# Engineering of steroid biotransformation in rhodococcus

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# Chapter 3

## Unmarked gene deletion mutagenesis of *kstD*, encoding 3-ketosteroid $\Delta^1$-dehydrogenase, in *Rhodococcus erythropolis* SQ1 using *sacB* as counter-selectable marker

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## Abstract

This paper reports the first method for the construction of unmarked gene deletion mutants in the genus *Rhodococcus*. Unmarked deletion of the *kstD* gene, encoding 3-ketosteroid $\Delta^1$-dehydrogenase (KSTD1) in *Rhodococcus erythropolis* SQ1, was achieved using the *sacB* counter-selection system. Conjugative mobilization of the mutagenic plasmid from *E. coli* S17-1 to *R. erythropolis* strain SQ1 was used to avoid its random genomic integration. The *kstD* gene deletion mutant, designated strain RG1, still possessed about 10% of the KSTD enzyme activity of wild type and was not affected in its ability to grow on the steroid substrates 4-androstene-3,17-dione (AD) and 9α-hydroxy-4-androstene-3,17-dione (9OHAD). Biochemical evidence subsequently was obtained for the presence of a second KSTD enzyme (KSTD2) in *R. erythropolis* SQ1. UV mutants of strain RG1 unable to grow on AD were isolated. One of these mutants, strain RG1-UV29, had lost all KSTD enzyme activity and also was unable to grow on 9OHAD. It stoichiometrically converted AD into 9OHAD in concentrations as high as 20 $g \cdot l^{-1}$. The two KSTD enzymes apparently both function in AD and 9OHAD catabolism. These isoenzymes have been inactivated in strain RG1 (KSTD1 negative) and strain RG1-UV29 (KSTD1 and KSTD2 negative), respectively.


## Introduction

The genus *Rhodococcus* is attracting strong interest in medical and biotechnological fields of research (Warhurst and Fewson, 1994; Bell *et al.*, 1998). Various rhodococci, exhibiting a diverse range of metabolic capabilities, are applied in bioremediation and biotransformation processes. Only a limited set of molecular tools for genetic engineering of *Rhodococcus* strains is currently available (Larkin *et al.*, 1998). Suitable strategies for the introduction of unmarked gene deletions have not been reported for *Rhodococcus* strains. Navas et al. (2001) recently described the first method for gene replacement in *Rhodococcus equi*, using the *aacC4* apramycin resistance gene from *Salmonella*. Mutants isolated via this method, however, retain the *aacC4* marker in their genome, excluding future use of this marker in such mutants. The unmarked gene deletion method described here for *R. erythropolis* overcomes this problem, since the technology can be used sequentially in constructed gene deletion mutants using the same vector and selection markers. Gene deletion or gene replacement methods have been described for other members of the *Actinomycetales*, namely *Streptomyces* (Hillemann *et al.*, 1991; Hosted and Baltz, 1997), *Corynebacterium* (Jäger *et al.*, 1992; Schäfer *et al.*, 1994) and *Mycobacterium* (Marklund *et al.*, 1995; Norman *et al.*, 1995; Sander *et al.*, 1995; Pelicic *et al.*, 1996; Knipfer *et al.*, 1997). Some of these methods make use of counter-selectable markers to screen for the rare second recombination event resulting in the actual gene deletion or gene replacement. In this respect, both *sacB* and *rpsL* proved to be useful reporter genes. The use of *rpsL* as a marker in *Rhodococcus* has not been reported, but *sacB* (encoding the *Bacillus subtilis* levansucrase) provides a potent positive selection marker in this genus (Jäger *et al.*, 1995; Denis-Larose *et al.*, 1998).

Insertional inactivation of the *kstD* gene, encoding 3-ketosteroid $\Delta^1$-dehydrogenase (KSTD1) in *R. erythropolis* SQ1, did not result in inactivation of steroid skeleton degradation (van der Geize *et al.*, 2000). Biochemical evidence for the presence of two KSTD isoenzymes in *R. erythropolis* SQ1 subsequently was obtained (van der Geize *et al.*, 2000). Inactivation of the genes encoding both these KSTD activities thus appeared necessary to obtain a strain completely blocked in steroid skeleton degradation (Fig. 1).

