Evolution of Mycolic Acid Biosynthesis Genes and Their Regulation during Starvation in *Mycobacterium tuberculosis*

Stevie Jamet,^{a,c} Yves Quentin,^{b,c} Coralie Coudray,^{a,c*} Pauline Texier,^{a,b,c} Françoise Laval,^{a,c} Mamadou Daffé,^{a,c} Gwennaele Fichant,^{b,c} Kaymeuang Cam^{a,c}

Centre National de la Recherche Scientifique, IPBS, UMR 5089, Toulouse, France^a; Centre National de la Recherche Scientifique, LMGM, UMR 5100, Toulouse, France^b; Université Toulouse, UPS, Toulouse, France^c

ABSTRACT

Mycobacterium tuberculosis, the etiological agent of tuberculosis, is a Gram-positive bacterium with a unique cell envelope composed of an essential outer membrane. Mycolic acids, which are very-long-chain (up to C₁₀₀) fatty acids, are the major components of this mycomembrane. The enzymatic pathways involved in the biosynthesis and transport of mycolates are fairly well documented and are the targets of the major antituberculous drugs. In contrast, only fragmented information is available on the expression and regulation of the biosynthesis genes. In this study, we report that the *hadA*, *hadB*, and *hadC* genes, which code for the mycolate biosynthesis dehydratase enzymes, are coexpressed with three genes that encode proteins of the translational apparatus. Consistent with the well-established control of the translation potential by nutrient availability, starvation leads to downregulation of the *hadABC* genes along with most of the genes required for the synthesis, modification, and transport of mycolates. The downregulation of a subset of the biosynthesis genes is partially dependent on Rel_{Mtb}, the key enzyme of the stringent response. We also report the phylogenetic evolution scenario that has shaped the current genetic organization, characterized by the coregulation of the *hadABC* operon with genes of the translational apparatus and with genes required for the modification of the mycolates.

IMPORTANCE

Mycobacterium tuberculosis infects one-third of the human population worldwide, and despite the available therapeutic arsenal, it continues to kill millions of people each year. There is therefore an urgent need to identify new targets and develop a better understanding of how the bacterium is adapting itself to host defenses during infection. A prerequisite of this understanding is knowledge of how this adaptive skill has been implanted by evolution. Nutrient scarcity is an environmental condition the bacterium has to cope with during infection. In many bacteria, adaptation to starvation relies partly on the stringent response. *M. tuberculosis*'s unique outer membrane layer, the mycomembrane, is crucial for its viability and virulence. Despite its being the target of the major antituberculosis drugs, only scattered information exists on how the genes required for biosynthesis of the mycomembrane are expressed and regulated during starvation. This work has addressed this issue as a step toward the identification of new targets in the fight against *M. tuberculosis*.

Mycobacterium tuberculosis, the etiological agent of tuberculosis, infects one-third of humans worldwide, with 9 million new cases and 1.5 million people dying each year from this disease (77). A long-standing challenge to the fight against tuberculosis is the ability of the pathogen to develop a nonreplicating, persistent, drug-tolerant form (1–3). In addition, the emergence of multi-drug-resistant (MDR), extremely drug-resistant (XDR) (4, 5), and total-drug-resistant (TDR) strains (6) underscores the urgent need to identify new therapeutic targets through a better understanding of the genetic programs developed by the bacterium to enable infection.

M. tuberculosis has an unusual cell envelope, characterized by an outer membrane (the mycomembrane) consisting largely of long-chain mycolic acids (MA) and a number of atypical lipids (7, 8). Most of these lipids are important to the viability and/or virulence of the bacteria (9), which is why much effort continues to be devoted to identifying, analyzing, and targeting enzymes involved in their metabolism (9–13). Consequently, much is known about the pathways responsible for biosynthesis of the mycolic acids and other components of the mycomembrane (Fig. 1A) (8, 14). This synthesis is centered on two fatty acid synthase (FAS) activities, the eukaryotic-type multifunctional single protein FAS-I and the

bacterial-type multiprotein complex FAS-II. FAS-I initiates *de novo* synthesis from an acetyl coenzyme A (acetyl-CoA) precursor of two products, a C₂₄-C₂₆ acyl-CoA at the origin of the MA α -branch and a C₁₆-C₁₈ acyl-CoA that is subsequently elongated by FAS-II to produce meromycolates. Various methyltransferases add functional groups to the meromycolates (8). A final conden-

Received 4 June 2015 Accepted 22 September 2015

Accepted manuscript posted online 28 September 2015

Citation Jamet S, Quentin Y, Coudray C, Texier P, Laval F, Daffé M, Fichant G, Cam K. 2015. Evolution of mycolic acid biosynthesis genes and their regulation during starvation in *Mycobacterium tuberculosis*. J Bacteriol 197:3797–3811. doi:10.1128/JB.00433-15.

Editor: O. Schneewind

Address correspondence to Gwennaele Fichant, Gwennaele.Fichant@ibcg.biotoul.fr, or Kaymeuang Cam, kaymeuang.cam@ipbs.fr.

* Present address: Coralie Coudray, Laboratoire de Biotechnologies Agroalimentaire et Environnemental, Auch, France.

Supplemental material for this article may be found at <http://dx.doi.org/10.1128/JB.00433-15>.

Copyright © 2015, American Society for Microbiology. All Rights Reserved.

FIG 1 Overview of mycolate biosynthesis and transport. (A) Biosynthesis pathway. The names of enzymes involved in the biosynthesis or transport are in bold (see the text). The dashed arrow indicates transport across the inner membrane. TMM, trehalose-mono-mycolate; TDM, trehalose-dimycolate; mAGP, mycolyl-arabinogalactan-peptidoglycan. (B) Genetic organization of genes involved in the biosynthesis. The operon organization is from the Database of Prokaryotic Operons (76) (<http://csbl.bmb.uga.edu/DOOR/>).

sation step forms the mature α -alkyl β -ketoacyl-MA, which is then transported across the cytoplasmic membrane as a trehalose-monomycolate (TMM) molecule by the inner membrane MmpL3 transporter. In the periplasmic compartment, the mycolyltransferase Ag85 either transfers the mycoloyl moiety of TMM to the envelope arabinogalactan layer or generates a trehalose-dimycolate (TDM) molecule (Fig. 1A). Although some issues are not settled (notably, the step at which the methyltransferases introduce the functional groups into the meromycolate chains), the biochemistry of MA synthesis is fairly well documented. In contrast, less is known about the regulatory circuits that control the expression of the ~30 genes involved in the biosynthesis of MA (Fig. 1B). A few transcription regulators that directly regulate the biosynthesis genes have been identified. FasR regulates the expression of the *fas* gene (15), while MabR (16) and FadR (17) regulate the expression of the *fabD-accD6* cluster, the *fasII* operon (Fig. 1B). However, the environmental cues that trigger these regulatory pathways and their physiological roles are not yet known. Several genome-wide microarray studies of cells challenged with various stresses, including nutrient deprivation (18–20) and those experienced by bacteria during infection (21), have shown that genes involved in MA synthesis are likely to be regulated. However, with the exception of a predictive analysis (22), none of these studies specifically addressed the regulation of the whole set of MA genes. Therefore, only a partial picture of the regulation of MA biosynthesis is available. Two genome-wide microarray studies reported the expression profile of *M. tuberculosis* genes during starvation (23, 24). However, as far as MA biosynthesis is con-

cerned, there were a number of discrepancies between these studies, confirming suggestions that conclusions based on genome-wide approaches should be experimentally verified (25).

Among the enzymes of the FAS-II multiprotein synthase, the dehydratases HadAB and HadBC were the last to be identified in *M. tuberculosis* (26) and in *Mycobacterium smegmatis* (27). The observation that the *hadABC* genes are localized downstream of genes encoding tRNAs and one ribosomal protein suggested a coordination of MA biosynthesis with translation potential. The stringent response (SR) is a universal adaptive response that regulates this potential under starvation conditions. The outcome of the SR is the upregulation of stress response genes and the down-regulation of genes encoding the translational machinery and, more globally, of genes involved in energy-consuming processes, such as DNA replication and the synthesis of peptidoglycan and phospholipids (28–31). The SR is required for virulence of many pathogens (32). For *M. tuberculosis*, the SR, although not essential for survival inside macrophages, is critical for survival during long-term *in vitro* starvation (33, 34), virulence in the guinea pig model (35), and persistence in mice and artificial granulomas (24, 36, 37).

In this study, we report that the *hadABC* genes are part of an operon that includes genes required for translation and that as such they are coregulated by the SR. By phylogenetic reconstruction, the key evolutionary steps that have shaped the genetic organization at the *hadABC* locus in *Actinobacteria* have been identified. Finally, an integrated picture of the regulation of all MA synthesis genes by starvation and the SR is presented.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and the plasmids used in this study are listed in Table S1. For liquid cultures of mycobacteria strains, Middlebrook 7H9 medium supplemented with 0.05% Tween (Sigma-Aldrich), glycerol (0.2%) and ADC (bovine albumin, fraction V; dextrose; catalase) or OADC (oleic acid plus ADC) was used. The incubation temperature was 37°C. For solid medium, no Tween was added and Middlebrook 7H10 and 7H11 were used for *M. smegmatis* and *M. tuberculosis*, respectively. When required, antibiotics were added: zeocin at 15 µg/ml, kanamycin (Kan) at 37.5 µg/ml, and hygromycin (Hyg) at 150 µg/ml (*M. smegmatis*) and 50 µg/ml (*M. tuberculosis*). In liquid medium, zeocin was omitted, as we noticed that resistance to zeocin was efficient only in solid medium. To induce, respectively, the *tetRO* and P_{AMIE} promoters, tetracycline (20 ng/ml) and acetamide (0.2%) were added. Luria-Bertani medium was used for the growth of *Escherichia coli* strains with antibiotics when required (Kan, 37.5 µg/ml; Hyg, 150 µg/ml).

