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New Insights into the Biosynthesis of Succinic Acid by *Actinobacillus succinogenes* with the Help of Its Engineered Strains

Chunmei Chen and Pu Zheng *

The Key Laboratory of Industrial Biotechnology, Ministry of Education, School of Biotechnology, Jiangnan University, Wuxi 214122, China; 7180201001@stu.jiangnan.edu.cn
* Correspondence: zhengpu@jiangnan.edu.cn; Tel./Fax: +86-510-8591-8156

Abstract: Succinic acid (SA), a C4 tricarboxylic acid cycle intermediate, is used as raw material for bulk chemicals and specialty chemicals, such as tetrahydrofuran and 1,4-butanediol, as well as also being used to synthesize the biodegradable biopolymers PBS (polymer poly (butylene succinate)). *Actinobacillus succinogenes*, which is facultative anaerobic and gram-negative, is one of the most promising natural SA-producing organisms, but genetic engineering of *A. succinogenes* is rare so far. In this study, a series of engineered strains was constructed using the pLGZ922 expression vector and a cytosine base editor (CBE) based on CRIPSR/Cas9; we found that phosphoenolpyruvate carboxylase (PEPC) was more important for the CO_2 fixation pathway than pyruvate carboxylase (PYC) in *A. succinogenes*, and the annotated oxaloacetic acid decarboxylase (Asuc_0301 and Asuc_0302) had little correlation with the SA synthesis pathway. The by-product pathway was closely related to cell growth, and overexpression of FDH was beneficial to growth, while the knockout of the *ackA* gene reduced the growth. For the first time, the hypothetic sugars and SA transporters were mined and identified in *A. succinogenes*, of which Asuc_0914 was responsible for glucose uptake, and Asuc_0715 and Asuc_0716 constituted SA exporters. This deepens the understanding of SA biosynthesis in *A. succinogenes* and is also valuable for SA production by fermentation.

Keywords: succinic acid; *Actinobacillus succinogenes*; genetic engineering; CO_2 fixation; transporter

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1. Introduction

Production of chemicals and fuels from renewable resources is a promising route for sustainable development in the future. The chemical synthesis of chemicals has a risk of environmental pollution, and the sustainable production of a microbial fermentation method is increasingly favored [1–3]. Succinic acid (SA), one intermediate of the tricarboxylic acid cycle (TCA), is a widely high-value chemical and is applied in numerous fields, including food, chemicals, agriculture, and pharmaceuticals [1,4]. It is also used as a precursor to produce other derivatives such as adipic acid, tetrahydrofuran, and 1,4-butanediol. Microbial fermentation produces SA mainly through three TCA-related pathways: the reduction branch of the TCA cycle (rTCA pathway), the oxidation branch of the TCA cycle (oTCA pathway), and the glyoxylic acid cycle [5]. Under anaerobic conditions, it occurs mainly through rTCA with SA as the end product. In this pathway, catalytic reactions between phosphoenolpyruvate carboxykinase (PCK) and PEP carboxylase (PEPC) or between phosphoenolpyruvate (PEP) and oxaloacetate (OAA) played an important role. OAA was converted to malic acid by malate dehydrogenase (MDH), then fumaric acid was converted by fumarase (FUM), and finally SA was obtained by fumarate reductase (FRD). Under aerobic conditions, SA was synthesized mainly through the oTCA pathway and the glyoxylate pathway (GAC pathway) [6]. SA appeared as an intermediate and was further converted to fumarate. To prevent the conversion of SA to FUM, the activity of succinate dehydrogenase (SDH) must be inhibited [7–11] (Figure 1).

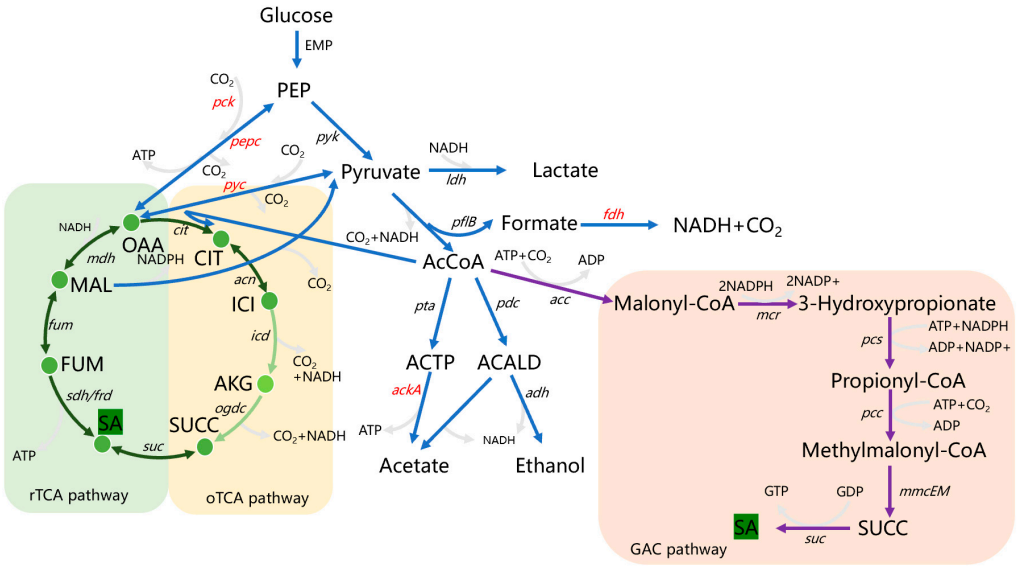

Figure 1. Biosynthetic pathway of SA. OAA: oxaloacetate; MAL: malate; FUM: fumarate; SA: succinic acid; SUCC: succinyl CoA; AKG: α-ketoglutarate; ICI: isocitrate; CIT: citrate; AcCoA: acetyl CoA; ACTP: acetyl phosphate; ACALD: acetaldehyde; *ackA*: acetokinase; *pyc*: pyruvic carboxylase; *pta*: phosphoacetyltransferase; *pdc*: pyruvate decarboxylase; *adh*: alcoholdehydrogenase; *pflB*: pyruvate formate-lyase; *fdh*: formate dehydrogenase; *ldh*: lactate dehydrogenase; *pyk*: pyruvate kinase; *pck*: phosphoenolpyruvate carboxykinase; *cit*: citrate synthase; *me*: malic enzyme; *mdh*: malate dehydrogenase; *fum*: fumarase; *sdh/frd*: succinate dehydrogenase/fumarate reductase; *suc*: succinyl-CoA thiokinase; *ogdc*: oxoglutarate dehydrogenase complex; *icd*: isocitrate dehydrogenase; *acn*: aconitase; *cit*: citrate synthase; *acc*: acetyl-coa carboxylase; *mcr*: malonyl-coa reductase; pcs: propionyl-CoA synthase; *pcc*: propionyl-CoA carboxylase; *mmcEM*: methylmalonyl-CoA exomerases and mutants.

