Discovery of a novel dehydratase of the fatty acid synthase type II critical for ketomycolic acid biosynthesis and virulence of *Mycobacterium tuberculosis*

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The fatty acid synthase type II (FAS-II) multienzyme system builds the main chain of mycolic acids (MAs), important lipid pathogenicity factors of *Mycobacterium tuberculosis* (*Mtb*). Due to their original structure, the identification of the (3R)-hydroxyacyl-ACP dehydratases, HadAB and HadBC, of *Mtb* FAS-II complex required in-depth work. Here, we report the discovery of a third dehydratase protein, HadD<sub>Mtb</sub>(Rv0504c), whose gene is non-essential and sits upstream of cmaA<sub>2</sub> encoding a cyclopropane synthase dedicated to keto- and methoxy-MAs. HadD<sub>Mtb</sub> deletion triggered a marked change in *Mtb* keto-MA content and size distribution, deeply impacting the production of full-size molecules. Furthermore, abnormal MAs, likely generated from 3-hydroxylated intermediates, accumulated. These data strongly suggest that HadD<sub>Mtb</sub> catalyzes the 3-hydroxyacyl dehydratation step of late FAS-II elongation cycles during keto-MA biosynthesis. Phenotyping of *Mtb* hadD deletion mutant revealed the influence of HadD<sub>Mtb</sub> on the planktonic growth, colony morphology and biofilm structuration, as well as on low temperature tolerance. Importantly, HadD<sub>Mtb</sub> has a strong impact on *Mtb* virulence in the mouse model of infection. The effects of the lack of HadD<sub>Mtb</sub> observed both in vitro and in vivo designate this protein as a bona fide target for the development of novel anti-TB intervention strategies.

Tuberculosis (TB) is the top infectious killer worldwide¹. The control of this disease has been challenged by the emergence of multidrug and extensively drug-resistant *Mycobacterium tuberculosis* (*Mtb*) strains. According to the WHO, the development of a new generation of drugs effective against these strains is urgently needed¹. The very thick lipid-rich envelope of the tubercle bacillus provides a vital protective coat against the attacks of the infected host. In particular, the mycolic acid (MA)-containing lipids, which play a strategic role in the envelope architecture and permeability, are essential to the mycobacterial survival and constitute important pathogenicity factors. As a consequence, their biosynthesis pathway represents one of the Achilles’ heels of the tubercle bacillus. It is the primary target of several anti-TB antibiotics, including the first line drug isoniazid²–³. Furthermore, recently discovered small molecules, that are showing great promise as TB therapeutics, affect their metabolism⁴. MAs, extremely long-chain α-alkylated β-hydroxylated fatty acids (FAs), are the major components of a highly efficient permeability barrier, the mycobacterial outer membrane (called mycomembrane)⁵, where they are covalently linked to the arabinogalactan layer or to polyol molecules such as trehalose⁶. Their production requires three distinct multienzyme systems, including the acyl carrier protein (ACP)-dependent fatty acid

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synthase type II (FAS-II) that is responsible for the synthesis of their main chain called ‘meromycolic chain’. The latter carries various types of chemical function (cyclopropane ring, double bond, methyl branch and oxygenated groups) that differentiate the MA subclasses and modulate their biological activities. While typical FAS-II systems found in plants, bacteria, parasites, and mitochondria perform de novo biosynthesis, the system from the Corynebacteriales order, which includes the Mycobacterium genus, elongates standard-size FAs (C\(_{16}-C_{20}\)). This unique property explains the success of the mycobacterial FAS-II system as a target for specific anti-TB drug therapy, as well as by the modes of action of the drugs isoniazid, ethambutone and thiacetazone. In mycobacteria, enzymes catalyzing the four main elongation steps have been characterized. The last enzymes identified in FAS-II were two heterodimeric (3β-hydroxacyl-ACP dehydratases (HADs), fabAB and fabBC. Belonging to the hydratase family, they have an original structure for HADs of FAS-II system, which are classically FabZ/FabA-type proteins.

In a recent survey, a combination of pull-downs, using the dehydratase HadAB of FAS-II as a bait, and proteomic analyses led to the discovery of a novel (R)-specific dehydratase, HadD\(_{\text{Msm}}\) from the FAS-II system from M. smegmatis, a non-tuberculous mycobacterium. HadD displays highly specific interactions with HadAB. Bioinformatic analyses showed that, unlike HadAB, there is no HadD\(_{\text{Msm}}\) ortholog in the genera of the Corynebacteriales order, such as Nocardia, Rhodococcus, and Gordonia, that produce medium-chain mycolic acids. This suggested that HadD\(_{\text{Msm}}\) might have a role during the late FAS-II elongation cycles. Consistent with this, the deletion of hadD\(_{\text{Msm}}\) (MSMEG_0948) gene quasi-totally abolished the biosynthesis of the long chain α- and epoxy-MAs, and the resulting mutant strain produced only the medium-size α'-MAs. Thus, HadD\(_{\text{Msm}}\) is most likely involved in building the third meromycolic segment leading to the synthesis of the full-size α- and epoxy-MAs. Importantly, hadD\(_{\text{Msm}}\) inactivation induced an upheaval of both the bacterial cell surface and envelope properties of M. smegmatis, strongly altering the interaction with the host cells and the assembly into colonies or biofilms and spread by sliding motility, critical for mycobacterial survival under stress and hostile conditions and for colonization. It also conferred a hypersensitivity to the first line TB drug rifampicin.