Starvation assay. For *M. tuberculosis*, standing cultures grown to mid-log phase in 7H9 plus Tween, glycerol, and ADC were harvested at mid-logarithmic growth phase, washed once, and resuspended in an equal volume of TBST buffer (50 mM Tris-HCl [pH 7], 150 mM NaCl, 0.05% Tween 80). The suspensions were then further incubated for 6 h before being processed for RNA preparation. Cultures for each strain were in triplicate (biological). For lipid preparation, the same growth conditions were used, except that Tween was replaced with 0.05% tyloxapol (Sigma-Aldrich) and the cell suspensions in TBST buffer were incubated for 96 h (a single biological and technical experiment).

Plasmid construction. pGBT was constructed by cloning the *C. glutamicum tetRO* region from the pMIND vector into the pGB9.2 plasmid. The *C. glutamicum tetRO* region, including the upstream tandem *rrn1* transcription terminators, was amplified by PCR, gel purified (Qiagen), cloned in pJET1.2 (Fermentas), digested with *AseI* and *SpeI* (Fermentas), gel purified, and cloned in pGB9.2 digested with the same restriction enzymes. Insertion was checked by sequencing (Eurofins). pLAM was constructed by replacing the Kan^r gene with the Hyg^r gene from pJV53H. pLAM-*rv0634A* was constructed by PCR amplification of the *rv0634A* open reading frame (ORF) from strain H37Rv using the primers Rv0634Aup and Rv0634Adown. The up primer contains a 5'-end *NdeI* restriction site, and the down primer contains a 5'-end *BamHI* restriction site. The PCR product was digested with *NdeI* and *BamHI* and cloned into pLAM digested with the same enzymes. pGBT-*rel_{Mtb}* was constructed by PCR amplification of the *rel_{Mtb}* ORF from strain H37Rv using RelMtbup and RelMtbdown primers. The up primer contains a consensus Shine-Dalgarno sequence upstream of an ATG start codon and a 5'-end *Bsu36I* restriction site. The down primer contains a 5'-end *BamHI* restriction site. Insertion was checked by sequencing (Eurofins). pGBG was constructed by cloning a Shine-Dalgarno *egfp* gene fragment between the *Bsu36I* and *SpeI* sites of pGBT. The *tetRO* promoter was then removed by an *XhoI*-*BamHI* deletion. Operon fusions were constructed by cloning PCR fragments between the *XhoI* and *Bsu36I* sites of pGBG.

Green fluorescent protein (GFP) activity measurements. *M. smegmatis* strains were cultivated for 2 days in 7H9-based medium; then 4 ml was centrifuged, and the pellet was washed twice with 1× phosphate-buffered saline (PBS) plus 0.1% Tween and resuspended in 1 ml of the same buffer. Fluorescence at 520 nm (excitation at 485 nm) and the optical density at 590 nm (OD₅₉₀) were measured in technical duplicates in a 96-well plate with the Clariostar reader (BMG). Relative fluorescence (in relative fluorescence units [RFU]) is the ratio of fluorescence to the OD value.

Construction of *rel_{Mtb}* and *rv0634A* deletion mutants. The mutants were constructed by the recombineering method, as described by van Kessel and Hatfull (38), with two modifications. The recombineering resulted in a hygromycin-resistant derivative (instead of the original kanamycin-resistant plasmid). The allelic exchange segment (AES) was generated with a two-step PCR procedure. In the first step, 3 PCRs were

performed to amplify two fragments ~500 to 600 bp long corresponding to the upstream and downstream regions of the ORF that was to be deleted and the ~700-bp zeocin resistance cassette (the *Streptoalloteichus hindustanus ble* [*sh ble*] gene). The three products were gel purified (Qiagen) and used in the second step for a three-fragment fusion PCR to generate the AES. The product was gel purified and used to transform a competent recombineering strain. Selection was performed on 7H10 medium containing zeocin at 15 µg/ml. Incubation was done at 37°C. PCR with cell lysates was performed to check for the replacement of the target gene by the zeocin cassette and for the absence of the wild-type region. The absence of a specific transcript was also checked by reverse transcription-quantitative PCR (RT-qPCR). The recombineering plasmid (pJV53) was cured from the mutant by successive growth without selection, and its loss was checked by plating on hygromycin-containing solid medium.

Total-RNA preparation. Cultures of the *M. tuberculosis* strains were grown to an A_{600} of ~0.6. Total RNA was extracted using the RNeasy kit (Qiagen) following the manufacturer's instructions, with slight modifications. Briefly, 20 ml of culture was centrifuged for 5 min at 3,500 rpm, and the pellet was resuspended in 1.2 ml of 0.1% β-mercaptoethanol containing RLT lysis buffer (Qiagen) along with 0.1-mm-diameter glass beads. Cells were then lysed by two 120-s pulses at full speed in a bead beater device. The sample was centrifuged at 14,000 rpm for 30 s and filtered twice through 0.22-µm filter units (Millipore) to sterilize it before releasing it outside the biosafety level 3 (BSL-3) laboratory. One volume of absolute ethanol was then added to the filtrate, and total RNA was purified with an RNeasy column following the manufacturer's procedure. To remove all genomic DNA contamination, the RNA sample was treated twice for 45 min successively with 3 U and 2 U of Turbo DNase (Turbo DNA free kit; Ambion). Biological triplicates were performed for each condition.

RT-qPCR. For each sample, 1 µg of RNA was reverse transcribed using random hexamers and Superscript III reverse transcriptase (Invitrogen) according to the manufacturer's instructions. qPCR of purified cDNA was performed using KAPA SYBRFAST qPCR master mix universal (CliniSciences) and primer sets (see Table S6 in the supplemental material). qPCR for each sample was performed in technical duplicate in a Bio-Rad CFX96 thermocycler with the following protocol: denaturation at 95°C for 3 min, followed by 40 cycles of denaturation at 95°C for 3 s and annealing and elongation with data collection at 60°C for 20 s. Standard curves and melting curves were obtained to check for the high amplification efficiency and the specificity of each primer pair, respectively. For qPCR, the mean threshold cycle (C_T) values were normalized against *sigA* C_T values to monitor the expression of the wild-type WT strain under test and control conditions. To calculate *rel_{Mtb}*/WT expression ratios, C_T values were normalized against *dnaJ1* C_T values, as *dnaJ1* was previously reported to be a suitable control to compare expression between the WT and *rel_{Mtb}* strains (24, 34). The difference in expression (*n*-fold) was calculated using the Pfaffl method (39). Statistical significance was determined by comparing C_T values using a two-tailed unpaired Student *t* test.

5' RNA linker-mediated random amplification of cDNA ends (5' RLM-RACE). For transcription start site (TSS) mapping, full-length mRNAs with 5'-triphosphate (5'-PPP) were enriched by incubating total RNA (3 µg) for 60 min with 3 U of Terminator 5'-phosphate-dependent exonuclease (Epicentre), which specifically digests degraded RNA with a 5'-monophosphate (5'-P). After a phenol extraction and ethanol precipitation, the remaining full-length 5'-PPP mRNAs were treated with 1 U of RNA 5' polyphosphatase (Epicentre) for 30 min in order to generate mRNA with a 5'-P end. The reaction was stopped by phenol extraction and ethanol precipitation. The GeneRacer kit (Ambion) RNA adapter was then ligated to the 5'-P end of the mRNA according to the manufacturer's instructions. The ligated mRNA was phenol extracted, ethanol precipitated, and reverse transcribed using a gene-specific primer (GSP) and Superscript III reverse transcriptase (Invitrogen). The amplification of 5' cDNA ends was performed using the GeneRacer 5' primer and a GSP, following by a second PCR amplification using the GeneRacer 5' nested

primer and a GSP to prevent nonspecific amplification. Each PCR product was gel purified (Qiagen), cloned in the pJET1.2 plasmid (Fermentas), and sequenced (Eurofins).

Phylogenomics analyses. The complete *Actinobacteria* genome entries were retrieved from the EBI (European Bioinformatics Institute; <http://www.ebi.ac.uk/genomes/bacteria.html>) and processed by a set of Perl programs into a MySQL database. An RPS (reversed-position specific) BLAST database was computed by retrieving the profiles corresponding to the Conserved Domain Database from the NCBI server (<http://www.ncbi.nlm.nih.gov/cdd>). We used the RPS BLAST program and the RPS BLAST database to annotate the conserved domains identified in the protein sequences. We computed pairs of one-to-one ortholog proteins with BLASTP as follows: proteins *a* and *b* from genomes A and B are considered orthologs if *a* is the best hit for *b* in genome A and vice versa, and if *a* (or *b*) has a paralogous protein named *c*, then the score of *a* versus *b* should be greater than the score of *a* (or *b*) versus *c*.

Identification of orthologous loci. By querying our *Actinobacteria* database, we retrieved, for each genome (one per species), the proteins orthologous to RPMG2 or HadB proteins from *M. tuberculosis*. The genetic organization around the *rpmG2* (*hadB*) gene was analyzed in all genomes in which orthologs of RPMG2 or HadB were identified. Genes involved in microsynteny were identified, and the conserved domains (clusters of orthologous groups [COG]) characterizing their gene products were retrieved. The microsynteny was thus described by a cluster of COGs (see Table S5 in the supplemental material) that are present between two anchor genes: *rpmG2* (upstream) and *rpoC* (downstream). Each *Actinobacteria* genome was screened for the presence of this cluster of COGs by delimitating the search in a region up to 40 kb between the two anchor genes. For each COG from our cluster, we verified that the corresponding genes identified at the locus in the different genomes form a set of orthologous genes.

