Characterization of a Second *Rhodococcus erythropolis* SQ1 3-Ketosteroid 9α-Hydroxylase Activity Comprising a Terminal Oxygenase Homologue, KshA2, Active with Oxygenase-Reductase Component KshB$^\nabla$

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Previously we have characterized 3-ketosteroid 9α-hydroxylase (KSH), a key enzyme in microbial steroid degradation in *Rhodococcus erythropolis* strain SQ1, as a two-component iron-sulfur monooxygenase, comprised of the terminal oxygenase component KshA1 and the oxygenase-reductase component KshB. Deletion of the *kshA1* gene resulted in the loss of the ability of mutant strain RG2 to grow on the steroid substrate 4-androstene-3,17-dione (AD). Here we report characteristics of a close KshA1 homologue, KshA2 of strain SQ1, sharing 60% identity at the amino acid level. Expression of the *kshA2* gene in mutant strain RG2 restored growth on AD and ADD, indicating that *kshA2* also encodes KSH activity. The functional complementation was shown to be dependent on the presence of *kshB*. Transcriptional analysis showed that expression of *kshA2* is induced in parent strain *R. erythropolis* SQ1 in the presence of AD. However, promoter activity studies, using β-lactamase of *Escherichia coli* as a convenient transcription reporter protein for *Rhodococcus*, revealed that the *kshA2* promoter in fact is highly induced in the presence of 9α-hydroxy-4-androstene-3,17-dione (9OHAD) or a metabolite thereof. Inactivation of *kshA2* in parent strain SQ1 by unmarked gene deletion did not affect growth on 9OHAD, cholesterol, or cholic acid. We speculate that KshA2 plays a role in preventing accumulation of toxic intracellular concentrations of ADD during steroid catabolism. A third *kshA* homologue was additionally identified in a *kshA1 kshA2* double gene deletion mutant strain of *R. erythropolis* SQ1. The developed degenerate PCR primers for *kshA* may be useful for isolation of *kshA* homologues from other (actino) bacteria.

Rhodococcus species display a wide range of metabolic capabilities, degrading a variety of environmental pollutants and transforming or synthesizing compounds with possible useful applications (3, 26, 30). A well-known feature of rhodococci is their ability to degrade a range of naturally occurring steroids, including cholesterol and phytosterols, e.g., β-sitosterol (26).

3-Ketosteroid 9α-hydroxylase (KSH) is a key enzymatic step in the microbial steroid catabolic pathway, acting on 4-androstene-3,17-dione (AD) or 1,4-androstadiene-3,17-dione (ADD) (Fig. 1). Introduction of the 9α-hydroxyl moiety into the steroid polyheterocyclic ring structure, combined with steroid Δ¹-dehydrogenation by 3-ketosteroid Δ¹-dehydrogenase (KSTD), initiates steroid B-ring opening due to the formation of chemically unstable 9α-hydroxy-1,4-androstadiene-3,17-dione (9OHADD) (Fig. 1). KSH of *Rhodococcus erythropolis* SQ1 is a two-component class IA monooxygenase comprised of the terminal oxygenase KshA1 and the oxygenase reductase component KshB (23). Classification of nonheme oxygenases is based on the number of constituent components and the nature of their redox centers (2, 6, 8–10, 13). By definition, class I oxygenases are two-component enzymes composed of a reductase component and a terminal oxygenase component. The reductase component of oxygenases contains either a flavin mononucleotide (class IA) or a flavin adenine dinucleotide (class IB), an NAD binding domain, and a [2Fe-2S] redox center situated either N terminally (class IB) or C terminally (class IA).

In previous work we have shown that gene inactivation of either *kshA1* (mutant strain RG2) or *kshB* (mutant strain RG4) in parent strain *R. erythropolis* SQ1 results in loss of 3-ketosteroid 9α-hydroxylation activity (24). Consequently, these mutants were unable to grow on both AD and ADD as sole carbon and energy sources. Such KSH mutant strains have industrial applications and can be used for the selective Δ¹-dehydrogenation of pharmaceutically important steroids by biotransformation (28). Interestingly, growth of strain RG2 on cholesterol was comparable to that of parent strain SQ1. Biotransformation of cholesterol by cultures of strain RG2 did not result in accumulation of the expected steroid pathway intermediates (i.e., AD and ADD), indicative of the presence of at least one additional degradation pathway. By contrast, the inactivation of a single *kshA* gene by transposon mutagenesis in *Mycobacterium smegmatis* mc²155 was recently shown to cause accumulation of AD and ADD from β-sitosterol (1). We thus anticipated the presence of additional gene homologues of *kshA* in *R. erythropolis*. Recent studies have shown that rhodococci often contain multiple homologues of catabolic genes, thereby drastically improving their catabolic capabilities (26). The *Rhodococcus jostii* RHA1 genome sequence revealed the presence of up to four distinct pathways for steroid catabolism, each containing a *kshA* gene homologue (11, 27).

