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ORIGINAL ARTICLE

Mycobacterium abscessus subsp. *massiliense* expressing bacterioferritin have improved resistance to stressful conditions

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Abstract

Aims: The importance of bacterioferritin in the virulence and pathogenicity of the genus *Mycobacterium* is still unclear. The aim of this study was to analyse if the expression of a recombinant bacterioferritin from *M. tuberculosis* (Mtb) by Mycma could improve the capacity of this bacillus to resist the host defence mechanisms.

Methods and Results: Recombinant Mycma, expressing bacterioferritin (Rv1876) from Mtb, was developed by transformation with pMIP12_Rv1876. To determine bacterioferritin influence on Mycma physiology and virulence, the mycobacteria growth was analysed *in vitro* and *in vivo*. It was observed that the expression of bacterioferritin improved the growth rate of recombinant Mycma_BfrA under iron excess and oxidative stress, as compared to the wild type. Furthermore, in the murine model of infection, it was observed that Mycma_BfrA-infected mice had higher bacillary load and a more pronounced lesion in the lungs when compared with the wild type.

Conclusion: This study showed that bacterioferritin confers additional resistance to stress conditions, resulting in increased pathogenicity of Mycma during mice infection.

Significance and Impact of the Study: This study provides new insights about the importance of bacterioferritin in the virulence and pathogenicity of the *Mycobacterium* genus.

Introduction

Iron is an essential nutrient for mycobacteria as it is a major cofactor for many proteins involved in vital metabolic processes of the bacilli (De Voss *et al.* 1999; Rodriguez and Smith 2003). However, although the absence of iron is deleterious for mycobacteria, the excess of free metal is potentially toxic for bacillus due to spontaneous Fenton reaction (Imlay *et al.* 1988; De Voss *et al.* 2000). Thus, mycobacteria need to tightly regulate the intracellular levels of iron by employing efficient iron acquisition and storage mechanisms (Rodriguez 2006). Ferritins are the main proteins involved in the regulation of intracellular iron levels among aerobic bacteria (Andrews 1998; Andrews *et al.* 2003).

In general, aerobic bacteria synthesize three types of ferritin-like proteins for iron storage: ferritin, bacterioferritin and Dps (DNA-binding protein from starved cells). It was demonstrated that ferritin and bacterioferritin have similar structures composed of 24 identical subunits of 18–20 kDa, respectively, and these subunits form a spherical shell, where up to 4500 atoms of Fe^{3+} can be stored (Andrews 1998). Although the ferritins and bacterioferritin have similar structures, their amino acid sequences present low identity also bacterioferritins have a haem group, proposing these proteins are distantly related in evolution (Andrews 1998).

The type and number of ferritin-like molecules varies among aerobic bacteria, while some bacteria have both ferritin and bacterioferritin, others have just two ferritins or two bacterioferritins (Andrews 1998). It was shown that *M. tuberculosis* (Mtb), the causative agent of tuberculosis, have one ferritin (BfrB) and one bacterioferritin (BfrA), coded by Rv3841 and Rv1876 genes respectively (Cole *et al.* 1998). The expression of both genes was shown to be modulated under various iron and oxidative stress conditions, and overexpressed in aminoglycoside resistant Mtb clinical isolates (Gold *et al.* 2001; Rodriguez *et al.* 2002; Kumar *et al.* 2013; Sharma *et al.* 2015). In addition, this expression modulation is orchestrated mostly by the Iron-dependent Regulator (IdeR), that binds to specific DNA regions called Iron box present in the promoters of their regulated genes (Gold *et al.* 2001; Rodriguez *et al.* 2002; Pandey and Rodriguez 2014; Kurthkoti *et al.* 2015).

Initial studies with the objective to determine the importance of these proteins in *M. tuberculosis* were made by Reddy *et al.* (2012), where double mutants for both Rv3841 and Rv1876 genes were incapable to growth in macrophages and in guinea pigs under low-iron conditions. It was suggested that the presence of only one of those two proteins was sufficient to confer normal *in vitro* or host Mtb grow (Reddy *et al.* 2012). However, the individual importance of each ferritin and bacterioferritin in iron storage, and consequently, virulence and pathogenicity of the bacillus was not determined. Another study, with Mtb mutants for each gene, demonstrated that the absence of BfrB is deleterious for the bacillus, whereas BfrA was shown to be dispensable. BfrB was shown to have a crucial role in the adaptation to iron limitation, resistance to oxidative stress, and the maintenance of iron, as well as persistence of Mtb in mice. Moreover, Mtb lacking BfrB was more susceptible to antimicrobials (Pandey and Rodriguez 2012).

The dispensable role of BfrA suggested by Pandey and Rodriguez raised the question as to why *M. tuberculosis* maintain both ferritins-like proteins. Recently, Khare *et al.* (2017) made a contribution to the understanding of the specific roles of both BfrB and BfrA in iron homeostasis of Mtb, using single and double mutants for these proteins. It was demonstrated that both ferritin and bacterioferritin have a nonredundant and crucial role in the iron homeostasis, but this function is related to the surrounding microenvironment of the cell. It was proposed that under low iron conditions, BfrA is the main source of iron while during high iron availability, BfrB is essential to reduce the oxidative stress produced by the iron. Furthermore, it was demonstrated that both proteins are essential for the bacillus survival under oxidative stress and hypoxia, but in the last condition, the BfrB seems to have a more important role (Khare *et al.* 2017). Another study showed that bacterioferritin present catalase and Dps-like activities suggesting these functions could be involved in the bacillus survival under oxidative stress (Mohanty *et al.* 2019). These studies bring new insights about the different roles of ferritin and bacterioferritin in the mycobacteria physiologic conditions *in vitro*, but the specific relevance of bacterioferritin in the growth and virulence in the host remains unclear.

Recently, we have demonstrated that *Mycobacterium abscessus* subsp. *massiliense* (Mycma), an emerging pathogen that causes nontuberculosis diseases (Esther *et al.* 2010; Medjahed *et al.* 2010; Bryant *et al.* 2016), do not possess a gene coding for bacterioferritin. In contrast, this *Mycobacterium* has two ferritin genes whose activity and structural analyses proved to code for a functional ferritins (Oliveira *et al.* 2018). The lack of bacterioferritin in *M. abscessus* makes this bacteria an appropriate model to improve our understanding about the role of bacterioferritin in the virulence and pathogenicity of mycobacteria. The aim of this study was to analyse if the expression of a recombinant bacterioferritin from Mtb by *M. abscessus* subsp. *massiliense* could improve the capacity of this bacillus to resist the host defence mechanisms.

