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Enhancement of 2,3-Butanediol Production by *Klebsiella pneumoniae*: Emphasis on the Mediation of sRNA-SgrS on the Carbohydrate Utilization

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Abstract: The demand for renewable energy is increasing. *Klebsiella pneumoniae* is one of the most promising strains to produce 2,3-butanediol (2,3-BD). Compared with chemical methods, the biological production of 2,3-BD has the characteristics of substrate safety, low cost, and low energy consumption. However, excessive glucose concentrations can cause damage to cells. Therefore, this study investigated the effect of sRNA-SgrS as a sugar transport regulator on the fermentative production of 2,3-BD by *K. pneumoniae* in response to sugar stress. We designed multiple mutants of *K. pneumoniae* HD79 to redistribute its carbon flux to produce 2,3-BD. It was found that the 2,3-BD yield of *sgrS* overexpressed strain decreased by 44% compared with the original strain. The results showed that a high concentration of sRNA-SgrS could accelerate the degradation of *ptsG* mRNA (encoding the glucose transporter EIICB^{Glc}) and downregulate the expression levels of the *budA* gene (encoding the α-acetyllactate decarboxylase) and the *budB* gene (encoding the α-acetyllactate synthase) and *budC* gene (encoding the 2,3-BD dehydrogenase) but had no effect on the *ack* gene (encoding the acetate kinase) and the *ldh* gene (encoding the lactate dehydrogenase). It provides a theoretical basis and a technical reference for understanding the complex regulation mechanism of sRNA in microorganisms and the genetics and breeding in industrial fermentation engineering.

Keywords: *Klebsiella pneumoniae*; sRNA-SgrS; 2,3-butanediol; glucose metabolism

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1. Introduction

2,3-Butanediol (2,3-BD) is a colorless, odorless liquid with numerous industrial applications. As an important raw chemical raw material, 2,3-BD has a wide range of uses in oil refining, dyes, adhesives, electronic component cleaning, printing ink, and other industries [1,2]. As an alcohol substance, various chemical reactions can occur with it, and the products of 2,3-BD have important applications in industry and daily life. In daily life, 2,3-BD is used in the manufacture of cosmetics, medicines, and detergents; in industry, it is used in the synthesis of liquid fuels and polymers [3–6]. Currently, there are two main methods for producing 2,3-BD: chemical synthesis and biotransformation. Compared with chemical methods, biological methods have low cost and low technical difficulty and significantly reduce pollution to the environment. This method meets the requirements of the low-carbon and environmentally friendly green chemical industry and is extremely sustainable [7,8]. Many bacterial species can be used to produce 2,3-BD, and the most efficient strains reported to date are *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Saccharomyces*, and *Escherichia aerogenes* [9–13].

K. pneumoniae has tremendous potential in industrial applications as a microbial cell factory. Due to its wide range of recipes, it can directly use various carbon sources, such

as glucose, arabinose, xylose, and fructose, to produce 2,3-BD and has the characteristics of thorough fermentation, fewer byproducts, and relatively high product concentrations and conversion rates [14–16]. An excessive glucose concentration will exert stress on the fermentation strain, and the high sugar-phosphate content inside the cell can inhibit the metabolic growth of the cell and even damage the DNA [17–19].

Therefore, we raised the following questions: based on the application of traditional metabolic engineering methods, does the yield of 2,3-BD not achieve the expected effect due to the limitation of substrate concentration? Could it be that increasing the tolerance of the strain to the substrate (glucose) could increase the metabolism to produce more 2,3-BD? After reviewing the literature, Negrete et al. found only one report demonstrating reduced acetic acid production of the *Escherichia coli* K-12 strain by overexpressing the *sgrS* gene in 2013 [20]. After 8 h of fermentation with 40 g/L glucose as a substrate, the acetic acid yield of the *sgrS*-overexpressing strain was close to 0 g/L, which was significantly lower than that of the original strain of 10 g/L [20]. Northern blot analysis showed that *ptsG* mRNA encoding $EIICB^{Glc}$ was significantly reduced in the *sgrS*-overexpressing strain but not in the original strain [20]. The results showed that sRNA-SgrS may affect the production of fermentation products by changing the uptake of sugars by fermentation strains and the concentration of sugar phosphate in the bacteria during the fermentation process with glucose as a carbon source. Therefore, sRNA-SgrS, as a sugar transport regulator in bacteria, became important.

Small RNA (sRNA) is an RNA molecule produced by genome transcription, but most sRNAs do not encode proteins [21]. In contrast to general sRNA, SgrS is a dual-function sRNA that regulates several mRNA targets through direct base pairing and encodes a short peptide, SgrT [22,23]. SgrS and SgrT solve the problem of sugar transport under sugar stress at two levels. At the protein level, the *sgrS* in most *Enterobacteriaceae* can translate a short peptide, SgrT, which can specifically bind to the $EIIC^{Glc}$ part of the carrier PtsG cell membrane [24]. It can shut down the transport function of glucose on transporter PtsG so that the bacteria can better survive in an environment where multiple carbon sources coexist [19]. At the transcriptional level, SgrS performs posttranscriptional regulation of multiple target mRNAs, thereby changing the number of intracellular-related target mRNAs. There are three regulation modes of sRNA-SgrS [25] (Figure 1). First, the *sgrS* gene participates in the stress process of glucose metabolism by inhibiting the translation initiation of the *ptsG* mRNA, reducing the $EIICB^{Glc}$ encoding the glucose transporter on the cell membrane, and inhibiting the entry of glucose [17,26]. Second, the *manXYZ* mRNA (encoding the mannose transporter ManXYZ, EII^{Man}) interacts with sRNA-SgrS, thereby inhibiting the translation of *manXYZ* mRNA. When sRNA-SgrS binds to two parts of the *manXYZ* mRNA at the same time, RNase E accelerates the degradation of the target mRNA [27,28]. In contrast to the above two genes, the *yigL* gene can encode a phosphatase located in the cytoplasm, which can dephosphorylate the phosphorylated glucose in the cell, and then transport the dephosphorylated glucose out of the cell through free diffusion and corresponding transport vectors, thus improving the tolerance of bacteria to sugar stress [29,30].

Currently, the research on sRNA-SgrS mostly focuses on the regulatory mechanism of sugar metabolism (*ptsG*, *manXYZ*, *yigL* mRNA) and other related metabolisms (*asd*, *adiY*, *folE*, and *purR* mRNA) [31]. Little attention has been given to the effect of this sRNA on metabolite production. This experiment explores how sRNA-SgrS, as a sugar transport coordinator, activates related target genes. This affects *K. pneumoniae* HD79 and *K. pneumoniae* HD79-02 (Δ*ldh*, Δ*ack*) to utilize glucose metabolism to produce 2,3-BD. It provides a theoretical basis and a technical reference for understanding the complex regulation mechanism of sRNA in microorganisms and the genetics and breeding in industrial fermentation engineering.

