Role of Porins in Iron Uptake by *Mycobacterium smegmatis*[▽]

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Many bacteria rely on siderophores to extract iron from the environment. However, acquisition of iron-loaded siderophores is dependent on high-affinity uptake systems that are not produced under high-iron conditions. The fact that bacteria are able to maintain iron homeostasis in the absence of siderophores indicates that alternative iron acquisition systems exist. It has been speculated that such low-affinity uptake of iron in Gram-negative bacteria includes diffusion of iron ions or chelates across the outer membrane through porins. The outer membrane of the saprophytic *Mycobacterium smegmatis* contains the Msp family of porins, which enable the diffusion of small and hydrophilic solutes, such as monosaccharides, amino acids, and phosphate. However, it is unknown how cations cross the outer membrane of mycobacteria. Here, we show that the Msp porins of *M. smegmatis* are involved in the acquisition of soluble iron under high-iron conditions. Uptake of ferric ions by a triple porin mutant was reduced compared to wild-type (wt) *M. smegmatis*. An intracellular iron reporter indicated that derepression of iron-responsive genes occurs at higher iron concentrations in the porin mutant. This was consistent with the finding that the porin mutant produced more siderophores under low-iron conditions than wt *M. smegmatis*. In contrast, uptake of the exochelin MS, the main siderophore of *M. smegmatis*, was not affected by the lack of porins, indicating that a specific outer membrane siderophore receptor exists. These results provide, to our knowledge, the first experimental evidence that general porins are indeed the outer membrane conduit of low-affinity iron acquisition systems in bacteria.

Due to its versatility as a redox cofactor, iron is an essential nutrient for the vast majority of organisms on earth (4, 41). It is the second most abundant metal in the earth's crust but is biologically unavailable due to the insolubility of the ferric ion in neutral and alkaline soils (4). Consequently, many bacteria produce low-molecular-weight, high-affinity chelators, termed siderophores, to extract iron from the environment (2). However, acquisition of iron-loaded siderophores relies on high-affinity transport mechanisms that are not expressed under high-iron conditions typically encountered in rich media (>10 μ M in *Escherichia coli*) (1, 40). Uptake of iron under these conditions is facilitated by low-affinity transport systems. The components of these systems are unknown, but they are speculated to involve diffusion of iron chelates through porins or nonspecific cotransport mechanisms (1, 13, 38).

Mycobacteria and corynebacteria are genera of the *Actinobacteria* class that are distinguished from other Gram-positive bacteria because they possess an outer membrane (14). The mycobacterial outer membrane presents a permeability barrier to the acquisition of nutrients (34) and the uptake of antibiotics (5, 16, 35) and offers pathogenic mycobacteria protection from antimicrobial peptides encountered during phagocytosis by macrophages (25). The soil-dwelling *Mycobacterium smegmatis* overcomes this permeability barrier by expressing water-filled porins in its outer membrane (36). MspA was discovered as the major porin (23) and was later found to be the most abundant protein of *M. smegmatis* (24). Deletion

of *mspA* reduced outer membrane permeability toward glucose (32), phosphate (42), and amino acids (34), indicating that MspA represents the major diffusion pathway in *M. smegmatis*. The loss of several Msp porins reduced the growth rate of *M. smegmatis* (34, 42), indicating that the influx of hydrophilic nutrients through porins is required for normal growth. The lack of specificity of these general porins implicates their involvement in the uptake of small inorganic ions; however, with the exception of phosphates (42), this has not been demonstrated. In particular, it is unknown whether binding of cations to the highly negatively charged constriction zone of the Msp porins of *M. smegmatis* (9) would impede rapid diffusion of further cations through the Msp channels.

Siderophore synthesis by *M. smegmatis* is repressed at high iron concentrations (21, 27). The maintenance of iron homeostasis in the absence of siderophores indicates that alternative transport processes must occur under these conditions. A low-affinity iron uptake system responsible for the uptake of iron from ferric citrate in *M. smegmatis* has been reported; however, the components of this system are unknown (18).

In this study, we examined the uptake of iron from ferric citrate in *M. smegmatis* by using a porin-deficient strain. The porin-deficient strain displays signs of iron deficiency even under high-iron conditions due to decreased uptake of iron from ferric citrate. As a consequence, this mutant upregulates iron-responsive genes, ultimately leading to the production of more siderophores than that by wild-type (wt) cells under low-iron conditions. These results revealed that porins are part of the low-affinity iron uptake system of *M. smegmatis*.

