Molecular and functional characterization of the *kstD2* gene of *Rhodococcus erythropolis* SQ1 encoding a second 3-ketosteroid Δ^1 -dehydrogenase isoenzyme

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Previously, *Rhodococcus erythropolis* SQ1 *kstD*, encoding ketosteroid Δ^1 -dehydrogenase (KSTD1) was characterized. Surprisingly, a *kstD* gene deletion mutant (strain RG1) grew normally on steroids. UV mutagenesis of strain RG1 allowed isolation of strains (e.g. strain RG1-UV29) unable to perform the Δ^1 -dehydrogenation of 4-androstene-3,17-dione (AD) and 9 α -hydroxy-4-androstene-3,17-dione (9OHAD). Functional complementation of strain RG1-UV29 with total genomic DNA of strain RG1 resulted in identification of a 1698 nt ORF (*kstD2*) showing clear similarity (35% identity at amino acid sequence level) with KSTD1. Expression of *kstD2* in *Escherichia coli* resulted in high KSTD2 activity levels. Single gene deletion mutants of either *kstD* (strain RG1) or *kstD2* (strain RG7) appeared unaffected in growth on the steroid substrates AD, 1,4-androstadiene-3,17-dione and 9OHAD. Strain RG7, but not strain RG1, showed temporary accumulation of 9OHAD during AD conversion. A *kstD kstD2* double deletion mutant (strain RG8) was constructed. Strain RG8 was unable to grow on steroid substrates, had undetectable steroid Δ^1 -dehydrogenation activity and efficiently converted AD into 9OHAD. Strain SQ1 thus employs two KSTD isoenzymes in steroid catabolism. Analysis of two null mutants in KSTD2 showed that the semi-conserved Ser325 and the highly conserved Thr503 play a role in KSTD enzyme activity.

Keywords: 3-ketosteroid dehydrogenase, isoenzymes, unmarked gene deletion, steroid

INTRODUCTION

3-Ketosteroid Δ^1 -dehydrogenase (KSTD) [4-ene-3-oxosteroid:(acceptor)-1-ene-oxidoreductase; EC 1.3.99.4] activity plays an important role in the microbial degradation of the polycyclic ring structure of steroids. KSTD and 3-ketosteroid 9 α -hydroxylase (KSH) together initiate opening of the steroid skeleton B-ring (Fig. 1). Limited information is available about the bacterial enzymes involved in steroid degradation. The industrial potential of KSTD enzymes in steroid biocatalysis has

stimulated research on KSTD proteins and their corresponding genes from different genera and species (Choi *et al.*, 1995; Drobic *et al.*, 1993; Fujii *et al.*, 1999; Itagaki *et al.*, 1990; Kaufmann *et al.*, 1992; Molnár *et al.*, 1995; Morii *et al.*, 1998; Plesiat *et al.*, 1991; Sih & Bennet, 1962; Wagner *et al.*, 1992; Wovcha *et al.*, 1979). Functional studies of the steroid Δ^1 -dehydrogenation system in the micro-organism itself and analysis of the metabolic effects of their inactivation, however, are limited (van der Geize *et al.*, 2000, 2001), probably due to the paucity of genetic tools for actinomycetes able to perform the KSTD reaction (e.g. *Rhodococcus*, *Arthrobacter*, *Nocardia*, *Mycobacterium*). Inactivation of *kstD*, encoding KSTD1 activity in *Rhodococcus erythropolis* SQ1, was shown to be ineffective to obtain mutants blocked in 3-ketosteroid Δ^1 -dehydrogenation (van der Geize *et al.*, 2000, 2001): growth on 4-androstene-3,17-dione (AD), 1,4-androstadiene-3,17-dione (ADD) or 9 α -hydroxy-4-andro-

Abbreviations: AD, 4-androstene-3,17-dione; ADD, 1,4-androstadiene-3,17-dione; DCPIP, 2,6-dichlorophenolindophenol; KSH, 3-ketosteroid 9 α -hydroxylase; KSTD, 3-ketosteroid Δ^1 -dehydrogenase; 9OHAD, 9 α -hydroxy-4-androstene-3,17-dione; PMS, phenazine methosulphate.

