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## Role of sigma factor RpoS in *Cronobacter sakazakii* environmental stress tolerance

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### ABSTRACT

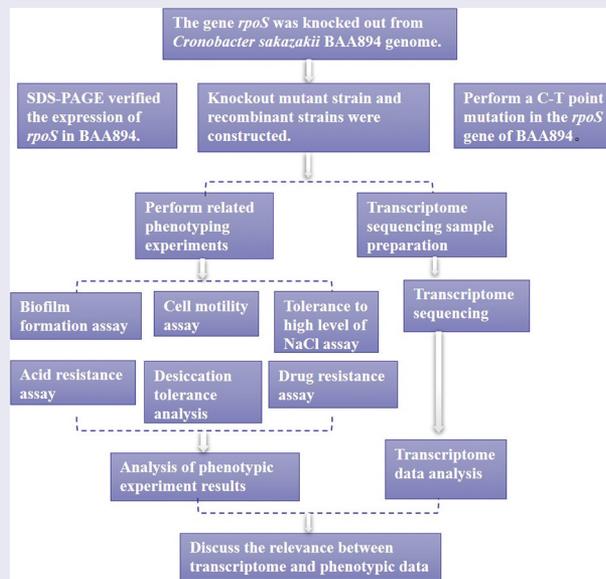
*Cronobacter sakazakii* is a food-borne, conditionally pathogenic bacterium that mainly infects neonates, especially premature infants. Previous studies have indicated that an important route of infection for *C. sakazakii* is through infant formula, suggesting a high stress resistance of the bacterium. RpoS is a  $\sigma$ -factor that is closely related to the bacterial resistance mechanisms. In this study, a *C. sakazakii* BAA894 model strain was used. An *rpoS*-deficient mutant strain  $\Delta rpoS$  was constructed using Red homologous recombination, and the differences between the mutant and the wild-type strains were compared. To investigate the functions of the *rpoS* gene, the membrane formation and cell wall properties of the strains were studied, and the tolerance of each strain to acid, osmotic pressure, desiccation, and drug resistance were compared. The results showed that the membrane formation ability in the mutant strain was increased, auto-aggregation was enhanced, motility, acid resistance and hyperosmotic resistance were alternated to different degrees, and desiccation resistance was stronger than observed in the wild type grown in LB medium but weaker than the wild type cultured in M9 medium. These results showed that *rpoS* is involved in environmental stress resistance in *C. sakazakii* BAA894. Finally, transcriptome analysis verified that the deletion of the *rpoS* gene caused differential expression of resistance-related genes and instigated changes in related metabolic pathways. These messenger RNA results were consistent with the functional experimental results and help explain the phenotypic changes observed in the mutant strain.

### ARTICLE HISTORY

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### KEYWORDS

*Cronobacter sakazakii*;  $\Delta rpoS$ ; tolerance to environmental stress; transcriptomic analysis



## Introduction

*Cronobacter sakazakii* is a gram-negative bacterium, which is a food-borne opportunistic pathogen from the enterobacteriaceae family. *Cronobacter* was previously called *Enterobacter sakazakii*. In 2007, Iversen et al. [1] proposed establishing a new genus including the original *Enterobacter sakazakii*, namely *Cronobacter* spp., and *C. sakazakii* as one of the seven species classified [2,3]. The genome of *C. sakazakii* BAA894 was the first sequenced in 2010 [4]. *C. sakazakii* BAA894 can parasitic in the intestinal tract of human and warm-blooded animals, and can cause meningitis, enterocolitis and septicemia, and sepsis in infants, once infected [5–9]. These infections were found to be related to infant formula milk (IFM), which is complex to process and has an extremely low water concentration [10]. The method by which *C. sakazakii* infects organisms illustrates that its pathogenicity is closely related to its abilities relating to stress resistance.

RpoS is a sigma factor ( $\sigma^{38}$ ) of RNA polymerase, and is responsible for regulating the general stress response [11]. It keeps bacteria alive in different external environments by inducing the expression of relevant genes and is believed to be extensively involved in cellular stress responses [12]. As reported in *E. coli*, RpoS is not only an indispensable regulator of glucose-repressed acid resistance [13], but it also plays regulatory roles in other resistance mechanisms, for example, RpoS can regulate the expression of the virulence genes [14,15]. At present, the gradual role of sigma factor RpoS in *C. sakazakii* has been focused upon, and RpoS has proved to be significantly important ensuring tolerance in *C. sakazakii* to acid, osmotic pressure, and oxidation [16–18]. RpoS also plays a crucial role in *C. sakazakii* tolerance in relation to long-term extremely low water activity ( $a_w$ ) [19]. It has also been reported that the expression of *rpoS*, *hfq* and *ompA* genes was up-regulated when *Cronobacter* enter the viable but nonculturable (VBNC) state after a 2 h period of desiccation tolerance [20]. By entering the VBNC state, *Cronobacter* can maintain its

virulence while escaping the typical colonies tests, and regulating *rpoS* affects bacterial abilities in relation to entering this state.

The *rpoS* gene has global regulatory significance, but its mechanism(s) of action in *C. sakazakii* BAA894 remains to be elucidated. In the present study, a new mutant strain  $\Delta rpoS$  was constructed by knocking out gene *rpoS* in *C. sakazakii* BAA894, the differences in environmental stress resistance between wild-type *C. sakazakii* BAA894 and the  $\Delta rpoS$  mutant were compared in terms of osmotic pressure, acid tolerance, desiccation tolerance, and drug resistance. Membrane formation and motility, which are supposedly related to bacterial adhesion and considered to be important for high stress enhance, were tested too. Transcriptomic analysis of  $\Delta rpoS$  and wild-type strains were conducted, and the pathways showing significant changes were analyzed. This study aimed to investigate the role of sigma factor RpoS in *C. sakazakii* in relation to environmental stress tolerance by studying the changes of the  $\Delta rpoS$  mutant in phenotypic experiments and transcriptomic analysis. The transcriptome data can also further clarify the mechanisms of RpoS in stress survival of this strain.

## Materials and methods

### Strains and media

Bacteria and plasmids used in this work are listed in Table 1. *C. sakazakii* cells were grown at 37°C in LB medium 5-g/L yeast extract, 10-g/L tryptone, and 10-g/L NaCl [21] or M9 medium (17.1 g/L  $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ , 3 g/L  $\text{KH}_2\text{PO}_4$ , 4 g/L glucose, 1 g/L  $\text{NH}_4\text{Cl}$ , 0.5 g/L NaCl, 0.24 g/L  $\text{MgSO}_4$ , and 0.011 g/L  $\text{CaCl}_2$ ) [22] with 200-rpm shaking.

### Reagents and primers

Kits: Plasmid DNA Miniprep Kit (Bio Basic Canada Inc), TIANamp Bacteria DNA Kit and agarose gel DNA recovery kit (Tiangen Biochemical Technology (Beijing) Co., Ltd.), and

**Table 1.** Strains and plasmids used in this study.

Strains and plasmids	Description	Sources
<b>Strains:</b>		
BAA894	Wild-type <i>C. sakazakii</i>	ATCC
BAA894 pKD46	BAA-894 harboring pKD46	This study
$\Delta rpoS$	<i>rpoS</i> deletion mutant of BAA894	This study
BAA894/pWSK29	BAA-894 harboring pWSK29	This study
$\Delta rpoS$ /pWSK29- $\Delta rpoSW$ ( <i>rpoSW/rW</i> )	$\Delta rpoS$ harboring pWSK29- <i>rpoS</i>	This study
$\Delta rpoS$ /pWSK29- $\Delta rpoSU$ ( <i>rpoSU/rU</i> )	$\Delta rpoS$ harboring pWSK29- <i>rpoSU</i>	This study
$\Delta rpoS$ /pWSK29- $\Delta rpoSD$ ( <i>rpoSD/rD</i> )	$\Delta rpoS$ harboring pWSK29- <i>rpoSD</i>	This study
$\Delta rpoS$ /pWSK29- $\Delta rpoSDTB$ ( <i>rpoSDTB/rDTB</i> )	$\Delta rpoS$ harboring pWSK29- <i>rpoSDTB</i>	This study
BL21	<i>E. coli</i> host for protein expression	Laboratory strain
JM109	<i>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1Δ(lac-proAB)<sup>f</sup> [traD36 proAB+ lacI<sup>q</sup> lacZΔM15]</i>	Laboratory strain
<b>Plasmids:</b>		
pKD46	ParaByβ <i>exo</i> , Repts, AmpR	[23]
pKD-Cre	ParaB <i>cre</i> , Repts, AmpR	[24]
pBlueScript II SK+	Cloning vector, ColE1, lacZ, AmpR	[25]
pDTW202	loxPLE- <i>kan</i> -loxPRE, AmpR, KanR	[24]
pWSK29	Expression vector	Stratagene
pWSK29- $\Delta rpoS$	pWSK29 harboring <i>rpoS</i>	This study
$\Delta rpoS$ /pWSK29- $\Delta rpoSU$	pWSK29 harboring <i>rpoSU</i>	This study
$\Delta rpoS$ /pWSK29- $\Delta rpoSD$	pWSK29 harboring <i>rpoSD</i>	This study
$\Delta rpoS$ /pWSK29- $\Delta rpoSDTB$	pWSK29 harboring <i>rpoSDTB</i>	This study
pET28a	Protein expression vector in BL21	Stratagene

SDS-PAGE Gel Quick Preparation Kit (Beyotime Institute of Biotechnology).

