The K\textsuperscript{+} uptake regulator TrkA controls membrane potential, pH homeostasis and multidrug susceptibility in *Mycobacterium smegmatis*

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**Background:** Rifampicin is an important first-line antibiotic for the treatment of mycobacterial infections. Although most rifampicin-resistant strains arise through mutations in the *rpoB* gene in bacteria, a proportion of such strains show no *rpoB* mutations. This suggests that alternative mechanisms are responsible for rifampicin resistance.

**Methods:** We have constructed and analysed a library of 11000 *Mycobacterium smegmatis* insertion mutants to find other possible rifampicin-resistance determinants.

**Results:** We found that disruption of *trkA*, a putative regulator of K\textsuperscript{+} uptake, leads to increased rifampicin resistance. Our data indicate that TrkA-mediated K\textsuperscript{+} uptake is essential for maintenance of the *M. smegmatis* growth rate, its pH homeostasis and membrane potential. In addition to increased rifampicin resistance, inactivation of *trkA* confers resistance to other hydrophobic agents, such as novobiocin, as well as increased susceptibility to isoniazid and positively charged aminoglycosides.

**Conclusions:** Our results suggest that *trkA* is a general regulator of antibiotic susceptibility, and the changes in the multidrug susceptibility/resistance pattern detected in the *trkA* mutant are associated with membrane hyperpolarization. This study sheds light on the role of ion transport activity in intrinsic and acquired antibiotic resistance in mycobacteria.

**Keywords:** antimicrobials, potassium uptake, drug susceptibility, mechanisms of resistance, mycobacteria

**Introduction**

With 9–10 million new infections annually and 2 million people dying each year from tuberculosis (TB), *Mycobacterium tuberculosis* infections are the leading bacterial cause of mortality worldwide.\textsuperscript{1} The composition and structure of the mycobacterial cell wall determines its impermeability to a wide number of agents, and thus it acts as an effective barrier to penetration by antibiotics.\textsuperscript{2} Resistance to antibiotics in mycobacteria is also associated with several mechanisms, including efflux pumps, detoxifying enzymes or changes in the antimicrobial targets.\textsuperscript{2,3} TB treatment requires long-course multidrug chemotherapy that is restricted to a limited number of antimycobacterial agents, including rifampicin, isoniazid, ethambutol and pyrazinamide. The efficacy of TB treatment is compromised by the increasing appearance of multidrug-resistant (MDR) and extensively drug-resistant (XDR) strains.\textsuperscript{4–6} To analyse the mechanisms of antibiotic resistance in mycobacteria, *Mycobacterium smegmatis* has been widely used as a model to identify new determinants involved in antibiotic resistance.\textsuperscript{7–11} Although drug susceptibility patterns sometimes are different among mycobacterial species, *M. smegmatis* is a valuable tool in the generation of mutant libraries that can be used to screen for strains with increased or reduced susceptibility to antibiotics.\textsuperscript{12–16}

Rifampicin is an essential first-line antibiotic for TB treatment, with powerful bactericidal activity against growing and stationary phase cells; furthermore, it reduces treatment failure and TB relapse rates due to its excellent sterilizing properties.\textsuperscript{17,18} Rifampicin binds to the RNA polymerase β-subunit and blocks RNA elongation, thereby inhibiting bacterial transcription. Most rifampicin-resistant strains therefore arise by mutations in the *rpoB* gene, which encodes the RNA polymerase β-subunit. It is estimated that ~95% of these mutations are clustered in a defined 81 bp region of the *rpoB* gene (codons 507–533), termed the rifampicin resistance-determining region.\textsuperscript{19} Many strains with high rifampicin resistance levels bear point mutations at codons 526 or 531, whereas others isolated at lower frequency, such as those with mutation at codon 516, show less resistance.\textsuperscript{20,21} Resistance to rifampicin and to isoniazid are frequently associated, leading to the emergence of MDR strains.\textsuperscript{5,6}
Although *rpoB* mutations are the major cause of rifampicin resistance, 5%-10% of *M. tuberculosis* rifampicin-resistant strains show no mutations in the *rpoB* gene, suggesting that alternative mechanisms contribute to the acquisition of resistance. In this work, we used *M. smegmatis* as a surrogate to identify new determinants whose disruption confers increased rifampicin resistance in mycobacteria. For this purpose, 11,000 *M. smegmatis* insertion mutants were obtained and analysed for their rifampicin susceptibility. Our data show that inactivation of the K+ transporter element TrkA produces rifampicin resistance in *M. smegmatis*. In addition, it causes hyperpolarization of the cytoplasmic membrane, imbalance in pH homeostasis and a high K+ requirement for normal growth. Moreover, TrkA acts as a general regulator that controls mycobacterial susceptibility to a variety of first- and second-line antibiotics.

