Deletion of the histone-like protein (Hlp) from *Mycobacterium smegmatis* results in increased sensitivity to UV exposure, freezing and isoniazid

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Adaptation to environmental stress is an important survival characteristic of any bacterial species. As a soil-dwelling saprophyte, *Mycobacterium smegmatis* is exposed to factors such as UV light and rounds of freezing and thawing that occur in temperate climates. Numerous studies in *Escherichia coli* have linked histone-like proteins to stress resistance and adaptation. We hypothesized that the 'histone-like' protein Hlp might likewise be involved in the stress response of *M. smegmatis*. The *hlp* gene was inactivated and the *M. smegmatis* Δhlp strain was found to be more susceptible to UV light and to the stress created by repeated cycles of freezing and thawing. In addition, loss of Hlp altered the colony morphology and allowed the organism to grow dispersed in the absence of a detergent, suggesting changes in the cell wall composition. As cell wall changes could affect permeability to certain antibiotics, the susceptibility of *M. smegmatis* Δhlp to kanamycin, rifampicin, ethambutol and isoniazid (INH) was tested. *M. smegmatis* Δhlp was more susceptible to INH, but loss of Hlp did not affect susceptibility to the other antibiotics tested. This suggests that the increased sensitivity of *M. smegmatis* Δhlp to INH was unlikely to be the result of alterations in cell permeability.

INTRODUCTION

Mycobacterium smegmatis is a non-pathogenic, fast-growing mycobacterial species that is a popular model for studying slow-growing mycobacterial pathogens. It is a soil-dwelling species that has also been isolated from water and vegetation found in sphagnum moss bogs (Kazda, 2000). In these environments, *M. smegmatis* is naturally exposed to environmental stresses that without adaptive survival mechanisms would be lethal. The lipid-rich cell envelope of *Mycobacterium* provides some protection against stress by acting as a barrier to the environment (Brennan & Nikaido, 1995). However, specific stress responses allow the cell to sense environmental insults and alter gene regulation to produce cellular and physiological changes that increase resistance. In bacterial species, a class of 'histone-like' proteins has been linked to the survival of numerous environmental stresses, such as UV light (Li & Waters, 1998; Miyabe *et al.*, 2000), cold shock (Wada *et al.*, 1988), heat shock (Wada *et al.*, 1988) and lack of nutrients (Claret & Rouviere-Yaniv, 1997).

Bacterial histone-like proteins are small, highly basic proteins that are typically found at high abundance in cells (Thanbichler *et al.*, 2005). These proteins display

similarities to eukaryotic histones in their ability to bind DNA and influence gene regulation (Arfin *et al.*, 2000; Parekh & Hatfield, 1996; Swinger & Rice, 2004). *M. smegmatis* possesses a histone-like protein called Hlp (also known as Hup, DNA-binding protein HU, CipMa and MDP1) (Katsube *et al.*, 2007; Lee *et al.*, 1998) that is encoded by locus *MSMEG_2389*. This protein contains an N-terminal domain with sequence homology to the HU proteins of *Escherichia coli* (Mukherjee *et al.*, 2008), the best-studied family of histone-like proteins in bacteria. Hlp differs from other bacterial histone-like proteins in that it contains a C-terminal domain composed of prolines, alanines and lysines, resembling the eukaryotic histone H1 family of proteins (Mukherjee *et al.*, 2008). Both the C-terminal and N-terminal domains of Hlp are involved in binding DNA with high affinity (Mukherjee *et al.*, 2008). Homologues of the *M. smegmatis* *hlp* have been found in most mycobacterial species, including pathogens such as *Mycobacterium tuberculosis* and *Mycobacterium leprae* (Shires & Steyn, 2001).

Hlp has been identified as the major protein upregulated during *M. smegmatis* dormancy (Lee *et al.*, 1998). It has also been identified as the major cold-shock-inducible protein in *M. smegmatis* (Shires & Steyn, 2001). Its homologue in *M. tuberculosis*, HupB, has been found to

Abbreviations: INH, isoniazid; TEM, transmission electron microscopy.

be upregulated by iron limitation (Yeruva *et al.*, 2006) and by the stringent response (Dahl *et al.*, 2003). Although HupB in *M. tuberculosis* is predicted to be an essential gene (Sasseti *et al.*, 2003), Lee *et al.* (1998) were able to generate an *hlp* knockout in *M. smegmatis*. This deletion was found to have no effect on the viability of cultures grown for 2 weeks (Lee *et al.*, 1998), but it did affect the ability of *M. smegmatis* to resume growth after being subjected to a 27 °C temperature reduction (Shires & Steyn, 2001).

