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Original Research Article

Improving the biotransformation efficiency of soybean phytosterols in *Mycolicibacterium neoaurum* by the combined deletion of *fbpC3* and *embC* in cell envelope synthesis

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ABSTRACT

Biotransformation of soybean phytosterols into 9α-hydroxy-4-androstene-3,17-dione (9-OHAD) by mycobacteria is the core step in the synthesis of adrenocortical hormone. However, the low permeability of the dense cell envelope largely inhibits the overall conversion efficiency of phytosterols. The antigen 85 (Ag85) complex encoded by *fbpA*, *fbpB*, and *fbpC* was proposed as the key factor in the combined catalysis of mycoloyl for producing mycolyl-arabinogalactan (m-AG) and trehalose dimycolate (TDM) in mycobacterial cell envelope. Herein, we confirmed that *fbpC3* was essential for the biotransformation of trehalose monomycolate (TMM) to TDM in *Mycolicibacterium neoaurum*. The deficiency of this gene raised the cell permeability, thereby enhancing the steroid uptake and utilization. The 9-OHAD yield in the *fbpC3*-deficient 9-OHAD-producing strain was increased by 21.3%. Moreover, the combined deletion of *fbpC3* and *embC* further increased the 9-OHAD yield compared to the single deletion of *fbpC3*. Finally, after 96 h of bioconversion in industrial resting cells, the 9-OHAD yield of 11.2 g/L was achieved from 20 g/L phytosterols and the productivity reached 0.116 g/L/h. In summary, this study suggested the critical role of the *fbpC3* gene in the synthesis of TDM in *M. neoaurum* and verified the feasibility of improving the bioconversion efficiency of phytosterols through the cell envelope engineering strategy.

1. Introduction

Biotransformation of low value-added phytosterols mainly extracted from vegetable oil processing waste or pine tree biomass to important steroidal intermediates is the key step in the semi-synthetic route of current steroidal pharmaceutical industry [1]. Actinobacteria, especially mycobacteria, play an essential role in the above bioconversion process [2,3]. From steroidal intermediates, almost all kinds of steroidal drugs can be produced by chemical modifications [4]. The inactivation of 3-ketosteroid-Δ^1-dehydrogenases (KstDs), which are key enzymes for initiating the degradation of steroidal nucleus, can block the sterol metabolic pathway of *Mycolicibacterium neoaurum*. Consequently, the obtained engineered strain could convert sterol substrates into an important steroidal intermediate, 9-OHAD [2]. However, due to its insufficient yield, the engineered strain was further improved to achieve a satisfying biotransformation efficiency of soybean phytosterols.

Sterols can be utilized as the sole carbon source and energy source for the basic physiological and metabolic activities of mycobacteria [5,6].

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The uptake of sterols by cells can be divided in two phases: the mass transfer of sterol particles to cell surface and the transport of sterol molecules through cell envelope [7,8]. Hence, cell envelope plays an essential role in the uptake of sterols. The outermost covering of cell envelope is typically composed of a variety of non-covalent binding capsular lipids, such as trehalose monomycolate (TMM), trehalose dimycolate (TDM), phenolic glycolipids, sulfolipids, and phospholipids [9,10]. A covalent binding layer of dense mycolyl-arabinogalactan-peptidoglycan (m-AG-PG) can be found on the inner side of the non-covalent layer (Fig. 1) [10,11]. This unique cell envelope leads to a low permeability and forms an effective barrier, which can prevent toxic compounds from entering mycobacterial cells [12,13]. However, the feature negatively affects the uptake of sterol substrates at the bioconversion to steroidal intermediates [8,14]. The deletion of *kasB* encoding a β-ketoacyl-acyl carrier protein synthase likely shortened the length of keto-mycolic acids, the key components of mycolic acid methyl esters (MAMEs) in the core m-AG-PG structure [8]. Moreover, the inactivation of arabinofuranosyltransferase gene *embC* caused a synthesis defect of the lipoarabinomannan from lipomannan [14,15]. The above two changes remarkably improved the cell permeability and enhanced the uptake of steroids. As a result, the accumulation efficiency of the target steroidal intermediate was further increased, whereas the inactivation of other dispensable genes (*hadA*, *hadC*, *mmaA1*, *mmaA3*, *mmaA4* or *pks13*) in the mycolic acid synthesis pathway did not show positive effects on steroid utilization [8].

TMM is the final product of mycolic acid synthesis pathway in the mycobacterial cytoplasm [16]. Synthesized TMM is then transported into periplasmic space for the mycoloyl residue transfer onto arabinogalactan to produce the m-AG, or used in the formation of the outer membrane cord factor TDM, the most abundant surface-exposed glycolipid of the mycobacterial cell envelope [10,16–19]. The complex of Ag85 composed of three secreted transesterases (Ag85A, Ag85B and Ag85C respectively encoded by *fbpA*, *fbpB*, and *fbpC*) was identified as the catalyzing enzyme for the assembly of the two lipids [20,21]. Up to now, at least two trehalose binding sites (active site and secondary site) in Ag85 enzymes were identified [20]. TMM firstly binds to secondary site for inducing a conformational change of the side chain at Phe^{232} in Ag85A/Ag85B and Leu^{230} in Ag85C after being transported through the membrane to periplasm [21]. Then, the molecule can enter active site to release a free trehalose, whereas the residual α chain of ester-linked mycolic acid is buried in a hydrophobic hole [21]. Finally, mycolic acid is selectively transferred onto 6′-hydroxy of TMM to form TDM or onto the 5-hydroxy of the terminal arabinose to yield m-AG [16]. Actually, a catalytic triad composed of a nucleophilic serine (Ag85C: Ser^{124}), histidine base (Ag85C: His^{260}), and glutamic acid (Ag85C: Glu^{228}) was employed to catalyze a ping–pong reaction mechanism for the mycolyl transfer [20,22,23]. A new potent inhibitor ebselen of *M. tuberculosis* Ag85 complex was identified to bind covalently to a Cys^{209} residue located near the Ag85C active site, thereby leading to a remarkably decreased enzymatic activity of Cys^{209} mutation [24,25]. Besides, nuclear magnetic resonance analysis revealed that another novel inhibitor I3-AG85 could bind Ag85C and thus block the biosynthesis of cord factor TDM in *M. tuberculosis* [17]. These studies suggested that the inactivation of *fbpC* suppressed the mycoloyl residue transfer in cell envelope synthesis of engineered *M. neoaurum* and might be a robust and efficient strategy to raise cell permeability. Since the *fbp* genes are likely synthetically lethal, the double deletion of them has not been reported [26]. It is necessary to evaluate the effects of the serial deletion of identified target genes involved in cell envelope synthesis of *M. neoaurum* [8,14]. As a consequence of these combined manipulations, the biotransformation efficiency of soybean phytosterols by the mutant strain might be boosted up to a new level.