Construction of a phylogenetic tree of *Actinobacteria*. The *Actinobacteria* phylogeny was inferred from a concatenated sequence alignment obtained for a sample of 56 conserved protein families selected as follows. We used RPS BLAST (40) to functionally annotate the whole protein set of each complete genome with the COG profiles downloaded from the NCBI CDD (Conserved Domains Database) repository (41). We selected a sample of 131 complete *Actinobacteria* genomes which includes only one genome per species. A first set of 149 COG families conserved in this sample were selected according to the quality of the alignments (an E value of $<1e^{-5}$ and an alignment coverage of at least 70% of the COG profile). The alignments for each COG family were created using the MUSCLE program (42) with the default parameters. We used the trimAl program (43) to remove spurious sequences and poorly aligned positions and to analyze the quality of the alignments according to gap numbers and residue conservation in the columns of the alignments. We used the automated parameters that were recommended by the authors to reconstruct maximum-likelihood trees. We retained the 56 top best sequence alignments. The maximum-likelihood trees were computed with PhyML (44), the ProtTest3 program, to select the optimal combinations of parameters (45). The most frequent combination was the LG model of sequence evolution with a γ correction (four categories of evolutionary rates) and shape parameter and proportion of invariant sites estimated from data. When a species did not have a record for a COG family, the missing sequence was replaced by gaps in the alignment. The aligned sequences of 56 COG families for the 131 species were concatenated to produce a single alignment of 16,329 aligned positions, including 2,685 sites without polymorphisms (16.44%). The concatenated tree was computed with PhyML with the parameters selected by ProtTest3, and the statistical branch support was inferred with the parametric bootstrap. The tree was rooted according to the work of Sen and collaborators (46). Our tree topology appears to be globally congruent with the one published by these authors. The tree was drawn with the online version of iTOL (47). Locus organizations were designed by using the protein domain symbolism provided by iTOL.

Phylogenetic tree of mycobacterium methyltransferases. Protein sequences homologous to *M. tuberculosis* methyltransferases were identified through a BLASTP (40) search against the protein sequences encoded by each mycobacterium genome of our selection. The homologous methyltransferases identified in the genomes of *Segniliparus rotundus*, *Tsukamurella paurometabola*, and *Nocardia brasiliensis* were added to the analysis. The multiple alignments were performed using the MUSCLE program (42) with the default parameters. The maximum-likelihood tree was computed with PhyML (44) with the optimal combination of parameter selected by ProtTest3 (45), i.e., an LG model of sequence evolution with a γ correction (four categories of evolutionary rates) and the shape parameter and the proportion of invariant sites estimated from the data. The statistical branch supports were inferred with the parametric bootstrap. The tree was drawn with the online version of iTOL (47).

RESULTS

***hadABC* is coexpressed with genes involved in translation.** The location of *hadABC* downstream of genes encoding two tRNAs (*thrT* and *metT*) and the L33 ribosomal protein (*rpmG2*) suggested coordination between the translation potential and the biosynthesis of mycolic acids. As a first step toward revealing this relationship, the transcription organization of the cluster was analyzed. To identify the transcription units, specific cDNAs from total RNA preparations were synthesized by PCR using a downstream primer matching the 3' end of *hadC* (R_{RT}) and various upstream primers along the cluster (Fig. 2). A single transcription unit encompassing the three *had* genes was detected with the F1-R1 primer pair, confirming that the three genes form an operon, in agreement with both the operon browser of the TbDB database (http://tuberculosis.bu.edu/tbdb_sysbio/operon) and the database of prokaryotic operons (<http://csbl.bmb.uga.edu/DOOR>). As expected, a single transcription unit encompassing the *hadABC* genes plus the three translation-related genes (*thrT*, *metT*, and *rpmG2*) was also detected (primers F2-R1 and F3-R1), as was an additional transcription unit extending to *rv0634A*, located just upstream of *thrT* (F4-R2 primers). No amplification was obtained with primers upstream of *rv0634A* (F5-R2 and F6-R2). These results indicated that the *hadA*, *hadB*, and *hadC* genes were part of an operon composed of at least seven genes, in agreement with the MycoRRdb database (<http://mycorrdb.uohbf.in>). To better define the expression pattern of the whole cluster, transcription start sites (TSSs) were determined by 5' RLM-RACE (Fig. 3). Five TSSs were mapped, one upstream of the *hadA* gene, two upstream of the *metT* gene, and two inside the *rv0634A* ORF. These results indicated that *hadABC* could be either independently expressed as a single operon (*had* operon), coexpressed with the translation genes (*tha* operon), or coexpressed with both the *tha* operon and the putative transcription regulator *rv0634A* gene (*rth* operon). The mapping of the most upstream TSS inside the *rv0634A* ORF suggested that the TTG codon start for this gene was misannotated. The ATG codon located 39 bp further downstream is likely the real start codon, with the TSS at the A of this codon (Fig. 3). The inside TSS mapped on the 74th nucleotide at a G. The *metT*-proximal +1 nucleotide is a G, the first nucleotide of the *metT* tRNA. The *had* operon +1 nucleotide is the G of the GTG start codon. The mapping of these TSSs was in agreement with the transcriptome shotgun sequencing (RNA-seq) global study reported in reference 48. Accordingly, a fairly conserved -10 box sequence could be found upstream these five TSSs (49). We identified two additional sites, 65 nucleotides before the end of *rv0634A* gene and 2 nucleotides downstream of the *thrT* sequence

FIG 2 The *hadABC* genes are coexpressed with translation process and transcription regulator genes. Transcription units were identified by RT-PCR. The forward (F1 to -6) and reverse (R1 and -2) primers used to identify the transcription units as well as the primer used to synthesize the cDNA (R_{RT}) are symbolized by arrows below the genetic map. Results of representative PCRs on reverse transcriptase-treated (+) or untreated (-) templates run on agarose gels are shown. M, DNA ladder.

(Fig. 3). However, neither a -10 box nor promoter activity (see below) was associated with these sites, suggesting that they were false-positive TSSs and likely resulted from natural or experimental RNA cleavages.

To correlate the transcription start sites with promoter activities, the whole region was subcloned downstream of two strong ribosomal T1T2 transcription terminators and upstream of the *gfp* reporter gene in a low-copy-number plasmid. The operon fusions were introduced into *M. smegmatis* and GFP fluorescence specific activities were measured in 2-day-old cultures (Fig. 4). The pGBG vector without any cloned fragment gave the back-

ground level. The F_A and F_B constructs indicated promoter activity specifically associated with the two TSSs located inside the predicted *rv0634A* ORF. In contrast, no promoter activity was detected in the F_C fusion, suggesting that the 5' end of the RNA detected at the 3' end of *rv0634A* likely resulted from a cleavage. The F_{12} and F_1 constructs confirmed the promoter activity associated with the 2 TSSs mapped upstream of *thrT*. The absence of any GFP activity in the F_2 construct suggested that the 5' end that mapped 2 nucleotides downstream of the *thrT* gene likely resulted from cleavage. The F_3 construct pointed to promoter activity associated with the TSS that mapped at the beginning of the *hadA*

FIG 3 Identification of five transcription start sites in the *rth* cluster. Transcription start sites (TSSs) mapped by 5' RLM-RACE are symbolized by broken arrows on the genetic map and are associated with promoter names. On the partial sequence extending from *rv0634A* to the 3' end of *hadA*, the TSSs are shown (+1). The question marks indicate detected 5'-end RNAs that are unlikely to correspond to TSSs. Putative -10 boxes, proposed start codons, and tRNA-encoding gene sequences (*thrT* and *metT*) are underlined.

FIG 4 Identification of five promoter activities in the *rth* cluster. (A) DNA fragments cloned in the GFP reporter vector are shown under the genetic map. Fragments which do not contain any promoter activity are in red. (B) The fluorescence specific activity is expressed in relative fluorescence units (RFU) and is the mean for biological triplicates. pGBG is the *gfp* promoterless control. Error bars show standard deviations.

sequence. In agreement with the absence of any other TSS in the *hadABC* sequence, no promoter activity was detected in fragments containing sequences beyond the beginning of *hadA* (F₄) (data not shown). Taken together, these results indicated that the *hadABC* genes could be transcribed from five promoters (Fig. 4).

The putative transcription regulator Rv0634A does not regulate the *rth* operon. Rv0634A belongs to the mycobacterial core genome (50), and its *Mycobacterium marinum* ortholog MMAR_0966 is annotated as a putative Arc/MetJ class transcription regulator (<http://mycobrowser.epfl.ch/marinolist.html>) (51). Many transcription regulators regulate neighboring genes as well as their own (52). The genetic organization at the *hadABC* locus suggested that Rv0634A might regulate the expression of the whole cluster. To test this possibility, the gene along with its promoters was deleted from the chromosome (Fig. 5A; also, see Materials and Methods). The deletion was verified by PCR (Fig. 5B and C), confirming the previous prediction that the gene is not essential (53), and the absence of *rv0634A* mRNA was verified by RT-qPCR (Fig. 5D). The amount of *rpmG2* and *hadA* mRNAs of the Δ *rv0634A* strain measured by RT-qPCR was not significantly different from that of the WT strain, indicating that neither the putative Rv0634A regulator nor the *rv0634A* promoter (*rthp* and

*thap*₃) contributes to the expression of the *thrT*-*hadC* cluster in log phase (Fig. 5D). When *rv0634A* was overexpressed 64-fold from a plasmid-borne acetamide-inducible promoter, no effect on the expression of either *rpmG2* or *hadA* was observed (Fig. 5D), indicating that Rv0634A does not regulate the *rpmG2*-*hadABC* operon. In addition, in *M. smegmatis*, the overexpression of *rv0634A* did not affect the expression of either the F_A *gfp* fusion, which indicates the absence of autoregulation of *rv0634A*, or the F_B fusion, showing that Rv0634A did not regulate the promoter inside the gene (Fig. 5E). Together, these results indicated that Rv0634A does not regulate the transcription of the *rv0634A*-*hadC* cluster.

