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Role for *Mycobacterium tuberculosis* Membrane Vesicles in Iron Acquisition

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***Mycobacterium tuberculosis* releases membrane vesicles packed with molecules that can modulate the immune response. Because environmental conditions often influence the production and content of bacterial vesicles, this study examined *M. tuberculosis* microvesicles released under iron limitation, a common condition faced by pathogens inside the host. The findings indicate that *M. tuberculosis* increases microvesicle production in response to iron restriction and that these microvesicles contain mycobactin, which can serve as an iron donor and supports replication of iron-starved mycobacteria. Consequently, the results revealed a role of microvesicles in iron acquisition in *M. tuberculosis*, which can be critical for survival in the host.**

The production of extracellular vesicles and vesicle-mediated communication is evolutionarily conserved among unicellular and multicellular organisms. Bacteria release membrane vesicles (MVs) containing proteins, genetic material, and lipids as a way to interact with prokaryotic and eukaryotic cells in their environment. Mycobacteria, including *Mycobacterium tuberculosis*, the causative agent of tuberculosis, release MVs in culture, in macrophages, and in the lungs of infected mice (1). MVs released by *M. tuberculosis* in culture are packed with immunologically active molecules that can modulate the immune response to the benefit of the bacterium (1). MV production by *M. tuberculosis* has recently been shown to be under genetic control (2).

Environmental factors, including those encountered by pathogens during infection, often influence the production and composition of outer membrane vesicles released by Gram-negative bacteria (3). Iron limitation is a well-recognized hallmark of the host environment. Due to its poor solubility in water in the presence of oxygen and at neutral pH, ferric iron is not found free but rather is sequestered in complexes with host iron binding proteins, such as transferrin, lactoferrin, and ferritin (4). For this reason, and because iron is essential for cell vitality, high-affinity iron acquisition systems are critical for pathogens to proliferate during infection. In fact, competition for iron deeply influences host-pathogen interactions. On one hand, rapid withdrawal of accessible iron is the most prominent example of "nutritional immunity"; on the other hand, iron deficiency in the host is a signal for pathogens to induce the expression of toxins and other virulence factors in conjunction with iron acquisition systems (5). To obtain iron, *M. tuberculosis* synthesizes and secretes high-affinity iron chelators or siderophores named mycobactins, which are essential for virulence (6, 7). Two forms of mycobactins are produced: carboxymycobactin, an amphiphilic molecule that is secreted into the medium, and mycobactin, a lipophilic molecule that remains cell associated (8). These two siderophores share a core but differ mainly in the length of an alkyl substitution; carboxymycobactin has a short one (2 to 9 carbons), whereas a long one (10 to 21 carbons) characterizes mycobactin. Carboxymycobactin effectively sequesters ferric iron from the environment and transfers it to mycobactin (9) or brings it into the cell via the iron-regulated transporter IrtAB (10, 11). The fate of iron bound by mycobactin, the precise location of this siderophore on the cell surface, and its overall contribution to iron uptake remain unclear.

In this work, we examined how iron limitation, such as that encountered by *M. tuberculosis* in the host, affects *M. tuberculosis* MV production. Our results show that iron deficiency stimulates MV release and influences the lipid content of MVs, suggesting potential complex patterns of MV-mediated communication modulated by the host environment. Significantly, we report that MVs produced during iron limitation can deliver iron and support proliferation of iron-deficient bacteria. The results unveiled a function for mycobactin and a novel way by which *M. tuberculosis* could overcome iron restriction and enhance its capacity to persist in the host.

MATERIALS AND METHODS

Media and bacterial cultures. *M. tuberculosis* strains were maintained in 7H10 agar (Difco) supplemented with 0.2% glycerol, 0.05% Tween 80, 0.5% bovine serum albumin (BSA), 0.2% dextrose, and 0.085% NaCl (ADN). Minimal medium (MM) was used to grow *M. tuberculosis* under iron-defined conditions. MM contains 0.5% (wt/vol) asparagine, 0.5% (wt/vol) KH_2PO_4, 0.2% glycerol, 10% ADN, and 0.05% Tyloxapol or Tween 80, with the pH adjusted to 6.8. To lower the trace metal contamination, the medium was treated with Chelex-100 (Bio-Rad) according to the manufacturer's instructions. Chelex was removed by filtration, and then the medium was supplemented with 0.5 mg $ZnCl_2$/liter, 0.1 mg/liter $MnSO_4$, 40 mg/liter $MgSO_4$, and 50 mg/liter ferric chloride. Low-iron minimal medium (LIMM) was supplemented with all metals except iron. This medium contains less than 2 μM residual iron, as determined by atomic absorption spectroscopy.

