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Published in:
Molecular Microbiology

DOI:
10.1111/j.1365-2958.2009.06915.x

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2009

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

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Download date: 23-10-2018
Cytochrome P450 125 (CYP125) catalyses C26-hydroxylation to initiate sterol side-chain degradation in Rhodococcus jostii RHA1

Kamila Z. Rosłoniec,1,1† Maarten H. Wilbrink,1,† Jenna K. Capyk,2 William W. Mohn,2 Martin Ostendorf,3 Robert van der Geize,1,* Lubbert Dijkhuizen1 and Lindsay D. Eltis2

1Department of Microbiology, Groningen Biomolecular Sciences and Biotechnology Institute (GBB), University of Groningen, PO Box 14, 9750 AA Haren, the Netherlands.
2Department of Microbiology and Immunology, Life Sciences Institute, University of British Columbia, Vancouver, V6T 1Z3, Canada.
3Schering-Plough, PO Box 20, 5340 BH, Oss, the Netherlands.

Summary

The cyp125 gene of Rhodococcus jostii RHA1 was previously found to be highly upregulated during growth on cholesterol and the orthologue in Mycobacterium tuberculosis (rv3545c) has been implicated in pathogenesis. Here we show that cyp125 is essential for R. jostii RHA1 to grow on 3-hydroxysterols such as cholesterol, but not on 3-oxo sterol derivatives, and that CYP125 performs an obligate first step in cholesterol degradation. The involvement of cyp125 in sterol side-chain degradation was confirmed by disrupting the homologous gene in Rhodococcus rhodochrous RG32, a strain that selectively degrades the cholesterol side-chain. The RG32Δcyp125 mutant failed to transform the side-chain of cholesterol, but degraded that of 5-cholestone-26-oic acid-3β-ol, a cholesterol catabolite. Spectral analysis revealed that while purified ferric CYP125RHA1 was < 10% in the low-spin state, cholesterol (Kο<sub>app</sub> = 0.20 ± 0.08 μM), 5α-cholestanol (Kο<sub>app</sub> = 0.15 ± 0.03 μM) and 4-cholestone-3-one (Kο<sub>app</sub> = 0.20 ± 0.03 μM) further reduced the low spin character of the haem iron consistent with substrate binding. Our data indicate that CYP125 is involved in steroid C26-carboxylic acid formation, catalysing the oxidation of C26 either to the corresponding carboxylic acid or to an intermediate state.

Introduction

Cytochromes P450 (P450s) are a widely distributed class of haem-containing monoxygenases that are present in all domains of life. Their essential roles in diverse metabolic pathways have also generated considerable interest for their use as biocatalysts (Julsing et al., 2008). Genome sequence data analysis has revealed that actinobacteria posses a remarkable number of genes encoding P450s compared with other prokaryotes (Lamb et al., 2006; McLean et al., 2006). For example, Rhodococcus jostii RHA1 harbours 29 genes predicted to encode P450s (McLeod et al., 2006). While the biological function of most of these monoxygenases is still unknown, several of them have been implicated in sterol/steroid catabolism.

The microbial degradation of cholesterol (5-cholestone-3β-ol; Fig. 1, compound I) involves two processes: sterol side-chain elimination and steroid ring opening (Van der Geize and Dijkhuizen, 2004). The order of these two processes in vivo is unknown and may vary between microorganisms. Generally, oxidation of the cholesterol 3β-hydroxyl moiety and isomerization of Δ5 into Δ4 is thought to initiate sterol degradation (Sojo et al., 1997; Chen et al., 2006; Chiang et al., 2008). This transformation is catalysed by either cholesterol oxidase (CHO; MacLachlan et al., 2000) or 3β-hydroxysteroid dehydrogenase (3β-HSD; Yang et al., 2007) and results in the formation of 4-cholestone-3-one (Fig. 1, compound II). Further degradation of 4-cholestone-3-one proceeds via hydroxylation at C26 to initiate side-chain degradation or oxidation of rings A and B analogous to ring degradation of 4-androstene-3,17-dione, resulting in the formation of 2-hydroxyhexa-2,4-diene-oic acid (Fig. 1, compound VI; Van der Geize et al., 2007). Microbial sterol side-chain degradation has been studied at the biochemical level in more detail in Nocardia species and Mycobacterium sp. strains NRRL B-3683 and NRRL B-3805 (Sih et al., 1968a,b; Marsheck et al., 1972; Fujimoto et al., 1982a,b). The latter two are capable of selectively degrading the 17-alkyl side-chains of cholesterol and phytosterols.
Microbial cholesterol side-chain degradation is initiated by C26 hydroxylation followed by further oxidation to the sterol C26-oic acid (Fig. 1, compound III). Subsequent degradation occurs via mechanisms similar to β-oxidation of fatty acids that leads to the formation of a steroid C22-oic acid intermediate (Fig. 1, compound IV) with the concomitant release of propionyl-CoA and acetyl-CoA. The remaining C3 side-chain is released as propionyl-CoA via a different mechanism (Sih et al., 1967; 1968b). Rhodococcus rhodochrous DSM43269 (synonym IFO3338) is able to selectively degrade the sterol side-chain in the presence of iron chelators, which inhibit 3-hydroxy-3β-sterol dehydrogenase (3βHSD) activity (Arima et al., 1978). This phenotype was replicated in a stable multiple gene deletion mutant strain of R. rhodochrous DSM43269 (strain RG32) lacking KSH activity (M.H. Wilbrink, L. Dijkhuizen and R. van der Geize, unpublished). Mutant strain RG32 is completely blocked in steroid ring degradation and capable of selective sterol side-chain degradation, thereby accumulating 1,4-androstadiene-3,17-dione (ADD) (Fig. 1, compound V) and 3-oxo-23,24-bisnorcholesta-1,4-dien-22-oic acid (Δ1,4-BNC) (Fig. 1, compound IV) from sterols. The strain RG32 phenotype thus allows us to specifically analyse sterol side-chain degradation.

