Iron is an essential nutrient for almost all living organisms. While ferric iron (Fe$^{3+}$) is nearly insoluble in aqueous solutions under aerobic conditions and neutral pH, the soluble ferrous iron (Fe$^{2+}$) is potentially toxic to the cell due to generation of deleterious oxygen radicals through participation in Fenton chemistry (1). Thus, the amount of soluble iron is tightly regulated within organisms (1). *Mycobacterium tuberculosis*, the causative agent of tuberculosis (TB), infects macrophages and resides within the low-iron environment of the phagosome (2, 3). To overcome iron starvation, *M. tuberculosis* produce high-affinity Fe$^{3+}$-chelating siderophores called mycobactin/carboxymycobactin (the latter a soluble variant of mycobactin; here we refer to both as mycobactins) to enhance iron uptake from the surroundings (4, 5). Nonpathogenic mycobacteria, like *Mycobacterium smegmatis*, produce a second structurally different siderophore, exochelin, in addition to the mycobactins (6, 7). When entering bacterial cells by means of siderophores, Fe$^{3+}$ is reduced to Fe$^{2+}$ for incorporation into mycobacterial iron-containing proteins, including iron storage proteins like bacterioferritin (1). Other known mycobacterial iron uptake mechanisms include heme uptake (8, 9), internalization of holo-transferrin (10), and low-affinity iron uptake through outer membrane porins (11). Guinea pigs and mice infected by *M. tuberculosis* strains deficient in siderophore synthesis or transport across the cell membrane show a significant reduction in bacterial burden compared to animals infected by wild-type (WT) *M. tuberculosis* (12, 13), demonstrating the need for iron in mycobacterial virulence.

TB is responsible for 1.5 million deaths per year, and the growing resistance toward current TB drugs calls for urgent identification of new mycobacterial drug targets (14–16). As part of a high-throughput drug target identification screen, we previously identified mutations within the *eccB3* gene of the mycobacterial *esx-3* gene cluster which conferred resistance to the novel antitubercular pyrazolopyrimidinone compound PZP {3-(4-chlorophenyl)-5-(cyclohexylmethyl)pyrazolo[1,5-α][pyrimidine-7(4H)]-one} (15) (Fig. 1A). For potential lead optimization, it is beneficial to unravel both the target and mechanism of action of a new compound at an early stage of development. In this study, we sought to identify the target and mechanism of PZP.

The *esx-3* gene cluster comprises a set of 11 genes, as indicated in Fig. 1B, that encode the ESX-3 type VII secretion system (17). The specific role of *eccB3* in ESX-3 function is currently unknown. Interestingly, ESX-3 is known to be required for the mycobactin-mediated siderophore iron uptake pathway (18–21). ESX-3 is essential in *M. tuberculosis* but not in *M. smegmatis*, which, in addition to mycobactins, produces exochelin that functions independently of ESX-3. However, ESX-3 is still absolutely required for mycobactin-mediated iron uptake in the latter species (18, 20). This nonessential nature of ESX-3 in *M. smegmatis* has previously proven to be of great advantage when resolving mechanisms re-
assessed by growing 10 ml of culture in 50-ml plastic tubes and measuring the OD_{600} using a standard spectrophotometer.