Many microorganisms, such as *Actinobacillus succinogenes*, *Anaerobiospirillum succiniciproducens*, *Mannheimia succiniciproducens*, *Escherichia coli*, *Corynebacterium glutamicum*, *Yarrowia lipolytica*, and *Saccharomyces cerevisiae*, have been developed for SA production [2,12–15]. *A. succinogenes* is facultative anaerobic and gram-negative, and is isolated from the bovine rumen [16]. It can utilize various kinds of carbohydrates, such as glucose, xylose, sucrose, fructose, lactose, galactose, and maltose, and can tolerate high concentrations of glucose and succinate [16,17]. The fermentation products are mainly SA, along with some acetic acid (AA) and a small amount of formic acid (FA). Therefore, it is considered to be one of the most promising strains for SA production. However, *A. succinogenes* also has some disadvantages as a SA production platform, such as numerous auxotrophies and acid sensitivity [18–20]. Engineering of *A. succinogenes* is a promising method for further increasing SA production [21]. The strategies to promote SA biosynthesis mainly include redirecting carbon flow, balancing the redox ratio (NADH/NAD+), and optimizing CO_2 supplementation [22–26]. The main by-products are AA and FA in *A. succinogenes*. Therefore, carbon flow is generally redirected by blocking the pathway of by-product synthesis and SA consumption. For example, AA can be effectively removed by inactivating the *ackA* gene and the inactivation of *plfB* effectively removes FA [27,28]. However, the removal of redundant metabolic branches resulted in the imbalance of the redox ratio ($NADH/NAD^+$), which affected the normal cell growth and the accumulation of SA. In order to improve the accumulation of SA, the key enzymes of the SA synthesis pathway including FUM, PCK, and MDH were overexpressed to enhance the main metabolic pathway of SA, but SA production was not significantly enhanced [27].

In this study, the effects of increasing the CO_2 fixation pathway, reducing the oxaloacetic acid outflow, and eliminating the production of the by-products acetic acid and formic acid in the synthesis of SA were investigated by using the pLGZ922 expression vector and a cytosine base editor (CBE) in *A. succinogenes*. In addition, genes associated

with sugar transporters and SA transporters were mined and identified, which will useful in improving the fermentative production of SA by *A. succinogenes*.

2. Materials and Methods

2.1. Strains, Plasmids, and Culture Conditions

The strains and plasmids used in this study are listed in Supplementary Materials: Table S1, and the primers are listed in Supplementary Materials: Table S2. *E. coli* JM109 was used as the host for the plasmid clone and cultured with LB medium at 37 °C or on LB agar plates with ampicillin (100 μg/mL) when necessary. *A. succinogenes* M 2012036 (ZK) was anaerobically cultured with TSB medium at 37 °C or on TSB agar plates with ampicillin (100 μg/mL) when necessary. The fermentation medium was described in reference [29]. The sgRNAs were designed online (https://chopchop.cbu.cib.no/ accessed on 1 September 2022), and the protospacer sequences are listed in Supplementary Materials: Table S3.

2.2. Plasmid Construction

The *pepc* and *pyc* gene fragment was amplified from the *Corynebacterium acetoacetophilus* genome, respectively. The *ppk* gene fragment was amplified from the *Pseudomonas aeruginosa* genome. The codon-optimized DVfdh and CBfdh were synthesized by GE-NEWIZ, Inc. The gene fragments were ligated, respectively, to the expression vector plGZ922 by ClonExpress II One Step Cloning Kit (Vazyme, China), yielding the expression vectors pLGZ-CBfdh, pLGZ-DVfdh, pLGZ-pepc, and pLGZ-pyc.

The cytidine base editor (CBE) base editor plasmid was constructed in a previous study (data not published). The linearized fragments with the target gene sgRNA were amplified using the CBE plasmid as a template by PCR, and transformed into *E. coli* JM109, yielding the plasmids Asuc_0914-Td-CBE, Asuc_0023-Td-CBE, Asuc_0715-Td-CBE, Asuc_0716-Td-CBE, Asuc_2056-Td-CBE, Asuc_0750-Td-CBE, Asuc_0301-Td-CBE, and Asuc_0302-Td-CBE.

2.3. Transformation and Gene-Silenced Mutant Screening of A. succinogenes

The method for the transformation refers to references [27,29].

After the gene-silenced plasmid was transformed into *A. succinogenes*, one colony was randomly picked and placed into TSB medium containing 100 μg/mL ampicillin and incubated anaerobically at 37 °C for 24 h. The cultures were spread on TSB agar plates until colonies were obtained. The colony PCR was performed by randomly picking 10 colonies, and the mutant was confirmed by DNA sequencing. A plasmid curing procedure was performed by subculture of mutants in TSB medium overnight and plating on TSBG agar without antibiotics. The target mutants that did not grow in the presence of antibiotics and that grew in the absence of antibiotics were the target strains.

2.4. Determination of Extracellular and Intracellular Glucose and SA Concentrations

A. succinogenes was cultivated anaerobically in 25 mL of TSB medium at 37 °C for 12–16 h, and a 10% *v*/*v* inoculum was added into shake flasks containing 50 g/L glucose fermentation medium with the pH regulator $MgCO_3$ powder. The cultures were centrifuged at 7000 rpm for 10 min. The supernatant was used to determine the extracellular glucose and SA concentrations. Cells were suspended with PBS solution and washed twice. Finally, cells were broken by adding 1.5 mL 10% H_2SO_4, and the broken solution was used to determine the intracellular glucose and SA concentrations.