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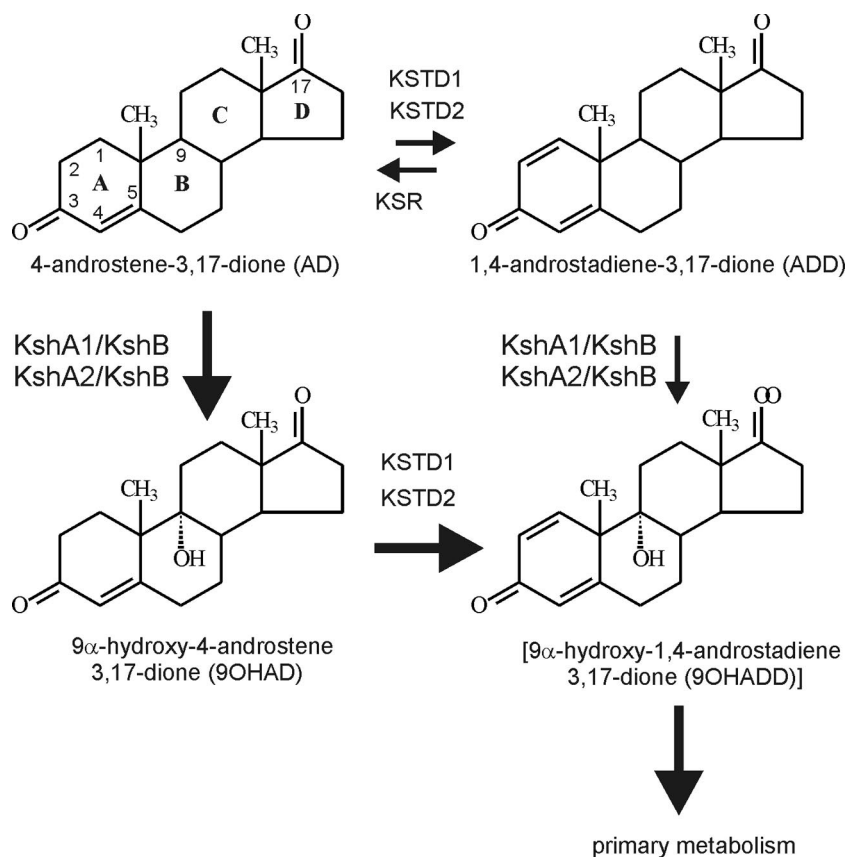
FIG. 1. Schematic representation of the key enzyme steps involved in the degradation of the steroid intermediates AD, ADD, 9OHAD, and [9OHADD] during steroid ring degradation in *R. erythropolis* strain SQ1. KSTD1 and KSTD2 represent KSTDs in SQ1 (25). KshA1, KshA2, and KshB represent two multicomponent KSHs of SQ1 (24; this study). KSR represents 3-ketosteroid Δ^1-reductase activity (see text). Thick arrows represent the proposed preferred route of steroid B-ring opening, aimed at keeping intracellular ADD concentrations low.

Currently, there is only very limited knowledge about KSH enzymes/genes, hampering industrial applications in the field of microbial steroid biotransformation. Studies of KSH enzymes furthermore are of medical importance. A gene cluster encoding the cholesterol catabolic pathway in *R. jostii* RHA1 was recently shown to be linked to the pathogenicity of *Mycobacterium tuberculosis* (27). *M. tuberculosis* is the causative agent of tuberculosis, causing millions of deaths every year. Notably, genes encoding enzymes involved in steroid ring opening, i.e., KSH, encoded by Rv3526, and KSTD, encoded by Rv3537, were identified as important for *M. tuberculosis* survival in macrophages (5, 17). The medical and applied aspects of KSH enzymes make them interesting enzymes to study in more detail.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Rhodococcus erythropolis* parent strain SQ1 (16) and mutant strains were cultivated at 30°C and 200 rpm. Mutant strains RG2 (*kshA1* mutant), RG4 (*kshB* mutant), and RG9 (*kshA1 kstD kstD2* mutant) were previously described (24). Complex rich medium (LBP) contained 1% (wt/vol) Bacto peptone (Difco), 0.5% (wt/vol) yeast extract (BBL Becton Dickinson and Company), and 1% (wt/vol) NaCl (21). Mineral medium (pH 7) consisted of 4.65 g/liter K$_2$HPO$_4$, 1.5 g/liter NaH$_2$PO$_4 \cdot$ H$_2$O, 3 g/liter NH$_4$Cl, 1 g/liter MgSO$_4 \cdot$ 7H$_2$O, and 1 ml/liter of fivefold-diluted Vishniac trace element solution (29). Cholesterol (1 g/liter) and cholic acid (1 g/liter) were added prior to autoclaving. Other steroids (0.5 g/liter) were solubilized in dimethyl sulfoxide (DMSO; 50 mg/ml) and added to autoclaved medium. The sucrose sensitivities of *Rhodococcus* strains were tested on LBP agar supplemented with 10% (wt/vol) sucrose. *Escherichia coli* strains were grown in Luria-Bertani (LB) broth at 37°C. Bacto agar (1.5% [wt/vol]; Difco) was added for growth on solid media.

