Comparative functional genomics of the acarbose producers reveals potential targets for metabolic engineering

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ABSTRACT

The α-glucosidase inhibitor acarbose is produced in large-scale by strains derived from Actinoplanes sp. SE50 and used widely for the treatment of type-2 diabetes. Compared with the wild-type SE50, a high-yield derivative Actinoplanes sp. SE50/110 shows 2-fold and 3-7-fold improvement of acarbose yield and acb cluster transcription, respectively. The genome of SE50 was fully sequenced and compared with that of SE50/110, and 11 SNVs and 4 InDels, affecting 8 CDSs, were identified in SE50/110. The 8 CDSs were individually inactivated in SE50. Deletions of ACWT_4325 (encoding alcohol dehydrogenase) resulted in increase of acarbose yield by 25% from 1.87 to 2.34 g/L, acetyl-CoA concentration by 52.7%, and PEP concentration by 22.7%. Meanwhile, deletion of ACWT_7629 (encoding elongation factor G) caused improvements of acarbose yield by 36% from 1.87 to 2.54 g/L, transcription of acb cluster, and ppGpp concentration to 2.2 folds. Combined deletions of ACWT_4325 and ACWT_7629 resulted in further improvement of acarbose to 2.83 g/L (i.e. 76% of SE50/110), suggesting that the metabolic perturbation and improved transcription of acb cluster caused by these two mutations contribute substantially to the acarbose overproduction. Enforced application of similar strategies was performed to manipulate SE50/110, resulting in a further increase of acarbose titer from 3.73 to 4.21 g/L. Therefore, the comparative genomics approach combined with functional verification not only revealed the acarbose overproduction mechanisms, but also guided further engineering of its high-yield producers.

1. Introduction

Acarbose (acarviosyl-1,4-maltose), an α-glucosidase inhibitor, is commercially used in the treatment of type-2 diabetes mellitus since 1990, which enables patients to better control the blood sugar level [1–3]. As the high incidence of type-2 diabetes mellitus is becoming a major health problem [4], the constant demand of acarbose and other antidiabetic drugs increases rapidly. The acarbose is produced in large-scale fermentation by strains derived from Actinoplanes sp. SE50 [1]. Therefore, improving the productivity of acarbose producers becomes very important nowadays.

Traditionally, strains with high productivity of acarbose used for industrial large-scale fermentation were obtained through multiple rounds of random mutagenesis and screening [5]. Actinoplanes sp. SE50/110 is a typical optimized industrial strain derived from SE50 [6]. Recently, its genome was sequenced and analyzed [7], leading to a rapid development of multi-omic analysis, such as transcriptome for comparative gene expression between cells grown in different media, proteome for localization of proteins encoded by the acarbose biosynthetic gene cluster (acb cluster), and genome-scale metabolic model for discovery of bottlenecks in acarbose production [8–12]. With the development of efficient genetic manipulation systems for acarbose producing strains [13–15], genetic engineering strategies for further improvement of acarbose productivity are in urgent need.

Comparative genome analysis is frequently used to decipher how the classical mutagenesis-and-screening strategy leads to an improved antibiotic production. The comparative genome study of the wild-type erythromycin producing strain Saccharopolyspora erythraea NRRL 2338 and a derived overproducer Px revealed that a considerable number of mutations, affecting genes encoding enzymes involved in central carbon and nitrogen metabolisms, biosynthesis of secondary metabolites, and basic transcription and translation machineries, contribute to erythromycin overproduction [16]. Meanwhile, 250 variations, affecting 227 coding sequences (CDSs), were identified in rifamycin B overproducer Amycolatopsis mediterranei HP-130 through comparative genome analysis with the reference strains S699 and U32, and the
mutations of mutB2 (coding for the large subunit of methylmalonil-CoA mutase) and argS2 (coding for arginyl-tRNA synthetase) were proved to be the causes for rifamycin overproduction [17]. Additionally, the deletions of large fragments including competitive gene clusters and several regulatory genes in salinomycin producer S. albus BK 3–25 resulted its overproduction [18]. These approaches take advantage of low-cost genome sequencing and lead to the identification of targets for further titer improvement by genetic engineering [19].

