Improving acarbose production and eliminating the by-product component C with an efficient genetic manipulation system of Actinoplanes sp. SE50/110

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The α-glucosidase inhibitor acarbose is commercially produced by Actinoplanes sp. and used as a potent drug in the treatment of type-2 diabetes. In order to improve the yield of acarbose, an efficient genetic manipulation system for Actinoplanes sp. was established. The conjugation system between E. coli carrying QC31-derived integrative plasmids and the mycelia of Actinoplanes sp. SE50/110 was optimized by adjusting the parameters of incubation time of mixed culture (mycelia and E. coli), quantity of recipient cells, donor-to-recipient ratio and the concentration of MgCl₂, which resulted in a high conjugation efficiency of 29.4%. Using this integrative system, a cloned acarbose biosynthetic gene cluster was introduced into SE50/110, resulting in a 35% increase of acarbose titer from 2.35 to 3.18 g/L. Alternatively, a pIJ101-derived replicating plasmid combined with the counter-selection system CodA(sm) was constructed for gene inactivation, which has a conjugation frequency as high as 0.52%. Meanwhile, almost all 5-flucytosine-resistant colonies were sensitive to apramycin, among which 75% harbored the successful deletion of targeted genes. Using this replicating vector, the maltoligosyltrehalose synthase gene treY responsible for the accumulation of component C was inactivated, and component C was eliminated as detected by LC-MS. Based on an efficient genetic manipulation system, improved acarbose production and the elimination of component C in our work paved a way for future rational engineering of the acarbose-producing strains.

1. Introduction

The α-glucosidase inhibitor acarbose (Fig. 1a) is produced on a large scale by strains derived from Actinoplanes sp. SE50 (ATCC31042; CBS 961.07) since 1990 [1]. The discovery of acarbose can be traced back to a screening program originated by Bayer AG to detect the inhibitors for mammalian intestinal α-amylase, sucrase, and maltase among the products of various actinomycetes in 1970 [1]. Subsequently, it was successfully marketed worldwide to treat type-2 diabetes, which enables patients to better control blood sugar concentrations when living with starch-containing diet [2]. Since diabetes becomes more prevalent [3], the demand of acarbose and other antidiabetic drugs increases rapidly. Therefore, improving the productivity of acarbose producers and reducing the production cost seem to be greatly important.

To increase the yield of acarbose, producing strains have been continuously selected by conventional mutagenesis and screening, along with appropriate optimization of fermentation processes, including media, osmolality, and fed-batch culture [4–7]. In addition, Actinoplanes sp. produce various acarbose analogs (Fig. 1a), of which component C is the main by-product and difficult to be removed by downstream purification process [1]. Usually, the accumulation of component C was reduced by the addition of C7-cyclitol-containing compounds, such as valienamine, validamycin and validoxylamine, or osmolality adjustment during fermentation process [8–10]. Component C was recently proved to be synthesized from acarbose by maltoligosyltrehalose synthase (TreY) in vitro [11]. The inactivation of the corresponding gene treY could probably eliminate the accumulation of component C.

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The 22 genes involved in acarbose biosynthesis are clustered and organized as several transcription units in the genome of *Actinoplanes* sp. (Fig. 1b). They encode proteins for the synthesis of C7-cyclitol and dTDP-4-amino-4,6-dideoxyglucose, glycosylation, export of acarbose, and utilization of extracellular starch [1,12,13]. Although the function of several enzymes (AcbC, AcbM, AcbO, AcbK, AcbD) had been established by biochemical reactions as previously reported [1,14–17], many other proteins are still putative, and the biosynthetic pathway of acarbose is not well elucidated, which limits further rational engineering of acarbose producers.

Recently, the complete genome sequence of *Actinoplanes* sp. SE50/110 was reported, which has a 9.24-Mb circular chromosome hosting 8270 putative protein coding sequences (CDSs) with a G+C content of 71.32% [18,19]. Comparative transcriptomics and proteomics were also applied to evaluate the different transcription and expression of *acb* cluster and other genes of SE50/110 cultivated in different media [20–23]. The abundant omics information would provide the potential targets for metabolic engineering of SE50/110.