The mycolic acid biosynthesis genes *hadABC* are negatively coregulated with the ribosomal protein gene *rpmG2* by the stringent response. The stringent response (SR) is a conserved adaptive response that downregulates translation potential in starvation conditions. The molecular hallmark of the SR is the synthesis of the alarmone (p)ppGpp by the RelA/SpoT homolog (RSH), which leads to dramatic reprogramming of cell transcription, including a rapid inhibition of stable RNA synthesis (54). The coexpression of the *hadABC* genes with genes coding for tRNAs and a ribosomal protein suggested that the *hadABC* genes might also be regulated by the SR. To test this proposition, the

FIG 5 The putative transcription regulator Rv0634A does not regulate the *tha* operon. (A) Genetic map showing the *rv0634A* and *sh ble* allelic exchange. Primers for the deletion analysis by PCR are symbolized by arrows. (B and C) PCR products with the a + b and a + c primer pairs to confirm the deletion of the *rv0634A* gene. Lysates of a $\Delta rv0634A$ strain (1) and of a WT clone (2) were used as PCR templates. (D) Monitoring by RT-qPCR of the expression (log₂) of the *rpmG2*, *hadA*, and *rv0634A* genes relative to the WT strain values (WT/pLAM) in the *rv0634A* mutant ($\Delta rv0634A$ /pLAM) and in the *rv0634A*-overexpressing background ($\Delta rv0634A$ /pLAM-*rv0634A*). Each strain was cultivated in the presence of 0.2% acetamide. Error bars show standard errors of the means (SEM) from three biological triplicates. (E) Expression, in relative fluorescent units (RFU), of *thap3::gfp* (F_A) and *thap2::gfp* (F_B) fusions in *M. smegmatis* carrying a plasmid expressing *rv0634A* from an acetamide-inducible promoter (pLAM12-*rv0634A*) or not (pLAM12). Each strain was cultivated in either the presence (+) or absence (−) of 0.2% acetamide. pGBG was the *gfp* promoterless control.

expression of genes in the *rv0634A*-*hadC* cluster was then measured by RT-qPCR after starvation for 6 and 24 h (approximately one-fourth and one generation time, respectively). As shown in Fig. 6A, in the WT background, the expression of *rel_{Mtb}* was up-regulated, as well as that of *sigE* and *mprA*, in agreement with previous reports (23, 24). In contrast, the five genes of the operon were downregulated, either after 6 h starvation only (*rv0634A*), 24 h starvation only (*rpmG2*) or both starvation periods (*hadA*, *hadB*, and *hadC*) (Fig. 6A). To determine the contribution of the SR to this regulation, we deleted *rel_{Mtb}*, the *M. tuberculosis* RSH-encoding gene. The construction was checked by PCR (Fig. 6B to D) and by RT-qPCR for the absence of *rel_{Mtb}* mRNA (see Tables S2 to S4 in the supplemental material). The expression level was then measured in the Δrel_{Mtb} background, and the mutant-to-WT expression ratio was determined (Fig. 6E). In log phase, the relative expression of *rv0634A*, *rpmG2*, and the three *had* genes in the Δrel_{Mtb} background was similar to that in the WT strain and in the complemented strain expressing *rel_{Mtb}* from a low-copy-number plasmid (pGBT-*rel_{Mtb}*). After starvation, a similar result was observed for *rv0634A*, whereas for *rpmG2*, *hadA*, *hadB*, and *hadC*, the expression ratio was higher for the Δrel_{Mtb} strain but not for the complemented strain (Fig. 6E). These results indicated that

rpmG2 and *hadABC* were negatively coregulated by the SR. The magnitude of the repression due to the SR for the four genes was similar (~2-fold) (Fig. 6E), suggesting that the SR exerted its control through the promoter(s) *thap₂* and/or *thap₁*, located just upstream of *thrT* (Fig. 4). Thus, in agreement with the genetic organization, these results suggested coordination of the MA biosynthesis with translation potential.

Starvation leads to a global downregulation of genes required for mycolic acid synthesis. One might have expected the regulation of the *hadABC* genes by starvation to be coordinated with that of other genes involved in the biosynthesis of MAs. Thus, in order to get an integrated picture of the regulation of MA biosynthesis, the expression of a subset of genes representative of each step of the biosynthesis and transport of MAs was measured after 6 and 24 h starvation. The results are presented in Fig. 7. The expression of *fas*, which is required for the synthesis of the MA α chain as well as the acyl-CoA substrates for the FAS-II complex (Fig. 1), was downregulated. The gene *fabH* acting at the intermediate step between FAS-I and FAS-II activities was not affected (Fig. 1) (55). Both *fabD* and *acpM*, which are required for the synthesis of FAS-II malonyl-acyl carrier protein (malonyl-ACP) elongation units, were downregulated (at 24 h starvation for *fabD*)

FIG 6 The *tha* operon is regulated by the stringent response. (A) Monitoring by RT-qPCR of the relative expression (log₂) of the *rv0634A*, *rpmG2*, *hadABC*, *rel_{Mtb}*, *mprA*, and *sigE* genes in the WT strain (WT/pGBT) upon 6 and 24 h of starvation. The values are expressed as a ratio to WT values in unstressed log-phase cultures. (B) Genetic map showing the *rel_{Mtb}* and *sh ble* allelic exchange. Primers for the deletion analysis by PCR are symbolized by arrows. (C and D) PCR products obtained with the a + b and a + c primer pairs to confirm the deletion of the *rel_{Mtb}* gene. Lysates of the WT strain (lanes 1) and of a Δrel_{Mtb} clone (lanes 2) were used as PCR templates. (E) Gene expression ratio relative to values for the WT/pGBT strain from log phase, upon 6 and 24 h of starvation, respectively. In panels A and E, error bars represent SEM from three biological triplicates. *P* values were <0.05 (*), <0.01 (**), <0.001 (***), and <0.0001 (****).

(Fig. 1). Along with *hadABC*, the genes involved in the four reaction steps driven by the FAS-II complex, *kasA*, *mabA*, and *inhA*, were also downregulated (Fig. 1). Of the two genes involved in the condensation of the FAS-I and FAS-II products, *fabD32* appeared not to be regulated, whereas *pks13* was downregulated after 6 h of starvation (Fig. 1). The genes required for the subsequent reduction step (*cmrA*) and the transport of TMM across the inner membrane (*mmpL3*) were also downregulated at 6 h starvation (Fig. 1). Among the three genes involved, the synthesis of the mycolyl-transferase antigen Ag85 complex gene, *fbpA*, was downregulated at 6 and 24 h, that of *fbpB* was downregulated at 6 h, and the expression of the third and most active Ag85 subunit gene, *fbpC* (Fig. 1) (56, 57), was upregulated at both 6 and 24 h. Among the six methyltransferase genes known to be involved in decorating the meromycotic chain in *M. tuberculosis* (Fig. 1), *pcaA* and *cmaA2* were upregulated (at 24 h for *cmaA2*), whereas *mmaA1* to -4 were downregulated (at 6 h for *mmaA1* and *mmaA3*). Therefore, upon starvation, the expression of most of the MA biosynthesis genes was affected, and with few exceptions, the expression was lower. To determine the contribution of the SR to this regulation, gene

expression was measured in the Δrel_{Mtb} mutant as well, and the ratio to the WT values was determined. The complete data are presented in Tables S2 to S4 in the supplemental material. With an arbitrary cutoff ratio of 1.4, Table 1 lists the genes whose expression was affected by the Δrel_{Mtb} mutation after 24 h starvation. This set included *acpM*, the FAS-II complex genes *hadABC* and *kasA*, the Ag85 *fbpA* gene, and the methyltransferase genes *mmaA2*, *mmaA3*, *cmaA2*, and *pcaA*. It is worth noting that *acpM* and *kasA* were the only genes to be positively regulated by the SR, despite the fact that the interplay between the different regulatory pathways that have been induced by our experimental starvation conditions ultimately led to the downregulation of both genes. Therefore, during severe starvation, most of the mycolate biosynthesis genes were regulated, whereas environmental signals specifically triggering the SR would regulate only a small gene subset. This later observation was in agreement with microarray studies of Dahl and coworkers showing that only 5 genes involved in MA biosynthesis were regulated by *Rel_{Mtb}* following 6 h of starvation (24). These genes also include *hadB*, *hadC*, and *fbpA*, along with the FAS-II gene *mabA* and the Ag85 gene *fbpC*.

FIG 7 Regulation of mycolic acid biosynthesis, transport, and regulator genes upon starvation. The WT strain (WT/pGBT), was grown to log phase and was starved for 6 or 24 h before total-RNA sampling. (A) The relative expression of the genes representative of the major steps of the mycolic acid biosynthesis and transport was measured by RT-qPCR. The values are expressed in $\log_2$ as a ratio to values for unstarved log-phase WT cultures. Error bars show SEM from three biological triplicates. *P* values were <0.05 (*), <0.01 (**), and <0.001 (***).

Few transcription regulators of MA genes have been characterized at the molecular level, namely, FasR, which activates the *fas* gene (15), MabR, which represses the *fasII* operon (*fabD-acpM-kasA-kasB-accD6*) and the *fas* gene (16), and FadR, a repressor of the *fasII* operon (17). As shown in Fig. 7, neither *mabR* nor *fadR* was regulated by starvation, whereas *fasR* was slightly upregulated during 6 and 24 h starvation independently of *Rel_{Mtb}*. The expression of *rel_{Mtb}* was reported to be indirectly activated by MprA, which regulates the response of the MprAB two-component system through the sigma factor SigE (58–60). Both the corresponding genes, *mprA* and *sigE*, were also upregulated by starvation, suggesting that these genes might also contribute to the upregulation of the *rel_{Mtb}* gene during starvation. Interestingly, the expression of both *mprA* and *sigE* was lower in the WT than in the *rel_{Mtb}* mutant (Table 1), suggesting negative feedback control of both genes by *Rel_{Mtb}*.

Phylogenetic reconstruction of the *rth* operon–*mmaA1-mmaA4* cluster. We undertook a detailed analysis of the *rth* locus

in 131 genomes of *Actinobacteria* (one strain per species) to understand the key evolutionary steps that have shaped the current genetic organization of this locus in mycobacterial genomes and, by extension, in *Actinobacteria* genomes. First, we retrieved for every genome the protein orthologous either to *M. tuberculosis* RpmG2 or to *M. tuberculosis* HadB when the *rpmG2* gene was absent from the studied genome. The genetic context of both genes in all genomes was further analyzed, and microsynteny was detected only downstream of them, with, in about 20 genomes, the following gene order conservation: *rpmG2-hadA-hadB-secE-nusG-rplK-rplA-rplJ-rplL-rpoB-rpoC*. The products of these genes are characterized by specific conserved domains (see the corresponding COG number in Table S5 in the supplemental material). This microsynteny could be disrupted by the insertion of a few other genes, among which *murB* was found at the locus in 54 genomes. In addition, three tRNA genes upstream of *rpmG2* were found to be conserved in order and nature (*tyrT-thrT-metT*) and one upstream gene, *secE* (*trpT*). Thus, we carried out a systematic search of this cluster of COGs on a chromosomal region whose maximal size was set to 40 kb using *rpmG2* or *hadA* as an upstream landmark and *rpoB* and *rpoC* as a downstream limit. The result of this search was plotted over our *Actinobacteria* species tree in order to identify the key evolutionary steps involved in shaping the locus genetic organization (Fig. 8; also, see Fig. S1 in the supplemental material).