The GenBank accession number for the sequence reported in this paper is AY078169

Fig. 1. Scheme of steroid catabolism in *R. erythropolis* SQ1. Two 3-ketosteroid Δ^1 -dehydrogenase (KSTD) isoenzymes, KSTD1 and KSTD2, and a two-component 3-ketosteroid 9 α -hydroxylase (van der Geize *et al.*, 2002), KSH (=KshA + KshB), are deployed for AD degradation. Subsequent steps involved in 9 α -hydroxy-4-androstadiene-3,17-dione (9OHADD) degradation are not shown.

stene-3,17-dione (9OHAD) was not affected. Biochemical data indicated the presence of a KSTD isoenzyme, named KSTD2. KSTD2 has been inactivated in *R. erythropolis* RG1-UV29, obtained via UV mutagenesis of a *kstD* mutant *R. erythropolis* RG1 (van der Geize *et al.*, 2001). Here we report the molecular characterization of *kstD2*, encoding KSTD2 in *R. erythropolis* SQ1, including the construction of *kstD2* gene deletion mutants from parent strain SQ1 and *kstD* mutant strain RG1. Two apparently distinct KSTD enzyme activities have been reported in *Mycobacterium fortuitum* (Wovcha *et al.*, 1979), but nucleotide sequences encoding these two activities are not available. The deduced amino acid sequence of an ORF (ORF3) located downstream of *ksdD* (encoding KSTD1) in *Arthrobacter simplex* (Molnár *et al.*, 1995) also displays clear similarities with several KSTD amino acid sequences (Dziadek *et al.*, 1998). Expression of ORF3 in *Escherichia coli* or *Streptomyces lividans*, however, did not result in steroid biotransformation activity.

The molecular characterization of *kstD2* in *R. erythropolis* SQ1 reported here provides the first unequivocal evidence for the functional involvement of two KSTD isoenzymes in microbial steroid catabolism. The results offer clear insights into the complexity of microbial steroid Δ^1 -dehydrogenation and allow rational construction of industrial strains for steroid production.

METHODS

Bacterial strains, plasmids and growth conditions. *R. erythropolis* strain SQ1 and derived mutant strains RG1, RG1-UV29, RG1-UV45, RG1-UV50, RG1-UV51 have been described previously (Quan & Dabbs, 1993; van der Geize *et al.*, 2001). *E. coli* DH5 α and pBlueScript (II) KS were used for general cloning steps. *E. coli* S17-1 (Simon *et al.*, 1983) was used for the conjugal mobilization of pK18mobsacB (Schäfer *et al.*, 1994) derivatives to *Rhodococcus* recipient strains. An *E. coli*–*Rhodococcus* shuttle vector, pRESQ (van der Geize *et al.*, 2002), was used in all complementation experiments. *Rhodococcus* strains were cultivated at 30 °C and 200 r.p.m. on a rotary shaker (G25; New Brunswick Scientific) in liquid medium (LBP) containing 1% (w/v) Bacto-Peptone (Difco), 0.5% (w/v) yeast extract (BBL) and 1% (w/v) NaCl. Mineral medium consisted of (g l⁻¹): K₂HPO₄, 4.65; NaH₂PO₄·H₂O, 1.5; NH₄Cl, 3; MgSO₄·7H₂O, 1, supplemented with Vishniac trace elements (pH 7.2). Steroids (50 mg ml⁻¹) were dissolved in DMSO and added to autoclaved medium. For growth on solid medium, 1.5% (w/v) Bacto-agar (Difco) was added. *E. coli* strains were grown in Luria–Bertani (LB) broth at 37 °C. BBL agar (1.5%, w/v) was added in the case of growth on solid medium. Sucrose sensitivity of *Rhodococcus* strains was tested on LBP agar supplemented with 10% (w/v) sucrose.

General molecular techniques. DNA modifying enzymes were purchased from Roche, New England Biolabs or Amersham Pharmacia Biotech and were used as described by the manufacturers. Isolation of DNA restriction fragments from agarose gels was done by using the Qiagen gel extraction kit

according to the manufacturer's protocol. All DNA manipulations were done according to standard protocols. *Rhodococcus* plasmid DNA was isolated using the Qiagen spin-prep kit with a slight modification: *Rhodococcus* cells were grown for 2–3 h in the presence of ampicillin ($600 \mu\text{g ml}^{-1}$) prior to lysozyme (2 mg ml^{-1}) treatment. PCR was performed under standard conditions using *Taq* polymerase (Roche) unless stated otherwise: 5 cycles of 1 min at 95°C , 1.5 min at 60°C and 1.5 min at 72°C , followed by 25 cycles of 1 min at 95°C , 1.5 min at 55°C and 1.5 min at 72°C .

