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Creation and Characterization of *Mycolicibacterium Smegmatis* mc^{2}155 with Deletions in Genes Encoding Sterol Oxidation Enzymes

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Abstract. The fast-growing saprotrophic strain *Mycolicibacterium smegmatis* mc^{2}155 is capable of utilizing plant and animal sterols and can be used for creation of genetically engineered strains producing biologically active steroids. Oxidation of the 3β-hydroxyl group and $\Delta^{5(6)}\rightarrow\Delta^{4(5)}$ double bond isomerization followed by formation of stenones from sterols are considered as the initial stage of steroid catabolism in some actinobacteria. The study of the mechanism of steroid nucleus 3β-hydroxyl group oxidation is relevant for the creation of a method of the microbiological production of valuable 3β-hydroxy-5-en-steroids. A mutant strain of *M. smegmatis* with deletions in three genes (*MSMEG_1604, MSMEG_5228* and *MSMEG_5233*) encoding known enzymes exhibiting 3β-hydroxysteroid dehydrogenase activity was constructed by homologous recombination coupled with double selection. The resulting mutant retained macromorphological properties and the ability to convert cholesterol. 3-Keto-4-en-steroids were found among the sterol catabolism intermediates. Experimentally obtained data indicate the presence of a previously undetected intracellular enzyme that performs the function of 3β-hydroxysteroid dehydrogenase/$\Delta^{5(6)}\rightarrow\Delta^{4(5)}$ isomerase.

1 Introduction

The study of the mechanisms of oxidation of the steroid nucleus remains relevant in the development of technologies for the microbiological production of biologically active steroids with a 3β-hydroxy-5-ene structure.

The strain of actinobacteria *Mycolicibacterium smegmatis* mc^{2}155 is characterized by rapid growth, absence of pathogenicity and high frequency of transformation, simplifying its genetic modification. The *M.smegmatis* strain is able to efficiently absorb and use sterols as a carbon source [1], has biotechnological potential for genetic engineering to create strains producing pharmaceutically valuable steroids [2-4]. The initial stage of sterol

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catabolism by actinobacteria starts with the 3β-hydroxyl group oxidation and the subsequent double bond $\Delta^{5(6)} \rightarrow \Delta^{4(5)}$ isomerization resulted in the formation of 3-keto-4-ene-steroids – stenones [5].

Three genes have been found in *M. smegmatis* that encode enzymes involved in the conversion of cholesterol into cholestenone. The constitutively expressed *MSMEG_1604* gene encoding cholesterol oxidase (ChoD) is similar to the *Rv3409c* gene of *Mycobacterium tuberculosis* cholesterol oxidase. 3β-Hydroxysteroid dehydrogenase/$\Delta^{5(6)}\rightarrow\Delta^{4(5)}$ isomerase (HsdD) encoded by the *MSMEG_5228* gene, on the contrary, is induced in the presence of cholesterol. Additional cholesterol-induced dehydrogenase/isomerase is a product of the *MSMEG_5233* gene. It is known that mutant strains *M. smegmatis* mc^{2}155 Δ(*choD*, *hsdD*) and *M. smegmatis* mc^{2}155 Δ(*hsdD*, *MSMEG_5233*) retain the ability to grow in the presence of sterols as the only carbon source, but exhibit reduced 3β-hydroxysteroid dehydrogenase activity [6, 7].

The aim of this study is to obtain a mutant strain of *M. smegmatis* with deletions in three genes encoding known enzymes of the initial stage of sterol catabolism, and to evaluate its cholesterol-transforming activity.

2 Materials and methods

2.1 Reagents

Steroids: cholesterol was obtained from the company "Solvay Duphar" (Netherlands); cholestenone, androstenedione (AD), androstadienedione (ADD) - from the company "Steraloids" (USA). Bactopepton, bactoagar, yeast extract ("Panreac", Spain), ampicillin hygromycin ("Thermo Fisher Scientific", USA), ethidium bromide ("Serva", Germany); methyl-β-cyclodextrin (MCD) ("Wacker Chemie", Germany) were used; all other reagents were of the highest purity grade and were purchased from domestic commercial suppliers (Russia).