Interestingly, a putative ortholog of HadD\(_{\text{Msm}}\) was found in all of the examined mycobacterial genomes, including those of the pathogen Mtb. Yet, Mtb produces a MA combination distinct from that of M. smegmatis, i.e. α-, keto- and methoxy-MAs, and has no α'- and epoxy-MAs. In this context, we sought to elucidate the role of the putative ortholog of HadD\(_{\text{Msm}}\) in Mtb, which in the reference strain H37Rv is represented by the yet uncharacterized protein Rv0504c. Furthermore, the importance of HadD\(_{\text{Msm}}\) for M. smegmatis physiology, we generated a Rv0504c knock-out Mtb mutant and examined the impact of Rv0504c inactivation on different physiological properties of Mtb in axenic cultures as well as on its virulence in the mouse model of infection.

**Results**

**M. tuberculosis holds a putative HadD ortholog that is not essential for survival.** Protein–protein BLAST searches performed against Mtb H37Rv genome, using the MSMEG_0948 (HadD\(_{\text{Msm}}\)) protein sequence as a probe, showed the presence of a potential ortholog of HadD\(_{\text{Msm}}\) with a sequence identity rate of 68% in Mtb, Rv0504c, which we named HadD\(_{\text{Mtb}}\) (Fig. 1A). The latter, annotated as ‘conserved protein’ and having a theoretical monomeric mass of 18.4 kDa, bears similarly to HadD\(_{\text{Msm}}\), a degenerate hydratase 2 motif ‘P-x(2)-a-x(2)-D-x(2)-P-x-H-x(5)-A’ containing the putative catalytic dyad Asp (D37) and His (H42) (Fig. 1A). The chromosomal region of hadD\(_{\text{Mtb}}\) gene is partially conserved between Mtb and M. smegmatis (Fig. 1B). Interestingly, cmaA2 (Rv0504c) gene sits downstream of hadD\(_{\text{Mtb}}\) (Rv0504c) on Mtb chromosome and is transcribed in the same direction. It encodes the mycic acid methyltransferase (MA-MT) CmaA2 that has a function of cyclopropane synthase and introduces a cyclopropane at the proximal position of both keto- and methoxy-MAs. The lack of a cmaA2 ortholog in M. smegmatis (Fig. 1B) is in agreement with the absence of these MA classes in this species.

The essentiality of hadD\(_{\text{Mtb}}\) gene was examined by generating an in-frame unmarked deletion (Fig. 1B) using a two-step homologous recombination method, so that it does not cause any polar effect on cmaA2 expression. The gene deletion was verified by PCR analysis (Fig. 1C). The viability of the resulting mutant strain showed that hadD\(_{\text{Mtb}}\) is not essential for the survival of Mtb in axenic culture, similar to the situation seen for hadD\(_{\text{Msm}}\) in M. smegmatis. This is consistent with the prediction of non-essentiality of hadD\(_{\text{Mtb}}\) made from a microarray-based study, but in discrepancy with a more recent survey using high-resolution phenotypic profiling where hadD\(_{\text{Mtb}}\) was predicted to be essential.

**HadD\(_{\text{Mtb}}\) deletion alters Mtb physiology and virulence.** In the aim of evaluating the importance of HadD\(_{\text{Mtb}}\) function in Mtb, phenotypic assays were realized. Although Mtb ΔhadD is viable, this mutant strain showed difficulties to grow in planktonic culture as compared to the wild type (wt) strain (Fig. 2A). HadD\(_{\text{Mtb}}\) inactivation also resulted in a strong alteration of the structuration of biofilms at the air-liquid interface, which appeared thinner with large clumps (Fig. 2B). There was a deep change of the colony morphology as well, with much larger, flat and spread colonies (Fig. 2C). The wt phenotype was restored in the mutant upon complementation meaning that these phenomena were linked to hadD\(_{\text{Mtb}}\) deletion. The lack of HadD\(_{\text{Mtb}}\) also conferred a high sensitivity to low temperature (Fig. 2D). In addition, Mtb ΔhadD was slightly more susceptible than Mtb wt to rifampicin, a first line antituberculous drug targeting the DNA-dependent RNA synthesis. In contrast, there was no significant difference in the sensitivity of both strains to isoniazid and ethambutol, two other first line TB drugs, and to ciprofloxacin, a broad spectrum fluoroquinolone, as well as to the SDS detergent (Supplementary Table S1).