**Construction of M. smegmatis mutant strains.** An *M. smegmatis* mutant unable to synthesize exochelin (ΔfxbA) was created by replacing the formyl transferase fxbA (MSMEG_0014 [SmegMalist, http://mycobrowser.epfl.ch/smegmalist.html]) with the hygromycin resistance (HygR) gene by recombineering (23). A fragment containing the 200 bp flanking fxbA separated by the linker ΔGATCTGCTTACTCAGG, where the underlined sequences are the restriction sites for BglII and Xhol, was synthesized and cloned into pUC57-simple (Genscript, USA) (see Fig. S1 in the supplemental material). The HygR cassette was amplified from pUV15TetORm (24) by using primers BamHIHygF (AG ATGGAATCTGAGCTCTCCTAGTTGTCCTG) and XhoIHygR (AG ATCTCGAGGCGCGCTTACGTTACTAATTGGT) and cloned into the BglII and Xhol sites of the flanking sequences. The flanked HygR cassette was amplified using primers KO_FxbA_F (TTCGGGATGTCGACC CGA) and KO_FxbA_R (CGTCTGTCGACGACGACG). Allelic exchange with fxbA and the HygR cassette was carried out by transforming the amplified product into an *M. smegmatis* strain expressing the mycobacteriophage recombinases gp60 and gp61 on a nitrile-inducible, counterselectable episomal plasmid. The recombineering plasmid was removed by counterselecting on 10% sucrose. Successful replacement of fxbA with the HygR cassette was confirmed by Southern blot analysis (data not shown).

The *M. smegmatis* point mutants eccB3(R14L), eccB3(N24H), and eccB3(N26) were constructed by single-stranded DNA recombineering (25) using the tetracycline-inducible, gp61-expressing vector pKM402 (kind gift from Kenan Murphy, University of Massachusetts Medical School, MA, USA). Tetracycline-induced *M. smegmatis* containing pKM402 was cotransformed with 1 μg of the oligonucleotide RpsL. (CGGGCGCCGAC CTTCCGAGGCGCGAGTTCGGCCTCCTCGGAGGTGTTTGTA AAACCGGCCTGACACCGC), which introduced a point mutation in the *M. smegmatis* rpsl locus, which confers resistance to streptomycin, and the oligonucleotide R14L. (CTCGTTGAGCCGGGTGGCAGGAC TGAAACGGGCGCGGCGTGTTGGAAGCGGCGGCGGTATCGC GCA), N24H (CGGGCCGGGTACTGACGCGCTGCGGTTTCTGCTG GGACGCGGTGCGCGCGCTGACACCGC), and the oligonucleotide N26 (CTGGGTTAGCCGGGTGGCAGGAC TGAAACGGGCGCGGCGTGTTGGAAGCGGCGGCGGTATCGC GCA), which introduced the specific point mutations in eccB3. RpsL mutants were selected on LB plates containing 20 μg/ml streptomycin, and a mismatch amplification mutation assay (MAMA)-PCR was used to identify the second desired mutation in the eccB3 gene (26). Positive mutants were confirmed by sequencing.

**Chemical materials.** PZP was synthesized at BioDuro Beijing Co., Ltd. Ferrozine [3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-p,p'-disulfonic acid monosodium salt hydrate], ferrous sulfate heptahydrate, and hydroxyamine hydrochloride were purchased from Sigma-Aldrich. PZP was dissolved in dimethyl sulfoxide (DMSO), ferrozine was dissolved in deionized water, and ferrous sulfate heptahydrate was freshly prepared in 0.1 M HCl. Hydroxyamine hydrochloride was dissolved in 2 N HCl (final concentration, 1.4 M). The solutions were kept at 4°C until use.

**Materials and cultures.** The parental bacterial strain used in this study was Mycobacterium smegmatis mc^2155 (22), and mutant strains used in this study are listed in Table 1. To monitor growth of *M. smegmatis* strains in iron-deplete or replete medium, the bacteria were first grown to stationary phase in Middlebrook 7H9 (BD Difco) supplemented with 0.2% glycerol, 0.05% Tween 80, and 10% albumin-dextrose-catalase (ADC) medium (5% [wt/vol] bovine serum albumin fraction V, 2% [wt/vol] dextrose, 145.5 mM NaCl, 0.003% [wt/vol] catalase). The cells were then centrifuged at 3,000 × g for 10 min and washed once in chelated Sauton’s medium prepared as previously described, using Chelsex100 (Bio-Rad) (18). When growth of the WT and eccB3(N26) mutant strains was compared (see Fig. 3C and D, below), cultures were not washed, in order to slightly relax the low-iron growth conditions. The cultures were diluted to an optical density at 600 nm (OD_{600}) of 0.01 in chelated Sauton’s medium with 100 μM 2,2’-bipyridine (Alfa Aesar) for iron-deplete (low-iron) conditions or with 150 μM FeCl_3 for iron-replete (high-iron) conditions. Growth was monitored in microplate honeycomb wells (Oy Growth Curves Ab Ltd.), with 200 μl in each well, using a Bioscreen growth curve reader (Oy Growth Curves Ab Ltd.). The growth was also