2.2 Bacterial strains and conditions for their maintenance

The bacterial strains used in the work are listed in Table 1. *M. smegmatis* cultures were maintained on M3 medium [8]. The growth of mycolicibacteria was evaluated on a minimal agar medium Middlebrook 7H9 Broth [9] in the presence of 0.5 g/l cholesterol and 8.5 g/l MCD. The strain of *Escherichia coli* DH5α was grown on LB medium [10].

Table 1. Bacterial strains, plasmids and oligonucleotide primers

Object	Description	Source / link
	Strains	
	Escherichia coli	
DH5α	F^-, *endA1*, *glnV44*, *thi-1*, *recA1*, *relA1*, *gyrA96*, *deoR*, *nupG*, *purB20*, φ80d*lacZ*ΔM15, Δ(*lacZYA-argF*)U169, hsdR17($r_K^- m_K^+$), λ^-	Invitrogen
	Mycolicibacterium smegmatis	
mc^{2}155, WT	Efficient at transforming by plasmids mutant variant mc^{2}6, *ept-1*	[11]
CH	Double mutant mc^{2}155 Δ(*choD, hsdD*)	[7]
CHE	Triple mutant mc^{2}155 Δ(*choD, hsdD, MSMEG_5233*)	This work
	Plasmids	
pJL020	Suicidal vector for knockout mutagenesis, *sacB*, Hyg^R	[12]

pJE	pJL020, carrying the *MSMEG_5233_AB* construct with the mutant allele of the *MSMEG_5233* gene	This work
Primers		
MSMEG_5233_A_F	5'-ccgagggatccagttcggttcccagcgcgatc-3', BamHI site (underlined)	This work
MSMEG_5233_A_R	5′-gagcagttgcggccagctgtggtgcccggttgctgatttgcggttggc-3′	This work
MSMEG_5233_B_F	5′-gccaaccgcaaatcagcaaccgggcaccacagctggccgcaactgctc-3′	This work
MSMEG_5233_B_R	5′-atccaccagcgcgcggcacagc - 3′	This work

2.3 Knockout of the *MSMEG_5233* gene

Mutant cells were obtained using the suicide vector pJL020 encoding hygromycin resistance and sucrose sensitivity due to the levansucrase *sucB* gene [12]. The DNA regions flanking the sequence of the *MSMEG_5233* gene upstream (A) and downstream (B) were amplified by PCR using oligonucleotide primers specified in Table 1 with the chromosomal DNA of *M. smegmatis* mc^{2}155. Mutual splicing of flank amplicons *MSMEG_5233_A* and *MSMEG_5233_B* was performed by overlapping PCR. The resulting fragment *MSMEG_5233_AB* was cloned in the vector pJL020 by the restriction site BamHI.

The pJE plasmid constructed in this way (Table 1) was used for directed mutagenesis. The competent *M. smegmatis* mc^{2}155 Δ(*choD*, *hsdD*) (CH) cells were transformed by electrtoporation with pJE plasmid according to a known protocol [13]. Selection of transformants was carried out on an agar nutrient medium M3 containing hygromycin (75 µg/ml). Transformants were grown in 5 ml of liquid medium M3 for the second act of recombination. The bacteria were selected for their ability to grow on a dense agar medium M3 containing sucrose (50 g/l) [12]. The presence of the deletion was confirmed by PCR using external primers MSMEG_5233_A_F and MSMEG_5233_B_R. The resulting strain of *M. smegmatis* (CHE) with deletions in three genes simultaneously *MSMEG_1604* (*choD*), *MSMEG_5228* (*hsdD*) and *MSMEG_5233* was used for further work.

2.4 Cholesterol biotransformation

Cultures of mutant *M. smegmatis* CH and CHE were inoculated to 50 ml of M3 medium containing 5 g/l cholesterol and 25.4 g/l MCD and grown under aerobic conditions at 37°C and 200 rpm. Products of cholesterol bioconversion were analysed by thin-layer chromatography (TLC).

2.5 Bioconversion of cholesterol by the cell-free culture liquid *M. smegmatis*

M. smegmatis CHE was cultured in 50 ml of medium M3. After 15 hours of growth, cholesterol (20 mg/l) was added as an inducer, cultures continued to grow for 9 hours. In the control, the inducer was not added. Bacterial cells were pelleted by centrifugation (40 min, 4200 g, 4°C). The supernatant was filtered; cholesterol was introduced into the filtrate to a final concentration of 0.05 g/l. Steroids were controlled by TLC as described earlier [8].