In this study, the relationship between the located *fbpC3* and the synthesis of mycolic acids-related components in *M. neoaurum* was firstly evaluated. The changes in cell permeability caused by the disordered assembly of cell envelope and the chain effect on steroid uptake and utilization were then determined. Then, the biotransformation efficiency of soybean phytosterols in the *fbpC3*-deficient 9-OHAD-producing *M. neoaurum* strain was measured. Finally, the combined manipulation of the previously screened gene targets that influenced the accumulation of steroidal intermediates [8,14] and the newly identified *fbpC3* was performed to further increase 9-OHAD yield.

2. Materials and methods

2.1. Strains, plasmids, primers, media, and culture conditions

The strains used in this study are listed in Table 1. *M. neoaurum* ATCC 25795 (Mn) and *E. coli* DH5α were respectively purchased from American Type Culture Collection (ATCC) and TIANGEN Biotech. Co., Ltd. (Shanghai, China). The steroid drug intermediate 9-OHAD producer Mn*ΔkstD1ΔkstD2ΔkstD3* (WI) was constructed by Kang Yao [2]. Plasmids and primers used for constructing the mutants are shown in Supplementary Table S1 and Table S2. Recombinant plasmids were transformed into *E. coli* DH5α by the heat shock method or into *M. neoaurum* by electroporation as described previously [27,28].

The media and culture conditions in previous descriptions were adopted in the study [28]. *E. coli* was cultured in Luria–Bertani (LB)

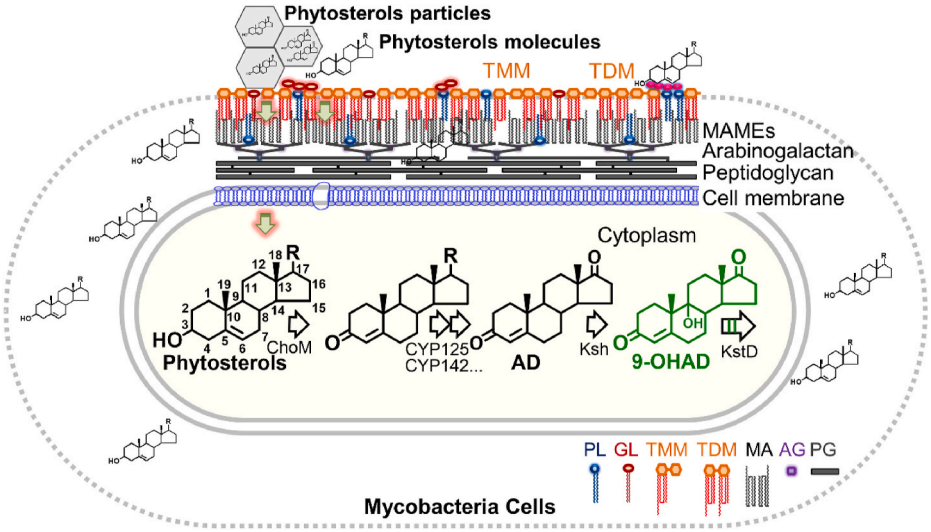

Fig. 1. Schematic diagram of the uptake and bioconversion of phytosterols by mycobacteria. The outer layer of cells is mainly composed of the non-covalent binding components, including TMM, TDM, glycolipid, phospholipid, etc. The core of the cell envelope is the dense covalent mycolyl-arabinogalactan-peptidoglycan layer. The uptake of sterols mainly relies on the direct contact between phytosterol substrates and cell surface. The steroidal drug intermediate (9-OHAD) can be produced through the incomplete metabolism of phytosterols in the engineered *M. neoaurum* strain. PL, phospholipid; GL, glycolipid; TMM, trehalose monomycolate; TDM, trehalose dimycolate; MA, mycolic acid methyl esters; AG, arabinogalactan; PG, peptidoglycan.

Table 1
Strains used in this study.

