Cytochrome P450 125 (CYP125) catalyses C26-hydroxylation to initiate sterol side-chain degradation in Rhodococcus jostii RHA1

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Published in:
Molecular Microbiology

DOI:
10.1111/j.1365-2958.2009.06915.x

Supplemental Figure 1. Partial amino acid sequence alignment of actinobacterial and mammalian cytochrome P450 proteins: *R. jostii* RHA1 CYP125 (accession number ABG96465), *R. rhodochrous* DSM43269 CYP125 (FJ824698), *M. tuberculosis* H37Rv CYP125 (NP_218062), bovine CYP11A1 (NP_788817), human CYP27A1 (NP_000775), human CYP3A4 (P08684) and human CYP46A1 (NP_006659). The alignment was generated using ClustalW, MEGA version 3.1 (Kumar *et al*., 2004). Specific residues discussed in the text in bold are indicated (V295, R300 and R343 of $Cyp125_{RHA1}$). Structure homology modeling of the CYP11A1:cholesterol complex revealed that the conserved arginine (R357, corresponding to R300 of $CYP125_{RHA1}$) points towards the docked cholesterol and is believed to be involved in catalysis (Storbeck *et al.,* 2007). Moreover, this residue was shown to be one of the active site residues in CYP46A1 (Mast *et al*., 2008) and has been recently identified in P450s (e.g. CYP3A4) as a heme-interacting residue involved in substrate regio-selectivity and specificity (Seifert and Pleiss, 2009). For its part, the conserved arginine of CYP27A1 (R415, corresponding to R343 of $CYP125_{RHA1}$) was shown to be connected via a tripartite salt-bridge with the ExxR motif, forming an ERR-triad as part of the active site cavity (Prosser *et al.,* 2006; Masuda *et al.,* 2007). Finally, in CYP27A1, V367 (corresponding to V295 of $CYP125_{RHA1}$) is an active site residue and appears to be crucial for regioselectivity of hydroxylation of both cholesterol and 5β-cholestane 3α,7α,12α-triol (Mast *et al.,* 2006). Conserved motifs of the P450s super-family (ExxR and PxxF), the oxygen-binding domain (GxxT) and the heme binding domain (F[x]xxGxxxCxG) (Mestres, 2005; Huang *et al*., 2008) are also shown.

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                                    GxxT
CYP125_RHA1       235 LSPEEFGFFVILLAVAGNETTRNAITHGMMAFLDHPD
CYP125_DSM43269   235 LAPEEFGFFFIVLAVAGNETTRNAITHGMAAFLDNPE
CYP125_H37Rv      253 LSDDEFGFFVVMLAVAGNETTRNSITQGMMAFAEHPD
CYP11A1           272 MLLEDVKANITEMLAGGVNTTSMTLQWHLYEMARSLN
CYP27A1h          287 LSPREAMGSLPELLMAGVDTTSNTLTWALYHLSKDPE
CYP3A4h           290 LSDLELVAQSIIFIFAGYETTSSVLSFIMYELATHPD
CYP46A1h          287 QDDEGLLDNFVTFFIAGHETSANHLAFTVMELSRQPE

                          ExxR    V295 R300                                  PxxF  R343
CYP125_RHA1       282 KTTADEIVRWATPVNSFQRTALEDTELGGVQIKKGQRVVMLYGSANFDEDAFENPEKFDIMR
CYP125_DSM43269   282 KTAADEIIRWATPVTSFQRTALEDTELGGQTIRKGERVVMLYASANNDEEVFENPREFDILR
CYP125_H37Rv      300 ETAADEIVRWATPVTAFQRTALRDYELSGVQIKKGQRVVMFYRSANFDEEVFQDPFTFNILR
CYP11A1           339 KASIKETLRLHPISVTLQRYPESDLVLQDYLIPAKTLVQVAIYAMGRDPAFFSSPDKFDPTR
CYP27A1h          354 KAVLKETLRLYPVVPTNSRIIEKEIEVDGFLFPKNTQFVFCHYVVSRDPTAFSEPESFQPHR
CYP3A4h           357 DMVVNETLRLFPIAMRLERVCKKDVEINGMFIPKGVVVMIPSYALHRDPKYWTEPEKFLPER
CYP46A1h          354 SQVLKESLRLYPPAWGTFRLLEEETLIDGVRVPGNTPLLFSTYVMGRMDTYFEDPLTFNPDR