Functional complementation experiments. Electrocompetent *Rhodococcus* cells of mutant strain RG1-UV29 were obtained as described previously (van der Geize *et al.*, 2000). A gene library (mean insert size 6 kb) of *R. erythropolis* strain RG1 was constructed by cloning *Sau*3A-digested chromosomal DNA into pRESQ (van der Geize *et al.*, 2002). Colonies of strain RG1-UV29 obtained after introducing this gene library by electrotransformation were replica-plated onto mineral agar medium containing AD (0.5 g l^{-1}) as sole carbon and energy source. Functional complementation was scored after 3 days incubation at 30°C . Colonies growing on AD mineral agar medium were cultivated in LBP medium for isolation of plasmid DNA that was subsequently reintroduced into strain RG1-UV29 to check for genuine complementation.

Nucleotide sequencing. Nucleotide sequencing analysis was done on an ALF-Express sequencing robot using dye-primers in the cycle sequencing method (Murray, 1989) with the thermosequenase kit RPN 2538 from Amersham Pharmacia Biotech. 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).

Expression of *kstD2* in *E. coli* BL21(DE3) and preparation of cell-free extracts. The *kstD2* gene was isolated from chromosomal DNA of parent strain SQ1 and mutant strains RG1-UV29, RG1-UV45, RG1-UV50 and RG1-UV51 by PCR, using *kstD2* forward primer 5'-GCGCATATGGCTAAGAATCA GGCACCC-3' and reverse primer 5'-GCGGGATCCCTAC TTCTCTGCTGCGTGATG-3'. For KSTD2 expression by the T7 RNA polymerase pET3 expression system (Novagen), *Nde*I (forward primer) and *Bam*HI (reverse primer) restriction sites were introduced (underlined) and used to clone *kstD2* into *Nde*I/*Bam*HI-digested pET3b (Novagen). The resulting plasmid, pKSD114, was introduced into *E. coli* BL21(DE3) (Studier & Moffatt, 1986). For expression of soluble active KSTD2, *E. coli* BL21(DE3)(pKSD114) was grown for 40 h at 25°C in LB broth (25 ml) supplemented with 0.5 M sorbitol. Cell pellets (7300 g, 30 min, 4°C) were washed with phosphate buffer (g l^{-1} : KH_2PO_4 , 2.72; K_2HPO_4 , 3.48; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 2.46; pH 7.2). Cells were suspended in 5 ml phosphate buffer and disrupted in a French pressure cell at 140 MPa. Cell extracts were centrifuged (40000 g, 20 min, 4°C) to remove cell debris. The supernatants (5–10 mg protein ml^{-1}) were used in KSTD activity assays.

Preparation of cell-free extracts of *E. coli* DH5 α containing the KSTD1 isoenzyme was done as described previously (van der Geize *et al.*, 2000).

Cloning of mutant *kstD2* genes. Mutant *kstD2* genes were isolated from four UV mutants of strain RG1 (strains RG1-UV29, RG1-UV45, RG1-UV50 and RG1-UV51) by PCR using *Pwo* polymerase (Roche) and *kstD2* expression primers. The obtained PCR products were cloned into pBlueScript (II) KS for nucleotide sequencing.

Gene deletion mutagenesis of *kstD2*. Unmarked *kstD2* gene deletions were carried out as described previously (van der

Geize *et al.*, 2001) using plasmid pKSD201 bearing the *sacB* counter-selection system. Southern analysis of *kstD2* gene deletion mutants was done using a digoxigenin (DIG)-labelled *kstD2* probe obtained by PCR (Boehringer PCR DIG probe synthesis kit 1636090) using primers developed for heterologous *kstD2* expression in *E. coli*.

Preparation of cell-free extracts of *R. erythropolis* strains. *Rhodococcus* strains were grown in mineral medium (100 ml) supplemented with 10 mM glucose. Cultures were induced with AD (0.5 g l^{-1}) for 10 h. Following centrifugation (7300 g, 30 min, 4°C), pellets of non-induced and induced cultures were washed with phosphate buffer, resuspended in phosphate buffer (1 ml) and disrupted by two passages through a French pressure cell at 140 MPa. Supernatants obtained after centrifugation (40000 g, 20 min, 4°C) were used for KSTD enzyme activity assays.

