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ORIGINAL RESEARCH PAPER

Engineering phytosterol transport system in *Mycobacterium* sp. strain MS136 enhances production of 9α-hydroxy-4-androstene-3,17-dione

Kun He · Hong Sun · Hao Song

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Abstract
Objectives To enhance the yield of 9α-hydroxy-4-androstene-3,17-dione (9-OHAD) from phytosterols, a phytosterol transport system was constructed in *Mycobacterium* sp. strain MS136.
Results 9-OHAD can be produced via the controlled degradation of phytosterols by *mycobacteria*. This involves an active transport process that requires trans-membrane proteins and ATP. A phytosterol transport system from *Mycobacterium tuberculosis* H37Rv was constructed in *Mycobacterium* sp. strain MS136 by co-expression of an energy-related gene, *mceG*, and two integrated membrane protein genes, *yrbE4A* and *yrbE4B*. The resultant of the *Mycobacterium* sp. strain MS136-GAB gave 5.7 g 9-OHAD l^{-1}, which was a 20% increase over 4.7 g l^{-1} by the wild-type strain. The yield of 9-OHAD was increased to 6.0 g l^{-1} by optimization of fermentation conditions, when 13 g phytosterols l^{-1} were fermented for 84 h in 30 ml biotransformation medium in shake flasks.
Conclusions Phytosterol transport system plays an active role in the uptake and transport of sterols, cloning of the system improved the mass transfer of phytosterols and increased the production of 9-OHAD.

Keywords 9α-Hydroxy-4-androstene-3,17-dione · *Mycobacteria* · Optimization · Phytosterols · Steroids · Transport system

Introduction

9α-Hydroxy-4-androstene-3,17-dione is the precursor of many steroidal pharmaceuticals, which are widely used as anti-tumor, anti-inflammatory, anti-microbial, anti-viral and anti-fungal drugs (Donova and Egorova 2012). *Mycobacteria*, *Rhodococcus* and *Nocardia* can use sterols as sole carbon and energy sources. The bacteria can then be used for the production of the steroid intermediate, 9-OHAD (Fernandes et al. 2003). The molecular mechanism of the degradation of phytosterol to 9-OHAD can be divided into three steps (Fig. 1). To obtain a high yield of 9-OHAD, previous studies have mainly focused on the modification of the steroidal nuclei, such as the overexpression of 3-steroid-9α-hydroxylation enzyme (*KSH*) and the knockout of 3-steroid-Δ1-dehydrogenase (*KstD*)

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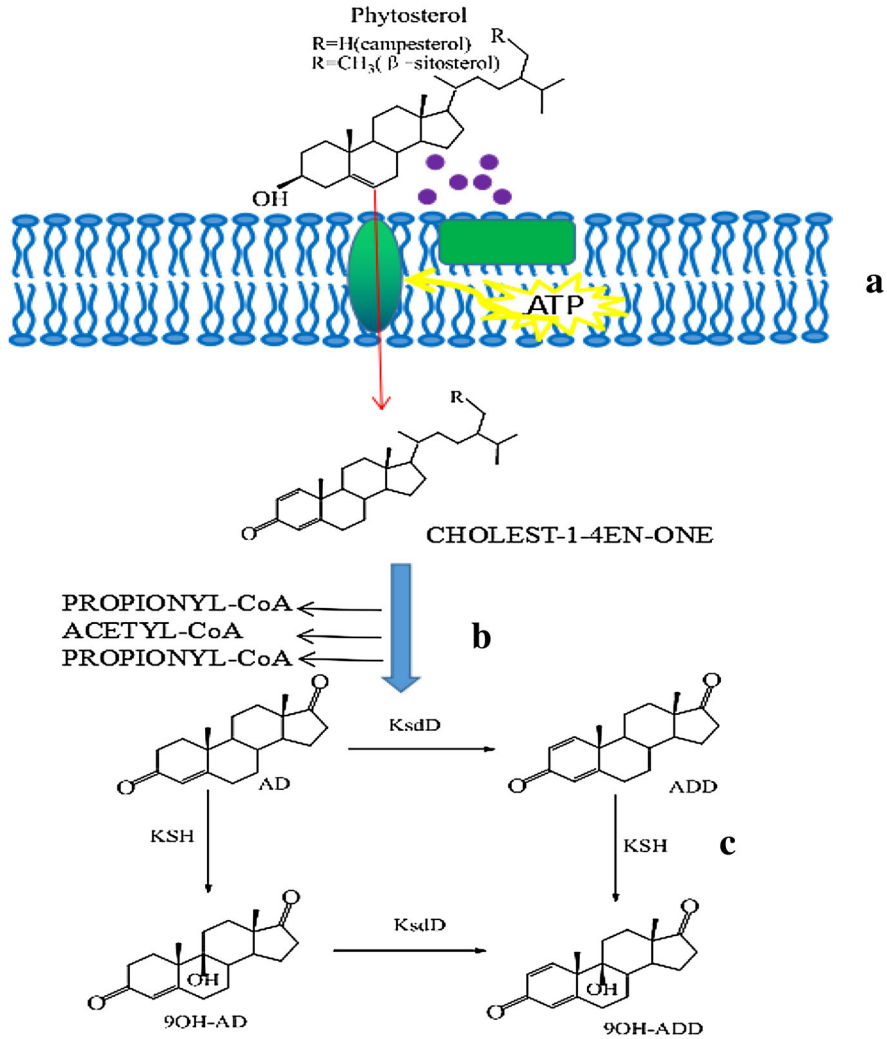

