

Revisiting the Assignment of Rv0241c to Fatty Acid Synthase Type II of *Mycobacterium tuberculosis*[∇]

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The fatty acid synthase type II enzymatic complex of *Mycobacterium tuberculosis* (FAS-II_{Mt}) catalyzes an essential metabolic pathway involved in the biosynthesis of major envelope lipids, mycolic acids. The partner proteins of this singular FAS-II system represent relevant targets for antituberculous drug design. Two heterodimers of the hydratase 2 protein family, HadAB and HadBC, were shown to be involved in the (3R)-hydroxyacyl-ACP dehydration (HAD) step of FAS-II_{Mt} cycles. Recently, an additional member of this family, Rv0241c, was proposed to have the same function, based on the heterologous complementation of a HAD mutant of the yeast mitochondrial FAS-II system. In the present work, Rv0241c was able to complement a HAD mutant in the *Escherichia coli* model but not a dehydratase-isomerase deficient mutant. However, an enzymatic study of the purified protein demonstrated that Rv0241c possesses a broad chain length specificity for the substrate, unlike FAS-II_{Mt} enzymes. Most importantly, Rv0241c exhibited a strict dependence on the coenzyme A (CoA) as opposed to AcpM, the natural acyl carrier protein bearing the chains elongated by FAS-II_{Mt}. The deletion of Rv0241c showed that this gene is not essential to *M. tuberculosis* survival *in vitro*. The resulting mutant did not display any change in the mycolic acid profile. This demonstrates that Rv0241c is a *trans*-2-enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydratase that does not belong to FAS-II_{Mt}. The relevance of a heterologous complementation strategy to identifying proteins of such a system is questioned.

In 2008, tuberculosis killed 1.8 million people, 30% of whom were human immunodeficiency virus positive. A total of 5% of all cases have multidrug-resistant tuberculosis that fails to respond to first-line drug therapy. Fighting this disease that affects mostly developing countries is one of the priorities of the World Health Organization (37). The development of new drugs effective against the resistant strains is particularly needed.

The tubercle bacillus, *Mycobacterium tuberculosis*, is protected by a highly impermeable envelope characterized by an outer membrane (14, 38) that holds unusual lipid components, mycolic acids. These very-long-chain α -alkylated β -hydroxylated fatty acids are the hallmark of the *Corynebacterineae* suborder. Each genus is typified by mycolic acids of different chain length. The simplest prototypes (C₃₂ to C₃₆), called “corynomycolic acids,” that result from an enzymatic condensation between two regular size fatty acids (C₁₆ to C₁₈) are found in *Corynebacterium*. The longest mycolates (C₆₀ to C₉₀) are biosynthesized by mycobacteria; they are the products of condensation between a very long meromycolic chain (C₄₀ to C₆₀) that has chemical functions at two main positions and a shorter α -chain (C₂₀ to C₂₄) (20). These molecules are essen-

tial to mycobacterial survival. Furthermore, they play a role in the virulence and persistence of *M. tuberculosis* within infected organisms (2, 8). The mycolic acid biosynthesis pathway represents a valuable source for potential new pharmacological targets (20).

Elongation cycles that include four main steps monitored by an acyl carrier protein (ACP)-dependent fatty acid synthase type II (FAS-II) system (3) lead to the formation of the meromycolic chain. FAS-II systems are found in plants, bacteria, parasites, and mitochondria (26). The dehydration step of FAS-II systems can be catalyzed by either a (3R)-hydroxyacyl-ACP dehydratase or a (3R)-hydroxyacyl-ACP dehydratase/isomerase that leads to the formation of unsaturated fatty acids due to its additional *trans* (E) Δ 2-*cis* (Z) Δ 3 isomerase activity (26). In *E. coli*, the corresponding enzymes are FabZ- and FabA-type enzymes, universally found in the known bacterial FAS-II systems (26). In contrast, the (3R)-hydroxyacyl-ACP dehydratase of yeast mitochondria is related to a different subfamily, the hydratases 2 (17). In both *E. coli* and yeast, the FAS-II system performs *de novo* fatty acid biosynthesis. The FAS-II system from *Corynebacterineae* is unique since it elongates long-chain fatty acids (C₁₂ to C₁₈) into very-long-chain fatty acids (3). In *M. tuberculosis*, we previously characterized two (3R)-hydroxyacyl-ACP dehydratases, HadAB and HadBC, involved in the third step of the cycle (28). These bacterial enzymes have the unique property of belonging to the hydratase 2 protein family. The latter also includes Rv0241c protein (5, 29), whose function is unknown and which has been annotated as a “conserved hypothetical protein” (6). Recently,

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however, it has been shown that *Rv0241c* gene was able to complement $\Delta htd2$ ($\Delta yhr067w$) yeast mutant lacking a functional mitochondrial FAS-II dehydratase (9). Gurvitz et al. concluded that *Rv0241c* encodes a bona fide FAS-II-like 3-hydroxyacyl-thioester dehydratase (9).

In order to determine whether *Rv0241c* behaved as a 3-hydroxyacyl-thioester dehydratase or as a 3-hydroxyacyl-thioester dehydratase-isomerase, we achieved complementation experiments in another model, *E. coli*, which holds both types of enzyme (36). Only the function of dehydratase and not that of dehydratase-isomerase could be restored by *Rv0241c* in *E. coli* mutants. To investigate the effective involvement of the *Rv0241c* protein in the *M. tuberculosis* FAS-II (FAS-II_{Mt}) system, we performed a detailed analysis of its substrate specificity with respect to the chain length and the nature of the acyl chain carrier using purified enzyme. Furthermore, the essentiality of the gene was studied by knocking it out in *M. tuberculosis*. The implication of *Rv0241c* in mycolic acid biosynthesis was examined through a phenotypic analysis of the deletion mutant.