*Unmarked gene deletion mutagenesis*

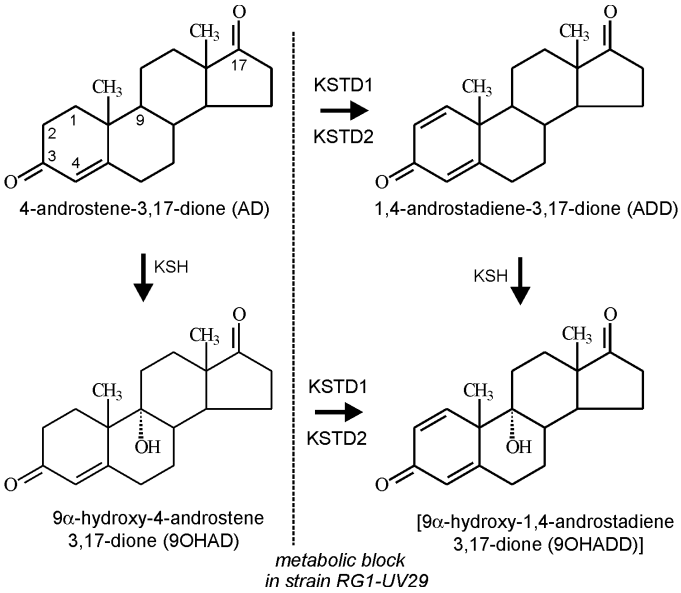


***Figure 1.*** Steroid catabolism in *R. erythropolis* strain SQ1 showing chemical structures of 4-androstene-3,17-dione (AD), 9α-hydroxy-4-androstene-3,17-dione (9OHAD), 1,4-androstadiene-3,17-dione (ADD) and 9α-hydroxy-1,4-androstadiene-3,17-dione (9OHADD) and the positions of KSTD1 and the identified isoenzyme KSTD2. Dotted line represents a metabolic block introduced by *kstD* gene deletion and KSTD2 inactivation. KSTD: 3-ketosteroid $\Delta^1$-dehydrogenase; KSH: steroid 9α-hydroxylase.

Mutant *Rhodococcus* strains devoid of KSTD activity are expected to accumulate steroid pathway intermediates, which are potential precursors in the synthesis of bioactive steroids (Kieslich, 1985; Mahato and Garai, 1997). The development of methods for the introduction of unmarked gene deletions for the step-wise inactivation of multiple KSTD genes, without being dependent on different vector/marker systems, became a high priority in our work. Moreover, the resulting strains would be genetically stable and free of heterologous DNA, which is advantageous for their industrial application.

Here we report methods for the introduction of unmarked gene deletions in *R. erythropolis* SQ1 involving conjugative transfer of a mutagenic plasmid carrying the *sacB* gene as counter-selectable marker. A *kstD* gene deletion strain was constructed, designated *R. erythropolis* strain RG1, lacking KSTD1 activity. The KSTD2 isoenzyme was subsequently inactivated by classical UV mutagenesis.

## Materials and methods

*Bacterial strains, plasmids and growth conditions*

*Rhodococcus erythropolis* SQ1 (Quan and Dabbs, 1993) and derived mutant strains were cultivated at $30^0$C and 200 rpm in liquid medium (LBP) containing 1% (w/v) bacto-pepton (Difco, Detroit, Mich.), 0.5% (w/v) yeast extract (BBL, Becton Dickinson and Company, Cockeysville , Md.) and 1% (w/v) NaCl. Mineral medium consisted of $K_2HPO_4$ 4.65 g·l$^{-1}$, $NaH_2PO_4 \cdot H_2O$ 1.5 g·l$^{-1}$, $NH_4Cl$ 3 g·l$^{-1}$, $MgSO_4 \cdot 7H_2O$ 1 g·l$^{-1}$, supplemented with Vishniac trace elements (pH 7.2). Steroids were

dissolved in dimethylsulfoxide (50 mg·ml$^{-1}$) 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 $^0$C. BBL agar (1.5% (w/v)) was added in case of growth on solid medium. Sucrose (suc) sensitivity of *Rhodococcus* strains was tested on LBP agar supplemented with 10% (w/v) sucrose (LBPS).
4-Androstene-3,17-dione (AD), 9α-hydroxy-4-androstene-3,17-dione (9OHAD) and 1,4-androstadiene-3,17-dione (ADD) were supplied by Diosynth bv (Oss, The Netherlands).

