The Rhodococcus erythropolis SQ1 kstD3 gene was cloned, heterologously expressed and biochemically characterized as a KSTD3 (3-keto-5α-steroid Δ1-dehydrogenase). Upstream of kstD3, an ORF (open reading frame) with similarity to Δ4 KSTD (3-keto-5α-steroid Δ1-dehydrogenase) was found, tentatively designated kst4D. Biochemical analysis revealed that the Δ1 KSTD3 has a clear preference for 3-ketosteroids with a saturated A-ring, displaying highest activity on 5α-AD (5α-androstane-3,17-dione) and 5α-T (5α-testosterone; also known as 17β-hydroxy-5α-androstane-3-one). The KSTD1 and KSTD2 enzymes, on the other hand, clearly prefer (9α-hydroxy)-4-androstene-3,17-dione as substrates. Phylogenetic analysis of known and putative KSTD amino acid sequences showed that the R. erythropolis KSTD proteins cluster into four distinct groups.

Interestingly, Δ1 KSTD3 from R. erythropolis SQ1 clustered with Rv3537, the only Δ1 KSTD present in Mycobacterium tuberculosis H37Rv, a protein involved in cholesterol catabolism and pathogenicity. The substrate range of heterologously expressed Rv3537 enzyme was nearly identical with that of Δ1 KSTD3, indicating that these are orthologous enzymes. The results imply that 5α-AD and 5α-T are newly identified intermediates in the cholesterol catabolic pathway, and important steroids with respect to pathogenicity.

Key words: actinomycete, 5α-androstane-3,17-dione (5α-AD), cholesterol degradation, 3-ketosteroid dehydrogenase, Rhodococcus, steroid.

INTRODUCTION

KSTD (3-ketosteroid Δ1-dehydrogenase) [4-ene-3-oxosteroid: (acceptor)-1-ene-oxoreductase; EC 1.3.99.4] is a flavoprotein catalysing the trans-axial elimination of the C-1(α) and C-2(β) hydrogen atoms of the A-ring from the polycyclic ring structure of 3-ketosteroids [1]. KSTD is a key enzyme in microbial steroid catabolism needed for opening of the steroid B-ring [2–5]. KSTD activities have been found in various actinobacteria, e.g. Arthrobacter [6], Mycobacterium [7] and Rhodococcus [4,5,8–10], as well as in Comamonas (formerly Pseudomonas) testosteroni [11,12]. Choi et al. [13] proposed to classify KSTD enzymes into two groups, on the basis of their ability to use 11-substituted steroids as substrate. KSTD of Ps. testosteroni is unable to desaturate C-11-hydroxy- or 11-ketosteroids [14], while KSTD of Nocardioida corallina acts on C-11-substituted steroids [15]. Cortisone (11-keto), but not 11β-cortisol, was found to be an excellent substrate. Characterized KSTD enzymes display quite a broad substrate range [7,13,15,16]. Highest Δ1 KSTD activities are observed with steroid substrates carrying the 3-keto-4-ene structure; (9α-hydroxy)-4-androstene-3,17-dione and 17β-hydroxy-4-androstene-3-one (testosterone), usually, are preferred substrates. Δ1 KSTD activity on saturated 3-ketosteroids, e.g. 5α-AD (5α-androstane-3,17-dione), has been observed in Ps. testosteroni [17]. However, no kinetic data for Δ1 KSTD enzymes and these saturated 3-ketosteroids are available.

In previous work, we have characterized the Rhodococcus erythropolis SQ1 Δ1 KSTD1 and Δ1 KSTD2 isoenzymes [5,9]. Analysis of a kstD1 kstD2 double gene deletion mutant R. erythropolis strain RG8 showed stoichiometric accumulation of 9OHAD (9α-hydroxy-4-androstene-3,17-dione) from 4-AD (4-androstene-3,17-dione) due to blocked opening of the steroid A-ring [5]. 4-AD is one of the proposed central pathway intermediates in the degradation of cholesterol. However, strain RG8 is still able to degrade and grow on cholesterol as the sole carbon and energy source without accumulation of steroid pathway intermediates (e.g. 4-AD and 9OHAD) (the present study). Our subsequent studies resulted in identification of a third Δ1 KSTD [KSTD3 (3-keto-5α-steroid Δ1-dehydrogenase)] enzyme in strain RG8.