Fig. 1 Conversion of phytosterol to 9-OHAD by the engineered *Mycobacterium* strain. The generation of 9-OHAD could be divided into three steps: **a** the uptake and transport; **b** the oxidation of the side chain; **c** the modification of steroidal nuclei at the 9-position

(Knol et al. 2008; Wei et al. 2010), whilst the uptake and transport of phytosterol were relatively rare.

Vancomycin antibiotics, glycine, ethambutol, polyethyleneimine and β-CD can be used to increase the transport of sterol (Wang et al. 2006), but none of these studies involved sterol transport regulated by genetics. With the discovery of microbial sterol-degrading gene clusters (Geize et al. 2007), a *Mce4* operon was discovered. This operon system encodes two integrin membrane proteins, which are closely related to the uptake and transport of sterols (Klepp et al. 2012). *Rhodococcus jostii* RHA1 mutants with deletions of the two integrin membrane proteins' *supAB* genes (homologous to *yrbE4AB*) will lose the ability to grow on cholesterol. In addition, the uptake activity of cholesterol was ATP dependent (Mohn et al. 2008). The *mceG* gene that encodes transporter ATPases has a significant effect on cholesterol uptake (Joshi et al. 2006; Pandey and Sassetti 2008). Therefore, steroid uptake and transport is an active transport process, which requires transport membrane proteins and ATP.

Steroid mass transfer is the first step in the microbial metabolism, which determines the metabolic flux of the process. Here, we used phytosterols as the substrate, which are slightly different from the cholesterol (Wilbrink et al. 2011), and can be degraded by *Mycobacteria*. The genes *mceG* (77%), *yrbE4A* (90%) and *yrbE4B* (88%) derived from *Mycobacterium tuberculosis* H37Rv were highly homologous to the genes of the wild-type strain, and these genes were overexpressed to construct an engineered *Mycobacterium* strain, enabling a high yield of 9-OHAD.

Materials and methods

Strains, plasmids and reagents

All strains used in this study are listed in Supplementary Table 1. A mixture of phytosterol containing β-sitosterol, campesterol, stigmasterol and brassicasterol (47.5:26.4:17.7:3.6) was purchased from Davi Biochemistry. The standard of 9-OHAD was obtained from Sigma. Other reagents and chemicals used in this study were of the highest grade available.

Culture media and growth conditions

Seed medium was used to cultivate the mycobacterium strains used in this study (10 g glycerol l^{-1}, 1.5 g $NH_4H_2PO_4$ l^{-1}, 10 g yeast extract l^{-1}, 0.5 g NaH_2PO_4 l^{-1}, 0.5 g Na_2HPO_4 l^{-1}, 0.5 g Tween-80 l^{-1}, pH 7.3). Slant medium was used for the passage of the mycobacterium strains. Steroid biotransformation medium were composed of 10 g glycerol l^{-1}, 1.8 g $(NH_4)_2SO_4$ l^{-1}, 13 g phytosterol l^{-1}, 0.3 g urea l^{-1}, 70 g β-cyclodextrin l^{-1}, 0.8 g NaH_2PO_4 l^{-1}, 0.5 g Na_2HPO_4 l^{-1}, and 0.2 g Tween-80 l^{-1}. *Escherichia coli* DH5a was used as a cloning host for plasmid replication. Kanamycin (50 mg l^{-1}) was supplemented into medium for the selection of mycobacterium transformants. *Mycobacteria* were cultivated at 30 °C with a shaking speed of 200 rpm.

