Molecular characterization of *ltp3* and *ltp4*, essential for C24-branched chain sterol-side-chain degradation in *Rhodococcus rhodochrous* DSM 43269

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INTRODUCTION

Actinobacteria, including members of the genera *Rhodococcus*, *Nocardia* and *Mycobacterium*, are renowned for their ability to metabolize a wide array of organic compounds, including steroids and sterols (Arima *et al.*, 1969; Martin 1977; van der Geize & Dijkhuizen, 2004). Two oxidative pathways are involved in microbial sterol degradation: steroid-ring oxidation and sterol-side-chain degradation (Dodson & Muir, 1958a, b; Sih *et al.*, 1968a, b). The mechanism of microbial sterol-side-chain degradation has been elucidated at the biochemical level by studying cell-free systems of *Mycobacterium* and *Nocardia* strains, as well as by the identification of pathway intermediates (Sih *et al.*, 1967, 1968a, b; Martin & Wagner, 1976; Fujimoto *et al.*, 1982a, b). Recently, a cholesterol catabolic gene cluster was identified in *Rhodococcus jostii* RHA1 containing a subset of genes (i.e. *ltp3* and *ltp4*). These results indicated a role for *ltp3* and *ltp4* in the removal of C24 branches specifically. Bioinformatic analysis of the encoded Ltp3 and Ltp4 proteins revealed relatively high similarity to thiolase enzymes, typically involved in β-oxidation, but the catalytic residues characteristic for thiolase enzymes are not conserved in their amino acid sequences. Removal of the C24-branched side-chain carbons of β-sitosterol was previously shown to proceed via aldolitic cleavage rather than by β-oxidation. Our results therefore suggest that *ltp3* and *ltp4* probably encode aldol-lyases rather than thiolases. This is the first report, to our knowledge, on the molecular characterization of genes with specific and essential roles in carbon–carbon bond cleavage of C24-branched chain sterols in *Rhodococcus* strains, most likely acting as aldol-lyases. The results are a clear contribution to our understanding of sterol degradation in actinobacteria.

Abbreviations: ADD, 1,4-androstadiene-3,17-dione; 1,4-BNC, 3-oxo-23,24-bisnorcholesta-1,4-dien-22-oic acid; CoA, coenzyme A.

The GenBank/EMBL/DDBJ accession number for the sequence data of *R. rhodochrous* DSM 43269 is HQ184439.

One supplementary table and one supplementary figure are available with the online version of this paper.
Fig. 1. Overview of the pathways involved in sterol degradation in actinobacteria (adapted from Sih et al., 1968b; Fujimoto et al., 1982b; van der Geize et al., 2007). (a) Cholesterol-side-chain degradation is initiated by formation of a cholesterol C26-oic acid (1), involving CYP125 (Roslonec et al., 2009). Next, the carboxylic acid is activated (2) by a CoA ligase, followed by a dehydrogenation step (3) by an acyl-CoA dehydrogenase and after one round of β-oxidation, propionyl-CoA is released (2) through thiolytic cleavage, catalysed by FadA5 in M. tuberculosis H37Rv (Nesbitt et al., 2010). After a second round of β-oxidation (3), acetyl-CoA is released followed by aldolytic cleavage of C19–20 (4). (b) The C24-branched side-chains of β-sitosterol and campesterol are also oxidized at position C26 (1), followed by CoA activation by CoA ligase FadD19 (2) (Wilbrink et al., 2011). Next, a double bond is introduced between C24 and C25 (3), which is followed by carboxylation of C28 (4). After hydration of the double bond (5), propionyl-CoA is released (6), probably through an aldol-lyase reaction involving ltp3 and/or ltp4. A CoA activation step is then followed by two rounds of β-oxidation (8 and 9) and formation of a C19 steroid by aldolytic cleavage (9), identical to cholesterol-side-chain degradation as shown above. (c) The enzyme steps for steroid ring opening are: oxidation of the 3-hydroxyl group into 3-keto, followed by isomerization of the C5–6 (Δ5) double bond to C4–5 (Δ4), catalysed by either cholesterol oxidase (CHO) or 3β-hydroxysteroid dehydrogenase (3β-HSD). Next, two activities, Δ1-dehydrogenation, mediated by 3-ketosteroid Δ1-dehydrogenase (KSTD), and 9z-hydroxylation, catalysed by 3-ketosteroid 9z-hydroxylase (KSH), yield an unstable intermediate which spontaneously rearranges, leading to opening of ring B and aromatization of ring A. R. rhodochrous mutant strain RG32 is devoid of KSH activity (indicated by crosses) and accumulates steroids with intact nuclei from sterol substrates.
formation of 4-androstene-3,17-dione (AD) and 1,4-androstanediene-3,17-dione (ADD) from cholesterol in M. tuberculosis H37Rv (Nesbitt et al., 2010). Detailed bioinformatic and biochemical analysis undisputedly proved that FadA5 is a thiolase. The fadA5 homologue in strain RH1A1, however, was not upregulated during growth on cholesterol (van der Geize et al., 2007). An aldol-lyase reaction ultimately leads to formation of 17-keto-steroids, such as ADD, from C22 steroids (Fig. 1a, compound V) (Sih et al., 1967, 1968a, b).

