

A New Steroid-Transforming Strain of *Mycobacterium neoaurum* and Cloning of 3-Ketosteroid 9 α -Hydroxylase in NwIB-01

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Abstract Using enrichment procedures, five strains that can utilize soybean phytosterols as the sole carbon source were isolated from steroids-contaminated soil samples. Among the isolated strains, the strain NwIB-01 with the highest steroid degradation ability was identified as *Mycobacterium neoaurum* by morphological, physiological, biochemical tests and 16S rRNA sequence analysis. Meanwhile, the key enzyme gene, which was involved in steroid metabolism and encoding 395-amino acid 3-ketosteroid 9 α -hydroxylase (KSH), was obtained from *M. neoaurum* NwIB-01 with the assistance of homology analysis and chromosome walking. To our best knowledge, this is the first report to the gene of key enzyme KSH from *M. neoaurum*. Strain NwIB-01 exhibited powerful ability of cleaving the side chain specifically from soybean phytosterols to accumulate 4-androstene-3,17-dione (AD) and 1,4-androstadiene-3,17-dione (ADD). It was showed that when cultured in 15 g/l phytosterols, the yield of ADD reached 4.23 g/l while accompanied by 1.76 g/l AD in 96-h-old culture (the molar yield of AD+ADD is 64.7%). The strain NwIB-01 can be applied as excellent phytosterols-transformation strains in potential industrial applications.

Keywords *Mycobacterium neoaurum* · Extraction chromatography · Screening · Phytosterols · 4-Androstene-3,17-dione (AD) · 1,4-Androstadiene-3,17-dione (ADD) · 3-Ketosteroid 9 α -hydroxylase (KSH)

Introduction

Steroid drugs play an important role in the pharmaceutical industry and have been widely used in clinical applications. Among the steroid drug intermediates, 4-androstene-3,17-dione (AD) and 1,4-androstadiene-3,17-dione (ADD) are the two major products for their

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essential value for production of various steroid medications, such as sex hormones, adrenal cortical hormones, and other steroids [1]. In contrast to the chemical synthesis, biotransformation provides an alternative method in the production of steroid intermediates and has been extensively used as a common and economical process in the pharmaceutical industry nowadays [1].

To develop an effective microbial transformation of phytosterols to AD(D) for industrial application, screening, isolation, and improvement of typical strains are the most important preconditions. So far, many authors have focused their efforts on isolating and screening of microorganisms for steroid transformation, and many microorganisms capable of producing AD(D) have been screened from the environment or isolated from the microbiologic mutation tests. *Arthrobacter simplex*, a gram-positive bacterium, has been used for 1-dehydrogenation because of its 3-ketosteroid-1-dehydrogenase activity since the 1960s [1]. Then, many other microorganisms that could utilize steroid as the sole carbon source were obtained. Dogra and Qazi reported that *Micrococcus roseus* RJ6 isolated from soil could degrade cholesterol and accumulate AD and ADD [2]. Later, they reported that another strain, *M. roseus*, isolated from animal feces, had a 5% enhanced ability to cholesterol degradation [3]. *Bacillus sphaericus*, which could accumulate AD by side chain cleavage of progesterone, was described by Wadhwa and Smith in 2000 [4]. Other strains such as *Nectria haematococca* [5] and *Mycobacterium neoaurum* [6] were also reported for the steroid degradation and AD(D) production.

In the process of steroid transformation, two key enzymes, 3-ketosteroid Δ^1 -dehydrogenase (KsdD) and 3-ketosteroid 9 α -hydroxylase (KSH), are essential for the production and accumulation of AD and ADD [7] (Fig. 1). Among the key enzymes, KSH catalyze the conversion of AD(D) into 9-hydroxy-AD(D). This gene is very important for strain improvement by genetic manipulation [8–10]. A number of mutants with KsdD or/and KSH inactivated by UV irradiation/chemical mutagens treatment or by genetic engineering could produce metabolic intermediates which are important in the pharmaceutical industry [8–10].