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**TABLE 1 Strains used in this study**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Reference</th>
<th>Siderophore(s) produced*</th>
<th>Functional siderophore* iron uptake pathway(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>mc^2155</td>
<td>22</td>
<td>M, E</td>
<td>M, E</td>
</tr>
<tr>
<td>Δesx-3</td>
<td>18</td>
<td>M, E</td>
<td>E</td>
</tr>
<tr>
<td>Δesx-3 fxbA:pS3E</td>
<td>18</td>
<td>M</td>
<td>None</td>
</tr>
<tr>
<td>ΔfxbA</td>
<td>This study</td>
<td>M</td>
<td>M</td>
</tr>
<tr>
<td>eccB3(R14L)</td>
<td>This study</td>
<td>M, E</td>
<td>M*, E</td>
</tr>
<tr>
<td>eccB3(N24H)</td>
<td>This study</td>
<td>M, E</td>
<td>M*, E</td>
</tr>
<tr>
<td>eccB3(N26)</td>
<td>This study</td>
<td>M, E</td>
<td>M*, E</td>
</tr>
</tbody>
</table>

* M, mycobactin/carboxymycobactin; E, exochelin; *, unverified.
**M. smegmatis** sensitivity assay. Determination of the **M. smegmatis** PZP MIC under iron-replete conditions was based on a resazurin microtiter assay (27). Briefly, *M. smegmatis* was grown to stationary phase before dilution to an OD_{600} of 0.001, added in triplicate to 96-well plates, and grown with shaking at 37°C in the presence of increasing concentrations of PZP for 24 h before addition of 0.02% of resazurin (Sigma). The cultures were incubated for another 16 h before the color of the cultures was analyzed. Pink and blue distinguish metabolically active and inactive **M. smegmatis** cultures, respectively. The PZP MIC under iron-deplete conditions was determined by monitoring growth in increasing concentrations of compound in a Bioscreen growth curve reader; the MIC was defined as the lowest concentration of PZP that prevented growth after 60 h growth with shaking at 37°C.

**Iron content analysis.** Whole-cell metal content was measured in **M. smegmatis** WT and eccB3ΔN26 mutant strains grown to the late exponential phase in low- or high-iron Sauton’s medium and in the presence or absence of 25 μM PZP for 24 h. Cells were washed three times in 5 ml of buffer containing 50 mM HEPES (pH 7.5) and 500 mM NaCl (all glassware was previously rinsed with deionized H2O plus 5% HNO3, trace metal grade). The samples were resuspended in 100 μl deionized H2O, and a 10-μl aliquot was used for protein content determination (28). The remaining 90 μl of each sample was acid digested with 200 μl HNO3 (trace metal grade) for 1 h at 80°C and overnight at 20°C. Digestions were concluded after addition of 60 μl of 30% H2O2 and dilution to 2 ml with water. Metal content in digested samples was measured by atomic absorption spectroscopy (Analyst 300; PerkinElmer, Foster City, CA, USA).

**RNA extraction and quantitative RT-PCR.** Total RNA extraction, cDNA synthesis, quantitative reverse transcription-PCR (RT-PCR), and analysis were performed as previously described by Siegrist et al. (18), using primers targeting **M. smegmatis** mtbB, mtbL, irA, and sigA (18).