To assess the influence of hadD\(_{\text{Mtb}}\) on the virulence level of Mtb, infection trials were performed with severe combined immunodeficiency (SCID) mice. This murine model provides a rapid and sensitive method for evaluating in vivo growth characteristics of mycobacterial strains during the acute phase of infection. Although over-expressing hadD\(_{\text{Mtb}}\) gene had no significant impact on the virulence level, we observed marked and reproducible
reductions of the bacterial loads in lungs (2 logs) and spleens of mice infected with the deletion mutant after 28 days of infection relative to controls (Fig. 3A). This effect correlated with a decrease of the spleen size (Fig. 3B). This phenomenon likely reflected a specific in vivo phenotype since the bacterial density of *Mtb* ∆hadD had reached that of the wt strain after 11 days of growth in axenic cultures (Fig. 2A). The complementation observed upon transformation of the mutant strain with a wt *hadDMtb* copy provides explicit evidence for a link between HadD function and *Mtb* virulence (Fig. 3A).

**Figure 1.** Analysis of HadD*mb* sequence, hadD chromosomic region and *Mtb* ∆hadD mutant. (A) Sequence alignment of HadD*mb* (Rv0504c) with HadD*sm* (MSMEG_0948) and HadB*mb* (Rv0636) proteins. Black and gray shadings indicate strictly conserved and similar residues, respectively. HadD*mb* shares a sequence identity of 63% with HadD*sm* (68% using BlastP alignment) and only 19% with HadB*mb* (Clustal Omega scores). HadD*mb* bears a degenerate hydratase 2 motif ‘F-x(2)-a-x(2)-D-x(2)-P-x-H-x(5)-A’ (uppercase: strictly conserved; lowercase: similar residue) indicated by blue stars; the putative catalytic Asp and His residues are labeled by red stars. The hydratase 2 motif ‘[YF]-x(1,2)-[LIVG]-[STGC]-G-D-x-N-P-[LIV]-H-x(5)-[AS]’ of HadB*mb* is indicated by black stars. Alignment was performed by using Clustal Omega program, and the figure was shaped with Box shade. Database accession numbers: HadD*mb*, P9WFK3 (166 aa); HadD*sm*, A0QR13 (177 aa); HadB*mb*, I6WYY7 (142 aa). (B) Genomic organization of hadD gene region in *M. smegmatis*, *Mtb* H37Rv and *Mtb* ∆hadD strains. Matching genes are drawn with identical colors. *Mtb* ∆hadD mutant strain was produced by an in-frame deletion of a 308 bp internal fragment (dashed lines) of hadDMtb gene (501 bp). In *Mtb*, *Rv0502* (1,077 bp), *cmaA2* (909 bp), *serB1* (1,122 bp) and *mmpS2* (444 bp) are annotated as encoding a conserved protein, the mycolic acid cyclopropane synthetase CmaA2, a possible phosphoserine phosphatase SerB1 and a probable conserved membrane protein MmpS2, respectively. The distinct genes in *M. smegmatis*, MSMEG_0946, MSMEG_0950 and MSMEG_0951, are annotated as encoding a NAD-dependent epimerase/dehydratase family protein, a hypothetical protein and the glutaredoxin 2, respectively. (C) Verification of hadDMtb gene deletion by PCR analysis. The primers (x and y; symbolized by black arrows in panel B) used for the PCR are located outside the hadDMtb gene. The genomic DNA of each strain was used as a template. HadD*mb* gene length: 501 bp; ∆hadDMtb gene length: 193 bp. L: DNA ladder.
In conclusion, HadD<sub>Mtb</sub> plays a key role in the faculty of the tubercle bacillus to invade and multiply within the infected host. Furthermore, it is also important for the fitness and the capacities of Mtb bacilli to assemble into colonies or biofilms, and for their tolerance to low temperature. The deep changes observed in had<sup>D</sup><sub>Mtb</sub>-deficient strain likely are the consequences of an alteration of the cell envelope composition and architecture.

**HadD<sub>Mtb</sub> influences the mycolic acid profile of Mtb.** Mtb produces three main MA classes, α-, methoxy- and keto-MAs (Fig. 4A). To examine the potential involvement of HadD<sub>Mtb</sub> protein in their biosynthesis, the MAs were extracted from Mtb ΔhadD<sub>Mtb</sub> strain and analyzed. The MA content, expressed as the ratio 'MA dry weight/delipidated bacterial residue dry weight', was similar between the wt (10.2 ± 1.1%) and the mutant (10.4 ± 1.7%) strains. Yet, the MA distribution was changed in the deletion mutant, as shown by HPTLC analysis of the MA methyl esters (MAMEs) (Fig. 4B,C). The abundance of keto-MAs was reduced by 63%. This was compensated by an increase in the α-MA content. The recovery of the wt profile upon complementation with a
wt hadD<sub>wt</sub> copy suggested that this gene is directly involved in keto-MA biosynthesis<sup>26</sup>. The overexpression of hadD<sub>wt</sub> in Mtb wt strain supported this conclusion, since it induced a strong increase (of 87%) in the keto-MA relative content (Fig. 4C). This had repercussions on the relative abundance of the biosynthetically affiliated methoxy-MAs, which increased slightly (Fig. 4C).