From the analysis of the phylogenetic distribution of the locus genes, the organization *rpmG2-secE-nusG-rplK-rplA-rplJ-rplL-rpoB-rpoC* should have been the ancestral locus organization present in the last common ancestor (LCA) of all current *Actinobacteria* species, as (i) those genes are found in almost all genomes, whatever their taxonomic classification (see Fig. S2 in the supplemental material), and (ii) this organization is observed in the genomes of the most ancient groups on the *Actinobacteria* species tree (*Conexibacteraceae* and the *Rubrobacterineae*) (see Fig. S1 in the supplemental material) as well as in genomes outside the *Actinobacteria*, notably in 123 of 219 *Firmicutes* genomes (see Fig. S3 in the supplemental material). From this ancestral organization,

TABLE 1 Genes regulated by the stringent response

Gene	Expression ratio (mean $\pm$ SEM) in strain with genotype ^a		
	WT (pGBT)	Δrel_{Mtb} (pGBT)	Δrel_{Mtb} (pGBT)- <i>rel_{Mtb}</i>
<i>rv0634A</i>	1 $\pm$ 0.04	0.61 $\pm$ 0.04	0.79 $\pm$ 0.02
<i>rpmG2</i>	1 $\pm$ 0.03	1.87 $\pm$ 0.10	1.39 $\pm$ 0.07
<i>hadA</i>	1 $\pm$ 0.04	2.01 $\pm$ 0.15	1.42 $\pm$ 0.09
<i>hadB</i>	1 $\pm$ 0.01	1.84 $\pm$ 0.15	1.33 $\pm$ 0.08
<i>hadC</i>	1 $\pm$ 0.02	1.80 $\pm$ 0.04	1.30 $\pm$ 0.09
<i>acpM</i>	1 $\pm$ 0.06	0.55 $\pm$ 0.01	1.05 $\pm$ 0.10
<i>kasA</i>	1 $\pm$ 0.02	0.46 $\pm$ 0.02	1.17 $\pm$ 0.14
<i>mmaA2</i>	1 $\pm$ 0.02	2.08 $\pm$ 0.29	1.26 $\pm$ 0.08
<i>mmaA3</i>	1 $\pm$ 0.01	1.42 $\pm$ 0.12	1.18 $\pm$ 0.08
<i>cmaA2</i>	1 $\pm$ 0.04	1.46 $\pm$ 0.12	1.13 $\pm$ 0.09
<i>pcaA</i>	1 $\pm$ 0.06	1.54 $\pm$ 0.14	1.23 $\pm$ 0.06
<i>fbpA</i>	1 $\pm$ 0.07	1.73 $\pm$ 0.02	1.19 $\pm$ 0.07
<i>mprA</i>	1 $\pm$ 0.07	1.55 $\pm$ 0.02	1.39 $\pm$ 0.09
<i>sigE</i>	1 $\pm$ 0.07	1.42 $\pm$ 0.02	1.10 $\pm$ 0.10

^a Expression relative to the wild-type value after 24 h of starvation.

December 2015 Volume 197 Number 24

at the origin of all taxonomic groups except *Conexibacteraceae* and *Rubrobacterineae*. In the branch leading to the LCA of *Mycobacterium*, a duplication of the *hadA/C* ancestor gene should have occurred, giving rise to the actual *hadA-hadB-hadC* locus organization found in those species. The absence of the genes *rpmG2*,

hadA, and *hadB* from the region upstream of *secE* in the *Corynebacterium* genomes corresponds clearly to a loss of these genes in the genome of their LCA, as the three tRNA genes, *thrT*, *thrT*, and *metT*, always located upstream of *rpmG2*, are found conserved and located just upstream of the tRNA gene *trpT*, invariably located upstream of *secE*. The absence of both *hadA/C* and *hadB* genes in the *Bifidobacteriaceae* genomes and of *rpmG2* in a group of *Kineosporiineae* genomes forming a subtree on the species tree (see Fig. S1 in the supplemental material) is probably due to the loss of those genes in the LCA genome of each group.

The gene *rv0634A* would have been acquired once on the branch leading to the LCA of the *Mycobacterium avium* and *M. tuberculosis* complexes and of *M. ulcerans*, *M. marinum*, and *M. leprae* (Fig. 8).

Finally, the four methyltransferase genes found at the *M. tuberculosis* locus were acquired through three independent events (Fig. 8). The gene *mmaA4* was acquired first after the speciation of *M. abscessus* on the branch leading to the LCA of the other mycobacterium species and was probably subsequently replaced in *M. smegmatis*. Then, *mmaA1* was gained on the branch leading to the LCA of the *M. avium* and *M. tuberculosis* complexes and *M. ulcerans*, *M. marinum*, and *M. leprae*. Finally, *mmaA2* and *mmaA3* were acquired on the branch leading to the LCA of *M. ulcerans*, *M. marinum*, and *M. leprae* and the *M. tuberculosis* complex. The different acquisition events do not result from successive duplications of the inserted genes, as the subtrees corresponding to *MmaA4*, *MmaA3*, *MmaA2*, and *MmaA1* gene products are not sister groups on the tree built on the homologous methyltransferase from mycobacterium (see Fig. S4A in the supplemental material). However, once acquired, those genes were inherited vertically, since both species and protein trees are congruent (see Fig. S4B in the supplemental material). In some species, some methyltransferase genes became pseudogenes (see Fig. S4B in the supplemental material).

The region extending from *rpmG2* to *rpoC* seems to be a hot spot of recombination, as it has suffered many gene gains and losses. Thus, in a few genomes, the *rpmG2* locus has been disrupted with the organization *secE-nusG-rplK-rplA-rplJ-rplL-rpoB-rpoC* found at a distant chromosomal location (see Fig. S1 in the supplemental material). However, those genomes are found scattered along the species tree, except for the *Bifidobacteriaceae* species, where the disruption events may have occurred in their last common ancestor.

DISCUSSION

The *M. tuberculosis* mycomembrane is a highly efficient permeability barrier containing many antigenic determinants and is essential to cell viability and virulence. During the process of infection, the bacteria must adapt to various environmental conditions, and little is known of how the biosynthesis of mycolic acids, the major components of the mycomembrane, responds to these conditions, notably to the nutrient scarcity that is believed to prevail inside the host (53, 61). About 30 genes, including *hadA*, *hadB*, and *hadC*, are required for the synthesis and transport of MAs. This study shows that upon starvation, the *had* genes are coexpressed and downregulated with three neighboring genes, *thrT*, *metT*, and *rpmG2*, involved in translation, in accordance with their genetic organization. This result suggests that MA biosynthesis is coordinated with the translation potential of the cell. The

regulation of synthesis of stable RNAs and ribosomal proteins is a hallmark of the stringent response (31), and accordingly, the downregulation was seen to be dependent on a functional *Rel_{Mtb}* protein, the key enzyme of the stringent response. The *hadABC* genes could also be coexpressed with *rv0634A*, the first gene of a seven-gene operon that includes *thrT*, *metT*, and *rpmG2*. Although the *M. marinum* Rv0634A homolog is recorded as a transcription regulator of the Arc/MetJ family (<http://mycobrowser.epfl.ch/marinolist.html>), we have no evidence that Rv0634A was involved in the regulation of this operon. The gene *rv0634A* was reported to belong to the mycobacterial core genes (50) and is conserved in only some slow-growing pathogenic and opportunistic mycobacterial species (Fig. 8; also, see Fig. S1 and S2 in the supplemental material) (62). Despite these observations, the deletion of the gene did not affect the fitness of the bacteria, including the ability to grow in human THP-1 macrophages (our unpublished results). The function of this gene remains to be discovered.

In coordination with the expression profile of the *hadABC* genes, starvation downregulated expression of most genes representative of each step of the synthesis and transport of the MAs. Because MA biosynthesis is a highly energy-consuming process, it makes physiological sense for the cell to slow down its synthesis during nutrient scarcity. Rohde et al. (21) reported that in a comprehensive transcription profiling study over the course of a 14-day infection in resting macrophages, one-third of the genes involved in MA biosynthesis were downregulated. These results agree with the pioneering observations of Lacave and coworkers, i.e., the arrest of MA biosynthesis in stationary phase (63), and are consistent with the observation that during infection, *M. tuberculosis* switches from a carbon to a lipid regimen during which the fatty acid catabolism and the storage compound triacylglycerol (TAG) synthesis pathways are activated instead (64, 65). The expression profile of genes involved in the first (*fas*, *fabH*, and *fabD*) and last (*fadD32*, *pks13*, *cmrA*, *mmpL3*, and *fbpBC*) steps of the biosynthesis pathway and of two of the six genes coding for methyltransferases (*mmaA1* and *mmaA4*) was independent of *Rel_{Mtb}* (Table 1; also, see Tables S2 to S4 in the supplemental material). In contrast, among the genes encoding FAS-II, the *hadABC* operon and *kasA* are regulated by the SR, with the latter being upregulated. Such an uncoupling between the expression of FAS-II genes and that of genes involved in upstream steps such as *fas* has been previously reported during growth arrest. This uncoupling would allow the rerouting of the carbon flux toward the synthesis of storage and stress-protective compounds, such as TAG and glutamate (65). A similar explanation might also apply to our results. A reverse enzymatic pathway during the SR primed at the *KasA* step would return the meromycolate intermediate products resulting from the activity of FAS-II to FAS-I for the synthesis of alternative fatty acids.