PCR screening of genomic library using degenerate primers. PCR was performed in a reaction mixture (50 μl) consisting of Tris-HCl (10 mM, pH 8) buffer, polymerase buffer, deoxynucleoside triphosphate (0.2 mM), DMSO (2%), KshAType-F primer (10 ng/μl), KshAType-R primer (10 ng/μl), and *Taq* or Vent polymerase (2 U) under the following conditions: 5 min at 95°C and 30 cycles of 1 min at 95°C, 45 s at 60°C, and 1 min at 72°C, followed by 5 min at 72°C. The previously described genomic library of *R. erythropolis* RG1, a *kstD* mutant of parent strain SQ1, was used as template (24).

RNA isolation and RT-PCR experiments. *Rhodococcus* cultures were grown in LBP medium (250 ml) until an optical density at 600 nm of approximately 2 was reached. When appropriate, cultures were induced for 4 h with steroids (0.5 g/liter) dissolved in DMSO. Following centrifugation (10 min, 7,300 × *g*, 4°C), the cell pellet was crushed by pestle and mortar in liquid N$_2$ and stored at −80°C until use. Total RNA was subsequently isolated using the RNeasy isolation kit (Qiagen). Prior to reverse transcriptase-PCR (RT-PCR), the isolated total RNA was treated with a DNase I amplification-grade kit (Invitrogen, The Netherlands) for 15 min at room temperature. DNase I was heat inactivated for 10 min at 65°C. RT-PCR was performed with approximately 0.5 μg total RNA, determined on a GeneQuant spectrophotometer, using the SuperScript III Platinum One Step GRT-PCR system kit (Invitrogen, The Netherlands). All kits were used according to the manufacturer's recommendations. cDNA made by the reverse transcriptase reaction (15 min at 50°C) was heated for 2 min at 95°C and used for PCR (40 cycles of 30 s at 95°C, 30 s at 60°C, and 1.5 min at 72°C). Gene-specific primers used for the RT-PCR experiments are listed in Table 1.

Construction of mutagenic plasmid pKSH310 for *kshA2* unmarked gene deletion. Gene deletion of *kshA2* was achieved with pKSH310. A 1.2-kb SalI fragment of pAR1 (this study; see Results), representing the upstream region of

TABLE 1. PCR oligonucleotides used in this study

Primer	Specificity	Amplicon size	Nucleotide sequence (5′–3′)[a]
KshAType-F	*kshA* homologue	0.3 kb	TGYCCITTCCAYGAYTGGCGITGGGGIGG
KshAType-R	*kshA* homologue		TGIACGTAAAGAAGRTGIGCCATRTC
KshA2-F	*kshA2, kshA3*	0.6 kb	CTCGATGCCTACTGCCGTCAC
KshA2-R	*kshA2, kshA3*		CGGATAGTGGCAGTTGATCAAAAC
KSHA2EX-F	*kshA2*	1,167 bp	GGCCATATGTTGACCACAGACGTGACGACC (NdeI)
KSHA2EX-R			GCCACTAGTTCACTGCGCTGCTCCTGCACG (SpeI)
RT-KSHA2-F	*kshA2*	469 bp	AGCCAGAGCGAATTGAAGGT
RT-KSHA2-R			ATGTCGTCGCGGTTGATACC
RT-KSTD1-F	*kstD1*	489 bp	CCAGGTGCAGGAACGCGCCG
RT-KSTD1-R			CGCCTTGATTCGCTGGGTTT
16SRNA-RHODO-F	16S rRNA	427 bp	GTCTAATACCGGATATGACCTC
16SRNA-RHODO-R	*R. erythropolis* (4)		GCAAGCNAGNAGTTGAGCTGCTGGT
delKSHA2-R	Upstream *kshA2*	NA[b]	TCGAATACTGGGGACACGAAGC
BLF	*bla*	809 bp	GCGCATATGCACCCAGAAACGCTGGTGAAAG (NdeI)
BLR			GCGACTAGTTACCAATGCTTAATCAGTGAGGC (SpeI)

[a] I, inosine; Y, C+T; R, A+G; N, any base. Underlined nucleotide sequences indicate incorporated restriction sites.
[b] NA, not applicable.

kshA2, was cloned into XhoI-digested pBluescript (II) KS and named pKSH307. The PCR product (2.2 kb), obtained with primers KSHA2-F and delKSHA2-R (Table 1) using strain SQ1 genomic DNA, was cloned into EcoRV-digested pZeRO-2.1 vector (Invitrogen), yielding pKSH308. A 1-kb SalI/SpeI fragment of pKSH308, containing the downstream region of *kshA2*, was subsequently ligated into SalI/SpeI-digested pKSH307, thereby producing pKSH309. The two ligated fragments, harboring the *kshA2* deletion, were then taken from pKSH309 by Asp718I/XbaI digestion, treated with Klenow enzyme, and cloned into SmaI-digested pK18mobsacB vector (18), thereby producing pKSH310 (Fig. 2).

