Identification and targeted disruption of the gene encoding the main 3-ketosteroid dehydrogenase in *Mycobacterium smegmatis*

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The catabolic potential for sterol degradation of fast-growing mycobacteria is well known. However, no genes or enzymes responsible for the steroid degradation process have been identified as yet in these species. One of the key enzymes required for degradation of the steroid ring structure is 3-ketosteroid Δ¹-dehydrogenase (KsdD). The recent annotation of the *Mycobacterium smegmatis* genome (TIGR database) revealed six KsdD homologues. Targeted disruption of the MSMEG5898 (*ksdD-1*) gene, but not the MSMEG4855 (*ksdD-2*) gene, resulted in partial inactivation of the cholesterol degradation pathway and accumulation of the intermediate 4-androstene-3,17-dione. This effect was reversible by the introduction of the wild-type *ksdD-1* gene into *M. smegmatis* Δ*ksdD-1* or overexpression of *ksdD-2*. The data indicate that KsdD1 is the main KsdD in *M. smegmatis*, but that KsdD2 is able to perform the cholesterol degradation process when overproduced.

**INTRODUCTION**

It has been known since the 1960s that fast-growing mycobacteria degrade natural sterols and use them as a source of carbon and energy. A number of *Mycobacterium* strains treated with mutagens have been reported to accumulate steroid biodegradation intermediates such as 4-androstene-3,17-dione (AD) and 1,4-androstadiene-3,17-dione (ADD) (Mahato & Garai, 1997; Martin, 1977; Szentirmai, 1990). These intermediates may be used as precursors for the production of steroid drugs and hormones (Sedlaczek, 1988). Two enzymes, 3-ketosteroid Δ¹-dehydrogenase (KsdD) and 3-ketosteroid 9α-hydroxylase (KsdH) are essential to initiate steroid ring degradation (Fig. 1). KsdD catalyses transhydrogenation of 3-keto-4-ene-steroid to 3-keto-1,4-diene-steroid, e.g. progesterone to ADD. *ksdD* genes have been isolated and overproduced from a number of micro-organisms (Molnar et al., 1995; Choi et al., 1995; Itagaki et al., 1998; Kaufmann et al., 1992; Morii et al., 1998), but the most abundant data about enzymes involved in steroid skeleton degradation have come from the study of *Comamonas testosteroni* (Plesiat et al., 1991; Florin et al., 1996; Horinouchi et al., 2001, 2003a, b). The inactivation of KsdD may lead to the accumulation of 9α-hydroxy-4-androstene-3,17-dione (9OHAD) from steroid compounds.

Studies on *Rhodococcus erythropolis* KsdD show two distinct enzymes with KsdD activity (Geize et al., 2000, 2001). The disruption of both genes was required to block effectively the AD degradation process (Geize et al., 2002). Reported mycobacterial activities in steroid biodegradation (Wilmanska et al., 1995; Wovcha et al., 1979) are not supported by the identification and molecular characterization of genes involved in this process. Here we report on the identification of the main *ksdD* gene in *Mycobacterium smegmatis* and its characterization by unmarked gene deletion.

**METHODS**

**Bacterial strains and culture conditions.** The following bacterial strains were used: *Escherichia coli* Top10 (Invitrogen), *Mycobacterium smegmatis* mc²155 (Snapper et al., 1990) and *Mycobacterium tuberculosis* H37Ra. The mycobacterial strains were cultured in Middlebrook 7H9 broth or 7H10 agar plates supplemented with albumin-glucose and 25 μg kanamycin (Kan) ml⁻1 (when required). For steroid bioconversion experiments, mycobacterial strains were cultured in NB broth [8 g nutrient broth (Difco) l⁻¹, 10-0 g glucose l⁻¹, supplemented with 0-2% Tween 80 (pH 6-0-6-2)] or in mineral medium [g l⁻¹: MgSO₄. 7H₂O, 0-5; Na₂HPO₄, 1-0;
Plasmid constructions. The plasmid DNA of mycobacterial transformants was recovered and analysed as described by Madiraju et al. (2000). Standard molecular biology protocols were used for this purpose (Sambrook et al., 1989). All PCR products were obtained using thermostable ExTaq polymerase (Takara) and were cloned initially into a TA vector (pGemTeasy; Promega), then released by digestion with appropriate restriction enzymes before cloning into expression vectors. To facilitate subcloning into expression vectors, restriction enzyme recognition sites (underlined, see below) were incorporated into the sequence of the primers. The plasmids used in this work are listed in Table 1.