3 Results and discussion

3.1 Knockout of the *MSMEG_5233* gene in *M. smegmatis* mc^{2}155 Δ(*choD*, *hsdD*)

For mutagenesis, plasmid pJE containing variant of the target gene with an internal deletion (Δ*MSMEG_5233*) was created. This plasmid was transferred to *M. smegmatis* mc^2155 Δ(*choD*, *hsdD*) cells. Hygromycin-resistant transformants were selected, as the products of the first crossing-over resulting in the integration of the pJE plasmid into the cell chromosome. Due to the presence of a counter-selection marker, the *sacB* levansucrase gene cassette, bacterial clones growing in the presence of sucrose and losing antibiotic resistance were selected.

The presence of the mutant allele Δ*MSMEG_5233* in the genome of double recombinants was confirmed by PCR (Figure 1) by detecting ~2.18 kb DNA fragment, whereas in the case of a wild-type gene, amplicons of ~2.92 kb are detected. Thus, a strain of *M. smegmatis* (CHE) was constructed with simultaneous deletions in the genes encoding enzymes catalysing oxidation of steroid nucleus 3β-hydroxyl group.

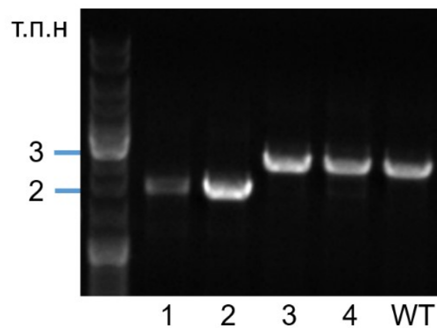

Fig. 1. Electrophoregram of PCR screening of mutant clones of *M. smegmatis* mc^2155 Δ(*choD*, *hsdD*), presumably carrying a deletion in the *MSMEG_5233* gene (1-4). WT – *M. smegmatis* mc^2155.

3.2 Oxidation of sterols by mutant mycolicibacteria

As can be seen from Fig.2, *M. smegmatis* cultures in the presence of cholesterol show more active growth regardless of the number of mutations. The triple mutant CHE has a similar colony macromorphology with both the precursor strain CH and the parent *M. smegmatis* mc^2155 (WT).

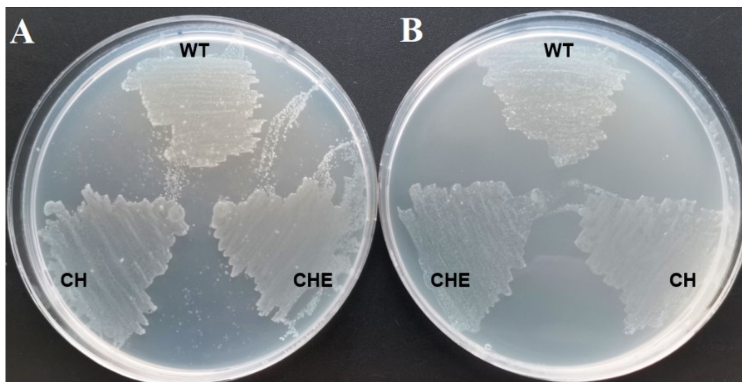

Fig. 2. Evaluation of the growth of mutant strains of *M. smegmatis* mc ^{2} 155 on minimal media. A – mineral medium with cholesterol. B — mineral medium without cholesterol. WT – *M. smegmatis* mc^2155, CH – *M. smegmatis* mc^2155 Δ(*choD*, *hsdD*), CHE - *M. smegmatis* mc^2155 Δ(*choD*, *hsdD*, *MSMEG_5233*).

Like a parental strain, the triple deletion mutant CHE demonstrated the preservation of the ability to utilize sterols (Figure 3) with a complete loss of the substrate within 72 hours of growth. Intermediates with 3-keto-4-ene structure, such as androstenedione and androstadienedione, typical for sterol catabolism by actinobacteria, were found among the conversion products [1, 14].