*General cloning techniques*
DNA modifying enzymes were purchased from Boehringer (Mannheim, Germany), New England Biolabs (Beverly, Mass.) or Amersham Pharmacia Biotech AB (Uppsala, Sweden) and were used as described by the manufacturer. Isolation of DNA restriction fragments from agarose gels was done using the Qiagen (Basel, Switzerland) gel extraction kit according to protocol. All DNA manipulations were done according to standard protocols (Sambrook *et al.*, 1989).

*Conjugative plasmid transfer from E. coli S17-1 to R. erythropolis SQ1*
Cells of the *R. erythropolis* SQ1 recipient strain were spread on LBP agar supplemented with 30 μg·ml$^{-1}$ nalidixic acid and grown for 5 days. The mutagenic plasmid pSDH422 was first introduced in *E. coli* S17-1 (Simon *et al.*, 1983) by transformation. Transformants (approx. 1000 per plate), obtained after growth overnight on selective media (kanamycin 25 μg·ml$^{-1}$), were incubated at room temperature for another 24 h to acquire additional cell material. Colonies of both *Rhodococcus* and *E. coli* strains were harvested from plate, and separately resuspended, each in a final volume of 1.5 ml of LBP. Aliquots of 750 μl of both strains were mixed together and gently pelleted by centrifugation. The pellet was suspended in 1 ml LBP and cells were spread on non-selective LBP agar in 200-250 μl aliquots. After growth overnight at 30$^0$C, the confluently grown material was harvested from plate, suspended in 2 ml LBP medium and 100 μl aliquots were spread on LBP agar supplemented with kanamycin (200 μg·ml$^{-1}$) and nalidixic acid (30 μg·ml$^{-1}$). *R. erythropolis* SQ1 transconjugants appeared after 3 days.

*Southern hybridization*
*Rhodococcus* total DNA was isolated as previously described (van der Geize *et al.*, 2000). Digested chromosomal DNA from *R. erythropolis* SQ1 was separated on an 1 % (w/v) agarose gel and blotted onto a high-bond nylon membrane supplied by Boehringer, via an alkaline transfer method (Sambrook et al., 1989). Southern hybridizations were done at 60 $^0$C using the complete *kstD* gene as a probe. Labeling of *kstD*, obtained by PCR using *kstD* primers as described previously (van der Geize *et al.*, 2000), was done using the random primed DIG-labeling kit from Boehringer. The membrane was washed at 60 $^0$C with 2xSSC (1xSSC is 0.15M NaCl and 0.015M sodium citrate), containing 0.1% (w/v) sodium dodecyl sulphate (SDS) for 5 min and twice with 0.1xSSC with 0.1% (w/v) SDS for 5 min.

*UV mutagenesis of R. erythropolis RG1*
*R. erythropolis* RG1 cells were grown in 10 mM glucose mineral medium (2·10$^8$ CFUs·ml$^{-1}$) and harvested in the late exponential growth phase. Sonic treatment of washed cells for a short period of time was performed to obtain single cells. Similar treatments were previously shown effective for the related actinomycete *Amycolatopsis methanolica* (Euverink *et al.*, 1996; Hektor and Dijkhuizen, 1996). Diluted (10$^4$) samples were spread on glucose mineral agar medium and irradiated for 15-20 sec with a UV lamp (Philips TAW 15W) at a distance of 27 cm, on average

resulting in 95% killing of cells. After 4 days of incubation, colonies that had appeared were replica plated on AD (0.5 $g \cdot l^{-1}$) mineral agar medium. AD growth deficient mutants scored after 3-4 days were further characterized for growth on ADD (0.5 $g \cdot l^{-1}$) and 9OHAD (0.5 $g \cdot l^{-1}$) mineral agar medium.

