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Genetic differences in *ksdD* influence on the ADD/AD ratio of *Mycobacterium neoaurum*

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Abstract *Mycobacterium neoaurum* TCCC 11028 (*MNR*) and *M. neoaurum* TCCC 11028 M3 (*MNR* M3) significantly differ in the ratio of androst-1,4-diene-3,17-dione (ADD) to androst-4-ene-3,17-dione (AD) produced. The large fluctuations are related to the dehydrogenation activity of 3-ketosteroid-Δ^1-dehydrogenase (KsdD). Analysis of the primary structure of KsdD showed that the Ser-138 of KsdD-*MNR* changed to Leu-138 of KsdD-*MNR* M3 because of C413T in the *ksdD* gene. This phenomenon directly affected KsdD activity. The effect of the primary structure of KsdD on dehydrogenation activity was confirmed through exogenous expression. Whole-cell transformation initially revealed that KsdD-*MNR* showed a higher dehydrogenation activity than KsdD-*MNR* M3. Then, *ksdD* gene replacement strain was constructed by homologous recombination. The results of steroid transformation experiments showed that the ability of the *MNR* M3Δ*ksdD*::*ksdD*-*MNR* strain to produce ADD was improved and it returned to the similar level of the *MNR* strain. This result indicated that the ADD/AD ratio of the two *M. neoaurum* strains was influenced by the difference in *ksdD*. The mechanism by which residue mutations alter enzyme activity may be connected with the crystal structure of KsdD from *Rhodococcus erythropolis* SQ1. As a key amino acid residue in the active center position, Ser-138 played an important role in maintaining the active center in the hydrophobic environment of KsdD. This study may serve as a basis for future studies on the structural analysis and catalytic mechanism of dehydrogenase.

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Introduction

Steroid hormones regulate the physiological functions in different organisms. Biotransformation is commonly used in the pharmaceutical industry to produce steroid medicine intermediates. Dehydrogenation can occur in $C_{1,2}$, $C_{4,5}$, $C_{7,8}$, $C_{9,11}$, $C_{14,15}$, and $C_{16,17}$, of which $C_{1,2}$ dehydrogenation is the most important, because many important steroid pharmaceutical substances contain 1,2-double bond. Many microorganisms exhibit $C_{1,2}$ dehydrogenation [4]. These microorganisms include *Arthrobacter* sp. [6], *Pseudomonas* sp. [1, 17], *Corynebacterium* sp., *Mycobacterium* sp. [3, 10], *Rhodococcus* sp. [8, 9], *Bacillus* sp., *Nocardia* sp. [12], and *Aspergillus* sp. These microorganisms can act on various types of steroid substrates. Mycobacteria can degrade the side chains of phytosterol (PS), leaving the pharmaceutically important steroid intermediates androst-4-ene-3,17-dione (AD) and androst-1,4-diene-3,17-dione (ADD) [20]. ADD is produced from AD by the $C_{1,2}$ dehydrogenation of 3-ketosteroid-Δ^1-dehydrogenase (KsdD). AD and ADD are simultaneously produced; thus, fluctuations in the ADD/AD ratio reduce industrial production efficiency and increase production costs because of their high structural similarity [5, 21].

To maintain the ADD/AD ratio at a stable level, understanding the behavior of the key enzyme KsdD in the reaction of AD to ADD is crucial. KsdD is a flavin adenine dinucleotide (FAD)-dependent enzyme that catalyzes the 1,2-desaturation of 3-ketosteroid; this enzyme also plays a key role in the microbial catabolism of steroids and the

