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Characterization and engineering of 3-ketosteroid-Δ^1-dehydrogenase and 3-ketosteroid-9α-hydroxylase in *Mycobacterium neoaurum* ATCC 25795 to produce 9α-hydroxy-4-androstene-3,17-dione through the catabolism of sterols

Kang Yao [1], Li-Qin Xu [1], Feng-Qing Wang *, Dong-Zhi Wei **

State Key Laboratory of Bioreactor Engineering, Newworld Institute of Biotechnology, East China University of Science and Technology, Shanghai 200237, China

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ABSTRACT

3-Ketosteroid-Δ^1-dehydrogenase (KstD) is a key enzyme involved in the microbial catabolism of sterols. Here, three homologues of KstD were characterized from *Mycobacterium neoaurum* ATCC 25795, showing distinct substrate preferences and transcriptional responses to steroids. Single deletion of any *MN-kstD* failed to result in a stable and maximum accumulation of 9-OHAD due to residual KstD activities. To develop stable 9-OHAD producers, all of these MN-KstDs were inactivated, which led to about 6.02 g l^{-1} of 9-OHAD from 15 g l^{-1} of phytosterols. However, the product was mixed with 1.55 g l^{-1} of AD as a major by-product. To transform AD, the oxygenase component of 3-ketosteroid-9α-hydroxylase (KSH), encoded by *kshA*, was overexpressed. As a result, the yield of 9-OHAD increased to 7.33 g l^{-1} with less than 0.31 g l^{-1} of AD and the selectivity of 9-OHAD production was improved to 95–97% among metabolites.

1. Introduction

A wide variety of natural sterols can be found in membrane-structured living systems. Among them, cholesterol, phytosterols and ergosterol have been mostly documented for their prominent roles in maintenance of the membrane physiology (Maier et al., 2009; Nicolaou et al., 2010; Souza et al., 2011). In some cases, sterols also serve as source of carbon and energy for microorganisms. Mycobacteria are well known for using natural sterols or some other steroidal compounds as sole carbon and energy source. Interruptions in the catabolic pathway of mycobacteria result in the excessive accumulation of some important intermediates, such as AD, ADD and 9-OHAD (Donova et al., 1996; Marsheck et al., 1972; Wovcha et al., 1978), which can be used as precursors to produce steroidal hormone pharmaceuticals (Donova, 2007; Donova and Egorova, 2012; Fernandes et al., 2003). Among them, the conversions of AD to ADD catalyzed by KstD and AD to 9-OHAD by KSH have been well-documented in the metabolic network of sterols. (Szentirmai, 1990). Under the combined action of KstD and KSH, no intermediates with intact steroid nucleus could be accumulated because of the destruction of steroid skeleton. To develop mycobacteria capable of accumulating steroid metabolites, therefore, the metabolic steps catalyzed by KstD and KSH must be dealt with. However, simple genetic modification of KstD and KSH was far from enough to produce desirable strains of industrial interest because of the deep redundancy of KstD and KSH homologues in those sterol-using microorganisms (Fernandez de lasHeras et al., 2012; Knol et al., 2008; Petrusma et al., 2011; van der Geize et al., 2001, 2002).

For a long time, mutation breeding by physical and chemical methods was the workable way to successfully develop some strains with KSH or KstD or both blocked, such as the AD-producing strain *Mycobacterium* sp. NRRL B-3805 and *Mycobacterium* sp. VKM Ac-1815D; the ADD-producing strain *Mycobacterium* sp. NRRL B-3683 and *Mycobacterium* sp. VKM Ac-1816D (Donova et al., 1996; Marsheck et al., 1972). However, the development of 9-OHAD-producing strains was not very successful. Nitrosoguanidine was once applied to mutate *Mycobacterium fortuitum* ATCC