Analysis of the genome of the human pathogen Mycobacterium tuberculosis H37Rv revealed the presence of a single gene (rv3537) encoding a putative Δ1 KSTD [4,18,19]. M. tuberculosis is the causative agent of tuberculosis, causing millions of deaths every year. The rv3537 gene is one of several essential pathogenicity genes recently identified as part of a gene cluster encoding cholesterol catabolism [19,20]. Biochemical characterization and phylogenetic analysis indicate that Rv3537 of M. tuberculosis H37Rv is the orthologue of Δ1 KSTD3 of R. erythropolis SQ1 (the present study). Phylogenetic analysis revealed clustering of Δ1 KSTD enzymes in at least four different groups. Here, we report the biochemical characterization of a
third and unique Δ^1 KSTD (KSTD3) of *R. erythropolis* SQ1 (and strain RG8) and its orthologue Rv3537 in *M. tuberculosis* H37Rv involved in cholesterol catabolism.

**EXPERIMENTAL**

**Bacterial strains and growth conditions**

*Rhodococcus* strains were cultivated in complex medium at 30°C and 200 rev./min. Complex medium (LBP) contained 1% (w/v) bacto-peptone (Difco), 0.5% (w/v) yeast extract (BBL Becton Dickinson and Co.) and 1% (w/v) NaCl. *Escherichia coli* strains were grown in LB (Luria–Bertani) broth (Sigma) at 37°C unless stated otherwise.

**General cloning techniques**

Recombinant DNA techniques were done according to standard protocols [21]. *E. coli* DH5α [22] was used as the general host for cloning. Transformation of *E. coli* strains was performed as described by Chung et al. [23]. DNA-modifying enzymes (restriction enzymes, ligase and polymerase) were purchased from Roche or New England Biolabs and were used as described by the manufacturer. Isolation of DNA restriction fragments from agarose gels was performed by using the Qiagen gel extraction kit according to protocol. DNA nucleotide sequencing was performed by AGOWA. These sequence data have been submitted to the GenBank®, EMBL, DDBJ and GSBD Nucleotide Sequence Databases under the accession number EU014895.

**Preparation of Rhodococcus cell extracts and KSTD activity staining on native PAGE**

Overnight cultures (250 ml) of wild-type *R. erythropolis* SQ1 [24] and kstD1 kstD2 double gene deletion mutant *R. erythropolis* RG8 [5] were grown in LBP medium until a D_{600} of 4 was reached and subsequently induced with steroids (1 g/l) for an additional 4 h. Cell pellets (centrifuged at 2500 g for 30 min at 4°C) were washed with 200 ml of phosphate buffer (2.72 g/l KH₂PO₄, 3.48 g/l K₂HPO₄, and 2.46 g/l MgSO₄ · 7H₂O, pH 7.2). The washed cell suspensions were concentrated to 4 ml and disrupted by passage through a French pressure cell (140 MPa) twice. Cell extracts were centrifuged at 25 000 g for 30 min to remove cell debris. The resulting cell-free extracts were used for analysis of KSTD activity on native PAGE (10% polyacrylamide). KSTD activity (~30–50 μg of protein loaded on to gel) was visualized by incubating native PAGE gels in 100 ml of 66.7 mM Tris buffer containing 3.1 mg of phenazine methosulfate, 2.9 mg of steroid (dissolved in ethanol) and 41 mg of NBT (Nitro Blue Tetrazolium) dissolved in 70% dimethylformamide.

