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ORIGINAL RESEARCH ARTICLE

The *mycma_1113* Gene from *Mycobacterium abscessus* subsp. *massiliense* is Related to Siderophore Synthesis

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Abstract Iron (Fe) homeostasis control is important for both pathogen and the host. During infection, the host reduces the access of microorganisms to iron, however, studies have shown that virulent pathogens are capable to sequester Fe from host proteins, and establish the infection. *M. abscessus* subsp. *massiliense* (Mycma), that is resistant to most drugs used against tuberculosis, was responsible for outbreaks around the world showing increased virulence when compared to other rapidly growing mycobacteria. The goal of this study was to determine whether Mycma produce siderophores and if the *mycma_1113* gene expression, a putative homolog of *M. tuberculosis mbtB* gene located in the *mbt* gene cluster, is related to the synthesis of these molecules. For that, the effect of different iron concentrations on the growth of Mycma, the expression of *mycma_1113* gene, and the production of siderophores was evaluated in vitro and in vivo. It is shown that Mycma produce siderophores under iron deprivation conditions and *mycma_1113* gene expression was influenced by iron availability. The *mycma_1113* gene expression was also increased after macrophage or in vivo infection indicating that mycobactin synthesis by Mycma could participate in the Fe sequestration from the host during infection. In conclusion, we show that Mycma produces siderophores under iron deprivation conditions and that the *mycma_1113* gene is involved in this process, furthermore, this gene expression is induced during infection.

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Introduction

Mycobacterium abscessus subsp. *massiliense* (Mycma) is clustered into the *M. abscessus* complex and has caused major concerns because of its emergency as hospital-associated infections worldwide [1–3]. Bacteria belonging to the *M. abscessus* complex are resistant to most antibiotics available, including those used in the treatment of tuberculosis, such as ethambutol and fluoroquinolone, making treatment of infections caused by this group extremely difficult [4].

Iron is an essential element for the bacterial metabolism and, during mammalian host infection, the availability of this metal is scarce due to nutritional immunity [5, 6]. Consequently, pathogenic bacteria exhibit a variety of mechanisms to compete for the insoluble form of the iron ion (ferric, Fe^{3+}) and establish infection [7]. One of these mechanisms is siderophore production, which acts as chelating molecules and bind with high affinity to iron [8]. *M. tuberculosis* (Mtb) has two types of siderophores known to be produced under conditions of iron scarcity: mycobactin and carboxymycobactin [9, 10]. The production of such molecules is carried out in *M. tuberculosis* and *M. smegmatis* by an apparatus encoded by two gene clusters, *mbtA-J* [11] and *mbtK-N* [12]. During infection of macrophages or mice, *M. tuberculosis* genes involved in the synthesis of mycobactins are up-regulated where *mbtB* gene was shown to be important for infection [13–17]. *M. tuberculosis* mutant without *mbtB* gene (Δ*mbtB*) was unable to produce mycobactin or carboxymycobactin

which impaired its ability to grow under low concentration of iron in vitro or in infected macrophages [18].

Mycma was shown to have increased virulence compared to other RGMs [19], thus this bacillus might have apparatus to resist the host's mechanism of defense [20]. Previous study showed that impairment of *M. abscessus* iron metabolism with inhibitory drugs was deleterious, supporting the essentiality of this ion for its development [21]. Our group previously demonstrated, by in silico studies, that Mycma could have genes involved with the production of siderophores [22]. However, the composition and organization of the genes that make up the genetic cluster, possibly involved in the synthesis of siderophores in the *M. abscessus* complex, differs significantly from other mycobacteria [23], where their function have already been characterized [6, 18]. However, it is not clear yet if the putative *mbt* gene cluster of Mycma is functional. The goal of this study was to determine whether Mycma produce siderophores and if the *mycma_1113* gene expression is related with the synthesis of these molecules.

Materials and Methods

In Silico Analysis

The *M. abscessus* subsp. *massiliense* GO06 genome [24] was used for analysis in this study and all the sequences analyzed were obtained from the NCBI database.

Iron Influence on *M. abscessus* subsp. *massiliense* Growth and *mycma_1113* Expression In Vitro

The influence of iron on Mycma growth and expression of the *mycma_1113* gene was done as previously described [25] using modified Sauton media. Iron excess condition was achieved with 150 µM of iron chloride ($FeCl_3$) supplementation, while iron deprivation condition was obtained with 1.25 mM deferoxamine mesylate (DFO) (Sigma-Aldrich, St Louis, MO, USA). Mycma was grown for five days at 35 °C and monitored spectrophotometrically at 600 nm. Bacterial culture concentration was determined by plating serial dilutions on Mueller Hinton agar. At the end of growth, bacterial cells were harvested by centrifugation and the pellet was used for RNA extraction.