These results altogether clearly demonstrate that HadD<sub>Mtb</sub> plays an important role in the biosynthesis of the keto-MAs. It is noteworthy that no major change in the profile of other lipids from Mtb was detected (Supplementary Fig. S1) suggesting that hadD<sub>Mtb</sub> is not involved in another lipid biosynthesis pathways. Therefore, the significant alteration of MA distribution in Mtb ΔhadD is most likely responsible for its loss of virulence (Fig. 3A).

Production of full-size keto-mycolic acids requires an active HadD<sub>wt</sub> protein. To investigate further the function of HadD<sub>wt</sub> in Mtb, the fine structures of MAs were analyzed using MALDI-TOF mass spectrometry (MS) and <sup>1</sup>H-NMR spectroscopy. The mass spectrum of the total MA mixture from Mtb ΔhadD displayed an increase of the relative intensities of α-MAMEs signals with respect to the wt strain (Fig. 5). More importantly, the size distribution of the keto-MAs changed significantly. The signal intensities of the long chain molecules (C<sub>82</sub>-C<sub>88</sub>) markedly decreased, while those of the short-chain keto-MAs (C<sub>78</sub>, C<sub>80</sub>) raised (Fig. 5). The same observations were made on the spectrum of keto-MAs purified from the deletion mutant (Supplementary Fig. S2). The wt profiles were partially recovered in the complemented strain Mtb ΔhadD::hadDMtb (Fig. 5, Supplementary Fig. S2), confirming the role played by hadD<sub>wt</sub> deletion in this phenotype. It is noteworthy that a reduction in the proportion of the longest molecules was also detected for the methoxy-MAs in Mtb ΔhadD (Fig. 5). Moreover, when hadD<sub>wt</sub> was overexpressed in Mtb wt, the relative content of the long-chain keto-MAs (C<sub>82</sub>-C<sub>86</sub>) strongly raised (Fig. 5).

Consistent with HPTLC analyses (Fig. 4B,C), these data altogether confirm the involvement of HadD<sub>wt</sub> in the keto-MA pathway. Furthermore, they show that HadD<sub>wt</sub> is implicated in the late elongation cycles during the biosynthesis of the longest chain keto-meromycolic chains. This is in agreement with the lack of HadD ortholog in other genera of the Corynebacteriales order, such as Nocardiia, Rhodococcus, and Gordonia, which produce...
medium-chain MAs\textsuperscript{16}. Despite a slight difference in the cis/trans cyclopropane distribution in the keto-MAs, there was no change in the type of unsaturations carried by the MAs in \(Mtb\) \(\Delta\)hadD, which remained cyclopropanes (Supplementary Table S2), whereas inactivation of \(cmaA2\) leads to accumulation of ethylenic oxygenated MAs in \(Mtb\) \(18\). Thus, as expected, the in-frame unmarked \(\text{hadDMtb}\) deletion performed had no visible polar effect on the expression of the adjacent \(cmaA2\) gene (Fig. 1B) encoding CmaA2 cyclopropane synthase.

