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Molecular and functional characterization of *kshA* and *kshB*, encoding two components of 3-ketosteroid 9α-hydroxylase, a class IA monooxygenase, in *Rhodococcus erythropolis* strain SQ1

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Summary

9α-Hydroxylation of 4-androstene-3,17-dione (AD) and 1,4-androstadiene-3,17-dione (ADD) is catalysed by 3-ketosteroid 9α-hydroxylase (KSH), a key enzyme in microbial steroid catabolism. Very limited knowledge is presently available on the KSH enzyme. Here, we report for the first time the identification and molecular characterization of genes encoding KSH activity. The *kshA* and *kshB* genes, encoding KSH in *Rhodococcus erythropolis* strain SQ1, were cloned by functional complementation of mutant strains blocked in AD(D) 9α-hydroxylation. Analysis of the deduced amino acid sequences of *kshA* and *kshB* showed that they contain domains typically conserved in class IA terminal oxygenases and class IA oxygenase reductases respectively. By definition, class IA oxygenases are made up of two components, thus classifying the KSH enzyme system in *R. erythropolis* strain SQ1 as a two-component class IA monooxygenase composed of KshA and KshB. Unmarked in frame gene deletion mutants of parent strain *R. erythropolis* SQ1, designated strains RG2 (*kshA* mutant) and RG4 (*kshB* mutant), were unable to grow on steroid substrates AD(D), whereas growth on 9α-hydroxy-4-androstene-3,17-dione (9OHAD) was not affected. Incubation of these mutant strains with AD resulted in the accumulation of ADD (30–50% conversion), confirming the involvement of KshA and KshB in AD(D) 9α-hydroxylation. Strain RG4 was also impaired in sterol degradation, suggesting a dual role for KshB in both sterol and steroid degradation.

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Introduction

3-Ketosteroid 9α-hydroxylase (KSH) is a key enzyme in the microbial steroid degradation pathway. Introduction of a 9α-hydroxyl moiety into the steroid polycyclic ring structure, combined with steroid Δ1-dehydrogenation, results in opening of the B-ring of the steroid skeleton (Fig. 1A). KSH activity has been found in many bacterial genera (Martin, 1977; Kieslich, 1985; Mahato and Garai, 1997), e.g. *Rhodococcus* (Datcheva *et al*., 1989; van der Geize *et al*., 2001), *Nocardia* (Strijewski, 1982), *Arthrobacter* (Dutta *et al*., 1992) and *Mycobacterium* (Wovcha *et al*., 1978). In previous work, we demonstrated the presence of KSH activity in *Rhodococcus erythropolis* strain SQ1: inactivation of both *kstD* and *kstD2*, encoding two 3-ketosteroid Δ1-dehydrogenase (KSTD) isoenzymes (KSTD1 and KSTD2 respectively) of *R. erythropolis* SQ1, results in the selective 9α-hydroxylation of AD, producing >90% 9OHAD (van der Geize *et al*., 2001). Well-characterized, stable bacterial mutant strains lacking KSH or KSTD activity, or both, are considered to be very important in sterol biotransformation, potentially producing valuable bioactive steroid molecules from sterols (Fig. 1B). Few efforts have been made to characterize the enzymes and genes involved in microbial steroid catabolism at the molecular level. As a result, limited data are currently available on the catabolic pathways of steroids and sterols, hampering genetic engineering approaches aimed at constructing molecularly defined mutant strains with desired properties. Knowledge of the KSH enzyme is extremely limited, mainly because of difficulties faced during enzyme purification procedures (Chang and Sih, 1964; Strijewski, 1982). A three-component KSH enzyme system has been partially purified from *Nocardia* sp. M117, which is composed of a flavoprotein reductase and two ferredoxin proteins (Strijewski, 1982). In *Arthrobacter oxydans* 317, 9α-hydroxylation of the steroid polycyclic ring structure appeared to be plasmid borne (Dutta *et al*., 1992). However, nucleotide sequence analysis of this plasmid has not been reported. In fact, no nucleotide sequences of genes encoding components of KSH have been reported so far.

The introduction of single hydroxyl groups on the aromatic ring, monohydroxylation, is generally catalysed by

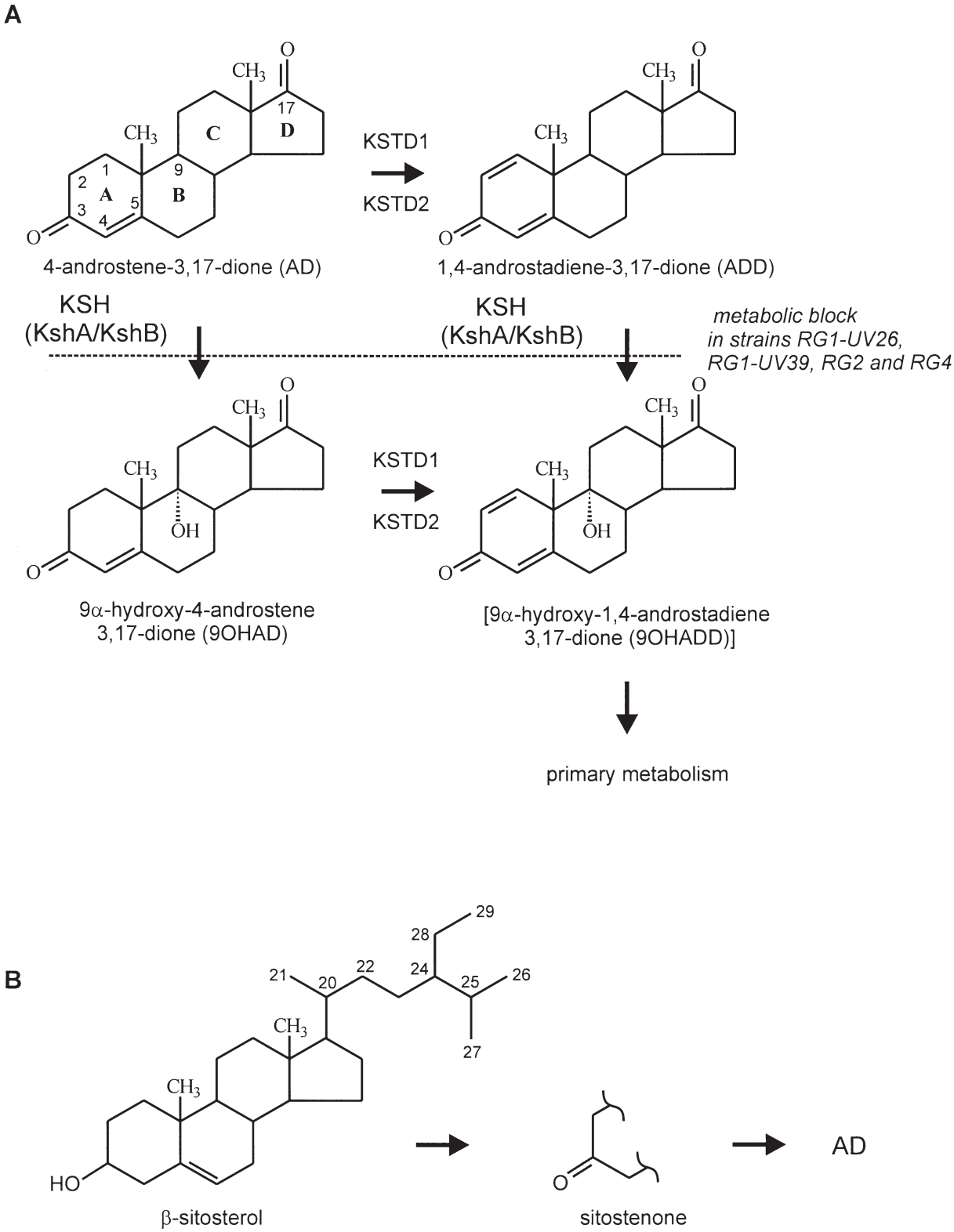

Fig. 1. A. Scheme of the initial enzymatic steps in steroid catabolism in *R. erythropolis* SQ1: 3-ketosteroid 9α-hydroxylase (KSH: KshA, terminal oxygenase component; KshB, oxygenase reductase component) and two 3-ketosteroid Δ1-dehydrogenase isoenzymes [KSTD1 encoded by *kstD* (van der Geize *et al.*, 2000); KSTD2 encoded by *kstD2* (van der Geize *et al.*, 2002)]. The four rings of the steroid polycyclic ring structure are indicated by A, B, C and D and numbered according to steroid nomenclature. The dotted line represents impaired KSH activity by inactivation of either *kshA* or *kshB* via either UV mutagenesis or gene deletion mutagenesis.
B. Biotransformation of the phytosterol 5-cholestene-24β-ethyl-3β-ol (β-sitosterol) and its stenone derivative 5-cholestene-24β-ethyl-3-one (sitostenone) into AD via side-chain degradation.