MATERIALS AND METHODS

Sequence analyses. Analyses of genome sequences were done via the TubercuList (6), National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/genomes/lproks.cgi), Wellcome Trust Sanger Institute (www.sanger.ac.uk/), and Comprehensive Microbial Resource (cmr.tigr.org/tigr-scripts/CMR/CmrHomePage.cgi) (24) web servers. Sequence alignments were performed by using BLAST algorithms (1) with default parameters.

Heterologous complementation assays. The *Rv0241c* gene was amplified by PCR from the BAC-Rv329 DNA (Institut Pasteur, Paris, France [4]) with the oligonucleotides 5'-TAAGCCGTCTAGATGACTCAACCCAGCGGCTGAA GA-3' and 5'-GATAACTCTCGAGCGTGGCCCGCTATAGACC-3', using the *Pfu* polymerase (Promega, Madison, WI). The PCR product was then cloned into the pBluescript II SK(+) (pBSK+) expression vector (Stratagene/Agilent Technologies, La Jolla, CA) between the *Xba*I and *Xho*I restriction sites downstream of a lactose-inducible promoter. Competent bacteria of *E. coli* CY50 (*fabA*-thermosensitive [Ts]) strain (*E. coli* Genetic Stock Center, New Haven, CT) were transformed by heat shock in the presence of either pBSK+ vector, the pBSK::*Rv0241c* plasmid, or the pDM5 plasmid (pBR322::wild-type *E. coli fabA* [*fabA*_{Ec}], kindly provided by D. de Mendoza) and screened on LB agar supplemented with carbenicillin at 30°C. At least 10 single colonies of each recombinant strain were used in the complementation assays. Each colony was cultivated in four different conditions: on rich broth (RB) agar supplemented with 50 µg of carbenicillin/ml either in the presence or in the absence of sodium oleate (0.1% [vol/vol]) and at either 30°C (permissive temperature) or 42°C (non permissive temperature). Assays were performed in the presence or in the absence of lactose (0.5% [vol/vol]), a positive regulator of the *lac* promoter in pBSK+; similar data were obtained in both conditions. Competent bacteria of *E. coli* HW7 (DY330 *fabZ*::*kan*/pBAD24::*fabZ* *Clostridium acetobutylicum*) strain (kindly provided by H. Wang [35]) were transformed by heat shock in the presence of either pBSK+ vector, pBSK::*Rv0241c*, or pBSK::*fabZ*_{Ec} and cultivated for 90 min in LB supplemented with 0.5% (vol/vol) L-arabinose at 30°C. After extensive washing with LB, a suspension of each recombinant strain was used to inoculate RB agar medium supplemented with 50 µg of carbenicillin/ml, 15 µg of kanamycin/ml, and either 0.5% (vol/vol) L-arabinose or 0.1% (vol/vol) D-fucose. The cultures were incubated at 30°C.

Protein production and purification. The *Rv0241c* gene was amplified by PCR from the total DNA of *M. tuberculosis* H37Rv by using the primer pair 5'-GCA ACGTCATTCTGCTCTG-3' (forward) and 5'-GATAACGTCGGATGGCTG AC-3' (reverse) and the high-fidelity polymerase *Pfu*Ultra (Stratagene/Agilent Technologies). In a second PCR, an N-terminal His₆ tag was introduced by using an alternative forward primer, 5'-ATG GCT CAT CAT CAT CAT CAT GGT ACT CAA CCC AGC GGC CTG AAG AAC-3'. After the addition of a 3'A overhang by incubation with *Taq* polymerase (New England Biolabs, Ipswich, MA), the DNA fragment was ligated to the pCR T7 TOPO vector (Invitrogen, Carlsbad, CA). Cloning was performed in *E. coli* TOP10 (Invitrogen), and the correctness of the isolated gene was verified by DNA sequencing. Expression was assessed in *E. coli* BL21(DE3) (Invitrogen). The cells were

cultured in LB medium supplemented with 50 µg of ampicillin/ml at 37°C. At an optical density at 600 nm of 0.4 to 0.7, the temperature was lowered to 24°C, and expression of the target gene was induced with 0.2 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 3 to 4 h. After harvesting by centrifugation, the cell pellet was resuspended in buffer A (50 mM NaH₂PO₄, 300 mM NaCl, 10% [vol/vol] glycerol; pH 8.0) supplemented with 10 mM imidazole, 0.5% (vol/vol) Triton X-100, 1 mg of lysozyme/ml, 10 µg of RNase A/ml, 20 µg of DNase I/ml, and 1 mM phenylmethylsulfonyl fluoride and lysed using a OneShot cell disruptor (Constant Systems, Ltd., Northants, United Kingdom). The soluble fraction was loaded onto a 1-ml preequilibrated Ni Sepharose FF column (GE Healthcare, Uppsala, Sweden). After being washed with 20 column volumes of buffer A plus 20 mM imidazole, the protein was eluted with 5 column volumes of 250 mM imidazole in buffer A. Fractions containing the protein were identified by SDS-PAGE, pooled, and further purified on a HiLoad 16/60 Superdex 75 prep-grade column (GE Healthcare) equilibrated with buffer A. The protein fractions identified by SDS-PAGE analysis were pooled and stored at -20°C after the addition of 50% (vol/vol) glycerol.