The siderophore-deficient *mbtB* mutant strain (ST142) provided by Cliff Barry (6) and the *ideR* mutant (ST22) (12) were grown in the presence of hygromycin (Hyg), 100 μg/ml, and kanamycin (Kan), 10 μg/ml, respectively.

When indicated, the iron chelator deferoxamine (DFO) (Sigma) was added at a final concentration of 50 μM.

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MV preparation. MVs were isolated as described previously (1). Briefly, 1,000 ml-cultures were centrifuged (3,450 × *g* for 15 min, 4°C), and the supernatants were filtered (0.45 μm; Millipore, Billerica, MA) and concentrated approximately 20-fold using an Amicon (Millipore) ultrafiltration system with a 100-kDa exclusion filter. Protein and other material aggregates formed during concentration were removed by centrifuging them at 15,000 × *g* (15 min, 4°C). MVs were then pelleted by centrifugation at 100,000 × *g* for 1 h at 4°C. The pellet was resuspended in 1 ml of 10 mM HEPES–0.15 M NaCl and mixed with 2 ml of Optiprep solution (Sigma) in 10 mM HEPES and 0.15 M NaCl (yielding a final Optiprep concentration of 35% [wt/vol]). The MV suspension was then overlaid with a series of Optiprep gradient layers with concentrations ranging from 30 to 5% (wt/vol). The gradients were centrifuged (100,000 × *g*, 16 h), and 1-ml fractions were removed from the top. The fractions were then dialyzed separately in phosphate-buffered saline (PBS) overnight, and MVs were recovered again by sedimentation at 100,000 × *g* for 1 h. Finally, the vesicles were suspended in PBS. The MV amount was determined based on protein concentration, which was measured using the Bradford reagent (Bio-Rad).

Dynamic light scattering. The average hydrodynamic radius (R_h) of vesicle samples was determined by dynamic light scattering using a 90 Plus Particle Sizing analyzer (Brookhaven Instruments Corp). The average R_h was calculated from 10 individual measurements. MV solutions were adjusted to a concentration of 0.1 mg ml^{-1} in PBS in a final volume of 100 μl.

MV labeling. To study MV production, 10 ml of MM or LIMM cultures was inoculated with *M. tuberculosis*. Cultures were grown to reach stationary phase and then diluted (1:100) in fresh MM or LIMM supplemented with 1 μCi ml^{-1} [1,2-^{14}C]acetate (50 to 62 mCi mmol^{-1}; Moravek Biochemicals, Brea, CA). Cultures were grown at 37°C for up to 12 days. Growth was monitored based on increase in the optical density (OD) at 550 nm. At different time intervals, the cells were harvested by centrifugation at 4,000 × *g* for 10 min. The supernatants were filtered (0.45 μm) and ultracentrifuged (60,000 g for 1 h). The MV-containing pellets were resuspended in PBS, and radioactivity was measured in a scintillation counter.

Growth assays. Frozen stocks of mycobacterial strains were inoculated onto 7H10 agar plates. An aliquot was taken to inoculate MM or LIMM to an initial OD at 540 nm of 0.05. The cultures were incubated at 37°C with agitation. The siderophore mutant (ST142) was preincubated in LIMM for 24 h before incubation with MVs (1 to 3 μg/ml). Cell growth was monitored by measuring the OD at 540 nm.

Electron microscopy. Transmission electron microscopy (TEM) was used to analyze the morphology and integrity of extracellular vesicles isolated from culture supernatants as well as mycobacterial cells to visualize vesicle release events. MVs were fixed with 2% glutaraldehyde in 0.1 M cacodylate at room temperature for 2 h and then incubated overnight in 4% formaldehyde, 1% glutaraldehyde, and 0.1% PBS. Fixed samples were stained for 90 min in 2% osmium tetroxide and then serially dehydrated in ethanol and embedded in Spurr's epoxy resin. Thin sections were obtained on an Ultracut UCT ultramicrotome (Reichert) and stained with 0.5% uranyl acetate and 0.5% lead citrate (Reichart, Depew, NY). Samples were observed in a JEOL 1200EX transmission electron microscope operating at 80 kV. For scanning electron microscopy (SEM), cells were fixed with 2.5% glutaraldehyde, 0.1 M sodium cacodylate, 0.2 M sucrose, and 5 mM $MgCl_2$ (pH 7.4) and dehydrated through a graded series of ethanol solutions. Critical point drying was assessed using liquid carbon dioxide in a Tousimis Samdri 795 critical point drier (Rockville, MD). Sputter was coated with gold-palladium in a Vacuum Desk-2 sputter coater (Denton, Cherry Hill, NJ). Samples were examined in a Zeiss Supra field emission scanning electron microscope (Carl Zeiss Microscopy, LLC North America), using an accelerating voltage of 5 kV. MV size distribution was calculated by measuring the diameter of isolated MV on EM pictures using the program ImageJ (U.S. National Institutes of Health [http://imagej.nih.gov/ij/index.html]).