monooxygenases, whereas the simultaneous introduction of two hydroxyl groups, dihydroxylation, is catalysed by dioxygenases. Classification of mono- and dioxygenases is based on the number of constituent components and the nature of their redox centres (Batie *et al*., 1991; Correll *et al*., 1992; Harayama and Kok, 1992; Mason and Cammack, 1992; Nakatsu *et al*., 1995). Class I oxygenases are two-component enzymes constituting a reductase component and a terminal oxygenase component. The reductase component of oxygenases contains either FMN (class IA) or FAD (class IB), an NAD-binding domain and a [2Fe-2S] redox centre situated either N-terminally (class IB) or C-terminally (class IA). Class II oxygenases are three-component enzymes in which the flavin (reductase component) and [2Fe-2S] redox centre (ferredoxin component) are separate (class IIA: plant-type ferredoxins; class IIB: Rieske-type ferredoxins). Class III oxygenases are three-component oxygenases in which the reductase component contains both a flavin and a [2Fe-2S] redox centre, but also requires a second [2Fe-2S] centre on a ferredoxin component for electron transfer to the third component, i.e. the terminal oxygenase.

Here, we describe for the first time the identification and molecular characterization of two genes, *kshA* and *kshB*, encoding components of the KSH enzyme involved in 9α-hydroxylation of AD and ADD in *R. erythropolis* strain SQ1. Based on conserved features within their deduced primary amino acid sequences, we conclude that KSH of *R. erythropolis* strain SQ1 constitutes a two-component [2Fe-2S] containing class IA monooxygenase. Inactivation of *kshA* and *kshB* separately rendered mutant strains unable to grow on AD(D), resulting in the accumulation of ADD from AD, confirming their role in the initial step of AD(D) degradation.

Results and discussion

Isolation of UV-induced mutants of R. erythropolis *blocked in the AD(D) 9α-hydroxylation reaction*

During selection for mutants of *R. erythropolis* strain RG1 (*kstD* mutant strain, Table 1) blocked in steroid Δ1-dehydrogenation (van der Geize *et al*., 2001), we additionally screened for AD growth-deficient mutants able to grow on 9OHAD mineral medium. Replica plate screening of UV-mutagenized *R. erythropolis* strain RG1 colonies yielded two mutants that were clearly impaired in the KSH reaction (AD^- $9OHAD^+$ growth phenotype). These mutants, designated strain RG1-UV26 and strain RG1-UV39, showed no growth after 3–4 days with either AD or ADD as sole carbon and energy source, whereas growth on 9OHAD mineral agar medium was normal.

Construction of a Rhodococcus–Escherichia coli *shuttle vector pRESQ*

A gene library of *R. erythropolis* strain RG1 was

Table 1. Strains and plasmids used in this study.

Strain or plasmid	Characteristics	Reference/origin
R. erythropolis SQ1	Mutant of ATCC4277-1 with increased transformability	Quan and Dabbs (1993)
R. erythropolis RG1	*kstD* mutant of strain SQ1	van der Geize *et al.* (2001)
R. erythropolis RG1-UV26	UV mutant of strain RG1 unable to grow on AD(D)	This study
R. erythropolis RG1-UV39	UV mutant of strain RG1 unable to grow on AD(D)	This study
R. erythropolis RG2	*kshA* gene deletion mutant of strain SQ1	This study
R. erythropolis RG4	*kshB* gene deletion mutant of strain SQ1	This study
R. erythropolis RG8	*kstD kstD2* double gene deletion mutant of strain SQ1	van der Geize *et al*., 2002
R. erythropolis RG9	*kshA kstD kstD2* triple gene deletion mutant of wild type: constructed from strain RG8	This study
E. coli DH5α	Host for general cloning steps	Bethesda Research Laboratory
E. coli S17-1	Strain for conjugal mobilization of pK18mobsacB derivatives to *Rhodococcus* strains	Simon *et al.* (1983)
E. coli Top10F′	Strain for general cloning steps with pZErO-2.1 and derivatives	Invitrogen
pBlueScript-II KS	*bla lacZ*	Stratagene
pZErO-2.1	*lacZ–ccdB* positive selection marker *aphII*	Invitrogen
pMVS301	*Rhodococcus–E. coli* shuttle vector	Vogt-Singer and Finnerty (1988)
pET3b	*E. coli* expression vector	Novagen
pK18mobsacB	*aphII sacB* oriT (RP4) *lacZ*	Schäfer *et al.* (1994)
pWJ5	*aphII sacB cat*	Jäger *et al.* (1992)
pRESQ	*Rhodococcus–E. coli* shuttle vector	This study
pKSH101	pRESQ containing ±6 kb *R. erythropolis* strain RG1 chromosomal fragment carrying *kshA*	This study
pKSH126	Construct used for introducing a *kshA* gene deletion in strains SQ1 and RG8	This study
pKSH200	pRESQ containing ±6.5 kb *R. erythropolis* strain RG1 chromosomal fragment carrying *kshB*	This study
pKSH212	Plasmid used for the construction of strain RG4, harbouring a *kshB* gene deletion	This study

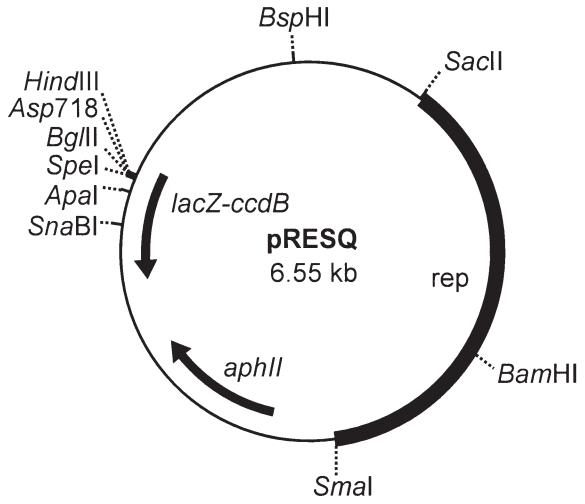

Fig. 2. The pZErO-2.1 (curved thin line)-based *Rhodococcus–E. coli* shuttle vector pRESQ used for constructing a genomic library of *R. erythropolis* RG1. rep, 2.5 kb region of pMVS301 coding for autonomous replication in *Rhodococcus* sp. (thick curved line). *lacZ–ccdB*, marker for positive selection in *E. coli*. *aphII*, kanamycin resistance marker for selection in *Rhodococcus* and *E. coli*.

constructed for complementation of the UV mutants obtained (see *Experimental procedures*). For this purpose, a *Rhodococcus–E. coli* shuttle vector, designated pRESQ (Fig. 2), was constructed. The pZErO-2.1-based shuttle vector contains a 2.5 kb fragment of pMVS301 (Vogt-Singer and Finnerty, 1988), encoding autonomous replication in *Rhodococcus* sp., and a unique *Bgl*II site in the *lacZ–ccdB* positive selection gene for easy cloning of *Sau*3A-digested chromosomal DNA of *Rhodococcus* sp. The unique *Bam*HI site present in pZErO-2.1 had to be replaced by *Bgl*II, as introduction of the 2.5 kb fragment of pMVS301 into pZErO-2.1 introduces an additional *Bam*HI restriction site that could not be removed. Destroying the latter *Bam*HI site by Klenow fill-in rendered plasmids unable to replicate in *R. erythropolis* strain SQ1 (data not shown).