To date, genes involved in sterol side-chain degradation have not been identified. Using transcriptomic analysis, we recently identified a cholesterol catabolic gene cluster in R. jostii RHA1 that includes two P450-encoding genes (Van der Geize et al., 2007). Interestingly, ro04679 (cyp125RHA1) was one of the most highly upregulated genes within this cluster during growth on cholesterol, suggesting an important role for this enzyme in cholesterol catabolism. In the RHA1 genome, cyp125 is located proximal to genes predicted to encode β-oxidation enzymes, and suggested to be involved in degradation of the alkyl side-chain of cholesterol (Van der Geize et al., 2007). Moreover, cyp125 is located within the ro04482-ro04705 gene cluster encompassing the mce4 genes, which encode the uptake system for cholesterol and related steroids with unsubstituted alkyl side-chains (Mohn et al., 2008).
Here we report the molecular characterization of CYP125 as a steroid 26-monoxygenase. The cyp125 gene was inactivated in each of *R. jostii* RHA1 and *R. rhodochrous* RG32 and the effect on cholesterol catabolism was elucidated. CYP125 in RHA1 was heterologously expressed and purified, and its binding to cholesterol and its analogues was investigated. This study provides novel insights into bacterial steroid degradation, revealing that degradation in *R. jostii* RHA1 is initiated by side-chain oxidation, not oxidation of the rings.

**Results**

CYP125 possesses conserved amino acid residues for interaction with sterols

Bioinformatic analysis revealed that CYP125 in RHA1 has high amino acid sequence identity with P450s from other actinobacteria, including *Nocardioides farricinacei* strain IFM10152 [Nfa5180, 79% (Ishikawa et al., 2004)] and *Mycobacterium tuberculosis* strain H37Rv [Rv3545c, 69% (Cole et al., 2002)]. These proteins belong to the uncharacterized CYP125 family (subfamily A) of P450 enzymes (Nelson et al., 1996), in which CYP125 in RHA1 has been assigned CYP125A14P (http://dmelson.utmem.edu/biblioe.html#125). These monoxygenases presumably transform lipid-like compounds, as the CYP125 family includes many actinobacterial proteins associated with lipid degradation (Ventura et al., 2007).

Bioinformatic analysis further revealed that the annotated sequence of CYP125 in RHA1 was about 50 residues longer than that of the annotated orthologues. Careful analysis of the cyp125 nucleotide sequence indicated that the start codon most likely is located 159 nucleotides downstream from that in the original annotation, and is preceded by a Shine–Dalgarno sequence (aggag). Thus, cyp125 is a gene of 1257 nucleotides, encoding a protein of 418 amino acids with a calculated molecular mass of 47.2 kDa. The re-annotated sequence of cyp125 (RHA1 genome co-ordinates 493090...4932156) was used in this study.

Amino acid sequence alignments revealed that the actinobacterial CYP125s share the conserved motifs characteristic for the P450 super-family, as well as key residues of cholesterol-transforming eukaryotic P450s (Fig. S1). The latter belong to various families, including: CYP3A4, which performs 4β-hydroxylation of cholesterol; CYP11A1, which transforms cholesterol to pregnenolone via C20–C22 bond-cleavage; CYP27A1, which hydroxylates cholesterol at C27; and CYP46A1, which transforms cholesterol to 24S-hydroxycholesterol (Mast et al., 2006; Pikuleva, 2006; Storbeck et al., 2007). The presence of these conserved residues in CYPs125 and in P450 enzymes known to interact with sterols suggests that sterols are substrates for CYP125.

**CYP125 is essential for growth on 3-hydroxy-sterols**

To elucidate the role of cyp125 in sterol/steroid catabolism, an unmarked single gene deletion mutant strain, RHA1Δcyp125, was constructed. Growth experiments in mineral medium (MM) supplemented with cholesterol revealed that the RHA1Δcyp125 strain was unable to grow on cholesterol (Table 1). To confirm that the observed phenotype was solely due to inactivation of cyp125, a complementation experiment was performed in which cyp125 was supplied in trans. The complemented strain, RHA1Δcyp125+pTip-QC1cyp125, displayed a restored wild-type growth phenotype in MM supplemented with cholesterol (Table 1). Wild-type RHA1 and RHA1Δcyp125 were subsequently grown in mineral liquid media on a range of other sterols, steroids and their metabolites as sole carbon and energy sources (Table S1). RHA1 grew readily on all tested compounds. By contrast, RHA1Δcyp125 failed to grow on epicholesterol, 5α-cholesterol and on the plant sterol mixture β-sitosterol/β-sitostanol/campesterol. Remarkably, growth of strain RHA1Δcyp125 on 3-ketone oxidized derivatives of two of these sterols, 4-cholestene-3-one and 5α-cholestone-3-one, was unimpaired, likely due to degradation of the steroid ring structure (Table S1, Fig. 1). We thus conclude that CYP125 is essential for 3-hydroxy-sterol degradation. The phenotype of RHA1Δcyp125 was investigated further by growing the mutant in mineral liquid media supplemented with cholesterol and an additional non-repressing carbon source (i.e. pyruvate or glycerol). In contrast to the wild-type strain, RHA1Δcyp125 did not significantly transform cholesterol under these conditions (Fig. 2).

