Increased drug permeability of a stiffened mycobacterial outer membrane in cells lacking MFS transporter Rv1410 and lipoprotein LprG

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Summary

The major facilitator superfamily transporter Rv1410 and the lipoprotein LprG (Rv1411) are encoded by a conserved two-gene operon and contribute to virulence in Mycobacterium tuberculosis. Rv1410 was originally postulated to function as a drug efflux pump, but recent studies suggested that Rv1410 and LprG work in concert to insert triacylglycerides and lipoarabinomannans into the outer membrane. Here, we conducted microscopic analyses of Mycobacterium smegmatis lacking the operon and observed a cell separation defect, while surface rigidity measured by atomic force microscopy was found to be increased. Whereas Rv1410 expressed in Lactococcus lactis did not confer drug resistance, deletion of the operon in Mycobacterium abscessus and M. smegmatis resulted in increased susceptibility toward vancomycin, novobiocin and rifampicin. A homology model of Rv1410 revealed a periplasmic loop as well as a highly conserved aspartate, which were found to be essential for the operon's function.

Introduction

The pathogenic bacterium Mycobacterium tuberculosis is responsible for approximately 1.7 million cases of death every year. In particular, the emergence of multidrug (MDR) and extensively drug-resistant strains are a global health threat. Mycobacteria are intrinsically resistant against many antibiotics due to their thick and complex outer membrane (Hoffmann et al., 2008). The main constituents of the mycobacterial cell wall are mycolic acids covalently connected to the arabinogalactan layer, and a plethora of glycolipids such as trehalose dimycolates (TDMs) and lipoarabinomannans (LAMs) that interact non-covalently with the mycolic acids (Dhiman et al., 2011). Due to its biological uniqueness, many TB-specific drugs are targeting the assembly of this cell envelope and the biosynthesis of its lipids. The composition and biosynthesis of this formidable cell barrier are well established. In contrast, much less is known about how these lipids are integrated into the cell wall or how the precursors are transported across the cytoplasmic membrane.

The major facilitator superfamily (MFS) transporter Rv1410 (also called P55) is located in the cytoplasmic membrane. Initial studies in Mycobacterium bovis have established that Rv1410 shares homology to multidrug efflux proteins and is encoded in a highly conserved two-gene operon together with the lipoprotein LprG (also named Rv1411 or P27) (Bigi et al., 2000). Overexpression of rv1410 in Mycobacterium smegmatis conferred amnoglycoside and tetracycline resistance, which could be reversed in the presence of the protonophore carbonyl cyanide m-chlorophenylhydrazone (CCCP), as well as
the efflux pump inhibitors verapamil and reserpine, suggesting that it functions as a proton-driven drug efflux pump (Silva et al., 2001). M. bovis Δrv1410 exhibited higher susceptibility toward a number of drugs including rifampicin and clofazimine (Ramon-Garcia et al., 2009), and deletion of the homologous operon in M. smegmatis resulted in increased susceptibility toward ethidium (Farrow and Rubin, 2008). Interestingly, the authors of these studies noted altered colony morphology, growth attenuation, cell clumping and hampered sliding motility, suggesting that lack of this operon leads to altered cell surface properties. Furthermore, it was found that LprG is needed for the normal functioning of Rv1410, suggesting that they operate in concert (Farrow and Rubin, 2008). Structural and functional analyses of the lipoprotein LprG permitted insights from a completely different angle. LprG was found to associate with the triacylated Toll-like receptor 2 (TLR2) agonists LAM, lipomannan and phosphatidylinositol mannoside (PIM). This notion was corroborated by a structure of LprG co-crystallized in complex with the LAM precursor Ac1PIM2, revealing a hydrophobic pocket accommodating the three alkyl chains of the lipid (Drage et al., 2010). Surface plasmon resonance (SPR) studies using purified LprG and triacylated lipids including LAM further confirmed this molecular interaction and revealed a secondary sugar-binding site outside of the hydrophobic pocket, resulting in a particularly high affinity of LprG for LAMs (Shukla et al., 2014). Very recent studies in addition revealed molecular interactions of purified LprG with TLR2 (Shukla et al., 2018). Virulence of M. tuberculosis ΔlprG in BALB/c mice was clearly attenuated and the mutant exhibits impaired macrophage entry and fails to inhibit phagosome–lysosome fusion (Bigi et al., 2004). Attenuated virulence was initially proposed to be caused by decreased surface localization of LAMs, implying that LprG transports LAMs from the plasma membrane into the cell envelope (Gaur et al., 2014; Shukla et al., 2014). However, a recent study refuted this hypothesis by showing that the deletion mutant also multiplies slower in mice lacking key elements of either the innate or adaptive immune system, which are thought to be triggered by LAMs (Martinot et al., 2016). Lipidome analyses revealed that deletion of the lprG-rv1410 operon leads to intracellular accumulation of triacylglycerides (TAGs) and that overexpression of the locus in turn increases the levels of TAGs in the culture medium (Martinot et al., 2016). This pointed at a metabolic defect in the deletion mutant being responsible for decreased virulence in mice and further lent support to the operon’s role in the transport of triacylated lipids. In a very recent study, LprG was found to interact with various periplasmic proteins, including mycolyltransferase Ag85A, which catalyzes the formation of TDM in the cell wall (Touchette et al., 2017).

The available data about the lprG-rv1410 operon of M. tuberculosis and the homologous operons of other mycobacterial species (henceforth called collectively lprG-mfs operons) so far mainly addressed the function of the lipoprotein LprG, namely its possible role in the transport of LAMs and TAGs from the plasma membrane into the outer membrane and the immunological consequences of a reduced LAM exposure at the surface of the mycobacterial cell. By contrast, comparatively little is known about the function of the proton-driven transporter Rv1410, in particular with regard to its suggested dual role of being a drug efflux pump as well as a lipid transporter. To shed light on the function of Rv1410, we cloned, expressed and purified Rv1410 and studied its interaction with the lipoprotein LprG in vitro. Deletion mutants of the lprG-mfs operon were constructed in M. smegmatis and in Mycobacterium abscessus and evaluated for drug susceptibility and transport of fluorescent dyes to resolve Rv1410’s role as drug efflux pump. A homology model of Rv1410 was generated, which formed the basis to functionally characterize a conserved aspartate and a periplasmic loop. Finally, cell surface properties and morphology of the M. smegmatis deletion mutant were studied using AFM and microscopy techniques offering insight into the biophysical role of the Rv1410 operon.

## Results

### Homology model of Rv1410

According to the transporter classification database (http://www.tcdb.org), Rv1410 belongs to the MFS subclass drug:H+ antiporter-2 (DHA2), which mainly consists of drug efflux pumps. DHA2 members all feature 14 transmembrane helices (TM)s, namely two bundles of six TM)s, which are common to all MFS transporters, and an additional helix pair placed between these bundles (6+2+6 TM)s (Reddy et al., 2012). Other well-characterized DHA2 family members are the drug efflux pumps LfA from M. smegmatis (Sander et al., 2000) and QacA from Staphylococcus aureus (Paulsen et al., 1996a). Currently, there is no structure available for a DHA2 transporter. The closest structural homologue of Rv1410 is PepT_{So2} of Shewanella oneidensis (sequence identity of 14.2% and coverage of 0.844 according to the SwissModel server), an MFS transporter belonging to the subfamily of proton-dependent oligopeptide transporters, which share the 6+2+6 helical arrangement with Rv1410 (Guettou et al., 2013; Newstead, 2015). Therefore, a homology model was generated based on the coordinates of PepT_{So2} (PDB ID: 4LEP) using the SwissModel server (Biasini et al., 2014) (Fig. 1A). The model contains the classical fold common to all MFS transporters comprising TM1–12 and...
the two additional helices (called HA and HB) connecting the two lobes of the transporter, whose functional role is currently unclear (Newstead, 2015). Interestingly, Rv1410 contains a periplasmic loop consisting of 34 amino acids placed between TM11 and TM12 (Fig. 1A), which is not present in any of the known MFS structures and whose function is unknown.