Plasmid and strain construction

The genes *mceG* (Genbank ID: 888081), *yrbE4A* (Genbank ID: 888320) and *yrbE4B* (Genbank ID: 888336) from *Mycobacterium tuberculosis* H37Rv were artificially synthesized according to the codon-optimized sequences. The single gene was digested and inserted into the *Bam*HI and *Hind*III sites of the expression vector pMv261. Then the digested gene segments and expression cassettes were ligated using T4 DNA ligase and transformed into *E. coli* DH5a. Selected clones were verified by colony PCR and restriction enzyme digestion, while the multiple genes were assembled by overlap extension PCR and then inserted into the expression vector pMv261. In this way, the series of gene expression vectors were constructed (Fig. 2).

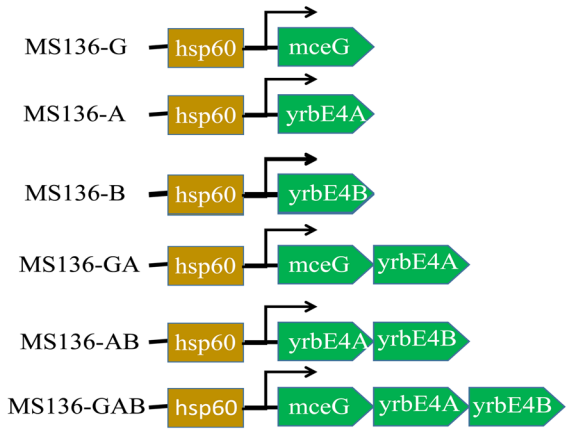

Fig. 2 Synthetic biology strategies for the construction of the phytosterol transport system in the recombinant *Mycobacteria*. The transport included one energy-related gene, *mceG,* and two integrated membrane protein genes, *yrbE4A* and *yrbE4B,* from *Mycobacterium tuberculosis* H37Rv. These gene assemblies were transformed into *Mycobacterium* sp. strain MS136, respectively, to construct a series of recombinant engineered strains, which were further examined whether the transport of phytosterol could be enhanced

Steroid bioconversion and analysis

The conversion of phytosterols was done in triplicate with cultures grown on 50 ml of transformation medium. After activation of the culture in seed medium for about 48 h at 30 °C and 200 rpm, 10% (v/v) culture broths were inoculated in the conversion reaction system. Bioconversion was monitored for 4 days and sampled every 12 h in flask fermentation at the beginning of the reaction.

The yield was detected by high-performance liquid chromatography (HPLC) method. A sample of the culture was transferred into a clean tube and centrifuged at 4000×*g* for 15 min. The supernatant was filtered through 0.22 μm pore membrane and then the part of filtrate was injected into the moderate amount of diluent that consists of acetonitrile/water (50:50, v/v). HPLC was performed on a C-18 reserved-phase HPLC column (250 mm × 4.6 mm) under condition at 240 nm with the flow rate 1 ml/min and the mobile phase consisted of acetonitrile/water (520:480) with 0.05% acetic acid. Peaks were compared to an authentic standard of 9-OHAD to determine the exact amount of product during sterol transformation (Supplementary Fig. 1).

Results and discussion

Overexpression of the transport system of phytosterols

A diversity of *mceG*, *yrbE4A* and *yrbE4B* can be usually observed in a few efficient sterol-utilizing strains, indicating its significant role in the sterol uptake. Therefore, we attempted to strengthen the expression of trans-membrane proteins and ATPase activity by overexpressing the sterols transport system in *Mycobacterium* sp. strain MS-136. The engineered strains could enhance the transformation ability of sterols in different degrees, and the gene *mceG* had a significant impact on the sterol assimilation. When the gene *mceG* was co-expressed with *yrbE4A* and *yrbE4B*, the yield of 9-OHAD was further improved (Fig. 3). Thus, we obtained a genetically engineered *Mycobacterium* sp. strain MS136-GAB, with a yield of 5.7 g 9-OHAD l^{-1}, which was a 20% increase over 4.7 g l^{-1} by the wild-type strain.