manufacture of steroid hormone medication [8, 13]. Structural information on the KsdD enzymes of *Mycobacterium* remains lacking, the dehydrogenation mechanism is not completely resolved, and previous studies have focused on the exogenous expression of the KsdD enzyme from *Mycobacterium* sp. [23]. Some scholars investigated the molecular, catalytic, and spectral characteristics of KsdD from various sources and identified some key residues [7, 12]. Rohman et al. [18] resolved the first 3D structures and catalytic mechanism of KsdD from *Rhodococcus erythropolis* SQ1 and found that the enzyme contains two domains, namely, an FAD-binding domain and a catalytic domain. They also confirmed that the active site contains four key residues, namely, Tyr-119, Tyr-318, Tyr-487, and Gly-491. The results of structural similarity search with the PDB database showed that KsdD-*MNR* and KsdD-*MNR* M3 were related to many FAD-binding proteins, with the highest similarity to KsdD from *R. erythropolis* SQ1. In addition, KsdD-*MNR* is also similar to the 3-ketosteroid-Δ^4-(5a)-dehydrogenase (Δ^4-(5a)-KsdD) from *Rhodococcus jostii* RHA1, which was reported by Oosterwijk et al. [14]. Moreover, they noted that their Tyr-319, Tyr-466, and Ser-468 play an essential roles in $C_{4,5}$ dehydrogenation.

Mycobacterium neoaurum TCCC 11028 (*MNR*) and its derived mutant strain *M. neoaurum* TCCC 11028 M3 (*MNR* M3) can degrade PS; however, *MNR* and *MNR* M3 significantly differ in the ADD/AD ratio, with 44.5:1 and 1:8.3, respectively [19]. Considering that the ADD/AD ratio is closely related to KsdD activity, we aim to explain this phenomenon by studying KsdD-*MNR* and KsdD-*MNR* M3. We investigated the differences in KsdD between the two strains through *ksdD-MNR* and *ksdD-MNR* M3 gene exogenous expression in *Escherichia coli* BL21 (DE3) and gene replacement by homologous recombination. Our data suggest that the slight genetic differences in *ksdD* can affect KsdD activity. This study may serve as a foundation for elucidating the structure and catalytic mechanism of the KsdD enzyme.

Materials and methods

Chemicals

Substrate phytosterol (PS; 98.4 % purity) was obtained from COFCO Tech Bioengineering Co. Ltd. (Tianjin). Standards of AD and ADD were purchased from Sigma-Aldrich Co. (USA). HP-β-CD (31.7 % degree of substitution, 1,523 average relative molecular mass) was procured from Xi'an Deli Biology and Chemical Industry Co. Ltd.

Bacterial strains and culture conditions

The bacterial strains, plasmids, and primers used in this study are listed in Table 1. *MNR* was obtained from Tianjin University of Science and Technology Culture Collection Center (TCCC), Tianjin, China. *MNR* M3 was a spontaneous mutant *MNR* strain. The cultivation and bioconversion

Table 1 Strains, plasmids, and primers used in this study

Strains, plasmids, or primers	Relevant properties	Source
E.coli strains		
T1	General cloning host	Transgen Biotech
DH5α	General cloning host	Transgen Biotech
BL21 (DE3)	General express host	Transgen Biotech
Plasmids		
pEASY-Blunt Simple	*E. coli* cloning vector, *amp*, *lacZ*	Novagen
pET-22b (+)	*E. coli* expression vector, *amp*	Novagen
p2NIL	Gene manipulation vector, *kan*	Dr. Parish
pGOAL19	*hyg* Pag_{85}-*lacZ* $Phsp_{60}$-*sacB Pac*I cassette vector, *amp*	Dr. Parish
Primers	Nucleotide sequence (5′–3′)	
E. coli expression		
ksdD-F	GTGTTCTACATGACTGCCCAGGACTA	
ksdD-R	TCAGGCCTTTCCAGCGAGATGCAAC	
*ksd*D-22F	GCCCCATGGTGTTCTACATGACTGCCCAGGA	
*ksd*D-22R	CCGAAGCTTGGCCTTTCCAGCGAGATGCAAC	
PCR for deletion and replacement		
ksdD-UF	CGCCTGCAGTCCGCCGGATTCAAAATGATGATC	
ksdD-UR	CCGAAGCTTTGGGCAGTCATGTAGAACACGTTATAG	
ksdD-DF	CCGAAGCTTGACCTGGGCACCAAGGGCGGTATT	
ksdD-DR	CGCGGATCCCACCCCCGAGAGCACCACGGTGTT	

of microorganisms, as well as the preparation and analysis of transformation products, were performed following the procedures described by Shen et al. [19].