**ESI-MS studies.** Electrospray-ionization-mass-spectrometry (ESI-MS) studies were carried out in an ESI-MS spectra recorder with an MSD SL Trap mass spectrometer (Agilent Technologies) equipped with an electrospray ionization source. Solutions were analyzed by direct injection. PZP was dissolved in water-methanol (1:1, vol/vol) to a final concentration of 10 μM, and a ferrous sulfate hexahydrate-water solution was added at a molar ratio 1:1. Data were analyzed with the Thermo Scientific Xcalibur software.

**UV-Vis titration of PZP with Fe^{2+}.** UV-Vis (UV-visible) spectroscopic studies were performed in a PerkinElmer Lambda 10 spectrometer at room temperature (25°C). Standard 1-cm quartz cuvettes were used to hold sample and reference solutions. PZP was titrated by sequential addition of increasing amounts of Fe^{2+} solution to the same cuvette. Briefly, to a starting 10 μM PZP solution in DMSO-potassium phosphate buffer (20 mM, pH 7.2), increments of 1 μM ferrous sulfate water solution (freshly made in HCl and hydroxylamine; 1 mM stock solution) were added until a total concentration of 10 μM Fe^{2+} was reached, and then in 2 μM increments until a total concentration of 20 μM Fe^{2+} was reached. All titrations were performed in 20 mM potassium phosphate buffer, pH 7.2. All solutions were scanned from 240 to 800 nm.

**Measurements of the binding constant.** UV-Vis spectroscopic methods were used for determining apparent binding constants for the formation of PZP-Fe^{2+} complex in 20 mM potassium phosphate buffer, pH 7.2. First, the molar stoichiometry of the complexes formed by titration of 10 μM PZP with increasing amounts of Fe^{2+} was determined by monitoring the change of absorbance at 400 nm (the wavelength where the complexes absorb while the ligand does not). The method used was previously described for the determination of the apparent binding constant of the quercetin-Fe^{2+} complex (29). The spectroscopic measurements registered at 400 nm were used for the calculations.

**Competition for Fe^{2+} between PZP and ferrozine.** A solution of 3:1 ferrozine-Fe^{2+} (60 μM:20 μM) mixture in 1 ml of 20 mM potassium phosphate buffer (pH 7.2) was prepared in a 1-cm-path-length quartz cuvette. Sixty microliters of PZP (60 μM) was added to this solution after 30 min. The absorbance of the complex was measured every 30 min for 1.5 h.

**RESULTS**

**M. smegmatis** is not susceptible to PZP under iron-replete conditions. Based on whole-genome sequencing of three **M. tuberculosis** mutants conferring resistance to PZP, we previously identified single mutations, all mapping to rv0283 (eccB3), a gene encoding a putative transmembrane protein of unknown function within the esx-3 gene cluster (15). Two of the clones encoded nonsynonymous EccB3 substitutions, R14L and N24H, while the third contained a frame-preserving 3-bp deletion that eliminated N26 from the primary EccB3 sequence (15). These residues are conserved among mycobacteria (Fig. 1C), and all three localize to the N-terminal cytosolic tail of EccB3, as predicted by the TMHMM software (30, 31) (Fig. 1D). **M. tuberculosis** is dependent on a functional ESX-3 system for growth (18, 20, 32–34), and all **M. tuberculosis** mutations conferring resistance to PZP were mapped to the esx-3 gene cluster (15). These findings prompted us to investigate whether PZP could interfere with the activity of ESX-3 or, alternatively, if ESX-3 could be involved in PZP uptake. ESX-3 is essential in **M. tuberculosis**, but not in **M. smegmatis** (18, 20). Thus, if EccB3 is the cellular target of PZP, **M. smegmatis** should not be susceptible to the compound. Indeed, a resazurin microtiter assay showed that growth was not affected by PZP when included in the medium up to a 1 mM concentration (results not shown), and we concluded that **M. smegmatis** is not affected by PZP under these conditions.