Abbreviations: AD, 4-androstene-3,17-dione; ADD, 1,4-androstadiene-3,17-dione; 9-OHAD, 9α-hydroxy-4-androstene-3,17-dione; 5α-AD, 5α-androstane-3,17-dione; T, 4-androstene-17β-ol-3-one; 5α-T, 5α-androstane-17β-ol-3-one; 1-(5α)-T, 1-(5α)-androstene-17β-ol-3-one; 9-OH-T, 9α-hydroxy-4-androstene-17β-ol-3-one; DHEA, 3β-hydroxyandrost-5-en-17-one; HBC, 22-hydroxy-23,24-bisnorchol-4-en-3-one; 4-BNC, 3-oxo-23,24-bisnorchol-4-en-22-oic acid; KstD, 3-ketosteroid-Δ^1-dehydrogenases; MN-KstD, 3-ketosteroid-Δ^1-dehydrogenase homologue in *M. neoaurum* ATCC 25795; KSH, 3-ketosteroid-9α-hydroxylase; MM, minimum medium; WT, wild-type strain *M. neoaurum* ATCC 25795

* Corresponding author. Fax: +86 21 64250068.
** Corresponding author. Fax: +86 21 64250068.
E-mail addresses: fqwang@ecust.edu.cn (F.-Q. Wang), dzhwei@ecust.edu.cn (D.-Z. Wei).

[1] These authors contributed equally to this work.

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6842 and resulted in the acquisition of a novel 9-OHAD producer, NRRL B-8119, which otherwise accumulated a mass of by-products, such as 9-OH-T, and two C22-ketosteroids, 9α-hydroxy-3-ketobisnorchol-4-en-22-ol (9-OH BN alcohol) and 9α-hydroxy-3-ketobisnorchol-4-en-22-oic methyl ester (9-OH BN acid methyl ester) (Wovcha, 1977; Wovcha et al., 1979a). The occurrence of C22-ketosteroids suggested an unexpected interruption of side chain cleavage (García et al., 2012). Besides, semi-continuous cultivation of naturally AD-producing strains in a mixture of sterols, ADD, and mutagens has succeeded in the development of 9-OHAD producer (Donova et al., 2005; Slijkhuis and Marx, 1994). Although the frequency of a desirable mutant was greatly improved in this way, special concern has to be paid to the result that several 9-hydroxylated metabolites derived from the unexpected interruption of side-chain cleavage process still co-existed with 9-OHAD. This may be ascribed to the close relationship between the KSH activity and some degradation steps of side chain (Capyk et al., 2011), which in return poses a great technical challenge to the development of 9-OHAD-producing strain.

As indicated above, the integrity of 9-OHAD is most at risk from KstD activities, which means any residual KstD activity may cause the decomposition of 9-OHAD. For example, residual KstD (St1DH) activity was still detected in *Mycobacterium* sp. VKM Ac-1817D, a well-known 9-OHAD producer, and therefore decreased the production of 9-OHAD (Sukhodolskaya et al., 2007). Evidence for two types of KstD activities has been reported in *M. fortuitum*, with one induced by AD and the alternate by 9-OHAD, which clearly showed a variety of KstD activity in mycobacteria (Wovcha et al., 1979b). Among rhodococci, the multiplicity of KstD isoenzymes has also been identified, such as three KstD homologues in *Rhodococcus erythropolis* strain SQ1 (Knol et al., 2008; van der Geize et al., 2000, 2002) and *Rhodococcus ruber* strain Chol-4 (Fernandez de lasHeras et al., 2012). Physiologically, this diversity was virtually derived from a kind of adaption mechanism which confers strains the ability to accommodate changing environments of different steroidal substrates. However, this adaptability has obviously complicated the metabolic evaluation of KstD roles and thus increased the difficulty to develop 9-OHAD producer by means of metabolic engineering. The *R. erythropolis* strain RG8, with a double deletion of *kstD* and *kstD2*, was able to produce 9-OHAD from AD but not cholesterol, indicating it was not an efficient 9-OHAD manufacturer (van der Geize et al., 2002). The elimination of all the known *kstD* genes in *R. ruber* strain Chol-4 did not preclude 9-OHAD from further cleavage, suggesting some other KstD isoenzymes still exist in the strain (Fernandez de lasHeras et al., 2012). By comparison, the inactivation of *rv3537*, the unique *kstD* gene in *Mycobacterium tuberculosis* H37Rv, for the first time resulted in the stable accumulation of 9-OHAD (Brzostek et al., 2009). However, it was evidently impossible to apply this pathogenic mutant of *M. tuberculosis* in the industrial production of 9-OHAD. All these attempts again stressed the major difficulty to develop 9-OHAD producers lied in the metabolic engineering of KstD.