Synthesis of fatty acid derivatives. The *trans*-2-enoyl-coenzymes A (CoAs) were synthesized as described previously (25, 29). *trans*-2-octenoyl-ACP_{Ec} and *trans*-2-acyl-AcpM were prepared enzymatically from *trans*-2-octenoic acid (Acros Organics, Geel, Belgium) and either *E. coli* holo-ACP (ACP_{Ec}, Sigma-Aldrich, St. Louis, MO) or *M. tuberculosis* AcpM using the *E. coli* acyl-ACP synthase as previously reported (27). The purification of *trans*-2-octenoyl-ACP_{Ec} was done as described previously (16) except that a Poros 10 HQ column (Applied Biosystems, Inc., Foster City, CA) was used instead of the DEAE-cellulose column. The purification of *trans*-2-acyl-AcpM was performed according to the same procedure used for the purification of holo-AcpM (see below) except that a first step of elution through a Hi-Trap Blue HP column (1 ml; GE Healthcare) with Tris 50 mM (pH 8) plus 0.5% Triton X-100 (wt/vol) was performed to eliminate the acyl-ACP synthase. Recombinant C-terminal His-tagged acyl-ACP synthase was produced and purified from *E. coli* C41(DE3) strain (kindly provided by J. E. Walker, [22]) transformed by the plasmid pAasH (kindly provided by J. Shanklin) as previously described (33), except that a Hi-Trap HP column (GE Healthcare) was used. Holo-AcpM was produced and purified from an *E. coli* C41(DE3) transformed by a pET-15b::*acpM* plasmid (kindly provided by Y.-M. Zhang) as described previously (19). The concentration of octenoyl-ACP was determined by measuring the ΔA at 340 nm in the presence of purified InhA protein and a saturating concentration of NADH as previously reported (25) or by both BCA protein assay and urea-PAGE. The gels were made of a stacking gel (4% [wt/vol] polyacrylamide, 125 mM Tris-HCl [pH 6.8], 0.5 mM EDTA, and 2.5 M urea) and a resolving gel (12% [wt/vol] polyacrylamide, 375 mM Tris-HCl [pH 9.0], 0.5 mM EDTA, and 2.5 M urea). The electrophoresis running buffer (pH 8.3) contained 25 mM Tris and 192 mM glycine. Samples were applied in a loading buffer containing 62.5 mM Tris-HCl (pH 6.8), 20% (vol/vol) glycerol, and 0.05% (wt/vol) bromophenol blue. After electrophoresis (at 30 mA/gel), the proteins were stained with Coomassie blue.

Enzyme assays and steady-state kinetics. Reactions were performed in a quartz cuvette in a total volume of 700 µl, at 25°C, in 100 mM sodium phosphate buffer (pH 7.0) in the presence of substrate; after equilibration of the baseline, reactions were started by adding a defined amount of enzyme, and measurements were performed for 1.5 to 3 min. Hydratase or dehydratase activities were monitored by spectrophotometry at 263 nm in the presence of *trans*-2-enoyl-CoA, *trans*-2-enoyl-ACP, *trans*-2-enoyl-AcpM, or 3-hydroxyacyl-CoA substrates (ΔA of 0.67 for a variation of concentration of 100 µM) using a thermostated Uvikon 923 spectrophotometer (Kontron Instruments, Milan, Italy). Assays in the presence of short-chain substrates (C₄), crotonoyl-CoA (*trans*-2-butenoyl-CoA), and 3-hydroxybutyryl-CoA (Sigma-Aldrich) were performed using 25 µM substrate and 8 or 80 nM enzyme, respectively. Measurements of the specific activities obtained for the different-chain-length *trans*-2-enoyl-CoAs (C₄ to C₂₀) or the different acyl chain carrier (CoA, AcpM, and ACP) were performed at fixed concentrations of substrate (2.5 µM) and enzyme (8 nM). Control experiments lacking the enzyme were also performed.

Construction of an *M. tuberculosis* deletion mutant of *Rv0241c*. *M. tuberculosis* was grown in Middlebrook 7H9 liquid medium containing 10% (vol/vol) oleic acid-albumin-dextrose-catalase (OADC) supplement (Becton Dickinson, Franklin Lakes, NJ) and 0.05% (wt/vol) Tween 80 or on Middlebrook 7H10 agar containing 10% (vol/vol) OADC. Concentrations of 100 µg of hygromycin/ml, 20 µg of kanamycin/ml, 10 µg of gentamicin/ml, 50 µg of X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside)/ml, and 2% (wt/vol) sucrose were used where appropriate. The *Rv0241c* gene deletion delivery vector was constructed by amplifying the regions upstream and downstream of the gene by using the primer pairs EUF11 (5'-AAGCTTGGGCTGCACGAGTTCTTTAC-3') and EUR11 (5'-GGATCCATGGAAAAGTCAACGCGAAC-3'); and EUF12 (5'-GGATC

TABLE 1. Orthologs of Rv0241c protein in *Corynebacterium*

Strain ^a	Protein ^b	Identity (%)	Similarity (%)	Length of alignment (aa) ^c	E-value ^d
<i>C. aurimucosum</i> ATCC 700975	Cauri_2166	42	58	236	8e-47
<i>C. genitalicum</i> ATCC 33030	ZP_05706810.1	43	59	283	5e-56
<i>C. jeikeium</i> ATCC 43734	ZP_05845933.1	43	56	259	2e-50
<i>C. jeikeium</i> K411	jk0821	43	56	244	4e-47
<i>C. kroppenstedtii</i> DSM 44385	Ckrop_0818	41	56	272	3e-51
<i>C. lipophiloflavum</i> DSM 44291	ZP_03978914.1	40	54	282	2e-46
<i>C. striatum</i> ATCC 6940	ZP_03936418.1	39	53	271	8e-50
<i>C. urealyticum</i> DSM 7109	Cur_1137	42	59	278	2e-57

^a Only strains holding an ortholog of the Rv0241c protein are displayed. Seven other *Corynebacterium* genomes were considered: *C. amycolatum* SK46 (incomplete genome), *C. diphtheriae* NCTC 13129, *C. efficiens* YS-314, *C. glutamicum* ATCC 13032 Bielefeld, *C. glutamicum* ATCC 13032 Kitasato, *C. matruchotii* ATCC 14266 (incomplete genome), and *C. tuberculostearicum* SK141 (incomplete genome).