RNA Extraction, cDNA Synthesis and Real-Time PCR

RNA extraction from bacterial cultures and cDNA synthesis were performed according to Oliveira et al. [25]. The RT-PCR amplification was carried out using the obtained cDNA as template. For that, oligonucleotide primers specific for the *mycma_1113* (target gene) and the 16S rRNA genes were used. The forward and reverse primers for *mycma_1113:* mycma_1113 Fwd (5′-CGGTAGAGCCCGATGTGAAG-3′) and mycma_1113 Rev (5′-CGTTGACCACCTCTGACTCC-3′) amplified a 141-bp fragment; for 16S rRNA, the primers 16S-Fwd (5′-AGCTCGTAGGTGGTTTGTCG-3′) and 16S-Rev (5′-AATCCTGTTCGCTACCCACG-3′) amplified a 214-bp fragment. As a negative control, RNA without AMV reverse transcriptase was used.

Real-time PCR was performed using the SybrGreen probe (Biorad, Hercules, CA, USA) for all samples. The reactions were carried out in the Rotor Gene thermocycler (Qiagen) with a temperature profile of 95 °C for 5 min, followed by 45 cycles at 95 °C for 15 s, 58 °C for 30 s, and 72 °C for 60 s. Melting curve of the PCR products were obtained at the end of PCR with temperatures ranging from 70 to 99 °C. Ct values were tabulated on an Excel 2011 spreadsheet, and the Delta Delta Ct (2^{-Ct}) method was applied using the expression of the 16s rRNA gene as normalizer. The calibrator group in this study was the experiment group grown in minimal media without DFO and $FeCl_3$, as these conditions contain sufficient iron levels to support mycobacteria growth. The values of (2^{-Ct}) obtained each sample was applied at log(2^{-Ct};2) formula for determined differential expression.

Overlay Chrome Azurol-S Assay (O-CAS)

Mycma was inoculated onto solid modified Sauton media supplemented or not with 150 µM of iron chloride. The CAS-medium (0.1 M chrome azurol S, 0.2 M HDTMA, 0.1 M Tris-HCl pH 6.8, 0.01 mM $FeCl_3$, 10 mM HCl and 9 g/L of agarose) was prepared according to Perez-Miranda et al. [26] with modifications. CAS overlay was applied over the bacterial culture and after a period of about 4 h the color change was observed.

Mice Air Pouch Formation and Mycma Infection

C57BL/6 mice were divided into 3 groups, with 5 animals per group. In DFO group, the mice were submitted to iron deprivation conditions by treatment with 1 mg/kg/day of

DFO. In Iron group, mice were submitted to excess iron conditions by treatment with 50 mg/kg/day of iron dextran solution (Tortuga, Santo Amaro, SP, Brazil) [27]. The control group (PBS) was treated with buffered phosphate saline. Each group was treated with intraperitoneal injections every other day for 19 days. On the 11th day, the animals received the sixth dose of treatment and the air pouches formation was initiated as described by Gaspar et al. [28]. The created pouches were allowed to settle for 3 days to permit wound healing. The pouch was then re-inflated with 5 mL of sterile air and left for 3 more days before infection. On day 17, the ninth treatment dose was administered and mice were infected with 3×10^6 Mycma per mouse through the formed pouches. After 3 days of infection (day 20), the animals were euthanized, the air pouches were washed with 3 mL of PBS, and the recovered fluid was stored in sterile tubes for RNA extraction.

Bone Marrow Derived Macrophages (BMMO) Obtention and Infection

To evaluate *M. abscessus* subsp. *massiliense* gene expression during macrophage infection, C57BL/6 mice were used to obtain BMMO according to Becker et al. [29], which were differentiated according to da Costa et al. [30]. Differentiated BMMO were plated in a 24 well plate at the concentration of 10^6 per well and infected with a fresh culture of Mycma at a MOI of 10. After 3 h of infection, extracellular mycobacteria were removed by washing the wells twice with PBS. To evaluate the expression profile of the *mycma_1113* gene during infection, the bacilli were recovered at three different times: 24, 48, and 72 h after infection. At these time points, RPMI medium was collected and nuclease-free water (Ambion Life Technologies, Carlsbad, CA, USA) was added to lyse macrophages. The lysate was centrifuged at $16{,}000 \times g$ for 10 min at 4 °C, the supernatant was discarded and the pellet separated for RNA extraction and gene expression quantification by RT-PCR. The expression of *mycma_1113* gene was determined with Ct using the expression of the 16s rRNA gene as the normalizer.