Names	Descriptions	Sources
E. coli DH5α	*E. coli* strain for cloning	TIANGEN Co., Ltd.
M. neoaurum ATCC 25795 (Mn)	Wild type strain, the starting strain	ATCC
Mn*ΔfbpC3*	*fbpC3* deleted in *M. neoaurum* ATCC 25795	This study
Mn*ΔfbpC3*+*fbpC3*	*fbpC3* complemented in Mn*ΔfbpC3* strain	This study
WI	*kstD1*, *kstD2* and *kstD3* deleted in *M. neoaurum* ATCC 25795, 9-OHAD-producing strain	Yao et al., 2014 [2]
WI*ΔfbpC3*	*fbpC3* deleted in WI strain	This study
WI*ΔfbpC3ΔkasB*	*fbpC3* and *kasB* deleted in WI strain	This study
WI*ΔfbpC3ΔembC*	*fbpC3* and *embC* deleted in WI strain	This study

medium at 37 °C and kanamycin (50 mg/L) or hygromycin (100 mg/L) was added in the corresponding media if necessary. *M. neoaurum* was firstly cultivated in LB medium and then inoculated in MYC/01 medium (20.0 g/L glycerol, 2.0 g/L citric acid, 2.0 g/L NH_4NO_3, 0.5 g/L K_2HPO_4, 0.5 g/L $MgSO_4{\cdot}7H_2O$, and 0.05 g/L ammonium ferric citrate (pH 7.5)) to obtain seed cells. For the purpose of determining growth phenotypes, the cells were inoculated into minimal medium (MM) (2.0 g/L NH_4NO_3, 0.5 g/L K_2HPO_4, 0.5 g/L $MgSO_4{\cdot}7H_2O$, and 0.05 g/L ammonium ferric citrate) containing 1 g/L cholesterol (purity >95.0%, Adamas Reagent, Co., Ltd., Shanghai, China). To assess the bioconversion efficiency, the cells were transferred into MYC/02 medium (10.0 g/L glucose, 2.0 g/L citric acid, 2.0 g/L NH_4NO_3, 0.5 g/L $MgSO_4{\cdot}7H_2O$, and 0.05 g/L ferric ammonium citrate (pH 7.5)) containing 5 g/L phytosterols. In order to determine the biotransformation efficiency of resting cells, the cells were inoculated into MYC/02 medium for 48 h–72 h. Then, the cultivated cells were harvested, washed with 20 mM KH_2PO_4, and resuspended to obtain 200 g/L suspensions. The transformation was performed with 100 g/L cells, 20 g/L phytosterols, and 80 g/L hydroxypropyl-β-cyclodextrin. The transformation experiment was carried out in shake flasks (30 °C and 200 rpm).

2.2. Gene manipulation in M. neoaurum

Target gene-deleted mutants were acquired through the allelic homologous recombination as previously described [28]. The plasmids p2NIL and pGOAL19 were employed to construct the homologous recombination plasmids. Briefly, the upstream and downstream sequences of a target gene were cloned, digested with restriction enzymes and ligated into p2NIL. A selection marker cassette from pGOAL19 was dissected and inserted into the recombinant p2NIL to construct the knockout-plasmids (Supplementary Table S1), which could be electroporated into *M. neoaurum* to obtain the corresponding deficient strains.

The coding frame of a wild-type target gene was firstly cloned, digested and then inserted into pMV261 and the constructed p261-gene was used to overexpress the function of the corresponding deficient gene. In addition, the expression cassette could be digested from the p261-gene and integrated into pMV306 to obtain a complementary p306-gene. The constructed plasmid could be then integrated into the mycobacterial chromosome DNA in a single copy.

2.3. Analysis of mycolic acid methyl esters

Mycobacterial cell MAMEs were extracted and determined as previously described [8]. Cultivated cells (50 mg, wet weight) were firstly collected at 12,000×*g* for 10 min. Then, after adding 0.5 mL of methanol and chloroform (2:1, v/v), collected cells were incubated at 60 °C for 2 h. Polar lipids were dissolved in the supernatant after the extraction solution was centrifuged at 12,000×*g* for 10 min.

MAMEs could be acquired from defatted cells by adding 500 μL of 10% tetrabutylammonium hydroxide (Sigma-Aldrich LLC., MO, USA) at 100 °C overnight. After cooling, 500 μL of ddH_2O, 250 μL of dichloromethane, and 62.5 μL of iodomethane (Sigma-Aldrich LLC., MO, USA) were added, then stirred for 30 min and centrifuged at 12,000×*g* for 10 min. The lower organic layer was washed respectively with 1.0 mL of hydrochloric acid (1 M) and 1.0 mL of ddH_2O. Crude MAMEs would be obtained after the organic layer was dried under a nitrogen stream. In order to further purify MAMEs, the resulting residue was dissolved in a mixture of toluene (0.2 mL) and acetonitrile (0.1 mL), followed by the addition of 0.2 mL of acetonitrile and incubation for 1 h at 4 °C. The mixture solution was centrifuged at 12,000×*g* for 10 min and then the obtained MAMEs were re-suspended in 200 μL of dichlormethane for further analysis.

Mycolic acids were analyzed by silica gel TLC plates. The mean grayscale intensity was analyzed with Quantity One (Version 4.6.6, Bio-Rad Laboratories, CA, USA). Relative abundances of polar mycolic acids (TMM and TDM) and nonpolar MAMEs were respectively calculated.

2.4. Analysis of cell permeability

After the manipulation of target genes, cell permeability was assessed according to the previous procedure [29]. Cultivated cells with the same wet weight were suspended in 4.5 mL of phosphate buffer (10^6 cells/mL), mixed with 0.5 mL of fluorescein diacetate (FDA) acetone solution (2 mg/mL) and vibrated at 32 °C for 10 min. Then, the fluorescence value of the obtained suspension was detected at the maximum excitation wavelength of 485 nm and the emission wavelength of 538 nm.

The quantity of cholest-4-en-3-one entering cells per unit time was determined as described [30]. The cells were cultured in MYC/02 medium containing 1.0 g/L cholest-4-en-3-one (purity >95.0%, Shanghai TITAN Scientific Co., Ltd., China) for 12 h and then 5 mL of the culture solution was harvested, centrifuged at 12,000×*g* for 10 min and washed with ddH_2O for two times, followed by the addition of 1.0 mL of the mixture of petroleum ether and ethyl acetate (6:4, v/v). Next, the cells were suspended in the mixture of acetonitrile and ddH_2O (7:3, v/v) (50 mg wet weight/mL). After adding 0.8 g of glass beads, the cells were destroyed with FastPrep-24 instrument (MP Biomedicals, CA, USA) and centrifuged at 12,000×*g* for 10 min. Then, steroids entering cells could be released and determined with a reversed-phase C18-column (250 mm × 4.6 mm) at 254 nm in HPLC system. The mixture of methanol and water (8:2, v/v) was used as the mobile phase.