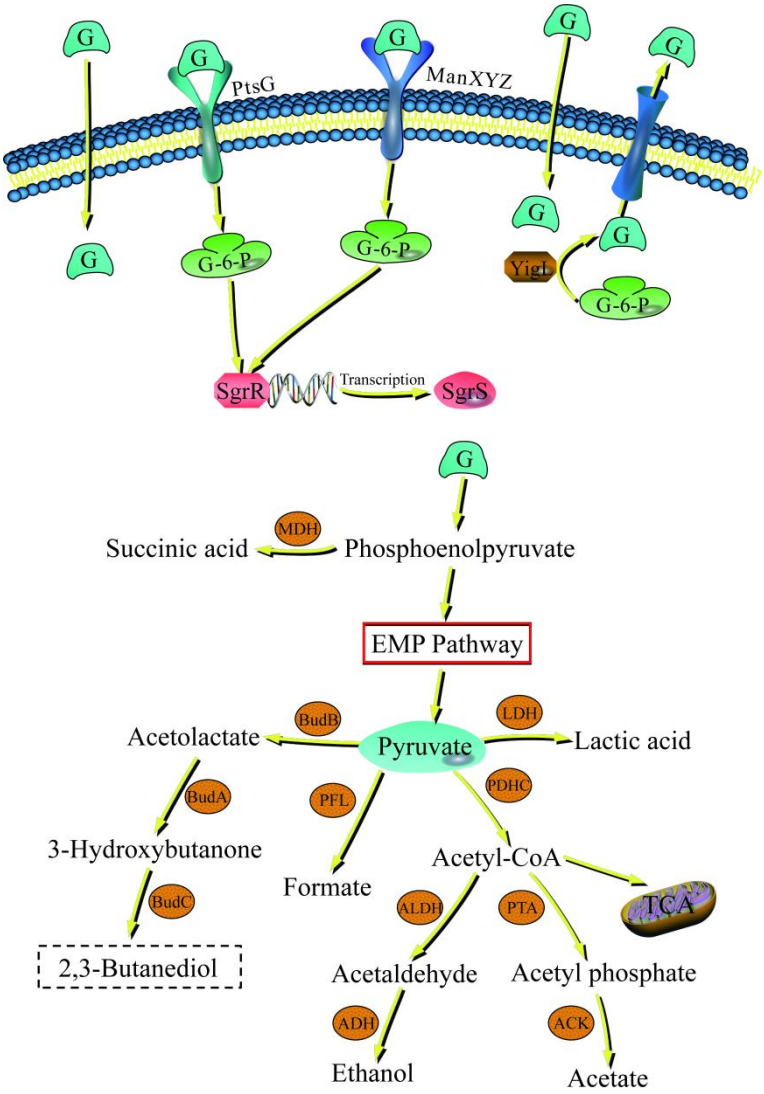

Figure 1. Carbohydrates in vitro enter cells and become G-6-P through transporters encoded by *ptsG* and *manXYZ*. G-6-P stimulates the transcription of SgrR into SgrS. sRNA-SgrS positively regulates *yigL* mRNA and negatively regulates *ptsG* mRNA and *manXYZ* mRNA. Intracellular glucose is used for bacterial fermentation to produce 2,3-BD.

2. Materials and Methods

2.1. Biochemical Reagents

5-Bromo-4-chloro-3-indole-β-D-galactoside (X-gal) and ampicillin (Amp) were purchased from Dalian Bao BioEngineering Co., Ltd. (Dalian, China). Ethylenediaminetetraacetic acid (EDTA) and agarose were purchased from Shanghai Bioengineering Co., Ltd. (Shanghai, China). Imported $CaCl_2$ was purchased from Amresco Company (Shanghai, China).

2.2. Strains, Media, and Growth Conditions

All strains used or created in this work, along with their sources and characteristics are shown in Table 1. *E. coli* DH5α was purchased from Takara Biotechnology Co., Ltd. (Dalian, China), which is used for gene cloning. For enrichment, *K. pneumoniae* HD79 and *K. pneumoniae* HD79-02, stored in −80 °C refrigerator, were inoculated in Luria-Bertani (LB) liquid medium (1% tryptone, 0.5% yeast extract, 1% NaCl; *w*/*v*, pH 7.0). and oscillated at 30 °C for 24 h at 150 r/min. It also was used for the growth of *E. coli* DH5α. Seed medium (SM, pH 7.0): 1 g/L $(NH4)_2SO_4$, 3.4 g/L $K_2HPO_4{\cdot}3H_2O$, 1.3 g/L KH_2PO_4, 0.2 g/L $MgSO_4$, 1 g/L yeast extract, 2 mL/L trace elements, 1 mL/L iron solution. The seed medium

was added to the sterilized glucose so that the glucose concentration in the medium was 30 g/L. It was used for the cultivation of cryopreserved 2,3-BD fermenting strains. Liquid fermentation medium (LFM, pH 7.0): 6.6 g/L $(NH_4)_2SO_4$, 8.7 g/L $K_2HPO_4 \cdot 3H_2O$, 6.8 g/L KH_2PO_4, 0.25 g/L $MgSO_4 \cdot 7H_2O$, 5 g/L yeast extract, 0.05 g/L $FeSO_4 \cdot 7H_2O$, 0.001 g/L $ZnSO_4 \cdot 7H_2O$, 0.001 g/L $MnSO_4 \cdot H_2O$, 0.001 g/L $CaCl_2 \cdot 2H_2O$. The LFM medium was added to the sterilized glucose so that the glucose concentration in the medium was 200 g/L. It was used for the metabolism test of 2,3-BD fermentation strains. Solid fermentation medium (SFM): 2× solid fermentation medium (13.2 g/L $(NH_4)_2SO_4$, 17.4 g/L $K_2HPO_4 \cdot 3H_2O$, 13.6 g/L KH_2PO_4, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 10 g/L yeast extract, 0.1 g/L $FeSO_4 \cdot 7H_2O$, 0.002 g/L $ZnSO_4 \cdot 7H_2O$, 0.002 g/L $MnSO_4 \cdot H_2O$, 0.002 g/L $CaCl_2 \cdot 2H_2O$, 4% agar powder; pH 7.0; autoclave at 121 °C for 15 min) and 2× saccharide aqueous solution (various sugars were doubled according to the required concentration, dissolved in sterile water, and sterilized by autoclaving at 108 °C for 20 min) were mixed in a ratio of 1:1, which was used to compare the tolerance of each strain to various carbohydrates.

Table 1. Strains used in this study.

Strain	Characteristics	Source/Reference
E. coli DH5α	*Amp*^{r}	Laboratory preservation
E. coli JM110	*Str*^{r}, *dam dcm* supE44Δ(*lac-proAB*)	Jiangsu Yugong Life
K. pneumoniae HD79	*Amp*^{r}, Original strain	Laboratory preservation
K. pneumoniae HD79-O	*Amp*^{r}, *Kan*^{r}, P_{T7lacO}- *sgrS*:: *RFP*	Mutant from gene disruption; this study
K. pneumoniae HD79-K	*Amp*^{r}, Δ*sgrS*:: *EGFP*	Mutant from gene disruption; this study
K. pneumoniae HD79-02	*Amp*^{r}, *Cm*^{r}, *Kan*^{r}, Δ*ldh* and Δ*ack*	Laboratory preservation
K. pneumoniae HD79-02-O	*Amp*^{r}, *Cm*^{r}, *Kan*^{r}, Δ*ldh* and Δ*ack* and P_{T7lacO}-*sgrS*:: *RFP*	Mutant from gene disruption; this study
K. pneumoniae HD79-02-K	*Amp*^{r}, *Cm*^{r}, *Kan*^{r}, Δ*ldh* and Δ*ack* and Δ*sgrS*:: *EGFP*	Mutant from gene disruption; this study