Molecular biology reagents: *Ex* Taq DNA polymerase and dNTPs (Bao Biological Engineering (Dalian) Co., Ltd), DNA restriction endonuclease, T4 DNA ligase and DNA Marker (Fermentas), GoldView II Nuclear Staining Dye (Beijing Solarbio Science & Technology Co., Ltd), agarose

(Shanghai Generay Biotech Co., Ltd). PCR experiments were performed using Mastercycler from Eppendorf (Hamburg, Germany). The sequences of all primers used in this study are listed in Table 2.

### Construction of gene knockout mutant of *Cronobacter sakazakii*

In this study, the target gene *rpoS* was knocked out using a *C. sakazakii* Red recombination system [26,27]. First, the upstream and downstream flanking sequences of the *rpoS* gene were amplified using the primer pairs *rpoS*-U-F/*rpoS*-U-R and *rpoS*-D-F/*rpoS*-D-R respectively retrieved from the *C. sakazakii* genome by PCR, and by introducing cleavage sites *Kpn* I (5') and *Bam*H I (3') to the upstream homology arm, and *Xba* I (5') and *Spe* I (3') to the downstream homology arm. Next, the kanamycin resistance cassette *kan* fragment was amplified by PCR using primer pair *kan*-F and *kan*-R with pDTW202 as template, and enzyme digestion sites *Bam*HI (5') and *Xba*I (3') were introduced to the product fragment. The three fragments obtained, *rpoS*-L, *rpoS*-R and *kan*, were digested with the corresponding DNA restriction endonucleases (Fermentas), and the recovered fragments were then ligated into the plasmid pBluescript II SK(+) digested by *Xho*I and *Pst*I. The ligation product is transferred to *E. coli* JM109; then, the resulting transformants were screened on agar plates supplemented with kanamycin to obtain the knockout recombinant plasmid pBluescript-*r*-U-*Fkan*-*r*-D, with enzyme digestion verification containing the upstream and downstream homology arm of *rpoS* gene, and the kanamycin resistance cassette. Finally, the *rpoS* knockout fragment *rpoS*-U-*Fkan*-*rpoS*-D was amplified using the forward primer *rpoS*-U-F which targeted the upstream homology arm and the reverse primer *rpoS*-D-R targeting the downstream homology arm. The amplified product was recovered following verification with agarose gel electrophoresis. The knockout fragment was transferred into BAA894 competent cells containing pKD46, and the *rpoS* on the genome

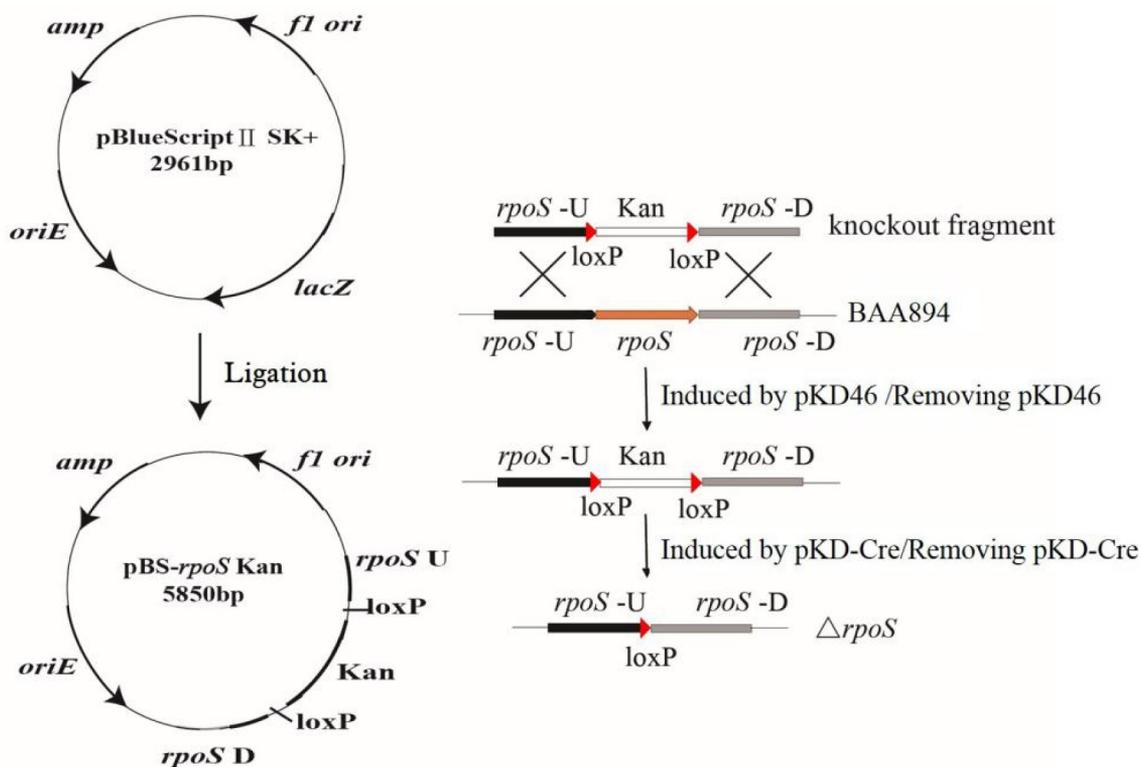
**Table 2.** Primers for PCR amplification used in this study.

Primer name	Sequence (5'-3')	Restriction site
<i>rpoS</i> -F	CCCCGGGAGGAGGATATACCATGAATCAGAATACGCT	<i>Sma</i> I
<i>rpoS</i> -R	CCCTCGAGTTATTCGCGAACAGCGCTTCG	<i>Xho</i> I
<i>rpoS</i> -U-F	GGGGTACCTGTTTACAATCGCAAGT	<i>Kpn</i> I
<i>rpoS</i> -U-R	CGGGATCCTATCGCTGCGGCAAAT	<i>Bam</i> H I
<i>rpoS</i> -D-F	GCTCTAGACACCATTCCCCTCAAAAGG	<i>Xba</i> I
<i>rpoS</i> -D-R	GACTAGTTTTCCCTCGGCTGCCTGTTGC	<i>Spe</i> I
RpoS-YZ-F	CAGCTCGATCTGCACAATATCTC	
RpoS-YZ-R	CTTTCCTGGATTAGCAACGCC	
<i>Kan</i> -loxP-F	ATGGATCCAATACGACTCACTATAGGGCG	<i>Bam</i> H I
<i>Kan</i> -loxP-R	ACCTCTAGAGCGCAATTAACCTCACTAAAG	<i>Xba</i> I I
<i>rpoS</i> DTB-F	GAGATTGCAGAACAGCTGGATAAAC	
<i>rpoS</i> DTB-R	AGCTGTTCTGCAATCTCTTCTGC	
RpoSU-R	CCCTCGAGCTATTTGGTCAACTGCTGCAATCGGC	<i>Xho</i> I
RpoSD-F	CCCAAGCTTAGGAGGATATACCATGCTGCGCCTGAACGAG	<i>Hind</i> III
pET28a_YZ-F	AGATCTCGATCCCGCGAAATTAATACG	
pET28a-YZ-R	CAAAAACCCCTCAAGACCCGTTTAG	
YZ_pWSK29-F	CGGGCCTCTTCGCTATTAC	
YZ_pWSK29-R	CCCAATACGCAAACCGCCTCTCC	

The restriction enzyme sites are underlined.

was replaced by *loxP*-*kan*-*loxP*. Following successful replacement, the temperature-sensitive plasmid pKD46 was removed via incubation at 42°C. Afterward, pKD-Cre was introduced into the cells, and the *loxP* site recombination was

induced by Cre recombinase to remove the *kan* resistance gene. The pKD-Cre plasmid was removed via incubation at 42°C, and the  $\Delta$ *rpoS* mutant was attained (Figure 1). The primers are listed in Table 2.