KSTD enzyme activity assay. Enzyme activities of KSTD1 or KSTD2 were measured in triplicate spectrophotometrically at 30°C using phenazine methosulphate (PMS) and 2,6-dichlorophenolindophenol (DCPIP). The reaction mixture (1 ml) consisted of 50 mM Tris/HCl, pH 7.0, 1.5 mM PMS, 40 μM DCPIP, cell-free extract and 500 μM steroid in methanol (2%). Activities are expressed as mean values $\pm$ SD in mU (mg protein) $^{-1}$; 1 mU is defined as the reduction of 1 nmol DCPIP min^{-1} ($\epsilon_{600} = 18.7 \times 10^3 \text{ cm}^{-1} \text{ M}^{-1}$).

Steroid bioconversion and steroid analysis. Steroid bioconversion was done (in duplicate) in shake flasks with *Rhodococcus* cultures grown in 100 ml YG15 (g l^{-1} : yeast extract, 15; glucose, 15) medium at 28°C (200 r.p.m. on a rotary shaker). After growth overnight till late exponential phase (OD_{600} of 5–9), AD or 9OHAD (5 g l^{-1} suspended in 0.1%, v/v, Tween 80) was added and bioconversion was followed for several days. Steroids were extracted from the medium (1 ml) with 3 ml methylene-chloride (GC) or 3 ml methylene-chloride/methanol (9:1) extraction (TLC). For HPLC analysis, samples were diluted five times with methanol/water (70:30) and filtered (0.45 μm). Steroids were analysed by HPLC [column, reversed phase Lichrosorb 10RP18, SS $250 \times 3 \text{ mm}$ (Varian Chrompack International)], UV₂₅₄ detection [liquid phase, methanol/water (60:40), 35°C], GC [column, J&W DB-5MS $30 \text{ m} \times 0.25 \text{ mm}$ i.d. $\times 0.25 \text{ mm}$ (Alltech): pre-column, $2 \text{ m} \times 0.25 \text{ mm}$ i.d. (Inter-Sciences): FID-40 detection at 300°C] or TLC [Kieselgel 60 F₂₅₄ $10 \times 20 \text{ cm}$ (Merck) in toluene/ethylacetate 1:1]. Retention times for HPLC (flow rate 0.8 ml min^{-1}) were $t_{\text{R},9\text{OHAD}} = 4.2 \text{ min}$, $t_{\text{R},\text{ADD}} = 7.0 \text{ min}$ and $t_{\text{R},\text{AD}} = 10.5 \text{ min}$, and for GC (carrier N₂, 100 kPa) $t_{\text{R},\text{AD}} = 10.8 \text{ min}$ and $t_{\text{R},\text{ADD}} = 11.1 \text{ min}$. Retention factors for TLC were $R_{\text{F},9\text{OHAD}} = 0.23$, $R_{\text{F},\text{ADD}} = 0.44$ and $R_{\text{F},\text{AD}} = 0.56$. Substrates used (AD, 9OHAD, ADD) were supplied by Diosynth.

In vitro conversion of AD by KSTD2. A reaction mixture (1 ml) consisting of Tris/HCl, pH 7.0 (50 mM), PMS (150 μM), AD (700 μM) and cell-free extract of *E. coli* BL21(DE3)(pKSD114) (approx. 25 μg protein) was incubated overnight at 30°C . Steroid analysis was done by HPLC as described above.

RESULTS AND DISCUSSION

Cloning and characterization of the *kstD2* gene

R. erythropolis strain RG1-UV29, isolated during a screening for 9OHAD growth-deficient mutants of UV mutagenized cells of *kstD* mutant *R. erythropolis* strain RG1 (van der Geize *et al.*, 2001), was used for cloning of the *kstD2* gene. A gene library of *R. erythropolis* strain RG1 was introduced into strain RG1-UV29 by electro-

Fig. 2. Construction of pKSD201 used for unmarked *kstD2* gene deletion in parent strain SQ1 and *kstD* mutant strain RG1, resulting in the isolation of *R. erythropolis* strain RG7 and *R. erythropolis* strain RG8, respectively. The 605 bp *kstD2* remnant, obtained after *MluI* digestion and self-ligation, is indicated by $\Delta kstD2$. ORFs are indicated with arrows: *kstD2*, 3-ketosteroid Δ^1 -dehydrogenase 2; *bla*, ampicillin resistance marker; *aphII*, kanamycin resistance marker; *sacB*, counter-selectable marker. Thick solid curved lines represent *R. erythropolis* SQ1 DNA.

transformation and transformants were replica-plated onto AD mineral agar medium to screen for complementation of the AD-negative growth phenotype. Plasmid pKSD101, isolated from a transformant that showed restored growth on AD mineral medium, was transformed into *E. coli* DH5 α for further analysis.