2.5. Fermentation and Analytical Methods

A. succinogenes was cultivated anaerobically in 25 mL of TSB medium at 37 °C for 12–16 h, and a 10% *v*/*v* inoculum was added into shake flasks containing 50 g/L glucose fermentation medium with the pH regulator $MgCO_3$ powder for 72 h. For a 3 L bioreactor, the temperature and agitation were 38 °C and 200 rpm, respectively. $MgCO_3$ was used as a pH regulator in the early stage of fermentation, and 3 M Na_2CO_3 was maintained at pH 6.5 in the late stage of fermentation. The initial glucose concentration was 35 g/L, and

we supplemented the glucose to maintain it between 10 and 30 g/L when the glucose fell to below 10 g/L.

The optical density of *A. succinogenes* was measured by spectrophotometry at 660 nm (OD_{660}). Glucose and organic acids were analyzed by HPLC. The special determination methods refer to reference [30].

3. Results and Discussion

3.1. Succinic acid Biosynthesis Pathway

3.1.1. Effect of Strengthening CO_2 Fixation Pathway

SA was synthesized by the reductive C4 pathway (rTCA) in *A. succinogenes*, and the by-products, such as acetic acid and formic acid, were synthesized by the C3 pathway [20,27]. A key CO_2-fixing enzyme has been reported to be phosphoenolpyruvate carboxykinase (PCK), which catalyzes the carboxylation of phosphoenolpyruvate with bicarbonate and ADP to generate oxaloacetic acid (OAA) and ATP. No gene encoding phosphoenolpyruvate carboxylase (PEPC) and pyruvate carboxylase (PYC) was found in *A. succinogenes* [20]. The heterogeneous expression of PEPC and PYC in *E. coli* and *C. glutamicum* enhanced the C flow of the SA synthesis pathway and increased the SA production [31,32]. The fixation of CO_2 in *Corynebacterium acetoacidophilum* depends on the fact that PYC catalyzes pyruvate and PEPC catalyzes phosphoenolpyruvate. Therefore, *pyc* and *pepc* genes from *C. acetoacidophilum* were introduced into *A. succinogenes* to strengthen the CO_2 fixation pathway, respectively, yielding the strains ZK::*pyc* and ZK::*pepc*.

To investigate the effect on *A. succinogenes* after introducing the *pyc* and *pepc* genes, cell growth, glucose, and organic acid concentrations were measured by shake-flask fermentation (Figure 2a). The results showed that the SA produced by strain ZK::*pyc* was similar to the wild-type ZK (SA 27.15 g/L), but the growth was slightly decreased and the residual glucose concentration was higher, indicating that the expression of exogenous PYC delayed the fermentation of SA. The SA production of strain ZK::*pepc* (38.63 g/L) was significantly higher than that of the wild type and increased by 39%, and its effect on cell growth was small. Although the by-products acetic acid and lactic acid are slightly elevated, the conversion rate of glucose and SA was also significantly increased. These results suggested that PEPC was a suitable precursor in the fixation of CO_2 of *A. succinogenes*, and overexpression of PEPC may enhance the carbon flow rate of the SA synthesis pathway, thus increasing the SA production.

Further, the above three strains were fed-batch fermented in a 3 L bioreactor (Figure 2b–d). The maximum OD_{660} of wild-type strain ZK was obtained at 7 h (up to 6.93), while that of strain ZK::*pyc* was 7.56 at 12 h, and strain ZK::*pepc* was 6.58 at 10 h, respectively. These results indicated that the overexpression of exogenous proteins delayed the growth of cells to a certain extent. After fermentation for 60 h, the SA concentration was 52.35 g/L and the conversion rate was 0.70 g/g in strain ZK, while the SA concentrations of strain ZK::*pyc* and strain ZK::*pepc* were 55.66 g/L and 59.47 g/L, and their conversion rates were 0.82 g/g and 0.79 g/g, respectively. This result was consistent with shake-flask fermentation, showing that the expression of PEPC from *C. acetoacidophilum* in *A. succinogenes* increased the conversion rate and SA production, indicating that increasing the expression of PEPC was beneficial to the SA synthesis of *A. succinogenes*.

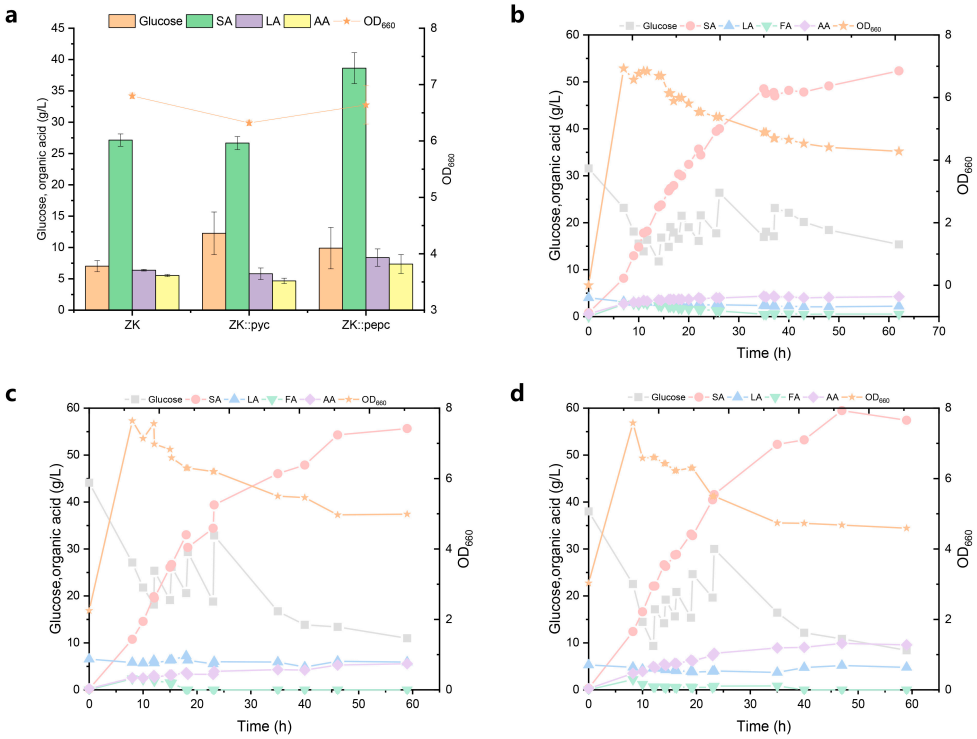

Figure 2. The results of shake-flask fermentations (**a**) and fed-batch fermentation by strains ZK (**b**), strains ZK::*pyc* (**c**), and strains ZK::*pepc* (**d**).