Materials and methods

Bacterial strains and growth conditions

Escherichia coli XL1-Blue and BL21 (DE3) pLysS were used for cloning and expression of the recombinant BfrA protein respectively. *Escherichia coli* strains and *M. abscessus* subsp. *massiliense* (Cardoso *et al.* 2008) were cultured in Luria Bertani (LB) (Himedia, Mumbai, India) broth (under 140 rev min^{-1} shaking) or agar at 37°C. For growth in different iron or H_2O_2 concentrations, the minimal medium (MM) containing 3·6 mmol l^{-1} of KH_2PO_4, 2·0 mmol l^{-1} of $MgSO_4 \cdot 7H_2O$, 6% (v/v) of glycerol, 30 mmol l^{-1} of L-asparagine, 0·006 mmol l^{-1} of $ZnSO_4$ and 0·05% (v/v) of Tween 80, pH 6·8 in iron free conditions was used. The antimicrobials kanamycin (Kan) and chloramphenicol (Cm) when used were supplemented at 20 μg ml^{-1}.

Animals

Female C57BL/6 IFN-γ-KO mice (4–8 weeks old), obtained from Institute of Tropical Pathology and Public Health at University Federal of Goiás, were maintained in ABSL-2 racks adapted with an HEPA air filter, with water and food provided *ad libitum*, at the animal-care facility of the Institute of Tropical Pathology and Public Health at University Federal of Goiás. The temperature was maintained at 20–24°C with a relative humidity of 40–

70%, and 12 h light/dark cycles. Wild-type C57BL/6 mice were used for derivation of macrophages from bone marrow cells. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Conselho Nacional de Controle de Experimentação Animal (CONCEA) and approved by the Research Ethical Committee of Federal University of Goiás (Permit Number: CEUA: 031/18).

Cloning, expression of Rv1876 gene in *E. coli* and recombinant rBfrA purification

The Rv1876 gene was PCR-amplified using *M. tuberculosis* H37Rv genomic DNA as template. The primers were designed using NCBI Primer designing tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast). The following primers were used to amplify the Rv1876 gene: forward 5′-CATATGCAAGGTGATCCCGATGTT-3′ that incorporates a *Nde*I restriction site (underlined) and reverse 5′-GGATCCTCAGGTCGGTGGGCGAGAGAC-3′ that incorporates a *Bam*HI restriction site (underlined). The amplicons were cloned into pGEM-T Easy (Promega Corporation, Madison, WI, USA) and then transferred to pET28a (Novagen, Madison, WI, USA) vector using their respective flanking sites. Recombinant plasmid was confirmed by sequencing. Recombinant protein expression was performed by transforming the recombinant plasmids into *E. coli* BL21 (DE3) pLysS cells. *Escherichia coli* BL21 (DE3) pLysS containing the recombinant plasmids were grown in LB containing kanamycin (20 μg ml^{-1}) and chloramphenicol (20 μg ml^{-1}) until OD_{550nm} reached 0·5. Then the culture was induced with 1 mmol l^{-1} of isopropyl-1-thio-β-D-galactopyranoside (IPTG) at 37°C for 4 h. Cells were then harvested by centrifugation at 4000 ***g*** for 20 min at 4°C. The pellet was used for protein extraction using the commercial protein purification QIAexpress-Ni-NTA Fast Start kit (Qiagen, Venlo, The Netherlands) according to the manufacturer's instructions. Eight microlitres from each collected fraction was analysed on 12% SDS-PAGE. The purified protein was used to produce the antibodies by subcutaneous inoculation in mice as described previously by Oliveira *et al.* (2018).

Recombinant *M. abscessus* subsp. *massiliense* expressing BfrA (Mycma_BfrA) construction

The Rv1876 gene encoding the Bacterioferritin (BfrA) was amplified by PCR using *M. tuberculosis* H37Rv genomic DNA as template. For the PCR amplification, a set of primers that allowed the creation of flanking restriction enzyme sites to facilitate posterior cloning into the pMIP12 plasmid (Kindly provided by Dr Brigitte Gicquel from Pasteur Institute) was designed. The following primers were used to amplify the Rv1876 gene: forward 5′-GGATCCATGCAAGGTGATCCCGATGTT-3′ that incorporates a *Bam*HI restriction site (underlined) and reverse 5′-GGTACCTCAGGTCGGTGGGCGAGAGAC-3′ that incorporates a *Kpn*I restriction site (underlined). The product of this amplification was cloned into the pGEM-T Easy vector (Promega). The cloned Rv1876 gene was removed by digestion with *Bam*HI and *Kpn*I and ligated into the *Mycobacterium/E. coli* shuttle vector pMIP12, previously digested with the same enzymes. The recombinant pMIP12_Rv1876 vector was then inserted into the *M. abscessus* subsp. *massiliense* cells and screened on LB medium containing kanamycin (100 μg ml^{-1}) to obtain the recombinant Mycma_BfrA strain. As control, *M. abscessus* subsp. *massiliense* cells were transformed with empty pMIP12 to obtain the Mycma strain.

Cell lysate obtention

Mycobacteria cultures at logarithmic growth were harvested at 6000 ***g*** for 10 min. The supernatant obtained was discarded and the pellet was processed for cell lysate preparations. The culture pellet was resuspended in PBS buffer and sonicated in an ice bath twice for 1 min to obtain cell lysate. The cell lysate was adjusted to 20% glycerol and stored at −20°C.

Western blotting

Protein preparations were resolved in a 12% SDS-PAGE and the separated proteins were electrotransferred to a nitrocellulose membrane. The membrane was blocked with an incubation of 2 h with 5% skimmed milk at room temperature. Then the membranes were incubated overnight at 4°C with the specific polyclonal antibody against rBfrA protein. The membrane was then washed three times with PBS buffer and incubated with 4 μg of secondary anti-Mouse-F (ab′) 2-xx-biotin (Molecular probes, Eugene, OR, USA) for 2 h at 37°C. Then, horse anti-mouse antibody conjugated with avidin-peroxidase (Sigma-Aldrich, St. Louis, MO, USA) was added and incubated for 1 h. The reaction was developed by adding 0·05% diaminobenzidine (DAB; Roche) in 10 ml of H_2O_2. The image was acquired with the help of Gel documentation system (Bio-Rad, Hercules, CA, USA) and analysed with Quantity One 4·5.6 software (Bio-Rad).