**Figure 1.** *rpoS* gene knockout procedure in *C. sakazakii* BAA894.

### **The C-T mutation at position 601 of *rpoS* in *C. sakazakii* BAA894**

The *rpoS* sequence in *C. sakazakii* BAA894 had a CAG-TAG mutation at position 601 compared with other *Cronobacter sakazakii*, and this TAG (stop codon) at positions 601–603 mutated to CAG (glutamine), resulting in the strain *rpoS*DTB. Meanwhile, *rpoS*U (*rpoS* up part 1–603 bp), *rpoS*D (*rpoS* down part 633–993 bp), *rpoS*W (*rpoS* whole part 1–993 bp) and *rpoS*DTB were overexpressed in BL21, expression was identified using SDS-PAGE (Beyotime Institute of Biotechnology).

### **Overexpression of *rpoS*U, *rpoS*D, *rpoS*W and *rpoS*DTB**

First, *E. coli* BL21 competent cells were prepared, transformed with the constructed pET28a-*rpoS*DTB, pET28a-*rpoS*U, pET28a-*rpoS*D and pET28a-*rpoS*W plasmids, and cultured onto LB agar plates supplemented with kan. After overnight incubation and colony PCR verification, the correct transformants were picked for plasmid extraction.

Total protein extractions from the five exponentially growing BL21 strains, BL21-pET28a-*rpoS*W, BL21-pET28a-*rpoS*U, BL21-pET28a-*rpoS*D, BL21-pET28a-*rpoS*DTB and BL21-pET28a, were collected via protein electrophoresis. 60  $\mu$ L culture was added to 12  $\mu$ L 5 $\times$  Loading Buffer, boiled for 10 min, separated by SDS-PAGE (5% stacking gel and 12% separation gel) [28,29] and electrophoresis (Tricine-SDS-PAGE was used due to the small size of *rpoS*D), and the gel was stained with Coomassie Brilliant Blue (Beijing Solarbio Science & Technology Co., Ltd).

### **Stress resistance of mutant strains**

#### **Biofilm formation assay**

In order to study the mutant strain change of membrane formation ability, the biofilm formation experiments were done according to some previous references [30–32]. The overnight culture in LB or M9 medium was transferred into 5 mL of LB medium or M9 medium at an initial OD<sub>600</sub> = 0.02; 1 mL of the culture was taken at OD<sub>600</sub> = 1.0, and incubated without shaking at 37°C for

72 h. The culture was checked every 12 h, and fresh sterile LB medium was added when necessary to prevent reduction of the medium. After the biofilm had formed, all the medium liquid was pipetted out and the tube was washed three times with deionized water. To conclude, 1 mL of 0.3% Crystal violet solution was added, incubated without shaking for 3 min at room temperature, washed in deionized water and the biofilm was observed.

For the determination of biofilm formation ability, the tubes were washed with deionized water until the eluted water showed no purple coloration. Finally, 1 mL of 30% acetic acid was added into the tube, sonicated for 30 min; then, the absorbance of the solution at a wavelength of 597 nm was measured with 30% acetic acid as the control, this was recorded as the biofilm formation rate. Three replicates were undertaken for each sample.

#### **Cell motility assay**

The motility experiment was performed following the methodology of Clemmer KM et al. [33], with modifications. Bacterial motility was measured on modified semi-solid LB agar plates and M9 agar plates. The agar content of the plate was 0.25% (w/v). The overnight culture was diluted to OD<sub>600</sub> = 0.2, and 1.5  $\mu$ L of this solution was added to the semi-solid agar plate. After complete absorption, the plate was incubated at 25°C for 48 h and then photographed to record the movement of the bacteria.

#### **Tolerance to different concentrations of NaCl**

In line with the method published by Fakruddin [15], a single colony was inoculated into 5 mL of LB liquid medium and incubated overnight. The overnight culture was then transferred into LB medium supplemented with different NaCl concentrations (1.0%, 2.0%, 4.0%, 6.0%, 8.0% and 10.0%) at an initial OD<sub>600</sub>  $\approx$  0.02, and incubated at 37°C, 200 rpm. The OD<sub>600</sub> value was measured at 12 h and 24 h, and three replicates were included for each group. These experiments were repeated twice.

#### **Acid resistance assay**

The acid resistance assay was performed following the methodology of Fakruddin [15] with

modifications. The growth curves under different acidity and medium conditions, and the pH changes of the media during growth were determined. The strains were inoculated at 37°C, 200 rpm, and different media pH values of 7.0, 6.0, 5.0 and 4.0 were initiated. The cultures were then incubated in shaking flasks and sampled every 1–2 h. The OD<sub>600</sub> of the samples were measured using UV spectrophotometry as the maximum absorption peak of *C. sakazakii* was 600 nm, and the valid range of the spectrophotometer was 0.3–0.9; samples with OD<sub>600</sub> out of this range were adjusted and re-measured. The growth curve was plotted according to the mean value of OD<sub>600</sub> at each sampling point, and the standard deviations were calculated and labeled. Meanwhile, the pH meter was used to measure acidity changes in each sample at all time points. The mean and standard deviation for each sample were calculated after obtaining stable readings, and the curves were plotted accordingly.

#### **Desiccation tolerance analysis**

This experiment was performed as previously published [19], with some modifications. As the dryness resistance abilities of thallus can change in different environments, tests were conducted in both LB and M9. The strains were cultured on plates; then, a single colony was cultured in 5 mL liquid LB or M9 broth until the cells reached the logarithmic phase. After OD<sub>600</sub> values had been measured in the different microbial cultures, a 100 mL portion of each culture was transferred into wells in a matched 96-well microtiter plate. Subsequently, the plate was transferred into a sterile dryer with dehydrated silica gel. The dryer was placed in a sterile incubator (DHP-2042BS, Huabei, Tianjin, China), which was constantly kept at 37°C. Six days later, the 96-well microtiter plates were placed onto a clean bench, and 100 mL/well of fresh LB or M9 medium was added; then, the plates were shaken at 200 rpm at 37°C for 3 h to re-culture. The liquid from each well was then transferred to a new 96-well microtiter plate, and the OD<sub>600</sub> was detected. Each strain was tested in triplicate, and each test was repeated three times. The desiccation tolerance was performed with inactivation rate and calculated using the following formula:

$$\text{Inactivation rate}(\%) = \frac{OD_0 - OD_1}{OD_0} \times 100\%$$

OD<sub>0</sub> represents the initial OD<sub>600</sub> of each strain, and OD<sub>1</sub> represents the OD<sub>600</sub> of each strain after re-cultured for 3 h.

#### **Drug resistance of BAA894-ΔrpoS**

The minimum inhibitory concentrations (MICs) were determined for each strain, according to the method reported [21,34]. Antibiotic stock solutions were prepared at the following concentrations: ampicillin 2.56 mg/mL, cefoxitin 2.56 mg/mL, norfloxacin 0.16 mg/mL, polymyxin 0.64 mg/mL, tetracycline 2.00 mg/mL, amoxicillin 50.00 mg/mL, vancomycin 0.64 mg/mL, gentamicin 0.64 mg/mL, cefoperazone 1.00 mg/mL, and novobiocin 50.00 mg/mL. The antibiotics were filtered and then diluted accordingly. Firstly, 198 μL of LB medium was added to the first well of the plate, and 100 μL of LB medium was added to the remaining wells. Then, 2 μL of stock solution was added to the first well, mixed well, and 100 μL of this solution was transferred into the second well; then 100 μL of that medium was transferred into the following well for sequential dilutions until the seventh well was reached, from which 100 μL of the medium was discarded. The eighth well was supplemented with no drugs and served as the growth control. Overnight cultures were inoculated into 5 mL LB liquid medium at an initial OD<sub>600</sub> = 0.02, incubated for around 2 h until OD<sub>600</sub> = 0.5 was achieved; then, the solution was diluted with LB medium at 1:1000, and 100 μL of this diluted culture was added to each well. After the plate had been sealed, it was incubated for 16–20 h in a water-proof incubator at 37°C, and the results were observed thereafter. Four replicates were included for each sample.

#### **Transcriptome analysis**

The main procedures for transcriptomic analysis included: total RNA extraction, DnaseI digestion, mRNA isolation, mRNA fragmentation, mRNA reverse transcription, cDNA synthesis and library quality checks. Qualified libraries were used for cDNA sequencing with Illumina HiSeq<sup>TM</sup>2000 technology. The RPKM (Reads per kilobase

transcriptome per million mapped reads) method [35] was used to calculate gene expression [11], and the obtained gene expression levels were used directly for differential gene expression analysis. In this study, differentially expressed genes were defined as genes with both an FDR  $\leq 0.05$  by Benjamini's test [36,37] and with a fold difference of higher than 2. After the differentially expressed genes were screened, KEGG [38] Pathway clustering analysis was performed on this basis.