**Materials and methods**

**Bacterial strains, media and growth conditions**

*M. smegmatis* wild-type strain mc² 155 and its mutant derivatives were grown at 37°C in Middlebrook 7H9 broth or Middlebrook 7H10 agar (Difco) containing 0.5% glycerol and 0.05% Tween 80, and enriched with 10% albumin/dextrase/catalase (Difco). For strain selection, media were supplemented with 25 mg/L kanamycin or 50 mg/L hygromycin B, when necessary. *Escherichia coli* DH5α strain was cultured at 37°C in Luria–Bertani (LB) medium containing 50 mg/L kanamycin or 100 mg/L hygromycin, when appropriate.

**Generation and screening of *M. smegmatis* ϕMycoMarT7 insertion library**

Transposon ϕMycoMarT7, a mariner-based system, was used to obtain a *M. smegmatis* mutant library of random insertions. The isolation of a thermosensitive transposon phage and preparation of high-titre phage stock have been described previously. For transduction, *M. smegmatis* mc² 155 cultures were washed with MP buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 10 mM MgSO₄ and 2 mM CaCl₂), mixed with the phage stock at a multiplicity of infection of 1:10 and incubated (37°C, 8 h), and enriched with 10% albumin/dextrase/catalase (Difco). For strain selection, media were supplemented with 25 mg/L kanamycin or 50 mg/L hygromycin B, when necessary. *Escherichia coli* DH5α strain was cultured at 37°C in Luria–Bertani (LB) medium containing 50 mg/L kanamycin or 100 mg/L hygromycin, when appropriate.

**Complementation of mutant strains**

A plasmid carrying the wild-type tra gene was constructed to restore the expression of the gene in the *tra* mutants. *tra* was amplified by PCR from *M. smegmatis* wild-type genomic DNA, using the forward primer 5'-GCCGCCCATATGGACGTCCGGCGTGCAG and the reverse primer 5'-CCGCGGTGCTGAGTTGGGGCGCTTGG. The PCR product was digested with Ndel and PsiI, and cloned directly into the pV16 vector to generate the complementation plasmid. The resulting plasmid, pV16-tra, was introduced into the *M. smegmatis* Δ*tra* mutant by electroporation. Transformants were selected on Middlebrook 7H10 agar supplemented with kanamycin (25 mg/L) and hygromycin (50 mg/L). Once single-crossover clones were obtained, they were grown in Middlebrook 7H9 broth without antibiotics to allow a second crossover event. Lastly, cultures were diluted and counterselected on Middlebrook 7H10 plates containing 10% sucrose. Candidate colonies were tested for kanamycin and hygromycin susceptibility, and analysed by PCR to confirm the unmarked deletion of *tra*.

**Generation of *tra* knockout mutant strain**

The *M. smegmatis* tra deletion was obtained by allelic replacement, as described previously. Briefly, 1.0 kb fragments up- and downstream of the target gene were amplified by PCR with appropriate primers: 5' fragment (5'-CCCCGTCCCAGGCTGTGTTGGGCGCCAGACA and 5'-CTCC AACGGTTGACCCGGCCGC); and 3' fragment (5'-CTCCAAGGGTCAGGATCGGAACTGCG CGGCGGCACGACCCGCGTGCG). Both fragments were cloned in-frame into the p2NIL vector. The resulting plasmid was digested with PciI to insert a hygromycin-resistance-sacB-lacZ cassette from pGOAL19 and verified by sequencing. The plasmid that harboured an in-frame deletion of the target gene, termed p2NIL-Δtra, was introduced into *M. smegmatis* wild-type strain mc² 155 and plated on Middlebrook 7H10 agar supplemented with kanamycin (25 mg/L) and hygromycin (50 mg/L). Once single-crossover clones were obtained, they were grown in Middlebrook 7H9 broth without antibiotics to allow a second crossover event. Lastly, cultures were diluted and counterselected on Middlebrook 7H10 plates containing 10% sucrose. Candidate colonies were tested for kanamycin and hygromycin susceptibility, and analysed by PCR to confirm the unmarked deletion of *tra*.

**Growth curves and competition experiments**

*M. smegmatis* wild-type and Δ*tra* mutants were cultured overnight and diluted (OD₆₀₀ 0.05) in 5 mL of Middlebrook 7H9 medium, and growth curves were performed in triplicate for 24 h at OD₆₀₀. The fitness of the *M. smegmatis* strains was quantified in vitro by competition assays in which overnight cultures of wild-type (rifampicin-susceptible) and Δ*tra* mutant (rifampicin-resistant) strains were diluted and adjusted to inoculate the same cell numbers in three mixed cultures of 5 mL of Middlebrook 7H9 medium (initial OD₆₀₀ 0.05). Competition cultures with the drug-resistant and -susceptible strains were incubated (37°C), and then 10-fold serial dilutions were plated after 0, 10 and 20 h on Middlebrook 7H10 agar plates, and selected on LB media supplemented with 25 mg/L kanamycin or 100 mg/L hygromycin, when appropriate.

