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Engineering of steroid biotransformation in rhodococcus

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Chapter 6

Summary and concluding remarks

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Bioengineering approaches on steroid biotransformation have been hampered by a lack of knowledge on the genetics and physiology of industrially interesting microorganisms. Many members of the family of *Actinomycetales* have been widely acknowledged as microorganisms able to rapidly degrade steroids and sterols. Pathway intermediates formed during sterol/steroid degradation may be used as precursor molecules in the synthesis of bio-active compounds. Important in steroid biotransformation is the availability of a microbial catalyst incapable of opening the poly-aromatic ring structure of the steroid molecule. Otherwise, steroid transformation by the biocatalyst will result in a substantial, if not complete loss of steroid substrate and steroid product. Ideally, such biocatalysts are genetically accessible, non-pathogenic strains blocked in steroid poly-aromatic ring degradation. 3-Ketosteroid 9 α -hydroxylase (KSH) and 3-ketosteroid Δ^1 -dehydrogenase (KSTD) are two key-enzymes involved in degradation of the steroid poly-aromatic ring structure. The combined efforts of these two enzyme activities results in the opening of the steroid B-ring.

This thesis describes a genetic engineering approach for the development of a microbial catalyst for steroid biotransformations. Molecular tools were developed for the actinomycete *Rhodococcus erythropolis* strain SQ1 and used for a molecular and physiological characterization of its steroid catabolic pathway and the construction of molecularly defined mutant strains blocked in steroid degradation.

Molecular tools for *Rhodococcus* species

Bioengineering of the steroid catabolic pathway at the molecular level requires a genetically accessible *Rhodococcus* strain capable of rapidly degrading sterols. *R. erythropolis* strain SQ1 was chosen in a comparative study of several *Rhodococcus* strains as a suitable (i.e. genetically accessible and sterol degrading) parent strain for this study (Chapter 2). An electrotransformation protocol allowed transformation efficiencies of up to $1 \cdot 10^6$ transformants per μg of plasmid DNA (pMVS301). This protocol was shown to be effective in obtaining targeted gene disruption mutants as well, using non-replicative plasmids containing the *aphII* kanamycin resistance marker (Chapter 2). Electrotransformation of *R. erythropolis* with such non-replicative plasmids, however, induced random plasmid integration events, resulting in antibiotic resistant transformants that did not have the expected gene disruption mutation. The rate of occurrence of this so-called illegitimate integration of plasmid DNA into the *Rhodococcus* genome appeared to be dependent on the constructs used in electrotransformation (data not presented). Intergeneric conjugal plasmid transfer proved an effective method for the mobilization and integration of mutagenic plasmids into the *Rhodococcus* genome with minimal random integration (Chapter 3). In the course of our study it became apparent that isoenzymes, encoded by different genes, were involved in steroid ring degradation, necessitating a gene inactivation system that could be used to inactivate multiple genes, successively. An unmarked gene deletion method, using *sacB* for counter-selection, was developed for *Rhodococcus* (Chapter 3). This allowed the straight-forward and easy construction of strains with single and multiple gene deletion mutations (Chapters 3, 4 and 5). Several genes involved in steroid degradation were cloned by functional complementation of different UV-induced mutants of *R. erythropolis* (Chapters 4 and 5). A novel and convenient *Rhodococcus*-*Escherichia coli* shuttle vector, named pRESQ, was developed for the construction of genomic libraries for functional complementation, as well as for gene identification experiments (Chapter 4 and 5). The

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pRESQ shuttle vector harbors the *ccdB* positive selection marker of *E. coli* cloning vector pZeRO-2.1 for easy cloning of genomic DNA and the *aphII* kanamycin resistance marker for maintenance in both *E. coli* and *Rhodococcus* species. Shuttle vector pRESQ stably maintains *Rhodococcus* DNA inserts in both *E. coli* and *R. erythropolis* and has been successfully applied for the construction of genomic libraries of *R. erythropolis* (this thesis) and *Rhodococcus rhodochrous* (data not presented).