^b The protein name or NCBI reference sequence number.

^c The number of amino acid (aa) residues aligned by the BLAST algorithm (1).

^d "Expect value" calculated by the BLAST algorithm.

CAATATCGAAGCCCGTTTCC-3') and EUR12 (5'-GGTACCGGTATCG GCCACTGAGATGT-3') and cloned into p2NIL (23) digested with HindIII-KpnI using the underlined restriction sites (HindIII-BamHI and BamHI-KpnI, respectively). The PacI cassette containing *lacZ*, *sacB*, and *hyg* from pGOAL19 (23) was introduced to construct the final vector pTACK0241. The plasmid was electroporated into *M. tuberculosis* and single crossovers were isolated. Double crossovers were isolated from the single crossover strain as previously described (23). Colonies were screened for the presence of the wild-type (wt) or deletion alleles by PCR using primers 0241D1 (5'-CATTGCGCATTAGATTGAA-3') and 0241D2 (5'-AATTGCGCACTCGAAACATC-3'). The deletion mutant was confirmed by Southern blotting.

Analysis of the lipid content of *M. tuberculosis* ΔRv0241c. Lipids were extracted, separated, and analyzed using previously reported protocols (8). The same procedure was applied to the parent strain *M. tuberculosis* H37Rv as a control. Briefly, extractable lipids were recovered by a series of three extractions with CHCl₃/CH₃OH (at 1:2, 1:1, and 2:1 ratios) and analyzed by thin-layer chromatography (TLC; silica gel G-60) using two types of eluent (CHCl₃/CH₃OH/H₂O, 60/16/2; CHCl₃/CH₃OH, 9/1). Delipidated bacilli and lipid extracts were saponified. Mycolic acids were extracted with diethyl ether, methylated with diazomethane, analyzed by analytical TLC, and purified by preparative TLC (silica gel G-60 plates; eluent, petroleum ether-diethyl ether [9/1]; five passages). Each type of mycolic acid was analyzed by matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectrometry (MS) (see below).

MALDI-TOF MS. MS analyses were performed in reflectron mode, on a 4700 Proteomics Analyzer (Voyager DE-STR; Applied Biosystems, Framingham, MA) equipped with an Nd:YAG laser (355-nm wavelength, 500-ps pulses, and 200-Hz frequency). A total of 2,500 shots were accumulated in positive ion mode, and MS data were acquired by using the instrument default calibration. Lipid samples were dissolved in chloroform and directly spotted onto the target plate, mixed with 0.5 μl of matrix solution (10 mg of 2,5-dihydroxybenzoic acid [Sigma-Aldrich]/ml in CHCl₃/CH₃OH, 1/1 [vol/vol]), and allowed to crystallize at room temperature.

RESULTS

**Conservation of Rv0241c protein among the *Corynebacteri-
neae*.** Rv0241c protein has a 280-amino-acid sequence and a theoretical mass of 30,163 Da. It is a putative (*R*)-specific enoyl hydratase/3-hydroxyacyl dehydratase belonging to the hydratase 2 protein family and it holds a well-conserved hydratase 2 motif, including the catalytic residues "D-x(4)-H" (5, 29). It is predicted to have a "double hot dog fold" structure (5, 29), where each of the two structural domains corresponds to a hot dog fold (mainly a long α-helix wrapped into a five-stranded antiparallel β-sheet [13]).

Sequence alignments using Rv0241c protein as a probe showed that the latter is conserved among all of the genomes (41 genomes) of mycobacteria sequenced thus far, a finding in

agreement with earlier observations on a smaller number of mycobacterial genomes (12 genomes) (29). An orthologous protein was also found in other *Corynebacteriineae*, namely, *Rhodococcus*, *Nocardia*, *Gordonia*, *Tsukamurella*, and *Brevibacterium* (data not shown). These data emphasize the importance of Rv0241c protein and its orthologs in the physiology of mycobacteria and related genera. All of these organisms contain a FAS-II system because of their production of mycolic acids holding a meromycolic chain longer than a regular size fatty acid (>C₁₈). To reinforce the putative correlation between the occurrence of Rv0241c and the production of mycolates, the presence of an Rv0241c ortholog was sought in the *Corynebacterium* genus that has the particularity to be devoid of FAS-II system. Indeed, no ortholog of FAS-II_{Mt} enzymes have been detected in *Corynebacterium* so far (28), a finding consistent with the fact that the meromycolic chain of its mycolic acids corresponds to a regular size fatty acid (mainly C₁₆ to C₁₈). Strikingly, we observed that about half of the 15 genomes examined coded for an ortholog of Rv0241c (Table 1). Thus, these data questioned the potential involvement of Rv0241c in mycolic acid biosynthesis and especially in FAS-II_{Mt} cycles.