**M. smegmatis** is susceptible to PZP under iron-depleted conditions. An **M. smegmatis** strain disrupted in a gene encoding a formyl transferase (*fxbA*) required for exochelin biosynthesis shows slight growth restriction compared to the WT under iron-limited conditions (18). When the esx-3 cluster, which is required for mycobactin-mediated iron uptake, is deleted in addition to the disrupted *fxbA* gene (Δesx-3 *fxbAːpES* mutant), **M. smegmatis** does not grow in low iron, since both the mycobactin and exochelin pathways are nonfunctional in this strain. However, an esx-3 knockout with an additional mutation that abolishes mycobactin synthesis is disrupted in the mycobactin pathway only and thus grows under low-iron conditions due to production of exochelin (18). These findings showed that ESX-3 is required for iron acquisition through the mycobactin pathway but not the exochelin pathway (18). If PZP targets mycobactin-mediated iron uptake via ESX-3/EccB3, we reasoned that an **M. smegmatis** mutant unable to produce exochelin (Δ*fxbA*) would behave similarly to the Δesx-3 *fxbAːpES* double mutant and not grow in the presence of PZP under low-iron conditions. To test this hypothesis, we grew the Δ*fxbA* mutant in iron-deplete medium (100 μM 2,2′-bipyridine) in the presence of PZP. Surprisingly, PZP inhibited the growth of all strains under these conditions, including the WT, which we hypothesized would grow due to the production of exochelin (Fig. 2A, left). All strains were rescued by iron repletion (150 μM FeCl3) (Fig. 2A, right). Thus, the action of PZP in **M. smegmatis** is dependent on low environmental iron.

**PZP does not target the ESX-3 secretion system.** Our results showed that production of exochelin could not rescue the cells from PZP, suggesting a mechanism of action independent of ESX-3. To further investigate whether the compound impairs mycobacterial growth independently of ESX-3, we analyzed the response of an Δesx-3 whole-locus deletion mutant to PZP. The
growth of the Δesx-3 mutant was slightly impaired at low iron levels but not at iron-replete levels compared to the WT strain (Fig. 2B). However, the growth of both Δesx-3 and WT strains was completely inhibited in the presence of PZP and rescued by the presence of iron (Fig. 2B). Furthermore, in low iron, the MICs for both WT and the Δesx-3 mutant were between 2.5 and 5 μM (Fig. 2C). Since the Δesx-3 mutant and WT strains showed similar sensitivities to PZP, we concluded that the compound does not target EccB3 or any other component of the ESX-3 system. In addition, ESX-3 does not appear responsible for uptake of the compound.