3.1.2. Effect of Preventing Oxaloacetic Acid Outflow by Inactivation of Oxaloacetic Acid Decarboxylase

In biological metabolism, OAA can be decarboxylated by oxaloacetic acid decarboxylase to produce pyruvate. Based on the *A. succinogenes* genome (GenBank: CP000746.1), *Asuc_0301* and *Asuc_0302* were annotated as genes encoding oxaloacetic acid decarboxylase. To investigate the presence of OAA outflow in the SA biosynthesis of *A. succinogenes*, we attempted to silence the gene encoding oxaloacetate decarboxylase using a cytosine base editor (CBE) based on CRIPSR/Cas9. The gene-silenced strains ΔAsuc_0301 and ΔAsuc_0302 were obtained by introducing terminators into the gene-coding region of *Asuc_0301* and *Asuc_0302* (Figure 3a). The shake-flask fermentation results showed that, compared with strain ZK, the SA production of strains ΔAsuc_0301 and ΔAsuc_0302 decreased slightly and their growth deteriorated, indicating that the inactivation of genes *Asuc_0301* and *Asuc_0302* not only did not promote the synthesis of SA, but also affected cell growth (Figure 3b). These results indicated that this pathway may be related to cell growth in *A. succinogenes*, and has little correlation with the biosynthetic pathway of SA.

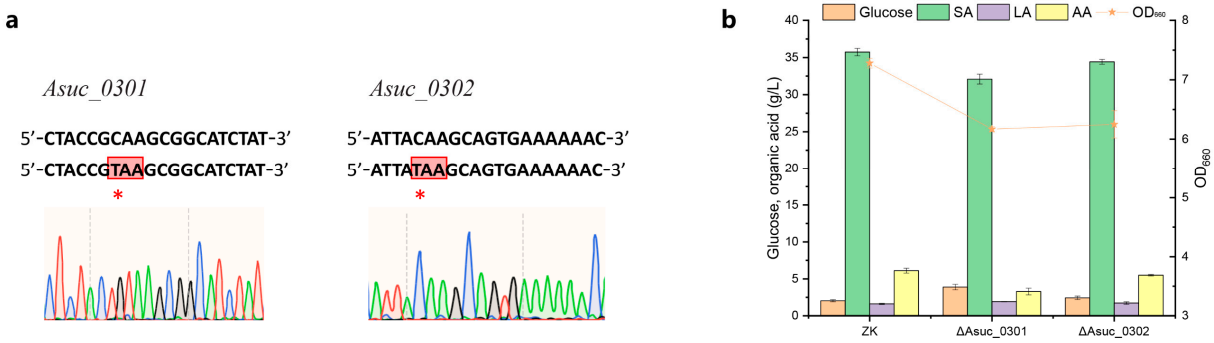

Figure 3. The sequencing results of STOP codons introduced in *Asuc_0301* and *Asuc_0302* (**a**) and the results of shake-flask fermentation (**b**). *: STOP codons.

3.2. Effect of Formic Acid Pathway

Formic acid is mainly catalyzed by pyruvate formic acid lyase to produce pyruvate in *A. succinogenes*. Although the knockout of the *plfB* gene blocked the formic acid synthesis pathway, deletion of *plfB* affected cell growth and did not promote the SA synthesis pathway [27,28]. In addition, it was found that *A. succinogenes* can produce formic acid in the initial stage of fermentation, but with the extension of fermentation, formic acid can be gradually consumed to produce NADH and release CO_2. To further investigate the effect of formic acid on the biosynthetic pathway of SA, formate dehydrogenase (FDH) of *Candida boidinii* and *Desulfovibrio vulgaris* was introduced into *A. succinogenes* to remove the formic acid, respectively, yielding strains ZK::CBfdh and ZK::DVfdh. The results of shake-flask fermentation are shown in Figure 4a. It was found that the SA yield of strain ZK::DVfdh was similar to that of strain ZK, indicating that the introduction of FDH from *D. vulgaris* had no significant effect on the SA synthesis of *A. succinogenes*. The SA production and OD_{660} of strain ZK::CBfdh were slightly higher than those of the wild type, and no formic acid production was detected, indicating that CBfdh might consume formic acid to generate NADH and CO_2, which promoted cell growth and SA production. In order to further investigate the effect of CBfdh expression on *A. succinogenes*, strain ZK::CBfdh was fed-batch fermented in a 3 L fermenter. As shown in Figure 4b, the maximum OD_{660} of strain ZK::CBfdh was obtained (7.76) at 7 h. After fermentation for 60 h, the SA concentration was 52.72 g/L, and the conversion rate was 0.67 g/g, but its growth was better than that of strain ZK (Figure 2b). Furthermore, the changes in the fermentation parameters of strain ZK::CBfdh were observed when formic acid was added during the fermentation process. As shown in Figure 4c, strain ZK::CBfdh obtained a maximum OD_{660} at 10 h, up to 6.68. Formic acid produced by the strain was exhausted at 10 h, and 12 g formic acid was added at 12 h, which could be exhausted at 24–34 h. After fermentation for 60 h, the SA concentration was 52.13 g/L and the conversion rate was 0.79 g/g. These results showed that the introduction of CBfdh into *A. succinogenes* could consume more formic acid but had no significant effect on the synthesis of SA, indicating that formic acid metabolic pathways may not account for a high proportion of carbon metabolic flow in *A. succinogenes*.