In this paper, we successfully isolated an actinomycete strain from soil, which could utilize soybean phytosterols and accumulated AD and ADD. The strain was identified as *M. neoaurum* by 16S rRNA and named *M. neoaurum* NwIB-01. Further, a KSH gene in *M. neoaurum* NwIB-01 was obtained by nested-polymerase chain reaction (PCR) and chromosome walking. The selected strain and the sequence of KSH gene from NwIB-01 provide a feasible way to perform strain improvement for potential industrial applications.

Materials and Methods

Strains, Culture Method, and Chemicals

Soil samples were collected from the soil near the sewage outfall in Zhejiang Xianju Pharmaceutical Co., Ltd, China, and Tianjin JINJIN biochemistry Co., Ltd, China. The bacteria used in this study were isolated from steroid-contaminated soil samples. Among the isolated bacteria, NwIB-01 was selected as the representative model to be studied extensively. The *M. neoaurum* ATCC 25795 was obtained from American Type Culture Collection (ATCC).

Enrichment culture medium contains (gram per liter): $K_2HPO_4 \cdot 3H_2O$, 1.0; $Na_2HPO_4 \cdot 3H_2O$, 1.0; $(NH_4)_2HPO_4$, 2.0; $NaNO_3$, 2.0; Tween 80, 0.3; soybean phytosterols, 10.0 (adjusted to pH 7.5). The seed medium contains (gram per liter): $K_2HPO_4 \cdot 3H_2O$, 0.5; citric

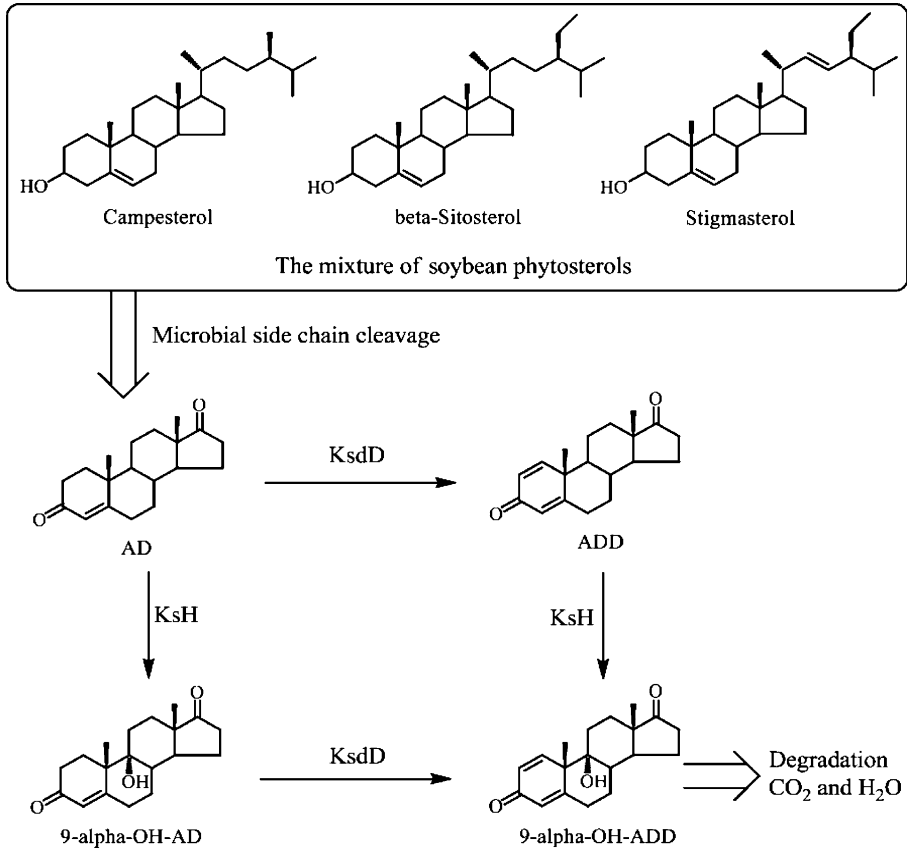


Fig. 1 Proposed pathway for phytosterol degradation. *KSH* 3-ketosteroid 9 α -hydroxylase, *KsdD* 3-ketosteroid Δ^1 -dehydrogenase, *AD* 4-androstene-3,17-dione, *ADD* 1,4-androstene-3,17-dione, *9-OH-AD* 9 α -hydroxy-4-androstene-3,17-dione, *9-OH-ADD* 9 α -hydroxy-1,4-androstadiene-3,17-dione