Statistical Analysis

Statistical significance between groups was determined by the two-tailed unpaired Student's *t* test or One-way ANOVA with Dunnett post-test using Prism software version 7 (Prism version 7a, GraphPad).

Ethics Statement

The protocols for animal use in this study were previously approved by the Ethical Committee (001/07 protocol) from Universidade Federal de Goiás, Brazil in accordance with the guidelines of the Conselho Nacional de Controle de Experimentação Animal (CONCEA- Ministério da Ciência e Tecnologia - Brazil).

Results and Discussion

Iron is Important for the Development of *M. abscessus* Subsp. *massiliense*

It has been demonstrated that iron is essential for mycobacteria growth and infection [5, 31, 32]. Thus, to determine if this ion is important for Mycma, the growth of this bacillus was evaluated in different iron concentrations. It was observed that under conditions of high concentrations of iron (150 µM) bacterial growth was significantly higher when compared to the metal deprivation conditions (DFO) (Fig. 1). After five days of culture, the bacteria that grew in medium supplemented with 150 µM of iron reached concentrations of 10^{10} CFU/mL, whereas in the absence of this ion this growth was 1000 times lower (10^7 CFU/mL). Thus, *M. abscessus* subsp. *massiliense* require iron for an appropriate development.

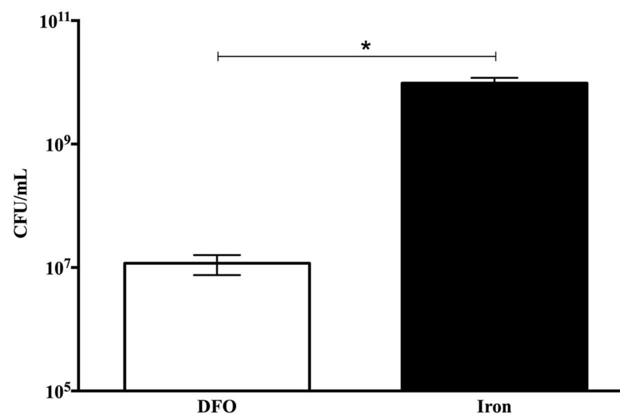

Fig. 1 Growth and survival of *M. abscessus* subsp. *massiliense* in depletion and in presence of iron. *M. abscessus* subsp. *massiliense* was grown in modified Sauton media with 150 µM $FeCl_3$ supplementation (Iron) or in deprivation iron condition obtained with 1.25 mM DFO (DFO). The bacterial culture concentration was determined by plating on Mueller Hinton agar and the colony forming units (CFU) was determined on the seventh day of growth in this plates. Data shown represents the mean ± SEM, n = 4. $*p < 0.001$ by *t* test

Under Iron Scarcity Conditions, *M. abscessus* subsp. *massiliense* Overexpressed the Gene Similar to mbtB and this Expression is Related to Siderophore Production

In order to determine whether Mycma could produce siderophores, searches were performed against the genome of the bacillus for genes with significant similarities to the *M. tuberculosis mbtA-J* gene cluster important for mycobactin synthesis. It was observed the Mycma has ten genes with similarities to *mbtA-J* cluster, but these genes have a different organization (Fig. 2). Moreover, this bacillus does not have the gene with similarity to the *mbt-J* gene, but instead has two copies of the *mbtE* gene. Mycma have a gene similar to the *mbtT* gene (Fig. 2) from *M. smegmatis*, shown to be important for the production of siderophores and absent in Mtb [23]. This analysis demonstrated the *M. abscessus* subsp. *massiliense* have similar *mbtA-J* gene cluster, but the composition and organization is different and more closely related to the *mbtA-J* gene cluster from *M. smegmatis* when compared with *M. tuberculosis* (Fig. 2). It has been demonstrated that *mbtB* gene expression plays a key role in siderophore synthesis [9, 18]. Thus, our analysis revealed that *mycma_1113* gene had 65% identity to the *mbtB* gene of Mtb. To determine if this putative *mbt* gene cluster found in the Mycma genome could be involved in the production of siderophore, the expression of *mycma_1113* gene was evaluated in vitro in different conditions of iron concentrations. It was observed that this gene was expressed both under conditions of scarcity and excess of Fe. Nonetheless, its expression was significantly higher during Fe deprivation (Fig. 3). These data suggest that the *mycma_1113* gene is modulated at different iron concentrations, being induced in the absence of this ion and repressed under conditions of excess (Fig. 3). Therefore, the *mycma_1113* gene expression is possibly regulated according to the iron availability in the medium. Possibly, *mycma_1113* gene could be negatively regulated under iron excess conditions in a similar way as iron-dependent regulator (IdeR) regulates *mbtB* gene