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MATERIALS AND METHODS

Chemicals and enzymes. Hygromycin B was purchased from Calbiochem. All other chemicals were purchased from Merck, Roth, or Sigma at the highest

TABLE 1. Strains used in this work^a

Strain	Parent strain; relevant genotype or phenotype	Source or reference
<i>E. coli</i> DH5α	<i>recA1 end gyrA96 thi; relA1 hsdR17(r_K⁻ m_K⁺) supE44 φ80ΔlacZΔM15 Δlac(ZYA-argF)UE169</i>	1
<i>M. smegmatis</i> SMR5	<i>M. smegmatis</i> mc ² 155; Sm ^r	3
ML16	<i>M. smegmatis</i> SMR5; Δ <i>mspA</i> :: <i>FRT</i> Δ <i>mspC</i> :: <i>FRT</i> Δ <i>mspD</i> :: <i>FRT</i> <i>attB</i> :: <i>loxP FRT</i>	5
ML56	<i>M. smegmatis</i> SMR5; Δ <i>mspA</i> :: <i>FRT</i> Δ <i>mspC</i> :: <i>FRT</i> Δ <i>mspD</i> :: <i>FRT</i> <i>attB</i> _{LS} :: <i>loxP</i> -p _{imyc} - <i>mspA-loxP</i>	This study

^a The annotations Sm^r and Hyg^r indicate that the strain is resistant to the antibiotic streptomycin or hygromycin, respectively. It should be noted that all strains were derivatives of SMR5 and are therefore resistant to streptomycin (this is not indicated in the table except for SMR5).

purity available. Enzymes for DNA restriction and modification were from New England Biolabs, Invitrogen, and Boehringer. Oligonucleotides were obtained from Integrated DNA Technologies. ⁵⁵FeCl₃ was purchased from Perkin-Elmer, and [1,5-¹⁴C]citric acid was purchased from GE Healthcare.

Bacterial strains, media, and growth conditions. All bacterial strains used in this study are listed in Table 1. Unless otherwise noted all mycobacterial strains were grown at 37°C in Middlebrook 7H9 liquid medium (Difco Laboratories) supplemented with 0.2% glycerol, 0.05% Tyloxapol, or on Middlebrook 7H10 agar (Difco Laboratories) supplemented with 0.2% glycerol. *Escherichia coli* DH5α was used for all cloning experiments and was routinely grown in LB medium at 37°C. Hygromycin was used when required at the following concentrations: 200 μg ml⁻¹ for *E. coli* and 50 μg ml⁻¹ for mycobacteria. For iron-dependent growth experiments a minimal medium consisting of 500 μM MgCl₂ · 6H₂O, 7 μM CaCl₂ · 2H₂O, 1 μM NaMoO₄ · 2H₂O, 2 μM CoCl₂ · 6H₂O, 6 μM MnCl₂ · 4H₂O, 7 μM ZnSO₄ · 7H₂O, 1 μM CuSO₄ · 5H₂O, 15 mM (NH₄)₂SO₄, 12 mM KH₂PO₄ (pH 6.8), 1% (vol/vol) glycerol was supplemented with ammonium ferric citrate as an iron source as indicated in the text and figures. Ferric citrate was made using a Fe³⁺/citrate molar ratio of 1:200. To minimize trace iron contamination, bottles containing medium stock solutions were washed in 6 M HCl and solutions were prepared with highly purified water (Barnstead Nanopure Diamond; 18.2 MΩ-cm). All low-iron growth experiments (see Fig. 1A and 4 below) were carried out in polystyrene culture tubes (Becton Dickinson) to avoid iron leaching from glass. Low-iron growth was operationally defined as iron-dependent growth with high IdeR activity and high chrome azurol S (CAS) activity compared to cultures grown at higher iron concentrations.

Construction and validation of an intracellular iron reporter for mycobacteria. An iron-regulated reporter was constructed similar to that described previously (8). The *M. smegmatis* gene *fxbA* and its corresponding *cis*-regulatory elements were transcriptionally fused to the codon-optimized *gfp_{m2+}* gene (33) by overlap extension PCR. A region encompassing the first 23 codons of *fxbA* down to -187 from the transcription start site of *fxbA* was amplified from the *M. smegmatis* genome by PCR using primers 1534 (5'-CAGCTCTAGATCGTTG ACCAGGACACG-3') and 1559 (5'-TCCTCGCCCTTCGAGATATCCATG ACCACGCGCACAGG-3') (the introduced restriction sites are underlined). *gfp_{m2+}* was amplified with a 5' *fxbA* overhang separated by an EcoRV restriction site by using the primers 1558 (5'-GTGCGCGTGGTCATGGATATCTCGAA GGGCGAGGAGCTG-3') and 1535 (5'-CCTGCGAAGCTTCTACTTGTACA GCTCGTCCATG-3') and pMN437 as a template (Table 2). The products of these two reactions were amplified using primers 1534 and 1535 to create the fusion construct, which was then cloned into pMS2 via the XbaI and HindIII sites to create pML1801.