acid, 2; ferric ammonium citrate, 0.05; glycerol, 20; MgSO₄·7H₂O, 0.5; NH₄NO₃, 2 (adjusted to pH 7.5). Fermentation medium contains (gram per liter): glucose, 10; K₂HPO₄·3H₂O, 0.5; citric acid, 2; ferric ammonium citrate, 0.05; MgSO₄·7H₂O, 0.5; (NH₄)₂HPO₄, 3.5; Tween80, 3 (adjusted to pH 7.5).

AD and ADD were obtained from Sigma (USA). 9 α -Hydroxy-4-androstene-3,17-dione (9-OH-AD) was obtained from Pfizer Pharmaceuticals Ltd. Phytosterol substrate used was a sterol mixture composed of 47.5% β -sitosterol, 26.4% campesterol, 17.6% stigmasterol, 3.6% brassicasterol, and 4.9% of a mixture of saturated sterols (Zhejiang DAVI biochemistry Co., Ltd, China). LA *Taq* polymerase and restriction and modification enzymes were purchased from Takara (Japan).

Enrichment and Screening

In this work, enrichment culture technique was applied. Soybean phytosterols as the sole carbon and energy source in medium were used to isolate potential bacterial strains [11, 12]. One gram of soil sample was added to a flask with 100 ml sterile distilled water. After

activation on a rotary shaker for 40 min, 0.1-ml activation culture broth was withdrawn and inoculated into 100-ml enriched medium in a flask, which then was shaken at 120 rpm, 30 °C for 48 h. After several rounds of enrichment, inocula was serially diluted and plated onto solid enrichment medium, which contained 10.0 g/l soybean phytosterols as the sole carbon source. After 48 h incubation at 30 °C, the growing microorganisms were isolated, purified, and transferred to maintenance slants.

Identification and Taxonomical Studies

The gene of 16S rDNA was amplified and sequenced using primers universal for the majority of prokaryotes (forward primer 5'-CCTACGGGAGGCAGCAG-3', reverse primer 5'-ACGGGCGGTGTGTAC-3'). The genomic DNA isolated from *M. neoaurum* was used as the template [13]. The PCR amplification was enforced with thermostable polymerase LA Taq in the GC buffer. The scheme of amplification included 30 cycles with the following conditions: initial denaturation at 94 °C for 4 min, followed by 30 cycles of denaturation (1 min at 94 °C), annealing (40 s at 55 °C), extension (90 s at 72 °C), and final elongation step (10 min at 72 °C). The target fragments with the approximate length with our hope were cloned into the pMD19-T vector, and the 16S rDNA gene was sequenced in two directions in Invitrogen Corporation, Shanghai, China.

The nucleotide sequence of the gene was aligned with the sequences of 16S rRNA genes of various mycobacterial species available in GenBank using the program CLUSTAL W [14, 15]. Then, an unrooted phylogenetic tree was constructed by using the PHYLIP program package (seqboot, dnamlk, and consense) [16].