In microorganisms, coordination between genes is important for gene function, and thus the functional properties of genes in metabolic process can be studied based on pathway clustering analysis. KEGG is a public database for metabolic pathway analysis. For differentially expressed genes, significant enrichment based on KEGG pathways can be tested in the context of the entire genome to highlight significantly enriched pathways. This analysis was performed by calculating the p-value of each Pathway, corrected by Benjamini's test, and by using a Q-value  $\leq 0.05$  as the threshold to determine significantly enriched pathways.

In this study, the biological functions associated with the differentially expressed genes were determined by clustering analysis of KEGG Pathways based on the significant differences in gene expression, and the involved metabolic pathways were used as references to study the mechanism of tolerance alteration in the mutant strain.

## Results

In order to clarify the function of RpoS related to stress resistance within *C. sakazakii* BAA894, an *rpoS* gene related DNA fragment deletion mutant strain was constructed using Red homologous recombination technology. The expression and function of the internal mutation of *rpoS* by these mutant strains was then analyzed. Following this the mutant strains were analyzed based on biofilm formation ability, motility, resistance to high permeability, acid resistance, and desiccation tolerance. The findings showed that the high permeability resistance and acid resistance of the  $\Delta rpoS$  mutant strain were weakened, dryness resistance increased in LB medium and decreased in M9 medium, and drug resistance

changed significantly. Finally, transcriptome analysis confirmed that the *rpoS* gene deletion resulted in differential expression of genes related to stress resistance and changes in related metabolic pathways, these were consistent with the other experimental results.

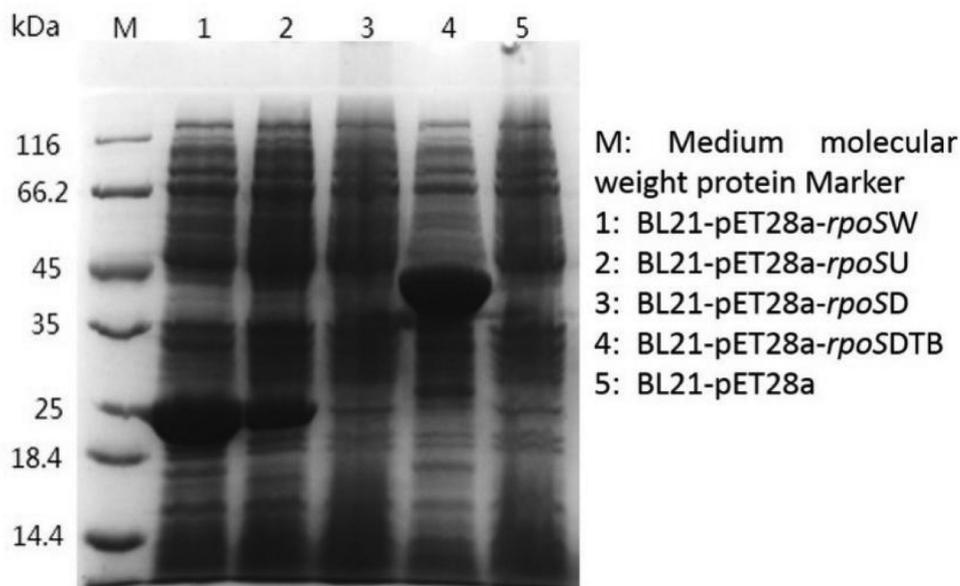
### **Verification of *rpoSU*, *rpoSD*, *rpoSW* and *rpoSDTB* overexpression**

The expression of *rpoS* in BAA894 cells and the effect of stop codon mutations on the expression levels were verified using SDS-PAGE (Figure 2). The protein sizes were determined using the protein marker sizes (14.4, 18.4, 25, 35, 45, 66.2 and 116 kDa). Lanes 2 and 3 show proteins with similar sizes of around 25 kDa, and the respective genes were inferred to be around 600 bp accordingly, which corresponds to the first 603 bp of *rpoS*. Lane 3 showed no significantly overexpressed proteins. The size of the entire RpoS protein is between 35 kDa and 45 kDa, which is about 40 kDa, and an obvious protein band of this size could be observed in lane 4 of BL21-pET28a-*rpoS* DTB. Based on the protein expression analysis, it was verified that the partial *rpoS* before the stop codon in BAA894 was overexpressed. When expressed using a strong promoter, the remaining part of RpoS, after the mutation site, showed a band at a corresponding position on the Tricine-SDS-PAGE, but the expression levels seemed to be lower than other overexpressed proteins, which therefore requires further verification.

### **Resistance-related phenotyping experiments**

#### **Bacteria biofilm formation ability**

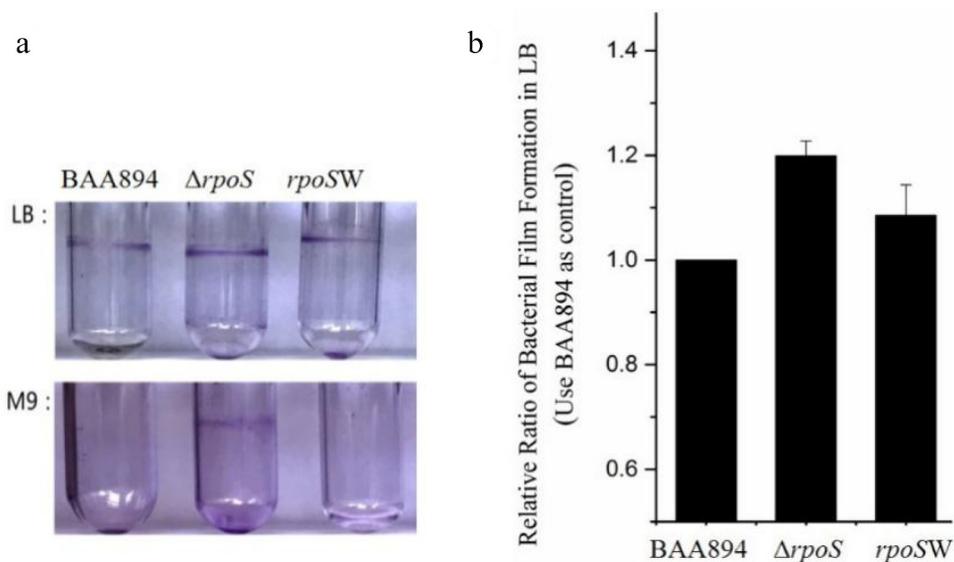
Biofilms are formed when bacteria cells interact with each other under changing culture conditions, which ensures the viability of the colony. The process of biofilm formation includes five stages: reversible adhesion, irreversible adhesion, microcolony expansion, development and maturation of biofilm, and dissociation of the biofilm. Following formation of the biofilm, a complex metabolic network is formed between the microbial community and the glycocalyx within the biofilm, which transports oxygen and nutrients



**Figure 2.** Protein Tris-SDS-PAGE electrophoresis of overexpressing strains.

into the membrane and removes the metabolic waste from the membrane. Therefore, some researchers regard the biofilm as a primitive circulatory system. In this study, the membrane formation abilities of the BAA894 wild type,  $\Delta rpoS$  mutant and *rpoSW* complementary strain were conducted in LB and M9 medium, respectively (Figure 3(a)).

In the LB medium (in which IPTG and Amp were supplemented to the complementary strain), the membrane formation abilities of the  $\Delta rpoS$  mutants were significantly enhanced comparing to that of BAA894, and the *rpoSW* complementary strain was able to complement the wild-type phenotype. In M9 medium (where both mutant strains were supplemented with IPTG and Amp), a blue



**Figure 3.** Analysis of the biofilm formation among the BAA894, mutant  $\Delta rpoS$  and recombinant strain *rpoSW*.

(a) Qualitative observation experiment. (b) Quantitative determination of the biofilm formation ability of each strain, the amount of biofilm formation was compared with wild type as a control.

band is shown on the wall of the test tube of  $\Delta rpoS$ , whereas both the wild type and complementary strains showed no obvious bands.

As shown in Figure 3(b), quantitative assays of biofilm formation ability were performed for the wild type,  $\Delta rpoS$  mutant and complementary strains in LB medium. As observed in M9 medium, the BAA894 and *rpoSW* strains had almost no biofilm formation, similar to results from the qualitative experiments. The wild type was used as a control with a value of 1. The results showed that membrane formation in the mutant strain was increased and the complementation of the entire BAA894 *rpoS* gene (*rpoSW*) achieved good complementary effects.