Removal of the C24–branches of β-sitosterol and campesterol is initiated by carboxylation of the C28 carbon, followed by a hydration reaction, and ultimately release of propionyl-CoA via cleavage of the C24–C25 bond by an aldol-lyase (Fig. 1b, Chen, 1985; Fujimoto et al., 1982b).

The aim of this study was to decipher the physiological roles of genes and enzymes involved in microbial sterol-side-chain degradation. Here, we report on the mutational analysis of various genes, including ltp3, ltp4 and fadA5, located within a rhodococcal cholesterol catabolic gene cluster in R. jostii RH1A1 and/or R. rhodochrous DSM 43269 mutant strain RG32 (Wilbrink et al., 2011) and propose physiological roles of these genes in sterol-side-chain degradation. The results are a clear contribution to our understanding of sterol degradation in actinobacteria.

**METHODS**

**Bacterial strains, plasmids and growth conditions.** R. jostii RH1A1 and R. rhodochrous DSM 43269 (and mutants of these strains) were grown in Luria–Bertani medium (LB; Sigma) at 30 °C with shaking (220 r.p.m.). To test growth of strain RH1A1 and mutant MW1 on sterile substrates, precultures were grown for 3 days at 30 °C with shaking (220 r.p.m.) in mineral medium (MM) (Masai et al., 1995) supplemented with 20 mM pyruvate and inoculated 1:50 into MM supplemented with cholesterol or β-sitosterol at 1 g l⁻¹ as sole carbon and energy source. Sterols were finely dispersed by sonication prior to autoclaving. Biomass production of the cultures was determined by measuring protein contents as described previously (Rosonieck et al., 2009).

All Escherichia coli strains were grown in LB at 37 °C. For growth on solid medium, 1.5 % (w/v) Difco agar (Becton) was added. E. coli DH5α and pBlueScript(II)KS were used for gene cloning. E. coli S17-1 was used for conjugation of pK18mobsacB-based deletion plasmids to Rhodococcus. If appropriate, ampicillin was added to a final concentration of 100 μg ml⁻¹. Kanamycin was used at a concentration of 25 μg ml⁻¹ (E. coli and R. rhodochrous) or 100 μg ml⁻¹ (R. jostii). Cholesterol (>95%, ash-free) and campesterol (65%) were obtained from Sigma. β-Sitosterol was purchased from Acros and was a mixture of β-sitosterol (75–80%), β-sitostanol (10–14%) and campesterol (6–9%).

**General cloning techniques.** Procedures for the manipulation and analysis of DNA were performed essentially as described by Sambrook et al. (1989). DNA-modifying enzymes (restriction enzymes, T4 DNA ligase and DNA polymerases) were purchased from Roche, New England Biolabs or Fermentas and were used according to the manufacturers’ protocols.

Primers were from Sigma-Aldrich. DNA sequencing was performed by LGC genomics.

