Transcriptome analysis of *Actinoplanes utahensis* reveals molecular signature of saccharide impact on acarbose biosynthesis

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Abstract

Different carbon sources lead to differential acarbose production in *Actinoplanes*. To uncover the underlying differentiation in the context of genes and pathways, we performed transcriptome sequencing of *Actinoplanes utahensis* ZJB-03852 grown on different saccharides, such as glucose, maltose, or the saccharide complex consisting of glucose plus maltose. The differentially expressed genes were classified into GO (gene ontology) terms and KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways for functional annotations. Key enriched modules were uncovered. Our data revealed that both maltose and its complex with glucose gave improved acarbose titer. Sugar transportation, cytochrome oxidase, protein synthesis and amino acid metabolism modules were enriched under the saccharide complex condition, while ferritin metabolism gene expressions were enriched in the glucose medium. Our results provided the foundation for uncovering the mechanism of carbon source on acarbose production in *A. utahensis*.

Keywords Acarbose · *Actinoplanes utahensis* · Transcriptome · Saccharide

Abbreviations

ACN	Acetonitrile
CDW	Cell dry weight
DEG	Differentially expressed genes
FPKM	Fragments per kilobase per million mapped reads
Glc	<i>A. utahensis</i> ZJB-03852 cultured with 80 g/L glucose as the carbon source
GM	<i>A. utahensis</i> ZJB-03852 cultured with 40 g/L glucose and 40 g/L maltose as the carbon source

GO	Gene ontology
KEGG	Kyoto Encyclopedia of Genes and Genomes
Mal	<i>A. utahensis</i> ZJB-03852 cultured with 80 g/L maltose as the carbon source
NRPS	Non-ribosomal peptide synthetase
PKS	Polyketide synthase
PPI	Protein–protein interaction
T2DM	Type 2 diabetes mellitus

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Introduction

Diabetes mellitus is a worldwide metabolic disease characterized by chronic hyperglycemia (American Diabetes Association 2019), which severely impairs the patients' life quality. In 2019, 463 million adults (20–79 years) were diagnosed with diabetes (Saeedi et al. 2019). There are four major types of diabetes: insulin-dependent diabetes mellitus (Type I), non-insulin-dependent diabetes mellitus (Type II), gestational diabetes mellitus (GDM) and others (American Diabetes Association 2019). Currently, over 90% of diabetes mellitus are type 2 diabetes mellitus (T2DM).

α -Glucosidase inhibitors are effective drugs to treat T2DM. Through inhibition of glucosidase activity in the small intestine and delay of the hydrolysis of polysaccharides, α -glucosidase inhibitors significantly reduce

postprandial blood sugar levels (Oki et al. 1999). As an α -glucosidase inhibitor, acarbose was widely used in the treatment of T2DM in clinical practice to reduce both blood sugar level and blood triglyceride (Mughal et al. 2000; Wehmeier and Piepersberg 2004).

In an industrial context, *A. utahensis* is the main acarbose producer in China. The fermentation conditions were continuously optimized to improve the yield of acarbose (Wang et al. 2011, 2012). As one of the most important components in the culture media, carbon source usually significantly influences the yield of microbial metabolites. Previous researches demonstrated that maltose containing media induced acarbose production (Wehmeier 2003), while glucose had a negative effect on acarbose production (Brunkhorst and Schneider 2005; Wang et al. 2011). However, the underlying changes in gene expression and pathway enrichment have not been fully clarified yet.

Transcriptome sequencing examines the whole transcriptome in cells to uncover gene expression profiles. It serves as a useful tool for uncovering the potential regulatory mechanism in the biosynthesis of microbial secondary metabolite. The transcriptome of *Actinoplanes* sp. SE50/110 was sequenced to improve the genome annotation, and fourteen novel biosynthetic gene clusters were discovered (Wolf et al. 2017a, b). The sporangium formation regulator TcrA in *A. missouriensis* (Mouri et al. 2018), and the transcriptional repressor AcrC in *Actinoplanes* sp. SE50/110 (Wolf et al. 2017a, b) were revealed by RNA-seq, which helped to discover the regulatory mechanism in *Actinoplanes* sp.

