Complete auxotrophy for unsaturated fatty acids requires deletion of two sets of genes in *Mycobacterium smegmatis*.

Cecilia B. Di Capua[1#], Mariana Doprado[1,2#], Juan Manuel Belardinelli[1,3], and Héctor R. Morbidoni[1*]

[1]Laboratorio de Microbiología Molecular. Facultad de Ciencias Médicas. Universidad Nacional de Rosario, Rosario, Argentina.

[2] Current address: Stago Diagnostics. Eng L.C Berrini 105, Sao Paulo, CEP 04571-010, Brazil

[3] Current address: Department of Microbiology, Immunology and Pathology. Colorado State University, Fort Collins, CO, United States

* Corresponding author

[#] Equal contributors

Running title: Unsaturated fatty acid synthesis in *M. smegmatis*

Key words: *Mycobacterium smegmatis*, *Mycobacterium tuberculosis*, unsaturated fatty acid synthesis, oleic acid synthesis, long-chain acyl-CoA desaturase

Tel: (54-341- 5689618)

Fax: (54-341-4390465)

E-mail: morbiatny@yahoo.com

Summary: The synthesis of unsaturated fatty acids in *Mycobacterium smegmatis* is poorly characterized. Bioinformatic analysis revealed four putative fatty acid desaturases in its genome, one of which, *MSMEG*_1886, is highly homologous to *desA3*, the only palmitoyl-stearoyl desaturase present in the *Mycobacterium tuberculosis* genome. A *MSMEG*_1886 deletion mutant was partially auxotrophic for oleic acid and viable at 37°C and 25°C, although with a long lag phase in liquid medium. Fatty acid analysis suggested that *MSMEG*_1886 is a palmitoyl/stearoyl desaturase, as the synthesis of palmitoleic acid was abrogated, while oleic acid contents dropped by half in the mutant. Deletion of the operon MSMEG_1741-1743 (highly homologous to a *Pseudomonas aeruginosa* acyl-CoA desaturase) had little effect on growth of the parental strain; however the double mutant MSMEG_1886-MSMEG_1741-1743 strictly required oleic acid for growth. The ΔMSMEG_1886-ΔMSMEG_1741 double mutant was able to grow (poorly but better than the ΔMSMEG_1886 single mutant) in solid and liquid media devoid of oleic acid suggesting a repressor role for ΔMSMEG_1741. Fatty acid analysis of the described mutants suggested that MSMEG_1742-43 desaturates C18:0 and C24:0 fatty acids. Thus, although the *M. smegmatis des*A3 homologue is the major player in unsaturated fatty acid synthesis, a second set of genes is also involved.

Introduction.

The genus *Mycobacterium* displays one of the most diverse and complex fatty acid metabolisms ever described in bacteria. Sequencing of the genomes of *Mycobacterium tuberculosis* and *Mycobacterium bovis*, the agents causing human and bovine tuberculosis respectively, revealed an impressive number of genes devoted to the synthesis, modification and catabolism of fatty acids (Cole *et al.*, 1998, Garnier *et al.*, 2003). The research on those fields during the last two decades was generously rewarded with a deeper understanding of the synthesis and role in pathogenicity of some of those extremely complex lipids. Over the last years, projects aimed at sequencing the genomes of Non Tuberculous Mycobacteria (NTM) also revealed a high degree of complexity of their fatty acid and lipid metabolism although at lesser extent than what was seen in *M. tuberculosis* complex (Heydari *et al.*, 2013, Hasan *et al.*, 2015, Stinear *et al.*, 2008, Zakham *et al.*, 2011, Sekizuka *et al.*, 2014). Mycobacteria contain two fatty acid synthesizing complexes known as FASI and FASII (Cole *et al.*, 1998). FASI, with a structural organization similar to the yeast fatty acid synthesis complex, displays a bimodal synthesis pattern, making medium chain (mainly C16:0 and C18:0) and long chain (up to C24:0 in *M. smegmatis* and C26:0 in *M. tuberculosis*) saturated fatty acids (Bloch, 1975, Bloch, 1977), while FASII containing all the required enzymatic activities in discrete polypeptides -as it is in the majority of the bacterial species- evolved to specialize its functions to make the very long α-alkyl β-hydroxy fatty acids known as mycolic acids (Barry *et al.*, 1998, Takayama *et al.*, 2005). In this genus, UFAs (present as palmitoleic and mainly oleic acids) are not only constituents of structural molecules such as the cell membrane phospholipids, glycolipids and glycans but also of important carbon and energy storage molecules such as triglycerides (TAGs) (Alvarez & Steinbuchel, 2002, Daniel *et al.*, 2004, Torrelles *et al.*, 2012). The *M. tuberculosis*

H37Rv sequencing project identified the genes encoding for a hypothetical fatty acyl desaturase (Rv3229c, named *des*A3) and its partner oxidoreductase (Rv3230c) (Cole *et al.*, 1998). Two other genes identified during the *M. tuberculosis* genome sequencing, *des*A1 and *des*A2, were found linked to mycolic acid biosynthesis, encoding enzymes that have been proposed to desaturate the very long fatty acyl chains precursor to the mature mycolic acid molecules (Singh *et al.*, 2016, Cole *et al.*, 1998). Subsequently, it was shown that DesA3 is a stearoyl coenzyme A (CoA) desaturase that works together with its cognate oxidoreductase Rv3230c to produce oleic acid, a constituent of mycobacterial membrane phospholipids and triglycerides (Chang & Fox, 2006). *des*A3 has been listed as part of the essential gene pool required for growth inside the macrophage, stressing the importance of oleic acid for the *M. tuberculosis* metabolism (Sassetti & Rubin, 2003). Much less is known on the synthesis of unsaturated fatty acids in other mycobacteria excepting for a few reports describing biochemical characterization of long-chain fatty acid desaturases and elongases (Fulco & Bloch, 1964, Kikuchi & Kusaka, 1986, Kikuchi & Kusaka, 1982). In this report we describe the construction and characterization of *M. smegmatis* strains carrying deletions of genes that have an impact on UFA synthesis, showing for the first time the involvement of MSMEG_1886 and MSMEG_1741-1743 in that process and shedding light into those vital pathways.

Results.

Mycobacterium smegmatis contains several genes encoding putative fatty acyl desaturases.

Genome sequencing of *M. tuberculosis* H37Rv revealed only one gene –designated *Rv3229c*- that was annotated as a hypothetical linoleoyl desaturase (Cole *et al.*, 1998).

Later on, it was simultaneously demonstrated that its function was of stearoyl desaturase and that its overexpression was involved in low level resistance to ISO (Phetsuksiri *et al.*, 2003). We wanted to characterize the pathway leading to UFA biosynthesis in *M. smegmatis*, thus we performed a bioinformatic analysis of the *M. smegmatis* genome searching for genes displaying homology to *M. tuberculosis Rv3229c*. That inspection revealed three genes, *MSMEG*_1211, *MSMEG*_1743 and *MSMEG*_6835, with an identity of 47%, 41% and 37%, respectively, in addition to the *M. tuberculosis des*A3 homologue, *MSMEG*_1886 displaying a 69% of identity (Fig 1A). Of those, *MSMEG*_1211 showed the closest relatedness to *MSMEG*_1886. Analysis of the protein sequences located the characteristic three His boxes required for enzymatic catalysis (Fig. 1B). Those boxes are features of the membrane bound acyl-CoA and acyl-lipid desaturases, in which histidine (His) residues are coordinating a Fe atom located in the enzyme active site. The first His box contains the motif HX_3H also described in acyl-lipid desaturases (Δ6, Δ12, Δ3) from plants and cyanobacteria. The second mycobacterial His box, HX_3HH, is common to Δ6 acyl-lipid desaturases from cyanobacteria and higher plants while in the third box, the first histidine residue is replaced by glutamine, with the disposition QX_2HH (Los & Murata, 1998). This disposition has some similarity to the third His box described in cyanobacteria and higher plants (QX_3HH), but has not been described until now in other prokaryotic systems. A fifth gene, *MSMEG*_3333, although displaying some similarity to *des*A3, was discarded from further analysis as it only contained the first two His boxes.

Fatty acyl desaturases require cognate oxidoreductases for their enzymatic catalysis; thus we examined the chromosomal regions adjacent to the putative desaturases in order to locate their partners (Fig 1A). Previous reports established the role of *Rv3230c* as an oxidoreductase in *M. tuberculosis* (Chang & Fox, 2006); our analysis showed that genes

encoding proteins with a possible oxidoreductase function are located upstream of *MSMEG*_1886, *MSMEG*_1743 and *MSMEG*_6835 in *M. smegmatis*. The putative oxidoreductase encoded by *MSMEG*_1885 is highly similar to *M. tuberculosis Rv3230c* (75% identity), while *MSMEG*_1742 shows a 35% of identity. Surprisingly, *MSMEG*_1211, the gene most similar to *MSMEG*_1886 (57% identity), does not have an adjacent partner oxidoreductase (Fig. 1A) suggesting the possibility that it could use one of the other three putative oxidoreductases or perform a different function.

The examination of mycobacterial genomes available in public databases showed that MSMEG_1211 is present in a few NTM such as *M. phlei*, *M. rhodesiae* and *M. intracelullare* while MSMEG_6835 is present in *M. tusciae*, *M. gilvum*, *M. vaccae* and *M. sinense*. It is of interest that a large group of opportunistic pathogens (such as *M. avium*, *M. ulcerans*, *M. abscessus*, *M. kansasii*, *M. chelonae*, among others) contains the MSMEG_1741-1743 operon, absent in the genomes of members of the *M. tuberculosis* complex (Fig. 2). Thus, a first analysis of NTM genomes pinpointed to various genes possibly involved in the biogenesis of UFAs in those mycobacteria.