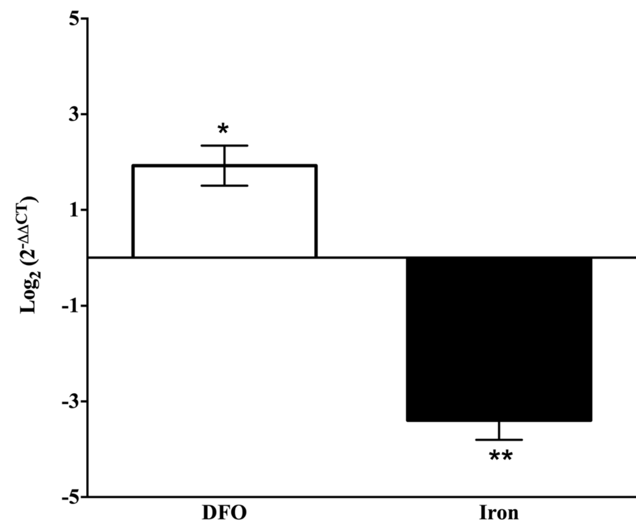

Fig. 3 In vitro *mycma_1113* relative expression from *M. abscessus* subsp. *massiliense* in different iron conditions. *M. abscessus* subsp. *massiliense* was grown in Sauton media with (DFO) or without iron chelator or in medium supplemented with 150 µM iron (Iron). After five days the bacteria culture was harvested, the RNA extracted and the expression of *mycma_1113* normalized by 16S rRNA expression was determined. The expression shown is relative to the control group grown in Sauton media without chelator or supplementary iron. Data shown represents the mean ± SEM, n = 4. *$p < 0.05$; **$p < 0.01$; difference from control group by One-way ANOVA with Dunnett post test

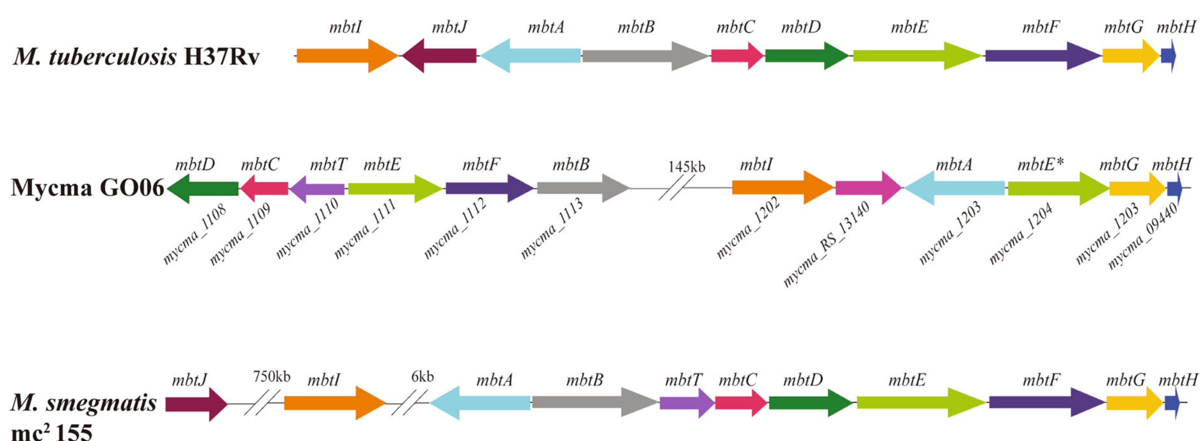

Fig. 2 Illustration of the predicted *mbt-I* gene cluster from Mycma and *mbt-I* gene clusters orthologs from *M. tuberculosis* H37Rv and *M. smegmatis* mc^2 155 strains. The possible *mbt-I* genes from Mycma are color coded according to the homolog gene from *M. tuberculosis mbt-I* gene cluster. The analysis in the genome of Mycma revealed a *mbt-I* gene cluster, but the genes are arranged differently. Moreover, Mycma does not have the gene with identity with *mbtJ*. Instead of that, it is observed the Mycma has a gene with identity to *mbtT* gene present in the *M. smegmatis* mc^2 155. Two possible copies of the gene with similarity to *mbtE* from Mtb was identified, *mbtE** (*mycma_1204*) and *mbtE* (*mycma_1111*)