| Table 1. Growth in mineral media supplemented with cholesterol (2.5 mM) as sole carbon and energy source of wild-type strain RHA1, mutant strain RHA1Δcyp125, complemented mutant strain RHA1Δcyp125 (RHA1Δcyp125+pTip-QC1cyp125), and RHA1Δcyp125 mutant strain harbouring null vector (RHA1Δcyp125+pTip-QC1) after 10 days of growth. |
|-----------------|-----------------|-----------------|
| Strain          | Protein content (mg l⁻¹) | Residual cholesterol (%) |
| RHA1            | 49 ± 6          | 56 ± 5          |
| RHA1Δcyp125     | 5 ± 3           | 112 ± 4         |
| RHA1Δcyp125+pTip-QC1cyp125 | 57 ± 4 | 55 ± 4         |
| RHA1Δcyp125+pTip-QC1 | 3 ± 3 | 116 ± 16       |
| Control (medium + cholesterol) | 0 | 100 ± 13       |

Non-inoculated mineral medium with cholesterol was included as a negative control. Values represent mean ± standard deviation (n = 3).
We hypothesized that CYP125RHA1 might have a specific role in sterol side-chain degradation. To substantiate this hypothesis, we used R. rhodochrous RG32, a mutant of R. rhodochrous DSM43269 which only degrades the side-chain of cholesterol, transforming it to ADD (Fig. 1, compound V) and Δ1,4-BNC (Fig. 1, compound IV) (Fig. 3A). First, we cloned cyp125 from R. rhodochrous DSM43269 by screening a genomic library of this strain with degenerate PCR primers based on conserved amino acid sequences found in actinobacterial CYP125s. A positive clone, containing 8.7 kb of insert DNA, was obtained, sequenced and analysed. The insert carried cyp125DSM43269, encoding a protein sharing 76% amino acid sequence identity with CYP125RHA1 (Fig. S1). Moreover, the cyp125 locus is similarly organized in R. jostii RHA1 and R. rhodochrous DSM43269. More specifically, the genes immediately downstream of cyp125 in DSM43269 encode proteins sharing 56%, 74% and 86% amino acid sequence identity to those encoded by ro04676, ro04677 and ro04678, respectively, in RHA1. Upstream of cyp125DSM43269, orthologues of ro04654 (82% identity) and ro04653 (82% identity) were located, as well as genes encoding hypothetical proteins that have no counterparts in RHA1.

We then specifically disrupted cyp125 in RG32, yielding mutant strain RG32cyp125. Whole-cell biotransformations of 3-hydroxy-sterols by RG32cyp125 revealed that the mutant was blocked in the ability to degrade sterol side-chains (Fig. 3). Cell cultures of RG32cyp125 incubated with cholesterol showed no formation of ADD or Δ1,4-BNC (Fig. 3B). Similar results were obtained when RG32cyp125 cell cultures were incubated with 5α-cholestanol and β-sitosterol (data not shown). Contrary to RHA1cyp125, cholesterol was rapidly converted by RG32cyp125 to 4-cholestene-3-one and 1,4-cholestenadiene-3-one, which accumulated in the medium (Fig. 3B inset). Indeed, cholesterol-induced cells of RG32 and RG32cyp125 contained high levels of 3β-hydroxy-cholesterol total oxidation activity using cholesterol as a substrate (0.34 and 0.73 μM min⁻¹ mg⁻¹ respectively). By contrast, no extracellular activity was detected in either strain. Reintroduction of cyp125DSM43269 into RG32cyp125 under its native promoter fully restored the ability of the strain to degrade the cholesterol side-chain (Fig. 3C). This excludes the possibility that side-chain degradation in RG32cyp125 was blocked by polar effects rather than by disruption of cyp125 directly.

CYP125 has a role in sterol side-chain degradation

To further investigate the initial cholesterol-transforming enzymes of RHA1, we assayed pyruvate-grown cultures of wild-type RHA1 and mutant strain RHA1Δcyp125 that had been induced with cholesterol for total 3β-hydroxy-steroid oxidation activity (Yang et al., 2007). These studies comprised assays for extracellular and intracellular activities arising from CHO and 3β-HSD. When cholesterol was used as a substrate in these assays, no activity was detected in either supernatants or cell lysates of these cultures, consistent with the lack of transformation of cholesterol by RHA1Δcyp125. By contrast, 3β-hydroxy-steroid oxidation activity was detected in lysates of cholesterol-induced cells of RHA1 (0.27 μM min⁻¹ mg⁻¹) and RHA1Δcyp125 (0.76 μM min⁻¹ mg⁻¹) when 5-pregnene-3β-ol-20-one was used as a substrate in the assay. Overall, these data indicate that CYP125 is essential for cholesterol degradation by RHA1, and that it catalyses an obligate first reaction in the cholesterol catabolic pathway.

Fig. 2. Cholesterol degradation by cell cultures of strains RHA1 and RHA1Δcyp125 grown in mineral liquid media supplemented with pyruvate (20 mM) and cholesterol (2.5 mM). The data represent averages of triplicates. Error bars indicate standard deviations.