MSMEG_1886 encodes a palmitoyl-stearoyl desaturase required for optimal growth.

Since among the hypothetical *M. smegmatis* fatty acid desaturase encoding genes, *MSMEG*_1886 displays the highest homology to *des*A3, the only fatty acyl desaturase proven as such in *M. tuberculosis*, we deleted it by using the "recombineering" technique described by van Kessel *et al.* (van Kessel & Hatfull, 2007). The *M. smegmatis* Δ*MSMEG*_1886 strain was easily obtained and confirmed by PCR of clones growing on solid media containing oleic acid (Fig. S1); however, testing of the auxotrophy on media not containing this supplement showed that the deletion strain was capable of growth yielding colonies of smaller size than the parental strain at 37°C (Fig.

3A). More surprisingly its growth in liquid medium was aberrant showing a very long lag phase of almost 24 h (Fig. 3B) after which growth started albeit at slower rate than the wild-type strain (duplication times 5 h and 3 h respectively). The lag phase was highly reproducible; moreover, this phenotype was maintained when mid-exponential growing cultures were diluted into fresh pre-warmed oleic-free medium. The same delay in the onset of growth (almost 72 h) took place at 25°C; at this temperature the duplication time for the mutant and wild type strains was of 14 h and 8 h respectively (Fig. S3). Those growth anomalies were corrected at both temperatures by the addition to the culture medium of oleic acid or palmitoleic acid in combination with albumin (designated OADS and PADS respectively), the usual way fatty acids are added to Middlebrook culture media formulations (Fig. 3C and 3D). As expected, expression of the deleted gene cloned in pMV306, an integrative plasmid (Snapper *et al.*, 1990), also corrected the phenotype, being sufficient to restore the wild-type growth at both temperatures (Fig. 3C, 3D and S3).

In order to evaluate the composition of the UFA pool in the deletion mutant, we performed GC-MS of extracted fatty acids. Our results showed that there was a 40% reduction in the content of C18:1 Δ9 (oleic acid) when compared to the wild type strain (Fig. 4 and Table S3). In line with those results, we determined that the mutant strain showed a large increase in stearic acid (C18:0, the precursor of oleic acid) that was quantitatively comparable to the loss of oleic acid. Interestingly, we observed that the C16:1 UFA pool in the parental *M. smegmatis* strain was comprised of three isomers as proven by adduct production (Table S4) and GC-MS analysis, namely C16:1 Δ7, C16:1 Δ9 and C16:1 Δ10, representing roughly 10% of the cellular fatty acids at either 37°C or 25°C (Table S3). The contents of the three isomers were dramatically reduced to less than 1% in the mutant strain, indicating that none of the other hypothetical desaturases

168 can fulfill the function of *MSMEG*_1886 under the experimental conditions used (Fig. 4
169 and Table S3). Thus our results indicate that *MSMEG*_1886 encodes a desaturase
170 capable of synthesizing C16:1 fatty acids and contributing to a substantial amount of the
171 total C18:1 synthesis. Interestingly, the synthesis of longer chain fatty acids was altered
172 with a relative (yet small) increase in C24:1 Δ15 (from 0.4% to 1.6% at 37°C) (Fig. 4
173 and Table S3). Moreover, adduct analysis showed that both the parental and the
174 ΔMSMEG_1886 deletion strains contained the same unsaturated fatty acid isomers, that
175 is, the gene deletion did not bring up a previously undetected minor unsaturated fatty
176 acid isomer (Table S4).
177 Comparable results were obtained from cultures grown at 25ºC although with a slightly
178 higher content of C16:0 in ΔMSMEG_1886 (Table S3) suggesting that there is no cold-
179 induced desaturating system as described for several other prokaryotes (Aguilar *et al.*,
180 1998, Los *et al.*, 1993, de Mendoza *et al.*, 1982).
181 These results underscore the importance of MSMEG_1886 in oleic acid and palmitoleic
182 acid production; at the same time they highlight the complexity of the mycobacterial
183 fatty acid metabolism since other functional pathway providing UFA synthesis has to be
184 present in *M. smegmatis*.
185
186 *Deletion of the MSMEG_1741-1743 operon in a ΔMSMEG_1886 genetic background*
187 *causes complete oleic acid auxotrophy in Mycobacterium smegmatis.*
188 Bioinformatics analysis pointed to MSMEG_1211 as a possible surrogate candidate for
189 MSMEG_1886 due to being the second most homologous gene to *M. tuberculosis*
190 *des*A3. However, MSMEG_1211 is not preceded by an oxidoreductase encoding gene
191 suggesting that it may use the one encoded by MSMEG_1885 or other yet unidentified
192 partner. In order to prove this, we cloned and overexpressed MSMEG_1211, using

pLAM12, an acetamide inducible vector, into the *M. smegmatis* ΔMSMEG_1886. Overexpression of MSMEG_1211 failed to restore growth and UFAs synthesis to normal levels in the mutant (data not shown) thus casting doubts about its function. What is more, subsequent over-expression of MSMEG_1743 and MSMEG_6835 also failed to improve growth of *M. smegmatis* ΔMSMEG_1886. Moreover, introduction by electroporation of the MSMEG_1886, MSMEG_1211 and MSMEG_6835 clones into *M smegmatis* mc^2155 followed by analysis of the fatty acid profile by GC-MS did not reveal substantial changes in the contents of the major saturated and unsaturated C16-C24 fatty acids upon induction of the expression (data not shown).

Based on the homology of MSMEG_1741-1743 to an acyl desaturase system studied in *P. aeruginosa*, we elaborated that such an activity could generate the required amounts of unsaturated fatty acids to maintain viability and proper cytoplasmic membrane fluidity in *M. smegmatis*. To test that, the entire MSMEG_1741-1743 operon was deleted in *M. smegmatis* mc^2155 using pPR27, a suicide temperature sensitive plasmid vector (Pelicic *et al.*, 1996). The knock out strain was easily obtained and the deletion confirmed by standard PCR analysis (Fig. S2). The constructed ΔMSMEG_1741-1743 strain was not affected for colony morphology or growth to an appreciable extent under the culture conditions used (Figs. 5A and B). We next used mycobacteriophage Bxz1 (Lee *et al.*, 2004) to transduce this deletion into the genetic ΔMSMEG_1886 background, obtaining colonies of the double mutant strain on culture medium supplemented with oleic acid. Confirming our hypothesis, the resulting strain ΔMSMEG_1886-ΔMSMEG_1741-1743 showed complete auxotrophy for oleic acid, not being able to grow on solid media devoid of this fatty acid in spite of very long incubation times (up to 15 days) at 37°C (Fig. 5C). The same phenotype was observed on liquid medium; with the double mutant being unable to grow on medium devoid of

UFA. The double mutant was able to grow when the media was supplemented with either OADS or PADS, although at a much lower rate than the parental strain only reaching an OD ≈ 2 on liquid media and producing smaller colonies on solid media (Figs. 5A and B). Introduction by electroporation of the integrative plasmid pML2300::1741-43 carrying the entire operon in the double mutant restored growth in the absence of exogenous UFA giving comparable growth on solid and liquid media to that of the single mutant ΔMSMEG_1886 (data not shown).

Fatty acid analysis by GC-MS was performed on the strains grown on media devoid of oleic acid (*M. smegmatis* mc^2 155, ΔMSMEG_1886 and ΔMSMEG_1741-1743 strains) or supplemented with 10% of the oleic acid content regularly present in OADS in the case of ΔMSMEG_1886-ΔMSMEG_1741-1743 (determined as the minimum amount of UFA required to support the growth of the double deleted mutant on liquid media). All the strains were grown to comparable OD before fatty acids extraction. Our results revealed that deletion of the MSMEG_1741-1743 operon in both, wild type or MSMEG_1886 background, resulted in decrease of C18:1, accumulation of C24:0 and complete loss of C24:1Δ15 (Fig. 6). The presence of oleic acid in the double mutant fatty acid profile by GC-MS analysis was mostly likely due to the supplement in the culture medium; a proof of that was that the same experiment using 1% PADS supplementation showed the absence of C18:1Δ9 along with high levels of C24:0 (data not shown). This observation proves the C18:0 desaturase activity displayed by MSMEG_1741-1743. Interestingly, the content of the C16:1 Δ7 isomer was unabated in the double mutant; even more, we found a detectable small increase of it that may suggest a third desaturase with a very minor role in the overall UFA synthesis and in *M. smegmatis* growth (Fig. 6). Thus, we have demonstrated for the first time that two

242 different sets of genes are in charge of growth supporting UFA synthesis in *M.*
243 *smegmatis*.
244
245 *In vivo labeling confirms that the MSMEG_1741-1743 operon encodes a fatty acid*
246 *desaturating system.*
247 To confirm that MSMEG_1741-1743 encodes components capable of generating the
248 required oleic acid, we performed "in vivo" labeling experiments as described in
249 Materials and Methods. Briefly, mid-log phase of cultures of *M. smegmatis* mc^2155
250 (parental strain) and its derivatives lacking MSMEG_1886, MSMEG_1741-1743 or
251 both MSMEG_1886 and MSMEG_1741-1743 were grown in media supplemented with
252 OADS, collected by centrifugation, washed and resuspended in fresh media devoid of
253 oleic acid. After three hours of depletion, a radiolabelled precursor (either [1-^{14}C] acetic
254 acid or [1-^{14}C] stearic acid) was added to 5 ml aliquots of each culture, growth was
255 continued for 3 hr after which the cells were collected by centrifugation. Extraction of
256 lipids from the cell pellets was carried out as described, and the content of SFA and
257 UFA was assessed by argentation TLC using silica plates presoaked in $AgNO_3$.
258 Inspection of TLC from [1-^{14}C] acetate labeled samples showed that both single knock
259 outs were able to synthesize UFA from this precursor while there was a total absence of
260 these fatty acids in the double knock out, thus confirming our previous results that both
261 desaturases contribute to UFA synthesis (Fig. 7 left panel). In addition to that,
262 radioactive stearic acid was very poorly converted into oleic acid in the
263 ΔMSMEG_1886 strain while the conversion in the ΔMSMEG_1741-1743 was
264 comparable to the conversion in the wild type strain suggesting that, as known,
265 MSMEG_1886 was capable of desaturating stearic acid while MSMEG_1743 did that
266 very poorly (Fig. 7 right panel). The fact that there is no detectable conversion of [1-