Herein, in order to decipher how the mutagenesis-and-screening method leads to an improved acarbose production, the genome of the wild-type strain SE50 was sequenced and compared with that of the high-yield SE50/110, and the genetic variations were identified. Subsequent functional verification revealed the critical variations responsible for the acarbose overproduction, and the underlying mechanisms were accordingly illustrated. The identified overproduction strategy was applied again in the high-yield strain and resulted in a further improved acarbose yield.

2. Materials and methods

2.1. Bacterial strains, plasmids and media

The strains, plasmids and primers used in this study are listed in Table S1, Table S2 and Table S3, respectively.

Actinoplanes sp. and their derivatives were grown on STY agar medium (sucrose 3%, tryptone 0.5%, yeast extract 0.5%, casin hydrolysate 0.1%, K₂HPO₄·3H₂O 0.1%, KCl 0.05%, FeSO₄ 0.005%, agar 2%, pH 7.2) at 30 °C for conjugation. For the isolation of total DNA, strains were cultivated in 30 mL SM broth (glucose 1.5%, maltose 1%, K₂HPO₄·3H₂O 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). The E. coli ET12567(pUZ8002) was used for conjugation. The E. coli cells were cultured in Luria-Bertani (LB) broth with corresponding antibiotics at 37 °C.

2.2. Genome sequencing and assembly of Actinoplanes sp. SE50

The genome of SE50 was sequenced by a combination of Illumina HiSeq 2500 sequencer and PacBio RS II System at Shanghai Biotechnology Corporation, generating one scaffold with 9,239,482 base pairs and providing a 100% coverage. No gap was formed and the obscure base Ns were replaced by PCR amplification with specific designed PCR primers. Putative protein-coding sequences (CDSs) were predicted by Prodigal v2.6.1 software. CDS annotation was based on the BLASTp program with NR, COG, and KEGG databases. The single nucleotide variations (SNVs) and Indels were identified by the BLASTp program with NR, COG, and KEGG databases. The whole genome of SE50 has been deposited at GenBank under the accession number CP023298.

2.3. Transcriptome sequencing and comparative analysis of SE50 and SE50/110

The RNA for transcriptome sequencing was sampled on the second day of fermentation. The cells were harvested from 1-mL fermentation culture by centrifugation at 4 °C, put in the liquid nitrogen for 0.5–1 h, and then stored at −80 °C. The RNA extraction, transcriptome sequencing and data analysis were performed by Shanghai Sinomics Corporation. The expression level of each gene was calculated as Fragments Per Kilobase of exon per Megabase of library size (FPKM).

2.4. Fermentation and HPLC analysis of acarbose

The mycelia from SM medium were transferred to 40-mL seed medium (1.5% maltose, 1% glucose, 4% soya flour, 1% glycerol, 1% soluble starch, 0.2% CaCO₃, pH 7.2) in baffled flask and cultivated for 20–22 h on rotary shaker at 220 rpm and 30 °C. Then, 7.5 mL seed culture was inoculated to 50 mL fermentation medium (5% maltose, 3% glucose, 1% soya flour, 0.3% glutamate, 0.1% K₂HPO₄·3H₂O, 0.05% FeCl₃ and 0.25% CaCO₃, pH 7.2) in 250-mL baffled flask and cultivated for 7–8 days. Additionally, 1 g of glucose and 1 g of maltose were added to each flask at 72 h. The supernatant of fermentation broth was obtained by centrifugation at 12,000 rpm for 10 min, diluted for 3 folds, and analyzed by HPLC (Agilent series 1260, Agilent Technologies, USA). Acarbose was 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 65:35 (v/v) with a flow rate of 1 mL/min, and detected at 210 nm.

