Combined enhancement of the propionyl-CoA metabolic pathway for efficient androstenedione production in *Mycobacterium neoaurum*  
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**Abstract**  
**Background**: The production of androstenedione (AD) from phytosterols by *Mycobacterium neoaurum* is a multi-step biotransformation process, which requires degradation of sterol side chains, accompanied by the production of propionyl-CoA. However, the transient production of large amounts of propionyl-CoA can accumulate intracellularly to produce toxic effects and severely inhibit AD production.

**Results**: In the present study, the intracellular propionyl-CoA concentration was effectively reduced and the productivity of the strain was improved by enhancing the cytosolic methyl-branched lipid synthesis pathway and increasing the expression level of *nat* operator gene, respectively. Subsequently, the application of a pathway combination strategy, combined and the inducible regulation strategy, further improved AD productivity with a maximum AD conversion rate of 96.88%, an increase of 13.93% over the original strain.

**Conclusions**: Overall, we provide a new strategy for reducing propionyl-CoA stress during biotransformation for the production of AD and other steroidal drugs using phytosterols.

**Keywords**: *Mycobacterium neoaurum*, Androstenedone, Propionyl-CoA metabolic, Pathway Associates

**Introduction**  
Androstenedione (AD) is an important steroid intermediate, and almost all steroid drugs such as adrenocorticotropic hormones, sex hormones and anabolic hormones can be produced from AD [1], and more than 100 kinds of related steroid hormone drugs have been synthesized from AD in the market, which has a broad market [2].

**Microbial conversion of phytosterols for AD production** is an environmentally friendly and low-cost production method to successfully replace the traditional chemical synthesis method [3]. *Mycobacterium* spp. are the main AD-producing genera due to their powerful sterol degradation system [4].

In the process of AD production by *Mycobacterium neoaurum* (MNR) using phytosterols as substrate, the initial oxidation and side-chain degradation will produce a large amount of propionyl-CoA in a short period of time, which makes the strain unable to degrade it rapidly through normal metabolism, thus leading to excessive accumulation of propionyl-CoA in the cell and thus inhibiting the growth of the bacterium [5–7]. Three
propionyl-CoA metabolic pathways exist in *Mycolicibacterium* spp.: the 2-methylcitrate cycle pathway (MCC), the methylmalonate cycle pathway (MMC) [5], and the cytosolic methyl-branched lipid synthesis pathway [5, 8]. Previous studies succeeded in reducing the intracellular propionyl-CoA content by enhancing the first two pathways, ultimately resulting in a 28.4% increase in transformation efficiency compared to the original strain [6, 7].

High concentrations of substrate (phytosterols) and product (AD) during AD production can be toxic to microorganisms [9–11]. In *Mycobacterium tuberculosis* (MTB), through the cytosolic methyl-branched lipid synthesis pathway, propionyl-CoA →acetyl-CoA carboxylase (ACC) catalyzes the intracellular malonyl-CoA and acetyl-CoA to methylmalonyl-CoA and malonyl-CoA [12, 13]. Later, in the presence of polyketide synthase (PikA1), the two undergo an esterification condensation reaction to produce nabolone, which is an important component in the synthesis of the cytosolic methyl-branched lipid phthiocerol dimycolates (PDIM) [14, 15]. When MTB infect the host, in order to improve its tolerance in the host, they produce sufficient acetyl-coenzyme A and malonyl coenzyme A by absorbing cholesterol from the host, and later express the ACC family genes at high levels to produce malonyl coenzyme A and methyl malonyl coenzyme A. The two, in the presence of a polyketide synthase complex, generate PDIM, which improves the tolerance of the strain [16]. Therefore, the cytosolic methyl-branched lipid synthesis pathway has the potential to be a potentially effective way to effectively reduce intracellular propionyl-CoA content and enhance the tolerance of the bacterium, however, there are few studies on the use of the cytosolic methyl-branched lipid synthesis pathway for AD production in MNR to date.