Gene Cloning of KSH

KSH is essential for the steroid metabolism and can catalyze the conversion of AD(D) into 9-hydroxy-AD(D), and then the steroid ring was degraded completely. To get the key enzyme in NwIB-01, protein/enzyme purification was attempted but defeated because microamount enzyme was contained, and no biocatalytically activity was determined after purification. Next, degenerate primers were designed related to a rieske-type [2Fe-2S] domain and non-haem iron-binding motif. To design degenerate primer, according to National Center for Biotechnology Information (NCBI) search, some gene sequences of confirmed KSH were analyzed as follows: *Mycobacterium vanbaalenii* PYR-1 (Mvan5225), *Mycobacterium tuberculosis* H37Rv (Rv3526), *Mycobacterium smegmatis* (MSMEG5925), and *Rhodococcus erythropolis* (AAL96829.1). Degenerate PCR primers were finally designed on the basis of the conserved sequence of KSH in NwIB-01 (forward primer 5'-TGCCCSTTCCACRACTG-3', reverse primer 5'-CAGYAGCGGRTTGTGCGAT-3'; Fig. 2). The gene fragment was amplified by PCR with degenerate primers and thermostable polymerase LA Taq in the GC buffer. The scheme of amplification included 30 cycles with the following temperature profile: DNA denaturation at 94 °C for 30 s, annealing of primers at 55 °C for 30 s, and elongation at 72 °C for 1 min. The targeted fragment with approximate length as we anticipated was sequenced in two directions in Invitrogen (China).

In succession, using TaKaRa LA PCR™ in vitro Cloning Kit (TaKaRa), unknown regions of genomic DNA (the whole sequence of *ksh*) were obtained by nested-PCR. Target DNA of upstream sequence of genes was specifically amplified with the following process: (1) completely digest target DNA using an appropriate restriction enzyme (*HindIII*, *PstI*, *BamHI*, and *EcoRI*); (2) ligate cassette with restriction site of corresponding restriction

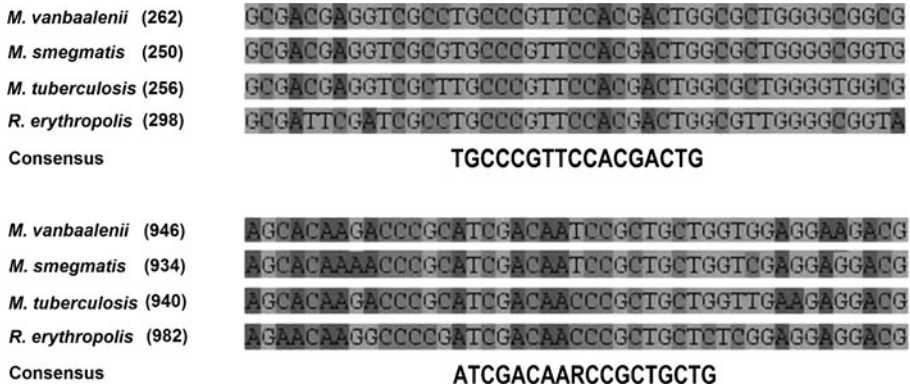


Fig. 2 Comparison of four reported *ksh* genes from different resources and the design of degenerate primers

enzyme; (3) perform the first PCR using cassette primer C1 (5'-GTACATATTGTCGTTA GAACGCGTAATACGACTCA-3') and primer S1 (5'-CCCTCGTGGTCATGCCAGAC GAAGAGCAG-3') specific to the known region; and (4) perform the second PCR using primers designed for inner sequences, cassette primer C2 (5'-CGTTAGAACGCGTAA TACGACTCACTATAGGGAGA-3') and primer S2 (5'-GTTTGGCGTAGGGGAC GAGCTTGCCTTG-3') of the known region. The process of getting downstream sequence target DNA was similar to the above and performed the first and second PCR using primer S1 (5'-GCCACTACCCGGTCAGTCAGGACTCCTT-3') and primer S2 (5'-CAGGACGTCGAGATCTGGAAGCACAAAGAC-3'), respectively. Nucleotide sequence was analyzed by Invitrogen. Protein and nucleotide sequence comparisons were performed by using Blast Search at NCBI.