                          FxxxGxxxCxG
CYP125_RHA1       349 VGFGGTGAHFCLGANLARLEIDL
CYP125_DSM43269   349 LAFGGTGAHYCLGANLARMEIDL
CYP125_H37Rv      367 VGFGGTGAHYCIGANLARMTINL
CYP11A1           414 LGF-GWGVRQCVGRRIAELEMTL
CYP27A1h          434 VPF-GYGVRACLGRRIAELEMQL
CYP3A4h           433 TPF-GSGPRNCIGMRFALMNMKL
CYP46A1h          428 FPF-SLGHRSCIGQQFAQMEVKV
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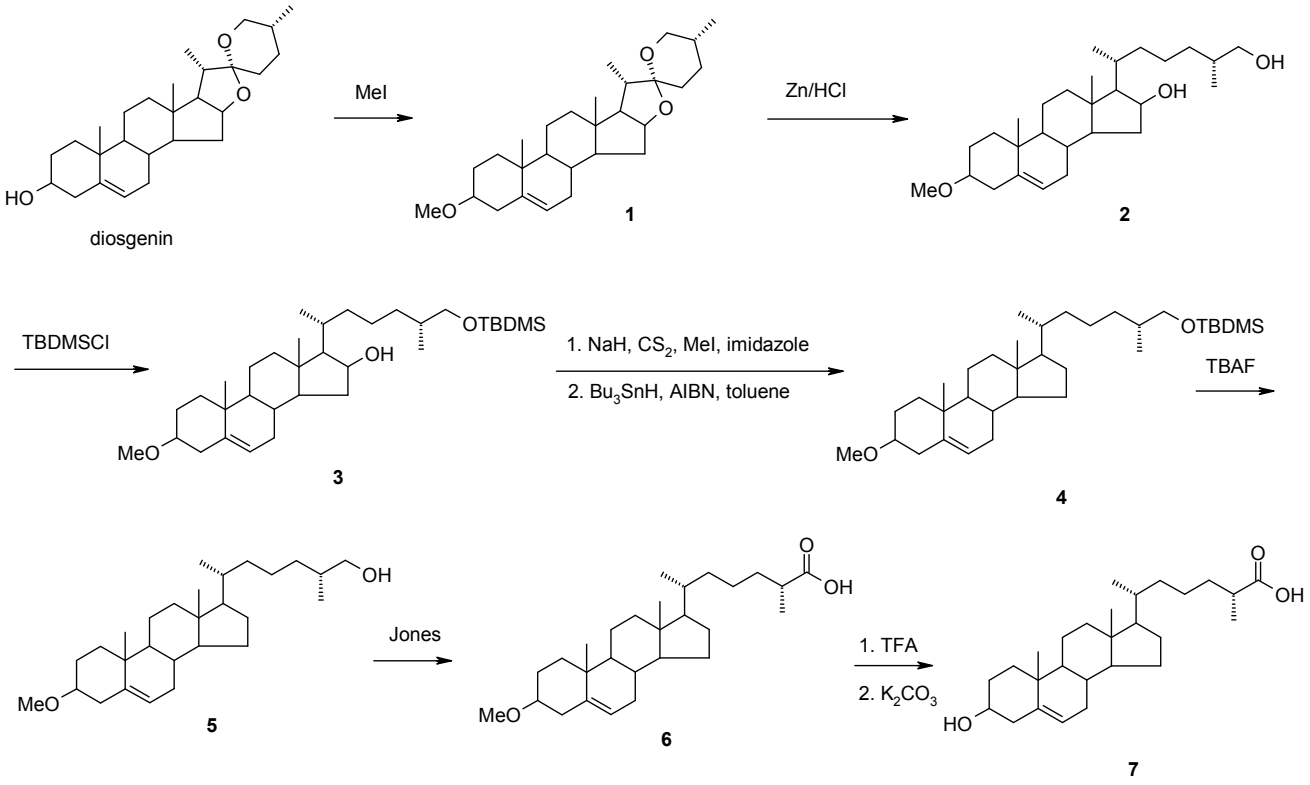
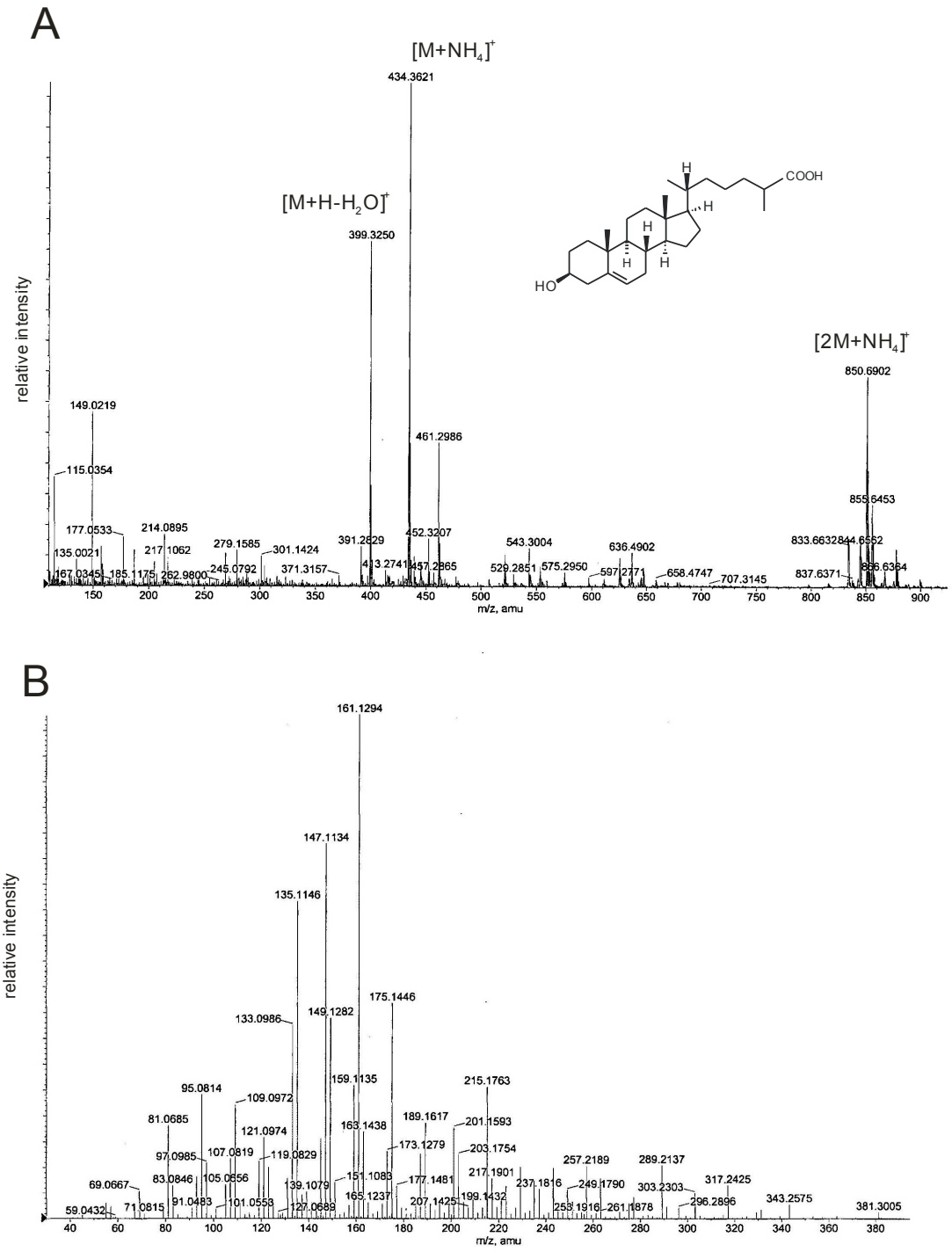
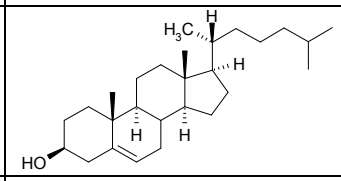
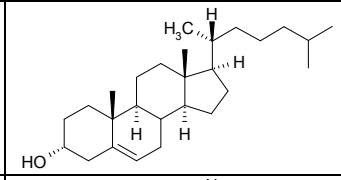
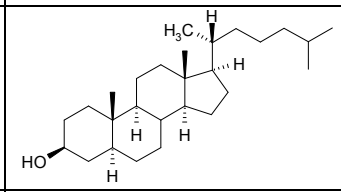
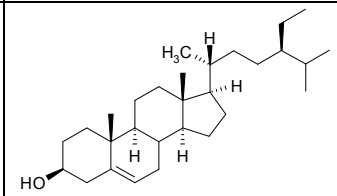
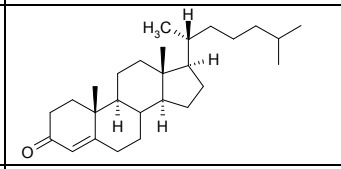
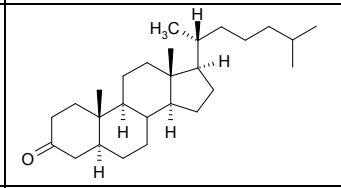
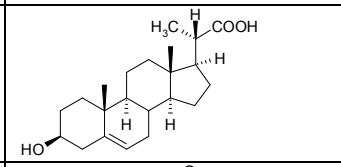
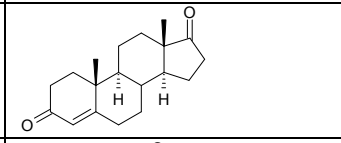
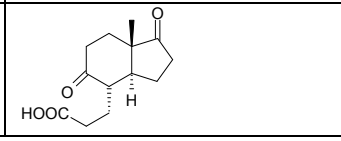
Supplemental Figure 2. Scheme of the chemical synthesis of 5-cholestene-26-oic-acid-3β-ol from diosgenin.

