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

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A previously identified sterol catabolic gene cluster is widely dispersed among actinobacteria, enabling them to degrade and grow on naturally occurring sterols. We investigated the physiological roles of various genes by targeted inactivation in mutant RG32 of *Rhodococcus rhodochrous*, which selectively degrades sterol side-chains. The *ltp3* and *ltp4* deletion mutants were each completely blocked in side-chain degradation of β-sitosterol and campesterol, but not of cholesterol. 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 aldolytic 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.

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. ro04678–ro04695) with a suggested role in sterol-side-chain degradation (van der Geize et al., 2007). Sterol-side-chain degradation is initiated by C26-hydroxylation (Zaretskaya et al., 1967) encoded by cyp125 (ro04679 in strain RHA1) (Rosloniec et al., 2009). Biochemical studies on the CYP125 homologue of *Mycobacterium tuberculosis* H37Rv revealed that CYP125_H37Rv catalyses hydroxylation of C26 or C27 followed by further oxidation to yield a sterol carboxylic acid (Capyk et al., 2009; McLean et al., 2009; Ouellet et al., 2010; Johnson et al., 2010) (Fig. 1, compounds II and VII for cholesterol and C24-branched sterols, respectively). Further degradation of the side-chain proceeds by a mechanism similar to β-oxidation of fatty acids (Sih et al., 1967, 1968a, b). This process is initiated by coenzyme A (CoA) activation of the carboxylic acid and is mediated by a sterol-CoA ligase (Ambrus et al., 1995). FadD19 of *Rhodococcus rhodochrous* DSM 43269 was characterized as a sterol-CoA ligase with a specific role in the degradation of C24-branched chain sterols (Fig. 1b) (Wilbrink et al., 2011). The CoA-activated side-chain is then shortened via a C24 carboxylic acid intermediate into a C22 carboxylic acid steroid (Fig. 1, compounds III and IV, respectively) involving thiolytic activity. Recently, the actinobacterial *fadA5* gene, encoding a putative thiolase, was shown to be required for the...
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 (Rosłoniec 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 campsterol 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-hydroxy 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 9α-hydroxylation, catalysed by 3-ketosteroid 9α-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.
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 RHA1 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 RHA1 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 RHA1 and mutant MW1 on sterol 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 (van der Geize et al., 2009). If appropriate, ampicillin was added to a final concentration of 100 μg ml⁻¹.

**Screening of an R. rhodochrous DSM 43269 genomic library for ltp3 orthologues.** A genomic library of R. rhodochrous DSM 43269 in pRESQ (Petrusma 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 DH5x 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 ltp3DSM 43269 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 RHA1 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.

**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.

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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 DH5x and pBlueScript(II)KS were used for gene cloning. E. coli S17-1 was used for conjugation of pK18mobscB-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 purchased from Acros and was a mixture of β-sitosterol (75–80 %), β-sitostanol (10–14 %) and campesterol (6–9 %).

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

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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 *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 EcoRV/Acc65I-digested PBS-Pkan (van der Geize et al., 2008), yielding PBS-Pkan-ltp3 and PBS-Pkan-ltp4, respectively. The Pkan-ltp3 and Pkan-ltp4 cassettes were then cloned into the *Rhodococcus*-E. coli shuttle vector pRESQ, using SphI/Acc65I digestion. The resulting constructs were named pCOMPltp3 and pCOMPltp4, respectively. The plasmids were mobilized to the respective mutants by electro-transformation as described previously (Wilbrink et al., 2011).

**HPLC and GC-MS analysis of sterol bioconversions.** For bioconversion experiments, *R. rhodochrous* cultures were grown overnight until mid-exponential phase (OD<sub>600</sub> 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<sub>600</sub> of 8.0–9.0. HPLC analysis was performed as described previously (Wilbrink et al., 2011), and standards of ADD and 1,4-BNC (3-oxo-23,24-bisnorchola-1,4-dien-20-oic acid) were used to quantify conversion rates.

For GC-MS analysis, samples were prepared by adding 50 µl of H<sub>2</sub>SO<sub>4</sub> (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 trimethylchlorosilane as described previously (Song 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 11 ml min<sup>−1</sup>. 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 et al., 2006). The obtained full-length protein sequences were subsequently aligned using CLUSTAL W (Thompson et al., 1994). Phylogenetic and molecular evolutionary analyses were performed using MEGA version 4 (Tamura et al., 2007).

**RESULTS**

**Deletion of the **ro04683-**ro04694** gene subset of the cholesterol gene cluster in *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 ro04683–ro04694. This suite of genes encodes all the enzymes required to perform a full cycle of β-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 β-sitosterol (1 g l<sup>−1</sup>) resulted in mean (±SD) protein contents of 43 (±4) and 53 (±1) mg l<sup>−1</sup>, respectively. These yields were comparable to those obtained with wild-type RHA1 [56 (±3) and 56 (±4) mg l<sup>−1</sup>, respectively].

**Cloning of **ltp3 and **ltp4** orthologues from *R. rhodochrous* DSM 43269**

*R. rhodochrous* strain RG32, a fivefold *kshA* deletion mutant blocked in 3-ketocholesterol-9α-hydroxy-hydroxylase activity, was previously engineered and shown to be capable of side-chain degradation of various sterols, such as cholesterol, β-sitosterol and campesterol (Wilbrink et al., 2011), thereby accumulating steroids 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*<sub>RHA1</sub>) and WFPEPMWL (aa 291–297 in *Ltp3<sub>RHA1</sub>*) 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 *R. rhodochrous* DSM 43269 are required for β-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 β-sitosterol. Both mutants were capable of cholesterol-side-chain degrada-tion, 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 β-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 m/z=124, typical for a 3-keto-4-ene steroid-ring structure (Brooks, 1979) and a molecular ion of m/z=412 (Fig. S1). These values are in agreement with 4-sitostene-3-one (m<sub>r</sub>=412), which is the predicted product of A-ring oxidation of β-sitosterol by cholesterol oxidase or 3β-hydroxysteroid dehydrogenase activity (Fig. 1c) present in *Rhodococcus* strains (van der Geize et al., 2007; Rosloniec 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 \textit{RG32\Delta ltp3} and \textit{RG32\Delta ltp4} were successfully complemented by providing the deleted gene \textit{in trans}, restoring the formation of ADD and 1,4-BNC from \textit{\beta}-sitosterol.