*Preparation of cell extracts of Rhodococcus*

Cells were grown in glucose (20 mM) mineral medium (250 ml) for 3 days and subsequently induced with 0.25 $g \cdot l^{-1}$ AD for 5 hours. Cell pellets (30 min; 7,300g; 4 $^0$C) were washed with 200 ml phosphate buffer ($KH_2PO_4$ 2.72 $g \cdot l^{-1}$; $K_2HPO_4$ 3.48 $g \cdot l^{-1}$; $MgSO_4 \cdot 7H_2O$ 2.46 g·l; pH7.2). Washed cell suspensions (5 ml) were disrupted by passage through a French pressure cell (140 Mpa) twice. Cell extracts were centrifuged for 20 min at 25,000 g to remove cell debris. The resulting supernatant (5-10 mg protein$\cdot ml^{-1}$) was used for KSTD enzyme activity assays or stored at -20$^0$C.

*KSTD enzyme activity assay*

Enzyme activities were measured spectrophotometrically at 30 $^0$C using phenazine methosulphate (PMS) and 2,6-dichlorophenolindophenol (DCPIP). The reaction mixture (1 ml) consisted of 50 mM TRIS pH 7, 1.5 mM PMS, 40 µM DCPIP, cell-free extract and AD (200 µM) in methanol (2%). Activities are expressed as mU$\cdot mg^{-1}$ of protein; 1 mU is defined as the reduction of 1 nmol$\cdot min^{-1}$ DCPIP ($\varepsilon_{600nm} = 18.7 \cdot 10^3$ $cm^{-1} \cdot M^{-1}$).

*In vitro conversion of AD by cell-free extracts*

A reaction mixture (1 ml) consisting of TRIS pH 7 (50 mM), PMS (150 µM), AD (700 µM) and AD-induced cell-free extract (approximately 25 µg of protein) was incubated overnight at 30$^0$C. Samples were diluted 5 times by addition of 4 ml methanol/water (70:30) and filtered (0.45 µm). Steroid content was analyzed by high-performance liquid chromatography (column: reversed phase Lichrosorb 10RP18, SS 250x3 mm (Varian Chrompack International, Middelburg, The Netherlands), $UV_{254\ nm}$ detection, liquid phase: methanol: water 60:40, 35 $^0$C) (van der Geize *et al.*, 2000).

*Bioconversion of AD by whole cells of strain RG1-UV29*

Strains SQ1, RG1 and RG1-UV29 were grown in medium containing yeast extract (25 $g \cdot l^{-1}$), glucose (10 $g \cdot l^{-1}$) and polypropyleneglycol (0.1 $ml \cdot l^{-1}$) at pH 7.5 in a 5 liter batch fermenter (28$^0$C, 650-850 rpm, airflow 0.1 $v \cdot v^{-1} \cdot min^{-1}$, 4 liter working volume). AD (20 $g \cdot l^{-1}$) suspended in Tween80 (0.1 % (v/v)) was added at $OD_{660}$ = 6 and bioconversion was followed for 48 h. Steroid content was analyzed as previously described (van der Geize *et al.*, 2000).