There are several types of MA species, as defined by the functional groups found at two positions on the meromycolate chain. In *M. tuberculosis*, six methyltransferase genes are involved in the introduction of these groups. Two, *pcaA* and *cmaA2*, were upregulated upon starvation, whereas *mmaA1*, *mmaA2*, *mmaA3*, and *mmaA4* were downregulated. Hence, upon starvation, all mycolic acid species should be enriched in decoration stemming from *PcaA* and *CmaA2* activities, i.e., with a cyclopropanation group at the proximal position (8, 66). Cyclopropanation by *PcaA* and *CmaA2* has been shown to be crucial for the virulence of *M.*

tuberculosis, implying that specifically enriching MAs in cyclopropanated species might contribute to the adaptive response during infection (67, 68). With the exception of *mmaA1* and *mmaA4*, the expression of the methyltransferase genes was negatively controlled by Rel_{Mtb} . Therefore, environmental signals specifically triggering the SR should lead to enrichment of MA species with a methyl group at the distal position of oxygenated species due to *MmaA4* activity and at the proximal position of *trans*-methoxy and *trans*-keto species due to *MmaA1* activity (8). The activity of *MmaA4* is crucial for envelope impermeability and for full virulence in mice (69). Loss of *MmaA1* has been shown to enhance the virulence-associated cording phenotype of *M. tuberculosis* (66). One unresolved issue in the biosynthesis of MA is the step at which decoration is added to the MA mero chain; addition could accompany elongation by either FAS-II or, following synthesis, the full-length meromycolate chain. The former possibility is favored by previous reports that methyltransferases interact with subunits of the FAS-II complex (70, 71). The observation that *mmaA1* to *-4* are clustered with the *hadABC* genes would also agree with the hypothesis of a functional coordination between methyltransferases and FAS-II. Moreover, it was recently shown that during macrophage infection by *M. bovis* BCG, the *hadABC* genes were the only ones contributing to the FAS-II complex to be regulated. Interestingly, this regulation was coordinated with that of the methyltransferase genes, arguing again for a close relationship between the HadABC enzymes and activity of the methyltransferases (72). Our observation that the regulation of both sets of genes—*mmaA2/mmaA3* and *hadABC*—is Rel_{Mtb} dependent further strengthens this relationship. Recently we have shown that mutations in the gene *hadC* influence the methyltransferase-dependent decoration profiles of meromycolate chains, reinforcing this hypothesis (our unpublished results).

There is a regulatory signaling cascade linking the two-component signal transduction system MprAB, the sigma factor SigE, and Rel_{Mtb} . Upon activation, the response regulator MprA directly upregulates *sigE*, which then directly stimulates expression of *rel_{Mtb}* (58, 73). Both *mprA* and *sigE* activate their own expression. Data from microarray analysis suggest additional positive feedback loops, with SigE upregulating *mprAB* (74) and Rel_{Mtb} activating its own expression (Table 1) (23, 24). The outcome of this complex regulatory circuit design is phenotypic heterogeneity, with two subpopulations expressing rel_{Mtb} at different levels (59, 60). This heterogeneity is part of a “bet-hedging strategy” contributing to the survival of the population (1). Our results, showing that Rel_{Mtb} might also negatively regulate the expression of both *mprB* and *sigE*, would therefore add two negative feedback loops to the positive feedback design of the *mprB-sigE-rel_{Mtb}* circuit. These negative loops may contribute to turning off the MprBA-SigE- Rel_{Mtb} signalization pathway once the stress is over.

For the MA biosynthesis genes whose regulation was partly or fully independent of Rel_{Mtb} , other transcription regulators must be involved. With the exception of the transcription activator FasR, which might contribute to the downregulation of its single target *fas* gene, neither MabR nor FadR, the two other well-characterized regulators, is likely to contribute to the regulation of the MA biosynthesis genes during starvation. Hence, further actors in the regulatory circuitry leading to global repression of MA synthesis genes during starvation remain to be identified.

Despite the downregulation of the MA biosynthesis genes, the

qualitative and quantitative profiles of the MAs were not affected after 96 h of starvation in either WT (see Fig. S5 in the supplemental material) or rel_{Mtb} (data not shown) strains, indicating that MAs are stable molecules. This result suggested that during starvation, the integrity of the mycomembrane would not be dramatically compromised by sparing the energy cost of synthesizing *de novo* MAs. The differential expression observed among the methyltransferase-encoding genes suggests that adaptation of the cell to starvation is mainly through subtle modifications of the meromycolic acid chains.

Following a comprehensive analysis of currently available actinobacterial genomes, we were able to draw an evolutionary scenario (Fig. 8) leading to the current organization of the locus encompassing *hadABC* and *mmaA1* to *-4* in *Mycobacterium* species. The *hadA* and *hadB* genes have been found to be associated in all the genomes analyzed so far. Conservation of the gene order in distantly related species has been shown to result from a functional constraint and could be a fingerprint of physical interaction between the encoded proteins (75). This is certainly the case for *hadA* or *hadC* and *hadB*, which are cotranscribed and for which physical interactions between their products have been described—the active complexes are either HadA-HadB or HadB-HadC heterodimers (26). The analysis of the methyltransferase tree (see Fig. S4 in the supplemental material) leads to the conclusion that the presence at the locus of the four neighboring methyltransferase genes (*mmaA1* to *-4*) is due to their acquisition through three independent events rather than the result of successive duplication events from an ancestral inserted gene. This intriguing observation suggests that further investigation will reveal the identity of recombination insertion sites. Considering the numerous rearrangements that have occurred, it is likely that this locus is a recombination hot spot. However, the microsynteny observed in the pathogenic mycobacterium genomes suggests that a functional constraint acts at this locus to maintain the methyltransferase genes in the vicinity of the *hadABC* genes. This is in agreement with the fact that their gene products are involved in the same biosynthesis pathway. The efficiency of interaction between the Had and methyltransferase proteins previously reported (70, 71) would certainly benefit from such a genetic organization, since clustering of genes at the same locus would increase the local protein concentrations. Furthermore, this is consistent with our proposed evolutionary scenario, as the duplication of the *hadA/C* gene predated the acquisition of the first methyltransferase gene at the locus.

The issue that triggered the present study was determining the significance, if any, of the localization of the *had* genes downstream of genes involved in the translation process. We have demonstrated that this organization has allowed both types of genes to be coregulated by the SR. Although the driving force for the gene rearrangement in this region was probably the recombinogenic nature of the locus, the coregulation together with the subsequent acquisition of the methyltransferase genes might have imposed a selection pressure strong enough to shape and maintain the current organization at this locus.

ACKNOWLEDGMENTS

We are grateful to Graham F. Hatfull for providing the mycobacterial recombineering system, Marcus A. Horwitz for the pGB9.2 plasmid, Brian D. Robertson for the pMIND plasmid, and Wladimir Malaga for the pJV53::hyg plasmid. We thank Ingrid Mercier, Alexandre Gouzy, Jeanne

Pilaire, Clément Carel, and Aïcha Bah for technical advice. We are grateful to Dave Lane for critical reading of the manuscript and to the members of the Daffé team for their constructive criticisms.

This work was supported by institutional grants from the CNRS (Centre National de la Recherche Scientifique). S.J. was supported by an MESR (Ministère de l'Enseignement Supérieur et de la Recherche) fellowship.