Identification and molecular characterization of kshA*, encoding a Rieske-type class IA terminal oxygenase after complementation of strain RG1-UV39*

The *R. erythropolis* strain RG1 gene library was introduced into strain RG1-UV39 by electrotransformation to complement its mutant phenotype. This resulted in the isolation of plasmid pKSH101, containing a 6 kb insert, able to restore growth of strain RG1-UV39 on AD mineral agar medium. Restriction enzyme mapping analysis of pKSH101, subcloning in pRESQ and subsequent complementation experiments resulted in the identification of a 1.8 kb *Bam*HI–*Sau*3A DNA fragment (pKSH106) that was still able to complement the strain RG1-UV39 mutant phenotype (Fig. 3A). A 2.6 kb insert of pKSH107 (Fig. 3A), including this 1.8 kb *Bam*HI–*Sau*3A fragment, was cloned into pBlueScript-II KS, and its nucleotide sequence was determined. Nucleotide sequence analysis revealed a single 1197 nucleotide open reading frame (ORF) (*kshA*; GC content, 60.2%), encoding a protein of 398 amino acids (KshA), within the 1.8 kb *Bam*HI–*Sau*3A complementing DNA fragment. Upstream of the *kshA* start codon, a Shine–Dalgarno (SD) consensus sequence for actinomycetes (Strohl, 1992) could be identified (GGAGGA). BLAST similarity searches and alignment of the deduced amino acid sequence of *kshA* revealed that KshA is similar to class IA terminal oxygenases (Fig. 4) (Batie *et al*., 1991). Among these are phthalate-4,5-dioxygenase (Pht3) (Nomura *et al*., 1992), vanillate demethylase (VanA) (Brunel and Davison, 1988; Priefert *et al*., 1997) and 3-chlorobenzoate 3,4-dioxygenase (CbaA) (Nakatsu *et al*., 1995). A hypothetical protein encoded by gene Rv3526 (DDBJ/EMBL/GenBank accession no. CAB05051) in *Mycobacterium tuberculosis* (Cole *et al*., 1998) showed significant similarity to the complete KshA amino acid sequence (58% identity; 84% similarity).

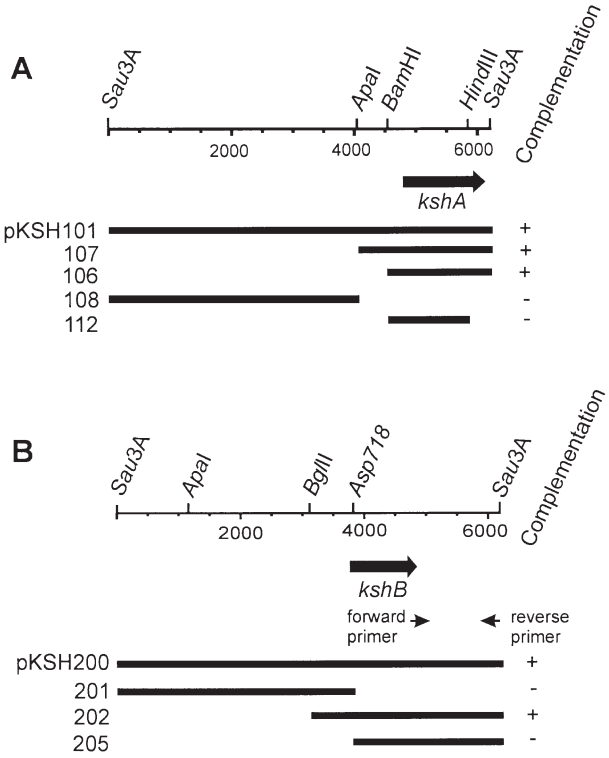

Fig. 3. Strategy for the separate cloning and identification of the *kshA* and *kshB* genes encoding KSH activity in *R. erythropolis* SQ1 by functional complementation of UV mutant strains RG1-UV39 (A) and RG1-UV26 (B), respectively, using several pRESQ-derived constructs. The arrows indicating forward primer and reverse primers (*kshB* complementation scheme) show the annealing positions of these primers to generate PCR product from pKSH202. The obtained PCR product was used to create pKSH212 for the construction of *kshB* unmarked gene deletion mutant strain RG4 (*Experimental procedures*).

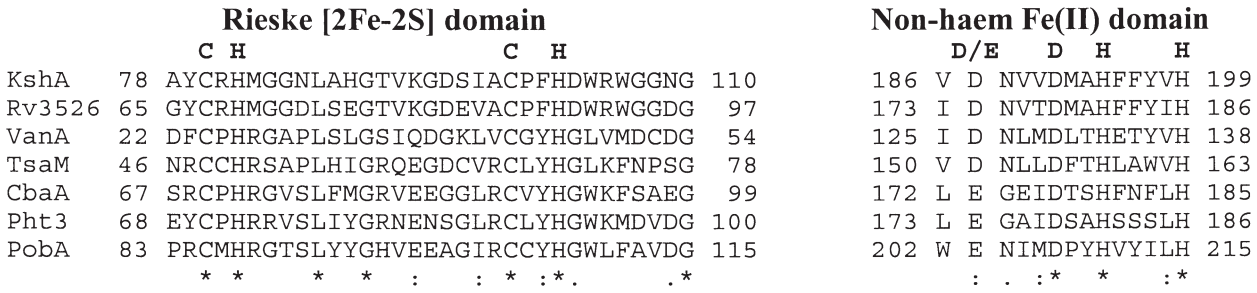

Fig. 4. Identification of a Rieske $[2Fe\text{-}2S]_R$ domain and the non-haem Fe(II) domain in the KSH terminal oxygenase KshA by alignment with known class IA terminal oxygenases (DDBJ/EMBL/GenBank accession no. between brackets): KshA (AY083508) 3-ketosteroid 9α-hydroxylase KshAB; Rv3526 (CAB05051), putative 3-ketosteroid 9α-hydroxylase *Mycobacterium tuberculosis* Rv3525 Rv3571 (Cole *et al*., 1998); VanA (O05616), vanillate demethylase VanAB (Priefert *et al*., 1997); TsaM (AAC44804), *p*-toluenesulphonate methyl-monooxygenase TsaMB (Junker *et al*., 1997); CbaA (Q44256), 3-chlorobenzoate 3,4-dioxygenase CbaAB (Nakatsu *et al*., 1995); PobA (Q52185), phenoxybenzoate dioxygenase PobAB (Dehmel *et al*., 1995); Pht3 (Q05183), phthalate 4,5-dioxygenase Pht23 (Nomura *et al*., 1992). (*) indicates identical residues. (:) and (.) indicate high and low similarity respectively.

Rv3526 is thus expected to be the orthologue of *kshA* in *M. tuberculosis*. Based on the alignment, a Rieske-type $[2Fe\text{-}2S]_R$ domain (C-X-H-X16-C-X_2-H) and the mononuclear, non-haem iron-binding motif (D/E-X_3-D-X_2-H-X_4-H) could be identified in KshA, both typically conserved in class IA terminal oxygenases (Nakatsu *et al*., 1995; Jiang *et al*., 1996). Interestingly, the proposed conserved glutamate residue in the mononuclear, non-haem iron-binding motif was substituted for an aspartate residue in KshA (Asp-187), as has been observed for the terminal oxygenases of vanillate demethylase (VanA) and *p*-toluenesulphonate monooxygenase (TsaM) (Fig. 4) (Brunel and Davison, 1988; Jiang *et al*., 1996; Junker *et al*., 1997). Whether this is of functional significance in class IA oxygenases remains to be determined. The mononuclear, non-haem iron-binding domain is thought to be involved in oxygen binding (Mason and Cammack, 1992; Jiang *et al*., 1996). The calculated molecular weight for KshA (44.5 kDa) resembles those found for other terminal oxygenases (48–55 kDa) (Mason and Cammack, 1992). Based on analysis of the primary amino acid sequence, we classify KshA as a class IA terminal oxygenase.