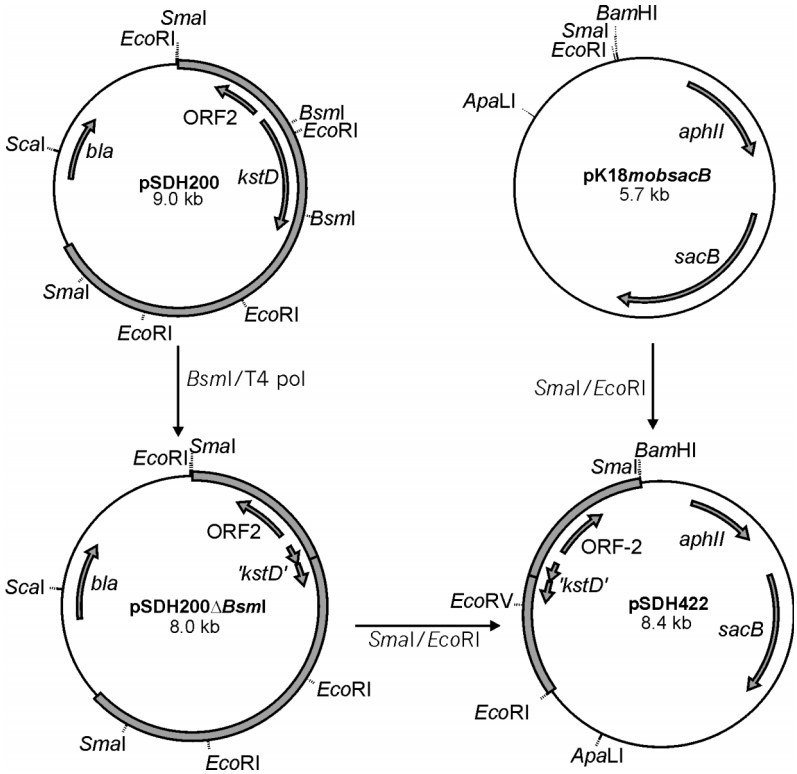


***Figure 2.*** Schematic representation of the construction of mutagenic plasmid pSDH422, containing the counter-selectable marker *sacB*, used for the introduction of a 1,062 bp unmarked *kstD* gene deletion in *R. erythropolis* strain SQ1.

## Results and discussion

*Unmarked kstD gene deletion in R. erythropolis strain SQ1*

Plasmid pK18*mobsacB* (Schäfer *et al.*, 1994), containing oriT of plasmid RP4 for conjugative mobilization, was used for the construction of a mutagenic plasmid, pSDH422 (Fig. 2), for the introduction of a *kstD* deletion in the *R. erythropolis* SQ1 chromosome. A 1,062 bp *Bsm*I fragment of pSDH200 (van der Geize *et al.*, 2000), encoding a large internal part of *kstD*, was deleted to construct pSDH200Δ*Bsm*I. This plasmid was subsequently used for construction of pSDH422 by cloning a 2,724 bp *Sma*I/*Eco*RI fragment of pSDH200Δ*Bsm*I, harboring the remaining 468 bp of *kstD* and its flanking regions, into the *Sma*I/*Eco*RI site of pK18*mobsacB*. The plasmid pSDH422 was introduced into *E. coli* S17-1 and mobilized to *R. erythropolis* SQ1 by conjugation. Conjugative plasmid transfer from *E. coli* strain S17-1 to *Rhodococcus* sp. has been shown to minimize random integration (Powell and Archer, 1998), a phenomenon commonly encountered in gene disruption experiments with *Rhodococcus* species (Desomer *et al.*, 1991; Barnes *et al.*, 1997; van der Geize *et al.*, 2000).