UPLC-MS analysis of membrane vesicle lipid constituents. Low- and high-iron vesicles were purified, their lipids were extracted with chloroform-methanol (2/1, vol/vol; 3 ml) overnight at room temperature and centrifuged at 1,500 rpm for 15 min, and the supernatants were removed and evaporated to dryness under nitrogen. Analysis was performed as published previously with minor modifications (13). Lipid extracts were dissolved in isopropanol-acetonitrile-water (2:1:1, vol/vol/vol; 0.75 ml). A Waters Acquity ultraperformance liquid chromatography (UPLC) system coupled to a Waters Synapt G2 quadrupole-time of flight hybrid mass spectrometer (MS) was used to separate and detect lipids using the method detailed in a Waters Application Note (14).

A C_{18} ethylene bridge hybrid (EBH) column, 1.7 μm (2.1 by 50 mm), was used instead of a C_{18} charged surface hybrid column.

The column was heated to 55°C and eluted at 0.4 ml/min with mobile phase A, acetonitrile-water (60:40, vol/vol), and mobile phase B, isopropanol-acetonitrile (90:10, vol/vol), both containing 10 mM ammonium formate and 0.1% formic acid. The gradient began at 40% B and was ramped to 43% B at 2 min, 50% B at 2.1 min, 54% B at 12 min, 70% B at 12.1 min, 99% B at 18 min, and then back to 40% B at 18.1 min until the end of the run at 20 min. The mass spectrometer was operated in positive-ion resolution mode with electrospray ionization source conditions: capillary voltage, 2 kV; cone voltage, 30 V; desolvation gas temperature, 550°C; flow, 900 liter/h; source temperature, 120°C. Mass spectra were acquired in MS^E centroid mode over the range 100 to 3,000 *m/z*. Scan time was 0.5 s, and 0.2 ng/μl leucine enkephalin was used for lock mass and detected at 556.2771 *m/z*.

Total ion chromatograms of three biological replicates of each sample type were aligned in Waters Masslynx software, and markers of exact mass-retention time pairs were extracted with intensities normalized to the total ion current. Waters/Umetrics Extended Statistics software was used to generate an orthogonal projections to latent structures discriminant analysis (OPLS-DA) model to determine the most differentially abundant lipid species between samples. Differentially abundant lipids were identified using the Mycomass database (15, 16) (see http://www.brighamandwomens.org/research/depts/medicine/rheumatology/labs/moody/default.aspx) and the Mtb LipidDB (17) (see http://www.mrl.colostate.edu).

RESULTS

Release of MVs by *M. tuberculosis* under iron-limiting conditions. To examine the influence of iron limitation in *M. tuberculosis* vesicle production, MVs were isolated from the culture supernatants of *M. tuberculosis* grown in low (LIMM) or high-iron medium (MM) as previously described (1). Transmission electron microscopic (TEM) analysis of vesicle pellets revealed intact spherical vesicles, with diameters ranging from 60 to 300 nm in low- and high-iron culture supernatants (Fig. 1). Samples were also analyzed by dynamic light scattering to get a size distribution of the vesicles in their native state. The average diameters (± standard deviation) of the vesicles measured by laser diffraction were 181.1 ± 14.1 nm and 177 ± 22.4 nm for MM- and LIMM-isolated MVs, respectively. Detailed observation of the bacteria, however, indicated enhanced vesiculation in *M. tuberculosis* cultured in LIMM (Fig. 2), suggesting that iron limitation stimulated vesicle generation. To confirm this, we monitored vesicle production in cells that were metabolically labeled with [C^{14}]acetate (as described in Materials and Methods) and assessed MV production based on the amount of radioactivity incorporated in the MV-containing supernatant. Radioactivity incorporated into vesicles generated in LIMM was considerably greater than in those prepared from cells grown in MM. Since the sizes of MVs released in MM and LIMM were similar, this result indicates increased MV