Comparison of the obtained nucleotide sequence of *kshA* with databases also revealed that *kshA* is nearly identical (97% over full-length sequence) to a hypothetical gene (ORF12) found by Maeda *et al*. (1995) in *R. erythropolis* strain TA421 (DDBJ/EMBL/GenBank accession no. D88013) upstream of *bphC1* (Fig. 5A). The *bphC1* gene encodes a *meta* cleavage dioxygenase with activity towards 2,3-dihydroxybiphenyl. However, the start codon proposed by these authors (corresponding to Met-83 in KshA) cannot be the correct codon, as it is located inside the $[2Fe\text{-}2S]_R$ domain of KshA (Fig. 4). In analogy with the molecular organization found in strain TA421, a hypothetical ORF11, identified in this strain downstream of ORF12, was also identified downstream of *kshA* in strain SQ1 (Fig. 5A). The nucleotide sequences of the DNA fragments of strain SQ1 and strain TA421 were therefore merged (Fig. 5B), and the resulting theoretical nucleotide sequence was used for the successful construction of an unmarked in frame *kshA* gene deletion mutant of *R. erythropolis* strain SQ1 and strain RG8 (see below).

Identification and molecular characterization of kshB*, encoding a class IA oxygenase reductase after complementation of* R. erythropolis *strain RG1-UV26*

Complementation of *R. erythropolis* strain RG1-UV26 with the strain RG1 gene library resulted in isolation of clone pKSH200, containing a 6 kb insert, able to restore growth of strain RG1-UV26 on AD mineral agar medium. Cross-complementation showed that pKSH200 was unable to complement the strain RG1-UV39 phenotype, indicating that a gene different from *kshA* had been cloned. Restriction mapping analysis of pKSH200, subcloning in pRESQ and subsequent functional complementation experiments allowed the identification of a 2.8 kb *Bgl*II–*Sau*3A fragment (pKSH202; Fig. 3B) that was still able to complement the strain RG1-UV26 mutant phenotype. This fragment was subcloned into pBlueScript-II KS (pKSH207), and its nucleotide sequence was determined. Analysis of the obtained nucleotide sequence revealed the presence of a single intact ORF of 1041 nucleotides. No functional complementation of the RG1-UV26 mutant phenotype was found with pKSH205 (Fig. 3B), lacking the 5′ part of the ORF. Hence, this ORF (*kshB*) is responsible for functional complementation of the strain RG1-UV26 mutant phenotype. *kshB* (GC content 62.3%) encodes a protein (KshB) of 346 amino acids with a calculated molecular weight of 37.1 kDa. Database similarity searches revealed that KshB shows high similarity to ferredoxin reductase components of multicomponent oxygenases. Highest similarity (56% identity; 85% similarity)

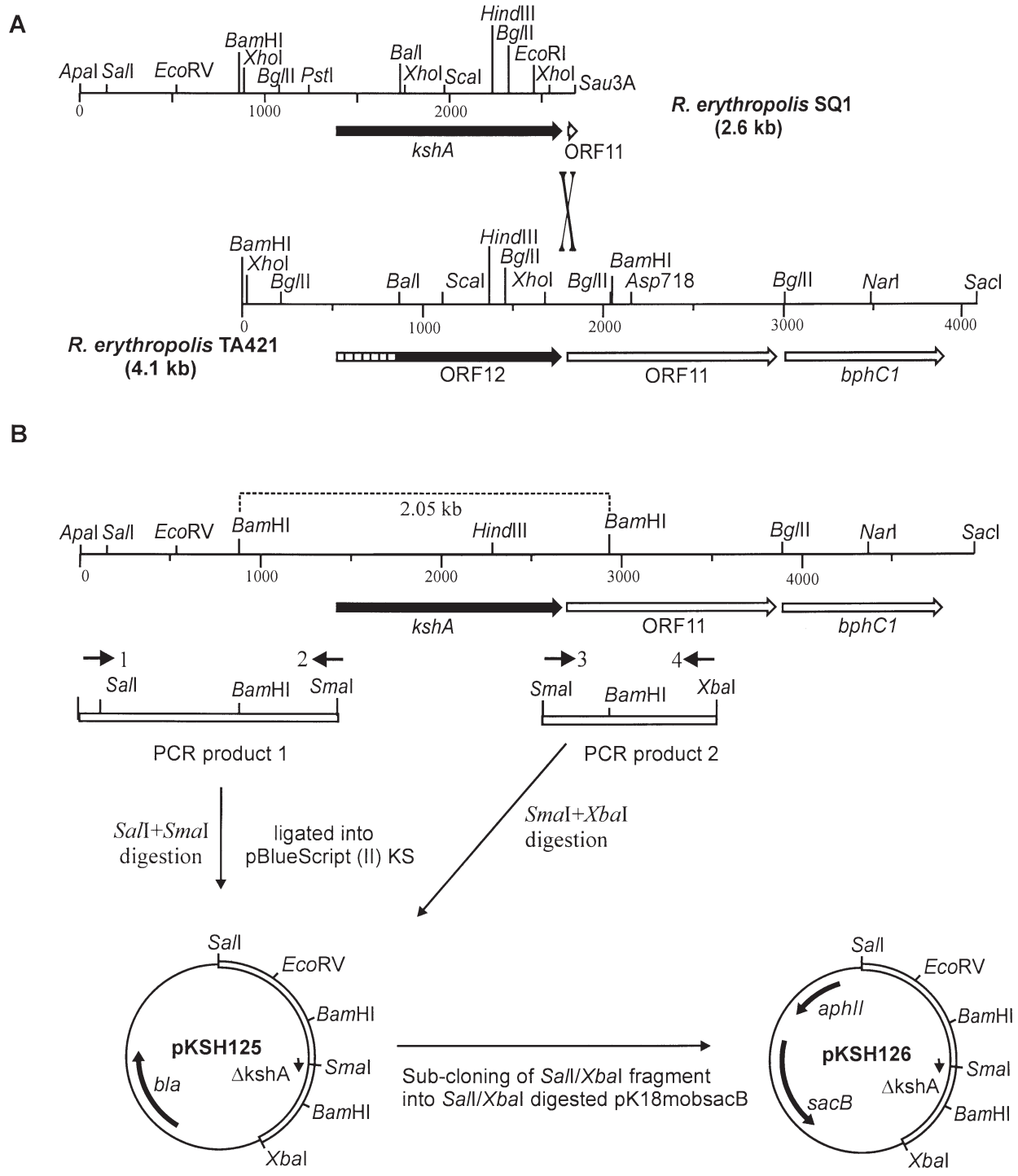

Fig. 5. A. Overview of the 2.6 kb DNA fragment of *R. erythropolis* SQ1 encoding *kshA* and its 4.1 kb counterpart in *R. erythropolis* TA421. The vertically striped bar of ORF12, together with the black arrow [proposed size of ORF12 from Maeda *et al.* (1995)], represents the actual size of ORF12 in *R. erythropolis* TA421, which is nearly identical (97%) to *kshA*. The 'X' indicates the point of merger of the two sequences (see *Results*).

B. Scheme of the theoretically merged nucleotide sequences of the DNA fragments of strain SQ1 and strain TA421 and its use in the construction of plasmid pKSH126 for unmarked in frame *kshA* gene deletion in strain SQ1 and strain RG8. Numbers 1–4 indicate the primers used to obtain PCR products 1 and 2 (see *Experimental procedures*).

was found to Rv3571 of *M. tuberculosis* (DDBJ/EMBL/GenBank accession no. A70606). Alignment of the amino acid sequence of KshB with several ferredoxin reductase components of oxygenases revealed the presence of domains typically conserved in class IA oxygenase ferredoxin reductases: a flavin mononucleotide (FMN) isoalloxazine-binding domain (RxYSL), an NAD-ribose-binding domain (GGIGITP) and a plant-type $[2Fe\text{-}2S]_{Fd}$ binding domain ($Cx_4Cx_2Cx_{29}C$) situated at the C-terminus of this component (Batie *et al*., 1991; Correll *et al*., 1992; Mason and Cammack, 1992; Nakatsu *et al*., 1995) (Fig. 6A and B). The KshB protein can thus be assigned to the class IA oxygenase ferredoxin reductases and represents the ferredoxin reductase component of KSH.

