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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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ABSTRACT

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 ØC31-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 $MgCl_2$, 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 maltooligosyltrehalose 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 C_7-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 maltooligosyltrehalose synthase (TreY) *in vitro* [11]. The inactivation of the corresponding gene *treY* could probably eliminate the accumulation of component C.

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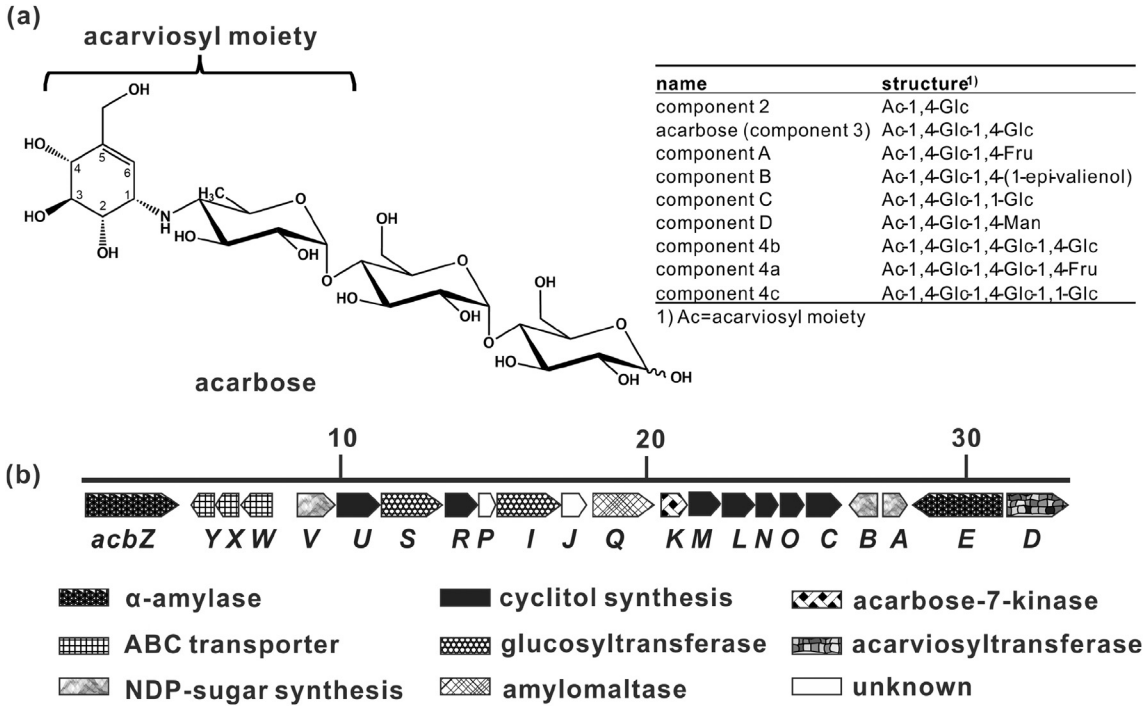

Fig. 1. Acarbose and its biosynthetic gene cluster. **a** Structures of acarbose and related metabolites. **b** Acarbose biosynthetic gene cluster (*acb* cluster) of *Actinoplanes* sp. SE50/110 (accession: Y18523).

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 C_7-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* 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-derivied 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%, $K_2HPO_4 \cdot 3H_2O$ 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%, $K_2HPO_4 \cdot 3H_2O$ 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% $K_2HPO_4 \cdot 3H_2O$, 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.

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

(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 10^6–10^7 CFU).