**Screening of an R. rhodochrous DSM 43269 genomic library for ltp3 orthologues.** A genomic library of R. rhodochrous DSM 43269 in pRESQ (Petruisma et al., 2009) was screened for the presence of ltp3 orthologues by using gene-specific degenerate primers based on conserved amino acids of Ltp3. The genomic library of R. rhodochrous DSM 43269 was transferred to E. coli DH5α by transformation and plated on LB with 25 μg kanamycin ml⁻¹. All colonies on a plate were resuspended in LB, the total plasmid pool was isolated and purified, and the presence of the target gene was verified by PCR analysis. The procedure of transformation, plating dilutions and PCR was repeated until a single clone was identified, named pRESQ4683, containing ltp3 comprising whose insert DNA was sequenced.

**Construction of targeted gene disruptions and unmarked gene deletions in rhodococci.** Disruption and unmarked gene deletion mutants of R. jostii RH1A1 and R. rhodochrous RG32 were constructed as described previously (van der Geize et al., 2001). Primers used to make gene deletion and disruption mutants are listed in Table S1 available with the online version of this paper.

Mutation MW1 of R. jostii RH1A1, containing a 13.5 kb deletion of ro04683–ro04694, was constructed using plasmid pDelBOX. Plasmid pDelBOX was constructed as follows. PCR amplimers of the flank regions (1.1 and 1.2 kb, respectively) were each cloned into EcoRV-digested pBlueScript(II)KS, yielding pBS_BOXup and pBS_BOXdown, for the upstream and downstream region, respectively. A 1.1 kb fragment of pBS_BOXup, obtained by BglII/XbaI digestion, was ligated into BglII/XbaI-digested pBS_BOXdown, yielding pBS_BOX. A 2.3 kb HindIII–XbaI fragment of pBS_BOX was then ligated into HindIII/XbaI-digested deletion vector pK18mobsacAB, generating pDelBOX.

A double deletion mutant of ltp3 and ltp4 in strain RG32 was constructed using pDelLtp3ltp4 as follows. The flanking regions were amplified by PCR and the obtained amplicons containing the upstream and downstream regions (1.1 kb each) were cloned into EcoRV-digested pBlueScript(II)KS, generating pBSltp3ltp4up and pBSltp3ltp4down, respectively. A 1.1 kb NdeI–XbaI fragment of pBSltp3ltp4up was then ligated into NdeI/XbaI-digested pBSltp3ltp4down, yielding pBSltp3ltp4. Following digestion of pBSltp3ltp4 with HindIII/XbaI, a 2.1 kb fragment was then ligated into HindIII/XbaI-digested plasmid pK18mobsacAB, generating deletion plasmid pDelLtp3ltp4.

A single deletion mutant of ltp3 was constructed in strain RG32 using pDelLtp3 as follows. Amplicons obtained by PCR amplification of the upstream and downstream regions (1.1 kb each) were cloned into EcoRV-digested pBlueScript(II)KS, generating pBSltp3up and pBSltp3down, respectively. A 1.2 kb BglII–XbaI fragment of pBSltp3up was then ligated into BglII/XbaI-digested pBSltp3down, yielding pBSltp3. Following digestion of pBSltp3 with HindIII/XbaI, a 2.3 kb fragment containing the ltp3 deletion cassette was ligated into HindIII/XbaI-digested plasmid pK18mobsacAB, generating deletion plasmid pDelLtp3.

An unmarked ltp4 deletion mutant was constructed in strain RG32 using pDelLtp4 as follows. The flanking regions of ltp4 were amplified by PCR and the 1.1 kb amplicons were cloned into EcoRV-digested pBlueScript(II)KS, generating pBSltp4up and pBSltp4down, respectively. A 1.1 kb NdeI–XbaI fragment of pBSltp4up was then ligated into NdeI/XbaI-digested pBSltp4down, yielding pBSltp4. Following HindIII/XbaI digestion of pBSltp4, a 2.2 kb fragment was then ligated into HindIII/XbaI-digested plasmid pK18mobsacAB, yielding pDelLtp4.

An fadA5 disruption mutant of strain RG32 was constructed using pTfada5. Plasmid pTfada5 was constructed by cloning a PCR amplicon (734 bp) of an internal fragment of fadA5 into SmaI-digested pK18mobsacAB.
All gene deletion mutants were confirmed by PCR analysis, using specific primers (Table S1), and chromosomal DNA was isolated from the mutants.