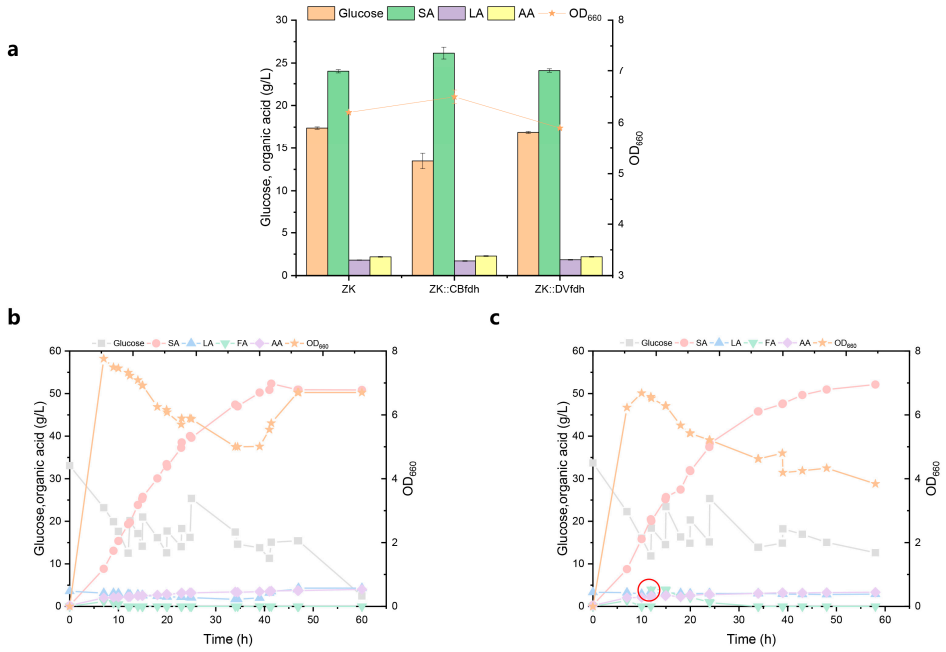

Figure 4. Evaluation of different fermentation and metabolic parameters in wild-type *A. succinogenes* and recombinants. (**a**) The shake-flask fermentation results of strains ZK, ZK::CBfdh, and ZK::DVfdh; (**b**) the fed-batch fermentation results of strain ZK::CBfdh; (**c**) the fed-batch fermentation results of strain ZK::CBfdh with 12 g FA. The added FA was marked with a red circle.

3.3. Effect of Acetic Acid Pathway

Although the deletion of the *ackA* gene can reduce acetic acid synthesis, it also affects the growth of strains [27]. In order to verify the role of acetokinase again, the strain ΔackA::ackA was constructed by expressing *ackA* into the knockout strain ΔackA. The shake-flask fermentation results showed that, compared with the knockout strain, the acetic acid production of the strain ΔackA::ackA was slightly increased and the growth was also restored to a certain extent, suggesting that the *ackA* gene was successfully expressed and partly complemented *ackA* function in *A. succinogenes* (Figure 5a). The insignificant complementary effect may be due to insufficient expression, but it also confirmed that the *ackA* gene was necessary for cell growth and acetic acid synthesis. The production of acetic acid is accompanied by the production of ATP, and it is speculated that ATP is associated with cell growth. Polyphosphate AMP phosphotransferase of *Pseudomonas aeruginosa* can promote ATP production. Therefore, we introduced the *ppk* gene into strain ΔackA to construct strain ΔackA::ppk, and investigated its effects on cell growth and SA synthesis. The results showed that, compared with strain ΔackA, strain ΔackA::ppk had a better growth, but there was no significant difference in the production of SA, indicating that the introduction of *ppk* may promote the production of ATP and thus promote cell growth, and there seemed to be little correlation with the synthesis carbon flow of SA. Indeed, it was indicated that the synthesis pathway of acetic acid may be more associated with cell growth through the production of ATP.

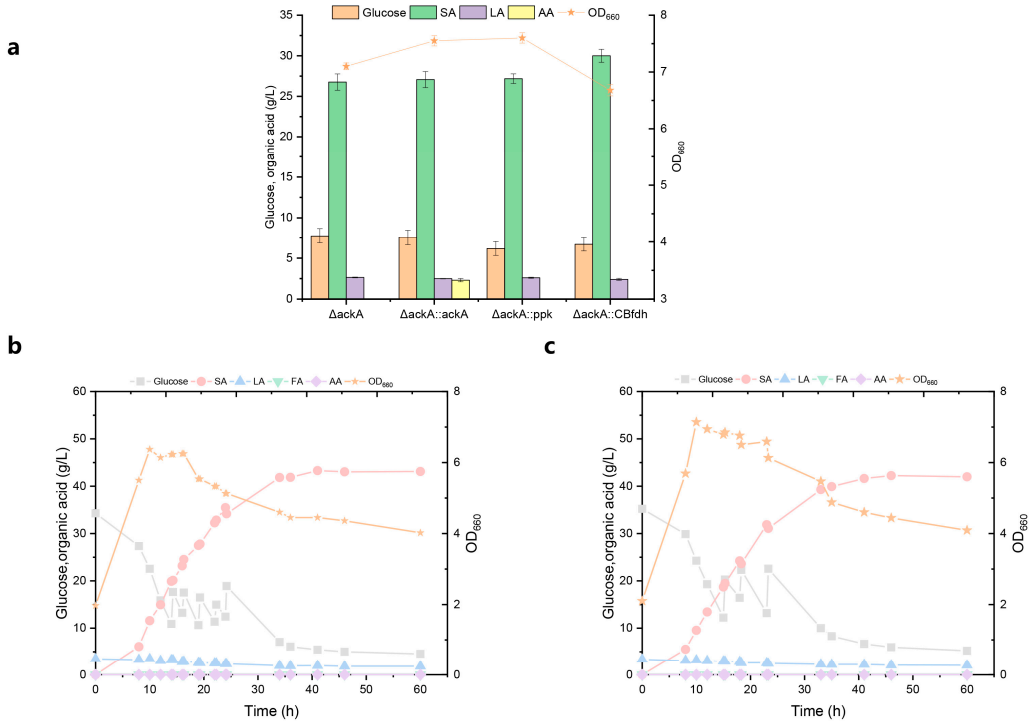

Figure 5. Evaluation of different fermentation and metabolic parameters in ΔackA and its derivates. (**a**) The shake-flask fermentation results of strain ΔackA and its derivates; (**b**) the fed-batch fermentation results of strain ΔackA; (**c**) the fed-batch fermentation results of strain ΔackA::CBfdh.

To further investigate the effect of the by-product pathway on the SA synthesis pathway, CBfdh was introduced into strain ΔackA to eliminate formic acid and acetic acid during fermentation, yielding strain ΔackA::CBfdh. The results of shake-flask fermentation showed that strain ΔackA::CBfdh could slightly increase the SA yield (Figure 5a). To study the effect of strain ΔackA::CBfdh on SA production, strain ΔackA and strain ΔackA::CBfdh were fed-batch fermented in a 3 L fermenter. As shown in Figure 5b,c, the residual glucose of strain ΔackA was 4.53 g/L after fermentation for 60 h, and the maximum OD_{660}, up to

6.37, was obtained at 14 h. No formic acid or acetic acid were detected in the fermentation process, and the yield of lactic acid was also reduced, with the concentration of SA production being 43.11 g/L and the conversion rate being 0.66 g/g. The results showed that the generation of acetic acid was eliminated after the *ackA* gene was knocked out, but the cell growth was affected, and the generation and conversion rate of SA were reduced. The residual glucose of strain ΔackA::CBfdh was 5.20 g/L after fermentation for 58 h, and the maximum OD_{660} was 7.14 when the strain was grown for 12 h. No formic acid was detected in the fermentation process, the acid production concentration was 42.24 g/L, and the conversion rate was 0.68 g/g. These results indicated that overexpression of CBfdh shortened the growth stagnation period of the strain, but slightly affected the SA production.