Heterologous complementation experiments of *E. coli* *fabZ* and *fabA* mutants using the Rv0241c gene. Heterologous complementation assays were realized in parallel using a *fabZ* knockout (KO) mutant, *E. coli* HW7, and a *fabA* Ts mutant, *E. coli* CY50. *E. coli* CY50 that has a Ts point mutation in *fabA* is able to grow at 30°C (permissive temperature) but becomes auxotrophic for unsaturated fatty acids, such as oleate (C_{18:1Δ9} acid), at 42°C (7). *E. coli* CY50 was transformed by Rv0241c carried by an expression plasmid (pBSK+) under the control of a lactose- and IPTG-inducible promoter. At a permissive temperature, the recombinant strain grew both in the absence and in the presence of oleate in the culture medium (Fig. 1). However, it was not able to grow at a nonpermissive temperature in the absence of oleate, whereas *E. coli* CY50 transformed by wt *fabA*_{Ec} actively grew in such conditions (Fig. 1). In *E. coli* HW7, the essential *fabZ* gene has been disrupted by a kanamycin resistance cassette. To keep the viability of the strain, the mutation has been complemented by the wt *fabZ* gene of *Clostridium acetobutylicum* (*fabZ*_{Ca}) under the control of an inducible arabinose promoter (35). Thus, this mutant

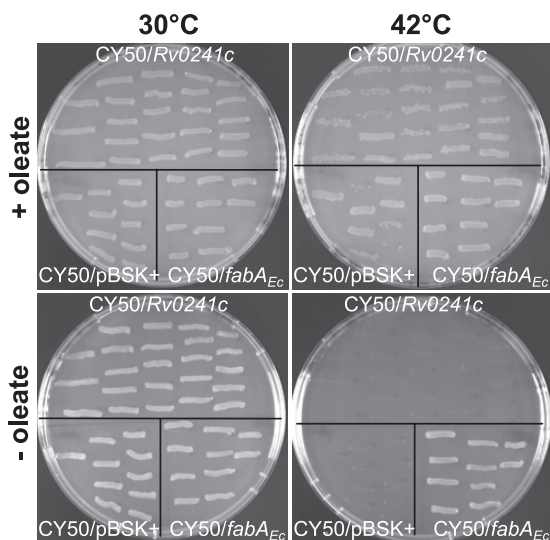


FIG. 1. Heterologous complementation assays of the *E. coli fabA* mutant. *E. coli* CY50 was transformed by either the *Rv0241c* gene, the expression vector (pBSK+) alone, or wt *fabA_{Ec}* and grown on RB agar in the presence or absence of oleate, at either 30 or 42°C, as indicated. The photographs are representative of three independent experiments.

grows in the presence of arabinose but is not viable in the absence of arabinose or when the promoter is repressed by fucose (Fig. 2). After transformation of *E. coli* HW7 by *Rv0241c*, we observed that the recombinant strain grew in the presence of either arabinose or fucose. This means that *Rv0241c* was able to restore the (3*R*)-hydroxyacyl-ACP dehydratase (HAD) function, although less efficiently than the wt *fabZ_{Ec}* gene itself (Fig. 2). For comparison, heterologous complementation assays were also performed using a yeast mutant, *Saccharomyces cerevisiae* $\Delta htd2$, deficient in the mitochondrial FAS-II (3*R*)-hydroxyacyl-ACP dehydratase Htd2 related to the hydratase 2 family (17). This mutant displays a respiratory deficiency phenotype; it can grow in the presence of glucose (both fermentable and respirable carbon source) but not of glycerol (usable only through the respiratory metabolism). Consistent with a previous report (9), we observed that *Rv0241c* also complemented this mutation, since it allowed the recovering of the respiratory ability of the strain (data not shown).

These data strongly suggested that *Rv0241c* protein is able on the one hand to work as an HAD *in vivo* by using the substrates available and processed by the *E. coli* and mitochondrial FAS-II systems, i.e., short- to long-chain ACP derivatives (C_4 - C_{16}) (11, 12), and on the other hand to perform coupled reactions with enzymes of these FAS-II systems.

Purification of *Rv0241c* protein and activity assays. The enzymatic activity of *Rv0241c* was studied in order to evaluate its putative contribution to the FAS-II_{Mt} system. For this purpose, *Rv0241c* gene was cloned into the pCR T7 TOPO expression vector, and the protein was produced in *E. coli* BL21(DE3) in fusion with a N-terminal polyhistidine tag (total molecular mass, 31.1 kDa) by induction with IPTG. Bacteria were lysed using a cell disruptor in the presence of lysozyme. The purification consisted in a two-step chromatography pro-

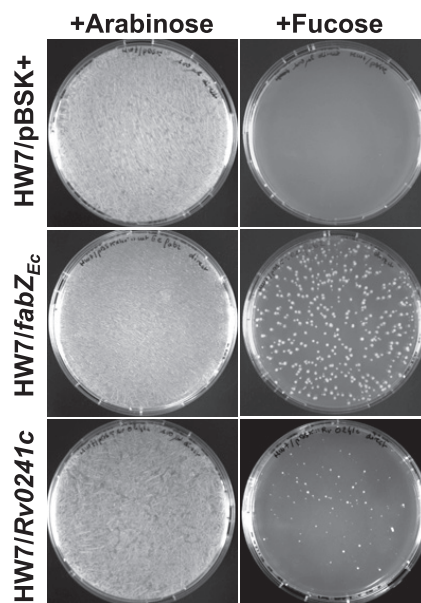


FIG. 2. Heterologous complementation assays of *E. coli fabZ* KO mutant. In *E. coli* HW7, the lethal disruption of *fabZ_{Ec}* has first been complemented by *fabZ_{Ca}* under the control of an arabinose promoter that is induced by arabinose and repressed by fucose (35). The strain was then cotransformed by either the *Rv0241c* gene, the expression vector (pBSK+) alone, or wt *fabZ_{Ec}* and grown on RB agar supplemented with arabinose or fucose, as indicated. The photographs are representative of three independent experiments.

cedure over a Ni Sepharose FF column, using an imidazole gradient, and a Superdex 75 gel filtration column (Fig. 3A).