Complementation of mutants RG32\ltp3 and RG32\ltp4. Chromosomal DNA of \textit{R. rhodochrous} DSM 43269 was used to amplify \ltp3 and \ltp4 using the primers listed in Table S1. The obtained PCR products of \ltp3\_DSM 43269 (1.2 kb) and \ltp4\_DSM 43269 (1.1 kb) were digested with Acc65I and cloned behind the \aphII promoter region of \textit{EcoRV}/Acc65I-digested pBs-Pkan (van der Geize \textit{et al.}, 2008), yielding pBs-Pkan\ltp3 and pBs-Pkan\ltp4, respectively. The Pkan-ltp3 and Pkan-ltp4 cassettes were then cloned into the \textit{Rhodococcus–E. coli} shuttle vector pRESQ, using SpeI/Acc65I digestion. The resulting constructs were named pCOMP\ltp3 and pCOMP\ltp4, respectively. The plasmids were mobilized to the respective mutants by electro-transformation as described previously (Wilbrink \textit{et al.}, 2011).

HPLC and GC-MS analysis of sterol bioconversions. For bioconversion experiments, \textit{R. rhodochrous} cultures were grown overnight until mid-exponential phase (OD\_\text{\text{600}} of 2.0–2.5) and sterols were added from a stock solution (25 mM in acetone) to a final concentration of 0.5 mM and were incubated for another 3 days at 30 °C with shaking (220 r.p.m.) when cell densities reached an OD\_\text{\text{600}} of 8.0–9.0. HPLC analysis was performed as described previously (Wilbrink \textit{et al.}, 2011), and standards of ADD and 1,4-BNC (3-oxo-23,24-bisnorcholest-1,4-dien-22-oic acid) were used to quantify conversion rates.

For GC-MS analysis, samples were prepared by adding 50 µl of H\textsubscript{2}SO\textsubscript{4} (10 %, v/v) to 0.5 ml of cell culture followed by extraction with 2 vols of ethyl acetate and vigorous shaking. The organic fraction was collected and dried under a stream of nitrogen and derivatized using trimethylsilylchloride as described previously (Song \textit{et al.}, 2003). GC separation was carried out using an HP 5890 II machine equipped with an HP-5 column (Agilent; 30 m × 0.25 mm × 0.25 µm) with helium as a gas carrier at a flow rate of 0.25 ml min\textsuperscript{-1}. MS analysis was performed using an Agilent 5973 with EI as ion source and a vaporizer temperature of 200 °C.

Phylogenetic tree construction. The amino acid sequences of thiolases encoded by strain RHA1 were obtained from the RHA1 genome website (www.rhodococcus.ca) by database searches for genes annotated as thiolase or thioesterase, or with a Cluster of Orthologous Groups (COG) prediction as acetyl-CoA acetyltransferase (McLeod \textit{et al.}, 2006). The obtained full-length protein sequences were subsequently aligned using CLUSTAL W (Thompson \textit{et al.}, 1994). Phylogenetic and molecular evolutionary analyses were performed using MEGA version 4 (Tamura \textit{et al.}, 2007).

RESULTS

Deletion of the \textit{ro04683–ro04694} gene subset of the cholesterol gene cluster in \textit{R. jostii} RHA1 does not affect growth on sterols

To assess the role of a subset of genes from the cholesterol catabolic gene cluster predicted to be involved in sterol-side-chain degradation, mutant strain MW1 of strain RHA1 was constructed, carrying a 13.5 kb deletion and lacking \textit{ro04683–ro04694}. This suite of genes encodes all the enzymes required to perform a full cycle of \beta-oxidation and was believed to be essential for growth of strain RHA1 on sterols. Growth experiments of mutant strain MW1 in mineral medium supplemented with cholesterol or \beta-sitosterol (1 g l\textsuperscript{-1}) resulted in mean (±SD) protein contents of 43 (±4) and 53 (±1) mg l\textsuperscript{-1}, respectively. These yields were comparable to those obtained with wild-type RHA1 [56 (±3) and 56 (±4) mg l\textsuperscript{-1}, respectively].