**Screening an *R. erythropolis* genomic library for 3-ketosteroid dehydrogenase genes**

Degenerate kstD<sup>deg</sup> PCR primers [forward 5′-tgctggctggacac(a/c)-gg(g/c)gg(g/c)tt-3′ and reverse 5′-tgcggccggctagagt(ch)-gt-3′] were developed on the basis of conserved sequences of amino acid sequences of Δ^1 KSTD proteins and the known nucleotide sequences of kstD1 and kstD2, and used to screen chromosomal DNA of *R. erythropolis* RG8 for the presence of novel 3-ketosteroid dehydrogenase genes. Specific kstD<sup>new</sup> primers (forward 5′-cttggctggctggagatg-3′ and reverse 5′-gacggtgccg-3′) were designed on the obtained 882 bp PCR fragment from RG8 and used to screen a genomic library of *R. erythropolis* strain RG1 in plasmid pRESQ [5] to get a clone containing the full-length kstD gene sequence. The genomic library of strain RG1 was introduced into *E. coli* DH5α and plated on LB with 25 μg/ml kanamycin. All colonies on a plate were used for plasmid isolation [25] and checked with PCR for positive signal with kstD<sup>new</sup> primers. PCR was performed using 30 cycles of 1 min at 94°C, 1 min at 60°C and 1 min at 72°C. This procedure of transformation, plating dilutions and PCR was repeated several times to identify a single clone, containing the pRESQ vector with a 7.4 kb insert containing kstD3, designated pJK2 (Figure 2).

**Heterologous expression of kstD3 in *E. coli* cells**

Total DNA of *R. erythropolis* was isolated as described in [4]. PCR on total DNA was performed using 30 cycles of 1 min at 94°C, 1 min at 60°C and 1.5 min at 72°C with forward primer kstD<sup>new</sup>-F (5′-ggatgtgagcaagccatagctg-3′) including start codon and NdeI restriction site (underlined) and reverse primer kstD<sup>new</sup>-R (5′-ggatctctagactttgctgctggcacc-3′) including stop codon and BlII restriction site (underlined). The resulting PCR product of kstD3 (1725 nt) was cloned into NdeI/BamHI-digested pET15b (Novagen), thereby introducing an N-terminal His tag into the protein. The pET15b-KSTD3 construct was introduced into *E. coli* strain BL21(DE3) (Invitrogen), resulting in recombinant strain BL21pET15bKSTD3. The KSTD1 (see for PCR [4] and kstD2 [5] genes were also cloned into the NdeI/BamHI site of pET15b and transformed into *E. coli* BL21(DE3), resulting in recombinant strains BL21pET15bKSTD1 and BL21pET15bKSTD2.

Precultures, diluted 100-fold, of recombinant *E. coli* strains were grown for 48 h at 16°C and 200 rev./min in LB broth supplemented with 0.5 M sorbitol and ampicillin (100 μg/ml). IPTG (isopropyl β-D-thiogalactoside) was added at a final concentration of 0.1 mM at inoculation (kstD1, kstD2 expression) or after 24 h of incubation for kstD3 expression. After 24 h (kstD3) or 48 h (kstD1, kstD2) induction, cells were harvested by centrifugation at 2600 g for 10 min and dissolved in phosphate buffer. Cells were disrupted by French press, and cell debris was precipitated by centrifugation at 25000 g for 30 min. Supernatants were used for kinetic studies with a range of substrates.

**Identification of steroids by HPLC**

Cell-free extract of *E. coli* expressing KSTD3 (50–200 μg of protein) was incubated with 200 μM DCPIP (2,6-dichlorophenol- indophenol) and 200 μM 5α-AD in 50 mM Tris buffer (pH 7.4) for 1 h at 30°C in a total volume of 0.5 ml. Subsequently, 2 ml of 80% (v/v) methanol was added and the sample was incubated for 10 min at room temperature (20°C) and analysed by HPLC.

Steroids were separated on an Alltech C18 column (250 mm × 4.6 mm, 5 μm) at 35°C, using a mobile phase consisting of methanol/water (80:20, v/v) and detected at a wavelength of 254 nm.

**KSTD enzyme activity standard assay**

The kinetics of the KSTD1, KSTD2 and KSTD3 enzymes were determined by incubating the respective cell-free extracts with 5α-AD (Steraloids), 5α-T (5α-testosterone; also known as 17β-hydroxy-5α-androstan-3-one) (Sigma), 4-AD (Organon Biosciences), 9OAHAD (Organon Biosciences), progesterone (ICN Biomedicals), 11β-cortisol (Sigma), 23,24-bisnor-5α-cholestan-3-one acid (Steraloids), 5β-androstan-3,17-dione (Steraloids), 1-(5α)-AD [1-(5α)-androsten-3,17-dione] (Steraloids), 11α-hydroxy-4-norandrostene-3,17-dione (Organon Biosciences), ADD (1,4-androstadiene-3,17-dione; Organon Biosciences),
Phylogenetic tree construction

Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 3.1 [26]. Amino acid sequences of different KSTD enzymes of different actinomycetes and of C. testosteroni were obtained from GenBank® or TIGR (The Institute for Genomic Research) databases. The amino acid sequences (accession number in parentheses) of KSTD enzymes of different actinomycetes and of C. testosteroni are listed below. The number of KSTD enzymes in R. erythropolis SQ1 (Figure 1) is even an underestimation, since two Δ^1 KSTD enzymes and one Δ^2 KSTD enzyme, although these inducing compounds are substrates for Δ^1 KSTD enzymes.

Figure 1 KSTD activity staining in R. erythropolis SQ1

KSTD activity was visualized by adding first 4-AD and subsequently 1-(5α)-AD as substrates, on native PAGE gel loaded with cell-free extracts (30–50 μg of protein) of wild-type R. erythropolis SQ1 (WT) and ksd1 ksd2 mutant strain R8 (R8) induced with 4-AD, 9OHAD or progesterone (P), revealing activity bands of Δ^1 3-ketosteroid dehydrogenases (Δ^1 KSTD) (the lower two bands, which appear after adding 4-AD) and a Δ^2 3-ketosteroid dehydrogenase (Δ^2 KSTD) (the upper band, which appears after adding 1-(5α)-AD). Although two Δ^1 KSTD-encoding genes have been knocked out in mutant strain R8, only one activity band has disappeared (KSTD1, [4]).

RESULTS AND DISCUSSION

Identification of additional KSTD activities in ksd1 ksd2 mutant R8

Deletion of the KSTD genes ksd1 and ksd2 in R. erythropolis SQ1 did not result in accumulation of steroid intermediates during incubation of strain R8 with cholesterol [5]. We now also observed that strain R8 still grows normally on cholesterol as the sole carbon source. Therefore we predicted the presence of additional KSTD enzymes in R. erythropolis SQ1. KSTD activity staining, first with 4-AD (a Δ^1 KSTD substrate) and subsequently with 1-5α-AD [Δ^2 KSTD (3-keto-5α-steroid Δ^1-dehydrogenase) substrate], visualized activity bands for two Δ^1 KSTD enzymes and for one Δ^2 KSTD enzyme in wild-type R. erythropolis SQ1 (Figure 1). Only KSTD1 enzyme activity band is missing in the ksd1 ksd2 mutant strain R. erythropolis R8 (Figure 1) (see also [5]). The Δ^1 KSTD and second Δ^2 KSTD enzyme have not been characterized yet. The steroids progesterone, 4-AD and 9OHAD each induce two Δ^1 KSTD enzymes and one Δ^2 KSTD enzyme, although these inducing compounds are substrates for Δ^1 KSTD enzymes.

Figure 1 thus demonstrates the natural diversity of 3-keto-steroid dehydrogenases in R. erythropolis. The number of KSTD activities shown in Figure 1 is even an underestimation, since some KSTD enzymes (for instance KSTD2) cannot use NBT as an electron acceptor and are not detected in this assay [5].

The two KSTD activities observed in ksd1 ksd2 mutant strain R8 (Figure 1) indicate that, in addition to KSTD1 and KSTD2, R. erythropolis harbours at least one more Δ^1 KSTD and at least one Δ^2 KSTD activity. In subsequent work, we have cloned and characterized additional ksd genes that might be responsible for the ongoing consumption of cholesterol in the ksd1 ksd2 mutant strain R8 (J. Knol, unpublished work).