M. smegmatis SMR5 (wt), ML16 (Δ*mspA* Δ*mspC* Δ*mspD*), and ML56 (Δ*mspA* Δ*mspC* Δ*mspD* *attB*_{LS}::p_{imyc}-*mspA*) were grown on 7H10 plates for 3 days. A single inoculum was prepared from plates by resuspending bacteria into a min-

imal medium without any added iron. This resuspension was used to inoculate 4 ml minimal medium containing 0.25, 0.5, 1.0, 5, 10, or 20 μM ferric citrate in 14-ml polystyrene tubes to an optical density at 600 nm (OD₆₀₀) of 0.01. Precultures were incubated for 30 h at 37°C, washed, and resuspended in minimal medium containing identical iron concentrations. When the cultures were in the mid-logarithmic growth phase (OD₆₀₀, 0.8 to 2.0), 650-μl aliquots were harvested by centrifugation. The cells were washed twice in Tris-buffered saline with Tween buffer, and green fluorescent protein (GFP) fluorescence intensities were determined in 96-well plates by using a Biotek Synergy HT plate reader with an excitation at 485 nm and a 528-nm ± 20-nm emission filter. Fluorescence intensities were normalized to the optical density of the same samples.

Photometric determination of secreted siderophores. The CAS assay was modified based on a previously published protocol (29). A working solution containing 100 mM potassium acetate (pH 5.5), 600 μM hexadecyltrimethylammonium bromide, 150 μM chrome azurol S, and 15 μM FeCl₃ · 6H₂O was prepared. For activity determinations media and culture filtrate were mixed in a 1:1 ratio with the working solution. Solutions were incubated for 30 to 45 min at 37°C, and absorbance was measured at 630 nm. The difference in the absorbance at 630 nm between medium and culture filtrates was normalized to the optical density of the cultures. All samples were analyzed in triplicate. The signal from the CAS assay is highest when cultures are grown with iron concentrations less than 5 μM for more than 16 generations, which presumably exhausts intracellular iron stores and promotes the production of siderophores. We chose 0.25 μM ferric citrate for our experiment (see Fig. 4B) to promote siderophore-dependent growth and limit the amount of interference from the 200-fold excess of citrate.

Uptake assays. Uptake experiments were done as published previously (42) with the following modifications. *M. smegmatis* SMR5 (wt), ML16 (Δ*mspA* Δ*mspC* Δ*mspD*), and ML56 (Δ*mspA* Δ*mspC* Δ*mspD* *attB*_{LS}::p_{imyc}-*mspA*) were grown on 7H10 plates for 3 days. Cells were scraped into 4 ml 7H9 medium and were filtered through a 5-μm filter to remove cell clumps. These cells were used to inoculate 4 ml of 7H9 Middlebrook medium. The preculture was grown overnight and used to inoculate 100 ml of 7H9 Middlebrook medium. This culture was grown to mid-logarithmic phase (OD₆₀₀, 0.6 to 1.0) and harvested by centrifugation (3,250 × g for 10 min at 4°C). Cells were washed twice in a buffer consisting of 50 mM Tris, pH 6.9 [⁵⁵Fe(Cit)₂⁵⁻ and Fe(¹⁴Cit)₂⁵⁻ uptake], or 50 mM KH₂PO₄, pH 7.0 (⁵⁵Fe-exochelin MS [ExoMS] uptake), supplemented with 15 mM KCl, 10 mM (NH₄)₂SO₄, 1 mM MgSO₄, or 200 μM sodium citrate [⁵⁵Fe(Cit)₂⁵⁻ and Fe(¹⁴Cit)₂⁵⁻ uptake only] and resuspended in the same buffer to an OD₆₀₀ of approximately 2.5 on ice. For uptake experiments 2 ml of cell suspension was equilibrated at 37°C for 5 min and shaken at ≈400 rpm. Radio-labeled substrates were added to cells as indicated in the figure legends and the text. Samples of 200 μl were removed at 1, 2, 4, 8, and 16 min and added to 400 μl of a killing buffer consisting of 100 mM LiCl, 50 mM EDTA in 4% formaldehyde in Spin-X filter microcentrifuge tubes. Cells were immediately centrifuged and washed twice in killing buffer. The radioactivity of the cells was quantified by liquid scintillation counting (Beckman Coulter LS6500). All experiments were performed in triplicate. Radioactive counts were normalized to the dry weights of cells by determining the dry mass of 4 ml of the washed cell suspensions.