Growth under excess of iron or other stressful conditions

A fresh inoculum, adjusted to OD_{590} 0·02 of recombinant Mycma_BfrA or Mycma strains in MM was incubated for 5 days at 37°C and the growth of mycobacteria under

different stress conditions was monitored by measuring OD_{590}. For growth analyses under high iron condition, the MM media were supplemented with 150, 300, 450 or 900 μmol l^{-1} of $FeCl_3$. For oxidative stress conditions, growth kinetics was monitored in the presence of 0·5, 1·5, 3 or 6 mmol l^{-1} of H_2O_2 with 50 μmol l^{-1} of $FeCl_3$.

Autophagy analysis

Bone marrow cells were obtained from femurs and tibias of C57BL/6 mice and seeded in Petri dishes containing 10 ml of DMEM supplemented with 10% foetal bovine serum (FBS), 20% L929 cell conditioned medium, 1% HEPES, 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin. Cells were maintained at 37°C and 5% CO_2. Fresh supplemented medium (10 ml) was added after 4 days of culture. At day 7 of culture, the cells had completely differentiated into macrophages. Bone-marrow-derived macrophage cells (BMDMs) from C57BL/6 mice were obtained and infected with Mycma or Mycma_BfrA at a MOI of 50 or stimulated with 15 μg ml^{-1} of rapamycin in a 24-well plate for 4 h (5×10^5 cells per well). After that, cells were washed and lysed using M-PER Mammalian Protein Extraction Reagent (Thermo fisher, Waltham, MA, USA) supplemented with 1 mmol l^{-1} sodium orthovanadate, 10 mmol l^{-1} NaF and 1 : 100 of protease inhibitor cocktail (Sigma-Aldrich), and stored at –80°C. Then 20 μg of total protein from each sample was resolved in a 15% SDS-PAGE and the separated proteins were electrotransferred to a nitrocellulose membrane. Endogenous LC3 conjugation and autophagic flux were evaluated by western blot analysis using antibodies against LC3-I/II, p62 and beta-actin (Cell Signaling Technology, Danvers, MA, USA). To determine the CFU concentration, cells were washed twice after 4 h of infection, lysed with 1 ml per well of 0·1% of saponin diluted in saline, serially diluted and plated on LB agar supplemented with 100 μg ml^{-1} of kanamycin.

Cell culture of THP-1 monocytic and infection

THP-1 monocyte cell line was cultured in RPMI 1640 containing 10% heat-inactivated FBS, 23·8 mmol l^{-1} sodium bicarbonate, 2 mmol l^{-1} glutamine, 100 U ml^{-1} penicillin and 100 μg ml^{-1} streptomycin, and maintained at 37°C under 5% CO_2. THP-1 monocytic cells (8×10^5 cells per ml) were differentiated into macrophage in 24-well tissue culture flask in the presence of 5 ng ml^{-1} of phorbol 12-myristate 13-acetate (PMA) for 48 h at 37°C under 5% CO_2. Following differentiation, PMA-containing media were replaced with fresh media, and cells were rested for 24 h. After that, the medium was replaced with fresh media and the cells were infected with Mycma_BfrA or Mycma at a MOI of 5 : 1. Three hours after infection, the medium was replaced with fresh RPMI media supplemented with 64 μg ml^{-1} of amikacin to inhibit extracellular replication of Mycma, and the cells were incubated at 37°C under 5% CO_2 for 30 min. Following incubation, the medium was replaced with fresh media, and the cells were maintained at 37°C under 5% CO_2. The CFU determination during infection was made by recovering the intracellular bacilli at four different time points: 3 (0), 24, 48 and 72 h postinfection. At these times, the wells were randomly selected and from them, the supernatant was removed and substituted with ice sterile water to lyse macrophages. The lysate was plated onto LB-agar medium and incubated at 37°C for 7 days.

Intranasal infection of C57BL/6 IFN-γ-KO with Mycma_BfrA or Mycma

C57BL/6 IFN-γ-KO female mice, 6–8 weeks of age was obtained from the Instituto de Patologia Tropical e Saúde Pública from Federal University of Goiás animal facilities. All animals were housed according to the Conselho Nacional de Controle de Experimentação Animal (CONCEA) under the supervision of a Veterinarian. Twelve C57BL/6 IFN-γ-KO mice were divided into two groups of six mice each: Mycma_BfrA and Mycma. The animals were maintained in a HEPA filtered rack with water and food available *ad libitum*. The animals were intranasally infected with 10^7 CFU of the respective fresh culture of recombinant mycobacteria prepared in PBS with 0·05% Tween 80. On the first day after the infection, one animal from each group was killed by cervical dislocation by a trained veterinarian and the lung was collected to determine the bacillary load by plating whole-organ homogenate onto the LB-agar medium. Ten days following infection all animals were euthanized by cervical dislocation by a trained veterinarian, to determine the bacillary load by plating partial lung homogenate onto the LB-agar medium, and the right caudal lobe of the lung was collected for histopathological analysis. Furthermore, the liver and spleen were collected for macroscopic analysis. The animals were routinely (twice daily) inspected for mortality during the experiment. Animal health was monitored for the following signs: loss of appetite, dehydration, prostration or lethargy. A humane endpoint protocol to perform euthanasia of animals presenting any of the above symptoms was approved the Ethical Committee on the animal use of Universidade Federal of Goiás. None of the animals presented any of the above signs or died during the experiments.

Histopathological analysis

For the histopathological analysis of the lungs, the right caudal lobe of the lungs from each mouse was collected 10 days after infection and fixed with 10% buffered formalin.

Samples were sectioned into 5-mm-thick slices and stained with haematoxylin and eosin (HE) for analysis via microscopy (Axio scope.A1—Carl Zeiss, Jena, Germany). The following parameters were evaluated under the microscope at 40×, 100× and 400× magnifications: the intensity of the inflammatory infiltrate and the presence or absence of foamy macrophages and necrotic areas. To calculate the percentage of lesion area, photomicrographs of the entire lung fragments were analysed using AxioVision 4.9.1 software (Carl Zeiss), where the area of each lesion was measured and compared to the total area of the lung fragment.