Fig. 3. HPLC profiles of whole-cell biotransformations of cholesterol by cell cultures of (A) R. rhodochrous strain RG32 showing the formation of 1,4-androstadiene-3,17-dione (ADD) and 3-oxo-23,24-bisnorcholest-1,4-dien-22-oic acid (Δ1,4-BNC) via selective sterol side-chain degradation, (B) mutant strain RG32Δcyp125 and (C) cyp125DSM43269 complemented mutant strain RG32cyp125. HPLC profiles of whole-cell biotransformations of 5-cholenic acid-3β-ol (D) and 5-cholene-26-oic acid-3β-ol (E) by cell cultures of R. rhodochrous mutant strain RG32cyp125 are also shown. Profiles of authentic ADD (50 μM, F) and Δ1,4-BNC (G), obtained by incubating authentic 3-oxo-23,24-bisnorcholest-4-en-22-oic acid (50 μM, Δ1,4-BNC) with purified Δ1-KST1 (Knol et al., 2008), are included as reference samples. Insets: GC profiles showing the accumulation of 4-cholestene-3-one (2), 1,4-cholestadiene-3-one (3) and 5α-cholestane-3-one (4) from cholesterol (1) by R. rhodochrous mutant strain RG32cyp125, but not strain RG32.
CYP125 initiates sterol side-chain degradation

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CYP125 is involved in formation of the sterol C26-oic acid intermediate

We then tested the ability of mutant strain RG32/cyp125 to convert each of two predicted sterol side-chain degradation pathway intermediates: 5-cholestene-26-oic acid-3β-ol (Fig. 1, compound III) and the C24-oic intermediate 5-cholenic acid-3β-ol. Whole cell biotransformations performed with cultures of mutant strain RG32/cyp125 resulted in conversion of both 5-cholenic acid-3β-ol and 5-cholestene-26-oic acid-3β-ol to ADD and Δ14,15-BNC (Fig. 3D and E). As predicted, RHA1/cyp125 was able to grow on both of these compounds (Table S1). Both diastereomers of 5-cholestene-26-oic acid-3β-ol appeared to be metabolized, because 75 mol% of the added substrate was converted into ADD and Δ14,15-BNC. These results show that CYP125 is essential for the conversion of cholesterol into the C26-oic acid catabolite during sterol side-chain degradation by both RG32 and RHA1.

Production and purification of CYP125RHA1

To biochemically characterize CYP125RHA1, we homologically produced and purified recombinant CYP125RHA1 with a 6-histidine tag. Expression of cyp125RHA1 was first attempted in Escherichia coli BL21(DE3) using T7 promoter-based expression vectors and conditions known to promote expression of P450 proteins, such as the addition of δ-aminolevulinic acid, FeCl₃, trace elements and thiamine (Parikh et al., 1997; Keizers et al., 2004). However, CYP125RHA1 was not produced in significant amounts in E. coli. By contrast, cyp125RHA1 was well expressed in R. jostii RHA1 using the pTip-QC1 vector (Nakashima and Tamura, 2004). Addition of δ-aminolevulinic acid and other additives, usually necessary to promote expression of properly folded and soluble P450 proteins in E. coli, was not needed for homologous production of CYP125RHA1 in R. jostii RHA1.

CYP125RHA1 was purified using Ni²⁺-NTA affinity chromatography and was determined by SDS-PAGE analysis to be in excess of 95% pure. The CO-difference spectrum of purified CYP125RHA1 displayed a maximum at 451 nm (Fig. 4, inset), indicating the haem iron thiolate ligation remained intact throughout the protein’s purification. The absorption spectrum of the purified ferric CYP125RHA1 had a maximum at 392 nm and a shoulder at 422 nm (Fig. 4). Based on analysis of this spectrum (see Experimental procedures), the preparation is estimated to contain ~93% high spin state haem iron.

Spectroscopic analysis of sterol binding

Spectroscopic assays were performed with purified CYP125RHA1 to investigate its binding to sterols. Following the addition of cholesterol (Fig. 5A) or 5α-cholestanol (data not shown) in a solution of 10% 2-hydroxypropyl-β-cyclodextrin, CYP125RHA1 exhibited a spectral change with a pronounced trough at 422 nm and a peak at 392 nm, consistent with the decrease in the low-spin character of the haem iron associated with substrate binding. The difference spectrum also exhibited a perturbation at 395 nm in comparison with the typical type I binding spectrum. A perturbation at the same wavelength was observed upon addition of 5-cholestene-26-oic acid-3β-ol and 5α-cholestane-3β-ol in 10% 2-hydroxypropyl-β-cyclodextrin, although the acid elicited no underlying type I spectral change at concentrations up to 20 μM (Fig. 5A). Cholesterol also induced a type I binding spectrum when added in the presence of other solubilizing agents, such as Triton WR1339 and dimethylsulphoxide. However, the spectral shifts were much weaker than in the presence of 2-hydroxypropyl-β-cyclodextrin (data not shown).

Using Eq. 1, apparent Kᵦ values for cholesterol, 5α-cholestan-3β-ol, and 4-cholestene-3-one were calculated to be 0.20 ± 0.08 μM, 0.15 ± 0.03 μM and 0.20 ± 0.03 μM respectively. The concentrations of enzyme calculated using this equation (4.0, 4.3 and 3.6 μM respectively) were within 15% of the enzyme concentration determined by spectrophotometry (4.0, 4.3 and 3.6 μM respectively) were within 15% of the enzyme concentration determined by spectrophotometry (Table S1). The concentration of enzyme calculated using this equation (4.0, 4.3 and 3.6 μM respectively) were within 15% of the enzyme concentration determined by spectrophotometry (4.0, 4.3 and 3.6 μM respectively) were within 15% of the enzyme concentration determined by spectrophotometry (4.0, 4.3 and 3.6 μM respectively) were within 15% of the enzyme concentration determined by spectrophotometry.
each binding experiment, indicating that the haem-thiolate ligation remained intact.