In the initial phase of fermentation, *A. succinogenes* obtains a brief energy boost by synthesizing acetic acid to initiate growth [33]. The effect of the overexpression of FDH on growth was not as large as that of the deletion of *ackA*. CBfdh catalyzed formic acid to produce NADH, and AA synthesis was accompanied by ATP generation, suggesting that ATP may be more necessary than NADH for cell growth and SA production. The acetic acid synthesis pathway is closely related to the SA synthesis pathway as it provides the ATP required for cell growth in *A. succinogenes*.

3.4. Mining and Identification of Glucose Transporter

A. succinogenes naturally accumulates SA from a wide variety of carbohydrates, but no associated transport genes have been characterized. The *ptsG* gene, which encodes glucose-specific permease of the phosphotransferase system, protein EIICBglc, was inactivated in *E. coli*, resulting in a strong shift in metabolic pathway to the SA synthesis pathway during glucose fermentation [34]. It was found that two proteins, *Asuc_0914* and *Asuc_1575*, were homologous with *ptsG* by BLAST analysis in *A. succinogenes* (Figure 6a). Among them, the *Asuc_1575* gene is annotated as triosephosphate isomerase and the *Asuc_0914* gene is annotated as the PTS system, sucrose-specific IIBC subunit. Therefore, these two genes were speculated to be involved in glucose transport. To investigate gene function, a stop codon was introduced into the coding region of the gene to yield a gene-silenced strain by using a cytosine base editor. However, no available target site was found in the coding region of gene *Asuc_1575*, so no *Asuc_1575* gene-silenced strain was obtained. The effects of the wild type and the gene-silenced strain ΔAsuc_0914 on SA synthesis were determined by shake-flask fermentation. As shown in Figure 6b, the strain ΔAsuc_0914 showed efficient glucose utilization and increased SA production, representing a 1.1-fold increase and indicating that the inactivation of *Asuc_0914* may promote the production of SA by participating in glucose transport.

To determine whether the *Asuc_0914* gene is involved in glucose transport, intracellular and extracellular glucose concentrations at different fermentation times were measured. The results are shown in Figure 6c; extracellular glucose concentrations of strain ΔAsuc_0914 were all higher than those of the wild type, indicating that Asuc_0914 was involved in glucose transport, and its inactivation affected glucose uptake. The intracellular glucose concentration of strain ΔAsuc_0914 was similar to that of the wild type during the whole fermentation process, which may be caused by the homeostasis of cell regulation. Similar to *E. coli*, the inactivation of Asuc_0914 in *A. succinogenes* affects the uptake of glucose by reducing the rate of glucose uptake and causing the metabolic pathway to shift to the SA biosynthesis pathway. The accumulation of unserviceable intermediates that did not effectively convert to the target product was caused by the rapid uptake of glucose.

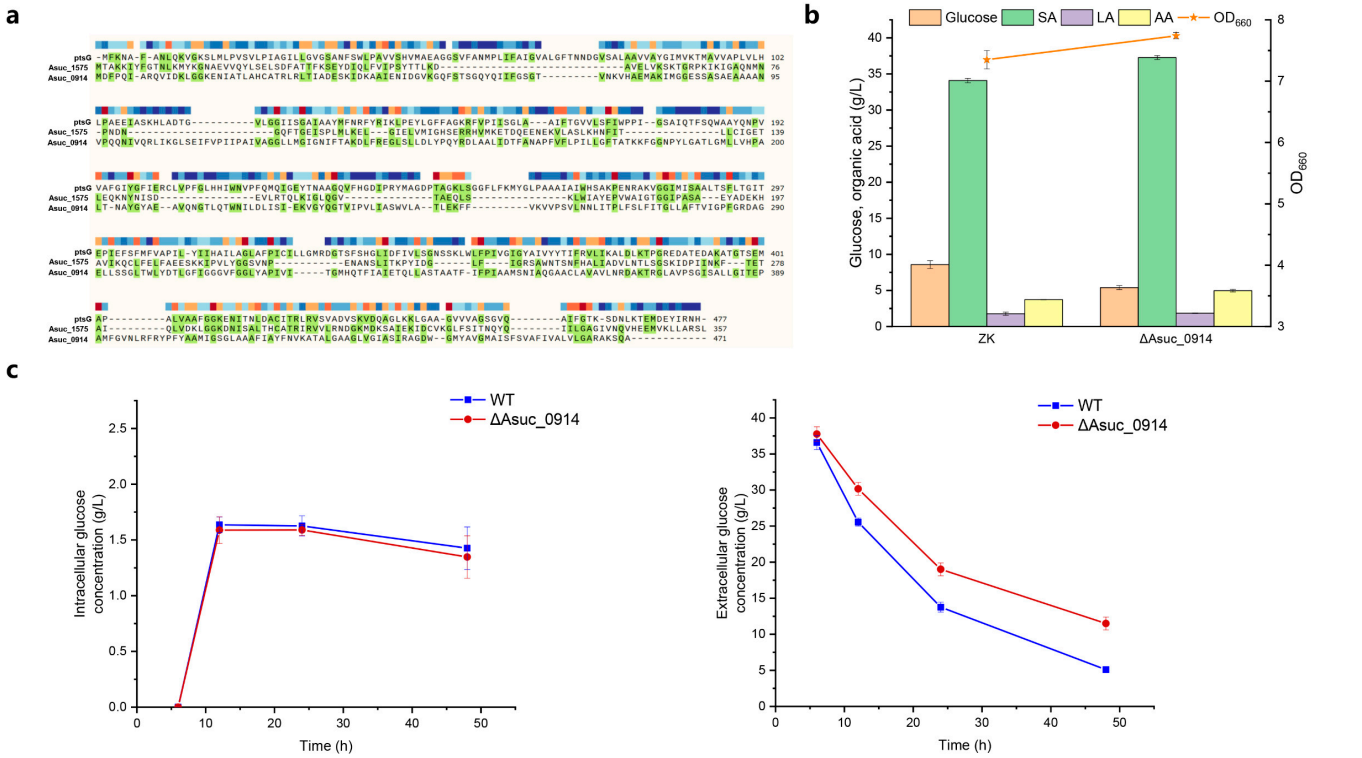

Figure 6. The results of amino acid sequence alignment, fermentation, and metabolic parameters in wild-type *A. succinogenes* and mutants. (**a**) The results of the amino acid sequence alignment of Asuc_1575, Asuc_0914, and ptsG; (**b**) the shake-flask fermentation results of strain ZK and its mutants; (**c**) the intracellular and extracellular glucose concentrations.