Gene replacement constructs. To perform unmarked deletions in ksdD-1 and ksdD-2 genes of M. smegmatis, suicidal recombination delivery vectors were constructed. In the first step the 5’ ends of the ksdD genes (40 bp ksdD-1; 19 bp ksdD-2) and upstream regions were amplified using primers MsD1GR1 (5’-AACTGACAGGAGTTCCGAGATCGATCGCG-3’) and MsD1GR2 (5’-CCAAAGCTTCCGAGACCCCAAAG-3’) for ksdD-1, and MsD2GR1 (5’-CCCAAGCTTCTGGGATGATCTCACTCCACCGCG-3’) and MsD2GR2 (5’-CCGGATCCCCAAGGAGAGCTCGCGGCACCGCG-3’) for ksdD-2, and cloned into the PstI/HindIII and HindIII/BamHI sites of p2NIL to create pTS6 and pAB44, respectively. Subsequently, the 3’ ends of the ksdD genes (418 bp ksdD-1; 1008 bp ksdD-2) and downstream regions were amplified using primers MsD1GR3 (5’-AACTGACAGGAGTTCCGAGATCGATCGCG-3’) and MsD1GR4 (5’-CCAAAGCTTCCGAGACCCCAAAG-3’) for ksdD-1, and MsD2GR3 (5’-CCGGATCCCCAAGGAGAGCTCGCGGCACCGCG-3’) and MsD2GR4 (5’-CCCGCGCCACCGAGATCGGCACCGCGG-3’) for ksdD-2, and cloned into the HindIII/BamHI sites of pTS8 and BamHI/KpnI sites of pAB45. The ligated 5’ and 3’ fragments of the ksdD-1 and ksdD-2 genes in the resulting vectors were out of frame. Finally, a 6 kb PacI marker cassette from pGOAL17 carrying lacZ and sacB genes was cloned into the PacI site of pTS8 and pAB45 to create pTS10 and pAB46, respectively.

Complementation and overexpression constructs. Two ORFs (MSMEG5898, ksdD-1; MSMEG4855, ksdD-2) were PCR-amplified using primers MsD1s (5’-GGAAGATCTATGACTGGACAGGAGTCC-3’) and MsD1r (5’-GCCTCACTGTAACGTTAGCTCCGAAAGG-3’) for ksdD-1, and MsD2s (5’-GGAAGATCTATGACTGGACAGGAGTCCGAAAGG-3’) and MsD2r (5’-GGAAGATCTATGTAACGTTAGCTCCGAAAGG-3’) for ksdD-2, and cloned into the PstI/HindIII and HindIII/BamHI sites of p2NIL to create pTS6 and pAB44, respectively. Subsequently, the 3’ ends of the ksdD genes (418 bp ksdD-1; 1008 bp ksdD-2) and downstream regions were amplified using primers MsD1GR3 (5’-AACTGACAGGAGTTCCGAGATCGATCGCG-3’) and MsD1GR4 (5’-CCAAAGCTTCCGAGACCCCAAAG-3’) for ksdD-1, and MsD2GR3 (5’-CCGGATCCCCAAGGAGAGCTCGCGGCACCGCGG-3’) and MsD2GR4 (5’-CCCGCGCCACCGAGATCGGCACCGCGG-3’) for ksdD-2, and cloned into the HindIII/BamHI sites of pTS8 and BamHI/KpnI sites of pAB45. The ligated 5’ and 3’ fragments of the ksdD-1 and ksdD-2 genes in the resulting vectors were out of frame. Finally, a 6 kb PacI marker cassette from pGOAL17 carrying lacZ and sacB genes was cloned into the PacI site of pTS8 and pAB45 to create pTS10 and pAB46, respectively.

![Proposed pathway for cholesterol degradation](image-url)

Fig. 1. Proposed pathway for cholesterol degradation (Martin, 1977; Sedlaczek, 1988). KSTH, Steroid 9α-hydroxylase; KSDD, 3-ketosteroid dehydrogenase; 9OHAD, 9α-hydroxy-1,4-androstadiene-3,17-dione.

KH$_2$PO$_4$, 0.5; NH$_4$NO$_3$, 2.5; CaCl$_2$.2H$_2$O, 0.001; Fe$_2$(SO$_4$)$_3$.6H$_2$O, 0.01; MnSO$_4$.nH$_2$O, 0.0001; Co(NO$_3$)$_2$.6H$_2$O, 0.00005; (NH$_4$)$_6$Mo$_7$O$_{24}$.4H$_2$O, 0.001. [A. Brzostek and others]
Disruption of ksdD-1 and ksdD-2 genes. The protocol of Parish & Stoker (2000) was used to disrupt ksdD-1, ksdD-2 or both genes at their native loci on the chromosome. Plasmid DNA (pTS10, pAB46) was treated with NaOH (0.2 mM) and integrated into the M. smegmatis mc\(^{155}\) chromosome by homologous recombination. The resulting single-crossover homologous recombinant (SCO) mutant colonies were blue, Kan\(^{\text{R}}\) and sensitive to sucrose. The resulting single-crossover homologous recombinant (SCO) mutant colonies were blue, Kan\(^{\text{R}}\) and sensitive to sucrose (2\%)

