Inactivation and Augmentation of the Primary 3-Ketosteroid- Δ^1 -Dehydrogenase in *Mycobacterium neoaurum* NwIB-01: Biotransformation of Soybean Phytosterols to 4-Androstene-3,17-Dione or 1,4-Androstadiene-3,17-Dione^{∇†}

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3-Ketosteroid- Δ^1 -dehydrogenase, KsdD_M, was identified by targeted gene disruption and augmentation from *Mycobacterium neoaurum* NwIB-01, a newly isolated strain. The difficulty of separating 4-androstene-3,17-dione (AD) from 1,4-androstadiene-3,17-dione (ADD) is a key bottleneck to the microbial transformation of phytosterols in industry. This problem was tackled via genetic manipulation of the KsdD-encoding gene. Mutants in which KsdD_M was inactivated or augmented proved to be good AD(D)-producing strains.

Steroid medications are widely used in clinical applications and are important in the pharmaceutical industry (5, 8). Among the steroidal derivatives, 4-androstene-3,17-dione (AD) and 1,4-androstadiene-3,17-dione (ADD) are two major products (3, 4). For industrial applications, microbial strains that can produce these chemicals are needed (9). To improve biotransformation, bacteria have been subjected to mutagenesis by UV/chemical treatments (10, 17, 18, 19, 21) and metabolic engineering (23, 24, 25, 27). However, most of the resulting strains produce AD and ADD simultaneously, which impedes their utility (11, 13).

Genes and pathways involved in phytosterol degradation have been identified in *Rhodococcus* and *Mycobacterium* species (1, 2, 6, 23, 24, 26). The enzyme that transforms AD to ADD is 3-ketosteroid- Δ^1 -dehydrogenase (KsdD) (4, 21) (Fig. 1). In this study, we identified the main gene encoding this enzyme in *Mycobacterium neoaurum* NwIB-01 (*ksdD_M*) and manipulated it to develop AD(D)-producing strains. This is the first successful use of gene augmentation to increase production of ADD.

Cloning of genes involved in phytosterol degradation by a new bacterial isolate. Using soybean phytosterols as the sole carbon source, strain NwIB-01 was isolated from steroid-contaminated soil samples and identified as *Mycobacterium neoaurum* (strain named *Mycobacterium neoaurum* NwIB-01) by 16S rRNA gene phylogenetic analysis and on the basis of its morphology. When cultured in fermentation medium containing 15 g/liter phytosterols for 96 h, NwIB-01 could produce

4.23 g/liter ADD and 1.76 g/liter AD simultaneously, showing it was a promising strain.

To obtain the genes involved in phytosterol degradation, the genomic fosmid library was built by using plasmid pCC2FOS, and fragments of the steroid degradation gene cluster, which is about 100 kb long and about 71% identical to the reported gene cluster in *Mycobacterium tuberculosis* (26), were selected using degenerate primers (7, 12, 16) (Table 1). The degenerate primers KsdD-SG-F/KsdD-SG-R, KshA-SG-F/KshA-SG-R, and KshB-SG-F/KshB-SG-R were used to amplify KsdD gene, 3-ketosteroid 9 α -hydroxylase (KshA) gene, and KshB gene conserved sequences, respectively.

The *ksdD_M* gene product serves as the enzyme for the steroid-1-dehydrogenation activity. To identify the *in vivo* function of the *ksdD_M* products, we disrupted *ksdD_M* by double-crossover homologous recombination in NwIB-01 (15) (see Fig. S2 in the supplemental material). Inactivation of *ksdD_M* (to create the NwIB-02 mutant) by the electrotransformation of suicide delivery vectors (pMN4) led to a significant change in the AD/ADD product ratio (see Fig. 3b).

The open reading frame (ORF) of *ksdD_M* (1,701 bp) uses GTG as the start codon with a possible ribosomal binding site (GAAAGG) 16 nucleotides upstream. Homology analysis revealed that KsdD_M is 88% identical to the protein MSMEG5941 in *Mycobacterium smegmatis* (the highest homology found by comparing with GenBank). An unrooted phylogenetic tree was constructed using the PHYLIP program package (22) (Fig. 2b), and the putative N-terminal flavin adenine dinucleotide (FAD)-binding motif in KsdD_M was consistent with the sequence as previously described, GSG(A/G)(A/G)(A/G)X17E (24) (Fig. 2a).