2.5. RNA extraction and quantitative real-time PCR (qRT-PCR) analysis

Mycelia of Actinoplanes spp. were collected at 48 h from fermentation broth, and the total RNA was extracted using Redzol according to the manufacturer's instruction (SBS Genetech, China) [20]. The quality of the RNA was checked by NanoDrop 2000 spectrophotometer. For qRT-PCR experiments, total RNA was reversely transcribed into cDNA using RevertAid™ H Minus First Strand cDNA Synthesis Kit (Thermo Fisher, USA). The qRT-PCR experiments were carried out with a 7500 Fast Real-time PCR system (Applied Biosystems, USA) using Maxima™ SYBR Green/ROX qPCR Master Mix (Thermo Fisher, USA) according to the manufacturer's procedure. The transcription of target genes was normalized to the housekeeping gene hrdB and quantified by the 2⁻ΔΔCT method [21].

2.6. DNA cloning and gene inactivation

Gene disruption was performed using double crossover homologous recombination strategy. In order to delete the gene ACWT_4325, two homologous arms, a 1.66-kb XbaI-EcoRI fragment of the left flanking region and a 1.49-kb EcoRI-HindIII fragment of the right flanking region were respectively amplified by primers ACWT_4325-L-F/ACWT_4325-L-R and ACWT_4325-R-F/ACWT_4325-R-R from the genome of SE50 and both ligated into XbaI/HindIII-digested pKTU1278-derived replicating vector pLQ903 (Table S2) to generate pLQ908. Then, pLQ903 was transferred into strain SE50 by conjugation from E. coli ET12567(pUZ8002). Exconjugants with single-crossover homologous recombination were selected for apramycin and trimethoprim resistance on STY plates. In order to promote double crossover homologous recombination in the exconjugants, the mycelia from STY plates 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, and the filtrate was diluted for 10⁻¹⁰⁻⁵ times. Subsequently, the apramycin-sensitive colonies were selected after cultivation for 4–5 days on STY plates without antibiotics. For further verification, the genotypes of the wild-type, pLQ903, and the mutant candidates were verified by PCR amplification with primers ACWT_4325-F/ACWT_4325-R. The mutants (A4325) gave a 1.07-kb amplified product, whereas the wild-type gave a 1.88-kb amplified product.

In order to delete the gene ACWT_7629, a 1.73-kb XbaI-EcoRI fragment of the left flanking region and a 1.33-kb EcoRI-HindIII fragment of the right flanking region were respectively amplified by primers ACWT_7629-L-F/ACWT_7629-L-R and ACWT_7629-R-F/ACWT_7629-R-R from the genome of SE50 and both ligated into XbaI/HindIII-digested pLQ903 to generate pLQ906. Then, pLQ906 was transferred into strain SE50 by conjugation from E. coli ET12567(pUZ8002). Exconjugants were picked, cultured and screened as described above. The genotypes of the wild-type, pLQ906 and the mutant candidates were verified by PCR amplification with primers ACWT_7629-F/ACWT_7629-R. The mutants (A7629) gave a 0.62-kb amplified product, whereas the wild-type gave a 0.81-kb amplified product.
product. Similar methods were used to delete other genes.

2.7. Concentration determination of intracellular ppGpp, acetyl-CoA and phosphoenolpyruvate (PEP)

Mycelia of Actinoplanes spp. were collected at 48 h from fermentation media, each sample was centrifuged for 3 min at 12,000 rpm to precipitate the cells. Cell pellets were resuspended in 1 mL 0.9% NaCl and washed by centrifugation for three times. The final pellet was resuspended in 200 mL 0.9% NaCl, to which was added 200 mL 20% formic acid to lyse the cells. The extraction was performed on ice with intermittent vortexing for 15 min and then placed on ice for 30 min. The supernatant of extraction was obtained by centrifugation at 12,000 rpm for 3 min, and 10 μL of supernatant was applied drop-wise onto a polyethyleneimine (PEI)-cellulose TLC plate (Sigma-Aldrich). As a marker, 0.1 nmol of GTP was applied on the same plate. Chromatography was performed in 1.5 M KH₂PO₄ (pH 3.2) until the buffer front reached the top of the membrane. The plate was dried, and the chromatograms were visualized under UV-light [17,22].