However, in order to elucidate the acarbose biosynthetic mechanism, eliminate the by-products and increase the yield of acarbose, an efficient genetic manipulation system is necessary. A conjugation system between acarbose producer *Actinoplanes* sp. 8–22 and *E. coli* ET12567 (pUZ8002) was firstly reported by Yu Z, et al. [24], in which the exconjugants were obtained with mycelia treated with heat shock at 37 °C. Then, Gren T, et al. [25] established a conjugation system using spores released from the sporangia of SE50/110 based on the protocol of Horbal L, et al. [26] by adjusting media, incubation time and ratio of donor/recipient cells. Recently, the CRISPR/Cas9 system was successfully applied for gene deletion in SE50/110 [27].

Herein, another efficient and convenient conjugation system between *E. coli* and the mycelia of SE50/110 was established and optimized with ØC31-derived integrative vector and pIJ101-derived replicating plasmid bearing counter-selection marker. Using this system, the yield of acarbose was improved via introducing an extra copy of the *acb* cluster, and the by-product component C was eliminated by deleting the *treY* gene in SE50/110.

2. Materials and methods

2.1. Strains, plasmids, culture conditions and general techniques

The strains, plasmids and primers used in this study are listed in Table S1. *E. coli* DH10B was used for gene cloning and *E. coli* ET12567 (pUZ8002) was used for intergeneric conjugation between *E. coli* and mycelia of SE50/110, which were cultured with LB broth or on LB agar plates at 37 °C. SE50/110 and their derivatives were incubated on STY agar medium (sucrose 3%, tryptone 0.5%, yeast extract 0.5%, casin hydrolysate 0.1%, K$_2$HPO$_4$ $\cdot$ 3H$_2$O 0.1%, KCl 0.05%, FeSO$_4$ 0.005%, agar 2%, pH 7.2) at 30 °C for 2–3 days, and then inoculated into 30 mL SM broth (glucose 1.5%, maltose 1%, K$_2$HPO$_4$ $\cdot$ 3H$_2$O 0.1%, glycerol 1%, maltose extract 1%, tryptone 0.5%, yeast extract 0.5%, casin hydrolysate 0.1%, pH 7.2) in 250 mL baffled flask for 36–48 h on rotary shaker (30 °C, 220 rpm) for DNA isolation. Medium for the seed culture contains 1.5% maltose, 1% glucose, 4% soya flour, 1% glycerol, 1% soluble starch and 0.2% CaCO$_3$, pH 7.2. The fermentation medium contains 5% maltose, 3% glucose, 1% soya flour, 0.3% glutamate, 0.1% K$_2$HPO$_4$ $\cdot$ 3H$_2$O, 0.05% FeCl$_3$ and 0.25% CaCO$_3$, pH 7.2. When needed, antibiotics were added to a final concentration of 50 mg/L for apramycin and kanamycin, 25 mg/L for chloramphenicol.

The strains, plasmids and primers used in this study are listed in Table S1. Synthesis of oligonucleotide primers and sequencing of PCR products were performed by Shanghai Generay Biotech Co., Ltd. Restriction endonucleases and alkaline phosphatase (FastAP) were purchased from Thermo Fisher Scientific. T4 DNA ligase and DNA polymerase were purchased from Takara. 5-flucytosine

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**Fig. 1.** Acarbose and its biosynthetic gene cluster. **a** Structures of acarbose and related metabolites. **b** Acarbose biosynthetic gene cluster (*acb* cluster) of *Actinoplanes* sp. SE50/110 (accession: Y18523).

| name    | structure$^1$ |
|---------|---------------|
| component Z | Ao1,4-Glc    |
| acarbose (component 3) | Ao1,4-Glc1,4-Glc |
| component A | Ao1,4-Glc1,4-Fru |
| component B | Ao1,4-Glc1,4-(1-epi-vallienol) |
| component C | Ao1,4-Glc1,1-Glc |
| component D | Ao1,4-Glc1,4-Man |
| component 4b | Ao1,4-Glc1,4-Glc |
| component 4a | Ao1,4-Glc1,4-Glc1,4-Fru |
| component 4c | Ao1,4-Glc1,4-Glc1,1-Glc |

1) Ac = acarbossyl moiety
(5-FC) was purchased from Adamas. Other biochemicals were purchased from Sinopharm Chemical Reagent Co., Ltd and Oxoid. CopyControl™ Fosmid Library Production Kit was purchased from Epicentre.