Construction of unmarked gene deletion mutants RG23, RG24, and RG27. Gene deletion mutants were constructed using the unmarked gene deletion method as described previously (23). The mutagenic plasmid pKSH310 was introduced into *R. erythropolis* SQ1 and RG2 by bacterial conjugation using *E. coli* S17-1 (19) to construct *kshA2* mutant strain RG23 and *kshA1 kshA2* mutant strain RG24. Mutagenic plasmid pKSH126 (23) was introduced into *R. erythropolis* RG4 to construct a *kshA1 kshB* mutant strain, RG27. Gene deletions were confirmed by PCR using gene-specific primers (Table 1).

Functional complementation experiments and construction of plasmids used. Electrocompetent *Rhodococcus* cells were prepared and transformed as previously described (22). *Rhodococcus* transformants were replica plated onto mineral agar medium containing AD or ADD (0.5 g/liter) as sole carbon and energy

source. Functional complementation for growth was scored after 3 days of incubation at 30°C. Plasmids pKSH312 and pKSH313 (Fig. 3), used for functional complementation experiments, were constructed as follows. The *kshA2* gene was amplified by PCR using *kshA2* primers KSHA2EX-F and KSHA2EX-R (Table 1) and subsequently cloned as an NdeI/SpeI fragment into NdeI/SpeI-digested pRESX (Fig. 3), resulting in plasmid pKSH312 (Fig. 3). Plasmid pKSH313, for simultaneous expression of *kshA2* and *kshB*, was constructed by ligating a 1.46-kb NsiI/SpeI fragment of pKSH207 (24) containing *kshB* into NsiI/SpeI-digested pKSH312.

Bioconversion experiments and analysis by gas chromatography and high-pressure liquid chromatography. Steroid biotransformations and steroid analysis were performed (in duplicate) in shaking flasks containing 100-ml LBP-grown cultures of *R. erythropolis* strains essentially as previously described (22).

***kshA2* promoter activity study and β-lactamase assay.** A leaderless *bla* gene (20) was amplified from pBluescript (II) KS by PCR using oligonucleotides BLF and BLR (Table 1). The obtained PCR product was digested with NdeI/SpeI and cloned into NdeI/SpeI-digested pRESX (Fig. 3). The leaderless *bla* gene, including rhodococcal ribosomal binding site sequence, was subsequently isolated from pRESX-BLAS following BstXI/SpeI digestion. The BstXI site was made blunt by T4 DNA polymerase treatment prior to SpeI digestion. The resulting DNA fragment was cloned under the control of the *kshA2* promoter into EcoRV/SpeI-
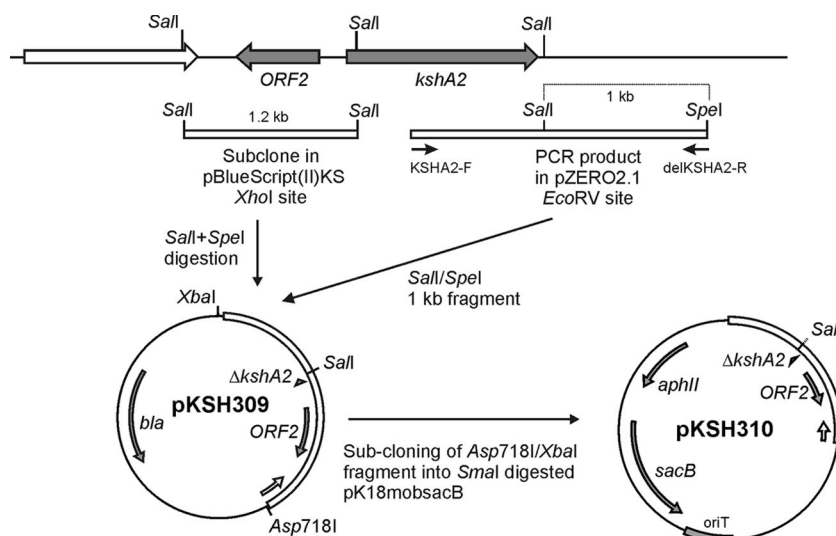

FIG. 2. Scheme of the genomic locus of *kshA2* of *R. erythropolis* SQ1 and the cloning strategy to construct mutagenic plasmid pKSH310 used for unmarked *kshA2* gene deletion in *kshA1* mutant strain *R. erythropolis* RG2. Upstream of *kshA2*, another open reading frame (*ORF2*) is found encoding a protein with signatures of IclR-type transcriptional regulators.
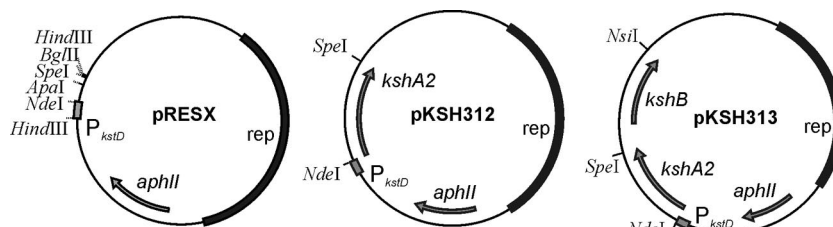
FIG. 3. Derivatives of *Rhodococcus* expression vector pRESX used in this study for expression of *kshA2* (pKSH312) and *kshA2* and *kshB* (pKSH313) in gene deletion mutants of *R. erythropolis* SQ1. The pRESX vector contains the *kstD1* promoter region providing constitutive gene expression in *R. erythropolis* (unpublished data).