A sequence alignment involving Rv1410, the L. lactis drug efflux pump LmrP and the Escherichia coli drug efflux pump MdfA – both belonging to the DHA1 family of MFS transporters containing 12 TMs – along with PepTSo2 and LfrA revealed a highly conserved aspartate (D70 in Rv1410) (Fig. 1B). This aspartate is part of the conserved motif A (consensus sequence GrLaDrfGrRRv) located at the cytoplasmic loop between TM2 and TM3 in all MFS transporters (Fig. 1A) (Paulsen et al., 1996b). Mutation of this aspartate into cysteine in LmrP or alanine in MdfA, respectively, was shown to completely abrogate drug efflux in these transporters (Mazurkiewicz et al., 2004; Sigal et al., 2006). A double electron–electron resonance...
(DEER) study of LmrP revealed that this aspartate plays a key role in coupling substrate transport to the influx of protons (Masureel et al., 2014). We therefore mutated this conserved aspartate into asparagine (henceforth called DtoN mutation), to obtain an inactive Rv1410 variant that is uncoupled from the proton gradient.

**Deletion of the lprG-mfs operon in M. abscessus and M. smegmatis increases drug susceptibility**

*Mycobacterium abscessus* is a major cause of infections in cystic fibrosis patients and is notoriously difficult to treat because of its intrinsic resistance toward many antibiotics including first-line TB drugs (Brown-Elliott et al., 2012; Mougari et al., 2016; Rominski et al., 2017; Luthra et al., 2018). Genetic deletion of the *lprG-mfs* operon (Δ*MAB_2806-07*, henceforth called dKO) resulted in a pronounced increase of antibiotic susceptibilities toward six antibiotics, including tetracycline, rifabutin and vancomycin (Table 1). Complementation studies of the deletion mutant were carried out using the integrative vector pFLAG (Arnold et al., 2018), harboring the coding sequences of LprG (MAB_2806), the MFS transporter (MAB_2807) or the entire operon under the control of a constitutively active promoter. Complementation with the entire operon fully rescued the dKO mutant. In contrast, MICs were identical to the vector control if the complementation vector carried LprG alone or the operon with the MFS transporter carrying the D82N mutation respectively. Interestingly, complementation with the MFS transporter alone almost completely reversed drug susceptibility.

The *lprG-mfs* operon was also deleted in *M. smegmatis* (Δ*MSMEG_3069-70*, henceforth called dKO), as described previously (Farrow and Rubin, 2008), and the resulting mutant was found to exhibit increased susceptibility toward vancomycin, rifabutin and novobiocin (Table 2). Complementation with the *MSMEG_3069-70* operon fully reversed drug susceptibility to wild-type level, whereas the D72N mutation introduced in the MFS transporter fully abrogated its function. Complementation with the MFS transporter *MSMEG_3069* partially reversed drug susceptibility, but complementation with *lprG* (MSMEG_3070) did not.

In summary, lack of the *lprG-mfs* operon strongly increases intrinsic drug susceptibility in *M. abscessus* and to a lesser degree in *M. smegmatis*. In both operons, the MFS transporters play a dominant role, because their expression partially reversed drug susceptibility.

**Periplasmic loop truncations strongly affect function of Rv1410**

The homology model of Rv1410 predicts a periplasmic loop (34 amino acids) between transmembrane helices 11 and 12, which is not present in published MFS transporter structures. To assess its functional role, we generated truncation mutants lacking 10, 18, or 26 amino acids of the loop respectively (Fig. 2A). The *M. smegmatis* dKO strain was complemented with the operon containing wild-type LprG and Rv1410 harboring different loop truncations, and was subjected to antibiotic susceptibility testing. Short and medium loop mutants of Rv1410 were as inactive as the deleterious D70N mutant (Fig. 2D–F). In contrast, the Rv1410 mutant containing the longest loop (i.e. the shortest deletion) was partially active. Its residual activity is similar to the one of LprG containing the previously described V91W mutation (Drage et al., 2010) (Fig. 2D–F). Western blot analysis revealed that the expression of the loop truncation mutants and wild-type Rv1410 were equally strong in *M. smegmatis* (Fig. 2C). Size exclusion chromatography analysis of purified mutant proteins suggested that these mutations did not alter the folding behavior of Rv1410 because the mutant proteins’ peaks coincided with the peak of wild-type Rv1410 on SEC (Fig. 2B, Rv1410 purification is explained in more detail in the subsequent section). Together, these results support the hypothesis that the periplasmic loop is involved in transport processes mediated by Rv1410 and LprG.

**Purified Rv1410 and LprG do not interact**

We hypothesized that the periplasmic loop of Rv1410 may physically interact with LprG, which is known to be attached to the outer leaflet of the cytoplasmic membrane via its lipid anchor. To study a potential interaction at the biochemical level, we individually expressed and purified Rv1410 and LprG. Rv1410 was overexpressed in *E. coli* with a His-tagged GFP fused to its C-terminus.
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The fusion protein was purified via the His-tag using the detergent β-DDM by immobilized metal ion affinity chromatography (IMAC) and GFP was cleaved off using HRV 3C protease. Subsequently, GFP and the His-tagged protease were removed by a reverse IMAC step, resulting in highly pure protein (Fig. 3A). Rv1410 was finally analyzed by size exclusion chromatography (SEC) and eluted as monodisperse peak at a retention volume corresponding to a Rv1410 monomer (Fig. 3B). Rv1410 was fused to an Avi-tag and purified for enzymatic biotinylation (Cull and Schatz, 2000) along with an Avi-tagged version of E. coli MdfA, which was used as negative control. SPR sensor chips were coated with biotinylated Rv1410 and MdfA, and purified LprG was injected as analyte at different concentrations. When injecting the highest three concentrations (22, 65 and 194 μM, respectively), LprG gave rise to a binding signal with Rv1410 (Fig. 3C). However, the same signal was also obtained with MdfA. Hence, the interaction is unspecific and presumably mediated through contacts of LprG with the detergent micelle of Table 1.

### Table 1. MIC determination of M. abscessus dKO (ΔMAB_2806-07).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Antibiotic</th>
<th>Tetracycline</th>
<th>Vancomycin</th>
<th>Rifabutin</th>
<th>Clofazimine</th>
<th>Novobiocin</th>
<th>Ofloxacin</th>
</tr>
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<tbody>
<tr>
<td>WT</td>
<td>Median</td>
<td>&gt;128</td>
<td>&gt;128</td>
<td>8</td>
<td>0.25</td>
<td>64</td>
<td>64</td>
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<tr>
<td></td>
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<td>(&gt;128)</td>
<td>(4–8)</td>
<td>(0.25–0.5)</td>
<td>(32–64)</td>
<td>(32–64)</td>
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<tr>
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<td>16</td>
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<td>0.06</td>
<td>1</td>
<td>4</td>
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<tr>
<td></td>
<td>Range</td>
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<td>(8–16)</td>
<td>(1)</td>
<td>(0.06–0.125)</td>
<td>(1–2)</td>
<td>(4)</td>
</tr>
<tr>
<td>EV</td>
<td>Median</td>
<td>16</td>
<td>16</td>
<td>0.5</td>
<td>0.125</td>
<td>0.5</td>
<td>4</td>
</tr>
<tr>
<td>c. mfs</td>
<td>Median</td>
<td>128</td>
<td>&gt;128</td>
<td>4</td>
<td>0.25</td>
<td>32</td>
<td>32</td>
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<tr>
<td></td>
<td>Range</td>
<td>(8–32)</td>
<td>(16–32)</td>
<td>(0.25–0.5)</td>
<td>(0.06–0.125)</td>
<td>(≤0.25–2)</td>
<td>(4–8)</td>
</tr>
<tr>
<td>c. lprG</td>
<td>Median</td>
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<td>16</td>
<td>0.5</td>
<td>0.06</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>c. lprG-mfs</td>
<td>Median</td>
<td>&gt;128</td>
<td>&gt;128</td>
<td>8</td>
<td>0.5</td>
<td>64</td>
<td>32</td>
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<td>c. lprG-mfs</td>
<td>Range</td>
<td>(128 to &gt;128)</td>
<td>(128 to &gt;128)</td>
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<td>(32–64)</td>
<td>(16–32)</td>
</tr>
<tr>
<td>c. lprG-mfs DtoN</td>
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<td>0.5</td>
<td>0.06</td>
<td>0.5</td>
<td>4</td>
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<tr>
<td></td>
<td>Range</td>
<td>(8–32)</td>
<td>(8–32)</td>
<td>(0.5–1)</td>
<td>(0.06–0.25)</td>
<td>(≤0.25–1)</td>
<td>(4–8)</td>
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</tbody>
</table>

Complementation (c.) was carried out with the genes of the lprG-mfs operon from M. abscessus. EV, empty vector control. DtoN, MAB2807 D82N. MIC values correspond to μg ml⁻¹ of the respective antibiotics used. Shown are the median and range of three biological replicates.