Isolation and cloning of additional ksd genes from R. erythropolis SQ1

Alignment of annotated (e.g. KSTD1 and KSTD2) and putative Δ^1 KSTD protein sequences identified by similarity searches in databases (http://www.ncbi.nlm.nih.gov/BLAST/) revealed the presence of conserved domains. Based on two conserved domains, the VVLAAGGF motif (amino acids 225–232 in KSTD1SQ1) and GLYAAG motif (amino acids 471–476 in KSTD1SQ1), a set of
degenerate PCR primers was designed and used for screening strain RG8 for the presence of additional \( kstD \) sequences. A PCR product of approx. 0.9 kb was obtained and sequenced. The deduced amino acid sequence was similar to, but not identical with, KSTD1 and KSTD2 from \( R. \) erythropolis SQ1, suggesting that a third \( kstD \) gene had been cloned. The full-length gene was subsequently isolated by screening a genomic library of \( R. \) erythropolis RG1 [5] with specific PCR primers based on the nucleotide sequence of the 0.9 kb PCR product. A positive clone (pJK2), carrying a 7.4 kb insert (GC-content 63.3\%), was identified and sequenced. Bioinformatic analysis of the nucleotide sequence revealed a total of six ORFs (open reading frames), identified and sequenced. Bioinformatic analysis of the nucleotide sequence revealed a total of six ORFs (open reading frames), identified and sequenced. Bioinformatic analysis of the nucleotide sequence revealed a total of six ORFs (open reading frames), identified and sequenced. Bioinformatic analysis of the nucleotide sequence revealed a total of six ORFs (open reading frames), identified and sequenced. Bioinformatic analysis of the nucleotide sequence revealed a total of six ORFs (open reading frames), identified and sequenced. Bioinformatic analysis of the nucleotide sequence revealed a total of six ORFs (open reading frames), identified and sequenced. Bioinformatic analysis of the nucleotide sequence revealed a total of six ORFs (open reading frames), identified and sequenced. Bioinformatic analysis of the nucleotide sequence revealed a total of six ORFs (open reading frames), identified and sequenced. Bioinformatic analysis of the nucleotide sequence revealed a total of six ORFs (open reading frames), identified and sequenced. Bioinformatic analysis of the nucleotide sequence revealed a total of six ORFs (open reading frames), identified and sequenced. Bioinformatic analysis of the nucleotide sequence revealed a total of six ORFs (open reading frames), identified and sequenced.

The genetic organization of this cluster is (semi)conserved in several other actinomycetes, e.g. in \( N. \) farcinica, \( Rhodococcus \) sp. RA1 and \( M. \) tuberculosis [10,18,28], indicating a highly relevant role in cholesterol catabolism.

**Heterologous expression of \( kstD3 \) in \( E. \) coli**

The \( kstD3 \) gene of strain \( R. \) erythropolis SQ1 was heterologously expressed in \( E. \) coli strain BL21(DE3) for further biochemical analysis. \( E. \) coli strain BL21(DE3), containing the pET15b-\( kstD3 \), expressed active KSTD3 at moderate levels (~5% of total protein, every lane contains approx. 50 \( \mu \)g of protein; Figure 3) when grown for 2 days at 16\( ^\circ \)C. The \( kstD3 \) gene encodes a protein of 574 amino acids with a calculated molecular mass of 61 kDa, which corresponds to the band of KSTD3 on the SDS/PAGE gel (Figure 3). The expression of KSTD3 is minimal compared with expression of KSTD1 (~50% of total protein) and KSTD2 (~20% of total protein). Expression at higher temperatures resulted in production of inactive KSTD3. Attempts to purify active His-tagged KSTD3 protein were unsuccessful, since KSTD3 activity was lost upon purification using an Ni-NTA (Ni\( ^{2+}\)-nitrilotriaceticate) column. Despite exhaustive attempts, using different elution agents (e.g. NaCl, imidazole and histidine) or additives (e.g. dithiothreitol, FAD, FMN and glyceral), we were unable to obtain purified active KSTD3 protein. Since the \( E. \) coli background lacks all KSTD activity, we decided to perform subsequent biochemical analysis of KSTD3 with cell-free extracts.

**KSTD3 displays 3-keto-5\( \alpha \)-steroid 1\( \Delta \)-dehydrogenase activity**

On the basis of sequence similarities to other 1\( \Delta \) KSTD proteins, KSTD3 is expected to encode 1\( \Delta \) KSTD activity. However, incubations of cell-free extracts containing KSTD3 with 4-AD (200 \( \mu \)M) did not reveal detectable initial activity in a standard assay using DCPIP as an artificial electron acceptor. Interestingly, 1\( \Delta \) KSTD enzymes characterized to date all display relatively high
KSTD3 uses O₂ as electron acceptor. Oxidase activity has been previously described for KSTD of *N. corallina* [15]. Intriguingly, aerobic dehydration by KSTD has been linked to transmembrane potential generation in *Arthrobacter globiformis* [29]. KSTD3, thus, may have a similar function in *Rhodococcus*.