1 mL of mycelial suspension (about 10^6–10^7 CFU) and 0.5 mL of *E. coli* suspension (about 10^7–10^8 CFU) were mixed and spread on SFM agar plate (mannitol 2%, full-fat soya flour 2%, agar 2%) containing 10 mM $MgCl_2$. 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 2–3 days, and then transferred to SM liquid medium with 50 mg/L apramycin and cultivated for 36 h to isolate total DNA. The mutants were confirmed by PCR amplification using primers *attB*-F/*attB*-R, *attB*-F/152-1 *and attB*-R/152-2. The primers *attB*-F/*attB*-R located both sides of $attB^{\phi C31}$ on the chromosome of SE50/110. 0.35-kb and 6.1-kb (containing whole sequence of pSET152) PCR products were expected to be amplified from total DNA of SE50/110 and SE50/110::pSET152, respectively (Fig. S2a). The primers 152-1/152-2 were reverse complement sequences within pSET152. Using the primers *attB*-F/152-1 *and attB*-R/152-2, approximately 4.1-kb and 2.0-kb fragments were expected to amplified from SE50/110::pSET152, respectively (Fig. S2b). The total length of these two fragments should be consistent with the fragment amplified by *attB*-F/*attB*-R.

2.4. Constructing genomic library of SE50/110 and screening for the fosmid containing 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-T1^R 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 *Afl*II and *Nhe*I restriction sites, was amplified from pSET152 using primers *apr*-750-F/R. The 986-bp *oriT* fragment between the *Nde*I restriction site and the terminator of *tsr* in pJTU1278 [30] was amplified using primers *oriT*-750-F/R with *Nde*I and *Afl*II restriction sites at both ends. These two fragments were respectively digested with *Afl*II/*Nhe*I and *Nde*I/*Afl*II, and inserted

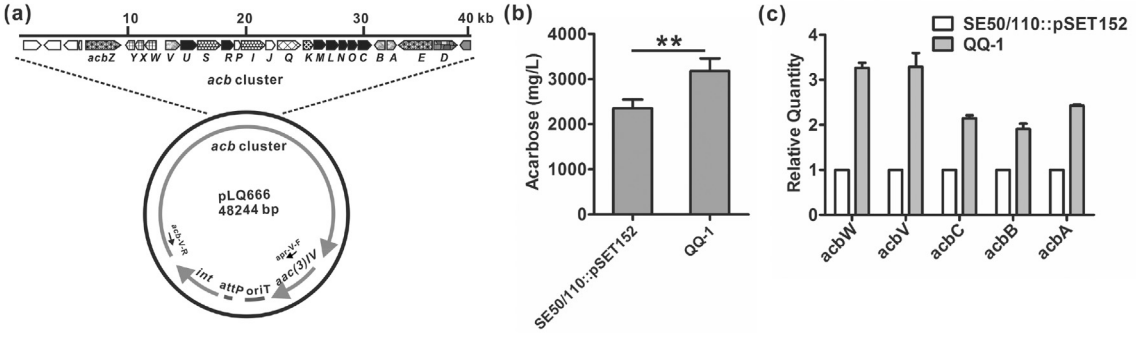

Fig. 2. Introduction of an extra copy of *acb* cluster into SE50/110. **a** pLQ666 with whole *acb* cluster and cassette of *int-attP-oriT-aac(3)IV* from pSET152. **b** Acarbose production of QQ-1 (with an extra copy of *acb* cluster introduced by integration of pLQ666) and SE50/110::pSET152 (control strain with integrated pSET152). **, $p < 0.05$. **c** The transcription pattern of *acbW*, *acbV*, *acbC*, *acbB*, *acbA* 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 *hrdB*. 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 *Nde*I/*Nhe*I-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 *Afl*II restriction sites, was amplified from pWHU2653 [31]. The amplified fragments were inserted into *Afl*II-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 *Bam*HI/*Hin*dIII-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 10^4-10^5 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 *kasO*p* 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 *Xba*I and *Bam*HI 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. The exconjugants were selected, cultured and verified by PCR using primers *treY*-E-1/*treY*-E-4 (Fig. S5).

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).