Ethical committee

The study was approved by the Ethics Committee for Animal Use (CEUA: Comitê de Ética no uso de animais; 031/18 of the Universidade Federal de Goiás (UFG), Goiânia, Brazil.

Statistical analysis

The results were tabulated with Excel (ver. 16.16.19, 2016 for Mac). Comparison between means was assayed for variance and Sidak's multiple comparison test using Prism software ver. 7.0c (GraphPad). Values of $P < 0{\cdot}05$ were considered statistically significant.

Results

Cloning and purification of rBfrA

The amplified gene encoding Mtb BfrA (Rv1876) (Fig. 1a) was cloned into plasmid pET28a and the recombinant BfrA (rBfrA) was expressed in *E. coli* BL21 cells transformed with pET28a_Rv1876. Expression of the recombinant bacterioferritin (rBfrA) was performed in the presence of IPTG, and rBfrA was localized in whole-cell lysates. Metal affinity chromatography was used for the purification of rBfrA protein using Ni-NTA column, and purified protein was analysed by 12% SDS-PAGE. As shown in Fig. 1b, rBfrA was highly expressed and detected with a molecular weight of approximately 20 kDa. Mice were immunized with the purified rBfrA to obtain anti-BfrA polyclonal sera. To determine the specificity of anti-BfrA polyclonal sera, immunoblot analysis using purified rBfrA was carried out, and then it was used for characterization of recombinant Mycma_BfrA.

Construction of recombinant Mycma_BfrA

Mycobacterium abscessus subsp. *massiliense* was transformed with pMIP12_Rv1876, the *Mycobacterium/E. coli* shuttle vector containing bacterioferritin gene, and the recombinant cells were screened on LB medium containing 100 μg ml^{-1} of kanamycin. To confirm the presence of the gene encoding Mtb BfrA in the recombinant clones, colony PCR using specific primers to Rv1876 was employed. As shown in Fig. 2a, all five selected clones presented PCR products with the expected size of approximately 492 bp, whereas the negative control, Mycma transformed with empty pMIP12 vector, did not present any amplification.

In order to check the expression of rBfrA by recombinant Mycma_BfrA, immunoblot analysis of the cell lysates was performed using anti-rBfrA polyclonal sera. It was observed that the anti-rBfrA serum recognized BfrA in cell lysates obtained from Mycma_BfrA as well as the *M. tuberculosis* H37Rv.

BfrA expression improved the resistance of Mycma to iron toxicity

First, we questioned if the expression of rBfrA by *M. abscessus* subsp. *massiliense* would improve the ability of

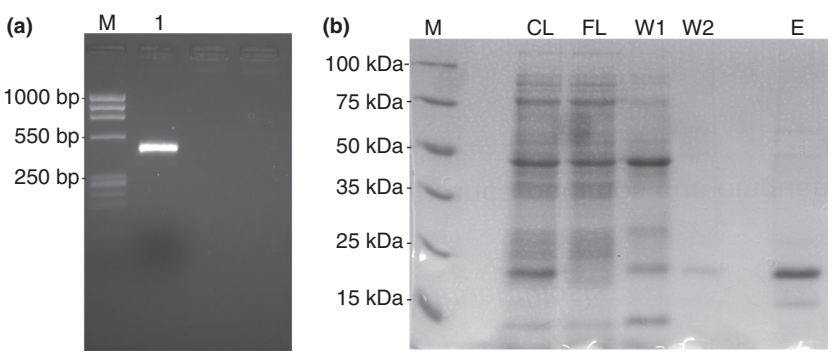

Figure 1 Cloning and purification of recombinant BfrA. (a) Amplified PCR product of the gene Rv1876 from *Mycobacterium tuberculosis* H37Rv was resolved and confirmed (~492 bps DNA sequence as expected) on 1·5% agarose gel. (b) 12% SDS-PAGE analysis of different purification fractions of rBfrA. M—protein marker Promega; CL—cells lysate; FL—affinity column flow-through fraction; W1 and W2: affinity column wash fractions; and E—elution fraction.

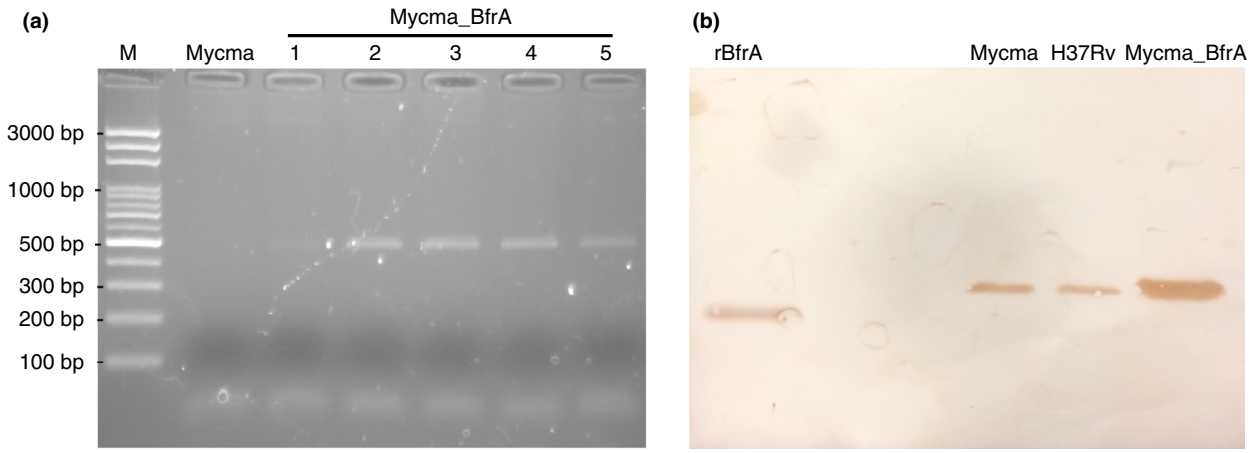

Figure 2 Recombinant Mycma_BfrA containing the Rv1876 gene express BfrA protein. (a) *Mycobacterium abscessus* subsp. *massiliense* recombinant clones transformed with pMIP12_Rv1876 were analysed for the presence of the Rv1876 gene by colony PCR. M—Amresco K180 DNA ladder; Mycma—*M. abscessus* subsp. *massiliense* transformed with empty pMIP12 vector; Mycma_BfrA 1-5: *M. abscessus* subsp. *massiliense* transformants with recombinant plasmid. (b) The expression of BfrA protein was detected in the Mycma_BfrA and *M. tuberculosis* H37Rv by the anti-BfrA polyclonal antibody. Cross-reactivity was observed by Mycma transformed with empty pMIP12 vector. [Colour figure can be viewed at wileyonlinelibrary.com]