**Localization of ϕMycoMarT7 transposon insertion site**

To determine the insertion position of the transposon cassette in the *M. smegmatis* chromosomal genome, purified genomic DNA from each resistant mutant was digested with BamHI and religated with T₄ ligase. Self-ligated plasmids contained the entire transposon plus flanking chromosomal DNA next to the insertion site. Recircularized plasmids were electroporated into *E. coli* DH5α x pir116 and selected on LB medium supplemented with kanamycin (50 mg/L). After purification, plasmids were sequenced using a MycoMar-specific primer (5'-CCCGAAGATCGCAA CTTAATTGTAAGCG) that hybridizes next to the transposon/chromosome junction. The DNA sequences adjacent to each insertion site were compared with the *M. smegmatis* genome sequence to identify the interrupted gene.
ratio of the number of generations of the rifampicin-resistant strain relative to that of the wild-type strain.

**MIC determination and survival rates to antibiotics**

MICs for the *M. smegmatis* wild-type and *trkA* mutant strains were determined in triplicate on Middlebrook 7H10 agar supplemented with 2-fold increasing concentrations of antibiotic. Each strain was grown in Middlebrook 7H9 broth to logarithmic phase. Cultures were diluted to yield a standard inoculum containing 10⁶ cfu/mL, and ~10⁴ viable cells were plated on drug-containing and on drug-free plates. The MIC of an antibiotic was defined as the lowest concentration at which no growth was visible after 3–5 days of incubation.

To measure survival rates in the presence of different antibiotics (rifampicin, isoniazid and ciprofloxacin), survivors were determined in triplicate by plating serial dilutions on Middlebrook 7H10 agar plates with the indicated antibiotic concentrations. Survival rates were recorded as the percentage of surviving cells (viable cells determined on antibiotic-containing plates divided by total viable cells x 100).

**Membrane potential assay**

For the membrane potential assay, three cultures of *M. smegmatis* wild-type and *trkA* mutant strains were grown to late logarithmic phase. The cultures were washed and adjusted to the same number of cells (~10⁷ cfu/mL). The cell membrane potential was estimated using the fluorescent probe rhodamine 123 (Sigma), a lipophilic cationic molecule that is taken up into mycobacterial cells in response to the level of electrical potential. Inside the cells, probe fluorescence is quenched. To measure the degree of fluorescence decay, rhodamine 123 was added to samples at a final concentration of 0.5 mg/L. Time courses of fluorescence decay were analysed by measuring the fluorescence decrease in the samples over a 10 min period, using a Tecan infinite F200 spectrofluorimeter (480 nm excitation, 530 nm emission). The rate of fluorescence decay was normalized to the initial fluorescence and the fluorescence decay of the probe itself.

**Results**

**Identification of *M. smegmatis* rifampicin-resistant insertion mutants**

To identify the mycobacterial genes implicated in rifampicin resistance, we generated an extensive transposon mutant library (~11000 independent clones) of *M. smegmatis* mc² 155 and screened this collection for mutants with reduced rifampicin susceptibility. Each insertion mutant was replicated on Middlebrook 7H10 agar plates supplemented with rifampicin (5, 10 or 20 mg/L). The growth of the *M. smegmatis* wild-type strain was completely inhibited at these antibiotic concentrations (MIC of rifampicin for *M. smegmatis* mc² 155, 2 mg/L); nonetheless, dozens of insertion mutants with increased rifampicin resistance were able to grow on plates containing 5 mg/L rifampicin. Among them, 16 mutants grew on plates supplemented with 10 mg/L rifampicin. The rifampicin resistance phenotype of these strains was confirmed by MIC. Lastly, the two transposition mutants with the highest degree of rifampicin resistance that grew well on 20 mg/L rifampicin were selected for further analysis.

To identify the disrupted gene in each strain, DNA sequences for each mutant were compared with the *M. smegmatis* genome sequence. The two selected mutants had independent insertions disrupting the same target gene (MSMEG 2771), which encodes a putative K⁺ transport regulatory protein (Figure 1a). One mutant harboured a transposon insertion near the translation start codon (TA dinucleotide at position +7); the other carried another transposon insertion in the middle of the gene (TA dinucleotide at position +321). Both mutants showed the same level of rifampicin resistance (MIC 32 mg/L), in accordance with a loss of gene function. Inactivation by transposition of a gene that encodes a K⁺ transport protein thus confers increased resistance to rifampicin in *M. smegmatis*.