KSH in R. erythropolis *SQ1 is classified as a two-component steroid 9α-monooxygenase*

Analysis of deduced amino acid sequences of both *kshA* and *kshB* revealed the presence of conserved domains that are typically found in class IA oxygenases (Figs 4 and 6), justifying the classification of KSH of *R. erythropolis* strain SQ1 as a member of the class IA multicomponent oxygenases. By definition, class I oxygenases consist of two components, implying that KSH of *R. erythropolis* strain SQ1 is made up of the terminal oxygenase component KshA and an oxygenase ferredoxin reductase component KshB (Fig. 7). The 9α-hydroxylation system in *R. erythropolis* strain SQ1 apparently differs fundamentally from the KSH system of *Nocardia* sp. M117, as this

A

	FMN-binding domain R YSL		NAD-ribose binding domain GGIGITP	
KshB	61	SVARCYSLASSP 72	123	LLFGAGSGITPVISI 137
Rv3571	69	SVARCYSLCSSP 80	131	LLLAAGSGITPIMSI 145
VanB	45	GLVRPYSLCNAP 55	108	LLFAGGIGITPILAM 122
TsaB	48	GLVRQYSLVNAT 59	113	LLLAGGIGVTPIYAM 127
CbaB	53	DLVRQYSLVKAS 64	116	VLICGGIGITPMVHM 130
Pht2	55	GSIRNYSLSNDP 66	118	IFVAGGIGITPILSM 132
PobB	53	GITRSYSLLNDP 64	116	VFIAGGIGITPLWSM 130
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$[2Fe\text{-}2S]_{Fd}$ binding domain

		C C C C	
KshB	298	YSCQEGECGSCACTVTEGEVQMDNSEILDAEDVANGYILGCKAKPITDRL	347
Rv3571	303	FSCREGHCGACACTLRAGKVNMGVNDVLEQQDLDEGLILACQSRPESDSV	352
VanB	267	LACEQGICGTCLTRVLDGEPEHRDSFLTDAERARNDQFTPCCSRARSACL	316
TsaB	264	WSCREGICGTCEAPVLEGEVQHLDYVLSPEERAEQRRMMVCVSRCGGGRL	313
CbaB	236	SSCRQGICGMCETTLISGVPDHRDRLLTDSEKASGRTMLICCSRALSPEL	285
Pht2	273	SSCESGTCGSCRTRLIEGDVEHRDMVLREDE--QHDQIMICVSRARNDVL	320
PobB	266	SSCQQGVCGICETAVLAGVPDHRDLVLSDQERAAGRTMMICCSGSKTAEL	315
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B

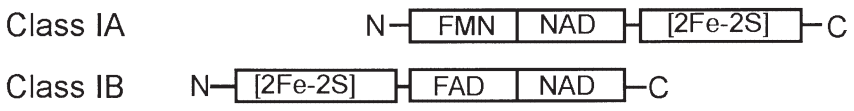

Fig. 6. A. Alignment of class IA oxygenase ferredoxin reductases and identification of the FMN, the NAD-ribose and the plant-type $[2Fe\text{-}2S]_{Fd}$ binding domains in KshB, classifying KshB as a class IA ferredoxin reductase: KshB (AY083509), Rv3571 (putative; CAB07145), VanB (O05617), TsaB (AAC44805), CbaB (Q44257), Pht2 (Q05182), PobB (Q52186). DDBJ/EMBL/GenBank accession numbers are shown in brackets.

B. Organization of the cofactor binding domains in class IA and class IB ferredoxin reductases (Batie *et al*., 1991; Correll *et al*., 1992; Nakatsu *et al*., 1995). (*) indicates identical residues. (:) and (.) indicate high and low similarity respectively.

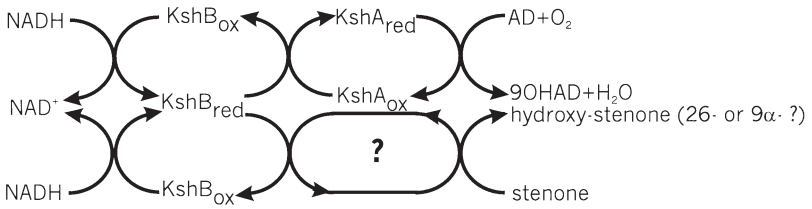

Fig. 7. Proposed functions of the terminal oxygenase KshA and the oxygenase ferredoxin reductase KshB in KSH activity in *R. erythropolis* SQ1. The KshB component participates in both 3-ketosteroid 9α-hydroxylation (KSH) and sterol catabolism (putatively 26- or 9α-hydroxylation of sterols).

system has been identified as a three-component oxygenase (Strijewski, 1982).

In contrast to many other structural genes encoding multicomponent oxygenases in bacteria, *kshA* and *kshB* were not found adjacent to each other. Nucleotide sequence analysis of flanking regions of both genes did not reveal their proximity to each other. Cross-complementation experiments, introducing pKSH101 into strain RG1-UV26 and pKSH200 into strain RG1-UV39, did not result in growth on AD(D), also showing that *kshA* and *kshB* are located on loci that lie at least several kilobases apart. However, this feature is not unique. *OxoO* and *oxoR* from *Pseudomonas putida* 86, encoding 2-oxo-1,2-dihydroquinoline 8-monooxygenase, are separated by ≈ 15 kb (Rosche *et al*., 1997). Also, the putative orthologues of *kshA* and *kshB* in the *M. tuberculosis* genome (Rv3526 and Rv3571 respectively) are separated by ≈ 50 kb (Cole *et al*., 1998).

Unmarked in frame gene deletion mutants confirm the involvement of kshA *and* kshB *in AD(D) 9α-hydroxylation*

An unmarked in frame *kshA* deletion mutant (strain RG2) was isolated from parent strain SQ1 using pKSH126 (Fig. 5B) via the *sacB* counterselection method (van der Geize *et al*., 2001), reducing the wild-type *kshA* gene to an ORF (Δ*kshA*) of 30 nucleotides encoding only nine amino acids (MALGPGTTS). Sucrose-resistant (Suc^r) colonies obtained via *sacB* counterselection were replica plated onto LBP agar plates containing 200 μg ml^{-1} kanamycin (Kan). A total of 12 Suc^r Kan^s colonies were identified among 96 Suc^r colonies (12.5%). Gene deletion of *kshA* was confirmed in three out of these 12 Suc^r Kan^s colonies (25%) by Southern analysis of *Bam*HI-digested chromosomal DNA using the 2 kb insert of pKSH126 as a probe: a 2.05 kb wild-type *Bam*HI DNA fragment (Fig. 5B) was reduced to 0.88 kb in the gene deletion mutant strain (data not shown). In frame gene deletion of *kshB* was achieved using pKSH212 (see *Experimental procedures*; Table 1). The isolation of a *kshB* mutant from parent strain SQ1 using pKSH212 (Table 1) was more difficult to achieve than the construction of *kshA* mutant strain RG2 for reasons unknown. No *kshB* mutants were identified among several dozen potential *kshB* mutants after *sacB* counterselection using our general screening methods [colony polymerase chain reaction (PCR), Southern analysis]. We thus decided to screen for potential *kshB* mutants by replica plating on AD mineral agar plates, enabling us to isolate a low percentage (<1%) of mutants unable to grow on AD. Southern analysis was performed on *Asp*718-digested chromosomal DNA from wild type and three AD growth-deficient mutants. Hybridization with the complete *kshB* gene showed that *kshB* was not present in the genome of the putative *kshB* mutants. A clear hybridization signal (4.3 kb fragment) was found exclusively with wild-type chromosomal DNA. Additional Southern analysis with an alternative probe, the 2.2 kb insert of pKSH212 (Table 1), comprising both flanking regions of *kshB*, also confirmed *kshB* gene deletion: a 4.3 kb *Asp*718 wild-type DNA fragment containing the *kshB* gene was reduced to 3.3 kb in strain RG4, demonstrating replacement of the 1041 bp *kshB* gene by a *kshB* in frame remnant of 30 nucleotides (encoding MTTVEVPIA).