REFERENCES

- Balaban NQ, Gerdes K, Lewis K, McKinney JD. 2013. A problem of persistence: still more questions than answers? *Nat Rev Microbiol* 11:587–591. <http://dx.doi.org/10.1038/nrmicro3076>.
- Gengenbacher M, Kaufmann SH. 2012. Mycobacterium tuberculosis: success through dormancy. *FEMS Microbiol Rev* 36:514–532. <http://dx.doi.org/10.1111/j.1574-6976.2012.00331.x>.
- Young DB, Gideon HP, Wilkinson RJ. 2009. Eliminating latent tuberculosis. *Trends Microbiol* 17:183–188. <http://dx.doi.org/10.1016/j.tim.2009.02.005>.
- Falzon D, Jaramillo E, Wares F, Zignol M, Floyd K, Ravigione MC. 2013. Universal access to care for multidrug-resistant tuberculosis: an analysis of surveillance data. *Lancet Infect Dis* 13:690–697. [http://dx.doi.org/10.1016/S1473-3099\(13\)70130-0](http://dx.doi.org/10.1016/S1473-3099(13)70130-0).
- Migliori GB, Sotgiu G, Gandhi NR, Falzon D, DeRiemer K, Centis R, Hollm-Delgado MG, Palmero D, Perez-Guzman C, Vargas MH, D'Ambrosio L, Spanevello A, Bauer M, Chan ED, Schaaf HS, Keshavjee S, Holtz TH, Menzies D. 2013. Drug resistance beyond extensively drug-resistant tuberculosis: individual patient data meta-analysis. *Eur Respir J* 42:169–179. <http://dx.doi.org/10.1183/09031936.00136312>.
- Velayati AA, Masjedi MR, Farnia P, Tabarsi P, Ghanavi J, Ziaiarifi AH, Hoffner SE. 2009. Emergence of new forms of totally drug-resistant tuberculosis bacilli: super extensively drug-resistant tuberculosis or totally drug-resistant strains in Iran. *Chest* 136:420–425. <http://dx.doi.org/10.1378/chest.08-2427>.
- Daffe M. 2015. The cell envelope of tubercle bacilli. *Tuberculosis* 95(Suppl 1):S155–S158. <http://dx.doi.org/10.1016/j.tube.2015.02.024>.
- Marrakchi H, Laneelle MA, Daffe M. 2014. Mycolic acids: structures, biosynthesis, and beyond. *Chem Biol* 21:67–85. <http://dx.doi.org/10.1016/j.chembiol.2013.11.011>.
- Daffe M, Crick DC, Jackson M. 2014. Genetics of capsular polysaccharides and cell envelope (glyco)lipids. *Microbiol Spectr* 2:MGM2-0021-2013.
- Guenin-Mace L, Simeone R, Demangel C. 2009. Lipids of pathogenic mycobacteria: contributions to virulence and host immune suppression. *Transbound Emerg Dis* 56:255–268. <http://dx.doi.org/10.1111/j.1865-1682.2009.01072.x>.
- Jackson M, McNeil MR, Brennan PJ. 2013. Progress in targeting cell envelope biogenesis in Mycobacterium tuberculosis. *Future Microbiol* 8:855–875. <http://dx.doi.org/10.2217/fmb.13.52>.
- Favrot L, Ronning DR. 2012. Targeting the mycobacterial envelope for tuberculosis drug development. *Expert Rev Anti Infect Ther* 10:1023–1036. <http://dx.doi.org/10.1586/eri.12.91>.
- Neyrolles O, Guilhot C. 2011. Recent advances in deciphering the contribution of Mycobacterium tuberculosis lipids to pathogenesis. *Tuberculosis (Edinb)* 91:187–195. <http://dx.doi.org/10.1016/j.tube.2011.01.002>.
- Goude R, Parish T. 2008. The genetics of cell wall biosynthesis in Mycobacterium tuberculosis. *Future Microbiol* 3:299–313. <http://dx.doi.org/10.2217/17460913.3.3.299>.
- Mondino S, Gago G, Gramajo H. 2013. Transcriptional regulation of fatty acid biosynthesis in mycobacteria. *Mol Microbiol* 89:372–387. <http://dx.doi.org/10.1111/mmi.12282>.
- Salzman V, Mondino S, Sala C, Cole ST, Gago G, Gramajo H. 2010. Transcriptional regulation of lipid homeostasis in mycobacteria. *Mol Microbiol* 78:64–77.
- Biswas RK, Dutta D, Tripathi A, Feng Y, Banerjee M, Singh BN. 2013. Identification and characterization of Rv0494: a fatty acid-responsive protein of the GntR/FadR family from Mycobacterium tuberculosis. *Microbiology* 159:913–923. <http://dx.doi.org/10.1099/mic.0.066654-0>.
- Provvedi R, Boldrin F, Falciani F, Palu G, Manganelli R. 2009. Global transcriptional response to vancomycin in Mycobacterium tuberculosis. *Microbiology* 155:1093–1102. <http://dx.doi.org/10.1099/mic.0.024802-0>.
- Boshoff HI, Myers TG, Copp BR, McNeil MR, Wilson MA, Barry CE, III. 2004. The transcriptional responses of Mycobacterium tuberculosis to inhibitors of metabolism: novel insights into drug mechanisms of action. *J Biol Chem* 279:40174–40184. <http://dx.doi.org/10.1074/jbc.M406796200>.
- Hampshire T, Soneji S, Bacon J, James BW, Hinds J, Laing K, Stabler RA, Marsh PD, Butcher PD. 2004. Stationary phase gene expression of Mycobacterium tuberculosis following a progressive nutrient depletion: a model for persistent organisms? *Tuberculosis* 84:228–238. <http://dx.doi.org/10.1016/j.tube.2003.12.010>.
- Rohde KH, Veiga DF, Caldwell S, Balazsi G, Russell DG. 2012. Linking the transcriptional profiles and the physiological states of Mycobacterium tuberculosis during an extended intracellular infection. *PLoS Pathog* 8:e1002769. <http://dx.doi.org/10.1371/journal.ppat.1002769>.
- Colijn C, Brandes A, Zucker J, Lun DS, Weiner B, Farhat MR, Cheng TY, Moody DB, Murray M, Galagan JE. 2009. Interpreting expression data with metabolic flux models: predicting Mycobacterium tuberculosis mycolic acid production. *PLoS Comput Biol* 5:e1000489. <http://dx.doi.org/10.1371/journal.pcbi.1000489>.
- Betts JC, Lukey PT, Robb LC, McAdam RA, Duncan K. 2002. Evaluation of a nutrient starvation model of Mycobacterium tuberculosis persistence by gene and protein expression profiling. *Mol Microbiol* 43:717–731. <http://dx.doi.org/10.1046/j.1365-2958.2002.02779.x>.
- Dahl JL, Kraus CN, Boshoff HI, Doan B, Foley K, Avarbock D, Kaplan G, Mizrahi V, Rubin H, Barry CE, III. 2003. The role of RelMtb-mediated adaptation to stationary phase in long-term persistence of Mycobacterium tuberculosis in mice. *Proc Natl Acad Sci U S A* 100:10026–10031. <http://dx.doi.org/10.1073/pnas.1631248100>.
- Wang Y, Barbacioru C, Hyland F, Xiao W, Hunkapiller KL, Blake J, Chan F, Gonzalez C, Zhang L, Samaha RR. 2006. Large scale real-time PCR validation on gene expression measurements from two commercial long-oligonucleotide microarrays. *BMC Genomics* 7:59. <http://dx.doi.org/10.1186/1471-2164-7-59>.
- Sacco E, Covarrubias AS, O'Hare HM, Carroll P, Eynard N, Jones TA, Parish T, Daffe M, Backbro K, Quemard A. 2007. The missing piece of the type II fatty acid synthase system from Mycobacterium tuberculosis. *Proc Natl Acad Sci U S A* 104:14628–14633. <http://dx.doi.org/10.1073/pnas.0704132104>.
- Brown AK, Bhatt A, Singh A, Saparia E, Evans AF, Besra GS. 2007. Identification of the dehydratase component of the mycobacterial mycolic acid-synthesizing fatty acid synthase-II complex. *Microbiology* 153:4166–4173. <http://dx.doi.org/10.1099/mic.0.2007/012419-0>.
- Boutte CC, Crosson S. 2013. Bacterial lifestyle shapes stringent response activation. *Trends Microbiol* 21:174–180. <http://dx.doi.org/10.1016/j.tim.2013.01.002>.
- Wolz C, Geiger T, Goerke C. 2010. The synthesis and function of the alarmone (p)ppGpp in firmicutes. *Int J Med Microbiol* 300:142–147. <http://dx.doi.org/10.1016/j.ijmm.2009.08.017>.
- Potrykus K, Cashel M. 2008. (p)ppGpp: still magical? *Annu Rev Microbiol* 62:35–51. <http://dx.doi.org/10.1146/annurev.micro.62.081307.162903>.
- Cashel M, Gentry DR, Hernandez VJ, Vinella D. 1996. The stringent response, p 1458–1496. In Neidhardt FC, Curtiss R, III, Ingraham JL, Lin ECC, Low KB, Magasanik B, Reznikoff WS, Riley M, Schaechter M, Umberger HE (ed), *Escherichia coli* and *Salmonella*: cellular and molecular biology. ASM Press, Washington, DC.
- Dalebroux ZD, Swanson MS. 2012. ppGpp: magic beyond RNA polymerase. *Nat Rev Microbiol* 10:203–212. <http://dx.doi.org/10.1038/nrmicro2720>.
- Primm TP, Andersen SJ, Mizrahi V, Avarbock D, Rubin H, Barry CE, 3rd. 2000. The stringent response of Mycobacterium tuberculosis is required for long-term survival. *J Bacteriol* 182:4889–4898. <http://dx.doi.org/10.1128/JB.182.17.4889-4898.2000>.
- Dahl JL, Arora K, Boshoff HI, Whiteford DC, Pacheco SA, Walsh OJ, Lau-Bonilla D, Davis WB, Garza AG. 2005. The relA homolog of Mycobacterium smegmatis affects cell appearance, viability, and gene expression. *J Bacteriol* 187:2439–2447. <http://dx.doi.org/10.1128/JB.187.7.2439-2447.2005>.
- Klinkenberg LG, Lee JH, Bishai WR, Karakousis PC. 2010. The stringent response is required for full virulence of Mycobacterium tuberculosis in guinea pigs. *J Infect Dis* 202:1397–1404. <http://dx.doi.org/10.1086/656524>.
- Karakousis PC, Yoshimatsu T, Lamichane G, Woolwine SC, Nuermberger EL, Grosset J, Bishai WR. 2004. Dormancy phenotype displayed by extracellular Mycobacterium tuberculosis within artificial granulomas in mice. *J Exp Med* 200:647–657. <http://dx.doi.org/10.1084/jem.20040646>.