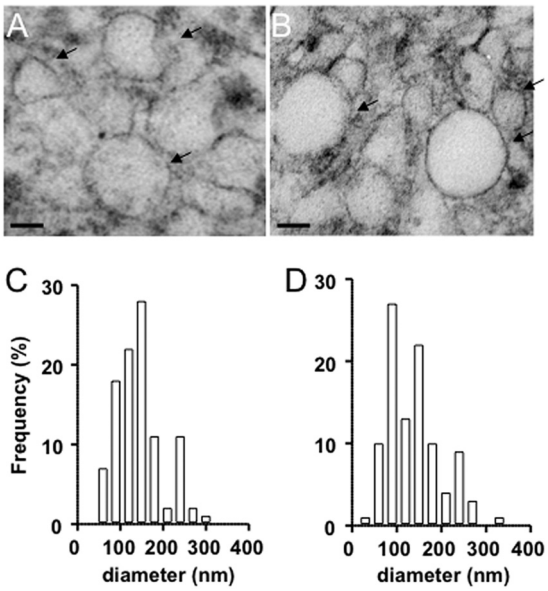

FIG 1 Mycobacterium produces membrane vesicles under iron limitation. (A and B) Transmission electron microscopy showing isolated membrane vesicles (arrows) from *M. tuberculosis* H37Rv cultured in LIMM (A) or MM (B) (scale bar, 50 nm). (C and D) Vesicle size distribution determined by measuring the diameter of 150 vesicles in TEMs of LIMM MVs (C) or MM MVs (D).

production by *M. tuberculosis* in response to iron limitation, in agreement with the microscopic observations.

MV lipid content. *M. tuberculosis* synthesizes mycobactins when facing low-iron conditions. Given our finding that iron limitation increased the release of MVs, we investigated whether MVs released under iron limitation contained mycobactin. Consequently, we analyzed the lipid content of MVs released in low- and high-iron medium by UPLC-MS. The results of these analyses demonstrated that LIMM MVs were enriched in Fe-loaded mycobactin (Fig. 3; also see Table 1 posted at http://www.phri.org/research/pdf/Suppl_Mat_Prados-Rosales_JB.pdf), whereas the mycobactin precursor dideoxymycobactin, which has low iron binding capacity, accounted for most of the siderophore found in MM MVs. This result suggests induced maturation of mycobactin under iron limitation. As mentioned before, the precise location of mycobactin in the cell is not known with certainty. Finding mycobactin associated with MVs supports the conclusions of early microscopic analysis that localized this siderophore in or in very close proximity to the plasma membrane (18). Regarding other lipids, LIMM MVs were enriched in diacyl and triacyl glycerides as well as phosphatidylethanolamines, whereas they were slightly depleted in diacyl trehaloses compared to MM MVs (see Fig. 1 posted at http://www.phri.org/research/pdf/Suppl_Mat_Prados-Rosales_JB.pdf).

MVs as iron transporters. The presence of iron-loaded mycobactin in LIMM MV and the enhanced vesicle generation under iron limitation lead us to hypothesize that *M. tuberculosis* can transport iron via MVs. To test this possibility, we examined whether MVs were active as iron delivery vehicles. For this purpose, we used an *mbtB* mutant (ST142) that cannot synthesize siderophores and consequently does not multiply in low-iron medium unless a suitable source of iron such as Fe^{+3}-siderophore is supplied exogenously (19). Addition of MVs purified from H37Rv LIMM cultures restored growth of ST142 in low-iron medium. In contrast, MVs recovered from MM H37Rv cultures failed to restore the growth of this strain (Fig. 4). These results demonstrate that MVs produced by *M. tuberculosis* under iron limitation could serve as iron donors and support the idea that mycobactin synthesized under iron limitation and packed into MVs can bind iron and provide it to other bacilli. To further investigate the role of mycobactin as the iron carrier in the vesicles, we recovered MVs from an *ideR* mutant strain (ST22). IdeR represses transcription of *mbt* genes in the presence of iron and thereby suppresses siderophore synthesis under iron sufficiency. As a consequence of the *ideR* mutation, ST22 produces mycobactin even when cultured in high-iron medium (12). In contrast to MVs produced by wild-type *M. tuberculosis*, the vesicles produced by ST22 in high-iron medium supported the growth of iron-deprived ST142 (Fig. 4). This result links mycobactin production to the iron-donating activity of the vesicles. Moreover, vesicles isolated from the siderophore mutant had no ability to serve as iron donors (Fig. 5), confirming that mycobactin is required to transport iron in MVs.