this bacillus to resist iron-mediated toxicity. It was observed that when the cells were grown under 150 μmol l^{-1} of $FeCl_3$, the presence of BfrA protein in the recombinant Mycma improved its growth rate, starting at 3 days of growth (Fig. 3a). Similar behaviour was observed when the cells were grown in MM with higher iron concentration (450 μmol l^{-1} $FeCl_3$) (Fig. 3b). These results suggest that the expression of BfrA contributes to the growth of Mycma under iron-rich conditions.

Recombinant BfrA expression influence on the resistance of Mycma to oxidative stress

The host uses the oxidative stress to combat infection by pathogenic micro-organisms, and it has already been reported that bacterioferritins provide a key mechanism of protection against this host defence (Khare *et al.* 2017). To determine if the presence of recombinant bacterioferritin reduced the harmful effects of oxidative stress, Mycma_BfrA and Mycma strains growth were analysed in MM supplemented with 50 μmol l^{-1} of $FeCl_3$ containing various concentration of H_2O_2. It was observed that the presence of rBfrA in Mycma improved the growth of this bacillus under 3 mmol l^{-1} H_2O_2 and 50 μmol l^{-1} $FeCl_3$ from the second day of culture until the end of the observed incubation period (Fig. 4a). When the cells were subjected to 6 mmol l^{-1} H_2O_2 and 50 μmol l^{-1} $FeCl_3$ the growth of Mycma_BfrA was more pronounced during all observed time points in comparison to the Mycma control strain (Fig. 4b). Additionally, it was noticed that under less oxidative stress conditions (0·5 and 1·5 mmol l^{-1} H_2O_2, data not shown) the growth of both strains were similar.

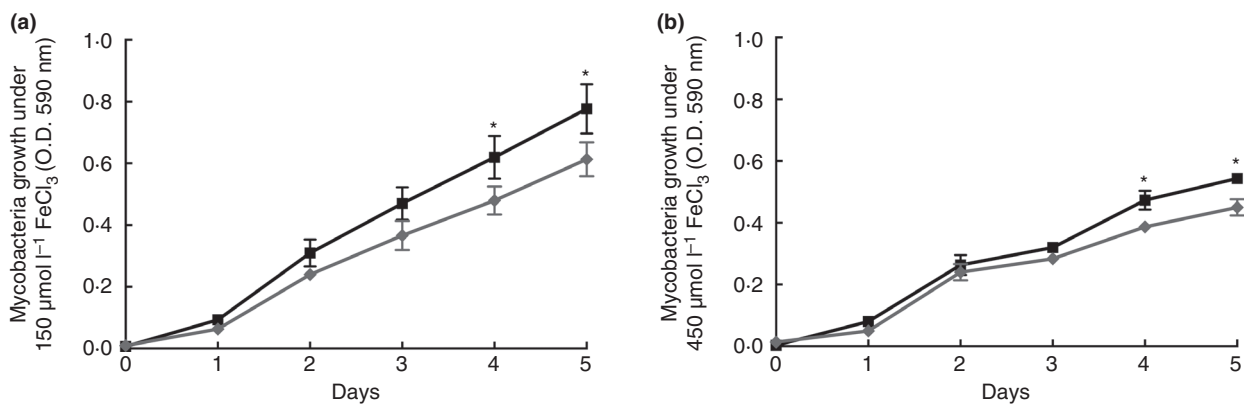

Figure 3 The influence of rBfrA expression on Mycma resistance to oxidative stress from iron. Mycma and Mycma_BfrA strains were grown in minimal medium supplemented with (a) 150 μmol l^{-1} $FeCl_3$ or (b) 450 μmol l^{-1} $FeCl_3$. The growth was monitored for 5 days by OD 590nm readings. **P* value <0·05 indicate statistically significant difference between groups (a,b: (—◆—) Mycma; (—■—) Mycma_BfrA).

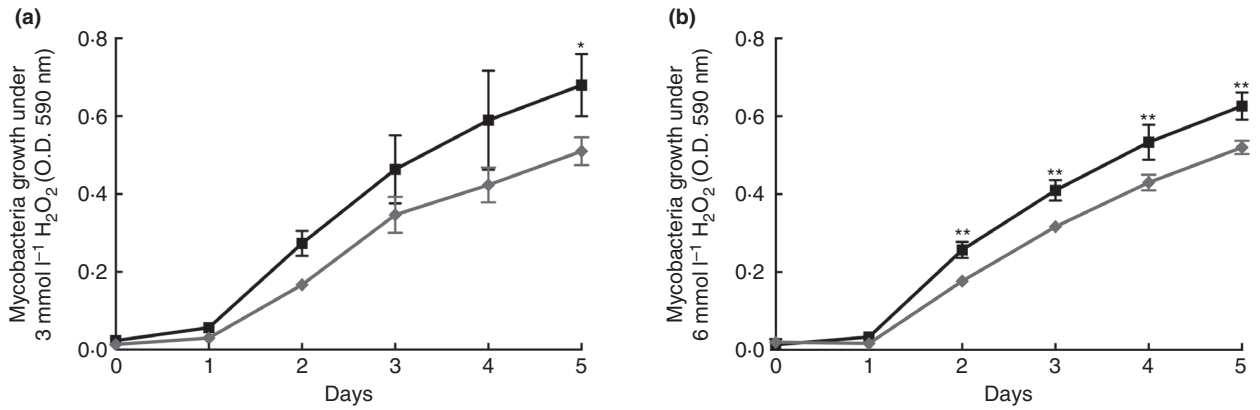

Figure 4 The expression of rBfrA by Mycma provides protection to bacillus against oxidative stress. Mycma and Mycma_BfrA strains were grown in minimal media supplemented with (a) 3 mmol l^{-1} H_2O_2 + 50 μmol l^{-1} $FeCl_3$ or (b) 6 mmol l^{-1} H_2O_2 + 50 μmol l^{-1} $FeCl_3$. The growth was monitored by OD 590nm readings. *, *P* value <0·05 and **, *P* value <0·01 indicate statistically significant difference between groups (a,b: (◆) Mycma; (■) Mycma_BfrA).