**KSTD3 displays a narrow substrate specificity compared with KSTD1 and KSTD2**

Cell-free extracts of *E. coli* B21(DE3), containing KSTD1, KSTD2, or KSTD3, were used to compare the substrate specificity of KSTD3 with that of KSTD1 and KSTD2 (Table 2). KSTD activity was not observed in control cell-free extracts of *E. coli* B21(DE3) harbouring the expression vector without a *kstD* gene. Clearly, KSTD3 has a very narrow substrate range compared with KSTD1 and KSTD2. KSTD3 activity was detected with 5α-AD, 5α-T and 5α-P, but not with 5β-H or 3-hydroxy isolomers tested. KSTD enzymes require a carbonyl oxygen at C-3 that interacts with an electrophilic residue, thereby facilitating the extraction of the C-2(β) hydrogen as a proton by a nucleophilic residue [30]. It is thought that an essential histidine residue is acting as a nucleophile [31].

5α-T appears to be the preferred KSTD3 substrate, with an approx. 2-fold lower *Kₘ* compared with 5α-AD. A 5α-H-3-keto configuration of the steroid substrate appears to be important for high KSTD3 activity. KSTD3 activity was not observed with 5α-steroids having a bulky side chain at C-17, indicating that only steroids carrying a small (5α-P) or no aliphatic side chain (5α-AD, 5α-T) are suitable substrates for KSTD3. Unlike KSTD1 and KSTD2, KSTD3 does not, or hardly at all, display activity with the common unsaturated Δ¹ steroids (e.g. 4-AD and 9OHAD), with the exception of a minor activity observed for 4-pregnene-3,20-dione (progesterone). The 5α-3-ketoestrogens 5α-AD and 5α-T are substrates for all three Δ¹ KSTD enzymes studied. KSTD3 can be distinguished from KSTD1 and KSTD2 by its preference for steroids with a saturated A-ring. KSTD3 and KSTD2 both cannot use NBT as an artificial electron acceptor; the

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**Table 2: Substrate profiles of KSTD1, KSTD2 and KSTD3 of *R. erythropolis* SQ1, and KSTD3 of *M. tuberculosis* H37Rv, expressed in cell-free extracts of *E. coli***

<table>
<thead>
<tr>
<th>Substrates</th>
<th>KSTD1 Rel <em>Vₘₐₓ</em></th>
<th><em>Kₘ</em> (app)</th>
<th>KSTD2 Rel <em>Vₘₐₓ</em></th>
<th><em>Kₘ</em> (app)</th>
<th>KSTD3*SQ1 Rel <em>Vₘₐₓ</em></th>
<th><em>Kₘ</em> (app)</th>
<th>KSTD3*H37Rv Rel <em>Vₘₐₓ</em></th>
<th><em>Kₘ</em> (app)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5α-T</td>
<td>84 ± 3%</td>
<td>36 ± 4</td>
<td>50 ± 3%</td>
<td>6 ± 1</td>
<td>97 ± 10%</td>
<td>36 ± 3</td>
<td>73 ± 14%</td>
<td>33 ± 10</td>
</tr>
<tr>
<td>5α-AD</td>
<td>100%</td>
<td>59 ± 3</td>
<td>100%</td>
<td>13 ± 3</td>
<td>100%</td>
<td>70 ± 12</td>
<td>100%</td>
<td>160 ± 16</td>
</tr>
<tr>
<td>4-AD</td>
<td>574 ± 14%</td>
<td>104 ± 5</td>
<td>249 ± 7%</td>
<td>5 ± 1</td>
<td>28 ± 9</td>
<td>29 ± 6</td>
<td>35 ± 6</td>
<td>65 ± 4</td>
</tr>
<tr>
<td>9OHAD</td>
<td>655 ± 30%</td>
<td>119 ± 11</td>
<td>186 ± 8%</td>
<td>58 ± 9</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Progesterone</td>
<td>340 ± 8%</td>
<td>18 ± 2</td>
<td>460 ± 6%</td>
<td>3 ± 1</td>
<td>25 ± 3%</td>
<td>29 ± 6</td>
<td>35 ± 6</td>
<td>65 ± 4</td>
</tr>
<tr>
<td>5α-P</td>
<td>22 ± 2%</td>
<td>21 ± 5</td>
<td>12 ± 1%</td>
<td>7 ± 1</td>
<td>47 ± 8%</td>
<td>14 ± 4</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>23,24-Bisnor-5α-cholestan-3-one</td>
<td>69 ± 2%</td>
<td>74 ± 4</td>
<td>60 ± 2%</td>
<td>26 ± 4</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>11β-Cortisol</td>
<td>88 ± 14%</td>
<td>557 ± 120</td>
<td>70 ± 2%</td>
<td>64 ± 5</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
</tbody>
</table>
Figure 4  Phylogenetic tree showing different KSTD enzyme clusters