2.3. Obtainment of sgrS Gene Knockout and Overexpression Strains

Bacterial genomic DNA (Mini) extraction kit (DP302-02) was used to extract the genomes of *K. pneumoniae* HD79 and *K. pneumoniae* HD79-02, cultured to the exponential stage. With *K. pneumoniae* HD79 and *K. pneumoniae* HD79-02 genomes as templates, the *sgrS* gene sequences (EMBL database, ID: URS0006F6AF4_573) were amplified by polymerase chain reaction (PCR) by using the following primers: *sgrS*-up (5′-CGGGGTACCGATGAAG CGAGGAGGTGAATA, *Kpn*I), *sgrS*-down (5′-CCGCTCGAGGAAAAAAGCCAGCAGAGGC, *Xho*I). To clone *EGFP*, pEGEP-C3 (Laboratory preservation) was used as a template with the following primers: *EGFP*-up (5′-CGCTGATCAGTAACAACTCCGCCCCATTG, *Bcl*I) and *EGFP*-down (5′-CGCTGATCAGGGAGGTGTGGGAGGTTTT, *Bcl*I). The *sgrS* and *EGFP* genes were cloned into pMD18-T (D101A, TaKaRa, Dalian, China) to construct pT-*sgrS* and pT-*EGEP*, respectively. pT-*sgrS* and pT-*EGEP* were digested with *Bcl*I, and the resulting *EGFP* gene fragments and linear pT-*sgrS* were linked to pXL-*sgrS*A1-*EGFP*-*sgrS*A2 by T4 DNA ligase. The ligation products were transformed into *E. coli* DH5α by heat shock. Then, the obtained positive clones were double digested by *Kpn*I and *Xho*I to obtain homologous recombination fragments, *sgrS*A1-*EGFP*-*sgrS*A2 (Figure 2a). The *sgrS*A1-*EGFP*-*sgrS*A2 was electroporated into *K. pneumoniae* HD79 and *K. pneumoniae* HD79-02 competent cells containing pKD46 (MLCC013, Wuhan Miaolingbio Bioscience & Technology Co., Ltd., Wuhan, China) and induced by L-arabinose to knock out the *sgrS* gene in the host bacteria.

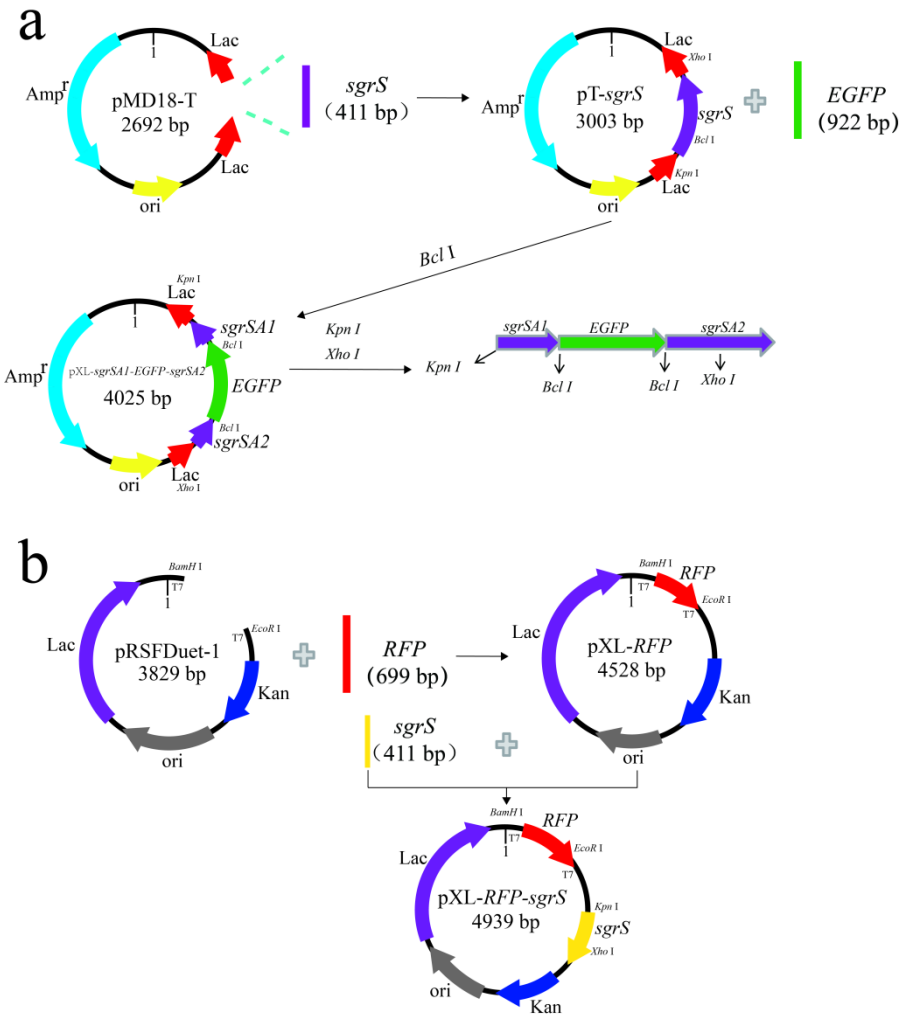

Figure 2. Diagram of the process of constructing a recombinant vector. (**a**) shows the whole strategy of homologus recombination fragment *sgrSA1-EGFP-sgrSA2*. (**b**) shows the construction process of recombinant overexpression vector pXL-*RFP-sgrS* containing target gene *sgrS*.

To clone *RFP*, pmCherry-3.1-*RFP* (provided by Harbin Medical University, Harbin, China) was used as a template with the following primers: *RFP*-up (5′- CGCGGATCCGGTCGCCACC ATGGTGAGC, *BamH*I) and *RFP*-down (5′-CCGGAATTCGGTGGAGTGGCGGCCCTC, *EcoR*I). The *RFP* was cloned into pRSFDuet-1 (Laboratory preservation), and the recombinant plasmid was called pT-*RFP*. The pT-*RFP* strain and pRSFDuet-1 were double digested with *BamH*I and *EcoR*I, and the vector after connecting them with T4 DNA ligase was pXL-*RFP*. The plasmids pT-*sgrS* and pXL-*RFP* were double digested with *Kpn*I and *Xho*I to obtain the *Kpn*I-*Xho*I-*sgrS* fragment and linear *Kpn*I-*Xho*I-pXL-*RFP*, which are related to T4 DNA ligase, and the recombinant plasmid was named pXL-*RFP-sgrS* (Figure 2b). The pXL-RFP-*sgrS* was electroporated *K. pneumoniae* HD79 and *K. pneumoniae* HD79-02 competent cells.

2.4. Sugar Tolerance Test

K. pneumoniae HD79, HD79-O, HD79-K, HD79-02, HD79-02-O, and HD79-02-K grown in LB liquid medium for 6–8 h to logarithmic growth phase (OD_{600nm} = 1.30 ± 0.5) were spread in SFM medium containing trehalose (150, 175, 200, 225, 250 g/L), glucose (150, 175, 200, 225, 250 g/L), galactose (150, 175, 200, 225, 250, 275 g/L), mannose (150, 175, 200, 225, 250, 275 g/L), arabinose (50, 62.5, 75, 100, 125, 150 g/L), xylose (50, 62.5, 75, 100, 125, 150 g/L) and fructose (50, 75, 100, 125, 150 g/L), respectively. The spread plate was incubated at 30 °C for 36 h. According to the growth of each strain in different kinds of

sugars at different concentrations, the critical concentration of different sugars was finally determined; that is, the sugar concentration of different strains under different growth conditions could be clearly distinguished.

2.5. Fermentation Kinetics

The bacterial liquid of 6 strains growing on SM medium was inoculated into FM medium with 5% inoculation amount. Batch fermentation was performed in 500 mL flasks containing 150 mL fermentation medium with glucose (200 g/L) as the substrate. Flasks were cultured at 30 °C with shaking at 150 rpm for 120 h. Samples were taken every 12 h until the end of fermentation, and transcriptome analysis was performed every 12 h from 24 h to 72 h. Each time, every 3 mL sample was taken to determine its pH value. At the same time, 1 mL sample was taken and diluted tenfold, and the absorbance value at OD_{600nm} was measured with the FM medium as a blank control. After the samples were processed, the concentrations of glucose and 2,3-BD were determined by high-performance liquid chromatography (HPLC). The HPLC analysis system (Shimadzu, Shanghai, China) of Shimadzu LC20A includes an LC-10ATvp pump, a RID-10A differential detector, an HPX-87H column, an injection volume of 20 μL, a mobile phase of 0.005 mol/L sulfuric acid, a flow rate of 0.8 mL/min, a differential detector temperature of 40 °C, a column temperature of 65 °C, and a fermentation product composition analysis time of 20 min.