Heterologous expression of KsdD in *E. coli*

On the basis of the *ksdD* gene sequence from *M. neoaurum* NwIBL-01 (GenBank: GQ228843.1), the complete *ksdD* gene was amplified from the genomic DNA of *MNR* and *MNR* M3. The resulting PCR product *ksdD* (1,701 bp) was cloned into *Nco* I/*Hin*d III-digested pET-22b(+), which includes a C-terminal His tag. The resulting construct pET-22b(+)-*ksdD* was introduced into *E. coli* BL21 (DE3) for heterologous protein expression.

A previously established protocol for KsdD expression was followed [21]. A 1 % inoculation from an overnight preculture of the recombinant *E. coli* strains was performed in 50 mL of Luria–Bertani in a 250 mL Erlenmeyer flask at 37 °C with shaking at 200 rpm. When the optical density at 600 nm reached 0.8, isopropyl-β-Dthiogalactopyranoside was added to a final concentration of 1.0 mM at inoculation. The cells were then grown for another 24 h with shaking at 200 rpm and 20 °C.

Whole cells were collected, resuspended in PBS (100 mM dipotassium hydrogen phosphate-potassium dihydrogen phosphate, 1 % glycerol, pH 7.3), and then incubated with AD at a final concentration of 1.2 g/L at 20 °C with shaking at 200 rpm. Samples were withdrawn at various time intervals and extracted with the same volume of ethyl acetate. The organic phases were collected and detected through thin layer chromatography (TLC), with *E. coli* BL21 (DE3)/pET-22b(+) as a control.

Replacement of the *ksdD* gene

Construction of delivery vectors

Suicidal recombination delivery vectors were constructed to perform unmarked deletions in the *ksdD* gene of *MNR* M3 [16]. For the unmarked *ksdD* deletion, two separate fragments were cloned into p2NIL. In the first step, the upstream region of the *ksdD* gene (a 1,543 bp *Pst* I/*Hin*d III fragment *ksdD*-up) was amplified and then cloned into the *Pst* I/*Hin*d III site of p2NIL to obtain pNGQ-1. Then, the downstream region of the *ksdD* gene (a 1,086 bp *Hin*d III/*Bam*H I fragment *ksdD*-down) was cloned into the *Hin*d III/*Bam*H I site to obtain pNGQ-2. Finally, an 8 kb *Pac* I marker cassette from pGOAL19 carrying the *hyg*, *lacZ*, and *sacB* genes was cloned into the *Pac* I site of pNGQ-2 to create pNGQ-3.

Suicidal recombination delivery vectors were constructed to perform gene replacement in the *ksdD* gene of *MNR* M3. In the first step, a 4,064 bp *Hin*d III/*Bam*H I fragment (harboring the upstream region of *ksdD-MNR* M3 and *ksdD-MNR*, as well as the downstream region of *ksdD-MNR* M3) was cloned into the *Hin*d III/*Bam*H I sites of p2NIL to create pNGR-1. Subsequently, an 8 kb *Pac* I marker cassette from pGOAL19 was cloned into the *Pac* I site of pNGR-1 to create pNGR-2.

Construction of replacement strain

The protocol of Parish and Stoker [16] was used to disrupt the *ksdD* gene at its native locus on the chromosome. Plasmid DNA (pNGQ-3) was treated with NaOH (0.2 mM) and integrated into the *MNR* M3 chromosome by homologous recombination. The resulting single-crossover (SCO) homologous recombinant colonies were blue, Kan^R, and sensitive to sucrose. The recombination site was confirmed by PCR. The SCO strains were further processed to select for double-crossover (DCO) mutants that were white, Kan^S, and resistant to sucrose (2 %). DNA sequencing was used to distinguish between wild-type and DCO mutants.