**PZP leads to iron accumulation in M. smegmatis.** Our observations suggested that PZP interferes with iron homeostasis. We hypothesized that the compound might interfere with iron uptake and expected that PZP treatment would result in decreased iron accumulation. To test this, we analyzed the whole-cell iron content by atomic absorption spectroscopy. *M. smegmatis* WT cells were grown in iron-replete or -deplete medium compared to those grown under iron-deplete conditions (Fig. 3A, blue bars, minus PZP). Surprisingly, however, iron accumulation increased in the presence of PZP under iron-rich conditions (Fig. 3A, blue bars, replete). No difference in iron content was observed in cells grown in low-iron medium in the presence or absence of the compound (Fig. 3A, blue bars, deplete). Since iron accumulated in *M. smegmatis* WT under iron-rich conditions, we compared the iron content in mycobacteria containing the eccB3 M. tuberculosis resistance mutations. The R14, N24, and N26 residues are conserved among mycobacteria (Fig. 1C), suggesting that a mutation in these residues in *M. smegmatis* would have the same effect as the *M. tuberculosis* mutations.
Hence, we constructed an \( M. \text{smegmatis eccB3} \) (\( \text{H9004 N26} \)) mutant strain and analyzed the iron levels. Increased iron accumulation was observed in the \( \text{eccB3} \) (\( \text{H9004 N26} \)) mutant in the presence of the compound under both iron-replete and -deplete conditions (Fig. 3A, white bars). We speculate that the mutations in \( \text{eccB3} \) increase the iron acquisition-related activity of ESX-3, leading to increased iron influx. However, this mutant strain did not show increased resistance to PZP (Fig. 3C and D). Similarly, increased resistance to the compound was not observed in \( M. \text{smegmatis eccB3 (R14L)} \) or \( \text{eccB3 (N24H)} \) strains (results not shown). Given the rather small shift in the MICs for these mutations that have been reported elsewhere for \( M. \text{tuberculosis} \) (only a 2.5-fold shift under iron-rich conditions) (15), it is likely that there is substantial interplay between the environmental iron concentration and resistance, and it remains possible that one could detect resistance of \( M. \text{smegmatis eccB3} \) mutants under optimal iron conditions. Attempts to recreate PZP-resistant \( \text{eccB3} \) mutants in \( M. \text{tuberculosis} \) were not successful, perhaps due to the selection of resistant recombinants under suboptimal iron conditions (15). Nevertheless, these results certainly demonstrated that iron accumulates in mycobacterial cells in the presence of PZP when sufficient iron is available in the medium.

\text{PZP-treated M. smegmatis upregulates iron acquisition genes.} Mycobacteria sense iron availability and upregulate acquisition systems in response to iron starvation (35). The increased iron accumulation seen in the presence of PZP suggested that cells sense iron starvation and should, therefore, show upregulated iron acquisition gene expression. Several genes are known to be iron regulated, including \( \text{mbtB} \) and \( \text{mbtL} \), both of which are involved in mycobactin synthesis but are located in separate gene clusters (36). Another iron-responsive gene, \( \text{irtA} \), encodes a mycobactin transporter suggested to couple siderophore import and iron assimilation together with IrtB (12, 37). Thus, we measured the expression levels of \( \text{mbtB} \), \( \text{mbtL} \), and \( \text{irtA} \) in \( M. \text{smegmatis} \) cells in the presence and absence of PZP. Under iron-deplete conditions, higher expression of iron uptake genes was observed than under iron-replete conditions (Fig. 3B). All three iron-responsive genes were, however, further upregulated in the presence of the compound under both high- and low-iron conditions (Fig. 3B). Thus, although cells accumulate increased amounts of iron in the presence of PZP, they simultaneously sense iron starvation.

\text{PZP chelates Fe}^{2+}. We found that cells treated with PZP accumulate iron but at the same time sense iron starvation. Aiming to resolve these seemingly contradictory observations, we noted that PZP has a pyrazolo-[1,5-a]pyrimidinone core that potentially possesses Fe\(^{2+}\)-chelating properties. Thus, we investigated whether the compound could complex with iron by using ESI-MS with direct injection of solutions of PZP and Fe\(^{2+}\). Figure 4 shows the ESI-MS spectra of PZP (Fig. 4A) and PZP plus 1 mole equivalent of Fe\(^{2+}\) (Fig. 4B). In addition to the free ligand PZP (m/z 364.17,
[PZP + Na]⁺, two Fe²⁺-PZP complexes were detected at m/z 551.92 and m/z 1078.00 (Fig. 4B) when we mixed PZP and Fe²⁺, with all displaying the typical iron isotopic pattern (Fig. 4B, insets). In both cases, species corresponding to the formation of the complex between PZP and Fe²⁺ showed a stoichiometry of 3:1 ([Fe²⁺(PZP)₂(PZP – H)Na]²⁺; m/z 1078.00 [Fe²⁺(PZP)₂(PZP – H)]⁺). All the other observed peaks did not fit with the classic iron isotopic pattern.