digested pKSH307, encompassing the 1.2-kb genomic SalI fragment (Fig. 2). Finally, the *kshA2* promoter and truncated *bla* gene were cloned into pRESQ, resulting in pRESQ307BLAS used for the *kshA2* promoter activity measurement. *Rhodococcus* cells harboring pRESQ307BLAS were grown in 100 ml glucose (20 mM) mineral medium supplemented with kanamycin (200 μg/ml) until the optical density at 600 nm reached 1.5 to 2.5. Induction (5 h) was performed using steroids at a final concentration of 0.25 g/liter where appropriate. β-Lactamase activity was measured at 37°C in 100 mM potassium phosphate buffer, pH 7.0, using 0.5 mg/ml nitrocefin ($\varepsilon_{486} = 15.6 \times 10^3$ M^{-1} · cm^{-1}) (14).

Nucleotide sequence accession numbers. The *R. erythropolis* SQ1 sequence data for *kshA2*, *ORF2*, and *kshA3* have been submitted to the DDBJ/EMBL/GenBank databases under accession numbers EU656136 (*kshA2* and *ORF2*) and FJ040874 (*kshA3*).

RESULTS

Designing degenerate PCR primers for identification of additional *kshA* homologues in *kshA1* mutant *Rhodococcus erythropolis* strain RG2. Degenerate *kshA* PCR primers KshA-Type-F and KshAType-R (Fig. 2; Table 1) were developed to screen the *kshA1* gene deletion mutant strain RG2 for the presence of *kshA* gene homologues. The degenerate primers were designed on the conserved Rieske [2Fe-2S] domain (99 CPFHDWRWG 107 in KshA1) and the nonheme Fe(II) binding domain (191 DMAHFFYVH 199 in KshA1) using an alignment of amino acid sequences of KshA1 and putative actinomycetal orthologues identified by BLAST in databases (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi) (24). PCR experiments with these degenerate primers on total DNA of strain RG2 resulted in a PCR product of the expected size (0.3 kb). Nucleotide sequence analysis revealed that part of a *kshA* homologue had been cloned that was very similar, but not identical, to that of *kshA1*; thus, it was designated *kshA2*.

Cloning of *kshA2* from *Rhodococcus erythropolis* SQ1. A genomic library of *R. erythropolis* strain RG1 (24) was screened using the degenerate *kshA1* PCR primers, and a clone was isolated, designated pAR1, that contained the *kshA2* gene. Nucleotide sequencing of this clone revealed that the 3′-end part of the *kshA2* gene was not present. The genomic library was thus screened again by PCR using newly designed *kshA2* primers KshA2-F and KshA2-R (Table 1) for an additional clone containing the 3′ end of *kshA2*. Primers KshA2-F and KshA2-R were designed on the conserved Rieske [2Fe-2S] domain (LDAYCRH) and the conserved VLINCHYP motif, respectively. The nucleotide sequence of the complete *kshA2* gene (1,167 nucleotides) was determined from sequencing data of both *kshA2*-positive clones.

kshA2 (GC content, 58.2%) encodes a protein (KshA2) of 388 amino acids with a calculated molecular mass of 44 kDa. KshA2 displays highest overall amino acid sequence identity to Ro05811 of *R. jostii* RHA1 (11) (63%). High sequence identity was also found with KshA1 of *R. erythropolis* SQ1 (60%). The typical class IA terminal oxygenase Rieske type [2Fe-2S]$_R$ domain (C-X-H-X$_{16}$-C-X$_2$-H) and the mononuclear, nonheme Fe(II) binding motif (D/E-X3-D-X2-H-X4-H), previously identified in KshA1, were also fully conserved in KshA2.

Transcriptional analysis revealed that *kshA2* is steroid inducible. To verify that *kshA2* is involved in steroid catabolism in *R. erythropolis* strain SQ1, we performed transcriptional analysis of *kshA2* gene expression by RT-PCR. Significant amounts of *kshA2* RNA transcript were observed in cell cultures of parent strain SQ1 grown in complex medium (LBP) following AD induction (Fig. 4, lane 2). A very low level of *kshA2* RNA transcript was found in control cell cultures without steroid induction (Fig. 4, lane 1). Thus, *kshA2* is steroid inducible and most likely part of the steroid catabolic pathway. The *kshA1* mutant strain RG2 is unable to grow on AD, apparently failing to use KshA2. KshA2 induction thus may require further conversion of AD, which is possible in wild-type strain SQ1 but not in strain RG2.