\(\text{HadD}\_\text{Mtb}\) and \(\text{HadD}\_\text{Msm}\) have distinct substrate specificities. Interestingly, HPTLC analysis showed that \(\text{hadD}\_\text{Msm}\) gene from \(M.\ text{smegmatis}\) expressed in \(Mtb\) \(\Delta\text{hadD}\) could restore a MA distribution similar to that of \(Mtb\) \(\text{wt}\) strain (Fig. 4B). Furthermore, the fine structures of the MAs in \(Mtb\ \Delta\text{hadD};\text{hadDMsm}\) were also largely recovered, as observed by MALDI-TOF MS (Fig. 5) and NMR spectroscopy (Supplementary Table S2). This suggested that the functions of \(\text{HadD}\_\text{Msm}\) and \(\text{HadD}\_\text{Mtb}\) proteins are similar. Indeed, the present work and previous data\textsuperscript{16} show that they are both involved in dehydration steps during late FAS-II elongation cycles. Yet, unlike \(\text{hadD}\_\text{Mtb}\), the \(\text{hadD}\_\text{Msm}\) gene was not able to restore the virulence level of \(Mtb\ \text{wt}\) in \(Mtb\ \Delta\text{hadD}\) strain (Fig. 3A,B). Furthermore, the reverse cross-complementation, \(i.e.\) the expression of \(\text{hadD}\_\text{Mtb}\) in \(M.\ text{smegmatis}\) \(\Delta\text{hadD}\), was far to be as successful in terms of MA profile and physiology. The MA distribution was much closer to that of \(M.\ text{smegmatis}\ \Delta\text{hadD}\) than that of \(M.\ text{smegmatis}\ \Delta\text{hadD};\text{hadDMsm}\) (Supplementary Fig. S3A,B). The same conclusions were drawn from the colony morphology observation (Supplementary Fig. S3C) as well as from the sensitivity assays to rifampicin (Supplementary Fig. S3D), towards which \(M.\ text{smegmatis}\ \Delta\text{hadD}\) exhibits a hypersensitivity\textsuperscript{16}. These data show that the functions of \(\text{HadD}\_\text{Mtb}\) and \(\text{HadD}\_\text{Msm}\) are not completely superimposable. This is in perfect agreement with the specificities of \(\text{HadD}\_\text{Msm}\) for the \(\alpha\)- and epoxy-MA biosynthesis\textsuperscript{16} and of \(\text{HadD}\_\text{Mtb}\) for the keto-MA pathway. The inactivation of \(\text{hadD}\_\text{Mtb}\) did not induce a reduction of \(\alpha\)-MA content in \(Mtb\) as in \(M.\ text{smegmatis}\) \(\text{hadD}\_\text{Msm}\). Therefore, although \(\text{HadD}\_\text{Mtb}\) and \(\text{HadD}\_\text{Msm}\) possess similar functions, they are not strict orthologs, and the substrate specificity of \(\text{HadD}\_\text{Mtb}\) seems important for its role in virulence.

**Accumulation of dehydration substrates triggered by \(\text{hadD}\_\text{Mtb}\) inactivation.** It is noteworthy that \(Mtb\ \Delta\text{hadD}\) accumulated a polar compound ‘Z’, undetectable in \(Mtb\) \(\text{wt}\) and hardly visible in \(Mtb\ \Delta\text{hadD};\text{hadDMtb}\) by HPTLC (Fig. 4B). This compound, appearing as a double band, likely corresponded to a heterogeneous mixture of molecules. After purification, the MALDI-TOF mass spectrum of compound Z displayed...
peak envelopes within a mass range from 1148 to 1362 Da (Supplementary Fig. S4A) reminiscent of the classical MAME mixture (Fig. 5), but with an addition of 16 mass units. Consistent with this, the mass increment of 84 Da (corresponding to two acetyl groups) observed after per-O-acetylation of compound Z indicated the presence of two hydroxyl groups in the intact compound (Supplementary Fig. S4A), which explained its low $R_f$ in HPTLC (Fig. 4B). This was confirmed by $^1$H-NMR spectroscopy of intact compound Z generating two signals at 3.85 ppm and 3.98 ppm assigned to methines bearing both hydroxyl groups (−CH$_{2}$OH−). After per-acetylation of compound Z, these signals shifted at 4.92 ppm and 5.13 ppm, corresponding to the chemical shifts of methines bearing O-acetyl groups (Supplementary Fig. S4B). The spin system in the $^1$H-$^1$H 2D NMR COSY spectrum of per-O-acetylated compound Z revealed that, in the intact compound, one hydroxyl group is carried by the C-3 like in the classical MAs, whereas the additional hydroxyl group is located on the C-5 (Supplementary Fig. S4C,D). These data altogether clearly showed that compound Z is a mixture of 5-hydroxylated MAs. These molecules are mostly cis-cyclopropanated like regular Mtb MAs (Supplementary Table S2).

The additional hydroxyl group on the C-5 in compound Z was located on the C-3 in the precursor meromycolic chains, before the mycolic condensation step (Supplementary Fig. S5). The 3-hydroxylated meromycolic acids were taken over by the mycolic condensation system leading to the synthesis of abnormal 5-hydroxylated MAs. The accumulation of 3-hydroxylated intermediates corresponding to dehydratase substrates strongly suggests that, in the absence of HadD$_{Mtb}$ protein, the dehydration step of the late FAS-II elongation cycles is partially blocked, preventing the formation of mature meromycolic chains (Supplementary Fig. S5). The 3-hydroxylated
α-meromycolic chains might come from the group of diethylenic precursors of oxygenated MAs (Fig. 6), which, accumulating abnormally, would later be cyclopropanated by default like the regular α-meromycolic chains.

In conclusion, HadD\textsubscript{Mtb} most likely catalyzes the 3-hydroxyacyl-ACP dehydratation step of the late elongation cycles during the biosynthesis of the oxygenated meromycolic chains.