**Discussion**

The current study presents several lines of evidence identifying CYP125 as a steroid 26-monoxygenase that catalyses the initial step in microbial sterol side-chain degradation (Fig. 1). First, a cyp125 deletion mutant of *R. jostii* RHA1 was unable to grow on or transform several 3-hydroxy-sterols with relatively long unactivated aliphatic side-chains. Second, a cyp125 disruption mutant of *R. rhodochrous* RG32 was completely blocked in cholesterol side-chain degradation. However, this mutant was still able to degrade the side-chain of 5-cholesten-26-oic acid-3β-ol (Fig. 1, compound III), an expected intermediate of cholesterol side-chain degradation. Mutant RG32cyp125 thus is unable to form the sterol C26-oic acid intermediate, strongly indicating that CYP125 catalyses the oxidation of the sterol at C26. Finally, CYP125RHA1 bound cholesterol, 5α,7α-cholestane-3β,5β-diol and 4-cholesten-3-one in a manner typical of P450 substrates: each compound induced a transition in the spin state of the haem iron and each bound with apparent submicromolar dissociation constants. The conclusion that CYP125 is a steroid 26-monoxygenase extends previous studies in which an NADH-dependent mixed function oxidase system was reported to be responsible for the first step in the mycobacterial sterol side-chain degradation pathway (Szentirmai, 1990) catalysing sterol C26-oxidation (Ambrus *et al.*, 1995).

Our data indicate that the oxidation of C26 is an essential first step of cholesterol degradation in *R. jostii* RHA1. The RHA1Δcyp125 mutant not only failed to detectably transform cholesterol, but grew on 3-oxo steroids, such as 4-cholesten-3-one and 5α-cholestan-3-one, as effectively as the wild-type strain. This indicates that in *R. jostii* RHA1, C26-oxidation precedes oxidation of the 3β-hydroxy moiety (Fig. 1). RHA1Δcyp125 is likely able to grow on 3-oxo steroids by degrading steroid A and B rings, resulting in the formation of 2-hydroxyhexa-2,4-diene-oic acid that is further metabolized to form pyruvate and propionyl-CoA for growth (Fig. 1; van der Geize *et al.*, 2007). Previously, it was suggested that the microbial catabolism of cholesterol was initiated by ring oxidation (Sojo *et al.*, 1997; Chen *et al.*, 2006; Chiang *et al.*, 2008). Indeed, *R. rhodochrous* RG32Δcyp125 is capable of performing ring oxidation in the absence of CYP125, illustrating that the order of ring oxidation and sterol side-chain oxidation may vary between different species of bacteria. Consistent with the conclusion that CYP125RHA1 initiates cholesterol degradation, genes encoding putative CHOs in RHA1 (ro03863, ro04305, ro06201) were not upregulated during growth on cholesterol and are located outside of the cholesterol catabolic gene cluster (McLeod *et al.*, 2006; Van der Geize *et al.*, 2007). Although 3β-HSD has not been definitively identified in RHA1, ro04707 encodes a protein sharing 43% amino acid similarity with 3β-HSD of *M. tuberculosis* (Rv1106c) and is located proximal to the genes encoding the Mcd4 steroid transporter (Mohn *et al.*, 2008). Indeed, ro04707 was upregulated in cholesterol-grown RHA1 cells (Van der Geize *et al.*, 2007). While no cholesterol-transforming 3β-HSD activity was detected in RHA1, a 3β-HSD was expressed that transformed 5-pregnene-3β-ol-20-one, a 3β-hydroxysteroid with a shortened C21 side-chain. 3β-HSDRHA1 thus appears to...
have a high substrate specificity for side-chain-degraded cholesterol metabolites. This is similar to 3β-HSD of *M. tuberculosis* (Rv1106c) which had threefold higher activity towards 5-pregnene-3β-ol-20-one compared with cholesterol (Yang *et al.*, 2007). It is possible that in RHA1, side-chain and ring degradation occur concurrently after C26 and C3-ol have been oxidized.

It is unclear whether CYP125 catalyses the oxidation of C26 to the corresponding carboxylic acid or only to an intermediate state. Various P450s have been reported to catalyse multistep oxidations (Helliswell *et al.*, 1999; 2001; Ro *et al.*, 2006), including a P450 from *Pseudomonas putida* PpG777 which catalyses two sequential oxidations of linalool, to 8-hydroxylinalool and 8-oxolinalool respectively (Ropp *et al.*, 1993). It was proposed that a second oxygenation step results in a transient gem-diol adduct that spontaneously dehydrates to a more stable carbonyl compound (Ullah *et al.*, 1990). Interestingly, CYP125RHA1 displays significant amino acid sequence identity (32%) with linalool 8-monooxygenase and thus might well catalyse the complete oxidation of the aliphatic sterol side-chain into the sterol 26-oic acid intermediate via a similar mechanism. Attempts to reconstitute the activity of CYP125RHA1 have so far proved unsuccessful despite using a variety of electron donors, including the spinach ferredoxin and ferredoxin-reductase electron transport chain and the peroxide shunt using cumene hydroperoxide (Hrycay *et al.*, 1975). The physiological reductase of CYP125RHA1 has not been identified yet.