**Table 1.** Plasmids used in this study

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description*</th>
<th>Source</th>
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</thead>
<tbody>
<tr>
<td><strong>Cloning vectors</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pGemTEasy</td>
<td>T/A cloning</td>
<td>Promega</td>
</tr>
<tr>
<td>pMV261</td>
<td>Shuttle vector carrying P(_{\text{hsp}}) promoter, Kan(^{\text{R}})</td>
<td></td>
</tr>
<tr>
<td>p2NIL</td>
<td>Recombination vector, non-replicating in mycobacteria, Kan(^{\text{R}})</td>
<td>Parish &amp; Stoker (2000)</td>
</tr>
<tr>
<td>pLam2</td>
<td>Shuttle vector carrying inducible P(_{\text{ami}}) promoter, Kan(^{\text{R}})</td>
<td>Triccas et al. (1998)</td>
</tr>
</tbody>
</table>

| **Vectors used for gene replacement** | | |
| pTS6 | 1060 bp PstI–HindIII fragment, including 5’ end of MSMEG5898 (40 bp) and its upstream region in p2NIL, Kan\(^{\text{R}}\) | This study |
| pTS8 | 1290 bp HindIII–BamHI fragment, including 3’ end of MSMEG5898 (418 bp) and its downstream region in pTS6, Kan\(^{\text{R}}\) | This study |
| pTS10 | pTS8 with PacI cassette from pGoal17, Kan\(^{\text{R}}\) | This study |
| pAB44 | 1550 bp HindIII–BamHI fragment, including 5’ end of MSMEG4855 (19 bp) and its upstream region in p2NIL, Kan\(^{\text{R}}\) | This study |
| pAB45 | 1999 bp BamHI–KpnI fragment, including 3’ end of MSMEG4855 (1008 bp) and its downstream region in pAB44, Kan\(^{\text{R}}\) | This study |
| pAB46 | pAB45 with PacI cassette from pGoal17, Kan\(^{\text{R}}\) | This study |

| **Overproduction vectors** | | |
| pTS1 | ksdD-1 (MSMEG5898) under P\(_{\text{ami}}\) promoter in pLam2, Kan\(^{\text{R}}\) | This study |
| pAB42 | ksdD-2 (MSMEG4855) under P\(_{\text{ami}}\) promoter in pLam2, Kan\(^{\text{R}}\) | This study |
| pAB43 | ksdD-1\(_{\text{ss}}\) (MT3641) under P\(_{\text{hsp}}\) promoter in pMV261, Kan\(^{\text{R}}\) | This study |
| pAB49 | ksdD-2\(_{\text{ss}}\) (MT0809) under P\(_{\text{hsp}}\) promoter in pMV261, Kan\(^{\text{R}}\) | This study |

* Amp, Ampicillin; Kan, kanamycin.

(5’-GAGAGTCATGACTGTCAGGACGGAGTTCG-3’) and TbD1r (5’-GAAATCTCCGCGTTCATGCCGATGAGC-3’) for ksdD-1\(_{\text{ss}}\) (MT3641) or TbD2s (5’-CCGGATCCGTCGTCGTTAAACCTGTAACCA-3’) and TbD2r (5’-CCACCGGCTGCAGATCCACTGGTCCCGGGCACGTGCTGTTCCG-3’) for ksdD-2\(_{\text{ss}}\) (MT0809). The PCR products were cloned into the BamHI/EcoRI (ksdD-1\(_{\text{ss}}\) or BamHI/HindIII (ksdD-2\(_{\text{ss}}\)) sites of pMV261 under control of the P\(_{\text{hsp}}\) promoter. The resulting constructs carrying ksdD-1\(_{\text{ss}}\) or ksdD-2\(_{\text{ss}}\) were named pAB49 and pAB43, respectively.

Steroid standards. \(\beta\)-Sitosterol (Triple Crown) and androst-1-ene-3,11,17-trione (Sigma) were used as internal standards for quantitative determination by GC (for cholesterol and AD, respectively). Additional standards were 9OHAD, AD and ADD (Koch-Light).