**Discussion**

The present study reports the existence in \textit{Mtb} of a putative ortholog, which we named HadD\textsubscript{Mtb}, of the recently discovered (3\textsubscript{R})-hydroxyacyl-ACP dehydratase HadD\textsubscript{Msm} of the FAS-II system from \textit{M. smegmatis}\textsuperscript{16}. The in-depth analysis of the distribution and fine structure of the MAs produced by \textit{Mtb} hadD\textsubscript{Mtb} deletion mutant revealed that HadD\textsubscript{Mtb} is involved in the oxygenated MA biosynthesis, and more particularly dedicated to the keto-MA pathway. Indeed, in the mutant, the overall keto-MA content was strongly reduced. The fact that the relative abundance of the longest keto-MAs decreased in \textit{Mtb} \textit{ΔhadD}_{Mtb} whereas it increased in \textit{Mtb} \textit{wt::hadD}_{Mtb} overexpression strain shows that HadD\textsubscript{Mtb} is involved in late FAS-II elongation cycles during the biosynthesis of the keto-meromycolic chains.
This conclusion is supported by the lack of HadD ortholog in the genera of the Corynebacteriales order that produce only intermediate size and not full size mycolic acids as found in mycobacteria. The abnormal 5-hydroxyxylated MAs observed in HadD\textsubscript{Mtb} deficient strain result from the condensation of 3-hydroxy-meromycoloyl chains with a carboxyacyl chain (Supplementary Fig. S5). Their accumulation together with the belonging of HadD\textsubscript{Mtb} to the hydratase 2 protein family and its high sequence similarity with HadD\textsubscript{Msm}, leads to the conclusion that HadD\textsubscript{Mtb} most likely catalyzes the 3-hydroxyacyl-ACP dehydration step of these FAS-II elongation cycles (Fig. 6). We had previously discovered two (3 R)-hydroxyacyl-ACP dehydratases of the FAS-II system from M. tuberculosis, HadAB and HadBC. Thus, HadD\textsubscript{Mtb} constitutes a third dehydratase of this system. It has been shown that HadAB is involved in the early elongation cycles common to both α and oxygenated MA pathways, while HadBC is required for the late cycles leading to the biosynthesis of the sole oxygenated MAs, which are 4–6 carbon longer than α-MAs\textsuperscript{3,27}. Since the keto- and methoxy-MA biosynthetically affiliated\textsuperscript{28}, the MA distribution in Mtb \( \Delta \text{hadC} \) strain was interpreted in terms of α/oxygenated ratio\textsuperscript{27}. Yet, it is noteworthy that, in this mutant, the keto-MA content remains stable whereas that of the methoxy-MAs drops dramatically\textsuperscript{27}. In the light of our new findings, this strongly suggests that HadBC is preferentially dedicated to the methoxy-MA biosynthesis and HadD\textsubscript{Mtb} to the keto-MA pathway. However, the functions of both enzymes are likely partially redundant since their individual inactivation does not totally inhibit the production of methoxy- or keto-MAs. This is supported by the variations in methoxy-MA content and size distribution observed in Mtb \( \Delta \text{hadD} \) and in Mtb \( \Delta \text{hadD} \textsuperscript{Msm} \), respectively (Figs. 4 and 5), and in keto-MA fine structure in Mtb \( \Delta \text{hadC} \) strain\textsuperscript{16}, which may also be partly due to a tight regulation between both biosynthesis pathways. These surveys allow us to draw a biosynthesis scheme for the three MA classes from Mtb, which details the specific roles of the three FAS-II dehydratases (Fig. 6).

The functions of HadD\textsubscript{Mtb} and HadD\textsubscript{Msm} in M. smegmatis appear closely related since they both catalyze the dehydration step during late elongation cycles\textsuperscript{16}. Yet, the protein from Mtb has a substrate specificity distinct from that of M. smegmatis, where it is required for the biosynthesis of α- and epoxy-MAs\textsuperscript{16}. The phenotypic analyses of the cross-complemented M. smegmatis \( \Delta \text{hadD} \) mutant in terms of MA profile, colony morphology and rifampicin sensitivity, and of the cross-complemented Mtb \( \Delta \text{hadD} \) mutant in terms of virulence level confirmed this conclusion and definitely showed that hadD\textsubscript{Mtb} and hadD\textsubscript{Msm} are not strict orthologs, despite the partial genomic conservation of their chromosomal regions. This suggests that there has been a functional divergence of both genes after the speciation event. Indeed, during its adaption to a distinct environment, the ortholog in a new species may undergo neofunctionalization, resulting in a species-specific function for this gene\textsuperscript{29}.