The concentrations of intracellular acetyl-CoA and phosphoenolpyruvate were determined by LC-MS/MS using a modified method [23]. Each strain was inoculated into 30 mL of fermentation medium in triplicate. 1 mL of culture was collected and used to quantify the concentrations of total intracellular proteins [24]. The remaining culture was centrifuged, and the mycelia were instantly quenched and extracted with acetonitrile/methanol/0.1% glacial acetic acid (45:45:10, v/v) to a final volume of 1 mL at 20 °C. The extraction was performed on ice with intermittent vortexing for 15 min, followed by a 3-min centrifugation at 12,000 rpm and 4 °C. The supernatant (10 μL) was injected for HPLC-MS/MS analysis.

Samples (10 μL) were analyzed by LC-QQQ MS (Agilent 1100 series LC/MSD Trap System) using an Agilent Eclipse TC-C18 column (5 μm, 4.6 × 250 mm). The mobile phase was composed of water with 20 mM ammonium acetate (phosphate buffer) and methanol with 20 mM ammonium acetate (solvent B). Elution was initiated with constant 25% solvent B for 5 min, and then with a linear gradient from 25% to 100% of solvent B in 10 min, followed by 100% solvent B for 5 min and re-equilibration to initial conditions for 5 min, at a flow rate of 0.5 mL/min [25]. Concentrations of Acetyl-CoA and PEP were measured in a multiple reaction monitoring (MRM) mode with the m/z of precursor ion > the m/z of product ion (acetyl-CoA 810 > 303, PEP 169 > 81). The ion-trap mass spectrometer was operated with an electro-spray ionization source in positive ion mode. The drying gas and ion source temperature were 375 °C and 300 °C, respectively. The nebulizer pressure was 30 lb/in² [23]. Acetyl-CoA and phosphoenolpyruvate purchased from Sigma were used as standards.

3. Results

3.1. Phenotypic differences between wild-type SE50 and high-yield SE50/110

Actinoplanes sp. SE50/110 is an acarbose-overproducing strain obtained by traditional mutagenesis-and-screening strategy from the wild-type strain SE50. To compare the phenotypic differences of these two producers, the acarbose production, biomass and transcription of genes in acb cluster were measured. The acarbose titers of SE50 and SE50/110 were 1.87 g/L and 3.73 g/L, respectively (Fig. 1A). The biomass of SE50 and SE50/110 were 14.60 g/L and 17.80 g/L, respectively (Fig. 1B). Also, the transcription of the genes in the acb cluster of SE50/110 was found to be 3–7 fold higher than that of SE50 (Fig. 1C). These results suggested that the substantial increase of acarbose titer of SE50/110 might be resulted from the higher transcription of genes in acb cluster.

3.2. Comparative genomics identified DNA variations in the genome of SE50/110

Comparative genome analysis of SE50 and SE50/110 was performed to reveal the genetic basis underlying the acarbose overproduction. While the genome of SE50/110 has been sequenced and submitted to the GenBank (Accession no. CP003170) [7], the whole-genome of SE50 was then fully sequenced with Illumina and PacBio sequencing technologies. After filtering the subreads, 204,594 reads containing 716,835,386 bases were obtained. When combined with the pair-end library sequencing, a single circular chromosome was successfully assembled by SPAdes-3.5.0, comprising 9,239, 482 bp with the average GC content of 71.73%. A total of 8252 protein-coding sequences (CDSs, locus tagged as ACWT) were identified by Prodigal v2.6.1 and further annotated by BLASTP-searching with the NR, COG, and KEGG databases. Additionally, 21 potential secondary metabolite clusters were found to be distributed in the genome of SE50 via antiSMASH analysis [26], and the acb cluster is located in the middle region of the chromosome (Fig. S1). Then, the completely annotated genome sequence was submitted to the GenBank (Accession no. CP023298).