We further analyzed the ability of PZP to form complexes with iron by using UV-Vis spectroscopy. The compound was titrated with increasing amounts of Fe²⁺ (Fig. 5A). The intensities of the two bands at 275 and 310 nm, which were observed for PZP alone, decreased after addition of Fe²⁺, whereas the absorbance in the region above 370 nm increased in intensity. Two isosbestic points were observed at 300 and 340 nm. These observations fit with the formation of a PZP-Fe²⁺ complex, since they can be explained by the delocalization of π electrons of the conjugated PZP ring system over the metal core upon PZP-iron binding. The titration curve confirmed the 3:1 stoichiometry (Fig. 5A, inset) determined in the ESI-MS spectrometry experiment. Figure 5B shows a possible chemical structure of the [Fe(PZP)₃]⁺ complex. The conditional binding constant of the compound with Fe²⁺ was estimated at 400 nm by applying a method established for measuring the binding constants of complexes between flavonoids and iron (29). Since a strong binding constant was determined for the [Fe(PZP)₃]⁺ complex (K, 3.65 × 10¹⁵ mM) (38), we performed competition experiments with ferrozine, a well-known Fe²⁺ chelator (K, 3.65 × 10¹⁵ mM) (39). Figure 5C shows the changes in absorbance of the Fe²⁺-ferrozine complex after addition of PZP. The peak at 562 nm (Fe²⁺-ferrozine) rose slightly in the first 30 min, possibly due to the formation of mixed ferrozine-Fe²⁺-PZP complexes; similar complexes have been reported for amino acids and ferrozine (39). Absorbance increases in the region between 390 and 475 nm indicate the formation of the [Fe(PZP)₃]⁺ complex. These findings indicate that the formation of this complex occurs while there is a dissociation of the ferrozine-Fe²⁺ complex. Altogether, these data confirm that PZP can strongly bind Fe²⁺ under physiological conditions.
DISCUSSION

Nearly all mycobacteria produce the siderophores mycobactins (4, 5). The saprophytic nonpathogenic mycobacterium M. smegmatis produces the siderophore exochelin in addition to the mycobactins (6). Mycobactin-mediated iron uptake is dependent on the type VII mycobacterial secretion system ESX-3, while exochelin-mediated iron uptake is ESX-3 independent (18). The M. tuberculosis esx-3 gene cluster is essential for in vitro growth (18, 20, 32–34), and M. tuberculosis is sensitive to PZP under standard iron conditions (15). In contrast, M. smegmatis ESX-3 is not essential for growth and M. smegmatis is protected against PZP in normal iron medium (18). Interestingly, although the species’ requirement for ESX-3 coincides with their susceptibilities to PZP, we show that ESX-3 is in fact not the cellular target of this compound. Instead, we found that PZP apparently enters mycobacterial cells and chelates Fe^{2+} and starves the bacteria for intracellular iron.

In the environment, iron is available as Fe^{3+}. Thus, siderophores have been optimized to complex this ion. Once taken up by the cells though, Fe^{3+} is reduced to Fe^{2+}. The affinity of siderophores for Fe^{3+} is extraordinarily high, ranging from binding constant (K) of $10^{30}$ to $10^{52}$ M (40), far higher than PZP ($K = 2 \times 10^{11}$ mM). Thus, it is unlikely that this compound could effectively compete for extracellular iron. Instead, we postulate that the compound enters cells and chelates intracellular Fe^{2+}. The affinities of mycobacterial iron storage molecules like ferritin and bacterioferritin for Fe^{2+} are, to our knowledge, unknown. However, the Fe^{2+} affinity of the Escherichia coli bacterioferritin is estimated to be lower than $K = 1.1 \times 10^7$ M, based on competition experiments with Zn^{2+} (41, 42). It is therefore likely that PZP binds Fe^{2+} intracellularly and deprives the cells of available iron storage and that the bacteria respond by upregulating the expression of iron acquisition systems. Given adequate access to extracellular iron, WT M. smegmatis can overcome the effects of starvation by increasing iron uptake. Similarly, mutations in eccB3 increase intracellular iron levels further. The role of ESX-3 in mycobactin-mediated iron uptake is currently unknown. We speculate that ESX-3
secretes effector molecules important for utilization of mycobactin-bound iron. EccB3 could play a role in regulating secretion of such effectors, and mutations in the cytosolic tail of EccB3 might alter effector secretion. Regardless, depriving cells of iron results in inhibition of cell growth by PZP.