In addition to this, another potential propionyl-CoA metabolic pathway emerged in our view. In MTB and *Mycolicibacterium smegmatis*, there is a nat operon that encodes the Hsa family protein HsaA-D and N-acetyltransferase (NAT) protein that are directly involved in the cholesterol degradation pathway of MTB [17], where NAT uses acyl-coenzyme A to degrade cholesterol cata- bolically metabolites, such as acetyl-CoA and propionyl-CoA, to enrich the cellular acyl pool for normal life activities such as cell growth [18, 19]. In *Mycolicibacterium smegmatis* nat operon, expression of the hsaA-D gene activates the transcription factor AraC. Nat gene is regulated by AraC, which recognizes free amino groups on various intracellular arylamine and hydrazine structural scaffolds, and catalyzes the transfer of acetyl groups from acetyl-CoA to the arylamine scaffold, releasing free CoA and re-producing acetyl-CoA, succinyl-CoA, propionyl-CoA and malonyl-coenzyme A, etc. [20]. It has been shown that increasing intracellular coenzyme A levels is effective in improving cell viability and thus the accumulation of target products [21, 22]. However, whether the action of this operon is beneficial for AD production by *Mycolicibacterium* spp., to our knowledge, has not been reported.

Based on these mechanisms, this study shows for the first time that enhancement of the cytosolic methyl-branched lipid synthesis pathway with the nat operon can improve AD production in *Mycolicibacterium* (Fig. 1). The changes in cell growth and AD production by *AccA1* and *AccD1*, key genes of the cytosolic methyl-branched lipid synthesis pathway, and nat, the key gene of the nat operon, as well as the transcription factor AraC, were investigated. Meanwhile, to further improve AD production, the two pathways were jointly enhanced to construct a tandem expression strain, and the expression of the key gene *AccA1* was regulated by modifying the existing plasmid using the tetracycline operator according to the growth status of the bacteria. The resulting optimal strain achieved a 96.88% AD transformation rate. The multi-pathway combination addressed the toxic effects of propionyl-CoA for efficient AD production.

**Results and discussion**

**Effect of enhanced cytosolic methyl-branched lipid synthesis pathway on biotransformation and strain physiology performances**

**Construction of cytosolic methyl-branched lipid synthesis pathway enhancing recombinant strains**

Previous studies have shown the presence of AccA1 and AccD1 in MTB, and they are key enzymes in the cytosolic methyl-branched lipid synthesis pathway of the MTB [12]. The *AccA1 D174_19730 and AccD1 D174_19735* were localized on the MNR VKM Ac-1815D (TAX:700508) genome by bioinformatics methods. Based on the functions of AccA1 and AccD1, their expression in MNR was aimed at enhancing the cytosolic methyl-branched lipid synthesis pathway and reducing the intracellular propionyl-CoA content. The recombinant strains QC M3-A and QC M3-D were successfully constructed according to the method described in 4.2, containing AccA1 and AccD1, respectively.

**Influence of AccA1 and AccD1 enhancement on cell growth indicators and conversion indicators**

The effects of *AccA1* and *AccD1* on cell growth were investigated and compared with the parental strains. All strains had typical growth curves, but the recombinant strain QC M3-A was severely affected and its biomass was lower than WT, with QC M3-A being the most severely affected (Fig. 2a). This is due to the over-expression of AccA1 leading to accelerated synthesis of cell membrane methyl branched lipids in the pre-growth...
phase of the strain, which generates growth stress. Previously reported results may also support this phenomenon in the opposite direction [23, 24]. Although the above recombinant strains showed a decrease in biomass, the highest molar transformation rates of recombinant strains QC M3-A and QC M3-D were increased by 9.7% and 6.3%. Compared to the starting strain, AD accumulation was $3.19 \pm 0.09$ g/L and $3.08 \pm 0.1$ g/L under 5 g/L sterol conditions, respectively (Fig. 2b).

**Influence of AccA1 and AccD1 enhancement on substrate tolerance of recombinant strains**

In the process of AD production by MNR, AD inhibits the growth and respiratory metabolic activities of
the bacterium, and the gradually increasing AD concentration will produce more and more severe stress on the bacterium, which will severely inhibit its ability to convert sterols. To address this phenomenon, it was found that the intensity of AD production could be effectively improved by increasing the tolerance of the strain to AD [25, 26]. The growth of the strain was measured at different AD concentrations (Fig. 3a), and it was found that although the biomass of QC M3-A was lower compared to WT, it was less affected by AD concentration and showed better substrate tolerance compared to WT, which may be due to the overexpression of AccA1, which thickens the cell wall of the recombinant strain (Fig. 3b). The changes in these indicators are one of the reasons for the enhanced biotransformation capacity of QC M3-A.