Induction of *hlp* homologues in response to stress and the apparent importance of Hlp in cold-shock adaptation suggest that the protein has a role in stress resistance. Because it can bind DNA and repress transcription *in vitro* (Matsumoto *et al.*, 2000), it has been suggested that Hlp could be involved in regulating gene expression during a stress response (Mukherjee *et al.*, 2008). In the current study we examined the effect of *hlp* deletion on the long-term survival of *M. smegmatis* in liquid culture and on the survival of cells exposed to environmental and chemical stresses.

METHODS

Bacterial strains, culture media and growth conditions. All *M. smegmatis* liquid cultures were grown in 7H9 (Difco) medium supplemented with 0.05 % Tween 80 and 0.2 % (v/v) glycerol, unless otherwise stated. For the long-term viability assay, cultures were gradually starved in 1.3 ml aliquots of 7H9+Tween 80 in Sarstedt tubes with gas-impermeable O-rings and little head space, and maintained in a 37 °C stationary incubator. At various time points, cells were gently vortexed with 0.1 mm diameter, sterile glass beads and serially diluted on 7H11 agar plates to determine c.f.u. ml⁻¹.

Deletion of *hlp* from *M. smegmatis* mc²155. Deletion of *hlp* from *M. smegmatis* mc²155 was performed by allelic replacement. Briefly, an approximately 700 bp PCR product (forward primer HusmF1, 5'-agtccacttaagcggttcgggtcgtgatct-3'; reverse primer HusmR1, 5'-agtccacttagacgatggtgtcgcacgacgttcc-3'), corresponding to a chromosomal region directly upstream of *hlp*, was cloned into the *Afl*III and *Xba*I restriction sites on pYUB854 (Jacobs *et al.*, 1987) on one side of a hygromycin resistance (Hyg^R) cassette. Another approximately 700 bp PCR product (HusmF2, 5'-agtccacatggagaaggccgccaagaag-3'; HusmR2, 5'-agtccaagatctgaccggtcgacaccaagacggtta-3'), corresponding to a region directly downstream of *hlp*, was inserted into the *Spe*I and *Hind*III restriction sites on the other side of the Hyg^R cassette. The resulting plasmid was named pDW150. A marker cassette containing P_{Ag85}-*lacZ*-P_{hsp60}-*sacB* was removed from pGoal17 (Parish & Stoker, 2000) and inserted into the *Pac*I site of pDW150. This plasmid, named pDW151, was then treated with 100 mJ UV light cm⁻² and electroporated into *M. smegmatis* mc²155.

Following plating on 7H11 agar containing 40 µg X-Gal ml⁻¹ and 50 µg hygromycin ml⁻¹, transformants were selected that represented a single-crossover event (blue Hyg^R colonies). The single-crossover transformants were then patched onto 7H11 plates with 50 µg hygromycin ml⁻¹ and 10 % sucrose to screen for loss of sucrose sensitivity and of the *lacZ* marker. The resulting Hyg^RSuc^R*lacZ*⁻ colonies were considered double recombinants and verified for loss of *hlp* by PCR and Southern blot analyses.

Complementation of *hlp* was performed using the integrative plasmid pMV306 (Stover *et al.*, 1991). An 833 bp PCR product (HusmCF, 5'-cgggtgtagccgaattcgccgccacct-3'; HusmCR, 5'-cgcggatccggcgctgct-

tttcggaat-3'; *Kpn*I restriction sites indicated by bold type) was generated to include both the entire *hlp* gene and approximately 150 bp upstream of the predicted translation start site. The PCR conditions were 94 °C for 3 min, and then 30 cycles of 94 °C, 30 s; 54 °C, 1 min; and 72 °C, 1 min. A final extension step of 72 °C for 7 min was included. This PCR product was then inserted into the *Kpn*I and *Bam*HI sites on pMV306 to create the construct for complementation called pMV306/*hlp*. This construct was electroporated into *M. smegmatis* Δ*hlp*, where it integrated onto the chromosome to generate *M. smegmatis* Δ*hlp*::pMV306/*hlp*.