Fermentation Experiments and Products Assay

The seed cells for the bioreactor were prepared in a 500-ml shake flask containing 100 ml seed medium. The shake flask was incubated at 30 °C for 24 h with shaking at 150 rpm and subsequently inoculated into the bioreactor at 10% (v/v). A 3.7-L bioreactor (KLF2000, Bioengineering, Switzerland) containing 2 L of fermentation medium was used during the batch stage. The pH was controlled by automatic addition of 5 M NaOH, and all fermentation experiments were carried out at 30 °C and 300 rpm with airflow at 0.5 vvm. Meanwhile, a general fermentation defoamer (Defoamer GPE-1) was added to control the foam during culture. After 24 h of the culture growth, soybean phytosterols (15 g/l in 0.5% v/v Tween 80) were added to the medium, and the bioconversion was monitored for several days. After substrate conversion, the samples were collected every 24 h and mixed with an equal volume of chloroform. Samples (500 µl) were extracted twice by shaking for 2 h, and the organic phase was pooled together. Then, by vacuum drying, the collected organic phase was evaporated to dryness. The solid residue obtained was resuspended in 500 µl of methanol and centrifuged (15,000×g, 15 min) or filtered (pore size, a 0.45-µm nylon syringe filter). Sample extracts were spotted in 2-µl aliquots onto thin-layer chromatography (TLC) plates [17]. Silica gel TLC plates with AE-ligarine (2:3) as its solvent system were used. The TLC plates were dyed by 20% sulfuric acid at 100 °C for 10 min. After washing with water carefully, the TLC plates were dried using hair dryer and scanned at

251 nm ultraviolet ray. Steroid products were observed as black spots on a yellow-green fluorescent background. Products were analyzed by high-performance liquid chromatography (HPLC) with the conditions as follows: HPLC column, Eclipse XDB-C18 (Agilent, USA); column temperature, 40 °C; mobile phase, methanol-water (80:20); flow rate of mobile phase, 1 ml/min. Analytes were detected with UV simultaneously at 254 nm. The liquid chromatography-mass spectrometry was performed with Agilent 1100 LC/MSD apparatus (Agilent Technologies) by using electron spray impact (ESI+) and the positive ionization mode.

Nucleotide Sequence Accession Number

The nucleotide sequences identified in this study were deposited into GenBank Data Library. The GeneBank accession numbers for the *M. neoaurum* NwIB-01 16S rDNA gene is GQ503243 and GQ476982 for the *M. neoaurum* NwIB-01 KSH gene.

Results and Discussion

Screening of the Strains

To obtain microorganisms capable of transforming soybean phytosterol to AD or/and ADD, some bacteria were isolated from steroidal contaminated soils collected around the places of steroid pharmaceutical companies using phytosterols as the sole carbon and energy source. Based on the qualitative screening on enrichment culture medium, five strains were firstly screened among these isolated microbes for their more powerful ability to degrade phytosterols or to produce AD and ADD, among which, an actinomycete stain (designated stain NwIB-01) was selected at last for its AD and ADD production from phytosterols.

Identification of Bacteria and Phenotypic Characterization

With the progress of the above research, 1,046 bp of 16S rDNA fragment in NwIB-01 was finally obtained. Based on the phylogenetic analyses with 16S rRNA, the morphology, and the culture characteristics, NwIB-01 was identified as *Mycobacterium* sp. In the phylogenetic tree, the 16S rRNA sequence of strain NwIB-01 displayed the similarity of 99.8–100% to the *M. neoaurum* (including the type strain of *M. neoaurum* ATCC 25795) and 95–99% to other species of *Mycobacterium*. The detected similarity degree between the sequences of NwIB-01 and *M. neoaurum* strains corresponded to the interspecies value (especially 100% to *M. neoaurum* ATCC 25795) and allowed the strain NwIB-01 to be ascribed to the species *M. neoaurum* (Fig. 3). Sequences were compared with other released sequences in the GenBank database by the BLAST program (NCBI) and the Ribosomal Database Project II [18]. The strain named *M. neoaurum* NwIB-01 was deposited at the China Center for Type Culture collection (<http://www.cctcc.org/>), Wuhan, China (accession number: CCTCC M 209094).