Cloning of \ltp3 and \ltp4 orthologues from \textit{R. rhodochrous} DSM 43269

\textit{R. rhodochrous} strain RG32, a fivefold \textit{kshA} deletion mutant blocked in 3-ketosteroid-9α-hydroxylase activity, was previously engineered and shown to be capable of side-chain degradation of various sterols, such as cholesterol, \beta-sitosterol and campesterol (Wilbrink \textit{et al.}, 2011), thereby accumulating sterols with intact nuclei (Fig. 1). The RG32 phenotype thus facilitates screening of mutants impaired in sterol-side-chain degradation by comparing their steroid-forming capacity with that of strain RG32 itself. As the genome sequence of strain DSM 43269 is unknown, the orthologues of the genes of our interest were cloned by screening a DSM 43269 strain genomic library with degenerate primers for \ltp3. These primers were based on the highly conserved amino acid sequences ESNAMWA (aa 119–125 in Ltp3\textit{RHA1} and WFPMWL (aa 291–297 in Ltp3\textit{RHA1}) present in actinobacterial Ltp3 homologues. An individual clone was identified carrying full-length \ltp3\_DSM 43269 on a 5.7 kb DNA insert in addition to several other orthologues of RHA1 genes. The genetic organization of the gene orthologues in strain DSM 43269 was identical to that in strain RHA1 (Fig. 2) and the proteins they encode share 74–92 % amino acid sequence identity with their RHA1 counterparts.

Both \ltp3 and \ltp4 of \textit{R. rhodochrous} DSM 43269 are required for \beta-sitosterol-side-chain degradation

Unmarked in-frame single gene deletion mutants of \ltp3 and \ltp4 were constructed in strain RG32, and were tested in whole-cell biotransformation assays for their ability to convert the side-chains of cholesterol and \beta-sitosterol. Both mutants were capable of cholesterol-side-chain degradation, producing ADD and 1,4-BNC at similar levels as observed with parent strain RG32 (Table 1). RG32\ltp3 and RG32\ltp4 were, however, blocked in \beta-sitosterol-side-chain degradation, as no detectable levels of ADD or 1,4-BNC were observed (Table 1). Furthermore, both mutants accumulated small amounts of an intermediate that was not observed in parent strain RG32. MS analysis of the intermediate revealed a base peak of \textit{m/z}=124, typical for a 3-keto-4-ene steroid-ring structure (Brooks, 1979) and a molecular ion of \textit{m/z}=412 (Fig. S1). These values are in agreement with 4-sitostene-3-one (\textit{M}_{r}=412), which is the predicted product of A-ring oxidation of \beta-sitosterol by cholesterol oxidase or \beta-hydroxysteroid dehydrogenase activity (Fig. 1c) present in \textit{Rhodococcus} strains (van der Geize \textit{et al.}, 2007; Rosloniec \textit{et al.}, 2009). The conversion of campesterol (Fig. 1) was also blocked in the \ltp3 and \ltp4
mutants (Table 1), suggesting that \textit{ltp3} and \textit{ltp4} are essential specifically for C24-branched-chain sterol-side-chain degradation.

Both RG32\textit{Δltp3} and RG32\textit{Δltp4} were successfully complemented by providing the deleted gene \textit{in trans}, restoring the formation of ADD and 1,4-BNC from \textit{β}-sitosterol.

### Table 1. Whole-cell biotransformations of sterols by \textit{R. rhodochrous} strain RG32 and mutants thereof, after 72 h of incubation

<table>
<thead>
<tr>
<th>Strain</th>
<th>\textit{β}-Sitosterol</th>
<th>Campesterol</th>
<th>Cholesterol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ADD (%)</td>
<td>1,4-BNC (%)</td>
<td>ADD (%)</td>
</tr>
<tr>
<td>RG32</td>
<td>7 ± 2</td>
<td>67 ± 7</td>
<td>1 ± 0</td>
</tr>
<tr>
<td>RG32\textit{Δltp3}</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>RG32\textit{Δltp4}</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>RG32\textit{Δltp3}+\textit{pCOMP}ltp3</td>
<td>4 ± 1</td>
<td>22 ± 3</td>
<td>ND</td>
</tr>
<tr>
<td>RG32\textit{Δltp4}+\textit{pCOMP}ltp4</td>
<td>7 ± 1</td>
<td>42 ± 5</td>
<td>ND</td>
</tr>
<tr>
<td>RG32\textit{fada5}</td>
<td>7 ± 1</td>
<td>70 ± 8</td>
<td>ND</td>
</tr>
</tbody>
</table>

The values represent the molar percentage of sterol conversion into ADD and 1,4-BNC using 0.5 mM of sterol substrates. ND, Not determined; –, below detection limit. Values are mean ± SD (n=3).
(Table 1). This conclusively demonstrated that the observed phenotypes were caused by deletion of the genes themselves, rather than by polar effects.