2.2. Pilot intergeneric conjugation between E. coli and SE50/110

A culture of the ET12567[pUZ8002] containing pSET152 was grown overnight in LB with 50 mg/L apramycin, 25 mg/L chloramphenicol and 50 mg/L kanamycin, and then inoculated (1/20, v/v) to fresh LB broth and grown to OD_{600} of 0.8–1.0. Cells were washed for three times with an equal volume of LB, re-suspended in 0.5 volume of LB (about 10^{7–10^8} CFU). SE50/110 was cultivated on STY plate for 2–3 days, inoculated to SM medium for a 36-h growth and subsequently transferred (1/10, v/v) to TSB medium for a further 8–12 h incubation. The mycelia were washed twice and re-suspended in equal volume of LB (about 10^9–10^7 CFU).

1 mL of mycelial suspension (about 10^6–10^7 CFU) and 0.5 mL of E. coli suspension (about 10^9–10^8 CFU) were mixed and spread on SFM agar plate (mannitol 2%, full-fat soya flour 2%, agar 2%) containing 10 mM MgCl₂. Then, SFM agar plates were incubated for 12 h at 30 °C and overlaid with 1 mL sterile water containing 1 mg apramycin and 0.5 mg trimethoprim. These plates were incubated at 30 °C for another 5–7 days.

2.3. Cultivation and verification of exconjugants

The exconjugants were streaked to STY plates with 50 mg/L apramycin and 25 mg/L trimethoprim, cultivated for 2 days, and then transferred to SM liquid medium with 50 mg/L apramycin and 25 mg/L kanamycin, and then inoculated (1/100, v/v) to fresh LB broth and grown to OD_{600} of 0.8

2.4. Constructing attB-F/attB-R contained fosmid for the acb cluster

The high-quality genomic DNA was isolated with the salting out procedure [28]. The DNA was sheared randomly to generate approximately 40 kb fragments. The end-pair of the sheared DNA was blunted, and 5’-end was phosphorylated by the CopyControl™ Fosmid Library Production Kit. The blunt-end DNA was purified from low-melting-point agarose and 40 kb fragments were isolated. The fragments were ligated to the cloning-ready CopyControl™ pCC1FOS™ fosmid vector and then packaged to transfect the EPI300-T1R™ plating cells. After overnight growth, about 3,000 clones were picked and transferred to 96-well plates. The first round of PCR screening used acbM in the middle of the acb cluster as a selection marker. Then, the clones with acbM gene were further selected with the boundary genes (acbZ and acbD). The fosmid (pLQ665) with acbM, acbZ and acbD was further verified by sequencing.

2.5. Introduction of an extra copy of the acb cluster

To overexpress the acb cluster (40.1 kb) on pLQ665, the cassette of int-attP-oriT-aac(3)IV was amplified by PCR using pSET152 as template DNA and primers pLQ666-F/R with 39-bp homologous arms at both ends. The cassette was subsequently introduced to the fosmid by PCR targeting [29] to generate pLQ666. pLQ666 was transferred to ET12567(pUZ8002) and subsequently introduced to SE50/110 by conjugation. The exconjugants (QQ-1) were selected, cultured, and verified by PCR using primers apr-F/acb-R (Fig. 2a, Fig. S3).

2.6. Construction of pJTU1278-derived replicating vectors

The 981-bp fragment with aac(3)IV and its promoter region, flanked by AffII and Nhel restriction sites, was amplified from pSET152 using primers apr-750-F/R. The 986-bp oriT fragment between the Ndel restriction site and the terminator of tsr in pJTU1278 [30] was amplified using primers oriT-750-F/R with Ndel and AffII restriction sites at both ends. These two fragments were respectively digested with AffII/Nhel and Ndel/AffII, and inserted