In this study, the effect of saccharides glucose, maltose and their combination on acarbose production in *A. utahensis* ZJB-03852 was examined. Thorough transcriptome profiling of *A. utahensis* ZJB-03852 cultured on the medium containing various saccharides was examined to illustrate the corresponding gene cascade alternation. Differentially expressed genes (DEG) and enriched pathways were analyzed. Our results revealed that acarbose yield was the highest on the saccharide complex. Sugar transport, cytochrome oxidase, protein synthesis and amino acid metabolism modules were enriched in the mixed medium culture, and ferritin metabolism gene expressions were increased in the saccharide complex medium. Our results provided the foundation for decoding the underlying mechanism of saccharides on acarbose production in *A. utahensis*.

Material and methods

A. utahensis ZJB-03852 cultivation and its biomass determination

The acarbose-producing strain *A. utahensis* ZJB-03852, a strain developed by our laboratory, was spread over agar

plates containing (g/L) sucrose 30.0, peptone 2.0, tyrosine 1.0, $K_2HPO_4 \cdot 3H_2O$ 1.0, KCl 0.5, $MgSO_4 \cdot 7H_2O$ 0.5, $MgSO_4 \cdot 7H_2O$ 0.5 and agar 20.0. The seed cultures were prepared by transferring a colony of 1 cm $\times$ 1 cm from the agar plates to seed medium per 50 mL, grown for 60 h at 28 °C and 200 rpm. The seed medium consisted of (g/L) corn starch 16.0, soybean flour 40.0, glycerol 20.0, $CaCO_3$ 2.0 and K_2HPO_4 0.5. A 5 mL (10%, v/v) of seed cultures was used for inoculation in 50 mL fermentation medium per 500-mL Erlenmeyer flask and incubated at 28 °C, 250 rpm for 7 d. Fermentation medium consisted of (g/L) soybean flour 16.0, $CaCO_3$ 2.5, $CaCl_2$ 2.0, $FeCl_3$ 0.5, $K_2HPO_4 \cdot 3H_2O$ 2.0, L-glutamic acid monosodium 3.0, glycerol 5.0, and various saccharides 80.0 with pH adjusted to 7.5. 80 g/L glucose (Glc), 80 g/L maltose (Mal), or a mixture of 40 g/L of both glucose and maltose (GM) were supplemented to the fermentation medium as carbon source, respectively.

The hyphae of different groups were harvested by centrifugation at 12,000 rpm for 15 min and washed twice with sterile water. Hyphae were then dried at 90 °C and weighted.

Quantification of acarbose from *A. utahensis* ZJB-03852 culture supernatants

Concentration of acarbose in the supernatant was quantified by HPLC (Waters, 2489 UV detector and 2414 differential refractive index detector) at 210 nm and 30 °C. An isocratic flow of 1 mL/min 75% acetonitrile (ACN) and 25% phosphate buffer (0.600 g/L KH_2PO_4 , 1.760 g/L $Na_2HPO_4 \cdot 12H_2O$) was applied on the HPLC column Luna 5 μ m NH_2 100 Å (4.6 mm $\times$ 250 mm, 5 μ m, Phenomenex).

Total RNA isolation from *A. utahensis* ZJB-03852 culture

Two biologically replicated samples grown on Glc, Mal, and GM groups were utilized for total RNA extraction. Day 4 fermentation broth was centrifuged at 4 °C at 12,000 rpm for 15 min and the supernatant was discarded. Total RNA was isolated using RNeasy Plus Mini Kit (74,106, QIAGEN) according to the protocol given under the manufacturer's recommendations. The RNA purity was determined by the OD260/OD280 ratio.

cDNA library construction and RNA sequencing

Isolated RNA samples were quantified and checked for RNA integrity number (RIN). The Ribo-zero kit was employed to remove the rRNA in the qualified RNA samples. RNA samples were fragmented, and cDNA was synthesized with random hexamers. Double-strand cDNA was synthesized using single-strand cDNA as the template. VAHTSTM DNA Clean Beads were utilized to purify double-strand cDNA

according to the manufacturer's instructions. The library preparations were sequenced on an Illumina HiSeq 2000 platform (Bioheng), and paired-end reads were generated.