Growth of wild-type and mutant M. smegmatis strains on cholesterol or AD. NB medium (100 ml in 1 l flasks) was inoculated with M. smegmatis and incubated overnight at 37°C with shaking at 130 r.p.m. The culture 20 ml was transferred to 180 ml fresh NB medium in 1 l flasks. At the time of inoculation, cholesterol or AD (0.1–0.3 g 1\(^{-1}\)) were added to the medium and the cultures were incubated on a shaker (130 r.p.m.) at 37°C. To determine the dry cell mass at the start of the experiments and at 24 h time intervals, samples (2 x 5 ml) were withdrawn from the culture, filtered through Sympor filters (pore diameter 0.2 μm) of known weight and the sediment was dried to constant weight.

To determine the progress in cholesterol or AD biotransformation, 2 ml culture samples were taken, mixed with \(\beta\)-sitosterol and androst-1-ene-3,11,17-trione as internal standards (each at 100 μg in 50 μl chloroform), and extracted three times with an equal volume of chloroform. The extracts were dried under vacuum, the residue was dissolved in 0.5 ml acetone and steroids were analysed by chromatography as described previously (Rumijowska et al., 1997).

To prepare cholesterol or AD, the substrate was dissolved in 5 ml 96% warm ethanol and an equal volume of sterile distilled water was added. Then the mixture was micronized for 20 min using a water bath-ultrasonic cleaner.

An overnight culture of M. smegmatis (100 ml in 1 l flasks) grown in NB medium was harvested by centrifugation for 20 min at 4000 r.p.m. The cells were resuspended in 10 ml mineral medium and a 2 ml sample was transferred to 198 ml fresh mineral medium in 1 l flasks. Cholesterol or AD was added as sole carbon source. The concentration of the substrate was determined in each experiment.

The enzymic activity of each strain was measured by using GC in at least three independent experiments.
Table 2. Similarity analysis of putative KsdD of *M. smegmatis* and *M. tuberculosis*

<table>
<thead>
<tr>
<th>Mycobacterial KsdD</th>
<th>Highest homology to non-mycobacterial KsdDs (excluding hypothetical enzymes) (%)</th>
<th>Most similar mycobacterial counterpart (%)</th>
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<tbody>
<tr>
<td>MSMEG2871</td>
<td>A. simplex (64)</td>
<td></td>
</tr>
<tr>
<td>MSMEG2873</td>
<td>R. erythropolis (60)</td>
<td></td>
</tr>
<tr>
<td>MSMEG4850</td>
<td>R. erythropolis (40)</td>
<td></td>
</tr>
<tr>
<td>MSMEG4855-KsdD2</td>
<td>R. erythropolis (29)</td>
<td></td>
</tr>
<tr>
<td>MSMEG5801</td>
<td>None</td>
<td></td>
</tr>
<tr>
<td>MSMEG5898-KsdD1</td>
<td>R. erythropolis (40)</td>
<td></td>
</tr>
<tr>
<td>MT0809</td>
<td>A. simplex (25)</td>
<td></td>
</tr>
<tr>
<td>MT3641</td>
<td>R. erythropolis (41)</td>
<td></td>
</tr>
</tbody>
</table>

RESULTS

The putative ksdD genes in *M. smegmatis*

The *M. tuberculosis* genome sequencing project (Cole et al., 1998) revealed a putative ksdD gene (Rv3537/MT3641) exhibiting about 34% identity with *Arthrobacter simplex* and *Rhodococcus rhodochrous* ksdD products at the amino acid level and an additional ORF (MT0809) with limited homology to a known KsdD (25% identity with KsdD of *A. simplex*). Recently the genome sequence of *M. smegmatis* was annotated. There are as many as six ORFs (MSMEG2871, 2873, 4850, 4855, 5801 and 5898) identified as putative ksdD genes, including those analysed in this paper. The most significant similarity values for each *M. smegmatis* and *M. tuberculosis* putative ksdD gene are shown in Table 2. KsdD is a flavoprotein carrying a conservative N-terminal FAD-binding domain with the consensus sequence GSG(A/G)(A/G)(A/G)GSG(A/G)(A/G)X17E (Geize et al., 2000). Such a motif can be identified in MSMEG5898, MSMEG4850 and MT3641, but not in the other five ORFs analysed (Fig. 2). Two putative ksdD genes of *M. smegmatis*, ksdD-1 (MSMEG5898) and ksdD-2 (MSMEG4855), displaying respectively high (78%) and low (33%) amino acid sequence identity with the putative ksdD gene of *M. tuberculosis* (MT3641), were selected for this study. The alignment of putative KsdDs revealed that ksdD-1 (MSMEG5898) is the most probable counterpart of the putative *M. tuberculosis* ksdD gene (MT3641) and that ksdD-2 (MSMEG4855) shows limited homology to both putative *M. tuberculosis* ksdD genes MT3641 and MT0809 (33 and 20%, respectively). Both *M. smegmatis* KsdDs contain the putative N-terminal FAD-binding motif with one exception, S to T in KsdD2, but we do not know the significance of this variation.