Purification of ferri-exochelin MS. ExoMS was purified from *M. smegmatis* as published previously (30). Briefly, 2 liters of a minimal medium supplemented with 0.25 μM ammonium ferric citrate and 50 μM sodium citrate was inoculated from an identical overnight preculture of the porin mutant *M. smegmatis* ML16 (34). The culture filtrate was isolated by filtration through a 0.2-μm filter after 72 h of growth and saturated with FeCl₃ until a precipitate formed. The iron-saturated culture filtrate was incubated at room temperature for 1 h to ensure formation of Fe-ExoMS. The mixture was filtered to remove insoluble iron and passed through cation exchange resin (AG50W X-8, NH₄⁺ form; 80 cm by 1.6 cm) by high-performance liquid chromatography at 2.5 ml min⁻¹, washed with

TABLE 2. Plasmids used in this work^a

Plasmid	Parent vector; relevant genotype and properties	Source or reference
pMS2	ColE1 origin, PAL5000 origin; Hyg ^r , 5,229 bp	2
pMN437	ColE1 origin, PAL5000 origin; Hyg ^r , p _{smyc} <i>gfp_{m2+}</i> , 6,236 bp	4
pML1801	ColE1 origin, PAL5000 origin; Hyg ^r , <i>fxbA23_ gfp_{m2+}</i> , 6,164 bp	This study

^a Up- and downstream homologous sequences of genes are shown as superscripts and subscripts, respectively. "Origin" refers to the origin of replication. Hyg^r indicates that the plasmid confers resistance to hygromycin.

FIG. 1. Loss of porins in *M. smegmatis* results in increased expression of iron-repressed genes. (A) Validation of a cytoplasmic iron reporter. *M. smegmatis* harboring an *fxbA-gfp* fusion (pML1801) was grown to mid-logarithmic phase in minimal medium containing different ferric citrate concentrations. (B) The porin mutant showed increased IdeR activity. *M. smegmatis* SMR5 (wt), ML16 (ΔmspA ΔmspC ΔmspD), and ML56 (ΔmspA ΔmspC ΔmspD *attB*_{L5}::*p*_{myc}-*mspA*) harboring the iron reporter were grown in a high-iron minimal medium containing 150 μM ammonium ferric citrate. In both panels, fluorescence intensities of *M. smegmatis* cells were determined in triplicate and normalized to the optical densities of the samples. Standard deviations are shown as error bars. AU, arbitrary units.

deionized water at 2.5 ml min⁻¹, and eluted with 1 M NH₄Cl-NH₄OH (pH 9.6) at 5 ml min⁻¹. Deep-orange fractions eluted after 70 ml and had a characteristic absorbance at 422 nm. Fractions with absorbance at 422 nm exceeding the baseline were pooled (~50 ml), evaporated to dryness, resuspended in approximately 5 ml deionized water, and desalted by gel filtration (Sephadex G-10). Desalted material was quantified by UV-visible spectroscopy with a λ_{max} of 422 nm and ϵ of 2,860 M⁻¹ cm⁻¹ as published elsewhere for exochelin MS (7). Iron was removed from this purified material by mixing with an appropriate amount of 8-hydroxyquinoline (8HQ) overnight in a 60:40 MeOH:H₂O solution (~50 ml; [Fe-ExoMS], ~100 μM ; [8HQ], ~500 mM). Fe-8HQ was extracted with 5 volumes of chloroform. The aqueous phase containing deferrated ExoMS was evaporated to dryness and resuspended in 2 ml deionized water and titrated with FeCl₃ to determine its concentration. This preparation yielded approximately 300 μg Fe-ExoMS per liter of culture of *M. smegmatis* ML16.

Preparation of radioactive ferric citrate. A ⁵⁵Fe(Cit)₂⁵⁻ solution was prepared that consisted of 50 mM Tris (pH 6.9), 15 mM KCl, 10 mM (NH₄)₂SO₄, 1 mM MgSO₄, 100 μM FeCl₃ (74 μCi ⁵⁵Fe), and 20 mM sodium citrate. The solution was centrifuged at 16,000 $\times g$ for 1 h to precipitate insoluble iron. This method takes advantage of the insolubility of Fe³⁺ at neutral pH and assumes that, in the presence of a 200-fold molar excess of citrate and at a pH between 6.0 and 9.0, the predominant species in solution is Fe(Cit)₂⁵⁻ (11, 18). To verify the formation of the ferric citrate complex, the above concentration of iron was added to a solution in the absence of citrate and to 0.5 M HCl. It was observed that 96% of iron precipitated in the absence of citrate, compared to the same amount of iron added to a 0.5 M HCl reference solution, indicating that iron is complexed by citrate under these conditions. An Fe([¹⁴C]Cit)₂⁵⁻ uptake solution was prepared consisting of 50 mM Tris (pH 6.8), 15 mM KCl, 10 mM (NH₄)₂SO₄, 1 mM MgSO₄, 30 μM FeCl₃, and 6 mM citrate (12.5 μCi [1,5-¹⁴C]citric acid). The pH of this solution was adjusted with NaOH to 6.8. This solution was diluted 30-fold for uptake experiments. A ⁵⁵Fe-ExoMS uptake solution was prepared that consisted of 50 mM KH₂PO₄ (pH 7.0), 15 mM KCl, 10 mM (NH₄)₂SO₄, 1 mM MgSO₄, 100 μM deferrated-ExoMS, and 100 μM FeCl₃ (269 μCi ⁵⁵Fe³⁺). All iron added to this mixture remained in solution after centrifugation.