Inactivation of KSH activity, by either *kshA* or *kshB* gene deletion, rendered *Rhodococcus* strains blocked in growth on AD(D) mineral agar medium: strains RG2 and RG4 were plated on mineral agar media containing AD, ADD or 9OHAD as sole carbon and energy source. Both strains showed no growth on AD(D), whereas growth on 9OHAD was comparable with that of strain SQ1. These phenotypes are in agreement with those found with strains RG1-UV26 and RG1-UV39. Bioconversion of AD (1 g l^{-1}) with parent strain SQ1, followed for 168 h, resulted in AD utilization, but not in the accumulation of ADD or other metabolites. Bioconversion of AD (1 g l^{-1}) by strain RG2 or strain RG4 was not complete, leaving 50–70% of the steroid substrate AD intact after 168 h of incubation. This resulted in comparable accumulation levels of ADD (varying between 0.3 and 0.5 g l^{-1} after 168 h). In these mutants, AD(D) 9α-hydroxylation had thus become blocked completely by inactivation of either *kshA* or *kshB*, demonstrating the essential role of both KshA and KshB in KSH activity in *R. erythropolis* strain SQ1. Growth experiments with *R. erythropolis* SQ1 on ADD mineral agar plates performed throughout this work have indicated that ADD is inhibitory to growth at higher concentrations (0.5 g l^{-1}). This ADD toxicity may explain the relatively low conversion of AD into ADD. Alternative

explanations are that either regulatory mechanisms or the presence of a 1-ene-steroid reductase activity, as found in *Mycobacterium* sp. (Goren *et al*., 1983), prevents complete conversion of AD into ADD.

Microbial degradation of phytosterols (e.g. β-sitosterol; Fig. 1B) is generally performed via two major processes, i.e. the selective removal of the sterol side-chain (side-chain degradation) and opening of the steroid polycyclic ring structure. These two processes proceed independently, either simultaneously or consecutively (Martin, 1977). No accumulation of AD(D) or other metabolites was found in phytosterol bioconversions with strain RG2. Degradation of phytosterols added to cultures of RG2 was not impaired, and rates of degradation were comparable with those of parent strain SQ1. This may involve a sterol-specific, steroid ring structure-degrading enzyme (i.e. sterol 9α-hydroxylase). However, phytosterol degradation had become blocked in the *kshB* mutant strain RG4. Phytosterols added to cultures of strain RG4 were oxidized to their stenone derivatives (Fig. 1B), but were not metabolized further, indicating that the side-chain degradation of the sterol molecule was blocked. The KshB protein component thus appears to be involved in both steroid and sterol catabolism (Fig. 7). Speculatively, KshB may either also be involved in 9α-hydroxylation of sterols as a component of the putative sterol 9α-hydroxylase or may be part of the sterol 26-hydroxylase enzyme system. The latter is generally thought to initiate sterol side-chain degradation (Murohisa and Iida, 1993; Ambrus *et al*., 1995) and would explain why no side-chain degradation is observed after the formation of stenone derivatives of phytosterols in a *kshB* mutant strain. Clearly, KshB is a very interesting multifunctional protein involved in microbial steroid and sterol catabolism.

Construction of kshA kstD kstD2 *triple gene deletion mutant strain RG9 verifies that KSH is an AD(D) 9α-monooxygenase*

Strain RG9, a *kshA kstD kstD2* triple gene deletion mutant, was derived from the 9OHAD-accumulating *kstD kstD2* mutant strain *R. erythropolis* RG8 (Table 1). *kshA* gene deletion was achieved by introducing plasmid pKSH126 into strain RG8, selecting for mutants as described for strain RG2. In AD bioconversion experiments with strain RG9, no decline in the initial AD concentration was observed. Similarly, no decline in the initial ADD concentration was detected when strain RG9 was incubated with ADD. This mutant strain thus confirms that *kshA* encodes both AD and ADD 9α-hydroxylase activity and that, in contrast to the KSTD isoenzymes, no further KSH isoenzymes for AD and ADD conversion are present in *R. erythropolis* strain SQ1.

Experimental procedures

Bacterial strains, plasmids and growth conditions

Plasmids and bacterial strains used are listed in Table 1. *Rhodococcus* strains were cultivated at 30°C and 200 r.p.m. Complex medium (LBP) contained 1% (w/v) Bacto peptone (Difco), 0.5% (w/v) yeast extract (BBL Becton Dickinson) and 1% (w/v) NaCl. Mineral medium consisted of 1 g l^{-1} NH_4NO_3, 0.25 g l^{-1} K_2HPO_4, 0.25 g l^{-1} $MgSO_4 \cdot 7H_2O$, 5 mg l^{-1} NaCl, 5 mg l^{-1} $FeSO_4 \cdot 7H_2O$ (pH 7.2). Steroids (0.5 g l^{-1}) were solubilized in DMSO (50 mg ml^{-1}) and added to autoclaved medium. For growth on solid medium, LBP was supplemented with 1.5% (w/v) Bacto agar (Difco). Sucrose (Suc) sensitivity of *Rhodococcus* strains was tested on LBP agar supplemented with 10% (w/v) sucrose (LBPS). *E. coli* strains (Table 1) were grown in Luria–Bertani (LB) broth at 37°C. BBL agar (1.5% w/v) was added for growth on solid medium.

General cloning techniques

DNA-modifying enzymes were purchased from Boehringer Mannheim, New England Biolabs or Amersham Pharmacia Biotech and used as described by the manufacturer. Isolation of DNA restriction fragments from agarose gels was done using the Qiagen gel extraction kit according to the manufacturer's protocol. All DNA manipulations were carried out according to standard protocols. *Rhodococcus* plasmid DNA was isolated using the Qiagen spin prep kit with slight modification: *Rhodococcus* cells were grown for 2–3 h in the presence of ampicillin (600 μg ml^{-1}) before lysozyme (2 mg ml^{-1}) treatment. PCR was performed under standard conditions using *Pwo* polymerase (Boehringer) unless stated otherwise: five cycles of 1 min at 95°C, 1.5 min at 60°C, 1.5 min at 72°C, followed by 25 cycles of 1 min at 95°C, 1.5 min at 55°C, 1.5 min at 72°C. Transformation of *Rhodococcus* strains for unmarked gene deletion experiments was performed by mobilization of the mutagenic vector (pKSH126 or pKSH212) from *E. coli* S17-1 to the *Rhodococcus* strain by conjugation as described previously (van der Geize *et al*., 2001).

Construction of the pRESQ shuttle vector

A pZErO-2.1 (Invitrogen) derivative was constructed in which the *Bam*HI site was replaced by a *Bgl*II site. A *Sac*I–*Stu*I fragment of pZErO-2.1, containing the *lacZ–ccdB* gene, was duplicated by PCR using a mutagenic forward primer (5′-ACCGAGCTCAGATCTACTAGTAACGGC-3′), containing the desired *Bgl*II restriction site (double underlined) and a *Sac*I restriction site (underlined), and reverse primer (5′-ATTCAGGCCTGACATTTATATTCCCC-3′) with a *Stu*I restriction site (underlined). The obtained PCR product was digested with restriction enzymes *Sac*I and *Stu*I and ligated in *Sac*I–*Stu*I-digested pZErO-2.1 (pRES14). The *aphII* gene from pWJ5 (Table 1) was cloned as a blunted *Hin*dIII–*Bam*HI fragment (Klenow fill-in) into *Eco*RV-digested pBlueScript-II KS to construct pBsKm1. The unique *Bgl*II site in pBsKm1 was destroyed by *Bgl*II digestion followed by Klenow fill-in (pRES4). The ampicillin cassette present in pRES4 was removed by self-ligation after *Bsp*HI digestion (pRES9). A

2.5 kb *Bgl*II–*Xba*I fragment of pMVS301 (Vogt-Singer and Finnerty, 1988), containing the region for autonomous replication in *Rhodococcus* sp., was subsequently ligated into *Bam*HI–*Xba*I-digested pRES9 to construct pRES11. Construction of pRESQ (6.55 kb; Fig. 2) was completed by ligating a 4.15 kb *Bsp*HI–*Nco*I fragment of pRES11 into *Bsp*HI–*Nco*I-digested pRES14.