2.6. Quantitative Real-Time PCR (qRT-PCR) to Measure Gene Expression

qRT-PCR was used to detect the expression of *sgrS*, *ptsG*, *manXYZ*, *yigL*, *ack*, *ldh*, *budA*, *budB,* and *budC* in *K. pneumoniae* HD79 and *K. pneumoniae* HD79-02 (Δ*ldh*, Δ*ack*) at 24 h, 48 h, and 72 h. The above reaction used SYBR Green on a 7500 Real-Time PCR System (Applied Biosystems, Inc., Waltham, MA, USA). The reaction primers were 16S-up (5′-GATGACCAGCCACACTGGAA) and 16S-down (5′-GGAGTTAGCCGGTGCTTCTT) and *ack*-up (5′-CGATACGGCGTTCCATCAGA) and *ack*-down (5′-GAAACAGAACCACCGT TGCC) and *ldh*-up (5′-TCATACCGATCGCATGCTCA) and *ldh*-down (5′-GACAGCGAAGAC TGCCAAAA) and *budA*-up (5′-TGATCGACCAGCAAATCCCC) and *budA*-down (5′-CGACG TTGATCCCCTGCATA) and *budB*-up (5′-AACAGTATCCGGTACGCCAG) and *budB*-down (5′-ATAAATGCGGCGTTGGCTTC) and *budC*-up (5′-ATGGCGGTGAAAGTGGATGT) and *budC*-down (5′-CGCCGTGACCCTCTTTCTTA) and *ptsG*-up (5′-AACTTTCGCTACATCCG CCA) and *ptsG*-down (5′-GCGTGCTGATTAAAGCCCTG) and *manXYZ*-up (5′-GCGTATCGA CCTTTCCCGAT) and *manXYZ*-down (5′-CACCACCAGCTGACTGCTTA) and *yigL*-up (5′-ACCTGCGATAAGCATGAGCA) and *yigL*-down (5′-TTCCGCATCGTTCATACCGT) and *sgrS*-up (5′-AACGCTACTTTTCTGCGACG) and *sgrS*-down (5′-AATCACGCAGCCCGAA CATA). The qRT-PCR results were analyzed through the DDCt method using the 16s rRNA gene as the reference gene. Results are presented as relative quantities.

2.7. Statistical Analysis

The results are shown as the average and standard deviation of three independent samples. OriginPro 2021b software was also used for statistical analysis and chart analysis. Structural equation modeling (SEM) was conducted by IBM SPSS AMOS (version 23.0, New York, NY, USA) All Pearson correlation analyses were performed using SPSS 25.0. Glucose consumption and 2,3-BD production between 0–24 h, 24–48 h, and 48–72 h of strain fermentation were analyzed and visualized by using the Lingbo Microclass online platform. (http://www.cloud.biomicroclass.com/CloudPlatform/SoftPage/GGCOR, accessed on 15 June 2022).

3. Results and Discussion

3.1. Glucose Tolerance of Each Strain

At a low concentration of glucose, the transcription level of the *sgrS* gene was lower, while that of the *ptsG* gene was higher [32]. For example, the experiments of these researchers indicated that no sRNA-SgrS was observed in *E. coli* K-12, DH5α, and IT1508 in a

10 g/L glucose environment [33–35]. This part was to confirm whether sRNA-SgrS can act as a sugar transport factor to help cells adjust sugar tolerance in the face of different high concentrations of sugar stress. The critical concentration of the *sgrS* gene deletion strains for different carbohydrates was 62.5 g/L arabinose and xylose, 125 g/L fructose, 200 g/L trehalose, 225 g/L mannose, and galactose; those for the *sgrS* gene overexpressed strains were 75 g/L arabinose and xylose, 150 g/L fructose, and 250 g/L mannose, and galactose and trehalose (Figure S1). After knocking out the *ack*, *ldh*, and *sgrS* genes, the tolerance of the strains to glucose decreased by 30% (Figure 3). The tolerance of the *sgrS*-overexpressing strain to sugar was higher than that of the wildtype strain and gene knockout strain. It can be concluded that sRNA-SgrS can regulate the entry into and exit from cells of various carbohydrates and enhance the tolerance of bacteria to carbohydrates. Negrete et al. showed that *E. coli* B (BL21) tolerates high glucose concentrations not only through its more efficient central carbon metabolism but also by controlling sRNA-SgrS-regulated glucose transport into cells [32]. Wadler and Vanderpool also that the expression of *sgrS* allows *E. coli* to recover from glucose-phosphate stress upon the intracellular accumulation of nonmetabolized sugars [36]. Thus, the conclusions of all studies are consistent.

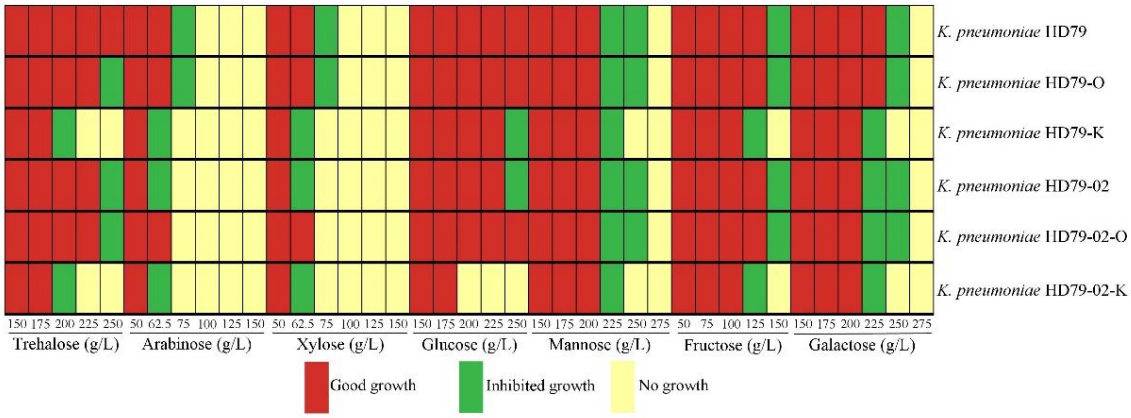

Figure 3. The growth of each strain on SFM plates containing different sugars and different concentrations. From left to right are trehalose, arabinose, xylose, glucose, mannose, fructose, and galactose. Red rectangles indicate good growth, green rectangles indicate inhibited growth, and yellow rectangles indicate no growth.

3.2. The Fermentation Results

The fermentation kinetics of *K. pneumoniae* HD79 series strains and *K. pneumoniae* HD79-02 series strains are shown in Figure 4a,b. The cell density of the original and knockout strains increased only slightly during the experiment. The culture density of the *sgrS*-overexpressed strain did not increase during the entire study period (Figure 4b), which may be one of the reasons why its 2,3-BD yield was lower than the original strain and the knockout strain. At a glucose concentration of 200 g/L, the glucose consumption rates of the control, *sgrS* knockout, and overexpression strains were similar regardless of the presence of the *ldh* and *ack* genes (Figure 4c). However, the overexpressed strain used the least amount of glucose. The theoretical yield of BD is 0.5 g/g glucose consumed. The reported yield in this study is around 0.2 to 0.25 g/g glucose at the highest level. This suggests that a significant amount of glucose is diverted to other products. Even when the *K. pneumoniae* HD79 strain knocked out *ack* and *ldh*, a drop in pH to about 4.5 indicated the production of other acids. After 72 h, the production of 2,3-BD might be inhibited by the production of other acids. Therefore, the 2,3-BD production in this study did not increase after reaching the peak at 72 h (Figure 4d). After the knockout of the *sgrS* gene, the production of 2,3-BD did not change significantly; after the overexpression of the *sgrS* gene, the production of 2,3-BD decreased significantly. The transformation rate of the strain after knocking out the *sgrS* gene was no different from that of the original strain, and

the transformation rate of the strain after overexpressing the *sgrS* gene was much lower than that of the knock-out strain and the original strains. Therefore, the ability of the *sgrS* overexpression strain to consume glucose and produce 2,3-BD was significantly lower than that of other strains.