Combining the results of both culture conditions, the absence of *rpoS* resulted in an increased membrane formation ability, indicating that the  $\Delta rpoS$  mutant may adhere more readily to the material surface. This increased capacity may be a compensatory swarming response by the bacteria when the regulatory effects of *rpoS* are reduced.

#### Motility assay of the wild-type, mutant and complementary strains

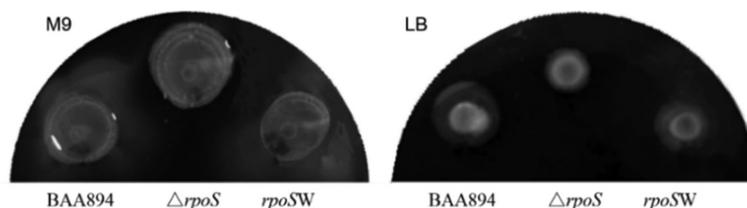
Pili structure can directly affect the interaction and adherence of cells, both of which affect the stress resistance of bacteria. Motility is one of the direct means by which changes in pili structure and number can be analyzed. In this study, the motility of wild-type,  $\Delta rpoS$  mutant and complementary strains were studied in different medium agar plates (Figure 4).

When the bacteria were in M9 medium (M9 + IPTG), it was observed that the concentric circles of the BAA- $\Delta rpoS$  colony were the largest and

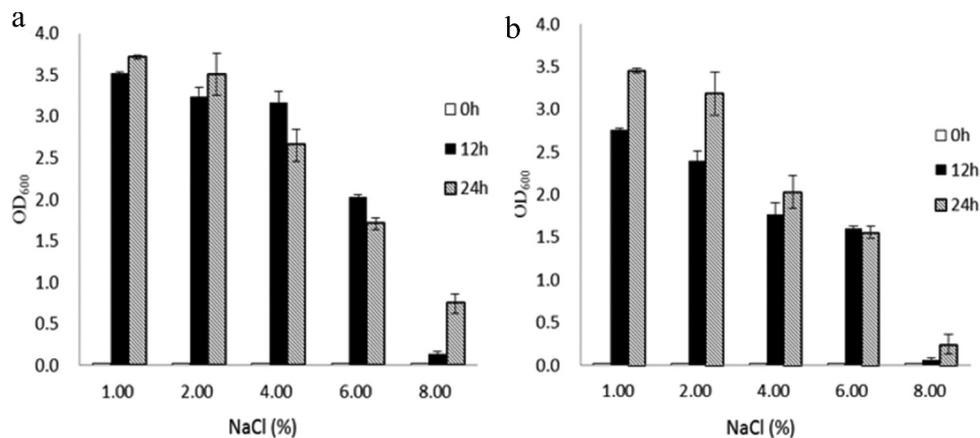
most obvious among the three strains. This size and shape indicated that motility was stronger. Additionally, the colony morphology was slightly flatter and more mucilaginous than that of the wild type, whereas the *rpoSW* complemented colony showed similar morphological and motility traits to those of the wild-type colonies. In comparison to the M9 medium, differing motilities between the strains growing on LB (LB+IPTG) agar plates were not obvious, and the motility of the mutant was weaker than that of the wild type.

#### Tolerance to high NaCl concentrations

As shown in Figure 5, when the medium contained normal concentrations of NaCl (i.e. 1.0% NaCl), the bacteria reached higher concentrations than those other high NaCl media. The growth of the BAA894 wild-type bacteria showed a decreasing trend with increased concentrations of NaCl. The bacteria showed basically no growth when the medium contained 8.0% NaCl, the highest density reached only 2.03 when 6.0% NaCl was supplemented. The highest density was observed at 12 h when supplemented with more than 4% salt, which decreased again at 24 h. In general, the growth of the wild-type bacteria decreased with increased NaCl concentrations.  $\Delta rpoS$  showed similar growth patterns to BAA894, but grew at a slower rate than BAA894. The growth rate of BAA894 was higher than that of  $\Delta rpoS$  under normal culture conditions, which was mainly caused by the longer adaptation stage of  $\Delta rpoS$ , resulting in a significantly lower OD value at 12 h than that of BAA894; however, the difference in OD value at 24 h had reduced. The  $\Delta rpoS$  mutant was less tolerant to high osmotic environments, which is consistent with the stress-resistance-



**Figure 4.** Swimming motility of the wild-type, *rpoS* null mutant and expression recombinant strains on M9 or LB medium.



**Figure 5.** Osmotic tolerance of wild-type BAA894,  $\Delta rpoS$  mutant strain. (a) wild-type BAA894, (b)  $\Delta rpoS$ .

related functions of the *rpoS* gene. This may be related to the transport or synthesis of ion or compatible solutes.

#### Acid tolerance assay

M9 medium was used to study the changes in acid tolerance in the mutant strains, the strains grow slowly in this medium; therefore, the differences in growth curves are more obviously reflected.

The growth of *C. sakazakii* BAA-894- $\Delta rpoS$  in the M9 medium under different pHs (7.0, 6.0, 5.0, 4.0) showed that the growth of the mutant was significantly slower than that of the wild type at pH 5.0 and pH 4.0. Medium measurements found that the mutant strain adjusted to the medium pH earlier than the wild type, but the pH of the wild type under pH 6.0 conditions increased rapidly after entering stationary phase and reached a higher final value than that of the mutant. The highest adjusted pH value of the wild type at pH 4.0 was higher than that of the mutant, which might explain the better growth of the wild type under low pH conditions (Figure 6).

#### Desiccation tolerance assay

Resistance to desiccation is one of the important characteristics of *C. sakazakii*. In order to investigate the role of RpoS in regulating the *C. sakazakii* desiccation resistance, the survival rates of the

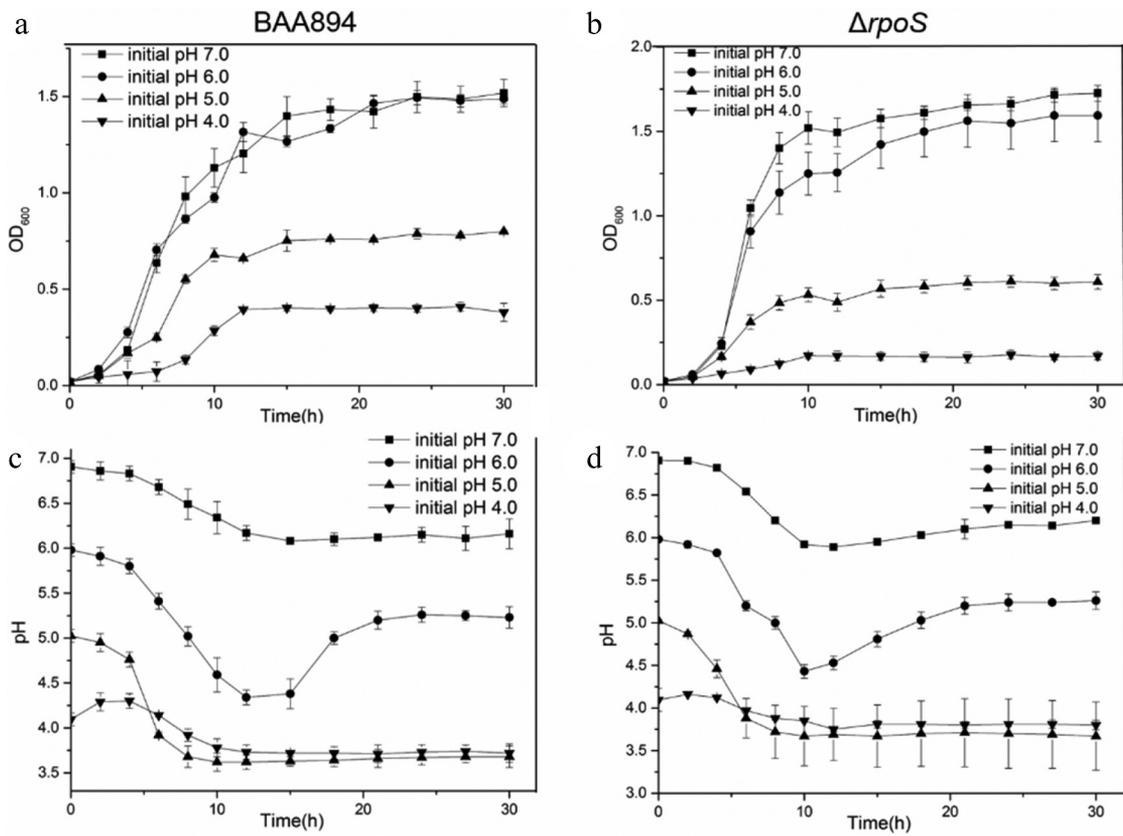
wild-type BAA894,  $\Delta rpoS$  mutant, and *rpoSW* were tested under varying desiccation conditions.