**Characterization of the gene involved in rifampicin resistance**

The target gene, MSMEG 2771, whose disruption increased *M. smegmatis* rifampicin resistance, encodes a TrkA protein with a K⁺ conductance regulatory domain (RCK). RCK proteins are subunits that control K⁺ transporters in Bacteria, Archaea and Eukarya. The core Trk system for K⁺ uptake in prokaryotes consists of two components: the integral membrane K⁺-translocating protein and the NAD⁺/NADH-binding peripheral membrane protein TrkA. TrkA binds to the cytoplasmic portion of the K⁺ membrane transporter, where it acts as an essential factor in K⁺ uptake. In *E. coli* TrkA-mediated K⁺ transport is driven by proton motive force (PMF) and also requires a high ATP concentration.

Analysis of the *M. smegmatis* TrkA sequence showed that the protein consists of two tandemly arranged subdomains (RCK-N and RCK-C terminal; Figure 1a). The N-terminal subdomain was predicted to form a Rossmann fold, similar to dehydrogenase enzymes. This region has a nucleotide-binding sequence (Figure 1a), previously identified as a flavin adenine dinucleotide-binding motif. This characteristic motif, also present in *E. coli* TrkA protein, binds NAD⁺ or NADH with high affinity. *M. smegmatis* TrkA protein shared considerable sequence identity with other TrkA domain proteins, such as *E. coli* TrkA N-terminal (23%) and *M. tuberculosis* CooB (Trk system potassium uptake protein) (74%) (Figure 1b).

**Deletion of the trkA gene decreases rifampicin susceptibility in *M. smegmatis***

To establish that the loss of TrkA activity was responsible for enhanced rifampicin resistance in *M. smegmatis*, we generated an in-frame ΔtrkA deletion mutant. Rifampicin MICs for the wild-type strain and the ΔtrkA mutant were determined in Middlebrook 7H10 agar (Table 1). The ΔtrkA mutant showed considerable rifampicin resistance (32 mg/L), with a 16-fold higher MIC of rifampicin than the wild-type strain. In agreement with the absence of a polar effect, the degree of resistance of the deletion mutant was similar to that of the two insertion mutants. In addition, analysis of survival curves confirmed that ΔtrkA was highly viable when mutant cells were exposed to antibiotic concentrations that killed the wild-type strain (Figure 2a).

The trkA gene from *M. smegmatis* wild-type strain mc² 155 was cloned into the shuttle vector pVV16 and the resulting plasmid, pVV16-trkA, was introduced into the ΔtrkA strain. Plasmid expression of the trkA gene in the deletion mutant restored rifampicin susceptibility (MIC 2 mg/L), but had no effect on the MIC for the wild-type strain, confirming that loss
of trkA expression was the only factor responsible for the rifampicin-resistance phenotype in M. smegmatis. These results demonstrate that trkA gene inactivation increases M. smegmatis resistance to rifampicin.

Deletion of the trkA gene confers isoniazid susceptibility in M. smegmatis

Overexpression of the M. tuberculosis TrkA orthologue CeoB confers isoniazid resistance in an isoniazid-susceptible E. coli oxyR mutant. It has been suggested that TrkA binds and sequesters isoniazid, preventing antibiotic attachment to its target. To examine the effect of trkA inactivation on isoniazid susceptibility, we determined the MIC of isoniazid for the wild-type strain and the ΔtrkA mutant (Table 1). Although M. smegmatis shows intrinsic isoniazid tolerance, the ΔtrkA mutant was more susceptible to isoniazid (MIC 32 mg/L), resulting in a 4-fold decrease in the MIC value compared with the wild-type strain (MIC 128 mg/L). This result was reflected by a sharp decrease in ΔtrkA viability when the mutant cells were exposed to low antibiotic concentrations; in contrast, wild-type cells were affected only moderately, even at high isoniazid concentrations (Figure 2b). This result supports a function for TrkA as an element that protects mycobacterial cells against isoniazid.

M. smegmatis multidrug susceptibility is dependent on TrkA activity

To explore the role of TrkA in the susceptibility pattern of M. smegmatis to distinct antimycobacterial agents, we determined the MIC of a large number of antibiotics for the ΔtrkA mutant; Table 1 shows the correlation of antibiotic activity with the predicted hydrophobicity coefficients (LogS). Large, hydrophobic antibiotics, such as rifampicin, penetrate cells by passive diffusion; reduced permeability to hydrophobic drugs could thus be responsible for the resistance phenotype in the trkA mutant. The MIC of novobiocin, another highly hydrophobic drug, was increased 4-fold for the ΔtrkA mutant compared with that for M. smegmatis MSMEG_2771.

