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Diterpene production in *Mycobacterium tuberculosis*

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Abstract

Diterpenes are a structurally diverse class of molecules common in plants, although they are very rarely found in bacteria. We report the identification in *Mycobacterium tuberculosis* (*Mtb*) of three diterpenes proposed to promote phagolysosome maturation arrest. MS analysis reveals that these diterpenes are novel compounds not previously identified in other organisms. The diterpene with highest abundance in *Mtb* has a mass fragmentation pattern identical to edaxadiene, which is produced *in vitro* from geranylgeranyl diphosphate by the enzymes Rv3377c and Rv3378c [Mann FM *et al.* (2009) *J Am Chem Soc* **131**, 17526–17527]. A second diterpene found in *Mtb* has a similar mass spectrum, and is always observed in the same proportion relative to edaxadiene, indicating that it is a side product of the Rv3378c reaction *in vivo*. We name this second diterpene olefin edaxadiene B. The least abundant of the three diterpenes in *Mtb* extracts is tuberculosinol, a dephosphorylated side-product of the edaxadiene pathway intermediate produced by Rv3377c [Nakano C *et al.* (2009) *ChemBiochem* **10**, 2060–2071; Nakano C *et al.* (2005) *Chem Commun (Camb)* **8**, 1016–1018]. A frameshift in Rv3377c in *Mtb* completely eliminates diterpene production, whereas expression of Rv3377c and Rv3378c in the nonpathogenic *M. smegmatis* is sufficient to produce edaxadiene and edaxadiene B. These studies define the pathway of edaxadiene and edaxadiene B biosynthesis *in vivo*. Rv3377c and Rv3378c are unique to *Mtb* and *M. bovis*, making them candidates for selective therapeutics and diagnostics.

Keywords

cyclase; edaxadiene; Rv3377c; Rv3378c; tuberculosinol

Introduction

With more than 40 000 members, terpenes (or isoprenoids) are among the most varied class of natural products [1]. These compounds occur throughout nature, ranging from essential metabolites such as sterols and hormones to unique secondary metabolites that serve in defense and communication [2]. In bacteria, several *Streptomyces* species have been shown to encode diterpene synthases [3–5], although very few diterpenes have been directly isolated from these organisms, and their functions are poorly understood [3,5,6]. Several recent studies have shown that *Mycobacterium tuberculosis* (*Mtb*) also contains genes

encoding diterpene synthases [7–9]. The demonstration that the *Mtb* enzymes Rv3377c and Rv3378c produce the unique diterpene edaxadiene from geranylgeranyl diphosphate (GGPP) *in vitro* showed that these enzymes are functional class II and I diterpene synthases, respectively [7]. Notably, Rv3377c and Rv3378c were identified in a transposon mutagenesis screen to identify *Mtb* genes involved in phagolysosome fusion arrest [10], suggesting a potential function for mycobacterial diterpenes.

Suggestive of a functionally integrated pathway, Rv3377c and Rv3378c are proximal to each other and several other lipid-metabolizing enzymes within the *Mtb* genome, including *dxs2* (Rv3379c), encoding a synthase for isoprenoid precursors, and *idsB* (Rv3383c), a predicted GGPP synthase. Rv3377c and Rv3378c, along with the hypothetical protein Rv3376, are co-inherited exclusively in *Mtb* and *M. bovis* and are not found in other *Mycobacterium* spp. These genes have a lower GC content than the rest of the *Mtb* genome, suggesting that they were acquired recently, apparently just prior to the divergence of *Mycobacterium bovis* and *Mtb* from their common ancestor.