We next examined whether MV utilization as an iron source was restricted to the siderophore mutant or whether a wild-type siderophore-producing mycobacterium could also benefit from iron in MVs. For this purpose, it was necessary to generate conditions in which even a siderophore-producing mycobacterium would experience iron deficiency. To achieve this, H37Rv was iron depleted by sequential passages in LIMM and then the iron chelator DFO was added to diluted cultures. DFO does not permeate the cells, is not utilized by *M. tuberculosis* as an iron donor, and effectively competes with carboxymycobactin for iron, which results in inhibition of *M. tuberculosis* growth in LIMM (Fig. 6). We then tested whether LIMM MVs produced by H37Rv could suppress the iron deficiency generated by DFO. Figure 6 shows that LIMM MVs efficiently delivered iron to wild-type *M. tuberculosis*, allowing proliferation under iron deprivation.

Taken together, the results presented here show that iron limitation prompts *M. tuberculosis* to release vesicles that are packed with mycobactin and capable of assisting other mycobacteria to overcome iron deficiency.

DISCUSSION

Studies of MVs produced by diverse bacteria indicate a common function: bacteria release vesicles to interact with prokaryotic and eukaryotic cells in their environment. Pathogens frequently release vesicles containing toxins, adhesins, immunomodulatory molecules, and other effectors that allow them to manipulate host-pathogen interactions to their own benefit. Bacteria also employ MVs for collaborative as well as competitive interactions with other bacteria. For instance, vesicle fusion can deliver bacteriolysins to eliminate competitors (20), transfer antibiotic-resistance enzymes to siblings (21), or mediate coaggregation in drug-tolerant biofilms (22). Previously, we demonstrated that *M. tuberculosis* has also adopted this secretory pathway for exporting molecules that can modulate the immune response. In this study, we showed that under iron limitation, *M. tuberculosis* also uses MVs to export concentrated mycobactin and enhance iron acquisition capacity.

Studies in *Escherichia coli* have shown that approximately 0.2% to 0.5% of outer membrane and periplasmic proteins are packed in vesicles (23). Considering that this is an energy-consuming process, it is likely that bacteria produce vesicles in a regulated