RNA-Seq analysis

Raw reads of the corresponding RNA samples were generated by sequencing. The preliminary quality check was performed with FastQC (Brown et al. 2017). Low-quality reads were filtered to generate clean reads. ASM23714v1 (RefSeq assembly accession No.: GCF_000237145.1) was utilized as the reference genome file. Bowtie2 was utilized for read mapping (Langmead and Salzberg 2012).

Pathway enrichment analysis

Gene ontology (GO) enrichment analysis was performed for biological process, cellular component, and molecular function (Ashburner et al. 2000, The Gene Ontology 2019). PANTHER classification system (<https://pantherdb.org/>) was employed by inputting the names of differentially expressed genes and exporting the results (Mi et al. 2019). $p < 0.05$ was considered statistically significant (Weng et al. 2020).

Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis was performed by the Database for Annotation, Visualization and Integrated Discovery (DAVID) version 6.8 (<https://david.ncifcrf.gov/>) (Huang et al. 2009).

Quantitative real-time PCR analysis

Total RNA was extracted from the three groups using RNeasy Plus Mini Kit (74,106, QIAGEN), and the first-strand cDNA was synthesized using the PrimeScript RT Reagent Kit (6210A, TaKaRa) with PrimeScript RT enzyme at 65 °C for 5 min, 42 °C for 60 min, and 70 °C for 15 min. Quantitative real-time PCR (qPCR) analysis was performed with three technical replicates. The amplification conditions were as follows: 50 °C for 5 min, 95 °C for 10 min, and 40 cycles of 95 °C for 15 s and 60 °C for 60 s. Relative expression in each sample was calculated using the $2^{-\Delta\Delta C_t}$ method. Melting curve analysis was used to verify the generation of a single product at the end of the assay.

Statistical analysis

All experiments were performed with triplicates. All values were denoted as mean $\pm$ standard deviation. The significantly differentially expressed genes were filtered as $\log_2(\text{Fold change}) \geq 4$ and $p \leq 0.01$. Positive values indicated increased expression; and negative values suggested decreased expression.

Results

Acarbose yield and acarbose gene expression in *A. utahensis* ZJB-03852 grown on various saccharides

All three groups can produce acarbose during *A. utahensis* ZJB-03852 fermentation (Fig. 1). The acarbose yield was highest in the GM group, and lowest in the Glc group (Fig. 1a). When glucose was utilized as the sole carbon source, the titer of acarbose was < 1 g/L during the whole fermentation. The acarbose titer for Mal reached its summit (1.34 g/L) on the 4th day of fermentation (Fig. 1a). Meanwhile, acarbose yield in the GM medium reached the highest (2.67 g/L) on the 4th day (Fig. 1a).

Additionally, cell dry weights of *A. utahensis* ZJB-03852 on the 4th Day were examined, and acarbose productivity (the acarbose formed per gram CDW, mg/g) was thus calculated for each group (Fig. 1b). Acarbose productivity characterized the acarbose yield per cell. It was high in the complex saccharide medium (65 mg/g), and low in the glucose only medium (16 mg/g).

Day 4 transcriptional levels of five acarbose synthesis genes, *acbD*, *acbK*, *acbL*, *acbN*, and *acbV*, were examined by real-time quantitative PCR to confirm the gene expression profile in the three groups. These genes demonstrated the highest expression levels in the complex saccharide group and lowest expression levels in glucose only group, which was consistent with the acarbose formation pattern (Fig. 1c).