Confirmation of *hlp* deletion from *M. smegmatis*. To confirm the loss of *hlp*, Southern blot analysis was performed as previously described (Sambrook *et al.*, 1989). Genomic DNA from both *M. smegmatis* mc²155::pMV306 and Δ*hlp*::pMV306 was digested with *Not*I and *Sca*I for 24 h before separation by gel electrophoresis and transfer to a nylon membrane. A 306 bp PCR product (HusmPrF, 5'-cgacatcttcgggggaacg-3'; HusmPrR, 5'-ccggatcagccgggagagt-3') was amplified from the region directly upstream of the *hlp* gene on the *M. smegmatis* chromosome. This product was then used as a template for a PCR using a Digoxigenin (DIG) labelling kit (Roche). This reaction incorporated DIG-labelled dUTP into the PCR product, generating a probe for Southern blotting. This probe was allowed to hybridize membrane-bound DNA, and detection of the probe was performed using a Nitro-Blue tetrazolium chloride/5-bromo-4-chloro-3'-indolylphosphate *p*-toluidine salt (NBT/BCIP) chromogenic reaction, as previously described (Wei *et al.*, 2000).

In addition to Southern blot analysis, deletion of *hlp* from the *M. smegmatis* chromosome was confirmed by PCR using primers that amplified a 208 bp internal region of *hlp* (HlpsmIntF, 5'-cgggttcggtgtcttcgagca-3'; HlpsmIntR, 5'-ggccttctggcgggaccag-3'). As *M. smegmatis* Δ*hlp* was generated using an allelic replacement strategy, this PCR was used to confirm the absence of the internal region of *hlp* from the chromosome. Coomassie-stained SDS-PAGE of late-exponential phase cultures was used to confirm the loss of the Hlp protein from *M. smegmatis* Δ*hlp*.

Transmission electron microscopy (TEM). TEM analysis was performed as previously described (Arora *et al.*, 2008). Two-week-old colonies were swabbed off 7H11 agar and suspended in PBS. Cell suspensions were spotted onto Formvar carbon-coated nickel grids, which were stained with 1 % uranyl acetate before viewing with a JEOL TEM 1200 EX electron microscope.

UV exposure. Aliquots (1 ml) of *M. smegmatis* cells grown to mid-exponential phase (OD₆₀₀ 0.6) were placed into sterile Petri dishes and exposed to increasing amounts of UV (5.0–15.0 J m⁻²) using a Bio-Rad GeneLinker UV box. Serial dilutions of treated cells were immediately performed and plated onto 7H11 agar. Results were reported as percentage survival compared with samples untreated with UV light.

Isoniazid (INH) sensitivity assays. Sensitivity to INH (Sigma) was examined using both the Kirby–Bauer technique and determination of MIC. First, disks soaked with concentrations of INH from 0–20 µg ml⁻¹ were placed on a lawn of cells swabbed from early exponential-phase cultures (OD₆₀₀ 0.2). After 48 h of incubation, diameters of the zones of inhibition were measured. The MIC was determined as described by Wallace *et al.* (1986). Briefly, cultures were grown to OD₆₀₀ 0.2 before diluting 1000-fold and placing 50 µl aliquots in triplicate into 96-well round-bottomed plates. Decreasing concentrations of INH (80–0.156 µg ml⁻¹) were then added to the wells in 50 µl aliquots. Following a 48 h incubation period, wells were examined using a dissecting microscope for growth or no growth. Sensitivities to rifampicin, ethambutol and kanamycin were also tested using the two methods described above.

Freeze–thaw assay. *M. smegmatis* cultures were grown to OD₆₀₀ 0.6 in 7H9+Tween 80. Aliquots (200 µl) were transferred to 1.8 ml cryovial tubes and frozen at –80 °C before removal and immediate thawing in a 37 °C water bath. This pattern was repeated 20 times before cells were serially diluted and plated onto 7H11 agar. c.f.u. were enumerated after 4 days growth and the percentage survival was calculated.