An insert of approximately 6.5 kb was identified in pKSD101 and subjected to restriction mapping analysis. A 3.6 kb *EcoRI* DNA fragment of pKSD101 was cloned into pBlueScript (II) KS (pKSD105; Fig. 2) and into *Rhodococcus*–*E. coli* shuttle vector pRESQ (pKSD106) for complementation. Plasmid pKSD106 was still able to restore the strain RG1-UV29 phenotype, whereas a 2.4 kb *Asp718* DNA fragment (located on this 3.6 kb fragment) in pRESQ could not. Nucleotide sequence analysis of the 3.6 kb *EcoRI* fragment revealed the presence of a large ORF (ORF1) of 1698 nt (GC content 63.9 mol%), encoding a putative protein of 565 aa (calculated mass 60.2 kDa). The deduced amino acid sequence of ORF1 showed clear similarity with KSTD1 (35% identity), encoded by *kstD* in *R. erythropolis* SQ1, and other known KSTD amino acid sequences present in databases (Fig. 3) and was thus designated *kstD2*. Highest similarity was found with the ORF3-encoded protein (63% identity) from *A. simplex* (Dziadek *et al.*, 1998) (GenBank accession no. Z93338). For alignment purposes, the ORF3 nucleotide sequence was edited to obtain a deduced amino acid sequence matching the KSTD consensus, since ORF3 obviously contained

several frame-shift errors. The alignment strongly suggests that *A. simplex* also contains at least two KSTD isoenzymes, encoded by *ksdD* (Molnár *et al.*, 1995) and ORF3 (suggested name *ksdD2*), respectively. Contrary to *kstD2* in *R. erythropolis* SQ1, ORF3 was found in close proximity to *ksdD* and *ksdI*, encoding KSTD1 and steroid 4,5-isomerase, respectively (Dziadek *et al.*, 1998; Molnár *et al.*, 1995). Analysis of nucleotide sequences directly upstream and downstream of the *kstD2* gene showed no direct evidence for the presence of additional (co-transcribed) nucleotide sequences involved in steroid metabolism. No overlap in nucleotide sequences upstream or downstream of the *kstD2* gene was found with sequences flanking *kstD* (van der Geize *et al.*, 2000).

The putative N-terminal FAD-binding motif of the *kstD2*-encoded protein (KSTD2) was similar to the previously suggested consensus amino acid sequence for FAD-binding in KSTD, GSG(A/G)(A/G)(A/G)X₁₇E (van der Geize *et al.*, 2000), although not completely conserved (Fig. 3).

Expression of *kstD2* in *E. coli*

Attempts to express *kstD2* from its own promoter in *E. coli* were unsuccessful. Alternatively, *kstD2* was cloned into the pET3b expression vector (pKSD114) for expression via the T7 RNA polymerase expression system in *E. coli* BL21(DE3). Highly active soluble KSTD2