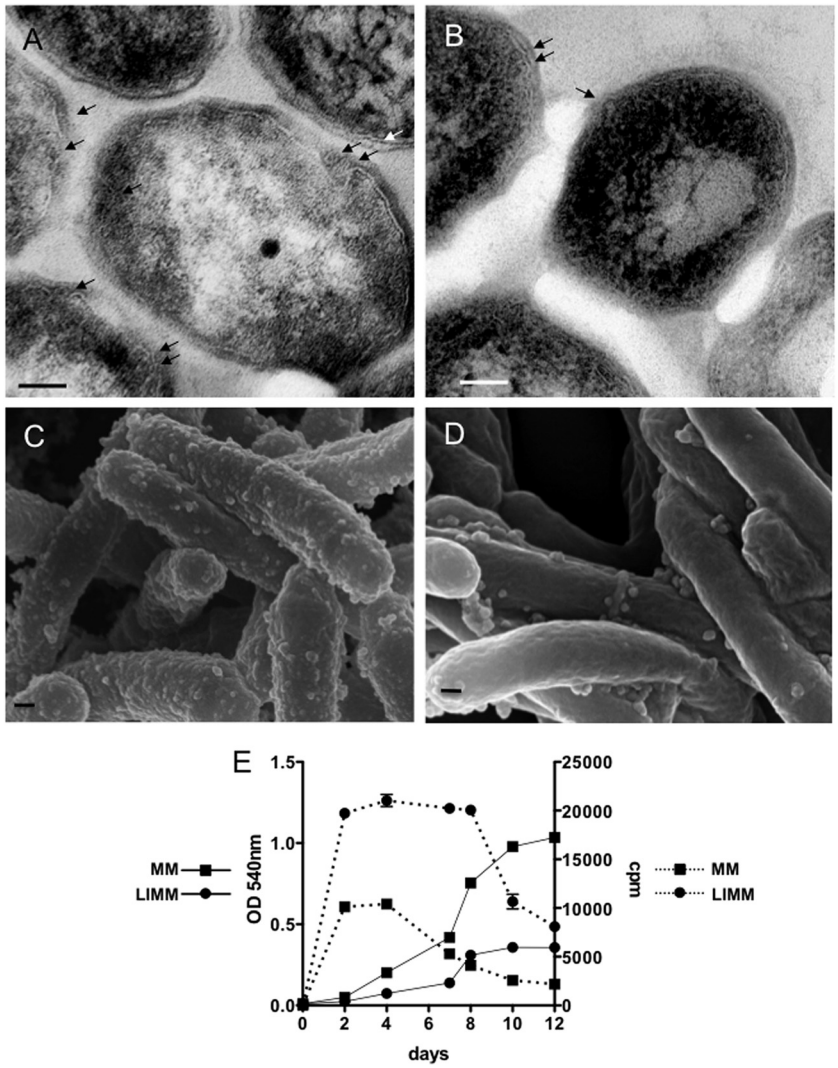

FIG 2 MV production is increased under low-iron conditions. (A and B) TEM showing production of MV by *M. tuberculosis* H37Rv in LIMM (A) or MM (B). Vesicles are seen budding from the surface of the bacteria (arrows) (scale bars, 100 nm). (C and D) SEM images of the preparations shown in panels A and B, respectively. The number of spherical vesicles associated with the mycobacterial surface was higher in LIMM cultures (C) than in MM (D) (scale bars, 200 nm). (E) Vesicle production was monitored by labeling LIMM and MM cultures with [^{14}C]acetate (2 mCi ml^{-1}). At the indicated time intervals, cell density (OD at 540 nm) and vesicle production (cpm) were assessed. The background level of radioactivity was determined on sterile medium containing [^{14}C]acetate (2 μCi ml^{-1}).

fashion in response to external and/or internal signals. Indeed, numerous examples support this hypothesis. Lipopolysaccharide (LPS) serotype switching, oxygen stress, and antibiotics are some of the factors that influence vesicle production and content in Gram-negative bacteria (24–26). Pathogenic bacteria generally produce more vesicles than their nonpathogenic counterparts (27, 28), which is consistent with the idea that vesicle production may be part of the adaptive response that promotes dissemination of virulence factors and propagation in the host.

Iron availability is a critical factor that affects all bacteria living within a host. Because basic cellular metabolic activities require iron, this metal is absolutely essential. However, due to its poor solubility and potential toxicity under aerobic conditions, free iron is not available in the host. Successful pathogens must be able to obtain iron and to adapt their metabolism according to iron availability. However, the influence of iron availability in pathogenic bacterial MV production has not been extensively examined. In one study, *Helicobacter pylori* vesicles produced in low-iron medium were found to contain reduced VacA toxin levels and increased concentrations of vesicle-associated proteases, although the production of MVs was not affected (29).

In this work, we initiated the characterization of *M. tuberculosis* MV production under iron limitation. An increase in vesicle production was detected when *M. tuberculosis* was cultured under iron deficiency, indicating that a higher rate of vesicle production is part of the strong response of *M. tuberculosis* to iron restriction. It is possible that reduced iron availability is a trigger for MV production in other bacteria as well. *E. coli*, *Vibrio cholerae*, and *Brucella melitensis* each manifest maximal production of vesicles at the end of log phase, a time that may coincide with decreased iron available in the medium (23, 30, 31).