Molecular characterization of the steroid poly-cyclic ring degradation pathway in *Rhodococcus erythropolis* strain SQ1

The steroid (i.e. 4-androstene-3,17-dione [AD]) degradation pathway of *R. erythropolis* SQ1 includes four enzymes involved in the two enzymatic steps that initiate opening of the steroid poly-aromatic ring structure (Fig. 1). Steroid 9 α -hydroxylation of AD and 1,4-androstadiene-3,17-dione (ADD) is performed by KSH, a two-component monooxygenase composed of KshA and KshB. Steroid Δ^1 -dehydrogenation of AD and 9 α -hydroxy-4-androstene-3,17-dione (9OHAD) involves two KSTD isoenzymes, named KSTD1 and KSTD2. These enzymatic steps together initiate opening of the steroid B-ring and are the first steps in full degradation of the steroid molecule.

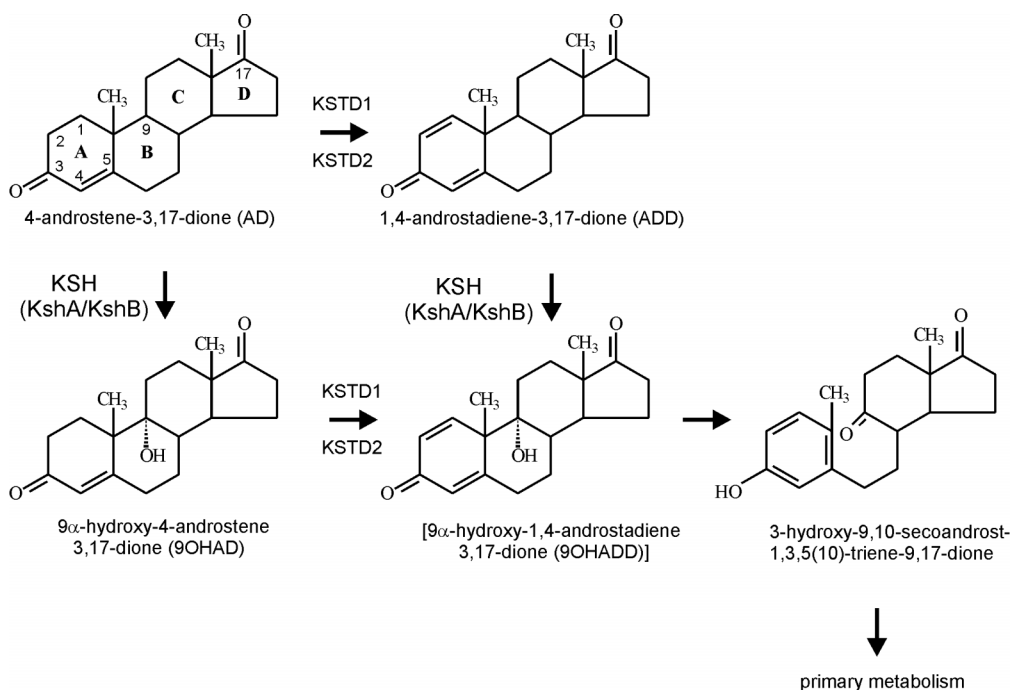


Figure 1. Four enzymes are involved in Δ^1 -dehydrogenation (KSTD1, KSTD2) and 9 α -hydroxylation (KshA, KshB) of the poly-aromatic ring structure of 4-androstene-3,17-dione (AD) in *Rhodococcus erythropolis* strain SQ1. KSTD1 and KSTD2 represent two 3-ketosteroid Δ^1 -dehydrogenase isoenzymes. KSH is a two-component 3-ketosteroid 9 α -monooxygenase comprised of KshA (terminal oxygenase component) and KshB (oxygenase reductase component).

Four structural genes, i.e. *kshA*, *kshB*, *kstD* and *kstD2*, have been identified in *R. erythropolis* strain SQ1, encoding the enzymes involved in AD degradation. The genomic loci of these genes appear to be scattered over the genome of *R. erythropolis* SQ1. No operon (-like) organization was apparent and therefore all four genes needed to be cloned individually. As an exception, a divergently transcribed TetR-type transcriptional regulatory gene (ORF2) was identified upstream of *kstD*, encoding a putative repressor (suggested name KstR) of *kstD* transcription (Chapter 2).