Construction of a gene library of R. erythropolis *strain RG1*

*Sau*3A-digested chromosomal DNA from *R. erythropolis* strain RG1 was sized by sucrose gradient centrifugation (6–10 kb) and ligated into *Bgl*II-digested pRESQ. Transformation of *E. coli* Top10F′ cells (Invitrogen) with the ligation mixture generated a genomic library of ≈ 15 000 transformants, in which ≈ 90% of the constructs contained an insert. An average insert size of 6 kb was estimated. No complications with stability or rearrangements were apparent. The gene library represents the complete genome ($P > 0.99$) of *R. erythropolis* strain RG1, assuming a genome size of ≈ 6 Mb (Bigey *et al*., 1995; Pisabarro *et al*., 1998; Redenbach *et al*., 2000).

Complementation experiments

Electrotransformation of competent cells of mutant *Rhodococcus* strains was done as described previously (van der Geize *et al*., 2000). Colonies obtained after introducing the genomic library of *R. erythropolis* strain RG1 by electrotransformation were replica plated on mineral agar medium containing AD (0.5 g l^{-1}) as sole carbon and energy source. Complementation was scored after 3 days of incubation at 30°C. Colonies growing on AD mineral agar medium were cultivated in LBP medium for isolation of plasmid DNA; this plasmid DNA was subsequently reintroduced into suitable mutant strains to check for genuine complementation.

Nucleotide sequencing analysis

Nucleotide sequencing analysis was performed using dye primers in the cycle sequencing method (Murray, 1989) with the thermosequenase kit RPN 2538 from Amersham Pharmacia Biotech. The samples were run on the ALF-Express sequencing robot. Protein and nucleotide sequence comparisons were performed using the facilities of the BLAST server (Altschul *et al*., 1990) at NCBI (National Library of Medicine, Washington, DC, USA). Alignments were done using CLUSTALW 1.7 (Thompson *et al*., 1994).

Construction of vectors for unmarked in frame gene deletion mutagenesis of kshA *and* kshB

pKSH126 was constructed for unmarked in frame gene deletion of *kshA* (Δ*kshA*). A 1.3 kb fragment (PCR product 1) was obtained from pKSH101 (Table 1) using a forward primer (Fig. 5B, primer 1) annealing to sequences upstream of *kshA* (5′-CGCGGGCCCATCGAGAGCACGTT-3′), and a reverse primer (Fig. 5B, primer 2) annealing to the 5′ end of the *kshA* gene (5′-GCGCCCGGGTCCGAGTGCCATGTCTTC3′) containing a *Sma*I site (underlined). PCR product 2 (840 bp) was obtained from SQ1 chromosomal DNA using primer 3 (Fig. 5B) annealing to the 3′ end of the *kshA* gene (5′-GC GCCCGGGACAACCTCCTGATTCGCAGTC-3′), including a *Sma*I restriction site (underlined), and primer 4 (Fig. 5B) annealing to ORF11 (5′-GCGTCTAGAGTGGAAGAGCATTC CCTCGCA-3′), including a *Xba*I restriction site (underlined) (Fig. 5B). Primer 4 for PCR product 2 was developed using the nucleotide sequence of the merged sequences of strain SQ1 and strain TA421 (DDBJ/EMBL/GenBank accession no. D88013) (Fig. 5; see *Results*). The *Sma*I restriction site was introduced to give an in frame deletion of *kshA* after ligation of this PCR fragment behind the 5′-truncated *kshA* gene from PCR product 1 in pBlueScript-II KS, yielding pKSH125 (Fig. 5B). Finally, a 2.05 kb *Sal*I–*Xba*I fragment from pKSH125 (Fig. 5B) was ligated into pK18mobsacB vector (pKSH126).

For unmarked in frame gene deletion of *kshB*, construct pKSH201 was fused to a PCR product (1275 bp) obtained from pKSH202 as template, using forward primer 5′-GCGGGTACCGATCGCCTGAAGATCGAGT-3′ and reverse primer 5′-GCGAAGCTTGCCGGCGTCGCAGCTCTGTG-3′ (Fig. 3B). At the 5′-terminal end of this PCR product, an *Asp*718 restriction site (see forward primer: underlined), preceding the stop codon of *kshB*, was introduced to ensure proper in frame deletion of *kshB* after ligation with the *Asp*718 restriction site of pKSH201. At the 3′-terminal end, a *Hin*dIII restriction site (see reverse primer: underlined) was added, compatible with the *Hin*dIII restriction site of pKSH201, for cloning purposes. The PCR product was ligated (blunt) into *Eco*RV-digested pBlueScript-II KS (pKSH210) for easy handling in subsequent cloning steps. A 1263 bp *Asp*718–*Hin*dIII fragment was isolated from pKSH210 and ligated into *Asp*718–*Hin*dIII-digested pKSH201 (pKSH211), thereby introducing the desired *kshB* in frame deletion. Finally, a 2.2 kb *Bam*HI–*Hin*dIII DNA fragment containing the *kshB* deletion was ligated into pK18mobsacB digested with *Bam*HI–*Hin*dIII (pKSH212).

Bioconversion experiments

Bioconversion experiments with AD(D) and phytosterols, as well as steroid analysis [high-performance liquid chromatography (HPLC), thin-layer chromatography (TLC)] and sterol/stenone analysis [gas chromatography (GC), TLC] were performed as described previously (van der Geize *et al*., 2000). Phytosterols (Henkel; 82.7% sterol content: mainly β-sitosterol, 40.4%; stigmasterol, 16.3%; campesterol, 22.4%) were supplied by Diosynth.

DDBJ/EMBL/GenBank database accession numbers

These sequence data have been submitted to the DDBJ/EMBL/GenBank databases under accession numbers AY083508 (*kshA*) and AY083509 (*kshB*).

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References

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., and Lipman, D.J. (1990) Basic local alignment search tool. *J Mol Biol* **215:** 403–410.

Ambrus, G., Ilkoy, E., Jekkel, A., Horváth, G., and Böcskei, Z. (1995) Microbial transformation of β-sitosterol and stigmasterol into 26-oxygenated derivatives. *Steroids* **60:** 621–625.

Batie, C.J., Ballou, D.P., and Correll, C.C. (1991) Phthalate dioxygenase reductase and related flavin-iron-sulfur containing electron transferases. In *Chemistry and Biochemistry of Flavoenzymes*, Vol. 3. Müller, F. (ed.). Boca Raton, FL: CRC Press, pp. 543–556.

Bigey, F., Janbon, G., Arnaud, A., and Galzy, P. (1995) Sizing of the *Rhodococcus* sp. R312 genome by pulsed-field gel electrophoresis. Localization of genes involved in nitrile degradation. *Antonie Van Leeuwenhoek* **68:** 173–179.

Brunel, F., and Davison, J. (1988) Cloning and sequencing of *Pseudomonas* genes encoding vanillate demethylase. *J Bacteriol* **170:** 4924–4930.

Chang, F.N., and Sih, C.J. (1964) Mechanisms of steroid oxidation by microorganisms. VII. Properties of the 9α-hydroxylase. *Biochemistry* **3:** 1551–1557.

Cole, S.T., Brosch, R., Parkhill, J., Garnier, T., Churcher, C., Harris, D., *et al*. (1998) Deciphering the biology of *Mycobacterium tuberculosis* from the complete genome sequence. *Nature* **393:** 537–544.