Fig. 2. Introduction of an extra copy of acb cluster into SE50/110. a pLQ666 with whole acb cluster and cassette of int-attP-oriT-aac(3)IV from pSET152. b Acarbose production of QQ-1 (with an extra copy of acb cluster introduced by integration of pLQ666) and SE50/110::pSET152 (control strain with integrated pSET152). **, p < 0.05. c The transcription pattern of acbH, acbV, acbC, acbB, acbM in acb cluster of QQ-1 and SE50/110::pSET152 at 48 h during the fermentation process. The Y-axis scale represents the expression value of genes relative to that of hdb. The average transcription of genes in SE50/110::pSET152 were set to 1 as standard, the transcription of genes in QQ-1 were accordingly calculated. Graphs depict means ± SD. Values represent average results from three independent experiments.
into NdeI/NheI-digested pJTU1278 to generate pLQ750 (9.2 kb) with 
*tsr* replaced by *aac(3)IV*.

The 1433-bp fragment with *codA* (sm) and its promoter, flanked by *AflII* restriction sites, was amplified from pVHU2653 [31]. The amplified fragments were inserted into *AflII*-digested pLQ750 to generate pLQ752 (10.6 kb).

### 2.7. Construction and selection of treY-deleted mutants

In order to delete *treY* gene, two homologous arms were respectively amplified with primers *treY*-1/*treY*-2 and *treY*-3/*treY*-4 from the genome of SE50/110 and cloned into *BamHI/HindIII*-digested plasmid pLQ750/pLQ752 to generate pLQ753/pLQ756, respectively.

The recombinant plasmids were transferred to ET12567(pUZ8002) and then introduced to SE50/110 by intergeneric conjugation. Exconjugants were streaked to STY plates with 50 mg/L apramycin and 25 mg/L trimethoprim for 2–3 days. In order to promote the DNA recombination in the exconjugants, the mycelia from STY plate were inoculated to SM broth. Then a 36-h culture was transferred (1/10, v/v) to fresh SM broth for another 36–48 h cultivation. The mycelia were diluted for 10 folds and filtered with non-absorbent cotton wool. The filtrate was diluted for 104–105 times and cultivated for 4–5 days on STY plates without antibiotics (for SE50/110::pLQ753) and with 50 mg/L 5-FC (for SE50/110::pLQ756). The apramycin-sensitive colonies were selected by replica plating and verified by PCR using primers TV-F/TV-R. The mutants with double-crossover recombination (QQ-2) gave a 0.70-kb amplified product, whereas the wild-type gave a 1.80-kb amplified fragment (Fig. 3a and b).

### 2.8. Complementation and overexpression of *treY* gene in QQ-2 and SE50/110

In order to overexpress *treY* gene, strong promoter of *kasOp* and *treY* gene were amplified from plasmid of pDR-4-K* [32] and the genome of SE50/110 by PCR with primers *treY*-E-1/*treY*-E-2 and *treY*-E-3/*treY*-E-4, respectively, both of which were ligated by overlapping PCR using primers *treY*-E-1/*treY*-E-4. This fragment was digested with *XbaI* and *BamHI* and inserted into pSET152 digested with both enzymes to generate pLQ758. The recombinant plasmid pLQ758 was transferred to ET12567(pUZ8002) and then introduced to QQ-2 and SE50/110 by intergeneric conjugation.

### 2.9. Fermentation and analysis of related compounds

To assess the acarbose production of SE50/110 and its derivatives, 3-mL mycelia from SM medium was transferred to 30 mL seed medium in 250 mL baffled flask and cultivated for 20–22 h on rotary shaker (30 °C, 220 rpm). Then, 7.5-mL seed culture was inoculated to 50 mL fermentation medium in 250 mL baffled flask and cultivated for another 7 days. Additionally, 1 g glucose and 1 g maltose was added to every flask in day 3.

The supernatant of fermentation broth was obtained by centrifugation at 12,000 rpm for 10 min, diluted for 3–5 folds, and analyzed by HPLC (Agilent series 1260, Agilent Technologies, USA).