Enzymes belonging to the (*R*)-specific enoyl hydratase/hydroxyacyl dehydratase family preferentially catalyze the hydration reaction when they are isolated from their enzymatic complex (26). Thus, their activities are most often studied in the presence of enoyl derivatives *in vitro*. The activity of *Rv0241c* was first measured spectrophotometrically (at 263 nm) in the presence of short-chain CoA derivatives, crotonoyl-CoA ($C_{4:1}$) and 3-hydroxybutyryl-CoA (C_4). At a 25 μ M concentration of substrate, the specific activities of the protein were 16.2 ± 1.0 and $0.93 \pm 0.07 \mu\text{mol min}^{-1} \text{mg}^{-1}$, respectively. The purified recombinant *Rv0241c* protein proved to be active and, as expected, its activity was greater in the presence of the enoyl-CoA substrate than in that of the 3-hydroxyacyl-CoA.

Substrate specificity of *Rv0241c*. The specificity of *Rv0241c* for the chain length of the substrate was then studied with a series of longer chain derivatives, C_8 to C_{20} *trans*-2-enoyl-CoAs. To minimize the solubility problems encountered with such amphipathic molecules, the experiments were performed at a fixed low substrate concentration (2.5 μ M). The enzyme displayed a broad chain length specificity since it worked in the presence of short-, medium-, and long-chain molecules, yet with a predilection for the C_8 to C_{12} substrates (Fig. 3B). For comparison, the FAS-II_{Mt} dehydratases HadAB and HadBC are not active in the presence of C_4 to C_8 *trans*-2-enoyl-CoAs, under the same experimental conditions, but use only longer-chain molecules, C_{12} to C_{20} (28).

To the best of our knowledge, FAS-II is the only system of fatty acid biosynthesis that is ACP-dependent in mycobacteria.

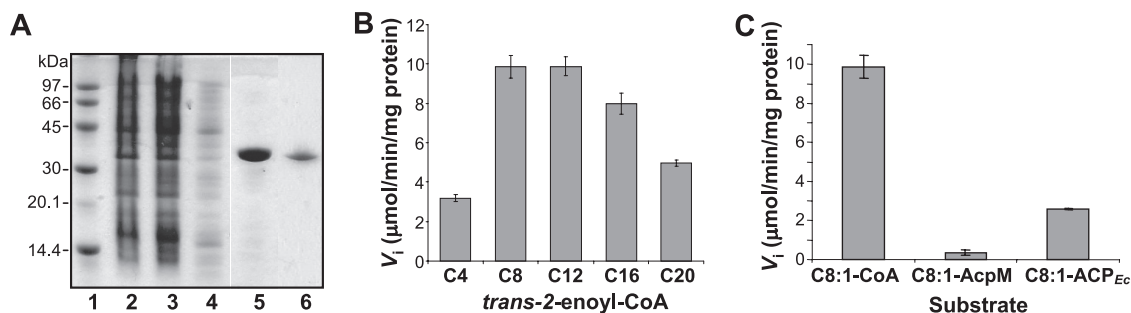


FIG. 3. Purification and enzymatic study of Rv0241c. (A) SDS-PAGE analysis of the purification steps. Lane 1, molecular weight markers; lane 2, cleared cell lysate; lanes 3 to 5, Ni Sepharose FF column (flowthrough, wash, and elution of Rv0241c with 250 mM imidazole); lane 6, Superdex 75 gel filtration column (elution fraction containing Rv0241c). Gels (20% polyacrylamide) stained with Coomassie blue are shown. The dividing lines separate different parts of the gel. (B) Chain length specificity profile. (C) Acyl chain carrier specificity. In panels B and C, the specific activities of Rv0241c were measured at fixed concentrations of substrate (2.5 μ M) and enzyme (8 nM) in 100 mM sodium phosphate buffer (pH 7.0). The data are means \pm the standard deviation.

It has been shown that it uses the AcpM protein as ACP unit (19, 31). Thus, an investigation of the ACP dependence of Rv0241c was decisive in order to assess its involvement in FAS-II_{Mt}. The behaviors of Rv0241c in the presence of AcpM or CoA derivatives were compared by using a fixed concentration of *trans*-2-octenoyl-AcpM, *trans*-2-octenoyl-ACP_{Ec}, or *trans*-2-octenoyl-CoA as substrates. The data clearly showed a strict dependence of Rv0241c on CoA as an acyl chain carrier (Fig. 3C). Indeed, the enzyme was not active in the presence of the AcpM derivative. Interestingly, however, Rv0241c was able to metabolize an enoyl chain carried by the heterologous ACP_{Ec}, although much less efficiently than the CoA derivative (Fig. 3C). For comparison, under the same experimental conditions, HadAB and HadBC work only in the presence of the ACP derivative (28).

Altogether, these experiments demonstrate that Rv0241c is a 2-*trans*-enoyl-CoA hydratase/3-hydroxyacyl-CoA dehydratase, with a relatively broad chain length specificity.

In vitro essentiality study of the Rv0241c gene. To examine the essentiality of Rv0241c enzyme, we used a two-step homologous recombination method to construct an in-frame, unmarked deletion of *Rv0241c* gene in *M. tuberculosis* H37Rv (Fig. 4). The mutant was viable, and its growth rate was similar to that of the wt strain (data not shown), showing that *Rv0241c* is not essential for *M. tuberculosis* survival *in vitro*, as predicted previously (30).