3.5. Mining and Identification of SA Transporter

3.5.1. Mining and Identification of SA Importer

In microbial fermentation production, the transport of products is also critical to the synthesis of target products. Two JEN family carboxylic acid transporters PkJEN2-1 and PkJEN2-2 of *Pichia kudriavzevii* can effectively transfer to SA [35]. BLAST analysis showed that Asuc_2056 had 18.99% homology with PkJEN2-1, and Asuc_0750 had 21.64% homology with PkJEN2-1 (Figure 7a). It was speculated that Asuc_2056 and Asuc_0750 may be SA transporters in *A. succinogenes*. Similarly, by introducing terminators into the coding regions of the *Asuc_2056* and *Asuc_0750* genes by cytosine base editor, respectively, the gene-silenced strains ΔAsuc_2056 and ΔAsuc_0750 were obtained. The effect of gene-inactivated strains on SA synthesis was determined by shake-flask fermentation. The results showed that the SA yields of the two gene-silenced strains were similar to that of the wild type, suggesting that Asuc_2056 and Asuc_0750 had no significant effect on the synthesis of SA, which may be due to the difference in genetic background (Figure 7b). This is consistent with our study that knocking out the JEN2 transporter in *Issatchenkia orientalis* also did not promote SA synthesis [14].

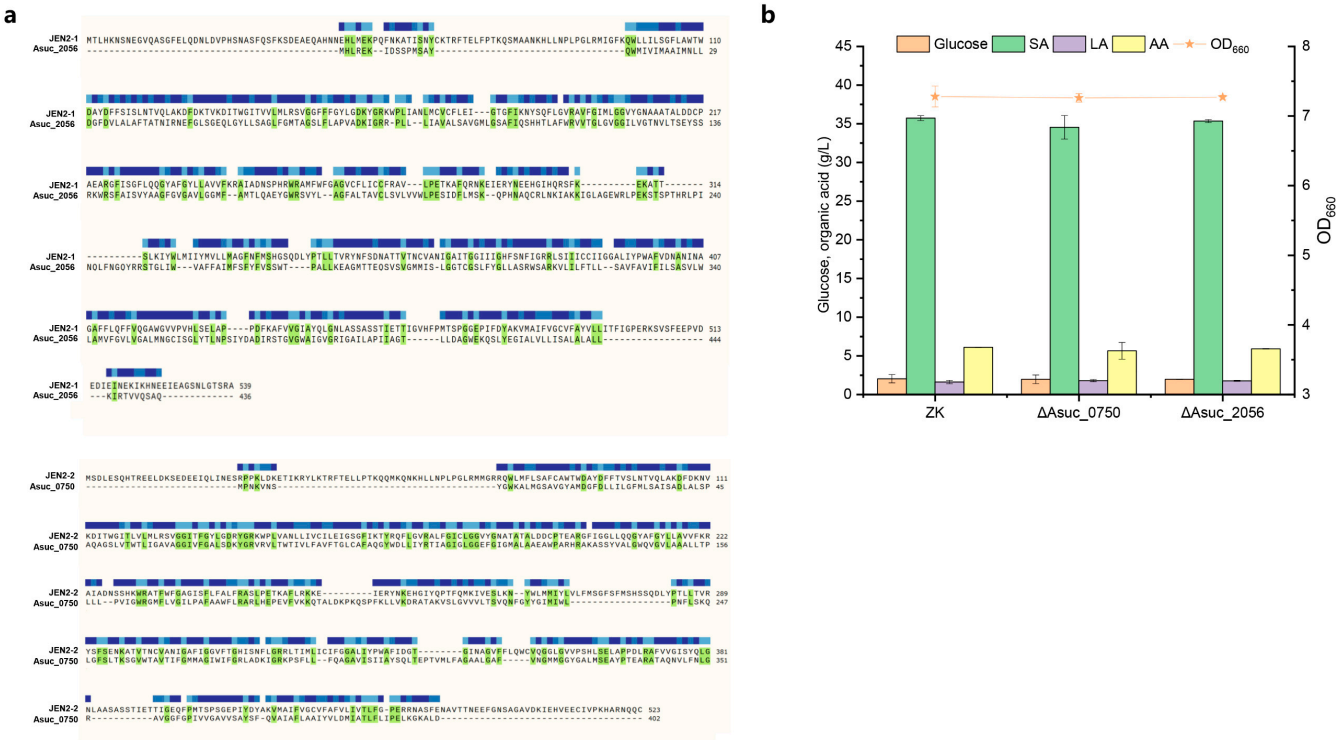

Figure 7. The results of amino acid sequence alignment, fermentation, and metabolic parameters in wild-type *A. succinogenes* and mutants. (**a**) The results of the amino acid sequence alignment of Asuc_2056 and JEN2-1, or Asuc_0750 and JEN2-2; (**b**) the shake-flask fermentation results of strain ZK and its mutants.