Diterpene synthases catalyze the cyclization of GGPP, the universal 20-carbon diterpene precursor, into cyclic products [11]. Diterpene synthases can be mechanistically divided into two groups, both of which use the formation of a highly-reactive carbocation to initiate cyclization [12]. Class I enzymes contain a characteristic DDxxD motif and catalyze diphosphate ionization-initiated reactions, whereas class II enzymes utilize a DxDD motif to catalyze protonation-initiated cyclization reactions [11,13]. Enzymes in both class I and II require a divalent ion for catalysis. Sequence homology to ent-copalyl diphosphate synthases, the occurrence of a conserved active-site motif, as well as the pyrophosphate in the diterpene product indicate that Rv3377c encodes a class II diterpene synthase. Recent studies indicate that Rv3378c is a class I diterpene synthase that converts the Rv3377c intermediate into the diterpene edaxadiene *in vitro* [7].

It is not known whether *Mtb* synthesizes diterpenes *in vivo* and the structures of any such compounds have not been described. To determine whether *Mtb* produces diterpenes, we examined the functions of Rv3377c and Rv3378c in diterpene synthesis in culture. In the present study, we show that *Mtb* produces three diterpenes from GGPP, all of which localize to cell membranes. Expression of Rv3377c and Rv3378c in the nonpathogenic species *Mycobacterium smegmatis* is sufficient to produce significant quantities of edaxadiene, suggesting that these genes constitute a committed biosynthetic pathway. Consistent with the idea that Rv3377c catalyzes the first step in the pathway, a frameshift mutation in Rv3377c in *Mtb* H37Rv abolishes all diterpene synthesis, and the introduction of Rv3377c on a plasmid complements this phenotype. Rv3378c catalyzes the second step in the pathway, yielding two products *in vivo*. The results obtained demonstrate that the products of two genes required to arrest phagolysosome fusion form a complete pathway that synthesizes novel diterpenes in *Mtb*.

Results

In vivo production and localization of *Mtb* diterpenes

To detect diterpenes in *Mtb*, we grew *Mtb* H37Rv to mid-log phase and extracted lipids using chloroform:methanol for analysis by GC/MS. From a GC/MS total-ion chromatogram, we detected several dozen low molecular weight species (Fig. 1A). When the same extract was analyzed for lipid compounds containing ions characteristic of diterpenes that have been generated *in vitro* using putative *Mtb* diterpene synthases (ions 80, 189 and 191), we identified three unique species (Fig. 1B). Analyzing the mass spectral fragmentation patterns of the three suspected diterpenes, we found that the most abundant species is identical to edaxadiene (Fig. S1A) and the least abundant diterpene was indistinguishable from

tuberculosinol (Fig. S1B). Unexpectedly, we also found an additional analyte with a similar retention time to edaxadiene, and a distinct but related mass fragmentation pattern (Figs 1B and S1C). The presence of a putative molecular ion of 272 (m/z) and a fragment ion of 257 indicate that this compound is a diterpene olefin related to edaxadiene, which we name edaxadiene B. Edaxadiene and edaxadiene B eluted earlier (at 23.2 and 23.8 min, respectively) from the GC column than tuberculosinol (30.3 min) and were present in significantly higher abundance.

To determine the cellular localization of these diterpenes, we prepared cell wall, cell membrane and cytosol fractions from γ -irradiated H37Rv cells and culture filtrate from live *Mtb* cultures. Lipids were extracted in either hexane (for cell wall) or chloroform:methanol (for culture filtrate, cytosol and cell membrane) and analyzed using GC/MS. Edaxadiene, edaxadiene B and tuberculosinol were detected in the cell membrane fraction. No diterpenes were detectable in the cytosol; the culture filtrate and cell membrane contained only trace amounts of edaxadiene (Table 1).