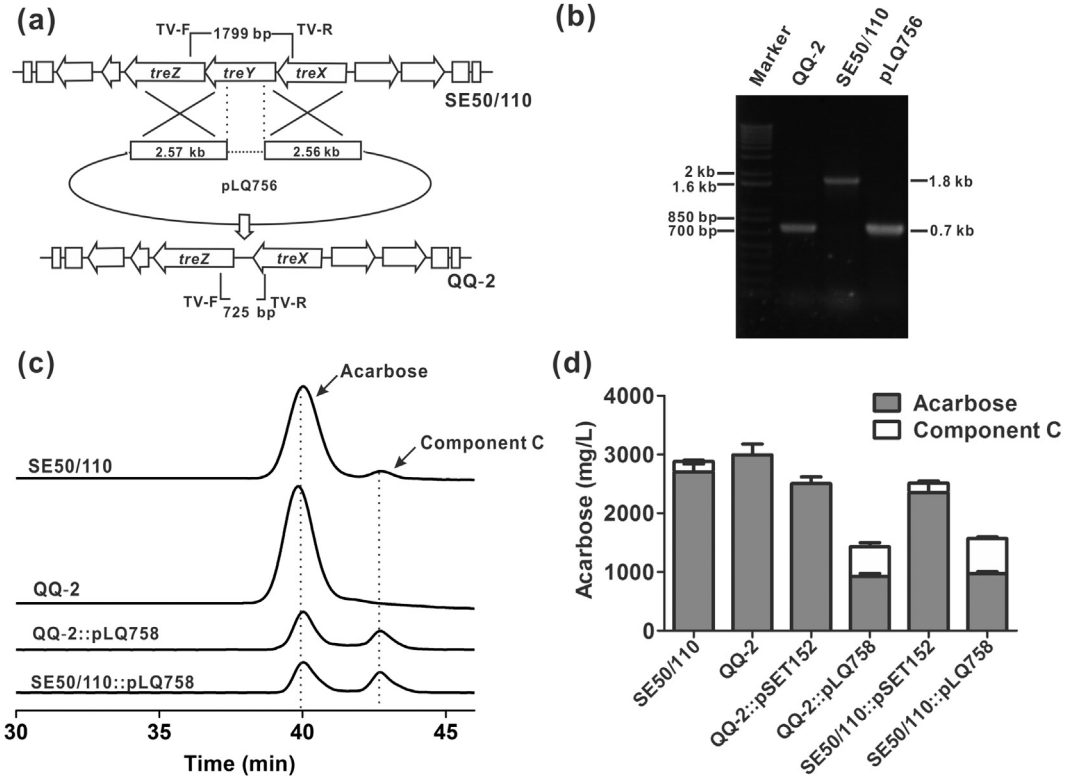

Fig. 3. Elimination of component C by deletion of *treY*. **a** Schematic representation of the gene deletion of *treY*. **b** Confirmation of the mutant QQ-2 by PCR amplification. Using primers TV-F and TV-R, approximately a 0.70-kb fragment was amplified using the total DNA of QQ-2 or the recombinant 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 NH_2 column (4.6 × 250 mm, particle size 5 μm) using an elution buffer composed of acetonitrile and phosphate buffer (0.70 g $Na_2HPO_4 \cdot 12H_2O$ and 0.60 g KH_2PO_4 in 1 L ddH_2O) 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 apramycin, kanamycin, nalidixic 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 ØC31-derived integrative plasmids (such as pSET152) have been verified to integrate specifically at the *attB* locus on the SE50/110 chromosome via ØC31 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 $MgCl_2$, 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 10^5 folds when the incubation time was increased from 8 h (1.35×10^{-6}) to 32 h (0.146).

Table 1
Effects of different incubation times of mixed culture and quantities of recipient cells on conjugation.