Incubation of *M. smegmatis* in a sphagnum moss bog. Cryovial tubes containing 1.3 ml aliquots of *M. smegmatis* strains in 7H9+Tween 80 were buried 20 cm deep in the brown layer of a local sphagnum moss bog (N 46°58.557', W 092°11.804') for several months in 2009–2010. On the 15th of each month, three vials of each strain were extracted from the bog and viable bacteria determined by enumerating c.f.u. ml^{–1} on 7H11 agar plates. This bog is located 5 miles from Duluth International Airport and daily surface temperatures are recorded there for submission to the National Oceanic and Atmospheric Administration (NOAA).

Acid-stress assay. Sensitivity of the *M. smegmatis* strains to low pH was determined by a method similar to that described by O'Brien *et al.* (1996). Briefly, cells were grown in Sauton's medium with 0.05 % Tween 80 adjusted to a pH of 6.8. At early exponential phase (OD₆₀₀ 0.015), cells were resuspended in 1 ml aliquots of either fresh Sauton's (pH 6.8) or Sauton's medium with a pH adjusted to a value of 5.0 or 4.0. Serial dilutions were performed on the pH 6.8 sample to determine c.f.u. ml^{–1} at the initial time point (0 h), which was designated 100 % survival. Samples were incubated for 1 day or 5 days before determining the c.f.u. ml^{–1} of each sample.

RESULTS

Deletion of *hlp* from *M. smegmatis* mc²155

Deletion of the *hlp* gene was completed by allelic replacement with a Hyg^R cassette. This replacement was confirmed by Southern blotting (Fig. 1a). The labelled probe bound to a 2.125 kb band in the digested DNA from *M. smegmatis* mc²155 and a 3.537 kb band in the DNA from the mutant strain. The larger product in the mutant strain corresponds to an expected increase in size due to the replacement of the *hlp* gene with the Hyg^R marker.

PCR analysis showed the absence of an internal region of *hlp* in the chromosome of *M. smegmatis* Δ *hlp* (Fig. 1b). A non-specific PCR product (upper band) served as an internal control. Absence of an *hlp*-specific PCR product from *M. smegmatis* Δ *hlp* corresponds with production of a Hyg^R-specific PCR product that was not generated from mc²155 (data not shown). Loss of the Hlp protein was also demonstrated by SDS-PAGE (Fig. 1c). Late exponential-phase cultures of *M. smegmatis* Δ *hlp*::pMV306 lacked an approximately 30 kDa protein (Fig. 1c, lane 3) that was present in mc²155::pMV306 (lane 1) and Δ *hlp*::pMV306/*hlp* (lane 2). Despite its predicted size of 21 kDa, Hlp has been shown to migrate more slowly on SDS-PAGE due to its highly basic nature (Lee *et al.*, 1998; Shires & Steyn, 2001).

Loss of *hlp* alters cell and colony morphology

Young colonies of the different strains showed marked differences in size. After 3 days growth, individual wild-type

Fig. 1. Confirmation of *hlp* deletion in *M. smegmatis*. (a) Southern blot analysis shows that the probe for a DNA sequence flanking *hlp* binds to a 2.125 kb chromosomal fragment in the wild-type mc²155 strain (lane 1). For the Δ *hlp* strain, this probe anneals to a fragment 1.4 kb larger, indicating the presence of the Hyg^R cassette within *hlp* (lane 2). (b) PCR reveals that a 200 bp internal region of *hlp* present in mc²155 (lower band in lane 1) is absent from the Δ *hlp* strain (lane 2). The upper band in lanes 1 and 2 is a non-specific PCR product that serves as an internal control. (c) Coomassie-stained 12 % SDS-PAGE of late exponential-phase cultures demonstrates elevated levels of Hlp protein (arrow) in mc²155::pMV306 (lane 1) and the complemented mutant strain Δ *hlp*::pMV306/*hlp* (lane 2), but not in Δ *hlp*::pMV306 (lane 3). An Invitrogen Benchmark pre-stained protein ladder was used as a molecular mass standard (lane M).

mc²155 colonies were much larger (Fig. 2a, upper inset), with confluent colonies showing large ridges (Fig. 2a, lower inset). Colonies of the Δ *hlp* strain were much smaller and confluent colonies lacked prominent ridges (Fig. 2c, upper and lower insets, respectively). As colonies aged, however, diameters of the two strains became more similar (compare Fig. 2b and d). Two-week-old mc²155 colonies were rough in appearance, with ridges forming across the entire surface (Fig. 2b). However, 2-week-old Δ *hlp* colonies had a smoother, flatter morphology (Fig. 2d). In addition, TEM analysis of 2-week-old cultures from agar surfaces showed that mc²155 cells were two- to threefold shorter than their