Rv3377c is necessary for diterpene synthesis *in vivo*

To define the biosynthetic pathway for diterpene production in *Mtb*, we compared diterpene production in several common laboratory *Mtb* strains. Chloroform:methanol extracts from five different *Mtb* isolates all showed the presence of the three diterpenes (Table S1). Surprisingly, one stock of H37Rv (#2) produced neither tuberculosinol, edaxadiene, nor edaxadiene B (Fig. 2A and Table S1). H37Rv #1 and #2 were obtained as separate glycerol stocks from the same source (M. Schelle, University of California, Berkeley, CA, USA) as the other *Mtb* strains analyzed, and they were grown under the same conditions. H37Rv #2, however, was subcultured in liquid medium for approximately 9 months longer than H37Rv #1. To determine how diterpene production is disrupted in H37Rv #2, we isolated mRNA and used it as a template for RT-PCR. *Rv3377c* transcripts were detected in the mutant strain (data not shown), although sequencing of the cDNA revealed two single-base insertions in *Rv3377c* (Fig. 2B). These two insertions disrupt the reading frame just upstream of the sequence encoding the catalytic-site motif and presumably eliminate production of the active enzyme. Notably, complementing this mutant H37Rv strain with a plasmid containing *Rv3377c* under the control of an inducible promoter restored the production of edaxadiene and edaxadiene B (Fig. 2C). These results indicate that the reaction catalyzed by *Rv3377c* is the first committed step in diterpene production in *Mtb*, and that *Rv3377c* is required to produce all three *Mtb* diterpenes.

Reconstitution of the edaxadiene pathway in *M. smegmatis*

To determine the full biosynthetic pathway of these diterpenes, we tested whether *Rv3377c* and *Rv3378c* are sufficient to produce all three diterpenes in *M. smegmatis*, a nonpathogenic mycobacterium species. *M. smegmatis* lacks orthologs of *Rv3377c* and *Rv3378c*, and GC/MS analysis of lipids extracted from wild-type *M. smegmatis* showed no evidence of diterpenes (Fig. 3A). We transformed wild-type *M. smegmatis* mc²115 with a vector harboring an inducible promoter driving both *Rv3377c* and *Rv3378c*. When transformed with vector alone, or when expression was not induced, *M. smegmatis* did not produce detectable levels of diterpenes (data not shown). When both *Rv3377c* and *Rv3378c* were expressed in *M. smegmatis* mc²115, edaxadiene and edaxadiene B were produced, whereas tuberculosinol was below detection levels (Fig. 3B). The diterpenes produced by *Rv3377c* and *Rv3378c* in *M. smegmatis* and those found in *Mtb* have identical GC retention times and mass spectra. These results support a working model of diterpene biosynthesis in *Mtb* whereby *Rv3377c* and *Rv3378c* act in sequence to convert GGPP to edaxadiene and the previously unidentified product, edaxadiene B.

Discussion

Lipids, including phthiocerol dimycoerate and sulpholipid-1, are critical mediators of *Mtb* virulence. Consistent with this role, the genes responsible for diterpene synthesis, *Rv3377c* and *Rv3378c*, were identified in a genetic screen for factors involved in initial fusion arrest events [10]. In the present study, we demonstrate for the first time that *Mtb* grown in broth culture produces membrane-associated diterpenes implicated in blocking phagolysosome fusion (Fig. 1 and Table 1). Thus, the proteins necessary for diterpene production are functional under normal growth conditions, even when the bacteria are outside the host macrophage. Observing diterpenes under laboratory culture conditions implies that they are typically present in the *Mtb* membrane, which appropriately situates the diterpenes to interact with the host cell upon contact. Inhibition of phagolysosome fusion is a fast process, taking place within 4 min of the bacteria engaging the host cell [14]. This time frame is too short for *Mtb* to synthesize new protein or the lipid factors necessary for arrest. The factors involved in early fusion arrest must therefore be present and active before the bacteria enters the host cell, and the diterpenes detected in *Mtb* membranes are well positioned to assist in this process.

Surprisingly, we identify three lipid species with mass spectral fragmentation patterns characteristic of diterpenes (Fig. 1B). Previous *in vitro* biochemical studies suggested that only a single diterpene, edaxadiene, would be present in *Mtb* [8,9]. The most abundant diterpene that we could detect in both *Mtb* membranes and whole-cell extracts shows a mass and fragmentation pattern identical to edaxadiene. The abundance of edaxadiene in *Mtb* extracts suggests that it is the major pathway product and that it is unlikely that there are subsequent modifications *in vivo*.