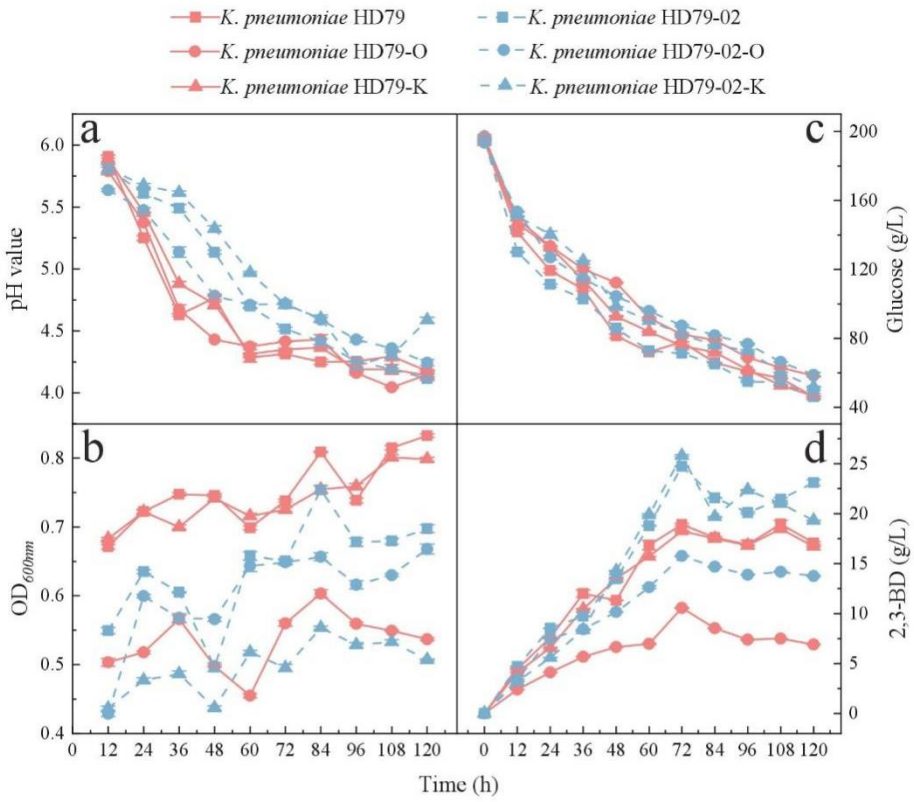

Figure 4. Fermentation of *K. pneumoniae* HD79 series strains and *K. pneumoniae* HD79-02 series strains. Variation curves of pH, OD_{600nm}, glucose residue, and 2,3-BD concentration are shown in (**a**–**d**), respectively. The pink box represents *K. pneumoniae* HD79 strain, the pink cycle represents *K. pneumoniae* HD79-O strain, the pink triangle represents *K. pneumoniae* HD79-K strain, the blue box represents *K. pneumoniae* HD79-02 strain, the blue cycle represents *K. pneumoniae* HD79-02-O strain, and the blue triangle represents *K. pneumoniae* HD79-02-K strain. The experiments were performed in triplicate, and the error bars represent the standard deviations.

At higher initial sugar concentrations, the cellular utilization of D-xylose, L-arabinose, D-galactose, D-mannose, and D-cellobiose decreased and reduced the production of butanediol [37]. In turn, G-6-P activates sRNA-SgrS as a sugar transport regulator to coordinate intracellular glucose content, thereby affecting 2,3-BD production. The expression of the *sgrS* gene was upregulated, the content of glucose that could be transported into cells was downregulated, and the production of 2,3-BD produced by bacterial metabolism was also downregulated.

In addition, after knocking out the *ack* and *ldh* genes, the production of 2,3-BD was upregulated, indicating that the *ack* and *ldh* genes can affect the production of 2,3-BD. In our previous study, *K pneumoniae* HD79 *ldhA* and *ack* knockout strain eliminated acid by-products and significantly increased the yield of 2,3-BD [38]. Guo et al. demonstrated that the double mutant strain with deletion of *adhE* and *ldhA* resulted in accelerated fermentation and higher 2,3-BD production [39]. Jung et al. showed that the *ldh* gene was deleted in *Enterobacter aerogenes* achieving a 2,3-BD concentration of 118 g/L by fed-batch fermentation when glucose was the carbon source [40]. Nguyen et al. showed that knockout of the *ldh*, *ack*, and *mdh* genes in *Mycobacterium chlorophila* 20Z resulted in a further increase in the 2,3-BD concentration from 35.66 mg/L to 68.8 mg/L [41]. The pH value of HD79-02 series strains decreased during fermentation compared with the initial pH value, indicating that

acid was produced during fermentation. Although this reduction was not as dramatic as in the HD79 series strain, it still indicated that acid was produced. It is very important to show the acetic acid and lactic acid profiles of the strain. After the gene encoding acetate kinase (Ack) and lactate dehydrogenase (Ldh) was knocked out, the metabolic pathway of glucose to produce acetate and lactate was blocked, which weakened the acid-producing ability of the strain. Glucose flowed to other pathways, increasing the production of 2,3-BD.

3.3. Analysis of Differentially Expressed Genes

This section further explores the regulatory mechanism of the *sgrS* gene on 2,3-BD biosynthesis at the gene transcription level. After knocking out the *sgrS* gene, the *sgrS* transcription level in the strain was significantly reduced; after the *sgrS* gene was overexpressed, the *sgrS* transcription level in the strain was significantly increased, and the difference was significant ($p < 0.01$). Compared with the original strain, the former was approximately 50% of the original strain, while the latter was approximately 2.5 times that of the original strain (Figure 5a–c). Under the condition of knocking out the *ack* and *ldh* genes, after knocking out the *sgrS* gene, the expression of the *sgrS* gene was downregulated by 58.5%, and after the overexpression of the *sgrS* gene, the expression of *sgrS* was upregulated by 163%. The expression level of *sgrS* in the strains that only knocked out the *ack* and *ldh* genes was much lower than that of the strains that only overexpressed or knocked out the *sgrS* gene ($p < 0.01$). Therefore, combined with the 2,3-BD production results of each strain, this result can better explain why sRNA-SgrS can regulate the entry and exit of sugars into and out of cells. The transport of internal sugars to the outside world reduces the intracellular glucose content and the product yield. Negrete et al. also conducted similar experiments and demonstrated that overexpression of *sgrS* enabled *E. coli* B (BL21) to reduce its acetate excretion by controlling the glucose transport [20].

After the knockout of *sgrS*, the expression of *ptsG* was significantly increased by 58%; after the overexpression of *sgrS*, the expression of *ptsG* was significantly decreased by 57%. The results demonstrated that the main regulatory target of sRNA-SgrS is the *ptsG* gene responsible for the intracellular transport of glucose. By negatively regulating it post-transcriptionally, the transporter encoded by the intracellular the *ptsG* gene is reduced, thereby reducing the uptake of glucose. Vanderpool and Gottesman showed that the overexpression of the *sgrS* gene directly promoted the degradation of *ptsG* mRNA during phosphate stress when glucose was the only carbon source [27]. Kimata et al. also found that when *E. coli* K-12 strains were grown in the presence of the nonmetabolized sugar α-methyl glucoside, *sgrS* transcript levels were increased, while *ptsG* mRNA was decreased [42]. Richards et al. demonstrated that SgrS helps to restore growth in part by inhibiting translation of the *ptsG* mRNA, which encodes the major glucose transporter $EIICB^{Glc}$ [43].