Fig. 2. Hlp affects colony and cell morphology. Strains $mc^2155::pMV306$ (a) and $\Delta hlp::pMV306$ (c) were grown for 3 days on 7H11 agar before visualization with a dissecting microscope. Insets show higher magnifications of individual colonies (upper insets) or of confluent colonies (lower insets). Bars, 1 mm. Single colonies of $mc^2155::pMV306$ (b) and $\Delta hlp::pMV306$ (d) were also allowed to continue growing for 2 weeks. TEM images ($\times 5000$) of 2-week-old cells are shown in the insets.

Δhlp counterparts (compare Fig. 2b and d, insets). The complemented Δhlp mutant had the typical rough colony appearance and shortened cell morphology of mc^2155 (data not shown).

Smooth colony phenotypes in *M. smegmatis* have been previously reported and linked with altered cell wall hydrophobicity (Arora *et al.*, 2008; Chen *et al.*, 2006). Without Tween 80, the hydrophobic nature of the *M. smegmatis* mc^2155 cell wall causes the cultures to aggregate in liquid. When *M. smegmatis* Δhlp was grown in the absence of this detergent, the cells were able to grow dispersed in solution without the clumping that was seen in *M. smegmatis* mc^2155 (results not shown).

Growth and long-term survival of *M. smegmatis* Δhlp

Growth of *M. smegmatis* Δhlp in 7H9 + Tween 80 was similar to that of *M. smegmatis* mc^2155 when grown at 37 or 45 °C (data not shown). Lee *et al.* (1998) examined the ability of an *M. smegmatis* hlp mutant strain to survive 2

weeks of bacterial dormancy by sustaining cultures in a low-oxygen environment. As mycobacteria are known to survive years of starvation (Sun & Zhang, 1999), we examined changes in long-term viability for up to 8 months. Loss of hlp had no effect on long-term viability in these 8-month-old, oxygen-starved cultures (results not shown).

M. smegmatis Δhlp has increased sensitivity to UV exposure

An earlier study showed that lack of the histone-like protein HU from *E. coli* leads to an increase in sensitivity to UV light (Li & Waters, 1998). This was due in part to an involvement in DNA repair processes (Miyabe *et al.*, 2000). As a DNA-binding protein, Hlp could have an involvement in DNA repair, which might be demonstrated by alterations in UV sensitivity upon deletion of the hlp gene. Deletion of hlp from *M. smegmatis* did result in an increase in sensitivity to UV light (Fig. 3). When exposed to up to 15 J m⁻² UV, *M. smegmatis* $\Delta hlp::pMV306$ had an eightfold decrease in survival compared with *M. smegmatis*

Fig. 3. Survival of *M. smegmatis* strains after exposure to UV light. Cell survival decreases with increased exposure to UV light. *mc*²155::pMV306 (□) and *Δhlp*::pMV306/*hlp* (◇) have eight-fold increased survival compared with *Δhlp*::pMV306 (◆). Error bars, SD for three samples.

*mc*²155::pMV306 or the complemented mutant strain *Δhlp*::pMV306/*hlp*.

M. smegmatis *Δhlp* has increased sensitivity to INH

Previous studies have linked alteration in cell wall composition and loss of a histone-like protein to alterations in antibiotic sensitivity (Arora *et al.*, 2008; Colangeli *et al.*, 2007; Jackson *et al.*, 1999; Lewin *et al.*, 2008). To determine whether loss of Hlp generates a similar effect on antibiotic sensitivity in *M. smegmatis*, isogenic strains were compared for sensitivity to various antibiotics. No differences were observed between the three strains for sensitivity to kanamycin, ethambutol or rifampicin (results not shown). However, *M. smegmatis* *Δhlp*::pMV306 showed increased sensitivity to varying concentrations of INH. The zones of INH inhibition of *M. smegmatis* *Δhlp* were significantly larger than that of the wild-type or the complemented mutant strains (Fig. 4). The MIC for INH of *M. smegmatis* *Δhlp* was determined to be 2.5 µg ml⁻¹, which was fourfold smaller than that observed for isogenic strains containing a functional *hlp* gene.