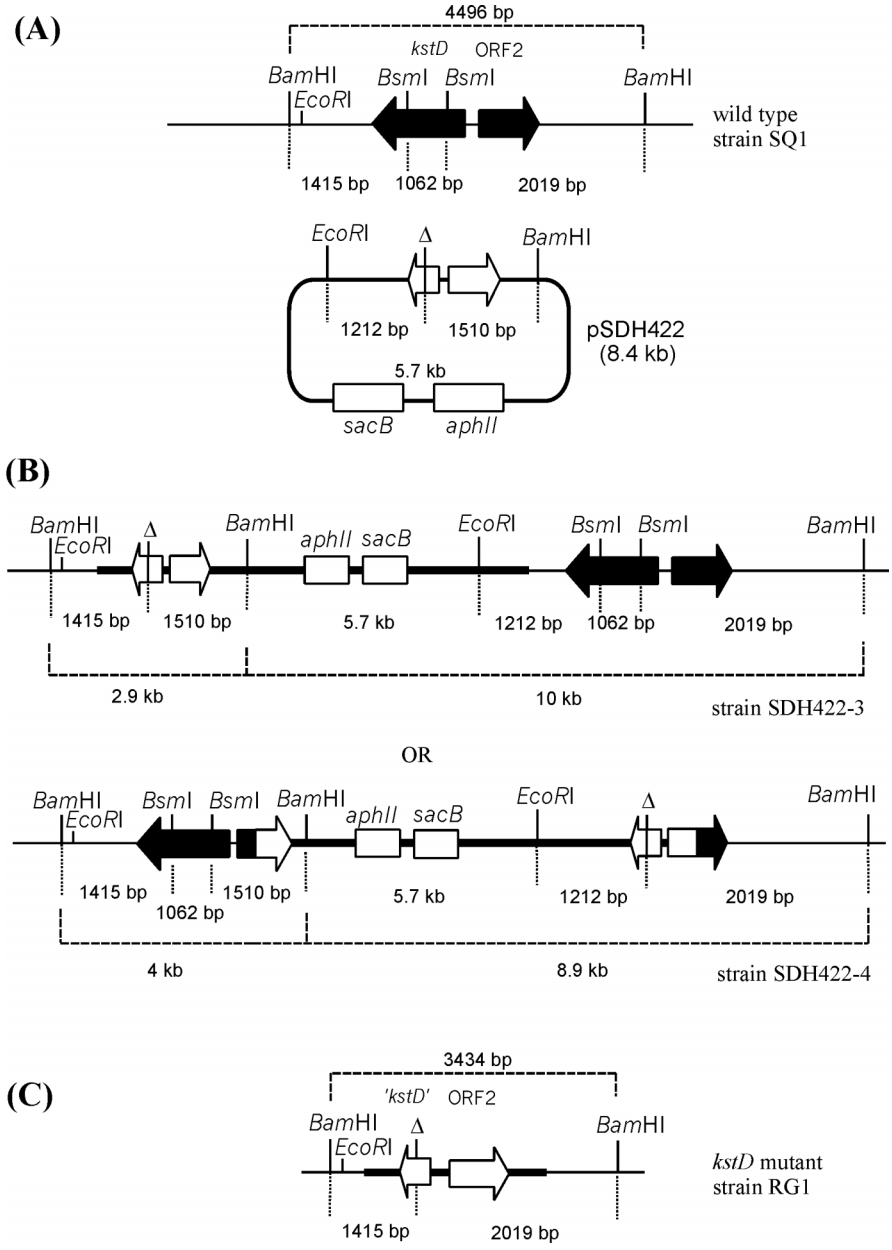


***Figure 3.*** Schematic overview of the molecular organization of the region encoding *kstD* in (A) wild type *R. erythropolis* strain SQ1, (B) after integration of pSDH422 by a single homologous recombination event at the targeted locus downstream (strain SDH422-3) and upstream (strain SDH422-4) of the *kstD* gene deletion, and (C) after *sacB* counter-selection for the second homologous recombination within the genetic organization of strain SDH422-3 (top scheme Fig.3B), resulting in *kstD* deletion mutant strain RG1. The result of the second homologous recombination within the genetic organization of SDH422-4 (lower scheme Fig.3B) is not shown, but would equally result in *kstD* gene deletion.

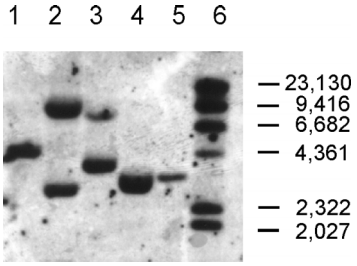


***Figure 4.*** Southern analysis of *R. erythropolis* chromosomal DNA digested with *Bam*HI of wild type (lane 1), strain SDH422-3 (lane 2), SDH422-4 (lane 3) and two individual *kstD* deletion mutants obtained from strain SDH422-3 after *sacB* counter-selection (lanes 4 and 5). The complete *kstD* gene, obtained by PCR, was used as a dig-labeled probe.