The *ksdD* gene deletion strain was used as the original strain. The *ksdD* gene replacement strain *MNR* M3Δ*ksdD*::*ksdD-MNR* was constructed by homologous recombination as described above, and pNGR-2 was used instead of pNGQ-3.

Results

Sequence analysis of KsdD

To obtain the amino acid sequence of KsdD, the complete sequence of the *ksdD* gene from *MNR* and *MNR* M3 was obtained by PCR. The *ksdD* gene sequence from *M. neoaurum* NwIBL-01 was used as a basis to design the primers. The resulting fragment containing 1,701 bp nucleotides is a complete open reading frame. The obtained sequences were BLAST searched in the NCBI. Results showed 100 % query cover and 99 % similarity to the *ksdD* gene from *M. neoaurum* NwIBL-01.

Sequence analysis of the *ksdD* gene showed 99.9 % similarity between *ksdD-MNR* and *ksdD-MNR* M3. The only difference lies in the 413 base. In particular, the 413 base of *ksdD-MNR* is C, whereas that of *ksdD-MNR* M3 is T (Fig. 1a). This base difference directly caused the Ser-138 of KsdD-*MNR* to change into the Leu-138 of KsdD-*MNR* M3 (Fig. 1b). The mutation in the amino acid sequence of KsdD directly led to the change in KsdD structure, which resulted in different enzyme activities. Therefore, we speculated that this residue is the key amino acid that exerts a direct effect on the activity center. This difference has a critical effect on the activity of the KsdD enzyme, thereby affecting the ratio of the related products. Bragin et al. [2]

Fig. 1 Sequence alignment analysis of the (**a**) nucleotide and (**b**) amino acid. **a** Sequence alignment analysis of the nucleotide, **b** Sequence alignment analysis of the amino acid

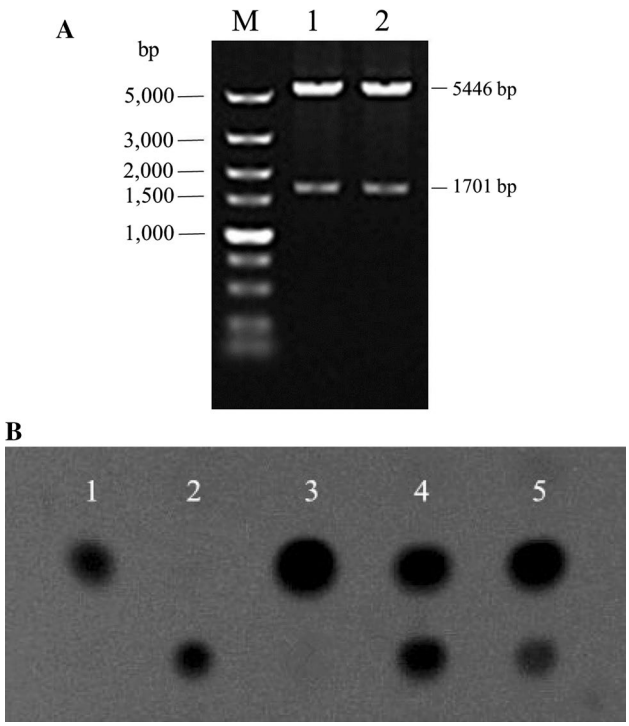

Fig. 2 Heterologous expression of KsdD in *E. coli*. **a** Identification of recombinant plasmids. The length of *ksdD* is 1,701 bp, the length of the vector pET-22b(+) is 5,446 bp. *M*: DL 5,000 DNA marker; *lane 1*: *E. coli* BL21 (DE3)/pET-22b(+)-*ksdD-MNR*; *lane 2*: *E. coli* BL21 (DE3)/pET-22b(+)-*ksdD-MNR* M3. **b** TLC analysis of 1.2 g/L AD conversion by whole-cell catalysis for 24 h. *Lane 1*: standard AD; *lane 2*: standard ADD; *lane 3*: AD transformed by *E. coli* BL21 (DE3)/pET-22b(+) as a control; *lane 4*: AD transformed by *E. coli* BL21 (DE3)/pET-22b(+)-*ksdD-MNR*; *lane 5*: AD transformed by *E. coli* BL21 (DE3)/pET-22b(+)-*ksdD-MNR* M3