Correll, C.C., Batie, C.J., Ballou, D.P., and Ludwig, M.L. (1992) Phthalate dioxygenase reductase: a modular structure for electron transfer from pyridine nucleotides to [2Fe-2S]. *Science* **258:** 1604–1610.

Datcheva, V.K., Voishvillo, N.E., Kamernitskii, A.V., Vlahov, R.J., and Reshetova, I.G. (1989) Synthesis of 9α-hydroxysteroids by a *Rhodococcus* sp. *Steroids* **54:** 271–286.

Dehmel, U., Engesser, K.H., Timmis, K.N., and Dwyer, D.F. (1995) Cloning, nucleotide sequence, and expression of the gene encoding a novel dioxygenase involved in metabolism of carboxydiphenyl ethers in *Pseudomonas pseudoalcaligenes* POB310. *Arch Microbiol* **163:** 35–41.

Dutta, R.K., Roy, M.K., and Singh, H.D. (1992) Role of plasmid pJL1 of *Arthrobacter oxydans* 317 in the degradation of β-sitosterol. *J Basic Microbiol* **32:** 317–324.

Goren, T., Harnik, M., Rimon, S., and Aharonowitz, Y. (1983) 1-Ene-steroid reductase of *Mycobacterium* sp. NRRL B3805. *J Steroid Biochem* **19:** 1789–1797.

Harayama, S., and Kok, M. (1992) Functional and evolutionary relationships among diverse oxygenases. *Annu Rev Microbiol* **46:** 565–601.

Jäger, W., Schäfer, A., Pühler, A., Labes, G., and Wohlleben, W. (1992) Expression of the *Bacillus subtilis sac*B gene leads to sucrose sensitivity in the gram-positive bacterium *Corynebacterium glutamicum* but not in *Streptomyces lividans*. *J Bacteriol* **174:** 5462–5465.

Jiang, H., Parales, R.E., Lynch, N.A., and Gibson, D.T. (1996) Site-directed mutagenesis of conserved amino acids in the alpha subunit of toluene dioxygenase: potential mononuclear non-heme iron coordination sites. *J Bacteriol* **178:** 3133–3139.

Junker, F., Kiewitz, R., and Cook, A.M. (1997) Characterization of the p-toluenesulfonate operon *tsaMBCD* and *tsaR* in *Comamonas testosteroni* T-2. *J Bacteriol* **179:** 919–927.

Kieslich, K. (1985) Microbial side-chain degradation of sterols. *J Basic Microbiol* **25:** 461–474.

Maeda, M., Chung, S.-Y., Song, E., and Kudo, T. (1995) Multiple genes encoding 2,3-dihydroxybiphenyl 1,2-dioxygenase in the Gram-positive polychlorinated biphenyl-degrading bacterium *Rhodococcus erythropolis* TA421, isolated from a termite ecosystem. *Appl Environ Microbiol* **61:** 549–555.

Mahato, S.B., and Garai, S. (1997) Advances in microbial steroid biotransformation. *Steroids* **62:** 332–345.

Martin, C.K.A. (1977) Microbial cleavage of sterol side chains. *Adv Appl Microbiol* **22:** 29–58.

Mason, J.R., and Cammack, R. (1992) The electron-transport proteins of hydroxylating bacterial dioxygenases. *Annu Rev Microbiol* **46:** 277–305.

Murohisa, T., and Iida, M. (1993) Some new intermediates in microbial side chain degradation of β-sitosterol. *J Ferment Bioeng* **76:** 174–177.

Murray, V. (1989) Improved double-stranded DNA sequencing using the linear polymerase chain reaction. *Nucleic Acids Res* **17:** 8889.

Nakatsu, C.H., Straus, N.A., and Wyndham, R.C. (1995) The nucleotide sequence of the Tn5271 3-chlorobenzoate 3,4-dioxygenase genes (*cbaAB*) unites the class IA oxygenases in a single lineage. *Microbiology* **141:** 485–495.

Nomura, Y., Nakagawa, M., Ogawa, N., Harashima, S., and Oshima, Y. (1992) Genes in PHT encoding the initial degradation pathway of phthalate in *Pseudomonas putida*. *J Ferment Bioeng* **74:** 333–344.

Pisabarro, A., Correia, A., and Martin, J.F. (1998) Pulsed-field gel electrophoresis analysis of the genome of *Rhodococcus facians*: genome size and linear and circular replicon composition in virulent and avirulent strains. *Curr Microbiol* **36:** 302–308.

Priefert, H., Rabenhorst, J., and Steinbüchel, A. (1997) Molecular characterization of genes of *Pseudomonas* sp. strain HR199 involved in bioconversion of vanillin to protocatechuate. *J Bacteriol* **179:** 2595–2607.

Quan, S., and Dabbs, E.R. (1993) Nocardioform arsenic resistance plasmid characterization and improved *Rhodococcus* cloning vectors. *Plasmid* **29:** 74–79.

Redenbach, M., Scheel, J., and Schmidt, U. (2000) Chromosome topology and genome size of selected actinomycetes species. *Antonie Van Leeuwenhoek* **78:** 227–235.

Rosche, B., Tshisuaka, B., Hauer, B., Lingens, F., and Fetzner, S. (1997) 2-Oxo-1,2-dihydroquinoline 8-monooxygenase: phylogenetic relationship to other multicomponent nonheme iron oxygenases. *J Bacteriol* **179:** 3549–3554.

Schäfer, A., Tauch, A., Jäger, W., Kalinowski, J., Thierbach, G., and Pühler, A. (1994) Small mobilizable multi-purpose cloning vectors derived from the *Escherichia coli* plasmids pK18 and pK19: selection of defined deletions in the chromosome of *Corynebacterium glutamicum*. *Gene* **145:** 69–73.

Simon, R., Priefer, U., and Pühler, A. (1983) A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in gram negative bacteria. *Bio/Technology* **1:** 784–791.

Strijewski, A. (1982) The steroid 9α-hydroxylation system from *Nocardia* species. *Eur J Biochem* **128:** 125–135.

Strohl, W.R. (1992) Compilation and analysis of DNA sequences associated with apparent streptomycete promoters. *Nucleic Acids Res* **20:** 961–974.

Thompson, J.D., Higgins, D.G., and Gibson, T.J. (1994) CLUSTALW: improving the sensitivity of progressive multiple sequence alignment through sequence weighing, position-specific gap penalties and weight matrix. *Nucleic Acids Res* **22:** 4673–4680.

van der Geize, R., Hessels, G.I., Van Gerwen, R., Vrijbloed, J.W., Van der Meijden, P., and Dijkhuizen, L. (2000) Targeted disruption of the *kstD* gene encoding a 3-ketosteroid Δ^1-dehydrogenase isoenzyme of *Rhodococcus erythropolis* strain SQ1. *Appl Environ Microbiol* **66:** 2029–2036.

van der Geize, R., Hessels, G.I., Van Gerwen, R., Van der Meijden, P., and Dijkhuizen, L. (2001) Unmarked gene deletion mutagenesis of *kstD*, encoding 3-ketosteroid Δ^1-dehydrogenase. *Rhodococcus erythropolis* SQ1 using *sacB* as counter-selectable marker. *FEMS Microbiol Lett* **205:** 197–202.

van der Geize, R., Hessels, G.I., and Dijkhuizen, L. (2002) Molecular and functional characterization of the KstD2 gene of *Rhodococcus erythropolis* SQ1 encoding a second 3-ketosteroid Δ1-dehydrogenase isoenzyme. *Microbiology* (in press).

Vogt-Singer, M.E., and Finnerty, W.R. (1988) Construction of an *Escherichia coli-Rhodococcus* shuttle vector and plasmid transformation in *Rhodococcus* spp. *J Bacteriol* **170:** 638–645.

Wovcha, M.G., Antosz, F.J., Knight, J.C., Kominek, L.A., and Pyke, T.R. (1978) Bioconversion of sitosterol to useful steroidal intermediates by mutants of *Mycobacterium fortuitum*. *Biochim Biophys Acta* **531:** 308–321.