Conversion of AD to ADD *in vitro*. The complete *ksdD_M* gene was amplified by PCR with *ksdD_M*-F/*ksdD_M*-R primers. After digestion by NcoI/HindIII, the *ksdD_M* gene was recombined and connected with the pET-22b(+) vector. After Ni-nitrilotriacetic acid purification, KsdD_M (His₆-tagged protein) activities were measured in triplicate spectrophotometrically at 30°C (25). The reaction mixture consisted of 50 mM

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FIG. 1. Microbial transformation pathway of phytosterols to AD(D).

Tris-HCl, pH 7.0, 1.5 mM phenazine methosulfate (PMS), 40 μ M DCPIP (2,6-dichlorophenolindophenol), cell extract, and 200 mM AD in 2% methanol. Activities were expressed as mean values in mU (mg protein)⁻¹; 1 mU is defined as the reduction of 1 nM DCPIP min⁻¹ ($\epsilon_{600} = 11.3 \times 10^3 \text{ cm}^{-1} \text{ M}^{-1}$).

Our results indicated that by low-temperature induction (22°C), the recombinant protein appeared partly as the soluble protein, especially after 24 h (see Fig. S3 in the supplemental material). The KsdD_M maximum activity of the intracellular soluble parts of KsdD_M was 0.8 U/mg.

Construction of KsdD overexpression mutants. To overexpress *ksdD_M* in *Mycobacterium neoaurum*, mycobacterial replicating vector pMV261 carrying the *Mycobacterium bovis* BCG Phsp60 promoter was used (20). The KsdD_M gene was first amplified by PCR with AG-*ksdD_M*-F/AG-*ksdD_M*-R primers and cloned into the BamHI/HindIII sites of pMV261 (14). Augmentation of *ksdD_M* (to create the NwIB-04 mutant) by the electrotransformation of shuttle plasmid pMV261-*ksdD_M* led to a significant increase of ADD in the product ratio.

TABLE 1. PCR primers used in this study

Primer	Specificity	Amplicon size (kb)	Nucleotide sequence (5'-3') ^a
KsdD-SG-F	<i>ksdD</i>	0.7	GTGTTCTACATGACTGMYC
KsdD-SG-R	<i>ksdD</i>	0.7	TGCGGATYCCGCCCTTG
KshA-SG-F	<i>kshA</i>	0.5	TGCCSTTCCACRACTG
KshA-SG-R	<i>kshA</i>	0.5	CAGYAGCGGRTTGTTCGAT
KshB-SG-F	<i>kshB</i>	0.6	CTTGCCCTGATGCTCGACGT
KshB-SG-R	<i>kshB</i>	0.6	TGYCGSSSGGAANCCGGTC
<i>ksdD_M</i> -F	<i>ksdD_M</i>	1.7	GCCCCATGGTGTCTACATGACTGCCAGGACTA
<i>ksdD_M</i> -R	<i>ksdD_M</i>	1.7	CCGAAGCTTTTCAGGCCTTTCCAGCGAGATGCAAC
QC- <i>ksdD_M</i> -UF	<i>ksdD_M</i> upstream region	1.2	CGCCTGCAGTCCGCCGATTCAAAATGATGATC
QC- <i>ksdD_M</i> -UR	<i>ksdD_M</i> upstream region	1.2	CCGAAGCTTTGGGCAGTCATGTAGAACACGTTATAG
QC- <i>ksdD_M</i> -DF	<i>ksdD_M</i> downstream region	0.8	CCGAAGCTTGACCTGGGCACCAAGGGCGGTATT
QC- <i>ksdD_M</i> -DR	<i>ksdD_M</i> downstream region	0.8	CGCGGATCCCCACCCCGAGAGCACCACGGTGTT
AG- <i>ksdD_M</i> -F	<i>ksdD_M</i>	1.7	CGGGGATCCGTGTTCTACATGACTGCCAGGACTA
AG- <i>ksdD_M</i> -R	<i>ksdD_M</i>	1.7	GGCAAGCTTTTCAGGCCTTTCCAGCGAGATGCAAC

^a Ambiguity codes: S, C or G; Y, C or T; M, A or C; R, A or G; N, A or T or G or C.