RESULTS

Loss of porins leads to increased IdeR activity by *Mycobacterium smegmatis*. Mycobacteria are routinely cultured in Middlebrook medium containing 150 μM ferric citrate (20). Given the small size and hydrophilicity of ferric citrate and the fact that these concentrations repress siderophore biosynthesis (27), we reasoned that the mechanism of iron penetration into cells under high-iron conditions occurs by diffusion through porins. If the number of porins were diminished, the cells

might sense an iron-deficient state and respond by upregulating iron acquisition genes to maintain homeostatic levels of iron. To test this hypothesis we constructed an iron-regulated reporter in which the *cis*-regulatory elements and the first 23 codons of the siderophore biosynthesis gene *fxbA* were fused to a *gfp* gene in a similar manner as published for an iron-responsive *lacZ*-based reporter (8). FxbA transcription is regulated by the global mycobacterial iron-dependent regulator IdeR, such that under high-iron conditions IdeR is bound to its operator, preventing the transcription of 92 genes involved in iron acquisition (43), and conversely, it derepresses the same genes under low-iron conditions (12). *M. smegmatis* transformed with the *fxbA-gfp* reporter construct displayed GFP fluorescence levels inversely proportional to the iron concentration in the medium, establishing that this reporter is indeed sensitive to extracellular iron concentrations (Fig. 1A). At iron concentrations greater than 5 μM , siderophore biosynthesis gene expression was minimal (Fig. 1A). This is consistent with previous observations that mycobactins are not detected at these concentrations (27), reaffirming that iron homeostasis is maintained in the absence of siderophores in mycobacteria. These results suggest (i) that an alternative route of entry for iron exists under these conditions and (ii) that the *gfp*-based reporter can be used as an iron sensor in *M. smegmatis*.

To examine whether diffusion of ferric citrate through porins is sufficient to meet the iron requirements of *M. smegmatis*, we measured iron reporter activity in the wt, the triple porin mutant (ΔmspA ΔmspC ΔmspD ; ML16 [Table 1]), and the triple porin mutant complemented with an integrated copy of *mspA* expressed from a constitutive promoter (ML56 [Table 1]). The strains were grown in minimal medium containing 150 μM ammonium ferric citrate, cells were harvested in the mid-logarithmic growth phase, and iron reporter activity was analyzed (Fig. 1B). The signal of the iron reporter in the porin mutant ML16 was increased by 64% compared to wt cells. Reporter activity was restored to wt levels by expressing *mspA*, indicating that lack of porins results in a derepression of IdeR-

FIG. 2. Uptake of ferric citrate by *M. smegmatis* is dependent on porins. Radioactive ferric citrate [$^{55}\text{Fe}(\text{Cit})_2\text{Na}_3$] was produced and added to wt *M. smegmatis* cells grown under high-iron conditions. Triplicate samples were removed at the indicated time points. The radioactivity of the cells was determined by liquid scintillation counting and normalized to the dry mass of cells. The amount of accumulated iron was calculated from the molar ratio of $^{55}\text{Fe}^{3+}$ to Fe^{3+} in the uptake stock solution. Standard deviations are shown as error bars.

regulated genes to increase iron uptake and thereby maintain iron homeostasis.

Porins are used by *M. smegmatis* to take up iron from ferric citrate. To rule out indirect effects of porins that might result in derepression of IdeR in the porin-deficient strain, uptake of ferric citrate was measured directly. The wt, ML16, and ML56 strains were grown overnight in minimal medium containing 150 μM ammonium ferric citrate. Cells were washed and incubated with ^{55}Fe -labeled ferric citrate (see Materials and Methods). The triple porin mutant ML16 accumulated approximately 40% less iron than wt *M. smegmatis* (Fig. 2). Expression of *mspA* in the porin mutant restored ferric citrate uptake to wt levels, demonstrating that the reduced uptake was caused by the loss of Msp porins and not by an indirect effect. These results showed that *M. smegmatis* does indeed acquire iron from ferric citrate in a porin-dependent manner under high-iron conditions. These experiments also validated the GFP-reporter construct as a reliable indicator of iron availability in *M. smegmatis*.