*Mycobacterium tuberculosis* contains a CYP125 encoded by *rv3545c* located within the recently described *igr* operon (Chang *et al.*, 2007; 2009). The bioinformatic data strongly suggest that the CYP125s of RHA1 and *M. tuberculosis* perform the same function: they are reciprocal best hits with 69% amino acid sequence identity that both occur in the cholesterol catabolic gene cluster (Van der Geize *et al.*, 2007). However, the recently reported phenotype of an *Δigr* mutant indicates that Rv3545c is not a steroid 26-hydroxylase (Chang *et al.*, 2009): the mutant appeared to partially degrade cholesterol and transform the cholesterol labelled with 14C at C26 into mycobacterial lipids. Additional studies are clearly required to definitively establish the role of CYP125 in *M. tuberculosis*. Indeed, while it is unclear if cholesterol degradation in mycobacteria occurs in the same manner as in *R. jostii* RHA1, two studies suggest that it does. First, *Mycobacterium* sp. NRRL B-3683, a mutant strain blocked in steroid ring degradation and able to selectively degrade the sterol side-chain, displayed a clear preference for substrates possessing a 3β-hydroxy-Δ5 ring configuration compared with the 3-keto-Δ4 configuration (Marsheck *et al.*, 1972). Second, 3β-HSD of *M. tuberculosis* (Rv1106c) showed threefold higher activity towards 5-pregnene-3β-ol-20-one, a sterol with a C21 side-chain, compared with cholesterol, suggesting that sterols with shortened side-chains are preferred substrates of 3β-HSD (Yang *et al.*, 2007). Regardless of the precise function of CYP125 in *M. tuberculosis*, its gene is upregulated during growth of *M. tuberculosis* in macrophages (Kendall *et al.*, 2004) and CYP125H37Rv is more resistant to nitric oxide than other P450s of H37Rv (Ouellet *et al.*, 2009). Moreover, the gene appears to be important for infection in mice (Chang *et al.*, 2007; 2009). CYP125 may thus be an interesting target for the development of novel antituberculosis drugs.

### Experimental procedures

#### Bacterial strains, plasmids and chemicals

Plasmids and bacterial strains used are listed in Table S2. 5-Cholestone-3β-ol, 5α-cholestone-3β-ol, 4-cholestone-3-one and 5-cholestone-24β-ethyl-3β-ol (75%) were obtained from Sigma-Aldrich. 5-Cholestone-3α-ol, 23,24-bisnor-5-cholestone-3β-ol, 5-choleneic acid 3β-ol and 1-(5α)-androstene-3,17-dione were obtained from Steraloids. 4-Androstene-3,17-dione and 9,17-dioxo-1,2,3,4,10,19-hexanorandrostane-5-ol acid were provided by Schering-Plough (Oss, the Netherlands).

#### Construction of *R. jostii* RHA1Δcyp125

A cyp125 unmarked single gene deletion mutant of *R. jostii* RHA1 was constructed using the sacB counter-selection system (Van der Geize *et al.*, 2001). Genomic DNA of *R. jostii* RHA1 was isolated as described (Van der Geize *et al.*, 2000). Mutagenic plasmid pDELcyp125RHA1 was constructed for cyp125 deletion, as follows. The upstream region of cyp125 was amplified by PCR using forward primer 5′-tcgac atcactgtgaagctcggcgc-3′ and reverse primer 5′-ccgACTAGTtgaccccctg-3′, resulting in a 1451 bp amplicon of the cyp125 downstream flanking region including the cyp125 stop codon was obtained using forward primer 5′-ccgACTAGTtagactccctgtaccgcggcggg-3′ (Spc restriction site) and reverse primer 5′-cgcAGCTTgagactccctgtaccgcggcggg-3′ (HindIII restriction site). This amplicon was digested with Spel/HindIII and ligated into Spel/HindIII linearized plK18mobsacB UPcyp125. A 1451 bp ampiclon of the cyp125 downstream flank region including the cyp125 stop codon was obtained using forward primer 5′-ccgACTAGTtagactccctgtaccgcggcggg-3′ (Spc restriction site) and reverse primer 5′-cgcAGCTTgagactccctgtaccgcggcggg-3′ (HindIII restriction site). This amplicon was digested with Spel/HindIII and ligated into Spel/HindIII linearized plK18mobsacB UPcyp125, resulting in pDELcyp125RHA1. Deletion of cyp125 from RHA1 was confirmed by PCR using forward 5′-gctctga gattactgtgagctg-3′ and reverse primer 5′-ctcggagacagaa ggaacagc-3′.

#### Functional complementation of mutant strain

RHA1Δcyp125 was performed by electrotransformation (Van der Geize *et al.*, 2000) of RHA1Δcyp125 cells with expression plasmid pTip-QC1cyp125RHA1 (see below).

#### Growth of *R. jostii* RHA1 and mutant RHA1Δcyp125 strain on sterols/stereoids

Pre-cultures of wild-type strain RHA1 and mutant strain RHA1Δcyp125 were grown for 3 days at 30°C with shaking...
(220 r.p.m.) in MM (Masai et al., 1995) supplemented with pyruvate (20 mM) and used to inoculate MM liquid media (1:50) supplemented with various sterols/steroids (1 g l⁻¹; Table S1) as sole carbon and energy source. Biomass production of R. jostii RHA1 cell cultures incubated with cholesterol were quantified by total protein content determination of sonicated cells (10 cycles of 30 s at 8 µm) using the Bradford protein assay (Bio-Rad, Hercules, CA) with BSA as protein standard.

### Biotransformation of cholesterol by R. jostii RHA1 and mutant RHA1Δcyp125

For biotransformation of cholesterol, precultures of RHA1 and RHA1Δcyp125 were grown in MM supplemented with pyruvate (20 mM) for 3 days at 30°C with shaking (220 r.p.m.). The precultures were used to inoculate MM liquid media (1:50) containing pyruvate (20 mM) and cholesterol (2.5 mM).