FIG. 2. Conserved sequence and phylogenetic tree analysis. (a) N-terminal FAD-binding motif of the putative KsdD. (b) Phylogenetic tree of KsdD proteins from *Mycobacterium* and *Rhodococcus* species.

Characterization of the producing properties of KsdD mutants. The mutants were first cultured in shaker flasks with 0.1 g/liter soybean phytosterols. Thin-layer chromatography (TLC) analysis revealed that NwIB-02 accumulated AD as the main product, while NwIB-04 accumulated ADD. Based

on band intensity from TLC analysis, the AD/ADD molar yields with NwIB-02 were about 10:1 and 1:2 with wild-type NwIB-01 (Fig. 3b). These results revealed that steroid-1-dehydrogenation activity was weakened by the KsdD_M gene knockout and enhanced by KsdD_M gene augmentation.

FIG. 3. Electrophoretic analysis of the mutants and TLC analysis of transformation products. (a) Identification of recombinant plasmids. The length of *ksdD_M* is 1,701 bp. The length of DNA fragment, including the *ksdD_M* upstream and downstream, is 4,031 bp in the wild-type strain and 2,525 bp in NwIB-02. The length of vector pMV261 is 4,492 bp. (b) TLC analysis of transformation products.

FIG. 4. AD(D) accumulation from soybean phytosterols. (a) HPLC analysis of product composition with 96-h-old culture. Peaks: I, wild-type NwIB-01; II, strain NwIB-02; III, strain NwIB-04. (b) Time course of phytosterol transformation to AD(D) by NwIB-01 and NwIB-04. The AD accumulation is indicated by open circles for NwIB-01 and filled circles for NwIB-04. The ADD accumulation is indicated by open squares for NwIB-01 and filled squares for NwIB-04.

To further quantify the producing properties, the scaleup from flask to fermentor was performed. A 3.7-liter bioreactor (KLF2000; Bioengineering, Switzerland) was used during the batch stage. All fermentation experiments were carried out at 30°C and 300 rpm with airflow at 0.5 volumes of air per unit of medium per minute (vvm). After 24 h, soybean phytosterols (15 g/liter in 0.5% [vol/vol] Tween 80) were added to the medium and the bioconversion was monitored for several days. The production composition was quantified by high-pressure liquid chromatography (HPLC) with an Agilent XDB-C₁₈ column (HPLC conditions: column temperature, 40°C; mobile phase, methanol-water [8.0:2.0]; flow rate, 1 ml/min.) (Fig. 4). For NwIB-01, the accumulation of AD(D) reached the maximum in 96 h after phytosterols (ADD, 4.23 g/liter; AD, 1.76 g/liter) were added. For strain NwIB-04, ADD reached the maximum 4.94 g/liter with only 0.096 g/liter AD (Table 2). The molar ratio of AD to ADD in products of strain NwIB-01 was 1:2.4, while it reached 11.5:1 in mutant NwIB-02 (data not shown in this paper) and 1:51.5 in mutant NwIB-04. Trace amounts of 9-OH-AD and substrate were also observed in our research and can be negligible for low 9-OH-AD and substrate contents. The final ADD purity was more than 95% after optimal fermentation conditions with NwIB-04. These results demonstrate that strain NwIB-04 is a successful ADD-producing strain.

In conclusion, the results presented above reveal that KsdD_M, first obtained from *M. neoaurum* NwIB-01, serves as the main KsdD for steroid metabolism. This study provides a

feasible way to achieve excellent phytosterol-transforming strains with high purity. KsdD_M in *M. neoaurum* NwIB-01 could be rationally modified by the method of metabolic engineering to achieve improved strains with high AD(D) production purity, which implies that other *Mycobacterium* strains transforming phytosterols to AD(D) could be improved by the same methods.

Nucleotide sequence accession numbers. The GenBank accession numbers for *M. neoaurum* NwIB-01 *ksdD_M*, *kshA*, and *kshB* identified in this study are GQ228843.1, GQ476982.1, and GU363925, respectively.