^{14}C] acetate or [1-^{14}C] stearic into [1-^{14}C] oleic acid in the double mutant ΔMSMEG_1886/ΔMSMEG_1741-1743 clearly demonstrates that the MSMEG_1741-1743 operon encodes a desaturase activity as suggested by the MSMEG_1743 sequence. Both desaturating systems under study do not play a role on mycolic acid synthesis as no significant quali- or quantitative changes are detected on their profile by 1D-TLC (data not shown) or on argentation TLC (Fig. 7, left panel). We thus conclusively show that no other gene(s) can provide UFA synthesis at growth supporting level under the conditions tested.

Deletion of MSMEG_1741 improves growth in the absence of oleic acid.

Our results strongly suggested that the putative fatty acyl desaturation system encoded by MSMEG_1741-1743 was able to partially sustain growth through unsaturated fatty acid production in a genetic background where the major Δ9 desaturase, MSMEG_1886, was deleted. However, the puzzling very long lag phase displayed by ΔMSMEG_1886 before it started growing suggested that the remaining desaturase activity was repressed, thus requiring intracellular metabolic changes to take place before induction. That encouraged us into looking at the role of MSMEG_1741, homologous to the *des*T gene that acts as a repressor of the *P. aeruginosa des*BCT operon. We successfully knocked out MSMEG_1741 in both the parental strain and in ΔMSMEG_1886 using the temperature sensitive plasmid pPR27 and gene deletion was further confirmed by PCR (Fig. S2). Both single (ΔMSMEG_1741) and double (ΔMSMEG_1886-ΔMSMEG_1741) mutants were further characterized by analysis of their growth in solid and liquid medium. Although the presence of oleic acid in solid medium allowed the growth of both strains to an extent comparable to the parental strain, the double mutant generated colonies of larger size than the ΔMSMEG_1886

single mutant in medium devoid of oleic acid but still smaller than the colonies produced by the parental strain (Fig. 8A). In liquid medium with no UFA supplementation the double mutant lacking the putative repressor MSMEG_1741 considerably reduced the lag phase observed in the ΔMSMEG_1886. Interestingly, the deletion of MSMEG_1741 in a wild type background caused a longer lag phase compared to the parental strain but still much shorter than the one displayed by the ΔMSMEG_1886 mutant (Fig. 8B). The addition of OADS to the medium allowed for growth of all the mutants although a lag phase longer than the one observed in the parental strain was still detectable (Fig. 8B).

Finally, complementation of the *M. smegmatis* ΔMSMEG_1886-ΔMSMEG_1741 double mutant with an integrative vector (pML2300) carrying a fragment encompassing MSMEG_1741 and an additional 400 bp of upstream sequence (thus covering the sequence corresponding to the intergenic region between MSMEG_1741 and MSMEG_1742) displayed the same growth behavior than the ΔdesA3 single mutant, both showing comparable lag phase lengths when grown in the absence of UFA supplementation (Fig. 8 B).

GC-MS analysis of the fatty acids present in the ΔMSMEG_1741 revealed that the deletion of the putative repressor did not alter the fatty acids profile when MSMEG_1886 was present; however, in the ΔMSMEG_1886-ΔMSMEG_1741 double mutant we detected a large increase in C24:0 and a small increase in C24:1 (Fig. 9A). Metabolic labeling experiments using [1-^{14}C] acetic acid demonstrated that the ΔMSMEG_1886-ΔMSMEG_1741 strain was capable of generating radiolabeled unsaturated fatty acids at comparable levels to the ones in the ΔMSMEG_1741 genetic background (Fig 9B); at the same time the ΔMSMEG_1886-ΔMSMEG_1741-1743 double mutant does not show any label in unsaturated fatty acids (Fig. 7, lane 4). Our

results strongly suggest that MSMEG_1741 is a functional repressor of MSMEG_1742-43, since once it is deleted, the release of the expression of MSMEG_1742-1743 partially restored growth of the Δ*des*A3 mutant in the absence of any exogenously added unsaturated fatty acid. The relief of the auxotrophy was enough for growth at a rate comparable to that of the wild-type strain; however it led the culture to a stationary phase at lower $O.D._{600nm}$ values (Fig. 8). A more clear understanding of the regulation of this pathway and its physiological relevance will require analysis of expression of the operon as well as the identification of the signal molecules behind its repression/induction.

Discussion

The genetic landscape of Unsaturated Fatty Acid synthesis in NTM.

Despite its importance in the bacterial cell membrane integrity and as part of the cell envelope through glycolipids and glycopeptidolipids, the study of the pathways of unsaturated fatty acid synthesis in mycobacteria remains poorly studied. There are few publications focused on the NTM desaturases; Lennarz *et al*. were the first researchers to describe an aerobic desaturase system producing UFAs in mycobacteria (Lennarz *et al.*, 1962). These authors demonstrated the desaturation of stearic acid to oleic acid in log phase cells of *M. phlei*, and its subsequent modification into 10-methyl stearic acid (tuberculostearic acid) in resting cells. Other publications reported the purification and biochemical characterization of activities capable of desaturating palmitoyl-CoA, stearoyl-CoA or lignoceroyl-CoA (C24:0) in *M. smegmatis* (Fulco & Bloch, 1964, Kikuchi & Kusaka, 1986).

Our studies revealed the presence of four genes in the *M. smegmatis* genome - *MSMEG*_1886, *MSMEG*_1743, *MSMEG*_1211 and *MSMEG*_6835- which displayed

342 homology to the *M. tuberculosis* stearoyl desaturase DesA3 encoded by *Rv3229c*. Their
343 sequence analysis showed the presence of the three histidine boxes characteristic of
344 membrane bound desaturases, although other enzymes such as hydroxylases and
345 monoxygenases also share this feature (Shanklin *et al.*, 2009). The disposition of the
346 histidines into the three boxes is similar to that found in cyanobacterial and higher plant
347 desaturases (Los & Murata, 1998). However, as previously pointed out for *M.*
348 *tuberculosis* DesA3 (Chang & Fox, 2006), the enzymes analyzed in this work share an
349 aminoacid spacing between His residues resembling that of Δ6 acyl-lipid desaturases
350 from plants and cyanobacteria instead of the one displayed by Δ9 acyl-lipid and acyl-
351 CoA desaturases of yeasts, animals and higher plants. Thus, as shown in Fig. 1B, the
352 overall signature motif is HX_3H, HX_3HH, QX_2HH, differing from the reported HX_4H,
353 HX_2HH, with HX_2HH and therefore becoming a possible feature of these mycobacterial
354 enzymes.
355 Genes encoding putative oxidoreductases are located upstream of the genes
356 *MSMEG*_1886, *MSMEG*_1743 and *MSMEG*_6835 but not of *MSMEG*_1211. Although
357 functional proof is yet to be provided, based on previous reports demonstrating the
358 oxidoreductase activity of Rv3230c in *M. tuberculosis*, we may propose identical
359 functions for *MSMEG*_1885, *MSMEG*_1742 and *MSMEG*_6836. The absence of an
360 oxidoreductase encoding gene for *MSMEG*_1211 suggests the possibility that it could
361 use any of the other partners mentioned as they are ubiquitous and not necessarily
362 restricted to a particular desaturase. However, it is also possible that this gene is not
363 encoding a true fatty acid desaturase.
364 During our work we have found that most of the NTM have more than one putative
365 fatty acid desaturase encoded in their genomes (Fig. 2). Surprisingly, the mycobacterial
366 enzymes analyzed did not exhibit significant aminoacid sequence homology to any

desaturase described out of the genus *Mycobacterium*, with one remarkable exception given by the *P. aeruginosa* desaturase DesB, an inducible acyl-CoA Δ9 desaturase (Zhu *et al.*, 2006). Interestingly, a second gene, *desC*, encoding the oxidoreductase partner, is located upstream of *P. aeuriginosa desB*; and a third gene, named *des*T (encoding a repressor belonging to the TetR family), is located on the opposite strand and overlapping the *des*B-*des*C operon (Zhu *et al.*, 2006). A similar organization is found in *M. smegmatis*; in which the *MSMEG*_1742-*MSMEG*_1743 operon contains an adjacent gene –*MSMEG*_1741- homologous to the *P. aeruginosa des*T regulator (Fig. 1).

Loss of the palmitoyl / stearoyl desaturase MSMEG_1886 unveils other players involved in fatty acid desaturation in M. smegmatis.