We showed that \( \text{Rv0504c} \) gene encoding HadD\textsubscript{Mtb} is not essential for the survival of the tubercle bacillus in axenic culture. However, we observed that it has an important impact on the fitness of bacteria, their organization into biofilms and colonies, as well as their tolerance to low temperature (30°C). This is certainly linked to the determinant role of MAs in the architecture and the fluidity of the envelope due to their strategic location within the mycomembrane\textsuperscript{30}. In agreement with our data, the growth of an Mtb strain overproducing \( \text{mmaA3} \) MA-methyltransferase gene and lacking keto-MAs was impaired at reduced temperature (32°C)\textsuperscript{31}. MAs are also known to be important for the formation of mycobacterial biofilms, where the extracellular matrix contains large amounts of free MAs\textsuperscript{32}. In particular, the production of keto-MAs in which HadD\textsubscript{Mtb} is involved is essential for biofilm growth\textsuperscript{31}. MAs represent also key pathogenic factors, their fine structures strongly potentiating the immune response to infection\textsuperscript{31}. Here, we show that HadD\textsubscript{Mtb} function greatly influences the virulence level of Mtb in the mouse model of infection. This is consistent with previous findings showing that a Mtb \( \text{hma} (\text{mmaA4}) \) mutant devoid of oxygenated MAs is attenuated in mice\textsuperscript{31} and that keto-MAs are critical for Mtb growth within the natural host cells\textsuperscript{31}. Additionally, oxygenated MAs play a role in the selective repression of macrophage IL-12p40 cytokine production aimed to evade elimination by the host immune system\textsuperscript{35}, and the \( \text{hma} \) gene required for their biosynthesis is actively expressed during human pulmonary tuberculosis\textsuperscript{36}. Importantly, the recently approved drug delamanid, active against MDR-TB, kills Mtb by blocking the oxygenated MA production\textsuperscript{37}. Altogether, these data indicate that targeting this metabolism would constitute a relevant strategy for the development of new therapeutics against Mtb. Because of its involvement in the Mtb-MA biosynthesis pathway, its role in Mtb virulence and its specificity to mycobacterial cells\textsuperscript{46}, HadD protein represents a promising pharmaceutical target. Besides, targeting non-essential enzymes of the tubercle bacillus should limit the occurrence of antibiotic resistance mechanisms.

**Methods**

**Bio-containment measures.** All of the experiments using Mtb living strains were performed either in a regular Biosafety Level-3 (BSL-3) laboratory or in an Animal Biosafety Level-3 (ABSIL-3) laboratory in the case of mouse infection experiments.

**Protein sequence analyses.** Sequence alignments were performed using BLAST and Clustal Omega software\textsuperscript{33,34}, with default parameters. The presence of a putative HadD\textsubscript{Mtb} ortholog in Mtb \( \text{H37Rv} \) was analyzed by BlastP searches against the fully sequenced genome available at NCBI website (www.ncbi.nlm.nih.gov/genomes/lproks.cgi), using MSMEG_0948 protein sequence (database accession number: A0QR13) as a probe. Genomic organization of hadD\textsubscript{Msm} and hadD\textsubscript{Mtb} gene regions in M. smegmatis mc\textsuperscript{155} and Mtb \( \text{H37Rv} \) respectively, was visualized at Mycobrowser website (https://mycobrowser.epfl.ch/)\textsuperscript{35}.

**Construction of the deletion mutant and complemented strains.** The unmarked deletion of \( \text{Rv0504c} \) (hadD\textsubscript{Mtb}) gene in Mtb \( \text{H37Rv} \) (ATCC 27294) strain was done using a previously described method\textsuperscript{35}. Briefly, the Rv0504c deletion delivery vector was constructed by amplifying the upstream (0.95 kb) and downstream (1.1 kb) regions of the gene using the primer pairs EUF24 (5’-GCTTCAGCTTCAAGTGGGACACAAC-3’) and EUR24 (5’-CAACGCTTGGCGATGCTGGGCTTCT-3’) and EUF25 (5’-CAACGCTTGGGACAGTC CGAAGCGAAATTT-3’) and EUR25 (5’-CGGTACCTTTCCGTCGACCATTT-3’), respectively.
PCR-amplified fragments were cloned into p2NIL using the restriction sites in the primers (PstI, HindIII and Kpn I, underlined in the above sequences) and was verified by the PCR. The plasmid was electroporated into Mtb and single crossovers were isolated. Double crossovers were isolated from the single crossover strain as previously described. Colonies were screened for the presence of the wild-type (wt) or deletion alleles by PCR using primers 0504D1 (′-AGCCTCTAGACGCCAATCAC-3′) and 0504D2 (′-GGCTCAAGGTTCAGCTTGTC-3′). The deletion was checked by PCR and sequencing. For complementation of Mtb ΔhadD strain, the wt copy of Rv0504c gene, containing its natural promoter (186 bp before the start codon), was amplified with the primer pairs 0504C1 (′-AGCCTCTAGACGCCAATCAC-3′) and 0504C2 (′-CAAGCTTCTGTCATTGAACGGACCCTAC-3′) that incorporates a HindIII restriction site and cloned into pSC-A (Agilent). The HindIII fragment from pUC-GM-Int containing the mycobacteriophage L5 integrase, att site, and Gm resistance was inserted to make the complementing vector. The resulting plasmid pUC-hadD was and the empty plasmid pUC (as a control) were used to transform both Mtb wt and Mtb ΔhadD strains (Supplementary Table S3). For cross-complementation experiments, pUC-hadD strain carrying a wt copy of MEG_0948 (hadD gene) was used to transform Mtb ΔhadD and pUC-hadD ΔSm plasmid was used to transform M. smegmatis ΔhadD strain previously constructed (Supplementary Table S3). Data were compared to those obtained for M. smegmatis mc²155 (wt) strain as well as M. smegmatis ΔhadD complemented by pUC-hadD strain and previously described.