However, *Mtb* also produces another diterpene with a distinct GC retention time but similar mass fragmentation pattern to edaxadiene (Figs S1B,C). This diterpene has the same putative molecular ion (272) as edaxadiene, as well as a 257 ion characteristic of diterpene olefins (M-CH₃), and is likely a minor product of *Rv3378c* not detected *in vitro*. We propose naming this side product edaxadiene B. Because of its low abundance and the difficulty in purifying sufficient quantities, the molecular structure of edaxadiene B remains unresolved. The production of more than one end product by a class I diterpene synthase is not uncommon in plants [13,15]. Several well-characterized plant enzymes, such as levopimaradiene/abietadiene synthase, produce multiple diterpenes [16]. In most cases, only one of the plant diterpenes is biologically active. It remains to be determined which form of edaxadiene is active *in vivo*.

The third diterpene that we detected in *Mtb* has a mass spectral fragmentation pattern identical to that of tuberculosinol, a dephosphorylated intermediate in the edaxadiene pathway. In the presence of *Rv3377c* alone *in vitro*, GGPP is converted into the intermediate halimadienyl diphosphate, which can be detected as tuberculosinol after dephosphorylation [8,9,17]. The presence of tuberculosinol in the *Mtb* membrane implies the existence of an endogenous phosphatase that can convert a small fraction of the halimadienyl diphosphate into tuberculosinol. This may act as a detoxifying strategy because high levels of prenyl phosphates are toxic to bacteria and are converted to less toxic alcohols by endogenous phosphatases [18]. The identity of the protein responsible for this dephosphorylation reaction remains unknown.

We investigated whether the three diterpenes identified are produced by common *Mtb* strains. All of the *Mtb* strains that we surveyed except one produce the three diterpenes with approximately the same abundance (Table S1). The exception is H37Rv #2, which does not produce any diterpenes (Fig. 2A and Table S1). This H37Rv strain, which was grown in

liquid culture for approximately 9 months longer than the other H37Rv strain analyzed, has two single-base insertions in *Rv3377c* that disrupt the reading frame upstream of the active site (Fig. 2B). Without active *Rv3377c*, none of the three diterpenes are produced. When a plasmid-encoded copy of *Rv3377c* is introduced into this diterpene mutant strain and protein expression is induced, edaxadiene and edaxadiene B are generated (Fig. 2C). These results confirm that *Rv3377c* performs the first committed step in the production of all three diterpenes in *Mtb*.

The related, nonpathogenic species *M. smegmatis* does not contain genes homologous to *Rv3377c* or *Rv3378c* and does not produce detectable levels of diterpenes *in vivo* as shown by GC/MS (Fig. 3A). However, when both *Rv3377c* and *Rv3378c* are expressed in *M. smegmatis* from a plasmid-based inducible promoter, edaxadiene and edaxadiene B are produced (Fig. 3B), indicating these are the only two proteins necessary for diterpene production in *Mycobacterium*. This two-step pathway from the substrate GGPP to final diterpene products, with no further modifications, is shorter than a typical diterpene pathway in plants or other microbes. An abbreviated pathway may reflect that *Rv3377c* and *Rv3378c*, as well as the adjacent gene *Rv3376*, were likely acquired recently through horizontal gene transfer. If recent acquisition is indeed the case and only these three genes were transferred, then it is likely that additional enzymes, such as P450 cytochromes, dehydrogenases or oxidoreductases that typically modify plant diterpenes, are not involved in diterpene synthesis in *Mtb*.