M. smegmatis *Δhlp* has increased sensitivity to freezing

An earlier report linked *hlp* with the ability of *M. smegmatis* to adapt to a cold shock of 10 °C and resume growth (Shires & Steyn, 2001). While this study looked at the ability of an *M. smegmatis* *hlp* mutant to gradually acclimatize to a temperature of 10 °C, here we tested the ability of *M. smegmatis* *Δhlp* to survive a rapid temperature

Fig. 4. Loss of *hlp* confers increased susceptibility to INH. Zones of inhibition were measured around disks soaked with increasing concentrations of INH. Black bars, *mc*²155::pMV306; grey bars, *Δhlp*::pMV306/*hlp*; white bars, *Δhlp*::pMV306. Error bars, SD for three experiments.

adjustment through repeated cycles of freezing and thawing (Fig. 5a). After 21 rounds of repeated freezing and thawing, *M. smegmatis* *Δhlp*::pMV306 had a 10-fold decrease in percentage survival compared with *mc*²155::pMV306 and *Δhlp*::pMV306/*hlp*.

Sphagnum peat bogs are environmental niches rich in mycobacterial diversity, and *M. smegmatis* can be found there (Kazda, 2000). Decaying sphagnum moss can act as a natural insulator to keep subsurface levels constant at 4–10 °C while daily surface temperatures fluctuate widely. During winter months, freezing can occur several centimetres downwards into the moss. Cryovial tubes containing different strains of *M. smegmatis* were incubated below the surface of sphagnum moss to let cultures be exposed to natural freezing and thawing cycles over a nine-month period. During the spring and summer months (March–July), there was no observed difference in survival of the three strains (Fig. 5b). However, during the autumn and winter months, cells lacking *hlp* experienced as much as a 100-fold decrease in viability compared with cells expressing *hlp* (Fig. 5c). This reduction in viability corresponds to surface and subsurface freezing temperatures. During the months of December 2009 and January 2010, average daily surface temperatures exceeded 0 °C only twice and not at all in February 2010.

Survival at acidic pH

Recently, Bi *et al.* (2009) showed that *E. coli* histone-like proteins (HU proteins) play an important role in allowing the bacteria to grow at low pH. To test whether *M. smegmatis* can survive at lower pH levels in an *hlp*-dependent fashion, isogenic strains were compared for viability after exposure to increasingly acidic media (Fig. 6). Although a decreasing viability corresponded to

Fig. 5. Viability of *M. smegmatis* strains after repeated rounds of freezing and thawing. (a) Following 21 cycles of controlled freezing and thawing, viabilities of cultures were compared by determining

c.f.u. ml⁻¹. $mc^2155::pMV306$ (black bar) and $\Delta hlp::pMV306/hlp$ (grey bar) have a 10-fold enhanced survival over $\Delta hlp::pMV306$ (white bar). Cultures were submerged in a sphagnum moss bog for several months in spring and summer (b) or autumn and winter (c). Samples were removed at the designated months and compared for cell viabilities. □, $mc^2155::pMV306$; ◆, $\Delta hlp::pMV306$; ◇, $\Delta hlp::pMV306/hlp$. Error bars, SD for three samples. Average monthly surface temperatures are also shown (b, insert).

Fig. 6. Comparison of *M. smegmatis* strain survival during acid stress. Cultures of $mc^2155::pMV306$ (□) and $\Delta hlp::pMV306$ (◆) were exposed to pH levels of 6.5 (a), 5.0 (b) and 4.0 (c) for increasing durations before testing for viability by measuring c.f.u. ml⁻¹ and recording this as the percentage survival of cells with respect to time 0. Error bars, SD for three experiments.

decreasing pH levels (compare Fig. 6a, b and c), there was no appreciable difference in acid tolerance between strains with or without *hlp*.

DISCUSSION

In this report we have examined the role that *hlp* plays in stress tolerance and antibiotic sensitivity for *M. smegmatis*. Loss of Hlp adversely affects the ability of the organism to survive UV radiation (Fig. 3) and freezing conditions (Fig. 5). Additionally, *M. smegmatis* Δhlp was more susceptible to the antibiotic INH (Fig. 4). However, the Hlp does not affect the growth rate of cells in liquid (Fig. 2a), the ability to survive hypoxia and nutrient deprivation (Fig. 2b), or the ability to withstand acidic conditions (Fig. 6).