**Culture conditions and phenotyping assays.** For biofilm growth and lipid analyses, Mtb strains were cultured as pellicles in glass bottles for 4–5 weeks at 37 °C in 7H9 broth (Difco) containing 0.2% glycerol, 10% Middlebrook ADC (Difco) and 10 µg/ml gentamycin. Cultures were inoculated with identical volumes of precultures in exponential growth phase grown in the same medium then adjusted at OD₆₀₀ ~ 1. For the growth curves, planktonic cultures were realized under shaking (120 rpm) in the above medium supplemented with 0.05% (w/v) Tween-80, and OD₆₀₀ was measured at different time points. For colony morphology and susceptibility to temperature assays on M. smegmatis or deletion alleles by PCR using primers 0504D1 (′-AGCCTCTAGACGCCAATCAC-3′) and 0504D2 (′-GGCTCAAGGTTCAGCTTGTC-3′). The deletion was checked by PCR and sequencing. For complementation experiments, pUC-hadD strain carrying a wt copy of MEG_0948 (hadD gene) was used to transform Mtb ΔhadD and pUC-hadD ΔSm plasmid was used to transform M. smegmatis ΔhadD strain previously constructed (Supplementary Table S3). Data were compared to those obtained for M. smegmatis mc²155 (wt) strain as well as M. smegmatis ΔhadD complemented by pUC-hadD strain and previously described.

**Lipid extractions, (HP)TLC analyses and purification.** The MAs and the total extractable lipids were extracted and analyzed as described previously, except that the (HP)TLC plates for MAME separation were developed in ether/diethyl ether (9:1, v/v, five runs). Purification of MAMEs and compound Z was performed by preparative TLC using silica gel 60 plates (Merck) developed in the above eluent; the compounds were then scraped off and extracted from silica gel three times with diethyl ether.

**Lipid structural analyses.** Peracetylation of compound Z was performed in pyridine:acetic anhydride 1:1 for 1 h at 100 °C. After drying, three extractions with H₂O:diethyl ether (1:1) were done; the ether phases were collected, washed three times with water and dried. MALDI-TOF MS analyses were performed in the positive ionization and reflectron mode, using the 5800 MALDI-TOF/TOF Analyzer (Applied Biosystems/ABSciex) equipped with a Nd:YAG laser (349 nm wavelength) as described previously. ¹H-NMR and ¹H-¹H-NMR COSY spectra were recorded in CDCl₃ at 298 °K using a 600-MHz Bruker Avance III spectrometer (Bruker Biospin) equipped with a TCI cryoprobe. Chemical shift values were referenced to CHCl₃ resonance (δH 7.26 ppm). The quantification of MAME unsaturations was performed with TopSpin 3.5 pl7 software.

**Mouse infection experiments.** Virulence studies were performed in SCID mice. Briefly, groups of 6 week old C.B-17/Icr SCID mice (Charles River) were intravenously infected with a bacterial suspension containing 1–2 × 10⁵ CFU/mouse. One day and 28 days after infection, mice were euthanized, and spleen and lungs were homogenized in 2 ml tubes containing 500 µl Sauton medium and 2.5 mm diameter glass beads using an MM300 apparatus (Qiagen). CFU numbers in target organs were determined by plating 5- or 10-fold serial dilutions of organ homogenates on solid medium and incubation at 37 °C. These studies were approved by the Institut Pasteur Safety Committee (Protocol 11.245; experimentation authorization number 75–1469), in accordance with European and French guidelines (Directive 86/609/CEE and Decree 87–848 of 19 October 1987), and implicating approval from local ethical committees (CETEA 2013–0036 and CETEA dab180023).

**Data availability**

All data generated or analysed during this study are included in this published article (and its Supplementary Information files).

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Author contributions
C.L., N.S., F.L.-V., M.Da and A.Q. designed and analyzed data of lipid structural analyses; W.F., R.B. and A.Q. designed and analyzed data of mouse infection experiments; C.L., P.C., M.Da and A.Q. designed and analyzed data of the other experiments. C.L., N.S., F.L.-V. and P.C. performed lipid extractions and structural analyses; W.F. performed mouse infection experiments; A.Q. performed protein sequence analyses; C.L. and P.C. performed the other experiments. F.L.-V., P.C., A.L., T.P. and N.E. contributed to experimental or analytical tools. A.Q. wrote the manuscript main text. C.L., R.B. and A.Q. wrote the Methods. W.F. and A.Q. prepared Fig. 3, and C.L. and A.Q. prepared the other figures and tables.

Competing interests
The authors declare no competing interests.

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