Incubation time (h)	Quantity of recipient cells (CFU)	Quantity of exconjugants[a] (CFU)	Conjugation frequency[b]
8	5.2×10^6	7 ± 2	$1.35 \pm 0.38 \times 10^{-6}$
12	5.2×10^6	537 ± 25	1.03 ± 0.04810^{-4}
16	5.2×10^6	1,500 ± 103	$2.88 \pm 0.20 \times 10^{-4}$
20	5.2×10^5	1,280 ± 69	$2.46 \pm 0.13 \times 10^{-3}$
24	5.2×10^4	535 ± 43	$1.03 \pm 0.08 \times 10^{-2}$
28	3.2×10^3	418 ± 12	0.130 ± 0.004
32	3.2×10^3	468 ± 47	0.146 ± 0.015
36	3.2×10^3	286 ± 27	$8.94 \pm 0.84 \times 10^{-2}$

The donor-to-recipient ratios were kept at 10:1–20:1, and 10 mM $MgCl_2$ was added to the SFM medium.
[a,b]Values represent average quantities or frequencies from three independent experiments.

10^3–10^4 CFU of recipient cells were sufficient to obtain numerous exconjugants when overlaid at 32 h.

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.

$MgCl_2$ is 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 $MgCl_2$ 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 $MgCl_2$. 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-int-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 *acbA* 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 pIJ101-derived replicating plasmid

pJTU1278, the replicating plasmid derived from pIJ101, contains multiple cloning sites, *lacZ* for convenient construction in *E. coli* and thiostrepton resistance gene (*tsr*) for the selection in actinobacteria

Table 2
Effects of donor-to-recipient ratio on conjugation.

Ratio (donor: recipient)[a]	Quantity of donor cells (CFU)	Quantity of exconjugants (CFU)	Conjugation frequency[a]
1:3	9.2×10^2	NE	0
3:1	9.2×10^3	NE	0
30:1	9.2×10^4	404 ± 18	0.13 ± 0.006
150:1	4.6×10^5	102 ± 22	$3.19 \pm 0.69 \times 10^{-2}$
300:1	9.2×10^5	77 ± 17	$2.41 \pm 0.05 \times 10^{-2}$
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 $MgCl_2$ was added to the SFM medium, and the mixed culture on SFM plates were incubated for 32 h.
NE, no exconjugant.
[a] Values represent average frequencies from three independent experiments.

Table 3
The efficiency of replicating vectors used for gene deletion in SE50/110.

Plasmids[a]	Conjugation frequency	ARC[b]/TSC (%)	GDM/TSC (%)	WT/TSC (%)
pLQ-753	1.10×10^{-2}	26/32 (81.25%)	4/32 (12.5%)	2/32 (6.25%)
pLQ-756	5.20×10^{-3}	0/24	18/24 (75%)	6/24 (25%)

ARC, apramycin resistant colonies; TSC, total selected colonies; GDM, gene deletion mutants; WT, wild-type.
[a] pLQ753 was derived from pJTU1278 with inserted flanking sequences of *treY*, in which *tsr* was replaced by *aac(3)IV*. pLQ756 was derived from pLQ753 with the insertion of *codA*(*sm*).
[b] The mutants still contained the delivery plasmid in replicating form or integrated into the chromosome by single-crossover recombination. Values represent average frequencies from three independent experiments.

[30,35]. Considering the resistance of SE50/110 to thiostrepton (Table S2), the gene *tsr* was replaced by *aac(3)IV* to generate pLQ750. In order to establish and optimize the replicating vector-based gene editing system, the *treY* possibly involved in the accumulation of component C was chosen as a target. Two fragments flanking *treY* were inserted into pLQ750 to generate pLQ753, which was transferred to SE50/110 by conjugation.

The conjugation frequency of pLQ753 was again optimized by using different quantities of recipient cells. As shown in Table S4, using 6.9×10^4 CFU of recipient cells resulted in large number of exconjugants and reached highest conjugation frequency (8.56×10^{-3}), although the frequency was 34-fold lower than that of the ØC31-derived integrative vectors (0.294).