We were also interested in whether the entire ferric citrate molecule was taken up into cells. It has been reported that citrate from ferric citrate is not taken up into *M. smegmatis* cells grown under low-iron conditions (18). It is possible that citrate was not taken up in these experiments due to the presence of *de novo*-synthesized siderophores, which would out-compete citrate as iron chelators. Hence, we analyzed the uptake of ^{14}C -labeled ferric citrate in *M. smegmatis* under high-iron conditions, in which siderophore biosynthesis activity was minimal (Fig. 1A). However, no accumulation of ^{14}C -labeled citrate was observed (Fig. 3).

The threshold for siderophore production is reduced in the porin mutant. To examine whether the iron deficiency experienced by the porin mutant ML16 would lower the threshold for siderophore production in order to compensate for reduced iron uptake (i.e., exhibit derepression of IdeR at higher iron concentrations), we compared iron reporter activities in wt, ML16, and ML56 grown in minimal medium containing ferric

FIG. 3. Citrate from ferric citrate is not taken up by *M. smegmatis* under high-iron conditions. 1,5- ^{14}C -labeled ferric citrate was added to *M. smegmatis* cells grown under high-iron conditions to a final concentration of 200 μM citrate (~ 420 nCi [^{14}C]citrate) and 1 μM FeCl_3 . Triplicate samples were removed at the indicated time points. The radioactivity of the cells was determined by liquid scintillation counting and normalized to the dry mass of cells. Standard deviations are shown as error bars.

citrate concentrations ranging from 20 μM to 0.25 μM . Cells were isolated from precultures containing identical iron concentrations. The iron reporter activity was then determined from cells in the mid-logarithmic growth phase. The reporter activity of all three strains increased above baseline levels at around 1 μM ferric citrate. However, basal expression of siderophore biosynthesis genes was higher in ML16 than in wt *M. smegmatis* and ML56 (Fig. 4A). Of note, ML16 induces full transcriptional activity from IdeR-regulated genes at higher iron concentrations than wt cells, indicating that the threshold for the production of siderophores is indeed reduced by fewer porins in the outer membrane of *M. smegmatis*.

To examine whether iron reporter activity correlates with siderophore production, we used the CAS assay to quantify siderophores in culture filtrates of *M. smegmatis* wt, ML16, and ML56. The CAS assay is based on the removal of iron from the iron-binding chromogenic dye CAS by siderophores (29). wt, ML16, and ML56 were grown in a minimal medium containing 0.25 μM ferric citrate for 27 h. These cultures were washed and used to inoculate fresh minimal medium and grown for another 27 h. Then the cells were harvested, and culture filtrates were analyzed for CAS activity. ML16 produced nearly twice the amount of siderophores than wt cells under these conditions (Fig. 4B). Expression of MspA in ML16 diminished CAS activity. These data indicate that the lowered threshold for siderophore production in ML16 indeed results in the production of more siderophores than from wt *M. smegmatis* when cells are grown over an equal time period.

The uptake of Fe-ExoMS by *M. smegmatis* does not depend on porins. The mechanism of uptake for the predominant siderophore of *M. smegmatis*, Fe-ExoMS, is not known. To rule out the possibility that porins of *M. smegmatis* mediate the passage of Fe-ExoMS across the outer membrane and thus account for the high CAS activity of ML16, the uptake of ^{55}Fe -labeled ExoMS was analyzed. ^{55}Fe -labeled ExoMS was prepared from low-iron culture filtrates of ML16 as published previously (30) (see Materials and Methods). *M. smegmatis* wt and ML16 were grown under high-iron conditions (150 μM

FIG. 4. Effects of the number of porins on the derepression of IdeR. (A) Threshold analysis. *M. smegmatis* wt, the triple porin mutant ML16 ($\Delta mspACD$), and the triple porin mutant ML56 constitutively expressing *mspA* ($\Delta mspACD::p_{myc}\text{-}mspA$) harboring the iron reporter were grown to mid-logarithmic phase in minimal medium containing the indicated ferric citrate concentrations. Fluorescence intensities of *M. smegmatis* cells were determined in triplicate and normalized to the optical densities of the samples. Percent maximal IdeR activity is reported as the percentage of the fluorescence intensity from cultures grown in 0.25 μM ferric citrate. (B) The porin triple mutant ML16 produces more siderophores than the wild type. Indicated strains were grown in minimal medium containing 0.25 μM ferric citrate. The culture filtrates were analyzed for siderophore activity in a CAS assay and normalized to the optical density of the samples. Error bars represent standard deviations from biological triplicates. AU, arbitrary units.

ammonium ferric citrate), and uptake of ^{55}Fe -labeled ExoMS was measured at a final concentration of 1 μM Fe-ExoMS (Fig. 5). Fe-ExoMS was taken up into cells under the conditions examined; however, there was no difference in the amount of accumulated iron between wt and ML16, indicating that Fe-ExoMS is shuttled across the outer membrane of *M. smegmatis* by a porin-independent process.