Figure 1. Analysis of the M. smegmatis trkA gene. (a) Vertical arrowheads indicate the location of transposon insertion in each rifampicin-resistant mutant (number of bp in the trkA gene) (top). Domain organization of the TrkA protein, showing the NAD(H)-binding motif sequence (bottom). (b) Alignment of TrkA sequences from M. smegmatis (MSMEG_2771), M. tuberculosis (Rv2691) and E. coli. Asterisks indicate identical residues.
the wild-type strain (Table 1). Less hydrophobic drugs with a large apolar core, such as fluoroquinolones (ciprofloxacin), were also less effective against the \( \Delta trkA \) mutant (Figure 2c), although there was no change in the MIC.

In prokaryotes, cationic antimicrobial peptides, which bind to the negative charge on the cytoplasmic membrane surface to disrupt the membrane, inhibit \( \Delta trkA \) mutants more efficiently than wild-type strains.37–39 Aminoglycosides, another group of positively charged, very hydrophilic antimycobacterial drugs, killed the \( \Delta trkA \) mutant more efficiently than the \( M. smegmatis \) wild-type strain. All aminoglycosides tested (kanamycin, amikacin, streptomycin and capreomycin) showed lower MIC values for the \( \Delta trkA \) mutant than for the wild-type strain, ranging from a 2- to 4-fold decrease (Table 1). \( M. smegmatis \) susceptibility to small hydrophilic drugs (ethambutol and ethionamide) or \( \beta \)-lactams was unchanged.

These data indicate that \( trkA \) deletion affected the drug susceptibility pattern of \( M. smegmatis \), increasing resistance to hydrophobic antibiotics and susceptibility to hydrophilic cationic agents. This suggests a TrkA effect on the \( M. smegmatis \) permeability barrier to antibiotics as a function of the chemical nature of the drug.

**Table 1.** MICs of the main group of antimycobacterial agents for \( M. smegmatis \) wild-type \( mc^2 155 \) and the \( \Delta trkA \) mutant; antibiotics are ordered by solubility values from hydrophobic to hydrophilic.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Molecular weight</th>
<th>LogS (^a)</th>
<th>MIC (mg/L)</th>
<th>( mc^2 155 )</th>
<th>( \Delta trkA )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Novobiocin</td>
<td>612</td>
<td>−4.80</td>
<td>16</td>
<td>64</td>
<td></td>
</tr>
<tr>
<td>Rifampicin</td>
<td>822</td>
<td>−4.09</td>
<td>2</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>Ofloxacin</td>
<td>361</td>
<td>−2.40</td>
<td>0.32</td>
<td>0.32</td>
<td></td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>331</td>
<td>−2.39</td>
<td>0.32</td>
<td>0.32</td>
<td></td>
</tr>
<tr>
<td>Ethanomide</td>
<td>166</td>
<td>−2.30</td>
<td>32</td>
<td>32</td>
<td></td>
</tr>
<tr>
<td>Ethambutol</td>
<td>204</td>
<td>−1.43</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Amikacin</td>
<td>585</td>
<td>−1.07</td>
<td>0.8</td>
<td>0.4</td>
<td></td>
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<tr>
<td>Streptomycin</td>
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<td>−0.96</td>
<td>0.8</td>
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<td>Kanamycin</td>
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<td>3.2</td>
<td>1.6</td>
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<tr>
<td>Capreomycin</td>
<td>1321</td>
<td>NA</td>
<td>6.4</td>
<td>1.6</td>
<td></td>
</tr>
<tr>
<td>Isoniazid</td>
<td>137</td>
<td>−0.59</td>
<td>128</td>
<td>32</td>
<td></td>
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</tbody>
</table>

\(^a\)Predicted hydrosolubility (DrugBank; http://www.drugbank.ca).

**Figure 2.** Survival (%) of \( M. smegmatis \) \( mc^2 155 \) wild-type and the \( \Delta trkA \) mutant under different concentrations of antibiotics. Percentages of survivors of the wild-type and the \( \Delta trkA \) mutant to rifampicin (a), isoniazid (b) and ciprofloxacin (c) at the indicated antibiotic concentrations are shown. The asterisks indicate a lack of growth on plates supplemented with these antibiotic concentrations, representing in all cases a survival of \( \leq 0.0001\% \).

**Growth, \( K^+ \) requirement and fitness in the \( M. smegmatis \) \( trkA \) mutant**

Growth curves of \( M. smegmatis \) wild-type strain \( mc^2 155 \) and the \( \Delta trkA \) mutant showed a notable growth defect for the deletion strain (Figure 3a). The mutant had a lower growth rate during the logarithmic phase than the wild-type strain, although both showed the same cell density at the stationary phase. The growth of the \( \Delta trkA \) strain was restored when additional \( K^+ \) was supplied to the medium (Figure 3b). Addition of \( KCl \) (10–100 mM) stimulated the growth of the \( \Delta trkA \) mutants, but had no effect on wild-type cells; at 200 mM \( KCl \), growth of the mutant and wild-type strains was the same. These results suggest a reduced \( K^+ \) uptake due to a lack of TrkA-mediated transport in the \( \Delta trkA \) mutant and that the mutant requires an additional \( K^+ \) supply to counterbalance its growth defect.