### Table 2. MIC determination of M. smegmatis dKO (ΔMSMEG_3069-70).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Antibiotic</th>
<th>Tetracycline</th>
<th>Vancomycin</th>
<th>Rifabutin</th>
<th>Clofazimine</th>
<th>Novobiocin</th>
<th>Ofloxacin</th>
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<td>(4–8)</td>
<td>(0.5)</td>
<td>(32)</td>
<td>(0.5)</td>
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<tr>
<td>dKO</td>
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<td>16</td>
<td>0.25</td>
<td>0.5</td>
<td>4</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>Range</td>
<td>(0.25–0.5)</td>
<td>(8–16)</td>
<td>(0.125–0.5)</td>
<td>(0.5)</td>
<td>(4)</td>
<td>(0.5)</td>
</tr>
<tr>
<td>EV</td>
<td>Median</td>
<td>0.5</td>
<td>32</td>
<td>0.125</td>
<td>0.5</td>
<td>4</td>
<td>0.5</td>
</tr>
<tr>
<td>c. mfs</td>
<td>Median</td>
<td>0.5</td>
<td>64</td>
<td>1</td>
<td>0.5</td>
<td>8</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>Range</td>
<td>(0.5)</td>
<td>(64–128)</td>
<td>(0.5–1)</td>
<td>(0.5)</td>
<td>(8)</td>
<td>(0.5)</td>
</tr>
<tr>
<td>c. lprG</td>
<td>Median</td>
<td>0.5</td>
<td>32</td>
<td>0.25</td>
<td>0.5</td>
<td>4</td>
<td>0.5</td>
</tr>
<tr>
<td>c. lprG-mfs</td>
<td>Median</td>
<td>0.5</td>
<td>512</td>
<td>4</td>
<td>0.5</td>
<td>16</td>
<td>0.5</td>
</tr>
<tr>
<td>c. lprG-mfs DtoN</td>
<td>Median</td>
<td>0.5</td>
<td>(0.5–1)</td>
<td>(256–512)</td>
<td>(4–8)</td>
<td>(0.5–1)</td>
<td>(16–32)</td>
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<tr>
<td></td>
<td>Range</td>
<td>(0.5)</td>
<td>(16)</td>
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<td>(0.5)</td>
<td>(4–8)</td>
<td>(0.5–1)</td>
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</tbody>
</table>

Complementation (c.) was carried out with the genes of the lprG-mfs operon from M. smegmatis. EV, empty vector control. DtoN, MSMEG3069 D72N. MIC values correspond to μg ml⁻¹ of the respective antibiotics used. Shown are the median and range of three biological replicates.
purified Rv1410 and MdfA. In summary, our biophysical experiments revealed that Rv1410 and LprG do not form a complex, at least not outside of the membrane context.

Lack of lprG-mfs operon leads to increased drug influx

*M. abscessus* and *M. smegmatis* dKO mutants were clearly more susceptible toward vancomycin, a drug which acts in the periplasm as a cell wall synthesis inhibitor (Perkins, 1969). Importantly, vancomycin does not need to enter the cytoplasm to inhibit cellular growth, and consequently, possible transport of vancomycin across the cytoplasmic membrane mediated by Rv1410 would not result in increased resistance toward this drug. This made us question the transporter's role as a drug efflux pump, which has been repeatedly proposed in the literature (Silva et al., 2001; Ramon-Garcia et al., 2009). We hypothesized that higher permeability of the
cell envelope due to the lack of transport of LAMs, TAGs and presumably additional triacylated lipids to their correct locations could cause increased influx of drugs. We tested the hypotheses of drug efflux mediated by Rv1410 (Fig. 4A) and increased drug influx via a defective cell wall (Fig. 4B) by monitoring the accumulation of fluorescent substrates (ethidium, BCECF-AM, calcein-AM) in energized and de-energized cells.

As a positive control, we included the well-studied ethidium efflux pump LfrA of *M. smegmatis* (Sander et al., 2000; Rodrigues et al., 2011). Akin to Rv1410, LfrA is a DHA2 family MFS transporter with 14 transmembrane helices. In the presence of the proton-motive force, LfrA and other active transporters mediating ethidium efflux lose their ability to extrude ethidium and thus the wild-type strain displays a phenotype identical to the ΔlfrA strain.

Similar results would be expected if Rv1410 and LprG formed a drug efflux system. However, *M. smegmatis* dKO accumulated more BCECF and calcein than wild-type cells, regardless whether they were in an energized or de-energized state (Figs 4D and S2A). This observation suggests that the dKO strain exhibits an increased

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**Fig. 3.** Interaction studies of purified Rv1410 and LprG.  
A. SDS-PAGE analysis monitoring the purification of Rv1410 expressed in *E. coli*. The following samples were loaded on the gel: (1) solubilized total membrane proteins, (2) elution from IMAC containing mainly His-tagged Rv1410-GFP fusion protein, (3) IMAC flow-through using wash buffer, (4) after cleavage with 3C protease, (5) flow-through of reverse IMAC after 3C cleavage containing tag-less Rv1410, (6) elution of reverse IMAC containing His-tagged GFP and His-tagged 3C protease, (7) highly pure Rv1410 after SEC.  
B. Analytical SEC using purified Rv1410 alone (red trace), LprG alone (green trace) and Rv1410 and LprG mixed at a 1:5 molar ratio (black trace).  
C. SPR analysis of Rv1410-LprG interaction. Rv1410 and control protein MdfA were immobilized and purified LprG served as analyte. [Colour figure can be viewed at wileyonlinelibrary.com]
dye permeability compared to wild-type cells. Hence, the data support the hypothesis that Rv1410 and LprG transport lipids required for the proper biogenesis of the mycobacterial outer membrane, which remained unaffected by the addition of CCCP within the short timeframe of our experiments (Fig. 4B). Of note, BCECF and...
calcein fluorescence levels were in general lower under de-energized conditions due to the dependence of dye fluorescence on pH (Ozkan and Mutharasan, 2002; Boens et al., 2006). When CCCP was added to cells energized with glucose, an immediate drop in fluorescence intensity was detected for BCECF (Fig. 4E) and calcein (Fig. S2B), respectively. This drop was caused by a sudden decrease of the cytoplasmic pH upon disruption of the proton motive force and shows that substantial amounts of BCECF-AM and calceine-AM were converted into their fluorescent forms by intracellular esterases.

In conclusion, our data strongly support the hypothesis that Rv1410 and LprG contribute to the integrity and low permeability of the mycobacterial cell envelope by transporting important lipid constituents. Further, direct experimental evidence is provided suggesting that Rv1410 does not export fluorescent substrates out of the cell.

**Rv1410 does not mediate drug efflux in L. lactis**

To further corroborate the lacking drug efflux activity of the MFS transporter, we expressed the rrv1410 gene of *M. tuberculosis* in the Gram-positive bacterium *L. lactis*, which is a widely used model system to investigate drug efflux pumps (Seeger et al., 2012; Hürlimann et al., 2016). First, we confirmed that the bona fide efflux pumps LmrP and MdfA actively export the dyes ethidium, Hoechst 33342 and in case of LmrP BCECF-AM (Figs 5, S3A and B), showing that Rv1410 appears to be unable to transport these dyes, which are common substrates for multidrug efflux pumps. Contrast, under the same experimental conditions Rv1410 and Rv1410 D70N exhibited nearly identical fluorescence traces (Figs 5A, S3A and B), showing that Rv1410 appears to be unable to transport these dyes, which are common substrates for multidrug efflux pumps. Further, we determined the minimal

![Fig. 4. Functional investigation of Rv1410 in M. smegmatis.](image)

A and B. Two hypotheses predicting the functional outcome of a transport experiment assuming that Rv1410 is a drug efflux pump (A) or a lipid transporter (B). Gray circles represent non-fluorescent BCECF-AM, which is converted by intracellular esterases into fluorescent BCECF (yellow stars). Drug efflux pumps (here Rv1410) export BCECF-AM prior to esterase cleavage. Addition of CCCP disrupts the proton gradient across the inner membrane and thereby inactivates Rv1410.

C. Ethidium transport in *M. smegmatis* wild-type and dKO cells, which follows the hypothesis shown in panel A. Ethidium was added to washed cells under energized (Glc) and de-energized (CCCP) conditions and ethidium uptake into the cell was monitored by fluorescence of ethidium intercalated into chromosomal DNA.

D. BCECF-AM transport in *M. smegmatis* wild-type and dKO cells, which follows the hypothesis shown in panel B. Non-fluorescent BCECF-AM was added to washed cells under energized (Glc) and de-energized (CCCP) conditions and was converted into fluorescent BCECF by intracellular esterases.