2.5. Analysis of sterol biotransformation

The change in the transformation efficiency of constructed strains was assessed in the vegetative cell and resting cell conversion systems [8,14]. Briefly, the cells in the suspension culture (0.5 mL) were extracted with the same volume of ethyl acetate (0.5 mL). The resting cell conversion suspension (0.1 mL) was extracted with 1.0 mL of ethyl acetate. After centrifugation at 12000*g* for 20 min, the upper organic phase containing steroids was analyzed with high-performance liquid chromatography (HPLC) or gas chromatography (GC).

A 1100 series HPLC system (Agilent Technologies, CA, USA) was used to analyze the target product 9-OHAD. The samples were determined with a reversed-phase C18-column (250 mm × 4.6 mm) (Agilent Technologies, CA, USA) at a wavelength of 254 nm. The mobile phase was the mixture of methanol and water (8:2, v/v). A GC system 7820A (Agilent Technologies, CA, USA) was used to determine sterol substrates. The samples were detected with a DB-5 column (30 m × 0.25 mm (i.d.) × 0.25 μm film thickness, Agilent Technologies, CA, USA). The oven temperature was controlled as follows: 200 °C for 2 min, 200 °C–280 °C within 4 min, 280 °C for 2 min, 280 °C–305 °C within 1.5 min, and 305 °C for 10 min. Inlet temperature and flame-ionization detector temperature were maintained at 320 °C. The flow rate of nitrogen carrier gas was 2 mL/min at 50 °C.

3. Results and discussion

3.1. FbpC3 is the key factor for the assembly of TDM in M. neoaurum

To further improve the bioconversion efficiency, the *fbpC* genes involved in the assembly of TMM after the transportation from cytoplasm to periplasm was explored. First of all, a gene *fbpC3* (GenBank Accession No. NZ_JMDW01000024.1; Region: 26,764 … 27,720, 957-bp) annotated for encoding the Ag85C protein was located in the genome of *M. neoaurum* ATCC 25795 (GenBank Accession No. NZ_JMDW00000000.1). Then, the gene was tentatively deleted from the wild-type strain for the subsequent phenotype identification. Briefly, a 1065-bp upstream sequence and a 1053-bp downstream sequence of the *fbpC3* coding sequence were cloned into the knockout plasmid for the allelic homologous recombination of the wild-type gene (Fig. 2A and B). Therefore, the gene *fbpC3* was theoretically mutated and the Mn*ΔfbpC3* strain was constructed accordingly. Interestingly, the deletion of *fbpC3* also accelerated cell growth in the presence of cholesterol (Fig. 2C), similar to the effect of the deletion of *kasB* and *embC* in the strain [8,14]. This phenotypic change might be ascribed to the changed FbpC3-responsible cell envelope assembly.

Next, the mycolic acids-related components in the mutant strain were extracted and analyzed. The polar lipids displayed significant differences in the Mn*ΔfbpC3* strain (Fig. 2D). The synthesis of TDM was thoroughly blocked, but the TMM component was not significantly changed. The relative abundances of polar TMM and TDM in the Mn*ΔfbpC3* strain were 96.0% and 4.0%, whereas the percentages of the two components in the wild-type Mn strain were respectively 86.2% and 13.8% (Fig. 2E). In a word, the TDM content was decreased by 245% approximately after the deletion of *fbpC3* and this phenotype was restored in the *fpbC3*-complemented strain, indicating that the located gene *fbpC3* empowered the strain to synthesize TDM. Additionally, MAMEs showed no obvious difference between the Mn*ΔfbpC3* strain and the parental strain (Supplementary Fig. S1), suggesting that *fbpC3* was possibly irrelevant to the MAMEs synthesis.

3.2. Deleting fbpC3 raised cell permeability of M. neoaurum

After the deletion of *fbpC3* in *M. neoaurum*, the Mn*ΔfbpC3* strain showed a determined defect in TDM assembly compared to that of its parental strain. It was speculated that this change might improve cell permeability and enhance the utilization of sterol substrates. To confirm this speculation, cell permeability of *fbpC3*-deficient strain was evaluated through the fluorescein diacetate (FDA) assay [8]. The mutant strain displayed an obvious improvement in fluorescence intensity (Fig. 3A). When incubation time increased from 10 to 30 min, the improvement in fluorescence intensity of Mn*ΔfbpC3* strain compared to that of the Mn strain increased from 15.0% to 23.3%, indicating that the penetrated FDA in the *fbpC3*-deficient cells was raised significantly. In other words, the permeability of the mutant strain might be improved by the deletion of *fbpC3*.

Based on the above results, the analog of cholesterol, cholest-4-en-3-one, was then used as a label to evaluate the cell permeability to steroids [30]. The determination of the steroids entering cells revealed that the enhanced cell permeability led to about an increase of 55% in the uptake of cholest-4-en-3-one by the Mn*ΔfbpC3* strain compared to that of the Mn strain (Fig. 3B). Then, the utilization efficiency of the sterol substrate in the *fbpC3*-deleted strain was further analyzed. The utilization efficiency of cholesterol in the Mn*ΔfbpC3* strain was raised indeed (Supplementary Fig. S2). Under the same cultivation conditions, the residual