observed a similar phenomenon between *Mycobacterium* sp. VKM Ac-1815D and *Mycobacterium* sp. VKM Ac-1816D. Their study suggested that a single point mutation may lead to differences in the accumulation of the main product.

Effect of amino acid mutations on KsdD activity

Whole-cell transformation experiments were carried out in genetically engineered bacteria to confirm that the difference in KsdD activity is caused by the amino acid differences in KsdD. KsdD was expressed in *E. coli* BL21 (DE3) by the expression vector pET-22b(+) to generate an expression strain (Fig. 2a). The results of TLC showed that AD was more easily transformed into ADD with genetically engineered bacteria *E. coli* BL21 (DE3)/pET-22b(+)-*ksdD-MNR* than with *E. coli* BL21 (DE3)/pET-22b(+)-*ksdD-MNR* M3 (Fig. 2b). Therefore, the activity of KsdD-*MNR* M3 was low, and the KsdD activity of the engineered strains was similar to that of the original *MNR* and *MNR* M3 strains. In the present study, the same host and vector were used as carriers of the heterologous gene expression of *ksdD-MNR* and *ksdD-MNR* M3 to guarantee the same level of transcriptional regulation. Therefore, the primary factor that influences KsdD activity is genetic differences in *ksdD*.

To eliminate the impact of regulatory factors in *M. neoaurum*, the gene knockout and replacement of *ksdD* were designed according to the two-step method of unmarked knockout principle [16] (Fig. 3a). The growth of engineered strains and wild-type *M. neoaurum* in the presence of HP-β-CD resulted in the fast degradation of the substrate PS. The ADD/AD ratios with *MNR* M3Δ*ksdD*::*ksdD-MNR* and wild-type *MNR* M3 were 31.54 and 0.15, respectively. It was confirmed that when *ksdD-MNR* M3 was replaced by *ksdD-MNR*, the ADD/AD ratio of *MNR* M3Δ*ksdD*::*ksdD-MNR* returned to similar level of the *MNR* strain (28.08) (Fig. 3b). The effect of S138L on the structure of the KsdD enzyme was considered the primary cause of the fluctuations in the ADD/AD ratio. Therefore, the activity differences between *ksdD-MNR* and *ksdD-MNR* M3 were indeed caused by the structure of the KsdD enzyme, and the nucleotide differences in the 413 base considerably affected KsdD activity.

Interestingly, the samples transformed by *MNR* M3Δ*ksdD* only produced AD, but no ADD was detected (Fig. 3b). This result indicated that the strain completely lost KsdD activity and there was no effect of other $C_{1,2}$ dehydrogenase enzymes.

Mechanism underlying the effect of amino acid mutations on KsdD activity

Single amino acid mutations significantly affect enzyme activity. Thus, residues play important roles in enzyme structure and dehydrogenation reactions. A structural similarity search was performed by NCBI BLAST with the PDB database and results showed that KsdD-*MNR* and KsdD-*MNR* M3 were related to many FAD-binding proteins, with the highest similarity to KsdD from *R. erythropolis* SQ1