The isolated strain NwIB-01 is a kind of nonmotile and nonsporulating bacteria. Colonies on solid medium are slightly rugose and flat with wavy edges and irregularly round with a diameter of up to about 4 mm after 7 days of incubation. NwIB-01 is liable to exhibiting pellucid and nonpigmented colonies in darkness, whereas under the exposure to light, it will gradually turn from light yellow to golden yellow. Optimum growth temperatures vary widely and range from 25 °C to over 45 °C. NwIB-01 can utilize

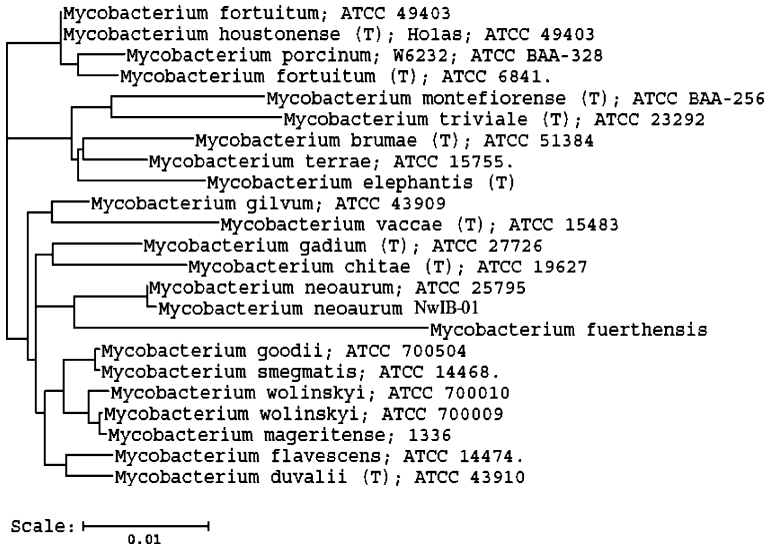


Fig. 3 Rooted phylogenetic tree of isolate NwIB-01 to other *Mycobacterium* species. Phylogenetic tree was constructed based on comparison of 16S rRNA sequences, demonstrating the position of NwIB-01 among closely related species

mannitol, sorbitol, L-glutamate, glycerol, acetamide, and citrate as sources of carbon but incapable of hydrolyzing cellulose or starch, liquefying gelatin, or degrading casein. It does not grow below 10 °C or over 45 °C, and no sign of sustainment is discerned on McConkey's agar, in the presence of 0.01% malachite green or in 5% NaCl. The growth rate refers to the length of time required to form mature colonies that are visible without magnification on solid media. Mycobacteria that form colonies clearly visible to the naked eye within 7 days on subculture are termed rapid growers, while those requiring longer periods are termed slow growers [19]. The strain NwIB-01 can form clearly visible colonies in 3 or 4 days and was identified as fast-growing Mycobacteria.

Biochemical tests and 16S rRNA sequence contrast are two main methods to identify an isolated strain. Biochemical tests to identify Mycobacteria are well standardized, reproducible, and inexpensive, but have two major limitations. Firstly, biochemical tests are reliable only for species that have been widely studied and do not apply to identify newly recognized species. Secondly, several kinds of biochemical tests are time-consuming. In addition to highly conserved primer binding sites, 16S rRNA sequences contain hypervariable regions, which can provide species-specific signature sequences useful for bacterial identification [20]. As a result, 16S rRNA gene sequencing has become prevalent in medical microbiology as a rapid, accurate alternative to phenotypic methods of bacterial identification.

KSH Gene Sequence Analysis

As key enzyme, KSH is essential for the steroid metabolism and catalyze the conversion of AD(D) into 9-hydroxy-AD(D). Then, the steroid core was degraded completely. To further confirm the veracity of NwIB-01 taxonomical classification and perform strain improvement by genetic engineering, we need to identify the KSH in the strain. By nested-PCR and chromosome walking, a 1,798-bp DNA fragment was cloned and sequenced, which