E. Traces of BCECF-AM uptake of energized (Glc) and de-energized (CCCP) cells are shown as in panel D. In addition, BCECF-AM uptake traces were recorded, in which CCCP was added to energized cells at time point 480 s (dotted lines, Glc-CCCP). CCCP addition leads to an immediate decrease of BCECF fluorescence, because the proton-motive force is eliminated resulting in a drop of intracellular pH. For the traces shown in panels C and D, average fluorescence intensities and 95% confidence intervals using a Student’s t distribution were calculated from the data of six biological replicates. [Colour figure can be viewed at wileyonlinelibrary.com]

**Fig. 5. BCECF-AM efflux mediated by Rv1410, LmrP and MdfA expressed in L. lactis.** BCECF-AM was added to the washed *L. lactis* cells expressing wild-type Rv1410 (A), LmrP (B) and MdfA (C) or transporters carrying the corresponding DtoN mutations. BCECF accumulation in the cytosol was monitored by fluorescence. Only LmrP exhibits BCECF-AM efflux activity. [Colour figure can be viewed at wileyonlinelibrary.com]

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inhibitory concentration for vancomycin, rifampicin and novobiocin using *L. lactis* cells expressing Rv1410, but did not find any difference between cells expressing the wild-type transporter versus the corresponding DtoN mutant (Fig. S3C). In order to assess expression levels, we fused GFP to the C-termini of the transporters and could show by in-gel fluorescence that all three transporters, including Rv1410, were well expressed (Fig. S3D). Of note, LmrP-GFP and MdfA-GFP were still capable of dye transport, whereas no efflux activity was observed for Rv1410-GFP (not shown). The lipid composition of *L. lactis* membranes is different from mycobacteria, which may cause incorrect folding and aggregation of the transporter. However, overexpressed Rv1410 purified from *L. lactis* membranes via its His-tag eluted as monodisperse peak from SEC at a retention volume corresponding to the size of a Rv1410 monomer, thereby excluding major folding and aggregation issues (Fig. S3E). In summary, Rv1410 is well expressed and properly folded in *L. lactis*, but lacks drug efflux activity.

**M. smegmatis** dKO cells are more rigid

We hypothesized that deletion of the *lprG-mfs* operon might also affect the mechanical properties of the cell surface and therefore investigated wild-type and dKO cells by AFM. Wild-type and dKO cells were mixed and imaged using correlated optical fluorescence microscopy (OFM) and AFM (Eskandarian et al., 2017). Fluorescently labeled Wag31 was expressed in the wild-type cells to distinguish them from dKO cells (Santi et al., 2013). Control experiments in which the dKO mutant was labeled with fluorescent Wag31 excluded the possibility that fluorescent protein expression affected the experimental outcome. The mean surface rigidity, represented as the elastic modulus, was calculated for wild-type and dKO cells from images obtained by AFM (Fig. 6A and B). Interestingly, the mean surface rigidity of the dKO mutant was found to be significantly higher than in wild-type cells (Fig. 6C).

**M. smegmatis** dKO has a cell separation defect

We used transmission electron microscopy (TEM) to investigate whether lack of the *lprG-mfs* operon influences cellular morphology. Interestingly, we noticed a cell separation defect as manifested by long cell chains divided by multiple septa (Fig. 7A), which appeared to be more prevalent in *M. smegmatis* dKO. In order to quantify this defect, we used fluorescence microscopy to analyze mycobacterial cells that were stained with the membrane dye SynaptoRed. In addition, the cells expressed the Wag31–GFP fusion protein, which localizes to septa formed during cell division and remains attached to the poles (Santi et al., 2013) (Fig. 7B). Overall, 1105 cell chains were counted. Because the experiments and data analysis were conducted blinded, the number of dKO strain and wild-type strain differed by a factor of around 2 (Fig. 7C). Upon data normalization, we observed 3 times more chains consisting of three or more cells in the dKO strain compared to the wild-type strain (Fig. 7D). Pearson’s *χ*² test showed significant difference in the distribution of the cell chains of different lengths between wild-type and dKO strains.
Increased drug permeability of a stiffened mycobacterial outer membrane

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These results suggest the presence of a cell separation defect after the cell division event. The extent of the cell separation defect was probably underestimated, because long cell chains are more likely to become entangled in cell clusters which were excluded from data analysis.

**Discussion**

Rv1410 and its operon partner LprG have been the subject of numerous studies in past years. Whereas LprG’s role is to bind triacylated lipids such as LAMs, PIMs and TAGs, Rv1410 was proposed to possess a
dual function, namely drug efflux as well as lipid transport (Farrow and Rubin, 2008; Ramon-Garcia et al., 2009). In this work, we observed that deletion of the lprG-mfs operon in M. smegmatis and M. abscessus resulted in a marked increase of vancomycin, rifampicin/rifabutin and novobiocin susceptibility, a result that is in full agreement with a previous study on M. bovis BCG (Ramon-Garcia et al., 2009). While rifampicin/ rifabutin and novobiocin target intracellular proteins, vancomycin has its target in the periplasm. Importantly, periplasmic vancomycin concentrations solely depend on the diffusion across the outer membrane, and consequently, Rv1410-mediated vancomycin export across the inner membrane cannot account for vancomycin resistance. Rather, the activity of Rv1410 appears to be required for the correct assembly of the mycobacterial outer membrane. In further support of this notion, cells lacking the operon were found to accumulate more BCECF and calcein dyes, irrespective whether they were in an energized or de-energized state. Hence, active dye efflux mediated by Rv1410, which depends on the proton-motive force, cannot explain this observation. Finally, Rv1410 expressed in L. lactis did not exhibit efflux activity toward BCECF-AM, ethidium and Hoechst 33342, nor did it confer resistance toward vancomycin, rifampicin or novobiocin, despite of the fact that the transporter was expressed at high levels and well folded.

We show in this work that Rv1410 critically depends on the proton-motive force, because substitution of a conserved aspartate by asparagine (DtoN mutation), a residue coupling substrate transport to the influx of protons, completely abrogated Rv1410’s capacity to confer resistance toward all drugs tested, including the periplasmic drug vancomycin. Hence, inclusion of protonophores in the growth medium does not only inhibit the potential drug efflux activity of Rv1410, but also its lipid transport activity and consequently the correct assembly of the outer membrane during the long growth period. Therefore, previous experiments performed in the presence of sub-inhibitory CCCP concentrations from which it was concluded that Rv1410 is an efflux pump for rifampicin and novobiocin are likely ambiguous (Ramon-Garcia et al., 2009). In order to differentiate between drug and lipid transport activity mediated by Rv1410, we chose an experimental design in which we added CCCP to washed cells shortly prior to the addition of the dyes BCECF-AM or calcein-AM. Thereby we avoided defects of the outer membrane and were able to truly distinguish drug influx from drug efflux. These experiments clearly revealed that increased BCECF and calcein accumulation in mutant cells is solely attributable to increased influx, unequivocally suggesting an outer membrane defect resulting in increased dye permeability. It is interesting to note that certain mutations affecting the lipopolysaccharide (LPS) biosynthesis of Gram-negative bacteria exhibit drug susceptibility profiles that are very similar to the lprG-mfs operon deletion mutants observed here and in M. bovis BCG (Ramon-Garcia et al., 2009). Most prominent in this context are the lpxC (originally called envA) mutations in E. coli, which are defective in lipid A biosynthesis resulting in periplasmic leakage of proteins and higher susceptibility toward larger drugs including rifampicin, novobiocin and vancomycin, that cannot be transported through outer membrane porins (Young and Silver, 1991; Young et al., 1995; Nikaido, 2005).

In addition to the increased outer membrane permeability, we observed a cell separation defect in M. smegmatis lacking the operon, as manifested by long chains of cells separated by already formed septa. Interestingly, such chain-forming phenotypes displaying septation and cell separation defects have been described for several Gram-negative bacterial mutants containing a defective outer membrane (Normark et al., 1969; Weigand et al., 1976; Stanley et al., 2001). Using state-of-the-art AFM, we could demonstrate that the cell surface of the deletion mutants is significantly more rigid. At first sight, a connection between increased membrane stiffness and higher permeability appears counterintuitive. Indeed, AFM studies on isolated membrane vesicles with defined lipid compositions revealed that membranes of higher rigidity are less permeable for calcein (Takechi-Haraya et al., 2017). However, lipid vesicles are several orders of magnitude softer than the complex mycobacterial outer membrane, suggesting that the physical principle underlying membrane permeability differs among them. Interestingly, an E. coli mutant unable to incorporate unsaturated fatty acid chains into lipid A became hypersusceptible toward vancomycin and rifampicin at 12°C, but not at 30°C, indicating that large drugs can enter the periplasm through a stiffened outer membrane (Vorachek-Warren et al., 2002). Our results suggest that lack of the lprG-mfs operon results in a rigidified mycobacterial outer membrane, which might contain imperfections and ‘cracks’ through which large drugs and dyes gain access to the periplasm.