Genomic comparison between SE50 and SE50/110 exhibited extremely high similarity in gene content and gene order, and no large fragment deletion occurred in the genome of SE50/110. A total of 115 variations and a 0.55 Mb rearrangement in SE50/110 were initially found and further confirmed by PCR amplification and sequencing. The verification results showed that only 15 mutated sites including 11 SNVs and 4 InDels occurred in the genome of SE50/110 (Fig. 2, Table 1). Other 100 nonexistent variations were attributed to the limited accuracy of the 454 pyrosequencing technology, which were also recently corrected by another research group in the database (GenBank Accession no. NC_017803). Among the 15 identified variations, 8 SNVs and 2 InDels located in coding regions, and 2 InDels and 3 SNVs occurred in intergenic regions, affecting 8 CDSs. The nature of every variation and their proposed functions were listed in Table 1.

Although only a few CDSs are affected in the genome of SE50/110, the comparative transcriptome analysis with SE50 showed obvious differences in gene expression. 1228 and 1337 (~31%) genes were significantly up-regulated and down-regulated (q < 0.05, fold-change > 2) in SE50/110, respectively (Fig. 3). Meanwhile, it was remarkable that the transcription of essential genes for acarbose biosynthesis was obviously improved, which was consistent with the results of qRT-PCR analysis (Fig. 1C). Additionally, the transcription of most genes involved in glycolytic pathway and TCA cycle was up-regulated, especially ACPL_1553 (gapA), ACPL_7704 (glpX), ACPL_549 (citA) and ACPL_7655 (sdhB), which might be the major cause for the higher biomass. These results suggested that these variations in the genome of SE50/110 result in prominent perturbation of gene expression and contribute to the cell growth and acarbose production.

Therefore, the roles of 8 affected genes, encoding LacI family transcription regulator (ACWT_6270), elongation factor G1 (ACWT_7629), alcohol dehydrogenase (ACWT_4325), bilirubin oxidase (ACWT_7246), ABC transporter (ACWT_2644), extracellular matrix-binding protein (ACWT_4230), and hypothetical proteins (ACWT_222 and ACWT_5166), were verified by individual deletion in SE50, and the corresponding mutants were named as Δ222, Δ2644, Δ4230, Δ4235, Δ5166, Δ6270, Δ7246 and Δ7629. As shown in Fig. S2, deletion of ACWT_4325 and ACWT_7629 resulted in dramatic improvements of acarbose titer. However, no obvious effect on the acarbose titer was observed from other 6 gene deletion mutants. Therefore, we focused on ACWT_4325 and ACWT_7629 to elucidate any possible mechanisms for acarbose titer improvement.
3.3. The metabolic perturbation caused by ACWT_4325 deletion resulted in increases of acarbose titer and intracellular acetyl-CoA and PEP concentrations

ACWT_4325 was predicted as alcohol dehydrogenase that catalyzes the interconversion between ethanol and acetaldehyde. Deletion of ACWT_4325 resulted in a 25% increase of acarbose titer from 1.87 g/L to 2.34 g/L and 15.5% improvement of the biomass from 14.6 g/L to 16.9 g/L (Fig. 4A–D). However, the transcription of acb cluster didn’t show obvious increase in mutant Δ4325 compared with that of SE50 (Fig. S3A). The acarbose titer and biomass were restored to the wild-type levels by trans-complementation of ACWT_4325 into the Δ4325 mutant (Fig. 4C and D). Meanwhile, overexpression of ACWT_4325 in SE50 resulted in simultaneous decrease of acarbose titer and biomass (Fig. S4). These results indicated that inactivation of ACWT_4325 is beneficial to both acarbose production and cell growth.

The alcohol accumulation branches from acetyl-CoA, a key intermediate bridging the glycolytic pathway and TCA cycle. Therefore, deletion of ACWT_4325 might increase the concentration of acetyl-CoA and direct the metabolic flux to TCA cycle, which in turn leads to a better growth. To address this hypothesis, the intracellular acetyl-CoA concentration was quantified and found to be improved by 52.7% in Δ4325 when compared with that of SE50, which was comparable to that of SE50/110 (Fig. 4E). To determine whether the increase of acetyl-CoA pool also affects the accumulation of phosphoenolpyruvate (PEP), which is involved in the biosynthesis of the acarbose precursor heptulose-7-phosphate, the concentrations of intracellular PEP were quantified and found to be increased by 22.7% in Δ4325 when compared with that of SE50 (Fig. 4F). These results suggested that the metabolic perturbation caused by the deletion of ACWT_4325 promotes both acarbose titer and biomass.