Targeted disruption of ksdD-1 results in accumulation of cholesterol degradation intermediates

Our preliminary study revealed that *M. smegmatis* has the ability to use cholesterol as a source of carbon and energy. Culturing of *M. smegmatis* in the presence of cholesterol results in fast (about 48 h) complete degradation of this substrate without accumulation of intermediate steroid compounds. The two-step recombination protocol of Parish & Stoker (2000) was used to obtain the unmarked deletion of the *ksdD-1* gene from the *M. smegmatis* chromosome as described in Methods. The resultant mutant was verified by PCR and Southern hybridization (Fig. 3). TLC analysis revealed the accumulation of AD when a mutant *M. smegmatis* strain, ΔksdD-1 (DCO1), but not a wild-type *M. smegmatis* strain, was cultured in the presence of cholesterol (data not shown). To quantify the results obtained, GC analysis, with androsterone and β-sitosterol as the internal standards, was performed. Growth of ΔksdD-1 mutant and wild-type *M. smegmatis* strains in the presence of cholesterol results in fast (48 h) degradation of the steroid substrate. However, the intermediates of cholesterol degradation were detected only in the mutant culture (Fig. 4). The expected accumulation of AD indicates the inhibition of the cholesterol degradation pathway at the KsdD step. Maximum AD accumulation was observed in a 48-h-old culture of the DCO1 mutant and its presence for the next 192 h indicated that ksdD-1 is essential for degradation of androstendione in *M. smegmatis* cultured...
under the conditions used. To confirm that the observed effect was due to unmarked deletion of the ksdD-1 gene, the ksdD gene of M. smegmatis was amplified and cloned in the pJam2 expression vector under the acetamidase promoter and introduced into the DCO1 mutant. The resultant complemented strain was cultured in the presence of cholesterol and acetamide (0–2 %) to induce the expression of the plasmid copy of ksdD-1 and the cholesterol degradation process was monitored by GC. Fast and complete degradation of cholesterol, without accumulation of detectable amounts of steroid degradation intermediates, was observed (data not shown). In the control culture (M. smegmatis ΔksdD-1) we found that acetamide per se has no effect on cholesterol degradation and accumulation of AD.

![Fig. 3. Replacement of the wild-type ksdD-1 gene with mutant sequence confirmed by PCR and Southern hybridization. (a) The chromosomal localization of the ksdD-1 gene is represented by a grey arrow. The PvuII and BglII recognition sites are denoted by single letters P and B, respectively. The PvuII–BglII distance, the ksdD-1 gene size and the size of the internal deletion in the ksdD-1 mutated copy are shown. The brick box represents the deleted part in the mutated copy of ksdD-1. (b) PCR amplification of the ksdD-1 gene with MsD1s and MsD1r primers based on plasmid DNA pTS10 (TS10) and chromosomal DNA isolated from wild-type and mutant strains (M. smegmatis mc2; single-crossover recombination mutant, SCO; double-crossover recombination mutant, mutDCO). M, 1 kb ladder (Promega). The ksdD (1701 bp) or ΔksdD (421 bp) PCR products are marked by black arrows. (c) Southern analysis of genomic DNA isolated from wild-type M. smegmatis (mc2) and recombinants (SCO and two mutDCOs). DNA was digested with BglII and PvuII, separated in 1 % agarose gel, transferred onto nylon membrane and hybridized with the PCR-amplified ΔksdD-1 probe (MsD1s, MsD1r primers and pTS10 as a template). The expected hybridization bands (2239 bp and 959 bp) are indicated by arrows.

![Fig. 4. Cholesterol degradation and AD/9OHAD accumulation by M. smegmatis mc2 (mc2), M. smegmatis ΔksdD-1 (DCO1) and M. smegmatis ΔksdD-1 carrying plasmid pTS1 with ksdD-1 under the control of Pami (DCO1-ksdD-1) cultured in rich medium (NB broth) and monitored by GC. 9OHAD comprised less than 20 % of the accumulated products at each time point. The cholesterol degradation rate is indicated by open squares (mc2), open circles (DCO1) and open triangles (DCO1-ksdD-1); the AD/9αAD accumulation rate is marked by filled squares (mc2), filled circles (DCO1) and filled triangles (DCO1-ksdD-1).]
KsdD2 exhibits low but detectable activity in the cholesterol degradation process