### Determination of intracellular and extracellular total 3β-hydroxysteroid oxidation activity

Total 3β-hydroxysteroid oxidation activity was determined by high-performance liquid chromatography (HPLC) analysis essentially as described by Yang et al. (2007). Cell cultures of RHA1 and RHA1Δcyp125 were grown in MM supplemented with pyruvate (20 mM) for 3 days to an OD₆₀₀ of 3. Cell cultures of RG32 and RG32Δcyp125 were grown overnight in Luria–Bertani (LB) medium. Grown cultures were induced for 16 h by adding 0.5 mM cholesterol from a 10 mM stock prepared in isopropanol. The cell cultures (50 ml) were pelleted and the resulting supernatants were filter-sterilized and used for assaying extracellular cholesterol oxidation. The cell pellets were washed twice with 50 mM phosphate buffer (pH 7) supplemented with 5% (v/v) isopropanol and resuspended in 2 ml of the same buffer. Cell lysates were prepared by bead-beating. Cell lysates were centrifuged to remove cell debris. The 3β-hydroxysteroid oxidation assay was performed in 100 mM triethanolamine hydrochloride buffer (pH 8.5) supplemented with 0.05% (v/v) Triton X-100, 3.5 mM NAD⁺ and either 200 µM cholesterol or 200 µM 5-pregnen-3β-ol-20-one and incubated at 30°C for several hours (Yang et al., 2007). 4-Cholesterol-3-one and 1,4-cholestadiene-3-one were incubated at 30°C for 24 h. Total 3β-hydroxysteroid oxidation activity was determined by HPLC-UV 254 nm using calibration curves.

### Steroid analysis

Steroid content of the cell cultures was analysed by HPLC and gas chromatography (GC). Culture samples (0.5 ml) were mixed with 2 ml of 80% methanol in water solution and filtered (0.2 µm) prior to analysis by HPLC-UV 254 nm. HPLC was performed on an Alltima C18 column (250 × 4.6 mm; Alltech, Deerfield, USA, 35°C) using a mobile phase consisting of methanol : water (80:20) supplemented with 1% formic acid at a flow rate of 1 ml min⁻¹. For analysis of 4-cholestene-3-one and 1,4-cholestadiene-3-one a mobile phase consisting of acetonitrile : tetrahydrofuran (75:25) at a flow rate of 2 ml min⁻¹ was used. Samples (0.5 ml) for GC analysis were mixed with 10% H₂SO₄ (10 µl) and ethyl acetate (2 ml) and the upper organic layer was subjected to GC. GC was performed on a (5% phenyl)-95% methoxypoly siloxane Heliflex AT-5 ms column (30 m × 0.25 mm, ID × 0.25 µm; Alltech, Deerfield, USA) with FID-40 detection at 300°C.

### Production of CYP125RHA1

The cyp125RHA1 gene was amplified by PCR on genomic DNA of RHA1 with forward primer 5'-CATATGGcgagcccaaattctcaggagg-3', containing an NdeI restriction site, and reverse primer 5'-GGATCTcagtgtctgccgcaacccg-3', containing a BamHI restriction site, such that the recombinant protein contains a 6-histidine tag. PCR was performed in a reaction mixture (25 µl) consisting of Tris-HCl (10 mM, pH 8), polymerase buffer, dNTP (0.2 mM), primers (0.8 µM) and Vent polymerase (0.1 U, New England Biolabs, Ipswich, MA) under the following conditions: 5 min 95°C, 30 cycles of 45 s 95°C, 45 s 65°C, 2 min 72°C, followed by 5 min at 72°C. A band of the expected size for cyp125RHA1 (1266 bp) was purified from agarose gel using GenElute Gel Extraction Kit (Sigma-Aldrich, Steinheim, Germany) and cloned into Smal-digested pBlueScript KS(II) (Strategene, La Jolla, CA, USA). The resulting plasmid was digested with NdeI and BamHI and the DNA fragment containing cyp125RHA1 was ligated into NdeI/BamHI-digested pTip-QC1.

CYP125RHA1 was homologously produced in R. jostii RHA1 using expression plasmid pTip-QC1cyp125RHA1. Cells were cultured in LB broth in the presence of 25 µM l⁻¹ chloramphenicol. R. jostii RHA1 cells were transformed with pTip-QC1cyp125RHA1 by electroporation and grown on LB-agar plates containing 25 µg ml⁻¹ chloramphenicol for 2 days, after which a single colony was used to inoculate 50 ml of liquid medium which was incubated at 30°C (200 r.p.m.). When OD₆₀₀ reached ~1.0 (~2–3 days) 2 l of medium inoculated with 20 ml of this preculture was incubated at 30°C. When the culture reached an OD₆₀₀ of 0.6, thioestropin was added to a final concentration of 50 µg ml⁻¹ and the cells were incubated for a further 20 h before harvesting by centrifugation (4600 g, 4°C, 10 min) and subsequent washing with 0.1 M potassium phosphate buffer, pH 8.0. Cell pellets were flash frozen in liquid nitrogen and stored at −80°C until use.

### Purification of CYP125RHA1

The cell pellets were suspended in potassium phosphate buffer (pH 7.4) (Lussenburg et al., 2005) containing DNase I (Roche diagnostics, IN). Cells were disrupted by bead beating and debris was removed by centrifugation at 10 000 g for 45 min at 4°C. The clear supernatant was passed through a syringe-driven 0.45 µm filter. Cell free extracts were loaded on a NTA column (Qiagen) equilibrated with 0.1 mM potassium phosphate, pH 7.4. The protein was washed with Buffer A containing 0.5 M NaCl and a brown fraction eluted with buffer A further supplemented with 50 mM L-histidine. The protein was exchanged into 0.1 M potassium phosphate, pH 7.4, concentrated to 20 mg ml⁻¹, flash frozen as beads in liquid nitrogen and stored at −80°C. P450 protein

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concentrations were calculated from the reduced CO-bound difference spectrum using the extinction coefficient $\varepsilon_{450-490} = 91 \text{ M}^{-1} \text{ cm}^{-1}$ (Omura and Sato, 1964).