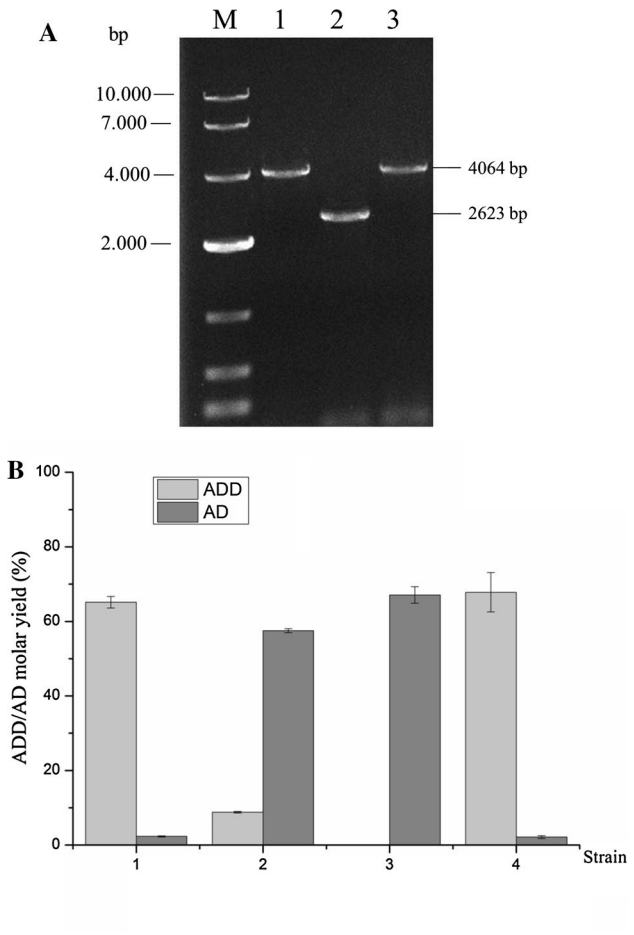

Fig. 3 Deletion and replacement of the *ksdD* gene in the *MNR* M3 strain**. a** Electrophoretic analysis of the engineered strains. The length of the upstream and downstream DNA fragments of *ksdD* is 4,064 bp in the wild-type strain and *ksdD* replacement strain, and 2,623 bp in the *ksdD* gene deletion strain. *M*: DL 10,000 DNA marker; *lane 1*: wild-type strain *MNR* M3; *lane 2*: *ksdD* gene deletion strain *MNR* M3Δ*ksdD*; *lane 3*: *ksdD* replacement strain *MNR* M3Δ*ksdD*::*ksdD-MNR*. **b** AD and ADD production with engineered strains of *M. neoaurum*. Tests were conducted with 10 g/L PS (after 7 days transformation). *Lane 1*: PS transformed by *MNR*; *lane 2*: PS transformed by *MNR* M3; *lane 3*: PS transformed by *MNR* M3Δ*ksdD*; *lane 4*: PS transformed by *MNR* M3Δ*ksdD*::*ksdD-MNR*

(PDB entry 4c3x; 38 % sequence identity), followed by 3-ketosteroid Δ^4-(5α)-dehydrogenase from *R. jostii* RHA1 (PDB entry 4at0; 26 % sequence identity). The structure of KsdD from mycobacteria has not yet been determined. The 3D structures of these two enzymes were referenced to analyze the role of amino acids in KsdD.

Based on the crystal structure of KsdD-*R. erythropolis* SQ1 resolved by Rohman et al. [18], we further analyzed its secondary structure using Discovery Studio 2.5 to elucidate the mechanism by which S138L causes the activity differences between KsdD-*MNR* and KsdD-*MNR* M3. Analysis of the amino acid sequence revealed that the Ser-138 of KsdD-*MNR* corresponded to the Ser-131 of KsdD-*R. erythropolis* SQ1. The effect of Ser-138 on KsdD-*MNR* activity was speculated by analyzing the role of Ser-131 in KsdD-*R. erythropolis* SQ1. Ser-131 is located in the active center of KsdD-*R. erythropolis* SQ1, extremely close to the substrate AD and the four key residues Tyr-119, Tyr-318, Tyr-487, and Gly-491. Ser-131 and its surrounding residues form a loop on a key area that is close to the active center (Fig. 4). Oosterwijk et al. [14] analyzed the function of the loop region in Δ^4-(5a)-KsdD. The active center is extremely hydrophobic because of the poor water solubility of the substrate AD. After the substrate binds to the active site, it needs to shield the FAD and substrate from the solvent to facilitate the catalysis. In the catalytic process of Δ^4-(5a)-KsdD, two loops (residues 170–182 and 256–268) that are poorly visible in the electron density map and close to the active site complete the function. The Ser-131 located in the former loop maintains the extremely hydrophobic environment of the active center. Compared with Leu residues, Ser residues have a hydroxyl group that can fix the movable loops by hydrogen bonds, and the fatty acid side chain of Leu due to steric hindrance may prevent the hydrophobic formation of the active center. Thus, Ser residues may directly affect the activity of dehydrogenase KsdD.