37. Weiss LA, Stallings CL. 2013. Essential roles for *Mycobacterium tuberculosis* Rel beyond the production of (p)ppGpp. *J Bacteriol* 195:5629–5638. <http://dx.doi.org/10.1128/JB.00759-13>.
38. van Kessel JC, Hatfull GF. 2007. Recombineering in *Mycobacterium tuberculosis*. *Nat Methods* 4:147–152. <http://dx.doi.org/10.1038/nmeth996>.
39. Pfaffl MW. 2001. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* 29:e45. <http://dx.doi.org/10.1093/nar/29.9.e45>.
40. Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* 25:3389–3402. <http://dx.doi.org/10.1093/nar/25.17.3389>.
41. Marchler-Bauer A, Lu S, Anderson JB, Chitsaz F, Derbyshire MK, DeWeese-Scott C, Fong JH, Geer LY, Geer RC, Gonzales NR, Gwadz M, Hurwitz DI, Jackson JD, Ke Z, Lanczycki CJ, Lu F, Marchler GH, Mullokandov M, Omelchenko MV, Robertson CL, Song JS, Thanki N, Yamashita RA, Zhang D, Zhang N, Zheng C, Bryant SH. 2011. CDD: a Conserved Domain Database for the functional annotation of proteins. *Nucleic Acids Res* 39:D225–229. <http://dx.doi.org/10.1093/nar/gkq1189>.
42. Edgar RC. 2004. MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res* 32:1792–1797. <http://dx.doi.org/10.1093/nar/gkh340>.
43. Capella-Gutierrez S, Silla-Martinez JM, Gabaldon T. 2009. trimAl: a tool for automated alignment trimming in large-scale phylogenetic analyses. *Bioinformatics* 25:1972–1973. <http://dx.doi.org/10.1093/bioinformatics/btp348>.
44. Guindon S, Dufayard JF, Lefort V, Anisimova M, Hordijk W, Gascuel O. 2010. New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. *Syst Biol* 59:307–321. <http://dx.doi.org/10.1093/sysbio/syq010>.
45. Darriba D, Taboada GL, Doallo R, Posada D. 2011. ProtTest 3: fast selection of best-fit models of protein evolution. *Bioinformatics* 27:1164–1165. <http://dx.doi.org/10.1093/bioinformatics/btr088>.
46. Sen A, Daubin V, Abrouk D, Gifford I, Berry AM, Normand P. 2014. Phylogeny of the class Actinobacteria revisited in the light of complete genomes; the orders 'Frankiales' and Micrococcales should be split into coherent entities: proposal of Frankiales ord nov, Geodermatophilales ord nov, Acidothermales ord nov and Nakamurellales ord nov *Int J Syst Evol Microbiol* 64:3821–3832.
47. Letunic I, Bork P. 2011. Interactive Tree Of Life v2: online annotation and display of phylogenetic trees made easy. *Nucleic Acids Res* 39:W475–478. <http://dx.doi.org/10.1093/nar/gkr201>.
48. Cortes T, Schubert OT, Rose G, Arnvig KB, Comas I, Aebersold R, Young DB. 2013. Genome-wide mapping of transcriptional start sites defines an extensive leaderless transcriptome in *Mycobacterium tuberculosis*. *Cell Rep* 5:1121–1131. <http://dx.doi.org/10.1016/j.celrep.2013.10.031>.
49. Newton-Foot M, Gey van Pittius NC. 2013. The complex architecture of mycobacterial promoters. *Tuberculosis (Edinb)* 93:60–74. <http://dx.doi.org/10.1016/j.tube.2012.08.003>.
50. Marmiesse M, Brodin P, Buchrieser C, Gutierrez C, Simoes N, Vincent V, Glaser P, Cole ST, Brosch R. 2004. Macro-array and bioinformatic analyses reveal mycobacterial 'core' genes, variation in the ESAT-6 gene family and new phylogenetic markers for the *Mycobacterium tuberculosis* complex. *Microbiology* 150:483–496. <http://dx.doi.org/10.1099/mic.0.26662-0>.
51. Aravind L, Anantharaman V, Balaji S, Babu MM, Iyer LM. 2005. The many faces of the helix-turn-helix domain: transcription regulation and beyond. *FEMS Microbiol Rev* 29:231–262.
52. Kepes F. 2004. Periodic transcriptional organization of the *E. coli* genome. *J Mol Biol* 340:957–964. <http://dx.doi.org/10.1016/j.jmb.2004.05.039>.
53. Zhang YJ, Ioerger TR, Huttenhower C, Long JE, Sassetti CM, Sacchettini JC, Rubin EJ. 2012. Global assessment of genomic regions required for growth in *Mycobacterium tuberculosis*. *PLoS Pathog* 8:e1002946. <http://dx.doi.org/10.1371/journal.ppat.1002946>.
54. Haurlyuk V, Atkinson GC, Murakami KS, Tenson T, Gerdes K. 2015. Recent functional insights into the role of (p)ppGpp in bacterial physiology. *Nat Rev Microbiol* 13:298–309. <http://dx.doi.org/10.1038/nrmicro3448>.
55. Choi KH, Kremer L, Besra GS, Rock CO. 2000. Identification and substrate specificity of beta-ketoacyl (acyl carrier protein) synthase III (mtFabH) from *Mycobacterium tuberculosis*. *J Biol Chem* 275:28201–28207.
56. Jackson M, Raynaud C, Laneelle MA, Guilhot C, Laurent-Winter C, Ensergueix D, Gicquel B, Daffe M. 1999. Inactivation of the antigen 85C gene profoundly affects the mycolate content and alters the permeability of the *Mycobacterium tuberculosis* cell envelope. *Mol Microbiol* 31:1573–1587. <http://dx.doi.org/10.1046/j.1365-2958.1999.01310.x>.
57. Belisle JT, Vissa VD, Sievert T, Takayama K, Brennan PJ, Besra GS. 1997. Role of the major antigen of *Mycobacterium tuberculosis* in cell wall biogenesis. *Science* 276:1420–1422. <http://dx.doi.org/10.1126/science.276.5317.1420>.
58. Sureka K, Dey S, Datta P, Singh AK, Dasgupta A, Rodrigue S, Basu J, Kundu M. 2007. Polyphosphate kinase is involved in stress-induced mprAB-sigE-rel signalling in mycobacteria. *Mol Microbiol* 65:261–276. <http://dx.doi.org/10.1111/j.1365-2958.2007.05814.x>.
59. Sureka K, Ghosh B, Dasgupta A, Basu J, Kundu M, Bose I. 2008. Positive feedback and noise activate the stringent response regulator rel in mycobacteria. *PLoS One* 3:e1771. <http://dx.doi.org/10.1371/journal.pone.0001771>.
60. Ghosh S, Sureka K, Ghosh B, Bose I, Basu J, Kundu M. 2011. Phenotypic heterogeneity in mycobacterial stringent response. *BMC Syst Biol* 5:18. <http://dx.doi.org/10.1186/1752-0509-5-18>.
61. Hingley-Wilson SM, Sambandamurthy VK, Jacobs WR, Jr. 2003. Survival perspectives from the world's most successful pathogen, *Mycobacterium tuberculosis*. *Nat Immunol* 4:949–955. <http://dx.doi.org/10.1038/ni981>.
62. Gutierrez MC, Supply P, Brosch R. 2009. Pathogenomics of mycobacteria. *Genome Dyn* 6:198–210. <http://dx.doi.org/10.1159/000235772>.
63. Lacave C, Laneelle MA, Daffe M, Montrozier H, Laneelle G. 1989. Mycolic acid metabolic filiation and location in *Mycobacterium aurum* and *Mycobacterium phlei*. *Eur J Biochem* 181:459–466. <http://dx.doi.org/10.1111/j.1432-1033.1989.tb14747.x>.
64. Russell DG, VanderVen BC, Lee W, Abramovitch RB, Kim MJ, Homolka S, Niemann S, Rohde KH. 2010. *Mycobacterium tuberculosis* wears what it eats. *Cell Host Microbe* 8:68–76. <http://dx.doi.org/10.1016/j.chom.2010.06.002>.
65. Shi L, Sohaskey CD, Pfeiffer C, Datta P, Parks M, McFadden J, North RJ, Gennaro ML. 2010. Carbon flux rerouting during *Mycobacterium tuberculosis* growth arrest. *Mol Microbiol* 78:1199–1215. <http://dx.doi.org/10.1111/j.1365-2958.2010.07399.x>.
66. Barkan D, Rao V, Sukenick GD, Glickman MS. 2010. Redundant function of cmaA2 and mmaA2 in *Mycobacterium tuberculosis* cis cyclopropanation of oxygenated mycolates. *J Bacteriol* 192:3661–3668. <http://dx.doi.org/10.1128/JB.00312-10>.
67. Rao V, Fujiwara N, Porcelli SA, Glickman MS. 2005. *Mycobacterium tuberculosis* controls host innate immune activation through cyclopropane modification of a glycolipid effector molecule. *J Exp Med* 201:535–543. <http://dx.doi.org/10.1084/jem.20041668>.
68. Corrales RM, Molle V, Leiba J, Mourey L, de Chastellier C, Kremer L. 2012. Phosphorylation of mycobacterial PcaA inhibits mycolic acid cyclopropanation: consequences for intracellular survival and for phagosome maturation block. *J Biol Chem* 287:26187–26199. <http://dx.doi.org/10.1074/jbc.M112.373209>.
69. Dubnau E, Chan J, Raynaud C, Mohan VP, Laneelle MA, Yu K, Quemard A, Smith I, Daffe M. 2000. Oxygenated mycolic acids are necessary for virulence of *Mycobacterium tuberculosis* in mice. *Mol Microbiol* 36:630–637.
70. Cantaloube S, Veyron-Churlet R, Haddache N, Daffe M, Zerbib D. 2011. The *Mycobacterium tuberculosis* FAS-II dehydratases and methyltransferases define the specificity of the mycolic acid elongation complexes. *PLoS One* 6:e29564. <http://dx.doi.org/10.1371/journal.pone.0029564>.
71. Veyron-Churlet R, Guerrini O, Mourey L, Daffe M, Zerbib D. 2004. Protein-protein interactions within the fatty acid synthase-II system of *Mycobacterium tuberculosis* are essential for mycobacterial viability. *Mol Microbiol* 54:1161–1172. <http://dx.doi.org/10.1111/j.1365-2958.2004.04334.x>.
72. Rienksma RA, Suarez-Diez M, Mollenkopf HJ, Dolganov GM, Dorhoi A, Schoolnik GK, Martins Dos Santos V, Kaufmann S, Schaap PJ, Gengenbacher M. 2015. Comprehensive insights into transcriptional adaptation of intracellular mycobacteria by microbe-enriched dual RNA sequencing. *BMC Genomics* 16:34. <http://dx.doi.org/10.1186/s12864-014-1197-2>.
73. Manganello R. 2007. Polyphosphate and stress response in mycobacteria.

- Mol Microbiol 65:258–260. <http://dx.doi.org/10.1111/j.1365-2958.2007.05819.x>.
74. Manganelli R, Voskuil MI, Schoolnik GK, Smith I. 2001. The *Mycobacterium tuberculosis* ECF sigma factor sigmaE: role in global gene expression and survival in macrophages. Mol Microbiol 41:423–437. <http://dx.doi.org/10.1046/j.1365-2958.2001.02525.x>.
75. Dandekar T, Snel B, Huynen M, Bork P. 1998. Conservation of gene order: a fingerprint of proteins that physically interact. Trends Biochem Sci 23:324–328. [http://dx.doi.org/10.1016/S0968-0004\(98\)01274-2](http://dx.doi.org/10.1016/S0968-0004(98)01274-2).
76. Mao F, Dam P, Chou J, Olman V, Xu Y. 2009. DOOR: a database for prokaryotic operons. Nucleic Acids Res 37:D459–D463. <http://dx.doi.org/10.1093/nar/gkn757>.
77. World Health Organization. 2014. Global tuberculosis report 2014. World Health Organization, Geneva, Switzerland.