contains a full-length open reading frame (ORF) consisting of 1,188 bp encoding a putative *ksh* (GQ476982) with an upstream region of 129 bp and a subsequent region of 481 bp. Sequence analysis revealed that the sequence of the *ksh* ORF uses GTG as the start codon and has 63.7% G+C content. Besides, a possible ribosomal binding site (GGGAGG) six nucleotides upstream was found. The KSH gene consists of 1,188 nucleotides and encodes a deduced protein of 395 amino acids. The molecular weight of KSH was estimated to be 45.37 kDa, and the pI value was calculated to be 5.46 by the ExPASy compute pI/Mw program algorithm (<http://www.expasy.org/cgi-bin/protparam>). Homology analysis revealed that KSH in *M. neoaurum* NwIB-01 is 89% identical to the protein encoded by Mvan5225 in *M. vanbaalenii* PYR-1 and 85% identical to the MSMEG5925 in *M. smegmatis* and 70% identical to AAL96829.1 in *R. erythropolis*.

As the high degree of homology to 16S rRNA in Mycobacteria (95–99% similarity in different *Mycobacterium* species), it is difficult and inadequate to identify new *Mycobacterium* species only relying on 16S rDNA sequence contrast. In our research, a new and specific KSH gene in steroid degradation was obtained, and the highest homology is just 89% at the amino acid level with other KSH in GenBank Data Library and shows about 96% identity with KSH in *M. neoaurum* ATCC 25795 (sequenced in this study).

Products Analysis of NwIB-01

It has been known that fast-growing Mycobacteria can degrade natural sterols and use them as a source of carbon and energy [7]. When cultivated on fermentation medium, strain NwIB-01 could utilize soybean phytosterols and accumulate AD and ADD by selective cleavage of the lateral chain. The TLC analysis revealed that when cultured in a shake flask (100 ml fermentation medium in 500 ml shake flask) with 0.1 g/l soybean phytosterol, the NwIB-01 could metabolize substrate efficiently and yield ADD as the primary product with few generation of AD. To quantify these products, 500 ml shake flask had been scaled up to a 3.7-L bioreactor with concentration of soybean phytosterol increased to 15 g/l in 0.1% v/v Tween 80. The HPLC data were consistent with the TLC analysis results. The molar ratio of AD:ADD in products of strain NwIB-01 is 1:2.4 (Fig. 4).

For NwIB-01, ADD accumulation was observed in 48-h-old culture with fermentation medium containing 15 g/l phytosterol and reached maximum in 96-h-old culture. At this time, the ADD accumulation reached 4.23 g/l and was accompanied by 1.76 g/l AD with a molar yield of 64.7% (AD+ADD). Fermentation time extension can result in product significant degradation. The ADD accumulation reached the maximum of 4.23 g/l in 96-h-old and decreased to 2.47 g/l in 144-h-old (Fig. 5). Trace of 9-OH-AD and substrate was also observed in our research and can be negligible for low content. The product ADD was degraded obviously, especially after 7 days in the end of fermentation culture, and about 40% product was degraded in the next 2 days after the ADD accumulation reached the maximum.

The serious steroid nucleus degradation in this strain led to the difficulty of product accumulation continued increase. This degradation was due to its 9 α -hydroxylase activity. This enzyme can catalyze the conversion of AD(D) into 9-hydroxy-AD(D), and finally, the steroid ring can be degraded completely [8]. In order to accumulate AD or ADD as intermediates, the activity of 9 α -hydroxylase must be deleted. The KSH gene sequence obtained in our research lays the foundation for further improved strain by genetic engineering and provides an available way to improve potential industrial applications.

Many Mycobacteria could degrade natural sterols and use them as a source of carbon and energy [7]. Also, some mutant by mutation breeding with UV or chemical mutagenic