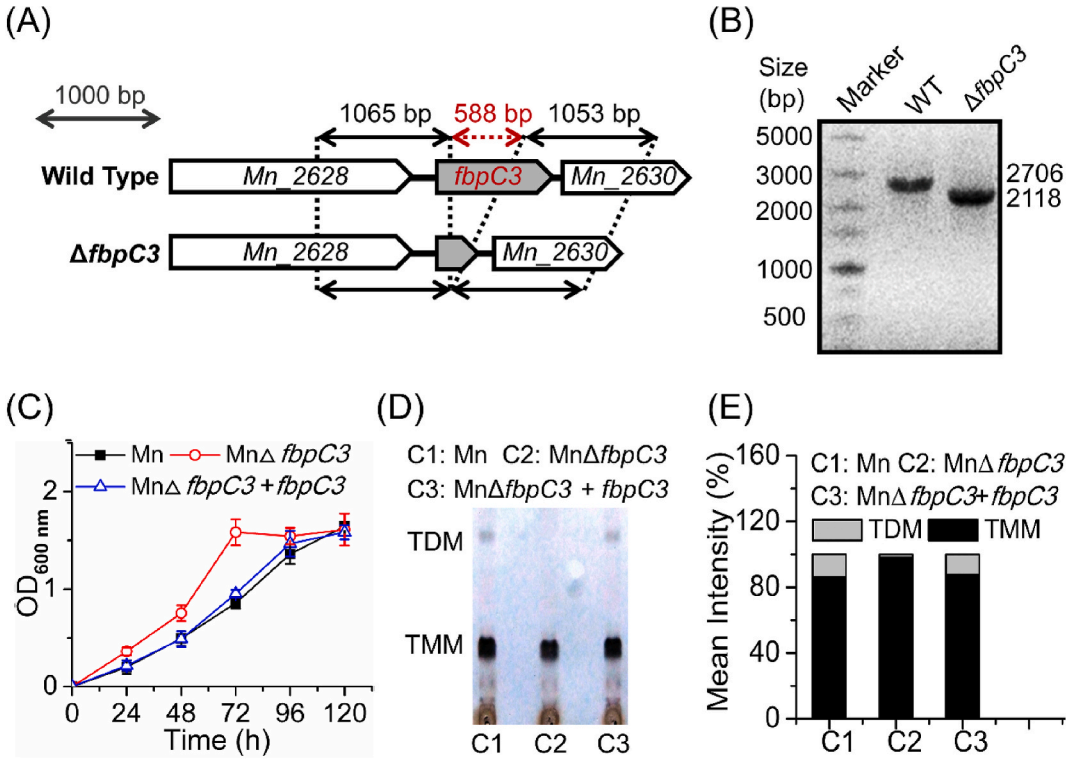

Fig. 2. Inactivation of *fbpC3* caused the assembly deficiency of TDM in *M. neoaurum*. (A) Schematic diagram of the deletion of *fbpC3* gene from the genome of *M. neoaurum* ATCC 25795. A 1065-bp upstream sequence and a 1053-bp downstream sequence were designed to replace the wild-type gene. (B) Validation results of allelic replacement at the located *fbpC3* in the strain. (C) Growth characteristics of the wide-type strain Mn, the *fbpC3*-deficient strain (Mn*ΔfbpC3*) and the *fbpC3*-complemented strain (Mn*ΔfbpC3*+*fbpC3*) in the MM containing 1.0 g/L cholesterol. (D) Content analysis of polar lipids (TMM and TDM) in the mycobacterial cell envelope. (E) Calculated percentage of the relative gray intensity of polar lipids. Deletion of *fbpC3* caused the synthesis deficiency of TDM in *M. neoaurum*. The relative abundances of TMM and TDM were 96.0% and 4.0% in the *fbpC3*-deleted strain, whereas the percentages of the two components in the Mn strain was 86.2% and 13.8%, respectively.

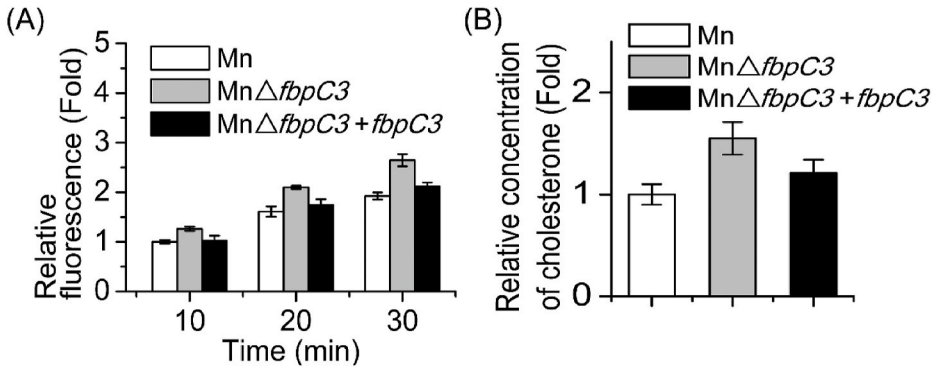

Fig. 3. Knockout of *fbpC3* increased cell permeability of *M. neoaurum*. (A) Determination of cell permeability in the *fbpC3*-deficient strain. Mycobacterial cells were stained with FDA and analyzed with a fluorescence spectrophotometer. (B) Effects of the deletion of *fbpC3* on the uptake of steroid cholest-4-en-3-one. Cells were cultivated in MM containing 1.0 g/L cholest-4-en-3-one. Data represent mean ± standard deviation of three measurements.

cholesterol content in the culture medium for the *fbpC3*-mutant strain was respectively 1.52 g/L at 48 h and 0.43 g/L at 96 h, whereas that of Mn strain was 1.72 g/L at 48 h and 0.66 g/L at 96 h. The phenotypic change might be interpreted as a chain effect caused by the improved cell permeability. In other words, it was confirmed that the improved cell permeability was associated with the deletion of *fbpC3*. Therefore, the uptake and utilization of steroids was accelerated.