**Reconstitution of the nat operon to promote intracellular acyl coenzyme A cycling**

**Construction of a nat operon overexpressing recombinant strain**

In order to achieve a high yield of the target product AD, the AD downstream transforming KsdD in the sterol metabolic pathway was knocked out so that the strain would not produce androsta-1,4-diene-3,17-dione (ADD), so the subsequent sterol parent nucleus degradation reaction would stop. In the presence of Hsa family genes, *Mycobacterium* accomplishes the complete degradation of the sterol parent nucleus. At the same time, the expression of Hsa family genes enhances the expression of the transcriptional regulator AraC [20, 27]. To reconstruct this operon and to investigate the role of AraC and Nat in sterol conversion, recombinant strains QC M3-C and QC M3-N were constructed, respectively. As shown in Fig. 4a, the transcriptional level of AraC in QC M3-C...
reached 19.64-fold higher than that of WT, and Nat transcription level increased nearly 40-fold. However, Nat did not show an upregulatory effect on AraC in QC M3-N. The regulation of the acyl-coenzyme cycle within the strain by the AraC gene relies mainly on the regulation of the expression of various downstream acyltransferases. The results of comparing the regulatory effect of AraC on Nat in different strains confirmed this conclusion that the expression of AraC does regulate the transcription of Nat, but no negative feedback regulation of intracellular Nat content on AraC was found for the time being.

**Growth indicators and conversion indicators**

The effect of nat operon enhancement on cell growth and AD transformation was investigated. It was found that although all strains had the same growth trend, the biomass of the recombinant strains was reduced (Fig. 4b). As shown in Fig. 4c, the AD production capacity of the recombinant strains was higher than that of the starting strains. Both QC M3-C and QC M3-N bio-maximal molar conversion ratios were higher than that of the starting strains, and AD accumulation was up to 3.13 ± 0.02 g/L and 3.08 ± 0.05 g/L. The above results indicated that the overexpression of the nat operon increased the AD production capacity of MNR, with the transcription factor AraC overexpression having the optimal effect.

**Effect of different pathways on intracellular propionyl-CoA**

The intracellular propionyl-CoA levels of the recombinant strains in both pathways were examined (Fig. 5), and the results showed that the recombinant strains and the starting strains had similar trends of change. Meanwhile, the propionyl CoA content of all recombinant strains was lower than that of WT. Among them, QC M3-C had the lowest intracellular propionyl coenzyme A level, and its intracellular propionyl-CoA level decreased by 38.70% compared with the starting strain at 96 h of fermentation. At the same time, due to the overexpression of AraC, which accelerated the metabolism of intracellular
propionyl-CoA and effectively promotes cellular coenzyme A circulation. This result could also well explain the better growth trend and AD productivity of QC M3-C relative to other recombinant strains. Combining the results of AD conversion rates of different strains in Figs. 2 and 4, we can find that all strains except recombinant strain QC M3-D had higher AD conversion rates than WT, although their biomass was lower than WT, which indicates that it is feasible to improve the conversion ability of strains by enhancing cytosolic methyl-branched lipid synthesis pathway and reconstitution of the nat operon.

**Combined enhancement of propionyl-CoA metabolism to improve transformation performance of strains**

Three new recombinant strains were constructed to further enhance the metabolism of propionyl-CoA and to obtain a more productive and rapidly growing strain. Firstly, to address the problem of \( \text{AccA1} \) inhibition, the promoter element in the pMV261 plasmid was modified and its expression was regulated using the tetO operator to construct the overexpression strain QC M3-A\text{TetO}.

Based on the consideration of the slow growth of the strain, \( \text{AccA1} \) was expressed in tandem with \( \text{AraC} \) to construct the tandem expression strain QC M3-AC. \( \text{AraC} \) could significantly improve the viability of the strain, and in order to cope with the problem of inhibition of strain growth by \( \text{AccA1} \), a strong RBS sequence (aaagaggtgaca) was added before the \( \text{AraC} \) in the construction of the tandem gene plasmid [28], hoping to use a high level of \( \text{AraC} \) expression to counteract the growth stress caused by \( \text{AccA1} \). In addition, the two schemes were combined to construct a regulated tandem expression strain, so that on the one hand, \( \text{AraC} \) relies on the hsp60 promoter for constitutive expression, allowing the bacterium to maintain high viability throughout all stages of fermentation, and on the other hand, the \( \text{AccA1} \) is expressed in a regulated manner by the administration of an inducer at mid-fermentation, so that the two genes can work together at the highest intracellular propionyl-CoA level at mid-fermentation.