The palmitoyl / stearoyl desaturase activity of *M. tuberculosis* DesA3 has already been shown by Phetsuksiri *et al.* (Phetsuksiri *et al.*, 2003) and by "*in vitro*" reconstitution experiments by Chang and Fox (Chang & Fox, 2006). We also confirmed this activity for *M. smegmatis* as the deletion of *MSMEG*_1886 caused a dramatic decrease of C16:1 and a 40-50% reduction in C18:1 content in the mutant strain. Given these results, and on the grounds of the published information mentioned above (Chang & Fox, 2006, Phetsuksiri *et al.*, 2003), we herein propose to rename MSMEG_1886 as *M. smegmatis des*A3. However, the *M. smegmatis* Δ*des*A3 deletion strain was viable on solid media with no addition of oleic acid, demonstrating that DesA3 is not the only enzyme capable of making UFAs in *M. smegmatis* and suggesting that one or more of the other putative desaturases encoded in the *M. smegmatis* chromosome were responsible for the remaining activity.

A very intriguing and important observation was the presence of a highly reproducible long lag phase of Δ*des*A3 in liquid medium after which growth was triggered although at a lower rate and reaching a lower final O.D. when compared to parental strain. That behavior was not suppressed or shortened when growing the mutant strain at low temperatures (Fig. 3B); thus suggesting against a role of temperature as a signal for fatty acid desaturase expression or activity in *M. smegmatis*. This could indicate that other UFA producing systems need to be activated through accumulation of or decrease of a specific signal. Overexpression of the remaning desaturases, namely, MSMEG_1211, MSMEG_1743 and MSMEG_6835 failed to increase production of oleic acid in both the wild-type and the ΔdesA3 strains (data not shown). However, our attempts to decipher the role of the putative desaturase genes in *M. smegmatis* may have been limited to some extent since we did not overexpress the oxidoreductase-desaturase pair (even when the MSMEG_1211 partner is unknown). Nevertheless, the fact that overexpression of *M. tuberculosis des*A3 alone increased 2-fold the synthesis of oleic acid implies that an overexpression strategy should have given a slight but detectable increase in UFAs that in this case we did not observe (Phetsuksiri *et al*., 2003).

Thus, we resorted to a different strategy and decided to delete the MSMEG_1741-1743 operon, which is highly homologous to the acyl desaturase *des*BCT operon in *P. aeruginosa*. The synthesis of UFAs in *P. aeruginosa* has been thoroughly dissected demonstrating that DesB desaturates exogenously provided saturated fatty acids (SFA) and its expression is regulated by DesT (Zhang *et al.*, 2007, Zhu *et al.*, 2006). In our hands the double Δ*des*A3-MSMEG_1741-1743 strain was unable to grow on solid or liquid medium if oleic acid was not present, confirming that this operon indeed encoded for the desaturase system supporting growth of the Δ*des*A3 mutant (Fig.5). Fatty acid analysis by GC-MS demonstrated that the MSMEG_1741-1743 operon was linked to

the synthesis of C18:1 and C24:1 (Fig. 6). However, since the need to supplement cultures of the double mutant with oleic acid may make difficult to trace the origin of the intracellular fatty acids detected (especially oleic acid contents), we used an "in vivo" radiolabeling approach using [1-^{14}C]-acetic acid and [1-^{14}C]-stearic acid (Fig. 7). Clearly, the double mutant lacks the synthesis of UFA as judged by the incorporation of the radioactive precursors "in vivo", indicating the involvement of both MSMEG_1886 and MSMEG_1741-1743 (Fig.7).

Taken together, we have undoubtedly established that a second fatty acid desaturation system is active in *M. smegmatis* and by extension in other NTM where this operon is present. On these grounds, we propose to adopt the genetic nomenclature existing for *P.aeruginosa* renaming the MSMEG_1741-1743 operon as *des*BCT. Based on our results we cannot rule out the existence of a third desaturase able to generate very small amounts of C16:1 Δ7 fatty acid (although not enough to support growth), a possibility that needs to be addressed.

DesT is the repressor of the long chain fatty acid desaturating system encoded by MSMEG_1742-1743.

The gene organization and protein homology between *P. aeruginosa des*BCT and *M. smegmatis des*BCT suggested that a plausible explanation for the low level generation of oleic acid in the Δ*des*A3 mutant could be due to the repression of *des*BC transcription by the putative repressor DesT. Our genetic evidence strongly supports that hypothesis since deletion of *des*T allowed for better growth of the Δ*des*A3-Δ*des*T double mutant compared to the growth of the Δ*des*A3 single mutant (Fig. 8A); moreover, the characteristic feature of this strain –its long lag phase preceding growth- was suppressed (Fig. 8B). In agreement with those observations we demonstrated that

441 introduction of *des*T cloned in an integrative plasmid restored the long lag phase in the
442 *des*A3-*des*T double mutant as expected (Fig 8B).
443 Interestingly, the same experiment carried on in medium containing OADS showed that
444 in spite of the exogenous surplus of oleic acid, there is a deleterious effect when *des*T is
445 deleted in an otherwise wild-type back-ground strongly pointing out that the major
446 product of *des*BC is somehow not convenient for balanced growth (Fig.8C).
447 Importantly, we observed some synthesis of oleic acid and a larger amount of C24:1 in
448 the *des*A3, Δ*des*T double mutant (Fig. 9 A). Adduct analysis identified it as C24:1 Δ15,
449 the only C24:1 isomer present in *M. smegmatis* mc^2155 (Table S4). Of note, the
450 bimodal activity of the mycobacterial Fatty Acid Synthase I (FAS I) yields mainly
451 C16:0 and C18:0 and C24:0 (C26:0 in the case of members of the *M. tuberculosis*
452 complex) (Bloch, 1975, Bloch, 1977), thus in the absence of a functional C16:0- C18:0
453 desaturase, it seems plausible that there would be a surplus of those saturated fatty acids
454 accessible to the *M. smegmatis des*BC system and possibly to the general –and highly
455 complex- mycobacterial fatty acid metabolism. Importantly, Kikuchi and Kusaka
456 described a cytosolic activity able to generate long chain monounsaturated fatty acids
457 (Kikuchi & Kusaka, 1986); in that report the authors showed that the purified enzymatic
458 complex desaturated mainly lignoceroyl-CoA yielding only Δ^{15} tetracosanoic acid; they
459 also demonstrated that stearoyl-CoA was also a substrate although less efficiently,
460 therefore our results are in agreement with their biochemical studies. Our analysis of
461 adducts of UFA present in the parental and the mutant strains agrees with those results
462 (Table S3), so it is tempting to suggest that *M. smegmatis des*BC introduces a double
463 bond in position 9 of stearoyl-CoA and with more efficiency a double bond in position
464 15 of lignoceroyl-CoA. An unexpected way to match those findings is *M. smegmatis*
465 *des*BC behaving as a methyl end desaturase, a hypothesis that would need to be

466 confirmed. The elucidation of the regioselectivity of the *M. smegmatis des*BC
467 desaturase as well as of its "in vivo" substrate (cytoplasmic saturated fatty acid on CoA
468 or ACP soluble carrier or cell membrane phospholipid) is beyond the scope of this
469 report and will be addressed separately.
470 It has previously been shown that DesT represses *des*BC in *P. aeruginosa*; moreover it
471 has also been shown that DesT is able to bind both UFAs and SFAs, but with a higher
472 affinity for UFAs, therefore becoming a means to sense unsaturated/saturated fatty acid
473 ratio in that species. Once bound to a fatty acid, DesT binds (when the ligand is an
474 UFA) or releases (when the fatty acid is a SFA) from the promoter regions of *des*C and
475 *fab*A, controlling the levels of UFAs cellular pool and linking aerobic and anaerobic
476 synthesis of unsaturated fatty acids (Zhang *et al.*, 2007, Zhu *et al.*, 2006). This could
477 explain the long lag phase observed in Δ*des*A3, in which expression of *M. smegmatis*
478 *des*T might not be triggered until a threshold amount saturated fatty acids have
479 accumulated.
480 The deletion of *des*T in *M. smegmatis* helped a *M. smegmatis* Δ*des*A3 mutant grow
481 better -although still not at wild type levels- in medium lacking oleic acid supplement
482 (Fig. 8); in addition to that, "in vivo" radiolabelling demonstrated a larger ability to
483 generate long chain unsaturated fatty acids including a small fraction of oleic acid. Thus
484 we concluded that MSMEG_1741 is the repressor of the MSMEG_1742-1743 operon,
485 giving grounds to our proposal of designating this operon as *M. smegmatis des*BCT.
486 There are several factors that could explain the inability of this desaturase system to
487 fully support growth. A possible explanation is that expression of *M. smegmatis des*BC
488 is not high enough to compensate for *des*A3. Another likely explanation could arise
489 from the fact that lignoceroyl-CoA and not stearoyl-CoA may be the preferred substrate
490 of *M. smegmatis des*BC, moreover C24:1 Δ15 is most likely not adequate to fulfill the

roles of oleic acid. Notwithstanding those possible explanations, the nature of the carrier of those fatty acyl moieties (CoA or Acyl Carrier Protein, ACP) is yet to be established. Besides those hypothesis, accumulation of long-chain fatty acyl-CoAs (mainly C24:0) may exert toxic effects on the cell metabolism. The combination of little oleic acid synthesis along with C24:1 Δ15 synthesis and an accumulation of C24:0 fatty acid would explain the hardship of growth of the different mutants constructed throughout this work.