3.3. Increasing the accumulation efficiency of 9-OHAD by deleting fbpC3

To assess the effect of the improved cell permeability caused by the decreased content of polar TDM on the bioconversion of target steroidal intermediates, a new WIΔ*fbpC3* strain was constructed based on the engineered 9-OHAD-producing strain WI [2]. In the biotransformation conditions, the vegetative cells of WIΔ*fbpC3* strain yielded 0.31 g/L 9-OHAD after 24 h of conversion, whereas its parental strain WI yielded 0.18 g/L 9-OHAD after 24 h of conversion. The accelerated accumulation of 9-OHAD was observed at all the subsequent three sampling time points. Ultimately, the deletion of *fbpC3* increased the 9-OHAD yield from 1.31 g/L to 1.59 g/L after 96 h of conversion. The transformation efficiency of WIΔ*fbpC3* strain was at least 21.3% higher than that of the WI strain in the above biotransformation conditions (Fig. 4A).

Subsequently, the resting cell transformation system was employed to measure the conversion efficiency of strains per unit wet weight. After 72 h of conversion, the highest increase was observed in WIΔ*fbpC3* strain and the yield reached 8.9 g/L (Fig. 4B), which was 35.1% higher than that of the parental strain. After 120 h of bioconversion, the WIΔ*fbpC3* strain produced 10.7 g/L 9-OHAD and the molar yield reached 68.2%, which was 20.3% higher than that of its parental strain WI. The above data showed that the deletion of *fbpC3* in the engineered 9-OHAD-producing strain indeed enhanced the bioconversion of soybean phytosterols to the target steroidal intermediate 9-OHAD.

3.4. Effects of the combined deletion of fbpC3 and other modified sites on the 9-OHAD yield

In previous studies [8,14], the independent inactivation of *kasB* or *embC* raised the productivity of 9-OHAD-producing strain. The combined deletion of screened target genes might have an additive enhancement effect on the yield of target intermediate. Then, we deleted the gene *kasB* of strain WIΔ*fbpC3* to obtain the WIΔ*fbpC3*Δ*kasB* strain. Unexpectedly, the yield of target product in the obtained strain WIΔ*fbpC3*Δ*kasB* did not show the additive enhancement effect compared to the WIΔ*fbpC3* strain. The 9-OHAD yield in the WIΔ*fbpC3*Δ*kasB* strain declined to about 0.79 g/L after 96 h of conversion under the same cultivation conditions (Fig. 4A). Therefore, the combined deletion of two genes (*fbpC3* and *kasB*) involved in the synthesis and assembly of mycolic acids-related components might not be an efficient method for further enhancing the bioconversion efficiency.

The gene *embC* is responsible for the assembly of lipoarabinomannan, which is one of the non-covalent lipoglycan component in the cell envelope [14]. Hence, *embC* was tentatively deleted from

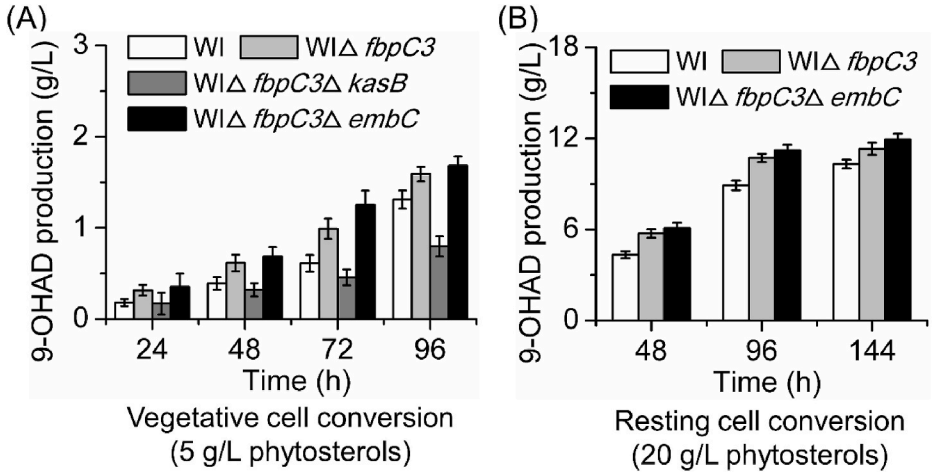

Fig. 4. Assessment of the influences of the deletion of multiple genes involved in the cell wall synthesis on the bioconversion of phytosterols to 9-OHAD. (A) Preliminary determination of 9-OHAD yield in the vegetative cell transformation system containing 5 g/L phytosterols. (B) Assessment results of the constructed 9-OHAD-producing strain with a resting cell conversion system containing 20 g/L phytosterols. Data represent mean ± standard deviation of three measurements.

the genome of WIΔ*fbpC3* strain to generate the strain WIΔ*fbpC3*Δ*embC*. Interestingly, this mutant strain displayed some additive effect on the biotransformation of soybean phytosterols to target product 9-OHAD in the shake flask experiments (Fig. 4A). The highest improvement was determined in the newly constructed WIΔ*fbpC3*Δ*embC* strain after 72 h of conversion and the 9-OHAD yield reached 1.25 g/L, which was about 105% higher than that of the WI strain. The serial deletion of *fbpC3* and *embC* finally increased the 9-OHAD to 1.68 g/L after 96 h of conversion. In addition, during the subsequent assessment with the resting cell bioconversion system, after 96 h of conversion, the 9-OHAD yield in the WIΔ*fbpC3*Δ*embC* strain increased to 11.2 g/L with the productivity of 0.116 g/L/h and the molar yield was 71.4%, which was higher than that the strain WIΔ*kasB* (69.5%) [8] and the strain WIΔ*fbpC3* (68.2%) (Fig. 4B). In a word, after 96 h of biotransformation, the 9-OHAD yield in the WIΔ*fbpC3*Δ*embC* strain was improved by 26% compared to that in the WI strain. When transformation time was increased to 144 h, the 9-OHAD yield of the WIΔ*fbpC3*Δ*embC* strain reached about 11.9 g/L, which was the highest in all the reported data of existing 9-OHAD-producing *M. neoaurum* strains [8,14,28,31].