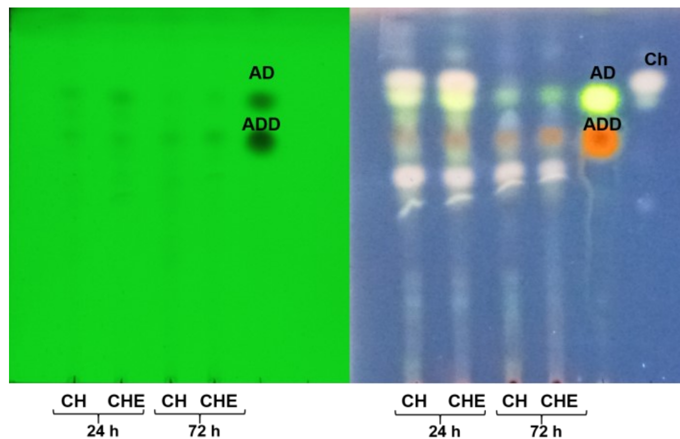

Fig. 3. Analysis of cholesterol bioconversion products by mutant strains of *M. smegmatis* mc[2]155 by TLC method. A – plate before staining. B – plate after staining with manganese chloride. CH – *M. smegmatis* mc[2]155 Δ(*choD*, *hsdD*), CHE - *M. smegmatis* mc[2]155 Δ(*choD*, *hsdD*, *MSMEG_5233*), AD – androstenedione, ADD – androstadienedione, Ch – cholesterol.

Thus, inactivation of all three known enzymes that catalyze the conversion of cholesterol into cholestenone does not lead to block this activity in cultures of mycolicibacteria. This fact is consistent with the conclusions of other authors [6, 7] about the presence in *M. smegmatis* cells of at least one additional protein that oxidizes the 3β-hydroxyl group of the steroid nucleus.

It was previously reported that the oxidation of sterols can be catalysed by extracellular bacterial oxidases [5, 15, 16]. The study of the cell-free culture liquid of the *M. smegmatis* CHE strain did not reveal cholesterol oxidase activity (Figure 4). This suggests the intracellular localization of an unknown enzyme functioning as 3β-hydroxysteroid dehydrogenase/$\Delta^{5(6)} \rightarrow \Delta^{4(5)}$ isomerase.

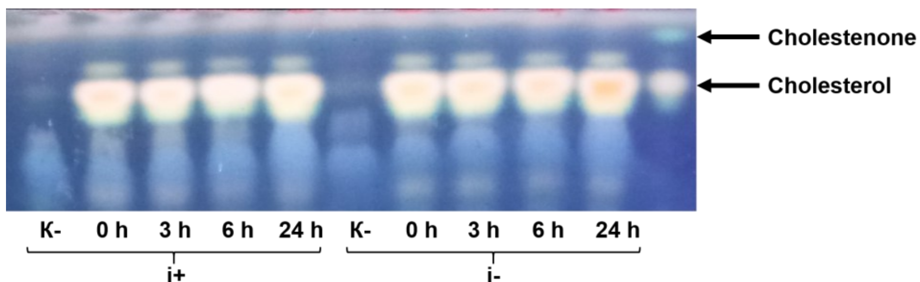

Fig. 4. Analysis of cholesterol bioconversion products by cell-free culture liquid by TLC. CHE – *M. smegmatis* mc[2]155 Δ(*choD*, *hsdD*, *MSMEG_5233*), K- – culture liquid without adding of cholesterol as a substrate, i+ – with cholesterol induction, i- – without cholesterol induction

4 Conclusion

A mutant *M. smegmatis* strain with deletions in three genes encoding known enzymes of the initial stage of sterol catabolism was obtained. Gene knockout did not affect the strain growth and its ability to convert 3β-hydroxy-5-ene steroids to 3-keto-4-ene derivatives. *In vitro* analysis of the culture liquid did not reveal the formation of cholestenone from cholesterol. The experimental data obtained indicate the presence of a previously undetected intracellular enzyme 3β-hydroxysteroid dehydrogenase/$\Delta^{5(6)} \rightarrow \Delta^{4(5)}$ isomerase.

Acknowledgments

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