The results suggest that the presence of BfrA may be an advantage for the bacillus growth mainly under high oxidative stress conditions.

The autophagy flux and intracellular growth in macrophages were similar between the Mycma strains

Macrophage is the principal host cell that harbours mycobacteria and has bactericidal ability of defence to destroy the bacilli, as for example, by production of oxidative stress and autophagy. Autophagy is a significant host defence mechanism against intracellular pathogens, and it has already been demonstrated that *M. abscessus* induces this response. However, *M. abscessus* inhibits the fusion of autophagosomes with lysosomes thereby favouring the bacterial growth inside of macrophages (Gutierrez *et al.* 2004; Roux *et al.* 2016; Kim *et al.* 2017; Kim *et al.* 2019). To assess if the expression of BfrA could improve the ability of inhibition of autophagosomes formation by Mycma, the autophagy analyses were performed. After 4 h of murine macrophage infection, it was observed an increase in LC3-II formation following Mycma strains infection, with an apparent superior accumulation by the cells infected with Mycma_BfrA as compared with Mycma (Fig. 5a). However, when the expression levels of p62 were analysed it was observed a high accumulation of this protein regardless of the presence of BfrA, demonstrating that both strains were capable to inhibit autophagosome–lysosome fusion (Fig. 5a). This result showed that the expression of BfrA by Mycma does not influence in the autophagy response or in the inhibition of autophagosome–lysosome fusion.

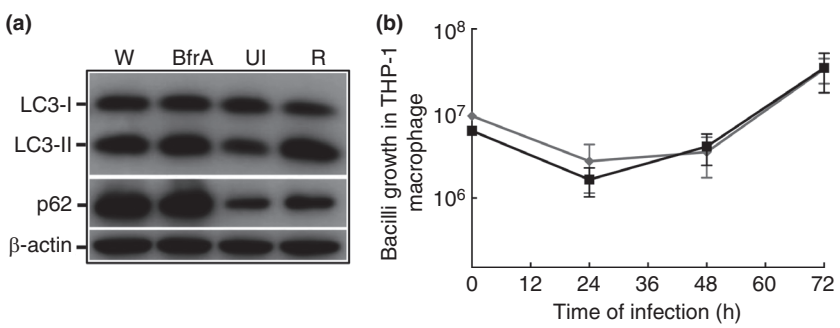

Figure 5 The autophagy induction and the inhibition of autophagy flux in murine macrophages were similar between the Mycma strains. BMDMs were infected with Mycma or Mycma_BfrA for 4 h at MOI 50. (a) After 4 h of infection the cells were washed and then were lysed for immunoblotting with anti-LC3, anti-p62 and anti-β-actin antibodies. W, Mycma wild-type; BfrA, Mycma_BfrA; UI, uninfected; R, rapamycin. (b) The growth of Mycma and Mycma_BfrA was similar during of infection. Differentiated THP-1 macrophage cells were infected with Mycma or Mycma_BfrA at a MOI of 5. After 3 h of infection the cells were harvested and the intracellular growth was quantified at 3 h (0 h), 24 h, 48 h and 72 h by plating on LB medium (b: (◆) Mycma; (■) Mycma_BfrA).

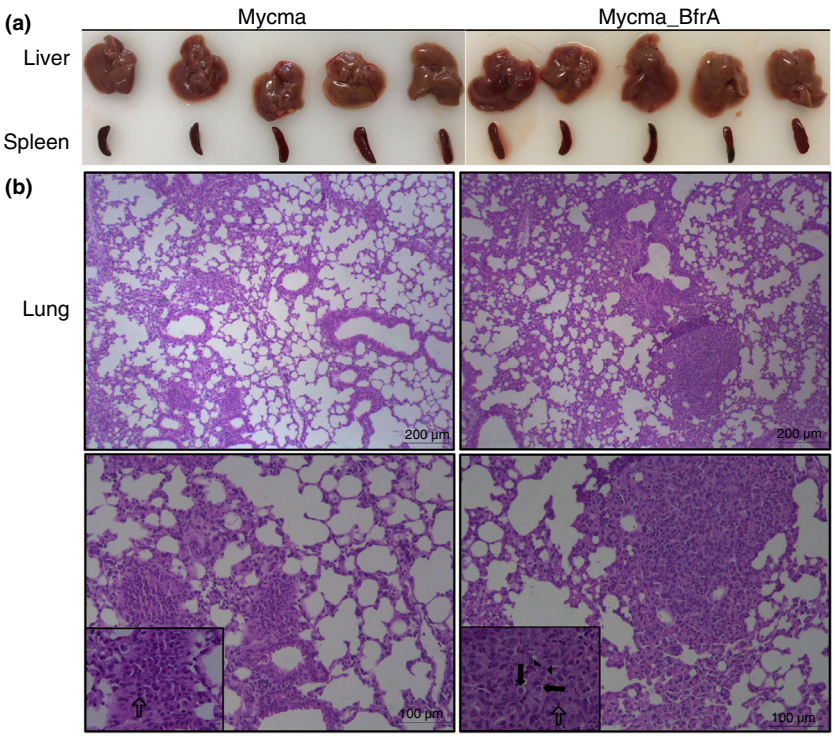

Figure 6 Mycma expressing bacterioferritin was more pathogenic in the mouse model of infection. After 10 days of intranasal infection, the C57BL/6 IFN-γ-KO mice were euthanized and the organs were collected for macroscopic and microscopic evaluation. (a) Macroscopic analysis of the liver and spleen from C57BL/6 IFN-y-KO mice 10 days after Mycma or Mycma_BfrA infection. Mice infected with Mycma_BfrA presented splenomegaly and coagulum after 10 days of infection. (b) Histological analysis the lungs of mice infected with Mycma (images at left) or Mycma_BfrA (images at right) after 10 days of infection. The inset is a high magnification (400×) demonstrating the distinct inflammatory infiltrate composition between the lungs of mice infected with Mycma and Mycma_BfrA. The lungs of Mycma_BfrA infected mice presented large inflammatory infiltrates with an accumulation of foamy macrophages (arrows), neutrophils (arrowhead) and mononuclear cells (open arrows), while the lungs of mice infected by Mycma presented mostly mononuclear cells (open arrows). [Colour figure can be viewed at wileyonlinelibrary.com]

To determine if the growth performance of Mycma_BfrA could be different, the THP-1 macrophage cells were infected and the bacillary load was determined. The intracellular growth of both mycobacteria was similar, suggesting that the expression of recombinant bacterioferritin by Mycma does not improve the capacity of this mycobacterium to subvert the defence mechanisms of host (Fig. 5b).