DISCUSSION

The uptake of iron from ferric citrate in *M. smegmatis* was examined by Messenger and Ratledge (18). In their study it was observed that iron from ferric citrate is taken up by a

FIG. 5. Porins do not affect uptake of Fe-ExoMS by *M. smegmatis*. Purified Fe-ExoMS was deferrated and labeled with $^{55}\text{FeCl}_3$. ^{55}Fe -labeled ExoMS was added to *M. smegmatis* cells grown under high-iron conditions at a final concentration of 1 μM (~ 50 mCi/mg Fe^{3+}). Triplicate samples were removed at the indicated time points. The radioactivity of the cells was determined by liquid scintillation counting and normalized to the dry mass of cells. The amount of accumulated iron was calculated from the $^{55}\text{Fe}^{3+}$ -to- Fe^{3+} molar ratio in the uptake stock solution. Standard deviations are shown as error bars.

low-affinity uptake system that is nonsaturable up to 125 μM and insensitive to electron transport chain inhibitors. Here we defined the outer membrane component of that low-affinity iron uptake system as the porins of *M. smegmatis*. This was demonstrated by the fact that the triple porin mutant ML16 accumulated smaller amounts of iron than wild-type cells when given a ^{55}Fe -labeled source of ferric citrate, a phenotype that was restored by expressing MspA (Fig. 2). The fact that iron uptake was not completely abolished in ML16 warrants an explanation. *M. smegmatis* contains four porin genes, *mspA*, *mspB*, *mspC*, and *mspD* (34). Deletion of *mspA*, *mspC*, and *mspD* resulted in compensatory expression of *mspB* (34). Additionally, it was observed that the deletion of *mspA*, *mspC*, and *mspD* resulted in an increase in IdeR activity as evidenced by the use of an iron reporter (Fig. 1B). This compensatory upregulation lowered the threshold for the biosynthesis of siderophores, resulting in the secretion of more siderophores than wt cells under iron-deficient conditions (Fig. 4). It is possible that additional iron uptake pathways are also derepressed in ML16. Bioinformatic analysis of the iron-regulated genes in *M. smegmatis* revealed the presence of a putative ferric uptake system displaying homology to the ferric uptake system of *Haemophilus influenzae*. The cumulative effect of an increase in *mspB* expression and the upregulation of a ferric uptake system could account for the accumulated iron observed in ML16.

Our data indicate that iron from ferric citrate is taken up into cells by diffusion through porins. However, citrate is not taken up into cells harvested at low iron concentrations (18) or at high iron concentrations, as shown in this study. Two hypotheses may explain this observation. Bioinformatic analysis of the genome of *M. smegmatis* revealed the presence of a putative citrate transport system (*ms4208-10*) displaying homology to the citrate transporter of the actinomycete *Corynebacterium glutamicum*, TctCBA (3). Expression of *tctCBA* of *C. glutamicum* is induced by citrate through a two-component regulatory system comprised of CitAB (homologous to Ms4211

and Ms4212) and requires the presence of Mg^{2+} or Ca^{2+} at concentrations greater than 5 mM to optimally utilize citrate (3). It is possible that this system was not induced under our experimental conditions due to the relative scarcity of citrate in 7H9 medium (0.5 mM) (20). The minimal citrate concentration necessary for the induction of the TctBCA uptake system is not known. However, Brocker and colleagues (3) reported that they used 50 mM citrate in all citrate utilization experiments. Additionally, the concentration of Mg^{2+} in our uptake solutions (1 mM) fell below the necessary concentrations that allow for citrate uptake by the TctCBA system in *C. glutamicum*. When taken together it seems reasonable to suggest that both the paucity of citrate in the growth medium and the low levels of Mg^{2+} in the uptake solution could account for the absence of citrate uptake under our experimental conditions.