**Fig. 3.** Elimination of component C by deletion of *treY*. a Schematic representation of the gene deletion of *treY*. b Confirmation of the mutant QQ-2 by PCR amplification. Using primers TV-F and TV-R, approximately a 0.70-kb fragment was amplified using the total DNA of QQ-2 or the recombinant plasmid pLQ756 as templates, whereas SE50/110 gave a 1.80-kb product. c HPLC profiles of SE50/110, *treY* mutant QQ-2, QQ-2::pLQ758 (complementation of *treY* gene in QQ-2) and SE50/110::pLQ758 (overexpression of *treY* gene in SE50/110). d Acarbose and component C production of SE50/110, QQ-2, QQ-2::pSET152 (control strain with the integration of pSET152 in QQ-2), QQ-2::pLQ758, SE50/110::pSET152 and SE50/110::pLQ758. Graphs depict means ± SD. Values represent average results from three independent experiments.
Acarbose and component C were separated with Agilent ZORBAX NH2 column (4.6 × 250 mm, particle size 5 μm) using an elution buffer composed of acetonitrile and phosphate buffer (0.70 g Na2HPO4·12H2O and 0.60 g KH2PO4 in 1 L ddH2O) at a ratio of 73:27 (v/v), a flow rate of 1 mL/min, and detected at 210 nm. In addition, the samples were also analyzed by UPLC-Q-TOF-MS (Agilent 1290—6500 Q-TOF) using elution buffer of acetonitrile and water at a ratio of 73:27 (v/v), a flow rate of 0.4 mL/min, and detecting in negative ion mode.

3. Results

3.1. Parameters affecting the efficiency of conjugation between E. coli and mycelia of SE50/110

To establish an efficient genetic manipulation system, the sensitivity of SE50/110 to different antibiotics at normally used concentrations (Table S2) was detected. As reported by Gren T, et al. [25], SE50/110 is sensitive to aminoglycosides, kanamycin, naldixic acid and resistant to thiostrepton. In addition, it was shown that SE50/110 was also sensitive to chloramphenicol (25 mg/L), streptomycin (25 mg/L) and resistant to spectinomycin (100 mg/L), trimethoprim (50 mg/L). Therefore, trimethoprim was chosen to substitute nalidixic acid to inhibit the growth of E. coli after conjugation.

The attP site of the OC31-derived integrative plasmids (such as pSET152) have been verified to integrate specifically at the attB locus on the SE50/110 chromosome via OC31 integrase (int) [25]. In our work, to improve the conjugation efficiency between E. coli and mycelia of SE50/110, various parameters, including mycelia cultivation, incubation time of mixed culture, the quantity of recipient cells, the donor-to-recipient ratio and the concentration of MgCl2, were optimized in detail.

To select mycelia with appropriate cultivation time, the mycelia from STY plate were inoculated to SM broth. Then, a 36-h culture was transferred (1/10, v/v) to TSB medium for another 28-h cultivation. The cells grew exponentially between 4 and 16 h and declined after 20 h in TSB medium (Fig. S1). Therefore, the mycelia were better to harvest at middle logarithmic phase (8—12 h) for conjugation.

The incubation time of mixed culture of donor and recipient cells, along with the quantity of recipient cells, might influence the conjugation frequency. Therefore, 10^3—10^7 CFU of recipient cells were used in conjugation, and all mixed cultures were incubated from 8 to 36 h before overlaid with antibiotics. As shown in Table 1, the conjugation frequency increased about 10^2 folds when the incubation time was increased from 8 h (1.35 × 10^{−6}) to 32 h (0.146).

### Table 1

| Incubation time (h) | Quantity of recipient cells (CFU) | Quantity of exconjugants* (CFU) | Conjugation frequency a |
|---------------------|----------------------------------|---------------------------------|-------------------------|
| 8                   | 5.2 × 10^6                       | 7 ± 2                           | 1.35 ± 0.38 × 10^−6     |
| 12                  | 5.2 × 10^6                       | 537 ± 25                        | 1.03 ± 0.04810^{−4}     |
| 16                  | 5.2 × 10^6                       | 1,500 ± 103                     | 2.88 ± 0.20 × 10^{−4}   |
| 20                  | 5.2 × 10^6                       | 1,280 ± 69                      | 2.46 ± 0.13 × 10^{−3}   |
| 24                  | 5.2 × 10^6                       | 535 ± 43                        | 1.03 ± 0.08 × 10^{−2}   |
| 28                  | 3.2 × 10^7                       | 418 ± 12                        | 0.130 ± 0.004           |
| 32                  | 3.2 × 10^7                       | 468 ± 47                        | 0.146 ± 0.015           |
| 36                  | 3.2 × 10^7                       | 286 ± 27                        | 8.94 ± 0.84 × 10^{−2}   |

The donor-to-recipient ratios were kept at 10:1—20:1, and 10 mM MgCl2 was added to the SMF medium.