3-ketosteroid Δ^1 -dehydrogenase activity involves two isoenzymes

Total KSTD activity in *R. erythropolis* strain SQ1 represents two isoenzymes, named KSTD1 and KSTD2. The *kstD* gene, encoding the KSTD1 isoenzyme, was cloned on a 6 kb *Bgl*III genomic fragment isolated from a partial (*Bgl*III) genomic library of *R. erythropolis* strain SQ1 using a degenerate *kstD* oligonucleotide as a probe in Southern analysis. The nucleotide sequence of the *kstD* oligonucleotide was based on a semi-conserved amino acid sequences region of known KSTD enzymes (Chapter 2). Deletion of the *kstD* gene yielded strain RG1 (Chapter 3). The *kstD2* gene, encoding the KSTD2 isoenzyme, was cloned on a 6.5 kb genomic DNA fragment isolated from a genomic library of *kstD* mutant *R. erythropolis* strain RG1. This gene was identified via functional complementation of UV-induced mutant strain RG1-UV29, a *kstD* gene deletion mutant (strain RG1 lacking KSTD1) with an inactivated KSTD2 (Chapter 4).

Amino acid sequence similarity between the KSTD1 and KSTD2 isoenzymes was only about 35% identity. Two amino acid residues important for KSTD activity were identified in KSTD2, i.e. the semi-conserved S325 and the fully conserved T503. S325F and T503I point mutations independently fully inactivated KSTD2 activity (Chapter 4). The KSTD1 isoenzyme displayed similar activities and substrate affinities towards AD and 9OHAD, whereas KSTD2 showed a 5-fold higher KSTD activity with AD compared to 9OHAD. KSTD2 substrate affinity for AD was even 10-fold higher than for 9OHAD (Table 1). Individually, KSTD1 and KSTD2 allowed growth of *R. erythropolis* SQ1 on AD and 9OHAD. In whole cell bioconversion experiments, however, KSTD1 seemed much less efficient in 9OHAD Δ^1 -dehydrogenation than KSTD2, since 9OHAD degradation proceeded at a much lower rate in the presence of KSTD1 (strain RG7) than in the presence of KSTD2 (strain RG1). KSTD2 thus seems to be the prime KSTD enzyme involved in AD Δ^1 -dehydrogenation (Chapter 4). The physiological significance of the employment of two isoenzymes by *R. erythropolis* strain SQ1 for AD degradation remains unknown. The presence of two KSTD isoenzymes may serve to prevent (in an unknown way) the accumulation of relatively high intracellular concentrations of ADD, which is mildly inhibitory to growth.

Our data on the general pathway of steroid poly-aromatic ring degradation shows that *R. erythropolis* SQ1 may metabolize AD either via ADD or via 9OHAD (Fig. 1). During AD degradation the ADD concentration may reach high intracellular levels, putatively inducing re-routing of AD degradation via 9OHAD by lowering KSTD enzyme activity levels. Consequently, little or no ADD is formed anymore, giving rise to steroid mixtures. Indeed, accumulating levels of ADD do not exceed 50% during AD bioconversion by KSH-negative mutant *Rhodococcus* strains blocked in AD(D) 9 α -hydroxylation and thus only able to perform Δ^1 -dehydrogenation (Chapter 5). Moreover, AD is almost exclusively converted into 9OHAD by mutant strain RG7, lacking the KSTD2 isoenzyme. Thus, in strain RG7, AD 9 α -hydroxylation by KSH proceeds much faster than AD Δ^1 -dehydrogenation by KSTD1, resulting in minimal ADD formation during AD degradation. Relative activity levels of KSTD1 and KSTD2 present in the cell may be relevant and may be regulated either at the transcriptional level, or by enzyme inhibition/activation mechanisms.

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Table 1. Comparison of substrate affinities and relative maximal reaction rates of KSTD1 and KSTD2 enzymes overexpressed in *E. coli*.

	Relative reaction rate (%) [#]		K _m _{app} (μM)	
	AD	9OHAD	AD	9OHAD
KSTD1	100	106	82	87
KSTD2	100	21	17	165

[#] Enzymatic activities measured with AD as a substrate for KSTD1 (0.45 μmol·min⁻¹·mg⁻¹) and KSTD2 (6 μmol·min⁻¹·mg⁻¹) were set at 100 %.

Interestingly, *kstD*, encoding KSTD1, is preceded by *kstR* (ORF2), a putative transcriptional regulator of *kstD* expression (Chapter 2). Speculatively, *kstR* could be part of a transcriptional regulatory system of KSTD enzyme activity, responding to changing concentration levels of ADD.