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REFERENCES

- Brzostek, A., B. Dziadek, A. Rumijowska-Galewicz, J. Pawelczyk, and J. Dziadek. 2007. Cholesterol oxidase is required for virulence of *Mycobacterium tuberculosis*. *FEMS Microbiol. Lett.* **275**:106–112.
- Brzostek, A., T. Sliwinski, A. Rumijowska-Galewicz, M. Korycka-Machala, and J. Dziadek. 2005. Identification and targeted disruption of the gene encoding the main 3-ketosteroid dehydrogenase in *Mycobacterium smegmatis*. *J. Gen. Microbiol.* **151**:2393–2402.
- Choi, K. P., I. Molnár, M. Yamashita, and Y. Murooka. 1995. Purification and characterization of the 3-ketosteroid-Δ¹-dehydrogenase of *Arthrobacter simplex* produced in *Streptomyces lividans*. *J. Biochem.* **117**:1043–1049.
- Donova, M. V. 2007. Transformation of steroids by actinobacteria: a review. *Appl. Biochem. Microbiol.* **43**:1–14.
- Fernandes, P., and J. M. S. Cabral. 2007. Phytosterols: applications and recovery methods. *Bioresour. Technol.* **98**:2335–2350.
- Horinouchi, M., T. Hayashi, T. Yamamoto, and T. Kudo. 2003. A new bacterial steroid degradation gene cluster in *Comamonas testosteroni* TA441 which consists of aromatic-compound degradation genes for seco-steroids and 3-ketosteroid dehydrogenase genes. *Appl. Environ. Microbiol.* **69**:4421–4430.
- Kim, B. Chul, J. M. Lee, J. S. Ahn, and B. S. Kim. 2007. Cloning, sequencing, and characterization of the pradimicin biosynthetic gene cluster of *Actinomyces hirsutus* P157-2. *J. Microbiol. Biotechnol.* **17**:830–839.
- Knol, J., K. Bodewits, G. I. Hessels, L. Dijkhuizen, and R. Van der Geize. 2008. 3-Keto-5α-steroid Δ¹-dehydrogenase from *Rhodococcus erythropolis* SQ1 and its orthologue in *Mycobacterium tuberculosis* H37Rv are highly

TABLE 2. AD(D) production with different strains^a

Strain	AD (maximum g/liter)	ADD (maximum g/liter)	AD/ADD	Molar yield (%)
Wild-type NwIB-01	1.76	4.23	1:2.4	57.8
<i>ksdD_M</i> gene augmentation NwIB-04	0.096	4.94	1:51.5	48.7

^a Tests were done in 3.7-liter bioreactors with 15 g/liter soybean phytosterols (96 h after addition of substrate).