As the data in Fig. 6a show, all three modes of tandem transformation strains, compared to QC M3-A strains can enter the logarithmic growth period quickly; in the late stage of fermentation, the biomass of the tandem strains were significantly increased, and remained basically the same as QC M3-C (Fig. 6b).

The tandem gene overexpression strains were constructed to improve the problem of the low AD conversion rate of \( \text{AccA1} \) overexpression strains in the pre-fermentation stage (Fig. 6a). As can be seen from Fig. 6c, all three tandem overexpression strains solved this problem well. Moreover, the conversion rate of the strains was improved with the addition of an inducer at 72 h. At 96 h, the AD conversion rate of QC M3-A\text{TetoC} had reached 90.70%, which was close to the highest conversion rate of the single gene overexpression strain at 168 h and greatly shortened the conversion cycle. The bacterium showed excellent performance in AD conversion ability, and its highest AD conversion rate reached 96.88% at 168 h, which was 13.93% higher than that of the original bacterium WT, the final AD yield was 3.34 ± 0.02 g/L at a substrate concentration of 5 g/L. Meanwhile, the production rate was 1.3 times that of the original strain (Fig. 6c, Table 1). Compared to our previous work on propionyl-CoA regulation, we obtained similar or even higher production rate [6, 7]. The production efficiency of QC M3-A\text{TetoC} was lower than that of MNR-Fpcc-Fndh [6]. Although this phenomenon may be due to the different transformation systems, at the same time we speculate that this phenomenon may be due to the synergistic effect of simultaneous regulation of the two cofactors (NAD\(^+\)/NADH and propionyl-CoA) in previous studies. Of course, in the subsequent work, we will also try to combine the regulation of multiple cofactors and explore the synergistic effect between cofactors to obtain higher conversion efficiency.

**Conclusion**

The aim of this study was to improve the AD production efficiency of the strain. A rational strategy was developed through genetic engineering and dynamic regulation methods. Overexpression of \( \text{AccA1, AccD1, AraC, and Nat} \) in MNR can reduce the intracellular propionyl-CoA content, while the combination of multiple pathways can better improve the transformation ability of the strain, which is an effective strategy to improve the catalytic
performance of the microorganism. For the first time, the inducible regulation strategy by tet operator in MNR was used for biotransformation, which provided a new idea to further improve the AD production efficiency of the strain.

Materials and methods

Strains, plasmids, and culture conditions

All strains and plasmids used in this study are listed in Table 2. *E. coli* DH5α was used for plasmid construction. Strain MNR was stored in Tianjin University of Science and Technology Culture Collection Center (TCCC), Tianjin, China, and used for constructing engineering strains. *E. coli* was cultured in an LB medium. The culture method and medium of MNR were performed as previously described [29]. The medium of recombinant *Mycobacterium* strains was supplemented with kanamycin (50 μg/mL). The pMV261 plasmid was modified using primers to construct an expression regulatory plasmid with a double promoter and containing a tet operator [28, 30], named pMV262, and the expression of the target gene was regulated by the addition of tetracycline during fermentation.

**Construction of recombinant strains**

The method of constructing recombinant strains has been reported in previous studies [7]. *AccA1, AccD1, AraC* and *Nat* were cloned from QC M3 genome and inserted into linearized pMV261 to construct recombinant plasmids named pMV261-AccA1, pMV261-AccD1, pMV261-AraC and pMV261-Nat, respectively, and the recombinant plasmids were dropped into the dedicated *Mycobacterium* strains to obtain recombinant strains, QC M3-N, QC M3-C, QC M3-A and QC M3-D. The tandem strain was constructed by inserting the fragment *AraC* into the linearized pMV261-AccA1 using the same method as in previous studies to construct the tandem recombinant plasmid pMV261-AraC-AccA1, and finally obtain...
the recombinant strain QC M3-AC. By using pMV262, AccA1 was regulated to construct pMV262-AccA1 and recombinant strain QC M3-AccA1TetO. Based on this, strong RBS was selected to construct pMV262-AraC-AccA1 and recombinant strain QC M3-AraC TetOC (Additional file 1: Fig. S1) [28].