All resulting kanamycin resistant ($Kan^r$) *Rhodococcus* transconjugants were sucrose sensitive ($Suc^s$), since no growth occurred after replica plating on LBPS agar supplemented with 200 $\mu g \cdot ml^{-1}$ kanamycin. Transconjugants were subsequently checked by Southern analysis. Fig. 3 schematically shows the theoretically expected molecular organization resulting from genomic integration of pSDH422 via homologous recombination into the *R. erythropolis* SQ1 chromosome. Integration by homologous recombination may occur at either side of the *kstD* deletion present on pSDH422, resulting in two types of transconjugants (Fig. 3). Southern analysis of chromosomal DNA digested with *Bam*HI (Fig. 4) of wild type (lane 1: single band of approx. 4.5 kb) and two transconjugants, SDH422-3 (lane 2: two bands of 2.9 kb and 10 kb) and SDH422-4 (lane 3: two bands of 4 kb and 8.9 kb) revealed that both types of transconjugants had been obtained and confirmed integration of one copy of pSDH422 at the targeted locus by a single homologous recombination event. Gene deletion of *kstD* was achieved by overnight growth of *R. erythropolis* strain SDH422-3 under non-selective conditions and subsequent plating on selective medium, i.e. LBPS agar. The $Suc^r$ colonies obtained were replica plated on selective media (LBPS) supplemented with 200 $\mu g \cdot ml^{-1}$ kanamycin, which revealed that 10% of all $Suc^r$ colonies were $Suc^r/Kan^r$. A $Suc^r/Kan^r$ phenotype indicates that an integrated copy of pSDH422 is still present in the chromosome. This phenotype therefore is presumably due to inactivation of the *sacB* gene by either a mutation, or an IS element as previously described for *Rhodococcus* (Jäger *et al.*, 1995). The remaining 90% of all $Suc^r$ colonies had lost kanamycin resistance ($Suc^r/Kan^s$). Colony PCR with *kstD* primers (van der Geize *et al.*, 2000) on 9 $Suc^r/Kan^s$ colonies resulted in 6 PCR products with fragment sizes of 468 bp, comprising the remaining part of the *kstD* gene, and 3 PCR products with fragment sizes of wild type *kstD*, representing 60% and 30% of the total $Suc^r$ population, respectively. Gene deletion was confirmed by Southern analysis performed on two potential *kstD* deletion mutants. A 4.5 kb *kstD* DNA fragment of wild type (Fig. 4, lane 1) obtained after *Bam*HI digestion of chromosomal DNA was reduced to 3.4 kb in the gene deletion mutants (Fig. 4, lanes 4 and 5), indicating deletion of the expected 1,062 bp *kstD* DNA fragment. The resulting strain, denoted *R. erythropolis* RG1, had regained wild type kanamycin sensitivity and sucrose resistance, confirming that an unmarked gene deletion in *R. erythropolis* SQ1 had been achieved.

*Characterization of kstD deletion mutant R. erythropolis strain RG1*

A substantial decrease in KSTD activity in cell-free extracts of strain RG1 (40 $mU \cdot mg^{-1}$) was observed compared to cell-free extracts of wild type strain SQ1 (450 $mU \cdot mg^{-1}$). Residual KSTD activity, however, was still detectable in the *kstD* gene deletion mutant strain RG1. Growth of strain RG1 on the steroid substrates AD or 9OHAD as sole carbon and energy sources was also not affected. These data indicate the presence of two KSTD activities in *R. erythropolis* SQ1 and would explain why inactivation of *kstD* alone does not have an effect on growth on steroid mineral medium. Conceivably, inactivation of the gene encoding the second KSTD enzyme in a *kstD* deletion mutant strain will generate a *Rhodococcus* strain completely devoid of KSTD activity.