Production and secretion of soluble siderophores are common

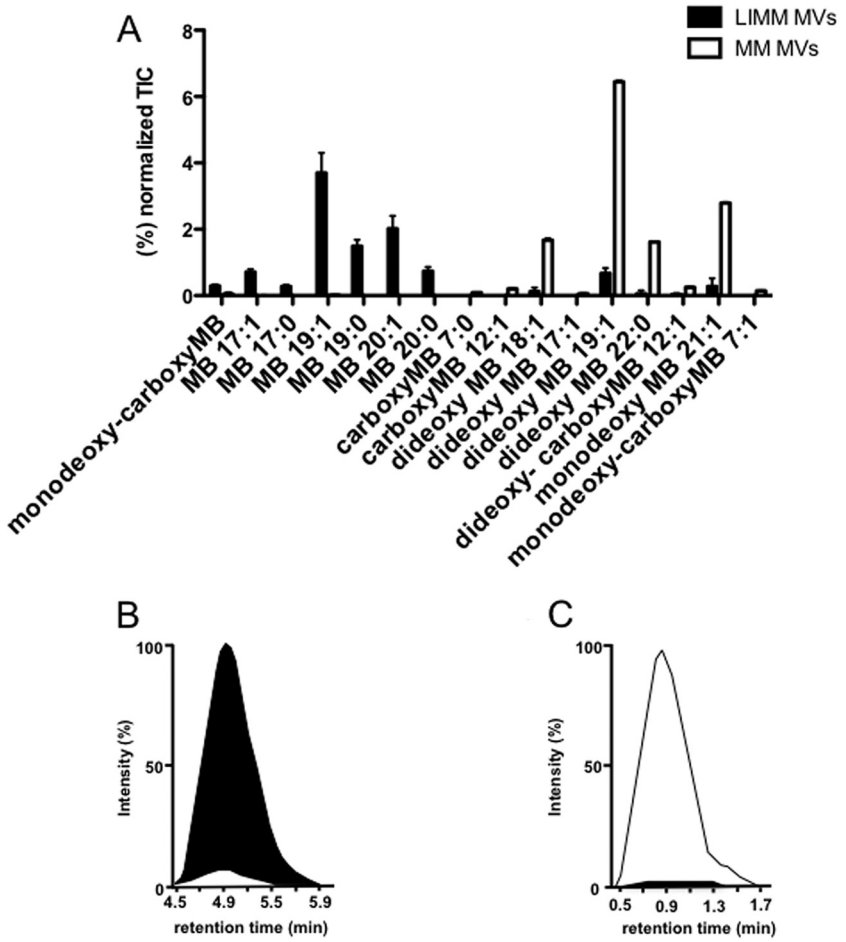

FIG 3 MV-associated siderophores. (A) Relative abundance of the MV-associated siderophores with differences between LIMM MVs and MM MVs, according to their normalized contribution to the total ion current (TIC). (B and C) Extracted chromatograms of the most prevalent and differentially abundant siderophores: mycobactin containing an alkyl substitution of 19 carbons with one unsaturation (19:1) (B) and dideoxymycobactin (19:1) (C) from LIMM MV (black) and MM MV (white).

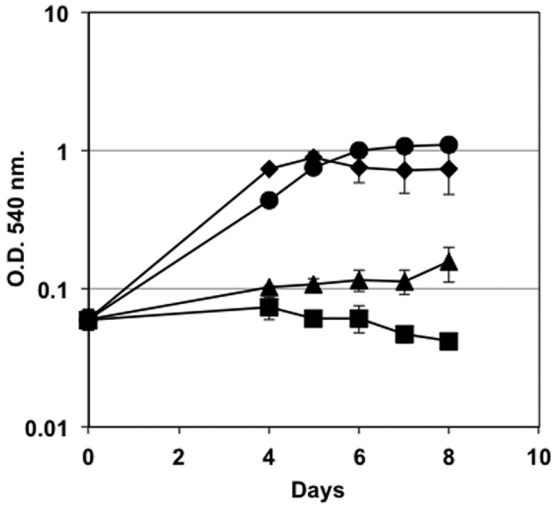

FIG 4 MVs can donate iron to a siderophore-deficient *M. tuberculosis* mutant. A siderophore synthesis mutant (ST142) was cultured in LIMM (■), alone or supplemented with MVs (3 μg/ml) isolated from H37Rv grown in low iron (●), H73Rv grown in high iron (▲), and the *ideR* mutant grown in high iron (◆). Error bars represent standard deviations from the means of biological triplicates.

to many microorganisms. However, production of a cell-associated siderophore is restricted mainly to mycobacterial and *Nocardia* species, suggesting an important role for this type of siderophore in these microorganisms. Mycobactin can bind iron transferred from carboxymycobactin or other iron chelators able to penetrate the cell envelope but is not required for utilization of Fe^{+3}-carboxymycobactin (19). In the absence of mutants lacking only mycobactin and not carboxymycobactin, it is not possible to determine the precise contribution of mycobactin to iron acquisition. Nevertheless, mycobactin was hypothesized to function as a temporary iron storage molecule (8), and liposomes packed with mycobactin have been shown to deliver iron when added to *M. tuberculosis*-infected macrophages (32). Our results suggest a very important role for mycobactin that makes use of its unique properties, i.e., high affinity for iron and hydrophobicity. Mycobactin synthesized during iron limitation reaches high local concentrations, and being in close proximity or associated with the cell membrane makes it well suited for incorporation into the MVs. Once secreted as part of the vesicle, mycobactin has access to extracellular iron and can deliver it via the MVs to the bacterium that released the vesicles or to its neighbors. MV-mediated iron acquisition may be critical during infection, especially in the context of