*M. smegmatis* KsdD2 exhibits limited homology to other KsdDs and its FAD-binding motif is not fully conserved. We asked the question whether KsdD2 can participate in the cholesterol degradation process in the wild-type *M. smegmatis* strain or in the DCO1 mutant. The ksdD-2 gene of *M. smegmatis* was PCR-amplified and cloned into the pJAm2 vector under control of the acetamidase promoter. The resulting construct (pJAm2-ksdD-2) was electroporated into the DCO1 mutant to obtain strain DCO1-ksdD-2. Culturing of this strain in the presence of cholesterol results in the degradation of substrate and accumulation of steroid degradation intermediate compounds which were monitored by GC (Fig. 5). Until the 96 h time point the accumulation of AD was observed, then the amount of AD began to decrease with a simultaneous increase in ADD. This result indicates that KsdD2 exhibits at least a low activity of KsdD after its accumulation in *M. smegmatis* cells. To test the significance of KsdD2 in *M. smegmatis* cholesterol degradation, a ksdD-2 gene knockout in wild-type *M. smegmatis* and the DCO1 mutant was performed. For unmarked ksdD-2 gene deletion mutagenesis, plasmid pAB46 was constructed and introduced into wild-type *M. smegmatis* and the DCO1 mutant. The selected ksdD-2 knock-out strains were analysed by PCR and Southern hybridization (Fig. 6). The obtained mutants, *M. smegmatis ΔksdD-2* (DCO2) and *M. smegmatis ΔksdD-1 ΔksdD-2* (dDCO1,2), were cultured in the presence of cholesterol. The degradation of cholesterol and accumulation of the steroid intermediates were monitored by GC. Both mutants were able to use cholesterol for 48 h as described for the wild-type strain (Fig. 5). The DCO2 mutant was shown to accumulate a small amount of AD [\(<1\ \text{mg}\ \text{l}^{-1}\ \text{g dry mass}^{-1}\)] and ADD in the first 24-48 h of the biotransformation process. After 48-72 h, steroids were not detectable, probably due to KsdD1 activity. The double mutant dDCO1,2 transformed cholesterol to AD, which was stably maintained until 192 h of culture (Fig. 5). Moreover, ADD was not detectable at any time point, suggesting that KsdD activity was completely inhibited in the dDCO1,2 mutant growing under the given conditions. Furthermore, the pJAm2-ksdD-2 construct was introduced into the dDCO1,2 mutant to confirm the activity of KsdD2. The dDCO1,2 mutant carrying the plasmid copy of ksdD-2 gene under a strong, inducible promoter restored KsdD activity manifested by the conversion of AD to ADD which was detectable after 96 h of culture as observed previously for the DCO1-ksdD-2 strain (Fig. 5).

**M. tuberculosis** genome also carries two *ksdD* genes

The genome of *M. tuberculosis* (TIGR) contains two putative KsdDs, MT3641 and MT0809. We tested if these genes are able to complement the *M. smegmatis ΔksdD-1 ΔksdD-2* double mutant. Both genes were PCR-amplified and cloned under control of the P\text{hsp} promoter. The resulting constructs were introduced into the mutant cells. GC analysis revealed that overproduction of ksdD-1 of *M. smegmatis* complements the ΔksdD-1 ΔksdD-2 double mutation (Fig. 7). Initially, cholesterol was biotransformed to AD (48 h) which was further degraded with temporary accumulation of ADD. The pAB43 construct (ksdD-2\text{p}) did not change the ability of the host strain to biotransform cholesterol in comparison to *M. smegmatis ΔksdD-1 ΔksdD-2*.

Other KsdD-like enzymes of *M. smegmatis* are activated when cholesterol or androstendione is the only source of carbon and energy

The above experiments performed in a rich medium would suggest that disruption of ksdD-1 and ksdD-2 in *M. smegmatis* is sufficient to inhibit completely further degradation of androstendione. In this case the dDCO1,2 mutant would not be able to grow in mineral medium with cholesterol (or AD) as the only source of carbon and energy. However, we found that the wild-type strain as well as the dDCO1,2 mutant can grow on mineral agar plates or in mineral liquid medium supplemented with cholesterol or AD, but not without these sources of carbon. Nevertheless, dDCO1,2 mutant growth was significantly delayed compared to the wild-type strain (data not shown).