		FAD-binding domain		
R.ery2 (100%)	MAKNQAPPATQAKDIVVDLLVIGSG-TGMAAALTANELGLSTLIVVEKTQYVGGSTARSG-	50		
R.ery1 (35%)	-----MQDWTSECDVLVVGSGGGALTGAYTAAAGLITIVLEKTRDFGGTSAYSG-	58		
R.rho (32%)	-----MAEWAEECDVLVVGSGAGGCCGAYTPAREGLSVILVEASEYFGGTTAYSGG	51		
A.sim (34%)	-----MDWAEEDVLVAGSGAGGMAGTYTAAREGLSVCLVEAGDKFGGTTAYSGG	50		
ORF3 (63%)	-----MSDTTVDLLVIGSG-TGLAALSAAREQGLHVLVVEKTEYVGGSTARS-	46		
M.tub (42%)	-----MTVQEFDDVVVGSGAAGMVAALVAHRGLSTVVVEKAPHYGGSTARSG-	48		
P.aer (32%)	----MTSAAVETTPLTCDVLVVGSGAAGLAAAVTAAWHGRRVVLVEKDPVFGGSAWSG-	55		
C.tes (29%)	-----MAEQEYDLIVVGSGAGACWAPIRAQEQLKTLVVEKTELPFGGTSALSG-	48		
		:: *** . . . * . ::* ****:*		
		#(F)	#(I)	
R.ery2	320 GSMDQSWWFFPAVASIKG-RFP	339	490 ALYAVKMTLSDLGTCGGVQ	508
R.ery1	275 ALLDQAWFCPGVEQPD--GSA	293	436 PFYAARIVLSDLGTKGGLV	454
R.rho	275 DLMQAWWSPGLTHPD--GRS	293	436 PFHAAQFGISDLGTKGGLR	454
A.sim	276 DLMGEAWWSPGLTHPD--GRS	294	437 PFHAAAFGISDLGTKGGLR	455
ORF3	306 TLMQDTWWFFPAVAPLPR-GTP	325	476 DLYAVKVVLSDLGTCGGLS	494
M.tub	313 DLMDDAWWGPTVPLV---GKP	330	484 PYYGAKMVPDGLGTKGGIR	502
P.aer	319 DLRSPIAWAPVSRVPHGDGET	339	491 PYYAVQVEPGCFGTAGLK	509
C.tes	315 DGLVLGRAHHGCSQGA--GLS	333	484 PFYAMRLWPGEIGTKGGLL	502
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Fig. 3. Alignment of known (AAF19054, AAL82579, AB007847, BAA07186, Q06401) and putative (CAB05041, Z93338, AAG05631) KSTD amino acid sequences (GenBank accession numbers are in parentheses): R.ery1, KSTD1 from *R. erythropolis* SQ1 (AAF19054); R.ery2, KSTD2 from *R. erythropolis* SQ1 (AAL82579); R.rho, KsdD from *Rhodococcus rhodochrous* (AB007847); A.sim, KsdD from *A. simplex* (BAA07186); ORF3, ORF3 from *A. simplex* (Z93338, sequence edited); M.tub, Rv3537 from *Mycobacterium tuberculosis* (CAB05041); P.aer, *P. aeruginosa* (AAG05631); C.tes, Δ 1-DH from *C. testosteroni* (Q06401). The alignment was generated using the CLUSTALW 1.74 program (Thompson *et al.*, 1994). Asterisks indicate identical residues. Colons and stops indicate high and low similarity, respectively. The percentage identity of each KSTD to *R. erythropolis* SQ1 KSTD2 is shown in parentheses at the beginning of the alignment. The putative FAD-binding domain [consensus GSG(A/G)(A/G)(A/G)X₁₇E] is shown. #(F) and #(I) indicate amino acid residue changes identified in KSTD2 mutants (deduced from mutant *kstD2* genes) from strains RG1-UV45 (Thr503Ile) and RG1-UV51 (Ser325Phe), respectively.

enzyme was obtained in extracts of *E. coli* cells grown at 25 °C. Incubation of AD with cell-free extracts (25 µg protein) of *E. coli* BL21(DE3)(pKSD114) resulted in its conversion into ADD, confirming that *in vivo* KSTD2 indeed performs the 3-ketosteroid Δ^1 -dehydrogenation reaction. ADD formation was not observed in controls with cell-free extracts without AD. Contrary to KSTD1, KSTD2 does not show any activity in the presence of nitro blue tetrazolium. KSTD2 thus could not be visualized by activity staining on native PAGE (van der Geize *et al.*, 2000). *In vitro* activity of KSTD2 was detected, however, in the presence of DCPIP and activity was further enhanced by addition of PMS. Optimal KSTD2 activity (6 ± 0.8 U mg⁻¹) was found at pH 7–8 at 30 °C using AD and the DCPIP/PMS system. A fivefold higher KSTD2 activity was observed with AD compared to 9OHAD. Substrate affinity of KSTD2 for AD ($K_{m,app} = 17$ µM) was 10-fold higher than for 9OHAD ($K_{m,app} = 165$ µM). In contrast, KSTD1 displays similar activities and affinities with AD ($K_{m,app} = 82$ µM) and 9OHAD ($K_{m,app} = 87$ µM).