In *K. pneumoniae* HD79, *K. pneumoniae* HD79-O, and *K. pneumoniae* HD79-K, knocking out the *sgrS* gene downregulated the expression of the *manXYZ* and *ack* genes, while the overexpression of *the sgrS* gene upregulated the expression of the *manXYZ* and *ack* genes. This result did not occur for the *K. pneumoniae* HD79-02 series strains. In *K. pneumoniae* HD79-02, *K. pneumoniae* HD79-02-O, and *K. pneumoniae* HD79-02-K, after the knockout of the *sgrS* gene, the expression of the *yigL* gene was downregulated, and after the overexpression of the *sgrS* gene, the expression of the *yigL* gene was upregulated. This phenomenon did not appear in the *K. pneumoniae* HD79 series strains. At 48 h, the *ldh* gene expression was upregulated after the *sgrS* knockout and downregulated after the *sgrS* overexpression. Rice and Vanderpool noted that the regulation of *manX* by SgrS is not as conserved as that of *ptsG* and that in some gut bacterial species, the major source of stress-causing sugar phosphates is PtsG, not ManXYZ [44]. sRNA-SgrS regulates different target genes under different substrate conditions [29]. *ptsG* mRNA and *yigL* mRNA are predominantly regulated by the exposure of cells to low concentrations of carbohydrates, and *manXYZ* mRNA and *ptsG* mRNA are predominantly regulated by the exposure of cells to 2-deoxyglucose stress [29].

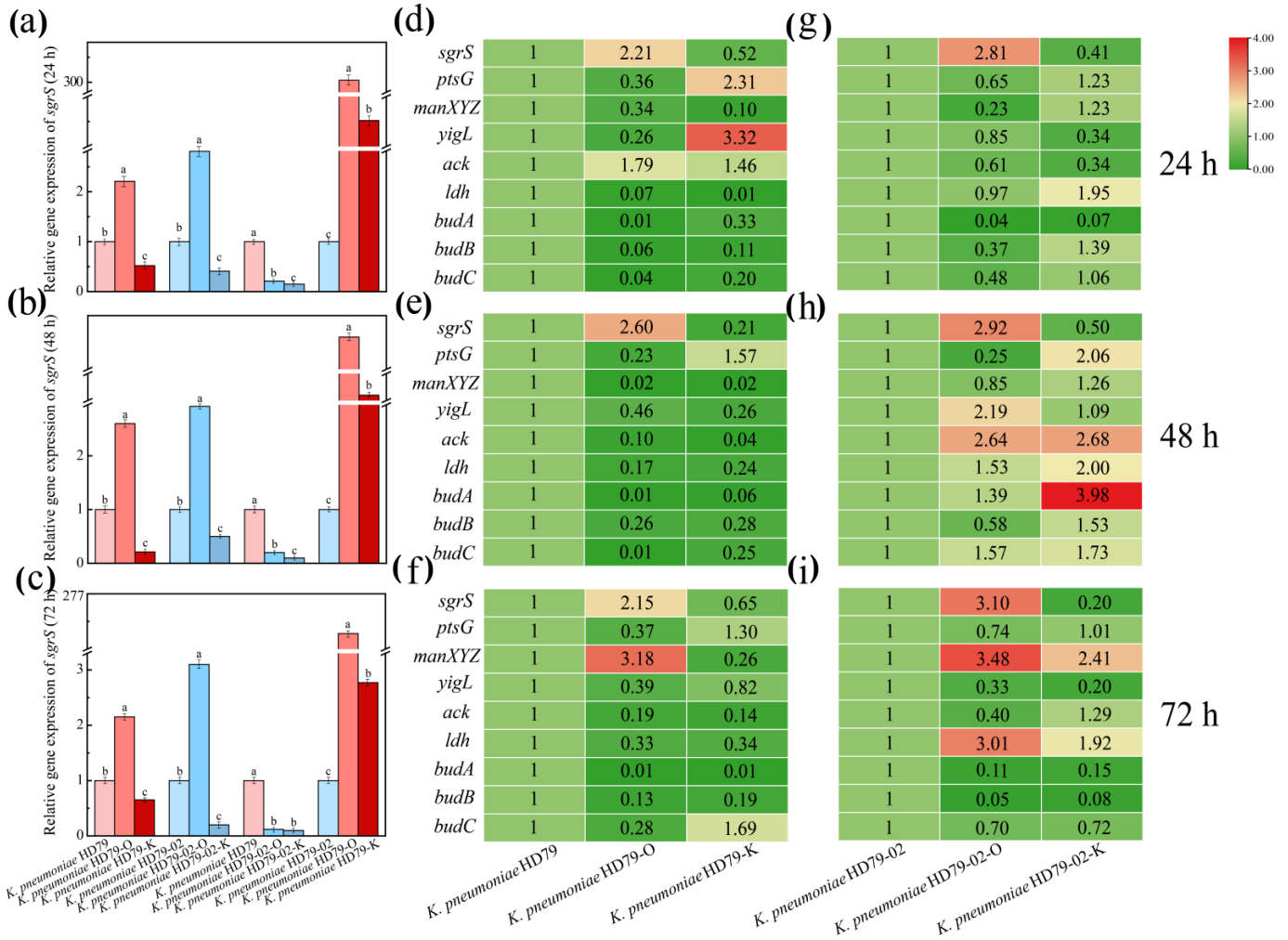

Figure 5. Changes of related genes in *K. pneumoniae* HD79 series strains and *K. pneumoniae* HD79-02 series strains at 24 h, 48 h, and 72 h of fermentation. (**a**–**c**) Relative expression levels of *sgrS* gene in different strains in three time periods; a, b and c on the bar chart are the results obtained by SPSS significance analysis. (**d**–**f**) Relative expression levels of each gene in HD79 series strains in three time periods; (**g**–**i**) Relative expression levels of each gene in HD79-02 series strains in three time periods.

As shown in Figure 5d–i, the *sgrS* gene indirectly affects the transcription levels of *budABC* genes. To explore the relationship between genes and glucose concentration and 2,3-BD concentration, we analyzed the correlation between gene expression, glucose metabolism, and 2,3-BD production. Different genes have various responsibilities and jointly participate in and regulate the production of 2,3-BD (Figure 6). It can be seen from Figure 6 that in this experiment, *manXYZ* was positively correlated with *ldh* ($p < 0.01$), *yigL* was positively correlated with *ack* ($p < 0.05$), *ack* was positively correlated with *budA* ($p < 0.01$), and *ldh* was positively correlated with *budC* ($p < 0.05$). Moons et al. showed that the deletion of the *budR* reduced the transcriptional levels of *ack* [45]. Wei et al. demonstrated that *K. pneumoniae* with the *budA* knockout produced almost no 2,3-BD and lower levels of organic acids [46].