Differential gene expression analysis

Heat map of differential genes

The expression pattern of differential genes under different saccharides was rendered by the heat map (Fig. 2a). Red indicated high expression of the gene, blue suggested low expression and white represented genes with no significant difference among different groups. The gene expression pattern in Glc group demonstrated substantial difference from those in Mal and GM, while the expression patterns in Mal and GM were similar to each other. Therefore, the difference between GM and Glc were mainly examined in our analysis (Fig. 2b).

Differentially expressed genes

Totally, 242 genes demonstrated significant expression difference in GM compared to Glc, including 71 upregulated genes and 171 downregulated genes (Supplementary Table 1).

Fig. 1 Day 4 acarbose yield of *Actinoplanes utahensis* ZJB-03852 under various carbon source cultures. Three carbon sources were utilized for culture: 80 g/L glucose (Glc), 80 g/L maltose (Mal) or combination of 40 g/L glucose and 40 g/L maltose (GM). The impacts of three carbon sources on **a** acarbose production, **b** acarbose per gram cell dry weight, and **c** acarbose synthesis gene expression were scrutinized. Mixture of glucose and maltose lead to substantial higher acarbose yield compared to the other groups. Quantitative real-time PCR for *acbD*, *acbK*, *acbL*, *acbN*, and *acbV* were performed to validate the *acb* gene expressions in different media. Values represent the means of triplicate measurements. Bars represent the standard deviation ($n=3$)

Fig. 2 Heat maps of differential genes in *Actinoplanes utahensis* ZJB-03852 grown under various carbon sources. **a** Heat map of differential genes among Glc, Mal and GM. **b** Heat map of differential genes between Glc and GM. Each column represents a sample and each row represents a gene. The genes were clustered. Red indicates high gene expression, and blue indicates low expression. Each group had two biological replicates

Differentially expressed gene annotation

Since the genomes of *A. utahensis* and *Actinoplanes* sp. SE50/110 were not fully annotated, the unannotated genes in DEGs were annotated by blastp search program using the non-redundant protein database (nr). Totally, 137 differential genes were annotated (Supplementary Table 2).

Gene ontology (GO) analysis

As the international standardized gene functional classification system, GO comprehensively describes the properties of genes and gene products in organisms. Three ontologies of molecular function, cellular component and biological process were examined for GO analysis.

The top 5 enriched terms in ontologies of biological process, molecular function, and cellular component were shown in Table 1. Differential genes in the biological process mainly lied in histidine catabolic process to glutamate and formate, thiamine diphosphate biosynthetic process, nucleotide salvage, cellular response to reactive oxygen, and ornithine metabolic process. Enriched genes in the molecular function performed anthranilate synthase activity, transferase activity, transferring pentosyl groups, tRNA binding, aminoacyl-tRNA ligase activity, and metal ion binding. No enriched genes in cellular component were discovered.

Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis

Enriched metabolic pathways between different groups were uncovered by KEGG analysis. Hypergeometric tests were applied to identify pathways enriched in differentially expressed genes compared to the entire genomic background. Sulfur metabolism and glyoxylate and dicarboxylate metabolism categories were evidently enriched in GM compared to Glc samples (Table 2). The enrichment degree in KEGG pathway was evaluated by the false discovery rate and the number of genes enriched in the pathway.

Table 2 Enriched KEGG pathways in GM group compared to Glc samples

Pathway	Description	Count in gene set	False discovery rate
ase00920	Sulfur metabolism	6 of 21	0.56×10^{-2}
ase00630	Glyoxylate and dicarboxylate metabolism	7 of 48	2.76×10^{-2}

Protein–protein interaction (PPI) and module analysis

PPI analysis was performed by the STRING platform. 111 connected nodes were identified in the comparison between GM and Glc samples (Fig. 3). Several modules were identified from the PPI network, including cytochrome oxidase module (qcrC, ctaE, cydA, cydB, ACPL_8067), protein synthesis module (rpmG, rpsM, rpsL, ACPL_123), ABC_type sugar transport system (iolD, ACPL_2916, ACPL_2917), and amino acid metabolism system (ACPL_3994, ACPL_3995, ACPL_3996) (Fig. 3).