Mycma_BfrA infection induces severe lesions in mice

Several other mechanisms than those involved in bacterial growth in macrophages are accounted for the host–pathogen responses. Thus, we hypothesize that the presence of bacterioferritin could improve the ability of Mycma to overcome the host distinct defences. To determine if the expression of bacterioferritin could increase the pathogenicity of Mycma, after 10 days of infection the liver, spleen and lungs were collected for macroscopic and microscopic analyses. Macroscopic analyses demonstrated that the spleen of the mice infected with Mycma_BfrA presented discrete splenomegaly and evident coagulum on the organ surface, whereas the group infected with the Mycma did not show apparent pathology (Fig. 6a). Histological analyses of the infected mice lungs showed that the infection by Mycma or Mycma_BfrA strains were capable of inducing diffuse lung inflammation, where the architecture was compromised (Fig. 6b). However, when the lung lesion area was analysed, it was observed that infection by Mycma_BfrA resulted in a significantly higher compromised lung tissue architecture in comparison with the lung of mice infected with Mycma (Fig. 7a). Furthermore, mice infected with Mycma_BfrA had large inflammatory agglomerates with mononuclear cells, foamy macrophages, neutrophils and necrosis (Fig. 6b). The results demonstrated that the expression of bacterioferritin by Mycma_BfrA increased its capacity to induce lesions in the lungs, suggesting that this protein could favour the pathology caused by mycobacteria.

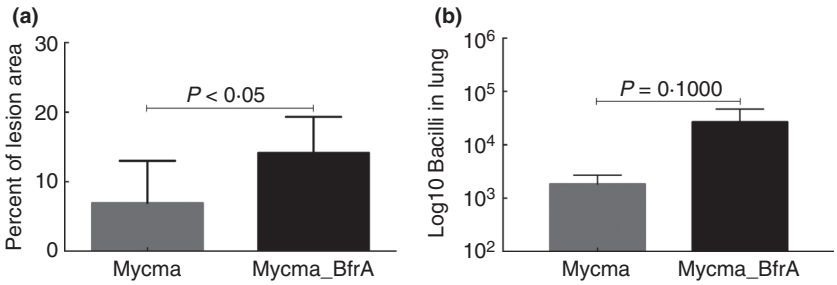

Figure 7 The expression of recombinant bacterioferritin by Mycma induces more lesions in the lungs and improved the bacterial growth in mice. C57BL/6 IFN-γ-KO mice intranasally infected with 10^7 CFU of Mycma_BfrA or Mycma were euthanized after 10 days of infection. (a) Histological score of the lesion area from all fields obtained by AxioVision 4.9.1 software, through ratio of lesioned and total field area. Data are presented as percentages. The lungs of mice infected by Mycma_BfrA presented more lesions as compared with the Mycma group ($P < 0{\cdot}05$). (b) The lungs of infected mice were collected and the bacillary load was determined by plating the homogenates on LB medium. It was observed that the bacillary load in the lungs from mice infected with Mycma_BfrA was higher as compared with Mycma strains ($P = 0{\cdot}1000$).

The increased lesions in the lungs could favour Mycma_BfrA growth and dissemination from the site of infection, consequently the bacillary load was determined. As shown in Fig. 7b, 10 days postinfection, the bacillary load of Mycma_BfrA in the lungs of C57BL/6 IFN-γ-KO mice was about 10 times higher than the observed for mice infected with Mycma. These results demonstrate that the presence of bacterioferritin in the Mycma could provide additional conditions for this bacillus to grow in the mouse model of infection.

Discussion

The importance of bacterioferritins in the virulence and pathogenicity of *Mycobacterium* remains unclear, although some studies have proposed a role of this protein in the iron homeostasis of the bacillus (Pandey and Rodriguez 2012; Reddy *et al.* 2012; Khare *et al.* 2017). The influence of bacterioferritin during host infection was suggested to be dispensable, as mutants for this protein presented similar behaviour to the parental strains in different models of infection (Pandey and Rodriguez 2012; Reddy *et al.* 2012). All studies to understand the role of bacterioferritin on the virulence and pathogenicity of mycobacteria conducted until now were done by generating knockout mutants for this protein (Pandey and Rodriguez 2012; Reddy *et al.* 2012). In this study, we developed a recombinant *M. abscessus* subsp. *massiliense* that expressed a recombinant bacterioferritin from Mtb, since Mycma does not have any gene encoding for bacterioferritin (Oliveira *et al.* 2018). Our results show that the expression of rBfrA by Mycma improved the ability of this mycobacterium to resist the harmful effects of excess iron and high levels of hydrogen peroxide. This improvement reflected in increased resistance of Mycma_BfrA to the mechanisms of defence during murine infection, resulting in worse lesions in the lung of mice.

Analyses made in the *M. abscessus* subsp. *massiliense* genome to find a gene homologous to *M. tuberculosis* Rv1876 showed that this *Mycobacterium* does not have a gene encoding bacterioferritin. Instead, we described previously that this bacillus has two functional ferritins (Oliveira *et al.* 2018). In this study, when immunoblot analysis was carried with the lysates derived from *M. tuberculosis* H37Rv and Mycma_BfrA using polyclonal anti-BfrA serum, it was observed the presence of a band with the same migration mobility expected for BfrA, confirming the expression of the recombinant protein (Fig. 2b). However, it was also observed some cross-reactivity with lysates from wild type Mycma, with the same mass to other lysates. We have shown by genomic annotation and PCR (Fig. 2a) that *M. abscessus* does not have a gene coding for bacterioferritin (Oliveira *et al.* 2018). Probably, Mycma have proteins with similar epitopes present in the bacterioferritin that were recognized by the raised anti-BfrA serum.