expression in Mtb [33]. Conversely, under iron scarcity conditions, the *mycma_1113* gene would be induced similarly to the genes involved in the synthesis of siderophores in *M. tuberculosis* [34, 35]. In this way, we suggest that the *mycma_1113* gene, which has similarity and expression profile similar to *M. tuberculosis mbtB*, could be involved in the siderophore production in Mycma. In order to determine whether Mycma actually produces siderophores, the Chrome Azurol assay (CAS) was performed at different iron concentrations. CAS assay confirmed the siderophore production by Mycma under iron scarcity conditions (Fig. 4). There was no siderophore production under conditions of excess of this ion (150 µM $FeCl_3$) (Fig. 4). In addition, the production of siderophores by Mycma was observed to occur concomitantly with the *mycma_1113* gene expression (Figs. 3, 4).

The *mycma_1113* Gene is Overexpressed Under In Vivo Iron Deprivation Conditions

It has already been demonstrated that during pathogen infection the host reduces available iron levels through nutritional immunity, while successful pathogens produce siderophores to sequester this ion [32, 31]. Thus, to determine if the *mycma_1113* gene could be involved in the subversion of this immunity and consequent access to iron, we evaluated the gene expression in in vivo conditions. A localized and controlled infection using the air pouch system was made where the environment conditions (such as iron availability) at the site infection could be easily made. Although the infection itself induces the host to restrict iron availability, we wanted to ensure that the mycobacteria would be in an environment without iron, and consequently mice were treated with DFO. It was observed that during infection under iron restriction conditions, the *mycma_1113* gene was induced eight times higher when compared to physiological conditions of this metal availability (Fig. 5). These results suggests the possible involvement of this gene during infection, in a similar way as observed in the expression of *mbtB* mRNA levels in mice infected with Mtb [16, 17].

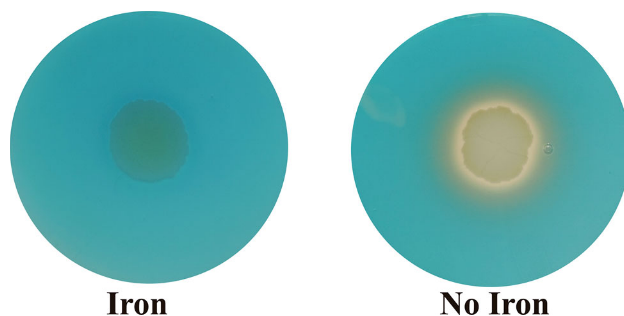

Fig. 4 Overlay Chrome Azurol-S (O-CAS) Assay was used for detection of siderophore production by *M. abscessus* subsp. *massiliense*. Bacteria were grown on modified Sauton agar plate with 150 µM $FeCl_3$ (left, Iron) or without iron supplementation (right, No Iron). After growth, O-CAS was added to the plates. The formation of a halo around the colonies due to overlay color change from blue to yellow indicates siderophores production in culture without iron supplementation (right)

The *mycma_1113* Gene is Expressed During Macrophage Infection

Several studies have demonstrated that macrophages are able to reduce iron levels during mycobacterial infection, making it difficult to establish infection [36]. Therefore, we evaluated the expression of the *mycma_1113* gene in a macrophage infection model at three different infection times. It was observed that the expression of the *mycma_1113* gene peaked during the first 24 h of the infection and that after this period it tended to decrease (Fig. 6), whereas the bacillary load increased as showed by Oliveira et al. [25]. Gold et al. [33] observed in their studies that in the first 24 h of infection, the *mbtB* gene expression from *M. tuberculosis* was also increased, but different from what we observed in this study, its expression remained increased 72 h after infection. The difference in expression observed between Mycma and Mtb is probably related to