***kshA2* encodes KSH activity and is under transcriptional control.** To determine whether *kshA2* can functionally complement the AD growth-negative phenotype of *kshA1* mutant strain RG2 and hence whether it encodes KSH activity, we forced expression of *kshA2* in strain RG2 by putting *kshA2* under the control of the promoter of *kstD* (22). The *kstD*
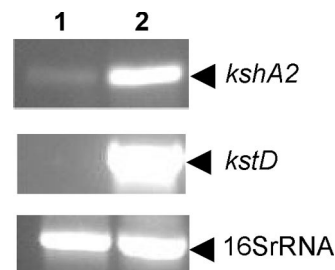

FIG. 4. Transcriptional analysis of *kshA2*, *kstD1* (positive control for AD induction), and 16S rRNA (control) by RT-PCR using total RNA isolated from noninduced cultures (lane 1) and AD-induced cultures of parent strain *R. erythropolis* SQ1 (lane 2). PCR primers used are RT-KSHA2-F and RT-KSHA2-R for *kshA2*, RT-KSTD1-F and RT-KSTD1-R for *kstD1*, and 16SRNA-RHODO-F and 16SRNA-RHODO-R for 16S rRNA (Table 1).
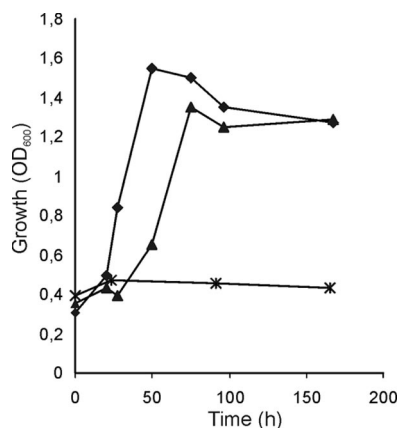
FIG. 5. Functional complementation of growth of *kshA1* mutant *R. erythropolis* RG2 by *kshA2* in AD mineral medium (average of duplicates). Growth curves represent parent strain (diamonds), *kshA1* mutant strain RG2 (crosses), and RG2 harboring *kshA2* on expression plasmid pKSH312 (Fig. 3) (triangles). OD_{600}, optical density at 600 nm.

FIG. 6. Promoter activity of *kshA2* following induction by AD, ADD, or 9OHAD in parent strain SQ (open bars) and *kshA1* mutant strain RG2 (solid bars), using leaderless β-lactamase as a reporter protein (14, 20).

promoter region, without its regulatory gene *kstR*, was cloned into pRESQ (24), yielding plasmid pRESX, and resulting in constitutive gene expression in *R. erythropolis* (Fig. 3) (unpublished data). The *kshA2* gene was cloned into pRESX under the control of the *kstD* promoter, and the resulting plasmid pKSH312 (Fig. 3) was introduced into mutant strain RG2 by electrotransformation. Functional complementation of the AD growth-negative phenotype of strain RG2 harboring pKSH312 was observed (Fig. 5), confirming that *kshA2* indeed is a *kshA1* homologue, encoding a second terminal oxygenase component (KshA2) with KSH activity in *R. erythropolis* strain SQ1. The results furthermore suggest that the *kshA2* promoter is under transcriptional control, since the *kshA2* gene's own promoter does not promote growth of RG2 on AD (Fig. 5) although the presence of AD induces *kshA2* expression in strain SQ1 (Fig. 4). Additionally, plasmid pKSH312 was introduced into the *kshA1 kstD1 kstD2* triple gene deletion mutant strain RG9, unable to form 9OHAD from AD (24). Introduction of a copy of the *kshA2* gene under the control of the *kstD1* promoter restored the capability of strain RG9 to produce 9OHAD during AD biotransformation, as determined by high-pressure liquid chromatography/UV analysis. Thus, KshA2 displays AD 9α-hydroxylase activity.

The *kshA2* promoter is induced by 9OHAD. Paradoxically, the *kshA1* mutant *R. erythropolis* strain RG2 cannot grow on AD as the sole carbon and energy source (24) despite the presence of a *kshA2* homologue, encoding an enzyme with AD 9α-hydroxylase activity. To determine whether the *kshA2* gene is actually transcribed in strain RG2, we checked *kshA2* promoter activity by fusing the *kshA2* promoter region to the *bla* gene, encoding β-lactamase activity. To the best of our knowledge, this is the first report on the use of *E. coli* β-lactamase as a convenient transcription reporter protein in *Rhodococcus*.

Noninduced cell cultures of parent strain SQ1 and *kshA1* mutant strain RG2 showed very low *kshA2* promoter activities (Fig. 6). Upon induction with AD, ADD, or 9OHAD, highly elevated levels of *kshA2* promoter activity were observed with strain SQ1. Cell cultur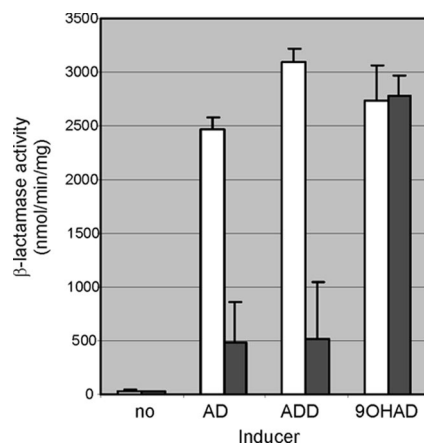es of RG2, however, showed a different induction pattern. The *kshA2* promoter activity in cell cultures of RG2 induced with 9OHAD was comparable to the levels found in parent strain SQ1 (Fig. 6), whereas RG2 cultures induced with AD or ADD showed greatly reduced (<20%) *kshA2* promoter activities compared to those of parent strain SQ1. These results indicate that neither AD nor ADD, but 9OHAD (or a metabolite thereof), is the actual inducer of *kshA2* expression. These observations also provide a plausible explanation for the AD/ADD growth-negative phenotype of the RG2 mutant. In strain RG2, *kshA2* expression is not sufficiently induced in the presence of AD to allow growth on this steroid substrate as a sole carbon and energy source.