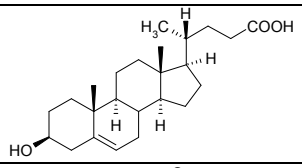
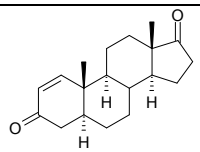
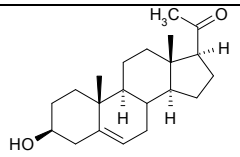
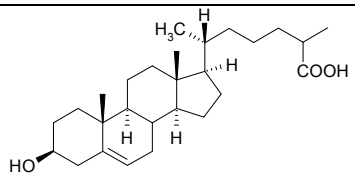


Supplemental Figure 3. (A) MS spectrum with fragmentation pattern of chemically synthesized 5-cholestene-26-oic acid-3β-ol. (B) MS-MS spectrum with fragmentation pattern of ion peak with m/z 399.



Supplemental Table 1. Growth of wild type *Rhodococcus jostii* strain RHA1 and mutant strain RHA1Δ*cyp125* on different sterols and related organic substrates. Growth is indicated with (+), whereas no growth is indicated with (-).

Steroid name	Steroid formula	RHA1	RHA1Δ*cyp125*
cholesterol (5-cholestene-3β-ol)		+	-
Epicholesterol (5-cholestene-3α-ol)		+	-
5α-cholestanol (5α-cholestane-3β-ol)		+	-
β-sitosterol* (5-cholestene-24β-ethyl-3β-ol)		+	-
Cholestenone (4-cholestene-3-one)		+	+
cholestanone (5α-cholestane-3-one)		+	+
23,24-bisnor-5-cholenic acid-3β-ol		+	+
4-androstene-3,17-dione		+	+
9,17-dioxo-1,2,3,4,10,19-hexanorandrostane-5-oic acid		+	+

5-cholenic acid-3β-ol		+	+
1-5α-androstene-3,17-dione		+	+
5-pregnene-3β-ol-20-one		+	+
5-cholestene-26-oic acid-3β-ol		+	+

* Mixture of β-sitosterol (>75 %) with β-sitostanol (10-14 %) and campesterol (6-9 %) was used.

Supplemental Table 2. Strains and plasmids used in this study.

Strain	Characteristics	Reference
Rhodococcus jostii RHA1	Wild type strain, PCB degrader	(Masai *et al*., 1995)
Rhodococcus rhodochrous DSM43269	Wild type strain; potent sterol degrader; identical to strain IFO3338	DSMZ culture collection
Rhodococcus rhodochrous RG32	Mutant strain of DSM43269 capable of selective sterol side chain degradation; carries 5-fold unmarked *kshA* gene deletion	Wilbrink *et al.,* unpublished
E. coli DH5α	General host for cloning	Bethesda Res. Laboratories
E. coli S17-1	Host strain for conjugal mobilization of pK18mobsacB derivatives to *Rhodococcus* strains	DSMZ culture collection
Rhodococcus jostii RHA1Δ*cyp125*	*cyp125* gene deletion mutant strain of RHA1; blocked side chain degradation	This study
Rhodococcus rhodochrous RG32Ω*cyp125*	*cyp125* gene disruption mutant strain of RG32; blocked side chain degradation	This study
Plasmid		
pK18*mobsacB*	Conjugative plasmid for gene mutagenesis in *Rhodococcus*; *aphII sacB oriT* (RP4) *lacZ*	(Van der Geize *et al*., 2001)
pBlueScript KS(II)	General *E. coli* cloning vector; *bla lacZ*	Stratagene
pTip-QC1	*Rhodococcus* expression vector; Chlr *bla PtipA repAB* (pRE2895)	(Nakashima and Tamura, 2004)
pRESQ	*E.coli-Rhodococcus* shuttle-vector; *aphII, lacZ-ccdB rep* (pMVS301)	(Van der Geize *et al*., 2002)
pBs-Apra-ori	Conjugative pBlueScript KS(II) derivative; *aacIV*, *oriT* (RP4) *lacZ*	(Van der Geize *et al.*, 2008)
pRRE1	*E. coli- R. rhodochrous* shuttle-vector	This study
pDEL*cyp125*$_{RHA1}$	pK18*mobsacB*-derived mutagenic plasmid for *cyp125*$_{RHA1}$ disruption in RHA1	This study
pTip-QC1*cyp125*$_{RHA1}$	*cyp125*$_{RHA1}$ expression plasmid used for CYP125$_{RHA1}$ production and functional complementation of RHA1Δ*cyp125*	This study
pRESQ4679	Genomic library clone DSM43269 carrying c*yp125*$_{DSM43269}$	This study
pΩ*cyp125*	pK18*mobsacB* derived mutagenic plasmid for *cyp125* gene disruption in RG32	This study
pCOMP*cyp125* $_{DSM43269}$	pRRE1-derived plasmid carrying *cyp125*$_{DSM43269}$; functional complementation of RG32Ωcyp125	This study

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