3.4. The stringent response caused by ACWT_7629 deletion resulted in acarbose titer and acb gene transcription improvements

Although the production of acarbose was greatly increased by the deletion of ACWT_4325, the mutant didn’t show obvious improvement in the transcription of genes in acb cluster, implying that the distinct difference on the transcription of acb cluster between SE50 and SE50/110 is caused by other mutations. Deletion of gene ACWT_7629,

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**Fig. 1.** The phenotypic differences between the wild-type SE50 and the high-yield SE50/110. (A) Acarbose titer of SE50 and SE50/110. (B) Dry cell weight of SE50 and SE50/110. (C) Transcription of genes in acb cluster of SE50 and SE50/110 at 48 h during the fermentation process. The Y-axis scale represents the expression of genes. The average transcription of genes in SE50 was set to 1, the transcription of genes in SE50/110 was accordingly calculated. Graphs depict means ± SD. Values represent average results from three independent experiments.

**Fig. 2.** Chromosome map of genetic variations distinguishing Actinoplanes sp. SE50/110 from SE50. From the outside in, Circle 1 and 2: (forward and reverse strands), predicted protein coding sequences colored according to COG function categories; Circle 3: distribution of secondary metabolic gene clusters; Circle 4: positions of variations between SE50/110 and SE50; Circle 5: GC content. Position of acarbose biosynthetic gene cluster was marked with arrow. S: synonymous variations. B: variations in intergenic regions.
ACWT_7629 cluster and cell growth were all restored to the wild-type levels by SE50 (Fig. S5). Subsequently, the acarbose titer, transcription of (Fig. 5D). However, the biomass of with that of SE50, which were comparable to that of SE50/110 overproduction, showing 36% improvement of acarbose titer from predicated as elongation factor G protein, was crucial for acarbose regulated in SE50/110), and others are marked in gray (not signi –Δ5-fold increase in Δ5-compared

### 3.5. Combined deletions of ACWT_4325 and ACWT_7629 further improved acarbose production

Since the acarbose titer was substantially improved in both Δ4325 and Δ7629 mutants, deletion of gene ACWT_4325 was further performed in the mutant Δ7629. The mutant was named as Δ4325/Δ7629, which showed further enhancement of acarbose titer, reaching 2.83 g/L (i.e. 76% of SE50/110) (Fig. 6A). Also, the biomass of Δ4325/Δ7629 was relatively higher than that of SE50, although it is less than that of Δ4325 (Fig. 6B). Additionally, the transcription of acb gene was substantially increased in the Δ4325/Δ7629 mutant when compared with that of SE50 (Fig. 6C), which was comparable to that of Δ7629 and SE50/110. Therefore, the metabolic perturbation and improved acb gene transcription caused by ACWT_4325 and ACWT_7629 mutations remarkably contributed to acarbose overproduction in SE50/110.

### 3.6. Enforced application of similar strategies for further acarbose titer increase in SE50/110

To further improve acarbose titer in SE50/110, similar overproduction strategies were utilized by inactivation of genes homologous to ACWT_4325 and ACWT_7629. According to the protein function annotation and gene transcription, ACPL_2718, ACPL_3172, ACPL_5664 and ACPL_6548 were chosen to be individually inactivated in SE50/110 to generate the corresponding mutants named as Δ2718, Δ3172, Δ5664 and Δ6548. Inactivation of ACPL_3172, showing certain similarity with ACWT_4325, resulted in a 12.9% improvement of acarbose titer from 3.73 g/L to 4.21 g/L (Fig. 7A). Besides, the biomass of Δ3172 mutant was higher than that of SE50/110 (Fig. 7B).
4. Discussion

In order to understand the mechanism underlying the higher acarbose productivity in SE50/110, the whole genome of SE50 was sequenced (Fig. S1) and compared with the genome of SE50/110, which was published in 2012 [7]. Even though 115 mutated sites were initially identified in the genome of SE50/110, only 15 mutated sites were confirmed to be existed in SE50/110 via intensive PCR amplification and sequencing verification (Fig. 2, Table 1). During the manuscript preparation, these errors were recently corrected, and the genome sequence of SE50/110 was refined by whole-genome resequencing [28], which confirmed the high accuracy of our sequencing data.