Mycolic acid profile of the mutant. To determine the impact of the mutation on the mycolic acid content of *Rv0241c* deletion mutant, the complex lipids holding mycolic acids, i.e., trehalose dimycolates and trehalose monomycolates, were obtained by successive CHCl₃/CH₃OH extractions from the bacterial pellets and analyzed. The same types of glycolipids and other lipids were detected in *M. tuberculosis* Δ Rv0241c mutant by comparison with the wt parent strain, *M. tuberculosis* H37Rv, as observed by TLC (Fig. 5). After saponification of the delipidated bacterial residues, the released mycolic acids were extracted, methylated, and analyzed by TLC. Very similar molecular profiles were observed for both the wt and the mutant strains (Fig. 5). The three types of mycolate were purified by preparative TLC and studied further by MALDI-TOF MS. Analysis of the mass spectra demonstrated that the α -, me-

thoxy-, and keto-mycolates held the same number of carbons in *M. tuberculosis* Δ Rv0241c as in the wt strain (Fig. 6).

In conclusion, the lack of Rv0241c enzyme does not affect the biosynthesis of all three types of mycolic acid in *M. tuberculosis*.

DISCUSSION

The purified Rv0241c protein displays a *trans*-2-enoyl hydratase/3-hydroxyacyl dehydratase activity with a strict speci-

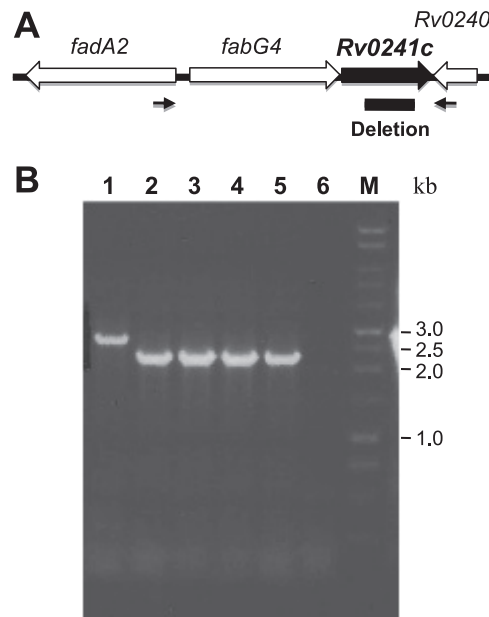


FIG. 4. Construction of the *Rv0241c* deletion mutant. (A) Chromosomal organization of the region. The deletion zone is drawn. Arrows indicate the primers used for PCR screening. (B) PCR confirmation of deletion strains. The primers indicated in panel A were used to amplify the *Rv0241c* region. Wild-type strains gave a product of 2.7 kb, the deletion strains gave a product of 2.2 kb. Lane 1, wt strain; lanes 2 to 5, deletion strains; lane 6, negative control; lane M, 1-kb markers. Deletions were verified by Southern hybridization.

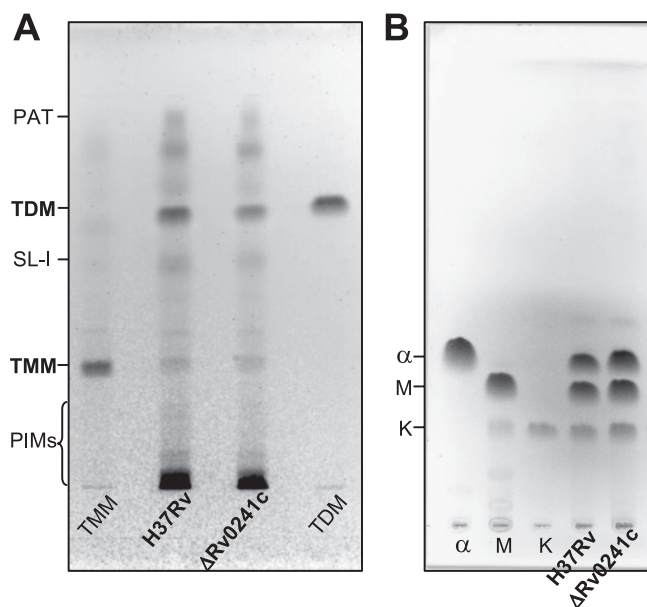


FIG. 5. Analysis of the mycolic acid content of *M. tuberculosis* $\Delta Rv0241c$. (A) TLC of the extractable lipids on silica gel G-60 plate using CHCl₃/CH₃OH/H₂O (60/16/2) as the eluent. Visualization by spraying with anthrone-H₂SO₄ and charring. No differences between the two strains were visible using another eluent (CHCl₃/CH₃OH, 9/1). PAT, polyacyltrehalose; TDM, trehalose dimycolates; SL-I, sulfolipid-I; TMM, trehalose monomycolates; PIMS, phosphatidylinositol mannosides. Standards of TDM and TMM were deposited. (B) TLC of the mycolic acid methyl esters on a silica gel G-60 plate using petroleum ether-diethyl ether (9/1, five passages) as the eluent. Visualization by spraying with molybdophosphoric acid and charring. α , α -mycolates; M, methoxymycolates; K, ketomycolates. The standards α -, methoxy-, and keto-mycolates were deposited. Identical quantities of total lipids from wt and $\Delta Rv0241c$ strains were deposited in panels A and B.

ficiency toward CoA as the carrier unit of the acyl chain, as opposed to AcpM. Furthermore, it shows a broad specificity for the chain length (C₄ to C₂₀) of the substrate, which is consistent with an earlier study predicting that this enzyme would have a substrate binding tunnel that could welcome long-chain substrates (5, 15). Rv0241c behavior is clearly distinct from that of FAS-II_{Mt} enzymes that display a marked specificity for C₁₂ to C₂₀ acyl chains carried by an ACP unit (21, 25, 28, 32). Therefore, the Rv0241c protein cannot belong to the FAS-II_{Mt} system, in sharp contrast to an earlier conclusion (9). Consistently, an Rv0241c ortholog was detected in the *Corynebacterium* genus, which is devoid of FAS-II system.