**Spectroscopic analysis**

UV-vis absorption spectra were recorded using a Cary 5000 spectrophotometer equipped with a thermostatted cuvette holder (Varian, Walnut Creek, CA). The CO-bound form of CYP125$_{DSM43269}$ was generated by first incubating samples with $-8 \text{ mM sodium dithionite for 10 min then slowly bubbling}$ them with CO for 30 s. The proportion of purified protein containing high-spin ferric haem iron was estimated by comparing the spectra of CYP125$_{DSM43269}$ to linear combinations of the spectra of CYP125$_{RHA1}$ and low spin states (Jung et al., 1991; Jefcoat, 1978) generated by adding 0.5% Triton X-100 and 40% methanol, respectively, to the sample. The same values were obtained when using substrate-free cytochrome P450$_{et al}$ from $P. putida$ as a low spin standard. Substrate-induced spectral responses were recorded in 0.1 mM KPI, pH 7.0 by titrating solutions of CYP125$_{RHA1}$ with 1.0 mM stock solutions of cholesterol, 5α-cholestan-3β-ol, and 4-cholesten-3-one in 10% 2-hydroxypropyl-$β$-cyclodextrin (Sigma). Equilibrium dissociation constants were calculated using Eq. 1.

\[
\Delta A = \frac{[S]_t + [E]_t + K_e - \sqrt{([S]_t + [E]_t + K_e)^2 - 4[S]_t[E]_t}}{2[E]_t} \Delta A_{max}
\]

(1)

In this equation, $\Delta A$ is the change in absorbance observed in the sample, $[S]_t$ is the total ligand concentration, $[E]_t$ is the total enzyme concentration, $K_e$ is the equilibrium dissociation constant, and $\Delta A_{max}$ is the change in absorbance at infinite ligand concentration. A non-linear least-squares fit of the data was obtained using the program R (http://www.R-project.org).

**Construction of R. rhodochrous RG32Δcyp125**

The cyp125 orthologue in $R. rhodochrous$ DSM43269 (cyp125$_{DSM43269}$) was identified using degenerate cyp125 primers (forward 5′ (a/g)ca(c/g)t/1gc(a/c/g)t/cc(a/c/g)t/at(a/c/t)tg/tgg.ta and reverse 5′-gg(a/g)/tt/c/ct/a/g/aa(a/c/g/t)g/cc(a/g)/ct/ct(a/g)) based on the deduced amino acid sequences T$^{34}$APIWWN$^{39}$ and $\text{D}^{321}$EDAFENP$^{336}$ from CYP125$_{RHA1}$; these sequences are highly conserved in Nfai5180 and Rv3545c from $R. farcinica$ IFM10152 and $M. tuberculosis$ H37Rv respectively. A genomic library of $R. rhodochrous$ DSM43269 in pRESO (Petrusma et al., 2009; Table S2) was screened by PCR using these degenerate primers. A single clone (pRESO4679) was identified containing an 8.7 kb DNA insert. Nucleotide sequencing confirmed the presence of full-length (1254 bp) cyp125 (DDBJ/EMBL/Genbank Accession No. FJ824698).

The cyp125 gene was disrupted in $R. rhodochrous$ strain RG32 essentially as described (Van der Geize et al., 2000). An internal cyp125$_{DSM43269}$ fragment of 811 bp was amplified by PCR using forward primer 5′-gcagaggtctgccgtggt and reverse primer 5′-cttttgcgccgctgccgct and ligated into EcoRV-digested pK18mobSacB, yielding pKCyp125.
temperature. After removal of the salts, extractive work up and a precipitation from acetone/water, the 16, 27-dihydroxylated product (product 2) was obtained in near 100% yield. A regio selective protection of the primary alcohol at C27 was carried out by reaction with tert-butylidimethylsilyl chloride and imidazole in N,N-dimethylformamide with 97% yield (product 3). For removal of the 16-hydroxy group, the Barton deoxygenation conditions were chosen. The C16-hydroxy group was transformed in the corresponding thiocarbonate with CS2 under the influence of NaH. The intermediate thiocarbonate anion was quenched with methyl iodide. Next, a radical reduction reaction was carried out using Bu3SnH and AIBN. After purification by silica gel column chromatography, the tert-butylidimethylsilyl-protected 3-methyl ether form of 27-hydroxycholesterol (product 4) could be isolated in approximately 95% and consisting of a 1:1 mixture of stereo-isomers. The structure was confirmed by protonation at C26 and a 1:1 mixture of diastereomers at C26. Apparently, during the strong acidic conditions used for the removal of the 3-methyl ether, enolization and protonation at C26 had occurred giving rise to a 1:1 mixture of stereo-isomers. The structure was confirmed by mass spectrometry (Fig. S3).

Acknowledgements

We are grateful to N. P. E. Vermeulen, J. Commandeur and B. van Vugt-Lussenburg (VU Amsterdam) for technical advice and support. We thank T. Tiemersma-Wegman (University of Groningen) for technical assistance in GC analyses. We thank T. Tamura for kindly providing pTip-QC1. This work was supported by grants from the Integration of Biosynthesis and Organic Synthesis program of Advanced Chemical Technologies for Sustainability and the Canadian Institutes for Health Research (to L.D.E.). J.C. is the recipient of studentships from NSERC of Canada and MSFHR.

References


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