![Fig. 5. GC analysis of accumulation of AD/9OHAD and ADD produced in cultures of *M. smegmatis* mutants (*M. smegmatis ΔksdD-1* harbouring plasmid pAB42 with ksdD-2 gene under the control of P\text{ani}, DCO1-ksdD-2; *M. smegmatis ΔksdD-2*, DCO2; *M. smegmatis ΔksdD-1 ΔksdD-2*, dDCO1,2; *M. smegmatis ΔksdD-1 ΔksdD-2* with pAB42 plasmid, dDCO1,2-ksdD-2); cultured in rich medium (NB broth) in the presence of cholesterol. The accumulation of AD/9OHAD is marked by filled squares (DCO1-ksdD-2), filled triangles (DCO2), filled circles (dDCO1,2-ksdD-2) and asterisks (dDCO1,2). The accumulation of ADD (if any) is marked by open squares (DCO1-ksdD-2), open triangles (DCO2) and open circles (dDCO1,2-ksdD-2). ADD was not detected in dDCO1,2 mutant culture. The AD/9OHAD ratio was about 1:1 in DCO2 and DCO2-ksdD-2 mutants. In the other cases there was as little as 0–20% 9OHAD in the AD/9OHAD mixture.](image-url)
Fig. 6. Gene replacement of the wild-type ksdD-2 with mutated sequence in M. smegmatis wild-type strain and M. smegmatis DCO1 mutant confirmed by PCR and Southern hybridization. (a) The chromosomal localization of ksdD-2 gene is represented by the grey arrow. The KpnI recognition site is denoted by single letter K. KpnI – KpnI distance, ksdD-2 gene size and size of internal deletion in ksdD-2 mutated copy are shown. The brick rectangle represents the deleted part in the mutated copy of ksdD-2. (b) PCR amplification of ksdD-2 gene with MsD2s and MsD2r primers based on plasmid DNA pAB46 (AB46) and chromosomal DNA isolated from wild-type and mutant strains (M. smegmatis mc² 155, mc²; single cross over recombination mutant, SCO; double cross over recombination mutant, mutDCO; double cross over recombination wild-type, wtDCO). M, 1kb ladder (Promega). The ksdD-2 (1641 bp) or ΔksdD-2 (1030 bp) PCR products are marked by arrows. (c) Southern hybridization of genomic DNA from wild-type M. smegmatis (mc²) and recombinants (SCO, mutDCO, wtDCO of M. smegmatis ΔksdD-2 and M. smegmatis ΔksdD-1, ΔksdD-2). The DNA was digested with KpnI restriction enzyme, separated in 1% agarose gel, transferred onto nylon membrane and hybridized with PCR amplified ΔksdD-2-probe (MsD2s, MsD2r primers and pAB46 DNA as a template). The expected hybridization bands (3607 bp and 2996 bp) are indicated by arrows.

Fig. 7. Cholesterol degradation and AD/9OHAD accumulation by M. smegmatis ΔksdD-1 ΔksdD-2 (dDCO1,2), M. smegmatis ΔksdD-1 ΔksdD-2 with pAB43 plasmid carrying ksdD-1tb gene under the control of P_hsp (dDCO1,2 ksdD-1tb); M. smegmatis ΔksdD-1 ΔksdD-2 with pAB49 plasmid carrying ksdD-2tb gene under the control of P_hsp (dDCO1,2 ksdD-2tb); cultured in rich medium (NB broth) and monitored by GC. The cholesterol degradation rate is marked by open squares (dDCO1,2), open triangles (dDCO1,2 ksdD-1tb) and open circles (dDCO1,2 ksdD-2tb). The accumulation of AD/9OHAD is marked by filled squares (dDCO1,2), filled triangles (dDCO1,2 ksdD-1tb) and filled circles (dDCO1,2 ksdD-2tb).
The GC analysis revealed that the dDCO1,2 strain cultured in mineral medium supplemented with cholesterol accumulated 9OHAD or AD as observed in the rich medium culture. However, the dDCO1,2 mutant growing in mineral medium with AD caused a decrease in the amount of substrate and accumulated ADD starting at the 48 h point. The use of AD by the mutant strain was significantly slower in comparison to the wild-type strain (Fig. 8). It is very likely that the mineral medium with AD or cholesterol as a sole source of carbon and energy induced the expression of ksdD-like genes (see Table 2) which are not expressed in rich medium. This expression would allow dDCO1,2 to convert AD to ADD which can be further degraded.