To our knowledge, *Mycobacterium* is only the second bacterial genus reported to produce diterpenes *in vivo*. A small number of other diterpenes, including terpentecin, have been isolated from several *Streptomyces* species [3–6]. The biological roles diterpenes play in bacteria are poorly understood because only the antibiotic function of terpentecin has been determined. The identification of edaxadiene and edaxadiene B as the endpoints of the biosynthetic pathway *in vivo* sets the stage for determining the biological functions and mechanism of action of the *Mtb* diterpenes. The diterpenes may create bacterial membrane microdomains that mediate signaling or, alternatively, they may transfer to host membranes to target host components. Discovering the biological target for the bioactive diterpene will further elucidate the pathways regulated by *Mtb* lipids during infection.

Experimental procedures

Amplification and cloning

A vector for expression of *Rv3377c* and *Rv3378c* from *M. tuberculosis* H37Rv (Colorado State University) was created using the Gateway System (Invitrogen, Carlsbad, CA, USA). A 2400 bp product was amplified by PCR containing primers 3378F (5'-CTGGTGCCACGCGTTCTCATATGAACTTGGTTAGCGAA-3') and 3377R (5'-GAAAGCTGGGTGAAGCTTTCATTGGTTACTCTCATC-3'), 5% dimethylsulfoxide and TurboCX polymerase (Stratagene, La Jolla, CA, USA). The PCR product was purified and used as a template for a second amplification using primers GatewayTHF and GatewayR. Recombinants of this second PCR product in the vector pDONR207 were obtained using BP clonase (Invitrogen) and were confirmed by DNA sequencing. *Rv3377c/Rv3378c* was moved from pDONR207 using LR clonase (Invitrogen) into destination vector pTETGW (a gift from C. Sassetti, University of Massachusetts, Worcester, MA, USA), a *Mycobacterium* expression vector containing Gateway cloning sites, a *Mycobacterium* origin of replication and a gene encoding hygromycin resistance. The LR reactions were transformed into *Escherichia coli* DH5a and confirmed by DNA sequencing.

***M. smegmatis* and *Mtb* strains and growth conditions**

Mtb strains were a kind gift from M. Schelle (University of California, Berkeley). *M. smegmatis* mc²115 and *Mtb* strains were grown in Middlebrook 7H9 broth (Difco, Franklin Lakes, NJ, USA) supplemented with 10% Middlebrook OADC Enrichment and 0.05% Tween 80. Strains harboring an Rv3377c expression plasmid, an Rv3377c/Rv3378c expression plasmid or vector only were grown in medium also containing 50µg·mL⁻¹ hygromycin B, and expression of Rv3377c or Rv3377c/Rv3378c proteins was induced with 50 ng·mL⁻¹ anhydrotetracycline (Atc; Sigma, St Louis, MO, USA). Strains were grown at 37 °C with shaking at 220 r.p.m.

Preparation of *Mtb* membranes and cytosol

γ-irradiated *Mtb* cells were obtained from the Colorado State University TB Vaccine Testing and Research Materials Contract. Irradiated *Mtb* H37Rv cells were resuspended in NaCl/Pi containing the protease inhibitors leupeptin (5µg·mL⁻¹) and 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (1 mM), sonicated on ice and centrifuged at 1500 g for 10 min. The supernatant was saved, and the cell pellet was resuspended, sonicated and centrifuged a second time. The two supernatants were combined and centrifuged at 26 000 g for 1.5 h to pellet the cell wall. The remaining supernatant was centrifuged at 100 000 g for 4 h to separate the cell membranes from the cytosol. The membrane fraction was resuspended in NaCl/Pi containing 1% *n*-octyl-β-D-glucopyranoside and 1 mM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride overnight at 4°C. Lipids were extracted from the cell membranes and cytosol in 5 mL of 1:1 chloroform:methanol by vortexing for 10 min at maximum speed and centrifugation at 3200 g for 10 min after partition with water. The organic phase was used for GC/MS analysis.