Resistance to antibiotics often has a fitness cost when the antibiotic is absent, which is shown by the reduced growth of mutants relative to the wild-type strain in mixed cultures.40 Competition experiments have shown that most rifampicin resistance mutations in the \( rpoB \) gene impose a cost in mycobacteria, although mutants without a fitness deficit have been isolated;41–43 we thus examined whether \( trkA \) inactivation had deleterious effects on bacterial fitness. As stated above, \( trkA \)
inactivation produces a notable growth defect. As expected, competition assays showed that the wild-type strongly outcompeted the rifampicin-resistant mutant during exponential growth in mixed cultures. In these experiments, the relative fitness value of the ΔtrkA mutant was 0.77 (Table 2).

### Table 2. Fitness of M. smegmatis mc² 155 and its ΔtrkA derivative during the logarithmic growth phase

<table>
<thead>
<tr>
<th>Strain</th>
<th>Competition cultures</th>
<th>relative fitness</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>number of divisions</td>
<td>doubling time (h)</td>
</tr>
<tr>
<td>mc² 155</td>
<td>6.87</td>
<td>2.91</td>
</tr>
<tr>
<td>ΔtrkA</td>
<td>5.32</td>
<td>3.76</td>
</tr>
</tbody>
</table>

Strains were grown in mixed cultures until late logarithmic phase (three independent experiments). The number of divisions and the doubling time of each strain were obtained from cfu counts on drug-containing and drug-free plates. The fitness of the ΔtrkA mutant was measured as the ratio of the number of generations of the rifampicin-resistant strain relative to those of the wild-type strain.

TrkA is necessary for pH homeostasis in M. smegmatis

K⁺ uptake maintains the intracellular ionic balance in prokaryotes. K⁺ accumulation is essential for intracellular pH homeostasis and a loss of K⁺ uptake can thus increase susceptibility to acidic conditions. We studied trkA knockout mutant growth over a range of pH values (pH 5–8; Figure 4a). At neutral pH, ΔtrkA mutant growth was reduced compared with that of the wild-type strain. At acidic pH, mutant growth was severely impaired, while wild-type strain growth was reduced only slightly. Wild-type cells were able to grow even at pH 5, at which ΔtrkA mutant growth was severely impaired. In contrast, at alkaline pH (pH 8), ΔtrkA mutant growth was nearly identical to that of wild-type cells, with no visible growth defect. The reduced growth of the ΔtrkA mutant at acidic pH was compensated when supplementary K⁺ was added to the medium. Figure 4b shows, as an example, how
intracellular pH, the dependent K$^+$ uptake rate has a direct influence on the electrical membrane potential, the main contributor to PMF when cells grow in a neutral environment. To explore the effect of trkA inactivation on cell membrane electrical properties in M. smegmatis, we estimated the electrochemical potential generated across the membrane by monitoring fluorescence quenching of rhodamine 123. Cells with higher membrane potential (increased interior negative charge) tend to increase intracellular pH, the K$^+$ uptake requirement decreases at higher pH.

### TrkA inactivation leads to hyperpolarization of the cytoplasmic membrane

PMF is an electrochemical ion gradient across the membrane, with an electrical component (Δψ, inside negative) and a chemical gradient (ΔpH, inside alkaline). The membrane is separated from the bulk aqueous phase by a barrier of electrostatic nature that could serve as storage for protons. The TrkA-dependent K$^+$ uptake is needed to maintain constant PMF values in prokaryotes. The K$^+$ uptake rate has a direct influence on the electrical membrane potential, the main contributor to PMF when cells grow in a neutral environment. To explore the effect of trkA inactivation on cell membrane electrical properties in M. smegmatis, we estimated the electrochemical potential generated across the membrane by monitoring fluorescence quenching of rhodamine 123. Cells with higher membrane potential (increased interior negative charge) accumulate the cationic probe rhodamine 123 more efficiently, leading to decreased intensity of fluorescence emission, the rate of fluorescence decay is thus proportional to the electrical membrane potential.

Compared with wild-type cells, the ΔtrkA cells showed increased rhodamine 123 uptake and, thus, enhanced fluorescence decay (Figure 5d); these data indicate that the trkA mutant has a hyperpolarized cell membrane. Loss of TrkA-dependent K$^+$ uptake is thus responsible for the increased electrical membrane potential in the M. smegmatis ΔtrkA strain. The higher negative charge of the intracellular membrane in the trkA mutant is thus consistent with its multidrug susceptibility pattern (susceptibility to cationic agents and resistance to hydrophobic drugs).