Actually, a total of four *fbpC* genes were annotated in the genome of *M. neoaurum* (Supplementary Table S3). However, the inactivation of *fbpC1*, *fbpC2*, or *fbpC4* in the *M. neoaurum* did not show any observable phenotype change (data not shown). Only the *fbpC3*-deficient strain displayed an expected enhancement on the 9-OHAD production, indicating that *fbpC3* possibly played an essential role in the synthesis of 9-OHAD. The serial inactivation of *fbpC3* and *kasB* theoretically caused defects on the synthesis of the polar TDM as well as the nonpolar meromycolic acids in the major frame of the core m-AG-PG structure in the *M. neoaurum* strain [8]. Consequently, the growth of the WIΔ*fbpC3*Δ*kasB* strain was extremely suppressed in the transformation medium in the presence of soybean phytosterols (data not shown). In contrast, the deletion of *fbpC3* and *embC* resulted in the deficient mutant on the assembly of TDM and lipoarabinomannan [14]. The combination of the two defects in the cell envelope synthesis displayed different superposition effects on the transformation production of the engineered strain. Thus, the excessive modifications on the synthesis and assembly pathway of similar components in *M. neoaurum* might be not a reasonable strategy for improving the bioconversion of soybean phytosterols. The intracellular metabolism rate is another critical factor influencing the overall transformation efficiency in the engineered strain, suggesting that the combined enhancement of the mass transfer, the transport of sterols into cells as well as the intracellular catabolism of sterols might be a promising breakthrough for evolving the transforming strain.

4. Conclusions

In summary, this study confirmed that *fbpC3* was a key gene responsible for the assembly of polar TDM in the cell envelope of *M. neoaurum*. The inactivation of this gene was proved to be an effective way to increase the production of the steroidal intermediate 9-OHAD. However, interestingly, the joint deletion of *fbpC3* and *kasB* did not display the desired promotion effect on 9-OHAD accumulation, whereas the combined deletion of *fbpC3* and *embC* achieved an expected additive enhancement effect on the production of 9-OHAD. Ultimately, in the WIΔ*fbpC3*Δ*embC* strain under the resting cell transformation system containing 20 g/L of phytosterols, the 9-OHAD yield increased to 11.9 g/L and the molar yield reached 75.8%.

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CRediT authorship contribution statement

Liang-Bin Xiong: Methodology, Formal analysis, Investigation, Writing – original draft, Funding acquisition. **Hao-Hao Liu:** Methodology, Validation, Formal analysis, Investigation, Writing – original draft. **Lu Song:** Methodology, Validation, Formal analysis. **Miao-Miao Dong:** Investigation. **Jie Ke:** Investigation. **Yong-Jun Liu:** Investigation. **Ke Liu:** Investigation. **Ming Zhao:** Writing – review & editing, Funding acquisition. **Feng-Qing Wang:** Conceptualization, Methodology, Formal analysis, Investigation, Supervision, Writing – review & editing, Funding acquisition. **Dong-Zhi Wei:** Supervision, Funding acquisition.

Declaration of competing interest

The authors declared that they have no conflicts of interest to this work. The manuscript is approved by all authors for publication. I would like to declare on behalf of my co-authors that the work described was original research that has not been published previously, and not under consideration for publication elsewhere, in whole or in part. All the authors listed have approved the manuscript that is enclosed.

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Appendix A. Supplementary data

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References

[1] Fernández-Cabezón L, Galán B, García JL. New insights on steroid biotechnology. Front Microbiol 2018;9:958.
[2] Yao K, Xu LQ, Wang FQ, Wei DZ. 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. Metab Eng 2014;24:181–91.
[3] Xu LQ, Liu YJ, Yao K, Liu HH, Tao XY, Wang FQ, et al. Unraveling and engineering the production of 23,24-bisnorcholenic steroids in sterol metabolism. Sci Rep 2016;6:21928.
[4] Donova MV, Egorova OV. Microbial steroid transformations: current state and prospects. Appl Microbiol Biotechnol 2012;94:1423–47.
[5] Pandey AK, Sassetti CM. Mycobacterial persistence requires the utilization of host cholesterol. Proc Natl Acad Sci Unit States Am 2008;105:4376–80.
[6] Fernández-Cabezón L, Galán B, García JL. Unravelling a new catabolic pathway of C-19 steroids in *Mycobacterium smegmatis*. Environ Microbiol 2018;20:1815–27.
[7] Donova MV, Nikolayeva VM, Dovbnya DV, Gulevskaya SA, Suzina NE. Methyl-β-cyclodextrin alters growth, activity and cell envelope features of sterol-transforming mycobacteria. Microbiology 2007;153:1981–92.
[8] Xiong LB, Liu HH, Zhao M, Liu YJ, Song L, Xie ZY, et al. Enhancing the bioconversion of phytosterols to steroidal intermediates by the deficiency of *kasB* in the cell wall synthesis of *Mycobacterium neoaurum*. Microb Cell Factories 2020;19:80.
[9] Draper P. The outer parts of the mycobacterial envelope as permeability barriers. Front Biosci 1998;3:D1253–61.
[10] Tahlan K, Wilson R, Kastrinsky DB, Arora K, Nair V, Fischer E, et al. SQ109 targets MmpL3, a membrane transporter of trehalose monomycolate involved in mycolic acid donation to the cell wall core of *Mycobacterium tuberculosis*. Antimicrob Agents Ch 2012;56:1797–809.
[11] Kaur D, Guerin ME, Skovierová H, Brennan PJ, Jackson M. Biogenesis of the cell wall and other glycoconjugates of *Mycobacterium tuberculosis*. Adv Appl Microbiol 2009;69:23–78.
[12] Nguyen L, Chinnapapagari S, Thompson CJ. FbpA-dependent biosynthesis of rrehalose dimycolate is required for the intrinsic multidrug resistance, cell wall structure, and colonial morphology of *Mycobacterium smegmatis*. J Bacteriol 2005;187:6603–11.