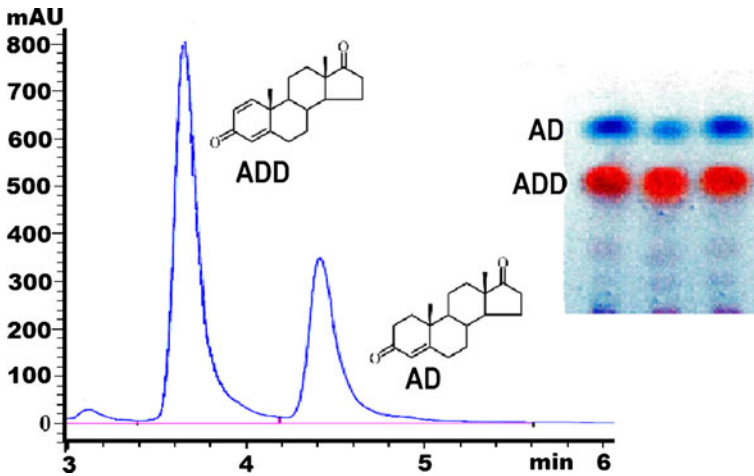


Fig. 4 High-performance liquid chromatography (HPLC) and thin-layer chromatography (TLC) analysis of *Mycobacterium neoaurum* NwIB-01 products with 96-h-old culture. HPLC mobile phase, methanol-water (80:20); TLC solvent system, AE-ligarine (2:3)

treatments was used to produce AD(D) and has been used in industrial application. *Mycobacterium* MB 3683 could convert 30 g/l phytosterols and accumulate AD in 80% molar yield [21]. *Mycobacterium* sp. NRRL B-3805 could utilize 1.0 g/l β -sitosterol to produce AD (molar yield 90%) [22]. *Mycobacterium* sp. NRRL B-3683 could utilize ergosterol as a substrate and accumulate ADD with 20% molar yield [23]. Compared with the industrial strain, the yield of AD(D) and the product purity of the isolated NwIB-01 is not so high. Also, the serious further degradation of AD(D) steroid nucleus for strain NwIB-01 is another important shortcoming to block their practical application. For in-depth

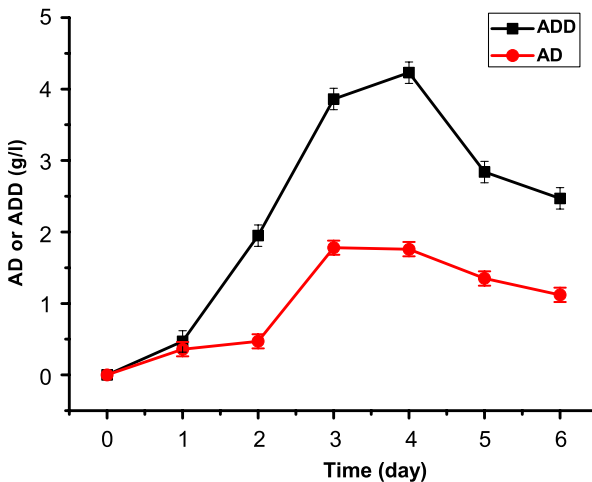


Fig. 5 Time course of 4-androstene-3,17-dione (AD)/1,4-androstene-3,17-dione (ADD) accumulation by *Mycobacterium neoaurum* NwIB-01 in 3.7 l bioreactor with 15 g/l soybean phytosterols. The pH was controlled to 7.2, and all fermentation experiments were carried out at 30 °C and 300 rpm with airflow at 0.5 vvm. The AD accumulation is indicated by circles, ADD accumulation with squares (time was calculated after addition of substrate)

study, we can knock out KSH and demonstrate increased ADD and AD production. Also, overexpressing KsdD may yield a greater ADD to AD ratio. Further optimization of the culture conditions may also result in increased production with NwIB-01.

Conclusions

In this study, we disclosed a bacterium, *M. neoaurum* NwIB-01, isolated from soil with microcrystalline phytosterols as the sole carbon source. The strain NwIB-01 could utilize soybean phytosterols to produce AD and ADD. In 3.7-L bioreactors, the strain could convert 15 g/l soybean phytosterols to 4.23 g/l ADD and 1.76 g/l AD under an unoptimized process in 96 h (molar yield of 64.7%), indicating that it is a promising strain. Meanwhile, a gene encoding 395-amino acid KSH involved in steroid metabolism was successfully obtained from *M. neoaurum* NwIB-01. To our best knowledge, this is the first report to the key enzyme KSH from *M. neoaurum*. The KSH gene obtained in this study provides a feasible way to rationally modify the phytosterol-transformation traits of strain by metabolic engineering.

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