The aggregated data on Rv1410 and LprG as outlined in previous works and here clearly suggest that these proteins form a tandem responsible for the transport of triacylated lipids for the correct biogenesis of the outer mycobacterial membrane (Fig. 8). But how do they work in concert? In this study, we showed that the MFS transporter plays the dominant role of the duo, because its expression as solitary protein in M. smegmatis and in particular in M. abscessus to a large degree reversed drug susceptibility. It is thus conceivable that Rv1410 and its mycobacterial orthologues can cooperate with lipoproteins other than LprG, which may partially substitute...
E. coli, the ABC transporter LolCDE powers the extraction of the lipoprotein from the inner membrane using the energy of ATP and delivers it to the periplasmic shuttling protein LolA (Yakushi et al., 2000). Currently, these possible mechanisms of Rv1410 are purely speculative and detailed biochemical in vitro studies are needed to further address the important question of how Rv1410 and LprG work in concert to shuttle lipids from the inner to the outer mycobacterial membrane.

**Experimental procedures**

**Bacterial strains and growth conditions**

*Mycobacterium smegmatis* mc² 155 and *Mycobacterium abscessus* (ATCC19977T) were used in this study. Mycobacteria were grown at 37°C in liquid Middlebrook 7H9 medium containing 0.05% Tween 80 or on solid Middlebrook 7H10 medium supplemented with OADC containing 4.5 ml L⁻¹ glycerol. *Escherichia coli* MC1061, the strain used during cloning and for heterologous protein expression, was grown in lysogeny broth medium (LB) or Terrific broth medium (TB) at 37°C or 25°C respectively. *Lactococcus lactis* ΔlmrA ΔlmrCD (Venter et al., 2008) was grown in M17 medium supplemented with 0.5% glucose (GM17) at 30°C without shaking (Hürlimann et al., 2016). Where required, the medium was supplemented with the following amounts of antibiotics: 100 μg ml⁻¹ of ampicillin (Amp100), 5 or 25 μg ml⁻¹ of chloramphenicol (Cm⁵/Cm²⁵) for *L. lactis* and *E. coli*, respectively, 25 μg ml⁻¹ of apramycin (Apr²⁵) for *M. smegmatis* or 50 μg ml⁻¹ apramycin (Apr50) for *E. coli* and *M. abscessus*, and 32 μg ml⁻¹ isoniazid for *M. abscessus*.

**Gene deletion of lprG-mfs (MSMEG_3069-70) and lfrA (MSMEG_6225) in M. smegmatis**

Gene deletions in *M. smegmatis* mc² 155 were generated by a two-step recombination approach. The flanking regions of the respective genetic loci were amplified by PCR from genomic DNA (gDNA) using primers listed in Table S1. Both fragments were purified via agarose gel, cut with BspQI, cloned in one step into pNIT (Cm²⁵) using fragment exchange (FX) cloning (Geertsma and Dutzler, 2011). After sequencing, the fragments were cut from pNIT and cloned into the mycobacterial suicide vector pKO, which was propagated in *E. coli* using Apr⁴⁰ (Arnold et al., 2018). For the first recombination step, the pKO vectors carrying the flanking regions were transformed into *M. smegmatis* by electroporation and plated on 7H10 plates containing Apr⁴⁰. Colonies appeared after 3–4 days incubation and were grown in 5 ml liquid 7H9 (Apr²⁵) for 2–3 days. gDNA was isolated (GenElute™ Bacterial Genomic DNA Kit, Sigma) and was analyzed by PCR for 5’ and/or 3’ integration. PCR-positive clones were grown in liquid 7H9 medium devoid of apramycin for 2–3 days and 1 μl was plated on 7H9 plates containing 0.5% 2-Deoxy-Galactose (2-DOG) and 10% sucrose for counterselection. Colonies were resuspended in 10 μl
dH₂O and screened by PCR to differentiate between gene deletions or wild-type revertants. gDNA of identified gene deletion mutants was isolated to confirm gene deletions by deletions or wild-type revertants. gDNA of identified gene operons and its single genes of lprG-mfs amplified. 

1. The genomic DNA (gDNA) as template, the following genes were Fidelity DNA polymerase (NEB) and if not stated otherwise by fragment exchange into the required destination vectors M. smegmatis mc2 155, M. abscessus end, a 1526 bp fragment upstream of the target genes was pSE-arp-katG-∆lprG-ΔMAB_2807. To construct the gene deletion mutant, gene deletion vector lprG-mfs ′ ∆ vector pSE-arp-katG-∆lprG-ΔMAB_2807. The vector was transformed into M. abscessus ATCC 19977T as previously described (Rominski et al., 2017). Putative single cross-over colonies were selected on Apr 50 and confirmed by Southern blotting. Double cross-over recombinants were subsequently selected on isoniazid (32 μg ml⁻¹) and confirmed by Southern blotting (Rominski et al., 2017).

Gene deletion of lprG-mfs (MAB_2806-07) in M. abscessus ATCC19977T

To construct the gene deletion mutant, gene deletion vector pSE-arp-katG-∆lprG-ΔMAB_2807 was generated. To this end, a 1526 bp fragment upstream of the target genes was amplified by PCR with the primers MAB_2806_07_5′_for and MAB_2806_07_5′_rev and a 1467 bp fragment downstream of the genes with the primers MAB_2806_07_3′_for and MAB_2806_07_3′_rev (primer sequences are found in Table S1). The primers were appended with Pscl, Xbal or HindIII recognition sites (underlined in Table S1). The PCR fragments were first cloned into pGEM-T easy (Promega), from which they were cut using the corresponding restriction enzymes and successively cloned into the linearized pSE-arp-katG vector to result in the final gene deletion suicide vector pSE-arp-katG-∆lprG-ΔMAB_2807. The vector was transformed into M. abscessus ATCC 19977T as described previously (Rominski et al., 2017). Putative single cross-over colonies were selected on Apr 50 and confirmed by Southern blotting. Double cross-over recombinants were subsequently selected on isoniazid (32 μg ml⁻¹) and confirmed by Southern blotting (Rominski et al., 2017).

Gene cloning into the FX cloning initial vector pINIT

All expression constructs were generated using the FX cloning method (Geertsm and Dutzler, 2011), which was recently expanded with a vector series suitable for myco-bacteria (Arnold et al., 2018). As a first step, the open reading frames of interest were cloned into a linearized vector (pINIT, Cm²), sequenced and then used for sub-cloning by fragment exchange into the required destination vectors (see below). Using primers specified in Table S2, Q5 High-Fidelity DNA polymerase (NEB) and if not stated otherwise genomic DNA (gDNA) as template, the following genes were amplified. (1) The lprG-mfs operons and its single genes of M. smegmatis mc² 155, M. abscessus ATCC19977T (gDNA) and M. tuberculosis H37Rv (bacmid library as PCR template (Brosch et al., 1998)); (2) a truncated version of lprG called lprG_trunc without signal sequence and lipobox as described previously (Drage et al., 2010) from M. tuberculosis for cytoplasmic expression; (3) lmRP from L. lactis; (4) mdfA from E. coli. PCR products were purified via agarose gel, cut with BspQI and cloned into pINIT using chemically competent E. coli MC1061 cells. The ORFs in the resulting pINIT constructs were completely sequenced. Mutations in the lipoprotein or the MFS transporter genes (pINIT) were introduced using the QuikChange site-directed mutagenesis protocol (primers listed in Table S3). Rv1410 loop truncations were generated by PCR amplification of pINIT_rv1410-11 (for complementation) or pINIT-rv1410 (for protein expression and purification) using primers listed in Table S3, resulting in a large blunt-ended PCR product lacking the nucleotides encoding for the loop. The PCR products were treated with DpnI (NEB), ligated with T4 ligase (Thermo Scientific), and transformed into E. coli MC1061. Loop truncations were confirmed by sequencing.