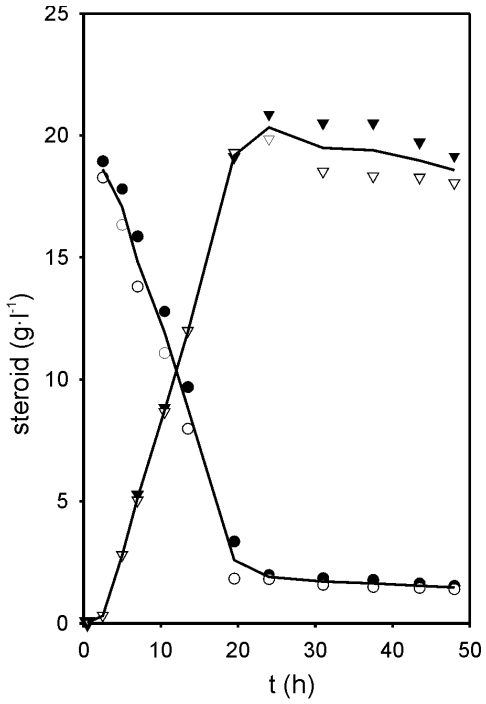


***Figure 5.*** Bioconversion of 20 g·l$^{-1}$ 4-androstene-3,17-dione (AD (circles); added at t=0 h) into 9α-hydroxy-4-androstene-3,17-dione (9OHAD (triangles)) by *R. erythropolis* RG1-UV29. Graph is the mean of two independent (open and closed symbols, respectively) bioconversion experiments.

*Inactivation of steroid Δ$^1$-dehydrogenation by UV mutagenesis of R. erythropolis RG1*

To isolate mutants of *R. erythropolis* RG1 blocked in Δ$^1$-dehydrogenation we used UV mutagenesis and screened for AD growth deficient mutants able to grow on ADD mineral medium. Sonic treatment of *Rhodococcus* cells prior to the UV mutagenesis treatment was shown to be effective to segregate cell clumps and in obtaining single cells. Replica plate screening of >10$^4$ CFU's of *R. erythropolis* RG1 after UV mutagenic treatment yielded 49 mutants unable to grow on AD mineral medium. UV mutant 29 (strain RG1-UV29) and three additional UV mutants were clearly impaired in the steroid Δ$^1$-dehydrogenation reaction: no growth occurred with either AD or 9OHAD as sole carbon and energy source, whilst growth on ADD mineral medium was normal. KSTD activities in cell-free extracts of strain RG1-UV29 were reduced to below detection levels.

*Bioconversion of AD by cell-free extracts and whole cells of R. erythropolis strains*

Overnight incubation of cell-free extracts (25 µg protein) of the *kstD* mutant strain RG1 with AD (700 µM) resulted in the nearly complete bioconversion of AD into ADD (85%), unambiguously indicating the presence of a second 3-ketosteroid Δ$^1$-dehydrogenation activity in *R. erythropolis*, a KSTD isoenzyme (KSTD2). No ADD formation was detected during AD bioconversion by cell-free extracts of strain RG1-UV29, demonstrating the absence of KSTD2 isoenzymatic activity in this mutant.

Whole cells of *R. erythropolis* RG1-UV29, completely impaired in steroid Δ$^1$-dehydrogenation, are expected to accumulate 9OHAD from AD as substrate. This mutant strain indeed carried out a stoichiometrical bioconversion of 20 g·l$^{-1}$ of AD into 9OHAD within 24 h with a 93% yield (Fig.

5). No accumulation of 9OHAD from AD was observed with either strain SQ1 or strain RG1. In comparison, Datcheva *et al.* (1989) reported the isolation of a *Rhodococcus* sp. converting AD in 9OHAD with a 70% yield at substrate concentrations of 1-3 $g \cdot l^{-1}$. With this strain, however, yields decreased significantly to 40% at higher concentrations of 5 $g \cdot l^{-1}$ AD.

*Conclusion*

We succeeded in constructing an unmarked gene deletion in *Rhodococcus* and used the technique in combination with classical mutagenesis to gain more insights in the rather complex metabolic pathways of steroid degradation in *R. erythropolis* strain SQ1. This complexity at least partly explains the moderate success in earlier random mutagenesis attempts to obtain stable and completely blocked mutant strains accumulating steroids. Only a rational approach, involving cloning of genes involved in steroid catabolism, and the step-wise unmarked deletion of these genes, may ultimately yield stable and genetically defined mutant *Rhodococcus* strains accumulating valuable steroid intermediates.

## Acknowledgements

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