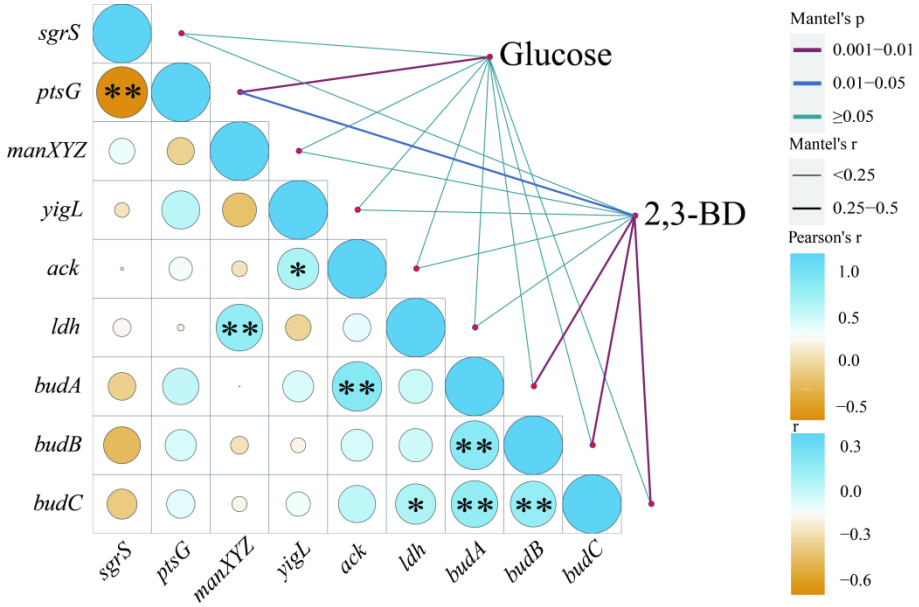

Figure 6. The lower left part of the figure is the heat map of correlation analysis between the relative expression levels of each gene at 24, 48, and 72 h. In this part, blue represents positive correlation and yellow represents negative correlation, * indicates $p < 0.05$; ** indicates $p < 0.01$. The upper right corner of the graph shows the correlation between each gene and glucose consumption and the yield of 2,3-BD at 24, 48, and 72 h. In this part, red represents $p < 0.01$ and blue represents $p < 0.05$, green represents no correlation.

The expression of the *ptsG* gene was significantly correlated with the glucose concentration ($p < 0.01$), and the *ptsG* gene was significantly correlated with the 2,3-BD concentration ($p < 0.05$). Therefore, *ptsG* can regulate glucose consumption and 2,3-BD production after transcription. In addition, the *ptsG* gene and the *sgrS* gene were negatively correlated ($p < 0.01$), indicating that the *sgrS* gene could regulate glucose concentration and 2,3-BD production by negatively regulating *ptsG* mRNA. This is consistent with the results above and with the results of Sun and Vanderpool. Under high stress, *ptsG* is mainly regulated, but not *yigL* and *manXYZ* [29]. The *budABC* genes were significantly correlated with the 2,3-BD concentration ($p < 0.01$); the *sgrS* gene repressed the transcription level of *budABC* genes (Figure 5d–i). Therefore, the *sgrS* gene affects the production of 2,3-BD by changing the expression level of the *budABC* genes.

In general, sRNA-SgrS is required for the cellular stress response to nonmetabolizable phosphoglucose molecules. In the presence of sugar stress, overexpression of the *sgrS* gene reduced the uptake of sugar by the bacteria, resulting in a decrease in the transcription level of the *ptsG* gene. This reduces the intracellular sugar stress, which weakens the ability of the strain to consume and utilize the substrate glucose (decreased transcript level of *budABC* genes), which ultimately leads to the reduction of 2,3-BD production by overexpressing the *sgrS* gene. Ultimately, the overexpression of the *sgrS* gene reduces the production of 2,3-BD. However, there was no significant effect on the transcription levels of byproducts in the metabolic process, such as the acetate kinase gene (*ack*) and the lactate dehydrogenase gene (*ldh*).

3.4. Effects of sRNA-SgrS on Carbon Metabolic Flux

As shown in Figure 7a, among all strains, *K. pneumoniae* HD79-O had the smallest glucose consumption, the largest sRNA-SgrS content, the smallest relative expression levels of the *ptsG* gene and *budABC* genes, and the lowest 2,3-BD production. The same was true for the overexpressing strains in the *K. pneumoniae* HD79-02 series (Figure 7b). Consistent with Vanderpool and Gottesman, we conclude that under the conditions of glucose phosphate accumulation, the synthesis of SgrS leads to the degradation of *ptsG* mRNA,

resulting in reduced production of the glucose transport machinery, thereby limiting the further accumulation of glucose phosphate [27]. Accordingly, the production of 2,3-BD will be downregulated, and the expression levels of *budABC* genes will be downregulated. Not only our results, Chou et al. aimed to improve cellular glucose uptake and reduce acetate excretion [47]. They minimized acetate excretion by knocking out the *ptsG* gene to reduce glucose uptake, thereby reducing the metabolic flux allocated to glycolysis [47].

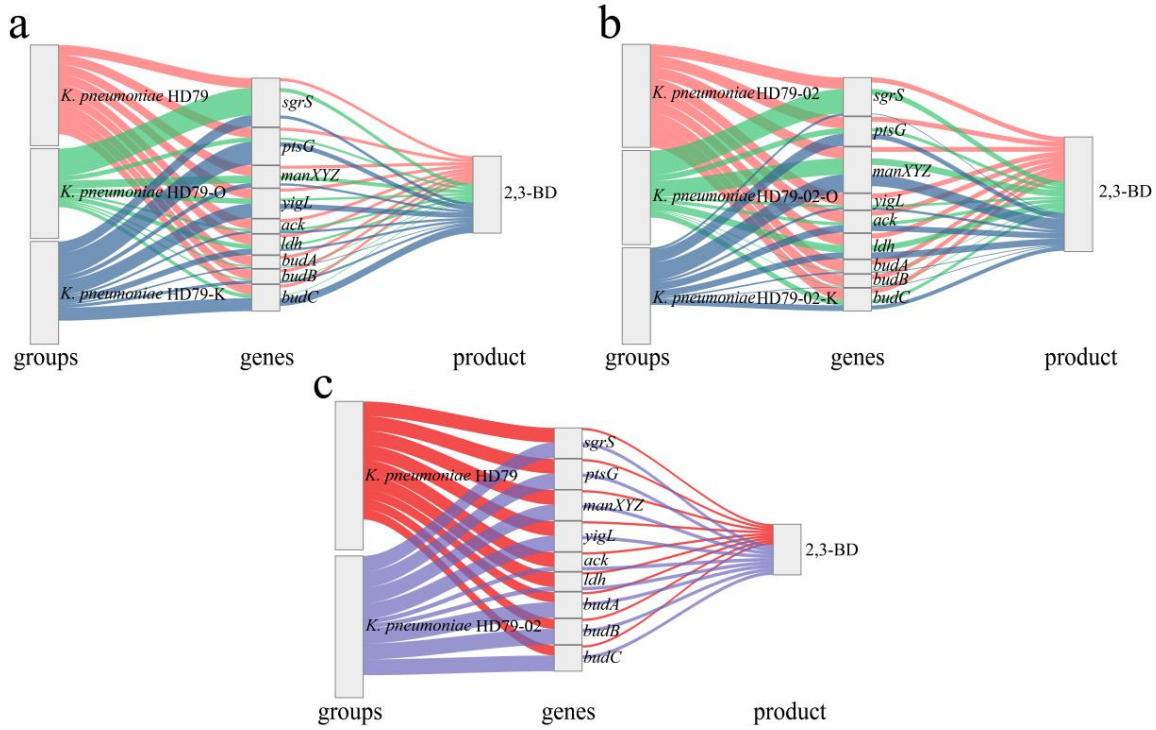

Figure 7. The distribution of carbon metabolism flow of each strain consuming glucose to produce 2,3-BD at 72 h of fermentation. A small cell in a Sankey diagram represents the amount of traffic allocated. The first column on the left describes the carbon flow allocated to each strain to represent glucose utilization, the second column describes the expression level of different genes in each strain, and the third column describes the carbon flow allocated to different strains to redistribute to the pathway producing 2,3-BD. (**a**) shows the *K. pneumoniae* HD79 series strains; (**b**) shows the *K. pneumoniae* HD79-02 series strains; (**c**) shows the *K. pneumoniae* HD79 strain and *K. pneumoniae* HD79-02 strain.