**FadA5**<sub>DSM 43269</sub> **is not essential for cholesterol degradation in R. rhodochrous RG32**

FadA5<sub>H37Rv</sub> was previously identified as a 3-ketoacyl-CoA thiolase essential for side-chain degradation of cholesterol in *M. tuberculosis* (Nesbitt et al., 2010). Bioinformatic analysis of a previously cloned genomic fragment (GenBank accession no. FJ824698) of *R. rhodochrous* DSM 43269 (Rosłoniec et al., 2009) identified a FadA5<sub>DSM 43269</sub> orthologue displaying 74 % aa sequence identity with FadA5<sub>H37Rv</sub>. To substantiate its role in sterol-side-chain degradation in *R. rhodochrous* DSM 43269, fadA5 was inactivated in strain RG32. Unsurprisingly, strain RG32<sup>FadA5</sup> was unimpaired in cholesterol or β-sitosterol-side-chain degradation (Table 1). This indicates that *fadA5* is not essential for side-chain degradation of cholesterol (or β-sitosterol) in *R. rhodochrous* DSM 43269.

**DISCUSSION**

Our research aims at elucidating the microbial degradation pathways of steroids and sterols. The current study reports on the molecular characterization of rhodococcal *ltp3* and *ltp4*, highlighting a crucial role for these genes in C24-branched sterol-side-chain degradation. Deletion of a large subset of genes from a cholesterol catabolic gene cluster including *ltp3* and *ltp4* in *R. jostii* RHA1 did not block growth on cholesterol or β-sitosterol. We anticipated that sterol-side-chain degradation might be impaired in this mutant, but that growth on sterol substrates could still be sustained through steroid-ring oxidation. Previous work showed that C26-oxidation, mediated by CYP125, is the first step in cholesterol degradation in strain RHA1 and that mutant RHA1<sup>Δcyp125</sup> was unable to grow on cholesterol, but able to grow on 3-ketone oxidized derivatives of cholesterol (Rosłoniec et al., 2009). As strain MW1 retained the *cyp125* gene, initiation of cholesterol-side-chain degradation is unhampered in this mutant, which is a prerequisite for further degradation by steroid-ring oxidation and opening, as depicted in Fig. 1(c).

These results prompted us to use another, more suitable *Rhodococcus* strain for studying the role of this set of genes in sterol-side-chain degradation specifically, i.e. mutant RG32 of *R. rhodochrous* DSM 43269 (Wilbrink et al., 2011). The phenotypes of the *ltp3* and *ltp4* deletion mutants in *R. rhodochrous* mutant strain RG32 led us to conclude that these genes are essential for the degradation of C24-branched sterol side-chains specifically. Sequence comparison of Ltp3<sub>DSM 43269</sub> and Ltp4<sub>DSM 43269</sub> with characterized proteins from the Swiss-Prot database revealed that they both show highest similarity with eukaryotic Sterol Carrier Protein x (SCPx) (26 and 24 % identity, respectively), a 3-ketoacyl-CoA thiolase acting on branched chain acyl-CoA substrates (Seedorf et al., 1994; Wanders et al., 1997). Despite being annotated as thiolases, sequence comparisons of Ltp3<sub>DSM 43269</sub> and Ltp4<sub>DSM 43269</sub> with various characterized thiolases revealed that none of the typical catalytic residues (Cys<sub>125</sub>, His<sub>375</sub> and Cys<sub>403</sub> in yeast thiolase) is highly conserved in catabolic thiolases (Haapalainen et al., 2006) and that the Pfam thiolase signature sequence motifs PF00108 and PF02803 (Finn et al., 2008) are not conserved in these proteins (Fig. 3).