There are substantial differences in the role of DesBCT in *P.aeruginosa* and in *M. smegmatis* as there are no fatty acids longer than C18 in that Gram (-) pathogen (Benamara *et al.*, 2014). However, it is tempting that –like *P. aeruginosa*- NTM may also use DesT to sense levels of UFA and SFA; future work will make clear whether this is the case in *M. smegmatis* and if there is a preference between stearoyl-CoA and lignoceroyl-CoA for the induction of the operon. In the first case, DesT may act as a general sensor of UFA/SFA ratio, but it is a very challenging proposal that in the second one may be sensing physiological uncoupling of FASI and FASII during growth or under stress. The absence of this operon in *M. tuberculosis* and *Mycobacterium bovis* is quite surprising; our search for genes homologous to MSMEG_1743 failed to detect any suitable candidate and inspection of the chromosomal location of putative members of the TetR regulators also failed to retrieve any gene associated to genes similar to MSMEG_1742-43. Given these results, further work to address the role of MSMEG_1741-1743 in NTM is warranted. However a note of caution is given by a recent publication describing that a *M. smegmatis fas*I *acp*S conditional mutant –thus incapable of making fatty acids- was able to make mycolic acids possibly at the expense of triglycerides (Cabruja *et al.*, 2017); this is a humbling demonstration that mycobacterial fatty acid synthesis and regulation is yet to be decipher in full and that

will require the identification and exhaustive studies of the players involved. Our report provides a working frame for the identification of the regulatory mechanisms underlying the expression of the *M. smegmatis des*BCT operon and its links to other parts of the mycobacterial fatty acid metabolism. Finally, *M. smegmatis des*BCT is present in the majority of the NTM species including opportunistic pathogens (Fig. 2); its ability to support growth in the absence of oleic acid synthesis makes it a valuable new target for drug development against NTM that would potentiate the effect of Isoxyl and other inhibitors of the stearoyl desaturase for treatment of infections due to those mycobacterial species.

Experimental procedures.

Strains, chemicals, culture media and growth conditions.

M. smegmatis mc^2 155, used as parental strain, and the mutants derived from it, were routinely grown in Middlebrook 7H9 broth (Difco) supplemented with 0.5 % (w/v) glycerol and 10 % (v/v) of either ADS (Albumin 50 g l^{-1}, Dextrose 20 g l^{-1}, NaCl 8.5 g l^{-1}), OADS (oleic acid 50 mg l^{-1} in ADS) or PADS (palmitoleic acid 10 µg l^{-1} in ADS). For some experiments, the concentration of oleic or palmitoleic acid was decreased to 1/10 by dilution with ADS. The same medium with the addition of 1.5 % (w/v) agar (Difco) was used as solid medium. Triton WR1339 (0.25 % v/v) was added to liquid cultures to avoid clumping of the cells. For sake of simplicity these media will be refered to as 7H9-Gly-ADS/OADS/PADS-Tr. *Escherichia coli* strain DH5α was maintained in Luria Bertani (LB) medium and propagated at 37°C, and used for routine cloning and transformation experiments.

Antibiotics, when required, were added at the following concentrations: for *E. coli*, Hygromycin 150 µg ml^{-1}, Ampicillin 100 µg ml^{-1}, Streptomycin 50 µg ml^{-1}, Kanamycin

50 µg ml^{-1}; for *M. smegmatis*, Hygromycin 50 µg ml^{-1}, Streptomycin 20 µg ml^{-1}, Kanamycin 20 µg ml^{-1}. All chemicals were from Sigma unless otherwise indicated. Radioactive precursors (from Perkin Elmer) were [1-^{14}C] acetic acid (specific activity 55.3 mCi $mmol^{-1}$) and [1-^{14}C] stearic acid (specific activity 56.1 mCi mmol-1).

Genetic manipulations.

Plasmids used in this study are listed in Table S1. Total genomic DNA of *M. smegmatis* was obtained as was described previously (Larsen, 2000) and used as template for gene amplification. The list of primers used throughout this study is presented in Table S2.

The deletion of *MSMEG*_1886 was achieved by the "*recombineering*" method developed by van Kessel and Hatfull (van Kessel & Hatfull, 2007). Briefly, upstream and downstream regions of *MSMEG*_1886 were amplified by PCR to generate the knock out mutant strain. To this end two 500 bp fragments containing 100 bp of the *MSMEG*_1886 *orf* and its adjacent 400 bp upstream or downstream were amplified using primers 1886Fw, 1886IntRev; 1886IntFw, 1886Rev. PCR conditions were as follows: denaturation (94°C, 5 min), followed by 30 cycles of denaturation (94°C, 1 min), annealing (64°C, 40 sec) and extension (68°C, 60 sec). The resulting DNA fragments were purified after agarose gel electrophoresis using a gel extraction kit (Promega, Madison, Wis.) following the manufacturer´s instructions (Fig. S2). The purified upstream and downstream fragments were digested with XbaI- BamHI and BamHI- EcoRI and sequentially ligated into the pLITMUS 28G *E. coli* vector. A Hygromicin cassette was obtained by HindIII digestion of plasmid pHP45ΩHyg (Blondelet-Rouault *et al.*, 1997), gel purified and cloned in between of the *MSMEG*_1886 upstream and downstream fragments, generating plasmid pLITΔ1886. A linear product obtained digesting pLITΔ1886 with XbaI and EcoRI was used to

transform electrocompetent cells of *M. smegmatis* mc^{2}155-pJV53 (van Kessel & Hatfull, 2007) previously induced by addition of acetamide 10% (w/v) to a final concentration of 0.2% (w/v). Selection of transformants was made on solid Middlebrook 7H9 OADS-Gly Hygromycin at 37°C for 5-7 days. Complementation of the deleted gene was achieved by cloning *MSMEG*_1886 into the integrative shuttle vector pMV306 (Stover *et al.*, 1991). To this end, *MSMEG*_1886 and 500 bp upstream were PCR amplified using primers C1886 Fw and C1886 Rev (which contained restriction sites for EcoRV and HindIII enzymes) using the following conditions: denaturation (94°C, 5 min), followed by 30 cycles of denaturation (94°C, 1 min), annealing (62°C, 40 sec) and extension (68°C, 90 sec); the amplification product of the expected size was gel purified and cloned into Zero Blunt® TOPO® PCR Cloning Kit. The DNA sequence of the insert was confirmed by sequencing, and subcloned into pMV306 by EcoRV-HindIII digestion. The newly constructed clones were transformed by electroporation into *E. coli* DH5α, plasmids extracted and subsequently electroporated into *M. smegmatis* Δ*MSMEG*_1886.

The deletion of *MSMEG*_1741 and *MSMEG*_1741-1743 were made using pPR27, a temperature sensitive suicide plasmid (Pelicic *et al*, 1997). Upstream and downstream fragments from both MSMEG_1741 and MSMEG_1741-1743 were cloned in pPR27 plasmid with a streptomycin cassette in between obtaining two plasmids pPR27UD41 and pPR27UD41-3, respectively (Fig. S2). These constructs were then introduced by electroporation into *M. smegmatis* mc^{2}155. Transformants were selected in Middlebrook 7H9 OADS-Gly supplemented with streptomycin (20µg ml^{-1}) at 30°C. After full growth, colonies receiving the constructed plasmid were detected by replica plating and spraying a solution of catechol (1 % w/v, Sigma). Yellow colonies were picked and patched into Middlebrook 7H9 OADS-Gly agar plates containing sucrose

(10% w/v) and streptomycin (20µg ml^{-1}), followed by incubation at 42ºC. Only insertional mutants that have lost the delivery plasmid grew in these conditions and remained white when sprayed with catechol solution. The deletions of the MSMEG_1741 and MSMEG_1741-1743 genes were confirmed by PCR using specific primer combinations. When oligonucleotides Fw1 and Rv1 (Table) were used, the absence of amplification confirmed the deletion of the target genes. On the other hand, amplification with Fw2-Rv2 oligonucleotides (for the MSMEG_1741 deletion) and Fw3-Rv2 oligonucleotides (for the MSMEG_1741-1743 deletion) demonstrated the insertion of the streptomycin resistance cassette in the deleted region. The PCR products were evaluated by standard agarose gel electrophoresis (Fig S2).

The complementing desT clone was constructed as follows: The MSMEG_1741-43 operon was amplified using two oligonucleotides 1743 Rv HindIII and 1741 Fw SpeI, generating a 3kb fragment that was cloned into the pGEM T Easy vector (Promega). Sequencing of several clones obtained confirmed the fidelity of amplification. One of the confirmed clones was named pGEM::1741-43 and used as source for the subcloning of the operon. To that end, pGEM::1741-43 was digested with SpeI and HindIII, the 3 kbp fragment purified by agarose gel electrophoresis and cloned into the integrative vector pML2300 (Huff *et al.,* 2010) yielding pML2300:: 1741-43. Digestion of the pGEM 1741-43 construct with SacI and HindIII restriction enzymes released a 1kb fragment containing MSMEG_1741 plus an additional 400bp upstream of its translation start site, the fragment was purified by agarose gel electrophoresis and cloned into pML2300 yielding pML2300::1741.