Wells et al. recently identified MmpS4/MmpL4 and MmpS5/MmpL5 as siderophore export systems in *M. tuberculosis* (13). An *M. tuberculosis* ΔmmpS4 ΔmmpS5 double mutant did not grow under iron-deplete conditions and was not fully rescued by adding hemoglobin as an iron source. However, the growth of *M. tuberculosis* ΔmmpS4 ΔmmpS5 with an additional mutation disrupting mycobactin synthesis was completely restored by hemoglobin, suggesting that the lack of mycobactin export leads to accumulation of the siderophore, potentially causing deleterious intracellular iron chelation (13). PZP could plausibly block exporters of mycobactins, perhaps by mimicking iron-chelating siderophores, and mutations in *eccB3* could make possible export of mycobactins through ESX-3. We have previously shown a small decrease in the pool of mycobactins and carboxymycobactins in the *M. smegmatis* Δess-3 supernatant filtrate (18). However, this moderate change in exported mycobactins does not likely account for the severe Δess-3-mediated growth impairment seen with low iron (18), making a model based on blocking siderophore export via ESX-3 improbable as a mechanism of PZP.

A streptomycin sensitivity assay recently suggested that intracellular iron decreases upon repression of ess-3 expression (19). Conversely, Du Toit Loots et al. used metabolomic approaches to speculate that an *M. smegmatis* ess-3 knockout mutant accumulates iron during iron-replete growth (43). It is likely that the resistant *eccB3* point mutations influence the iron uptake/assimilation-related properties of ESX-3, leading to increased influx of iron. However, this alone cannot explain the elevated iron accumulation by the *eccB3* mutant on low iron, as we did not see accumulation in the absence of PZP.

Although the *eccB3*ΔN26 mutant accumulates iron also when grown under low-iron conditions, none of the three *M. smegmatis* eccB3 mutants conferred resistance to PZP. However, we postulate that *eccB* mutations enhance ESX-3-mediated iron uptake or assimilation, and it is likely that the subtle resistance effect (2.5-fold) seen in *M. tuberculosis* was not detectable in *M. smegmatis* simply because the cells were grown under iron-deficient conditions and lacked sufficient metal in the medium to rescue the cells from the compound.

A previous study on structure-activity relationships of compounds structurally related to PZP identified molecules with an inhibitory effect on *M. tuberculosis* 1-deoxy-d-xylulose 5-phosphate (DXS), an enzyme that combines the mechanisms of pyrurate decarboxylase and transketolase (44). Interestingly, DXS activity is absolutely dependent on divalent cations (45). It would be interesting to investigate PZP’s activity on DXS as well as the chelating properties of these structurally related compounds.

The potential of iron chelators in TB therapeutics has been investigated previously. Chelators are able to restrict growth of *Mycobacterium avium* (46–48), while synthetic mycobacin analogues selectively inhibit *M. tuberculosis* growth (49). Additionally, iron chelation has proved to inhibit the cytotoxic effect of mycolactone, a macrolide toxin produced by *Mycobacterium ulcerans*, the causative agent of the necrotizing skin disease Buruli ulcer (50). A wide spectrum of chelators have furthermore been designed and synthesized in the search for the treatment of diseases ranging from acute myocardial infarction to neurodegenerative diseases and cancer (51–53). Together, these findings demonstrate a wide range of potential medical applications for iron chelators that is not restricted to antimycobacterial therapy.

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