DISCUSSION

We identified MSMEG5898 (KsdD-1) as the main KsdD in M. smegmatis. The disruption of ksdD-1 results in the accumulation of AD or 9OHAD in the cholesterol degradation process. However, the M. smegmatis genome contains five other putative ksdD genes. Slight perturbations in cholesterol degradation were also observed due to unmarked deletion of MSMEG4855 (ksdD-2). Moreover, the overexpression of this gene in the M. smegmatis ΔksdD-1 mutant complemented the mutant ksdD-1 gene and restored KsdD activity. The double mutant, lacking both ksdD-1 and ksdD-2 genes, accumulated AD in the cholesterol degradation process performed in rich medium, but was able to grow in mineral medium with cholesterol (or AD) as the sole source of carbon and energy. This clearly showed the presence of at least one additional KsdD enzyme responsible for AD dehydrogenation. Two KsdDs were previously described in R. erythropolis and each enzyme alone was able to support the degradation of 9OHAD (Geize et al., 2002). However, the double disruption of both ksdD and ksdD2 genes resulted in a metabolic block at the level of 9OHAD. It is interesting that disruption of ksdD or ksdD2 genes in R. erythropolis resulted in the accumulation of 9OHAD (Geize et al., 2002). In the case of M. smegmatis the 9OHAD was observed exclusively when cholesterol but not AD was used as a substrate (Wovcha et al., 1979). This would suggest differences in the steroid degradation pathways in R. erythropolis and M. smegmatis.

Two distinct KsdD activities have also been reported in Mycobacterium fortuitum. Crude extracts of cells induced by AD exhibited Δ₁ dehydrogenation activity to AD and much weaker activity (four times) to 9OHAD. In contrast, cultures induced with 9OH-progesterone were found to show a two-times higher level of Δ₁ dehydrogenation activity to 9OHAD than to AD. However, the particular genes encoding these enzymes remain unknown (Wovcha et al., 1979). An extra ksdD-like gene (ORF3) was also reported in the A. simplex genome, but its activity was not confirmed (Dziadek et al., 1998).

In contrast to M. smegmatis, the genome of M. tuberculosis contains only two putative ksdD genes, MT3641 and MT0809 (TIGR). Moreover, the N-terminal FAD-binding motif is not conserved in MT0809. Since the amino acid identity is over 80 %, it is likely that the counterpart of MT3641 in the M. smegmatis genome is MSMEG5898 (ksdD-1). We also found that MT3641 (but not MT0809) was able to complement KsdD activity when over-produced in M. smegmatis dDCO1,2. The genome of M. smegmatis is much bigger in comparison to M. tuberculosis (6–9/4–4 Mb). It is likely that the number of genes has decreased in the course of evolution, since the huge enzymic machinery important for environmental strains could probably be decreased in pathogenic bacteria growing in much more defined conditions. The presence of MT0809 in the M. tuberculosis genome and its low level of homology to known non-mycobacterial ksdD genes would suggest a specific enzyme activity distinct from KsdD1 of M. smegmatis or M. tuberculosis. The most likely counterpart of MT0809 in the M. smegmatis genome is MSMEG5801 (78 % identity at amino acid level) which also lacks the conserved N-terminal FAD-binding sequence. However, further investigations would be necessary to identify the significance of MT0809 and MSMEG5801 for steroid bioconversion.

The significant accumulation of AD by M. smegmatis ΔksdD-1 in the biotransformation of cholesterol suggests that the other KsdD-like enzymes (including KsdD2) exhibit low activity or a low expression level in rich medium. The overproduction of KsdD2 in the M. smegmatis ΔksdD-1 mutant resulted in the accumulation of AD in the first 72 h of the cholesterol degradation process and subsequent bioconversion of AD to ADD. Our previous investigations showed that the time of maximal activation of P₃₉₃ in the presence of 0-2 % acetamide is 3–6 h. Moreover, the activated P₃₉₃ was able to produce about 250 000 FtsZ molecules per M. smegmatis cell (Dziadek et al., 2002). On the other hand, the complementation of M. smegmatis

![Fig. 8. The bioconversion of AD to ADD by the M. smegmatis wild-type strain (mc²) and the M. smegmatis ΔksdD-1 ΔksdD-2 (dDCO1,2) mutant growing in mineral medium with AD as the only source of carbon and energy, monitored by GC. The AD and ADD level is marked by open and filled squares (mc²) or open and filled circles (dDCO1,2), respectively. 9OHAD was not detected in this experiment.](image-url)
ΔkSD-1 by kSD-1, expressed under control of the same promoter (P\text{ami}), resulted in fast (48 h) degradation of cholesterol without the accumulation of steroid intermediates as observed in the wild-type strain. The low activity of KSD2 and potentially of the other KSD-like enzymes of \textit{M. smegmatis} could be due to different substrate specificity of these enzymes. It is likely that a particular KSD-like enzyme uses a different substrate for dehydrogenation and the other substrates are less preferred. We observed the accumulation of an unrecognized product (probably C\textsubscript{22}-24 acids) in the cholesterol degradation process carried out by strains lacking kSD-2 which did not accumulate in \textit{M. smegmatis} ΔkSD-1. However, to understand the significance of all putative KsDs in steroid biotransformation and their substrate specificity, further investigations, including construction of mutants, protein purification and analysis, would be required.

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