Phage transduction: Bxz1, a generalized transducing phage (the kind gift of WR Jacobs, Jr., AECOM, NYC) was used to construct double mutants by transduction of selected deletion into the chosen genetic background. *M. smegmatis* strains mc^2155 Δ1741 and

616 Δ1741-43 were infected with Bxz1 and a high titer transducing lysate was obtained as
617 described (Lee *et al.*, 2004). Transduction was carried on by infecting the recipient
618 strain *M. smegmatis* mc^{2}155 MSMEG_Δ1886. Transductants were selected by plating
619 the transduction mix into Middlebrook 7H9 OADS-Gly agar plates containing
620 streptomycin (20μg ml^{-1}) and hygromycin (50μg ml^{-1}) and grown at 42ºC. Colonies in
621 each transduction experiment were checked by PCR (Fig. S2); the phenotype was
622 confirmed by replica plating each of the double mutant strains (*M. smegmatis* mc^{2}155
623 Δ1886-Δ1741 and *M. smegmatis* mc^{2}155 Δ1886-Δ1741-43) on Middlebrook 7H9-Gly
624 agar streptomycin (20μg ml^{-1}) and hygromycin (50μg ml^{-1}) plates with our without
625 addition of OADS.
626
627 *Over-expression of genes MSMEG_1211, MSMEG_1743 and MSMEG_6835.*
628 Coding sequences of *MSMEG*_1211, *MSMEG*_1743 and *MSMEG*_6835 were PCR
629 amplified using primers 1211Fw, 1211Rev; 1743Fw, 1743Rev; 6835Fw and 6835Rev
630 (Table S3). PCR conditions were as follows: denaturation (94°C, 5 min), followed by
631 30 cycles of denaturation (94°C, 1 min), annealing (60°C, 40 sec) and extension (68°C,
632 1.5 min). The PCR products were purified after agarose gel electrophoresis using a gel
633 extraction kit (Promega, Madison, Wis) following the manufacturer´s instructions and
634 then were cloned on Zero Blunt® TOPO® (InVitrogen). The putative desaturase
635 encoding fragments were obtained by restriction with NdeI- EcoRI (for *MSMEG*_1211)
636 and NdeI- HindIII (for *MSMEG*_1743 and *MSMEG*_6835) and then cloned under the
637 control of the acetamidase promoter P*ami* present in the mycobacterial expression
638 vector pLAM12 (Triccas *et al.*, 1998). The plasmids obtained (designated
639 pLAM12::1211, pLAM12::1743 and pLAM12::6835) were introduced by
640 electroporation into both the wild type strain *M. smegmatis* mc^{2}155 and its derived

mutant Δ*MSMEG*_1886 and selected on Middlebrook 7H9-ADS-Kanamycin agar plates. The analysis of the putative desaturase overexpression was carried on in 7H9-Succinate-Km in absence or presence of acetamide (0.2% w/v) at 25°C and 37°C and their optical density was followed every 6 and 3 h at 25°C and 37°C, respectively.

Phenotypic characterization of the M. smegmatis ΔMSMEG_1886, ΔMSMEG_1741, ΔMSMEG_1741-1743, ΔMSMEG_1886-ΔMSMEG_1741 and ΔMSMEG_1886-ΔMSMEG_1741-1743 mutant strains.

Colony morphology was determined by plating dilutions of freshly grown stationary phase cultures of wild-type and mutant strains on 7H9-Gly-ADS/OADS agar plates. After 5 days at 37 ºC, plates were visually inspected followed by optical microscopy examination at low magnification (2x) using a Zeiss-Stemi 2000 binocular scope. Growth characteristics in liquid medium were analyzed as follows: saturated cultures in Middlebrock 7H9-Gly-OADS-Tr were diluted into the fresh medium to an $O.D._{600nm}$ = 0.1- 0.2 and grown at chosen temperatures (25°C or 37ºC, with the addition of ADS, OADS or PADS when necessary). Growth was followed by reading the OD at 600nm at different time points.

Analysis of cellular fatty acids.

M. smegmatis parental and mutant strains were grown at 25°C or 37°C in Middlebrook 7H9-Gly-ADS-Tr broth until an $O.D._{600nm}$= 1- 1.3 was reached. Cells were harvested by centrifugation at 5,000 x g, washed twice with distilled water and kept frozen until extraction. Exceptionally, the double mutant strain *ΔMSMEG_1886-ΔMSMEG_1741-1743* was grown in 7H9-Gly-ADS-Tr broth supplemented with 1% OADS or 1% PADS to alleviate its UFA auxotrophy.

"In vivo" labeling with [1-^{14}C] acetic acid (0.5 μCi ml^{-1}) or [1-^{14}C] stearic acid (0.4 μCi ml^{-1}) were performed by growing cultures in 7H9-Gly-OADS-Tr broth until O.D.$_{600nm}$= 0.5-0.6. At this point cultures were gently centrifuged and resuspended in the same volume of 7H9-Gly-ADS-Tr. After 3hs [1-^{14}C] acetic acid or [1-^{14}C] stearic acid were added and incubated for 3 hours. Cells were harvested by centrifugation and kept frozen at –80°C until used.

The extraction and analysis of fatty acids was performed as previously described (Vilcheze *et al.*, 2000). In brief, cells were subjected to alkaline hydrolysis in 15 % (w/v) tetrabutylammonium hydroxide (TBAH, Fluka) at 100ºC overnight, followed by the addition of 2 ml of CH_2Cl_2 and 100 μl of CH_3I. The entire reaction mixture was then mixed on a rotator for 1 h and centrifuged, and the upper aqueous phase was discarded. The lower organic phase was then washed with water, and dried at 55ºC under a nitrogen stream. The resulting pellet was extracted with ethyl ether and dried again. The fatty acid methyl esters (FAMEs) obtained were analyzed by GC-MS on an Agilent 7890B Gas Chromatograph equipped with a capillary column (30 m long x 0.25 mm internal diameter x 0.25 mm thick stationary phase) HP5 MS coupled to Mass spectrophotometer 5977A (Agilent Technologies). The column temperature was programmed to increase from 5°C min^{-1} from 160 to 320ºC. The results were analyzed using GCMS Analysis Postrun software (AgilentChemStation).

The position of the double bonds in the UFAs was determined by GC-MS after conversion of extracted FAMEs into dimethyl disulfide (DMDS) adducts as described by Yamamoto *et al.* (Yamamoto, 1991). This was achieved by addition of 4 mg of iodine flakes and 0.3 mL of DMDS to the extracted and dried FAMEs, followed by incubation for 30 min at 35°C and addition of 1 ml of hexane: ether (1:1 v/v) and 1 ml of sodium thiosulfate, 10% (w/v). The mixtures were centrifuged 5 min at 12,000 x g.

The supernatants were dried under nitrogen and then suspended in 0.5 ml of hexane for analysis.

FAMEs extracted from labeled cultures were separated by thin layer chromatography (TLC) in silica gel 60 F_{254} plates (Merck) soaked with $AgNO_3$ 10% (w/v) (Ag-TLC) and developed 3 times using petroleum ether and diethyl ether (85:15 v/v) as mobile phase. Radioactivity was revealed by autoradiography in X-OMAT film (Kodak).

Bioinformatic analysis.

Promoter prediction was carried out by the program BPROM (Prediction of bacterial promoters) (Solovyev, 2011). Palindromic sequences were analyzed using the program *einverted* (http://emboss.bioinformatics.nl/). Multiple alignments were carried out with ClustalX2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) and MEME (http://meme-suite.org/tools/meme). The phylogenetic tree was made using the program ClustalX2 (www.clustal.org) and draw using TreeView program.

Acknowledgements. M.D., J.M.B. and C.DC were fellows of CONICET (Consejo Nacional de Investigaciones Científicas y Tecnológicas), Argentina. H.R.M. is Principal Investigator of CIUNR (Consejo de Investigaciones de la Universidad Nacional de Rosario), Argentina. This work was funded by grants from ANPCyT (Agencia Nacional de Promoción Científica y Tecnológica), Argentina to H.R.M. Colony morphology observations were performed at the Microscope Facility, IBR-CONICET (Rosario, Argentina) under the advice of Dr. E. Morales. The excellent technical assistance of Ms. M. Hourcade (GC facility, Facultad de Ciencias Bioquímicas y Farmacéuticas, UNR, Rosario, Argentina) is greatly appreciated. The authors state that there is no conflict of interest regarding this manuscript.

716

717 **Authors contribution:** HRM conceived and design the study and performed the
718 analysis, MD, CBDC, JMB carried on the experimental work, data adquisition and its
719 analysis. All the authors participated in the writing of the manuscript.

720

REFERENCES.

Aguilar, P.S., J.E. Cronan, Jr. & D. de Mendoza, (1998) A *Bacillus subtilis* gene induced by cold shock encodes a membrane phospholipid desaturase. *Journal of bacteriology* **180**: 2194-2200.

Alvarez, H.M. & A. Steinbuchel, (2002) Triacylglycerols in prokaryotic microorganisms. *Applied microbiology and biotechnology* **60**: 367-376.

Barry, C.E., 3rd, R.E. Lee, K. Mdluli, A.E. Sampson, B.G. Schroeder, R.A. Slayden & Y. Yuan, (1998) Mycolic acids: structure, biosynthesis and physiological functions. *Progress in lipid research* **37**: 143-179.

Benamara, H., C. Rihouey, I. Abbes, M.A. Ben Mlouka, J. Hardouin, T. Jouenne & S. Alexandre, (2014) Characterization of membrane lipidome changes in *Pseudomonas aeruginosa* during biofilm growth on glass wool. *PloS one* **9**: e108478.

Bloch, K., (1975) Fatty acid synthases from *Mycobacterium phlei*. *Methods in enzymology* **35**: 84-90.

Bloch, K., (1977) Control mechanisms for fatty acid synthesis in *Mycobacterium smegmatis*. *Advances in enzymology and related areas of molecular biology* **45**: 1-84.

Blondelet-Rouault, M.H., J. Weiser, A. Lebrihi, P. Branny & J.L. Pernodet, (1997) Antibiotic resistance gene cassettes derived from the omega interposon for use in *E. coli* and *Streptomyces*. *Gene* **190**: 315-317.

Cabruja, M., S. Mondino, Y.T. Tsai, J. Lara, H. Gramajo & G. Gago, (2017) A conditional mutant of the fatty acid synthase unveils unexpected cross talks in mycobacterial lipid metabolism. *Open biology* **7**.