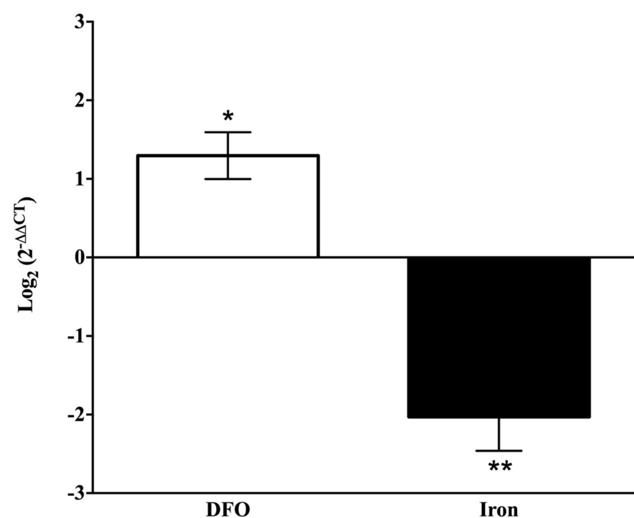

Fig. 5 Relative expression of *mycma_1113* in vivo. C57BL/6 mice were subjected to iron depletion (DFO), iron dextran supplementation (Iron) or a PBS control treatments. Mice air pouches were infected with *M. abscessus* subsp. *massiliense* as described in methods. After 3 days of infection the bacteria was harvested, the RNA extracted and the expression of *mycma_1113* normalized to the 16S rRNA expression was determined. The expression shown is relative to the group treated with PBS. Data shown mean ± SEM, n = 4. *$p < 0.05$; **$p < 0.01$; difference from PBS group by One-way ANOVA with Dunnett post test

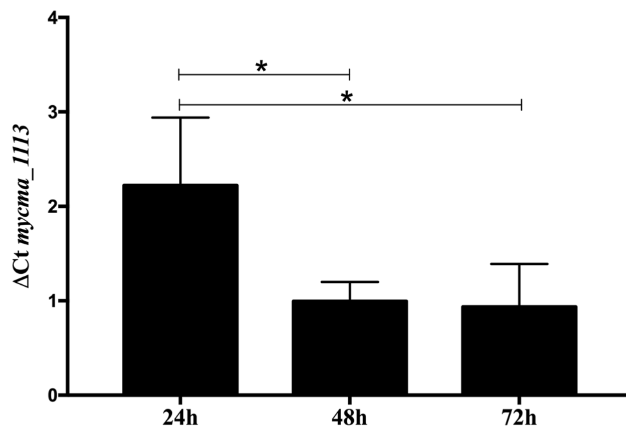

Fig. 6 Expression of *mycma_1113* gene during Mycma infection of BMMO. Macrophages were infected at a multiplicity of infection of 1:10 with *M. abscessus* subsp. *massiliense,* and total RNA was extracted at 24, 48 and 72 h after infection. Expression of *mycma_1113* was analyzed by quantitative RT-PCR with SYBR green. The data are expressed as the relative quantity of the *mycma_1113* mRNA normalized to 16S ribosomal RNA. $*p < 0.05$ difference from 24 h of macrophage infection by One-way ANOVA with Dunnett post test

the generation time of each *Mycobacterium sp.*, the former have a much shorter time than the latter. It was shown that mycobacterial physiological growth rate is directly related to the time of establishment of mycobacterial infection in macrophages, and it was observed that after 24 h of *M. abscessus* infection the bacillary load rapidly increased within the macrophages, while that of Mtb remained constant even after 48 h of infection [37].

During infection, mycobacteria are maintained within phagosomes with limited iron availability due to the Nramp-1 action [38, 39]. However, it was also shown that 48 h after infection with *M. abscessus* there was a significant reduction of membrane integrity of the macrophages [37] what could favor the access to iron released by injured macrophages. Thus, we believe that after 24 h of Mycma infection, there was an increase in the mycobacterial load that could have led to the disintegration of the macrophages membrane, facilitating access to iron. The expression of genes involved in the uptake of iron is directly related to the concentration of this metal in the microenvironment, and these genes are repressed under sufficient iron conditions by the action of the IdeR [33–35] thus the expression of the *mycma_1113* gene may not be required later in infection (Fig. 6), as is during the initial stages of infection (24 h), due to iron availability in result of membrane damage. These data suggest that the capacity of Mycma in infection establishment may be related in part to its ability to survive under conditions of iron limitations imposed by the host, especially during the first 24 h of infection, through the siderophore production.

Conclusion

In conclusion, we show that *M. abscessus* subsp. *massiliense* produce siderophores under iron deprivation conditions, and that the *mycma_1113* gene is involved in this process. The *mycma_1113* gene expression occurs during infection indicating that it may participate in the Fe sequestration from the host.

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