[13] Jankute M, Grover S, Rana AK, Besra GS. Arabinogalactan and lipoarabinomannan biosynthesis: structure, biogenesis and their potential as drug targets. Future Microbiol 2012;7:129–47.
[14] Xiong LB, Liu HH, Song XW, Meng XG, Liu XZ, Ji YQ, et al. Improving the biotransformation of phytosterols to 9*α*-hydroxy-4-androstene-3,17-dione by deleting *embC* associated with the assembly of cell envelope in *Mycobacterium neoaurum*. J Biotechnol 2020;323:341–6.
[15] Korkegian A, Roberts DM, Blair R, Parish T. Mutations in the essential arabinosyltransferase EmbC lead to alterations in *Mycobacterium tuberculosis* lipoarabinomannan. J Biol Chem 2014;289:35172–81.
[16] Marrakchi H, Lanéelle M-A, Daffé M. Mycolic acids: structures, biosynthesis, and beyond. Chem Biol 2014;21:67–85.
[17] Warrier M, Tropis M, Werngren J, Diehl A, Gengenbacher M, Schlegel B, et al. Antigen 85C inhibition restricts *Mycobacterium tuberculosis* growth through disruption of cord factor biosynthesis. Antimicrob Agents Ch 2012;56:1735–43.
[18] Grzegorzewicz AE, Pham H, Gundi VA, Scherman MS, North EJ, Hess T, et al. Inhibition of mycolic acid transport across the *Mycobacterium tuberculosis* plasma membrane. Nat Chem Biol 2012;8:334–41.
[19] Belisle JT, Vissa VD, Sievert T, Takayama K, Brennan PJ, Besra GS. Role of the major antigen of *Mycobacterium tuberculosis* in cell wall biogenesis. Science 1997; 276:1420–2.
[20] Anderson DH, Harth G, Horwitz MA, Eisenberg D. An interfacial mechanism and a class of inhibitors inferred from two crystal structures of the *Mycobacterium tuberculosis* 30 kDa major secretory protein (Antigen 85B), a mycolyl transferase. J Mol Biol 2001;307:671–81.
[21] Goins CM, Dajnowicz S, Smith MD, Parks JM, Ronning DR. Mycolyltransferase from *Mycobacterium tuberculosis* in covalent complex with tetrahydrolipstatin provides insights into Antigen 85 catalysis. J Biol Chem 2018;293:3651–62.
[22] Ronning DR, Klabunde T, Besra GS, Vissa VD, Belisle JT, Sacchettini JC. Crystal structure of the secreted form of antigen 85C reveals potential targets for mycobacterial drugs and vaccines. Nat Struct Biol 2000;7:141–6.
[23] Ronning DR, Vissa V, Besra GS, Belisle JT, Sacchettini JC. Mycobacterium tuberculosis antigen 85A and 85C structures confirm binding orientation and conserved substrate specificity. J Biol Chem 2004;279:36771–7.
[24] Favrot L, Grzegorzewicz AE, Lajiness DH, Marvin RK, Boucau J, Isailovic D, et al. Mechanism of inhibition of *Mycobacterium tuberculosis* antigen 85 by ebselen. Nat Commun 2013;4:2748.
[25] Goins CM, Dajnowicz S, Thanna S, Sucheck SJ, Parks DR, Ronning DR. Exploring covalent allosteric inhibition of antigen 85C from *Mycobacterium tuberculosis* by ebselen derivatives. ACS Infect Dis 2017;3:378–87.
[26] Armitige LY, Jagannath C, Wanger AR, Norris SJ. Disruption of the genes encoding antigen 85A and antigen 85B of *Mycobacterium tuberculosis* H37Rv: effect on growth in culture and in macrophages. Infect Immun 2000;68:767–78.
[27] Yao K, Wang FQ, Zhang HC, Wei DZ. Identification and engineering of cholesterol oxidases involved in the initial step of sterols catabolism in *Mycobacterium neoaurum*. Metab Eng 2013;15:75–87.
[28] Xiong LB, Liu HH, Xu LQ, Wei DZ, Wang FQ. Role identification and application of SigD in the transformation of soybean phytosterol to 9*α*-hydroxy-4-androstene-3,17-dione in *Mycobacterium neoaurum*. J Agric Food Chem 2017;65:626–31.
[29] Shen YB, Wang M, Zhang LT, Ma YH, Ma B, Zheng Y, et al. Effects of hydroxypropyl-β-cyclodextrin on cell growth, activity, and integrity of steroid-transforming *Arthrobacter simplex* and *Mycobacterium* sp. Appl Microbiol Biotechnol 2011;90:1995–2003.
[30] Xiong LB, Liu HH, Xu LQ, Sun WJ, Wang FQ, Wei DZ. Improving the production of 22-hydroxy-23,24-bisnorchol-4-ene-3-one from sterols in *Mycobacterium neoaurum* by increasing cell permeability and modifying multiple genes. Microb Cell Factories 2017;16:89.
[31] Xiong LB, Sun WJ, Liu YJ, Wang FQ, Wei DZ. Enhancement of 9α-hydroxy-4-androstene-3,17-dione production from soybean phytosterols by deficiency of a regulated intramembrane proteolysis metalloprotease in *Mycobacterium neoaurum*. J Agric Food Chem 2017;65:10520–5.