Phylogenetic tree with different amino acid sequences of (putative) 3-ketosteroid dehydrogenases of well-known cholesterol-degrading actinomycetes and the Gram-negative bacterium C. testosteroni. A clear separation between $\Delta^1$ KSTD and $\Delta^4$ KSTD enzymes is apparent. The $\Delta^1$ KSTD enzymes cluster in at least four different groups: $\Delta^1$ type 1 orthologues of KSTD1 of R. erythropolis SQ1, $\Delta^1$ type 2 orthologues of KSTD2 of R. erythropolis SQ1, $\Delta^1$ type 3 orthologues of KSTD3 of R. erythropolis SQ1, and a yet unknown type of $\Delta^1$ KSTD (tentatively designated $\Delta^1$ type 4) so far without a homologue of R. erythropolis SQ1. The $\Delta^4$ KSTD enzymes cluster in at least three different groups, of which only one group contains a $\Delta^4$ KSTD of R. erythropolis SQ1. Abbreviations: KSTD, R. erythropolis SQ1; Tes, C. testosteroni; MAP, M. avium subsp. paratuberculosis str. k10; MSMEG, M. smegmatis; Rv, M. tuberculosis H37Rv; nfa, N. farcinica IFM10152; ro, Rhodococcus sp. RHA1; orf, R. equi; KsdDAs, A. simplex; KsdDRr, R. rhodochrous.

unknown $\Delta^1$ KSTD activity band in Figure 1 is therefore not the KSTD3 protein characterized in the present paper.

The genomic co-location of kstD3 with other cholesterol catabolic genes in R. erythropolis SQ1 (Table 1; Figure 2), as well as strong up-regulation of its homologue in cholesterol-grown cells of Rhodococcus sp. RHA1 [19], indicates a role for KSTD3 in cholesterol degradation. Unexpectedly, the preferred substrate of KSTD3 is not 4-AD, the commonly accepted central intermediate in sterol/steroid degradation, but the 5α-reduced form of 4-AD. The results thus suggest that 5α-T and 5α-AD are pathway intermediates preceding ADD during cholesterol catabolism. The conversion of 5α-AD into ADD requires the action of a $\Delta^1$ KSTD, encoded by kstD3, and a $\Delta^4$ KSTD, putatively encoded by kst4D. Both activities appear to be encoded by the cloned DNA fragment (Table 1; Figure 2).