3-Ketosteroid 9α-hydroxylase is a two-component monooxygenase composed of KshA and KshB displaying AD(D) 9α-hydroxylase activity

Steroid 9α-hydroxylation of AD and ADD in *R. erythropolis* strain SQ1 is catalyzed by a two-component monooxygenase enzyme system composed of KshA and KshB (Chapter 5). The genes encoding KshA and KshB were isolated and identified following functional complementation of two KSH UV-induced mutant strains (strain RG1-UV39 and RG1-UV26, respectively) using a genomic library of *kstD* mutant *R. erythropolis* strain RG1. Contrary to many other structural genes encoding microbial multi-component oxygenases, *kshA* and *kshB* are located at different genomic loci separated by at least several kilobases. The *kshA* gene was cloned and identified on a 6 kb genomic DNA fragment by functional complementation of strain RG1-UV39. Complementation of strain RG1-UV26 allowed the cloning and identification of *kshB* on a 6 kb genomic DNA fragment. The deduced amino acid sequences of *kshA* and *kshB* showed that KshA and KshB both are [2Fe-2S] containing enzymes, harboring highly conserved domains typically found in class IA terminal oxygenases and class IA oxygenase-reductases. Class IA oxygenases by definition are two-component oxygenases, classifying KSH of *R. erythropolis* strain SQ1 as a two-component enzyme system. In this system, KshA constitutes the terminal oxygenase component of KSH, performing the actual 9α-monooxygenation of AD(D), whereas KshB is the oxygenase-reductase component, transferring electrons, generated by NAD(P)H oxidation, to the KshA oxygenase component. A P-450 cytochrome dependent enzyme system apparently is not involved in steroid 9α-hydroxylation in this strain. The two-component enzyme system of KSH furthermore differs fundamentally from the three-component enzyme system reported for *Nocardia* sp. M117 (Strijewski, 1982), suggesting that there may be several different classes of microbial KSH enzymes in nature. Inactivation of either the KshA or KshB component of KSH in parent strain SQ1 completely blocked AD and ADD 9α-hydroxylation. Mutant strains RG2 (KshA-negative) and RG4 (KshB-negative) did not grow on AD and ADD as sole carbon and energy source, not even after 3-4 days of incubation. KSH displays both AD and ADD 9α-hydroxylase activity: strain RG9, a *kshA kstD kstD2* triple gene deletion mutant, which was incapable of 9α-hydroxylation of AD and ADD in bioconversion experiments (Chapter 5). No AD(D) 9α-hydroxylase isoenzymatic activities were apparent in *R. erythropolis* strain SQ1, contrary to the KSTD isoenzymes found in this strain.

Table 2. Molecularly defined gene deletion mutant strains of *Rhodococcus erythropolis* strain SQ1 blocked at the level of 3-ketosteroid 9 α -hydroxylation (*kshA*, *kshB*) and 3-ketosteroid Δ^1 -dehydrogenation (*kstD*, *kstD2*).

Strain	Mutant phenotype	Steroid catabolism	AD biotransformation
SQ1	parent strain	+	+, no accumulation
RG1	<i>kstD</i>	+	+, no accumulation
RG2	<i>kshA</i>	AD ⁻ , ADD ⁻	+, ADD accumulation
RG4	<i>kshB</i>	AD ⁻ , ADD ⁻	+, ADD accumulation
RG7	<i>kstD2</i>	+	+, temporarily 9OHAD accumulation
RG8	<i>kstD kstD2</i>	AD ⁻ , 9OHAD ⁻	+, 9OHAD accumulation
RG9	<i>kstD kstD2 kshA</i>	AD ⁻ , ADD ⁻ , 9OHAD ⁻	-, completely blocked