Expression of gene clusters for ferritin metabolism

The ferritin genes *bfrA* and *bfd* encode ferredoxin, which regulates the iron content in the cells. When *A. utahensis* ZJB-03852 was cultivated in Mal or GM medium, *bfrA* expression was repressed (Fig. 4a). The expression of *bfrA* was undetectable under all the three groups.

The gene cluster *efeUOB* participates in the iron intake. *EfeU* is an iron permease, but the functions of *EfeO* and *EfeB* have not been identified yet. The expression level of *efeUOB* gene cluster in *A. utahensis* ZJB-03852 grown on

Table 1 Enriched GO pathways in GM group compared to Glc samples. Only the top five most significantly enriched items were shown

Term ID	Term description	Observed gene count	Over/under	Fold enrichment	False discovery rate
Molecular function					
GO:0004049	Anthranilate synthase activity	2	+	85.31	3.57×10^{-2}
GO:0016763	Transferase activity, transferring pentosyl groups	3	+	24.37	2.76×10^{-2}
GO:0000049	tRNA binding	3	+	24.37	2.63×10^{-2}
GO:0004812	Aminoacyl-tRNA ligase activity	3	+	18.96	4.50×10^{-2}
GO:0046872	Metal ion binding	15	+	4.67	8.00×10^{-5}
Biological process					
GO:0010033	Histidine catabolic process to glutamate and formate	3	+	> 100	1.57×10^{-4}
GO:0009229	Thiamine diphosphate biosynthetic process	3	+	> 100	3.52×10^{-4}
GO:0,043,173	Nucleotide salvage	2	+	56.87	1.57×10^{-2}
GO:0034614	Cellular response to reactive oxygen species	2	+	56.87	1.56×10^{-2}
GO:0006591	Ornithine metabolic process	2	+	56.87	1.55×10^{-2}

“+” in the “Over/Under” column indicates overexpression in GM samples

lism and cytochrome oxidase. Pink connections indicated experimentally verified connections; green connections indicated predicted interactions based on gene neighborhood; blue interaction represented predicted interaction based on gene co-occurrence; red interaction represented predicted connection based on gene fusions

proteins. *nrps2A*, *nrps2B* and *nrps2C* encode non-ribosomal peptide synthetases. *tetR* and *afsR* are regulatory factors. *ACPL_6137* and *ACPL_6151* genes are unannotated. *pks3A*, *pks3B* and *pks3C* encode polyketide synthases. *ACPL_6159*, *ACPL_6160* and *ACPL_6161* are used for drug delivery. The other genes have modified function. When *A. utahensis* ZJB-03852 was cultured in GM medium, the genes with the highest expression were *ACPL_6137*, *tetR*, *ACPL_6153*, *afsR*, *trpE* and *nrps2C* (Fig. 5). The highest expression level of polyketide synthase encoded by *pks3A*, *pks3B* and *pks3C* was in GM group, and the lowest was in Glc group (Fig. 5). The combined carbon source was more conducive to the production of metabolites.

The antibiotic biosynthesis gene cluster *cACPL_4* consists of three non-ribosomal peptide synthetases (NRPS), three polyketide synthases (PKS) and other accessory

Fig. 4 The gene expressions of **a** *bfrA*, **b** *efeUOB* and **c** *yfe* clusters in *Actinoplanes utahensis* ZJB-03852 grown under various carbon sources. The y-axis represented the fragments per kilobase per million mapped reads (FPKM) value of each gene in Glc, Mal and GM. Each group had three biological replicates. Bars represent the standard deviation ($n = 3$)

Discussion

Our results suggested *A. utahensis* ZJB-03852 grown on Mal and its complex with glucose gave high acarbose titer, while grown on Glc gave low acarbose titer, indicating Glc is necessary for cell growth, and maltose not only supports cell growth but also serves as the precursor of acarbose. Therefore, the lack of either Glc or Mal lead to a decrease of acarbose yield. In the Glc group, *A. utahensis* ZJB-03852 grew continuously, but can only synthesize little acarbose because of lack of precursor; in the Mal only medium, cells

switched to acarbose synthesis in the early stage. However, in the complexed saccharide medium, cells grew vigorously because of the abundance of Glc, and plentiful Mal ensured synthesis of a large amount of acarbose. Therefore, the maximum yield of acarbose in saccharide complex medium was in a later stage compared to that in Mal medium.