Cloning of transporters for expression in L. lactis

Transporters genes encoding for Rv1410, LmrP and MdfA and the corresponding DtoN mutants were sub-cloned from the pINIT constructs into E. coli vectors pREXC3H and pREXC3GH (Geertsm and Dutzler, 2011) and from there via vector-backbone exchange (VBE) cloning into the L. lactis expression vector pNZ8048, which is under the control of the nisin-inducible promoter (Geertsm and Poolman, 2007). For transport experiments, the transporters were cloned into pNZ8048 via vector pREXC3H, resulting in a fusion of a HRV 3C protease cleavage site and a His₉₀-tag to the C-terminus of the proteins. For protein detection and purification, cloning into pNZ8048 via pREXC3GH was performed, which adds a 3C site, a GFP and His₉₀-tag to the C-terminus of the expressed transporters.

Construction of mycobacterial complementation vectors

For complementation, the integrative mycobacterial vector pFLAG was used (Arnold et al., 2018). It contains a constitutive tet-promoter and adds a 3xFLAG sequence to the C-terminus of the cloned genes for detection by Western blotting. The lprG-mfs operons, its single genes as well as site directed mutants and loop truncation mutants were sub-cloned from pINIT (see above) into pFLAG and propagated in E. coli (Apr 25). The resulting pFLAG expression vectors were integrated into the genome of M. smegmatis ∆lprG-mfs (∆MSMEG_3069-70) by co-electroporation with suicide plasmid pMA_Int harboring the L5 bacteriophage integrase (Arnold et al., 2018). Cells were plated on 7H10 (Apr 25) and colonies were picked and grown in 7H9 (Apr 25). Of note, because pFLAG does not contain the integrase gene, the integration is stable also in the absence of the selection marker apramycin (Springer et al., 2001).

Western blotting to detect loop mutant production in M. smegmatis

 Cultures of M. smegmatis dKO complemented with empty vector pFLAG, and pFLAG harboring Rv1410, Rv1410 D70N, or the three Rv1410 loop truncation mutants were grown into stationary phase. The OD₆₀₀ of the cultures were adjusted to an identical value and harvested. The cell pellets were resuspended in 500 μl 20 mM Tris/HCl pH 6.8, 50% glycerol, 100 mM DTT, 2% SDS (w/v), NaCl, a spatula tip of acid-washed glass beads ≤106 μm (Sigma) was added, and then the cells were lysed with a FastPrep-24 Classic cell lysis machine (MP Biomedicals) for 3 cycles of 60 s at 6 m s⁻¹. The lysate was spun 5 min at 8,000 rpm at 4°C with a F-45-30-11 rotor (Eppendorf) to pellet glass beads and cell debris. 100 μl of supernatant were mixed with 25 μl of 5x SDS loading dye (120 mM Tris/ HCl pH 6.8, 50% glycerol, 100 mM DTT, 2% SDS (w/v), 0.1% bromophenol blue (w/v), of which 10 μl were loaded.
on a 4%–20% SDS-PAGE gel (ExpressPlus™ PAGE gel, GenScript). Separated proteins were transferred to a membrane (Immobilon-PSQ, Merck) by soaking the gel and membrane in transfer buffer (2.9 g glycine, 5.8 g Tris, 0.1% SDS (w/v) and 20% methanol (v/v) in 1 liter), and transferring with a Trans-Blot® SD Semi-Dry Electrophoretic Transfer Cell (BIO-RAD) for 1 h at 12 V. Then the membrane was blocked in PBS buffer containing 5% (w/v) milk powder and 0.05% (v/v) Tween20 for 1 h at RT, incubated in blocking buffer with anti-FLAG antibody (Sigma, F3165) diluted 1:10,000 (v/v) for 1 h at RT while rotating, and washed 3× with washing buffer (PBS containing 0.05% (v/v) Tween20). The membrane was incubated with α-mouse-HRP antibody with washing buffer (PBS containing 0.05% L-arabinose overnight. Cells were harvested in TBS (pH 7.5) containing 10% glycerol. Membranes were collected by ken cells and cell debris were removed by centrifugation for 30 min at 15,000 rpm in a Beckman Coulter ultracentrifuge at 4°C using a Beckman Ti45 rotor. The supernatant was loaded on Ni²⁺-NTA columns, washed with 50 mM imidazole (pH 7.5), 200 mM NaCl, 10% glycerol, 0.03% β-DDM. In order to remove the C-terminally attached GFP/His₆₅-Tag the buffer of the protein preparation was first exchanged (20 mM Tris/HCl pH 7.4, 150 mM NaCl, 0.03% β-DDM) via a PD-10 desalting column. In a second step 3C protease cleavage was performed overnight. In case of LprG_trunc, unbroken cells and cell debris were removed by centrifugation for 30 min at 15,000 rpm in a Beckman Coulter ultracentrifuge at 4°C using a Beckman Ti45 rotor. The supernatant was loaded on Ni²⁺-NTA columns, washed with 50 mM imidazole (pH 7.5), 200 mM NaCl, 10% glycerol and eluted with 200 mM imidazole (pH 7.5), 200 mM NaCl, 10% glycerol. In order to remove the attached C-terminal His₁₀-tag and get rid of the imidazole the affinity-purified protein was dialyzed against 20 mM Tris/HCl pH 7.4, 150 mM NaCl and simultaneously cleaved by 3C protease. Finally, cleaved Rv1410 and LprG_trunc were again loaded on a Ni²⁺-NTA column to remove GFP/His₁₀-tag and the His-tagged 3C protease and were separated by size exclusion chromatography (SEC, Superdex 200 10/300 GL) in 20 mM Tris/HCl pH 7.4, 150 mM NaCl, 0.03% β-DDM. Protein concentrations were determined by measuring A₂₈₀ using NanoDrop 2000c.

**Expression and purification of biotinylated Rv1410_AviC and MdfA_AviC containing a C-terminal Avi-tag**

The purification of Avi-tagged versions of Rv1410 (Rv1410-AviC) and MdfA (MdfA-AviC) was performed the same way as described above for Rv1410 with the exception that an additional biotinylation step was introduced between the Ni²⁺-NTA purification and the buffer exchange using a PD-10 column. The eluted protein from the Ni²⁺-NTA column was concentrated to approximately 250 μl. A final concentration of 5 mM ATP, 10 mM MgOAc, a 2-fold excess of biotin over the Avi-tag concentration, 16 μg ml⁻¹ BirA, 200 mM NaCl and 10% glycerol was added to the protein to reach a final volume of 2.5 ml. The biotinylation reaction was incubated for 16–18 h at 4°C. After this step the standard purification described above was performed.

**Analytical size exclusion chromatography (SEC) to investigate Rv1410-LprG interaction**

The main elution fractions of the monodisperse SEC peaks of the lipoprotein LprG_trunc and Rv1410 were first separated individually by SEC (Superdex 200 10/300 GL) in 20 mM Tris/HCl pH 7.4, 150 mM NaCl, 0.03% β-DDM. Then, the collected fractions of the main peak of each protein were pooled and the two proteins were mixed together. LprG_trunc was added at a fivefold excess over the transporter Rv1410, incubated for 15 min on ice and analyzed by SEC.

**Surface plasmon resonance**

The affinity of the MFS transporter Rv1410 to the lipoprotein LprG_trunc was assessed by SPR analysis on a ProteOn XPR36 protein interaction array system.

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**Construction of E. coli vectors for the expression of Rv1410, LprG_trunc, Rv1410-AviC and MdfA_AviC**

By subcloning genes from pNIT (see above), the following arabinose-inducible pBX-vectors (Geertsma and Dutzler, 2011) for protein expression and purification were constructed: (1) pBXC3H_LprG_trunc encoding for LprG_trunc (no signal sequence and lipobox, as described in (Drage et al., 2010))/3C protease-cleave site/His₁₀-tag, (2) pBXC3GH_Rv1410 encoding for Rv1410/3C/GFP/His₁₀-tag, (3) pBXCA3GH_Rv1410 encoding for Rv1410/AviC/3C/GFP/His₁₀-tag, (4) pBXCA3GH_MdfA encoding for MdfA_AviC. The latter two constructs contain an Avi-tag for enzymatic biotinylation after protease cleavage (Bukowska et al., 2015).