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

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 (\(n=3\)).

\begin{table}[h]
\centering
\begin{tabular}{|l|c|c|c|c|c|}
\hline
\textbf{Strain} & \textbf{\textit{\beta}-Sitosterol} & & & & \\
 & ADD (%) & 1,4-BNC (%) & ADD (%) & 1,4-BNC (%) & ADD (%) & 1,4-BNC (%) \\
\hline
\textit{RG32} & 7 ± 2 & 67 ± 7 & 1 ± 0 & 48 ± 10 & 3 ± 1 & 73 ± 12 \\
\textit{RG32\Delta ltp3} & – & – & – & – & 4 ± 1 & 70 ± 3 \\
\textit{RG32\Delta ltp4} & – & – & – & – & 3 ± 1 & 71 ± 2 \\
\textit{RG32\Delta ltp3+pCOMP\textit{ltp3}} & 4 ± 1 & 22 ± 3 & ND & ND & ND & ND \\
\textit{RG32\Delta ltp4+pCOMP\textit{ltp4}} & 7 ± 1 & 42 ± 5 & ND & ND & ND & ND \\
\textit{RG32\Delta fadA5} & 7 ± 1 & 70 ± 8 & ND & ND & 4 ± 2 & 74 ± 9 \\
\hline
\end{tabular}
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FadA5\textsubscript{DSM 43269} is not essential for cholesterol degradation in \textit{R. rhodochrous} RG32

FadA5\textsubscript{H37Rv} was previously identified as a 3-ketoacyl-CoA thiolase essential for side-chain degradation of cholesterol in \textit{M. tuberculosis} (Nesbitt \textit{et al.}, 2010). Bioinformatic analysis of a previously cloned genomic fragment (GenBank accession no. FJ824698) of \textit{R. rhodochrous} DSM 43269 (Rosłoniec \textit{et al.}, 2009) identified a FadA5\textsubscript{DSM 43269} orthologue displaying 74\% aa sequence identity with FadA5\textsubscript{H37Rv}. To substantiate its role in sterol-side-chain degradation in \textit{R. rhodochrous} DSM 43269, fadA5 was inactivated in strain RG32. Unexpectedly, strain RG32\textsubscript{fadA5} was unimpaired in cholesterol or \(\beta\)-sitosterol-side-chain degradation (Table 1). This indicates that \(\text{fadA5}\) is not essential for side-chain degradation of cholesterol (or \(\beta\)-sitosterol) in \textit{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 \textit{ltp3} and \textit{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 \textit{ltp3} and \textit{ltp4} in \textit{R. jostii} RHA1 did not block growth on cholesterol or \(\beta\)-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\textsubscript{Δcyp125} was unable to grow on cholesterol, but able to grow on 3-ketone oxidized derivatives of cholesterol (Rosłoniec \textit{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 \textit{Rhodococcus} strain for studying the role of this set of genes in sterol-side-chain degradation specifically, i.e. mutant RG32 of \textit{R. rhodochrous} DSM 43269 (Wilbrink \textit{et al.}, 2011). The phenotypes of the \textit{ltp3} and \textit{ltp4} deletion mutants in \textit{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\textsubscript{DSM 43269} and Ltp4\textsubscript{DSM 43269} 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 \textit{et al.}, 1994; Wanders \textit{et al.}, 1997). Despite being annotated as thiolases, sequence comparisons of Ltp3\textsubscript{DSM 43269} and Ltp4\textsubscript{DSM 43269} with various characterized thiolases revealed that none of the typical catalytic residues (Cys\textsubscript{125}, His\textsubscript{375} and Cys\textsubscript{403} in yeast thiolase) is highly conserved in catabolic thiolases (Haapalainen \textit{et al.}, 2006) and that the Pfam thiolase signature sequence motifs PF00108 and PF02383 (Finn \textit{et al.}, 2008) are not conserved in these proteins (Fig. 3).

Strikingly, in a phylogenetic analysis of annotated thiolase proteins of strain RHA1 and characterized thiolases (Peretó \textit{et al.}, 2005), Ltp3\textsubscript{DSM 43269} and Ltp4\textsubscript{DSM 43269} cluster separately from most of the previously characterized thiolase enzymes (Fig. 4). These data strongly suggest that \textit{ltp3} and \textit{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 \(\beta\)-carbon, essential for thiolase activity, is chemically impossible. Fujimoto \textit{et al.} (1982a) suggested that sterol C24-branched are cleaved off by an aldol-lyase reaction, similar to that of other tertiary \(\beta\)-hydroxy-CoA esters, such as (\(\beta\)-methyl-)malyl-CoA (Hacking & Quayle, 1974) and hydroxymethylglutaryl-CoA (Stegink & Coon, 1968).

We hypothesize that \textit{ltp3} and \textit{ltp4} encode such aldol-lyase activity towards C24-branched sterol side-chains, with an
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 (Uhia 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 FadA5RHA1 and FadA5H37Rv (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 *R. rhodochrous* 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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