Cole, S.T., R. Brosch, J. Parkhill, T. Garnier, C. Churcher, D. Harris, S.V. Gordon, K. Eiglmeier, S. Gas, C.E. Barry, 3rd, F. Tekaia, K. Badcock, D. Basham, D. Brown, T. Chillingworth, R. Connor, R. Davies, K. Devlin, T. Feltwell, S. Gentles, N. Hamlin, S. Holroyd, T. Hornsby, K. Jagels, A. Krogh, J. McLean, S. Moule, L. Murphy, K. Oliver, J. Osborne, M.A. Quail, M.A. Rajandream, J. Rogers, S. Rutter, K. Seeger, J. Skelton, R. Squares, S. Squares, J.E. Sulston, K. Taylor, S. Whitehead & B.G. Barrell, (1998) Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. *Nature* **393**: 537-544.

Chang, Y. & B.G. Fox, (2006) Identification of Rv3230c as the NADPH oxidoreductase of a two-protein DesA3 acyl-CoA desaturase in *Mycobacterium tuberculosis* H37Rv. *Biochemistry* **45**: 13476-13486.

Daniel, J., C. Deb, V.S. Dubey, T.D. Sirakova, B. Abomoelak, H.R. Morbidoni & P.E. Kolattukudy, (2004) Induction of a novel class of diacylglycerol acyltransferases and triacylglycerol accumulation in *Mycobacterium tuberculosis* as it goes into a dormancy-like state in culture. *Journal of bacteriology* **186**: 5017-5030.

de Mendoza, D., J.L. Garwin & J.E. Cronan, Jr., (1982) Overproduction of cis-vaccenic acid and altered temperature control of fatty acid synthesis in a mutant of *Escherichia coli*. *Journal of bacteriology* **151**: 1608-1611.

Fulco, A.J. & K. Bloch, (1964) Cofactor Requirements for the Formation of Delta-9-Unsaturated Fatty Acids in *Mycobacterium Phlei*. *The Journal of biological chemistry* **239**: 993-997.

Garnier, T., K. Eiglmeier, J.C. Camus, N. Medina, H. Mansoor, M. Pryor, S. Duthoy, S. Grondin, C. Lacroix, C. Monsempe, S. Simon, B. Harris, R. Atkin, J. Doggett, R. Mayes, L. Keating, P.R. Wheeler, J. Parkhill, B.G. Barrell, S.T. Cole, S.V.

Gordon & R.G. Hewinson, (2003) The complete genome sequence of *Mycobacterium bovis*. *Proceedings of the National Academy of Sciences of the United States of America* **100**: 7877-7882.

Hasan, N.A., R.M. Davidson, V.C. de Moura, B.J. Garcia, P.R. Reynolds, L.E. Epperson, E. Farias-Hesson, M.A. DeGroote, M. Jackson & M. Strong, (2015) Draft Genome Sequence of *Mycobacterium chelonae* Type Strain ATCC 35752. *Genome announcements* **3**.

Heydari, H., W.Y. Wee, N. Lokanathan, R. Hari, A. Mohamed Yusoff, C.Y. Beh, A.H. Yazdi, G.J. Wong, Y.F. Ngeow & S.W. Choo, (2013) MabsBase: a Mycobacterium abscessus genome and annotation database. *PloS one* **8**: e62443.

Huff, J., A. Czyz, R. Landick & M. Niederweis, (2010) Taking phage integration to the next level as a genetic tool for mycobacteria. *Gene* **468**: 8-19.

Kikuchi, S. & T. Kusaka, (1982) New malonyl-CoA-dependent fatty acid elongation system in Mycobacterium smegmatis. *Journal of biochemistry* **92**: 839-844.

Kikuchi, S. & T. Kusaka, (1986) Isolation and partial characterization of a very long-chain fatty acid desaturation system from the cytosol of Mycobacterium smegmatis. *Journal of biochemistry* **99**: 723-731.

Larsen, M.H., Bardarov, (2000) *Molecular Genetics of Mycobacteria*. ASM Press.

Lee, S., J. Kriakov, C. Vilcheze, Z. Dai, G.F. Hatfull & W.R. Jacobs, Jr., (2004) Bxz1, a new generalized transducing phage for mycobacteria. *FEMS microbiology letters* **241**: 271-276.

Lennarz, W.J., G. Scheuerbrandt & K. Bloch, (1962) The biosynthesis of oleic and 10-methylstearic acids in Mycobacterium phlei. *The Journal of biological chemistry* **237**: 664-671.

Los, D., I. Horvath, L. Vigh & N. Murata, (1993) The temperature-dependent expression of the desaturase gene desA in Synechocystis PCC6803. *FEBS letters* **318**: 57-60.

Los, D.A. & N. Murata, (1998) Structure and expression of fatty acid desaturases. *Biochimica et biophysica acta* **1394**: 3-15.

Pelicic, V., J.M. Reyrat & B. Gicquel, (1996) Generation of unmarked directed mutations in mycobacteria, using sucrose counter-selectable suicide vectors. *Molecular microbiology* **20**: 919-925.

Phetsuksiri, B., M. Jackson, H. Scherman, M. McNeil, G.S. Besra, A.R. Baulard, R.A. Slayden, A.E. DeBarber, C.E. Barry, 3rd, M.S. Baird, D.C. Crick & P.J. Brennan, (2003) Unique mechanism of action of the thiourea drug isoxyl on Mycobacterium tuberculosis. *The Journal of biological chemistry* **278**: 53123-53130.

Sassetti, C.M. & E.J. Rubin, (2003) Genetic requirements for mycobacterial survival during infection. *Proceedings of the National Academy of Sciences of the United States of America* **100**: 12989-12994.

Sekizuka, T., M. Kai, K. Nakanaga, N. Nakata, Y. Kazumi, S. Maeda, M. Makino, Y. Hoshino & M. Kuroda, (2014) Complete genome sequence and comparative genomic analysis of Mycobacterium massiliense JCM 15300 in the Mycobacterium abscessus group reveal a conserved genomic island MmGI-1 related to putative lipid metabolism. *PloS one* **9**: e114848.

Shanklin, J., J.E. Guy, G. Mishra & Y. Lindqvist, (2009) Desaturases: emerging models for understanding functional diversification of diiron-containing enzymes. *The Journal of biological chemistry* **284**: 18559-18563.

Singh, A., C. Varela, K. Bhatt, N. Veerapen, O.Y. Lee, H.H. Wu, G.S. Besra, D.E. Minnikin, N. Fujiwara, K. Teramoto & A. Bhatt, (2016) Identification of a

Desaturase Involved in Mycolic Acid Biosynthesis in Mycobacterium smegmatis. *PloS one* **11**: e0164253.

Snapper, S.B., R.E. Melton, S. Mustafa, T. Kieser & W.R. Jacobs, Jr., (1990) Isolation and characterization of efficient plasmid transformation mutants of Mycobacterium smegmatis. *Molecular microbiology* **4**: 1911-1919.

Solovyev, V.S., A, (2011) Automatic Annotation of Microbial Genomes and Metagenomic Sequences. In: Metagenomics and its Applications in Agriculture, Biomedicine and Environmental Studies. R.W. Li (ed). Nova Science Publishers, pp. 61-78.

Stinear, T.P., T. Seemann, P.F. Harrison, G.A. Jenkin, J.K. Davies, P.D. Johnson, Z. Abdellah, C. Arrowsmith, T. Chillingworth, C. Churcher, K. Clarke, A. Cronin, P. Davis, I. Goodhead, N. Holroyd, K. Jagels, A. Lord, S. Moule, K. Mungall, H. Norbertczak, M.A. Quail, E. Rabbinowitsch, D. Walker, B. White, S. Whitehead, P.L. Small, R. Brosch, L. Ramakrishnan, M.A. Fischbach, J. Parkhill & S.T. Cole, (2008) Insights from the complete genome sequence of Mycobacterium marinum on the evolution of Mycobacterium tuberculosis. *Genome research* **18**: 729-741.

Stover, C.K., V.F. de la Cruz, T.R. Fuerst, J.E. Burlein, L.A. Benson, L.T. Bennett, G.P. Bansal, J.F. Young, M.H. Lee, G.F. Hatfull & et al., (1991) New use of BCG for recombinant vaccines. *Nature* **351**: 456-460.

Takayama, K., C. Wang & G.S. Besra, (2005) Pathway to synthesis and processing of mycolic acids in Mycobacterium tuberculosis. *Clinical microbiology reviews* **18**: 81-101.

Torrelles, J.B., P.A. Sieling, N. Zhang, M.A. Keen, M.R. McNeil, J.T. Belisle, R.L. Modlin, P.J. Brennan & D. Chatterjee, (2012) Isolation of a distinct Mycobacterium tuberculosis mannose-capped lipoarabinomannan isoform responsible for recognition by CD1b-restricted T cells. *Glycobiology* **22**: 1118-1127.

Triccas, J.A., T. Parish, W.J. Britton & B. Gicquel, (1998) An inducible expression system permitting the efficient purification of a recombinant antigen from Mycobacterium smegmatis. *FEMS microbiology letters* **167**: 151-156.

van Kessel, J.C. & G.F. Hatfull, (2007) Recombineering in Mycobacterium tuberculosis. *Nature methods* **4**: 147-152.

Vilcheze, C., H.R. Morbidoni, T.R. Weisbrod, H. Iwamoto, M. Kuo, J.C. Sacchettini & W.R. Jacobs, Jr., (2000) Inactivation of the inhA-encoded fatty acid synthase II (FASII) enoyl-acyl carrier protein reductase induces accumulation of the FASI end products and cell lysis of Mycobacterium smegmatis. *Journal of bacteriology* **182**: 4059-4067.

Yamamoto, K., Shibahara, A., Nakayama, T., and Kajimoto, G., (1991) Determination of Double-Bond Positions in Methylene-Interrupted Dienoic Fatty Acids by GC–MS as Their Dimethyl Disulfide Adducts. *Chem. Phys. Lipids* **60**: 39-50.