The studies made to define the involvement of the bacterioferritin in maintaining the homeostasis of Mtb has shown that this protein does not have a crucial role in the resistance to oxidative stress generated by high levels of iron (Pandey and Rodriguez 2012; Khare *et al.* 2017). In this study, we observed a positive influence of bacterioferritin expression on the growth of Mycma under normal and high levels of iron (Fig. 3). Feasibly, the different phenotype observed in our study is related to BfrA expression levels in the recombinant Mycma_BfrA. In *M. tuberculosis* the expression of bacterioferritin is under control of two promoters, one responds to low iron levels (P_{Low}), whereas the other to high levels (P_{High}). It was observed that the expression of BfrA induced by IdeR binding at P_{High} under iron excess is modest as compared to BfrB (Gold *et al.* 2001; Rodriguez *et al.* 2002). Moreover, it was reported that an IdeR mutant does not express BfrA or BfrB, which increased the sensibility to

iron excess. Interestingly, when the IdeR mutant was transformed with a plasmid carrying Rv1876 transcribed from an IdeR independent promoter, the ability of the mutant to grow in high iron was restored (Pandey and Rodriguez 2014). That observed phenotype suggested that when BfrA is expressed constitutively, the protein level is sufficient to reduce the iron toxicity, whereas expression induced by IdeR at P_{High} seems to reach unsatisfactory protein levels. This modest expression of BfrA could explain why the presence of this bacterioferritin alone is not capable to maintain the growth of the BfrB-KO mutant under iron excess (Pandey and Rodriguez 2012; Khare *et al.* 2017). In this study, the Rv1876 was cloned into the pMIP12 plasmid which has the up-regulated *Mycobacterium fortuitum* promoter pBlaF* and an optimized Shine-Dalgarno sequence (Mega SD) that allows strong ribosomal attachment and consequent expression (Lagranderie *et al.* 1997; Le Dantec *et al.* 2001). We hypothesized that in this condition, the levels of BfrA expression was satisfactory to confer protection against iron toxicity for Mycma_BfrA (Fig. 3).

When the impact of BfrA expression on the growth rate of Mycma_BfrA under high concentrations of hydrogen peroxide was investigated, it was noticed that there was an improved resistance to those oxidative stress conditions that was greater when the cells were subjected to 6 mmol l^{-1} of H_2O_2 and 50 μmol l^{-1} of $FeCl_3$. Bacterioferritin was shown to confer protection to bacilli against oxidative stress deleterious effects (Khare *et al.* 2017). Recently, a study demonstrated that BfrA from Mtb has catalase and DNA protection activities that could be involved in the resistance of Mtb to oxidative stress (Mohanty *et al.* 2019). In this study it was observed that expression of rBfrA by Mycma resulted in enhanced growth under stressful conditions, supporting the probable Dps-like and catalase action of this protein to reduce damage against the bacilli. Also, it has been described that ferritins have the important function to reduce the oxidative damage by hydrogen peroxide in Mtb (Pandey and Rodriguez 2012, 2014; Khare *et al.* 2017). In our study the protective effect of bacterioferritin was only observed with high hydrogen peroxide concentrations leading us to believe that the presence of two ferritins in Mycma may confer a higher threshold level of resistance to stressful conditions (Oliveira *et al.* 2018). During macrophage infection, an oxidizing environment is created that favours the growth of *M. abscessus* (Oberley-Deegan *et al.* 2010), possibly due to the presence of the ferritins.

Our results imply that the expression of rBfrA by Mycma_BfrA confers advantages to the bacillus growth under extremely stressful *in vitro* conditions, such as in high iron and hydrogen peroxide concentrations. To address if these advantages could result in improved resistance of Mycma_BfrA to the host-innate immunity defences during infection, the capacity of this bacillus to establish infection was evaluated. The autophagy is a crucial host defence mechanism against intracellular pathogens, and it was demonstrated that different strains of *M. abscessus*, showing different phenotypes, induces distinct autophagy responses and has a variable intracellular growth success in macrophages, that were associated with the mycobacterial virulence (Gutierrez *et al.* 2004; Roux *et al.* 2016; Kim *et al.* 2017; Kim *et al.* 2019). The endogenous LC3 conjugation is a marker of autophagy induction, and the p62 protein degradation is related to the autophagosome–lysosome fusion and with mycobacterial killing (Gutierrez *et al.* 2004; Ponpuak *et al.* 2010; Watson *et al.* 2012). Here we have demonstrated that Mycma_BfrA infection induces slightly increased levels of endogenous LC3-II protein in murine macrophage, as compared to Mycma. Thus, this stronger autophagy response indicator by Mycma_BfrA could also be related to more inhibition of autophagosome–lysosome fusion. However, infection with either Mycma or Mycma_BfrA strains presented similar accumulation of p62 protein in murine macrophages. Consequently, Mycma and Mycma_BfrA intracellular survival were similar in murine and THP-1 macrophages (Fig. 5b), showing that BfrA expression by Mycma did not improve the resistance of this *Mycobacterium* to the initial host defence mechanism. Remarkably, Mycma_BfrA was capable to induce more macroscopic and microscopic changes in the spleen and lungs tissues, as compared to wild-type Mycma (Figs 6 and 7a). Furthermore, the growth of Mycma_BfrA in the lungs of intranasal model of infection was higher than Mycma (carrying the empty plasmid) (Fig. 7b), although the difference was not statistically significant. This result suggested that expression of recombinant BfrA by Mycma during infection apparently increased the virulence of this bacillus.

Based on our findings, it is reasonable to propose that the bacterioferritin has a role in the virulence of mycobacteria. The studies made until now have little divergencies in their results, but almost all assume that BfrA has a dispensable role for the mycobacterial growth in the host, whereas the lack of BfrB impaired the establishment of infection by Mtb (Pandey and Rodriguez 2012; Reddy *et al.* 2012; Pandey and Rodriguez 2014). Our work brings new insights about the role of bacterioferritin in the virulence of mycobacteria using Mycma as a model, through genetic approaches not used until now for this propose. Nonetheless, more studies are necessary to define the mechanism by which the bacterioferritin contributes to the mycobacterial virulence. In conclusion, expression of recombinant bacterioferritin by Mycma conferred advantages to the bacilli growth under

stressful conditions *in vitro* and increased the pathogenicity *in vivo*.

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Conflict of Interest

The authors declare no conflict of interest.

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