Additionally, the cells were pre-incubated for 30 min with increasing K$^+$ concentrations (50, 100 and 200 mM KCl). The addition of K$^+$ to the medium depolarizes the bacterial membrane, leading to a strong decrease in the diffusion of the probe inside the cells and, consequently, to a reduced fluorescence decay in the samples (Figure 5b). As expected, the trkA mutant maintains higher levels of membrane potential when compared with wild-type cells at each KCl concentration.

We have studied the effect of an H$^+$ ionophore, carbonyl cyanide m-chlorophenyl hydrazone (CCCP), against M. smegmatis wild-type and the trkA mutant. CCCP destroys membrane potential by eliminating the proton H$^+$ gradient. Although the M. smegmatis trkA mutant had a hyperpolarized membrane, the susceptibility of both strains to CCCP was similar (MIC 4 mg/L). Finally, we analysed the effect of a K$^+$ ionophore, nigericin. This compound has a very high affinity for monovalent cations, such as K$^+$, and catalyses the electroneutral exchange of intracellular K$^+$ for H$^+$ (antiporter). Therefore, nigericin reduces intracellular K$^+$ levels, disrupts membrane potential and decreases intracellular pH. As expected, the trkA mutant showed a hypersusceptibility to the K$^+$ ionophore nigericin (MIC 4 mg/L), compared with the wild-type cells (MIC 16 mg/L). The trkA mutant was unable to counterbalance the K$^+$ efflux due to its impaired K$^+$ uptake, leading to severe defects in the membrane potential and intracellular pH.

### Discussion

To identify mechanisms that could contribute to the acquisition of drug resistance in mycobacteria, we analysed a M. smegmatis...
mutant library for rifampicin resistance. We studied two independent insertion mutants with a rifampicin-resistance phenotype; both carried insertions within the same target gene, trkA (MSMEG_2771), predicted to encode a regulator of K+ uptake. The level of antibiotic resistance was identical (16-fold increase) in the ΔtrkA in-frame deletion mutant, which was complemented by the wild-type trkA gene.

Our results show that TrkA is necessary for K+ uptake in M. smegmatis, in accordance with studies that indicate it is essential for K+ accumulation in prokaryotes, which need a high intracellular K+ concentration (0.1–1 M) for survival. Here, we show that the M. smegmatis trkA mutant requires an additional K+ supply to counterbalance its growth defect and its susceptibility to acidic pH. Given the K+ deficiency in ΔtrkA cells, we suggest that TrkA-dependent ion acquisition is central to the maintenance of adequate K+ levels in M. smegmatis.

In bacteria, proteins involved in basic physiological functions have an essential role in intrinsic resistance to antibiotics and the acquisition of antibiotic resistance. Here, we demonstrate that in addition to increased rifampicin resistance, the M. smegmatis ΔtrkA mutant exhibits enhanced isoniazid susceptibility. Interestingly, overexpression of CeoB, the M. tuberculosis TrkA homologue, confers isoniazid resistance in an E. coli oxyR mutant. This effect was suggested to be due to TrkA sequestration of isoniazid, as it is chemically similar to the NAD+ nucleotide. This hypothesis is supported by a recent study in which TrkA showed high affinity for active isoniazid adducts in complex with NAD(P)+. Another group suggests that flavin adenine dinucleotide-binding proteins also influence NADH/NAD+ levels, and, hence, modify isoniazid activation and binding to its main target, InhA, through nucleotide competition. Our results indicate that the mycobacterial protein TrkA protects cells from the first-line antibiotic isoniazid, increasing tolerance of this drug by M. smegmatis. In addition to the previously proposed mechanisms, our results suggest that the chemical properties of isoniazid (high solubility and polarity) influence its penetration inside M. smegmatis and, thus, it could contribute to explain why the trkA mutant is more susceptible (see below).

TrkA is necessary for intrinsic resistance to a number of positively charged antibiotics in prokaryotes, such as polycationic antimicrobial peptides in E. coli, Salmonella enterica and Vibrio vulnificus or aminoglycosides in Pseudomonas aeruginosa. We found that loss of TrkA activity modifies M. smegmatis susceptibility to a wide variety of antibiotics. The M. smegmatis ΔtrkA mutant showed hypersusceptibility to cationic agents, such as aminoglycosides, and increased resistance to large hydrophobic antibiotics, including rifampicin, novobiocin and fluoroquinolones. In E. coli, Trk proteins, including TrkA, are also associated with a general function in antibiotic susceptibility. Disruption of the Trk system increased antibiotic tolerance when E. coli cells were exposed to sublethal concentrations of several classes of antibiotics with different targets (naldixic acid, piperacillin, tetracycline and doxycycline), although the molecular basis of this effect was not identified.