Zakham, F., L. Belayachi, D. Ussery, M. Akrim, A. Benjouad, R. El Aouad & M.M. Ennaji, (2011) Mycobacterial species as case-study of comparative genome analysis. *Cell Mol Biol (Noisy-le-grand)* **57 Suppl**: OL1462-1469.

Zhang, Y.M., K. Zhu, M.W. Frank & C.O. Rock, (2007) A Pseudomonas aeruginosa transcription factor that senses fatty acid structure. *Molecular microbiology* **66**: 622-632.

Zhu, K., K.H. Choi, H.P. Schweizer, C.O. Rock & Y.M. Zhang, (2006) Two aerobic pathways for the formation of unsaturated fatty acids in Pseudomonas aeruginosa. *Molecular microbiology* **60**: 260-273.

Legends to Figures

Figure 1. Desaturases from *M. smegmatis*, *M. tuberculosis* and *P. aeruginosa.*

A. Schematic representation of desaturases and its cognate oxido-reductases genes from *M. tuberculosis* H37Rv, *M. smegmatis* mc^2155 and *P. aeruginosa* PAO1. The intergenic regions are shown in base pair (bp). The predicted promoter of the desaturases (P_1) and oxido-reductases (P_2) are represented by arrows. **D**: desaturase, **OR**: oxidoreductase, **HP**: Hipotetical Protein.

B. Sequence alignment of the His boxes of the four putative desaturases from *M. smegmatis* mc^2155 and DesA3 from *M. tuberculosis* was done using MEME (Multiple Em for Multiple Elicitation http://meme-suite.org/tools/meme).

Figure 2. Phylogenetic tree of fatty acid desaturases of the genus *Mycobacterium*. Multiple alignments of amino acid sequences was performed using the ClustalX2 software and the tree was constructed by the method of nearest neighbors NJ with 1000 bootstrap iterations with ClustalX2 and draw with TreeView program. Desaturases of *M. smegmatis* mc^2155 and DesA3 of *M. tuberculosis* are shown in boxes.

Figure 3. Growth of *M. smegmatis* strains mc^2155 and Δ*MSMEG*_1886 on solid and liquid medium.

A. *M. smegmatis* strains mc^2155 and Δ*MSMEG*_1886 were grown on Middlebrook 7H9-OADS-Gly-Tr broth till saturation. Cultures were diluted and plated on solid Middlebrook 7H9-Gly medium supplemented with either ADS or OADS to obtain isolated colonies. Plates were incubated at 37°C for 5 days and colonies were observed and photographed with a Zeiss-Stemi 2000 lens at 2x increase.

B. Growth curves of *M. smegmatis* strains grown at 37°C in the absence of exogenous UFAs.

C. Growth curves of *M. smegmatis* strains grown at 37°C in the presence of exogenous oleic acid (OADS).

D. Growth curves of *M. smegmatis* strains grown at 37°C in the presence of exogenous palmitoleic acid (PADS).

M. smegmatis mc^2155 (●),Δ*MSMEG*_1886 (△)

and complemented Δ*MSMEG*_1886C (◆) . Aliquots of each culture were taken at the indicated times and the $O.D._{600nm}$ was measured. An average of three independent replicates is shown.

Figure 4. Fatty acid composition of *M. smegmatis* mc^2155 and Δ*MSMEG*_ 1886. Cultures were grown at 37°C in Middlebrook 7H9-Gly-ADS-Tr broth until $O.D._{600}$=1. FAMEs were extracted and analyzed using GC-MS. An average value of three independent repetitions with their standard deviation is shown.

Figure 5. Growth of *M. smegmatis* strains mc^2155, Δ*MSMEG*_1741-1743 and Δ*MSMEG*_1886-Δ*MSMEG*_1741-1743 on solid and liquid medium.

A. All three strains under study were grown on Middlebrook 7H9-Gly-OADS-Tr. Saturated cultures were diluted and plated on the same medium supplemented with ADS (left) or OADS (right) to obtain isolated colonies. Plates were incubated at 37°C for 5 days and colonies were observed and photographed with a Zeiss-Stemi 2000 lens at 2x increase.

B. Growth curves of *M. smegmatis* strains growing at 37°C in the presence or absence of exogenous UFAs. *M. smegmatis* mc^2155 (●:ADS; ○: OADS; ▼: PADS),

Δ*MSMEG*_1741-1743 (△: ADS; ■: OADS □: PADS) and Δ*MSMEG*_1886-Δ*MSMEG*_1741-1743 (◆: ADS; ◇: OADS; ▲: PADS). Aliquots of each culture were taken at the indicated times and the $O.D._{600nm}$ was measured. An average of three independent replicates is shown.

C. Saturated cultures of *M. smegmatis* mc^2155, Δ*MSMEG*_1886, Δ*MSMEG*_1741-1743 and Δ*MSMEG*_1886-Δ*MSMEG*_1741-1743 were loaded in Middlebrook 7H9-Gly-agar media supplemented with ADS (left), OADS (center) or PADS (right).

Figure 6. Fatty acid composition of *M. smegmatis* mc^2155, Δ*MSMEG*_ 1886, Δ*MSMEG*_1741-1743 and Δ*MSMEG*_1886-Δ*MSMEG*_1741-1743 strains. Cultures were grown in Middlebrook 7H9-Gly-ADS-Tr broth at 37°C until $O.D._{600}$=1. Exceptionally, the double mutant strain Δ*MSMEG*_1886-Δ*MSMEG*_1741-1743 was grown in the same medium but supplemented with 10% of the usual OADS used in Middlebrook 7H9-Gly-OADS-Tr. FAMEs were extracted and analyzed using GC-MS. An average value of three independent repetitions with their standard deviation is shown.

Figure 7. "In vivo" labeling of *M. smegmatis* mc^2155, Δ*MSMEG*_ 1886, Δ*MSMEG*_1741-1743 and Δ*MSMEG*_1886-Δ*MSMEG*_1741-1743 strains. Cultures were grown in Middlebrook 7H9-Gly-OADS-Tr broth at 37°C until $O.D._{600}$=0.5-0.6. After centrifugation cells were resuspended in 7H9-Gly-ADS-Tr and incubated for 3 hours. Then 1[^{14}C] acetic acid or 1[^{14}C] stearic acid were added and after 3 hours of labeling cultures were centrifuged. Fatty acids were extracted and separated in $AgNO_3$ embebed TLC. *M. smegmatis* mc^2155 (lane 1); Δ*MSMEG*_ 1886 (lane 2);

949 Δ*MSMEG*_1741-1743 (lane 3); and Δ*MSMEG*_1886-Δ*MSMEG*_1741-1743 (lane 4).
950 SFA: Saturated fatty acids; UFA: Unsaturated fatty acids.
951
952 **Figure 8.** Growth of *M. smegmatis* strains mc^{2}155, Δ*MSMEG*_1886, Δ*MSMEG*_1741
953 and Δ*MSMEG*_1886-Δ*MSMEG*_1741 on solid and liquid medium.
954 **A**. The *M. smegmatis* mc^{2}155 wild type and mutant strains Δ*MSMEG*_1886 and
955 Δ*MSMEG*_1886-Δ*MSMEG*_1741 were grown on Middlebrook 7H9-Gly-OADS-Tr
956 medium. Saturated cultures were diluted and plated on the same medium supplemented
957 with ADS (left) or OADS (right) to obtain isolated colonies. Plates were incubated at
958 37°C for 5 days and colonies were observed and photographed with a Zeiss-Stemi 2000
959 lens at 2x increase.
960 **B.** Growth curves of *M. smegmatis* strains at 37°C in the absence of exogenous UFAs.
961 *M. smegmatis* mc^{2}155 (●), Δ*MSMEG*_1886 (○), Δ*MSMEG*_1741 (▼),
962 Δ*MSMEG*_1886-1741 (△), ΔMSMEG_1886-1741C (■). Aliquots of each culture were
963 taken at the indicated times and the O.D.$_{600nm}$ was measured. An average of three
964 independent replicates is shown. C denotes complementation of the strain by a plasmid
965 carrying MSMEG_1741.
966 **C.** Growth curves of *M. smegmatis* strains at 37°C in the presence oleic acid. *M.*
967 *smegmatis* mc^{2}155 (●), Δ*MSMEG*_1886 (○), Δ*MSMEG*_1741 (▼), Δ*MSMEG*_1886-
968 1741 (△), ΔMSMEG_1886-1741C (■). Aliquots of each culture were taken at the
969 indicated times and the O.D.$_{600nm}$ was measured. An average of three independent
970 replicates is shown. C denotes complementation of the strain by a plasmid carrying
971 MSMEG_1741.
972
973

Figure 9. Fatty acids analysis of *M. smegmatis* Δ*MSMEG*_1741 and Δ*MSMEG*_1886-Δ*MSMEG*_1741.

A. GC-MS fatty acids analysis. Cultures were grown at 37°C in Middlebrook 7H9-Gly-ADS-Tr broth until $O.D._{600nm}$=1. FAMEs were extracted and analyzed using GC-MS. An average value of three independent repetitions with their standard deviation is shown.

B. "In vivo" labeling of the studied strains with 1[^{14}C] acetic acid. Cultures were grown in Middlebrook 7H9-Gly-ADS-Tr broth at 37°C until $O.D._{600}$=0.5-0.6 and incubated with the radioactive compound for 3 hours. Fatty acids were then extracted and separated in argentation TLC; *M. smegmatis* Δ*MSMEG*_1741 (lane 1) and Δ*MSMEG*_1886-Δ*MSMEG*_1741 (lane 2).