**Expression and purification of M. tuberculosis Rv1410 and LprG_trunc**

_E. coli_ MC1061 precultures harboring pBXC3GH_Rv1410 or pBXC3H_LprG_trunc were directly inoculated from glycerol stocks in LB, Amp 100 and grown at 37°C overnight. The precultures were diluted 1:40 (v/v) into fresh expression medium (TB, Amp 100) and grown for 2 h at 37°C and an additional hour at 25°C before induction of protein expression with 0.002% L-arabinose overnight. Cells were harvested for 20 min at 6,000 rpm in a Fibrelite F10-4X1000 LEX centrifuge rotor at 4°C. Membranes were prepared by disrupting the cells with a Microfluidizer (Microfluidics) at 30 kpsi in 20 mM Tris/HCl pH 8.0, 200 mM NaCl supplemented with 3 mM MgSO₄ and traces of DNaseI on ice. In case of Rv1410, unbroken cells and cell debris were removed by centrifugation for 30 min at 8,000 rpm in a Sorvall SLA-1500 rotor at 4°C. Membranes were collected in a Beckman Coulter ultracentrifuge using a Beckman Ti45 rotor at 38,000 rpm for 1 h at 4°C and resuspended in TBS (pH 7.5) containing 10% glycerol. Membranes were solubilized for 2 h using 1% β-DDM (w/v); insolubilized material was removed by ultracentrifugation. The supernatant was loaded on Ni²⁺-NTA columns, washed with 50 mM imidazole (pH 7.5), 200 mM NaCl, 10% glycerol, 0.03% β-DDM and eluted with 200 mM imidazole (pH 7.5), 200 mM NaCl, 10% glycerol, 0.03% β-DDM. Each protein was further purified by size exclusion chromatography (SEC, Superdex 200 10/300 GL) in 20 mM Tris/HCl pH 7.4, 150 mM NaCl and 0.03% β-DDM. Protein concentrations were determined by measuring A₂₈₀ using NanoDrop 2000c.
Expression tests of GFP-fused transporters in L. lactis and purification of Rv1410-GFP from L. lactis

L. lactis NZ9000 ΔlmrA ΔlmrCD cells (Venter et al., 2008) harboring plasmids encoding Rv1410-GFP, LmrP-GFP or MdfA-GFP were grown in GM17 Cm\(^5\) at 30°C. Expression was induced at an OD\(_{600}\) of 0.4-0.6 with nisin (culture supernatant of L. lactis NZ9700 for 2 h (1:1,000 (v/v))). 4 ml of cells were harvested in 2 ml tubes and resuspended in 350 \(\mu\)l 20 mM Tris/HCl pH 8, 200 mM NaCl. A spatula tip of acid-washed glass beads ≤106 μm (Sigma) was added and the cells were subsequently lysed with a FastPrep-24 Classic cell lysis machine (MP Biomedicals) for 3 cycles of 60 s at a force of 6 m s\(^{-1}\). The lysate was spun 15 min at 14,000 rpm at 4°C with a F-45-30-15 rotor (Eppendorf) to pellet glass beads and cell debris. The supernatant containing membrane vesicles was pelleted by ultracentrifugation and the membrane pellet was dissolved in 10 \(\mu\)l of 5x SDS loading dye (120 mM Tris/HCl pH 6.8, 50% glycerol, 100 mM DTT, 2% SDS (w/v), 0.1% bromophenol blue (w/v)), and separated by a 10% SDS-PAGE gel. The GFP-fusions were detected by in gel fluorescence with an ImageQuant LAS 4000 instrument (GE Healthcare).

For the purification of Rv1410, Rv1410-GFP was expressed in L. lactis NZ9000 ΔlmrA ΔlmrCD as described above using 4 liters of medium. The cell pellet was resuspended in PBS containing 15 mM K-EDTA and protease inhibitor. The cells were then disrupted using a Benchtop Microfluidizer M110PS (Microfluidics) 3 times at 30 kpsi. The cell lysate was centrifuged at 8,000 rpm at 4°C for 10 min (Centrifuge 5804R, rotor: A-4-44 by Eppendorf) and the supernatant was incubated with 20 mM MgCl\(_2\) and DNase I (1:1,000 v/v) on ice for 30 min. Subsequently, ultracentrifugation was performed at 42,000 rpm and 4°C for 1 h using a Beckman Ti45 rotor. The pellet membrane was resuspended in TBS containing 10% glycerol. Solubilization, protein purification via Ni\(^{2+}\)-NTA, 3C protease cleavage and SEC was performed exactly as for Rv1410 purified from E. coli (see above).

Hoechst 33342, ethidium and BCECF-AM transport in L. lactis

L. lactis NZ9000 ΔlmrA ΔlmrCD cells (Venter et al., 2008) harboring plasmids encoding wild-type or mutant Rv1410, LmrP or MdfA were grown in GM17 Cm\(^5\) at 30°C. Expression was induced at an OD\(_{600}\) of 0.4-0.6 with nisin (culture supernatant of L. lactis NZ9700) for 1 h (1:1,000 (v/v)). Cells were washed and resuspended using fluorescence buffer (50 mM K\(_2\)P, pH 7.0, 5 mM MgSO\(_4\)) adjusted to an OD\(_{600}\) of 0.5 and energized by adding 0.5% glucose. Accumulation of 5 μM ethidium, 0.5 μM Hoechst 33342 at 25°C or 0.2 μM BCECF-AM at 30°C was followed for 600 s using a Fluorescence Spectrometer LS-55 (Perkin Elmer). Excitation and emission wavelengths and slit widths were set at 520, 10 and 595 nm, 15 nm for ethidium; 355, 5 and 457 nm and 5 nm for Hoechst 33342; 502, 2.5 and 525 nm, 4 nm for BCECF. Transport assays were conducted twice in two technical replicates. For clarity of discussion, one representative dataset is shown.

Ethidium, BCECF-AM and calcein-AM transport in M. smegmatis

Wild-type M. smegmatis, M. smegmatis dKO (ΔMSMEG_3069-70) and M. smegmatis ΔlmrA (ΔMSMEG_6225) were inoculated into 25 ml of 7H9 medium and incubated at 37°C while shaking at 160 rpm until the cultures reached mid-log growth phase (OD\(_{600}\) = 0.5–1.0). The cultures were harvested by centrifugation, cell pellets were resuspended in 1 ml of 50 mM potassium phosphate (KPi) buffer (pH = 7.0) and kept on ice. Prior to the experiment, cells were diluted using KPi buffer kept at 37°C to a final OD\(_{600}\) = 0.2. 5 ml of cell suspension was transferred to a Quartz SUPRASIL® cuvette (Hellma Analytics, 101-QS) (also warmed to 37°C). The cuvette was promptly placed in a LS 55 Fluorescence Spectrometer (PerkinElmer) that was connected to a MultiTemp III water bath (Pharmacia Biotech) to keep the temperature of the cuvette at 37°C and to a magnetic stirrer (Rank Brothers Ltd, model 300) to ensure the homogeneity of the diluted culture. Before each measurement, either 0.4% w/v glucose or 500 μM carbonyl cyanide m-chlorophenyl hydrazone (CCCP) was added to the cells and incubated for 3 min while stirring. The fluorescent dyes ethidium (5 μM), BCECF-AM (0.2 μM), and calcein-AM (0.2 μM) were added 10 s after the start of fluorescence intensity measurements.

Excitation and emission wavelengths and slit widths were set at 520, 10 and 595 nm, 15 nm for ethidium; at 495, 12 and 520 nm, 15 nm for BCECF; at 502, 2.5 and 525 nm, 4 nm for calcein. In the experiments shown in Fig. 4E, Glc\(_6\) was added, as described above, to energize the cells, but after 8 min 500 μM CCCP was added to the same sample to determine whether the presence of CCCP alters the fluorescence properties of BCECF and calcein. Average fluorescence intensities and 95% confidence intervals (using a Student’s t distribution) were calculated from the data of six biological replicates.

Minimal inhibitory concentration (MIC) measurements in M. abscessus and M. smegmatis

All antibiotics were purchased as powder from Sigma-Aldrich (Buchs, Switzerland) and stock solutions were prepared. Novobiocin and vancomycin were dissolved in water, rifabutin and clofazimine in DMSO, tetracycline in ethanol and ofloxacin in a 0.1 M HCl solution. The MICs for M. abscessus and M. smegmatis were performed in 96-well plates as previously described (Dal Molin et al., 2018). The
MICs were determined on day 5 for *M. smegmatis* and *M. abscessus*.