3.5.2. Mining and Identification of SA Exporter

SucE1 of *C. glutamicum* and yjjPB of *E. coli* were identified as SA exporters [36,37]. BLAST analysis showed that Asuc_0023 had 29.93% homology with SucE1, Asuc_0716 had 49.23% homology with yjjP, and Asuc_0715 had 50.96% homology with yjjB (Figure 8a). Asuc_0716 and Asuc_0715 are also in one operon and have 49.88% homology with yjjPB. It is speculated that Asuc_0716 and Asuc_0715 may be SA exporters of *A. succinogenes*. Similarly, introducing terminators into the coding regions of *Asuc_0023*, *Asuc_0716*, and *Asuc_0715*, respectively, yielded the strains ΔAsuc_0023, ΔAsuc_0716, and ΔAsuc_0715. The effect of the gene-inactivated strain on SA synthesis was determined by shake-flask fermentation. As shown in Figure 8b, there was no significant difference in the SA production of strain ΔAsuc_0023 compared with the wild type, indicating that Asuc_0023 may not be involved in the transport of SA. The growth of the ΔAsuc_0715 and ΔAsuc_0716 strains deteriorated, and the yield and conversion rate of SA decreased significantly, indicating that Asuc_0715 and Asuc_0716 may be SA transporters which are involved in SA output. After the inactivation of the two genes, the intracellular SA could not be transported out to affect cell homeostasis, resulting in poor cell growth and SA synthesis. To further determine the effect of Asuc_0715 and Asuc_0716 on SA synthesis, a double gene-silenced strain ΔAsuc_0715-0716 was constructed. The shake-flask results showed (Figure 8b) that, compared with the wild type, the double gene silencer had poorer growth and lower SA production, indicating that Asuc_0715 and Asuc_0716 could affect SA synthesis. However, there was no significant difference in the growth and SA production of the double gene-silenced strains compared with the single gene-silenced strains, which may be due to the fact that cells can regulate the intracellular SA concentration to maintain cell homeostasis.

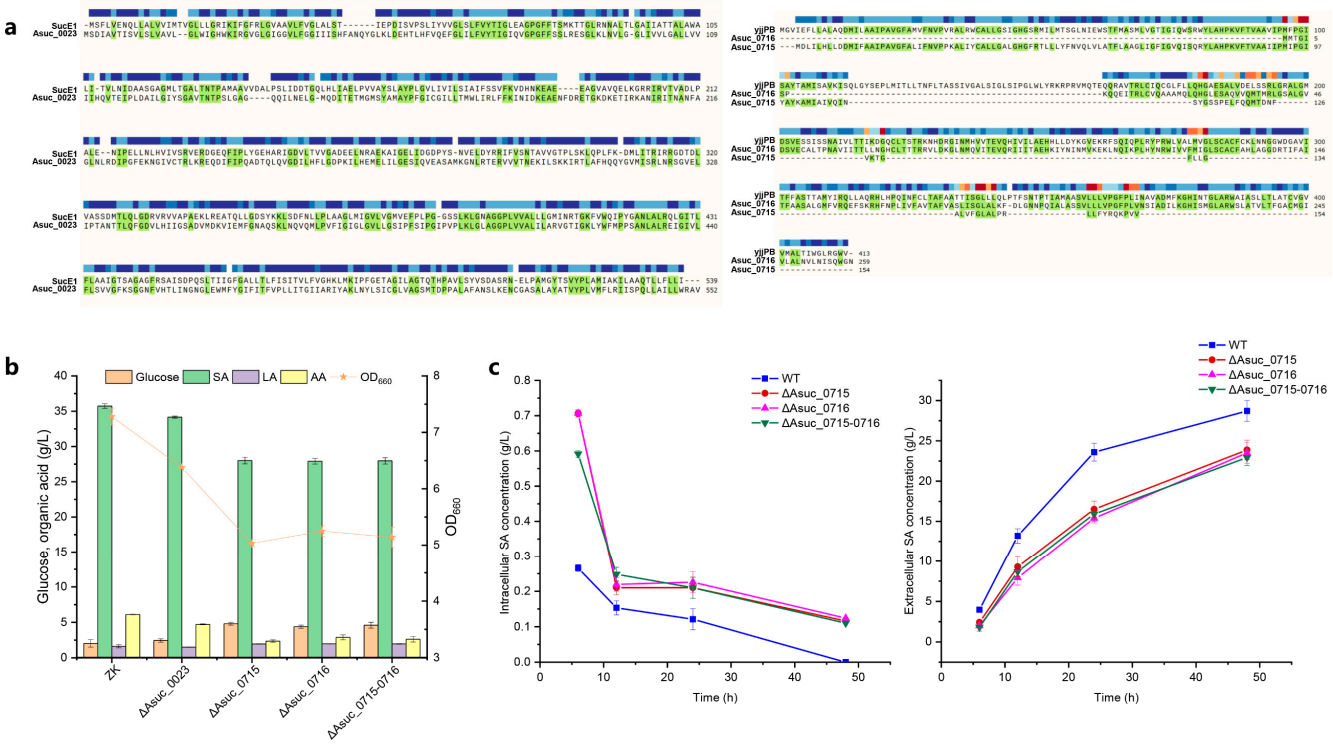

Figure 8. The results of amino acid sequence alignment, fermentation, and metabolic parameters in wild-type *A. succinogenes* and mutants. (**a**) The results of the amino acid sequence alignment of Asuc_0023 and SucE1, or Asuc_0715-0716 and yjjPB; (**b**) the shake-flask fermentation results of strain ZK and its mutants; (**c**) the intracellular and extracellular SA concentrations.

To identify whether Asuc_0715 and Asuc_0716 were SA exporters, the intracellular and extracellular SA concentrations of the three mutant strains at different fermentation times were measured. The results are shown in Figure 8c; extracellular SA concentration of the three mutants was lower than the wild type, while intracellular succinic acid concentration was higher than the wild type, indicating that the inactivation of Asuc_0715 and Asuc_0716 affected the exportation of intracellular SA, resulting in the accumulation of intracellular SA. The intracellular and extracellular SA concentrations of the three mutants were similar, suggesting that either Asuc_0715 or Asuc_0715 is important for SA exportation. Taken together, these results suggested that Asuc_0715 and Asuc_0716 were SA exporters and that any one of them was necessary for SA export in *A. succinogenes*.

4. Conclusions

The expression of PEPC and PYC from *C. acetoacidophilum* suggested that *A. succinogenes* tended to fix CO_2 and produced SA through PEPC carboxykinase. Overexpression of FDH from *Candida boidinii* was beneficial to cell growth and removed the by-product formic acid. The deletion of the *ackA* gene can remove acetic acid, but is unfavorable to cell growth due to insufficient ATP supply. By silencing hypothetic glucose and SA transporter genes by base editor, it was found that Asuc_0914 was responsible for glucose uptake, and Asuc_0715 and Asuc_0716 constituted SA exporters that were important for SA production in *A. succinogenes*.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation9121026/s1, Table S1: the strains and plasmids used in this study; Table S2: the primers used in this study; Table S3: the PAM sequences used in this study.

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