Construction of single and double gene deletion mutants

For *kstD2* unmarked gene deletion mutagenesis, plasmid pKSD201, with a *kstD2* remnant ($\Delta kstD2$) of 605 bp, was obtained after deletion of a 1093 bp *Mlu*I internal fragment of *kstD2* (Fig. 2). Plasmid pKSD201 was introduced into *E. coli* S17-1 and mobilized by conjugation to both parent strain *R. erythropolis* SQ1 and *kstD* mutant strain RG1. Using the *sacB* counter-selection system we were able to isolate mutant strains

RG7 (*kstD2* mutant) and RG8 (*kstD kstD2* mutant). Southern analysis, using DIG-labelled *kstD2* gene as probe, on *Asp*718-digested chromosomal DNA of parent strain SQ1, strain RG7 and strain RG8, confirmed deletion of *kstD2* in the latter two strains: a 2.4 kb *Asp*718 DNA fragment of strain SQ1 hybridizing with the *kstD2* probe was reduced to a 1.3 kb DNA fragment in both mutant strains (data not shown). PCR performed on chromosomal DNA of strains RG7 and RG8 with the *kstD2* primers resulted in PCR products with sizes of 0.6 kb, further demonstrating *kstD2* gene deletion.

Characterization of mutant strains RG1, RG7 and RG8

Growth on AD, ADD or 9OHAD mineral agar medium was unaffected in both single gene deletion mutant strains RG1 and RG7. Growth on steroid substrates AD and 9OHAD was blocked in the *kstD kstD2* double gene deletion strain RG8, while growth on ADD was not affected, confirming inactivation of the steroid Δ^1 -dehydrogenation reaction. Thus, both KSTD1 and KSTD2 are involved in steroid degradation by *R. erythropolis* SQ1 and the presence of only one of these enzymes is sufficient to allow degradation of the steroid skeleton to proceed. KSTD enzyme activities could not be detected in extracts of cells of parent strain SQ1 and mutant strains RG1, RG7 and RG8 grown on glucose without steroid induction. Upon steroid (AD) induction, KSTD enzyme activities (using AD as substrate) were found in cell-free extracts of parent strain SQ1 (280 ± 40 mU mg⁻¹), strain RG1 (80 ± 5 mU mg⁻¹) and strain RG7 (285 ± 30 mU mg⁻¹), indicating that both KSTD1 and KSTD2 are inducible proteins. No KSTD

Fig. 4. Graphs of steroid (5 g l⁻¹) bioconversion by *R. erythropolis* strain SQ1 (●), *kstD* mutant strain RG1 (○), *kstD2* mutant strain RG7 (▼) and *kstD kstD2* mutant strain RG8 (▽), showing (A) 9OHAD accumulation from AD and (B) 9OHAD degradation.

activity was detected in cell-free extracts of the double gene deletion mutant strain RG8.

Bioconversion of AD

Incubations of parent strain SQ1 and three mutants, i.e. RG1, RG7 and RG8, with 5 g AD l⁻¹ (17.5 mM) or 5 g 9OHAD l⁻¹ (16.6 mM) were performed (Fig. 4). Comparable rates of AD degradation (via KSTD and/or

KSH activities; Fig. 1) were observed for all strains (1.0–1.7 mmol AD l⁻¹ h⁻¹). No 9OHAD accumulation from AD was observed with strains SQ1 and RG1 (Fig. 4A). Strain RG7 initially accumulated 9OHAD (1.8 ± 0.1 mmol 9OHAD formed l⁻¹ h⁻¹), stoichiometrically derived from AD (1.7 ± 0.1 mmol AD degraded l⁻¹ h⁻¹), suggesting low, or even absence, of KSTD activity during the early phase of bio-transformation. With strain RG7, maximal 9OHAD accumulation was found within 20 h after addition of AD; 9OHAD was subsequently slowly degraded again (0.23 ± 0.03 mmol 9OHAD l⁻¹ h⁻¹). Similar rates of 9OHAD degradation (0.27 ± 0.04 mmol 9OHAD l⁻¹ h⁻¹) were found during incubation of strain RG7 with 9OHAD. Much higher rates of 9OHAD degradation were observed for parent strain SQ1 and *kstD* mutant strain RG1 (1.3 ± 0.2 mmol 9OHAD l⁻¹ h⁻¹). These experiments show that 9OHAD degradation proceeds differently in an environment with only KSTD2 present, compared to a situation in which KSTD1 is the only available KSTD enzyme. With solely KSTD2 present (strain RG1), steroid degradation of 9OHAD proceeds at the same rate as in parent strain SQ1. In contrast, the absence of KSTD2 (strain RG7) results in significant temporary accumulation of 9OHAD during AD bioconversion, and in low rates of 9OHAD degradation. *In vivo*, KSTD1 thus is considerably less efficient in Δ^1 -dehydrogenation of 9OHAD than KSTD2. Incubation of strain RG8 with AD resulted in high accumulation levels of 9OHAD (75% of AD converted into 9OHAD) within 24 h after addition of AD. The 9OHAD formed was not degraded during further incubation. Thus, inactivation of both *kstD* and *kstD2* is necessary to inactivate KSTD activity in *R. erythropolis* SQ1 and to block 9OHAD degradation.