### Table 2

| Ratio (donor:recipient) | Quantity of donor cells (CFU) | Quantity of exconjugants (CFU) | Conjugation frequency b |
|-------------------------|-------------------------------|---------------------------------|-------------------------|
| 1:3                     | 9.2 × 10^2                    | NE                              | 0                       |
| 3:1                     | 9.2 × 10^3                    | NE                              | 0                       |
| 10:1                    | 9.2 × 10^4                    | 404 ± 18                        | 0.13 ± 0.006            |
| 150:1                   | 4.6 × 10^6                    | 102 ± 22                        | 3.19 ± 0.69 × 10^{−2}   |
| 300:1                   | 9.2 × 10^5                    | 77 ± 17                         | 2.41 ± 0.05 × 10^{−2}   |
| 1,500:1                 | 4.6 × 10^6                    | 1                              | 3.13 × 10^{−4}          |
| 3,000:1                 | 9.2 × 10^6                    | NE                              | 0                       |

The number of recipient cells were kept at 3.2 × 10^3 (CFU). 10 mM MgCl2 was added to the SMF medium, and the mixed culture on SMF plates were incubated for 32 h. NE, no exconjugant.

### Values represent average frequencies from three independent experiments.

To optimize the ratio between donor and recipient cells, 10^3—10^7 CFU of donor cells were mixed with 3.2 × 10^3 CFU of recipient cells. As shown in Table 2, using 9.2 × 10^2 and 9.2 × 10^3 CFU of donor cells resulted in no exconjugant, but the conjugation frequency reached peak (0.136) by further increasing the number of donor cells to 9.2 × 10^4 CFU. When 150—1,500-fold excess donor cells were used, the conjugation frequency decreased dramatically, and nearly no exconjugant was obtained with 3,000-fold excess donor cells. Thus, the optimal donor-to-recipient ratio was about 30:1.

MgCl2 was usually added to the media to increase the conjugation efficiency, albeit the optimal concentration is different for different actinobacteria strains [33,34]. In our study, 2—30 mM MgCl2 was added to the SFM medium, and the conjugation frequency was shown in Table S3. The highest conjugation frequency of 0.294 was obtained by adding 20 mM MgCl2. However, higher concentration (>30 mM) would slightly inhibit the growth of exconjugants (data not shown).

After conjugation, about 100 exconjugants of SE50/110::pSET152 were randomly picked and verified. All selected exconjugants were confirmed to be successfully integrated with pSET152 (Fig. S2), suggesting that the conjugation system is efficient and reproducible.

3.2. Improvement of acarbose production by introducing a cloned acb gene cluster

A pCC1FOS-derived fosmid with the 40.1-kb intact acb cluster was selected from the genomic library of SE50/110. Subsequently, the cassette of attP-integ-oriT-aac(3)IV was amplified from pSET152 and inserted into the fosmid by PCR targeting to construct plQ666 (Fig. 2a). Plasmid plQ666 was then introduced into the SE50/110 to generate QQ-1 (Fig. S3), which resulted in an increase of acarbose production from 2.35 g/L to 3.18 g/L (about 35%) and slightly lower biomass (Fig. 2b, Fig. S4). Meanwhile, the transcription of acbw, acbv, acbc, acbb and achA in acb cluster of QQ-1 were improved 1.9—3 folds as compared with SE50/110::pSET152 (Fig. 2c). These results suggested that improvement of the copy number or expression of acb cluster would be beneficial to acarbose production.