K+ uptake via the Trk system has a major role in bacterial physiology, as it has a function in osmotic stress tolerance, internal pH maintenance, the regulation of protein activity and the control of bacterial virulence. M. smegmatis maintains intracellular pH near neutral and a constant PMF through interconversion of the membrane electrical potential to a pH gradient. Here, we show that TrkA activity counteracts the effect of extracellular acidic pH in M. smegmatis and, hence, participates in controlling intracellular ionic balance. Our results strongly suggest that in the absence of TrkA-dependent K+ uptake, pH homeostasis fails and intracellular pH decreases to lethal levels in acidic conditions. Furthermore, it has been demonstrated that pH could affect K+ flux and regulate the activity of K+ uptake systems in prokaryotes. Therefore, the effect of pH on K+ transport in M. smegmatis needs to be explored in detail.

Although a connection between TrkA-dependent K+ transport and cell physiology has been established in prokaryotes, its association with antibiotic tolerance remains to be analysed in detail. General alterations in antibiotic susceptibility are frequently caused by permeability changes in the mycobacterial cell envelope due to electrostatic perturbations. Our results suggest that the mechanism by which TrkA controls multidrug susceptibility is similarly based on regulation of K+ transport, and its effect on pH homeostasis and membrane permeability. K+ uptake is essential for the maintenance of a constant PMF through the interconversion of membrane electrical potential to a pH gradient in Actinobacteria. Impaired K+ uptake by inactivation of the Trk system or K+ insufficiency increase membrane potential in E. coli. In the yeast Schizosaccharomyces pombe, changes in membrane potential are associated with pleiotropic changes in the susceptibility to chemotherapeutic agents due to impaired K+ uptake. Here, we demonstrate that the lack of TrkA-dependent K+ uptake in M. smegmatis leads to increased membrane potential. The hyperpolarized membrane of the M. smegmatis trkA mutant could attract and facilitate the penetration of positively charged antibiotic molecules into bacterial cells, but might also reduce the diffusion of large hydrophobic drugs, such as rifampicin or novobiocin. Additionally, both membrane potential and intracellular pH are key components that control the activity of PMF-dependent multidrug efflux pumps in prokaryotes. Therefore, an impaired K+ uptake could also indirectly modify the activity of efflux pumps in M. smegmatis and may influence the susceptibility to antibiotics. However, our results showed a correlation between the physicochemical properties of the antibiotics with their activities against M. smegmatis, suggesting a direct effect of K+ uptake rates on bacterial permeability to antimycobacterial agents.

The acquisition of rifampicin resistance in M. smegmatis by trkA inactivation entails a conspicuous growth defect and a clear loss of fitness, probably due to the impact on cell physiology of ionic imbalance and membrane hyperpolarization. In addition, trkA mutants are attenuated for virulence in several bacterial pathogens, including M. tuberculosis, suggesting a low frequency of trkA (ceoB) mutations among rifampicin-resistant M. tuberculosis clinical isolates; however, the occurrence of different trkA mutations in M. tuberculosis strains remains to be explored.

Our results indicate that by modifying cell permeability, alterations in ion transport promote a change in the M. smegmatis susceptibility pattern to antibiotics. K+ supply might be an important element in this effect; variations in K+ levels could influence antibiotic diffusion and produce changes in drug susceptibility. In addition to trkA, the M. smegmatis genome contains two other genes, MSMEG_2769 (trkB) and MSMEG_1945, which encode TrkA-like proteins. Mycobacteria also encode another main K+-uptake system, named Kdp, a P-type ATPase.
Kdp is an inducible system with high affinity for K⁺ and requires ATP hydrolysis to promote K⁺ uptake in E. coli. Furthermore, ABC components could also develop a regulatory role in Trk-mediated K⁺ uptake. As other proteins involved in ion transport might also influence antibiotic effectiveness, it would be of interest to explore the role of ion transporters in intrinsic resistance and the acquisition of drug resistance in mycobacteria. On the other hand, K⁺ uptake is inhibited by some compounds with antimycobacterial activity, such as the riminophenazine clofazimine. Our data, nonetheless, indicate that the inhibition of K⁺ uptake could induce a complex pattern of phenotypes, including increased resistance to antimycobacterial drugs such as rifampicin. Such collateral resistance should be carefully considered when combined treatments are designed.

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Transparency declarations
None to declare.

References
21 Cummings MP, Segal MR. Few amino acid positions in rpoB are associated with most of the rifampin resistance in Mycobacterium tuberculosis. BMC Bioinformatics 2004; 5: 137.


65 Steel HC, Matlola NM, Anderson R. Inhibition of potassium transport and growth of mycobacteria exposed to clofazimine and B669 is associated with a calcium-independent increase in microbial phospholipase A2 activity. J Antimicrob Chemother 1999; 44: 209–16.