The structure of KsdD from mycobacteria remains unresolved. Thus, comprehensive data must be gathered to support this hypothesis. The methods used to analyze the structure of *Rhodococcus* KsdD provide a good guidance for the KsdD structural analysis of mycobacteria. This study is significant for the analysis of the key amino acids of KsdD.

Discussion

Previous studies isolated the derivative mutant *MNR* M3 from the ADD-producing strain *MNR*; the principal product of PS degradation by *MNR* M3 is AD, and the ADD/AD ratio strongly shifts to the AD direction in the mutant strain [19]. Analysis of the *ksdD* gene sequence showed that the only difference between the two strains lies in the 413 base. This base difference directly caused Ser-138 to change to Leu-138. This single residue mutation resulted in a considerable difference in activity, suggesting that this residue is the key amino acid that exerts a direct effect on the activity center.

The PS degradation pathway by mycobacteria remains unresolved. Potentially valuable strains such as *M. neoaurum* ATCC 25795 (GenBank: JMDW00000000.1) and *Mycobacterium* sp. VKM Ac-1815D (GenBank: CP006936.1) were subjected to whole-genome sequencing to understand metabolic pathways. All of the data support that KsdD is a key enzyme in the degradation of steroid

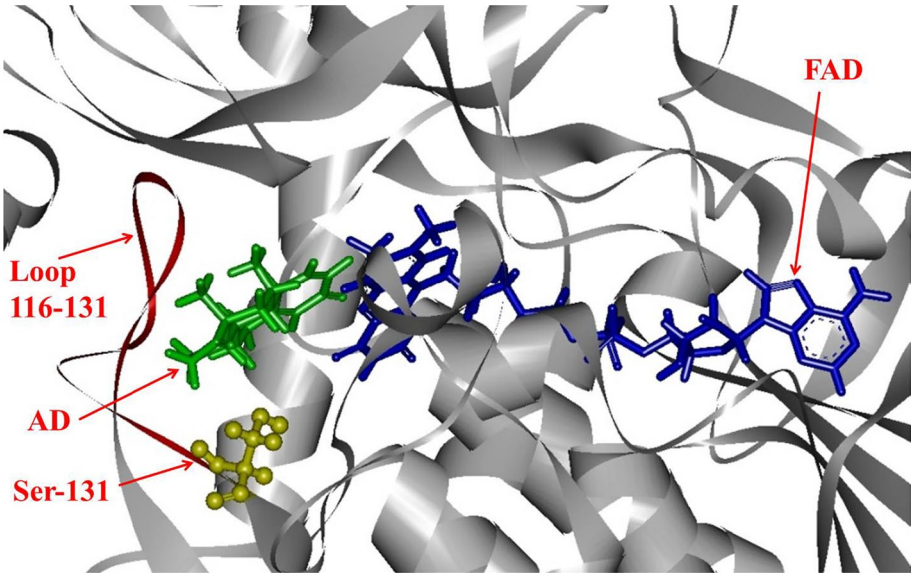

Fig. 4 Position of mutating amino acid in KsdD with AD. Discovery Studio 2.5 was used to analyze the mutation site in the base of the crystal structure of KsdD-*R. erythropolis* SQ1(4c3y)

nucleus. Previous research on KsdD enzymes focused on heterologous expression, molecular characterization, and catalytic mechanism.