The biosynthetic pathways of 2,3-BD, lactic acid, and acetate are the major consumption pathways of NADH in *K. pneumoniae*. Therefore, if the biosynthetic pathway of 2,3-BD is blocked, the distribution of carbon is naturally redirected to other pathways. Kim et al. increased carbon flux in 2,3-BD by knocking out *ldhA* and overexpressing *budA* and *budB* [48]. The *K. pneumoniae* HD79 and *K. pneumoniae* HD79-02 strains consumed similar concentrations of glucose, but the *K. pneumoniae* HD79-02 strain knocked out *ack* and *ldh*, resulting in a much larger expression of 2,3-BD-producing key enzyme genes (*budABC*) than the *K. pneumoniae* HD79 strain (Figure 7c). Therefore, the 2,3-BD production of *K. pneumoniae* HD79 was less than that of *K. pneumoniae* HD79-02. The knockout of *ack* and *ldh* significantly blocked the pathways of intracellular acetate and lactate production and altered the intracellular state. Compared with the original strain, the carbon flux from glucose to 2,3-BD increased by 31% in strains with *ack* and *ldh* genes knocked out and by 36% in strains lacking *ack*, *ldh*, and *sgrS* genes. Therefore, under the knockout of *ack* and *ldh* genes, the control of SgrS is of great significance in regard to increasing the production of 2,3-BD.

3.5. Relationship of sgrS, Sugar Transport Genes (ptsG, manXYZ), and budABC with Glucose Metabolism and 2,3-BD Production

The relative expression of the *sgrS* gene was significantly negatively correlated with the relative expression of sugar transport genes, glucose metabolism, and 2,3-BD production capacity but had no significant relationship with the relative expression of *budABC* genes (Figure 8a). This result suggests that the *sgrS* gene mainly negatively regulates sugar transport genes to affect the amount of glucose uptake by cells. In addition, 2,3-BD production and sugar transport genes were significantly positively correlated with metabolic glucose and the expression levels of *budABC* genes. Therefore, sugar transport genes positively regulate glucose metabolism, and *budABC* genes positively regulate 2,3-BD production. It is currently known that in bacteria, SgrS negatively regulates two target mRNAs, *ptsG* and *manXYZ* encoding $EIICB^{Glc}$ and EII^{Man} carriers, respectively. In fact, *sgrS* regulates the cellular utilization of various carbon sources, such as glucose, mannose, and fructose, by activating the expression of these two genes. The elimination of *ptsG* transcripts would halt the new synthesis of $EIICB^{Glc}$, further reducing the transport and phosphorylation of glucose [19]. Therefore, although *sgrS* cannot directly regulate the genes involved in the synthesis of 2,3-BD, it can indirectly affect 2,3-BD production.

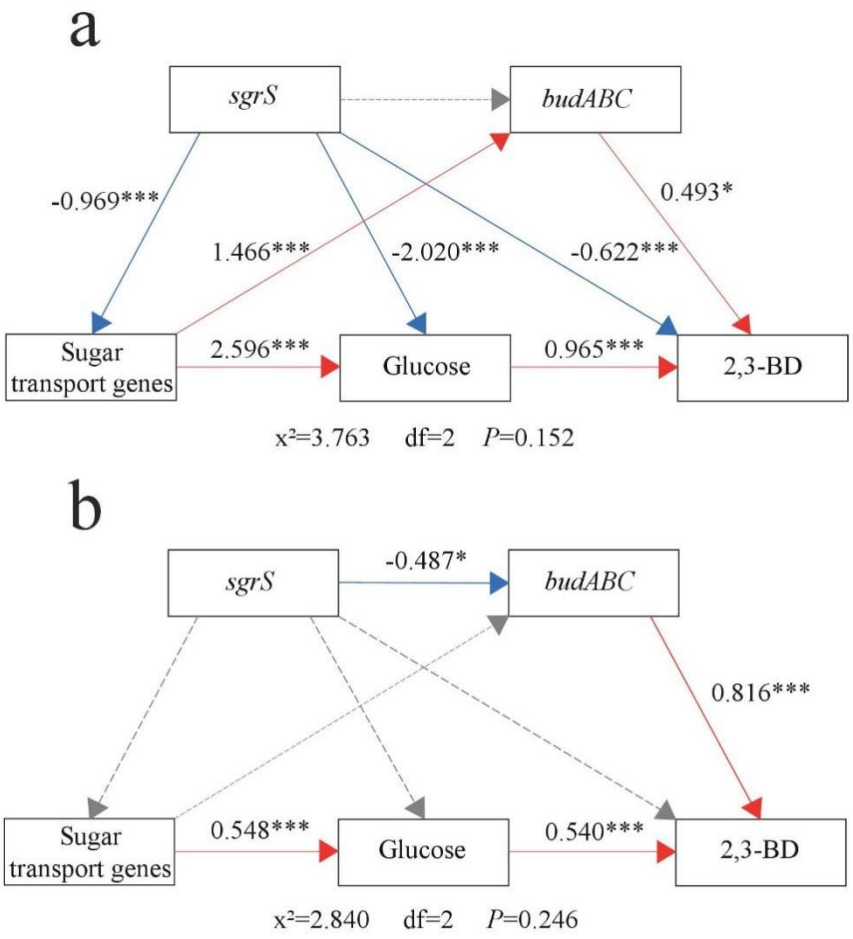

Figure 8. Structural equation models (SEM) showing the direct and indirect effects of the *sgrS* gene, sugar transport regulatory genes, *budABC* genes, glucose metabolism, and 2,3-BD production, during fermenting. (**a**) shows the relationship of indexes in *K. pneumoniae* HD79 series strains; (**b**) shows the relationship of indexes in *K. pneumoniae* HD79-02 series strains. The line outlined in gray indicates no significance ($p > 0.05$). The solid red line shows a positive association, and the solid blue line shows a negative association (* $p < 0.05$, *** $p < 0.001$). Numbers on arrows were standardized pathways coefficients indicating the degree of influence for relationships.

After knocking out *ack* and *ldh*, the *sgrS* gene only played a negative regulatory role on the *budABC* genes and had no significant relationship with the relative expression of sugar transport genes, glucose metabolism, or 2,3-BD production. 2,3-BD production and sugar transport genes were still significantly positively correlated with glucose metabolism and the expression levels of *budABC* genes (Figure 8b). Therefore, the *ack* and *ldh* genes affect the regulation of intracellular glucose content by *sgrS* on sugar transport genes. However, it is unclear whether this effect exists and what its mechanism of action is, and further characterization is needed.

4. Conclusions

To develop the economic industry of biorefineries, the efficient utilization of biomass is necessary. In this study, we knocked out or overexpressed the *sgrS* gene, which encodes the sugar transport regulator sRNA-SgrS, and observed how *K. pneumoniae* increased the production of 2,3-BD in the presence of high-concentration sugar fermentation. To effectively utilize the high substrate concentration of glucose, after overexpression of the *sgrS* gene, the transcription level of the *ptsG* gene decreased, and the content of glucose ingested by mutants was downregulated. This enhanced the tolerance of bacteria to sugar and indirectly inhibited the transcription level of *budABC* genes, resulting in the downregulation of 2,3-BD production. We also found that the knockout of *ack* and *ldh* reduced the carbon flux allocated to the acetate and lactate metabolic pathways and increased the carbon flux of the 2,3-BD metabolic pathway, further increasing the production of 2,3-BD. The transformation of *K. pneumoniae* utilizing genetic engineering provides a scientific basis for how to effectively utilize carbohydrates for the purpose of increasing the production of 2,3-BD.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation8080359/s1, Figure S1: Sugar tolerance of *K. pneumoniae* HD79 series strains and *K. pneumoniae* HD79-02 series strains.

Author Contributions: R.S.: Methodology, Data curation, Writing—original draft, Writing—review & editing. J.K.: Formal analysis. X.W.: Investigation. B.X.: Validation. W.P.: Conceptualization, Supervision. J.G.: Resources, Supervision. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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