3.3. Construction and optimization of the gene editing system based on plf101-derived replicating plasmid

pJTU1278, the replicating plasmid derived from plf101, contains multiple cloning sites, lacZ for convenient construction in E. coli and thiostrepton resistance gene (tsr) for the selection in actinobacteria.
plasmids are more stable in colonies that were selected by replica plating. Only 12.5% colonies were of the ØC31-derived integrative vectors (0.294). Therefore, pIJ101-derived replicating round of nonselective growth[35].

3.5. Elimination of component C by inactivation of treY

SE50/110 produced about 177 mg/L component C and 2.7 g/L acarbose after 7-day fermentation in shake flasks (Fig. 3d). However, component C was not detected in the fermentation broth of treY deleted mutant (QQ-2) by HPLC or LC-MS (Fig. S5). And acarbose production was increased slightly from 2.70 g/L to 2.99 g/L (Fig. 3d). To further verify the involvement of TreY in the accumulation of component C, pLQ753, with the gene treY under the control of strong promoter kasOp*, was constructed and introduced to SE50/110 and QQ-2 (Fig. S5). Whereas the yield of component C in the control strains SE50/110::pSET152 was 155 mg/L, its yields in SE50/110::pLQ758 and QQ-2::pLQ758 were remarkably increased to 594 mg/L and 501 mg/L, respectively. Meanwhile, the overexpression of treY resulted in a decreased acarbose yield about 59%–63% (Fig. 3c and d, Fig. S6). Therefore, 93–112 folds improvement of treY transcription in SE50/ 110::pLQ758 and QQ-2::pLQ758 resulted in the synthesis of large amount of component C from acarbose (Fig. S7). These results proved that component C is generated by the catalysis of TreY, and it is thus eliminated by the deletion of treY gene.

4. Discussion

The genus of Actinoplanes form characteristic sporangia bearing motile spores [37]. The generation of sporangia mainly depends on the features of different strains, components of media and culture conditions, and usually requires long time (about 7–15 days) in the laboratory [25,33]. The sporangia are usually enveloped by hydrophobic membrane, which need to burst under the condition with sufficient moisture for enough time [38]. However, the mycelia of Actinoplanes sp. are easier to obtain in short period. Therefore, the utilization of mycelia in conjugation system of Actinoplanes sp. would shorten the conjugation period, which is also appropriate for other actinobacteria with less or no spores. Moreover, the optimized conjugation system showed high efficiency of 0.294 (Table S3), which is even better than that reported in S. lividans (8 × 10⁻³) [39] and other Actinoplanes strains (3 × 10⁻³–6 × 10⁻³) [25,33].

In addition, compared to the conjugation systems established by Yu Z, et al. [24] and Gren T, et al. [25], the conjugation parameters of our system, such as mycelia cultivation, incubation time of mixed culture, number of recipient cells, recipient-to-donor ratio and MgCl₂ concentration, were systematically evaluated and set as a good reference for establishing genetic manipulation system for other actinobacteria. Meanwhile, trimethoprim was selected as a substitute of nalidixic acid to inhibit the growth of E. coli after conjugation or during cultivation of the exconjugants, which significantly simplified the construction process of mutants.

pl101-derived replicating vectors are widely used for gene inactivation in actinobacteria, due to its high copy numbers (>50) providing abundant DNA sequences for homologous recombination and genetic instability for easy loss without antibiotic selection [30,35]. In this work, pl101-derived vector (pLQ750) was successfully transferred to SE50/110 with high conjugation efficiency of 8.56 × 10⁻³ (Table S4), which is comparable to S. lividans (about 10⁻³) with another pl101-derived plasmid pJTU412 [35]. However, different to Streptomyces, pLQ750 was difficult to lose in acarbose-producing Actinoplanes sp.

CodA (sm), the D314A mutant of cytosine deaminase (CodA) that efficiently converts 5-FC to 5-fluorouracil (5-FU) with high toxicity, was developed as a valuable counter-selection marker for the actinobacteria [31,36]. When CodA(sm) was introduced into...
HPLC method of separating these two compounds was optimized in produced by of acarbose yield. However, it is not sure if component C is still component C production was decreased from 0.514% to 0.056% [24] had deleted by high-resolution UPLC-Q-TOF-MS (Fig. S6). It was con

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