**Determination and analytical methods**

The desired product was extracted from the fermentation broth according to the previously described method, and propionyl-CoA was extracted from the cells and detected by HPLC [7]. Measurements were performed using an HPLC system equipped with a UV detector. Chromatographic separation was performed on a reversed-phase C18 column (4.6 mm × 250 mm, 5 μm). AD detection was performed using 80% methanol aqueous solution as mobile phase and buffer A (acetonitrile) and buffer B (100 mM ammonium acetate, pH 5.8) as mobile phases for the detection of propionyl-CoA. The molar product yields of androstenedione were calculated using the following equation: androstenedione molar yield % = (CAD/MAD)/(CPS/MPS) × 100%, where CAD and CPS are the product concentrations (g/L) of AD and phytosterol, respectively; MAD and MPS are the molar masses of AD and phytosterol, respectively.

The intracellular propionyl CoA content was calculated using the external standard method with reference to the method of Zhang et al. [7]. Calibration curves were plotted with propionyl CoA standard working solution, and sample propionyl CoA concentrations were calculated from the standard curves using the interpolation method. Finally, the relative concentration of intracellular propionyl coenzyme A was calculated based on the cell dry weight of each sample.

**RNA extraction and Real-time quantitative PCR (qRT-PCR) analysis**

For qRT-PCR analysis, cells were cultured for 48 h and collected by centrifugation at 8000×g for 10 min at 4 °C. RNA isolation was performed according to the method described in our previous description [7]. qRT-PCR analysis was performed according to the previously described method. Primers for qRT-PCR are listed in Table 2. 16S rRNA gene messenger RNA (mRNA) levels were used as housekeeping genes (internal controls) to normalize sampling errors. Relative gene expression levels were calculated by comparing the Ct method (2^−ΔΔCt method) [31].

**Transmission electron microscope observation of cell structure**

Fixation and observation of MNR using transmission electron microscopy (TEM) [32]. Ultrathin sections (90 nm) were obtained using a Leica EM U27 ultrathin sectioning machine and a Leica EN KMR3, and the sections were stained and observed on a Hitachi HT7700 transmission electron microscope. The cell wall thickness measurement using ImageJ [33].

**Statistical analysis**

Data represent the mean and standard deviation (SD) of three independent experiments. Student’s t-test were used to determine significant differences between the data. Differences between two groups were measured by the Student’s t-test. A p value less than 0.05 was defined statistically significant difference.

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**Table 1** Comparison of the impact of various regulation strategies on AD production

| Strains     | Regulation Strategies                  | Conversion yield (%) | Production rate (g/L/days) |
|-------------|----------------------------------------|----------------------|----------------------------|
| QC M3-A     | Overexpression AccA1                   | 92.6                 | 0.457                      |
| QC M3-D     | Overexpression AccD1                   | 89.2                 | 0.44                       |
| QC M3-N     | Overexpression NAT                     | 89.3                 | 0.441                      |
| QC M3-C     | Overexpression AraC                    | 90.7                 | 0.447                      |
| QC M3-AC    | Co-expression AccA1 and AraC           | 94.1                 | 0.464                      |
| QC M3-A_{TetO} | Inducible regulation AccA1          | 95.1                 | 0.469                      |
| QC M3-A_{TetO}C | Inducible regulation AccA1 and overexpression AraC | 96.9 | 0.655 |
| MNR-prpR [7] | MCC enhancement                        | 90.6                 | 0.628                      |
| MNR-prpDBC/ΔglnR [7] | GlnR knockout and MCC enhancement    | 94.3                 | 0.654                      |
| MNR-Fpcc [6] | MMC enhancement                        | 25.4                 | 0.176                      |
| MNR-Fpcc-Fndh [6] | Overexpression NDH-II and MCC enhancement | 96.4 | 0.669 |
Table 2  Strains, plasmids, and primers used in this study