The desiccation tolerance assay showed that, in LB medium, the desiccation resistance of the mutant strain decreased than the wild type and the inactivation rate reached 4 times that of the wild type. The mutant strain desiccation resistance in M9 medium was slightly higher than that of the wild type, whilst its inactivation rate decreased by about a third (Figure 7). In addition, *rpoSW* rates complemented the desiccation resistance observed in the mutant strain.

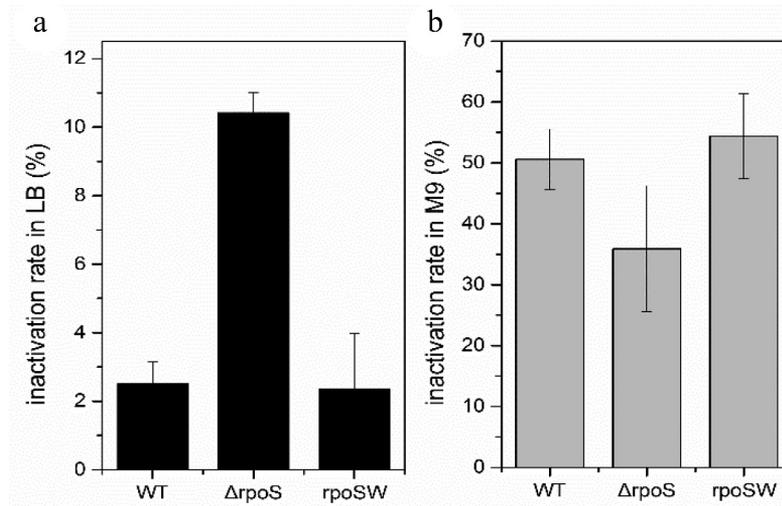
#### Drug resistance assays on BAA894 and $\Delta rpoS$

Minimum inhibitory concentrations (MICs) were determined for the wild-type and  $\Delta rpoS$  mutant strains, respectively.

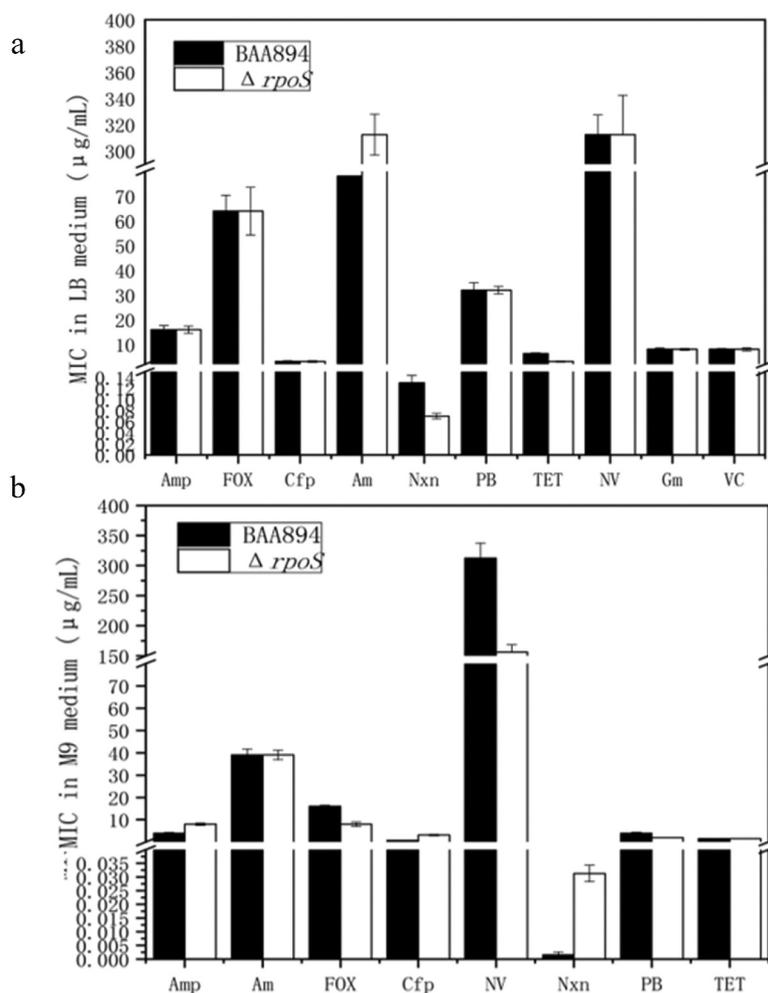
The mutant strain was tested for drug resistance in both LB and M9 medium conditions (Figure 8). In the M9 medium, compared with the wild-type BAA894, the mutant strain showed reduced resistance to the cephalosporins cefoxitin and coumarins neomycin, and increased MIC to norfloxacin, which prevents bacterial DNA replication by blocking its DNA gyrase. When grown in LB media, the mutant strain showed increased sensitivity to norfloxacin and tetracycline, and decreased sensitivity to amoxicillin. The mutant strain showed higher susceptibility to polymyxin B in both LB and M9 media. The reduced MIC to



**Figure 6.** Growth curves and the extracellular pH changed curves of BAA894 and  $rpoS$  null mutant  $\Delta rpoS$  in M9 medium. (a) BAA894 growth curves, (b)  $\Delta rpoS$  growth curves, (c) BAA894 extracellular pH changed curve, (d)  $\Delta rpoS$  extracellular pH changed curve.



**Figure 7.** Desiccation tolerance of the wild type (WT),  $rpoS$  null mutant strains  $\Delta rpoS$  and complementation strains  $rpoSW$  on (a) LB and (b) M9 medium.



**Figure 8.** MIC determination of different antibiotics for BAA894,  $\Delta rpoS$  in different cultural media. (a) LB and (b) M9.

$\beta$ -lactam and peptide antibiotics in the mutant strain suggested that the *rpoS* deletion may have caused some structural changes in the cell membrane and wall, making it more fragile.

### Transcriptome analysis under different conditions

#### Overall comparison of transcriptomes of BAA894 and $\Delta rpoS$ mutant strains

In LB medium, the total number of annotated genes was 3927 for ATCC BAA894 and 3900 for the  $\Delta rpoS$  mutant strain. A total of 3973 annotated genes were obtained from the two strains, with 3854 crossed, of which 1778 genes were up-regulated and 2195 genes were down-regulated in the mutant. After accounting for the FDR correction, 123 genes were significantly up-regulated,

and 260 genes were significantly down-regulated. When cultured in the M9 medium, 74 genes were significantly up-regulated and 19 genes were significantly down-regulated after accounting for the FDR correction.

#### Overall pathways enrichment of differentially expressed genes in BAA894 and $\Delta rpoS$ mutant strains

In LB medium, the three most significantly enriched pathways in the  $\Delta rpoS$  mutant strains were flagellar assembly, microbial metabolism in diverse environments and nitrogen metabolism, and all three pathways were significantly down-regulated when compared to the wild type. Among them, the pili synthesis pathways were all down-regulated, and over 75% of the genes in metabolic pathways of different environments