Gene deletion of *kshA2* does not affect growth on 9OHAD, cholesterol, or cholic acid. Since *kshA2* is induced predominantly by 9OHAD, we considered the possibility that the AD 9α-hydroxylase activity observed for KshA2 is not its main enzymatic activity. Instead, KshA2 might have an essential function in the further degradation of 9OHAD. To test this hypothesis, *kshA2* gene deletion mutant strain RG23 and *kshA1 kshA2* double gene deletion mutant strain RG24 were constructed from parent SQ1 and *kshA1* mutant strain RG2, respectively. Growth of RG23 or RG24 in 9OHAD mineral medium was comparable to that of RG2 and parent strain SQ1, indicating that *kshA2* is not essential for further 9OHAD degradation. Also growth of RG23 and RG24 on alternative classes of steroid substrates, i.e., sterols (cholesterol) and bile acids (cholic acid), as sole carbon and energy sources was similar to that of the parent strain. *kshA2* therefore is not essential for the degradation of either of these steroid substrates. Growth of RG24 on cholic acid mineral medium was impaired compared to that of SQ1, but this was shown to be solely due to the inactivation of *kshA1*, since RG2 was impaired in growth on cholic acid to the same extent.

Functional complementation of RG2 by *kshA2* is dependent on the presence of *kshB*. KshA2 expectedly is part of a class IA two-component enzyme system, raising the question whether KshA2 could form an active enzyme complex with the previously identified KshB class IA oxygenase-reductase component (24) as part of a putative KshA2/KshB two-component en-

zyme. Alternatively, KshA2 may form a two-component enzyme system with a different, yet unknown, oxygenase-reductase component.

Functional complementation of growth of *kshA1* mutant RG2, *kshB* mutant RG4 (24), and *kshA1 kshB* double mutant RG27 on ADD mineral agar plates by the *kshA2* gene was therefore tested. Following the introduction of *kshA2* expression plasmid pKSH312 (Fig. 3) into these strains, functional complementation of growth on ADD was observed only with mutant strain RG2, harboring an intact copy of the *kshB* gene. KshA2 thus indeed is active in the presence of the KshB component, indicating that KshB can function as an oxygenase-reductase for both KshA1 and KshA2 (Fig. 1). The formation of an active KshA2/KshB two-component enzyme system was furthermore supported by the observed functional complementation of growth on ADD mineral agar medium of strain RG27 harboring pKSH313, a pRESX derivative carrying both *kshA2* and *kshB* (Fig. 3).

Identification of a third homologue, *kshA3*, in a *kshA1 kshA2* double mutant. In order to determine whether strain SQ1 contains more *kshA* homologues in addition to *kshA1* and *kshA2*, PCR was performed on total DNA of the *kshA1 kshA2* mutant strain RG24 using the degenerate *kshA* PCR primers (KshAType-F and KshAType-R, Table 1). Indeed, a PCR product of the expected size (0.3 kb) was obtained and nucleotide sequence analysis revealed that an additional *kshA* homologue had been amplified, designated *kshA3*. We additionally succeeded in obtaining a 0.6-kb PCR product of *kshA3* from RG24 using PCR primers originally developed for *kshA2* (KSHA2-F and KSHA2-R, Table 1). The nucleotide sequences of the 0.3-kb and 0.6-kb PCR products were 100% identical in the overlapping region, indicating that both PCR products originated from the same gene (*kshA3*). The deduced amino acid sequence of the partial KshA3 protein encoded by the 0.6-kb PCR product (199 amino acids) displayed the highest similarity with Ro04538 (89% identity over 199-amino-acid stretch) from *R. jostii* RHA1 (11). KshA3 is also highly similar to the same stretch of amino acids of KshA1 (69% identity) and KshA2 (66% identity). *R. erythropolis* strain SQ1 thus contains at least three *kshA* homologues.

DISCUSSION

Two enzymatic activities, i.e., KSTD and KSH, are involved in the opening of the steroid B-ring during microbial steroid degradation. In previous work, we have identified one KSH activity, comprised of KshA1 and KshB, and two KSTD activities, KSTD1 and KSTD2, in *R. erythropolis* SQ1 that are involved in the degradation of AD (22, 23, 24, 25). Here, we report the identification and characterization of a second terminal oxygenase component, KshA2, in *R. erythropolis* SQ1 as part of the AD catabolic pathway (Fig. 1).