Since the genome of *Actinoplanes* sp. was not fully annotated, the gene symbol and functions of many genes were not clarified, which hindered the throughout analysis of *Actinoplanes* sequencing data. We annotated the uncharacterized proteins in our DEGs by comparing their sequence with the nr database using the protein–protein blast (blastp) program (Supplementary Table 2). Totally, 137 genes were annotated, which provided the foundation for further analysis of the functions of these genes. However, the functions of 52 genes still cannot be clarified after blastp examination in the nr database, since none of their functions was explained in any organism. The functions of these genes need to be elucidated by further motif analysis combined with an experimental confirmation.

The DEGs between GM and Glc were enriched in sugar transport, protein synthesis, amino acid metabolism and cytochrome oxidase in the context of GO and KEGG analyses. As expected, the modules of sugar transport, protein synthesis and amino acid metabolism were enriched in the GM group compared to that in Glc samples (Fig. 3). Since acarbose yield was higher in the GM group, the biosynthesis-related enzymes and sugar transport proteins were actively expressed for the acarbose biosynthesis and transport. Cytochrome oxidase *bd* is encoded by the *cydABCD* gene cluster, and is responsible for the detoxification of dioxygen through reduction to water as the terminal of the respiratory chain. The biosynthesis of acarbose includes several steps of transfer of PO_3^{2-} . The PO_3^{2-} donor may be ATP, which was derived from the β -oxidation in mitochondria. As an enzyme in the respiratory electron transport chain of cells, cytochrome oxidase plays an important role in the oxidation in mitochondria. In that way, cytochrome oxidases may indirectly regulate acarbose biosynthesis. The *cydABCD* cluster also encodes two additional ABC-type transporter proteins required for the assembly of the complex (Das et al. 2005). Since *cydABCD* expression is generally induced under oxygen-limiting conditions, upregulation of *cydABCD* in *A. utahensis* ZJB-03852 cultured in the GM medium indicates that the culture lacks oxygen as a result of increased cell density in the GM medium (Fig. 3).

Ferritin metabolism genes (*bfrA*, *efeU*, *efeO*, *efeB*) demonstrated higher expressions in the glucose-only group, which is consistent with the previous research (Schwientek et al. 2013). However, the expressions of metal transport system-associated genes (*yfeA*, *yfeB*, *yfeC*, *yfeD*) were different from the previous report (Schwientek et al. 2013). This indicates that both ferritin metabolism and transport

Fig. 5 The transcriptions of NRPS/PKS gene cluster of *Actinoplanes utahensis* ZJB-03852 in three different cultivation media. The expression of each gene is represented by three colored bars, corresponding to the Glc medium, the Mal medium, and the GM medium. The level of expression is given as fragments per kilobase per million mapped reads (FPKM). Bars represent the standard deviation ($n=3$)

may participate in the biosynthesis of acarbose. In Glc only medium, cells grew fast and little acarbose were secreted; therefore, ferritin may be required for cell growth in the logarithmic phase, and is unnecessary for acarbose biosynthesis.

In most cases, genes in the antibiotic biosynthetic gene cluster demonstrated high expressions in the groups of Mal and GM, which is consistent with the yield of acarbose. This is in line with the previous research that most of the active secondary metabolites in *Actinomycetes* were synthesized by the PKS pathway and the NRPS pathway (Wang et al. 2010). These enzymes separately bind the different building blocks at discrete reaction sites and further catalyze condensation and modification reactions (Weber 2014).

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Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest.

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