- specific enzymes that function in cholesterol catabolism. *Biochem. J.* **410**: 339–346.
9. Malaviya, A., and J. Gomes. 2008. Androstenedione production by biotransformation of phytosterols. *Bioresour. Technol.* **99**:6725–6737.
 10. Marsheck, W. J., S. Kraychy, and R. D. Muir. 1972. Microbial degradation of sterols. *Appl. Environ. Microbiol.* **23**:72–77.
 11. Martin, C. K. A. 1977. Microbial cleavage of sterol side chains. *Adv. Appl. Microbiol.* **22**:29–58.
 12. Meyer, J. D. F., W. Deleu, J. Garcia-Mas, and M. J. Havey. 2008. Construction of a fosmid library of cucumber (*Cucumis sativus*) and comparative analyses of the eIF4E and eIF(iso)4E regions from cucumber and melon (*Cucumis melo*). *Mol. Genet. Genomics* **279**:473–480.
 13. Molchanova, M. A., V. A. Andryushina, T. S. Savinova, T. S. Stytsenko, N. V. Rodina, and N. E. Voishvillo. 2007. Preparation of androsta-1,4-diene-3,17-dione from sterols using *Mycobacterium neoaurum* VKPM Ac-1656 strain. *Russ. J. Bioorganic Chem.* **33**:354–358.
 14. Papavinasundaram, K. G., M. J. Colston, and E. O. Davis. 1998. Construction and complementation of a recA deletion mutant of *Mycobacterium smegmatis* reveals that the intein in *Mycobacterium tuberculosis* recA does not affect RecA function. *Mol. Microbiol.* **30**:525–534.
 15. Parish, T., and N. G. Stoker. 2000. Use of a flexible cassette method to generate a double unmarked *Mycobacterium tuberculosis* tlyA plcABC mutant by gene replacement. *J. Gen. Microbiol.* **146**:1969–1975.
 16. Rezwan, M., M. A. Lanéelle, P. Sander, and M. Daffé. 2007. Breaking down the wall: fractionation of mycobacteria. *J. Microbiol. Methods* **68**:32–39.
 17. Rodina, N. V., M. A. Molchanova, N. E. Voishvillo, V. A. Andryushina, and T. S. Stytsenko. 2008. Conversion of phytosterols into androstenedione by *Mycobacterium neoaurum*. *Appl. Biochem. Microbiol.* **44**:48–54.
 18. Rumijowska, A., K. Lisowska, A. Ziolkowski, and L. Sedlaczek. 1997. Transformation of sterols by *Mycobacterium vaccae*: effect of lecithin on the permeability of cell envelopes to sterols. *World J. Microbiol. Biotechnol.* **13**: 89–95.
 19. Spassov, G., V. Pramatarova, R. Vlahov, and G. Reinhold. 1993. Bioconversion of sitosterol to androstene-dione by mutants of *Mycobacterium vaccae*. *Dokl. Akad. Nauk.* **46**:123–126.
 20. Stover, C. K., V. F. Cruz, T. R. Fuerst, J. E. Burlein, L. A. Benson, L. T. Bennett, G. P. Bansal, J. F. Young, M. H. Lee, G. F. Hatfull, S. B. Snapper, R. G. Barletta, W. R. Jacobs, and B. R. Bloom. 1991. New use of BCG for recombinant vaccines. *Nature* **351**:456–460.
 21. Szentirmai, A. 1990. Microbial physiology of sidechain degradation of sterols. *J. Ind. Microbiol.* **6**:101–116.
 22. Taylor, M. J., and B. A. Peculis. 2008. Evolutionary conservation supports ancient origin for Nudt16, a nuclear-localized, RNA-binding, RNA-decapping enzyme. *Nucleic Acids Res.* **27**:1–14.
 23. Van der Geize, R., G. I. Hessels, and L. Dijkhuizen. 2002. Molecular and functional characterization of the kstD2 gene of *Rhodococcus erythropolis* SQ1 encoding a second 3-ketosteroid Δ^1 -dehydrogenase isoenzyme. *J. Gen. Microbiol.* **148**:3285–3292.
 24. Van der Geize, R., G. I. Hessels, R. Van Gerwen, J. W. Vrijbloed, P. Van der Meijden, and L. Dijkhuizen. 2000. Targeted disruption of the kstD gene encoding 3-ketosteroid Δ^1 -dehydrogenase isoenzyme of *Rhodococcus erythropolis* SQ1. *Appl. Environ. Microbiol.* **66**:2029–2036.
 25. Van der Geize, R., G. I. Hessels, R. Van Gerwen, P. Van der Meijden, and L. Dijkhuizen. 2001. Unmarked gene deletion mutagenesis of kstD, encoding 3-ketosteroid Δ^1 -dehydrogenase, in *Rhodococcus erythropolis* SQ1 using sacB as counter-selectable marker. *FEMS Microbiol. Lett.* **18**:197–202.
 26. Van der Geize, R., K. Yam, T. Heuser, M. H. Wilbrink, H. Hara, M. C. Anderton, E. Sim, L. Dijkhuizen, J. E. Davies, W. W. Mohn, and L. D. Eltis. 2007. A gene cluster encoding cholesterol catabolism in a soil actinomycete provides insight into *Mycobacterium tuberculosis* survival in macrophages. *Proc. Natl. Acad. Sci. U. S. A.* **104**:1947–1952.
 27. Van der Geize, R., P. Van der Meijden, G. I. Hessels, and L. Dijkhuizen. July 2003. Identification of 3-ketosteroid 9 α -hydroxylase genes and microorganisms blocked in 3-ketosteroid 9 α -hydroxylase activity. Patent WO 03/070925, Akzo Nobel N.V. (NL).