The *kshA2* gene codes for a second terminal oxygenase component, KshA2, in *R. erythropolis* SQ1 with AD 9α-hydroxylase activity. In parent strain SQ1, expression of *kshA2* is induced by AD. However, analysis of induction of *kshA2* promoter activity, using β-lactamase as a convenient reporter protein, showed that 9OHAD serves as a much better inducer than does AD or ADD. Thus, 9OHAD, or a metabolite thereof, is the actual inducer of *kshA2* expression. The *kshA2* promoter

study also provided an explanation for the observation that *kshA1* mutant strain RG2 is unable to grow on AD or ADD, despite the presence of a functional *kshA2* gene. In RG2, *kshA2* is unable to take over the function of *kshA1*, because expression of *kshA2* is not sufficiently induced in the presence of AD or ADD and also because 9OHAD is not formed.

kshA2 clearly is under transcriptional control: *kshA2* episomally expressed under the control of a constitutive promoter (*kstD1*) yielded KSH activity that could restore the growth of the mutant strain RG2 on AD. Interestingly, *kshA2* is preceded by an open reading frame (*ORF2*) (Fig. 2), encoding a 238-amino-acid protein displaying similarity to IclR-type transcriptional regulators (ScanProsite, www.expasy.ch). The *ORF2*-encoded protein contains an IclR-type helix-turn-helix domain (PS51077) and an IclR-type effector domain (PS51078). The protein also displays similarity to the IclR-type regulator identified upstream of the *bphC1* gene in *Rhodococcus rhodochrous* K37 and KsdR, a putative DNA binding regulatory protein of *ksdD* encoding KSTD in *Arthrobacter simplex* (12, 21). The *bphC1* gene of *R. rhodochrous* K37 is induced by the steroid testosterone (21). Based on these similarities, *ORF2* might encode a transcriptional regulator for *kshA2* expression. Most IclR-type transcription regulators are repressors of specific catabolic genes in the absence of specific substrates, while excess of a specific effector triggers derepression (Prosite documentation PDOC00807, www.expasy.ch). If the *ORF2*-encoded protein indeed acts as a repressor of *kshA2* expression, inactivation of *ORF2* should restore growth of RG2 on AD. However, when we performed a gene deletion of *ORF2* in *R. erythropolis* strain RG2, growth of the resulting double mutant on AD was not restored (data not shown). Thus, the *ORF2*-encoded protein most likely is not a repressor of *kshA2* expression.

The terminal oxygenase KshA2 activity is observed only in the presence of KshB, suggesting that KshA2/KshB constitute a second KSH enzyme activity (KSH2), in addition to the KSH activity of KshA1/KshB in *R. erythropolis* SQ1. KshB has been classified as a class IA oxygenase-reductase (24). Therefore, we can classify KshA2 as a class IA terminal oxygenase. Strain SQ1 most likely contains only one oxygenase-reductase, KshB, supplying reducing equivalents to multiple terminal oxygenases. This is in agreement with the observed phenotype of *kshB* mutant RG4, which is fully blocked in AD and cholesterol degradation (24), likely due to a lack of supply of reducing equivalents toward KshA1, KshA2, and additional homologues, e.g., KshA3.

Inactivation of *kshA2* did not result in blocked growth on 9OHAD, cholic acid, or cholesterol. The *kshA2* gene thus does not have an essential role in the degradation of either of these different classes of steroids. Speculatively, KshA2 might play a role in limiting the metabolic flux through ADD during AD catabolism. Keeping the intracellular levels of ADD low by regulating KSTD and KSH activities would be advantageous to the cell, since ADD is known to have toxic effects (15). Interestingly, ADD biotransformation experiments with *R. erythropolis* cell cultures generally result in a temporal accumulation of AD (data not shown), indicating the presence of 3-ketosteroid Δ^1-reductase activity (KSR, Fig. 1). Δ^1-Reductase activity has also been observed in *Mycobacterium* sp. strain NRRL B-3805, and the activity increased significantly upon induction

of cultures by ADD (7). The above-mentioned data suggest a preference for steroid catabolism via 9α-hydroxylated rather than Δ^1-desaturated steroid pathway intermediates. Degradation of AD via the 9OHAD intermediate and not via the moderately toxic intermediate ADD would be beneficial to the cell, necessitating the activation of an additional KSH activity, i.e., KshA2 (Fig. 1).

A third *kshA* gene (*kshA3*) was successfully identified by PCR in *kshA1 kshA2* mutant RG24 by using the developed degenerate primers for *kshA1*, indicating that these are suitable PCR primers for the amplification of *kshA* gene homologues. These primers can thus be used to screen for, and clone, additional *kshA* genes from other interesting (actino)bacteria. The KshA3 protein sequence displayed highest identity with Ro04538 from *R. jostii* RHA1. Interestingly, the ro04538 gene is part of the cholesterol catabolic gene cluster recently identified in *R. jostii* RHA1 (27), suggesting that *kshA3* encodes the KSH oxygenase component involved in cholesterol catabolism in *R. erythropolis* SQ1.

ACKNOWLEDGMENTS

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