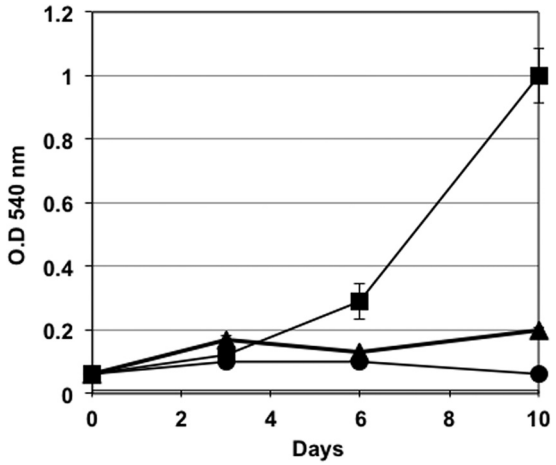

FIG 5 Siderophore mutant MVs do not donate iron. The siderophore synthesis mutant (ST142) was cultured in LIMM (●) or LIMM with MVs isolated form H37Rv grown in low iron (■) or MVs isolated from ST142 cultured in MM (▲). Error bars represent standard deviations from the means of biological triplicates.

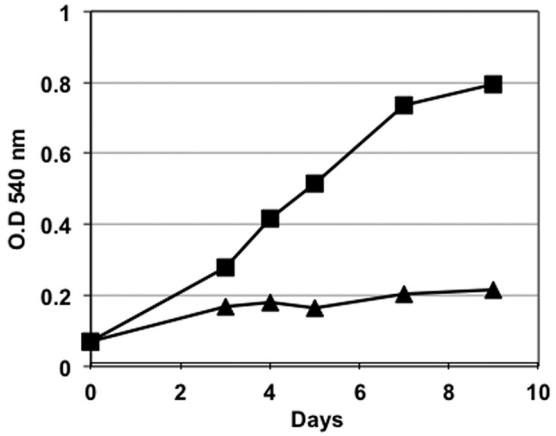

FIG 6 Wild-type, iron-deficient mycobacteria can utilize iron from MVs. H37Rv was cultured in LIMM in the presence of DFO (▲) or in the same medium supplemented with LIMM H37Rv derived MVs (■). Shown are the results from one representative experiment. The experiment was repeated twice with similar results.

intense iron deprivation, as it may take place in the granuloma where activated macrophages restrict bacterial access to iron and free iron is not available. In addition, immune cells release siderocalin, which binds carboxymycobactin (33) and thereby compromises its role in iron acquisition. It is possible that vesicular mycobactin is not accessible to siderocalin. If that were the case, by "disguising" mycobactin in the MVs, *M. tuberculosis* would be able to overcome the interference of siderocalin with iron acquisition.

Although we focused here on the presence of Fe-mycobactin in low-iron-medium-produced MVs, it is worth noting that iron availability also influenced the content of other lipids in the MVs such as triacyl glycerols and diacyl trehaloses, which may be relevant for host-pathogen interactions. The lipid content of MVs from both MM and LIMM cultures was consistent with their plasma membrane origin, but surprisingly, lipids from the outer membrane (mycomembrane) were not detected in MVs regardless of the concentration of iron in the medium. At this time we have no explanation for this observation and recognize the need for additional studies to address the mechanism of vesicle synthesis.

The presence of dideoxymycobactins in MM-derived vesicles suggests that the cell maintains mycobactin precursors even under iron sufficiency and that maturation is induced under iron deficiency. This may serve to increase the efficacy of siderophore production during iron limitation. Interestingly, dideoxymycobactins are antigens presented by CD1 molecules (34). Considering the possibility that bacterial MVs may deliver their cargo to host cells as well as to other bacteria, antigen-presenting cells could obtain and present MV-derived antigens. Therefore, the antigenic content of MVs could be a relevant determinant for stimulation of the immune system. This may have implications in the design of ways to deliver antigenic molecules, which when presented to T cells induce a protective immune response. Additionally, it is tempting to speculate that bacteria residing in different microenvironments could release MVs whose content could transmit a signal when taken up by other bacteria. Thus, MV release could potentially be a way by which mycobacteria communicate.

In summary, this study unveiled the influence of iron availability on the production and content of *M. tuberculosis* MVs and revealed the ability of MVs to transport the essential nutrient (Fe). This constitutes the first demonstration of a role for *M. tuberculosis* MVs that can benefit the local community, potentially contributing to the survival and persistence of this pathogen in the host.

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