Recently, some successes of metabolic engineering on enzymes involved in the steroid catabolism of *Mycobacterium neoaurum* cells have been made (Wei et al., 2010; Yao et al., 2013). Herein, we attempted to rationally modify KstD and KSH activities in *M. neoaurum* ATCC 25795 and establish a broad highway to produce 9-OHAD.

2. Materials and methods

2.1. Bacterial strains, plasmids, and chemicals

All strains used in this study are listed in Table 1. Oligonucleotides and plasmids were supplemented in Supplementary Table S1. *Mycobacteria* were aerobically cultivated in MYC/01 and fermented with 2 g l^{-1} of cholesterol or 0.5 g l^{-1} of AD in flask of MYC/02 medium. Bioreactor fermentation with 15 g l^{-1} of phytosterols in MYC/02 medium for a large-scale application was performed as described previously (Yao et al., 2013). RT-qPCR studies of *kstD* genes were carried out in MM (minimal medium) with 2.0 mM cholesterol, 1.5 mM AD, 1.5 mM 9-OHAD or 18 mM glycerol (blank control). Strains were collected at their mid-exponential phase. The strains *Escherichia coli* DH5α and *E. coli* BL21(DE3) carrying recombinant pET-28a plasmids were used for cloning and expression, respectively.

Phytosterols, AD, 9-OHAD, ADD, 5α-T, cholic acid, epiandrosterone and DHEA were purchased from J&K Scientific Ltd. (Beijing, China). T, progesterone, and 4-cholesten-3-one were obtained from Sigma-Aldrich (Shanghai, China). 1-(5α)-T and HBC was from Steraloids (Newport, RI, USA). Total RNA preparation system was purchased from MP Biomedicals (Santa Ana, CA). cDNA synthesis and RT-qPCR kits were obtained from Beijing Dingguo Changsheng Biotechnology Co., Ltd., (Beijing, China). Other reagents and chemicals used in this study were of the highest grade available.

2.2. Bioinformatics

The genome was isolated from *M. neoaurum* ATCC 25795 and sequenced as indicated in Supplementary Method. BLASTp package was employed to analyze protein identity against the GenBank nr database with filtering parameter *E*-value $< e^{-40}$ at NCBI

Table 1
Strains used in this study.

Strains	Description
E. coli	
E-*k1*/*k2*/*k3*/*kshA*	Recombinant BL21 (DE3) cells, possessing pET28a-*MN-kstD1*/*MN-kstD2*/*MN-kstD3*/*kshA*
M. neoaurum	
ATCC 25795	Sterol consumer with no detectable intermediates[a]
NwIB-01	Soil isolate, AD(D) producer[b]
$Mut_{MN\text{-}kstD1}$	*kstD1* deleted mutant of *M. neoaurum* ATCC 25795
$Mut_{MN\text{-}kstD3}$	*kstD3* deleted mutant of *M. neoaurum* ATCC 25795
$Mut_{MN\text{-}kstD1\&3}$	*kstD1*&3 deleted mutant of *M. neoaurum* ATCC 25795
$Mut_{MN\text{-}kstD1\&2\&3}$	*kstD*-null mutant of *M. neoaurum* ATCC 25795
NwIB-C	$Mut_{MN\text{-}kstD1\&2\&3}$ carrying a vacant pMV261
NwIB-yV	KshA over-expression in $Mut_{MN\text{-}kstD1\&2\&3}$ via pMV261-*kshA*

[a] *M. neoaurum* ATCC 25795 was a soil isolate by Tsukamura and Mizuno (1972) deposited in ATCC (American Type Culture Collection, accession number ATCC 25795) and the sterol transformation profile was provided in Supplementary Fig. S-1 by Yao et al. (2013) as well as in Supplementary Fig. S4 of this study.
[b] Wei et al. (2010).

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