Phylogeny of (putative) 3-ketosteroid dehydrogenases of several actinomycetes

The genome sequences of several actinomycetes were analysed for KSTD homologues by searching for high hits with the amino acid sequences of KSTD1, KSTD2, KSTD3 and $\Delta^4$ KSTD. Construction of a phylogenetic tree, based on the complete amino acid sequences of the different (putative) KSTDs, revealed that the $\Delta^1$ KSTDs cluster into at least four distinct groups (Figure 4). Three of the four groups also have a $\Delta^1$ KSTD representative of R. erythropolis. M. smegmatis has six putative $\Delta^1$ KSTDs [32], one of which does not belong to any of the groups in this phylogenetic tree (Figure 4). This suggests that the natural diversity of $\Delta^1$ KSTDs is even more elaborate than four groups. Among the $\Delta^1$ KSTDs, the identity of the KSTD3 amino acid sequences is slightly more conserved (64–73%) as compared with KSTD1 (40–70%) and KSTD2 (58–70%). The KSTD enzymes display significant similarity to Frds (fumarate reductases; 20–24%). Alignment of amino acid sequences of Frd of Shewanella putrefaciens with those of $\Delta^1$ KSTDs revealed four conserved regions (I–IV; Figure 5) in addition to the conserved FAD-binding domain in the N-terminal part of the protein [6,17]. Region I has been suggested to comprise the active-site residues of KsdD of A. simplex, based on an alignment with the active site of Frd [6]. However, our alignment indicates that the active-site residues His$^{165}$, Glu$^{178}$ and Arg$^{381}$ of Frd do not align with region I, as was proposed for KsdD of A. simplex, but are scattered around conserved region II. Active-site residue Arg$^{402}$ of Frd, responsible for donation of a proton to the substrate [33–36], is also found near region II and, interestingly, is occupied by a tyrosine residue in most KSTDs (Figure 5). Tyrosine residues have been implicated as important for KSTD activity [37]. Residues Tyr$^{164}$ and Tyr$^{116}$
biochemical characterization of 3-keto-5α-steroid Δ1-dehydrogenase

Figure 5 Alignment of amino acid sequences of Δ1 KSTD enzymes and Frd reveals four conserved regions

Alignment of the four conserved regions (I–IV) present in all Δ1 KSTD enzymes and Frd of S. putrefaciens MR-1 (Frd, accession number 1D4D_A). KSTD1, KSTD2 and KSTD3 indicate the three different Δ1 KSTD enzymes of R. erythropolis. KsdD As represents KsdD of A. simplex, and KsdD Rr represents the KsdD of R. rhodochrous. See the Experimental section for accession numbers.

in KsdD of R. rhodochrous, thought to be involved in steroid substrate binding, however, are not conserved in the different types of Δ1 KSTDs. Residue Tyr121 in KsdD of R. rhodochrous, on the other hand, is conserved among all Δ1 KSTDs and was shown to be essential for catalysis [37]. Conserved region IV includes two key substrate-binding residues identified in Frd, i.e. His504 and Arg544 [33–36]. Residue His508 in region IV may be involved in modulation of the pK_a of His504, while residues Thr506, Met507, Gly508, Glu534 and Thr536 in Frd are important for co-ordinating a sodium ion positioned close to both the FAD cofactor and the active site of Frd [38]. The threonine residue, corresponding to Tyr506 of Frd in region IV, is fully conserved among KSTD enzymes and was suggested to be important for substrate binding or catalysis in KSTD2 of R. erythropolis SQ1 [5]. Interestingly, the cysteine residue located next to this conserved threonine residue seems to be characteristic for KSTD2 enzymes.

Rv3537 of M. tuberculosis H37Rv is a KSTD3 orthologue

Remarkably, among the Δ1 KSTDs, only the KSTD3 clusters with a putative KSTD of M. tuberculosis (Figure 4). The putative 3-ketosteroid dehydrogenase Rv3537 of M. tuberculosis H37Rv is essential for survival in the macrophage [20]. To analyse whether the biochemical characteristics of different KSTDs in one distinct cluster are similar, the substrate profiles of Rv3537 from M. tuberculosis H37Rv was expressed in E. coli B121(DE3), by using the same conditions as for KSTD3 expression. Δ1 KSTD enzyme assays and HPLC product analysis revealed a highly similar substrate range and apparent substrate affinities for Rv3537 compared with KSTD3 (Table 2). The substrate range for Rv3537 was even more limited than for KSTD3, since its Δ1 KSTD activity towards 5α-P was below detection limits.

Similarity searches and phylogenetic analysis show that Rv3537 of M. tuberculosis H37Rv is the only Δ1 KSTD enzyme present in H37Rv [18]. Rv3537 plays an important role in macrophage survival of M. tuberculosis [20] and in the cholesterol catabolic pathway [19], suggesting that 5α-AD and/or 5α-T are important steroid intermediates with respect to pathogenicity. KSTD exclusively occurs as a bacterial enzyme, i.e. there are no known human orthologues, marking KSTD as an interesting new drug target to fight tuberculosis.
REFERENCES