Hlp is a small, basic, DNA-binding protein that was first characterized in *M. smegmatis* as a major protein induced in response to cellular dormancy (Lee *et al.*, 1998). Hlp shares similarity to other prokaryotic histone-like proteins through its N-terminal domain, and to the eukaryotic histone H1 proteins through its C-terminal domain (Prabhakar *et al.*, 1998). As a histone-like protein, Hlp might play roles in DNA compaction and gene regulation necessary for stress survival. The ability of Hlp to act as a transcriptional repressor in *M. smegmatis* *in vitro* means that it may regulate gene activity (Mukherjee *et al.*, 2008), although at present the full significance of Hlp in *M. smegmatis* has not been elucidated. Hlp homologues have been found localized to the cytosol and nucleoid (Mukherjee *et al.*, 2008; Prabhakar *et al.*, 1998), and to the cell wall (Aoki *et al.*, 2004; Soares de Lima *et al.*, 2005; Yeruva *et al.*, 2006). This localization suggests a complex and multifunctional role for these structurally unique mycobacterial histone-like proteins.

We have recently reported that loss of the histone-like protein Lsr2 from *M. smegmatis* produces a smooth colony (Arora *et al.*, 2008; Chen *et al.*, 2006). The deletion of *hlp* from the *M. smegmatis* chromosome also results in a smoother colony morphology (Fig. 2c, d). This suggests changes in the cell envelope composition. Using scanning electron microscopy, Katsube *et al.* (2007) showed that stationary-phase cells of an *M. smegmatis* *hlp* mutant had crenellated surfaces, unlike the smooth cell surface seen in wild-type *M. smegmatis*. These authors also demonstrated that Hlp was involved in the downregulation of cell surface components in stationary phase (Katsube *et al.*, 2007). These results all appear to confirm that loss of Hlp affects the composition of the cell envelope. Such changes often coincide with differences in cell hydrophobicity (Chen *et al.*, 2006; Ojha *et al.*, 2005). A decrease in the hydrophobicity of *M. smegmatis* Δhlp was evident from its ability to grow dispersed in liquid media without the addition of the detergent Tween 80 (results not shown). Given that Hlp homologues are found localized to the cell envelope (Katsube *et al.*, 2007; Soares de Lima *et al.*, 2005) and that Hlp can bind externally to *Mycobacterium bovis* (Katsube *et al.*, 2007), it is possible that Hlp is involved in

cell-to-cell aggregation of *M. smegmatis*. Lewin *et al.* (2008) found that reducing expression of the Hlp homologue in *M. bovis* decreased cell aggregation.

Because *hlp* is upregulated in response to bacterial dormancy, Lee *et al.* (1998) examined the effect of the loss of Hlp on the short-term viability of oxygen-starved cultures left stationary for up to 2 weeks. While they did not observe any decrease in viability without *hlp*, *M. smegmatis* can exist in a dormant state for years. Therefore, we repeated this experiment for up to 8 months but were unable to observe any effect on long-term viability in oxygen-starved cultures due to loss of *hlp* (results not shown). Lewin *et al.* (2008) have reported that there is no correlation between Hlp levels and growth rates of *M. bovis* under low-oxygen conditions. However, differences in protein production were observed if Hlp levels were reduced, suggesting that Hlp plays a role in protein expression of *M. bovis* BCG in hypoxic conditions. Further work is needed to determine whether the loss of Hlp in *M. smegmatis* affects gene expression under low oxygen.

M. smegmatis Δhlp had an eightfold decrease in survival when subjected to UV light (Fig. 3). This is in contrast to an *M. smegmatis* Δsmc mutant, which shows no change in levels of repair of UV-induced lesions (Güthlein *et al.*, 2008). This is despite the role of SMC (structural maintenance of chromosomes) proteins in repair of DNA damage in different species of bacteria. In *E. coli*, deletion of the heterodimer formed by the histone-like proteins HU α and HU β leads to an over 100-fold decrease in UV survival (Li & Waters, 1998). This susceptibility to UV light in *E. coli* is attributed to a deficiency in RecA-dependent DNA repair and SOS induction (Miyabe *et al.*, 2000). Although Hlp appears to play less of a protective role in *M. smegmatis* than in *E. coli* (eightfold compared with 100-fold), it is possible that Hlp of *M. smegmatis* is also involved in DNA repair. A recent publication has demonstrated that Hlp enhances DNA end-joining in *M. smegmatis* *in vitro* (Mukherjee *et al.*, 2008). An additional reason for the increased susceptibility to UV seen here in the Δhlp strain could be the alteration in the cell envelope structure. As a major barrier against the environment, changes in the cell envelope could affect UV sensitivity. For example, the loss of genes involved in cell envelope composition in *Lactococcus lactis* leads to an increase in UV sensitivity (Duwat *et al.*, 1997).