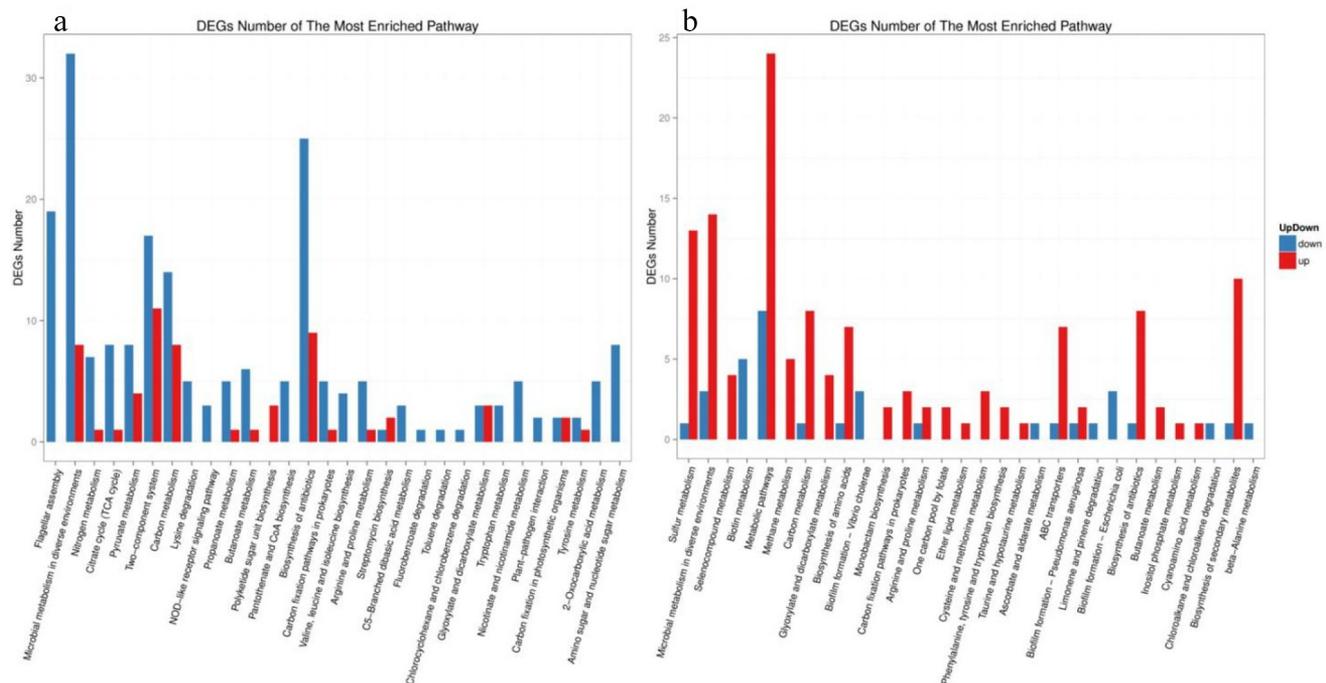
were downregulated. Few nitrogen-metabolism-related genes were identified, but the genes in this pathway were significantly down-regulated, and the number of down-regulated genes was more than 6 times that of up-regulated genes. The overall observations showed that the differentially expressed genes caused by *rpoS* deletion in LB medium were mainly down-regulated, and all of the pathways also showed a trend of down-regulation (Figure 9(a)).

In M9 medium, sulfur metabolism, microbial metabolism in diverse environments and seleno-compound metabolism were the most significantly altered pathways, they were all up-regulated. Among them, all the genes within the selenium-containing compound metabolism pathway were up-regulated. Figure 9(b) shows that the differentially expressed pathways caused by *rpoS* deletion in M9 medium were mostly up-regulated, but in general, the number of genes significantly differentially expressed in M9 media was less than that in LB media.

### Analysis of significant differential pathways

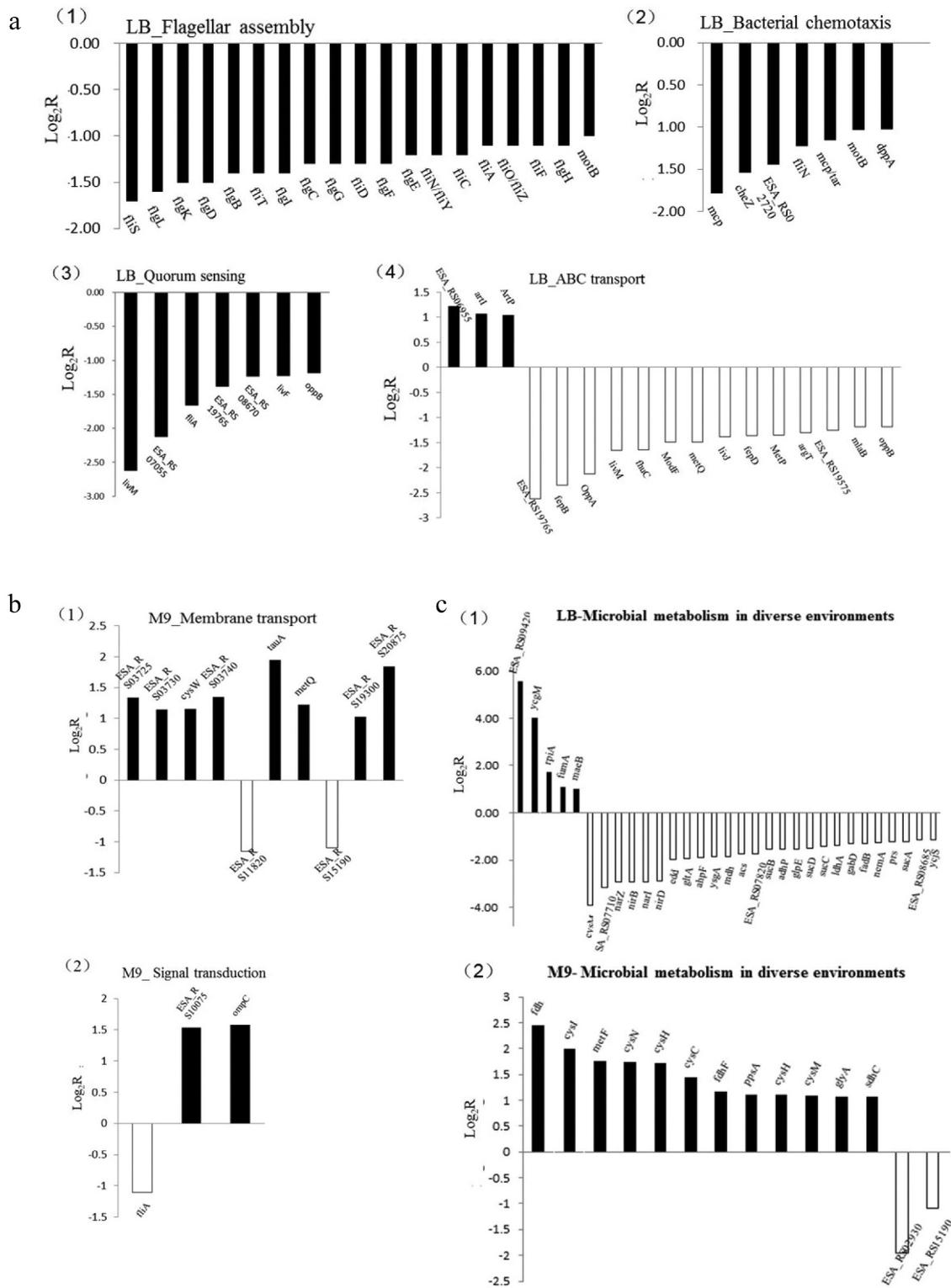
Bacteria can improve population tolerance through cell-to-cell interactions in stressful environments. Cell surface hydrophobicity, pili, and transport proteins can directly affect tolerance, while chemotaxis and population effect signaling molecules are involved to reflect the changes in tolerance through intercellular communication within the cell population. In this study, we analyzed the transcription of genes involved in flagella assembly, cellular chemotaxis, quorum sensing and ABC transport in LB medium, as they were significantly regulated pathways. Meanwhile, the membrane transport and signal transduction pathways were analyzed in M9 medium. The comparison between  $\Delta rpoS$  mutant and wild type, and the results are shown in Figure 10(a,b).

The mutant strain showed reduced motility in LB medium (Figure 10(a(1))), and transcriptome analysis also showed down-regulation of all genes involved in the pili synthesis pathway in the mutant strain cultured in LB medium. The pili



**Figure 9.** KEGG pathway cluster analysis of differentially expressed genes in  $\Delta rpoS$ , using BAA894 as a control.

The significantly enriched pathway categories ( $p$ -value $\leq 0.05$ ) in the differentially expressed genes were shown. The Y-axis represents the numbers of differentially expressed genes in each category. Red bars denote the up-regulated genes, and the blues denote down-regulated genes. The culture media are (a) LB and (b) M9.



protein FliS presented as the most significantly down-regulated gene, followed by the hook proteins FlgL and FlgR, the pili basal-body rod protein FlgB, and the motility switch protein. These consistent down-regulations of flagellar assembly and function-related proteins were also consistent with the reduced motility observed in the *rpoS* mutant strain when in LB medium. The mutant strains were also downregulated in all bacterial chemotaxis and quorum sensing pathways, including MCP and protein phosphatase CheZ (Figure 10(a(2), (3))), and some ABC transport-related proteins such as substrate binding protein OppA in the population sensing, which are actually related to cell perception, environmental response and motility. Most of the differential genes are also involved in the ABC transport system of membrane active transport, and as shown in Figure 10(a(4)), the ABC transporter pathway was highlighted as one of the significantly down-regulated pathways.

In M9 medium, the pathways related to membrane transport and signal transduction were significantly up-regulated in the mutant (Figure 10(b)). Genes *tauA* and *metQ* encode taurine ABC transporter substrate binding protein and methionine ABC transporter substrate binding protein, respectively, and *cysW* was associated with sulfate permease to enhance active sulfide transport, in fact, the differential enrichment of the sulfur-metabolism-related pathway was the most significant seen in the M9 medium. In the signal transduction pathways, OmpC was associated with outer membrane permeability, and its upregulation indicated enhanced membrane permeability. In addition, the upregulation of motility-related *ESA\_RS10075* (*trg*) in bacteria grown in M9 medium indicated increased motility, which was consistent with the results of the motility assay. It was also of interest that the pathways related to the cell secretion system were also upregulated under M9 environments.