An alternative possibility for the lack of citrate uptake observed in our experiments is that Fe^{3+} complexed with citrate is reduced by an extracellular/cell surface-attached reductase to aqueous Fe^{2+} , which would then diffuse through porins. While there have been no reports of extracellular ferric reductase activity in *M. smegmatis*, such an activity has been recovered from spent culture supernatants of *Mycobacterium paratuberculosis* (15). However, *M. paratuberculosis*, a facultative intracellular pathogen, has a markedly different lifestyle than the saprophytic *M. smegmatis* (39). The ferric reductase activity found in *M. paratuberculosis*, much like that seen in the facultative intracellular pathogen *Listeria monocytogenes* (6), is dependent on NADH and Mg^{2+} , suggesting that these cofactors are available to these pathogens in their intracellular niches. It follows that *M. smegmatis*, being nonpathogenic, would have to supply its own electron donors into the environment in order to reduce the available iron. Thus, the existence of an extracellular ferric reductase in *M. smegmatis* seems unlikely, but it cannot be completely discounted, considering the findings that bacteria can indeed secrete electron donors into the environment to acquire iron (28, 37, 38).

The mechanism of uptake for the predominant siderophore of *M. smegmatis*, ExoMS, is not known. One explanation for the high CAS activity of ML16 grown under iron-deficient conditions (Fig. 4B) is that exochelin MS might penetrate cells through Msp porins. However, by monitoring the uptake of radiolabeled ExoMS in ML16 we observed that Fe-ExoMS was taken up across the outer membrane of the cell independently of porins. Fe-ExoMS most likely enters cells by a yet-to-be-identified Fe-ExoMS receptor.

Taken together our data suggest that under high-iron conditions the iron requirements of the cells are met by diffusion of ferric citrate complexes through porins. When the iron concentration decreases below a certain threshold, or the number of porins is reduced, *M. smegmatis* compensates by upregulating iron acquisition genes. *M. smegmatis* produces two disparate siderophores for high-affinity iron acquisition (26). The predominant siderophore, exochelin MS, is secreted earlier and to a higher level under low-iron conditions, while the mycobactins appear later in the growth period (26). Uptake of mycobactins is dependent on the ESX-3 secretion system (31). Here, we showed that iron-loaded exochelin MS is taken up across the outer membrane of the cell independently of porins, suggesting its uptake is mediated by a yet-to-be-identified Fe-exochelin MS receptor (Fig. 6), as pointed out recently (22).

FIG. 6. Model of iron transport in *M. smegmatis*. The model of the mycobacterial cell envelope is based on cryo-electron microscopy (14). Mycolic acids are depicted in red. Under high-iron conditions ($[Fe^{3+}]$, $>5 \mu M$), the uptake of ferric ions through porins is sufficient to meet the iron requirements of *M. smegmatis*. When the availability of iron decreases, either by decreasing the number of pores in the outer membrane or by decreasing iron concentrations, *M. smegmatis* compensates for low iron levels by producing siderophores. Exochelin MS (yellow dots) is the main siderophore of *M. smegmatis* and strongly binds iron (black dots). Uptake of iron-loaded exochelin MS probably requires binding to an unknown receptor in the outer membrane and the FxuABC system in the inner membrane. Exochelin MS is secreted by ExiT, probably in combination with a yet-unknown outer membrane channel protein. The uptake of mycobactins depends on the ESX-3 secretion system (31) but was omitted from our model for reasons of clarity. See the text for more details.

Once Fe-exochelin MS passes the mycobacterial outer membrane, the putative periplasmic-binding protein FxuD (44) likely shuttles Fe-ExoMS to the ATP-dependent iron permease system FxuABC, where it is then actively imported into the cytoplasm (10). It is generally agreed that Fe-ExoMS is deferrated by reduction once it enters the cytoplasm. This is supported by the fact that the affinity of exochelin MS for Fe^{2+} is ~ 19 orders of magnitude less than that for Fe^{3+} (7) and that ferric reductase activity has been reported from *M. smegmatis* lysates (17). The deferrated siderophores are then either degraded by a yet-to-be-identified esterase or recycled and secreted along with *de novo*-synthesized exochelin MS in a process involving ExiT (45) and an unknown outer membrane excretion channel. More experiments are obviously needed to identify the missing components of the iron acquisition systems of *M. smegmatis*.

Here we have defined the outer membrane component of a low-affinity iron uptake system in *M. smegmatis*. Porins have been implicated in low-affinity iron uptake in *Helicobacter pylori*.

lori (38) and *Pseudomonas aeruginosa* (19); however, the corresponding outer membrane components have not been characterized. Low-affinity iron transport has also been postulated to include nonspecific cotransport by magnesium transporters in *E. coli* (13). By the use of a porin-deficient strain of *M. smegmatis* we have shown that the major porins of *M. smegmatis* are responsible for the uptake of iron from ferric citrate across the outer membrane. Thus, we provide for the first time experimental evidence for the long-standing assumption that general porins constitute the outer membrane component of low-affinity iron transport systems.

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