Interestingly, we observed that the Rv0241c enzyme could metabolize an enoyl chain linked to ACP_{Ec}, although with a weak activity. This feature and the broad chain length specificity of Rv0241c may account for its ability to artificially replace the HADs of *E. coli* and *S. cerevisiae* mitochondria *in vivo* during complementation experiments. Indeed, the latter proteins take part into *de novo* fatty acid biosynthesis pathways, as classical FAS-II systems usually do. Thus, in *E. coli*, FabZ has a predilection for short- to medium-chain length intermediates (11). In mitochondria, Htd2 displays a broad chain length (C₂ to C₁₆) specificity for the substrate (12).

We have previously shown that the *hadABC* cluster was essential to *M. tuberculosis* survival *in vitro* (28), like most of the genes encoding FAS-II_{Mt} enzymes. In contrast, the *Rv0241c* KO mutant is viable, meaning that either the Rv0241c enzyme has a function redundant with that of another *M. tuberculosis* enzyme or that it catalyzes a nonessential step of a metabolic pathway. A BLAST search against *M. tuberculosis* predicted proteins, using as a probe the Rv0241c sequence, revealed the absence of a paralog or closely related protein (data not shown). In particular, Rv0241c has a poor sequence

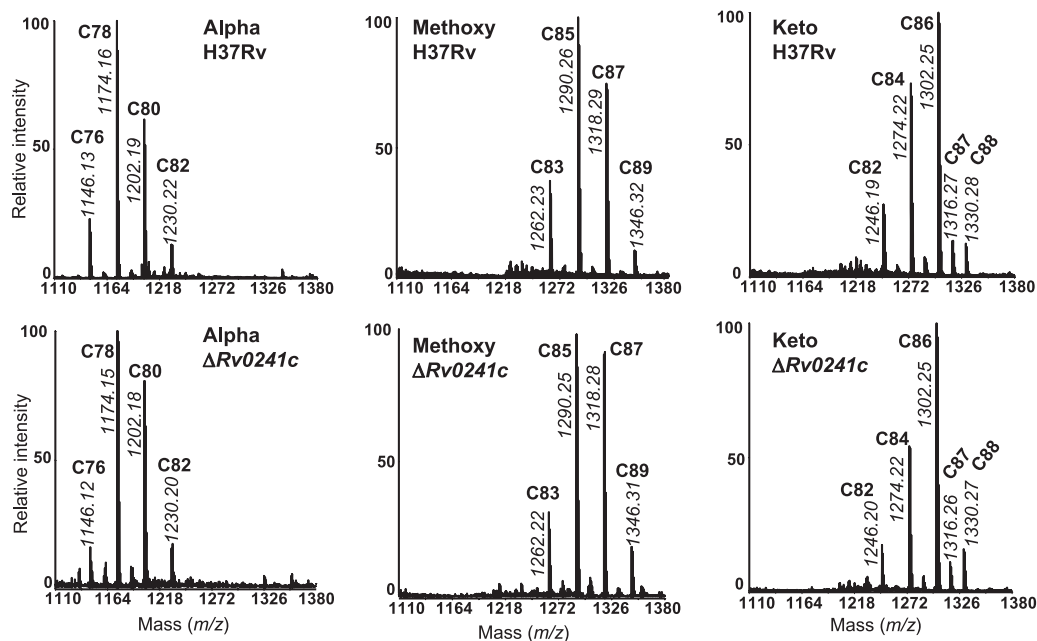


FIG. 6. MALDI-TOF MS analysis of the mycolic acids of *M. tuberculosis* $\Delta Rv0241c$. Mass spectra of purified mycolic acid methyl esters from the mutant strain and the wt parent strain. The peaks correspond to $[M+Na]^+$ ions. The total carbon number of each molecular species is mentioned.

identity with HadA, HadB, and HadC subunits (6 to 19% over the whole sequences). Furthermore, *Rv0241c* KO mutant has no detectable change in the mycolic acid profile compared to the parent strain. These data are consistent with the enzymatic study of Rv0241c and with the conclusion that this enzyme is not implicated in the mycolic acid biosynthesis pathway. Rv0241c may belong to another mycobacterial lipid metabolism such as, for example, the potential CoA-dependent fatty acid biosynthesis systems (18, 34).

The present study illustrates the fact that giving a positive result of heterologous complementation of HAD-deficient mutants of *E. coli* or *S. cerevisiae* is not a sufficient condition for a gene to encode a HAD homolog. Other *M. tuberculosis* proteins of the hydratase 2 family, Rv0130 and Rv3389c, were proposed to possess a HAD function and consequently to be part of FAS-II_{MT} system based on the complementation of *S. cerevisiae* Δ htd2 mutant (9, 10). However, it has previously been shown that Rv3389c is a CoA-dependent enzyme (29), like Rv0241c. Furthermore, Rv3389c is not ubiquitous among mycobacteria (no ortholog in *M. smegmatis* and a pseudogene in *M. leprae*), whereas FAS-II enzymes are very well conserved due to their essential function. Rv0130 is not conserved through the *Mycobacterium* genus either (absent in *M. leprae*). Furthermore, it has an ortholog in *Corynebacterium* (28), like Rv0241c. Finally, a soluble protein extract of a recombinant yeast strain overexpressing *Rv0130* exhibits 5- and 16-fold lower specific activities, respectively, in the presence of decenoyl-CoA compared to crotonoyl-CoA and *trans*-2-hexenoyl-CoA (10), suggesting that Rv0130 has a predilection for short-chain substrates. These data together are incompatible with an involvement of either Rv0130 or Rv3389c in the FAS-II_{MT} system, similarly to Rv0241c.

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