M. smegmatis Δhlp was tested for its susceptibility to four antibiotics: INH, rifampicin, ethambutol and kanamycin. No change in the susceptibility to rifampicin, ethambutol or kanamycin was observed. This result is similar to that seen in *M. bovis*, where reduced expression of the Hlp homologue has no effect on the rifampicin susceptibility (Lewin *et al.*, 2008). The loss of Hlp from *M. smegmatis* did lead to a fourfold increase in susceptibility to INH (Fig. 4). INH is a front-line drug for the treatment of tuberculosis, and is a small hydrophilic prodrug that enters into the cell by passive diffusion, where it is activated by the action of

KatG (Zhang & Telenti, 2000). Activated INH can inhibit enzymes involved in the synthesis of mycolic acids (Zhang & Telenti, 2000). Further cellular damage can occur as a result of the action of reactive oxygen radicals, which are produced during prodrug activation (Zhang *et al.*, 1996). With the loss of Hlp, *M. smegmatis* may be more susceptible to the free radicals produced by INH activation, suggesting an involvement of Hlp in the resistance of *M. smegmatis* to oxidative damage. It is possible that alterations in the cell wall of *M. smegmatis* Δhlp increase cell permeability to INH. However, this does not seem likely, since no Hlp-dependent differences were seen in the sensitivities to the other antibiotics tested, including ethambutol, a similarly small hydrophilic antibiotic.

Tolerance to freeze–thaw activity is a complex mechanism involving the accumulation of trehalose, the production of molecular chaperones, and the composition of the bacterial cell wall (Tanghe *et al.*, 2003). *M. smegmatis* Δhlp has decreased tolerance to repeated freezing and thawing (Fig. 5). An earlier study demonstrated the inability of an *M. smegmatis* *hlp* mutant to resume growth after cultures were shifted from 37 to 10 °C (Shires & Steyn, 2001). This study examined cellular viability after repeated rounds of freezing and thawing. As a major cold-shock-inducible protein, Hlp could be involved in stabilizing mRNA transcripts and allowing transcription initiation to occur after a cold shock (Shires & Steyn, 2001). However, the method used here suggests a different role for Hlp in freeze tolerance as the cells were rapidly shifted to a state of metabolic inactivity (Fig. 5a). In lactobacilli, the fatty acid composition of the cell membrane has been linked to the tolerance of freezing and thawing (Gomez Zavaglia *et al.*, 2000), and it is possible that the altered composition of the *M. smegmatis* Δhlp cell envelope lowers freeze tolerance.

Attempts were made in this study to test in the laboratory the conditions that *M. smegmatis* would naturally encounter in a peat bog. Sphagnum moss bogs are acidic, oligotrophic, anoxic environments with poor microbial growth. Typically these environments are located in boreal and arctic regions subject to periodic annual snow cover. Although these environments provide numerous physical hindrances to microbial growth, mycobacteria are found in great abundance and diversity in the slowly decaying dead layer (grey–brown layer) several centimetres below the green, actively growing surface of the moss. Mycobacteria are not present in the green canopy of the moss because it is extremely acidic as a result of cation antiport, in which moss cells export protons in order to take up essential cations (Brennan & Nikaido, 1995). Hpl does not appear necessary for *M. smegmatis* to tolerate low oxygen, low nutrients or low pH, but Hpl is likely to help *M. smegmatis* withstand repeated bouts of freezing as well as penetrating UV light.

The pleiotropic phenotype of the *hlp* mutant suggests that some regulator mechanism has been altered. It is possible

that alterations in a single cell envelope moiety could be responsible for all of the phenotypes reported here, but this seems less feasible than changes in the regulation of multiple genes due to loss of Hlp. Further study of this possibility will be conducted through microarray comparisons of the wild-type and Δhlp strains.

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