Enhancing the production of 9-OHAD by passaging and condition optimization

Many industrial strains need passaging to enhance the productivity. The wild-type *Mycobacterium* strain was passaged four times to enable a highest yield of 9-OHAD, while the passaging of engineered strains remained unclear. Therefore, we selected the *Mycobacterium* sp. strain MS136-GAB as the optimal strain to examine the effect of the passage numbers on the production of 9-OHAD. The experiments showed that the engineered strains had the strongest transformation ability in the third generation, which could thus reduce the total fermentation time to a certain extent (Fig. 4a). Excessive substrate could be toxic to the bacteria, we thus varied the concentration of substrate and found that a concentration of 13 g phytosterol l^{-1} were optimal for the bioconversion reactions to produce 9-OHAD (Fig. 4b).

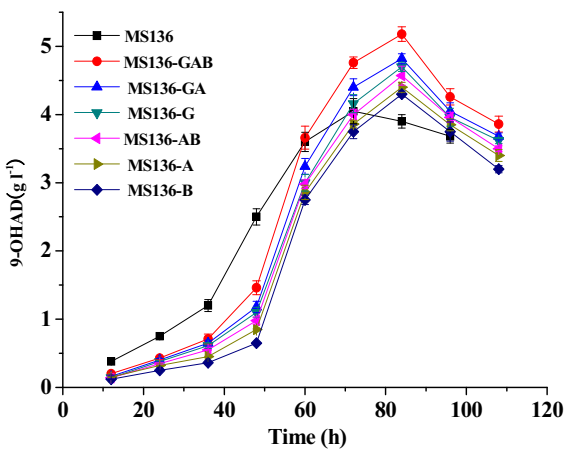

Fig. 3 Time course of the production of 9-OHAD from phytosterol by the engineered *Mycobacterium* strains. The authentic standard of 9-OHAD was used to determine the exact amount of 9-OHAD during sterol transformation by HPLC. The error bars indicated standard deviation with three replicates

Mycobacteria belong to actinomycetes, which are mostly aerobic. The dissolved oxygen level plays a crucial role in bioconversion reactions performed with high substrate concentrations (Gao 2016). In typical shake flask experiments, the oxygen-supply conditions were determined by the system volume. We examined the effect of system volume on the production of 9-OHAD. With the increase in the amount of liquid in the flask, the yield of 9-OHAD was gradually reduced (Fig. 4c). Therefore, 30 ml system was selected as the experimental fermentation system in the subsequent experiments.

Evaluation of the 9-OHAD producer

Considering the importance of phytosterol transport system in *Mycobacteria*, we engineered the *Mycobacterium* strain to enhance its phytosterol transport system for increased productivity of 9-OHAD. Initially, we achieved an optimal genetic-engineered strain *Mycobacteria* sp. strain MS136-GAB by co-expression of the gene *mceG* and two integrated membrane protein genes, *yrbE4A* and *yrbE4B*. The yield of 9-OHAD reached 5.7 g l^{-1} after three passages, which led to a 20% increase over 4.7 g l^{-1} by the wild-type strain. Moreover, the yield of 9-OHAD was increased to 6.0 g l^{-1} by optimization of fermentation conditions (Fig. 4d). When the production of 9-OHAD reached the maximum, the degradation of 9-OHAD also occurred (Fig. 2). According to previous studies, the integrity of 9-OHAD is most at risk from 3-steroid-Δ1-dehydrogenase (*KstD*), which means the *KstD* activity may cause the decomposition of 9-OHAD. The inactivation of the *KstD* gene in *Mycobacteria* could result in a stable accumulation of 9-OHAD, suggesting the degradation of 9-OHAD is closely related to the