Cells can sense stress by recognizing the signal receptors in response to the external environment. These are then transmitted by messenger molecules such as ppGpp to stress proteins and  $\sigma^S$  regulators, and the transcriptional regulator  $\sigma^S$  regulates the transcription function of RNA polymerase (RNAP) by binding to the promoter or the

nearby region to achieve the transduction of signal to final activation of gene expression in vivo. In this study, we compared and analyzed the differentially expressed genes in microbial metabolism in diverse environment pathways both in LB and M9 medium using transcriptomic analysis (Figure 10(c)). When compared to the wild type, the genes that were down-regulated the mutant strain in LB medium mainly included cysteine synthase *cysM*, 2-oxoglutarate dehydrogenase *sucB*, nitrite reductase catalytic subunits *nirB*, *nirD*, and nitrate reductase subunits *narH*, *narZ*, indicating a decrease in the metabolism of nitrogen and sulfur by the bacterium. Up-regulated genes were mainly related to formic acid metabolism. *ESA\_RS09420* formic acid dehydrogenase subunit and *ybcF* carbamate kinase were up-regulated in LB medium. Meanwhile in the M9 medium, sulfite reductase subunit *cysHIJN* and other sulfite synthesis and transformation-related genes were up-regulated, while those down-regulated included dimethylallyl transferase and membrane protein *ESA\_RS11100*. The significant changes in this pathway demonstrate that *rpoS* removal can cause corresponding changes in cellular responses to the environment.

## Discussion

RpoS is an important global regulator, and Alvarez et al. [5] showed that  $\sigma$ -factor is the main signal switch in response to environmental stress, besides which, it is also an important gene which influences *C. sakazaki* to enter the VBNC state after extreme stress. During the process of our research, we also found that bacterial culturability decreased in the absence of *rpoS* gene expression. As the main  $\sigma$ -factor during the stationary phase of the cell, its effect and mechanism on environmental stress in *C. sakazakii* requires further investigation.

The biofilm formation abilities and cell motility are indirectly related to environmental tolerance. Microorganisms cannot stay in a specific environment during growth, but more often grow as a population within the environment; therefore, the study of bacterial tolerance should consider some properties related to cellular interactions. As a product of bacterial stress regulation in

response to changing environmental conditions, the biofilm plays an important role in the survival and growth of bacteria under less favorable conditions [39]. The adhesion of bacteria to materials depends more on pili, and the bacterial adhesion not only affects the resistance of multiple external shocks, but also is related to the host invasion, which determines the virulence of bacteria to a certain extent.

The increased ability of the mutant strains in this study to form biofilm indicates an increased adhesion property within the mutant strain [40]. However, in the motility assay, mutant strain mobility was reduced in LB, suggesting that the increased auto-aggregation ability and membrane formation ability in this medium may not be caused by the pili synthesis, but may be related to other cell membrane alterations. *rpoS* gene modulates expression of 10% of the genome in Gram-negative bacteria, including flagella and fimbriae synthesis genes, membrane transport genes, and signal transmission genes, etc. Based on these regulations, a large part of genes involved in biofilm formation and motility are affected, especially in harsh environments. PmrA/PmrB (polymyxin resistance A/B) regulatory system is one of the two-component regulatory systems (TCS), and it is also known as an important stress tolerance and biofilm-related gene in *C. sakazakii* [41–43]. PmrA/PmrB mainly affect the biofilm formation by regulating genes (*lpxA*, *eptAB*, *cpxAR*) related to lipopolysaccharide (LPS) modify [44]. Unlike PmrA/PmrB regulatory system, *rpoS* not only affects virulence-related stress tolerance, but also affects many metabolic reactions and survival states of the strains.

In this study, the tolerance assay to different concentrations of NaCl indicated a reduced tolerance of the mutant strain  $\Delta rpoS$  to high osmotic pressure. The acid tolerance assay showed that the acid tolerance of the  $\Delta rpoS$  deletion mutant strain was reduced, and the growth assay in M9 medium indicated that the mutant strain grew worse than the wild type under acidic conditions. The desiccation tolerance of the mutant strain decreased in LB medium and increased in M9 medium. In LB medium, the desiccation tolerance of the cells might be mainly derived from the synthesis of compatible solutes, and the deletion of *rpoS* gene

thus leads to reduced tolerance due to its positive regulation of a variety of intracellular synthesis and transport pathways. However, in M9 medium, the cells reduced the intensity of many cellular metabolism pathways and slowed growth down due to limited carbon and nitrogen sources. Meanwhile, the cells consumed a large amount of carbon sources to synthesize extracellular secretions to protect the cells from the stress environments, which also shifted the way of desiccation tolerance from intracellular regulation to the structural changes of membrane walls and appendages. Therefore, the regulatory effect of RpoS in the restricted medium was not as important as observed in normal medium, and the increased desiccation resistance of the mutant strain in M9 medium may be related to the survival status of the organism in different environments.

The drug resistance of the mutant strain was tested in both LB and M9 media. In the M9 medium, the mutant strain  $\Delta rpoS$  showed reduced resistance to cefoxitin from the cephalosporins and neomycin from the coumarins, and increased MIC to norfloxacin, which prevents bacterial DNA replication by blocking the DNA gyrase. The mutant strain grew slower than the wild type, and its DNA replication rate may be lower than that of the wild type, thus showing lower resistance to these antibiotics. The reduction in MIC to  $\beta$ -lactam and peptide antibiotics suggests that the *rpoS* deletion may have caused some structural changes in the cell membrane and cell wall, making them more fragile.

In this study, we conducted biofilm formation ability, motility, acid resistance, desiccation tolerance, high permeability and antibiotic resistance phenotypic experiments. In the transcription analysis, we indicated that cell surface hydrophobicity, pili, and transport proteins directly affected tolerance, while chemotaxis and population effect signaling molecules were involved to reflect the changes in tolerance through intercellular communication within the cell population. Therefore, we analyzed the transcription activities of genes involved in flagella assembly, cellular chemotaxis, quorum sensing and ABC transport in LB medium, as they are significantly regulated pathways in LB medium (Figure 10(a)). Meanwhile, the membrane transport and signal transduction pathways

were analyzed in M9 medium, as they can help explain the performance of the mutant strains in M9 medium (Figure 10(b)). We also compared and analyzed the differentially expressed genes in microbial metabolism in diverse environment pathways both in LB and M9 medium using transcriptomic analysis (Figure 10(c)). Cells can sense stress by recognizing the signal receptors in response to the external environment, and the transcriptional regulator  $\sigma^S$  regulates the transcription function of RNA polymerase (RNAP) by binding to the promoter or the nearby region to achieve the transduction of signal to final activation of gene expression in vivo. Analysis of the significantly differentially expressed genes of this pathway in the mutant strain can explain the results of the acid tolerance and high permeability experiments.

## Conclusion

In this article, we investigated the overall regulatory  $\sigma$  factor RpoS for environmental stress resistance in *C. sakazakii* BAA894. First, the gene *rpoS* was knocked out of the genome by Red homologous recombination; then, the trials with acid, osmotic pressure, desiccation and drug tolerance were carried out. The results showed that mutant strain  $\Delta rpoS$  membrane formation ability significantly enhanced, motility became enhanced, hyperosmotic resistance increased, acid tolerance decreased, whilst drying tolerance decreased in LB medium and increased in M9. Finally, we analyzed the transcriptome of the mutant to confirm the experiment results. These experiments concluded that RpoS played a critical regulatory role in *C. sakazakii* BAA894 in relation to withstanding environmental stresses. All these conclusions are meaningful toward understanding the infection mode of *C. sakazaki* in newborns and help to prevent these infections.

## Highlights

- 1) Constructed *rpoS* deletion mutant strain using Red homologous recombination technology.
- 2) The  $\Delta rpoS$  biofilm formation was enhanced, and motility changed oppositely in LB or M9 media.

3)  $\Delta rpoS$  exhibited resistance changes in relation to hypertonicity, acid, dryness, and antibiotics.

4) Transcriptomic analysis results were mostly consistent with the other experiments.

## Compliance with ethics requirements

This article does not contain any studies with human or animal subject.

## Disclosure statement

The authors declare no conflict of interest.

## Funding

Raw transcriptome sequence data are available from the NCBI Sequence Read Archive (SRA) under BioProject accession code [PRJNA714757].

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