Preparation of *Mtb* culture filtrate

A 100 mL culture of *Mtb* H37Rv was grown until an D_{600} of 0.8 was reached in 7H9 medium supplemented with 10% OADC and 0.05% Tween-80. The cells were removed by centrifugation at 3200 g for 15 min. The supernatant (culture filtrate) was sterilized by passing it through a 0.22µm filter twice, before removal from the biosafety level three facility. The culture filtrate was lyophilized to dryness and lipids were extracted in 8 mL of 1:1 chloroform:methanol with vortexing for 10 min. A water partition separated the phases, and samples were centrifuged at 3000 g for 10 min. The lower organic phase was used for GC/MS analysis.

Lipid extraction from cell pellets

The cell pellet from a 50 mL culture of *Mtb* or *M. smegmatis* was resuspended in 4 mL of 1:1 chloroform:methanol and transferred to a glass vial. The cell suspension was vortexed for 10 min. One milliliter of ddH₂O was added and the samples were vortexed for an additional 1 min. Cell suspensions were centrifuged at 3000 g for 10 min. The lower, organic phase was removed and used for GC analysis.

GC/MS

Chloroform extracts were transferred to 2 mL glass vials, containing glass inserts for smaller volumes as appropriate, and run directly on a gas chromatograph-mass spectrometer (GC model 6890, MS model 5973 inert; Agilent Technologies Inc., Santa Clara, CA, USA). An aliquot of the sample (1 µL) was injected into a Cyclosil-B column (Agilent Technologies Inc.) operating at a helium flow rate of 1 mL·min⁻¹. The oven temperature was held at 100 °C for 2 min after injection, and was then ramped to 250 °C at 5 °C·min⁻¹, and finally held at 250 °C for 2 min. The MS ion source was held at 230 °C throughout, with the quadrupole

at 150 °C. Full mass spectra were generated for metabolite identification by scanning within the m/z range of 45–350.

RNA extraction from *Mtb*

Mtb H37Rv (30 mL) was grown until an D_{600} of 0.8 was reached. Cells were harvested by centrifugation, and the cell pellet was frozen on dry ice and resuspended in 1 mL of Trizol reagent (Invitrogen). The suspension was transferred to a 2 mL screw cap tube containing 0.5 mL of 0.1 mm glass beads, and cells were disrupted using three 30 s pulses in a bead beater. After 5 min of inversion at room temperature, samples were centrifuged at 13000 g for 1 min. The Trizol solution was transferred to a tube containing 500 μ L of heavy phase lock gel (5 Prime 3 Prime, Inc., Boulder, CO, USA) and 300 μ L of chloroform was added followed by rapid inversion for 2 min and centrifugation at 13 000 g for 5 min. The aqueous layer was transferred to a tube containing 270 μ L of isopropanol and mixed with 270 μ L of 0.8 M sodium citrate/1.2 M NaCl prior to removal from the biosafety level three facility.

RNA was precipitated overnight at -20 °C and then centrifuged at 13 000 g for 10 min at 4 °C. The pellet was washed once with 70% ethanol in diethylpyrocarbonate-treated water, centrifuged for 5 min as above and air-dried. After resuspension of RNA pellets in 90 μ L of diethylpyrocarbonate-treated water, 10 μ L of 10 \times DNase I buffer and 4 units of DNase I (Invitrogen) were added and samples were incubated for 30 min at room temperature. Final purification of RNA was carried out using an RNeasy column in accordance with the manufacturer's instructions (Qiagen, Valencia, CA, USA).

cDNA synthesis and RT-PCR

cDNA was synthesized from 3 μ g of purified RNA using random primers (Invitrogen) and Thermo-X reverse transcriptase (Invitrogen). This cDNA was used as a template for PCR with primers complementary to sequences near the 5' end of *Rv3377c* [RT3377F (5'-TGTCGAAGTTTGGACG-3') and RT3377R (5'-CTTGGCAATCCACAAC-3')], creating a product of approximately 450 bp. Primers [SigAF (5'-ACATGGTTCGAGGTGAT-3') and SigAR (5'-ATGGTCTTGGACTCGAT-3')] complementary to sequences in the SigA gene were used as a positive control. To confirm the sequence of *Rv3377c* from the H37Rv strain, the complete ORF was amplified from cDNA using primers 3377F (5'-CTGGTGCCACGCGTTCTCATATGGAGACTTTCAGGACT-3') and 3377R (5'-GAAAGCTGGGTGAAGCTTTCATTGGTTACTCTCATC-3') using PFU Turbo CX polymerase (Invitrogen). The 1500 bp product was visualized on a 1% agarose gel, gel purified and sequenced directly.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