Strikingly, in a phylogenetic analysis of annotated thiolases of strain RHA1 and characterized thiolases (Peretó et al., 2005), Ltp3<sub>DSM 43269</sub> and Ltp4<sub>DSM 43269</sub> cluster separately from most of the previously characterized thiolase enzymes (Fig. 4). These data strongly suggest that *ltp3* and *ltp4* do not encode thiolase activity, but another enzyme function. Furthermore, due to the structure of C24-branched chain sterols, formation of a ketoacyl-CoA at the β-carbon, essential for thiolase activity, is chemically impossible. Fujimoto et al. (1982a) suggested that sterol C24-branched are cleaved off by an aldol-lyase reaction, similar to that of other tertiary β-hydroxy-CoA esters, such as (β-methyl)methyl-CoA (Hacking & Quayle, 1974) and hydroxymethylglutaryl-CoA (Steigink & Coon, 1968).

We hypothesize that *ltp3* and *ltp4* encode such aldol-lyase activity towards C24-branched sterol side-chains, with an
Fig. 4. Phylogenetic tree comprising full-length protein sequences annotated as thiolases (or thioesterases) in the R. jostii RHA1 genome (www.rhodococcus.ca) and the characterized thiolases from various organisms: E. coli FadA (AAC76848); Homo sapiens mitochondrial 3-oxoacyl thiolase, HMOT (BAA03800); Rattus norvegicus peroxisomal thiolase I, RPO (BAA14108); Xenopus laevis cytosolic thiolase II, XCAT (AAD34967); Arabidopsis thaliana thiolase I (NP_171965) and thiolase II (NP_199583); Bacillus subtilis YusK (BG14023); and H. sapiens sterol carrier protein 2, SCPx (NP_002970).

essential role in β-sitosterol and campesterol degradation. Interestingly, ltp3 and ltp4 and their genetic organization were found to be highly conserved among (sterol-degrading) actinobacteria (Fig. 2). Possibly, their encoded proteins form (hetero) multimeric structures as in other aldol-lyase enzymes, such as malyl-CoA lyase of Methylobacterium extorquens (formerly Pseudomonas AM1) (Hacking & Quayle, 1974) and citrate lyase of Leuconostoc mesenteroides (Bekal et al., 1998).

Additional biochemical studies are required to establish the exact biochemical role of Ltp3 and Ltp4 in sterol-side-chain degradation. However, such studies are hampered by the lack of availability of the required sterol substrates (Fig. 1b, compound VIII) for the reaction(s) expected to be catalysed by these enzymes.

Our results showed that targeted inactivation of fadA5 in strain RG32 did not result in inactivation of
cholesterol-side-chain degradation, in contrast to the situation in M. tuberculosis H37Rv (Nesbitt et al., 2010). Conceivably, enzyme redundancy or enzyme compensation for FadA5 activity occurs in R. rhodochrous DSM 43269. Candidate thiolase genes in the genome of strain RHA1 that might compensate for fadA5 loss are ro04488 and ro05815. A homologue of ro04488 (i.e. MSMEG_5990) was highly upregulated during growth of Mycobacterium smegmatis on cholesterol (Uhía et al., 2012). However, this gene also has a homologue in M. tuberculosis, where no compensation was observed when fadA5 was inactivated. On the other hand, ro05815 encodes a protein with high amino acid similarity to FadA5_RHA1 and FadA5_H37Rv (67 and 64 %, respectively, Fig. 4) and this gene was not found in the mycobacterial strains, although reciprocal best hits of Ro05815 could be identified in all available rhodococcal genomes with high protein sequence similarities (82–92 %). Therefore, an ro05815 homologue is probably present in the genome of Rhodococcus and compensates for loss of FadA5 function in this strain. Analogously, R. rhodochrous DSM 43269, whose genome sequence is not known, may also contain such a gene homologue, thus explaining the lack of phenotype in the fadA5-inactivated mutant. The fact that fadA5 (ro04678) in R. jostii RHA1 was not upregulated during growth on cholesterol (van der Geize et al., 2007) combined with the lack of a phenotype in the fadA5-inactivated mutant in strain RG32 suggests that rhodococcal FadA5 enzymes may have different physiological roles from FadA5 of M. tuberculosis H37Rv.

In conclusion, this is the first report, to our knowledge, on the identification and characterization of genes (ltp3 and ltp4) with specific and essential roles in C24-branched sterol-side-chain degradation. The results are a clear contribution to our understanding of sterol degradation in actinobacteria.

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