1322A</td>
<td>Conserved hypothetical protein</td>
<td>Putative GntR family transcriptional regulator of <em>Streptomyces coelicolor</em></td>
</tr>
<tr>
<td>A6</td>
<td>Rv1233c</td>
<td>Conserved hypothetical membrane protein</td>
<td>Putative alpha oxygenase of <em>Nitrosomonas europaea</em></td>
</tr>
<tr>
<td>M13</td>
<td>Rv3487c/lipF</td>
<td>Probable esterase/lipase</td>
<td>Hypothetical protein of <em>Mycobacterium avium</em></td>
</tr>
<tr>
<td>M6</td>
<td>Rv3035</td>
<td>Conserved hypothetical protein</td>
<td></td>
</tr>
</tbody>
</table>

*ORF numbers, gene names and gene products are as annotated in the *M. tuberculosis* H37Rv genome sequence (Cole et al., 1998).
expression. No previous reports have documented the existence of this putative phosphonate transporter in *M. smegmatis* or in other mycobacterial species. The promoter of *phnD* contained a putative SigF binding signature (i.e. GTTT-N\(^17\)-GGGTAT) that was similar to the mycobacterial consensus sequence GTTT-N\(^17\)-GGGTAT (Manganelli et al., 2004), suggesting that expression was dependent on the stress-response sigma factor SigF. Moreover, A2 and A4 displayed a survival defect in stationary phase, suggesting that this putative phosphonate/phosphate-scavenging mechanism may be important in starvation survival of *M. smegmatis*.

CCCP has been shown to have other effects on the cell than just equilibrating internal pH with external pH (Foster & Bearson, 1994; Gage & Neidhardt, 1993a), and therefore a screen based on this compound could in fact identify mutants that were not acid-sensitive *per se*, but rather were sensitive to CCCP at acidic pH. For example, some mutations may increase membrane permeability to this protonophore. Mutants of Gram-positive bacteria that are resistant to CCCP exhibit changes in fatty acid composition of membrane lipids (Kruwich et al., 1990; Quirk et al., 1994). Notwithstanding this, a screen using the protonophore DNP identified genes important for acid resistance in *S. typhimurium* (Foster & Bearson, 1994). In *S. typhimurium*, six genes with obvious roles in mediating protection from acid stress were identified (e.g. the *atp* operon encoding the proton translocating ATPase). *M7* contained a Tn\(^611\) insertion in a gene encoding a putative DNA binding protein with homology to the XRE family of transcriptional regulators. This family of regulatory proteins is poorly characterized, with most members assigned a hypothetical function only. However, some regulators of this family have been found to regulate stress responses in bacteria (Liu et al., 2003). CCCP is a xenobiotic compound, and therefore the gene disrupted in M7 may mediate resistance to xenobiotics. However, M7 was no more sensitive than the wild-type strain to CCCP at pH 7-0, suggesting that the effect of CCCP was pH dependent. *Rv0474* (Table 2) has also been shown to be upregulated at high temperatures in *M. tuberculosis*, suggesting a potential role in heat shock (Stewart et al., 2002). Furthermore, the uncoupler DNP has been shown to induce heat-shock proteins in *E. coli* (Gage & Neidhardt, 1993a), and therefore mutants in such proteins may be more sensitive to protonophores. Mutant M4 was shown to have an insertion in the gene *metB* encoding a probable cystathionine \(\gamma\)-synthase. Genes involved in methionine biosynthesis (e.g. *metC*) have been identified in other screens for DNP-sensitive mutants of *S. typhimurium* (Foster & Bearson, 1994). A potential link between M7 and M4 may therefore exist. *E. coli* mutants (glyA) have been isolated that are unable to induce the heat-shock response in the presence of DNP (Gage & Neidhardt, 1993b). The phenotype could be reversed by the addition of exogenous methionine to the growth medium (i.e. the heat-shock response occurred in the presence of DNP). Based on these findings, the results in *M. smegmatis* are comparable: the *metB* mutant (M4) and the mutant defective in putative heat-shock proteins (M7, *Rv0474*) are sensitive to CCCP at acidic pH.

**Conclusions**

We believe this is the first report of genes that contribute to acid resistance in *M. smegmatis*. In our experiments, complete inhibition of mutant growth at pH 5-0 was conditional upon the presence of CCCP. Several of the acid-sensitive mutants displayed impaired growth (slow doubling times) at pH 5-0 in the absence of CCCP, and their survival at lethal pH values was diminished. Some of the genes identified here (e.g. lipF, fatty acid metabolism) have been implicated in combating acid stress in *M. tuberculosis* (Fisher et al., 2002; Saviola et al., 2003), but several of the genes identified are unique (e.g. *phnDCE* and *phnF*), and also appear to be important in stationary-phase survival of *M. smegmatis*. Furthermore, our study identified genes that may represent a response to CCCP at acidic pH, as reported in similar studies with bacterial responses to protonophores (e.g. *metB*, *Rv0474*). Future work is aimed at defining the precise role of these gene products in the physiology of *M. smegmatis* at acidic pH.

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