The mycelia of exconjugants from STY plate were inoculated to SM liquid medium for two rounds of nonselective growth to promote DNA recombination. Then, the mycelia were diluted, filtered and cultivated on STY agar plates. The apramycin-sensitive colonies were selected by replica plating. Only 12.5% colonies were with *treY* gene deleted, whereas 81.25% colonies were still resistant to apramycin (Table 3). Therefore, pIJ101-derived replicating plasmids are more stable in *Actinoplanes* sp. than in *Streptomyces*, in which about 95% colonies lost the antibiotic resistance after one round of nonselective growth [35].

3.4. Utilization of negative selection marker codA

In order to promote the loss of replicating plasmid after recombination, a negative selection system using cytosine deaminase (CodA) [36] was employed. The *codA*(*sm*) gene was cloned from pWHU2653 and inserted into pLQ750 to generate pLQ752. Then, the homologous arms for *treY* deletion were inserted into pLQ752 to construct pLQ756 (Fig. 3a). The exconjugants were cultured, diluted and cultured on the STY agar plate supplemented with 50 mg/L 5-FC. Almost all colonies were sensitive to apramycin, while 75% colonies were with *treY* deletion and 25% colonies were reverted to the wild-type (Table 3 and Fig. 3b), indicating that the counter-selection system CodA(*sm*) was efficient in *Actinoplanes* sp.

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. 3c, Fig. S6), 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, pLQ758, with the gene *treY* under the control of strong promoter *kasO*p*, 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^{-2}) [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 $MgCl_2$ 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.

pIJ101-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, pIJ101-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 pIJ101-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

pLQ750, almost all the tested colonies growing on STY plates with 50 mg/L 5-FC lost the resistance to apramycin (Table 3). This efficiency is relatively higher than that of temperature-sensitive plasmid pCRISPomyces-2 bearing CRISPR/Cas9 system, which is with 6%–61% colonies lost the resistance to apramycin after two rounds cultivation at 37 °C [27]. Meanwhile, the frequency of successful deletion mutants per apramycin sensitive colonies were comparable between pLQ756 (75%) (Table 3) and pCRISPomyces-2 derivative (80%) [27]. The conjugation frequency of pLQ756 (5.2×10^{-3}) was lower than pLQ753 (1.1×10^{-2}) (Table 3), which might be resulted from the constitutive expression of *codA* (*sm*), converting intracellular 5-FC to 5-FU and showing toxicity to the exconjugants. Thus, this frequency could be improved by replacing the constitutive promoter of *codA* (*sm*) with inducible promoter, such as thiostrepton-inducible *tipA* promoter [25] or oxytetracycline-inducible Potr* system [40].

TreY, responsible for biosynthesis of trehalose from maltooligosaccharides, was proved to catalyze the formation of component C from acarbose by its proposed isomerase activity [11]. Yu Z, et al. [24] had deleted *treY* in acarbose producer *Actinoplanes* 8–22 and component C production was decreased from 0.514% to 0.056% of acarbose yield. However, it is not sure if component C is still produced by *treY* deleted mutant based on the poor separation of acarbose and component C in the HPLC profiles [24]. Therefore, the HPLC method of separating these two compounds was optimized in this work (Fig. 3c), and the two compounds were further analyzed by high-resolution UPLC-Q-TOF-MS (Fig. S6). It was confirmed that component C couldn't be detected after *treY* deletion. In addition, the remarkable improvement of component C after overexpression of *treY* under the control of strong promoter *kasO*p* (Fig. 3c and d, Figs. S6 and S7) genetically indicated that TreY plays an important role in the formation of component C. However, it couldn't be excluded that the trehalose is able to incorporate in acarbose biosynthetic pathway instead of maltose.

In conclusion, the efficient genetic manipulation system with both integrative and replicating vectors was established and optimized for acarbose-producing *Actinoplanes* sp.. The improvement of acarbose production by overexpression of *acb* cluster implied the necessity and urgency in elucidating the regulatory mechanism of *acb* cluster for rationally engineering of the producers. Meanwhile, elimination of component C by deletion of *treY* would benefit to the separation of acarbose in industrial production.

Conflict of interest

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.synbio.2017.11.005.

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