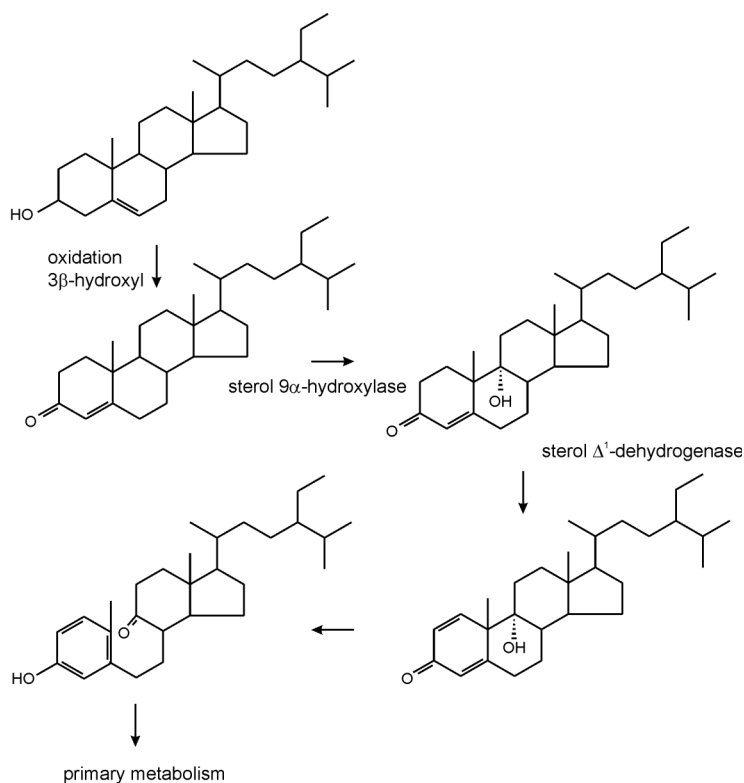


Figure 2. Proposed scheme for sterol (i.e. in this case β -sitosterol) degradation in *R. erythropolis* strain SQ1. Following oxidation of the 3 β -hydroxyl group by cholesterol oxidase (or -dehydrogenase), the poly-aromatic steroid ring structure of the sterol molecule is attacked by a sterol specific 9 α -hydroxylase and a sterol Δ^1 -dehydrogenase (or vice versa). KSTD1, KSTD2 and the KshA component do not participate in sterol degradation.

Bioengineering of the steroid catabolic pathway

The research described in this thesis has resulted in isolation of several gene deletion mutants of *R. erythropolis*, blocked in opening of the steroid poly-aromatic ring (Table 2). Bioengineering of *R. erythropolis* mutant strains capable of selectively performing steroid 9 α -hydroxylation demands the inactivation of both KSTD1 and KSTD2 enzyme activities (Chapter 2, 3 and 4). Such mutants, fully blocked in steroid Δ^1 -dehydrogenation, are able to efficiently perform bioconversions of AD into 9OHAD in high yields (Table 2; Chapter 3). Inactivation of only one of the KSTD isoenzymes, however, results in degradation of either substrate, or the hydroxylated product, or both. On the other hand, selective steroid Δ^1 -dehydrogenation can easily be achieved by inactivation of either the KshA component or the KshB component of the KSH enzyme system (Chapter 5). However, in this case no complete conversion of AD into ADD occurs, most likely caused by regulatory systems as discussed before.

Sterol catabolism in Rhodococcus erythropolis SQ1

Bioengineering of the steroid degradation pathway of *R. erythropolis* was aimed to construct mutant strains able to selectively degrade the sterol side chain, but which were unable to open the steroid poly-aromatic ring structure. The mutants that have been isolated so far were blocked in opening of the poly-aromatic ring structure of AD, but unfortunately did not accumulate (9OH)AD(D) from sterols. The identified genes involved in steroid degradation thus do not participate in sterol degradation, with the exception of *kshB*. KshB appears to be a multi-functional enzyme involved in both steroid and sterol degradation (Chapter 5). Inactivation of KshB completely blocks steroid and sterol degradation, indicating that KshB plays a key role in both sterol/steroid catabolism, most likely providing reducing power for several sterol/steroid terminal oxygenases. Among these, we expect the presence of a sterol 9 α -hydroxylase (Fig. 2). The terminal oxygenase component of the putative sterol 9 α -hydroxylase most likely is a KshA homolog, interacting with KshB. Analogously, we propose the involvement of a sterol Δ^1 -dehydrogenase in sterol catabolism (Fig. 2). In addition to KSTD1, a second KSTD activity band was observed on PAGE (Chapter 2). This activity was not based on KSTD2, which shows no activity with PMS/NBT used to stain PAGE slabs (Chapter 4). The observed KSTD activity band thus may represent a putative sterol Δ^1 -dehydrogenase with minor, in vivo insignificant, activity towards the steroids (9OH)AD (KSTD3). *R. erythropolis* strain SQ1 therefore is expected to contain multiple enzyme systems, specialized in either opening the poly-aromatic ring structure of steroids or sterols.