GGPP	geranylgeranyl diphosphate
<i>Mtb</i>	<i>Mycobacterium tuberculosis</i>

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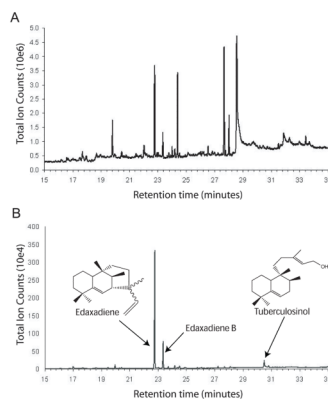
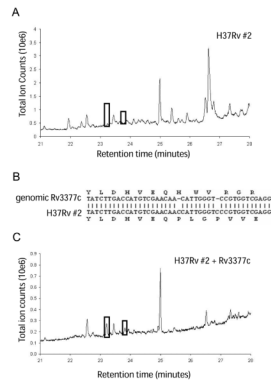


Fig. 1. GC trace of *Mtb* chloroform:methanol extracts. Lipids from *Mtb* cells grown to mid-log phase were extracted in chloroform:methanol and analyzed by GC/MS to detect low-molecular weight compounds. (A) Trace showing all species detected by GC/MS. (B) GC trace showing only those species containing ions characteristic of *Mtb* diterpenes (ions 80, 189 and 191). Chemical structures of diterpenes are adapted from previous studies [7,17] (Fig. S1).

**Fig. 2.**

Frameshift mutations in *Rv3377c* disrupt diterpene production in *Mtb*. The strain H37Rv #2 does not produce any diterpenes. (A) GC trace from H37Rv #2. Black boxes indicate where edaxadiene and edaxadiene B are expected to elute. (B) Sequencing of *Rv3377c* from H37Rv #2 shows two single-base insertions in the gene, upstream of the active site. (C) GC trace of H37Rv #2 complemented with a plasmid expressing *Rv3377c*. Black boxes indicate the retention times of edaxadiene and edaxadiene B.

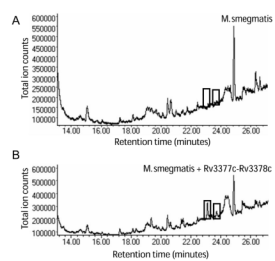


Fig. 3. Engineering diterpene production in *M. smegmatis*. (A) GC trace of wild-type *M. smegmatis* mc²115, which lacks orthologs of *Rv3377c* and *Rv3378c*. Boxes indicate the elution times of edaxadiene and edaxadiene B. (B) GC trace of *M. smegmatis* overexpressing *Rv3377c* and *Rv3378c*. Boxes show edaxadiene and edaxadiene B.

Table 1

Localization and relative abundance of *Mtb* diterpenes. γ -irradiated *Mtb* cells (Colorado State University) were separated into cell wall, cell membrane and cytosol fractions, and lipids were extracted using hexane (for cell wall) or chloroform:methanol (for cytosol and cell membrane). Culture filtrate prepared from live *Mtb* cultures was lyophilized, and lipids were extracted in chloroform:methanol. Tuberculosinol, edaxadiene and edaxadiene B were detected by GC/MS and the relative abundances are indicated.

Fraction	Tuberculosinol	Edaxadiene	Edaxadiene B
Cell membrane	+	+++	++
Cell wall	-	+	-
Cytosol	-	-	-
Culture filtrate	-	+	-