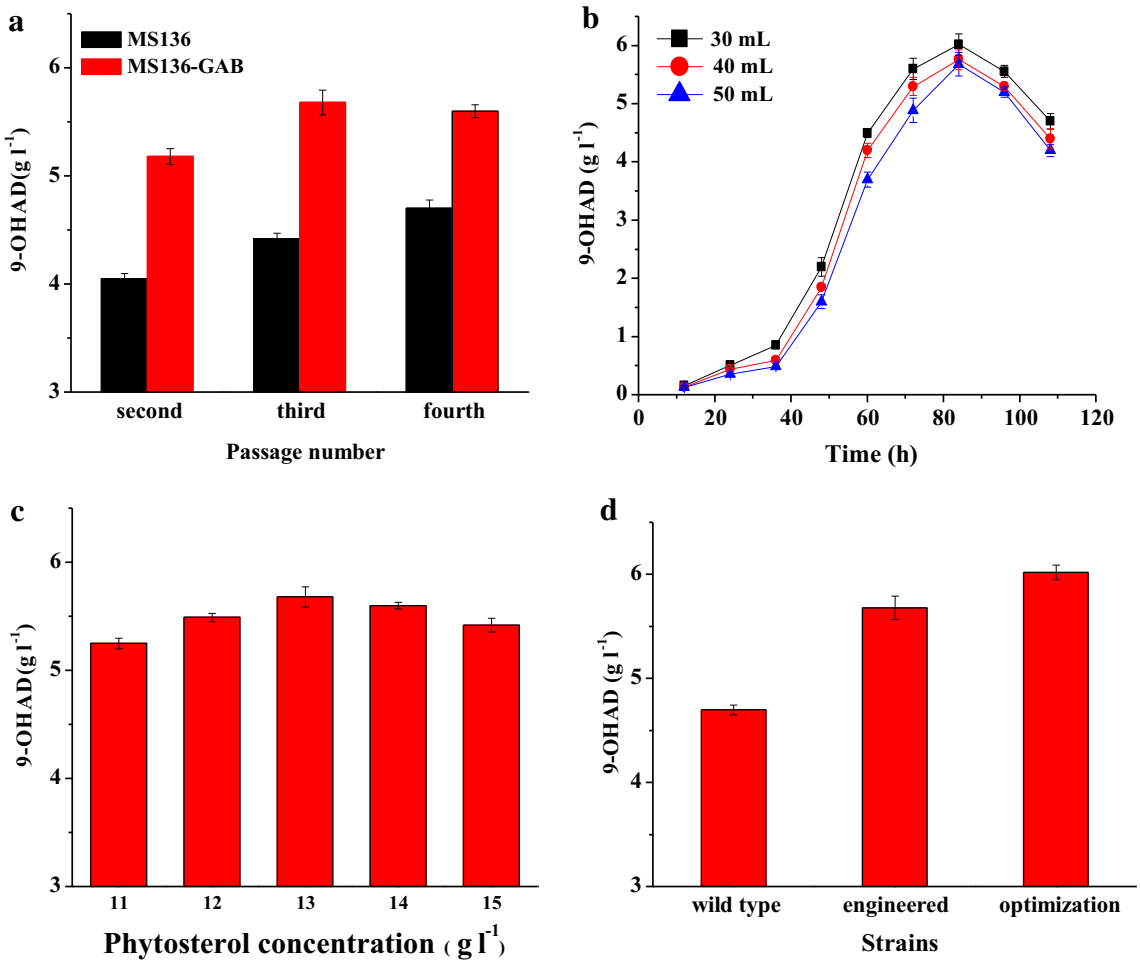

Fig. 4 The 9-OHAD production analyses by different improvements. **a** The 9-OHAD production differences of *Mycobacterium* sp. strain MS136 and MS136-GAB under different passages; **b** effects of system volume on 9-OHAD production by the *Mycobacterium* sp. strain MS136-GAB; **c** effects of phytosterol concentrations on 9-OHAD production by the *Mycobacterium* sp. strain MS136-GAB; **d** the maximum production of 9-OHAD by genetic engineering and optimization of fermentation conditions. The error bars were calculated from triplicate experiments

expression of the *KstD* gene (Wei et al. 2010). Thus, deletion of the gene *KstD* may further improve the productivity of 9-OHAD.

Conclusions

Steroid uptake and transport is an active transport process that requires the assistance of trans-membrane protein and ATP. The gene *mceG* encoding a transporter ATPase and the two integrin membrane protein genes, *yrbE4A* and *yrbE4B,* were identified through the amino acid homology analysis. These genes were heterologously overexpressed to obtain an engineered *Mycobacterium* sp. strain MS-136-GAB, which could improve the 9-OHAD productivity compared with the wild-type strain. Subsequently, through the passage analysis and the optimization of fermentation conditions, we succeeded in maximizing the yield of 9-OHAD, increasing it from 4.7 to 6.0 g l^{-1}. In conclusion, the important role of the phytosterol transport system for sterol uptake and metabolism was confirmed.

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Supporting information Supplementary Table 1—Strains, primers and plasmids used in this study.

Supplementary Fig. 1—HPLC analysis of 9-OHAD produced by engineered strains.

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