**Cellular growth assays in complemented M. smegmatis dKO cells**

We developed a high-throughput growth assay in a 96-well format to assess the functional diversity of Rv1410 and LpRG in *M. smegmatis* dKO cells. All tested strains (wild-type, dKO and dKO complemented with pFLAG vectors as described above) were grown to late stationary phase in 7H9 medium in the absence of antibiotics (this was possible because the pFLAG vector is stably integrated in the genome). From these pre-cultures, cells were diluted 1,000-fold into 7H9 medium containing the following drug concentrations: for the experiments assessing the operons from different mycobacteria (Figs 1D and S1): vancomycin (0.3 μg ml⁻¹), rifampicin (0.4 μg ml⁻¹), or novobiocin (0.5 μg ml⁻¹); for the experiments assessing the loop mutants (Fig. 2D–F): vancomycin (0.0125 μg ml⁻¹), rifampicin (0.075 μg ml⁻¹), or novobiocin (0.05 μg ml⁻¹). One milliliter of these cultures were transferred in 4–6 technical replicates into 96-well deep well plates and were incubated at 37°C, shaking at 300 rpm, for approximately 50 h. After removing 50 μl of each culture into 96-well plates at indicated time points, OD₆₀₀ was measured (BioTek). The presented growth curves are representatives of 3 biological replicates and error bars denote the standard deviation of technical replicates.

**Transmission electron microscopy**

Wild-type and dKO cells were grown to stationary phase and washed in buffer. Cells were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.35) for at least 1 h and sequentially treated with 1% OsO₄ for 1 h at 0°C in 0.1 M sodium cacodylate buffer and 2% uranyl acetate in H₂O for 1 h at 4°C. Pellets of bacteria were embedded in 2% of Agar, subsequently dehydrated in an ethanol series and embedded in Epon/Araldite (Sigma-Aldrich, Buchs, Switzerland). Ultrathin (50 nm) sections were contrasted with lead citrate (Reynolds) and imaged by atomic force microscopy (AFM) using a Park Instruments XE-100 instrument with a silicon cantilever (Olympus). The images were analyzed with ImageJ software.

**Fluorescence microscopy**

500 μl of stationary phase cultures of wild-type or dKO *M. smegmatis* carrying pIS220_Wag31-GFP (Santi et al., 2013) were inoculated into 10 ml of 7H9 medium with kanamycin (15 μg ml⁻¹) (Sigma-Aldrich) and SynaptoRed C2 membrane dye (10 μg ml⁻¹) (Sigma-Aldrich) and incubated overnight at 37°C while shaking at 160 rpm until the cultures reached stationary phase (OD₆₀₀ = 3–5). Experiments were conducted blinded until the last data analysis step. The stationary phase cultures were diluted 1:10 in 7H9 medium and sonicated 3 times to reduce cell clumping (10 s 0 × 10% cycle at 80% power, Bandelin Sonopuls HD2200 sonicator). 200 μl cells were transferred on coverslips in 24 well plates and centrifuged for 10 min at 4,000 rpm and room temperature (Heraeus Multifuge 4 KR centrifuge, Thermo Scientific) to attach the mycobacterial cells to the coverslips. Cells were fixed with 4% paraformaldehyde (PFA; Electron Microscopy Sciences) for 10 min at room temperature, washed twice with 2 ml of PBS buffer, mounted on glass-slides using ProLong Diamond Antifade Mountant (Invitrogen, Thermo Fisher Scientific) and incubated at 4°C overnight. The samples were analyzed in 3D on the next day with a Leica SP8 inverse confocal laser scanning microscope (HC PL APO CS2 63x/1.4 OIL; Leica Microsystems) at excitation wavelengths of 488 and 561 nm to detect GFP and SynaptoRed respectively. The pictures were analyzed in a semi-automated workflow employing Fiji image processing package (Schindelin et al., 2012) and a custom script by Dr. Moritz Kirschmann (ZMB, UZH, Switzerland). In this image analysis pipeline, consecutive poles or septa (labeled by fluorescence via Wag31-GFP fusion protein) of individual cell chains were marked manually. Each cell (chain) was saved as a region of interest (ROI) and the program calculated the number of cells per cell chain. Only cells that had been stained by SynaptoRed and harbored at least two Wag31-GFP foci on the cell poles were counted. Cells that were clustered or did not have defined boundaries on the 3D pictures were excluded from the analysis. After having counted the cells per cell chains from two biological replicates, the samples were de-blinded and data analysis was conducted using statistics software R (www.R-project.org). The cell chains, whose length was longer than 2 cells, were binned together and Pearson’s χ² test was performed to evaluate the difference of distribution of cell chain lengths in the two strains.

**Correlated optical fluorescence microscopy (OFM) and atomic force microscopy (AFM)**

For AFM experiments, *M. smegmatis* wild-type and dKO strains were grown in Middlebrook 7H9 liquid medium (Difco) supplemented with 0.5% albumin, 0.2% glucose, 0.085% NaCl, 0.5% glycerol and 0.05% Tween-80. In order to conduct AFM measurements of wild-type and dKO strains in parallel under identical experimental settings, wild-type *M. smegmatis* was differentiated from dKO cells by expressing Wag31-GFP or Wag31-mCherry fusion protein from an ectopic locus, integrated at attB, as described previously (Santi et al., 2013). Polydimethylsiloxane (PDMS)-coated coverslips were prepared by spin-coating a mixture of PDMS at a ratio of 15:1 (elastomer:curing agent) with hexane (Sigma 296090) at a ratio of 1:10 (PDMS:hexane) (Thangawng et al., 2007; Koschwanez et al., 2009). An aliquot of bacteria was grown to mid-exponential phase and 2–5 ml of culture was filtered (5 μm pore size PVDF filter – Millipore) to select for individual cells and concentrated to 50 μl. Wild-type cells expressing Wag31-GFP or Wag31-mCherry were mixed with the non-fluorescent dKO mutant, deposited onto the PDMS-coated coverslip and incubated for ~20 min to increase surface interactions between the hydrophobic...
bacterial surface and the coverslip. Correlated optical fluorescence and AFM images were acquired as described in Eskandarian et al. (2017). Optical fluorescence images were acquired with an electron-multiplying charge-coupled device (EMCCD) iXon Ultra 897 camera (Andor), mounted on an IX81 inverted optical microscope (Olympus), and equipped with an UPLFLN100XO2PH × 100 oil immersion objective (Olympus). Transmitted light illumination was provided by a 12V/100W AHS-LAMP halogen lamp. A U-MGFPHQ fluorescence filter cube for GFP with HQ-lon-coated filters was used together with a bandpass barrier filter, exciter filter BP460-480HQ, a dichroic beam splitter DM485, and barrier filter BA495-540HQ to detect green and red fluorescence originating from Wag31-GFP and Wag31-mCherry fusion protein respectively. The AFM was mounted on top of the inverted microscope and 7H9 medium (~3 ml) was supplied to the sample to immerse the bacterial sample and the AFM cantilever in fluid. Images were acquired with a Dimension Icon scan head (Bruker) using ScanAsyst fluid cantilevers (Bruker) with a nominal spring constant of 0.7 N m⁻¹ in Peak Force QNM mode at a force setpoint ~1 nN and typical scan rates of 0.5 Hz.Indentation on the cell surface was estimated to be ~10 nm with a range of ~5 nm in the Z-axis. Height, peak force error, DMT modulus, and log DMT modulus were recorded for all scanned images in the trace and retrace directions. Images were processed using Gwyddion (Department of Nanometrology, Czech Metrology Institute - http://gwyddion.net). ImageJ was used for extracting bacterial cell profiles in a tabular form. The data were analyzed using a two-sided Wilcoxon rank sum test with continuity correction and confidence level of 95% using statistics software R (www.R-project.org).

**Data availability**

AFM raw data are available at: https://figshare.com/s/088396e3e85b87530455, DOI:10.6084/m9.figshare.7364810.

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**Author contributions**

MH, SR, PS and MAS conceived the project. MAS generated the homology model. MH generated *M. smegmatis* dKO and cloned all genes into the respective complementation and expression vectors for *E. coli* and mycobacteria and MH and SR carried out mutagenesis. MH purified Rv1410, Rv1410 loop truncations and LprG and conducted SPR experiments. SR determined MIC and performed growth experiments in *M. smegmatis*. MDM generated *M. abscessus* dKO and performed MIC determinations in *M. abscessus*. SR carried out dye transport experiments in *M. smegmatis* and *L. lactis*. HAE performed AFM experiments. SR performed and analyzed fluorescence microscopy experiments. MH prepared the samples for TEM. FMA generated *M. smegmatis ΔlfrA*. LMH and AK cloned transporters into *L. lactis*, performed transport experiments in *L. lactis*, determined expression levels of GFP-fusion proteins and purified Rv1410 from *L. lactis*. MH, SR, HAE, MDM, LMH, AK, PS and MAS analyzed and interpreted the data. MH, LMH, GEF, PS and MAS supervised students and postdocs. MH, SR, HAE and MAS wrote the paper. MDM and PS edited the paper.

**References**


**Supporting information**

Additional supporting information may be found online in the Supporting Information section at the end of the article.