| Strains, plasmids, and primers | Significant properties | Source or purpose |
|--------------------------------|------------------------|------------------|
| **Strains**                    |                        |                  |
| Escherichia coli DH5α          | General cloning host   | Transgen Biotech |
| M3                             | Wild type Mycobacterium neoaurum TCCC 11u978 (MNR) | Tianjin University of Science and Technology Culture Collection Center (TCCC) |
| QC M3                          | Deletion of KsdD in Mycobacterium neoaurum TCCC 11u978 | Lab of Systematic Microbiology and Biomanufacturing Engineering, Tianjin University of Science and Technology |
| QC M3-N                        | QC M3 containing plasmid pMV261-Nat | This work |
| QC M3-C                        | QC M3 containing plasmid pMV261-AraC | This work |
| QC M3-A                        | QC M3 containing plasmid pMV261-AccA1 | This work |
| QC M3-D                        | QC M3 containing plasmid pMV261-AccD1 | This work |
| QC M3-AC                       | QC M3 containing plasmid pMV261-AraC-AccA1 | This work |
| QC M3-ArobR                    | QC M3 containing plasmid pMV262-AccA1-AraC | This work |
| **Plasmids**                   |                        |                  |
| pMV261                         | Shuttle vector, hsp60, Kan R | Dr. W. R. Jacobs Jr |
| pMV262                         | Shuttle vector, Tet O, Tet R, Kan R | This work |
| pMV261-Nat                     | Nat was connected to pMV261 | This work |
| pMV261-AraC                    | AraC was connected to pMV261 | This work |
| pMV261-AccA1                   | AccA1 was connected to pMV261 | This work |
| pMV261-AccD1                   | AccD1 was connected to pMV261 | This work |
| pMV262-AccA1                   | AccA1 was connected to pMV262 | This work |
| pMV261-AraC-AccA1              | AraC and AccA1 were Series connected to pMV261 | This work |
| pMV262-AraC-AccA1              | Adding tet operon between AraC and AccA1 at pMV262-AraC-AccA1 | This work |
| **Primers**                    |                        |                  |
| AccA1-f                        | gcggatccagctgcagaattc | AccA1 amplification |
| AccA1-r                        | gcggatccagctgcagaattc | AccA1 amplification |
| AccD1-f                        | gcggatccagctgcagaattc | AccD1 amplification |
| AccD1-r                        | gcggatccagctgcagaattc | AccD1 amplification |
| Nat-f                          | gcggatccagctgcagaattc | Nat amplification |
| Nat-r                          | gcggatccagctgcagaattc | Nat amplification |
| AraC-f                         | gcggatccagctgcagaattc | AraC amplification |
| AraC-r                         | gcggatccagctgcagaattc | AraC amplification |
| TetO-f                         | gcggatccagctgcagaattc | TetO amplification |
| TetO-r                         | gcggatccagctgcagaattc | TetO amplification |
| 16S-RT-F                       | GTAGGAGTCCAGCTTGGTC   | Quantitative RT-PCR |
| 16S-RT-R                       | GGTCTTCAGTACTGCGAGAG | Quantitative RT-PCR |
| AraC-RT-F                      | GGTCTTCAGTACTGCGAGAG | Quantitative RT-PCR |
| AraC-RT-R                      | GGTCTTCAGTACTGCGAGAG | Quantitative RT-PCR |
| Nat-RT-F                       | GGTCTTCAGTACTGCGAGAG | Quantitative RT-PCR |
| Nat-RT-R                       | GGTCTTCAGTACTGCGAGAG | Quantitative RT-PCR |

**Abbreviations**
- AD: Androstenedione
- ADD: Androsta-1,4-diene-3,17-dione
- MNR: Mycobacterium neoaurum
- MCC: 2-Methylcitrate cycle pathway
- MMC: Methylmalonyl cycle pathway
- MTB: Mycobacterium tuberculosis
- ACC: Propionyl-CoA / acetyl-CoA carboxylase
- PikA1: Polyketide synthase
- PDIM: Phthiocerol dimycolates
- NAT: N-acetyltransferase
- DCW: Dry cell weight
- LB: Luria–Bertani
- CoA: Coenzyme A
- qRT-PCR: Quantitative Reverse Transcription-PCR
- mRNA: Messenger RNA
- HPLC: High-performance liquid
- TEM: Transmission electron microscopy
Supplementary Information
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Additional file 1. Plasmid profiles of recombinant strains

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Author contributions
ZS and ZZ contributed equally to this work. ZS and ZZ performed most of the experiments, data analyses, and interpretation. YS designed and supervised the research and edited the manuscript aspects of this work. YJ and CY designed and participated in the experimental aspects of this work. LS and XZ helped edit the manuscript. MW supervised the research and edited the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
All data for this study are included in this published article and its additional file.

Declarations

Ethics approval and consent to participate
This article does not contain any studies involving human or animal participants.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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