In this work, deletion of carbohydrate metabolism related gene ACWT_4325 led to the improvement of acarbose titer, biomass, and intracellular concentrations of acetyl-CoA and PEP (Fig. 4C–F). This finding suggested that metabolic perturbation caused by ACWT_4325 deletion played a vital role in acarbose production. To further correlate the metabolite concentrations and the acarbose production, concentration of 2-epi-5-epi-valiolone in the fermentation broth, as the first intermediate in acarbose biosynthesis, was determined. Although no obvious change was found at 48 h of fermentation, the concentration of 2-epi-5-epi-valiolone of Δ4325 was improved for 3 folds after 7-day fermentation, suggesting that deletion of ACWT_4325 increased the flux from acetyl-CoA via PEP to the ultimate acarbose biosynthesis (Fig. S3B).

The guanosine tetraphosphate (ppGpp) [30,31], as the signaling molecule of stringent response, has been proved to play a central role in triggering the onset of secondary metabolism in actinomycetes [32]. For example, the production of glycopeptide antibiotic A40926 in Actinomadura sp. ATCC 39727 was dependent on the control of gene transcription by the stringent response [33]. In addition, specific mutations in the rpoB gene (encoding the RNA polymerase β-subunit) of S. lividans exerted function at the transcription level by activating directly or indirectly key regulatory genes, including actII-ORF4 and red, to achieve the productions of actinorhodin and/or prodigiosins, which was proposed to mimic the ppGpp-bound form in activating the secondary metabolism [34]. Meanwhile, the mutation of translation elongation factor Tu (EF-Tu) has been proved to affect the cell growth and increase the ppGpp concentration in Salmonella typhimurium [22]. In our work, deletion of EF-G in Actinoplanes spp. was confirmed to increase intracellular ppGpp and then lead to the enhancement of acb gene transcription.

In summary, our work disclosed the genetic mechanisms underlying the acarbose overproduction in SE50/110 through comparative genomics and functional verification. The metabolic perturbation and stringent response caused by mutations resulted in substantial increases of cell growth, supply of precursor, and transcription of genes in acb cluster. Additionally, enforced application of one of these two strategies resulted in a further increased acarbose titer in SE50/110. The comparative genome approach and mechanisms underlying acarbose overproduction reported in this work would shed lights on the overproduction and genetic engineering of other microbial secondary metabolites.
The genome sequence of Actinoplanes sp. SE50 has been deposited at GenBank under the accession number CP023298.

The authors declare that they have no competing interests.

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Fig. 5. The effect of deletion of ACWT_7629 on acarbose titer, acb cluster transcription and ppGpp concentration. (A) Schematic representation of homologous recombination of ACWT_7629 deletion. (B) Confirmation of Δ7629 by PCR amplification. The mutant Δ7629 gave a 0.62-kb product, whereas the wild-type SE50 gave a 0.81-kb product. (C) Acarbose titer of SE50, Δ7629, Δ7629 ΔSET152, Δ7629 ΔSET152 and SE50/110. (D) Transcription of genes in acb cluster of SE50, Δ7629 and SE50/110. (E) The relative intracellular concentrations of ppGpp in SE50, Δ7629 and SE50/110. Graphs depict means ± SD. Values represent average results from three independent experiments.

Fig. 6. Effects of combined deletions of ACWT_4325 and ACWT_7629 on acarbose titer, biomass and transcription of genes in acb cluster. (A) Acarbose titer of SE50, Δ4325, Δ7629, Δ4325 Δ7629 and SE50/110. (B) Biomass of SE50, SE50/110 and these mutants. (C) Transcription of genes in acb cluster of SE50, SE50/110 and mutants. Graphs depict means ± SD. Values represent average results from three independent experiments.
Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.sysbio.2019.01.001.

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