Patrich et al. [17] analyzed the heterologous expression of KsdD from *Pseudomonas testosterone* expression in *E. coli* in 1991. Then, the *ksdD* gene from multiple sources, such as *Arthrobacter*, *Rhodococcus*, and *Mycobacterium* species, was used for heterologous expression studies, with *E. coli* being the host in heterologous expression. Zhang et al. [23] used pUC18 as an expression vector and achieved the heterologous expression of KsdD from *M. neoaurum* NwIBL-01 in *E. coli* DH5a. Oosterwijk [14, 15] and Rohman [18] used the expression vector pET-15b and the expression host *E. coli* BL21 (DE3) to obtain the pure KsdD enzyme of *Rhodococcus*. Crystallization of the enzyme was also performed to reveal the crystal structure. In the present study, pET-22b(+) served as the expression vector and *E. coli* BL21 (DE3) as the expression host. The obtained engineered strains showed $C_{1,2}$ dehydrogenase activity; in addition, the KsdD activity of the engineered strains was similar to that of the original *MNR* and *MNR* M3 strains. Knockout of the *ksdD* gene in *MNR* M3 completely blocked the production of ADD even in the presence of HP-β-CD. This result indicates the complete loss of the dehydrogenase activity and the lack of effect of the other $C_{1,2}$ dehydrogenase enzymes. A gene replacement was designed in accordance with the two-step method of unmarked knockout principle to eliminate the effect of regulatory factors [16]. The ability of the *MNR* M3Δ*ksdD*::*ksdD-MNR* strain to produce ADD improved and it returned to the similar level of the *MNR* strain. This result indicates that the activity differences between KsdD-*MNR* and KsdD-*MNR* M3 are caused by the structure of the KsdD enzyme. Furthermore, the nucleotide differences in the 413 base significantly affected KsdD activity.

KsdD is a membrane-bound enzyme [13], and the hydrophobic transmembrane domains complicate the procurement of pure protein [11]. Thus, the progress of research on the structure and catalytic mechanism of this enzyme is slow. The research team from the University of Groningen [14, 15, 18] has successfully resolved two KsdD enzymes from *Rhodococcus* and further identified numerous key residues by site-directed mutagenesis. Oosterwijk et al. [14, 15] established a method for analyzing membrane protein structure and used this method to successfully resolve the crystal structure of Δ^4-(5a)-KsdD-*R. jostii* RHA1. Rohman et al. [18] used the same method to resolve the crystal structure of KsdD-*R. erythropolis* SQ1. This structural analysis method is suitable for KsdD from mycobacteria and is expected to achieve a breakthrough in the near future.

Aside from the differences in KsdD enzyme structure caused by gene differences, the expression levels of the *ksdD* gene also affected KsdD enzyme activity. The expression levels of *ksdD-MNR* and *ksdD-MNR* M3 remain to be evaluated. The RT-PCR method constructed by Yao et al. [22] can be used to detect the expression levels of the *ksdD* gene. The expression levels of the *MNR*, *MNR* M3, and *MNR* M3Δ*ksdD*::*ksdD-MNR* strains were compared by conducting RT-PCR experiments, with *ksdD-MNR* M3 as a control and a housekeeping gene as an internal reference.

This study demonstrated that Ser-138 is a critical amino acid of the KsdD enzyme. The large fluctuations in the ADD/AD ratio between *MNR* and *MNR* M3 were caused by the structural differences in KsdD that resulted from the nucleotide differences in *ksdD*. The mutated residues in the

active center of KsdD affected its dehydrogenation activity. These residues may lead to product migration issues in industrial production strains. Therefore, these key residues must be identified to clarify the catalytic mechanism of the KsdD enzyme.

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