KSTD2 mutants Ser325Phe and Thr503Ile

In addition to strain RG1-UV29, three other mutants (strains RG1-UV45, RG1-UV50 and RG1-UV51) were identified during screening for mutants blocked in Δ^1 -dehydrogenation. Conceivably, these four mutant strains possess mutant *kstD2* genes, containing point mutations as a result of UV irradiation, resulting in inactive KSTD2 enzymes. Nucleotide sequencing revealed that *kstD2* from RG1-UV29 contained an opal mutation at amino acid residue Trp122. Mutant *kstD2* gene from strain RG1-UV45 contained the Thr503Ile mutation. Thr503 is a fully conserved residue among all KSTD enzymes known to date (Fig. 3). Replacement of the Thr503 residue by isoleucine, a hydrophobic residue of comparable size, renders KSTD2 non-functional, suggesting that the hydroxyl group of threonine is important in either substrate binding or in catalysis. The *kstD2* gene of strain RG1-UV51 contained the Ser325Phe mutation (Fig. 3), replacing a small hydroxyl group by a relatively large phenyl moiety. Ser325 is positioned in a poorly conserved region of KSTD enzymes, although the low similarity is mostly due to the aberrant amino acid sequence of KSTD from *Comamonas testosteroni* and *Pseudomonas aeruginosa* in this region. Amino acid

sequences of actinomycetal KSTD enzymes, however, are rather conserved over a stretch of 8 aa (322-MDQSWWFP-329). Amino acid residue position 325 in KSTD2 thus is important for activity in actinomycetal KSTD enzymes. Ser325 of KSTD2 is occupied by an alanine or threonine in other actinomycetal KSTD enzymes, indicating that a small residue at this position is favoured. The presence of a phenyl moiety in KSTD2 could inactivate KSTD activity, for instance by steric hindrance of substrate binding. No mutation was found in *kstD2* of strain RG1-UV50. Inactivation of KSTD2 in this strain could be the result of a mutation in sequences needed for transcription (e.g. promoter sequences), which was not further investigated.

Heterologous expression of mutant KSTD2 protein encoded by *kstD2* (Thr503Ile) or *kstD2* (Ser325Phe) in *E. coli* BL21(DE3) resulted in full-length protein on SDS-PAGE having no detectable KSTD enzyme activity.

Conclusions

R. erythropolis SQ1 employs two KSTD isoenzymes that *in vivo* may have different functional roles in AD degradation. AD degradation may occur via either ADD or 9OHAD (Fig. 1). Considering the observed mild toxicity of ADD to *R. erythropolis* SQ1 [growth on agar medium is considerably slower in the presence of higher concentrations (0.5 g l^{-1}) of ADD], we speculate that the presence of two KSTD enzymes may serve to prevent accumulation of relatively high cellular concentrations of ADD. A regulatory system thus is expected that differentially regulates KSTD2 and KSTD1 cellular activity levels in response to ADD levels. *kstD* is preceded by a putative regulatory gene (*kstR*) that may fulfil just this role (van der Geize *et al.*, 2000). Regulation of KSTD1 and KSTD2 activities and/or expression levels, and the role of *kstR* herein will be at the focus of our future research.

Complete inactivation of steroid Δ^1 -dehydrogenation by gene deletion of *kstD* and *kstD2* in *R. erythropolis* SQ1 (strain RG8) has resulted in a metabolic block at the level of steroid degradation and a stoichiometrical accumulation of 9OHAD from AD. Such mutants are of industrial interest. Accumulation of steroid intermediates (e.g. AD, 9OHAD, ADD) from sterol substrates may produce bioactive (precursor) molecules for medical use.

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